Biological control against bacterial wilt and colonization of mulberry by an endophytic Bacillus subtilis strain

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Received 8 November 2007; revised 23 May 2008; accepted 26 May 2008.
First published online 10 July 2008.
DOI:10.1111/j.1574-6941.2008.00543.x

Editor: Christoph Tebbe

Keywords
biological control; bacterial wilt; mulberry; endophyte; Bacillus subtilis; colonization.

Abstract
Forty-five bacterial isolates were collected from surface-sterilized leaves of mulberry (Morus alba L.). By screening their antagonistic activities against Ralstonia solanacearum in vitro, four isolates showed a remarkable inhibitory effect. The evaluation of the antagonistic strains against bacterial wilt of mulberry indicated that the strain Lu144 effectively reduced disease incidence. In the greenhouse, Lu144 displayed effective biological control against bacterial wilt of mulberry when it was applied to sterile or nonsterile soil before the infection by the pathogen. Based on bacteriological properties and 16S rRNA gene sequencing, Lu144 was identified as a strain of Bacillus subtilis. The endophytic population and infection process of Lu144 in mulberry seedlings was explored following recovery of the green fluorescent protein (GFP)-labeled Lu144 and examination of the labeled strain by confocal laser scanning microscopy. Interestingly, the infection of GFP-labeled Lu144 cells into the mulberry seedlings occurred through the cracks formed at the lateral root junctions and the zone of differentiation and elongation, and the cells were able to develop and transfer in mulberry and mainly in the intercellular spaces of different tissues. The population of the GFP-labeled Lu144 inoculant was larger and more stable in leaves than that in roots and stems.

Introduction
Mulberry (Morus alba L.) is the sole food plant of the silkworm (Bombyx mori L.) during its entire larval period and is often affected by a number of diseases prevalent around the year in different agroclimatic zones (Kumar & Gupta, 2004). Among the diseases affecting mulberry, bacterial wilt of mulberry has recently been recognized as one of the most destructive bacterial diseases (Wang et al., 2007). This vascular disease, which is difficult to control, is caused by the soilborne bacterium Ralstonia solanacearum (Smith) (Yabuuchi et al., 1992), a genetically diverse soilborne pathogen with a wide host range (Hayward, 1991; Ji et al., 2005). However, as mulberry leaves are used to feed silkworms, the improper use of agrochemicals to treat this disease could be hazardous to the worms. Therefore, the use of agrochemicals has not gained wide acceptance. Biological control of plant pathogens using antagonistic bacteria is a promising strategy for plant protection from soilborne diseases and has attracted considerable attention with the aim to reduce the use of agricultural chemicals (Kloepper et al., 1999; Mizumoto et al., 2007).

Some biological agents have been intensively studied, including strains of Gram-negative Pseudomonas (Kraus & Loper, 1995; Walsh et al., 2001; Haas & Defago, 2005) and Gram-positive Bacillus subtilis (Krebs et al., 1998; Touré et al., 2004; Leclere et al., 2005; Ongena et al., 2005; Stein, 2005; Kapley et al., 2007). Previous studies have shown the potential of root-colonizing microorganisms to inhibit or displace soilborne pathogens at the root–soil interface (Anuratha & Gnanamanickam, 1990). However, because these microorganisms are affected by biotic and abiotic factors, biological control in fields by soil microorganisms has often been inconsistent (Tsuda et al., 2001). Recently, endophytic bacteria, which reside in plant tissues mainly in intercellular (rarely in intracellular) spaces, and inside vascular tissues without causing symptoms of disease (Sessitsch et al., 2002), have received more attention. One
of the advantages of using endophytes is that, once inside the host, they are better protected against environmental stress and microbial competition. Furthermore, the use of endophytic Bacillus strains as biological control candidates has the advantage that they form endospores that can easily be formulated and stored (Ednar et al., 2002). Because of these advantages, the application of endophytic Bacillus strains for biological control of plant diseases caused by soilborne pathogens has been explored (Pleban et al., 1995; Asaka & Shoda, 1996; Raupach & Kloeper, 1998; Chen & Wu, 1999; Harris & Adkins, 1999; Szczech & Shoda, 2004; Ongena et al., 2005) and a number of wild-type B. subtilis strains have been collected (Podile, 1994; Pleban et al., 1995; Wulff et al., 2002; Szczech & Shoda, 2004; Okigbo, 2005; Ongena et al., 2005; Kapley et al., 2007).

