Sphaceloma coryli: A Reemerging Pathogen Causing Heavy Losses on Hazelnut in Southern Italy

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Abstract

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In 2013 to 2014, a disease causing severe crop losses was observed in several growing areas of the Campania region in southern Italy. Disease symptoms consisted of necrotic spots on leaves, bracts, and shells along with vein necrosis. Nuts were necrotic or aborted and symptomatic fruit dropped prematurely. A fungus was consistently isolated from symptomatic tissues and morphologically identified as *Sphaceloma coryli*. The fungus was first reported in France and Italy (Campania) over 30 years ago and was not subsequently documented until 2006 in the Latium region of Italy. When artificially inoculated on healthy hazelnut plants,

disease symptoms were reproduced. The teleomorph of *S. coryli* was recorded for the first time on overwintering tissues of hazelnut trees. It was also induced in vitro. The morphology of asci and ascospores together with phylogenetic analysis based on internal transcribed spacer sequences indicated that the teleomorph is an undescribed species within the genus *Elsinoë* for which the name of *Elsinoë coryli* is proposed. Optimum temperatures for ascospore and conidium germination and mycelium growth were determined in vitro. The inhibition effect of some commercial fungicides on *S. coryli* was also evaluated in vitro.

Hazelnut (*Corylus avellana* L.) is a deciduous, monoecious, multistemmed bush that can be grown as a single trunk tree for commercial purposes. The nut is edible, rich in protein, and with a significant amount of B vitamins. The use of hazelnut as a food source has been found on several European Mesolithic sites dating about 9,000 years ago. Hazelnuts are sold as in-shells or kernels. In-shell hazelnuts are sold for fresh consumption while shelled ones have many uses, including raw material, confectionary, and bakery food. Furthermore, due to its beneficial health attributes, hazelnut has also been valued by cosmetic and pharmaceutical companies (Kris-Etherton et al. 1999). Nearly 90% of the harvested yield is sold to processing companies, whereas fresh consumption represents the residual 10%. Italy is the second-largest hazelnut producer in the world (22%), far behind Turkey (70%), followed by the United States (5%) and Spain (3%) (FAOSTAT 2013). Hazelnut cultivation is spread all along Italy, from north to south, with main production sites in the Piedmont (13%), Latium (38%), Campania (37%), and Sicily (9%) regions (ISTAT 2010). In 2013, the total Italian production reached 128,940 metric tons (FAOSTAT 2013). Only 15 to 20% of the production of hazelnut in Campania is destined to direct consumption; the remainder is used by industry.

Since 2013, hazelnut growers in Campania have been suffering heavy yield losses due to a premature nut drop associated with necrotic spots on fruit bracts and shells, necrosis or abort of nuts, and necrotic spots and vein necrosis of leaves. Among the cultivars grown

in Campania, ‘San Giovanni’ was the most susceptible, followed by ‘Camponica’ and ‘Mortarella’; ‘Tonda Tardiva’ was the least susceptible. Yield loss has been estimated to be 50 to 100% depending upon the area, management practices, cultivars, and other factors. Total yield loss was observed in some San Giovanni trees grown in unmanaged orchards with high humidity. Several pathogens may infect hazelnut, causing spot symptoms on leaves and fruit or fruit drop. Among them, some fungi (*Monilinia fructigena*, *M. laxa*, *Piggotia coryli*, *Sphaceloma coryli*, and *Fusarium lateritium*) and bacteria (*Xanthomonas arboricola* pv. *corylina*) have been reported in Italy (Librandi et al. 2006).

Initially, the disease observed in Campania was attributed to hazelnut bacterial blight caused by *X. arboricola* pv. *corylina*, based on symptomatology (Miller et al. 1940; Vauterin et al. 1995). The bacterium spread throughout Europe in the last decade, being occasionally detected in Campania (Noviello 1968) (A. Zoina, personal communication) while causing significant yield loss in Central Italy (Lamichhane et al. 2012; 2013). However, more detailed analysis have ruled out bacterial involvement (A. Zoina, personal communication). The bacterium was, in fact, never isolated from *C. avellana* symptomatic tissues, and symptoms typical of *X. arboricola* pv. *corylina*, such as shoot dieback and canker formation, have never been observed on the symptomatic trees.

Another pathogen causing symptoms similar to those observed in Campania is *S. coryli*, one of the agents of hazelnut anthracnose. The fungus was first reported in France and Italy (Campania) over 30 years ago (Bourgeois and Vegh 1967; Gardan and Bourgeois 1982; Ragozzino 1983) and was not subsequently documented until 2006 in the Latium region of Italy (Librandi et al. 2006). The fungus had never been associated with economically important losses. In addition, symptoms attributed to it were leaf and fruit spots but not fruit drop.

Fungal species belonging to the genus *Sphaceloma* de Bary produce hyaline, unicellular, not septate, ellipsoidal conidia in subepidermal acervuli. The perfect stage of *Sphaceloma* belongs to *Elsinoë* Racib., the type of Elsinoaceae, a family recently revisited (Jayawardena et al. 2014; Wijayawardene et al. 2012). They are responsible for anthracnose diseases of numerous crop and ornamental plants such as citrus, grape, mango, pepper, legumes, rose, and pit-spore, on which they cause diseases of great economic importance (Agrios 2005; Condé et al. 1997; Hyun et al. 2001; Mchaur

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et al. 1998; Ridley and Ramsfield 2005; Ugglia and Carlson-Nilsson 2009).

