

Full Length Research Paper

# Screening of endophytic fungi that promote the growth of *Euphorbia pekinensis*

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This study explored a strategy to use endophytic fungi for promoting the growth of the medicinal plant, *Euphorbia pekinensis*. The growth of *E. pekinensis* was examined in pot culture following inoculation of *E. pekinensis* with endophytic fungal strains (*Fusarium* spp.) from *E. pekinensis* (E4 and E5) and those not from *E. pekinensis* (B3, B6 and S12). The results showed that plants treated with E4 or E5 exhibited significant increase in growth and all tested growth parameters, as compared to the control (CK). The biomass of E4- and E5-treated plants was 2.08 and 1.50 fold higher than that of CK. Analysis of superoxide dismutase (SOD) activity, the indicator of unsaturated fatty acids (IUFA), showed a similar trend as the growth biomass, indicating that the treatments of E4 and E5 benefited the growth of the host plantlets. Indole acetic acid (IAA) and gibberellin (GA) were found in the fermented broth of E4 and E5. *E. pekinensis* cell suspensions demonstrated similar responses to treatment with mycelia extract or filtrate of E5 culture medium. Replacement of MS culture medium components with E5 extracts in host cell suspensions suggested that E5 extract could replace auxin to enhance the plant growth. Therefore, strain E5 could be used as a growth-promoting strain for *E. pekinensis*. The method used in this study could be applicable to similar studies on the relationship between endophytic fungi and their host plants. This method provides a technique to accelerate the growth of *E. pekinensis* plantlets and suspension cells.

**Key words:** *Euphorbia pekinensis*, endophytic fungi, *Fusarium* spp., growth promotion, phytohormone.

## INTRODUCTION

Some plants from the genus of *Euphorbia*, such as *Euphorbia fischeriana* Steud, *Euphorbia ebracteolata* Hayata, and *Euphorbia pekinensis* Rupr., are important folk medicine materials, and they can be found in almost every tome of Chinese traditional medicine (Liu et al., 2004a, b; Ma et al., 1997). These plants are bitter in taste, and may be toxic if not administered properly. However, the root of the plant has been widely used to treat dropsy, hepatocirrhosis, and ascite infections, liquid accumulation caused by tuberculo-conjunctivitis, furuncle and boils. *E. pekinensis* is a representative species of these medicinal plants. Traditionally, the root of *E. pekinensis* is collected from wild plants. The cultivation of the

plant has been hampered by its slow maturation of this perennial persistent-root plant. In addition, there are serious diseases and insect pests in seedlings of *E. pekinensis*, of which the prevention and cure have not been thoroughly investigated. The unsteady yield of the plants limits the supplies of the medicinal materials domestically and internationally. Therefore, a study on endophytic fungi on plantlets of *E. pekinensis* can greatly benefit the industrialization of the planting.

It has been reported that some endophytic fungi can promote host plant growth and protect the host from microbes and insects (Keith, 1998; Strobel et al., 1996). Similar studies have been conducted on endophytic fungi from Euphorbiaceae (Dai et al., 2003, 2005, 2006a, 2006b). We concluded that the inhibition activities of endophytic fungi benefited the host (*E. pekinensis*) (Dai et al., 2005). As the inhibition activity of fungi from genus

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of *Fusarium* is a usual behavior of the plant, it is important to understand whether endophytic fungi are capable of promoting the growth of plantlets of this important species.

In this study, five types of endophytic fungi were determined. Two of them (*Fusarium* strains E4 and E5) were screened from *E. pekinensis*. The other three were strains *Phomopsis* sp. (B3) and *Ceratobasidium stevensii* (B6) from *Bischofia polycarpam*, and strain *Alternaria* sp. (S12) from *Sapium sebiferum*. They were used for comparison.

## MATERIALS AND METHODS

### Plant

*E. pekinensis* were obtained from Langyashan Forest Park of Anhui Province, China. It was identified by Dr. Xu Zenglai from Jiangsu Plant Institute, China, through personal communications.

