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Original Research Article

Effect of Endophytic Bacterium Inoculation on Total Polyphenol and Flavonoid Contents of Tartary Buckwheat Sprouts

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ABSTRACT The effects of endophytic microbial inoculation and temperature on the phenolic content of tartary buckwheat (TP) sprouts were investigated. TP seeds were inoculated with *Herbaspirillum* spp. at concentrations (%v/v) of 0 (control), 10, 20, and 40% at 20, 25, and 30ºC in a growth chamber for seven days. It was observed that the phenolic content (PC) including flavonoid, rutin, and tanin increased with an increase in inoculant rate at 20ºC, whereas the PC content increased with an increase in temperature regardless of the inoculant rate. Therefore, it is suggested that increasing the inoculant rate is effective at achieving higher phenolic contents when plants are grown at lower temperatures.

Keywords : endophytic bacterium, seed inoculants, tartary buckwheat, total phenol, total flavonoid, tanin

Buckwheat (*Fagopyrum* spp.) is an annual plant belongs to Polygonacae family. *Fagopyrum* spp. consists of at least 18 species distribution in the world wide (Ohnishi, 1995). However, only Common buckwheat (*Fagopyrum esculentum* Moench) and Tartary buckwheat (*Fagopyrum tatarucum* L. Gaertn.) are of economically important and contain higher nutritional value. This plant contain proteins, carbohydrates, lipids, fibers, vitamins, amino acids and minerals as their basic components. Moreover, buckwheat grains has no gluten, so they are safe for people with celiac disease (Halbrecq *et al*., 2005; Skerritt, 1986). Tartary buckwheat has higher rutin content than common buckwheat (Kitabayashi *et al*., 1995a, b). Many people have interest in utilizing of Tartary buckwheat grains and sprouts because of their nutritional and pharmaceutical values. Nowadays, fresh and dried Tartary buckwheat sprouts are attracting a lot of attention from pharmaceutical and food companies, and are applied in the prevention of obesity and diabetes (Lee *et al*., 2006; Lin *et al*., 2008). Tartary buckwheat sprouts contents phenolic compounds significantly higher during sprouting stage than matured stage (Sharma *et al*., 2012; Kim *et al*., 2008). Rutin including quercetin and catechin is the main polyphenol present in buckwheat (Park *et al*., 2005).

Tannins is a polyphenolic compound which is proven as antioxidant and possesses a chemo-protective potential found in plant food (Saxena *et al*., 2013). Gadzo *et al*. (2010) reported that light exposed leaves had higher concentration of tannins in Tartary buckwheat. On the other hand, the tannin contents significantly increased in the sprouting Tartary buckwheat compared to grains (Lee *et al*., 2004).

Endophytes are microorganisms that colonize living internal tissues of plants without causing any harm to their host. All types of microorganisms (fungi, bacteria and actinomycetes) have been discovered as endophytes. Studies have revealed the ubiquity of these microbes, with an estimate of at least one million species of endophytic microorganisms residing in plants with protect their hosts from infectious agents and adverse conditions by secreting bioactive secondary metabolites

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ⓒ 본 학회지의 저작권은 한국작물학회지에 있으며, 이의 무단전재나 복제를 금합니다.
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(Hyde & Soytong, 2008; Strobel, 2003). Moreover, there is enormous potential for exploiting these endophytes for medicinal, agricultural and industrial uses (Aly *et al.,* 2010). Therefore, the aim of this study was to investigate the secondary metabolites content of Tartary buckwheat sprouts inoculated with endophytic bacterium *Herbaspirillum rubrisubalbicanse* isolate at different temperature.

