

COMPARISON OF SCREENING METHODS FOR THE EVALUATION OF OLIVE RESISTANCE TO *VERTICILLIUM DAHLIAE* KLEB.

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SUMMARY

Three inoculation methods for screening olive germplasm for resistance to *Verticillium dahliae* Kleb. were compared: (i) dipping soil-free washed roots into inoculum (WRI); (ii) dipping root balls with soil into inoculum (RBI); (iii) direct introduction of the inoculum into a stem incision (SII). The effectiveness of the methods was compared in their capacity to differentiate levels of resistance/susceptibility to *Verticillium* wilt in olive germplasm. Genetically homogeneous clones of the olive cvs Frantoio, Coratina and Leccino, which have different degrees of resistance/susceptibility to wilt, were inoculated with one defoliating and two non-defoliating *V. dahliae* pathotypes. Results indicate that the three screening methods had different sensitivity in identifying variable levels of olive resistance to *Verticillium* wilt. Inoculation of washed roots (WRI) was the most drastic and may be used to identify higher levels of resistance (such as that of cv. Frantoio), but was not sensitive enough to detect lower levels of resistance. Inoculation of root balls with soil (RBI) was less destructive and allowed detection of lower resistance expression (such as that of cv. Coratina), not detected by WRI. Stem incision inoculation (SII) failed to discriminate between resistant (cvs Frantoio and Coratina) and susceptible (cv. Leccino) germplasm.

Key words: *Olea europaea*, inoculation methods, defoliating and non defoliating pathotypes, *Verticillium* wilt, screening for resistance.

INTRODUCTION

Wilting disease of olive caused by *Verticillium dahliae* Kleb. is widespread wherever this crop is grown. *Verticillium* wilt of olive was first reported in Sicily (Ruggieri, 1946), and subsequently in many other areas of Italy (Cirulli, 1975, 1981) and European countries, the

Middle East and the USA (Jiménez-Díaz *et al.*, 1998). The disease is most damaging on young plants, while on older plants, including those over 100-year-old, the disease does not normally kill the plant, but reduces vegetation and causes partial defoliation of one or more branches. At present, control is based essentially on preventive methods such as the use of pathogen-free plants and soil when planting new olive orchards. Chemical fungicides such as benzimidazoles are not effective (Biris and Thanassoulopoulos, 1980; Cirulli, 1981). Soil solarization with the crop in progress, whether alone or followed by applications of the natural antagonist *Talaromyces flavus* (Klöcker) Stolk Samson to the soil, has been fairly effective in containing the disease (Skoudridakis and Bourbos, 1989; Tjamos *et al.*, 1991; Tjamos, 1993; Lopez-Escudero and Blanco-Lopez, 1997).

Use of resistant olive varieties is the most effective, economically feasible and ecologically sustainable means of control. Research in Italy and elsewhere has shown that the cvs Frantoio, Coratina, Frangivento, Oblonga and Kalamon have interesting resistance properties (Wilhelm and Taylor, 1965; Hartmann *et al.*, 1971; Schnathorst and Sibbett, 1971; Cirulli and Montemurro, 1976; Tjamos, 1993; Lopez-Escudero *et al.*, 2004), while cvs Ascolana, Cellina, Leccino, Manzanillo, Chemlalie, Konservolia, Mission and Picual are susceptible (Wilhelm and Taylor, 1965; Cirulli and Montemurro, 1976; Wilhelm, 1981; Tjamos, 1993; Rodriguez-Jurado *et al.*, 1993; Lopez-Escudero *et al.*, 2004).

In finding and characterizing plant germplasm resistant to the disease, the screening procedure is of primary importance, in addition to physiological races, inoculum concentration, plant age and environmental parameters such as temperature and humidity. For a better comparison of results on *Verticillium* wilt resistance in olive, a simple, effective and standardized inoculation method is needed. Up to now, different screening methods have been used with a multitude of variables such as inoculation technique, form and concentration of inoculum, age and type of plant (grafted, rooted cuttings, seedlings), growing environment (growth chamber or greenhouse), and disease evaluation scales (Wilhelm and Taylor, 1965; Schnathorst and Sibbett, 1971; Cirulli and Montemurro, 1976; Rodriguez-Jurado *et al.*, 1993).

