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Microbial community responses associated with the development of *Fusarium oxysporum* f. sp. *cucumerinum* after 24-epibrassinolide applications to shoots and roots in cucumber

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Abstract Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *cucumerinum* (FO), is one of the major diseases in cucumber (*Cucumis sativus*) production. Root and foliar applications of 24-epibrassinolide (EBL), an immobile phytohormone with antistress activity, were evaluated for their effects on the incidence of Fusarium wilt and changes in the microbial population and community in roots of cucumber plants. EBL pre-treatment to either roots or shoots significantly reduced disease severity followed by an improved plant growth regardless of the treatment methods applied. EBL applications decreased the *Fusarium* population on root surfaces and in nutrient solution, but increased the population of fungi and *actinobacteria* on root surfaces. PCR-DGGE analysis showed that FO-inoculation had significant effects on the bacterial community on root surfaces as expressed by a decreased diversity index and evenness index, but EBL applications alleviated these changes. Moreover, several kinds of decomposing bacteria and growth-promoting bacteria were identified from root surfaces of FO-inoculated plants and EBL-pre-treated plants, respectively. Overall, these results show that the microbial community on root surfaces was affected by a complex interaction between phytohormone-induced resistance and plant pathogens.

Keywords *Fusarium oxysporum* · PCR-DGGE · Phytohormone · Resistance

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Introduction

Brassinosteroids (BRs) are recognised as a novel group of phytohormones to regulate a broad range of responses in plants such as seed germination, stem elongation, cell division and expansion, xylem differentiation, plant growth and apical dominance (Clouse and Sasse 1998; Sasse 2003). Besides these, BRs have an ameliorative role in plants under biotic stress, such as heat, cold, drought and salt (Khripach et al. 2000; Kagale et al. 2007). Evidence suggests that BRs also play an important role in pathogen defence. BR-induced disease resistance was noted in several plants including barley, potato and cucumber plants under field conditions (Khripach et al. 2000). Recently, a series of experiments confirmed that BRs could induce disease resistance in tobacco and rice against a broad range of pathogens (Nakashita et al. 2003).

Plants have a natural array of defence mechanisms to protect themselves from pathogenic organisms. A vast array of natural products from plant roots plays a critical role in plant resistance to diseases (Richard 2001). Survival of the delicate and physically unprotected root cells under continual attack by pathogenic microorganisms depends on a continuous secretion of phytoalexins, defence proteins and other as yet unknown chemicals (Flores et al. 1999). *Arabidopsis*, *Lithospermum erythrorhizon* and *Medicago truncatula* are, collectively, rich sources of antimicrobial products such as rosmarinic acid, indole, terpenoid and flavonoid/isoflavonoid (Brigham et al. 1999; Bais et al. 2002; Richard 2001).

Root–microorganism interactions are involved in disease suppression both in soil systems and soilless systems. Many plant growth-promoting bacteria or pathogen-suppressive microorganisms have been isolated from soils and identified in term of their roles in disease suppression (Landa et al. 2002). Recently, Postma et al. (2005) found that disease suppression was highly correlated with the cultural number of filamentous *actinobacteria* and the bacterial composition. It is unclear, however, whether phytohormone-induced resistance to root diseases is associated with the rhizospheric microbial community. There is a need to further investigate the behaviour of pathogens and associated changes in the microbial community.

Cucumber (*Cucumis sativus*) is one of the major greenhouse vegetables in the world, and is vulnerable to Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *cucumerinum* (FO; Ahn et al. 1997; Ye et al. 2004). The aim of the present study was to evaluate the effects of BR application to roots or shoots on the development of Fusarium wilt and changes in the microbial community on roots of cucumber plants. Subsequently, we determined to what extent these changes could be associated with resistance.

Materials and methods

Greenhouse experiments

The cucumber cv. Jinyan No. 4 was used because of its known susceptibility to Fusarium wilt (Ye et al. 2004). Seeds were sterilised in 2.5% NaClO and germinated in perlite. After emergence, batches of eight seedlings were grown hydroponically in a plastic tank (13 l, not sterilised) filled with 10 l half-strength Enshi nutrient solution (Yu and Matsui 1997). Plants were incubated in a greenhouse maintained at 32°C/22°C (day/night) with a relative humidity of 85% and a photoperiod of 16 h with a photosynthetic flux of 1,000 μmol m^{-2} s^{-1}.