However, no attempt has been made to control R. solanacearum infection in mulberry using endophytic Bacillus strains as biological agents. In the present paper we address the isolation of endophytic bacteria from mulberry, evaluate the use of these endophytic strains for control against bacterial wilt of mulberry and investigate the colonization of the antagonistic Lu144 strain in mulberry. The objectives of this study were to select endophytic bacteria that control the bacterial wilt of mulberry and to clarify the specific localization of the selected endophytic bacterium within mulberry.

**Materials and methods**

**Collection of mulberry leaves and isolation of bacteria**

Mulberry (M. alba L.) leaves were collected from 20 healthy adult trees in May 2006 near the town of Tai’an, Shandong province of China. Leaves were washed with running water and then cut into several disks c. 5 mm in diameter. Five disks from each leaf were randomly selected, washed and rinsed in 0.1% Tween 20 for a few seconds and surface-sterilized by dipping successively into 70% ethanol for 1 min and 11% aqueous sodium hypochlorite for 5 min, followed by washing six times with sterile distilled water. Leaf disks were placed in Petri dishes containing 2% potato dextrose agar (PDA) medium (Tsuda et al., 2001). Plates were incubated in darkness at 26 ± 2 °C and checked every day until the emergence of endophytic bacteria on the fourth day. Bacteria colonies were picked and purified by single-colony streaking on three successive King’s medium B (Gray et al., 2006) plates. Strains with distinct colony morphologies were kept for further study. To confirm leaf surface sterility, 0.1 mL of the last surface-wash water was spread on PDA media plates, and the plates were incubated at 26 ± 2 °C for 7 days. When no contamination was found on the plates, the leaf surface sterility was confirmed. Isolates were retained only from leaves that had been confirmed to be surface-sterilized.

**Screening bacterial isolates for antagonistic activity against R. solanacearum in vitro**

The Rs-M07 strain of R. solanacearum was obtained from the Department of Plant Protection of Shandong Agricultural University and maintained on tetrazolium chloride medium (Swanson et al., 2005). The bacteria were cultured in Luria–Bertani (LB) liquid medium (Wang et al., 2000) at 30 °C for 24 h and the inoculum concentration was adjusted to 10⁸ CFU mL⁻¹. Five hundred microliters of R. solanacearum was mixed evenly in 15 mL of melted nutrient agar (NA: 3 g beef extract, 5 g peptone and 15 g agar in 1000 mL distilled water) in a Petri dish and allowed to solidify. Four small wells (4 mm × 2 mm) were cut in the plate using a cork borer. All isolates of endophytic bacteria were added to LB liquid medium and incubated at 37 °C with shaking until a logarithmic phase was reached. The culture was spun down and the supernatant was filtered through a 0.20-μm filter to obtain a cell-free culture supernatant fluid. Fifty microliters of cell-free supernatant fluid along with water as a control were separately added to the small wells. Three plates were used for each strain tested and three test spots were placed on each plate. The plates were incubated at 30 °C and observed for inhibition zone after 2 days.

