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# Biological control of the latent pathogen *Gnomoniopsis smithogylvyi* in European chestnut grafting scions using *Bacillus amyloliquefaciens* and *Trichoderma atroviride*

**Abstract:** A search for endophytes in *Castanea sativa* Miller (Fagales: Fagaceae) grafting scions showed that a latent pathogenic fungus *Gnomoniopsis smithogilvyi* (Diaporthales: Gnomoniaceae) was present as the major component of the endophytic flora. Initially, the goal of this study was to develop a biological control method of *Cryphonectria parasitica* (Diaporthales: Valsaceae), the chestnut blight agent, by soaking chestnut scions before grafting in antagonists suspension. However, the healthy chestnut material used in *in vitro* and glasshouse experiments turned out to be naturally infected by a pathogen. At first view, the symptoms looked very similar to those caused by *C. parasitica* but some differences were noticed. DNA sequencing and application of Koch's postulates revealed that *G. smithogilvyi* was the agent responsible of those symptoms. Preventive biocontrol experiments were carried out with chestnut tree scions soaked overnight in a liquid suspension of *Bacillus amyloliquefaciens* (Bacillales: Bacillaceae). This bacterium was then frequently found in the lower parts of scions (CF of 100% between 3.1 and 6 cm) and up to a height of 18 cm. It was observed that when *B. amyloliquefaciens* was present, the endophytic and opportunistic pathogenic fungus *G. smithogilvyi* was not present. Conversely, the parts not colonized by the bacteria were always naturally infected by the endophytic fungus. This would indicate that the endophytic behavior of *B. amyloliquefaciens* inhibited the growth of *G. smithogilvyi* and reduced its presence in scions. A similar experiment, carried out with the *Trichoderma atroviride* (Hypocreales: Hypocreaceae), led to similar observations. *Trichoderma atroviride* was frequently isolated in the lower parts of scions (CF of 100% until 6 cm) and up to a height of 27 cm. Inoculating *B. amyloliquefaciens* and *T. atroviride* as part of a preventive biocontrol treatment would allow these biological control agents to colonize the plant as endophytes and prevent the development of *G. smithogilvyi*.

**Keywords:** biocontrol; endophyte; chestnut scion, chestnut rot

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## Introduction

Initially involved in the search for an alternative biological control method of *C. parasitica* (Murrill) M.E. Barr (Diaporthales: Valsaceae) in Ticino and Geneva in Switzerland, we conducted a survey of endophytes in chestnut plants, to test a treatment less aggressive than the biological control with hypovirulent strains of *C. parasitica*. Indeed, tests conducted by the Agroscope Changins-Wädenswil (ACW) Cadenazzo and the Swiss Federal Institute for Forest, Snow and Landscape Research (WSL) which consisted to introduce hypovirulent strains of *C. parasitica* in chestnut grafting have failed. Mortality increased sharply since the hypovirulent strain was too aggressive to the plant (Jermini, pers. com.). Healthy grafted plants for a glasshouse experiment in Ticino and scions collected from a chestnut tree in Geneva for an *in vitro* experiment showed to be naturally infected by an unexpected fungus. We described it elsewhere as the cause of canker symptoms very similar to those caused by *C. parasitica* on twigs and scions and documented its natural presence in healthy grafted plants as well as in scions (Pasche *et al*. 2016). The fungus isolated from asymptomatic tissues and fungal fructifications was identified as *G. smithogilvyi* L.A. Shuttlew., E.C.Y. Liew & D.I. Guest (Diaporthales: Gnomoniaceae) (Shuttleworth et al. 2012a), which has been known in Italy, France, Switzerland, New Zealand and Australia for causing chestnut rot, one of the many diseases affecting chestnuts, and canker in India. In Australia, this fungus caused million dollar losses for the chestnut industry in 2010 (Shuttleworth et al. 2012b). The fungus overwinters as a saprobe on dead burrs and branches of *Castanea* sp. in its sexual phase. It was isolated as an endophyte from asymptomatic flowers, leaves and stems and from rotten chestnut kernels (Shuttleworth et al.; 2012a, 2012b). Visentin et al. (2012) observed its massive presence as an endophyte in one- and two-year-old shoots but they reported this fungus as a symptomless chestnut endophyte. More recently. Dar & Rai (2015) also reported *G. smithogilvyi* as a canker agent on chestnut trees.

