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Enzymatic response of olive varieties to parasitism by *Xiphinema index* (Nematoda: Longidoridae)

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Summary. Phenols content, phenylalanine ammonia lyase (PAL) and peroxidase (POD) activities were determined in leaves and roots of one cultivar (FS-17) and one rootstock (DA-12I) of olive (*Olea europaea* L.), uninfected and infected by different population densities of *Xiphinema index* 6 months after inoculation. The role of phenols and peroxidase in the response of olive to feeding by nematodes was confirmed by the experiment. Phenols content and PAL activity were higher in uninfected roots of DA-12I, whereas phenols increased and PAL decreased in the roots of both varieties in relation to the nematode inoculum density. The variation of phenols qualitative profile in the roots of DA-12I suggested a translocation of oleuropein from the leaves to the roots in response to *X. index* feeding. POD activity increased in roots and leaves of DA-12I, but only in the leaves of FS-17. Analysis of kinetic parameters showed that the same POD isozyme is present in both varieties.
Key words: ascorbic acid, *Olea europaea,* peroxidase, phenols, phenylalanine ammonia-lyase, resistance, *Xiphinema index.*

Plant resistance to root-knot nematodes has been reported to be associated with high levels of phenolic compounds, phenylalanine ammonia lyase (PAL) and peroxidase (POD) (Bajaj & Mahajan, 1977; Zacheo *et al.*, 1982, 1983; Ouyang & Xue, 1988). The physiological role of POD in plants is unknown, although the basic reactions of this enzyme have been determined.

Peroxidase has been reported as being involved in many host-pathogen interactions, and particularly in the protection of plants against parasites (Fric, 1976; Hammerschmidt *et al.*, 1982). Abnormal production of active oxygen species (AOS, O_2^-, H_2O_2) occurs in plants exposed to a number of extreme environmental conditions, in senescing plants, and in plants infected by pathogens (Sutherland 1991). A number of oxidoreductases (*e.g.* xanthine oxidase, NADH oxidase, peroxidases) can generate AOS as catalytic byproducts (Monk *et al.*, 1989). Plants have evolved protective scavenging or antioxidant systems, both enzymatic and non enzymatic, against AOS (Scandalios, 1993). Among the enzymatic systems, POD protects cells from the harmful effects of H_2O_2.

Plants hypersensitive to parasites also elicit metabolic changes activated in the phenylpropanoid pathway. The initial reaction resulting in the biosynthesis of a wide variety of phenolic compounds is catalyzed by the enzyme PAL to yield trans-cinnamic acid and NH_4^+. This metabolic pathway is involved in plant resistance mechanisms, *i.e.* cytotoxic activity exerted by quinones (Ponz & Bruening , 1986), deposition of phenolics in the cell wall (Kimmis & Wuddah, 1977), and constitution of a non-degradable mechanical barrier resulting from cell wall deposition of lignins (Vance *et al.*, 1980).

Phenols, PAL and POD were found to be closely correlated with the reaction of olive to parasitism by *Meloidogyne incognita* and *M. javanica* (Ridolfi *et al.*, 1998). *Xiphinema index,* which is most commonly associated with fig and grapevine, has been recovered from the rhizophere of olive in Italy (M.I. Coiro, pers. comm.) and

Greece (Vlachopoulos, 1991), and has been found to affect the growth of some olive cultivars (Sasanelli *et al.*, 1999). However, information is not available on the effect of *X. index* on metabolic activity in olive. A glasshouse experiment was established to determine variations in phenols content, PAL and POD activity, the potential role of these variations in plant resistance mechanisms, and possible interactions between roots and leaves in a cultivar and a rootstock of olive subjected to several population densities of *X. index*. The results of the experiment are reported here.

