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Review

Water-immiscible dissolved oxygen carriers in combination with Pluronic F 68 in bioreactors

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The supply and availability of dissolved oxygen (DO) in aerobic bioprocesses is often a limiting factor for the scaling up, improvement and general performance of these bioprocesses. The use of different DO carriers, particularly the use of perfluorocarbons as oxygen carriers, is discussed in this review. It also highlights interactions of microbial cultures with the surfactant, Pluronic F 68. Although oxygen carriers have been used extensively in the medical field, this review only focuses on their use in microbial bioprocess used for the production of high-value bioproducts. The use of water-immiscible compounds in combination with Pluronic F 68 in bioprocesses is discussed with the intention of analysing their combined effect where bioreactor and biomass performance is affected by DO limitations, nutrient starvation, high concentrations of trace element ions, oxidative stress and cell death from mechanical stress.

Key words: Dissolved oxygen, oxygen carriers, perfluorocarbon, Pluronic F 68, surfactant.

DISSOLVED OXYGEN TRANSPORT LIMITATIONS IN BIOREACTORS

In aerobic bioprocesses, dissolved oxygen (DO) transport from the gas phase into the liquid medium is one of the critical parameters for effective bioprocess operations. In bioreactors such as membrane gradostat reactors (MGRs), where biofilms are attached to membrane surfaces, low shear aeration conditions are employed in order to reduce biofilm sloughing. This particular bioreactor uses biofilms system so that the different parts of the biofilm can experience different nutrient concentrations, such that a gradient could be established. This cannot occur if the biofilm sloughs off. However, under these conditions, the overall DO mass transfer into the immobilised biofilms is restricted and controlled by a liquid film at the gas-liquid-biomass interface (Ju et al., 1991a). This adversely affects overall biofilm- and reactor performance. Furthermore, DO transfer is hampered by extracellular polymeric substances (EPS) produced or stored by microbial biomass during fermentation. In fungal bioprocess systems where high glucose concentrations are used in the nutrient medium, the production of EPS as storage carbohydrates further hampers DO transport because of the availability of excess glucose, necessitating the use of pressurised bioreactor systems. Although the use of pressurised bioreactors might improve DO transport, this is also likely to increase the rate of biofilm metabolism and the generation of carbon dioxide. Furthermore, the possibility of carbon dioxide entrapment in the fermenting biomass will increase because of EPS production associated with high DO transport into the biofilms. EPS production is also reported to be promoted by the high partial pressures of oxygen used during aeration, limited nitrogen availability and low pH conditions that are prevalent in bioreactors used to produce secondary metabolites, such as the MGR system (Ahimou et al., 2007; Ryu and Beuchat, 2004; Damiano and Wang, 1985; Jarman et al., 1978).

As oxygen has a low solubility in aqueous medium, DO transfer becomes an important design aspect for any aerobic bioprocess design. Inefficient DO transfer in-

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fluences the scale-up and economic value of the fermentation processes, as it increases operational costs and reduces recoveries related to the aerobic biosynthesis of high-value bioproducts (Cascaval et al., 2006). Furthermore, respiratory activity of the immobilised microorganism can be inhibited by bioproducts when the biomass is suspended in the product solution. DO cannot be increased by heating the water-based medium, as it has been found that the oxygen-transfer rate (OTR) remained constant between 20 and 55°C (Vogelaar et al., 2000). The presence of dissolved salts in most fermentation media has also resulted in decreased solubility and transfer of DO into microbial cells during the course of bioreactor operations (Anke and Weber, 2006). For example, an increase in the salinity of freshwater from 0 to 8% resulted in the reduction of DO from 200.4 to 192.3 □mol $O_2.l^{-1}$ (Unisense, 2008).

Spargers, agitators and technical-grade oxygen (pressurised ~100% O_2) have been used to improve the availability of DO and the performance of biomass in fermentation broths hampered by DO transfer (Murhammer and Goochee, 1990). However, the use of these devices and aeration sources can result in cell rupture as a result of the mechanical stress caused by mixing and continuous aeration. Conditions that can also limit biomass performance include: hyperoxia resulting from the availability and the use of high partial pressures of oxygen and the generation of reactive oxygen species (ROS), which cause lipid peroxidation and unwanted by-product formation as the biomass tries to protect itself from environmental stressors in the bioreactors.

DO carriers are used to alleviate DO limitations in different bioprocesses. They have been used extensively in the medical field (Goorha et al., 2003; Lowe, 2003; Riess, 2002) with limited applications within membrane-based bioreactors. To alleviate DO limitations, the use of water-immiscible DO carriers becomes necessary to optimise biomass performance. Some of these water-immiscible organic compounds have greater solubilization capacity for oxygen than aqueous media (Junker et al., 1990). These materials include haemoglobin derivatives (polymerised, polymer conjugated, intramolecular cross-linked, recombinant and lipid-based vesicle haemoglobin) and organic oils based on synthetic, highly fluorinated organic compounds named perfluorocarbons (PFCs) (Goorha et al., 2003; Lowe, 2003). The advantage of these PFCs over haemoglobin-based oxygen carriers is that haemoglobin is slowly oxidised by oxygen into methaemoglobin (Adlercreutz and Mattiasson, 1982), thus limiting their potential to be recovered and recycled in highly oxidative processes. The presence of trace element ions can further increase the oxidation of haemoglobin-based DO carriers. The presence of a DO carrier in the liquid phase can have an improved effect on the rate of oxygen transfer in fermentation processes, thus improving the performance of the biomass and bioproduct yield.

WATER-IMMISCIBLE GAS CARRIERS: THEIR APPLICATION AND BENEFITS TO DIFFERENT CELL CULTURES

Gas saturated water-immiscible DO carrier droplets can be used to enhance oxygen supply by liquid-liquid and liquid-biomass contact. Some are heavier than and immiscible in, aqueous medium and can be collected at the base of the vessel for reuse in subsequent fermentations (Richardson et al., 2002; Lowe et al., 1998; Kabalnov et al., 1990). Some DO carriers, such as PFCs, have higher solubilities for carbon dioxide than ordinary aqueous media. Therefore, they can also carry metabolically produced carbon dioxide, removing it from the fermentation broth and thus improving overall biomass and bioreactor performance, especially for continuous bioprocess systems. One such example was the inclusion of 50% (v/v) perfluorodecalin in the fermentation medium using *Streptomyces coelicolor* A3 (2). This resulted in a five-fold increase in the maximum actinorhodin production. The use of water-immiscible DO carriers can also improve the maximum specific growth rates of micro organisms with increasing concentrations (Amaral et al., 2007); while the specific death rate decreases with DO availability. These phenomena clearly suggest that the additional oxygen supplied by inclusion of water-immiscible gas carriers could be readily utilised by aerobic submerged cultures resulting in the improved performance of the fermentation system (Elibol and Mavituna, 1999). Examples of other water-immiscible DO carriers are hexanol (Koide et al., 1985), olive and lard oil (Liu et al., 1994) and soybean and silicone oil (Morao et al., 1999).

The benefits of using gas carriers in microbial culture systems include (1) a reduction in mechanical damage to biomass by eliminating the effects of conventional aeration through sparging or continuous stirring, (2) the provision of a multi-phased interface for effective DO transfer; (3) prolonged survival rates of micro organisms, (4) ease of sterilisation by means of autoclaving or filtering) and (5) ease of recovery and recycling (Lowe et al., 1998). Microbial cultivation in these dispersions is attrac-tive because maximum DO transfer rates in some cases were increased by over 400% when using oxygen carriers (McMillan and Wang, 1988). In the majority of these cases, the emulsions have not been used in volume fractions exceeding 40% (v/v) (Junker et al., 1990), thus limiting fermentation broth viscosity close to that of water. Table 1 lists examples of applications and benefits for different water-immiscible gas carriers in different submerged microbial cultures.

PERFLUOROCARBONS AS DISSOLVED OXYGEN CARRIERS

The use of perfluorinated organic oils as oxygen carriers

Table 1. Application and benefits of water-immiscible oxygen carriers in submerged cultures.

Process	Oxygen carrier Surfactant	Micro organism	Consequence	Reference
Dihydroxyacetone production	0.85 mM oxyhaemoglobin	*Gluconobacter oxydans*	> 4.0 mM dihydroxyacetone production	(Adlercreutz and Mattiasson, 1982)
Dihydroxyacetone production	32.4% (v/v) FC-72	*Gluconobacter oxydans*	4 - 5 x dihydroxyacetone production	(Adlercreutz and Mattiasson, 1982)
Dihydroxyacetone production	20-80 mM*p*-benzoquinone	*Gluconobacter oxydans*	70% increase in dihydroxyacetone production	(Adlercreutz and Mattiasson, 1984)
IgG production	Fluorinert FC-40	*Mouse-mouse hybridoma, 4C10B6*	High cell density attained	(Hamamoto et al., 1987)
Escherichia coli and *S. cerevisiae* culture	20% Fluosol-DA (F-DA) Perfluorodecalin	*E. coli HB101* *Saccharomyces cerevisiae*	*E. coli* and *S. cerevisiae* inhibited by F-DA *E. coli* not affected by 15 to 30% PFC	(Chandler et al., 1987)
Hybridoma cell culture	perfluoromethyldecalin	*Mouse-mouse hybridoma, cell line* (#824)	Increased oxygen transfer Increased cell density when compared to batch culture	(Cho and Wang, 1988)
Penicillin fermentation	0. 5, 2, 5% *n*-hexadecane	*Penicillium chrysosgenum, Wis.* 54 to 1255	Increased cell growth Increased penicillin production	(Ho et al., 1990)
Aerobacter aerogenes culture	54.9 $mg.l^{-1}$ n-dodecane and 118 $mg.l^{-1}$ F66E 12.5 mg/ml Pluronic F 68	*A. aerogenes*	3.5 x K_La achieved	(Rols et al., 1990)
E. coli culture	15% (v/v) $FlurO_2$	*E. coli K-12*	2.55 x enhance in biomass concentration	(Ju et al., 1991a)
Protoplast culture	Perfluorodecalin 0.01% (w/v) Pluronic F 68	*Petunia hybrida*	52% plating efficiency than control	(Anthony et al., 1994)
E. coli culture	50% (v/v) Foralkyl 10 mg/ml Pluronic F 68	*Escherichia coli*	0.17 g $O_2.l^{-1}h^{-1}$ supply and 0.23 g $CO_2.l^{-1}h^{-1}$ extraction	(Martin et al., 1995)
C. acetobutylicum culture	18.5% Forane F66E	*Clostridium acetobutylicum*	9% extraction of total CO_2 produced	(Percheron et al., 1995)
Anthraquinone production	0.11% (w/v)n-hexadecane	*Morinda citrifolia*	2 x Anthraquinone production than control	(Bassetti and Tramper, 1995)
E. coli culture	50% (v/v) Foralkyl 10 mg/ml Pluronic F 68	*Escherichia coli*	0.17 g $O_2.l^{-1}h^{-1}$ supply and 0.23 g $CO_2.l^{-1}h^{-1}$ extraction	(Martin et al., 1995)
C. acetobutylicum culture	18.5% Forane F66E	*Clostridium acetobutylicum*	9% extraction of total CO2 produced	(Percheron et al., 1995)
Anthraquinone production	0.11% (w/v)n-hexadecane	*Morinda citrifolia*	2 x Anthraquinone production than control	(Bassetti and Tramper, 1995)

was initially investigated using mice. The oils were shown to assist the animals with liquid breathing (Clark and Gollan, 1966). Over the years, they have also been shown to be effective as DO carriers in bioprocesses using different microorganisms. Recently, Amaral et al. (2007) demonstrated the effects of concentrations of 10 to 20% (v/v) perfluorodecalin in *Yarrowia lipolytica* under agitation conditions. The result was an increased glucose consumption and specific growth rate for the culture. As PFCs are non-corrosive, odourless and colourless (Lowe, 2002), they were also shown to be suitable for the development of synthetic blood (Inayat et al., 2006; Goorha et al., 2003; Lowe, 2003; Riess, 2002). Other examples of PFC-emulsion applications are listed in Table 1.

PFCs are chemically inert compounds consisting of fluorine-substituted hydrocarbons in which most or all of the hydrogen atoms have been replaced by fluorine. A

Table 1. (continued)

Process	Oxygen carrier Surfactant	Micro organism	Consequence	Reference
Actinorhodin production	10% (v/v) Perfluorodecalin 4% (w/v) Pluronic F 68	*S. coelicolor* A3(2)	2 x increase in actinorhodin production compared to 10% PFC (/v) without Pluronic	(Elibol and Mavituna, 1996)
Mycobacteria parafortuitum culture	26% (v/v) FC-40 18.2% (v/v) FC-40	*M. parafortuitum*	1.8 x KLa achieved 1.2 x KLa achieved	(Cesario et al., 1996)
Post thaw culture	Perfluorodecalin 0.01% (w/v) Pluronic F 68	*Oryza sativa cv.*	21% increase in post thaw viability	(Anthony et al., 1997)
Actinorhodin production	50% (v/v) Perfluorodecalin	*Streptomyces coelicolor* A3(2)	5 x increase actinorhodin production	(Elibol and Mavituna, 1997)
Hybridoma antibody production	Natural bovine haemoglobin Erythrogen™-1 Formula-1™ Polyethylene glycol cross-linked haemoglobin perfluorocarbon	*Hybridoma 3C11*	No antibody production increase for natural bovine haemoglobin 104% antibody production increase using Erythrogen™-1 20% antibody production increase using glycol cross-linked haemoglobin 78% antibody production increase using perfluorocarbon	(Shi et al., 1998)
S. cerevisiae culture	Perfluorodecalin 4% (w/v) Pluronic F 68	*Saccharomyces cerevisiae*	Significant increases in K_La values	(Elibol, 1999)
Actinorhodin production	50% (v/v) Perfluorodecalin	*S. coelicolor A3(2)*	5 x increase in actinorhodin production	(Elibol and Mavituna, 1999)
Lactate production	Fluorinert FC-40	*Spodoptera frugiperda sf9*	Increased cell density, growth yield and lactate yield	(Gotoh et al., 2001a)
Virus infected *S. frugiperda* culture	Fluorinert FC-40	*S. frugiperda; sf9*	Cell density achieved was higher than that in surface aeration Recombinant protein yield increased 1.6 x K_La achieved	(Gotoh et al., 2001b)
Actinorhodin production	10% Perfluorodecalin	*S. coelicolor* A3(2)	3.0 x K_La achieved	(Elibol, 2001)
Aspergillus terreus culture	n-dodecane	*Aspergillus terreus ATCC* 20542	1.4 x increase in lovastatin production	(Lai et al., 2002)
Egg hatching after storage	Fluorinert-77	*Oncorhynchus mykiss*	> 75.1% hatching/ compared to 14.3 % in control	(Richardson et al., 2002)
Ropionibacterium shermanii culture	5 -20% (v/v) n-dodecane	*P. shermanii*	3.5 - 5 x KLa achieved	(Cascaval et al., 2006)
S. cerevisiae culture	5 -20% (v/v) n-dodecane	*Saccharomyces cerevisiae*	3.5 - 5 x KLa achieved	(Cascaval et al., 2006)
A. chroococcum culture	5%(v/v) perfluorodecalin	*Azotobacter chroococcum ACB 121*	> 5 x increase in cell concentration 3.4 x nitrogenase activity 4.5 x increase in nitrogen content	(Bakulin et al., 2007)

progressive substitution of fluorine for hydrogen led to an increase in molecular mass, resulting in liquids that are much heavier than other hydrocarbon oils such as mineral oil. The oils typically have specific gravities, approximately twice that of water (Lowe et al., 1998). They are stable, non-toxic and can store and release oxygen at a greater rate (Goorha et al., 2003; Lowe, 2003; Richardson et al., 2002; Riess, 2002). Oxygen solubility in these oils is related to the molecular volume of the dissolving gas and decreased in the following

Table 2. Characteristics of PFC liquids compared to those of water at standard pressure and temperature (Lowe et al., 1998; Ju et al., 1991a).

Liquid	Oxygen	Carbon dioxide	Density[c]	Boiling point[d]	Molecular weight[e]
Water	2.2[a]	57[a]	1.0	100	18
FC-40	37[b]	142[b]	1.87	155	650
FC-43	36[b]	140[b]	1.88	174	670
FC-77	56[b]	214[b]	1.78	97	415
FC-84	59[b]	224[b]	1.73	80	388
Bis-(Perfluorobutyl) ethene	44.0[a]	203[a]	1.41	60	464
Perfluorobutyl tetrahydrofuran	51[b]	209[b]	1.77	102	416
Perfluorodecalin	35.5[a]	125[a]	1.92	142	462
Bis-Perfluorohexyl ethene	37.9[a]	159[a]	1.77	195	664
Perfluoro-n-hexane	65[b]	248[b]	1.68	59	340
Perfluorooctyl bromide	44.0[a]	185[a]	1.93	142	499
Perfluorotributylamine	35.2[a]	123[a]	1.85	155	671
Perfluorotripentylamine	31[b]	117[b]	1.94	215	821
Perfluorotripropylamine	39.6[a]	146[a]	1.82	130	521

[a]Gas dissolving capacity in mM at 25°C; [b]Gas dissolving capacity in ml gas/100 ml PFC at 37°C; [c]Density in $g.cm^{-3}$; [d]Boiling point in °C; [e]Molecular weight in g/mol.
FC 40/43/77/84-are Fluorinert electronic liquids, products of 3M Company.

order: $CO_2 > O_2 > N_2$ (Lowe et al., 1998), making them suitable for oxygen delivery and metabolic CO_2 removal from fermentation systems.

PFCs have the following advantages: 1) they do not react chemically with oxygen or other gasses; 2) oxygen solubility is not subject to the effects of pH; 3) they are not susceptible to dissolved salts in the fermentation medium and 4) they facilitate an effortless transfer of oxygen. Oxygen solubility here is inversely proportional to the molecular weight and directly proportional to the number of fluorine atoms in the oils (Goorha et al., 2003). When comparing traditional bioprocesses with PFC-supplemented bioprocesses, it can be seen that the OTR is enhanced without the need for supplementary energy consumption for mixing. However, the use of these oxygen carriers needs to be further analysed to determine their compatibility with microbial strains that are chosen for a defined bioprocess (Cascaval et al., 2006). The characteristics of commonly used PFC liquids at standard pressure (1 atm.) and temperature (25°C) are listed in Table 2.

There are several disadvantages relating to the application of pure PFCs in cell cultures, such as: 1) a reduction in biomass generated and 2) increased lag phases in the fermentation (Amaral et al., 2007). In order to use PFCs as oxygen carriers, their effects on the physiological state and performance of microorganisms need to be determined. The consensus is that PFCs have different effects on prokaryotic and eukaryotic cells. PFCs in their emulsion form have no apparent effect on prokaryotic cells, whilst some eukaryotic cells show ultrastructural changes after treatment with pure PFCs (Elibol, 2001; Chandler et al., 1987). Problems may also occur in batch PFC-mediated aeration cultures, where ventilation is only carried out through PFCs, as volatile organic compounds (VOCs) can be produced and thus hamper the metabolic activity of micro organisms (Gotoh et al., 2001b). VOCs are organic chemical compounds, mostly carbon-based molecules such as aldehydes and ketones, with high pressures, which enable them to rapidly vaporise and enter the atmosphere. PFCs cannot dissolve electrolytes and other organic compounds, except for fluorine-substituted compounds. The VOCs can, therefore, accumulate in the medium without being removed, especially in batch cultures (Gotoh et al., 2001b).

PREPARATION OF PERFLUOROCARBON EMULSIONS

Being virtually insoluble in water, PFCs are usually formulated as submicron emulsions. PFC emulsions can be prepared using different types and concentrations of surfactants as nano- or micro-emulsions. Nano-emulsions (droplets covering the size range of 100 to 600 nm) are preferable, as they have increased stability (Bouchemal et al., 2004). The formulation of an emulsion requires a surfactant capable of ensuring adequate dispersion, homogeneity, reproducibility, stability and biocompatibility (Riess, 2002).

The droplet size of the dispersed PFC phase can be controlled with the concentration of the surfactant. After preparation, the initial oil-particle size decreases with increasing surfactant concentration. PFC emulsions containing 10% (w/w) PFC were determined to be stable at surfactant concentrations as low as 0.5% (w/w) (Magdassi and Siman-Tov, 1990). In the preparation of PFC

Table 3. Hydrophilic-lipophilic balance of perfluorochemicals and surfactant (Weers, 1993).

Perfluorochemical	HLB value	Surfactant	HLB value
Perfluorodecalin	9.5	Lecithin	8.0
Perfluorooctyl bromide	6.0	Potassium oleate	18.0
Perfluorotributylamine	10.3	Pluronic F 68	24.0 - 29.0

HLB value = 3 to 6 (water-in oil surfactant); HLB value = 8 to 15 (oil-in-water surfactant); HLB value = > 15 (solubiliser).

Table 4. Surfactants most commonly used in PFC-based emulsions (Floyd, 1999).

Surfactants and/or their combinations	Range/ratio
Glycerol/propylene glycol	30 - 70% (w/w individually)
Egg lecithin	1 - 3% (w/w)
Pluronic F 68, 88, 108	1.5 - 10% (w/v)
Polysorbate 80	0.4% (w/w)

emulsions, dispersions with surfactant-PFC ratios of < 2% were determined to be surfactant limited, whereas those with a ratio of > 5% were energy-input limited (De Vleeschauwer and Van der Meeren, 1998). Although, several disadvantages of supplying pure PFCs in cell cultures are evident, the use of PFC emulsions with lower oil concentrations outweigh any disadvantages determined when cells are cultured in pure PFC dispersions (Ju et al., 1991b).

Emulsions are usually prepared with a mixture of surfactants with different hydrophobicity. The concept of hydrophilic-lipophilic balance (HLB) was introduced as an indication of the relative strength of the hydrophilic-lipophilic portions of the surfactant molecule and can be characterised by the relative affinity of surfactants for the aqueous and organic parts of the surfactant molecule (Griffin, 1949). The HLB arbitrary scale range is 1 to 30. As a result, surfactants with high HLB values (> 15) tend to stabilise oil-in-water (O/W) emulsions, while surfactants with low HLB values (< 10) tend to stabilise water-in-oil emulsions. As most PFC emulsions used in bioprocesses are O/W emulsions, surfactants with high HLB values are preferred in the preparation of these emulsions (Sajjadi, 2006). Examples of the HLB values of several PFCs and commonly-used surfactants are listed in Table 3, indicating that PF 68 with an HLB value of 24 to 29 is suitable for O/W emulsions.

PLURONIC F 68 AS A SURFACTANT AND A BIOMASS PROTECTOR

Polymeric surfactants have been extensively used in various applications ranging from personal care products to pharmaceutical and industrial use (Plucktaveesak et al., 2000). Pluronic F 68 (Poloxamer 188/ PF 68) is a non-ionic block copolymer of polyethylene glycol and polypropylene glycol (Elibol, 1999), which has been used to emulsify PFCs for use in oxygen-transport fluids (Johnson et al., 1990). This surfactant has been used as a growth-promoting additive to animal and microbial cultures (Lowe et al., 1994). When a PF 68 concentration of 10mg.ml^{-1} was used in Perfluorooctyl bromide (PFOB) emulsions, the emulsion was extremely stable and remained in this state for more than 7 days. This was advantageous for cultures, as the emulsions were homogeneous, but led to great difficulty in PFOB recovery. The total recovery percentage was low (78%) after centrifugation. When a PF 68 concentration of 5 mg.l^{-1} was used, destabilisation occurred rather rapidly, but the recovery of PFOB was easier, resulting in a 91% recovery rate (Martin et al., 1995).

The mass transfer characteristics of PF 68 were investtigated in a MYGP (malt extract-yeast extract-glucose-peptone) medium using Saccharomyces cerevisiae (NCYC 239) and was found to reduce the volumetric oxygen transfer coefficient ($K_L a$). This was not the case in the presence of PFC oils, where final biomass concentrations were unaffected (Elibol, 1999). Typical usage levels of surfactants in an emulsion system are: 1) 1 to 5% (w/v) for water-in-oil emulsions and 2) 5 to 10% (w/v) for oil-in-water emulsions (Floyd, 1999). Table 4 shows concentration levels for some of the surfactants most commonly used in PFC-based emulsion formulations, as compared to PF 68.

During PFC-based emulsion preparation, the particle size of the oil decreased when a combination of PF 68 and Tween 80 were used as hydrophilic surfactants. It was concluded that PF 68 greatly influenced the particle-size distribution profile in the PFC-based dispersions (Bouchemal et al., 2004). Emulsions prepared with PF 68 have a lower probability of stability loss due to coalescence resulting in homogeneous emulsions with an

Table 5. Examples of different applications of Pluronic F 68.

Surfactant	Micro organism	Consequence	Reference
0.2% (w/v) Pluronic F 68	*Spodoptera frugiperda* Sf9	Cell protection	(Murhammer and Goochee, 1990)
20% (w/v) Pluronic F 68	*Saccharomyces cerevisiae*	Improved fluorescein diacetate uptake	(King et al., 1991)
0.1 - 1.0% (w/v) Pluronic F 68	*Saccharomyces* cerevisiae (X 2180 1B)	No adverse effect on growth kinetics	(Laouar et al., 1996)
0.01% (w/v) Pluronic F 68	*Tetrahymena thermophila*	Protection against chemical/physical stress	(Hellung-Larsen et al., 2000)

Note: Quantities of reagents are listed at their final concentration in the nutrient medium.

Even distribution of DO. When coalescence and ripening are suppressed, the emulsion might remain stable for years (Mason, 1999).

PF 68 has also been used as a cytoprotectant and growth promoting additive to animal cell and microbial cultures (Lowe et al., 1994). Microbial cell damage arising from gas sparging is considered to be a major obstacle in the operational longevity of large-scale bioprocesses (Wu, 1995), thus requiring the application of protective additives in the culture medium. PF 68 is one of the most recognised and commonly used additives, as it has been shown to have strong protective effects in microbial cultures (Wu, 1995). Several other poloxamers (F88, F108 and L35) have also shown varying degrees of protective effects in agitated and aerated fermentation systems (Wu, 1995).

PF 68 has been determined to protect cells by coating the membranes of micro organisms, thus directly altering the cell membrane and resulting in the reorganisation of membrane lipids. The surfactant affects lipid-lipid and lipid-protein interactions, thus improving the survival rate of micro organisms by inhibiting damaging interactions between the cell membrane, fermentation broth and the air-liquid interface (King et al., 1991; Murhammer and Goochee, 1990). PF 68 was also shown to protect and prolong the survival of low concentrations of cell suspensions during nutrient starvation. Furthermore, the surfacetant prevented death caused by concentrations of Ca^{2+}, prolonging the survival of cells exposed to higher ion concentrations of Ca^{2+}, Na^{+} and K^{+}. It was effective in the postponement of death caused by trace element ions like Zn^{2+}, Fe^{3+} and Cu^{2+} and death caused by shearing forces, while prolonging the survival of cells exposed to hyperthermia (Hellung-Larsen et al., 2000). The surfactant protects cells by regulating the permeability and loss of ions from the affected cells (Laouar et al., 1996). Table 5 summarises examples of microbial cultures and the effects caused by the addition of PF 68 to these cultures, showing that PF 68 has mostly positive effects on microbial cultures. In addition, no quantitative results were reported in literature as to whether PF 68 improves microbial growth when used as an additive to synthetic media.

CONCLUSIONS

As new technologies are developed to continuously produce high-value biopharmaceuticals in order to meet increasing demand, the effective use of water-immiscible DO carriers and additives to improve bioreactor operational efficiency and longevity remains overlooked. This review clearly shows the positive effects of adding DO carriers to different microbial systems.

PFCs have been shown to be effective in carrying and delivering oxygen to biological cells in storage or culture systems. The general consensus was that PFCs, espe-cially those in emulsions, can have different effects on prokaryotic and eukaryotic cells, but their use in many instances showed that they increased the overall biomass performance and in some systems, they were shown to increase the yields of commercially important cellular products such as antibiotics. However, their application in bioprocessing systems needs to be evaluated in order to avoid harmful effects. Therefore, determining adequate concentration levels for a predetermined bioprocesses is important.

The promising capabilities of PFC and PF 68-based emulsions for providing culturing conditions suitable for a general improvement in microbial biomass performance and extended product formation have been illustrated. PF 68's ability to improve the functioning of individual cells in fermentation systems, while protecting the cells against trace element toxicity, shearing effects, hypothermia and product inhibition, will provide for increased biofilm and bioreactor performance in fixed-film bioreactors, thus improving the economic viability of these continuous systems.

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Review

Increasing trend of metronidazole resistance in the treatment of *Helicobacter pylori* infection: A global challenge

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Helicobacter pylori **are gram negative spiral bacteria that colonize the human stomach. Infection with *H. pylori* is associated with chronic gastritis, peptic ulcer, gastric adenocarcinoma and gastric mucosa-associated lymphoid tissue (MALT) lymphoma. Antibiotic resistance is an ever increasing problem with the treatment of most microbial infections including *H. pylori*; and has become a growing problem worldwide with the eradication of this organism. In recent years, several treatment regimens have been proposed for *H. pylori* eradication. However, the only conditions for which such treatment is strongly recommended on the basis of unequivocal supporting evidence are peptic ulcer disease and low grade gastric MALT lymphoma. Success of antimicrobial regimens for *H. pylori* eradication depends on patient compliance and lack of antimicrobial resistance. Metronidazole (Mtz) containing regimens have been shown to limit effectiveness because of increasing prevalence of resistance to this drug. A high prevalence (> 90%) of Mtz resistance in *H. pylori* has been reported especially in developing countries. Mtz resistance may be mediated through an inability of Mtz-resistant strains to remove oxygen from the site of Mtz reduction, thereby preventing Mtz activation. This has been attributed to a mutation on the *frxA* and/or *rdxA* genes resulting in strains of the organism with defective nitro-reductases coded by these genes. Infection by Mtz or amoxicillin resistant strains is an important factor leading to treatment failure; subjecting all *H. pylori* clinical isolates to susceptibility testing most especially to Mtz is recommended. If not possible, a program to survey the prevalence of resistance should be implemented in a given area or population. This increasing emergence of antimicrobial resistance in *H. pylori* treatment posses serious public health problems and is therefore necessary that new drug regimens be examined.**

Key words: *Helicobacter pylori*, drug resistance, metronidazole, gene mutations, public health.

INTRODUCTION

Helicobacter pylori are gram negative spiral bacteria that colonize the human stomach. Infection with the organism is associated with chronic gastritis, peptic ulcer, gastric adenocarcinoma and gastric MALT lymphoma (Suerbaum and Michetti, 2002; Ndip et al., 2004, 2008). Simple blood, breath and stool tests can determine if one is infected with *H. pylori*. The most accurate way to diagnose *H. pylori*, however, is through upper endoscopy of the oesophagus, stomach and duodenum (Ables et al., 2007). Eradication of the organism has been shown to result in ulcer healing, prevention of peptic ulcer recurrence and may also reduce the prevalence of gastric cancer in high risk populations (Sepulvedo and Coelho, 2002; Tanih et al., 2009).

Antibiotic resistance is an ever increasing problem with

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Abbreviations: MALT, Mucosa-associated lymphoid tissue; **Mtz,** metronidazole; **PPIs,** proton pump inhibitors; **MIC,** minimum inhibitory concentration; **CLSI,** clinical laboratory standard institute; **Mtz^S,** Mtz sensitive; **Mtz_R,** Mtz resistant.

the treatment of most microbial infections including *H. pylori* infection (Hoffman, 1999; Thyagarajan et al., 2003). New antimicrobial agents are therefore being developed to overcome the problem of antibiotic resistance in bacterial pathogens, such as combination of antibiotics with plant extract and other natural products that possess antimicrobial activity (Chaudhuri et al., 2003; Ndip et al., 2007a,b; Tanih et al., 2009). Combinations of drugs have often been used for the treatment of drug resistant infections as this takes advantage of different mechanisms of action (Chaudhuri et al., 2003).

Antibiotic resistance with regard to *H. pylori* eradication has become a growing problem both in the developing and in developed countries. Mtz was initially used against a diversity of anaerobic microorganisms but was later established to have activity against certain microaerophilic organisms such as *H. pylori*. At present, Mtz is a cornerstone of many triple-therapy formulations for the eradication of *H. pylori*. However, resistance approaches 90% in many developing countries and even in Western Europe it ranges from 5 to 50% (Alarcon et al., 1999). In some countries, the prevalence of Mtz resistant strains approaches 70-100% and is associated with prior exposure to the drug (Noach et al., 1994; Goddard and Logan, 1996; Ndip et al., 2008). While several triple and quadruple-therapy formulations use more than one antibiotic, Mtz resistance has a profound effect on the efficacy of these regimens (van der Wouden et al., 1997, 2000, 2001).

Based on findings of several studies, the currently recommended therapy for the eradication of *H. pylori* infection include proton pump inhibitors (PPIs) such as omeprazole, lanoprazole, rabeprazole, or pantoprazole together with clarithromycin and either Mtz or amoxicillin (Salcedo and Al-Kawas, 1998; Veldhuyzen et al., 1998; Chaudhuri et al., 2003; Ndip et al., 2008). Success rates of cure with the use of these combination therapy ranges from 85 to 95%. However resistance to Mtz or clarithromycin results in an increased failure rate of therapies (Megraud, 1997). The emerging resistance to Mtz limits its use in the treatment of infections; and this problem is encountered more in Africa (Asrat et al., 2004; Ndip et al., 2008; Tanih et al., 2009). The resistance mechanisms in anaerobic organisms and *H. pylori* vary. Mtz, a synthetic nitroimidazole (Figure 1), is a prodrug and becomes active when reduced in the cytosol of the microorganism to a toxic metabolite. Unstable Mtz radicals react rapidly with proteins, RNA and DNA, eventually resulting in cell death. Under the conditions of low-redox potential in anaerobic organisms, drug activation can be catalyzed by nitroreductases such as pyruvateflavodoxin reductase by means of a single electron transfer event. *H. pylori* possesses this enzyme, but owing to its microaerophilic nature, molecular oxygen is also present and can compete with the Mtz radical for electrons in a futile cycle that restores the prodrug along with super oxide. Instead, a separate mechanism seems to account for most Mtz sensitivity in *H. pylori* (Goodwin et al., 1998). A non-

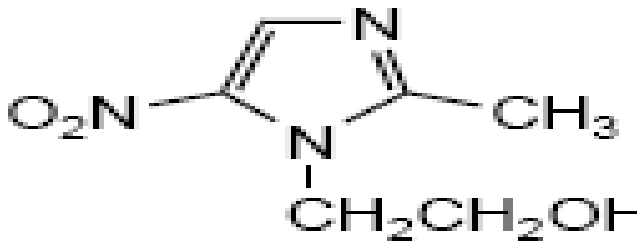

Figure 1. The chemical structure of metronidazole (Mendz and Megraud, 2002).

oxygen-sensitive NADPH nitroreductase encoded by the *rdxA* gene reduces Mtz by a two-electron transfer step into a toxic metabolite that cannot be retransformed to its parent by molecular oxygen. The vast majority of clinically isolated (Tankovic et al., 2000) or experimentally induced (Jenks et al., 1999) Mtz-resistant clones contain a mutation somewhere in the *rdxA* coding sequence. However, there have been reports that mutation of a second reductase NAD(P)H-flavin oxidoreductase encoded by *frxA* could also confer low-level Mtz sensitivity in some strains (Kwon et al., 2001) and a role for oxygen sensitive reductases has not been formally excluded. Sequencing candidate genes, such as the reductases (mentioned above), in sensitive and resistant isolates has provided support for the idea of these genes playing a role in resistance, but reports also show that frameshift mutations in *frxA* occur with similar frequencies in sensitive and resistant strains (Chisholm and Owen, 2004). Jeong et al. (2000), concluded that most Mtz resistance in *H. pylori* depend on *rdxA* inactivation, of which mutations in *frxA* can enhance resistance, and that genes conferring Mtz resistance without *rdxA* inactivation are rare or nonexistent in *H. pylori* populations. Although null mutations in a *rdxA* gene that encodes oxygen-insensitive NAD(P)H nitroreductase was reported in Mtz-resistant *H. pylori*, an intact *rdxA* gene has also been reported in Mtz-resistant *H. pylori*, suggesting that additional Mtz resistance mechanisms exist in *H. pylori* (Kwon et al., 2000a). In this paper, we present an overview of Mtz resistance as well as the roles of the putative genes *frxA* and *frxA* implicated in Mtz resistance.

TREATMENT OF *H. PYLORI* INFECTION

In recent years, several treatment regimens have been proposed for the eradication of *H pylori*. However, the only conditions for which such treatment is strongly recommended on the basis of unequivocal supporting evidence are peptic ulcer disease and low grade gastric MALT lymphoma (Graham, 1998). Success of antimicrobial regimens for *H. pylori* eradication depends on patient compliance and lack of antimicrobial resistance. Thus, complicated regimens or those associated with side effects or both may result in non-compliance and failure

to eradicate the organism (Lesbros-Pantoflickova et al., 2007; Ndip et al., 2008). Theoretically, therapies have improved such that regimens should demonstrate 85 to 95% efficacy. Single-agent therapy should not be used because of the unacceptably low eradication rates. Clinical trials have shown that multidrug regimens (triple therapy) are the most effective treatment with eradication rates of up to 96% (Lamouliatte et al., 1996; Salcedo and Al-Kawas, 1998; Kwon et al., 2001; Aboderin et al., 2007). Despite these high eradication rates, the problem of drug resistance is still well established. The regimen of choice is a PPI or ranitidine bismuth citrate (Tritec) plus two antibiotics for 14 days (Megraud and Lehours, 2007; Ndip et al., 2008). Mtz, clarithromycin, amoxicillin and tetracycline are the major antibiotics prescribed for *H. pylori* eradication (Goddard et al., 1996; Kobayashi et al., 2001; Megraud, 2004). These agents have superior efficacy to and are better tolerated than the standard bismuth based triple therapy consisting of bismuth subsalicylate, metronidazole (flagyl) and tetracycline hydrochloride (Graham et al., 1991; Meurer and Bower, 2002).

METRONIDAZOLE

Mtz is a nitroimidazole used principally for the treatment of anaerobic and parasitic infections. Mtz is stable at a low pH and is actively secreted into the gastric juice (Edwards, 1993). Active secretion of Mtz is reduced when it is given with a proton pump inhibitor (Goddard et al., 1996) and has been reported to have a half-life of 8 to 12 h. The most common adverse effects of Mtz are a metallic taste in the oral cavity, nausea and epigastric discomfort. Mtz has been reported to produce a disulfiram like reaction when taken in combination with alcohol. In the past two decades, Mtz has become a stronghold in the treatment of *H. pylori* infection. In Western countries where Mtz use is very minimal, more than 70% of *H. pylori* isolates are sensitive to it (Glupczynski, 1992); however, more recent data has indicated an Mtz resistance of 10-50% of all adult patients infected with *H. pylori* in the developed world (Lopez-Brea et al., 1997; Osato et al., 1999; Adamek et al., 1998). In fact, in developing countries where Mtz use is more common, there have been reports that more than 80% of *H. pylori* isolates are Mtz resistant (Bell et al., 1992; Tanih et al., 2010). *H. pylori* eradication is rarely achieved when it is administered as a single agent. Therefore, Mtz is always given in combination with one or more antibiotics, that is, multiple therapies (Chowdhurger et al., 2002). Mtz resistance may be mediated through an inability of Mtz - resistant strains to remove oxygen from the site of Mtz reduction, thereby preventing Mtz activation. This has been attributed to a mutation on the *frxA* and/or *rdxA* genes resulting in a strain of *H. pylori* with defective nitroreductases coded by these genes (Cederbrant et al., 1992; Kwon et al., 2000a; Paul et al., 2001). Resistance is defined by a cut off value, which depends on the minimum inhibitory concentration (MIC) of the strains and on the concentration of antibiotic that can be achieved in the tissue by a given therapeutic dose (Megraud and Doermann, 1998; Thyagarajan et al., 2003). Although the Clinical Laboratory Standard Institute (CLSI) has not designated a MIC breakpoint for Mtz, values of ≥ 8 µg/ml have been proposed (Vasques et al., 1996; Osato, 2000; CLSI, 2007). In a recent study in the Eastern Cape Province of South Africa, we reported a MIC value of > 10 µg/ml for Mtz (Tanih et al., 2010).

TRENDS IN METRONIDAZOLE RESISTANCE

A systematic review of the data on antibiotic resistance published in the last 5 years highlighted regional differences in resistance pattern for Mtz and clarithromycin (Megraud, 2004). Mtz containing regimens have been shown to limit effectiveness because of increasing prevalence of resistance to this drug (Ching et al., 1996; Wang et al., 2000; Al-Quarashi et al., 2001). In a study in Egypt, a universal high-level primary Mtz resistance in children, compared to lower resistances to other selected antibiotic was reported by Sherif et al. (2004), which is consistent with other reports in Africa (Wang et al., 2000; Al-Quarashi et al., 2001; Ndip et al., 2008; Tanih et al., 2009). Very high resistances to Mtz and amoxicillin have been reported in Nigerian, Kenyan and Cameroonian patients (Abdulrashed et al., 2005; Lwai-lume et al., 2005; Aboderin et al., 2007; Ndip et al., 2008). Eradication failures in children are mostly due to non compliance because of adverse effects or due to resistance to Mtz and clarithromycin. In Cameroon, Ndip et al. (2008) revealed a very high antimicrobial resistance rate of 93.2% for Mtz. A similar study in Western Nigeria documented 100% resistance of *H. pylori* strains to Mtz (Smith et al., 2001). Equally, in a study conducted in Ethiopia, Asrat et al. (2004) found 76% of their strains resistant to Mtz.

Mtz containing regimens have been shown to limit effectiveness because of increasing prevalence of resistance to this drug (Wang et al., 2000; Al-Quarashi et al., 2001; Abdul et al., 2001). Patients originating from Africa have been reported to harbour resistant strains significantly more than those from other parts of the world (Megraud, 2004). Resistance rates as high as 75 - 98% have been reported in some areas of South Africa (Wong et al., 2000; Tanih et al., 2010) and up to 100% in Ethiopia. The problem of Mtz resistance was thought to be ameliorated when the drug was used in combination with clarithromycin (Hardin and Wright, 2002). However, high resistance to clarithromycin has been reported in patients originating from Africa (Loffeld et al. 2003). Banatvala et al. (1994) in their study documented that women born in the United Kingdom, were more likely to harbour Mtz-resistant *H. pylori* strains than men (54 v 18% respectively) and more likely to have a history of previous nitroimidazole ingestion (41 v 11% respectively);

and patients previously exposed to either Mtz or tinidazole were more likely to harbour resistant strains (84 v 41%).

Mtz consumption appears to be an important risk factor for this resistance. A marked difference has been found between the rate of resistance to nitroimidazoles in developed and developing countries. This difference may be linked to the high level of general use of the drug in developing countries to treat parasitic infections such as amoebiasis (Alarcon et al., 1999) because it is inexpensive and also commonly sold in the street corners heralding untold resistances to it. The cause of this resistance may also be linked to the use of these compounds for genital infections, especially trichomonasis and therefore, strains isolated from women are more likely to be resistant than strains isolated from men (Banatvala et al., 1994; Alarcon et al., 1999; Cameron et al., 2004; Ndip et al., 2008). Another possible cause may be the use of these compounds to treat dental infections (Megraud, 1997).

Studies of *H. pylori* antibiotic resistance in South Africa are lacking. This could be a serious problem owing to the fact that susceptibility patterns are changing globally, and rapidly too. As a result, eradication failures will be frequent as some of the drugs given to the patients may fail to produce the desired effects and yet left on the shelf. However, we reported an Mtz-resistance of 95.5% in a recent study we conducted in South Africa (Tanih et al., 2010) which seems to corroborate earlier reports made in other African countries, thus, confirming high Mtz-resistance in the developing world.

THE ROLE OF *RdxA* NITROREDUCTASE IN METRONIDAZOLE RESISTANCE

The elemental discovery two decades ago by Goodwin et al. (1998) that *H. pylori* Mtz resistance may be a result of loss of activity of an oxygen independent NADPH nitroreductase encoded by the gene *rdxA* marked the establishment of a renewed and intense interest in gaining a full understanding of the causes of resistance to this drug. Mutational inactivation of *rdxA* was initially proposed as the cause of naturally acquired Mtz resistance in *H. pylori* (Goodwin et al., 1998), and various features of *rdxA*, such as the polypeptide it encodes, were suggested as the molecular bases for a low redox potential capable of reducing Mtz (Kwon et al., 2000b). However, the results of further studies to verify this hypothesis revealed other enzyme activities with biological functions different from nitroreductases that appear to modulate Mtz reduction and were thus proposed as potential candidates to cause resistance to this drug in *H. pylori* (Jeong et al., 2001). As a follow up to these data, successive modifications were proposed to the initial hypothesis, with the result that the association of *rdxA* in susceptibility to Mtz is accepted currently, but its specific role is unclear to many researchers (Kwon et al., 2001). The principal role on the involvement of *rdxA* nitroreductase in the Mtz sensitive (Mtz^S) phenotype of *H. pylori* and the specification of *rdxA* inactivation as the necessary and sufficient cause of Mtz resistance in this bacterium, were proposed by Goodwin et al. (1998).

Goodwin et al. (1998) demonstrated that intrinsically resistant *E. coli* transformed with functional *H. pylori rdxA* became sensitive to Mtz, and assays of the reduction of the drug by one of the clones demonstrated the functionality of the protein expressed in *E. coli*. The study showed that introduction of *rdxA* into Mtz resistant (Mtz^R) *H. pylori* rendered them Mtz^S, that isogenic *rdxA* knockout *H. pylori* mutants of an Mtz^S parent became Mtz^R and that *rdxA* genes originating from matched pairs of Mtz^S and Mtz^R strains differed from one another by several base substitutions (Goodwin et al., 1998). It was also demonstrated that in *rdxA* alleles that showed extensive deletions, insertions of transposable elements (Debets-Ossenkopp, 1999; Tankovic et al., 2000; Kwon et al., 2000b) or mutations leading to stop codons which could result in premature translation, termination and truncated polypeptides (Jenks et al., 1999; Tankovic et al., 2000), would lead to a situation whereby the corresponding *rdxA* will not be expressed or functional. Several studies have also demonstrated the large heterogeneity of point mutations present in the *rdxA* genes of resistant strains, with no particular nucleotide mutation or amino acid substitution that could be linked to Mtz resistance with the exception of mutations generating stop signals (Goodwin et al., 1998; Debets-Ossenkopp, 1999; Jenks et al., 1999; Tankovic et al., 2000; Kwon et al., 2000a; Solca et al., 2000; Jeong et al., 2000; Kwon et al., 2001; Paul et al., 2001; Jorgensen et al., 2001). Other studies have also revealed the existence of unchanged *rdxA* in sensitive and resistant isolates and of different *rdxA* in Mtz^S strains (Jenks et al., 1999; Tankovic et al., 2000). In contrast with resistance mechanisms for other antibiotics, the resistance mechanism of Mtz is complex (Mendz and Megraud, 2002; Chisholm and Owen, 2003). Clearly, alterations of the *rdxA* gene are of prime importance but it has not been possible to identify a clear panel of point mutations which could explain the phenomenon. Moreover, research data still question the unequivocal linkage between *rdxA* mutations and the resistant phenotype. Additionally, other genes such as *frxA* also seem to be involved (Marais et al., 2003).

THE ROLE OF *FrxA* NITROREDUCTASE IN METRONIDAZOLE RESISTANCE

Studies of the role in resistance of other enzymes potentially capable of reducing Mtz have yielded mixed results (Hoffman et al., 1996; Kaihovaara et al., 1998; Kwon et al., 2000b). The discovery of frameshift mutations in the gene *frxA,* encoding an NAD (P) H: flavin oxidoreductase, suggested the potential role of frxA in Mtz resistance in

H. pylori (Kwon et al., 2000b).

In a study by Kwon et al. (2000b), confirmation was obtained by transforming Mtz^S strains with the mutated *frxA,* and by inactivation of this gene in sensitive strains. Furthermore, an *E. coli* resistant strain transformed with the *frxA* gene of an *H. pylori* Mtz^S strain was capable of reducing Mtz, and was rendered sensitive to the drug. Several studies have demonstrated that inactivation of *rdxA* or *frxA* led to increase in the MIC for Mtz and simultaneous inactivation of both genes led to higher MIC than the inactivation of either gene (Kwon et al., 2000a), thus providing a possible explanation for the heterogeneity of resistance observed in the wide range of MIC for Mtz measured in *H. pylori* strains. These findings have motivated several studies designed to verify the role of *frxA* in Mtz resistance and a synergy of the interpretations of the data obtained for *frxA* and *rdxA,* revisiting the methods used in the studies of *rdxA* (Jeong and Berg, 2000; Kwon et al., 2001; Jeong et al., 2000, 2001). Interestingly, there is general agreement that both *rdxA* and *frxA* have roles in Mtz resistance, but there are some differences in two important points:

The roles of *frxA* and *rdxA* in Mtz resistance, and their relative contributions to the MIC for Mtz. In one analysis, deficiencies in either *rdxA*, *frxA* or both explained the resistant phenotype, even though with mutations in *frxA* alone yielding low-level Mtz resistance in clinical isolates, and moderate-to-high-level Mtz resistance in laboratory mutants with a disrupted gene (Jeong et al., 2000).

In contrary to this, it has been observed that *H. pylori* strains become resistant to Mtz in two ways: by inactivation of *rdxA* (type I) or by inactivation of both *rdxA* and *frxA* (type II), and rarely, if ever by inactivation of *frxA* alone, disruption of *rdxA* alone can produce Mtz resistance at all levels, and mutations in *frxA* can enhance the level of resistance of type I strains (Jeong et al., 2000; Jeong and Berg, 2000; Jeong et al., 2001). In spite of the common argument shared by both views, there are significant differences in the data and their interpretations; it has not been possible to identify a clear panel of mutations which could explain the phenomenon (Chisholm and Owen, 2003).

CONCLUSION

Infection by Mtz or amoxicillin resistant strains is an important factor leading to treatment failure; subjecting all *H. pylori* clinical isolates to susceptibility testing most especially to Mtz is recommended (Megraud, 2004). If not possible, a program to survey the prevalence of resistance should be implemented in a given area or population. This increasing emergence of antimicrobial resistance in *H. pylori* treatment posses serious public health problems and is therefore necessary that new drug regimens be examined. Studies are currently underway in our group to study the role of Mtz in the treatment of *H. pylori* in our environment (Tanih et al., 2010). We have already determined the prevalence of Mtz resistance and successfully amplified the two putative resistance genes (*rfxA* and *frxA*) implicated in Mtz resistance. Sequencing of these genes, which is our next line of experiments, is expected to add to existing global knowledge the types of mutations involved and consequently the roles of these genes in Mtz resistance in *H. pylori* strains circulating in the Eastern Cape Province of South Africa.

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Full Length Research Paper

Functional analysis of a gene encoding threonine synthase from rice

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Threonine synthase (TS) is a pyridoxal phosphate dependent enzyme that catalyzes the formation of threonine (Thr) through *O*-phosphohomoserine (OPH) from the aspartate family pathway in plants. The properties of the TS enzyme have been evaluated in many bacteria and few plants. Sequence analysis of the cDNA from rice revealed that it harbors a full-length open reading frame for *OsTS* encoding for 521 amino acids, corresponding to a protein of approximately 57.2 kD. The predicted amino acid sequence of OsTS is highly homologous to that of *Arabidopsis* TS and many bacterial TS encoded by *thrC* gene. The OsTS protein harbors a signature binding motif for pyridoxal- 5' -phosphate at the amino terminus. A *thrC* mutant strain of *Escherichia coli* was complemented by *OsTS* expression. *OsTS* expression was correlated with the survival of the *thrC* mutant, which is affected by the supplementation of an aspartate pathway metabolite, methionine.

Key words: *Oryza sativa*, methionine, threonine, threonine synthase.

INTRODUCTION

Threonine (Thr) is an essential amino acid in animals, including humans. The biosynthetic pathway of Thr is initiated from aspartate (Asp) and is called the Asp family pathway in plants. The aspartate-derived amino-acid pathway from plants is well suited for analyzing the function of the allosteric network of interactions in branched pathways (Curien et al., 2009).

Thr is synthesized via a branched pathway that includes lysine (Lys) and methionine (Met) (Azevedo et al., 1997; Bryan, 1980). The carbon skeleton of Thr is derived from Asp as the amino acids Lys and Met. The common precursor for the synthesis of Thr and Met in the branching point is *O*-phosphohomoserine (OPH) (Figure 1). In plants and microorganisms, Thr synthesis is a component of the multibranched biosynthetic pathway originnating with Asp and resulting in the synthesis of Lys, Met, Thr and isoleusine (Curien et al., 1996). Threonine synthase (TS: EC 4.2.99.2) is a fold type II pyridoxal 5' -phosphate (PLP)-dependent enzyme and catalyses the final step of Thr formation (Curien et al., 2008; Mas-Droux et al., 2006). TS is involved in the essential amino acids pathway derived from aspartate and catalyzes the conversion of *O*-phosphohomoserine (OPH) into Thr and inorganic phosphate via a PLP dependent reaction (Mas-Droux et al., 2006; Casazza et al., 2000). The TS activity has been identified, purified and described in a variety of microorganisms, such as *Neurospora crassa* (Flavin and Slaughter, 1960), *Escherichia coli* (Farrington et al., 1993; Parsot et al., 1983), *Corynebacterium glutamicum* (Eikmanns et al., 1993), *Crytococcus neoformans* (Kingsbury and McCusker, 2008), *Streptococcus* sp. (Tang et al., 2007) and *Mycobacterium tuberculosis* (Covarrubias et al., 2008). The corresponding gene was isolated from a number of bacteria (Parsot, 1986; Han et al., 1990; Motoyama et al., 1994; Omori et al., 1993; Clepet et al., 1992). The characterization and analysis of several plant genes have been reported, including those of *Arabidopsis thaliana* (Curien et al., 1998; Avraham and

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Abbreviations: TS, Threonine synthase; ***OsTS*, *Oryza sativa*** threonine synthase gene; **Thr,** threonine; **Met,** methionine; **Lys,** lysine; **OPH,** O-phosphohomoserine; **cDNA,** complementary deoxyribonucleic acid; **SAM,** S-adenosylmethionine; **CGS,** cystathionine-γ-synthase; **EST,** expressed sequence tag; **PCR,** polymerase chain reaction; **ORF,** open reading frame; **IPTG,** isopropyl β-D-thiogalactopyranoside; **BLAST,** basic local alignment search tool; **EMBL,** European molecular biology laboratory.

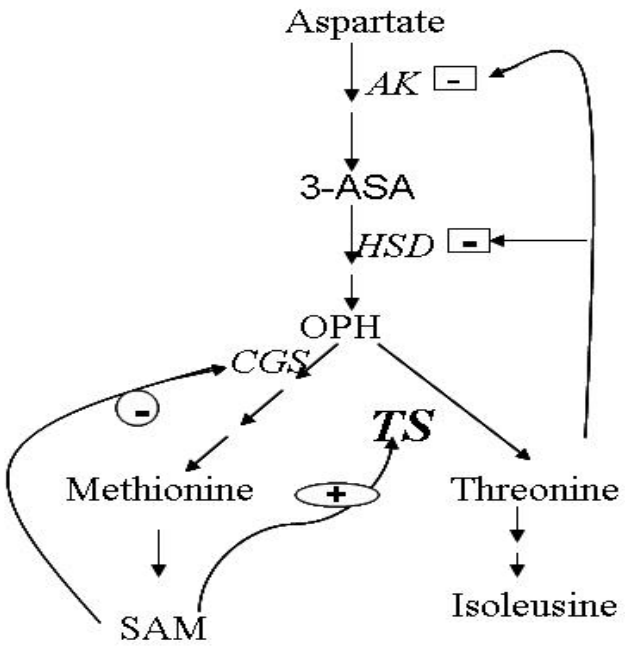

Figure 1. Scheme of the threonine biosynthesis pathway of aspartate family in plants. The abbreviations are AK, aspartate kinase; 3-ASA, 3-aspartic semialdyde; HSD, homoserine dehydrogenase; OPH, *O*-phosphohomoserine; SAM, *S*-adennosylmethionine, CGS, cystathionine g-synthase; TS, threonine synthase; TDH, threonine dehydratase. Symbols are indicated: ; allosteric activation, ; feedback repression and ; feedback inhibition.

Amir, 2005, Lee et al., 2005; Laber et al., 1999), *Solanum tuberosum* L (Casazza et al., 2000) and *Sorghum bicolor* (Ferreira et al., 2006).

The synthesis of aspartate-derived amino acids is subject to complex regulation. The key to pathway control is feedback inhibition of aspartate kinase by Lys and/or Thr, or by Lys in concert with S-adenosylmethionine (SAM) (Rinder et al., 2008). Aspartate kinase, the first enzyme in the pathway, is inhibited allosterically by Lys and Thr (Lee et al., 2005). TS compete with the first enzyme required for subsequent Met biosynthesis, cystathionine-γ-synthase (CGS), for their common substrate OPH (Thompson et al., 1982). TS enzyme activity is activated by S-adenosyl-methionine (SAM) and inhibited by cysteine (Madison and Thompson, 1976; Giovanelli et al., 1984; Curien et al., 1996). SAM is, in turn, directly synthesized from Met; therefore, increasing Met levels will result in increases in the concentration of SAM and subsequently affect TS activity (Casazza et al., 2000). *In vitro* studies have showed that SAM stimulates TS activity in an allosteric manner (Curien et al., 1998). A number of studies have also documented a dynamic interaction between CGS and TS in the control of Met biosynthesis (Amir et al., 2002; Hesse and Hoefgen, 2003). Over expression of CGS resulted in elevated free Met levels, but did not significantly affect Thr levels (Chiba et al., 2003; Inaba et al., 1994; Kim et al., 2002). Here, we report the analysis and characterization of a gene for the TS enzyme from rice (*Oryza sativa*), an important crop plant and the influence on its activities by an Asp pathway metabolite, probably SAM.

METHODS AND MATERIALS

Strains and plasmids

Two *E. coli* strains were used in this study, Gif41 [*thrC1001, λ14-, e14-, relA1, spoT1, th-1 thi-1*] (Theze et al., 1974) and Sφ415 [*Udk-2*, *upp-11*, *rclA1*, *rpsL254* (*strR*), *metB1*] (Hammer-Jespersen and Munch-Petersen, 1973). The source of both strains was the *E. coli* Genetic stock Center (CGSC) at Yale University, USA. An EST clone (Genbank Accession Number AK101669 and clone name J033058D04) was used in this study.

DNA sequence analysis

An EST clone (GenBank Accession No. AK101669 and clone name J033058D04) used in this study was obtained from the Rice Genome Resource Center (RGRC), National Institute of Agrobiological Science (NIAS), Japan. The clone was derived from a rice cDNA library (Osato et al., 2002) from developing seeds prepared in pBluescript SK-. DNA sequencing was conducted using an automatic sequencer (A1Fexpress DNA sequencer, Pharmacia Biotech. Inc., UK) with synthetic oligonucleotide primers. Nucleotide sequences and amino acid sequences were compared with the sequences in the GenBank and EMBL databases and analyzed via BLAST (Wheeler et al., 2003) and the Clustal W multiple sequence alignment program (Thompson et al., 1994) or Biology WorkBench 3.2 (http://workbench.sdsc.edu; San Diego Supercomputer Center; University of California San Diego, USA). Sequence comparisons were conducted at the nucleotide and amino acid levels. Motifs were searched by the GenomeNet Computation Service at Kyoto University (http://www.genome.ad.jp) and phylogenic tree with bootstrap value prepared by the Mega 4.1 program (Kumer et al., 2008).

Polymerase chain reaction (PCR) and recombinant constructs

Our sequence analysis showed the presence of an ATG start codon located in-frame at -99 positions upstream from the translation-starting site. Therefore, the specific primers were designed from the sequence information around the translational start and stop codons of OsTS to amplify the full-length open reading frame (ORF) and to over express the gene product in *E. coli*. Polymerase chain reaction (PCR) (Sambrook and Russell, 2001) was conducted to amplify the full-length ORF. After the EST was purified from a pellet harvested from a liquid culture containing ampicillin (Amp), the ORF of OsTS was amplified from the EST clone as a template and the following primers were designed from the *OsTS* sequence: OsTS-F (5'- AAA GCT TTC ACT CAC TCC CTA AAA CCC-3') and *OsTS*-R (5' AAA GCT TCA CAC TTC AGA GCT TAC CCT -3') using Ampli TaqGold polymerase (Perkin-Elmer, U.S.A). The underlined bases in the OsTS-F and OsTS-R primers are the designed restriction sites for *Hin*dIII to facilitate subcloning, respectively. The polymerase chain reaction was conducted using a MYCyler™ PCR system (BioRad, U.S.A) for 35 cycles with 95°C for 1 min, 55°C for 1 min and 72°C for 2 min, with 10 µM primers. The PCR products were analyzed on 1% (w/v) agarose gel. The amplified fragment (1.5 kb) was then subcloned into pGEM-T-easy vector (Promega) and finally subcloned into pBluescript II KS+ (Stratagene Inc.,

U.S.A) as a *Hin*dIII fragment, to give *pB::OsTS*. Restriction analysis was conducted in an effort to confirm the recombinant DNA construct of *pB::OsTS* with the right orientation for over expression.

Functional complementation and growth assay

The competent *thrC* mutant of *E. coli* strain, Gif41, was transformed with *pB::OsTS* via electroporation (ECM399, BTX, USA) using a cuvette with a 0.1 cm electrode gap, then plated on LB medium (20 g/l) with Amp (100 µg/ml). The growing culture was tested for growth retardation in M9 minimal medium containing Amp (25 µg/ml), 20% glucose, 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) and 19 amino acids (Sigma, Germany) each at a concentration of 25 µg/ml, excluding Thr. Bacterial growth was then assessed by measuring optical density at 595 nm at one-hour interval. After 12 h, the diluted culture was plated and incubated overnight at 37°C.

Growth inhibition assay of OsTS in *E. coli*

The *E. coli* mutants harboring the *pB::OsTS* construct, control plasmid and wild-type with control plasmid were grown at 37°C in M9 minimal medium (5 x M9 salts (200 ml/l), 1 M $MgSO_4$ (2 ml/l), 1 M $CaCl_2$, 0.1 ml/l IPTG, 20% glucose (20 ml/l), containing 19 amino acids and Amp (25 µg/ml), excluding Thr and the same medium was used with all the reagents kept constant, but an additional supplementation of 10-fold high Met. The bacterial growth was monitored via optical density measurements every hour using a spectrophotometer (UV1101, Biochrom, England) at 595 nm (OD_{595}).

RESULTS AND DISCUSSION

Sequence analysis of *OsTS*

An expressed sequence tag (EST) clone (GenBank Accession number AK101669, clone name J033058D04 and clone ID 212512) obtained from the Rice Genome Resource Center (RGRC) was analyzed to determine the nucleotide sequence using the designed primers. The cDNA (*OsTS)* sequence harbored a full-length open reading frame consisting of 1563bp, encoding for a protein of approximately 57.2 kDa. The expected isoelectric point of the protein was 6.60. Data analysis revealed that the *OsTS* sequence was identical to the genomic region located in chromosome V. Comparisons of the amino acid sequence of the *OsTS* and the homologous sequences from maize (*Zea mays*) and Arabidopsis (*A. thaliana*) revealed high identity, at 91 and 71%, respectively (Figure 2).

Analysis of the *OsTS* amino acid sequence revealed a signature binding motif for PLP in the N-terminal region (189-203) (Figure 2). The motif sequence (HCGISHTGSF KDLGM) was highly homologous to the consensus [DESH]-x(4,5)-[STVG]-{EVKD}-[AS]-[FYI]-K-[DLIFSA]-[RLVMF]-[GA]-[LIVMGA], where the underlined amino acids were well conserved. The binding motif for PLP is present in bacterial TSs and Serine/threonine dehydratases that utilize PLP as a cofactor. The exact PLP binding site seemed to be K-199 and was identified via comparison with the binding site of bacterial TS. This result indicates that the OsTS product utilizes PLP as a co-factor. Phylogenic analysis of the related sequence further indicated that OsTS is grouped with several plant sequences and is divergent and evolved from ancestor bacterial TS (Figure 3).

OsTS expression in *E. coli*

The recombinant DNA, *pB::OsTS*, was constructed using the ORF of a PCR-amplified *OsTS* fragment. After the transformation of *E. coli* with the recombinant DNA, *OsTS* activity was monitored *in vivo* in a medium containing IPTG and 19 amino acids, excluding Thr. Functional complementation was performed using the *TS* mutant of *E. coli* to confirm the enzyme activity of the gene product of *OsTS*. To assess the viability of *E. coli* cells by OsTS activity, the *OsTS*-expressing cells were cultured for 12 h with shaking and the diluted portion was plated on agar medium containing the 19 amino acids and Amp (25 µg/ml) without Thr (Figure 4). The *thrC* mutant of *E. coli* with the *OsTS* construct could grow under the conditions above in which the mutant without *OsTS* could not. The result revealed that the OsTS is able to complement with functional TS activity.

Expression of *OsTS* can complement the *thrC* mutant of *E. coli*

A growth study was performed to determine whether the *OsTS* gene would increase the sensitivity of bacterial cells to Thr. The *pB::OsTS* construct was transformed into the *thrC* mutant *E. coli* Gif41. A control plasmid was also transformed into wild-type (Sφ415) and the *thrC* mutant Gif41. The *pB::OsTS* activity was monitored via a growth assay in the absence of Thr. Bacterial cells were grown in M9 minimal medium with IPTG and Amp and 19 amino acids excluding Thr. The wild-type *E. coli* strain Sφ415 harboring the control plasmid grew normally and evidenced an S-shaped classical growth curve in the medium without Thr (Figure 5A). The Sφ415 strain could synthesize Thr itself and thus grew normally in the medium. The *thrC* mutant strain Gif41 expressing *pB::OsTS* also grew normally and evidenced an S-shaped classical growth curve in the same medium, but grew slightly more slowly than the wild-type strain containing the control plasmid (Figure 5A), although the Gif41strain harboring the control plasmid in the same medium without Thr evidenced dramatically retarded growth. In this case, the *thrC* mutant *E. coli* strain Gif41 could not synthesize Thr itself and thus grew dramatically less rapidly; however, the same *E. coli* strain Gif41 containing *pB::OsTS* grew well because the *thrC* mutant *E. coli* was able to synthesize Thr using TS expressed by the *pB::OsTS* plasmid (Figure 5A). This is a consequence of *pB::OsTS* activity.

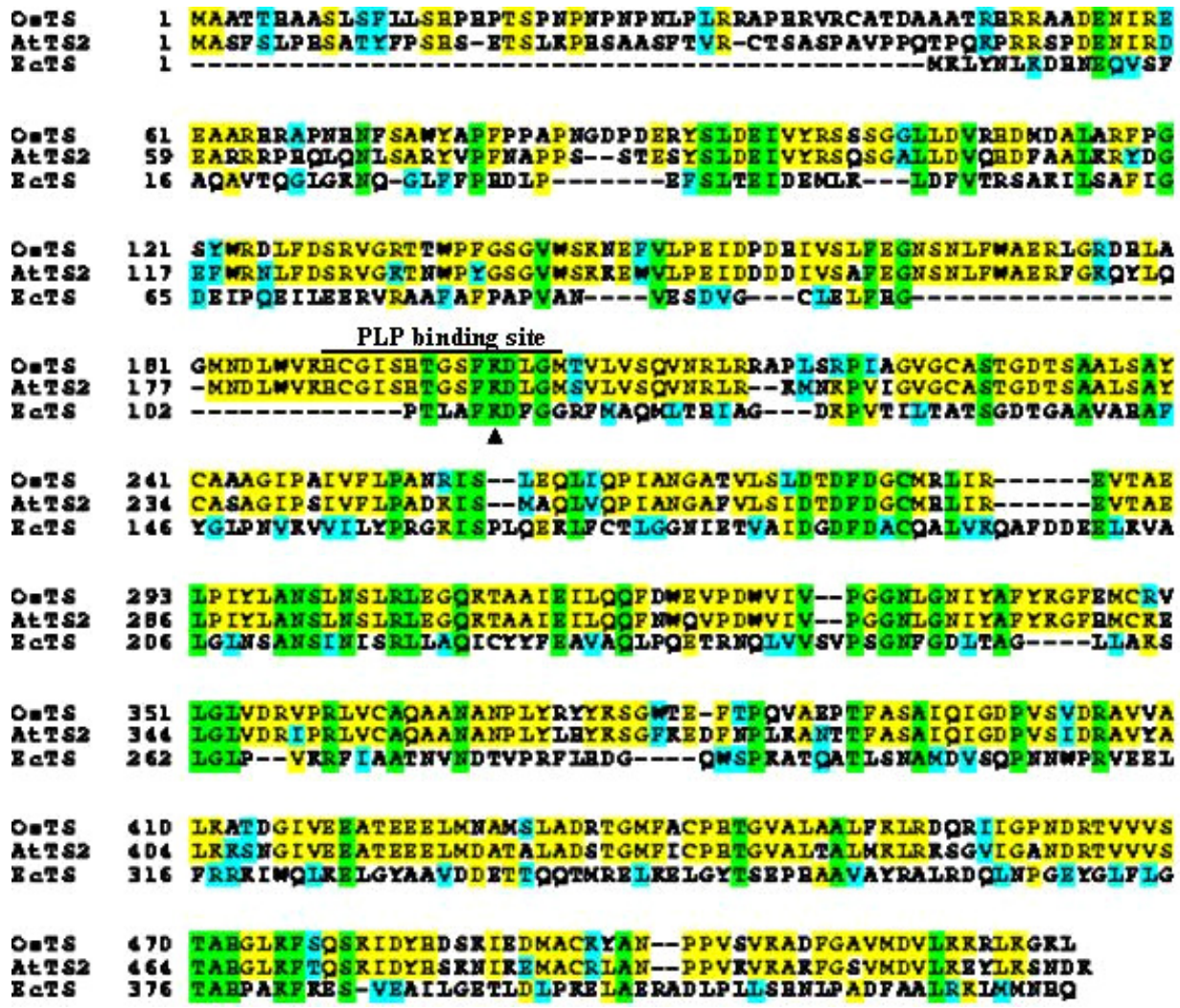

Figure 2. Amino acid sequence alignment of TS from *Oryza sativa* (OsTS), *Arabidopsis thaliana* (AtTS2) and *Escherichia coli* (EcTS). Shaded residues represent amino acids that are identical among at least three of the three amino acids. GenBank Accession Numbers; AK101669 (OsTS), Q9SSP5 (AtTS2) and NP_414545 (EcTS).

From the above finding, it was concluded that *OsTS* expression can functionally complement the *thrC* mutant *E. coli*.

The growth of the *thrC* mutant of *E. coli* was influenced by the expression of *OsTS* in high levels of methionine

The growth pattern of the *thrC* mutant of *E. coli* complemented with *pB::OsTS* was also assessed in the presence of high Met levels. The wild-type *E. coli* strain Sφ415 harboring the control plasmid grew normally and evidenced an S-shaped classical growth curve in M9 minimal medium with 19 amino acids (excluding Thr, containing 1 mM IPTG and supplemented with additional 10-fold high Met). The *E. coli* strain Gif41 grew and evidenced an S-shaped classical growth curve in the same medium, but the growth pattern was much more vigorous than in the medium without Met (Figure 5B). In this case, when a high level of Met was added, the Met was converted to SAM and the SAM allosterically activated TS activity--this is why the *thrC* mutant of *E. coli* grew so vigorously. This result is consistent with previously reported results in studies of bacteria and plants (Giovanelli et al., 1984; Curien et al., 1996, Casazza et al., 2000 and Ferreira et al., 2006). The principal feature of plant TS, in contrast to its bacterial counterpart, may be allosteric regulation by SAM, which induces a dramatic stimulation of TS activity (Hesse et al., 2004). However, the Gif41 strain harboring the control plasmid also evidenced dramatically retarded growth in the same medium owing to a lack of Thr, even when 10-fold high Met was added (Figure 5B). This finding indicates that Met has a marked influence on *OsTS* activity in rice plants.

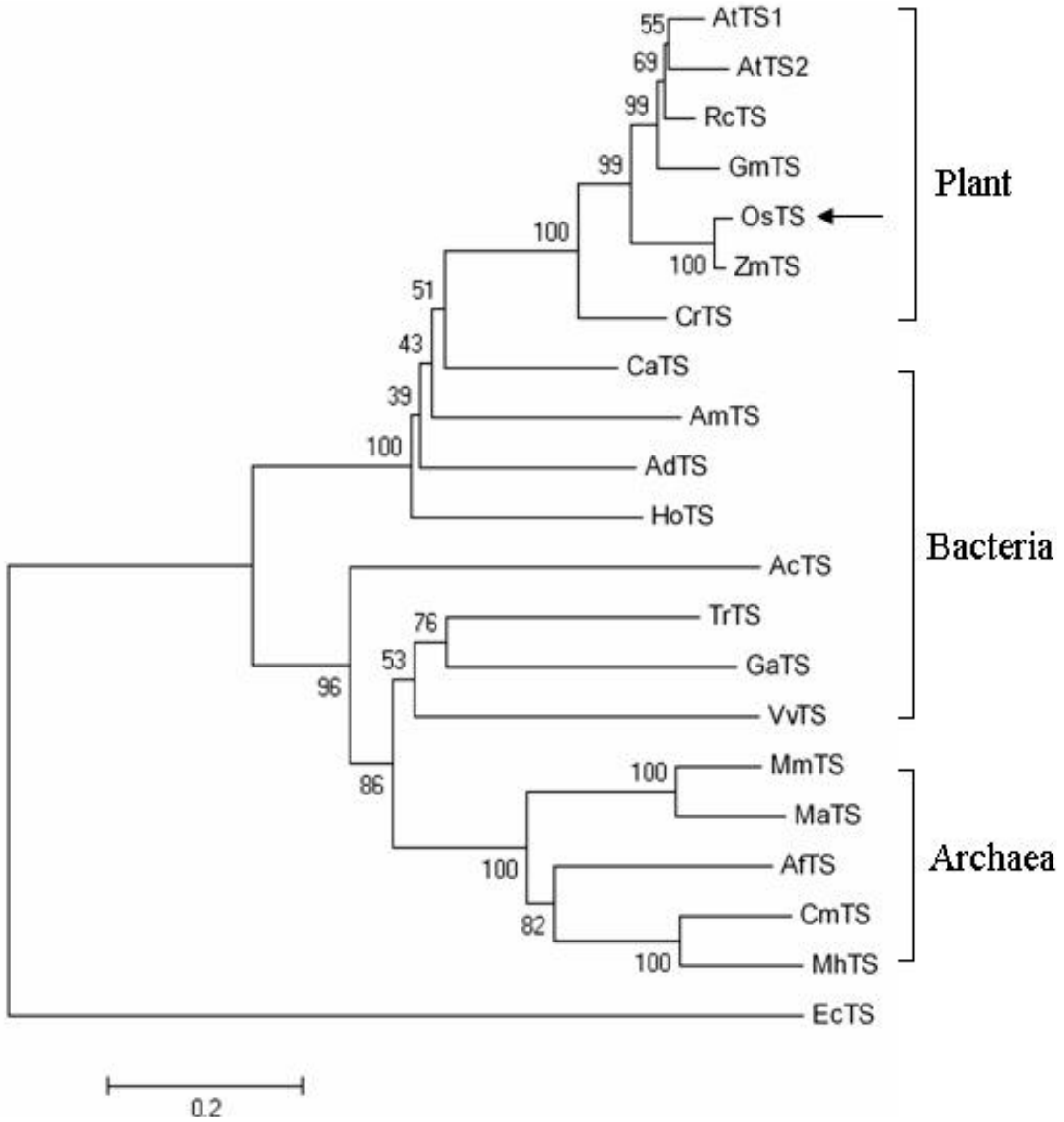

Figure 3. Phylogenetic tree: Phylogenetic analysis of OsTS related proteins using Clustal W and Mega 4.1 program. GenBank accession numbers are as follows: AK101669 (OsTS from *Oryza sativa*), XP_002514088 (RcTS; *Ricinus communis*), Q9S7B5 (AtTS1; *Arabidopsis thaliana*), Q9SSP5 (AtTS2; *A. thaliana*), ABC00741(GmTS; *Glycine max*), ACG39080 (ZmTS; *Zea mays*), XP_001698517 (CrTS; *Chlamydomonas reinhardtii*), YP_001515596 (AmTS; *Acaryochloris marina*), YP_002463167(CaTS; *Chloroflexus aurantiacus*), YP_003264969 (HoTS; *Haliangium ochraceum*), YP_002492618 (AdTS; *Anaeromyxobacter dehalogenans*), YP_002753372 (AcTS; *Acidobacterium capsulatum*), YP_002522459 (TrTS: *Thermomicrobium roseum*), YP_002760880 (GaTS; *Gemmatimonas aurantiaca*), ZP_01923848 (VvTS; *Victivallis vadensis*), YP_001330351 (MmTS; *Methanococcus maripaludis*), NP_070145 (AfTS; *Archaeoglobus fulgidus*), YP_002466596 (CmTS; *Candidatus Methanosphaerula*), YP_503069 (MhTS; *Methanospirillum hungatei*) and NP_414545 (EcTS; *Escherichia coli*).

Attempts are currently underway to obtain some important information about the substrate specificity of the enzyme by purifying recombinant *OsTS* in *E. coli and* to assess the physiological functions of this novel enzyme for Thr metabolism by screening T-DNA insertion mutants in which the *OsTS* gene is knocked out in rice. Our reports regarding the cloning and characterization of the cDNA encoding for TS from rice have generated bioinformatic predictions, as well as motifs and complementation, in a *thrC* mutant of *E. coli*. These results may constitute a starting point for investigations at the molecular level to investigate Thr biosynthesis in rice, which might eventually be applied to modify the nutritional compositions of crop plants.

