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Effects of Soaking Cucumber and Tomato Seeds in Paclobutrazol Solutions on Fruit Weight, Fruit Size, and Paclobutrazol Level in Fruits

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Abstract. **Determination of plant growth regulator accumulation in fruits and vegetables for human consumption is an important safety issue even when it is applied to seeds. Paclobutrazol accumulated preferentially in the seedcoats when soaking cucumber (*Cucumis sativus* L., cv. Poinsett 76SR) seeds in 1000 or 4000 mg·L^{-1} paclobutrazol. Cucumber plants grown from seeds soaked in 1000 mg·L^{-1} paclobutrazol had lower average fruit weights than the control plants. Individual fruit length in cucumber was reduced by 40% when seeds were soaked in 1000 mg·L^{-1} paclobutrazol solutions for 180 minutes. Soaking tomato (*Solanum lycopersicum* L., cv. Sun 6108) seeds in 0 to 1000 mg·L^{-1} paclobutrazol did not reduce average fruit weight or diameter per treatment. Paclobutrazol residue was not detected in cucumber and tomato fruits harvested from plants grown from seeds soaked in 1000 mg·L^{-1} paclobutrazol for 180 minutes. Soaking seeds in paclobutrazol solutions represents a promising method of applying plant growth regulators to tomato and cucumber without accumulation of paclobutrazol residue in fruits.**

Application of plant growth regulators (PGRs) influences all stages of plant development, including flowering and maturation of seeds (Han and Kim, 1999; Pressman and Shaked, 1988). Synthetic inhibitors of gibberellin biosynthesis in plants (e.g., paclobutrazol) may increase (Nishizava, 1993) or reduce (Knurshid et al., 1999) fruit yield when applied to plants as soil or foliar treatments. However, current regulations of the U.S. Environmental Protection Agency (EPA) prohibit paclobutrazol applications to vegetables (EPA, 2004). Other countries and institutions mandate maximum residual levels of paclobutrazol at 0.5 mg·kg^{-1} in apples (Food and Agriculture Organization of the United Nations), 0.01 mg·kg^{-1} in stone fruits (Australia), or 0.5 mg·kg^{-1} in dry beans and small berries (Thailand) (EPA, 2004). These levels have been established based on analysis of plants subjected to soil or foliar paclobutrazol applications (Davis et al., 1988; Singh and Ram, 2000).

Presoaking seeds with PGRs is an alternative method of PGR application to plants (Pasian and Bennett, 2001; Pill and Gunter, 2001; Still and Pill, 2003). Soaking seeds in 50 (Still and Pill, 2006) or 500 (Pasian and Bennett, 2001) mg·L^{-1} paclobutrazol effectively controlled plug height in tomato, whereas soaking seeds in 500 mg·L^{-1} paclobutrazol was the most effective treatment in growth retardation of cucumber (Cho et al., 2002). However, there are no available data on how treating seeds with paclobutrazol affects fruit weight and paclobutrazol level in fruits. Quantitative analysis of PGR accumulation in fruits harvested on plants grown from PGR-treated seeds allows determination of whether this application method is suitable for food destined for human consumption. Measuring paclobutrazol level in fruits would also help to clarify the movement of seed-associated PGR into plants during and after seed germination. The objectives of the present study were 1) to determine whether tomato and cucumber fruits harvested from plants obtained from paclobutrazol-treated seeds had any detectable growth regulator residue and 2) to determine the effect of soaking tomato and cucumber seeds in paclobutrazol solutions on fruit weight and size.

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Materials and Methods

Plant materials and chemicals. The plant growth regulator used in this study was paclobutrazol (β-[(4-chlorphenyl)methyl]-α-(1,1-dimethylethyl)-1H-1,2,4-triazole-1-ethanol) in the Bonzi formulation (Syngenta Crop Protection, Greensboro, N.C.) containing 0.4% a.i.

