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Enhancement of phenolic compounds in olive plants (*Olea europaea* L.) and their influence on resistance against *Phytophthora* sp.

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Abstract

The total phenol levels in different olive organs and tissues are studied. The HPLC-MS studies point to the presence of oleuropein, catechin and tyrosol as some of the main phenolic compounds in these extracts. The effect of Brotomax treatment on phenolic compound levels in this plant and the possible role of these compounds as antifungal agents against *Phytophthora* sp. are also studied. An increase in the total phenol content of leaves and stems was observed 120 days after treatment with 0.3% Brotomax. The cortex was the stem tissue which showed the greatest accumulation of these secondary compounds. An in vitro study of the inhibitory effect of these compounds on fungal growth revealed that tyrosol was the most active agent, followed by catechin and oleuropein, their fungitoxic effect being greater when they acted synergically.

Keywords: Antifungal agents; Catechin; Brotomax; Oleuropein; Tyrosol

1. Introduction

In previous studies we examined the phenolic composition of olive (*Olea europaea* L.) fruits from some Spanish varieties and evaluated the beneficial effect of 0.3% Brotomax treatment on fruit size, oil content, the levels of these phenolic compounds and the Trolox-equivalent antioxidant activity (Botía et al., 2001). The increases observed in the levels of these polyphenolic compounds in olive, following Brotomax treatment, agreed with the results obtained with Brotomax in *Citrus* and *Vitis vinifera*, in which an increased expression of flavanones and coumarins (Fuster et al., 1995; Ortuño et al., 1997) and polyphenols (Del Río et al., 2001), were described.

The interest in these phenolic compounds derives from the fact they are considered responsible for the special organoleptic properties of the oil as well as its resistance to autoxidation (Baldioli, Servili, Perretti, & Montedoro, 1996; Benavente-García, Castillo, Lorente, Ortuño, & Del Río, 2000; Perrin, 1992). In addition, they are considered to have an ecologial role, and, although scarce, some studies suggest that they are related to plant defence mechanisms against pathogenic attack (Del Río et al., 2000; Marsilio & Lanza, 1998; Ruiz-Barba, Garrido Fernández, & Jiménez Díaz, 1991).

Phytophthora sp. is one of the most abundant of soil fungi. It can infect both via the roots and air, causing roots and the basal part of the stem to rot and it wounds the stem cortex, especially in woody species such as olive.

The objective of this study was to complement the information available on the synthesis and accumulation of these phenolic compounds in different plant organs (leaves, stems and roots) and stem tissues (cortex and pith) and to establish the effect of Brotomax treatments on them. We also assayed the inhibitory effect of the phenolic compounds present in olive extracts on the in vitro growth of *Phytophthora* sp.

2. Material and methods

2.1. Plant materials and Brotomax treatment

The study was carried out with 8 year-old olive plants (*Olea europaea* L., var. Picual) grown in a commercial plantation located in Jaén (Spain). Fifty days after

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anthesis, 20 trees of this variety were sprayed with an aqueous solution of 0.3% Brotomax, using 5 l/tree (treated) and a separate group of 20 trees was left untreated. Leaves and stems were harvested from around the middle part of treated and untreated olive trees at 140, 155 and 170 days after anthesis (90, 105 and 120 days post-treatment, respectively). In each case, secondary roots were collected and, in some experiments, the cortex and pith tissues from the stems were separated.

2.2. Chemicals

The standard phenolic compounds catechin, caffeic acid, and Folin Ciocalteu's phenol reagent were purchased from Sigma (St. Louis, MO, USA). Tyrosol was from Aldrich (Madrid, Spain) and oleuropein from Extrasynthèse, S.A. (Genay, France). The compound Brotomax was supplied by Agrométodos S.A. (Madrid, Spain).

2.3. Extraction and measurement of phenolic compounds

The plant materials (leaves, stems, roots, cortex and pith) collected from the different trees (untreated and treated), were mixed and divided, in each case, into three lots. These were ground and shaken with dimethylsulphoxide (DMSO) (150 mg of fresh weight/ml) for 1.2 h for extraction.

The corresponding extracts were filtered through a 0.45-μm nylon membrane before analysis by: (1) spectrophotometry, using a UNICAM UV–vis spectrometer UV2 (Unicam Limited, Cambridge, UK), to estimate total phenols, expressed as caffeic acid/100 g FW, by the Folin Ciocalteu Method (Singleton & Rossi, 1965); (2) HPLC-MS with a Hewlett-Packard liquid chromatograph (model HP 1050) (Hewlett-Packard Co., Palo Alto, CA, USA) fitted with a diode array detector (range scanned: 220–500 nm) to quantify and identify compounds (Botía et al., 2001). In the HPLC analysis, the stationary phase was a Sherisorb ODS-2 column (250 mm×4 mm i.d.) with a particle size of 5 μm, thermostatted at 30 °C. The solvent was a mixture of acetonitrile (A) and acetic acid/water (2.5:97.5) (B): 25–95% of A in 50 min.

