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Bacterial endobionts in the big non-mycorrhizal roots of Scots pine (*Pinus sylvestris* L.)

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Abstract

Subsurface bacterial growth occurred in an N-free medium inoculated with interior tissues of big non-mycorrhizal roots (7 to 8 mm diameter) of 15–20 years-old Scots pine (*Pinus sylvestris* L.) growing on sand dunes at the Baltic Sea of Poland. The bacteria were not N_2 fixers as determined by the acetylene reduction method. Light microscopic and scanning electron microscopic observations revealed massive bacterial clusters residing in the cortical cells underlying epidermis and parenchyma. The bacteria produced yellow-green pigments on King's medium, which fluoresced under ultraviolet (UV) irradiation at 366 nm wavelength, and could be a siderophore-producing *Pseudomonas*.

Key words: *Pinus sylvestris* – bacteria – root endophytes – endobiont

Introduction

Wilson (1995) defined endophytes as "fungi or bacteria which, for all part of their life cycle, invade the tissues of living plants and cause unapparent and asymptotic infections entirely within plant tissues but cause no symptoms of disease." However, none of the examples in his paper involved bacteria (Chanway 1996). O'Dell and Trappe (1992) used the term endophyte for mycorrhizal fungi. But mycorrhizal fungi reside only partly inside plant tissues; they form external hyphae emanating into the soil. Mycorrhizal fungi have long been recognized as important microorganisms that promote tree growth and survival (Smith and Read 1997). Nitrogen-fixing bacteria have been isolated from within sporocarps of mycorrhizal fungi or mycorrhizas (Li and Castellano 1987; Li *et al.* 1992).

Bacteria have been isolated from root, stem, leaf, and other tissues of healthy, symptomless agricultural and forest plant species (Kloepper *et al.* 1992; Bell *et al.* 1995; Schippers *et al.* 1987; Shishido *et al.* 1999). Most of the bacterial endophytes isolated are members of common soil bacteria such as *Pseudomonas*, *Bacillus*, *Azospirillum* (Li and Castellano 1987; Chanway 1996; Shishido *et al.* 1996). Plants such as sugar cane, rice, wheat, and maize may contain endophytes involved in nitrogen fixation (James and Olivares 1997; Reinhold-Hurek and Hurek 1998).

In this paper, we describe the occurrence of siderophore-producing bacteria within big nonmycorrhizal, suberized roots of 15–20-years-old Scots pine (*Pinus sylvestris* L.) growing on sand dunes near the coastal Baltic Sea of Poland.

Materials and methods

Nonmycorrhizal, suberized roots with 7 to 8 mm in diameter were collected from a 15–20-years-old Scots pine (*Pinus sylvestris* L.) forest on sand dunes (near Karwia, Poland) at the Baltic Sea. The nonmycorrhizal roots with similar size were also collected from a 40-years-old Scots pine forest on the sandy loam soil near Nicolaus

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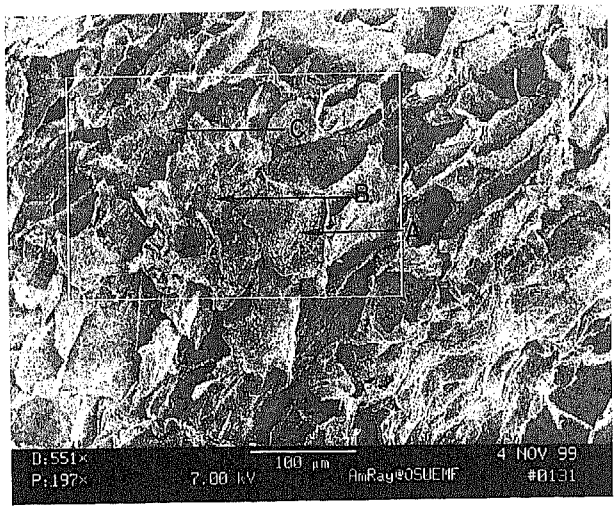

Fig. 1. Scanning electron micrographs of a cross section of a *P. sylvestris* root. The cells (A, B and C) are colonized by bacterial clusters.

