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Observations on the non-native thousand cankers disease of walnut in Europe's southernmost outbreak

Salvatore Moricca [a, *], Matteo Bracalini [a], Alessandra Benigno [a], Luisa Ghelardini [a], Edson Luiz Furtado [b], Celso Luis Marino [c], Tiziana Panzavolta [a]

[a] Department of Agricultural, Food, Environmental and Forestry Science and Technology (DAGRI), Plant Pathology and Entomology Section, University of Florence, Italy
[b] Plant Protection Department, Faculdade de Ciências Agronômicas (FCA), Universidade Estadual Paulista (UNESP), Botucatu, São Paulo, Brazil
[c] Instituto de Biociências, Departamento de Genética, Universidade Estadual Paulista Júlio de Mesquita Filho (UNESP), Botucatu, São Paulo, Brazil

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ABSTRACT

The ascomycete fungus *Geosmithia morbida* (GM) and the walnut twig beetle (WTB), *Pityophthorus juglandis*, constitute a recently discovered fungal–insect complex responsible for the harmful Thousand Cankers Disease (TCD) in walnut trees. Key aspects of the epidemiology and ecology of the fungus and its insect vector were investigated in a disease outbreak found in central Italy, currently the southernmost introduction area of TCD on the Old Continent. Walnut trees with symptoms of crown dieback and epicormic branching with beetle galleries were sampled for pathogen isolation, while funnel traps were used to catch beetles and obtain adult flight curves. Growth-temperature relationships were determined for the fungus; optimum growth temperature for GM was 25°C, but it thrived up to 37°C. The phloem-boring bark beetle showed a longer flight period than in northern Italy. The beetle haplotype identified in Tuscany (H1) differed from haplotypes H2 and H21 found in northern Italy, suggesting possible multiple anthropogenic introductions. The substantial high temperature tolerance shown by the two organisms suggests they will not be constrained by the high temperatures of warmer areas. As a consequence, the impact of TCD in southern Europe, where the native *Juglans regia* is widely cultivated for its nuts and wood, would potentially be disastrous to local economies. In fact, although *J. regia* is not considered as susceptible as *J. nigra*, it can nevertheless be attacked in conditions of high bark beetle population density, a harbinger of high fungus' propagule pressure.

1. Introduction

The complex of *Geosmithia morbida* Kolařik (Ascomycota, Hypocreales, Bionectriaceae) and its vector, the bark beetle *Pityophthorus juglandis* Blackman (Coleoptera, Curculionidae, Scolytinae) is responsible for Thousand Cankers Disease (TCD) in walnut trees. The fungus lives in close association with this beetle species, also known as the walnut twig beetle (WTB),

* Corresponding author.
E-mail address: salvatore.moricca@unifi.it (S. Moricca).

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which is the principal known vector of the pathogen, although other beetle species have recently been found to carry *G. morbida* propagules (EPPO, 2015; Karandeep et al., 2019). TCD results in a progressive crown decline, with the fungus colonizing the copious feeding and reproductive galleries of the phloem-boring bark beetle. The outcome is the development of numerous, coalescent cankers that girdle first small branches, then larger ones and, finally, the stem, until they kill the tree (Newton et al., 2009) (Fig. 1). Due to this disease progression, symptoms become usually visible only 3–4 years after initial insect infestation: flag-like brown leaf wilting (Fig. 2), cankers on branches and the stem, and general crown dieback (Grant et al., 2011).

TCD is a recently discovered disease of North American origin, with the first report appearing at the beginning of this century (Tisserat et al., 2009; Utley et al., 2013). The original description of walnut twig beetle dates back to 1928 in New Mexico (Lincoln County), where it was presumably associated with the native *Juglans major* Torr. (A. Heller) (Cranshaw, 2011). The beetle was found later, in 1959, on black walnut (*Juglans nigra* L.) and California walnut (*J. californica* S. Watson) in Los Angeles County (Bright, 1981). However, until the massive outbreaks that struck in the 2000s in various southwestern states in the US, the bark beetle was considered a secondary pest of walnut trees (Tisserat et al., 2009). Before these outbursts, in fact, the widespread phenomena of branch dieback and mortality in walnut species, observed at various sites in the above-mentioned areas, had erroneously been ascribed to physiological impairment caused by drought or adverse soil conditions (Tisserat et al., 2009). The involvement of this beetle/fungus complex in the gradual deterioration and mortality of walnut species was not definitively ascertained until 2008 (Kolařik et al., 2011). Currently, its distribution range includes many of the western (Arizona, California, Colorado, Idaho, New Mexico, Nevada, Oregon, Utah, and Washington) and some eastern (Indiana, Maryland, North Carolina, Ohio, Pennsylvania, Tennessee, and Virginia) U.S. states (Seybold et al., 2016).

