

LABORATORY MANUAL FOR PHYSIOLOGICAL STUDIES OF RICE

SHOUICHI YOSHIDA • DOUGLAS A. FORNO • JAMES H. COCK • KWANCHAI A. GOMEZ

THIRD EDITION

IN COOPERATION WITH THE INTERNATIONAL RICE RESEARCH INSTITUTE

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MAIL ADDRESS P.O. BOX 933 MANILA, PHILIPPINES

FOREWORD

This manual is primarily intended for students of crop physiology and agronomy.

The procedures given in the text of this manual are particularly suited for routine chemical analysis and physiological studies of the rice plant. Considerable attention is given to technical matters involved in these procedures.

The equipment section of each chapter lists only special items needed for the procedures discussed. Ordinary laboratory equipment is not listed.

Shouichi Yoshida Douglas A. Forno James H. Cock Kwanchai A. Gomez

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Equipment Scissors, paper bags, marking pen, drying oven, weighing scales, grinding mill with a sieve of 1 mm screen size.

Time of sampling When to sample depends largely on what you are studying.

When you want to diagnose nutritional disorders, take the sample when the plants are showing symptoms of the disorder. For example, symptoms of iron toxicity and zinc and phosphorus deficiency are usually seen 3 to 4 weeks after transplanting (Tanaka and Yoshida 1970). A chemical analysis of the plants at this stage is very helpful for diagnosing these disorders.

If you are studying nutrient uptake, take the samples at different stages of growth: at transplanting time, when the seedlings have recovered from transplanting, during the vigorous tillering stage, at panicle initiation, during stem elongation, at flowering, at the milky and dough stages, and at maturity (Ishizuka 1964).

When you are studying the total nutrient uptake by a crop, take the whole plant samples at maturity. Sampling at this time sometimes underestimates the nutrient uptake because some of the older leaves may have fallen off and because rain may have leached nutrients such as nitrogen and potassium from the leaves before maturity (Tanaka and Navasero 1964).

What plant part to analyze To analyze the level of some constituent in the rice plant, use the whole plant, the leaf blade, or the Y-leaf (the most recently matured leaf blade) as the sample. These parts have been widely studied (Tanaka and Yoshida 1970, Mikkelsen 1970) and the critical contents for deficiency, sufficiency, or toxicity have been established so that you can use them as standards for comparing your results (see Table 1). When you sample at early stages of growth, take the whole plant.

Remember that such critical contents may vary according to the criteria by which the disorders are defined, growth stages of the plant, varieties, climatic conditions, etc. So use the critical levels listed in the following table with care.

Remember also that "percent content" is an intensity factor while "total amount absorbed" is a capacity factor. Hence the content of an element often may be affected by the growth statu. of a plant, which in turn is affected by many other factors. For example, the silica content of rice straw can be changed greatly by nitrogen application, which means that the silica content in the rice straw is not always a good index of the availability of silica in a soil.

The total silica uptake by the plant may be a better index in this case (Imaizumi and Yoshida 1958). Such considerations are particularly important in interpreting the chemical analyses of plant tissue from pot experiments.

Table 1. Critical contents of various elements for deficiency and toxicity in the rice plant.

Element	Deficiency (D) or toxicity (T)	Critical content	Plant part analyzed	Growth stage
N	D	2.5%	Leaf blade	Tillering
P	D	0.1%	Leaf blade	Tillering
	T	1.0%	Straw	Maturity
K	D	1.0%	Straw	Maturity
	D	1.0%	Leaf blade	Tillering
Ca	D	0.15%	Straw	Maturity
Mg	D	0.10%	Straw	Maturity
S	D	0.10%	Straw	Maturity
Si	D	5.0%	Straw	Maturity
Fe	D	70 ppm	Leaf blade	Tillering
	T	300 ppm	Leaf blade	Tillering
Zn	D	10 ppm	Shoot	Tillering
	T	> 1,500 ppm	Straw	Maturity
Mn	D	20 ppm	Shoot	Tillering
	T	>2,500 ppm	Shoot	Tillering
B	D	< 3.4 ppm	Straw	Maturity
	T	100 ppm	Straw	Maturity
Cu	D	< 6 ppm	Straw	Maturity
	T	30 ppm	Straw	Maturity
Al	T	300 ppm	Shoot	Tillering

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Sampling and sample preparation

1. Uproot the plant and wash the roots and the basal part of the shoot with tap water. If micronutrients are to be measured, wash the roots and basal part of the shoot with distilled or demineralized water. Remove the roots with scissors. This may be done after the sample is dried (below).
2. Place the sample in a paper bag and mark the date and location of sampling on the bag. Write relevant information about the sample on the bag at the time sampling begins.
3. If you are going to analyze particular plant parts you can remove them in the field. But it may be more convenient to remove the whole plant from the field and separate the individual parts later. Wash the sample with tap water and then, if necessary, with distilled or demineralized water.
4. Dry the samples in a draft-oven at 80 C until a constant dry weight is obtained (about 48 hours). Avoid packing the oven too full because the samples will dry unevenly causing errors in measuring dry weight. When analyzing organic compounds in the dry tissue, dry the tissue as soon as possible after sampling. For precise analysis, kill fresh tissue by placing it in boiling alcohol for 3 minutes, or dry the tissue using the freeze-dry technique.

5. Record the oven-dry weights when drying is completed. Do not expose the samples to the atmosphere for very long before weighing them because they will quickly take up moisture. If samples are broken, it is advisable to weigh each sample in the bag in which it was dried. Then remove the sample and obtain the weight of the bag. In recording dry weights, three effective figures are sufficient for routine analysis.

6. Cut the samples into small pieces and then grind them in a mill fitted with a sieve of 1-mm screen size. Be sure that the mill is free of grease and thoroughly cleaned between each sample grinding. As an extra precaution when analyzing minor elements, grind the samples suspected of containing the lowest concentration of the element in question first. (Since the sieve in the mill is usually brass, it must be considered a source of copper and zinc contamination.) If the sample is less than 1 g, cut it into fine pieces and weigh it for chemical analysis as such or use a suitable smaller mill. When analyzing starch, grind the sample further in a ball mill.

7. Store the ground samples in glass bottles with tight stoppers. Envelopes can be used for small samples. Store the envelopes in a polyethylene bag. When analyzing boron, store the samples in soft-glass containers; don't use Pyrex containers because Pyrex is a borosilicate and may be a source of boron contamination. Store the samples in a cool, dark place. Be sure the samples are properly labeled before storing: dates of sampling are essential.

8. Before weighing samples for chemical analyses, redry the container of ground tissue at 80 C for 24 hours.

Source of error

Five major sources of error occur in sample analysis: Contamination, sample variation, analytical variation, person-to-person and laboratory-to-laboratory variation, and carelessness. Contamination usually comes from soil particles, dust, the researcher's hands, and grinding. These problems have been thoroughly discussed by Hood et al. (1944) and Mitchell (1960).

The variation between analyses of the same sample is usually much less than the variation among different samples. Hence close attention should be paid to the sampling technique.

Yanagisawa and Takahashi (1964) have computed the number of samples which should be taken to give the desired precision at a given coefficient of variation. In general, if a precision of 10 percent is desired, collect 10 to 20 samples from the same field.

Person-to-person and laboratory-to-laboratory error is difficult to assess but it may be quite large. One method of assessing the magnitude of these variations is the use of standard samples (Bowen 1965).

Table 2 is an example to demonstrate magnitude of errors in chemical analysis of plant samples when many people analyzed subsamples from the Same sample bottle. Each analyst made four determinations on the subsamples, following the procedures described in this manual.

Table 2. Analysis of standard plant sample by seven persons.

Analyst	K (%)	Mg (%)	Mn (ppm)	Cu (ppm)	Zn (ppm)
A	2.52±0.03	—	17.1±0.2	6.7±0.7	28.4±1.1
B	2.55±0.01	—	18.2±0.5	7.3±1.1	29.0±0.7
C	2.46±0.01	0.178±0.000	17.5±0.0	5.6±0.1	29.4±0.5
D	2.42±0.01	0.121±0.009	20.0±0.0	5.0±0.1	35.0±0.4
E	3.16±0.08	0.176±0.003	15.6±1.3	9.1±2.3	39.7±6.3
F	2.76±0.03	0.162±0.025	14.1±0.9	3.7±0.1	31.3±1.0
G	2.44±0.01	—	17.6±0.1	—	28.9±0.2

Within the person, the error was small but between the persons it was large, particularly for copper. Therefore, caution must be taken to make a straight comparison of analytical values reported by different persons or different laboratories. The above data were obtained when each analyst knew he was participating in analytical trial for accuracy and precision. Hence, it would be likely that we would encounter larger errors than the above in our routine analysis.

Errors are often larger when a large number of samples are being prepared and analyzed. Under these circumstances, include several standard samples in the analysis.

References

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Tanaka, A. and S. Yoshida. 1970. Nutritional disorders of rice in Asia. *Int. Rice Res. Inst. Tech. Bull.* 10. 51 p.

Yanagisawa, M. and J. Takahashi. 1964. Studies on the factors related to the productivity of paddy soils in Japan with special reference to the nutrition of the rice plants. *Bull. Natl. Inst. Agr. Sci., Ser. B* 14:41-171.

This manual assumes the reader is familiar with inorganic and analytical chemistry at the undergraduate level.

No attempt is made to describe the principles underlying the procedures used, therefore the student is expected to both understand the principles involved and be able to derive the various formulae and constants given in the text.

The following are the recommended procedures for chemical analysis of rice tissues.

Element or constituent	Digestion or extraction	Method of analysis
N	Kjeldahl method	Volumetric
P	Ternary mixture digestion	Colorimetric
Al	Ternary mixture digestion	Colorimetric
Fe	Ternary mixture digestion	Colorimetric
Si	Ternary mixture digestion or dry ashing	Gravimetric
K } Na } Ca }	HCl extraction or water extraction	Flame photometric
Mg } Mn } Zn } Cu }	HCl extraction	Atomic absorption spectrophotometric
B	HCl extraction	Colorimetric
Cl	Water extraction	Volumetric
Chlorophyll	Acetone extraction	Colorimetric
Sugar	Alcohol extraction	Colorimetric
Starch	Perchloric acid extraction	Colorimetric

At the end of each section, references are given for specific topics mentioned in that chapter. Some general references on analytical procedures are listed below.

References

- Black, C. A. (Ed.) 1965. Methods of soil analysis. Part 2. Chemical and microbiological properties. American Society of Agronomy, Inc., Madison, Wisconsin. 1572 p.

- Chapman, H. D. and P. F. Pratt. 1961. Methods of analysis for soils, plants, and waters. University of California, Riverside. 309 p.
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Equipment Micro-Kjeldahl distillation apparatus (obtained from Arthur H. Thomas Co., Philadelphia 5, Pa., U. S. A.), 100-ml Kjeldahl flasks, 125-ml Erlenmeyer flask, quick delivery 10-ml pipettes.

Sample preparation

Reagents

- 1) Concentrated sulfuric acid.
- 2) Salt mixture. Mix 250 g K_2SO_4 or Na_2SO_4 with 50 g $CuSO_4 \cdot 5H_2O$, and 5 g metallic selenium (i.e. 50:10:1 ratio).

Procedure

Place 200 mg of dried sample in a 100-ml Kjeldahl flask. Add approximately the same weight of salt mixture and 3 ml of concentrated H_2SO_4 . Place the Kjeldahl flask in an empty tin can of suitable size and heat over a flame to digest the sample. When the sample is clear, cool it and then add 10 ml of distilled water. Mix thoroughly and allow the sample to cool again.

Sample analysis

Reagents

- 1) Boric acid, 4 percent. Dissolve 40 g H_3BO_3 in 1 liter of distilled water. The concentration of this reagent need not be precise as long as the amount of boric acid is more than chemically equivalent to the amount of ammonia to be absorbed.
- 2) Mixed indicator. Dissolve 0.3 g of bromcresol green and 0.2 g methyl red in 400 ml of 90 percent ethanol. The indicator color will change from red in acid solution to blue in alkaline solution.
- 3) Sodium hydroxide, 40 percent. Under a fume hood, dissolve 400 g of technical grade NaOH in a beaker containing 600 ml of distilled water. Place the beaker in a cold water bath to dissipate the heat produced. When cool, store the solution in a screw-top bottle.
- 4) Sodium carbonate. Transfer 10 to 20 g of AR grade Na_2CO_3 to a Pyrex beaker and heat at 270 C for 3 hours. Cool the beaker in a desiccator.
- 5) Methyl orange indicator. Dissolve 0.1 g of methyl orange in 100 ml of distilled water.
- 6) Standard hydrochloric acid, 0.1 N. Dilute 9 ml of concentrated HCl to 1 liter with distilled water. Standardize this approximate 0.1 N HCl solution as follows: Dissolve exactly 0.530 g of the sodium carbonate reagent 20 ml of distilled

water. Dilute to 100 ml. Transfer 10 ml of this 0.1 N sodium carbonate solution to a 125-ml Erlenmeyer flask. Add two drops of methyl orange indicator. Titrate the approximate 0.1 N HCl solution into the 0.1 N sodium carbonate until the methyl orange indicator turns reddish orange. Boil the solution gently for 1 minute and then cool to room temperature by running tap water over the outside of the flask. If the color changes back to orange, titrate more HCl until the first faint but permanent reddish-orange color appears in the solution.

Calculation

$$\text{Normality of HCl} = \frac{0.1 \times 10}{\text{ml of HCl titrated}}$$

7) Standard hydrochloric acid, 0.05 N. Transfer 500 ml of the standardized 0.1 N HCl to a 1-liter volumetric flask and make up to volume with distilled water.

Procedure

Distillation. Empty the Kjeldahl flask containing the digested sample into the micro-Kjeldahl distillation apparatus. Rinse the flask three times with distilled water, each time emptying the rinse water into the distillation apparatus. Use a minimum amount of water. Then with a quick delivery pipette, add 10 ml of the 40 percent NaOH to the distillation apparatus.

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Prepare a 125-ml Erlenmeyer flask containing 10 ml of 4 percent boric acid reagent and three drops of mixed indicator. Place the flask under the condenser of the distillation apparatus, and make sure that the tip of the condenser outlet is beneath the surface of the solution in the flask.

Allow steam from the boiler to pass through the sample, distilling off the ammonia into the flask containing boric acid and mixed indicator solution.

Distill the sample for 7 minutes. Then lower the flask and allow the solution to drop from the condenser into the flask for about 1 minute. Wash the tip of the condenser outlet with distilled water.

Titration. Titrate the solution of boric acid and mixed indicator containing the "distilled off" ammonia with the standardized HCl.

Note

- a) Use the standardized 0.1 N HCl for samples containing 1.5 to 4 percent nitrogen.
- b) Use the standardized 0.05 N HCl for samples containing less than 1.5 percent nitrogen.
- c) Try to have 3 titration value of more than 2 ml so that the titration error will be negligible.

d) Determine the titration value of a blank solution of boric acid and mixed indicator.

