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First report of the isolation of fungal strains causing disease in *Castanea sativa* Mill. Tree in Northern of Algeria

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ABSTRACT

Castanea sativa Mill. is a species belonging to the family *Fagaceae*, a woody tree that is very common and useful in Mediterranean areas, however, an epidemic from 1990s is causing a tremendous harm in Skikda (Province, Algeria) that was occasionally fatal due to unknown factors.

These symptoms led us to carry out an in-depth diagnosis in order to determine the causal agent of this disease, suggesting that fungi could be responsible. For this purpose, isolation was carried out on a PDA (potatoes dextrose agar) medium at 28°C from the soil, roots and leaves of the trees.

After macroscopic and microscopic identification of the fungal isolates, pathogenic species used as an inoculum during the growth of seeds determined that ten species belonging to major nine genera of different phylum were isolated and identified. *Verticillium* sp. was prevailed with 30% in soil, 45% in roots, and 53% in leaves of trees.

The identification of the causal agents using inoculation divulged the presence of *Verticillium* sp. as the highest probable cause of this damage and *Fusarium* sp. with lower probability.

Keywords: *Castanea sativa* Mill. Epidemic, fungi, Inoculation, Plant Pathogen, Disease, *Verticillium*.

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INTRODUCTION

Castanea sativa is one of the most important and common species belonging to the family *Fagaceae* in the Mediterranean, occupying an area of more than 1.6 million ha (Bourgeois et al., 2005; Alinho et al., 2021). When cultivated, the tree is long lasting, up to 1000 years (Bounous, 2014; Rob Jarman et al., 2019).

The chestnut tree offers food products and maintain a major role in the agroforestry system. Besides having edible nuts, the trees are of high value in the production of tannins, honey, and timber...etc. (Fernández-López and Alía, 2003; Conedera and Krebs, 2008) (Fig. 1). Chestnuts are a prized food for an increasingly large market and differs from other nuts in their low fat content, which makes them ideally suited for high complex carbohydrate and low fat diets (Becca et al., 2019). In addition, it is considered as natural resource for the wood and fruit industries (Alinho et al., 2021). The diversity and richness of part of these tree's biomolecules were proved and their biological activities such as antioxidants were also demonstrated (Formato et al., 2022).

In Algeria, the distribution data may lack symmetry and exactitude, the latest report by IRD and INRF (2007) indicates that *C. sativa* is very predominantly based in the Northern region. It plays a very important role for humans and their environment as a source of high quality timber, food, medicine, and for the preservation of biodiversity. Over the last ten years, production and exploitation have decreased due to biotic and abiotic factors (Bourgeois et al., 2005).

In Georgia, researchers have seriously considered that this tree, which covers almost 75% of forests, requires special protection because of its low potential for resistance to diseases, especially fungal diseases (Gaidamashvili et al., 2021).

Phytopathogenic fungi are filamentous fungi that can cause quantitative and qualitative damage to plants (Sieber et al., 2007). *C. sativa* is particularly threatened by two fungi, *Phytophthora cambivora* and *Cryphonectria parasitica*, which cause irreversible damage to the tree and its fruit, contributing significantly to the worldwide decline in chestnut production (Robin et al., 2006; Karadžić et al., 2019; Kovács et al., 2021). *Castanea* species have different susceptibility levels to these pathogens that affect its production (Fernandes et al., 2022).

Nearby the Skikda area, Northern of Algeria, *C. sativa* occupies a very important surface with more than 100000 trees. Since the 1990s, an epidemic with unknown factors has caused

damages to these trees; more than 95% are diseased. Thus far, no research was conducted to identify the causative agent and to eliminate it. Therefore, the aim of our work is to determine the phytopathogenic fungi that can cause damage to *C. sativa* trees in this area using the symptoms observed in the trees under monitoring (rotting, partial and total tree death).



Figure 1. Overview of *Castanea sativa* tree with its important organs (Conedera et al., 2016).