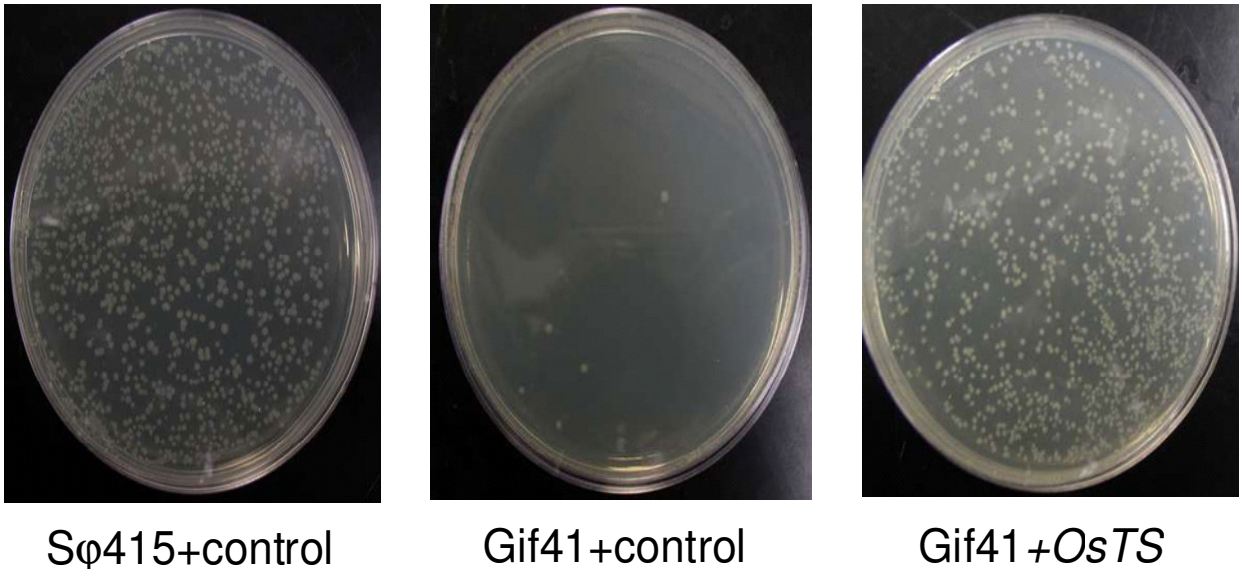

Figure 4. Functional complementation assay. The *thrC* mutant strain of *E. coli* Gif41 containing *pB::OsTS* and pBluescript II KS+ and wild-type *E. coli* Sφ415 containing pBluescript II KS+ as a control plasmid.

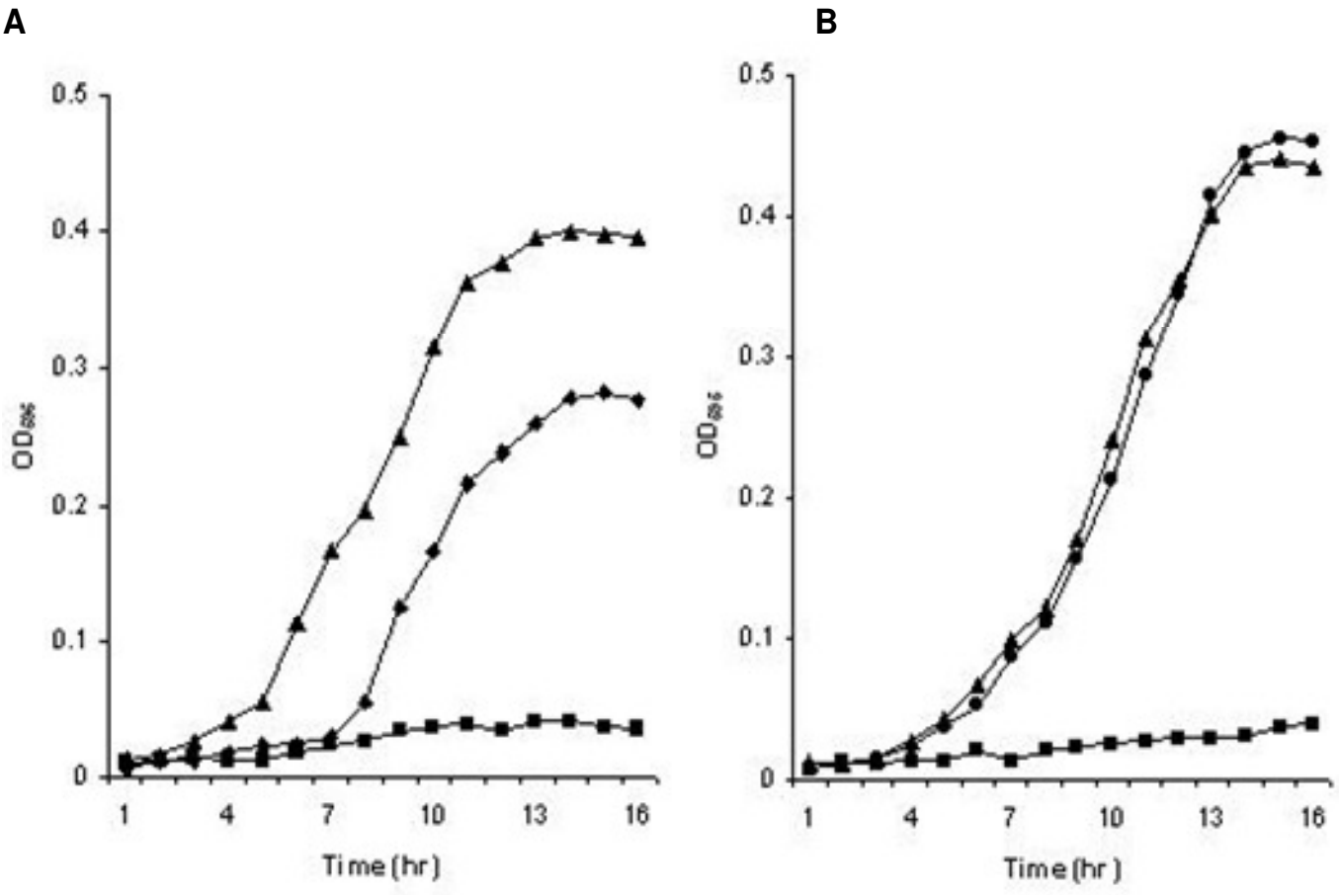

Figure 5. Growth curves of *E. coli* mutant Gif41 and Sφ415 harboring *OsTS* without Met (A) and supplementing Met (B). Bacterial cells were grown at 37°C in M9 minimal medium containing 19 amino acids except Thr (A) or the same medium supplemented with an additional 10 times high Met. Growth was monitored via optical density measurements at 595 nm (OD_{595}). Symbols: ♦, Gif41+ *pB::OsTS*; ▲, Sφ415+ pBluescript II KS+; ■, Gif41+ pBluescript II KS+.

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Full Length Research Paper

Effect of endophytic *Fusarium oxysporum* on paralysis and mortality of *Pratylenchus goodeyi*

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Three bioassays were conducted to investigate the antagonistic effect of secondary metabolites produced by 5 endophytic *Fusarium oxysporum* isolates from banana (*Musa* spp.) plants in Kenya, against *Pratylenchus goodeyi.* Percentage paralyses were recorded 3, 6 and 24 h after exposure to culture filtrates. Percentage mortality was evaluated after 48 h. All isolates caused significantly higher percentage paralysis (17.5 - 25.9%) and percentage mortality (62.3 - 72.8%) of *P. goodeyi* motile stages compared to the control (8.4 - 10.9% and 17.3 - 34.6%, respectively). Percentage paralysis of motile stages of *P. goodeyi* decreased as the length of time exposure to culture filtrates increased, while mortality increased as length of nematodes exposure to culture filtrates increased. Kenyan isolates performed equally as good as the Ugandan isolate (V5W2) in causing paralysis and mortality. Results from this study demonstrated that endophytic *F. oxysporum* antagonizes *P. goodeyi* through production of secondary metabolites.

Key words: Banana, endophyte, *Fusarium oxysporum*, mortality, paralysis.

INTRODUCTION

Banana (*Musa* spp.) is an important staple food for millions of people in developing countries of the tropics (INIBAP, 1991). In Kenya, it is an important food and cash crop for millions of rural farmers in banana producing areas, grown on an estimated 2% of total arable land predominantly by small-scale farmers (Seshu et al., 1998). Production is mainly characterised by low input application, with no or limited use of pesticides and fertilizers (Swennen and Vulysteke, 2001). Consequently, banana production in Kenya has been on a very rapid decline over the last 2 decades (MOALD, 1994). This sharp decline in yield has been due to the combined effect of pests and diseases, including nematodes, which have reduced average Kenyan banana yields on traditional farms to 14 ton/ha, less than one-third of the crop's potential under humid tropical conditions (Karamura, 1998).Bananas in East Africa are attacked by a complex of nematodes which include burrowing nematodes (*Radopholus similis* (Cobb) Thorne), the root lesion nematode (*Pratylenchus goodeyi* (Sher and Allen)) and the spiral nematode (*Helicotylenchus multicinctus* (Cobb) Golden) (Gowen and Quénéhervé, 1990a; Karamura, 1993). The lesion nematode *P. goodeyi* (Sher and Allen) has been observed in many banana growing region in Kenya and can cause yield losses of over 85% (Bridge et al., 1997; Gichure and Ondieki, 1977; Gowen and Quénéhervé, 1990a; Gowen and Quénéhervé, 1990b). Damages caused by *P. goodeyi* lead to stunting, lengthened vegetative cycles, a reduction in number of leaves and bunch weight and eventual toppling over, especially in soils with low nutritional content (Bridge et al., 1997). The use of contaminated planting material to establish new fields is the main source of *P. goodeyi* infestation in banana fields (Sarah, 1989). Due to high cost of nematicides combined with their related health and environmental concerns, there is need for alternative methods of plant-parasitic nematode management.

The potential of endophytic *Fusarium oxysporum* isolates, naturally occurring inside banana plants, has gained attention as an alternative to nematicides (Athman et al., 2006; Athman, 2006; Dubois et al., 2006; Dubois et al., 2004; Sikora et al., 2003). Endophytes are microorganisms that spend part or all of their life cycle residing

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benignly inside host plant tissues (Wilson, 1995). Many endophytes form mutualistic relationships with their host plants, from which they obtain nutrients and in turn confer protection against biotic and abiotic stresses to the plant (Schulz and Boyle, 2005). When inoculated into otherwise sterile banana tissue-cultured plants, populations of and damage caused by *Radopholus similis* (Cobb) Thorne is reduced (Athman, 2006; Dubois et al., 2004; Paparu et al., 2004; Paparu et al., 2006). Endophytic *F. oxysporum* protects banana plants by induction of systemic resistance (Paparu et al., 2006) and production of secondary metabolites with nematicidal properties, causing *R. similis* mortality and paralysis (Charbonneau, 1997; Dubois et al., 2004). Among several Ugandan isolates tested, V5w2 caused the highest percentage *R. similis* paralysis (100%) and mortality (100%) (Athman, 2006) and is currently being investigated in on-farm experiments (REF). Although extensive research has been carried out on the use of endophytic *F. oxysporum* in management of *R. similis* in bananas, no study has investigated the potential of endophytic fungi against *P. goodeyi*. The objective of this study was to 1) investigate the effect of secondary metabolites produced by 5 endophytic F. oxysporum isolates from banana plants in Kenya, on mortality and paralysis of P. goodeyi and 2) compare it to that of the Ugandan strain V5w2, which is antagonistic to *R. similes.*

MATERIALS AND METHODS

Endophytes

Five endophytic *F. oxysporum* isolates (4M0C321, 2MR23, 5JTOC134 and 5MR11) originating from banana plants in Meru, Kenya (Mwaura et al., 2008) and one endophytic *F. oxysporum* isolate (V5w2), originating from banana plants in xx, Uganda (REF) were included in the study. The isolates had been preserved on filter paper in sterile 2.5 ml Eppendorf tubes at 4°C. For each isolate and under sterile conditions, a filter paper was placed on sterilized (autoclaved at 121ºC for 15 min) synthetic nutrient agar medium (SNA) (1 g KH_2PO_4, 1 g KNO_3, 0.5 g $MgSO_47H_2O$, 0.5 g KCl, 0.2 g glucose, 0.2 g sucrose, 0.6 ml NaOH (1 M), 13.2 g agar/l sterile distilled water (SDW)) in 90 mm diameter petri dishes. Petri dishes were incubated in the laboratory (-25°C with a natural photoperiod of -12:12 L: D h) for 7 days. For each isolate, 3 blocks of SNA (1 cm^3) containing spores and mycelium were aseptically inoculated into 250 ml sterile Erlenmeyer flasks containing 100 ml of sterilized half strength potato dextrose broth medium (PDB) (12 g PDB/l SDW; Sigma-Aldrich, City, Country) and flasks containing inoculum were incubated for 7 days in the laboratory. Filtrates from each isolate were obtained by filtering PDB though cheese cloth into sterile 40 ml plastic bottles and centrifuged at 2,012 g for 15 min. One ml of each filtrate was transferred aseptically into 7 ml Bjorn glass bottles, after which pH was recorded.

Nematodes

Nematode-infested roots were obtained from Cavendish banana fields (Juja, Kenya), transported in a cool box to the laboratory. Roots were washed under running tap water to remove soil, cut into 1 cm long pieces and macerated in using a Kenwood blender (Kenwood, UK) for 15 s. The macerated suspension was collected in a modified Baermann dish and nematodes extracted overnight (Luc et al., 1990). Nematodes that collected at the bottom of the dish were transferred into a 10 ml beaker, concentrated using a 28 µm (aperture) sieve and counted per sex (approximately the composition was, 21 female, 16 male and 12 juveniles) male female juvenile ratio and mention adjusted to 50 mixed stages/ml by either concentrating the suspension or diluting the suspension containing the nematodes.

Bioassays

A total of 3 repeated bioassays were conducted. Bioassays consisted of 6 treatments: 4 treatments comprised the fungal isolates, while 2 treatments were controls (SDW and PDB with pH adjusted to the average pH from all the fungal culture filtrates). Each bioassay was arranged in a complete randomized design and conducted in the laboratory. Within each bioassay, each treatment was repeated 3 times, using 3 bottles. Culture filtrates were inoculated with 50 mixed stages (female, male and juveniles) of *P. goodeyi* suspended in 100 µl SDW.

Mortality and paralysis was determined after 3, 6 and 24 h of exposure to culture filtrates. Active nematodes were considered those with normal sigmoid shape and exhibiting active movement; paralysed nematodes were considered those that were not active and that were curved and never in a straight position even after poking them with a fine sterile needle, while dead nematodes were considered those that were straight (uncurved) after poking them with fine needle, the percentage dead and paralyzed nematodes were calculated based on the original numbers of nematodes.

After 24 h exposure to culture filtrates, nematodes were concentrated through a 28 µm sieve, rinsed with SDW and transferred into sterile 7 ml Bjorn bottles containing 2 ml SDW. The nematode cultures were left in the laboratory for an additional 24 h. Nematodes were probed with a fine needle under a compound microscope (magnification × 20) and those that were elongated and remained immotile even after probing with a fine needle were considered dead. Percentage mortality and paralysis were calculated using Abotts corrected mortality formula (Abbott, 1925).

Statistical analysis

Using percentages from the water control treatment, percentages mortality and paralysis from each of the other treatments were corrected using Abott's formula (Abbott, 1925). Corrected percentages mortality and paralysis were arcsine-square root transformed before analysis of variance (ANOVA) to obtain equal variances among treatments and normal distributions. Two way ANOVA was used to demonstrate interaction effects between treatments and exposure time. When interactions were significant, the effects of one factor were analysed at each level of the interacting factor. If significantly different, treatment means were compared using Tukey test (SAS, 2001).

RESULTS

Effect of isolates culture filtrates on *P. goodeyi* paralysis

Percentage paralysis (immobilization) caused by culture filtrates of different *F. oxyposrum* isolates differed among the 3 bioassays ($P < 0.001$) with interaction between

Table 1. Percentage paralysis (immobilization) (mean ± S.E.) of *P. goodeyi* mixed stages (males, females and juveniles) in culture filtrates of 6 endophytic *F. oxysporum* isolates after exposure times of 3, 6 and 24 h.

	Bioassay 1			Bioassay 2			Bioassay 3		
Isolate	**3 h**	**6 h**	**24 h**	**3 h**	**6 h**	**24 h**	**3 h**	**6 h**	**24 h**
5JTOC134	34.8 ±3.4^{a}	31.0 ±0.8^{a}	19.9±1.1^{ab}	34.9 ± 3.4^{a}	31.0 ± 0.8^{a}	19.9 ± 1.0 ^{ab}	27.6 ± 5.5^{b}	10.8 ± 5.9^{cd}	6.30 ± 3.5^{a}
4M0C321	28.2 ±2.9^{ab}	24.5 ±1.4^{a}	23.3 ±2.6^{a}	28.6 ± 2.9^{a}	26.2 ± 2.1^{ab}	24.4 ± 3.3^{a}	28.2 ± 0.3^{b}	21.7 ± 1.2^{abc}	16.7 ± 2.0^{a}
5MR11	27.8 ±4.6^{ab}	27.2 ±5.7^{a}	17.6±3.3^{ab}	27.9 ± 4.5^{ab}	27.2 ± 5.7^{a}	17.6 ± 3.2^{ab}	41.9 ± 1.7^{a}	30.9 ± 1.3^{a}	15.5 ± 3.3^{a}
V5W2	16.2 ±8.7^{ab}	16.1 ±1.0^{a}	6.4 ± 6.4 ^{b}	26.4 ± 4.6^{ab}	24.8 ± 2.5^{ab}	16.4 ± 2.8^{ab}	29.9 ± 2.1^{ab}	24.9 ± 0.8^{ab}	20.3 ± 0.4^{a}
2MR23	11.8 ± 2.5^{b}	17.8 ±0.9^{a}	20.7±1.5^{ab}	11.9 ± 2.4^{bc}	18.0 ± 0.8^{abc}	20.9 ± 1.7^{ab}	28.1± 2.8^{b}	16.5 ± 1.6^{bcd}	10.8 ± 5.4^{a}
PDB	12.4 ± 2.7^{b}	12.9 ±3.1^{a}	12.7±1.7^{ab}	12.4 ± 2.7^{bc}	12.9 ± 3.1^{bc}	12.7 ±1.7 ^{b}	9.9 ± 1.5^{c}	12.9 ± 1.4^{bcd}	14.0 ± 2.1^{a}
Water	10.3 ± 1.7^{b}	10.4 ±1.8^{a}	13.4±0.8^{ab}	10.3 ± 1.7^{c}	10.4 ± 1.8^{c}	13.3 ± 0.8^{ab}	4.5 ± 2.3^{c}	6.7 ± 3.8^{d}	6.10 ± 3.3^{a}

treatments and bioassays (F = 3.5, DF = 12, P = 0.001). In individual bioassays, percentage parlaysis of *P. goodeyi* was influenced by the 3 different time of exposure (F = 15.0, DF = 2, P < 0.001). As the length of exposure time increased, percentage paralysis decreased.

In all the 3 bioassays conducted, percentage *P. goodeyi* nematodes immobilized by some *Fusarium* culture filtrates were significantly higher than the control treatments (Table 1). In bioassay 1, after 3 h exposure, percentage paralysis by fungal filtrates of did not differ from each other neither did they differ from the control (P = 0.0054). However, compared to bioassay 3, after 3 h of exposure, all the fungal isolates except 2MR23 differed from one control (Water) (Table 1). Over all the isolate 5JTOC134 resulted in nematode immobilization rates similar to that of the reference isolate *V5W2* (Table 1) across bioassays.

Effect of isolates culture filtrates on *P. goodeyi* mortality

Fusarium isolates caused variable (P < 0.001) mortality levels on motile stages of *P. goodeyi* after 48 h of exposure to culture filtrates of the isolates. Interaction between the treatments and bioassays was significant (F = 11.9, DF = 12, P < 0.001) meaning that the same isolates caused varied mortality levels across the three repeat bioassays. In bioassay 1, the reference isolate V5w2 caused significantly higher mortality (75.9%) compared to the controls (42.1 and 22.2%) PDB and water respectively. The 2 controls had significant differences, with more mortality in PDB control as opposed to water control. Although V5w2 had the highest effect on the mortality of the nematodes, its effects did not differ from the Kenyan isolate 5JTOC134 in causing mortality (Figure 1). In bioassay 2, isolate 2MR23 caused the highest mortality (77.5%) and again, the isolate did not significantly differ from the tester isolate V5w2 (Figure 1). However all the isolates differed significantly from the 2 controls. In bioassay 3, isolate 5JTOC134 had significantly higher mortality (81.4%), but had similar effect to the tester strain V5w2 (Figure 1). Control treatment (Water) had lower nematode mortality compared with the pH adjusted PDB control in bioassays 1 and 2, while in bioassay 3, the controls did not differ from each other (P = 0.05).

DISCUSSION

The study revealed that all the culture filtrates of the 5 endophytic *F. oxysporum* isolated from banana plants in Kenya had *in vitro* mortality and paralysis effects on *P. goodeyi*. Although previous experiments focused more on effects of endophytic *F. oxysporum* against exotic nematodes in Africa, no research has focused on the effect of endophytic fungi isolated from Kenyan bananas against the African native lesion nematode, *P. goodeyi*. It is clear that isolates tested in the study produced nematicidal or nematotoxic compounds against *P. goodeyi*, with results independent upon parameters such as the fungal isolate, pH or the concentration of the culture filtrates.

All efforts were taken to maintain constant conditions for the 3 repeat bioassays, but there were variations that occurred within treatments in different bioassays. There are a number of factors that could have contributed to the inconsistencies,

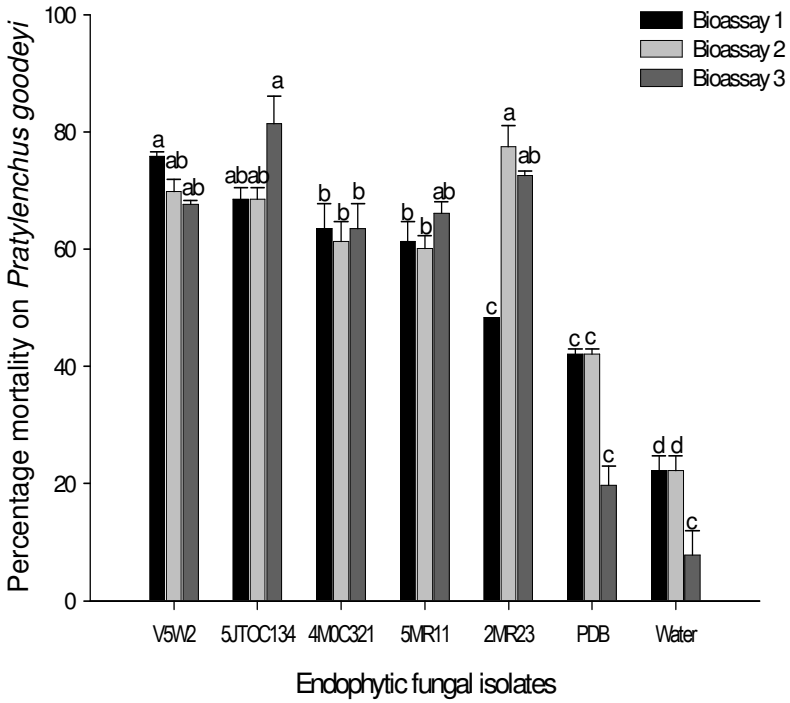

Figure 1. Percentage mortality **(**mean ± S.E.) of *P. goodeyi* mixed stages (males, females and juveniles) in culture filtrates of 5 endophytic *F. oxysporum* isolates after an exposure time of 24 h, n = 3, in 3 repeat bioassays.

such as the type of culture medium, age of the fungal culture filtrates, culture filtrate concentration and the time of nematodes exposure to culture filtrates (Cayrol et al., 1989). The pH of the fungal filtrates on nematodes inhibition seems to have an effect since there was differences in mortality between the pH adjusted control and water control. Partly, the effect could be attributed to the to the levels of osmotic pressure created by the dextrose in PDB, which could have lead nematodes desiccation and eventual death due to osmotic pressure difference. However, there is need to evaluate further the effect of using the PDB as a control as compared to other suitable controls.

Isolates 5JTOC134 and 5MR11 resulted in consistency in causing both mortality and paralysis of *P. goodeyi* and were comparable to the tester strain V5W2, which has been reported to be the best isolate against *R. similis* (Athman, 2006; Dubois et al., 2004). This also demonstrates that fungal endophytes e.g. V5W2 produces compounds which can cause mortality and paralysis to multiple hosts (*R. similis* and *P. goodeyi*) as opposed to earlier studies on effects on nematocidal properties of culture filtrate of the nematophagous fungus *Paecilomyces zilacinus,* which was only found to have specific effects on nematodes of Heteroderidae family (*Meloidogyne, Heterodera*) (Cayrol et al., 1989).

The time of exposure had a vital role in influencing both the mortality and paralysis of the nematodes. It was evident that the toxic effects of the isolates after 24 h of exposure to culture filtrates of the isolates were not reversible even after rinsing the nematodes with clean sterile distilled water. However toxic effects of some endophytic *F. oxysporum* have been reported to have a reversibility effect if nematodes were exposed to 6 h or less (Athman, 2006). Percentage paralysis of the nematodes decreased as time of exposure increased. The total number of paralysed nematodes translated into dead nematodes as time length increased. This indicates that nematodes are first paralysed before they eventually die off as a result of toxic nature of the culture filtrates of endophytic fungi. The fact that the isolates caused both mortality and paralysis to all the motile stages of *P. goodeyi* is of great importance since both sexes of the nematodes are invasive to banana plants (Gichure and Ondieki, 1977).

Currently there is very limited information on the nematodes-inhibiting nature of the fungal culture filtrates, especially those antagonistic to the nematodes. However, it was evident that the compounds in the culture filtrates used in this study were toxic to *P. goodeyi* since the filtrates did not contain any spores and mycelia. PDB used as the control in this experiment had negligible effects on the nematodes mortality since it varied significantly with all the isolates at different times of exposure, indicating that the effects were independent of the PDB effect.

The study revealed the potential of endophytic fungi as biological control agent against the lesion nematode, *P.*

goodeyi. The study revealed that all the culture filtrates of the 5 endophytic *F. oxysporum* isolated from banana plants had *in vitro* mortality and paralysis effects on *P. goodeyi*. *F. oxysporum* as control agent of banana nematode *P. goodeyi*.

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Full Length Research Paper

Effects of basal media, salt concentrations, antioxidant supplements and co-effects on the *Agrobacterium*-mediated transformation efficiency in maize

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Transformation efficiency enhancement in maize *Agrobacterium*-mediated transformation was tested using four different basal media, five levels of N6 salts, two antioxidants and copper sulfate. In the absence of the antioxidants L-cysteine and dithiothreitol (DTT), the frequencies of transient GUS expression was higher using Linsmaier and Skoog (LS) and Murashige and Skoog (MS) media as an alternative to Chu (N6) and Ducan (D). N6 basal medium exhibited better performance in the presence of antioxidants than MS, LS and D basal media. Five different levels of N6 medium salts (10, 30, 50, 70 and 100%) were tested, and the highest transformation efficiency was 15.9% under a 50% salt concentration, followed by 6.4% transformation efficiency with 70 and 3.2% under 100% salt conditions. More than 95% of infected immature embryos exhibited GUS staining under 10 and 30% salt concentrations, however none of the embryos developed into embryogenic callus, indicating that low salt levels favored T-DNA delivery, but not stable transformation. Additions of DTT or L-cysteine, or a combination of L-cysteine and DTT, showed a significant improvement in the frequency of transient GUS expression, however increases were not observed with independent $CuSO_4$ treatments. Polymerase chain reaction (PCR) and Southern-blot analysis confirmed T-DNA integration into the maize genome.

Key words: Maize (*Zea mays* L.), *Agrobacterium tumefaciens*, transformation.

INTRODUCTION

The maize genome project has been completed and the draft sequence represents a vast repository of genomic data that is now readily available (Gore et al., 2009).

However, research continues to annotate sequences to ascertain gene function in maize. Genetic transformation is considered one of the most direct and effective strategies to determine gene function in plants. Microprojectile bombardment (Gordon-Kamm et al., 1990) and *Agrobacterium tumefaciens*-mediated transformation (Ishida et al., 1996) are two popular methods that are currently used in maize transformation. Many studies have shown that high-copy number and extensive rearrangement of foreign DNA are frequently identified in transgenic plants by microprojectile bombardment, which often leads to transgenic silencing and unstable inheritance (Register et al., 1994; Shou et al., 2004). Therefore, high transformation frequency of maize mediated by *A. tumefaciens* has been reported as the method of choice for the delivery of

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Abbreviations: **N6,** Chu medium; **MS,** Murashige and Skoog medium; **LS,** Linsmaier and Skoog medium; **D,** Ducan medium; **2,4-D,** 2,4-Dichlorophenoxyacetic acid; **MES,** 2-(N-Morpholino) ethanesulfonic acid; **DTT,** Dithiothreitol; **NAA,** α-Naphthalene acetic acid; **6-BA,** N6-benzylaminopurine; **CTAB,** Cetyltrimethyl ammonium bromide; **DIG,** Digoxin; **PVP,** Polypyrrolidone; **GUS,** β-Gluceronidase gene; **Bar,** Bialaphos resistance gene.

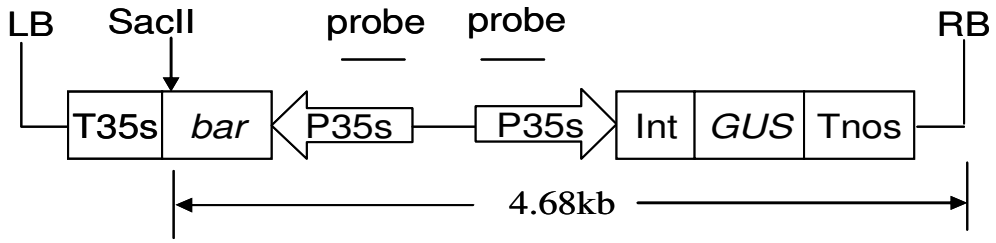

Figure 1. pCAMBIA3301. LB and RB diagram: left and right borders of the T-DNA; P35s: CaMV35s promoter; T35s and Tnos: CaMV35s terminator and nopline synthase gene terminator; *bar*: bialaphos resistance gene; *GUS*: β-gluceronidase gene.

exogenous genes into the maize genome.

A. tumefaciens-mediated transformation is the pre-eminent method for plant genetic transformation due to several advantages, including high efficiency, low-copy number, large DNA segments, low rearrangement rate and low cost (Ishida et al., 1996; Hansen and Wright, 1999). Initially, this method was only successfully applied in dicotyledonous plant genetic transformation, because monocotyledonous plants were not naturally susceptible to *A. tumefaciens*. Consequently, efforts to extend the host range of *Agrobacterium* to monocotyledonous plants were achieved and *A. tumefaciens*-mediated transformation was applied in monocots (Hernalsteens et al., 1984; Hooykaas-Van Slogteren et al., 1984). Subsequently, it was successfully implemented in several economically important monocotyledonous plants, such as rice (Hiei et al., 1994), wheat (Cheng et al., 1997), maize (Ishida et al., 1996) and barley (Tingay et al., 1997). High throughput genetic transformation systems in maize were developed using super binary vector systems and transformation frequency was approximately 40% (Zhao et al., 2001). However, transformation frequency was still low relative to standard binary vector systems, even under the improved co-culture conditions in maize (Frame et al., 2002). Therefore, further improvements in transformation efficiency in standard binary vector systems are still necessary.

Several factors can affect *A. tumefaciens*-mediated transformation efficiency, including co-cultivation period and temperature, co-cultivation medium pH, host genotype, explant type and source, *Agrobacterium* strain and vector, and components of the medium (Huang and Wei, 2005; Zhang et al., 2001; Frame et al., 2002; Zhao et al., 2001; Ishida et al., 1996; Frame et al., 2006). Two of the key factors in transformation efficiency are host genotype and medium components. Maize remains highly recalcitrant to *A. tumefaciens*-mediated transformation, but genotype A188, Hi hybrid and H99 are three genotypes recommended for successful transformation (Zhao et al., 2001; Ishida et al., 1996; Negrotto et al., 2000). Although there is an increasing interest in elite genotypes or hybrids, inbred line A188 or derivatives of A188 remain the line of choice for *A. tumefaciens*-mediated transformation. This is because type or friable embryogenic callus is easily produced from these lines. Furthermore, A188 is a model genotype widely used in *A. tumefaciens*-mediated transformation, with a reported transformation frequency of 5 to 50% (Ishida et al., 1996; Ishida et al., 2007).

Medium components are also key factors in successful transformations. Many basal media, including N6, MS, LS and D media have been used in maize transformation (Vega et al., 2008; Frame et al., 2006; Ishida et al., 2007; Yang et al., 2001). Previous studies determined that antioxidant supplemented media, such as L-cysteine, DTT and PVP (polypyrrolidone), and low salt concentration media, improved *A. tumefaciens*-mediated transformation frequency in several crops species (Perl et al., 1996; Olhoft and Somers, 2001; Paz et al., 2004). However, few reports of transformation efficiency using multiple factors and the interactive effects of these factors are available for maize.

In the present study, the effects of antioxidants, basal media, salt concentration and copper sulfate on transformation efficiency was evaluated, and focused was also on optimizing these factors to establish a high-frequency transformation system in maize.

MATERIALS AND METHODS

Plant material

The maize inbred line, A188, was used as initial material and cultivated on the Agronomy farm at China Agricultural University, Beijing, China. Following self-pollination at approximately 9 - 12 days, the ears were collected and sterilized with 70% ethanol for 5 min. Kernels of normal and healthy morphology were sampled for the study. Immature embryos approximately 1.0 to 1.5 mm in length were isolated.

Agrobacterium strain and vector

Agrobacterium strain LBA4404 (Hoekema et al., 1983) was used to harbor a standard binary vector pCAMBIA3301 (CAMBIA, Australia) that contained a CaMV35s promoter-*bar* and a CaMV35s promoter-*gus*-intron reporter gene cassette (Figure 1). *Bar* was used as the selectable marker gene and *uidA* (GUS) included an intron as the reporter gene in the binary vector. The pCAMBIA3301 vector was mobilized into *Agrobacterium* by a direct DNA transfer method (An et al., 1988) and its integrity in *Agrobacterium* cells was confirmed

Table 1. Media composition used in *Agrobacterium*-mediated transformations.

Infection medium[a]	N6, MS, LS , D medium basal salt and their vitamin, 68.5 g/l sucrose, 36.0 g/l glucose, 0.7 g/l L-proline, 0.1 g/l myo-inositol, 0.5 g/l MES, 2.0 mg/l 2,4-D, pH 5.2. Add 100 µM acetosyringone[a] before using.
Co-cultivation medium	Infection medium without glucose, reduce sucrose to 30 g/l and supplemented with 0.05 µM copper sulfate, 0.4 g/l L-cysteine[a], 0.15 g/l DTT[a], 8 g/l agar, 100 µM acetosyringone[a], pH 5.8.
Resting medium	Cocultivation medium without copper sulfate, L-cysteine[a] and DTT[a], supplemented with 0.85 mg/l silver nitrate[a] and 0.1 g/l carbenicillin[a], 2.5 g/l gelrite, pH 5.8.
Selection medium	Resting medium supplemented with 2.0 g/l bialaphos, pH 5.8.
Regeneration medium	MS basal salt and vitamin, supplemented with 30 g/l sucrose, 0.1 g/l myo-inositol, 3.5 mg/l 6-BA, 3.0 g/l gelrite, pH 5.8.
Rooting medium	MS basal salt and vitamin, supplemented with 25 g/l sucrose, 0.5 mg/l NAA, 1.2 g/l gelrite, pH 5.8.

[a] Infection medium, acetosyringone, L-cysteine, DTT, silver nitrate carbenicillin and bialaphos were filter sterilized.

by restriction enzyme analysis.

Medium

N6, MS, LS and D media were chosen and the compositions are listed in Table 1. Methods followed Ishida et al. (2007) and Vega et al. (2008) with several modifications, including: (1) The addition of $CuSO_4$, L-cysteine and DTT into the co-cultivation medium; (2) Replacing cefotaxime by carbenicillin in the resting and selection media; (3) Supplementing with silver nitrate in co-cultivation, resting and selection media; (4) Autoclaving basal salt, sucrose and glucose; and (5) Filter-sterilizing infection medium, antioxidants, vitamins and antibiotics.

Transformation procedure

Agrobacterium cells were incubated at 19°C for 3 days in YP solid medium. The pre-infection preparation, bacterium inoculation, co-cultivation, selection and plant regeneration were conducted according to Ishida et al. (2007). Isolated immature embryos were immersed in 1.5 ml liquid infection medium in a 2.0 ml micro-centrifuge tube. The medium was removed and replaced with 1.5 ml fresh liquid infection medium when approximately 200 immature embryos were collected. The tube with collected immature embryos was centrifuged at 20,000 g at 4°C for 10 min. The supernatant was subsequently replaced by 1 ml *Agrobacterium* suspension (OD660 = 1.0). The tube was vortexed and the isolated immature embryos were incubated for 5 min at room temperature. Infected embryos were plated with scutellum side up on co-cultivation medium, which was overlaid by a piece of sterile filter paper. Following 3 days of incubation at 19°C in the dark, the embryos were transferred to a resting medium and incubated at 25°C. The following 7 days, all embryos were transferred into a selection medium containing 1.5 mg/l bialaphos. After 3 weeks, bialaphos was increased to 2.0 mg/l for 6 weeks to strengthen selection, followed by 2.5 mg/l bialaphos for 6 weeks. After 2 months, emergent resistant calli were transferred to a regeneration medium and incubated at 25°C under a 16/8h (light/dark) photoperiod. Plantlets regenerated from the resistant callus within 2 - 3 weeks were transferred to a tube containing rooting medium. Plantlets with fully-grown roots were transplanted into soil and grown under greenhouse conditions.

Histochemical GUS assays

GUS assays were conducted on infected immature embryos after 3 days of co-cultivation (Jefferson et al., 1987). One hundred infected immature embryos were randomly chosen for each assay and three replicates were conducted. Ten resistant calli and shoots which developed from resistant callus were also submitted for assay. The frequency of transient GUS expression (%) was determined as the percentage of the number of infected immature embryos that exhibited GUS staining after 3 days of co-cultivation. The transformation frequency (%) was the percentage of the number of positive transgenic plants against the total number of immature inoculated embryos.

Transgene analysis

Total genomic DNA from regenerated plant leaves was extracted following the CTAB isolation procedure (Saghai-Maroof et al., 1984), and used for polymerase chain reaction (PCR) and Southern blot analysis. PCR primers for the *bar* genes were forward primer: 5′-ACTTCAGCAGGTGGGTGTAGAGCGT-3′ and reverse primer: 5′-GCACCATCGTCAACCACTACATCGA-3′. The PCR parameters included one cycle of 95°C 3 min; 35 cycles of 95°C 30 s, 61°C 30 s, 72°C 45 s and one cycle of 72°C 10 min; samples were stored at 4°C. PCR products were 273 bp and separated on 2% (w/v) agarose gels. 20 mg of each DNA sample was digested with Sac at 37°C overnight. Digested products were fractionated on 0.8% agarose gels and subsequently transferred to nylon membranes. DNA was fixed to the membrane at 80°C for 2 h. The CaMV35s promoter probe was DIG labeled applying PCR and forward primer: 5′-TACCCGAGCAATAATCTCCAGG-3′ and reserve primer: 5′-CGGCAGAGGCATCTTCAACGA -3′. Southern blot analysis was conducted according to the Roche Southern blot Kit protocol (Roche Applied Science, Mannheim Germany).

Transgenic plants regenerated from resistant calli were selfed or back-crossed to the wild-type A188 plants. For progeny analysis, five sets of progeny were analyzed using PCR assays to examine the *bar* gene segregation patterns. Progeny segregation analysis of

Table 2. The effects of media, antioxidants and N6 salts levels on infection frequency.

Type of medium	Treatment		Number of infected immature embryos	Number of GUS staining embryos	Average frequency of transient GUS expression	Number of infected immature embryos	Number of resistant callus	Number of regeneration plants	Number of positive plants	Transformation frequency
MS	1		300	113	37.7±5.0	723	8	7	0	0.0
	2		300	188	62.6±4.5	1132	39	35	23	2.0
LS	1		300	121	40.3±4.7	847	13	10	0	0.0
	2		300	170	56.6±4.1	1498	57	51	33	0.9
D	1		300	101	33.6±4.5	426	5	5	0	0.0
	2		300	163	54.3±4.7	792	31	26	14	1.8
N6	1		300	82	27.3±3.5	551	6	6	0	0.0
	2	10%	300	295	98.3±0.6	877	0	--	--	--
		30%	300	287	95.6±1.5	784	0	--	--	--
		50%	300	278	92.6±3.2	653	112	107	104	15.9
		70%	300	246	82±4.0	574	47	44	37	6.4
		100%	300	217	72.3±3.7	872	44	41	28	3.2

Treatment 1 = Absence of 0.4 g/l L-cysteine and 0.15 g/l DTT.
Treatment 2 = Presence of 0.4 g/l L-cysteine and 0.15 g/l DTT.
10, 30, 50, 70 and 100% mean levels of N6 medium basal salts.

the *bar* gene was statistically analyzed for goodness-of-fit to simple Medelian expectations using a Chi-square test.

RESULTS

Effects of basal medium and antioxidants

The absence of 0.4 g/l L-cysteine and 0.15 g/l DTT resulted in a decreased frequency of transient GUS expression in N6 medium (27.3%) compared with LS (40.3%), MS (37.7%) and D media (33.7 %) (Table 2). GUS activity increased with the addition of both 0.4 g/l L-cysteine and 0.15 g/l DTT. Among the four different basal media, transient GUS expression improved most with N6 basal medium; 72.3% infected immature embryos displayed GUS staining, approximately three fold higher than in the absence of L-cysteine and DTT (27.3%). Consequently, 0.4 g/l L-cysteine and 0.15 g/l DTT were added to the N6 salt co-cultivation medium for subsequent experiments.

Transformation frequency was also affected by basal medium and antioxidants. The inclusion of 0.4 g/l L-cysteine and 0.15 g/l DTT in the co-cultivation medium increased the transformation frequency of the N6 medium (3.2%) compared to MS (2.0%), LS (0.9%) and D (1.8%) media (Table 2).

Effects of N6 medium under different salt concentrations

The differences in the frequency of transient GUS expression were exhibited among the five different basal salt levels of N6 medium. The following 3 days of co-cultivation, over 95% of infected immature embryos displayed GUS staining in 10 and 30% N6 basal salts medium. Unfortunately, most infected immature embryos did not develop further and died, indicating that low salt concentrations in the medium could have negative effects on the embryogenic capacity of immature maize embryos, although salt concentration facilitated high frequency of T-DNA delivery. The frequency of transient GUS expression reached 92.7% with 50% N6 basal salts, but with the increased salt concentration, transient GUS expression decrea-sed by 82.0% in 70% basal salt and 72.3% in 100 % basal salts (Table 2). Therefore, salt concentration in basal medium likely affected the transformation frequency and high salt concentration resulted in a low transformation rate.

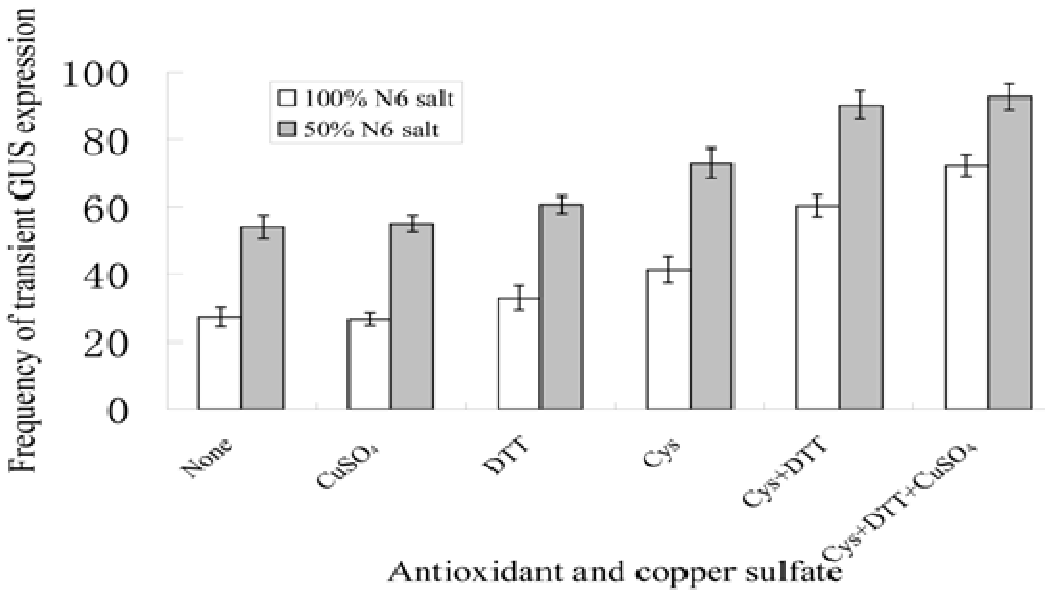

Figure 2. The effects on infection frequency of copper sulfate and antioxidants added to the co-cultivation medium. Data shown are mean ± SE of three experiments; each experiment consisted of N = 100. L-cysteine at 0.4 g/l, DTT at 0.15 g/l and copper sulfate at 0.05 µM.

The application of 50% N6 salt concentration resulted in a transformation frequency increase as high as 15.9%, followed by 6.4% with a 70% salt level and 3.2% with a 100% salt concentration (Table 2). These results suggested that basal salt concentration greater than 50% was not favorable for the transformation process, probably suppressed infection and subsequent integration of T-DNA into the plant genome. As a result, a 50% salt concentration, 0.4 g/l L-cysteine, 0.15 g/l DTT and 0.05 µM $CuSO_4$ for *A. tumefaciens*-mediated transformation in the system was used.

Effects of individual and combinations of antioxidants and copper sulfate

In N6 basal salt and absence of copper sulfate co-cultivation medium, 27.3% of the immature embryos displayed GUS staining. However, a copper sulfate supplement of 0.05 µM decreased the frequency of transient GUS expression to 26.7% (Figure 2). Addition of DTT alone resulted in a higher level of GUS expression with 33% in 100% N6 salt and 60.7% in 50% salt. L-cysteine separately showed an increase in transient GUS expression by 41.3% in 100% N6 salt and 73% in 50% N6 salt, indicating that L-cysteine was more favorable to DTT alone in the co-cultivation medium. The combined use of both L-cysteine and DTT in the co-cultivation medium demonstrated a rise in the level of GUS expression to 60.3% in 100% N6 salt and 90.3% in 50% N6 salt. Including copper sulfate with the combined use of L-cysteine and DTT resulted in higher GUS activity with a transient GUS expression of 72.3% at a 100% salt level and 92.7% at 50% N6 salt.

Transient GUS expression was not increased at 100% N6 salt and $CuSO_4$ alone. However, a significant improvement in transient GUS expression was observed when DTT was added alone ($p < 0.05$) (Table 3). Addition of L-cysteine alone and the combination of L-cysteine and DTT in the co-cultivation medium resulted in significant increase in transient GUS expression ($p < 0.01$) (Table 3). Similar results were obtained when 50% N6 salt concentration was applied to the co-cultivation medium (Table 3).

Transgenic plant recovery and molecular and genetic characterization

Resistant calli began to emerge from the immature infected embryos following approximately two months of bialaphos selection (Figure 3). The plants regenerated from these calli appeared normal morphologically (Figures 4a and b). Ten resistant calli and plantlets were randomly chosen for GUS assays, respectively and all samples displayed blue GUS staining (Figures 5a - c). These results indicated the *uidA* gene was integrated and expressing in the calli and plantlets.

Total genomic DNA was extracted from leaf material of 332 putative plantlets for PCR analysis. Primer pairs specific to detect the *bar* gene were employed. 239 of 332 putative plantlets tested positive with 72.0% selection efficiency (Figure 6), suggesting our three-step selection regime (beginning with 1.5 mg/l for 3 weeks; increased to

Table 3. T-test analysis: Effects of different antioxidants and copper sulfate on the frequency of transient GUS expression.

N6 Salt concentration	Treatment	Average frequency of transient GUS expression	Variance	P value	Significance level
100%	none	27.33 ± 3.51	12.33		
	$CuSO_4$	26.67 ± 2.51	6.33	0.401241	NS
	DTT	33.00 ± 2.65	7.00	0.044696	*
	L-cys	41.33 ± 4.51	20.33	0.006618	**
	DTT+L-cys	60.33 ± 4.04	3.00	0.000218	**
	DTT+L-cys+$CuSO_4$	72.33 ± 3.79	14.33	5.62E-05	**
50%	none	54.00 ± 3.00	9.00		
	$CuSO_4$	55.00 ± 2.00	4.00	0.328023	NS
	DTT	60.67 ± 3.51	12.33	0.033383	*
	L-cys	73.00 ± 4.00	16.00	0.0013794	**
	DTT+L-cys	90.33 ± 3.21	10.33	6.92258E-05	**
	DTT+L-cys+$CuSO_4$	92.67 ± 3.21	10.33	5.42E-05	**

100 and 50% N6 basal salts were used in co-cultivation medium for every treatment.
Data shown are mean ± SE of three experiments; each experiment included N = 100.
L-cysteine at 0.4 g/l, DTT at 0.15 g/l and copper sulfate at 0.05 mM.

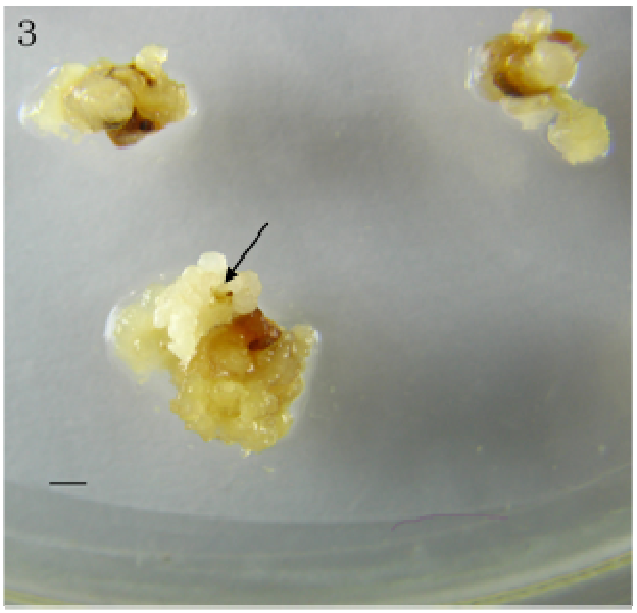

Figure 3. Resistant embryogenic callus emergent from an immature maize embryo A188 showing vigorous growth on selective medium with 2.5 mg/l bialaphos (scale bar = 1 mm).

2.0 mg/l for 6 weeks and 2.5 mg/l for 6 weeks) resulted in a low escape rate.

Six genomic DNA samples were obtained from PCR positive plants and used for Southern blot analysis. Figure 7 indicates that four transgenic plants possessed a single copy of the transgene; one possessed two copies of the transgene, revealing that the T-DNA was successfully integrated into the genome, and the different banding patterns suggested each was from independent transgenic events.

Five transgenic events with large numbers of seeds were chosen to examine *bar* gene segregation in the progeny. Two transgenic events (T83 and T141) were selfed and three events (T127, T189 and T206) were backcrossed to the wild-type A188 plants. T83 and T141 displayed a 3:1 segregation ratio and T127, T189 and T206 displayed a 1:1 ratio, which indicated the presence of single locus integration in all five transgenic lines (Table 4).

DISCUSSION

Maize transformation frequency has primarily been affected by basal medium in *A. tumefaciens*-mediated transformation (Ishida et al., 1996; Frame et al., 2006). However, to date the reason for this limitation is not well understood. The production of type or friable embryogenic callus from maize immature embryos is clearly related to the nature of the basal medium, and the efficiency of embryogenic callus formation is different when various basal media are used (Armstrong and Green, 1985; Ducan et al., 1985; Carvalho et al., 1997). The present results demonstrated that for maize A188 inbred line, LS medium performed better than N6 medium when L-cysteine and DTT were absent from the co-cultivation medium. This result is consistent with that reported by Ishida et al. (1996). However, when a 50%

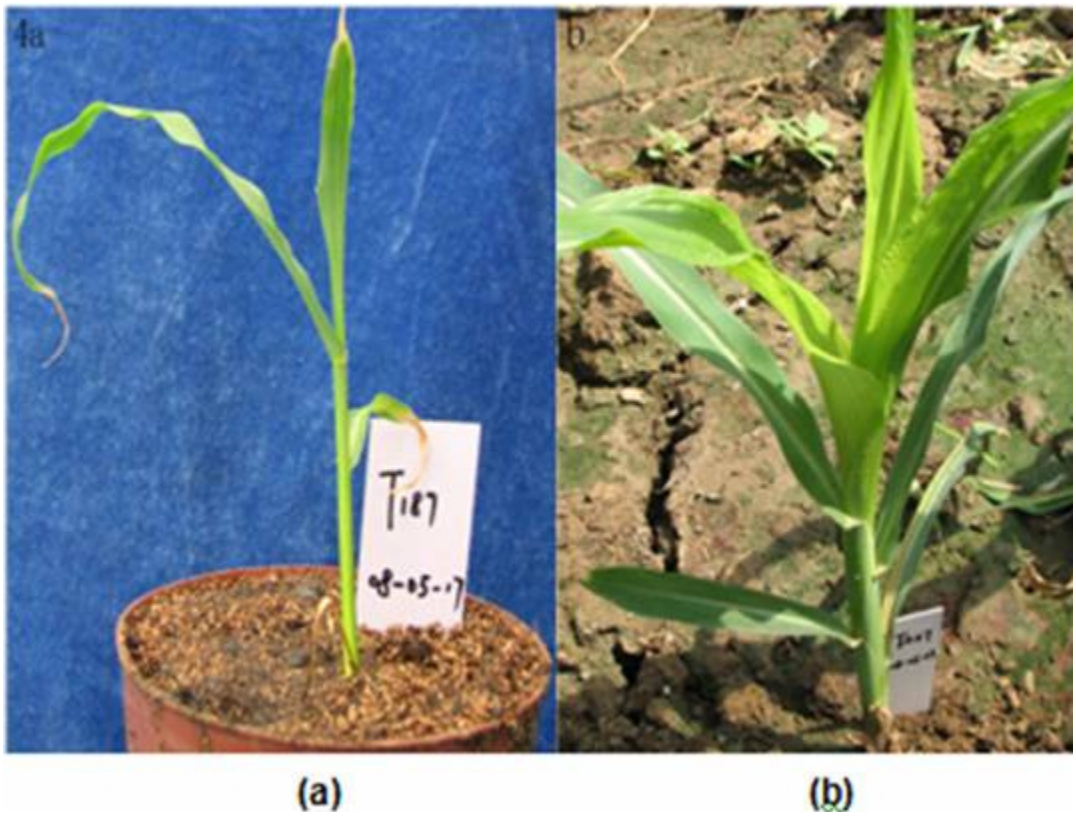

Figure 4. The regenerated transgenic plants transferred to soil. **a:** Transgenic plant (T_0) transferred from tissue culture to pot. **b:** Transgenic plant grown in the field and under greenhouse conditions.

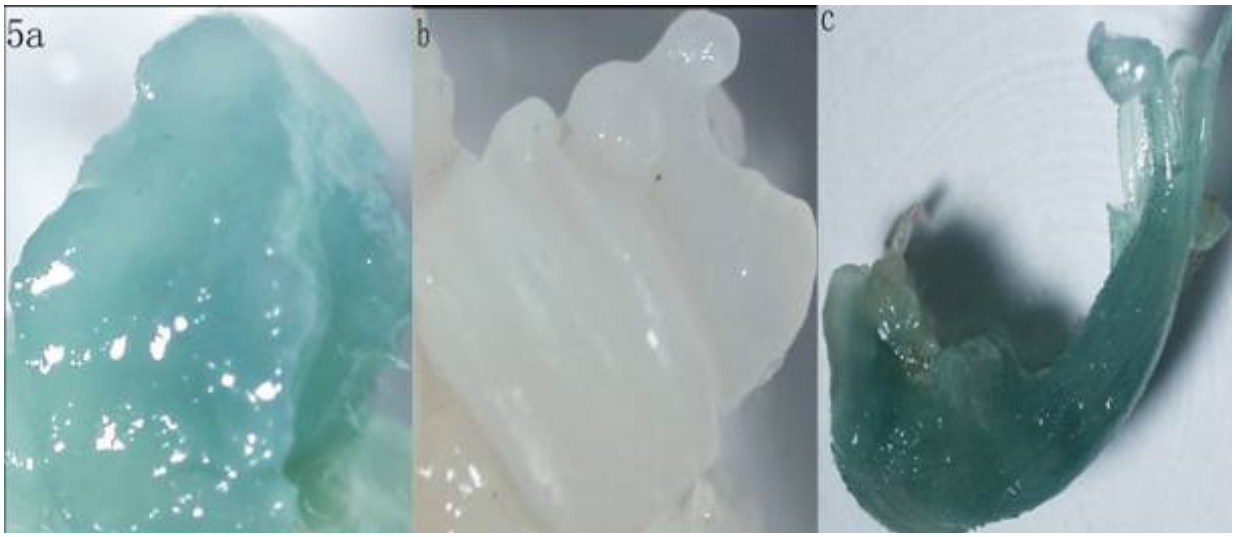

Figure 5. Stable GUS expression was observed in resistant callus and shoots. **a:** Bialaphos resistant callus. **b:** Normal A188 callus (control). **c:** Shoot regenerated from resistant callus.

instead of full strength basal salt was used in conjunction with both 0.4 g/l L-cysteine and 0.15 g/l DTT in the co-cultivation medium, a high frequency of transient GUS expression was observed and transgenic plants were obtained from N6 basal medium. These results are incongruent from those reported by Ishida et al. (1996), who showed that no positive transgenic plants were obtained when using N6 as a basal medium. In addition,

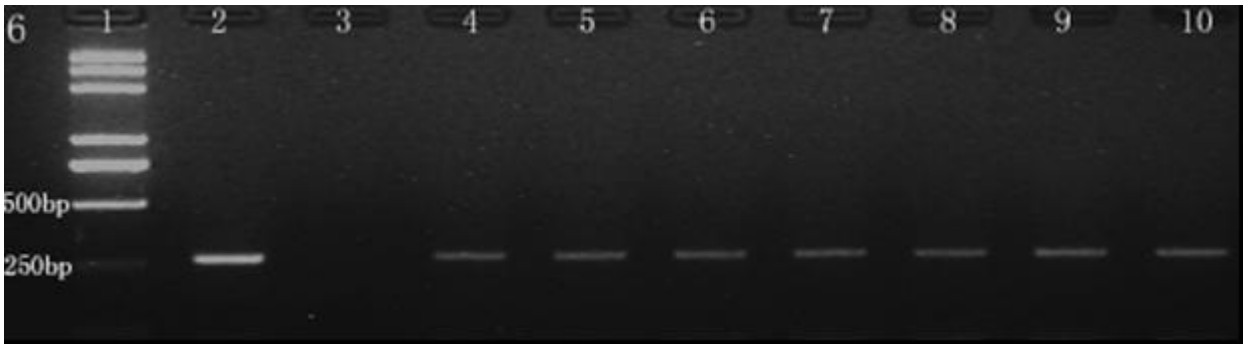

Figure 6. PCR amplification using bar primers. Lane 1: DL2000 plus DNA maker. Lane 2: Plasmid DNA. Lane 3: Non-transgenic plant. Lanes 4 - 10: transgenic plants.

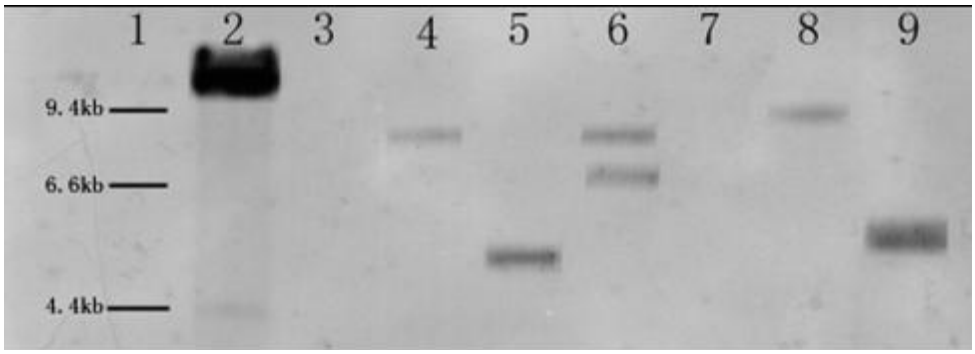

Figure 7. Southern-blot analysis of transformed plants. Lane 1: λDNA/Hind maker. Lane 2: pCAMBIA3301 digested with Sac. Lane 3: Non-transgenic plant. Lane 4 - 6 and 8 - 9: Different PCR-positive plants. Lane 7: False PCR-positive plant.

Table 4. Progeny segregation analysis of primary transgenic A188 maize events.

Events	Total progeny	Selfing or back crossing	Positive	Negative	Segregation ratio	χ^2
T83	39	Selfing	28	11	3:1	0.214
T127	52	Back crossing	24	28	1:1	0.308
T141	26	Selfing	19	7	3:1	0.051
T189	91	Back crossing	44	47	1:1	0.099
T206	63	Back crossing	29	34	1:1	0.397

$\chi^2 = 3.84$ (0.05, 1 df).

antioxidants were integral in *A. tumefaciens*-mediated transformation of maize (Frame et al., 2002, 2006; Vega et al., 2008). The role of antioxidants may minimize cell death caused by a hypersensitive response (Frame et al., 2002). This may enable increased survival and embryogenesis of infected embryogenic-competent cells, resulting in improved transformation frequency. In the system used in this study, 0.4 g/l L-cysteine and 0.15 g/l DTT as a supplement to the N6 co-cultivation medium resulted in increased transformation frequency.

Gao and Bao (2004) found that salt concentration influenced gene transfer by influencing *A. tumefaciens* growth. However, the reason transformation frequencies improved under low concentration remains unclear. Most GUS staining appeared on the embryo axis sides and the edges when a 100% N6 basal salt concentration was used in the co-cultivation medium. However, more than 50% GUS staining displayed in the scutellum area when the salt concentration was below 50% (data no show). Callus is typically derived from the scutellum area of immature embryos and rarely from the axis side or edges. This likely explains why the transformation frequency was improved in low salt concentrations. However, very low salt concentrations of 10 and 30% exhibited negative effects on transformation frequency. Infected immature embryos severely damaged, did not

grow further into embryogenic calli, and consequently no resistant calli were recovered. N6 medium with 50% salt concentration showed the highest frequency of transient GUS expression, and also facilitated further development of infected immature embryos without negative impact, resulting in increased transformation efficiency.

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Full Length Research Paper

Identification of rust resistance genes Lr10 and Sr9a in Pakistani wheat germplasm using PCR based molecular markers

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Wheat (*Triticum aestivum* L.) rusts are the most widespread and destructive among all other diseases of wheat because of their wide distribution, and their capacity to form new races that can attack previously resistant cultivars which result in serious yield losses. Stem rust caused by *Puccinia graminis* f. sp*. tritici* and leaf of orange rust caused by *Puccinia recondita* are more effective and durable when several rust resistance genes are pyramided into a single line. Molecular survey was conducted to screen altogether 44 Pakistani wheat germplasm lines consisted of 23 cultivated varieties for the presence of *Lr10* and *Sr9a* genes by using co-dominant STS and SSR primers specific for respective alleles. The polymorphic survey revealed that out of 44 germplasm lines obtained from Ayub Agriculture Research Institute, 31 genotypes were observed with *Lr10* gene (including C-228, C-245, C-288), while 13 germplasm lines did not show the presence of *Lr10* gene. Of the 23 cultivated varieties, 21 varieties observed *Lr10* gene (including Auqab 2000, Chenab-70, Punjab-76, Kohsar-95, Parvaz-94, Pasban-90, Chenab-2000, Satluj-86, Shahkar-95), while only Kohsar 95 and PARI 73 did not show the presence of *Lr10* gene. Molecular survey for *Sr9a* gene depicts that 33 genotypes were observed for stem rust resistance like C-248, LR26, C-250, C-271, C-273, C-288 and C-518, while 11 genotypes (including C-228, C-245, C-247 and C-271) showed no such fragment. Of 23 conventional cultivated varieties, 21 varieties showed the presence of *Sr9a* stem rust resistance gene like Punjab-76, Pak-81, Kohistan-97, Kohsar-95, Parvaz-94, Pasban-90 etc and 4 varieties showed the absence of *Sr9a* stem rust resistance gene like Shahkar-95, Parvaz-94, Chenab-2000 and Chenab-79. The identification of *Lr10* and *Sr9a* in Pakistani wheat germplasm will help in accelerating the breeding program in future, including pyramiding of different wheat resistant genes in wheat varieties.

Key words: Stem rust, leaf rust, *Sr9a, Lr10*, wheat, molecular markers.

INTRODUCTION

Wheat is the most widely used staple food grain of the world. The world wheat output declined from 591.9 to 589.1 million tons in the last couple of years. Pakistan and India account for 20% of the annual world wheat production. In Pakistan, wheat being the staple diet is the most important crop and cultivated on the largest acreages in almost every part of the country. It contributes 14.4% to the value added in agriculture and 3.0% to GDP. Pakistan has been divided into ten production zones because of great agro ecological areas where wheat is grown. The zoning is mainly based on cropping pattern,

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disease prevalence and climatological factors. Improved semi-dwarf wheat cultivars available in Pakistan have genetic yield potential of 6-8 ton/ha whereas the national average yields are about 2.5 ton/ha. A large number of experiment stations and on-farm demonstrations have repeatedly shown high yield potential of the varieties. There are progressive farmers of irrigated area who are harvesting 6 to 7 tonnes yield per hectare. However, farmers yield ranges from 0.5 to 1.3 ton/ha depending on the amount of rainfall in rain-fed areas and in irrigated areas it ranges from 2.5 to 2.8 ton/ha depending upon the amount of water available and other factors.

Diseases, especially rusts (stripe/yellow rust, stem rust, leaf rust and emerging scenario of powdery mildew) are major biotic stresses of wheat crop that inflict heavy losses when in epidemic form. Therefore, breeders and pathologists are given priority to develop disease resistant and high yielding varieties in order to cope with threats created by ever changing rust races by using approaches like durable resistance. In the last few decades, disease epidemic of rust inflicted heavy losses to the wheat production in the country. As a consequence, it was realized that there is need of strengthening the agricultural research in the country.

The rusts caused by *Puccinia* species are some of the most important diseases of wheat and these deadly pathogens affected the balance of its trade in under-developing countries like Pakistan. Leaf rust caused by *Puccinia triticina* (*P. recondita f. sp. tritici*) is a major foliar disease of wheat, resulting in severely damaged wheat production and yield loss throughout the world (Eversmeyer and Browder, 1974; Kolmer, 1996). The disease is most damaging when the upper leaves of infected plants become severely rusted. Heavy rusting causes early loss of these leaves, which reduces the grain filling period and results in smaller kernel size. Stem rust of wheat caused by *Puccinia graminis* Pers.:Pers. *f. sp. tritici* Eriks and *E. Henn* is another destructive disease of wheat, reaching devastating epidemic levels in most wheat-growing areas of the world (Knott, 1989). To date, more than 45 stem rust resistance (Sr) genes have been identified against different races of this fungus (McIntosh et al., 2003).

As the name implies, it infects the stem but is not always confined to the stem. It can infect leaves, sheaths, glumes and awns. The host epidermics is ruptured by the elongate, reddish brown (brick-red) pustule with ragged margins, giving the stems and sheaths a rough-textured surface. Stem rust pustules are larger than leaf rust pustules and often erupt on both upper and lower leaf surfaces.

Knowledge of genes for resistance to diseases is important to estimate the risk of the disease according to the virulence in the population of the pathogen. Although, single gene resistance may overcome new races of pathogens, the use of resistant varieties is still the most commercial and economic method to reduce yield losses (McIntosh, 1988). To avert rust crisis, it is essential for plant breeders to use rust resistant genes, for the choice of the most suitable resistance genes and their combinations for the breeding process. Pyramiding of several rust resistant genes in a single line after following molecular markers could dramatically improved yield losses. Moreover resistant varieties are the best control for biotic stress. So for this reason, it is necessary to screen Pakistani wheat lines that are considered effective against all races of pathogen and provide broad spectrum resistance. Marker assisted selection provides an indirect selection of a genetic determinant(s) of a trait of interest. Microsatellite markers (SSR) are PCR-based markers and are located in repetitive deoxyribonucleic acid (DNA) sequences (Roder et al., 1998). Only a few SSR markers are sufficient to discriminate among even closely related wheat breeding lines (Roder et al., 1995; Plaschke et al., 1995; Bryan et al., 1997). Thus, it is important to investigate, in wheat, whether mapped microsatellite markers are randomly distributed or clustered in certain regions of chromosomes (Roder et al., 1998).

The wheat genome is large, hexaploid, (1.6×10^{10} bp) and (80%) highly repetitive sequences. Diversity of resistance to leaf rust caused by *P. triticina* can be enhanced in wheat cultivars through a better knowledge of resistance genes that are present in important cultivars and germplasm. To date, more than 45 stem rust resistance (*Sr*) markers (genes) (McIntosh et al., 2003) and nearly 58 leaf rust resistance markers have been identified against different races of this fungus. Leaf rust resistance genes are designated as *Lr1* through *Lr58* (McIntosh et al., 2005; Kuraparthy et al., 2007), but most varieties have only a few *Lr* genes. *Lr10* is a single-copy gene on chromosome 1AS (Feuillet et al., 1997). *Lr10* confers enhanced resistance to leaf rust; *Lr10* has similarities to *RPM1* in *Arabidopsis thaliana* and to resistance gene analogs in rice and barley. A collection of 68 cultivars of common wheat has been screened for leaf rust resistance genes with the use of molecular markers (Urbanovich et al., 2006).

The potential benefits of molecular marker-assisted selection (MAS) have been widely discussed (Melchinger, 1990; Paterson et al., 1991; Young, 1996; Mohan et al., 1997; Anderson, 2003), especially to provide solutions to overcome some of the problems faced by classical phenotypic screening approaches in plant breeding programs. For example, to facilitate breeding for durable resistance to stem rust, molecular markers are useful tools in developing resistant cultivars and especially, pyramiding several disease resistance genes (Anderson, 2003). Marker-assisted selection can be used at an early stage of plant development when multiple DNA markers are used to screen several genes simultaneously.

The objective of this work was to address this issue by using SSR markers and to verify the application of the molecular marker for the determination of the gene cluster *Sr9a* and *Lr10* in Pakistani wheat germplasm to develop wheat lines that are considered effective against

Table 1. Primer sequences of Lr10 and Sr9a.

Locus name	Location (Chromosome #)	Primer sequence	Reference
Lr10	1AS	Frw: 5′GTGTAATGCATGCAGGTTCC3′ rev: 5′AGGTGTGAGTGAGTTATGTT3′	Stepien et al. (2003).
Sr9a	2BL	SSR marker Xgwm47 Frw: 5′TTGCTACCATGCATGACCAT3′ rev: 5′TTCACCTCGATTGAGGTCCT3′	Tsilo et al. (2007).

all races of stem and leaf rust pathogens. This information will be directly useful to wheat breeders and plant pathologists to help develop wheat cultivars with high levels of leaf and stem rust resistance.

MATERIALS AND METHODS

Plant materials

Seeds of 44 wheat genotypes obtained from the Ayub Agricultural Research Institute (AARI), Faisalabad, Pakistan comprising 23 conventional cultivars were grown in pots at Institute of Biotechnology (IBT), Bahauddin Zakariya University (BZU), Multan Pakistan. List of all these lines along with their parentage is given in Table 1. All these were tested with PCR-based DNA markers for, leaf rust and stem rust resistance genes *Lr10*, *Sr9a*, respectively.

DNA extraction

Total genomic deoxyribonucleic acid (DNA) from individual genotype was extracted from young leaves at seedling stage by CTAB (cetyl trimethtyl ammonium bromide) method (Doyle and Doyle, 1990). Fresh leaves (five plants of each accession) were ground in mortar with a pestle with continuous addition of liquid nitrogen and transferred to a 50 ml falcon tube. The 15 ml of hot 2x CTAB was added and incubated for 30-45 min at 65°C with occasional swirling. Equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed gently by inverting the tube to form an emulsion, then spun at 11, 000 rpm for 10 min. The supernatant was collected into a new 50 ml tube and a nucleic acid was precipitated with 0.6 volume of chilled isopropanol and left in the refrigerator for 2 h. Nucleic acid was pelleted at 12, 000 rpm for 5 min and supernatant was discarded and the Pellet was washed with 70% ethanol and air dried before resuspending in 0.5 ml 0.1x TE buffer. The suspension was transferred to 1.5 ml Eppendorf tube, added 7 µL of RNase and incubated at 37°C for 1 h. DNA concentration was measured by flouremeter DyNA Quant^{TM}200 and the DNA were diluted to 10 ng/µL using sterilized distilled water and stored in microfuge tubes at 4°C for further use.

PCR Amplification of Lr10 and Sr9a

To screen rust resistance genotypes, polymerase chain reaction (PCR) was performed as described by Chen et al. (1997) in a 96-well plate of Gene Amplification PCR System 9700 Applied Biosystems.

Amplification reaction were carried out with a 15 µL of final reaction mixture containing 50 ng genomic deoxyribonucleic acid, 1 µL of each of primer, 100 µM dATP, dCTP, dGTP and dTTP, 1 unit of Taq polymerase (fermentas), 1x Taq polymerase buffer and 2.5 Mm magnesium chloride. DNA amplification reaction was performed in a Gene Amplification PCR System 9700 of Applied Bioystems programmed for *Sr9a* as under; initial denaturation of 5 min at 94°C, 45 cycles of 94°C for 1 min (denaturation), 60°C for 1 min (annealing), 72°C for 2 min and for *Lr10* as under; initial denaturation of 5 min at 94°C, 35 cycles of 94°C for 1 min (denaturation), 55°C for 1min (annealing), 72°C for 2 min. The amplified products were resolved at 8% denaturing polyacrylamide gels followed by staining with $AgNO_3$ (Heukeshoven and Dernick, 1985; Budowle et al., 1991) and scored for the presence and absence of *Lr10* and *Sr9a* linked DNA fragments.

Data analysis

The amplified fragments of all wheat genotypes for *Lr10* and *Sr9a* were observed and compared with band size describe by Stepien et al. (2003) and Tsilo et al. (2007).

RESULTS AND DISCUSSION

The wheat cultivars become susceptible to rusts due to their narrow genetic base for resistance and the rapid rate of evolution of the pathogen, making it necessary to search for new source(s) for resistance. Genetic resistance is important to control many phytopathogenic epidemics. Thus, the wheat production has been largely dependent on the development and the use of resistant cultivars having diverse and well characterized genes. Resistance based on single major gene is often considered short-lived due to the genetic shifts or the emergence of new virulence in the pathogen population in response to selection imposed by the host. It is believed that, in wheat, certain gene combinations give better and long lasting resistance to rust diseases than given by any of the genes individually (Dyck and Samborski, 1982). Molecular marker SSR offers an effective way of assessing the resistance of a set of genotypes and these markers are widely distributed throughout plant genomes and provide discrete data about morphological traits. Similar kind of work was conducted by Arif et al. (2008) on screening of bacterial blight resistant line in rice and practically showed the significance of molecular markers.

Amplification of DNA using *Lr10* F primer and R primer has been described as a way to detect *Lr10* gene and the amplified fragments corresponded to leaf rust resistance,

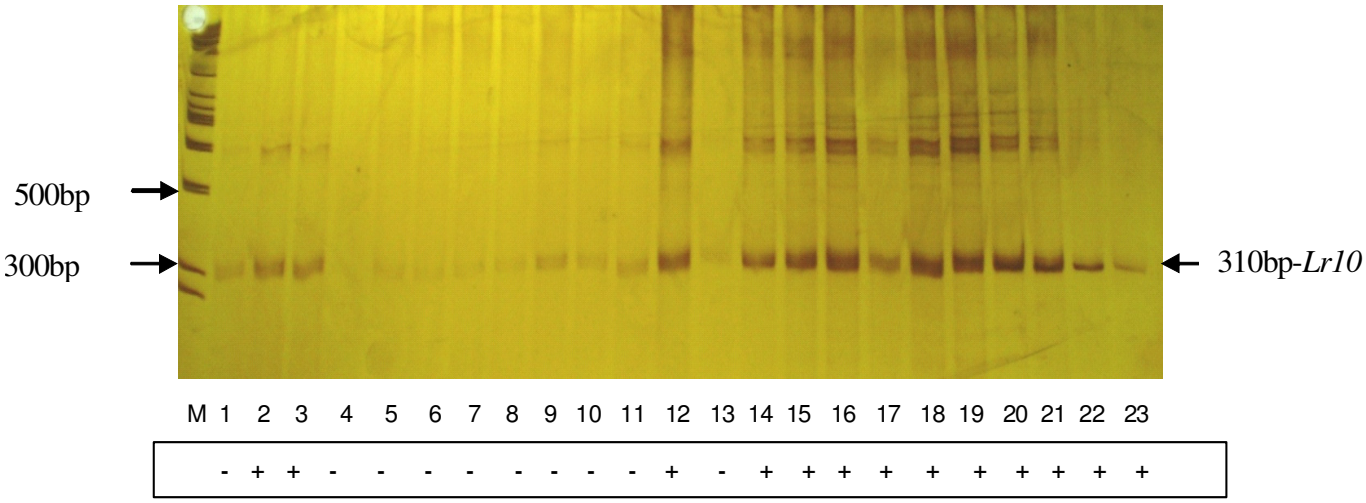

Figure 1. Banding patterns showing the presence and absence of *Lr10* gene in germplasm of wheat line amplified 310 bp size fragment. + sign represent presence of band whereas - sign indicates absence of *Lr10* gene. Arrow is showing amplified gene (310 bp).M = 1000 bp size marker.

