



Full Length Article

Isolation and Characterization of Bacterial Endophytes from Ramie (*Boehmeria nivea*)

Xiang-ping Sun^{1*}, Meng-ya Chen², Liang-bin Zeng¹, Jian-jun Li¹, Li Yan¹

¹Institute of Bast Fiber Crops, Chinese Academy of Agricultural Sciences, Changsha 410205, Hunan Province, China

²College of Plant Protection, Hunan Agricultural University, Changsha 410128, Hunan Province, China

*For correspondence: sunxiangping@caas.cn

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Abstract

Endophytic bacteria provide several advantages to the host plant by promoting growth as well as conferring resistance against pathogens and toxic compounds. More than 90% of the cultivation of ramie [*Boehmeria nivea* (L.) Gaud] is contributed by China. In our study, 265 isolates of bacterial endophytes were obtained from ramie plants. The isolates were then subjected to PCR-based screening. Four endophytic bacterial phyla were identified using 16S rDNA sequencing. The identified dominant microorganisms were *Janibacter melonis*, *Moraxella*, and *Bacillus pumilus* in the root, stem, and leaf, respectively. The results suggest that these endophytes isolated from the ramie have the potential to act as biocontrol agents against pathogens such as *Sclerotinia*, *Colletotrichum*, and *Phytophthora*. However, further study is required to examine the potential application of bacterial endophytes, particularly as biological control agents. © 2021 Friends Science Publishers

Keywords: Endophytes; Host plant; Isolate; Biological-control

Introduction

Endophytes are microorganisms that live asymptotically inside the tissues of a wide variety of host plants (Shubhpriya *et al.* 2020). Endophytes have also been known to produce a large variety of metabolites with unique structures often beneficial to plants (McInroy and Kloepper 1995). Therefore, such microbe-host interactions play a major role in creating plant biodiversity (Goda *et al.* 2006). The first reports of endophytes associated with plant analyzed ecotypes of alkali grass (Chlebicki and Lembicz 2001). A wide variety of novel metabolites produced by endophytes have exhibited diverse biological activities (Pimentel *et al.* 2011). The endophytic microbiome can serve as a reservoir of important secondary metabolites, including antibiotics, anticancer molecules, and antioxidants (Qin *et al.* 2020). Many reports suggest that bacterial endophytes have great potential as plant growth promoters (Barac *et al.* 2004; Chauhan *et al.* 2013; Balla *et al.* 2019). There are many reports on the presence of endophytes in plants, which were isolated from different parts of the plant rather than the seed of different plants (McInroy and Kloepper 1995; Strobel and Daisy 2003; Balla *et al.* 2019). Few studies have reported the intracellular presence of endophytes in different tissues of many plants (McInroy and Kloepper 1995; Strobel and Daisy 2003). The relationship between the host plant and

endophytes has been influenced by many factors, such as genotype, plant growth stage, physiological state, and environmental conditions (Davitt *et al.* 2011; Afzal *et al.* 2019). Rosenblueth and Martinez-Romero (2006) found that since bacterial endophytes reside inside the plant tissues, they have been more resistant to biotic and abiotic stresses compared to the rhizospheric bacteria in the environment. However, the use of endophytes has resulted in an excessive loss of propagules, which makes them expensive. Several endophytes are being used in agricultural cropping systems for the biological control of phytopathogens (Sturz *et al.* 1998; Lugtenberg *et al.* 2002).

Ramie [*Boehmeria nivea* (L.) Gaud], known as “China grass”, is an important fiber crop and a herbaceous perennial crop belonging to Urticaceae, the nettle family (Qin *et al.* 2020). China contributes more than 90% of the total production of ramie (Qin *et al.* 2020). Ramie fibers are famous for their excellent properties, such as great tensile strength, high thermal conductivity, silky luster, good ventilation, high moisture absorption, antibacterial properties, etc. (Qin *et al.* 2020). In this study, symptoms of the disease of ramie included stunted plants and a reduced number of ramets per plant (Goda *et al.* 2006). The study aimed to (1) isolate and characterize the endophytes from ramie and (2) evaluate the biocontrol potential of isolated endophytes against various fungal pathogens.

