Chapter 3

Effect of Low Temperature on Flowering, Physiological and Biochemical Changes

3.1 Introduction

Low temperature is one of the most important factors affecting plant growth and distribution (Larcher, 1995). Optimum low temperatures and cold period are required to complete vernalization, which however varies among species. For example, mango requires around 15°C for 30 days, whereas litchi needs 39 days of transfer to low temperature (Batten and McConchie, 1995). In litchi, the temperatures directly effected dormancy and shoot development (O'Hare, 2002). A cool winter is also generally considered to be a pre-requisite for adequate flowering (Menzel, 1983; Chaikiattiyos *et al.*, 1994; Menzel and Simpson, 1994; O'Hare, 2002). Moreover, temperatures also affect the rate of reproductive developments, by which panicles emerge earlier at 15/10°C than at 20/15°C, but taking longer time to reach anthesis (Menzel, 2002a).

In addition, the temperature also affects sensitivity of biochemical reactions of photosynthesis (Taiz and Zeiger, 1998). Moderately low temperature are known to limit the photosynthetic productivity of sub-tropical fruit trees (Schaffer and Anderson, 1994; Allen *et al.*, 2000), it may because low temperatures reduce enzyme-catalysed reactions such as inhibition of sucrose synthesis, which reduces photosynthesis (Stitt and Hurry, 2002). Moreover, dark chill causes a combination of chill-induced inhibition of photosynthetic metabolism and stomatal closure (Martin *et al.*, 1981).

For endogenous hormones response to low temperature, Naphrom (2004) revealed in mango trees after held in cool temperature of 13°C that auxin concentrations declined in plant tissue, except in terminal buds, whereas Z/ZR concentrations increased during days 13-29 of treatment. Flower buds also occurred within three months. Moreover, Morris (1979) proposed that cold temperature might inhibit IAA polar transport out of the pea shoot tip. Nowadays, it is widely accepted that the partitioning of assimilates between photosynthetic source and utilizing sink organs is regulated by endogenous plant hormones (Baker, 2000).

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In this study, experiments were preliminarily conducted to follow the effect of low temperature (15/10°C day/night temperature) on the physiological activities and biochemical substances content, i.e. endogenous hormones and assimilate substances.

3.2 Materials and methods

3.2.1 Plant materials acclimatization and experimental design

Experiments were conducted during June to October 2002 at Lampang Agricultural Research and Training Centre, Rajamangala Institute of Technology, Thailand based upon completely randomized design on one and a half year-old 'Hong Huay' litchi trees propagated by air-layering. Stem diameter of studied plants were around 2.0-2.5 cm with around 90 day-old fully mature leaves on terminal shoots. Plants were randomly divided into two groups of which 90 plants and cultivated in two different environments. The first ninety trees were cultivated in two growth chambers (Fitotron SGC970, Sanyo, U.K.) under diurnal day/night temperature regime of 15/10°C with a 12/12 h light/dark period (Figure 3.1 A, 3.2 and 3.3). The other half number of the tree were as control treatment grown in open field under warm temperature range of 34.4-36.6°C at day time and 25.1-28.4°C at night time (Figure 3.1 B and 3.4). To prevent cold shock and to acclimatize the studied plants, temperature in growth chambers were stepwise decreased from 25/20°C to be 15/10°C within 7 days and kept the temperature constant in accordance to the temperature regime for another 28 days. Each tree was watered 100 ml every morning and received slow released fertilizer monthly, which containing: 25%N, 30%P, and 25%K.

After 28 days of the cold treatment (15/10°C day/night temperature), the two chambers were separately operated in two different temperature regimes in accordance to Batten and McConchie (1995) and Menzel (2002b). The first chamber was kept at 15/10°C for another 10 days, whereas the second chamber the temperature was stepwise warming up. Temperature was slowly increased to arrived 27.5/17.5°C within 14 days (Figure 3.2 and 3.3). This adaptation to warmer temperature is need to allow plants for bud break and flushing (O'Hare, 2004; Batten and McConchie, 1995). The similar temperature warming up pattern was also followed for the first chamber. Thereafter, plants were transferred to nursery for flowering observation.

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A. Low temperature treatment

Plant materials

Growth chamber

B. Warm temperature treatment

Plant materials

Field grown plants

Figure 3.1 Plant materials grown in a growth chamber and in the field

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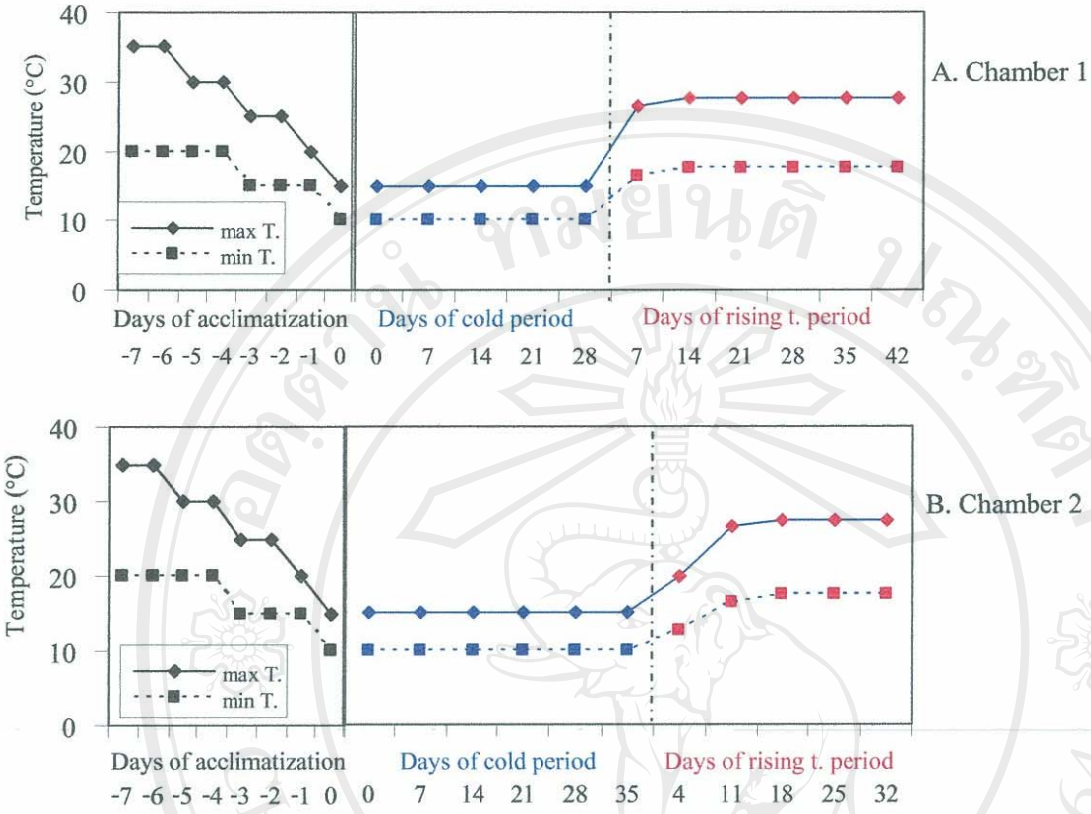

Figure 3.2 Stepwise change of ambient temperature in chamber 1 (28 days cold duration) and chamber 2 (38 days cold duration) starting from acclimatization period, cold treatment and warming up period

