Different Flower-Inducing Conditions Elicit Different Responses for Free Polyamine Levels in Olive (*Olea europaea*) Leaves

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In various plant species, polyamines have been implicated in regulating developmental phenomena as well as responses to environmental stimuli. The role of polyamines in regulating developmental phenomena in olive trees, such as flowering, is poorly understood, although seasonal changes and temperature effects on polyamine levels in olive trees have been reported. In this study, levels of free polyamines (putrescine, spermine, and spermidine) in the leaves of trees kept under non-inducing conditions were compared with polyamine levels in trees that were induced to flower under chilling and non-chilling conditions. Putrescine and spermine levels were much higher in leaves kept under inductive chilling conditions compared to control trees kept vegetative, but such increased levels of polyamines did not occur in trees that were induced to flower under non-chilling conditions. These results clearly differentiated between the effects of temperature versus the effect of developmental change on free polyamine levels in olive leaves. The results show that changes in free polyamine levels in leaves have little relevance to flowering in olives. Free polyamine levels within auxiliary buds increased when vegetative buds transformed into flowering buds and then declined when buds developed into flowers. Compared to floral buds, immature and mature fruits contained much smaller amounts of polyamines.

Key Words: olive, polyamine, putrescine, spermidine, spermine.

Introduction

Polyamines have long been known to play an important role in the regulation of various plant developmental phenomena including flowering and fruiting in a number of plant species (Alcázar et al., 2005; Kakkar and Rai, 1993; Kaur-Sawhney et al., 2003; Liu et al., 2006; Wang and Kao, 2006; Yamasaki and Cohen, 2006; Ziosi et al., 2006). In olive cultivars, spray application of putrescine was reported to increase fruit set and yield (Iannotta et al., 1996; Rugini and Mencuccini, 1985). Except for putrescine, increased levels of different polyamines were observed in olive cultivars during the flower induction period (Prista and Voyiatzis, 2004). Polyamines also promote rooting in olive cuttings (Ozkya and Celik, 1994; Rugini et al., 1990); however, studies on the role of polyamines in regulating developmental processes in olive cultivars are limited (Prista and Voyiatzis, 2004).

Polyamine levels also change in response to environmental stress and stimuli, including chilling (Kuznetsov et al., 2007; Shen et al., 2000; Tattini et al., 1993; Urano et al., 2004; Yamaguchi et al., 2007). Pan et al. (1999) reported increased synthesis of putrescine, spermine, and spermidine in olive fruits stored at cold temperatures versus room temperature. Since flowering in olives is attributed to several days of chilling before flower differentiation (Hartmann and Whisler, 1975), it is important to determine if the changes observed in polyamines at the time of flowering result from a developmental change or simply from the effect of chilling.

Using our locally designed growth chambers (Malik and Bradford, 2005a), we recently showed that flowering in the ‘Arbequina’ cultivar of olives could be induced under chilling (Malik and Bradford, 2004) and non-chilling conditions (Malik and Bradford, 2005b). This system seemed ideal to determine if there was a correlation between flowering and polyamine levels in olive leaves independent of the effects of chilling. Free polyamine levels were determined under chilling and non-chilling conditions that induce flowering in the ‘Arbequina’. The results of this study provided interesting insights into the relation between flowering and polyamine levels in leaves and buds.
Materials and Methods

Plant material

‘Arbequina’ trees, approximately four-years old, were grown in 16-L pots (containing equal parts of sand, peat, medium vermiculite and coarse perlite) at the USDA-ARS facility in Weslaco, TX, USA, N 26.16° latitude, 97.96° longitude. Trees were supplied with 50 g of all-purpose Miracle-Gro slow-release fertilizer (10(N)-10(P2O5)-10(K2O)) (Miracle-Gro Products Inc., Marysville, OH, USA) once in two months. In addition, the trees were sprayed with water-soluble fertilizer (15(N)-30(P2O5)-15(K2O)) (Miracle-Gro Products) after 2–3 weeks at the rate of 20 g/gallon. Ten replicate trees were used for each treatment in the experiment. The trees were subjected to the following temperature regimes from mid-November until mid-February:

1. Warm, non-inducing conditions (Control 1; C1).

   The trees were kept in a greenhouse with average daytime (12 h) temperatures maintained at 29 ± 1°C and average nighttime (12 h) temperatures maintained at 26 ± 1°C. Under these conditions, flowering in olive trees does not occur.

2. Open-field non-inducing conditions in Weslaco (non-flowering trees; C2).

   Potted trees were grown in an open area in Weslaco. Ambient temperatures varied from day to day but usually nighttime temperatures were not sufficiently cold to provide enough chilling (temperatures below 7.2°C), and frequently daytime temperatures are warmer than 24°C. Normally, olive trees in Weslaco do not flower.

