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Short Notes

Alternaria alternata causing necrosis on leaves, fruits, and pedicels of olive plants in Italy

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Summary. This paper is the first report of symptoms caused by *Alternaria alternata* on aboveground organs of olive plants in Italy. On leaves, symptoms included spots and necroses frequently associated with damage caused by the olive thrips (*Liothrips oleae*). On fruits, symptoms included browning and necroses of pedicels, necroses of fruitlets soon after fruit set, and rot and mummification of mature fruit. Several isolates of *A. alternata* with identical morphological features and DNA sequences were associated with all the different symptoms. The impacts of *A. alternata* on olive production can be severe, and infections to fruit pedicels are particularly relevant as they cause severe fruit fall soon after fruit set.

Keywords. *Olea europaea*, leaf spot, fruit rot.

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INTRODUCTION

The presence of *Alternaria spp.* on olive plants (*Olea europaea* L.) was first associated with infections of drupes and high yield losses in 2001 in the Languedoc-Roussillon region of France (Alaux, 2002). In 2008, *Alternaria alternata* (Fries) Keissler was isolated in Spain and was associated with soft rot of mature olive drupes showing grey-white skins and mummification (Moral *et al.*, 2008). A survey conducted in in Morocco showed presence of *A. alternata* in all the sampled olive orchards (Chliyeh *et al.*, 2014). More recently, *A. alternata* has been identified as a causal agent of olive leaf spots in Turkey (Basım *et al.*, 2017), leaf and fruit spots in Pakistan (Alam and Munis, 2019), bud and blossom blight in Greece and Bosnia and Herzegovina (Lagogianni *et al.*, 2017; Crnogorac *et al.*, 2023), and necroses on olive tree cuttings in Greece (Tziros *et al.*, 2021). In the last 8-10 years, in Calabria (Southern Italy), similar symptoms have been observed in olives, which did not match any disease previously reported in Italy. These Symptoms had different frequencies and severities, depending on microclimatic

