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Full Length Research Paper

Isolation, quantity distribution and characterization of endophytic microorganisms within sugar beet

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The present investigation was undertaken in order to document the spectrum of endophytes colonizing healthy leaves of sugar beet cultivars in Xinjiang Province (China) and to determine the degree of colonization at three growth stages. From the 360 sugar beet leaf and root segments incubated, 221 bacterial isolates, 34 fungal isolates and 5 actinomycete isolates were obtained. Of all the isolates, 7 bacterial species and 6 fungal species were identified. The actinomycete isolates were characterized as *Streptomyces griseofuscus* and *Streptomyces globisporus*. There were significant differences between microorganisms, stages of growth, and stages of microorganism interaction. The number of microorganisms isolated increased during the growth period of the sugar beet. At the same time, the number of microorganisms affecting different parts of the sugar beet tissue was quite different. The greatest number of microorganisms was found in the secondary root emergence zone of the sugar beet tissue. Endophytic microorganisms in sugar beet promote growth and increase the yield of the beet.

Key words: Isolation, quantity distribution, characterization, endophytic microorganisms, sugar beet.

INTRODUCTION

Endophytic microorganisms are present in various plants species, and rarely produce any symptoms of disease (Yates et al., 1997; Holderness et al., 2000). Symptomless internal colonization of healthy plant tissues by microorganisms is a widespread and well-documented phenomenon. They may originate from indigenous species that occur either naturally in soil or they may be introduced through agricultural practices (Gordon and Okamoto, 1992). Endophyte is an all-encompassing topographical term that includes all organisms which during a variable period of their life-cycle colonize the living internal tissues of their hosts without producing symptoms of disease (Petrini, 1991). Such 'endophytes' are widely assumed to be present in virtually all land plants (Carroll, 1986; Schulz et al., 1999). Common endophytes include a variety of bacteria, fungi and action mycetes, and they can be isolated from wild (Brooks et al., 1994) or cultivated crops (Liu and Tang, 1996) of either the monocotyledonous (Fisher et al., 1992) or dicotyledonous plant groups (El-Shanshoury et al., 1996). The presence of endophytic bacteria within healthy sugar beet roots and endophytic fungi in healthy beet leaves has been demonstrated (Jacobs et al., 1985; Larran et al., 2004). Little is known about the endophytic microorganisms which colonize cultivated sugar beet during the growing season.

Endophytes are important in epidemiology because certain endophytic associations lead to the enhancement of the pathogen resistance of the plant (White and Cole, 1985) and an increase in vegetative growth (Clay, 1987) when compared to similar uninfected plants. The role of endophytes as plant growth promoters has long been established to be due to the presence of common endobacteria such as Rhizobium and Azorhizobium, particularly in leguminous and non-leguminous plants (Cavalcante and Dobereiner, 1988; Chanway, 1996). Host plants benefit in terms of enhanced growth and reproduction and the development of resistance towards abiotic and biotic stresses (Postma and Rattink, 1991; Larkin et al., 1996). These endo-symbionts enhance the absorption of nutrients by the host plant, leading to improved vegetative growth. As plant growth promoters,

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endophytes enhance growth including the formation of increased numbers of lateral roots and root hairs (Pillay and Nowak, 1997) in addition to an increase in plant height, shoot weight and shoot diameter (Yates et al., 1997). Little is known about the effect of the association of endophytic microorganisms with the sugar beet cultivars. It is unclear whether these endophytic microorganisms contribute to the vigorous and prolific growth of sugar beet in the crops fields in Xinjiang. In this study, the species and quantitative changes produced by endophytic microorganisms and their influence on sugar beet seedling growth were studied. The results will provide theoretical guidance for discovering and using microorganisms to promote growth in sugar beet plants, thus increasing the yield of sugar.

