

APPENDIX

APPENDIX A

1. Yeast Mannitol Broth (YMB); YMA*

Mannitol	10 g
K ₂ HPO ₄	0.5 g
MgSO ₄ ·7H ₂ O	0.2 g
NaCl	0.1 g
Yeast extract	0.5 g
Distilled water	1.0 liter

Adjust to pH 6.8. Autoclave at 121°C and 15 lbs. For all media every time in autoclave according to volume:

Up to 500ml	20 minutes
1000ml	30 minutes
2000-4000ml	40 minutes
5000-8000ml	60 minutes

For Yeast Mannitol Agar (YMA)*

Yeast mannitol broth	1.0 liter
Agar	15 g
Congo red 1%	2.5 ml

* Taken from Somasegaran and Hoben (1994)

2. Inhibitory Model Agar-2 (IMA-2)*

Glucose	5 g
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Soluble starch	5 g
Beef extract	1 g
Yeast extract	1 g
Nz-case	2 g
NaCl	2 g
CaCO ₃	1 g
Agar	20 g
Distilled water	1.0 liter

* Taken from Shimizu *et al.*, 2000

3. N-free nutrient solution*

stock solutions	element	M	form	MW	g/l	M
1	Ca	1000	CaCl ₂ .2H ₂ O	147.03	294.1	2.0
2	P	500	KH ₂ PO ₄	136.09	136.1	1.0
3	Fe	10	Fe-citrate	355.04	6.7	0.02
	Mg	250	MgSO ₄ .7H ₂ O	246.5	123.3	0.5
	K	250	K ₂ SO ₄	174.06	87.0	0.5
	Mn	1	MnSO ₄ .H ₂ O	169.02	0.338	0.002
4	B	2	H ₃ BO ₃	61.48	0.247	0.004
	Zn	0.5	ZnSO ₄ .7H ₂ O	287.56	0.288	0.001
	Cu	0.2	CuSO ₄ .5H ₂ O	249.69	0.100	0.0004
	Co	0.1	CoSO ₄ .7H ₂ O	281.12	0.056	0.0002
	Mo	0.1	Na ₂ MoO ₄ .2H ₂ O	241.98	0.048	0.0002

*Broughton and Dillworth (1970) cited by Somasegaran and Hoben (1994)

For each 10 liters of full strength culture solution, take 0.5 ml each of solution 1 to 4, then add to 5.0 liters of water, then dilute to 10 liters. Use 1N NaOH to adjust the pH to 6.6-6.8.

For plus N control treatments, KNO_3 (0.05%) is added given an N concentration of 70ppm.

4. Soil Testing Kit (Central laboratory, Faculty of Agriculture Chiang Mai University)

Soil sample preparation

- Collect the same amount of soil from each sport. Mixed well with soils from the same sampling area.
- Air dry the sample. Do not dry the sample under the sun or in the oven in order to prevent the change of plant nutrient content.
- Grid and sieve the soil.

4.1 Soil pH testing procedure

- Fill up half of 2 wells in the pH testing tray with a soil sample
- Add pH indicator No. 2 into the soil in the first well until the soil is saturated and then add 2 more drops.
- Mixed well the soil and indicator by using wooden stick. Leave for a while until the soil is settle down.
- Read the pH value from color chart having the same color as the indicator in the well.
- If the color of indicator in the well is yellow checking pH of the soil in second well by using pH indicator No.1.

- If the color of indicator in the well is purple checking pH of the soil in second well by using pH indicator No.3.
- Read the pH value from color chart having the same color as the indicator in the well.