When the cucumber seedlings were at the two-leaf stage (8 days after emergence), 24-epibrassinolide (EBL) was applied to shoots (sEBL) or roots (rEBL) 2 days before FO inoculation. There were four treatments: Control, FO, sEBL + FO and rEBL + FO. EBL was dissolved in ethanol and was applied either to shoots by spraying on leaves (0.2 μM, 10 ml per plant), or to roots by adding to the nutrient solution (0.1 μM), respectively. Our preliminary experiment showed that EBL at 0.2 μM had highest physiological activity for spraying while EBL concentration >0.1 μM in a nutrient solution induced phytotoxicity (Yu et al. 2004). The final concentrations of ethanol in nutrient solution and spraying solution including the control was 0.1% (*v*/*v*), at which concentration ethanol has a negligible effect on cucumber plants (Yu and Matsui 1997). Meanwhile, the same concentration of ethanol was applied to roots or shoots that had not been subjected to EBL treatments. After 2 days of EBL treatments, all EBL pre-treated and half of EBL-untreated plants were inoculated with FO by adding a conidial suspension into the nutrient solution. FO suspension was prepared by culturing the *Fusarium* pathogen in a potato sucrose liquid medium at 28°C for 6 days (Yu and Komada 1999) and added to the nutrient solution at 10^4 conidia ml^{-1}. The day for FO inoculation was designated 0 days. Each treatment had 16 plants and was done in triplicate. The experiment was terminated at 12 days after inoculation when control plants had approximately ten leaves and FO plants showed wilting symptoms with dead plants or yellowing leaves.

Measurement of Fusarium wilt and the *Fusarium* population

At the end of the experiment, the percentage of leaf yellowing or wilting plants was measured. Each plant was harvested for the measurement of the root rot and vascular bundle browning on a scale of 0–4 as follows: 0, healthy with no browning; 1, white root with slight browning; 2, light root rot and browning;

3, mild root rot and browning; 4, severe root rot and browning (Ye et al. 2004). Dry weights per plot were then determined after drying the plants at 80°C for 3 days.

Root microflora was collected using a method similar to that described by Khalil and Alsanius (2001). In general, at 0, 4, 8, 12 days after FO-inoculation, 5 g of fresh roots were added to 20 ml sterile distilled water (SDW) and shaken at 60 rpm for 3 h. The suspension containing the microbial community on root surfaces was collected, and diluted with the nutrient solution using sterilised water. The diluted suspension was plated onto a selective Komada medium for the counting of the pathogenic *Fusarium* population (Komada 1975; Yu and Komada 1999). After 6 days at 28°C, the number of colony-forming units (CFU) appearing on plates was counted and the number of viable cells on root surfaces and in nutrient solution was estimated (Yu and Komada 1999). CFU on root surfaces and in nutrient solution were calculated gram^{-1} roots and ml^{-1}, respectively.

Microorganism population analysis

The suspension prepared from roots was also used to enumerate different microbial populations on the roots. Diluted suspension was plated onto Beef extract-peptone agar medium, Martin agar medium and GAOSHI I agar medium for the enumeration of bacterial, fungal and *actinobacterial* populations, respectively (Wollum 1982). After 2 days at 37°C for bacteria, 4 days at 28°C for fungi and 4 days at 28°C for *actinobacteria*, respectively, the number of CFU appearing on plates was counted and the number of viable cells estimated.

DNA extraction and PCR-DGGE analysis

DNA for the microorganisms on the root surfaces was extracted with 3S DNA isolation Kit V2.2 (Sangon, Shanghai, China). The suspension was prepared in the same way as that used to enumerate the microbial populations on the roots. The quality and quantity of extracted DNA were checked with 1% agarose gels.