**Evaluation of the antagonistic strains for biological control against bacterial wilt of mulberry under greenhouse conditions**

The healthy mulberry seeds were washed in running tap water for 5 min. They were then surface-disinfected by shaking in 20% (w/v) hydrogen peroxide for 3 min, washed in 70% (w/v) ethanol for 90 s and finally soaked for 3 min in a solution of 10% (w/v) sodium hypochlorite in 0.01% (v/v) Tween 20 and then were placed on moist filter paper and incubated at 25 °C for 5–6 days in Petri dishes. When the roots were c. 25-mm long, the seedlings were transplanted into 18-cm-diameter plastic pots filled with autoclaved soil or nonautoclaved soil and inoculated with the antagonistic strains by drenching the soil at a dose of 12 mL of the suspension (10⁶ CFU mL⁻¹) per 100 g soil. The soil used in this study was collected from the upper 30 cm of a mulberry field where the strains were isolated. The soil was sieved through a 1.5-mm sieve and put into a sterilizable polypropylene bag and autoclaved for 60 min at 121 °C four times at 12-hour intervals. Then the soil was brought to 1. Five hundred microliters of 1.0 g beef extract, 5 g peptone and 15 g agar in 1000 mL distilled water) in a Petri dish and allowed to solidify. Four small wells (4 mm × 2 mm) were cut in the plate using a cork borer. All isolates of endophytic bacteria were added to LB liquid medium and incubated at 37 °C with shaking until a logarithmic phase was reached. The culture was spun down and the supernatant was filtered through a 0.20-μm filter to obtain a cell-free culture supernatant fluid. Fifty microliters of cell-free supernatant fluid along with water as a control were separately added to the small wells. Three plates were used for each strain tested and three test spots were placed on each plate. The plates were incubated at 30 °C and observed for inhibition zone after 2 days.

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replication and three plants per pot. Seedlings treated with sterile distilled water served as control. Seedlings were grown in a growth chamber at 26 °C, humidity 90% and under 12 h of light. Ten days after transplantation, the seedlings were challenge-inoculated by drenching the soil at a dose of 12 mL of the R. solanacearum suspension (10^6 CFU mL^{-1}) 100 g^{-1} soil. The plants were maintained by suitable watering. After maintenance for 60 days, the plant height was measured from the base to the tip of the seedling and the fresh weight was determined by weighing the uprooted plant. The disease was assayed by the percentage of seedlings with any wilt symptom. Protective value as a degree of suppression was calculated by the following formula: protective value = (1 – percentage of bacterial wilt in treatment/ percentage of bacterial wilt in disease control) × 100 (Tsuda et al., 2001).

Further experiments were performed in plastic pots containing sterilized and nonsterilized soils prepared as described above. When the roots were c. 25 mm long, the seedlings were transplanted into the plastic pots and were challenge-inoculated by R. solanacearum at the same dose as described above at the same time. Application of the selected bacteria Lu144 was performed as follows: (1) 7 days before transplantation; (2) the same time mixed with R. solanacearum; (3) 7 days after transplantation. In each experiment, treatment consisted of three replicates with five pots per replication and three plants per pot. Seedlings treated with sterile distilled water at the same time served as control. All the pots were arranged in a completely randomized design in a greenhouse as described above and maintained by suitable watering. After maintenance for 60 days the plant height, fresh weight and the disease were assayed as described above.

**Identification of Lu144**

The biochemical and physiological characterizations of Lu144 were determined and it was further identified using the method described previously (Sneath, 1986). The 16S rRNA gene was PCR amplified from genomic DNA isolated from pure Lu144 bacterial colonies following standard protocols (Haldal-Alija & Johnston, 1999). The primers used for PCR were pF (5’-AGA GTT TGA TCC TGG CTC AG-3’) and pR (5’-AAG GAG GTG ATC CAG CCG CA-3’). The PCR products with the expected size (about 1500 bp) were purified using a DNA Gel Extraction Kit and cloned into pMD18-T vector followed by sequencing. Sequence analysis was performed using the BLAST algorithm (http://www.ncbi.nlm.nih.gov). Bacterial identifications were based on 16S rRNA gene sequence similarity. Neighbor-joining phylogenetic trees were generated based on partially sequenced 16S rRNA genes and closely related sequences.