Various species of the genera *Juglans* and *Pterocarya* have been reported as hosts of TCD, with variable levels of susceptibility (Utley et al., 2013). Among walnuts, some species, namely *J. ailantifolia* and *J. major*, are considered resistant; others, such as *J. hindsii*, are recognized as susceptible; *J. nigra* is considered highly susceptible. On English walnut (*J. regia* L.) and Manchurian walnut (*J. mandshurica* Maxim.), both exotic in the US, TCD can cause disease, though these species are considered less susceptible hosts (Utley et al., 2013; Hefty et al., 2018). This information is particularly important in regards to the risk TCD poses to English walnut in Europe, where it is extensively cultivated for both its valuable wood and its nuts, harvested for local consumption and export.

Due to the dangerousness of TCD, both the fungus and its vector are regulated as quarantine pests in various US states and in Europe (EPPO A2 List). Italy is at present the only country where TCD has been reported outside the North American range. This alien insect-fungal complex was reported for the first time on black walnuts in northern Italy (Veneto) in 2013 (Montecchio and Faccoli, 2014) and, later, on English walnut trees (Montecchio et al., 2014). The bark beetle alone was caught with pheromone traps in other areas of northeastern Italy (Friuli-Venezia Giulia), whereas in Piedmont, Lombardy and Emilia-

Fig. 1. A black walnut tree killed by TCD in a young plantation.

Fig. 2. A shoot with typical, flag-like wilting of foliage.

Romagna (northwestern Italy) both the insect and the fungus were identified in 2015 (Faccoli et al., 2016; Bosio and Cooke-McEwen, 2018; EPPO, 2019).

Until 2018, TCD reports had all been concentrated in northern Italy. The geographical distribution of these reports, spanning from the east to the west of northern Italy, had generated the false expectation that TCD would not move southwards – beyond the natural barrier of the northern Apennine mountains – where walnut cultivation is of much greater economic importance. On the contrary, in 2018 the beetle/disease complex was reported just below this mountain range, in northern Tuscany (Moricca et al., 2018). Since this is the southernmost finding of the disease not only in Italy but on the entire European continent, the objectives of the present study were: a) to investigate some physiological requirements (e.g. thermotolerance) of the fungus; b) to elucidate the voltinism of the beetle in central Italy; and c) to deepen knowledge on the bioecology of the causal organisms associated with TCD. This knowledge might allow scientists to make more accurate inferences (a Pest Risk Analysis has been elaborated five years ago; EPPO, 2015) about the epidemic risk of TCD in those southern territories of European Union where the climate is similar to that of the native range of this beetle/fungus complex.

2. Materials and methods

2.1. Study area

The TCD outbreak occurred at Rosano, in the municipality of Rignano sull'Arno, near Florence (Italy), on a walnut plantation located on a hill sloping towards the valley bottom, with a north/northwest exposure. The plot (43°46′ N, 11°25′ E, about 115 m above sea level) covered an area of about 1.5 Ha, was roughly wedge shaped, and extended for about 350 m (from end to end), with a maximum width of 63 m. The plantation was surrounded by farmland and it was the only one in the area known to be attacked by TCD. The plantation included 281 trees (about 90% of *J. nigra* with a small percentage, around 10%, of *J. regia*), about 25 years old, with diameters averaging between 15 and 35 cm. More than half of the black walnut trees were symptomatic, with symptoms ranging from a generalized dieback with crown transparency to leaf yellowing and withering. Necrotized leaves, folded in a flag-like shape, remained attached to the branch. Branches showed a multitude of entrance and exit holes bored by *P. juglandis* adults, within and around which *G. morbida* killed tissues producing cankers.

