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内生真菌 *Berkleasmium* sp. 寡糖对盾叶薯蓣培养细胞和幼苗防御相关酶活性的影响

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摘　要:从内生真菌 *Berkleasmium* sp. Dzf12 多糖(EPS、WPS 和 SPS)分别制备出相应的寡糖(EOS、WOS 和 SOS),研究寡糖对宿主盾叶薯蓣(*Dioscorea zingiberensis*)培养细胞和幼苗防御相关酶活性的影响。对于培养细胞,浓度为 0.25 mg/mL 的 EOS 处理 24 h,苯丙氨酸解氨酶(PAL)和多酚氧化酶(PPO)活性达最大值,分别为对照的 6.19 倍和 7.16 倍;浓度为 0.05 mg/mL 的 EOS 处理 48 h,过氧化物酶(POD)活性达最大,是对照的 4.63 倍。对于幼苗,浓度为 2.5 mg/mL 的 EOS,处理 48 h,PAL 活性达最大值,为对照的 6.21 倍;处理 24 h,PPO 活性达最大值,为对照的 4.80 倍;处理 48 h,POD 活性达最大值,为对照的 6.09 倍。在 3 种寡糖中,EOS 是最有效的诱导子。结果表明,用内生真菌 *Berkleasmium* sp. Dzf12 寡糖处理宿主盾叶薯蓣培养物能有效提高防御相关酶的活性,激活其防卫机制。

关键词:寡糖;内生真菌;*Berkleasmium* sp. Dzf12;盾叶薯蓣;防御相关酶;培养物

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Effects of Oligosaccharides from Endophytic Fungus *Berkleasmium* sp. Dzf12 on Activities of Defense-related Enzymes in Cell and Seedling Cultures of *Dioscorea zingiberensis*

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Abstract: Three oligosaccharides (EOS, WOS and SOS) were prepared from their corresponding polysaccharides (EPS, WPS and SPS) of *Berkleasmium* sp. Dzf12, an endophytic fungal strain of *Dioscorea zingiberensis*. The effects of oligosaccharides on the activities of defense-related enzymes phenylalanine ammonia lyase (PAL), polyphenoloxidase (PPO) and peroxidase (POD) in suspension cell and seedling cultures of *D. zingiberensis* were investigated. For the suspension cell cultures, the highest activities of PAL and PPO were induced by 0.25 mg/mL of EOS at 24 h after treatment, which were 6.19-and 7.16-fold of control, respectively. While the POD activity was increased to the maximum by 0.05 mg/mL of EOS at 48 h after treatment, which was 4.63-fold of control. For the seedling cultures, the highest activities of PAL, PPO and POD were induced by 2.5 mg/mL of EOS at 48, 24 and 48 h, respectively, which were 6.21-, 4.80-and 6.09-fold of control, separately. Among three oligosaccharides, EOS was the most effective elicitor to induce activities of defense-related enzymes in *D. zingiberensis* cultures. The results demonstrated that the increased activities of PAL, PPO and POD elicited by the oligosaccharides may be related to the activation of defensive mechanisms of *D. zingiberensis* suspension cell and seedling cultures.

Key words: oligosaccharides; endophytic fungus; *Berkleasmium* sp. Dzf12; *Dioscorea zingiberensis*; defense-related enzymes; cultures

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Introduction

It has been well demonstrated that several defense mechanisms in plants against microbial pathogens are induced in response to diverse biotic and abiotic stimu-

li, such as hypersensitive response, the induction of phytoalexin biosynthesis, cell wall reinforcement by deposition of lignin, induced synthesis of pathogenesis-related proteins, hormone signaling crosstalk, and accumulation of soluble phenolics[1,2]. The induction of defense-related enzymes such as phenylalanine ammonia lyase (PAL), polyphenoloxidase (PPO), peroxidase (POD), glucanase and chitinase, is an obviously ubiquitous feature of plant defensive response to pathogen attack[3]. In plants, PAL is an entrypoint enzyme in phenylpropanoid metabolism, and it regulates the biosynthesis of a wide range of secondary metabolites including flavonoids, lignins, furanocoumarins, isoflavonoids and hydroxycinnamic acid esters acting as phytoalexins and protectants[4]. PPO, which is widely distributed in plants, can oxidize monophenols, diphenols or trihydric phenols to their corresponding quinines with great toxicity to pathogens[5]. POD can assist superoxide dismutase (SOD) and catalase (CAT) to scavenge the excessive superoxide radical ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (·OH) in plant cells, which could ensure plant healthy growth[6].