**Tagging of the strain Lu144 with green fluorescent protein (GFP)**

The strain Lu144 was sensitive to neomycin (7 μg mL^{-1}) and ampicillin (100 μg mL^{-1}). The plasmid, pGFP4412, containing one copy of constitutively expressed gfp, neomycin and ampicillin resistance genes in tandem, was kindly donated by the College of Agronomy and Biotechnology, China Agricultural University, Beijing, China. This plasmid was introduced into Lu144 by electroporation as described by Liu et al. (2006). The transformed cells were immediately transferred to 1 mL of LB medium and incubated for 1 h at 30 °C with shaking at 180 r.p.m., and then plated on selective medium (LB medium containing 7 μg mL^{-1} neomycin). Transformants, which emitted green fluorescence, were screened with a confocal laser scanning microscope with an excitation wavelength of 488 nm. The stability of the GFP-labeled Lu144 was determined as described by Ramos et al. (2002).

**Microscopy observation of colonization in mulberry plant**

Mulberry seeds were pregerminated as described before. When the roots were c. 25 mm long, the seedlings were transplanted into 18-cm-diameter plastic pots filled with autoclaved soil prepared as described above and incubated in a growth chamber at 26 °C, humidity 90% and under 12 h of light. When the seedlings were c. 10 cm in height, they were inoculated with tagged Lu144 by drenching the soil at a dose of 12 mL suspension (10^6 CFU mL^{-1}) 100 g^{-1} soil. The control seedlings were inoculated with sterile distilled water. The seedlings were maintained in a greenhouse as described above. Root, stem and leaf samples were obtained at different times after inoculation and were surface disinfected as described by Martínez et al. (2003), and then directly inspected and/or dissected under a stereomicroscope in the transverse and longitudinal directions. Aggregations of fluorescent bacterial cells on plant material were visualized under a Bio-Rad MRC1024 laser confocal microscope with excitation wavelengths of 488 and 633 nm. Emitted light was collected in the range of 510–560 nm for GFP fluorescence. Images were obtained using LEICA CONFOCAL SOFTWARE, version 2.477. For each sampling point, six plants were examined. Images were collected from 10–20 sections.

**Quantification of endophytic population**

The surface-sterilized samples obtained as described above were triturated with a sterile mortar and pestle in potassium phosphate buffer (PB). Serial dilutions of the triturate in PB were plated on plates supplemented with King’s medium B containing neomycin (7 μg mL^{-1}) and ampicillin (100 μg mL^{-1}). The plates were incubated at 28 °C for
48–72 h and colony counts were recorded. For each sampling date, the average value of triplicate plates of a particular dilution was taken for calculation of the number of viable cells in 1 mL of suspension.

Results

Screening and evaluation of the strains for biological control against bacterial wilt

Using the isolation procedure described above, most endophytic bacteria gradually became visible 7–10 days after the onset of incubation. Based on the colony color, size and morphology, a total of 45 isolates were successfully collected from the samples. By testing their ability to control *R. solanacearum*, four isolates displaying a remarkably inhibitory effect were selected (Table 1). The four isolates proved to be antagonistic to *R. solanacearum* when they were evaluated for biological control against bacterial wilt of mulberry under growth chamber conditions, which were not detrimental to plant growth in either sterile or nonsterile soils. The results showed that, among the four isolates, the protective value of the Lu144 strain was over 50 (Table 2). In addition, the height and weight of seedlings treated with the Lu144 strain were higher than those of untreated controls, and the plants grew better than those that survived in the disease control plots. Therefore, the Lu144 strain was selected to further test its biocontrol against bacterial wilt. Under greenhouse conditions, Lu144 significantly protected mulberry plants against bacterial wilt when it was applied no later than the plant inoculated by *R. solanacearum* whether in sterile soil or nonsterile soil (Table 3). The protective values of these treatments were all > 50, and the seedlings with these treatments were all taller and heavier than the disease controls. However, when Lu144 was applied 7 days after inoculation by *R. solanacearum*, no significant protection was observed either in sterile soil or nonsterile soil, and there were no differences in seedling growth. These results indicated that the Lu144 strain played an important role in the biological control against bacterial wilt of mulberry in a greenhouse, and it should be applied no later than infection by *R. solanacearum*.

