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Effects of Polysaccharide Elicitors from Endophytic *Bionectria pityrodes* Fat6 on the Growth and Flavonoid Production in Tartary Buckwheat Sprout Cultures

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This study was to examine the effects of four fungal polysaccharides, namely exo-polysaccharide (EPS), water-extracted mycelia polysaccharide (WPS), sodium hydroxide-extracted mycelia polysaccharide (SPS), and hydrochloric-extracted mycelia polysaccharide (APS) obtained from the endophytic fungus *Bionectra pityrodes* Fat6, on the sprout growth and flavonoids production of *Fagopyrum tataricum*. Without obvious changes in the appearance of the sprouts, the exogenous polysaccharide elicitors notably stimulated the sprout growth and functional metabolites accumulation, and the stimulation effect was mainly depended on the polysaccharide species along with its treatment dose. With application of 150 mg/l of EPS, 150 mg/l of WPS and 200 mg/l of SPS, the total rutin and quercetin yield of buckwheat sprouts was effectively increased to 49.18 mg/(100 sprouts), 50.54 mg/(100 sprouts), and 52.27 mg/(100 sprouts), respectively. That was about 1.57- to 1.66-fold in comparison with the control culture of 31.40 mg/(100 sprouts). Moreover, the present study revealed the accumulation of bioactive flavonoids resulted from the stimulation of the phenylpropanoid pathway by fungal polysaccharide treatments. It could be an efficient strategy for improving the nutritional and functional quality of tartary buckwheat sprouts applied with specific fungal elicitors.

Keywords: polysaccharide, endophyte, *Bionectra pityrodes* Fat6, tartary buckwheat, flavonoid, elicitation

Introduction

Tartary buckwheat [*Fagopyrum tataricum* (L.) Gaertn], an important medicinal and edible plant, has been widely planted in the southwest of China, northern India, Bhutan and Nepal (Zhang et al. 2012). It is rich in protein, amino acids, dietary fiber, vitamins, iron, zinc, selenium and other trace elements, as well as various bioactive phytochemicals. The major functional components of tartary buckwheat have been demonstrated to be flavonoids, polyphenols, phytosterols, fagopyrins, D-*chiro*-inositol and D-fagomine. Recent

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investigations revealed that these compounds had notable antioxidant, hypocholesterolemic, antidiabetic, antimicrobial and antitumor activities (Inglett et al. 2011; Ren et al. 2014), and this has increased many researchers' interest. There have been a large variety of buckwheat-based food products available in the market such as buckwheat flour, noodles, bread, tea, vinegar, wine and sprouts (Qin et al. 2011; Zhang et al. 2012).

Buckwheat sprouts (Fig. S1*), which have a soft and slightly crispy texture and an attractive fragrance, have been regarded as a nutritional and health vegetable (Kim et al. 2004). Through sprouting, the protein quality and fatty acid composition of tartary buckwheat can be efficiently improved, and the buckwheat sprouts are more nutritional than their seeds. Moreover, the accumulation of many functional metabolites such as rutin, γ-aminobutyric acid and D-*chiro*-inositol, which have a variety of interesting pharmacological effects, are also effectively enhanced (Wang et al. 2013).

As the biosynthesis of many secondary metabolites in plants is usually a defence response of plants to biotic or abiotic stresses, their accumulation can be effectively stimulated by various biotic or abiotic elicitations. Nowadays, the application of pathogenic or non-pathogenic fungal preparations and chemicals as elicitors has become one of the most important and successful strategies for functional metabolites production in plant tissue cultures (Zhou and Wu 2006). These elicitors mainly consisted of living or autoclaved fungi mycelia, crude extracts, peptides, proteins, as well as fungal carbohydrates. There are many valuable bioactive components production has been successfully stimulated by fungal elicitors such as silymarin in *Silybum mariamum* cell cultures (Sanchez-Sampedro et al. 2005), tanshinone in *Salvia miltiorrhiza* cell cultures (Zhao et al. 2010), and diosgenin production in *Dioscorea zingiberensis* cell cultures (Li et al. 2011).

