

EVIDENCE OF GROWTH STIMULATION BY LOW CONCENTRATION OF GIBBERELLIN SYNTHESIS INHIBITORS

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ABSTRACT

There are several compounds used in ornamental production systems and in the care and maintenance of trees in urban landscapes because they are known to inhibit the synthesis of gibberellins via the isoprenoid pathway when applied at labeled rates. The consequence of this effect on plants is typically reduction of vegetative growth with a re-partitioning of assimilates to reproductive growth and fine root development. However, at low concentrations that may arise due to environmental degradation or misapplication of applied compounds, growth stimulation may occur. Examples of stimulation of growth in plants and a possible explanation for this seemingly anomalous response related to energy production in mitochondria via the electron transport chain are presented.

INTRODUCTION

There are a number of gibberellin synthesis inhibitors, frequently referred to as growth retardants, which are used in ornamental plant production systems and in the maintenance of urban trees. The major modifications to plants induced by growth retardants are shorter internodes, less height, reduced cambial growth, and somewhat smaller, darker green leaves (Bai et al, 2004; Davis and Curry, 1991). These effects are thought to arise from inhibition of various steps in the isoprenoid pathway leading to gibberellins (Fletcher et al., 2000; Rademacher, 2000) (Fig. 1). Additional benefits to plants of treatment with many of these growth retardants also have been reported and include increased tolerance to drought and resistance to fungal diseases. For these plant responses, the mode of action involves further alterations in the isoprenoid biosynthetic pathway related to inhibition of sterols and promotion of abscisic acid (Chaney, 2002). Users of the so-called growth retardants have become conditioned to expect the plant responses mentioned above. If something other than this occurs, it is frequently discounted as an anomaly related to weather conditions, an unusual soil situation, or possibly a mistake in mixing solutions or calibrating equipment.

Although there are many gibberellin synthesis inhibitors as indicated in Fig. 1, the remainder of this discussion will focus on paclobutrazol and flurprimidol. Both of these compounds were formulated for use on trees in urban areas until about 2.5 years ago when flurprimidol tablets for implanting into the trunks of trees were removed from the market. Flurprimidol is still available for use in ornamental production and turf management.

Several experiments were designed to investigate the potential of stimulation of growth of intact plants and of electron transport by the growth retardants paclobutrazol and flurprimidol at concentrations lower than that which reduces these same processes.

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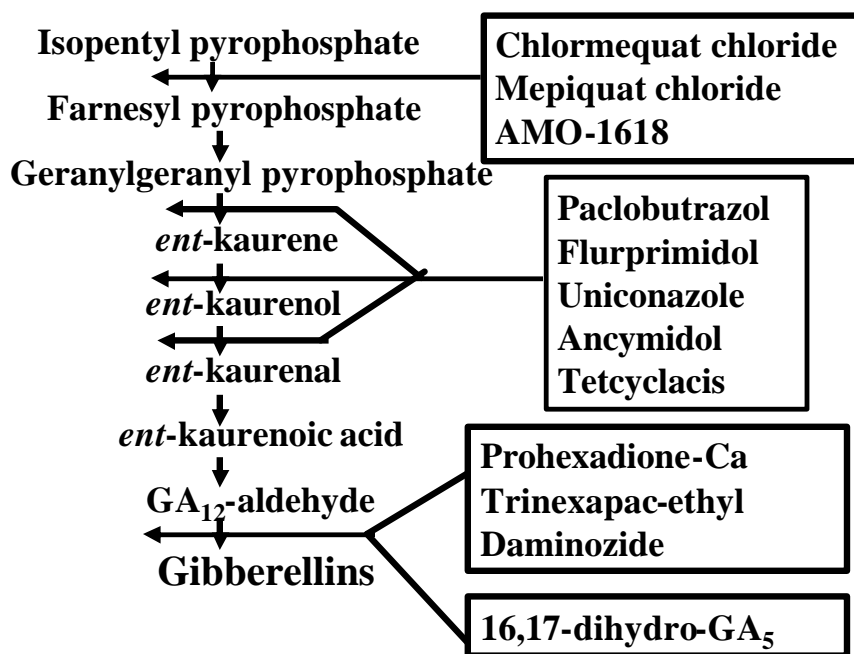


Figure 1. Isoprenoid pathway for synthesis of gibberellins and sites of inhibition by various growth retardants.