Lack of information on the pathogen involved in the recent outbreaks of the hazelnut disease recorded in Campania has prompted the need to (i) identify the agent associated with the disease on the basis of morphological and molecular characteristics, (ii) determine the in vitro effect of temperature on ascospore and conidial germination and colony growth, and (iii) evaluate the in vitro efficacy of some fungicides in order to develop an effective disease management strategy.

Materials and Methods

Field observation, sample collection, and light microscopy. In 2013 to 2014, symptomatic trees (12 per location) of San Giovanni or Mortarella were chosen at random in two orchards located in San Paolo Belsito, two orchards in Nola (Naples Province), and one orchard in Domicella (Avellino Province). From the beginning of January to the end of September, samples (female flowers, buds or leaves, and fruit) were collected monthly. In total, 540 samples, consisting of 480 symptomatic (115 female flowers, 105 buds, 110 leaves, and 150 fruit) and 60 symptomless (15 female flowers, 15 buds, 15 leaves, and 15 fruit), were collected. Symptomatic samples consisted of plant parts showing necrotic lesions. Furthermore, symptomatic fruit (bracts or shells) and leaves, five for each area and each tissue, were collected in June from six orchards located in Caianello and Presenzano (Caserta), Liveri, Palma Campania and Roccarainola (Naples), and Cava dei Tirreni (Salerno) (Fig. 1). During spring, dried symptomatic fruit bracts, overwintering on trees, were collected from trees (two per locations) located in Caianello, Cava dei Tirreni, Domicella, Nola, Presenzano, and San Paolo Belsito. Fresh symptomatic (9% leaves and 7% fruit) and symptomless (33% leaves and 33% fruit) tissues and all dried symptomatic fruit bracts collected were embedded in 50% optimal cutting temperature (O.C.T.) compound (Miles) for 24 h. The specimens were mounted onto an aluminum stub and sectioned at 25 μ m by Cryostat 1720 digital (Leitz). Sections were observed using an Orthoplan microscope (Leitz).

Pathogen isolation and maintenance. Symptomatic and asymptomatic buds, female flowers, and fruit were washed in tap water (60 s), disinfected superficially with ethanol 70% (30 s), then washed with 1% sodium hypochlorite solution (30 s), and, finally, rinsed three times in sterilized distilled water (10 s). Leaves were quickly wiped with cotton wool impregnated with 70% ethanol and passed through a Bunsen burner flame.

For fungal isolations, 10 to 20 small pieces (1 to 2 mm² each) of material for each sample were excised from the selected tissues and transferred aseptically to 2% potato dextrose agar (PDA; Difco, Bacto), or to 2% malt extract agar (MEA; Difco, Bacto), then incubated under a photoperiod of 8 h of darkness and 16 h of light at 20 $\pm$ 1°C. Colonies were subcultured and, after a month, pure cultures were identified at genus and species level on the basis of morphological characteristics.

For long-term storage, fungal isolates were placed on PDA covered with filter paper for 30 days. Mycelia were then desiccated using anhydrous calcium chloride desiccant (Sigma-Aldrich) and stored at 4°C in a sterile glass vial sealed with Parafilm.

Morphological study. Fungal colonies of four isolates (two Mortarella and two San Giovanni, one from San Paolo Belsito and one from Nola, respectively) were grown at 20 $\pm$ 1°C for a month. Shape, length, and width of 50 conidia per isolate were measured, and mean length and width were calculated using ImageJ (<http://imagej.nih.gov/ij>). Experiments were repeated two times.

To evaluate the effect of temperature on conidial and ascospore germination, mycelia were collected from cultures of the same four isolates and crushed in sterile distilled water. Spore suspension was filtered through cheesecloth to remove hyphal fragments and diluted to a final concentration of approximately 50 conidia or ascospores per microliter. For each isolate, 12 1- μ l drops of spore suspension were placed onto petri dishes containing water agar (2% agar) and placed at 5, 10, 15, 20, 25, 30, and 35 $\pm$ 1°C, respectively. Percent spore germination was determined after 24 and 48 h by observing 50 spores selected from each drop. Conidia or ascospores were

considered as germinated when the length of the germ tube was at least one-half of the spore diameter.

Mycelial growth of the four isolates was assessed by placing a 4-mm² mycelium fragment on MEA or PDA media and incubating at 5, 10, 15, 20, 25, 30, and 35 $\pm$ 1°C under a photoperiod of 8 h of darkness and 16 h of light. Every mycelium fragment used in this study was excised from the expanding margin of a fresh colony. Eight colony areas for each isolate were measured weekly (for 4 weeks) using ImageJ software (<http://imagej.nih.gov/ij>).

Molecular characterization. *DNA isolation and purification.* Mycelia (100 mg) were harvested from the agar surface and ground with a pestle in a mortar containing liquid nitrogen. Total DNA was extracted from 24 isolates obtained from different tissues (buds, leaves, fruit bracts, and stalks) and six locations (Caianello, Cava dei Tirreni, Domicella, Nola, Presenzano and San Paolo Belsito) using a Qiagen DNeasy Plant Mini Kit (Qiagen Inc.), following the manufacturer's instructions.