PCR on DNA was performed by using a GeneAmp™ PCR System 9700 (Roche Molecular System, Inc., Alameda, CA, USA) with primers for bacterial groups. The primers used for PCR were 341fGC (5′-GC Clamp [CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G]-CCT ACG GGA GGC AGC AG-3′) and 534r (5′-ATT ACC GCG GCT GCT GG-3′; Muyzer et al. 1993). Each PCR reaction mixture consisted of 6 μl of 10×PCR buffer, 0.6 μl of 10 mM deoxynucleoside triphosphate mixture, 1 μl of each 10 μM primer, 0.6 μl of Taq DNA polymerase (Sangon, Shanghai, China), 1 μl of template DNA (about 5–15 ng), and SDW to supplement the reaction mixture to a final volume of 60 μl. PCR amplification was performed at 94°C for 5 min, followed by 35 thermal cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 30 s, and a final single extension at 72°C for 5 min. The size of the PCR product was visualised by electrophoresis in 1% agarose gels after ethidium bromide (EB) staining. Strong bands of approximately 230 bp were subjected to DGGE analysis.

DGGE was performed with Dcode™ Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Fifty microliters of PCR product were loaded onto 10% polyacrylamide gels in 1×TAE (9 mM Tris and 2 mM EDTA). Polyacrylamide gels with denaturant-gradient ranging from 30–60% (100% denaturant contains 7 M urea and 40% deionised formamide) were run at 180 V and 60°C for 270 min. After electrophoresis, gels were stained with EB for 20 min and photographed under UV with Tanon equipment (Tanon Technology, Shanghai, China). Photographs were analysed with the Tanon GIS gel photograph management system (Tanon Technology, Shanghai, China). Functional diversity from DGGE data was evaluated. Shannon-Weaver diversity index (H) was calculated as follows: $H = -\sum (p_i)(\log_2 p_i)$, where p_i is the proportion of ith phylotype. Evenness index (E) was calculated from H/H_{max} where H_{max} is equal to ln(S), where S is the total number of phylotypes (Shannon and Weaver 1963). The Simpson's diversity index (D) was calculated using the formula $D = 1 - \sum (p_i)^2$, and the results are reported as the reciprocal (1/D; Simpson 1949).

Gel strip of a band was excised from the DGGE gel into a 1.5-ml tube and DNA was eluted in 50 μl TE (pH8.0) at 37°C for 2 h. The DNA fragments were amplified from the eluted solution by PCR. The primer pair without GC clamp (341f and 534r) was used in the template amplification by PCR for the subsequent cycle sequencing. Bands were all cloned into pGEM-T easy vectors (Promega, USA) and sequenced at Sangon Company (Shanghai, China).

Table 1 Effects of 24-epibrassinolide (EBL) applications on the biomass accumulation and incidence of fusarium wilt in cucumber plants with or without inoculation of *Fusarium oxysporum* f. sp. *cucumerinum* (FO)

Treatment	Root DW (g per plant)	Shoot DW (g per plant)	Total DW (g per plant)	Disease severity (% of plants wilted)	Browning index of vascular bundle
Control	0.65±0.03 a	5.81±0.26 a	5.46±0.35 a	0 c	0 c
FO	0.22±0.05 c	2.04±0.20 c	2.27±0.40 c	89.68±9.01 a	2.54±0.28 a
sEBL + FO	0.49±0.05 b	3.58±0.45 b	4.12±0.53 b	25.00±8.50 b	0.54±0.19 b
rEBL + FO	0.45±0.06 b	3.34±0.12 b	3.80±0.07 b	33.33±7.22 b	0.56±0.26 b

24-epibrassinolide was applied 2 days before FO inoculation. Samples were taken at the end of the experiment (12 days). The results were similar after two independent experiments and only the result for one experiment is shown here. Data are the means of three replications within an experiment with standard errors. Bars sharing the same letter are not significantly different across the treatments within the same plant parts as determined by Duncan's multiple range test (P=0.05)

DW dry weights, *Control* plants without FO inoculation, *FO* plants with FO inoculation, *sEBL + FO* plants receiving foliar spray of 24-epibrassinolide at 0.2 μM 24-epibrassinolide before FO inoculation, *rEBL + FO* plant roots were fed with 0.1 μM 24-epibrassinolide before FO inoculation

Sequences recovered from excised bands were submitted to the National Centre for Biotechnology Information (NCBI) for BLAST analysis. Bacterial classification was determined by a sequence match programme (Ribosomal Database Project II-Release 9 website).

Statistical analysis

Plants were arranged in three randomised blocks with three replicates per treatment. Data obtained for the incidence of Fusarium wilt, the *Fusarium* population, microbial population and bacterial diversity indices from DGGE analysis were subjected to ANOVA. Mean values of three replicates were compared by using Duncan's multiple range test (P<0.05).

