菌物学报

jwxt@im.ac.cn | 15 September 2015, 34(5): 996-1006
Http://journals.im.ac.cn | Mycosystema ISSN1672-6472 CN11-5180/Q

研究论文 Research paper DOI: 10.13346/j.mycosystema.150027

Endophytic fungal community of *Illicium verum* with different star anise yields in southern China

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Abstract: Chinese anise (*Illicium verum*), a traditional spice and medicinal plant in China, is endemic in southern China and northern Vietnam. Endophytic fungi may play an important role in promoting plant growth and yield, however, their relationship with star anise yield of *I. verum* remains enigmatic. In this study, foliar endophytic fungi from *I. verum* trees with different star anise yields were investigated in order to reveal the relationship between endophytic fungal incidence and star anise yield in Guangxi, southern China. A total of 412 fungal strains were isolated from 60 individuals of *I. verum*, and 22 fungal taxa were identified based on morphology and molecular biological techniques. *Colletotrichum gloeosporioides*, Dermateaceae sp., and *Phyllosticta* sp. 1 were found to be dominant in the endophytic fungal community. The mean colonization rate of endophytic fungi was 41%, with a range from 6 to 100%. Regression analysis showed that endophytic fungal colonization rate was positively correlated with star anise yield in *I. verum* individuals with low and moderate yield, but negatively correlated with star anise yield in individuals with high yield.

Key words: endophytic fungi, molecular identification, colonization, star anise yield, *Illicium verum*

我国南方不同产量的八角树内生真菌群落研究

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摘 要：八角 *Illicium verum* 原产于我国华南和越南北部，是我国传统的香料和药用植物。内生真菌可提高植物生长与产量。然而，八角的产果量与其内生真菌间的关系尚未有人报道。为揭示内生真菌与八角产量的关系，本文分析了广西壮族自治区不同八角产量植株的内生真菌定殖率和群落组成。从 60 棵植株的叶片中分离到 412 株内生真菌，并根据形态学和分子生物学方法将其鉴定为 22 个真菌分类单元。内生真菌群落的优势类群分别为 *Colletotrichum gloeosporioides*、Dermateaceae sp.和 *Phyllosticta* sp. 1。内生真菌定殖率的变化范围为 6%–100%，平均值为 41%。内生真菌的定殖率与八角

Supported by the Knowledge Innovative Program of the Chinese Academy of Sciences (KSCX2-EW-Z-6-2) and the National Natural Science Foundation of China (31100016, 31470151).
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Received: 02-02-2015, accepted: 10-05-2015

产量的回归分析表明，在较低和中等产量的八角植株中，内生真菌定殖率与产量呈正相关，而在高产的八角植株中，内生真菌定殖率与产量呈负相关。

关键词：内生真菌，分子鉴定，定殖，八角产量，八角

INTRODUCTION

The term “endophyte”, originally introduced by de Bary (1866), refers to any organisms occurring within plant tissues, distinct from the epiphytes that live on plant surfaces. Carroll (1986) defined endophytes as mutualists that colonize aerial parts of living plant tissues and do not cause symptoms of disease, from which pathogenic and mycorrhizal fungi are excluded. Petrini (1991) proposed an expansion of Carroll’s definition to include all organisms inhabiting plant organs that, at some time in their life, can colonise internal plant tissues without causing apparent harm to the host. Therefore, latent pathogens known to live symptomlessly inside host tissues that have an epiphytic phase in their life cycle are also endophytes (Guo 2001; Sun & Guo 2012). Endophytic fungi, as an important component in forest ecosystem, have been widely reported from numerous plant species, contributing significantly to diversity in natural ecosystems (Hyde & Soytong 2008; Suryanarayanan *et al*. 2011; Li *et al*. 2007; Wang & Guo 2007; Sun *et al*. 2011, 2012). Endophytic fungi can benefit their host plants in varied ways, such as growth promotion (Mucciarelli *et al*. 2003; Ting *et al*. 2008; Yates *et al*. 2005), heavy metal and drought stress tolerance (Monnet *et al*. 2001; Rudgers & Swafford 2009), and improved resistance against fungal pathogens (Arnold *et al*. 2003), virus (Lehtonen *et al*. 2006), nematodes (Richmond *et al*. 2004; Sikora *et al*. 2008), and insects (Gange *et al*. 2012; Miranda *et al*. 2011). Particularly, some endophytic fungi have been proved positive on tomato fruit yield. For example, endophyte *Leptodontidium orchidicola* doubled biomass of tomato and increased glucose content by 17% at early age (Andrade-Linares *et al*. 2011), and *Piriformospora indica* inoculation led to a significant increase in tomato fruit yield (Sarma *et al*. 2011).

