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Changes of free polyamines in the leaves and stems of 'Kinnow' mandarin tree as affected by alternate bearing

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Abstract:

In order to investigate the seasonal changes and the possible role of the free polyamines in the leaves and stems on the alternate bearing habit of the 'Kinnow' mandarin (*Citrus reticulata* Blanco) trees, a comparative study was conducted to analyze the levels of free polyamines (putrescine, spermidine and spermine) in the leaves and stem tissues of "on" and "off" trees during the flower bud formation period. Samples were taken from trees in one month interval period in a commercial orchard in the City of Darab, Fars Province from Nov. 2010 till Mar. 2011 according to the stages of flower bud phenology in citrus. The experiments were designed as a randomized complete block with three replications and two trees per each replication. Total polyamines (putrescine, spermidine and spermine) levels in leaves were higher in "off" trees than in "on" trees throughout the experimental period but only the differences of spermine contents were significant during the most of season. The contents of spermidine and spermine in the stems of "off" trees were significantly higher than in "on" trees most of the season. Putrescine levels and polyamine ratio (putrescine/spermidine+spermine) in the stems were significantly higher in "on" trees than in "off" ones. Time of sampling had no significant effect on the stem total polyamines, putrescine and polyamine ratio in "off" trees. Results could not show a relationship between polyamine fluctuations in stems and leaves with processes of flower bud formation in mandarin trees.

Keywords: Alternate bearing, Flower bud formation, Free polyamines, 'Kinnow' mandarin, Putrescine, Spermidine, Spermine

Introduction:

Alternate bearing (biennial or uneven bearing) is the tendency of a fruit tree to produce a heavy crop in one year (on-year), followed by a light crop or no crop production in the next (off-year). This phenomenon is widespread among fruit trees including both deciduous and evergreens (Goldschmidt and Golomb, 1982). Alternate bearing is a major problem in citrus fruit production all over the world especially within the so-called easy-peeling groups (mandarin and their hybrids) (Wheaton, 1997). In many cases, alternate bearing can become almost complete and in certain cultivars ('Wilking' and 'Morcott') can lead to decline or even collapse of trees after an extremely heavy crop (Monselise and Goldschmidt, 1982). The alternate bearing habit in citrus cultivars is known to be due to lack of flowering in the next spring following a heavy crop in previous year and not due to a negative effect of the heavy crop on fruit set (Goldschmidt and Golomb, 1982). Under natural conditions, citrus flower bud induction in subtropical areas of northern hemisphere occurs during the winter months (December to January) and floral differentiation continues uninterrupted during the next two months until anthesis in mid-March till the end of April (Davenport, 1990).

Many attempts have been made to explain this phenomenon. It has been suggested that the competition for assimilates and nutrients between fruits and the flower buds is responsible for this phenomenon (Golomb and Goldschmidt, 1987; Rosecrance *et al.*, 1998; Vemmos, 1999; Ulger *et al.*, 2004; Baninasab *et al.*, 2007; Smith, 2009; Mirsoleimani *et al.*, 2014). However, other researchers have indicated that the unbalanced nutrition was not the primary cause of the inhibition of flower bud formation and attention should be given to the involvement of plant growth regulators (Monselise and Goldschmidt, 1982; Tromp, 2000; Baktir *et al.*, 2004; Koshita and Takahara, 2004; Al-Shdiefat and Qrunfleh, 2008). Interesting correlations between the reduction in flower induction due to heavy fruit load and levels of chemical and hormones have been reported in many diverse species. However identifying the exact structure of a certain chemical is not an easy task (Samach and Smith, 2013). It has been found that high fruit load affects the next year's flowering and yield by inhibiting the expression of FT gene in the leaves of mango and citrus (Samach and Smith, 2013). In the "on" year, there is an early and prolonged negative effect of high crop load on shoot and root growth of olive trees (Beya-Marshall and Fichet, 2017) and shoot growth of "Pixi" mandarin trees (Verreynne and Lovatt, 2009). It has been

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indicated that shoot growth is limited by fruit development in "Moncad" mandarin and C utilization by new flushes depend on crop load whereas fruit N consumption does not limi vegetative growth (Martinez-Alcantara *et al.*, 2015).

