Chapter 3

Seasonal effects on the changes in some endogenous hormones, other biochemical substances and photosynthetic rate of *Curcuma alismatifolia* Gagnep.

3.1 Introduction

In Thailand, the off-season (OS) production of *Curcuma alismatifolia* Gagnep is practiced by growers to increase their income, because the price for regular season (RS) crops is usually the cheapest of the year. Although *Curcuma* plants can be cultivated in wide areas including tropical and temperate regions, OS cropping may reduce the quality of flowers or rhizomes. It is postulated that changes in environmental conditions, such as temperature, humidity, photoperiod and light intensity, in different seasons affect the rhythm of the growth cycle and plant growth. The growth patterns and quality of crops may be influenced by modifications of endogenous developmental programs through changes in the levels of plant hormones, metabolites (Thomas and Vince-Prue, 1997), biochemical substances, such as free sugars content, nutritional status and free amino acids content, and photosynthesis (Ribeiro *et al.*, 2009). Endogenous phytohormones generally play an important role in regulating plant development and metabolism. Several phytohormones,

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such as ABA, CKs, IAA, GAs and ethylene, have also been implicated in the tuberization of potatoes (Rodriguez-Falcon *et al.*, 2006) and stem swelling in tulips (Nishijima *et al.*, 2005). Rietveld *et al.* (2000) reported that auxin caused a rapid elongation of the stalk in tulips under low temperature. In *C. roscoeana* and *C. alismatifolia*, the application of a plant growth retardant reduced plant height and flower stalk length (Thohirah *et al.*, 2005). Khuankaew *et al.* (2009) reported that application of GA_3 solution increased shoot height in *Curcuma* plants. Many previous reports showed that environment could affect plant hormone (Yoo and Kim, 1996), sugar accumulation (Souza *et al.*, 2010; Walton *et al.*, 2007; Yasin and Buller, 2007; Yoo and Kim, 1996), mineral content and free amino acids (Sagisaka, 1974; Srivastava *et al.*, 1997) which led to different plant growth and development.

The present study aimed to compared the levels of some endogenous hormones and other biochemical substances in various parts of *Curcuma* plant during cultivation between off-season cropping and regular season cropping, in relation to plant growth patterns and to clarify the possible role of ABA, *t*-ZR and IAA and other biochemical substances in the regulation of plant growth and development.

3.2 Materials and methods

3.2.1 Plant materials and experimental conditions

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Stubbed rhizomes of *C. alismatifolia* 'Chiang Mai Pink', 1.8 - 2.5 cm in diameter and with 4 storage roots were purchased from a commercial nursery (Ubonrat Garden, Chiang Mai, Thailand). They were stored at 15°C and 70% RH until used (4 months for OS). One rhizome was planted in a 6 X 12 inch plastic bag containing a mixture of soil: rice husk: rice husk charcoal (ratio 1:1:1), and 7.5 g of 16-16-16

(N-P_2O_5-K_2O) fertilizer was applied twice monthly from the first fully expanded leaves until the flowering stage. Then 7.5g of 13-13-21 (N-P_2O_5-K_2O) fertilizer was supplied twice monthly from the flowering stage until the rhizome-forming stage.

Each pot was irrigated daily with 450 ml of tap water throughout the growth periods. Plants were grown in two seasonal cultivations; i.e. 1) regular season (RS) crops in which plants were cultivated on 22 July 2006 and rhizomes were harvested on 18 January 2007, and 2) off-season (OS) crops in which plants were cultivated on 22 November 2006 and rhizomes were harvested on 22 May 2007.

To examine the changes in endogenous hormonal levels (ABA, *t*-ZR and IAA), plant materials were analyzed at 6 stages of growth; i.e. 1) sprouting, 2) 5-10 cm shoot length, 3) fully expanded leaves in the first shoot, 4) the 1st floret opening, 5) the last flower senescence and 6) dormancy (aboveground parts dried). For RS cropping, the dates of growth stages were as follows; 1) Aug 11, 2) Aug 18, 3) Aug 30, 4) Sep 22, 5) Nov 3 and 6) Jan 18. For OS cropping, the dates were 1) Dec 20, 2) Dec 27, 3) Jan10, 4) Feb 15, 5) Mar 22 and 6) May 22. Growth and development were measured in terms of plant height, leaf fresh weight (FW), rhizome FW, storage roots FW, flower quality and rhizome yield.

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3.2.2 Hormone analysis

ABA analysis

The extraction, purification and quantification of ABA were performed as described by Walker-Simmons *et al.* (2000). Plants were separated into leaves, rhizomes, storage roots, inflorescence, new rhizomes and new storage roots, washed with water and weighed (FW). Then parts were frozen in liquid nitrogen. They were freeze-dried, weighed, ground into a fine powder and stored at -20 °C prior to analysis. Next, 100 mg dry weight (DW) of each sample was extracted with 10 mL of extracting solvent (100% methanol with 500 mg L^{-1} citric acid monohydrate and 100 mg L^{-1} BHT: butyl hydroxyl toluene) overnight in the dark at 4 °C. The crude extract supernatant was adjusted to 70% methanol by diluting 200 µL of sample with 800 µL of 62.5% methanol. Each diluted extract was filtered through a Sep–Pak® C18 cartridge (Water, Milford, Mass U.S.A.) that had been washed twice with 2 mL of 100% and 1 mL of 70% methanol, respectively. The filtrate was evaporated overnight using a speed-vacuum concentrator, dissolved in absolute methanol, and refrigerated at 4 °C until it was assayed. An internal ABA standard was occasionally added to parallel samples to re-calculate % recovery after ELISA. The rate of recovery of ABA was 62%.

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CK (t-ZR) analysis

Samples were prepared as in the ABA analysis, except that the extraction process was modified as follows (Potchanasin *et al.*, 2009). A total of 100 mg DW of freeze-dried sample powder was extracted with 50 mL of cold 80% methanol overnight at 4 °C. The extract was filtered through a G-4 glass-inter-filter (max. pore size 10-16 μm). The filtrate was evaporated dry at 40 °C, and the residue was re-dissolved in 12 mL of 0.01 M ammonium acetate (pH 7.5) and frozen at -20 °C overnight. After thawing, the extracts were centrifuged at 22,000 rpm for 25 min at 4 °C. The supernatant was purified by passing through a pre-conditioned column filled with about 10 mL of polyvinyl-poly-pyrrolidone (PVP) followed by a Sep–Pak cartridge. The CK was eluted directly from the removed Sep-Pak with 4 mL of 30% methanol in 0.01 M acetic acid, evaporated overnight, re-dissolved in 0.2 mL of methanol and refrigerated at 4 °C until it was assayed for CK. The rate of recovery of CK ranged between 77% and 80%.