Results

Plant growth

Plants grew vigorously in the absence of the *Fusarium* pathogen while FO inoculation resulted in severe plant wilting or even death of plants. FO inoculation significantly decreased the biomass accumulation of plants; this reduction in biomass, however, was greatly alleviated by the EBL treatments (Table 1). Compared with the FO treatment, root weights increased by 122.7% and 104.5% from EBL applications to shoots and roots, respectively. Similar trends were also observed in the changes of shoot dry weights. However, there was no significant difference in dry weights between the two EBL applications.

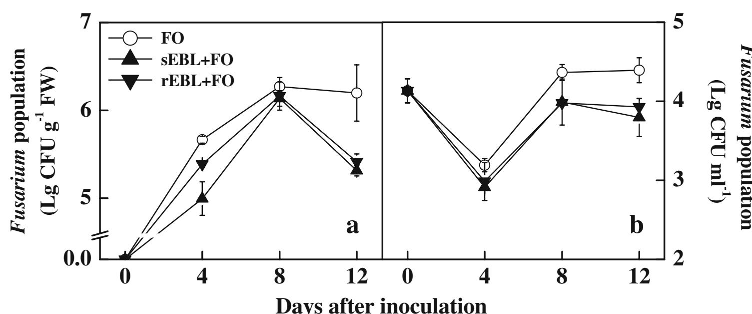

Fig. 1 Effects of 24-epibrassinolide (EBL) applications on the pathogenic *Fusarium* population on root surfaces (*a*) and in nutrient solution (*b*) caused by *Fusarium oxysporum* f. sp. *cucumerinum* (FO) in cucumber plants. *Control*, plants without FO inoculation; *FO*, plants with FO inoculation; *sEBL + FO*, plants receiving foliar spray of 24-epibrassinolide at 0.2 μM 24-epibrassinolide before FO inoculation; *rEBL + FO*, plant roots were fed with 0.1 μM 24-epibrassinolide before FO inoculation. 24-Epibrassinolide was applied 2 days before FO inoculation. Data are the means of three replications within an experiment with standard errors

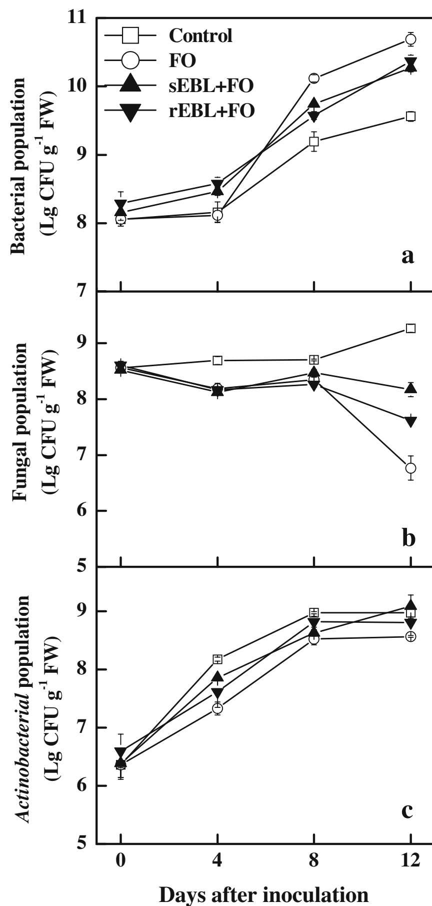

Fig. 2 Effects of 24-epibrassinolide (EBL) applications on bacterial (*a*), fungal (*b*) and *actinobacterial* (*c*) populations on root surfaces of cucumber plants with or without inoculation of *Fusarium oxysporum* f. sp. *cucumerinum* (FO). Data are the means of three replications within an experiment with standard errors. *Control*, without FO inoculation; *FO*, FO inoculation; *sEBL + FO*, foliar spray of 24-epibrassinolide at 0.2 μM 24-epibrassinolide before FO inoculation; *rEBL + FO*, roots fed with 0.1 μM 24-epibrassinolide before FO inoculation

Incidence of Fusarium wilt and changes in the *Fusarium* population

FO inoculation resulted in plant wilting; however, EBL pre-applications greatly decreased the percentage of wilted plants and vascular bundle browning index. At the end of the experiment, wilting percentage decreased by 59.06% and 47.79% while the vascular bundle browning index decreased by 51.51% and 45.20% from EBL applications to shoots and roots, respectively (Table 1). The incidence of Fusarium wilt could be alleviated by EBL pre-treatments either to shoots or roots.

