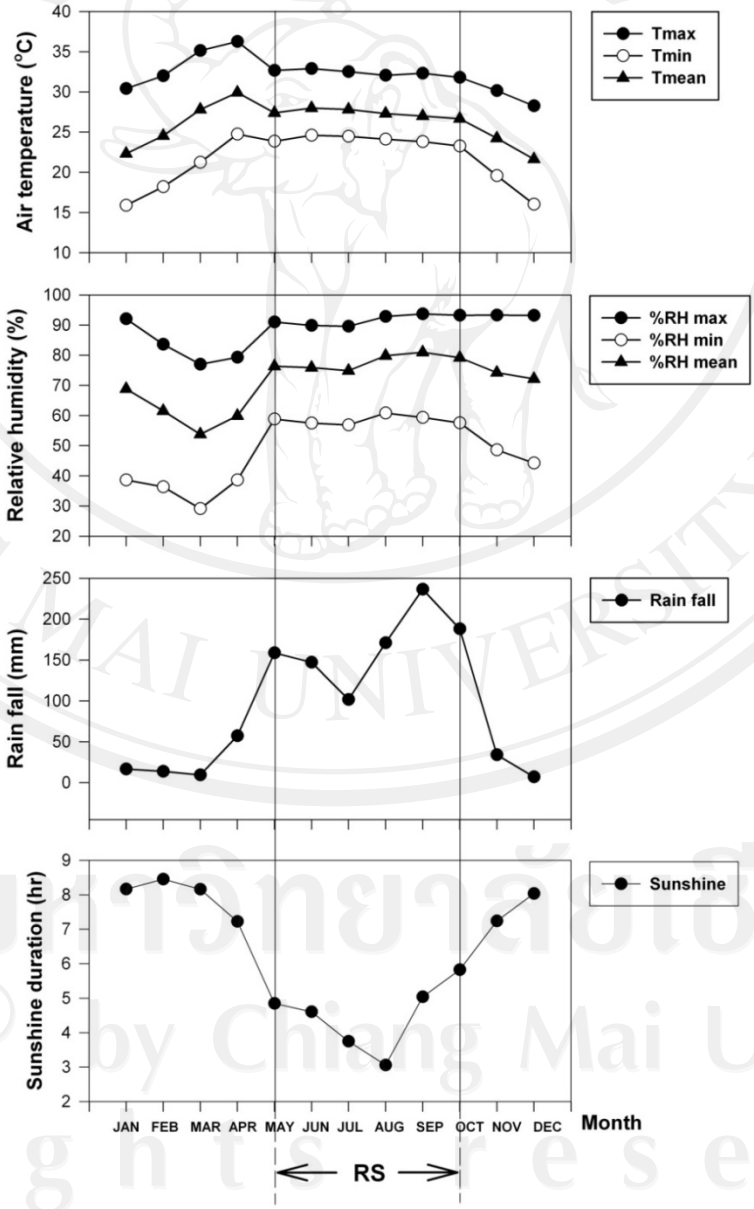
APPENDICES

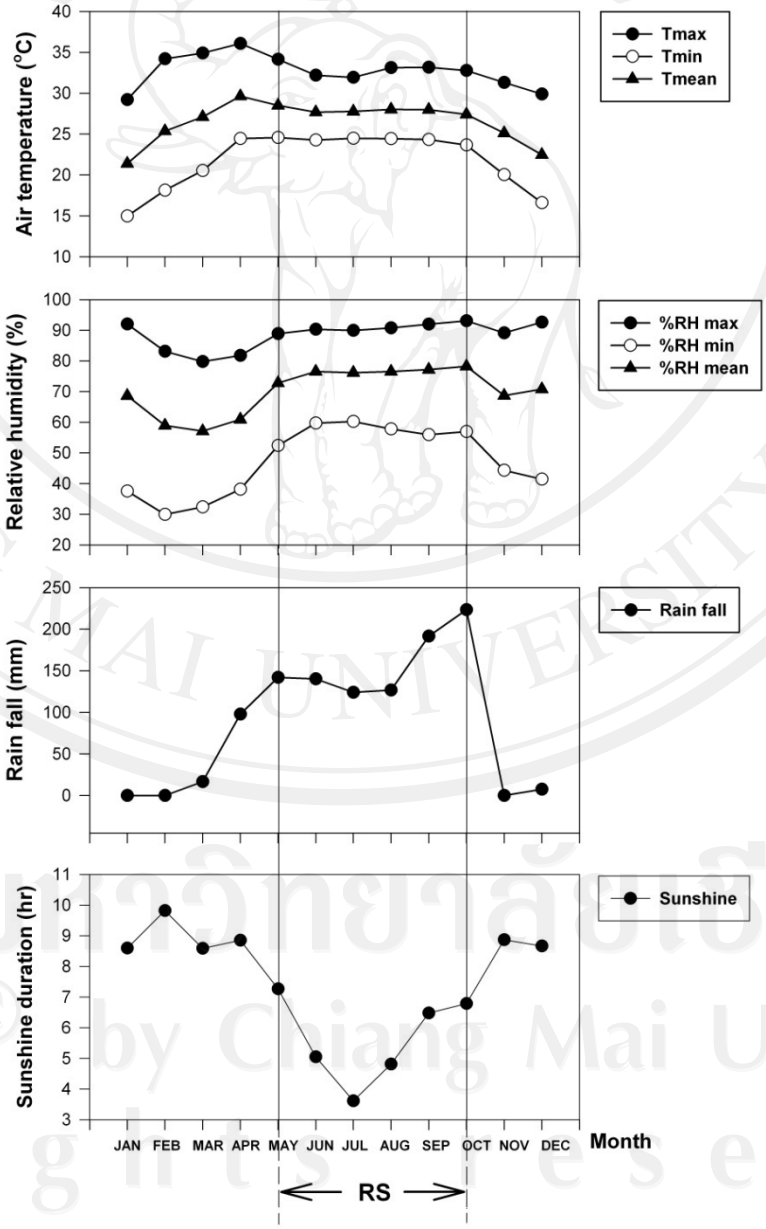
APPENDIX 1

Macroclimate referred to meteorological data automatically recorded between regular seasons (RS) covering the period from May, 2008 to October, 2008. Air temperature (T max, T min and T mean), relative humidity (RH max, RH min and RH mean), rain fall (Rf) and sunshine duration.