Calculation

$$\% \text{ nitrogen in sample} = \frac{(\text{sample titer} - \text{blank titer}) \times \text{normality of HCl} \times 14 \times 100}{\text{Sample weight (g)} \times 1000}$$

Equipment Spectrophotometer, 75-ml Pyrex test tubes graduated at 50 ml, filter funnels, and Whatman filter papers Nos. 1 and 44, pH meter.

Sample preparation

Reagent

Acid mixture. Prepare a mixture containing 750 ml concentrated HNO_3 , 150 ml concentrated H_2SO_4 , and 300 ml 60 to 62 percent HClO_4 .

Procedure

Put 1.00 g of dried, ground, plant material into a 75-ml Pyrex test tube. Add 10 ml of acid mixture and allow to predigest under a fume hood for at least 2 hours. Then heat over a low gas flame. If you heat the test tube too rapidly, some of the sample may be lost from the test tube due to excessive frothing. Gradually increase the heat until the mixture becomes clear. Do not evaporate to dryness. Cool and fill the test tube up to the 50-ml mark with distilled water. Filter the sample extract through an acid-washed filter paper (Whatman No. 1).

Note

- a) Phosphorus will be lost if you allow the digestion to go to dryness.
- b) If aluminum is to be determined, continue digesting the mixture until the volume has been reduced to 0.5 ml to remove as much acid mixture as possible.
- c) If silica is to be determined, use ashless Whatman filter paper No. 44, and then keep the filter paper and residue for the crude silica determination.

Sample analysis :
Phosphorus

Reagents

- 1) Molybdate-vanadate solution. Dissolve 25 g ammonium molybdate ($(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$) in 500 ml of distilled water. Dissolve 1.25 g ammonium vanadate (NH_4VO_3) in 500 ml of 1 N HNO_3 . Then mix equal volumes of these two solutions. Prepare a fresh mixture each week.
- 2) Nitric acid, 2 N. Dilute 10 ml concentrated HNO_3 to 80 ml with distilled water.
- 3) Standard phosphorus solution. Dissolve 0.110 g monobasic potassium phosphate (KH_2PO_4) in distilled water and dilute to 1 liter. This solution contains 25 ppm phosphorus.

Prepare each of the standards (below) by placing the amount of 25-ppm solution indicated in a 10-ml test tube. Add 2 ml of 2 N HNO₃ to each tube and then dilute to 8 ml with distilled water.

P standards (ppm)	Milliliter of 25-ppm P solution to add to a 10-ml tube
2.5	1
5.0	2
7.5	3
10.0	4
12.5	5
15.0	6

Procedure

Put 1 ml of the sample extract into a 10-ml tube. Add 2 ml of 2 N HNO₃ and dilute to 8 ml with distilled water. To tubes containing sample extract or standards, add 1 ml of the molybdate-vanadate solution and then make up to 10 ml with distilled water. Shake and allow the tubes to stand for 20 minutes. Measure absorbance at 420 m μ and compare with the absorbance of the phosphorus standards.

Comments

Temperature and acidity affect the color intensity. Absorbance values of the sample and standard cannot be compared if their colors are developed at temperatures differing by 10 C or more. Instead of using 2 N HNO₃ in the above procedure you can use 2 N HClO₄ but the standards must then be made up in 2 N HClO₄. The final acidity for color development should be in the range from 0.3 to 0.8 N. The above procedure gives an acidity of 0.4 N. Hence the inclusion of additional nitric or perchloric acid of less than 0.1 N from the extraction procedure can practically be neglected.

This method is best suited for samples of high phosphorus content such as in rice tissue.

References

- Black, C. A. (Ed.) 1965. Method of soil analysis. Part 2. Chemical and microbiological properties. American Society of Agronomy, Inc., Madison, Wisconsin. 1572 p.
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Sample analysis:
Iron

Reagents

- 1) Hydroquinone. Prepare a 1 percent solution in distilled water. If color develops, discard and make a new solution.
- 2) Sodium citrate. Dissolve 250 g sodium citrate in water and dilute to 1 liter with distilled water.
- 3) Ortho-phenanthroline. Dissolve 0.5 g ortho-phenanthroline in distilled water and dilute to 100 ml. Warm the flask in a water bath to dissolve the chemical faster. Store the solution in a dark bottle or in a dark place. If color develops, discard and make a new solution.
- 4) Iron standards. Place 0.100 g electrolytic iron in a 100-ml beaker. Cover with a watch glass and then add 50 ml of 1:3 (v/v) HNO₃. Boil until the brown fumes of nitrous oxide are no longer evolved. Cool and dilute to 1 liter with distilled water. This solution contains 100 ppm Fe. Take a 10-ml aliquot of this solution and make up to 100 ml with distilled water. This solution contains 10 ppm Fe. Prepare each of the iron standards (below) by placing the amount of 10-ppm solution indicated in a 25-ml volumetric flask and make up to volume with distilled water.

Fe standards (ppm)	Milliliters of 10-ppm Fe solution to add to a 25-ml volumetric flask
0	0
0.4	1
0.8	2
2.0	5
4.0	10

Procedure

Put 10 ml of the sample extract or standard into a 25-ml volumetric flask. Add 1 ml of hydroquinone reagent and 1 ml of the orthophenanthroline reagent. Add the predetermined (see note) amount of sodium citrate required to bring the pH to 3.5. Then make up to volume with distilled water. Heat the flask in a water bath for 1 hour to completely reduce the iron. Read absorbance at 508 mμ and compare with the absorbance of the iron standards.

Note Using an aliquot of the sample and the standard, determine the amounts of sodium citrate required to bring the pH to 3.5.

Comments

An orange-red complex forms when ortho-phenanthroline reacts with ferrous iron. The color intensity is independent of acidity between pH 2.0 to 9.0. The standards and sample solutions should have the same final pH and are therefore buffered with sodium citrate at pH 3.5. The colored complex will remain stable for several months.

Reference

Sandell, E. B. 1950. Iron, p. 522-554. In Colorimetric determination of traces of metals, 3rd ed. rev. Interscience Publishers, Inc., New York. 1032 p.

Sample
analysis:
Manganese

Reagents

- 1) Acid solution. Mix 400 ml concentrated HNO₃ with 200 ml of distilled water. Dissolve 75 g HgSO₄ in this solution and then add 200 ml of 85 percent H₃PO₄. Dissolve 0.035 g AgNO₃ in this solution and make up to 1 liter with distilled water.
- 2) Ammonium persulfate. Store the ammonium persulfate crystals [(NH₄)₂S₂O₈] in a desiccator.
- 3) Manganese stock solution. Place 3.08 g AR grade MnSO₄·H₂O in a 100-ml beaker. Dissolve by carefully adding 50 ml of 1:1 (v/v) HCl. Transfer the solution to a 1-liter volumetric flask and make up to volume with distilled water. This solution contains 1000 ppm Mn.

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Prepare each of the standards (below) by placing the amount of 1000-ppm solution indicated in a 100-ml volumetric flask and make up to volume with distilled water.

Mn standards (ppm)	Milliliter of 1000-ppm Mn solution to add to a 100-ml volumetric flask
0	0
2	0.2
4	0.4
6	0.6
8	0.8
10	1.0
12	1.2

Procedure

Transfer 10 ml of the sample extract or standard to a 50-ml volumetric flask. Add 2.5 ml of the acid solution and make up to 40 ml with distilled water. Then add 0.5 g of ammonium persulfate. Place the flask in boiling water for 5 minutes. Then cool under running water and make up to 50-ml volume with distilled water. Read absorbance at 530 m μ and compare with the absorbance of the manganese standards.

Comment

Phosphoric acid is included in the procedure to avoid precipitation of ferric iron and manganese and to decolorize iron by complex formation.

Reference

Sandell, E. B. 1950. Manganese, p. 606-620. In Colorimetric determination of traces of metals, 3rd. ed. rev. Interscience Publishers, Inc., New York. 1032 p.

Sample analysis:
Aluminum

Reagents

- 1) Aluminum reagent. In separate beakers, dissolve 0.75 g ammonium aurine tricarboxylate, 15 g gum acacia, and 200 g ammonium acetate in distilled water. When each is dissolved, mix them together and add 190 ml concentrated HCl. Mix, filter, and dilute to 1.5 liters with distilled water.
- 2) Thioglycollic acid. Add 1 ml of thioglycollic acid (AR grade) to a 100-ml volumetric flask and make up to volume with distilled water.
- 3) Phenolphthalein. Dissolve 1 g phenolphthalein in 60 ml of absolute ethanol and 40 ml of distilled water.
- 4) Ammonium hydroxide (1:9). Dilute 10-ml concentrated NH_4OH to 100 ml with distilled water.
- 5) Aluminum standards. Prepare a 1000-ppm aluminum stock solution by dissolving 8.95 g $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ in 1 liter of distilled water.

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Transfer 1 ml of this stock solution to a 100-ml volumetric flask and make up to volume. This solution contains 10 ppm aluminum. Prepare each of the standards (below) by placing the amount of 10-ppm solution indicated in a 50-ml volumetric flask and make up to volume with distilled water.

<u>Al standards (ppm)</u>	<u>Milliliters of 10-ppm Al solution to add to a 50-ml volumetric flask</u>
0	0
0.2	1
0.4	2
0.6	3
0.8	4
1.0	5
1.2	6
1.4	7

Procedure

Transfer 1 to 5 ml of the sample extracts or standards (depending on amount of Al suspected) to test tubes graduated at 50-ml volume. Add a 2 to 3 drops of phenolphthalein and then add ammonium hydroxide (1:9) until the first pink color develops. Dilute to 20 ml with distilled water and add 2 ml of the thioglycollic acid solution. Mix and add 10 ml of the aluminum reagent. Then mix again.

Heat in a boiling water bath for 16 minutes. Use the same method of heating for both the standards and the samples. Cool for at least 1.5 hours and then make up to volume with distilled water. Mix and measure absorbance at 465 m μ or 537 m μ . The latter is more sensitive for samples containing low aluminum.

Note If the sample has too much iron (Fe precipitates out during the neutralization with NH₄OH), proceed as follows: Take some of the sample extract and neutralize it with excess 40 percent NaOH. Place the sample in a boiling water bath for 5 minutes, then centrifuge it and remove the supernatant by suction. Add 2 to 3 drops of phenolphthalein to the supernatant and then add HCl until the pink color just disappears. Then dilute to 20 ml with distilled water and proceed as described above by adding 2 ml of the thioglycollic acid solution, etc.

Comments

The aluminum reagent (ammonium salt of aurine tricarboxylic acid) reacts with aluminum to give a colored complex that is used as a basis for a colorimetric determination of aluminum. The method is very sensitive and is suitable for determining small amounts (as low as 5 μ g) of aluminum in plants and soil extracts.

Thioglycollic acid reacts with iron to form a colorless complex. This prevents interference from iron if the ratio of iron to aluminum does not exceed 20 to 1. The color of the aluminum complex will remain stable for 24 hours but then fades. There is always some color in the blank.

References

Chenery, E. M. 1948. Thioglycollic acid as an inhibitor for iron in the colorimetric determination of aluminum by means of "Aluminon." Analyst 73:501-502.

Sandell, E. B. 1950. Aluminum p. 219-253. In Colorimetric determination of traces of metals, 3rd ed. rev. Interscience Publishers, Inc., New York. 1032 p.

Sample
analysis :
Crude silica

Procedure

Dry the filter paper and residue of the sample extract in an oven at 80 C. Then char the paper with a naked flame under a fume hood and allow it to turn to ash by placing it in a muffle furnace for 2 hours at 550 C. Cool the ash in a desiccator for at least 2 hours before weighing. This gives an estimate of crude silica in 1 g of the dry plant sample.

Calculation

$$\text{Crude silica \%} = \frac{\text{Wt of crude silica (g)} \times 100}{\text{Wt of sample (g)}}$$

CHAPTER 5. An EDTA method for routine determination of calcium and magnesium in plant tissue and soil solution.

Equipment Tall 100-ml Pyrex beakers, oven, burette, centrifuge, and 15-ml centrifuge tubes .

Sample extraction

Reagents

- 1) Concentrated hydrochloric acid, 12 N A. R.
- 2) Hydrochloric acid, 2 N. Dilute 100 ml concentrated HCl to 600 ml with distilled water.
- 3) Ferric chloride (3 mg Fe/ml). Dissolve 3.66 g $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 250 ml of distilled water containing 1 ml concentrated HCl.
- 4) Sodium acetate, 10 percent. Dissolve 100 g sodium acetate trihydrate in distilled water and dilute to 1 liter.
- 5) Sodium hydroxide, 0.4 N. Dissolve 8 g NaOH in distilled water and dilute to 500 ml.
- 6) Bromine water. Prepare a saturated solution of bromine by adding AR grade Bromine to distilled water until droplets of excess bromine can be seen in the solution.
- 7) Ammonium chloride, 25 percent. Dissolve 250 g NH_4Cl in distilled water and dilute to 1 liter.
- 8) Ammonium hydroxide, 0.6 N. Dilute 42 ml of concentrated NH_4OH (specific gravity 0.89), to 1 liter with distilled water.

Procedure

Place 2 g of oven-dried ground plant material in a tall, 100-ml Pyrex beaker and heat in a muffle furnace for 2 hours at 550 C. Then add 10 ml concentrated HCl to the ash and evaporate to dryness. Then add 5 ml of 2 N HCl and dilute to 50 ml with distilled water.

Depending on the calcium or magnesium concentration expected in the sample, pipette 2 or 4 ml of the sample extract (either plant or soil solution) into a 15-ml centrifuge tube and add 0.5 ml ferric chloride solution. Spin the tube by hand and then add 2 ml sodium acetate solution. Spin the tube by hand again and then add 2 ml 0.4 N sodium hydroxide. Add 1 ml bromine water and digest for 1 hour at 95 C to flocculate the manganese dioxide and to expel the excess bromine.

Add 2 ml of 25 percent ammonium chloride. Spin the tube by hand and then digest for 15 minutes at 80 C. Add 1.0 ml of 0.6 N ammonium hydroxide. Spin the tube by hand again and digest for another 5 minutes at 80 C to flocculate the precipitate. Then while hot, centrifuge the tube for at least 5 minutes. Decant the sample extract into a 250-ml beaker.

Sample
analysis:
Calcium

Reagents

- 1) KOH, 8 N. Dissolve 448.9 g KOH in 1 liter distilled water.
- 2) Dotite NN (Wako Jun Yaku, Kogyo Co., Ltd., Tokyo, Japan).
- 3) Calcium stock solution (5.00 mg/ml). Place 6.244 g of dried reagent grade CaCO₃ in a 500-ml beaker. Cover with a Pyrex watch glass and add 100 ml of distilled water. Then slowly add 150 ml of 1 N HCl. When the CaCO₃ has dissolved, boil gently for 3 minutes. Allow the beaker to cool and then transfer the solution to a 500-ml volumetric flask. Make up to volume with distilled water. This solution contains 5.00 mg/ml Ca.

Dilute 10 ml of this solution to 100 ml with distilled water to obtain a standard solution containing 0.50 mg/ml Ca.