The first elicitor to induce phytoalexin synthesis was an oligosaccharide from the cell walls of the plant pathogen *Phytophthora sojae*[7]. The elicitors from pathogens have been frequently reported recently, such as live pathogens, fragments of cell walls, crude extracts of mycelia, saccharides (*i. e.* oligosaccharides and polysaccharides), and proteins, could activate plant disease resistance system[8,9]. Plant endophytic fungi, belonging to non-pathogenic fungi, show symbiotic relationships with their host plants, and induce no visual symptoms to host plants[10]. *Berkleasmium* sp. Dzf12 was an endophytic fungus isolated from the healthy rhizomes of medicinal plant *Dioscorea zingiberensis*[11]. Twenty-two bioactive spirobisnaphthalenes have been isolated from this fungus[12,13]. It was found to be a high-yield producer of spirobisnaphthalenes[14]. Furthermore, three polysaccharides, namely exopolysaccharide (EPS), water-extracted mycelial polysaccharide (WPS) and sodium hydroxide-extracted mycelial polysaccharide (SPS), were prepared from *Berkleasmium* sp. Dzf12, and their *in vitro* antioxidant activities were evaluated[15]. The effects of EPS, WPS and SPS along with their corresponding oligosaccharides EOS, WOS and SOS on growth and disogenin accumulation of *D. zingiberensis* suspension cell and seedling cultures were also investigated[16]. It has been well demonstrated that the polysaccharide or oligosaccharide from fungi were in favor of induction of plant defensive responses[17]. Therefore, we aimed to investigate the effects of oligosaccharide elicitors from endophytic fungus *Berkleasmium* sp. Dzf12 on the defense-related enzymes including PAL, PPO and POD in suspension cell and seedling cultures of its host plant *D. zingiberensis*. By investigating the effects of elicitors from each endophyte on its host plant defensive system, it will provide information for development of agrochemicals, and integrated utilization of the saccharides, aside from the production of active spirobisnaphthalenes, as well as further understanding the interactions between *Berkleasmium* sp. Dzf12 and its host *D. zingiberensis*.

Materials and Methods

Plant materials

The calli of *D. zingiberensis* were induced from its root explants and subcultured at an interval of 30 d in darkness as the previous report[18]. Murashige and Skoog (MS) medium supplemented with 6-benzyladenine (5.0 mg/L) and kinetin (2.0 mg/L) was used to induce callus redifferentiation to form seedlings. The obtained *D. zingiberensis* seedlings were subcultured on MS medium at 25 ℃ with an interval of 30 d under 12 h daily illumination of 2000 lux. When the seedlings were subcultured for 5 generations, they could be used as the experimental materials. The medium pH was adjusted to 5.8 before autoclaving for 15 min at 121 ℃. All experiments were carried out in 125-mL Erlenmeyer flasks. The suspension cell cultures were maintained in liquid medium on a rotary shaker at 120 rpm in darkness at 25 ℃. Three seedlings (about 1.0 g fresh weight) were inoculated in a 125-mL Erlenmeyer flask containing 50 mL MS solid medium.

Cultivation of endophytic fungus *Berkleasmium* sp. Dzf12

Endophytic fungus *Berkleasmium* sp. Dzf12 was isolated from the healthy rhizomes of *D. zingiberensis* as previous reports[11]. The fermentation culture was carried out in 1000-mL Erlenmeryer flasks containing modified Sabouraud broth medium (300 mL for each flask) composed of glucose (40 g/L), peptone (10 g/L), KH_2PO_4 (1.0 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L), $FeSO_4 \cdot 7H_2O$ (0.05 g/L)[14]. The initial pH of medium was adjusted to 6.5 before autoclaving. All flasks were cultured on a rotary shaker at 150 rpm and 25 ℃ for 12 d. All the fermentation broth was combined and centrifuged at 7,741 × *g* for 20 min to obtain mycelia and supernatant.