MATERIALS AND METHODS

Endophytic bacterium *Herbaspirillum rubrisubalbicans* was isolated from Tartary buckwheat stem at the Laboratory of Applied Microbiology, Department of Biology, Faculty of Science, Chiang Mai University, Thailand. The taxonomic studies of the bacteria isolate was determined as described in Bergy's manual of systematic bacteriology. Gram staining was performed after 24 h of bacteria cultivation at 37°C on nutrient agar media. Endospore formation was assessed with spore-staining method using malachite green (Schaeffer & Fulton, 1993). Colony formation was assessed by spread plate technique on nutrient agar media. The biochemical test including oxidase test, catalase test, methyl red test, Voges-Proskauer test and citrate test were performed according with the method described by MacFaddin (2000). The growth of endophytic bacterium *Herbaspirillum rubrisubalbicans* was evaluated by using nutrient broth (NB) and nutrient agar (NA) media (Atlas, 2010). Endophytic bacterium cultivation was conducted at 37°C on 150 rpm rotary shaker. Sample was taken every 1h and measured at 600 nm by spectrophotometer. Viable cell was determined by the spread plate method on nutrient agar (NA) media and all plates were incubated at 37°C for 24h. Colony formation was counted and the value was expressed as cfu/ml of sample.

A single colony was selected and inoculated into NB medium, then incubated at 37°C for 8-10 h under shaking conditions. Exponentially growing cells were harvested by centrifugation at 6000 rpm for 20 min, and re-suspended in sterilized normal saline (0.85% NaCl) solutions to obtain the final cell densities of 10^8 cfu/ml. The inoculums were prepared with the proper diluents by using sterile distilled water to obtain a final concentration of 10, 20 and 40% (v/v), respectively. Tartary buckwheat seeds were surface-sterilized with 2.5% (v/v) sodium hypochlorite for 3 min, and rinsed thoroughly in sterile distilled water for 4 times with 1 min duration. Seeds were soaked in liquid suspension of endophytic bacterium (10^8 cfu/ml) by separating each treatment for 0, 4, 8 and 12 h, at 30°C (Fig. 1).

Seven days-old Tartary buckwheat sprouts obtained from endophytic bacterium-treated seeds were harvested, cleaned and dried in hot air oven at temperature of 50°C for 24h. The dried samples were ground in a mortar and pestle into powder. The powdered samples (2.0 g) sprouts were taken and 100 ml of 80% methanol was added to each and incubated overnight in a shaker followed by filtration using filter paper No.2. The extract was dried using a rotatory evaporator (Eyela N-100, Japan) at a temperature of 40°C. The extracts were vacuum freeze dried to remove the remaining moisture. The extracts were stored in a refrigerator at -20°C for further analysis.

Chemicals and reagents

The following reagents were used: Folin-Ciocalteu's phenol reagent (Fluka), Folin-Denis reagent (Fluka), Gallic acid (Sigma-Aldrich), Tannic acid (Fluka), Sodium carbonate (Fisher), Aluminum nitrate nanohydrate (ACS reagent, Sigma-Aldrich), Potassium acetate (Fisher), Methanol (AR, ACI Labscan), Ethanol 95% (AR, ACI Labscan), Deionized water (Thermoscientific), Rutin (Sigma-Aldrich), Quercetin (Sigma-

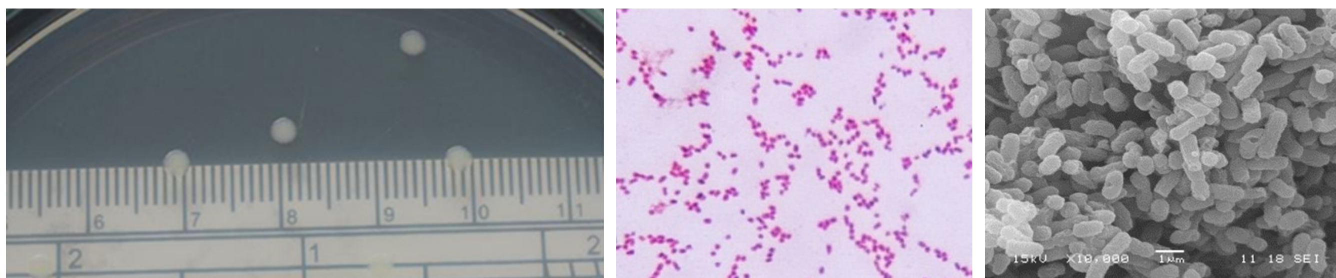

Fig. 1. Colony formation (left), Gram staining (middle) and cell morphology (right) of *Herbaspirillum rubrisubalbicans*.