The term endophyte usually encompasses a broad spectrum of potential plant hosts and inhabitants, including fungi, bacteria, algae and insects (Schulz & Boyle 2005). Any organ of the plant can be colonized and endophytic interaction could range from facultative saprobic to parasitic interactions, and from exploitive to mutualistic interactions. In many cases endophytes showed a good ability to protect the plant against pathogens (Schulz & Boyle, 2005; Porras-Alfaro & Bayman, 2011) and could therefore represent a huge potential for plant protection. *Trichoderma* spp. and *Bacillus* spp. have been reported as such potential endophytes (Hanada et al., 2008; Harman et al., 2004; Bacon & Hinton, 2002; Wang et al., 2014; White et al., 2014). *Trichoderma* spp. are easily isolated from decaying wood, any forms of plant organic matter and soil. *Trichoderma* species properties usually include induction of defence response in plants, mycoparasitism, antibiotic production, competition trough rhizosphere, enzymes production, metabolism of germination stimulants, plant growth promoting activity and resistance to biotic and abiotic stress (Howell, 2003; Shoresh *et al,*. 2010). Strains of *T. atroviride* P. Karsten (Hypocreales: Hypocreaceae) showed a good efficiency as biocontrol agents for a large range of economically important plant pathogens from air and soil (Brunner et al., 2005). The endophytic bacteria *Bacillus amyloliquefaciens* (Bacillales: Bacillaceae) (ex Fukumoto 1943) Priest et al. 1987 emend. Wang et al. 2008, first considered as a subspecies of *Bacillus subtilis* (Bacillales: Bacillaceae) due to a very similar phenotype, has since proved to be a separate species as it differs metabolically and secretes different enzymes (Priest et al., 1987). This bacterium produces a protein designated as baciamin which shows an antifungal activity against a broad spectrum of various phytopathogens (Wong et al., 2008). The *B. amyloliquefaciens* strain D747 is the active ingredient in two end-use pesticide products used against several diseases in agricultural and greenhouse crops due to microorganisms such as *Alternaria*, *Botrytis cinerea*, *Fusarium* and *Rhizoctonia* (Gagliardi et al., 2011).

The objectives of this study were to assay the potential of *B. amyloliquefaciens* and *T. atroviride* to adopt an endophytic behaviour by soaking chestnut tree scions in biological suspensions and to observe if the antagonists were able to colonize the rootstocks and remain persistent after grafting. The experiments included isolating and identifying the endophytic fungal flora. Withdrawing *G. smithogilvyi* from chestnut tree scions could be an advantageous strategy for a preventive biocontrol treatment of wood grafting material.

## Methods

### Plant material

The chestnut tree material used in the first experiment was composed of one-year-old scions obtained from a mother tree of the cultivar Monti Cimini (Viterbo, Italy). This cultivar, very sensitive to chestnut blight, is especially known under the name Viterbo in Ticino and has been used there for restoring chestnut production. The one-year-old rootstocks originated from chestnut tree seedlings, collected under specific cultivars of nut-bearing *C. sativa* trees. Scions were grafted on rootstocks on 11th May 2013 and used for

a glasshouse biological control experiment carried out at the Cadenazzo Research Centre of Agroscope (Ticino). All chestnut tree material came from the nursery "Vivaio forestale cantonale di Lattecaldo" (Ticino). For the second experiment, one-year-old scions were sampled from an ungrafted chestnut tree of unknown variety (*C. sativa*) in Geneva. These scions were cut into 20 centimetre-long segments and used for an *in vitro* biological control experiment in Geneva. Each extremity of scions was soaked in melted wax to prevent desiccation and the scions were kept in 25 centimeter-long glass tubes, with plastic caps, in a climatic chamber, set up with the following conditions: temperature 26°C± 2°C, relative air humidity of 70%, a 16 h light / 8 h dark photoperiod with a light intensity of approximately 270 $\mu$E $m^{-2}$ $s^{-1}$ 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 rootstocks twigs coming from 4 different rootstocks (*C. sativa*), from the nursery of the Centre for Professional Training in Nature and Environment (CFPNE) in Jussy (Geneva), were used to carry out Koch's postulates, and also kept in glass tubes in a climatic chamber under the same previously described conditions.

## Biological treatment

Fungal propagules suspensions were obtained by scraping 7 day-old cultures of *T. atroviride* strain ITEC (ITEC, France) grown on fresh potato glucose agar medium (PGA; Carl Roth, Switzerland) at room temperature in the dark. Suspensions were made in 0.02% Tween 20 in ultrapure water (Siemens, Switzerland) and then spread on PGA medium at different dilutions to adjust the concentration of viable propagules at a final concentration of $10^7$ cfu/ml. Bacterial aqueous suspensions were obtained from 2 day-old *B. amyloliquefaciens* strain UASWS BA1 (Lefort et al., 2014) grown in fresh Luria Bertani broth (LBB; Carl Roth, Switzerland) at room temperature in the dark under shaking at 150 rpm. Bacteria concentration of the liquid culture was first assessed with a Thoma haemocytometer. The liquid culture was then spun and the bacterial pellet re-suspended in ultrapure water at a final concentration of $10^7$ cfu/ml.

