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Distribution in plant, substrate and leachate of paclobutrazol following application to containerized *Nerium oleander* L. seedlings

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Abstract

Paclobutrazol (PBZ) is a retardant often used in potted ornamental plants to control their growth and compactness. The distribution in plant, substrate and leachate of PBZ applied to containerized oleander seedlings were studied after a single liquid drench application to the substrate surface (20 mg a.i. per plant) in a nursery dedicated to pot production in SE Spain. PBZ significantly reduced all growth parameters, providing more compact plants of good commercial value and confirming its ability to reduce the vertical growth of native oleander in the nursery. The level of PBZ residues in leaves was higher at the end of pot cultivation (156 days post PBZ application) than at the first sampling time (30 days after PBZ application), whereas the level in stems decreased for the same period of time. PBZ residues in roots remained constant during the experimental period. The high level of PBZ residues detected in the substrate 30 days after application had decreased significantly (by 67%) by the end of the experiment. A greater leaching fraction was detected for PBZ-treated seedlings and so a greater amount of PBZ was leached into the nursery soil with the irrigation water at the end of the experiment. This greater amount of PBZ leached into the soil represents an important way of contamination in the nursery and a substantial risk to the environment.

Additional key words: contamination, cultar, nursery, oleander, residues, uptake.

Resumen

Distribución en planta, sustrato y drenaje de paclobutrazol aplicado a plántulas de *Nerium oleander* L. en contenedor

Paclobutrazol (PBZ) es un retardante del crecimiento frecuentemente utilizado en el cultivo de plantas ornamentales en maceta para el control de su crecimiento y compacidad. La distribución en el sustrato, planta y drenaje de PBZ aplicado a plántulas de adelfa en contenedor fue estudiada tras la aplicación de una única dosis a la superficie del sustrato de la maceta (20 mg i.a. por planta) en una plantación comercial de un vivero localizado en el Sureste de España. PBZ redujo significativamente todos los parámetros del crecimiento, proporcionando plantas más compactas de buen valor comercial y conformando su capacidad para reducir el fuerte crecimiento vertical de la adelfa nativa en el vivero. El nivel de residuos de PBZ en hojas resultó más elevado al final del ciclo de cultivo en maceta (156 días tras la aplicación) que a los 30 días de la aplicación, mientras que la cantidad de residuos presentes en el tallo disminuyó para el mismo periodo de tiempo. Los residuos de PBZ en las raíces permanecieron constantes durante todo el período experimental. El elevado contenido de residuos de PBZ detectados en el sustrato 30 días después de la aplicación se redujo significativamente (en un 67%) al final del experimento. Una mayor fracción de drenaje fue detectada en las plántulas tratadas con PBZ, a la vez que una mayor cantidad de PBZ fue drenado en el suelo del vivero conjuntamente con el agua de riego al final del experimento. Esta mayor cantidad de PBZ drenado en el suelo representa una vía importante de contaminación en el vivero así como un riesgo importante de contaminación medioambiental.

Palabras clave adicionales: absorción, adelfa, contaminación, cultar, residuos, vivero.

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Introduction

In Spain, nursery production and the marketing of containerized ornamental plants have undergone a huge increase in the last ten years, making a significant economic contribution to the horticulture sector. In 2005 over 245 million ornamental plants were produced in Spain (MAPA, 2006). Many of them are wild native species that are largely produced for xerogardening and landscape restoration projects in semi-arid environments, because they are well adapted to adverse environmental conditions (Fernández *et al*., 2006). Oleander (*Nerium oleander* L.) is a native plant of the Mediterranean regions widely grown as a drought-adapted shrub in landscape designs and as a flowering pot plant. Nursery techniques are crucial for obtaining a quality product in the nursery. The application of plant growth retardants during nursery production has become an extensive practice in horticulture. Among them, paclobutrazol [(2RS,3RS)-1-(4-clorofenil)-4,4-dimetil-2-(1H-1,2,4-triazol-1-il) pentano-3-ol], is one of the most important representatives of triazoles and is used both as fungicide and as plant growth regulator. PBZ has demonstrated its usefulness for controlling traits of agronomic interest in several crops including cereals, vegetables, fruit trees and ornamentals (Greene, 2002; Rademacher and Bucci, 2002). Among these traits PBZ is used for reducing the size of plants, improving compactness (Bañón *et al*., 2001), and for its ability to resist abiotic stresses (Bañón *et al*., 2006). In oleander pot production PBZ is used to reduce its strong stem elongation (Bañón *et al*., 2001) and to advance flowering (Singh *et al*., 2004).