Plant endophytic fungi are an important and novel resource of natural bioactive compounds with great potential applications in agriculture, medicine and food industry (Zhao et al. 2011). Research on fungal endophytes has become a hotspot all around the world in recent years. However, there were few reports about the effects of endophytic fungi as elicitors on the growth and functional metabolites production of their host plants. In our previous investigation, the crude mycelia extract and polysaccharide of endophyte *B. pityrodes* Fat6 exhibited strong stimulation effect on flavonoid production of tartary buckwheat sprout cultures (Zhao et al. 2014). The purpose of this study was to evaluate the effects of four kinds of polysaccharides (EPS, WPS, SPS and APS) prepared from endophyte *B. pityrodes* Fat6, on the growth and functional flavonoids production of tartary buckwheat sprout cultures. Moreover, the intracellular phenylalanine ammonia lyase (PAL) activity of buckwheat sprout cells induced by the fungal polysaccharides was examined further, and the potential relationship to plant stress response was also discussed.

* Further details about the Electronic Supplementary Material (ESM) can be found at the end of the article.

Materials and Methods

Cultivation of endophytic fungus Bionectria pityrodes *Fat6*

The endophytic fungus *Bionectria pityrodes* Fat6 (GenBank accession number KC218450) was isolated from the healthy roots of *F. tataricum* as reported previously (Zhao et al. 2014). The living culture has been maintained on potato dextrose agar (PDA) slants at 4 °C, and in 40% glycerol at –70 °C at the CCPC of Chengdu University. After the fungal mycelia were grown on PDA medium in Petri dishes at 25 °C for 4–6 days, two to three agar plugs with mycelia were transplanted and grown in a 500-ml Erlenmeyer flask containing 200 ml liquid potato dextrose (PD) medium. All shake flasks were maintained on a rotary shaker at 150 rpm and 25 °C for 10 days. A total of 30 l fermentation broth was obtained and centrifuged at 3000 rpm for 15 min. Then, the supernatant and mycelia were collected separately.

Preparation of EPS, WPS, SPS and APS

The exopolysaccharide of endophyte *B. pityrodes* was prepared from the supernatant mentioned above. Briefly, the supernatant was concentrated under vacuum at 55 °C by a rotary evaporator to a suitable volume and mixed with three volumes of 95% ethanol, and allowed to precipitate for 48 h at 4 °C in a refrigerator. Then, the solution was centrifuged at 8000 rpm for 15 min, and the precipitate was collected as crude EPS which was further subjected to deproteinization with Sevag reagent, decolorization with 3% H_2O_2, and removal of small molecule impurities by dialysis. Polysaccharide mixture with molecular weight great than 8,000–14,000 Da was kept in dialysis tube. After lyophilization, the purified EPS (7.58 g) was stored in a desiccator at room temperature. The carbohydrate concentration of EPS was determined by the anthrone test using glucose as a reference (Zhao et al. 2012), and its content was determined as 87.2%.

The collected mycelia of *B. pityrodes* was washed twice with deionized water, and then lyophilized. The lyophilized mycelia was powdered and subjected to heat circumfluence extraction at 50 °C by 95% ethanol-petroleum ether at 1:1 (v/v) to remove the lipid, monosaccharide and disaccharide. The ratio of mycelia powder (g) to refluxing solvent (ml) was 1:6 (w/v). Defatted mycelia powder was obtained by centrifugation and drying at 40–45 °C to a constant weight. Afterwards, the pretreated mycelia powder was immersed in distilled water, and extracted at 85 °C for 90 min with the ratio of the material (g) to water (ml) as 1:20 (w/v). After that, centrifugation was carried out at 8,000 rpm for 15 min to separate the supernatant and the residue. The supernatant was concentrated to a certain volume, and mixed with three volumes of 95% ethanol, and then kept in a refrigerator at 4 °C for 48 h. The following procedure for polysaccharide purification was the same as the treatments of EPS. The gained polysaccharide (8.15 g) was named as water-extracted mycelial polysaccharide (WPS). The residue not containing WPS was further extracted with 1 M sodium hydroxide (NaOH) solution at root temperature for 24 h. The remaining steps were the same as the treatments of WPS. The obtained polysaccharide (8.62 g) was designated as sodium hydroxide-extracted mycelial polysaccharide (SPS).

The residue not containing WPS and SPS was further extracted with 1 M HCl solution at root temperature for 24 h. The remaining steps were the same as the treatments of WPS mentioned above. The obtained polysaccharide (6.58 g) was designated as acid-extracted mycelial polysaccharide (APS). The carbohydrate content of WPS, SPS and APS was determined as 86.5%, 78.6% and 82.5%, respectively.

