APPENDICES

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
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Appendix A

Phytodetek for ABA or *t*-ZR Test Kit (Agdia, Inc. Elkhart, IN.)

Competitive ELISA, for the quantitative determination of Ascisic acids or *trans*-Zeatin Riboside

Catalog number: PDK 09347/0096 for ABA and PDK 09348/0096 for *t*-ZR

List of contents

Lot number Item 96 wells

1. Anti-*t*-ZR coated testwells 96 testwells
2. *t*-ZR tracer, alkaline phosphatase 3 vials – 1 mL lyophilized
3. TBS buffer, 20X concentrate 1 bottle – 60 mL
4. PNP substrate tablets 1 vial – 6 tablets x 5 mg
5. Substrate diluent (*contains 0.02% sodium azide*) 1 bottle – 30 mL
6. PBST wash buffer, 20X concentrate, 50 ml 3 pouches
7. Instructions 1
8. Plate sealers 2

The above items should be stored at 4° C.

Use within 18 months of receiving

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Materials required, but not provided

- (+) cis/trans Abscisic Acid standard - Sigma Cat. No. A 4906 or *trans*-Zeatin Riboside standard - Sigma Cat. No. Z0375
- Absolute methanol - diluent for *t*-ZR standard
- Vertical light path photometer for microtiter plates, strips or wells with 405 nm filter
- 37° C Incubator - Forced air microplate incubator recommended
- Refrigerator 4° C
- Airtight container for incubations
- Test tubes for standard dilution
- Test tube rack
- Distilled water
- Paper towels
- Timer
- Additional TBS buffer (ACC 00580) - for sample preparation (see buffer formulation preparing)
- Reservoirs - You will need 3 small containers to prepare and hold substrate, wash and tracer solutions

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• Pipette tips

• Pipettes

- Transfer
- 1 mL volumetric
- 5 mL serological
- 100 µL single channel
- 50-200 µL multichannel

Principle

Phytodetek enzyme immunoassays are convenient tests for the quantitative determination of plant hormones. The Abscisic acid (ABA) or *trans*-Zeatin Riboside (*t*-ZR) test utilizes an anti- ABA or *t*-ZR monoclonal antibody, respectively and are sensitive in the range of 0.032 – 0.16 picomloes ABA/ mL or 0.2 - 100 picomoles *t*-ZR /mL. The assay principle uses the competitive antibody binding method to measure concentrations of ABA or *t*-ZR in plant extracts. The ABA or *t*-ZR -tracer are labeled with alkaline phosphatase and then added along with the plant extract to the antibody coated microwells.

A competitive binding reaction is set up between a constant amount of the ABA or *t*-ZR -tracer, a limited amount of the antibody and the unknown sample containing ABA or *t*-ZR. The ABA or *t*-ZR in the sample competes with the ABA or *t*-ZR-tracer for antibody binding sites. The unbound ABA or *t*-ZR -tracer are washed away before adding the substrate. The yellow color produced is inversely proportional to the

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amount of hormone in the sample. The intensity of color is related to the sample ABA or *t*-ZR concentration by means of a standard curve.

Warnings

Phytodetek ABA or *t*-ZR kit is for research use. Some reagents in the kit contain 0.02% sodium azide as a preservative. Consult manual guide "Safety Management No. CDC-22, Decontamination of Laboratory Sink Drains to Remove Azide Salts" (Center for Disease Control, Atlanta, Georgia, April 30, 1976).

Limitations

Storage: The kit is temperature sensitive and must be stored at 4° C.

Expiration: This test should be used within 18 months of purchase. Do not use reagents after the kit expiration.

ABA or *t*-ZR tracer: Reconstituted ABA or *t*-ZR-tracer are stable for 7 days at 4° C. Precise pipetting of the sample and ABA or *t*-ZR -tracer is critical to the accuracy and reproducibility of the assay.

Substrate: Dissolve PNP tablets completely before using. The working solution is stable for 8 hours at 4° C.

PBST wash buffer: Once the PBST wash buffer has been diluted to the working concentration, azide should be added to make a 0.02% solution if long term stability is desired.