Soil drenches of PBZ have been found to be more effective than spray applications to reduce plant size (Li *et al*., 1989; Barret *et al*., 1994, 1995; Singh, 2000; Bañón *et al*., 2002; Pardos *et al*., 2005; AlKhassawneh *et al*., 2006). This effectiveness may be directly related to its high persistence in the soil (Jacyna and Dodds, 1999; Sharma and Awasthi, 2005) and in plant organs (Gent, 1997; Latimer *et al*., 2003; Pateli *et al*., 2004). Gent and McAvoy (2000) indicated that PBZ persists in annuals, herbaceous perennials and, especially, woody ornamentals. Although the persistence of PBZ has been investigated in several ornamental plants, including oleander (Syros and Economou, 2000; Bañón *et al*., 2001), it has not been determined analytically from the viewpoint of environmental contamination. PBZ treatments to substrate in containerized oleander production (as well as other woody ornamentals) may result in contamination of the nursery soil surface through leaching; the nearby groundwater may in turn be contaminated and represent a potential hazard to human and animal health. In addition, the materials used in the nursery could be contaminated and this could affect the growth of successive crops, as indicated by Adriansen and Odgaard (1997). In this respect, no studies have so far been carried out to asses the level of PBZ residues in containerized oleander during nursery production in the Mediterranean area.

The purpose of this study was to assess the extent of PBZ in plant and substrate of containerized oleander production after a single drench and to estimate the extent of contamination through leaching as a consequence of the treatment.

Material and methods

Location and plant material

A nursery experiment was conducted at the Agricultural Experimental Station of the Universidad Politécnica de Cartagena (UPCT) on the south eastern Mediterranean coast of Spain (37º 45' N; 0º 57' W).

Seedlings of oleander 'Ajauque' (a native of the province of Murcia, SE Spain) were used. The most homogeneous selection of seedlings possible (14-16 cm in height) was made avoiding, to the greatest extent possible, variations resulting from sexual propagation.

Nursery conditions and experimental details

Oleander seedlings were grown in round pots (14 x 14 cm) filled with a substrate of black peat, perlite and clay-loam soil (1:1:2, v/v) amended with Osmocote plus (Scotts Co., Ohio, USA) (2 g L^{-1} substrate). These were placed in a metal-framed structure (2.8 m height) covered with a white plastic shade screen (85% transmissivity). The soil used for preparing the substrate had 78.88% sand, 8% silt and 13.12% clay, an electrical con-

Abbreviations used: a.i. (active ingredient), EC (electrical conductivity), df (capilar column film diameter), PBZ (paclobutrazol), UPCT (Universidad Politécnica de Cartagena).

ductivity (EC) of 1.6 $dS.m^{-1}$ and pH 7.25. Oleander seedlings were potted on 15 May 2003 and each seedling was pinched above the sixth node 30 days after potting, giving 9±1.5 cm (mean ± sd) high plants. The following conditions prevailed during the 156 days nursery growing period: minimum temperature 12-21ºC and maximum 25-43ºC; minimum relative humidity 20-74% and maximum 73-99%.

The 20 mg a.i. per plant of PBZ (Cultar 25% SC, w/v) was applied to the substrate surface as a single 45 mL liquid drench on the morning of 5 June 2003, 2 h after the last watering. Untreated plants acted as control. Seedlings were drip-irrigated three days per week with a 1 L h^{-1} emitter per plant, with a total volume of approximately 180 mL per plant. The leaching fraction in untreated plants was 0.20 from transplanting to PBZ application and it was adjusted to 0.16 from PBZ application to the end of the experiment. The EC of the irrigation water was 1.73 dS m^{-1}. The leachate was determined in 10 pots per treatment placed at random and was measured after each irrigation. The total amount of water applied per seedling in each treatment throughout the whole period was 9.20 L.