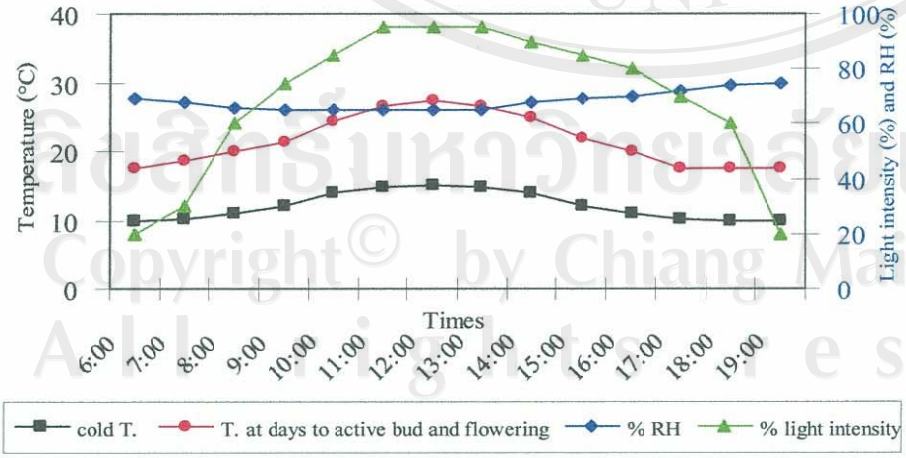

Figure 3.3 Diurnal changes of temperature, light intensity and relative humidity (RH) at the day of visible active bud and of flowering

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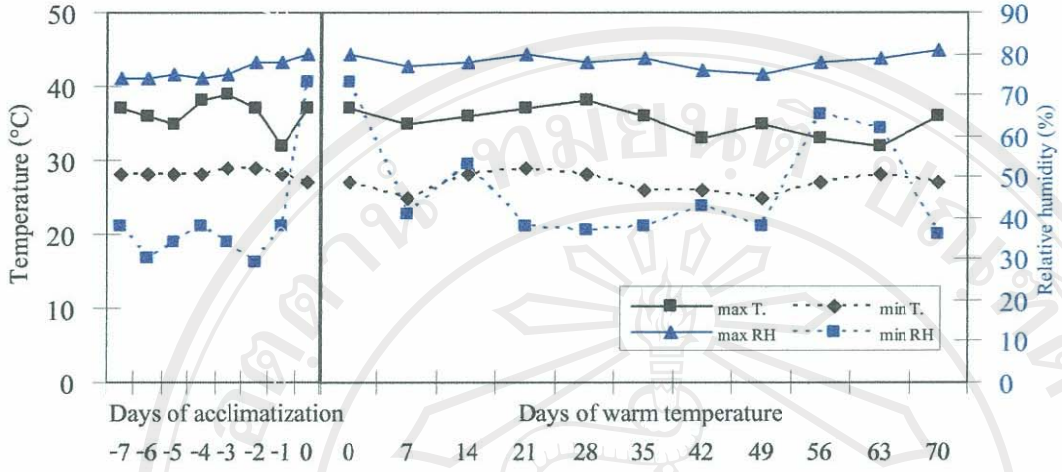

Figure 3.4 Maximum and minimum temperatures and relative humidity at opened field in Lampang Agricultural Research and Training Centre during experimental period

3.2.2. Data collection

3.2.2.1 Effect of low temperature on flowering

Immediately after 28 days (chamber 1) or 38 days of cold period (chamber 2), temperature was rising up to reach 27.5/17.5°C, which took 42 days for chamber 1 and 32 days for chamber 2. Plants were then transferred to nursery for flowering observation. Five litchi trees per treatments were observed. Percentage and days to visible active bud as well as percentage and days to start flowering were collected, compared to trees kept in the field as warm temperature treatment.

3.2.2.2 Effect of low temperature on physiological activities

To compare the physiological change of litchi leaves as affected by different temperature regimes, chlorophyll fluorescence (*Fv/Fm*) was measured at the third leaf from shoot apex by using plant efficiency analyzer (Model PEA, Hansatech Instruments, U.K.). On the same leaf, photosynthetically active radiation (*PAR*), photosynthetic rate (*Pn*), transpiration rate (*Tr*), stomatal conductance (*Gs*) and leaf temperature (*Tl*) were measured by using Portable

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Photosynthesis System (Model CIRAS-1, PP System, U.K.). These parameters were measured at 7 days interval during 1 month of cold treatments and during 10:30-12:00 o'clock (1.5 h). One diurnal change at day 24 of cold treatments was measured for following parameters:

1) Chlorophyll fluorescence

Chlorophyll fluorescence reflected the maximum photochemical yield of photosystem II (PS II), was using portable saturation pulse modulation chlorophyll fluorescence. *Fo* was estimated after exposing the leaf to a weak modulated measuring beam (< 0.1μmol m^{-2} s^{-1}) when the PS II reaction centers were oxidized. To record *Fm*, the leaf was exposed to a 800 ms saturation pulse of white light to produce a reduction of the PS II reaction centers. Variable fluorescence (*Fv* = *Fm-Fo*) was defined as the increase of fluorescence from *Fo* to *Fm*. This allowed the determination of the maximum quantum efficiency of PS II primary photochemistry, given by *Fv/Fm* = (*Fm-Fo*)/*Fm* (Kock, 1994). Therefore, the value of *Fv/Fm* was allowed to study the different functional levels of photosynthesis indirectly (processes at the pigment level, primary light reactions, thylakoid electron transport reactions, dark-enzymatic stroma reactions and slow regulatory processes).

The most basal leaflet of the third leaf from shoot apex were selected and measured for chlorophyll fluorescence at 10:30-11:00 o'clock from 8 trees.

2) Photosynthetic efficiency

As in photosynthesis, plant uses solar energy to oxidize water, thereby releasing oxygen, and to reduce CO_2 into organic compounds, primary sugars. The complex series of reactions that culminate in the reduction of CO_2 include the thylakoid reactions and the carbon fixation reactions. The thylakoid reactions of photosynthesis take place in the specialized internal membranes of the chloroplast called "thylakoids". The reaction centers are called PS I and PS II, which PS I is found exclusively in the non-stacked stroma membranes, PS II largely in the stacked grana membranes. PS II and PS I carry out noncyclic electron transport, oxidize water to molecular oxygen, and reduce $NADP^+$ to NADPH. The end products of these thylakoid reactions are the high-energy compounds ATP and NADPH, which are used for the synthesis of sugars in the carbon fixation reactions. The reduction of CO_2 to carbohydrate *via* the carbon linked

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reactions of photosynthesis (Calvin cycle) is coupled to the consumption of NADPH and ATP synthesized by the light reactions of thylakoid membranes (Taiz and Zeiger, 1998).

According to measurement of photosynthetic rate by using portable photosynthetis system, the photosynthetic rate is calculated from the difference in the CO_2 concentration entering, that leaving and the flow rate through the cuvette. The CO_2 readings are assumed to be corrected for water vapor, temperature, and atmospheric pressure. Also, the addition of water vapor by transpiration in the leaf cuvette dilutes the outgoing air, and this must be compensated for in the calculation (PP Systems, 1996).

Measuring was operated on mature leaflets at the opposite side of leaflet that used for chlorophyll fluorescence measurement. Eight plants were selected and measured at 10:30-12:00 o'clock by using a handy leaf cuvette connected to a CIRAS-1 Portable Photosynthesis System. Photosynthetically active radiation (μmol m^{-2} s^{-1}), photosynthetic rate (μmol CO_2 m^{-2} s^{-1}), transpiration rate (mmol m^{-2} s^{-1}), stomatal conductance (mmol m^{-2} s^{-1}) and leaf temperature (°C) were recorded.

3.2.2.3 Hormones analysis

1) Sample collection

Five aerial plant parts, e.g. terminal bud including two lateral buds, bark, wood, xylem exudates, and leaf diffusate were sampled at 7, 14, 21 and 28 days of cold treatment for hormones analysis. Bark is presumable containing phloem sap, whereas wood presumably contains hormone transport from root to shoot like xylem sap. Leaf diffusate was collected for phloem transport of plant hormone from leaf. The plant tissues were immediately frozen in liquid nitrogen, freeze dried and stored at -20°C until analysis. For collection of xylem-sap, four litchi stems per treatment were cut at about 10 cm above the root/shoot junction. The basal part with root was placed into a pressure chamber (Model 3005, Soilmoisture Equipment Corp., U.S.A.). By raised up the pressure in the chamber up to 3.5 MPa, xylem exudates at the amount of 1.0 ml was collected from cut surface with silicon tube and immediately frozen in liquid nitrogen and stored at -20°C until analysis.