Polyamines (PAs) mainly putrescine (Put), spermidine (Spd) and spermine (Spm) are organic polycation nitrogenous bases that are widely distributed in plant tissues and have been shown to be related to some of physiological and morphogenetic events in plants including reproductive development, floral induction and development (Zhu *et al.*, 1999; Pritsa and Voyiatzis, 2004), inhibition of fruit abscission (Aziz, 2003), stress tolerance mechanisms (Gill and Tuteja, 2010). In these roles, PAs interfere with plant hormones and other compounds such as nitrogen (Tiburcio *et al.*, 2001). Polyamines play a crucial roles in abiotic stress tolerance such as salinity and increase in the level of polyamines are correlated with stress tolerance in plants (Gupta and Huang, 2014). A better understanding of the seasonal changes in metabolites in general and of specific regulators such as polyamines in particular, might be of major importance in controlling and regulating the bearing habit of citrus trees.

'Kinnow' mandarin is a hybrid of 'King' and 'Willow leaf' mandarin cultivars that has vigorous growth and heavy yield and great tolerance capability. It has attractive fruit color, size, good eating quality with aromatic and distinctive flavor. This is an alternate bearer cultivar with seeded fruits (Altaf *et al.*, 2008)

The aim of this study was to determine the seasonal variation in endogenous polyamine contents of the leaves and stems of bearing and non-bearing 'Kinnow' mandarin trees and the possible involvement in flower bud formation stages in these trees.

Materials and Methods:

Plant materials and sampling: This study was conducted on 7-year old 'Kinnow' mandarin (*Citrus reticulata* Blanco) trees (without any annual pruning) grafted on 'Mexican' lime (*Citrus aurantifolia*) rootstock grown in a commercial orchard in the City of Darab, Fars Province, Iran. Trees were irrigated by micro-sprinkler and standard commercial cultural practices were performed during the experiment. Twelve uniform size 'Kinnow' mandarin trees (six on- and six off-year) were selected in Sep. 2010. The experiments were designed as a randomized complete block with three replications and two trees per each replication. Samples for chemical analysis were taken from "on" and "off" trees in approximately monthly intervals from Nov. 2010 till Mar. 2011. Each time, fifteen current season growth branches were collected for each replication. At first, fully expanded leaves were taken from the tip portion of these shoots and then the rest of the leaves were removed to collect the stems. Leaves and stems were washed with water and kept in liquid nitrogen and brought to the laboratory. Mature fruits were harvested in the first week of Feb. 2011. Minimum, and average air temperatures of experimental orchard are given in table 1.

Floral bud development: Ten shoots (15-20 cm long) from each tree were sampled at one month intervals. In order to study the flower bud development, the shoots were defoliated and sprayed with 100 mg/l benzyladenine and placed in a vase for three weeks to grow in a growth chamber kept at 28 °C and light condition. Eventually the numbers of total buds, sprouted buds including vegetative (inflorescences with leaf only) and reproductive (inflorescences with flower, with or without leaf) were counted (Yahata *et al.*, 2006).

Sample preparation and derivatization: Extraction, separation and quantification of polyamines were made according to the method described by Marce *et al.* (1995) with some modifications.

Samples were powdered with liquid nitrogen in the presence of 1,7-diaminoheptan as internal standard using pestle in a mortar in 5% (v/v) cold percholoric acid (PCA). After incubation in 4 °C for 30 mins. homogenates were centrifuged at 20000 rpm (67000 g) for 20 mins. at 4 °C. Supernatants (100 µl) were mixed with 200 µl of saturated sodium carbonate and 400 µl of dansyl chloride in a tapered reaction vial. Mixtures were incubated at 60 °C for 60 mins. A 100 µl aliquot of L-prolin solution was then added. After incubation in 60 °C for 30 mins. dansylated PAs were extracted with 500 µl toluene. Then 400 µl of this solution was taken and dried under nitrogen flow. The residue was dissolved in 800 µl acetonitrile (HPLC grade) and analyzed by HPLC.