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ABA or *t*-ZR standard: It is important that a standard curve be included in each test run.

Results: Test is not valid unless Bo reads greater than 0.750 O.D. If the value is below this, increase the substrate incubation time until the desired O.D. is obtained (not to exceed 30 additional minutes).

Sample Preparations

Sample preparation procedures may vary with different types of plant materials. Results may be influenced by compounds such as terpenoids, phenolics, pigments or other plant components. Review the pertinent literature to determine whether extraction protocols have been established for the species of interest. It is important that the final extract contain no more that 10% organic solvent in TBS buffer. All samples require dilution in TBS buffer.

Prepare Buffers

1. The TBS buffer and PBST wash buffer are concentrated and must be diluted prior to use. Prepare only as much as will be needed for one day. Mix thoroughly, stirring each buffer for 15 to 30 minutes.

2. To prepare 100 mL of 1X TBS buffer, mix 5 mL 20X TBS buffer with 95 mL of distilled water.

3. Prepare PBST wash buffer by diluting one 20X pouch of PBST wash buffer with 950 mL of distilled water.

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Directions for use

1. Prepare tracer solution: (Note: Each ABA or *t*-ZR-tracer vial contains sufficient materials for 32 testwells. Standards and samples should be run in duplicate. Diluted ABA or *t*-ZR-tracer can be stored at 4° C for up to 7 days).

Add 5 mL of 1X TBS buffer to each ABA or *t*-ZR tracer vial you will need. Replace the cap and mix the contents by inverting the bottle several times. Let the solution rest for 5 minutes before use.

2. Weigh 26.43 mg of 2-cis-(S)-ABA and dissolve in 10.0 mLof Absolute methanol. If an enantiomeric ABA compound is used, weigh 52.86 mg of the compound. Add 100 μL of this solution to 9.90 ml of Absolute methanol. This makes a stock solution (SS) with a concentration of 0.1 μmole ABA/ml. Store this stock solution in an amber bottle, in the dark at -20° C or lower.

Weigh 17.57 mg of *t*-ZR and dissolve in 10.0 ml of absolute methanol. Add 200 μL of this solution to 9.80 ml of absolute methanol. This makes a stock solution (SS) with a concentration of 100,000 picomoles *t*-ZR/mL. Store this stock solution in an amber bottle, in the dark at -20° C or lower.

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3. Following the chart below, prepare the standards by diluting the standard stock solutions in 1X TBS buffer. New standards should be prepared each time the test is run.

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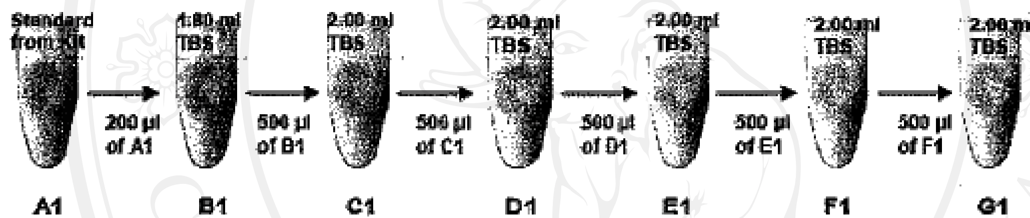
Stock Solution (SS) = 100,000 picomoles/mL, NSB=Nonspecific Binding, Bo=100% Binding

The standard included in this kit contains an enantiomeric mixture of (+/-) ABA in the concentration indicated in the Certificate of Analysis. The kit recognizes (+) ABA or half of the standard solution concentration indicated in the certificate.

