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Changes in Endogenous Abscisic Acid and Cold Hardiness in *Actinidia* Treated with Triazole Growth Retardants

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Abstract. Soil drench of either paclobutrazol or uniconazole (0.4 mg/pot) was applied to plants of *Actinidia arguta*, "Ananasnaja," to determine the effect on endogenous abscisic acid measured in November, January, and March, and the concomittant cold hardiness of the treated plants. Both paclobutrazol and uniconazole treatments significantly increased cold hardiness. Paclobutrazol was more effective in increasing ABA levels compared to uniconazole. Abscisic acid was found to be highest in January, corresponding to deep dormancy, and least in March when plants were undergoing vegetative bud break. Paclobutrazol delayed vegetative bud break by 6.3 days, while uniconazole delayed bud break by 2.9 days.

The triazole plant growth retardants are reported to protect a number of plants from environmental stresses, including chilling and freezing (Fletcher and Hofstra 1985). Whether or not this protectant role is mediated by changes in endogenous plant hormones, particularly abscicic acid and the gibberellins, is not known.

The triazole growth retardants act as gibberellin biosynthesis inhibitors by blocking the oxidative steps from ent-kaurene to ent-kaurenoic acid. The physiological result is long-term retardation of internode elongation in both monocots and dicots (Couture 1982). The role of the triazole growth retardants is less clear with respect to abscisic acid (ABA). The triazole paclobutrazol at 0.1 micromolar concentrations inhibited ABA synthesis by the fungus *Cercospora* by 33% (Norman et al. 1986). In studies with higher plants, the triazole triadimefon increased ABA in *Phaseolus* (Asare-Boamah et al. 1986). ABA was unchanged in *Pennisetum* treated with paclobutrazol (Rajasekaran et al. 1987). ABA levels increase during dormancy and cold hardening (Lalk and Dorffling 1985, Daie and Campbell 1981) and it is known to play a physiological role in chilling and freezing resistance in a number of plants (Bornman and Jonson 1980, Chen and Gusta 1985, Daie et al. 1981).

This study was undertaken to investigate the effect of two triazoles, paclobutrazol and uniconazole, on cold hardiness and endogenous ABA levels in *Actinidia arguta* during a dormancy cycle.

Materials and Methods

Plant Material and Experimental Conditions

Actinidia arguta "Ananasnaja" plants were propagated from hardwood cuttings and grown in Fafard-mix no. 2 (Cassco, Montgomery, AL, USA) in 20 cm diameter plastic pots for 9 months. They were then divided into two uniform groups of 36 plants each. In September 1991, plants in the first group were placed in a shade house under 30% shade on the campus of Alabama A&M University (Normal, AL, USA). This group was exposed to the natural progression of winter during 1991–1992. Plants in the other group were placed in a Rheem constant environment growth chamber (Puffer-Hubbard Environmental, Weaverville, NC, USA). The growth chamber was adjusted to simulate natural weather conditions, using monthly weather means calculated from the prior 27 year periods from September to March. Environmental conditions in the growth chamber were as follows: (a) respective monthly temperatures of 23.5, 17.5, 12, 8, 7, 8, and 11.5°C for the months of September to March; (b) respective monthly relative humidities (%) of 80, 79, 81, 78, 63, 72, and 56; and (c) 14, 13, 13, 12, 11, 12, and 13 h of daylight. Day and night temperatures were the same. Light intensity provided by fluorescent tubes were 250 μmol m^{-2} s^{-1}. Each group was subdivided into three groups of 12 plants each for growth regulator treatment. Paclobutrazol, [PAC: (2RS, 3RS)-1-(4-chlorophenyl)-4,4-dimethyl-2-1,2,4-triazol-1yl)pentan-3-ol,] and uniconazole [UNI: (E)-1-(p-cholorophenyl)-4,4-dimethyl-2(1,2,4-triazol-1-yl)-1-penten-3-ol] were applied as a soil drench at the rate of 0.4 mg/pot in mid-September 1 week after potting.