Leaf diffusates were collected from three leaves per terminal shoot by detached the leaves and dipped immediately the petioles cut surface into multititer culture plates filled with 3.1

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ml phosphate buffer (0.1M, pH 6.2). It was incubated under 100% RH in darkness at 25°C for 20 h to facilitate the diffusion process. Thereafter the leaves were removed and the plates of hormones samples were frozen at -20°C until analysis. The above mentioned system was used to study the IAA contents. For diffusion of CKs and GAs, 200 mM ethylenediamine tetraacetic acid (EDTA) was added to the phosphate buffer solution in order to prevent the closure of the phloem's open surface.

Hormone analysis was carried out at The University of Hohenheim, Stuttgart, Germany using radio-immunoassay method. Three groups of endogenous hormones were measured, i.e. cytokinins (Z/ZR and i-Ado/i-Ade), auxin (IAA) and gibberellins ($GA_{1, 3, 20}$).

2) Hormone extraction and purification (Naphrom, 2004)

2.1) From plant tissues

All bud samples (approximately 200 mg DW) were homogenized in 20 ml 80% cold methanol. Bark and wood were pre-cooled in liquid nitrogen and blended in a frozen blender then 500 mg DW of the samples were weighted and extracted in 50 ml of 80% cold methanol. The plant extracts were kept in the darkness at 4°C overnight. The extracts were evaporated then filtered through G4-glassinter-filters (max. pore size 10-16 µm). After that the extracts were evaporated to almost complete dryness in a rotary evaporator under vacuum at 40°C under low pressure and dissolved 3 times with 4 ml 0.01 M ammonium acetate, pH 7.5, by using an ultrasonic bath. The extracts were pooled (approx. 12 ml) and subsequently frozen at –20°C overnight. After thawing, the extracts were centrifuged at 22,000 rpm at 4°C for 25 min. According to purification, the supernatant from the centrifugation step was passed through a combination of preconditioned column, filled with polyvinylpyrrolidone (PVP; Sigma Chemical Co., Deisenhofen, Germany), DEAE-Sephadex A-25 (Pharmacia, Freiburg, Germany) followed by a C_{18} Sep-Pak cartridge (Water, Dreieich, Germany), arranged as described in Figure 3.5 (modified from Bertling and Bangerth, 1995).

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2.2) From leaf diffusate samples

IAA: For IAA leaf diffusate purification, six replications were performed in the experiment. Four blocks of buffer solution were pooled, adjusted to pH 3 with acetic acid and passed through a C_{18} Sep-Pak cartridge (reverse phase, Waters, Eschborn, Germany). ^{14}C-IAA was used as internal standard. After that the cartridge was washed with 4 ml 0.1 M acetic acid then washed again with 4 ml 20% methanol in 0.1 M acetic acid. Thus IAA was eluted with 4 ml 40% methanol in 0.1 M acetic acid. Aliquots of this elute were pipetted into small vials in triplicates and evaporated overnight in a vacuum concentrator. RIA was used to determine the IAA concentration (Bohner and Bangerth, 1988).

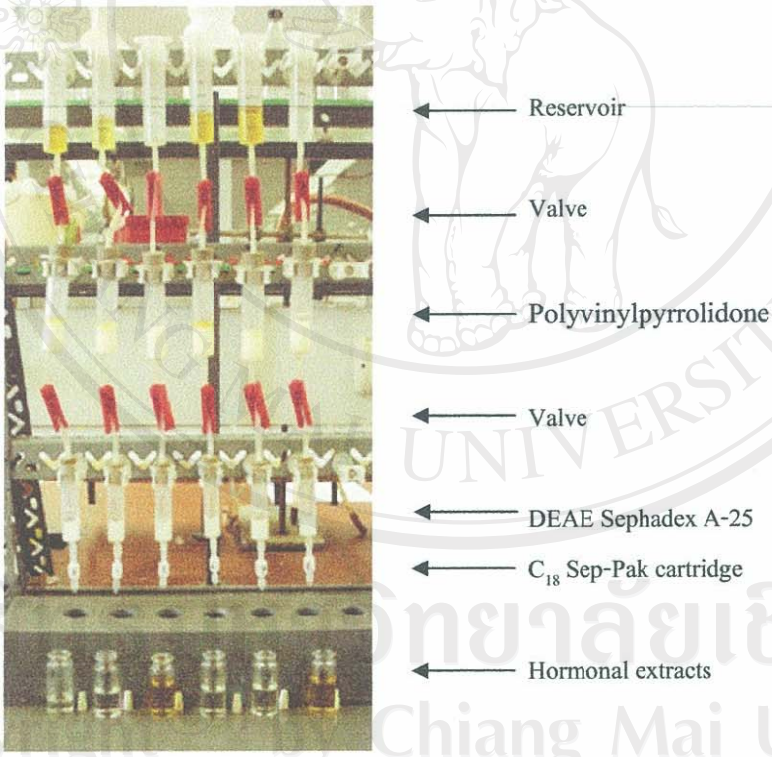

Figure 3.5 Column systems used for hormone purification

CKs and GAs: According to purification of leaf diffusate samples, two replications were performed. Twelve blocks (cavities) of buffer solution were pooled, adjusted to pH 3 with acetic acid and first purified by C_{18} Sep-Pak cartridge to eliminate EDTA. Then the

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Sep-Pak was washed with 4 ml 15% methanol in 0.1 M acetic acid. Thus Z/ZR were eluated with 4 ml 30% methanol in 0.1 M acetic acid. GAs were eluted with 65% methanol in 4 ml 0.1 M acetic acid. I-Ado/i-Ade were also eluted with 80% methanol in 4 ml 0.1 M acetic acid. Aliquots of this elution were pipetted into small vials in triplicate and evaporated overnight in a vacuum concentrator. GAs, Z/ZR and i-Ado/i-Ade were determined by RIA.

2.3) From xylem sap samples

The frozen xylem sap, which had been stored in silicon vial was thawed then weighted and adjusted to pH 8.5 with 0.4 M NaOH. After that the xylem sap was passed through a PVP column and collected in a 10 ml glass bottle. The plastic vial was rinsed with 2 ml distilled water, which were passed again through the PVP column. Thus the xylem sap and distilled water were pooled then adjusted to pH 3.0 with 3 M acetic acid. The pooled solution was then loaded onto a C_{18} Sep-Pak cartridge, which was preconditioned for CKs. After that the Sep-Pak was washed with 4 ml 0.1 M acetic acid and 10% methanol in 0.1 M acetic acid, respectively. Then Z/ZR and i-Ado/i-Ade were eluted with 4 ml 30% and 80% methanol in 0.1 M acetic acid, respectively. Aliquots of this elution were also pipetted into small vials in triplicate. Then they were evaporated overnight in a vacuum concentrator. CK concentrations were also determined by RIA.

3) Quantification of hormones

Acidic hormone extraction; IAA, GAs were methylated with diazomethane before analysis. In all cases hormone analysis was quantified by radio-immunoassay with polyclonal antibodies. Antibodies used were raised in rabbits against free IAA, Z/ZR, i-Ado/i-Ade, and $GA_{1,3,20}$. Radio-immunological hormone qualification was performed according to Bohner and Bangerth (1988). Cross-reactions of the GA_3 antibody used were determined by Bertling and Bangerth (1995), to be about 90% with GA_1 and GA_{20}. Therefore, the GA determined by means of this antibody are expressed as GA_3 equivalents and called GAs in the following text.