After a gradual increase, the *Fusarium* population on root surfaces remained almost unchanged for the FO treatment (Fig. 1a). EBL pre-treatments resulted in a decrease in the *Fusarium* population on root surfaces. At the end of the experiment, the pathogenic *Fusarium* number on root surfaces of FO-inoculated plants was 16.76% and 14.40% higher than that of sEBL + FO and rEBL + FO plants. In the nutrient solution, the *Fusarium* population greatly decreased within 4 days after FO inoculation and then increased to a relatively stable value (Fig. 1b). By contrast, the EBL treatments always had a lower *Fusarium* population throughout the experiment. At the end of the experiment, compared with FO-inoculated plants, the pathogenic *Fusarium* population in the nutrient solution of sEBL + FO and rEBL + FO decreased by 11.36% and 10.11%, respectively. Similarly, there was no significant difference between the two EBL treatments.

Changes in the microorganism population

The bacterial population gradually increased in all treatments and the increase was most significant in FO, followed by rEBL + FO and sEBL + FO (Fig. 2a). At

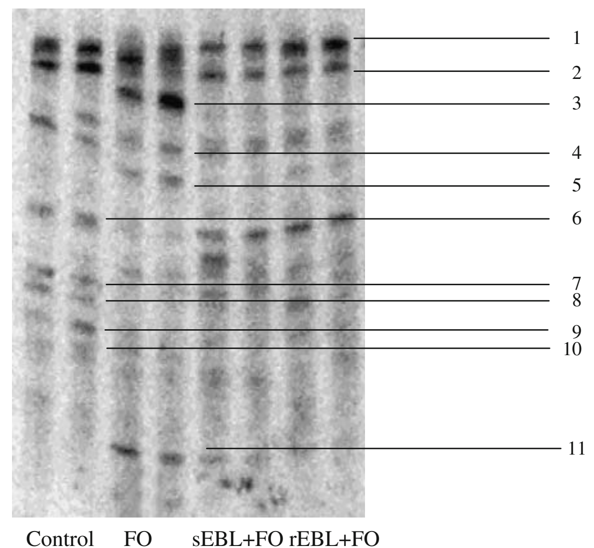

Fig. 3 Denaturing gradient gel electrophoresis analysis of bacteria from root surface samples of cucumber. *Control*, without FO inoculation; *FO*, FO inoculation; *sEBL + FO*, foliar spray of 24-epibrassinolide at 0.2 μM 24-epibrassinolide before FO inoculation; *rEBL + FO*, roots fed with 0.1 μM 24-epibrassinolide before FO inoculation. There were two samples for each treatment

the end of the experiment, the bacterial population in the FO, rEBL + FO and sEBL + FO treatments increased by 11.82%, 8.47% and 7.43%, respectively, compared with the control. The number of fungi on root surfaces of control plants remained little changed during the first 8 days but increased slightly at the end of the experiment (Fig. 2b). For FO-inoculated plants, the fungal population declined slightly during the first 4 days and dramatically declined at the end of the experiment. A similar trend was also observed on root surfaces of EBL-treated plants. However, the most significant decline was found on root surfaces of FO-inoculated plants. As compared with the control, the fungal population of FO, rEBL + FO and sEBL + FO treatments decreased by 27.08%, 22.65% and 11.87%, respectively. The *actinobacterial* population gradually increased during the experiment (Fig. 2c). At the end of the experiment, the population on root surfaces of EBL-treated plants was similar to that of the control plants but higher than that of FO-treated plants.

Changes in bacterial community structure

DGGE analysis (Fig. 3) showed that FO inoculation resulted in a decrease of the visible band numbers of the DGGE profile from 12.75±0.50 to 9.25±0.96 (Table 2). However, DGGE patterns of EBL treatments were similar to the control when expressed as visible bands. There were significant decreases in the diversity (*H* and 1/*D*) index and evenness (*E*) index of the bacterial community after FO inoculation. However, the decreases were significantly alleviated by EBL pre-treatments either to shoots or to roots. Compared with FO, the Simpson index (1/*D*) for sEBL + FO and rEBL + FO treatments increased by 55.46% and 44.40%, respectively.