Materials and Methods

Plant sampling and isolation of endophytic bacteria

Samples from different tissues of five ramie plants, including root, stem, and leaf, were collected at the mature stage from ramie fields in Yuanjiang (E: 112.33, N: 28.16), Hunan province, in autumn 2018. The plant tissues were washed under tap water with a paintbrush to remove any debris or dirt from the surface of the plant. Samples were then surface-sterilized according to the protocol developed by Shyam *et al.* (2020). Different surface disinfection processes were performed to isolate the endophytes from different plant tissues (Sun *et al.* 2016). These methods were as follows: ramie leaves were treated with 70% ethanol for 2 min and 1 g L⁻¹ silver nitrate for 1 min; stems were subjected to 70% ethanol for 2 min and 20 g L⁻¹ potassium permanganate; likewise, root samples were immersed in 70% ethanol for 2 min followed by 2% sodium hypochlorite for 1 min. The water blank was used as the control in this study. The stems, leaves, and roots of ramie plants were cut into 2-cm pieces and thoroughly ground using a pestle and mortar in the Bio-Clean bench.

The ground samples were then serially diluted, and a 0.1 mL aliquot was spread on the LB (Luria-Bertani) medium. Firstly, the cultures of endophytes were incubated at 30°C for 24 h. Secondly, morphologically different endophyte colonies were selected and purified on the nutrient agar medium (NA) after three days of incubation at 37°C. Finally, individual purified colonies were stored at 4°C on the nutrient agar medium (NA), and their rates of growth were analyzed using MS office 2010.

16S rRNA gene amplification and sequencing analysis of bacterial endophytes

The genomic DNA of single bacterial endophytes was isolated following the manufacturer's instructions using the GeNeiPure™ bacterial DNA purification kit (GeNei™, Bengaluru, India). The DNA extraction, PCR amplification of 16S rRNA genes, and gene sequencing of selected endophytic actinobacterial isolates were carried out according to instructions. The amplicons were purified and quantified at 260 nm using calf thymus DNA as a control. Universal eubacterial primers (B27F: 5'-AGAGTTTGATCCTGGCTCAG-3' and U1492R: 5'-GGTTACCTTGTTACGACTT-3') were used to amplify a region of about 1500 bp of the 16S rRNA gene sequence using a thermal cycler (BioRad, USA). PCR amplified products of bacterial endophytes were resolved on a 1.5% agarose gel and visualized using a gel documentation system. The 16S rDNA purified partial amplicon was sequenced using an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems®, USA).

Analysis of endophytic bacterial 16S rDNA sequences

Sequences of the bacterial endophytes isolated from the

ramie were compared with the bacterial sequences obtained from the National Center for Biotechnology Information (NCBI), and sequences showing > 99% similarity were retrieved from Nucleotide Basic Local Alignment Search Tool (N BLAST) program available on the NCBI BLAST server (www.ncbi.nlm.nih.gov/BLAST).

Scanning electron microscopy (SEM)

The roots, stems, and leaves of the ramie plant were fixed in 4% glutaraldehyde in 0.1 M sodium cacodylate buffer. The tissues were then post-fixed in 2% osmium tetroxide containing 0.1 M cacodylate buffer. The samples were rinsed with buffer, dehydrated using ethanol, exposed to drying until the critical point was reached, mounted on specimen holders, coated with gold-palladium, and examined by an SEM (JSM-6360LV, NEC).

Screening of endophytic antagonistic bacteria

The pure cultures of the pathogenic fungi including *Phytophthora capsici* (*Linum usitatissimum*), *Rhizoctonia solani* (*Solanum tuberosum*), *R. solani* (*Oryza sativa*), *Colletotrichum linicolum*, *Fusarium oxysporum* f. sp. *lini*, *F. oxysporum* f. sp. *cucumerinum* and *Sclerotinia sclerotiorum*, were provided by the Chinese Academy of Agricultural Sciences, Institute of Bast Fiber Crops. These phytopathogenic fungi were then cultured at 25°C for 5~7 days. The antagonistic activities of endophytic bacterial isolates were evaluated on PDA plates through the dual culture plate method (Ren *et al.* 2012). The isolated strains were tested in vitro for their antagonistic activity against the pathogenic fungi. A 5-mm diameter pathogen disk was placed at the center of the 9-cm PDA plate, and the plate was incubated at 25°C for 2~3 days. Endophytic bacterial isolates were spot-inoculated on the surface of the agar plate 2.5 cm away from the fungal disc at 25°C. PDA plates without the antagonistic strain served as the control. Treatments were replicated three times.