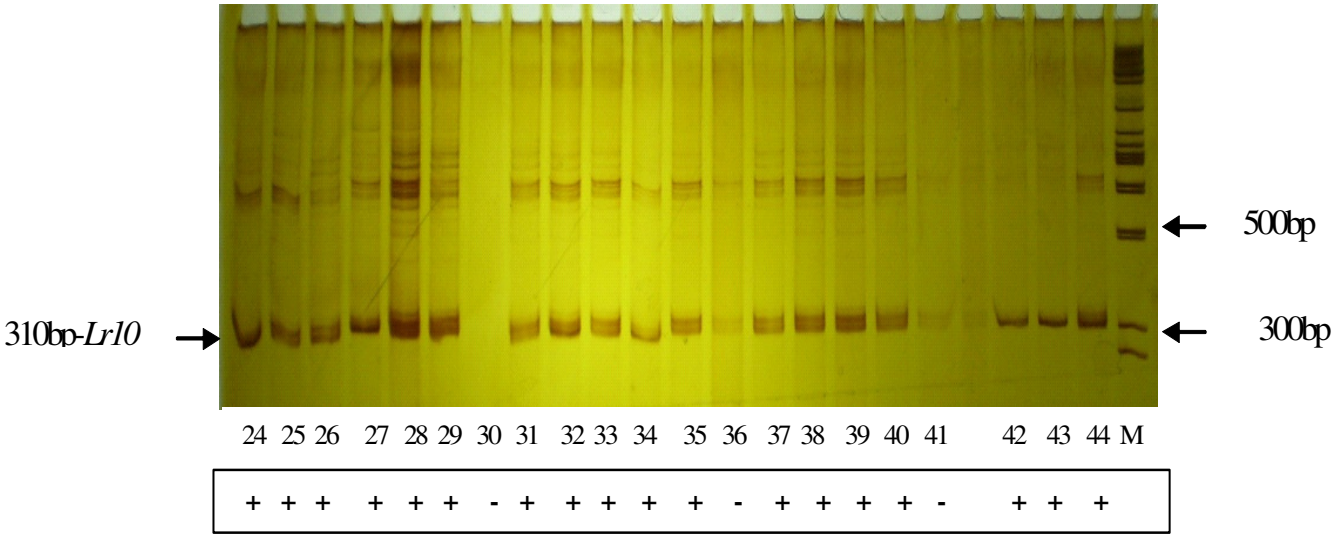

Figure 2. Banding patterns showing the presence and absence of *Lr10* gene in germplasm of wheat line amplified 310 bp size fragment. + sign represent presence of band whereas - sign indicates absence of *Lr10* gene. Arrow is showing amplified gene (310 bp) M = 1000 bp size marker.

results were compared with the work which has already been done on *Lr10* (Stepien et al., 2003). The size of the band corresponds to *Lr10* was 310 bp. Varieties possessed 310 bp size of band thought to be resistant against leaf rust whereas lines which lack 310 bp of band which are susceptible to leaf rust carry no *Lr10* gene (Figures 1 and 2). The amplified fragments corresponded to stem rust resistance and the results were compared with the work which has already been done on *Sr9a* (Tsilo et al., 2007). The size of the band corresponding to *Sr9a* was 190bp and varieties having 190 bp size of band are found to be resistant against stem rust whereas lines having 165 bp of band which are susceptible to stem rust carry no *Sr9a* gene. The presence and absence of *Lr10* has shown in Figures 1 and 2 and *Sr9a* in Figures 3 and 4 while the screening of 44 wheat genotypes with *Lr10* and *Sr9a* markers whose loci are already mapped, provide information about presence and absence of resistant genes in Pakistani germplasm and varieties (Table 2).

This polymorphic survey revealed that out of 44 germplasm lines obtained from Ayub Agriculture Research Institute, 31 genotypes were observed with *Lr10* gene, namely: C-228, C-245, C-288, while 13 germplasm lines did not showed the presence of *Lr10* gene. Of the 23

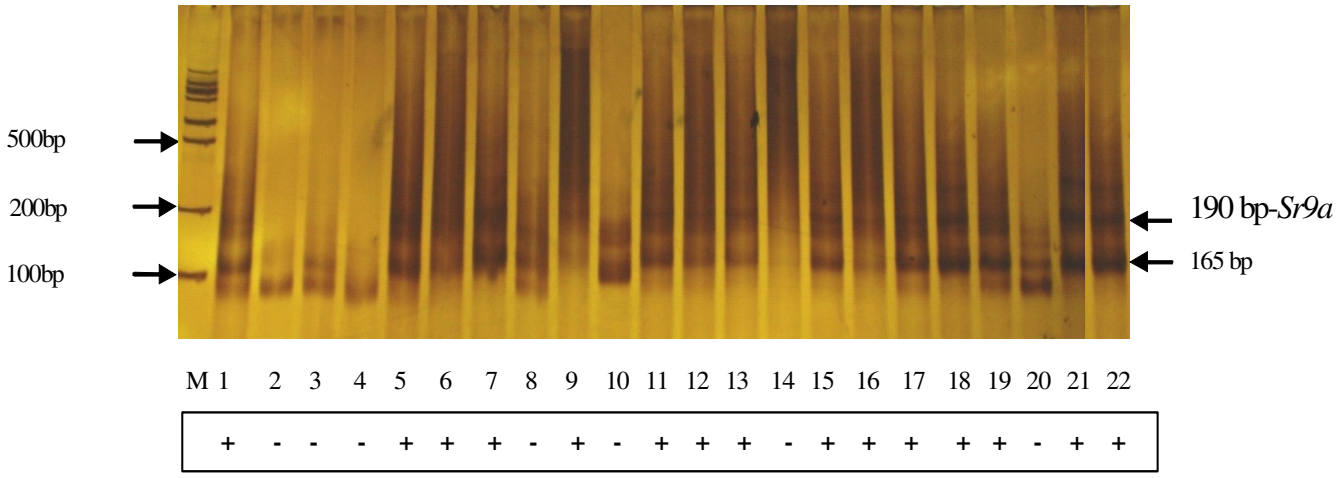

Figure 3. Banding patterns showing the presence and absence of *Sr9a* gene in germplasm of wheat line amplified 190 bp and 165 bp size fragments respectively. + sign represent presence whereas - sign indicates absence of *Sr9a* gene. Arrow is showing amplified gene (190 bp) M = 100 bp size marker.

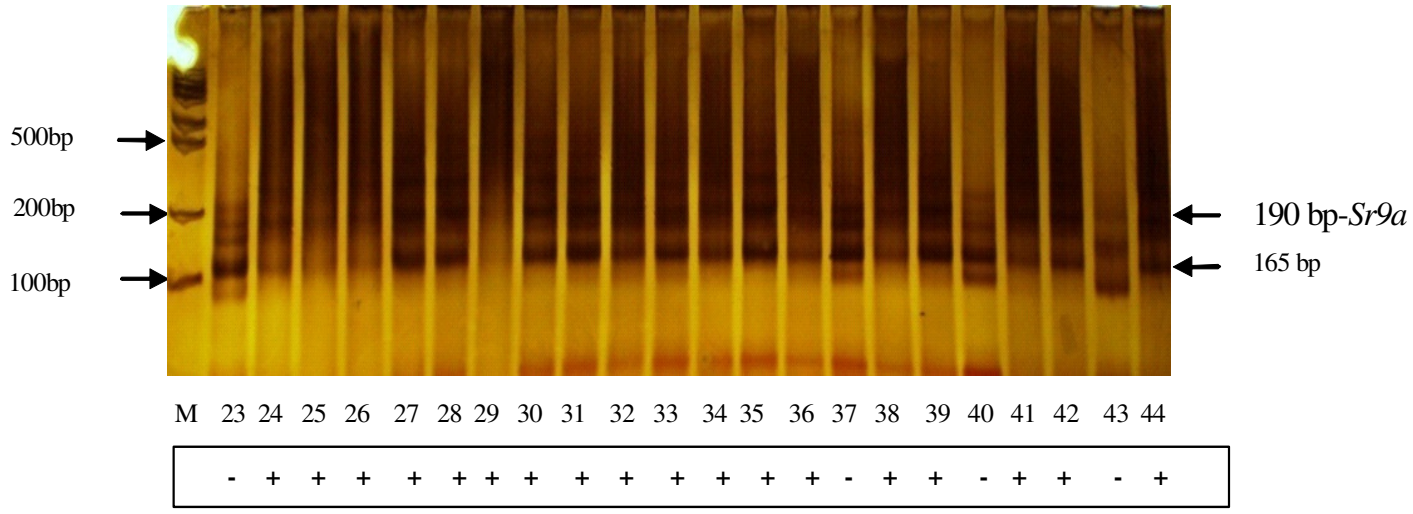

Figure 4. Banding patterns showing the presence and absence of *Sr9a* gene in germplasm of wheat line amplified 190 bp and 165 bp size fragments, respectively. + sign represent, presence whereas - sign indicates absence of *Sr9a* gene. Arrow is showing amplified gene (190 bp) M = 100 bp size marker.

cultivated varieties, 21 varieties observed *Lr10* gene which includes: Auqab 2000, Chenab-70, Punjab-76, Kohsar-95, Parvaz-94, Pasban-90, Chenab-2000, Satluj-86, Shahkar-95, etc, and only Kohsar 95 and PARI 73 did not show the presence of *Lr10* gene. Molecular survey for *Sr9a* gene depicts that 33 genotypes were observed for stem rust resistance like C-248, LR26, C-250, C-271, C-273, C-288 and C-518, while 11 genotypes showed no such fragment like C-228, C-245, C-247 and C-271, etc. Of 23 conventional cultivated varieties, 21 varieties showed the presence of *Sr9a* stem rust resistance gene like Punjab-76, Pak-81, Kohistan-97, Kohsar-95, Parvaz-94, Pasban-90, etc, and 4 varieties showed the absence of *Sr9a* stem rust resistance gene like Shahkar-95, Parvaz-94, Chenab-2000 and Chenab-79 (Table 2). The identification of *Lr10* and Sr9a in Pakistani wheat germplasm will help in accelerating the breeding program in future, including pyramiding of different wheat resistant genes in wheat varieties.

Abbreviations:

MAS, molecular marker-assisted selection; **SR,** stem rust resistance; **CTAB,** cetyl trimethtyl ammonium bromide; **PCR,** polymerase chain reaction; **LR,** leaf rust resistance; **DNA,** deoxyribonucleic acid; **SSR,** microsatellite markers; ***Sr,*** stem rust resistance gene; ***Lr,*** leaf rust resistance gene.

Table 2. List of wheat germplasm along with their parentage showing presence and absence of *Lr10* and *Sr9a* genes.

S/N	Variety code	Pedigree / Parentage	*Lr10*	*Sr9a*
1	IBT-1	C-217 (C-516 x C-591)	-	+
2	IBT-2	C-228(Hard Federation x 9D)	+	-
3	IBT-3	C-245	+	-
4	IBT-4	C-247	-	-
5	IBT-5	C-248,LR26, 14A	-	+
6	IBT-6	C-250Hard Federation x 9D	-	+
7	IBT-7	LR30, 14A	-	+
8	IBT-8	C-256 LR10, 23, 30	-	-
9	IBT-9	C-258	-	+
10	IBT-10	C-271(C220xIP165)	-	-
11	IBT-11	C-273,(C209 x C-591)	-	+
12	IBT-12	C-288	+	+
13	IBT-13	C-518, (T9 x 8A)	-	+
14	IBT-14	C-591(T9 x 8B)	+	-
15	IBT-15	AS-2002 = WD-97603KHP/D.31708//CMH74A.370/ 3/CNO79/4/RL6043/*4NAC.PBD.795-23A-1A-0A	+	+
16	IBT-16	AUQAB 2000CROW'S'/NAC//BOW'S' PB 22138-3A-0A-0A-234A-0A	+	+
17	IBT-17	BHAKKAR-2000P102/PIMA//F3.71/TRM/3/PVN	+	+
18	IBT-18	BLUE SILVER = SONALIKAII53.388/AN//YT54/N10B/3/LR/4/	+	+
19	IBT-19	CHENAB 70	+	+
20	IBT-20	CHENAB79,PB76/CH70	+	-
21	IBT-21	CHAKWAL86,FORLANI/ACC//ANA 75	+	+
22	IBT-22	CHAKWAL 97,BUC'S'/FCT'S'	+	+
23	IBT-23	CHENAB-2000, CBRD (CHUM18/BAU), CM92991-59M-0Y-0M-5Y-0B	+	-
24	IBT-24	FAISALABAD83, FURY//KAL/BB, CM 37138-48Y-1M-5Y-1M-4Y-5Y-0A	+	+
25	IBT-25	FAISALABAD85,MAYA/'MON'S'//KVZ/TRM,CM 44083-N-3Y-1M-1Y-1M-1Y-0B	+	+
26	IBT-26	GA-2002, DWL 5023/SNB//SNB, CM 84986-H-1M-3M-2B-0Y	+	+
27	IBT-27	INQILAB 91	+	+
28	IBT-28	IQBAL2000,BURGUS/SORT1213//KAL/BB/3/PAK.81,PB21912-11A-0A-0A-59A-0A,KOHINOOR 83,OREF1158/FDL//MFN/2*TIBA63/3/COC	+	+
29	IBT-29	KOHISTAN97,V-1562//CHRC'S'/HORK/3/KUFRA-1/4/CARP'S'/BJY'S',PB.24883B-1A-0A	+	+
30	IBT-30	KOHSAR 95	-	+
31	IBT-31	LU 26,BLS/KHUSHAL 69	+	+
32	IBT-32	LYP 73,BB/NORTENO.67	+	+
33	IBT-33	MEXIPAK 65,PJ62/GB55,II.8156-0PAK	+	+
34	IBT-34	MH 97 = ATTILA, ND/VG 9144//KAL/BB/3/YACO/4/VEE#5,CM. 85836-50Y-0M-0Y-3M 0Y	+	+
35	IBT-35	PAK 81,KVZ/BUHO//KAL/BB= VEE#5	+	+
36	IBT-36	PARI 73,BLUEBIRD,CNO//SN64/KLRE/3/8156	-	+
37	IBT-37	PARWAZ 94, V 5648/PRL'S' (V-87189), PB. 20089-7A-4A-0A	+	-
38	IBT-38	PASBAN90,INIA66/A,DISTT//INIA 66/3/GEN 81	+	+
39	IBT-39	PUNJAB76,NAI 60/CB 151//S 948/3/MXP	+	+
40	IBT-40	SA 42,C 271/LR 64//SON 64	+	-
41	IBT-41	SA 75, NAI 60/CB 151//S 948/3/MXP	-	+
42	IBT-42	SATLUJ 86,CMT/YR//MON'S'	+	+
43	IBT-43	SHAHKAR95,WL711//F3.71/TRM,PB 20371-20A-4A-0A	+	-
44	IBT-44	INQ.91/FINK'S', SH-2002, PB. 25553-1A-0A-1A-0A	+	+

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Full Length Research Paper

In vitro culture and plant regeneration derived from ray florets of *Chrysanthemum morifolium*

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Nine cultivars of *Chrysanthemum morifolium* were screened using the ray floret explants to determine the capability for plant regeneration on four media protocols and subsequently to find out the best genotype source linked with the optimum medium conditions for the high potentiality of shoot formation. The results indicated that all *in vitro* culture traits were highly significantly influenced by the differences in genotypes, medium protocols and their interaction. The percentage of explants which developed calli ranged from 73.83% "Ping Pong" to 25.67% "Palisade White" among the cultivars across the four medium protocols with an average of 48.28%. The highest percentage of embryogenic callus, shoot formation and mean value of shoot length was produced by cultivar "Delistar White" when calli were differentiated on medium protocol B. The medium protocol B showed the greatest potential for shoot length across the cultivars and it was significantly superior to all other medium protocols except the medium protocol A. The present study indicated that the medium protocol "B" and then "A" appear to be the best protocols for plant regeneration. The cultivar "Delistar White" with the medium protocols B and then A, could be successfully utilized for further *in vitro* mutagenesis investigations.

Key words: *Chrysanthemum morifolium, in vitro* culture, plant regeneration.

INTRODUCTION

Chrysanthemum (*Chrysanthemum morifolium* Ramat), currently classified as *Dendranthema x grandiflora* (Anderson, 1987) belonging to the Asteraceae family, was initially classified as a Compositae (Salinger, 1991). It is also known as florist's chrysanthemum or Higo-giku (in Japanese). The name originally came from the Greek word *krus anthemon*, meaning gold flower which originnated in China (where it has been cultivated for over 3000 years). This culturally rich flower is globally the second economically most important floricultural crop following rose and it is one of the most important ornamental species. Its popularity as cut flower had led to the introduction of thousands of new cultivars of large flower diversity (Cockshull, 1985).

For modern and industrialized horticulture, the cut flower industry, perhaps different from any other industry, is always in demand and in need of new varieties to routinely attend the continuous flower consumer demands. Consumer preferences change and show new and sometimes uncommon features. Therefore, the priority of the flower and ornamental plant biotechnology segments should be the generation of novel plant and flower types (Hutchinson et al., 1982). Chrysanthemum *in vitro* culture was extremely useful for producing a huge number of explants in a short time as stated by Dao et al. (2006). Tissue culture studies in chrysanthemum are being done as a tool for mutation induction and as a means of micropropagation. However, the ability to regenerate plants from a single cell of florets is a useful approach to establish a mutant in pure form and facilitate the production of a wide range of new flower cultivars as stated by Mandal et al. (2000).

The aim of the present study was to determine the capability of nine chrysanthemum cultivars for plant regeneration on four medium protocols and subsequently, to find the best genotype source linked with the optimum

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Table 1. *Chrysanthemum morifolium* cultivars* used in the *in vitro* regeneration experiment and their characteristics.

S/N	Cultivar*	Colour*	Form[1]	Response time[2]	Vigour[3]	Ø[4]
1	Delistar white	White	Spider	7	5	100/20
2	Delistar yellow	Yellow	Spider	7	5	100/20
3	Palisade white	White	Decorative	8.5	5.5	160/20
4	Palisade yellow	Yellow	Decorative	8.5	5.5	160/20
5	Ping Pong	White	Pompon	8	4.5	70/15
6	Ping Pong Golden	Yellow	Pompon	8	4	70/15
7	Rodet	Orange	Decorative	11	5.5	180/20
8	Cassandra	Purple	Decorative	10.5	4	150/20
9	SunnyCassandra	Bronze	Decorative	10.5	4	150/20

Form[1] = The most important characteristics of each variety are given under each form; Response time[2] = indicates the number of weeks between the beginning of the short day period and the flowering date; Vigour[3] = group is a scale of '7' which is used for the most vigorous varieties and the lower numbers for the less vigorous ones; Ø[4] = Diameter of flower (ray florets) mm / diameter of flower centre (disc florets) mm.

Table 2. Composition of the four medium protocols used for callus initiation, plant regeneration and root induction in chrysanthemum cultivars.

Protocol components	Callus Induction	Shoot differentiation	Root Induction	Callus Induction	Shoot differentiation	Root induction
	Protocol (A)			**Protocol (B)**		
MS basic medium	1X	1X	1/2 X	1X	1X	1/2 X
BAP	0.5 mg/l	0.5 mg/l	-	1.0 mg/l	1.0 mg/l	-
NAA	0.2 mg/l	0.2 mg/l	0.02 mg/l	0.5 mg/l	0.5 mg/l	0.1 mg/l
Sucrose	30 g/l	30 g/l	15 g/l	30 g/l	30 g/l	15 g/l
Agar	5.5 g/l	5.5 g/l	5.5 g/l	5.5 g/l	5.5 g/l	5.5 g/l
	Protocol (C)			**Protocol (D)**		
MS basic medium	1X	1X	1/2 X	1X	1X	1/2 X
IAA	0.5 mg/l	0.5 mg/l	_	-	-	-
BAP	0.2 mg/l	0.2 mg/l	0.02 mg/l	-	-	-
NAA	-	-	-	0.2 mg/l	0.2 mg/l	-
Kinetin	0.1 mg/l	-	-	2.0 mg/l	2.0 mg/l	-
Sucrose	30 g/l	30 g/l	15 g/l	30 g/l	30 g/l	15 g/l
Agar	5.5 g/l	5.5 g/l	5.5 g/l	5.5 g/l	5.5 g/l	5.5 g/l

medium conditions for the high potentiality of shoot formation.

MATERIALS AND METHODS

Plant material and *in vitro* culture response

Flower heads of nine *C. morifolium* cultivars were used in this study. A list of these cultivars and their characteristics is given in Table 1. Ray florets were collected from the inner whorl of a half bloom flower head of the *C. morifolium* cultivars, after approximately 110 days of planting the cuttings in the greenhouse. Ray florets of chrysanthemum explants were surface sterilized by immersing in 70% ethanol containing one drop of Tween 20, for 2 min, followed by immersing in 0.1% mercuric chloride for 4 min, then, washed with six changes of sterile distilled water.

The ray florets were, aseptically, placed in Petri dishes containing 25 ml of culture medium. Full details of the four medium protocols used for callus induction, differentiation and root induction are given in Table 2. Each dish with five explants was considered as a replication. Cultures were incubated in a growth chamber at 25 ± 2°C under 16 h illuminations (200 Lux, daylight florescent tubes). After four weeks of incubation, callus induction response was recorded and the following determinations were made for each Petri dish: Callus induction, Callus weight and the percentage of somatic embryogenesis derived from the ray floret base explants.

Calli with somatic embryogenesis, derived from the ray floret explants, were transferred to jars each containing 30 ml of the callus differentiation sequence media (Table 2) and the following determinations were made for each jar after 6 weeks of incubation: Shoot formation, number of shoots per explant and shoot length derived from the ray floret base explants. Regenerated shoots were

Table 3. Analysis of variance for callus induction (%), callus weight (gm/explant) and somatic embryogenesis (%) as affected by chrysanthemum cultivars, media protocols and their interactions.

S.O.V.	D.F.	M.S.		
		Callus Induction (%)[a]	Callus Weight (gm)	Somatic embryogenesis (%)[a]
Blocks	9	875.20 $^{N.S.}$	0.004 $^{N.S.}$	150.130 $^{N.S.}$
Cultivars (A)	8	8615.03 **	0.1527 **	1589.31 **
Media (B)	3	65143.58 **	1.1374 **	3645.60 **
A x B	24	2988.34 **	0.0496 **	397.69 **
Error	315	345.83	0.009	105.93

[a] Data were subjected to arcsine transformation; **highly significant at 0.01 probability level; $^{N.S.}$ not significant at 0.05 probability level.

transferred to jars with half strength Murashige and Skoog (1962) medium with different growth regulators for root induction.

Statistical analysis

Data were statistically analyzed as a 2- factor experiment (cultivars and medium protocols) in a randomized complete block design (RCBD) with ten replicates. Data with percentage were subjected to arcsine transformation prior to statistical analysis (Steel and Torrie, 1980). Comparisons among means were made using the least significant differences test (LSD). The data were analyzed, using statistical analysis system (SAS) programme, version 6 (1985).

RESULTS AND DISCUSSION

In vitro culture and plant regeneration of chrysanthemum cultivars

Establishing reliable *in vitro* plant regeneration is a pre-requisite step before conducting any *in vitro* selection experiments. If a particular plant species shows no competence for *in vitro* regeneration, the chances are that regeneration from useful variant cell lines in the same species may also be unsuccessful. Once a tissue culture system capable of plant recovery has been established, the next step is to apply the developed system to *in vitro* mutagenesis studies. Having this as a principle, the present work was initiated with the aim of finding a tissue culture system competent to regenerate plants from cultured tissue of chrysanthemum.

Response of explants to callus induction

Data on production of callus, derived from ray floret explants of nine cultivars of chrysanthemum (*C. morifolium),* were recorded after four weeks of incubation. These explants were incubated on media previously developed and successfully employed by other investigators for chrysanthemum. The analysis of variance, presented in Table 3 indicates that callus induction was highly significantly influenced by differences within chrysanthemum cultivars and medium protocols. The two - way- interaction was, also, highly significant.

Callus formation varied widely among chrysanthemum cultivars. The percentage of explants that developed calli ranged from 73.84% (Ping Pong) to 25.67% (Palisade White) among the cultivars across the four medium protocols with an average of 48.28% (Table 4).

The response of callus induction varied according to the medium. Table 4 indicates that the medium protocols A and B gave the highest average of callus induction, 68.83 and 74.09% respectively, across cultivars with significant differences from the other protocols.

The interaction was highly significant between cultivars and medium protocols. The cultivars “Delistar White” and "Cassandra" gave the highest callus induction (90%) with medium protocol B, while cultivar "Delistar White" had significantly the lower callus induction percentage (7.97 and 21.92%) when its explants were cultured on medium protocols C and D, respectively. On the other hand, cultivars "Cassandra" and "Sunny Cassandra" had the significantly lowest callus induction percentage (5.31%) and (2.66%), respectively, when its explants were cultured on medium protocol D (Table 4).

The results in Table 4 shows that calli were not observed with the cultivar Delistar yellow on the medium protocol C as well as the cultivar Palisade white on the medium protocols C and D. The callus was light green in colour and began to develop darker green regions within the callus for many genotypes on different media.

Callus weight

Callus growth and development are influenced by a complex relationship between the genotypes and the constituents of the medium protocol. The estimates of significance for the effects of cultivars, medium protocols and their interaction, on callus weight, are presented in Table 5. The analysis of variance indicated that the callus weight was highly significantly influenced by cultivars,

Table 4. Means of callus induction (%) as influenced by chrysanthemum cultivars, media and their interactions.

Cultivar	Callus induction (%)				
	Medium protocol				Cultivar mean (%)
	A	B	C	D	
Delistar Yellow	58.60	56.18	0.0	23.08	34.47
Delistar White	84.69	90.00	7.97	21.92	51.15
Palisade White	45.12	57.57	0.0	0.0	25.67
Palisade Yellow	44.08	54.00	33.47	22.16	38.43
Sunny Ping Pong	83.42	82.03	9.24	42.34	54.26
Ping Pong	84.69	78.11	57.92	74.66	73.84
Rodet		82.03		14.55	52.38
Cassandra	80.76	90.00	70.50	5.31	61.64
Sunny Cassandra	76.72	76.84	14.55	2.66	42.69
Medium mean (%)	68.83	74.09	27.25	22.96	48.28

L. S. D. $_{(0.05)}$ for cultivar means = 8.182; L. S. D. $_{(0.05)}$ for medium means = 5.454; L. S. D. $_{(0.05)}$ for cultivar x medium protocol interaction = 16.38.

Table 5. Means of callus weight (g /explant) as influenced by chrysanthemum cultivars, media and their interaction.

Cultivar	Callus weight (g/explant)				
	Medium protocol				Cultivar mean (g/explant)
	A	B	C	D	
Delistar Yellow	0.162	0.110	0.0	0.059	0.083
Delistar white	0.168	0.233	0.006	0.101	0.127
Palisade White	0.075	0.333	0.0	0.0	0.102
Palisade Yellow	0.150	0.215	0.051	0.0	0.108
Sunny Ping Pong	0.226	0.339	0.011	0.049	0.156
Ping Pong	0.261	0.393	0.185	0.106	0.236
Rodet	0.084	0.139	0.105	0.019	0.087
Cassandra	0.323	0.458	0.216	0.003	0.249
Sunny Cassandra	0.087	0.374	0.024	0.021	0.127
Medium mean (g/explant)	0.171	0.288	0.066	0.042	

L. S. D. $_{(0.05)}$ for cultivar means = 0.0415; L. S. D. $_{(0.05)}$ for medium means = 0. 0277; L. S. D. $_{(0.05)}$ for cultivar x medium protocol interaction = 0.083.

medium protocols and their interaction. The growth rate of callus was dependent on the cultivar and the medium employed. The cultivar “Cassandra” produced the highest callus weight (0.249 gm/explant) across medium protocols (Table 5) and it was significantly different from all other cultivars except Ping Pong cultivar. On the other hand, the cultivars “Delistar Yellow” and "Rodet" had the significantly lowest callus weight (0.082 and 0.087 gm/ explants, respectively). Among medium protocols, B medium protocol gave the highest callus weight (0.288 gm/explant) and was significantly different from all other medium protocols (Table 5). The cultivar “Cassandra”, significantly, gave the highest response to callus weight for all medium protocols.

Finally, it can be suggested that before utilizing tissue culture techniques as tools in crop improvement, it is necessary to determine the factors influencing callus formation, its quality during induction and maintenance and subsequently shoot regeneration from callus. The previous results provide an indication of the relative importance of genotype, medium protocols and explant effect in culture response. These results showed that the growth rate of the callus was dependent on the genotypes and the culture medium employed. The efficiency of callus induction and callus growth rate have been reported to be in part genotype dependent (Ohishi and Sakurai, 1988; Kaul et al., 1990; Tanaka et al., 2000; Mandal and Datta, 2005; Barakat, 2008). They compared

Table 6. Means of embryogenic callus (%) as influenced by chrysanthemum cultivars, media and their interaction.

Cultivar	Embryogenic callus (%)				
	Medium protocol				Cultivar mean (%)
	A	B	C	D	
Delistar Yellow	9.51	20.17	0.0	0.0	7.42
Delistar White	32.66	36.47	0.0	9.58	19.68
Palisade White	0.0	0.0	0.0	0.0	0.00
Palisade Yellow	0.0	6.17	0.0	0.0	1.54
Sunny Ping Pong	0.0	0.0	0.0	0.0	0.00
Ping Pong	11.32	9.24	5.66	0.0	6.55
Rodet	12.16	15.47	0.0	0.0	6.91
Cassandra	11.82	28.63	7.97	0.0	12.10
Sunny Cassandra	8.66	10.73	0.0	0.0	4.85
Medium mean (%)	9.57	14.10	1.51	1.07	

L. S. D. $_{(0.05)}$ for cultivar means = 4.528; L. S. D. $_{(0.05)}$ for medium means = 3.019; L. S. D. $_{(0.05)}$ for cultivar x medium protocol interaction = 9.0.

the effect of plant genotype and media modifications on culture behavior. Their results provide an indication of the relative importance of genotype and media effects on culture responses.

Somatic embryogenesis

Statistical analysis of embryogenic callus was highly significant influenced by differences between cultivars, medium protocols and their interaction (Table 3). The highest percentage of embryogenic callus, across the four medium protocols resulted from "Delistar White" cultivar (19.68%) and it was significantly different from all other cultivars (Table 6). On the other hand, no embryo-genic callus was observed with any medium protocol for the "Palisade White" cultivar (Table 6). Medium protocol B produced the highest percentage of embryogenic callus (14.10%) across the chrysanthemum cultivars and was significantly different from all other medium protocols (Table 6).The interaction between cultivars x medium protocols was highly significant. The variation ranged from 36.47% for cultivar "Delistar White" with medium protocol B to 0.0% for the cultivar "Palisade white" with all medium protocols (Table 6). These results indicate that somatic embryogenesis of chrysanthemum was induced by high concentrations of both BAP (6-benzyl amino purine) and NAA (naphthalene acetic acid) in medium protocols A and B. These results agree with previous studies (May and Trigiano, 1991; Pavingerona et al., 1994; Urban et al., 1994, Tanaka et al., 2000; Mandal and Datta, 2005). They reported that successful somatic embryogenesis and subsequently plant regeneration of chrysanthemum were obtained from different callus cultures. It seemed that both BAP (1.0 mg/L) and NAA (0.5 mg/L) concentrations were more effective for the somatic emberyogenesis of chrysanthemum.

Morphogenetic response

Shoot formation

The ability to regenerate large number of shoots from cultured tissues is important for the success of most of biotechnological techniques such as *in vitro* mutagenesis. In the present investigation, the callus derived from chrysanthemum ray floret explants, which was induced on the induction media (Table 2), was sub-cultured on a wide range of differentiation media. When calli were placed on differentiation media, some calli differentiated into shoots and some did not differentiate. Shoots were subcultured on rooting medium. Plants were established *ex-vitro* and then established into soil till flowering.

Analysis of variance for the formation of shoots derived from the induced callus in chrysanthemum indicated that the effects of cultivars and medium protocols and their interaction were highly significant (Table 7).

The results in Table 8 showed that the cultivar "Delistar White" produced the highest mean value of shoot formation (21.90%) across medium protocols. The medium protocol B (15.68%) was significantly better than all other medium protocols across the cultivars. The results also revealed that there was a significant interaction between cultivars and medium protocols. For instance, Delistar White and Cassandra cultivars gave the highest percentage of shoot formation (41.43 and 33.58%, respectively) when calli differentiated on medium protocol B (Figure 1). However, the same protocol resulted in low percentage of shoot formation for some other cultivars such as Palisade

Table 7. Analysis of variance for the effect of chrysanthemum shoot formation (%), number of shoots per explant and shoot length (cm) as influenced by cultivars, media and their interaction.

S.O.V.	D.F.	M.S.		
		Shoot formation (%)[a]	Number of shoots per explant	Shoot Length (cm)
Cultivars (A)	8	2125.32 **	2.619 **	3.279 **
Media (B)	3	4395.65 **	5.129 **	8.027 **
A x B	24	507.58 **	0.746 **	0.8868 **
Error	315	126.11	0.1247	0.269

[a]Data were subjected to arcsine transformation; ** highly significant at 0.01 probability level.

Table 8. Means of shoot formation (%) as influenced by chrysanthemum cultivars, media and their interaction.

Cultivar	Shoot formation (%)				
	Medium protocol				Cultivar mean (%)
	A	B	C	D	
Delistar Yellow	9.51	20.17	0.0	0.0	7.42
Delistar White	36.58	41.43	0.0	9.58	21.90
Palisade White	0.0	0.0	0.0	0.0	0.00
Palisade Yellow	0.0	6.16	0.0	0.0	1.54
Sunny Ping Pong	0.0	0.0	0.0	0.0	0.00
Ping Pong	11.31	9.58	5.66	0.0	6.64
Rodet	12.16	16.74	0.0	0.0	7.23
Cassandra	15.85	33.58	12.92	0.0	15.59
Sunny Cassandra	8.66	13.50	0.0	0.0	5.54
Medium mean (%)	10.45	15.68	2.06	1.06	

L. S. D. $_{(0.\ 05)}$ for cultivar means = 4.94; L. S. D. $_{(0.\ 05)}$ for medium means = 3.29; L. S. D. $_{(0.\ 05)}$ for cultivar x medium protocol interaction = 9.89.

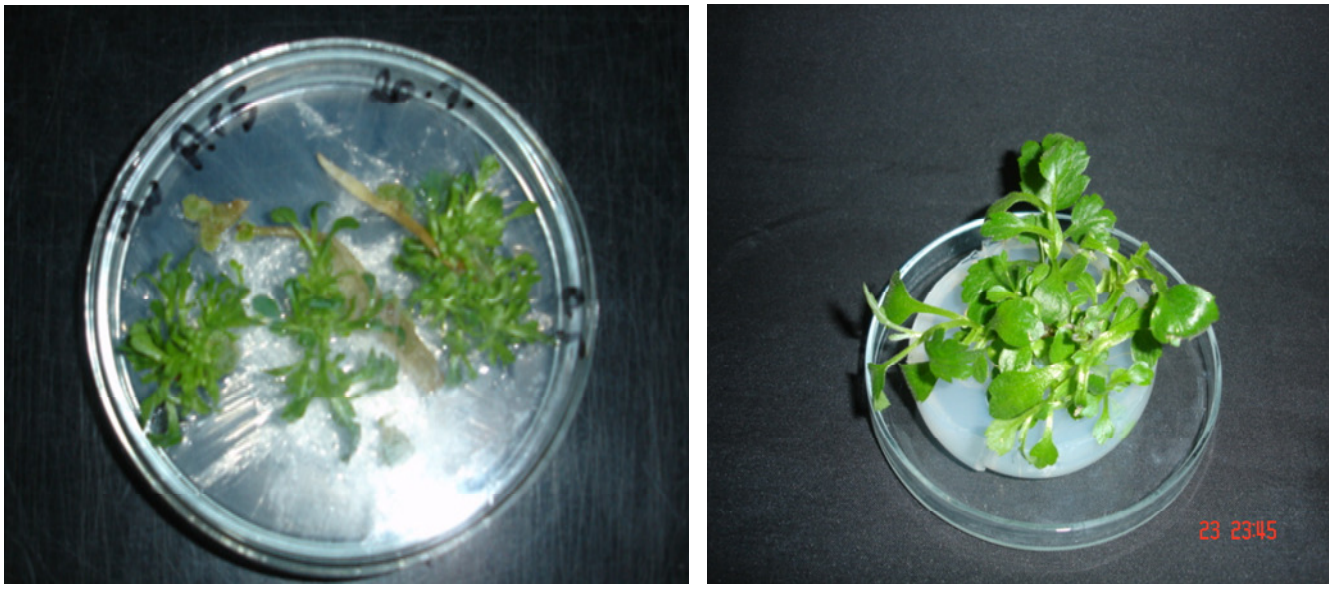

Figure 1. Shoot formation derived from ray floret explant.

Table 9. Means of shoot number per chrysanthemum explant as influenced by cultivars, media and their interaction.

Cultivar	Number of shoots				
	Medium protocol				Cultivar mean Shoot No.
	A	B	C	D	
Delistar Yellow	0.30	0.60	0.0	0.0	0.23
Delistar White	1.40	1.70	0.0	0.20	0.83
Palisade White	0.0	0.0	0.0	0.0	0.00
Palisade Yellow	0.0	0.20	0.0	0.0	0.05
Sunny Ping Pong	0.0	0.0	0.0	0.0	0.00
Ping Pong	0.40	0.30	0.20	0.0	0.23
Rodet	0.50	0.70	0.0	0.0	0.30
Cassandra	0.40	0.80	0.30	0.0	0.38
Sunny Cassandra	0.30	0.30	0.0	0.0	0.15
Medium mean shoot no.	0.37	0.51	0.06	0.02	

L. S. D. $_{(0.05)}$ for cultivar means = 0.155; L. S. D. $_{(0.05)}$ for medium means = 0.104; L. S. D. $_{(0.05)}$ for cultivar x medium protocol interaction = 0.317

Yellow, Ping Pong and Sunny Cassandra (6.16, 9.58 and 13.80%, respectively) and gave no shoot formation at all by the cultivars Palisade White and Sunny Ping Pong.

In addition, the ability of fresh callus to differentiate into plantlets depends on the hormone level of the initial callus induction medium, as well as on the cultivar or the donor plant. The cultivar factors, which are considered important for eliciting success in morphogenesis, have been listed by Thorpe (1980). These factors included: (a) selection of the organ to be used as the tissue culture source, (b) the appropriate physiological and ontogenetic age of the organ, (c) the suitable season in which explant is obtained, (d) the size of the explant and (e) the overall quality of the plant from which explants are derived.

Results from the present investigation showed that there was significant genotype x medium interaction for morphogenetic response. The probable reasons for differences in morphogenetic response *in vitro* may be attributed to (a) genetic differences among the genotypes used (b) differences in the growth regulators, or (c) differences in the growth conditions and age of the source of explants.

Prasad et al. (1983) reported that the rate of shoot multiplication is genotypic dependent in *Dendranthema grandiflorum*. Bhattacharya et al. (1990) studied the influence of different growth regulators on the *in vitro* morphogenesis of chrysanthemum. They reported that a combination of 0.1 mg/l IAA and 0.2 mg/l BAP was most appropriate for callus formation and for the regeneration of shoots from callus. Rademaker and de Jong (1990) reported that cultivar and explant type had a greater effect on regeneration than the type of medium.

Number of shoots per explant

Statistical analysis of the shoot number per explant derived from callus of chrysanthemum ray floret explant (Table 7) revealed highly significant differences among cultivars, medium protocols and their interaction. Results in Table 9 shows that the cultivar "Delistar White" produced the highest mean value of shoot number (0.83shoot/explant) across medium protocols which were significantly different from all other cultivars. The medium protocol B showed the highest potential for shoot number (0.51shoot/explant) and it was significantly higher than the other protocols (Table 9). The cultivar x medium protocol interaction was highly significant. Higher mean values for the number of shoots in Delistar White, Rodet and Cassandra (1.7, 0.7 and 0.8% respectively) were gotten in the medium protocol B. However, lower mean values for the number of shoots in the same cultivars were obtained in the medium protocols C and D (Table 9).

Shoot length

The analysis of variance, presented in Table 7 indicates that shoot length was highly significantly influenced by chrysanthemum cultivars, medium protocols and their interaction.

Results in Table 10 shows that the cultivar "Delistar White" produced the highest mean value of shoot length (0.78 cm) across medium protocols. The medium protocol B showed the greatest potential for shoot length

Table 10. Means of shoot length (cm) as influenced by chrysanthemum cultivars, media and their interaction.

Cultivar	Shoot length (cm)				
	Medium protocol				Cultivar mean (cm)
	A	B	C	D	
Delistar Yellow	0.33	0.28	0.0	0.0	0.28
Delistar White	1.54	0.78	0.0	0.33	0.78
Palisade White	0.0	0.00	0.0	0.0	0.00
Palisade Yellow	0.0	0.09	0.0	0.0	0.09
Sunny Ping Pong	0.0	0.00	0.0	0.0	0.00
Ping Pong	0.52	0.32	0.35	0.0	0.32
Rodet	0.89	0.52	0.0	0.0	0.52
Cassandra	0.92	0.69	0.48	0.0	0.69
Sunny Cassandra	0.40	0.20	0.0	0.0	0.20
Medium mean (cm)	0.51		0.09	0.04	

L. S. D. $_{(0.05)}$ for cultivar means = 0.228; L. S. D. $_{(0.05)}$ for medium means = 0.152.

(0.63 cm) across cultivars and it was significantly superior to all other medium protocols except the medium protocol A. The data, also, revealed that cultivars x medium protocols interaction was highly significant. In "Delistar White" cultivar, protocols A and B were significantly superior to C and D protocols in shoot length. On the other hand, in "Palisade White" and "Sunny Ping Pong" cultivars, none of the applied protocols showed response in shoot length in comparison to the other protocols. Moreover, protocol B showed significant increase in shoot length in "Cassandra", "Rodet" and "Delistar Yellow" cultivars (1.35, 1.18 and 0.77, respectively) compared to other protocols (Table 10). The results of the present investigation indicated that the cultivar "Delistar White" with the medium protocols A and B could be successfully utilized for further *in vitro* mutagenesis to select several unique traits relevant to chrysanthemum.

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Full Length Research Paper

Arbuscular mycorrhizal fungi improve the growth of olive trees and their resistance to transplantation stress

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Two native Algerian mycorrhizal fungi (*Glomus mosseae* and *Glomus intraradices*) were tested for their effect on the growth of micropropagated olive tree (*Olea europaea* L.). The effect of inoculation of plantlets with *G. mosseae* was also compared with chemical fertilization using osmocote. Specific molecular techniques were then used to detect the presence of the two fungi. Highly significant increases in growth were evident for inoculated plants compared with uninoculated ones. For a slightly lower shoot growth, *G. mosseae* doubled the root growth of the inoculated plantlets, compared to that of the fertilized plants. This change in the root: shoot ratio permitted greater utilization of soil resources and strengthened the plant's capacity to resist transplantation shock and water stress. The abundance of the two fungi in the roots of wild olives just as in the inoculated olives is indicative of the predominance of *G. intraradices* when the natural microflora is present.

Key words: Arbuscular mycorrhizal fungi, olive tree, inoculation, chemical fertilizer, transplantation, growth.

INTRODUCTION

The olive tree is a particularly mycotrophic plant (Roldan-Fajardo and Barea 1986) and plays an important part in the economy of several countries in the Mediterranean region where there is predominant water stress. These regions have a long dry season where low water availability has a large impact, notably in the transport and the uptake of the solutes needed for vegetative growth (Monneveux and This, 1997).

Mycorrhizal fungi, which are active in the rhizosphere, take part in the cycles and transfer of the mineral elements in the soil and into the roots (George et al., 1992). Certain minerals such as phosphorus, iron, zinc and copper are of very limited mobility in the soil and are only found in extremely low concentrations in soil solution. Their use by plants may be increased by the presence of symbiotic microflora, notably mychorrhizal fungi, which assist their nutrition, growth (Gianinazzi et al., 1982; Smith and Read, 1997; Jeffries et al., 2003; Duponnois et al., 2005) and their tolerance to different types of biotic and abiotic stress (Rosendahl and Rosendahl, 1991; Caravaca et al., 2003a; Al-Karaki and McMichael, 2004; Selosse et al., 2004).

In vitro production of micro-plantlets of the olive is a developing biotechnology that is well-suited to new methods of plant production. The aim of the technique is to eliminate pathogenic microorganisms, but it also eliminates beneficial ones such as mycorrhizal fungi, which help plants cope with transplantation stress (Ruiz-Lozano et al., 1996; Porras-Soriano et al., 2009) once they are placed into normal culture conditions. Routine inoculation of young plants in the nursery has been a suggested strategy (Plenchette, 2000).

The inoculation of *in vitro* plantlets with arbuscular mycorrhizal (AM) fungi to improve their growth has been studied for many different cultures (Lovato et al., 1996;

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Abbreviations: AM, *Arbuscular mycorrhizal*; **MD,** mycorrhizal dependence; **PCR,** polymerase chain reaction; **ITS,** internal transcribed spacer; **MF,** mycorrhizogenic fungi; **rDNA,** ribobosomal deoxyribonucleic acid.

Verma and Arya 1998; Caravaca et al., 2003a; Ouahmane et al., 2007). Results vary according to the mycorrhizal strain and plant cultivars (Azcon-Aguilar and Barea, 1997; Calvente et al., 2004; Soriano et al., 2006; Binet et al., 2007). This emphasizes the importance of testing the effectiveness of different mycorrhizal fungi to create the appropriate inoculum for production of top-quality olive plants.

To study the potential of the mycorrhizal fungi *Glomus mosseae* and *Glomus intraradices* to stimulate the growth of micropropagated olive plants and to compare their capacity to resist water stress after re-potting with that of plantlets receiving chemical fertilizer (osmocote), controlled experiments were carried out using strains of *G. mosseae* and *G. intraradices* isolated from Algerian olive plantations. Molecular tools were used in parallel to assist in the search for the appropriate inoculum capable of co-existence with the natural field community, by detecting the different taxons of native Glomeromycetes that colonize the root system of the wild olive *Olea oleaster* (Hoffm and Link) grown in nurseries. This latter approach is widely-used as root-stock in commercial olive growth in this country. A molecular detection was also carried out on *in vitro* plantlets of the Aglandau variety of the olive-tree with an aim of comparing the presence of *G. mosseae* and *G. intraradices* inoculated separately in controlled conditions and in absence of the native microflora.

The study objectives were to evaluate the mycorrhizogenic potential of *G. mosseae* and *G. intraradices* on the growth of micropropagated olive plants, compare the capacity to resist water stress after re-potting of *in vitro* cultivated olive plantlets that received osmocote with that of plantlets inoculated with *G. mosseae* and to detect mycorrhizogenic fungi by molecular methods for their study in the field.

MATERIALS AND METHODS

Extraction of spores of arbuscular fungi and controlled inoculation

G. mosseae and *G. intraradices* spores used in these experiments were initially extracted by wet sieving (Gerdemann and Nicholson, 1963) using soil from Algerian olive groves situated in the commune of Bekouche Lakhdar, wilaya de Skikda (36° 71' N and 7° 29' E). *G. mosseae* (Nicol. and Gerd.) Gerdemann and Trappe and *G. intraradices* Schenck and Smith identified both by morphological and molecular criteria, were chosen according to their abundance in the olive tree rhizosphere and their abundance after trapping and monospecific multiplication on the leek (*Allium porrum* L.). They were inoculated in a sample of olive plantlets (variety Aglandau) produced by the experimental group of INRA *in vitro* (Dijon, France). After 2 weeks of acclimatization in a greenhouse, the plantlets were potted into individual 400 ml pots filled with a sterile mix of soil and gravel (3:1, v:v). The soil had been previously γ-irradiated radiation (10 kGy) and the gravel steam-disinfected in an autoclave (120°C for 2 h). The soil (pH 8.0; 20 ppm P_2O_5) was from the domaine of Epoisses (INRA Center at Dijon-Bretenieres). At the time of re-potting, each plant was inoculated with 100 spores of *G. mosseae* or *G. intraradices*; five duplicates were made for each treatment and uninoculated plants were kept as a control. The experiment was carried out in controlled greenhouse conditions (under normal daylight for around 12 h, average daily temperature 18 - 22°C, 60 - 70% relative humidity). The plants were watered daily with distilled water. After 9 months growth in individual pots, the height, number of inter-nodes and aerial and root biomass (fresh and dry; drying at 70°C for 1 week) were measured. The efficiency of the mycorrhizal inoculum was calculated as the percentage increase in height, number of inter-nodes, root fresh mass, aerial fresh mass, root dry mass and aerial dry mass produced by each AM fungus over the uninoculated control. The mycorrhizal dependence (MD) of the plants was found using the formula of Plenchette et al., (1983): 100 × [(dry mass of mycorrhizal plants -dry mass of control plants) ÷ dry mass of mycorrhizal plants].

Test of resistance to water stress

After acclimatization in the greenhouse and at the time of re-potting into the 400 ml pots, 10 olive plantlets (variety Aglandau) were inoculated with 25 carpophores of *G. mosseae* per plant. Ten other plants were treated with chemical fertilizer; for this, a granule of 2.5 g of osmocote was placed on the soil surface in each pot. The substrate was the same as that used in the previous experiment. Daily watering was made according to the needs in the field. The osmocote fertilizer was composed of 15% nitrogen, 10% anhydrous phosphate, 15% potassium oxide, 2% magnesium oxide, 4.5% sulphur, 0.02% boron, 0.05% copper, 0.15% iron, 0.075% manganese and 0.015% zinc. After 6 months growth, all plants were again re-potted, this time into 1 L pots with the same soil mixture and the osmocote granules were removed. From this point onwards watering was reduced. The soil water content was kept at 10% of the field capacity. A month after re-potting, the fresh mass of the aerial and root growth was measured and the ratio of root: shoot growth was recorded.

Staining and estimation of mycorrhizal colonization

Endomycorrhizal colonization was estimated on the set of inoculated plantlets used in the experiment. Five repetitions of 1 g of root samples taken from each plant were incubated in a 10% solution of KOH at 90°C for 1 h and then stained with Trypan Blue following the method of Phillips and Hayman (1970). AM colonization was estimated for 30 root fragments of 1 cm length mounted in a drop of glycerol and observed in a photonic microscope, with annotation according to the method of Trouvelot et al. (1986) using the MYCOCALC computer programme (www.dijon.inra.fr/mychintec/Mycocalc-prg/download.html).

Statistical analysis

Results are presented as the mean ± standard deviation. Comparisons of means between inoculated and uninoculated plants were made for all the parameters tested and were distinguished by the Dunnett test ($P < 0.05$). Comparisons between plants inoculated with *G. mosseae* and those inoculated with *G. intraradices* were made by the Turkey test ($P < 0.05$). Between group comparisons of those plants inoculated with *G. mosseae* and those fertilized with osmocote were made using Student's T test at a significance level of 5%.

Table 1. Effect of inoculation with *G. mosseae* and *G. intraradices* native to Algeria on the growth of olive plants.

Inoculation	Parameters measured [z]							
	H (cm)	NInt	RFM (g)	AFM (g)	RDM (g)	ADM (g)	M (%)	MD (%)
GM	51.87 ± 5.32*a*	18.25 ± 4.03 *a*	6.66 ± 1.25 *a*	9.44 ± 1.74 *a*	2.78 ± 0.70 *a*	4.89 ± 1.31 *a*	51.06 ± 7.4	73.9
GI	46.9 ± 6.99 *a*	20.4 ± 3.51 *a*	4.09 ± 1.10 *a*	7.75 ± 1.33 *a*	1.80 ± .0.54 *a*	3.37 ± 0.55 *a*	69.96 ± 4.5	67.3
UI	9.25 ± 1.76	5.25 ± 0.96	1.42 ± 0.34	1.96 ± 0.26	0.44 ± 0.12	1.12 ± 0.15	/	/

GM: *G. mosseae*; GI: *G. intraradices*; UI: uninoculated; H: Height; NInt: number of internodes; RFM: root fresh mass; AFM: aerial fresh mass; RDM: root dry mass; ADM: aerial dry mass; M: intensity of colonization by arbuscular mycorrhizal fungi; MD: mycorrhizal dependency.
[z]Mean ± standard error, n = 5; *a* indicates results that are not significantly different (P < 0.05).

Molecular detection of arbuscular fungi

With the aim of using molecular tools to detect inoculated fungi and those existing naturally in the soil, two groups of plants were created. One group was comprised of olive plants (Aglandau variety) obtained from *in vitro* culture growth on sterile soil for 9 months after inoculation with *G. mosseae* or *G. intraradices* spores (100/plant). Another group was comprised of oleaster plants, *Olea oleaster* (Hoffm. and Link) grown in polyethylene bags containing 5 kg of natural soil from the Belkhir nursery (Algeria). Root samples were taken from all plants in each group and were mixed to make one single sample for each experimental group. From this sample, five sub-samples of 100 mg each of root material were used for the extraction of ribosomal DNA (rDNA) and approximately 1 g was used to estimate the level of mycorrhizal colonization (M%).

Extraction and purification of rDNA

One hundred milligrams (100 mg) of each root sample was crushed in 1.5 ml Eppendorf tubes using a micro-pestle. The DNA was extracted and purified using a Macherey-Nagel NucleoSpin kit, following the protocol furnished by the supplier and collected in 50 µl of ultra-pure water.

Polymerase chain reaction (PCR) amplification of rDNA

Double amplification (nested PCR) allowed amplification of a targeted part of the large subunit covering domains D1 and D2 of the 5' region and the internal transcribed spacer (ITS) region of the rDNA root samples. Nested PCR was carried out using the universal primer ITS3 (White et al.,1990) and the primer specific to fungi, FLR2 (Trouvelot et al., 1999) at the first PCR and the specific primers for the different genera and species of fungi AM at the second PCR, which were FLR3/FLR4 (Gollotte et al., 2004) for the detection of the set of mycorrhizogenic fungi (MF), GloITS/LR3rev for the detection of the *Glomus* genus, 8.24/FLR3 (Farmer et al., 2007) for the detection of *G. intraradices*, 5.25/FLR4 (van Tuinen et al., 1998) for the detection of *G. mosseae*, 23.5/FLR4 mixed primers for the detection of *Scutellospora*/*Gigaspora* and Acaul/FLR4 for the detection of *Acaulospora*. Five repeats were made. For a reaction volume of 20 µl, the PCR product contained 2 µl of 10× buffer (100 mM Tris-acetate pH 9, 500 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100 in phosphate buffer saline (PBS), 2 mg/ml bovine serum albumin, 2 µl of 1 mM dNTP, 0.1 µl of each primer (100 mM), 0.05 µl of Taq polymerase (15 U/µl, Qbiogene) and 14.85 µl of ultra-pure water. Root DNA extracts were undiluted of diluted (1:10, 1:100, 1:1000, 1:10 000) and 1 µl of each suspension was added to 19 µl of PCR mixture. To check on the effectiveness of PCR, a control using water was used for the first and second PCR runs. The amplification was carried out in a Biometra thermocycler programmed in 15 cycles. The PCR programme consisted of an initial denaturation for 5 min at 95°C, then each cycle included denaturation of the double stranded DNA (94°C for 1 min), hybridization (58°C for 1 min) and polymerization (72°C for 1.5 min) for cycles 1 - 14. The last cycle was followed by a final elongation at 72°C for 10 min. The amplified product was used in 1 µl volumes for a second amplification. The second PCR programme involved 30 cycles with the same steps as before. PCR amplimers were separated by electrophoresis in a 1.4% agarose gel using 7 µl mixed with one drop of the appropriate buffer and placed into the wells of the gel. Three microliters (3 µl) of 1 kb ladder marker were coincidentally used. After migration at 100 V for 25 min, the gel was stained with ethidium bromide and observed under ultraviolet transilluminance. The DNA was checked by observation of fluorescent bands.

RESULTS

Effectiveness of *G. mosseae* and *G. intraradices* on olive plant

The data in Table 1 demonstrate the strong dependence of the olive *(Olea europea* L.) on mycorrhizae and the positive effect that these had on the species. After 9 months growth, all olive plants inoculated with *G. mosseae* or *G. intraradices* showed greater development than uninoculated plants. The former displayed greater growth in height, number of inter-nodes and fresh and dry weights for all aerial parts than the uninoculated plants. *G. mosseae*, while having a significantly lower level of colonization (51.06%) than *G. intraradices* (69.96%), had a marked effect on growth of the olive plant. The values for gain in total fresh weight were 376% for *G. mosseae* and 226% for *G. intraradices* (Table 2). But presently, despite the strong mycorrhizal dependence for *G. mosseae* (Table 1), the one-sided analysis of variance did not show any significant difference for all the measured parameters in the two groups of plants inoculated with these fungi. Both proved to be beneficial for growth. It should also be noted that the positive effect of these two species of fungi could arise from ecological compatibility. Fungi collected in the rhizosphere of olive groves seem to

Table 2. Efficiency of inoculation by *G. mosseae* and *G. intraradices*.

Inoculation	H (%)	Nint (%)	TFM (%)	RFM (%)	AFM (%)	RDM (%)	ADM (%)
GM	460	247	376	369	381	531	336
GI	407	288	226	188	295	309	200

GM: *G. mosseae*; GI: *G. intraradices*; UI: un-inoculated; H: Height; NInt: number of internodes; TFM: total fresh mass; RFM: root fresh mass; AFM: aerial fresh mass; RDM: root dry mass; ADM: aerial dry mass.

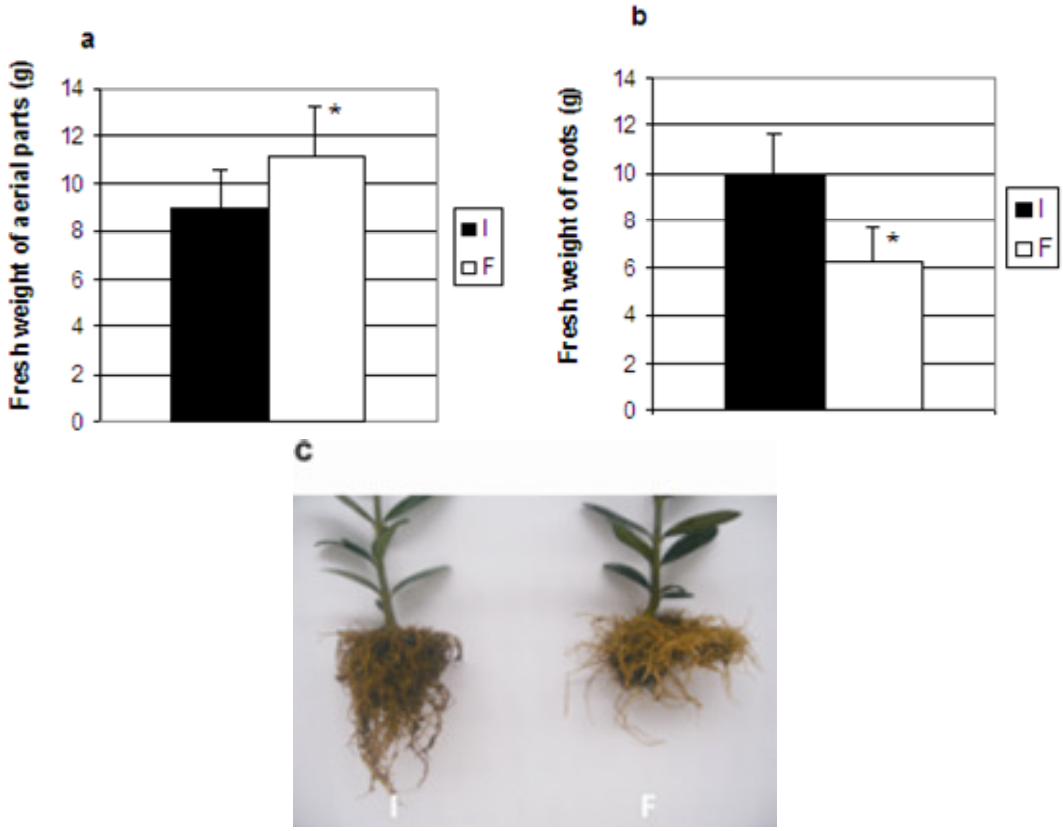

Figure 1. Fresh weight of shoot (a) and roots (b) and morphology of the root system (c) of plants inoculated with *G. mosseae* (I) and those fertilized with osmocote (F). *: I vs. F ($P < 0.05$).

act beneficially on the genotypes of the trees.

Effect of arbuscular mycorrhization on resistance to water stress after re-potting

Comparison of plants inoculated with *G. mosseae* with those given chemical fertilizer (osmocote) allowed the positive effects of mycorrhization to be assessed in several aspects. One aspect concerned the effect on root development. For significantly lower development of aerial parts (Figure 1a), the root system of mycorrhizal plants was more developed (nearly double) compared to plants receiving osmocote (Figure 1b) and presented a different morphology (Figure 1c). Another aspect concerned the effect on tolerance to the stress of re-potting. A week after re-potting, observations made on plants inoculated with *G. mosseae* and those with chemical fertilizer showed a clear difference in reaction to repotting shock. Plants receiving fertilizer seemed to have experienced a shock that led to loss of weight, withering of aerial parts and a loss of leaves, while inoculated plants maintained their turgidity and vigour (Figure 2). These results could be explained by the presence of the fungus, which had a positive effect on the root to shoot ratio (Figure 3a). The effect was significantly higher (almost double) for mycorrhizal plants. A more highly developed and dense root system allowed the plant greater exchange between the roots and the aerial parts and more rapid water uptake (Figure 3b). The consequence of this was greater

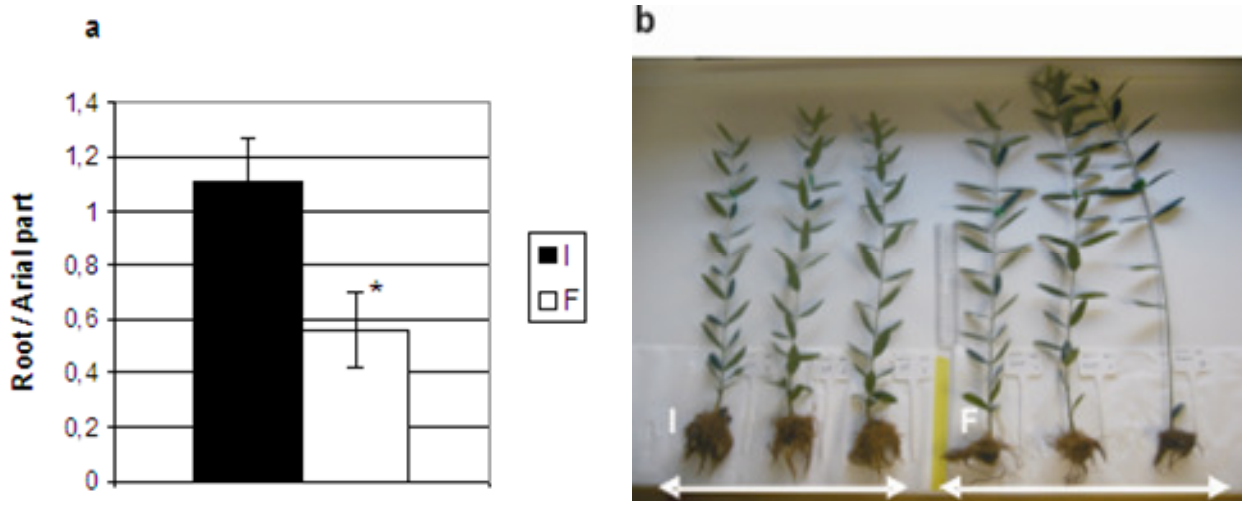

Figure 3. Relationship between root and aerial growth in plants inoculated with *G. mosseae* (I) and fertilized plants (F). * I vs. F ($P < 0.05$).

Figure 2. Signs of shock in a chemically-fertilized plant (F) compared with a plant inoculated with *G. mosseae* (I).

resistance both to the stress of re-planting and to water stress.

Molecular detection of mycorrhizal fungi in the wild olive and in the Aglandau variety of cultivated olive

The use of molecular probes for the detection of the set of mycorrhizogenic fungi on the roots of the wild olive revealed fluorescent bands at a dilution of 1:100 for the five biological repeats (Figure 4a). At the same time, M% was estimated at 19.72. The use of specific molecular probes for the *Glomus* genus revealed that the roots of the wild olive were colonized by this genus; a positive signal was obtained for the five repetitions carried out (Figure 4b). Also, *G. intraradices* was more abundant than *G. mosseae*, producing a positive signal at a 1:100 dilution for the five repetitions, while with *G. mosseae*, the fluorescent bands were apparent in only the initial sample dilution and then in only two repetitions (Figures 4c and d). A positive signal was also observed with the use of mixed probes specific to *Scutellospora/Gigaspora* until dilution 1/100 for two repetitions only. At the three other repetitions, there is no signal after dilution 1:10 (Figure 4e).

No band was detected for the genus *Acaulospora* (Figure 4f). In contrast, using the same approach with DNA extracted from the roots of micropropagated Aglandau and inoculated in pots with spores of *G. mosseae*, a positive signal was obtained up to a dilution of 1:10 000 (Figure 5a). The use of a specific probe for *G. intraradices* confirmed our first interpretation. A more intense signal was obtained at the 1:10 000 dilution in Aglandau inoculated into pots with spores of *G. intraradices* (Figure 5b). The degree of mycorrhizal colonization by the latter was 69.96%. The observations were consistent with the suggestion that *G. intraradices* appears more colonizer than G. *mosseae*.

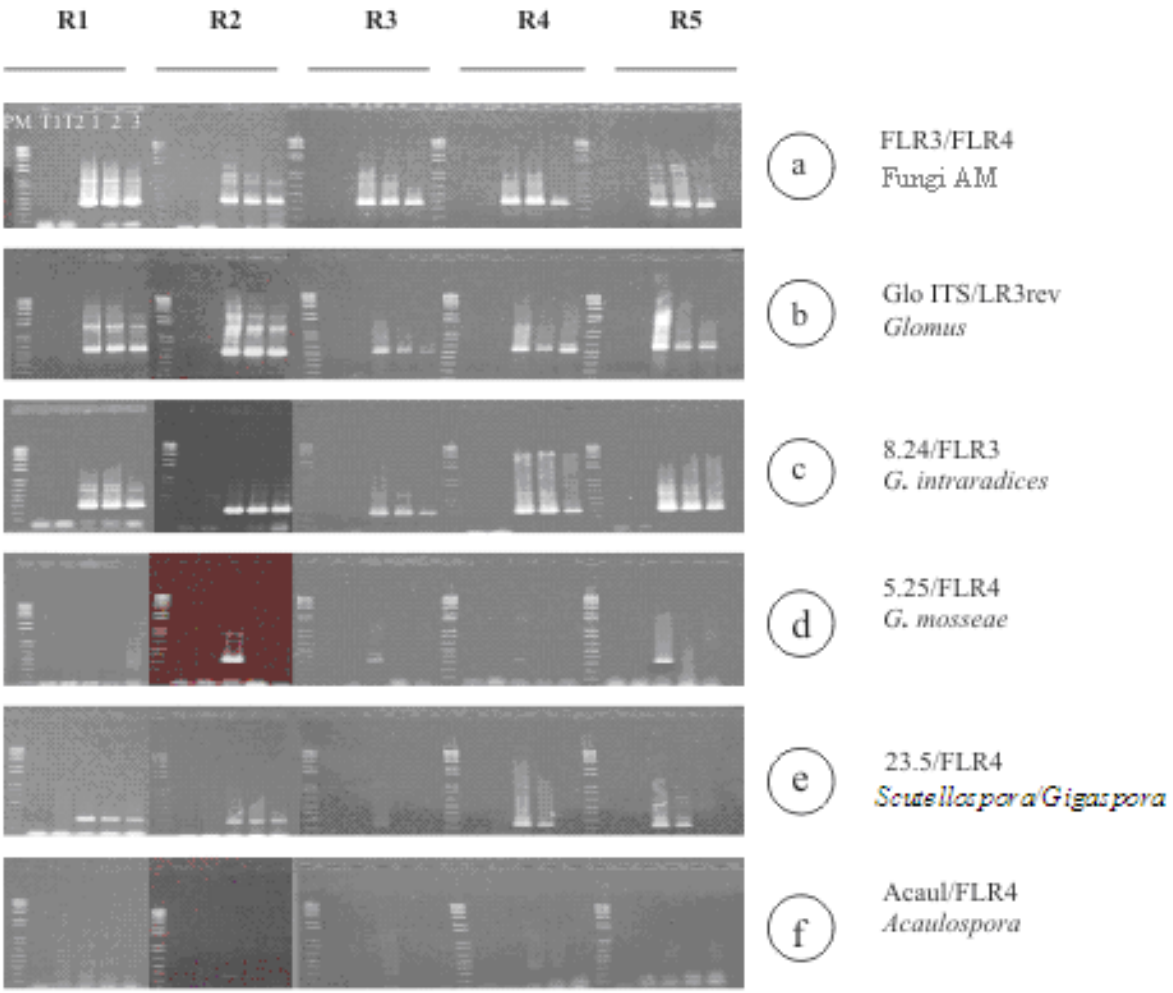

Figure 4. PCR detection of native AM fungi in oleasters. The detection was carried out using the FLR3/FLR4 primers for the entire set of mycorrhizogenic fungi, MF, GloITS/LR3rev (Genus *Glomus)*, 8.24/FLR3 *(G. intraradices)*, 5.25/FLR4 *(G. mosseae*), 23.5/FLR4 *(Scutellospora*/*Gigaspora*) and Acaul/FLR4 *(Acaulospora*). PM: molecular weight marker 1kb ladder; T1: water control for first PCR; T2: water control for second PCR. For each repetition (R1-R5), there is T1, T2 and three preparations (undiluted, 1:10, 1:100).

DISCUSSION

The inoculation with *G. mosseae* or *G. intraradices* showed a better development of the inoculated plantlets compared to the controls. These results agree with those of Caravaca et al. (2003b) who tested effects of the two fungi on the olive and those of Porras-Soriano et al. (2009) who studied the efficiency of the same fungal species on the Cornicabra variety of olive. However, the high level of AM colonization (> 50%) for these two strains of *Glomus* could bear no actual direct linkage to the efficiency of the fungi (Guissou et al., 1998; Requena et al., 2001; Calvente et al., 2004).

Several studies on other varieties of olive tree (Binet et al., 2007; Porras-Soriano et al., 2009) demonstrated the superiority of *G. mosseae.* This shows the importance of controlled mycorrhization in the micropropagation of olives after their transfer from *in vitro* conditions to growth in the soil.

Much research is currently aimed at improving the quality of vegetative production while at the same time preserving the environment. Inoculation with effective mycorrhizogenic fungi could represent a worthwhile alternative to treatment with chemical fertilizer. Underscoring this potential, we presently observed that inoculation of olive plantlets with the two strains of native Algerian mycorrhizal fungi produced increases in production of total vegetative matter by as much as 376% for plants inoculated with *G. mosseae* and 226% for those inoculated with *G. intraradices*. Although these values were superior to *G. mosseae* and the latter fungus was more advantageous in promoting growth of the different

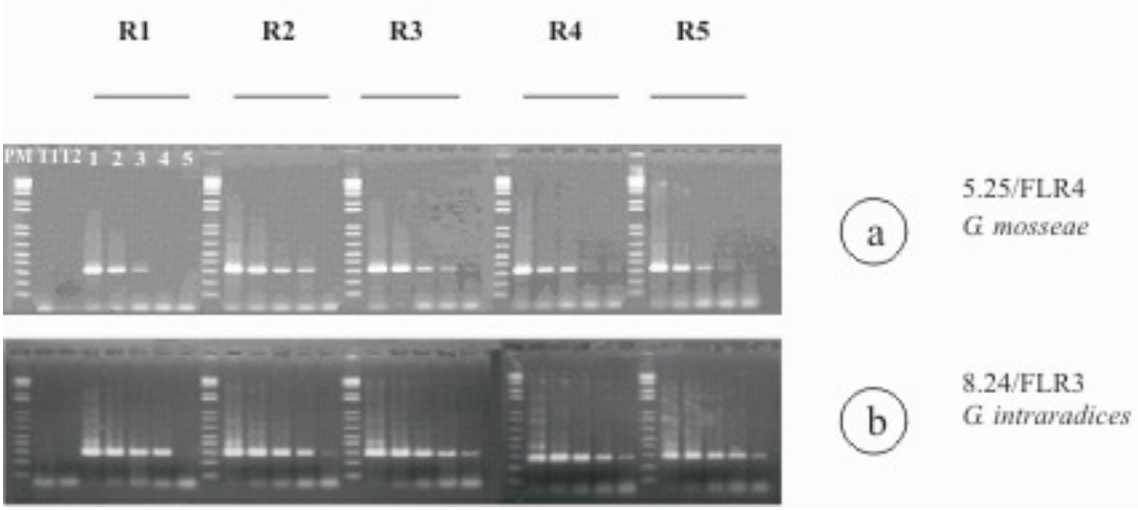

Figure 5. PCR detection of *G. mosseae* and *G. intraradices* inoculated into the cultivated olive (variety Aglandau) following 6 months of growth. The detection was carried out using the specific probes 8.24/FLR3 (*G. intraradices*) and 5.25/FLR4 (*G. mosseae*). PM: molecular weight marker 1kb ladder; T1: water control for first PCR; T2: water control for second PCR. For each repetition (R1-R5), there is T1, T2 and three preparations (undiluted, 1:10, 1:100, 1:1000, 1:10 000).

parameters measured when compared with *G. intraradices*, the differences were not significant. As far as root growth was concerned, no significant differences were currently evident between plants inoculated with *G. intraradices* and uninoculated control plants, whereas plants inoculated with *G. mosseae* displayed highly significant differences from the controls. Nevertheless, the high degree of colonization by *G. intraradices* (M% approximately 70%) certainly reflects its major hyphal network in the rhizosphere, which ensures better transport of nutrients to the host plant and causes the classic stimulation of aerial growth. It appears that the growth of aerial parts of the plant is linked to a correlation between the size of the root system and the degree of mycorrhizal colonization.

Further comparison of the results obtained by two culture techniques aimed at improving plant growth, mycorrhization and fertilization, indicated the clear superiority of mycorrhization. In fact, although the development of aerial parts of those plants inoculated with *G. mosseae* was significantly weaker than that of plants receiving osmocote, the root system showed the opposite effect. Inoculated plants showed a greater root to shoot ratio than did plants that had received the osmocote. Similar observations have been reported in other models (Tisserant et al., 1991; Berta et al., 1995; Dalpe, 2005; Porras-Soriano et al., 2009).

Tobar et al. (1994) have conclusively demonstrated that the root to shoot ratio reflects the degree of efficiency of AM fungi. Mycorrhization allows the plant to have a high root: shoot ratio, causing better hydro-mineral nutriation and thereby reinforcing the capacity to resist stress, especially the stress of transplantation (Caravaca et al., 2003b; Marschner, 1995). On the other hand, in the case of chemical fertilization, fertilizer placed near the roots makes the minerals immediately available for the plant, which reduces the need for extensive root development, resulting in a low root to shoot ratio. When the latter plants are transplanted into natural soil conditions without fertilizers, the low root to shoot ratio causes an abrupt fall in the uptake of soil solution by the plant and/or in the growth rate. This is what generally happens when young plants are moved from the nursery into the field. As Smith and Read (1997) suggest, mycorrhizal symbiosis may improve the quality of the root system, increasing the survival rate of young plants moved into the field. The suggestion was confirmed by Guissou et al. (2001) for fruit trees; mycorrhization does not improve the stress tolerance in this case, but stimulates mineral uptake and growth.

Finally, molecular biology studies of rDNA confirmed the presence of mycorrhizal fungi in the roots of olive trees, this being true for both wild olives and for the cultivated olive. The genus *Glomus* appears to be dominant over the other genera, *Scutellospora*/*Gigaspora* and *Acaulospora*, in the roots of the oleasters. *G. intraradices* is the dominant species and is the most commonly detected. *G. mosseae* varies from rare to completely absent. It is only present in trace amounts with the set of microflora in the roots of the oleasters. However, in monoculture, it is a very good root colonizer. The same result was found with *G. intraradices*; it was more frequent in monoculture than when in the natural soil microflora. These observations have been confirmed for sweet potato (Farmer et

al., 2007). Molecular detection of indigenous mycorrhizal populations in the roots of the olive tree in natural conditions is needed to link the success and the resistance that a given AM fungus can produce following its introduction. It is essential that the best inoculum be chosen to optimize the chances of root colonization and preserve the full mycorrhizogenic potential of the selected fungus.

Conclusion

This study affirms the importance of introducing mycorrhizal fungi onto olive plants produced *in vitro* and of developing the biotechnology to improve resistance to water stress after re-potting. The use of mycorrhization seems to be an extremely valid alternative to the use of chemical treatments. Trial inoculation of two mycorrhizal fungi (*G. mosseae* and *G. intraradices*) produced significantly increased growth over that of uninoculated controls. The success of the mycorrhization process appears not to depend so much on the degree of mycorrhization as on the efficiency of the fungus. This can be seen with *G. mosseae*, which, despite its lower level of colonization than *G. intraradices*, is just as good at improving growth of the olive plant.

Further, the use of mycorrhization and chemical fertilization demonstrates a clear superiority for mycorrhization, which allows the plants to obtain better nutrition from the soil, notably better water uptake and increases the capacity of plants to resist water stress after transplantation.

PCR examination of root colonization of the wild olive by the Glomeromycetes has shown the genus *Glomus* to be dominant in the roots of the oleasters. *G. intraradices* is the dominant species and is more frequently detected, with *G. mosseae* being less prominent when the microflora is complete. Both fungi colonize olive roots better when in monoculture.

Our results indicate the feasibility of *G. mosseae* and *G. intraradices* use in the production of olive plants, since both fungi are efficient at improving plant growth. However, being that *G. intraradices* appears to be more efficient and better at colonizing the roots of oleasters in the field, it would be interesting in future work to co-inoculate the two fungi into the olive and look at possible synergic effects on plant growth.

