

Xiaojie HOU, Zhengnan LI, Dangyue HAN, Qiuxian HUANG, Longxian RAN

Presence of indigenous endophytic bacteria in jujube seedlings germinated from seeds *in vitro*

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Abstract The presence of indigenous endophytic bacteria in tissue-cultured seedlings germinated from seeds of *Zizyphus jujuba* var. Fupingdazao was investigated using cultivation, light microscopy and scanning electronic microscopy (SEM). In addition, bacteria specific 16S rDNA PCR followed by denaturing gradient gel electrophoresis (DGGE) was used. No cultivable bacteria could be detected on plates or in liquid cultures. However, large quantities of endophytic bacteria in jujube seedlings were observed under the light microscope. The bacterial cells were round (measuring $1.5\ \mu\text{m}$ – $1.8\ \mu\text{m}$), short rods ($2.2\ \mu\text{m}$ – $2.9\ \mu\text{m} \times 1.1\ \mu\text{m}$ – $1.9\ \mu\text{m}$) and rod shaped ($3.7\ \mu\text{m}$ – $4.4\ \mu\text{m} \times 1.6\ \mu\text{m}$ – $1.9\ \mu\text{m}$). Rod-shaped bacterial cells (measuring $3.5\ \mu\text{m}$ – $4.0\ \mu\text{m} \times 1.5\ \mu\text{m}$ – $2.0\ \mu\text{m}$) were observed by SEM as well. A 16S rDNA fragment of 1.5 kb could be amplified from the total DNA of seedlings of jujube using the bacterial primer pair 27F/1525R. The V3 fragment of 16S rDNA was separated by DGGE, and the 16S rDNA-PCR-DGGE showed that there were at least six dominant bands within the seedlings of *Z. jujuba* var. Fupingdazao. It can be concluded that endophytic bacteria that cannot be detected by cultivation are present with high densities in jujube seeds.

Keywords DGGE, optical microscope, scanning electron microscopy (SEM), unculturable bacteria, *Zizyphus jujuba*

1 Introduction

Zizyphus jujuba Mill., i.e. jujube, belonging to the family

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Xiaojie HOU, Dangyue HAN, Qiuxian HUANG, Longxian RAN (✉)
Section Forest Pathology, Forestry College, Agricultural University of Hebei, Baoding 071001, China
E-mail: longxianran@hebau.edu.cn

Zhengnan LI
National Engineering Research Center for Agriculture in Northern Mountainous Areas, Baoding 071000, China

Rhamnaceae, is the most widely grown dried-fruit-oriented tree species in China and used for medicinal purposes and grown as a cash plant. It is distributed throughout China except for Heilongjiang Province, and 98% of the *Zizyphus* species resources are in China (Qu and Wang, 1993). The leaves, fruit-bearing branches, bark and wood of jujube are rich in vitamin, raw tannage materials, tannin, and microelements, such as iron and zinc. Its flowers are excellent feed stock for honey, and the fruits, rich in vitamin C, can be used both as food and medicinal components. Jujube trees, with strong adaptability to arid conditions, and main economic benefits for farmers in rural areas, have played an important role in agricultural production. However, with the steady increases of jujube plantations, damages due to different diseases are becoming the biggest obstacle for jujube production. Of all the diseases, jujube witches' broom, jujube fruit-shrinking disease and mosaic leaf disease are the most serious problems, and no effective control measures are available yet (Bi, 2009). Therefore, use of endophytic bacteria to control these diseases is worthwhile to be investigated.

Endophytic bacteria are characterized by colonizing the plant tissues internally without causing substantive harm to the plant (Hallmann et al., 1997). Once inside the plant, an endophyte occupies a niche with a favorable microenvironment protected by plant tissues, relatively low competition from other microorganisms, and sufficient carbon and nitrogen sources (Han and Song, 2004). These culturable bacteria can be isolated from roots, leaves, stems, and fruits of different crops (Mukhopadhyay et al., 1996; Reiter et al., 2002; Yan et al., 2004; Li et al., 2005). Upon inoculation, some of the bacterial endophytes were able to directly promote plant growth or to inhibit pathogen growth and/or activity (Fisher et al., 1992; Bacilio-Jiménez et al., 2001; Mercado-Blanco and Bakker, 2007). The presence of culturable bacteria within seeds of various plant species has also been reported (Mundt and Hinkle, 1976; Bacilio-Jiménez et al., 2001; Cankar et al., 2005; Mano et al., 2006).