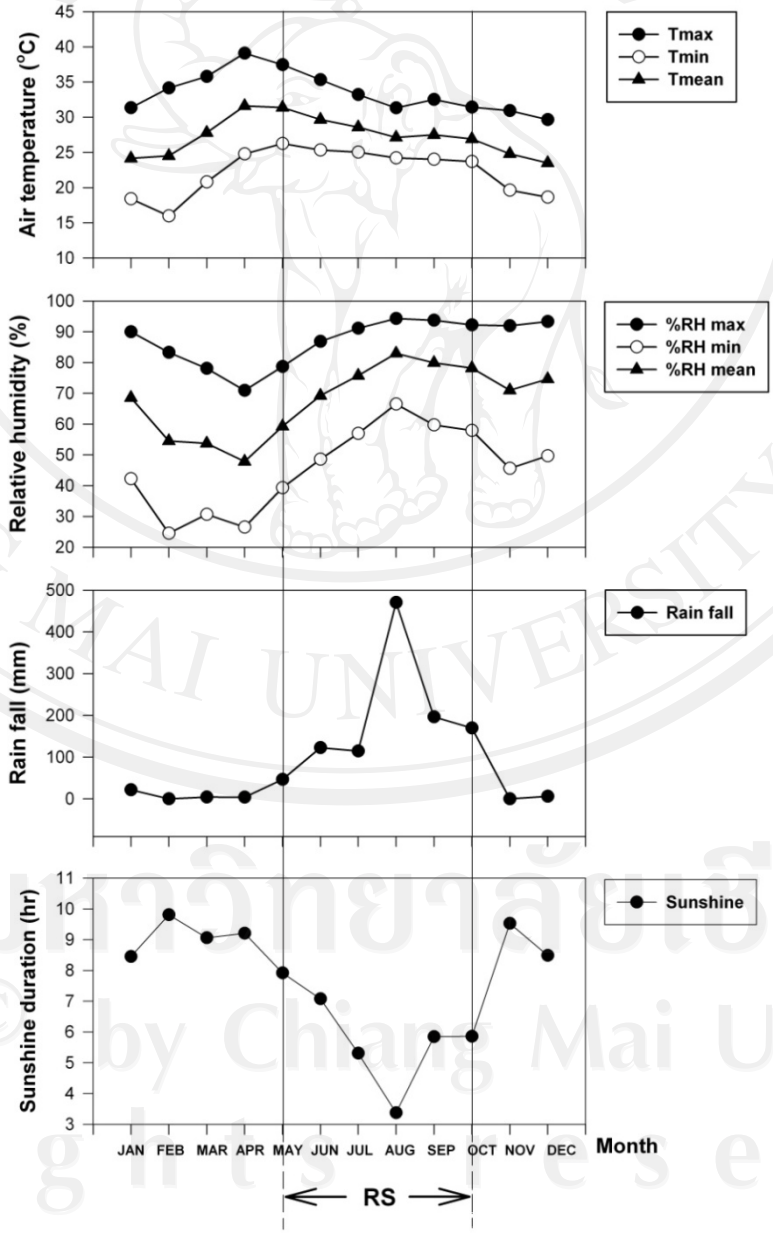
APPENDIX 2

Macroclimate referred to meteorological data automatically recorded between regular seasons (RS) covering the period from May, 2009 to October, 2009. Air temperature (T max, T min and T mean), relative humidity (RH max, RH min and RH mean), rain fall (Rf) and sunshine duration.