## Glasshouse experiment

Three modalities were tested for the glasshouse experiment. Chestnut tree scions with an average length of 1.20 m, were soaked overnight: 10 scions in 1l of the *T. atroviride* propagules suspension, 10 scions in 1l of the *B. amyloliquefaciens* suspension and 10 control scions in 1l of sterile distilled water. After soaking, 3 scions of each modality, not used for grafting, were immediately put on PGA for the detection of antagonists in the wood tissues. The higher parts of the other scions were cut to a length of approximately 15 cm leaving two buds, grafted on rootstocks and placed in a glasshouse the 11th May 2013. To prevent any potential loss due to the grafting, 30 grafted plants were produced for each modality. The chestnut material used in this work came from the nursery "Vivaio Forestale Cantonale di Lattecaldo" (Ticino). One-year-old rootstocks had been produced as seedlings from several cultivars of nut-bearing *C. sativa* trees. The one-year-old scions were obtained from a mother tree of the cultivar Monti Cimini. These grafted plants were cultivated in the glasshouse under natural light and without control of climatic conditions. The outdoor temperature varied between 10°C at the end of May to 25°C until early August and the roof and the sides of the glasshouse were kept opened. The plants were watered by drip irrigation twice per day during 15 minutes with a flow of 2l/h. After 3 months in the glasshouse, 3 grafted plants of each modality were used to detect the presence of antagonists in the wood tissues from scions and rootstocks.

## *In vitro* experiment

In the *in vitro* experiment, the three same modalities were tested. One-year-old scions were sampled from an ungrafted chestnut tree of unknown variety (*C. sativa*) in Geneva. These scions were cut into 20 cm long segments and soaked overnight: 23 scions in the fungal propagules suspension, 23 scions in the bacterial suspension and 23 control scions in ultrapure water. Immediately after soaking, 3 scions of each modality were put on PGA to detect if the endophytes had colonized the wood tissues. For the other scions, both ends were soaked in melted wax in order to prevent desiccation and the scions were then 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 $\mu$E $m^{-2}$ $s^{-1}$ provided by cool white fluorescent (Sylvania LUXLINE PLUS F58W/840 – T8, 1500 mm) and purple photosynthetic lamps (Sylvania GROLUX F58W/GRO, 1500 mm).

## Screening for antagonists as endophytes in treated scions

Prior to the isolation of antagonists, scions and rootstocks were surface disinfected during 1 min. in ethanol 95%, 45 min. in NaOCl 2.6%, followed by 30 sec in ethanol 95%, washed twice in ultrapure water for 5 min. and 10 min., respectively. Scions were then

cut into 1 cm thick slices. These slices were then put on PGA medium cast in Petri dishes. Following the method described by Sunayana & Prakash (2012), 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. 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. The variance of the isolation frequency of the pathogen in the presence of antagonists was analysed by the ANOVA procedure with the software Minitab 16 (Ryan et al. 2013).

## DNA extraction, PCR amplification, sequencing and sequence alignments

The biological control agents *B. amyloliquefaciens* and *T. atroviride* which developed on Petri dishes were first identified visually. Then, DNA from three isolates of each modality was extracted and amplified. Bacteria single colonies were directly collected from the Petri dish using an inoculating loop and mixed with 50 $\mu$l of PCR reaction mixture. The DNA of fungal isolates was extracted using a protocol adapted from Lefort & Douglas (1999). The quantity and quality of resulting DNA was estimated with a Nanodrop spectrophotometer (Thermo Scientific NanoDrop ND1000, USA). DNA was finally diluted to a final concentration of 50 ng/$\mu$L. PCR were performed using the primers ITS4 and ITS5 (White et al. 1990) for amplification of the rDNA ITS region for *T. atroviride* and other fungi. For bacteria identification, a 16S rDNA gene partial sequence was amplified using the primers 341F (Muyzer *et al.,* 1993) and 907R (Lane et al., 1985).

The 50$\mu$l reaction mixture total volume was as follows: 50 ng fungal or bacterial genomic DNA, 1x reaction buffer, 4mM $MgCl_2$, 200$\mu$M of each dNTP, 0.4 $\mu$M of each primer, 1 U Taq polymerase (Biotools B&M Labs, Spain) in sterile distilled water. PCR reactions were carried out in a thermocycler T1 (Biometra GmbH, Germany) and included an initial denaturation step at 95°C for 5 min., followed by 39 cycles (denaturation at 95°C for 1 min., annealing at 56°C for 45 sec. and extension at 72°C for 1 min.), terminated a final extension step at 72°C for 5 min. and 50 sec. The PCR products were analyzed by electrophoresis on 1% agarose gels in TBE pH8, stained with GelRed (Biotium, Inc., USA) and visualized under UV light. PCR products were then purified with the Wizard SV gel and PCR Clean-Up System (Promega, AG, Switzerland). PCR products were finally sequenced following Sanger's method for identity confirmation (Macrogen, The Netherlands). Other endophytes which were frequently found on Petri dishes were also sequenced following the same protocol. BLAST searches through the NCBI nucleotide database allowed for the identification of the microorganisms.