Seed soaking. Tomato or cucumber seeds were soaked in water solutions of 0, 50, 200, 500, or 1000 mg·L^{-1} paclobutrazol for 5, 45, or 180 min at 20 °C, whereas untreated (non-soaked) seeds represented the controls. For mass spectrometric analysis (LC- MS) only, cucumber seeds were soaked in 0, 1000, or 4000 mg·L^{-1} paclobutrazol solutions for 5 or 180 min. In each treatment, 100 seeds were placed in a glass beaker with 50 (tomato) or 100 (cucumber) mL paclobutrazol solution or water. While soaking the seeds, the beakers were constantly agitated (40 rpm) on an orbital shaker Koala-Ty AP06502 (Accurate Chemical and Scientific Corp., Westbury, N.Y.). After soaking, seeds were transferred to a sieve and then dried on the surface of a filter paper (Whatman International Ltd., Maidstone, U.K.) for 24 h on an open bench at 20 °C.

Plant growth. Within 2 to 4 d after treatment application, tomato or cucumber seeds were sown one seed per cell in plastic 288 (5.9-cm^3 volume) or 164-cell (9.7-cm^3 volume) plug trays, respectively, filled with plug growth media Sunshine LP5 (Sun Gro Horticulture, Bellevue, Wash.). Each treatment consisted of 100 seeds divided into four replicates of 25 seeds each. Each replicate was randomly distributed within the plug trays. Seeds were covered with a small portion (5-mm depth) of the same substrate and placed under intermittent mist at 25 °C for 1 d. Plug trays were then moved to a 25 °C greenhouse at The Ohio State Univ., Columbus, and randomly arranged on a wet capillary mat under natural central Ohio summer conditions. Plugs were watered as needed with municipal water and fertilized with a Peters Professional water-soluble fertilizer 20N–8.7P–16.7K (Scotts-Sierra Horticultural Products Co., Marysville, Ohio) at a rate of 2 g·L^{-1} every third irrigation.

At 33 or 34 d after planting (DAP) of cucumber or tomato, respectively, subsamples of eight plants were randomly selected in each treatment and transplanted into 3.8-L plastic containers (one plant per container) filled with Metro-Mix 360 (The Scotts Co.). Flower number was estimated in five plants of each treatment of cucumber and tomato. Commercially mature fruits having yellow tips (cucumber) or pink color (tomato) were manually harvested in 7-d intervals from 40 to 68 DAP (cucumber) or in 10-d intervals from 62 to 92 DAP (tomato). Fruit weight and length of all fresh fruits in tomato and cucumber were manually measured at harvest. Fresh fruits were stored at –18 °C for further measurements of paclobutrazol residues.

Quantitative analysis of paclobutrazol in plant tissues. Cucumber seeds were manually separated into the seedcoats and the rest of the seed (embryonic axis plus cotyledons). Seed