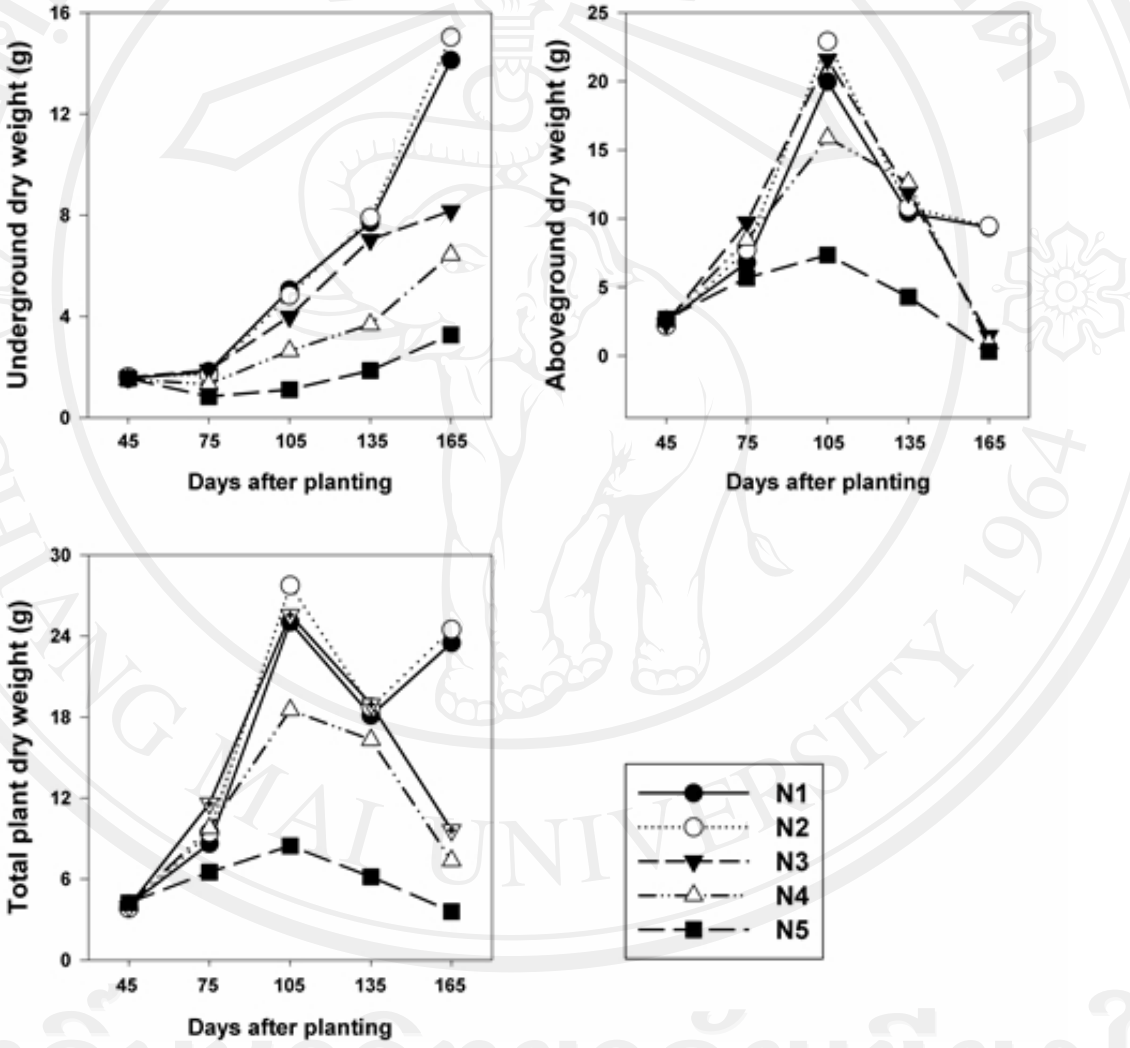
APPENDIX 3

Macroclimate referred to meteorological data automatically recorded between regular seasons (RS) covering the period from May, 2010 to October, 2010. Air temperature (T max, T min and T mean), relative humidity (RH max, RH min and RH mean), rain fall (Rf) and sunshine duration.