MATERIAL AND METHODS

M. Oleifera Lam. cultivar originally from Tebelbella (400 km south of Bechar, Algeria) (Fig.01). The seeds were generously donated by a commercial farmer. The seeds used in this experiment collected in June 2022. Seeds collected directly from plants at the mature seed stage; they were stored in paper bags, labelled with the name of the species, the date and the place of collection, until they used.

1. Presentation of the studied area

The area of Skikda is located in the North-East of Algerian coast (Fig. 2). Characterized by a Mediterranean climate, summer is hot and dry and winters are mild and rainy with a high rate of precipitation. The dry season extends from the months of June, July and August, while the rainy season extends over the rest of the year.



Figure 2. Map of sampling site in Northern of Algeria (Google Map, 2023)

2. Sampling

Sampling was taken place in three sites (S1, S2, S3) using the stratified random sampling method preconised by Mathio and Pieltain (2003). The soil samples were collected with a sterile burrow in depths of 10 cm. The root sampling was performed after removal of all other debris. All the samples were labelled and transported to the laboratory at 4°C to be analysed within 24h.

For each site, three plots were selected in accordance to the health status of the trees (Tab.1).

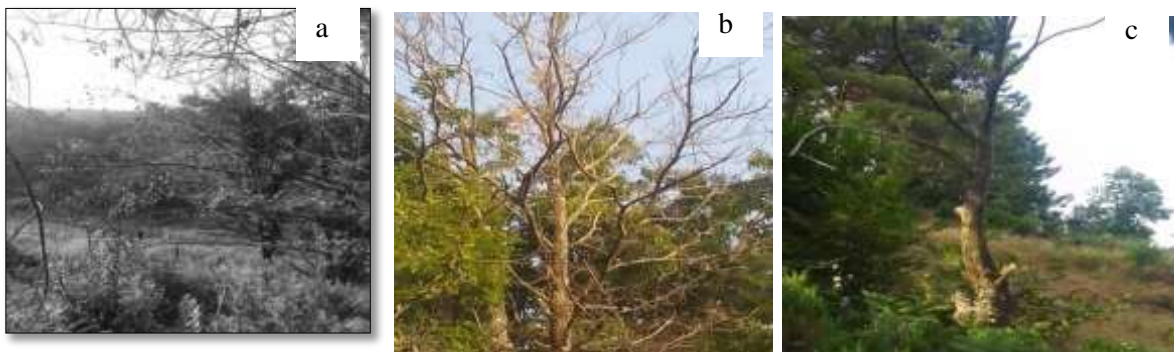


Figure 3. Symptoms of the disease of *Castanea sativa* Mill. Tree with, **a.** low degree, **b.** high degree, **c.** death.

Table 1. The distribution of samples including on three different plots.

Plots	Health status	Number of trees	Number of samples	Repetition	Total
Plot 01	Dead with 100%	03	05	03	45
Plot 02	Dead with 50%	03	05	03	45
Plot 03	Health with 100%	03	05	03	45

3. Fungal isolation

- **From soil:** Fungal isolation from soil was performed by the serial dilution method, with the aim of reducing the microbial load of samples. Ten grams of each soil sample were added to 90 ml of sterile physiological water. A series of dilutions was prepared, and 0.1ml from the 10^4 dilution was spread onto Petri plates containing PDA. Triplicate were prepared for each dilution (Davet and Rouxel, 1997).

- **From roots:** The collected roots were cut into small pieces, 1 to 3 mm size, and disinfected with sodium hypochlorite solution for 30 seconds. Afterward, the roots were washed three times with sterile distilled water and placed on Petri dishes containing PDA medium supplemented with 10% gentamicin antibiotic (Davet and Rouxel, 1997).

- **From leaves:** The isolation from leaves was carried out by cutting small pieces or the infected leaves of 5-10 mm square shape using a sterile scalpel blade. These fragments were also placed in sodium hypochlorite solution for 30 seconds. Each fragment was then aseptically washed three times with sterile distilled water and dried with a sterile filter paper (Agrios, 2005; Davet and Rouxel, 1997).