Elicitation treatment of buckwheat sprout cultures

Stock polysaccharide solutions were prepared by dissolving each polysaccharide in distilled water, and sterilized by filtering through a microfilter (0.45 μm). Effects of the four polysaccharide elicitors (EPS, WPS, SPS and APS) on *F. tataricum* sprout growth and flavonids production were investigated as following elicitation treatments. The healthy buckwheat seeds (cultivar Chuanqiao-01) were pre-surface-sterilized for 5 min in 2.5% sodium hypochlorite solution followed by a quick deionized-H_2O rinse for three times. Then, they were immersed in each polysaccharide solution at the following six concentrations (50, 100, 150, 200, 300 and 400 mg/l) for 16 h, respectively, and transplanted into commercial germination boxes (120 × 120 × 50 mm). The buckwheat sprouts were cultivated in illumination incubators at 25 ± 1 °C and 70% relative humidity, and harvested on day 9 or 10 for measuring their germination rate, sprout length, biomass, and main functional flavonoid content. In addition, the kinetics of optimal PS-treated buckwheat sprouts growth and flavonoids accumulation was also investigated. All treatments were performed in triplicate.

Measurement of germination rate, sprout biomass and flavonoid content

At the end of the culture period, the germination rate of buckwheat seeds was counted. The percentage (%) of seed germination was determined as $(G/t) \times 100$, where G is an average number of three replicates of germinated seeds, and t is an average value of three replicates of the total seeds in each test. For measurement of the sprout biomass, they were harvested and rinsed thoroughly with distilled water, and blotted dry by paper towels to obtain the fresh weight (fw), and then dried at 40–45 °C in an oven to attain the constant dry weight (dw).

For determination of the main flavonoids (rutin and quercetin) content of buckwheat sprout cultures, the dried spouts were ground into powder, and the extraction was performed by mixing sample (0.1 g) with methanol-water (25 ml, 70%, v/v) solution in a conical flask under sonication for 30 min. After removal of the solid, the filtrates were transferred into a 25 ml volumetric flask and the volume adjusted to 25 ml with 70% methanol. The contents of rutin and quercetin were analyzed by high performance liquid chromatography (HPLC), according to our previous method (Zhao et al. 2014).

Measurement of PAL activity

The phenylalanine ammonia lyase (PAL) was extracted from the fresh buckwheat sprouts with borate buffer (pH 8.8). The sprouts were ground in the buffer (0.2 g/ml) for 2 min with a pestle and mortar on ice, and then centrifuged at 8,000 rpm and 4 °C for 20 min to obtain a solid-free extract. The PAL activity was determined based on the conversion of *L*-phenylalanine to cinnamic acid according to the previous method (Zhao et al. 2010).

Results

Effects of EPS, WPS, SPS and APS on the growth of tartary buckwheat sprout cultures

Table 1 listed the effects of four fungal polysaccharide elicitors (EPS, WPS, SPS and APS) on seed germination and sprout growth of tartary buckwheat, which were dependent both on the PS species and elicitation dose. In general, three polysaccharide elicitors EPS (50–100 mg/l), WPS (50–200 mg/l) and SPS (50–300 mg/l) could stimulate the seeds germination effectively. However, the APS elicitor had no positive effect on the buckwheat seed germination. Among these elicitation treatments, the highest germination rate was 96.33% when the seeds treated with 300 mg/l of SPS. That was about 1.12-fold in comparison with the control of 86.33%. The buckwheat sprout length was efficiently promoted by both WPS and APS elicitors, and their length was between 11.37 cm and 13.07 cm, about 1.01- to 1.16-fold compared to the control of 11.30 cm. Nevertheless, both the EPS and SPS elicitors exhibited a slight or negative effect on the sprout length of tartary buckwheat.

For the sprout biomass, the EPS, WPS and SPS elicitors could enhance the sprout growth, except for APS, which had a slightly inhibition on the sprout growth. Treated with these effective elicitors, the fresh weight of buckwheat sprout was from 14.42 g/(100 sprouts) to 16.29 g/(100 sprouts), about 1.04- to 1.17-fold in comparison with the control of 13.92 g/(100 sprouts). Correspondingly, the maximum dry weight of buckwheat sprout was 1.19 g/(100 sprouts), when treated with 300 mg/l of WPS. That was about 1.24-fold in comparison with the control culture of 0.96 g/(100 sprouts).