- 4) Standard EDTA, 0.005 M. Dissolve 1.861 g disodium ethylene-diaminetetraacetate (Na₂ EDTA • 2H₂ O) in distilled water and dilute to 1 liter. Store in a polyethylene bottle. Standardize the EDTA solution as follows: Transfer 5 ml of the 0.50 mg/ml Ca standard solution to a 200-ml Erlenmeyer flask. Add 80 ml of distilled water and 5 ml 8 N KOH to adjust the pH to 12. Add a pinch of Dotite NN to the flask and then titrate the EDTA solution against the Ca standard until a permanent blue color develops. Obtain the blank titration value by titrating the EDTA solution against the blank containing 5 ml 8 N KOH, 80 ml distilled water and a pinch of Dotite NN until a permanent blue color develops.

Correct the titration value of the Ca standard solution by subtracting the titration value of the blank. Calculate the molarity of the EDTA solution.

Note One milliliter of a 0.005 M EDTA solution is equivalent to 0.2204 mg Ca.

Procedure

Add 80 ml of distilled water and 5 ml 8 N KOH to the sample extract to adjust the pH to 12. Add a pinch of Dotite NN and then titrate with standard EDTA until a permanent blue color develops. Correct for a blank carried throughout the entire procedure.

Calculations

For a 2-ml sample

$$\% \text{ Ca} = \frac{\text{0.005 M EDTA titer} \times 0.250}{\text{}} \times 100$$

For a 4-ml sample

$$\% \text{ Ca} = 0.005 \text{ M EDTA titer} \times 0.125$$

Sample
analysis:
Magnesium
plus calcium

Reagents

- 1) Buffer solution, (pH 10). Dissolve 67.5 g NH_4Cl in 400 ml of water. Add 570 ml concentrated NH_4OH and dilute to 1 liter with distilled water.
- 2) Superchrome Black TS or Erichrome Black T (Merck Aktiengesellschaft, 61 Darmstadt, Germany). Dissolve 50 mg Superchrome Black TS or Erichrome Black T in 20 ml of distilled water. Prepare a fresh solution daily.
- 3) Magnesium stock solution (5.00 mg/ml). Put 2.500 g unoxidized Mg metal (reagent grade) in a 500-ml beaker. Cover the beaker with a Pyrex watch glass and add 150 ml of distilled water. Then carefully add 20 ml 1:1 (v/v) HCl. Although the magnesium dissolves in the acid very rapidly, the solution should be boiled gently-for 3 minutes to make sure this process is complete. Transfer this solution to a 500-ml volumetric flask and make up to volume with distilled water. This solution contains 5.00 mg/ml Mg. Dilute 10 ml of this solution to 100 ml with distilled water to obtain a standard solution containing 0.50 mg/ml Mg.
- 4) Standard EDTA, 0.005 M. Prepare as outlined in reagents for sample analysis of calcium (above). Standardize the EDTA solution as follows: Transfer 5 ml of the 0.50 mg/ml Mg standard solution to a 200-ml Erlenmeyer flask. Then add 80 ml of distilled water and 5 ml of buffer solution (pH 10).

Add six drops of superchrome Black TS or Erichrome Black T and titrate with the EDTA solution until a permanent blue color develops. Obtain the blank titration value by titrating the EDTA solution against the blank containing 5 ml buffer solution (pH 10), 80 ml of distilled water and six drops of Superchrome Black TS or Erichrome Black T, until a permanent blue color develops.

Correct the titration value of the Mg standard solution by subtracting the titration value of the blank. Calculate the molarity of the EDTA solution.

Note One milliliter of 0.005 M EDTA solution is equivalent to 0.1216 mg Mg.

Procedure

Add 80 ml of distilled water and 5 ml of buffer solution (pH 10) to the sample extract. Stir and add six drops of superchrome Black TS or Erichrome Black T. Titrate with standard EDTA until a permanent blue color develops. Correct for a blank carried throughout the entire procedure. This titration determines the sum of calcium and magnesium.

Calculations

For a 2-ml sample

$$\% \text{ Mg} = [(0.005 \text{ M EDTA titer for Ca + Mg}) - (0.005 \text{ M EDTA titer for Ca})] \times 0.125$$

For 4-ml sample

$$\% \text{ Mg} = [(0.005 \text{ M EDTA titer for Ca + Mg}) - (0.005 \text{ M EDTA titer for Ca})] \times 0.076$$

Comments

Many procedures are available for the EDTA titration method in which iron and manganese are complexed with cyanide and triethanolamine, and in which phosphate is removed by ion-exchange resin. The most reliable results, however, are obtained by completely removing these extraneous ions chemically. The above procedure may seem very tedious, but if the pipetting system can be improved by use of automatic dispensing burettes, it is relatively easy.

Barrows and Simpson (1962) have proposed separating calcium from magnesium by precipitating calcium sulfate in alcohol and directly titrating calcium and magnesium.

References

- Barrows, H. L. and E. C. Simpson. 1962. An EDTA method for the direct routine determination of calcium and magnesium in soils and plant tissue. *Soil Sci. Soc. Amer., Proc.*, 26:443-445.
- Cheng, K. L. and R. H. Bray. 1951. Determination of calcium and magnesium in soil and plant material. *Soil Sci.* 72:449-457.
- Greweling, T. 1960. The chemical analysis of plant materials. Cornell University, Ithaca, N.Y., U. S. A. (mimeographed).

CHAPTER 6. Procedures for routine analyses of zinc, copper, manganese, calcium, magnesium, potassium, and sodium by atomic absorption spectrophotometry and flame photometry.

Equipment Atomic absorption spectrophotometer Perkin Elmer Model 303 for analysis of Zn, Cu, Mn, Ca, and Mg, EKO Flame Photometer for analysis of K and Na, 100-ml polystyrene bottles, filter funnels, Whatman No. 1 filter paper, pipettes, vials graduated at 40-ml, and 10-ml graduated tubes. Bottles of compressed air and acetylene, ion exchanger, research model (Illinois Water Treatment Co., Rockford, Illinois, U.S.A.).

Sample extraction

Reagents

- 1) Deionized-distilled water. Prepare by passing distilled water through the ion exchanger (max. flow rate, 315 cc/min). Deionized-distilled water is the only type of water used in the entire procedure.
- 2) Hydrochloric acid, 1 N. Prepare in batches of 18 liters at a time. Add 1.5 liters of concentrated HCl to 16.5 liters of deionized-distilled water. The same acid should be used by all operators.

Procedure

Place 1.00 g of dried, ground plant sample into a dry 100-ml polystyrene bottle that has been rinsed with 1 N HCl. Add 25 ml of 1 N HCl from an automatic dispensing burette. Be sure the acid thoroughly wets the sample. Avoid shaking the bottle so as to keep all of the sample in the acid. Allow to stand for 24 hours. Then shake briefly and filter through Whatman No. 1 filter paper. Collect in a 100-ml polystyrene bottle. Prepare a 25-ml 1 N HCl blank at the same time using the same procedure.

Sample preparation:
Calcium

Reagent

Lanthanum solution, 5 percent. Weigh exactly 58.65 g La_2O_3 . Wet with 100 ml of deionized-distilled water and then very slowly add 250 ml of 1:1 (v/v) HCl. When the lanthanum has dissolved, transfer the solution to a 1-liter volumetric flask and make up to volume with deionized-distilled water.

Procedure

Pipette 1 ml of the sample extract into a 10-ml graduated tube, Pipette 2 ml of the 5-percent lanthanum stock solution into the tube and dilute to the 10-ml mark on the tube with 1 N HCl. Seal the tubes with plastic stoppers. This solution is used for the analysis of calcium.

Sample preparation:
Magnesium,
potassium,
and sodium

Procedure

Pipette 2 ml of the sample extract into a vial graduated at 40 ml. Dilute to 40 ml with 1 N HCl. Seal the vial with a plastic lid.

Sample preparation:
Zinc,
copper, and
manganese

Procedure

The remaining sample extract can be used without further dilution for the analysis of Zn, Cu, and Mn.

Comment

Additional dilution may be necessary when the concentration of any element in the sample to be analyzed is exceptionally high.

Stock solutions and standards

1) Zinc, 1000-ppm stock solution. Put 1.000 g of zinc metal (AR grade) into a 100-ml beaker. Cover the beaker with a Pyrex watch glass and dissolve the zinc by slowly adding 50 ml of 1:1 (v/v) HCl. Boil the solution gently for 3 minutes to be sure the zinc is completely dissolved, then put the solution into a 1-liter volumetric flask. Make up to volume with deionized-distilled water.

From this 1000-ppm stock solution, transfer exactly 10 ml to a 100-ml volumetric flask and make up to volume with 1 N HCl. This gives a 100-ppm zinc solution. Use this solution for preparation of working standards.

Prepare each of the standards (below) by placing the amount of 100-ppm solution indicated in a 100-ml volumetric flask. Make-up to volume with 1 N HCl.

Zn standards (ppm)	Milliliters of 100-ppm Zn solution to add to a 100-ml volumetric flask
0	0
0.25	0.25
0.50	0.50
0.75	0.75
1.00	1.00

2) Copper, 100-ppm stock solution. Put 0.100 g of copper metal (AR grade) into a 100-ml beaker. Cover the beaker with a Pyrex watch glass and carefully add 50 ml of 1:1 (v/v) nitric acid. Gently boil the solution for a few minutes until brown fumes of nitrous oxide are no longer evolved. Allow the beaker to cool and then transfer the copper solution to a 1-liter volumetric flask. Make up to volume with deionized-distilled water.

Prepare each of the standards (next page) by placing the amount of 100-ppm solution indicated in a 100-ml volumetric flask. Make up to volume with 1 N HCl.

Cu standards (ppm)	Milliliters of 100-ppm Cu solution to add to a 100-ml volumetric flask
0	0
0.5	0.5
1.0	1.0
1.5	1.5

3) Manganese, 1000-ppm stock solution. Place 3.08 g of AR grade $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ into a 100-ml beaker. Dissolve by carefully adding 50 ml of 1:1 (v/v) HCl. Transfer the solution to a 1-liter volumetric flask and make up to volume with deionized-distilled water.

Prepare each of the standards below by placing the amount of 1000-ppm solution indicated in a 100-ml volumetric flask. Make up to volume with 1 N HCl.

Mn standards (ppm)	Milliliters of 1000-ppm Mn solution to add to a 100-ml volumetric flask
0	0
5	0.5
10	1.0
15	1.5
20	2.0

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4) Magnesium, 1000-ppm stock solution. Put 1.000 g of unoxidized Mg metal (reagent grade) into a 100-ml beaker. Cover the beaker with a Pyrex watch glass and very carefully add 50 ml of 1:1 (v/v) HCl. Boil the solution gently for a few minutes to ensure that the magnesium dissolves completely. Then transfer the solution to a 1-liter volumetric flask and make up to volume with deionized-distilled water.

Prepare each of the standards (below) by placing the amount of 1000-ppm solution indicated in a 100-ml volumetric flask. Make up to volume with 1 N HCl.

Mg standards (ppm)	Milliliters of 1000-ppm Mg solution to add to a 100-ml volumetric flask
0	0
2	0.2
4	0.4
6	0.6
8	0.8
10	1.0

5) Calcium, 1000-ppm stock solution. Put 2.497 g CaCO_3 (AR grade) into a 100-ml beaker. Cover the beaker with a watch glass and very carefully add 50 ml of 1 N HCl. The reaction is quite vigorous and care should be taken to

prevent any loss by splattering. Gently boil the solution for 3 minutes. Allow the beaker to cool and then transfer to a 1-liter volumetric flask. Make up to volume with deionized-distilled water.

Prepare each of the standards (below) by placing the amount of 1000-ppm solution indicated in a 100-ml volumetric flask. Add 20 ml of the 5 percent lanthanum stock solution, then make up to volume with 1 N HCl.

<u>Ca standards (ppm)</u>	<u>Milliliters of 1000-ppm Ca solution to add to a 100-ml volumetric flask</u>
0 in 1% La	0
5 in 1% La	0.5
10 in 1% La	1.0
15 in 1% La	1.5
20 in 1% La	2.0

6) Potassium, 1000-ppm stock solution. Dissolve 1.907 g of dried AR grade KCl in 1 liter of deionized-distilled water.

Prepare each of the standards (below) by placing the amount of 1000-ppm solution indicated in a 100-ml volumetric flask. Make up to volume with 1 N HCl.

<u>K standards (ppm)</u>	<u>Milliliters of 1000-ppm K solution to add to a 100-ml volumetric flask</u>
0	0
20	2.0
40	4.0
60	6.0
80	8.0
100	10.0

7) Sodium, 1000-ppm stock solution. Dissolve 2.542 g dried AR grade NaCl in 1 liter of deionized-distilled water.

Prepare each of the standards (below) by placing the amount of 1000-ppm solution indicated in a 100-ml volumetric flask. Make up to volume with 1 N HCl.

<u>Na standards (ppm)</u>	<u>Milliliters of 1000-ppm Na solution to add to a 100-ml volumetric flask</u>
0	0
20	2.0
40	4.0
60	6.0
80	8.0
100	10.0

Note Do not contaminate the stock solutions in any way. When preparing working standards, pour a small amount of the stock solution into a beaker and pipette the required amount from this. Store all stock solutions and standards in polystyrene or Pyrex bottles.

Specifications
for individual
element
analysis

Use the atomic absorption spectrophotometer according to the instruction manual.

Zinc

Standards (ppm): 0, 0.25, 0.50, 0.75, 1.0

Range: UV

Wavelength: 214

Source: 15 ma (according to lamp operating current)

Slit: 5

Scale: 5

Filter: OUT

Meter response: 2

Zero knob position: 2.20

Burner height: 0.4

Air flow: 9.0

Fuel flow: 9.0 (Acetylene)

Plot percent absorption against the standards.

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Copper

Standards (ppm): 0, 0.5, 1.0, 1.5

Range: UV

Wavelength: 324.6

Source: 30 ma (according to lamp operating current)

Slit: 4

Scale: 10

Filter: OUT

Meter response: 2

Zero knob position: 1.00

Burner height: 0.4

Air flow: 9.0

Fuel flow: 9.0 (Acetylene)

Plot percent absorption against the standards.

Manganese

Standards (ppm): 0, 5, 10, 15, 20

Range: UV

Wavelength: 279.9

Source: 30 ma (according to lamp operating current)

Slit: 4

Scale: 2

Filter: OUT

Meter response: 2

Zero knob position: 1.01
Burner height: 0.4
Air flow: 9.0
Fuel flow: 9.0 (Acetylene)

Divide the percent absorption by 2. Convert to absorbance using the tables provided with the instrument, and plot absorbance against the standards.

Calcium

Standards (ppm): 0, 5, 10, 15, 20
Range VIS
Wavelength: 211.9
Source: 15 ma (according to lamp operating current)
Slit: 4
Scale: 2
Filter: OUT
Meter response: 2
Zero knob position: 0.57
Burner height: 0.3
Air flow: 7.5
Fuel flow: 9.0 (Acetylene)

Divide percent absorption by 2, convert to absorbance using tables provided with the instrument and plot absorbance against the standards.

