Kasetsart J. (Nat. Sci.) 45 : 189 - 200 (2011)

Paclobutrazol, Water Stress and Nitrogen Induced Flowering in 'Khao Nam Phueng' Pummelo

Thiwaporn Phadung[1,2], Krisana Krisanapook[1]* and Lop Phavaphutanon[1]

ABSTRACT

Thailand can export pummelo for only a short period from August to September each year. However, the demand is increasing and lasts almost all year round. Hence, success in controlling the flowering time will expand the export window. In this study, flower induction of 2-year-old pummelo trees cv. Khao Nam Phueng was investigated using five treatments: T1) daily watering (control), T2) water stress by withholding water for 12 d in December, T3) spraying with 750 mg/L paclobutrazol (PBZ) twice at an interval of 15 d, T4) spraying with 750 mg/L PBZ as in T3 combined with withholding water for 12 d and T5) urea fertilizer (46-0-0) application 4 times every 2 wk at the rate of 25 g/tree before the experiment started combined with withholding water for 12 d. The results showed that with the water stress treatments, the soil moisture in the root zone decreased from 28 to 14% and the leaf water potential (LWP) decreased from -0.66 to -2.17 MPa at the end of water stress and returned to normal after rewatering. PBZ application was highly effective in reducing shoot length and could reduce the effect of water stress by maintaining LWP. Urea application combined with water stress could induce early flowering 1 wk after rewatering, while the control trees did not flower. The water stressed trees had the greatest amount of inflorescence. The inflorescence types were classified into four types and most inflorescence had many flowers with leafy shoots. Water stress and PBZ increased the leaf total non-structural carbohydrates (TNC) and the C/N ratio at the end of water stress, which then slightly decreased until the flushing period, while total nitrogen (TN) decreased and tended to increase until flowering. The results indicated that trees treated with PBZ had comparable flowering and TNC and C/N ratio values to the water stressed trees, while urea applied before water stress stimulated early flowering. Therefore, the application of PBZ and urea combined with water stress to induce flowering of pummelo trees may be a useful development for commercial production.

Keywords: Khao Nam Phueng pummelo, flowering, C/N ratio, water stress, paclobutrazol

INTRODUCTION

Thailand has been producing pummelo in great quantities and exports are tending to increase every year (Office of Agricultural Economics, 2009). In 2009, 12,000 metric ton of pummelo was exported, with a value of 100 million baht. The major pummelo-importing countries are China, Hong Kong, Singapore, the Netherlands and Canada (Thai Customs

[1] Department of Horticulture, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University, Nakhon Pathom 73140, Thailand.
2 Present address: Agricultural Production Sciences Research and Development Office, Department of Agriculture, Ministry of Agriculture and Cooperatives, Bangkok 10900, Thailand.
* Corresponding author, e-mail: krisana.k@ku.ac.th

Received date : 23/06/10 Accepted date : 19/11/10

Department, 2009). The well known commercial cultivars are Thong Dee and Khao Nam Phueng .

Flowering is an important step in fruit production. The price of fruit depends on market demand, so therefore, induction of flowering at certain times is necessary. In the central part of Thailand, flowering induction of pummelo begins in late November to December and anthesis normally occurs in January with the peak harvest in August and September (Somsri and Vichitrananda, 2007). It is known that the flowering process is controlled by both internal and external factors such as carbohydrate, hormones, temperature, water stress and nutrition (Albrigo and Sauco, 2004). Among these factors, carbohydrate is one of the internal factors that affects flowering in citrus species since it is necessary for growth, regulation of the plant metabolism and development (Goldschmidt, 1999). Ito *et al.* (2004) reported that a large amount of carbohydrates was consumed during flower initiation and the consequent development of floral organs. Goldschmidt (1999) also reported that reserved carbohydrates were utilized in the early stages of reproductive development. Thus, it is likely that a sufficient level of carbohydrate is a prerequisite for flower bud differentiation in citrus.