- All the PDA medium used were supplemented with gentamicin and all the plates were incubated at 28°C for 7 days (Jiang et al., 2021).

4. Identification of fungal strains. The identification was mainly based on the observation of macroscopic and microscopic characters (Jiang et al., 2021).

5. Rate of fungal isolates

The percentage of infection was determined based on each tree as follows:

Rate of each fungus (%) = $(n/N) \times 100$

Whereas:

n: number of fungus in each plot.

N: number of genus in the total plots

6. Inoculation of pathogenic fungi

The pathogenic fungi were inoculated into twice-autoclaved soil containing disinfected seeds accordingly to methods of Yvonne Lechner et al. (2023) and Vermeulen et al. (2022), with slight modifications.

RESULTS AND DISCUSSION

1. Isolation and identification

Macroscopic and microscopic identification characters made it possible to identify the different species of isolated genera according to Botton et al. (1990) and Watanabe (2002).

- **From soil.** Results from soil samples revealed ten strains belonging to nine different genera of different phylum (Fig. 4) and other species with low rates (<1%) including the genus *Chalara*, *Alternaria*, *Botrytis*, *Monilia*, *Phoma* and *Pythium*. Although their pathogenicity has increased, these rates were not significant.
- *Verticillium* sp. gave the most significant rate with 30.2 %, 22% and 4% for P1, P2 and P3 respectively. This was followed by S1 and S2 identified as *Fusarium* sp. with a rate of 20%, 18.9% and 18.6% for P1, P2 and P3 in order for the first site; while *Fusarium* sp. was present in the second site with 19%, 15% and 18.6% for P1, P2 and P3 respectively.
- Furthermore, *Stemphyllium* sp. showed a result of 12%, 13% and 14.5% for P1, P2 and P3 subsequently. While *Penicillium* sp., *Aspergillus* sp., *Rhizopus* sp., *Trichoderma* sp. were reported by lower rates (Fig. 4).
- In addition to that, *Fusarium* sp.2 was isolated from P1, P2 and P3 with a rate of 20%, 18.9% and 18.6% respectively. While *Stemphyllium* sp. was present with 12% in P1; 13% in P2 and 14.5% in P3.

- Others species were isolated with lower rates, such as *Penicillium sp.*, *Aspergillus sp.*, *Rhizopus sp.*, *Trichoderma sp.*

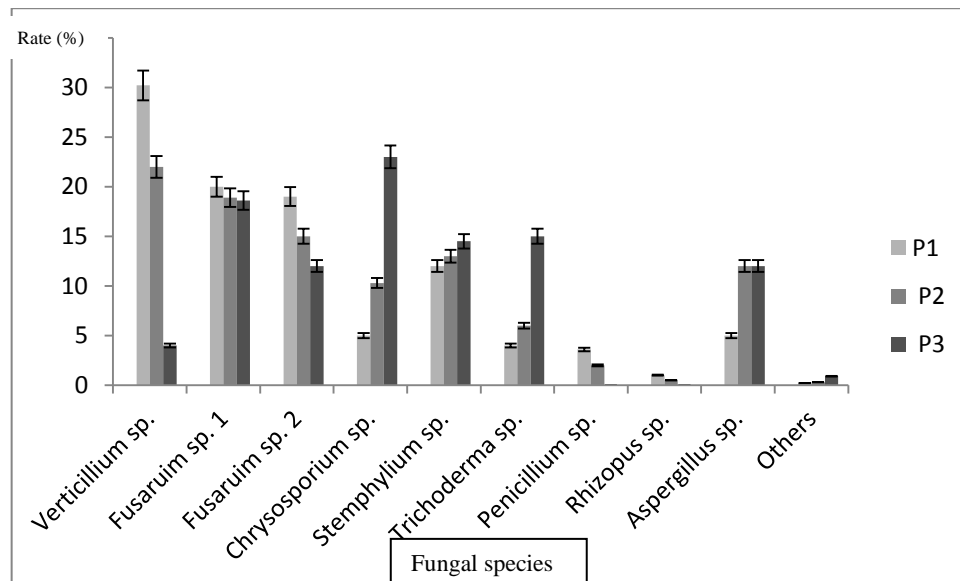


Figure 4. Rates of isolated fungal species from soil at the three plots.