Note: Mix each dilution well

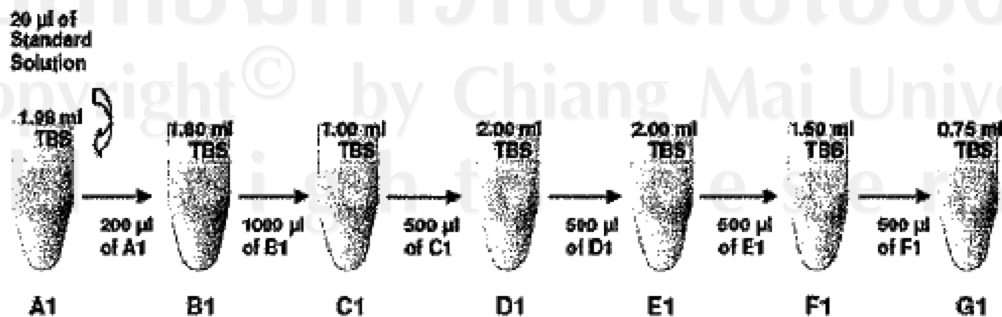
ABA Standard 09347
Note: Mix each dilution well

Plate Position	ABA Solution	1X TBS Buffer	[ABA] picomoles/mL	Dilution
A1= NSB	Standard; One strip		1,000	
B1	200 µl of A1	1.80 ml	100	1:10
C1	500 µl of B1	+2.00 ml	20	1:5
D1	500 µl of C1	+2.00 ml	4	1:5
E1	500 µl of D1	+2.00 ml	0.8	1:5
F1	500 µl of E1	+2.00 ml	0.16	1:5
G1	500 µl of F1	+2.00 ml	0.032	1:5
H1=B₀	0	100 µl	0	



t-ZR Standard 09348
Note: Mix each dilution well

Plate Position	t-ZR Solution	1X TBS Buffer	[t-ZR] picomoles/mL	Dilution
A1= NSB	20 µl of SS	+1.98 ml	1000	1:100
B1	200 µl of A1	+1.80 ml	100	1:10
C1	1000 µl of B1	+1.00 ml	50	1:2
D1	500 µl of C1	+2.00 ml	10	1:5
E1	500 µl of D1	+2.00 ml	2	1:5
F1	500 µl of E1	+1.50 ml	0.5	1:4
G1	500 µl of F1	+0.75 ml	0.2	1:2.5
H1=B₀	0	100 µl	0	



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4. Remove the desired number of test wells from the pouch and place them in the test well holder. Reseal the pouch, making sure the desiccant is still present, and return it to the refrigerator.

5. Add 100 μL of standard or sample extract to each well. Standards and samples should be run in duplicate.

6. Add 100 μL diluted tracer prepared in step 1 to each well using a multichannel pipette. Make sure the tips do not touch the solutions in the well so that cross contamination does not occur.

7. Mix the contents by gently swirling the plate on the bench top. Cover test wells with plate sealer and place in a humid box (airtight plastic box lined with damp paper towel). Make sure the humid box has been pre cooled to 4°C for 30 minutes before use.

8. Incubate test wells in the refrigerator at 4° C for 3 hours.

9. Prior to the end of the incubation period, prepare the substrate solution: Dissolve 1 substrate tablet in 5 mL of substrate diluents. Please be sure that the substrate tablet is completely dissolved and mixed before use.

10. After the 3 hour incubation, remove the test wells from the refrigerator and expel the contents of the test wells into the sink. For efficient expelling of the samples, while squeezing the long sides of the frame to hold the test wells in place, use a quick flipping motion to empty the contents of the wells into a sink or waste container.

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11. Fill wells completely with 1X PBST wash buffer, and then quickly empty them again. Repeat 5 times. Grasp the test well holder upside down then firmly tap it on a paper towel to remove remaining wash solution.

12. Before adding substrate, make sure that a humid box has been preheated to 37° C for 30 minutes. Add 200 µL of substrate solution to each well using a multichannel pipette.

13. Cover the test wells with the plate sealer and place them in a humid box.

14. Incubate at 37° C for 60 minutes.

15. Read the absorbance values at 405 nm. Test is not valid unless Bo reads greater than 0.750 O.D. If the value is below this, increase the substrate incubation time until the desired O.D. is obtained (not to exceed 30 additional minutes).

Calculations

1. Calculate the means of the optical densities of duplicate standards or samples.

2. Calculate the % Binding for the standard and sample with the following equation:

Definition of Symbols

NSB = Well A1 = 0% Binding.

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Bo= Well H1 = 100% Binding.