In addition, nitrogen plays an important role in flowering because high concentrations of carbohydrates accumulated with low nitrogen cannot promote flowering. In peach, high flowering intensity was characterized by a high concentration of ammomium-nitrogen and glucose (González-Rossia *et al.*, 2008). Optimizing nitrogen in citrus trees is necessary to regulate vegetative growth and to promote flower induction and bud differentiation, as well as increase fruit set (Menino *et al.*, 2003). Davies and Albrigo (1994) suggested that maintaining leaf nitrogen in the optimum range (2.5˘2.7%) resulted in a moderate number of flowers and produced the greatest fruit set and yield, while a high level of leaf nitrogen induced excess vigor and vegetative growth rather than flowering. Moreover, ammonium nitrogen may directly affect flowering via regulation of ammonium and polyamine levels in the bud, which play a role in the meristematic activity involved in flower bud differentiation (Lovatt *et al.*, 1988). González-Rossia *et al.* (2008) reported that high ammonium-nitrogen content correlated well with the flowering intensity in peach. Furthermore, the influences of carbohydrate and nitrogen on citrus flowering have been shown in several reports and the results differed depending on the species, growth conditions or cultural practices (Garcia-Luis *et al.*, 1995). Although both carbohydrates and nitrogen are essential, the limitations of carbohydrates and nitrogen on pummelo flowering are still not clear. Therefore, understanding the role of carbohydrates and nitrogen is beneficial in controlling pummelo production to meet the market demand.

Besides internal factors, citrus crops could be induced to flower by environmental factors and cultural practices (Albrigo and Sauco, 2004). In Thailand, withholding water for a short period followed by rewatering heavily is a common practice to regulate pummelo flowering in the dry season (Somsri and Vichitrananda, 2007). There have been some reports that water stress affected flower bud formation in citrus (Southwick and Davenport, 1986; Albrigo and Sauco, 2004). Water stress stopped vegetative growth, resulting in carbohydrate accumulation in trees (Subhadrabandhu *et al.*, 1997). In neck orange, Chuchird (2004) found that water stress produced higher foliar carbohydrate and a higher C/N ratio when compared to sufficiently watered plants. Thus, water stress may enhance the C/N ratio and induce flowering of citrus including pummelo, as reported for other fruit trees. (Lovatt *et al.*, 1988). If an enhanced C/N ratio via water stress is an important requirement for flowering of pummelo, an increase in the plant nitrogen status by over-application of nitrogen fertilizer will inhibit or delay flowering. However, an application of urea during the stress period to Washington navel orange trees increased the number of flowers per tree (Davies and Albrigo, 1994), whereas foliar application of KNO_3 stimulated flowering of mango (Yeshitela *et al*., 2004). It is possible that KNO_3 increased cell division and enlargement in the meristematic zone (Protacio, 2000).

Although, water stress is effective in pummelo flower induction, severe water stress may also cause more leaf fall, less flowering and reduce tree productivity (Mataa *et al*., 1998). Therefore, other means that can induce flowering should be considered. In recent years, paclobutrazol (PBZ) has been used with considerable success to induce flowering in several fruit crops such as apple (Zhu *et al*., 2004), mango (Phavaphutanon *et al*., 2000) kumquat (Iwahori and Tominaga, 1986) and citrus (Yamashita *et al*. (1997). PBZ inhibits gibberellin biosynthesis (Sterrett, 1985), reduces vegetative growth and induces water-stress tolerance (Chaney, 2005) as well as increases total non-structural carbohydrates (TNC) (Subhadrabandhu *et al*., 1997; Yeshitela *et al*., 2004). However, there is still no report on using PBZ in pummelo for flower induction. Therefore, this study aimed to investigate the effect of water stress, PBZ and nitrogen application on flowering, TNC, TN and the C/N ratio in Khao Nam Phueng pummelo.

