
Changes in cytokinins concentrations during induction period of longan cv. Daw in sand culture

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Floral induction in longan (*Dimocarpus longan* Lour.) trees by potassium chlorate (KClO₃) treatment or low temperature (LT), is partially acted by their interference with the hormonal concentration. Therefore, the effect of KClO₃ and LT on cytokinins (CKs) concentration was investigated in these experiments. The experiment was performed with 4 years old longan cv. Daw. The completely randomized design with 7 replications (of each 1 tree) and 3 treatments were performed namely 1) control, 2) potassium chlorate concentration of 400 ppm and 3) low temperature (day temperature is 17 °C and night temperature is 12 °C). Cytokinins were examined by competitive enzyme-linked immunosorbent assay (ELISA) in the apical buds, leaf and roots: *trans*-zeatin/zeatin riboside (*tZ/ZR*) and isopentenyl-adenine/isopentenyl-adenosine (*iP/iPA*) during induction period. The results showed that applying KClO₃ at the concentration of 400 ppm lead to floral bud initiations faster than maintaining the longan trees at LT (day temperature is 17 °C and night temperature is 12 °C). However the control treatment did not show flowering. The cytokinins content alterations in both *iP/iPA* and *tZ/ZR* of all tissues enhanced until prior to flowering in potassium chlorate, compared to the control and low temperature treatment.

Key words: Cytokinins, potassium chlorate, longan

Introduction

At floral induction natural habitat longan trees show irregular flowering and fruit bearing which is the most critical obstacle for the farmer to achieve regular yields and income (Thunyarpar, 1998). Longan trees require an appropriate period of low temperatures (usually ≤ 18 °C) for good flowering induction. Variation in temperatures, the factor that strongly influences

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flowering. Recently, applying chemical substance beside has been used to overcome with this insufficient low temperature (LT) problem, thereby enabling farmers to produce out-of-season (off-season) longan. Longan is the exclusive member of the Sapindaceae family which responds to flower induction by potassium chlorate (KClO₃) at any time of the year (Manochai *et al.*, 2005; Subhadrabandhu and Yapwattanaphun, 2001).

The application of KClO₃ is rather succeeded and most favorable to the grower. However, role of KClO₃ of physiological changes during flowering of longan is not be clearly understood, especially the balancing of endogenous hormone. Hegele *et al.* (2004) studied the effect of hormone balancing on flowering of longan and found that before flowering period, gibberellin and auxins (CKs inhibiting hormones) decreased while CKs (flower promoting hormone) and ethylene increased, therefore induce flowering of plants. This is due to the inhibitory effect of auxins (i.e. IAA) that inhibit CKs. The application of KClO₃ or LT treatment on flowering of longan is suggested to effects the translocation of CKs from leaf to apical buds, The CKs also increased, which could be detected during flowering of longan (Sringarm *et al.*, 2009). Therefore, the focus of the following experiment was done in KClO₃ and LT affected CKs concentration changes during induction period.

Materials and methods

Plant material and temperature conditions

Dimocarpus longan Lour. cv. Daw with an age of four years old were randomly selected and the total of twenty one plants were used in this experiment. They were grown in plastic box (ϕ 12 inches), using sand as growing media. Plants nutrition was prepared according Hoagland and Arnon (1950), and was applied once a week and watering twice a day (in the morning and the afternoon). This experiment was conducted at open field of Department of Plants Science and Natural Resources, Faculty of Agriculture, Chiangmai University, Chiangmai, Thailand. The experiment designs was completely randomized design (CRD), three treatments, seven replications and on plant per treatment. First treatment represented control, second treatment represented KClO₃ at concentration of 400 ppm and third treatment represented low temperature at 17/12°C (day/night temperature). For the 3 treatments, plants were grown under low temperature at 17/12 °C (day/night temperature). The fully grown plants were used for the control and KClO₃ application treatments which were transferred into greenhouse (covered with plastic). Plants were exposed of day/night temperature at average of 35/27 °C. For KClO₃

application was applied during plant nutrition application. For the last treatment, plants were transferred to the growth chamber. The temperature was set at 20 °C, and decreased 2 °C for everyday until it reached 17 °C, count up day-0 of the pre-flower. After that, the temperature setting was started up at 1 °C for everyday until 27 °C reached and then transferred to the constant day/night temperature at 35/27 °C (Fig. 1).