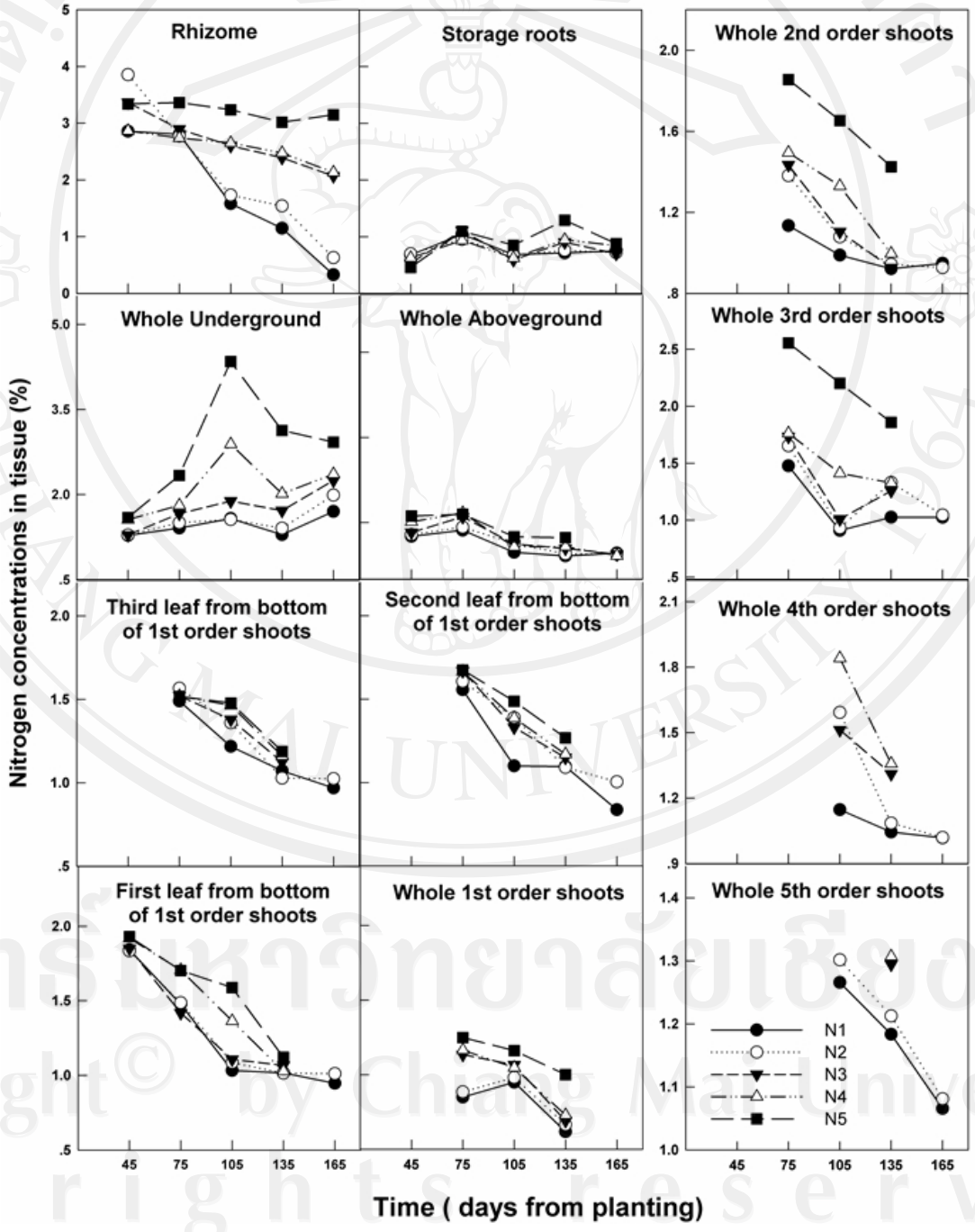
APPENDIX 4

Effect of nitrogen supply on underground dry weight, aboveground dry weight and total plant dry weight in *C. alismatifolia* 'Chiang Mai Pink'.



APPENDIX 5

Effects of nitrogen supply on nitrogen concentrations in selected plant parts of *Curcuma alismatifolia* 'Chiang Mai Pink'.



APPENDIX 6

Total nitrogen analysis by modified Kjeldahl method (Ohyama, 1985)

Reagent

1. **Reagent A** (EDTA reagent);

   Dissolve 6 g of EDTA (ethylenediaminetetra acetic acid disodium salt) into 80 ml of deionized water, adjust pH about 7, mix well and dilute to a final volume of 100 ml.

2. **Reagent B** (1 M of KH_2PO_4);

   Dissolve 136.09 g KH_2PO_4 and 2.75 g benzoic acid into 1 L of deionized water.

3. **Reagent C** (Phenol-nitroprusside reagent);

   Dissolve 100 mg sodium nitroprusside into 10.25 ml phenol, dilute to a final volume of 1 L with deionized water (Use the sodium nitroprusside as a catalyst).

4. **Reagent D** (Buffer hypochlorite reagent);

   Put 10 g NaOH (adjusts pH 10 by 10 N of NaOH), 7.06 g Na_2HPO_4 and 31.8 g $Na_3PO_4.12H_2O$ into a 500 ml beaker, dissolve in deionized water and transfer to 1 L of volumetric flask, add 10 ml of sodium hyperchlorite, dilute to 1 L of flask with deionized water.

5. **Standard ammonium solution**;

   Dissolve 471.7 mg $(NH_4)_2SO_4$ in 1 L of 0.5 N H_2SO_4 for 100 mg/L of a stock solution. Make standard concentration 0 - 0.7 mg/L.