Therefore, the results obtained have in some cases been contradictory (Cirulli and Montemurro, 1976; Pennisi *et al.*, 1993). In the present study different screening methods have been evaluated for their effectiveness in discriminating different characteristics of resistance/susceptibility in olive germplasm.

MATERIALS AND METHODS

Inoculation methods. Three inoculation methods were evaluated for their effects on the appearance of Verticillium wilt:

(i) Shaking soil from the roots, washing them in running water, and immersing roots for 2 min in the inoculum (WRI). Plants were then repotted in the same soil. Control plants were subjected to the same treatment but were immersed in tap water.

(ii) Immersion of root balls with soil into inoculum, after making two parallel vertical slashes on opposite sides of the plant 5 cm from the surface the pot (RBI). Control plants were subjected to the same treatment but were immersed in tap water.

(iii) Direct introduction of 30 ml inoculum into a horizontal cut in the stem made with a scalpel 10 cm from the top of the soil (SII). To stimulate inoculum uptake, the plants were not watered for 24 h before inoculation. Control plants were subjected to the same treatment using tap water.

Plants. Olive cultivars tested were the susceptible Leccino (Cirulli and Montemurro, 1976; López-Escudero *et al.*, 2004) plus Frantoio and Coratina, which possess different levels of resistance to Verticillium wilt (Wilhelm and Taylor, 1965, Cirulli and Montemurro, 1976). Since cross-pollination occurs in olive, to overcome the genetic variability of seedlings, genetically homogeneous one-year-old rooted cuttings were used. The three variables, olive cultivar, inoculation method and fungal isolate were combined. For each combination (cultivar, pathotype and inoculation method) 42 plants distributed in three randomized blocks were used. Fourteen plants constituted non inoculated controls for each inoculation method. Plants were grown in plastic boxes 15×15×20 cm (3.5 l) in a mixture of soil (three parts) and peat (one part) steamed at 80°C. Inoculated plants were kept in a greenhouse at 24±5°C.

Inoculum. Non-defoliating and defoliating pathotypes of *V. dahliae* vary in aggressiveness and symptomatology on olive (Schnathorst and Sibbett, 1971; Rodriguez-Jurado *et al.*, 1993). In this study two non-defoliating isolates (Vd 311, Vd 315) from diseased olive and one defoliating isolate (Vd 312) obtained in southern Italy from diseased cotton (M. Cirulli, unpublished information) were used.

For the first two screening methods each isolate was grown on Petri dishes (9 cm diameter) for a week on potato dextrose agar (PDA) in the dark at 25±0.2°C. Entire colonies of each isolate plus the agar were then comminuted separately in a Waring Blender. The final inoculum concentration for each fungal isolate was adjusted to 2×10⁶ conidia/ml. The mycelial part and microsclerotia of the inoculum were not evaluated.

The inoculum used in the third inoculation method was prepared using Petri dishes and growing medium as above. Plates with 7-day-old cultures were submerged in sterile water and the surface of the colonies was scraped with a spatula. The final concentration of inoculum thus obtained for each of the three isolates was adjusted to 6×10⁶ conidia/ml. The other components of the inoculum were not evaluated.