Effects of EPS, WPS, SPS and APS on rutin and quercetin accumulation of buckwheat sprouts

The effects of four polysaccharide elicitors on rutin and quercetin production are shown in Fig. 1. As displayed in Fig. 1A, the rutin accumulation of buckwheat sprouts was effectively stimulated by all PS elicitors except APS. The highest rutin content of *F. tataricum* sprouts was 44.92 mg/g when treated with 200 mg/l of SPS, about 1.42-fold in comparison with the control of 31.59 mg/g. After treatment with 150 mg/l of WPS, the sprouts rutin content was efficiently increased to 43.09 mg/g. And application with 150 mg/l of EPS, the rutin content of buckwheat sprouts was as much as 42.65 mg/g.

Table 1. Effects of polysaccharides EPS, WPS, SPS and APS on the sprout growth of tartary buckwheat

Treatment	Polysaccharide concentration (mg/l)	Germination rate (%)	Sprout length (cm)	Fresh weight (g/100 sprouts)	Dry weight (g/100 sprouts)
Control	0	86.33±1.53^{efgh}	11.30±0.36^{k}	13.92±0.26^{i}	0.96±0.01^{hij}
EPS	50	89.33±2.08^{cde}	11.07±0.25^{lm}	14.47±0.26^{h}	1.02±0.04^{efgh}
	100	91.33±1.53^{bcd}	10.87±0.21^{m}	15.22±0.18^{ef}	1.07±0.03^{cdef}
	150	87.67±1.53^{defg}	10.67±0.15^{n}	15.62±0.34^{cd}	1.11±0.01^{bcd}
	200	84.67±0.53^{fghi}	10.37±0.23^{o}	15.98±0.29^{b}	1.15±0.04^{ab}
	300	82.67±1.53^{ijk}	9.90±0.36^{q}	15.87±0.25^{b}	1.13±0.03^{abc}
	400	79.33±2.52^{kl}	9.67±0.21^{r}	15.54±0.32^{de}	1.10±0.02^{bcde}
WPS	50	91.00±1.73^{bcd}	11.37±0.55^{k}	14.42±0.20^{h}	1.01±0.02^{fgh}
	100	93.67±1.53^{abc}	11.57±0.32^{ij}	15.21±0.12^{ef}	1.07±0.02^{cdef}
	150	94.67±1.53^{ab}	11.83±0.51^{gh}	15.77±0.15^{bcd}	1.12±0.02^{abc}
	200	89.67±1.53^{cde}	12.87±0.45^{b}	15.92±0.21^{b}	1.14±0.01^{abc}
	300	83.67±1.15^{hijk}	13.07±0.38^{a}	16.29±0.28^{a}	1.19±0.03^{a}
	400	80.33±2.08^{jkl}	12.67±0.47^{c}	15.76±0.23^{bcd}	1.12±0.02^{abc}
SPS	50	88.33±1.53^{def}	11.64±0.78^{hi}	15.09±0.13^{g}	1.04±0.06^{defg}
	100	90.67±1.53^{bcd}	11.22±2.19^{kl}	15.25±0.12^{ef}	1.09±0.05^{bcde}
	150	91.33±1.15^{bcd}	10.59±0.87^{n}	15.89±0.18^{b}	1.16±0.03^{ab}
	200	93.67±0.58^{abc}	10.26±1.18^{op}	15.73±0.38^{bc}	1.12±0.04^{abc}
	300	96.33±1.15^{a}	10.14±1.02^{p}	15.14±0.33^{fg}	1.07±0.03^{cdef}
	400	84.33±1.15^{fghij}	9.57±1.28^{r}	15.02±0.30^{g}	1.04±0.04^{defg}
APS	50	86.33±1.53^{efgh}	11.40±0.10^{jk}	13.91±0.11^{i}	0.97±0.01^{ghi}
	100	85.67±1.53^{fghi}	11.93±1.43^{fg}	13.82±0.09^{i}	0.95±0.01^{hij}
	150	82.33±0.58^{ijk}	12.43±0.47^{de}	13.78±0.07^{ij}	0.91±0.01^{ijk}
	200	79.67±1.53^{kl}	12.57±1.70^{cd}	13.57±0.10^{j}	0.89±0.03^{jkl}
	300	76.67±1.53^{l}	12.30±1.00^{e}	13.08±0.20^{k}	0.86±0.03^{kl}
	400	71.67±1.53^{m}	12.10±0.40^{f}	12.64±0.20^{l}	0.83±0.01^{l}

Note: Values represent mean ± standard deviation (n = 3). Different letters (i.e., a–r) in each column indicated significant differences among the treatment at p = 0.05 level.