Analytical method

1. Pipette sample solution of the H_2SO_4 digested solution 0.1 – 2 ml into a 25 ml of volumetric flask, add 0.5 ml of reagent A and 0.5 ml of reagent B.
2. Add a small amount of 2 N NaOH, for pH adjust, until color changed, add 2.5 ml of reagent C, follow by 2.5 ml of reagent D, and then fill up flask to volume with deionized water and mix well.
3. Maintain the flask at 30^{o}C for 3 hrs and determine the absorbance of the colored complex at a wavelength of 625 nm. Do the same method for blank solution and standard.
4. Determine the NH_4^+-N concentration of the sample by reference to a calibration curve plotted form the results obtained with a standard curve.

APPENDIX 7

Nitrate and Ammonium concentration test by Reflectometer RQflex® 10

Reflectoquant®

Sample preparation

Homogenize the sample in a blender. Weigh a representative sample into a beaker and add distilled water. Cover the beaker with a watch glass and boil for approx. 15 minutes. Allow to cool, transfer to a volumetric flask and make up to the final volume with distilled water.

Nitrate test

1. **Method:** Nitrate ions are reduced to nitrite ions by a reducing agent. In the presence of an acidic buffer, these nitrite ions react with an aromatic amine to form a diazonium salt, which in turn reacts with N-(1-naphthyl)-ethylenediamine to form a red-violet azo dye that is determined reflectometrically.

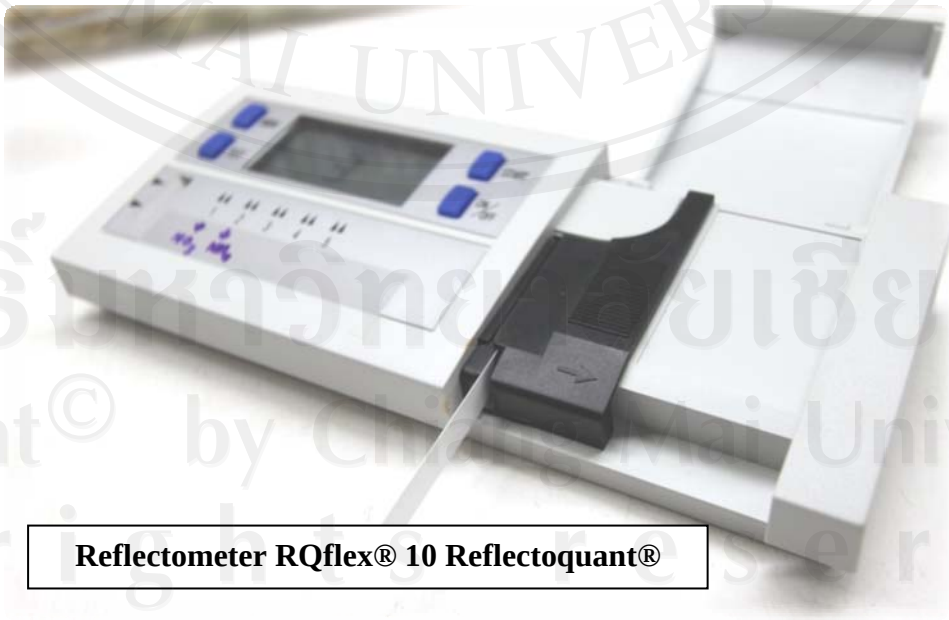
2. **Reagents and auxiliaries**

Package contents: Nitrate test Cat. No. 1.16995.0001

- Tube containing 50 test strips
- 1 bar-code strip

Other reagents:

- Nitrite Test Method: colorimetric with test strips 2 - 5 - 10 - 20 - 40 - 80 mg/L NO_2 Merckoquant®, Cat. No. 110007
- Amidosulfonic acid GR for analysis, Cat. No. 100103
- Nitrate Test Method: colorimetric with test strips 10 - 25 - 50 - 100 - 250 – 500 mg/L NO_3 Merckoquant®, Cat. No. 110020
- pH-indicator strips pH 0 - 14 Universal indicator non-bleeding pH 0 - 1 - 2 – 3- 4 - 5 - 6 - 7 - 8 - 9 - 10 - 11 - 12 - 13 -14**,** Cat. No. 109535
- Sodium acetate anhydrous for analysis, Cat. No. 106268
- L (+) - Tartaric acid GR for analysis, Cat. No. 100804
- Nitrate standard solution CertiPUR®, 1000 mg/l NO3-, Cat. No. 1.19811.0500



Reflectometer RQflex® 10 Reflectoquant®

3. Preparation:

- Extract solid sample materials by an appropriate method.
- Check the nitrite content with the Merckoquant® Nitrite Test. If necessary, eliminate interfering nitrite ions:To 5 ml of sample (pH < 10) add 5 drops of a 10 % aqueous amidosulfonic acid solution and shake several times, then boil briefly and allow to cool.
- Check the nitrate content with the Merckoquant® Nitrate Test. Samples containing more than 90 mg/L NO_3^- or 225 mg/L NO_3^- must be diluted with distilled water.
- The pH must be within the range 1 - 12. If the pH is lower than 1, buffer the sample with sodium acetate; if greater than 12, adjust to approx. 3 - 5 with tartaric acid.