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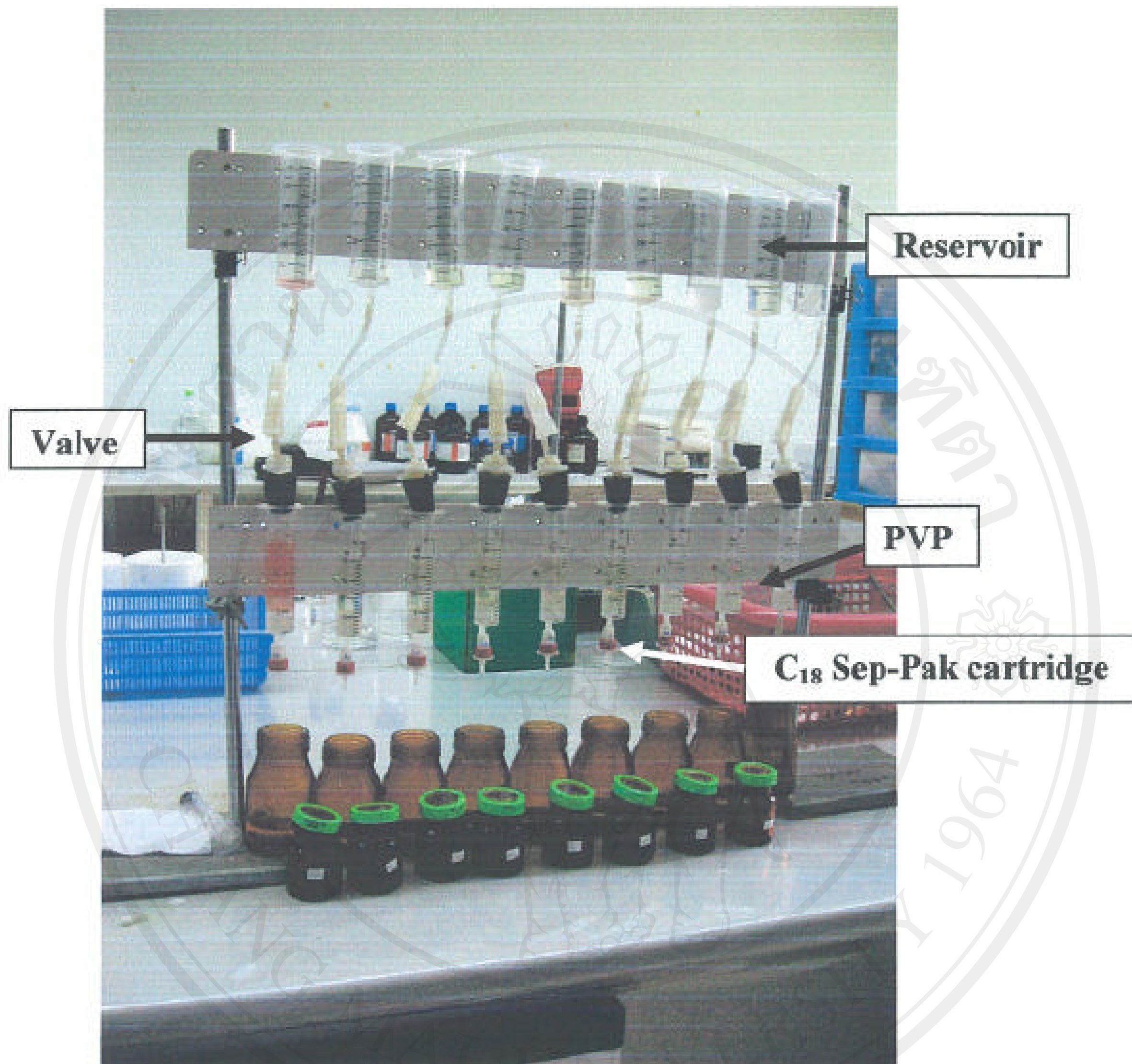

Figure 3.1 The column set up system used for hormone purification

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The measurements of ABA and CK (*t*-ZR) were carried out by competitive ELISA, using Phytodetek test kits, monoclonal antibodies (Agdia, Inc. Elkhart, IN.) (See details in appendix). The *t*-ZR values were designated as *t*-ZR equivalents.

Diffusible IAA

Leaf diffusates were collected from the cut basal end of leaves for the quantification of IAA, as described by Gruber and Bangerth (1990) with some modifications. All the leaves were cut off from the shoots and the cut basal end of leaves were placed into falcon test tubes (one plant per tube), which contained 5.0 mL of 0.10 M phosphate buffer at pH 6.20. The tubes were placed in sealed ice boxes and incubated in the dark at 22 °C, and 100% RH for 20 h. Then, the leaves were removed and the tubes were kept at -20 °C until used for the IAA analysis.

IAA analysis

The extraction and purification of IAA was performed as described by Koshita *et al.* (1999). Briefly, all (approximately 5 ml) of the thawed samples were adjusted to pH 2.5 by adding 4 M acetic acid, then extracted in diethyl ether, and subjected to solvent partitioning. The diethyl ether fraction was collected and evaporated dry with N_2 gas. The dried sample was dissolved in 200 µL of 50% methanol containing 0.10 M acetic acid. The samples were further purified by high performance liquid chromatography (HPLC): column SC-150 (150 x 4.6 mm), PRONTOSIL 120-5-C18, H 5.0 µm, BISCHOFF Chromatography; flow rate 1.0 mL min^{-1}; mobile phase was solvent A; 0.1 M acetic acid in water, solvent B; 0.1 M acetic acid in methanol; gradient profile; 0-12 min 45-85% of B, 12-15 min 85-100% of B, 15-18 min 100-45% of B and 18-22 min 45-45% of B. Under these conditions, the retention time of IAA was at 7.10 min as quantified from the calculated peak area related to a standard peak. The limit of detection and limit of quantification was 1 ng mL^{-1} and 6.25 ng mL^{-1}, respectively. The recovery rate ranged from 67% to 70%.

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3.2.3 Photosynthesis and physiological variables

Measurements of leaf gas exchange in fully expanded leaves were carried out between 10.00 and 12.00 AM at the flowering stage (12 WAP) for both RS and OS production. The evaluation was performed on a clear day without clouds. Physiological variables, i.e. the photosynthetic rate (*A*), stomatal conductance (*gs*), stomatal resistance (*rs*), transpiration rate (*E*), photosynthetical active radiation: P.A.R. on leaf surface (*Q leaf*) and leaf surface temperature (*T leaf*), were determined at the flowering stage by using a leaf gas chamber analyzer (Model LCA4, ADC Hoddessdon, Herts, England). Chlorophyll fluorescence (*Fv/Fm*) was measured at the flowering stage by using an exciting chlorophyll fluorescence meter (Handy-PEA Type, Hansatech Model, UK).

3.2.4 Free sugar contents

Free sugars (glucose, fructose and sucrose) in plant parts were determined by Gas Liquid Chromatography technique as described by Ohyama *et al.* (1986) (see appendix B).

3.2.5 Total free amino acids concentration

Amide-N (total amino acids concentration) was determined by ninhydrin method (see more detail in appendix C).

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3.2.6 Plant nutrient analysis

Total nitrogen was determined by a modified indophenol method using a Kjeldahl-digested solution (Ohyama *et al.*, 1991), phosphorus was quantified by the ammonium molybdate method (Davidescu and Davidescu, 1972) and potassium was measured by atomic absorption spectrophotometry (Perkin-Elmer 3100, Waltham, Massachusetts, USA.) using a modified $HClO_4$-HNO_3 digestion method (Mizukoshi *et al.*, 1994).

3.2.7 Statistical analysis

Data were analyzed for statistical significance using Statistic 8 analytical software (SXW Tallahassee, FL.). Student's *t*-test was used to determine significant differences in growth, photosynthesis (with 10 replicates per treatment), and hormones (3 replicates, 4 plants per replicate).

3.3 Results

3.3.1 Comparison of climatic conditions between RS and OS cultivation periods

The macroclimate data were shown in Table 3.1, and were significantly different between the RS and OS seasons. Air temperature (*T air*) and rain fall (*Rf*) were the lowest in December to February during OS cultivation. The minimum temperature (*T min*) in OS was 13.8 °C in February, and was about 8 °C lower than that in RS (22.1 °C) in October. Average day length during RS was 12.4 h, and it was significantly longer than that during the OS period, 11.1 h (Table 3.1).

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Table 3.1 Environmental conditions during the experimental period, considering the variation in macroclimate between regular season (RS) production and off-season (OS) production.

	Month	*Tair-max* (°C)	*Tair-min* (°C)	*Tair-mean* (°C)	*RH max* (%)	*RH min* (%)	*RH mean* (%)	*Rain fall* (mm)	Sunshine duration (h)
RS	Jul	32.9	24.2	27.9	86.5	67.3	77.3	119.0	13.0
	Aug	33.2	23.7	27.7	90.9	64.8	79.0	205.0	12.1
	Sep	33.3	23.9	27.9	89.0	66.2	78.3	156.0	12.7
	Oct	32.4	22.1	26.5	92.2	59.5	75.9	93.0	11.6
	AVG.	**32.9**	**23.5**	**27.5**	**89.7**	**64.5**	**77.6**	**143.1**	**12.4**
OS	Nov	29.8	18.5	23.3	92.3	54.4	74.1	59.4	11.1
	Dec	30.9	15.2	21.9	91.9	43.8	69.0	0.0	10.9
	Jan	32.7	15.7	23.0	80.2	30.4	56.7	0.0	11.4
	Feb	30.9	13.8	21.1	89.8	36.8	64.0	0.0	11.0
	AVG.	**31.1**	**15.8**	**22.3**	**88.6**	**41.4**	**65.9**	**14.9**	**11.1**

Macroclimate refered to meteorological data automatically recorded at the Multiple Cropping Center: MCC, Faculty of Agriculture, Chiang Mai University weather station: Air temperature (*T air-max*, *T air-min* and *T air-mean*), relative humidity (*RH max*, *RH min* and *RH mean*), rain fall (*Rf*) and sunshine duration.