O.D. = Optical Density / Absorbance value

$$\% \text{ Binding} = \frac{(\text{Standard or Sample } \mathbf{O.D.} - \text{NSB } \mathbf{O.D.})}{(\text{Bo } \mathbf{O.D.} - \text{NSB } \mathbf{O.D.})} \times 100$$

3. After % Bindings have been calculated, calculate the Logit value for the % Binding of standards and samples. See the equation below. Calculate the natural log for each standard concentration. Plot the Logit values on the y-axis and the correlating standard concentrations (in natural log values) on the x-axis. Calculate the y-intercept and slope from the linear curve generated with the standard data.

Logit equation for standard Logit and sample % Binding values:

$$= \text{Ln}\left(\frac{\%\text{Binding}}{100-(\%\text{Binding})}\right)$$

4. Use the following equation for the calculation of samples *t*-ZR concentration:

$$[\text{Sample Concentration}] = e^{(\text{logit}-(\text{y-intercept})) / \text{slope})}$$

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Percent (%) cross reactivity

For monoclonal antibody ABA-15-I –C-5

Compound	Cross-Reactivity*
*2-cis-(*S)-ABA	100
2-cis-(S)-ABA methylester	Less than 0.1
2-cis-(R)-ABA	0
2-trans-(S)-ABA	0
2-cis-(S)-ABA-B-D-glucopyranosyl ester	0
2-cis-(S)-ABA-cis-diol	0
Phaseic acid	Less than 0.1
Dihydrophaseic acid	Less than 0.1
Xanthoxin	0
All-*trans*-Farnesol	0

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*Cross reactivities were determined from tracer displacement curves at 50% displacement on molar basis.

Important Note: If you are trying to determine the concentration of *cis* (+) ABA when using *cis/trans* (+/-) ABA as an analytical standard, the effective concentration of

cis ABA is one half the value of *cis/trans* ABA. For example: 10 picomoles *cis/trans* ABA is 5 picomoles *cis* ABA.

For monoclonal antibody *t*-ZR J3-I-B3

Compound	Cross-Reactivity*
trans-Zeatin Riboside	100
Dihydrozeatin	2.3
Dihydrozeatin Riboside	1.2
cis-Zeatin Riboside	0.8
cis-Zeatin	0.4
trans-Zeatin	47.3
Zeatin Riboside-5'-monophosphate	95.2
Dihydrozeatin Riboside-O-glucoside	0.07
Dihydrozeatin-O-glucoside	0.8
Zeatin-O-glucoside	7.7
Zeatin Riboside-O-glucoside	0.8
Isopentenyl Adenosine	0.5
Isopentenyl Adenine	0.9
6-Furfurylaminopurine (kinetin)	0.06
6-n-Hexylaminopurine	0.2

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Compound	Cross-Reactivity*
6-Benzylaminopurine-9-glucoside	0.7
6-Benzylaminopurine-7-glucoside less than	0.01
6-Benzylaminopurine-3-glucoside	0.06
6-Amino-3-dimethylallylpurine	0.7
Adenosine less than	0.01
Adenine less than	0.01
Guanine less than	0.01
Guanosine-5'-triphosphate less than	0.01
Cytosine less than	0.01
Cytidine	0
Inosine-5'triphosphate	0.01
6-Piperidino-1-purine less than	0.01

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*Cross reactivities were determined from tracer displacement curves at 50% displacement on molar basis.

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Buffer formulations: For reference only, concentrated versions are supplied in your kit.

Substrate Diluent Dissolve in 800 ml distilled water:

Magnesium chloride	0.1 g
Sodium azide	0.2 g
Diethanolamine	97.0 ml

Adjust pH to 9.8 with hydrochloric acid. Adjust final volume to 1000 ml with distilled water. Store at 4° C.

TBS Buffer (1X) Dissolve in 800 ml distilled water:

Trizma base	0.53 g
Trizma hydrochloride	3.25 g
Sodium chloride	5.84 g
Magnesium chloride hexahydrate	0.20 g
Sodium azide, optional*	0.20 g

Adjust pH to 7.5. Adjust final volume to 1000 mL with distilled water. Store at 4° C.

*Add sodium azide if you will need long term stability for storing unused buffer.