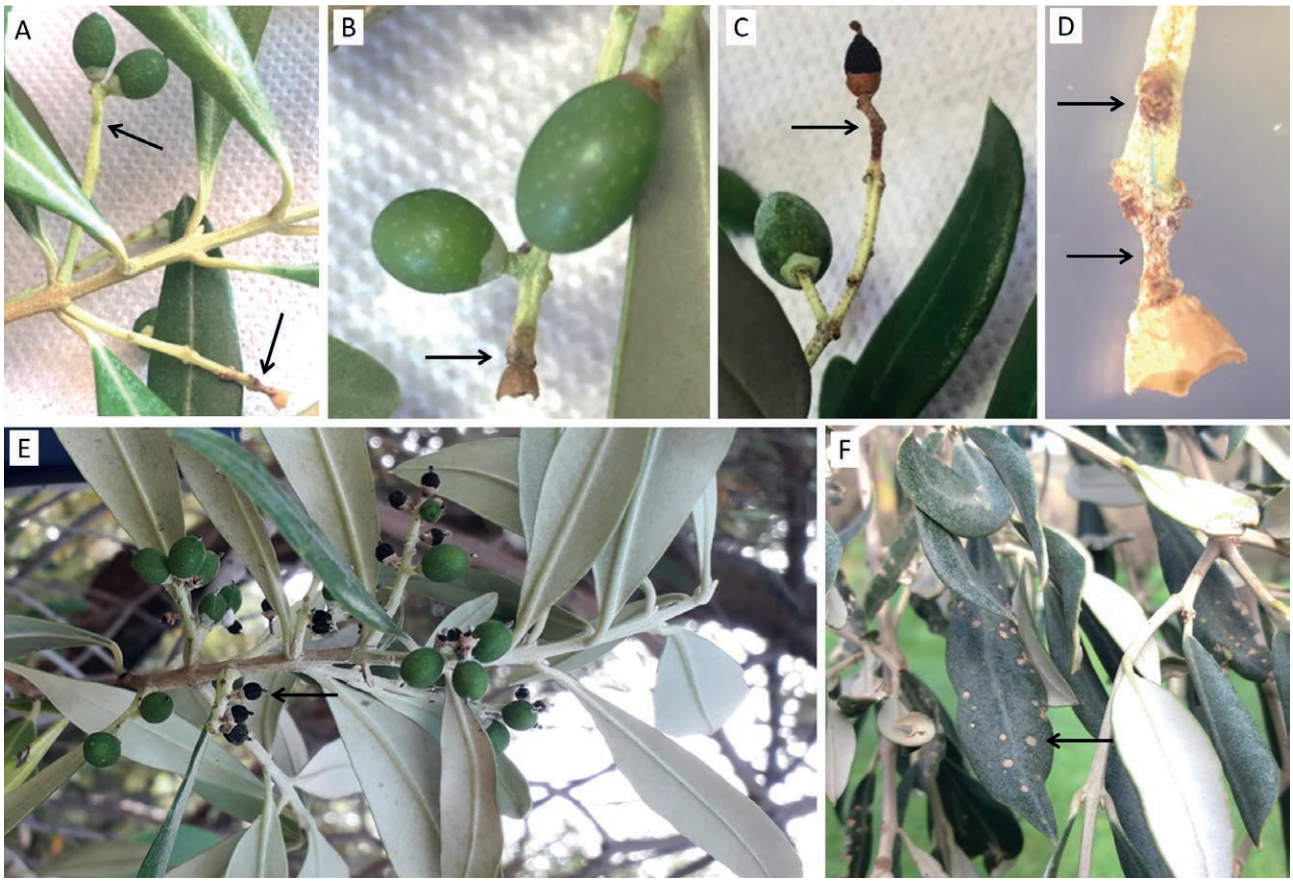

Figure 1. Symptoms caused by infections of *Alternaria alternata* on pedicels and leaves of *Olea europaea*. (A) Localized browning (top arrow) and extended necrosis (bottom arrow) of the pedicel. (B) Necrosis of the apical part of a pedicel and detachment of the fruitlet. (C) Extended necrosis of a pedicel and consequent death of the fruitlet, which remained attached to the plant. (D) Close-up of a necrotic pedicel. (E) Olive branch with dead fruitlets due to infections of *A. alternata* on the pedicels. (F) Leaf spots caused by *A. alternata*, probably in association with *Liothrips oleae*.

areas (orchards), olive cultivars and years. Overall, however, symptoms were observed in a large area extending for more than 150 km in the east coastal area of Catanzaro and Reggio Calabria. Often, whole production areas were lost. Symptoms included (i) browning and necroses of fruit pedicels (Figure 1 A, B, C, D, E); (ii) leaf spots and necroses frequently associated with damage caused by the olive thrips, *Liothrips oleae* (Figure 1 F); (iii) necroses on fruitlets soon after setting (Figure 2A, B); immature fruits (Figure 2C); and (iv) rots of mature fruits with grey-white skins and subsequent mummification (Figure 2 D).

The present study aimed to determine if *A. alternata* was responsible for all the symptoms described above, carrying our *in vitro* isolations and re-inoculations to confirm the role of this fungus as a pathogen of olive plants.

MATERIALS AND METHODS

Pathogen isolation

Samples of symptomatic olive tree organs (leaves, pedicels, fruitlets, and mature fruits) were collected in 2017 and 2022 from a representative orchard of cv. Carolea located in Sellia Marina, Catanzaro, southern Italy (GPS 38.894225, 16.716885), and *in vitro* isolation of the causal agent was attempted. Olive tree samples were surface sterilized by dipping them for 15 s in a 2% sodium hypochlorite solution, rinsed twice in sterile distilled water, and then dried on sterile absorbent paper. Small pieces of tissues were dissected and placed on Potato Dextrose Agar (PDA) plates, containing ampicillin and streptomycin (200 μg mL^{-1}) to prevent bacterial growth. The plates were then incubated at 25±1°C under a 12 h