MATERIALS AND METHODS

Collection of sugar beets

Sugar beets were collected from border rows of a research plot in Xinjiang, China. Twenty-two sugar beets were harvested by hand during the growing season of 2006 (July to October) to determine the number and species of endophytes present in the root tissue. At least two sugar beets were taken at each sampling. Endophyte populations in sugar beet roots were first enumerated during the growth season. Bacterial populations were monitored for the next 7 weeks. Beets were harvested shortly after the 7th week of testing, at which time the plants were about 15 cm at the crown.

Isolation of endophytic microorganisms

Freshly harvested sugar beets were washed thoroughly with tap water to remove adhering soil and debris, soaked in 70% ethanol for 4 min followed by immersion in 1.05% solution of commercial bleach and shaken by hand for 5 min. The surface-disinfested Samples were then rinsed ten times (5 min each rinse) in sterile phosphate buffer (PB). To confirm that the surface disinfestation process was successful and to verify that no biological contamination from the surface of the beet was transmitted into the root tissues during maceration, sterility checks were carried out for each sample to monitor the effectiveness of the disinfestation procedures. For these checks, Samples impressions were taken and 0.1 ml from the final rinse was plated out on Petri plates of tryptic soy agar (TSA), potato dextrose agar (PDA) and Gause's No.1 synthetic medium agar. The absence of bacterial and fungal, after 6 days of incubation in the sterility checks was taken to confirm that sterility and microbial that was isolated was considered to be endophytic.

Samples were aseptically taken from core, periphery, and secondary root emergence zone (crease) areas. Each sample of root tissue was aseptically weighed, then added to 100 mL of sterile saline (0.85%), and blended for 2 min in a Waring blender. The blended samples were initially diluted to standardize all preparations (weight per volume). This was followed by additional serial decimal dilution in sterile phosphate buffer (PB). Endophytic bacteria were isolated using an aerobic spread-plate method. Dilution volumes of 0.1 mL were spread with a sterile glass rod over the surface of nutrient agar (NA) supplemented with 2.0 g/L sucrose. Endophytic fungi were isolated by plating root tissues on potato dextrose agar (PDA). Petri plates were incubated at 28°C for 7 days and colony counts recorded by standard methods (Brazi et al., 1972). Pure cultures were subsequently isolated: fungalisolate were maintained as filter paper cultures (Correll et al., 1986), and bacterial isolates were stored on agar slants.

Identification of endophytes

Pure cultures of the bacterial and fungal isolates were prepared for identification purposes. The fungal isolates F1, F2, F3, F4, F5, F6 and F7 were characterized and identified by their morphological characteristics. The bacterial isolates B1, B2, B3, B4, B5, B6 and B7 were identified using the Vitek AutoMicrobic System (Vitek AMS; Vitek Systems, Inc., Hazelwood, MO.). The Vitek test was repeated twice. Isolates were characterized by Vitek AMS, colony morphology, catalase production, oxidase test, and gram stain (Matthews et al., 1990).

Phylogenetic analysis of the isolated bacterial strains

The partial 16S rDNA was amplified with genomic DNA of the isolated bacterial strains as a template by PCR, using forward primer PA (5′-AgAgTTTgATCCTggCTCAg-3′) and reverse primer PB (5′-AAggAggTgATCCAgCCgC-3′). After amplification, the 16S rDNA sequence was determined using primers PA and PB, respectively. A phylogenetic tree was constructed using the neighbour-joining method (Saitou and Nei, 1987).

Screening of the growth-promoting properties of endophytic microorganisms in sugar beet

The impact of the endophyte–sugar beet plantlet association was assessed in an experiment carried out in the glasshouse with a day/night temperature of 30/25°C and with 200 µmol photons m^{-2} s^{-1} of light supplied for 12 h during the daytime; the experiment used healthy sugar beet plantlets to test the role of endophytes in promoting the growth of sugar beet. For the experiment, sugar beet seeds were obtained from the sugar-refinery of Changji in Xinjiang. The seeds were maintained in Hoagland's plant nutrient solution (Dhingra and Sinclair, 1985) for 2 weeks in order to induce root formation to facilitate inoculation.

