Plant Pathol. J. 24(3) : 352-356 (2008)

The Plant Pathology Journal

Differential Structural Responses of Ginseng Root Tissues to Different Initial Inoculum Levels of *Paenibacillus polymyxa* GBR-1

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(Received on May 23, 2008; Accepted on August 14, 2008)

Root discs of 4-year-old ginseng, *Panax ginseng* C. A. Meyer, were inoculated with the higher (10^8 colony-forming units (CFU)/ml) and lower (10^6 or 10^5 CFU/ml) initial inoculum levels of a plant-growth promoting rhizobacterium (PGPR), *Paenibacillus polymyxa* GBR-1 to examine rot symptom development and bacterial population changes on the root discs. At the higher inoculum level, brown rot symptoms developed and expanded on the whole root discs in which the bacterial population increased continuously up to 4 days after inoculation. In light and electron microscopy, ginseng root cells on the inoculation sites were extensively decayed, which were characterized by dissolved cell walls and destructed cytoplasmic contents. However, no rot symptoms were developed and the bacterial population increased only during the initial two days of inoculation at the lower inoculum level (10^6 CFU/ml) of *P. polymyxa* GBR-1. At the lower inoculum level (10^5 CFU/ml), boundary layers with parallel periclinal cell divisions, structurally similar to wound periderm, were formed internal to the inoculation sites, beneath which the cells were intact containing numerous normal-looking starch granules and no disorganized cell organelles, suggesting that these structural features may be related to the suppression of symptom development, a histological defense mechanism.

Keywords : ginseng root, inoculum level, *Paenibacillus polymyxa*, structural changes, symptom development, wound periderm.

Ginseng (*Panax ginseng* C. A. Meyer), a deciduous perennial plant belonging to Araliaceae family, is cultivated under shade for 4-6 years to produce marketable roots. In ginseng cultivation, the roots are continuously exposed to and influenced by biotic and abiotic agents of soil. Fungal pathogens predominantly affect adversely the root growth, among which *Cylindrocarpon destructans* is the most responsible for ginseng root rot and is also known as one of the main causes of replant problem (Chung, 1975; Lee, 2004; Park, 2001; Yu, 1987; Yu and Ohh, 1993). Little has been known about the occurrences of bacterial root diseases in field conditions even though *Erwinia* and *Pseudomonas* spp. are listed as causal agents of root rots (Yu and Ohh, 1993), and *Agrobacterium* spp. as crown gall (Jeon et al., 2008).

There may be a variety of non-pathogenic microorganisms in the rhizosphere of ginseng root, having neutral or beneficial relations with the ginseng plant. In our previous study (Jeon et al., 2003), out of 345 bacterial isolates obtained from the decaying ginseng roots, 81 isolates were bacillus-like bacteria, among which 20 were identified as *Paenibacillus polymyxa*, a plant growth-promoting rhizobacterium (PGPR). This bacterium has a good potential as a biocontrol agent especially for plant-parasitic nematodes (Son et al., 2007; Khan et al., 2008), and also is antagonistic to various pathogens including *Fusarium oxysporum* f. sp. *lycopersici* and *Phytophthora capsici* (unpublished data). In other studies, *P. polymyxa* has been used as a potential biocontrol agent for plant diseases (Dijksterhuis et al., 1999; Helbig, 2001; Li et al., 2007; Mavingui and Heulin, 1994; Shishido et al., 1996). However, there are reports that this bacterium causes a rot of germinating seeds and seedling blight of tomato (Caruso et al., 1984).

In the previous study we found that ginseng roots were rotten at the higher initial inoculum levels of *P. polymyxa*, but not at the lower inoculum concentrations (Jeon et al., 2003). For efficient use of this bacterium as a potential biocontrol agent in the control of ginseng root diseases, it should be addressed what the pathological responses of ginseng root tissues are in relation to different inoculum levels. Therefore, this study examined population changes on and histological changes of ginseng root tissues inoculated with different inoculum levels of *P. polymyxa*.

Materials and Methods

Bacterium and inoculum preparation. *Paenibacillus polymyxa* GBR-1, which was isolated from a rotten ginseng root (Jeon et al., 2003), was grown in brain heart infusion

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(BHI) agar at 28°C for 2 days. The bacterial colonies were suspended in sterilized distilled water to make a standard concentration of 10^8 colony forming units (CFU)/ml adjusting an optical density of 0.8 at 590 nm by a Ultraspec 4000 Spectrophotometer (Pharmacia Biotech Ltd., UK). Lower bacterial inoculums were made from diluting the standard bacterial suspension 100× and 1000× for concentrations of 10^6 CFU/ml and 10^5 CFU/ml, respectively.