Extraction and Purification of Endogenous Abscisic Acid

Pieces of stem from secondary growth were excised from plants in each replication in November, January, and March, minced using a razor blade into paper thin pieces, and then 1 g fresh wt of stem was extracted at 4°C for 72 h with 10 ml of 80% methanol containing 10 mg/L butylated hydroxytoluene (BHT) as an antioxidant. The extract was centrifuged for 15 min at 3000 *g* in a Beckman TJ-6 refrigerated centrifuge. The pellet was discarded and the supernatant was passed through a premoistened Sep-Pak disposable chromatography cartridge. The eluent was evaporated to dryness under nitrogen. The residue was dissolved in 1 ml of 25 mM Tris-buffered saline (25 mM Tris-HCl, 100 mM NaCl, 1 mM $MgCl_2$, 0.1% sodium azide, pH 7.5) and refrigerated at 4°C until assayed for ABA.

Enzyme Immunoassay for Endogenous ABA

A commercially available monoclonal antibody immunoassay for ABA which is sensitive to 0.02 pmol was used in this study to quantify ABA. Aliquots of the purified extracts (100 µl) from each treatment were removed and their ABA levels were determined using a Phytodetek TM monoclonal antibody enzyme immunoassay (Idetek, Inc. San Bruno, CA, USA). ABA was first labeled with alkaline phosphatase (tracer), then added with the plant extract to the antibody-coated microwells provided in the kit. A competitive binding reaction was thus initiated between a constant amount of the tracer, a limited amount of the antibody, and the unknown sample containing ABA. In this process, the ABA in the sample competes with the tracer for binding site with antibody. The unbound tracer is washed away using the wash solution before the substrate is added. Hence, the intensity of the yellow color produced is inversely proportional to the amount of ABA in the sample.

The desired number of strips with microwells coated with antibodies were removed from −20°C storage and placed in a strip holder. Aliquots (100 µl) of standards or sample were added to each well using a multichannel pipette. Then, 100 µl of tracer was added to each well and the solutions were mixed gently, covered with plate sealer, and incubated at 4°C for 3 h. After incubation, the solutions were decanted. Wash solution (200 µl) was placed in each well, decanted, and repeated twice. To each well was then added 200 µl substrate solution (PNPP: P-nitrophenol phosphate). The microwells were covered and incubated at 37°C for 60 min in a forced air TM Microplate Incubator (Idetek Inc., San Bruno, CA, USA). The strips were removed from the incubator and one drop of stopping reagent was added to each well. After 5 min, absorbance was read at 405 nm on a Titertek Miniskan vertical-path, single channel photometer (Flow Laboratories, Inc., McLean, VA, USA). The optical densities were recorded and converted to percentage binding (B/Bo) using the equation:

$$B/B_0\% = \frac{\text{Standard or Sample OD} - \text{NSB OD}}{B_0\ \text{OD} - \text{NSB OD}} \times 100$$

where

B_0 = 100% Binding
NSB = Nonspecific binding = 0% Binding

The measured optical densities for each sample were related to the ABA concentration by means of the following standard curve: $y = -0.405 - 0.675x$, $r^2 = 0.98$.

Evaluation of Cold Hardiness

Stem sections of 1 cm length were collected from secondary growth from plants in each replication in early January and immediately used in controlled freezing tests, followed by evaluation of electrical conductivity to determine their cold hardiness. An LT-50 low temperature circulating bath (Neslab Instruments, Dublin, CA, USA) containing absolute methanol and equipped with an ETP-30 Electronic Temperature Programmer was used to cool stem samples which had been placed in glass vials (1.5 × 4 cm), from temperatures of 2.5°C to −20°C at a rate of 2.5°C/h.