Eleven representative bands were excised from DGGE profiles and the recovered sequences were analysed by BLAST. The highest identity from NCBI and putative phylum are shown in Table 3. Bands 1 and 2 were detected in the profiles of all the samples and identified as unclassified-Gamma proteobacteria and *Chitinophaga*. Four additional populations, represented by bands 3, 4, 5 and 11 were detected to varying extents in FO samples and identified as unclassified-Bacteroidetes, *Flavobacterium*, unclassified-Gamma proteobacteria and *Flectobacillus*, respectively. Bands 6, 7, 8, 9 and 10 were detected in the profiles of the control and EBL-treated plants but not in FO-inoculated plants. These bands belonged to *Acidovorax*, *Flavobacterium*, *Roseateles*, *Verrucomicrobium* and unclassified-Gamma proteobacteria, respectively.

Discussion

It has been well documented that BRs can induce resistance against a broad range of pathogens including viral, bacterial and fungal pathogens in tobacco, rice, barley, potato and cucumber (Khripach et al. 2000; Nakashita et al. 2003). However, in these earlier studies, increased resistance to foliar diseases was generally obtained by applications to shoots while the resistance to root diseases was obtained by applications to roots. Here we showed that EBL applications either to roots or shoots significantly increased resistance to FO as indicated by the decreased occurrence of Fusarium wilt (Table 1). This implied that EBL induced not only local resistance but also systemic resistance. As an immobile phytohormone (Symons et al. 2008), it is therefore interesting to know how the

Table 2 Numbers of visible bands and diversity indices based on DGGE analysis of total bacteria on the root surfaces of cucumber

Treatment	Numbers of visible bands	Shannon–Wiener index (*H*)	Evenness (*E*)	Simpson index (1/*D*)
Control	12.75±0.50 a	2.50±0.01 a	0.98±0.01 a	11.62±0.14 a
FO	9.25±0.96 b	2.06±0.06 c	0.89±0.03 b	6.87±0.67 c
sEBL + FO	12.25±0.50 a	2.43±0.02 b	0.98±0.01 a	10.68±0.34 ab
rEBL + FO	12.50±0.58 a	2.38±0.04 b	0.96±0.01 a	9.92±0.46 b

Samples were taken at the end of the experiment (12 days). Data are the means of three replications within an experiment with standard errors. Bars sharing the same letter are not significantly different as determined by Duncan's multiple range test ($P<0.05$).

Control without FO inoculation and 24-epibrassinolide treatment, *FO* FO inoculation, *sEBL + FO* foliar spray of 24-epibrassinolide at 0.2 μM 24-epibrassinolide before FO inoculation, *rEBL + FO* roots were fed with 0.1 μM 24-epibrassinolide before FO inoculation

Table 3 Partial sequence analysis of bacteria 16S rDNA genes from rhizosphere samples of cucumber inoculated with *Fusarium oxysporum* f. sp. *cucumerinum* (FO) and 24-epibrassinolide (EBL) treatments