Population changes of *P. polymyxa* GBR-1 on ginseng root discs. The standard (10^8 CFU/ml) and 100 x diluted (10^6 CFU/ml) bacterial suspensions were inoculated on root discs of 4-year-old ginseng roots (20 μl on each root disc of about 2.2 cm (diameter)×0.8 cm (thickness)) which were purchased from a commercial market. Inoculated root discs were placed in Petri dishes with sufficient moisture supplied by the inclusion of a water-soaked cotton swab, and were incubated at 23-25°C in an incubation chamber. Symptom development and bacterial population changes on ginseng root discs were examined daily up to 6 days after inoculation. For examining the bacterial populations, whole root discs were ground in 5 ml of 0.01 M phosphate buffer solution (pH 7.0), diluted serially, plated on BHI agar, and incubated at 28°C. After 2-3 days, colonies formed on the plates were counted and converted to log CFU per root disc. Each treatment was replicated three times.

Histological responses of ginseng root tissues to *P. polymyxa* inoculation. As the above experiment, the bacterial suspensions (the same for the higher inoculum level, 10^5 CFU/ml for the lower inoculum level instead of 10^6 CFU/ml to exclude possible intermediary responses between high and low inoculum levels) were inoculated in the pith region at the center of 4-year-old ginseng root discs (ca. 2.2 cm (diameter)×0.8 cm (thickness)), and incubated at 23-25°C. At 5 days after inoculation, root tissue segments from infection sites were cut and fixed in Karnovsky's fixative in cacodylate buffer (pH 7.0) for 4 h (Karnovsky, 1965). The segments were rinsed with the same buffer three times for 20 min each, and post-fixed in 1% osmium tetroxide for 2 h. The samples were washed briefly in distilled water, *en bloc* stained in 0.5% uranyl acetate at 4°C overnight, and then dehydrated in an ethanol series and embedded in Spurr's epoxy resin (Spurr, 1969). For light microscopy, the embedded specimens were sectioned 1 μm in thickness with a glass knife on an MT-X ultramicrotome (RMC, Inc., Tucson, AZ), and observed under a compound light microscope (Axiophot, Zeiss, Germany) after staining with 0.1% toluidine blue O. For electron microscopy, the embedded specimens were sectioned (80-90 nm thickness) with a diamond knife on the ultramicrotome. Sections were stained with 2% uranyl acetate and lead citrate for 7 min each and examined under a JEM-1010 electron microscope (JEOL, Ltd., Japan) at 80 kV.

Results

Population changes of *P. polymyxa* GBR-1 on ginseng root discs. *P. polymyxa* GBR-1 induced rot symptoms on the ginseng root discs inoculated at the higher inoculum level of 10^8 CFU/ml, but no symptoms were developed at the lower level of 10^6 CFU/ml (Fig. 1). At the higher inoculum level, brown rot symptoms began to appear on the ginseng root discs from one day after inoculation, which rotted completely at 3 days after inoculation. At the lower inoculum, the bacterial population increased about 100-fold or 10-fold during the first two days after inoculation and then remained or decreased for the remaining days on ginseng root discs (Fig. 2). On the other hand, the bacterial

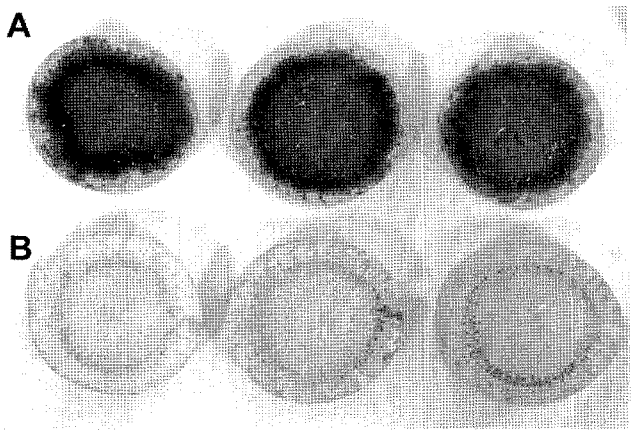

Fig. 1. Symptoms developed on 4-year-old ginseng root discs 3 days after inoculation with the higher (10^8 CFU/ml) (A) and lower (10^6 CFU/ml) (B) inoculum levels of *Paenibacillus polymyxa* GBR-1, showing that ginseng root discs are completely rotted at the higher inoculum level, but no rot symptoms were developed at the lower inoculum level.