Disease evaluation. Disease reaction was assessed 70 and 100 days after inoculation. In the first recording the severity of external symptoms was calculated using an arbitrary scale in which 0 = healthy plant; 1 = light foliar symptoms in 1 to 9% of the plant canopy; 2 = moderate foliar symptoms and light defoliation (10-25%); 3 = severe foliar symptoms and moderate defoliation (26-50%); 4 = total defoliation and 5 = dead plant. In the second recording, severity of external symptoms and vascular browning were assessed. In the case of RBI and WRI inoculation methods, vascular browning was evaluated on a cross-section taken on the main stem 10 cm above the soil. For the SII inoculation method vascular browning was assessed on a stem cross section 5 cm above the inoculation cut. Vascular browning was evaluated using a scale based on the percentage of wood discoloration in which 0 = absence of browning; 1 = 1-10% of cross section with browning; 2 = 11-25% of cross section with browning; 3 = 26-75% of cross section with browning; 4 = 76-100% of cross section with browning and dead plant.

At the end of the experiment, re-isolations of *V. dahliae* were attempted from cross sections of the stem made 5 cm above soil level. Samples were taken from five randomly selected plants including symptomless plants. Bark was removed from the cross sections and woody tissues were sterilized in 3.5% sodium hypochlorite for 60 sec, placed on PDA plates and incubated in the dark at 24±0.2°C. Colonies grown on plates for seven days were identified according to their macroscopic and microscopic characteristics.

Statistical analysis. The experiment was repeated twice with similar results, thus only data from the final trial are presented. The effects of the main factors (inoculation method, *V. dahliae* isolate and olive cultivar) as well as their interactions were estimated using ANOVA (SAS Institute Inc., 1987), means were separated using the Waller-Duncan test. Each of all the possible combi-

nations between inoculation method, *V. dahliae* isolate and olive cultivar were considered as a single treatment instead of comparing among them by considering each factor individually. Thus, for statistical purposes, all combinations were compared.

RESULTS

No *Verticillium* wilt symptoms were observed in non-inoculated control plants, nor was the pathogen reisolated from any of them.

The two methods of root inoculation (WRI and RBI) were effective in differentiating cvs Frantoio, Coratina and Leccino for resistance/susceptibility to *Verticillium* wilt but the SWI method was not effective (Figs. 1 and 2).

Frantoio was only slightly affected by the disease. Values of external symptoms observed on this cultivar 100 days after inoculation were the lowest and there were no significant differences among inoculation methods and *V. dahliae* pathotypes. In contrast, in the susceptible cv. Leccino, isolate Vd 312 caused severe defoliation extending to the entire plant, which eventually died. On the same cultivar, the non-defoliating pathotypes Vd 311 and Vd 315 caused typical leaf yellowing, necrosis and infrequent defoliation but in no case death of the plants.

The WRI method induced the strongest wilting. At

70 and 100 days after inoculation, on cvs Coratina and Leccino plants inoculated by this method with the defoliating pathotype Vd 312, the mean values of external symptoms were significantly higher than those observed on the same cultivars inoculated with the same pathotype but using the RBI and SWI methods. At 100 days after inoculation, cv. Frantoio showed the least external symptoms (0.4). At the same time, cvs Coratina and Leccino showed the highest values of external symptoms but these did not significantly differ from one another (4.3 and 4.7, respectively). Plants of these last two cultivars, inoculated with the two non-defoliating pathotypes Vd 311 and Vd 315, showed mean severities of external symptoms not statistically different from those observed on the same olive cvs inoculated using RBI and SWI.

The SWI method was less drastic. With the defoliating pathotype Vd 312, the mean severities of external symptoms recorded at 100 days from inoculation were significantly different among the three olive cultivars: Frantoio showed the lowest disease severity (0.3), Coratina had an intermediate value (2.6), and Leccino showed the highest (3.7). At the same time, with the non-defoliating pathotypes (Vd 315 and Vd 311) this method caused only very slight symptoms on all inoculated plants (Figs. 1 and 2).