2.2. Field samplings

On April 6, 2018, two black plastic traps (eight-unit Lindgren multiple funnel traps - Contech Enterprises Inc., Victoria, BC, Canada) baited with WTB lure (Alpha Scents Inc., Portland, OR) were placed on the plantation and maintained until no

catch was recorded in two consecutive inspections. Trap cups, filled with 50 ml of a 30% propylene glycol solution, were inspected at 15-day intervals. At each inspection, cups were emptied, and the liquid was replaced; catches were preserved in 96% ethanol. The lure was changed at 30-day-intervals, as indicated by the manufacturer. Samples were taken from 70 *J. nigra* trees exhibiting symptoms typical of TCD. Four symptomatic branches were cut from each tree, each was put separately in a polyethilene bag, and all were taken to the laboratory for further processing. Field samplings were limited to the year 2018 because immediately afterwards, following our reporting of the two quarantined organisms to the local Plant Health Inspection Service, eradication was carried out, with destruction of the whole plantation by burning. Meteorological data from the nearby Pontassieve weather station were kindly provided by SIR (Servizio Idrografico Regionale) of the Tuscany Region.

2.3. GM and WTB identification

2.3.1. GM isolation and culturing

Branch samples were examined under a Wild M8 stereoscope (Leica Microsystems, Heerbrugg, Switzerland) for disease symptoms (i.e. staining and necroses around galleries) and for precise localization of WTB galleries. Pieces of branches (no longer than 20–30 cm) were surface-sterilized by three-step immersion: in 96% ethanol (30 s), 4% NaOCl (4 min), followed by another 96% ethanol (30 s). After removing the bark with a sterile scalpel, 5 to 15 small pieces (2–4 mm long) of internal necrotic/necrotizing wood tissue were excised from each branch (1–2 mm around galleries) and transferred onto 9-cm-diameter plastic Petri dishes filled with Potato Dextrose Agar (39 g PDA, 5 g Agar, in 1L deionized water) (Liofilchem srl, Roseto degli Abruzzi, Teramo, Italy). The nutrient medium was amended with Ampicillin (Polycillin-N) and Rifampicin (Rifamycin), to prevent the growth of Gram-positive and Gram-negative bacteria and of mycobacteria. Such an antibiotic-enriched medium was necessary because fungus isolation was attempted also from WTB larvae and adults in order to verify if both stages carried the fungus, since they are known to be contaminated by a plethora of bacterial, archaeal, and eukaryotic microorganisms (Douglas, 2015). After 7 days of incubation at 24°C in the dark, emerging colonies were individually transferred in purity to newly prepared PDA medium (without antibiotics) and incubated again at 24°C in the dark. After 10 days of incubation, when cultures had almost covered the entire surface of the Petri dishes, colony phenotypes were visually determined and recorded, and each individual colony was subjected to microscopic examination. The growth patterns of colonies deriving from tissue pieces and of their derivatives (subcultures) were also ascertained. Tufts of mycelium were picked up from colonies and mounted in lactophenol blue or in lactic acid. Details of hyphae, conidiophores and conidia were obtained by using a Zeiss light microscope (ZEISS, Jena, West Germany) at x100, ×400 and ×1000 magnifications.

2.3.2. WTB conventional identification

Traditional beetle identification was performed by observing morphological characters (Wood, 1982; LaBonte and Rabaglia, 2015) under a Wild M8 stereoscope (Leica Microsystems, Heerbrugg, Switzerland) equipped with magnifying lenses to reach ×100 magnifications. Beetles were also sexed according to Wood (1982). Voucher specimens were stored at the Plant Pathology and Entomology Section of the Department of Agricultural, Food, Environmental and Forestry Science and Technology (DAGRI) of the University of Florence.

2.3.3. DNA isolation, amplification and sequencing

Seven-day-old colonies from *G. morbida* isolates ROS1, ROS2 and ROS3 grown on PDA were utilized for DNA extraction. The pure fungal mycelium was gently scraped from the agar surface by means of a sterile scalpel, put into sterile 1.5-ml microfuge tubes, and placed in the freezer for at least 12 h. Genomic DNA was extracted according to the protocol recommended by the GenElute plant Genomic DNA Miniprep extraction kit (Sigma Aldrich, St. Louis, Missouri, USA) and, finally, stored at −20°C. The ITS1-5.8S-ITS2 region from the rRNA was PCR-amplified by using the universal primers ITS-6 (5′-GAAGGTGAAGTCG-TAACAAGG-3′) (Cooke et al., 2000) and ITS-4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990). Cycling conditions were as follows: 95°C for 3 min; 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min; with a final extension at 72°C for 5 min (Ginetti et al., 2014).