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PBST Buffer (Wash Buffer) (1X)

Dissolve in distilled water to 1000 ml

Sodium chloride	8.00 g
Sodium phosphate, dibasic (anhydrous)	1.15 g
Potassium phosphate, monobasic (anhydrous)	0.20 g
Potassium chloride	0.20 g
Tween-20	0.50 g
Sodium azide, optional*	0.20 g

Adjust pH to 7.4.

*Add sodium azide if you will need long term stability for storing unused buffer.

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Appendix B

Free sugar analysis (Ohyama *et al.*, 1986)

The analysis of free sugar (Fructose, Glucose and Sucrose) used by Gas Liquid Chrsomatography.

Analytical method

1. Standard preparation;

Dissolve standard sugars which combined with 100 mg of Rhamnose, 100 mg of Fructose, 100 mg of Glucose and 100 mg of Sucrose into 25 mL 80% ethanol.

2. Pipette 100 μL of 80% ethanol extracted sample and 50 μL of rhammose (100 mg of rhammose in 25 mL of 80% ethanol) as internal standard, put into a vial, evaporate until dry, then dehydrate by vacuum for 1 – 2 h.

3. Pipette 50 μL of standard solution, puts into a vial, then evaporate and dehydrate.

4. Add 50 μL of TMSI-H (N-trimethylsilylimidazole) into the vial of sample, and standard. Cap with the parafilm, leave it overnight at room temperature.

5. Inject 10 μL of sample to Gas Chrsomatograph (GC).

6. The analytical conditions of GC

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Column for Fructose OV-17

Column initiation temperature	120°C
Column final temperature	280°C
Temperature rate	10°C/min
Injection temperature	300°C

Note : Use column SE-30 for Glucose and Mannose analysis

Initiation temperature	110°C
Temperature rate	5°C/min

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Appendix C

Amide-N or total free amino acids analysis (Ohyama, 1985)

Extraction of plant materials for amid-N by 80%ethanol

1. Plant samples: Freeze dry sample should be ground to be fine powder. Sample can be kept at -20°C until analysis.
2. Weight about 50 mg of sample and record the weight in 1.5 mL centrifuge tube.
3. Add 1 mL of 80% ethanol to centrifuge tube and vortex. (Preparation of 80% ethanol: mix 400 mL of ethanol plus 100 mL of pure water and keep in a dark 500 mL bottle)
4. Incubate at 60°C for 15 min to stop enzyme activity, and shake 30 min. Then keep the sample in a refrigerator overnight.
5. Shake them for 15 min and centrifuge at 10,000 rpm for 15 min (4°C).
6. Transfer the extract (supernatant) to a 10 mL falcon tube. Keep in refrigerator.
7. Repeat 4 times of washing the residues
8. Add 1 mL of 80% ethanol to centrifuge tube and shake them for 15 min. Centrifuge at 10,000 rpm for 15 min (4°C). Transfer the extract (supernatant) to a 10 mL falcon tube. Keep in refrigerator. Final volume of 80% ethanol extract is 5 mL.
9. Keep the 80% ethanol extract in freezer at -20 °C.

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10. Dry residue in ventilation oven 60°C over night, and weight the tube + residue. Keep it in freezer at -20°C (The dry sample residues were used for analysis the sugar concentration).

Amide-N (total free amino acids concentration) was determined by ninhydrin method.

Reagents

1. Citrate buffer: Dissolve 56 g of citrate and 21.3 g of NaOH in 1L of water
2. Ninhydrin solution: Dissolve 0.958 g of ninhydrin and 33.4 mg of ascorbate in 3.2 mL of water. Then add 2-methoxyethanol 100 mL.
3. Standard solution: Dissolve 165 mg of asparagines (or 188 mg of asparagines monohydrate) plus 183 mg of glutamine in 250 mL water, which contains 280 µg N mL^{-1}.

Procedure

1. Take 100 µL of 80% ethanol extraction into a test tube, and 1.5 mL of citrate buffer.
2. Then add 1.2 mL ninhydrin solution and heat in boilng water for 20 min with glass ball.
3. Add 3% mL of 60% ethanol, mix and then cool to room temperature. After 10 min incubation, measure OD 570 by optical spectrometry.
4. Measure diluted standard solution simultaneously with sample.