Sampling

Plant samples from ten PBZ treated seedlings were collected 30 and 156 days after PBZ application. The soil was gently washed from roots and the plants were divided into leaves, stems and roots. The samples were cut, mixed and homogenized using a high speed Waring blender for 2 min; 50 g of the homogenized samples were drawn and processed for extraction separately. The dry weight of each plant sample was determined at each analysis time by drying at 70ºC until they reached a constant mass.

Substrate samples were collected from the pots 30 and 156 days after PBZ application. A homogeneous substrate sample was drawn from 10 randomly distributed pots of each PBZ-treated or untreated seedlings. Substrate was collected from the whole volume of the pots (0-14 cm height). The substrate collected from each pot was air-dried, clods broken, mixed thoroughly and sieved through a 2 mm sieve. Six 50 g substrate samples were taken from the above for extraction. In addition, leachate samples were collected from the leaching trays at 30 and 156 days after PBZ application. All the samples were processed immediately and analyzed within 14 days.

Extraction and quantitative determination of PBZ

The analytical method followed was adapted from Reed (1988) and Stahly and Buchanan (1986). A 50 g sampled of homogenized leaves, stems, roots or 50 g substrate sample was placed in an Erlenmayer flask and extracted with 70 mL of chilled methanol using a rotary mechanical shaker for 1 h at 200 rpm. The extract was filtered using a Buchner funnel. The sample was re-extracted along with the filter paper with 2 x 50 mL of chilled methanol. The extracts were filtered and all the filtrates were combined. The combined filtrate was diluted with 30 mL water and evaporated to aqueous phase in a rotary vacuum evaporator at 35ºC. The pH of the extract was adjusted to 11 with 1N NaOH.

The above aqueous layer was partitioned against 3 x 50 mL of dichloromethane in a 500 mL separating funnel. The lowest dichloromethane layers were collected each time, passed through anhydrous sodium sulphate and combined. The combined extract in dichloromethane thus obtained was evaporated under vacuum to 5 mL.

A glass column (50 x 1.3 cm) was packed with 5 g florisil in diethyl ether and the above extract was added slowly to the column. The column was eluted with 10 mL of diethyl ether + methanol (97+3, v/v), while the eluate was collected and evaporated to dryness in a rotary evaporator. The residue, including 5 mL of an internal standard (dichlobutrazol), was made up to 75 mL for GC-MS analysis.

PBZ was estimated quantitatively using a Varian ASC GC (Varian Inc., CA, USA) equipped with a mass selective detector S-2000. A low bleed-ms Varian db5CP Sil 8 CB capillary column (30 m x 0.53 mm, df = 0.25 m) was used. The limit of quantification (LOQ) of PBZ residues in plant and substrate was 0.1 μg g^{-1} and 1 μg mL^{-1} in leachate. Selected ion monitoring was used for quantitation (mass: charge ratio=236 for dichlobutrazol). The results were expressed as μg g^{-1} of substrate or plant sample dry weight and as μg mL^{-1} of leachate.

Growth and development measurements

At the end of the experimental period (156 days after PBZ application), the pot substrate was gently washed from the roots and the plants were divided into shoots (stems and leaves) and roots. These were oven dried at 70ºC until they reached a constant mass to measure the respective dry weight. Plant height, stem diameter,

internode length, average leaf area, total leaf area, leaf dry weight, stem dry weight and root dry weight were measured in 30 randomly distributed plants per treatment. Average leaf area and total leaf area were measured using a Delta-T Leaf Area Meter (Device Ltd., Cambridge, UK).

Statistical analysis

The pot experiment was a completely randomized block design. There were six replicates with five plants per replicate in both PBZ-treated and untreated plants. All data were analysed by one-way ANOVA using Statgraphics Plus for Windows. Treatment means were separated with Duncan's Multiple Range Test (p£0.05).

