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Full Length Research Paper

Effects of endophytic fungal elicitor on two kinds of terpenoids production and physiological indexes in *Euphorbia pekinensis* suspension cells

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Treating *Euphorbia pekinensis* suspension cultures with endophytic fungal elicitor from *Fusarium* sp. E5 resulted in accumualtion of isoeuphpekinensin and euphol, activated defense-related enzymes, and increased biomass. The elicitor was prepared from extract of fungal endophyte *Fusarium* sp. E5 mycelium, and added into 21 day-old cell suspension cultures. The results showed that the biomass of culture after elicitor treatment was increased by 19.35%, whereas the isoeuphpekinensin and euphol contents were 5.81 and 3.56 times greater than those of the control, respectively. The activity of 1-deoxy-D-xylulose 5-phosphate synthase (a key enzyme of isoeuphpekinensin biosynthesis) was not increased significantly, while the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (a key enzyme of euphol) was increased by 1.67-fold compared with the control. Furthermore, the elicitor enhanced intracellular and extracellular polyphenol production and the activities of phenylalanine ammonia-lyase, peroxidase and catalase, therefore improved the plant defense ability. In addition, the nuclei of elicitor-treated cells were not apoptotic, and the culture was consistently healthy with increasing biomass. Our results demonstrate that endophytic fungal elicitor promotes both terpenoid biosynthesis and biomass of plant cultures for the first time, and thereby the information from this study will be helpful for large-scale medicinal plant suspension culture.

Key words: *Euphorbia pekinensis*, endophytic fungal elicitor, terpenoids, *Fusarium*.

INTRODUCTION

Plant secondary metabolites are widely studied because they often have some efficacy in treating human diseases such as paclitaxel to cancer (Wang et al., 2001; Zhang et al., 2002) and shikonin to HIV (Wu et al., 2009). Plant cell suspension culture is one of the methods to produce plant secondary metabolites (Cheng et al., 2008), and this method has advantages of stabile quality, insensitive commercial production by plant cell culture is still rare due to low productivity. The use of biotic and abiotic elicitors has been one of the most promisingly effective strategies for improvement of the productivity of plant cell culture (Roberts and Shuler, 1997). Fungal elicitor prepared from pathogenic microorganism can be added into plant cell suspension to enhance target substances. For example, *Candida albicans* and *Staphylococcus aureus* (acted as elicitors) increased the production of bilobalide and ginkgolides in *Ginkgo biloba* cell suspension cultures (Kang et al., 2009). However, one of the big obstacles is that, with the yield improvement, the growth of plant cells is significantly inhibited after the incubation with pathogenic fugal elicitor (Yuan et al., 2002; Kang et al., 2009).

Endophytic microbes are an intriguing group of

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Abbreviations: CAT, Catalase; **DW,** dry weight; **DXS;** 1-Deoxy-D-xylulose 5-phosphate synthase; **FW,** fresh weight; **HMGR,** 3-Hydroxy-3-methylglutaryl coenzyme A reductase; **NAA,** naphthaleneacetic acid; **PAL,** phenylalanine ammonia lyase; **POD,** peroxidase; **SOD,** superoxide dismutase; **6-BA,** 6-Benzyladenine.

organisms associated with various tissues and organs of terrestrial and some aquatic plants, whose infections are inconspicuous, and the infected host tissues are at least transiently symptom-less (Stone et al., 2000). Fungal endophytes are synergism symbiosis with plant that can promote the growth of the plant and enhance its resistance to phytopathogen (Glenn et al., 1996; Wilhelm et al., 1998). However, little has been done on influence of endophytic fungal elicitors on host plant suspension cultures.

Euphorbia pekinensis which contains euphol, β–sitosterol, and methylenecyclo artanol, is one of traditional Chinese medicinal herbs. The roots of the plant from genus of *Euphorbia* have been widely used to treat dropsy, hepatocirrhosis, and ascite infections (Dai et al., 2008). Isoeuphpekinensin is the major diterpene, while euphol is the major triterpene in *E. pekinensis.* Both of diterpene and triterpene are terpenoids which are the characteristic compounds of *E. pekinensis* (Kong et al., 2002; Liang et al., 2008). However, with the environmental contamination, the supply of medicinal materials becomes insufficient to meet the demand. In our early study, we found that *E. pekinensis* endophytes E4 and E5 (*Fusarium* spp.) played a beneficial role on the growth and disease resistance of host plant (Dai et al., 2005a, b). Further study found that content of isoeuphpekinensin and euphol in E5-inoculated one-year-old plant was 105.32 and 241.38% more than control (Yong et al., 2009). However, the mechanism that fungal endophyte E5 enhancing both plant biomass and terpenoids is still unknown. To study effects of endophytic fungal elicitor on terpenoids production and physiology in *E. pekinensis* suspension culture, we added elicitor prepared from fungal endophyte *Fusarium* sp. E5 to plant cultures to investigate the changes in the content of terpenoids and biomass of the cells. The other secondary metabolite polyphenol and defense-related enzymes including phenylalanine ammonia lyase, peroxidase, catalase, and superoxide dismutase were also measured.