Unculturable endophytic bacteria in seeds and axenic

culture were reported recently as well (de Almeida et al., 2009). Thomas et al. (2008) demonstrated that normally non-culturable endophytes gradually turned cultivable after 8–20 subcultures in *in vitro* grown banana. By using culture-independent methods, i.e. electron microscopy and PCR-DGGE, unculturable endophytic bacteria were demonstrated to be present in axenic culture of pineapple (Abreu-Tarazi et al., 2010) and axenically grown seedlings of *Eucalyptus urophylla* (Shen et al., 2010), confirming the ubiquitous existence of unculturable indigenous bacteria in *in vitro* culture of plants.

In our previous study, we found that some endophytic bacterial strains, isolated from eucalypt and tomato, could colonize the root of *in vitro* grown jujube seedlings germinated from seeds; however, their colonization decreased gradually with time. It was also found that the population of *Pseudomonas poae*, an endophytic bacterium isolated from tomato, was 100 times higher in tetracycline-treated jujube seedlings than in control jujube seedlings (Hou, 2007). This might be caused by suppression or elimination of indigenous bacteria in the *in vitro* germinated jujube seedlings by the tetracycline (Ran et al., unpublished data). Liu et al. (2005) found that leaf-tip necrosis of micropropagated static plantlets, a serious problem in commercial laboratories in Taiwan, was associated with the existence of endophytic bacteria, and it could be controlled by subculturing affected plantlets on antibiotic-amended medium. It has been reported that endophytic bacteria in seed or plants can inhibit colonization of other bacteria (Bacilio-Jiménez et al., 2001); thus, we hypothesize that indigenous endophytic bacteria in jujube seedlings can hamper the colonization of exogenous biocontrol bacterial strains. In this study, we explored the presence of indigenous endophytic bacteria in jujube seedlings using cultivation-dependent dilution plating and cultivation-independent microscopical and molecular techniques.

2 Materials and methods

2.1 Materials

Air-dried fruits of *Z. jujuba* var. Fupingdazao, stored at 4°C in the refrigerator for 3 years, were used.

2.2 Cultivation of seedlings

The thoroughly dried endocarp of the fruits of *Z. jujuba* var. Fupingdazao was knocked open with a sharp plier, and seeds were taken out, surface-sterilized by successively dipping in 70% ethanol for 30 s and 0.1% acidic mercury chloride for 3.5 min, rinsing with sterile distilled water four times and then drying on sterile towel paper. The surface-sterilized seeds were placed in an Erlenmeyer flask containing 50-mL solid MS medium for germination.

Solid MS medium consisted of MS basal medium, vitamins (Murashige and Skoog, 1962), 3% sucrose, and 0.8% agar at pH 5.8. Then the germinated seedlings were incubated in a growth chamber, maintained at 25°C daytime and 23°C nighttime with a 12-h photoperiod, and a relative humidity of 70%. The sprouts of seedlings at the height of about 3 cm were subcultured into the same medium, respectively. In this way, the identical seedlings with the same background of endophytes were achieved.

2.3 Isolation of endophytic bacteria

Three *Z. jujuba* var. Fupingdazao seedlings were ground in a mortar with a pestle by using 1-mL sterile water, and 100-μL aliquot was plated on modified nutrient agar (NA) (Zheng et al., 2008), respectively. Plates were incubated at 28°C, and the colonies were counted after 2 weeks. Alternatively, 200-μL aliquot was transferred to each Erlenmeyer flask with liquid NA medium and incubated in a shaking incubator at 150 r·min⁻¹ and 28°C for 2 weeks.