Eluent flow rate was 1 ml/min. The absorbance changes were recorded in the UV–vis diode array detector at 280 and 353 nm. The amounts of the principal phenolic compounds were determined from the area given by the integrator, using the response factor of the corresponding standards. The main phenolic compounds in these extracts were collected with a fraction collector (Pharmacia LKB Biotechnology, Uppsala, Sweden) at the exit of the HPLC column for identification by means of a Hewlett Packard mass spectrometer (Model 5989).

2.4. Fungal cultures and antifungal activity of olive plants extracts

An isolate of the fungus *Phytophthora* sp. was selected from the collection of the Centro de Investigación y Desarrollo Agroalimentario (CIDA), Murcia, Spain, and cultured on potato dextrose agar (PDA) medium, at 25 °C, to serve as inoculum.

The antifungal activities of these phenolic extracts (obtained from different organs and tissues of untreated and Brotomax-treated plants) against *Phytophthora* sp. were determined by in vitro assay. In each case, a 5 mm disk of culture medium, containing mycelium of the fungus, was placed in PDA culture medium (control) and in the same PDA culture medium to which a phenolic extract had been added at a final concentration of 1 ml/20 ml culture medium. Mycelial growth inhibition (%) in each assay, with respect to the corresponding control, was calculated after 160 h.

The inhibition index (IC_{50}) was expressed as the concentration (g/l) of tyrosol, catechin and oleuropein (isolated from olive plants) required to cause 50% inhibition of radial growth (millimeters) at 100 h. The IC_{50} was determined by linear regression.

3. Results and discussion

3.1. Modulation of total phenols in different organs and tissues of olive plants by Brotomax treatment

Fig. 1 shows the total phenols in different organs from untreated and 0.3% Brotomax-treated olive plants (var. Picual), 140, 155 and 170 days after anthesis. The results show that the highest phenol levels were detected after 170 days in leaves, stems and roots (in that order). This is because leaves are the principal producers of phenolic compounds, the pathway of shikimic acid, which acts as the precursor of phenolic compounds, beginning in their photosynthetic cells. Several studies in different plant species have shown that various phenolic compounds are synthesized and accumulate in different tissues of the leaf (Jähne, Fritzen, & Weissenböck, 1993; Weissenböck, Hedrich, & Sachs, 1986).

One hundred and twenty days after treatment with 0.3% Brotomax (170 days after anthesis), increases of 15.6 and 8.8% in total phenol levels were measured in the leaves and stems, respectively, compared with the corresponding controls (Fig. 1). These findings agree with observations made for the increased levels of phenolic compounds observed in the fruit of Brotomax-treated olive varieties (Botía et al., 2001; Del Río et al., 2000), and in different organs of other plant species (Del Río et al., 2001; Fuster et al., 1995; Ortuño et al., 1997). When the distribution of total phenols was analysed in the stem tissues, the levels recorded in the cortex were

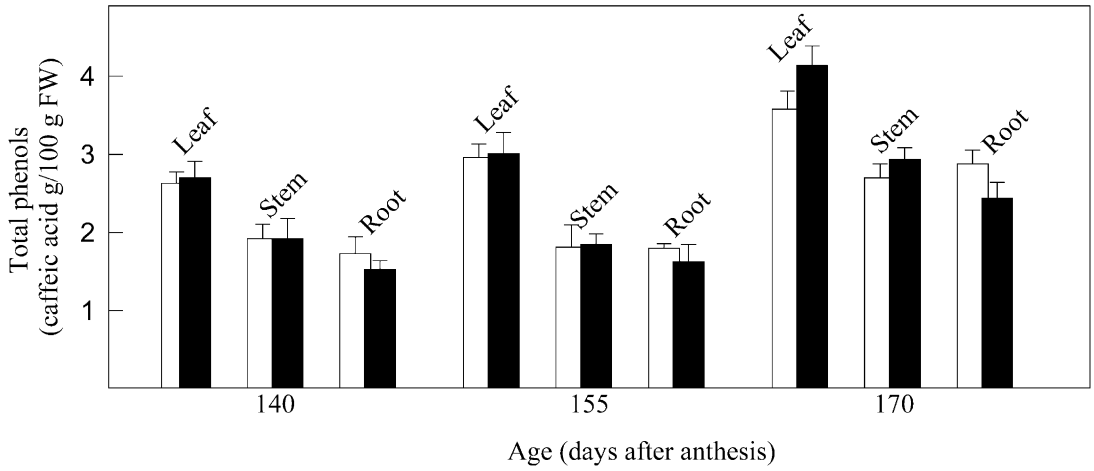

Fig. 1. Effect of Brotomax on total phenol levels in different organs of the olive (var. Picual). The data correspond to mean values (caffeic acid g/100 g FW) in untreated (□) and 0.3% Brotomax-treated plants (■) at 140, 155 and 170 days after anthesis. Vertical bars denote ± SE ($n=3$).