MATERIALS AND METHODS

Height Growth of Intact Plants

Mature silver maple (*Acer saccharinum* L.) trees (42.4 cm mean diameter), located under electric distribution wires in Griffith and Gary, Indiana, were treated in early spring with paclobutrazol (Profile 2SC[®]) by soil injection or flurprimidol (Cutless TI[®]) by trunk implantation using recommended rates (0.7 g paclobutrazol per cm diameter and 0.2 g flurprimidol per cm diameter). Seeds from three silver maple trees treated with flurprimidol and from two untreated trees were collected in May, one-month after treatment. One year after treatment, seeds were collected in May from untreated and both paclobutrazol and flurprimidol treated trees. Seeds of silver maple mature shortly after leaves emerge in the spring, do not have a dormant period, and will germinate immediately. The seeds were germinated shortly after collection and the seedlings were grown in a greenhouse in 10-cm-diameter pots containing Fafard #2 rooting medium. Height of the seedlings was measured at two-week intervals for 16 weeks beginning when seedlings were four weeks old. There were 12 replications of each treatment in a completely randomized experimental design. Results were analyzed using analysis of variance for a completely randomized design and differences between means at $p=0.05$ determined using Tukey's w procedure.

Electron Transport

Six mature European black alder (*Alnus glutinosa* L.) trees (4-8 cm diameter) in a plantation at the Martell Experimental Forest, Purdue University, West Lafayette, Indiana, USA, were selected for their vigorous growth and condition. Fully expanded leaves were collected from the trees in mid-summer and their mitochondria isolated and purified using standard procedures (Hamasur et al., 1990). Flurprimidol and paclobutrazol concentrations including 0, 1.0×10^{-5} , 5.0×10^{-5} , 1.0×10^{-4} , 5.0×10^{-4} , 1.0×10^{-3} , 1.5×10^{-3} , 2.0×10^{-3} , and 2.5×10^{-3} mg L⁻¹ were applied to an assay media containing the isolated mitochondria to determine the *in vitro* effect of the growth retarding compounds on electron transport as evident by NADH oxidation and cytochrome c reduction. There were 12 replications of each assay.

Seedlings of silver maple (*Acer saccharinum* L.) were exposed to a range of flurprimidol concentrations including 0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1.0, 2.0 and 5.0 mg L⁻¹ based on growing medium volume. The growth retardant was applied suspended in an aqueous solution to the surface of the growing medium 15 days after the seeds were planted in 10-cm-diameter pots.

Another set of silver maple seedlings was treated one week after planting in 10-cm-diameter pots with a range of paclobutrazol concentrations including 0, 0.0001, 0.0003, 0.0005, 0.0007, 0.001, 0.005, 0.01, 0.05, and 0.1 mg L⁻¹ based on growing medium volume.

NADH oxidation and cytochrome reduction for mitochondria isolated from mature leaves of these seedlings were measured to determine the effect of *in vivo* exposure to the growth retarding compounds on electron transport. The experimental design for both of these experiments was a randomized complete block with 12 replications.

For NADH oxidation and cytochrome c reduction assays of the mitochondria, the reaction mixture consisted of 50 mM Tris buffer (pH 7.4), 25 mM MgCl₂, 40 mM KCl, and 0.1 M sucrose, and either 100 μM NADH or 0.1 mg ml⁻¹ cytochrome c. Changes in optical density were recorded with a BioSpec-1601 UV-visible spectrophotometer (Shimadzu) at 340 nm for NADH oxidation or 550 nm for cytochrome c reduction. Extinction coefficients of 6.22 ($E_{340} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$) and 19 ($E_{550} = 19 \text{ mM}^{-1} \text{ cm}^{-1}$) were used to calculate NADH oxidation and cytochrome c reduction rates, respectively. Both were expressed as nmoles per mg protein per minute (Crane et al., 1988). At least three measurements were made for each sample.

Data were analyzed using ANOVA and differences between means at $p=0.05$ determined with Tukey's *w* procedure.