MATERIALS AND METHODS

Woody cuttings, at the four leaf stage, (15-20 cm long and 8-10 mm thick) of the C.N.R.'s patented olive (*Olea europaea* L.) cultivar FS-17 and rootstock DA-12I, previously dipped in talcum powder (Magnesium silicate) containing 2,000 ppm (by weight) indolebutyric acid, were planted in perlite and maintained in a mist chamber with bottom heating at 23±2 °C. Two months later, rooted cuttings were transplanted into 250 ml plastic pots filled with steam-sterilized sandy soil, and placed in a glasshouse at 25±2 °C until required (Fontanazza, 1993). Subsequently, one year old self-rooted olive cuttings of the two selected olive cultivars were individually transplanted into 500 ml clay pots filled with steam sterilized sandy soil. After two weeks, hand-picked non-gravid female *X. index*, obtained from an Italian population reared on fig (*Ficus carica* L.) in a glasshouse, were inoculated into two holes around the seedling roots at inoculum levels 0, 10, 20, 40 and 80 females/pot. The pots were arranged in a randomized block design on benches in a glasshouse at 25±2 °C, with ten replicates for each inoculum level.

After six months, the plants were uprooted and the top and root fresh weights were recorded. Also, the effect of *X. index* on plant growth was assessed by calculating the percentage increase of stem length, stem diameter at soil level, and the number of nodes on the main shoot with respect to their initial values at transplanting.

Damage by the nematodes on the root systems was assessed according to a 0-3 scale (Kunde *et al.*, 1968), in which 0 - no symptoms; 1 - few localized swollen or curved tips; 2 - general swelling of root tips, and 3 - deformation and reduction of the root system.

Final nematode population density in each pot was determined by recovering the *X. index* from 100 ml soil by a decanting and sieving procedure (Brown & Boag, 1988).

For enzyme extraction, leaves and root samples were washed in distilled water and stored at -80 °C. Subsequently, ice-cold (0 - 2 °C) samples were homogenized in an Ultra-Turrax for 1 minute with 0.05 M sodium-phosphate pH 6.55 buffer at a 1:5 (w/v) ratio. The extraction buffer contained 1% (v/v) 2-mercaptoethanol, 1 mM PMSF and 3% (w/v) PVP. The homogenate was filtered through four layers of gauze. The filtrate was stirred at 4 °C for 30 minutes and then centrifuged at 500 rpm for 10 minutes. The supernatant was further centrifuged at 14,000 rpm for 45 minutes. The pellet from a second centrifugation was discarded and the supernatant was frozen at -80 °C.

Subsequently, root extracts were precipited with ammonium sulphate to 70% saturation, kept at 4 °C for 3 hours, and then centrifuged at 12,000 rpm for 30 minutes. The pellet was dissolved in a minimal volume of 0.05 M sodium-phosphate pH 6.55 buffer and then dialyzed overnight against a 1,000 volume of 0.05 M sodium-phosphate pH 7 buffer containing 0.1 M KCl at 4 °C. No precipitation occurred with dialysis at this pH.

Leaf extracts were immediately dialyzed overnight against a 1,000 volume of the same buffer solution, without a previous precipitation (Montalbini & Raggi, 1974).

The reaction mixture for PAL assay consisted of 0.1 ml enzymatic extract, 2 ml of 0.2 M sodium-borate buffer at pH 8.8 and 1 ml of 0.1 M L-phenylalanine. In the reference mixture L-phenylalanine was replaced by water. The activity of PAL was measured by recording the production of trans-cinnamic acid after 1 minute, at 40 °C. Cinnamate was detected at 290 nm. PAL activity was expressed in unit/g of fresh weight. A unit of PAL was defined as a 0.001 increment of absorbance/min. For the POD assay a guaiacol oxidation was monitored in 2 ml of an assay mixture containing 0.05 M sodium-phosphate buffer (pH 6.5), 5 mM of guaiacol dissolved in ethanol, 2 mM of H_2O_2, and 200 µl of enzyme extract, at 470 nm. The reaction was carried out at 25 °C against a control containing all the components, but without guaiacol. POD activity was expressed in unit/g of fresh weight. A unit of POD was defined as a 0.001 increment of absorbance/min. Lineweaver-Burk (double reciprocal) plots were used to calculate kinetic parameters of cytoplasmatic POD contained in DA-12I and FS-17 controls (Lineweaver & Burk, 1934): affinity of peroxidase towards guaiacol (Km) and maximum reaction velocity at the enzyme saturation (Vmax) were determined. The assay mixture for guaiacol

oxidation contained different concentrations of guaiacol (1-5 mM) and 2 mM of H_2O_2 in 2 ml of 0.05 M phosphate-buffer (pH 6.5). The oxidation of guaiacol was monitored at pH 4, 5, 6, 6.5, 7, 8 and 9 using 0.05 M sodium-phosphate buffer. In each experiment the assay mixture contained 1.595 ml of buffer, 105 µl of H_2O_2, 200 µl of enzymatic extract and 100 µl of guaiacol.