Stem samples were removed from the low temperature bath after being exposed to three test temperatures (−10, −15, and −20°C) for 1 h. After samples were allowed to equilibrate for 1.5 h at room temperature, 10 ml of distilled water were added to each tube containing a stem section. Samples were then allowed to sit 24 h at 20°C with intermittent stirring. Initial conductivity values of the resultant solutions were then determined with a YSI Model 32 conductivity meter (Yellow Springs Instrument Co., Yellow Springs, OH, USA). The stem samples were then boiled for 5 min, brought up to the original volume (10 ml) with distilled water, and again allowed to sit 24 h with intermittent stirring before a final reading was made on the conductivity meter. Percent electrolyte leakage (EL) at each temperature was defined as

$$EL\ (\%) = (\text{Initial EC/Final EC}) \times 100$$

where EC is electrical conductivity (Ketchie et al. 1972).

Statistical Analysis

All data were subjected to analysis of variance as a randomized complete block design of four replications and three growth regulator treatments with three repeated measurements over time (November, January, and March) within each of two environments (growth chamber and shade house). An analysis of variance was performed for main effects and interactions of growth regulator treatment, location, and time of sampling using SAS (SAS Institute 1982). Means were separated using Tukey's HSD procedure at the 5% level of significance.

Results and Discussion

Figure 1 illustrates the endogenous ABA levels extracted from stem sections of *Actinidia arguta* "Ananasnaja" treated with no growth regulator versus that treated with either uniconazole or paclobutrazol and then grown under natural winter conditions under 30% shade or in a growth chamber under simulated winter conditions. There was no significant effect (Fig. 1) of the location (growth chamber vs. shade house) on the levels of ABA, but

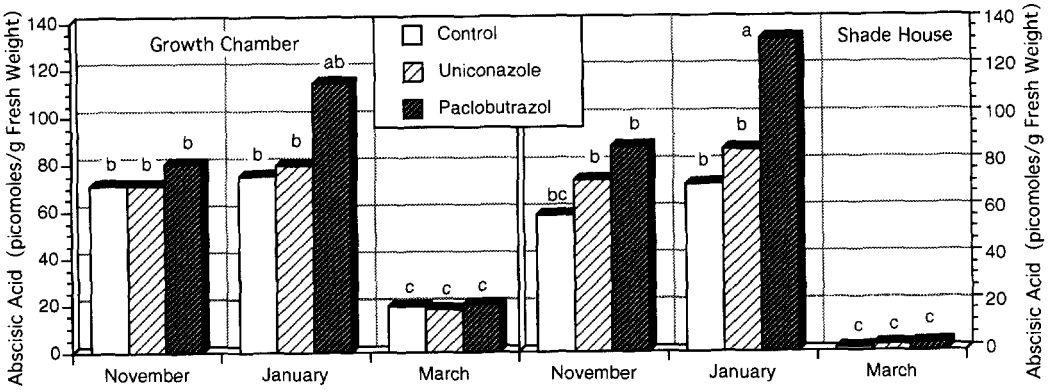

Fig. 1. Endogenous abscisic acid levels for November, January, or March in stem tissue of *Actinidia arguta* treated with a 0.4 mg soil drench of either uniconazole or paclobutrazol and then grown in the growth chamber or the shade house. Significance of the ANOVA with L = Location, GR = growth regulator, M = month: L^{ns}, $Rep(L)^{ns}$, GR***, L × GR^{ns}, Rep × $GR(L)^{ns}$, M***, L × M*, M × GR***, L × M × GR^{ns}, where ns = nonsignificant and *, **, and *** are significant at the 0.05, 0.01, and 0.001 levels, respectively. Means were separated using Tukey's HSD procedure at the 5% level of significance.

there was a highly significant effect of collection date, indicating the expected changes in endogenous ABA with the progression through the simulated winter season. ABA levels were highest in January during deep dormancy and least in March when plants were initiating spring growth. Growth regulators also exerted very highly significant effects on ABA levels. In each case, the triazole paclobutrazol enhanced the endogenous ABA content, particularly in January when plants were still in deep dormancy. This resulted in a highly significant growth regulator × month interaction. Uniconazole has been reported to have a greater effect than paclobutrazol in decreasing height growth of marigold (Gilbertz 1991). In this study on its influence on endogenous ABA levels, paclobutrazol had a greater effect than uniconazole enhancing ABA levels relative to controls.