Fungus identification by polymerase chain reaction. The universal primers intergenic transcribed spacer ITS1-F and ITS4 (Gardes and Bruns 1993; White et al. 1990) were used to amplify part of the nuclear ribosomal DNA spanning the 3' end of the 18S ribosomal RNA (rRNA) gene (small subunit), the first ITS region (ITS1), the 5.8S rRNA gene, the second ITS region (ITS2), and the 5' end of the 28S rRNA gene (large subunit). Polymerase chain reaction (PCR) mixture consisted of 1 μ l of total genomic DNA, 0.5 μ l of 10 pmol each primer, 0.5 μ l of 0.2 mM dNTP, 2.5 μ l of 10x reaction buffer, 0.75 μ l of 1.5 mM MgCl₂, and 0.5 U of Platinum Taq (Invitrogen). Amplification conditions, performed on a Mastercycler gradient (Eppendorf), consisted of an initial denaturation (5 min at 94°C) and 35 cycles of PCR (94°C for 1 min, 48°C for 1 min, and 72°C for 1 min), followed by a final extension period at 72°C for 10 min. PCR amplicons were separated electrophoretically (100 V for 40 min) on 1% agarose gels prepared with Tris-borate-EDTA buffer amended with ethidium bromide at 10 mg/ml and visualized under UV light. PCR amplicons of the expected size (approximately 600 bp) were excised from the gel, purified using the Nucleospin Extract Kit (Macherey-Nagel), and, finally, sequenced in both directions at BMR Genomic.

Genetic analysis. All obtained sequences were compared with the sequences present in GenBank through a BLASTn search (Altschul et al. 1990) and the identity of samples was determined based on the sample's maximum identity to sequences available in database.

Sequences were aligned using Clustal W (Thompson et al. 1994) and all alignments were manually examined to check for single apparent misalignment. In addition to the sequences generated in this study, interspecific multiple alignments included the ITS1-5.8S-ITS2 sequences obtained from GenBank; in particular, 5 *Sphaceloma*

Fig. 1. Distribution of hazelnut anthracnose disease in Campania, Southern Italy. Dots indicate the areas where the disease was observed and samples collected.

spp. and 37 *Elsinoë* spp. sequences, with *F. proliferatum* as the outgroup taxon. Phylograms based on ITS1-5.8S-ITS2 sequence comparisons were constructed using the neighbor-joining, maximum-parsimony, and maximum-likelihood methods implemented in MEGA6 (Molecular Evolutionary Genetic Analysis Software Version 4 for Windows; Tamura et al. 2013). Bootstrap analyses with 1,000 replicas were performed to test the significance of the nodes and the robustness of the trees (Felsenstein 1985). A bootstrap threshold of 50% was retained for all branches.

Pathogenicity bioassays. Two-year-old healthy potted hazelnut plants (San Giovanni) were spray inoculated with four isolates (the same used for morphological studies) obtained during this study. Fungal inocula were prepared by crushing 30-day-old pure colonies cultured on PDA at $20 \pm 1^\circ\text{C}$ in sterile distilled water. Control plants were spray inoculated with sterile distilled water. Experiments were performed by directly spraying buds, fully expanded leaves, stalks, and immature fruit with fungal suspension (approximately 20 ml/branch, 50,000 conidia/ml). In a second experiment, wounds were made by puncturing tissues of 50 buds, 30 leaves and petioles, and 30 immature fruit per plant by a sterile needle before inoculation. For each isolate, one plant was used. Inoculation experiments were repeated two times. All the plants were covered with plastic bags for 7 days to maintain a high relative humidity, then maintained in the greenhouse at 25°C for a month. Tissues of each plant were visually inspected every day and the appearance of disease symptoms was recorded. Reisolations were performed by incubating symptomatic tissue fragments on PDA at $20 \pm 1^\circ\text{C}$.

In vitro fungicide sensitivity. Nine fungicides belonging to five different chemical families (Table 1) were tested in vitro to determine their effect on fungus growth. Fungicide concentrations were chosen considering the commercial minimum and maximum doses recommended by manufacturers to control fungi on nut or fruit trees. An intermediate dose was also used. Fungicide stock suspensions were prepared and added to sterile MEA. The suspensions were mixed and poured into 9-cm-diameter petri dishes. Using a 2-mm sterile cork borer, fragments of 30-day-old cultures of four isolates grown on MEA at $20 \pm 1^\circ\text{C}$ were removed, placed at the center of plates, and incubated at $20 \pm 1^\circ\text{C}$, with a photoperiod of 8 h of darkness and 16 h of light. For each fungicide treatment, five replicates per concentration were used. MEA without the fungicide amendment was used as control. The fungus growth (colony area) was measured weekly for a month. Colony areas were measured using a public domain software (ImageJ).

Statistical analysis. For repeated experiments in the laboratory, the variance was tested by variance analysis ($P \leq 0.05$) using Statistical Package for Social Science 17.

Results

Field observation, sample collection, and light microscopy. The disease was observed throughout 2013 to 2014 in several orchards located in Avellino, Caserta, Naples, and Salerno Provinces (Campania) (Fig. 1). Early symptoms appeared in January as small, dark-brown, sunken lesions on female flowers that evolved into small cankers causing partial flower distortion. Similar lesions were observed on buds. After 2 months, reddish-purple spots adjacent to the midribs and vein necrosis were observed on leaves. As the season progressed,

leaf vein necrosis and leaf spots increased, often coalescing (Fig. 2); necrotic spots with a purple border and a depressed lighter color center began to develop on fruit bracts and shells. Necrotic lesions also developed on stalks, causing wilting and consequent premature fruit dropping and nut abortion. Symptoms were prevalent on trees in areas with high relative humidity and where cultural practices (sucker removal or pruning) were not adopted.

