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EFFECT OF BRASSIN-LIKE SUBSTANCE ON THE QUALITY OF EARLY GERMINATED ARABICA COFFEE BEAN (*Coffea arabica* L.)

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ABSTRACT

Arabica coffee beans were monitored to evaluate the changes of chemical compositions during early germination period after soaking with brassin-like substance (BS). The experiment was designed as 4×5 factorial in completely randomized design (CRD) by soaking coffee seeds into BS at 4 different concentrations (0 (control), 0.5, 1.0 and 2.0 mg/L), which considered as factor 1. Chemical compositions were determined at 5 germination times (before soaking, day 2, 4, 6 and 8 after soaking), which considered as factor 2. Three replications were made on each combination and each replication consisted of 100 coffee seeds. The results showed that chemical compositions and antioxidant activity were clearly effected by BS and germination time. Caffeine content significantly declined upon BS concentrations. Furthermore, the content also gradually decreased during early germination. Interestingly, soaking coffee seeds in 2.0 mg/L BS could enhanced the greatest amount of chlorogenic acid content within 4 days after soaking. The compound, however, sharply declined afterward. BS could decrease total phenolic compounds in germinated coffee seeds. BS applications at concentrations of 1.0 and 2.0 mg/L also showed greater antioxidant activity than those seeds applied by BS 0.5 mg/L and the control. Moreover, radical scavenging activity of coffee seed crude extracts also indicated that the activity tended to increase during early germination.

Keywords: Early germination, chemical compositions, brassin-like substance, arabica coffee

1. INTRODUCTION

The most important beverage crop nowadays that serves millions of people is coffee. Vietnam, Indonesia and Thailand are the major countries that provide coffee products to Asia's market. The office of agricultural economics revealed an export value which reached over 3.1 million USD. Arabica coffee (*Coffea arabica* L.), a typical coffee species. Coffee beans and beverage products have been studied and proofed their health-promoting ability. Through variety selections, appropriate agricultural practices and better postharvest techniques, coffee bean quality has improved continuously. Several publications reported on health bene-fits of long-term consumption due to coffee's nutritive specialty. To date, coffee is considered to be a functional food since it contains high amount of notable bioactive compounds which exhibit high level of antioxidant activity (Farah, 2008). In addition, it has unique aroma and tastewhich elicited by several volatile compounds once being roasted (Yeretzian et al., 2003).

Seed has high nutritional values, however, germinated one has been found to be even greater quality (Wang and Fields, 1978). Seed in germination stage has become a popular choice in food industry worldwide due to the improvement of chemical compositions and other nutritive substances during certain phenomena (Frias et al., 2007). The process involves in several crucial biological processes which result in the production of nutrition, such as proteins, fats and carbohydrates, as well as other bioactive compounds (Marcone, 2004; Gulewicz et al., 2008). To date, only little work has investigated coffee seed germination in term of its regulation. Moreover, an application of plant growth regulators was immature, somewhat provided negative effects on coffee seed germination such as the application of exogenous gibberellic acid (GA_3) and abscisic acid (ABA) (Valio, 1976; Da Silva, et al., 2008).

Brassinosteroids (BRs) are the class of natural plant steroid hormones that control growth and development including cell division and seed germination. The plant growth regulators were recently also found to enhance antioxidant activity through the synthesis of secondary metabolites, such as flovonoids, phenolics, anthocyanins and tannins in plant seeds (Bajguz and Hayat, 2009; Choudhary et al., 2011; Swamy and Rao, 2011; Xi et al., 2013). Likewise BRs, brassin-like substance (BS), a synthetic brassinosteriod, has been studied and believed to enhance seed germination, growth and development, and metabolic process in several plants. Thus, the purposes of this study were

(i) to evaluate the effect of brassin-like substance and (ii) to determine the influence of germination process through the changes of chemical compositions during Arabica coffee seed germination.