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Magnesium

Standards (ppm): 0, 2, 4, 6, 8, 10
Range: UV
Wavelength: 285.4
Source: 15 ma (according to lamp operating current)
Slit: 5
Scale: 2
Filter: OUT
Meter response: 2
Zero knob position: 1.05
Burner height: 0.3
Air flow: 8.0
Fuel flow: 9.0 (Acetylene)

Divide the percent absorption by 2. Convert to absorbance using the tables provided with the instrument, and plot absorbance against the standards.

Note The zero knob and burner positions specified above are only approximate to help you check your settings. Although the instrument does not have any scale for setting the zero knob, the positions specified for individual element analysis can be obtained as follows. For example, for magnesium, you must set the zero knob at 1.05 (see above). First turn the zero knob counterclockwise until it stops. Then turn the knob to the 5-minute position (i.e. read the second figure as you read a clock). Using zinc as another example, the specified setting for the zero knob is 2.20. Turn the zero knob counterclockwise until it stops.

Then, turn the knob two complete clockwise revolutions. Next turn the knob to the 20-minute position.

Wavelength setting: This may vary slightly from the figure listed. Always set at the position which gives maximum deflection of the energy needle to the right.

Potassium
(Flame
photometer)

Standards (ppm): 0, 20, 40, 60, 80, 100

Use the EKO Photometer according to the instruction manual. Plot the meter reading against the standards.

Sodium
(Flame
photometer)

Standards (ppm): 0, 20, 40, 60, 80, 100

Use the EKO Flame Photometer according to the instruction manual. Plot the meter reading against the standards.

Comments

The 1 N HCl extraction procedure (Kushizaki 1968) is excellent in its simplicity and reproducibility. The HCl extraction procedure and the standard acid digestion procedure agree with each other within the acceptable range of errors.

For samples such as grains which contain high phosphorus but low calcium, remove phosphorus interference either by an ion exchange method (Leyton 1954, Hemingway 1956, Hinson 1962) or by addition of magnesium and sulfuric acid (David 1959) prior to calcium determination.

References

David, D. J. 1959. Determination of calcium in plant material by Atomic absorption spectrophotometry. *Analyst*. 84:536-545.

Eiko-Seiki Co., Ltd. Instruction manual for the EKO flame photometer. Otomachi 2-4, Chiyoda-ku, Tokyo, Japan.

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Hinson, W. H. 1962. Ion exchange treatment of plant ash extracts for removal of interfering anions in the determination of calcium by atomic absorption. *Spectrochim. Acta*. 18:427-429.

Kushizaki, M. 1968. An extraction procedure of plant materials for the rapid determination of Mn, Cu, Zn, and Mg by the Atomic absorption analysis. *J. Sci. Soil Manure, Japan* 39:489-490.

Leyton, L. 1954. Phosphate interference in Flame photometric determination of calcium. *Analyst.* 79:497-500.

Perkin-Elmer Corp. 1971. Analytical methods for atomic absorption spectrophotometry. Norwalk, Connecticut, U. S. A.

Equipment Four-liter and 125-ml Pyrex, Glass-stoppered, separating funnels and racks for holding them, 1-liter Pyrex bottle, filter funnel and Whatman filter paper No. 1.

Purification
of 0.05%
dithizone
solution

Reagents

- 1) Dithizone (Diphenylthiocarbazone).
- 2) Carbon tetrachloride (AR grade).
- 3) Ammonium hydroxide (0.02 N). Add 1 liter of deionized-distilled water (see Chapter 6) to a clean 4-liter separating funnel and then add 1.25 ml of 58 percent W/V NH₄OH (AR grade).
- 4) Concentrated hydrochloric acid (AR grade).
- 5) Ethanol (AR grade)

Procedure

Dissolve 0.5 g dithizone in 250 ml of carbon tetrachloride. Filter this solution through a Whatman No. 1 filter paper into a 4-liter separating funnel containing 1 liter of approximately 0.02 N ammonium hydroxide solution. Shake the separating funnel for 10 minutes until most of the dithizone passes into the aqueous phase and becomes bright orange in color. Discard the carbon tetrachloride phase.

Add 50 ml of carbon tetrachloride and shake for 5 minutes. Once again, discard the carbon tetrachloride phase. Add another 50 ml of carbon tetrachloride and repeat the procedure until the carbon tetrachloride phase is a true green color. Run out and discard the carbon tetrachloride phase after each shaking.

Then add 1 liter of carbon tetrachloride to the aqueous phase. Carefully add 1.5 ml of concentrated HCl to produce an acid reaction approximately equivalent to 0.02 N. When acidified, the aqueous phase will change color from orange to dark purple. Shake the separating funnel for 10 minutes to extract the purified dithizone into the carbon tetrachloride phase.

Put about 10 ml of this dithizone solution into a 1-liter Pyrex, glass-stoppered bottle. Swirl the green solution around to completely cover the inside of the bottle. Then rinse the bottle several times with carbon tetrachloride. If any trace of red develops on the inside of the bottle, continue rinsing it with more dithizone solution followed by carbon tetrachloride until only a pale-green dithizone solution can be seen. This indicates that the bottle is free from heavy metal contaminants.

Finally run the remaining dithizone solution from the separating funnel into the Pyrex bottle. Discard the aqueous phase. Add a few drops of ethanol to the dithizone solution to prevent the accumulation of carbonyl chloride.

Then store the dithizone solution in a cool, dark place. It should remain good for several months if stored under the above conditions.

The concentration of the dithizone is approximately 0.05 percent.

Dithizone
test for
heavy
metals

Reagents

- 1) Purified dithizone solution (0.05 percent). Prepared according to the preceding procedure.
- 2) Carbon tetrachloride (AR grade).
- 3) Ammonium acetate, 0.5 M. Dissolve 19.27 g ammonium acetate (AR grade) in 500 ml of deionized-distilled water (see Chapter 6) and store in a Pyrex glass-stoppered bottle previously cleaned with dithizone solution and carbon tetrachloride. (Heavy metal contaminants in this solution can usually be neglected. However, if necessary the solution can be extracted with the dithizone solution using the following procedure.)

Procedure

Calibrate a glass-stoppered, Pyrex separating funnel at the 5, 10, and 100-ml volume levels. Carefully clean the inside of the separating funnel with several rinsings of dithizone solution and carbon tetrachloride as described in the procedure for purifying the dithizone solution. Finally rinse with carbon tetrachloride until the rinsing solution is colorless. Discard this solution.

Put 10 ml of carbon tetrachloride into the separating funnel and then add 0.1 ml of the purified dithizone solution. Place a stopper in the separating funnel and shake for 1 minute. Then run out to the 5-ml level. Next add the solution for testing to the separating funnel to make the volume up to the 100-ml level. Add 1 ml of the 0.5 M ammonium acetate solution. Then stopper and shake the separating funnel vigorously for 5 minutes.

If the carbon tetrachloride phase remains green then the test is negative, indicating heavy metal contamination is less than 0.001 ppm. When the carbon tetrachloride phase is faintly pink, the test is positive. If the carbon tetrachloride phase turns a definite pink or red-brown, heavy metal contamination is more than 0.02 ppm.

Comments

The above procedure is adapted from Hewitt (1966). The dithizone reacts with many metal ions to form color complexes. This complex formation is highly pH dependent (Sandell 1950). Among the elements encountered in routine plant and

soil analysis, cupric, zinc, cobalt and ferrous ions are dithizone reactive. However, the dithizone test is commonly used to give information on the presence or absence of copper and zinc. This is possible because the cobalt in plant tissue and water is quite low and the ferrous iron can easily be oxidized to ferric iron.

References

Hewitt, E. J. 1966. Sand and water culture methods used in the study of plant nutrition. Technical Communication No. 22 (Revised) of the Commonwealth Bureau of Horticulture and Plantation Crops, East Malling, Maidstone, Kent, England. Commonwealth Agricultural Bureaux. 547 p.

Sandell, E. B. 1950. Colorimetric determination of traces of metals, 2nd ed., Interscience Publishers, Inc., New York. 673 p.

Equipment Polyethylene centrifuge tubes, 15-ml capacity, with polyethylene stoppers; level shaker with rack for 24 centrifuge tubes; automatic dispensing burettes; white porcelain spot plates with 1-ml capacity depressions; polyethylene rod; nylon filter cloth; oven; boron-free, soft glass beakers; deionized distilled water (see Chapter 6); infra-red lamp.

Sample extraction

Reagent

Hydrochloric acid, 0.5 N. Transfer 42 ml of concentrated HCl to a 1-liter volumetric flask and make up to volume with deionized distilled water. Store in an automatic dispensing burette.

Procedure

Put 0.25 g of ground plant sample into a polyethylene centrifuge tube. Add 10 ml of 0.5 N HCl and then close the tube with a Polyethylene stopper. Shake the tube for 2 hours on a level shaker using the rack especially made for this purpose. Centrifuge the tubes at 2,000 rpm for 10 minutes and then filter the contents through a nylon cloth to obtain the sample extract.

Sample analysis

Preparation of the spot plate

Wash the spot plate with detergent and rinse it thoroughly with deionized distilled water. Apply a thin coating of 2 percent silicone oil (Shinetsu Chemicals Co., Tokyo) to the surface of the plate. Dry at 120 C for 30 minutes.

Reagents

1) Alcohol solution of glycerine. 20 percent. Add 10 ml of glycerine to a 50-ml volumetric flask and make up to volume with 99 percent ethyl alcohol. Store in a polyethylene bottle.

2) Curcumin-oxalic acid solution. Dissolve 0.1 g of curcumin (Eastman Kodak Co., Rochester, N.Y.) and 12.5 g of oxalic acid in 95 percent ethyl alcohol. Dilute to 250 ml with the ethyl alcohol. Store in a polyethylene bottle in a refrigerator. Prepare a fresh solution each week.

3) Boron standards. Prepare a 1000-ppm boron stock solution by dissolving 5.716 g of AR grade boric acid in deionized distilled water. Transfer to a 1-liter volumetric flask and make up to volume with deionized distilled water. Transfer 10 ml of this 1000-ppm boron stock solution to a 1-liter volumetric flask and make up to volume with deionized distilled water. This gives a 10-ppm boron solution. Prepare each of the boron standards by placing the amount of

10-ppm solution indicated (below) in a 50-ml volumetric flask and make up to volume with deionized distilled water.

<u>B standard (ppm)</u>	<u>Milliliters of 10-ppm B solution to add to a 50-ml volumetric flask</u>
0	0
0.10	0.5
0.3	1.5
1.0	5.0
1.5	7.5
3.0	15.0
5.0	25.0

Note Store stock solutions and standards in polyethylene bottles.

Procedure (Plant tissue analysis)

Transfer 0.1 ml of the sample extract and 0.1 ml of each of the standards to the spot plate. Add 0.25 ml of curcumin-oxalic acid solution to each depression that contains sample or standard. Then evaporate in an oven at 55 C. After the solution has evaporated continue heating for 15 minutes. Remove the spot plate from the oven and cool to room temperature.

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Dissolve the colored residue in each depression with 0.5 ml of the 20 percent alcohol solution of glycerine and stir thoroughly with a polyethylene rod. Compare the color of the sample with the colors developed at the same time from the standard boron solutions.

Calculation

$$\text{Concentration of boron in plant sample (ppm)} = \frac{\text{concentration of comparable boron standard}}{\text{standard} \times 40}$$

Procedure (water analysis)

Transfer 1 ml of the water sample and 0.1 ml of each of the standards to the spot plate and evaporate under an infra-red lamp. Then cool to room temperature and add 0.1 ml of 0.5 N HCl to each depression. Add curcumin-oxalic acid solution as described for plant tissue analysis (above) and complete the steps listed there.

Calculation

$$\text{Concentration of boron in water sample (ppm)} = \frac{\text{concentration of comparable boron standard}}{10}$$

Note Dilute sample extracts containing more than 5 ppm boron before developing the color. When boron concentrations exceed 5 ppm, comparing colors is difficult.

References

- Yoshida, Y. and S. Yoshida. 1964. An improved spot test for boron in plant tissue and waters. *J. Sci. Soil Manure, Japan* 35:408.
- Yoshida, Y. and S. Yoshida. 1965. An extraction procedure for rapid determination of boron in plant tissues. *J. Sci. Soil Manure, Japan* 36:45-48.

CHAPTER 9. Determination of chlorine in plant tissue.

Equipment Muffle furnace, silica or porcelain evaporating basin, hot plate, burette, stirring rods, filter funnel, Whatman No. 1 filter paper.

Sample extraction

Reagent

Calcium oxide, AR grade. Use freshly opened AR grade reagent.

Procedure

Place a 1 to 2 g sample of dried ground plant material in a silica or porcelain evaporating basin. Mix the sample with about one-quarter of its weight of calcium oxide and sufficient distilled water to make a thin paste. Place in a muffle furnace and gradually raise the temperature to 550 C. Keep at this temperature for at least 90 minutes.

Remove the sample from the muffle furnace and cool. Add 15 ml of hot distilled water while warming the evaporating basin on a hot plate. Then break up the ash with a large diameter, blunt stirring rod and filter the mixture into a 250-ml Erlenmeyer flask. Rinse the residue in the evaporating basin with five 10-ml portions of hot water and filter into the 250-ml Erlenmeyer flask containing the sample extract. Allow the extract to cool.

Sample analysis

Reagents

- 1) Acetic acid. Dilute 200 ml of concentrated acetic acid with 800 ml of distilled water.
- 2) Potassium chromate. Prepare a 1-percent solution.
- 3) Sodium chloride, 0.1 N. Place 10 g AR grade NaCl in a porcelain crucible and heat for 30 minutes in a muffle furnace at 500 C. Then cool in a desiccator for 40 to 60 minutes. Dissolve 5.845 g of this sodium chloride in distilled water and dilute to 1 liter.
- 4) Standard silver nitrate, 0.05 N. Dissolve 8.5 g AR grade AgNO₃ in distilled water. Transfer to a 1-liter volumetric flask and make up to volume with distilled water. Put the solution in a glass-stoppered amber colored bottle and store in the dark. Standardize as follows: Put 10 ml of the 0.1 N sodium chloride standard into an Erlenmeyer flask and add 50 ml of distilled water. Titrate with the prepared silver nitrate solution in the same manner as described in the procedure below.

$$1 \text{ ml } 0.05 \text{ N AgNO}_3 = 1.77 \text{ mg Cl}$$

Procedure

Add drops of the acetic acid solution to the filtrate until the solution is about pH. 6 to 7. Check pH using an appropriate pH indicator paper. Then add five drops of the potassium chromate solution and titrate with standardized 0.05 N silver nitrate until the first permanent reddish-brown color appears.

Calculation

For a 1-g sample:

$$\% \text{ Cl} = \text{ml of } 0.05 \text{ N AgNO}_3 \times 0.177$$

Reference

Chapman, H. D. and P. F. Pratt. 1961. Chlorine, p. 97-100. In H. D. Chapman and P. F. Pratt [ed.] Methods of analysis for soils, plants, and waters. University of California, Riverside.

Equipment Mortar and pestle, volumetric flasks, Whatman No. 42 filter paper, spectrophotometer.

