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Gnomoniopsis smithogilvyi causes chestnut canker symptoms in *Castanea sativa* shoots in Switzerland

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ABSTRACT

A screening of *Castanea sativa* scions for grafting for the presence of endophytes showed that the opportunistic fungal pathogen *Gnomoniopsis smithogilvyi* was the most abundant member of the endophytic flora. This fungus is known as a pathogen affecting chestnut fruits in Italy and Australia. Here, we present evidence that it causes cankers very similar to the ones due to *Cryphonectria parasitica* infection on twigs and scions of chestnut trees. We found natural infections of *G. smithogilvyi* in healthy grafted plants as well as in scions from chestnut trees. The identity of the fungus isolated from asymptomatic tissues was verified by applying Koch's postulates and corroborated by DNA sequencing of four different gene regions. In contrast to *C. parasitica* that appears on the bark as yellow to orange pycnidia, stromata and slimy twisted tendrils, *G. smithogilvyi* forms orange to red and black pycnidia, gray stromata and cream-colored to beige slimy twisted tendrils on the bark. These Swiss strains are closely related to *G. smithogilvyi* strains from Australia and from New Zealand, *Gnomoniopsis* sp. and *Gnomoniopsis castanea* from New Zealand, Italy, France and Switzerland. While the strains from Ticino are genetically very close to *G. smithogilvyi* and *G. castanea* from Italy, the differences between the strains from Ticino and Geneva suggest two different origins. The present study supports the hypothesis that a single species named *G. smithogilvyi*, which is known to be the agent of chestnut rot, also causes wood cankers on chestnut.

1. Introduction

The autarchic "chestnut civilisation", dominant in the Swiss Alp region until the 18th century, began to slowly decrease in parallel to the rise of other commercial opportunities and the introduction, at the beginning of the 19th century, of maize and potatoes as staple foods. This trend accelerated after World War II when traditional chestnut cultivation was virtually abandoned because of the emergence of chestnut blight caused by *Cryphonectria parasitica* (Conedera, 1998; Conedera and Krebs, 2009).

C. parasitica attacks the bark of the trunk and branches of chestnut trees (Heiniger, 1997). The first observation in Europe dates back to 1938, when *C. parasitica* was found on *Castanea sativa* in the back country of Genova in Italy. In Switzerland, the fungus was observed for the first time in Ticino in 1948 near Monte Ceneri. In 1952, the pathogen was reported in various parts of Sottoceneri, Bellinzona, and Locarno. Nine years later, the fungus had colonized all chestnut orchards in Ticino, threatening their survival. The first observation of the pathogen north of the Swiss Alps dates from 1986. Currently, chestnut blight is a quarantine disease whose appearance north of the Alps must be announced (Prospero, 2009).

In recent years, a high mortality of young chestnut trees exhibiting cankers at the grafting point was observed in Ticino. The symptoms were similar in color and appearance to chestnut blight and therefore allocated to chestnut blight without further screening. Up to 12.5% of the trees died during the first two years of plantation to go up to 40% after 3 years (data not shown). This suggests that infections occurred already in the nursery.

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We have conducted a survey of endophytes in chestnut plants with the initial goal of developing a biological control method against *C. parasitica*. Interestingly, experimental plant material such as healthy grafted plants and scions from a chestnut tree turned out to be naturally infected by a pathogen. The symptoms resembled those caused by *C. parasitica.* However, the fungus we isolated from asymptomatic tissues and fungal fructifications was identified as *Gnomoniopsis smithogilvyi*, known in Australia and Italy for causing chestnut brown rot, one of the many diseases affecting chestnut fruits (Crous et al., 2012). In Australia too, the determination of the correct identity of the pathogens causing chestnut rots posed problems at the beginning. The fungal agent was first identified as the ascomycetous fungus *Phomopsis castanea* Sacc (Höhn) (anamorph)/*Diaporthe castaneti* Nitschke (teleomorph). Thereafter, the pathogen was reclassified informally as *Gnomonia pascoe* sp. nov. Smith and Ogilvy. Shuttleworth finally came to the conclusion that the pathogen belonged to yet another genus, describing it as a novel taxon, *G. smithogilvyi* based on morphological examination and phylogenetic analysis of representative isolates collected from various farms in Victoria and New South Wales (Liew, 2010). Visentin et al. (2012) also reported a new species: *Gnomoniopsis castanea*. It was isolated from rotten nuts from north-western Italy, south-eastern Switzerland and south-eastern France. A morphological and phylogenetic analysis helped to highlight that *G. smithogilvyi*, *G. castanea* and *G. pascoe* are one species, namely *G. smithogilvyi* (Shuttleworth et al., 2013). Maresi et al. (2013) were also able to confirm a complete homology with *G. castanea* and *G. smithogilvyi*. In Switzerland, the agents causing such a disease phenotype were first reported to be *Phomopsis endogena* and *Amphiporte castanea* (Conedera et al., 2004) whereas Dennert et al. (2015) identified *G. castanea* as the main agent of chestnut rot.