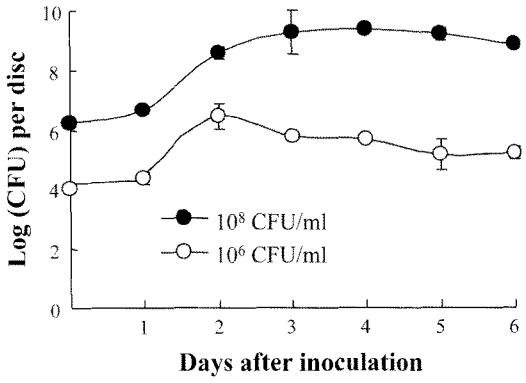

Fig. 2. Population changes of *Paenibacillus polymyxa* GBR-1 on 4-year-old ginseng root discs inoculated with the higher (10^8 CFU/ml) and lower (10^6 CFU/ml) bacterial inoculum.

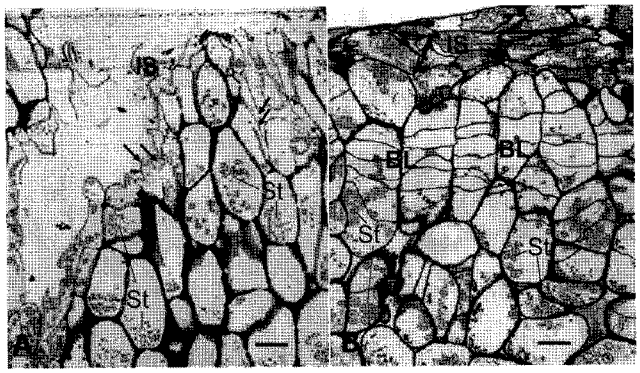

Fig. 3. Light micrographs of 4-year-old ginseng root tissues 5 days after inoculation with the higher (10^8 CFU/ml) (A) and lower (10^5 CFU/ml) (B) inoculum of *Paenibacillus polymyxa* GBR-1, showing dissolved cell walls (arrows) and degraded starch granules (St) interior to the inoculation site (IS) in (A) and formation of boundary layers (BL) and intact starch granules in healthy-looking cells beneath the inoculation site (IS) in (B). Bars =50 μm (A, B).

population increased continuously up to 4 days after inoculation and remained constant for the rest of the days at the higher inoculum level.

Histological responses of ginseng root tissues to *P. polymyxa* inoculation. Light microscopy of ginseng root tissues showed extensively decayed cells characterized by dissolved cell walls and decomposed cell organelles such as starch granules at the higher inoculum level of *P. polymyxa* GBR-1 (Fig. 3A). At the lower inoculum level, however, boundary layers with periclinal cell divisions, indicated by parallel-formed cell walls, were formed internal to the inoculation sites, beneath which the cells were intact containing numerous normal-looking starch granules (Fig. 3B).

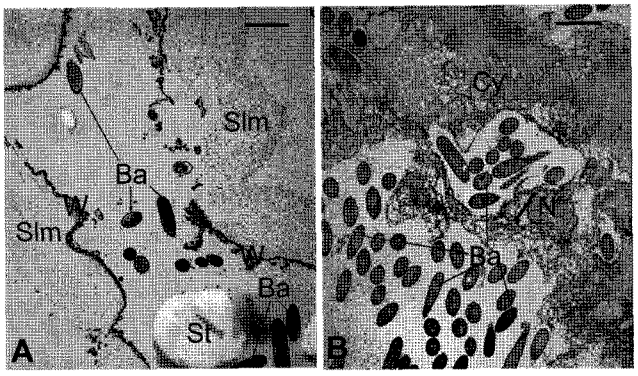

Fig. 4. Electron micrographs of 4-year-old ginseng root parenchyma tissues inoculated with the higher inoculum (10^8 CFU/ml) of *Paenibacillus polymyxa* GBR-1, showing disrupted cell walls (W), disorganized cytoplasm (Cy) and numerous inter and intracellular bacterial cells (Ba). Note slime-like material (Slm) probably from dissolution of cell wall (W). St=starch granule, N=nucleus. Bars=2 μm (A, B).

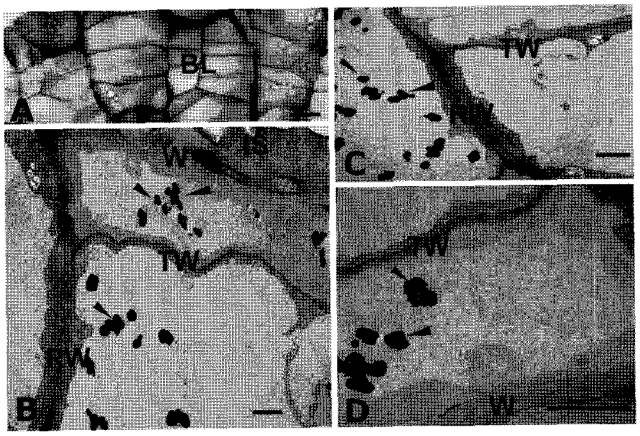

Fig. 5. Light micrograph (A) and electron micrographs (B-D) of root parenchyma tissues of 4-year-old ginseng 5 days after inoculation with 10^5 CFU/ml of *Paenibacillus polymyxa* GBR-1, showing formation of the boundary layer (BL) by periclinal cell divisions indicated by newly formed tangential cell walls (TW) beneath the inoculation site (IS). Note thickened radial cell wall (RW) and electron dense materials (arrowhead) in central vacuoles (V). W=cell wall. Bars=25 μm (A) and 3 μm (B-D).