Illicium verum Hooker fil., native to southern China and northern Vietnam, is a traditional spice and medicinal plant in China (Chempakam & Balaji 2008). Guangxi is the main and the largest star anise production area in China, yielding 60 000–80 000kg of dry fruit per year (Ma *et al*. 2011). Several studies have been carried out to enhance the star anise yield, such as cultivation of artificially dwarfed individuals (Huang & Wei 2008), selection of cultivars (Qin *et al*. 2004), fertilization (Deng *et al*. 2008), and refinement of integrated planting management (Ma *et al*. 2004). However, we know little about endophytic fungi associated with *I. verum*, except that Li *et al*. (2005) have screened some components with antitumour and antifungal activities from endophytic fungi associated with *I. verum*.

In order to investigate the potential effect of endophytic fungi on star anise yield, we selected 60 *I. verum* individuals with different star anise yields in Shanglin county of Guangxi, southern China. The endophytic fungi were isolated from the leaves and identified based on morphology and internal transcribed spacer (ITS) sequence data. The aims of

this study were to investigate the colonization and community composition of endophytic fungi associated with leaves of *I*. *verum*, and to understand the potential relationship between endophytic fungi and star anise yield in an *I*. *verum* plantation in southern China.

1 MATERIALS AND METHODS

1.1 Sampling sites and procedures

The sampling sites were located in an *I*. *verum* plantation on a hill near Dongchun Village (23°25'N, 108°32'E) in Shanglin County of Guangxi, southern China. The mean annual temperature was 20.9°C and the mean annual precipitation 1 789.2mm in 2011. The *I*. *verum* trees were planted in 1956, and were categorized into low and high production areas according to historical star anise yields. One transect was established in low and high production areas, respectively, with an interval of 2km between transects. Three 30m×30m plots were set up in each transect, and 10 plant individuals at intervals of 5m were randomly chosen in each plot in late August 2011. Ten healthy leaves were randomly collected from each individual and were immediately placed in plastic bags, labeled, and transported to the laboratory. Samples were stored at 10°C and processed within 4 days of collection. The star anise yield of each sampled individual was measured after harvest.

1.2 Isolation and identification of endophytic fungi

The sampling regime was designed with the intention of isolating as many endophyte species as possible from leaf samples. Selected leaves were cut into discs with 5mm in diam., and 16 leaf discs were randomly selected from each tree. In total, 960 discs (60 trees × 16 leaf discs) were used in this study.

Surface sterilization follows the method of Guo *et al*. (2000). Leaf discs were surface-sterilized by consecutive immersion for 1min in 75% ethanol, 3min in 3.25% sodium hypochlorite, and 30s in 75% ethanol. Four discs were then evenly placed in each 90mm Petri dish containing malt extract agar (MEA, 2%). Benzylpenicillin sodium (50mg/L, North China Pharmaceutical Group Corporation, China) was added to suppress bacterial growth. Petri dishes were sealed, incubated for 2 months at 25°C, and examined periodically. When colonies developed, they were transferred to new Petri dishes with potato dextrose agar (PDA, 2%) for purification. Vouched pure strains were transferred to PDA slants for further study. Subcultures on PDA were examined periodically and sporulating isolates were identified based on their morphological characteristics. The non-sporulating cultures were selected for further molecular identification (Guo *et al*. 2000).

1.3 DNA extraction, amplification and sequencing

Total DNA of each non-sporulating culture was extracted from fresh cultures following the protocol of Guo *et al*. (2000). The ITS (ITS1, 5.8S, ITS2) region was amplified using primer pairs ITS4 and ITS5 (White *et al*. 1990). Amplification was performed in a 50μL reaction volume which contained PCR buffer (20mmol/L KCl, 10mmol/L $(NH_4)_2SO_4$, 2mmol/L $MgCl_2$, 20mmol/L Tris-HCl, pH8.4), 200μmol/L of each deoxyribonucleotide triphosphate, 15 *p*mols of each primer, *c*. 100ng template DNA, and 2.5 units of *Taq* DNA polymerase (Biocolor BioScience & Technolgy Company, Shanghai, China). The thermal cycling program was as follows: 3min initial denaturation at 95°C, followed by 35 cycles of 40s denaturation at 94°C, 50s primer annealing at 52°C,

1min extension at 72°C, and a final 10min extension at 72°C. A negative control using water instead of template DNA was included in the amplification process. Four microliters of PCR products from each PCR reaction were examined by electrophoresis at 75V for 2h in a 0.8% (*W*/*V*) agarose gel in 1× TAE buffer (0.4mol/L Tris, 50mmol/L NaOAc, 10mmol/L EDTA, pH 7.8) and visualized under UV light after staining with ethidium bromide (0.5μg/mL).