For the quercetin biosynthesis, it was effectively enhanced by all the four polysaccharides. Treated with these effective elicitors, the quercetin content of buckwheat sprouts was from 1.16 mg/g to 1.75 mg/g, about 1.04- to 1.56-fold compared to the control culture of 1.12 mg/g (Fig. 1B). Correspondingly, the highest rutin and quercetin yield of tartary buckwheat sprouts was 52.27 mg/(100 sprouts), 50.54 mg/(100 sprouts), and 49.18 mg/(100 sprouts), when the sprouts treated with 200 mg/l of SPS, 150 mg/l of WPS,

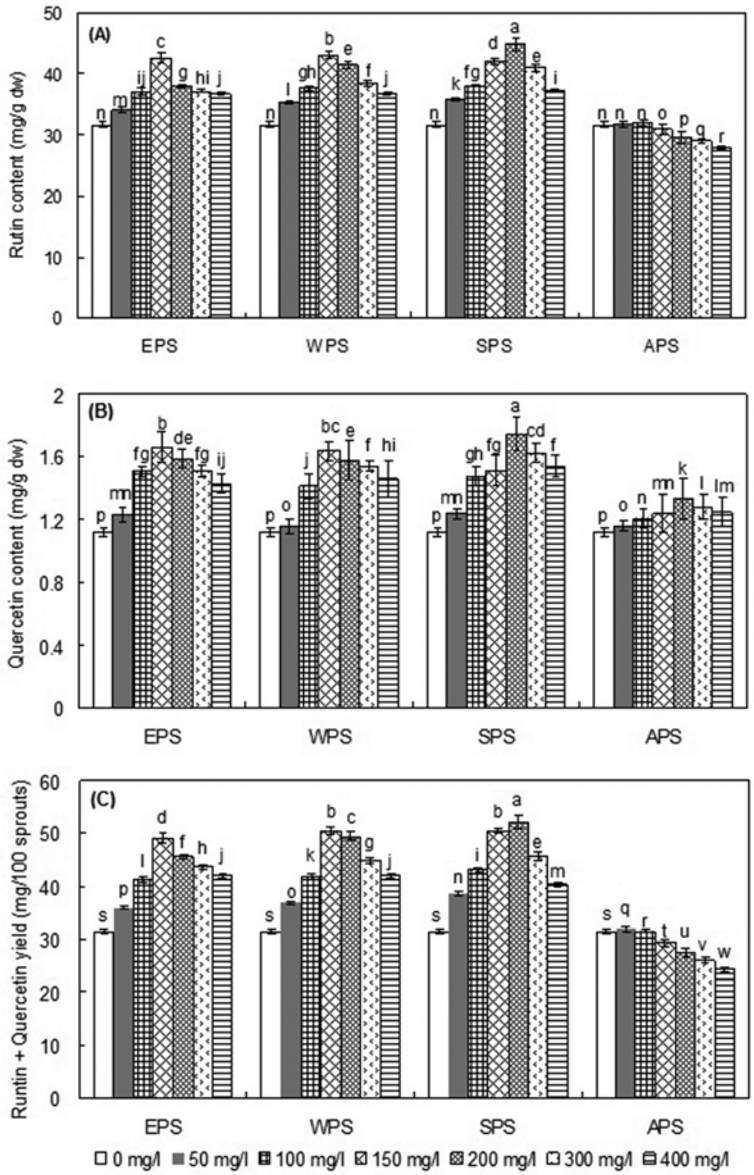

Figure 1. Effects of EPS, WPS, SPS and APS (25, 50, 100, 200, and 400 mg/l) of endophyte *Bionectria pityrodes* Fat6 on rutin content (A), quercetin content (B), and total rutin and quercetin yield (C) of *F. tataricum* ($n = 3$)

and 150 mg/l of EPS, respectively, about 1.57- to 1.66-fold compared with the control culture 31.40 mg/(100 sprouts) (Fig. 1C). The typical HPLC profiles for the ruin and quercetin analysis of control culture, EPS, WPS and SPS treated buckwheat sprout samples are shown in Fig. S2.