2. MATERIALS AND METHODS

2.1 Plant material: Fully ripened Arabica coffee (*Coffea arabica* L. cv. Catimor) fruits were harvested from a Highland Research and Training Center (Khun Chang Kien Station). The fruits were de-pulped by hand and left for 48 hours in fermented container. Mucilage was subsequently removed afterward, and then the seeds were washed by running tab water and dried until seed moisture content declined as low as 12%. Lastly, each seed was individually removed from its endocarp manually.

2.2 Germination procedure: Prior to germination procedure, all coffee seeds were submerged into 1% sodium hypochlorite (NaOCl) solution for 2 minutes, and rinsed off by distilled water after ward. Four treatments were made on this experiment by soaking (24 hours) coffee seeds into 4 different solutions, namely, (i) distilled water (control treatment), (ii) 0.5mg/L brassin-like substance (BS) (iii) 1.0 mg/L brassin-like substance and (iv) 2.0 mg/L brassin-like substance. Each treatment consisted of 3 replications, in which each replication had 100 coffee seeds. Germination procedure was conducted as explained by Valio (1976). Coffee seeds were placed between 2 layered-paper in the aluminum tray (width×length = 18.0×29.0 cm). Each tray was moistened by spraying distilled water at an amount of 2.5 times of dry paper weight (20 mL). Subsequently, all trays were incubated in a germination chamber (30.0±1.0ºC, relative humidity (RH) of 85.0±5.0 %) (Model 620 RHS, P6 Contherm, New Zealand) under dark lighting condition. At day 0 (before soaking brassin-like substance), day 2, 4, 6 and 8 after soaking, coffee seeds (100 seeds) from each treatment were randomly sampled for bioactive compounds and antioxidant activity determinations.

In order to acquire the effect of BS on coffee bean during germination, the experiment was designed as 4×5 factorial in completely randomized design (Factorial in CRD) with 3 replications, and 100 coffee seeds were used in each replication.

2.3 Determination of bioactive compounds and antioxidant activity

2.3.1 Caffeine content: Caffeine content was determined by a high performance liquid chromatography (HPLC) as described by Wang et al., (2000). One gram of dried ground green coffee was dissolved in 10 mL of absolute methanol solution (Labscan, Thailand). The mixture was consistently shaked for 1 hour at room temperature and left overnight at the same condition. Supernatant was collected and filtered through a 0.45 µm, 13 mm nylon filter. Filtered solution was kept in brown vial at -20ºC waiting for caffeine content analysis. The sample was subjected to HPLC (SCL 10 AVP, Shimadzu, Japan) equipped with a C18 column (Platinum EPS 100A 3µ (53.0×7.0 mm)) (Grace, USA) with a flow rate of 20 mL/min at 25ºC. The mobile phase was isocratic: acetonitrile in water (13:87 v/v) and trifluoro acetic acid 0.5 mL/L. A UV/Vis diode array detector (Shimadzu, Japan) was used at a wavelength of 210 nm. Caffeine content was quantified against caffeine standard and expressed as mg/gDW of dried coffee bean.

2.3.2 Chlorogenic acid content: Chlorogenic acid content was extracted and quantified as described by (Ky et al., 1997). One gram of dried ground green coffee seed was dissolved in 100 mL of 70% methanol solution with 0.5mL of 0.5% Na_2CO_3. The sample was consistently stirred (125 rpm) under dark lighting condition at 4ºC for overnight. Then, the extract was obtained by filtering the aforementioned mixture through a sterile cotton cloth. Two mL of Carrez reagent (Ajaxh, Australia) was added into 50 mL of the extract solution and stirred thoroughly. The obtained mixture was subjected to HPCL apparatus, SPD-M10 AVP (Shimadzu, Japan) equipped with an Ultra C18 5 µm column (250.0×4.6 mm) (Restek, USA) with a flow rate of 0.8 mL/min at 25ºC. Two mM phosphoric acid in 5% methanol was used as a mobile phase. A UV/Vis diode array detector (Shimadzu, Japan) was used at a wavelength of 325 nm. Chlorogenic acid content was quantified against its standard and expressed as mg/gDW of dried coffee bean.