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3.2.2.4 Carbohydrate analysis

Twelve leaves were sampled from the same leaves of leaf diffusate studies and used for carbohydrate analysis. The roots from five trees of each treatment were also sampled to determine carbohydrate content. TNC and RS were measured at Faculty of Agriculture, Chiang Mai University, Thailand.

Leaf and root samples were cleaned with distilled water and dried at 70°C for 72 h using hot air oven before grinded and kept in desiccators. Before analysis samples were dried again in oven at 70°C for 8 h, then acid extracted for TNC (Smith *et al.*, 1964 modified by Chaitrakulsup and Subhadrabandhu, 1983). RS was also measured using the ethanolic extraction method described by Yemm (1935), and Hodge and Hofreither (1962).

3.2.2.5 Statistical analysis

Statistical analysis of data was done by using Analysis of variance and paired samples T-Test from the SPSS version 11.5 for windows. Least significant difference (Lsd) was used to examine significant difference of means ($P<0.05$ and $P<0.01$).

In addition to bud and xylem sap, hormonal determination of the other materials, the tiny structure and small volume of materials and the very expensive procedures of plant hormone evaluation only three analytical replications but no biological replicates could be performed. Similar to bark, wood and leaf diffusate materials, five analytical replications were also interpreted.

3.3 Results

3.3.1 Effect of low temperature duration on flowering

Figure 3.6 showed the development of the litchi terminal bud after experience with low temperature in growth chambers. Plants grown under open air condition (warm treatment) developed young vegetative flushing (Figure 3.6 A) at only 29 day of the treatment (Table 3.1). In the opposite response, plants with cold treatment developed flower buds. An active swelling green terminal bud was observed even during low temperature treatment (Figure 3.6 B and Table 3.1) then the buds further developed to start flowering (Figure 3.6 C). At this stage, duration of

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low temperature treatments showed a clear effect on flowering habitat. Plants kept under 15/10°C for 38 days tended to start flowering earlier within 27 days of rising temperature period, and increased flowering up to 60% compared to plant grown under cold temperature for 28 days. Those plants kept cold only 28 days produced a few flowers of only 20% and required long period of warm climate to urge the flower bud development of up to 37 days (Table 3.1).

Table 3.1 Effects of temperatures on leaf flushing and flowering

(5 replications)

Parameters	Temperature		
	Low temperature treatment		Warm temperature
	28 days	38 days	treatment (control)
Days to start flushing (days)	0 b	0 b	29 a
Days to visible active bud (days)	18 b	21 a	0 c
Percentage of active buds (%)	60 a	100 a	0 b
Days to start flowering (days)	37 a	27 a	0 b
Percentage of flowering (%)	20 a	60 a	0 b

a, b means in the same row followed with the same letter does not significant difference at $\alpha = 0.05$ by Lsd.

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A. Vegetative flushing B. Active-swelling buds C. flowering

Figure 3.6 Stage of the vegetative flushing (A), active-swelling buds (B) and flowering (C) (Scale = 0.2 cm)

3.3.2 Physiological change during cold treatment

3.3.2.1 Diurnal photosynthetic activity

As shown Figure 3.7, measuring of diurnal photosynthetic rate was carried out every hour during 7:30-17:30 o'clock at day 24 of cold treatment compared to warm treatment. At warm temperature regime, *Fv/Fm* of litchi stayed relative constant during the day which ranged between 0.7-0.8. Diurnal trends of *Pn* and *PAR* values seemed to be bell-shape and paralleled together. *Pn* values increased from 3.0 μmol CO_2 m^{-2} s^{-1} at 7:30 o'clock to reach a maximum (7.5 μmol CO_2 m^{-2} s^{-1}) at 12:30 o'clock then decreased slowly after *PAR* declined in the afternoon. Even though *PAR* reached the maximum about 1,000 μmol m^{-2} s^{-1} at 11:30 o'clock, after that it slowed down. At low temperature condition, *Fv/Fm* was slightly lower than those of warm temperature, which ranged at 0.25-0.45, whereas *Pn* values were greatly lower than under warm temperature. It remained relative constant at 0.1-0.7 μmol CO_2 m^{-2} s^{-1}. Its trend seemed similar to *PAR* trend, even though its values were not shown significantly different. Therefore, *Pn* may response to *PAR*, whereas *Fv/Fm* as in term of plant efficiency could not predict exactly *Pn* values. That means not only low temperature affected *Fv/Fm* and *Pn* in leaves, but also *PAR*.

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From Figure 3.7, RH at warm temperature regime declined from 75 %RH at 7:30 o'clock to minimum value (37% RH) at 13:30 o'clock, and increased thereafter. In contrast, *Gs* and *Tr* values were changed in the opposite direction to RH. *Tr* values changed parallelly to *Gs* values as a bell shape. In the morning RH declined, while *Gs* and *Tr* values increased slowly. After that RH reached to minimum at 12:30 o'clock, whereas *Tr* and *Gs* values reached to maximum at 12:30 o'clock (2.01 mmol m^{-2} s^{-1}), and at 10:30 o'clock (87.5 mmol m^{-2} s^{-1}), respectively. At low temperature regime, RH stayed, under growth chamber condition, consistently during a day as compare to those of warm temperature. *Gs* values were also parallel line as *Tr* values, which their values reached the maximum values at 8:30 and 16:30 o'clock. Normally, stomata open whenever the weather has moderate air humidity and light intensity and close whenever less or excess of those values. Data in this experiment supported that information. Moreover, it may conclude that transpiration rate depend on stomatal conductance and air humidity, and low temperature also effects on reduction of transpiration rate and stomatal activity.

In the case of leaf temperature (Figure 3.7), litchi leaves kept their *Tl* values at around 22–35°C as same as ambient temperature in the opened field. In low temperature condition leaves were warmed up to arrive 20-27°C, even though ambient temperature was only 10-14°C.

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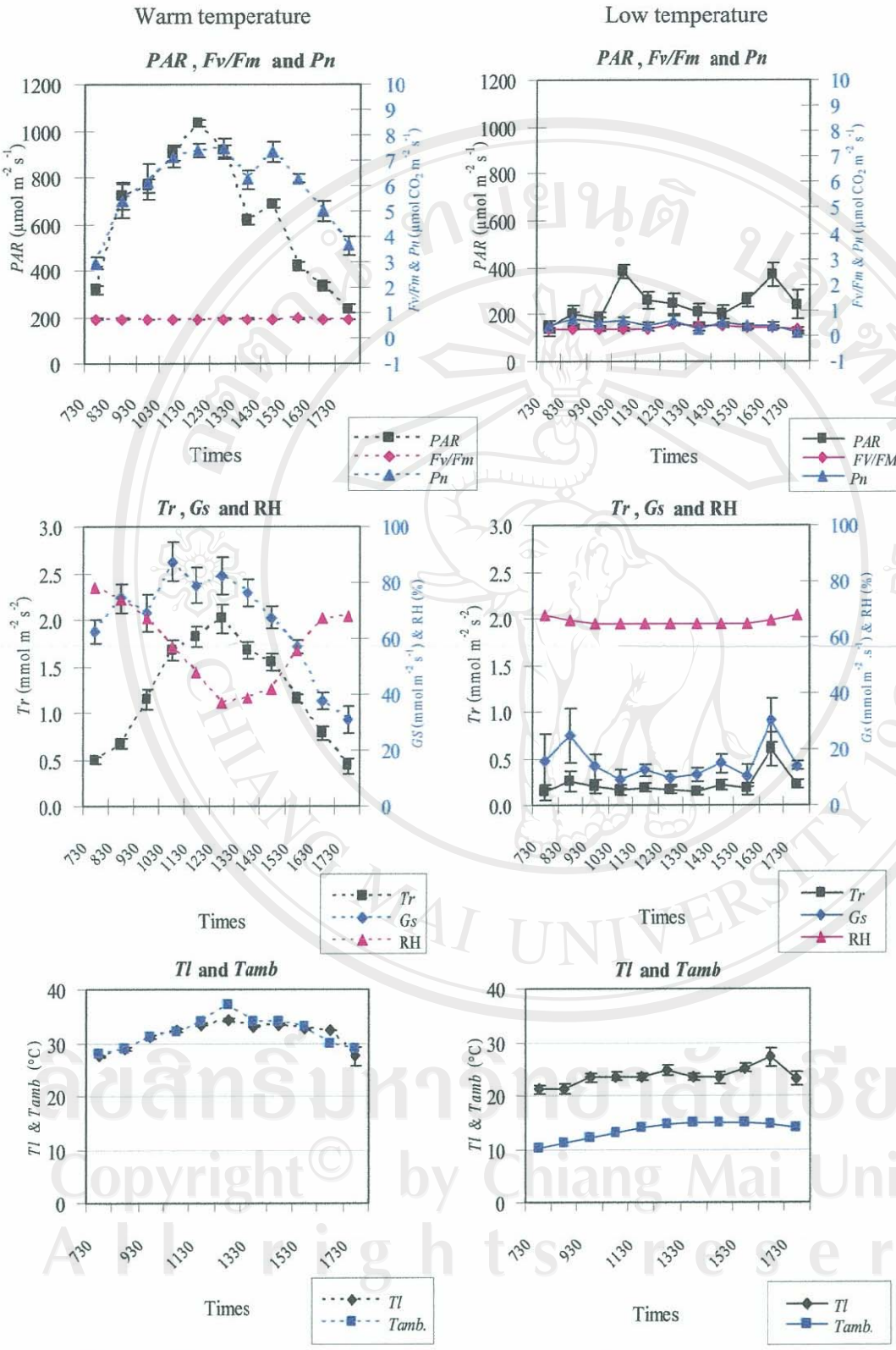