Mean severities of vascular browning on cv. Frantoio was the lowest and without significant difference among

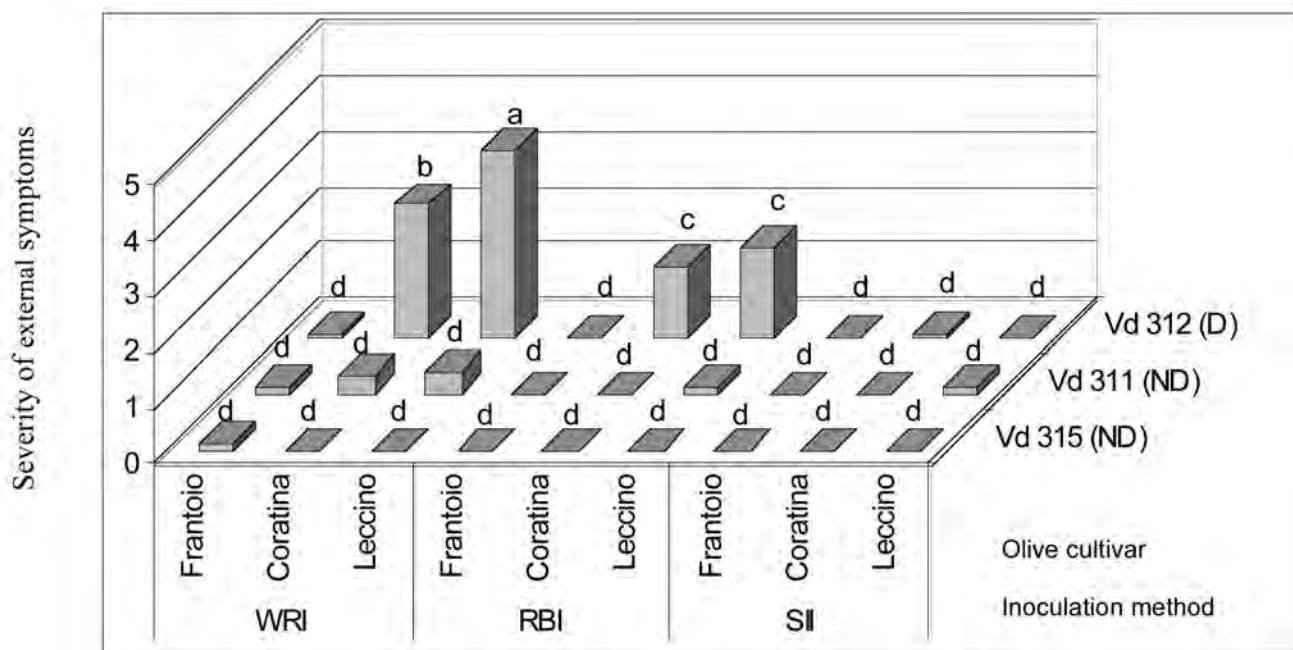


Fig. 1. Effect of inoculation methods on the severity of *Verticillium* wilt external symptoms on olive cultivars Frantoio, Coratina and Leccino. Readings made 70 days after inoculation. Columns with the same letter are not significantly different ($P \leq 0.05$) according to the Waller Duncan Test. WRI = Washed roots inoculation, RBI = Root ball inoculation, SWI = Stem wound inoculation. D = Defoliating pathotype; ND = Non-defoliating pathotype.