Principle Measuring absorbance of the chlorophyll solution at two wavelengths allows the use of the following simultaneous equations:

$$D_{663} = 82.04 C_a + 9.27 C_b \quad (1)$$

$$D_{645} = 16.75 C_a + 45.6 C_b \quad (2)$$

Where:

D_{663} = absorbance at 663 m μ

D_{645} = absorbance at 645 m μ

C_a = concentration of chlorophyll a in grams per liter

C_b = concentration of chlorophyll b in grams per liter

82.04, 9.27, 16.75, and 45.6 are specific absorption coefficients of chlorophyll a and b at wavelengths 663 and 645 m μ , respectively.

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Solving equations (1) and (2) gives

$$C_a = 0.0127 \cdot D_{663} - 0.00269 \cdot D_{645} \quad (3)$$

$$C_b = 0.0229 \cdot D_{645} - 0.00468 \cdot D_{663} \quad (4)$$

Therefore:

$$\begin{aligned} \text{Total chlorophyll C (grams per liter)} &= C_a + C_b \\ &= 0.0202 \cdot D_{645} + 0.00802 \cdot D_{663} \quad (5) \end{aligned}$$

When equation (5) is expressed in mg per liter, it becomes

$$C = 20.2 \cdot D_{645} + 8.02 D_{663} \quad (6)$$

This is the equation used for calculation of total chlorophyll content.

For a simplified procedure, the following equation can be used:

$$\text{Total chlorophyll (C)} = \frac{D_{652}}{34.5} (\text{grams per liter}) \quad (7)$$

i.e. $C = \frac{D_{652} \times 1000}{34.5} (\text{mg per liter})$

At 652 m μ , chlorophyll a and b intersect, and 34.5 is the specific absorption coefficient for both pigments at this wavelength.

Sample preparation

Reagent

Acetone, 80 percent.

Procedure

Cut the fresh leaves into pieces and put 2 g of fresh tissue into a mortar. Crush thoroughly with a pestle. Add acetone so that final concentration of the acetone becomes 80 percent (the leaf blade of rice contains approximately 80% water). Add enough acetone to allow the tissue to be thoroughly homogenized. Continue to homogenize the tissues and then decant the supernatant through a filter paper into a 100-ml volumetric flask. Add 80 percent acetone to the residue in the mortar and repeat the extraction procedure. Then make up to volume with 80 percent acetone. Transfer 5 ml of this solution into a 50-ml volumetric flask and make up to volume with 80 percent acetone.

Sample Measure the absorbance of the leaf tissue extract at 663 m μ and 645 m μ or at 652 m μ .

Calculation example

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Observed absorbance:

$$D_{645} = 0.187$$

$$D_{663} = 0.506$$

$$D_{652} = 0.275$$

From equation (6) and taking the dilution factor into consideration:

$$C = (20.2 \times 0.187 + 8.02 \times 0.506) \times \frac{50}{1000} \times \frac{100}{5} \times \frac{1}{2}$$

$$= 3.90 \text{ mg chlorophyll/g fresh weight sample}$$

Alternatively, from equation (7)

$$C = \frac{0.275 \times 1000}{34.5} \times \frac{50}{1000} \times \frac{100}{5} \times \frac{1}{2}$$

$$= 4.00 \text{ mg chlorophyll/g fresh weight}$$

Comment

For routine measurement of total chlorophyll, use equation (7).

References

- Amon, D. I. 1959. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in Beta vulris. Plant Physiol. 24:1-15.
- MacKinney, G. 1941. Absorption of light by chlorophyll solutions. J. Biol. Chem., 140:315-332.

Equipment Ball mill, water bath, 50-ml beakers, centrifuge, and 15-ml centrifuge tubes, 50-ml Pyrex test tubes, stirring rods, spectrophotometer.

Sample extraction

Reagents

- 1) 80 percent ethanol
- 2) Perchloric acid, 9.2 N. Dilute 793 ml of 70 percent HClO₄ to 1 liter.
- 3) Perchloric acid, 4.6 N. Dilute 397 ml of 70 percent HClO₄ to 1 liter.

Procedure

Grind the dried sample finely in a ball mill. Place 100 mg of dried sample (see note) into a 15-ml centrifuge tube and add 10 ml of 80 percent ethanol. Place a glass ball on top of the tube and keep in a water bath at 80 to 85 C for 30 minutes. Centrifuge and decant into a 50-ml beaker. Repeat this extraction three more times.

Evaporate the alcohol extract on a water bath at 80-85 C until most of the alcohol is removed (e.g. reduce the volume to about 3 ml). Make up to 25 ml with distilled water. Analyze sugars in this sugar extract. (See note.)

Dry the residue left in the centrifuge tube in an oven at 80 C for starch extraction.

Add 2 ml of distilled water to the centrifuge tube containing the dried residue. Put the tube in a boiling water bath for 15 minutes and stir occasionally. Allow the tube to cool and add 2 ml 9.2 N HClO₄ while stirring constantly. Then stir the solution occasionally for 15 minutes. The suspension is then made up to about 10-ml and centrifuged.

Collect the supernatant and add 2 ml 4.6 N HClO₄ to the residue. Stir this suspension for 15 minutes and then make up to 10-ml with distilled water. Centrifuge and then combine the supernatants. Make up to 50-ml with distilled water. Analyze starch in this starch extract.

Note

- a) Use only 50 mg of dried sample when analyzing starch in panicles harvested at or after the milk-ripe stage.
- b) sugar should be analyzed while the residue of the sugar extract is being dried.

Sample analysis:
Sugars

Reagents

1) Anthrone. Dissolve 2 g of anthrone in 1 liter of concentrated sulfuric acid. Store in a refrigerator. Prepare a fresh solution every 2 days.

2) Glucose stock solution. Put 0.100 g of dried glucose into a 1000-ml volumetric flask. Make up to volume with distilled water. This solution contains 100 ppm glucose. Prepare each of the standards (below) by placing the amount of 100-ppm solution indicated in a Pyrex test tube and make up to 5 ml with distilled water.

Glucose standards (mg/tube)	Milliliters of 100-ppm glucose solution to be added to Pyrex test tubes
0	0
0.1	1.0
0.2	2.0

Note Glucose stock solutions and standards should be prepared daily and kept cool.

Procedure

Transfer 5 ml of sugar extract to a 100-ml volumetric flask and make up to volume with distilled water. Put 5 ml of this diluted sugar extract into a Pyrex test tube and then put this tube and the tubes containing the standards into an ice bath. To each tube slowly add 10 ml of the anthrone reagent, allowing the reagent to run down the side of the test tube. Stir slowly with a glass rod.

Put the tubes into a boiling water bath for exactly 7.5 minutes. Then immediately cool in ice. When cool, measure the absorbance at 630 m μ .

Sample analysis:
Starch

Reagents

1) Anthrone. Prepare as for sugar analysis.

2) Perchloric acid, 0.46 N. Dilute 10 ml of the 4.6 N HClO₄ to 100 ml with distilled water.

3) Glucose stock solution. Prepare a 100-ppm glucose stock solution as for sugar analysis.

Prepare each of the standards (below) by placing the amount of 100-ppm solution indicated in a Pyrex test tube. Then add 0.6 ml 0.46 N HClO₄ to each tube and make up to 5-ml with distilled water.

Glucose standards (mg/tube)	Milliliters of 100-ppm glucose solution to be added to Pyrex test tubes
0	0
0.1	1.0
0.2	2.0

Note Glucose stock solutions and standards should be prepared daily and kept cool.

Procedure

Put 5 ml of starch extract into a 50-ml volumetric flask and make up to volume with distilled water. Put 5 ml of this diluted starch extract into a Pyrex test tube (see note), and then put this tube and the tubes containing the standards into an ice bath. To each tube, slowly add 10 ml of the anthrone reagent, allowing the reagent to run down the side of the test tube. Stir slowly with a glass rod.

Put the tubes in a boiling water bath for exactly 7.5 minutes. Then immediately cool in ice. When cool, measure the absorbance at 630 m μ .

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Note This method can be used when starch and sugar concentrations are between 5 and 20 percent. If the concentration is outside this range, dilute the extract appropriately.

The concentration of HClO₄ in the starch standards must also be adjusted so that the standards contain the same amount of HClO as the sample.

The usual range of sugar and starch in rice tissue is shown in Table 1.

Table 1.

Plant part	Seeding to panicle initiation	Panicle initiation to 1 week after flowering	Maturity
Sheath plus culm	0 - 5% sugar	5 - 15% sugar	0 - 5% sugar
	2 - 8% starch	5 - 15% starch	0 - 5% starch
Leaf		5 - 10% sugar	0 - 5% sugar
Panicle			0 - 5% sugar 60 - 80% starch

Comments

The size and thickness of the test tubes influence the color development (Morris 1948), It is essential therefore to use similar test tubes for all samples and standards.

Anthrone gives only a very slight color reaction with pentoses (McCready et al. 1950), however in rice only a small fraction of the total sugar is present as pentoses (Murayama et al. 1961). Precipitation of amino acids before measuring sugars had no effect on the determination.

The anthrone reagent gives values for sugar close to those obtained using the procedure of Somogyi (1945).

References

McCready, R. M., J. Guggolz, V. Silveira, and H. S. Owens. 1950. Determination of starch and amylose in vegetables. *Anal. Chem.* 22:1156-1158.

Morris, D. L. 1948. Quantitative determination of carbohydrates with Drywood's anthrone reagent. *Science* 107:254-255.

Murayama, N., S. Tsukahara, and M. Oshima. 1961. Studies on metabolism of rice plant during the ripening period. (Part 5) Photosynthetic products and their translocation. *J. Sci. Soil and Manure, Japan* 32:256-260.

Pucher, G. W., C. S. Leavenworth, and H. B. Vikery. 1945. Determination of starch in plant tissues., *Anal. Chem.* 20:850-853.

Somogyi, M. 1945. A new reagent for the determination of sugars. *J. Biol. Chem.* 160:61-68.

Equipment Packard Tri-Carb Model 314 EX Liquid Scintillation Spectrometer, Coleman Model 33 carbon hydrogen analyzer modified by attaching a piece of Polyvinyl chloride (PVC) tubing to the end of the CO_2 absorption tube. The CO_2 absorption tube is kept empty and instead the CO_2 is absorbed by bubbling it through hydroxide of Hyamine contained in test tubes. In order to produce small bubbles, a piece of glass tubing of 1 mm diameter outlet is fitted into the end of the PVC tubing.

Carbon hydrogen analyzer preparation Prepare the combustion tube, the absorption tube, the pretreat absorption tube and the H_2O absorption tube as described in the instrument instruction manual. Insert an empty CO_2 absorption tube into its holder and fit the PVC tubing and glass outlet tip onto the end of the CO_2 absorption tube. Place the glass outlet of the PVC tubing into a test tube containing distilled water. The outlet should be close to the bottom of the test tube.

Preliminary settings of instrument:

Line switch: ON
Cycle delay: OFF
Auxiliary timer: ZERO
Combustion cycle: STANDBY
Upper furnace: 800 C
Low furnace: 800 C
Sweep control: 100 ml/min for "purge," "final combustion," and "sweep."
10 ml/min for "first combustion"

Sample preparation

Reagent

Hydroxide of Hyamine (Packard Instrument Co., Ltd., Downer Grove, Ill., U. S. A.).

Procedure

Place 50 mg of dried ground tissue into an aluminum boat (standard equipment for carbon hydrogen analyzer) and put in the combustion tube. Place 10 ml of hydroxide of Hyamine into a test tube and insert the PVC tube and glass outlet as described previously.

Turn the combustion cycle control to 'start' and allow to complete one cycle; During the 'first combustion,' the 'final combustion,' and the 'sweep,' bubbles of gas will pass through the hydroxide of Hyamine in the test tube. When the cycle is complete, remove the glass tube outlet from the test tube and stir the hydroxide of Hyamine solution. Seal the tube and keep for the determination of total ^{14}C activity. Prepare a blank by repeating the entire procedure using non-labeled plant tissue.

Sample analysis

Reagents

1) Toluene ethanol scintillator. Mix 70 parts toluene containing 0.4 percent PPO and 0.01 percent dimethyl POPOP with 30 parts absolute ethanol (PPO and dimethyl POPOP obtainable from Packard Instrument Co., Ltd. see above).

2) ¹⁴C-sodium bicarbonate standards (for the determination of counting efficiency). Prepare a stock solution of NaH ¹⁴CO₃ containing 0.05 µc/ml in 'alkaline' distilled water.

Prepare the standards (below) by placing the amount of NaH ¹⁴CO₃ stock solution indicated in a glass vial of the type used in the liquid scintillation spectrometer. Make up to 2 ml with hydroxide of hyamine and mix with 10-ml of toluene ethanol scintillator.

<u>NaH ¹⁴CO₃ standards (µc/vial)</u>	<u>Milliliters of NaH ¹⁴CO₃ stock solution/vial</u>
0 (zero blank)	0
1 x 10 ⁻³	0.02
2 x 10 ⁻³	0.04
3 x 10 ⁻³	0.06
4 x 10 ⁻³	0.08

Allow the standards to cool for at least 2 hours in the liquid scintillation spectrometer and then determine the activity of each sample in cpm using the same procedure as that described below for the sample.

$$\text{Counting efficiency} = \frac{\text{count/min standard} - \text{count/min zero blank}}{2.22 \times 10^6 \times \mu\text{c NaH}^{14}\text{CO}_3 \text{ in standard}}$$

Calculate the average counting efficiency using the above standards.

Procedure

Mix 2 ml of the hydroxide of Hyamine solution with 10 ml toluene ethanol scintillator in the glass vial. Also prepare a blank solution by mixing 2 ml of the hydroxide of Hyamine solution used in collecting CO₂ from the unlabeled plant tissue (above) with 10-ml toluene ethanol scintillator. Place the glass vials in the liquid scintillation spectrometer and allow to cool for 2 hours. Adjust the settings of the spectrometer according to the operation manual and then determine the activities of the hydroxide of Hyamine sample, blank and standard (above) solutions in counts per minute (cpm).

Calculation

$$\begin{aligned} \text{Activity of } ^{14}\text{C in plant tissue (dpm/mg tissue)} &= \frac{\text{count/min sample solution} - \text{count/min blank solution}}{\text{mg sample combusted}} \\ &\times \frac{1}{\text{counting efficiency}} \end{aligned}$$

Comment

This method gives counting efficiencies greater than 50 percent with blank counts of less than 50 count/min. There was no detectable difference in quenching between 0.08 ml and 0.02 ml of aqueous NaH ¹⁴CO₃.

References

Lian, S. and A. Tanaka. 1967. Behavior of photosynthetic products associated with growth and grain production in the rice plant. *Plant and Soil* 26:333-347.

Operation Manual Model 314 EX-2 Tri-Carb Liquid Scintillation Spectrometer System. Packard Instrument Co., Ltd. 32 p.

Passman, J. M., N. S. Rading, and J. A. D. Cooper. 1966. Liquid scintillation technique for measuring carbon-14-dioxide activity. *Anal. Chem.* 28:484-486.