Figure 3.7 The diurnal photosynthetic apparatus of litchi leaves at warm and low temperature conditions at days 24 after treatments

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3.2.2.2 Photosynthesis, transpiration and stomatal movement

At warm temperature, *Fv/Fm* values were constant at range of 0.66-0.78 during day 7-24 of the cold treatment, whereas *Pn* values fluctuated at 5.0-8.1 μmol CO_2 m^{-2} s^{-1} (Figure 3.8). In contrast, *Fv/F*m of litchi trees held in low temperature declined from 0.62 at day 7 of cold treatment to 0.39 at day 24. Photosynthetic rate also drastically decreased during cold treatment from 0.5 μmol CO_2 m^{-2} s^{-1} to be less than 0.1 μmol CO_2 m^{-2} s^{-1} within 14 days, and remained constant low throughout the cold period.

From Figure 3.8, in litchi trees kept under warm temperature, *Gs* values tended to decline during day 7-14 of warm treatment then their values were stable from day 17 to day 24. *Tr* values also decreased during day 7-17 of warm treatment, after that they increase through day 24 of warm treatment whereas *Tl* values were consistent with 28.0-34.4°C. Litchi trees under low temperature had significant lower *Tr* and *Gs* values than those of warm temperature. However, reduction of *T*r depended on *Gs* as in litchi tree under warm temperature.

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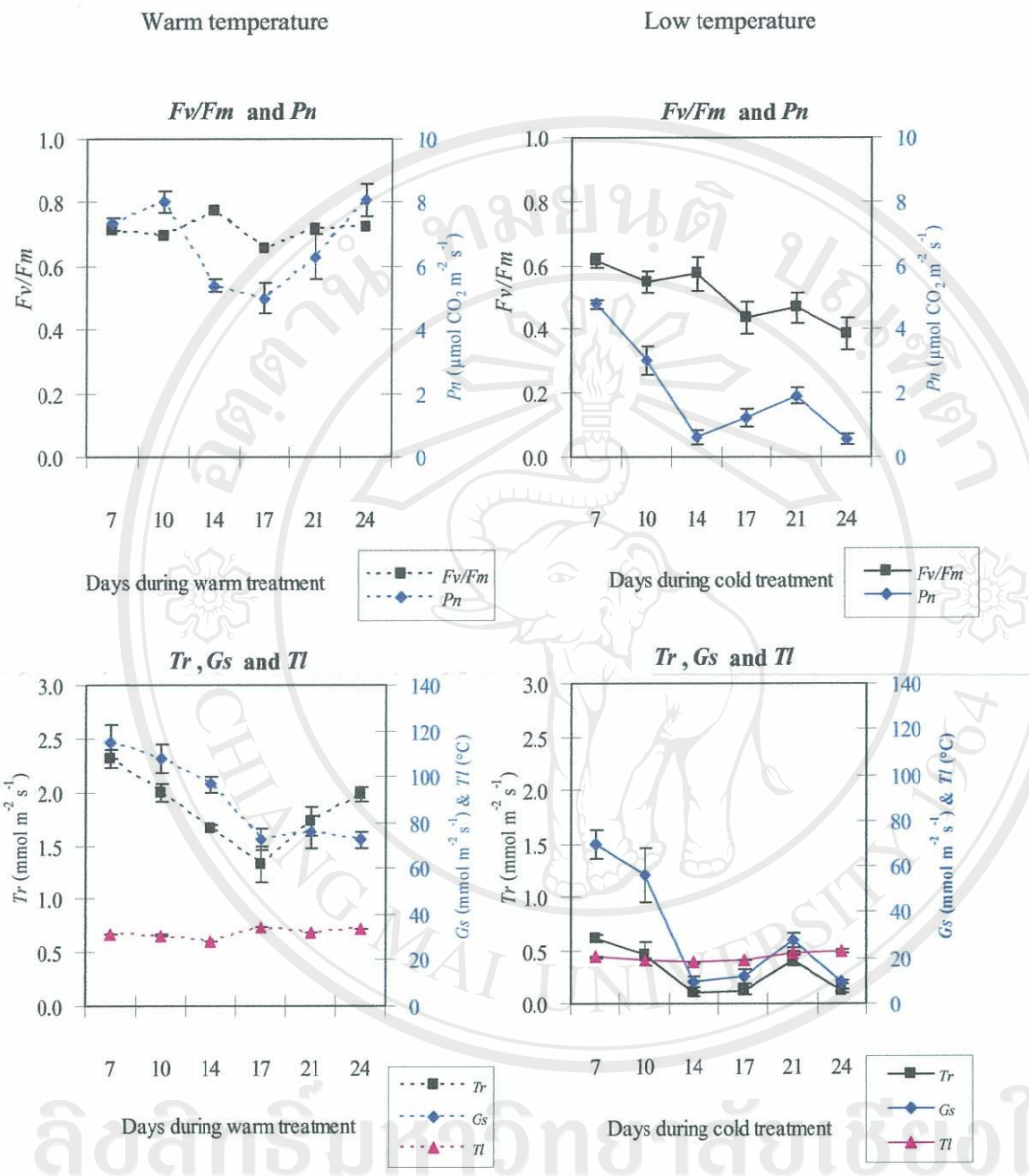

Figure 3.8 *Fv/Fm*, *Pn*, *Tr*, *Gs* and *Tl* of litchi leaves at warm and low temperature conditions during 24 days of treatments

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3.3.2.3 Assimilate distribution

As shown in Figure 3.9 amount of TNC (in form of mg glucose g^{-1} DW) accumulate in leaves tended to increase from days 7 to days 28 of cold treatment and also increased significantly under low temperature compared to warm temperature treatment, whereas trend of RS concentrations were similar in leaves treated with warm and low temperature. Under low temperature condition, TNC and RS in roots tended to increase slowly as same as warm temperature treatment. Therefore, TNC and RS in roots were not affected by cold treatment and remained relatively constant throughout the 28 days of cold period.