Criteria for soil pH measurement

pH	Level of soil reaction	Effects on plants
4.5 and lower	Extremely acidic	Too acid for most plants
4.6 – 5.2	Very acidic	Too acid for most plants
5.3 – 6.0	Moderately acidic	Too acid for some plants
6.1 – 6.9	Slightly acidic	Suitable for most plants
7.0	Neutral	Suitable for most plants
7.1 – 7.5	Slightly basic	Suitable for most plants
7.6 – 8.2	Moderately basic	Too basic for some plants
8.3 – 9.0	Very basic	Too basic for many plants
9.1 and higher	Extremely basic	Too basic for most plants

4.2 Measurement of Lime Requirement (LR)

4.2.1 Lime requirement testing kit

- Buffer solution
- Plastic tube with cap
- Plastic spoon and wooden stick
- Plastic droppers 3ml. and 1ml.
- pH indicator No. 3.
- Lime requirement testing color chart

4.2.2 Testing of lime requirement of soil

- Soil 3 spoons + 3 ml of buffer solution
- Leave for 30 minutes
- Shaking every 10 minutes (2 times)
- Take the clear supernatant by using 1ml dropper
- Add 4 drops into the well in the tray
- Soil solution (yellow)
- Add 1 drop of pH indicator No.3.
- The color of soil solution change to green
- Read the figure from the LR testing color chart and estimate the amount of lime needed from the table of the LR color chart.

Lime Requirement Estimation

pH of buffer	amount of lime need (kg/ha)		
	CaCO ₃	dolomite lime stone	burned lime
6.8	1,057	969	781
6.6	2,112	1,944	1,562
6.4	3,169	2,912	2,344
6.2	4,356	4,006	3,225
6.0	5,412	4,981	4,006
5.8	6,731	6,194	4,981
5.6	8,319	7,650	6,156
5.4	10,425	9,594	7,719
5.2	13,462	12,388	9,962
5.0	17,294	15,906	12,794

Note: use only one kind of lime

4.3 Testing of available P content of the soil

Available P testing kit

4.3.1 Extraction of available P from the soil

- P1 solution
- Plastic spoon with wooden stick
- Paper for soil sample
- Plastic bottle for soil extraction 1 bottle/sample
- Plastic bottle for filtration 1 bottle/sample
- Plastic funnel for and filter paper 1 set/sample
- 10 ml. plastic syringe 1 unit

4.3.2 Kits for color development

- A set of standard P solution (low, high, very high)
- Color development powder 1 tube (should be used within 24 hours after mixing with P2 solution)
- P2 solution 1 bottle
- P3 solution 1 bottle
- Glass test tube 3 tubes for standard P
- Glass test tube for soil sample 1 tube/sample
- 10 ml. plastic syringe
- 3 ml. plastic dropper 1 unit
- 1 ml. plastic dropper

4.3.3 Measurement of available P testing

- Soil sample 1 spoon
- 10 ml. P1 solution
- Shake 1 minute

- Filter
- Add P2 solution 10 ml. in color development powder

4.3.4 Color development

- Add 1 ml. from each of standard P solution into each of class test tube by starting with the lowest concentration
- Add soil extracting solution into one glass test tube
- Add 1ml. of color developing solution per tube
- Add 3ml. of P3 solution
- Shake slightly and leave for 15 minutes
- Compare the color of soil sample with the color of a set of standard P

Criteria of available P measurement

color comparison with STD P	level of available P	content of available P in soil (mg P/kg soil)
P sample < P std 1	Low	< 10
P std 1 < P sample < P std 2	Moderate	10 – 40
P std 2 < P sample < P std 3	High	> 40
P std 3 < P sample	Very High	> 100

4.4 Method for testing of exchangeable K in the soil

Exchangeable K testing kit

4.4.1 Soil extraction

- Plastic spoon with wooden stick
- Paper for soil sample

- Plastic bottle for soil extraction 1 bottle/sample
- Plastic bottle for filtration 1 bottle/sample
- K1 solution one bottle
- Funnel for and filter paper 1 set/sample
- 12 ml. syringe 1 unit

4.4.2 K testing by turbidity

- A set of standard K solution (low, high, very high)
- K2 solution 1 bottle (should kept in refrigerator)
- K3 solution 1 bottle
- Turbidity testing chart

4.4.3 Method of exchangeable K testing

- Soil sample 3 spoon
- 12 ml. K1 solution
- Shake 15 minutes
- Filter
- Add 1 ml. from each of standard K solution into each of glass test tube by starting with the lowest concentration

- Add soil extracting solution into one glass test tube
- Add K2 solution into each tube (10-15 drops)
- Add 1ml. of K3 solution