Identification of Lu144

Under the electron microscope, cells of strain Lu144 were observed to be small, regular, short rods (0.9–1.2 μm wide and 1.8–2.4 μm long) with peritrichous flagella and the ability to form spores. Physiological and biochemical analysis indicated that the Lu144 strain was Gram-positive with an optimal growth temperature of 25–28 °C, and could grow in 7% NaCl and produce caseinase, catalase and oxidase, suggesting that it is similar to *B. subtilis* (Sneath, 1986). To characterize Lu144, we performed strain identification using both biochemical and molecular approaches. The results showed that Lu144 was a Gram-positive organism with all the characteristics of a *Bacillus* sp. Moreover, an expected 1513-bp DNA fragment of the 16S rRNA gene was obtained from the Lu144 strain by PCR amplification. BLAST analysis of the amplicon sequence indicated that it shared highest similarities of over 99% with 16S rRNA gene sequences from other *B. subtilis* strains, which is higher than the generally accepted value (≥97%) for members in the same species. The sequence was deposited in the GenBank database (accession no. EU118756). To clarify the phylogenetic position of Lu144, a phylogenetic

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Table 1. Inhibition of the isolates from mulberry on growth of *Ralstonia solanacearum*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Diameter of inhibition zone (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LA</td>
<td>8.0 b</td>
</tr>
<tr>
<td>LB</td>
<td>7.5 c</td>
</tr>
<tr>
<td>LC</td>
<td>6.4 d</td>
</tr>
<tr>
<td>Lu144</td>
<td>9.6 a</td>
</tr>
</tbody>
</table>

Data are the average of nine test spots and they were analyzed using Student’s t-test (*P* ≤ 0.05). The lowercase letters indicate values, with ‘a’ being the highest, and ‘d’ the lowest value.

Table 2. Control efficiency of the four isolates on bacterial wilt of mulberry used in different soils in greenhouse

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage of surviving seedlings with bacterial wilt (%)</th>
<th>Protective value</th>
<th>Height (cm)</th>
<th>FW (g per plant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LA-S</td>
<td>66.1 b</td>
<td>11.4 d</td>
<td>14.9 c</td>
<td>1.10 c</td>
</tr>
<tr>
<td>LB-S</td>
<td>59.2 c</td>
<td>20.6 c</td>
<td>15.1 c</td>
<td>1.14 c</td>
</tr>
<tr>
<td>LC-S</td>
<td>46.1 d</td>
<td>38.2 b</td>
<td>16.2 b</td>
<td>1.24 b</td>
</tr>
<tr>
<td>Lu144-S</td>
<td>22.3 e</td>
<td>70.1 a</td>
<td>17.4 a</td>
<td>1.31 a</td>
</tr>
<tr>
<td>Disease control-S</td>
<td>74.6 a</td>
<td>0 e</td>
<td>13.1 d</td>
<td>1.00 d</td>
</tr>
<tr>
<td>LA-NS</td>
<td>54.4 c</td>
<td>16.6 c</td>
<td>15.4 c</td>
<td>1.17 bc</td>
</tr>
<tr>
<td>LB-NS</td>
<td>55.6 b</td>
<td>14.7 d</td>
<td>15.3 c</td>
<td>1.15 c</td>
</tr>
<tr>
<td>LC-NS</td>
<td>41.1 d</td>
<td>37.0 b</td>
<td>16.2 b</td>
<td>1.22 b</td>
</tr>
<tr>
<td>Lu144-NS</td>
<td>20.4 e</td>
<td>68.7 a</td>
<td>17.6 a</td>
<td>1.34 a</td>
</tr>
<tr>
<td>Disease control-NS</td>
<td>65.2 a</td>
<td>0 e</td>
<td>13.9 d</td>
<td>1.06 d</td>
</tr>
</tbody>
</table>

*Protective value = (1 – percentage of bacterial wilt in a treatment/percentage of bacterial wilt in a disease control) × 100.

