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Increasing Resistance against *Phytophthora citrophthora* in Tangelo Nova Fruits by Modulating Polymethoxyflavones Levels

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The effect of 6-benzylaminopurine on polymethoxyflavone levels in tangelo Nova fruits and the possible participation of these secondary metabolites in defense mechanisms against *Phytophthora citrophthora* are studied. The in vitro study of the inhibitory effect of these compounds on fungal growth reveals that nobiletin is the most active agent followed by sinensetin, heptamethoxyflavone, and tangeretin. Treatment with 100 ppm of 6-benzylaminopurine increased the levels of these polymethoxyflavones in this *Citrus* hybrid and also enhanced the in vivo resistance of the fruit to the fungus by ~60%.

KEYWORDS: 6-Benzylaminopurine; *Citrus*; heptamethoxyflavone; nobiletin; sinensetin; tangeretin

INTRODUCTION

The genus *Citrus* contains a series of polymethoxyflavones, which are characteristic of each species (*1*, *2*). They occur in minimal concentrations or are not found in other plant genera. In a previous paper it was demonstrated that the tangelo Nova [(*Citrus reticulata* B) × tangelo Orlando (*Citrus reticulata* × *Citrus paradisi*)] fruits produce nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone), sinensetin (5,6,7,3′,4′-pentamethoxyflavone), tangeretin (5,6,7,8,4′-pentamethoxyflavone), and 3,5,6,7,8,3′,4′-heptamethoxyflavone. The degree of expression of these compounds in this *Citrus* hybrid is related to different stages of cell growth. For example, the highest levels of nobiletin, sinensetin, and tangeretin are associated with young developing fruits, whereas 3,5,6,7,8,3′,4′-heptamethoxyflavone comes into full accumulation toward the end of the linear phase of growth (*3*).

Developmental processes in plants are regulated by the action and balance of different plant growth regulators. However, there is little available information concerning the possible involvement of these compounds in secondary metabolism, although it has been demonstrated that some of them activate or inhibit the synthesis and/or accumulation of phenolic secondary metabolites (*4*−*10*). Commercial interest in these compounds is great because the polyphenolic profile has been used to characterize citric juices and to determine which citric juices have been used in the manufacturing process (*11*, *12*). The polymethoxyflavones of *Citrus* are also of potential pharmacological interest (*13*−*17*). Several studies have revealed that these compounds are located only in the peel and support the idea that this superficial localization plays a part in protecting the fruit from possible pathogenic attacks (*18*−*23*).

Phytophthora citrophthora causes serious losses in *Citrus* fruits through brown rot lesion. Treatment with chemical fungicides is widely used to prevent the development of this pathogen. However, treatment with fungicides may produce problems due to accumulation of residues on the fruit (*24*).

The objective of this study was to analyze the individual effect of these polymethoxyflavones (isolated and purified from tangelo Nova) on the in vitro growth of *P. citrophthora.* Changes in the resistance of this *Citrus* against the fungus were studied after in vivo enhancement of the endogenous levels of the polymethoxyflavones by applying a growth cytokinin regulator (6-benzylaminopurine).

MATERIALS AND METHODS

Chemicals. The following standards were used: sinensetin and tangeretin, purchased from Extrasynthèse S.A. (Genay, France). Heptamethoxyflavone and nobiletin were isolated by semipreparative HPLC and identified by MS (*22*). The concentrations of these standards were 0.5 and 20 mg mL^{-1} for HPLC and MS analyses, respectively.

Fungal Cultures and Estimation of IC$_{50}$. An isolate of the fungus *P. citrophthora*, obtained from the Spanish Collection of Type Culture (Valencia, Spain) (CECT 2353), was cultured on potato dextrose agar (PDA) medium at 25 °C to serve as inoculum. A 6-mm-diameter disk of culture medium containing mycelium of this fungus was then placed in PDA culture medium (control) and in the same PDA culture medium to which sinensetin (0.2, 0.4, and 0.6 g L^{-1}), nobiletin (0.05, 0.1, and 0.4 g L^{-1}), heptamethoxyflavone (0.1, 0.2, and 1 g L^{-1}), and tangeretin (0.1, 0.5, and 1 g L^{-1}) were added. The antifungal activities of these polymethoxyflavones (isolated and purified from tangelo Nova fruits) against *P. citrophthora* were determined by an in vitro assay (*22*). The

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inhibition index (IC_{50}) was expressed as the concentration (millimolar) of these compounds required to provide 50% inhibition of radial growth (millimeters) at 100 h. The IC_{50} was determined by linear regression.