For the experiment, the sixteen endophytes tested were individually inoculated onto the plantlets. Sterilized liquid medium (SLM) was inoculated as the control (CK). Each treatment comprised fifteen replicates (each plantlet constitutes a replicate). At inoculation, the plantlets were 1 weeks old and of a uniform height (2 cm, two-leaf stage). Each plantlet was inoculated by soil-drenching with 50 ml of fungal (10^5 cfu/mL) or bacterial (10^6 cfu/mL) endophytes. The inoculated plantlets were maintained in the glasshouse and were watered twice daily with sterilized distilled water. The individual plant height, fresh weight, dry weight and leaf number of sugar beet were examined four weeks after germination.

Experimental design and analysis

The experiment was conducted in a randomized complete block design to assess the growth-promoting role of the endophytes in sugar beet. The data collected were analyzed using analysis of variance (ANOVA) and means were compared using Tukey's studentizedrange test [HSD (0.05)].

RESULTS AND DISCUSSION

Identification of endophytic isolates

During this investigation, 360 sugar beet root and 60 sugar beet leaf segments were incubated and 221 bacterial isolates, 34 fungal isolates and 5 actinomycetes were obtained. From all the isolates, 7 bacterial species,

Table 1. Identification of strains of endophytes isolated from sugar beet grown on the north slope of Xinjiang Tianshan Mountain.

Strain number	Area of sugar beet tissue	Number of isolates	Endophyte
B1	LR*	84	*Pseudomonas fluorescens*
B2	R	43	*Bacillus flexus*
B3	R	18	*Pseudomonas fulva*
B4	R	46	*Bacillus pumilus*
B5	R	10	*Paenibacillus Polymyxa*
B6	L	2	*Chryseobacterium indologene*
B7	L	18	*Enterococcus faecalis*
F1	LR	15	*Alternaria alternate*
F2	LR	6	*Fusarium oxysporum*
F3	LR	3	*Pythium aphanidermatum*
F5	LR	4	*Penicillium expansum*
F6	LR	3	*Plectosphaerella cucumerina*
F7	LR	3	*Phoma betae*
S1	R	2	*Streptomyces griseofuscus*
S2	R	3	*Streptomyces globisporus*

*L indicates leaf; R indicates root.

6 fungal species and 2 actinomycete species were identified (Table 1). The seven bacteria isolates were characterized as *Pseudomonas fluorescens*, *Bacillus flexus*, *Pseudomonas fulva*, *Bacillus pumilus*, *Paenibacillus Polymyxa, Chryseobacterium indologene* and *Enterococcus faecalis*. Most of the endophytic fungi isolated are primarily anamorphs of fungi belonging to the genus-Deuteromycotina,-including-several yphomycetes, whereas Ascomycotina were comparatively scarce. Similar colonization patterns were reported on an annual species of *Juncus microflora* by Cabral et al. (1993). Most of the fungal isolates from sugar beet leaves belonged to genera which have already been described as endophytes of sugar beet and others plants from temperate zones and from the tropics (Larran et al., 2004). Cabral et al. (1993) point out that *Alternaria alternata* are confined to the immediate subestomata area, where they may benefit from some nutrient leakage from the host, or are afforded some protection from desiccation or from mycophagous invertebrates. Other fungi isolated included both *Fusarium oxysporum* and penicillium species. These two genera have been reported to be present as endophytes in other plants (Spurr and Welty, 1975; Fisher et al., 1992; Cabral et al., 1993).

To detect any phylogenetic relationship with reported strains, a partial 16S rDNA sequence of the isolated bacterial strains was determined and compared with available 16S rDNA sequences (Figures 1 and 2). The partial 16S rDNA sequence of these strains showed high homology with those strains (98 – 100%).

The species isolated in this work may be classified to three groups:

i.) Well-known and economically important pathogens of beet, that is, *F. oxysporum*.