MATERIALS AND METHODS

Plant materials and treatments

Fifteen 2-year-old Khao Nam Phueng pummelo plants propagated by air-layering from mature pummelo trees were planted in 80 cm diameter pots filled with a mixture of sand, coir dust, rice hulls, rice hull charcoal and manure (1:1:1:1:1 by volume) and applied with 10 g/ tree of 15-15-15 controlled release fertilizer every 2 wk. The pummelo trees were grown outdoors and maintained under a uniform culturing schedule throughout the experimental period. The experiment was arranged in a completely randomized design with five treatments: T1) water to container capacity at 24 L/ day during the experimental period (control); T2) withhold water for 12 d (water stress) in mid December 2008, then rewater to container capacity at 24 L/d; T3) spray with 750 mg/L of PBZ twice at 15 d intervals (early-mid December 2008) and water as in T1; T4) spray with PBZ as T3 combined with water stress; and T5) applications of 100 g urea nitrogen (46% N) which were separated into four doses at the rate of 25 g/tree every 2 wk before withholding water. All treatments were carried out in triplicate.

Leaf water potential and soil moisture

The leaf water potential (LWP) was measured using a pressure chamber (Soilmoisture Equipment Corp., USA). Two fully expanded leaves at the fourth position around the canopy of each experimental tree were sampled. LWP was measured between 6:00 and 7.30 a.m. one day before water stress, the day at the end of water stress, 1 wk after the rewatering, and during the flushing period. Soil moisture was determined using a gravimetric method and soil samples were taken on the same dates as the LWP measurements.

Vegetative growth and reproductive growth

After rewatering, the number of new shoots and their lengths and numbers of inflorescence were monitored. The flowering period was recorded from when inflorescence emerged until the end of flowering. Inflorescence types were classified into five main types according to Moss (1969). The number of inflorescence per tree and number of flowers per inflorescence were counted at 15 d after rewatering until the end of flowering and expressed as an average.

Carbohydrate and nitrogen concentration

Leaf samples from each tree were

collected from the fourth to the sixth fully expanded leaves one day before water stress, the day at the end of water stress, 1 wk after the rewatering, and during the flushing period. Leaf samples were dried at 65 °C for 72 h and then ground through a 40 mesh Wiley mill. TNC was determined using acid extraction following the method described by Smith *et al.* (1964) and Nelson s reducing sugar procedure (Hodge and Hofreiter, 1962). Leaf total N concentration (TN) was measured by a combustion method using a Nitrogen Determinator (FP-528 Leco Crop. USA). The C/N ratio was calculated from TNC and TN in the same period.

Statistical analysis

The data were subjected to analysis of variance and the means were separated by Duncan s multiple range test (DMRT) at $P < 0.05$.

RESULTS

Soil moisture and leaf water potential (LWP)

The average soil moisture in the non-water stressed trees (control and PBZ) remained high (around 26˘31%) throughout the experimental period, while the average soil moisture in the trees under water stress treatments decreased to 14% at the end of water stress and returned quickly to the original level after rewatering (Figure 1).

The change in LWP before and at the end of water stress was a consequence of the change of soil moisture. The LWP in the water stressed trees and in the trees receiving the urea application combined with water stress dropped sharply to -2.09 and -2.17 MPa, respectively, at the end of water stress and returned to normal after rewatering, whereas the LWP in non-water stressed trees (control and PBZ) remained high at -0.5 MPa throughout the experiment. Meanwhile, LWP in