## Assessment of chestnut canker symptoms on chestnut material

After 3 months growing period in a glasshouse, grafted plants that showed canker symptoms were sampled for observation under a dissecting microscope and for molecular identification of microorganism fructifications stemming from symptomatic tissues onto PGA medium. Observations were performed according to the protocol of "Laboratoire national de la protection des végétaux" (Anonyme, 2005) for the evaluation of *C. parasitica* cankers. In parallel, the pathogen causing canker symptoms on chestnut material was identified using Koch's Postulates. Scions were sampled from an asymptomatic chestnut tree in Geneva and kept in vitro for 3 weeks until they exhibited infection symptoms such as slimy twisted cirrhi and orange to red pycnidia. Slimy twisted cirrhi were sampled from 10 infected scions and put on PGA Petri dishes for cultivation and microorganism isolation. Resulting pure cultures of the fungus were inoculated on 8 green healthy rootstock twigs. According to the method of Arisan-Atac et al. (1995), 3 holes were drilled at equal distance between adjacent holes in healthy chestnut rootstock twigs previously disinfected with ethanol 70% in order to insert PGA plugs (5 mm diameter) containing the isolated fungus cut from the margin of a 7 day-old culture. For the negative control, four healthy rootstock twigs were inoculated with non-inoculated PGA agar plugs. The development of symptoms was observed and the fungus was re-isolated from the developed cirrhi, as described above, and then visually compared with the first isolation. Finally, the organism was identified by PCR amplification and sequencing. The penetration of the pathogen inside the scions was observed by microscopy. Infected tissues of scions were hand cut with a razor blade and immersed in lactophenol cotton blue or acetic cotton blue according to the protocol of Baar & Lecomte (2011). Digital images were recorded with an Axiocam ICc3 camera (Zeiss, Germany) mounted on a Leitz Ortholux microscope (Leitz microscope, Germany) and images were processed with the software Axiocam 4.6 LE (Zeiss, Germany).

## Isolates

All different fungal cultures retrieved more than once from scions and grafted plants were identified. The strains of *G. smithogilvyi*, Ti1 from Ticino and Ge1 from Geneva, were labelled UASWS1315 and UASWS1319 respectively in our collection, and deposited in the collection DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) under the reference numbers DSM 100093 and DSM 100042. The other microorganisms isolated in this study were labelled according to Table 1.

Table 1. Labels of microorganisms isolated in this study

| Isolate | Label* |
|---|---|
| *Trichoderma atroviride* | UASWS1400 |
| *Alternaria alternata* | UASWS1401 |
| *Aureobasidium pullulans* | UASWS1402 |
| *Botryosphaeria dothidea* | UASWS1403 |
| *Diaporthe eres* | UASWS1404 |
| *Trichoderma hamatum* | UASWS1405 |

* UASWS = University of Applied Sciences Western Switzerland.

# Results

## Glasshouse experiment

In the scion slices which were directly plated on to PGA after soaking, *B. amyloliquefaciens* was frequently isolated from the lower parts of scions and up to a height of 18 cm. Between 3.1 and 6 cm, its CF was 100%. In the first 3 cm and between 6.1 and 9 cm it was present at 66.7% and at 33.3% between 12.1 and 18 cm, representing finally 45% of retrieved microorganisms (Tab. 2). For each slice colonized, only one organism was isolated per slice and the CR was 74.07%. DNA sequencing after isolation identified *G. smithogilvyi*, which represented 50% of dominant endophytes. Noticeably that fungus was not present when *B. amyloliquefaciens*, which represented 45% of dominant endophytes. For the scions treated with *T. atroviride*, its CF was 100% up to 6 cm. Up to 12 cm, and between 18.1 and 24 cm, it was 66.7%. Between 12.1–5 cm and 24.1–27 cm it was 33.3%. Between 15.1 and 18 cm it was present at 55.6% while *G. smithogilvyi* was present at 44.4%. The antagonist had not reached the parts higher than 27 cm in the scions and represented 70.7% of endophytes. *G. smithogilvyi* (DE of 29.3%) was found when *T. atroviride* was absent (Tab. 3). The CR was 92.59%. For the scions treated with ultrapure water, the antagonists *B. amyloliquefaciens* and *T. atroviride* were not found in the control scions. The fungus *G. smithogilvyi* was frequently present in control scions (DE of 84.1%), with a CF at least at 66.7% by section and until the end of scions (Tab. 4) for a CR at 88.89%. The presence of *G. smithogilvyi* was strongly reduced with the treatments ($P<0.01$).

On grafted plants, which were soaked in biocontrol suspension treatment before grafting and spent 3 months in a glasshouse, the antagonists *B. amyloliquefaciens* and *T. atroviride* were never isolated, but the latent pathogen *G. smithogilvyi* was frequently found. Its CFs were 73% for rootstocks, 64.8% for scions, 38.9% for rootstocks in contact with scions and 33.3% for scions in contact with rootstocks. It was the dominant fungi (52.5%), followed by the fungus *Alternaria alternata* (Fr.) Keissl (Pleosporales: Pleosporaceae) (18.5%) especially found in scions in contact with rootstocks for a CF at 38.9%. The fungi *Diaporthe eres* Nitschke (Diaporthales: Diaporthaceae), *Trichoderma hamatum* (Bonord.) Bainier (Hypocreales: Hypocreaceae), *Botryosphaeria dothidea* (Moug.