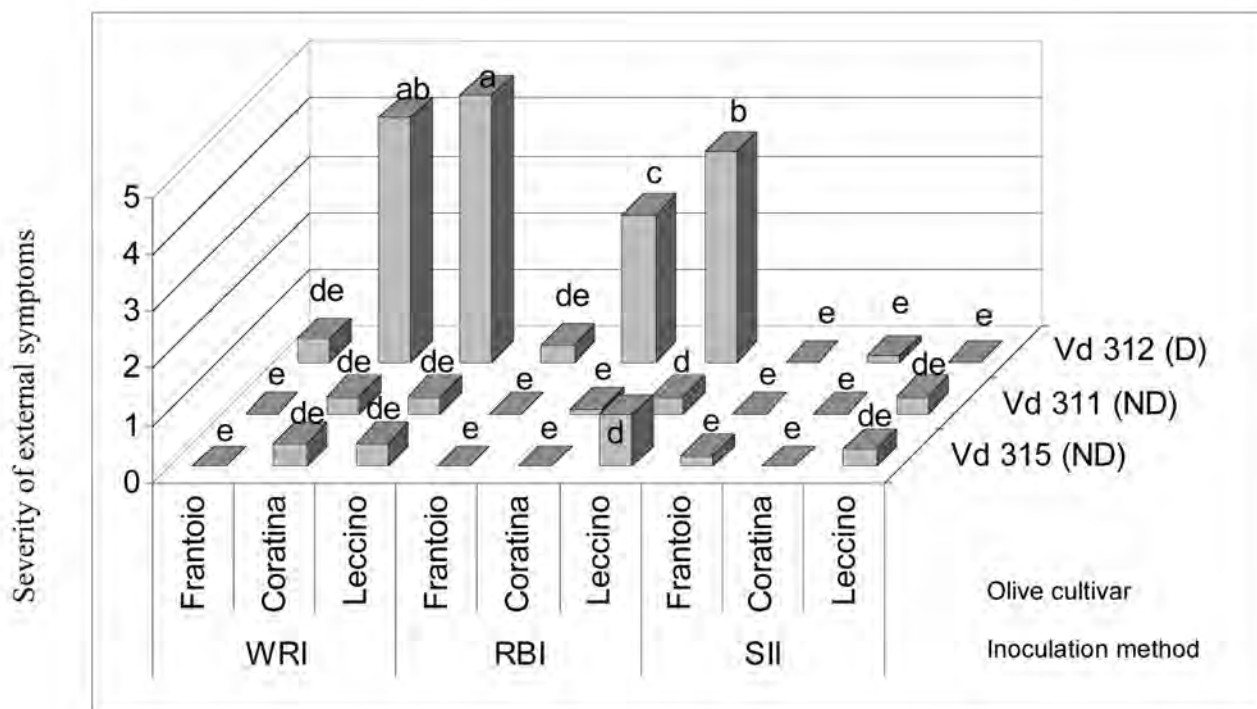


Fig. 2. Effect of inoculation methods on the severity of *Verticillium* wilt external symptoms on olive cultivars Frantoio, Coratina and Leccino. Readings made 100 days after inoculation. Columns with the same letter are not significantly different ($P \leq 0.05$) according to the Waller Duncan Test. WRI = Washed roots inoculation, RBI = Root ball inoculation, SWI = Stem wound inoculation. D = Defoliating pathotype; ND = Non-defoliating pathotype.

the fungal pathotypes (Fig. 3). Vascular discoloration observed in cvs Coratina and Leccino inoculated by WRI or RBI with the defoliating pathotype were significantly the highest (Fig. 3).

The mean levels of positive re-isolations of *V. dahliae* are given in Fig. 4. The lowest percentage was obtained from plants inoculated with the non-defoliating isolate Vd 311. The highest percentage of re-isolations from the three olive cultivars was obtained in plants exposed to root inoculation (WRI and RBI).

DISCUSSION

Our results have confirmed the previously recorded high level of resistance of cv. Frantoio (Wilhelm and Taylor, 1965; Cirulli and Montemurro, 1976; López-Escudero *et al.*, 2004) and the high susceptibility of cv. Leccino (Cirulli and Montemurro, 1976; López-Escudero *et al.*, 2004) toward the non-defoliating *V. dahliae* pathotype. Coratina was also confirmed as partially resistant as shown in previous tests carried out in Italy (Cirulli and Montemurro, 1976) but only against the non-defoliating pathotypes. This emphasizes the importance of using different *V. dahliae* pathotypes in screening for resistant germplasm, as these may induce different resistance/susceptibility reactions. In this study, cv. Frantoio proved to be highly resistant also to the defoli-

ating *V. dahliae* pathotype, while in other studies this cultivar was found only moderately susceptible to this pathotype (López-Escudero *et al.*, 2004).