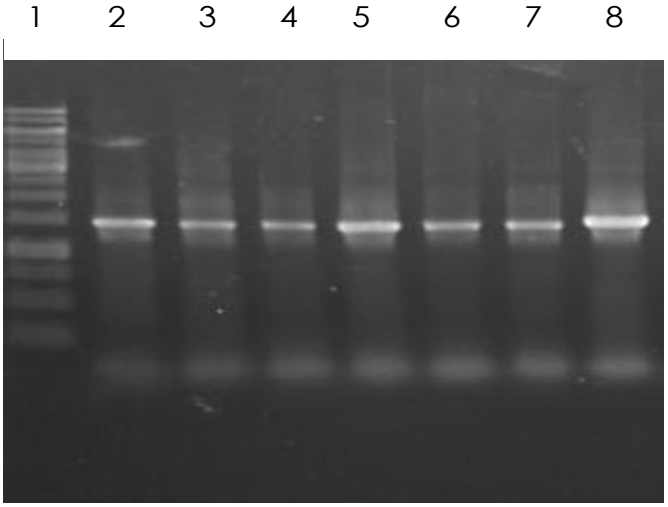

Figure 1. PCR patterns of endophytic bacterial of 16S rDNA fragments retrieved from sugar beet samples of profile. (a) Lane 1, marker; Lane 2, B1; Lane 3, B2; Lane 4, B3; Lane 5, B4; Lane 6, B5; Lane 7, B6; Lane 8, B7

ii.) Commonly abundant phylloplane fungi which are considered to be primary saprophytes and minor pathogens (Zillinsky, 1984) that is, *A. alternata*.

iii.) Species which are occasionally present in beet, that is, *Bacillus* sp.

There were significant differences between microorganisms, beet growth stages, and growth stages. *A. alternata*; *F. oxysporum*, and *Pythium aphanidermatum* were the fungi most frequently isolated from sugar beet. The other microorganisms were present at intermediate or low frequencies. Further studies are needed to firmly establish whether the presence of endophytes is the

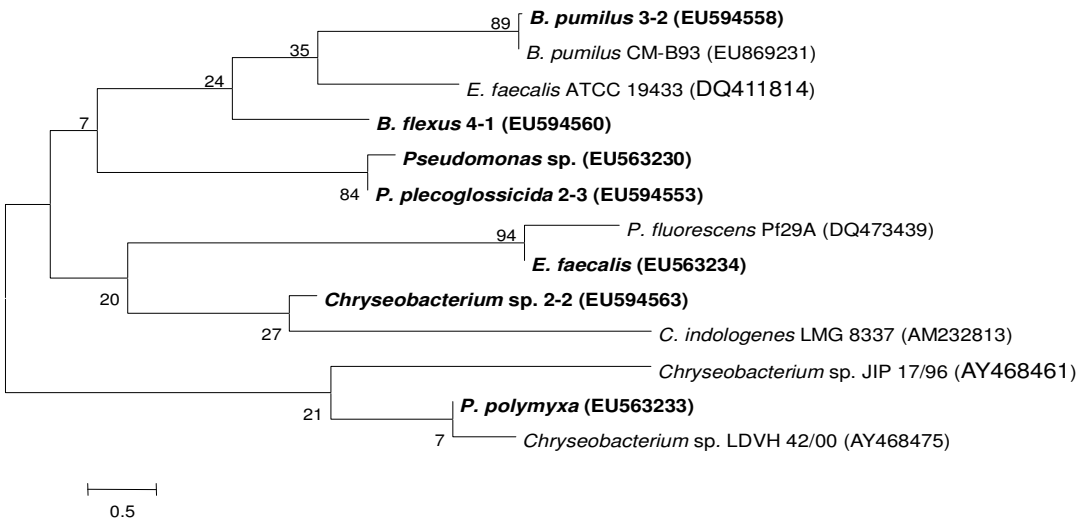

Figure 2. Phylogenetic analysis of isolated endophytic bacterial 16S rDNA from sugar beet samples. Numbers in parentheses represent the sequences accession number in GenBank. The number at each branch points is the percentage supported by bootstrap. Bar, 50% sequence divergence.

result of a mutually beneficial relationship with the host (sugar beet) or of competitive colonization.