MATERIALS AND METHODS

Cell suspension culture

The suspended cell line was obtained from the procedures described in our previous report (Dai et al., 2005a). The culture medium was MS medium (Murashige and Skoog, 1962) supplemented with 0.4 $mg \cdot l^{-1}$ naphthaleneacetic acid (NAA), 2.0 $mg \cdot l^{-1}$ 6-benzyladenine (6-BA), and 30 $g \cdot l^{-1}$ sucrose. The medium pH was adjusted to 5.8 before autoclaving. Cultures were shaken at 120 rpm in darkness at 25°C in 100 ml Erlenmeyer flasks and subcultured every 2 weeks.

Endophytic fungal elicitor preparation and treatment

The endophytic fungi E5 (*Fusarium* sp.) was isolated from *E. pekinensis*, cultured on potato dextrose agar, and incubated at 28°C. From 7 day-old cultures, 1 cm^2 mycelia was transferred to a 250 ml Erlenmeyer flask containing 80 ml of potato dextrose medium, and the mycelia was kept on the medium at 150 rpm, 28°C until harvest. When harvesting, the mycelia was filtered and ground with a mortar and a pestle. The mixture was then dissolved in water (10 $g \cdot l^{-1}$), and autoclaved for 20 min at 121°C. The autoclaved fungal suspension was used as the elicitor (Yu et al., 2001). The amount of fungal extract was determined by the phenol-sulfuric acid method using glucose as a standard (Dubois et al., 1956). Elicitor treatments of the 21 day-old *E. pekinensis* cultures were at the rate of 7.85 $mg \cdot l^{-1}$ carbohydrate equivalents. In the meantime, a control was inoculated with equal volume of sterile double distilled water. All treatments were performed in triplicate to examine the reproducibility, and the results were represented by their mean ± standard error (SE).

Determination of fresh weight of cells

The cells in the suspension cultures were filtered through a Whatman filter paper under vacuum. The dry weight (DW) was obtained by drying the fresh cell mass at 50°C in an oven until constant weight, and both the dry weight and the fresh weight (FW) were recorded with the help of a physical balance. All the experiments were repeated three times.

Extraction and analysis of terpenoids and phenolics

Dried cell sample (500 mg) was powdered, and sonicated for 30 min in 30 ml methanol. The extract solution was filtered and evaporated, and the residue was dissolved in 1 ml methanol. The solution samples were transferred to an Eppendorf tube, and centrifuged at 12000 × g for 5 min. The supernatant was filtered through a 0.45 µm membrane, and transferred to clean glass vials for HPLC analysis. The isoeuphpekinensin and euphol content was determined by HPLC using a reverse-phase column (Hedera Packing Material Lichrospher 5-C18, 4.6 × 200 mm, 5 µm). The mobile phases were methanol: H_2O (80:20, v/v) at 1 $ml \cdot min^{-1}$ for isoeuphpekinensin and acetonitrile: H_2O (95:5, v/v) at 1 $ml \cdot min^{-1}$ for euphol. Isoeuphpekinensin was detected at 268 nm at 30°C, and euphol was detected at 217 nm at 25°C. The standard substances of isoeuphpekinensin and euphol were obtained from Dr. Qiao-Li Liang (Liang et al., 2008). The intracellular phenolic content was determined using Foline-Ciocalteu method adapted from Dicko et al. (2002).

Cells from different treatments were ground to fine powder using a pestle and a pre-cooled mortar, and the powder was extracted three times with 80% methanol at 4°C under continuous stirring for 20 min. The homogenate was centrifuged at 7000 × g for 3 min, and the supernatants were analyzed using a spectrophotometer (UNIC 2802S) at 760 nm. A calibration curve was generated with freshly prepared solution of gallic acid. Results were calculated as mg equivalent gallic acid per gram of fresh weight (mg eq. gallic acid/g FW). Extracellular phenolic compounds were extracted from the culture medium with ethyl acetoacetate (EAc) (ca. 2 ml/100 ml) by sonication for 30 min at room temperature. The extraction solvent (5 ml) was then evaporated under vacuum, and the residue was re-dissolved in 3 ml 75% (v/v) ethanol. The supernatant was collected, and the absorbance was measured at 280 nm. The quantity of phenolic compounds was expressed as OD280/ g FW.