samples consisting of 5 to 8-g seedcoats and 22 to 26 g of decoated seed parts were used for paclobutrazol extraction. Fruits obtained from seed treatments with the lowest (0 mg·L^{-1}) and highest (1000 mg·L^{-1}) paclobutrazol rates were analyzed in both crops. For paclobutrazol analysis, fruits were randomly selected from different plants of each treatment. Each treatment was analyzed in three replicates with each replicate represented by an individual fruit of tomato or cucumber. Paclobutrazol was extracted from seeds or fruits according to the protocol developed by Jia et al. (2001). All samples were homogenized and extracted twice using a blender with 200 mL methanol/water (75/25, v/v %) and 1.5 mL H_2SO_4 for 10 min. The combined extracts were filtered through Celite 545. The Celite 545 cakes with plant residues were washed with 20 mL of the same solvent mixture. The filtrates were concentrated to 50 to 60 mL on a vacuum rotary evaporator under reduced pressure at 45 °C and cleaned out from the precipitates by suction. The filtrates were adjusted to pH 10 with 2.0 M KOH and centrifuged at 6000 rpm for 15 min. The supernatant was aspirated and twice subjected to liquid–liquid extraction with equal volume of methylene chloride. The organic phase was separated, evaporated, dissolved in 2 mL cyclohexane/ether (50/50, v/v %), and applied to a 10.5-mm i.d. glass column packed with 10 g Florisil (0.15–0.25-mm particle size, 5% deactivated) (Fisher Scientific, Fair Lawn, N.J.). The elution scheme for fraction cleanup on Florisil column at 2.5 mL·min^{-1} was: 1) 25 mL ether/cyclohexane (50/50, v/v %); 2) 25 mL ether; and 3) 25 mL ether/methanol (96/4, v/v %). The last fraction was collected, evaporated to 100 to 200 µL, dissolved in 200 µL acetonitrile/water (35/65, v/v %), and subjected to combined liquid chromatography–mass spectrometry at the OSU Campus Chemical Instrument Center, Columbus. The liquid chromatographic/autosampler system consisted of a Waters Alliance 2690 Separations Module (Waters, Milford, Mass). A 1.0 × 250-mm C_{18} column was used. The mobile phase was acetonitrile/water (35/65, v/v %) with 0.1% formic acid. The flow rate was 0.05 mL·min^{-1}. The eluant from the column was injected directly into the Micromass LC-Tof II (Micromass, Wythenshawe, U.K.) mass spectrometer equipped with an orthogonal electrospray source (Z-spray) operated in the positive ion mode. Sodium iodide was used for mass calibration in a range of 100 to 2000 m/z. Optimal electrospray ionization conditions were 3000 V capillary voltage, 110 °C source temperature, and 55 V cone voltage. The ionization gas was nitrogen. All ions transmitted into the TOF analyzer were scanned with a 1-second integration time and data were acquired in continuous mode during the LC run. Analytical grade imidacloprid (1-[(6-chloro-3-pyridinyl)methyl]-*N*-nitro-2-imidazolidinimine) was used as an internal standard. Preliminary data indicated that up to 90% paclobutrazol was extracted from the plants when using the sample preparation procedure described in this research (data not shown).

Paclobutrazol concentrations (mg·kg^{-1} DW) were measured in the seedcoats and decoated seeds and used to calculate paclobutrazol amounts (milligrams per dry weight of seed part) in the seedcoats and decoated seeds. The latter amounts were added to receive paclobutrazol amount (milligrams per dry weight of one seed) in whole seeds, which was further used to calculate paclobutrazol concentration (mg·kg^{-1} DW) in whole seeds. Dry weight of seeds was determined by drying seed parts at 60 °C for 48 hours.

Statistical analysis. Paclobutrazol level in seeds or fruits, total fruit yield, average fruit weight, and fruit length or diameter as a response to paclobutrazol concentration and soaking time were analyzed to test for significant linear effects using regression analysis with the general linear model procedure in SAS (SAS Institute, Cary, N.C.). Mean comparisons by *t* test were used to evaluate the effect of water treatments (water-treated seeds vs. untreated seeds) on total fruit yield, average fruit weight, and fruit length or diameter.

Results

Paclobutrazol levels in cucumber seeds and fruits of cucumber and tomato. LC-MS analysis revealed that paclobutrazol accumulated preferentially in the seedcoats (Table 1). Paclobutrazol concentrations increased in all seed parts when seeds were soaked during longer times. However, for the highest concentration (4000 mg·L^{-1} paclobutrazol) tested, the ratio between paclobutrazol concentration in the seedcoats and the rest of the seed decreased from ≈70 to 17 with increasing soaking time from 5 to 180 min (Table 1). Paclobutrazol was not detected by LC-MS in cucumber or tomato fruits harvested on plants grown from seeds soaked in 1000 mg·L^{-1} paclobutrazol solution for 180 min (data not shown).

Seedling emergence percentages in each treatment were 95% or 90% for cucumber or tomato, respectively. No differences in seedling emergence percentages between seeds treated with various paclobutrazol rates were found (data not shown).