The two methods of root inoculation were both effective in identifying sources of resistance to *Verticillium* wilt of olive. With these methods it was possible to discriminate the different levels of susceptibility/resistance in the cultivars used.

Inoculation by washed root immersion (WRI) induced higher disease severities than the other two methods. It was rapid and effective, showing that cv. Frantoio is more resistant than cvs Coratina and Leccino, although it did not allow differentiation between the latter two. The WRI method produced a differential reaction in a short, effective incubation period. In fact, at 100 days from inoculation the defoliating pathotype, when inoculated on the susceptible cv. Leccino, caused severe defoliation of 100% of all tested plants and 70% death.

The WRI method was successful, also in comparison with other inoculation systems, in studies of resistance to *Verticillium* wilt in cauliflower (Koike *et al.*, 1994), broccoli (Bhat and Subbarao, 2001), horseradish (Atibalentja and Eastbur, 1997), olive (Colella *et al.*, 2005) and artichoke (Jiménez-Díaz *et al.*, 2006).

Inoculation by immersion of whole root balls with attached soil (RBI) proved to be less drastic, allowing discrimination among the olive cultivars used. It showed that cv. Coratina possesses resistance intermediate be-

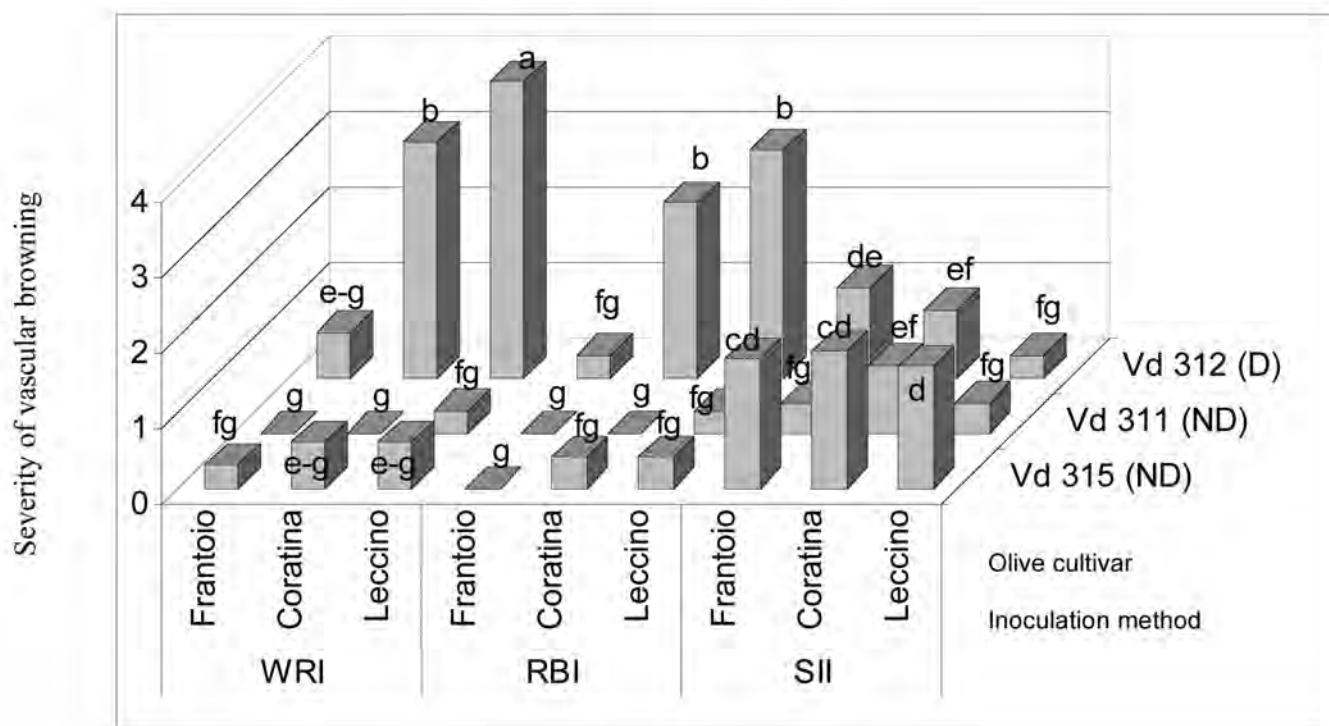


Fig. 3. Effect of inoculation methods on the severity of *Verticillium* wilt vascular browning on olive cultivars Frantoio, Coratina and Leccino. Readings made 100 days after inoculation. Columns with the same letter are not significantly different ($P \leq 0.05$) according to the Waller Duncan Test. WRI = Washed roots inoculation, RBI = Root ball inoculation, SWI = Stem wound inoculation. D = Defoliating pathotype; ND = Non-defoliating pathotype.

tween the most resistant cv. Frantoio and the highly susceptible cv. Leccino. With this inoculation method, differential reactions began to appear 70 days post inoculation and were completely established at 100 days. The defoliating isolate Vd 312 on cv. Leccino inoculated by RBI caused severe defoliation of 100% of the plants, 57% of which died. In this connection, it is worth mentioning that the RBI method proved also effective for screening germplasm resistant to *V. dahliae* in *Prunus* spp. (Cirulli *et al.*, 2001), strawberry (Amenduni *et al.*, 2003), and artichoke (Cirulli *et al.*, 1994).