Sequenced bands	GeneBank submission number	Fragment length (bp)	Strains or clones having the highest identity from NCBI	Putative Phylum
1	EU 369177	192	Aquatic bacterium R1-G9 gene for 16S ribosomal RNA (98%)	Phylum Proteobacteria
			Uncultured bacterium clone EV818SWSAP31 16S ribosomal RNA gene (98%)	Class Gamma-proteobacteria
			Rheinheimera sp. 114NP12 gene for 16S rRNA (97%)	Unclassified-Gammaproteobacteria
2	EU 369178	188	Uncultured bacterium gene for 16S rRNA, clone:TS66 (98%)	Phylum Bacteroidetes
			Uncultured bacterium clone WCB40 16S ribosomal RNA gene (97%)	Class Sphingobacteria
			Uncultured bacterium PHOS-HE31 16S ribosomal RNA gene (97%)	Order Sphingobacteriales
				Family Crenotrichaceae
				Genus *Chitinophaga*
3	EU 369179	188	Uncultured bacterium gene for 16S ribosomal RNA, clone:d03 (98%)	Phylum Bacteroidetes
			Uncultured proteobacterium clone R7C31 16S ribosomal RNA gene (98%)	Unclassified-Bacteroidetes
			Uncultured bacterium gene for 16S rRNA, clone:Niitsu39-49 (98%)	
4	EU 369180	188	*Flavobacterium* sp. GH1-10 16S ribosomal RNA gene (98%)	Phylum Bacteroidetes
			Flavobacterium sp. HI-M4 16S ribosomal RNA gene (98%)	Class Flavobacteria
			Bacterium RBA-1-24 16S ribosomal RNA gene (97%)	Order Flavobacteriales
				Family Flavobacteriaceae
				Genus *Flavobacterium*
5	EU 369181	194	Aquatic bacterium R1-G9 gene for 16S ribosomal RNA (99%)	Phylum Proteobacteria
			Uncultured bacterium clone EV818SWSAP31 16S ribosomal RNA gene (99%)	Class Gamma-proteobacteria
			Rheinheimera sp. 114NP12 gene for 16S rRNA (98%)	Unclassified-Gammaproteobacteria
6	EU 369182	194	Bacterium SRMC-33-4 16S ribosomal RNA gene (100%)	Phylum Proteobacteria
			Bacterium SRMC-11-10 16S ribosomal RNA gene (100%)	Class Betaproteobacteria
			Acidovorax sp. I-F1 16S ribosomal RNA gene (100%)	Order Burkholderiales
				Family Comamonadaceae
				Genus *Acidovorax*
7	EU 369183	190	Uncultured yard-trimming-compost bacterium clone S-72 16S ribosomal RNA gene (100%)	Phylum Bacteroidetes
			Uncultured lake bacterium P38.17 16S ribosomal RNA gene (100%)	Class Flavobacteria
			Uncultured bacterium clone 25ds5 16S ribosomal RNA gene (98%)	Order Flavobacteriales
				Family Flavobacteriaceae
				Genus *Flavobacterium*
8	EU 369184	195	Uncultured bacterium gene for 16S rRNA, clone: RB018 (93%)	Phylum Proteobacteria

Table 3 (continued)

Sequenced bands	GeneBank submission number	Fragment length (bp)	Strains or clones having the highest identity from NCBI	Putative Phylum
			Uncultured bacterium clone CEB2 16S ribosomal RNA gene (91%)	Class Beta-proteobacteria
			Uncultured earthworm intestine bacterium clone ew63 16S ribosomal RNA gene (91%)	Order Burkholderiales
				Family Incertae sedis 5
				Genus *Roseateles*
9	EU 369185	194	Unidentified bacterium clone W4-B59 16S ribosomal RNA gene (98%)	Phylum Verrucomicrobia
			Uncultured bacterium clone aab52e08 16S ribosomal RNA gene (97%)	Class Verrucomicrobiae
			Uncultured bacterium clone aab56b01 16S ribosomal RNA gene (97%)	Order Verrucomicrobiales
				Family Verrucomicrobiaceae
				Genus *Verrucomicrobium*
10	EU 369186	194	Aquatic bacterium R1-G9 gene for 16S ribosomal RNA (99%)	Phylum Proteobacteria
			Uncultured bacterium clone EV818SWSAP31 16S ribosomal RNA gene (99%)	Class Gamma-proteobacteria
			Rheinheimera sp. 114NP12 gene for 16S rRNA (98%)	Unclassified-Gammaproteobacteria
			Uncultured bacterium clone mek64c06 16S ribosomal RNA gene (100%)	
11	EU 369187	189	Uncultured bacterium clone mek63d08 16S ribosomal RNA gene (100%)	Phylum Bacteroidetes
			Uncultured bacterium clone mek63c11 16S ribosomal RNA gene (100%)	Class Sphingobacteria
				Order Sphingobacteriales
				Family Flexibacteraceae
				Genus *Flectobacillus*

BR signal is translocated to roots when it is applied to shoots, and how these signals affect rhizospheric microbial communities.