Table 2. Frequency (%) of *B. amyloliquefaciens* UASWS BA1 isolated as endophyte in scions not used for grafting for a CR of 74.07%

| Endophytic fungi | Treatment with *B. amyloliquefaciens* | | | | | | | | | % of dominant endophyte |
|---|---|---|---|---|---|---|---|---|---|---|
| | % of colonization frequency for different length (cm) | | | | | | | | | |
| | 0–3 | 3.1–6 | 6.1–9 | 9.1–12 | 12.1–15 | 15.1–18 | 18.1–21 | 21.1–24 | 24.1–27 | |
| *B. amyloliquefaciens* | 66.7 | 100.0 | 66.7 | 0.0 | 33.3 | 33.3 | 0.0 | 0.0 | 0.0 | 45.0 |
| *G. smithogilvyi* Ti1b | 33.3 | 0.0 | 33.3 | 66.7 | 0.0 | 66.7 | 66.7 | 0.0 | 66.7 | 50.0 |
| Unknown bacteria* | 0.0 | 0.0 | 0.0 | 33.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 5.0 |

*Microorganisms isolated just once and not sequenced

Table 3. Frequency (%) of *T. atroviride* UASWS1400 isolated as endophyte in scions not used for grafting for a CR of 92.59%

| Endophytic fungi | Treatment with *T. atroviride* ITHEC | | | | | | | | | % of dominant endophyte |
|---|---|---|---|---|---|---|---|---|---|---|
| | % of colonization frequency for different length (cm) | | | | | | | | | |
| | 0–3 | 3.1–6 | 6.1–9 | 9.1–12 | 12.1–15 | 15.1–18 | 18.1–21 | 21.1–24 | 24.1–27 | |
| *T. atroviride* | 100.0 | 100.0 | 66.7 | 66.7 | 33.3 | 55.6 | 66.7 | 66.7 | 33.3 | 70.7 |
| *G. smithogilvyi* Ti1t | 0.0 | 0.0 | 33.3 | 33.3 | 33.3 | 44.4 | 33.3 | 0.0 | 66.7 | 29.3 |
| Unknown fungi* | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |

*Microorganisms isolated just once and not sequenced.

Table 4. Frequency (%) of microorganisms isolated as endophytes in control scions in scions not used for grafting for a CR of 88.89%

| Endophytic fungi | Control treatment | | | | | | | | | % of dominant endophyte |
|---|---|---|---|---|---|---|---|---|---|---|
| | % of colonization frequency for different length (cm) | | | | | | | | | |
| | 0-3 | 3.1-6 | 6.1-9 | 9.1-12 | 12.1-15 | 15.1-18 | 18.1-21 | 21.1-24 | 24.1-27 | |
| *G. smithogilvyi* Ti1c | 77.8 | 66.7 | 66.7 | 66.7 | 100.0 | 66.7 | 66.7 | 66.7 | 66.7 | 84.1 |
| Unknown fungi* | 0.0 | 33.3 | 33.3 | 33.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 13.0 |
| Unknown bacteria* | 22.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 2.9 |

* Microorganisms isolated just once and not sequenced.

Table 5. Frequency (%) of microorganisms isolated as endophytes in grafted plants spending 3 months in glasshouse

| Endophytic fungi | Grafted plants | | | | % of dominant endophyte |
|---|---|---|---|---|---|
| | % of colonization frequency | | | | |
| | Rootstock | Rootstock in contact with scion | Scion in contact with rootstock | Scion | |
| *G. smithogilvyi* Ti4 | 73.0 | 38.9 | 33.3 | 64.8 | 52.5 |
| *A. alternata* TiC | 6.3 | 22.2 | 38.9 | 6.5 | 18.5 |
| *A. pullulans* TiE | 9.5 | 0.0 | 0.0 | 4.6 | 3.5 |
| *B. dothidea* TiB | 0.0 | 5.6 | 5.6 | 7.4 | 4.6 |
| *D. eres* TiA | 1.6 | 11.1 | 11.1 | 7.4 | 7.8 |
| *T. hamatum* TiF | 3.2 | 11.1 | 11.1 | 1.9 | 6.8 |
| Unknown fungi* | 6.3 | 11.1 | 0.0 | 7.4 | 6.2 |
| Unknown bacteria* | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |

* Microorganisms isolated just once and not sequenced.

Table 6. Frequency (%) of *B. amyloliquefaciens* UASWS BA1 isolated as endophyte in *in vitro* experiment and other microorganisms isolated

| Endophytic fungi | Treatment with *B. amyloliquefaciens* | | | | % of dominant endophyte |
|---|---|---|---|---|---|
| | % of colonization for different length (cm) | | | | |
| | 0–5 | 5.1–10 | 10.1–15 | 15.1–20 | |
| *B. amyloliquefaciens* | 50.0 | 26.7 | 0.0 | 0.0 | 19.2 |
| *G. smithogilvyi* Ge1b | 50.0 | 33.3 | 66.7 | 66.7 | 54.2 |
| *A. alternata* GeD | 0.0 | 33.3 | 33.3 | 0.0 | 16.7 |
| *A. pullulans* GeA | 0.0 | 0.0 | 0.0 | 20.0 | 5.0 |
| *D. eres* GeC | 0.0 | 0.0 | 0.0 | 13.3 | 3.3 |
| Unknown fungi* | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Unknown bacteria* | 0.0 | 6.7 | 0.0 | 0.0 | 1.7 |

* Microorganisms isolated just once and not sequenced.

ex Fr.) Ces. & De Not. (Botryosphaeriales: Botryosphaeriaceae) and *Aureobasidium pullulans* (De Bary) G. Arnaud (Dothideales: Dothioraceae) were found several times, but represented respectively only 7.8%, 6.8%, 4.6% and 3.5% of microorganisms isolated (Tab. 5) for a CR of 100%.