From roots

In total, five strains belonging to four different genera were isolated within the three plots (Fig.5). *Verticillium sp.* up to 45%, 18%, 15% in Plot 1, 2, 3 respectively. *Fusarium sp.1* exhibited 21%, 34%, 15% in the three plots in order. While, *Fusarium sp.2* was found with a rate of 19%, 15%, 12% in P1, P2, P3 correspondingly. *Chrysosporium sp.* presented 7%, 22% and 47% while *Stemphyllium sp.* gave lower percentages, 8% in P1 and 11% in both P2 and P3.

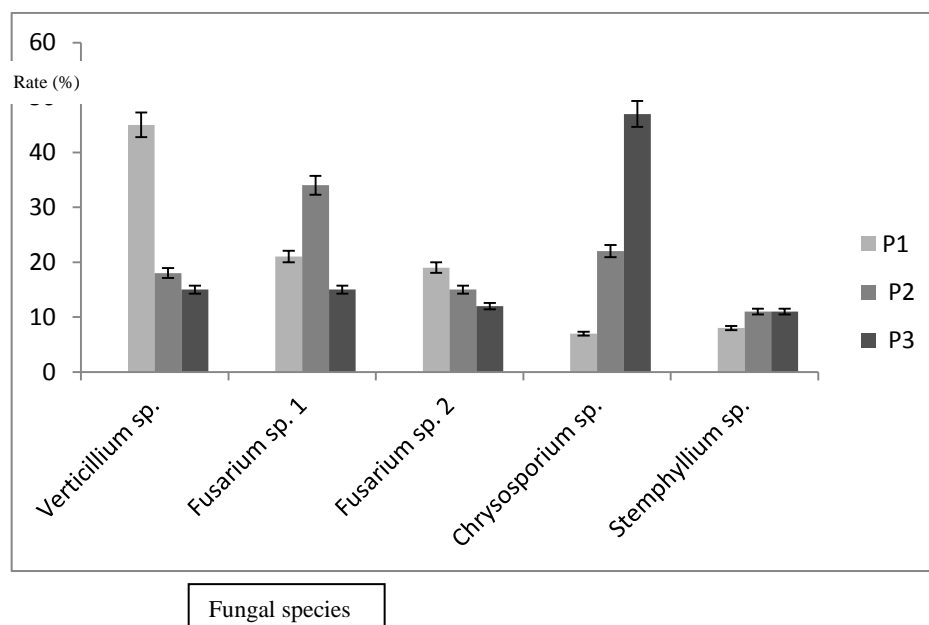
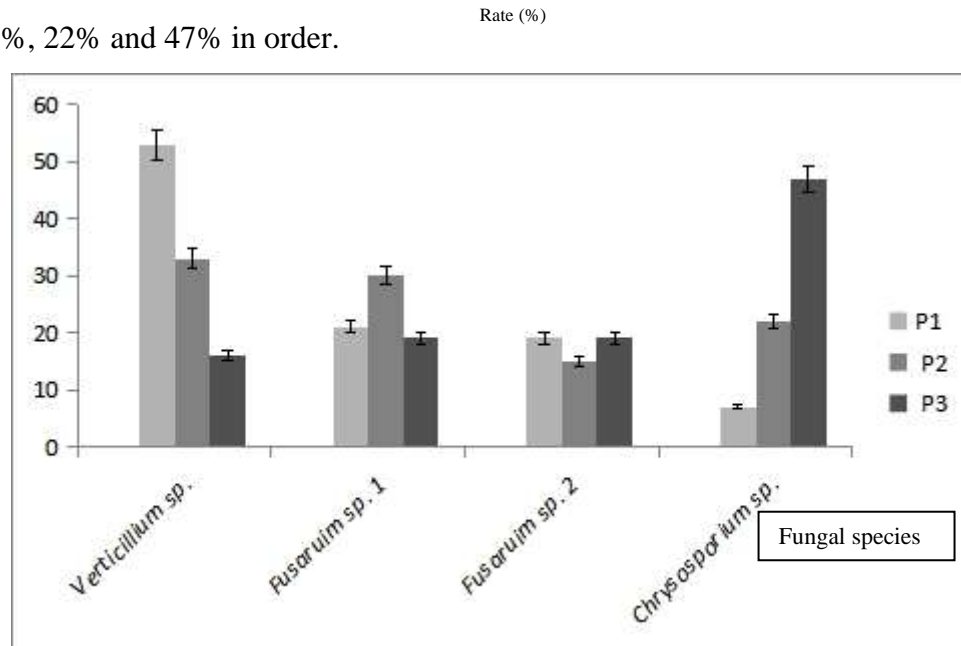