Plant Material and Hormonal Treatments. Seven-year-old trees of tangelo Nova, a mandarin [(*C. reticulata* B) × tangelo Orlando (*C. reticulata* × *C. paradisi*)] hybrid, were used. The trees were cultivated in an experimental plantation of the Centro Regional de Investigaciones Agrarias in Alhama (Murcia, Spain). At fruit set, four plots of 20 trees were divided into five subplots and sprayed with an aqueous solution of 0, 20, 50, 100, or 250 ppm of 6-benzylaminopurine (Sigma, St. Louis, MO) in 0.1**%** polyethylene glycol as a wetting agent, using 5 L/tree. In the different assays, none of the subplots of four trees was adjacent to another subplot.

Extraction of Polymethoxyflavones and Chromatographic Analysis. Four whole tangelo Nova fruits were used in each assay. The control and treated fruits were taken at different stages of development and immediately dried at 50 °C to constant weight. The dried fruits were ground, and 40 mg of dry weight mL^{-1} was shaken with dimethyl sulfoxide for 24 h. The resulting extracts were filtered through a 0.45 μm nylon membrane before analysis.

The conditions used for the HPLC and MS analyses of the polymethoxyflavones present in these extracts were similar to those described previously (*22*).

Inoculation and Evaluation of Fruit Resistance. Two 6-mm-diameter sections of flavedo (from opposite points in the equatorial zone) were removed from control fruit and from fruits treated with 100 ppm of 6-benzylaminopurine (both 160 days post-treatment) by means of a hollow glass tube. In each case 30 fruits were subdivided into three subgroups of 10 fruits. A disk of similar diameter of PDA culture medium with mycelium of *P. citrophthora* was then placed in each wound. Inoculation was carried out immediately after wounding, and the wounds were sealed by adhesive plastic strip (Cellotape). The respective fruits were kept in a chamber at 20 °C and 85% relative humidity. The resistance of fruit to infection was determined by measuring the lesion area (square centimeters) 1, 3, and 5 days after inoculation.

RESULTS AND DISCUSSION

Effect of Sinensetin, Nobiletin, Heptamethoxyflavone, and Tangeretin on in Vitro Growth of *P. citrophthora*. Polymethoxyflavones isolated from tangelo Nova fruit were individually active in inhibiting *P. citrophthora* growth in the range of concentrations used (**Figure 1**).

The IC_{50} for each of these compounds, as an average of three experiments similar to those depicted in **Figure 1**, revealed that nobiletin ($IC_{50} = 0.59 \pm 0.07$ mM) is more active than sinensetin ($IC_{50} = 0.91 \pm 0.05$ mM), heptamethoxyflavone ($IC_{50} = 1.04 \pm 0.07$ mM), and tangeretin ($IC_{50} = 1.72 \pm 0.13$ mM). All of these flavonoids acted as fungistatic agents at the concentration assayed, causing marked abnormalities in hyphal morphology and a reduction in their size (data not shown), which explains the reduced radial growth mentioned above.

Our findings suggest for the first time that the polymethoxyflavones from tangelo Nova fruit (nobiletin, sinensetin, heptamethoxyflavone, and tangeretin) could act individually as natural and in situ inhibitors against *P. citrophthora* attack in this plant material, where they may play a protective role as agents localized in the outermost tissue of the fruit (*3*, *30*).

A comparison of the IC_{50} value obtained for tangeretin against *P. citrophthora* and that obtained against *Penicillum digitatum* ($IC_{50} = 6.45 \pm 0.53$ mM) (*23*) shows that this polymethoxyflavone is more effective against *Phytophthora*. The fungistatic effect of other phenolic compounds from different plant materials has been described (*25*).