Results

Identification of the dominant endophytic bacteria isolated from ramie tissues

The numbers of bacterial endophytes isolated from ramie tissues were 4.4×10², 35×10², and 40×10² CFU g⁻¹ fresh weight in root, stem, and leaf, respectively. Therefore, the number of bacteria in roots was lower than in leaves and stems. The results of our study suggest that the behavior of bacterial endophytes could be different in different plants, depending on the host and environmental conditions.

Phylogenetic analysis of endophytic bacteria isolated from ramie tissues

Endophytic bacterial isolates of different tissues of ramie plants were identified based on morphological, biochemical,

Table1: Dominant endophytic bacterial genera inhabiting tissues of ramie

Host organ	Genus	Number of isolates
Root	<i>Janibacter melonis</i>	16
	<i>Paenibacillus</i>	6
	<i>Bacillus</i>	2
	<i>Actinomadura</i>	1
	<i>Streptomyces</i>	1
	Unidentified genera	2
Stem	<i>Moraxella</i>	146
	<i>Moraxella osloensis</i>	3
	<i>Brevundimonas</i>	3
	<i>Rhizobium</i>	2
	<i>Bacillus</i>	1
	<i>Microbacterium</i>	1
	<i>Nocardioidea</i>	1
	<i>Geodermatophilus</i>	1
	Unidentified genera	7
	Leaf	<i>Bacillus</i>
<i>Sphingomonas</i>		11
<i>Staphylococcus</i>		5
<i>Microbacterium</i>		6
<i>Rhizobium</i>		3
<i>Janibacter melonis</i>		3
<i>Rhodococcus</i>		4
<i>Xanthomonas</i>		2
<i>Quadrisphaera granulorum</i>		1
Unidentified genera		11

and molecular characteristics. Isolates were subjected to 16S rDNA sequencing for molecular identification of isolated bacterial strains. The diverse bacterial populations were identified by analyzing the 16S rDNA clone libraries (Table 1). A total of 265 endophytic bacteria were isolated from various healthy tissues, including roots (27), stems (175), and leaves (88), of the ramie plant. Four phyla of bacterial endophytes were identified. However, many endophytic bacterial isolates could not be identified in this study (Table 1). The distribution of species collected in this study varied between regions.

Many endophytes isolated from ramie have exhibited predatory characteristics and can inhibit the growth of other bacteria. Among all the isolates identified, the dominant endophytes were *Janibacter melonis*, *Moraxella* spp., and *Bacillus pumilus* in the root, stem, and leaf, respectively. The *Bacillus* spp. were found in all tissues of the ramie plant. They present a wide distribution of natural product biosynthetic gene clusters. All bacterial endophytes isolated from different tissues of ramie by culture methods belonged to a total of 4 genera. Among these four genera, *Moraxella* spp. were more abundant than other genera. In this study, among all isolated endophytes from ramie, *Moraxella* was the predominant genus. Several bacterial endophytes in the plant form spores and other dense refractive structures to survive periods of nutrient depletion. Some strains that could not be identified formed dense refractive structures in culture. The species identified in our study have been reported as endophytes isolated from different plants in other studies.

Physio-biochemical characterization

In this study, ramie endophytic bacterial variation could reflect variation in mean conidial lengths of isolates of different tissues of plants. SEM allowed the visualization of bacterial cells inside different plant tissues and studying their distribution patterns and sizes.

The endophytes present in the root, stem, and leaf mainly exist in the forms of colonies, vascular bundles, and intercellular spaces, respectively (Fig 1). The life cycles of all the organisms occur inside the ramie tissues, without the appearance of symptoms of the disease at different growth stages of ramie.

The antagonistic effect of isolated bacterial endophytes

Different endophytic strains, including Y1 (*Sphingomonas* sp.), Y2 (*Bacillus cereus*), Y9 (*Bacillus* sp.), Y23 (*Bacillus* sp.), G12 (*Bacillus pumilus*), and their extracts, exhibited a wide range of activities against *S. sclerotiorum* (Lib.) de Bary, *C. linicolum*, *Cucumerinum owen*, and *Phytophthora capsici* Kuhn (*Linum usitatissimum*). In our study, the endophytic bacteria isolated from the ramie act as biocontrol agents (Fig. 2). However, the complex mechanisms and inter-species signaling pathways involved in biocontrol activities have not been elucidated.