- Shake slightly and leave for 3 minutes

- Compare the turbidity of color extract solution with those color of a

set of standard K by placing the turbidity testing chart at the back of the tube

Criteria of exchangeable K measurement

turbidity comparison with STD K	level of exchangeable K	content of exchangeable P in soil (mg K/kg soil)
K sample < K std 1	Low	< 60
K std 1 < K sample < K std 2	Moderate	60 – 100
K std 2 < K sample < K std 3	High	> 100
K std 3 < K sample	Very High	> 300

5. Measurement of Nitrogen Fixation by leguminous

5.1 Determination of Allantion

(Young and Conwey, 1942)

Reagents:

- 1- NaOH (0.5 M)
- 2- Phenylhydrazine hydrochloride (0.33%)

(to be made fresh on each day of analysis and store in a brown bottle)

- 3- Potassium ferricyanide (0.833%)

(to be made fresh on each day of analysis and store in a brown bottle)

- 4- HCl (0.65 M)

- 5- HCl (10 M) store at 0°C

- 6- Allantion standard (1.0 μ mole/ ml)

Dilute stock for peppering standard curve

- (1) 10 n mole/ ml (2) 20 n mole/ ml
 (3) 30 n mole/ ml (4) 40 n mole/ ml
 (5) 50 n mole/ ml

Note: Always includes 2.5 ml distilled water blank with standard during analysis. A full set of standards should be run through (0-125 n mole; take 2.5 ml from each dilute stock) to check linearity of response.

Procedure:

- 1 Pipette 0.05-0.01 ml of sap sample into each test tube and dilute to 2.5 ml with distilled water.
- 2 Add 0.5 ml of 0.5 M NaOH
- 3 Mix and place tubes in boiling water bath for 10-15 min.
- 4 Remove tubes and allow to cool to room temperature, then add 0.5 ml of 0.65 M HCl and 0.5 mL of 0.33% Phenylhydrazine hydrochloride to each tube.
- 5 Place tubes in boiling water bath for 2-4 min.
- 6 Cool tubes immediately water bath for 15 min.

(The rapidity of cooling is an important factor in technique, rapid cooling increased the intensity of the final color and lower temperatures also increase the color intensity)

- 7 After remove tubes from ice bath, add 2 ml of 10 N HCl (chilled 0°C) then 0.5 mL of potassium ferricyanide. (mix immediately after each addition of potassium ferricyanide).
- 8 Stand at room temperature for 10 min then measure the absorbance at 525 nm.

The color is not stable, in 60 minutes there is 8-15% fading of color intensity, therefore the measurement of sample should be finished within 20 min)

5.2 Determination of Amino Acids with Ninhydrin

(Yemm and Cocking, 1955)[An adaptation of the method by Herridge, 1984]

Reagents:

- 1- Citrate buffer pH 5 (citric acid 16.8% (w/v), NaOH 6.4% (w/v).
- 2- Ninhydrin reagent (ninhydrin 0.96% (w/v), ascorbic acid 0.033% (w/v) in 2-methoxyethanol)
- 3- Ethanol 60% (w/v)
- 4- Asparagine standard (2.5 μ mole/ ml)

Dilute stock for peppering standard curve

- | | |
|---------------------------|---------------------------|
| (1) 50 <i>n</i> mole/ ml | (2) 100 <i>n</i> mole/ ml |
| (3) 150 <i>n</i> mole/ ml | (4) 200 <i>n</i> mole/ ml |
| (5) 250 <i>n</i> mole/ ml | |

Note: Always includes 0.5 ml distilled water blank with standard during analysis. A full set of standards should be run through (0-125 *n* mole; take 0.5 ml from each dilute stock) to check linearity of response.

Procedure:

- 1- Pipette 0.05 ml of sap sample into each tube and dilute to 0.5 ml with distilled water.
- 2- Add 0.5 ml of citrate buffer.
- 3- Add 1.2 ml of ninhydrin reagent and mix well.
- 4- Place tube sin boiling water bath for 25 min.