Enzyme assays

The activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) was determined by the method of Toroser and Huber (1998). The enzyme extract was added (ca.75 mg protein per ml) to a 50 mM of Tris–HCl assay buffer (pH 7.0) containing 0.3 mM of HMG-CoA (Sigma, Cat. H6132), 0.2 mM of NADPH and 4 mM of

dithiothreitol. NADPH oxidation in the reaction solution was monitored at 25°C by the decreasing absorbance at 340 nm against the solution free of HMG-CoA as a blank. One HMGR enzyme unit is equivalent to the oxidation of 1 mM of NADPH per minute.

The activity of 1-deoxy-D-xylulose 5-phosphate synthase (DXS) was determined by the fluorometric method of Querol et al. (2001), which is based on the reaction of 1-deoxy-D-xylulose 5-phosphate with 3, 5-diaminobenzoic acid in an acidic medium to form a highly fluorescing quinaldine derivative. The reaction mixture contained 40 mM of Tris–HCl (pH 7.5), 2.5 mM of $MgCl_2$, 5 mM of β-mercaptoethanol, 1 mM of thiamin diphosphate, 10 mM of sodium pyruvate, and 20 mM of DL-glyceraldehyde 3-phosphate as well as the enzyme extract (ca. 75 mg protein per ml reaction solution). The reaction solution was maintained at 37°C for 1 h, stopped by heating at 80°C for 5 min, and then spun down at 13,000 rpm for 5 min to remove the denatured proteins. The supernatant was mixed with 1 ml of 10 mM 3, 5-diaminobenzoic acid in 5 M phosphoric acid, and then heated in a boiling water bath for 15 min. The fluorescence intensity of the reaction product, which is proportional to DXS activity, was determined with the excitation at 396 nm and emission at 510 nm using a fluorescence spectrophotometer (Shimadzu RF-540, Shimadzu, Japan).

The activity of phenylalanine ammonia lyase (PAL) was analyzed followed the method of Cheng and Breen (1991) with some modifications. Cells of 500 mg were homogenised for 2 min in 5 ml 0.1 M borate buffer (pH 8.8) containing 600 mg polyvinylpyrrolidone (PVP), 5 mM β-mercaptoethanol, and 2 mM EDTA. The homogenate was centrifuged for 15 min at 14,000 × g and the supernatant collected for enzyme activity determination. The activity of PAL was measured by incubating 0.5 ml of supernatant with 2 ml of 0.1 M borate buffer (pH 8.0) containing 3 mM L-phenylalanine for 1 h at 30°C. The increase in absorbance at 290 nm due to the formation of trans-cinnamate was measured spectrophotometrically. PAL activity was expressed as the change in OD290 per hour per gram of fresh weight. The activity of peroxidase (POD) was analyzed followed the method of Upadhyaya et al. (1985) with some modifications. Fresh cells (500 mg) were homogenized with 10 ml of 0.1 M phosphate buffer (pH 7.8), 1 mM EDTA and 1.5% w/w polyvinylpyrrolidone. The homogenate was centrifuged at 15,000 × g for 20 min at 4°C. The supernatant was used as the crude extract for the assay of enzyme activities. The POD activity was measured following the change of absorbance at 470 nm due to guaiacol oxidation. The activity was assayed for 1 min in a reaction solution (3 ml final volume) composed of 100 mM potassium phosphate buffer (pH 7.0), 20 mM guaiacol, 10 mM H_2O_2 and 50 µl of crude extract. One unit POD activity was defined as an absorbance change of 0.01 unit min^{-1}. Catalase (CAT) activity was determined by the consumption of H_2O_2 at 240 nm for 30 s (Aebi, 1984). The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.0), 15 mM H_2O_2 and 50 µl cell extract in 3 ml volume. CAT activity was expressed as an absorbance change in OD240 per minute per gramma fresh weight.

Activities of superoxide dismutase (SOD) were assayed by measurement of its capacity of inhibiting the photochemical reduction of nitro-blue tetrazolium (NBT) (Beauchamp and Fridovixh, 1971). Three milliliters of reaction mixture contained 50 mM phosphate buffer (pH 7.8), 10 mM methionine, 1.17 mM riboflavin, 56 mM NBT and 30 µl enzyme extract. The absorbance of solution was measured at 560 nm. One unit of SOD activity was defined as the amount of enzyme which caused 50% inhibition of photochemical reduction of NBT. All the results expressed by the means of three replicate samples ± SE.

Protein determination

The soluble protein concentration was determined by the method of standard.

Microscopic analysis

Microscopic analysis was conducted in this study based on the method of Nishikawa et al. (2006). Cell suspension cultures were collected in the 14th day after the treatment, and were incubated in solution of 4', 6-diamidino-2-phenylindole (DAPI; Sigma Inc., 50 µg·ml^{-1}) for 5 to 10 min in the dark at 25°C. The cultures were viewed with a fluorescence microscope (Zeiss Axio Imager A1, MetaMorph 7.0, Germany). DAPI was used for staining in the fluorometric analysis of DNA. The stained nuclei exhibited blue fluorescence under UV excitation.