## *In vitro* experiment

*B. amyloliquefaciens* did not colonize much tissue because it was found in 2 scions, up to 5 cm in one and up to 10 cm in the other. The fungus *G. smithogilvyi* was present in all 3 scions. It was the dominant fungus, representing 54.2% of microorganisms isolated, followed by the bacterium *B. amyloliquefaciens* (19.2%). The fungus *A. alternata* represented 16.7% of endophytes followed distantly by *A. pullulans* (5%) and *D. eres* (3.3%) (Tab. 6) for a CR at 100%. For the scions treated with *T. atroviride*, the CR was 91.67 and the antagonist has colonized the tissues in 3 scions up to 15 cm out of a 20 cm final length. It represented 78.2% of endophytes, followed by *G. smithogilvyi* (21.8%). *G. smithogilvyi* was hardly present between 10.1 and 15 cm, then more frequently between 15.1 and 20 cm (Tab. 7). For the control scions, the CR was 75% and the antagonists were not found in the scions. Surprisingly, the fungus *G. smithogilvyi* was present throughout scion 1 but not in scion 2 and only at a height between 15.1 and 20 cm for scion 3. In total, it represented 44.5% of endophytes, followed by *A. pullulans* (20%) (Tab. 8). However, there was no statistical difference between treated and control scions about the presence of *G. smithogilvyi* ($P>0.1$).

Three weeks after the beginning of the *in vitro* experiment, canker symptoms were observed on 3 scions (15%) treated with *T. atroviride*, on 5 scions (25%) treated with *B. amyloliquefaciens* and on 15

Table 7. Frequency (%) of T. atroviride UASWS1400 isolated as endophyte in in vitro experiment

| Endophytic fungi | Treatment with *T. atroviride* ITHEC | | | | % of dominant endophyte |
|---|---|---|---|---|---|
| | % of colonization for different length (cm) | | | | |
| | 0–5 | 5.1–10 | 10.1–15 | 15.1–20 | |
| *T. atroviride* | 100.0 | 100.0 | 86.7 | 0.0 | 78.2 |
| *G. smithogilvyi* Ge1t | 0.0 | 0.0 | 13.3 | 66.7 | 21.8 |
| Unknown fungi* | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |

* Microorganisms isolated just once and not sequenced.

Table 8. Frequency (%) of microorganisms isolated as endophytes in control scions in *in vitro* experiment

| Endophytic fungi | Control treatment | | | | % of dominant endophyte |
|---|---|---|---|---|---|
| | % of colonization for different length (cm) | | | | |
| | 0–5 | 5.1–10 | 10.1–15 | 15.1–20 | |
| *G. smithogilvyi* Ge1c | 20.0 | 20.0 | 26.7 | 66.7 | 44.5 |
| *A. pullulans* GeB | 46.7 | 13.3 | 0.0 | 0.0 | 20.0 |
| Unknown fungi* | 33.3 | 33.3 | 0.0 | 33.3 | 33.3 |
| Unknown bacteria* | 0.0 | 0.0 | 6.7 | 0.0 | 2.2 |

* Microorganisms isolated just once and not sequenced.

control scions (75%) (Fig. 1a). After 7 weeks, all scions were infected. The same symptoms were observed on grafted plants in a glasshouse in Ticino, where all the chestnuts were finally infected. Using Koch's postulates and observation under binocular and microscopy, the pathogen responsible for these symptoms could be identified as *G. smithogilvyi*. The fungus grew from the slimy twisted cirrhi (Fig. 1b) put on Petri dishes (Fig. 1c). Once inoculated on the healthy rootstock twigs, pycnidia were observed on the surface with hyphae colonized through the intercellular spaces of the bark (Fig. 1d) and medullary ray (Fig. 1e). Although the symptoms looked like those caused by *C. parasitica* at first observation, they were still different. Indeed, Heiniger (1997) reported, that *C. parasitica* generated on the bark yellow to orange pycnidia, stromata and slimy twisted cirrhi (Fig. 2a and b), which was confirmed by our own observations, while we showed in this study that *G. smithogilvyi* formed orange to red and black pycnid-