Discussion

Since the identification of *Paenibacillus* around twenty years ago, many endophytic species isolated from different plants, with the potential to contribute to plant growth promotion and the use in biological control of plant pathogens, have been identified (Guo et al. 2008). Several species of bacterial endophytes isolated from different plants, including *Theobroma*, *Penicillium*, *Pseudozyma*, *Paraphaeosphaeria*, *Microsphaeropsis*, and *Talaromyces*, have been reported to be able to promote the growth of the host plant (You et al. 2016) and could also induce resistance to environmental stress and act as antimicrobial agents (You et al. 2016). In this study, we identified several endophytic bacterial populations colonizing all three tested tissues of the ramie plant, including roots, stems, and leaves. The distribution of endophytic species is often patchy owing to several host-related and environmental factors. Numerous species of endophytic bacteria could positively influence the root growth and morphology of the host plant by improving plant nutrient uptake (Tailor and Joshi 2014). Furthermore, the discovery of genome mapping techniques used in endophyte studies has allowed the identification and characterization of genes that encode important ecological information in the plant, especially the ramie, which may be due to the specific structure of ramie. Around 60 genera of endophytic bacteria have been identified from almost 30 kinds of plants, including rice, wheat, cotton, peanut, potato, tomato, lemon, and orange, among which about 2/3 were

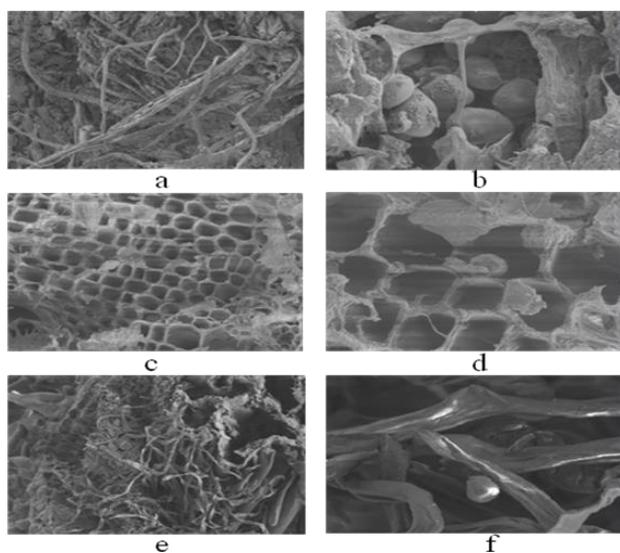


Fig 1: The SEM images of endophytic bacteria colonizing tissues of ramie

a: ramie root (200 μm); b: endophytes within the root tissue (20 μm); c: ramie stem (100 μm); d: endophytes within the stem tissue (20 μm); e: ramie leaf (30 μm); f: endophytes within the leaf tissue (10 μm)

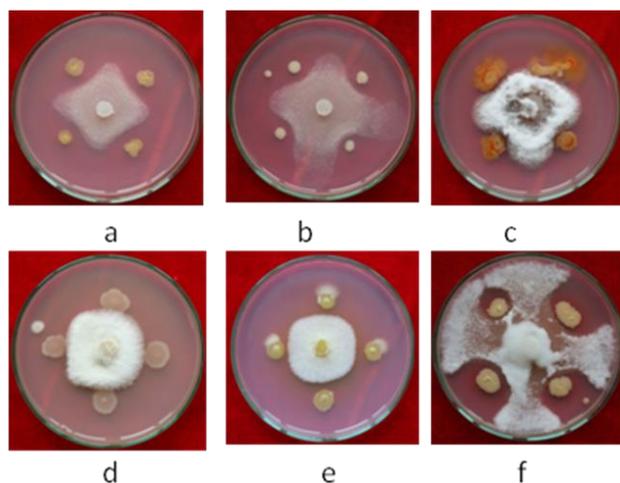


Fig. 2: The effect of endophytes isolated from ramie tissues on the growth of phytopathogenic fungi

a: *Sclerotinia sclerotiorum* (Lib.) de Bary (G12); b: *Sclerotinia sclerotiorum* (Lib.) de Bary (Y2); c: *Cucumerinum owen* Tochinai (Y1); d: *Cucumerinum owen* (Y9); e: *Cucumerinum owen* (G12); f: *Phytophthora capsici* Kuhn (*Linum usitatissimum*) (Y23)