PAs separation and quantification by HPLC: Separation and determination of free PAs were performed in an HPLC system equipped with a fluorescence detector with excitation at 252 nm and emission at 500 nm. The sample was subjected to a gradient elution in acetonitrile and water. The initial conditions were 70% acetonitrile and 30% water pumping at a flow-rate of 1.5 ml/mins. The mixture was pumped for 4 mins; then the concentration of acetonitrile was raised to 100%. This concentration of acetonitrile was kept constant for 4 mins, and finally returned to the initial conditions. Data were evaluated using a Chemstation Software (Agilent Technologies) data processing system. The separation was achieved on an Agilent, XDB-C18, 5 µm, 4.6×150 mm column.

Statistical analysis: The experiments were designed as a randomized complete block with three replications and two trees per each replication. Statistical analysis was performed using the SAS software (Statistical Analysis System) (V9) (SAS Institute Inc. Cary, NC, USA). Differences among the mean values were detected by Least Significant Differences (LSD) test at %5 level.

Results:

Flower bud development: Table 2 shows that the percentage of bud-break in "on" trees was very low and all those buds broken on fruiting shoots were vegetative.

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Table 1. Information of air temperature in experimental orchard.

	2010			2011		
Temperature (°C)	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.
Average of minimum	17.3	10.1	3	3.5	4.9	8.4
Average	26	18.5	13.2	10.8	9.9	15.1
Average of maximum	34.7	27	23.4	18	15	21.8

Table 2. Flower bud development in the excised shoots of 'Kinnow' **mandarin trees.**

Date of sampling	Tree cropping state	Percentage of bud break (%)	Type and number of buds formed per 100 nodes	
			Vegetative	Generative
Oct. 7	ON	1.6	1.6	0
	OFF	6.45	6.45	0
Nov. 4	ON	2.77	2.77	0
	OFF	9.64	9.64	0
Dec. 8	ON	1.58	1.58	0
	OFF	16.74	16.74	0
Jan. 6	ON	3.82	3.82	0
	OFF	13.65	13.65	0
Feb. 7	ON	3.81	3.81	0
	OFF	22.85	21.30	1.54
Mar. 5	ON	12.04	12.04	0
	OFF	23.88	20.85	3.03

Table 3: Variations of putrescine contents[1] (µg/g FW) in the leaves and stems of "on" and "off" 'Kinnow' mandarin trees

Sampling Dates	Leaves		P [3]	Stems		P
	ON	OFF [2]		ON	OFF	
Nov. 4	8.27 [b]	15.94 [a]	***	81.69 [a]	31.18 [a]	***
Dec. 8	9.58 [b]	13.47 [ab]	*	70.99 [ab]	35.15 [a]	**
Jan. 6	14.28 [a]	12.79 [abc]	ns	64.50 [abc]	39.83 [a]	*
Feb. 7	12.82 [ab]	10.44 [bc]	ns	49.96 [c]	33.58 [a]	ns
Mar. 5	11.03 [ab]	9.05 [c]	ns	53.64 [bc]	38.34 [a]	ns
	ns	*		*	ns	

1 - Average of six "on" or "off" trees for each sampling time, 2- off-year trees in 2010 cropped heavily in 2009 and vice versa, 3- ns, *, **, *: not significant at P < 0.05, significant at P < 0.05, 0.01, 0.001, respectively by LSD test.**

Generative shoots appeared only in the last two sampling dates and were only present in non-fruiting trees.

Changes in Free PAcontents of the leaves: As can be concluded from table 3, leaves Put contents in both "on" and "off" trees were affected by sampling time. Leaves from non-fruiting trees exhibited much higher Put than that of fruiting ones in the first two sampling dates but in the next sampling dates Put contents in the leaves of fruiting trees were higher than that of non-fruiting trees.