Many plants are capable of developing resistance when they are under stress conditions; it is hypothesized that adaptation to such conditions is hormonally mediated, particularly by ABA (Boussiba et al. 1975). Endogenous ABA prevents chilling injury in some plants (Rikin and Richmond 1976, Rikin et al. 1979) and increases freezing tolerance (Chen and Gusta 1985, Chen et al. 1983). Reports relating the influence of triazoles on ABA levels are limited and conflicting. Paclobutrazol at 0.1 μM inhibits ABA synthesis by 33% in the fungus *Cercospora rosicala* (Norman et al. 1986). In contrast, Rajasekaran et al. (1987) reported that paclobutrazol did not affect endogenous ABA content in *Pennisetum purpureum*. The results reported here with the woody species *Actinidia arguta* agree with the finding of Asare-Boamah et al. (1986) who reported increased ABA content in triazol-treated *Phaseolus vulgaris*, a herbaceous species. The conflicting results reported with the fungus *Cercospora* are not hard to reconcile since it is possible that ABA biosynthesis pathway differs between lower and higher plants as suggested by Davis (1988).

The amount of electrolyte leakage from stem segments that had been exposed to different temperatures in controlled freezing tests was proportional to the amount of cold damage experienced by the stem segments (Fig. 2). The lower the temperature, the greater the amount of damage and hence leakage measured from the stem segments. Control plants experienced the greatest amount of damage and the triazole-treated plants the least. There was a highly significant growth regulator by location interaction which reflected the greater efficacy of paclobutrazol in the growth chamber study and uniconazole in the shade house.

Even though triazoles appear to be effective in increasing cold hardiness of *Actinidia*, a woody liana, they may not increase cold hardiness of other woody plants. There are reports of triazole-treated herbaceous plants, such as *Phaseolus* (Asare-Boamah and Fletcher 1986, Lee et al. 1985) and *Cucumis* (Wang 1985), expressing increased low temperature tolerance or delayed chilling injury symptoms. In contrast, reports on woody plants indicate either more winter injury on paclobutrazol-treated cherry and peach (Proebsting and Mills 1985) or no effect on *Vitis* (Ahmedullah et al. 1986) or peach (Walser and Davis 1986). If these divergent results were due to a difference between chilling and freezing protection as suggested by Davis et al. (1988), then there would have been no difference in cold hardiness of the treated *Actinidia* in this study. It may be that *Actinidia arguta*, a woody

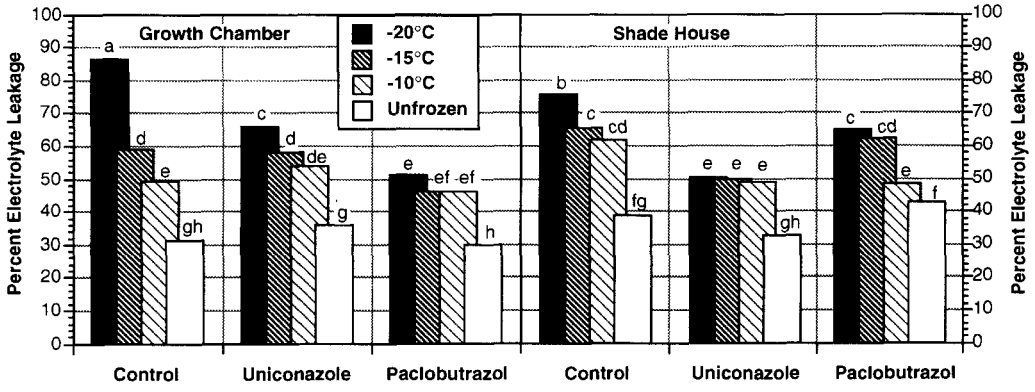

Fig. 2. Percent electrolyte leakage from stem sections exposed to controlled freezing tests. Plants had been treated with a 0.4 mg soil drench of either uniconazole or paclobutrazol and then grown in the growth chamber or the shade house. Significance of the ANOVA with L = Location, GR = growth regulator, T = temperature: L***, Rep(L)^{ns}, GR***, GR × L***, Rep × GR(L)^{ns}, T***, T × L***, GR × T***, GR × T × L***, where ns = nonsignificant and *** is significant at the 0.001 level. Means were separated using Tukey's HSD procedure at the 5% level of significance.