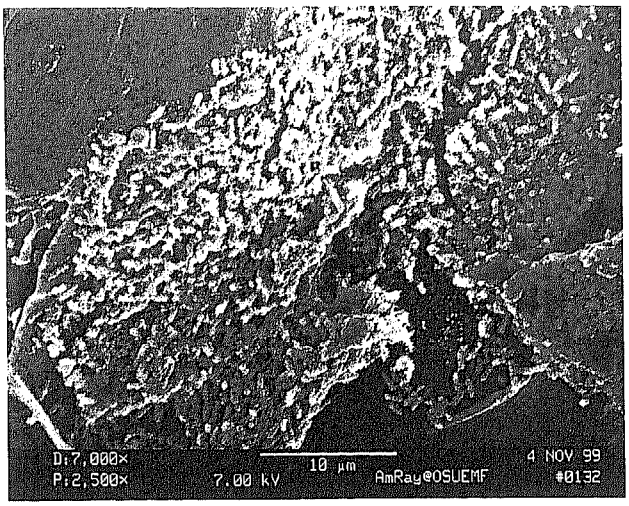

Fig. 3. Enlargement of the bacteria-colonized cell (B). Note the massive bacterial clusters.

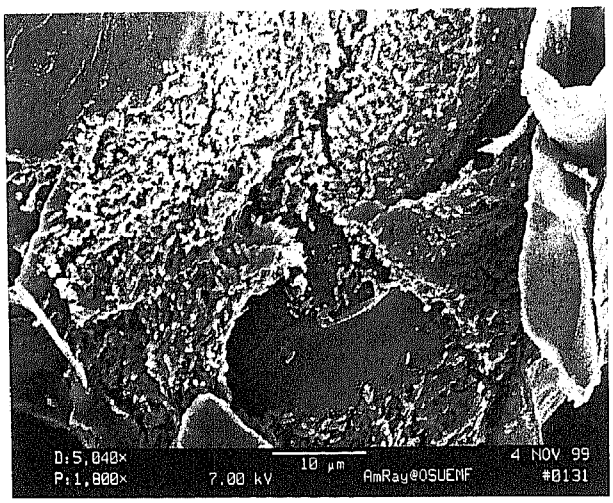

Fig. 2. Enlargement of the bacteria-colonized cell (A). Note the massive bacterial clusters.

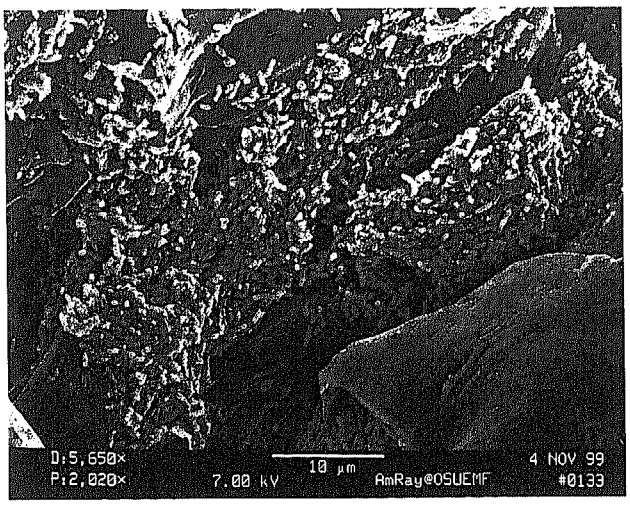

Fig. 4. Enlargement of the bacteria-colonized cell (C). Note the massive bacterial clusters.

Copernicus University, Torun, Poland. The trees in both places are healthy and showed no disease symptoms. The roots were sterilized for 25 min on a magnetic stirrer with a 2.6% aqueous solution of sodium hypochlorite with 1 drop of Tween 20. Then they were rinsed 5 times with sterile distilled water. The roots were aseptically split longitudinally. Interior tissues from various locations were introduced into the N-free semisolid medium in tubes (Rennie 1981). The tubes with subsurface growth of bacteria were sealed with rubber stoppers and injected with 10% acetylene. Acetylene reduction was determined with a gas chromatograph equipped with a flame ionization detector and a 2.0 m × 2/1 mm stainless steel column packed with Porapak R on 80 to 100 mesh chromosorb W. Acetylene reduction was also determined with thin sections of the excised roots (Li *et al.* unpublished results). Bacteria in Rennie's medium were isolated by repeatedly streaking the culture on King's medium (Geels and Schippers 1983).