Preparation of oligosaccharides from *Berkeleasmium* sp. Dzf12

Exopolysacccharide (EPS) was prepared from the supernatant by ethanol (95%) precipitation method, and water-extracted mycelial polysaccharide (WPS) and sodium hydroxide-extracted mycelial polysaccharides (SPS) were prepared from the lyophilized mycelia according to the previous method[15]. The three oligosaccharides, EOS (EPS-derived oligosaccharide), WOS (WPS-derived oligosaccharide) and SOS (SPS-derived oligosaccharide), were respectively prepared by hydrolysis of their corresponding polysaccharides, EPS, WPS and SPS using trifluoroacetic acid (2.17 mol/L) at 85 ℃ for 4 h[16]. By TLC detection, the crude oligosaccharide contained a series of oligosaccharide monomers with their degrees of polymerization ranged from 5 to 12. The carbohydrate content of each crude oligosaccharide was analyzed by the method of anthrone-sulfuric acid spectrophotometry using glucose as a reference[19].

Elicitation experiments

Each oligosaccharide stock solution was prepared using sterile distilled water, and was then conducted by filtration through a sterile filter membrane (pore size, 0.45 μm). The oligosaccharide concentrations were prepared as 0.5, 2.5 and 5.0 mg/mL.

When *D. zingiberensis* suspension cell cultures were cultured for 20 d, 3 mL of oligosaccharide elicitor solution (0.5, 2.5 and 5.0 mg/mL) was separately added. Therefore, the final addition concentrations of oligosaccharide elicitor were 0.05, 0.25 and 0.50 mg/mL. Addition of 3 mL sterile distilled water was used as control. The treated suspension cell cultures were respectively harvested at 12, 24, 48, 72 and 96 h after elicitor addition, and then collected by vacuum filtration. The obtained fresh cell cultures were immediately used for extraction of crude defense-related enzymes. Each treatment was carried out in triplicate.

The *D. zingiberensis* seedlings cultured for 20 d were placed in the 125-mL flasks. Each 125-mL flask containing 20 mL oligosaccharide solution (0.5, 2.5 and 5.0 mg/mL) was inoculated three seedlings (about 1.0 g fresh weight). Sterile distilled water was used as control. All the treated seedlings were kept in the culture room at 25 ℃ under 12 h daily illumination of 2000 lux. The seedlings were separately harvested at 12, 24, 48, 72 and 96 h after oligosaccharide treatment. Water adhering to the surface of seedlings was removed by absorbent papers. The harvested fresh seedlings were immediately used for extraction of crude defense-related enzymes. Each treatment was carried out in triplicate.

Extraction and detection of defense-related enzymes

The PAL extraction and detection were conducted according to the method as described previously with some modifications[20]. The harvested suspension cells or seedlings (0.5 g in fresh weight, fw) were homogenized in pre-cooling 5 mL 0.05 M sodium borate buffer (pH 8.8) containing 5.0 mM β-mercaptoethanol. The homogenate was then centrifuged at 12,000 g for 15 min at 4 ℃, and the supernatant was collected as the enzyme extract of PAL. The reaction mixture consisted of 50 μL enzymatic extract, 100 μL 0.02 M L-phenylalanine and 50 μL 0.05 M sodium borate buffer (pH 8.8). After incubation at 40 ℃ for 60 min, 50 μL 2 M HCl was added to stop the reaction. The absorbance at 290 nm of the reaction mixture was recorded by a micro-plate spectrophotometer. One unit (U) of PAL activity is defined as a change of 0.01 OD at 290 nm per minute per gram fresh weight. The PAL activity results were presented as U/min g fw.

The crude enzymes PPO and POD were extracted according to the method as described previously with

some modifications[21]. The harvested suspension cells or seedlings (0.5 g fw) were homogenized in pre-cooling 5 mL 0.05 M sodium phosphate buffer (pH 6.8) containing 1% polyvinyl polypyrrolidone (PVPP). The homogenate was centrifuged at 12,000 g for 15 min at 4 ℃ and the supernatant was used for PPO or POD activity assay.

The reaction mixture for PPO assay contained 50 μL crude enzymatic solution, 100 μL 0.05 M catechol and 50 μL 0.05 M PBS (pH 8.8), and it was monitored by measuring the change of absorbance at 398 nm for 2 min. One unit (U) of PPO activity is defined as a change of 0.01 OD at 398 nm per minute per gram fresh weight. The PPO activity results were presented as U/min/g fw.

For the POD activity assay, 10 μL crude enzymatic solution was mixed with 25 μL 1% guaiacol (g/mL), 25 μL 1% H_2O_2 (v/v) and 150 μL 0.05 M PBS (pH 8.8). After the reaction lasted for 10 min at 37 ℃, the absorbance of the solution at 470 nm was recorded by a micro-plate spectrophotometer. One unit (U) of PPO activity is defined as a change of 0.01 OD at 470 nm per minute per gram fresh weight. The POD activity results were presented as U/min/g fw.

