Use of Endophytic Bacteria as Biocontrol Agents Against Chestnut Blight*

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ABSTRACT. Chestnut blight is a severe disease of European *(Castanea sativa)* and American chestnut *(Castanea dentata).* Biocontrol by application of hypovirulent *Cryphonectria parasitica* strains has been limited because of vegetative compatibility between the fungal strains. Further, in Austria there is no evidence for the existence of hypovirulent strains. Therefore, a search was made for antagonistic bacteria.

Selected bacterial strains from xylem sap of healthy chestnut, leaves of lilac and roses had an inhibitory effect *in vitro* on the growth of the fungus. More than 80 bacterial isolates were screened for their antagonistic activity. About 50% inhibited the growth of *C. parasitica in vitro.* Upon further investigation, the most efficient isolate from the xylem fluid was characterized as *Bacillus subtilis.*

In vitro shoots of different micropropagated chestnut clones were inoculated with the selected *B. subtilis.* These shoots were infected after one and/or two wk with the Austrian *C. parasitica* isolates. Growth of the fungus was severely retarded in stems pre-inoculated with bacteria. Differences in susceptibility to the pathogen between chestnuts also were noted.

Preliminary greenhouse tests are under way and show similar results. The mechanisms that are responsible for these effects could either be the production of fungistatic substances by *B. subtilis* or the induction of systemic resistance in the plant.

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The first appearance of *Cryphonectriaparasitica* (Murr.) Barr in Austria was reported by Donabauer (1). In recent years, chestnut blight has become an increasing problem in Austria. The incidence of *C. parasitica* in Austria is related to the natural range of chestnut, which is in the warmer southeastern regions of Syria and Burgenland close to the borders of Hungary and Slovenia (Figure 1). The application of hypovirulent strains is not feasible because, up to now, there is no evidence of hypovirulence in Austria.

Investigations into antagonistic bacteria have shown a potential use to control plant diseases (3). The report presented here describes an approach for screening bacterial isolates of various sources for antagonistic effects on the growth of *C. parasitica in vitro.*

MATERIAL AND METHODS

Screening and testing of antagonistic bacteria. *C. parasitica* was isolated from more than 30 diseased *Castanea sativa* Mill. trees of different regions in Styria and Burgenland. Fungal isolates were grown on potato dextrose agar (PDA) at 25 C. Surface bacteria from leaves of lilac, birch, roses and chestnut, as well as endophytic bacteria of the

Figure 1. Disease centers of *C. parasitica* in Austria.

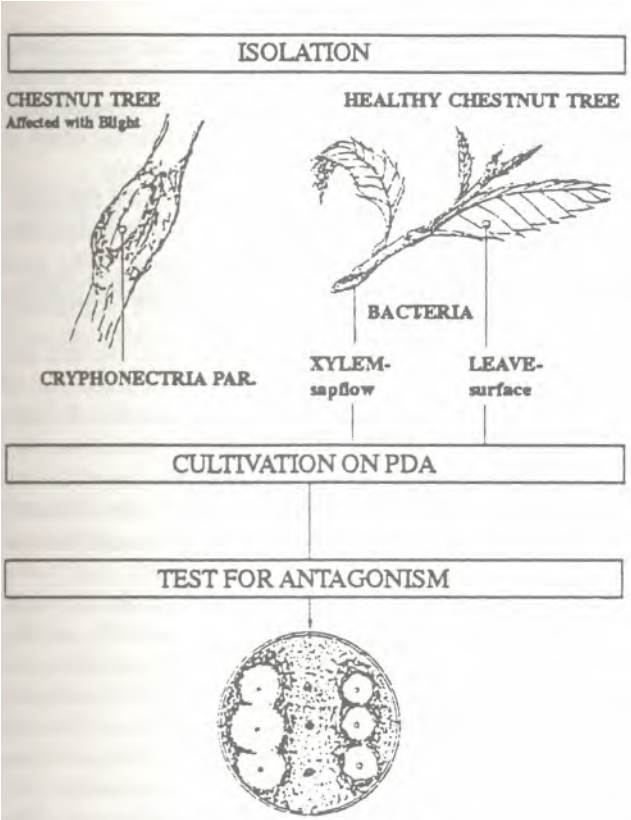

Figure 2. Scheme for isolation and co-inoculation.

xylem sap of a healthy chestnut were isolated on selective media after Sands (4). PD-broth or PD-agar was used for further cultivation of the bacterial isolates (Figure 2).