Molecular identification of the beetle was performed by extracting DNA from 19.31 mg of dead individuals using disposable 1.5-ml microfuge tubes and pestles. The homogenized material was then processed using the same GenElute plant Genomic DNA Miniprep protocol used for the fungus, and the resulting DNA was stored at −20°C until use. The partial CoxI mitochondrial gene of *P. juglandis* was PCR-amplified with LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAAT 3′) primers (Folmer et al., 1994). Amplification was through 35 cycles of 1 min at 95-°C, 1 min at 40°C, and 1.5 min at 72°C, followed by a final extension at 72°C for 7 min (Folmer et al., 1994). Amplicons from both DNA regions were then purified and sequenced.

2.4. Fungus growth rates and cardinal temperatures

Growth of *G. morbida* colonies was determined under a spectrum of six temperatures to define their limits of growth and development, and the optimum temperature for the fastest growth. For this purpose, three isolates of the fungus

were utilized, taken at the two ends (isolates ROS1 and ROS3) and in the middle (isolate ROS2) of the plantation, from walnut trees growing at a distance not less than 150 m from each other. Agar plugs of 3-mm-diameter were taken from mature mycelia with a sterile scalpel and placed in the center of a Petri dish containing PDA. Three replicate plates per isolate were then incubated in the dark at each of the following temperatures: 21, 25, 29, 33, 37 and 41°C. Radial growth rates of the colonies were recorded at two-day intervals along two lines intersecting the center of the inoculum at right angles.

3. Results

A brown discoloration was observed around beetle galleries in branch samples. Necrotized areas were restricted to the phloem and outer bark and may reach superficially the sapwood. Beetle galleries and associated cankers were numerous; therefore, they often coalesced and surrounded branches, resulting in their dieback. The fungus was always found on both beetle adults and larvae, as well as in galleries from all sampled branches. The interior of the galleries was often covered with a characteristic whitish mycelial felt.

3.1. Identification

3.1.1. GM morphology

Colonies on PDA were from lobate to sublobate, with a plane surface topography and a compact, hyaline, dense, monilioid mycelium. Colony appearance and morphology varied greatly depending on growth temperatures and exhibited a pigmentation that ranged from whitish to brownish. At lower incubation temperatures (from 21 to 25°C), colonies were flat, with a yeast-like appearance and a highly lobed margin. At higher temperatures (from 29°C), the colony had a more cottony surface texture, a white and sparse aerial mycelium, a regular or slightly lobed margin, and cream sporulation.

Conidiophores were roughened to sharply warty, up to 200 μm long, 2–3 μm thick, penicillate (penicilli 28–63 μm), terverticillate or quaterverticillate, symmetric or asymmetric and beared metulae (9–11 x 2–2.5 μm) and phialides (8–15 x 2–2.5 μm). Phialides in clusters of 3–7 per conidiophore, normally bore conidia, but in some instances produced further elongating hyphae-generating conidia. Conidia yellow-ochraceous, narrow cylindrical to ellipsoid, 5.5 × 2 μm, in persistent chains, aggregated in tough, translucent masses in young colonies. Profuse conidiogenesis gave later these slimy colonies a dusty appearance. A monilioid mycelium with budding and inflated cells frequently formed at the basis of colonies.

3.1.2. Molecular identification

Sequences of both GM and WTB were compared for their closest homologies respectively with those from the fungus and the beetle deposited in the database (GenBank). The ROS1, ROS2 and ROS3 isolates shared complete sequence homology. A BLAST nucleotide sequence comparison of 601 bp from GM isolate ROS1 (GenBank accession no. MH620784), including the 5.8S gene and flanking ITS1 and ITS2 spacers, revealed a ≥99% identity with numerous isolates from North America and with the only GM isolate from northern Italy (Veneto) present in the GenBank database. The barcode region of the CoxI mitochondrial gene of *P. juglandis* gave a 727 bp fragment. BLAST analyses of this fragment showed 100% identity to *P. juglandis* haplotype H1 from the US (Rugman-Jones et al., 2015) and from Piedmont (Italy) (Bosio and Cooke-McEwen, 2018). The WTB sequence was deposited in GenBank (accession no. MH666050).