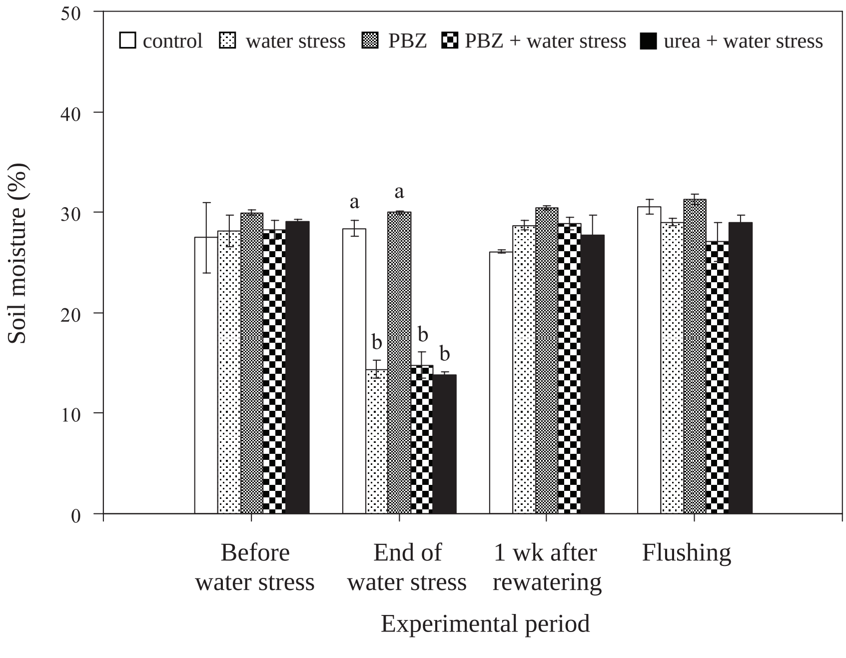

Figure 1 Changes in soil moisture during the experimental period. There is no significant difference ($P < 0.05$) between columns in a treatment having the same letter. Vertical bars represent the SD.

the trees receiving the PBZ application combined with water stress was not statistically different from non-water stressed trees (Figure 2).

Vegetative growth and reproductive growth

The number of shoots on the pummelo trees at 6 wk after rewatering was not different among treatments; however, a difference in shoot length was found. The water stressed trees produced the longest shoot length (23.23 cm) but it was not different from the control trees and the trees receiving the urea application combined with water stress, while the PBZ application showed its effect by decreasing shoot length significantly (Table 1).

For reproductive growth, the control trees did not flower and still grew vegetatively until the end of experiment. After rewatering, the trees receiving the urea application combined with water stress flowered about 1 wk earlier than other treatments. In this study, four types of inflorescence were found in Khao Nam Phueng pummelo: 1) many flowers with leafy shoots, 2) many flowers with a few leaves, 3) one flower with a leafy shoot and 4) many flowers with leafless shoots (Figure 3). The most common inflorescence type was many flowers with leafy shoots (Figure 3A). There were 15.7 inflorescence/tree in the water stress treatment whereas the other inflorescence types had between 0.3 and 2.0 inflorescence/tree. The number of inflorescence per tree and number of flowers per inflorescence were not significantly different among all treatments. Nevertheless, the water stressed trees still produced the highest amount of inflorescence while the trees receiving the PBZ application combined with water stress produced the highest number of flowers per inflorescence (Table 1).

Carbohydrate and nitrogen concentration

The TNC in leaves of Khao Nam Pheung pummelo was significantly different before and at the end of water stress but it was not significantly different among treatments after the