Results

PBZ reduced all growth parameters by a statistically significant degree compared with the control (Table 1). Both shoot (51%) and root (46%) dry weight were reduced 156 days after PBZ application compared with the control, confirming the greater sensibility of the shoots to this retardant (Table 1). Plant height was significantly inhibited by PBZ (37% reduction compared with the control), while the stem diameter was reduced to a lesser extent (by 24%). As results of these reductions, the native oleanders obtained were of the suitable commercial size and value. A summary of the PBZ amounts detected in the different plant parts, the substrate and the leachate are presented in Table 2.

Table 1. Effects of paclobutrazol on the growth of oleander seedlings 156 days after treatment (end of nursery period)

Growth parameters	Paclobutrazol (mg a.i./plant)	
	0	20
Height (cm)	48.0 b	29.8 a
Stem diameter (mm)	10.0 b	7.6 a
Internode length (mm)	2.0 b	1.1 a
Average leaf area (cm^2)	51.0 b	30.0 a
Total leaf area (cm^2)	595.0 b	290.0 a
Shoot dry weight (g)	49.2 b	24.1 a
Leaves dry weight (g)	30.1 b	13.5 a
Stem dry weight (g)	19.1 b	10.6 a
Root dry weight (g)	55.5 b	30.1 a

Different letters within a row indicate means are significantly different by Duncan´s multiple range (p<0.05). Each value is the mean of 30 plants.

Thirty days after application, PBZ was seen to have translocated to all parts of the plant. Most of PBZ was retained in the stems (51% of the total amount recovered in plant) while leaves contained the lowest level of PBZ (23%). The roots contained lower PBZ (40.9 μg g^{-1}) than stems but more than leaves (Table 3). The amount of PBZ in leaves increased by a statistically significant degree 156 days after PBZ application compared with the levels determined at 30 days, whereas in stems the amount decreased significantly (Table 3). At this time, it was found that 61% of the PBZ recovered in plant were located in leaves, while 17% were in stems (Table 3). PBZ in roots (21% of the total amount recovered in plant) did not vary statistically during the experimental period.

At 30 days, the substrate contained high PBZ residue levels (13.0 μg g^{-1}), but decreased significantly by 67% at the end of the experiment (156 days) (Figure 1). Thirty days after PBZ application, there was little difference between the quantity of leachate in both treatments, but by the end of the experiment the leachate from the PBZ-treated plants was significantly higher (Figure 2).

The level of PBZ residues decreased significantly in the leachate from 38 μg mL^{-1} at 30 days to 25 μg mL^{-1} at 156 days after the application (Table 4). However, the amount of PBZ leached with the irrigation water was higher at the end of the nursery period, due to the higher leachate volume per pot.

Discussion

At the rate of 20 mg a.i. per plant PBZ decreased native oleander plant growth particularly stem elongation, confirming its usefulness for obtaining plant of adequate commercial value by pot production, aspects that have previously been reported (Syros and Economou, 2000; Bañón *et al*., 2001).

The present study showed that 30 days after PBZ application, most of PBZ was accumulated in the substrate, while a low amount was absorbed by the different plant parts (leaves, stems and roots) (Table 2). At the end of the experiment the amount of PBZ was mainly accumulated in the plants, while decreased considerably in the substrate. The highest accumulation of PBZ took place in the leaves. A quite important amount of PBZ was leached into the environment at the end of the experiment which represents a significant environmental problem.

Table 2. Summary of paclobutrazol distribution in leaves, stems, roots, substrate and leachate, 30 and 156 days after the application of 20 mg (a.i.) in oleander seedlings

Days after application	Sample	DW (g)	Volume (mL)	Residues		
				µg g^{-1} DW	µg mL^{-1}	mg
30	Leaves	3.1	-	36.7	-	0.1
	Stems	1.6	-	81.3	-	0.1
	Roots	7.2	-	40.9	-	0.3
	Substrate	906.0	-	13.0	-	11.8
	Leachate		25.2	-	38	0.9
156	Leaves	13.5	-	91.6	-	1.2
	Stems	10.6	-	26.2	-	0.3
	Roots	30.1	-	32.2	-	1.0
	Substrate	889.0	-	4.3	-	3.8
	Leachate		57.6	-	25	1.4

Each value is the mean of 30 plants in leaves, stems and roots measurements, and the mean of 10 pots in substrate and leachate measurements. DW: dry weght.