Both methods of root inoculation are therefore effective, but the choice depends on specific purposes. WRI screening appears more suitable when searching for highly resistant germplasm to be used as rootstock for which resistance nearing immunity is essential. The RBI method seems more appropriate for the identification of partial resistance in olive germplasm destined for production of rooted cuttings or scions for grafting on highly resistant rootstocks.

Screening by stem incision inoculation method (SII) was technically more difficult because the stem of 1-year-old olive plants is thin (0.5 cm diameter) and difficult to handle. The disease values were the lowest with this method and did not show up any differences in disease reaction between cvs Frantoio, Coratina and Leccino. However in evaluating resistance/susceptibility in

two-year-old olive cultivars, inoculated by root immersion and by stem incision, good results were obtained with both methods (Pennisi *et al.*, 1993).

Direct introduction of the pathogen into the vascular system using methods such as stem incision, drill injection or puncturing, has been used to identify sources of resistance to *Verticillium* wilt in ash (Hiemstra, 1995), maple (Hiemstra, 1997), cocoa (Resende *et al.*, 1995), avocado (Zentmeyer, 1949), cotton (Bolek *et al.*, 2005; Jimenez-Diaz *et al.*, 2006), and olive (Blanco-López *et al.*, 1984) but this method is limited conceptually in that it does not allow activation of resistance mechanisms present in the root system, which is the main point of entry for *V. dahliae*.

Previous studies (Cirulli and Montemurro, 1976) indicated a correspondence in susceptible cultivars between the severity of external symptoms and percentage of pathogen re-isolation from artificially inoculated olive plants. However, the data obtained in this study show that the differences in resistance/susceptibility of the three olive cultivars tested are not related to the percentage of positive re-isolations obtained. Similar results were also obtained by López-Escudero *et al.* (2004).

V. dahliae was not obtained from all inoculated plants of cvs Coratina, Leccino and Frantoio. These results are similar to those obtained by Cirulli and Montemurro (1976) who, like Wilhelm and Taylor (1965), found that