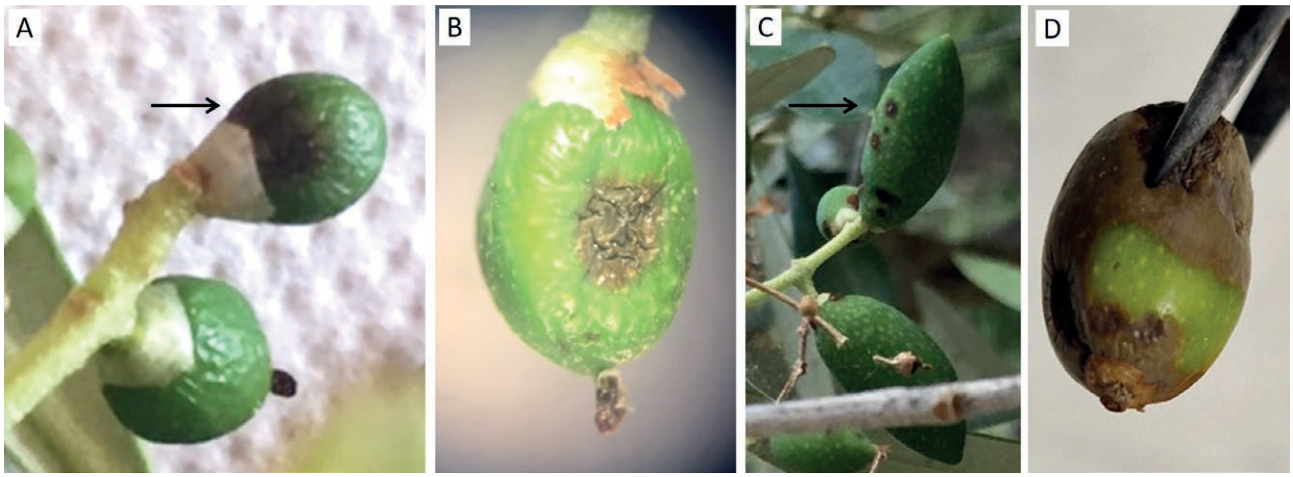

Figure 2. Symptoms caused by *Alternaria alternata* infections on olive fruits in different growing phases. (A and B) Necrotic areas around the floral calyx and in the equatorial zone of young fruits soon after setting. (C) Necrotic areas on an immature fruit. (D) Rotten mature fruit.

light 12 h dark regime. Dark mycelium developed from most of the dissected tissues, and mycelium from the margins of these colonies was transferred to fresh PDA plates to establish pure cultures. Stock cultures of these isolates are maintained at 4°C and at -20°C at the University of Reggio Calabria.

Morphological, molecular and phylogenetic analyses of isolated fungi

The morphology of colonies, hyphae and conidia of sixteen representative isolates (four from each of the sampled olive organs) was observed after growing pure cultures on PDA at 23°C for 10–15 d. Four isolates (one from each sampled olive organ) were identified by Sanger-sequencing six gene regions (ITS, GAPDH, RPB2, TEF1, ALTA1, and ENDOPG) commonly used as barcode genes for *Alternaria* sect. *Alternaria* (Woudenberg *et al.*, 2015). DNA extraction, amplification, and sequencing were carried out as described by Schena *et al.* (2014), and sequences of each gene were manually curated using the software ChromasPro v.2.1.10.1.

A phylogenetic tree was built with concatenated sequences of the six genes, using representative reference sequences of *Alternaria* sect. *Alternaria* (Woudenberg *et al.*, 2015). In addition, sequences were aligned and compared with available sequences of *A. alternata* previously associated with olive diseases (Alam and Munis, 2019; Tziros *et al.*, 2021; Crnogorac *et al.*, 2023). Sequences were aligned with MUSCLE, and were used to build a phylogenetic tree using the Maximum Likelihood method and the Tamura–Nei model with 1,000 bootstraps, as implemented in MEGA 11 (Tamura *et al.*, 2021).