In electron microscopy of ginseng root tissues inoculated with the higher inoculum level of *P. polymyxa* GBR-1, disorganized cytoplasm, dissolved cell walls and destructed cell organelles such as nucleus were found (Fig. 4). Numerous bacterial cells colonized inter- and intracellular spaces of the disrupted cells containing broken and indistinct structures and slime-like materials probably derived from cell wall degraded by the bacterial cells. No such cellular destructions as in the higher inoculum infection were noted in ginseng root tissues inoculated with the lower inoculum level of *P. polymyxa* GBR-1 (Fig. 5). Tangential cell walls, an indication of periclinal cell divisions for boundary layer formation, were found beneath the inoculation sites. In these cells, neither cell wall dissolution nor cytoplasmic disruption as in the higher inoculum level was found, but electron dense materials, probably phenolic materials, were deposited in central vacuoles. Unlike the higher inoculum level, no bacterial cells were found interior to the inoculation sites (interior to the boundary layers) with the lower inoculum level.

Discussion

Ginseng root discs were rotten extensively by the higher inoculum (10^8 CFU/ml) of *P. polymyxa* GBR-1, but not by the lower inoculum (10^6 CFU/ml). Accordingly, the bacterial population in the higher inoculum increased more greatly than that in the lower inoculum. The bacterial population in the lower inoculum increased for the first two days as high as the initial bacterial population of the higher inoculum on

ginseng root tissues, probably in the intercellular spaces of root cut surface tissues; however, the bacterial population somewhat decreased thereafter, indicating that defense mechanisms may be activated in the inoculation sites. This suggests that rot symptom development in the inoculation site may be governed by the initial population of *P. polymyxa* GBR-1 but not by the intermediate or final populations. Natural bacterial populations rarely exceed the lower inoculum level of 10^6 CFU/ml under storage conditions (Mavingui and Heulin, 1994) or in ginseng fields (Jung et al., 2002). Therefore, root rot symptoms of ginseng may hardly be produced in natural conditions by *P. polymyxa* GBR-1, even though its population may increase to antagonize against organisms other than the host plant cells.

In our study, boundary layer formation was observed internal to the inoculation sites 5 days after inoculation by the lower inoculum of *P. polymyxa* GBR-1. The layer was formed by periclinal cell divisions adjacent to the inoculation (wounding) sites, consequently separating the healthy tissue underneath from the infected or wound tissue above. This boundary layer is structurally similar to wound periderm of the sweet potato (*Ipomoea batatas*) (Morris and Mann, 1955). Similar structural changes are also found in resistant chili fruit at the later stage of infection after *Colletotrichum gloeosporioides* inoculation, which are also suggested to be wound periderm formed as a resistant response (Kim et al., 2004). Thus, the boundary layer formed at the *P. polymyxa* GBR-1 inoculation site in ginseng root tissues is wound periderm.

Wound periderms are detectable in the wound healing processes in dicotyledons and certain monocotyledons (Esau, 1977), and formed in response to wounding and parasite invasion, which function as histological defense structures (Agrios, 2005; Biggs and Britton, 1988; Kim et al., 2004; Mullick, 1977, Rittinger et al., 1987). Resistance of white-pine trees to the blister rust is related to the wound periderm formation (Struckmeyer and Riker, 1951). Combined inoculations of the perennial peach canker pathogen *Leucostoma cincta* and epiphytes result in wound healing similar to the epiphyte alone, suggesting the inhibition of canker development and the potential use of the epiphytes as biocontrol agents against *L. cincta* (Biggs and Alm, 1992). In our study, the wound healing might have been somewhat accelerated or not significantly influenced by the inoculation of the lower concentration of *P. polymyxa* GBR-1, because by wounding alone, definite wound periderms were observed in root tissues at the time similar to or somewhat later than (after 6 days of wounding) by the bacterial inoculation (unpublished data). Therefore, the antagonistic potential of *P. polymyxa* GBR-1 to invading pathogens may not be attenuated but rather strengthened by the plant responses for inhibiting disease development on the wounding site, an important infection court for soilborne plant pathogens. Considering all of these aspects, *P. polymyxa* GBR-1 may be a potent biocontrol agent for ginseng root rots.

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