Interactions between rhizoplane bacteria and a phytopathogenic Peronosporomycete *Aphanomyces cochlioides* in relation to the suppression of damping-off disease in sugar beet and spinach

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Abstract: We investigated the modes of root colonization and antibiosis of *Lysobacter* sp. strain SB-K88 and other rhizoplane bacteria of spinach and sugar beet antagonizing a Peronosporomycete pathogen, *Aphanomyces cochlioides.* The SB-K88 has huge long brush-like fimbriae at one pole of the sessile bacterial rod. Scanning electron microscope (SEM) analysis of two weeks old seedlings of sugar beet and spinach upon inoculation of seeds revealed that SB-K88 densely colonized to the root and cotyledon surfaces of plants in a perpendicular fashion using polar fimbriae and developed biofilm-like structures on roots covered by root mucigel. Seed treated with either SB-K88 or its culture fluids significantly suppressed damping-off disease in both sugar beet and spinach caused by *A. cochlioides*. In dual culture assay, SB-K88 and other rhizoplane bacteria caused excessive branching, swelling and loss of radial growth in the approaching hyphae of *A. cochlioides*. TEM also visualized remarkable ultrastructural alterations in the affected hyphae. Interestingly, zoospores of *A. cochlioides,* were rendered immotile within 1 min of exposure to cell suspension or cell free culture supernatant or EtOAc extracts or pure xanthobaccin A (**1**) isolated from SB-K88, and subsequent lysis occurred within 30 min. Our observations provide the convincing evidence that *Lysobacter* sp. exerts a direct inhibitory effect on *A. cochlioides* and suppresses damping-off disease in sugar beet and spinach through a combination of antibiosis and high root colonization.

Key words: *Lysobacter* sp., root colonization, zoospore lysis, *Aphanomyces cochlioides,* sugar beet

Introduction

The members of Peronosporomycetes (Oomycetes in the old classification) genera such as *Aphanomyces, Phytophthora,* and *Pythium* are the devastating pathogens of many economically important crops. Root rot and damping-off caused by *Aphanomyces cochlioides, Pythium ultimum* and *Rhizoctonia solani* are the serious diseases of sugar beet all over the world. *A. cochlioides* also infects spinach, feather cockscomb and some other members of Chenopodiaceae and Amaranthaceae. The biflagellated zoospores of *A. cochlioides* liberated from the mycelium locate host roots guided by a host-specific flavonoid signal, cochliophilin A (5-hydroxy-6,7-methylenedioxyflavone) released from the roots, after which they undergo a series of morphological changes before penetrating the host tissues (Islam et al., 2003).

Disruption of any of these homing events eliminates the potential for pathogenesis. Homma et al. (1993) isolated a number of antagonistic bacterial strains against *Pythium* sp. from the rhizoplane of sugar beet where one of the isolates, *Lysobacter* sp. strain SB-K88 (tentatively identified as *Xanthomonas* or *Stenotrophomonas* sp.) has shown promise as a control agent of root rot and damping-off diseases in sugar beet through production of a major metabolite, xanthobaccin A (**1**) (Nakayama et al., 1999). However, the effect of xanthobaccin A against zoospores (infecting agents) of *A. cochlioides*, and the ability of colonization of SB-K88 on the host roots have not yet been investigated. Therefore, the objectives of the present work were to i) investigate the modes of attachment and patterns of bacterial colonization to host roots upon inoculation to the seed; ii) test the effects of live bacteria and their secondary metabolites on the approaching hyphae, and survival and homing events of zoospores; iii) examine the host range of SB-K88 with respect to root colonization; and iv) search other potential rhizoplane biocontrol bacteria against *A. cochlioides*.

Materials and methods

Culture condition and extraction

The culture fluid of *Lysobacter* sp. strain SB-K88 cultivated in liquid medium at 25 °C for 15 days with shaking was centrifuged and then the supernatant was freeze-dried. The residue was subsequently extracted with EtOAc and MeOH and then concentrated *in vacuo*.

Zoospore production and bioassay

A. cochlioides (AC-5) was cultured on corn meal agar on a glass Petri dish (9 cm i.d.), and the zoospores were produced as reported before (Islam et al., 2003). Zoospore bioassay of live bacteria, freeze-dried materials, EtOAc extracts, water solubles, and pure xanthobaccin A (**1**) (Nakayama et al., 1999) were done by homogeneous solution method (Islam et al., 2002).