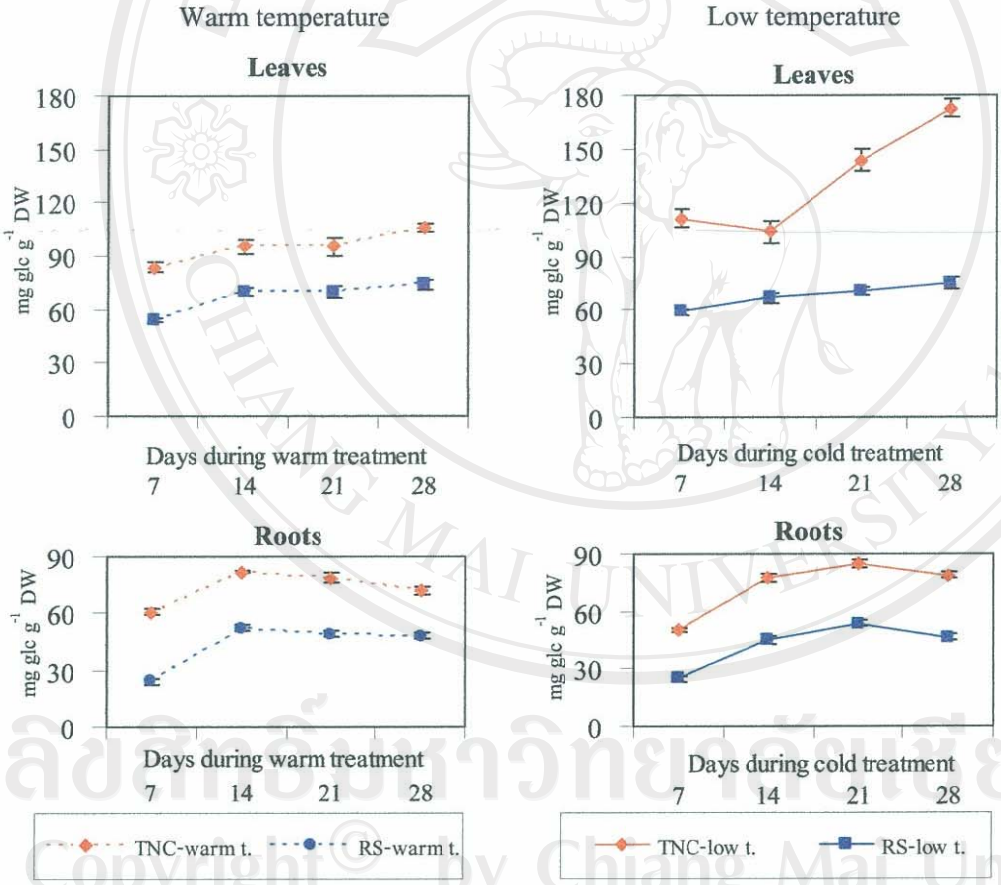

Figure 3.9 TNC and RS contents in leaves and roots of litchi plants grown under warm and low temperature conditions

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3.3.3 Change in hormonal content during cold treatment

This experiment, in general, the highest amounts of endogenous hormones were detected in terminal bud followed by in bark and wood, whereas only trace amount was found in xylem sap and leaf diffusate (Figure 3.10). In terminal buds of warm temperature treatment, Z/ZR was hormone with the highest concentrations of around 104.6-146.0 ng g^{-1} DW compared to i-Ado/ i-Ade (32.4-44.9 ng g^{-1} DW), IAA and GAs, by which the last two hormones were similar in amount of about 10-20 ng g^{-1} DW. Z/ZR was the hormone, which significantly strongest affected by low temperature. It decreased from average value at 120 ng g^{-1} DW in warm temperature to be only around 35 ng g^{-1} DW (29% decreasing) in low temperature regime. The same pattern of endogenous hormonal content and hormone reduction as affected by low temperature was also observed in wood, bark and xylem sap; where Z/ZR content was the highest and strongly reduced due to low temperature treatment. These effects of low temperature were already detected even within 7 days of the treatment.

In leaf diffusate, auxin (IAA) was found to be the major component diffused out of leaf. Its concentrations were 0.15 ng $leaf^{-1}$ on the 7 days of the warm treatment and increased drastically during the study warm period of 28 days. From field observation this dramatically increase was follow by new flushing. Therefore increase in GAs and CKs were also detected on day 28 of warm period. Low temperature treatment could decrease the amount of IAA in leaf diffusate. The amount of IAA content decreased from 0.38 ng $leaf^{-1}$ on day 21 of warm treatment to only 0.11 ng $leaf^{-1}$ (71% decreasing) on the same day of cold treatment.

From this study, it can be concluded that Z/ZR is the major endogenous plant growth regulator found in bud, wood and bark and strongly decreased under cold treatment. In leaf, auxin is however the major hormone with high content and as well as the strongest decreased through low temperature treatment.

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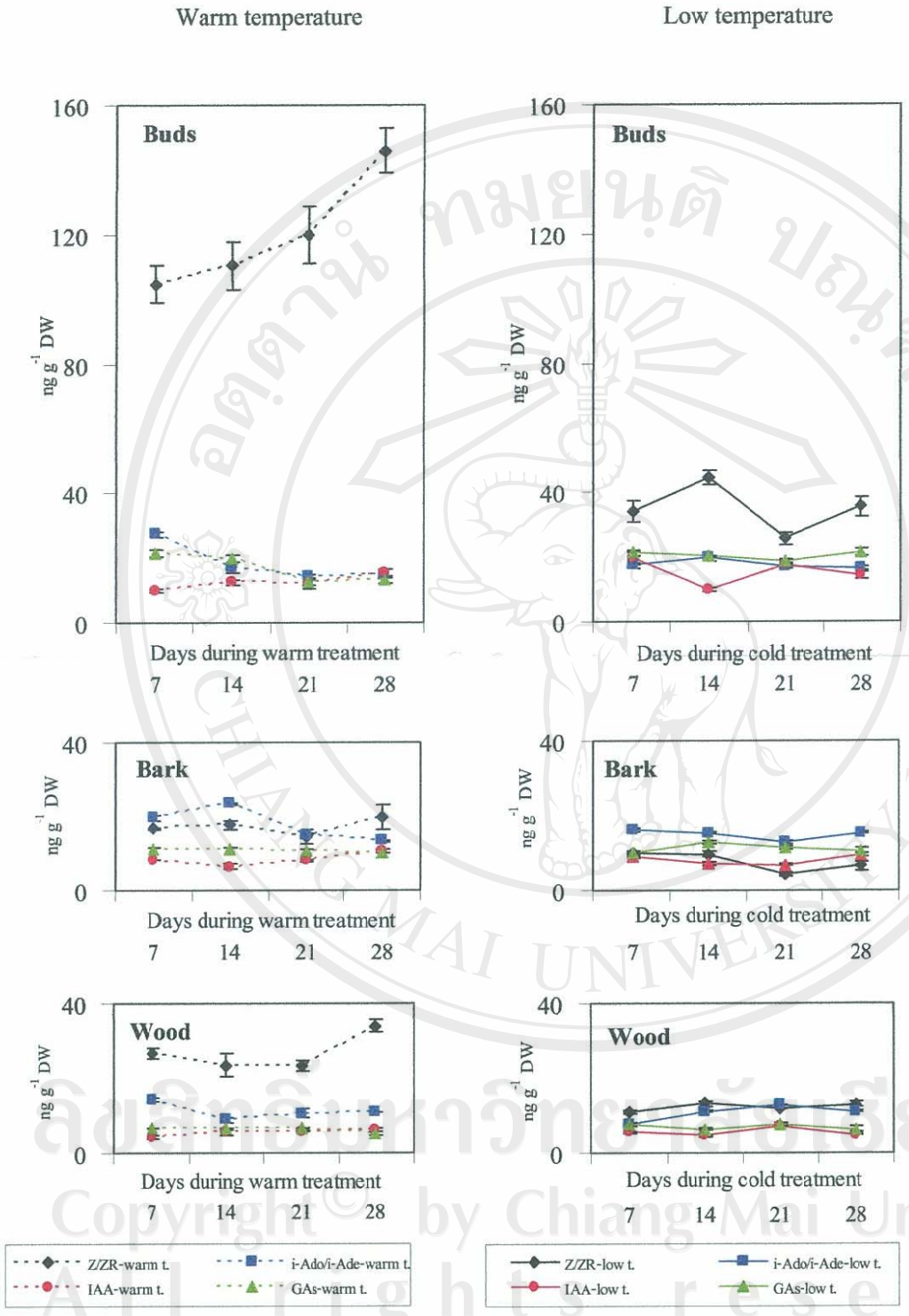