To assess ascorbic acid inhibition in the peroxidase activity reaction 200 µl ascorbic acid (200 µM) was substituted by buffer, following the same reaction to determine peroxidase activity, after 2 minutes incubation at 25 °C.

Soluble phenols were determined by extracting three different samples of roots (0.5 g) and leaves (1 g) in an Ultra-Turrax for 2 minutes with 15 ml of MeOH-H_2O (80-20 v/v). After shaking for 10 minutes, the slurry was centrifuged at 3,000 rpm for 20 minutes and the supernatant was then collected. This procedure was then repeated. Polyphenols were quantified by the Folin-Ciocalteau method (Colowich & Kaplan, 1964) at 765 nm, using 400 µl and 100 µl of extract, respectively, for roots and leaves. The qualitative profile of phenols was assessed in the control and at the lowest and highest nematode densities. Qualitative extract was evaporated to dryness in a rotary evaporator at 35 °C and re-dissolved in 5 ml of extraction solution. The phenolic extract was analysed by HPLC (Varian mod. 9010, Sugar Land TX, U.S.A.) equipped with a reverse phase column (Erbasil 100-S-C18, 230 x 8 x 4 mm, Carlo Erba, Milan, Italy) and a UV detector operating at 240 nm. The column was eluted (1 ml/min) with H_2O-AcOH (99:1, v/v) and MeOH by the following linear gradient: 0 min, 0% MeOH; 2 min, 5% MeOH; 10 min, 25% MeOH; 45 min, 60% MeOH; 55 min, 85% MeOH; and 60 min, 100% MeOH.

All data were statistically analysed and means compared using the Student's *t* test, and Duncan's multiple range test.

RESULTS

Final fresh top and root weights of inoculated plants were not significantly ($P<0.05$) different from the control with FS-17, whereas the presence of *X. index* caused a significant ($P<0.05$) reduction of root weight with DA-12I. Percentage increases of stem diameter, length and node number were found to be reduced at nematode densities > 20 females/pot with DA-12I, but only at the highest inoculum density with FS-17 (Table 1).

Reproduction of *X. index* was very low with both varieties, but always higher with FS-17 than with DA-12I (Table 2). Root damage was not particularly evident, and only at the highest inoculum density was the gall index of DA-12I significantly ($P<0.01$) lower than that of FS-17.

Phenols content and PAL activity in non inoculated roots of DA-12I were higher ($P<0.01$) than with FS-17, whereas no significant difference was found in leaves (Tables 3-4). Presence of *X. index* caused a significant ($P<0.01$) increase of phenols in roots and leaves of DA-12I and FS-17 at the highest nematode inoculum densities. The phenols profile varied significantly ($P<0.01$) only in the roots of DA-12I, in which a substantial increase of oleuropein was found in plants subjected to 80 females/pot, as compared with the control and the lowest nematode inoculum density plants (Fig. 1).

PAL activity always decreased in roots subjected to feeding by *X. index* with the decrease being greater with DA-12I than with FS-17 plants, whereas it significantly ($P<0.01$) increased in the leaves of DA-12I plants, but did not change in FS-17 plants (Table 4).

POD increased in roots and leaves of DA-12I plants, but the difference from the control was statistically significant only at the highest nematode inoculum density (Tables 3-4). No significant difference ($P<0.01$) was found with the roots of FS-17 plants, but POD activity increased ($P<0.01$) in the leaves of FS-17 plants subjected to 20 females/pot.

Analysis of kinetic parameters of both varieties showed that Km did not differ significantly ($P<0.01$) in roots and leaves (10 and 6 with DA-12I and 6 and 7.8 with FS-17 plants). However, values of Vmax were higher ($P<0.01$) in leaves (400 and 555) of DA-12I and FS-17 plants, respectively, than in roots (312 and 238) (Fig. 2).