Figure 5. Rates of isolated fungal species from roots at the three plots.

▪ Isolation from leaves

As displayed in Fig. 6, *Verticillium* sp. was widespread with 53.7%, 33.7%, and 16.7% in the three plots respectively. *Fusarium* sp.1 was present with 21%, 30% and 19% while *Fusarium* sp.2 gave a lower rate, 19%, 15%, and 19% in the three plots. Finally, *Chrysosporium* sp. showed 7%, 22% and 47% in order.

**Figure 6.** Rates of isolated fungal species from leaves at the three plots.

2. Inoculation results

The isolation of *Verticillium* sp. increased from 12% to 34% during the first six months, while the symptoms did not appear yet until month 9 with a fungal rate of 39% (Fig.7.). After 15 months, the symptoms appeared in 75% of case.

The isolation rate of *Fusarium* sp.1 increased from 10% to 50% during the first nine months, but the symptoms only manifested themselves at the 9th month with a low and stable rate degree (25%) in the rest of inoculation period (Fig.8).

The *Fusarium* sp.2 isolation increased from 10% to 25 % during the first six months, but decreased from 17 to 7% in the remaining period.

However, all the infected samples with *Fusarium* sp. seemed to be asymptomatic (Fig. 9).

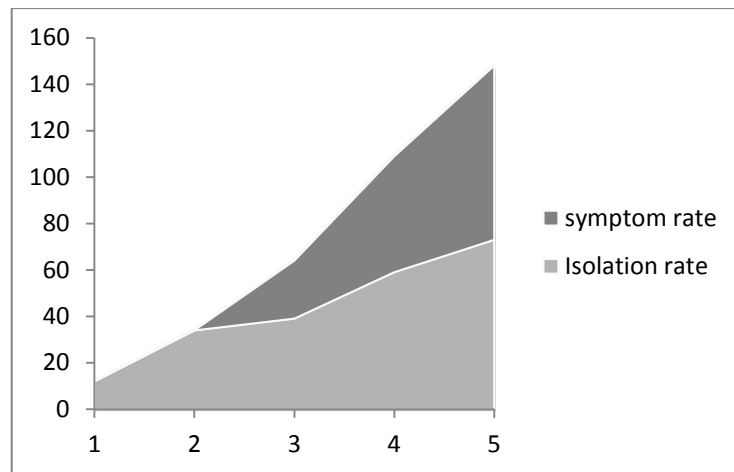


Figure 7. Symptom rate after inoculation of *Verticillium* sp.