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3.3.2 Comparisons of photosynthesis between RS and OS cultivations

At the flowering stage, the net photosynthetic rate (*A*) of *C. alismatifolia* was higher in RS plants, 8.19 ± 0.89 μmol $m^{-2}s^{-1}$ than that in OS plants, 5.78 ± 0.20 μmol $m^{-2}s^{-1}$ (Table 3.2).

Table 3.2 Physiological variables related to the photosynthesis between the regular season (RS) and off-season (OS) cropping of *C. alismatifolia* plants at flowering stage (12 WAP).

Physiological Variables/1		Season		t-test
		Regular season	Off-season	at $p < 0.05$
A	(μmol $m^{-2}s^{-1}$)	8.19 ± 0.89	5.78 ± 0.20	*
Gs	(mol $m^{-2}s^{-1}$)	0.06 ± 0.00	0.08 ± 0.00	*
Rs	(mol $m^{-2}s^{-1}$)	17.7 ± 1.28	12.2 ± 0.52	*
E	(mol $m^{-2}s^{-1}$)	1.92 ± 0.08	2.42 ± 0.06	*
Q leaf	(μmol $m^{-2}s^{-1}$)	1130 ± 47.3	570 ± 18.8	*
T leaf	(° C)	39.3 ± 0.14	35.5 ± 0.06	*
Fv/Fm		0.80 ± 0.00	0.75 ± 0.01	*

Net photosynthetic rate (*A*), stomatal conductance (*gs*), stomatal resistance (*rs*), transpiration rate (*E*), P.A.R. on leaf surface (*Q leaf*), leaf surface temperature (*T leaf*) and chlorophyll fluorescence (*Fv/Fm*) as affected by regular season (RS) and off-season (OS) at 10.00 am-12.00 pm. Data were means (n = 10, one plant per replicate) ± SE.

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Both stomatal conductance (*gs*) and the transpiration rate (*E*) were significantly greater in OS. The P. A. R. on leaf surface (*Q leaf*) was significantly lower in OS plants, 570 ± 19 μmol $m^{-2}s^{-1}$ than that in RS plants, 1130 ± 50 μmol $m^{-2}s^{-1}$. Leaf surface temperature (*T leaf*) and *Fv/Fm* values were also significantly lower than those in OS leaves than RS leaves of *C. alismatifolia* at flowering stage.

A negative correlation was found between *A* and *E* (R = -0.22), while positive correlations were found between *A* and *Q* leaf (R = 0.47), *A* and *T leaf* (R = 0.49), *Q leaf* and *T leaf* (R = 0.95). There was no correlation between *A* and *gs* or *rs* (Table 3.3).

Table. 3.3 Correlations among the physiological variables related to photosynthetic between the regular season (RS) and off-season (OS) croppings of *C. alismatifolia* at flowering stage.

Parameter (X axis vs. Y axis)	Regression equation	*R*	Correlation test at $p<0.05$	N
A vs. E	y = -0.03x + 2.39	-0.22	*	20
A vs. Q leaf	y = 62.20x + 415.50	0.47	*	20
A vs. T leaf	y = 0.41x + 34.53	0.49	*	20
A vs. gs	y = -0.00x + 0.08	-0.22	ns	20
A vs. rs	y = 0.48x + 11.57	0.27	ns	20
Q leaf vs. T leaf	y = 0.01x + 32.21	0.95	*	20

Net photosynthetic rate (*A*), stomatal conductance (*gs*), stomatal resistance (*rs*), transpiration rate (*E*), P.A.R. on leaf surface (*Q leaf*) and leaf surface temperature (*T leaf*). The linear regression and correlation coefficient *R* are presented at $p>0.05$. Data were calculated from means at the flowering stage.

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3.3.3 Plant growth and development

OS plants sprouted about 3 weeks later than RS plants. The height of RS plants reached a maximum at 58.5 ± 1.3 cm 40 days after planting (DAP) and remained constant thereafter, while that of OS plants reached a maximum only at 27.3 ± 1.0 cm 86 DAP (Fig. 3.2A and Fig. 3.3A). The rate of increase was 1.60 cm d^{-1} in RS and 0.36 cm d^{-1} in OS.

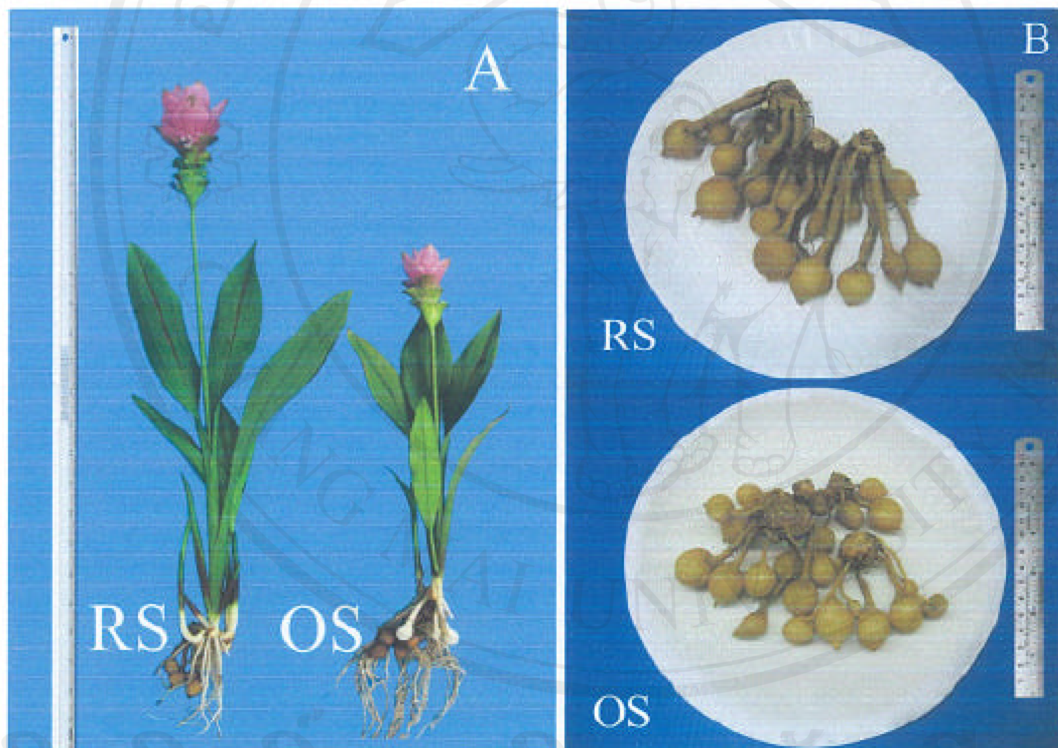

Figure 3.2 Plant height and flower quality (A) and rhizome yields (B) of *Curcuma alismatifolia* 'Chiang Mai Pink' grown in the regular season (RS) and off-season (OS) at the flowering and dormancy stage, respectively.

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The first floret opening was at 60 DAP when the plant produced 3-4 fully expanded leaves in RS, but 90 DAP in OS. Flower quality (spike length, stalk length, number of green bracts and number of pink bracts) was better in RS than OS at stage 4 (Table 3.4 and Fig. 3.2A).

Table 3.4 Flower quality and rhizome yields in *C. alismatifolia* Gagnep. grown in different seasons at the flowering and dormancy stage, respectively.

Yields	Production period		*t*-test at $p<0.05$
	Regular season	Off-season	
Flower quality			
Spike length (cm)	16.10 ± 0.48	10.70 ± 0.33	*
Stalk length (cm)	52.10 ± 1.81	28.50 ± 0.69	*
No. of green bracts	09.40 ± 0.40	06.80 ± 0.37	*
No. of pink bracts	13.00 ± 0.32	09.60 ± 0.75	*
Rhizomes yields			
New rhizomes			
Total numbers	02.06 ± 0.17	04.75 ± 0.40	*
Fresh weight (g)	11.60 ± 0.80	17.30 ± 1.50	*
Diameter (cm)	02.22 ± 0.04	02.10 ± 0.07	ns
New storage roots			
Total numbers	09.88 ± 0.86	13.90 ± 1.13	*
Fresh weight (g)	55.20 ± 4.77	72.00 ± 7.06	ns
Diameter (cm)	02.32 ± 0.06	02.10 ± 0.05	*

Data were means (n = 10, one plant per replicate) ± SE.