Results and Discussion

Effects of oligosaccharides on the activity of PAL

The effects of oligosaccharides EOS, WOS and SOS on the activity of PAL in suspension cell and seedling cultures of *D. zingiberensis* are presented in Fig. 1. When the cultures were elicited by any oligosaccharide elicitor, the activity of PAL showed an increasing trend at first and then decreased with the elongation of elicitation time. When suspension cell cultures were treated with 0.25 mg/mL of EOS, the activity of PAL was increased to the highest at 24 h, which was 6.19-fold of control (Fig. 1A). The enhancement of PAL activity in suspension cell cultures was also induced by 0.50 mg/mL of WOS at 48 h, which was 5.12-fold of control (Fig. 1B). For the seedlings, the highest PAL activity was also induced by EOS. When the seedlings were treated with 2.5 mg/mL of EOS, the PAL activity was increased to the maximum at 48 h, which was 6.21-fold of control (Fig. 1D). Meanwhile, an obvious enhancement on PAL activity in the seedlings was also observed by 2.5 mg/mL of WOS at 24 h, which was 4.07-fold of control (Fig. 1E). However, SOS produced relatively weak improvement on PAL activity in seedling cultures (Fig. 1F). On the whole, oligosaccharide EOS showed the most excellent improving effect on PAL activity in both suspension cell and seedling cultures of *D. zingiberensis*.

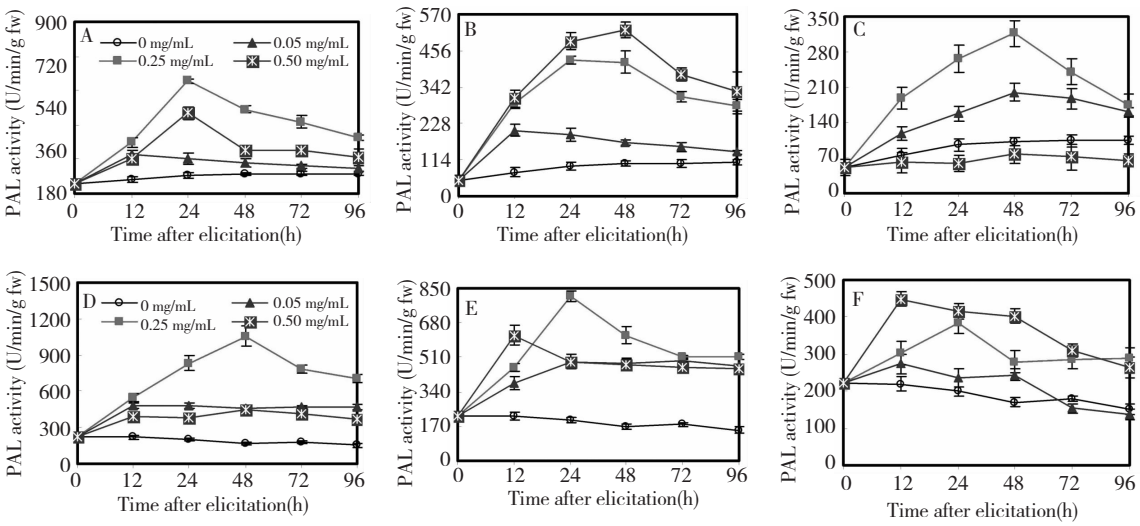

Fig. 1 Effects of oligosaccharides EOS, WOS and SOS on PAL activities respectively in suspension cells (A, B and C) and seedlings (D, E and F) of *D. zingiberensis*

Note: Each value was expressed as mean ± standard deviation ($n = 3$)

Effects of oligosaccharides on the activity of PPO

Fig. 2 displayed the effects of oligosaccharides EOS, WOS and SOS on PPO activity in *D. zingiberensis* suspension cell and seedling cultures. As presented in Figs. 2A to 2C, the most effective elicitor to increase the PPO activity in suspension cell cultures was EOS. When suspension cell cultures were elicited by 0.25 mg/mL of EOS, the activity of PPO was improved to the highest at 24 h, which was 7.16-fold of control (Fig. 2A). Though WOS produced slightly weaker enhancement than that of EOS, the activity of PPO was improved to the maximum at 24 h, which was 5.19-fold of control in suspension cell cultures (Fig. 2B). For the seedlings, EOS produced the strongest enhancement on PPO activity. When the seedlings were treated with 2.5 mg/mL of EOS, the activity of PPO was improved to the maximum at 24 h, which was 4.80-fold of control (Fig. 2D). When the seedlings were treated with 0.5 mg/mL of WOS at 24 h, the maximum PPO activity was observed that was 3.09-fold of control (Fig. 2E). As displayed in Figs. 2E and 2F, SOS did not produce any evident enhancement on PPO activity in suspension cell or seedling cultures of *D. zingiberensis*.