Kinetics of sprout growth and flavonoid accumulation after treatment with EPS, WPS and SPS

According to the previous investigation, the EPS (150 mg/l), WPS (150 mg/l), and SPS (200 mg/l) elicitors exhibited strong promoting effects on the buckwheat flavonoid production. Therefore, the kinetic studies of sprout growth, rutin and quercetin accumulation

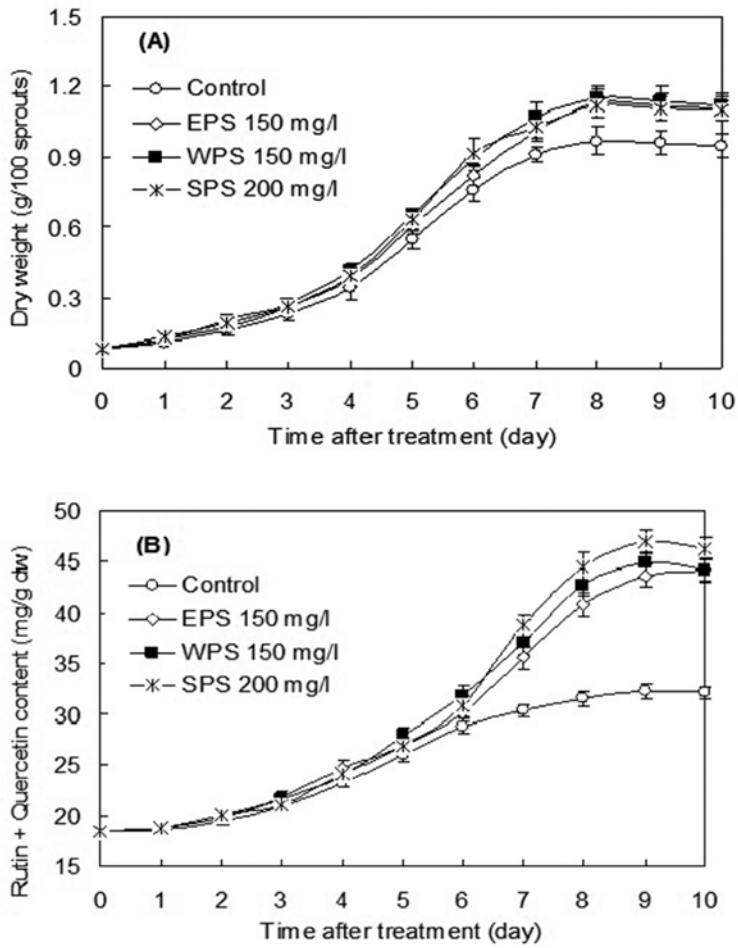

Figure 2. Kinetic studies of sprout growth (A), rutin and quercetin accumulation (B) of *F. tataricum* after treatment with 150 mg/l of EPS, 150 mg/l of WPS, and 200 mg/l of SPS of endophyte *Bionectria pityrodes* Fat6 compared with the control culture (n = 3)

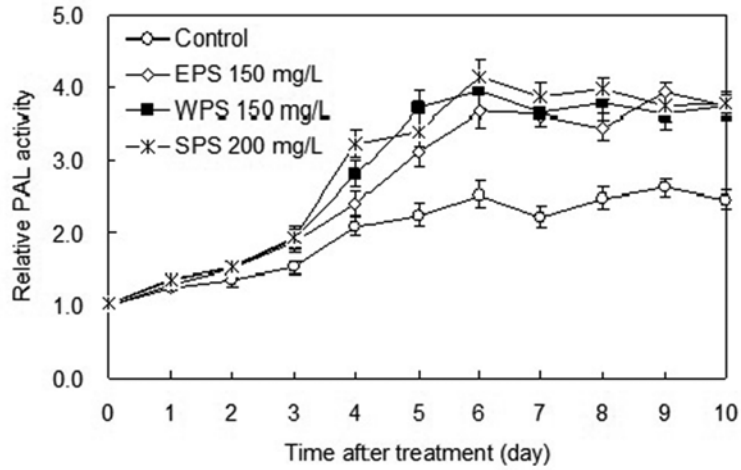

Figure 3. Time courses of PAL activity of *F. tataricum* after treatment with 150 mg/l of EPS, 150 mg/l of WPS, and 200 mg/l of SPS of endophyte *Bionectria pityrodes* Fat6 compared with the control culture (n = 3)