There are two secondary root emergence zones (SREZ) on opposite sides of a sugar beet root. The emergence of roots from these SREZ causes wounding, which may provide a natural path of entry for bacteria. We found that counts of root bacteria from the SREZ increased 100- to 1000-fold over the 7-week period (Table 2). During the same period, no significant increase in bacterial numbers was demonstrated in the peripheral or core tissues. The increased bacterial population in the SREZ was possibly due to an increase in tissue invasion made possible by the natural wounding process resulting from the emergence of secondary roots. This was the only period of time during which a change in the bacterial population was observed.

Effect of endophyte–host association on the growth of sugar beet plantlets

In the experiment, the growth of sugar beet plantlets infected with endophytes (B2, B3, F1, F3, F5, S1 and S2) was similar to that of the SLM control plantlets for all of the parameters assessed except plant height (Table 3). Plantlets responded better to the bacterial endophytes (B2 and B3) than to the fungal endophytes (F4), with the average growth values given by the former higher than those of produced by the latter (Table 3). The bacterial endophyte B2 demonstrated the most potential as an efficient plant growth promoter. The association of isolate B2 with sugar beet plantlets resulted in the highest growth values: 4.1 cm (height), 0.242 g (dry weight) and 1.7 (total number of leaves plant^{-1}), relative to the association with any of the other endophytes and the SLM control (Table 3), followed by the fungal endophyte F6, which also induced growth in terms of increased pseudostem diameter, root mass and total number of leaves. A comparison between isolates B1, B6 and F4 revealed that plantlets inoculated with all isolates generated a similar growth response, although plantlets inoculated with B1 showed slightly poorer growth. Among the bacterial endophytes, Isolate B2 was the most efficient followed by F6 and F1. In addition, their growth values for plant height, fresh weight, dry weight and total number of leaves were not significantly different from those of the control plantlets.

This study underlines the potential positive effect of indigenous endophyte species isolated from sugar beet on the growth of the plant. Further tests are required to determine the compatibility of these endophytes with other commercial cultivars, although similar positive responses are expected, primarily attributable to the non-host specificity of the endophytes, their growth-promoting properties and the amenability of beet plants to endophyte infection (Pan et al., 1997).

Most endophytes form either a beneficial, neutral or detrimental association with their host plant. In this study, the endophytes tested formed a neutral–beneficial association with the beet plantlet, as the growth of the inoculated plantlets was either similar or better than that of plantlets which were not colonized by endophytes (control, SLM). As a result, in the absence of endophytes poorer plant growth may occur due to the disrupted flow of essential nutrients and water through blocked vessels. The endophytes functioned as growth promoters in plantlets.

The mechanism of growth improvement is beyond the scope of our investigation, but the improved growth of the inoculated plantlets is believed to be mainly attributable

Table 2. Average number of microorganisms isolated from sugar beet tissue (cells per gram tissue).