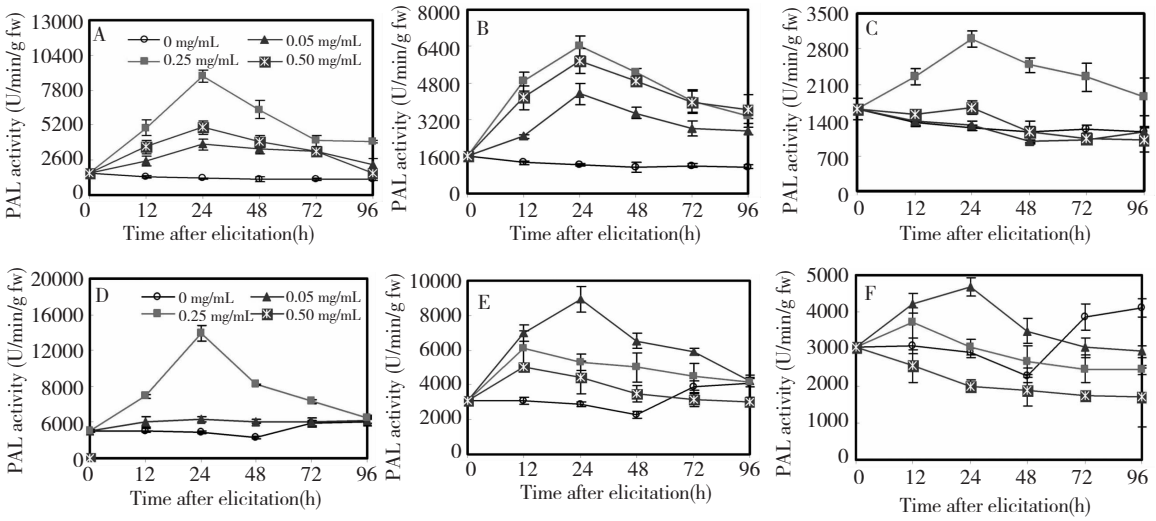

Fig. 2 Effects of oligosaccharides EOS, WOS and SOS on PPO activities respectively in suspension cells (A, B and C) and seedlings (D, E and F) of *D. zingiberensis*

Note: Each value is expressed as mean ± standard deviation (n = 3).

Effects of oligosaccharides on the activity of POD

The effects of oligosaccharides EOS, WOS and SOS on POD activity in *D. zingiberensis* suspension cell and seedling cultures are presented in Fig. 3. For suspension cell cultures, the highest POD activity was induced by 0.05 mg/mL of EOS at 48 h, which was 4.63-fold of control (Fig. 3A). When the suspension cell cultures were elicited with 0.25 mg/mL of WOS, the POD activity was 3.49-fold of control at 24 h (Fig. 3B). The highest POD activity in suspension cell cultures induced by SOS was observed at 24 h with the concentration of 0.25 mg/mL, which was just 2.20-fold of control and far lower than that of EOS or WOS (Fig. 3C). For the seedlings, EOS showed excellent effects on enhancement of POD activity. The highest POD activity in the seedlings was produced by 2.5 mg/mL of EOS at 48 h, which was 6.09-fold of control (Fig. 3D). Though the improving effect of WOS was slightly weaker than that of EOS, an obvious enhancement of POD activity with 5.18-fold of control at 24 h was also induced by 2.5 mg/mL of WOS (Fig. 3E). In general, oligosaccharide EOS showed the best enhancement on POD activity in both suspension cells and seedlings of *D. zingiberensis*.