Pathogenicity tests

Pathogenicity tests were carried out on detached fruits and leaves of three olive varieties (Coratina, Leccino, and Nocellara etnea). Fruits and leaves (n = 40 per organ and cultivar) were surface sterilized by immersion in a 1% sodium hypochlorite solution for 1 min, washed twice with tap water, air-dried, and then wounded in the equatorial zones with a needle (fruits) or a scalpel (leaves). Half of the fruits and leaves (n = 20 for each cultivar) were each inoculated with 20 µL of a conidium suspension (≈10^5 conidia mL^{-1}) of the pathogen, while the other half (n = 20 for each cultivar) were inoculated with sterile water. These detached leaves and fruits were then placed in plastic boxes to maintain high relative humidity. A similar set of tests were carried out for leaves of 2-year-old potted plants (n = 3) of the olive cultivar Carolea. Due to practical difficulties for surface sterilizing potted plants, leaves (n = ten per plant) were each wounded with a scalpel without preliminary sterilization, and were then inoculated as above and covered with a transparent plastic bag. A set of ten leaves for each plant were inoculated with sterile water as inoculation controls. Lesion diameters on detached fruits and leaves was recorded 7 d after inoculation, and differences between plant varieties were assessed tested using ANOVA of these data. Data analyses were carried out with R v4.4.0 (R Core Team, 2020), using *Base R* and *stats* packages for statistical tests and the *ggplot2* package (Wikman, 2009) for data visualization.

RESULTS AND DISCUSSION

Five days after isolation, white mycelium developed from most dissected tissues, and this turned dark later due to abundant sporulation (Figure 3 A). Developing conidiophores were dark green to brown and were short, septate, and branched. Conidia were ellipsoidal each with a short beak and many transverse septa. Sixteen representative isolates (four from each of the sampled olive organs) had identical morphological features of colonies, hyphae, and conidia, were tentatively identified as *Alternaria* spp. (Simmons 2007).

The molecular analyses of the four sequenced isolates gave identical results for the ITS, GAPDH, RPB2, TEF1, ALTA1, and ENDOPG genes (GenBank Accession number, respectively, PP762481, PP783612, PP783614, PP783613, PP783615, and PP783616). BLAST analyses of the sequences showed 100% identity with several corresponding sequences of reference strains of *Alternaria alternata* (GenBank Accession numbers KP124341, KP124195, KP125117, KP124809, KP123889, and KP124042). The phylogenetic analysis confirmed the identity of the isolates from olive (Figure 3 B). After trimming sequences to an even length, ITS, TEF, and ENDOPG sequences of the isolates were identical across all isolates previously reported on olive (Alam and Munis, 2019; Tziros *et al.*, 2021; Crnogorac *et al.*, 2023). Furthermore, ALT1 sequences were identical to three of five previously reported sequences in Bosnia and Herzegovina (OP972865, OP972866) (Crnogorac *et al.*, 2023) and Greece (MN512439) (Lagogianni *et al.*, 2017), differing for a single base (C instead of T) compared to the other two Greek sequences (MN512440, MN512441). Greater diversity was found within the RPB2 gene, as seven bases differentiated the present study isolates from two other isolates from Bosnia and Herzegovina (OP038921 and OP038922) (Crnogorac *et al.*, 2023).