RESULTS

Height Growth of Intact Plants

Seeds collected one month after treatment of the parent trees had accumulated enough flurprimidol that the growth of seedlings was reduced (Fig. 2). However, one year after treatment of the parent trees with flurprimidol, seedlings grown from seeds produced that year grew taller than seedlings grown from seeds collected from untreated trees (Fig. 3). The growth stimulation was presumably due to degradation and diminished translocation of the growth retardant in the second growing season such that the concentration accumulated in the developing seeds was so low that it stimulated rather than reduced growth.

Electron Transport

Electron transport in mitochondria isolated from European black alder leaves and treated *in vitro* with a range of concentrations of flurprimidol or paclobutrazol varied in response from stimulation to reduction. Concentrations of 5.0×10^{-5} mg L⁻¹ flurprimidol or 5.0×10^{-4} and $1.0 \times$

10^{-3} mg L⁻¹ paclobutrazol applied directly to the assay medium stimulated the rate of NADH oxidation (Figure 4A, B). In contrast, higher concentrations of flurprimidol from 1.0×10^{-3} to 2.5×10^{-3} mg L⁻¹ or paclobutrazol from 2.0×10^{-3} to 2.5×10^{-3} mg L⁻¹ applied directly to isolated mitochondria of European black alder reduced NADH oxidation (Figure 4A, B).

A similar pattern of response was found for cytochrome c reduction in mitochondria. Flurprimidol was stimulatory at 5.0×10^{-5} mg L⁻¹ and inhibitory at concentrations higher than 1.5×10^{-3} mg L⁻¹ (Figure 4C). Three concentrations of paclobutrazol applied (5.0×10^{-5} , 1.0×10^{-4} , and 5.0×10^{-4} mg L⁻¹) stimulated cytochrome c reduction whereas only the highest concentration used (2.5×10^{-3} mg L⁻¹) suppressed it (Figure 4D).

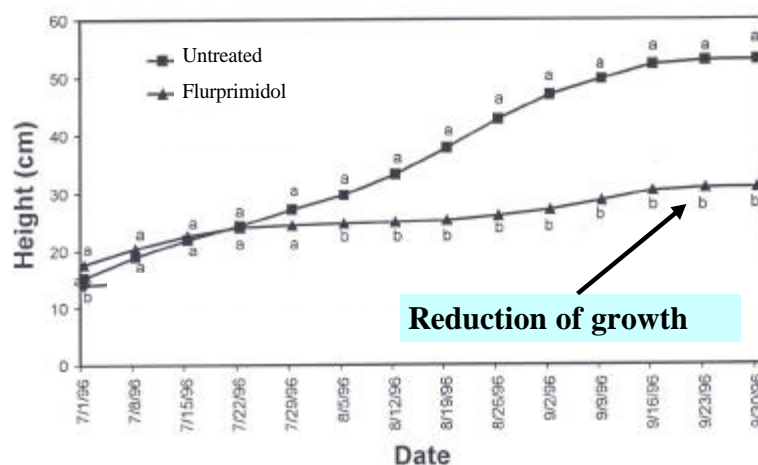


Figure 2. Height growth of silver maple seedlings grown from seeds one month after treatment of parent trees with flurprimidol. Heights at the same date labeled with the same letter are not statistically different at $p=0.05$.

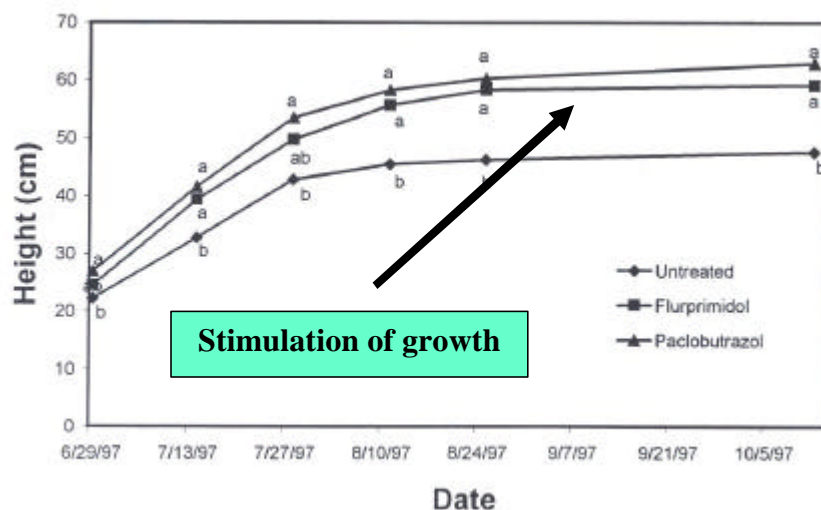


Figure 3. Height growth of silver maple seedlings grown from seeds one year after treatment of parent trees with paclobutrazol or flurprimidol. Heights at the same date labeled with the same letter are not statistically different at $p=0.05$.