Studies of the PBZ levels in plant during the experimental period showed that PBZ was taken up through the roots and translocated via stems to the leaves, where it accumulated. These results support the assumption that PBZ is translocated acropetally via xylem (Hamid and Williams, 1997), although some phloem translocation has also been reported (Wang *et al*., 1986; Witchard, 1997). In addition, higher levels of PBZ were found in *Prunus persica* (Early and Martin, 1989) and apple (Wang *et al*., 1986) leaves than in other parts of these species. Curry and Reed (1989) established that PBZ is accumulated mainly in leaves and roots in *Malus communis* seedlings, although the greatest restrictive effect was in stem elongation. In this experiment, leaf dry weight was the parameter most reduced by PBZ treatment (Table 1). Lehman *et al*. (1990) assumed that PBZ could be stored in perennial plant tissues and was responsible for long-term growth suppression. The restrictive effect of PBZ on shoot growth persisted up to seven months in gardenia (Malorgio *et al.*, 1993), 13 months in *Pyracantha* (Ruter, 1994), 1 year in native oleander (Bañón *et al*., 2001), 2 years in *Eucalyptus globulus* (Hasan and Reid, 1995) and *Rhododendron catawbiense* (Gent, 2004) and 3 years in *Mangifera indica* (Salazar and Vázquez, 1997).

The amount of residue left in plant parts appear to depend on the method of application, quantity applied and crop species. Mishra and Mishra (2006) reported that the application of PBZ through a soil drench was more effective than foliar spray in reducing the aboveground biomass of China aster. PBZ applied as a container medium drench at 5 mg a.i. per pot was excessive

Table 3. Uptake and persistence of paclobutrazol distribution in shoots (leaves and stems) and roots in oleander seedlings

Days after paclobutrazol application	Mean levels of paclobutrazol (µg g^{-1})		
	Leaves	Stems	Roots
30	36.7 a	81.3 b	40.9 a
156	92.0 a	26.2 a	32.2 a

Different letters within a column indicate means are significantly different by Duncan´s multiple range (p<0.05). Each value is the mean of 10 plants.

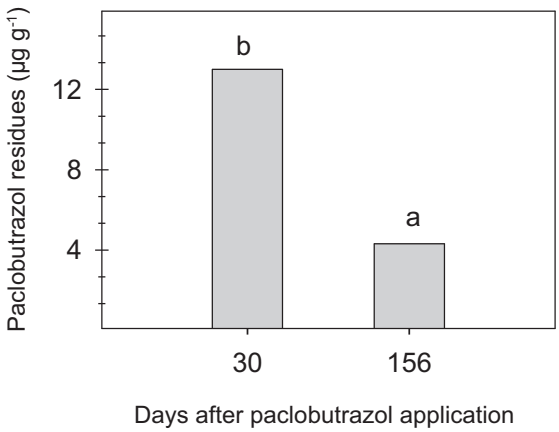

Figure 1. Persistence of paclobutrazol residues in pot substrate. Different letters indicate significant differences (p<0.05) based on the Duncan's multiple range test.