For the pathogenicity tests, after 6 d at 20 ± 2°C, all detached leaves (Figure 4 A) and fruits (Figure 5) inoculated with *A. alternata* developed dark lesions resembling those of natural infections, while non-inoculation control fruits and leaves remained asymptomatic. *Alternaria alternata* was re-isolated from symptomatic samples, fulfilling Koch's postulates. Similarly, for the second set of tests on potted plants, all the inoculated leaves developed necroses (Figure 4 B), while all the non-inoculated control leaves were asymptomatic. ANOVA revealed statistically significant differences of sensitivity to inocu-

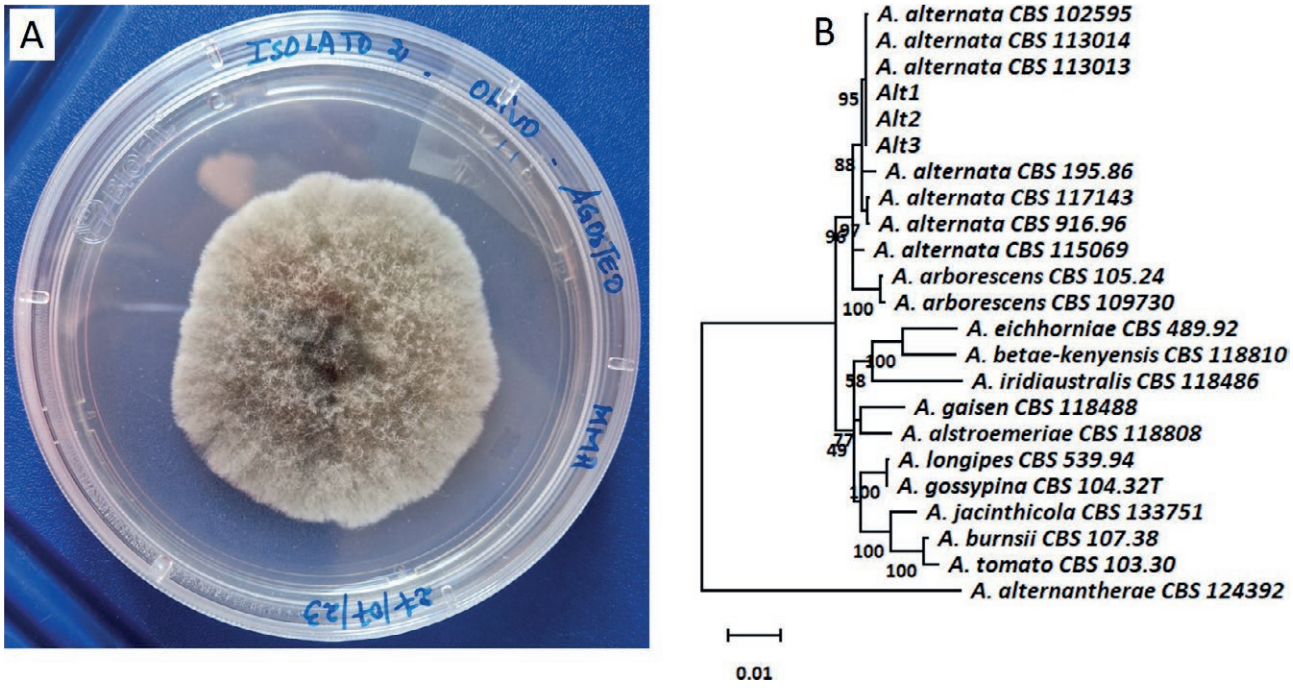

Figure 3. (A) Representative colony of *Alternaria alternata* isolated from a young fruit of *Olea europaea* on potato dextrose agar after 4 d incubation. (B) Phylogenetic tree constructed using concatenated sequences of ITS, GAPDH, RPB2, TEF1, ALTA1, and ENDOPG gene regions (Woudenberg *et al.*, 2015). Representative sequences obtained in the present study (Alt1, Alt2, and Alt3) were analyzed with reference sequences of *Alternaria* sect. *Alternaria* (Woudenberg *et al.*, 2015). Sequences were aligned with MUSCLE and used to build a phylogenetic tree using the Maximum Likelihood method and the Tamura–Nei model with 1,000 bootstraps, as implemented in MEGA 11 (Tamura *et al.*, 2021).