4. Procedure

Press the START button of the reflectometer and - this is imperative - at the same time immerse both reaction zones of the test strip in the pretreated sample (59 – 86 °F) for 2 sec. Carefully allow excess liquid to run off via the long edge of the strip onto an absorbent paper towel. Approx. 10 sec before the end of the reaction time, insert the strip all the way into the strip adapter with the reaction zones facing the display. After the end of the reaction time, read off the result from the display in mg/L NO_3^-

Calculation:

$$\text{Nitrate content [mg/kg]} = \frac{\text{Measured value [mg/L] x Vol. of water [ml]}}{\text{Weight of sample [g]}}$$

Ammonium test

1. **Method:** Ammonium ions react with a chlorinating agent to form monochloramine. This in turn reacts with a phenol compound to form a blue indophenols derivative that is determined reflectometrically.
2. **Reagents and auxiliaries**

Package contents: Ammonium test Cat. No. 1.16892.0001

- Tube containing 50 test strips
- 1 bar-code strip
- 1 bottle of reagent NH_4-1
- 1 bottle of reagent NH_4-2
- 1 test vessel with stopper

Other reagents:

- Merckoquant®, Ammonium Test: Cat. No. 1.10024.0001, measuring range 10- 400 mg/L NH_4^+
- pH-indicator strips pH 0 - 14 Universal indicator non-bleeding pH 0 - 1 - 2 – 3- 4 - 5 - 6 - 7 - 8 - 9 - 10 - 11 - 12 - 13 -14**,** Cat. No. 109535
- Sodium hydroxide solution 1 mol/L, Cat. No. 109137
- Sulfuric acid 0.5 mol/L, Cat. No. 109072

- Ammonium standard solution CertiPUR®, 1000 mg/L NH_4^+, Cat. N0. 1.19812.0500

3. Preparation

- Extract sample materials by an appropriate method.
- Check the ammonium content with the Merckoquant®, Ammonium Test. Samples containing more than 7.0 mg/L NH_4^+ must be diluted with distilled water.
- The pH must be within the range 4-13. Adjust, if necessary, the sodium hydroxide solution or sulfuric acid.

4. Procedure

Rinse the test vessel several times with the pretreated sample

Pretreated sample (20 – 30 °C)	5 ml	Fill the test vessel to the 5-ml mark.
Reagent NH_4-1	10 drops	Add and swirl.
Reagent NH_4-2	1 level blue microspoon (in the cap of the NH_4-2 bottle)	Add, close the test vessel, and shake vigorously until the reagent is completely dissolved.

- Press the START button of the reflectometer and – this is imperative – at the same time immerse both reaction zones of the test strip in the measurement sample for 8 min.
- Approx. 10 sec before the end of the reaction time remove the strip from the measurement sample and carefully allow

excess liquid to run off via the long edge of the strip onto an absorbent paper towel.

- Immediately insert the strip all the way into the strip adapter with the reaction zones facing the display.
- After the end of the reaction time, read off the result from the display in mg/L NH_4^+.

Calculation:

$$\text{Ammonium content [mg/kg]} = \frac{\text{Measured value [mg/L] x Vol. of water [ml]}}{\text{Weight of sample [g]}}$$

APPENDIX 8

Nitrate reductase activity analysis (Truax *et al.* 1994)

Method: Spectrophotometric Stop Rate Determination

Reagent

1. Incubating solution: Dissolve 17.418 g of KH_2PO_4 and 4.044 g of KNO_3 in water and add 12 ml of 1-propanal then adjust pH to 7.5 and adjust final volume to 1000 ml with distilled water.
2. 0.77 mM N-(1-Naphthyl) ethylenediamine dihydrochloride solution (NED): Dissolve 19.9 mg of NED in 100 ml of distilled water.
3. 58 mM Sulfanilamide solution: Dissolve 0.1 g of sulfanilamide in 100 ml of 3M HCl.
4. Nitrate Standard Solution: Dissolve 5 mg of $NaNO_3$ in 50 ml of incubating solution.

Analytical method

Plant samples were rinsed and then dried of surface water before sampling

↓

Plant samples were cut and place 0.2g fresh sample in test tube with 3 ml of **incubating solution*** and 0.1 g of **PVP**

(*100mM phosphate buffer [pH 7.5], 40 mM KNO_3, 1.2% 1-propanol)

↓

Seal + place in dark for 1hr at room temperature.