PCR products were purified using PCR Cleanup Filter Plates (MultiScreen® $PCR_{\mu 96}$, Millipore, USA) according to the manufacturer's protocol. Purified PCR products were directly sequenced with primer pairs as mentioned above in the ABI 3730-XL DNA sequencer (Applied Biosystems, Inc., USA).

1.4 Data analysis

The ITS sequences generated in this study were used as query sequences to search similar sequences in GenBank with Blastn program to provide at least tentative identification for the fungi. A value of 97% ITS identity was used as a DNA barcoding threshold (Guo *et al*. 2000; Schoch *et al*. 2012).

The colonization rate was calculated as the total number of plant tissue segments infected by fungi divided by the total number of segments incubated (Petrini *et al*. 1982). Relative abundance was calculated as the number of isolates of particular taxon divided by the total number of isolates of all taxa to determine the potential relationship between endophytic fungal community and star anise yield. Frequency was calculated as the number of host individuals of a fungal taxon isolated divided by the total number of all host individuals. Linear and non-linear regressions were carried out to explore the potential relationship between endophytic fungal colonization and star anise yield.

2 RESULTS

2.1 Endophytic fungi

The mean colonization rate of foliar endophytic fungi was 41%, ranging from 6% to 100% (Fig. 1). A total of 412 fungal strains were isolated from 960 leaf discs of 60 *I*. *verum* trees, in which 399 sporulating strains were identified into 10 fungal taxa according to morphological characteristics, and 19 non-sporulating strains were identified into 13 OTUs based on the ITS sequence analysis (Table 1).

A total of 22 endophyte taxa were recovered in the current study (Fig. 2). Of these fungi, *Colletotrichum gloeosporioides*, *Phyllosticta* sp.1 and Dermateaceae sp. were dominant, accounting for 90% of toal strains. *C*. *gloeosporioides* was also harbored in 85% of host individuals, while *Phyllosticta* sp.1 and Dermateaceae sp. colonized in

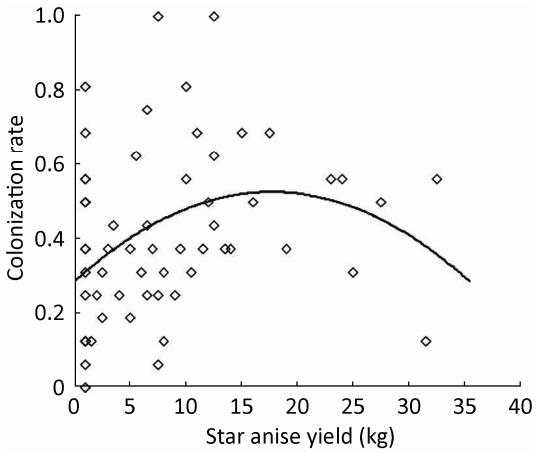

Fig. 1 Quadratic regression indicated an positive effect between endophyte colonizaiton in majority of *Illicium verum* individuals and the star anise yield, and the decreasing trend in several high yield individuals (n=60). [R^2= 0.120, P= 0.026, y= 0.283+0.027x - 0.001x^2].