During the first floret opening, the fresh weight of RS leaves increased at the rate of 0.43 g $d^{-1}$and reached a maximum at 39.50 ± 1.20 g, then declined. The fresh weight of OS leaves increased continuously at a rate of 0.31 g $d^{-1}$from stages 1 to 5 and a reached maximum of 30.90 ± 2.40 g at 120 DAP (stage 5), then the leaves became senescent at stage 6, approximately 180 DAP (Fig. 3.3B). A sharp decline in the FW of old rhizomes was observed in OS plants, from 4.36 ± 0.20 g at stage 3 (50 DAP) to 3.30 ± 0.10 g at stage 6 (183 DAP), at a rate of - 0.01 g d^{-1} (Fig. 3.3C).

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In addition, the FW of old storage roots in OS plants dropped from19.60 ± 0.60 g at stage 4 (86 DAP) to 1.90 ± 0.60 g at stage 5 (122 DAP), at a rate of - 0.50 g d^{-1} (Fig. 3.3D).

During flowering (stage 4), the formation of new rhizomes was initiated in both RS and OS plants by swelling of the basal pseudo stem. OS plants showed a significantly higher number of new rhizomes per plant (2.10 ± 0.20 in RS and 4.80 ± 0.40 in OS) and FW of new rhizomes (11.60 ± 0.80 g in RS and 17.30 ± 1.50 g in OS), although the diameter did not change (2.22 ± 0.00 cm in RS and 2.10 ±0.10 cm in OS), compared with RS plants (Table 3.4). The rate of production was 0.04 g d^{-1}in RS and 0.04 g d^{-1}in OS (Fig. 3.3E).

New storage roots were formed at 90 DAP in OS plants *versus* 110 DAP in RS plants. Their fresh weights reached a steady level at 120 DAP in OS plants with a growth rate of 0.40 g d^{-1}, while in RS plants, they increased from 120 to 180 DAP at 0.30 g d^{-1} (Fig. 3.3F). The number and fresh weight of new storage roots increased in OS, but less so in RS, at harvest (stage 6) (Table 3.4 and Fig 3.2B).

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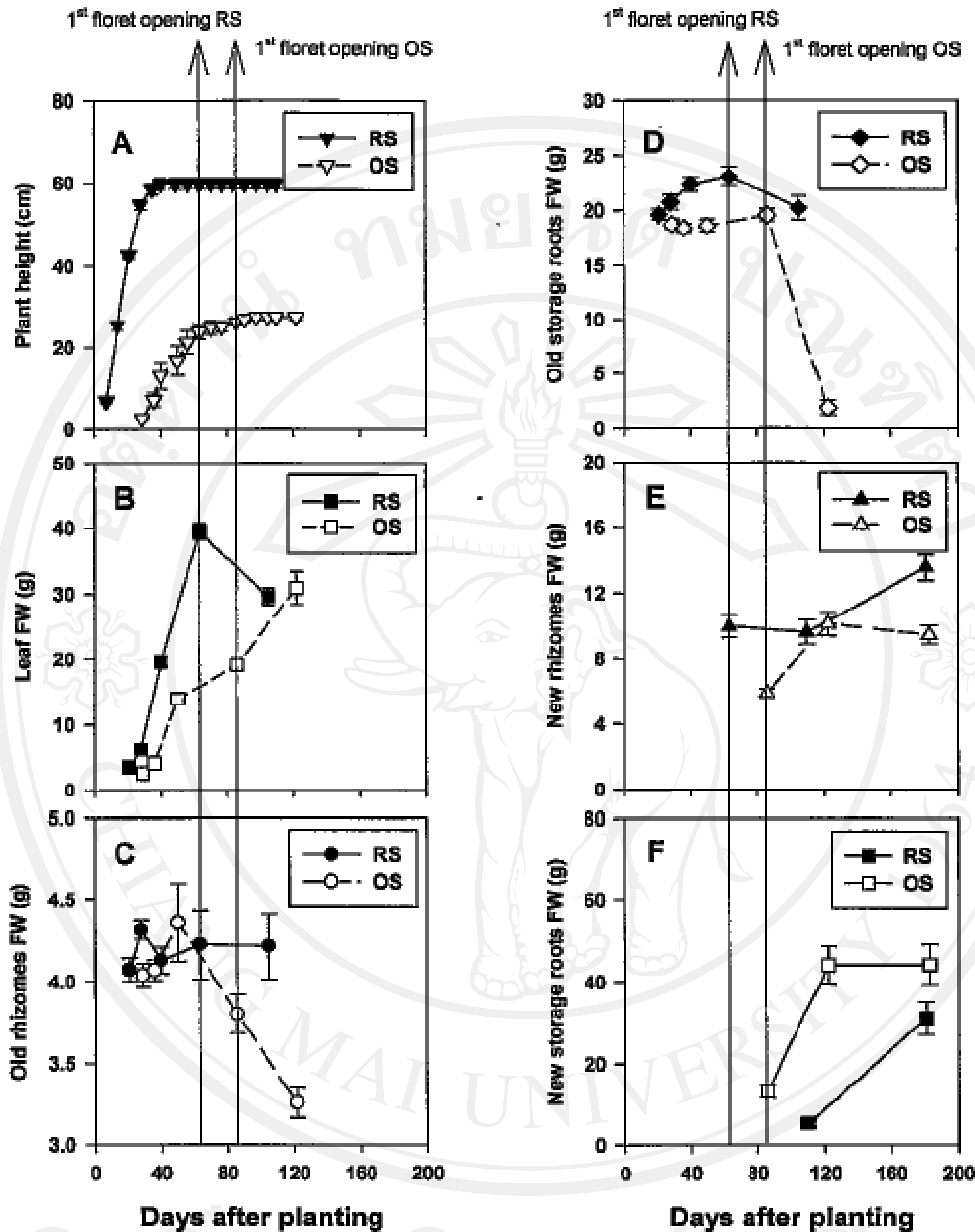

Figure 3.3 Changes in plant height (**A**), leaf FW (**B**), old rhizomes FW (**C**), old storage roots FW (**D**), new rhizomes FW (**E**) and new storage roots FW (**F**) during the growth cycle of *C. alismatifolia* 'Chiang Mai Pink' in the regular season (RS, solid symbols) and off-season (OS, open symbols) cropping. Data were means (n = 3, 4 plants per replicate) ± SE. Arrows indicated the 1st floret opening stage.

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3.3.4 Comparison on the levels of endogenous hormones between RS and OS plants

ABA concentrations

The ABA concentrations in leaves, old rhizomes and old storage roots of *C. alismatifolia* were higher at stage 3 to 6 in OS plants than in RS plants (Fig. 3.4A to C). The ABA concentration in RS leaves reached a maximum of 1.10 μg gDW^{-1} at stage 3, and declined thereafter. In contrast, that in OS leaves peaked of 2.20 ± 80 μg gDW^{-1} at stage 4, and sharply declined thereafter (Fig. 3.4A). ABA concentrations in RS rhizomes were relatively constant at about 0.50 to 0.60 μg gDW^{-1} through all stages. Those in old rhizomes of OS plants were initially low at sprouting; however, they gradually increased and were greater than in RS plants from stage 3 until stage 6. The maximum was 1.30 μg gDW^{-1} (Fig. 3.4B). The ABA concentrations in old storage roots of RS plants were about 0.50 to 0.60 μg gDW^{-1}, except for stage 3. The concentrations in OS plants continuously increased after stage 4 and reached a maximum of 0.10 μg gDW^{-1} at stage 6 (Fig. 3.4C). A decline in the ABA concentration in RS inflorescence was observed at stage 5. On the other hand, a strong increase from 1.2 to 2.20 μg gDW^{-1} was found in OS inflorescence at stage 5 (Fig. 3.4D). The ABA concentrations in new rhizomes and new storage roots were higher in OS plants than RS plants (Fig. 3.4E and F).