Aldrich), Methanol (HPLC grade), Formic acid (Reagent grade, Sigma-Aldrich). 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich), Ferric chloride (Fluka), Ferrozine [Iron (II) chloride ($FeCl_2$)] (Fisher), Potassium ferricyanide (III) (RANKEM), Trichloroacetic acid (ACS reagent, Sigma-Aldrich), Ferric chloride (Fluka), disodium hydrogen phosphate Anhydrous (RANKEM), Sodium dihydrogen phosphate Anhydrous (UNIVAR).

Determination of total polyphenol and flavonoids content

Total polyphenol (TP) content was determined by the Folin-Ciocalteu assay method as described by Eom *et al*. (2008) with slight modifications. Briefly, an aliquot of 200 µl was added to a test tube containing 200 µl of phenol reagent (1 M). The volume was increased by adding 1.8 ml of distilled deionized water (DDW). Further 400 µl of Na_2CO_3 (10%, w/v) was added and adjusted final volume to 4 ml by adding DDW. The absorbance was measured at 725 nm. The TP content was expressed as mg of Gallic acid equivalents (GAE) per gram of dried sample.

Total flavonoid (TF) content was determined according to the method described by Eom *et al*. (2008) with slight modification. Briefly, an aliquot of 0.5 ml was mixed with 0.1 ml of aluminum nitrate (10%, w/v) and 0.1 ml of potassium acetate (1 M). Thereafter, 3.3 ml of 80% methanol was added to make the total volume 4 ml. The mixture was vortexed and incubated for 40 min and the absorbance was measured at 415 nm. Quercetin was used as a standard and the value of TF content was expressed as mg of quercetin equivalent (QE) per gram of dried sample.

Determination of total tannin content

Total tannin content was determined by Folin-Denis method (Saxena *et al*., 2013). 1 ml of extract was mixed with 7.5 ml of standard solution of tannic acid (10-50 µg/ml). Then 0.5 ml of Folin-Denis reagent and 1 ml of Na_2CO_3 (10%, w/v) solutions were added. The volume was made up to 10 ml with distilled deionized water. The absorbance was measured at 700 nm in spectrophotometer. Total tannins content was expressed as mg of tannic acid equivalent (TAE) per gram of extract.

Determination of rutin and quercetin content by HPLC

The quantitative estimation of rutin and quercetin were performed by HPLC (Agilent 1100 Series, HPLC Value System, Germany). The analysis was performed according to the procedure reported by Rangkadilok *et al*. (2005) and Zvezdanovic *et al*. (2012). The HPLC instrument and operating conditions for rutin and quercetin was as follows: injection volume 10 µl, flow rate 1.0 ml/ min, UV detector 270 nm and Zorbax C_{18} ABS (150 × 4.6 ㎜. i.d., 5 µm) reversed phased column with two mobile phase (A): Formic acid (0.4%) and (B): Methanol (100%).

Statistical analysis

Statistical analysis of all tests were carried out using Statistix software version 8.0. FL. Data was analyzed with ANOVA and Least significant difference (LSD) test at $P \leq 0.05$ level was used to separate the means when the ANOVA F-test indicated a significant effect of the treatments.

RESULTS AND DISCUSSION

Identification and growth of endophytic bacterium

The morphological characteristics and biochemical test of *Herbaspirillum rubrisubalbicans* were shown in Fig. 1 and Table 1. Cells of *H. rubrisubalbicans* exhibited gram-negative staining and negative staining endospore. For biochemical tests, negative test in methyl red test, indole test and oxidase test, while the positive test occurred with Vogas-Prokauer test, citrate utilization test and catalase test. However, the morphological characteristics, they generally have a circular form, convex elevation, entire margin and rode shape (Table 1). Colony formation with a small moist white colony and smooth with rode shapes was found in NB medium (Fig. 1). The optimal condition for *H. rubrisubalbicans* growth was performed using nutrient broth and nutrient agar medium. It is reported that endophytes have beneficial effects on plant growth, improvement of plant resistance to environmental stress and enhance N_2 fixation (Spiering *et al*., 2006; Briatia *et al*., 2016). The exponential growth phases was 8-10h of incubation with a cell density 10^8 cfu/ml, moreover, viable cell was more than 300 cfu/ml after 48h of incubation times (data not shown).