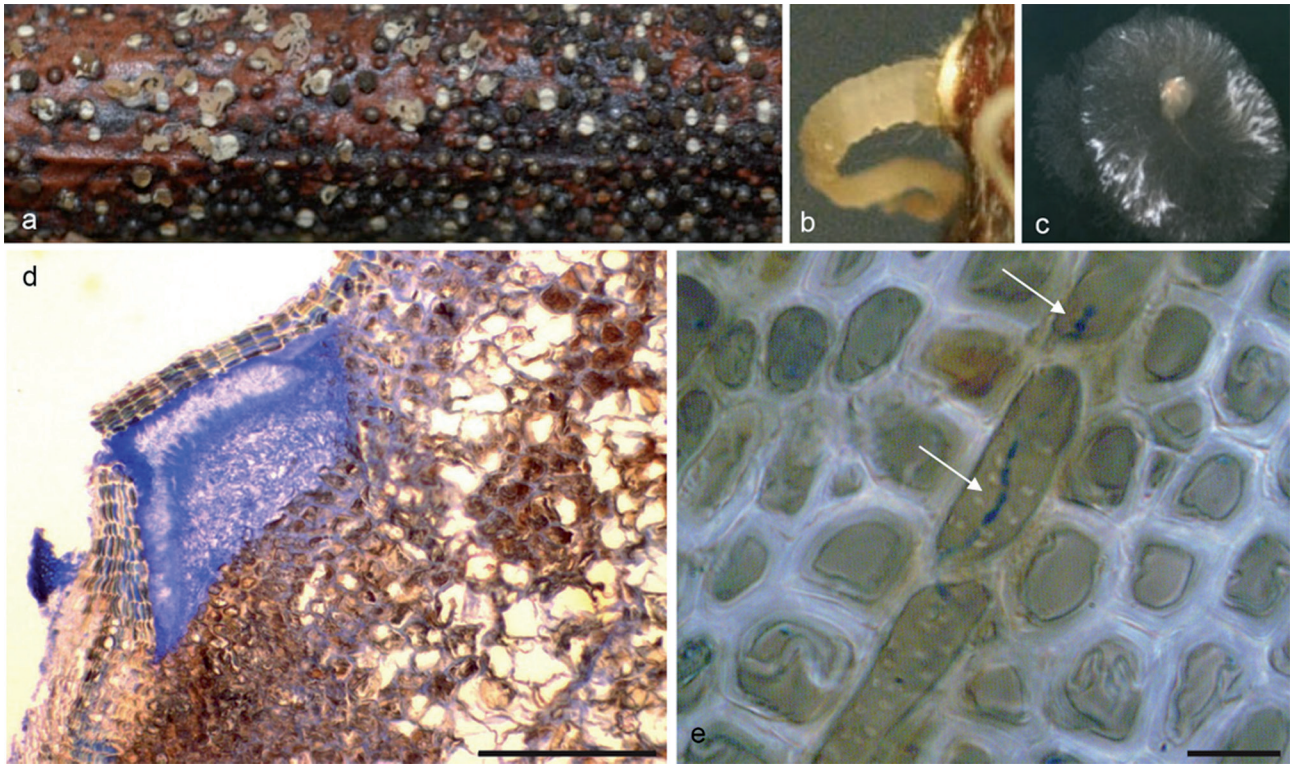


Fig. 1. *Gnomoniopsis smithogilvyi* **a.** Control scion infected by *G. smithogilvyi* **b.** Slimy twisted cirrhus **c.** *G. smithogilvyi* grew from cirrhus on PGA **d.** Pycnidium with hyphae propagated through the intercellular spaces of the bark **e.** Hyphae in the medullary ray of a scion. Scale bars: d = 200 $\mu$m and e = 10$\mu$m