5- Remove from boiling water bath and cool to room temperature then add 3 ml of 60% ethanol.

6- Measure the absorbance 570 nm.

5.3 Determination of Nitrate

(Catadol et al., 1975)

Reagents:

1- 2 N NaOH

2- 5% (w/v) Salicylic acid in concentrated H₂SO₄ (SA- H₂SO₄).

(5 g of salicylic acid in 100 ml of con. H₂SO₄ make fresh at least once each week and store in brown bottle)

3- Nitrate standard (2.5 μ mole KNO₃/ ml)

Dilute stock for preparation of standard curve

(1) 2.50 μ mole KNO₃/ ml

(2) 5.00 μ mole KNO₃/ ml

(3) 7.50 μ mole KNO₃/ ml

(4) 10.00 μ mole KNO₃/ ml

(5) 12.50 μ mole KNO₃/ ml

Note: Always includes 0.5 ml distilled water blank with standard during analysis. A full set of standards should be run through (0-0.5 μ mole; take 0.5 ml from each dilute stock) to check linearity of response.

Procedure:

1- Pipette 0.05 ml of sap sample into test.

2- Add 0.2 ml of 5% SA- H₂SO₄ to sap sample and mix well.

3- Stand at room temperature for 20 min, then add 4.75 ml of 2 N NaOH into tubes slowly (to raise the pH about 12).

4- Cool to room temperature and measured the absorbance at 410 nm.

5.4 Relative Ureide Index

$$\text{Relative ureide index (\%)} = \frac{\text{UreideN}}{\text{TotalsapN}} \times 100$$

Since one ureide molecule contains 4 N-atoms, ureide is calculate as 4 X ureide molar concentration. Total sap N is estimated as 4 X ureide + amino acid + nitrate.

The relative ureide index can be calculated as:

$$\text{Relative ureide index (\%)} = \frac{4 \times \text{ureide}}{(4 \times \text{ureide} + \text{amino acid} + \text{nitrate})} \times 100$$

5.5 Plant sample digestion

(Novosamsky et al., 1974)

Reagents

- 1- Sulphuric acid, 96% (w/w), c (H₂SO₄) = 18 mol/l (d = 1.84g/ml).
- 2- Hydrogen peroxide 30% (w/w)
- 3- Salicylic acid, powder.
- 4- Digestion mixture. Put 18 ml water in a 250 ml Erlenmeyer flask. While cooling, add in small portions 100 ml of sulphuric acid (1) (CAUTION). Then dissolve 6 g of salicylic acid (3) with the aid of a magnetic stirrer.

Remark

- 3- Hydrogen peroxide must be of analytical quality; a lower grade may be stabilized with EDTA, phosphate, or other interfering compounds.
- 4- Since the dried sample still contains about 10% (w/w) water, the sulphuric

acid is somewhat diluted to prevent the temperature from raising too high during addition of the digestion mixture which otherwise would result in loss of nitrate.

Procedure

Weigh, to the nearest 0.001 g, approximately 0.3 g of the dried plant material sample in a metal weighing funnel and transfer the sample to a 50 ml volumetric flask. Take care that all the plant material comes below the neck of the flask. Add 3.3 ml of the digestion mixture (4) and swirl carefully until all the plant material is moistened. Allow to stand overnight. Prepare also two blank digestions.

Heat the flask on a hot plate at 180°C for about one hour. Remove the flask from the plate, let cool, and add 5 drops of hydrogen peroxide (2). Place the flask on the hot plate and increase the temperature to about 280°C. Heat 5-10 minutes until the water has evaporated (appearance of white vapours). Remove the flask from the plate, let cool, add 5 drops of hydrogen peroxide (2), and heat again for 5-10 minutes to appearance of white vapours. Repeat this treatment until the digest has turned colourless.

Remove the flask from the plate and cool to room temperature. Add about 10 ml water and mix; swirl until most of the precipitate has dissolved. Make up to the mark with water, mix well and filter over coarse filter paper.

Remark

5- After moistening the sample with the digestion mixture (4), at least 2 hours are needed to form the nitro-salicylic acid compounds. A longer period (overnight), however, will prevent foaming later on.

