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An unknown trees die back caused by *Pseudomonas* species in Switzerland

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Abstract

A model for tree pathogen diagnosis – *Prunus domestica* L. has been studied against pathogenic bacteria. An orchard of 110 trees of *P. domestica* showed dying back symptoms in May 2009 and nineteen of these trees were eradicated and burnt for prophylaxis. No symptoms correlated with those caused by pathogens previously observed in stone fruit die back in Europe or elsewhere (*Pseudomonas syringae* pv *syringae* van Hall, *Pseudomonas syringae* pv *morsprunorum* Lazarowtz, *Phytophthora* sp., *Diaporthe perniciosa* Marchal., phytoplasma or viruses) were not found. Interestingly, cutting the trunk in transversal sections allowed the observation of stem heart necrosis which was mostly important at the grafting point. Isolations from necrotic stem heart allowed to identify a not yet described *Pseudomonas* species not related to *P. syringae*. The method described in the paper for isolation of pathogenic bacteria and their quick an reliable identification can be also applied for detection of pathogens in forest tree plantations.

Keywords

PCR and sequencing diagnostic, *Prunus domestica*, *Pseudomonas*, rootstock, stone fruit die back

Introduction

Bacteria are microscopic, unicellular, prokaryotic organisms so far neglected in forest pathology because of dificulties with their identification. Modern, inovative technology has opened the new detection and identification possibilities on the basis of DNA analisis. Bacterial diseases produce symptoms on leaves and shoots like: water-soaked lesions that become necrotic and macerated or slimy. Pathogens enter plant through stomates, wounds in leaves and roots, or via insect vectors and cause e.g. bacterial canker of cherry, crown gall (hypertrophy of stem and root tissue), fire blight (wilted, necrotic leaves, wetwood, slimy flux from branch stubs and cracks), yellows (witches' brooming, small, malformed, wavy and yellow leaves; branch dieback; and stunting).

Bacteria importance in forest pathology is steadly growing, especially in the light of observed climatic changes (Lambais *et al*. 2006) found an extraordinary

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level of bacterial biodiversity in the tree leaf canopy of the Atlantic forest by using culture-independent molecular methods. Their survey suggests that each tree species selects for a distinct microbial community. Analysis of the bacterial 16S ribosomal RNA gene sequences revealed that about 97% of the bacteria were unknown species and that the phyllosphere of any one tree species carries at least 95 to 671 bacterial species. The tree canopies of tropical forests likely represent a large reservoir of unexplored microbial diversity. Some of them might be pathogenic and cause problems in forest protection (Scortichini *et al.* 1991). Many bacterial species, most of them anaerobic, have been isolated from forest trees showing symptoms of wetwood (*Abies alba* Mill., *Populus deltoides* Bartram, Ex Marshall., and *Ulmus americana* L.). From discolored woody tissues (*Quercus* sp.) *Clostridium* is the genus most frequently isolated. From woody tissues of declining *Q. cerris* trees showing epicormic twigs and bleeding on the trunk several species were isolated from discolored subcortical: *Erwinia herbicola* (the most common), *Bacillus cereus* Frankland, *Staphylococcus sciuri* Kloos, *Micrococcus luteus* Kloos, and *Kluyvera criocrescens* Oliver.

Another known bacterium – *Pseudomonas savastanoi* Janse is a Gram-negative plant pathogen that infects a variety of plants and causes ash canker. A pathovar of greatest economical significance is *Pseudomonas savastanoi pv. Oleae* Janse, responsible for the olive knot disease. Symptoms include formation of galls on infected trees; tumour formation is induced by indoleacetic acid biosynthesis by the bacteria, in a similar manner to the well-studied crown gall pathogen, *Agrobacterium tumefaciens* Smith and Townsend. The purpose of this work is focussed on the detection techniques of likely new emerging pathogenic bacterium causing dieback of plum trees.

Materials and methods

Nineteen trees of *Prunus domestica* out of an orchard of 110 trees, located in Lullier (Canton of Geneva) were affected by stone fruits die back symptoms (Fig. 1) in May 2009. Trees were 3 years old. Ninety trees were grafted with the cultivar Quetsche. Two lines, 60 trees totally, showed mild to severe die back symptoms. Out of these 60 trees, 30 were directly grafted on rootstock Ishtara whereas the 30 others were grafted on Fellenberg overgrafted on rootstock Ishtara. Sixteen dead trees out of these 60 were removed in June 2009. Besides that, 10 trees of cultivar Reine Claude Verte grafted on rootstock Plumina showed the same symptoms and 3 dead trees were eradicated, while ten other trees of cultivar Mirabelle grafted on rootstock Jaspi showed no symptoms.

Additionally, a third line of 30 trees of cultivar Quetsche, grafted on Fellenberg, overgrafted on Richard. Early overgrafted on rootstock Saint-Julien showed no symptoms at all and looked normally vigourous. Necrotic zones of cross sections of the graft point were carefully sampled, after removing the superficial zone, in the laboratory under optimal sterility conditions. Cross sections were made on the cultivar scion, on the rootstock trunk at different heights and at the graft point of diseased trees.

