APPENDICES

1. APPENDIX I

Yeast mannitol broth (YMB); YMA*

Mannitol	10.0 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	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 vary time in autoclave according to volume:

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

For Yeast Mannitol Agar (YMA)*

Yeast Mannitol Broth	1 liter
Agar	15 g

* Taken from Somasegaran and Hoben, 1994

Inhibitory Mold Agar-2 (IMA-2)*

Glucose	5 g
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 liter

* Taken from Shimizu *et al*., 2000

N- free nutrient solution*

Stock Solutions	Element	M	Form	MW	g/l	M
1	Ca	1000	$CaCl_2.2H_2O$	147.03	294.1	2.0
2	P	500	KH_2PO_4	136.09	136.1	1.0
3	Fe	10	Fe-citrate	355.04	6.7	0.02
	Mg	250	$MgSO_4.7H_2O$	246.5	123.3	0.5
	K	250	K_2SO_4	174.06	87.0	0.5
	Mn	1	$MnSO_4.H_2O$	169.02	0.338	0.002
4	B	2	H_3BO_3	61.84	0.247	0.004
	Zn	0.5	$ZnSO_4.5H_2O$	287.56	0.288	0.001
	Cu	0.2	$CuSO_4.7H_2O$	249.69	0.100	0.0004
	Co	0.1	$CoSO_4.7H_2O$	281.12	0.056	0.0002
	Mo	0.1	$Na_2MoO_2.2H_2O$	241.98	.048	0.0002

* Broughton and Dillworth, (1970) cited by Somasegaran and Hoben, (1984)

For each 10 liters of full strength culture solution, take 5.0 ml each of solutions 1 to 4, then add to 5.0 liters of water, then dilute to 10 liters. Use 1 N 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 70 ppm.

Measurement of Nitrogen fixation by leguminous

Determination of Allantoin

(Young and Conway, 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) stored at 0˚C
6. Allantoin standard (1.0 µmole/ 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 nmole; 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. 7. After removed tubes from ice bath, add 2 mL of 10 N HCl (chilled to 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 a 8-15% fading of color intensity, therefore the measurement of sample should be finished within 20 min)

Determination of Amino acids with Ninhyrin

(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 µmole/ mL)

   Dilute stock for peppering standard curve,

   (1) 50 *n* mole/ mL (2) 100 *n* mole/ mL

   (3) 150 *n* mole/ mL (4) 200 *n* mole/ mL

   (5) 250 *n* 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 tubes in 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 at 570 nm.

Determination of Nitrate

(Cataldo *et al*., 1975)

Reagent:

1. 2 N NaOH

2. 5% (w/v) Salicylic acid in concentrated H_2SO_4 (SA- H_2SO_4)

   (5 g of salicylic acid in 100 mL of con. H_2SO_4, make fresh at least once each week and store in a brown bottle)

3. Nitrate standard (25 µmole KNO_3 / mL)

   Dilute stock for peppering standard curve

   (1) 2.50 µmole KNO_3 / mL (2) 5.00 µmole KNO_3 / mL

   (3) 7.50 µmole KNO_3 / mL (4) 10.00 µmole KNO_3 / mL

Note: Always includes 0.05 mL distilled water blank with standard during analysis. A full set of standards should be run through (0-0.5 µmole, take 0.05 mL from each dilute stock) to check linearity of response.

Procedure:

1. Pipette 0.05 mL of sap sample into test tube.
2. Add 0.2 mL of 5% SA- H_2SO_4 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 above 12).
4. Cool to room temperature and measured the absorbance at 410 nm.

Relative ureide index

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

Since one ureide molecule contains 4 N-atoms, ureide N is calculates 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:

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

Analysis of Total Nitrogen

(Novozamsky *et al*., 1974)

Measure shoot dry weight sample about 0.2000 to 0.2009 g and put it into tube (1 or 2 tubes) per sample.

1. 1000 ml of H_2SO_4 (conc:) mix with 3.5g Selenium and then heating with 300^{o} C with Yellow MAG HS 10 Machine until black liquid turn to white color. It takes about 3 or 4 hrs. Take 100 ml of this solution mix well with Salicylic Acid 7.2 g by using glass rod.
2. Add 3 ml of above solution per tube
3. Shake with Vortex Genie 2 Machine until melted well
4. Leave all tube into Heating Machine (Hot plates oven) for one night and then turn the Heating Machine on
   a) Heating with 150˚ C for 2 hours after that takes all tube out off heating machine.
   b) Add 1 ml of Hydrogen Peroxide for 3 times
   c) Bring all tube back into Heating Machine and then put it on the heating machine again.
   i) 200˚C for 2 hours
   ii) 250˚C for 2 hours
   iii) 300˚C for 1 hour
   iv) 350˚C for 1 hour
   v) 380˚C until liquid in all tube turns into white color
5. By adding pure water into all tube and to adjust 50 ml volumetric flask and then pour into plastic bottle by using filter paper and keep in refrigerator for measuring

NH_4^+-N Test by Colorimeter Method

Reagents:

1. 10 M NaOH
2. Salicylic Acid 110 g in 10 M NaOH 105 ml mixed together and adjust until 250 ml
3. Na_2HPO_4 (anhydrous) [buffer pH 12.3]

   13.35 g of Na_2HPO_4 + 10 M NaOH (5 ml), it dissolves in distilled water and adjust pH 12.3 until 1000 ml for final volume
4. 4 % EDTA

   EDTA 4 g/ 100 ml of distilled water
5. Sodium hypochloride 1 M in 0.1 M NaOH

   - Sodium hypochloride 258.66 ml
   - 10 M NaOH 5.0 ml
   - adjust 500 ml in volumetric flask