Equipment Packard Tri-Carb 314 EX Liquid Spectrometer and appropriate glass vials, ion exchange columns (prepared according to standard procedure).

Sample preparation

Reagents

- 1) Sodium chloride, 1 N. Dissolve 59 g NaCl in 1 liter distilled water.
- 2) Silver nitrate, 1 percent. Dissolve 1 g AgNO_3 in 100-ml distilled water.
- 3) Hydrochloric acid, 1 N. Dilute 90 ml concentrated HCl to 1 liter with distilled water.
- 4) Amberlite IRA 400 (Cl^- form). 20–50 mesh. This anion exchange resin is usually supplied in the strongly basic form and can be converted to the Cl^- form by first washing the resin with 1 N NaCl and then with distilled water. Continue washing with distilled water until no more chloride can be detected in the wash solution. (Test for chloride by adding a few drops of wash solution to 1 ml of 1 percent silver nitrate. Chloride is indicated by the formation of a white precipitate.)

Note After each sample is passed through the resin, reconvert the resin to the Cl^- form as described above.

- 5) Amerlite IR120 (H^+ form) 20-50 mesh.

Note After each sample is passed through the resin, reconvert this cation exchange resin to the H^+ form by washing it first with 1 N HCl and then with distilled water until the pH of the wash solution becomes neutral.

Procedure

Extract sugar from the plant sample as described in Chapter 11. Dilute 25-ml of the sugar extract to 50 ml with distilled water. Pass the diluted sugar extract first through a column of the Amberlite IR120 (H^+ form) resin and then through a column of Amberlite IR400 (Cl^- form) resin. Discard the first 10 ml of the eluent passed through the columns and keep the remainder for the determination of ^{14}C -sugar and total sugar concentration (i. e. labeled and unlabeled).

Sample analysis

Reagents

- 1) Brays scintillator. Dissolve 60 g naphthalene, 4 g PPO, 0.2 g POPOP in 100 ml of absolute methanol and add 20 ml ethylene glycol. Dilute this solution with an equal volume of p-dioxane (PPO and POPOP obtainable from Packard Instrument Co., Ltd., Downer Grove, Ill., U. S. A.).

2) ^{14}C -glucose standard. (For determination of counting efficiency.) Prepare a stock solution of ^{14}C -glucose containing $0.01 \mu\text{c/ml}$ in distilled water.

Prepare the standards (below) by placing the amount of ^{14}C -glucose stock solution indicated in a glass vial of the type used in the liquid scintillation spectrometer. Make up to 2 ml volume with distilled water and mix with 10-ml of Brays scintillator.

^{14}C -glucose standards ($\mu\text{c/vial}$)	Milliliters of ^{14}C -glucose stock solution/vial
0 (zero blank)	0
0.005	0.5
0.010	1.0
0.015	1.5
0.020	2.0

Allow the standards to cool for at least 2 hours in the liquid scintillation spectrometer and then determine the activity of each standard in count per minute using the sample procedure as that described below for the sample.

$$\text{Counting efficiency} = \frac{\text{count/min standard} - \text{count/min zero blank}}{2.22 \times 10^6 \times c \text{ } ^{14}\text{C-glucose in standard}}$$

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Calculate the average counting efficiency using the above standards.

Note The same procedure is used for finding the counting efficiency in the analysis of ^{14}C -starch (Chapter 14).

Procedure

Mix 2 ml of the sample eluent with 10 ml of Brays scintillator in a glass vial. Also prepare a blank by mixing 2 ml distilled water with 10 ml Brays scintillator.

Place the glass vials in the liquid scintillation spectrometer and allow to cool for 2 hours. Adjust the setting of the spectrometer according to the operation manual and then determine the activities of the sample eluent, blank and ^{14}C -glucose standards (above) in counts per minute.

In addition, the concentration (mg/ml) of sugar (i. e. labeled and unlabeled) is determined in the sample eluent and the diluted sugar extract using the procedure described in Chapter 11.

Calculations

$$\begin{aligned} \text{Specific activity of sugar} &= \frac{\text{count/min eluent} - \text{count/min blank solution}}{\text{concn sugar in sample extract (mg/ml)}} \\ \text{in eluent (dpm/mg sugar)} &= \frac{1}{\text{counting efficiency}} \end{aligned}$$

Calculate the percent sugar in the plant tissue from the sugar determination in the diluted sugar extract.

$$\text{Activity of sugar in plant tissue (dpm/mg tissue)} = \frac{\text{specific activity of sugar} \times \% \text{ sugar in tissue}}{100}$$

Comments

The hydroxyl form of a strongly basic ion exchange resin such as Amberlite IRA400 or Dowex 1 retains a considerable amount of sugar. This sugar can be eluted with sodium chloride, acetic acid, and sodium phosphate. Hence the hydroxyl form of a weakly basic ion exchange resin (e.g., Amberlite IR45) and the formate or acetate form of a strongly basic exchange resin are often used for separation of sugars from acidic compounds (Roseman et al. 1952).

The chloride form of Amberlite IRA400 appears to retain less than 10 percent of the sugar passed through it.

If the alcohol extract is strongly colored, remove the pigments by warming the diluted extract at 40 C for 5 minutes with 1 g activated charcoal. Then filter to remove the charcoal. However the charcoal does absorb some of the sugar.

The ratio of the count/min on the red and green scaler of the liquid scintillation spectrometer, should be constant for all samples and standards. If the ratio varies, the individual samples have different degrees of quenching. In this case, use an internal ¹⁴C-glucose standard to determine the counting efficiency of each sample as described in the spectrometer operation manual.

This method provides counting efficiency greater than 40 percent with blank count rates less than 50 count/min.

References

- Bray, G. A. 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. *Ad. Biochem.* 1:279-285.
- Operation Manual, Model 314-EX-2 Tri-Cart, Liquid Scintillation Spectrometer System. Packard Instrument Co., LM. 32 p.
- Roseman, S., R. H. Abeles, and A. Dorfman. 1952. Behavior of carbohydrates toward strongly basic ion exchange resins. *Arch. Biochem. Biophys.* 36: 232-233.
- Splittstoesser, W. E. 1969. Arginine metabolism by pumpkin seedlings. Separation of plant extracts by ion exchange resins. *Plant and Cell Physiol.* 10:87-94.
- Yamamoto, T. 1967. The distribution pattern of carbon-14 assimilated by a single leaf in tobacco plant. *Plant and Cell Physiol.* 8:353-362.

Equipment Packard Tri-Carb Model 314 EX Liquid Scintillation spectrometer and appropriate glass vials, centrifuge, 50-ml centrifuge tubes, mortar and pestle.

Sample preparation

Reagents

- 1) Sodium chloride, 20 percent. Dissolve 200 g NaCl in 1 liter distilled water.
- 2) Iodine-potassium iodide reagent. Put 7.5 g iodine and 7.5 g potassium iodide with 150 ml of distilled water in a mortar and grind with a pestle. Dilute to 250 ml with distilled water and filter through a Whatman No. 4 filter paper. Store in the dark.
- 3) Alcoholic sodium chloride. Mix 350 ml absolute ethanol with 80 ml distilled water and 50 ml of 20 percent NaCl. Dilute to 500 ml with distilled water.
- 4) Alcoholic sodium hydroxide. Dissolve 5 g NaOH in a mixture of 350 ml absolute ethanol and 100 ml distilled water. Dilute to 500 ml with distilled water.

Procedure

Extract starch with perchloric acid as described in Chapter 11. Immediately dilute the starch extract to 50 ml with distilled water and place 10 ml of this diluted solution in a 50-ml centrifuge tube. Add 5 ml of 20 percent sodium chloride and 2 ml of iodine-potassium iodide reagent. Mix and allow to stand for 20 minutes. Centrifuge and discard the supernatant. Suspend the precipitate in 5 ml of alcoholic sodium chloride. Tap the tube gently, centrifuge and discard the supernatant.

Add 2 ml of alcoholic sodium hydroxide to the precipitate and shake (do not stir with a glass rod) until all blue color disappears. Centrifuge and discard the supernatant. Suspend the precipitate in 5 ml of alcoholic sodium chloride. Tap the tube gently, then centrifuge and discard the supernatant. Add 10 ml of distilled water to the precipitate and shake gently to dissolve the starch. ^{14}C -labeled starch is analyzed in this starch solution.

Sample analysis

Reagents

- 1) Brays scintillator. Dissolve 60 g naphthalene, 4 g PPO, and 0.2 g POPOP in 100-ml absolute methanol and add 20 ml ethylene glycol. Dilute the solution with an equal volume of p-dioxane. (PPO and POPOP obtainable from Packard Instrument Co., Ltd., Downer Grove, Ill., U. S. A.).

2) ^{14}C -glucose standards, For determination of counting efficiency. Prepare, measure activity and determine counting efficiency as described in Chapter 13.

Procedure

Mix 2 ml of the starch solution with 10 ml of Brays scintillator in the glass vial. Also prepare a blank by mixing 2 ml of distilled water with 10-ml Brays scintillator.

Place the glass vials in the liquid scintillation spectrometer and allow to cool for 2 hours. Adjust the settings of the spectrometer according to the operation manual and then determine the activity of the starch solution and blank solution in counts per minute (cpm).

In addition, the concentration (mg/ml) of starch (i. e. labeled and unlabeled) in the starch solution is determined using the procedure described in Chapter 11.

Calculations

$$\begin{aligned} \text{Specific activity of starch (dpm/mg starch)} &= \frac{\text{count/min starch solution} - \text{count/min blank solution}}{\text{concn starch in starch solution (mg/ml)}} \\ &\times \frac{0.5}{\text{counting efficiency}} \end{aligned}$$

$$\text{Activity of starch in plant tissue (dpm/mg tissue)} = \frac{\text{specific activity of starch} \times \% \text{ starch in tissue}}{100}$$

Comments

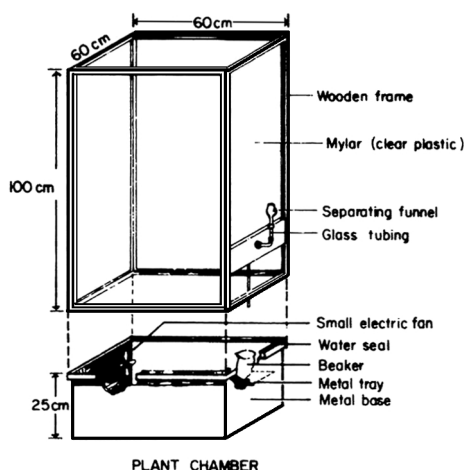
After extracting the starch with perchloric acid, the starch will be rapidly hydrolyzed to sugars which are not precipitated by iodine-potassium iodide reagent. Therefore precipitate the starch immediately after extraction. The determination of starch by anthrone is not affected by this hydrolysis.

References

Operation Manual, Model 314-EX Tri-Carb Liquid Scintillation Spectrometer System. Packard Instrument Co., Ltd. 32 p.

Whelan, W. J. 1955. Starch, glycogen, fructasans and similar polysaccharides, p. 145-196. In K. Paech and M. V. Tracey (ed.) Modern Methods of Plant Analysis. Springer Verlag. Berlin.

Equipment Plant chamber (see diagram), beakers, separating funnels, L-shaped glass tubes, rubber stoppers.



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Experimental procedure

Reagents

- 1) $\text{NaH}^{14}\text{CO}_3$ solution. (See comment.)
- 2) Lactic acid, 1 N.
- 3) KOH, 2.5 N. Dissolve 15 g KOH in 100 ml of water.

Procedure

Place the base of the chamber over the plants, taking care not to damage the plants. Be sure the bottom of the metal base is completely submerged by the water in the field. Fill the water seal with water. Place a 100-ml beaker containing the $\text{NaH}^{14}\text{CO}_3$ on the metal tray. Fit the wooden mylar frame onto the base so that the tube from the separating funnel is in the beaker containing the $\text{NaH}^{14}\text{CO}_3$ solution. Switch on the fan. Add sufficient lactic acid to the separating funnel to acidify the bicarbonate. Allow lactic acid to run into the $\text{NaH}^{14}\text{CO}_3$ and then close the tap of the separating funnel. Allow the plants to assimilate the $^{14}\text{CO}_2$ for 2 hours.

At the end of this period, add 50 ml of 2.5 N KOH to the separating funnel and allow to run into the beaker. Leave for 10 minutes to absorb most of the excess $^{14}\text{CO}_2$. Then remove the wooden-mylar frame.

Harvest the plants according to the requirements of the experiment.

Comments

The total activity of the $\text{NaH}^{14}\text{CO}_3$ solution to be used will depend on the purpose of the experiment. Adding 200 μC per chamber 10 days before flowering gives adequate activity for measuring starch, sugar and total ^{14}C activity until grain maturity.

Be Sure the $\text{NaH}^{14}\text{CO}_3$ is made up in alkaline distilled water to prevent loss of $^{14}\text{CO}_2$.

Equipment Microscope, glass slides, and coverslips.

Sample preparation

Reagents

1) Safranin-phenol. Add five drops of 0.01 percent safranin aqueous solution to 20 ml of reagent grade phenol. Since the phenol (m.p. ca. 40 C) is solid at room temperature, warm it in a water bath until liquid.

2) Ethanol, 70 percent (v/v)

Procedure

Removal of chlorophyll pigments. Soak the fresh rice samples in 70 percent ethanol for 3 days or more.

Staining. Select tissue in which silicified cells are to be examined and cut a piece of tissue about 1 cm long. Place the sample piece in a beaker containing 20 ml of the safranin-phenol reagent and boil it gently for 1 minute.

Microscopic examination. Remove the sample piece from the beaker and place it on a slideglass with 2 drops of hot safranin-phenol reagent. Cover the tissue with a coverglass and examine under a microscope. The optimal magnification usually ranges from x50 to x100.

The silicified cells are seen as rectangular-shaped, clear to grey areas in the leaf tissue. The bulliform cells generally silicify at a late stage in the silicification process. These cells when silicified are seen as quite large, round, clear to grey areas.

Comments

Phenol alone may be substituted for the safranin-phenol reagent.

Crystallization of the safranin-phenol reagent during the microscopic examination can be prevented by keeping the slideglass warm. This can be easily done by placing a small electric heater near the microscope. If the phenol still tends to crystallize, substitute a mixture of equal volumes of phenol and glycerol for the safranin-phenol reagent.

Equipment Porcelain or plastic pots with appropriate bamboo culture baskets, 4-cm to 1.5-cm diameter stones, 6-liter tray and germination frame, demineralized water. To prepare new culture baskets soak them in tap water for 1 week. Renew the water daily. Then thoroughly dry the baskets and coat the entire basket with a plastic vinyl paint. From time to time the baskets will need repainting. Select well-weathered river stones ranging from 1.5 to 4-cm diameter. Rinse them thoroughly with demineralized water and then coat them with plastic vinyl plant.

Preparation of seedlings

Reagents

- 1) Mercuric chloride, 0.1 percent. Dissolve 1 g HgCl₂ in 1 liter of water.
- 2) Formalin solution. Transfer 16 ml of formaldehyde to a 1-liter volumetric flask. Add 4 ml of methanol and make up to volume with demineralized water.