The POD activity profile at different pH values showed only one peak at 6.5, with roots and with leaves of DA-12I and FS-17 plants (Fig. 3).

Addition of 200 µM ascorbic acid to the enzymatic extracts inhibited 80% of POD activity.

DISCUSSION

Growth of FS-17 plants was not affected by the presence of *X. index*, whereas DA-12I plants were only slightly damaged at high nematode population densities. From the low nematode reproduction rates (Pf/Pi: 0.1 – 0.4 and 0.4 - 1.2 for DA-12I and FS-17 plants, respectively) these plants can not be considered good hosts for *X. index*. The lower reproduction rate with DA-12I may be attributed to a higher phenols content in the uninfested roots, as previously reported for tomato and olive roots infested by root-knot nematodes (Bajaj & Mahajan, 1977; Ridolfi *et al.*, 1998).

Table 1. Effect of *Xiphinema index* on plant growth of a cultivar (FS-17) and a rootstock (DA-12I) of olive.

Nematode density (females/pot)		Mean weight (g)[1]			Mean increase with respect to initial values (%)[1]			
		Top fresh	Root	Total	Stem diam	Shoot diam	Shoot length	Node number
DA-12I	0	100[2] a[3]	100a	100a	100a	100a	100a	100a
	10	93a	80b	86b	86a	91a	82a	81ab
	20	85ab	76b	80b	74ab	62b	72ab	67bc
	40	89ab	78b	83b	75ab	61b	43b	53bc
	80	71b	63c	67c	44b	53b	39b	46c
FS-17	0	100a	100a	100a	100a	100a	100a	100a
	10	97a	97a	97a	70ab	85ab	108a	107a
	20	87a	84a	85a	73ab	102a	73ab	95a
	40	81a	80a	80a	73ab	93a	84ab	98a
	80	76a	79a	77a	43b	62b	50b	80a

(1) Data are expressed as per cent of the control (=100);
(2) Each value is a mean of ten replicates;
(3) Data with the same letter in a column are not statistically different according to Duncan's multiple range test (P = 0.05).

Table 2. Reproduction of *Xiphinema index* in the rhizosphere of a cultivar (FS-17) and a rootstock (DA-12I) of olive.

Nematode density (females/pot)	Mean root gall index			Mean n° females/l soil			Mean n° juveniles/l soil			Mean reproduction rate ($r = Pf/Pi$)		
	DA-12I	FS-17	t[3]	DA-12I	FS-17	t	DA-12I	FS-17	t	DA-12I	FS-17	t
0	0[1]a[2]	0a	–	0a	0a	–	0a	0a	–	0a	0a	–
10	0.7b	0.6b	–	5.0ab	7.0ab	–	3.6ab	15.0ab	*	0.4d	1.2b	–
20	0.6b	1.0bc	–	7.4b	10.8b	–	4.8ab	32.6bc	**	0.3cd	1.1b	*
40	0.8b	0.6b	–	11.2b	8.0ab	–	6.2b	33.0bc	**	0.2bc	0.5a	–
80	0.8b	1.4c	**	7.4b	25.0c	*	4.8ab	39.0c	**	0.1ab	0.4a	**

(1). Each value is a mean of ten replicates;
(2) Data with the same letter in a column are not statistically different according to Duncan's multiple range test (P = 0.05);
(3) Statistically different according to Student's *t* test. * for P = 0.05; ** for P = 0.01.

Table 3. Effect of *Xiphinema index* on phenols content, PAL, and POD in roots of a cultivar (FS-17) and a rootstock (DA-12I) of olive.