Harvest date month/day/year	Cross section of root	Adhering soil	Peripheral tissue	Middle tissue	Core tissue	Secondary root emergence zone
8/7/2007	Top third	6.2×10^{8}	5.6×10^{4}	7.8×10^{4}	5.9×10^{5}	3.7×10^{3}
	Middle third	5.3×10^{8}	4.6×10^{4}	5.1×10^{4}	4.9×10^{4}	4.4×10^{3}
	Bottom third	1.5×10^{9}	1.4×10^{5}	1.7×10^{5}	2.0×10^{5}	5.7×10^{3}
8/22/2007	Top third	7.1×10^{8}	5.2×10^{6}	4.3×10^{6}	3.8×10^{6}	4.2×10^{4}
	Middle third	5.1×10^{8}	6.7×10^{6}	5.2×10^{6}	4.8×10^{6}	3.6×10^{4}
	Bottom third	1.1×10^{9}	8.7×10^{6}	9.1×10^{6}	1.1×10^{7}	6.1×10^{4}
8/30/2007	Top third	8.2×10^{8}	5.2×10^{6}	4.7×10^{6}	3.8×10^{6}	6.8×10^{4}
	Middle third	4.1×10^{8}	6.7×10^{6}	5.2×10^{6}	4.8×10^{6}	4.7×10^{4}
	Bottom third	8.1×10^{8}	8.7×10^{6}	9.2×10^{6}	1.1×10^{7}	7.9×10^{4}
9/12/2007	Top third	9.4×10^{8}	1.6×10^{6}	1.3×10^{6}	1.1×10^{6}	3.4×10^{5}
	Middle third	3.7×10^{8}	1.0×10^{6}	3.5×10^{5}	6.0×10^{4}	1.2×10^{5}
	Bottom third	7.8×10^{8}	4.1×10^{6}	0	0	5.1×10^{5}
9/18/2007	Top third	1.2×10^{9}	2.5×10^{6}	6.2×10^{5}	1.4×10^{6}	6.5×10^{5}
	Middle third	2.5×10^{8}	1.0×10^{6}	0	0	4.3×10^{5}
	Bottom third	5.2×10^{8}	1.1×10^{6}	2.8×10^{5}	0	1.8×10^{6}
9/27/2007	Top third	3.6×10^{9}	5.6×10^{4}	6.2×10^{4}	5.9×10^{5}	2.3×10^{6}
	Middle third	1.2×10^{8}	4.6×10^{4}	4.5×10^{4}	4.9×10^{4}	1.9×10^{6}

Table 3. Effects of proportion of endophytes on plant height, fresh weight, dry weight and leaf number.

Treatment	Plant height (g)	Fresh weight (g)	Dry weight (g)	Total number of leaves plant^{-1}
B1	7.8b	1.5d	0.142c	3.6b
B2	14.5a	2.3a	0.242b	3.8b
B3	11.3a	1.8c	0.189b	3.3c
B4	8.4b	1.6d	0.153c	3.5c
B5	9.7ab	1.9c	0.189b	3.4c
B6	8.1b	2.5a	0.267a	3.5c
B7	8.9b	2.0b	0.193b	3.5c
F1	11.6a	1.9c	0.195b	3.9a
F2	9.7ab	1.9c	0.192b	3.5c
F3	10.3a	1.7c	0.182b	3.6b
F4	7.2b	1.5d	0.132c	3.3c
F5	10.6a	1.5d	0.141c	3.6b
F6	13.5a	2.1b	0.225a	4.1a
F7	13.5a	2.1b	0.225a	4.1a
S1	10.7a	1.9c	0.184b	3.6b
S2	10.5a	1.8c	0.176b	3.7b
CK	10.4a	1.9c	0.182b	2.1d

Values are means of fifteen replicates, and the values with the same lower case letter within a column are not significantly ($P > 0.05$) different according to Fisher's Protected LSD Test.

to the production of plant growth regulators by the endophytes (Porter et al., 1979). Auxins and cytokinins are the two most cited growth promoters associated with the induced growth of plants as a response to endophytic infection. Auxins stimulate cell division (Daly and Inman, 1958), thus explaining the increase in root mass and accelerated formation of root hairs, while cytokinins induce root elongation (Daly and Inman, 1958), thereby also increasing root mass. Cytokinins are also reportedly responsible for enhancing nutrient accumulation and transportation (Kiraly et al., 1967; Dekhuijzen and Overeem, 1971; Sziraki et al., 1975; Vizarova, 1979),

thus contributing to overall improved plant growth.

Conclusion

We have demonstrated here that endophytes isolated from sugar beet can promote the growth of sugar beet plantlets. These isolates are amenable to artificial inoculation, and their non-host specificity enabled them to infect and colonize new host plants. The beneficial association of endophytes with sugar beet plantlets may be extended to other commercial cultivars. However, further testing is necessary to determine this. The application of endophytes is strongly recommended at the nursery stage on tissue-cultured clones in order to allow the establishment of endophytes prior to transplanting to the field. There is also a need to determine the mechanisms of growth promotion and to optimize the conditions for endophyte application so that the endophytes, particularly B2 and F6, not only serve as growth promoters, but also as a strategy for increasing the production of sugar.

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