For a general *in vitro* screening of antagonistic bacteria multi-plates containing 200 ,u1 PD-broth were inoculated with a 1µl loopful of bacteria colonies and cultivated on a shaker for 24 hat 30 C. Twenty ul of each bacterial solution was spotted three times in a petri dish on PD-agar.

After 7-day growth (30 C in the dark) the cultures were overlayed with conidial suspension (2 x 10^6 conidia/ml) of a reference strain of *C. parasitica* from a German culture collection (DSM). Seven days later, the petri dishes were evaluated for antagonistic effects.

The five most efficient endophytic bacterial isolates were selected and used for further screening with the Austrian *C. parasitica* isolates. Each bacterial isolate (about 1 x 10^6 cfu/ml) was spotted on a petri dish as described above. The most efficient isolate was characterized as *Bacillus subtilis* (Ehrenberg) Cohn, and designated as L25 (Figure 3). This isolate was used for all further experiments.

In vitro* inoculations.** ***In vitro shoots (5–6 cm) of two chestnut clones, GG 12 (Austrian Gross Gelb) and C 125 *(C. sativa x C. crenata* provided from A. Ballester, CSIC, Spain) were pierced and a small piece of agar with isolate L25 was applied to the wound.

After 1 and 2 wk, respectively, the inoculated shoots and control shoots were challenged with 1 ,u1 conidial solution (appr. 40 x 10^3 conidia/ml) of a virulent fungus strain (BF 5) (Figure 4). Twelve replications per treatment were

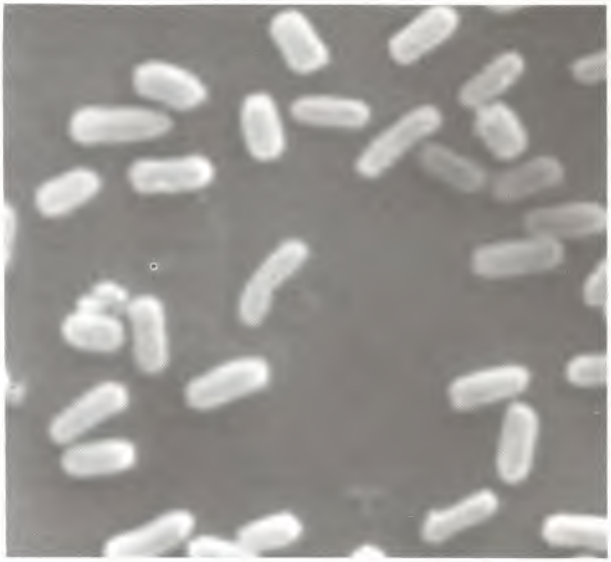

Figure 3. *Bacillus subtilis* isolate L25. SEI × 6000.

made. Disease symptoms were scored for each shoot in weekly intervals up to 6 wk, using the following rating scheme:

0 no symptoms
1 hyphae at the wound
2 hyphae along the stem
3 massive growth of hyphae
4 massive necrosis
5 death of explant

Shoots were cultivated in glass tubes, 12 ml Gresshof and Doy media (2) with 3% sucrose, 0.8% agar plus 0.2 mg/1 BAP at a temperature of 25 C with 16-h fluorescent light. Cultures were transferred every 3 days to fresh media to prevent fungal growth on the media.

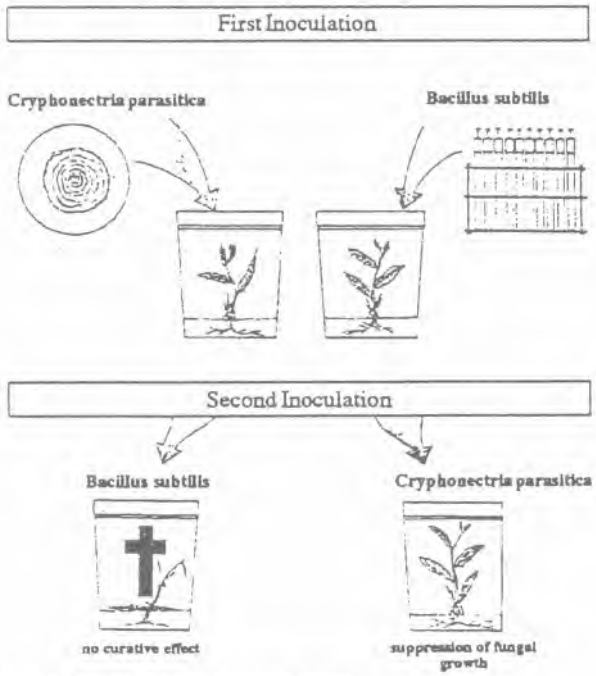

Figure 4. *In vitro* inoculation experiments.