Pulvinate, superficial, brown, globose to subglobose multiloculate pseudoascostromata were observed by light microscope in all dried symptomatic fruit bracts collected. Pseudoascostromata presented multiascus locules. Asci were bitunicate, globose, or subglobose and contained eight irregularly arranged, light-brown, oblong, bicellular ascospores slightly constricted at the median septum, sized 8.9 ± 1.3 by $5.3 \pm 0.8 \mu\text{m}$ (Fig. 3A and B). Rarely, ascospores with two or three septa were observed.

Subepidermal acervula producing colorless, one-celled, ovoid, and cylindrical oblong conidia of 5.0 ± 0.5 by $2.0 \pm 0.3 \mu\text{m}$ developed on symptomatic leaves and petioles from June, as observed by analyzing the symptomatic hazelnut tissues collected monthly and embedded in O.C.T. compound.

Morphological characterization. In total, 7,950 fungal isolates were obtained from symptomatic and asymptomatic female flowers, buds, leaves, petioles, stalks, and fruit bracts and shells. No fungus was isolated from necrotic or aborted nuts.

Independent of geographical area and cultivar, a fungus (80%, 7,375 isolates) was prevalently isolated from all symptomatic tissues collected from all orchards; the remaining recovered fungi were all saprophytes. Only saprophytes were isolated from asymptomatic tissues. The prevalent isolated fungus formed irregular, strongly folded, convoluted, compact, and elastic colonies (Supplementary Fig. S1). The fungal colony appearance varied according to the media. After a month, on PDA, colonies appeared purple-brown with a yellow-ochre margin, highly raised with a deeply wrinkled or wrinkled surface; on the reverse side of the plate, mycelium appeared purple-brown with a flat or hollow center. On MEA, colonies were yellow-ochre with a lighter margin and yellow-ochre with a flat or hollow center on the reverse side. Colony area reached 90 mm^2 on PDA and 110 mm^2 on MEA after 30 days at 20°C (8 h of darkness and 16 h of light). Only limited color changes in colony appearance were observed between isolates. Hyphae from the upper culture surface were branched, septate, and hyaline and cells of filamentous hyphae measured 3.8 ± 0.5 by $8.7 \pm 1.1 \mu\text{m}$ while those of intramatrix hyphae were 7.1 ± 0.7 by $6.2 \pm 0.5 \mu\text{m}$. Along the mycelium, swollen, rounded, polygonal cells, often thick walled, were observed. They measured 9.0 ± 0.9 by $5.0 \pm 1.0 \mu\text{m}$. Conidiophores were hyaline, cylindrical, usually unicellular, and single (not aggregated). Conidia were hyaline and unicellular of about 6.0 ± 0.5 by $3.2 \pm 0.4 \mu\text{m}$. On the basis of morphological criteria such as conidia size and shape and colony appearance, the fungus was ascribed to the species *S. coryli* Vegh & M. Bourgeois, a fungal species described on hazelnut in France and Italy (Jalloux and Doussaud 2000 a,b; Librandi et al. 2006; Ragozzino 1983; Vegh and Bourgeois 1976). Stromata containing numerous spherical, bitunicate asci were produced on partially dried MEA and PDA media. Asci harbored eight hyaline, oblong bicellular ascospores, 8.5 ± 3.0 by $6.9 \pm 0.5 \mu\text{m}$, with a central septum (Fig. 3C and D).

Table 1. Fungicides tested in the in vitro screening for their ability to control *Sphaceloma coryli*

Active ingredient	Commercial name	Formulation ^a	Chemical family	Manufacturer	Dose
Boscalid and pyraclostrobin	SIGNUM	WG	Anilids and strobilurins	Basf	0.5, 1.0, 1.50 g/liter
Copper sulfate pentahydrate	...	...	Copper compounds	Sigma-Aldrich	0.05, 0.25, 0.5, 1.0 g/liter
Mancozeb	Penncozeb DG	WDG	Ethylenebisdithiocarbamates	Cerexagri Italia	1.5, 2.5, 3.2 g/liter
Methyl thiophanate	Enovit Metil FL	CS	Benzimidazoles	Sipcam Italia	1.50, 1.60, 1.75 ml/liter
Tebuconazole	Folicur WG	WG	Triazoles	Bayer CropScience	0.40, 0.55, 0.75 g/liter
Tetra copper oxychloride	Coprantol WG	WG	Copper compounds	Syngenta Italia	2.5, 3.0, 5.0 g/liter
Tetra copper oxychloride	Neoram Blu	WP	Copper compounds	Isagro	3.0, 5.0, 7.0 g/liter
Tribasic copper sulfate	Cupravit Bio Evolution	WG	Copper compounds	Ambechem	1.75, 1.80, 1.85 g/liter
Tribasic copper sulfate	TBCS 30WG	WG	Copper compounds	Cinkarna	1.0, 3.0, 8.0 g/liter

^a CS = concentrated suspension, WG or WDG = water-dispersible granules, and WP = wettable powder.