Spd content in leaves from non-fruiting shoots were higher than that of "on" shoots but only the differences in the samples of Nov. and Jan. were significant (Table 4). Leaves Spd contents in "on" trees were significantly affected by sampling date while the Spd levels in the leaves from "off" trees were not affected by date of sample preparation.

Spm contents in the leaves of non-fruiting trees, after an initial significant (P < 0.0001) decrease during the first month, stayed almost stable till the end of experiment (Table 5). As shown in table 5, Spm contents exhibited a significant decrease in leaves from fruiting shoots during the season. The leaves from "off" shoots had much higher Spm contents than the "on" ones, during most of the experimental period.

Total PAs content in the leaves of "on" trees exhibited a significant fluctuation with sampling time, as seen in table 6, while time had not significant effect on the total PAs measured in the leaves of "off" trees. Total PAs levels in the leaves were not significantly affected by the fruiting state of trees, although in the beginning of the season, leaves taken from non-fruiting trees had significantly higher total PAs contents than those obtained from fruiting ones (Table 6).

As can be concluded from table 7, time of sampling had a significant effect on the PA ratio in the leaves of both "on" and "off" trees. From the second sampling date onwards, leaves taken from fruiting trees displayed a much higher PA ratio than those removed from non-fruiting trees.

Changes in Free PA contents of the stems: There were no significant differences in the stems of non-fruiting trees concerning their Put contents throughout the experiment. Put contents in the stems of "on" trees decreased significantly with time till the end of

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Table 4: Variations of spermidine contents[1] (µg/g FW) in the leaves and stems of "on" and "off" 'Kinnow' mandarin trees

Sampling Dates	Leaves		P [3]	Stems		P
	ON	OFF [2]		ON	OFF	
Nov. 4	10.35 [b]	31.74 [a]	***	7.19 [c]	10.38 [c]	ns
Dec. 8	21.32 a	28.41 [a]	ns	11.45 [b]	16.15 [b]	*
Jan. 6	22.61 [a]	34.78 [a]	*	13.90 [b]	21.55 [a]	***
Feb. 7	14.67 [b]	21.28 [a]	ns	15.10 [b]	24.27 [a]	***
Mar. 5	15.46 [b]	25.30 [a]	ns	19.16 [a]	21.43 [a]	ns
	**	ns		**	***	

1- Average of six "on" or "off" trees for each sampling time.
2- off-year trees in 2010 cropped heavily in 2009 and vice versa.
3- ns, *, **, *: not significant at P < 0.05, significant at P < 0.05, 0.01, 0.001, respectively by LSD test.**

Table 5: Variations of spermine contents[1] (µg/g FW) in the leaves and stems of"on" and "off" 'Kinnow' mandarin trees

Sampling Dates	Leaves		P [3]	Stems		P
	ON	OFF [2]		ON	OFF	
Nov. 4	7.68 [a]	17.25 [a]	***	5.61 [a]	7.82 [a]	***
Dec. 8	6.91 [ab]	8.81 [b]	ns	4.89 [ab]	7.30 [a]	***
Jan. 6	5.88 [abc]	8.29 [b]	*	3.22 [dc]	5.29 [b]	**
Feb. 7	5.50 [bc]	7.97 [b]	*	2.48 [d]	4.74 [b]	**
Mar. 5	4.73 [c]	8.10 [b]	**	4.09 [bc]	7.12 [a]	***
	*	***		***	***	

1 - Average of six "on" or "off" trees for each sampling time, 2- off-year trees in 2010 cropped heavily in 2009 and vice versa, 3- ns, *, **, *: not significant at P < 0.05, significant at P < 0.05, 0.01, 0.001, respectively by LSD test.**

Table 6: Variations of total polyamine contents[1] (µg/g FW) in the leaves and stems of "on" and "off" 'Kinnow' mandarin trees