RESULTS

Effects of elicitor on cell growth and terpenoids accumulation

The effect of endophytic fungal elicitor on plant cell growth is shown in Figure 1. There is little difference between the treatment and the control in the first few days. At the 8th day, however, the fresh weight of the control was lower than the treatment. The fresh weight of treated cells at the 14th day was 19.25% greater than that of the control, suggesting that elicitor might prolong the stationary phase of *E. pekinensis* suspension culture (Figure 1a). Isoeuphpekinensin reached the maximum at the 6th day after the onset of elicitor treatment, and the content of isoeuphpekinensin was 5.81 times of that of the control (Figure 1b). Euphol in treated cells was 3.56 times of that of the control in 6th day, and reached the maximum 2 days earlier than the control (Figure 1c).

For terpenoids biosynthesis, the elicitor did not have much impact on the activities of DXS (a key enzyme to synthesize diterpene), while HMGR (a key enzyme to synthesize triterpene) were only 1.44 to 1.67 times of the control, respectively, from 6th to 14th day (Figure 1d and e). The reason that the activity of DXS did not increased distinctively might be the result from indirect relationship between the enzyme and elicitor.

The effects to intracellular and extracellular polyphenol and PAL activities

The content of intracellular and extracellular polyphenol was immediately increased after the addition of elicitor (Figure 2a and b). The content of intracellular polyphenol, was about 19% more than that of the control between 0 and 8th days after the onset of eliciting treatment, but phenolics appeared to fluctuate after the 10th day (Figure 2a). The content of polyphenol in treated culture medium, however, gradually increased after the treatment, was 64.5% more than the control at the 10th day (Figure 2b). PAL is the first key enzyme of phenol biosynthesis. As positive Bradford (Bradford, 1976) using bovine serum albumin (BSA) as a shown in Figure 2c, the activity of PAL was 1.44 to 1.67 times of the control, which suggests PAL played an important role in polyphenol production induced by endophytic elicitor.