subtropical liana, has a different mechanism regulating cold hardiness that is responsive to triazole treatment. The pattern of response with respect to both cold hardiness and ABA levels could indicate a possible mechanism for regulation of this protective effect. Foliarly applied ABA has been reported to increase cold hardiness and corresponding soluble carbohydrate levels in treated *Actinidia* (Tafazoli and Beyl 1993). A positive relationship has been reported between high soluble carbohydrates, particularly glucose and fructose in *Actinidia chinensis* and *A. arguta*, and freezing tolerance (Kim and Kim 1986). They also reported that a high amount of unsaturated relative to saturated fatty acids and elevated levels of ABA were correlated to freezing tolerance.

There was a highly significant effect of growth regulator on speed of vegetative bud break (Fig. 3). Plants treated with paclobutrazol had an average delay in bud break of 6.3 days relative to the controls. The effects of uniconazole were intermediate between the control and paclobutrazol, with an average of 2.9 days delay. There was also a highly significant effect of location on vegetative bud break with plants in the shade house exhibiting bud break an average of 6 days earlier. Plants in the shade house were exposed to the normal fluctuations of temperature occurring during a natural sequence of winter in north Alabama where our winters are characterized by many periods of warm temperature interspersed with colder temperatures. These oscillations make fruit production in the area risky for some crops. The delay in vegetative bud break caused by triazole treatment particularly paclobutrazol would be beneficial in reducing the risk inherent in premature bud break. Delayed flowering

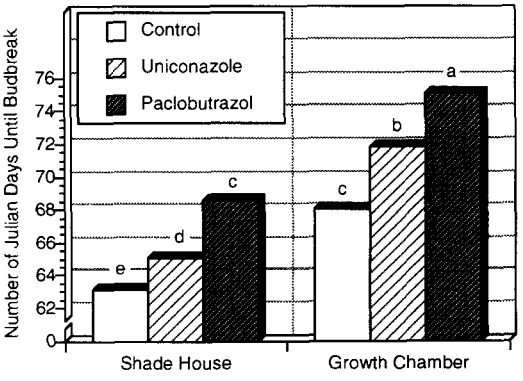

Fig. 3. Delay of vegetative bud break from *Actinidia arguta* plants treated with a 0.4 mg soil drench of either uniconazole or paclobutrazol and then grown in the growth chamber or the shade house. Significance of the ANOVA with L = Location, GR = growth regulator: L***, GR***, L × GR**, where ** and *** are significant at the 0.01 and 0.001 levels, respectively. Means were separated using Tukey's HSD procedure at the 5% level of significance.

induced by foliar application of paclobutrazol has been reported in apple (Stinchcombe et al. 1984).

This research reports significant increases in cold hardiness induced by triazole treatment in *Actinidia arguta*, a woody subtropical vine related to kiwifruit. Paclobutrazol was more effective with respect to increasing endogenous ABA levels and delaying vegetative bud break than was uniconazole. These results suggest that regulation of ABA levels may be one of the means that the triazole's confer stress protection.