Effect of temperature on in vitro ascospore and conidium germination and mycelium growth. The effect of temperature on ascospore and conidium germination and mycelium growth was evaluated on four *S. coryli* isolates. Because no significant differences were observed in the response of the four isolates tested, the mean values of each parameter were reported. Ascospores germinated at temperatures

between 5 and 25°C. Germination started after 12 h at 20 to 25°C (data not shown) and, after 2 days, 5.0% of ascospores germinated at temperatures of 5°C, 17.2% at 10°C, 43.9 at 20°C, and 45.8 at 25°C. The greatest percentage of germination (55.3%) was recorded at 15°C (Supplementary Fig. S2A). No ascospore germination was observed at 30 to 35°C.

Fig. 2. Symptoms observed on hazelnut in Campania fields. Necrotic spots on **A**, buds; **B**, female flowers; **C**, petioles and young shoots; and **D**, nut bracts and shells. Presence of necrosis on the midrib and secondary veins of the lower leaf surface is visible (**C**).

Fig. 3. *Sphaceloma coryli*: **A**, ascostroma and asci and **B**, ascospores in nut bract sections. **C** and **D**, Asci and ascospores produced on a partially dried malt extract agar medium. Scale bars represent 10 μ m.

Fig. 4. Maximum-parsimony analysis with 1,000 bootstrap replicates of *Sphaceloma* taxa derived from the alignment of the intergenic transcribed spacer ITS1-5.8S-ITS2 ribosomal DNA of 29 *Sphaceloma* spp. isolates, including those of this study (black box) and 37 *Elsinoë* spp. The tree is rooted with the outgroup *Fusarium proliferatum* (GenBank accession: X94171).

Conidia germinated at higher temperatures than ascospores. After 2 days, 21.5% of conidia germinated at temperatures of 15°C. Approximately 45% of conidia germinated between 20 to 25°C and 53.7% at 30°C (Supplementary Fig. S2B). At lower (5 to 10°C) and higher (35°C) temperatures, no germination was observed.

Mycelia grew between 5 and 30°C, with an optimum at 20°C (Supplementary Fig. S2C). At the optimum temperature, the mean growth rate was 27.5 mm²/week on MEA. Fungal growth was completely inhibited at 35°C but colonies remained viable and would grow if subsequently incubated at 25°C. After 21 days, isolates produced abundant conidia at 25 and 30°C, whereas production was significantly inhibited at 10 and 15°C. No conidia were produced at 5°C. The shape and the size of conidia were affected by the incubation temperatures. The conidia produced at 25 and 30°C were spherical and larger whereas those at 10 and 15°C were smaller and more elongated.

Pathogenicity bioassays. The four isolates tested on hazelnut buds, leaves, stalks, and immature fruit were pathogenic. Necrotic lesions appeared on wounded and unwounded hazelnut tissues. Lesions on buds developed 7 days after fungus inoculation whereas necrotic spots and vein necrosis appeared 3 days after inoculation on 70% of the inoculated leaf blade (Supplementary Fig. S3). Necrotic spots appeared on stalks and fruit after 15 to 20 days and fruit drop was observed on 10% of symptomatic fruit after 30 to 45 days. A pathogen with the same characteristics in terms of colony morphology, growth form, and color as the inoculated fungus was reisolated from all symptomatic tissues, fulfilling Koch's postulates. The hazelnut tissues in the control set remained asymptomatic and no fungus was reisolated.

Molecular characterization and phylogenetic analyses. PCR amplification with universal primers ITS1-F and ITS4 directed the synthesis of amplicons of the expected size (approximately 600 bp). Amplicons of 24 isolates obtained from different hazelnut tissues (buds, leaves, fruit bracts, and stalks) and six localities were sequenced. The ITS sequences (GenBank accession numbers KT001427 to KT001450) showed 99% identity to each other. A BLAST search showed the closest similarity to *Elsinoë brasiliensis* (GenBank accession number JN943501, 97% identity), *S. arachidis* (GenBank accession numbers JN943484 and JN943485, 97% identity), *S. araliae* (GenBank accession number EF600972, 97% identity), and *E. araliae* (GenBank accession number JX462672, 97% identity).

In total, 66 *Sphaceloma* spp. and *Elsinoë* spp. ITS1-5.8S-ITS2 sequences, including 24 *S. coryli* sequences derived from this study, 5 *Sphaceloma* spp., and 37 *Elsinoë* spp. sequences retrieved from GenBank, were aligned. The ITS1-5.8S-ITS2 sequence from *F. proliferatum* was included as outgroup. The alignment was used to construct a phylogenetic tree (Fig. 4) which produced three clades: one with *E. fawcettii* and *E. eucalypticola*; another group with *E. australis*, *E. pyri*, and *E. eucalyptorum*; and another with eight species, including the 24 *S. coryli* isolates from Campania that formed a unique subgroup (bootstrap 98%).

In vitro sensitivity to fungicides. Nine fungicides (Table 1) were tested in vitro to evaluate their effect on mycelial growth. Boscalid-pyraclostrobin mix, mancozeb, methyl thiophanate, and tebuconazole were the most effective fungicides. On media containing boscalid-pyraclostrobin at 0.5, 1.0, or 1.5 g/liter, the growth was fully inhibited. Similarly, total growth inhibitions were observed when mancozeb, methyl thiophanate, or tebuconazole were used even at minimal concentrations of 1.5, 1.0, and 0.4 g/liter, respectively. The two formulations of tribasic copper sulfate presented different effects on mycelial growth. Cupraviv Bio Evolution did not inhibit mycelium growth, while TBCS 30 WG inhibited growth at the intermediate and high doses. Copper sulfate decreased fungal growth at 1 g/liter while the two tested formulations of tetra copper oxychloride did not show any effect.