Sampling Dates	Leaves		P [3]	Stems		P
	ON	OFF [2]		ON	OFF	
Nov. 4	26.29 [c]	64.93 [a]	***	94.49 [a]	49.38 [a]	**
Dec. 8	37.80 [ab]	50.69 [ab]	ns	87.33 [ab]	58.60 [a]	*
Jan. 6	42.76 [a]	55.87 [ab]	ns	81.62 [ab]	66.67 [a]	ns
Feb. 7	33.00 [abc]	39.69 [b]	ns	67.89 [b]	62.58 [a]	ns
Mar. 5	31.21 [bc]	42.45 [b]	ns	76.88 [ab]	66.88 [a]	ns
	*	ns		ns	ns	

1 - Average of six "on" or "off" trees for each sampling time, 2- off-year trees in 2010 cropped heavily in 2009 and vice versa, 3- ns, *, **, *: not significant at P < 0.05, significant at P < 0.05, 0.01, 0.001, respectively by LSD test.**

Table 7: Variations of put/ (spd+spm) ratio[1] in the leaves and stems of "on" and "off" 'Kinnow' mandarin trees

Sampling Dates	Leaves		P [3]	Stems		P
	ON	OFF [2]		ON	OFF	
Nov. 4	0.46 [ab]	0.33 [ab]	ns	6.92 [a]	1.70 [a]	***
Dec. 8	0.34 [b]	0.37 [a]	ns	4.35 [b]	1.48 [a]	**
Jan. 6	0.51 [ab]	0.29 [bc]	**	3.93 [b]	1.49 [a]	*
Feb. 7	0.63 [a]	0.36 [a]	***	2.78 [b]	1.16 [a]	ns
Mar. 5	0.56 [a]	0.28 [c]	***	2.30 [b]	1.34 [a]	ns
	*	**		**	ns	

1 - Average of six "on" or "off" trees for each sampling time, 2- off-year trees in 2010 cropped heavily in 2009 and vice versa, 3- ns, *, **, *: not significant at P < 0.05, significant at P < 0.05, 0.01, 0.001, respectively by LSD test.**

experiment (Table 3). During the most of the entire sampling period (first three sampling dates) the fruiting shoots indicated much higher Put concentration than the non-fruiting ones.

There were significant differences (except for the beginning and the end of the experiment) in Spd contents between stems from "on" and "off" 'Kinnow' mandarin trees (Table 4). Time had a significant effect on the Spd contents in the stems of both types of trees. Table 4 shows that stem Spd contents exhibited increase in "on" and "off" trees all over the period of experimentation and reached to 2.0 and 2.7 time higher than initial value in "off" and "on" shoots at the end of season, respectively.

As can be seen in table 5, concentration of Spm in the stems of "on" and "off" trees were significantly

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affected by the fruiting state of shoots and sampling time. Stem Spm levels after an initial significant decrease during the first four months, exhibited a significant increase, reaching initial content at the end of season in both types of trees. Stems from non-fruiting trees had much higher Spm contents than that of fruiting ones for all of sampling dates.

Our results also showed that both the contents of total PAs as well as that of the PA ratio in the stems of non-fruiting trees were not influenced by the time of sampling. On the other hand the total PA contents of the stems in "on" trees displayed a gradual and non-significant decrease throughout the season but PA ratio in these shoots decrease significantly during the experiment. There were significant differences between stem PA ratio in "on" and "off" trees in the most period of the experiment but the same was true for the total PAs only for the first two sampling dates (Tables 6 and 7).

Discussion

The data presented in table 2 show that the presence of fruits on "on" trees inhibited flower bud formation, decreased total sprouted buds and resulted in the reduction of vegetative and reproductive growth of these trees. The effects of crop load on the inhibition of citrus flowering supported by our results have been reported by several researchers. Verreynne and Lovatt (2009) reported that for the 'Pixie' mandarin in California, the alternate bearing cycles appear to be a crop load-dependent inhibitory effect of fruit on bud break.