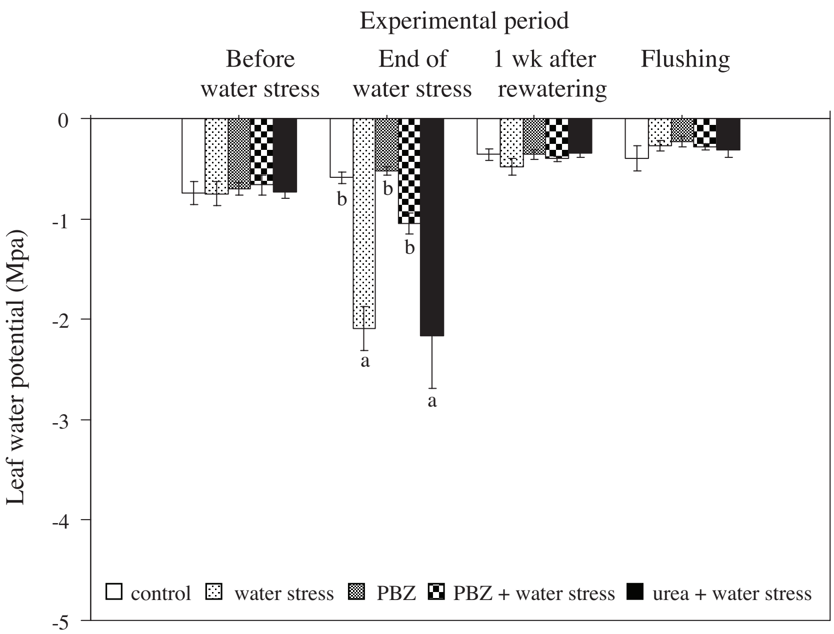

Figure 2 Changes in leaf water potential (LWP) of Khao Nam Phueng pummelo during the experimental period. There is no significant difference (P < 0.05) between columns in a treatment having the same letter. Vertical bars represent the SD.

Table 1 Effects of PBZ, water stress and urea application on vegetative growth and reproductive growth of Khao Nam Phueng pummelo after rewatering.

Treatment	Vegetative growth		Reproductive growth		
	Shoot number	Shoot Length (cm)	Flowering period (day)	Number of inflorescence per tree	Number of flowers per inflorescence
Control	25.0±4.6	20.6±2.6^{a}	-[1/]	-[1/]	-[1/]
Water stress	26.7±7.9	23.2±3.8^{a}	13.2±2.4	18.0±5.2	7.5±1.9
PBZ	11.0±5.6	10.7±4.2^{b}	15.7±4.9	7.7±2.3	5.5±1.2
PBZ ± water stress	23.0±6.6	8.8±1.0^{b}	15.5±0.7	11.3±3.8	8.1±3.6
Urea ± water stress	31.7±8.5	18.9±0.4^{a}	7.9±0.7	13.7±4.1	6.3±1.6
F-test	ns	**	ns	ns	ns

[1/] The control treatment did not flower and was not included in the statistical analysis.
Values are shown as mean ± SD; n=3.
Means within the same column followed by the same superscript letter are not significantly different at the 5% level by DMRT.
Flowering period was recorded when inflorescence emerged until the end of flowering.
ns = not significantly different.
** = significantly different at $p < 0.01$.

Figure 3 Inflorescence types of Khao Nam Phueng pummelo found in this study: A) many flowers with leafy shoots; B) many flowers with a few leaves; C) one flower with leafy shoots; and D) many flowers with leafless shoots.

rewatering and flushing periods. An increase in the TNC was found in leaves at the end of the withholding water period, especially in the water stressed trees. The TNC rapidly increased from 92.19 to 134.30 mg/ g dry weight. Afterwards, the TNC began to decrease after rewatering until the flushing period, whereas the TNC in the control trees decreased slightly from the beginning until the end of experiment. In addition, the treatment involving the urea application combined with water stress gave the lowest leaf TNC throughout the experiment when compared with other treatments (Figure 4A).