Shoot tips, leaves and root of each treatment were harvested at 0, 5, 10, 15, 25 and 30 days after treatment. All samples were immediately grounded in liquid nitrogen, and freeze-dried prior to hormone extraction and analysis.

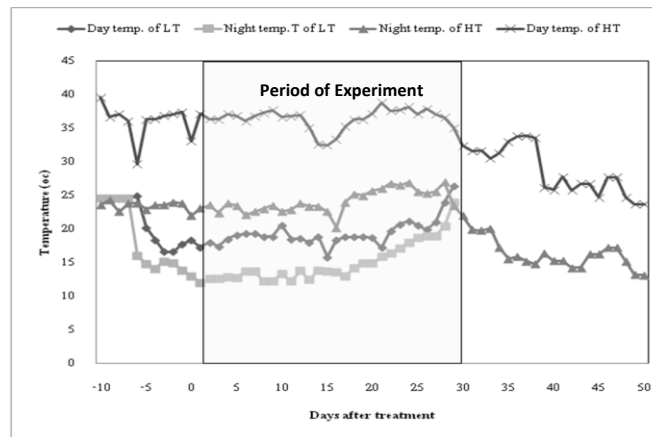


Fig. 1. This figure indicates day and night temperature for control, KClO_3 400 ppm and low temperature (17/12°C) treatment.

Hormone extraction (Sringarm, 2008)

Plant tissue (0.5 g) was ground into fine powder using liquid nitrogen. Powder samples were extracted in 80% cold methanol (50 ml) overnight at 4°C. The samples were filtered through a G4-grass sinter filter (Schott, Mainz, Germany) and the filtrate evaporated below 40°C on a rotary evaporator. The residue was dissolved in 4 ml of 0.01 M ammonium acetate (pH 7.5) and kept frozen at -20°C for 16 h. Samples were thawed at room temperature prior to centrifugation at 22,000 rpm at 4 °C for 25 min. The supernatant of each sample was transferred into a small glass bottle before loading through the column.

The above supernatants were purified on the following series of column combinations as follows:- reservoir, polyvinylpyrrolidone, DEAE-Sephadex A25 and C_{18} -Sep-Pak[®] cartridge. The series of columns were conditioned with 15 ml 1.0 M ammonium acetate (pH 8.5) followed by 20 ml 0.01 M ammonium

acetate (pH 7.5). Between both conditioning steps the preconditioned Sep-Pak cartridges for cytokinins were attached to the 10 ml syringes. Afterwards the extracts were loaded to the first syringe (30ml) and each sample was eluted with 3 times of 10 ml 0.01 ammonium acetate (pH 7.5). Cytokinins passed through the Sep-Pak[®] cartridge. After that, taken up Sep-Pak[®] cartridge from the column system was eluted *tZ/ZR* with 4 ml 30% methanol in 0.1 M acetic acid and *iP/iPA* with 4 ml of 80% methanol in 0.1 M acetic acid. Cytokinins were evaporated under speed vacuum concentrator prior to hormone analysis.

Hormonal quantification by competitive ELISA

The standard curve for iP/iPA and determination levels

ELISA system used polyclonal antibody against *iP/iPA* (pAB-*iP/iPA*) that produced at Central Laboratory, Faculty of Agriculture, Chiangmai University by Viroidsakul (2009). ELISA was performed with 96 well plate coated with 100 µl/well of 1:200 pAB-*iP/iPA*, in coating buffer (50 mM NaHCO₃, pH 9.6) and incubated at 4°C overnight. After washing, the wells were incubated for an additional period of 60 mins at 37°C with 200 µl of a 0.4% of BSA in coating buffer. The wells were filled with 50 µl of *iPA* standard (0-10 ng dilution range) or extracts and incubated together with *iPA*-Alkaline Phosphatase in TBS at the rate 1 : 200, followed by 20 µl/ well. The microplate was incubated for 90 minutes at 37°C and washed 3 times. The phosphatase activity was measured by adding 100 µl of a *p*-nitrophenyl phosphate solution (1mg.ml⁻¹ 50mM NaHCO₃, pH 9.6) to each well. The reaction was stopped by adding 100 µl 5N KOH and the absorbency of each well was measured at 405 nm. All concentration of hormones were adjusted with the corresponding recovery values for each sample.