↓

Remove plant tissue (stop enzyme reaction)

↓

Take 1 ml aliquot from tube

↓

Mixed with 1 ml **NED** and 1 ml **Sulfanilic acid**

↓

After 30 minutes, measure at **A540** nm

Standard curve of nitrate

Standard solution + 1 ml NED + 1 ml Sulfanilic acid

↓

Measure at 540nm

From above measurements, following equation could be made

y=Ax+B (y=Absorbance, x=nitrate concentration)

↓

x = (y-B)/A μmol/ml

APPENDIX 9

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

Ninhydrin method

Reagents

1. Citrate buffer: Dissolve 56 g of citrate and 21.3 g of NaOH in 1 L 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 boiling 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.

APPENDIX 10

Free amino acids analysis by ACQUITY UPLC™ amino acid analyzer

Extraction of plant materials for free amino acids analysis by 80% Ethanol

(Three times extraction with 0.5 ml of 80% ethanol)

1. Weigh accurately about 25 mg of freeze-dried sample powder (Sample kelp at -20˚C) in a 1.5 ml Eppen tube.
2. Add 0.5 ml of 80% ethanol (Mix Ethanol 80 ml and water 20 ml) into the tube and vortex well.
3. Heat the tube at 60 ˚C in aluminum block for 15 min to loose the enzymatic activity.
4. Centrifuge at 10,000 rpm for 10 min. Pour the extract to another 2.0 ml Eppen tube.
5. Add 0.5 ml of 80% ethanol to the residues in the tube, then vortex well. Keep the mixture in refrigerator overnight.
6. Centrifuge at 10,000 rpm for 10 min. Pour the extract to 2.0 ml Eppen tube which used at first time.
7. Add 0.5 ml of 80% ethanol to the residues in the tube, then vortex well.
8. Centrifuge at 10,000 rpm for 10 min. Pour the extract to previous 2.0 ml Eppen tube and store at -20˚C.

Evaporation the ethanol extraction and re-dissolve in water.

1. Add the 0.5 ml of 80% ethanol extract as above into a 1.5 ml Eppen tube.
2. Evaporate the ethanol until dry by centrifuge evaporator (Tomy Centrifugul Concentrator CC-105).
3. Dissolve the dry residues with 0.5 ml of water
4. Centrifuge at 10,000 rpm for 10 min. Pour the extract to another 1.5 ml Eppen tube.
5. Store the water soluble fraction at -20 ˚C.

Determination free amino acids composition by the AccQ·Tag amino acid analysis Method (UPLC).

The AccQ·Tag amino acid analysis Method (UPLC)

The Water AccQ·Fluor™ Reagent Kit used to perform the amino acid analysis method. A set consists of one vial each of:

1. AccQ·Fluor Borate Buffer (Vial 1)
2. AccQ·Fluor Reagent Powder (Vial 2A). The reagent powder is the 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) derivatizing reagent.
3. AccQ·Fluor Reagent Diluent (Vial 2B). Use to the diluent (acetonitrile) to reconstitute the reagent for derivatization.

Reconstituting AccQ·Fluor Reagent Preparation

1. Preheat a heating block to 55˚C
2. Tap Vial 2A lightly before opening to ensure all AccQ·Fluor Reagent Powder is at the bottom of the vial.
3. Rinse a clean micropipette by drawing and discarding 1 ml of AccQ·Fluor Reagent Diluent from Vial 2B.
4. Transfer 1.0 ml of AccQ·Fluor Reagent Diluent from Vial 2B to the AccQ·Fluor Reagent Powder in Vial 2A.
5. Cap Vial 2A on top of the heating block, vortex occasionally, until the powder dissolves.

Storage: Store reconstituted AccQ·Fluor Reagent at room temperature in a desiccator for up to one weeks. (Seal the container tightly when not in use)

Amino acid analysis by using the Water AccQ·Fluor™ Reagent Kit

Preparation

1. Extract sample.
2. Amino acid calibration standard
3. AccQ·Fluor Borate Buffer (Vial 1)
4. Reconstituted AccQ·Fluor Reagent.
5. 20 μM AABA

Derivatizing standard and sample

1. Preheat a heating block at 55˚C.
2. **Standard:** Use a clean syringe to deliver 10 µL of calibration standard to the bottom of a 1.5 ml Eppen tube (standard tube) and use a micropipette to add 70 µL of Borate Buffer (Vial 1) and vortex briefly.
3. **Sample:** Use a clean syringe to deliver 10 µL of extraction sample to the bottom of a new 1.5 ml Eppen tube (sample tube) and use a micropipette to add 5 µL of AABA and vortex briefly. Then add 55 µL of Borate Buffer and vortex.
4. Add the reconstituted AccQ·Fluor Reagent 20 µL to the standard and 30 µL to the sample tube, respectively. Vortex immediately for several seconds.
5. Allow to incubate for 1 min at room temperature.
6. Heat the tube in a heating block for 10 min at 55˚C.
7. Transfer the contents of the standard and sample tube to the bottom of an auto sampler vial limited volume insert (LVI). Cap with a silicone-lined septum.
8. Analysis amino acid by ACQUITY UPLC™ with ACQUITY UPLC™ TUV.