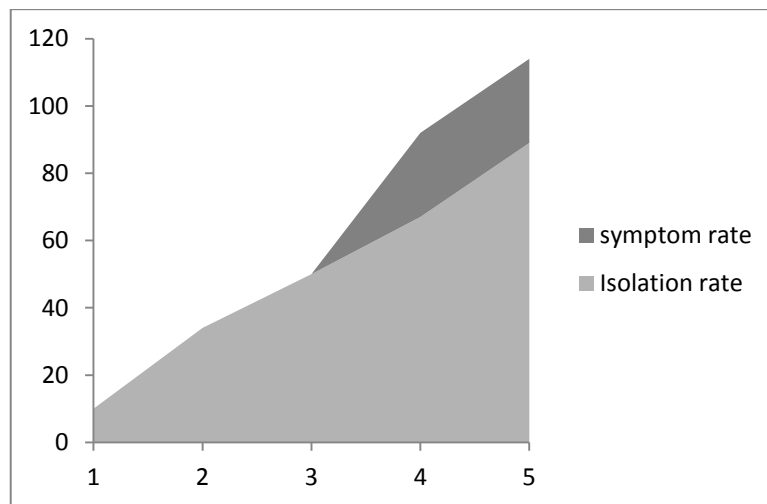


Figure 8. Symptom rate after inoculation of *Fusarium* sp. 1.

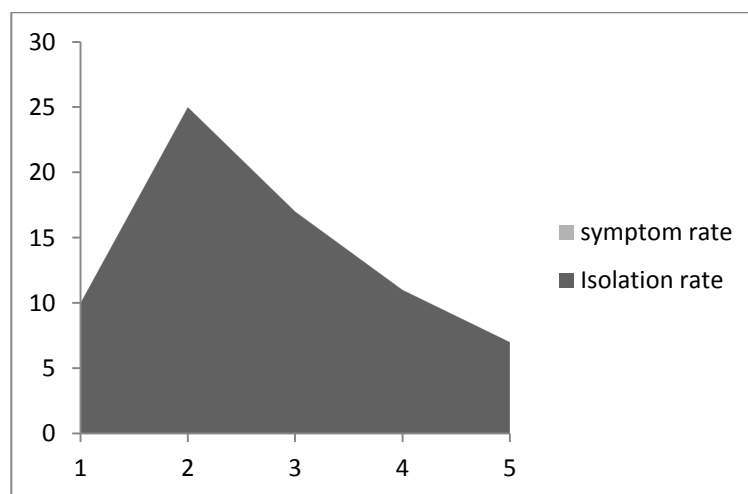


Figure 9. Symptom rate after inoculation of *Fusarium* sp. 2.

Discussion

C. sativa trees are probably one of the most important forest covering in the studied region of Skikda. Véla and Benhouhou (2007) have described this region as one of thirty-four “hot spots” encompassing almost half of the world's biodiversity, characterised by a proven taxonomic richness, a high plant endemism but a growing anthropogenic threat.

A few recent data are available on the distribution and factors affecting these trees in the region of Skikda or in Algeria. Chestnuts tree has principal damage are moulding or rotting caused by fungi and insects. All of its parts are benefits for the population, but the epidemic that touched them has become a threat. The aim of this study was revealing the cause behind this disaster in order to protect them. The symptoms of the observed epidemic were similar to Ink disease described by Fernandes et al. (2022).

In this research, several fungi were isolated around *C. sativa* tree and from its biological structure; some of them were considered pollutants and spoiled, especially for fruits such as *Penicillium* spp. and *Aspergillus* spp. Others were lower pathogenic, while others were highly pathogenic.

Further threats for chestnut trees include the ink disease (*Phytophthora* spp.), as well as the spread of the newly introduced chestnut blight (*Cryphonectria parasitica*), and the impact of the Chinese gall wasp (*Dryocosmus kuriphilus*). The latter is a pest introduced in 2002 in Piedmont and is currently spreading to other regions, although successfully limited by the specific antagonist *Torymus sinensis* where this biological control was applied. Further sources of economic loss for the chestnut growers are fruit damaging insects such as the chestnut weevil (*Curculio elephas*) and tortrices (*Cydia splendana*; *Cydia faggladana*; *Pammene fasciana*). The chestnut blight fungus, *Cryphonectria parasitica*, is an important plant pathogenic ascomycete (Eusebio-Cope et al., 2015).