Discussion

In recent years, premature dropping of hazelnuts has become of increasing concern, threatening to decimate hazelnut industry in Campania, Southern Italy. The severe yield losses and the fast spread of the disease necessitated pathogen identification.

In the present study, we demonstrated that *S. coryli* is the pathogen responsible for the emerging disease problem in Campania. The fungus was consistently isolated from the symptomatic tissues collected throughout the growing season from different geographical areas, and collected isolates successfully fulfilled Koch's postulates. Fungus identification was based on the characteristics of colony and on the morphology and the size of mycelia and conidia of its anamorph form according to the description of *S. coryli* given by Vegh and Bourgeois in 1976.

The discovery of a teleomorph of *S. coryli* has allowed a more reliable and accurate fungus characterization. On the basis of pseudoascostromata, asci, and ascospore observations, *Elsinoë* is the perfect stage found to be associated with *S. coryli* (Cheewangkoon et al. 2009; Hyde et al. 2013; Jayawardena et al. 2014; Rogerson 1970; von Arx and Müller 1975; Wijayawardene et al. 2012). The genus *Elsinoë* is characterized by few to numerous globose to subglobose, fissitunicate and bitunicate asci in locules scattered in the upper part of pseudoascostromata which form scab-like blemishes on leaves and fruit. Ascospores within asci are irregularly arranged, oblong or fusiform, with one to three transverse septa. Several *Elsinoë* spp. are reported in Index Fungorum (2013) but none infect hazelnut or present morphological characteristics (numerous asci per locule and ascospores of 8.9 ± 1.3 by 5.3 ± 0.8 μm with one septum) similar to those of the present fungus.

Sequence analysis of the ITS region is widely used for the identification of *Elsinoë* spp. (Hyun et al. 2007; Swart et al. 2001; Tan et al. 1996). The 24 isolate nucleotide sequences of our collection group in a well-supported (98%) clade, markedly divergent from the rest of *Sphaceloma* and *Elsinoë* spp. retrieved from GenBank, further supporting the idea that *S. coryli* and its teleomorph are different from the species previously described within the genus *Elsinoë*. Thus, on the basis of the distinctive morphological and molecular characteristics, the teleomorph of *S. coryli* is a new species of the genus *Elsinoë*, for which the name of *E. coryli* is proposed.

The reasons why *E. coryli* caused a disease outbreak on hazelnut 30 years from its first report in Campania (Ragozzino 1983) are not known. A number of factors may have contributed to disease development. The infections of *Elsinoë* spp. are favored by high moisture and abundant rainfall (Agrios 2005; Brook 1973; Chung 2011), whereas temperature range requirements depend on fungus species (Agrios 2005). *E. coryli* requires 5 to 25°C for ascospore germination, with an optimum at 15°C, whereas temperatures of 5 to 30°C, with an optimum at 20°C, are required for mycelium growth. Therefore, the climatic conditions observed in the years 2013 to 2014 in Campania, characterized by frequent rainfalls and high humidity and temperature during female flowering and early fruit production periods, may be one of the reasons of the *E. coryli* outbreak in our region. To provide some information about the climatic conditions occurred in Campania before 2013 to 2014, we analyzed the meteorological data available at the Archivio Meteo Napoli website (<http://www.ilmeteo.it/portale/archivio-meteo/Napoli>) concerning the weather trend in Naples (25 km away from the diseased hazelnut areas) during the last 20 years. Data show a clear increasing trend of minimum temperatures and rain days, especially in the period of January to February (hazelnut female flowering) and April to May (early fruit production). The effect of climate changes on disease development has been widely documented for other plant pathogens such as *F. graminearum* and *Phytophthora ramorum* in recent decades (FAO 2008; Pautasso et al. 2012).

In addition to climatic conditions, other factors may have contributed to the recent hazelnut disease outbreak. First of all, inadequate cultural field management has been observed in many orchards during the 2013 to 2014 survey. The recent practice of minimal or no pruning of dead twigs and branches, foliage, and fruiting structures likely makes the environment more favorable for inoculum production. Moreover, the rise of inoculum could have also been caused by the hazelnut chemical management regulated by the Campania phytosanitary guideline (DM 242/ST 31-01-2005), which was based only on the use of copper compounds. Thus, cultural practices may have created a new environment favorable to the emergence of

E. coryli. Finally, a more virulent strain of the fungus may also have developed. Unfortunately, strains isolated 30 years ago have been lost and no comparison was possible with those obtained in this study.

Among the nine fungicides tested in vitro for their effectiveness to reduce or suppress the mycelial growth of *E. coryli*, thiophanate-methyl, tebuconazole, boscalid-pyraclostrobin mix, and mancozeb are the most effective. Their efficacy is independent of the concentration used; also, at the lowest concentration recommended by manufacturers, *E. coryli* growth is suppressed.

Even though copper fungicides have been reported as protective against *Sphaceloma* spp. (Chung 2011; Mazzone and Ragozzino 2006), our in vitro studies indicate that their efficacy is dependent on formulation or concentration. The best effects were obtained by TBCS 30 WG at 3.0 g/liter.