Elicitors, especially saccharides (*i. e.* polysaccharide and oligosaccharide) from fungi, have been frequently reported to induce defensive responses in plants[22]. However, the oligosaccharide elicitors were mostly

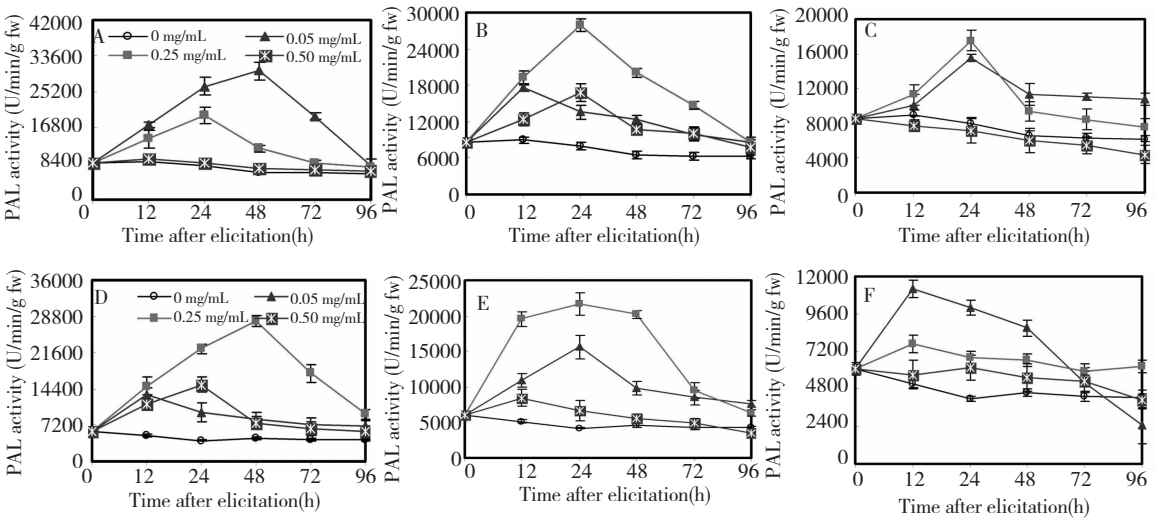

Fig. 3 Effects of oligosaccharides EOS, WOS and SOS on POD activities respectively in suspension cells (A, B and C) and seedlings (D, E and F) of *D. zingiberensis*

Note: Each value is expressed as mean ± standard deviation ($n = 3$).

prepared from pathogenic fungi[17]. It is rarely reported to induce defense-related enzymes in plants by oligosaccharide elicitors from endophytic fungi. Increases of PAL, PPO and POD activities have been proved to be one of the earliest defensive responses of plants against fungal stimuli[23]. Accompanied by the enhancement of defense-related enzyme activities in plants, some compounds related to plant defensive response have also be observed, such as the accumulation of phenolic compounds and PR-proteins[24]. In the present work, the crude oligosaccharides (EOS, WOS and SOS) prepared from their corresponding polysaccharides EPS, WPS and SPS from the endophytic fungus *Berkleasmium* sp. Dzf12 were observed to significantly increase the activities of defense-related enzymes in suspension cell and seedling cultures of its host *D. zingiberensis*. Results demonstrated the oligosaccharides could trigger the enhancement of the activities of defense-related enzymes in *D. zingiberensis* cultures. The variations of PAL, PPO and POD activities in *D. zingiberensis* cultures were influenced by the categories and concentrations of oligosaccharides as well as the elicitation time, which were in accordance with the previous studies[9]. However, the structural characterizations of the oligosaccharides (*i. e.* purification of oligosaccharide monomers, monosaccharide composition, monosaccharide linkages of each oligosaccharide monomer) as well as their structure-activity relationships and more specific defensive mechanisms are not clear and worth investigating. Furthermore, the relations between disease resistance and defense-related enzyme activities by treatment with oligosaccharides *in vivo* are also needed for studying in detail.

Conclusion

In this study, the effects of the oligosaccharides EOS, WOS and SOS from the endophytic fungus *Berkleasmium* sp. Dzf12 on the activities of the defense-related enzymes PAL, PPO, and POD in suspension cell and seedling cultures of *D. zingiberensis* were investigated. The maximum values of PAL, PPO and POD activities were mostly observed at 24 h or 48 h after oligosaccharide treatment, which showed significant enhancements as compared with those of control. Among three oligosaccharides, EOS was the most effective elicitor to induce activities of defense-related enzymes in *D. zingiberensis* cultures. The present work might be contributed to further understand the interaction between endophytic *Berkleasmium* sp. Dzf12 and its host *D. zingiberensis*, which will provide an idea for the research and development of saccharide agrochemicals as plant defense-related elicitors.

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