For light microscopic and scanning electron microscopic (SEM) studies the roots were fixed in FAA solution (50% ethyl alcohol: 5% glacial acetic acid: 10% formaldehyde) by intermittent vacuum. Afterwards the roots were cut into 4 mm segments and dehydrated in an ethanol series (50%, 70%, and 95%, with two

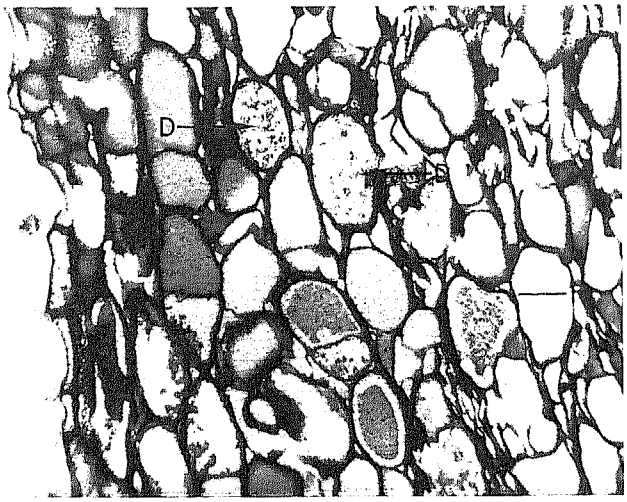

Fig. 5. Light micrograph of a cross section of a *P. sylvestris* root. Note the bacteria inside the cells (D) underlying the epidermis. Bar = 51 µm.

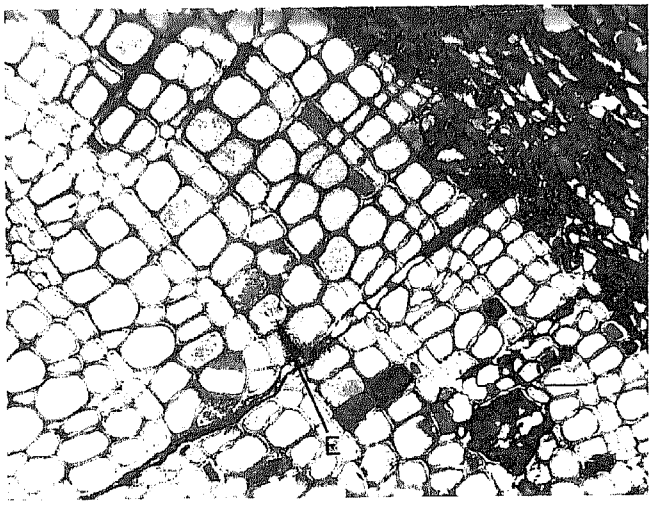

Fig. 7. Light micrograph of a cross section of a *P. sylvestris* root. Note the bacteria inside the cell (E) underlying the parenchyma. Bar = 70 µm.

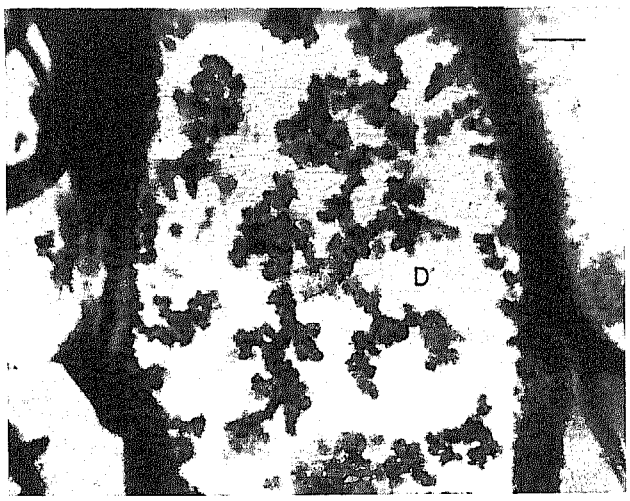

Fig. 6. Enlargement of the cell (D) underlying the epidermis. Note the bacterial clusters inside the cell. Bar = 11.5 µm.