Table 1. Biochemical test for endophytic bacterium *Herbaspirillum rubrisubalbicans* isolate.

Biochemical test	Observation results
Colony forming	Moist, smooth and small white colonies
Colony characteristic	Form (circular), elevation (convex) and margin (entire)
Cellular shape	rod shaped
Gram staining	Negative gram staining
Endospores	Negative staining endospore (-)
Methyl red test	Negative test (-): yellow color (glucose is converted into neutral end products, indicating no acid production).
Vogas-Proskauer test	Positive test (+): red color (glucose is converted into product).
Indole test	Negative test (-): failure to see a red layer (indole was not formed from tryptophan).
Citrate utilization test	Positive test (+): blue color (meaning the bacteria metabolized citrate utilize the ammonium salts releasing ammonia and increasing the pH of the medium, produce an alkaline end product).
Catalase test	Positive test (+): visible bubbles (meaning the organism splits H_2O_2 to oxygen and water using the enzyme catalase).
Oxidase test	Negative test (-): light pink (meaning no color change) indicate the absence of oxidase.

Phenolic compound and flavonoid content

It is shown that seed inoculation significantly increased total polyphenol (TP) and flavonoid content of Tartary buckwheat sprouts compared to control at 20°C growth temperature. However, increasing temperature enhanced TP content at lower inoculant rate (Table 2). Generally, temperature has an enormous positive effect to accumulation of secondary metabolites in plant (Shiow and Zheng, 2001). However, Chen *et al.* (2010) reported that plant inoculated with *S. cinereus* had the highest total polyphenols content of tea leaves. Aquaculture buckwheat sprouts significantly enriched total polyphenolics content higher than that of solid-phase cultured to 13% (Peng *et al*., 2009).

The content of total flavonoid of sprouts was high in order, 40% > 20% > 10% > control at 20°C, however, the order is different where 10% and 20% inoculation has same trends for flavonoid content at 25 and 30°C (Table 3). From these results it is commercially economic to consider lower growth temperature regarding inoculation ratio. Tao *et al*. (2004) shown that Tartary buckwheat production by application of microbial inoculants *(Bacillus megaterium)* effectively increased flavonoids content. Furthermore, they suggested that *Streptomyces* strain could be adopted in the production of Tartary buckwheat sprout to get better quality buckwheat tea. Randhir *et al*. (2008) found lower phenolic content in buckwheat seedling when grown under high temperature. Li *et al*. (2010) reported that after treated Tartary buckwheat by endophyte strains N_3, the content of flavonoids reached 4.59% and the production of flavonoids was 15.3% higher than the control.

Table 2. Total polyphenol content of tartary buckwheat sprouts (mg/g dw).

Inoculants (% v/v)	Sprouts growth temperature		
	20°C	25°C	30°C
Control	38.13 ± 0.51c	47.99 ± 0.06b	42.67 ± 0.13b
10%	42.31 ± 1.29b	50.54 ± 0.19a	44.13 ± 0.51a
20%	42.99 ± 0.19b	50.31 ± 0.13a	44.90 ± 0.32a
40%	45.35 ± 0.45a	44.13 ± 0.26c	43.31 ± 1.41b

The results are expressed as means ± SD (n=3). Values with the same letter in columns are not significantly different at P < 0.05.

Table 3. Total flavonoid content of tartary buckwheat sprouts (mg/g dw).

Inoculants (% v/v)	Sprouts growth temperature		
	20°C	25°C	30°C
Control	77.15 ± 1.08c	93.78 ± 0.92b	74.11 ± 0.46c
10%	91.00 ± 0.31b	96.93 ± 1.08a	84.87 ± 0.31a
20%	92.04 ± 1.23b	98.67 ± 1.08a	80.96 ± 0.46ab
40%	99.17 ± 1.54a	86.28 ± 0.77c	77.15 ± 0.46b

The results are expressed as means ± SD (n=3). Values with the same letter in columns are not significantly different at P < 0.05.