### Endophytic fungi strains

Endophytic fungi were isolated and stored according to the procedures reported previously (Dai et al., 2006b). The endophytic fungi strains E4 (*Fusarium* sp.) and E5 (*Fusarium* sp.) were originally isolated from *E. pekinensis*. The endophytic fungi strains B3 (*Phomopsis* sp.) and B6 (*C. stevensii*) were isolated from *B. polycarpam*. The endophytic fungi strain S12 (*Alternaria* sp.) was isolated from *Sapium sebiferum*.

### Culture medium

Endophytic fungi strain samples were cultured and stored on potato dextrose agar (PDA). *E. pekinensis* tissue culture was performed in phytohormone-producing medium (MS medium, Murashige and Skoog, 1962) supplemented with 2.0 mg L<sup>-1</sup> 6-benzyladenine (BA), 0.4 mg L<sup>-1</sup> naphthalene acetic acid (NAA), 3% sucrose and 1% agar, which had been formerly adjusted to pH 5.8 using 0.2 mol L<sup>-1</sup> KOH or 0.2 mol L<sup>-1</sup> HCl and sterilized at 121°C for 20 min.

### Plant culture, fungal inoculation and measurements

For the propagation of plantlet, plants with buds were cut into 5-cm pieces, and placed in moist soil supplemented with 1 mg urea every 2 days for every plant. The plantlets grew for about 30 days, and we obtained 62 plantlets from 70 pieces (88.6%). A total of 36 plantlets of similar size were selected, and transplanted to soil pots; 1 plantlet for each pot. Every 6 plantlets were inoculated with one kind of the 5 different endophytic fungus strains. Another 6 plantlets received no inoculation, and served as the control (CK). The inoculation was performed by spraying 5 ml water containing 10<sup>6</sup> spores ml<sup>-1</sup> of endophytic fungi onto the leaves of every plantlet. The inoculation was repeated 10 days after the first inoculation. The plantlets were harvested 40 days after the first inoculation. For the determination of growth characteristics, seedlings were separated from the soil, washed, and towel dried. The fresh weight was measured and recorded. The superoxide dismutase (SOD) activities of the plantlets were determined according to the method reported by Robert et al. (1980), and the analysis of fatty acids was performed as reported previously (Liu et al., 2006; Choi et al., 1992). The remnant plantlet tissue were dried at 105°C for 15 min, and then kept at 60°C until constant weight to determine the dry

weight. The biomass of the plantlets was calculated by the fresh weight and water ratio.

### Tissue culture

Stems with buds were washed 3 times with tap water, sterilized with 70% ethanol for 2 min and 0.1% mercuric chloride for 5 min, and then washed with axenic water three times. The stems and buds were cut into 0.5 ~ 1 cm pieces, and cultured in MS medium containing 2.0 mg L<sup>-1</sup> 6-BA and 0.4 mg L<sup>-1</sup> NAA at 28°C for more than 14 days until callus was formed. For preparation of cell suspensions, the *E. pekinensis* calluses were ground with glass pestle lightly, and suspended in liquid MS culture medium. The most actively growing cells were inoculated to a new culture medium after 14 days. A stable cell strain was achieved after 5 series of inoculations, and the reduced sugar was determined by Fehling method.

### Phytohormone determination

Various endophytic fungus strains were inoculated to MS liquid medium, and cultured for 20 days at 20°C, with shaking at 100 rpm. Phytohormones were extracted from the mycelia and filtrates, and determined by the Phytohormones Laboratory of Nanjing Agricultural University according to the methods previously reported (Morits et al., 1991; Nakajima et al., 1991).

### Statistical analysis

All results reported here are the means of replicates. Data were subjected to the analysis of variance (ANOVA) using STATGRAPHICS Plus 5.1 statistical software (Statistical Graphics Corp., Princeton, NJ).

## RESULT

### The effect of endophytic fungi on soil-planted *E. pekinensis*

For the plantlets inoculated with various strains of endophytic fungi, the analysis of the leaves after 20 days indicated that the inoculations were successful. Every inoculated plantlet could screen the inoculated endophytic strains.