Germination

Surface-sterilize seeds for 1 minute with a 0.1 percent mercuric chloride solution or soak them in a formalin solution for 15 minutes. Then wash thoroughly with several changes of demineralized water.

Allow the seeds to soak for 24 hours in a beaker of demineralized water.

Then spread the seeds on a nylon net stretched over a wooden frame and float in a tray containing complete culture solution at pH 5.0 using mixed indicator.

Transplanting

Transplant seedlings in pots 2 weeks after germination. Support selected uniform seedlings in the culture basket with small stones so that the roots dip into the culture solution. Generally plant two to three seedlings together in each 4-liter pot.

Table 1. Preparation of stock solutions.

Element	Reagent (AR grade)	Preparation (g/10 liters of distilled water)
N	NH ₄ NO ₃	914
P	NaH ₂ PO ₄ · 2H ₂ O	403
K	K ₂ SO ₄	714
Ca	CaCl ₂	886
Mg	MgSO ₄ · 7H ₂ O	3240
Mn	MnCl ₂ · 4H ₂ O	15.0
Mo	(NH ₄) ₆ · MO ₇ O ₂₄ · 4H ₄ O	0.74
B	H ₃ BO ₃	9.34
Zn	ZnSO ₄ · 7H ₂ O	0.35
Cu	CuSO ₄ · 5H ₂ O	0.31
Fe	FeCl ₃ · 6H ₂ O	77.0
	Citric acid (monohydrate)	119

} Dissolve separately;
then combine with
500 ml of concen-
trated H₂SO₄. Make
up to 10 liter vol-
ume with distilled
water.

Store in glass or polyethylene carboys.

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Preparation
of culture
solution

Reagents

- 1) Sodium hydroxide, 1 N. Dissolve 40 g NaOH in 1 liter of demineralized water.
- 2) Hydrochloric acid, 1 N. Put 83 ml of concentrated HCl in a 1-liter graduated beaker and make up to volume with demineralized water.
- 3) Mixed indicator. Dissolve 0.3 g of bromcresol green and 0.2 g of methyl red in 400 ml of 90 percent ethanol. This indicator is red below pH 5.0, purple at pH 5.0 to 5.1 and blue above pH 5.1.
- 4) Composition of culture solution.

Table 2.

Element	Milliliters of stock solution per <u>four</u> <u>liters</u> of culture solution	Concentration of element in nutrient solution (ppm)
N	5	40
P	5	10
K	5	40
Ca	5	40
Mg	5	40
Mn	} 5	0.5
Mo		0.05
B		0.2
Zn		0.01
cu		0.01
Fe		2

Note

a) The nitrogen level may be varied as follows:

40 ppm	Until 3 weeks after transplanting
80 ppm	At maximum tiller number stage
40 ppm	At 2 weeks after flowering
0 ppm	At maturity

b) If addition of silica is desirable, use water glass (sodium silicate). Fifty to one hundred ppm SiO_2 is adequate for rice.

c) If tap water is used for preparation of culture solution, analyze the water for calcium and magnesium to see if these elements can be omitted in the preparation of the culture solution.

Procedure

For every 4 liters of culture solution to be prepared, add 5 ml of each of the stock solution (as set out in Table 2) to 1 liter of water in a plastic bucket. For example, to fill twenty 4-liter pots, add 100 ml of each of the stock solutions to 20 liters of demineralized water in the plastic bucket.

Prepare a few extra liters of solution in case of spillage. Stir the solution after adding each reagent to avoid any precipitation.

Then, using mixed indicator, adjust the pH of the solution to 5.0 by adding 1 N NaOH. Stir the solution continuously while adding the sodium hydroxide.

Add 1 liter of this solution per 4-liter pot and then fill the pots with demineralized water to within 3 cm of the top.

Comments

Rice can grow well in a wide range of composition of culture solutions. The Kimura B solution has been widely used in Japan (Baba and Takahashi 1956) and, in the author's experience, a diluted Hoagland and Snyder solution (Hewitt 1966), is also satisfactory. Tables 3 and 4 show the composition of these two culture solutions.

Table 3a. Nutrient concentration of Kimura B solution.

Element	Concentration of element in nutrient solution (ppm)
N	23.0
P	5.6
K	21.4
Ca	14.6
Mg	13.3
Fe	1.4-3.5

Table 3b. Preparation of Kimura B solution.

Reagent	Preparation (mg/liter of culture solution)
$(\text{NH}_4)_2\text{SO}_4$	48.2
KH_2PO_4	24.8
KNO_3	18.5
K_2SO_4	15.9
$\text{Ca}(\text{NO}_3)_2$	59.9
MgSO_4	65.9
Ferric citrate	

Table 43. Nutrient concentration of Hoagland and Snyder solution.^{1/}

Element	Concentration of element in nutrient solution (ppm)
N	210
P	32
K	235
Ca	200
Mg	48

^{1/}This solution is used at appropriate dilution.

Table 4b. Preparation of Hoagland and Snyder solution.^{1/}

Reagent	Preparation (mg/liter of culture solution)
KNO_3	510
$\text{Ca}(\text{NO}_3)_2$	820
KH_2PO_4	136
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	490
Ferric tartarate	1 ml of 0.5% solution
Micronutrients	A-Z solution

^{1/}This solution is used at appropriate dilution.

If ammonia is the sole source of nitrogen, the pH of the culture solution decreases as the rice plant absorbs the ammonium ion. Regularly correct the pH of the culture solution or the growth of the rice roots will be disturbed

because of the low pH. When nitrate is the sole source of nitrogen, the pH of the culture solution increases and the high pH may cause iron deficiency, particularly when the rice plants are young.

Both ammonium and nitrate are equally effective sources of nitrogen (Tanaka et al. 1959) but the ammonium ion is absorbed much faster than the nitrate ion. Hence, when both these forms of nitrogen are present in the culture solution, the pH of the solution decreases at first and then begins to increase as the ammonium ion is absorbed and the plant begins to take up the nitrate ion.

Management
and care of
growing
plants

pH

Using mixed indicator adjust the pH of the culture solution to 5.0 every second day with either 1 N NaOH or 1 N HCl. When the indicator turns a purple color, the pH is about 5.0.

Renewal of culture solution

Change the culture solution once a week at early growth stages and twice a week from active tillering until flowering.

From 2 weeks after flowering to maturity, replace the culture solution with tap water. Adjust the pH of the tap water to 5.0.

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Daily water requirement

Fill the pots with water at least twice daily to supplement loss by evapotranspiration.

Insect control

Spray plants and surroundings at least once a week.

Rat and cat control

Young cats can be more damaging than the rats. Protect plants from rat and cat damage.

Common symptoms and correction of bad management

A detailed description of visual symptoms of nutrient deficiencies and toxicities of the rice plant is given by Tanaka and Yoshida (1970).

Some of the problems commonly encountered when growing rice plants in water culture are listed below.

1) Symptoms. Entire leaves become chlorotic and may turn white. The young emerging leaf is affected first. These symptoms occur readily in young seedlings.

Reason. Iron deficiency. This indicates the pH of the culture solution is too high.

Correction. Lower the pH to a little below 5.0. This should effect recovery in 3 days. For faster recovery, spray the seedlings with a fine mist of 0.5 percent $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ solution, pH 4.4, containing two drops of the sticker Tween 20.

2) Symptoms. The plant becomes light green in color. The older leaves are noticeably chlorotic and begin to turn yellowish-orange and die from the tip.

Reason. Nitrogen-deficiency. The culture solution is not being changed regularly enough.

Correction. Change the culture solution at least twice a week and add nitrogen to give a concentration of 80 ppm in the culture solution.

3) Symptoms. The roots are stunted and very branched.

Reason. Low pH. The culture solution has been substantially below pH 4.0 for some time.

Correction. Adjust the pH to 5.0 more regularly.

4) Symptoms. The pot smells strongly of hydrogen sulfide and in severe cases, the roots may turn black. This often occurs in the period from the emergence of the flag leaf until flowering.

Reason. Associated with a low level of nitrogen in the culture solution.

Correction. Change the culture solution more regularly and add nitrogen to give a concentration of 80 ppm in the culture solution.

References

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- Tanaka, A., S. Patnaik, and C. T. Abichandani. 1959. Studies on the nutrition of the rice plant (Oryza sativa L.). Proc. Indian Acad. Sci. Section B, 49:386-396.
- Tanaka, A. and S. Yoshida. 1970. Nutritional disorders of rice in Asia. Int. Rice Res. Inst. Tech. Bull. 10. 51 p.

Equipment	Toshiba Illuminometer No. 5 (Daiichi Boeki Shokai Co., Tokyo). Attach the light receiving part to a bamboo stick.
Light intensity	Select proper filter, adjust the correction setting as indicated in the instructions and read the intensity with the switch on. Ensure that the light detector is kept horizontal while readings are being taken.
Light transmission ratio (LTR)	Use two photometers. Adjust the correction settings as indicated in the instructions accompanying photometer. Place one photometer above the canopy and the other at the ground surface. Read the light intensity on both instruments at the same time. Keep the light detectors horizontal while the readings are being taken.

$$\text{LTR (\%)} = \frac{I_i}{I_o} \times 100$$

I_o = light intensity above the canopy

I_i : light intensity at the ground surface

Make 5 to 10 measurements in one canopy and average the values obtained.

Comments

The light transmission ratio (LTR) will differ on cloudy and clear days. Solar altitude will influence it on clear days. In the tropics where solar altitude may vary from 0–90°, diurnal changes in LTR may be large. Consequently when recording LTR, also record both the amount of cloud and the time of day.

At noon on clear days it is difficult to measure LTR accurately with the above instrument because of the presence of sun specks at ground level.

On days when the sky is partially overcast, I_o is extremely variable, and measurements of LTR are also quite difficult.

Although LTR is most easily measured either early in the morning or late in the afternoon, the photosynthesis of the canopy is small at these times and the LTR measurements may not be very meaningful.

References

- Clegg, M. D., W. W. Biggs, J. D. Eastin, J. W. Maranville, and C. Y. Sullivan. 1969. Visible radiation studies, 1968, p. 35-51. In Annual Report No. 3: The physiology of yield and management of sorghum in relation to genetic improvement. Coop. Res. by Univ. Nebraska, The Rockefeller Foundation, and Crops Res. Div. ARS, USDA. 199 p.
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Introduction The measurement of leaf area index (LAI), the area of leaf surface per unit area of land surface, involves two techniques: measuring the area of a leaf and selecting the correct leaf samples so that leaf area per plant can be adequately estimated.

Methods for selecting leaf samples:
1. With leaves removed

Sample selection

Select at random six hills per plot. Make sure that the hills are surrounded by living hills.

Procedure

Remove the six hills from the soil. Take precautions to keep the leaves from drying and curling before the leaf area is measured.

From each hill, use the second topmost tiller as the sample tiller. Determine the leaf area of all leaves on the sample tiller by any of the three methods described below. It is convenient to place the sample leaves in a test tube containing a small amount of water before measuring leaf area. Remove the other green leaves from the hill. Dry the sample leaves and the other leaves and weigh separately.

2. Without removing leaves

Sample selection

Use the same selection procedure as above.

Procedure

From each selected hill, count the number of tillers, measure the length and maximum width of each of the leaves on the middle tiller, and compute leaf area using the length-width method.

Methods for measuring leaf area:
1. Length-width method

Procedure

For each sample leaf, measure the length and the maximum width and compute the area:

$$\text{Leaf area} = \underline{K} \times \text{length} \times \text{width}$$

where \underline{K} is the "adjustment factor." \underline{K} varies with the shape of the leaf which in turn is affected by the variety, nutritional status, and growth stage of the leaf. Experimental studies at IRRI (IRRI, 1972) have indicated, however, that the

value of 0.75 can be used for all stages of growth except the seedling stage and harvest where the value of 0.67 should be used. Values found by other investigators are shown in Table 1.

Table 1. List of K values reported by different writers.

Adjustment factor	Variety	Growth stage	References
0.725	26 varieties	All stages of growth	Tsunoda (1964)
0.670 ± 0.021	Sasanishiki	Seedling	Murata (1967a)
0.750 ± 0.074	Sasanishiki	Maximum tillering	Murata (1967a)
0.690 ± 0.041	Norin No. 29	Booting	Murata (1967a)
0.802	2 varieties	Vegetative	Bhan and Pande (1966)

2. Blueprint method

Equipment

Photographic paper with accompanying developing and fixing chemicals, glass sheet, 375-watt infra-red lamp.

Procedure

Lay the leaf blades flat on a sheet of photographic paper in partial darkness. Also lay a piece of paper, 10 x 10 cm in dimension, on a photographic paper. With a pencil, write the sample identification on one corner of the photographic paper. Flatten the samples with a glass sheet and pass the infra-red lamp across the sheet several times.

Develop and fix the photographic print according to the instruction. Dry the sheets in air and cut out the blueprints of the leaf blades and the 10 x 10 cm paper sample. Place the leaf blades and their blueprints in envelopes. Oven-dry them at 80 C overnight, cool in a desiccator, and weigh. Treat the blueprint of the 10 x 10 cm sample sheet in the same way.

Calculation

$$\text{Area of leaves (sq cm)} = \frac{\text{wt of leaf blade blueprints (mg)}}{\text{wt of 1 sq cm of blueprint paper}}$$

3. Automatic area method (Murata, 1967b)

Equipment

Automatic area meter, Model AAM-4 (Hayashi-Denko Co., Ltd., Tokyo, Japan), 1-cm x 25-cm section paper.

Procedure

Adjust the sensitivity of the meter using the section paper so that the error is less than 1 percent. Place sample leaves on the transparent plastic belt and read digital value from the meter.

Comment

Keep the transparent plastic belt as clean as possible.

Calculations
of leaf area
index:

$$\text{Leaf area/hill} = \frac{\text{total leaf area of sample tillers} \times \text{dry wt of all leaves}}{\text{dry wt of leaves from sample tillers}}$$

1. With leaves
removed

where dry wt of all leaves = dry wt of sample leaves + dry wt of remaining leaves

$$\text{LAI} = \frac{\text{sum of leaf area/hill of six hills (sq cm)}}{\text{area of land covered by six hills (sq cm)}}$$

Comment

If the blueprint method or an automatic area meter is used, the area of all leaves from the sample tiller of each hill can be determined together. This practice will save time.

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2. Without
removing
leaves

Leaf area/hill = total leaf area of middle tiller x total no. of tillers

$$\text{LAI} = \frac{\text{sum of leaf area/hill of six hills (sq cm)}}{\text{area of land covered by six hills (sq cm)}}$$

Leaf
thickness

Direct measurement of leaf thickness is tedious. Therefore, in routine growth analysis, leaf thickness is usually expressed in terms of area and dry weight. Data on area and dry weight are available from the previous measurements. Specific leaf area is defined as area per unit dry weight (cm^2/g). On the other hand, leaf dry matter index (aerial weight) is defined as weight per unit area (g/cm^2). These two measurements can be used as a measure for leaf thickness.

Leaf thickness of rice varieties as expressed by aerial weight ranges from about 3 to 6 mg/cm^2

References

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Equipment Large ruler, protractor, marking pen, large sheet of paper.