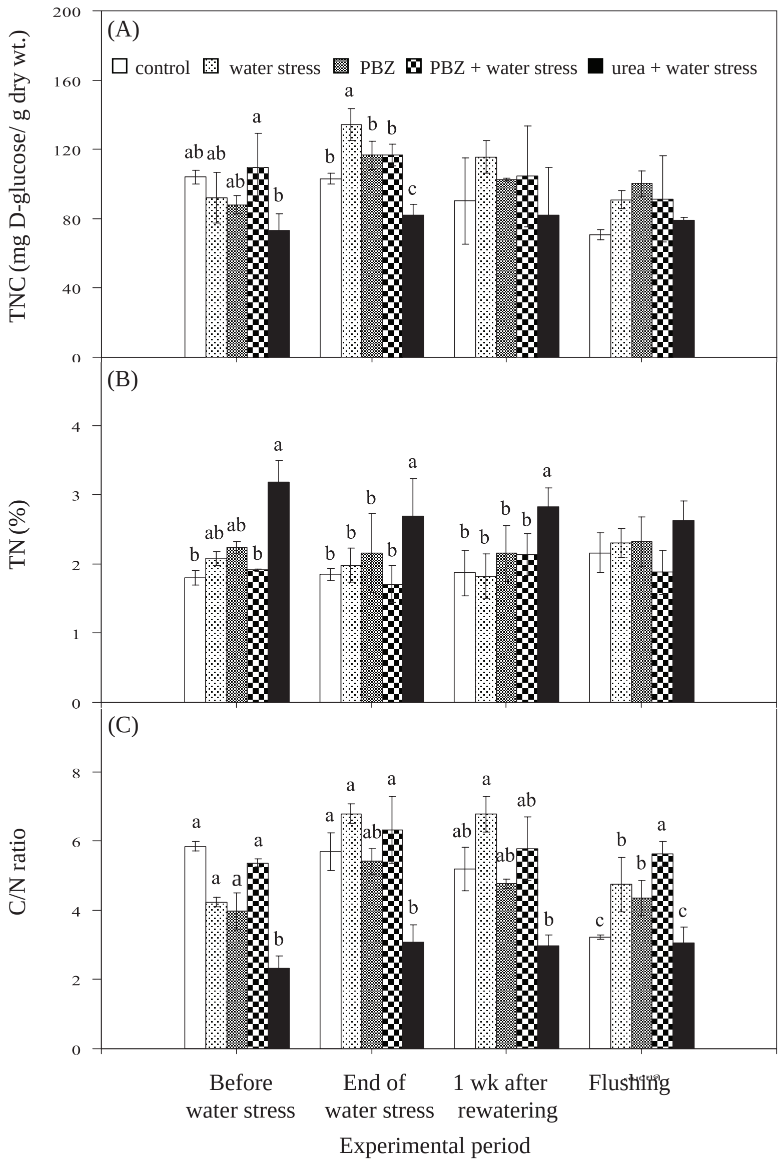

Figure 4 Changes in: A) total non-structural carbohydrate (TNC); B) total nitrogen (TN); and C) C/N ratio in leaves of Khao Nam Phueng pummelo during the experimental period. There is no significant difference (P < 0.05) between columns in a treatment having the same letter. Vertical bars represent the SD.

The pattern of TN in leaves was rather stable except in the treatment combining the urea application with water stress that produced the highest leaf TN during the experimental period when compared with other treatments. In the two treatments of water stress and PBZ applied alone, TN decreased slightly from the beginning until after rewatering and tended to increase in the flushing period. In the two treatments of PBZ applied alone and of the urea application combined with water stress, TN decreased from the beginning to the end of the water stress period and increased after rewatering, then gradually decreased again during the flushing period, whereas TN in the control trees increased slightly from the beginning until the end of experiment (Figure 4B).

The pattern of C/N ratio variation was similar to the change in the TNC in leaves. There were significant differences in the C/N ratio throughout the experiment. An increase in the C/N ratio in all treated trees was found in leaves just before rewatering and the water stressed trees had the highest C/N ratio (6.78) but it was not significantly different from trees in the treatment involving the PBZ application combined with water stresses. In contrast, trees treated with the urea application combined with water stress had the lowest C/N ratio when compared with other treatments; then the C/N ratio began to decrease after rewatering until the flushing period. The C/N ratio in the control trees decreased slightly from the beginning until the end of experiment (Figure 4C).

DISCUSSION

Application of water stress, PBZ and urea fertilizer affected vegetative growth and reproductive growth of Khao Nam Phueng pummelo trees. In water stressed trees, decreasing the soil moisture led to a decrease in LWP. The results agreed with previous studies involving Valencia and neck orange that reported a reduction in LWP that was sensitive to water stress and varied according to soil moisture during the progression of water stress (Chuchird, 2004). When PBZ was applied in combination with water stress, the LWP was not different from non-water stressed trees. This may have been due to PBZ reducing the effect of water stress by promoting the production of ABA that may cause stomatal closure, reducing water loss from the leaves through transpiration and maintaining LWP values (Chaney, 2005). Similar results have been shown in apple (Zhu *et al*., 2004) and ponkan (Mataa *et al*., 1998).