- 6- When heating at 180°C, the sample will turn black and foam may be formed. If this reaches the neck of the flask, 1 or 2 drops hydrogen peroxide (2) should be added.
- 7- A precipitate may be formed when cooling after completing the digestion. The addition of 10 ml water then produces enough heat to dissolve the precipitate rapidly.
- 8- The digest is filtered to remove any SiO₂ that will otherwise dissolve gradually and then interfere in the determinations.
- 9- Do not use the volumetric flasks for other purposes.
- 10- Near the end of the digestion, the liquid becomes yellow due to the last portions of organic matter, or to Fe. If the yellow colour persists after extra addition(s) of H₂O₂, then the presence of Fe should be assumed and the digestion considered as completed.

5.6 Determination of nitrogen in digested plant samples by colorimetry

Field of application

This determination may be carried out on

digest 2.2 (H₂SO₄-salicylic acid-H₂O₂-Se)

digest 2.2 (H₂SO₄-salicylic acid-H₂O₂)

If for any reason, this determination cannot be carried out, then determination 3.23 may be done.

Apparatus

Colorimeter or spectrophotometer

Reagents

- 1- Sodium hydroxide solution, $c(\text{NaOH}) = 10 \text{ mol/l}$. Dissolve 200g of sodium hydroxide, NaOH , in about 400 ml water; allow to cool and make up to 500 ml.
- 2- Salicylate solution. Dissolve 110 g of salicylic acid, $\text{C}_7\text{H}_6\text{O}_3$ in 150 ml of sodium hydroxide solution (1) and make up directly with water to 250 ml. Prepare just before use.
- 3- Buffer solution pH 12.3. Dissolve 26.70 g sodium hydroxide phosphate dehydrate, $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ in some water in a 2 liters volumetric flask. Add 10 ml of the sodium hydroxide solution (1) and dilute to volume with water. Measure the pH and adjust if necessary.
- 4- EDTA solution. Dissolve 4 g of disodium ethylene diamine tetra acetate dehydrate, $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ in 100 ml water.
- 5- Hypochlorite solution. A stock solution, containing approximately 1 M sodium hypochlorite in 0.1 M NaOH , should be purchased commercially. Dilute 20 ml of this stock solution with water to 100 ml. Prepare fresh daily.
- 6- Nitroprusside solution. Dissolve 50 ml of sodium nitroprusside dehydrate, $\text{Na}_2\text{-}[\text{Fe}(\text{CN})_5\text{NO}] \cdot 2\text{H}_2\text{O}$, in 100 ml water. Prepare just before use.
- 7- Mixed reagent I. Mix 50 ml of the salicylate solution (2) with 100 ml of the nitroprusside solution (6) and 5 ml of the EDTA solution (4).
- 8- Mixed reagent II. Mix 200 ml of the buffer solution (3) with 50 ml of the hypochlorite solution (5).

- 9- Stock solution, N concentration 2500 mg/l. Dissolve 11.793 g of ammonium sulphate, $(\text{NH}_4)_2\text{SO}_4$, in water in a 1000 ml volumetric flask and make up to volume with water.

Remark

- 4- The final solution of sodium hypochlorite should contain $0.7\% \pm 0.1\%$ of active chlorine. This will be true for solutions purchased from a supplier of chemicals; if, however, the hypochlorite was bought as bleach in a supermarket, a check on its concentration is recommended.
- 5- The mixed stock solution of 4.5.4 can be used.

Standard series

Pipette into 100 ml volumetric flask, that contain already about 50 ml water, 4.5 ml of concentrated sulphuric acid (96%). Mix and let cool. Then add 0 - 1.00 - 2.00 - 3.00 - 4.00 - 5.00 - 6.00 ml of the stock solution (9) and dilute to volume with water.

Dilute this standard series 1 + 9 (v/v) with water; this should be done together with the diluting of the digests. The diluted standard series has N concentrations of 0 - 2.5 - 5 - 7.5 - 10 - 12.5 - 15 mg/l.

Procedure

Dilute all digests 1 + 9 (v/v) with water.