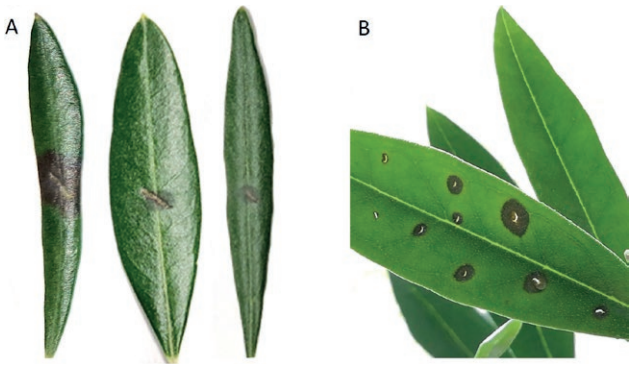

Figure 4. (A) Symptoms on detached leaves of cultivars Coratina (left), Leccino (middle), and Nocellara etnea (right), 6 d after inoculation with *Alternaria alternata*. (B) Symptoms on leaves of a potted olive plant cultivar Carolea 6 d after inoculation *Alternaria alternata*.

lation among the cultivars, on leaves ($F_{2, 57}$ = 68.65, P < 0.001) and fruits ($F_{2, 57}$ = 62.78, P < 0.001). Tukey's post-hoc contrasts showed that on fruits, mean lesion diameters (Figure 6, mean ± sd) were greatest for the cultivar Leccino (11.50 ± 0.32 mm), followed by Coratina (7.42 ± 0.72 mm) and Nocellara etnea (4.08 ± 0.16 mm). Leaves of Coratina were more sensitive (mean lesion diameter = 11.09 ± 0.97 mm) than those of Leccino (3.97 ± 0.12 mm) and Nocellara etnea (1.19 ± 0.42 mm).

As indicated above, previous studies have reported *A. alternata* as the cause of diseases in olive plants. However, this is the first report of infections on olive fruit pedicels. This is also the first report of the occurrence of *A. alternata* on olive plants in Italy. The same pathogen has been shown to cause all the previously reported symptoms on olive (Moral *et al.*, 2008; Basım *et al.*, 2017; Lagogianni *et al.*, 2017; Alam and Munis, 2019; Crnogorac *et al.*, 2023).

The present study results also showed differences in susceptibility to *A. alternata* for different olive varieties. The close similarity between sequences of the present study isolates with those previously reported indicated that observed differences in symptom incidence and severity are likely to be related to environmental conditions and host genotype rather than *A. alternata* strain. This emphasises the importance of the cultivar choice for to prevent the spread of this disease. Although further investigations are required, the impacts of disease caused by this pathogen were severe in the region surveyed in the present study. Infections of fruit pedicels