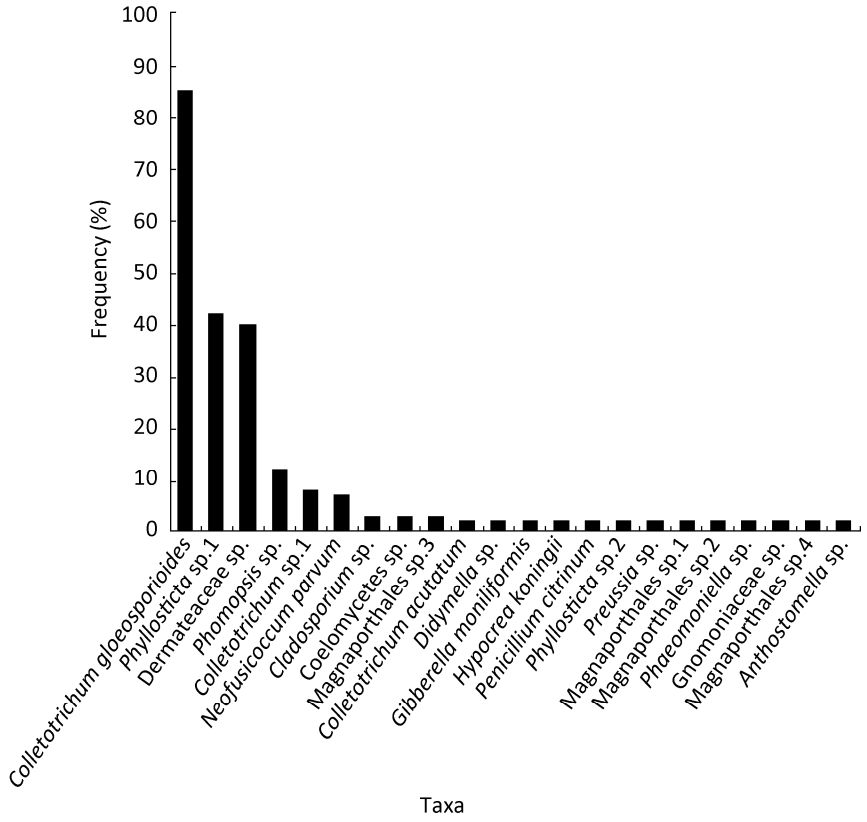

Fig. 2 Frequency of foliar endophytic fungi associated with *Illicium verum.*

42% and 40% of individuals, respectively. Thirteen taxa were rare in mycoflora (Frequency<2%), indicating that endophytic fungal community consisted of a few dominant and major rare taxa in the leaves of *I*. *verum*.

2.2 Relationship between endophytic fungi and star anise yield

The star anise yields varied from 1 to 32.5kg per individual in present study (data not shown). Regression results showed that endophytic colonization rate formed a quadratic relationship with star anise yield (R^2= 0.120, *P*= 0.026. Fig. 1). However, there was no significant relationship between relative abundance of endophytic fungi and star anise yield (data not shown).

3 DISCUSSION

3.1 Endophytic fungal community

The endophytic fungal community in the leaves of *I*. *verum* was composed of a few dominant and major rare taxa, which was accordant with previous studies (Petrini *et al*. 1982; Sun *et al*. 2012). In the three dominant species, *C*. *gloeosporioides* has been reported as endophyte with wide distribution and host range, such as several tree species in Guyana (Lu *et al*. 2004), *Camellia japonica* in Japan (Osono 2008), *Theobroma cacao* in Panama (Rojas *et al*. 2010), *Hevea* spp. in Amazonian region (Gazis *et al*. 2011), *Vitex negundo* in India (Arivudainambi *et al*. 2011), *Abies beshanzuensis* in China (Yuan *et al*.

2011), and *Cinnamomum camphora* in subtropical China (He *et al*. 2012). Actually, *C*. *gloeosporioides* was one of the most common and widely distributed plant pathogen in the world (Hyde *et al*. 2009), infecting multiple host plants in China (Zhuang 2001, 2005). The dominant endophyte *Phyllosticta* sp.1 was a common plant pathogen and endophyte (Wikee *et al*. 2011). It has been reported as endophyte associated with *Citrus* in Brazil, New Zealand, Southeast Asia, and China (Glienke *et al*. 2011; Wang *et al*. 2012), with various host plants in southern India (Murali *et al*. 2007; Suryanarayanan *et al*. 2011), and with *Gingko biloba* in Japan (Thongsandee *et al*. 2012).

Other fungi isolated in this study also have been reported as endophytes in some previous studies, such as *Cladosporium* sp. (Sun *et al*. 2011), *Phomopsis* sp. (Sun *et al*. 2012; Sun *et al*. 2011), and *Trichoderma koningii* (teleomorph *Hypocrea koningii*) (Samuels *et al*. 2006). *Anthostomella* members were recovered as endophytic fungi from *Suaeda fruticosa* in Dorset, UK (Petrini *et al*. 1987), *Pinus sylvestris* and *Abies alba* in Poland (Kowalski 1993; Kowalski & Andruch 2012).

Neofusicoccum species, previously placed in the genus *Botryosphaeria* (Sakalidis *et al*. 2011), have often been reported as endophytes and pathogens of woody hosts, such as *Neofusicoccum parvum* infecting *Eucalyptus* in South Africa, Chile, Ethiopia, Uganda, Uruguay (Pérez *et al*. 2010; Slippers *et al*. 2009), and Guangxi (Chen *et al*. 2011) of China. It is suggested that *N*. *parvum* might be associated with *I*. *verum*.