Preliminary greenhouse test. Chestnut seedlings 25 to 30 cm length were inoculated with bacteria (L25) by cutting the bark in form of a T and applying agar with bacteria underneath the bark. The wound was covered with a rubber band. Each stem was inoculated three times. After 1 wk the seedlings were infected with the fungus by applying growing mycelium into an artificial wound.

RESULTS AND DISCUSSION

In total, 171 bacterial isolates were screened for their antagonistic effects on the growth of the fungus. The xylem fluid from chestnut yielded 81 bacterial isolates. Forty isolates exerted growth suppression on *C. parasitica.* Tweny-five are of endophytic origin from a healthy chestnut (Figure 5). Growth suppression ranged from small inhibition zones to nearly total growth inhibition.

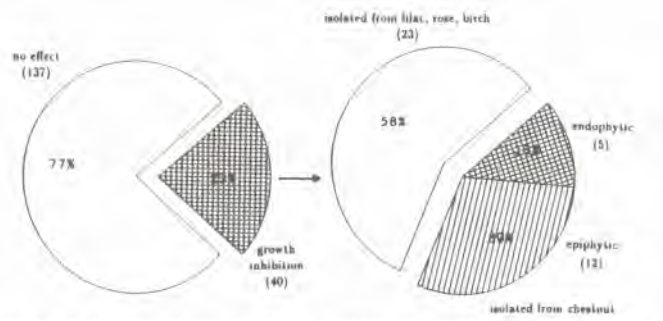

Figure 5. *In vitro* effects of isolated bacteria on *Cryphonectria parasitica.*

Experiments with *in vitro* chestnut exhibited a distinct growth retardation on the fungus after inoculation with bacteria. No curative effect of the bacteria on diseased *in vitro* chestnuts could be observed.

Responses of chestnut clones are different. Clone 125 was more susceptible to the pathogen than clone GG 12. Pre-inoculation with bacteria 1 wk before fungal infection suppressed mycelium development considerably more compared to a 2-wk interval (Figure 6).

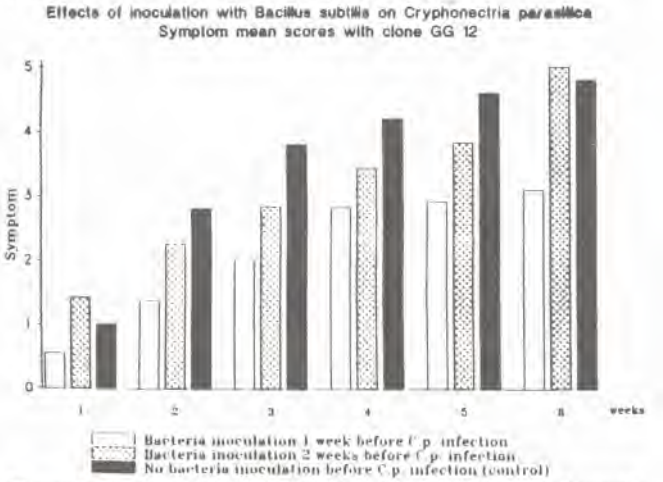

Figure 6. Effects of inoculation with *Bacillus subtilis* on *Cryphonectria parasitica*. Symptom mean scores with clone GG 12.

Preliminary results from greenhouse tests were similar to those of the results of the *in vitro* inoculation tests. In late summer extremely high temperatures in the greenhouse, combined with the wounding stress resulted in the death of all main shoots including those used as controls.

Closer evaluation of the experiment showed that plant regeneration assessed by appearance of stump sprouts, was much better from pre-inoculated chestnuts (70%) than noninculated plants (10%). From controls without bacteria and fungus, 100% regenerated.

Mechanisms for these observed effects are still unknown. One possibility is the production of antibiotics of *B. subtilis.* This bacterium is a known producer of cyclic peptide antibiotics such as Iturin, Bacillomycin, etc. Schreiber et al. (6) suggested that the thickening of the cell wall of *Ceratocystis ulmi (= Ophiostoma ulmi* [Buism.] Nannf.) might be due to the inhibition of chitin polymerization caused by the antibiotics of *B. subtilis.*

Another explanation could be a change in the host plant metabolism caused by the inoculation with *B. subtilis.* Scheffer (5) discussed the probability that microbial compounds may trigger the host's defenses either by acting as elicitors or by producing them. The ultimate effect of a biological control treatment may be an increased resistance of the host, referred to as "induced resistance."

Further investigation into a practical application of the endophytic bacteria is necessary. If successful this protective method may be considered as a step towards a biocontrol system against *C. parasitica.*

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