Effect of Benzylaminopurine Treatment on the Polymethoxyflavones Content of Tangelo Nova Fruits. The most effective 6-benzylaminopurine concentration in increasing the

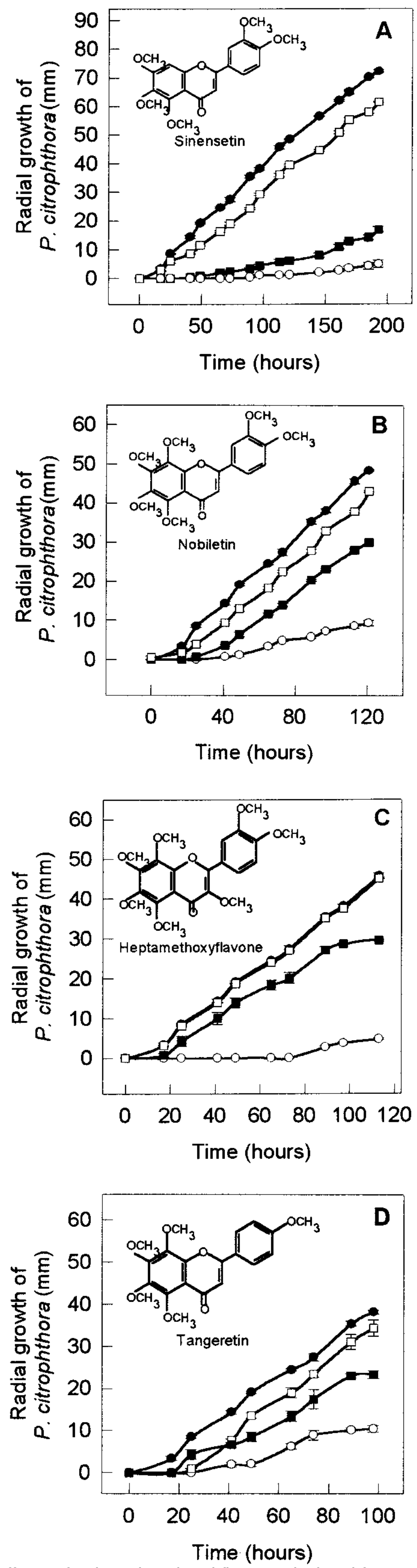

Figure 1. Effects of polymethoxylated flavones isolated from tangelo Nova fruits on the growth of *P. citrophthora*: (A) sinensetin, 0.2 g L^{-1} (□), 0.4 g L^{-1} (■), 0.6 g L^{-1} (○); (B) nobiletin, 0.05 g L^{-1} (□), 0.1 g L^{-1} (■), 0.4 g L^{-1} (○); (C) heptamethoxyflavone, 0.1 g L^{-1} (□), 0.2 g L^{-1} (■), 1 g L^{-1} (○); (D) tangeretin, 0.1 g L^{-1} (□), 0.5 g L^{-1} (■), 1 g L^{-1} (○). The control is represented by ●. The data correspond to mean values of mycelial diameters in millimeters. Vertical bars denote ± SE ($n = 3$) when larger than symbols.

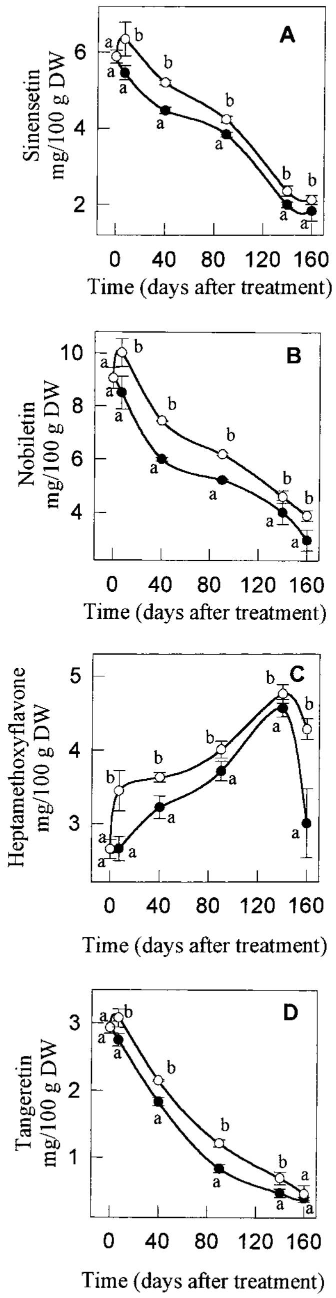

Figure 2. Effect of 6-benzylaminopurine on polymethoxyflavones levels in tangelo Nova fruits. Data represent the mean values in milligrams per 100 g of dry weight of fruit for sinensetin (A), nobiletin (B), heptamethoxyflavone (C), and tangeretin (D), and the vertical bars denote ± SE ($n = 4$) when larger than symbols. Treatments with 100 ppm of benzylaminopurine were carried out on recently set fruit. Values corresponding to control fruits (●) and treated fruits (○) were compared by Duncan's multiple-range test ($p = 0.1$). In (C), the control data (●) overlap the data of heptamethoxyflavone 0.1 g L^{-1} (□).

polymethoxyflavone levels was 100 ppm (**Figure 2**). The higher dose (250 ppm) did not result in any additional increase in the levels of these compounds (data not shown).