APPENDIX 11

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

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

Catalog number: 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

Materials required, but not provided

• *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

• 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 *trans*-Zeatin Riboside (*t*-ZR) test utilizes an anti- *t*-ZR monoclonal antibody, respectively and are sensitive in the range of 0.2 - 100 picomoles *t*-ZR /ml. The assay principle uses the competitive antibody binding method to measure concentrations of *t*-ZR in plant extracts. The *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 *t*-ZR -tracer, a limited amount of the antibody and the unknown sample containing *t*-ZR. The *t*-ZR in the sample competes with the *t*-ZR-tracer for antibody binding sites. The unbound *t*-ZR -tracer are washed away before adding the substrate. The yellow color produced is inversely proportional to the amount of hormone in the sample. The intensity of color is related to the sample *t*-ZR concentration by means of a standard curve.

Warnings

Phytodetek *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.

***t*-ZR tracer**: Reconstituted *t*-ZR-tracer are stable for 7 days at 4° C. Precise pipetting of the sample and *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.

***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.

Directions for use

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

Add 5 ml of 1X TBS buffer to each *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 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.

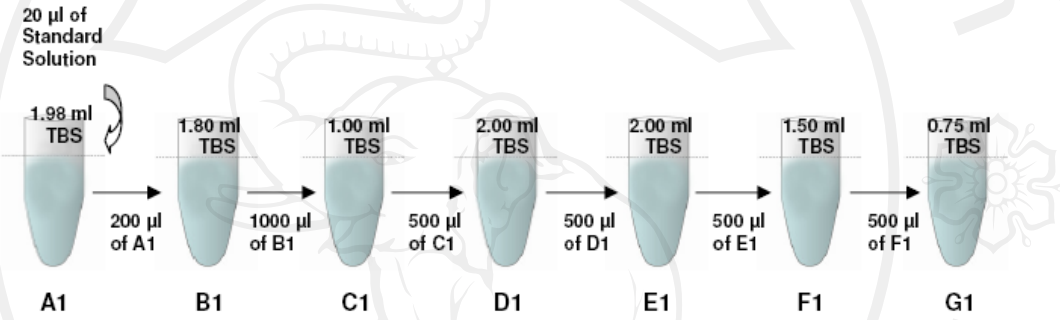
3. Following the chart below, prepare the standards by diluting the standard stock solutions in 1X TBS buffer. New standards should be prepared each time the test is run.

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

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=B_o	0	100 µl	0	



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.

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.

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 100-(%Binding) 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:

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

Percent (%) cross reactivity

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
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

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

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.

CURRICULUM VITAE

Name Mr. Chaiartid Inkham

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Education background

2002 – 2005 B.Sc. (Horticulture) with 2nd class honors, Department of Horticulture, Faculty of Agriculture, Chiang Mai University, Chiang Mai, Thailand

1996 – 2001 Secondary School, Rangsee Vittaya School, Chiang Mai, Thailand

1989 – 1995 Primary School, Ban Mae Saw School, Chiang Mai, Thailand

Scholarship

2008 – 2011 Thailand Research Fund scholarship through the Royal Golden Jubilee Ph.D. Program (Grant No. PHD/0088/2550)

2009 – 2012 The Center of Excellence on Agricultural Biotechnology, Science and Technology Postgraduate Education and Research Development Office, Office of Higher Education Commission, Ministry of Education (AG-BIO/PERDO-CHE)

Training

March – May, 2010	Analysis of free amino acids in *Curcuma* plant by using UPLC technique, Department of Applied Biological Chemistry, Faculty of Agriculture, Niigata University, Niigata, Japan.
December – January, 2011	Training for activity analysis of glutamine synthetase (GS) and glutamate synthetase (GOGAT) in plant, Department of Applied Biological Chemistry, Faculty of Agriculture, Niigata University, Niigata, Japan.

Publications

Inkham, C., K. Sueyoshi, N. Ohtake, T. Ohyama, S. Ruamrungsri. 2011. Critical nitrogen level derermination in *Curcuma alismatifolia* Gagnep. Europ. J. Hort. Sci. 76(5/6): 188–193.

Inkham, C., K. Sueyoshi, N. Ohtake, T. Ohyama, S. Ruamrungsri. 2010. Effects of temperature and nitrogen sources on growth and nitrogen assimilation in *Curcuma alismatifolia* Gagnep. Thai J. Agric. Sci. 44(3): 145-153.

Academic conferences

Inkham C., K. Sueyoshi and S. Ruamrungsri. 2010. Effect of temperature and nitrogen supply on growth and development of *Curcuma alismatifolia* Gagnep. 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)

Inkham C., K. Sueyoshi and S. Ruamrungsri. 2009. Optimum nitrate and ammonium ratio for growth and rhizome qualities of *Curcuma alismatifolia* Gagnep. production. 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)

Inkham C., K. Sueyoshi and S. Ruamrungsri. 2009. Effects of NO_3^-: NH_4^+ ratios on growth and nitrate reductase activity 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)