1No wilted plants in each treatment, means of 40 random plants.

2FW, fresh weight of the seedling at 60th day after transplantation.

Means of the surviving plants.

3Data are the average of three experiments for nine test spots and they were analyzed using Student’s t-test (*P* ≤ 0.05). The lowercase letters indicate values, with ‘a’ being the highest, and ‘d’ the lowest value. The same letters within a column mean that no significant differences exist between the numbers.

S, sterile soil; NS, nonsterile soil.
Means of the surviving plants.

Localization of the Lu144 strain in mulberry

Table 3. Control efficiency of the Lu144 strain on bacterial wilt of mulberry used in different soils in greenhouse

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage of surviving seedlings with bacterial wilt (%)</th>
<th>Protective value*</th>
<th>Height (cm)</th>
<th>FW (g per plant)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-S</td>
<td>24.1 b**</td>
<td>67.5</td>
<td>17.7 a</td>
<td>1.38 a</td>
</tr>
<tr>
<td>Disease control A-S</td>
<td>74.2 a</td>
<td>0</td>
<td>12.7 b</td>
<td>0.99 b</td>
</tr>
<tr>
<td>B-S</td>
<td>25.2 b</td>
<td>66.2</td>
<td>17.6 a</td>
<td>1.38 a</td>
</tr>
<tr>
<td>Disease control B-S</td>
<td>74.6 a</td>
<td>0</td>
<td>12.5 b</td>
<td>1.00 b</td>
</tr>
<tr>
<td>C-S</td>
<td>71.1 b</td>
<td>6.7</td>
<td>12.7 a</td>
<td>1.02 a</td>
</tr>
<tr>
<td>Disease control C-S</td>
<td>76.2 a</td>
<td>0</td>
<td>12.5 a</td>
<td>0.97 a</td>
</tr>
<tr>
<td>A-NS</td>
<td>21.4 b</td>
<td>68.4</td>
<td>17.1 a</td>
<td>1.25 a</td>
</tr>
<tr>
<td>Disease control A-NS</td>
<td>67.7 a</td>
<td>0</td>
<td>13.6 b</td>
<td>1.02 b</td>
</tr>
<tr>
<td>B-NS</td>
<td>20.9 b</td>
<td>68.6</td>
<td>17.2 a</td>
<td>1.27 a</td>
</tr>
<tr>
<td>Disease control B-NS</td>
<td>66.5 a</td>
<td>0</td>
<td>13.7 b</td>
<td>1.02 b</td>
</tr>
<tr>
<td>C-NS</td>
<td>61.1 b</td>
<td>10.5</td>
<td>13.9 a</td>
<td>1.07 a</td>
</tr>
<tr>
<td>Disease control C-NS</td>
<td>68.3 a</td>
<td>0</td>
<td>13.6 a</td>
<td>1.01 a</td>
</tr>
</tbody>
</table>

*Protective value = (1 - percentage of bacterial wilt in a treatment/percentage of bacterial wilt in a disease control) × 100.

†No wilted plants in each treatment, means of 40 random plants.

‡FW, fresh weight of the seedling at 60th day after transplantation. Means of the surviving plants.

Data are the average of three experiments for nine test spots and they indicate values, with the letter ‘a’ being the highest, and ‘b’ the lowest value. The same letters within a column mean no significant differences exist between the numbers.

A, 7 days before transplantation; B, the same time mixed with Ralstonia solanacearum; C, 7 days after transplantation; S, sterile soil; NS, nonsterile soil.

Biological control against bacterial wilt

Fig. 1. 16S rRNA gene-based tree showing the relationships between Lu144 and 10 other strains of closely related genera. Numbers at the nodes are the bootstrap confidence values obtained after 1000 replicates. The number of substitutions per site is indicated by the bar at the top left of the phylogeny. Phylogenetic trees were generated by the neighbor-joining method based on the two-parameter Kimura correction of evolutionary distances. Sporolactobacillus inulinus was used as the outgroup. The GenBank accession numbers for nucleotide sequence data are shown in the brackets.