Fruit weight, length, and diameter. When cucumber plants were grown from seeds treated with the highest paclobutrazol rates, flowering was delayed by ≈7 to 10 d. Increase in paclobutrazol concentration was associated with a reduction in average fruit weight or fruit length (Table 2) of cucumber when seeds were soaked for 5 or 180 min, but not 45 min. Fruit length (Table 2) was reduced by 40% when planting seeds soaked in 1000 mg·L^{-1} paclobutrazol solution for 180 min as compared with water-soaked seeds. When cucumber seeds were treated with increasing paclobutrazol rates, no harvest-ready fruits were present for some treatments indicating delays in fruit maturation (data not shown). Flowering and fruit maturation were not delayed (data not shown), and fruit weight and diameter were unaffected in tomato plants grown from seeds soaked in paclobutrazol (Table 3).

Discussion

Quantitative analysis by LC-MS demonstrated that paclobutrazol penetrated cucumber seedcoats during seed soaking. The data in Table 1 indicate that the seedcoats inhibited paclobutrazol penetration into seeds mainly during a short soaking period (5 min). Soaking cucumber seeds for a longer time (180 min) reduced the difference between paclobutrazol concentration in the seedcoats and the rest of the seed.

Table 1. Paclobutrazol concentration (mg·kg^{-1} DW) in cucumber seedcoats and the rest of the seed (embryonic axis plus cotyledons) measured by mass spectrometry.[z]

	Seed coats			Rest of the seed			Whole seed		
Treatment	5 min	180 min	L^x	5 min	180 min	L^x	5 min	180 min	L^x
0 mg·L^{-1}	ND	ND		ND	ND		ND	ND	
1000 mg·L^{-1}	0.106	0.208	y	0.002	0.012	y	0.022	0.060	y
4000 mg·L^{-1}	0.419	0.716	x	0.006	0.042	y	0.100	0.192	y
L^y	y	y		x	y		y	x	
Untreated	ND			ND			ND		

[z]Seeds were soaked in 0, 1000, or 4000 mg·L^{-1} paclobutrazol solutions for 5 or 180 min, dried, and manually separated into parts.
[y,x]Significant at $P \leq 0.001$, ≤ 0.01, respectively.
L^x, L^y, Linear models for the soaking time and paclobutrazol concentration effects, respectively.
ND, nondetectable amounts.

Table 2. Average cucumber fruit fresh weight (g) and fruit length (mm) of plants grown from seeds soaked in 0, 50, 200, 500, or 1000 mg·L^{-1} paclobutrazol solutions for 5, 45, or 180 min.[z]

	Fruit fresh wt (g)				Fruit length (mm)			
Treatment	5 min	45 min	180 min	L^x	5 min	45 min	180 min	L^x
0 mg·L^{-1}	258	234	277	NS	210	220	182	NS
50 mg·L^{-1}	253	275	222	NS	200	208	206	NS
200 mg·L^{-1}	248	246	252	NS	218	226	186	NS
500 mg·L^{-1}	202	250	210	NS	182	202	152	NS
1000 mg·L^{-1}	163	283	196	NS	168	182	110	*
L^y	**	NS	*		*	NS	**	
Untreated	252	240	262		204	216	194	

[z]Fruits were harvested from 40 to 68 d after planting.
[NS,*,**]Nonsignificant or significant at $P \leq 0.05$, or ≤ 0.01, respectively.

Soaking seeds in 250 to 2000 mg·L^{-1} paclobutrazol inhibited not only seedling growth (Cho et al., 2002), but also flowering and fruit growth in cucumber, which might be associated with inhibition of GA biosynthesis. Delays in flowering resulting from inhibition of GA biosynthesis were earlier reported in sunflower and almond plants sprayed with paclobutrazol solutions (Almeida and Pereira, 1996; Koukourikou-Petridou, 1996). Reduction in fruit yield in paclobutrazol-treated plants was also likely incited by inhibition of GA biosynthesis (Davis et al., 1988).

An important question to be answered is whether paclobutrazol was present in plant tissue at the time when flowering and fruit maturation were delayed. LC-MS analysis indicates that paclobutrazol was not present in fruits or its concentration was below the detection limit of 0.025 mg·kg^{-1} DM. Therefore, reductions in fruit weight or length were likely the result of action of paclobutrazol at the level of seeds or seedlings rather than at later stages of plant growth. Delays in flowering and reduced fruit weight were observed after transplanting plants into a new growing medium indicating that paclobutrazol was not located in the substrate. However, it cannot be excluded that at the time of flowering and fruit growth, paclobutrazol was present in the stems, leaves, or rhizosphere.