Nematode density (females/pot)	Phenols content (mg/g f.w.)			PAL (U/g f.w.)			POD (U/g f.w.)		
	DA-12I	FS-17	t[3]	DA-12I	FS-17	t	DA-12I	FS-17	t
0	9.4[1]b[2]A	7.2aA	**	344aA	232aA	**	36aA	323aA	**
10	9.2abA	7.2aA	–	226bB	201bB	*	55abA	309aA	–
20	8.6aA	7.2aA	–	210bcBC	192bB	*	171bcA	175bB	–
40	8.9abA	8.3bB	**	173cdBC	164cC	–	194cA	293aA	–
80	11.3cB	7.9bAB	–	164dC	160cC	**	2,833dB	302aA	–

(1) Each value is a mean of three replicates;
(2) Data with the same letter in a column are not statistically different according to Duncan's test (lower case letters, P=0.05; capital letters, P=0.01);
(3) Significantly different according to Student's *t* test (* for P=0.05 and ** for P=0.01). Comparison between DA-12I and FS-17 plants were made on absolute values for the control (population density = 0) and on percentage variation from the control for the other densities.

Table 4. Effect of *Xiphinema index* on phenols content, PAL, and POD in leaves of a cultivar (FS-17) and a rootstock (DA-12I) of olive.

Nematode density (females/pot)	Phenols content (mg/g f.w.)			PAL (U/g f.w.)			POD (U/g f.w.)		
	DA-12I	FS 17	$t^{(3)}$	DA-12I	FS-17	t	DA-12I	FS-17	t
0	24.3[(1)]a[(2)]A	21.9aA	–	2,575aAB	2,032aA	–	428abA	1,986aA	**
10	27.8bB	22.0aA	–	2,331aA	2,077abA	–	421aA	4,830dD	**
20	27.0bAB	22.9aAB	–	3,223bB	3,433cB	**	495bA	5,433eE	**
40	26.9bAB	27.9bB	–	3,033bB	2,295bA	–	497bA	3,861cC	*
80	33.8cC	27.8bB	–	4,877cC	1,889aA	**	655cB	3,116bB	–

(1) Each value is a mean of three replicates;
(2) Data with the same letters in a column are not statistically different according to Duncan's test (lower case letters, P=0.05; capital letters, P=0.01);
(3) Significantly different according to Student's *t* test (* for P=0.05 and ** for P=0.01). Comparison between DA-12I and FS-17 plants were made on absolute values for the control (population density = 0) and on percentage variation from the control for the other densities.