Seed pelleting, zoospore inoculation and disease intensity

Sterilized seeds (Daigaku-Noen, Tokyo) of sugar beet and spinach were pelleted with SB-K88 (*ca.* 10^7 CFU/seed) (Nakayama *et. al*, 1999), and grown in 0.2% gellan gum containing 1/5 Hogland's S medium in test tubes or in sterilized soils in 36-cells small plastic pack. On day 12, each seedling was inoculated with 1 ml of an *A. cochlioides* zoospore suspension containing 1×10^4, 1×10^3 or 1×10^2 zoospores. Data for frequency of healthy seedlings (%) were recorded after 2 weeks of zoospore inoculation. Each treatment was replicated thrice.

SEM and TEM observations

Colonization of bacteria to the roots grown in gellan gum or soils, and zoospore lytic activity of xanthobaccin A (**1**) were observed by scanning electron microscopy (SEM) on day 14 of cultivation as described previously (Islam et al., 2003). Ultrastructural changes of approaching hyphae of AC-5 toward bacterial colony of SB-K88 in the PDA medium were observed by transmission electron microscopy (TEM).

Results and Discussion

Characteristic attachment and colonization of* Lysobacter *sp. SB-K88

TEM observation revealed that *Lysobacter* sp. strain SB-K88 has huge long (~6 μm) brush-like fragile fimbriae on one pole of the sessile bacterial rod (~1 μm) (Fig. 1a). Our SEM observation visualized that upon seed inoculation, SB-K88 highly colonized and attached perpendicularly on the roots of both hosts (spinach and sugar beet) and nonhosts (tomato, *Amaranthus gangeticus* and *Arabidopsis thaliana*) of *A. cochlioides* when plant seedlings were grown in the gnotobiotic systems containing gellan gum or soil media (Fig. 1b-g). In the case of host roots, i) SB-K88 densely colonized to the root and cotyledon surfaces in a perpendicular fashion using polar fimbriae (Fig. 1b,d); ii) a stable biofilm-like structure covered by root mucigel (Fig. 1c); and iii) micro-colonies localized mainly at the junction between primary and secondary roots with bacterial numbers declining from the top to down the root. However, no root mucigel covered stable biofilm was observed in the case of nonhost plants. The distinct features of *Lysobacter* sp. colonization to plants were the perpendicular attachment and the development of a stable biofilm on the root. Similar phenomenon was observed in opportunistic pathogen *Pseudomonas aeruginosa* strain PA14 to the surface of human respiratory epithelial cells and also to plants (Plotnikova et al., 2000). Effective colonization of both foliar and subterranean plant parts has also been observed in *L. enzymogenes* (Sullivan *et al.,* 2003).

Motility inhibition and lysis of zoospores

In addition to mycelial growth inhibition, the zoospores of *A. cochlioides,* were rendered immotile (100%) within 1 min of exposure to cell suspension (10^9 bacteria/ml) or cell culture supernatant (100 μg/ml) of the SB-K88, and subsequent lysis (~40%) occurred within 30 min. Crude EtOAc extract (MIC 10 μg/ml) of the culture supernatant or pure xanthobaccin A (**1**) (MIC 0.01 μg/ml) also caused identical motility inhibition (100%) followed by lysis of zoospores in a dose dependent manner (Fig. 1k). Chemical fractionation of crude extracts revealed that xanthobaccin A (**1**) (Fig. 1) produced by the SB-K88 was primarily responsible for mycelial growth inhibition and lysis of zoospores. Production of zoospores from the *A. cochlioides* mycelia was remarkably reduced (~50%) in the presence of SB-K88 at a dose of 10^7 cells/ml. Lytic activity against other microorganisms is a generic characteristic of *Lysobacter* spp. (Christensen and Cook, 1978). However, motility inhibition followed by lysis of zoospores shown in this report has not been claimed so far.

Morphology and ultrastructural alterations in approaching hyphae

Excessive branching, irregular swelling and loss of apical growth in the *A. cochlioides* hyphae approached to the bacterial colony (dual culture in PDA) were observed by light microscopy (Fig. 1l). Remarkable ultrastructural alterations including considerable thickening of hyphal

cell walls, extensive vacuolization, accumulations of lipid bodies and degeneration of the hyphal cytoplasm were observed in the approaching hyphae by TEM (Fig. 1n).