Tree was constructed based on the 16S rRNA gene sequence homology (Fig. 1). The result also implied that the endophytic strain Lu144 belonged to the B. subtilis family.

Localization of the Lu144 strain in mulberry

GFP-labeled Lu144 was constructed by transferring pGFP4412, an Escherichia coli–Bacillus cereus shuttle vector containing the gfp (mut3a) gene, into Lu144. The GFP-labeled Lu144 cells emitted green fluorescence with excitation wavelengths of 488 and 633 nm and could be detected by laser confocal microscopy. The constructed plasmids were stably maintained in Lu144 strains even in the absence of selective pressure. After 40 generations in the absence of antibiotic pressure, 60% of GFP-labeled Lu144 strain cells were neomycin- and ampicillin-resistant and had GFP fluorescence. Furthermore, the abundance of GFP-labeled bacteria colonizing the mulberry seedling after 30 days of inoculation (Fig. 2) also suggests significant stability of the plasmids. The optical sections of mulberry seedlings were examined under a laser confocal microscope at different times after inoculation with GFP-labeled Lu144. One day after inoculation, the bacterial cells were found to colonize the surfaces of the primary roots in the root hair zone (Fig. 2a), the zone of differentiation and elongation of the root tip (Fig. 2b) and the lateral root junction (Fig. 2c), suggesting that these are probably the sites at which Lu144 enters the mulberry roots. Three days after inoculation, the majority of the bacteria were found to live in the intercellular spaces of the cortical parenchyma and the root hair, and a few bacterial cells were located within the epidermis (Fig. 2d and e). The bacteria had progressed towards the inner cortex of the primary root at the fifth day (Fig. 2f) and penetrated into the xylem vessels and piths of the primary roots at the seventh day (Fig. 2g and h). Nine and 11 days after inoculation, the bacteria were found in cell aggregates in the tissues of the root (Fig. 2i and j), indicating that the bacteria had finished the process of infecting the roots within 7 days.

The presence of the bacteria in the xylem vessels and parenchyma cells of the stem by day 14 (Fig. 2k and l) indicated that the bacteria had finished migrating from the roots to the stems. Sixteen days after inoculation, the bacteria could be observed in the vascular bundles at the

FW, fresh weight of the seedling at 60th day after transplantation.
junction of the stem and leafstalk (Fig. 2m) and the vascular bundles of leaves (Fig. 2n), indicating that they had invaded into the leaves through the vascular bundles. The bacteria were found in the leaf veins (Fig. 2o) on day 20 after inoculation and in the intercellular spaces of the leaves on day 30 after inoculation (Fig. 2p). There was no apparent variability in bacterial colonization progression between the sampling plants. In contrast, no GFP-labeled Lu144 cells
were found in the control plants. Together, our results indicated that the GFP-labeled bacterial cells infected the roots through the zone of differentiation and elongation and the cracks formed at the lateral root junctions, and then penetrated into the cortex, xylem, and pith and migrated slowly from the roots to the stems and leaves. The Lu144 strain was mainly located in the intercellular spaces.

Quantification of endophytic population

To quantify the endophytic population, the labeled strain of Lu144 was isolated from the surface-disinfected roots, stems and leaves of mulberry seedlings (Fig. 3). The results showed that the bacteria could be reisolated from surface-sterilized roots and stems and could successfully establish their incidence in roots by the 7th day after inoculation. Fourteen days after inoculation, small amounts of the bacteria were found in the leaves, which indicated they had already spread to all plant parts. Bacterial growth in the plant during that time was as follows: after an initial increase, there was a decrease in the number of bacteria. Although the strain maintained high persistence in roots all the time, the stems and leaves were more heavily colonized by the bacteria than the roots by the 28th day after inoculation. This suggests that Lu144 could transfer from roots into stems and then to leaves, and the leaves may be more suitable as a microbial habitat for the bacteria during plant development. The bacteria could be recovered from the roots, stems and leaves of the plants even after 42 days without causing any visible damage, confirming that Lu144 was a true endophyte. The number of bacteria was around \(10^3\)–\(10^6\) CFU g\(^{-1}\) of fresh plant tissue. In contrast, the control seedlings did not yield bacterial colonies when their surface-disinfected roots, leaves and stems were plated on King’s medium B containing neomycin and ampicillin.