3. Flower-inducing conditions under optimum chilling temperatures (Flowering trees; F1).

   Trees were kept in a growth chamber where average nighttime temperatures were maintained at 2 ± 1°C and average daytime temperatures where maintained at 18 ± 1°C. This temperature regime is considered optimum for flowering in olives (Denney and McEachern, 1983). These conditions produced extensive flowering.

4. Flower-inducing conditions under non-chilling temperatures (i.e., minimum temperature above 7°C) (Flowering trees; F2).

   Trees were kept in a growth chamber where average nighttime temperature was kept at 9 ± 1°C and the average daytime temperature was maintained at 16 ± 1°C (reaching 18°C for two hours). These conditions produced extensive flowering.

5. Sub-zero nighttime temperatures (S1).

   Trees were kept in a growth chamber where the average nighttime temperature was kept at −2 ± 1°C and the average daytime temperature was kept at 16 ± 1°C (reaching 18°C for two hours). These conditions strongly inhibited flowering; i.e., only a few flowers were produced on 3 trees.

At the completion of the induction period (mid-February), samples were taken at random from fully expanded leaves of replicate trees in each treatment. Three composite leaf samples from each treatment were immediately frozen and stored at −80°C. Several hundred floral buds at different stages (Fig. 1) were excised from the axils of leaves from a number of ‘Arbequina’ trees at Moro Creek Ranch in Carrizo Springs, TX, USA. Vegetative buds (1–2 mm length) were collected from trees grown in a field in Weslaco, where trees normally do not flower. Two samples of fruits; i.e. immature (3–4 mm diameter) and mature (15 mm diameter) fruits were also collected from ‘Arbequina’ trees in Carrizo Springs. Flower and fruit samples were brought from the field in dry ice and then stored at −80°C until used for analyses.

Extraction of polyamines

Plant samples were pulverized in liquid nitrogen to a very fine powder as described earlier (Malik and Bradford, 2005c). Free polyamines were extracted in 5% perchloric acid (Flores and Galston, 1982). Briefly, 0.25 g of finely powdered plant material was mixed with 8 mL of 5% perchloric acid. The mixture was sonicated for 30 s, shaken overnight at 4°C and then centrifuged at 5000 × g for 45 min. Free polyamines in the supernatant liquid were purified by ion exchange chromatography according to the procedure described by Corbin et al. (1989).

HPLC analyses of polyamines

The PA fraction was reacted with dansyl chloride to obtain fluorescent polyamines (Flores and Galston, 1982). Derivatized polyamines from olive tissue extracts...
results. The column was eluted at a flow rate of 1 mL·min⁻¹ with the gradient of the solvent system of HPLC grade methanol (solvent A) and HPLC grade water (solvent B). Initially, the gradient was composed of 60% A and 40% B, then solvent A was linearly increased to 95% in 23 min, and then maintained isocratic for the next 7 min. A 10 µL aliquot of the derivatized extract or standard solution was injected for each run and elution profiles were detected with a fluorescent detector at 365 nm wavelength for excitation and 510 nm wavelength for emission. For quantitative measurements, standard regression curves were developed for each of the known standard compounds.

Statistical analysis
Leaves and buds were randomly collected from 10 replicate trees in each treatment. A minimum of three replicate extractions were performed from each treatment followed by a minimum of three replicate HPLC analyses from each extract. Statistical analyses were conducted to compare treatment means using the t-test procedure of the InStat software (version 3.0, GraphPad, San Diego, CA, USA).

Results and Discussions
Levels of free putrescine and spermidine, but not spermine, increased considerably (561% and 222%, respectively) in the leaves of ‘Arbequina’ trees when subjected to chilling conditions (from mid-Nov. to mid-Feb.) appropriate for the induction of flowering (F1) compared to similar trees kept in non-inducing conditions, such as a warm greenhouse (C1), that did not flower (Fig. 2). It is interesting to note that the greatest increase occurred in putrescine levels (561%), thus supporting an earlier report that the exogenous application of putrescine to olive trees increased fruit set (Rugini and Mencuccini, 1985). Trees kept outside under ambient conditions that lacked sufficient chilling temperatures to induce flowering (C2) showed a small increase in putrescine (116% as opposed to 561% in F1 trees) and a modest increase in spermidine (75% as opposed to 221% in F1 trees). These results appear to be in line with the general concept that polyamines are perhaps involved in the regulation of flowering.

In contrast to the increase in free polyamine levels in leaves observed above for the chilling conditions that induced flowering (F1), trees that did flower extensively under non-chilling conditions (F2) (Malik and Bradford 2005b, 2006) did not show any substantial increase in any of the polyamines studied compared to the control trees kept under non-inducing conditions in the greenhouse (Fig. 2). Since the trees exposed to non-chilling but flower-inducing conditions (F2) flowered extensively, it appears that the sharp rise in polyamine levels in leaves seen under the F1 chilling condition was related only to the temperature effect of the environment and not to the developmental change from vegetative to flowering state.