Total tannin content

Results of total tannin content is presented in Table 4. At 20°C growth temperature total tannin content significantly increased when the inoculation rate was 40%. However tannin content increased with increasing temperature regardless inoculant rate. Buckwheat sprout is known as a rich source of tannin (Kreft *et al*., 2002). Previous report explored that tannin content highly affected by *Bradyrhizobium* inoculation in cowpea seeds (Musa *et al.*, 2011). Elsheikh (2001) also demonstrated that *Rhizobium* inoculation of legumes seed significantly increased tannin content. However, higher tannin content was achieved in 25°C temperature in our study. Similar results were obtained by Wu *et al.* (2016), they found higher tannin content of sorghum grain when grown under high temperature. In the same way Trifoli leaf was accumulated higher condensed tannin when grown in high temperature (Lees *et al.*, 1994).

Table 4. Total tannins content of tartary buckwheat sprouts (mg/g dw).

Inoculants (% v/v)	Sprouts growth temperature		
	20°C	25°C	30°C
Control	23.80 ± 0.57c	27.35 ± 0.21b	24.05 ± 0.35b
10%	26.45 ± 0.35b	30.10 ± 0.14a	25.40 ± 0.14a
20%	26.65 ± 0.07b	29.56 ± 0.07a	25.50 ± 0.28a
40%	28.30 ± 0.42a	25.05 ± 0.07c	24.88 ± 0.14ab

The results are expressed as means ± SD (n=3). Values with the same letter in columns are not significantly different at $P < 0.05$.

Rutin and quercetin content

It is observed that rutin content was highest at 40% inoculation rate at 20°C temperature, however, quercetin content was increased at 20% inoculation rate at 30°C temperature (Table 5). The chemical structure shows that rutin has sugar moiety in their ring, on the other hand, quercetin form without this moiety. High temperature may destructed the sugar moiety from rutin therefore, rutin converted to quercetin therefore enhanced quercetin was achieved. Report showed that seed inoculation with microbes (*Streptomyces jingyangesis*) significantly increased rutin content in tartary buckwheat (Tao *et al.*, 2004). Similar results were obtained by Singh *et al.* (2016) in rutin content from rice. They observed that seed inoculation with *Bacillus subtilis* greatly increased rutin and other phenolic content from rice seed.

The enriched phenolic compounds can be the reason for the enhanced biofilm formation which has been implicated to play a key role in efficient root colonization. Exerting chemical from microbes in plant root zone might have beneficial effects on plant secondary metabolites. Study showed that effective *microbial* colonization in *Arabidopsis thaliana* roots was triggered by certain plant polysaccharides (Beauregard *et al.*, 2013). Similarly, in another study L-malic acid (MA) secreted from roots of *A. thaliana* and recruited the beneficial rhizobacterium *B. subtilis* thus accelerate to accumulates plant secondary metabolites.

Table 5. Rutin and quercetin contents of tartary buckwheat sprouts (mg/g dw).

Contents (mg/g dw)	Inoculants (% v/v)	Sprouts growth temperature		
		20°C	25°C	30°C
Rutin	Control	54.52 ± 1.63d	56.77 ± 0.97b	62.06 ± 0.41b
	10%	62.72 ± 1.59b	62.68 ± 2.02a	64.31 ± 1.15a
	20%	58.56 ± 1.32c	62.11 ± 1.75a	65.91 ± 0.37a
	40%	67.06 ± 1.13a	57.40 ± 0.37b	61.32 ± 1.72b
Quercetin	Control	0.66 ± 0.05c	0.66 ± 0.13b	1.07 ± 0.09c
	10%	0.78 ± 0.03a	0.88 ± 0.03a	1.18 ± 0.12b
	20%	0.73 ± 0.02ab	0.84 ± 0.06a	1.41 ± 0.07a
	40%	0.71 ± 0.01b	0.81 ± 0.03ab	1.15 ± 0.07b

The results are expressed as means ± SD (n=3). Values with the same letter in columns are not significantly different at $P < 0.05$.

CONCLUSION

It is observed from our findings that inoculation rate is depended upon plant growth environment. Higher inoculant rate is effective when sprouts are grown at lower temperature. Inoculated plant grown under elevated temperature is giving same output as low temperature dose. As therefore, it might be suggested that Tartary buckwheat inoculated with *Herbaspirillum* spp should be grown under lower temperature in order to get higher secondary metabolites.

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