orchideous negative bacteria (Gardner *et al.* 1982; Wongphatcharachai *et al.* 2015). In this study, we examined the distribution of endophytes inside tissues of the ramie plant using SEM. Our SEM observations indicate that endophytes, which are ubiquitous and may establish complex interactions with their host plants, live within plant tissues (Nair and Padmavathy 2014). Several studies have reported that the intercellular space in the plant is the most suitable niche for endophytic colonization (Monteiro *et al.* 2012). The distribution of endophytic bacteria in the plant

was firstly observed by Gardner *et al.* (1982), who identified several bacterial endophytes in the Florida citrus tree. The endophytes live inside the plant tissues due to the more stable environment than in soil (Gouda 2016). In the literature, the internal tissues of host plants provide a uniform and protective environment for bacterial endophytes in response to extreme environmental conditions (Taghavi and Lelie 2013). Bacterial endophytes could produce a variety of bioactive metabolites with antifungal properties (Strobel 2003). According to the literature review, 51% of new bioactive substances are derived from endophytes in host plants, whereas soil microbes produce only 38% of these compounds (Hyde and Soyong 2008). In our study, several endophytes isolated from the ramie plant were tested for biocontrol activity and were effective against plant diseases such as *Sclerotinia sclerotiorum* (Lib.) de Bary, *Colletotrichum linicolum*, *Cucumerinum owen* and *Phytophthora capsici* (*Linum usitatissimum*). In the present study, endophytic strain of G12 controlled *S. sclerotiorum* and *Cucumerinum owen* in pots by 82 and 88%, respectively and helped form callus to close wounds in the host plant. The successful application of bacterial endophytes with considerable biotechnological potential was reported by Barac *et al.* (2004). The scope of potential applications of endophytic microbes seems to be broad. The novel application of bacterial endophytes for improving plant growth through metabolizing compounds associated with the chemical wastes in host plants has been reported (Gouda 2016). Therefore, further research is required to better understand the mechanisms of interaction between endophytic microorganisms and plants. We tested 265 endophytic bacteria for their antagonistic effects against common phytopathogens such as *Phytophthora parasitica*, *S. sclerotiorum*, and *Colletotrichum* sp. The species of *Bacillus* were the most common isolated endophytic bacteria found in many plants (Suhandono *et al.* 2016). They also act as biocontrol agents against plant diseases and promote plant growth (Suhandono *et al.* 2016). Some *Bacillus* species reported as endophytic microbes could produce IAA (Indole-3-acetic acid, β -indoleacetic acid, and heteroauxin) and siderophores and improve plant growth by producing auxin and gibberellin in host plants. Endophytes produce many compounds with physiological activities similar to secondary metabolites released by the host plant (Guo *et al.* 2008; Chandra 2012; Uzma *et al.* 2018). Endophytes may reside and multiply in the host plant grown at high concentrations of salt or in a low ionic strength by conferring salt tolerance to the host (Suhandono *et al.* 2016; Uzma *et al.* 2018). The development of successful application technologies depends on improving our understanding of how bacterial endophytes enter and colonize plants in endophytic studies (Suhandono *et al.* 2016). Further research should be conducted to develop a suitable formulation and effective application techniques for maximizing plant productivity. An ecological awareness of the role of endophytes inside the host plants provides clues

for targeting the proper type of endophytic bioactivity with great potential for bioprospecting (Suhandono et al. 2016).

Conclusion

Ramie is an economically important crop plant of China. With a view to exploring potential benefits conferred by the endophytes of ramie, we identified and characterized endophytes found within ramie plants in the Hunan province of China. Our study identified few of these to have potential to become effective biocontrol agents. The dominant bacteria identified within ramie are also known to be present within other crop plants. Our study makes a significant contribution by laying the groundwork to further explore the roles of these classes of endophytes and their possible uses in plant or crop breeding techniques.

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Author Contributions

Xiang-ping Sun, Meng-ya Chen and Li Yan planned the experiments, Xiang-ping Sun and Meng-ya Chen interpreted the results, Jian-jun Li made the write up, Xiang-ping Sun statistically analyzed the data and made illustrations.

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