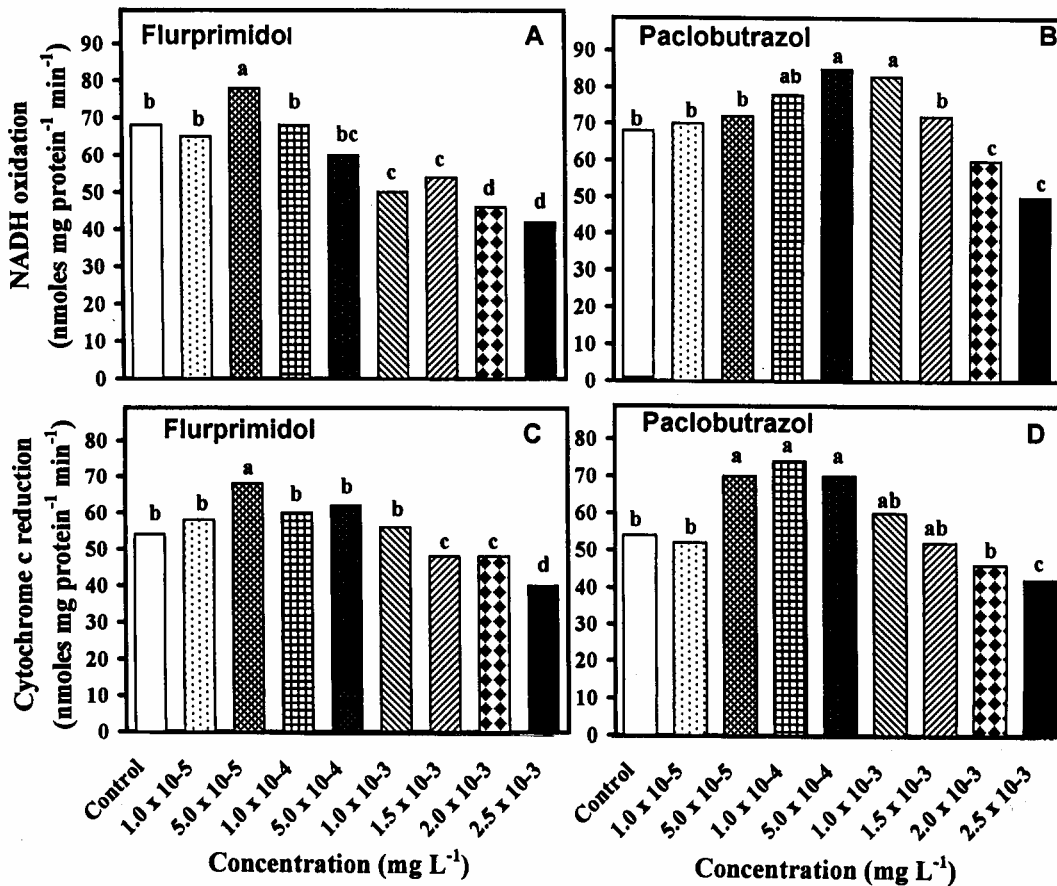


Figure 4. Electron transport in isolated mitochondria of European black alder treated *in vitro* with a range flurprimidol or paclobutrazol concentrations. Bars within a chart with the same lower case letter are not significantly different at $p=0.05$. (A) NADH oxidized in response to flurprimidol. (B) NADH oxidized in response to paclobutrazol. (C) Cytochrome c reduction in response to flurprimidol. (D) Cytochrome c reduction in response to paclobutrazol.