To determine the effect of various endophytic fungi on the growth of *E. pekinensis* plant, we monitored the plantlet growth for 40 days after the inoculation (Table 1). We found that inoculation with E4 and E5 resulted in 2.08 and 1.50 fold increase in biomass of *E. pekinensis* in comparison to the control. The differences were significant for E5 treatment, and highly significant for E4 treatment. In contrast, the inoculations with B3, B6, and S12 did not exhibited statistical difference from CK.

Because the medicinal part of the plant is the root, we analyzed three root growth indices; the root dry weight, the ratio of root to the plantlets, and the ratio of root mass of treated plantlet to that of CK. The results showed that root weights of the plantlets treated with E4, E5, and B3 were significantly greater than the control. In terms of the

**Table 1.** Growth comparison of *Euphorbia pekinensis* plantlets inoculated with various endophytic fungi .

Parameter	E5	E4	S12	B3	B6	CK
Biomass (mg)	143.44±10.97*	198.71±40.96**	55.37.91±8.78*	121.08±32.31	79.37±15.61	95.40±26.92
Root dry weight (mg)	29.05±5.87*	51.09±9.65**	9.91±3.44	27.60±2.88*	15.0±2.84	13.87±2.80
Root/ plantlet (%)	20.25	25.71	17.70	22.79	18.90	14.50
Root/CK (w/w)	2.09	3.68	0.71	1.99	1.08	1

The parameters were determined from 12 leaves (there are 2 leaves for every plantlet and 6 plantlets for each treatment).

\*P < 0.05 = significant difference; \*\*P<0.01 = extremely significant difference.

**Table 2.** The leaves fatty acid composition of *Euphorbia pekinensis* plantlets inoculated with endophytic fungi.

Fungus	14:0	16:0	16:1	18:0	18:1	18:2	18:3	IUFA
E5	4.09	14.85	6.32	2.25	7.72	14.78	50.0	193.58
E4	1.43	17.17	3.73	3.62	9.95	14.71	49.40	191.30
S12	2.52	19.26	0	5.80	12.79	20.19	39.43	171.47
B3	0	16.08	0	0	11.92	21.44	50.57	206.50
B6	4.08	16.84	0	7.43	7.92	12.83	50.89	186.26
Ck	2.42	17.38	2.25	3.74	7.80	16.18	50.23	193.10

14:0 = Myristic acid; 16:0 = palmitic acid; 16:1 = palmitoleic acid; 18:0 = stearic acid; 18:1 = oleic acid; 18:2 = linoleic acid; 18:3 = linolenic acid; IUFA = index of unsaturated fatty acid (or degree of lipid unsaturation) was calculated according to the method of Choi and coworkers .ie: IUFA = (1 × monoene (%) + 2 × diene (%) + 3 × triene (%) + .....) sum of all know fatty acids.

Fatty acids were determined from 12 leaves (there are 2 leaves for every plantlet and 6 plantlets for each treatment).

ratio of root weight of treated to the control, all treated groups outperformed the control.

All these results suggested E4 and E5 were beneficial to the growth of *E. pekinensis*, and B3 appeared not harmful to the growth of plantlets. In contrast, the B6 and S12 treatments did not benefit the growth of *E. pekinensis*.

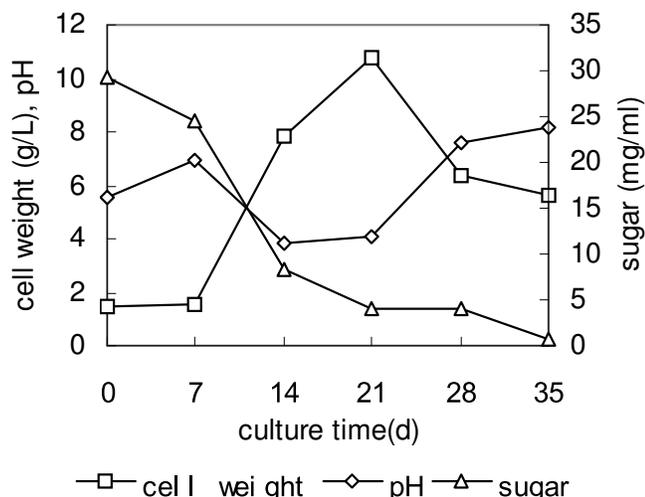
### Physiological effects of endophytic fungal inoculation on *E. pekinensis* plantlets