Compared to healthy chestnuts fruits whose flesh is creamy yellow, chestnut fruits infected with *G. smithogilvyi* display brown lesions on the endosperm and embryo (Crous et al., 2012; Shuttleworth et al., 2013). Black pycnidia of *Gnomoniopsis* sp. erupt on the external episperm surface from brown rot tissue and they may also be present on galls induced by the Asian chestnut gall wasp, *Dryocosmus kuriphilus* (Maresi et al., 2013). However, symptoms are not always visible on the external episperm, which is problematic for growers and consumers (Shuttleworth et al., 2013).

G. smithogilvyi is said to overwinter as a saprobe on dead burrs and branches of *Castanea* sp. in its sexual stage. It has been isolated as an endophyte from asymptomatic flowers, leaves and stems and from rotten chestnut kernels (Crous et al., 2012). *G. castanea* seems to be not only a nut pathogen, but also a symptomless chestnut endophyte (Visentin et al., 2012). Walker et al. (2010) also reported the endophytism of *Gnomoniopsis* spp. Maresi et al. (2013) reported a massive presence of this fungus as endophyte in one- and two-year-old shoots but it was not related to bark alteration (cankers or necrosis) and no fructification was observed on bark tissue in this study, while Magro et al. (2010) isolated *Gnomoniopsis* sp. from necrotic leaves and galls. More recently, *Gnomoniopsis* sp. was also isolated from chestnut blight cankers in India (Dar and Rai, 2013).

2. Material and methods

2.1. Plant material

The chestnut material used in this work was composed of one-year-old scions, obtained from a mother tree of the cultivar Monti Cimini (Viterbo, Italy), known under the name Viterbo in Ticino and very sensitive to chestnut blight. This cultivar has been used in Ticino for restoring the chestnut production. The one-year-old rootstocks were grown from wild chestnuts that were collected under defined cultivars of nut-bearing *C. sativa* trees. Scions and rootstocks were grafted on May 11, 2013, and used for a biological control experiment in the greenhouse carried out at the Swiss Centre of Excellence for Agricultural Research Agroscope in Cadenazzo (Ticino). These grafted plants were kept for 3 months in a glasshouse under natural light and without climate control. The roof and the sides of the glasshouse were left open to be close to natural conditions. The temperature varied between 10 °C at the end of May to 25 °C in early August. The plants were watered twice per day by drip irrigation for 15 min with a flow of 2 L/h. All chestnut material came from the nursery "Vivaio Forestale Cantonale di Lattecaldo" (Ticino). One-year-old scions were also sampled from an ungrafted chestnut tree of unknown variety (*C. sativa*) in Geneva. These scions were cut into 20 cm long segments and used for an *in vitro* biological control experiment in Geneva. Both extremities of the scions were soaked in melted wax to prevent desiccation and the scions were kept in 25 cm long glass tubes with plastic caps in a growth chamber at 26 °C ± 2 °C, with a relative air humidity of 70% and under a 16 h light/8 h dark photoperiod with a light intensity of approximately 270 μE m^{-2} s^{-1} provided by cool white fluorescent (Sylvania LUXLINE PLUS F58W/840 – T8, 1500 mm) and purple photosynthetic lamps (Sylvania GROLUX F58W/GRO, 1500 mm). Green healthy twigs coming from 4 different rootstocks (*C. sativa*) from the nursery of Centre for professional training Nature and Environment (CFPNE) in Jussy (Geneva), were used for carrying out the pathogenicity testing and also kept in glass tubes in the growth chamber. The leaves used for the pathogenicity testing came from these same rootstocks.

2.2. Isolation of endophytes from asymptomatic scions

Twelve asymptomatics scions from Ticino and twelve from Geneva were sterilized according to Wadia et al. (2000). The bark was separated from the wood under sterile conditions and samples were placed individually on fresh potato glucose agar medium in Petri dishes (PGA; Carl Roth, Switzerland) to monitor for fungal growth or on Luria Bertani agar (LBA; Carl Roth, Switzerland) for bacteria. Emerging colonies were grown in pure culture. Fungal and bacterial isolates were kept at room temperature (22 °C) in the dark for 7 days. The organisms occurring most frequently were identified by molecular means (see below). The colonization rate (CR) was calculated by dividing the total number of slices yielding fungi in a given sample by the total of slices in a sample × 100 and the isolation rate (IR) was calculated by dividing the total number of isolates obtained from a given sample by the total number of slices of that sample (Sunayana and Prakash, 2012). The relative frequency of colonization (CF) of a single endophyte species was equal to the number of slices colonized by the endophyte, divided by the total number of slices observed × 100. Then, the percentage of dominant endophyte (% DE) was calculated as CF for a specific endophyte divided by the sum of CF of all endophytes × 100.

2.3. Assessment of chestnut canker symptoms on grafted plants

After growing for 3 months in a greenhouse, grafted plants expressing canker symptoms were observation under a dissecting microscope followed by molecular identification of material stemming from symptomatic tissues grown on PGA medium. Observations were performed according to the protocol of "Laboratoire national de la protection des végétaux" (2005) for the evaluation of *C. parasitica* cankers.

2.4. Pathogenicity testing

Fungi retrieved from chestnut canker fructifications from symptomatic grafted plants in Ticino or from asymptomatic scions were