Flurprimidol concentrations of 0.1, 0.5, 1.0, 2.0, or 5.0 mg L⁻¹ reduced growth of silver maple seedlings so much that leaf biomass was insufficient for isolation of mitochondria. Treatment of silver maple seedlings with 0.01 and 0.05 mg L⁻¹ flurprimidol applied to the surface of the growing medium was found to reduce NADH oxidation in mitochondria isolated from fully expanded leaves by 15 to 18 percent. Lower concentrations of 0.001 or 0.005 mg L⁻¹ had no effect (Figure 5A). In contrast, cytochrome c reduction was increased 24 percent by treatment of seedlings with 0.001 mg L⁻¹ flurprimidol whereas the other concentrations applied did not change cytochrome c reduction from that of mitochondria isolated from untreated controls (Figure 5B).

Electron transport was more sensitive to paclobutrazol. Low concentrations (0.0003, 0.0005, and 0.0007 mg L⁻¹) stimulated NADH oxidation by as much as 32 percent, whereas 0.001, 0.005, and 0.01 mg L⁻¹ had no effect. High concentrations of paclobutrazol (0.05 and 0.01

mg L⁻¹) reduced NADH oxidation by 12.5 percent (Figure 6A). Concentrations of 0.0005, 0.0007, 0.001, 0.005, and 0.01 mg L⁻¹ stimulated cytochrome c reduction from 15 to 69 percent. In contrast, both lower (0.0003 mg L⁻¹) and higher (0.05 and 0.1 mg L⁻¹) concentrations of paclobutrazol applied to the growing medium had no effect on cytochrome c reduction in mitochondria isolated from the silver maple seedlings growing in pots (Figure 6B).

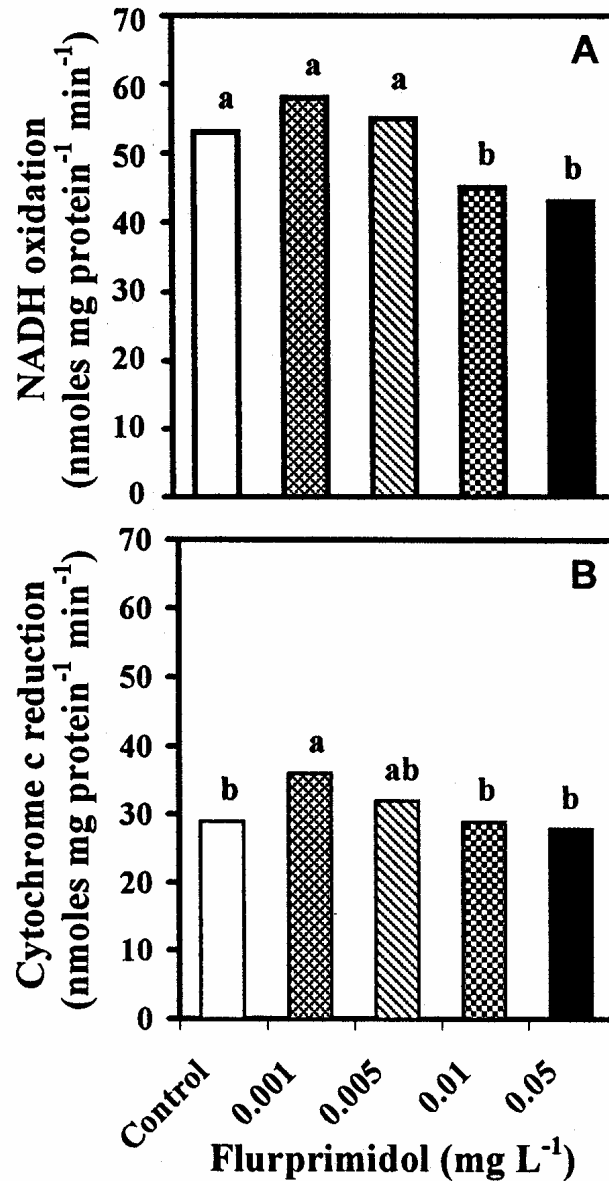


Figure 5. Electron transport in leaf mitochondria isolated from silver maple seedlings grown in pots treated with a range of flurprimidol concentrations. Bars within a chart with the same lower case letter are not significantly different at $p=0.05$. (A) NADH oxidation (B) Cytochrome c reduction.