Bacteria isolation was carried out from these necrotic sections on LB medium and fungal isolation on PGA Ampicillin medium. Subcultures of growing bacteria on KB medium appeared to be fluorescent under UV

Fig. 1. Stone fruit die back symptoms on Quetsche cultivars

Fig. 2. Cross section at the graft point between Fellenberg cultivar and Ishtara rootstock – black necrosis of the stem heart is clearly visible

light orienting the diagnostic towards a *Pseudomonas sp.* A single colony was picked up from a LB subculture and submitted to direct amplification with the kit Extract-N-Amp (Sigma Aldrich, Buchs, Switzerland.) using primers 341F (Muyzer *et al.* 1993) and 907rM (Muyzer *et al.* 1998). DNA amplification was checked by electrophoresis and PCR products were purified using the PCR Clean-Up kit (Sigma-Aldrich) prior to sequencing.

Besides the identification work, the nineteen diseased trees were removed and burnt in order to destroy a possible disease focus.

Results and discussion

On diseased trees, leaves died on affected twigs where other remaining leaves were healthy and not chlorotic. Buds died and did not produce leaves and flowers on affected branches, similarly to abortion of flowering buds in *Pseudomonas syringae* infections of plum trees (Roos and Hattingh 1987, Hinrichs-Berger 2004) but no bacterial cankers or gummosis were observed on any diseased or healthy trees of the orchard, as it was reported in case of plum or cherry tree die back due to *P. syringae* pv. *syringae* or *P. syringae* pv. *morsprunorum* strains (Hinrichs-Berger 2004, Latorre and Jones 1979). Cambium was dying causing necrosis in buds and shoots. Twig ends emanated an alcoholic smell when broken.

Some trees were pulled out and roots, collar and collar roots appeared to be healthy, withdrawing the hypothesis of a possible *Phytophthora* infection, which was recently reported in several regions of Switzerland on rootstock Maxma 14 (Bosshard *et al.* 2003). No symptoms could be correlated with previous record of plum trees die back involving MLOs (mycoplasme like organism) and virus (Pilotti *et al.* 1995) or the fungus *Diaporthe perniciosa* (Harris 1998). Figure 2 clearly shows dark necrotic zones of the stem heart which might explain dying back of above branches, by stopping or delaying xylem and phloem exchanges. Necrosis was most important at the graft point and necrotic surface decreased in cross sections lower and upper to the graft point.

No fungus was isolated from the laboratory test on PGA Ampicillin medium. The bacterial sequence obtained after the DNA amplification (Genbank accession number GQ327986, NCBI, Bethesda, MD, USA) showed 99% identity with many previously found unknown and not yet identified *Pseudomonas* spp. isolated or directly amplified from diverse environmental samples. Consequently the bacterium isolated from necrotic stem heart of dying back plum trees belongs to the genus *Pseudomonas*, but is no related to *Pseudomonas syringae*, with which it only shared 96% to 97% identity on a 536 base pair (bp) sequence of the 16S rDNA (when compared to *P. syringae* or *P. syringae* pv. *syringae* or *P. syringae* pv *morsprunorum* sequences (Genbank accessions GQ160904, FJ971872, CP000075, EU708321 and AB001445).

Collected data about the orchard and plantation history would point to a possible interaction between soil conditions and rootstock sensitivity. Most affected trees were on Ishtara and Plumina rootstock while Jaspi and St-Julien rootstock were not affected. Interestingly, all plant material came from the same plant nursery. Orchard soil is a cold, humid and heavy argillaceous limestone soil which is known as a soil condition favouring stone fruit die back.

In general, the role of bacteria in forest pathology has been underestimated for many years because of lack of appropriate methods of detection and identification.

Recent development of molecular biology opens a new possibilities in forest protection. Bacteria may overwinter in host or in soil. As many plants are to be considered as well as forest as ornamental species, the exchange of seedlings between nurseries (and soil) take place quite often and regularly. Plant pathogenic bacteria have no spores, and cannot be wind-disseminated. Thus soil may be an important source of infection. Nowadays, the geographical distance (isolation) is not longer a problem having diverse transportation means and lack of borders (e.g. bonsai and ornamental plant are often grown and transported in containers with soil). In such circumstances the risk of transfer of alien invasive species between continents or countries becomes an emerging problem of the contemporary forestry, floriculture and pomology. In Poland, similar symptoms in seed plantations have been observed several times in the past when death of many lime, maple and pine trees had been reported. In Switzerland fungal infection was confirmed in 1980 or in 2006 while searching the reason of decline 7–10 year-old trees. Necrosis were readily visible at the graft point and necrotic surface decreased in cross sections lower and upper to the graft point. However, at that time dark necrotic zones at the level of grafted stems had not been tested against bacteria. The appliance of new, modern molecular techniques in forest science may put a new light on unexplained reasons of tree diebacks observed in the past, usually several years after grafting.

Conclusion

- Applied model of fruit tree sampling, bacteria culturing and identification with the use of molecular techniques when transferred to forest research will open new possibilities of bacteria monitoring in forest nurseries.
- The founding of new pathogenic bacterium species (probably soil borne) proves the necessity of the monitoring of nurseries (both forest and ornamental species).
- Closer co-operation between foresters and horticulturists in this respect is urgently needed because exchange of plant materials (with soil) may introduce pathogens into forest ecosystems with all its negative consequences.
- The achieved foundings may help to elaborate an efficient detection system against bacterial disease to be applied in forest nurseries.
- Whether bacteria are the cause of the disease, whether they originate from soil or were inoculated during grafting remain pending questions. This last hypothesis should pull more attention on prophylaxis to be applied in the future when grafting.

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