2.4 Microscopic observation of endophytic bacteria

2.4.1 Light microscopy (LM)

Three seedlings of *Z. jujuba* var. Fupingdazao were pestled as described previously. The trituration suspensions were collected into a sterile centrifuge tube and centrifuged at 1141 × *g* for 5 min. The supernatant was transferred into a new sterile centrifuge tube and centrifuged at 10146 × *g* for 5 min. The final precipitate was suspended in 100-μL sterile water. A droplet of the suspensions was mounted onto a glass slide, observed and measured using a light microscope at a magnification of 1600 times.

2.4.2 Scanning electron microscopy (SEM)

The stem segments of seedlings (3–5 mm) were fixed for 12 h in glutaraldehyde solution (2.5%, pH 7.4) at 4°C, rinsed three times for 30 s each using double distilled water and further dehydrated in a graded ethanol series (50% for 10 min, 70% for 10 min, 80% for 10 min, 90% for 10 min). Then the samples were further dried by ter-butanol (75% for 10 min, 100% 10 min) at 4°C and finally put into a vacuum drier. The samples were treated with CO₂ and mounted on an aluminum cylinder with silver paste, and covered with a steam of carbon and ionized gold using ion plating equipment IB-3. Metal-coated specimens were observed under a SEM (Hitachi S-3500N) at an accelerating voltage of 20 kV.

2.5 Amplification of bacterial 16S rDNA

DNA was extracted from seedlings of *Z. jujuba* var. Fupingdazao using a CTAB protocol (Li et al., 2005). The fragment of 16S rDNA was amplified using the universal

bacterial primer pairs, 27F (5'- GAG TTT GAT CCT GGC TCAG-3') and 1525R (5'-GAA AGG AGG TGA TCC AGCC-3' (Zhang et al., 2000), with polymerase chain reaction (PCR) in a volume of 25 μL :2.5 μL 10 $\times$ PCR buffer (contained 20 $\text{mmol}\cdot\text{L}^{-1}$ Mg^{2+}), 0.5 μL template DNA, 12.5 $\text{mmol}\cdot\text{L}^{-1}$ dNTP mixture, 1 U Taq DNA polymerase, and 10 pmol of each bacterial primer. Amplification was done with an initial denaturation for 10 min at 95°C followed by 30 cycles of 30 s at 95°C, 30 s at 54°C, and 1.5 min at 72°C, with a final extension of 5 min at 72°C. The PCR products were ascertained by electrophoresis on 1.2 % (w/v) agarose gels. The expected size of the amplified fragment was 1500 bp.

2.6 PCR- DGGE analysis

The V3 fragment of 16S rDNA was amplified using the universal bacterial primer pair with a GC clamp, i.e., F357GC (5'- CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGGG CCT ACG GGA GGC AGC AG-3') and R518 (5'-ATT ACC GCG GCT GCT GG-3') (Xing et al., 2006), and the amplified product was a short fragment of 230 bp, which was more suitable for DGGE separation. PCR products were subsequently analyzed by denaturing gradient gel electrophoresis (DGGE), with the model D-Code Universal Mutation Detection System (BioRad, Hercules, CA) on 16 cm $\times$ 16 cm $\times$ 1 mm gels. DGGE analysis was performed as previously described (Muyzer et al., 1993). The polyacrylamide gels were made with denaturing gradients ranging from 45% to 70%, and run for 7 h at 130 V and 60°C, followed by silver staining and photographing.

3 Results and analysis

3.1 Cultivation of indigenous endophytic bacteria in jujube seedlings

No bacterial growth was observed either in the modified NA agar plates or in the liquid modified NA medium after 2 weeks of incubation.

3.2 Microscopic observation of endophytic bacteria

3.2.1 Light microscopy

The concentrated suspensions of *in vitro* growing jujube seedlings were observed under optical microscope (Fig. 1). The presence of indigenous endophytic bacteria, which can be easily distinguished from the plant organelles by their identical shapes, was obvious in jujube seedlings, and their appearances were spherical, rod-shaped and club-shaped with the sizes of 1.5 μm –1.8 μm , 2.2 μm –2.9 μm $\times$ 1.1 μm –1.9 μm and 3.7 μm –4.4 μm $\times$ 1.6 μm –1.9 μm .