It can be concluded that seeds soaked in paclobutrazol solutions did not absorb and retain a significant amount of paclobutrazol to be retained in fruit. In paclobutrazol-treated seeds, embryo-associated paclobutrazol remains in closer proximity to the vascular system of embryonic axis than coat-located paclobutrazol. Therefore, paclobutrazol transport from internal seed parts into the shoot may begin directly from the embryonic phloem. However, the amount of paclobutrazol in the internal seed parts (Table 1) did not exceed the paclobutrazol detection limit of 0.025 mg·kg^{-1} DM fruit and, therefore, could not significantly contribute to paclobutrazol accumulation in fruits.

It is possible that coat-located paclobutrazol—the main potential source of paclobutrazol in cucumber seeds (Table 1)—diffused from the seedcoats into substrate and was absorbed by the root (Pasian and Bennett, 2001). However, further paclobutrazol movement from roots into fruits through xylem seems to be insignificant as a result of the small contribution of xylem sap to fruit growth (Ho and Adams, 1994). Additionally, small fruits had lower sink demand and less xylem area in the pedicel as compared with large fruits (Starck et al., 1990). Therefore, low accumulation of paclobutrazol through xylem could be expected in small cucumber fruits such as those collected from plants grown from seeds treated with increased paclobutrazol rates (Table 1). Root-absorbed paclobutrazol may also move from the xylem to phloem and eventually penetrate into fruits (Singh and Ram, 2000). However, phloem–xylem connections in roots (Steudle and Peterson, 1998), stems (Welbaum and Meinzer, 1990), and fruits (Patrick, 1997) would likely contribute to losses of paclobutrazol during its movement from the xylem into the phloem. After entering the phloem, paclobutrazol would be unlikely transported into fruits in sufficient amounts, because it is characterized by limited phloem mobility (Davis et al., 1988; Fletcher et al., 2000). Therefore, both locations and concentration patterns of paclobutrazol in cucumber seeds (Table 1) provided unfavorable conditions for paclobutrazol accumulation in fruits. Tomato seeds have smaller volume and surface area than cucumber seeds and, therefore, may carry even less paclobutrazol amount than cucumber seeds when treated with the same paclobutrazol rates.

It could be concluded (Table 1) that cucumber seeds soaked in 200 mg·L^{-1} paclobutrazol for 180 min may accumulate ≈0.016 mg·kg^{-1} DW paclobutrazol. Soaking seeds in such paclobutrazol rate is suggested for effective plug height control in cucumber (Cho et al., 2002) or tomato (Pasian and Bennett, 2001). Results of our study correspond well to international standards establishing zero tolerance for paclobutrazol residue in fruits (EPA, 2004). Therefore, soaking seeds in paclobutrazol solutions represents a promising method of controlling plug height of tomato and cucumber without accumulation of paclobutrazol residue in fruits.

Table 3. Average tomato fruit fresh weight (g) and fruit diameter (mm) of plants grown from seeds soaked in 0, 50, 200, 500, or 1000 mg·L^{-1} paclobutrazol solutions for 5, 45, or 180 min.[z]

	Fruit fresh wt (g)				Fruit diam (mm)			
Treatment	5 min	45 min	180 min	L[x]	5 min	45 min	180 min	L[x]
0 mg·L^{-1}	169	141	158	NS	52	54	56	NS
50 mg·L^{-1}	158	136	124	NS	54	56	54	NS
200 mg·L^{-1}	137	126	138	NS	56	54	54	NS
500 mg·L^{-1}	134	125	138	NS	52	58	56	NS
1000 mg·L^{-1}	152	150	158	NS	56	56	53	NS
L[y]	NS	NS	NS		NS	NS	NS	
Untreated	162	140	150		52	56	54	

[z]Fruits were harvested from 62 to 92 d after planting.
L[x], L[y]: Linear models for the soaking time and paclobutrazol concentration effects, respectively.
[NS]Nonsignificant.

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