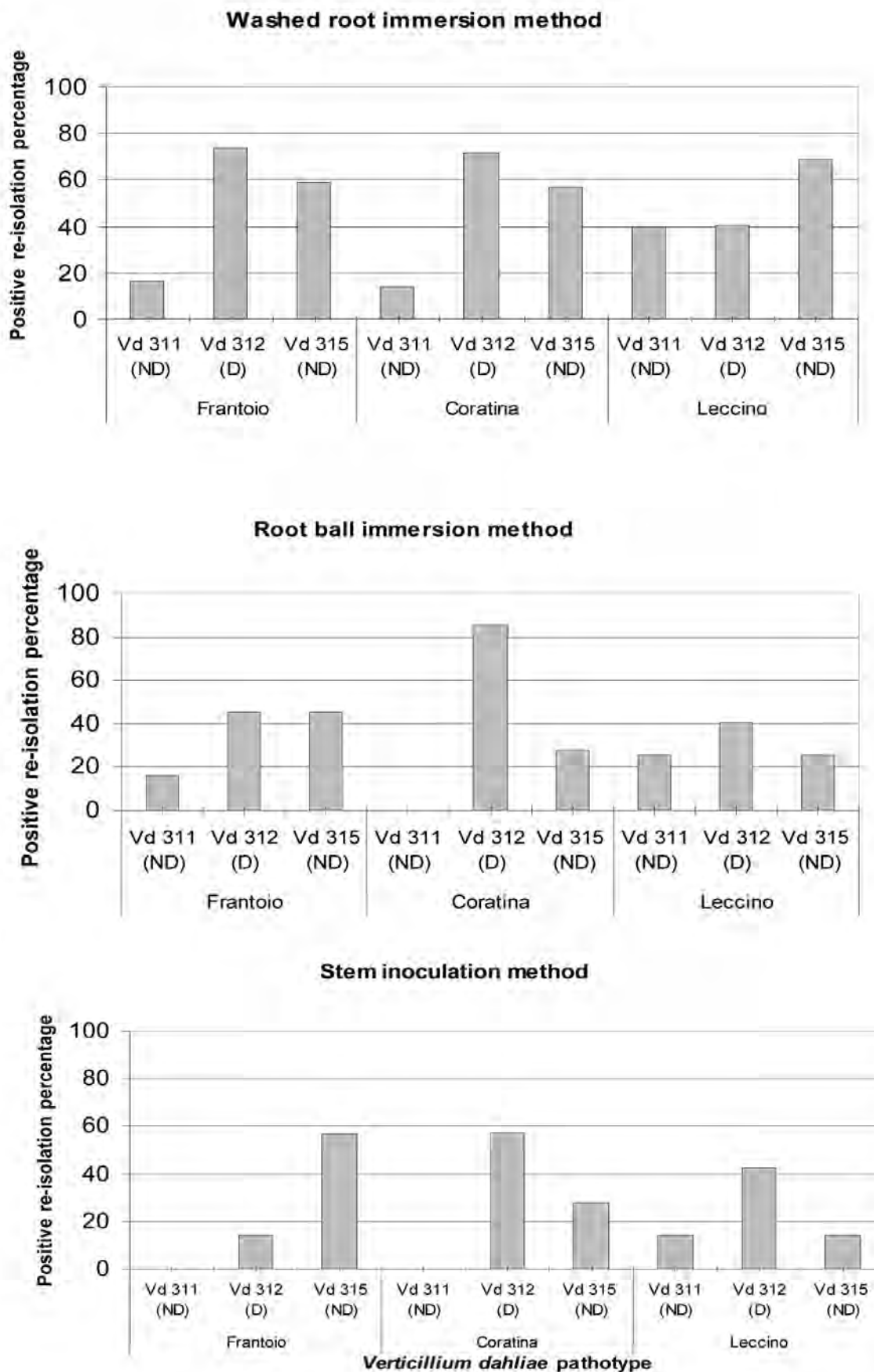


Fig. 4. Percentage of positive *Verticillium dahliae* re-isolations from cvs Frantoio, Coratina and Leccino. D = Defoliating pathotype, ND = Non-defoliating pathotype.

different levels of resistance and tolerance to infection occur in olive. Failure to detect the fungus in stems of inoculated plants does not exclude the presence of the fungus in the lower parts of the plants, for example in the root system.

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