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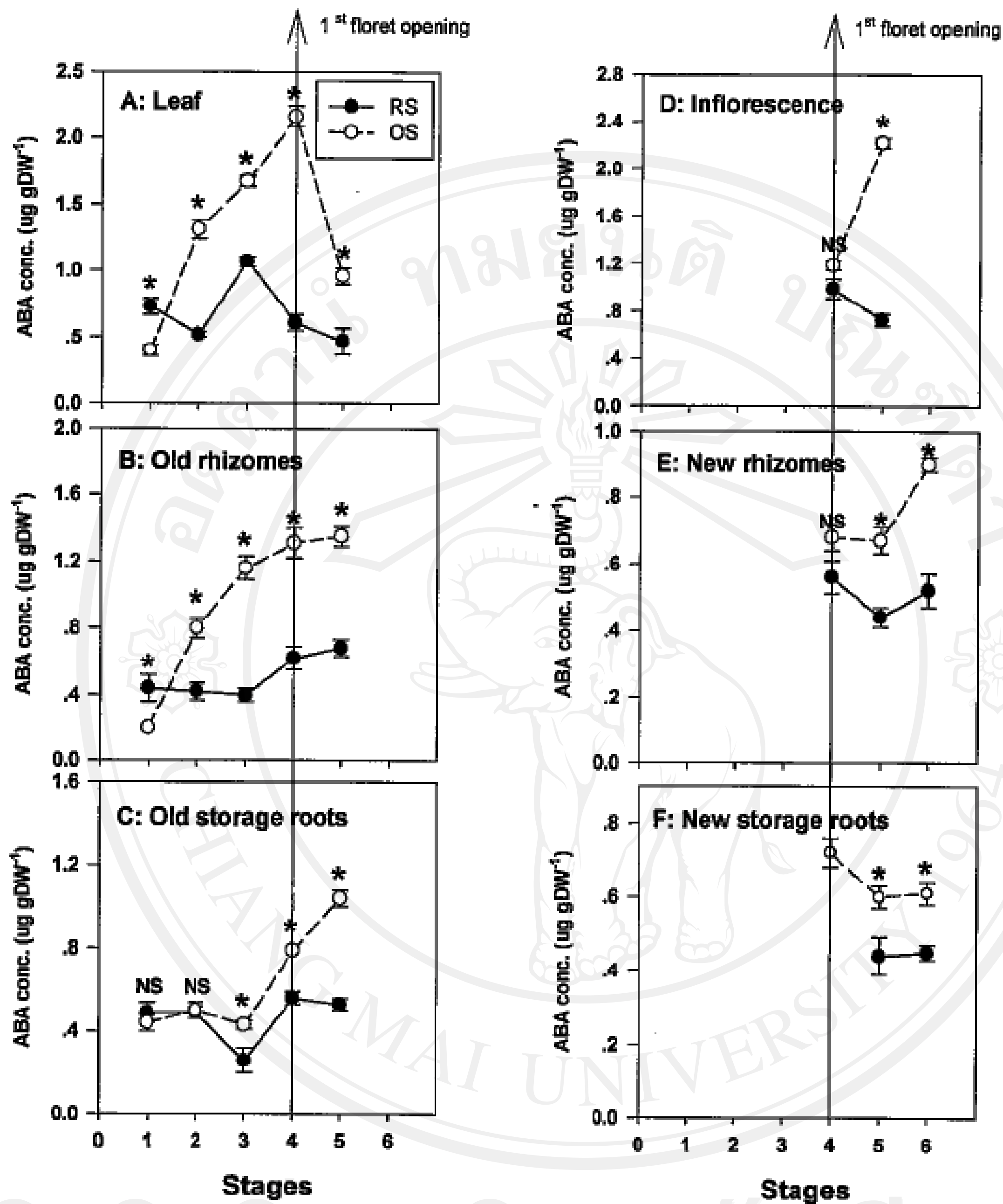

Figure 3.4 Changes in endogenous ABA levels (μg gDW^{-1}) in leaves (A), rhizomes (B), storage roots (C), inflorescence (D), new rhizomes (E) and new storage roots (F) during the growth cycle of *C. alismatifolia* 'Chiang Mai Pink' in the regular season (RS, solid circles) and off-season (OS, open circles) croppings. Data were means ± SE. (n=3). *, significantly different at $p < 0.05$ at the same stage. NS; not significantly different. Arrows indicate the 1st floret opening stage.

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***t*-ZR concentrations**

t-ZR concentrations were significantly higher in OS leaves than RS leaves of *C. alismatifolia* at stages 1, 3, 4 and 5 (Fig. 3.5A). The similar finding was also occurred in old rhizomes (Fig. 3.5B). *t*-ZR levels in old storage roots were significantly higher in OS than those in RS at stages 1, 2 and 5 (Fig. 3.5C). From stage 4 to 6, the *t*-ZR levels in inflorescence were also higher in OS than in RS plants (Fig. 3.5D). A decline in *t*-ZR concentrations in new rhizomes from stage 4 to 6 occurred in RS plants. The opposite was found in OS new rhizomes, and a maximum was reached at 72.70 ± 6.60 ng gDW^{-1} (Fig. 3.5E). From stage 5 to 6, *t*-ZR levels in new storage roots of OS were fairly constant at approximately 30-40 ng gDW^{-1} in OS plants, while they slightly declined in RS plants (Fig. 3.5F).

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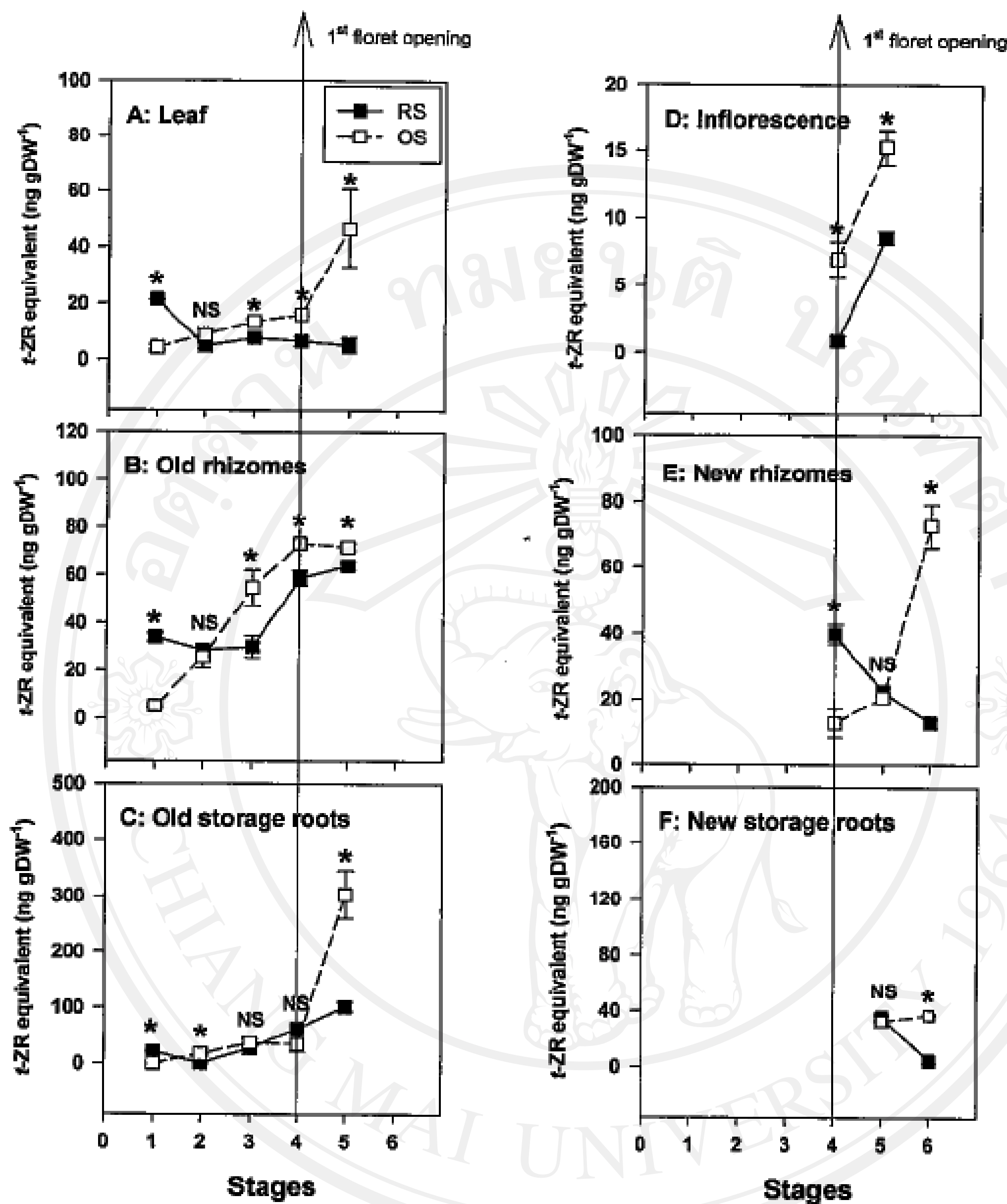