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Full Length Research Paper

Antimicrobial activity of the aqueous, methanol and chloroform leaf extracts of *Cissus multistriata*

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Antimicrobial activity of aqueous, methanol and chloroform leaf extracts of *Cissus multistriata* were investigated against 8 bacterial and 2 fungal test organisms, using the tube dilution and agar ditch diffusion methods. Aqueous leaf extract had no activity against both the bacterial and fungal test organisms. Both the methanol and chloroform leaf extracts inhibited all the test organisms with chloroform leaf extract showing the highest zone of inhibition against *Escherichia coli* (diameter 25 mm) and least against *Staphylococcus aureus* (diameter 13 mm). The methanol leaf extract was least inhibitory against *Salmonella typhi* (diameter 8 mm) and most inhibitory against *S. aureus* (diameter 15 mm). The methanol leaf extract of *C. multistriata* show more antifungal activity compared with chloroform leaf extract, with *Candida albicans* being more susceptible than *Aspergillus niger* to both methanol and chloroform leaf extracts. The minimum inhibitory concentration (MIC) of methanol leaf extract show least activity against *Yersinia enterocolitica* and *Pseudomonas aeruginosa* (MIC = 100 mg/ml) and higher activity of MIC at 50 mg/ml against the other bacterial test organisms. The chloroform leaf extract MIC of 100 mg/ml had least activity against *Proteus mirabilis* and *P. aeruginosa* and MIC of 20 mg/ml most inhibitory against *E. coli, Klebsiella pneumonia* and *S. typhi*. The antimicrobial activity of the heated extracts persisted after exposure to various temperatures between 30°C to 121°C for 15 to 30 min. However, the extract activity decreased as the temperature increased. The killing rate of the MBC of chloroform extract on *E. coli* was 1 cfu/3 min while on *S. typhi* was 1 cfu/3.8 min.

Key words: *Cissus multistriata*, antimicrobial, extract, inhibition, susceptible.

INTRODUCTION

Over the years there has been an incessant and alarming report of drug resistance in medically important strains of susceptible bacteria and fungi (CDC, 1995). This rapid emergence of resistant strains among susceptible pathogens coupled with the fact that many of the present day antibiotics in use are fast losing their potency are some of the reasons for the unabated search for effective and affordable antimicrobial drugs from local medicinal plants which could provide a source of new possible antimicrobial drugs (Egah et al., 1999). It is important that clinicians continue to keep a step ahead of these emerging resistant strains (Okafor, 1987). One of the ways to achieve this objective is through sustained search for new antibiotics and other antimicrobial agents. Consequently, there has been an upsurge in the interest in herbal remedies in several parts of the world with many being incorporated into orthodox medical practice (Okwuzua, 1973).

As part of the unabated search for antimicrobial drugs and in view of the fact that there are still plants whose medicinal uses have not been ascertained, this study was carried out on *Cissus multistriata* to establish its antimicrobial activity. It is a plant of choice because of its traditional use for treatment of Kwashiorkor, Marasmus and other ailments in children among Ibaji herbal healer in Kogi State, Nigeria.

MATERIALS AND METHODS

Plant source, identification and preparation

*C. multistria*ta plant was collected from Mr. Joshua, a peasant farmer in Idah, Kogi State. The plant was identified by the Forestry Research Institute of Nigeria (FRIN) Jos, Nigeria. The leaves were

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washed and air dried at room temperature 27 ± 2°C for several days in the Microbiology Laboratory of Kogi State University until the leaves become crispy. They were ground into a fine powder using sterile mortar and pestle. This was stored in air tight glass container protected from direct light and heat until required for analysis.

Test organisms and culture media

The isolates used as test organisms were obtained from the Department of Microbiology, Federal College of Veterinary and Medical Laboratory Technology, Vom, Plateau State. They include *Staphylococcus aureus, Escherichia coli, Yersinia enterocolitica, Proteus mirabilis, Shigella sonnei, Pseudomonas aeruginosa, Klebsiella pneumonia, Salmonella typhi, Candida albicans* and *Aspergillus niger.* The bacterial isolates were grown at 37°C on nutrient agar (oxoid), confirmed using the standard techniques as described by Cowan and Steel (1965) and maintained on nutrient agar (oxoid) at 4°C while the fungi isolates were cultured and maintained on potato dextrose agar (PDA) to which 0.05% chloramphenicol was added.

Nutrient broth and agar were used as culture media for bacteria while potato dextrose broth and agar were used for fungi. The broths were used for MIC determination while the nutrient agar and PDA were used for the MBC and MFC determination respectively. The media were prepared and sterilized according to the manufacture's instruction. About 5 ml of the molten agar was poured into 90 mm diameter sterile Petri dishes to give a depth of 4 mm.

Nutrient broth was inoculated with bacterial colonies of the isolates and incubated at 37°C for 18 – 24 h while the fungi isolates were inoculated in potato dextrose broth and incubated for 72 h at room temperature 27 ± 2°C. Vandepitte et al. (1991) method was used to adjust the turbidity.

Crude extract preparation

50 g of the ground leaf powder was weighed and extracted with 95% methanol, chloroform and water respectively, in a ratio of 1:3 of powdered leaves to each solvent. The extraction of the leaves was done by gentle but continuous agitation of each mixture for 3 h using an orbital shaker at 20 rpm. Each mixture was then filtered using dried Whatman filter paper and the filtrate concentrated.

The solvent extract of methanol and chloroform were evaporated to dryness in an evaporating dish placed on water bath at a temperature of 70°C while the aqueous extract was evaporated to dryness in a Gallenkamp oven at temperature of 110°C until a constant weight was obtained.

Each solid extract was reconstituted in their respective solvents to obtain a stock solution of 200 mg/ml. The stock solution obtained was sterilised using a Millipore Membrane Filter (0.45 um pore diameter).

Determination of antimicrobial activity of the extracts

The antimicrobial activity of the leaf extracts were determined by the agar ditch - diffusion method (Collins and Lyne, 1979; Baron and Finegold, 1999; Conz et al., 1999). Sterile nutrient agar and potato dextrose agar were prepared and aseptically poured into sterile Petri dishes to test antibacterial and antifungal activity respectively. Five to six hours old standardised culture of isolates were used to flood the media surfaces (Onaolapo, 1997). A sterile cork borer of 5 mm diameter was used to make ditches on each plate. Then 0.15 ml of the stock extract (equivalent of 30 mg extract) was dropped into each ditch and appropriately labelled. Incubation was made at 37°C for bacterial isolates and 25°C for fungal isolates for 24 h. Zones of inhibition (diameter) produced after incubation were examined and recorded in millimetre. The test organisms were tested separately.

Determination of minimum inhibitory concentration (MIC)

Using the tube dilution technique 1 g of the extract was dissolved in 5 ml nutrient broth; this gave 200 mg/ml. Thereafter two fold serial dilutions were made from the original stock of 4 ml, using nutrient broth for bacteria and potato dextrose, broth for fungi to achieve the following concentrations: 200, 100, 50 and 25 mg/ml (Egorov, 1985; Scott, 1989; Baron and Finegold, 1990). Having obtained different dilution and concentrations, 0.1 ml of standardized test organisms were inoculated into the dilutions and incubated at 37°C for 24 h for bacterial and 25°C for 7 days for fungal isolates (Tilton and Howard, 1987; National Committee for Clinical Laboratory Standard, 1990 and 2002). Using solvents and test organisms without extract, positive controls were equally set up. The lowest concentration of the extracts that inhibited the growth of the test organism was recoded as the MIC.

Bacterial tubes showing no visible growth from the MIC test were subculture into nutrient agar and incubated at 37°C for 24 h. For fungi, it was subcultured on Potato Dextrose Agar and incubated at 25°C. The lowest concentration of the extracts yielding no growth on subculturing was recorded as the minimum bactericidal concentration (MBC) for bacteria and minimum fungicidal concentration (MFC) for fungi.

Effect of temperature on stability/potency of crude extracts

The crude leaf extracts were heated in a water bath at different temperatures as follows 30, 60, 80 and 100°C for 30 min and in an autoclave at 121°C for 15 min. After cooling, the activity of the heated leaf extract samples were tested against the test organisms.

Determination of killing rate of MBC on most susceptible and least susceptible clinical bacterial isolates

Time-kill studies of the chloroform leaf extract of *C. multistriata* on the most susceptible and least susceptible bacterial isolates used (*E. coli* and *S. typhi* respectively) was carried out. This method involved mixing 0.5 ml of 10^5 cfu/ml of the test organism with 4 ml of MBC of chloroform extract. Then 0.1 ml of the mixture was taken and plated out on sterile nutrient agar at interval of 30 min up till 300 min. The plates were incubated at 37°C for 24 h. The number of surviving bacterial colonies on each plate at the time intervals were counted and recorded against time (Lesly and Maurer, 1974; NCCL, 1999).

RESULTS

The diameters of zones of inhibition of the test organisms using the aqueous, methanolic and chloroform leaf extracts of *C. multistriata* are shown in Table 1. The aqueous leaf extract had no activity against both the bacterial and fungal test organisms. The highest zone of inhibition was observed in the chloroform leaf extract of *C. multistriata* against *E. coli* (diameter 25 mm). The methanol leaf extract was least effective against *S. typhi* (diameter 8 mm) and most effective against *S. aureus* (diameter 15 mm) for bacterial test organisms, while the chloroform leaf extract was least effective against *S. aureus* (diameter 13 mm) and most effective against the *E. coli* (diameter 25

Table 1. Activity of crude extracts from the leaf of *Cissus multistriata* on test organisms.

Test organism	Diameter of zones of inhibition (mm)					
	Extract			Control		
	Aqueous	Methanolic	Chloroform	Aqueous	Methanol	Chloroform
Staphyllococcus aureus	0	15	13	0	7	10
Escherichia coli	0	9	25	0	5	12
Yersinia enterocolitica	0	12	20	0	6	8
Proteus mirabilis	0	10	15	0	6	7
Shigella sonnei	0	12	18	0	5	7
Pseudomonas aeruginosa	0	7	16	0	5	6
Klebsiella pneumonia	0	10	21	0	7	10
Salmonella typhi	0	8	10	0	6	6
Candida albicans	0	14	11	0	6	4
Aspergillus niger	0	11	6	0	5	4

Table 2. Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of extracts of *Cissus multistriata.*

Test organism	MIC (mg/ml)		MBC & MFC (mg/ml)	
	Methanol	Methanol	Chloroform	Chloroform
Staphyllococcus aureus	50	100	100	50
Escherichia coli	50	100	50	25
Yersinia enterocolitica	100	100	50	50
Proteus mirabilis	50	100	100	100
Shigella sonnei	50	50	100	50
Pseudomonas aeruginosa	100	200	100	100
Klebsiella pneumonia	50	200	50	25
Salmonella typhi	50	200	200	25
Candida albicans	50	100	100	100
Aspergillus niger	50	100	200	100

mm). The methanol leaf extract show more antifungal activity compared with the chloroform leaf extract with *C. albicans* being more susceptible than *A. niger* for both methanol and chloroform leaf extracts (Table 1).

Table 2 show the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) values for the methanol and chloroform leaf extracts of *C. multistriata*. The methanol leaf extract showed activity against all the test organisms with least activity against *Y. enterocolitica* and *P. aeruginosa* (MIC = 100 mg/ml) while the other test organisms had higher activity of MIC (50 mg/ml). The chloroform leaf extract was least inhibitory against *P. mirabilis* and *P. aeruginosa* at 100 mg/ml and most inhibitory against *E. coli*, *K. pneumonia* and *S. typhi* at 25 mg/ml. The MIC pattern for *S. aureus, E. coli, Y. enterocolitica*, and *K. pneumonia* were the same with that of the MBC while the pattern of activity for the other bacterial test organisms were not the same with the MBC (Table 2).

The effect of temperature on the stability and potency of the leaf extract of *C. multistriata* is shown in Table 3. The antimicrobial activity of the heated extracts persisted after exposure to various temperatures between 30 to 121^{o}C for 15 to 30 min. However, the extract activity decreased as the temperature increased.

Figure 1 shows the death rate of the most inhibited bacterial test organism *E. coli* and the least inhibited *S. typhi*. There was no surviving cell in the MBC after 240 min exposure for *E. coli* unlike *S. typhi* that still had some surviving cells after 300 min. There was a sharp drop in the number of surviving cells of *E. co*li within 30 min of exposure to MBC of the leaf extract. The sharp drop was also observed between 60 and 90 min of exposure compared to *S. typhi* in which the reduction in number of surviving cells was gradual till 120 min exposure after which a sharp drop was observed, between 120 and 150 min exposure to MBC of the leaf extract.

DISCUSSION

The methanol and chloroform leaf extracts of *C. multistriata* have shown antibacterial and antifungal activity

Table 3. Effect of temperature on the stability/potency of crude extracts of *Cissus multistriata*.

Test organism	Zones of inhibition (mm) of extract heated at different temperatures (℃)				
	30	**60**	**80**	**100**	**121**
Staphyllococcus aureus	13	12	12	10	5
Escherichia coli	15	15	13	12	10
Yersinia enterocolitica	20	18	18	16	15
Proteus mirabilis	16	15	15	14	12
Shigella sonnei	6	6	5	5	4
Pseudomonas aeruginosa	20	20	18	18	15
Klebsiella pneumonia	7	7	6	6	5
Salmonella typhi	22	21	21	19	17
Candida albicans	10	8	8	8	6
Aspergillus niger	12	10	10	10	8

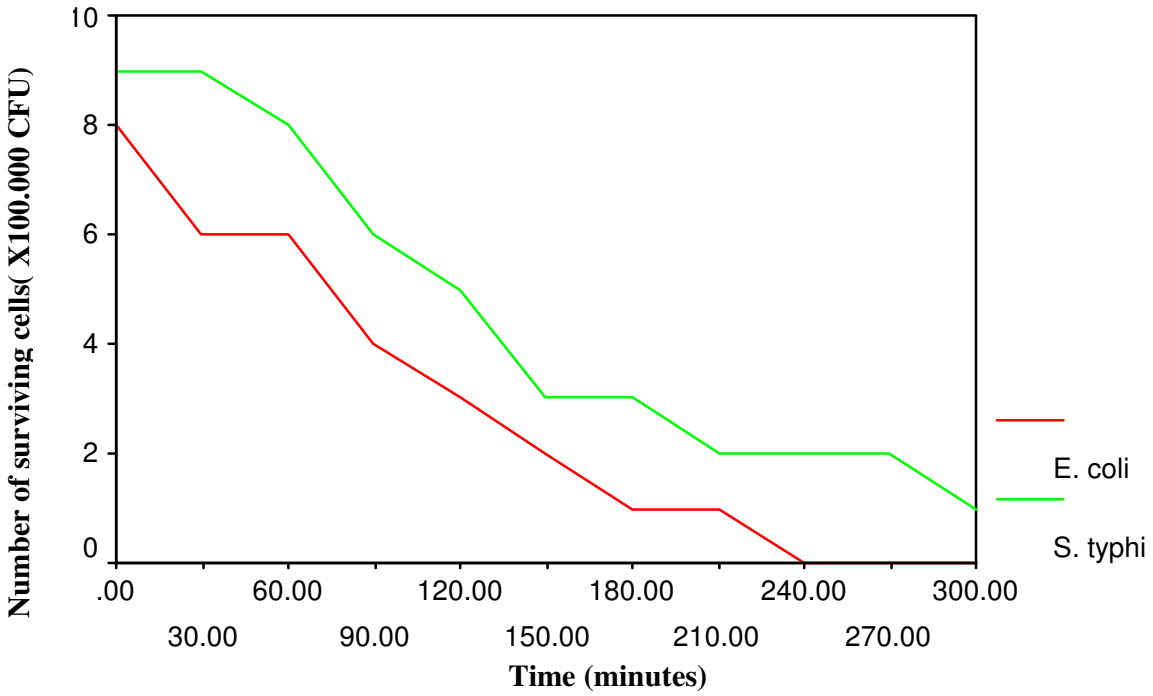

Figure 1. Death rate of E. coli S. typhi in MBC of C. multistrata chloroform leaf extract.

against the test organisms. This finding corroborates some investigators report that flowering plants contain antimicrobial substances (Zaria et al., 1995; Ibekwe et al., 2001). The extracts showed varying degrees of antibacterial and antifungal activity. The fact that both the methanol and chloroform extracts inhibited the test organisms differently could imply that the plant contain active principles that can be extracted differently depending on the ability of the extracting solvent (Kafaru, 1996). All the bacterial test organisms are highly susceptible to the methanol and chloroform extract of *C. multistriata*. However, *S. aureus* was observed to be the most inhibited and *S. typhi* and *P. aeruginosa* were the least inhibited by methanol extract while *E. coli* was observed the most inhibited and *S. aureus* least inhibited by chloroform leaf extract of *C. multistriata*.

With the appreciable level of inhibition exhibited by the leaf extract of *C. multistriata* against the test organisms, it is obvious that the plant is a potential source of novel antimicrobial drugs. The susceptibility of both gram positive and gram negative bacteria to the extract show its potential of broad-spectrum activity (Adebayo et al., 2001). It should be noted that herbal healers in Ibaji, Kogi State, Nigeria use the leaf in treating kwashiorkor and other ailments in children.

The least activity of the extracts against *P. aeruginosa*

and *S. typhi,* both gram negative bacteria was not unexpected, since resistance of gram negative bacteria to most antibacterial agents is well known (Irvin et al., 1981). *E. coli* which is the most inhibited of the test organisms has been implicated in the aetiology of many ailments (Adeyemo et al., 1994; Nedolisa, 1998; Ebie et al., 2001).

Temperature treatment had effect on the leaf extract activity of *C. multistriata*. Generally, the potency of the extracts reduced with increased temperature. The implication of this is that the extract should be properly stored for maximum efficiency. However, the antimicrobial activity persisted even after exposure to heat at 121^{o}C for 15 min. It is worthy to note that heat treatment increased the activity of the extracts against the least inhibited bacterial test organisms, *P. aeruginosa* and *S. typhi.* The death rate of the most inhibited test organism *E. coli* was high with no surviving cells observed after 240 min exposure to MBC of the leaf extract while it was gradual for the least inhibited bacterial test organism, *S. typhi*, which still had some surviving cells after 300 min exposure to MBC of the leaf extract.

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Full Length Research Paper

Isolation and characterization of a bacterial strain for aniline degradation

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Aniline, a serious environmental threat and health risk to living organisms is being released into the soil and water bodies owing to its expanded use in industry. The objective of the present study was to isolate a strain from rhizospheric soil samples of wheat (*Triticum aestivum* L.) taken from an agricultural site near the industrial area of Faisalabad, with the capability of degrading aniline with its maximum activity. The isolated strain was identified as *Staphylococcus aureus* ST1 a newly reported strain for aniline degradation. The strain ST1 showed tolerance up to 2000 ppm for aniline on mineral salt media plates and its degradative ability was checked through shake flasks experiments using HPLC. The strain was capable of degrading aniline and utilizing it as a sole source of carbon and energy. Maximum reduction of aniline concentration in medium up to 59.65% was observed after 72 h. An enhancement in biodegradation was observed using glucose as an additional growth substrate. The degradative products analyzed by HPLC were catechol, phenol and some other unknown compounds. Plasmid curing showed the involvement of plasmid encoded genes which was later followed by the isolation of plasmid DNA, which was found to be a large one of ~40 kb having restriction sites for enzymes (*Eco*RI, *Bam*HI, *Cla*I, *Stu*I, *Pst*I, and *Hind*III) used.

Key words: Aniline, biodegradation, *Staphylococcus aureus*, HPLC, plasmid curing, restriction sites.

INTRODUCTION

Rapid industrialization and improper discharge of industrial effluents, wastes, accidental spills or deliberate release of certain hazardous chemicals that are mutagenic, carcinogenic and recalcitrant, pose a serious threat to environment including soils, groundwater as well as open water bodies (Tani et al., 1998). These effluents have a variety of unusual chemicals including a range of aromatic hydrocarbons and their derivatives (Van der Meer et al., 1992) which the microbes enzymatically decompose and utilize in cellular metabolism (Phale et al., 2007).

Aniline is a toxic chemical present in the effluent of many industries as it is widely used as a raw material in the manufacturing of a number of products such as dyes, plastics, resins, pharmaceuticals, petro-chemicals, herbicides, pesticides among others. Its other source is through natural microbial transformation of several nitro aromatic compounds (Hallas and Alexander, 1983; Kinouchi and Ohnishi, 1983).

Aniline is a major breakdown product of diphenylamine and p-aminoazobenzene (Zissi et al., 1997). To treat wastewaters and affected soils containing aniline and most of its derivatives, remedies often used are photodecomposition, auto-oxidation, electrolysis, resin adsorption or ozone oxidation but the cost of such treatments limits their application to small scale facilities (Gheewala and Annachhatre, 1997). Many herbicides are transormed to anilinic compounds whose fate in soil and other environments is only partially clarified and when such anilinic compounds are applied to soil, a greater portion of them is apparently bound both by physical and chemical adsorption (Laanio and Blattmann, 1978; Chung and Boyd,

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Table 1. Growth of seven bacterial isolates at different concentrations of aniline in mineral salt media with and without glucose.

Bacterial strains		Concentration of aniline (ppm)														
		100	200	300	400	500	600	700		100	200	300	400	500	600	700
ST1	PNR-aniline	+++	+++	+++	+++	+++	+++	+++	PNR-G-aniline	++	+++	+++	+++	+++	+++	+++
ST2		+++	+++	+++	++	++	++	++		+++	++	++	++	++	++	++
ST3		++	+	+	-	-	-	-		++	+	+	-	-	-	-
ST4		++	+	-	-	-	-	-		++	+	-	-	-	-	-
ST5		++	+	-	-	-	-	-		+	-	-	-	-	-	-
ST6		++	+	-	-	-	-	-		+	+	-	-	-	-	-
ST7		++	+	+	-	-	-	-		++	++	+	-	-	-	-

+++ = Rich growth; ++ = good growth; + = poor growth; - = no growth.

1987). Aniline and its derivatives are considered to be a health risk due to their strong toxicity and mutagenicity (Saint et al., 1990; Holder, 1999).

Biological treatment facilities are better suited to deal with affected soils and for large volumes of wastewaters where the traditional activated sludge process is not effective in treating aniline (Gheewala et al., 2004). Activated sludge results in the production of derivatives of aniline which are difficult to degrade and inhibit the biodegradation of other chemicals (Wang et al., 2006). O' Neill et al. (2000) isolated a consortium of bacteria capable of degrading aniline found in wastewaters produced by oil-fields, marine mud, acid peat bog water and soils. The majority of the bacteria which metabolize aniline belong to the genera *Rhodcocci, Alkaligenes*, *Pseudomonas* and *Bacilli.* Under anaerobic conditions, the biodegradation of aniline and substituted anilines by *Paracoccus* sp occurs (Wang et al., 2006), while under aerobic conditions, biodegradation of pentylaniline and aniline by *Pseudomonas* sp was reported by Bollag and Russel (Bollag and Russel, 1976).

Anilines are metabolized to the corresponding catechols via one or more oxidative reactions catalyzed by aniline dioxygenases, liberating ammonia and subsequently undergoing metabolic transformations (Liodl et al., 1990; Fuchs et al., 1991). Bacterial degradation of aromatic compounds has frequently been shown to be plasmid encoded (Chakrabarty, 1976). The first degradative phenotypes attributed to catabolic plasmids were found in *Pseudomonas* and were for camphor, octane and salicylate (Chakrabarty, 1972). Plasmids encoding the ability to utilize aniline have been recognized such as pCITI in *Pseudomonas* sp. strain CITI, pTDN1 in *Pseudomonas putida* UCC22 and pYA1 in *Acinetobacter* sp. strain YAA (Saint et al., 1990; Fujji et al., 1997).

The objectives of the present study were the isolation and selection of strain that harbour maximum concentrations of aniline and to check the biodegradative ability of the strain for aniline and ultimately its fate into its different degradative products (catechol, phenol among others).

MATERIALS AND METHODS

Soil Samples

Rhizospheric soil samples of wheat (*Triticum aestivum* L.) were taken from an agricultural site near Faisalabad (31.36 °N 72.99 °E) where industrial effluent was thrown. The soil samples were further processed immediately after it reached the laboratory.

Selection, adaptatiion and screening of aniline degrading bacteria

Seven different strains of bacteria (ST1, ST2, ST3, ST4, ST5, ST6 and ST7) were isolated from the soil samples by streaking on nutrient agar plates. The bacterial colonies of these seven strains were streaked on sterilized mineral salt (PNR) media with 100 ppm of aniline concentration and the strains showing growth at this concentration were then restreaked on higher concentrations of aniline up to 700 ppm (Table 1) of aniline with and without glucose for the screening of the most tolerant strains. Maximum growth showing strains were further restreaked on PNR media at aniline concentrations up to 2000 ppm with and without glucose (Table 2) to make them adopted for higher concentrations of aniline and selection of the final one for further experiment.

Mineral salt media of the composition, 13.6% KH_2PO_4, 2.4% $(NH_4)_2SO_4$ and 2.5% NaOH for PN salt (20×) and 8.0% $MgSO_4$, 0.2% Fe_2SO_4 and 4% HCl for R salt, was used to check the growth with 4-aminophenol as sole source of carbon.

Characterization of colonies

Individual colonies were characterized on the basis of colony morphology (shape, size, texture and colour), gram staining and conventional biochemical tests. Streak plate method was used to obtained single and pure colony. The isolated colony was streaked on nutrient agar slants and incubated at 37°C for 24 h to obtain optimum growth. The preliminary characterization was based on colony morphology on nutrient agar plates after 2 - 3 days at 37°C of incubation. Each isolate was subjected to gram staining (Baker, 1962) and examined for cellular morphology.

Bacterial suspensions for the API-50-CHB/E test were prepared using isolates pregrown on nutrient agar. Colonies were picked up and suspended in sterile CHB medium to get turbidity equivalent to 2 McFarland. The suspension was then added to the tubes according to the manufacturer's instruction. Reading of reaction within each test was taken after 24 and 48 h incubation at 37°C. The

Table 2. Tolerance of ST1 and ST2 on different concentrations of aniline with (PNR-G) and without glucose (PNR).

Bacterial Strain	Concentration of aniline (ppm)											
	100	300	500	700	900	1000	1300	1500	1600	1800	1900	2000
PNR-G-aniline												
ST-1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
ST-2	+++	+++	++	++	+	+	-	-	-	-	-	-
PNR-aniline												
ST-1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
ST-2	+++	+++	++	++	+	+	-	-	-	-	-	-

+++ = Rich growth; ++ = good growth; + = poor growth; - = no growth.

results were then interpreted for bacterial identification according to the manufacturer's instruction and also using the results of conventional biochemical tests (Holt et al., 1994).

Quantification by high performance liquid chromatography (HPLC)

Staphylococcus sp. ST1 was cultured overnight in nutrient broth and was used as inoculum (10% v/v) for 100 ml media with and without glucose having different concentrations of aniline (100, 200 and 300 ppm) and was incubated at 37°C in shaking incubator at 150 rpm. Samples taken at different time intervals (0/start time and after 72 h) of incubation were spun at 10,000 rpm for 10 min and supernatant was filtered by 0.2 µm filter and analyzed by HPLC. HPLC analysis was carried out with a Waters instrument and isocratic separations were done on a C-18 reverse phase column (thermo Hypersil 150 x 4.6 mm 5µ Hypersil) with the mobile phase composition (ACN: H_2O = 78:22 v/v), at a flow rate of 0.8 ml/min at room temperature. The aniline eluted out was detected with a UV detector at 254 nm. Qualitative and quantitative data were obtained by comparing the peaks area of the chromatograms obtained.

Plasmid curing

Before isolation of plasmid having susceptible degradation gene, plasmid curing suggested by Hardy (1993) was performed to ensure that the gene degrading aniline is plasmid encoded or chromosomal encoded. For this, the strain was cultured in 4 ml nutrient broth overnight. Then 0.2 ml of culture was added in 4 ml nutrient broth and placed in shaking incubator at 29°C for 2 - 4 h. The log phase 200 µL culture was then added in each tube with 2 ml broth containing different concentrations of ethidium bromide (20 µg/ml to 10 mg/ml). Positive control containing only cells (without the curing agent) while negative control containing only ethidium bromide were also run and all tubes were incubated (in dark) at 29°C overnight. Tubes with highest concentrations of ethidium bromide (in which growth was manifested) and bacterial growths were selected. These were then serially diluted with sterilized distilled water. Following the spreading technique, equal volumes of inoculum from different dilutions were spreaded on nutrient agar plates and also on PNR media containing aniline of 1400 ppm (in which rich growth and clear zones of hydrolysis of culture were manifested). Plates were then incubated at 37°C for 24 h and then colonies were counted. Colonies were then replica plated on nutrient agar and transferred to selective media containing aniline.

Plasmid isolation

A single colony from fresh plate was inoculated in nutrient broth (10 ml) and was incubated at 37°C. The next day, the inoculum was spun at 14,000 rpm for 1 min and pallet of bacterial cells was obtained. Finally, the pallet of bacterial cells was resuspended in 100 µl of solution 1 and 200 µl of solution 2 and placed for 5 minutes after each addition at room temperature. 150 µl of solution 3 was then added, placed for 20 - 30 min at room temperature and spinned at 14,000 rpm for 5 min. Subsequently, the supernatant was extracted three times with an equal volume of chloroform: Isoamyl alcohol (24:1) and by centrifuging at 14,000 rpm for 5 min. The supernatant was then taken and mixed with twice the volume of ethanol and allowed to stand at -70°C for 30 min following spinning at 14,000 rpm for 10 min. The DNA pallet obtained was air dried, resuspended in 20 µl TE buffer and stored at 4°C (Mini prep DNA isolation procedure). The plasmid isolation was also done using Gene Jet Miniprep Kit, Fermentas according to the instructions given by manufacturer.

Restriction analysis of plasmid DNA

The dilutions of DNA were made (0.2 – 1 µg of DNA in 20 µl of ddH_2O). 2.0 µl of dilution was taken in PCR tubes and then 2 µl of digestion buffer (10X), 1U of restriction enzyme making a total volume of 20 µl by adding deionized water with mixing by tapping tube was added. Incubation was at 37°C for 1 - 2 h and the reaction was stopped by adding 0.5 M EDTA (pH 7.5). The quality and quantity of the genomic DNA was also inspected by running a small aliquot of plasmid DNA on 1% agarose gel (Sambrook and Russell, 2001). A total of 10 µl (6 µl sample + 4 µl loading dye) was loaded in gel pockets and run for 1.5 h at 80 V. Finally, the gel was analyzed in gel documentation system (Bio Rad Instruments, Italy).

RESULTS

In the present study, the effect of different concentrations of aniline with and without glucose was studied on seven bacterial strains (ST1, ST2, ST3, ST4, ST5, ST6 and ST7) and it was found that these all were capable of growing on aniline (100 ppm). When the bacterial colonies of these strains were streaked on mineral salt media (PNR) with 700 ppm of aniline with and without

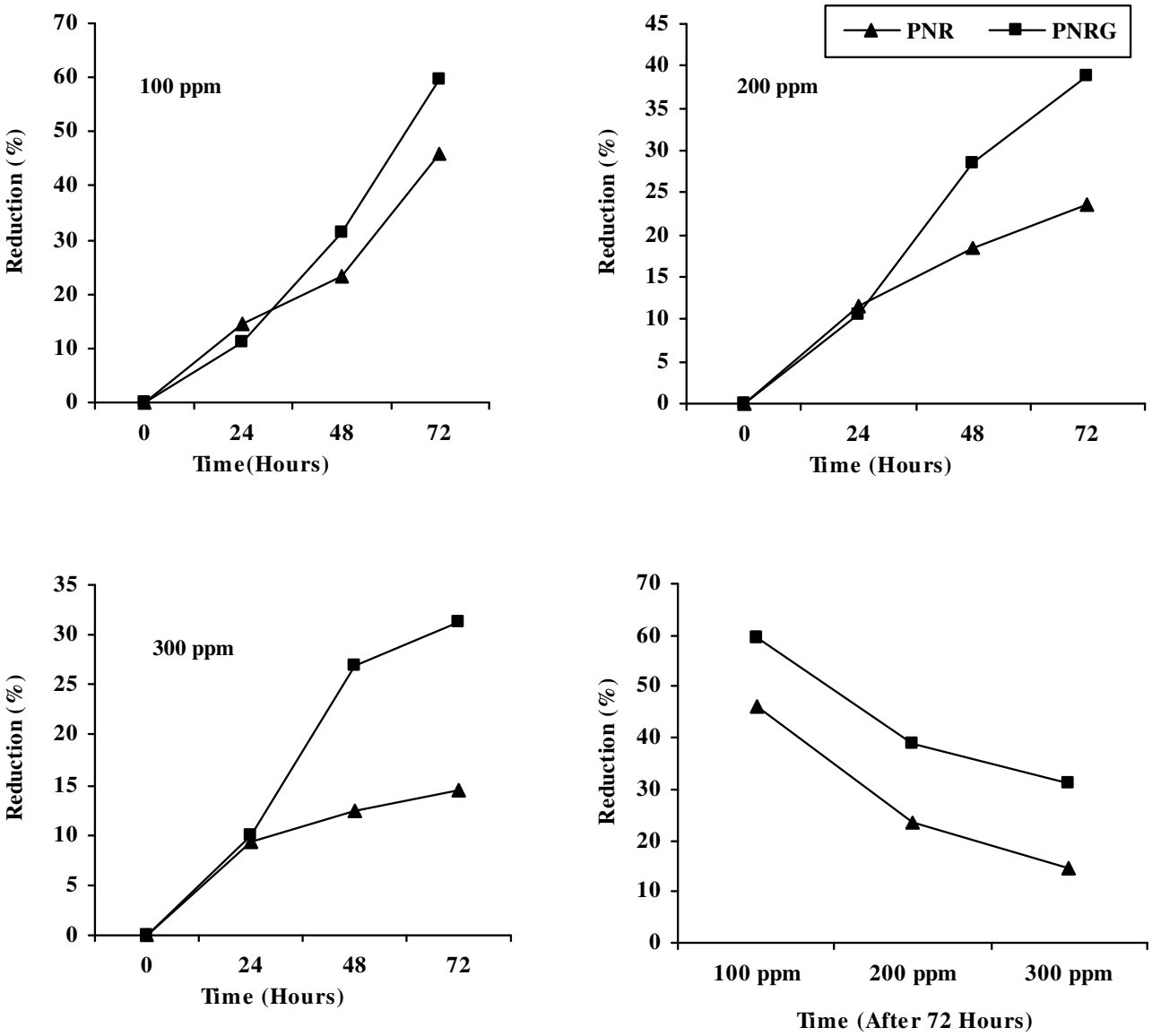

Figure 1. Percentage reduction of aniline at different aniline concentration after 72 h by *Staphylococcus* sp. ST1 determined by HPLC.

glucose, it was observed that of these all only two strains (ST1, ST2) gave rich growth in the 2 to 3 days of incubation (Table 1). These two strains (ST1 and ST2) when restreaked on PNR media containing higher concentrations of aniline with and without glucose for adaptation, strain ST1 showed good and maximum tolerance at all concentrations of aniline while ST2 showed its maximum tolerance at 1000 ppm (Table 2) and as such, strain ST1 was selected for further experiments.

Morphologically, the ST1 gave round to some extent pin point, raised edged, irregular, lobate margin and opaque white to off-white colonies on agar plates while by gram staining, the cells were gram positive cocci forming clusters (bunches) (Holt et al., 1994). The ST1 was also identified on the basis of biochemical tests using API 50 CHB/E kits (Biomerieux, France) and the results were interpreted (Figure 1) and finally the strain was identified as *Staphylococcus* sp. (*Staphylococcus aureus*).

When the effect of aniline alone and aniline with glucose on the degradative efficiency of *S*T1 was observed, aniline reduction in the PNR media containing 100, 200 and 300 ppm of aniline with glucose was more than with no glucose as an additional substrate for each of the concentrations. This is quite obvious from their comparisons individually after different intervals of time (Figure 1). Ultimately with HPLC (Figure 2), overall reduction levels observed after 72 h showed a reduction in aniline of

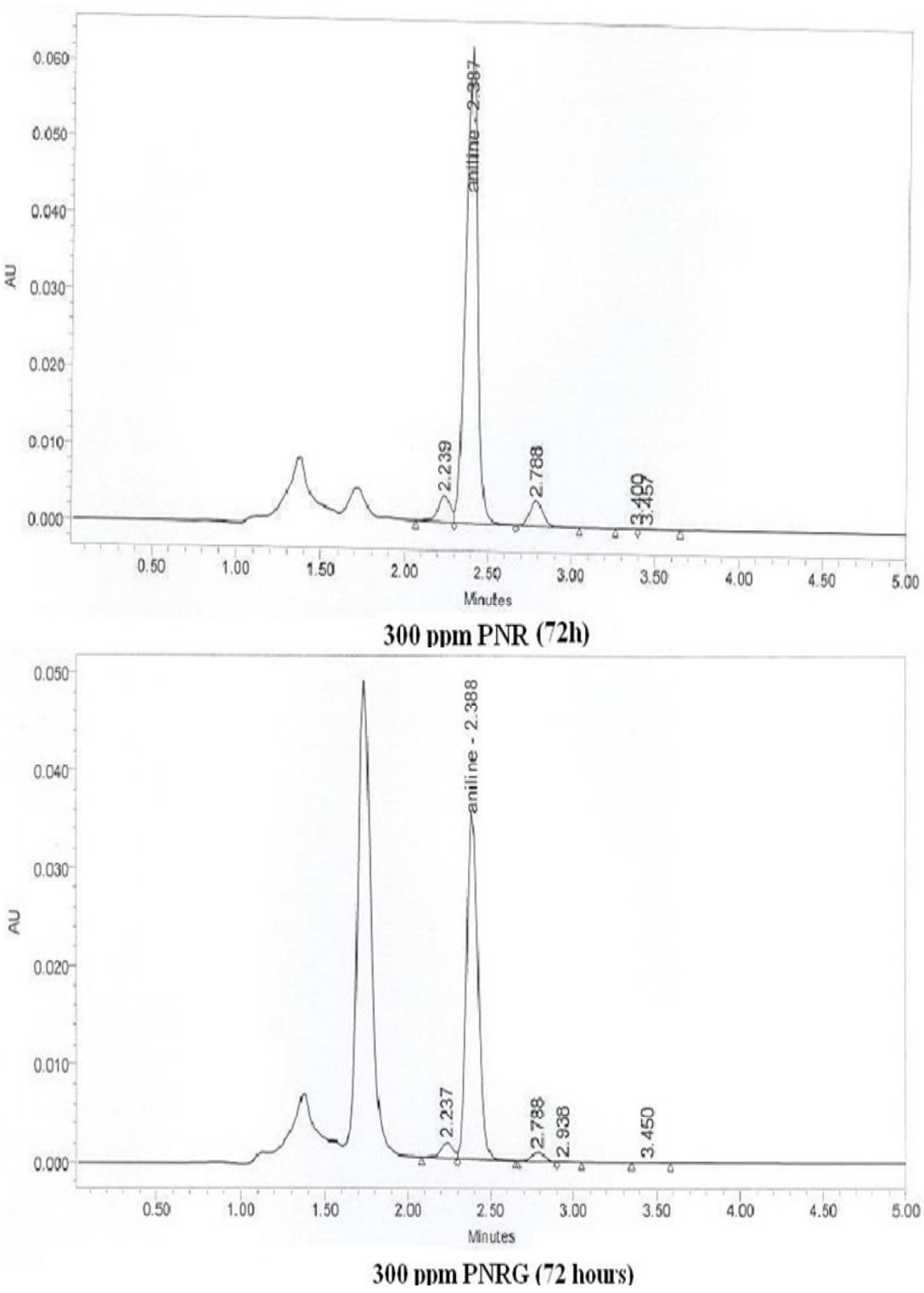

Figure 2. HPLC chromatogram for reduction of aniline at 300 ppm of aniline with and without glucose after 72 h by *Staphylococcus* sp.

45.97% without glucose and 59.65% with glucose at 100 ppm whereas 23.56% reduction without glucose and 38.75% reduction with glucose were observered for 200 ppm. For higher concentration, that is, 300 ppm, 14.5%

reduction without glucose and 31.23% reduction with glucose was seen (Figure 1). The individual and collectively observed levels showed more reduction with glucose as an additional growth substrate.

Plasmid curing, isolation and restriction analysis

In order to determine the involvement of plasmid-encoded genes in aniline degradation, plasmid curing was performed in which ethidium bromide, a mutagenic compound that rendered the plasmid genes inactive was used. It was observed that 200 out of 250 colonies mutated were unable to utilize aniline and thus failed to grow. So it was concluded that the genes involved in aniline degradation were plasmid encoded. The plasmid was isolated and the product obtained was electrophoresed and compared with standard to calculate its molecular weight which was found in the range of ~40 kb. Multiple cloning sites in isolated plasmid DNA of ST1 were also analyzed using restriction endonucleases (*Eco*RI, *Bam*HI, *Cla*I, *Stu*I, *Pst*I and *Hind*III) and two bands for *Eco*RI, *Cla*I and *Stu*I were found showing more than one restriction sites for each but one restriction site for *Bam*HI, *Hind*III and *Pst*I was found as they produce linear or single bands.

DISCUSSION

Wide varieties of aromatic compounds which are being released into the environment through different human activities are metabolized by soil bacteria. This is of great importance in environmental cleanup technologies (Watanabe et al., 1996). Efficient bioremediation process is very important. This is because the bacteria involved perform a complete degradation pathway to eliminate toxic metabolites from soil (Kazunga et al., 2001). In the present study, a bacterial strain *Staphylococcus* sp. (ST1) isolated from rhizospheric soil of wheat crop, was tested for its ability to degrade aniline, a toxic chemical which is present in the effluents of many industries. The strain has been reported in same area in wheat rhizosphere by Arshad et al. (2006) while it is being reported for the first time that the isolated strain has a potential for aniline degradation. The strain grew well in selected media containing aniline with glucose and without glucose showing that it readily decomposed it and utilize it as a sole source of carbon and energy. Similar findings on *Pseudomonas* sp. (PN1001) that actively utilized and degraded pentylaniline and aniline as carbon source with no glucose/starch degradation has being reported by Wang et al. (2006).

Transformation processes are accelerated by adaptation to aromatic compounds which are basic criterion for microbial selections (Bastos et al., 1995). Shake flask transformation of aniline was studied and selection of ST1 was done by adapting it to higher concentration (2000 ppm) of aniline with and without glucose. It was observed that cell growth was increased with time and aniline concentration was decreased as traced by HPLC.

It was found that the total percent reduction was decreased with increase in aniline concentration. As for the lowest concentration (100 ppm), more reduction (59.65% with glucose) was seen after 72 h than with higher concentrations of 200 and 300 ppm which was 38.75 and 31.23%, respectively with glucose. It showed that at higher concentration of aniline, there could be an inhibi-tory effect on the strain which was clear by the less degradation of aniline by the strain at higher concentrations.

HPLC analysis also showed some other smaller peaks of degradative products of aniline which were also studied and were confirmed as benzene, phenol and catechol by their respective standards. The fate of other carbon ring system remained unclear, because benzene (as a product of reductive cleavage), phenol (as a product of hydrolytic cleavage) were not observed in the experiment with the method employed (Drzyzga and Blotevogel, 1997). The 1,2-dichlorobenzene can be co-metabolized (biodegraded) in the presence of glucose by *Pseudomonas* and *Staphylococcus* species. The catabolic end products can be analyzed in the culture medium (Ziagova and Liakopoulou-Kyriakides, 2006).

Aniline transformation in the presence of glucose showed promising results. Without glucose, less reduction (45.97 %) in aniline concentration was observed after 72 h which is less than aniline with glucose (59.65%) for 100 ppm. The same pattern of aniline reduction was seen for 200 and 300 ppm when glucose was used as an additional supplement. This indicated that the glucose posed a stimulatory effect on the microbes to degrade aniline faster. The induction of aniline oxidizing activity in *Rhodococcus erythropolis* AN-13 was accelerated in the presence of glucose with an increase in cell growth (Aoki et al., 1983). Shukat et al. (1983) reported that *Rhodococcus* sp. AN117 was able to co metabolize 2- and 3-chloroaniline in the presence of glucose and that the addition of supplemental glucose did not have any inhibitory effect on the rate of aniline degradation.

The bacterial transformation of aromatic compounds has frequently been shown to be plasmid encoded as reported for many microbial strains (Chakrabarty, 1972; Dunn and Gunsalus, 1973; Williams and Murray, 1974; Anson and Mackinnon, 1984; Saint et al., 1990). Plasmid curing of ST1 showed that 90% of the colonies failed to grow on PNR-aniline agar plates which was possibly due to fact that aniline catabolic pathway genes were plasmid encoded.

The plasmids of ST1 were of larger size of more than 20 kb approximately in the range of 30 - 40 kb. This was confirmed by comparing with DNA marker of known size and by the slow movement of plasmid DNA on agarose gel. The isolated plasmid DNA was digested with restric-

tion endonucleases (*Eco*RI, *Bam*I, *Cla*I, *Stu*I, *Pst*I and *Hind*III) and the resultant restriction fragments were separated by electrophoresis on an agarose gel against the standard to calculate the molecular weight of different restriction DNA fragments. Two bands for *Eco*RI, *Cla*I and *Stu*I have more than one restriction sites and hence results in digests of smaller size 8 - 10 kb, while linear bands were observed for, *Bam*HI, *Pst*I and *Hind*III. These had one s restriction site for each.

From the present study, it is concluded that *Staphylococcus aureus* ST1 is a newly reported bacterial strain that is capable of utilizing aniline as a growth substrate with a catabolic rate that is up to 59.65%. Glucose on the other hand, proved to be an additional carbon constituent to influence and increase the activity of the strain. The genes responsible for aniline degradation are plasmid encoded with different restriction sites.

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Full Length Research Paper

Enhanced production of intracellular dextran dextrinase from *Gluconobacter oxydans* using statistical experimental methods

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Optimization of the fermentation medium for DDase production by *Gluconaobacter oxydans* M5 was carried out in the shake flasks using two kinds of statistical methods. Four variables, namely glucose, tryptone, yeast extract and sodium chloride, were found to influence DDase production significantly by the Plackett-Burman screening. A four-factor five-level central composite design (CCD) was chosen to explain the combined effects of the four medium constituents. The optimum medium consisted of glucose (17.670 g/L), maltobiose (30 g/L), tryptone (12.198 g/L), yeast extract (13.528 g/L), ammonium nitrate (15 g/L), copper sulfate (0.01 g/L), zinc sulfate (0.01 g/L), and sodium chloride (0.009 g/L); the initial pH 6.0 was set prior to sterilization. The DDase yield obtained from optimized medium increased by 17-fold (0.238 U/mL) or so. Under these optimal conditions, the experimental values agreed with the predicted values, indicating that the chosen method of optimization of medium composition was efficient, relatively simple, time reducing and material saving.

Key words: Dextran dextrinase, *Gluconobacter oxydans,* medium optimization, Plackett-Burman design, central composite design.

INTRODUCTION

Dextran, which is synthesized from sucrose by the dextran sucrase (EC 2.4.1.5, DSase) of *Leuconostoc mesenteroides*, is widely used in pharmaceutical and biochemical fields (Mountzouris et al., 1999; Robyt and Walseth, 1979; Sidebotham, 1974). Dextran dextrinase (EC2.4.1.2), produced by *Gluconobacter oxydans*, could also convert maltooligosaccharides to dextran (Hehre, 1951), which have consecutive α(1,6)-linked glucose residues in the main chains and a wealth of α(1,4), α(1,3) and α(1,2)-branch linkages (De Muynck et al., 2007; Mountzouris et al., 1999). Yamamoto et al. purified the intracellular DDase from *G. oxydans* ATCC11894 and reported three different transglucosylation modes of the DDase enzyme (Yamamoto et al., 1992, 1993a).

The difference in structure between *Gluconobacter* and *Leuconostoc* dextran had been further studied and *G.* dextran could be used as a dietary fibre (Yamamoto et al., 1993b). Furthermore, *G.* dextran was used not only as a fat replacer in acceptable low-fat foods but also for taste improvement of the bitter-sweet compound stevioside (Sims et al., 2001; Yamamoto et al., 1994). What's more, *G.* dextran displayed shear-thinning flow behavior (Naessens, 2003). According to the former research, the novel polysaccharide displayed lower viscosity than *L. mesenteroides* dextran of similar molecular weight as a consequence of its higher degree of branching, and it might be suitable for certain food applications not asso-

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Abbreviations: DDase, Dextran dextrinase; **CCD,** central composite design; **DSase,** dextran sucrase; **NPG,** *p*-nitrophenyl-α-D-glucopyranoside; **R^2,** determination coefficient; **RSM,** response surface methodology.

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ciated with thickening functionality, such as a source of dietary fibre or a low-calorie bulking agent for sweeteners (Naessens et al., 2005). Above all, *G. oxydans* DDase could promise alternatives to *L. mesenteroides* DSase as biocatalysts for the synthesis of dextran and oligodextrans. But the enzyme system had been studied far less extensively than the glucosyltransferase of *L. mesenteroides*.

In our group, *G. oxydans* have been studied for about 20 years in the applications of incomplete oxidation, such as synthesizing L-ribulose, D-tagatose, miglitol and chiral aldehydes (Gao et al., 2006; Gao and Wei, 2006; Yang et al., 2008). In the process of these studies, we have found the strain also has the potential ability of interconverting between maltodextrins and dextran. It is known to all that changing environmental factors, such as the medium composition and the concentration of nutrient, can lead to an increase of DDase production. The development of an economically productive medium requires selecting carbon, nitrogen, ion and trace element sources. And there were two ways by which the problem of medium component limitations may be addressed: classical and statistical (Mao et al., 2007). As for the classical experimental design, there are several limitations towards complete optimization. Moreover, medium optimization by single dimensional search needs a great deal of experiments to determine optimum levels, which are unreliable. Furthermore, it was hard to evaluate the relative significance and the presence of complex interactions of several affecting factors (Kammoun et al., 2008; Mao et al., 2007; Tan et al., 2010). Compared with the classical method, the statistical experimental design in the fermentation medium optimization exhibits its advantages, including more advanced results with less process variability, closer confirmation, less development time and less overall costs.

In this study, medium optimization for production of intracellular DDase by *G. oxydans* was reported to make it clear that fermentation factors influenced the DDase yield under statistical experimental design.

MATERIALS AND METHODS

Microorganism

The strain *G. oxydans* M5 was stored as 1 mL aliquots in 20% glycerol at -72°C. The frozen cultures were plated periodically to control their viability.

Medium and growth conditions

The *G. oxydans* grew in a medium containing 80 g of D-sorbitol, 20 g of yeast extract, 2 g of KH_2PO_4 and 0.5 g of $MgSO_4 \cdot 7H_2O$ in 1 L of deionized water. The initial pH was set at 6.0 prior to sterilization.

The organism was incubated at 30°C at 200 rpm in a rotary shaker for 24 h. After 24 h cultivation, 5 mL of the seed culture was used to inoculate 50 mL of production medium in a 250 mL glass flask and this culture was grown under the same conditions as the seed culture. In the optimization process, the amounts of every component were changing in different experimental processes. All the experiments were carried out in three parallel tests.

DDase assay

The extraction method of *G. oxydans* DDase was as described in the former research by Yamamoto et al. (1992). The *G. oxydans* DDase activity was determined in accordance with the method described by Naessens et al. with some modifications (Naessens et al., 2005). It was based on the release of nitrophenol from *p*-nitrophenyl-α-D-glucopyranoside (NPG), mimicking G2 utilization by the transglucosidase, which was developed. The DDase specificity of the test was proven via an NPG zymogram. All results represented an average of three separate determinations. One unit of DDase activity was defined as the amount of enzyme which liberates 1 µmol of nitrophenol from NPG per min under the standard assay conditions.

Experimental design and response surface methodology

Screening for the important factors

For screening, the Plackett-Burman design was applied with which 12 runs would enable us to estimate all main effects for up to 11 factors. Table 1 showed the Plackett-Burman design used in this study (Plackett-Burman, 1946). Since there were only 9 factors in this case, less than the maximum allowable factors, the remained 2 factors became “dummy factors” which would be used to estimate errors. The signs -1 and +1 represented the lower and higher levels of the corresponding components under investigation. In this experiment, the higher levels of the components were chosen to equal 1.25 times of their lower levels (Table 2).

Central composite design (CCD) method

Sodium chloride, tryptone, glucose and yeast extract were four effective nutrients in the Plackett-Burman design. These variables were selected to find the optimum condition for higher DDase production using central composite design (CCD). In this case, a 2^4 full factorial central composite design for four independent variables each at five levels with eight star points and six replicates at the centre points was employed to fit a second order polynomial model, in which 30 experiments were required in this procedure (Box et al., 1978, Box, 1951). Each variable was designed at five levels (–2, –1, 0, 1, 2) and the lowest and the highest concentration were: sodium chloride, 0 and 0.02 g/L; tryptone, 2 and 18 g/L; glucose, 5 and 55 g/L; yeast extract, 2 and 18 g/L , respectively. Table 3 showed the CCD design for the values and the observed response for the DDase activity. The central values (zero level) chosen for experimental design were same as the lower level (designated -1) in the Plackett-Burman design.

For statistical calculation, the relationship between the coded and actual values is described as the following equation:

$$X_i = \frac{U_i - U_i^0}{\Delta U_i} \quad (1)$$

where X_i is the coded value of the ith variable, U_i is the actual value of the ith variable, U^0_i is the actual value of the ith variable at the center point and ΔU_i is the step change of variable.

The response variable (DDase activity) suitable to a quadratic

Table 1. Plackett-Burman design for 11 variables with coded values.

S/N	A	B	C	D	E	F	G	H	J	K	L	DDase (U/mL)
1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	0.171
2	-1	1	1	-1	1	-1	-1	-1	1	1	1	0.174
3	1	1	-1	1	1	-1	1	-1	-1	-1	1	0.193
4	1	-1	-1	-1	1	1	1	-1	1	1	-1	0.178
5	1	-1	1	1	-1	1	-1	-1	-1	1	1	0.192
6	-1	-1	1	1	1	-1	1	1	-1	1	-1	0.171
7	1	1	1	-1	1	1	-1	1	-1	-1	-1	0.144
8	-1	1	1	1	-1	1	1	-1	1	-1	-1	0.166
9	1	1	-1	1	-1	-1	-1	1	1	1	-1	0.153
10	-1	1	-1	-1	-1	1	1	1	-1	1	1	0.165
11	-1	-1	-1	1	1	1	-1	1	1	-1	1	0.138
12	1	-1	1	-1	-1	-1	1	1	1	-1	1	0.169

Table 2. Assigned concentrations of variables at different levels in Plackett-Burman design.

Factor	Variables with designate	Lower level (-1) (per litre)	Higher level (+1) (per litre)
1	A Maltobiose	30 g	37.5 g
2	B Glucose	30 g	37.5 g
3	C Tryptone	10 g	12.5 g
4	D Yeast extract	10 g	12.5 g
5	E Ammonium nitrate	15 g	18.75 g
6	F Copper sulfate	0.01 g	0.0125 g
7	G Zinc sulfate	0.01 g	0.0125 g
8	H Sodium chloride	0.01 g	0.0125 g
9	J Prior pH	6	7.5
10	K Dummy		
11	L Dummy		

equation for the variables was as following:

$$Y = b_0 + \sum_i b_i x_i + \sum_i \sum_j b_{ij} x_i x_j + \sum b_{ii} x_i^2 + e \quad (2)$$

where Y is the measured response, X_i, X_j are input variables which influence the response variable Y; b_0 is a constant; b_i is the ith linear coefficient; b_{ii} is the squared coefficient and b_{ij} is the ijth cross-product coefficient.

The 'Design Expert' software (version 7.1.6, Stat-Ease, Inc., Minneapolis, USA) was utilized to analyze the significance of experimental results. The central composite design (CCD) employed to fit a second order polynomial model, had four variables, of which everyone was at five levels with eight star points and six replicates at the centre points and the result indicated that 30 experiments were required for this procedure (Cochran, 1957).

RESULTS AND DISCUSSION

Screening for the important factors

The effects of different carbon sources on the growth of *G. oxydans* and DDase activity were studied in the media containing yeast extract (2%) as the nitrogen source. The results showed that glucose and maltobiose were better than other carbon sources for the DDase production; the effects of nitrogen sources on the DDase production by *G. oxydans* were investigated using glucose (2%) as carbon source and it exhibited that tryptone was the best and yeast extract and ammonium nitrate was inferior (data not shown). Then the DDase yield was determined for fermentation containing glucose (2%) and tryptone (1%) as carbon and nitrogen sources and one of the following ions (0.001, 0.01 and 0.1%) were used as additives: KCl, NaCl, $MgCl_2 \cdot 6H_2O$, $FeCl_3 \cdot 6H_2O$, $CoCl_2 \cdot 6H_2O$, $BaCl_2 \cdot 2H_2O$, $CaCl_2$, $FeSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, $ZnSO_4 \cdot 7H_2O$ and $MnSO_4 \cdot H_2O$. High yield of DDase was observed when $CuSO_4$ or $ZnSO_4$ or NaCl was used as trace mineral "cocktails" for fermentation (data not shown). According to above study, which was under the premise of keeping the other factors constant, we optimized the composition of the production medium as follows: glucose 30 g/L, maltobiose 30 g/L, tryptone 10 g/

Table 3. The CCD design for the values in coded and the observed values in response.

S/N		Sodium chloride (g/L)	Tryptone (g/L)	Glucose (g/L)	Yeast extract (g/L)	DDase (U/mL)
1	CENTER	0.01	10	30	10	0.177
2	CENTER	0.01	10	30	10	0.173
3		0	10	30	10	0.114
4		0.005	14	17.5	14	0.207
5		0.005	6	17.5	14	0.202
6	CENTER	0.01	10	30	10	0.171
7		0.01	10	30	18	0.141
8		0.015	6	42.5	14	0.077
9		0.005	6	42.5	6	0.092
10		0.01	10	30	2	0.068
11		0.005	14	17.5	6	0.178
12		0.01	18	30	10	0.148
13		0.005	14	42.5	14	0.103
14	CENTER	0.01	10	30	10	0.175
15		0.005	6	17.5	6	0.115
16	CENTER	0.01	10	30	10	0.170
17		0.02	10	30	10	0.105
18		0.01	2	30	10	0.113
19		0.015	14	42.5	14	0.102
20		0.005	6	42.5	14	0.099
21		0.015	14	17.5	6	0.171
22		0.015	6	42.5	6	0.069
23	CENTER	0.01	10	30	10	0.177
24		0.015	14	17.5	14	0.184
25		0.015	14	42.5	6	0.078
26		0.01	10	5	10	0.157
27		0.015	6	17.5	14	0.166
28		0.005	14	42.5	6	0.097
29		0.01	10	55	10	0.067
30		0.015	6	17.5	6	0.111

L, yeast extract 10 g/L, ammonium nitrate 15 g/L, copper sulfate 0.01 g/L, zinc sulfate 0.01 g/L, sodium chloride 0.01 g/L and the initial pH 6.0 was set prior to sterilization. The culture was incubated at 30°C at 200 rpm in a rotary shaker for 62 h.

After above optimization, the Plackett-Burman technique was used to select the most important factors which would influence the DDase yield. In this section, twelve experiments were minimum runs to screen the importance of nine complications, namely glucose, maltobiose, tryptone, yeast extract, ammonium nitrate, copper sulfate, zinc sulfate, sodium chloride and pH. The Model F-value of 20.84 implied the model was significant. There was only a 4.66% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicated model terms were significant. The experimental date analysis showed that four variables, namely glucose, tryptone, yeast extract and sodium chloride out of nine variables influenced the DDase yield significantly.

Central composite design (CCD) method

With the central composite design method, the experiments with different combination of glucose, tryptone, yeast extract and sodium chloride were performed. A four-factor five-level central composite design was used based on the "Design Expert" software (version 7.1.6) and experimental data is presented in Table 3.

By statistical analysis, a mathematical model was obtained to describe the relationship between the DDase production (Y) and the test variables in coded factors (concentration of glucose, tryptone, yeast extract and sodium chloride, respectively):

Table 4. ANOVA for response surface quadratic model.

Source	Sum of Squares	DF	Mean Square	F Value	R^2
Model	0.052	14	3.695×10^{-3}	13.82	0.9280
Residual	4.011×10^{-3}	15			
Lack of Fit	3.970×10^{-3}	10	2.674×10^{-4}	48.97	
Pure Error	4.054×10^{-5}	5	3.970×10^{-4}		
Cor Total	0.056	29	8.109×10^{-6}		

Table 5. The least-squares fit and coefficient estimate.

Factor	Coefficient estimate	Standard error	95% CI Lower limit	95% CI Upper limit	F Value	p-value Prob > F
Intercept	0.17	6.676×10^{-3}	0.16	0.19		
X_1	-6.386×10^{-3}	3.338×10^{-3}	-0.014	7.290×10^{-4}	3.66	0.0750
X_2	0.011	3.338×10^{-3}	3.700×10^{-3}	0.018	10.50	0.0055
X_3	-0.033	3.338×10^{-3}	-0.040	-0.026	99.46	< 0.0001
X_4	0.016	3.338×10^{-3}	8.584×10^{-3}	0.023	22.12	0.0003
$X_1 X_2$	2.243×10^{-3}	4.088×10^{-3}	-6.470×10^{-3}	0.011	0.30	0.5913
$X_1 X_3$	3.192×10^{-4}	4.088×10^{-3}	-8.394×10^{-3}	9.033×10^{-3}	6.095×10^{-3}	0.9388
$X_1 X_4$	-1.888×10^{-3}	4.088×10^{-3}	-0.011	$6.826\times10^{-}$	0.21	0.6509
$X_2 X_3$	-6.383×10^{-3}	4.088×10^{-3}	-0.015	2.331×10^{-3}	2.44	0.1393
$X_2 X_4$	-5.339×10^{-3}	4.088×10^{-3}	-0.014	3.375×10^{-3}	1.71	0.2113
$X_3 X_4$	-8.808×10^{-3}	4.088×10^{-3}	-0.018	-9.439×10^{-3}	4.64	0.0479
X_1^2	-0.014	3.122×10^{-3}	-0.020	-7.089×10^{-3}	19.38	0.0005
X_2^2	-8.551×10^{-3}	3.122×10^{-3}	-0.015	-1.896×10^{-3}	7.50	0.0152
X_3^2	-0.013	3.122×10^{-3}	-0.020	-6.466×10^{-3}	17.66	0.0008
X_4^2	-0.015	3.122×10^{-3}	-0.022	-8.330×10^{-3}	23.03	0.0002

CI = Confidence interval; Prob = probabaility; F = frequency.

$$Y = 0.17 - 6.386\times10^{-3}X_1 + 0.011X_2 - 0.033X_3 + 0.016X_4 + 2.243\times10^{-3}X_1X_2 + 3.192\times10^{-4}X_1X_3 - 1.888\times10^{-3}X_1X_4 - 6.383\times10^{-3}X_2X_3 - 5.339\times10^{-3}X_2X_4 - 8.808\times10^{-3}X_3X_4 - 0.014X_1^2 - 8.551\times10^{-3}X_2^2 - 0.013X_3^2 - 0.015X_4^2 \quad (3)$$

The coefficient values of the model and factors were calculated by Design Expert Software and their values are shown in Tables 4 and 5. The results of the second order response surface model fitting in the form of ANOVA were given in Table 4.

In Table 5, the Model F-value of 13.82 implied that the model was significant. There was only a 0.01% chance that a "Model F-Value" this large could occur due to noise. The "Lack of Fit F-value" of 48.97 implied the Lack of Fit was significant. The well fit of the model was checked by determination coefficient (R^2). In this case, the value of the determination coefficient (R^2 = 0.9280) indicated that it was reasonable to use the regression model to analyze the tendency (Akhnazarove, 1982; Khuri and Cornell, 1987). Table 5 shows the significance of each coefficient determined by F-value and P-value. Values of "Prob > F" less than 0.0500 indicate model terms were significant. In this case X_2, X_3, X_4, X_3 X_4, X_1^2, X_2^2, X_3^2, X_4^2 were significant model terms.

Figures 1 - 6 shows the response surface for the variation in the production of DDase, from which the values of DDase activity for different concentrations of the variables could be predicted. Each contour curve represented an infinite number of combinations of two test variables with another maintained at their zero level. Figures 1 - 3 show that increasing the sodium chloride concentration from 0.003 to 0.01 g/L increased DDase production. The maximum production was obtained with the concentration of sodium chloride at about 0.01 g/L. The two nitrogen sources, tryptone and yeast extract, were important to increase the DDase yield. From the response surface contour plot, the concentrations of tryptone and yeast extract were around 12 - 14 g/L, respectively, to result in the maximum DDase yield. In this study, glucose was also a significance parameter. Figures 2, 4 and 6 have shown that the maximum DDase activity appeared at the concentration of 17.5 - 18 g/L.

According to the optimized mathematical model, the optimal values of the test variables in coded unit were as follows: X_1 = -0.156, X_2 = 0.549, X_3 = -0.986, X_4 = 0.882

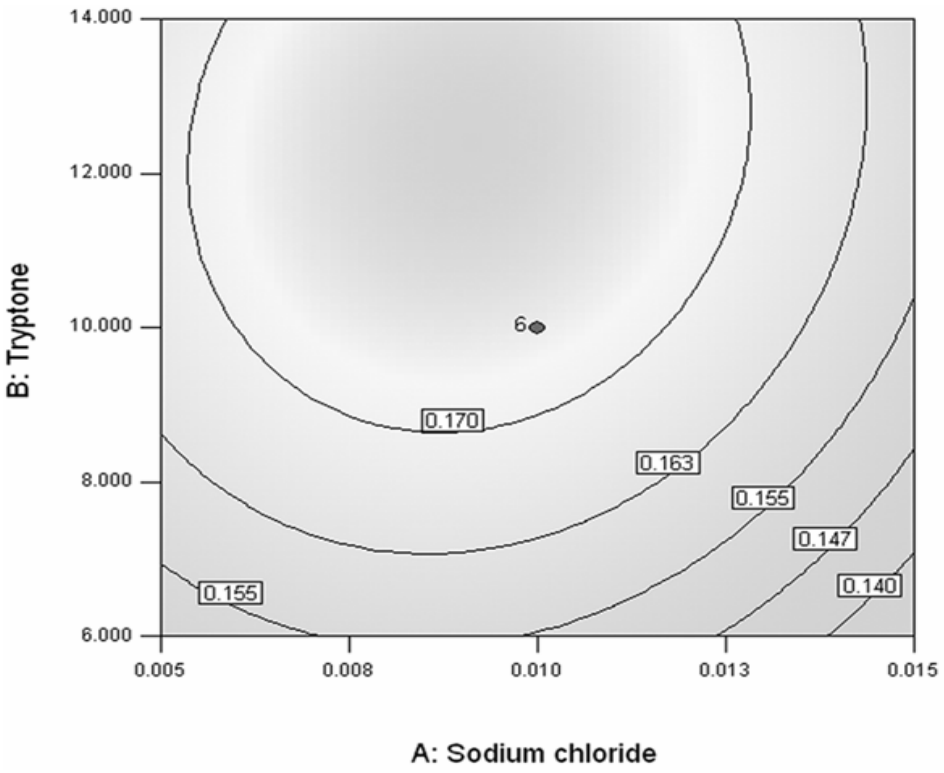

Figure 1. Effect of sodium chloride and trypotone concentration on the DDase production by *G. oxydans*. Other variables are held at zero level.

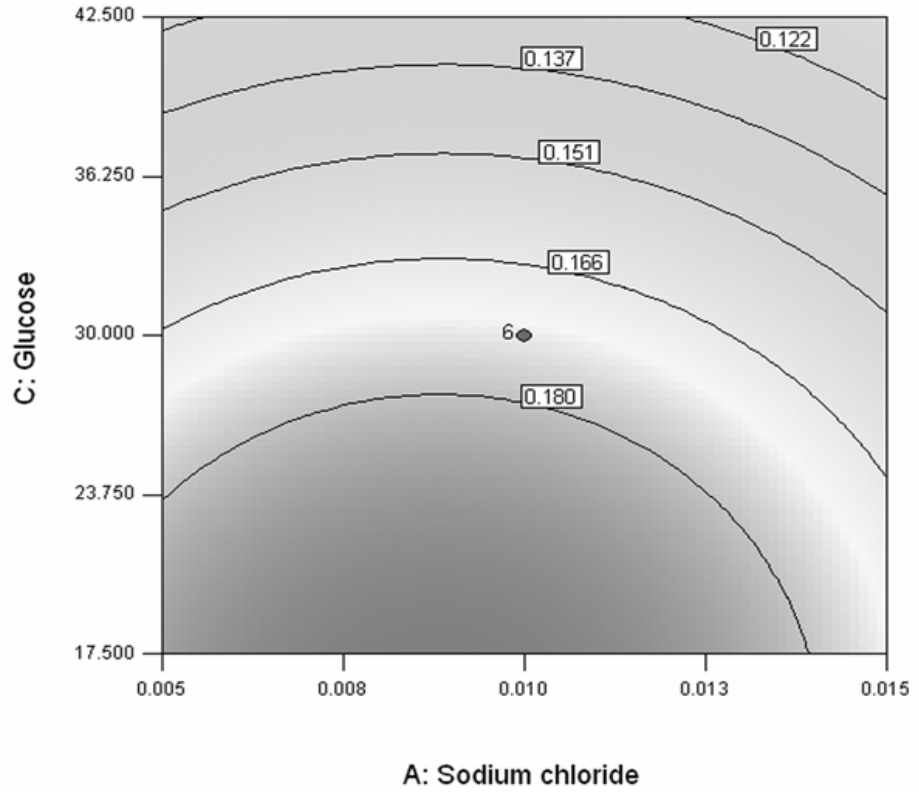

Figure 2. Effect of sodium chloride and glucose concentration on the DDase production by *G. oxydans*. Other variables are held at zero level.

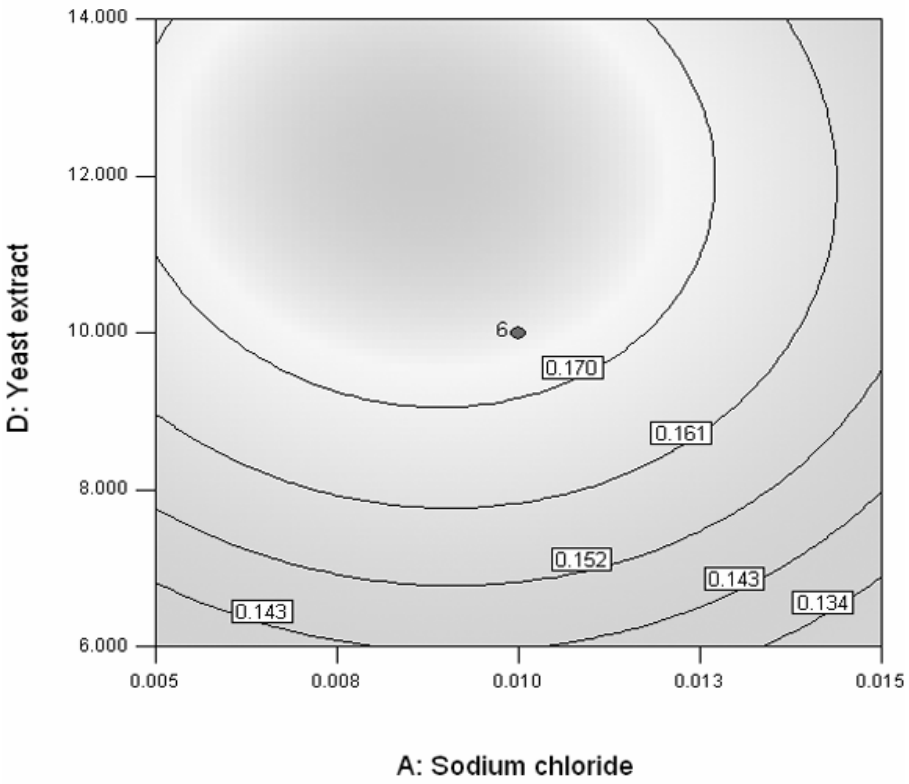

Figure 3. Effect of sodium chloride and yeast extract concentration on the DDase production by *G. oxydans*. Other variables are held at zero level.

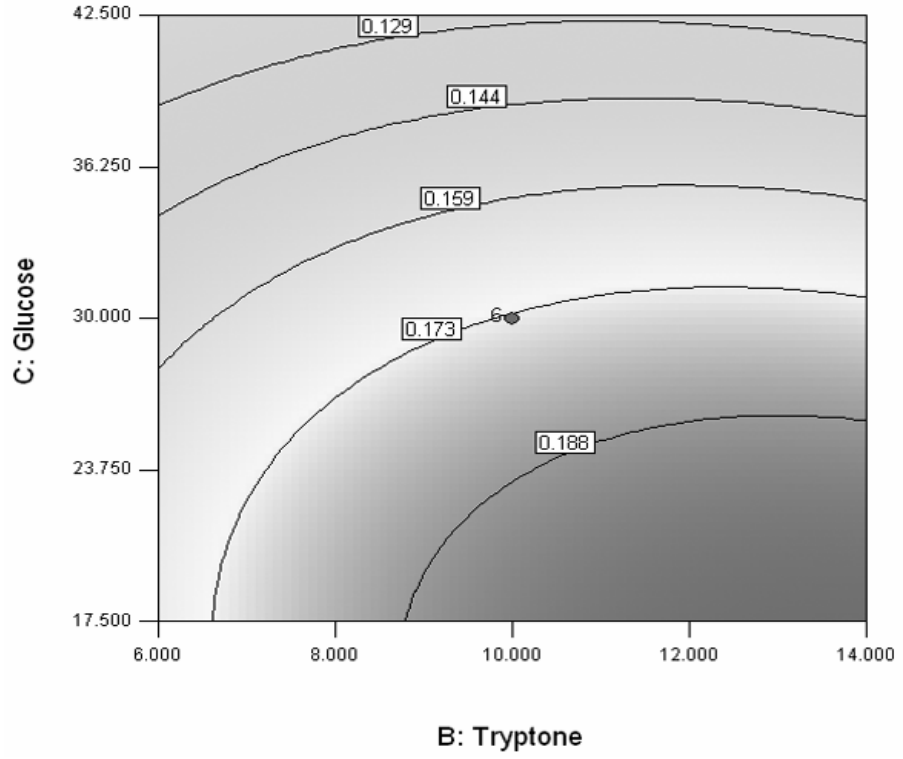

Figure 4. Effect of trypotone and glucose concentration on the DDase production by *G. oxydans*. Other variables are held at zero level.

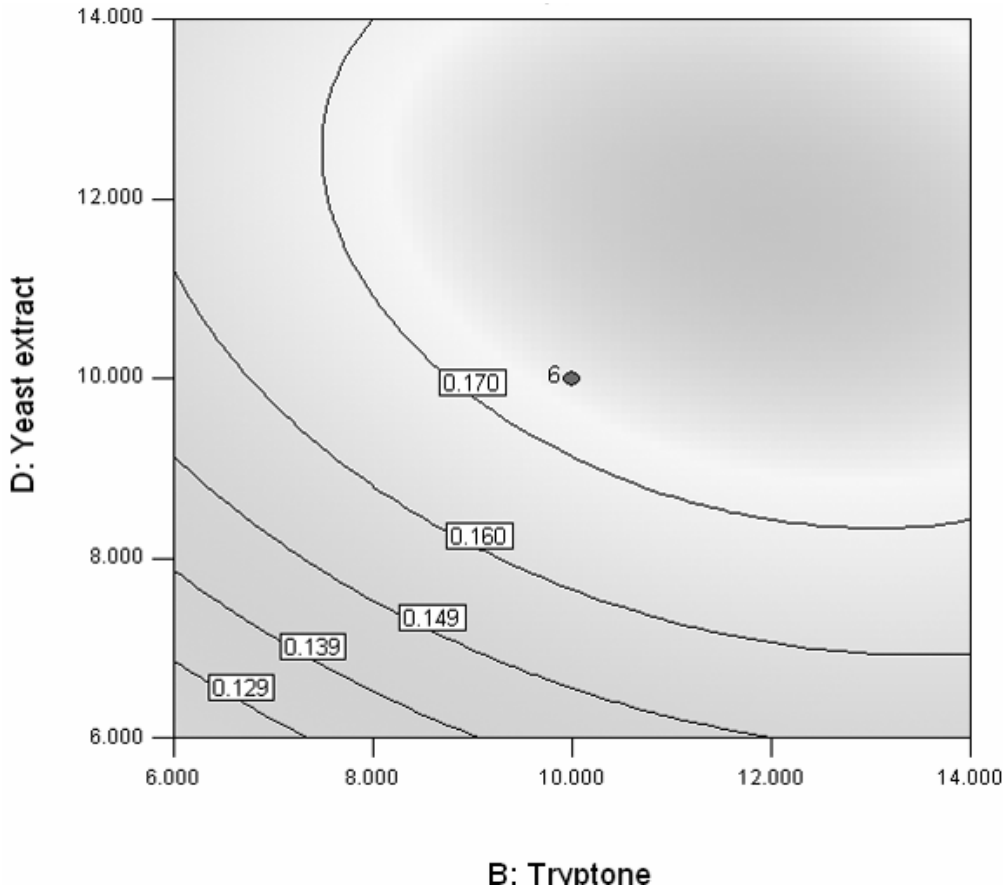

Figure 5. Effect of trypotone and yeast extract concentration on the DDase production by *G. oxydans*. Other variables are held at zero level.

with the corresponding maximum DDase production Y = 0.209 U/mL (a possible variation is between 0.17 and 0.25 mg/L) in the confidence range of 95%. Putting the respective values of X_i in Equation (1), the concentration of sodium chloride, tryptone, glucose and yeast extract were at 0.009, 12.198, 17.670 and 13.528 g/L, respectively. It was clear that the optimal values from response surface plots were almost consistent with those from optimized mathematical equation.

In order to verify the predicted results, the experiment was performed with the optimized medium and the maximum DDase production was found to be 0.238 U/mL, which was obvious in close agreement with the model prediction. After optimization, compared with the minimum medium, the production of DDase was enhanced about 17 fold, experimentally (Table 6). To our best knowledge, the DDase production in this research is higher than any other reported results (Naessens et al., 2005).