Superoxide dismutase (SOD) is often used to evaluate the harmful effect of environmental factors to the plant (Scandalions et al., 1997). In this study, we measured SOD activity 40 days after the inoculation to examine the impact of endophytic fungi to the host. The results showed that the SOD activities of the plantlets treated with endophytic fungi were higher than those of control plants. The highest was observed in the plantlets treated with S12 (1533.87±336.55 U), followed by B3 (511.47±94.94 U) and B6 (542.19±63.92 U). The SOD activities of plantlets treated with endophytic fungi derived from *E. pekinensis* were 376.04±74.29 U for E5 and 277.52±60.91 U for E4, as compared to 254.69±62.67 U for the control. Thus, for the plantlets treated with endophytic fungi, their growth was inversely related to their SOD activities. However, it is not clear whether the low SOD activity in the host plants is the reason of slow growth or the result of the slow growth.

External conditions can induce changes in the saturated/unsaturated fatty acid composition of plant cells, an index to reflect the change of cell membrane (Liu et al., 2006). Our experiments revealed some consistency between SOD and leaf fatty acid results. The fatty acid composition remained relatively unchanged when *E. pekinensis* plantlets were treated with *E. pekinensis*-derived endophytic fungi (E4 and E5). In contrast, the fatty acid unsaturation degree increased slightly in response to B3, indicating low-degree damage to the plantlets. In B6-treated plantlets, the fatty acid unsaturation degree declined slightly, while the degree decreased sharply in S12-treated plantlets (Table 2). These results suggest that the ability to adjust fatty acid composition was damaged in 40 day-old plantlets treated with S12. Despite the increase of SOD activity, it appeared that unsaturated fatty acid oxidation continued in S12-treated plants, indicating that S12 was not an effective endophytic fungus of *E. pekinensis*. These results were consistent with our growth index data (Table 1).

### Phytohormones produced by *E. pekinensis* - stimulating endophytic fungi

To determine how E4, E5 and B3 stimulated the growth of *E. pekinensis*, we cultured these three fungi, and determined their phytohormone contents using immunochemical methods. The results showed that all three



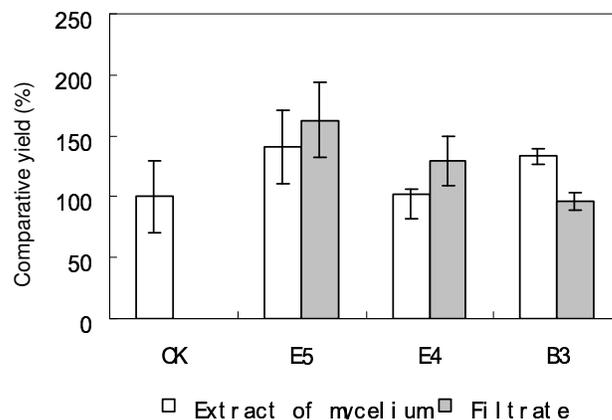
**Figure 1.** The growth curve of suspended *Euphorbia pekinensis* cell cultures over time.

strains produced varying levels of IAA ( $24615.85 \mu\text{mol L}^{-1}$  for E4,  $10257.95 \mu\text{mol L}^{-1}$  for E5, and  $6213.53 \mu\text{mol L}^{-1}$  for B3). E4 and E5 also produced  $3748.40 \mu\text{mol L}^{-1}$  and  $87279.80 \mu\text{mol L}^{-1}$  gibberellin, and B3 produced  $2.51 \mu\text{mol L}^{-1}$  abscisic acid.