Figure 3.5 Changes in endogenous *t*-ZR equivalent (ng gDW^{-1}) in leaves (A), rhizomes (B), storage roots (C), inflorescence (D), new rhizomes (E) and new storage roots (F) during the growth cycle of *C. alismatifolia* 'Chiang Mai Pink' in the regular season (RS, solid squares) and off-season (OS, open squares) cropping. Data were means (n = 3, 4 plants per replicate) ± SE. *, significantly different at $p < 0.05$ at the same stage. NS; not significantly different. Arrows indicated the 1st floret opening stage.

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Leaf diffusible IAA concentrations

The IAA concentration in leaf exudates of *C. alismatifolia* did not significantly differ between the cropping seasons at stages 1, 3 and 4. However, at stage 5, it was higher in RS than in OS plants (Fig. 3.6). The maximum concentration of IAA in RS plants was 659.00 ± 24.00 pg leaf^{-1} 20 h^{-1}, while it was only 60.00 ± 5.40 pg leaf^{-1} 20 h^{-1} in OS plants (Fig. 3.6).

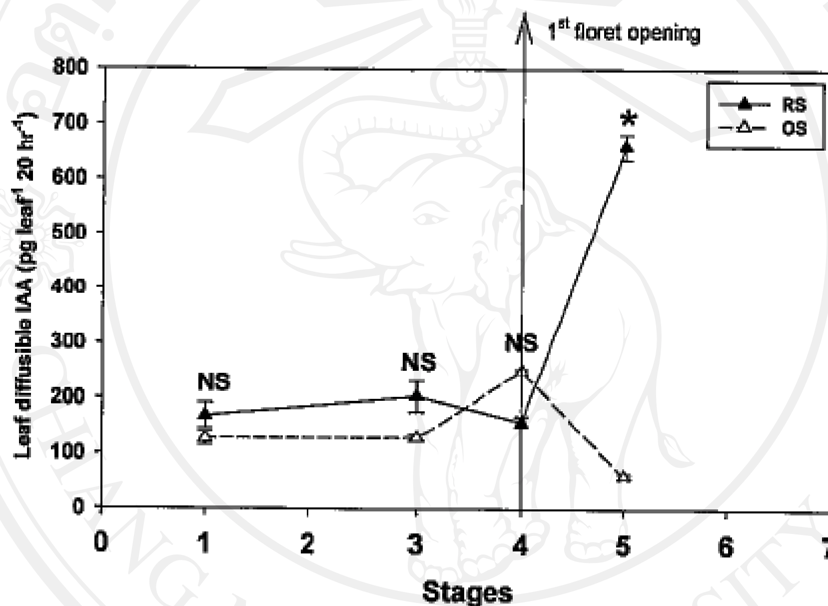

Figure 3.6 Changes in leaf diffusible IAA (pg leaf^{-1} 20 h^{-1}) during the growth cycle of *C. alismatifolia* 'Chiang Mai Pink' in the regular season (RS, solid triangle) and off-season (OS, open triangle) croppings. Data were means (n = 10, one plant per replicate) ± SE. *, significantly different at $p < 0.05$ at the same stage. NS; not significantly different. Arrows indicated the 1st floret opening stage.

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3.3.5 Correlation between concentration of endogenous hormones and plant growth

During the growth cycle (stages 1 to 6), negative correlations were found between plant height and ABA levels in leaves (R = -0.79) and old rhizomes (R = -0.94). Leaf FW was also negatively correlated with ABA levels in leaves (R = -0.81) and rhizomes (R = -0.67). Plant height was negatively correlated with the levels of ABA in leaves (R = -0.79). The positive correlations were found between endogenous ABA levels in leaves (R = 0.90) and in old rhizomes (Table 3.5). Positive correlations were also found between *t*-ZR concentrations in old rhizomes and number of new rhizomes (R = 0.66), and *t*-ZR in leaves and *t*-ZR in old storage roots (R = 0.81) (Table 3.5).

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Table 3.5 Correlations between endogenous hormones in plant parts and some growth parameters (X value vs. Y value) of *C. alismatifolia*. The linear regression equations and coefficients, *R* were presented at $p < 0.05$.

Parameter	Regression equation	*R*
X value		
ABA in old rhizomes		
Y value		
Plant height	y = -0.04x + 86.60	-0.94
Leaf fresh weight	y = -0.01x + 43.50	-0.67
No. of new rhizomes	y = 0.00x + 0.04	0.73
Diameter of new Storage roots	y = -0.00x + 2.52	-0.61
X value		
ABA in leaves		
Y value		
Plant height	y = -0.02x + 63.90	-0.79
Leaf fresh weight	y = -0.01x + 40.00	-0.81
No. of new rhizomes	y = -0.00x + 1.11	0.90
No. of new storage roots	y = -0.01x + 5.29	0.87
X value		
t-ZR in old rhizomes		
Y value		
No. of new rhizomes	y = 0.17x – 8.04	0.66
X value		
t-ZR in leaf		
Y value		
t-ZR in old storage roots	y = 4.53x + 40.80	0.81

Data were calculated from the means of growth parameters and hormone levels ($n = 30$) within six growth stages (1 to 6).

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3.3.6 Free sugars content

The high levels of free sugars content were found at flowering stage (stage 4) of *C. alismatifolia* in both cropping seasons (Fig.3.7). Sucrose (ranged between 36.33 to 147.34 mg gDW^{-1}) was the major sugar in both RS and OS whole plants in all 3 stages of growth (Fig. 3.7C), followed by glucose (ranged between 10.79 to 139.94 mg gDW^{-1}) (Fig. 3.7B) and fructose (ranged between 9.93 to 123.91 mg gDW^{-1}) (Fig. 3.7A).

At flowering stage, the inflorescence and new storage roots were the major organs which contained the higher levels of free sugars content than the other organs. Fructose and glucose concentrations at stage 4 were significantly greater in OS whole plant than those in RS plant, but their levels were low in stage 1 and 6 (Fig. 3.7A and B) while, sucrose was significantly greater in OS whole plants than that in RS at stage 1 and 6 (Fig. 3.7C).

However, fructose and glucose concentrations in OS plant were in traced amount to detect when compared with those in RS plant at dormancy stage (Fig. 3.7A and B).