Seven days after application, during the exponential growth stage of fruit (*9*), there was a significant increase in the levels of heptamethoxyflavone (29%), sinensetin and nobiletin (~20%), and tangeretin (12%) (parts C, A, B, and D of **Figure 2**, respectively). At longer postapplication times (until 160 days post-treatment), increased polymethoxyflavones levels continued to be observed, even after the fruits had reached maturity

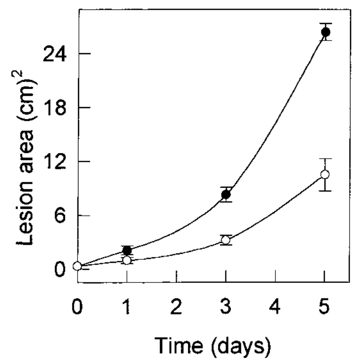

Figure 3. Lesion development after inoculation (160 days after anthesis) of tangelo Nova fruits with *P. citrophthora*: (○) fruits treated with 100 ppm of benzylaminopurine; (●) untreated fruits (control). The fruits were inoculated immediately after wounding. Data represent mean values of lesion area (square centimeters) at different days after inoculation, and the vertical bars denote ± SE ($n = 3$) when larger than symbols.

(**Figure 2**). The significant increase (~43%, **Figure 2C**) observed in the concentration of heptamethoxyflavone at 160 days was probably due to the fact that this compound is synthesized mainly during the maturation phase in tangelo Nova fruits (*3*).

The increase in polymethoxyflavones levels in tangelo Nova fruits following the application of 100 ppm of 6-benzylaminopurine suggests that this cytokinin further activates some of the enzymes that take part in the biosynthetic pathway of polymethoxyflavones. Along these lines, other authors have also suggested that cytokinins may act as direct regulators of enzyme activity (*26*), and cytokinins have also been found to affect the accumulation of other phenolic compounds in fruits (*9*, *27*, *28*).

Influence of Enhancement the Polymethoxyflavone Levels in Tangelo Nova Fruits on the in Vivo Growth of *P. citrophthora*. On the basis of the size of the brown rot lesions formed on tangelo Nova fruits inoculated with *P. citrophthora* 160 days after treatment with 100 ppm of 6-benzylaminopurine, fungal growth was inhibited by ~60% at 3 and 5 days after inoculation (**Figure 3**) compared with the untreated fruits.

These results reveal that the fruits treated with 100 ppm of 6-benzylaminopurine show greater resistance to attack by *P. citrophthora* than the control fruits (untreated), probably due to the increase in polymethoxyflavone levels resulting from the 6-benzylaminopurine treatment (see **Figure 2**).

The effective polymethoxyflavone levels for inhibiting fungal growth in vitro (see **Figure 1**) were higher than those detected in vivo (see **Figure 2**). However, it should be noted that these in vivo levels are a mean of those found in the whole fruit, whereas polymethoxyflavones are in reality found only in the peel (*3*). Furthermore, it must be borne in mind that not just one polymethoxyflavone was present but a mixture of these compounds, so that the increased response to the fungus may have been due to a synergic effect, although it cannot be discounted that other phenolic compounds may act in the defense mechanisms of this plant material, as has been described in other *Citrus* species (*31*, *32*).

On the basis of these results, we suggest that 6-benzylaminopurine can be used in preharvest hormonal treatments as an alternative or in combination with the use of chemical fungicides, because this plant growth regulator induces resistance in tangelo Nova fruits by increasing the concentration of polymethoxyflavones. These flavonoids may play an active role in protection against pathogen attack (*22*, *29*), acting as a first line of defense, because they are mainly localized in the outermost tissue of the fruit, the flavedo, or exocarp (*3*, 3*0*).

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