Disease suppression activity

Seeds treated with either SB-K88 (10^8 cells/seed) or its cell free culture supernatant (100 μg/g soil) significantly suppressed damping-off disease in both sugar beet (65% healthy plants) (Fig. 1p) and spinach (85% healthy plants) (Fig. 1o) when seedlings were artificially infested with the zoospores (10^3 zoospores/seedling) of *A. cochlioides*. Our observations provide the convincing evidence that *Lysobacter* sp. exerts a direct inhibitory effect on *A. cochlioides* and suppresses damping-off disease in sugar beet and spinach through a combination of antibiosis and high root colonization.

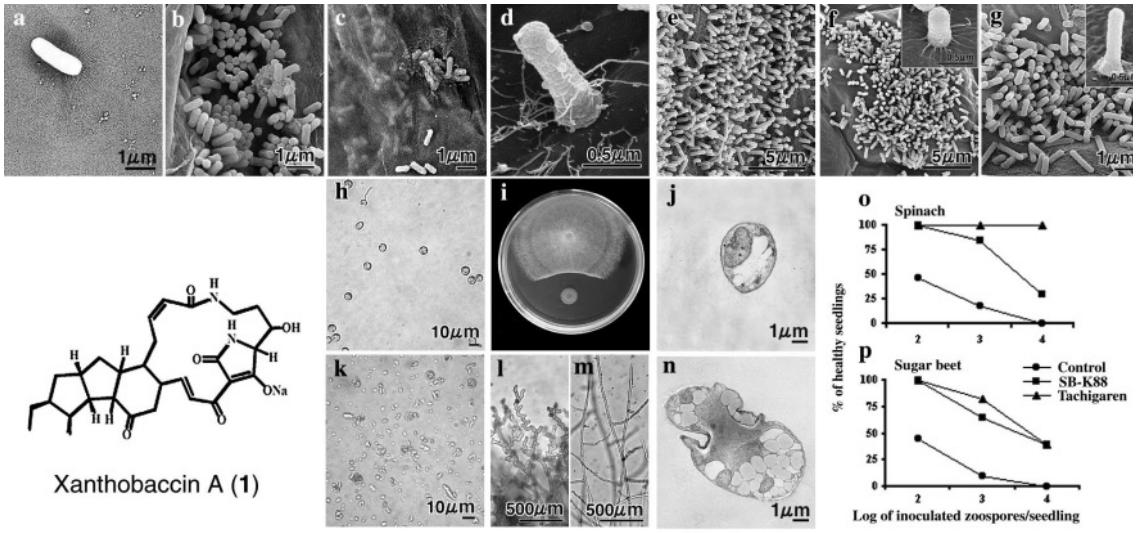

Figure 1. Colonization of *Lysobacter* sp. (SB-K88) to plant surfaces, and antagonistic activities toward *Aphanomyces cochlioides*.

a. huge fimbriae at one end of bacterium; **b**. & **d**. colonization by perpendicular attachment to sugar beet root (**b**) and cotyledon (**d**); **c**. bacterial biofilm covered by root mucigel of sugar beet; e. colonization of the tomato root; **f**. & **g**. colonization by perpendicular attachment (insets) to *Arabidopsis thaliana* leaf (**f**) and root (**g**); **h**. & **k**. normal cystospores (**h**) (control) and lysed zoospores (**k**) by xanthobaccin A (**1**) at 1 ppm; **i**. hyphal growth inhibition by SB-K88 (4 days); **j**. & **n**. ultrastructure of control (**j**) and affected (**n**) hyphae; **l**. & **m**. control (**m**) and affected hyphae (**l**) approaching towards SB-K88; **o**. & **p**. damping-off disease suppression in spinach (**o**) and sugar beet (**p**). Tachigaren: a commercial fungicide.

Other antagonistic rhizoplane bacteria

We screened 150 rhizoplane bacterial isolates from the host and nonhost plants, and evaluated the antagonistic activity towards the growth of *A. cochlioides*. Some of the strains (~5%) of *Pseudomonas* spp., *Stenotrophomonas* spp., *Klebsiella* spp., *Delftia* sp. and *Bacillus* sp., exhibited characteristic hyphal morphological alterations: excessive branching, spiral

growth, longer and pointed tips formation, and cytoplasmic disintegration on PDA medium, all of which was highly correlated with *in vivo* disease suppression activity of the respective bacteria in sugar beet and spinach. These results suggest that hyphal morphological alterations could be considered as a novel criterion for screening potent biocontrol rhizobacteria.

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