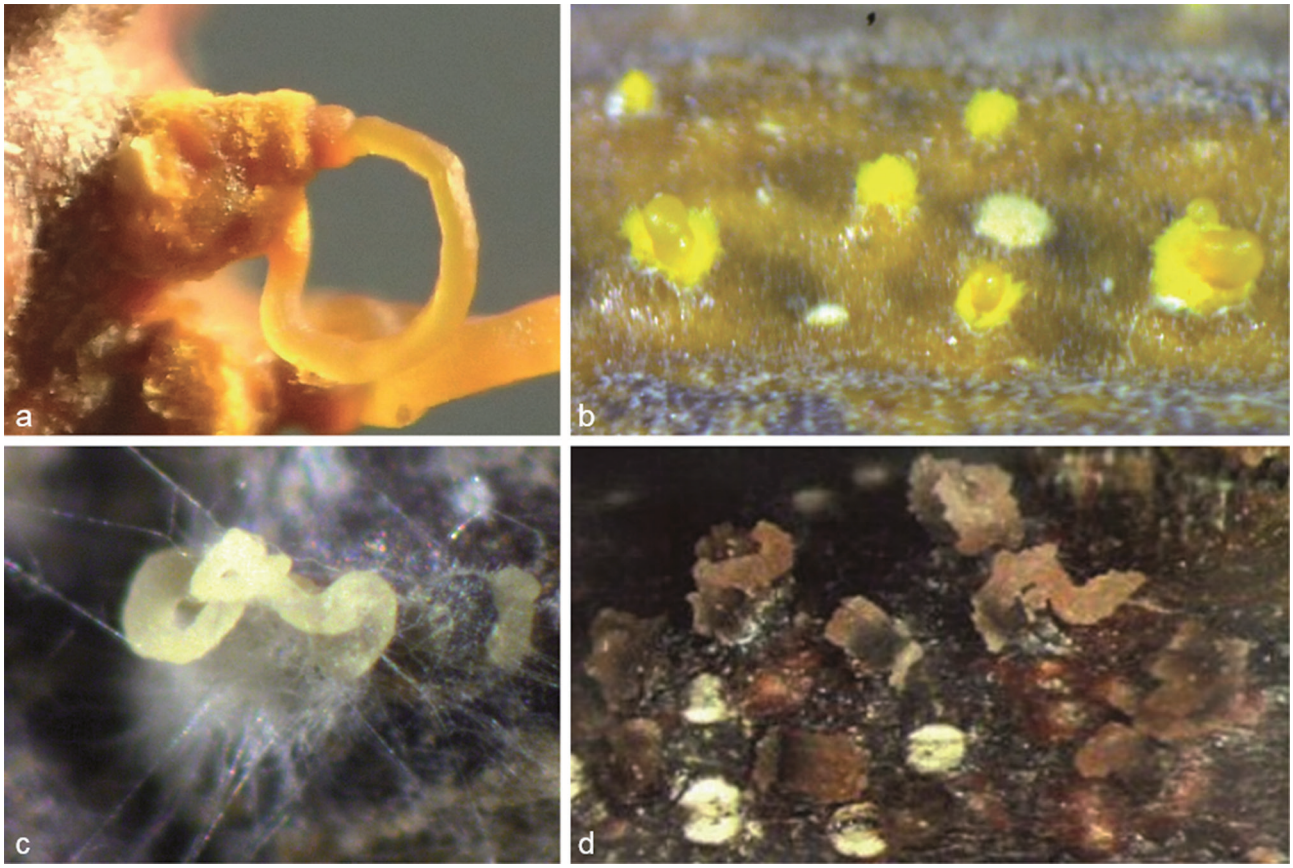


Fig. 2. Comparison between *C. parasitica* and *G. smithogilvyi* **a.** Stroma and cirrhus of *C. parasitica* **b.** Bark infected by *C. parasitica* **c.** Stroma and cirrhus of *G. smithogilvyi* **d.** Bark infected by *G. smithogilvyi*

ia, grey stromata and cream-colored to beige slimy twisted cirrhi on the bark (Fig. 2c and d).

## Discussion

The observation that antagonists were never found in control scions support the hipothesis that their originated from the treatment. It thus seems that these antagonists have adopted an endophyte behaviour. Indeed, *T. atroviride* was found up to 27 cm and *B. amyloliquefaciens* up to 18 cm on scions which were plated on to PGA directly after soaking in the glasshouse experiment and respectively up to 15 cm and 10 cm in the *in vitro* experiment. These interesting results should now be confirmed by further works with an emphasis on endophytism, inoculation methods, inoculum dosage and application timing. The absence of antagonists in the highest parts of the scions can be explained. Indeed, as the upper parts of scions were the ones used for grafting, the antagonists were not able to colonize the internal wood tissues through the scions, which measured about 1m20. In the *in vitro* experiment, canker symptoms were aggressive because all scions were finally infected and the presence of *G. smithogilvyi* was not statistically different between the scions treated and the control scions. Endophytism of *T. atroviride* was not efficient enough because it was not recorded higher than 15 cm. In the last 5 cm, where it was no longer present, *G. smithogilvyi* was found at 66.7%. So even if *T. atroviride* represented 78.2% of endophytes, it left enough space to *G. smithogilvyi* to develop in the scions. It was nonetheless observed that canker symptoms developed more slowly when antagonists were present because 75% of control scions have showed infection symptoms while only 15% of scions treated with *T. atroviride* and 25% of scions treated with *B. amyloliquefaciens* showed symptoms of an infection by *G. smithogilvyi*. Moreover, all the grafted plants in glasshouses were infected when the antagonists were not present. In all cases, the slices from which these antagonists were isolated, were never colonized by other organisms and the presence of *G. smithogilvyi* between treated and control scion was statistically different for the scions not used for grafting. These results prompt to think that *B. amyloliquefaciens* and *T. atroviride* had an influence on the development of *G. smithogilvyi*. It is thus likely that if the antagonists colonized the totality of woody tissues, they should then able to prevent the development of the pathogen and would eventually stop its growth. Longer soaking times and for shorter scions should therefore be more efficient, however more testing is needed.

Since the fungus *G. smithogilvyi* was strongly present in scions and rootstocks, further investigations should be made to determine the source of infection. Nevertheless, microscope observations demonstrated its endophyte behaviour. This would suggest that the fungus was initially present in the wood tissues as a latent pathogen. Other fungi, which are known as pathogens (*A. alternata, B. dothidea* and *D. eres*), were rarely isolated with a DE much lower than *G. smithogilvyi*.

The use of endophytic antagonists for preventive biocontrol could be a promising strategy provided the inoculation method is improved. For example, only scions already cut for grafting should be soaked, or roots of the rootstocks could be soaked and for a longer time. Further field investigations would also be required over the long term, for assessing the persistence, the viability and the suppressive effect on the pathogen. This control method could be, however, very advantageous for the producer, since it is cheap, easy to implement and requires no chemicals.

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