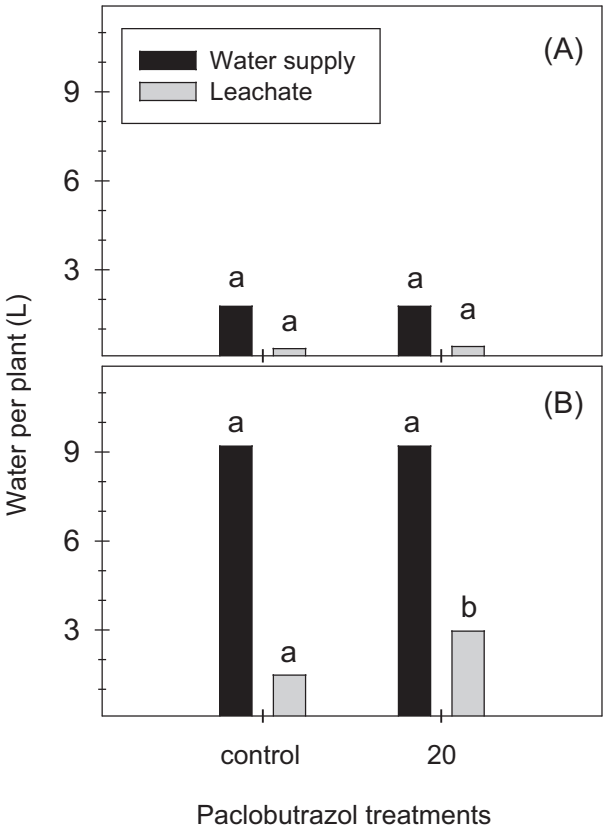

Figure 2. Total water applied and leachate in paclobutrazol treated and untreated plants (A) 30 days and (B) 156 days after treatment. Each histogram represents the mean of 10 values. Different letters in the same bar of the histogram represent significant differences.

during nursery production of *Pyracantha* and *Juniperus* species (Ruter, 1994) while the same dose did not significantly affect native oleander growth (Bañón *et al*., 2001).

In order to minimize residue levels as much as possible it is important to know the lowest dose that will give the desired growth retardation. In this experiment, at the dose required for adequate oleander growth control (Syros and Economou, 2000; Bañón *et al*., 2001) the residues in the leachate were high. As a result, the plants treated with PBZ showed increased leachate levels, as a consequence of their lower growth compared with the non-treated plants. The greater leaching resulting from PBZ treatment has been previously reported by Navarro *et al*. (2007a) in strawberry tree. This increase in the leachate per pot was accompanied by a substantial decrease in substrate residue concentration as the experiment progressed. This fact implies an enhancement of PBZ degradation in the substrate. PBZ is known to be

Table 4. Leaching of paclobutrazol residues per pot and per irrigation in treated oleander seedlings 30 and 156 days after paclobutrazol application

Days	Mean paclobutrazol concentrations in leachate (µg mL^{-1})	Mean leachate volume per pot and irrigation (mL)	Paclobutrazol leached per pot and irrigation (mg)
30	38 b	25.2 a	0.9 a
156	25 a	57.6 b	1.4 b

Different letters within a column indicate means are significantly different by Duncan´s multiple range (p<0.05). Each value is the mean of 10 plants.

stable to degradation in the pH range of 4 to 9 (Roberts, 1996), when it is relatively immobile and shows high persistence in soil (Sharma and Awasthi, 2005). Adriansen and Odgaard (1997) observed a strong adsorption of PBZ to peat based substrate in *Kalanchoe* pot production. Moreover, Milfont *et al*. (2008) have recently determined that the sorption of PBZ in soil is predominantly controlled by organic matter. However, in this experiment it was only observed a strong sorption to substrate 30 days after application that may be related to the bioavailability of the molecule and its concentration in the soil (Navarro *et al*., 2007b).

While the greater amount of PBZ residues were detected in leaves, the results of the present study establish that PBZ residue levels found in oleander seedlings were responsible for their restricted growth, which resulted in a greater leaching fraction. This circumstance could have played an important role in the mobility of PBZ residues in the substrate. Consequently, when PBZ is applied regularly in the nursery there may be a risk of environmental contamination. In addition, this increase in leachate could imply an important way of contamination since PBZ may be adsorbed to the materials in the nursery. This suggests that contamination may spread through containers (especially when they are reused) as well through plastics or benches under the pots, and this could impact negatively on the growth of successive crops (Laermann *et al*., 1991; Grimstad, 1993; Adriansen and Odgaard, 1997). Bañón *et al*. (2002) found that the application of 0.25 mg of PBZ per pot was sufficient to clearly reduce the growth of potted carnation.

The findings of this study suggest that alternative methods to the soil drench application of PBZ are recommended for controlling oleander growth. Application of alternative plant growth regulators or a combination

(providing a synergistic effect and reducing the quantity needed to control growth) or the combination of these with DIF (differential between day/night temperature) methods or photoselective netting would represent a way of reducing this problem.

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