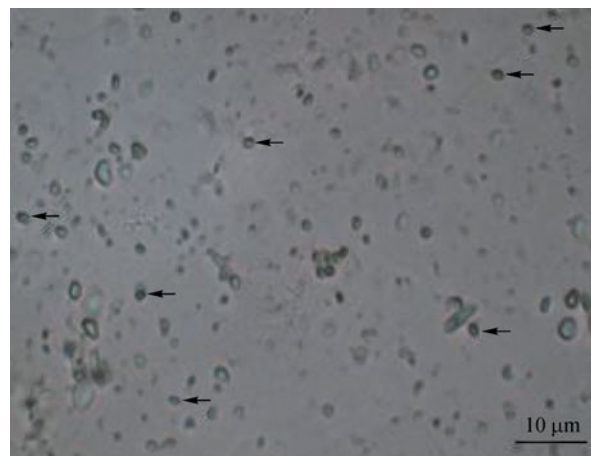


Fig. 1 Presence of endophytic bacteria in seedlings germinated from seeds of *Zizyphus jujuba* var. Fupingdazao observed by optical microscope

3.2.2 Scanning electron microscopy (SEM)

The intracellular endophytic bacteria aggregated in the phloem cell of the stem segment of jujube seedling were observed under SEM. They were rod-shaped with the size of 3.5 μm –4.0 μm $\times$ 1.5 μm –2.0 μm (Fig. 2). The number of endophytic bacteria was few in stems of jujube seedlings, and they were not evenly distributed.

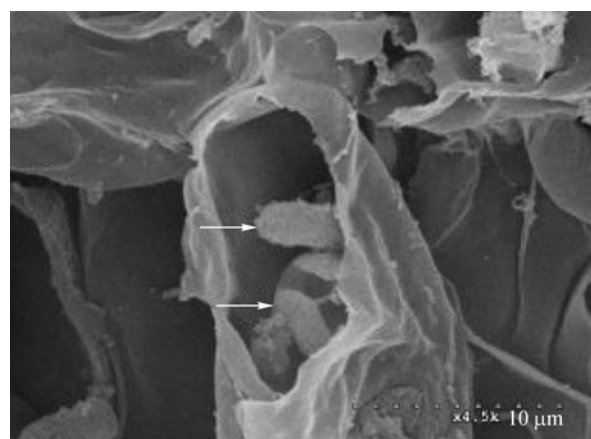


Fig. 2 Observation of endophytic bacteria inside the phloem cell of seedling germinated from seeds of *Z. jujuba* var. Fupingdazao by SEM

3.3 Amplification of endophytic bacterial 16S rDNA

A single band of 16S rDNA at the size of 1500 bp in all tested samples was amplified using the universal bacterial primer pairs 27F/1525R (Fig. 3). Therefore, the PCR strategies proved that there were indigenous endophytic bacteria colonizing the tissue of seedlings in *Z. jujuba* var. Fupingdazao.

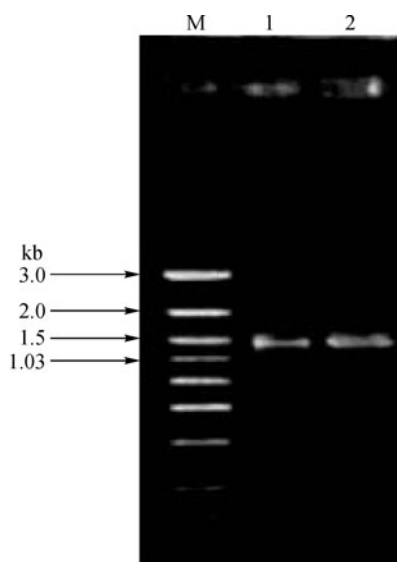


Fig. 3 Amplification of 16S rDNA fragments of endophytic bacteria in jujube seedlings germinated from seeds
Note: M is Marker DL3000; Lanes 1 and 2 represent two seedlings germinated from seeds of *Z. jujuba* var. Fupingdazao.