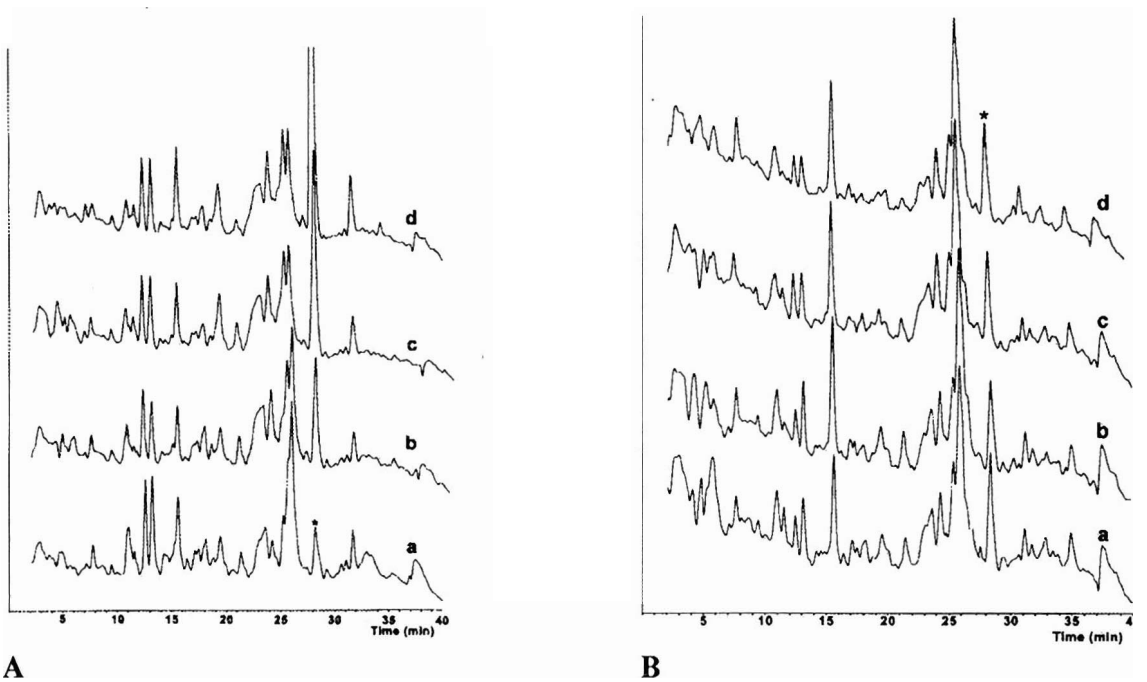

Fig. 1. Phenols chromatographic profile in the roots of DA-12I (A) and FS-17 (B) plants at different population densities of *Xiphinema index*: a, uninoculated soil; b, 10 females/pot; c, 40 females/pot; d, 80 females/pot; Asterisk indicates the peak relative to oleuropein.

The decrease of PAL content in roots subject to nematode feeding possibly demonstrates that the reaction in olive to *X. index* is not related to PAL activity. Similar observations were reported for the reaction of olive and peach to root-knot nematode infection (Yongbing *et al.*, 1994; Ridolfi *et al.*, 1998). Moreover, the variation of phenols chromatographic profile, due specifically to an increase of oleuropein content, may be indicative of plant resistance not being related to a local phenols synthesis in the roots by the means of PAL. The response may be due to a translocation of oleuropein from the leaves, as oleuropein is normally present at high concentration in olive leaves, and is characterized by a high translocability.

Peroxidase activity, as with phenols content, increased in DA-12I plants relative to the nematode inoculum density, and a similar reaction has been observed with olive and tomato plants infested with root-knot nematodes (Zacheo *et al.*, 1982, 1983; Ridolfi *et al.*, 1998). Values of kinetic

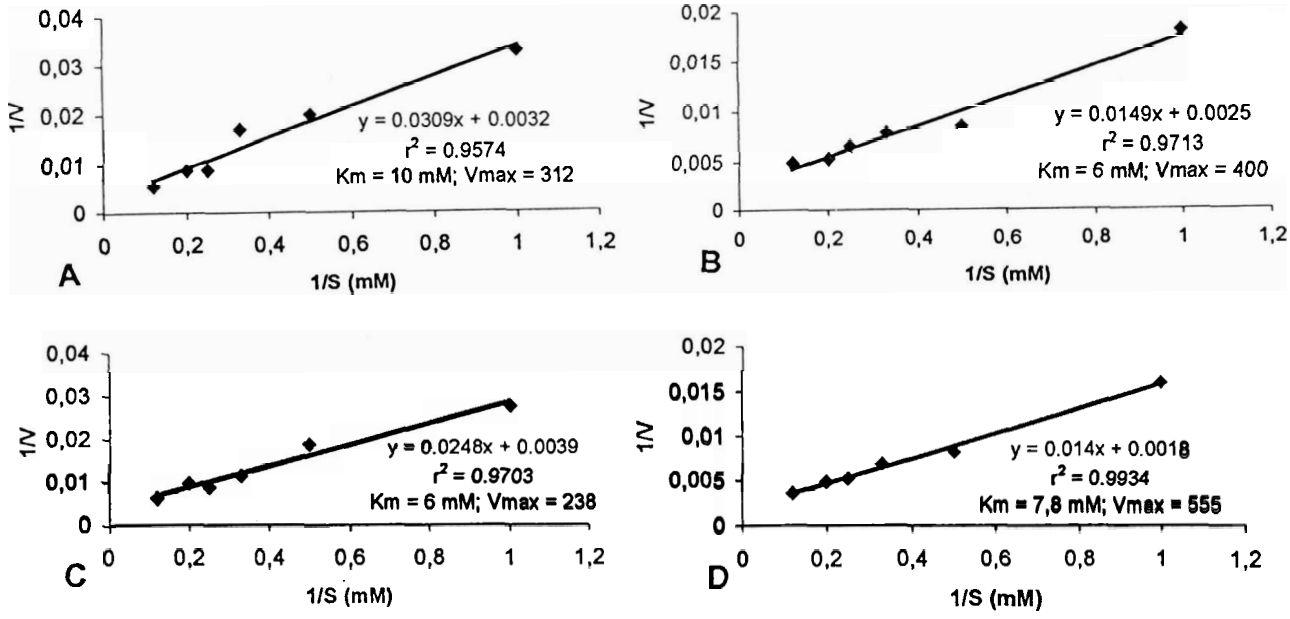

Fig. 2. Double reciprocal plots of the initial rate (v) substrate oxidation by peroxidase from roots and leaves of DA-12I (A and B, respectively) and FS-17 plants (C and D, respectively).