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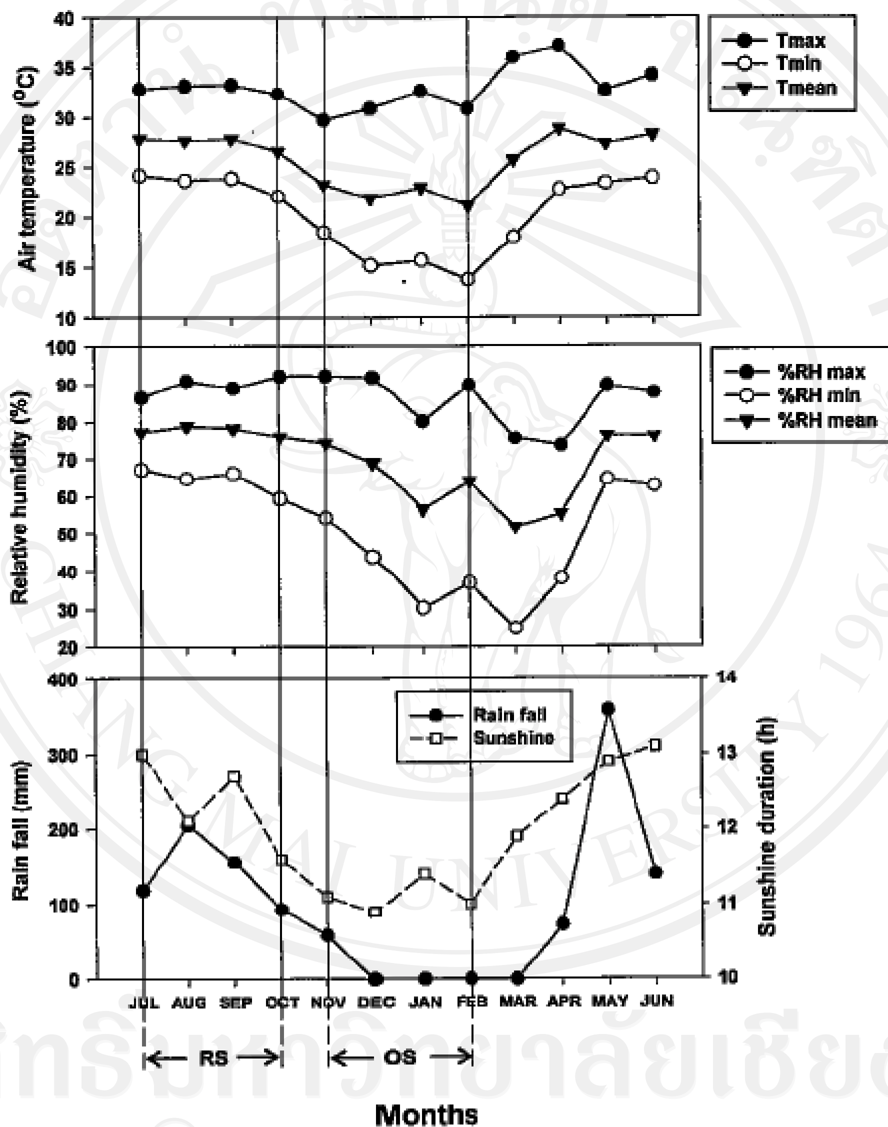
Appendix D



Macroclimate referred to meteorological data automatically recorded between regular season (RS) cover from July, 2006 to October, 2006 and off-season (OS) cover from November, 2006 to February, 2007: Air temperature (T max, T min and T mean), relative humidity (RH max, RH min and RH mean), rain fall (Rf) and sunshine duration.

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Curriculum Vitae

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Date of birth 17 January 1981

Address 269/60 Kholak district, Muang Prachuab Khiri Khan, Prachuab Khiri Khan, Thailand 77000

Education background

1999 - 2003 B.Sc. (Agriculture) with 1st **class honors (GPA 3.64),** Department of Horticulture, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand

Special problem title: Effects of Shading on the Growth of *Polyscias fruiticosa* (L.) Harm.