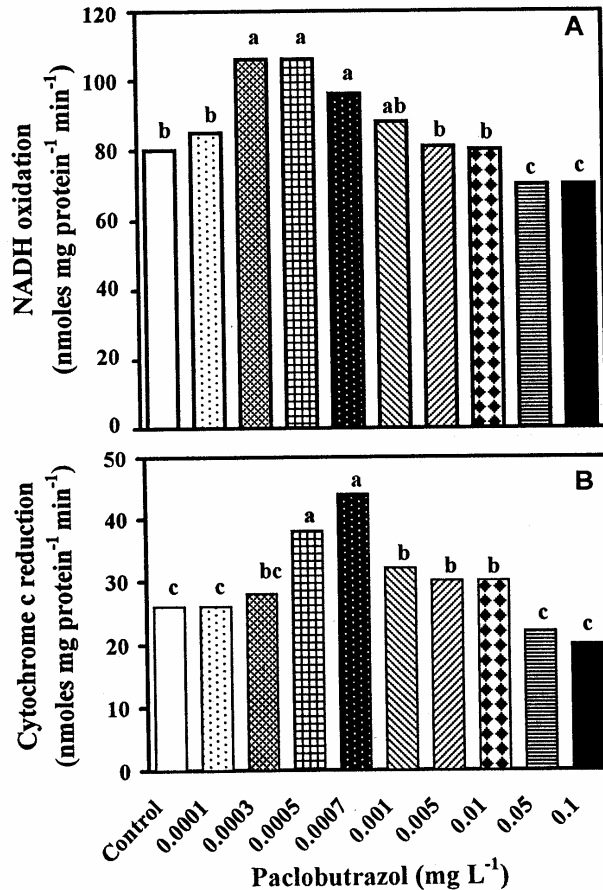


Figure 6. Electron transport in leaf mitochondria isolated from silver maple seedlings grown in pots treated with a range of paclobutrazol concentrations. Bars within a chart with the same lower case letter are not significantly different at $p=0.05$. (A) NADH oxidation (B) Cytochrome c reduction.

DISCUSSION

Stimulation of growth by low concentrations of growth retardants such as paclobutrazol and flurprimidol has been observed before, but only a few published reports can be found, probably not because it rarely occurs but rather because the effect is subtle and most experiments have been designed to test for growth retardation. An incidental observation was made by Arnold and Davis (1994) that 800 mg/L paclobutrazol applied to the foliage of Chinese chestnut (*Castanea mollissima*) increased internode elongation. Little significance was given to the results even though Estabrooks (1993) and Tromp (1987) had observed shoot growth stimulation in apple trees treated with paclobutrazol. Driessche (1990, 1996) also found enhancement of shoot dry weight at low concentrations of paclobutrazol in lodgepole pine (*Pinus contorta*), white spruce (*Picea glauca*), and Douglas-fir (*Pseudotsuga menziesii*). When one-inch-caliper red maple (*Acer rubrum*) were treated with flurprimidol by soil injection at a rate of 0.5 g a.i./diameter inch in March 1983, height growth was reduced by 25% that year. In the 1984

growing season, height growth was reduced by only 12%. In the third season after treatment, height growth increased 26% compared to untreated control trees (Gilliam et al., 1988).

Another example of growth stimulation was found in a study with cutting of Americana 'Red' and 'Orange', and Eclipse 'Salmon' varieties of geraniums to which paclobutrazol was applied to the foliage, the effect on height 51 days after treatment ranged from 5.0% increase at 2 ppm paclobutrazol to decreases of 3.9% and 27.6% at 4 ppm and 8 ppm paclobutrazol, respectively (Barcel, 1997). Arnold and McDonald (2001) found 5 to 10 mg/L concentrations of paclobutrazol as a substrate drench in the nursery production phase resulted in increased vegetative growth and flowering of blue plumbago (*Plumbago auriculata*) when these were transplanted to the landscape. In yet another example of growth stimulation, flurprimidol was found to inhibit growth in height at concentrations as low as 0.2 mg/L applied to the soil in which zinnias were grown, but concentrations of 0.04, 0.008, and 0.0016 mg/L stimulated growth. The growth of tobacco callus tissue also was stimulated at low concentrations of flurprimidol (Premachandra et al., 1996).