### The effect of mycelium extracts of endophytic fungi on the growth of suspended *E. pekinensis* cells

To confirm the relationship between endophytic fungal phytohormones and the growth of *E. pekinensis*, the effect of fungal extracts on the growth of suspended cells was investigated. In this experiment, *E. pekinensis* calluses were mashed and suspended in liquid MS culture medium. Successive cultures (at least 5) were used to establish stable cell lines. The resulting growth curves indicated that the highest cell weight appeared at 21 days post-inoculation ( $10.82 \text{ g L}^{-1}$ ), as the sucrose resources were depleting with the time. The culture pH did not change significantly at the beginning of the incubation, but declined during the high growth stage (due to metabolism), and then increased at the latter stages (due to cell autolysis). The growth curve of *E. pekinensis* suspending cells was showed in Figure 1.

We then examined the effects of endophytic fungal extracts on the growth of these cell suspensions using the method described by Wen et al. (1994) and  $7.85 \text{ mg L}^{-1}$  sugar as measurement index. Our results showed that mycelium extracts and filtrates from strain E5 stimulated *E. pekinensis* growth, improving the culture yields by 141 and 162%, respectively (Figure 2). In contrast, E4 showed a less significant stimulatory effect than the others, producing only 102.34% yield (no stimulation) and 112.52% yield (mild stimulation), respectively, when cultures were tested with the mycelium extract and filtrate. However, B3 showed the opposite trend, yielding 133%

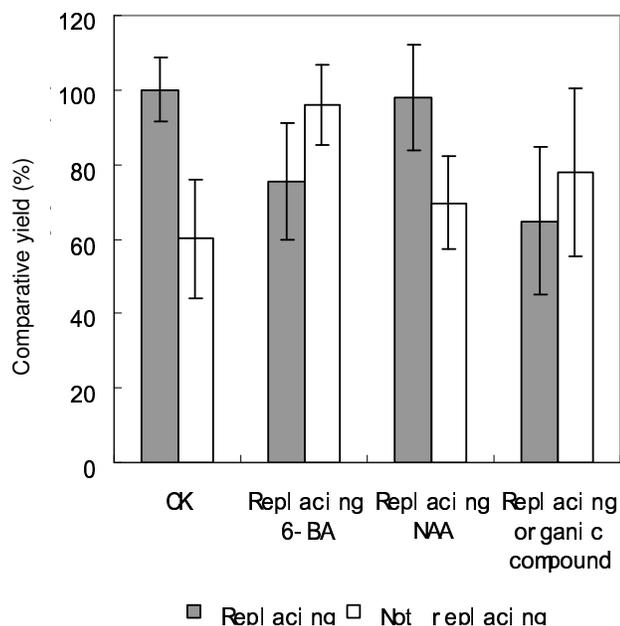


**Figure 2.** The growth of *Euphorbia pekinensis* cell suspensions in the presence of endophytic fungal extracts.

of stimulation in cultures treated with mycelium extract, but no stimulation (96%) in cultures that were treated with filtrate. These results demonstrated that E5 had the greatest stimulatory effect on the cultures, and these results prompted us to use this strain in the following experiments.

### Replacement of MS medium with E5 extract

To determine what components the endophytic fungi produced stimulated the host growth, we examined whether E5 extract could replace certain key components of MS culture medium. MS culture medium (Murashige and Skoog, 1962) contains nitrogen, carbon source, and following organic materials: 1) 6-BA (cytokinin-like), (2) NAA (auxin-like); and (3) organic compounds (glycine, inositol, vitamins B1 and B6). We successively replaced the three components above with E5 fungal extract, and then monitored the growth curves of the suspended cells. Our results revealed (Figure 3) that lack of any organic materials reduced the biomass to 59.92% of the control, indicating that these supplements are important to the growth of the suspended cells. When the culture medium lacked 6-BA, the biomass reached 96% that of CK. When 6-BA was replaced with E5 extract, the biomass reached 71% of CK, indicating that the mycelium extract did not replace the full effect of cytokinin. When the MS culture medium lacked organic components, the biomass reached 78% of the CK biomass. In contrast, replacement of these factors with E5 mycelium extract yielded only 69.38% of the CK biomass, indicating that the extract could not adequately replace the organic components in MS medium. When NAA (auxin) was not present in the culture, the biomass reached 69.57% of the CK biomass. In contrast, the biomass reached 97.92% of the CK when the E5 mycelium extract was added. Replacement of NAA with gibberellin yielded just 71.68% of the CK yield. These results indicated that the extract functions acted as an auxin.