Discussion

*Bacillus subtilis*, the type species of the genus, is commonly found in soil, water sources and in association with plants (Chun & Sook, 2000). Some *B. subtilis* isolates have been reported to be endophytes in the internal tissues of plants; they are effective in the biocontrol of multiple plant diseases caused by soilborne pathogens and have been used in commercially available biocontrol products (Ongena et al., 2005). However, *B. subtilis* has not been reported as a biological control agent against bacterial wilt of mulberry. Our present results demonstrated that the strain *B. subtilis* Lu144 is an endophyte that can colonize root, stem and leaf tissues of mulberry seedlings rapidly and efficiently following the application of the bacteria by soil drenching. The colonization pattern was similar to many other endophytes (An et al., 2001; Liu et al., 2003, 2006). To our knowledge, this is the first detailed report of the colonization pattern for Gram-positive endophytic *B. subtilis* in mulberry.

The presence of the endophyte in mulberry tissues had positive effects on suppression of infection by *R. solanacearum*, resulting in reduced incidence of bacterial wilt. The exact mechanisms underlying these biological responses remain unclear. Biofilm formation is one of the critical adaptations used by microorganisms to resist and survive adverse conditions (Emmert & Handelsman, 1999; Davey & O'Toole, 2000) and can protect the host plant from pathogens (Bais et al., 2004). In this study, biofilm formation was not found on the mulberry seedling roots (Fig. 2), implying that biofilm formation was not the main operating mechanism. Kloepper et al. (1999) reported that induced systemic resistance (ISR) might be one of the most important operating mechanisms when dealing with biocontrol of systemic plant pathogens. Other investigators proved that ISR is triggered by bacterial inoculation (Van Peer et al., 1991; Benhamou et al., 1996; Wilhelm et al., 1998). We suggest that disease suppression might also be due to the induction of resistance in the mulberry plant, given that the bacterial populations established on roots (Fig. 3) were over the threshold necessary to trigger ISR (Raaijmakers et al., 1995).

The colonization of the Lu144 strain might modulate gene expression in plants and trigger the plant defense mechanism to enhance resistance toward pathogen ingress. When the pathogen *R. solanacearum* infects the host, it first invades host roots through wounds, notably those created by lateral root emergence, aggregates in the intercellular spaces.
of cortex tissues, and then ingresses into vascular bundles and spreads in xylem vessels to induce the wilt symptom (Tans-Kerssen et al., 2001). The process and parasitized sites of *R. solanacearum* are similar with the Lu144 strain in mulberry. Therefore, the systemic distribution of Lu144 throughout the plant may be partially responsible for the competitive exclusion of *R. solanacearum* from internal mulberry tissues. In our study, we found that cultures of Lu144 grown on PDA medium plates produced a metabolite(s) that was released into the medium and inhibited *R. solanacearum* (data not shown). This inhibitory activity may be partially attributed to the production of antibiotic compounds.

Genetic and biochemical studies will be conducted to determine the mechanisms by which Lu144 suppresses disease caused by *R. solanacearum*. Although Lu144 showed promising potential for significantly reducing the incidence of disease in plants that were artificially infested with *R. solanacearum* in a greenhouse, the beneficial traits have not been confirmed by field trials. Large-scale field trials will be conducted under varying environmental conditions to demonstrate the efficacy of this bacterium for the suppression of disease caused by *R. solanacearum* on mulberry.

References


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