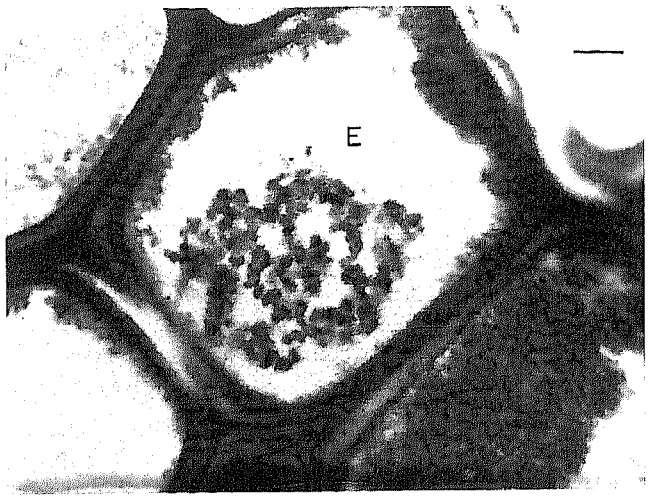

Fig. 8. Enlargement of the cell (E) underlying the parenchyma (× 1000). Note the bacterial clusters inside the cell. Bar = 11.5 µm.

changes in 100%). The fixed and dehydrated root specimens were imbedded in glycol methacrylate plastic (Feder and O'Brien 1968), and cut (5 to 8 µm thick cross sections) with a rotary microtome. The specimens were mounted on slides, stained with 0.05% toluidine blue in 0.02 M sodium benzoate buffer, pH 4.4 (Sidman *et al.* 1961), and examined by using a differential interference contrast (DIC) microscope. For SEM observations, the fixed 4-mm root segments were dehydrated in ethanol and absolute acetone, followed by thin sectioning (100 µm) with a steel blade. The metal for coating the thin sections was 60% gold, 40% palladium by weight %, applied by sputter coating.

Results and discussion

The excised big nonmycorrhizal roots collected from the forests on coastal sand dunes and near the Nicolaus Copernicus University did not reduce acetylene. However, subsurface bacterial growth occurred in tubes of N-free semi-solid Rennie (1981) medium inoculated with tissues removed aseptically from pine roots of coastal sand dunes. Also the tissue did not reduce acetylene. Thus, the subsurface culture in the tube is not an N_2-fixing microbe. No microbial growth occurred in tubes of this medium inoculated with tissues removed aseptically from pine roots collected near the University. The sub-

surface bacterial culture in the tube was a siderophore-producing *Pseudomonas*; it produced yellow-green pigments on King's medium, which fluoresced under UV irradiation at 366 nm (Geels and Schippers 1983).

Both light microscopic and SEM studies of the roots revealed massive bacterial clusters residing within the cortical cells underlying the epidermis and parenchyma tissues of pine growing on sand dunes (Figs. 1–8). These bacterial clusters could correspond to those we isolated.

Of the bacterial endophytes in agricultural crops, few were reported to be associated with forest trees. Shishido *et al.* (1995) detected plant growth-promoting *Bacillus polymyxa* inside the root of young lodgepole pine (*Pinus contorta* var. *latifolia* (Dougl.) Engelm.) seedlings and *P. fluorescens* within stem tissue of young hybrid spruce (*Picea glauca* × *P. engelmannii*) seedlings (Shishido *et al.* 1999). Both bacterial species can colonize cortical cells and stem vascular tissues of the hybrid spruce upon inoculation with these two bacteria (Shishido *et al.* 1999). In contrast, the endophytes we discovered were inside the big nonmycorrhial and suberized roots of 15–20-years-old Scots pine growing on the Polish coastal sand dunes of Baltic Sea, but not in the older Scots pine growing near the University.

The ecological role of this siderophore-producing *Pseudomonas* sp. needs to be explored. Beneficial responses of siderophore-producing *Pseudomonas* have been reported, including plant growth promotion (Shishido *et al.* 1996; Schroth and Becker 1990), suppression of plant pathogens (Schroth and Becker 1990), and bioremediation of recalcitrant compounds (Leahy and Colwell 1990).