3.2. GM growth-temperature relationships

Growth temperature for all GM isolates was optimal at 25°C, suboptimal at 29°C, scanty at 33°C, markedly poor at 37°C. Some colonies were slow growing but still viable at 37°C, whereas they ceased growth at 41°C (Fig. 3). Re-incubation at 25°C at the end of the trial (for viability testing) revealed that colonies that had been incubated at 29, 33, 37°C resumed growth, while those at 41°C did not, proving colony death at this temperature. Colonies tended to develop a smoother and more regular margin at higher temperatures.

3.3. WTB flight curve

In the plantation, the bark beetle was caught constantly for a seven-month period, from April 20, 2018 (first control date) to November 16, 2018 (Fig. 4a). Mean catches per trap ranged from about 34 individuals, on the first control date, to about 595 beetles on October 19, 2018. In the whole trapping period, mean catches never fell below 34 individuals. Two flight peaks were recorded, one on June 15 and another one on October 19, whereas three negative flight peaks were observed, respectively on June 29, August 10 and October 5. Two out of three of these negative peaks occurred with high daily mean wind speed (Fig. 4b). The catch trend was the same for males and females; however, the sex ratio favored females, in fact, 55.39% of all adults caught were females.

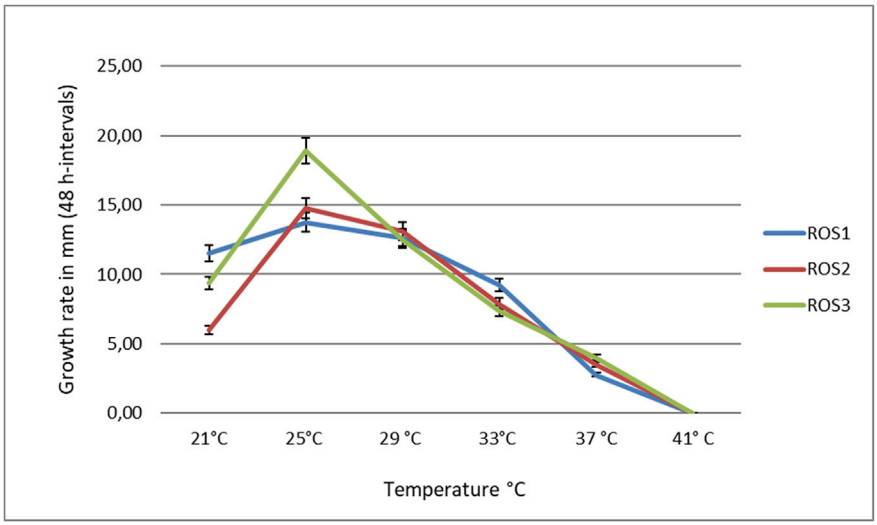


Fig. 3. Average diameter growth of *G. morbida* ROS1, ROS2 and ROS3 isolates on PDA at six different temperatures. Bars represent standard errors.