Figure 3.10 Hormonal concentrations in plant tissues at warm and low temperature conditions during the first month of treatments

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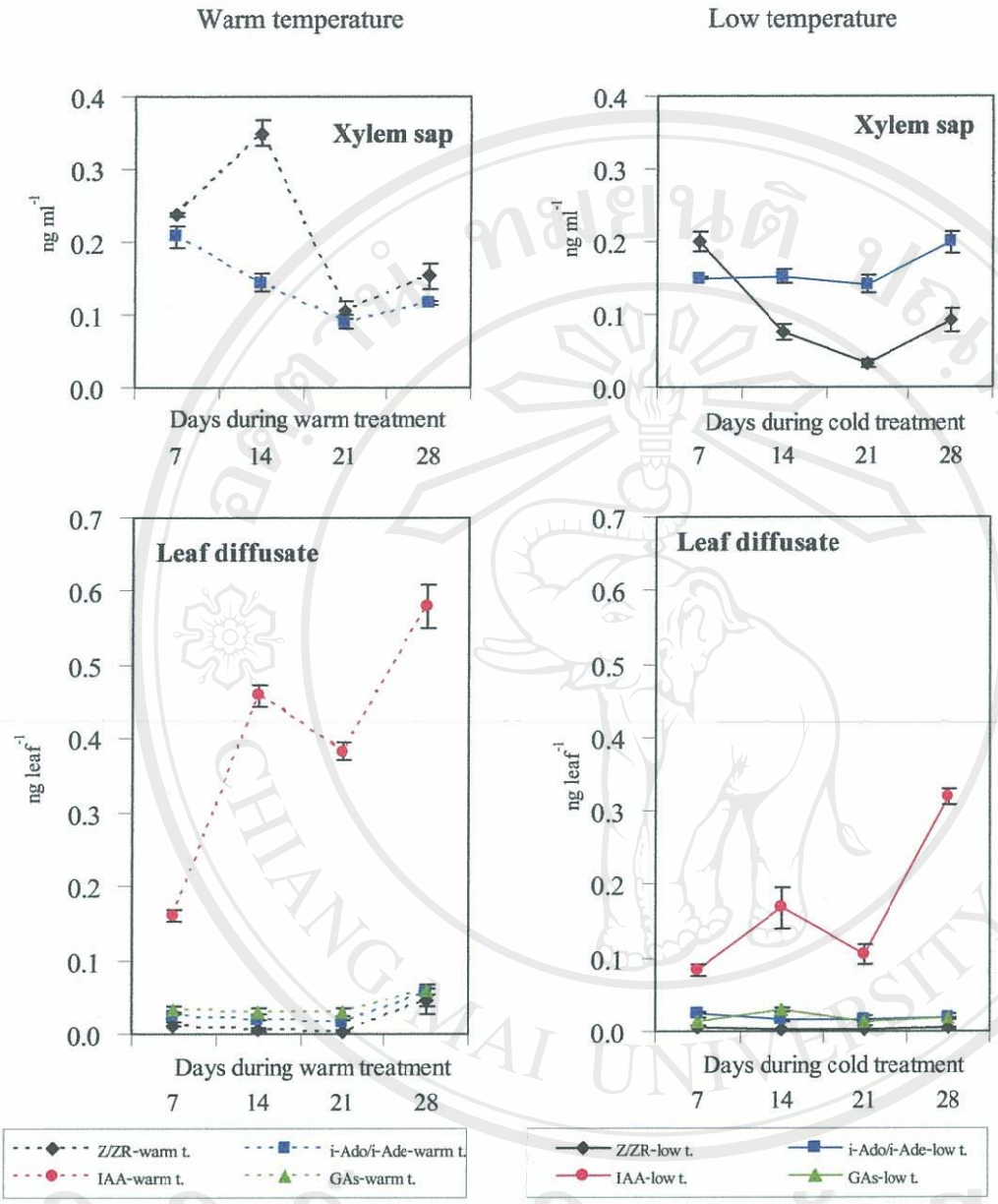

Figure 3.10 (Continued)

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3.4 Discussion

3.4.1 Effect of low temperature regime on flowering

Two major points could be concluded from this experiment, that firstly under warm temperature studied plants produced only new flushing. Secondly, longer cold period of 38 days promoted higher percentage active buds, fast developed young bud and also higher percentage of

flowering than a shorter cold period of 28 days. This means that effect of low temperature on flowering of litchi tree was relied on the duration of cold period treatment.

This result is confirmed the work of Menzel and Simpson (1995), which showed that 'Wai Chee' litchi, kept at 15°C for 4 and 10 weeks produced partial (22%) and full (90%) flowering, respectively. Menzel and Simpson (1988) also reported that 7 cultivars of litchi trees grown under an optimum temperature regime of 15/10°C (day/night temperature) showed 100% of terminal branches flowering and induction, floral initiation and panicles emerged within only 4 weeks whereas those with 20/15°C day/night temperature showed panicles emerge after 6 weeks. Batten and McConchie (1995) also reported that floral induction occurred relatively quickly in 'Salathiel' litchi, floral initials were visible within only 39 days after transfer to low temperature.

In addition to floral inducing, in generally, litchi trees require lower temperature and longer cool period than mango and longan trees. Whenever temperature in winter is not cool enough for litchi to enhance flowering, mango and longan trees flower. Similarly, Whiley *et al.* (1989) reported that temperature below 20°C enhanced floral induction in mango, whereas longan trees require 15-22°C in winter season for 2-3 months (Wong and Ketsa, 1991). Litchi trees produce vegetative shoots rather than flowers when winter maximums are above 25°C or minimum above 20°C (Menzel and Simpson, 1994). Furthermore, at least 3 weeks of low temperatures is the best condition for flowering (Menzel, 2002b). According to duration of low temperature, mango requires around 15°C for 30 days, whereas litchi need 39 days of transfer to low temperature (Batten and McConchie, 1995).

3.4.2 Physiological response of litchi plant to low temperature regime

In this chapter preliminary studies on the effect of low temperature were carried out to use as the basic information for the next experiments. It was found generally that diurnal change of *Pn*, *Tr* and *Gs* were on bell shape; increase the morning to arrived the peak at noon time and decreased steadily in the afternoon. Low temperature to 15°C decreased all the values of *Pn*, *Tr* and *Gs*; which remained relatively low and without diurnal change the whole daytime (Figure 3.7).

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In Figure 3.8, responses of plant to cold duration were also shown. Reduction in *Pn*, *Tr* and *Gs* were already detected within 10 days and arrived the lowest value within 14 days. However, based on the curve trend, which decreased drastically from day 7 of cold treatment, it might be expected that plant responded to low temperature much earlier than 10 days even within 7 days of cold treatment. These results also supported the finding of several physiologists which reported the low temperature limited the photosynthetic productivity of plant species such as in mango and subtropical woody species (Schaffer and Anderson, 1994; Nir *et al.*, 1997; Allen *et al.*, 2000; Stitt and Hurry, 2002; Hendrickson *et al.* 2004).