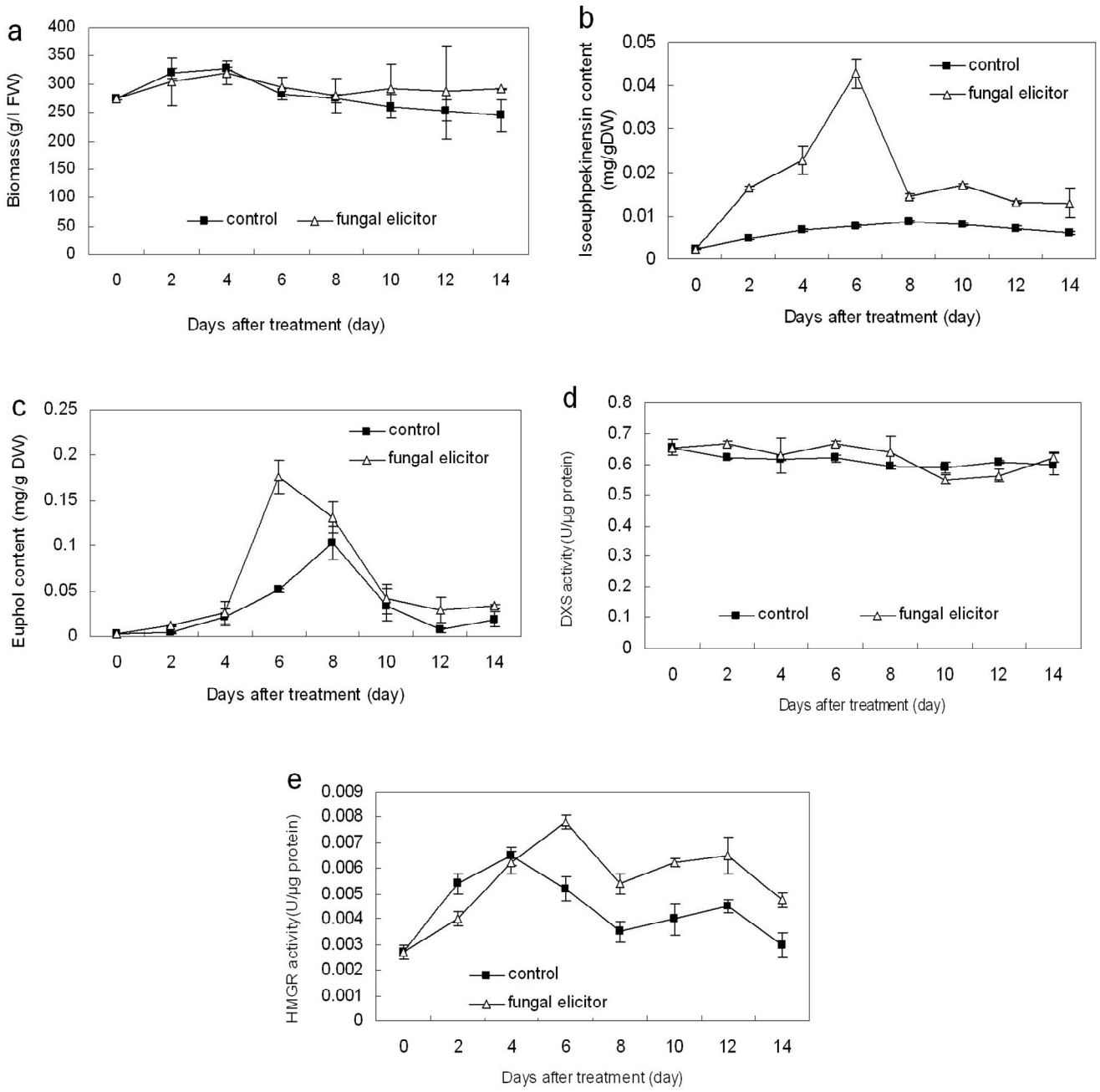

Figure 1. The effects of elicitor on the growth, terpenoids accumulation, and activities of HMGR and DXS of *E. pekinensis* cultures. The 21 day-old cells treated with 7.85 mg·l^{-1} elicitor or sterile distilled water were harvested after various periods. (a) Effects to cell growth, (b) effects to the accumulation of isoeuphpekinensin; c, effects to the accumulation of euphol, (d) effects to the activity of DXS, (e) effects to the activity of HMGR.

The effects to POD, CAT and SOD and intracellular protein content

In order to understand the mechanism of secondary metabolites biosynthesis induced by elicitor, the activities of antioxidant enzymes including POD, SOD and CAT were determined. As shown in Figure 3, the activity of POD was 1.36 to 11.03 times of the control, while the activity of SOD was not significant changed after elicitor treatment. The activity of CAT was increased at the second day after elicitor treatment and reached the top at 6th day, which suggests H_2O_2 might be produced. The intracellular protein content immediately enhanced after treatment, and kept the high levels before the 8th day.