Consistent with the decreased incidence of Fusarium wilt, the *Fusarium* population on root surfaces or in nutrient solution dramatically decreased from EBL application to roots or shoots, suggesting that EBL induced special root exudates to suppress pathogen growth and colonisation. Flavonoids and sesquiterpenes in root exudates are known as signalling compounds in a number of pathogenic and symbiotic plant–microbe interactions (Richard 2001). It seems likely that secondary signals in the BRs signalling system induced the expression of defence-related genes leading to an increased resistance to pathogens including FO. Recently, we found that EBL applications resulted in a significant increase in the transcript level of defence-related genes such as *WRKY 30*, *PR*, *PAL* and *APX* in plants (unpublished data). It is also possible that EBL induced a change in the metabolism of roots, leading to changes in the composition of root exudates and associated microbial communities.

Introduction of pathogens or chemical treatments to plants may influence the microbial community because of the variability in chemical composition of the exudates (Christensen 1989). Oomycete pathogen introduction increased microbial bacterial levels in roots of hydroponic tomato (Calvo-Bado et al. 2006), also in the present study (Fig. 2). However, the roots of EBL-pre-treated plants had higher populations of fungi and *actinobacteria* than those of FO-inoculated plants. Positive correlations between microbial density and soil suppression have been well established (Janvier et al. 2007). Several kinds of *actinobacteria* have

biological activities against pathogens by producing many secondary metabolites including antibiotic and extracellular enzymes (Inbar et al. 2005). An array of fungi that produce different type of antibiotics are also being used to control many plant pathogens (Whipps 2004). An increased number of microorganisms are believed to be effective colonisers on roots, and could prevent the roots from being colonised by pathogens (Postma et al. 2005). In our study, efficient colonisation of roots by fungi and *actinobacteria* induced by EBL applications and competition for root exudates might be an effective mechanism to prevent the roots from being infected by the *Fusarium* pathogen. Taken together, the general increases in the microbial population on root surfaces of EBL-treated plants might play an important role in the suppression of Fusarium wilt development.

Bacterial diversity is greater than the diversity of any other group of organisms in an agroecosystem, and pathogen introduction could decrease both the biomass and diversity of the bacterial community in the soil (Karin et al. 2006). Similarly, FO inoculation resulted in decreases in DGGE band numbers, bacterial diversity and evenness in the current study (Fig. 3; Table 2). However, EBL applications alleviated these FO-induced changes in the bacterial community structure. Numbers of visible bands and the evenness index (E) of EBL-pre-treated plants were not significantly different from those of control plants. Maintenance of a good bacterial population and community structure is highly important, since the diversity and evenness of the soil microbial community is well correlated with the capacity of a soil to suppress the soil-borne potato pathogen, *Rhizoctonia solani* AG3 (van Elsas et al. 2002). Soils with a greater diversity of bacterial functional communities were more suppressive to the pathogen *Sclerotium rolfsii* (Liu et al. 2007). All these results indicate that the bacterial community structure on root surfaces of EBL-pre-treated plants was less disturbed by the FO introduction, and this might be one of the reasons for the lower disease incidence.

Sequencing results showed that FO inoculation brought about a scarcity of Burkholderiales (bands 6 and 8), and EBL applications restored this type of bacteria. Burkholderiales can preferentially use oxygen as an electron acceptor and promote plant growth (Macrae et al. 2001). Furthermore, the bacterial structure on root surfaces of FO-inoculated plants was characterised by an increased population of *Flavobacterium* and *Flectobacillus* (Bands 4 and 11). *Flavobacterium* can decompose several polysaccharides which are available as carbon sources or nutrients for microorganisms such as pathogens (Bernardet et al. 1996). *Flectobacillus* is widely distributed in an aquicolous environment and when isolated from a highly eutrophic pond (Hwang and Cho 2006). It is known that there is a shift in the bacterial communities on roots from r-strategists, which are species with fast growth rates and capacities to utilise simple substrates, to k-strategists, which are species with relatively slow growth rates and capacities to degrade more complex substrates during plant growth (Morgan et al. 2005). In agreement with these earlier studies, FO inoculation might induce plant senescence, even death, by inducing the emergence of these decomposing bacteria, whilst EBL applications induce the occurrence of plant growth-promoting bacteria and a decreased incidence of Fusarium wilt.

Overall, our study showed that EBL applications to either shoots or roots is effective in reducing the incidence of Fusarium wilt. FO inoculation induced a disturbed rhizospheric microbial structure but EBL pre-treatments alleviated this disturbance. The microbial community composition on root surfaces was affected by a complex interaction between phytohormone-induced resistance and plant pathogens.

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