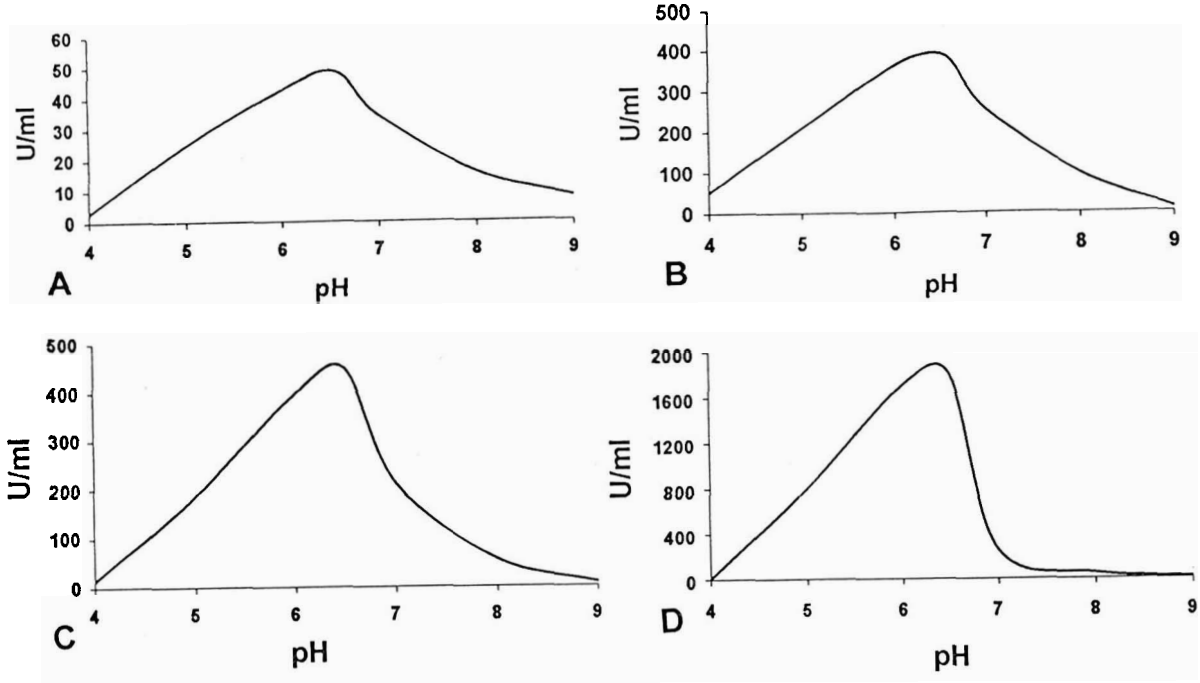

Fig. 3. Peroxidase activity profile at different pH values in roots and leaves of DA-12I (A and C, respectively) and FS-17 plants (B and D, respectively).

parameters showed that the same isozyme was present in DA-12I and FS-17 plants, and it was more active in leaves than roots. This was confirmed also by the relationship between POD and pH, in which a peak at the same 6.5 pH value was recorded in leaves and roots. Addition of ascorbic acid showed that olive POD was inhibited by this antioxidant and that POD activity values should be attributed more specifically to this enzyme catalysis.

In conclusion, the work confirms the role of phenols and POD in the response of olive to feeding by nematodes (Huang, 1985). More specifically, the apparent increased resistance of DA-12I plants to feeding by *X. index* may result from high POD activity following nematode infection

allowing a rapid transformation of phenols to insoluble phenolic polymers at feeding sites that causes giant cells necrosis.

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