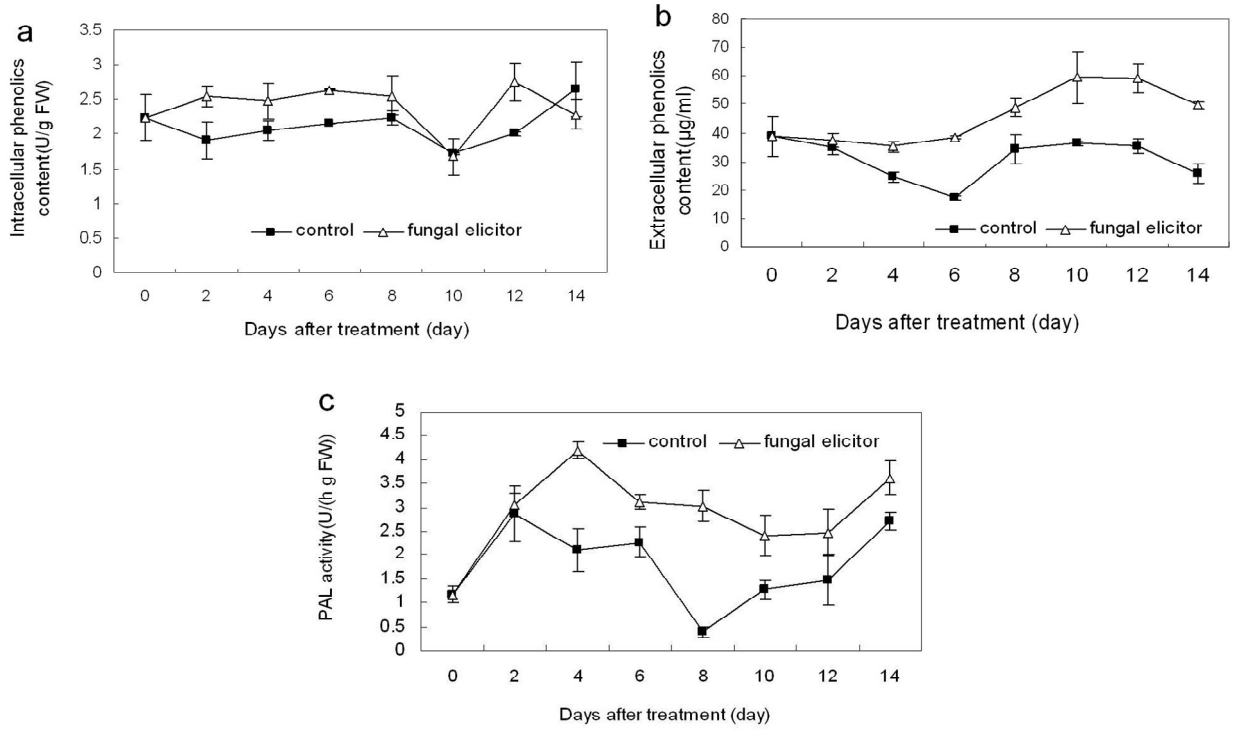

Figure 2. The effects of elicitor on external and intracellular polyphenol and PAL activities. The 21 day-old cells treated with 7.85 mg·l^{-1} elicitor or sterile distilled water were harvested after various periods. (a) Intracellular polyphenol, (b) extracellular polyphenol, (c) PAL activities.

A correlation between the increasing of protein content (Figure 3c) at early period (0 to 6 days) and the increasing of enzyme activities (Figures 2c, 3a, and b) was observed.

The effect to morphous of nucleus

Nuclei were observed by fluorochrome. The result showed that the nucleus was normal 14 days after the treatment, while cells were live (Figure 4). Endophytic fungal elicitor did not exhibit harmful effect to the plant cell. This result was distinctly different from the result reported by Wang et al. (2002). These date indicated that fungal endophyte elicitor *Fusarium* sp. E5 is low intensive to *E. pekinensis* while endophytic elicitor in Wang's study is high intensive to *Artemisia annua* L. hairy root and triggered programmed cell death.

DISCUSSION

It is widely accepted that secondary metabolites are produced by plants to protect themselves against the attacks from insects, herbivores and pathogens, or to survive under other biotic and abiotic stresses (Zhao et al., 2005). Therefore, many fungal components can be used as elicitor, which can induce productions of various types of secondary metabolites. Although pathogenic fungal elicitor immediately induced plant secondary metabolites production in suspension cultures, biomass and viability of cells decreased after elicitation (Baker and Orlandi, 1995; Yuan et al., 2002; Kang et al., 2009), which suggests that the duration of high level production induced by pathogenic fungal elicitor might be un-sustainable to the commercial demand. Li and Tao (2009) added the fungal endophyte culture supernatants to the suspension cultures of *Taxus cuspidata*, and found that the highest yield of paclitaxel treated by elicitor was 1.8 fold of the yield from the controls with decreased biomass of cultures. Their results showed that some fungal endophytes would impair host cells. However, in our study, endophytic elicitor treatment did not change the morphology of cells and nuclei (Figure 4a and b), indicating that endophyte *Fusarium* sp. E5 would probably not lead to *E. pekinensis* cell death. Fungal endophyte elicitor promoted not only the growth of cells, but also the content of terpenoids (Figure 1a, b, and c), which was far from other elicitors. With the long-term co-evolution, endophyte–host interactions might be based on mutual exploitation and therefore exhibited little harmful effect to the plant cells.