Conclusions

Plackett-Burman screening and response surface methodology (RSM) for medium optimization were employed for the DDase production from *G. oxydance*. The method used was effective in the screening for nutritional requirements in a limited number of experiments. It enabled us to screen a large number of experimental factors and was useful to determine the optimum levels of medium components concentration that significantly influenced the DDase production in a perfect way. The chosen method of optimization of medium composition was efficient, relatively simple, time reducing and material saving. The final composition of definite medium after the optimization was as follows: glucose 17.670 g/L, maltobiose 30 g/L, tryptone 12.198 g/L, yeast extract 13.528 g/L, ammonium nitrate 15 g/L, copper sulfate 0.01 g/L, zinc sulfate 0.01 g/L, sodium chloride 0.009 g/L, respectively, and the initial pH was set at 6.0. The optimization of the medium resulted in an about 17-fold (0.238 U/mL) increase in DDase yield. This work would provide a stable foundation for the industrialization of DDase.

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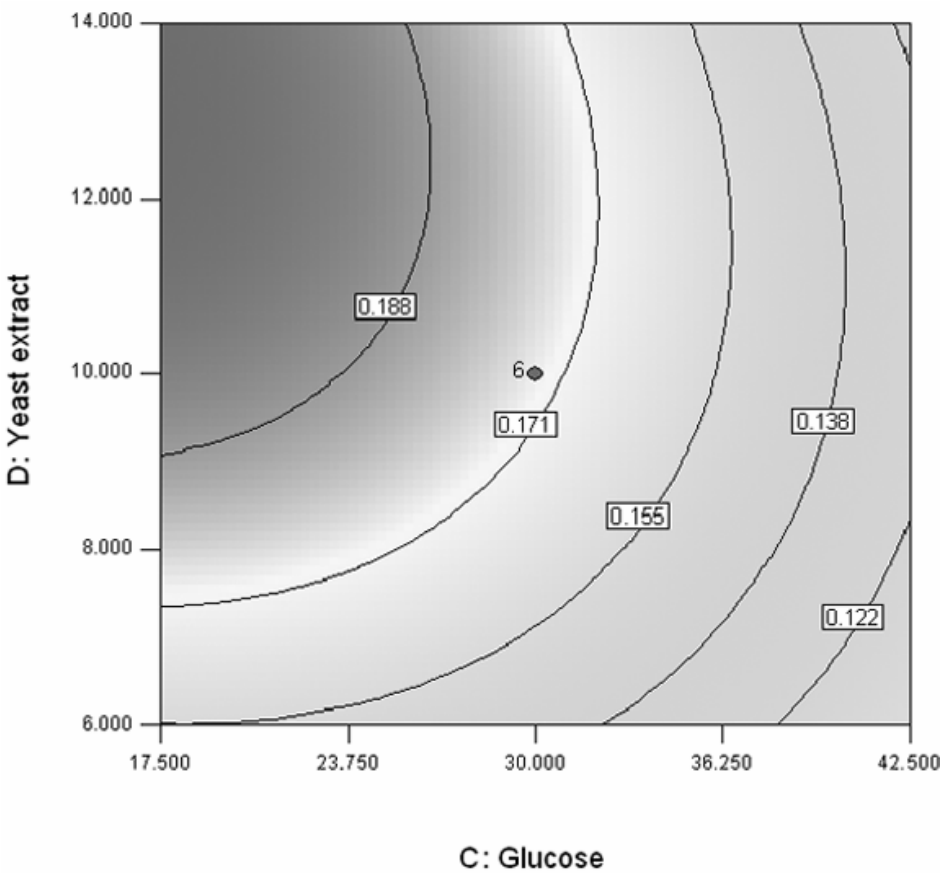

Figure 6. Effect of glucose and yeast extract concentration on the DDase production by *G. oxydans*. Other variables are held at zero level.

Table 6. Experimental verification of combined effect of optimized medium on the response of DDase production.

Variables	Levels after optimization		DDase production (U/mL)		
	Coded	Uncode (g/L)	Unoptimized medium	New medium	
				Predicted	Experimental
Sodium chloride	-0.156	0.009	0.013	0.209	0.238
Tryptone	0.549	12.198			
Glucose	-0.986	17.670			
Yeast extract	0.882	13.528			

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Full Length Research Paper

Combinations of nisin with salt (NaCl) to control *Listeria monocytogenes* on sheep natural sausage casings stored at 6°C

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This study evaluated the effect of combinations of nisin with salt (NaCl) to control *Listeria monocytogenes* on sheep natural sausage casings. Casings were inoculated with 3.0 x 10^5 cfu/g final inocula of *L. monocytogenes*, stored at 6°C in different solutions of nisin at 0, 100, 150 and 200 µg/g. Each combined with salt at 0, 4, 7 and 12% (w/v). Samples were taken at day 0, 10, 20, 35, 60 and 90 post-inoculation and the number of bacteria present was determined. The bactericidal effect of nisin against *L. monocytogenes* cells was evident where nisin was applied in combination with salts. In all treatments, nisin/salt mixtures induced a bacterial growth inhibitory effect greater than salt alone. These results indicate that nisin and salt synergistically and significantly inhibit the growth of *L. monoctogenes* in sheep natural casings. The use of nisin combined with salt as antibacterial agent will be appropriate for applications on natural sausage casing industries as natural preservatives to control foodborne pathogens. They can be used as growth inhibitors of *L. monocytogenes,* an important foodborne pathogens and spoiling bacterium. The main reason for their appropriateness is their natural origin, which consumers find comforting. These beneficial characteristics could increase casings safety and shelf life.

Key words: Nisin, salt, casings, biopreservative, *Listeria monocytogenes*.

INTRODUCTION

Natural casings from sheep, hog and beef have been used for thousands of years as an edible container for sausage. They are derived from the gastrointestinal tracts of the respective species (Bakker et al., 1999; Madhwaraj et al., 1980; Pearson and Gillett, 1999). Casings are usually preserved by salting, curing and/or drying (Fischer and Schweflinghaus, 1988) to reduce the microbial contamination, but enteric or exogenous microorganisms in the natural casing are inevitable and also the number of microorganisms increases during processing and distribution, especially under unhygienic treatment or high storage temperature (Trigo and Fraqueza, 1998). By their nature, natural casings are contaminated with bacteria (10^4 to 10^7 cfu/g) (Bakker et al., 1999; Byun et al., 2001; Ockerman and Hansen, 2000; Gabbis and Silliker, 1974). Generally, adequately salted casings are considered microbiologically fully acceptable. *Listeria monocytogenes* is an important human food-borne pathogen which causes febrile gastroenteritis in healthy individuals (Piana et al., 2005) and life-threatening invasive infections in susceptible individuals (Mead et al., 2006), such as the young, the old, the pregnant and the immune-compromised, the so called "YOPI" (De Cesare et al., 2006). In Europe it has an incidence of 0.3 cases/year/100,000 population (European Food Safety Authority, 2006), this pathogen has the ability to growth over a wide range of pH values (4.3 - 9.6) (Lou and Yousef, 1999) and can survive under salt concentrations as high as 10% NaCl (McClure et al., 1989). Due to the ubiquitous nature of this pathogen in the slaughterhouse and the meat packaging environments, it is not surprising that the incidence and behavior of this pathogen in meat products are receiving increasing attention.

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Table 1. Explanation of group names, nisin and NaCl Concentrations.

Salt (%)	Nisin (µg/g)			
	0	100	150	200
0	A	B	C	D
4	E	F	G	H
7	I	J	K	L
12	M	N	O	P

Presence and survivability of this pathogen were studied by using fresh and salted beef, sheep and hog casings. *L. monocytogenes* was confirmed in beef and fresh hog casings after 30 days of storage in dry-salted at 15°C (Bockemühl, 2000).

L. monocytogenes was positively identified after the mandatory 30 day preservation period for natural casings at a water activity level of 0.85 or lower (Wijnker et al., 2006). Casings may be used in the manufacture of either fresh sausages, fermented sausages, or cooked/sterilized sausages. Eventually pathogens like *L. monocytogenes* occasionally survive in fermented sausages (Encinas et al., 1999; Levine et al., 2001), which are generally consumed in an uncooked condition. Our research activities focus on the identification of biopreservatives that could improve and partly replace salt/brine methods, which are currently applied, to decrease environmental impact of large quantity of salt used by the casing industry and to improve the casing's technical characteristics. A likely substance to obtain this effect is nisin, the best known and studied bacteriocin produced by lactic acid bacteria (LAB). It is the only bacteriocin that has been approved as a food additive in Europe and it is considered safe as bacteriocin in the United States (Delves-Broughton, 1990; Food and Drug Administration, 1998; Montville et al., 2001). Nisin is effective in controlling a wide range of gram-positive pathogenic bacteria, including *L. monocytogenes* (Ming et al., 1997; Siragusa et al., 1999; Coma et al., 2001). The inhibition of Listeria by nisin has been demonstrated in culture media as well as in different foods such as cottage cheese (Ferreira and Lund, 1996), ricotta-type cheeses (Davies et al., 1997), fresh pork sausages (Scannell et al., 1997), cold-smoked salmon (Nilsson et al., 1997) and Ice cream (Dean and Zottola, 1996). Therefore, the aim of this study was to evaluate nisin applied singly or in combination with salt as bio-conservative solutions, for control of *L. monocytogenes* introduced on sheep natural casings and storage at 6°C.

MATERIALS AND METHODS

L. monocytogenes strain and inoculums preparation

The *L. monocytogenes serovar* 4b CECT 4032 strain was used to artificially contaminate sheep natural casing before treatment, packaging and storage. Bacteria were grown in 10 ml BHI agar (Difco) for 24 h at 37°C, diluted and counted and adjusted to a final inoculum of approximately 3.0 x 10^5 cfu/g of sheep natural casings when 0.25 ml of the inoculum was applied to each part of casing, as described below.

Sample preparation

Dry salted Australian sheep casings (AA 20 - 22) were obtained from BOA Company, (Boyauderie d'Atlas Tangier Morocco). These casings were previously cleaned scarped selected and salted according to the Company Standard Operating Procedures. For the experimental treatment, eight hanks were transferred to the microbiology laboratory. Casings were first desalinated for 30 min with flowing lukewarm tap water (temperature 25 ± 2°C). This reduces the residual salt level of dry-salted casings to less than 3 wt% (Lee et al., 1994). Thereafter casings were drained overnight at 3°C to reduce water level. The next day, the casings were divided and weighed of approximately 100 g. Each part was then transferred to the glass recipients for inoculation, treatment, storage and testing, as described below.

Preservation solutions

To preserve casing under controlled conditions, 16 different solutions with specific salt (Salt: 0, 4, 7 and 12%) and nisin concentrations (0, 100, 150 and 200 µg/g) and all possible combinations were prepared with sterilized water. The pH of all solutions was adjusted to 5.8 with HCl (Sigma-Aldrich, Darmstadt, Germany).

Microbiological media and chemicals

PALCAM media and its supplements were purchased from BIOKARD (Beauvais, France). For serial dilutions, TRYPTONE SALT BROTH (BIOKARD) was used. Nisin from *Lactococcus lactis* (subsp lactis) was obtained from Sigma-Aldrich (N5764). Nisin was solubilised in 0.02 M HCl at a concentration of 10 mg/ml with heating (60 - 70°C) to aid solubilisation. The solution was sterilised by filtration through 0.22 µm membrane filters (Millex, Millipore) prior to use and this solution was used throughout.

Product inoculation and treatment with antimicrobials

The desalinated and drained casings were divided into sixteen groups (100 g of each part), placed on glass recipients under a biohazard hood and 0.25 ml of the inoculum was deposited. Inoculated casings were homogenized thoroughly and kept at 6°C for 1 h for inoculum attachment. Thereafter, 100 ml of each antimicrobial solution were added. Each of the groups, were treated separately with different nisin and salt concentrations as showed in Table 1.

Microbiological analysis

Microbiological analysis were realised according to AFNOR V08-055 method, Samples were analyzed for microbiological counts at time-points; 0, 10, 20, 35, 60 and 90 days of storage at 6°C. 10 g of casing for each treatment were transferred into individual sterile plastic bags (Whirl-Paks, Paris, France), mixed with 90 ml of TRYPTONE SALT BROTH (BIOKARD, Beauvais, France) using a Stomacher (Stomacher/Lab Blender 400) for 2 min. Appropriate serial decimal dilutions were made in TRYPTONE SALT BROTH.

The number of *L. monocytogenes* was determined on PALCAM agar (BIOKARD) after incubation at 37°C for 24 h and for further analyses, examination of the TSAYE (BIOKARD) plate with an oblique Henry illumination system, Gram staining, examination for catalase activity, tumbling motility, hemolysis zone on blood agar (BIOKARD), CAMP test were performed according to Harrigan (1998). All treatments were conducted in triplicate. Microbiological counts were expressed as log10 cfu.

Statistical evaluation

Analyse of variance of the data was performed using the ANOVA-MANOVA using statistical software STATISTICA 6.0 (1997 edition). Significant differences ($p < 0.05$) between mean values of triplicate sample were determined.

RESULTS AND DISCUSSION

No *L. monocytogenes* was detected in the natural casings before inoculation. *L. monocytogenes* populations exceeded 6.3 $\log_{10}$ cfu/g in no treated sample - group A (Figure 1). This demonstrates the ability of the pathogen to undergo abundant growth on sheep natural sausage casings without antimicrobials even when stored under refrigeration and acidified solutions. Nisin (100, 150 and 200 µg/g) used alone had no antilisterial effect before 20 days ($p > 0.05$). However, at days 20 to 90 nisin reduced the *L. monocytogenes* population in the all nisin treated simples when compared to the control ($p < 0.001$) (Figure 1). This reduction may be due to acidification of solution of treatment due to lactic acid production by *L. monocytogenes,* which led to a significant acidification (Conner et al., 1986). Nisin is known to act better at pH 5.5 or below (Buncic et al., 1995; De Martinis et al., 1997; Ukuku and Shelef, 1997). The inhibitory action of nisin increased with decreasing pH values .This response could be attributed to acidic damaging effects on target cells concomitant with the higher stability and solubility of nisin (Liu and Hansen, 1990) and with the increase in net positive charge of nisin (Jack et al., 1995). The effect of a combination of nisin (0, 100, 150 and 200 µg/g) with 4% salt to control *L. monocytogenes at* 6°C in casings was studied. At 4% of salt and without nisin, the colonies number of *L. monocytogenes* increased from an initial 5.4 $\log_{10}$ cfu/g to 5.8 $\log_{10}$ cfu/g of casing after 10 days. The bactericidal effect of nisin against *L. monocytogenes* cells was evident in all simple where nisin was applied in combination with 4% (w/v) of salts, regardless of the nisin concentrations, when compared to the control (Figure 2). A decrease was approximately 1 $\log_{10}$ cfu/g at 90 days.

The bactericidal effect of salt at 7% (w/v) against *L. monocytognes* was evident in all groups regardless of the presence of nisin (Figure 3). However, the inhibitory effect was more pronounced in group L, where 200 µg/g of nisin was used ($p < 0.001$). This inhibition reaches 1.2 $\log_{10}$ at 10 days compared to group I (0 µg/g nisin), J (100 µg/g nisin), K (150 µg/g nisin) where the number of

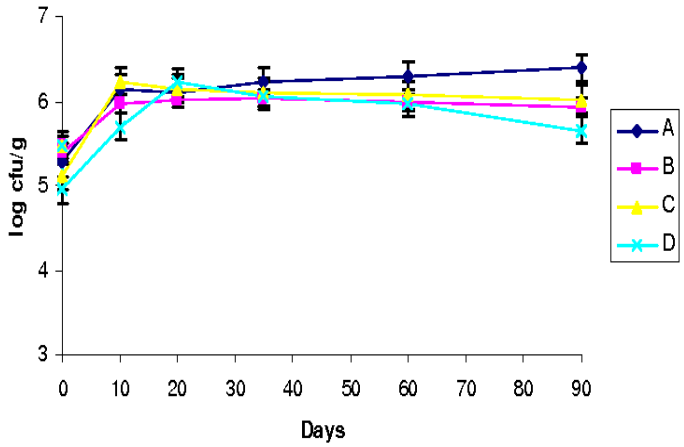

Figure 1. Survival/growth (n = 3) of inoculated *L. monocytogenes* (PALCAM agar) in natural sheep casing treated with (A) 0 µ/g of nisin (B) 100 µg/g of nisin (C) 150 µg/g of nisin and (D) 200 µg/g. Each point represents the mean of three experiments. Error bars represent standard deviation of the means.

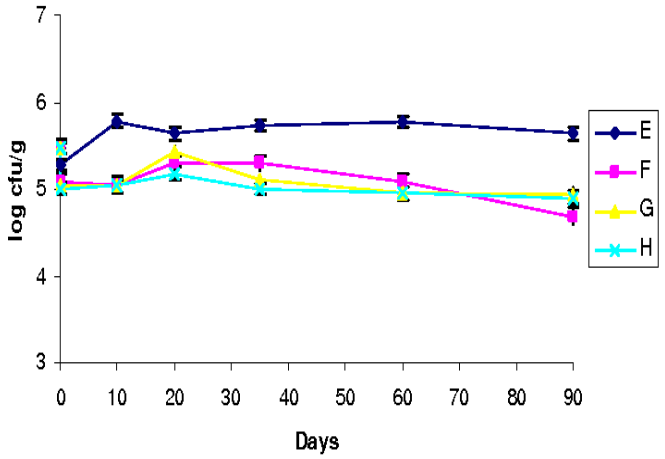

Figure 2. Survival/growth (n = 3) of inoculated *L. monocytogenes* (PALCAM agar) in natural sheep casing treated with 4% of NaCl combined to (E) 0 µg/g of nisin, (F) 100 µg/g of nisin, (G) 150 µg/g and (H) 200 µg/g of nisin. Each point represents the mean of three experiments. Error bars represent standard deviation of the means.

colonies formed by *L. monocytogenes* decrease by 0.4 $\log_{10}$ cfu/g (Figure 3).

A considerable decrease of *L. monocytogenes* was shown when casings were treated with 12% of salt alone or in combination with various nisin concentrations. The decrease reached 2.2 $\log_{10}$ in simple L0 µg/g nisin after 90 days, compared with no nisin treated simple with only 0.7 $\log_{10}$ cfu/g decreases (Figure 4).

In all experiments, nisin added to the same salt concentration induced an inhibitory effect greater than salt alone. Georgnaras et al. (2004a) investigated the antilisterial effects of nisin on commercial bologna and ham. They contaminated the samples with *L. monocytogenes*

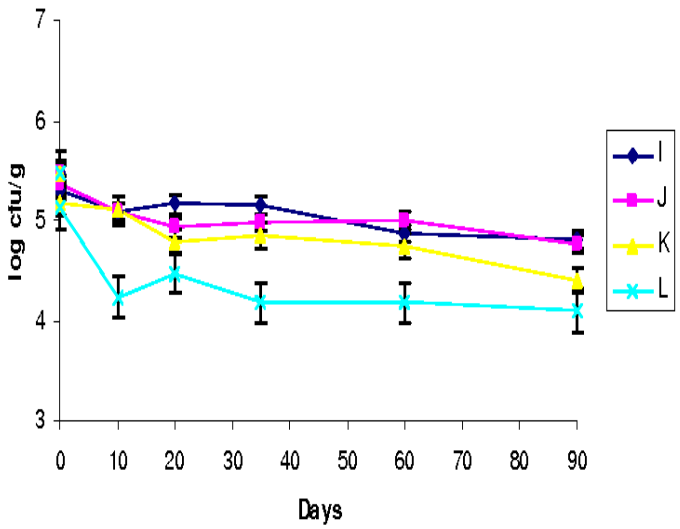

Figure 3. Survival/growth (n = 3) of inoculated *L. monocytogenes* (PALCAM agar) isolated from natural sheep casing treated with 7% of NaCl combined to (I) 0 µg/g of nisin, (J) 100 µg/g of nisin, (K) 150 µg/g and (L) 200 µg/g of nisin. Each point represents the mean of three experiments. Error bars represent standard deviation of the means.

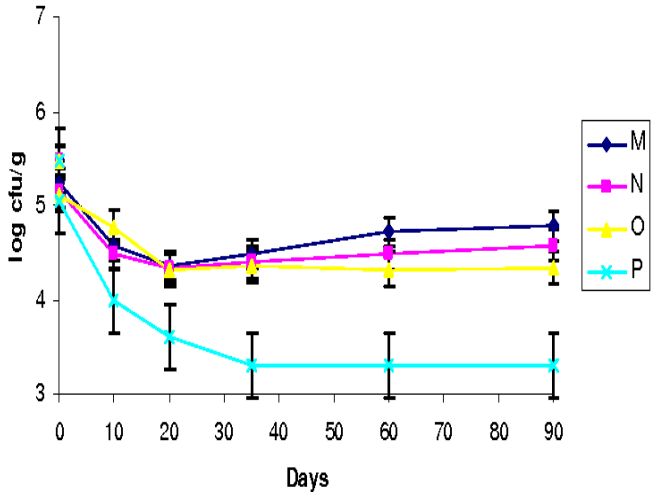

Figure 4. Survival/growth (n = 3) of inoculated *L. monocytogenes* (PALCAM agar) in natural sheep casing treated with 12% of NaCl combined to (M) 0 µg/g of nisin. (N) 100 µg/g of nisin. (O) 150 µg/g and (P) 200 µg/g of nisin. Each point represents the mean of three experiments. Error bars represent standard deviation of the means.

at 3 - 4 log/cm^2 and immersed them in nisin solution (0.5%). They found that nisin reduced the count of *L. monocytogenes* by 2.4 - 2.6 log/cm^2. These researchers performed another study on frankfurter sausages contaminated with *L. monocytogenes* at 3 - 4 log/cm^2 and dipped in nisin solution (0.5%) and found that nisin reduced the count of *L. monocytogenes* by 2.4 - 3.5 log/cm^2 (Georgnaras et al., 2004b). Thus nisin has an antilisterial activity in meat and meat products.

Pawar et al. (2000) found that the growth of *L. monocytogenes* in the treated groups was significantly inhibited compared to the controls. Also, the degree of inhibition increased with increasing concentrations of nisin (Samelis et al., 2005).

Sahl and Bierbaum (1998) proposed that the reaction between nisin and the listerial cell membrane was caused by hydrophobic interaction between the amino acid residues of nisin and the fatty acids of the membrane phospholipids (Henning et al., 1986). It was further suggested that the electrostatic attraction between nisin molecules and the negatively charged phospholipids is involved in the antilisterial effect. Ming and Daeschel (1993) compared the sensitivity to nisin of cells of two strains of *L. monocytogenes* Scott A: one with significantly decreased phospholipid content compared to the parental strain. They observed low antilisterial activity of nisin in the strain with the low phospholipid content. The nisin resistant cells were found to bind less nisin and release less phospholipid than the sensitive cells when treated with the same concentrations of nisin.

Thomas and Wimpenny (1996) and Parente et al. (1998) found that the presence of NaCl enhances nisin action. The emergence of *L. monocytogenes* as an important food-borne pathogen has led to a resurgence of interest in antimicrobials suitable for its control. At the same time, consumer demand for foods that contain fewer preservatives, are less processed, free from artificial additives and perceived as fresh and more natural food stuffs has increased (Gould, 1992). In the present study, it was found that nisin with various combinations of salt has a great influence as an antilisterial effect in sheep natural casings.

Conclusion

In the present study, we found that the combination of nisin with salt significantly inhibited the growth of *L. monoctogenes* in sheep natural casings. Forthcoming investigations should focus on evaluating antimicrobial activity of nisin against other pathogens founding in natural casings to support a potential application for nisin in natural casing industries as potential biopreservative.

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Full Length Research Paper

The incidence and antibiotics susceptibility of *Escherichia coli* O157:H7 from beef in Ibadan Municipal, Nigeria

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The incidence of *Escherichia coli* 0157: H7 was assessed in meat samples from slaughtered cattle in Ibadan metropolis by culturing on sorbitol MacConkey agar and confirmed using serological agglutination kits. The isolates were tested for susceptibility to seven commonly used antimicrobial agents. Out of the total of the 116 non-sorbitol fermenting (NSF) *E. coli* isolated from the samples, 71 (comprising of 18.4, 2.0, 3.2 and 4.8% from Bodija abattoir, Bashorun, Apata and Iwo Road slaughter slabs, respectively) were confirmed as *E. coli* O157:H7 serotype. Antibiotics susceptibility profile showed that all the isolates were resistant to one or multiple antibiotics, resulting in eight different resistance patterns. Tetracycline resistant isolates were the highest with 91.4% incidence. The results are of public health significance confirming cattle as major reservoir of EHEC and antimicrobial-resistant organisms. The high level of carcass contamination with microorganisms may be due to unhygienic slaughtering and meat processing engaged in these abattoir and slabs. Indiscriminate and misuse of antimicrobials on livestock in Nigeria could also be responsible for the multiple resistance pattern of the organism. Application of food hygiene practices such as HACCP is recommended for high quality farm to fork wholesome and safe meat for public consumption in Nigeria.

Key words: EHEC, *Escherichia coli*, beef, food safety Ibadan, Nigeria.

INTRODUCTION

Escherichia coli is a widespread intestinal commensal organism found in human and animal resulting from fecal contamination or contamination during food animal slaughter it is often found in soil, water and foods. Shiga toxin-producing *E. coli* (STEC) O157 has emerged as a public health threat following its initial identification as a pathogen in a 1982 outbreak of illness associated with the consumption of undercooked ground beef (Riley et al., 1983). There are many pathogenic strains causing a variety of illness in man and animals with associated clinical features and virulence factors depending on the serogroups from a food safety perspective, the EHEC groups are most important. Specifically, *E. coli* O157:H7 and O157: NM (non-motile) is recognized as major etiologic agent in hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS) in humans (Thielman and Guerrant, 1999).

In the recent years, there are growing concern of bacterial adaptation and evolution resulting in the emergence of a number of zoonotic microorganisms in the food and water. Food-borne disease is a global public health concern. Mead et al. (1999) reported an estimated food-borne 76 million illnesses, 325,000 hospitalizations and 5,000 deaths annually in United States and in the United Kingdom, an estimated 2.37 million cases of food-borne gastroenteritis occurred in 1995 (Adak et al., 2002). Available data from United States Department of Agriculture Food Safety and Inspection Service indicated that 13 million Kg of ground beef were contaminated with *E. coli* O157:H7 on August 12, 1997 and 9.5 million Kg of beef trimmings and ground beef potentially contaminated with *E. coli* O157:H7 on July 19, 2002 (Sofos, 2008).

Abbreviations: NSF, Non-sorbitol fermenting; **HC,** hemorrhagic colitis; **HUS,** hemolytic-uremic syndrome; **LGAs,** local government authorities.

Table 1. Result of screening of slaughtered cattle from ibadan abattoir and slaughter slabs.

Abattoir/slaughter slabs	Number of samples	Number of NSF *E. coli* isolated	Number of *E. coli* O:157 isolated
Bodija	100	65 (26.0%)	46 (18.40%)
Basorun	50	13 (5.2%)	05 (2.00%)
Apata	50	16 (6.4%)	08 (3.20%)
Iwo road	50	22 (8.8%)	12 (4.80%)
Total	250	116 (46.4%)	70 (28.00%)

Transmission of pathogens to humans may occur from contaminated foods or water, or from infected persons, environments or animals. Food animals, in particular mature cattle are usually asymptomatic carriers of *E. coli* O157, including STEC (Meng et al., 1998) can also serve as reservoirs of antimicrobial-resistant bacteria. Resistance to antibiotics is highly prevalent in bacterial isolates worldwide, particularly in developing countries including Nigeria (Hart and Kariuki, 1998; Aibinu et al., 2007; Okeke et al., 2005; Ojo et al., 2009).

Carcass contamination from hides, skin and gut contents of animals can occur during bleeding, handling and processing of meat which include slaughtering, scalding, eviscerating and washing (Ikeme, 1990). Unhygienic floor dressing of carcasses is a common practice in this part of the world resulting in carcass contamination and isolation of pathogenic microorganisms from meat and slaughtering facilities in Nigeria (Umolu et al., 2006, Ojo et al., 2009).

Ibadan in Oyo State, Nigeria is located on geographic grid reference longitude 3° 5E, latitude 7° 20N with a population of over 2 million (Filani, 1994) and having Federal, State and Local Government participation in meat processing hygiene and inspection. The local government authorities (LGAs) are allowed legally to own slaughter slabs and abattoirs within their boundaries, subject to the approval of the supervising State Veterinary Division. This study investigated the incidence of STEC and characterize antimicrobial susceptibility of the isolates of *E. coli* O157 obtained from the meat samples from the main municipal abattoir and 3 other slaughter slabs at different LGAs to assess the wholesomeness and safety of this product resulting from contamination with STEC, a zoonotic food-borne pathogen.

MATERIALS AND METHODS

Two hundred and fifty replicates meat samples were randomly obtained from 800 carcasses of cattle during slaughter for public consumption at Bodija abattoir, Bashorun, Iwo Road and Apata slaughter slabs within Ibadan metropolis for a period of four weeks. The samples were aseptically collected using the method of carcass scraping adopted by Adams et al. (1980) in sterile sample bags and immediately transported to the laboratory for processing and culture. One gram of meat samples were suspended in 9 ml of peptone water and vortexed. After which, 0.1 ml of the same buffer was spread onto the surface of MacConkey agar and incubated for 24 h at 37°C and sub-cultured onto sorbitol-MacConkey agar plates. The non-sorbitol fermenting colonies were picked and characterized using standard biochemical tests. The sorbitol negative colonies were serologically typed using latex agglutination kit for *E. coli* O157:H7 (Oxoid DRO 120 M, UK). Hemolytic activity of the isolates was tested by culturing the isolates in 7% sheep blood agar (Oxoid Columbia blood agar) and incubating at 37°C for 24 h. Colonies with morphologic characteristics of *E. coli* were confirmed by conventional biochemical tests. The standard disk diffusion method was used for susceptibility testing (according to NCCLS, 1990). The antibiotic disks used include Nitrofurantoin 200 µg, Cefuroxime 25 µg, Norfloxacin 30 µg, Cotrimoxazole 25 µg, Gentamycin 10µg, Tetracycline 30 µg, Ciprofloxacin 25µg, Nalidixic acid 30 µg, Chloramphenicol 30 µg and Ampicillin 25 µg (Abtek Biological Ltd, England). *E. coli* NCTC 10418 and K-12 C600 were used as controls.

RESULTS AND DISCUSSION

E. coli O157:H7 serotypes were isolated from a 28.4% of the samples from the four meat processing center comprising of 18.4, 2.0, 3.2 and 4.8% from Bodija abattoir, Bashorun, Apata and Iwo Road slaughter slabs, respecttively (Table 1). Antibiotic susceptibility profile showed that all the isolates were resistant to one or multiple antibiotics. Tetracycline resistance was the highest in 91.4% of the isolates, while 72.9% resistance to nitrofurantoin and Chloramphenicol, 65.7% to cefuroxime, 44.3% resistance to cotrimozole, 35.7% resistance to nalidixic acid, 11.4% resistance to gentamicin (Figure 1). Eight different resistance patterns were observed (Table 2).

Contamination of meat and other animal products with entero-pathogenic bacteria and their contribution to the spread of antibiotic resistant bacteria to humans via food-chain is a global food safety concern. The results of this investigation showed high incidence microbial contamination of meat in the city as indicated by high incidence of *E. coli* (46.4%) which is usually an indicator organism. The high level of carcass contamination was envisaged due to the unhygienic slaughtering and meat processing engaged in these abattoir and slabs, where butchering of meat are done on concrete floor with inadequate slaughtering basic facilities including lack of potable water. The study also confirms cattle as a major reservoir of EHEC and antimicrobial-resistant organisms for meat consumers which have also been isolated from live cattle, meat and milk from other parts of the country by different researchers (Ojo et al., 2009; Aibinu et al., 2007; Lugal et

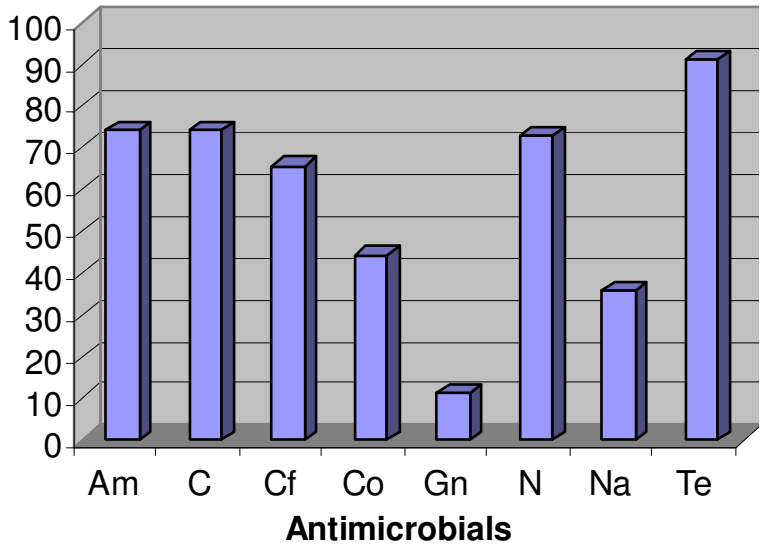

Figure 1. Percentage resistance of STEC isolates to common antimicrobials.

Table 2. Antibiotic resistance patterns of *E. coli* isolated from beef.

Pattern of resistance	Bodija	Bashorun	Apata	Iwo Road	Numbers of resistance isolates
Am,C,Cf,Co, Na,Nf,Te	8	0	2	3	13
Am,C,Cf,Co,Gn,N,Te	5	1	0	2	8
Am,C,Cf,N,Na,Te	10	1	0	1	12
Am C,Cf,N,Te	3	1	1	2	7
Am C,Co,N,Te	8	0	2	0	10
Am,C,N,Te	5	0	0	1	6
Am,C,Cf,N	4	0	1	1	6
Te	3	2	1	2	8
Total	46	5	7	12	70

Key: Am = Ampicillin, C = Chloramphenicol, Cf = Cefuroxime, Co = Cotrimoxazole, Gn = Gentamycin, N = Nitrofurantoin, Na = Nalidixic acid, Nf = Norfloxacin, Te = Tetracycline.

al., 2007; Umolu et al., 2006; Amosun et al., 2005). This study also confirms the widespread resistance to most commonly used antimicrobial agents in both human and animal health practice in Nigeria. The public health significance of these findings is that antimicrobial-resistant bacteria from food animals may colonize the human population via the food chain, contact through occupational exposure, or waste runoff from meat production facilities to the neighborhood. Indiscriminate and misuse of antimicrobials among livestock producers and marketers in Nigeria could also be responsible for the resistance pattern obtained in this study. Tetracycline resistance was the highest in 91.4% of the isolates; it is the most commonly available for use as growth promoter and routine chemoprophylaxis among livestock in Nigeria. The high prevalence of antibiotic resistance in bacteria in Nigeria and other developing countries has been associated with several factors including indiscriminate use due to unregulated access of non-professional to different classes of antimicrobial over-the-counter (Hart and Kariuki, 1998; Okeke et al., 1999). Application of food hygiene practices such as, Good Veterinary Practice and HACCP are therefore recommended for high quality farm to fork wholesome and safe meat processing for public consumption in Nigeria. There is also the need for further surveillance of resistant food borne pathogens and their genetic materials in animal and man ecosystem in order to achieve the global one health aspiration.

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Full Length Research Paper

Effect of processing on iodine content of some selected plants food

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Effect of processing on iodine content of some selected plants food was investigated. Results show significant reduction ($p < 0.05$) in the iodine content of the processed food compared with the raw forms. The iodine value of 658.60 ± 17.2 µg/100g observed in raw edible portion of *Discorea rotundata* was significantly higher compared with the value of 448.60 ± 2.46, 248.60 ± 2.46 and 300.05 ± 5.66 µg/100g dry matter observed in the boiled yam, pounded yam and yam flour, respectively. The result also indicates that the iodine value of 592.50 ± 8.22 µg/100g dry matter observed in the raw form of cassava was higher when compared with that of garri (366.03 ± 3.82 µg/100g) and cassava flour (216.90 ± 1.03 µg/100g dry matter). Similarly, raw Z*ea mays* contain higher iodine (112.24 ± 0.42 µg/100g) compared with boiled maize (79.44 ± 0.64 µg/100g dry matter) and fresh ogi (45.07 ± 1.24 µg/100g dry matter). The result also indicates that raw *Vigina uguicnlata* and *Arachis hypogea* contain higher iodine content (112.22 ± 0.22 and 119.62 ± 0.22 µg/100g dry matter, respectively) when compared with their boiled forms (97.33 ± 1.53 and 83.12 ± 1.35 µg/100g, respectively). Results obtained in this study thus indicate that processing significantly reduces iodine content of food products, hence consideration must be given to different processing methods when accessing Iodine intake from different processed foods.

Key words: Processing, iodine content, food products, fermentation, boiling, roasting.

INTRODUCTION

Iodine is an essential trace element of great importance in human nutrition. The element is an integral part of the thyroid hormones (Dunn and Dunn, 2001). Recommended daily allowance of dietary iodine is 180-200 µg for adults, > 100 µg for children and the daily intake during pregnancy should be at least 230 µg iodine (Horst et al., 2005). As iodine is essential for normal brain development (Delange, 2000), it is particularly important that the foetus and young children have adequate intakes (Venturi et al., 2000).

The term iodine deficiency disorder is used to describe the wide range of effects low iodine status can have on health. Iodine deficiency disorders due to iodine decrease or loss during processing or cooking is still a major public health problem in several areas of the world, especially in developing countries (Ozdemir et al., 2009; Wisnu, 2008). One of the serious health effects of iodine deficiency disorders is goiter (Roti and Uberti, 2001; Rasmussen et al., 2002). In very severe iodine deficiency, stunted growth and mental retardation can occur in children (Laurberg, 2004). A number of studies have reported adverse effects on hearing capacity, motor and cognitive function in children associated with moderate and severe iodine deficiency (Venturi et al., 2000; Ruwhof and Drexhage, 2001; Mann and Aitken, 2003).

Food processing is one of the earliest technologies that man has been using. It has the advantage of insuring food supply and in many cases as a necessary step before consumption for a variety of reason, such as increasing stability, improving flavour, decreasing possibility of toxicity and introducing functioning ability.

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Some of these processing methods are fermentation, frying and cooking (Raghuuath and Belavady, 1997; Prablivathi and Narasinga, 1979). These processing also introduce chemicals that affect nutritional values adversely. Certain food processing practices often increase the amount of iodine in foods. For example, the addition of potassium iodide to table salt to produce "iodized" salt has dramatically increased the iodine intake of people in developed countries (Clark et al., 2002; Diosady et al., 1997). In addition, iodine-based dough conditioners are commonly used in commercial bread-making, which increases the iodine content of the bread (Connolly et al., 1970). When in elemental form iodine readily sublime and is then rapidly lost to the atmosphere (Laurberg, 2004), its iodate form such as potassium iodate can be reduce to elemental iodine by a variety of reducing agents (Diosady et al., 1997). The absorption and/or utilization of iodine are inhibited by components of certain foods. These food components, called goitrogenic compounds, are found primarily in cruciferous vegetables (for example, cabbage and broccoli), soybean products, cassava root, peanuts, mustard, and millet. Over consumption of these foods may lead to thyroid problems by reducing the amount of available iodine for the manufacture of thyroid hormones (Kontras et al., 2002; Soetan, 2008). It is believed that cooking can inactivate the goitrogenic compounds in these foods, thereby eliminating their negative impact on iodine status (Kontras et al., 2002).

In our previous study, we reported on the iodine content of some tubers, fruit and vegetables locally grown and consume in Ijebu North Local Government Area of Ogun State, Nigeria (Salau et al., 2008). Till date, very few studies have been conducted to assess the losses of iodine during cooking procedures. As with most chemical reactions, an increase in temperature (as encountered in cooking and drying) increases rate of reaction that forms elemental iodine and increase its evaporation rate. In light of the above we set to investigate effect of some local processing methods on iodine content of some selected plants food grown and consumed in Ijebu-North local government of Ogun state, Nigeria.

MATERIAL AND METHODS

Sample collection

Yam, cassava tubers, maize, cowpeas and groundnuts were purchase from two major markets in Ijebu-Igbo and Ago-Iwoye in Ijebu-North Local government. Six samples, from each market were purchased randomly. The sample weight varied between 1-5 kg. The six samples for each product were thoroughly mixed together.

Sample preparation

Yam

The edible portion was prepared by cleaning the dirt with water and removing the peel with knife. The moisture and iodine content was then determined. The edible portion was further cut into pieces and later divided into six portions for moisture and iodine content determination. The first two (2) portions were mixed together and boiled. The data generated from this was recorded for boiling. The other two (2) portions were first boiled and used for pounded yam. The last two (2) portions were used for yam flour following the method of Song (1992).

Cassava

The edible portion was also prepared as described for yam. Two other portions were mixed together and used for cassava flour (lafun). The last two portions were used for cassava meal (garri). The garri was prepared using local method. The edible portion was first grated, dewatered, fermented, sieved and then fried (Song, 1992).

Maize

The maize was cleaned and washed in water. 100 g of sample was collected from each portion for moisture and iodine content determination. They were later divided into six portions. Three (3) portions each were mixed together into two parts. One part was boiled and the other part used for ogi following local methods as described by Augustine (1991).

Cowpea and groundnuts

Edible portions were prepared by cleaning and washing with water. The portions were later divided into two parts. One part was used as raw sample while the other part was boiled in water. Samples were collected in six replicate for each analysis

Sample analysis

Moisture Content

Thirty (30) g of each sample were taken (6 replicate) into 200 ml crucible, dried in an oven at 105°C for 24 h and moisture content was thereafter determined.

Ashing

Five (5) g of each dried sample was pulverized using mortar and pestle. Two (2) g of powdered sample was taken and placed in ash crucible. They were then mixed with five (5) g of Na_2CO_3, 5 ml of 0.5 M NaOH and 10 ml ethanol. The sample was placed in the steam bath at 100^{o}C for about 20 min and later transferred to carbolite furnace for about 15 min at 500^{o}C.

Iodine content

The iodine content was analysed according to Elmsie Caldwells methods as modified by Diosady and Fitzgerald (1983).

Statistics

The experimental design was completely randomized. Data were analyzed using the SPSS. Significant difference between the data was determined at $p < 0.05$ using Duncan multiple range test.

Table 1. Iodine content of yam and its products.

Yam and its Products	Moisture content (g %)	Iodine content (µg/100g dry matter)	% difference in iodine content
Fresh yam (edible portion)	68.80 ± 0.20	658.65 ± 17.26[a]	Not determined
Roasted yam	24.60 ± 0.09	592.48 ± 5.82[b]	-9.94
Boiled yam	74.91 ± 2.31	448.60 ± 2.46[c]	-31.89
Yam flour	12.00 ± 1.68	307.40 ± 3.66[d]	-53.33
Pounded yam	64.53 ± 1.82	248.60 ± 2.16[e]	-62.26

Values are expressed as mean of 6 determinations ± SEM.
Values in the same column with thee same superscript are not significantly different from each others.

Table 2. Iodine and Moisture content of cassava (*Maintial esculena*) and its products.

Cassava and its product	Moisture content (g %)	Iodine content (µg/100g dry matter)	% difference in iodine content
Fresh cassava tuber (edible portion)	58.25 ± 1.18	592.50 ± 8.22[a]	Not determined
Cassava flour (lafun)	14.15 ± 0.22	216.90 ± 1.23[b]	-63.39
Cassava meal (garri)	18.32 ± 0.38	366.03 ± 3.82[c]	-38.22

Values are expressed as mean of 6 determinations ± SEM.
Values in the same column with the same superscript are not significantly different from each others.

Table 3. Iodine and iodine content of maize (*Zea maize*) and its product.

Maize and its products	Moisture content (g %)	Iodine content (µg/100g)	% difference in iodine content
Dry maize (edible portion)	0.05±0.88	112.24±1.42[a]	Not determined
Boiled maize	51.01±1.23	79.44±2.64[b]	-29.22
Ogi	66.04±1.21	45.01±1.23[c]	-59.90

Values are expressed as mean of 6 determinations ± SEM.
Values in the same column with the same superscript are not significantly different from each others.

RESULT

Shown in Table 1 is the result of the moisture and iodine content of yam and yam products. Iodine content of all the processed products were significantly ($p < 0.05$) lowered than the raw food. The least reduction (9.94%) was observed in roasted yam, while the highest reduction (62.26%) was observed in pounded yam. The result of moisture and iodine content of cassava and its products is shown in Table 2. The two processed products were significantly lowered ($p < 0.05$) in iodine content when compared with the raw edible portion. The least reduction in iodine content was observed in garri (38.22%) while lafun has the highest reduction (63.39%)

Table 3 is the result of the moisture and iodine contents of maize and its products. Boiling of maize and processing to ogi was observed to significantly reduce the iodine content when compared with the raw edible portion. Highest loss of iodine (59.90%) was observed in ogi whereas as a percentage iodine reduction of 29.22% was observed in the boiled maize.

The result of the moisture and iodine content of cowpea and groundnut (raw edible portions and their products) are shown in Table 4. The result indicates that boiling significantly reduced iodine content by 29.95% in cowpea and 18.63% in groundnut. No significant difference was observed between the iodine content of raw groundnut and cowpea whereas the iodine content of boiled groundnut was significantly higher than that of the boiled cowpea.

No significant correlation was observed between the iodine content and moisture content of all the products.

DISCUSSION

The iodine content of foods is generally reflective of background levels as well as processing technology and manufacturing practices. For example, the high iodine content of milk and dairy products has been attributed to the use of iodine-containing supplements in feed for dairy cattle, iodophor-based medications, teat dips and udder washes as well as iodophors used as sanitizing agents in

Table 4. Iodine and Moisture content iodine content of cowpea (*Vigina uguiculata*), groundnut (*Arachis hypogea*) and their products.

Raw processed form	Moisture content (g %)	Iodine content (µg/100g)	% difference in iodine content
Raw cowpea (edible portion)	11.15 ± 1.33	112.22 ±1.25[a]	Not determined
Boiled cowpea	55.46 ± 2.92	83.12± 1.35[b]	-29.93
Raw groundnut edible portion	21.12 ± 1.30	119.62±3.11[a]	Not determined
Boiled groundnut	61.21 ± 2.48	97.33±1.54[c]	-18.63

Values are expressed as mean of 6 determinations ± SEM.
Values in the same column with the same superscript are not significantly different from each others.

dairy processing establishments (Fischer and Giroux, 1987; Park et al., 1981; Pennington et al., 1986). The elevated iodine levels found in grain and cereal products are related to endogenous iodine in ingredients but, in addition, likely reflect the use of iodine-containing food additives, such as iodate dough conditioners (Varo et al., 1982; Katamine et al., 1986).

In the present study, the major processing methods used were boiling, frying, drying and fermentation. The study indicates that fermentation leads to the greatest loss in iodine content as observed in yam (53.3%), cassava flour (63.39%) and ogi (59.90%). The reduction could be attributed to leaking of iodine into the soaking water and also possibly by evaporation especially during drying of yam and cassava flour. This is in agreement with previous study which reported reduction in certain nutrients during fermentation and drying (Binita and Khetarpaul, 1997; Varo et al., 1982; Nelson and Philip, 1985).

Few older studies with rather inconsistent results exist on the influence of household preparations and processing on the iodine content of foods (Manthey, 1989; Varo et al., 1982; Montag and Grote, 1981). Manthey et al. (1989) found an increase of iodine after cooking and frying of saithe portions of fish, whereas Montag and Grote (1981) reported a considerable decrease in the iodine content after household preparation of plaice and haddock. Our study indicates that cooking reduced iodine content of roasted yam by 10.04%, whereas boiling reduced the iodine content of yam by 31.89%, maize by 29.22%, cowpea by 29.93% and groundnut by 18.63%. Our result indicates a percentage reduction in iodine content that varies from 18.63 to 31.89% as a result of roasting and boiling. The difference observed may be due to differences in cooking time and the nature of plant material. In a previous study, it was reported that roasting of meatballs for 15 min resulted in a 5% loss of iodine. Extension of thermal requirement to 30 min resulted in an increase, almost threefold, of iodine loss (Ozdemir et al., 2009). The same study also reported that the decrease of iodate content and conversion to other iodine species is affected by acidity, moisture content, heating during cooking process and is also influenced by the type of cooking spices and raw materials used.

The observed difference in iodine content between the boiled and pounded yam could be attributed to the release of iodine during pounding.

Conclusion

The result of this study indicates that local processing method reduced iodine content of the selected plant food grown and consumed in Ijebu-north Local Government Area of Ogun State, Nigeria. Though root and tuber contain high level of iodine, the final content at consumption stage is very low. Thus, improved method of processing of the local plant food available in this area in order to ensure that the dietary requirement of iodine is met by people leaving in this area is met may be advocated.

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Full Length Research Paper

Selected cultivars of cornelian cherry (*Cornus mas* L.) as a new food source for human nutrition

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The aim of this work was to determine antioxidant activity in 12 cultivars of cornelian cherry (*Cornus mas* L.). Two assays based on ion reduction of ABTS (2,2´-azinobis(3-ethylbenzothiazoline-6-sulphonate) and DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals were used for antioxidant activity. Total phenolic content of the fruit was analysed by Folin-Ciocelteau colorimetric method and ascorbic acid content was analysed using column chromatography - electrochemical detector (Coulochem III). The highest amounts of total phenolic content were found in cultivars ´Vydubeckij´ and ´Titus´ as 7.96 and 8.11 g gallic acid kg^{-1} of fresh mass (FM). High correlation between polyphenols and antioxidant activity in fruits of the cultivars was observed (r^2 = 0.970 for DPPH test and r^2 = 0.978 for ABTS test). The highest total content of ascorbic acid was determined in cultivar ´Olomoucky´, with the value of 3.11 g kg^{-1} FM. This study attempts to contribute to the knowledge of human nutritional properties of these cornelian cherry cultivars and may be useful for the evaluation of dietary information and further propagation of cultivation and utilization of this fruit in the world.

Key words: Cornelian cherry, phenolics, antioxidant activity, ascorbic acid.

INTRODUCTION

Cornelian cherry (*Cornus mas* L.) belongs to the family Cornaceae. It is a tall deciduous shrub or small tree from 5 to 8 m high. This plant is popular in southern Europe with the northern limit being southern Belgium and central Germany (Mamedov and Craker, 2004). In the Czech and Slovak Republic, it is spread in the area of the White Carpathian Mountains (Tetera, 2006).

Cornelian cherry is a widely distributed species in Europe and it grows up to 1400 m. The species is highly tolerant to diverse abiotic and biotic conditions. The bloom time in Central European conditions begins early in the spring. The fruits are very valuable for fresh consumption and for processing to produce syrups, juices, jams (Brindza et al., 2007), spirits and other traditional products (Tesevic et al., 2009). The stone fruits are in full maturity continuously during early autumn (Kutina, 1991).

The cultivation of cornelian cherry in the Caucasus and Central Asia has occurred for centuries, mainly for food and medicine, but also as an ornamental plant (Mamedov and Craker, 2004). Nowadays, Turkey is an important centre of cornelian cherries (Ercisli et al., 2008), especially in northern Anatolia (Ercisli, 2004). Generally, the historical areas of cornelian cherry occurrence are important for an adaptation of some genotypes to different local conditions in different regions of several countries (Yilmaz et al., 2009a).

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Abbreviations: ABTS, 2,2´-Azinobis(3-ethylbenzothiazoline-6-sulphonate); **DPPH,** 2,2-diphenyl-1-picrylhydrazyl; **FM,** fresh mass.

The aim of our work was to monitor antioxidant activity, total phenolic content and the content of ascorbic acid in cultivars of cornelian cherry. The consumption of food-stuffs with a high amount of antioxidant compounds has a positive impact on human health, particularly the prevention of cancer and other inflammatory diseases. Only little information about antioxidant activity and main bioactive components in particular cultivars of cornelian cherries is available in scientific works. 12 cultivars of cornelian cherries were investigated. The cultivars used were ´Elegantnyj´, ´Jalt´, ´Kijevskij´, ´Lukjanovskij´, ´Vydubeckij´ which are Russian in origin, ´Devin´, ´Olomoucky, ´Ruzynsky´, ´Sokolnicky´, ´Titus´ which are Czech and Slovak in origin and ´Joliko´ and ´Fruchtal´ which are Austrian in origin (Tetera, 2006). The main aim of our work was to popularize this fruit species for propagation in other continents in the world and draw attention to the potential of European cultivars as a new promising fruit species.

MATERIALS AND METHODS

Locality description and collection of samples

Fruits were harvested in experimental orchards of Tomas Bata University Zlin within the period of 2007 – 2009. These orchards are situated in the south-western part of the White Carpathians near Zlin, the Czech Republic. The average altitude is 340 m above sea level, and the mean annual temperature and precipitation are 7.9°C and 760 mm, respectively. The soil type was classified as the Mesotrophic Cambisol.

Fruit were harvested in consume ripeness from five trees of each cultivar under study (thus each year in 5 replications) in the course of September. 20 randomly chosen fruits from each tree were used for analyses (that is, altogether 100 per each cultivar).

Sample processing

The fruit of individual trees were processed immediately after the harvest (not later than within two days). Harvested fruits were poured in a mixer and the average sample was obtained by means of quantitation. Each parameter was measured in five replications from the fruit taken from each tree of particular cultivars (n = 25). The results were expressed as average of a three-year experiment.

Total phenolic content (TPC) and free radical scavenging assay

The extraction was performed according to the method described by Kim et al. (2003a), using 10 g of fresh sample which were homogenized for 10 s in an extraction mixture of hydrochloric acid : methanol : water in the ratio 2:80:18. For measurement of TPC, Folin-Ciocalteau reagent was used. The resulting absorbance was measured in the spectrophotometer LIBRA S6 at the wavelength of 765 nm against a blind sample, which was used as reference. The results were expressed as grams of gallic acid (GAE) kg^{-1} of fresh mass (FM).

Antioxidant activity was measured using the ABTS radical scavenging method described by Sulc et al. (2007). This test is based on monitoring of the course of inactivation of the cation $ABTS^{+}$, which is produced during the oxidation of 2,2´-azinobis(3-ethylbenzothiazoline-6-sulphonate). $ABTS^{+}$ shows a strong absorbance in the visible region of the electromagnetic spectrum (600-750 nm); this solution is green and its antioxidant activity can be easily measured by means of spectrophotometry. DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay was carried out according to the method of Brand-Williams et al. (1995). This test is based on reduction of $DPPH^{+}$ radical. In its radical form, $DPPH^{+}$ absorbs light at 515 nm, but upon reduction by an antioxidant or a radical species, the absorption disappears (Thaipong et al., 2006). In both methods the calculated activity was converted using a calibration curve of the standard and expressed in ascorbic acid equivalents (AAE) (Rupasinghe et al., 2006).

Ascorbic acid content assay

The determination of ascorbic acid content was carried out by a modified method of Miki (1981). 5 g of the sample were extracted in an extraction mixture (methanol:H_2O:H_3PO_4 in the ratio 99:0.5:0.5). The instrument used for ascorbic acid analysis consisted of a solvent delivery pump (ESA Inc., Model 582), guard cell (ESA Inc., Model 5010A, working electrode potential K1 = 600 mV, K2 = 650 mV), chromatographic column - Model Supelcosil LC8 (150.0 x 4.6 mm), 5 µm particle size and an electrochemical detector (Coulochem III). Chromatographic conditions were constant: 30°C, as a mobile phase methanol was used: H_2O:H_3PO_4 = 99:0.5:0.5, (filtrated through a filter Nylon, 0.2 µm), type of elution was isocratic, the flow rate of the mobile phase was 1.1 ml min^{-1}, and retention time 1.9 - 2.0 min. The content of ascorbic acid was calculated as g kg^{-1} of FM.

Statistical analysis

The data obtained were analysed statistically by an analysis of variance (ANOVA) and Tukey´s multiple range test for comparison of means (Snedecor and Cochran, 1968) using the statistical package Unistat, v. 5.1.

RESULTS AND DISCUSSION

The results of chemical analyses of samples of cornelian cherry cultivars are shown in Tables 1 and 2.

The total phenolic content ranged from 2.61 to 8.11 g of gallic acid kg^{-1} of fresh mass. In connection with the decrease of total phenolic content, the free radical scavenging activity of the fruits extracts (a measure of the antioxidant activity) also reduced (see Table 1). The correlation coefficient between the total phenolic content and free radical scavenging activity of DPPH and ABTS were r^2 = 0.970 and 0.978, respectively (see Figures 1 and 2). Quite a number of authors (Rupasinghe et al., 2006; Moyer et al., 2002; Jurikova and Matuskovic, 2007) refers to high correlation dependence of polyphenols and antioxidant activity in different fruit species. What is more interesting, the highest content of phenolic substances and antioxidant activity was measured in the cultivars ´Titus´ and ´Vydubeckij´, which are Russian in origin. Minor differences in the ascorbic acid contents between

Table 1. Free radical scavenging activity (grams of ascorbic acid equivalent kg^{-1} FM) of extracts of fruits of different cultivars of cornelian cherry (*Cornus mas* L.), n = 25.

Cultivar name	DPPH radical scavenging activity	ABTS radical scavenging activity
Devin	3.30 ± 0.20^{a}	3.65 ± 0.28^{a}
Elegantnyj	4.11 ± 0.28^{b}	4.62 ± 0.32^{b}
Fruchtal	5.02 ± 0.25^{c}	5.75 ± 0.24^{c}
Jalt	4.61 ± 0.31^{bc}	5.02 ± 0.27^{b}
Joliko	5.27 ± 0.24^{c}	5.95 ± 0.25^{c}
Kijevskij	6.83 ± 0.29^{d}	7.16 ± 0.32^{d}
Lukjanovskij	4.58 ± 0.24^{b}	5.04 ± 0.30^{b}
Olomoucky	6.94 ± 0.28^{d}	7.51 ± 0.25^{d}
Ruzynsky	6.85 ± 0.27^{d}	7.41 ± 0.38^{d}
Sokolnicky	4.61 ± 0.30^{bc}	4.94 ± 0.26^{b}
Titus	8.90 ± 0.25^{e}	9.64 ± 0.31^{e}
Vydubeckij	9.54 ± 0.32^{e}	10.28 ± 0.34^{e}

Different superscripts in each column indicate the significant differences in the mean at $P < 0.05$.

Table 2. Total phenolic content (grams of gallic acid.kg^{-1} FM) and ascorbic acid content (grams of ascorbic acid.kg^{-1} FM) of extracts of fruits of different cultivars of cornelian cherry (*Cornus mas* L.), n = 25.

Cultivar name	Total phenolic content	Ascorbic acid
Devin	2.61 ± 0.21^{a}	1.75 ± 0.22^{a}
Elegantnyj	3.41 ± 0.34^{b}	2.13 ± 0.31^{a}
Fruchtal	4.45 ± 0.32^{c}	1.48 ± 0.29^{a}
Jalt	4.00 ± 0.41^{bc}	2.52 ± 0.36^{ab}
Joliko	4.80 ± 0.30^{d}	3.01 ± 0.40^{b}
Kijevskij	5.37 ± 0.21^{d}	2.15 ± 0.28^{a}
Lukjanovskij	3.95 ± 0.30^{bc}	1.75 ± 0.27^{a}
Olomoucky	5.55 ± 0.32^{d}	3.11 ± 0.31^{b}
Ruzynsky	5.34 ± 0.34^{d}	3.05 ± 0.25^{b}
Sokolnicky	4.08 ± 0.42^{bc}	2.70 ± 0.38^{ab}
Titus	7.96 ± 0.42^{e}	2.91 ± 0.37^{ab}
Vydubeckij	8.11 ± 0.40^{e}	2.77 ± 0.33^{ab}

Different superscripts in each column indicate the significant differences in the mean at $P < 0.05$.

cultivars were observed (see Table 2).

The results of measurements performed in 12 cultivars showed a variability of the total phenolic content which ranged from 2.61 to 8.11 g GAE kg^{-1} FM. It is also of interest that the variability of total antioxidant activity was likewise high (3.30 to 9.54 g AAE kg^{-1} FM). Regarding the fact that all cultivars were grown under identical conditions and in the same locality, it is possible to conclude that one can clearly see the varietal variability, which is quite typical of fruits (Kim et al., 2003b). High antioxidant activity of fruit species is influenced by a number of chemical compounds. Amongst all of such active compounds, flavonols is one of the most important (Chun et al., 2003). Our results showed high values of total phenolic content and antioxidant activity in comparison with other results conducted on cornelian cherries (Yilmaz et al., 2009b; Gulcin et al., 2005). A high content of ascorbic acid and antioxidant activity (e.g. in comparison with strawberries, orange fruits, kiwi fruits, etc) was demonstrated in other work, too (Tural and Koca, 2008). In our work on cornelian cherries high contents of ascorbic acid were observed with the values ranging from 1.48 (the cultivar ´Fruchtal´) to 3.11 g kg^{-1} (the cultivar ´Olomoucky´).

Vitamin C can also be held for a substance which shows antioxidant activity. Nevertheless, in this case the

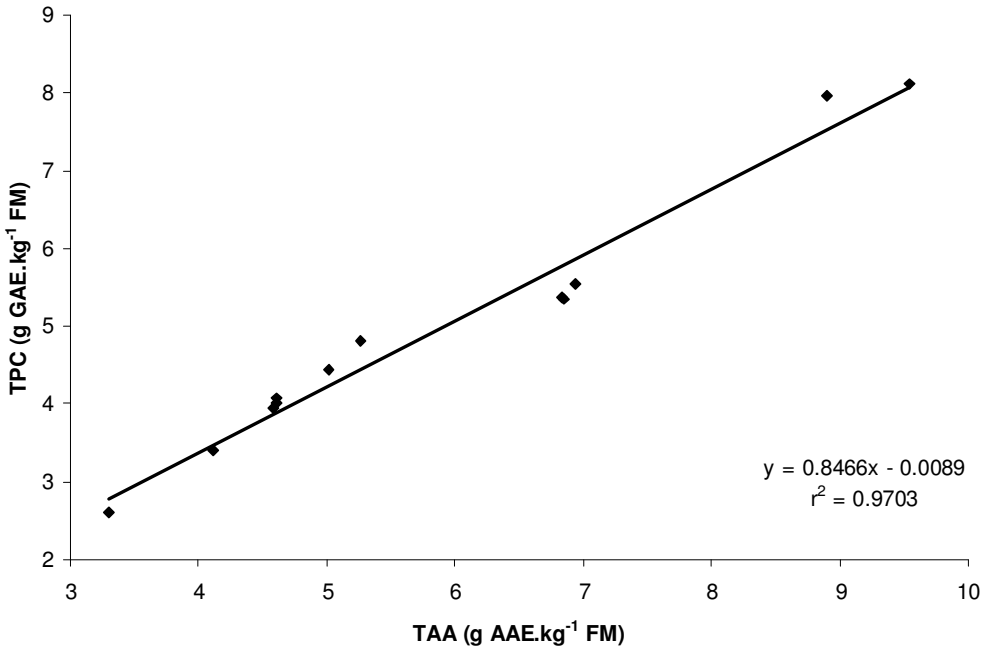

Figure 1. Relationship between total phenolic content (g GAE.kg^{-1} FM) and DPPH radical scavenging activity (g AAE kg^{-1} FM) in 12 cultivars of cornelian cherry (*Cornus mas* L.).

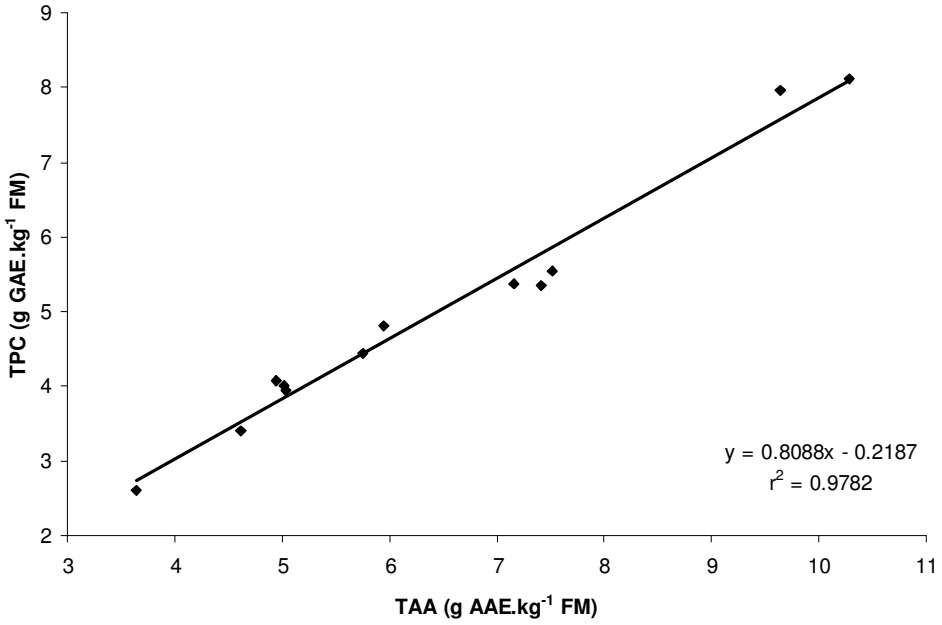

Figure 2. Relationship between total phenolic content (g GAE.kg^{-1} FM) and t ABTS radical scavenging activity (g AAE kg^{-1} FM) in 12 cultivars of cornelian cherry (*Cornus mas* L.).

effect on total values of antioxidant activity is problematic (Gil et al., 2002). This can also be associated with a considerably variable content of vitamin C due to the effect of the year, the degree of ripeness, manipulation, processing, etc (Piga et al., 2003). In addition, it is possible that the levels of other components participating in the formation of antioxidant activity may be rather variable and for that reason the momentary content of

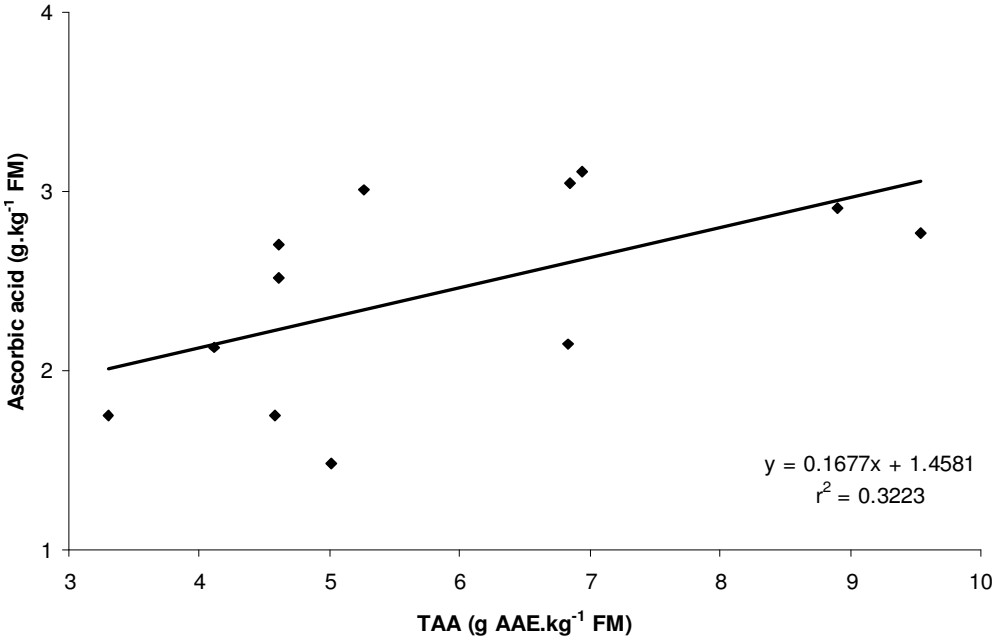

Figure 3. Relationship between total ascorbic acid content (g.kg^{-1} FM) and DPPH radical scavenging activity (g AAE kg^{-1} FM) in 12 cultivars of Cornelian Cherry (*Cornus mas* L.).

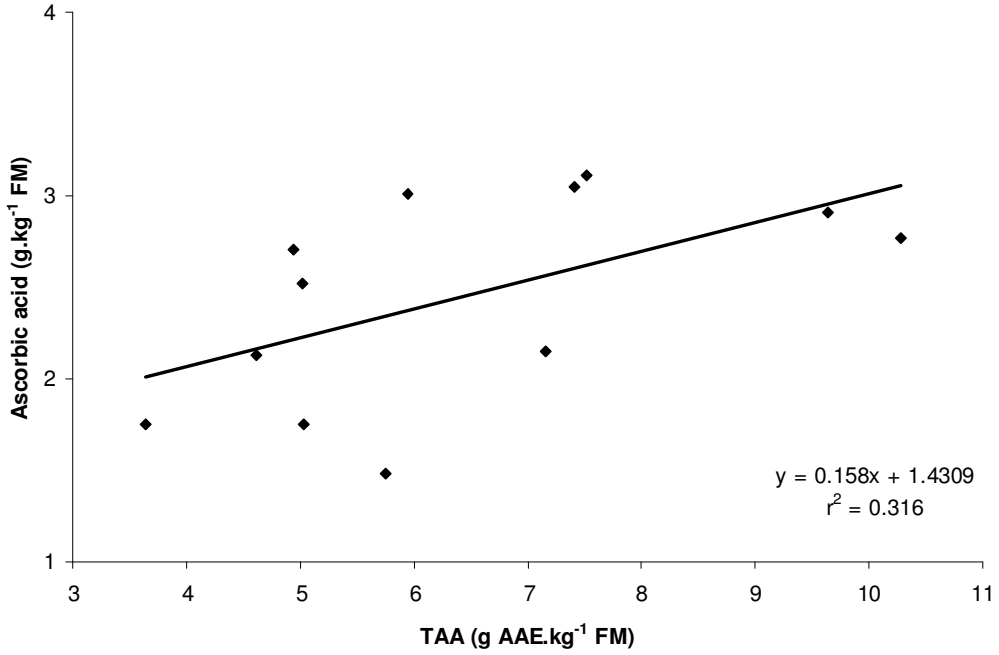

Figure 4. Relationship between total ascorbic acid content (g.kg^{-1} FM) and ABTS radical scavenging activity (g AAE kg^{-1} FM) in 12 cultivars of Cornelian Cherry (*Cornus mas* L.).

phenolic substances is considered to be the most important coefficient of correlation with antioxidant activity in fruits (Gil et al., 2002; Chun and Kim, 2004; Vizzotto et al., 2006) and vegetables (Valsikova and Belko, 2004). Regarding our work, the same is shown in Figures 3 and 4 where we determined the correlation coefficient between the total ascorbic acid content and free radical scavenging activity of DPPH and ABTS to be $r^2 = 0.322$ and

0.316, respectively.

Nevertheless, as it follows from our measurement, within the scope of one fruit species there exist big differences in the content of chemical compounds in the fruit of particular cultivars, which is quite typical of fruit after all (Rop et al., 2009). Central European cultivars of cornelian cherry are generally considered to be a valuable source of substances with high antioxidant activity (Pantelidis et al., 2007). In comparison with other cultivars grown, e.g. in Turkey (Tural and Koca, 2008), they can be a suitable food supplement in relation to a positive impact on human health.

Conclusion

The main contribution of our work was to popularize and draw attention to Central European cultivars of cornelian cherries so that they could become a part of worldwide production and enrich the range of fruit commodities on a global scale. Cornelian cherry cultivars have high biological efficiency-antioxidant activity, total phenolic content and the content of ascorbic acid, which was confirmed in our measurement. This means that in future some cornelian cherry cultivars could be used as a source of new health sources when improving nutritional properties of the world's less traditional fruit species.

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Full Length Research Paper

Hepatoxicity of aqueous extract and fractionated methanol extract of *Phytolacca americana* by isolated rat liver perfusion system

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Phytolacca americana **is a perennial plant native to North America and other parts of the world. It is well known for several medicinal properties despite being considered to have digestive toxicity (especially hepatotoxicity). Our objective is to examine whether extract of aerial parts of *P. americana* could produce biochemical changes by Isolated Rat Liver Perfusion (IRLP) system, which is ideal for studying biochemical alterations of chemicals with minimum neural-hormonal effects. In this study, the liver was perfused with Kerbs-Henseleit buffer, containing different concentration of aqueous extract of the aerial parts of *P. americana* (10, 20, 40, 50,100 mg/kg) and $CHCl_3$, ethanol and methanol fraction (10, 20, 40 mg/kg) were added to the buffer and perfused for 2 h. During the perfusion, many factors including amino-transferase activities were assessed as indicator of liver viability. Consequently, sections of liver tissue were examined for any histo-pathological changes. The results showed that histo-pathological changes in liver tissues were related in a dose-dependent manner to methanol extract of aerial parts *Phytolacca americana* concentrations. Doses of 50, 100 mg/kg caused significant ($P < 0.05$) histo-pathological changes.**

Keywords: *Phytolacca americana*, Liver perfusion, Kerbs-Henseleit buffer, histo-pathological changes, amino-transferees activity.

INTRODUCTION

Phytolacca americana, Pokeweed is a medical herb form Phytolaccaceae, shrubby perennial plant growing up to 10 feet in height native to eastern North America and other parts of the world. One hundred and fifty species of *Phytolacca* are available in western and northwestern of Iran, mainly in the coastal and forest areas; however, it is rarely and in most cases, accidentally used in Iran (Zargari, 1981; Mirhaydar, 1994). the leaves are alternate with coarse texture with moderate porosity and can reach nine inches in length. Leaves are medium green and with an unpleasant odor and mostly the boiled leaves, are used in a popular salad (called grandmother salad), in the American diet.

The flowers have 5 regular parts with upright stamens and are up to 0.2 inches wide. They have white petal-like sepals without true petals. A shiny dark purple berry held in racemous clusters on pink pedicles with a pink peduncle. Pedicles are without berries have a distinctive rounded five part calyx. It also has tan cortex, white pulp, moderate number of rootlets. Transversely cut root slices show concentric rings, and has no nitrogen fixation ability (Santillo, 1993; Murray and Pizzorno, 1999).

Known constituents are alkaloids (betanidine, betanine, phytolaccine, prebetanine), triterpene saponins (phytolaccoside A, B, C, D, E, F, G or esculentoside E, Phytolaccagenin, jaligonic acid, esculentic acid, 3 -oxo- 30-carbomethoxy-23-norolean-12-en-28-oic acid, esculentic acid,

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phytolaccagenic acid, oleanolic acid), tripertine alcohols (alpha spinasterol, alpha spinasteryl-beta-D-glucoside, 6 palmityl-delta 7-stigmasterol-delta-D-glucoside, 6 palmytityl-alpah-spinasteryl-6-D-glucoside), and others (phytolaccatoxin, canthomicrol, astragalin, protein PAP-R, pokeweed nitrogen glycoprotein's (pa^1-pa^5), pokeweed antiviral protein (PAP), caryophyline, histamine, GABA, tannin, starch) (Newall et al., 1996; Takahashi et al., 2001; Tyler, 1987).

P. americana or poke root (common name) has been most commonly used for its laxative properties. The dried root has found application in relieving pain, reducing inflammation, treating rheumatism and arthritis along with various skin diseases (Woo and Kang, 1976), Modern researchers are investigating the plant to determine if it possesses any anti-viral, anti-cancer, antifungal, or immune stimulant properties (Goldestein et al., 1973), despite being considered to have digestive toxicity especially hepato-toxicity (Harkness et al., 2003; Stein, 1975; Heinrich et al., 2004).

In this study, isolated rat liver perfusion was employed to evaluate *P. americana* hepato-toxicity and its correlation to biochemical changes.

MATERIALS AND METHODS

Animals

Male albino Wistar rats (6 to 8 weeks), weighing 200 - 250 g were used for all experiments. They were housed individually in standard rat cages in a room on a 12- h light- dark cycle at 22°C (22 ± 1°C) and 50 ± 5% with relative humidity, including food and water *ad libitum*. The animals were adapted to the condition for 7 days prior to the beginning of the experiments (Woo and Shin, 1976).The experiments were performed during the day time (08:00 - 16:00 h). Each animal was used once only. A research proposal was prepared according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The Institutional Animal Ethical Committee (IAEC) of Mazandaran University of Medical Sciences approved the proposal.

Plant

Aerial parts (flowered browse) of *P. americana* were collected from Mazandaran (a Northern state in Iran) in April 2007 and identified and confirmed by Department of Pharmacognosy. A voucher specimen (No.0506-16) has been deposited in Tehran School of Pharmacy Herbarium. Aerial parts were dried at room temperature and powdered before extraction. One hundred grams of the powdered sample was extracted at room temperature by percolation with methanol/water (80:20, 400 ml × 3 times). The resulting extract was concentrated over a rotary vacuum evaporator until a solid extract sample was obtained (Samsamshariat et al., 1981). The extract was prepared in phosphate buffer (pH = 7.4) for pharmacological studies.

Experimental design

Rats were divided into five treatment group and control group. Each group contained four male rats and their livers were perfused by a single dose of 10, 20, 40, 50 and 100 mg/kg of aqueous extract of aerial parts of *P. americana* with $CHCl_3$, ethanol and methanol fraction (10, 20, 40 mg/kg) respectively (total = 16 groups). Control livers were perfused with the perfusion buffer. Following the preliminary study, the dose of 100 mg/kg was chosen for the remaining of the study in order to evaluate the hepato-toxicity of *P. americana* (Dehpour et al., 1999).

Buffer

Perfusion fluid was made of Kerbs-henseleit buffer. The perfusion medium consisted of 118.9 mM Na Cl, 4.76 mM KCl, 1.19 mM KH_2PO_4, 2.55 mM $CaCl_2$ and 24.8 mM $NaHCO_3$, at 37°C.Glucose (1%, w/v) is usually added (Jeong et al., 2004; Wolkoff, 1987), The perfusion medium was gassed continuously with carbogen (95% O_2, 5% CO_2) (Figure 1).

Perfusion conditions and parameters of liver viability

Temperature, perfusion pressure, flow rate and perfusion fluid pH were closely monitored during the perfusion, particularly, during the first 30 min of equilibration (Wolkoff, 1987). These parameters were initially checked every 10 to 15 min and the experiment did not begin until they had reached constant and acceptable values. The temperature in the perfusion system was also set and maintained at 37°C. Perfusion pressure was not raised above 10 - 15 cm of water with a flow rate of approximately 2 ml/min/g liver weight, to provide adequate oxygenation. The perfusion fluid pH was always set between 7.2 and 7.4 by adjusting the CO_2 gases. As soon as perfusion began, the liver developed an even, light-brown color, was soft and kept moistened. Serum amino-transferees activities (ALT and AST) serve as indicators of liver viability during perfusion which was determined in samples of perfusion medium.