According to the effect of low temperature, firstly changes may be on altering of enzymes activity. Normally, most enzymes have their greatest activity somewhere 25°C and 37°C corresponding to the temperature of most cellular environment (Davies and Littlewood, 1979). For chemical reaction, it will be taking place first at a temperature of T° and then at T+ 10° as called "Q_{10}". Whenever temperature decreases 10°C the rate of a chemical reaction is depleted such as Q_{10} value of hydrolysis of sucrose, which is catalyzed by invertase is about 1.5 (Yudkin and Offord, 1973). In this experiment photosynthesis may be affected by low temperatures as described in above including enzymatic steps of electron transport chain in chloroplast thylakoids (Brüggemann and Dauborn, 1993), coupling to photoshosphorylation, enzymes in the carbon reduction cycle (Byrd *et al.*, 1995; Kingston-Smith *et al.*, 1997; Stitt and Hurry, 2002) and transport mechanisms of the photosynthetic products from the chloroplasts (Öquist, 1983).

Furthermore, Van Heerden *et al.* (2003) reported that the inhibition of photosynthetic rate was characterized by a simultaneous decrease in stomatal conductance and intercellular CO_2 concentration in 'Maple Arrow' soybean. It was concluded that dark chilling-induced limitation on photosynthesis were predominantly due to metabolic restriction rather than to direct effects on electron transport reactions and that stromal fructose-1,6-bis phosphatase was particularly susceptible to dark chilling. In this experiment, decrease in photosynthetic rate under low temperature may be the concurrent activities response to low temperature both by decrease the stomatal aperture and deplete in enzymatic activities.

Even though a low *Pn* was observed, leaves of cold treated plants, however, contained higher TNC than the warm treated ones. Although no effect on RS was measured. This litchi

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result was similar to the finding of Thonglem (2000), which TNC in stem apex of "Hong Huay" litchi (during November 1999-January 2000) was high in the 8th week before flowering and decreased in the 6th week prior to flowering and increased again in the 4th week prior to flowering. In this study plants flowered within 27-37 days after cold treatment.

Generally, plants produce assimilate through photosynthesis by leaf and green tissue and translocate to accumulate in sink organs, e.g. young leaf, root, stem and branch. Success of the activities relies on many factors such as genetics and environmental factors. Normally, carbohydrates are important in the growth of woody plants and account for over 65% of dry matter in tree crops (Kozlowski and Keller, 1966). Menzel *et al.* (1995) reported clearly that starch reserves about half of litchi tree were found in small and medium branches, which were used for new leaf and fruit growth. Temperature is one of the important factors influencing assimilate production and distribution through affect on enzyme activity and solute transporting efficiency. According to the effect of temperature on phytochemical substances in plants, low temperatures induce many other biochemical changes in addition to increased soluble carbohydrate levels (Farrarrr and Gunn, 1996; Strand *et al.*, 1997). On the other hand, it may be because the low temperature inhibited in night-time mobilization of leaf starch (Paul *et al.*, 1992). Therefore, during low temperature, increasing in leaf TNC may occur even though photosynthesis decrease.

How low temperature decreased the *Pn*, *Tr*, *Gs* of litchi tree, is not yet clearly understanding. However a so called "Feedback system photosynthetic rate inhibition" (Brüggemann, *et al.*, 1992; Sun *et al.*, 1999) phenomena may be included as one of the main reason. A low mobilizing rate of sucrose out of leaf as affected by low temperature (Brüggemann *et al.*, 1992; Farrarrr and Gunn, 1996; Sun *et al.*, 1999) caused an accumulation of photosynthetic-produced carbohydrate in leaf tissue, especially in palisade cell. Starch granule increased, which it is proposed that large starch grains could damage chloroplast or restrict CO_2 diffusion (Iglesias *et al.*, 2002), so that water potential decrease. Similar to a phenomena as in rice seeds, when they accumulate starch granule from rice milk (Sultana *et al.*, 1999). Furthermore, Brüggemann *et al.* (1992) reported that starch formation in tomato plant was impaired during chilling at 6°C, soluble sugar contents increase and reached to 8 fold the values found in unchilled plants within 2 weeks. It was suggested that the feedback inhibition by

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internal sugar accumulation, the low activity of Rubisco can play role in the strong decrease of photosynthetic capacity during long-term chilling in tomato as same as in wheat (Savitch *et al.*, 1997) and citrus (Iglesias *et al.*, 2002). As concurrent, stomata closed due to less turgidity, *Gs* decrease followed by a reduction in *Tr* and *Pn*. A negative effect of low temperature on enzyme activity in leaf can be excluded, since the leaves kept their temperature as optimum of 20-25°C even under low temperature treatment.

3.4.3. Response of litchi plant to low temperature at the hormone level

Two endogenous plant growth regulators are supposed to be affected by low temperature treatment; namely cytokinin (especially Z/ZR) and auxin (IAA), by which level of both hormones decreased significantly. O'Hare (2004) reported in litchi, that cold root treatment reduced root growth and CKs production, so that a reduction in CK/ABA ratio was observed. In this experiment a reduction in Z/ZR was also detected in wood and xylem sap. This confirmed the above mentioned finding of O'Hare (2004). Furthermore, Itai and Vaddia (1965) also reported earlier that decrease in cytokinin from root exudates due to drought stress of sunflower may be resulted from less synthesis from root or from transport inhibition from root. Zhu *et al.* (2004) additionally reported that breakdown of cytokinin increased under drought stress and drought with paclobutrazol.

The result of this experiment confirmed the studies of Chen (1991) and Naphrom *et al.* (2001). Naphrom *et al.* (2001) reported that low CK-like concentrations were found during 6-8 weeks prior to flowering of 'Hong Huay' litchi trees grown in Doi Pui orchard, Chiang Mai. The concentration of CK-like substance increased at four weeks to reach a maximum two weeks prior to flowering.

Naphrom *et al.* (2004) reported a contradictory effect of cold temperature on hormonal level in mango cv. Tommy Atkins. A cold temperature of 13°C increased Z/ZR level in terminal buds, bark and wood compared to mango trees grown under 25°C. This measurement was conducted during 13-29 days of cold treatment, whereas the mango plants started flowering at 85-90 days after cold treatment. This contrast results reminded the necessity to repeat this study on litchi cold temperature treatment. A different response of different studied plant species;

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mango and litchi, is also cannot yet be ruled out. The relation between hormonal level and stage of bud development may also another factor to be studied.

In the case of auxin, it was found that leaves treated with low temperature (15/10°C) diffused out less amount of IAA compared to these treated with high temperature (35/27°C), even when kept for diffusation under the same conditions of 25°C and 100% RH. The difference in IAA concentrations in leaves of both treatments at the time of detach from plants was the major reason. Looking into the environmental condition of plant growth in this experiment, it must be declared that plants were treated with different temperature and light regime. Plants in growth chambers received the temperature of 15/10°C regime and light intensity (26%), *PAR* (260 μmol m^{-2} s^{-1}), whereas outside received 35/27°C temperature regime and light intensity (maximum *PAR* of around 1,000 μmol m^{-2} s^{-1}). Although it is still too early to discussed on the effect of warm temperature and low temperature on IAA content in leaf diffusate, but both of the environmental factors seem to play an important role on auxin level in leaves and a better response of full sunny shoot of longan to $KClO_3$ (Sruamsiri *et al.*, 2003).

3.5 Conclusion

A preliminary study on effect of low temperature treatment on flowering, physiological and biochemical change in litchi showed that:

1) A long, steady low temperature regime (15/10°C) of 38 days was more effective to promote flowering compared to 28 days of cold duration.

2) Low temperature reduced diurnal change of photosynthetic rate, transpiration rate and stomatal conductance as well as chlorophyll fluorescence. In these parameters, plants showed response as fast as 7 days after treatments.

3) Low temperatures decrease Z/ZR level in terminal buds, bark, wood and xylem sap. A reduction in IAA level in leaf diffusate was also observed. Less effects of cold temperature on i-Ado/i-Ade and GAs were confirmed.

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