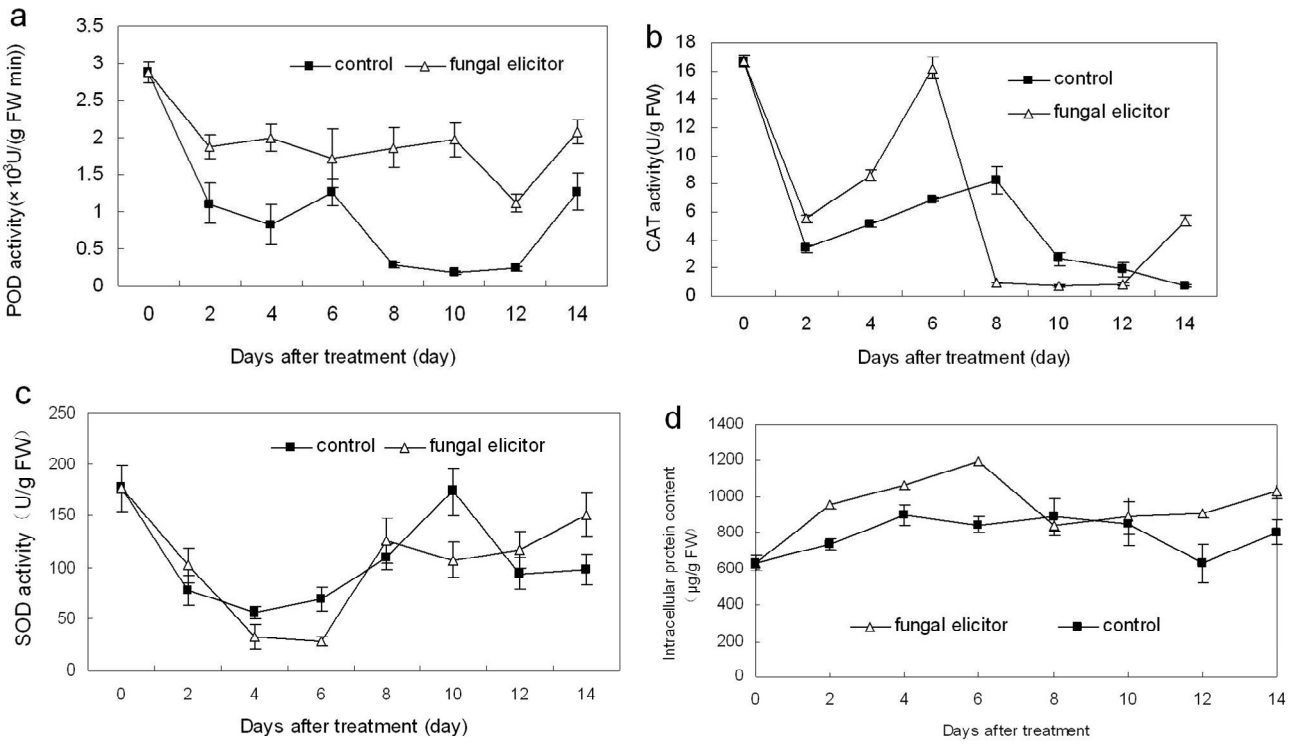

Figure 3. The effects of elicitor to the activities of antioxidant enzymes and intracellular protein content in suspending cells of *E. pekinensis*. The 21 day-old cells treated with 7.85 mg·l^{-1} elicitor or sterile distilled water were harvested after various periods. (a) the activities of POD, (b) the activities of CAT; **c**, the activities of SOD, (d) intracellular protein content.

However, some endophytic elicitor will still cause plant cell apoptosis (Wang et al., 2002), the different mechanisms need to be explored in the future.

The synthesis of many secondary metabolites in plants is widely accepted to be part of the responses of plant defense reaction. Endophytes within the Clavicipitaceae can accumulate several classes of fungal metabolites that serve as relief mechanisms to grasses resisting biotic and abiotic stresses including fungal diseases (Kuldau and Bacon, 2008). In this study, endophytic elicitor could stimulate not only terpenoids regarded as characteristic metabolites, but also external and intracellular polyphenol production, implying that the induction of this type of elicitor could be widely effective for secondary metabolites production. The activities of PAL, POD, SOD, and CAT are usually used to evaluate physiological and biochemical responses of plants to biotic and abiotic stresses and the plant systemic acquired resistance (SAR) (Gechev et al., 2003; Peltonen et al, 1997). PAL is the first enzyme in the general phenylpropanoid pathway, and was reported to be up-regulated in *Catharanthus roseus* cell cultures induced by *Aspergillus niger* elicitor (Xu and Dong, 2005). POD participates in a variety of plant defense mechanisms, and is involved in plant resistance to diseases (Silva et al., 2008; Dutsadee and Nunta, 2008). CAT and SOD, which play important role in the metabolism of reactive oxygen species (ROS), could be induced by environmental stresses including fungal elicitor (Pachten and Barz, 1999; Tanabe et al., 2008). The fact that adding endophyte elicitor enhances the activities of PAL, POD and CAT suggests that endophyte *Fusarium* E5 can improve the resistance of *E. pekinensis* cells, and thus the extensive defensive reaction of the plants as well as the content of secondary metabolites. However, all of activities of these enzymes induced by endophytic fungal elicitor is low intensity with long period (Figures 2c, 3a, b, and c), which is much different from pathogenic fungal elicitor (Yuan et al., 2002; Dutsadee and Nunta, 2008).

As noted in the introduction, fungal endophyte *Fusarium* sp. E5 plays a beneficial role in both biomass and terpenoids content of one-year-old *E. pekinensis* plantlet. However, the isoeuphpekinensin enhancement in cell cultures (nearly 6 times of the control) was much higher than that in plantlet (about 2 times of the control). The reason might be that other microorganisms existed in the experimental pots and contributed the changes of terpenoids content. However, single stimulation in cell cultures caused isoeuphpekinensin content to increase significantly after eliciting. Our previous research indicated that *Fusarium* sp. E5 extract functions acted as an auxin, and that the mechanism of fungal endophyte stimulating the host growth might be attributed to the phytohormone effect produced by endophytic fungi (Dai