Measurement Separate the main tiller from the rest of the plant. Immediately place the tiller against a vertical board covered with paper. The culm is the vertical axis. With the leaves drooping normally from the axis, mark the positions of the tip and collar of each leaf on the paper. Draw a line between the two points and measure the angle between the line and the vertical axis with a protractor.

Comments

There are two different ways of expressing leaf angle: 1) angle from the horizontal line and 2) angle from the vertical line. Hence, be sure to define which angle you have measured.

Reference

Yoshida, S., S. A. Navasero, and E. A. Ramirez. 1969. Effects of silica and nitrogen supply on some leaf characters of the rice plant. *Plant and Soil* 31:48-56.

Equipment Thresher, cleaner, balance, moisture-tester

Harvest area The plot size commonly used in rice field experiments is 10 to 25 sq m. After discarding border areas on all sides of the plot to avoid competition effects, harvest as much area as possible but not less than 5 sq m.

Procedure

Harvest all plants in the harvest area. Thresh, clean, dry, and weigh the grains. Determine the moisture content of the grains and adjust grain weight to 14 percent moisture, using the following formula:

$$\text{Adjusted weight} = \frac{100 - \underline{M}}{86} \times \underline{W}$$

where W is the weight of the grains in grams and M is percent moisture content of the grains.

Comments

Make sure that at least two rows on each side of the plot are excluded from the harvest area to avoid border effects.

If one or more hills are missing all hills immediately adjacent to a missing hill must be excluded from the harvest. If the reduction in the number of hills harvested, due to the presence of missing hill in any plot, is not more than 20 percent, compute the grain yield per plot as

$$\frac{\text{Grain wt from harvested hills}}{\text{Total no. of harvested hills}} \times \text{Total no. of hills in normal plots}$$

If the reduction in number of hills harvested is more than 20 percent, do not harvest from this plot. Treat it as "missing value."

References

Gomez, K. A. and S. K. De Datta, 1971. Border effects in rice experimental plots. I. Unplanted borders. *Experimental Agr.* 7:87-92.

Gomez, K. A. and R. C. Alicbusan. 1969. Estimation of optimum plot size from rice uniformity data. *Philippine Agr.* 52:586-601.

Two methods can be used to measure panicle number per hill, average number of filled grains per panicle, percentage of unfilled grains, and 100-grain weight. The manual method does not involve any special equipment. The mechanical method is applicable when a seed-counter and a seed separator are available.

Manual
method

Equipment

Balance.

Sample selection

Select two representative four-hill (2 x 2 hills) sampling units. These hills must not come from the two border rows on any side of the plot, from hills adjacent to a missing hill, or from hills that were replanted,

Procedure

1. Count the number of panicles from each hill and total them for eight hills (P).
2. From each hill, separate the topmost panicle from the rest of the panicles. From these eight panicles, thresh and bulk the grains, and separate the unfilled grains from filled grains. Then count the number of filled grains (f) and unfilled grains (u) and weigh the filled grains (w).
3. From the rest of the panicles of the eight hills, thresh the grains and separate unfilled grains from filled grains. Then count the number of unfilled grains (U) and weigh the filled grains (W).

Calculations

$$\text{No. of panicles/hill} = \frac{P}{8}$$

$$\text{No. of filled grains/panicle} = \frac{f}{w} \times \frac{W + w}{P}$$

$$\text{Percentage unfilled grains} = \frac{\frac{U + u}{\frac{f(W + w)}{w} + (U + u)}}{1} \times 100$$

$$\text{100-grain weight} = \frac{w}{f} \times 100$$

Comments

Weigh the grains in steps 2 and 3 simultaneously to ensure that the two samples have a similar moisture content. If the determination of yield components is intended for comparing computed yield with measured yield, in addition to obtaining the weight of the filled grains in step 3, determine percent moisture content of the filled grains (\underline{M}). The computation of 100-grain weight at 14 percent moisture content then becomes

$$\frac{100 - \underline{M}}{86} \times \frac{\underline{w}}{f} \times 100$$

To separate unfilled grains from filled grains, salt-water (sp gr 1.06) method can be used. As shown in Table 1, manual separation gives a higher value for percentage filled grains than the use of salt-water.

Table 1. Comparison of methods for measuring percentage of filled grains and weight of 1,000 grains at different nitrogen levels. IRRI, 1969 dry season.

Nitrogen level (kg/ha)	Panicles/hill (no.)	Spikelets/panicle (no.)	Filled grains ^{a/} (%)		1,000-grain wt ^{a/} (g)		
			M	S	M	S	B
0	6.7	100	93	83	28.3	27.9	29.3
50	9.1	113	93	85	28.8	28.8	30.5
100	10.2	122	94	84	30.1	31.8	30.7
200	13.4	120	94	81	29.9	29.6	30.6
Mean	9.9	116	94	83	29.3	29.5	30.3

^{a/} M = manual method; S = salt-water method; B = blown twice by Almaco seed cleaner.

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Mechanical method

Equipment

Seed counter, seed separator, balance.

Procedure

1. Count the number of panicles from each hill and total them for the eight hills (\underline{P}).

2. Thresh grains from all sample hills. Separate unfilled grains from filled grains using the seed separator.

3. Using the seed counter, count the number of filled grains (F) and the number of unfilled grains (U).

4. Weigh the filled grains (W).

Calculations

$$\text{No. of panicles/hill} = \frac{\underline{P}}{8}$$

$$\text{No. of filled grains/panicle} = \frac{\underline{F}}{\underline{P}}$$

$$\text{Percentage unfilled grains} = \frac{\underline{U}}{\underline{F} + \underline{U}} \times 100$$

$$\text{100-grain weight} = \frac{\underline{W}}{\underline{F}} \times 100$$

Principle Identification of unfertilized grains is based on the fact that unfertilized spikelets do not contain starch at all and hence give negative color reactions to the iodine reagent.

Reagents

1) Iodine solution, 1%

Dissolve 1 g of iodine (I₂) in 100 ml of 5% potassium iodide (KI) solution. Prior to use, dilute with distilled water to the specified concentrations as indicated in the procedure.

2) Ethanol, 70% (v/v)

3) HCL, concentrated

4) Xylene

Procedure 1

At early stages of ripening. Soak sample grains in the ethanol for sometime to remove chlorophyll, then transfer to 0.3% iodine solution and allow to stay for 20 minutes. Wash the grains with the ethanol to remove the iodine. Examine the grains in the light. Absence of black color indicates "unfertilized grains."

For more precise work, soak the grains in xylene before the examination. This step makes the husk transparent, thereby making the examination much easier.

Procedure 2

At maturity. Soak sample grains in warm water (about 50 C) overnight.

Soak the grains in concentrated HCl for 30 minutes, then transfer to 0.5% iodine solution, and allow to stay for 20 minutes. Proceed to the subsequent steps as in procedure 1.

Reference

Matsushirna, S. and Tanaka, T. 1960. Analysis of developmental factors determining yields and its application to yield prediction and culture improvement of lowland rice. LV. Early discrimination of non-fertilized rice grains. Proc. Crop Sci. Soc. Japan 28:365-366.

APPENDIX 1. Abbreviations used in this manual and their meanings

AR	analytical reagent
C	degree(s), Celsius
ca	about (<u>circa</u>)
cc	cubic centimeter(s)
cm	centimeter(s)
cm ² , sq cm	square centimeter(s)
concn	concentration
count/min	counts per minute
dpm	disintegration per minute
EDTA	ethylenediaminetetraacetate
g	gram(s)
LAI	leaf area index
LTR	light transmission ratio
<u>M</u>	molar (mole per liter)
ma	milliampere(s)
max	maximum
mg	milligram(s)
ml	milliliter(s)
mμ	millimicron(s)
μc	microcurie(s)
<u>N</u>	normal
ppm	parts per million
rpm	revolutions per minute
sp gr	specific gravity
UV	ultraviolet
VIS	visible
v/v	volume/volume (concentration)
wt	weight
w/v	weight/volume (concentration)

APPENDIX 2. A list of chemical suppliers.^{a/}

ALDRICH	Aldrich Chemical Co., Inc. 940 West St. Paul Avenue Milwaukee, Wisconsin 53233 U. S. A.	MALLINCKRODT	Mallinckrodt Chemical Works Laboratories Products 2nd and Mallinckrodt Streets St. Louis, Missouri 63160 U. S. A.
BAKER	J. T. Baker Chemical Co. 222 Red School Lane Phillipsburg New Jersey 08865 U. S. A.	MANN	Mann Research Laboratories Mountainview Avenue Orangeburg, New York 10962 U. S. A.
BDH	BDH Chemicals Ltd. Overseas Division Poole, England, BH12 4 NN	MCB	Matheson Coleman & Bell 2909 Highland Avenue Nenwood, Ohio 45212 U. S. A.
	Local Distributor: "MEDIC" a division of Mercury Drug Corporation P. O. Box 1847 Manila, Philippines	MERCK	E. Merck D 61 Darmstadt Germany
BIO-RAD	Bio-Rad Laboratories 32nd & Griffin Avenue Richmond, California U. S. A.		Local distributors: NECO Trading 1158 Magdalena St. Manila, Philippines
CALBIOCHEM	Calbiochem, Inc., Los Angeles, California 9005 U. S. A.		Belman Laboratories 741 Cordillera St. Corner Quezon Blvd. Ext. Quezon City, Philippines
EASTMAN	Eastman Kodak Co. Eastman Organic Chemical Rochester, New York 14650 U. S. A.	SIGMA	Sigma Chemical Co. 3500 Dekalb St. St. Louis, Missouri 63118 U. S. A.
FISHER	Fisher Scientific Co. International Division 52 Fadem Road, Springfield	THOMAS	Arthur H. Thomas Co. Vine Street at 3rd P. O. Box 779 Philadelphia Pennsylvania 19105 U. S. A.
INHELDER	Inhelder 41 Pioneer St., Mandaluyong Rizal, Philippines		
K&K	K & K Laboratories, Inc. 121 Express St., Engineers Hill Plainview, N.Y. 11803, U. S. A.	WAKO	Wako Junyaku Kogyo Co., Ltd. Do-shu cho 3-10 Higashi-ku, Osaka Japan

^{a/}The addresses listed here are those of companies from whom IRRI has purchased products. Listing here does not constitute an endorsement. Products of other suppliers may be equally satisfactory.

Atomic absorption spectro-photometer	Perkin Elmer Model 303 Perkin-Elmer Corporation P. O. Box 2539 Church St., Station New York 8, New York U. S. A.	Furnace	Hevi-Duty Heating Equipment Division Basic Products Corporation Watertown, Wisconsin U. S. A.
Automatic area meter	Model AAM-4 Daiichi Boeki Shokai Co. 4-5, 2-Chome Higashi-Shinbashi Minato-ku, Tokyo Japan <u>Also available from:</u> Hayashi Denko. Ltd. Kanda Zipboh-cho 1-32 Chiyoda-ku, Tokyo Japan	Incubators	Precision Scientific Co. 3737 West Cortland Street Chicago 47, Illinois U. S. A. Labline Instrument Inc. Labline Plaza Melrose Park, Ill. 60160 U. S. A. Local distributor: Greuter & Matile, Inc. 1176 C-D-E Pasong Tamo Makati, Rizal, Philippines
Balance	Mettler Instruments Corp. 20 Nassau Street Princeton, N. J. 08540 U. S. A. Toledo Scale Model 4644 (capacity 12.5 kg) Toledo Scale Co., Toledo Ohio, U. S. A.	Kjeldahl apparatus	Arthur H. Thomas Co. Vine Street at 3rd P. O. Box 779 Philadelphia Pennsylvania 19105 U. S. A.
Carbon-hydrogen analyzer	Coleman Model 33 Coleman Instruments 42 Madison Street Maywood, Illinois 60153 U. S. A.	Light meter	Toshiba Illuminator SPI-No. 5 Daiichi Boeki Shokai Co. 4-5, 2-Chome Higashi-Shinbashi Minato-ku, Tokyo Japan
Centrifuge and accessories	International Equipment Co. 360 Second Avenue Needham Height Massachusetts 02194 U. S. A.	Liquid scintillation spectrometer	Packard Instrument Co., Inc. 2200 Warrenville Road Downers Grove, Ill. 60515 U. S. A.
Flame photometer	EKO Flame Photometer Model N Daiichi Boeki Shokai Co. 4-5, 2-Chome, Higashi-Shinbashi Minato-ku, Tokyo, Japan	Magnetic stirrer	Mini-stirrers: Toyo Roshi Kaisha Ltd. 7, 3-Chome Nihonbashi-Honcho Chuo-ku, Tokyo Japan

Mixer	Omni-mixer: Ivan Sorvall Inc. Norwalk, Connecticut 06852 U. S. A.	Thresher	Vogel Thresher Bills Welding Co., Ltd. South Grand St. Pullman, Washington U. S. A.
Moisture	Steinlite Electric Moisture Tester D. L. Model RCT Fred Stein Laboratories, Inc. 121 North Fourth St. Atchinson, Kansas 66002 U. S. A.	Willey mill	Arthur H. Thomas Co. Vine Street at 3rd P. O. Box 779 Philadelphia, Penn. 19105 U. S. A.
pH meter	Coleman Model Metrion III Coleman Instruments 42 Madison Street Maywood, Illinois 60153 U. S. A.		
	TOA Model HM-5A TOA Electronics Ltd. 235 Suwa-cho Shinjuku-ku, Tokyo, Japan		
Seed cleaner	Almaco Seed Cleaner Allan Machine Co. Ames, Iowa, U. S. A.		
Seed counter	Fujimoto Kagaku Kogyo, Co. Ltd. 2-15 Uchikanda 3-Chome Chiyoda-ku, Tokyo, Japan		
Seed separator	Fujimoto Kagaku Kogyo, Co. Ltd. 2-15 Uchikanda 3-Chome Chiyoda-ku, Tokyo, Japan		
Shaker	Burell Corporation 2223 Fifth Avenue Pittsburgh 19, Pennsylvania U. S. A.		
Spectro-photometer	Coleman Model 101 Coleman Instruments 42 Madison Street Maywood, Illinois 60153 U. S. A.		

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APPENDIX 4. A list of isotope suppliers.^{a/}

1. The Radiochemical Centre
Isotope Production Unit
AERE Hamell, Didcot
Berkshire, England

or

Amersham/Searle Corporation
2636 S Clearbrook Drive
Arlington Heights, Illinois 6005
U. S. A.

2. New England Nuclear
575 Albany Street
Boston, Massachusetts 02118
U. S. A.
3. Nuclear Equipment Chemical Corporation
165 Marine Street
Farmingdale, New York 11735
U. S. A.
4. International Chemical & Nuclear Corporation
Chemical and Radioisotope Division
2727 Campus Drive
Irvine, California, U. S. A.

or

1601 Trapelo Road
Waltham, Massachusetts, U. S. A.

5. Schwarz BioResearch, Inc.
Orangeburg, New York 10962
U. S. A.
6. Research Products Division
Miles Laboratories, Inc.
Box 272, Kankakee, Illinois 60901
U. S. A.

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