Biochemical determinations

The activities of aspartate aminotransferase (AST) activity and alanine aminotransferase (ALT) activity in the perfusion fluid were assayed using a commercial Kit of Zist himie (Tehran, Iran).

Histological studies

The liver was completely excised and freed of any extraneous tissue. Multiple samples were then taken from each liver (mean 3 mm) and placed in 10% neutral buffered formalin. The liver was cut into small pieces, sections prepared and stained by Eosin-Hematoxylin and examined blind for histo-pathological changes.

Surgery

The rats were anesthetized with ether. Heparin (500 unit; I.P.) was used to prevent blood clotting prior to anesthesia (Cheung et al., 1996). An incision was made along the length of the abdomen to expose the liver. Sutures were placed loosely around the common bile duct, which then was annulated with PE-10 tubing and secured. Satures were then placed loosely around the inferior vena cava, above and below the renal veins. The distal suture around the vena cava was tightened and an 18 g polyethylene catheter was inserted and placed above the renal vein. The diaphragm was incised and the inferior vena cava ligated suprahepatically. Following attachment of the perfusion tubing to the cannulate, the liver was perfused in situ through the portal vein (Cheung et al., 1996).

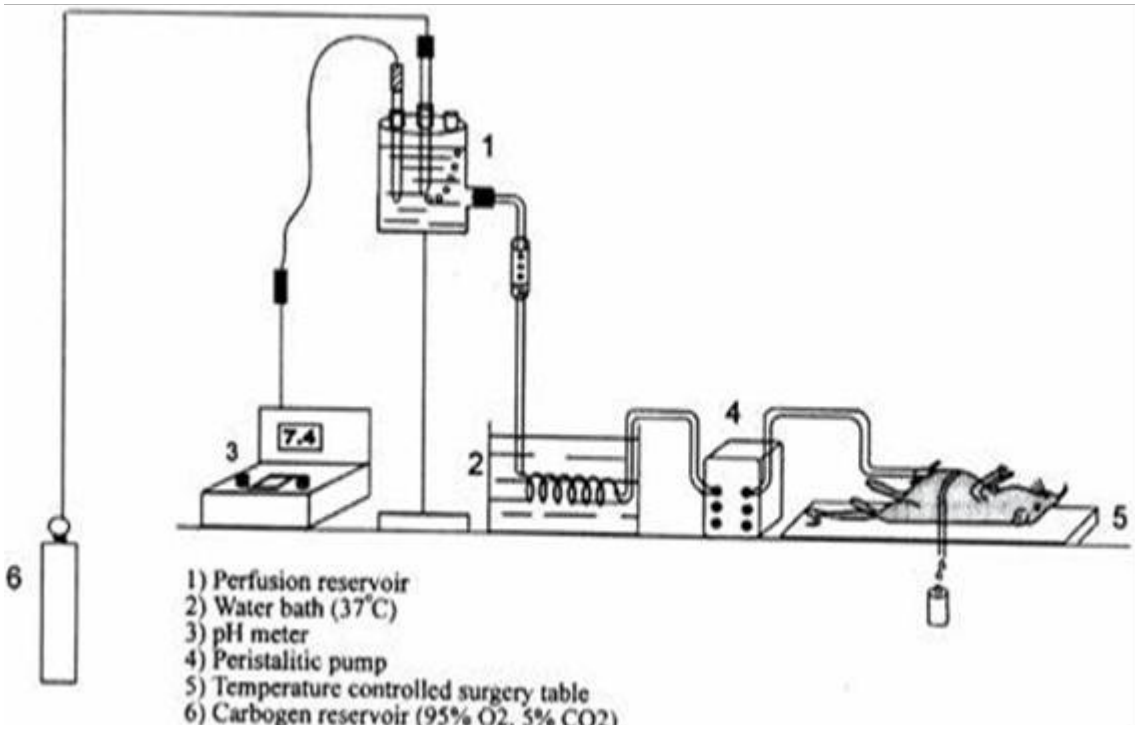

Figure 1. Schematic diagram of a rat liver perfusion system.

Analyses of the data

Statistical analysis was performed using SPSS for Windows (Ver.10, SPSS, Inc., Chicago, USA). All values were analyzed by one-way analysis of variance (ANOVA) and expressed as mean ± standard error in the mean of 4 rats (S.E.M). Student-Newman-Keuls test were used to evaluate the significance of the obtained results. $P < 0.05$ was considered to be significant (Bermeyer and Bernt, 1980).

RESULTS

Activity of serum amino-transferees enzymes changes

The results of present study showed that the aqueous extract of aerial parts (flowered browse) of *P. americana* significantly increased the activity of amino-transferase enzymes in a dose-dependent mannar ($P < 0.01$) at 60^{th} min, in comparison with control at single dose of 10, 20, 40, 50 and 100 mg/kg (Figure 2). Furthermore, the activity of enzymatic methanol extract fractionates increased compared with the control group (Figures 3A and B).

Light microscope observation

Histo-pathological studies using a light microscope showed significant hepatocellular damage including necrosis and infiltration, due to aqueous extract of aerial parts (flowered browse) of *P. Americana* (Figure 4b) when compared to control group (Figure 4a). In addition, other histo-pathological parameters including the number of Kupffer and mononuclear cells, edematous cells and cell degeneration changed significantly with aqueous extract and $CHCl_3$, ethanol and methanol fractions of aerial parts (flowered browse) of *P. americana* respectively (Table 1).

DISCUSSION

The liver has been identified as the most important tissue target for *P. americana* in rats (Jeong et al., 1997). The pain relief composition is prepared from roots of the Phytolacca family, particularly the species of Phytolacca Americana (Potter and Clarke, 1900). On the other hand the anti-cancer effects, appear to work primarily based upon anti-tumor and anti-inflammatory properties, along with immune stimulant functions (Larson, 2007), Phytolaccatoxin and related triterpene saponins, believed to be the primary toxic constituents, are present within berry juice and other parts. Other toxic constituents have also been identified including the alkaloid phytolaccine (in small amounts), the alkaloid phytolaccotoxin, as well as a glycoprotein and histamines (Armstrong and Yo, 2007; Winston, 2004; Rossini et al., 1976). Our data showed that administration of aqueous extract and methanol extract fractionates of aerial parts (flowered browse) of *P. Americana* causes edema which can be assessed by histo-pathological examination (Table 1).

Our findings are in agreement with the fact that an

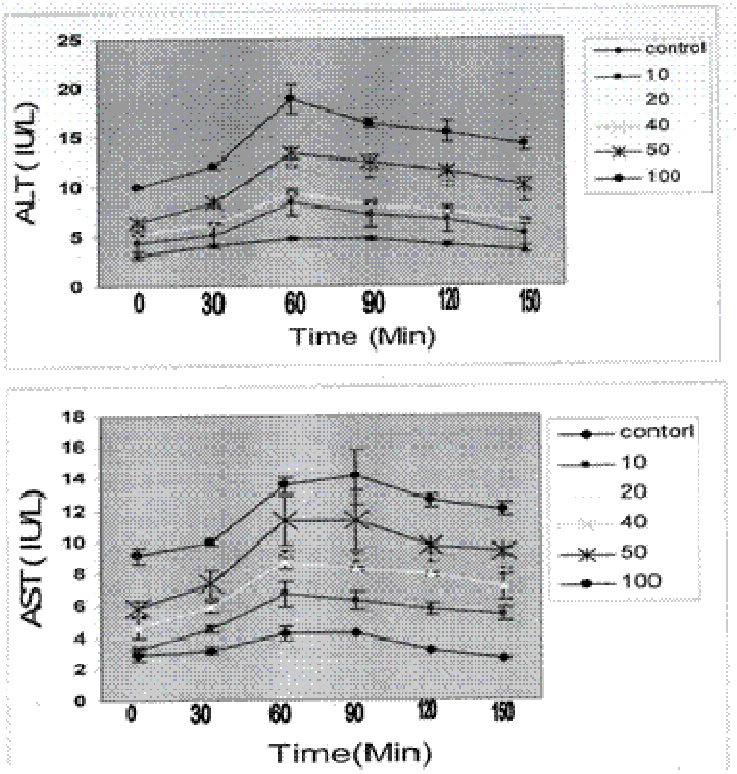

Figure 2. Activity of ALT (alanine transferase) and AST (asparate transferase) enzymes aqueous extract of aerial parts of *Phytolacca Americana* at differences times. Values are presented as mean ± SEM (N = 4), ***P < 0.001 with respect to control (ANOVA followed by Newman-Keuls multiple comparisons test).

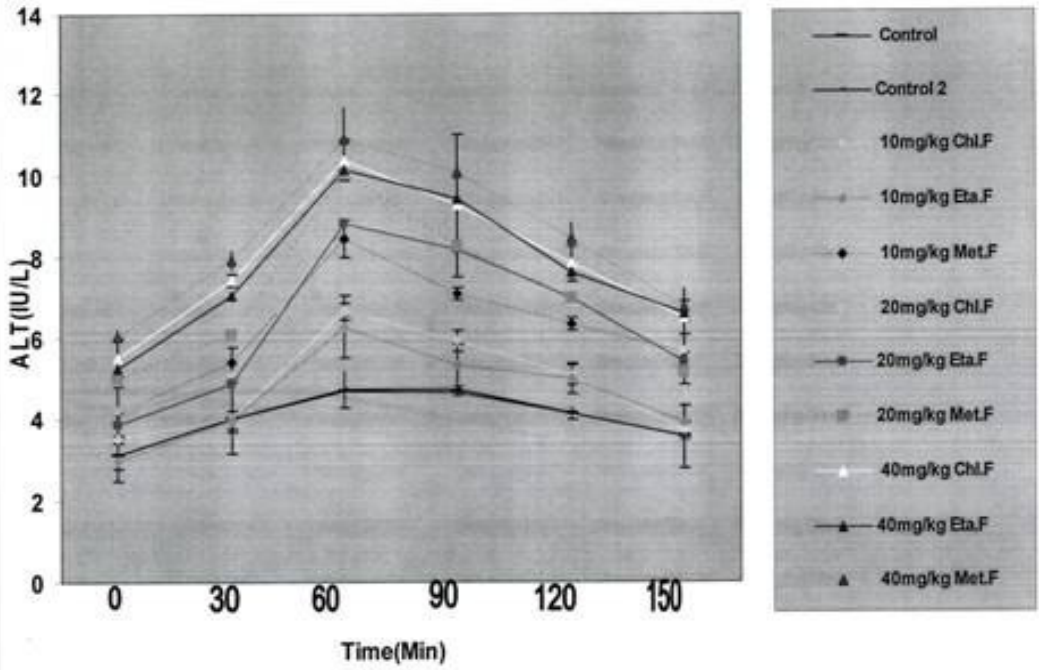

Figure 3A. Activity of ALT (alanine transferaes) enzyme fractionated methanol extract of aerial parts of *Phytolacca Americana* at differences times. Values are presented as mean ± SEM (N = 4), ***P < 0.001 with respect to control, (ANOVA followed by Newman-Keuls multiple comparisons test).

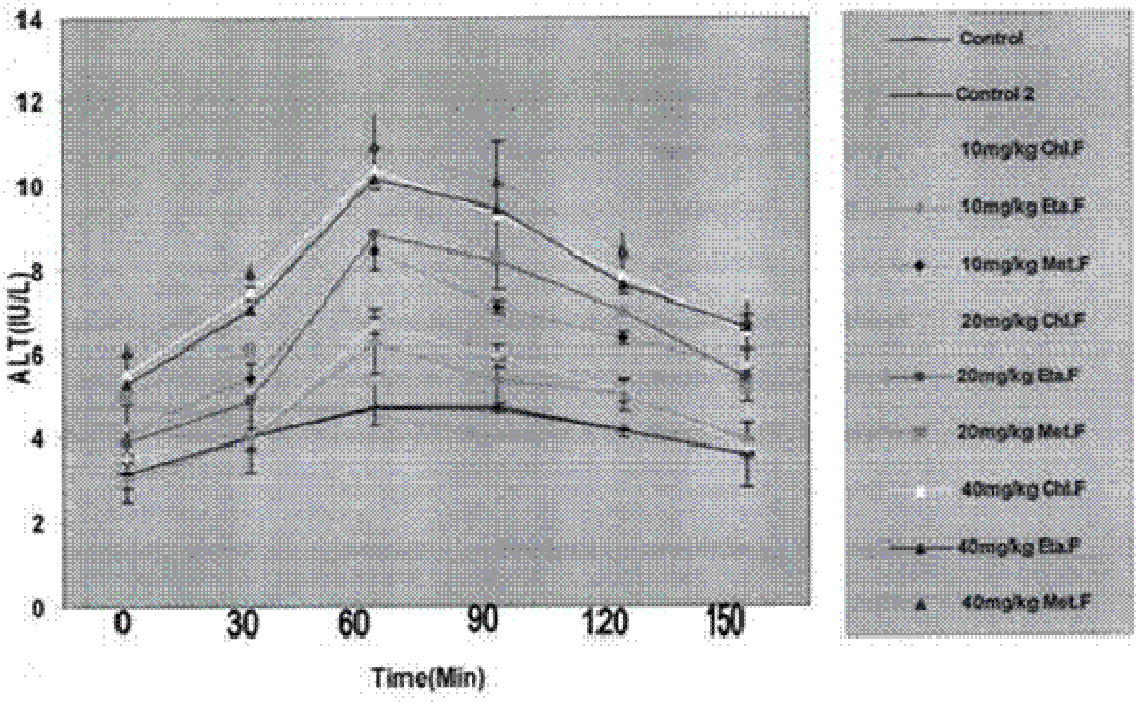

Figure 3B. Activity of AST (asparate transferaes) enzyme fractionated methanol extract of aerial parts of *Phytolacca Americana* at differences times. Values are presented as mean ± SEM (N = 4), ***P < 0.001 with respect to control (ANOVA followed by Newman-Keuls multiple comparisons test).

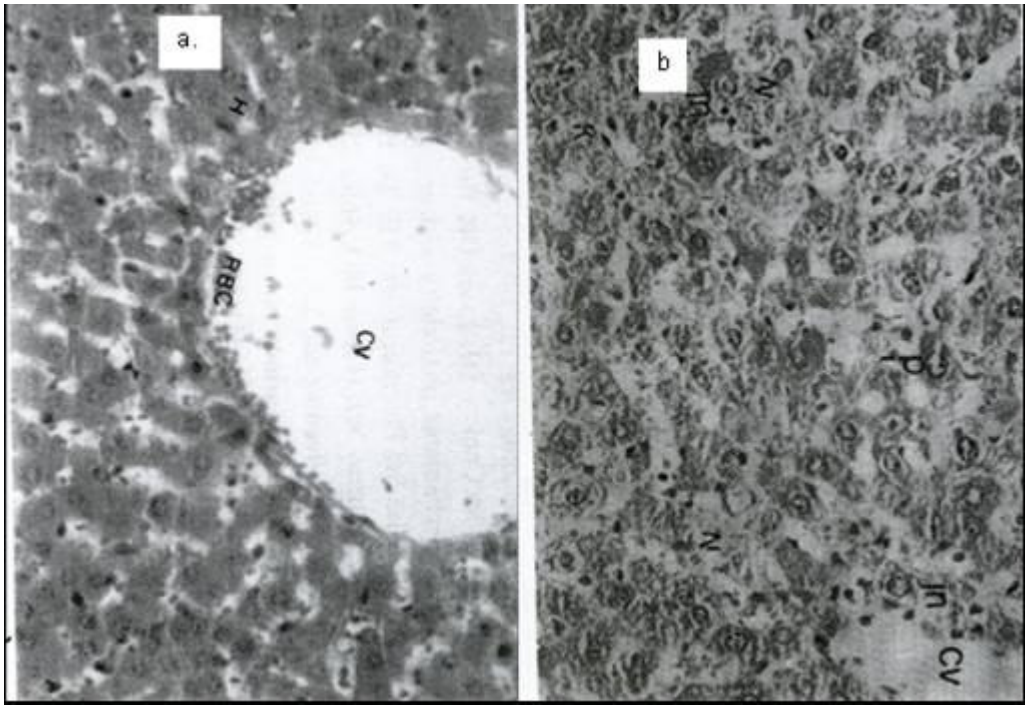

Figure 4. Photomicrograph of lobules from control group and *Phytolacca americana* - treated liver. Control showed red blood cells (RBC), hepatocytes (H) and central veins (CV). Staining shows that cytoplasm was acidophilic and surround by a bright basophilic nucleus (a). *Phytolacca americana* perfused liver (50 mg/kg) showed limited changes in lobules of liver and hepatocellular necrosis (N), with infiltration (IN) of mononuclear cells and accumulation of necrotic Kupffer cells (K), with pyknotic (P) nuclei (b).

Table 1. Histo-pathological effects of aqueous extract and $CHCl_3$, ethanol and methanol fractionated extract of aerial parts of *Phytolacca americana*.

Histopathological parameters	Control	*Phytolacca Americana* (mg/kg)					$CHCl_3$ fraction (mg/kg)			Ethanol fraction (mg/kg)			Methanol fraction (mg/kg)		
		10	20	40	50	100	10	20	40	10	20	40	10	20	40
Kupffer cells	+	+	+	++*	+++**	+++**	+	+	++*	+	+	++*	+	++*	+++**
Edematous cells	+	+	+	+	++*	+++**	+	+	++*	+	+	++*	+	++*	+++**
Mononuclear cells	+	-	+	+	+++*	+++**	+	+	+	+	+	++*	+	++*	+++**
Degeneration	-	+	+	+	++*	+++**	-	+	+	-	+	++*	+*	++*	+++**
Necrosis	-	-	-	+	++*	+++**	-	+	++**	-	+	++*	+*	++*	+++**

- = No effect, + = minor effect, ++ = medium effect, +++ = major effect.
*P < 0.05, **P < 0.01, significantly different from control using Fisher' exact test. Data are means of three replicates

oncotic agent can cause increase in pressure (Chen and Cosgriff, 2000). In addition, isolated organs have a time-dependent tendency to absorb water, as with a relatively protein-free medium water which gradually escapes from the vascular space and therefore interstitial edema develops (Wolkoff, 1987). Histo-pathological examination revealed significant hemolysis as assessed by the hemolytic index (Figure 4b).

This can be due to altered calcium homeostasis concomitant with a significant increase in cytosolic calcium, which has been previously reported for *P. americana* in liver (Jeong et al., 2004). Moreover, the disturbances of intracellular calcium homeostasis have been shown to be associated with a variety of toxicological and pathological processes (Cheung et al., 1996). Accumulation of *P. americana* in the liver as the target organ has been shown to cause degeneration (Goldestein et al., 1973). In a similar manner the results of this study also showed liver degeneration (Table 1). This in fact could be a result of *P. americana* recep-tor binding, which is sufficient to affect different cells. In this study, significant necrosis was also observed in the liver at *P. americana* doses of 50 and 100 mg/kg. *P. Americana* induces formation of reactive oxygen species and an oxidative stress, resulting in lipid peroxidation (Karami et al., 2001). This may explain the observed necrosis (Figure 4b). We have also observed cell death followed by cell proliferation with hyperplasia nodules in Kupffer cell by *P. americana*. Therefore, the results of our study, in agreement with others (Goldestein et al., 1973; Heinrich et al., 2004), demonstrate that liver perfusion is a suitable model in order to study the hepatotoxicity of chemicals (such as *P. americana*). More studies, however, are needed to further elucidate the exact mechanism by which *P. americana* induces hepatotoxicity.

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Full Length Research Paper

Acute and subacute toxicity study of ethanolic extract of the stem bark of *Faidherbia albida* (DEL) A. chev (Mimosoidae) in rats

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***Faidherbia albida* (Del.) A. Chev (mimosoidae) is widely used in African traditional medicine (ATM) for management of fever, diarrhoea and human trypanosomiasis. Acute and sub-acute toxicity profiles of ethanolic stem bark extract of *F. albida* were evaluated in wistar albino rats. The acute toxicity was studied using the method of Lorke (1983). In the sub-acute toxicity study, four groups of six rats per group were used. The control group (1) received 10 ml normal saline/kg body weight while groups 2, 3 and 4 received oral daily doses of 125, 250 and 500 mg extract/kg body weight respectively for 21 days. The effects of the extract on clinical signs, feed and water intake, body weight changes, haematology, plasma biochemical parameters, relative organ weight (ROW) were evaluated. The oral LD_{50} of the extract was estimated to be greater than 5000 mg/ kg body weight. The extract produced slight increase in body weight of rats given 125 mg extract/kg body weight. However, dose-dependent highly significant ($P < 0.01$) decrease in body weight was observed at 250 and 500 mg/ kg-treated rats in weeks 2 and 3 of the study. Feed and water intake was not affected by the treatment. ROW for all organs was not affected by the treatment except significant ($P < 0.05$) increase in the testes of rats treated with 250 and 500 mg extract/kg body weight. Although the treatment elicited highly significant ($P < 0.01$) changes in the levels of the hepatic and some of the haematological parameters, they were within the normal reference range for rats. This study revealed that while the stem bark of the plant may be considered relatively safe when used sub-acutely, further investigation is needed to ascertain its effect on the male reproductive system as well as its effect on chronic administration.**

Key words: *Faidherbia albida,* toxicity, haematology, liver function, testes.

INTRODUCTION

Faidherbia albida (Del.) A. Chev (mimosoidae) also known as A*cacia albida* Del is a leguminous woody species distributed throughout the arid and semi arid lands of Africa. It is found in western, eastern and southern Africa (Vandenbelt, 1991). It is also found in the Middle East, Arabia and in Palestine (Wickens, 1969). It has been introduced into India, Pakistan, Nepal, Peru, Cyprus, Cape Verde and the Ascension Islands. *F. albida* is a large thorny tree that may reach 31 m in height with wide and rounded crown. The trunk is thicker at the base. The bark is light grey and smooth when young, but becomes cracked with age. *F. albida* is characterised by a long, deep taproot that can reach 7 m long. It possesses pairs of spines up to 2 cm. Its leaves are compound, bipinnate, blue green with 3-12 pairs of pinnae carrying 6-23 pairs of leaflets up to 12 mm long and 5 mm wide and the flowers are fragrant in dense cream-yellow spikes about 10 cm in length (Mulofwa, 1994). *F. albida* is valued for its green manure and fodder. It sheds its

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leaves at the beginning of the rainy season providing nutrients for new crops thus reducing the shade of the canopy. It is a nitrogen-fixing tree and therefore can enrich the soil and improve crop yield.

In northern Senegal, the leaves are boiled in water to make a cough mixture (Boury, 1962). The seeds can be boiled and eaten. The pods may be dried and ground into edible flour. They are said to have been used as fish poison and are worn as charm by African women and children to avert smallpox (Irvine, 1961). In Malawi, the root and bark are used as a poison to stupefy fish (Fanshawe, 1962). The stem bark exudes a gum which is sometimes collected in Nigeria and used for its emollient and emulsifying properties (Howes, 1949). The Pulaar people of Senegal use this gum as an aphrodisiac to treat 'impotence' (Kerharo and Adam, 1962 a). In Nigeria, an infusion of the bark is taken for fever, cough and to assist in child birth (Dalziel, 1937 and Singha, 1965). It is added to a potion to treat chest pain by the Fulanis of Nigeria (Jackson, 1973). A decoction of the bark is used in cleansing fresh wounds in a manner similar to that of potassium permanganate (Berhaut, 1975). Combined with other herbs, it is used to treat 'madness' (Kerharo and Adam, 1962 b, Berhaut, 1975). A decoction of the bark is also used as an emetic in fevers by the Masai people of East Africa, taken for diarrhoea in Tanganyika (Irvine, 1961) and for colds, haemorrhage, leprosy and ophthalmia in West Africa. A liniment made by steeping the bark is used for bathing and massage in pneumonia. The bark is employed in dental hygiene, strips used as dental floss in Namibia and its extract is employed in the treatment of toothache. In northern Nigeria, West Africa, the cattle-rearing nomads take a decoction of the stem bark orally for the management of the sleeping sickness (Trypanosomiasis).

Kubmarawa et al. (2007) reported its antimicrobial activity against *Pseudomonas aeruginosa, Bacillus subtilis* and *Salmonella typhii*. Ethanolic extract of the stem bark was shown by Tijani et al. (2008) to possess antipyretic, anti-inflammatory and anti-diarrhoeal properties in rats. Tijani et al. (2009) also reported the trypanostatic as well as anti-haemolytic effects of the extract in *Trypanosoma brucei brucei*-infected rats, thus explaining the basis for its use in folkloric medicine. The present studies were carried out to evaluate the sub-acute toxicity of *F. albida* in rats.

MATERIALS AND METHODS

Plant material

The leaves and stem bark of *F. albida* were collected from Gyamso ward in Toro Local Government Council of Bauchi State, Nigeria, in September, 2008. They were identified and authenticated by Mrs Jemilat Ibrahim of the Department of Medicinal Plant Research and Traditional Medicine of National Institute for Pharmaceutical Research and Development (NIPRD). A voucher specimen (number NIPRD/H/6151) was deposited at NIPRD herbarium for future reference. The stem bark was cleaned, air-dried for 7 days and pounded into fine powder using mortar and pestle. The powder was stored in an airtight container and kept in a cool, dry place.

Extract preparation

200 g of the powdered stem bark were weighed and macerated in 2 l of water and ethanol in ratio 1:1 for 48 h. The mixture was filtered using muslin cloth followed by Whatman filter paper (No. 1). The resultant filtrate was evaporated to dryness on steam bath to give a dark brown extract. The percentage yield was calculated as:

Yield (%) = [wt of extract (g)/wt of plant material (g)] x 100

The crude extract was stored at -4°C until required for use. Aliquot portions of the crude extract residue were weighed and suspended with 2.5% tragacanth in distilled water for use on each day of the experiment.

Animals

Male wistar albino rats (200 - 250 g) obtained from Animal Facility Centre, National Institute for Pharmaceutical Research and Development, Abuja, Nigeria were used in the study. The rats were fed standard laboratory diet, given water *ad libitum* and maintained under laboratory conditions of temperature 22 ± 1°C, relative humidity 14 ± 1% and 12 h light and 12 h dark cycle. All experiments were performed according to the "Principles of Laboratory Animal Care" (NIH Publication No. 85; rev. 1985) and NIPRD Standard Operating Procedure for Animal Care.

Acute toxicity study

Acute toxicity study was carried out using the method of Lorke (1983). In the first phase, nine rats randomly divided into three groups of three rats per group were given 10, 100 and 1000 mg extract/kg body weight orally (via a cannula), respectively. The rats were observed for signs of adverse effects and death for 24 h and then weighed daily for 14 days. Based on the results of the phase-one study, the procedure was repeated using another set of three rats randomly divided into three groups of one rat each, given 1600, 2900 and 5000 mg extract/kg body weight, respectively. For 14 days, the rats were observed for signs of toxicity which include but not limited to paw-licking, salivation, stretching, rubbing of nose on the floor and wall of cage, change in body weight and death. The surviving animals were sacrificed under chloroform anaesthesia, autopsied and examined macroscopically for any pathological changes. The number of deaths in each group within 24 h was recorded and the final LD_{50} values were calculated as the geometric mean of the highest non-lethal dose (with no deaths) and the lowest lethal dose (where deaths occurred).

Sub-acute toxicity study

The sub-acute toxicity study was carried out using standard operating procedure on toxicity testing adopted by pharmacology and toxicology department, NIPRD. Twenty four rats were randomised into four groups of six rats each. Rats in control group were given normal saline (3 ml/kg), while those in the treatment groups received 125, 250 and 500 mg extract/ kg body weight via the oral route for 21 days. Water and feed intake were measured daily while body weights of the rats were determined twice weekly and at the end of the study (day 21). After 24 h (day 22), the rats were euthanized in an airtight glass chamber saturated with chloroform

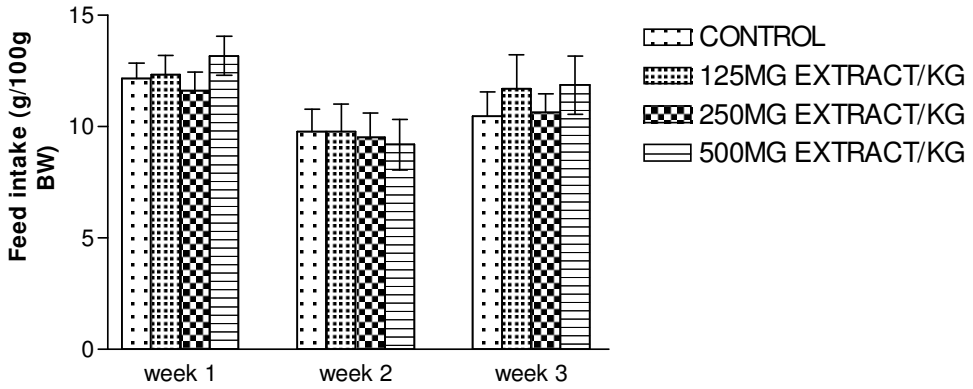

Figure 1. Effect of ethanolic extract of *F. albda* on weekly feed intake in rat (n = 6).

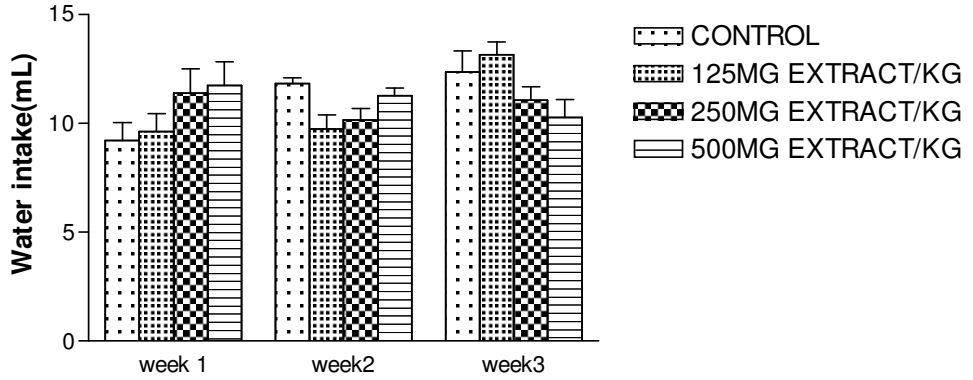

Figure 2. Effect of ethanolic extract of *F. albda* on water intake in rat (n = 6).

and after opening up the rats surgically, blood samples were collected by cardiac puncture into ethylenediaminetetraacetic acid (EDTA) bottles for the analysis of haematological parameters [white blood cell(WBC), red blood cells (RBC) haemoglobin (HGB), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), platelets (PLT), lymphocytes (LMP), monocytes (MXD), neutrophils (NEUT) and eosinophils (EOS)] using Sysmex KX-21N automated hematology analyzer (Sysmex America Inc, USA). Another portion of blood was collected into lithium heparin sample bottles, centrifuged to obtain the plasma and analysed for liver enzymes [alaninine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP)], bilirubin, liver proteins, creatinine and urea levels using Hitachi 902 analyser (Roche Diagnostic, GmbH, Germany). The plasma was also analysed for electrolytes (sodium, potassium, chloride and bicarbonate ions) levels using Ilyte machine auto analyser. Different organs mainly the brain, heart, lungs, kidneys, liver, intestine, stomach, spleen and testes were removed and weighed. The relative organ-body weight ratio (ROW) of each rat was then calculated as:

ROW = wt of organ (g)/body wt of animal (g)

Statistical analysis

Results were expressed as the mean ± standard error of mean (SEM). Statistical analysis of data was carried out using one-way analysis of variance (ANOVA) followed by student's t-test. Differences in mean were considered to be significant when $P < 0.05$.

RESULTS

The yield of the extract was 8.0% (w/w). In the acute toxicity study, the behavioural signs of toxicity observed in the rats at 2900 and 5000 mg extract/kg body weight were salivation, rubbing of nose and mouth on the floor of the cage and restlessness. Gross pathological study showed no abnormality in all the organs examined. Absence of death at all doses up to 5000 mg extract/kg showed that the LD_{50} of the ethanolic stem bark of *F. albida* is greater than 5000 mg extract/ kg body weight.

Effect of the extract on food and water intake

In the sub-acute toxicity study, there was no significant difference in the water and food intake in all the treatment groups when compared to the control (Figures 1 and 2).

Effect of the extract on body weight

The extract at 250 mg/kg body weight elicited a highly significant ($P < 0.01$) reduction in body weight in weeks 2 and 3. Significant ($p < 0.05$) reduction was observed in week 1 in the 250 mg extract/kg group and at 500 mg

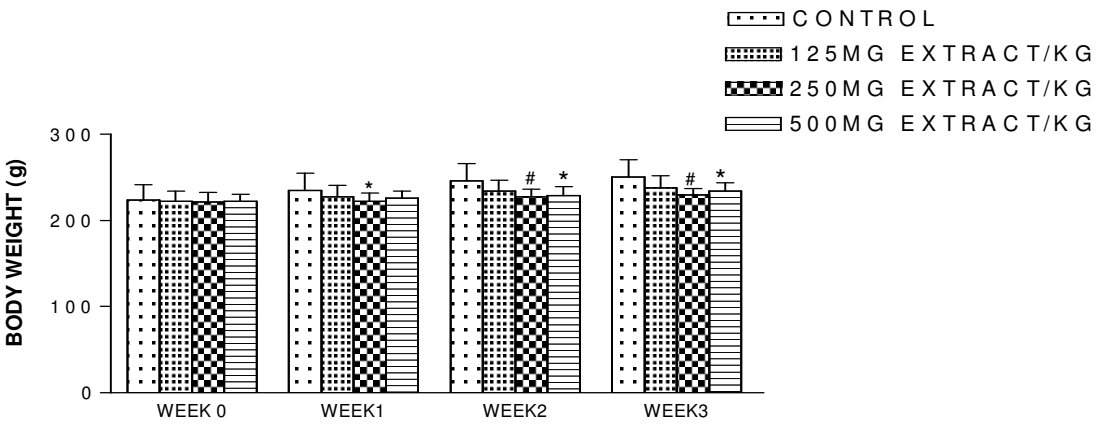

Figure 3. Effect of ethanolic extract of *F. albda* on weekly body weight of rat. n = 6; *significantly different from control at P < 0.05; #significantly different from control at P < 0.01.

Table 1. Effect of ethanolic extract of *F. albida* on haematological parameters in rats.

Parameter	Control	125 mg/kg	250 mg/kg	500 mg/kg
WBC	4.73 ± 1.18	7.42 ± 0.69	9.53 ± 1.03**	6.22 ± 0.99
RBC	7.12 ± 0.31	7.74 ±0.19	7.71 ± 0.15	8.02 ± 0.10
HGB	12.77± 0.59	13.65 ± 0.19	13.67 ±0.27	13.83 ± 0.18
HCT	45.67 ± 2.56	46.50 ±0.81	49.17 ± 0.93	47.67 ± 0.69
MCV	63.70 ±1.71	60.15 ± 2.42*	63.87 ± 1.24	59.53 ± 0.39*
MCH	17.93 ± 0.20	17.70 ± 0.20	17.73 ± 0.23	17.25 ± 0.18
MCHC	28.23 ± 0.61	29.43 ± 0.28	27.80 ± 0.36	29.33 ± 0.18
PLT	708 ± 23.79	675.17 ± 59.05**	737.33 ± 43.03**	860.83 ± 19.63**
LMP (%)	76.17 ± 2.92	66.33 ± 2.77**	69.50 ± 1.61**	75.00 ± 2.45
MXD (%)	1.67 ± 0.38	1.67 ± 0.45	1.67 ± 0.38	1.00 ± 0.24
NEUT (%)	20.50 ± 2.71	29.67 ± 2.31**	26.67 ± 1.47*	22.17 ± 2.52
EOS (%)	1.83 ± 0.44	2.17 ± 0.55	2.17 ± 0.28	1.83 ± 0.55

n = 6; *significantly different from the control at p<0.05; **significantly different from the control at P < 0.01.

extract/kg in the 2nd and 3rd weeks of the study (Figure 3).

Effect of ethanolic extract of *F. albida* on haematological parameters in rats

There was highly significant (p < 0.01) increase in the white blood cell count in the groups treated 250 mg extract/kg and a highly significant (p<0.01) reduction in the lymphocyte levels in the 125 and 250 mg extract/ kg groups. There was a highly significant (p<0.01) increase in platelet count in all the treated groups when compared with the control. There was a highly significant (p < 0.01) increase in the neutrophil count in the 125 mg group and a significant (p < 0.05) increase at 250 mg extract/kg. There were no significant changes for all the treated groups in red blood cells, packed cell volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, monocytes and eosinophils (Table 1).

Effects of ethanolic stem bark extract of *F. albida* on liver enzyme levels in rats

The extract produced a highly significant (p < 0.01) increase in the levels of aspartate and alanine transaminases at all dose levels and in the level of alkaline phosphatase at 125 and 500 mg extract/kg but there was no significant change in the levels of the total and direct bilirubin in all the treated groups (Table 2).

Effects of ethanolic stem bark extract of *F. albida* on liver proteins in rats

Significant (p < 0.05) increase in protein concentration

Table 2. Effects of ethanolic stem bark extract of *F. albida* on liver enzyme levels in rats.

Parameter	Control	125 mg Extract/kg BW	250 mg Extract/kg BW	500 mg Extract/kg BW
Aspartate transaminase (u/l)	273.17 ± 50.04	446.50 ± 118.41**	451.67 ± 119.19**	318.17 ± 62.72**
Alanine transaminase(u/l)	60.17 ± 5.45	90.50 ± 18.02**	132.17 ± 47.39**	79.67 ± 9.46**
Alkaline phosphatase (u/l)	109.17 ± 11.70	122.33 ± 5.36**	118.67 ± 9.53	128.17 ±9.07**
Total bilirubin(u/l)	3.12 ± 0.38	3.70 ± 0.24	3.88 ± 0.31	3.55 ± 0.18
Direct bilirubin(u/l)	1.52 ± 0.11	1.88 ± 0.08	1.92 ± 0.16	1.67 ± 0.13

n = 6; *significantly different from the control at p<0.05; **significantly different from the control at P < 0.01.

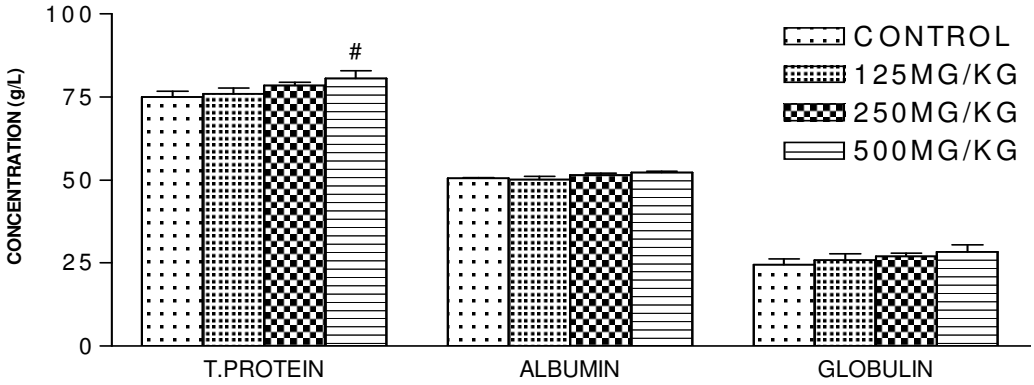

Figure 4. Effect of ethanolic extract of *F. albida* on liver protein in rat, n = 6; #significantly different from control at P < 0.01.

was produced in rats given 500 mg extract/kg while there was no significant difference in albumin and globulin in all the treated groups (Figure 4).

Effect of ethanolic extract of *F. albida* on renal indices of rats

No significant change was observed in urea, potassium, bicarbonate, sodium and chloride ions for all the treated groups (Table 3). However, there was a highly significant (P < 0.01) reduction in the concentration of creatinine in all the extract-treated groups (Table 3).

Effects of ethanolic stem bark extract of *F. albida* on relative organ weight (Row) of rats

There was a significant (P < 0.05) reduction in the row in the testes at 250 and 500 mg extract/kg body weight when compared with the control (Table 4).

DISCUSSION

The lack of death at oral treatment of over 5000 mg extract/kg body weight obtained suggests that the ethanolic stem bark extract of *F. albida* is practically non-toxic acutely (Cobett et al., 1984). It is therefore safe acutely for oral use in the ethno-therapeutic management of trypanosomasis, fever and diarrhoea. The high safety profile obtained may have been responsible for its wide spread use in different ethno-therapeutic interventions. The reduction in the body weight of the treated rats especially in weeks 2 and 3 at 250 and 500 mg/kg may not be due to the food and water intake as there was no significant decrease in these parameters among the treated groups. The decrease may have been due to direct systemic toxic effect of the extract resulting in muscle wastage of the rats. Lymphocytes are mediators of the specific immune response against pathogens, while neutrophils are responsible for phagocytosis (Sacher and McPherson, 1991). An increase in neutrophil count is associated with acute insult to the body whether in the form of infection or not. Use of drugs such as corticosteroids, histamine and epinephrine are known to cause an increase in neutrophils count (Sacher and McPherson, 1991). It is likely that the extract produced an effect similar to any of the above drugs. Platelets are responsible for haemostasis —a process aimed at reducing blood loss and repairing vascular injury (Dahlback, 2007). An increase in platelet count - thrombocytosis is usually a result of a reactive process or a myelo-proliferative disorder (Miller, 1996). The increase observed in the platelet count may indicate that the plant extract stimu-

Table 3. Effect of ethanolic extract of *F. albida* on renal indices of rats.

	Control	125 mg Extract/kg BW	250 mg Extract/kg BW	500 mg Extract/kg BW
CREATININE (µmol/L)	60.83 ±8.83	45.33 ±1.31**	47 ±2.54**	41 ±0.47**
UREA(mmol/L)	8.88 ±0.35	9.63 ±0.38	8.3 ±0.52	8.98 ±0.39
Na^+(mmol/L)	141.83 ±0.96	140.83 ±0.37	141.83 ±0.83	143.5 ±0.69
K^+(mmol/L)	5.31 ±0.52	6.5 ±0.39	6.05 ±0.82	6.43 ±0.83
HCO^-_3(mmol/L)	24.5 ±0.46	25.33 ±0.81	24.33 ±0.45	24.67 ±0.45
Cl^-(mmol/L)	102.17 ±0.93	102 ±0.47	102.33 ±1.04	103.5 ±1.07

Table 4. Effect of ethanolic stem bark extract of *F. albida* on Relative Organ Weight (ROW) in rats.

Organs	Control	125 mg Extract/kg BW	250 mg Extract/kg BW	500 mg Extract/kg BW
HEART ($X10^{-3}$)	3.32± 0.008	3.32± 0.12	3.59± 0.11	3.32± 0.088
LUNG ($X10^{-3}$)	6.67± 0.13	7.42± 0.28	8.26± 0.45	7.68± 0.699
KIDNEY ($X10^{-3}$)	7.23± 0.21	5.8± 0.81	7.05± 0.13	6.89± 0.56
INTESTINE ($X10^{-3}$)	6.08± 0.74	5.55± 0.34	5.29± 0.56	5.03± 0.58
LIVER ($X10^{-3}$)	30.54± 0.94	29.27± 0.89	32.59± 1.02	30.53± 1.63
TESTES ($X10^{-3}$)	16.99± 2.4	13.82± 1.70	12.47± 0.47*	11.62± 0.56*
STOMACH ($X10^{-3}$)	9.52± 0.73	12.46± 1.14	10.81± 1.06	12.68± 1.60
BRAIN ($X10^{-3}$)	5.7± 0.51	5.69± 0.32	6.38± 0.27	6.67± 0.40
SPLEEN ($X10^{-3}$)	3.14± 0.6	2.07± 0.20	3.57± 0.50	1.86± 0.18

n=6; *- significantly different from control group at $p<0.05$.

lates the biosynthesis of clotting factors by the liver and may therefore be useful in the treatment of haemorrhage. These observations may however not result in any adverse effect since the platelet count is still within the normal range for rats (Mitruka and Rawnsley, 1993). The mean corpuscular volume (MCV) is an erythrocyte index that measures the average volume of erythrocytes (Morris and Davey, 1996). It is decreased in microcytic anaemia and increased in macrocytic anaemia (Herfindal and Gourley, 2000). The observed decrease in MCV values may also not indicate that the extract elicits haemolytic anaemia since the values obtained in this experiment falls within the reference range for MCV in laboratory. That this is not indicative of anaemia is shown in the increases observed in the red cell count and the packed cell volume. Tijani et al. (2009) reported that this plant stimulates erythropoiesis in cases of anaemia. Alanine amino transferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) are markers of liver function. These enzymes are only released in significant amounts into the bloodstream from the cytosol and subcellular organelles when hepatic injuries occur (Lu, 1996). ALT is more hepato-specific than AST because it is more sensitive to hepatic damage (Herfindal and Gourley, 2000) and there is a good correlation between its level and severity of hepatic necrosis (Lu, 1996). Increase in ALP level is also observed in bone disorders involving osteoblastic activity. However, an elevation in ALP activity between 2 and 8 fold the normal value usually is the first clue to intrahepatic and extra hepatic cholestasis (biliary obstruction) (Moss, 1987, Young and Holland, 1995). The observed increases in the values of ALT and AST in this study fall within the normal range, while AST was slightly higher than the upper limit of the experimental animal values (Picus et al., 1996). The significant increase in the total plasma protein concentration observed at the highest dose used for the study may indicate that the liver function was not adversely affected by the extract. This observation is further confirmed by the report of an earlier study by Tijani et al. (2009) which showed that the ethanolic stem bark extract of *F.albida* significantly increased the serum protein level in rats infected with *T. brucei brucei*. It may therefore be suggested that the extract increases the protein synthesis function of the liver in cases of injury resulting in enhanced haemostasis, prevention of ascites and deposition of fats in the liver. Reduction in creatinine level is observed in cases of muscle wasting as seen in malnutrition (Pincus, 1996). The highly significant ($P < 0.01$) reduction in creatinine concentration in all the extract-treated groups indicates that the extract does not exert deleterious effect on the renal function and that the decrease in body weight of rats at 250 and 500 mg extract/kg body weight might be

due to muscle wasting possibly due to toxic effect of the extract. Reduction in the relative organ weight for the testes may be due to deleterious effect of the extract with possible adverse effect on its physiological function of spermatogenesis.

Conclusion

These results suggest that ethanolic stem bark extract of *F. albida* is relatively safe when used sub-acutely in rats. In view of the adverse effect of the extract on the testes, it is recommended that a reproductive study be carried out on male rats. Clinical assays have to be done as to confirm the low toxicity of the extract in humans.

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Full Length Research Paper

Anti-inflammatory and analgesic activity of the methanol extract of *Malva parviflora* Linn (Malvaceae) in rats

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Malva parviflora **Linn Malvaceae is a medicinal plant used for the treatment of wounds and other related ailments by the Xhosa people of South Africa. The anti-inflammatory activity of the stem extract of this plant was assessed using carrageenan-induced paw oedema and histamine-induced paw oedema. The analgesic effect was determined using the acetic acid writhing method as well as formalin test. The extract at 100 and 200 mgkg^{-1} body weight reduced significantly, the formation of oedema induced by carrageenan and histamine. In the acetic acid-induced writhing model, the extract showed a good analgesic effect characterized by reduction in the number of writhes when compared to the control. The extract caused dose-dependent decrease of licking time and licking frequency in rats injected with 2.5% formalin, signifying its analgesic effect. These results were also comparable to those of indomethacin, the reference drug used in this study. Since the plant extract reduced significantly the formation of oedema induced by carrageenan and histamine as well as reduced the number of writhes in acetic acid-induced writhing models and dose-dependent decrease of licking time and licking frequency in rats injected with 2.5% formalin, it is concluded that the use of *M. parviflora* for the treatment of inflammed purulent wounds, swellings, bruises and broken limbs may have been justified.**

Key words: Anti-inflammatory, analgesic, carrageenan, histamine, *Malva parviflora*

INTRODUCTION

Inflammation is a pathophysiological response of mammalian tissues to a variety of hostile agents including infectious organisms, toxic chemical substances, physical injury or tumor growth leading to local accumulation of plasmic fluid and blood cells (Sobota et al., 2000). Although a defense mechanism, the complex events and mediators involved in the inflammatory reaction can induce, maintain and aggravate many disorders. The use of non-steroidal anti-inflammatory drugs (NSAIDs) in the treatment of diseases associated with inflammatory reactions has adverse effects which pose a major problem in their clinical use. Hence, new anti-inflammatory and analgesic drugs lacking such effects are being searched for as alternatives to NSAIDs. According to Kumara (2001), plant based drugs in traditional medicine are being paid much attention because of their minimal side effects, cheapness and also the fact that 80% of the world population still relies on them.

Malva parviflora Linn (family: Malvaceae), a prostrate perennial herb, with a deep strong tap root system, found in Europe, has become a cosmopolitan weed species in gardens throughout South Africa and Namibia (Henderson and Anderson, 1966). The leaf of this plant is used in the treatment of boils (Grierson and Afolayan, 1999) and inflammed purulent wounds (Watt and Breyer-Brandwijk) by the Xhosa people of South Africa. The poultice from its leaves is also used to treat wounds and swellings. In Lesotho, the plant is incorporated into a lotion to treat bruises and broken limbs and the dried powder or an infusion made from the leaves and roots is used by herbalists to clean wounds and sores (Shale et al., 1999). In Ethiopia, the root of *M. parviflora* is used in

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the treatment of asthma and wounds (Abate, 1989). Grierson and Afolayan (1999) showed that *M. parviflora* possessed an inhibitory effect on some fungi but was ineffective against some species of bacteria. In contrast, Shale et al. (1999) reported the antibacterial activity of the hexane and methanol extracts of the roots but noted poor activity of the methanol leaf extract.

The present study was undertaken to investigate the anti-inflammatory and analgesic potentials of *M. parviflora* in experimental animals in order to validate its folkloric use in the treatment of inflammed purulent wounds.

MATERIALS AND METHODS

Plant material and preparation of extracts

Young plant of *M. parviflora* was collected from the Nkonkobe municipality of the Eastern Cape province, South Africa in May 2007. The plant was identified by Prof. D. Grierson of the Department of Botany, University of Fort Hare, Alice, South Africa and a voucher specimen (S/N Aboyade 001) deposited at the Giffens Herbarium of the same institution. The plant material was air-dried at room temperature, ground to a powder (200 g) and extracted in methanol (1 l) by shaking for 24 h. The extract was filtered using a Buchner funnel and Whatman no 1 filter paper and concentrated to dryness under reduced pressure at 40°C.

Animals

The animals used in this study were male wistar rats weighing between 120 and 290 g. They were maintained at the experimental animal house of the Agricultural and Rural Development Research Institute (ARDRI), University of Fort Hare in rat cages and fed on commercial rabbit cubes (EPOL Feeds, East London, South Africa). The animals were allowed free access to clean fresh water in bottles *ad libitum*. All experimental protocols were in compliance with University of Fort Hare Ethics Committee on research in animals as well as internationally accepted principles for laboratory animal use and care.

Chemicals

Carrageenan, Tween 80, carboxymethylcellulose and acetic acid were obtained from Sigma-Aldrich (Chemie Gmbh, Steinheim, Denmark). The standard drugs used in the various experiments were indomethacin and histamine obtained from Sigma-Aldrich. All the chemicals and drugs used were of analytical grade.

Anti-inflammatory activity

Carrageenan-induced paw oedema

16 animals used in this study were divided into 4 groups of 4 animals per group. The first group served as the control, the second, third and fourth group received respectively indomethacin (10 $mgkg^{-1}$ body weight) and the *M. parviflora* extract at 2 doses of 100 and 200 $mgkg^{-1}$. The plant extract was suspended in carboxymethylcellulose while indomethacin was suspended in 3% Tween 80 in normal saline. Carrageenan solution (0.1 ml of 1% carrageenan solution) was injected into the sub plantar region of the right hind paw of the rats 1 h after intraperitoneal administration of 3% Tween solution, indomethacin and extract (Moody et al., 2006). The paw volume was measured at 0 h and at 1, 2 and 3 h after administration of drug and extract using a micrometer screw gauge.

The anti-inflammatory effect of the extract was calculated by the following equation; anti-inflammatory activity (%) = (1 - D/C) x 100, where D represented the average paw volume after the extract was administered to the rats and C was the paw volume in the control groups. The percentage inhibition of the inflammation was calculated from the formula: % inhibition = D0 - Dt/D0 x 100 where D0 was the average inflammation (hind paw oedema) of the control group at a given time and Dt was the average inflammation of the drug treated (that is, extracts or reference indomethacin) rats at the same time (Gupta et al., 2005; Sawadogo et al., 2006).

Histamine induced paw oedema

Using the method of Perianayagam et al. (2006), the paw oedema was produced by sub-plantar administration of 0.1% freshly prepared solution of histamine into the right hind paw of the rats. 16 rats divided into 4 groups of 4 rats per group were used. The paw volume was recorded before 0 and 1 h after the histamine injection. The 4 groups of the rats were pretreated with the plant extract (100, 200 $mgkg^{-1}$), 2 $mlkg^{-1}$ of normal saline (vehicle control) or 10 $mgkg^{-1}$ indomethacin (reference drug). These were administered intraperitoneally 1 h before eliciting paw oedema. The anti-inflammatory activity was calculated as described for carrageenan-induced oedema.

Analgesic activity

Acetic acid-induced writhing in rats

The acetic acid-induced writhing test measures abdominal constrictions together with stretching of the hind limbs resulting from intraperitoneal (i.p.) injection of 0.7% acetic acid (10 $mlkg^{-1}$). This was carried out according to the procedures described by Sawadogo et al. (2006). 4 groups of 4 animals per group were used in this study comprising the control (2 $mlkg^{-1}$ normal saline solution), indomethacin (10 $mgkg^{-1}$), or plant extract (100, 200 $mgkg^{-1}$). After 30 min, acetic acid was administered i.p. The number of writhing movements was counted for 30 min.

Formalin test in rats

The procedure was essentially similar to that described by Correa and Calixto (1993). Formalin solution (0.05 ml of 2.5% formalin) was injected into the sub-plantar of the right hind paw. The number of times and the time spent licking the injected paw was recorded and was considered as indicative of pain. The animals were pretreated with indomethacin and plant extract (100 and 200 $mgkg^{-1}$) 30 min before being administered with formalin and the responses were observed for 30 min. The control group was pre-treated with normal saline.

Statistical analysis

The data were expressed as mean ± S.D. Where applicable the difference in response to test drugs was determined by student's t-test. $P < 0.05$ was considered significant.

RESULTS

Anti inflammatory activity

Carrageenan-induced paw oedema

When compared with the control, the extract and indome-

Table 1. Anti-inflammatory activity of the methanol extract of *M. parviflora* on carrageenan-induced oedema in the right hind-limb of rats. Data is presented as mean ± S.D., n = 4.

Time (h)	Control	Extract ($mgkg^{-1}$)		Indomethacin ($10\ mgkg^{-1}$)
		100	200	
1	21 ± 0.3	11 ± 0.5 (48.0)	0.7 ± 1.0 (96.6)	4.0 ± 0.5 (81.0)
2	28.9 ± 0.1	9.7 ± 0.7 (66.6)	5.8 ± 0.2 (80.0)	5.1 ± 0.4 (82.5)
3	30.1 ± 0.1	14.5 ± 0.9 (51.9)	4.8 ± 0.7 (84.1)	3.1 ± 0.4 (89.6)

Percentage inhibitions of the carrageenan-induced inflammation (oedema) are in parenthesis.

Table 2. Anti-inflammatory activity of the methanol extract of *M. parviflora* on histamine-induced oedema in the right hind-limb of rats. Data is presented as mean ± S.D., n = 4.

Time (h)	Control	Extract ($mgkg^{-1}$)		Indomethacin ($10\ mgkg^{-1}$)
		100	200	
1	18.5 ± 0.3	18.2 ± 0.5 (1.6)	13.0 ± 0.5 (29.5)	17.0 ± 0.3 (7.9)
2	14.4 ± 0.2	14.2 ± 0.5 (1.5)	5.5 ± 0.1 (62.1)	11.7 ± 0.2 (18.7)
3	9.7 ± 0.1	7.9 ± 0.9 (19.0)	5.5 ± 0.2 (43.8)	3.4 ± 0.12 (65.0)

Percentage inhibitions of the carrageenan-induced inflammation (oedema) are in parenthesis.

Table 3. Influence of methanol extract of *M. parviflora* and indomethacin on rat writhing reflex induced by acetic acid.

Group	Dose ($mgkg^{-1}$)	Number of writhing per 20 min	Inhibition (%)
Control	3 ml/kg	50.4 ± 2.2	0
Indomethacin	10	0 ± 0.0	100
Extract	100	0 ± 0	100
Extract	200	0 ± 0	100

Data is presented as mean ± S.D., n = 4.

thacin significantly reduced the paw oedema hours after carrageenan injection. For instance, the 100 $mgkg^{-1}$ produced its highest effect at 2 h (67%) after carrageenan injection while the 200 $mgkg^{-1}$ was more effective 1 h (97%) after injection. The reference drug produced time-dependent reduction as the effect was more pronounced at 3 h (90%) of carrageenan administration (Table 1).

Histamine-induced paw oedema

Inhibition of histamine-induced oedema was observed in this study. The antihistaminic activity of the 200 $mgkg^{-1}$ extract was most pronounced at 2 h (62%) while that of the 100 $mgkg^{-1}$ and indomethacin was at 3 h (Table 2).

Anti-nociceptive activity

Acetic acid-induced writhing

The effect of the methanol extract of *M. parviflora* on writhing response in rats showed that the extract at 100 and 200 $mgkg^{-1}$ caused 100% inhibition on the writhing response induced by acetic acid. A similar inhibition was observed with indomethacin, the reference drug used in the study (Table 3).

Formalin test

In this study, the extract caused a dose-dependent decrease in licking time and licking frequency by the rats injected with formalin. The indomethacin group showed better analgesic effect than the 2 doses. The analgesic effects of these groups were significantly different from that of control at $P < 0.05$ (Table 4).

DISCUSSION

The results of this investigation suggest that the methanol extract of *M. parviflora* possesses a dose dependent activity against carrageenan and histamine induced paw oedema in rats. The activity of the 200 $mgkg^{-1}$ of the

Table 4. Analgesic effect of methanol extract of *M. parviflora* and indomethacin on formalin test on mice.

Group	Dose ($mgkg^{-1}$)	Licking time (s)	Licking frequency/30 min
Control	0	15 ± 2.5	25.3 ± 0.5
Indomethacin	10	5.8 ± 0.5	13.8 ± 0.3[a]
Extract	100	6.4 ± 0.3	15.4 ± 0.6[a]
Extract	200	2.3 ± 0.5	14.5 ± 0.2[a]

Data is presented as mean ± S.D., n = 4.
[a] Superscript is significantly different from the control at $P < 0.05$.

extract was comparable to that of indomethacin. The carrageenan-induced inflammation model which is a predictive test for anti-inflammatory agents acts by inhibiting the mediators of acute inflammation (Ozaki, 1990; Mossai et al., 1995; Silva et al., 2005; Sawadogo et al., 2006). Therefore, these results suggest that *M. parviflora* may be effective in the treatment of acute inflammatory disorders.

In the histamine-induced paw oedema, 200 mg kg^{-1} of the extract exhibited a higher inhibitory effect than the reference drug at 2 h. It should be noted that the early phase (1 - 2 h) in the induced paw oedema model is mainly mediated by histamine, serotonin and the increase of prostaglandin (PG) synthesis in the surroundings of the damaged tissues. The late phase on the other hand, is mainly mediated by bradykinin, leukotrienes, polymorphonuclear cells and PGs produced in tissue macrophages (Antonio and Souza Brito, 1998; Cuman et al., 2001; Linardi et al., 2002; Vasudevan et al., 2007). The results of the present study suggest that the suppression of inflammation at the early phase was as a result of the antihistamine activity of the plant extract.

The acetic acid induced writhing test, a non specific but nevertheless sensitive method widely used for analgesic screening (Le Bars et al., 2001), indicated a 100% inhibition of writhing in the animals at both doses of the extract and also of the reference drug used in this study. Acetic acid has been found to cause an increase in peritoneal fluid levels of prostaglandins (PGE_2 and PGF_2), hence causing inflammatory pain by inducing capillary permeability (Amico-Roxas et al., 1984). The observed effects in the present study suggest that *M. parviflora* had an inhibitory effect on prostaglandins synthesis.

The formalin test has been described as a convenient method for producing and quantifying pain in rats (Dubuisson and Dennis, 1977; Tjolsen et al., 1992). The test employs an adequate painful stimulus to which the animals show a spontaneous response and it is sensitive to commonly used analgesics. The pain stimulus, a continuous rather than a transient one, may have resemblance to some kinds of clinical pain and observations are made on animals which are restrained only lightly or not at all (Hunskaar et al., 1985; Ghannadi et al., 2005). Intraplantar injection of 2.5% formalin evoked a characteristic licking response in the Wistar rats. In this study, the extract caused a dose-dependent decrease in licking time and licking frequency by the rats injected with formalin signifying the analgesic effect of the extract. Although the active doses of the plant extract were higher than those of the reference drug, it should be noted that the extract is made up of different compositions of several substances.

It is concluded that the plant extract reduced significantly the formation of oedema, reduced the number of writhes and dose-dependent decrease of licking time and licking frequency in rats. These results may have validated the basis for the traditional use of *M. parviflora* against inflammed purulent wounds, swellings, bruises and broken limbs among the Xhosa people of South Africa.

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Full Length Research Paper

Frequency of *cryptosporidium* infection in children under five years of age having diarrhea in the North West of Pakistan

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Cryptosporidium **species are minute, coccidian protozoan parasites that have been associated with enterocolitis. It has worldwide distribution and has emerged as an important cause of diarrhea, particularly in children less than 5 years of age and in immunocompromised individuals. Waterborne transmission is particularly troublesome because *Cryptosporidium parvum* oocysts are not eliminated by chlorination or domestic disinfectants. In the present study, single stool specimens from young children (< 5 years) presented with diarrhea were collected in Khyber teaching hospital, Peshawar, Pakistan. Wet mount preparation and modified Ziehl-Neelsen staining were used for identification of oocysts in stool specimens. *Cryptosporidium* oocysts were found in 18 (9.0%) out of 200 children suffering from diarrhea. Infection was common in children between 1 - 24 months of age and associated with abdominal cramps (50%), vomiting (61.1%) and prolonged duration of diarrhea (88.9%). Direct and indirect contact with animals was present in most of *C. parvum* infected children (83.3%). Most of *C. parvum* infected children were consumers of well water (77.8%). *Cryptosporidium* spp. are highly infectious causes of diarrheal illness around the world. It is an important cause of diarrhea in children. Clinician and laboratories should be encouraged to include *C. parvum* diagnostic techniques while dealing with diarrheal stool samples of young children.**

Key words: *Cryptosporidium parvum,* cryptosporidiosis, modified Ziehl-Neelsen staining, immunocompromised, immunocompetent, diarrhea, Pakistan.

INTRODUCTION

Cryptosporidium spp. are tiny microscopic parasites that cause cryptosporidiosis. It is classified as an emerging pathogen by the Center for Disease Control and prevention (CDC) (Guerrant, 1997). Using genotyping tools, five species of *Cryptosporidium*, that is, *Cryptosporidium hominis, Cryptosporidium parvum, Cryptosporidium meleagridis, Cryptosporidium felis* and *Cryptosporidium canis* have been shown to be responsible for most human infections. Of these five species, *C. hominis* and *C. parvum* are the most common species involved in clinical infections (Sulaiman et al., 2005).

C. parvum is an obligate intracellular parasite that infects the epithelial lining of luminal surfaces of gastrointestinal and respiratory tract in a wide variety of hosts. *C. parvum* ingested as an oocyst, undergoes excystation and the sporozoites parasitizes the host. Infection can occur in areas of esophagus and any portion of gastrointestinal tract can be involved. It usually starts in the lower small intestine. Other areas include the gall bladder, bile ducts, pancreas and respiratory tract. Infection induces symptoms of diarrhoea, abdominal cramps, vomiting, low grade fever, generalized malaise, weakness, fatigue, loss of appetite and nausea (Armson et al., 2003).

In immunocompetent individuals, the organism is primarily localized in the distal small intestine and proximal colon, whereas in immunocompromised hosts, the parasite has been identified throughout the gut, biliary and respiratory tracts. After the ingestion of oocyst of *C. parvum*, immuno-competent persons can either experience

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asymptomatic infection or self limiting diarrhoea. Those patients, who have defect in innate, humoral or cellular immunity, can experience severe or prolonged illness. The life threatening potential of *C. parvum* in immunocompromised and immunosuppressed individual has increased the importance of cryptosporidiosis as a global public health problem. The infection by *C. parvum* is usually of short duration and is self-limiting in immunocompetent individual (Moghaddam, 2007).

Cryptosporidium is transmitted through multiple routes. The infection may be transmitted by direct person to person, contact with infected animal or by ingestion of contaminated food or water (Khan et al., 2004). The oocyts are highly resistant to common household disinfectants and survive for long periods in the environment. It has major public health implication because infection can result from exposure to low doses of *Cryptosporidium* oocysts (Gatei et al., 2006).

According to Ajjampur et al. (2007), cryptosporidiosis is a major cause of diarrhoea in children with or without human immunodeficiency virus (HIV) in developing countries. AIDS and protein energy malnutrition (PEM) severely impair immune system (Ajjampur et al., 2007). According to study by Banwat et al. (2004), there is a high prevalence rate of *Cryptosporidium* in young children suffering from protein energy malnutrition (PEM) (Banwat et al., 2004).

The parasites exhibit tropism for the jejunum and ileum in immunocompetent persons. When the immune response is weak such as in patients with AIDS, the infection is more widespread. It may involve stomach, duodenum, colon and the biliary tract. In such patients, cell mediated immunity is low. It means that cell mediated immunity is the major component in eliminating the infection. This is evident by correlation between lower CD4 T cells count, risk and severity of cryptosporidiosis. Interferon-α, interleukin-12 (IL-12) and tumor necrosis factor-α (TNF- α) are involved in protection against cryptosporidiosis (Wyatt et al., 1997). HIV infected person with CD4 count <50/cubic mm, when exposed to *Cryptosporidium* are at high risk for biliary symptom and for death within a year after infection (Vakil et al., 1996).

This is the first report of prevalence of *Cryptosporidium* infection in children from Peshawar, North West of Pakistan. The aim of this study was to find out frequency of *Cryptosporidium* infection and risk factors in children under five years of age having diarrhoea with the objective of creating awareness among the population and in caretakers of the diarrheal disease patients, to highlight the importance of detection of this parasite in routine stool examination and to improve and modify the treatment of these patients.

MATERIALS AND METHODS

A total of 200 stool specimens from children (under 05 years of age) suffering from diarrhoea were examined for the presence of *C. parvum* oocyst. This study was carried out prospectively at clinical laboratory of Khyber Teaching Hospital (KTH) Peshawar and in the pathology department of Khyber Medical College (KMC), Peshawar.

This was a simple descriptive study carried out to find the frequency of *C. parvum* infection in young children with diarrhoea less than 5 years of age. The study was conducted from March, 2007 to April, 2008. Random survey was done with non probability convenience sampling. A proforma was designed with inclusion and exclusion criteria to record the information of patients.

Inclusion criteria

1 All children under 5 years of age with diarrhoea, whether immunocompetent or immunocompromised.
2 Duration of diarrhoea greater than 5 days.
3 Children already on anti diarrhoeal treatment but not receiving antibiotics.

Exclusion criteria

4 Patients who refused submission of specimen.
5 Specimen delayed for more than six hours after collection.
6 If pus cells were reported or any other parasitic infection was detected in stool examination.

The parents/guardians were interviewed using designed proforma including demographic and clinical history. Results were entered in computer database and evaluated. All relevant nurses and technical staff were guided regarding collection and processing procedure of the specimen. Inclusion and exclusion criteria were laid down, before collection of stool specimens. Faecal specimens meeting the inclusion criteria were collected in clean, dry, leak proof glass/plastic containers. Each sample was examined for appearance (or by naked eye for consistency), color, presence of mucus or blood. Samples were kept at 4°C and processed in 6 - 8 h.

According to Morgan et al., (1998), microscopy is a reliable diagnostic method than immunologic based methods for detection of *C. parvum* oocysts (Morgan et al., 1998), hence wet mount preparation, modified Z-N staining and microscopy was carried out for the detection of *C. parvum* oocysts in stool specimens. The specimens were examined on the same day of collection, using wet mount preparation and modified Z-N staining technique. Wet mount preparation was carried out along with Lugol's iodine (01%). This method helped in the differentiation of yeasts cells from *C. parvum* oocysts. Yeasts cells appeared deep yellow by accepting iodine while *C. parvum* oocysts do not accept iodine stain and appeared as transparent discs (Mahgoub et al., 2004).

RESULTS

Out of 200 stool specimens collected from indoor patients of two pediatric units of KTH, a total of 18 cases (9.0%) were found positive for the oocysts of *C. parvum* using modified Z-N staining (Figure 1). The gender distribution of the positive cases was 13 (72.2%) male and 5 (27.8%) were female.

Majority of the children with *C. parvum* infection were less than 24 months of age (Table 1). Mean age of the patients with *C. parvum* infection was 20 months with standard deviation of 15.29 months (Min = 1 month and max = 60 months).

Mean duration of diarrhoea was 11 days in positive cases.

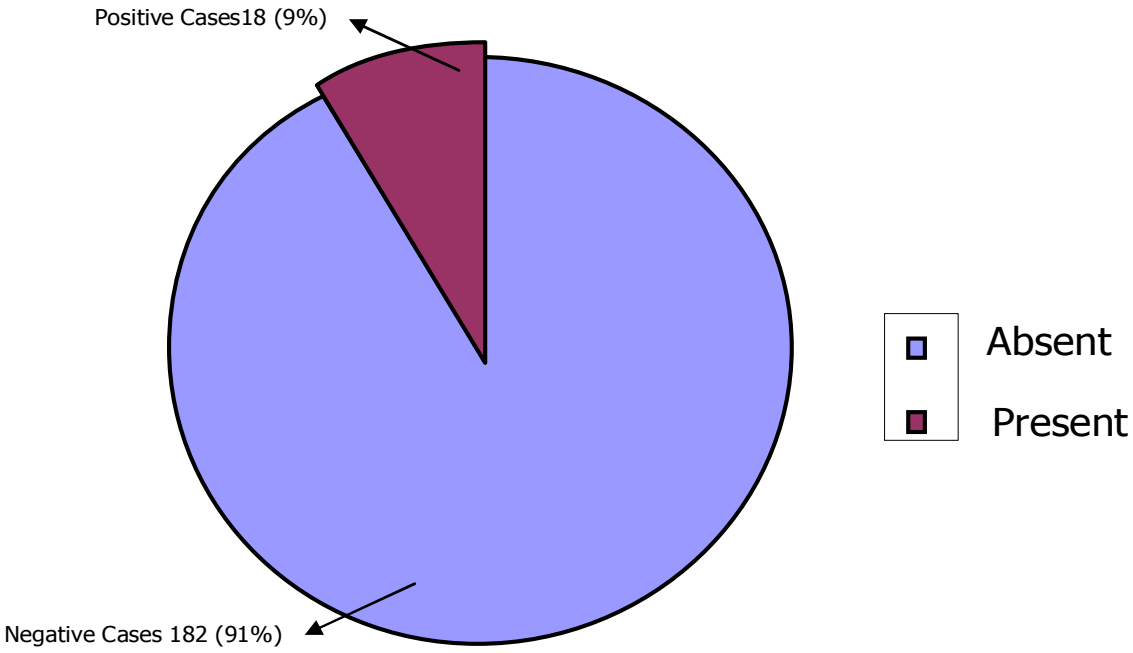

Figure 1. Frequency of *C. parvum* in diarrhoeal Stool (n = 200).

According to the onset of illness, (diarrhoea) 16 cases (88.9%) presented with persistent diarrhoea and the remaining 2 cases (11.1%) are of children that had acute diarrhoea (Table 1).

Abdominal pain was presented in 9 (50%) patients (Table 1). Fever was present in 15 (83.3 %) children with *C. parvum* associated diarrhoea. Only 1 child (5.6%) had blood in his stool (Table 1).

Moderate dehydration was present in 15 (83.3%) and only 3 (16.7%) children had mild dehydration at the time of presentation (Table 1). Watery diarrhoea mixed with mucus was present in 11 (61.1%) children (Table 1). Watery diarrhoea with abdominal pain was the most frequent presenting symptom in all cases.

Majority of infected children, that is, 17 (94.4%) were from poor socioeconomic group and only one case (5.6%) came from satisfactory group.

The frequency of cryptosporidiosis has strong association with large family size 15 (83.3%) as compared to small family size 3 (16.7% p value < 0.03). The history of animal contact was present in 15 (83.3%) children. The source of drinking water was well water in 14 (77.8%) children with *C. parvum* infection and only 4 (22.2%) children were using municipal water supply.

DISCUSSION

C. parvum is an endemic, zoonotic parasite that is highly prevalent in developing countries. Cryptosporidiosis is a serious disease in these countries, because it increases morbidity and mortality associated with poverty and malnutrition (Shoaib et al., 2003; Laubach et al., 2004). In developing countries, it is a leading cause of persistent diarrhoea especially in children. Microscopic analysis of Z-N stained faecal smear is the most commonly used method for screening stool specimens for *C. parvum* oocysts. Although microcopy is considered to be a reliable diagnostic tool for the detection of cryptosporidial infection, it is however, not practiced routinely in Pakistan.

Different epidemiologic studies have demonstrated the prevalence rate of *C. parvum* varies in different parts of Asia. In this study, it was found that 18 (9.0%) out of 200 children under five years of age had *C. parvum* oocysts in their faeces. They had watery diarrhea (61.1%) and few complained of abdominal colic (50%) as well. They were clinically dehydrated (83.3%) while other associated symptoms were fever (83%) and vomiting (61.1%). The incidence of *C. parvum* infection in the present study is consistent with a previous study from Rawalpindi, Pakistan by Iqbal et al. (1999). This study showed similar rate of cryptosporidial infection in children with diarrhea (10.3%) than in children without diarrhoea (3.3%) (Iqbal et al., 1999). The prevalence rate in China is 3.6% (Wang et al., 2002). The low infection rate in China is likely due to better living and sanitary conditions. However, an earlier study from Karachi, Pakistan by Shoaib et al. (2003) showed significantly low rate of *C. parvum* infection (1.7%) in children presented with persistent diarrhoea. Failure to detect *Cryptosporidium* oocysts in the stool of infected person may be due to seasonal variation, while intermittent nature of excretion of this parasite in stool, can also lead to low detection rate (Nimri and

Table 1. Frequeny of *C. parvum* in children <05 years of age having diarrhoea (n = 200).

Characteristics		Cases	%Age	Controls	% Age	O.R.	95% C.I.	P-Value
	Present	11	61.1	98	53.8			
Age	1 - 36 Months	15	83.3	105	57.7	3.67	0.95 - 16.55	0.06
	37 - 60 Months	03	16.7	77	42.3			
Sex	Male	13	72.2	100	54.9	2.13	0.67 - 7.18	0.05
	Female	05	27.8	82	45.1			
Socio-Economic Status	Poor	17	94.4	112	61.5	218.88	0.01	0.05
	Satisfactory	01	5.6	70	83.5			
Hydration	Moderate	15	83.3	81	44.5	6.23	1.62 - 28.14	0.001
	Mild	03	16.7	101	54.5			
Family Size	Large	15	83.3	100	54.9	4.1	1.08 - 18.50	0.03
	Small	03	16.7	82	45.1			
Source of Drinking Water	Well	14	77.8	88	48.9	3.7	1.08 - 13.89	0.03
	Municipal	04	22.2	93	51.1			
Duration of Diarrhoea	11 - 20 days	16	88.9	100	54.9	6.56	1.39 - 42.58	0.01
	5 - 10 days	02	11.1	82	45.1			
Abdominal Cramps	Present	09	50	115	63.2	0.84	0.29 - 2.43	0.38
	Absent	09	50	67	36.8			
Animal Contacts	Yes	15	83.3	89	48.9	5.22	1.34 - 23.8	0.01
	No	03	16.7	93	51.1			
Blood	Absent	17	94.4	170	93.4	1.2	0.15 - 26.17	0.74
	Present	01	5.6	12	6.6			
Fever	Present	15	83.3	11	60.9	3.2	0.83 - 14.45	0.1
	Absent	03	16.7	71	39.1			
Mucus	Absent	07	38.9	84	46.2	0.74	0.25 - 2.19	0.73

Batchoun, 1994). Other studies have reported prevalence rate of 17% in Egypt, 9 - 13% in Tanzania, 5.9% in Uganda and 14.2% in Gaza (Gatei et al., 2006).

Despite its wide distribution in the environment and obvious relevance to public health, there is very little awareness about this protozoan parasite in Pakistan. In this study, *C. parvum* oocysts were more frequently detected in children between 13 - 24 months of age, the mean age group being 20 months (SD = 15.29). Children in this age group were found to be more vulnerable when exposed to contaminated environment, food and water. Immunity is less than optimal at both ends of life, that is, in new born and in the elderly. New born babies appeared to have less T-cell function and antibodies are provided primarily by the transfer of maternal IgG crossing the placenta. Maternal antibodies decay over time (little remains by 3 - 6 months of age) and the risk of infection in children is high after the age of six months (Levinson and Jawetz, 2003).

In this study, many patients were Afghan refugees with large families. They were mostly uneducated and lived in poor hygienic conditions. These children were not properly looked after and hand washing was not practiced. The crawling children who are more exposed to dirty surfaces are therefore more vulnerable to these oro-faecal infections. Similar observations were made in the study conducted by Nagamani et al. (2007) in Indonesia and Ghana (Nagamani et al., 2007). The increased infection rate in this age group could be explained by their immunologic immaturity and other factors as mentioned above. Results from study by Nagamani et al. (2007) suggested that infections were more common in young children. Children in this age group were found to be more vulnerable because of weaning. Contaminated food and water could be the source of infection in these children.