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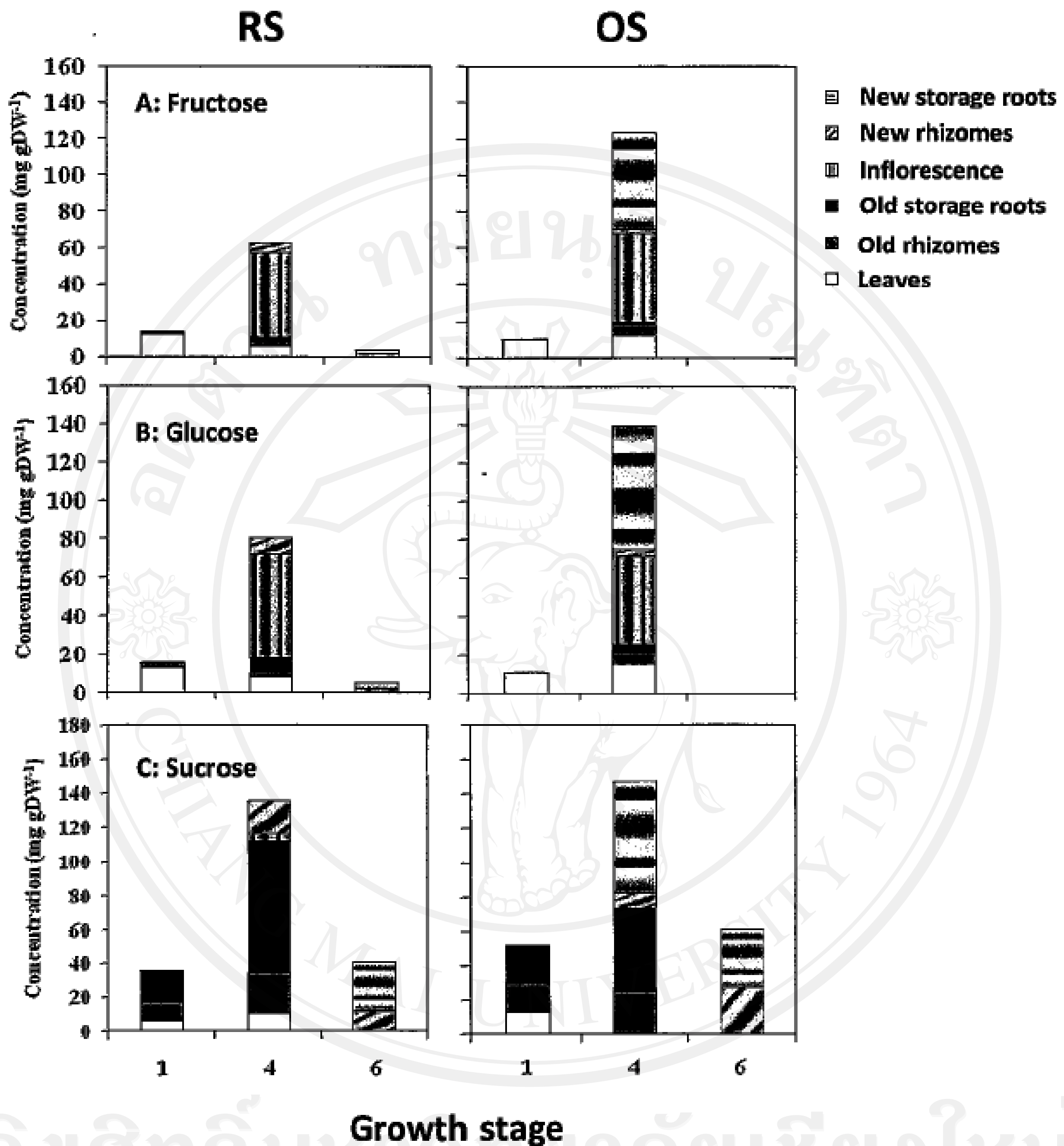

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Figure 3.7 Changes of free sugars contents (fructose (A), glucose (B) and sucrose (C)) in various parts of *C. alismatifolia* plant grown in regular season (RS) and off-season (OS) cropping at 3 growth stages; 1) sprouting, 4) first floret opening and 6) dormancy stage. Data were means ± SE ($n = 4$).

3.3.7 Amide-N (total free amino acids concentration)

At sprouting, total free amino acid concentrations in leaves, old rhizomes and old storage roots in OS plant (8.84, 4.60 and 3.75 mg gDW^{-1}, respectively) were significantly higher than those in RS plant (5.14, 1.99 and 1.53 mg gDW^{-1}, respectively). Leaf was the major organ that contained the highest total free amino acid, followed by old rhizomes and old storage roots in this stage of *C. alismatifolia* (Table 3.6).

At flowering, the amino acid concentrations only in old rhizomes of both seasonal croppings did not significantly differ. However, the concentrations in other organs (leaves, old storage roots and inflorescence) of OS plant were greater than those of RS plant (Table 3.6) while, its concentration in new rhizomes of OS plant was lower than that of RS. RS condition caused the highest free amino acid concentration in new rhizomes by 9.39 mg gDW^{-1} (Table 3.6).

At dormancy stage, both new rhizomes and new storage roots of OS plant had higher free amino acid concentrations (5.32 and 7.12 mg gDW^{-1}) than those of RS plant (2.82 and 5.17 mg gDW^{-1}) (Table 3.6).

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Table 3.6 Total free amino acid concentration in various organs of *Curcuma* plants between regular season (RS) and off-season (OS) cropping.

Organs	Treatment	Total free amino acids (mg gDW^{-1})		
		Growth stages		
		1	4	6
Leaves	RS	5.14±0.20	0.71±0.08	-
	OS	8.48±0.63	3.25±0.23	-
t-test		*	*	
Old rhizomes	RS	1.99±0.10	5.21±0.15	-
	OS	4.60±0.27	5.29±0.30	-
t-test		*	ns	
Old storage roots	RS	1.53±0.07	2.17±0.10	-
	OS	3.75±0.18	2.68±0.13	-
t-test		*	*	
Inflorescence	RS	-	2.45±0.14	-
	OS	-	4.36±0.11	-
t-test			*	
New rhizomes	RS	-	9.39±0.13	2.82±0.37
	OS	-	4.04±0.24	5.32±0.14
t-test at			*	*
New storage roots	RS	-	-	5.17±0.02
	OS	-	7.26±0.33	7.12±0.02
t-test			-	*

Data were means ($n = 4$) ± SE.

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3.3.8 Nutrient status

At flowering stage, the nutrient contents were analyzed. OS condition increased N content in most underground parts (old rhizomes, old storage roots and new rhizomes), but it decreased the content in leaves. The highest N content was observed in leaves under RS condition (353.23 mg plant^{-1}). Only N content in inflorescence did not significantly different between RS and OS condition (165.45 and 172.46 mg plant^{-1}) (Table 3.7).

P content significantly increased in leaves and inflorescence under the RS condition (87.95 and 60.84 mg per plant) as compared with those under OS condition (64.70 and 50.89 mg plant^{-1}). On the other hands, OS condition contained less P content in new rhizomes (9.68 mg per plant) than those under RS condition (25.52 mg per plant) (Table 4). Old rhizomes and old storage roots contained similar P contents within either the seasonal croppings (28.01 and 22.68 mg plant^{-1} for RS and 46.38 and 44.90 mg plant^{-1}for OS, respectively) (Table 3.7).

K content significantly decreased in leaves, old rhizomes and new rhizome under the OS condition (228.26, 14.19 and 2.11 mg plant^{-1}) as compared with that under RS condition (340.91, 18.91 and 67.58 mg plant^{-1}). Neither inflorescence (197.64 and 192.52 mg per plant) nor old storage root (93.96 and 113.27 mg per plant) contained difference in K content within both seasonal croppings (Table 3.7).

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Table 3.7 Nitrogen (N), phosphorus (P) and potassium (K) contents (mg plant^{-1}) in various organs of *Curcuma* plants grown in regular season (RS) and off-season (OS), at the flowering stage (12 WAP).

Organs	Nutrient content (mg plant^{-1})					
	N		P		K	
	RS	OS	RS	OS	RS	OS
Aboveground						
Leaves	353.23 ± 8.06	295.86 ± 14.47*	87.95 ± 3.64	64.70 ± 0.47*	340.91 ± 18.37	228.26 ± 7.87*
Inflorescence	162.45 ± 8.03	172.46 ± 5.54^{ns}	60.84 ± 2.02	50.89 ± 2.48*	197.64 ± 3.68	192.52 ± 2.43^{ns}
Underground						
Old rhizomes	89.85 ± 1.16	114.70 ± 4.56*	28.01 ± 2.23	22.68 ± 1.76^{ns}	18.91 ± 1.03	14.19 ± 1.11*
Old storage roots	41.78 ± 1.57	60.75 ± 2.19*	46.38 ± 3.39	44.90 ± 2.25^{ns}	93.96 ± 2.18	113.27 ± 14.0^{ns}
New rhizomes	108.66 ± 4.10	120.43 ± 1.93*	9.68 ± 1.57	25.52 ± 1.79*	67.58 ± 3.68	26.11 ± 2.43*
New storage roots	-	66.36 ± 1.22	-	10.35 ± 0.59	-	114.0 ± 8.29
Total	755.98±15.71	830.56±20.78*	232.86±6.75	219.05±3.49^{ns}	719.02±12.96	688.35±21.48^{ns}

*Significant by Student's *t*-test at $p<0.05$. Data are means ± SE ($n=3$).