* Usage: 20 ml of sodium hypochloride and adjust until 100 ml with distilled water

6. Sodium nitroprusside solution 0.05 %

dissolve 0.05 g of Sodium nitroprusside in distilled water 100 ml

*Prepare just before use.

standard

dissolve $(NH_4)_2SO_4$ 11.739 g in distilled water in volumetric flask 1000 ml

= 2500 mg/L (stock solution)

Standard concentrate 0 2.5 5.0 7.5 10.0 12.5 15.0 mg/L

Procedure:

1. Use diluted sample 0.2 ml (both Blank and Standard) per tube

2. Solution I (3 ml) : [reagent 2: 50 ml + reagent 6: 100 ml + reagent 4: 5 ml] mixed well
3. Solution II (5 ml) : [reagent 3: 200 ml + reagent 5: 50 ml] mixed well

Wait 2 hrs for color development. After that it can measure within 20 hrs.

Measure the absorbance 660 nm by using Spectrophotometer.

2. APPENDIX II

Appendix table 1.

ANOVA table for Nodule, Root and Shoot Dry weight of Myanmar Soybean Variety at V6 and R 3.5

Source of variance	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	2	0.00618*	0.53256^{NS}	4.55823*	0.01268**	0.70816^{NS}	4.33634**
treat	11	0.00740*	0.38095^{NS}	2.78496*	0.01771**	0.43771^{NS}	6.20731**
Error	22	0.00327	0.25105	1.04118	0.00497	0.29692	1.82727

^{NS} = Non-significant

* = Significant at P<0.05

** = Significant at P<0.01

Appendix table 2.

ANOVA table for Nodule, Root and Shoot Dry weight of Thailand Soybean Variety at V6 and R 3.5

Source of variance	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	2	0.00437*	0.61470^{NS}	4.04424**	0.02316*	3.9033^{NS}	30.9264**
treat	11	0.01589*	0.29674^{NS}	3.74426**	0.03907*	1.7121^{NS}	17.4918**
Error	22	0.00562	0.20140	0.76742	0.01646	0.57431	5.1054

^{NS} = Non-significant

* = Significant at P<0.05

** = Significant at P<0.01

Appendix table 3.

ANOVA table for Nodule, Root and Shoot Dry weight of Cambodia Soybean Variety at V6 and R 3.5

Source of variance	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	2	0.01430*	0.08310^{NS}	3.39547**	0.00416*	0.8895^{NS}	18.8769*
treat	11	0.01928*	0.08516^{NS}	3.71701**	0.01168*	0.2067^{NS}	5.6947*
Error	22	0.00872	0.07128	0.57584	0.00497	0.26723	2.7461

^{NS} = Non-significant

* = Significant at P<0.05

** = Significant at P<0.01

Appendix table 4.

ANOVA table for relative ureide index (%), P-fix (%), Nitrogen accumulation and amount of fix N of Myanmar Soybean Variety at R 3.5

Source of variance	DF	MS			
		Relative ureide index (%),	P-fix (%)	Nitrogen accumulation	Amount of fix N
block	2	63.640**	92.425**	11832.5**	15802**
treat	11	567.235**	823.478**	78302.4**	103964**
Error	22	83.007	120.504	19157.7	22481

** = Significant at P<0.01

Appendix table 5.

ANOVA table for relative ureide index (%), P-fix (%), Nitrogen accumulation and amount of fix N of Thailand Soybean Variety at R 3.5

Source of variance	DF	MS			
		Relative ureide index (%),	P-fix (%)	Nitrogen accumulation	Amount of fix N
block	2	107.694*	163.992*	76544.4**	47910.6**
treat	11	407.051*	603.039*	53056.6**	58234.4**
Error	22	166.058	242.833	6756.6	6404.9

* = Significant at P<0.05

** = Significant at P<0.01

Appendix table 6.

ANOVA table for relative ureide index (%), P-fix (%), Nitrogen accumulation and amount of fix N of Cambodia Soybean Variety at R 3.5

Source of variance	DF	MS			
		Relative ureide index (%),	P-fix (%)	Nitrogen accumulation	Amount of fix N
block	2	323.694**	509.07**	33936.1**	21604.8**
treat	11	708.020**	1048.86**	31486.9**	46395.4**
Error	22	163.937	238.18	7330.1	4170.3

** = Significant at P<0.01

Appendix table 7.

ANOVA table for pods per plant, seeds per pod and seed yield of Myanmar Soybean Variety at harvest stage

Source of variance	DF	MS		
		Pods per plant	Seeds per pod	Seed yield
block	2	4.6730**	0.02672**	15.7449**
treat	11	33.4864**	0.25788**	51.1412**
Error	22	2.4109	0.06132	7.2809

** = Significant at P<0.01

Appendix table 8.

ANOVA table for pods per plant, seeds per pod and seed yield of Thailand Soybean Variety at harvest stage

Source of variance	DF	MS		
		Pods per plant	Seeds per pod	Seed yield
block	2	35.1533**	0.01605*	32.6390**
treat	11	38.0003**	0.27028*	39.3630**
Error	22	9.3392	0.12194	6.4208

* = Significant at P<0.05

** = Significant at P<0.01

Appendix table 9.

ANOVA table for pods per plant, seeds per pod and seed yield of Cambodia Soybean Variety at harvest stage

Source of variance	DF	MS		
		Pods per plant	Seeds per pod	Seed yield
block	2	4.20084^{NS}	0.13593*	11.4562*
treat	11	5.29497^{NS}	0.33562*	4.6184*
Error	22	3.66111	0.11899	1.5002

^{NS} = Non-significant

* = Significant at $P<0.05$

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