The standard curve for tZ/ZR and determination levels

ELISA system used polyclonal antibody against *tZ/ZR* (pAB- *tZ/ZR*) that produced at Central Laboratory, Faculty of Agriculture, Chiangmai University by Viroidsakul (2009). ELISA was performed with 96 well plate coated with 100 µl/well of 1: 1000 pAB- *tZ/ZR*, in coating buffer (50 mM NaHCO₃, pH 9.6) and incubated at 4°C overnight. After washing, the wells were incubated for an additional period of 60 mins at 37°C with 200 µl of a 0.4% BSA in coating buffer. The wells were filled with 50 µl of *ZR* standard (0-5000 pg dilution range) or extracts and incubated together with *ZR*-Horseradish peroxidase in PBS at the rate 1:200, followed by 20 µl/ well. The microplate was incubated for 90 minutes at 37°C and washed 3 times. The enzyme activity was measured

by adding 100 μl of a OPD solution ($1\text{mg}\cdot\text{ml}^{-1}$ 50mM NaHCO_3 , pH 9.6) to each well. The reaction was stopped by adding 100 μl 4N H_2SO_4 and the absorbency of each well was measured at 492 nm. All concentration of hormones were adjusted with the corresponding recovery values for each sample.

Statistical analysis

The data were analyzed for statistical significance using Statistic 8 analytical software and Least Significant Difference (LSD) test was applied at $\alpha = 0.05$ significance level.

Results

Effect of KClO_3 and LT on flower induction

KClO_3 and LT gave some impact on flower induction of longan. The result suggested that KClO_3 400 ppm and LT could induce flowering in sand culture but the control did not flower (Fig.4 and Table 1). KClO_3 induce flower in 22 days which was faster than LT and percentage of flowering (94.81%) was significantly higher. However, LT showed an important factor for natural flowering induction of longan, but the percentage of flowering was low (29.14%).

Table 1. Duration and the percentage of flowering as affected by the treatment

Treatment	Duration of flowering (day) ^{1/}	The percentage of flowering ^{1/}
Control	No flower	00.00c
KClO_3 400 ppm	22	94.81a
Low temperature (17/12°C)	44	25.14b
Significant	-	*

^{1/}Means values with the same letter in a column are not significantly different at $P < 0.05$ by Least Significant Difference (LSD).

The changes of cytokinins: iP/iPA and tZ/ZR type in the tissues of apical buds leaf and root tips had effect of KClO_3 at 400 ppm and low temperature

Flowering process of longan changes CKs levels. This research observed CKs levels changed in apical buds, leaf and root tips at every 5 days for 1 month and detected by competitive ELISA.

From the standard curve of iP/iPA concentration found that the sensitivity at 50% binding was 1.8ng/50ul (Fig. 2). For iP/iPA levels in apical buds,

KClO₃ were increased during induction stage (day 10-15) as compared with those of control and LT treatment (Fig. 5). Those KClO₃ treatment gave the highest iP/iPA levels at day 25 which was the day of flowering after that iP/iPA levels decreased as found on day 30. For those LT treatments, it was found that iP/iPA levels were increased during 20-30 after treatment.

The iP/iPA levels in leaf, KClO₃ treatment increased at day 15 and decrease thereafter. And then iP/iPA levels in root tips, KClO₃ treatment were increased on day 15 as compared with LT treatment and iP/iPA tendency decrease, thereafter. Moreover, the iP/iPA levels of those LT increased on day 25-30 as compared to those of other treatments.

The standard curve of *tZ/ZR* found the sensitivity at 50% binding was 70.3ng/50ul (Fig. 3). The *tZ/ZR* levels in apical buds, KClO₃ were increased on day 25 as compare with those control and LT (Fig. 5). For *tZ/ZR* levels in leave, the KClO₃ increased at day 15 but not significantly difference. After day 25, the KClO₃ gave flower and *tZ/ZR* levels were decreased. While, LT treatment, *tZ/ZR* had low level. Those controls did not give any flower.

For *tZ/ZR* levels in root tips, KClO₃ and LT treatment were increased during induction stage. The LT treatment had the highest of *tZ/ZR* levels.