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3.4 Discussion

The lower *Rf* and average % RH (Table 3.1) and higher *gs*, lower *rs* and higher *E* (Table 3.2) induced stomatal opening in OS plants. The lower value of *A* under OS conditions was probably caused by the lower *Q leaf* and lower average *T air*. A similar response was found when low temperature treatment decreased photosynthetic activity in pea cultivars (Georgieva and Lichtenthaler, 2006). The lower photosynthetic rate in OS apparently caused the reduction in plant height and flower quality (Table 3.4, Fig. 3.2A), because the daily gains in carbon derived from photosynthesis mostly accumulate in leaves as structural carbons and soluble sugars or starch, for using in plant growth (Khuankaew *et al.*, 2009). However, the longer storage of rhizomes before planting was expected to have reduced food reserves and altered plant growth in OS. In this result, the total free sugars (fructose, glucose and sucrose) in sprouting rhizome with storage roots seemed to be higher in OS. Interestingly rhizome yields were significantly higher in OS than RS, although shoot growth was strongly depressed, indicating the competition between aboveground (leaves and inflorescences) and underground organs. The higher free sugars contents (fructose, glucose and sucrose) in new storage roots at flowering stage and higher sucrose concentration in new rhizomes and storage roots at dormancy stage, could be indicated that both organs became the strength sink in OS, since the developing of storage organs could act as a major sink for assimilates (Xu *et al.*, 2008).

The accumulation of ABA is generally considered to be a physiological response to adverse conditions; such as drought, cold, heat, or salinity (Zeevaart, 1999). Under these conditions, levels rise and suppress the growth process (Kondrat' Eva *et al.*, 2009). Although the roots of *Curcuma* plants were sufficiently watered in

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both seasons, the FW of leaves was significantly lower in OS than RS, indicating a loss of water in OS plants which might have responded by raising levels of ABA. The significant correlations between plant height and leaf fresh weight and endogenous ABA levels in leaves suggested that the decrease of shoot growth in OS was not only caused by a low photosynthetic rate, but also due to the influence of a high level of ABA in shoot organs. Exogenous ABA has been reported to inhibit shoot growth and stem elongation in tulips (Saniewski *et al.*, 1990), and shoot elongation and floral development in Dutch iris (Doss *et al.*, 1983). In this experiment, the OS plants produced more and heavier rhizomes than those of RS plants. The ABA levels in leaves and old rhizomes were positively related to the number of new rhizomes and number of new storage roots, although it was negatively correlated with leaf FW (Table 3.5).

A low temperature and a high ABA content are considered to be favorable conditions for the formation of bulbs in plants (Okubo, 2000). In addition, ABA was related to thickening and dry matter accumulation in tuberous roots of sweet potatoes (Nakatani and Komeichi, 1991). Therefore, the significant increase in rhizome yields in OS plants might be caused by low temperature and the higher levels of ABA in rhizomes. In addition, the depression of shoot growth might also reflect the sink-source relationship, with more photoassimilates transported to the underground parts under severe depression of shoot growth. The reduction of ABA concentrations in OS rhizomes at sprouting (stage 1) might be due to the longer storage at 15°C before planting the same as found in lily (Xu *et al.*, 2006) and tulip (Geng *et al.*, 2007). The ABA levels in old rhizomes were similar to those in leaves in both RS and OS plants (Fig. 3.4). The increase of ABA and *t*-ZR in old rhizomes from stage 2 to 6 might

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indicate that old rhizomes possibly played the role in storing reserves of endogenous hormones in the growth cycle.

CKs are considered to be synthesized mainly in root tips and exported to other organs by the transpiration stream (Hare *et al.*, 1997; Shashidhar *et al.*, 1996). The *t*-ZR levels in various organs were significantly higher in OS than RS plants (Fig. 4). There have been many reports that *t*-ZR is involved in root formation by controlling the rate of meristematic cell differentiation (Dello Ioio *et al.*, 2007; Kyozuka, 2007) and the formation of storage organs by creating a sink and regulation on the expression of genes implicated in assimilate partitioning and source-sink regulation (Roitsch and Ehneβ, 2000). Since old rhizomes and old storage roots are still active, the increase of *t*-ZR in them and the positive correlation between *t*-ZR in leaves and old storage roots, leading to speculate that these organs are also functioned as temporary storage tanks for *t*-ZR. The positive correlation between new rhizome yield and the level of *t*-ZR in old rhizomes may support this hypothesis (Table 3.5).

Auxin had been implicated in tuber physiology but the level of endogenous IAA in buds was positively related to the termination of dormancy (Sorce *et al.*, 2000) and would not be directly responsible for the inhibition of sprouting in tuber potatoes (Sorce *et al.*, 2009). That, there were no differences in leaf diffusible IAA levels of OS and RS plants in this experiment, suggested that the inferior shoot growth was not attributed to the decrease in IAA concentrations in shoots of OS plants (Fig. 3.6).

At flowering, OS condition seemed to reduce N, P and K contents in almost aboveground organs. This occurrence might explain by the macroclimate effect in OS, as low temperature and short day condition probably reduced the rate of nutritional up take by root, since the low temperature in root zone had been reported to lower the up

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take rate of both NH_4^+ and NH_3^+ (Mengel and Kirkby, 1987) and reduced K^+ influx outside of root (Ching and Barbers, 1979). While the reduction of photoperiods from 16 to 8 h had also been reported to referential decrease N up take in *Medicaco sativa* (Noquet *et al.*, 2003). N accumulation in underground parts of OS plant was found to be higher than that of RS plant, and this led to higher accumulation of total amino acids in this organ under OS condition. Ruamrungsri *et al.*, (2001) reported that most N in new rhizomes and storage roots during dormancy was the 80% ethanol insoluble fraction of high molecular weight constituents, such as protein, etc.

Some evidences showed that low temperature usually more severely inhibited the uptake of P in corn (Bravo-F and Uribe, 1981; Engels *et al.*, 1992), although P up take and translocation did not involved in the demanding control of shoot (Engels *et al.*, 1992). Thus, this result could be expected to have low levels of P content which caused by the low night temperature in OS conditions, but it was found the increase of P content only in new rhizomes and in new storage roots under the OS condition. This finding might be considered to be the function of P as the role in energy conserved and transferred in cell (Marschner, 1995), brought about the greater storage roots yields by more carbon partitioning, and sink-source competition between root and shoot under OS than RS condition. Khuankaew (2010) reported that P in storage organs of *C. alismatifolia* was used during the initial of plant growth and flowering. In potatoes, the tuber (storage organs) became the principal site of phosphate accumulation (Poder *et al.*, 1988), therefore the suitable P content could be beneficial for better plant growth. However, P deficiency also promoted root growth and reduced shoot growth by increasing root-shoot mass ratio and root surface in several plants (Wittenmayer and Merbach, 2005).

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The decreased K content in leaves, old rhizomes and new rhizomes under low night temperature in OS condition might be explained by the finding of Ching and Barbers (1979) who reported that low temperature reduced K^+ influx outside the root and brought to less growth in *Zea may*. Beside this reference above, reducing K content could be partially related to the decrease in above ground growth and development of OS plant, due to the important role of K in meristematic growth by the contributor of K^+ to the osmotic potential of the cell (Marschner, 1995). Nevertheless, the various concentrations of K supplied to *C alismatifolia* did not affect any plant growth parameters, except on the number of green bracts (Ruamrungsri *et al.*, 2005).

3.5 Conclusion

The unfavorable conditions in OS, such as low temperature, short day length, water loss etc., induced a decrease in the photosynthetic rate, increased total free sugar contents in storage organs, increased total free amino acids, ABA and *t*-ZR concentrations and fluctuated in N, P, and K contents in various organs at different growth stages. The response of ABA was much stronger than that of *t*-ZR and brought about decreases in plant height and flower quality, but stimulated the formation of rhizomes in *C. alismatifolia*. The distinct factors involved in growth regulation via changes of endogenous hormones and other biochemical substances should be further studied.

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