of tartary buckwheat stimulated by these effective elicitors were further investigated (Fig. 2). Generally, the promoting effects of three optimal PS elicitors on the sprout growth could be observed after 4 days elicitation treatments (Fig. 2A). The highest sprout biomass was 1.15 g/(100 sprouts) for 150 mg/l of WPS, about 1.2-fold in comparison with control culture of 0.97 g/(100 sprouts). The maximum buckwheat sprout dry weight was 1.13 g/(100 sprouts) for 150 mg/l of EPS, and 1.12 g/(100 sprouts) for 200 mg/l of SPS achieved on day 8. As shown in Fig. 2B, the stimulation effects of three fungal PS elicitors on rutin and quercetin accumulation could be significantly noticed after 5 days treatment, and then followed a steady increase to the end of the culture period. The highest rutin and quercetin content of *F. tataricum* sprouts was 46.93 mg/g obtained on day 9 for 200 mg/l of SPS, about 1.5-fold in comparison with the control culture of 32.25 mg/g, and that was 43.68 mg/g for 150 mg/l of WPS, and 44.85 mg/g for 150 mg/l of EPS, respectively.

The intracellular PAL activity of tartary buckwheat sprout cells was also effectively induced by the three fungal PS (150 mg/l of EPS, 150 mg/l of WPS and 200 mg/l of SPS) elicitation treatments, from 1.01- to 1.74-fold of the control lever over the culture periods, which are displayed in Fig. 3.

Discussion

Plant endophytic fungi are the fungal microorganisms that live asymptomatically within plant tissues. During the long period of co-evolution, a friendly relationship has developed between each endophyte and its host plant (Strobel et al. 2004). The fungal endophytes could produce many valuable components such as antimicrobial, insecticide, cytotoxic, and growth regulator agents. They would prevent the host from pathogen attacks,

promote host growing, improve adaptation and help the host to keep healthy (Rodriguez et al. 2009). The present study showed that the fungal polysaccharides obtained from endophyte *B. pityrodes* Fat6 could effectively promote the buckwheat sprout growth and flavonoids accumulation. As the PAL is a key enzyme at the entrance step in the phenylpropanoid pathway in plants, and its activity increase induced by elicitors is suggestive of an enhanced secondary metabolism in the plant cells (Kim et al. 2011). According to the results obtained from this research, it could be speculated that the phenylpropanoid pathway was closely associated with the flavonoid biosynthesis in buckwheat sprouts. That was in accordance with those found in previous investigations (Liu et al. 2006; Kim et al. 2011; Zhao et al. 2014). These valuable findings provide further evidence for the elicitor activity of fungal polysaccharide in stimulating the stress responses and secondary metabolism of buckwheat sprouts.

In conclusion, this study demonstrated that three exogenous fungal polysaccharides EPS, WPS, and SPS obtained from the endophyte *B. pityrodes* Fat6 exhibited strong promoting effects on the sprout growth and flavonoids production of tartary buckwheat. In addition, the present research also revealed the accumulation of flavonoids in *F. tataricum* sprout cultures was caused by the stimulation of the phenylpropanoid pathway by PS treatments. These findings indicate that polysaccharides from *B. pityrodes* Fat6 are promising candidates for potent elicitors and growth-promoting components for the buckwheat sprout cultures. Although, the chemical composition of these polysaccharides, the structure-activity relationship, the physiological responses and biochemical reactions of the sprout induced by polysaccharides, the process optimization of polysaccharide production, as well as the effects of PS on the growth and flavonoids biosynthesis of tartary buckwheat in soil need to be further clarified and studied.

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Electronic Supplementary Material (ESM)

Electronic Supplementary Material (ESM) associated with this article can be found at the website of CRC at http://www.akademiai.com/content/120427/

Electronic Supplementary *Figure S1*. The sprout cultures of tartary buckwheat

Electronic Supplementary *Figure S2*. Typical HPLC profiles for the rutin and quercetin analysis: control sprout cultures of *F. tataricum* (A), EPS (150 mg/l) treated sprouts (B), WPS (150 mg/l) treated sprouts (C), and SPS (200 mg/l) treated sprouts (D)