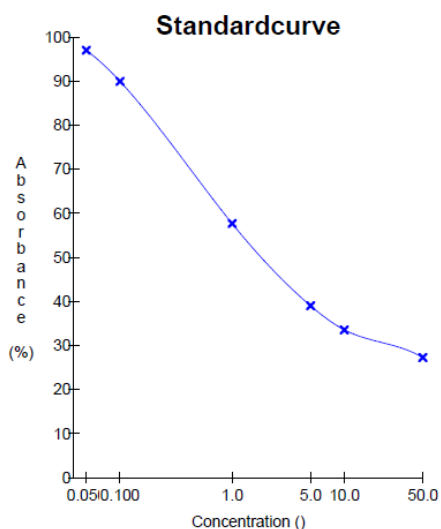


Fig. 2. Standard curve of iP/iPA

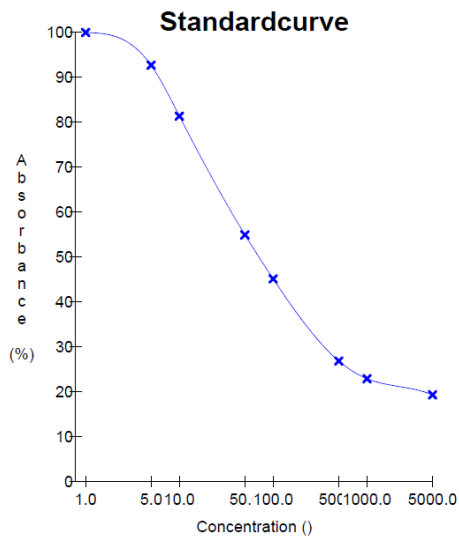
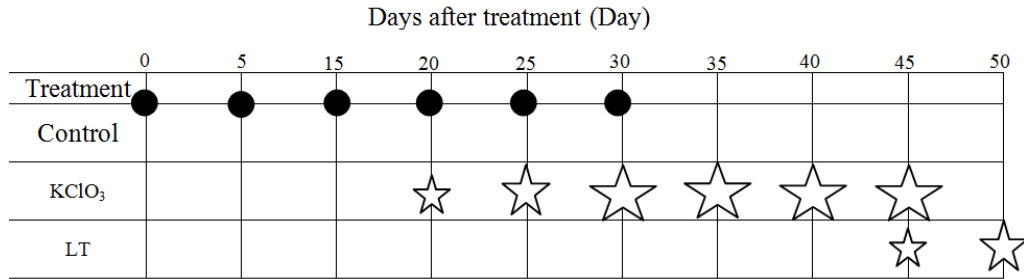


Fig. 3. Standard curve of *tZ/ZR*



● = Sampling times; ☆ - ☆ = 25% to 95% flowering buds

Fig. 4. Diagram showing the flowering time of longan tree during experiment in control, potassium chlorate (KClO₃) and low temperature (LT) treatments.