Fruit load had significant effect on development of flowers per shoot for both 'Valencia' and 'Hamlin' sweet orange trees (Valiente and Albrigo, 2004). The presence of fruits, on branches of "on" trees had an inhibitory effect on the sprouting of new shoots and the basipetal appearance of lateral buds in citrus trees (Garcia-Luis *et al.*, 1986; Koshita *et al.*, 1999; Martínez-Fuentes *et al.*, 2010). The presence of fruits on shoots may reduce the sensitivity of buds to inductive conditions (Valiente and Albrigo, 2004) or may be related to competition for carbohydrates and inorganic elements (Goldschmidt, 2005) or related to imbalance in plant hormones (Garcia-Luis *et al.*, 1986).

Polyamines are positively charged aliphatic nitrogen-containing compounds of low molecular weight that are widely distributed in living organisms. The polycationic nature of polyamines at physiological pH is one of the main properties believed to mediate their biological activity. They are able to bind with several negatively charged molecules such as DNA, RNA, proteins, membrane phospholipids and proteins and pectic polysaccharides. Due to their properties, polyamines are involved in the regulation of plants growth and development events such as flower bud induction and formation in fruit trees (Tiburcio *et al.*, 2001).

In accordance with the findings of Roussos *et al.* (2004), our results showed that Spd and Spm contents of both leaves and stems and total polyamines contents of leaves were affected by fruiting state of shoots and these were higher in "off" than "on" trees. On the other hand Put concentration in shoots of "on" trees decrease rapidly during the season and reached the same level of "off" trees at the end of season. It seems that, in fruiting trees, PAs translocate to the fruits. There are some reports of polyamine translocation among organs and it is believed that parallel to the increase in the number of fruits, PAs and other N compounds are diluted between developing fruits (Arias *et al.*, 2005).

Plant polyamines metabolism is very sensitive to the adverse environmental conditions (Flores and Galston, 1982; Galston and Sawhney, 1990) thus, polyamines are considered as suitable stress markers in plant tissues. Plants under stress are characterized by showing high photorespiration rates leading to increased NH_4 levels that are toxic for plant. Polyamines accumulation under stress could be related to a detoxification mechanism preventing NH_4 accumulation (Pedrol and Tiburcio, 2001; Gill and Tuteja, 2010). In agreement with previous reports, in present study, Spd levels of stems in both types of trees increased with a decline in temperature (Table 1). This indicated a possible involvement of Spd in chill-protecting mechanism (Pritsa and Voyiatzis, 2004; Shen *et al.*, 2000). Polyamines may act as storage sites of carbon and nitrogen in plant tissues (Aziz, 2003).

Spermidine was found to be the only PA that increased in buds of mature peach (*Prunus persica*) at the beginning of spring growth (Fraga *et al.*, 2004). Rey *et al.* (1994) also reported that in hazel (*Corylus avellana*) buds, high levels of free Spd and Spm were found to positively correlated with bud burst in spring and their levels were decreased in autumn. In agreement with these reports, we found that Spd concentration in the stems of both types of trees increased gradually throughout the experimental period. In addition, Spd level was higher in the stems of "off" trees than "on" ones, and this is in agreement with the data of table 2. This table showed that the amount of bud break was higher in "off" trees than "on" ones.

There is some evidence to support the hypothesis that a high Put to Spd and Spm ratio correlate with cell division (Kushad *et al.*, 1990) and shoot growth (Pritsa and Voyiatzis, 2004) of plants. In contrast to above reports, we could not find any relationship between cell division and shoot growth with PAs ratio in the leaf and stem of 'Kinnow' mandarin trees.

Conclusion

In conclusion our results indicated that in 'Kinnow' mandarin trees, fruiting state of trees were affected on the polyamine content of trees, especially in the shoots. In most cases, PAs concentrations were much lower in organs of fruiting trees than non-fruiting ones. It is possible that declining in polyamine concentrations in these organs is because of translocation to the fruits. We

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could not find support for the view that variation in PAs in leaf and stem were involved in flower bud formation. Further research is needed in order to elucidate the role of all forms of polyamines in relation to flower bud formation stages in citrus trees.

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