**Figure 3.** The effect of E5 mycelium extract in replacing components of MS culture medium

## DISCUSSION

The results from this study demonstrated that endophytic fungi from *E. pekinensis* could effectively promote the growth of the host plantlets and suspending cells. Although the mechanism was fully illuminated from this study, our results gave some insight about the endophytic fungi and the host.

Fungus strains from genus *Fusarium* can often be found within the inner part of plants (Blodgett et al., 2000; Jacobson et al., 1998; Zou and Tan, 2001). However, those strains can also act as pathogenic fungi (Sobowale et al., 2005). To our knowledge, there is still no publication that addresses whether *Fusarium* spp. are endophytic fungi using the strict criteria. We believe that *E. pekinensis*, a perennial persistent-root plant, may contain *Fusarium* spp. in its leaves and stems. We examined the relationships between the host and two isolated *Fusarium* strains, and compared our results with those from interactions between the plant and other non-*E. pekinensis*-derived endophytic fungi. Our results showed that the two *E. pekinensis*-derived strains stimulated the growth of the host plantlet more effectively than those of non-*E. pekinensis*-derived endophytic fungi. Based on our unpublished data and the results from this study, we can conclude that *Fusarium* strains E4 and E5 act as endophytic fungi according to the strict criteria. The relation is mutualistic symbiont between the host and the endophytic fungi *Fusarium* spp. from *E. pekinensis*. Thus, our work has increased the list of strict endophytic fungi to include these two members of *Fusarium*. The mechanisms on which endophytic fungi stimulate the host growth

can be attributed to the phytohormone effect produced by endophytic fungi because phytohormones are capable of stimulating host growth.

Although fungi could be generally isolated from the plant, to determine which is endophytic and which is not can be very challenging due to existence of saprophytic and latent pathogenic fungi in normal healthy plants (Luceroa et al., 1997). Therefore, some physiological indexes and tissue method are needed to differentiate them. Endophytic fungi should not stimulate the host exquisitely and not virulently. As SOD and fatty acid composition are sensitive indexes for stress stimulation (Liu et al., 2006), we used these two indexes for the first time to distinguish the endophytic fungi.

Many strains from genus of *Fusarium* and other endophytic fungi could produce phytohormone (Chen et al., 1997; Nassar et al., 2005). The effect of phytohormone to plants is complicated, and some of phytohormones such as ABA can inhibit the growth of host plants. However, there are several kinds of phytohormones for one strain, and there are many different strains in a plant at natural conditions. How can we determine what is the base material of the endophytic fungi that stimulate the growth of the host? In our study, we used tissue culture method to demonstrate that *Fusarium* E5 stimulated the growth of *E. pekinensis* probably via phytohormone pathway, in which phytohormone acted as an auxin-like hormone. Although *Fusarium* sp. could produce gibberellins, we found that gibberellin was not the material for the symbiosis relation between the endophytic fungi and the host. The material is something that could replace the action of auxin, maybe it is IAA. Thus, we were able to elucidate the relationship between the particular endophytic fungus and its host. These techniques may be useful for studies of other endophytic strains. It is the first time that these techniques have been used in this manner. Also, our results may be relevant to the propagation of *E. pekinensis*, which is traditionally grown from seeds. It is possible that the techniques, in addition to growth stimulation by endophytic fungi, may contribute to new large-scale propagation of this important medicinal plant.

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