4. Discussion

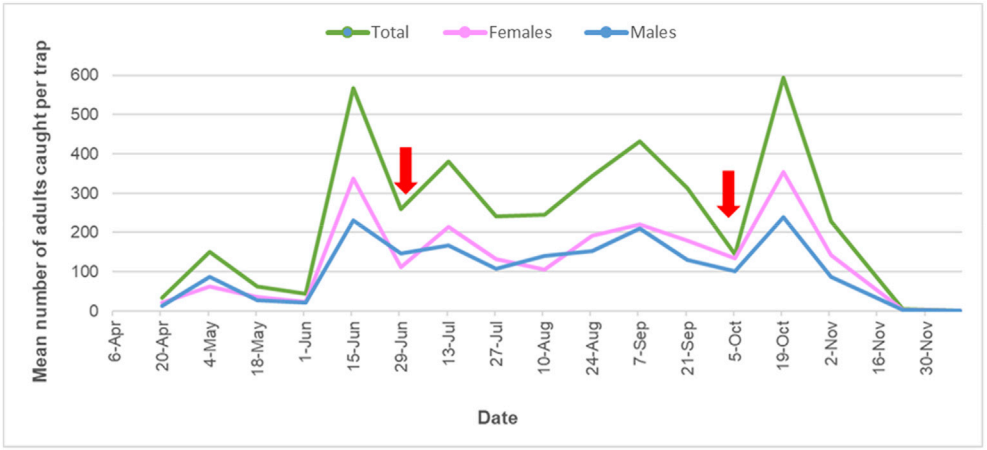
While GM found in Tuscany did not show complete sequence homology with any of the sequences contained in the GenBank repository, the WTB from Tuscany corresponded to the American mitochondrial haplotype H1, one of the most likely ancestral haplotypes in the US. According to Rugman-Jones et al. (2015) this is, together with H2 and H17, the most abundant haplotype throughout North America, whereas Zerillo et al. (2014) found that the most common haplotypes in the US were H2 and H3, particularly in California. The H1 haplotype differed from H2 and H21 haplotypes, which had also been found in north Italy, for a single nucleotide substitution, with a C→T transition and a G→A transition, respectively (Bosio and Cooke-McEwen, 2018; Faccoli et al., 2016). These data suggest that more than one introduction event may have occurred in Italy, even if the possibility of a single, simultaneous introduction of all three haplotypes cannot be ruled out (Dlugosch and Parker, 2008).

GM showed an optimal growth around 25°C, which is already a fairly high value for fungi, which generally have an optimum lower temperature (around 18–21°C). However, this ascomycete also thrived at moderate tending-to-high temperatures, since it also grew at 37°C. Whereas Kolařik et al. (2011) had reported that the fungus was still viable at 41°C, our isolates at this temperature ceased growth and died. Genotypic variability of the fungus might account for this discrepancy and, hence, it would be worth repeating tests on growth/temperature relations on a greater number of isolates, possibly originating from different geographical areas.

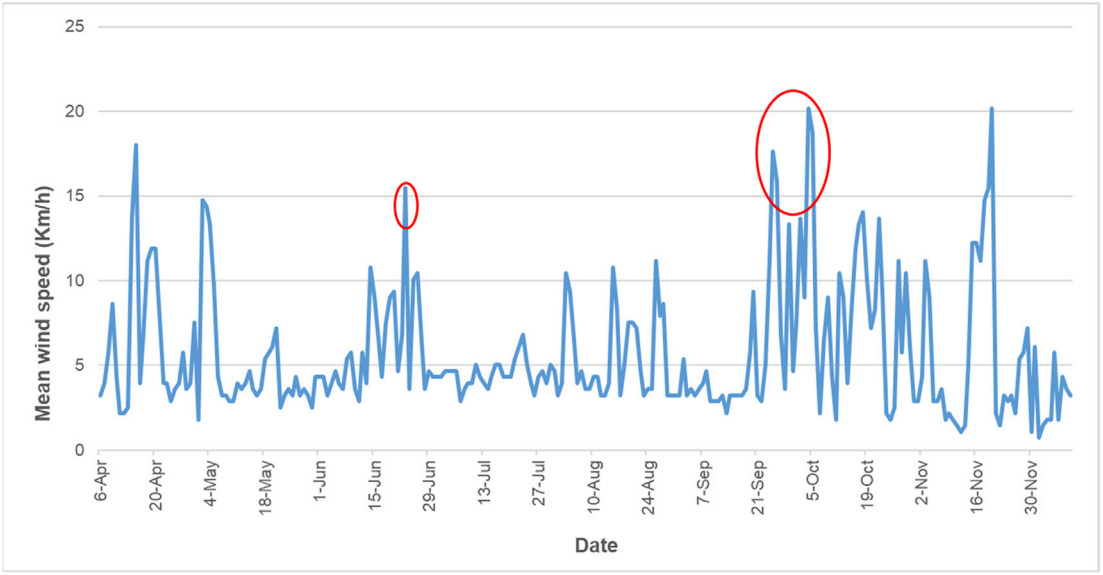
P. juglandis' flight period in Tuscany differed from that observed in northern Italy, whereas it was similar to that of the mild areas of its North American range. More in detail, it was longer than that observed in northern Italy (Faccoli et al., 2016): our catches began earlier (20 April in Tuscany versus mid-May in northern Italy) and finished later (16 November in Tuscany versus 24 October in northern Italy). In addition, at our site flight probably began before 20 April, since the first control date already had a mean of 34 specimens per trap. This agrees with catches in mild climates, such as in Chen and Seybold (2014) who, in northern California, caught adults even in January/March and up to the end of November. Both in the US and in northern Italy at least two overlapping generations have been hypothesized (Tisserat et al., 2009; Faccoli et al., 2016). In agreement with these authors, our flight curve had two main peaks, one in June and the other in October. However, according to Cranshaw and Tisserat (2012), adults were caught during the whole flight period and distinct peaks were actually not very clear. In fact, two out of three negative peaks (29 June and 5 October) in Tuscany occurred at higher wind speed, indicated by Chen and Seybold (2014) as a negative impacting factor; thus, they were likely not correlated to insect voltinism.