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References

Bell, C. R., Dickie, G. A., Harvey, W. L. G., Chan, J. W. Y. F. (1995): Endophytic bacteria in grapevine. Can. J. Microbiol. **41,** 46–53.

Chanway, C. P. (1996): Endophytes: they're not just fungi! Can. J. Bot. **74,** 321–322.

Feder, N., O'Brien, T. P. (1968): Plant microtechnique: some principles and new methods. Amer. J. Bot. **55,** 123–142.

Geels, F. P., Schippers, B. (1983): Selection of antagonistic fluorescent *Pseudomonas* spp. and their root colonization and persistence following treatment of seed potatoes. Phytopath. Z. **108,**193–206.

James, E. K., Olivares F. L. (1997): Infection and colonization of sugar cane and other graminaceous plants by endophytic diazotrophs. Crit. Rev. Plant Sci. **17,** 77–119.

Kloepper, J. W., Schippers, B., Bakker P. A. H. M. (1992): Proposed elimination of the term endorhizosphere. Phytopathol. **82,** 726–727.

Leahy, J. G., Colwell, R. R. (1990): Microbial degradation of hydrocarbons in the environment. Microbiol. Rev. **54,** 305–315.

Li, C. Y., Castellano M. A. (1987): *Azospirillum* isolated from sporocarps of the mycorrhizal fungi: *Hebeloma crustuliniforme* and *Rhizopogon vinicolor*. Trans. Br. Mycol. Soc. **88,** 563–565.

Li, C. Y., Massicotte, H. B., Moore, L. V. H. (1992): Nitrogen-fixing *Bacillus* sp. associated with Douglas-fir tuberculate ectomycorrhizae. Plant Soil **140,** 35–40.

O'Dell, T. E., Trappe, J. M. (1992): Root endophytes of lupine and some other legumes in northwestern USA. New Phytol. **122,** 479–485.

Reinhold-Hurek, B., Hurek, T. (1998): Interactions of graminaceous plants with *Azoarcus* spp., and other diazotrophs: identification, localization, and perspectives to study their function. Crit. Rev. Plant Sci. **17,** 29–54.

Rennie, E. M. (1981): A single medium for the isolation of acetylene-reducing (nitrogen fixing) bacteria in soils. Can. J. Microbiol. **27,** 8–14.

Schippers, B., Bakker, A. W., Bakker, P. A. H. M. (1987): Interactions of deleterious and beneficial rhizosphere microorganisms and the effects of cropping practices. Annu. Rev. Phytopathol. **25,** 339–358.

Schroth, M. N., Becker, J. O. (1990): Concepts of ecological and physiological activities of rhizobacteria related to biological control and plant growth promotion. In: Biological control of soil-borne plant pathogens (Ed.: D. Hornby). CAB International, Wallingford, United Kingdom, pp. 389–414.

Shishido, M., Loeb, M. B., Chanway, C. P. (1995): External and internal root colonization of lodgepole pine seedlings by two growth-promoting *Bacillus* strains originated from different root microsites. Can. J. Microbiol. **41,** 707–713.

Shishido, M., Petersen, D. J., Massicotte, H. B., Chanway, C. P. (1996): Pine and spruce seedling growth and mycorrhizal infection after inoculation with plant growth promoting *Pseudomonas* strains. FEMS Microbiol. Ecol. **21,** 109–119.

Shishido, M., Breuil, C., Chanway C. P. (1999): Endotrophic colonization of spruce by plant-promoting rhizobacteria. FEMS Microbiol. Ecol. **29,** 191–196.

Sidman, R. L., Mottla, P. A., Feder, N. (1961): Improved polyester wax embedding for histology. Stain Technol. **36,** 279–284.

Smith, S. E., Read, D. J. (1997): Mycorrhizal symbiosis. 2nd ed., Academic Press, Cambridge, p. 605.

Wilson, D. (1995): Endophyte – the evolution of a term, and clarification of its use and definition. Oikos **73,** 274–276.