The vegetative growth of pummelo trees was observed 6 wk after rewatering. Shoot number was not different but shoot length showed significant differences among treatments. Water stress resulted in greater shoot lengths as well as a greater number of flowers while the control trees did not flower. The water stressed trees produced the highest number of inflorescence per tree. This result was supported by the study of Southwick and Davenport (1986) who reported that flowering in Tahiti lime was induced after 2 wk of water stress and the highest percentages of flowering shoots and flowers per plant were found after 5 wk of water stress.

In the present study, PBZ application with or without water stress was effective in reducing shoot length since the PBZ inhibits GA biosynthesis by blocking the step in the oxidation of ent-kaurene to ent-kaurenoic acid (Sterrett, 1985) resulting in unelongated shoots, even though cell division still occurs. Similar results have been reported in kumquat (Iwahori and Tominaga, 1986), ponkan (Mataa *et al*., 1998) and apple (Zhu *et al*., 2004). In addition, PBZ could also induce flowering in pummelo trees because it reduced vegetative growth (Chaney, 2005). The involvement of the GA in citrus flowering was also demonstrated by Yamashita *et al*. (1997) who reported that GA acts as a flowering inhibitor.

The treatment involving urea application combined with water stress was also effective in inducing early flowering in Khao Nam Phueng pummelo trees 1 wk after rewatering. This can be explained by the induction of flowering perhaps being related to ammonium levels in the leaves because TN before flowering in this treatment was higher than in other treatments. It is possible that the accumulation of ammonium during the stress period could lead to an increase in polyamines biosynthesis that plays a role in the meristematic activity involved in flower bud differentiation in citrus (Lovatt *et al.*, 1988). The accumulation of ammonium also caused the pummelo trees to flower earlier (by about 1 wk) than trees applied with PBZ or water stress alone. This was similar to Ndung u *et al.* (1997) who reported that the effect of water stress on bud break and flowering of grapevines might be mediated through nitrogen metabolism.

Inflorescence on the pummelo trees in this study was classified into four types, with the majority being of the type with many flowers with leafy shoots. Short inflorescence was observed for pummelo trees sprayed with PBZ. Similar results have been shown in mango (Yeshitela *et al.*, 2004) and ponkan (Mataa *et al.*, 1998). Nevertheless, the PBZ application combined with water stress trees produced the highest number of flowers per inflorescence. This result was supported by Yeshitela *et al.* (2004) who found that PBZ produced the highest number of flowers per panicle in mango. However, the intensity and duration of flowering also varies with climatic region and environmental factors, and in particular, sufficient irrigation and the temperature can regulate the time, types of flower and the extent of flowering in citrus trees (Davies and Albrigo, 1994).

In the treatments subjected to either water stress or the PBZ application combined with water stress, the TNC in the leaves of trees increased at the end of water stress and decreased after rewatering until the flushing period. Pummelo trees under water stress had the highest TNC because utilization and translocation of carbohydrate out of the leaves was reduced (Kameli and Losel, 1996). Moreover, the TNC could be increased by PBZ which was in agreement with the findings of Yeshitela *et al.* (2004) who reported that the foliar application of PBZ at rates of 5.50 and 8.25 g active ingredient per tree produced higher TNC in mango shoots before flowering. Since PBZ reduced vegetative growth, it is assumed that the treated trees consumed less TNC in the growth process than usual, leading to higher carbohydrate levels before withholding water. Similarly, Subhadrabandhu *et al.* (1997) observed that PBZ retarded vegetative growth and produced short terminal shoots in mango trees which then had higher food reserves than untreated trees. The decrease in the TNC after rewatering until the flushing period agreed with the previous report of Sivaci (2006) who stated that carbohydrate levels began to decrease during bud break and the expansion of shoots and new leaves. The decrease in carbohydrate could be explained by the conversion of stored carbohydrate into structural carbohydrates to form growth of the new organs. The urea application combined with water stress and the control trees had lower TNC than other treatments because they produced a vegetative flush all the time (data not shown) and used carbohydrate to produce new tissues.