In the present study, it was found that only one case of *C. parvum* oocysts in diarrheal stools of children below 6 months of age was presented. The *p* value (< 0.05) was marginally significant and this may be due to small sample size. In the present set up, this age group was usually breast fed. Colostrum contains antibodies especially secretary IgA, which protects newborn babies against various respiratory and gastrointestinal infections. Studies have shown that persistent diarrhoea is uncommon in breast fed babies (Meremikwu et al., 1997; Mihrshahi et al., 2007). In developing countries including Bangladesh, cryptosporidiosis has been reported in non breast fed babies (Khan et al., 2004).

In this study, the frequency of *Cryptosporidium* infection

in male and female was statistically significant (p value < 0.05). The high prevalence rate of this intestinal parasite observed among males could be attributed to the fact that males are more active, mobile and integrated to the environment. Moreover, it may involve gender specific immunological differences (Agnew et al., 1998).

Socioeconomic status (SES) plays a major role in health. This, not only affects the level of health care but also affects diet, housing and environmental conditions that can affect health. Intestinal parasitic infections are associated with poor socioeconomic group of people, unsanitary environments and inadequate personal hygiene. In this study, there was high incidence of cryptosporidial infection among low socioeconomic group, p value was highly significant (< 0.01).

C. parvum is one of the most common waterborne human parasitic protozoa (Casemore and Roberts, 1993). There have been eleven outbreaks of crypto-sporidiosis in the United States during 1991 - 2000 due to contamination of drinking water (Roy et al., 2004). Contaminated drinking and recreational water are associated with outbreaks of cryptosporidiosis. In this study, many patients belonged to rural areas having low socioeconomic conditions. There was no awareness regarding cleanliness, sterilization and disinfection. These children were more at risk from *C. parvum* infection. Source of drinking was well water in 14 (77.8%) infected children and only 4 (22.2%) children were using municipal water supply. Most of human infections are caused by contaminated water supply. Because of small size of *C. parvum* oocysts and its ability to survive chlorination, it can contaminate municipal water supply with ease.

The results showed that the mean duration of diarrhoea was 11 days in children infected with *C. parvum*. Sixteen out of 18 (89%) children presented with persistent diarrhoea at the time of admission to the hospital. In developing countries, persistent diarrhoea is an important child health problem but is given less attention regarding its control and prevention. Cryptosporidial infection usually leads to chronic watery diarrhea associated with dehydration. Moderate dehydration occurred in 15 (83.3%) children and only 3 (16.7%) children had mild dehydration at the time of presentation, p-value was highly significant (< 0.001).

Epidemiology of cryptosporidiosis in human is not completely understood because of multiple transmission routes such as person to person, animal to person, water and possible air borne transmission (Ajjampur et al., 2007). Many outbreaks of gastroenteritis by *C. parvum* are caused by contaminated water and food (Verweij et al., 2004). A similar association at risk by acquiring infection of *C. parvum* due to consumption of contaminated water and *C. parvum* infection was observed in various studies from Pakistan, Bangladesh, Kuwait and India (Iqbal et al., 1999; Sulaiman et al., 2005).

According to Arrowood (2002), cryptosporidiosis has worldwide distribution and affects a wide range of reptiles, avian, fishes and mammals (Arrowood, 2002). In Kuwait and UK, genotyping of *Cryptosporidium* isolates from human cases revealed zoonotic transmission involving livestock (Nagamani et al., 2007). In this study, majority of children (83.3%) had history of contact with animals either within the house or in close neighborhood. These animals could have been the reservoir of transmission of infection. This parasite is not host specific and infection can spread from infected animals to humans (Laubach et al., 2004).

Highly variable clinical manifestations of cryptosporidiosis in healthy population and in disease endemic areas were observed. These clinical manifestations can be attributed in part to the different species of *Cryptosporidium*. Results showed that molecular mechanisms may play an important role with clinical manifestations of human cryptosporidiosis (Cama et al., 2008). Clinical disease and morbidity is determined by combination of host related factors such as nutritional status, local and systemic immunity.

Diarrhoea due to *C. parvum* was recognized to have seasonal variation showing increased incidence during wet and hot months of the year (Gatei et al., 2006). In this study, most of the cases occurred during rainy season of July and August. The weather during these months in Peshawar is usually hot and humid.

Although only 9.0% of patients in the present study were shown to be excreting *C. parvum* oocysts, it is significant from the public health point of view, as specific diagnostic methods are not routinely used in stool examination (especially from patients suffering from chronic gastroenteritis). Therefore, it seems reasonable to test apparently healthy people with undiagnosed chronic diarrhea, particularly the animal handlers, travelers to endemic areas, hospital workers, household contacts of infected persons and children in day care centers. The present study signifies that *C. parvum* is associated with early childhood diarrhoea. The focus should be to reduce the infectious period, length of illness, risk of dehydration and to prevent complication and death. Health education, personal hygiene and awareness of clinician about importance of cryptosporidiosis testing is useful. This is due to the fact that *C. parvum* will not be detected unless specific diagnostic tests are use by clinicians. Facilities for the detection of *C. parvum* oocysts should be made available as an investigation of diarrheal stool specimens that come to the laboratory. Routine testing of stool will help to explore the gravity of the situation even further. Vigilant surveillance and control measures will help to improve overall health of children.

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Full Length Research Paper

Phytochemical screening and *in vitro* anticandidal activity of extracts and essential oil of *Curculigo pilosa* (Schum and Thonn) Engl. Hypoxidaceae

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Curculigo pilosa **is commonly used for herbal preparations as a purgative and also in the management and treatment of hernia, infertility and gonorrhea in Southwestern Nigeria. Owing to reported resistance of** ***Candida albicans*** **to toxic expensive anticandidal agents such as azoles and its implication for promoting opportunistic fungal infections of immunosuppressed patients, the anticandidal activity of** ***C. pilosa*** **was studied. The phytochemical screening of its powdered rhizomes was done using standard procedure. The extracts and essential oil were prepared using Soxhlet and Clavenger-type apparatus respectively. Ten** ***C. albicans*** **isolates from vagina cotton swabs were obtained from three hospitals in Ibadan, Nigeria. The isolates were tested against extracts and essential oil for any anticandidal activity using agar-well diffusion method. The minimum inhibitory concentration (MIC) was determined using broth dilution method. The phytochemicals found in** ***C. pilosa*** **were alkaloids, saponins, tannins, cardenolides and traces of anthraquinones. The ethanol extracts (500 mg/ml) and undiluted essential oil exhibited anticandidal activity while the water extract (1000 mg/ml) was inactive against isolates. The MIC exhibited by the ethanol extract against the tested isolates range between 0.020 and 1.500 mg/ml. The isolation and identification of the active compounds of** ***C. pilosa*** **could lead to the discovery of anticandidal phytomedicine.**

Key words: *Curculigo pilosa, Candida albicans,* phytochemical screening, extracts, essential oil, anticandidal activity.

INTRODUCTION

The prevalence of *Candida albicans* in candidiasis has been reported by many authors. Osho (2000) studied the antimicrobial effects of some medicinal plants on *Candida* species isolated from human oral mucosa and reported that *C. albicans* constituted 64.8% of the 128 isolates in the six species of *Candida* obtained by him. Other species encountered in the study were *C. tropicalis, C. glabrata, C. krusei, C. stellatoidea* and *C. parapsilosis.* The species most frequently causing human candidiasis are *C. albicans*, *C. tropicalis* and *C. glabrata* whilst the others may also be of medical importance (Jones, 1985). The global human immunodeficiency virus (HIV) epidemic has resulted in an increase in severely ill immunocompromised hospitalized patients, accompanied by more reports of fungal infections. The most common fungal pathogens associated with invasive disease in humans are opportunistic yeasts (e.g. *Candida albicans*) (Toscano and William, 1999). Unfortunately the limited number of antifungal agents available in the market is toxic, expensive and *C. albicans* has developed resistance to commonly used antifungals (Perea et al., 2001). Due to this reason, there has been a search for newer generation of drugs to combat such complex mycotic pathogens. This has attracted the researchers to search for new antifungal agents of herbal origin which are relatively economically affordable, safer and easily available to common men (Rai et al., 2003).

Curculigo pilosa belongs to Hypoxidaceae and is an herbaceous plant with stout, erect rhizomes bearing a cluster of grass-like leaves to 60 cm long and flower shoots to 20 cm at the end of the dry season. It is found

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in seasonally marshy savanna. It is widely dispersed from Senegal to West Camerouns and over much of tropical Africa and Madagascar (Burkill, 1985). In Nigeria, it is found in Mubi, Abuja, Igboho and Erin-odo (UIH). In the Yoruba traditional medicine of Southwestern Nigeria *C. pilosa* is used as a purgative as well as for the management and treatment of hernia, infertility, genital infections and sexually transmitted infections especially gonorrhea.

A survey of literature indicates that many investigators have studied herbal anticandidal agents in recent past. Giordani et al. (2001) reported the *in vitro* susceptibility of C. *albicans* to *Euphorbia characias* latex using the macrobroth dilution method. Runyoro et al. (2006) reported that twenty-eight (28) out of the sixty-three (63) aqueous methanolic extract, belonging to 27 plant species and constituting 48% of the Tanzanian medicinal plants collected exhibited activity against *C. albicans*. Ajaiyeoba and Sama (2006) reported that the leaf and stem redistilled hexane and ethanol extract of *Capparis thonningii* showed inhibitory activity against *C. albicans* and *Aspergillus flavus*. The concentrations of extract used were 250, 500 and 1000 mg/ml.

This work examined the anticandidal activity of *C. pilosa* against 10 clinical isolates of *C. albicans,* to produce scientific insight for the use of the plant in ethnobotany and widen the spectrum of activity against *Candida*.

MATERIALS AND METHODS

Plant material

Fresh rhizomes of *C. pilosa* were purchased from a local market in Ibadan, Nigeria in the month of July and were identified in the University of Ibadan Herbarium (UIH). The rhizomes were thoroughly washed with tap water, air-dried, ground into powder, weighed and stored in an air-tight glass container for further use.

Phytochemical screening

The powdered plant material was screened for the presence of natural products using standard procedures in the laboratory of the Department of Pharmacognosy, University of Ibadan, Ibadan, Nigeria.

Preparation of extracts and essential oil

Water extract: 200.0 g of the dried powdered rhizome was soaked in 1000 ml of sterile distilled water for 48 h. The mixture was filtered and the filtrate was freeze dried. 5 g of the extract was reconstituted in 5 ml sterile distilled water to obtain a solution of 1000 mg/ml, which was used for the anticandidal screening.

Ethanol extract

500 g of powdered sample was extracted in 1.5 litre of ethanol (95 % w/v) for 24 h using Soxhlet apparatus. The extract was transferred into sample holder of the rotary vacuum evaporator, where the extract was concentrated to dryness at 50°C and then air-dried to constant weight. The extract was refrigerated at 4°C prior to use. 5 g of the extract was reconstituted in 10 ml sterile distilled water to obtain a solution of 500 mg/ml, which was used for the anticandidal screening.

Essential oil

Essential oil was extracted from 300 g of the plant sample (4 h) by hydrodistillation using a Clavenger - type apparatus designed to the British pharmacopoeia specification (1980). The essential oil was stored in the refrigerator at 4°C prior to use. The undiluted oil was used for the anticandidal screening.

Identification of *C. albicans* isolates

The *C. albicans* isolates were identified according to the methods used by Gbadamosi and Egunyomi (2008).

Screening of plant extract for anticandidal activity

The extracts were tested for their anticandidal activity using agar well diffusion method. Each was suspended in sterile malt extract broth (Difco Laboratories, USA), incubated at 35 ± 2°C for 18 h. Different concentrations of each isolate were prepared from the broth in sterile distilled water to give a range of concentrations at 10^{-1} to 10^{-6} colony forming unit (cfu) per ml. One millilitre of each concentration was added and thoroughly mixed with 19 ml of sterile liquid Mueller Hilton agar (LAB M, UK.) and poured into sterilized Petri dishes (100 mm in diameter). The agar was left to solidify, from each of these plates 9 mm diameter wells were cut out from the agar using sterile cork-borer. Each of these wells was filled with 50 µl of plant extract using a micro pipette. The plates were left at room temperature, long enough for diffusion of the extract into agar. Subsequently, the plates were incubated at 35 ± 2°C for 18 - 36 h. Zones of inhibition were measured in millimetres. A control plate containing the test organism without any plant extract was also incubated. Each examination was carried out in triplicates for all isolates.

Minimum inhibitory concentration (MIC) of ethanol extract

The MIC was also determined using broth dilution method. The dilutions of the ethanol extract to be tested were prepared in 5.0 ml volumes of sterile nutrient broth to give a range of concentration from 5,000 to 0.020 mg/ml. After preparation of suspensions of test organisms Ca. 10^{-6} organisms per ml, 0.1 ml was added to the extract/broth dilutions (Atalay et al., 1998). For control experiment, 200 mg tablet of metronidazole (May and Baker, Nigeria) was dissolved in 200 ml of sterile distilled water to give a concentration of 1 mg/ml, The dilutions of metronidazole to be tested were prepared in 5.0 ml volumes of sterile nutrient broth to give a range of concentration from 1 to 0.020 mg/ml, that was used for the MIC test. After 18 h incubation at 35 ± 2°C, the tubes were then examined for growth.

Assay of essential oil by agar-well diffusion method

All overnight cultures of isolates were grown in malt extract broth at 35 ± 2°C for 18 h. The inoculum load was adjusted to 1 x 10^{-6} organisms per ml using serial dilution method prior to use. 1 ml of this concentration of inoculum was added and thoroughly mixed with 19 ml of sterile liquid. Mueller Hilton agar and poured (aseptically) into sterilized Petri-dishes. The agar was allowed to solidify. From each plate 9 mm diameter wells (two wells per Petri

Table 1. Phytochemical screening of rhizome of *C. pilosa.*

Phytochemical constituents	Powdered rhizomes
Alkaloids	+
Anthraquinones	±
Cardenolides	+
Saponins	+
Tannins	+

+ = Present; ± = trace amount present

Table 2. Inhibitory behaviour of ethanol extract of rhizome of *C. pilosa* against *C. albicans* isolates at different concentrations of inoculum.

C. albicans isolate code	Inoculum load (cfu/ml) / zone of inhibition (mm)					
	1.0×10^{-1}	1.0×10^{-2}	1.0×10^{-3}	1.0×10^{-4}	1.0×10^{-5}	1.0×10^{-6}
C1	*20.00 ± 0.00^{a}	17.50 ± 3.53^{a}	19.00 ± 1.41^{a}	16.50 ± 2.12^{a}	17.00 ± 2.82^{a}	19.00 ± 1.41^{a}
C2	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	25.00 ± 0.00^{b}	17.50 ± 2.12^{b}	25.00 ± 0.07^{b}	25.00 ± 0.00^{b}
C3	19.00 ± 1.41^{a}	21.50 ± 4.95^{a}	22.50 ± 2.12^{ab}	27.00 ± 1.41^{abc}	32.50 ± 3.53^{bc}	35.00 ± 0.07^{c}
C4	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
C5	21.00 ± 1.41^{a}	21.00 ± 1.41^{a}	20.00 ± 0.00^{a}	23.50 ± 0.70^{a}	25.00 ± 0.07^{a}	23.50 ± 2.12^{a}
C6	15.00 ± 0.00^{a}	12.50 ± 0.70^{a}	19.00 ± 1.41^{a}	18.50 ± 4.95^{a}	27.50 ± 3.53^{b}	36.00 ± 0.00^{c}
C7	21.00 ± 1.41^{a}	22.00 ± 0.00^{a}	25.50 ± 0.70^{b}	25.00 ± 0.00^{b}	35.50 ± 0.00^{c}	50.00 ± 0.00^{d}
C8	20.00 ± 0.00^{a}	20.00 ± 0.00^{a}	20.00 ± 0.00^{a}	22.50 ± 3.53^{ab}	27.50 ± 3.53^{b}	27.50 ± 3.53^{b}
C9	19.50 ± 0.70^{a}	19.50 ± 0.70^{a}	24.50 ± 0.70^{b}	24.50 ± 0.70^{b}	29.00 ± 1.41^{c}	27.00 ± 1.41^{bc}
C10	19.50 ± 0.70^{a}	19.50 ± 0.70^{a}	22.50 ± 0.70^{a}	20.50 ± 0.70^{a}	35.50 ± 0.70^{b}	52.00 ± 2.82^{c}

Diameter of the cork borer = 9.00 mm.
Values represent Mean ± SD. (n = 3).
Values in the same column followed by the same letter are not significantly different ($p > 0.05$) from each other. They differ significantly ($p \leq 0.05$) with values that do not share a similar letter.
0.00 = Resistant.

dish) were cut from the agar using sterile cork-borer, each of these wells was aseptically filled with 50 µl of undiluted essential oil of plant samples or sterile nutrient broth (control). The plates were incubated at 35 ± 2°C for 18 - 38 h and zones of inhibition were recorded in millimetres (mm) (Jennie et al., 2003).

Statistical analysis

Analysis of variance and comparison of means were carried out on all data using Statistical Analysis System (SAS). Differences between means were assessed for significance at $P \leq 0.05$ by Duncan's multiple range test (DMRT).

RESULTS AND DISCUSSION

The percentage yields of the extracts were 17.83% (ethanol), 22.76% (aqueous) and 0.17% (essential oils). The phytochemicals in *C. pilosa* extracts are shown in Table 1. All isolates were identified as *C. albicans*. The aqueous extract of *C. pilosa* showed no anticandidal activity. Table 2 shows the inhibitory activity of the ethanol extract on *C. albicans* isolates. The extract was active on 9 out of 10 tested isolates. The highest activity was on isolate C10 with an inhibition zone of 52.00 mm at 10^{-6} cfu/ml inoculum load, the least activity was on isolate C6 with a diameter of inhibition of 12.50 mm at an inoculum concentration of 10^{-2} cfu/ml. Thus the ethanol extract of *C. pilosa* was most active on isolate C10 and least active on isolate C6, while it was inactive on isolate C4 at all inoculum concentrations used. The result of the MIC tests is presented in Table 3. The essential oil of *C. pilosa* exhibited inhibitory activity against all screened isolates of *C. albicans* with inhibition zones of 31.00 - 59.00 mm. The oil was most active on isolate C6 and least active on isolates C3 and C8 (Table 4).

The phytochemical analysis of the plant material revealed the presence of alkaloids, traces of anthraquinones, cardenolides, saponins and tannins (Table 1). Many vegetable drugs owe their therapeutic action to phytochemical constituents (Oliver-Bever, 1986). Many well known puragative drugs such as aloes, senna and others contain di-tri or tetra-hydroxymethyl anthraquinones which occur in the plants either free or in the form of glycosides (Oliver, 1960). This finding justifies the use of *C. pilosa* as a purgative.

The extraction of the plant sample with water and

Table 3. Minimum inhibitory concentration (MIC) of ethanol extract of rhizomes of *C. pilosa.*

Test drug	***C. albicans* isolates / minimum inhibition concentration (mg/ml)**									
	C1	**C2**	**C3**	**C4**	**C5**	**C6**	**C7**	**C8**	**C9**	**C10**
C. pilosa	0.020 ± 0.00	0.100 ± 0.00	0.100± 0.00	0.100± 0.00	0.020 ± 0.00	0.100± 0.00	0.020 ± 0.00	0.020 ± 0.00	0.020 ± 0.00	1.500 ± 0.00
Metroni-dazole	0.040 ± 0.00	0.040 ± 0.00	0.040 ± 0.00	0.020 ± 0.00	0.040 ± 0.00	0.040 ± 0.00	0.040 ± 0.00	0.020 ± 0.00	0.020 ± 0.00	0.020 ± 0.00

Values represent Mean ± SD. (n = 3).

Table 4. Inhibitory behaviour of essential oil of rhizome *C. pilosa* on *C. albicans* isolates.

Test oil	***C. albicans* isolates (10^{-6} cfu/ml) / zone of inhibition (mm)**									
	C1	**C2**	**C3**	**C4**	**C5**	**C6**	**C7**	**C8**	**C9**	**C10**
C. pilosa	37.00 ± 1.80	42.50 ± 1.80	31.00 ±1.80	43.50 ± 1.80	43.50 ± 1.80	59.00 ± 1.80	37.50 ± 1.80	31.00 ± 1.80	52.50 ± 1.80	32.50 ± 1.80

Diameter of the cork borer = 9.00 mm.
Values represent Mean ± SD. (n = 3).

ethanol gave different percentage yields of extracts, which did not have any relationship with the anticandidal activity of the plant. Although the yield of aqueous extract was higher, the extract was inactive on *C. albicans*. That the ethanol extract exhibited a relatively high degree of anti-candidal activity while no activity was shown by the aqueous extract is significant. This finding can be correlated with the traditional preparation of herbs in which alcoholic drinks are used to extract the active plant components.

Based on the results of antimicrobial screening, it is evident that the ethanol extract of *C. pilosa* was very active (90%) on *C. albicans* isolates (Table 2). As shown in Table 3 the ethanol extract of *C. pilosa* gave the MIC values (0.02 – 1.5 mg/ml) and metronidazole inhibited all the tested isolates with varied MIC values (0.02 - 0.04 mg/ml). The MIC of ethanol extract of *C. pilosa* on *C. albicans* isolate C1 and C7 was 0.02 mg/ml, a value which was lower than the MIC of metroni-dazole (0.04 mg/ml). Also the essential oil showed varied degree of anticandidal activity. The oil of *C. pilosa* exhibited 100% anticandidal activity against all isolates (Table 4). A great many of essential oil have a slight antibiotic action and are used in the treatment of infections (Oliver, 1960).

Other pharmacological activities of *C. pilosa* have being reported. Palazzino et al. (2000) iso-lated two benzyl benzoate diglucosides, piloside A and piloside B and a glucosyl-fused norlignan, pilosidine, previously obtained as tetra-o-methyl derivative from the rhizome of *C. pilosa*. Pilosidine showed facilitating effect on adrenaline evoked contraction. Also Cometa et al. (2001) reported the reversible hypertensive effect of total extract of *C. pilosa,* its butanolic fraction (0.5 - 100 microg) and the most active compounds structurally similar to adrenaline, pilosidine (10 µg – 1 mg/kg) in anaesthetized rat.

Conclusion

The significant anticandidal activity exhibited by the ethanol extract and essential oil of *C. pilosa* is an indication that active compounds from this plant could be a source of anticandidal agent. Also tincture, ointment, cream and soap could be prepared from the plant for treatment of candidiasis and fungal infections of the skin. The results from this work form a basis for isolation and identification of phytochemical compounds responsible for the observed anticandidal activity.

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Full Length Research Paper

Phytotoxic, insecticidal and leishmanicidal activities of aerial parts of *Polygonatum verticillatum*

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The aim of the present study was to explore the aerial parts of the *Polygonatum verticillatum* for various biological activities such as phytotoxic, insecticidal and leishmanicidal properties. Outstanding phytotoxicity was observed for the crude extract and its subsequent solvent fractions against *Lemna acquinoctialis* Welv at tested doses of 5, 50 and 500 µg/ml. Complete growth inhibition (100%) was demonstrated by the crude extract and aqueous fraction at maximum tested dose (500 µg/ml). Among the tested insects, moderate insecticidal activity was recorded against *Rhyzopertha dominica*. However, neither crude extract nor its solvent fraction registered any significant (> 100 µg/ml) leishmanicidal activity against *Leishmania major*. Based on the phytotoxicity, the aerial parts of the plant could be a significant source of natural herbicidal for sustainable weed control.

Key words: *Polygonatum verticillatum*, phytotoxicity, insecticidal activity, leishmanicidal activity.

INTRODUCTION

Polygonatum verticillatum [L.]. All. (Nooreallam) is a perennial rhizomatous herb belongs to family *Convallariaceae* (Tamura, 1993; Monika et al., 2006). In the traditional system of treatment, *Polygonatum* has been used for thousands of years. The rhizomes of *P. verticillatum* are indicated in the treatment of pain, pyrexia, burning sensation and for phthisis (Amrit, 2006). As a polypharmacy, it has been practiced to promote urine discharge (diuretic) and attenuate painful urination (Ballabh et al., 2008). Some of the other documented uses of the plant are as emollient, aphrodisiac, galactagogue (increases milk release), weakness, appetizer and tonic (Alam, 2004). The antinociceptive activity of the rhizomes of the plant has been recently reported (Khan et al., 2010). Affinity chromatography has led to the purification of lectins from fresh rhizomes of the plant and was estimated 120 mg/kg (Antoniuk, 1993). While considering the diverse folk uses of the plant, the present study was designed to analyze the crude extract of the aerial parts of plant and its subsequent solvent fractions for various biological activities such as phytotoxic, insecticidal and leishmanicidal activities.

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Abbreviations: FBS, Fetal bovine serum; **DMSO,** dimethylsulfoxide; **IC_{50}**, 50% inhibitory concentrations; **LD_{50}**, half maximum lethal dose.

MATERIALS AND METHODS

Plant material

The whole plant, *P. verticillatum* [L.] All. was collected from District Swat N.W.F.P, Pakistan, in July - August, 2007. The botanical identity of the plant material was done by the Taxonomy Department of PCSIR Laboratories Peshawar and a specimen with catalogue No: 9970 (PES) was deposited in the herbarium of PCSIR Laboratories Peshawar.

Plant extraction and fractionation

The aerial parts of the plant (10 kg) were air dried in shade, chopped into small pieces and powdered. The extraction of plant material was carried out by soaking in methanol at ambient temperature for 14 days. The methanolic extract was filtered through filter

Table 1. Phytotoxic activity of the crude extract and subsequent fractions of aerial parts of *Polygonatum verticillatum* against the *Lemna acquinoctialis* Welv.

Name of extracts		No of fronds					
		5 µg/ml		50 µg/ml		500 µg/ml	
	Control	Sample	GI (%)	Sample	GI (%)	Sample	GI (%)
Crude extract	19.33	18	6.88	15	22.4	10	48.26
n-hexane	19.33	18	6.88	10	48.26	5	74.13
Chloroform	19.33	17	12.05	9	53.44	Nil	100
Ethyl acetate	19.33	18	6.88	10	48.26	4	79.3
n-butanol	19.33	15	22.40	9	53.44	Nil	100
Aqueous	19.33	18	6.88	11	43.09	6	68.96

GI = % Growth Inhibition, Standard drug = Paraquat (3.142µg/ml).

paper and the marc obtained was again macerated with methanol. The same process of extraction was repeated three times and the combined filtrates were concentrated under vacuum at low temperature (40°C) using rotary evaporator (Khan et al., 2007). Finally, the crude methanolic extract (2.410 kg, 24.10% w/w) was obtained. The crude extract (1.8 kg) was dissolved in distilled water and sequentially partitioned with various solvents to obtained n-hexane (275 g), chloroform fraction (295 g), ethyl acetate fraction (210 g), n-butanol fraction (317 g) and aqueous fraction (445 g).

In vitro phytotoxicity assay

In vitro phytotoxicity assay was carried out for the crude extract and subsequent solvent fractions against *Lemna acquinoctialis* Welv (Atta-ur-Rahman, 1991; Finney, 1991). The medium was prepared by mixing various inorganic components in 100 ml of distilled water and KOH solution was added for the adjustment of pH at 6.0 - 7.0. The medium was autoclaved at 121°C for 15 min. Test samples (15 mg) dissolved in ethanol (1.5 ml) served as stock solution. Nine flasks (three for each dilution) were inoculated with 1000, 100 and 10 µl of the stock solution for 500, 50 and 5 ppm. The solvent was then evaporated overnight under sterilized conditions. Each flask was supplemented with 20 ml of the medium. Thereafter, 10 plants each containing a rosette of three fronds, were added to each flask. One other flask, supplemented with solvent as control and reference plant growth inhibitor (Paraquat), served as a standard phytotoxic drug. The flasks were plugged with cotton and placed in growth cabinet for 7 days. On the 7th day, the number of fronds per flask was counted. Results were analyzed as growth regulation in percentage, calculated with reference to the negative control.

In vitro insecticidal activity

In vitro insecticidal assay was carried out for the crude extract and its various fractions against *Tribolium castaneum*, *Sitophilus oryzea*, *Rhyzopertha dominica and Callosobruchus analis* following method available in literature (Tabassum et al., 1997). The test sample was prepared (200 mg of crude extract was dissolved in 3 ml of methanol and served as stock solution). The sample (1572.7 µg/ cm^2) was loaded over the filter paper of appropriate size (9 cm or 90 mm) on Petri plate using micropipette. The plate was left overnight (24 h) to evaporate the solvent. Next morning, 10 healthy and active insects of each species of same size and age were added to each plate including control (methanol) and standard drug (Permethrin, 393.17µg/cm^2). Thereafter the plates were incubated in growth chamber at 27°C for 24 h with 50% relative humidity. For calculation, the number of survived insects was counted and the mortality (%) was determined using following formula. Results were the mean of three different experiments.

In vitro leishmanicidal activity

Leishmania major (DESTO) promastigotes were cultured at 22 - 25°C in RPMI-1640 (Sigma). The medium was supplemented with 10% heat-inactivated (56°C for 30 min) fetal bovine serum (FBS). Promastigote culture in the logarithmic phase of growth was centrifuged at 2000 rpm for 10 min, and washed with saline three times in the same condition. Parasites were diluted with fresh culture medium to a final density of 10^6cells/ml. In a 96-well micro titer plate, 180 µl of medium was added in first row and 100 µl of medium was added in others wells. Test extracts (20 µl) was added in medium and serially diluted. 100 µl of parasite culture was added in all wells. One row was used for control (DMSO) which received medium while one for standard drugs (Amphotericin B, Pantamidine). The plate was incubated at 21-22°C for 72 h and the numbers of surviving parasites were counted microscopically in Neubauer chamber. Results are the replicates of three different experiments. The 50% inhibitory concentrations (IC_{50}) were calculated by a Windows based EZ-Fit 5.03 Perrella Scientific Software.

RESULTS AND DISCUSSION

The practice of herbal treatment is well established in Pakistan like most other developing countries of the world. Large number of Hakims and Tabbies are involved in this practice especially in the rural areas of the country. The dynamic features of local system of treatment are their safety, affordability and availability to large population. Traditional health care systems using medicinal plants can be recognized and used as a starting point for the development of novelties in drugs (Khan et al., 2008). Therefore, the current study was designed to explore some of the biological properties of the aerial parts of the *P. verticillatum* in the light of established *in vitro* protocols.

The results of the phytotoxic assay are presented in Table 1. It is evident from the results that the crude form of the aerial parts showed 6.88, 22.40 and 48.26% growth inhibition at 5, 50 and 500 µg/ml, respectively. Upon fractionation, prominent increase in the inhibitory

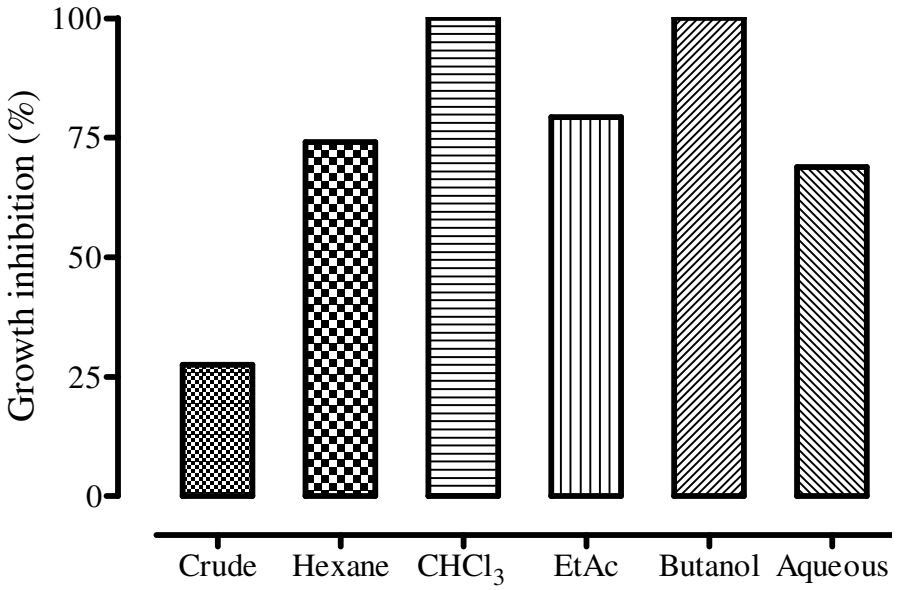

Figure 1. Phytotoxic activity of the crude extract and its subsequent solvent fraction of aerial parts of *Polygonatum verticillatum* at 500 µg/ml.

Table 2. Insecticidal activities of crude extract and various fractions of aerial parts of *Polygonatum verticillatum*.

Name of insect	Mortality (%)							
	Std	NC	Crude	Hexane	Chloroform	Ethyl acetate	Butanol	Aqueous
Rhyzopertha dominica	100	Nil	Nil	50	30	Nil	Nil	Nil
Tribolium castaneium	100	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Callosdruchus analis	100	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Sitophilus oryzae	100	Nil	Nil	Nil	Nil	Nil	Nil	Nil

NC = negative control; SP = Sample (1019.9 µg/cm^2); Std = Standard (Permethrin) = 235.9 µg/cm^2.

activity was found in some of the fractions. Complete growth inhibition (100%) of *L. acquinoctialis* Welv was demonstrated by the chloroform and butanol fractions at 500µg/ml as shown in Figure 1. In addition to that, the phytotoxic potential of hexane and ethyl acetate fractions were also worth mentioning: 74.13 and 79.30% at 500µg/ml, respectively. The aqueous fraction, however, produced only 68.96% activity at 500µg/ml.

Interference of weeds obviously reduces the quality and quantity of agricultural crops and is responsible for huge economic losses all-over the world. It is estimated in US alone that weeds cause a loss of around 12% costing to nearly US$ 33 billion and the situation is more alarming in developing countries (Piment et al., 2001). Synthetic herbicides are extensively used for the control of weeds in agricultural sectors. However, various factors that restricted the use of synthetic herbicides include water and soil pollution, herbicide-resistant weed populations, herbicide residues and detrimental effects on non-target (Li et al., 2003). In recent times, more emphasis has been laid on the natural allelochemicals from plants for weed control in crop production especially to cope with the problem of weed resistance. It has been proved that the phytotoxicity of the plant reduced the growth of weeds without any negative effect on the crops growth and overall yield under normal field conditions, rather significant increase has been recorded in crops production (Batish et al., 2007). It is therefore, assumed on the basis of results that the phytotoxic principle(s) of the aerial parts of the plant could be a significant source of natural herbicides for weeds control in a sustainable manner for better crop production.

Regarding the results of insecticidal activity of crude extract and its various solvent fractions, as depicted in Table 2, moderate activity was exhibited by hexane (50 %) and chloroform fraction (30%) against *R. dominica*. On the other hand, none of the tested samples showed any activity against various insects used in the assay. Similarly, the experimental findings of leishmanicidal assay are posted in Table 3. Neither crude extract nor its various solvent fractions were able to produced any significant (LD_{50}: > 100 µg/ml) activity against *L. major*.

Table 3. *In vitro* antileishmanial activity of the methanol extract and fractions of aerial parts of *Polygonatum verticillatum* against *Leishmania* major.

Test organism	Extracts/Fractions	IC_{50} (µg/ml)
Leishmania major DESTO)	Crude methanol extract	> 100
	n-Hexane	> 100
	Chloroform	> 100
	Ethyl acetate	> 100
	n-Butanol	> 100
	Aqueous	> 100
	Amphotercin-B	0.50 ± 0.02,
	Pentamidine	2.56 ± 0.02

Incubation period was 72 h at 22°C.

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Full Length Research Paper

A survey of indigenous herbal diarrhoeal remedies of O.R. Tambo district, Eastern Cape Province, South Africa

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Indigenous health system and the use of herbal plants have been recognized as pivotal in primary health care and a system to reckon with in achieving one of the targets of the millennial goals on health. An ethnobotanical survey was conducted to identify indigenous herbal remedies for diarrhoea and associated stomach ailments in rural areas of the O.R. Tambo district municipality in the Eastern Cape province of South Africa. The main objective of the study was to gather ethnomedical data on potentially valuable indigenous medicinal plants for the eventual development of new pharmaceuticals and also emphasize the role of ethnomedicine in primary health care. The use of herbal remedies in the treatment of diarrhoea and dysentery was investigated using interviews. The survey was conducted among traditional healers and knowledgeable local elders who use medicinal plants to treat common illnesses. Data from the survey indicated the names of plants commonly used in the treatment of diarrhoea and associated ailments, the methods of preparation, parts used and administration. A total of 32 plant species belonging to 26 families were reportedly used as diarrhoea remedy in the study area. The most predominant families of medicinal plants employed and most frequently recommended were Fabaceae (16.67%), followed by Hyacinthaceae and Hydnoraceae (8.33% each). The most commonly utilized portions of plants for medicinal purposes included roots and leaves. Other parts were corms, bulbs, tubers, fruits and bark. The methods of preparation often employed were decoctions and infusions whilst medication was frequently administered orally or as enema. Some of the plants were used singly or mixed with other plant(s) while some edible ones are consumed as food. The survey documented a diversity of plants employed as remedy for diarrhoea. Integration of this form of health care system into western medicine is warranted. The propagation of such medicinal plants is vital for sustainable use of these medicinal plants.

Key words: Ethnobotany, herbal remedy, indigenous, diarrhoea, primary health.

INTRODUCTION

According to the World Health Organization (WHO,1978), traditional medicine has been described as one of the surest means to achieve total health care coverage of the world's population, yet this form of health care system has long been relegated to a marginal place. Recently, WHO commitment to the Millennium declaration has been reaffirmed by its governing bodies (WHO, 2002a, 2002b) and Ministers of Health of the WHO African Region (2007) have also made a declaration to recognize the role of traditional medicine in primary health care.

In the rural and remote parts of most African countries, hospitals and clinics are often sparsely located far away from dwellers and where there is accessibility to clinics, other factors such as finance and mobility make orthodox medicine far fetched from these people. It has been estimated that up to 80% of the world's rural populations depend on plants for their primary health care, since western pharmaceuticals are often expensive, inaccessible

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Figure 1. Map of O.R. Tambo District Municipality. Source: http://isrdp.dplg.gov.za/documents/IDP/ISRDP/OR_Tambo_IDP.pdf.

or unsuitable (Cunningham, 1993; WHO, 1978). Considering the relative ratios of traditional practitioners and university-trained doctors in relation to the whole population in African countries, traditional healers and remedies made from plants play an important role in the health of millions of people. The people are reliant on traditional healers who usually reside among them trusting their ingenuity on the use of herbs and some other cultural and traditional beliefs. These facts thus provide a role for traditional healers among the rural dwellers' trust.

It is becoming increasingly urgent to document the medicinal use of African plants because of the rapid loss of the natural habitat for some of these plants due to anthropogenic activities. The migration factor especially among the youths to urban areas as well as the demise of most of the local practitioners along with their wealth of knowledge are sources of threat to the future of most of the important cultures including knowledge on the use of plant species (Akerele et al., 1991; Bodeker, 1994; Schlage et al., 1999).

Several studies have been conducted in the Eastern Cape to identify and document biodiversity and ethnomedicinal value of the province (Dold and Cocks, 2001; Kambizi and Afolayan, 2008; Mucina and Rutherford, 2006; Van Wyk and Smith, 2001). Nevertheless, exploration of the plant biodiversity of the province is inexhaustive due to the vastness of the vegetation hence the survey of O.R. Tambo district municipality (ORTDM). This paper presents findings of a survey of the indigenous herbal diarrhoeal remedies in the O.R. Tambo District of the Eastern Cape, South Africa. The main objective of the study was to gather and document ethnomedical data on potentially valuable indigenous medicinal plants for the eventual development of new pharmaceuticals and also emphasize the role of ethnomedicine in primary health care.

A brief description of O.R. Tambo district municipality

O.R. Tambo district municipality (ORTDM) is located in the east of the Eastern Cape Province along the Indian Ocean coastline of South Africa. It is situated in the former Transkei homeland area of the province which falls within the latitudes 30° 00' and 34° 15' South and longitudes 22° 45' and 30° 15' East. ORTDM is bordered by the Alfred Nzo district to the North, Ukhahlamba district to the Northwest, Chris Hani to the West and Amathole district to the Southwest (Figure 1). It is more than 2,700 m above sea level and descends southward from the great interior plateau to form a relatively narrow coastal plain along the Indian Ocean (http://isrdp.dplg.

gov.za/documents/IDP/ISRDP/ OR_Tambo_IDP.pdf).

The population of the district municipality stands at 1.7 million persons and covers an area of 16,617 square km^2. A total of 93% of the district municipality's population reside in rural areas while an estimated 77% of the population is unemployed (STATSSA, 2001). The mother-tongue of the majority of the dwellers is *isiXhosa*, an Nguni language while the rest of the people speak Afrikaans and English.

The region is predominantly rural with large tracts of arable land. Nevertheless, agriculture in ORTDM is inadequately developed and largely subsistence. Traditionally, Eastern Cape is known for rearing livestock which represents 70% of the province's gross agricultural income. (http://www.britannica.com/EBchecked /topic/ 176933/Eastern-Cape).

This study area includes moderate and high rainfall areas. ORTDM has a diversity of vegetation, from grasslands and thicket to forests and bushveld including coastal and marine habitats. The district is considered to have the richest natural resources and the most fertile areas in the country, with good soils and climatic condition (http://isrdp.dplg.gov.za/documents/IDP/ISRDP/OR_Tambo _IDP.pdf). The vegetation of the study region has been previously described (Cawe et al., 1994; Clark et al., 2008; Mucina and Rutherford, 2006; Van Wyk and Smith, 2001).

However, due to the rich flora of this study area coupled with the disappearing traditional knowledge on medicinally useful plants, a lot of grounds still needs to be covered in the documentation of the indigenous knowledge on medicinal plants. Thus, it is critical to preserve the plants and knowledge of their uses.

Diarrhoeal diseases are often associated with low living standards, poor sanitation infrastructure and poor access to potable water sources. The predominantly rural Eastern Cape Province (ECP) is noted for lack of proper sanitation and piped or clean water (ECDOH, 2009). Hence, water-borne diseases are not far-fetched from ORTDM with its predominant rural dwellers depending largely on spring, pond or river water which are often shared with domestic animals. According to a previous study in South Africa (Obi et al., 2007), incidence of diarrhoea could be linked to poor quality of household drinking water. A recent Eastern Cape Department of Health statistics (2009) confirmed a significant number of deaths due to diarrhoea among children, with the highest number of deaths reported in the ORTDM. Of grave health consequences is cholera which recently ravaged Zimbabwe and rapidly spread into South Africa. Kwa-Zulu Natal (KZN) Province of South Africa was designated as endemic for cholera (Henninger and Snell, 2002). ORTDM shares border with KZN and as indicted in Figure 2, it is worth noting that the blue arrow indicating direction of spread of cholera is towards ORTDM. A recent outbreak of cholera in South Africa between November 2008 and April 2009 claimed 65 lives whilst there was >12, 000 case definitions of the disease (Archer et al., 2009). With the high incidence of HIV/AIDS and multidrug resistant TB in South Africa (Cohen, 2006; Jones et al., 2008), diarrhoea can pose a serious challenge to the public health in terms of burden of diseases.

MATERIALS AND METHODS

An ethnobotanical survey on medicinal plants employed in the treatment of diarrhoea and associated ailments was conducted within ORTDM. Areas visited included Port St. Johns, Lusikisiki, Flagstaff, Tabankulu, Bizana, Ugie and Coffee bay from June 2008 to February 2009. The investigation was carried out using interviews among traditional healers and knowledgeable local elders who use medicinal plants to treat common illnesses. Questionnaires were administered through personal contact discussions. This method proved to be a very viable and an effective option of data collection. The choice to employ this particular method was heavily influenced by the literacy levels, remote locations visited and willingness of the respondents that participated in this survey. The traditional healers consulted were trained practitioners of repute within the various communities. The survey was to elicit information on the names of plants commonly used in the treatment of diarrhoea and associated ailments, the methods of preparation, parts used and administration. Interviews were conducted in isiXhosa, the local language of the informants and were later translated to English. In most cases the interviews often started in the form of informal discussions to gain the confidence of the interviewees.

With the assistance of the local practitioners, samples of the plant material used as diarrhoeal remedy were collected from the wild. Scientific identification of samples was aided by staff of the herbarium of Walter Sisulu University, where voucher specimens were deposited. Further characterization of plants and their usage was established by consultation of literatures and monographs (Hutchings et al., 1996; Pooley, 1993, 1998). For data analysis, plant species were grouped into their respective families along with local and common names. An inventory of plant species was compiled from this fieldwork.

RESULTS

Ethnobotanical information obtained from the study area on medicinal plants used in the treatment of diarrhoea revealed 33 medicinal plants scattered in 26 families. Table 1 shows the diverse families of the various indigenous medicinal plants. Among the families, Fabaceae provided the highest proportion of medicinal plants prescribed at 16.67% followed by Hyacinthaceae and Hydnoraceae, 8.33% each.

About 30 visits were conducted involving 15 traditional healers and knowledgeable elders with more than one visit per interviewee in most cases. The rationale behind the use of some of the medicinal plants listed according to the traditional healers was that the information about the plants were revealed to them by their ancestors in their dreams or knowledge about the plants was passed onto them by parents or experienced mentors. Most traditional healers claimed never to use cultivated plants but depended on sourcing from the wild. The usage and preparation as described by the interviewees are shown

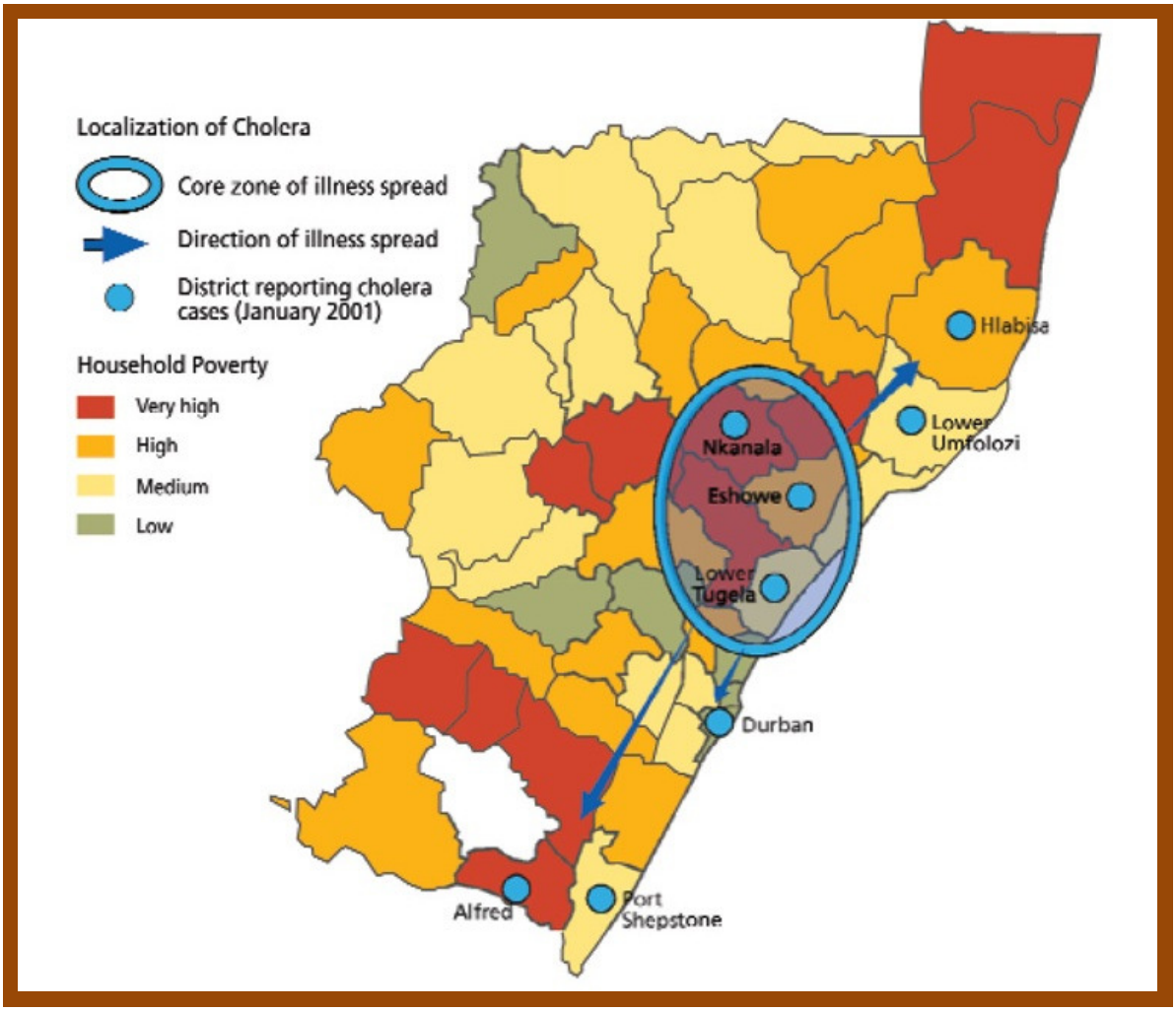

Figure 2. WHO cholera endemic zone. Source: www.who.int/entity/heli/tools/en/gridmap2.jpg; Henninger and Snell (2002).

on Table 1.

The frequently utilized portions of plants for medicinal purposes included roots, corms, leaves and bark but roots were the mostly used preparation of medicine for the treatment of diarrhoea, followed by the use of leaves. The methods of preparation varied considerably from one healer to the other. Plant remedies were often utilized in the form of decoctions and infusions. Extraction may be hot by boiling in water or mere soaking plant parts in cold water. Plant parts may be eaten raw in some cases whilst medication is frequently administered orally or as enema. Some of these plants were used singly or mixed with other plant(s) or even with western mixtures such as salt, vinegar and in a particular instance Amsphogel (Aluminum hydroxide) was mixed with plant part by the traditional healer. The survey also revealed that dosage of plant extracts was not consistent. The analysis revealed the diversity of indigenous plants used as diarhoeal remedy in OR Tambo. These medicinal plants which are of value in the treatment of diarrhoea are also used to treat different other ailments while some edible ones are consumed as food.

DISCUSSION

Traditional medicine is the most widely used medical system in the rural setting of ORTDM. Orthodox medicine is costly and often inaccessible. Not only is ethno-medicine popular and acceptable due to their important role in primary health-care delivery systems, but also, in many areas, it has been the only system available. Likewise, medicinal plants need more attention because it forms an essential component of the total well-being of humans particularly the rural dwellers whose major sources of food, shelter, energy and medicines are forest plants (Akerele, 1988; Hamayun et al., 2003). Furthermore, the emerging global problem of multidrug resistant pathogens (Alekshun and Levy, 2007; Bisi-Johnson et al.,

Table 1. Herbal remedies used for the treatment of diarrhoea and associated ailments.

FAMILY/SCIENTIFIC NAMES COMMON NAME	LOCAL NAMES	PLANT PART	USAGE & PREPARATION	OTHER USAGE
ALLIACEAE *Tulbaghia alliacea* L.f. Wild Garlic	Umwelela (X) ivimba-mpunzi (X) Sikwa (Z)	Bulb	Stomach ailments, bulb infusion taken orally to treat fever.	Remedy for tuberculosis (TB) and influenza, as an antihypertensive or to expel intestinal worms (W & B-B, 1962). As a medicated bath to treat paralysis, rheumatism and reduce fever temperature (W & B-B, 1962).
AMARANTHACEAE *Hermbstaedtia odorata* Wild Cockscomb Rooi-aarbossie	Ubuphuphu (X,Z)	Leaves	Leaves eaten as food and infusion for diarrhoea.	Root cleansing stomach wash alone or with *Acaccia xanthophloea* and *Cappa* (Hutchings et al., 1996).
AMARYLLIDACCEAE *Scadoxus puniceus* (=*Hemanthus magnificus, H. natalensis*) Bloody Lily, Snake Lily	Umphompho –wezinja, Isiphompo, umgola (Z)	Bulb & roots	Stomach ailments, diarrhoea, nausea.	Bulb poisonous, use for poultices (Batten & Bokelmann, 1966). Coughs (Bryant, 1966), headaches, poisoning antidote (Hutchings et al., 1996).
ANACARDIACEAE *Protorhus longifolia* (Bernh. ex C. Krauss) Engl. Red Beech	Izintlwa, ikubalo, umkupati (X)	Bark	Bark dried powdered and guava and Qangazani boiled & taken orally for diarrhoea and bloody stool.	Heart burn and stomach bleeding (Hutchings et al., 1996).
APOCYNACEAE *Acokanthera oppositifolia* (Lam.) Codd= *A. venenata* Common Poison-bush	inHlungunye mbe Intlungunyem be (X, Z)	Leaves	Leaf decoction for stomach ache, diarrhoea, antihelmintic	Treat snakebite (Gerstner, 1939). Spider bite, aches, intestinal worms, cold (Pooley, 1993). Powder from dried roots as snuff for headache (Bhat & Jacobs, 1995).
ASCLEPIADACEAE *Xysmalobium undulatum* (L.) W.T.Aiton Milkwort, Uzura	Ishongwe (X,Z), Itsongwe	Roots	Boil root for diarrhoea, stomach ailments or pain.	Dysentery, Headaches (Pujol, 1990). Flower and seed decoction for colic, poison antidote (W & B-B, 1962)
ASPHODELACEAE *Aloe candelabrum* Berger. Candelabrum Aloe *Bulbine abyssinica,* Bushy Bulbine,	Ikhalana Inkalane (X) Uphondonde (Z) Utswelana Intelezi (X) Ibhucu (Z), Incelwane (X);	Leaves Leaves, tubers	Leaf decoction for diarrhoea. Boil leaves for vomiting, diarrhoea, TB.	 Treat bilharzias, dysentery, cracked lips, skin ailments, urinary complaints, rheumatism, as a charm (Pooley, 1998). Tuber decoctions-antispasmodic to quell vomiting (Hutchings et al., 1996).
ASTERACEAE *Bidens bipinnata* Spanish-black jack, Spanish needle	Uvelemampo ndweni uvelegoli	Leaves	Leaves edible, infusion for diarrhoea.	Rheumatism (W & B-B, 1962). Hemorrhage, reduce cancer, flu, cold, fever (Pooley, 1998).
CORNACEAE *Curtisia dentata* (Burm.f.) C.A.Sm.= *C. faginea* Assegaai	Umlahleni (X,Z), Unsirayi (X), Umgxina, Umlahleni (Sefile), Uzintlwa	Bark, Root	Diarrhoea, stomach ailments.	Stomach ailments including diarrhoea, blood strengthener, aphrodisiac (Pajol, 1990).

Table 1. Contd.

EUPHORBIACEAE *Euphorbia cooperi* N.E.Br. ex. Berger Euphorbia or Milkweed, Spurge, Transvaal Candelabra Tree	Umhlonhlo (X)	Bark of root	Bark of root ground dry boiled, then mix with a sachet epsom salt, cool & add 2 spoons vinegar for diarrhoea, stomach disorders. For infants, mix equal portion of decoction with Amsphojel to flavour milk.	
FABACEAE subfamily **MIMOSACEAE** *Acacia mearnsii* De Wild Blackwood,Black Wattle	Ublekwana (X) Udywabasi (X, Z) Indywabasi	Bark	Bark infusion taken orally for diarrhoea, dysentery	Sore throat, coughs, children fever, tooth ache (Hutchings et al., 1996). Syphilis, stop bleeding (Jacot Guillardmod, 1971), chest complaints, heart conditions (W & B-B, 1962). Fever, ulcers, dysentery, diarrhoea, dysmenorrhoea (Bryant, 1966).
FABACEAE **(LEGUMINOSAE**) *Elephantorrhiza elephantina* (Burch.) Skeels Elephant's Root	Intolwane (X,Z)	Root, stem	Boil equal part plant and *Acokanthera oblongifolia* for diarrhoea, stomach ailment. Infusion of ground stem alone for diarrhoea and menstrual disorder.	
GERANIACEAE *Pelargonium sidoides* Rose-scented Pelargonium	Umsongelo (X)	Leaves, roots	Infusion of leaves or root enema for diarrhoea, dysentery and vomiting.	Bruised leaves soothes skin rashes, in tea to treat kidney & bladder ailments, nausea, gonorrhoea, root decoction severe diarrhoea in Transkei (Hutchings et al., 1996). Leaf paste for wound, powdered root mixed with food for dysentery (W & B-B, 1962).
P. luridum Wild geranum	Umsongelo, ishwaqa	Leaves	Eaten raw as vegetable, treat dysentery, nausea, vomiting, fever	
HYACINTHACEAE *Eucomis autumnalis* (Mill.) Chitt. Common Pineapple Flower	Ubuhlungu becanti Isithithibala (X) Umathunga (Z)	Bulb	Boil bulb for abdominal problems.	Decoctions of bulb and roots for coli, flatulence (Cunningham, 1993). Syphilis (W & B-B, 1962). Treat urinary & pulmonary ailments, fever (Hutchings et al., 1996; Roberts, 1990).
Scilla nervosa (Burch.) Jessop White Scilla	Umagaqana, magaqana (X), Imbizankulu ingema (Z)	Bulb Root	All purpose herb. For diarrhoea, TB, various diseases, cleans blood.	Purgative, sprain, fracture, cancers (W & B-B, 1962). Rheumatic fever, dysentery (Rood, 1994; Silayo et al. 1999).
Scilla sp.	Umasixabane (X)		Infusion of ground tuber and *H. africana* and *Curtisia dentate* for diarrhoea	
HYDNORACEAE *Hydnora africana* Warty Jackal Food, Jakkalskos Kanip	Ubuklunga (X) Umavumbuka(Z), Umafumbuka (X)	Fruits, tuber, leaves	Boil handful of ground dried tubers and blackwood and peach for diarrhea	Parasitic on *Euphorbia* roots. Fruit pulp like potato eaten by people birds jackal, plant dried ground raw for dysentery, amenorrhoea. Swollen glands or inflamed throat (W & B-B, 1962).

Table 1. Contd.

HYPOXIDACEAE *Hypoxis latifolia* Hook. African potato (Eng.) Small Yellow star-flower *H. hemerocallidea = H. rooperi* Star-flower	Inongwe Ilabateka (X) Inongwe Ilabateka (X)	Tuber	Boil ground dried tuber for diarrhoea.	Treat benign prostrate (van Staden, 1981). Headaches, dizziness, mental disorders, inflammation, HIV (Singh, 1999; van Wyk, 2000).
IRIDACEAE *Gladiolus sericeo-villosus* Hook.f. *forma sericeo-villosus* Natal Lily, parrot's beak gladiolus	Umnunge (X)	Corm	Corm's decoction for cold and dysentery, TB (use with care)	Corms for dysentery oral and enema ((Hutchings et al., 1996). Impotence (W & B-B, 1962). Menstrual pain (Bryant, 1966).
MESEMBRYANTHEMACEAE *Carprobotus edulis* Hottentot's fig, ghaukum, rankvy	Ikhambi-lamabulawo, umgongozi, Igcuthuma Unomatyumtyum, igcukuma	Leaves	Various diseases, diarrhoea	Allergies, diabetes, sore throats (Hutchings et al., 1996). Juice from pounded leaves as gargle for sore throats, thrush, diphtheria, treat digestive troubles, diarrhea & dysentery (W & B-B, 1962).
MYRTACEAE *Psidium guajava* L. Guava	Ugwava (X,Z)	Leaves	Leaves boiled for alone or mixed with other plants for diarrhea.	Infusion of leaves for bloody diarrhea (Hutchings et al., 1996). Roots for veneral disease by Vhavenda (Mabogo, 1990 The Ethnobotany of the Vhavenda. Unpublished Master of Science Thesis, University of Pretoria).
OLEACEAE *Olea europaea* subsp. *Africana* Wild Olive	Uzintlwa (X)	Fruit	Infusion for diarrhoea and bloody Stool	Leaves for urinary and bladder infections (Roberts, 1983; Pooley, 1993). Immature fruits as astringents against diarrhea (Iwu, 1993).
POLYGONACEAE *Rumex obtusifolius* Dock	Idololenkonyane (X,Z)	Leaves	Leaf extract for diarrhea	Infertility in women (W & B-B, 1962). Leaf decoction for worms (W & B-B, 1962). Scabies, powdered root as gargle for laryngitis
ROSACEAE *Prunus africana* (Hook.f.) Kalkman Red Stinkwood, Bitter Almond, Peach	Umkhakhazi (X), Umkakase (X)	Roots	Root of peach and bark of blackwood and leaves of guava and roots of umswaninge for diarrhoea, abdominal ailments.	The bark extracts have become popular in Europe for the treatment of benign prostate hypertrophy (Van Wyk *et al.* 1997)
RUBIACEAE *Pentanisia prunelloides* (Klotzsch ex Eckl. & Zeyh) Walp Broad-leaved Pentanisia	Icishamlilo, Icimamilo (X,Z)	Roots, leaves, bulb	Boil grated dried bulb, a spoon taken orally to stop vomiting, diarrhoea in children. For adult, a wine shot 3 times daily. Expose face to steam from boiling herb fro pimples. Rub leaves to soothe swollen body.	A range of ailments, root as enema for stomach pain (Hutchings et al., 1996). Hemorrhoids, snakebite, rheumatism (Bryant, 1966; Gerstner, 1941).
RUBICEAE Pavetta – Bride's Bush *Psychotria capensis* (Eckl.) Vatke Black Bird-berry	Isithitibala (Z), UmGono-gono (X)	Fruits	For diarrhoea and vomiting.	Unspecified part for tuberculosis (Batten & Bokelmann, 1966). Leave paste for wound. For gastric complaints and root infusions are taken to cause vomiting (Hutchings et al., 1996).

Table 1. Contd.

SAPINDACEAE Atalaya – Krantz Ashes *Hippobromus pauciflorus* (L.f.) Radilk. False Horsewood	Ulwathile (X)	Bark, root, leaves	Diarrhoea, dysentery	Coughs, catarrh related headaches (Bryant, 1966). Eye problems (W & B-B, 1962).
SCOPHULARIACEAE *Physalis peruviana* Cape gooseberry	Igquzu (X)	Leaves	Leaf edible, stomach disorders.	Leaf infusion as enema to relieve abdominal ailment in children (W & B-B, 1962). Treat high blood pressure, diabetes (W & B-B, 1962).
SOLANACEAE *Solanum aculeastrum* Dun Apple of Sodom, poison apple, Goat apple	Umthuma (X,Z)	Fruits, roots, leaves.	Fruit decoction orally for haemorrhoids & dysentery, fruit as enema for diarrhoea.	Fruit pulp as enema. Rheumatism, ringworm in cattle (Pooley, 1993). Root & leaves for coughs, fever, sore throats, colic, indigestion, abdominal pain, venereal diseases (W & B-B, 1962; Kokwaro, 1976).
VERBENACEAE *Clerodendrum glabrum* E.mey = *C. rehmannii* Cat's Whiskers or Verbena Tree or Tinderwood	Umqangazani Uqangazana (X), iNunkisiqaqa(X) Umqangazane	Leaves	Bloody stool, chest infections.	Snakebite (Roberts, 1990). Leaf infusion for intestinal parasites (W & B-B, 1962). Leaf and root of *Cymbopogon marginata* (Steud.) for roundworms, threadworms, cough, fevers (Hutchings et al., 1996).

KEY: X = Xhosa; Z = Zulu; W & B-B = Watt and Breyer-Brandwijk (1962).

2005; Levy, 2005; Obi et al., 2007) and the need for the discovery of lasting and sustainable therapy to combat diseases such as HIV/AIDs, malaria and cancer which have defied available treatments has led to a paradigm shift to natural herbal product for succor.

This study documented a diverse list of plants used as remedy for diarrhoea in ORTDM. Previous reports have also linked some of the plants encountered in the course of this survey with remedy of diarrhoea, dysentery or stomach ailments (Bigalke, 1967; Hutchings, 1989; Van Wyk and Gericke, 2000; Van Wyk and Wink, 2004; Watt and Breyer-Brandwijk, 1962). The parts of the plants commonly used were roots and leaves. The problem of inconsistent dosaging is a critical set back which is crucial in the standardization of medicinal plants. Another factor which may impact on the traditional therapy standardization is the work place hygiene and the quality of water used in preparation particularly with cold extraction. Most of the traditional healers boast of treating some ailments that have defied modern medical practice. While some of the healers embrace the idea of both western medicine and traditional medicine complementting each other, a few others do not. Some of the traditional healers tend to hide the information on plants used for different ailments largely for fear of losing patronage to the investigators or interested persons. By way of mystifying the native trade, the vast majority of plants are collected from the wild and cultivation of the plants is often not encouraged by the traditional healers. Some participants believed that cultivated plants would have been attacked by evil spirit and hence will not be potent for use. This is similar to the findings of Keirungi and Fabricius (2005). Scientific evaluations of the therapeutic claims as well as toxicological data are still underprovided for many of the plant species. This study forms the basis for microbiological and phytochemical research on selected diarrhoeal medicinal plants and work is in progress.

In the course of this study, some unsustainable methods of harvesting of medicinal plants were observed. For instance, root excavation and bark striping of plants which pose a threat to the continued existence of plants were used to harvest plants such as *Pelargonium sidoides* and *Acacia* spp. These two methods have been reported as most harmful harvesting methods for plants (Akerele et al., 1991; Cunningham et al., 2002). The tendency for extinction of scavenged species is obviously going by the unsustainable handling and the habit of not cultivating medicinally valuable species. For conservation to be effected, planting of designated valuable herbal plants in small gardens in the homesteads is strongly recommended. Also, large scale farming of commercially viable plants should be encouraged whilst scientists are implored to undertake studies on various factors affecting growth of plants such as soil conditions, temperature and seasonal variations and disseminate best propagation methods. Conclusively, it is pertinent for scientists to urgently salvage this cheap and alternative health care system from extinction, help preserve indigenous knowledge and conserve nature.

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Full Length Research Paper

Thalassemia mutations in Gaziantep, Turkey

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Ninety-eight postnatal and six prenatal cases of thalassemia were studied by the reverse dot-blot hybridization technique in the city of Gaziantep, Turkey. We found the following mutations: IVS 1.110 (G>A) in 29.1%, IVS 2.1 (G>A) in 12.3%, IVS 1.1 (G>A) in 7.7%, Codon 8 (–AA) in 5.6%, -30 (T>A) in 4.6%, IVS 1.6 (T>C) in 4.6%, Codon 39 (C>T) in 3.6%, Codon 44 (-C) in 3.1%, IVS 2.745 (C>G) in 1.5%, Codon 8/9 (+G) in 2.1%, Codon 36/37 (-T) in 2.1%, IVS 1.5 (G>C) in 2.1%, Codon 22 (7pb del) in 0.5%, Codon 5 (-CT) in 0.5% while 20.9% were undetermined. 54 of the thalassemia patients were homozygotes, 12 were compound heterozygous and 31 were heterozygotes. In one allele of 5 thalassemia patients, α-thalassemia mutation (3.7 single gene deletions in 1 patient, anti-3.7 gene triplication in 4 patients) was determined at the same time. Finally, this is the first comprehensive study in this region and percentage of α and β- globin genes mutation is 2.6 and 79.4%, respectively.

Key words: α- thalassemia, β -thalassemia, DNA, mutation, polymerase chain reaction.

INTRODUCTION

β–Thalassemia (OMIM 141900) is one of the most common single gene defects in the world and it results from decrease in lack of β-globin chain production. It is common in the Mediterranean, Middle East, Africa, India, South Asia and South China (Kuliev, 1988; Birgens and Ljung, 2007). In β^0-thalassemia, there is no production of the β-globin chain and is an important cause of morbidity and mortality being associated with severe hypochromic hemolytic anemia, the requirement of frequent blood transfusions and lifetime iron-chelating treatment; in β^+-thalassemia, the β-globin chain is produced to a lesser extent than normal (Kuliev, 1988; Borgna-Pignatti and Galanello, 2003).

α-Thalassemia (OMIM 141800) is the most common inherited disorder of hemoglobin (Hb) synthesis in the world, with gene frequencies varying between 1 and 98% throughout the tropics and subtropics. It can occur in all ethnic groups but is more common in those of Southeast Asian descent. The American College of Obstetricians and Gynecologists recommends hemoglobinopathy screening for those of African, Southeast Asian and Mediterranean descent. More than 95% cases of recognized α-thalassemia involve deletion of one or both α-globin genes from chromosome 16p13.3 (www.labcorp.com/datasets 6300.htm - 05.08.2009).

The number of affected births is higher than expected in Turkey because of a large and young families that have many children and the number of consanguineous marriages especially first cousins are above 60% in the Eastern and Southern parts of Turkey (Basak, 2008). Genetic counseling and prenatal diagnosis programs play an important role in overcoming the problem.

We have aimed the frequency β and α-thalassemia mutations among 98 patients and 6 prenatal cases.

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MATERIALS AND METHODS

We looked for α and β-thalassemia mutations in 196 chromosomes of 98 thalassemia patients (52 male, 46 female) between the ages

Table 1. Frequency of β-thalassemia mutations (%) in Turkey.

Mutation	Gaziantep	Cukurova	Diyarbakir	Aegean	Denizli	Turkey
IVS 1.110 (G>A)	29.1	57.3	27.8	44.1	36.4	39.2
IVS 2.1 (G>A)	12.3	3.4	8.3	2.7	3.6	5.4
IVS 1.1 (G>A)	7.7	8.3	2.8	28.2	16.4	5.5
Codon 8 (–AA)	5.6	4.7	11.1	-	3.6	6.1
-30 (T>A)	4.6	5.7	2.8	-	-	3.1
IVS 1.6 (T>C)	4.6	5.7	11.1	13.3	7.3	9.5
Codon 39 (C>T)	3.6	6.4	2.8	2.4	9.1	3.8
Codon 44 (-C)	3.1	0.7	NS	-	-	1.3
IVS 2.745 (C>G)	1.5	2.8	NS	9.3	7.3	4.6
Codon 8/9 (+G)	2.1	0.4	3	-	1.8	1.5
Codon 36/37 (-T)	2.1	0.7	1.2	-	-	<1
IVS 1.5 (G>C)	2.1	0.4	NS	-	1.8	1.1
Codon 22 (7bp del)	0.5	-	-	-	-	-
Codon 5 (-CT)	0.5	-	-	-	-	-
Undetermined	20.9	?	?	?	?	?
Reference		11	12	14	13	5

-: not studied.

Table 2. Thalassemia patients who carry both α and β-globin gene mutations.

Patient Number	Gender	Age	β-globin gene mutation	α-globin gene mutation
1	Female	1	heterozygote - IVS 2.1 (G>A)	anti-3.7 gene triplication
2	Female	27	Heterozygote - IVS 1.1 (G>A)	anti-3.7 gene triplication
3	Male	34	Homozygote - Codon 8 (–AA)	anti-3.7 gene triplication
4	Female	39	Homozygote - IVS 1.6 (T>C)	anti-3.7 gene triplication
5	Male	26	Homozygote - IVS 1.110 (G>A)	anti-3.7 gene deletion

of 1 and 52 years (mean: 15.1 ± 10.9 years) and 12 chromosomes of 6 fetuses whose parents' mutations have been known to have the β-thalassemia trait. Hematological parameters were obtained with an automated cell counter. Consent from patients and family members were obtained before collection of blood and amniotic fluid samples. Genomic DNA was extracted from peripheral blood leukocyte using the salting out method techniques (Miller et al., 1988). Fetus DNA was extracted from 3 ml amniotic fluid by using NucleoSpin Isolation Kit. Detection of known β-globin gene mutations was done using a commercialized reverse dot blot platform (Strip A ViennaLab. Diagnostics, Vienna, Austria) which included the following: IVS 1.110 (G>A), IVS 1.116 (T>G), IVS 2.1 (G>A), IVS 1.2 (T>A), IVS 1.1 (G>A), -30 (T>A), IVS 1.6 (T>C), Codon 39, Codon 44, Codon 22 , Codon 8/9, IVS 1.5 (G>C) , Codon 36/37, Codon 30, IVS 2.745 (C>G), IVS 1-25 (25bp del), -87 (C>G), Codon 5, Hemoglobin C (HbC), HbS, Codon 6 and Codon 8 (Samara et al., 2007). Detection of known α-globin gene mutations was done using a commercialized reverse dot blot platform (Strip B VienneLab. Diagnostics, Vienna, Austria) which included the following mutations: 3.7 single gene deletion (SGD), 4.2-SGD, MED double gene deletion (DGD), SEA-DGD, THAI-DGD, FIL-DGD, 20.5 kb -DGD, anti-3.7 gene triplication, α1 cd 14 (TGG>TAG), α1 cd 59 (GGC>GAC) (Hb Adana), α2 init cd (ATG>ACG), α2 cd 19 (–G), α2 IVS1 (-5 nt), α2 cd 59 (GGC>GAC), α2 cd 125 (CTG>CCG) (Hb Quong Sze), α2 cd 142 (TAA>CAA) (Hb Constant Spring), α2 cd 142 (TAA>AAA) (Hb Icaria), α2 cd 142 (TAA>TAT) (Hb Pakse), α2 cd 142 (TAA>TCA) (Hb Koya Dora), α2 poly A-1 (AATAAA-AATAAG) and α2 poly A-2 (AATAAA-AATGAA) (Hadavi et al., 2007). Maternal contamination was eliminated using ChromoQuant version 2 kit (CyberGene AB, Sweden).

RESULTS AND DISCUSSION

The frequency of β-thalassemia mutations is shown in Table 1. The ratio of β^+ to β^o mutations was 0.58 (36/62). We detected 14 different mutations, of which six were present in 63.9% of the chromosomes. The IVS 1.110 mutation, the most frequent mutation, was present in 29.1% of all chromosomes. In Table 2, five thalassemia patients who carry both α and β-globin gene mutation are summarized. Also we identified the ratio of the parents' consanguinity is very high (49%).

In Turkey, however a broad range of mutations had been observed, probably because of Turkey's location at the three continents and of the influence of different cultures over the course of history. Although the mutations that we identified in Gaziantep, were similar to those seen elsewhere in our country, we detected some

Table 3. Frequency of β-thalassemia mutations in the Eastern Mediterranean.

Mutation	This study	Turkey	Cyprus	Greece	Syria	Palestine	Bulgaria	Azerbaijan	Iran	Iraq
IVS 1.110 (G>A)	29.1	39.3	79.7	42.1	24.1	17.6	24.2	20.2	4.8	1.9
IVS 2.1 (G>A)	12.3	4.7	-	3.3	4.2	2.9	-	-	33.9	18.3
IVS 1.1 (G>A)	7.7	5.0	5.9	12.8	17.0	9	3.1	2.0	2.9	8.7
Codon 8 (–AA)	5.6	5.5	0.2	0.8	0.7	-	5.5	21.2	4.5	2.9
-30 (T>A)	4.6	3.1	-	-	-	2.1	-	-	-	-
IVS 1.6 (T>C)	4.6	10.1	6.2	8.1	4.2	28.7	10.2	7.1	1.1	8.7
Codon 39 (C>T)	3.6	3.8	2.4	18.8	6.4	4.6	21.9	2.0	1.7	8.7
Codon 44 (-C)	3.1	1.3		-	0.0	-	-	-	2.6	12.5
IVS 2.745 (C>G)	1.5	5.0	5.5	6.3	-	0.3	6.9	3.1	-	-
Codon 8/9 (+G)	2.1	1.3	-	-	1.4	1.4	0.3	2.0	4.8	7.7
Codon 36/37 (-T)	2.1	0.1	-	-	-	-	-	-	-	-
IVS 1.5 (G>C)	2.1	1.1	-	-	0.0	1.1	-	-	7.6	6.7
Codon 22 (7bp del)	0.5	0.1	-	-	0.0	-	-	-	3.0	1.0
Codon 5 (-CT)	0.5	-	-	-	-	-	-	-		
Reference		15	13	17	22	18	19	20	21	22

different frequencies between the regions (Table 1) (Cavdar and Arcasoy, 1971; Gurbak et al., 2006; Curuk et al., 2001; Ince et al., 2003; Yildiz et al., 2005; Golesken et al., 2000). The IVS 2.1 (G>A) mutation is the second most frequent type (12.3%) in our study. This mutation occurs in 3.4% of the population in the Cukurova region and 8.3% in Diyarbakir (Gurbak et al., 2006; Ince et al., 2003). In Turkey as a whole, Başak et al. found this mutation frequency to be 5.4% (Basak, 2008). This mutation was the most frequent one in Iran (33.9%) and Kuwait (29.2%); however, it is difficult to explain why this mutation is frequent in Diyarbakir, Cukurova and Gaziantep as compared to Turkey in general (Table 3) (Baysal et al., 1992; Boussiou et al., 2008; Kyriacou et al., 2000; Darwish et al., 2005; Petkov and Efremov, 2007; Curuk et al., 1992; Peykar et al., 2007; Al-Allawi et al., 2006).

IVS 1.1 (G>A) is the third most frequent mutation in our study at 7.7%. It was the second most frequent mutation in Cukurova (8.3%) the Aegean (28.2%) and Denizli (16.4%) (Curuk et al., 2001; Golesken et al., 2000; Yildiz et al., 2005). It was the fourth most frequent (5.5%) mutation in Diyarbakir and in Turkey as a whole in accordance with the findings of Başak et al. (2008) and Ince et al. (2003). This mutation was seen mainly in the β-thalassemia zone, from Hungary, Yugoslavia, Greece and Cyprus to Czechoslovakia (Boussiou et al., 2008; Ringelhann et al., 1993; Dimovski et al., 1990; Efremov, 2007; Indrak et al., 1992). In Turkey, it was common in Turkish people originating from Marmara, Aegean and Balkans (Basak, 2008).

Codon 8 (-AA) was the fourth most frequent mutation in our study (5.6%) with a ratio that was lower than the studies reported from Diyarbakir. In Turkey as a whole, Başak et al. reported this mutation as the third most frequent with a ratio of 6.1 (Basak, 2008). This mutation is most frequent in East Anatolia and the Marmara region of Turkey (Basak, 2008). Among the neighboring countries, it was most frequent in Azerbaijan (21.2%) (Curuk et al., 1992). IVS 1.6 (T>C) and -30 (T>A) were the fifth most frequent mutation (4.6%) in Gaziantep. Başak et al. reported that IVS 1.6 (T>C) mutation was the second most frequent one in Turkey as a frequency of 9.5% (Basak, 2008).