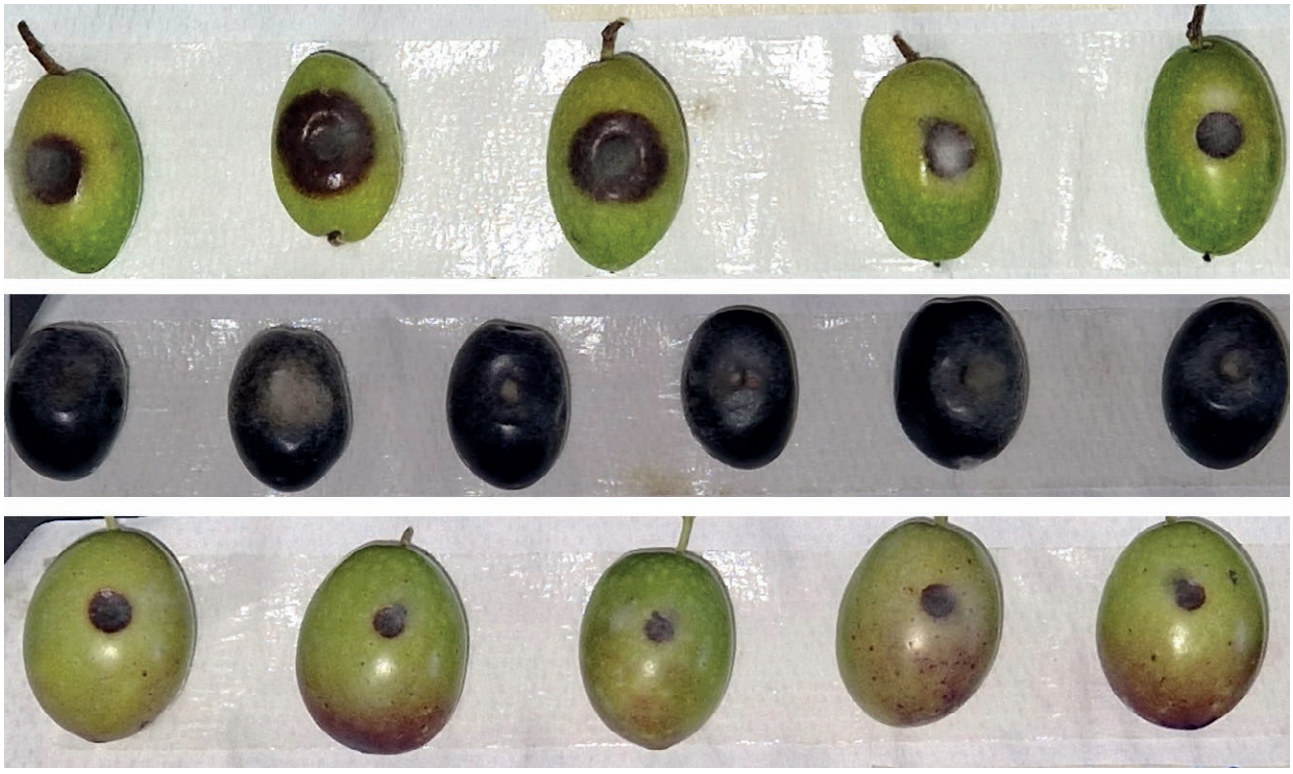

Figure 5. Symptoms on fruits of Coratina (top), Leccino (middle), and Nocellara etnea (bottom) 6 d after inoculation with *Alternaria alternata*.

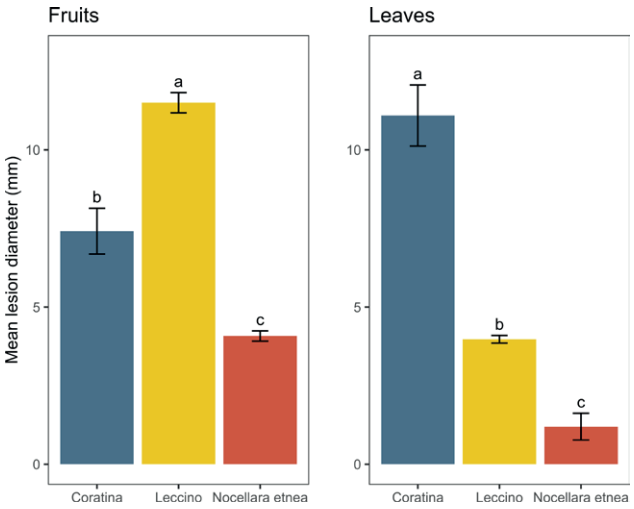

Figure 6. Mean lesion diameters on olive fruits (left) and leaves (right) of cultivars Coratina, Leccino, and Nocellara etnea 6 d after inoculation with *Alternaria alternata*. Barplots are means ± sd for each group. Pairwise comparisons are indicated as letters on top of each barplot (Tukey's multiple comparison procedure).

were the most relevant, as these caused many fruits to fall from trees during the first developmental phases.

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