3.4 DGGE analysis of 16S rDNA V3 amplicons

The 16S rDNA V3 fragment of endophytic bacteria amplified with the universal primers in *Z. jujuba* var. Fupingdazao was separated by DGGE. In this way, we could distinguish the microbial community structure and biodiversity of the samples. Each band corresponded approximately to a dominant microbial community or an operational taxonomic unit, and more bands suggested richer biodiversity. Moreover, a brighter band signal suggested a higher richness of the species. Therefore, we could obtain information about the number of dominant species and quantity of microbes in the samples (Zhao et al., 2008). The DGGE patterns were showed in Fig. 4. At least six dominant bands (a, b, c, d, e and f) could be observed. Samples 1 and 2, extracted from two seedlings obtained from the same seed, respectively, had almost the same endophytic bacteria patterns, indicating that *in vitro* propagation through subculture of axillary buds or shoot tips for two or three times did not change the dominant populations of endophytic bacteria.

4 Conclusions and discussions

In this study, all tested plant materials were propagated by subculturing from one seedling germinated from the jujube seed, and seedlings with the same population background of endophytic bacteria were obtained. No culturable bacteria were recovered from the triturate of the seedlings from the fourth or fifth subculture using both plating and liquid medium cultures. However, lots of bacterial cells, most of them with short-rod shape, were observed under

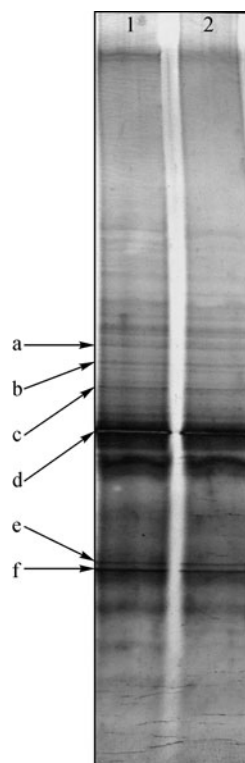


Fig. 4 DGGE profiles of 16S rDNA of endophytic bacteria in jujube seedlings germinated from seeds
Note: Lanes 1 and 2 represent two seedlings germinated from *Z. jujuba* var. Fupingdazao seeds.

optical microscope. Because the optical observation is easily interfered by tissue particles, little investigations were performed to study the infection and colonization of endophytic bacteria in the cellular level (Dong et al., 1994; James et al., 1994; Döbereiner et al., 1995; Taechowisan et al., 2003). To confirm the existence of endophytic bacteria in the tissue of jujube seedlings, scanning electron microscopy was used to detect endophytes in the jujube plantlets. Bacterial cells were observed inside phloem cells in cross sections of the stem tissue.

Molecular detection of endophytic bacteria was performed using universal primers to amplify bacterial 16S rDNA, and a clear band with the size of 1.5 kb could be seen in all tested plant materials, indicating that endophytic bacteria were present in seeds of the Fuping jujube cultivar.

The majority of microorganisms in nature cannot be cultured, and it is difficult to detect the presence of endophytic bacteria using conventional microbiological methods (Amann et al., 1995). Endophytic bacteria in seeds that cannot be cultured might have important ecological and economic effects (Tyson et al., 2004). Analysis of the diversity and dynamics of unculturable endophytic bacteria species in plant requires the application of molecular detection methods. DGGE or temperature gradient gel electrophoresis (TGGE) is capable of estimating or determining the variations of endophytic

bacteria between different plants or the number of endophyte species in the same plants. At present, DGGE and TGGE are widely used for the study of microbial diversity (Wu et al., 2006; Zhao et al., 2008; Zhang et al., 2009). Based on the DGGE patterns of 16S rDNA of endophytic bacteria amplified from the total DNA of jujube seedlings, at least six clear bands were observable; thus, we can infer that more than six dominant species populations intrinsically coexisted within the jujube seeds.

This research confirmed the intrinsic existence of endophytic bacteria within jujube seed seedling. The plant is a complex system containing a variety of microbes, and the metabolites of endophytic bacteria not only have effects on the plant itself, but may also affect plant pathogens (Li and Luo, 2003; He et al., 2004). The possible involvement of these endophytic bacteria in disease resistance and their application in agricultural practice needs further investigations.

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