Preliminary observations under field conditions have indicated the effectiveness of thiophanate-methyl, tebuconazole, mancozeb, or boscalid-pyraclostrobin mix in significantly limiting disease development. For example, two treatments with thiophanate-methyl (one in October to November and the other in April to May) drastically reduced (90%) disease incidence. In plots where copper compounds were used or trees were not treated, disease incidence was higher. On the basis of these experiments, the Campania region authorized the temporary use of thiophanate methyl on hazelnut for the year 2015.

Further studies are needed to determine the fungal life cycle, to clarify the role of acervula and conidia in fungus biology, and to evaluate the efficacy in the field of fungicides with different properties, modes of action, and applications beyond copper-based products. This information would be useful for formulating and realizing integrated control strategies.

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Literature Cited

- Agrios, G. N. 2005. Plant Pathology, 5th ed. Elsevier Academic Press, Burlington, MA.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. 1990. Basic local alignment search tool. J. Mol. Biol. 215:403-410.
- Bourgeois, M., and Vegh, I. 1967. Anthracnose du noisetier (*Sphaceloma* sp.). Page 12 in: Les Champignons parasites des arbres fruitiers à noyau. P. Bondoux, ed. M. Ponsot, Paris.
- Brook, P. J. 1973. Epidemiology of grapevine anthracnose, caused by *Elsinoë ampelina*. N. Z. J. Agric. Res. 16:333-342.
- Cheewangkoon, R., Groenewald, J. Z., Summerell, B. A., Hyde, K. D., To-anun, C., and Crous, P. W. 2009. *Myrtaceae*, a cache of fungal biodiversity. Persoonia 23:55-85.
- Chung, K. R. 2011. *Elsinoë fawcettii* and *Elsinoë australis*: The fungal pathogens causing citrus scab. Mol. Plant Pathol. 12:123-135.
- Condé, B. D., Pitkethley, R. N., Smith, E. S. C., Kulkarni, V. J., Thiagalingam, K., Ulyatt, L. L., Connelly, M. L., and Hamilton, D. A. 1997. Disease notes or new records: Identification of mango scab caused by *Elsinoë mangiferae* in Australia. Australas. Plant Pathol. 26:131.
- FAO. 2008. Climate Change: Implications for Food Safety. Online publication. Food and Agriculture Organization of the United Nations, Rome. <http://www.fao.org/home/en/>
- FAOSTAT. 2013. Online, retrieved from <http://faostat.fao.org>
- Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783-791.
- Gardan, L., and Bourgeois, M. 1982. Nouvelles manifestations d'une maladie fongique du noisetier due à *Sphaceloma coryli* Vegh et Bourgeois. P.H.M. Rev. Hortic. 224:39-41.
- Gardes, M., and Bruns, T. D. 1993. ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. Mol. Ecol. 2:113-118.
- Hyde, K. D., Jones, E. B. G., Liu, J. K., Ariyawansa, H., Eric, B., Boonmee, S., Braun, U., Chomnunti, P., Crous, P. W., Dai, D., Diederich, P., Dissanayake, A., Doilom, M., Doveri, F., Hongsanan, S., Jayawardena, R., Lawrey, J. D., Li, Y. M., Liu, Y. X., Lücking, R., Monkai, J., Nelson, M. P., Phookamsak, R., Muggia, L., Pang, K. L., Senanayake, I., Shearer, C. A., Wijayawardene, N., Wu, H. X., Thambugala, K. M., Suetrong, S., Tanaka, K., Wikee, S., Zhang, Y., Aguirre-Hudson, B., Alias, S. A., Aptroot, A., Bahkali, A. H.,