The frequency of Codon 39 (C>T) were found in Gaziantep, Cukurova and Diyarbakir as 3.6, 6.4 and 2.8%, respectively (Gurbak et al., 2006; Curuk et al., 2001). It was the most frequent mutation in Italy 49%, Spain 64%, Portugal 33.5%, Bulgaria 21.9% and Tunisia 49% (Petkov and Efremov, 2007; Ferrara et al., 2001; Amselem et al., 1988; Faustino et al., 1999; Fattoum et al., 2004). Başak et al. found the average frequency in Turkey as a whole to be 3.8% (Basak, 2008). This mutation is usually the most common one that was obseved in the West Mediterranean. In contrast, it is rarely seen in Cyprus, Lebanon, Iran, Palestine and Azerbaijan (Bozkurt et al., 1992; Darwish et al., 2005; Curuk et al., 1992; Peykar et al., 2007; Chehab et al., 1987). It has been suggested that the distribution pattern of this allele correlates with the early migrations of Phoenicians and Carthaginians, who spread the β-thalassemia allele(s) in the Mediterranean Basin (Birgens and Ljung, 2007; Cavalli-Sforza et al., 1996).

This is the first study to determine the frequencies of α and β- thalassemia mutations in the city of Gaziantep. When a patient is born with β-thalassemia, there is no effective treatment option. Therefore, the best method for dealing with β-thalassemia disease is prenatal diagnosis. In the current study, six β-thalassemia families who carry the β-globin gene mutation were given genetic consultation for prenatal diagnosis based on DNA analysis. In

conclusion, after we have identified the most frequent mutations in β-thalassemia patients living in the city of Gaziantep, the frequency of prenatal diagnosis will promptly increase. Therefore, this will provide important benefits to population health and the national economy as well.

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Full Length Research Paper

Validated high performance liquid chromatographic (HPLC) method for analysis of zerumbone in plasma

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Zerumbone (ZER) is a sesquiterpene derived from Zingiber zerumbet smith, family Zingiberaceae. It has been shown to possess anti-cancer and apoptosis-inducing properties against various human tumour cells as well as *in vivo* against a number of induced malignancies in mice. In this study a simple, specific and accurate high performance liquid chromatographic method for determination of ZER in micro-volumes human plasma (| 1.5 ml) was developed and validated. ZER and its analogue α-Humulene as internal standard were easy recovered by simple one step plasma protein precipitation using acetonitrile and separated in isocratic mobile phase, on reverse phase-C_{18} column. The effluent was monitored by Photodiode Array (PDA) detector and at a flow rate of 1.0 ml/min. The linearity of proposed method was 2 – 15 µg/ml. The intra-day and inter-day coefficient of variation and percent error values of the method were less than 15% and mean recovery was more than 90% for both ZER and α-Humulene. This method was found to be precise, specific, accurate and robust for detection and analysis of ZER in human plasma.

Key words: ZER, humulene, HPLC, human plasma.

INTRODUCTION

Zingiber zerumbet (L.) smith, known as lempoyang, is a member of the family Zingiberaceae, used in the traditional medicine as a cure for swelling, sores, loss of appetite and worm infestation in children (Somchit and Nur-Shakirah, 2003). In some Southeast Asian countries, the rhizomes of the plant are employed in traditional medicine as anti-inflammation, while the young shoots and inflorescence are used as condiments (Murakami et al., 2002). This plant has been shown to have anti-tumor (Sakinah et al., 2007), anti-inflammatory (Murakami et al., 2002) and cyclooxygenase-2 suppressant properties (Tanaka et al., 2001). Zerumbone (ZER) is a bioactive crystalline monocyclic sesquiterpene derived from zingiber zerumbet rhizomes. It exhibits variety of interesting reactions, such as region-and regeoselective conjugates additions, transannular ring contraction, cyclization and several regeospecific reactions which cleave the 11-membered ring (Kitayama et al., 1999, 2001; Ohe et al., 2000). This bioactive component has its unique structure, with cross-conjugated ketone in an 11-membered ring, as well as remarkable biological activities. It has been reported that ZER constitutes about 37% of *Z. zerumbet* (Matthes et al., 1980; Sakinah et al., 2007). Moreover, this compound showed a potential candidate for the development of anti-cancer treatment of natural origin.

ZER has been previously identified as distinct suppressor of tumor promoter 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-induced Epstein-Barr virus

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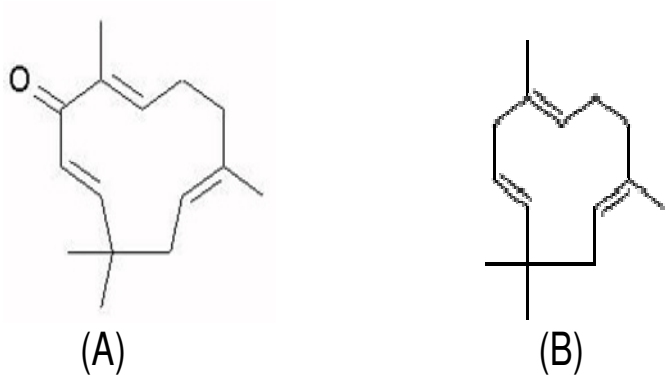

Figure 1. Chemical structure of Zerumbone (A) and α-Humulene (B).

(EBV) activation in Raji cells (Murakami et al., 1999). Murakami et al. (2004) reported that ZER inhibits the proliferation of colon and skin cancer cells through apoptosis, while having less effect on normal cells proliferation (Murakami et al., 2002; Murakami et al., 2004). Recently, ZER was reported to suppress the development of cervical intraepithelial neoplasia in mouse (Abdul et al., 2008). ZER has gained a great attention due to its activity towards many diseases *in vitro* and *in vivo*. Very recently, Sung et al. (2009) reported ZER as modulator for osteoclastogenesis induced by Receptor Activator for Nuclear Factor κ B Ligand (RANKL) and breast cancer (Sung et al., 2009). In addition ZER was reported to effectively suppress mouse colon and lung carcinogenesis through multiple modulatory mechanisms (Kim et al., 2009).

However, to the best of our knowledge, no chromatographic method has been described in literature for ZER determination in human plasma as well as other biological fluids. Here, we present for the first time an easy, HPLC method for determination of ZER in human plasma which requires minor laboratory efforts. This method is one step plasma protein precipitation followed by injection into HPLC with Photodiode Array (PDA) detector which is available in most analytical laboratories. The method will reduce the experimental work to almost 50%.

MATERIALS AND METHODS

Chemicals

ZER was extracted from *Zingiber zerumbet* rhizomes obtained from the wet market in Kuala Lumpur, Malaysia. The purity of the extracted ZER was > 98%. Structurally similar, α-Humulene (HUM) (Figure 1 (A) and (B)) was used as internal standard (IS) and was purchased from Sigma Chemical Co. (Street Louis, MO, USA). HPLC grade acetonitrile, methanol and potassium dihydrogen orthophosphate monobasic were purchased from Fisher Scientific (USA). Ultra pure water was used throughout the experiment.

Standard preparation and quality control

Stock and working solutions of ZER and HUM (100 µg/ml) was prepared by dissolving in acetonitrile. Standard solutions of ZER in human plasma were prepared by spiking the diluted stock solution, to give the final concentrations of 2, 4, 6, 8, 10 and 15 µg/ml. The internal standard HUM solution 50 µg/ml was also prepared.

HPLC apparatus and chromatographic conditions

HPLC system consist of Alliance separation module model e2695 with PDA detector, the signals were processed by Empower™ software (Waters, Milford, MA, USA). The mobile phase composed of acetonitrile: methanol: 0.01 M potassium dihydrogen orthophosphate (25: 55: 20). The analytical column used was C_{18} (Symmetry, 250 X 4.6 mm ID, 5 µm particles size) at an ambient temperature. The elute was monitored by PDA detector at a flow rate of 1 ml /min in wavelength of 254 nm.

Sample preparation

Ten microliters of internal standard solution 50 µg/ml were added to 150 µl plasma sample in microcentrifuge tube. The tube was vortex for 1 min and 200 µl of acetonitrile was added and vortexed for 2 - 4 min to precipitate the plasma protein, then the sample was centrifuged at 10000 rpm for 15 min. The supernatant was withdrawn in 300 µl insert vials and 10 µl was injected into HPLC system.

Validation procedure

The validation parameters obtained were specificity, linearity, accuracy, precision, stability and robustness. The method was validated according to FDA guidelines.

Specificity

Specificity was obtained by comparing chromatograms of 6 different batches of blank plasma obtained from 6 different subjects and plasma samples spiked with ZER and HUM.

Linearity and lower limit of quantification (LOQ)

Calibration plots were constructed from blank plasma spiked with HUM and six concentrations of ZER (2 – 15 µg/ml). The linearity of each calibration curve was determined by plotting the peak area ratio(y) of ZER to HUM vs the nominal concentration (x) of ZER. The calibration curves were constructed by weighted (1/x) least – square linear regression method. The lower limit of quantitation (LOQ) is the lowest concentration of the analyte that can be determined with acceptable precision and accuracy.

Precision and accuracy

The intra-day and inter-day precision and accuracy of the method were determined by percent coefficient of variation (% CV) and percentage relative error (% RE) respectively, according to the reported guidelines (FDA guidance for industry, 2001; Shah et al., 2000). Quality Control (QC) samples containing 3, 7 and 12 µg/ml concentrations were spiked for determination of precision and

Table 1. Intra- and Inter-day precision and accuracy of ZER in human plasma.

Nominal Conc. (µg/ml)	Calculated Conc. (µg/ml)		C.V.%		% Error	
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
3	2.98	2.85	3.47	7.46	-0.62	-4.89
7	7.13	6.77	2.43	5.71	1.79	-3.26
12	12.27	11.87	2.81	8.11	2.28	-1.05

accuracy. Three replicates at each concentration were processed as described in the sample preparation on day 1, 2, 3 and 15 to determine intra-day and inter-day precision and accuracy. The % RE values were calculated by the following equation:

[(CC - AC) / AC] X 100

Where: CC = Calculated concentration, AC = Added concentration

Recovery

The recovery of ZER was determined for QC samples at the three samples pools 3, 7, 12 µg/ml. Three replicates of each QC sample were treated as mentioned in the sample preparation previously and injected into HPLC system. The extraction recovery was calculated by the following equation:

Recovery = [(peak area after extraction) / (peak area after direct injection)] X 100

Stability

The stability studies were carried out at the three different concentrations of the QC values (3, 7, 12 µg/ml) stored at room temperature (bench top stability) was evaluated for 24 h and compared with freshly prepared extracted samples. Freeze and thaw stability for three cycles was determined at the same concentrations (QC), by thawing at room temperature for 2 - 6 h and then refreezing at -20°C for 12 - 24 h. The long term stability of ZER at the same QC values was assessed by carrying out the experiment after 15 days of the storage in -30°C. The concentration of ZER after each storage period was related to the initial concentration as determined for the samples that were freshly prepared.

Robustness

The robustness of a method is its capability to remain unaffected by small, but deliberate variations in method parameters. The effect of percent organic strength as well as the buffer on resolution was studied by varying acetonitrile from -1 to +1%, methanol from -2 to +2% and 0.01 M KH_2PO_4 from -1 to +1% while the other mobile phase components were remained constant as stated in chromatographic condition. The flow rate was changed by ± 0.2 units while the other mobile phase components were remained constant as stated in chromatographic condition. The column temperature was also studied in such a way that the temperature was studied at ambient, 25 and 30°C.

RESULTS AND DISCUSSION

The chromatographic conditions and sample preparation for the proposed analytical procedure were optimized to be suitable for preclinical pharmacokinetic studies. The main challenge here is to use the smallest plasma volume as much as you can in order to avoid the risk of contamination and poor recovery as well. Unlike solid phase extraction for purification of plasma samples for HPLC analysis that needs more plasma volumes (Cao et al., 2008), protein precipitation by acetonitrile is simple, not tedious and less risky for the analyst. As shown in the chromatograms of human plasma (Figure 2), the retention times were 4.5 and 8.89 min for HUM and ZER respectively, with a total run time of less than 10 min. The analytical process of ZER and HUM were resolved with good symmetry. No interfering peaks were observed in individual blank plasma at the retention time of ZER and HUM, thus confirming the specificity of the method. System suitability parameters for the method were as follows: the theoretical plates of ZER and HUM were 8530, 4608, respectively. Tailing factors was less than 1.6 for both ZER and HUM and resolution between ZER and HUM was 11.6.

The peak area ratio of ZER to HUM was used for the quantification of ZER in human plasma samples. Figure 3, the mean of five calibration curves made over a period of 14 days, each calibration curve originating from a new set of extraction. The mean calibration curve was linear in the concentration range of 2 - 15 µg/ml and equation of the six points was y = 0.1948X – 0.0862 with correlation coefficient (r) of 0.9978. The accuracy and precision were evaluated with QC samples at concentrations of 3, 7 and 12 µg/ml. The intra-day precision (expressed as percentage error RE) was deter-mined by the analysis of three replicates of QC samples at three different concentrations. The inter-day accuracy and precision were determined on three different days and the results were shown in Table 1. The intra-day and inter-day of the QC values were satisfactory with C.V% less than 10% and accuracy with RE within ±3%.

The lower LOQ was calculated by determining the concentration of four spiked calibration standards and was found to be 2 µg/ml for ZER in human plasma with CV of less 20% and the accuracy of 88 - 112%. The limit of detection (LOD) was determined to be 1.5 µg/ml based on signal to noise ratio (s/n) ratio of 3:1. The extraction recovery was determined by standard addition at three different concentrations (3, 7 and 12 µg/ml) for ZER and one concentration (10 µg/ml) for HUM and was found to be 95 ± 3, 97 ± 1 and 96 ± 2 for ZER and 92 ± 3 for HUM, Table 2. The recovery of ZER using the described

Table 2. ZER recovery and accuracy of the assay.

Conc.(µg/ml)	Absolute recovery				Accuracy (%)		
	Conc. (µg/ml) (Mean ± SD)	Mean (%)±SD (n = 3)	Range (min - max)	C.V.%	Mean ±SD (n = 3)	Range (min - max)	C.V.%
3	2.71 ± 0.24	95.44 ± 3.4	93 - 99	4	94 ± 6	90 - 100	6
7	6.69 ± 0.12	97.00 ±1.2	89 - 92	1	97 ± 3	94 - 101	3
12	11.39 ± 0.20	92.11 ± 3.1	90 - 97	3	93 ± 4	92 - 99	4

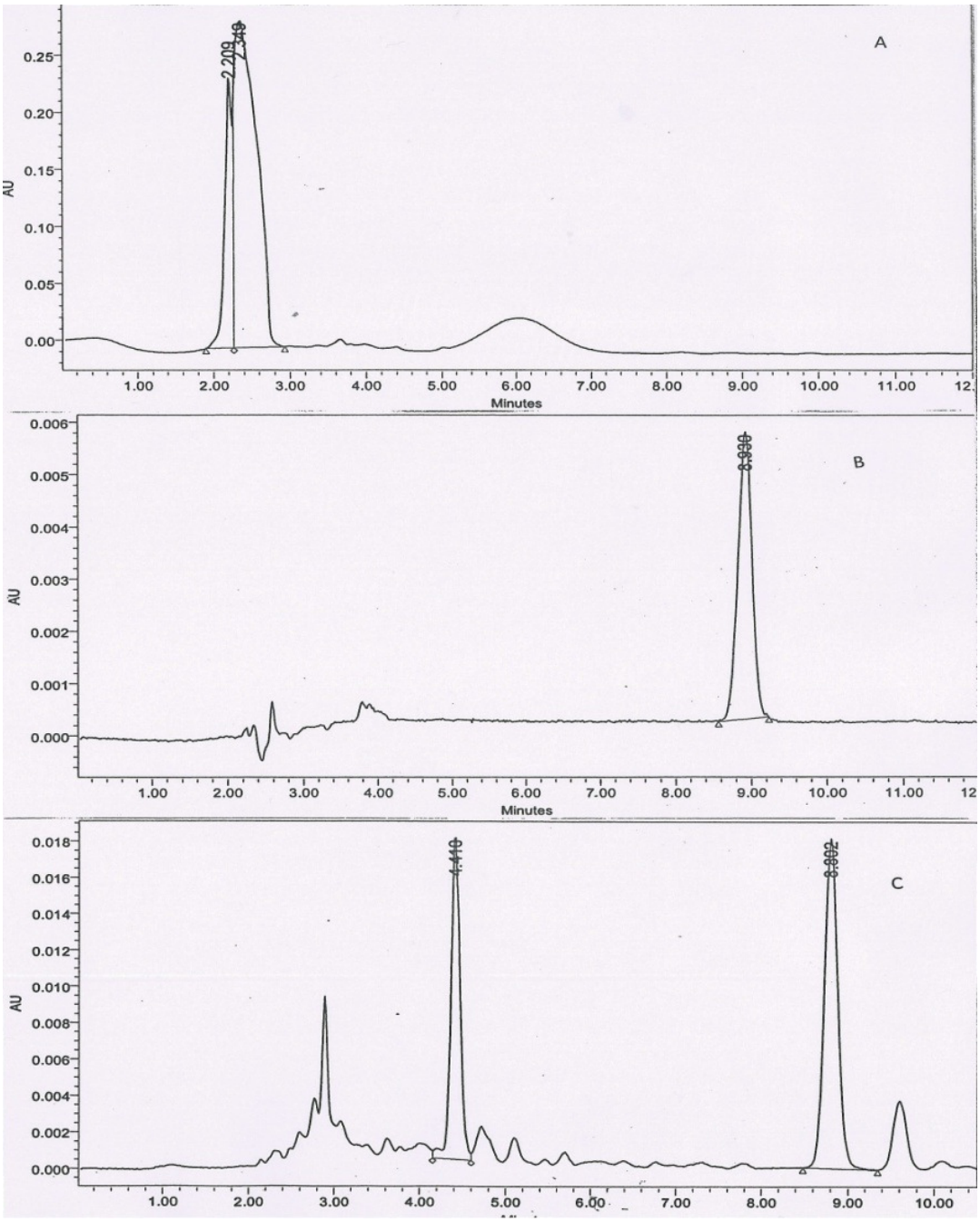

Figure 2. Typical chromatograms of human plasma (drug free) (A), blank plasma spiked with ZER (2 µg/ml) (B) and human plasma spiked with 10 µg/ml of ZER and 10 µg/ml of HUM.

Table 3. Stability of ZER in three different concentrations at three different conditions (bench top, freezing and thawing cycles and long term storage).

Stability	Spiked Conc. (µg/ml)	Calculated Conc (µg/ml) Mean ±SD(n = 3)	Average %	% C.V.
Bench Top[a]	3	2.99 ± 0.07	96	2
	7	6.64 ± 0.50	95	8
	12	10.27 ± 0.14	88	1
Freeze and Thaw[b]	3	2.97 ± 0.04	97	1
	7	6.63 ± 0.57	94	9
	12	10.79 ± 0.70	90	3
Long Term[c]	3	NT[d]	NT[d]	NT[d]
	7	6.5 ± 0.30	93	5
	12	10.4 ± 0.70	87	7

[a]After 24 h at room temperature.
[b]After three freeze thaw cycles.
[c] After 15 days at -30°C.
[d] Not tested.

Table 4. The robustness data of the assay method.

Parameter	Modification	RT (min)		Tailing Factor		Plates		Resolution
		ZER	HUM	ZER	HUM	ZER	HUM	
Mobile phase ratios MeOH:ACN:Buffer	53:26:21	9.27	4.41	1.2	1.3	7511	3844	13.80
	55:25:20	8.75	4.39	1.5	1.0	6615	3721	12.00
	57:24:19	10.09	4.9	1.3	1.3	12723	4624	14.60
Flow rate (ml/min)	0.80	11.09	5.54	1.2	1.0	10404	5929	10.06
	1.00	8.75	4.39	0.7	1.4	9526	6615	9.00
	1.20	7.36	3.69	1.3	1.5	6790	4624	11.56
Column Temp. (°C)	Ambient	8.89	4.44	1.5	0.8	6833	3844	12.40
	25	8.69	4.38	1.3	1.0	9526	3721	13.33
	30	8.19	4.22	1.0	1.2	6834	4547	13.18

method was consistent and efficient.

Analysis of the stock solution was performed at 200 µg/ml and after storage for two weeks at 4°C and at room temperature for 24 h, more than 99% of ZER remained unchanged, based on the peak areas in comparison with freshly prepared solution of ZER (200 µg/ml). Thus, ZER in standard solution was stable at least 14 days when store at 4°C and for 24 h at room temperature. Bench top stability of ZER was investigated at the concentration of 3, 7 and 12 µg/ml and the results revealed that the ZER in plasma was stable for at least 24 h with average percentage of 96, 95 and 88% respectively. Repeated freezing and thawing (three cycles) of plasma samples spiked with ZER at three levels (3, 7, 12 µg/ml) showed a mean percentage concentration of 97, 94 and 90%, respectively. Long term stability of ZER in plasma at -30 was also performed after 15 days of storage at three levels (3, 7, 12 µg/ml), which showed mean percentage concentration of (non-tested, vial was broken), 93, 87% respectively. The results of the stability study was presented in Table 3 and indicated that ZER was stable in the studied conditions.

The robustness results are shown in Table 4. It can be seen that the chromatographic conditions in accordance with established value (Shah et al., 2000). A change in mobile phase composition (acetonitrile 25 ± 1% v/v), (methanol 55 ± 2% v/v), (0.01 M KH_2 PO_4 20 ± 1% v/v), flow rate (1 ± 0.2 units) and column temperature (ambient, 25 and 30°C) had no impact on chromatographic performance. The tailing factor for both ZER and HUM was less than 2 and the two analytes (ZER and HUM) were well separated under the conditions carried out. The resolution between ZER and HUM was ranged 9.0 - 14.6. Considering the result of modifications in the system suitability parameters and the specificity of the method, it could be concluded that the method conditions are robust.

Conclusion

The newly developed HPLC method for determination of

ZER in micro-volumes human plasma (| 1.5 ml) was found to be simple, accurate, specific and robust. The method consisted of one step plasma protein precipitation using acetonitrile, followed by chromatographic separation in photodiode detector. No interference peaks were observed at the retention time of both ZER and HUM. This method could therefore be recommended for *in vivo* analysis of ZER for preclinical pharmacokinetic study and may also be applied for the estimation of ZER in other biological fluids such as serum and tissues after partial validation.

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Full Length Research Paper

Laboratory studies on vegetative regeneration of the gametophyte of *Bryopsis hypnoides* Lamouroux (Chlorophyta, Bryopsidales)

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Vegetative propagation from thallus segments and protoplasts of the gametophyte of *Bryopsis hypnoides* Lamouroux (Chlorophyta, Bryopsidales) was studied in laboratory cultures. Thallus segments were cultured at 20°C, 20 µmol photons m^{-2} s^{-1}, 12:12 h LD); protoplasts were cultured under various conditions, viz. 15°C, 15 µmol photons m^{-2} s^{-1}, 10:14 h LD; 20°C, 20 µmol photons m^{-2} s^{-1}, 12:12 h LD; and 25°C, 25 µmol photons m^{-2} s^{-1}, 14:10 h LD. Microscope observation revealed that the protoplast used for regeneration was only part of the protoplasm and the regeneration process was complete in 12 h. The survival rate of the segments was 100% and the survival rate of protoplasts was around 15%, regardless of culture conditions. Protoplasts were very stable in culture and were tolerant of unfavorable conditions. Cysts developed at the distal end or middle portion of gametophytic filaments under low illumination (2 - 5 µmol photons m^{-2} s^{-1}), the key induction factor. Cysts formed several protoplast aggregations inside or the cyst as a whole detached directly from the matrix and all the units were able to develop directly into new gametophytes. Regeneration directly from protoplasts and thallus segments were also discovered in the field. A relatively completed life history of *B. hypnoides* is established with newly discovered propagation methods, namely protoplast regeneration.

Key words: *Bryopsis hypnoides*, protoplast, thallus segment, propagation method, life history.

INTRODUCTION

Bryopsis is globally distributed and has been identified in Japan, China (Yellow Sea coast), North Carolina, Southern Australia, Mediterranean Sea, Western North America and other regions. So far, studies of *Bryopsis* have been mostly focused on four aspects: (1) Ultrastructural observations and life history, contributing to knowledge of the fine structures of organelles and sexual reproduction (Hori and Ueda, 1967; Neumann, 1969; Urban, 1969; Burr and West, 1970; Morabito et al., 2003); (2) The wound healing or regeneration of the protoplast (Fritsch, 1935; Tatewaki and Nagata, 1970; Kobayashi and Kanaizuka, 1985; Menzel, 1988; Pak et al., 1991; Kim et al., 2001; Ye et al., 2005); (3) Natural products with important bio-activity against acquired immune deficiency syndrome (AIDS) opportunistic infections (Hamann et al., 1996; Becerro et al., 2001); and (4) other aspects, such as phototropic properties (Iseki et al., 1995a, 1995b; Ye et al., 2006).

The life history of *Bryopsis* is diplo-haplontic. Data on the life history of *B. hypnoides* Lamouroux were obtained in the 1970s in Banyuls, France (Neumann, 1970; Rietema, 1971a, b). There is also life history information on *Bryopsis plumosa* (Hudson) C. Agardh from Naples (Schussnig, 1930; Zinnecker, 1935; Rietema, 1970) and Banyuls (Rietema, 1970; Kermarrec, 1975) and *Bryopsis muscosa* Lamouroux (Kermarrec, 1975) from Banyuls. In those studies, gametophytes, either monoecious or dioecious, were reported to alternate with prostrate microthalli (sporophytes), which, after a variable period of dormancy, were able to follow two pathways: some isolates holo-carpically produced stephanokont zoospores, which

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Abbreviations: LD, Light/dark; **FFB,** free floating branch; **MA,** macrothallus.

developed into gametophytes; while in others, the gametophyte arose directly from sporophytes (Morabito et al., 2003). Populations of *B. plumosa* from Naples exhibit both patterns (Rietema, 1970).

Later works (Bartlett and South, 1973; Tatewaki, 1973; Kornmann and Sahling, 1976; Tanner, 1982; Rietema, 1969; 1972; 1975; Neumann, 1969; Richardson, 1982; Harper and Pienaar, 1985; Diaz-Piferrer and Burrows, 1974; Burr and West, 1970; Kermarrec, 1980; Brück and Schnette, 1997) revealed six different pathways from microthallus to macrothallus and two transitions needed require validation, viz. the steps from zygote to macrothallus and from gametangium to microthallus directly (Brück and Schnette, 1997).

Regeneration of protoplasts from *Bryopsis* has been studied for about 40 years. Protoplasts of *Bryopsis* can be easily obtained and regenerated into new individuals; however, there are only few studies that make use of a naturally occurring propagation method (Diaz-Piferrer and Burrows, 1974; Richardson, 1982; Brück and Schnetter, 1993) and pertinent information is still limited. Laboratory studies allow a better understanding of the life histories of Bryopsidaceae and have led to a taxonomic revaluation of this group (Morabito et al., 2003).

The present investigation is a contribution to the knowledge of the strategies of reproduction, growth pattern, as well as the life history of *Bryopsis* from the intertidal zone of Qingdao, China.

MATERIALS AND METHODS

Materials and culture

Gametophytic specimens of *B. hypnoides* were collected between August and November 2005 from the intertidal zone (35.35°N, 119.30°E, 20-50 cm depth) of Zhanqiao Wharf, Qingdao, China. Seawater was collected at the time of sampling using a pump placed 2 m depth under the water surface and filtered with nested plankton nets (200 µm net with 20 µm net inside). Collected water was autoclaved and made up into ES enriched seawater (Mclachlan, 1979). In the laboratory, the thalli were examined and those that were intact were isolated, washed several times with sterile seawater, sterilized with 1% sodium hypochlorite for 2 min and then rinsed with autoclaved seawater. The sterilized material was placed into a sterile aquarium (d = 40 cm, h = 30 cm) containing enriched seawater and maintained at 20°C, with 20 µmol photons m^{-2} s^{-1} provided by cool-white fluorescent tubes, with a photoperiod of 12:12 h LD.

Thallus segments and protoplast culture

For regeneration investigations, *B. hypnoides* thalli were sterilized again with 1% sodium hypochlorite for 2 min and rinsed with autoclaved seawater and the surface moisture was removed at once with 3 layers of absorbent paper. The clean algae were cut into segments (about 4 mm) and protoplasts were squeezed out with 8 layers of muslin into 10 ml Eppendorf tubes. Some of the initially extruded protoplasts were cultured in Petri dishes (d = 10 cm, h = 1.5 cm) containing autoclaved ES enriched seawater (1:30, v/v). The culture conditions included 3 combinations: 15°C, 15 µmol photons m^{-2} s^{-1}, 10: 14 h LD; 20°C, 20 µmol photons m^{-2} s^{-1}, 12:12 h LD; 25°C, 25 µmol photons m^{-2} s^{-1}, 14: 10 h LD. The survival rate of protoplasts was calculated after 1 week in culture. Ten Petri dishes were included in every treatment.

Longer gametophytic filaments were selected and cut into 1 cm long segments using a sapphire knife and cultured in Petri dishes (d = 6 cm, h = 1 cm) containing enriched ES seawater. 1200 segments were cultured; segments were evenly distributed in 80 Petri dishes. The culture condition was as follows: 20°C, 20 µmol photons m^{-2} s^{-1}, 12: 12 h LD. The induction experiment was performed under various low light conditions (1 - 10 µmol photons m^{-2} s^{-1}) combined other with conditions, viz. 15°C, 10:14 h LD; 20°C, 12:12 h LD; and 25°C, 14:10 h LD. All the culture media, including seawater used in the protoplasts culture, were changed once a day. Observations were also carried out every day.

For stress culture, one of the treatments was to break the primary envelop using a fine stainless needle 5 min after onset of culture; another was to culture the protoplasts under 20°C, 20 photons m^{-2} s^{-1} and 12:12 h LD without changing or adding any of the culture media during the whole experiment, leaving the protoplast in a high salinity environment caused by evaporation; the final treatment was culture of the protoplasts 4°C, 20 µmol photons m^{-2} s^{-1} and 12:12 h LD.

For induction culture, thallus regenerated plants were cultured at 20°C, 20 µmol photons m^{-2} s^{-1} and 12:12 h LD for 2 weeks, then transferred to a 4 µmol photons m^{-2} s^{-1} circumstance without changing other conditions. When the cysts evacuated their matrices or the protoplasts started to germinate, all of them were taken out and cultured under normal conditions (20°C, 20 µmol photons m^{-2} s^{-1}, 12:12 h LD).

Fixation and dyeing

For observation of the forming of the cell membrane, the fixative (ethanol: acetic acid: formalin: seawater = 3:1:1:3, v: v) and 0.125% commassie brilliant blue R250 (CBB R250) dye solution (ethanol: acetate acid: seawater = 40:5:55) were prepared. Protoplasts cultured in autoclaved seawater were fixed with the fixative at intervals. The fixation process in every treatment lasted for 30 min and then the material was dyed with 0.125% CBB R250 solution for 30 min.

Microscopy and photography

Observations were performed with a light microscope (Nikon, ECLIPSE 50i, Tokyo, Japan). Sizes of protoplasts were determined with an eyepiece micrometer and the number, including the survival rate, was counted randomly with an inverted microscope (ZEISS, HBO 50, Jena, Germany) at a magnification of × 200. Six microscope fields of view were selected randomly on each Petri dish and at least 3 Petri dishes were included in each batch under all culture conditions and all protoplasts in the field of view were included. Survival rate (%) = the number of protoplasts (or thallus segments) that developed into thalli one week after onset of culture/all the protoplasts observed (or all thallus segments cultured) × 100%. For stress culture, the viability and intactness of the protoplasts were routinely checked using neutral red and Evans blue one week after onset of culture.

RESULTS

Thallus regeneration

The wild mature thalli of *B. hypnoides* growing along the coastline of Yellow Sea were usually about 15 cm in

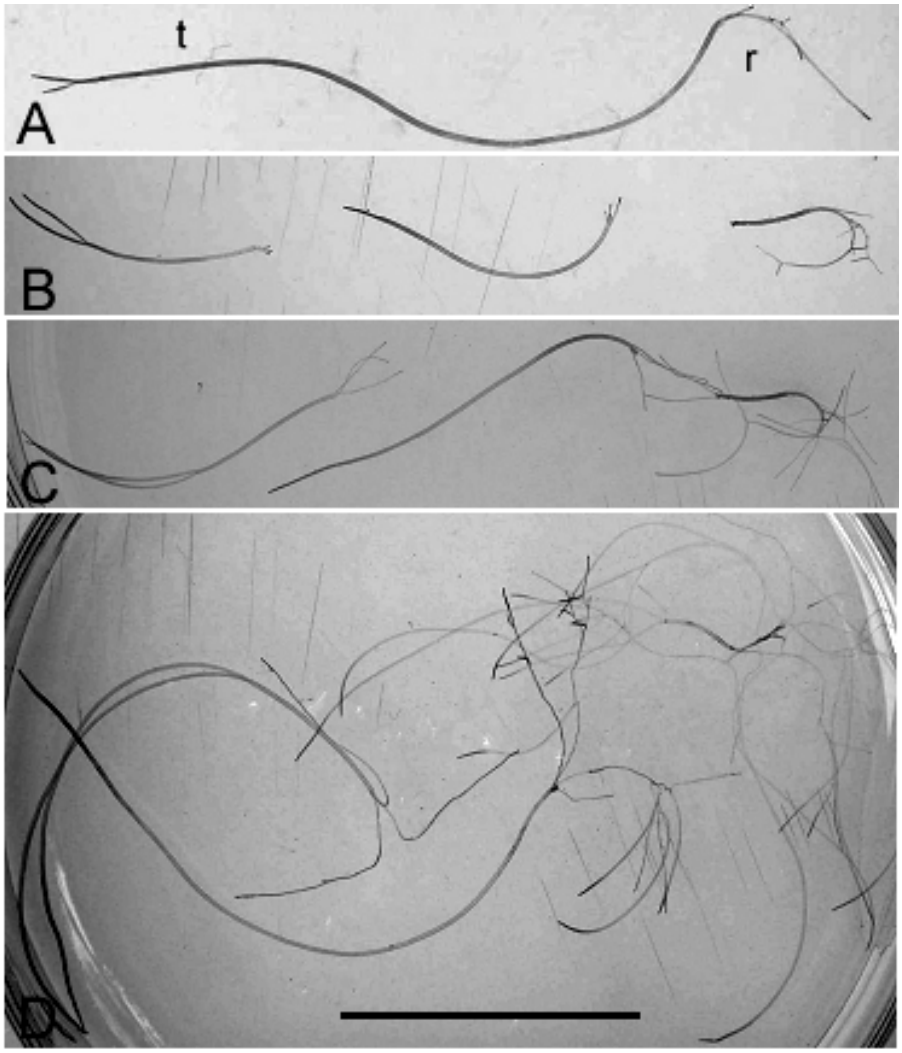

Figure 1. *Bryopsis hypnoides*: Thallus segment regeneration to the mature gametophytic phase. Scale bar, 2 cm. **(A)** A gametophytic filament selected for thallus segment regeneration. t, thallus; r, rhizoid. **(B)** Thallus segments showing newly developed thalli and rhizoid from the cuts. Segments were cut in sequence from the filament in B using a surgical blade, two days after the culture was initiated. **(C)** Developing gametophytic thallus, one week after culture initiation. **(D)** Developed gametophyte with many newly developed thalli branching from the rhizoid, 2 weeks after the culture was initiated.

length, appearing through the period of summer and autumn every year. The erect thalli are usually gracile, bearing rhizoids (Figure 1A). The thalli are fragile and easily broken by mechanical force. All segments survived and the regeneration process needed only a few days. The scar of the section healed within few hours after cutting and the ends protruded new erect thalli or rhizoid. After two days in culture, the newly generated thalli and rhizoid could be seen with the unaided eye (Figure 1B). Interestingly, rhizoids developed at the base of the segment, while a thallus formed distally (Figure 1C). Segments in culture always maintained this apico-basal orientation, indicating the existence of polarity. As a result of the rapid growth rate, the regenerated thallus developed into filaments in few weeks. Rhizoids developed into irregularly branched thalli that consisted of a tightly attached prostrate and detached filament (Figure 1D).

Aggregation process

The results from the experiment with the dye CBB R250 solution are shown in Figure 2. At the beginning of the culture, hydrophobic protein granules in blue color were distributed among the protoplasts (Figure 2A). The protein granules dispersed and moved to the outer layer

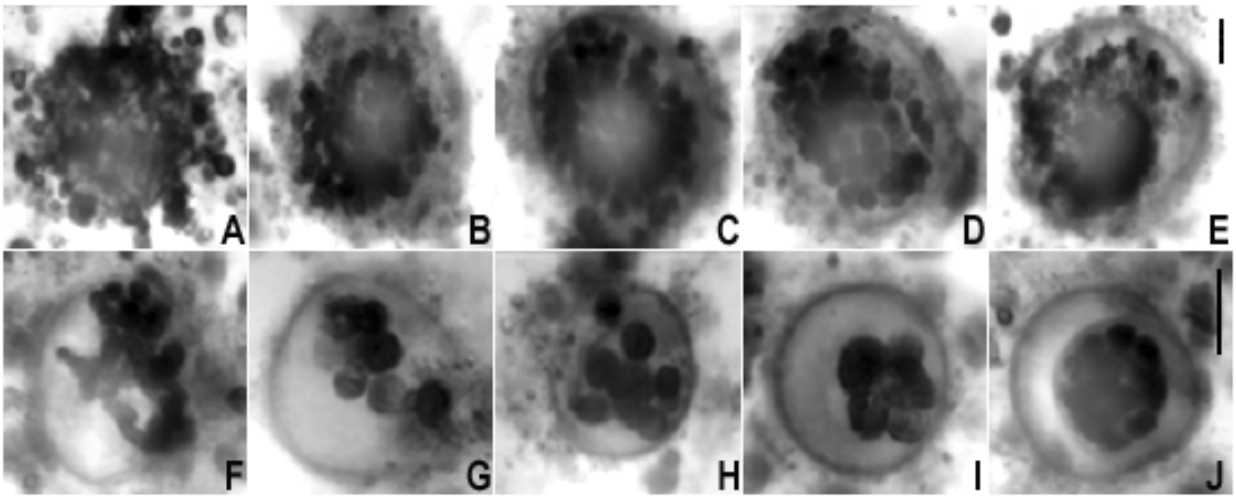

Figure 2. The formation of the cell membrane of sub-protoplast of *B. hypnoides*. Protoplasts cultured in autoclaved seawater were fixed with the fixative at intervals. The fixation process in every treatment lasted for 30 min and then the material was dyed with 0.125% CBB R250 solution for 30 min. Bar, 20 µm. **A** was fixed after 10 s onset of the culture. **B - J** were fixed every 40 min in turn and the total fixation period lasted for 6 h.

of the aggregate with the other organelles, such as chloroplasts, which were concentrated in the center (Figure 2B). Later, most of the protein granules dispersed and distributed around the aggregation and a faint pre-membrane (Figure 2C). With the passage of time, the pre-membrane turned clear and gradually became compact (Figures 2D - I). Finally, the second membrane formed at the periphery of the sub-protoplast (Figure 2J).

Culture of protoplasts

The culture conditions did not seem to affect the morphological expression or the development of the reproductive protoplasts. Most of the protoplasts dropped into seawater aggregated into protoplasts and the diameters of newly-formed protoplasts were from 10 to 100 µm (n > 110, each batch). Each protoplast produced a visible envelope within 2 to 10 min. Protoplasts adhered to the glass plates firmly and could not be dislodged when the culture medium was changed. About 60% of protoplasts were from 20 to 30 µm in diameter, regardless of culture conditions (Figure 3A). Protoplasts cultured at lower temperatures (15°C) aggregated slowly in comparison with those cultured under higher temperatures (20 and 25°C). However, survival rates were equivalent, with about 15% difference between temperatures (n > 600, each batch). One week later, the survivors began to generate (Figure 3B). The germlings of the protoplasts grew into thalli one month after the onset of culture.

Stress culture

Protoplasts with the protection of the newly-formed cell membrane and wall had high tolerance of unfavorable conditions (Figure 4). The newly-formed cell membrane was flimsy and easily broken by external mechanical forces. When seawater penetrated into the envelope through the interstices, the inner parts gathered again to quickly form a pre-membrane (Figure 4A). Changing the culture media periodically ensures suitable conditions for protoplast development; however, a few protoplasts did survive the stressful salinity conditions (Figure 4B). Six Petri dishes containing protoplasts were cultured under 4°C, 20 µmol photons m^{-2} s^{-1} and 12:12 h LD. Protoplasts were able to aggregate into protoplasts though 99.78% of them died (n = 1819) and others became dormant (Figure 4C).

Regeneration in the wild

In the field, an injured plant may persist through production of protoplasts and thallus segments within the shelter of the original cell wall (Figure 5). Although the primary cell wall plays an important role protecting the protoplasts from mechanical stresses or predators, it does restrict their further development (Figures 5A and B). Regenerated plants from broken thalli are readily found in the field (Figure 5C).

Induction experiment

Various cultivations at reduced light levels (1~10 µmol photons m^{-2} s^{-1}) were carried out and the best induction conditions were found to be 4 µmol photons·m^{-2} s^{-1}, 20°C and 12:12 h LD. Cyst induction and subsequent development are shown in Figure 6. The frequency of the new

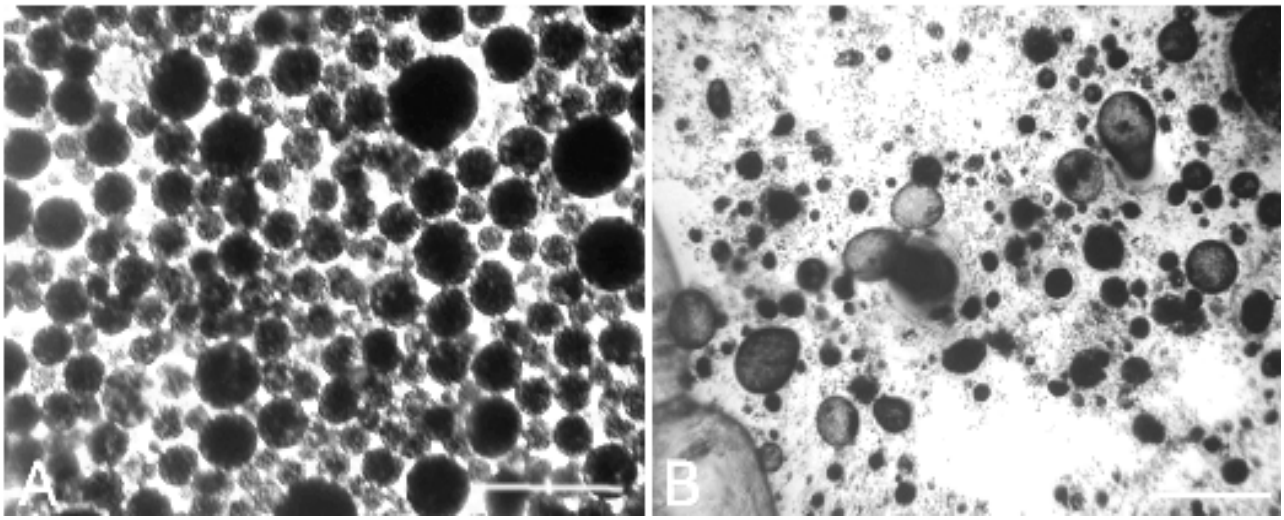

Figure 3. Protoplasts cultured under 20°C, 20 µmol photons m^{-2} s^{-1} and 12:12 h LD. Scale bar, 50 µm. **(A)** Aggregations appearing as rimless globes 2 min after the cultures was initiated. **(B)** Survivors starting to germinate, 1 week after the culture was initiated.

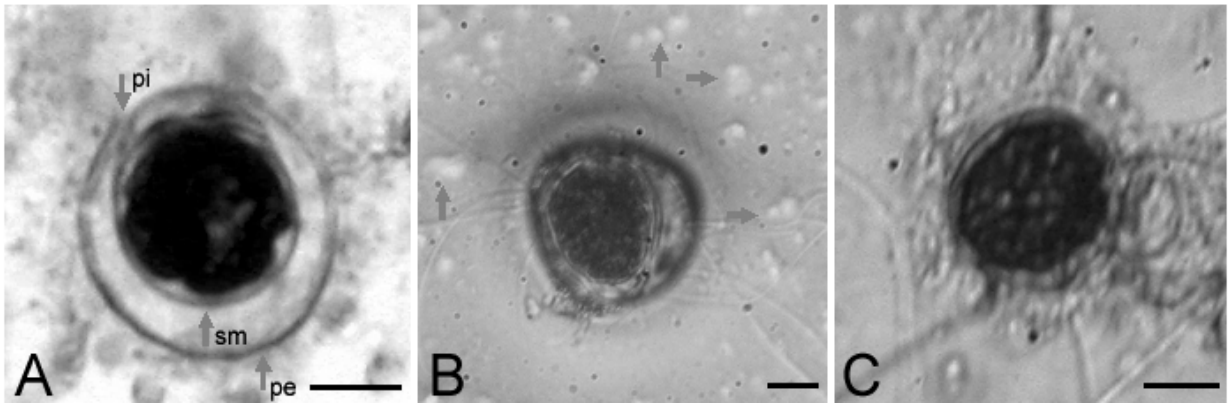

Figure 4. Protoplasts cultured under stress conditions. Scale bar, 30µm. **(A)** A protoplast covered with newly formed pre-membrane. pe, primary envelope; sm, second membrane. **(B)** A protoplasts cultured in a high salinity environment by without changing or adding culture media. Arrows show crystalloid salts from the seawater produced by evaporation, one week after onset of culture. **(C)** A protoplast cultured at 4°C, three months after the onset of culture. The other conditions were 20°C, µmol 20 photons m^{-2} s^{-1} and 12:12 h LD, except for the temperature in C.

form of propagation was less than 10% of the total population (n = 230, each batch) under the best induction conditions. Two weeks after, small capsule-like sacs called cysts formed at the distal ends or middles of erect thalli and rapidly became swollen (Figures 6A and B); this did not occur under normal culture conditions. Small cysts (sub-cysts) also developed from the primary cysts (Figure 6C). Some of the cysts developed from the distal end of the thalli expanded to 400 µm in length and 200 µm in width; in the cysts, newly-formed protoplasts developed into germlings (Figure 6D). Most of the cysts exited their matrixes (Figures 6E and F). As with the protoplasts, separate cysts developed into mature gametophytes several weeks later (Figures 6G, H and I).

DISCUSSION

Since *Bryopsis* is unicellular, it faces major problems when its cell wall is damaged. In multicellular algae, trauma is not as serious because the damage will extend only to those cells that have been ruptured. In *Bryopsis* and other siphonous algae, even the smallest tear in the cell wall and membrane risks spillage of all cell contents. Fritsch (1935) first reported that *Bryopsis* has a special clotting factor, similar to that of human blood, which is a combination of multiple small protein; when the cell wall is torn, these proteins collect at the opening and form a new cell membrane. Burr and Evert (1972) described wound healing of *B. hypnoides* with regards to thalli

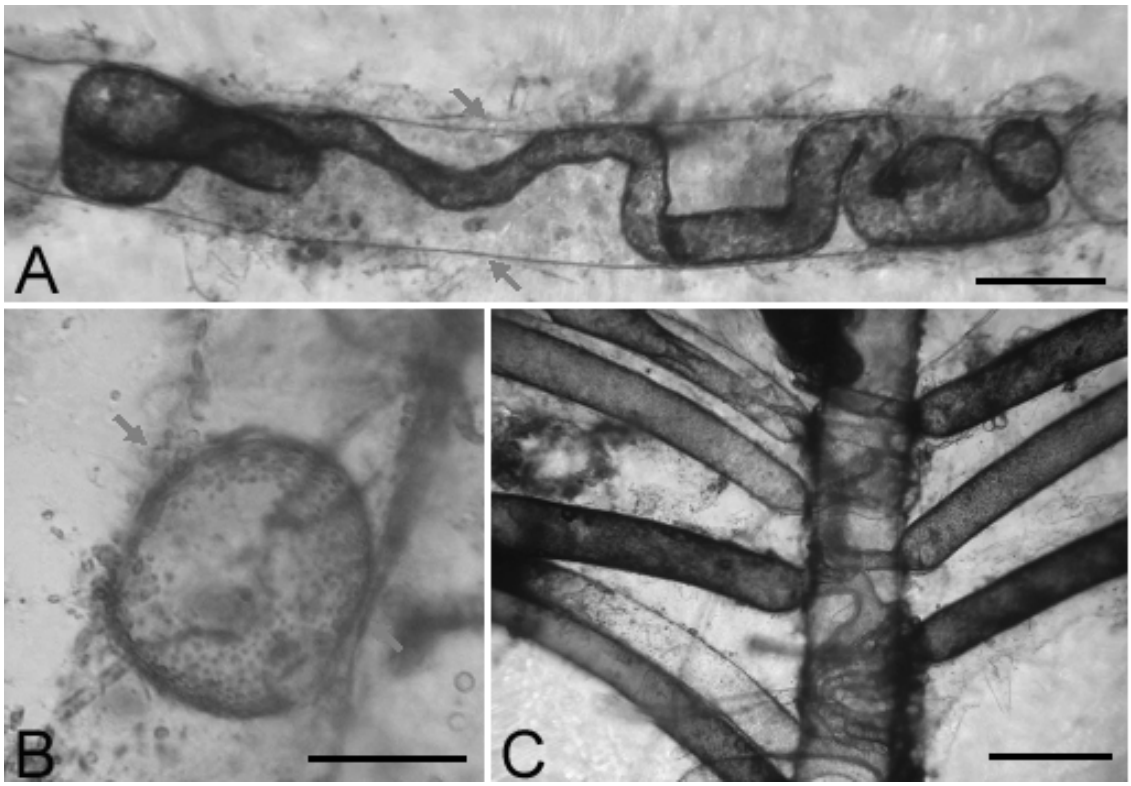

Figure 5. Regeneration of protoplasts and thalli in the field. Scale bars, 50 µm in A and B, 200 µm in C. **(A)** Protoplasts in a dead thallus. Arrows show the perimeter of the dead thallus. **(B)** A newly formed protoplast without germination. Arrows show the perimeter of the dead thallus. **(C)** New individuals derived from the branches, showing newly developed rhizoid at the bases of the segments.

regeneration from cut off segments and found whose wound-healing materials are proteinaceous substances. Kim and Klochkova (2005) recorded the aggregation process of protoplasts from *B. plumosa* using scanning electronic microscope and found a certain form of lectin that led to the protoplast formation. Clotting factors also aid *Bryopsis* in a form of secondary reproduction. If a piece of the plant is cut off by turbulence or grazing, clotting factors in the segment will seal the opening and the broken off segment can then drift and settle in a new location. In this way, *Bryopsis* may disperse. Our previous data showed that one of the protoplast-regenerated individual grew into thalli of 58 cm, 3-fold longer than the wild thalli and found that the total soluble proteins and the different ratios of chlorophyll *a* to chlorophyll *b* between the regenerated algae and wild ones were different (Ye et al., 2005).

Kim et al. (2001) pointed out that the survival rate of protoplasts was about 40%, much higher than in our experiment. The reasons behind the discrepancy may be as follows: (1) In our study, the survival rate were calculated one week after regeneration, while Kim et al. (2001) took readings 24 h after initiation; (2) The materials used belong to different species, *B. plumosa* and *B. bryopsis*, respectively. Ye et al. (2005) reported that on the diatom *Amphora coffeaeformis* (C. Ag.) Kützing could prevent protoplast germination; however, when the diatoms were removed, the protoplasts immediately geminated. More tolerant characters of protoplasts were found in the present study.

Light intensity is the key factor causing phase conversion. The cyst phase has a higher resistance to unfavorable conditions than the extruded protoplast because it is protected by the cell membrane and cell wall. It is not necessary for a cyst to undergo a dormancy period in order to germinate. Zygotes, on the other hand usually need some months to a year, or even more of dormancy to germinate (Rietema, 1969, 1970, 1971a, 1971b, 1975; Kermarrec, 1972; Diaz-Piferrer and Burrows, 1974; Jónsson, 1980; Morabito et al., 2003). The low light levels in the induction experiment are not suitable for long-term culture of the gametophytic filaments because there is no net photosynthetic gain. We believe that when a wild gametophyte of *B. hypnoides* is shaded in semi-darkness the filaments will change their life phase to produce cysts. Cysts can exit the filaments and disperse to an illuminated site. Illumination stress is common and could bring about life cycle phase shifts in *B. hypnoides*.

On the basis of our work and earlier studies, we are able to propose a relatively complete life cycle for *B. hypnoides*. Other parts except for Parts A and B have been described by Brück and Schnetter (1993, 1997).

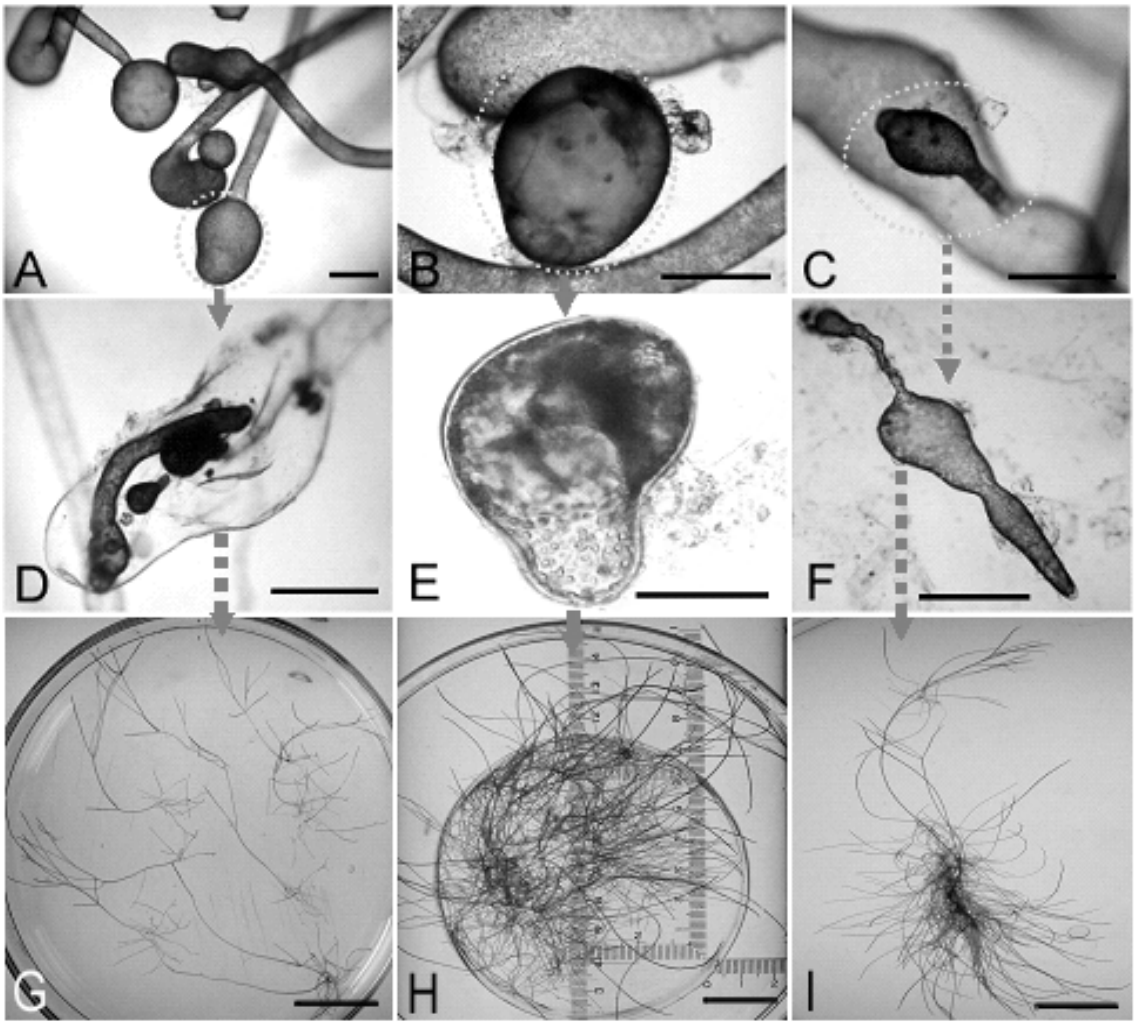

Figure 6. Induced cysts and their development. Scale bars, 100 μm in A, B, C, D, E and F; 2 cm in G, H and I. The dashed circles enclose developing under inducting conditions; arrows indicate the derivation and development of the cysts. **(A)** Cysts developing at the distal end of a thallus. Some cysts bear sub-cysts. **(B)** A cyst formed in the middle of the filament. **(C)** A sub-cyst developing from a mother cyst. **(D)** Protoplasts generating in a dead giant cyst. **(E)** A swelling cyst. **(F)** A germinating sub-cyst. **(G)** Plants regenerated from protoplasts derived from a giant cyst, 2 weeks after germination. **(H)** Gametophytic filaments regenerated from a cyst derived from the middle of a filament, one month culture after induction. **(I)** A gametophytic plant regenerated from a sub-cyst, 3 weeks after induction.

The direct development of free floating branch (FFB) con-firms the importance of the macrothallus (MA) generation for the survival of *Bryopsis* species. Further experiment needs to be conducted to validate the variation from MA to protoplast (SP) directly.

ACKNOWLEDGEMENTS

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Full Length Research Paper

Physicochemical equivalence of some brands of Nifedipine retard tablets available in Nigeria

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This research evaluated the physicochemical equivalence of some samples of Nifedipine 20 mg Retard Tablets available in Nigeria. Seven samples were randomly procured from various zones of the country and standard protocols applied to evaluate their tablet weight uniformity, dimensions, hardness, disintegration time, content of active Ingredient and *in vitro* drug release profile. Results showed that all the samples tested were chemically, but not physically equivalent. Although within each sample, compendial requirement for tablet weight uniformity was met, there were significant differences in the mean tablet weights, diameters and thickness of the samples studied ($p < 0.05$). Furthermore, tablet hardness and disintegration time varied much among the samples, but not within each sample. All the samples met the compendial requirement for content of active ingredient and released more than 80% of the drug within 4 h. It is therefore pertinent that manufacturers of this product be advised to formulate tablets that are equivalent in size, as different tablet sizes may impart negative psychological effects on clinicians and their patients when the need arises for switch over from one product to another, since the availability of particular products is never guaranteed at all times in Nigeria, a largely import dependent nation.

Key words: Nifedipine 20 mg Retard tablets, physicochemical equivalence, *in-vitro* drug release.

INTRODUCTION

In Nigeria, all the brands of Nifedipine 20 mg retard tablets available in the market, except one, are imported. As a result of the high cost of branded products, prescription and use of generic drug products is advocated in order that essential drugs would be affordable to all that need them.

However, unscrupulous importers, in order to ensure that their products compete commercially with other products, may sometimes compromise quality. As a result, healthcare professionals sometimes wonder whether those generics are equivalent to their original counterparts and whether patients are put at risk (Wiyada et al., 2002). Nifedipine is a calcium channel blocker used in the management of various cardiovascular diseases (Neal, 1995). It belongs to the dihydropyridine class of calcium channel blockers and is chemically known as dimethyl-1, 4-dihydro-2, 6-dimethyl-4- (2-nitrophenyl) pyridine-3, 5-dicarboxylate. It is light sensitive and its photo-reaction products (nitrosophenylpyridine and nitrophenylpyridine derivatives) possess highly diminutized pharmacological activity. It is a yellow crystalline powder, with melting point range of 171 – 175^{o}C (Al-Turk et al., 1989; Kennis et al., 2001; Martindale, 2002; USP/NF, 2002; BP, 2003).

The effects of nifedipine are evident within 30-60 min of an oral dose (Kennis et al., 2001). The elimination half-life for the immediate release formulation is 2-5h (CPS 2002). Apart from the relatively short half-life of the immediate release formulation, they have other short comings such as flushing, dizziness, palpitation and reflex tachycardia. These have necessitated the formulation of longer acting dosage forms and in some countries, the withdrawal of immediate release formulations from their markets (Brown et al., 1997; Minami et al., 2004; Emdex, 2006). In nifedipine retard, the rate of drug release is reduced by increasing particle size or forming insoluble crystals (MeRec, 2000).

Other applications of nifedipine are in the management of exercise induced asthma (Barnes et al., 1981), primary Raynaud syndrome (Kennis et al., 2001), prevention of

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Table 1. Identities of the Nifedipine 20 mg retard samples used in the study.

Code	Country of Origin	Shelf Life Status	Batch No.	NAFDAC No.
A	Nigeria	Not Expired	2676T	04-3161
B	India	Not Expired	MOI	
C	Israel	Not Expired	06E86	04-4499
D	India	Not Expired	K51025	04-7584
E	Austria	Not Expired	P-909	
F	Slovenia	Not Expired	31951054	04-0766
G	India	Not Expired	MNB-101	047943

atherosclerosis (Hirata et al., 2000), amelioration of endothelium injury in patients with systemic sclerosis (Allanore et al., 2004) and enhancement of the activity of anti-cancer drugs in colon cancer treatment (Yang and Friedlander, 2001).

MATERIALS AND METHODS

Materials

The samples (Table 1) studied were purchased from Lagos, Owerri, Onitsha, Ilorin, Suleja and Abuja. Nifedipine RS was purchased from Sigma-Aldrich Cheme GmbH, Germany. All other reagents were of analytical grade and water was double distilled.

Tablet weight, dimensions and hardness measurements

The weights of 20 tablets selected randomly from each sample were determined at room temperature using an electronic balance (Mettler Toledo B 154, Switzerland), while the dimensions (diameter and thickness) of 10 randomly selected tablets were measured using the Mitutoyo gauge (Model 10C-1012 EB Japan). The hardness values of another set of 20 tablets randomly selected from each sample were determined at room temperature using the Monsanto hardness tester (Monsanto Chemical Co. USA). The results reported are the means and standard deviations.

Disintegration time

The disintegration times of the tablets were determined in distilled water at 37 ± 0.5°C using the disintegration tester (Manesty, Model: MK 4, UK). Thereafter, all other tests were carried out under subdued light.

Calibration curve for Nifedipine RS

Various concentrations of Nifedipine RS (1.5, 2.5, 5.0, 10.0, 15.0, 20.0 and 25.0 µg/ml) were prepared in 0.1 M HCl. Their absorbances were read at 350 nm against 0.1 M HCl using a UV-Visible spectrophotometer (UV-160 A Shimadzu Corporation Japan). The values of absorbance were plotted against the corresponding concentrations. Data used in plotting the curve were mean values of at least duplicate determinations.

Content of active drug

Five tablets were randomly selected from each sample and finely crushed in an agate mortar. An amount of powder equivalent to 20 mg of nifedipine was dissolved in 50 ml of methanol in a 100 ml volumetric flask and shaken vigorously for 15 min. Thereafter, the flask was made up to volume with more methanol. The solution was filtered through a Whatman No 1 filter paper and 2.5 ml of the clear filtrate was pipetted into a 50 ml volumetric flask and made up to volume with more methanol. The absorbance of the solution was measured at 350 nm using methanol as blank. The content of active drug was calculated based on the absorbance of 10.0 µg/ml of Nifedipine RS in methanol at the same wavelength.

Dissolution rate

The dissolution rate study was carried out using the USP XXIII (1995) basket method (Erweka dissolution tester, Type DT 80, Germany), operated at 50 rpm. The dissolution medium was 750 ml 0.1 M HCl maintained at 37 ± 0.5ºC. Three tablets selected at random from each sample were used simultaneously for the study. A 5 ml volume of leaching fluid was withdrawn at various time intervals, filtered and appropriately diluted and its absorbance read at 350 nm against 0.1 M HCl as blank. The dissolution medium was replenished with fresh 5 ml aliquots at the same temperature after each sampling was done. The percentage of drug released was then calculated from the equation ($y = 0.0082x + 0.0022$) obtained from the calibration curve.

Statistical analysis

The Fisher test (F-test) was employed to test whether there were significant differences between the means (µ) of the diameters, thicknesses, or weights of the samples studied. The post-hoc test: Fisher's Least Significant Difference (FLSD) was carried out to locate the cause of the rejection of the null hypothesis (the means of the diameters, thicknesses, or weights of the tablets studied were equal) whenever it occurs.

$F_{calculated}$ = Treatment Mean Square / Error Mean Square

$FLSD = t_{critical} \sqrt{s^2 (1/ N_A + 1/ N_B)}$

where N_A and N_B are the sizes of the two treatment groups, s^2 is the pooled variance, estimated using the Error Mean Square term in the F-test and $t_{critical}$ is the critical t statistic associated with the experimental design (Jones, 2002). The $F_{calculated}$ values were evaluated using Microsoft Excel 2007 ANOVA: Single Factor Analysis Tool Pack.

RESULTS AND DISCUSSION

All the Nifedipine 20 mg Retard tablets studied were within their shelf lives (Table 1). Each had a labeled

Table 2. Tablet weights of the Nifedipine 20 mg retard samples used in the study (mg).

A	B	C	D	E	F	G
81.4	174.2	205.5	83.8	189.0	180.2	202.8
82.0	145.8	203.7	83.0	195.1	180.5	205.0
80.6	174.7	203.1	82.2	185.3	179.5	202.4
83.8	168.5	207.5	87.6	194.9	178.6	200.6
80.0	169.1	200.8	81.3	194.2	179.2	206.5
80.4	170.4	204.1	86.0	192.8	177.9	203.0
82.1	165.3	204.5	83.9	191.5	180.0	202.9
81.1	178.8	205.2	83.4	188.0	180.2	203.8
79.7	169.4	203.1	84.1	190.0	179.2	201.8
82.1	180.9	205.0	86.7	193.7	178.1	202.7
80.1	182.0	203.0	85.3	198.1	179.0	203.5
81.6	174.2	207.8	80.7	198.1	177.9	204.8
81.7	167.8	200.4	82.8	189.8	180.7	201.6
79.8	193.6	204.7	85.0	195.4	178.5	203.2
82.0	180.2	202.0	83.3	199.7	180.2	198.9
82.0	182.1	201.9	81.3	197.2	179.5	201.6
83.0	187.4	204.7	82.5	194.6	181.4	199.6
81.7	167.7	206.3	80.6	189.3	176.9	203.8
83.8	186.1	203.0	81.3	198.2	178.2	204.3
81.3	180.5	206.2	85.2	182.4	177.5	203.7
μ: 81.51	174.94	204.13	83.50	192.87	179.16	202.83
s: 1.191	10.290	2.023	2.007	4.630	1.197	1.812

μ = Mean weight; s = standard deviation.

Table 3. Some physicochemical characteristics of the Nifedipine 20 mg Retard samples studied.

Code	Tablet weight* (mg)	Tablet dimensions (mm)		Tablet hardness* (Kg)	Tablet disintegration time*(min)	Content of nifedipine (%)
		Diameter*	Thickness*			
A	81.51(1.191)	5.66(0.010)	3.10(0.018)	5.65(1.631)	7.91(1.197)	101.1
B	174.94(10.290)	7.95(0.019)	3.70(0.079)	4.95(0.945)	2.17(0.621)	104.3
C	204.13(2.023)	8.14(0.009)	4.07(0.020)	5.05(0.510)	1.08(0.132)	95.7
D	83.50(2.007)	5.64(0.004)	3.14(0.047)	5.70(0.571)	17.39(2.355)	98.9
E	192.87(4.630)	8.16(0.010)	3.79(0.066)	7.80(0.894)	7.06(0.822)	106.5
F	179.16(1.197)	8.20(0.008)	4.11(0.030)	7.50(0.607)	38.79(5.398)	102.2
G	202.83(1.812)	8.12(0.005)	3.80(0.042)	>11.00(-)	47.41(4.107)	94.6

* Standard deviation in parenthesis.

strength of 20 mg nifedipine as a slow release formulation. Of the seven (7) samples studied, two (2), B and E, were not registered with the National Agency for Food and Drug Administration and Control (NAFDAC). Only one (1) sample, A, was formulated in Nigeria.

Tables 2 and 3 show some of the physicochemical characteristics of the samples studied. The compendial requirement (BP, 2004) for tablet weight uniformity is met by all the samples in that not more than two of the individual weights from each sample deviated from the mean weight by more than 7.5% (Table 2). This compliance is important since the uniformity of dosage unit can be demonstrated by either weight variation or content uniformity study (USP/NF, 2003). These either reflect indirectly or measure directly the amount of drug substance in the tablet (Alderborn, 2002). The compliance within each sample notwithstanding, it is observable that the mean weight of the various samples varied widely and on application of ANOVA (Fisher's-test) and its least significance difference (LSD) post hoc test, significant differences were revealed, with the mean tablet weights (μ_w) related as follows: $\mu_{wA} = \mu_{wD} \neq \mu_{wC} = \mu_{wG} \neq \mu_{wB} \neq \mu_{wE} \neq \mu_{wF}$ ($p < 0.05$) (Tables 4c and 5). The test also revealed significant differences among the samples mean tablet

Table 4a. ANOVA results for tablet diameters.

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	87.05378	6	14.50896	137453.3	1.1229E-127	2.246407983
Within Groups	0.00665	63	0.000106			
Total	87.06043	69				

SS = sum of squares; df = degree of freedom; MS = mean square; F = calculated F- value.

Table 4b. ANOVA results for tablet thicknesses.

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	9.980789	6	1.663465	717.8456	5.34E-56	2.246408
Within Groups	0.14599	63	0.002317			
Total	10.12678	69				

SS = sum of squares; df = degree of freedom; MS = mean square; F = calculated F- value.

Table 4c. ANOVA results for tablet weights.

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	349280.56	6	58213.43	2878.133	3.9E-138	2.167423
Within Groups	2690.072	133	20.22611			
Total	351970.63	139				

SS = sum of squares; df = degree of freedom; MS = mean square; F = calculated F- value.

diameters and thicknesses, with the post hoc identifying that: $\mu_{dA} = \mu_{dD} \neq \mu_{dB} \neq \mu_{dF} \neq \mu_{dG} \neq \mu_{dE} = \mu_{dC} = \mu_{dG}$, $\mu_{tA} \neq \mu_{tB} \neq \mu_{tC} \neq \mu_{tD} \neq \mu_{tF} \neq \mu_{tE} = \mu_{tG}$ for mean tablet diameters and thicknesses respectively ($p < 0.05$) (Tables 4a, 4b, and 5). These differences in the tablet sizes (that is, weight, diameter and thickness) may actually have some negative psychological effects on clinicians and their patients since they could raise doubts on the general equivalence of the different brands of nifedipine 20 mg retard tablets available. Although the WHO Model Formulary (2002) advised that a patient be placed on a particular brand, probably due to pharmacokinetic and psychological reasons, in Nigeria where the availability of a particular brand for the patient concerned is never guaranteed at all times, it would be advisable that manufacturers of this product formulate tablets of equivalent sizes in order to assuage patients' worry regarding the identity and efficacy of the different brands because of the wide differences in tablet sizes.

Table 3 shows that the mean tablet hardness for the samples ranged from 4.95 to > 11.00 (Kg Sufficient tablet hardness is essential to ensure resistance to damage by handling, packaging and transportation. Tablet hardness of 4 kg is considered to be the minimum for a satisfactory tablet (Rudnic and Schwartz, 2000). The disintegration times of the samples varied widely too and cannot be predicted from the tablet hardness values. This is not unusual since different manufacturers adopt different formulation techniques to manipulate the disintegration and release properties of nifedipine retard tablets (MeRec, 2000). The content of active ingredient in all the samples is within the range required by the USP/NF (2003), that is, not less than 90.0% and not more than 110.0% of the labeled content. Thus, all the samples studied are chemically equivalent.

The *in-vitro* drug release profile (Figure 1) depicts that all the samples released more than 80% of their labeled contents within 4 h. The profiles were different however, in that some products released up to 50% of their contents within the first 1 h, while three of them (D, F and G) did not. These differences in the patterns of release must have been caused by the manufacturers' choice of method of achieving reduction or delay in the rate of drug release (either increase in particle size or formation of insoluble crystals) (MeRec, 2000). The need to release up to 50% within 1 h is important because there is benefit in attaining a good blood pressure control level within 1 h and then maintaining it for at least the next 2 h (Kennis et al., 2001). On the whole, all the Nifedipine Retard tablets tested performed creditably and may perform even better *in vivo*, in the presence of some biosolvents that may enhance their release profiles.

Table 5. Fisher's LSD test on the Samples at 0.05 level (two tailed) with critical values = 0.03[d], 0.04[t], 2.81[w].

Diameter			Thickness			Weight		
Compar.	**Diff.**	**Signif.**	**Compar.**	**Diff.**	**Signif.**	**Compar.**	**Diff.**	**Signif.**
F – D	2.56	F≠ D	F – A	1.01	F ≠ A	C – A	122.62	C ≠ A
F – A	2.54	F ≠ A	F – D	0.97	F ≠ D	C – D	120.63	C ≠ D
F – B	0.25	F ≠ B	F – B	0.41	F ≠ B	C – B	29.19	C ≠ B
F – G	0.08	F ≠ G	F – E	0.32	F ≠ E	C – F	24.97	C ≠ F
F – C	0.06	F ≠ C	F – G	0.31	F ≠ G	C – E	11.26	C ≠ E
F – E	0.04	F ≠ E	F – C	0.04	F ≠ C	C – G	1.30	C = G
E – D	2.52	E ≠ D	C – A	0.97	C ≠ A	G – A	121.32	G ≠ A
E – A	2.50	E ≠ A	C – D	0.93	C ≠ D	G – D	119.33	G ≠ D
E – B	0.21	E ≠ B	C – B	0.37	C ≠ B	G – B	27.89	G ≠ B
E – G	0.04	E ≠ G	C – E	0.28	C ≠ E	G – F	23.67	G ≠ F
E – C	0.02	E = C	C – G	0.27	C ≠ G	G – E	9.96	G ≠ E
C – D	2.50	C ≠ D	G – A	0.70	G ≠ A	E – A	111.36	E ≠ A
C – A	2.48	C ≠ A	G – D	0.66	G ≠ D	E – D	109.37	E ≠ D
C – B	0.19	C ≠ B	G – B	0.10	G ≠ B	E – B	17.93	E ≠ B
C – G	0.02	C = G	G – E	0.01	G = E	E – F	13.71	E ≠ F
G – D	2.48	G ≠ D	E – A	0.69	E ≠ A	F – A	97.65	F ≠ A
G – A	2.46	G ≠ A	E – D	0.65	E ≠ D	F – D	95.66	F ≠ D
G – B	0.17	G ≠ B	E – B	0.09	E ≠ B	F – B	4.22	F ≠ B
B – D	2.31	B ≠ D	B – A	0.60	B ≠ A	B – A	93.43	B ≠ A
B – A	2.29	B ≠ A	B – D	0.56	B ≠ D	B – D	91.44	B ≠ D
A – D	0.02	A = D	D – A	0.04	D ≠ A	D – A	1.99	D = A

d = Diameter, t = thickness, w = weight.

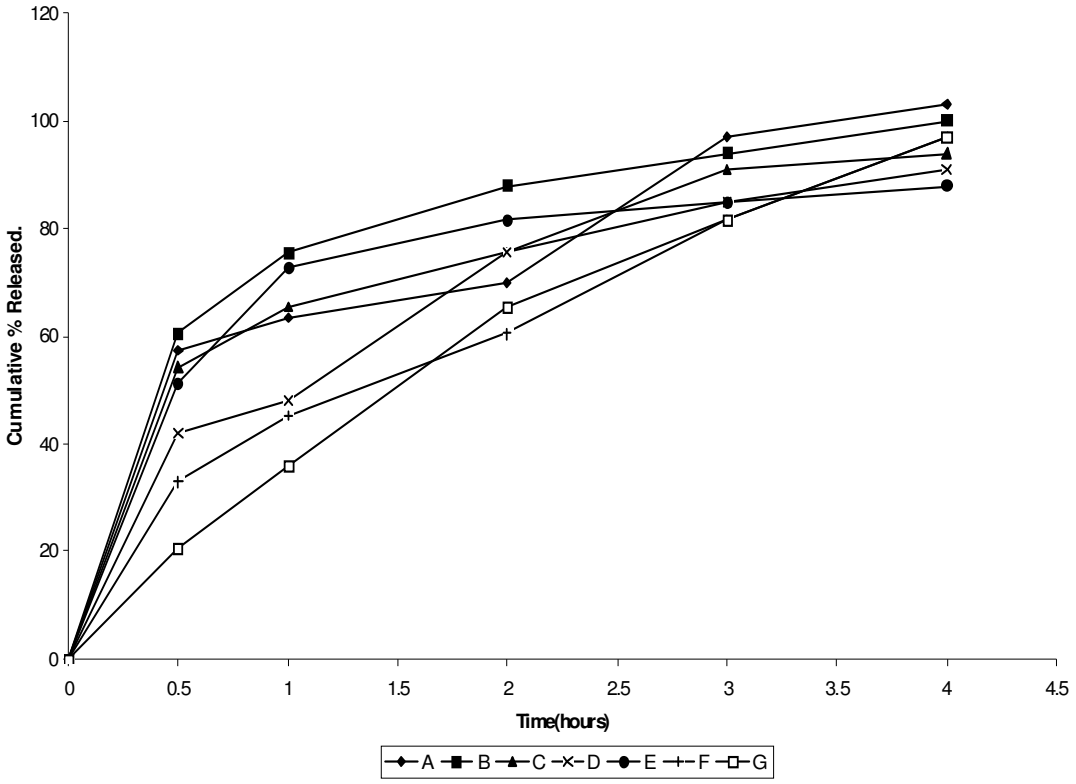

Figure 1. Dissolution profile of Nifedpine 20 mg retard tablets.

Conclusion

All the samples of Nifedipine retard tablets studied performed well *in vitro*. However, manufacturers of nifedipine retard tablets and other sensitive drugs should be advised to formulate products that are identical in size and colour so that patients, especially non-literate ones would not doubt the similarity in effectiveness of the different available brands.

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