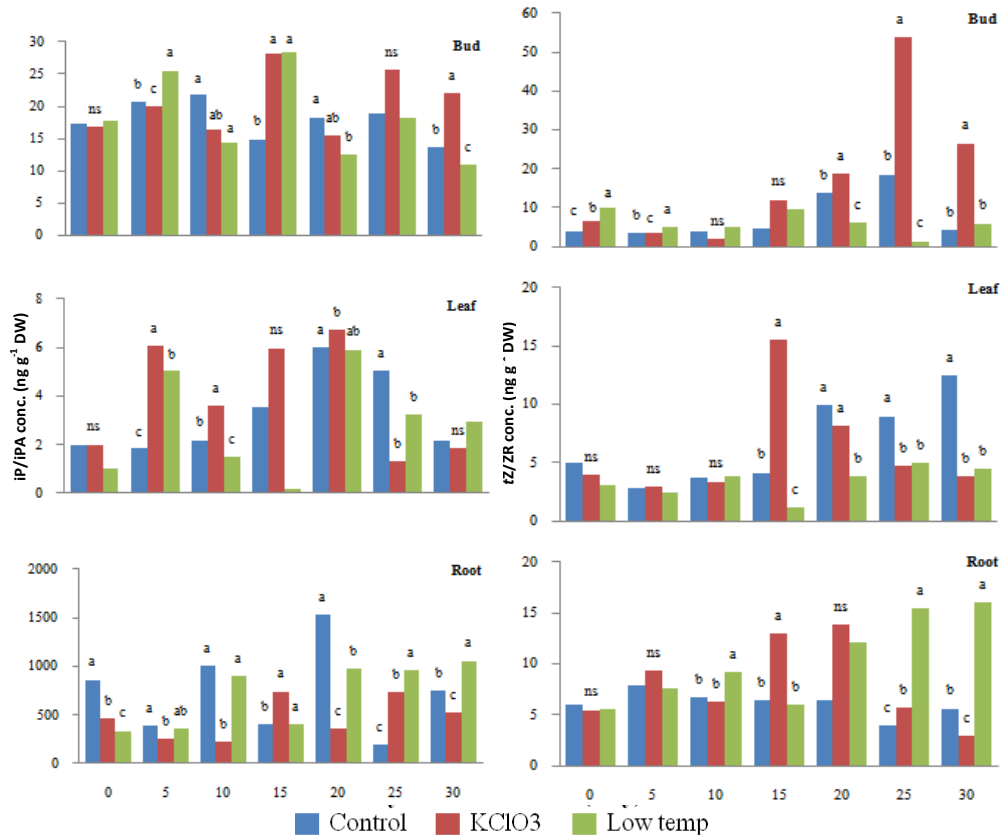


Fig. 5. The CKs concentrations in apical buds, leaf and root tips as affected by treatments. ns = not significant. Different letters between treatments but within the same sampling date indicate significant difference at $P < 0.05$.

Discussion

In the experiments shown above, visible flowering occurred approximately 22 days after the application of KClO_3 (Fig 4). These results is similar to work of Wechpibal (2009) who reported that KClO_3 12 gram per tree could induce flower in 20 days and percentage of flowering was 91 % as compared to control (non-treated KClO_3). While LT treatment, flower visibility on day 44th after the application (Fig 4). These results confirmed the finding of Davenport and Stern (2005) who reported that LT conditions induced floral induction in longan whereas only vegetative development occurred at high temperature conditions.

CKs are involved in the process of floral induction in trees. The key result of these experiments was the finding that *tZ/ZR*-type CKs, compared with *iP/iPA*-type CKs, were detected in apical buds, leaf and root tips. The CKs changed during induction period might be an important factor on flowering induction of longan, even though previous reports suggested that *iP/iPA* might not be related on flowering of other orchards or longan when treated with KClO_3 . Sringarm *et al.* (2009) suggested that *iP* type CKs were specifically exporting from leaves via phloem to shoot apical as well as subapical tissue. Besides being the hormones exported at significant quantities by longan leaves, *iP*-type CKs were also significantly affected by the LT treatment. However the conversion caused transient accumulate of *iP/iPA* in leaf which is the immediate precursors in *Z/ZR* biosynthesis before they are translocated to other organs as well as shoot tips to evocate of flower buds (Sringarm *et al.*, 2009; Potchanasin *et al.*, 2009).

KClO_3 application must have the ability to provide *iP* as well as *ZR*, CKs from alternative, non-leaf, sources and accumulate them in the shoot apical bud or the ability to facilitate the biosynthesis of these CKs in sub apical or apical tissue. As preferably *Z*-type CKs accumulated in the shoot apical bud, and because *Z*-type CKs are considered to be the active ones, a conversion of the accumulated *iP*- into *Z*-type CKs would be required in the shoot apical bud or within the tissue (Sringarm *et al.*, 2009). Similarly, Sringarm (2008) found that *tZ/ZR* was increased prior to flowering and flowering stage after treated with KClO_3 . Wangsin (2002) also found that cytokine-like substances has high tendency in prior flowering stage. Group of shoots were treated with KClO_3 which had cytokine-like substances levels higher group of controls at every week. Chen *et al.* (1997) found high levels of conjugated cytokinins (generally O-glucosides) during the dormant stage of longan trees which declined in favour of “free cytokinins” preferably of the *Z*-type and *iP*-type CKs in the shoot apical bud during the time of flower bud initiation.

Beside that NADPH in leaf might act as central messenger on process of CKs synthesis which relate to conversion iP/iPA to *tZ/ZR* by hydroxylation at isoprenoid side chain and cytochrome P450 moxygenase as catalyzation (Takei *et al.*, 2001). Generally, natural flowering when longan receive LT found the increasing of CKs levels in shoots which was related to iP/iPA translocation from leaf to shoots via the phloem to switch of flower, while KClO₃ application on off season flowering of longan, the increasing of CKs levels in shoots could not assign the source and iP export by leaves was greatly stimulated by LT and KClO₃ (Sringarm *et al.*, 2009).

In conclusion, KClO₃ can completely replace cool temperatures as flower inducing agent in longan trees mainly to induce off season flowering induction. Several studies have been performed to optimize this KClO₃ treatment. However, only few studies concerning the changes in the concentration of cytokinins. Our experiment suggested that both applied treatments, (KClO₃, LT) are able to initiate floral induction in longan trees and affected the hormone concentrations of CKs which KClO₃ increased the floral promoter CK.

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