If we assume that the increased polyamine levels observed in F1 tree leaves was due to chilling temperatures, rather than to flower induction, then the question arises: Do polyamines increase under chilling conditions if flowering is suppressed? To test this, trees were kept under S1 conditions where nighttime temperatures were kept at −2 ± 1°C. These conditions suppressed flowering so that only 3 out of 10 trees flowered with three- to five inflorescences. However, putrescine and spermidine levels were 854% and 358% higher than greenhouse control trees (C1), respectively; i.e., more than the levels seen under F1 and F2 conditions where trees flowered extensively (Fig. 2). Spermine levels in leaves showed little change under different environmental conditions in this study (Fig. 2).

Thus, based on polyamine levels and the flowering pattern from olive trees kept under F1, F2, and S1 conditions, it can be concluded that there is little correlation between free polyamine levels in the leaves of ‘Arbequina’ and the production of flowers in this cultivar (Fig. 2). To our knowledge, this is the first time metabolic changes in olives have been studied when trees were exposed to different flower-inducing temperatures under controlled environmental conditions (i.e., same developmental change under different environmental conditions). These experimental conditions, however, provide critical information regarding...
whether or not a certain metabolic change is involved in regulating a particular developmental change when the two occur in parallel under natural conditions. Indeed, the results presented here demonstrate that increased levels of free polyamines in olive leaves may not be necessary to induce flowering that typically occurs during winter just before the trees flower. This information is important for future studies on the regulation of flowering in olives because it helps researchers to be cautious about correlating seasonal changes in leaves with developmental phenomena such as flowering.

Here, a question can be raised as to whether leaves play any role in the regulation of flowering in olives. It has been shown that the removal of olive leaves inhibits flowering in olives, although it was also possible to induce flowering in olives by wrapping rubber tubes around lateral buds with chilled water circulating in the tubes while leaves were exposed to non-inducing conditions (Hackett and Hartmann, 1964). At this time there is no clear understanding of the contribution of various metabolic factors in the leaves that participate in the regulation of flowering in olives, but it seems apparent from this study that fluctuations of polyamine levels in leaves have little effect on the production of flowers.

The determination of free polyamine levels in vegetative buds and reproductive structures of various stages showed that putrescine levels were 310, 280, and 430 percent higher in stages 1–3 flower buds, respectively, compared to vegetative buds (Fig. 3). Putrescine in opened flowers (Stage 4) was significantly lower (40% less) than in Stage 3 flower buds, and a similar trend was seen with spermidine (52% less) and spermine levels (77% less) (Fig. 3). Since all of the floral bud and flower samples were collected at the same time from the same site (i.e., subjected to the same environmental conditions), the differences in free polyamine levels between flower buds and opened flowers represent developmental changes rather than the effects of environmental temperatures.

The dramatic rise in free polyamine (putrescine, spermidine, and spermine) levels after transition from vegetative to Stage 1 floral buds appear to represent a developmental change for the following reasons even when samples were taken from two different sites (Fig. 3). First, the trees at both sites remained under non-chilling conditions for over a month prior to taking the samples and we have seen above that polyamine levels did not increase in leaves of trees that were kept under non-chilling conditions even when they flowered (Fig. 2). In addition, a previous report (Prista and Voyiatzis, 2004) indicated a sharp decline in polyamine levels in olive buds within 3 weeks after the temperatures increased from above 12 to 25°C at both sites for the 2–3-week period prior to sampling floral and vegetative buds, yet polyamine levels were significantly higher in floral buds compared to vegetative buds (Fig. 3). Thus, increased levels of polyamines in floral buds compared to vegetative (both under no-chilling conditions) must be due to developmental changes in the buds rather than an environmental effect. These results indicate that changes in free polyamine levels within lateral buds may play an important role in differentiating flowering buds from vegetative buds.

Putrescine levels modestly, but significantly, increased from immature to mature fruits that are consistent with earlier findings that the exogenous
application of putrescine increased fruit yield (Iannotta et al., 1996; Rugini and Mencuccini, 1985); however, further detailed studies are needed to establish a pattern from early fruit set through various stages of fruit development to determine the role of polyamines in the fruit developmental process.

Different flower-inducing conditions in olives elicit different changes in free polyamine levels in leaves and, therefore, free polyamine levels in leaves do not correlate with the development of axillary buds into flowers. Thus, seasonal changes in free polyamines in olive leaves do not necessarily represent their involvement in reproductive development even if the timing of the two changes coincides. Early development of flowering buds involves higher levels of free polyamines compared to vegetative buds, thus polyamine levels within axillary buds may be involved in the transition from vegetative to flowering state. Free polyamine levels decline with the opening of flowers. Additional detailed studies are needed to study the role of polyamines in olive fruit developmental processes.

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