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References

Ahmedullah M, Kawahami A, Sandidge CR III, Wample RL (1986) Effect of paclobutrazol on the vegetative growth, yield, quality, and winterhardiness of buds of Concord grape. HortScience 21:273–274

Asare-Boamah NK, Fletcher RA (1986) Protection of bean seedlings against heat and chilling injury by triadimefon. Physiol Plant 67:353–358

Boussiba S, Rikin A, Richmond AE (1975) The role of abscisic acid in cross adaptation of tobacco plants. Plant Physiol 56:337–339

Bornman CH, Jonson E (1980) *Nicotiana tobacum* callus studies × ABA increases resistance to cold damage. Physiol Plant 48:491–498

Chen HH, Gusta LV (1985) Abscisic acid induced freezing tolerance in cultured plant cells. Plant Physiol 73:71–75

Chen HH, Li PH, Brenner ML (1983) Involvement of abscisic acid in potato cold accumulation. Plant Physiol 71:362–365

Couture RM (1982) PP333: A new experimental growth regulator from ICI. Proc Plant Growth Reg Soc Am 9:59

Daie J, Campbell WF, Seeley SD (1981) Temperature stress induced production of ABA and dihydrophaseic acid in warm and cold season crops. J Am Soc Hort Sci 106:11–13

Daie J, Campbell WF (1981) Response of tomato plants to stress temperatures. Plant Physiol 67:26–29

Davis ID, Steffens GL, Sankhla N (1988) Triazole plant growth regulators. Hort Rev 10:63–105

Fletcher RA, Hofstra G (1985) Triadimefon, a plant multiprotectant. Plant Cell Physiol 26:238–243

Gilbertz DA (1991) Cultivar response of marigold to triazole compounds. Proc Plant Growth Reg Soc Am 18:70–71

Ketchie DO, Beeman CH, Ballard CH (1972) Relationship of electrolytic conductance to cold injury and acclimation in fruit trees. J Am Soc Hort Sci 97:403–406

Kim HY, Kim KR (1986) Relationship between organic compound contents and freezing tolerance in kiwifruit plant. Res Rep Rural Devel Admin (Suweon) 28:95–103

Lalk I, Dorffling K (1985) Hardening ABA proline and freezing resistance in two winter wheat varieties. Physiol Plant 63:287–292

Lee EH, Byun JEK, Wilding SJ (1985) Paclobutrazol induced sulfur dioxide, chilling, and high temperature tolerance in snap bean plants (abstract). Proc Plant Growth Reg Soc Am 12:78

Norman SM, Bennett RD, Poling SM, Marier VP, Nelson MD (1986) Paclobutrazol inhibits abscisic acid biosynthesis in *Cercospora rosicola*. Plant Physiol 80:122–125

Proebsting EL, Mills HH (1985) Cold resistance in peach, apricot, and cherry as influenced by soil applied paclobutrazol. HortScience 20:88–90

Rajasekaran K, Hein MB, Vasil IK (1987) Endogenous abscisic acid and indole-3-acetic acid and somatic embryogenesis in cultured leaf explants of *Pennisetum purpureum* Schem. Plant Physiol 84:47–51

Rikin A, Atsmon D, Giltler C (1979) Chilling injury in cotton (*Gossypium hirstum*) by abscisic acid. Plant Cell Physiol 20:1537–1546

Rikin A, Richmond AE (1976) Amelioration of chilling injuries in cucumber seedlings by abscisic acid. Physiol Plant 38:95–97

SAS Institute (1982) SAS user's guide: statistics. SAS Institute Inc., Cary, North Carolina

Stinchcombe GR, Copas E, Williams RR, Arnold G (1984) The effect of paclobutrazol and daminozide on the growth and yield of cider apple trees. J Hort Sci 59:323–327

Tafazoli E, Beyl CA (1993) Foliarly applied abscisic acid and its effect on cold hardiness and carbohydrates in *Actinidia arguta*. Iran J Agri (in press)

Walser RH, Davis TD (1986) Cold hardiness, rest, and growth of peach trees as influences by paclobutrazol. Proc Plant Growth Reg Soc Am 13:177–181

Wang CY (1985) Modification of chilling susceptibility in seedlings of cucumber and zucchini squash by the bioregulator paclobutrazol (PP 333). Sci Hort 26:293–298