Members in *Didymella* are common plant pathogens, nevertheless, they are meanwhile reported as endophyte in *Holcoglossum flavescens* (Orchidaceae) in Yunnan, China (Tan *et al*. 2012). *Fusarium verticillioides* (teleomorph *Gibberella moniliformis*) was reported as endophyte and pathogen in maize (Brown *et al*. 2008; Pereira *et al*. 2011). Endophyte *Penicillium citrinum* has been found in many plant species and was widely used as biocontrol agent (Khan *et al*. 2008; Ting *et al*. 2012; Vega *et al*. 2006), and endophytic *Preussia* species were proved to be a productive source of new spirobisnaphthalene analogues (Chen *et al*. 2009).

3.2 Relationship between endophytic fungi and star anise yield

In present study, strong linkages between *I*. *verum* fruit yield and the endophyte colonization were observed. The majority of *I*. *verum* individuals produced moderate or low star anise yield, but the yield rose as the endophyte colonization increased. Nevertheless, the colonization of endophyte decreased as the yield increased in several high yield individuals. The endophyte colonization of *I*. *verum* individuals appeared a significant quadratic regression relationship (Fig. 2).

Endophytic fungi influence the growth of host in different ways, promoting the resistance of host to disease (Arnold *et al*. 2003; Lehtonen *et al*. 2006). Previous studies revealed that endophytes antagonize against various pathogens (Kilani-Feki & Jaoua 2011; Mejia *et al*. 2008; Rocha *et al*. 2011). Mejia *et al*. (2008) suggested that endophytic fungi antagonize pathogens through competing for substrate and producing antibiotic. *C*. *gloeosporioides*, the predominant species in the community, was proved to be an endophyte with antibiotic activity and a biocontrol agent (Arivudainambi *et al*. 2011; Mejia *et al*. 2008). The endophyte colonization might improve star anise yield by promoting the host resistance against pathogens.

Table 1 Endophytic fungi identified by morphology and molecular technique in this study

Fungi	GenBank accession No.	Closest blast match (GenBank accession No.)	Query/reference ITS length (Similarity %)
Anthostomella sp.	JX014386	*Anthostomella sepelibilis* (AY908989)	408/445 (92)
Cladosporium sp.	JX014379	*Cladosporium* sp. (EU167592)	677/683(99)
Didymella sp.	JX014389	*Ascochyta rabiei* (EU167600)	668/687(97)
Hypocrea koningii	JX014378	*Hypocrea koningii* (EU722404)	724/728 (99)
Neofabraea sp.	JX014387	*Neofabraea alba* (AF141190)	631/681(93)
Neofusicoccum parvum	JX014381	*Neofusicoccum parvum* (JN135282)	630/634(99)
Preussia sp.	JX014396	*Preussia* sp. (FJ210521)	523/527(99)
Phaeomoniella sp.	JX014388	*Phaeomoniella* sp. (JN225891)	490/537(91)
Gnomoniaceae sp.	JX014385	*Gnomonia rubi* (EF212847)	329/369(89)
Magnaporthales sp.1	JX014392	*Preussia africana* (JQ031265)	223/230(97)
Magnaporthales sp.2	JX014394	*Clavariopsis aquatica* (FJ804122)	222/226(98)
Magnaporthales sp.3	JX014391	Magnaporthales sp. (JN198467)	290/307(94)
Magnaporthales sp.4	JX014395	Magnaporthales sp. (JN198468)	545/555(98)

Endophytic fungi have been proved to be beneficial to host in promoting growth (Mucciarelli *et al*. 2003; Ting *et al*. 2008; Yates *et al*. 2005). For example, inoculated endophytic fungi accelerated the growth of *Euphorbia pekinensis* (Dai *et al*. 2008) and elicited two kinds of terpenoids produced in *E. pekinensis* cell suspension cultures (Gao *et al*. 2011). Endophytic fungi were also capable of sitimulating growth of non-host plants. *Phomopsis* sp., a *Bischofia polycarpa* endophyte established inducible mutualism with *Oryza sativa*, and promoted its growth, antioxidant enzyme activity, and photosynthesis (Yuan *et al*. 2007). Therefore, endophyte colonization might be a key reason in yield variation of star anise.

The present study also demostrated a negtive effect between yield and endophyte colonization in individuals with high productivity. The individuals with high yield and low endophyte colonization were probably influenced by the occasional nutrient accumulation in the field, since the high N-fertilization would increase star anise yield (Ma *et al*. 2004) and inhibit endophyte colonizaion (Fuentes-Ramírez *et al*. 1999).

Current result could bring inspiration to biocontrol, cultivation, and field management for *I. verum*. However, we still cannot conclude that whether the difference in endophyte pattern caused the varied yield, or the difference in endophyte pattern and fruit yield was jointly caused by some unknown reason. Further studies are needed to reveal more details of the interaction between *I. verum* and its endophytes.

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