Pipette 0.20 ml of the diluted sample digest, the diluted blank digests and the diluted standard series into test tubes. Add 3.0 ml of mixed reagent I (7) and mix. Then add 5.0 ml of mixed reagent II (8) and mix. Allow to stand for at least 2 hours. Measure the absorbance in a 1-cm cuvette at a wavelength of 660 nm. Plot a calibration curve, and read the N concentrations.

Remark

- 5- In 2 hours the blue color reaches its maximum intensity; it is stable for at least 10 hours.
- 6- The digests obtained according to 2.2 or 2.3 are 0.7 – 0.9 M in H₂SO₄. Any further dilutions (if the N concentrations would be higher than the highest standard) should be made with zero standard solution.
- 7- The test tubes must be used exclusively for this N determination. They must first be cleaned by taking a blank determination; there after they should be cleaned only with water.

Calculation

The nitrogen content of the dried plant material, expressed in mmol/kg N, is calculated by

$$0.714 * (a-b) * \frac{V}{W}$$

in which

- a : concentration of nitrogen in the diluted sample digest, in mg/l;
- b : concentration of N in the diluted blank digest, in mg/l;
- v : total volume of digest at the end of the digestion procedure, in ml;
- w : weight of plant material sample, in g.

APPENDIC B

Appendix Table 1.

ANOVA table for nodule, shoot and total plant dry weight, %Ndfa and shoot N uptake of DT 84 soybean variety in control room

sov	df	MS			
		nodule dry weight	shoot dry weight	total dry weight	total plant N uptake
block	4	0.00193	0.47313	0.53102	5.209E - 04
tr.	15	0.00473 **	0.77281 **	0.81004 **	8.930E - 04 **
error	60	0.00051	0.12321	0.13216	1.279E - 04

Appendix Table 2.

ANOVA table for nodule, root and shoot dry weight of DT 84 soybean variety
at V₆ and R_{3,5} stage

sov	df	MS					
		V ₆			R _{3,5}		
		nodule dry weight	root dry weight	shoot dry weight	nodule dry weight	root dry weight	shoot dry weight
block	4	0.991E - 05	0.00738	0.05089	0.00128	0.05430	0.21736
tr.	11	3.266E - 04 **	0.02022 **	0.18688 **	0.00231 ns	0.17023 **	1.03575 **
error	44	7.533E - 05	0.00373	0.03904	0.00149	0.03299	0.26265

Appendix Table 3.

ANOVA table for relative ureide index (%), %Ndfa, shoot N uptake and amount of fixed N of DT 84 soybean variety at R_{3,5} stage

sov	df	MS			
		relative ureide index (%)	%Ndfa	shoot N uptake	Amount of fixed N
block	4	165.00	275.51	0.4685E - 04	0.00040
tr.	11	1215.67 **	2150.03 **	0.3114E - 04 ns	0.00130 **
error	44	100.00	173.11	1.616E - 04	0.00019

Appendix Table 4.

ANOVA table for shoot and root dry weight, no. of pods per plant and seed yield per plant of DT 84 soybean variety at harvest stage

sov	df	MS			
		shoot dry weight	root dry weight	pods per plant	seed yield
block	4	0.12394	0.05337	19.7508	3.25469
tr.	11	0.66782 **	0.13689 **	40.5366 **	5.44028 **
error	44	0.24142	0.03238	9.0361	0.95148

CURRICULUM VITAE

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2007-2010	Master of Science (Agriculture) in Agronomy, Department of Plant Science and Natural Resources, Faculty of Agriculture, Chiang Mai University, Thailand.
1993-1998	Bachelor of Science in Animal Production and Health of Royal University of Agriculture, Phnom Penh, Cambodia.
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Scholarship Awarded	
2007-2010	Thailand International Development Cooperation Agency (TICA).
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Work Experiences	
2002-Present	Assistant to Excellency It Nody, Under Secretary of State of the Ministry of Agriculture, Forestry and Fisheries (MAFF).
2009-Present	High Technical Official of the Department of International Cooperation (DIC) of MAFF.
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