Compared with the results from other authors, our catches were very high in number. In northern Italy Faccoli et al. (2016) caught 2042 specimens per trap from May to October; in northern California Chen and Seybold (2014) found 713 specimens per trap from May to September. In contrast, we captured a mean of 4069 specimens per trap from April to November, even though we may have missed the onset of the first flight. In addition, Seybold et al. (2012) in northern California observed a mean of 1.83 ± 0.16 males/trap/day and 2.78 ± 0.24 females/trap/day, while we had 7.32 ± 0.03 males/trap/day and 9.09 ± 0.03 females/trap/day. Although traps are not completely reliable for estimating population density (Nix, 2013), our catches were higher than those from either northern Italy or the United States. These results, together with the high proportion of symptomatic and dead trees on the plantation, indicate a longer lasting outbreak. In fact, Cranshaw and Tisserat (2012) have suggested that many years (sometimes a decade or more) are needed for TCD to kill a black walnut.

Both the fungus and the bark beetle are thermotolerant. This results from several lines of evidence: their center of origin from hot and dry states of the southwestern U.S. (Seybold et al., 2019); their broad ecological valence, as clearly emerges from



(a)



(b)

Fig. 4. WTB flight curve and wind speed in the study period. (**a**) Mean number of *Pityophthorus juglandis* adults caught per trap; (**b**) Mean wind speed from the nearby Pontassieve weather station. The red arrows (figure a) and rings (figure b) indicate negative flight peaks and corresponding periods with high wind speed. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

their adaptation to a range of climatic zones with heterogeneous sites and varied ecological conditions (Seybold et al., 2016); our findings on their infection biology and bio-ecology. Given the ecological amplitude of TCD, the high temperatures of warmer regions of Europe, where its southernmost offshoots are located, certainly do not represent a limiting factor. The fungus' survival at relatively high temperatures and the beetle's longer activity throughout the year in warm areas might favor disease development in southern Europe, where it could have a devastating impact on the numerous walnut plantations. Here, indeed, the cultivation of *J. regia* has recently become important for nut production, due to the nutraceutical properties of the fruit (Banel and Hu, 2009), resulting in a source of considerable income for plant growers. Extreme disease severity and high beetle population density, such as those currently found in the Tuscan outbreak, raise concerns that TCD may be also damaging to *J. regia*. In fact, although this species is at present considered only relatively susceptible, it has been demonstrated that it is attacked in the case of high WTB population densities (Cranshaw and Tisserat, 2012).

5. Conclusions

In the last few years, the cultivation of walnut trees has increased significantly in Europe, especially in its central-southern regions, where extensive plantations have been made benefiting from EU funds aimed at boosting the cultivation of valuable hardwoods. The disease outbreaks occurring in various Italian regions show that in the Mediterranean basin the conditions

suitable for the TCD beetle/fungus complex to thrive do exist. Major factors normally contributing to disease development seem indeed to co-occur in Mediterranean Europe: 1) a large pabulum, provided by an extensive occurrence of available hosts; 2) a lack of host/pathogen coevolution; 3) an environment conducive to disease. We should therefore expect TCD expansion in this area.

Author contributions

S.M. and T.P. designed the experiments, analyzed and interpreted the data and wrote the manuscript. M.B., A.B., L.G., E.L.F. and C.L.M. contributed with field sampling, laboratory analyses and data collection.

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Declaration of competing interest

None.

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