- Bezerra, J. L., Bhat, J. D., Binder, M., Camporesi, E., Chukeniroti, E., Hoog, S. D., Guedan, C., Hawksworth, D. L., Hirayama, K., Kang, J. C., Knudsen, K., Li, W. J., Liu, Z. Y., Mapook, A., Raja, H. A., Tian, Q., Scheuer, C., Schumm, F., Taylor, J., Yacharoen, S., Tibpromma, S., Wang, Y., Yan, J. Y., and Zhang, M. 2013. Families of Dothideomycetes. *Fungal Divers.* 63:1-313.
- Hyun, J. W., Peres, N. A., Yi, S. Y., Timmer, L. W., Kim, K. S., Kwon, H. M., and Lim, H. C. 2007. Development of PCR assays for the identification of species and pathotypes of *Elsinoë* causing scab on citrus. *Plant Dis.* 91:865-870.
- Hyun, J. W., Timmer, L. W., Lee, S. C., Yun, S. H., Ko, S. W., and Kim, K. S. 2001. Pathological characterization and molecular analysis of *Elsinoë* isolates causing scab diseases of *Citrus* in Jeju Island in Korea. *Plant Dis.* 85:1013-1017.
- Index Fungorum. 2013. Online, retrieved from <http://www.indexfungorum.org>
- ISTAT. 2010. Online, retrieved from <http://www.istat.it/it/censimento-agricoltura/agricoltura-2010>
- Jalloux, F., and Doussaud, G. 2000a. L'antracnose du noisetier: *Gloeosporium coryli* (Desm.) Sacc. Biologie du champignon et action in vitro de fongicides sur la germination des spores et la croissance mycélienne. *Arboric. Fruit.* 535:19-23.
- Jalloux, F., and Doussaud, G. 2000b. L'antracnose du noisetier: *Sphaceloma coryli* Vegh et Bourgeois. *Arboric. Fruit.* 541:19-23.
- Jayawardena, R. S., Ariyawansa, H. A., Singtripop, C., Li, Y. M., Yan, J., Li, X., Nilthong, S., and Hyde, K. D. 2014. A re-assessment of *Elsinoaceae* (*Myriangiales*, *Dothideomycetes*). *Phytotaxa* 176:120-138.
- Kris-Etherton, P. M., Yu-Poth, S., Sabaté, J., Ratcliffe, H. E., Zhao, G., and Etherton, T. D. 1999. Nuts and their bioactive constituents: Effects on serum lipids and other factors that affect disease risk. *Am. J. Clin. Nutr.* 70:504S-511S.
- Lamichhane, J. R., Fabi, A., Ridolfi, R., and Varvaro, L. 2013. Epidemiological study of hazelnut bacterial blight in central Italy by using laboratory analysis and geostatistics. *PLoS One* 8:e56298.
- Lamichhane, J. R., Fabi, A., and Varvaro, L. 2012. Severe outbreak of bacterial blight caused by *Xanthomonas arboricola* pv. *corylina* on hazelnut, cv. Tonda di Giffoni, in central Italy. *Plant Dis.* 96:1577.
- Librandi, I., Galli, M., and Belisario, A. 2006. Le patologie del frutto del nocciolo in Italia, con particolare riguardo alla zona del Viterbese. *Petria* 16:125-134.
- Mazzone, P., and Ragozzino, A. 2006. Le principali avversità del nocciolo in Campania. *Petria* 16:19-30.
- Mchau, G. R. A., Crous, P. W., and Philips, J. L. 1998. Molecular characterization of some *Elsinoë* isolates from leguminous hosts. *Plant Pathol.* 47:773-779.
- Miller, P. W., Bollen, W. B., Simmons, J. E., Gross, H. N., and Barss, H. P. 1940. The pathogen of filbert bacteriosis compared with *Phytophthora juglandis*, the cause of walnut blight. *Phytopathology* 30:713-733.
- Noviello, C. 1968. Osservazioni sulle malattie parassitarie del nocciolo con particolare riferimento alla Campania. *Ann. Facolt. Sci. Agrar. Univ. Napoli* 4:3-31.
- Pautasso, M., Döring, T. F., Garbelotto, M., Pellis, L., and Jeger, M. J. 2012. Impacts of climate change on plant diseases—opinions and trends. *Eur. J. Plant Pathol.* 133:295-313.
- Ragozzino, A. 1983. Le malattie del nocciolo. (Hazelnut diseases). Pages 22-24 in: *Atti Convegno Internazionale Nocciolo* (Proc. Int. Conf. Hazelnut), Avellino, Italy.
- Ridley, G. S., and Ramsfield, T. D. 2005. *Elsinoë takoropuku* sp. nov. infects twigs of *Pittosporum tenuifolium* in New Zealand. *Mycologia* 97:1362-1364.
- Rogerson, C. T. 1970. The Hypocrealean fungi (Ascomycetes-Hypocreales). *Mycologia* 62:865-910.
- Swart, L., Crous, P. W., Kang, J.-C., Mchau, G. R. A., Pascoe, I. A., and Palm, M. E. 2001. Differentiation of species of *Elsinoë* associated with scab disease of *Proteaceae* based on morphology, symptomatology, and ITS sequence phylogeny. *Mycologia* 93:366-379.
- Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Mol. Biol. Evol.* 30:2725-2729.
- Tan, M. K., Timmer, L. W., Broadbent, P., Priest, M., and Cain, P. 1996. Differentiation by molecular analysis of *Elsinoë* spp. causing scab diseases of citrus and its epidemiological implications. *Phytopathology* 86:1039-1044.
- Thompson, J. D., Higgins, D. G., and Gibson, T. J. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22:4673-4680.
- Uggla, M., and Carlson-Nilsson, B. U. 2009. Rose hip fly (*Rhagoletis alternata* fallen) and leaf spot fungus (*Sphaceloma rosarum* (pass.) Jenkins)—Possible threats against rose hip production? *Acta Hortic.* 814:857-862 (ISHS).
- Vauterin, L., Hoste, B., Kersters, K., and Swings, J. 1995. Reclassification of *Xanthomonas*. *Int. J. Syst. Bacteriol.* 45:472-489.
- Vegh, I., and Bourgeois, M. 1976. *Sphaceloma coryli* Spec. Nov. sur noisette (*Corylus avellana* L.). *Rev. Mycol.* 40:273-281.
- von Arx, J. A., and Müller, E. 1975. A Re-evaluation of the Bitunicate Ascomycetes with Keys to Families and Genera. *Studies in Mycology*, no. 9. Centraalbureau voor Schimmelcultures, Baarn, Holland.
- White, T. J., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in: *PCR Protocols: A Guide to Methods and Applications*. M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds. Academic Press, New York.
- Wijayawardene, D. N. N., McKenzie, E. H. C., and Hyde, K. D. 2012. Towards incorporating anamorphic fungi in a natural classification—Checklist and notes for 2011. *Mycosphere* 3:157-228.