Although rare, cambial growth too can be stimulated by growth retardants. It was reported that trunk cross-sectional area of four-year-old pear trees was increased after treatment with 1000 or 2000 mg/L paclobutrazol (Costa et al. 1995). Although this result is not consistent with others showing cambial growth suppression following treatment with paclobutrazol (Bai et al., 2004), it shows that in some species growth retardants can promote cambial growth.

It is not unusual for substances with hormonal activity to have both inhibitory and stimulatory effects over a range of concentrations. Auxin is a good example. At low concentrations auxins mediate many physiological processes essential to growth. At high concentrations, however, auxins can be toxic to plants (Leopold and Kriedemann, 1975). The herbicide 2,4-D, for example is a synthetic auxin compound. It can be added at low concentrations (ca. 1.0 ppm) to growth media to stimulate growth of cell cultures (George, 1993), but of course, its ability to kill weeds at about 2000 ppm is well known. Another example is the cotton defoliant thidiazuron, a potent cytokinin, which has become an important tool at low concentrations to induce morphogenesis of many species of plants in tissue cultures (Murthy et al., 1998).

Both paclobutrazol and flurprimidol bind to the protoheme iron of cytochrome P₄₅₀ dependent monooxygenases, inhibiting their activity and blocking the synthesis of gibberellins (Grossmann, 1992). Degradation of ABA, which is catalyzed by P₄₅₀ dependent monooxygenase, is inhibited in a similar way by these growth retardants (Rademacher, 2000).

In plant mitochondria, cytochrome oxidase, the final electron carrier in the proton-pumping assembly of the electron transport chain, catalyzes the transfer of electrons from cytochrome c to molecular oxygen, the final acceptor (Berg et al., 2002). Carbon monoxide, potassium cyanide, and sodium azide are effective inhibitors of this process, blocking electron flow in cytochrome oxidase by binding to the iron atoms of the enzyme (Caughey et al., 1993). It is proposed that paclobutrazol and flurprimidol may bind to the iron of cytochrome oxidase in the mitochondria respiratory pathway in the same manner because of their ability to complex with the heme moiety of cytochrome P₄₅₀ dependent enzymes in the gibberellin synthesis pathway. Oxidation of NADH, the first step of the electron transport chain, also is dependent on an iron-sulfur protein and P₄₅₀ (Lewis, 1996).

Results showing stimulation of height growth at low concentrations could have implications for use of growth retardants. Growth inhibition achieved in the first few weeks or even years after treatment could revert to growth stimulation as the concentration of retardants in

annual plants or the crowns of trees declines due to lower rates of uptake and movement from the soil and/or degradation in soil and plant tissues. The mode of action for growth stimulation is not at all clear, but stimulation of electron transport and release of metabolic energy may be one of the explanations.

Accidental discovery and serendipity are very important and have led to many of the most significant advancements in science. Louis Pasteur's comment, "*In the field of observation chance favors only the mind that is prepared*", should always be remembered and might lead to better understanding or even new applications for growth regulators currently in use.

CONCLUSIONS

Only a few of the many examples of unexpected plant responses to applications of growth retardants are presented here. There are probably even more observations made in commercial greenhouses and fields that have just been discounted as anomalies. Some of these observations may in actuality be anomalies and flukes, but many may be significant plant responses trying to bubble to the surface as the pot of plant growth regulator science is boiled and stirred.

Results of the experiments presented here suggest that the response of plants to growth retardants such as paclobutrazol and flurprimidol involve more than just the inhibition of gibberellin biosynthesis and the reduction of cell elongation and growth in height. Effects on the production of metabolic energy via the electron transport chain in the final phase of respiration could be an additional mode of action for growth retardants. Although the mode of action for reduction of electron transport is not clearly understood, it is likely that paclobutrazol and flurprimidol act like the known inhibitors of cytochrome oxidase, since both growth retardants complex with the heme moiety of cytochrome P₄₅₀ in other metabolic processes. A mechanism for the stimulation of electron transport by paclobutrazol and flurprimidol presented here is not apparent. No similar stimulation of electron transport by low concentrations of CO, KCN, or NaN₃ has been reported. Further research needs to be conducted to elucidate the mode of action for the stimulatory effect of paclobutrazol and flurprimidol at low concentrations on electron transport and its relation to promotion of growth.

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