1993 – 1998 Secondary School, Prachuab Wittayalai School, Prachuab Khiri Khan, Thailand

Scholarship: Thailand Research Fund scholarship through the Royal Golden Jubilee Ph.D. Program (Grant No. PHD/0121/2548)

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Work experience

2004 - 2005 Agricultural technologists, Flowers and Ornamental Plant Production Promotion Group, Bureau of Agricultural Commodities Promotion and Management, Department of Agricultural Extension, Bangkok, Thailand.

2003 – 2004 Agricultural technologists, Commodities Standard Promotion Group, Bureau of Agricultural Product Quality Development, Department of Agricultural Extension, Bangkok, Thailand.

Training

Research and analysis of ABA concentrations in *Curcuma* plant by using GC-MS and ELISA test kit techniques, Analysis of free amino acids in *Curcuma* plant by using UPLC technique, Department of Applied Biological Chemistry, Faculty of Agriculture, Niigata University, Niigata, Japan. 1 March to 31 May, 2007. c/o Prof. Dr. Takuji Ohyama

Research and analysis of carbohydrate concentrations (glucose, fructose and sucrose) in *Curcuma* plant by using GC technique, Department of Applied Biological Chemistry, Faculty of Agriculture, Niigata University, Niigata, Japan.18 March to 29 May, 2010. c/o Prof. Dr. Takuji Ohyama

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Publication

Hongpakdee, P., P. Siritrakulsak, N. Ohtake, K. Sueyoshi, T. Ohyama, S. Ruamrungsri. 2010. Changes in endogenous abscisic acid, *trans* -Zeatin riboside, indole-3-acetic acid levels and the photosynthetic rate during the growth cycle of *Curcuma alismatifolia* Gagnep. in different production seasons. ***Europ. J. Hort. Sci.*** 75(5): 204–213.

Hongpakdee, P. and S. Ruamrungsri. 2010. Effect of night interruption and PGRs application on growth and ABA levels in *Curcuma alismatifolia* Gagnep. off-season production. ***J. Agri.*** 26(1): 7-14. (In Thai)

Hongpakdee, P., N. Ohtake, K. Sueyoshi, T. Ohyama, S. Ruamrungsri. 2010. Effects of low night temperature and short day length on some phytohormones and nutrient status in *Curcuma alismatifolia* Gagnep. ***Thai J. Agric. Sci.*** 43(3): 163-173.

Presentation

Hongpakdee, P., T. Ohyama and S. Ruamrungsri. 2010. Physiological responses of *Curcuma alismatifolia* Gagnep. to exogenous ABA foliar spraying. Paper presented at The 3rd Joint Symposium between Chiang Mai University and Kagawa University organized by Chiang Mai University, Chiang Mai, Thailand. 24-26 August 2010. **(poster presentation)**

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Hongpakdee, P., T. Ohyama and S. Ruamrungsri. 2009. Comparison of growth, flower qualities and endogenous ABA levels in *Curcuma alismatifolia* Gagnep. under the different seasonal product. Paper presented at The 2nd International Meeting for Development of International Network for Reduction of Agrochemical Use: Food Safety Technology in Southeast Asia, Chiang Mai, Thailand. 22-23 September 2009. **(poster presentation)**

Hongpakdee, P., T. Ohyama and S. Ruamrungsri. 2009. Seasonal effects on the changes of abscisic acid concentration in *Curcuma alismatifolia* Gagnep. Paper presented at the National Horticultural Congress IIX organized by Department of Horticulture, Faculty of Agricultural Production, Maejo University. Chiang Mai, Thailand. 6-9 May 2009. **(oral presentation)**

Hongpakdee, P., T. Ohyama and S. Ruamrungsri. 2009. Effects of production season on growth, flower qualities and endogenous ABA levels in *Curcuma alismatifolia* Gagnep. Paper presented at Royal Golden Jubilee Ph.D Congress X organized by Thailand Research Fund. Pattaya, Thailand. 3-5 April 2009. **(poster presentation)**

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