Fusarium sp. a filamentous fungus recognised as a significant agricultural pathogen, exhibited rapid development of the chemical characteristics of the soil. This genus is most prevalent in areas experiencing disease outbreaks. James et al. (2007) and Berger (2010) noted that citrus and palm rots cause deep cracks and necrosis in the bark of the trees, and that the death of these trees is unavoidable if the disease is severe. Agrios (2005) indicated that parasitic fungi including *Fusarium* genus is responsible for the degradation of the vascular system in trees. Therefore, Delforme (2009) reported that *Fusarium* sp. leads to multitudes of leaf spots, wilting and root rot. In our study, *Fusarium* was isolated from all organs and in all plots.

The observed symptoms on *C. sativa* trees in this research were mainly represented by partial or complete mortality and bark necrosis (cracking). These observations strongly suggested *Fusarium* sp. as the causal agent.

In addition to that, another soilborne fungus that invades its host's roots and colonises the xylem as soon as it has entered. *Verticillium dahliae* produces conidiospores that spread upwards throughout the plant with the sap stream (Fradin & Thomma, 2006). This pathogenic agent causes branch and stem desiccation as its major symptoms. Soaking of xylem vessels is a common diagnostic technique used to identify this fungus (Agrios 2005). The presence of severe and moderate dieback in olive trees is the result of *V. dahliae* action (Guechi et al., 2005).

Verticillium wilt is considered the most serious biotic threat to olive trees and is commonly found in almost every region where olive is cultivated (López-Escudero and Mercado-Blanco, 2011). In our study, *Verticillium* sp. was isolated from different parts in multiple plots. The symptoms targeted the branches of *C. sativa* trees and were similar to those caused by this agent in other trees. Microscopic and macroscopic identification of this genus is in accordance to Watanabe (2002).

Verticillium nonalfalfae is a well-known biological control agent against the invasive Tree of Heaven (*Ailanthus altissima*). During the observation period, in 2018, the recorded maximum daily temperatures on the test area exceeded 30 °C on 13 days and even reached 35 °C during 3 days. This might be optimal or at least suitable for growth of the fungus *V. nonalfalfae* according to Yvonne Lechner et al., (2023).

Verticillium sp. produces infectious hyphae that emerge from microsclerotia (multicellular, long-lived structures in the soil) and colonise plant roots. It penetrates through the root via vascular tissue and then spreads upwards in the vascular system (Beckman, 1987). Symptoms of *Verticillium* wilt include stunting, wilting, chlorosis, foliar desiccation, defoliation, and plant death. The stress imposed by *Verticillium* wilt decreases the rate of photosynthesis (Ayele et al., 2020). Vermeulen et al. (2022) conclude that there is a scarcity of major resistance genes against *V. dahliae*. We hypothesise that the behaviour of this fungus in nature is mainly caused by the endophytic agent, with very limited levels of damage to its hosts in natural vegetation. Although *V. dahliae* is not very offensive to specific plant species in nature, it can still survive, thanks to its wide host range and lack of clear specialisation. In 2019, Beccaro et al. listed

almost all fungal species, which are pathogenic, that contain the isolated fungi mainly *Fusarium* and *Verticillium* species.

Conclusion

We concluded that a major pathogenic agent of *C. sativa* must be composed from one species of *Verticillium* genus and two species of *Fusarium* sp. playing a second role. We hypothesise that this was caused by the behaviour of this fungus in nature, with very limited levels of damage to its hosts in natural vegetation. The high level of damages may have prevented the need of development of major resistance in *C. sativa* trees. These fungi need detailed studies to know their genomics, conditions of infection in order to develop effective protection and prevention strategies for *C. sativa* trees.

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