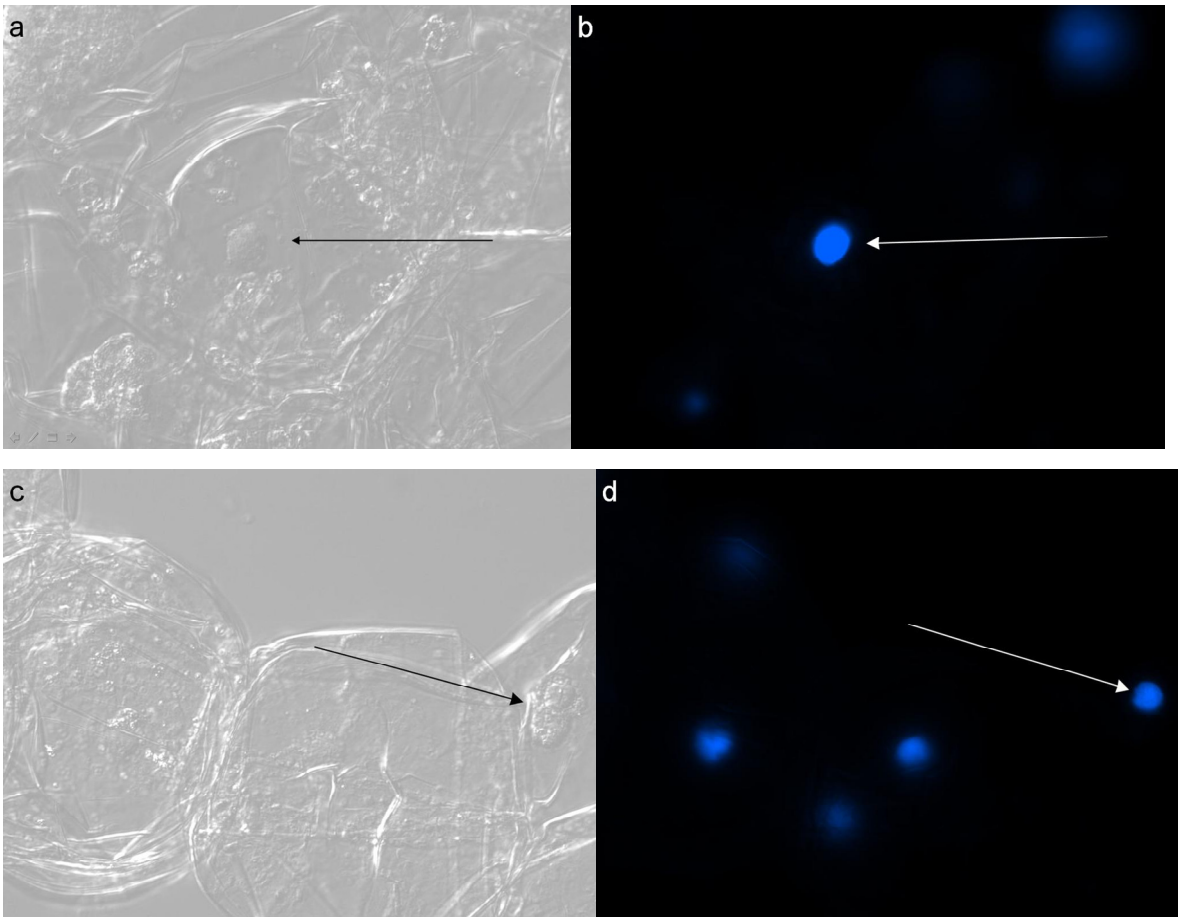

Figure 4. The morphology of *E. pekinensis* cells and nuclei under fluorescence microscopy (64×). (a) the morphology of cells and nuclei treated with endophytic fungal elicitor, arrow shows nuclei in normal light, (b) the morphology of cells and nuclei treated with endophytic fungal elicitor, arrow shows nuclei in fluorchrome, (c) the morphology of cells and nuclei treated with sterile distilled water in normal light, (d) the morphology of cells and nuclei treated with sterile distilled water in fluorchrome.

et al., 2008). Other similar reports indicated that the genus from *Fusarium* and other endophytic fungi could produce phytohormone (Chen et al., 1997; Nassar et al., 2005). This may be the real reason of increasing biomass by elicitor in this study. As the elicitor used in this study was fungal extract, there must be many components including saccharides. Li and Tao (2009) found the major elicitor element that could enhance the yield of paclitaxel in *Taxus* cells was an oligosaccharide of 2 kDa. We suspect that the effective element of the elicitor inducing terpenoid production may be saccharides. Thus, the mechanism of the enhancement of biomass and terpenoids induced by elicitor may be different. The former may be due to the phytohormone produced from fungal endophyte, and the latter may be due to saccharide components from fungal fragment. The signaling pathway of how endophytic elicitor increases terpenoids in *E. pekinensis* remains unclear. Further exploration is needed for precise mechanism of secondary metabolites production induced by fungal endophytes in *E. pekinensis* cultures.

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