2.4 times higher than in the pith. The results obtained indicate that the above mentioned increase in the stem tissues, after the 0.3% Brotomax treatment (Fig. 1), was due solely to increase in total phenols in the cortex, where they increased by 16.9% with respect to untreated plants (Table 1). This increase as a result of Brotomax treatment suggests an increase in the transport of these phenolic substances from the leaves and through the phloem (which is included in the cortex in our conditions).

HPLC-MS studies point to the presence of oleuropein, catechin and tyrosol among the most abundant phenolic compounds in the cortex of untreated and treated plants (Table 2). Increases of 24.6, 10.3 and 30.9%, respectively, were measured in the levels of each of these compounds in treated plant compared with those in untreated plants (Table 2).

3.2. *Antifungal action of phenolic compounds present in olive plants against* Phytophthora *sp.*

When the phenolic compounds present in the extracts of different organs (leaves, stems and roots) and tissues (cortex and pith) from untreated and treated olive plants were added to the PDA culture medium, an inhibition in the mycelial growth of this fungus was observed (Table 3). The greatest inhibition of mycelial growth was observed for the extracts obtained from the root and stem (around 61% in untreated and 62% in treated plants) compared with the 50 and 58% inhibition observed in the extracts obtained from untreated and treated leaves.

As regards the stem tissues analysed, greater mycelial growth inhibition was observed in the extracts obtained from the cortex (61% in untreated and 66% in treated plants) as opposed to the 55 and 60% (untreated and treated plants, respectively) inhibition for the extracts obtained from pith. The greater inhibition observed with cortex extracts is probably related to its higher total phenol content compared with pith (Table 1).

The IC_{50} obtained for the principal compounds present in these extracts reveal tyrosol to be the most active, followed by catechin and oleuropein (2.7±0.3, 3.3±0.2 and 4.0±0.2 g/l, respectively). The fact that higher concentrations of these compounds are necessary to inhibit fungal growth when they are incorporated in the medium individually (above mentioned IC_{50} data) suggests that they have a synergic effect when they act together (Table 3), as has been suggested for other plant materials (Ortuño, Arcas, Botía, Fuster, & Del Río, 2002).

Phytophthora sp. enters the plant through the roots before being distributed to the rest of the plant by means of the conducting tissues. The results obtained in

Table 1
Distribution of total phenols in olive stem tissues (var. Picual). The data correspond to mean values of total phenols (as caffeic acid g/100 g FW) ± SE ($n=3$) in cortex and pith from untreated and 0.3% Brotomax-treated plants at 170 days after anthesis

Stem tissues	Total phenols	
	Untreated	Treated
Cortex	3.90±0.28	4.56±0.32
Pith	1.61±0.15	1.53±0.14

Table 2
Levels of tyrosol, catechin and oleuropein (mg/100 g FW) in olive cortex stem (var. Picual)

Phenolic compound	Untreated	Treated
Tyrosol	4.2±0.3	5.5±0.2
Catechin	8.7±0.5	9.6±0.3
Oleuropein	523±17	651±21

The data correspond to mean values ± SE (n = 3) from untreated and 0.3% Brotomax-treated plants at 170 days after anthesis.

Table 3
Inhibition (%) of *Phytophthora* sp. growth with leaf, stem, root, cortex and pith extracts from untreated and 0.3% Brotomax-treated plants of the olive (var. Picual) at 170 days after anthesis

Growth inhibition (%)	Organs			Stem tissues	
	Leaf	Stem	Root	Cortex	Pith
Untreated	49.6±3.1	60.6±1.8	61.2±2.2	61.2±3.7	54.6±1.2
Treated	57.8±2.0	62.3±2.7	62.9±1.6	65.9±1.3	59.6±3.5

In each case, 1 ml of extract (150 mg FW/ml DMSO) was added to 20 ml of culture medium PDA. The data correspond to mean values ± SE ($n=3$).

this study are of great interest, as the roots had a high capacity to inhibit mycelial growth (Table 3). This is presumably a resistance mechanism that the plant has developed in an attempt to hinder access of the fungus and to prevent its proliferation in the rest of the plant. It is known that *Phytophthora* sp. attacks the stem cortex, producing rot and necrosis in this tissue, where an accumulation of phenolic compounds is needed (Table 1) to help inhibit the growth of the fungus (Table 3).

This hypothesis on the involvement of phenolic compounds in the defence mechanisms of olive plants against *Phytophthora* sp. is in accordance with the results obtained, in which we have seen that the increase in phenolic compound levels brought about by Brotomax treatment (Fig. 1 and Tables 1 and 2), increases the inhibition of mycelial growth (Table 3). It also accords with the results obtained by other authors in in vivo studies in several species, where the phenolic content of different organs was observed to increase in plants infected with pathogens (Arcas, Botía, Ortuño, & Del Río, 2000; Cahill & McComb, 1992).

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