The change in TN in leaves was not significantly different among treatments, though the treatment combining the urea application with water stress had the highest TN throughout the experiment. Nevertheless, the amount of inflorescence and the number of flowers were not different from other treatments. Thus, it is possible that the application of urea in this study could induce flowering. The result was in agreement with Lovatt *et al.* (1988) who reported that leaf ammonium concentration and flower intensity have been found to be closely related, since ammonium that was accumulated under water stress was able to regulate flower initiation in citrus (Lovatt *et al.*, 1988). TN in the treatments involving water stress and the PBZ treatment slightly decreased at the end of water stress period and tended to increase after rewatering. The decrease in TN during water stress may have been caused by the reduction in the nitrogen uptake and translocation in plants that resulted in limited vegetative growth and promoted carbohydrate accumulation in the storage organs (Ndung u *et al.*, 1997). Some previous studies reported that the application of PBZ did not affect the TN content in mango (Yeshitela *et al.*, 2004), whereas Arzani and Roosta (2004) reported that PBZ decreased the nitrogen level in apricot leaves. On the other hand, mango trees receiving PBZ had a higher percentage of TN in their leaves (Subhadrabandhu *et al.*, 1997).

The C/N ratio plays an important role in the differentiation of buds into the vegetative or flowering phase. A high C/N ratio enhances flower bud differentiation, whereas a high nitrogen content results in vegetative growth (Lovatt *et al.*, 1988). In the present study, the change in the C/N ratio was similar to the pattern of change in the TNC, with the former increasing at the end of water stress and then decreasing until the flushing period. These results agreed with the experiment of Chuchird (2004), who reported that water stress increased flowering, foliar carbohydrate and the C/N ratio in neck orange. Changes in the C/N ratio in PBZ treated trees were similar to the changes in the C/N ratio in the water stressed trees. However, the present study found that the urea application combined with water stress trees gave lower C/N ratios than in the control trees, but flowering was not different from other flowering trees with greater C/N ratios. Sernthaisong (2002) found that mandarin did not flower although the C/N ratio was in the range 4.15˘5.56. It may be possible that the C/N ratio was not the key factor but only a supporting factor for the flowering process. Nakajima *et al.* (1993) reported that the increase in flowers of pummelo trees exposed to water stress may have been caused by sugars in the leaves, whereas the C/N ratio of the leaves ranged from 4.75 to 5.10 and this range was not different from that in unstressed trees. Lovatt *et al.* (1988) also proposed that carbohydrate and nitrogen do not influence flower initiation directly but serve as substrates for the synthesis of key metabolites that act alone or work with plant hormones to initiate the flowering process. However, the relationship between reserve carbohydrate and cultural practices on flowering in pummelo should be further investigated to develop more effective techniques to control pummelo flowering.

CONCLUSION

Water stress treatments decreased LWP in Khao Nam Phueng pummelo, followed by noticeable flowering after rewatering. Meanwhile, flowering also occurred in trees treated with PBZ without any noticeable decrease in LWP which indicated that this chemical can induce flowering of pummelo without causing much stress through withholding water. The TNC in the leaves increased after withholding water and decreased after rewatering and then further decreased during flushing, while TN decreased slightly after withholding water and increased again in the flushing period. The change in the C/N ratio was similar to the change in the TNC. The C/N ratio in PBZ treated trees was not different from water stressed trees throughout the experiment. The urea application combined with water stress also induced early flowering regardless of the higher leaf TN than in other treatments.

ACKNOWLEDGEMENTS

This research was financially supported by the Thailand Research Fund (TRF) and the Southeast Asian Regional Center for Graduate Study and Research in Agriculture (SEARCA).

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