2.3.3 Total phenolic contents: Folin-Ciocalteu colorimetric assay (Folin-Ciocalteu, 1927) was used to determine total phenolic compounds in coffee seed. One gram of dried ground seed was added into 10 mL of absolute ethanol (Merck, Germany), stirred thoroughly for 1 hour at room temperature, and kept under dark lighting condition for 24 hours. Supernatant (crude extract) was collected afterward, and kept at 4ºC for further analysis. One hundred µl of crude extract was added into 1 mL of phenol stock solution (Folin-Ciocalteu's reagent:distilled water; 1:1) (Fulka, Switzerland). Subsequently, 1 mL of 10% Na_2CO_3 (Ajax, Austria) and 4 mL of distilled water were added into the aforementioned mixture. The sample was left at room temperature under dark

lighting condition for 45 minutes prior to measuring an absorbance at 760 nm by spectrophotometer, Genesys20 (Thermo Scientific, USA). Total phenolic content was expressed in milligram gallic acid equivalent per gram dry weight (mg GAE/gDW).

2.3.4 Antioxidant activity: Free radical scavenging activity of the germinated coffee seed was studied using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity assay described by Brand-Williams et al., (1995). Crude extract was obtained as described in the aforementioned paragraph. DPPH solution was prepared by dissolving 31.36 g of DPPH (Fluka, Switzerland) in 15 mL of absolute ethanol and adjusted to a final volume of 100 mL with distilled water. Trizma buffer solution was prepared by dissolving Triss (hydroxymethy) aminomethane (Sigma, Switzerland) 6.057 g and potassium chloride (Ajax, Switzerland) 17.184 g in 900 mLof distilled water. The mixture was adjusted its pH to 7.4 by adding 5 N hydrogen cyanide (HCN) (Merck, Germany) prior to making up a final volume of 1 l using distilled water. As for antioxidant activity analysis, 600 µl of crude extract was added into 600 µl of DPPH solution, Trizma buffer solution and 85% ethanol (totaling 2.4 mL). The sample was incubated under dark condition for 30 minutes at room temperature. An absorbance of solution was measured at 525 nm and quantified against its Trolox solution standard.

2.4 Statistical Analysis: Means of each data combinations were analyzed by SPSS v.18.0 software (SPSS Inc, IL, USA). In order to inter pret significant among the means (p<0.05), Duncan's multiple range test (DMRT) was used.

3. RESULTS AND DISCUSSION

3.1 Caffeine content: During early germination, caffeine content was found to decrease gradually (Figure 1). The content remained about half of the initial values (non-germinated seed) at day 8 of germination. Pretreatment with BS concentrations of 1.0 and 2.0 mg/L had lower caffeine content than the seeds applied by BS concentrations of 0 and 0.5 mg/L (Table 1). Nonetheless, there was no significant interaction among BS concentration and germination time. Although several researches have reported about positive effects of BS on seed germination and plant growth, the effects on caffeine in early germinated coffee seed is very limited. Petermann and Baumann (1983) found that caffeine content in coffee seed endosperm was unchanged during cotyledon development, meaning the compound was neither degraded nor additionally formed during seed germination. In contrast with this study, Baumann and Gabriel (1984) found that caffeine content markedly increased during initial germination throughyoung seedling. However, in our study, the declines of caffeine content in all treatments especially for BS applications might occurred by the translocation and catabolism of caffeine in seed endosperm. In order to avoid autotoxicity of endogenous caffeine to harm embryonic growth and differentiation during an initial germination (up to 2 weeks), considerable amounts of caffeine might be translocated and/or catabolized naturally (Friedman and Waller, 1983). The BS applications in high concentrations (1.0 and 2.0 mg/L) might typically enhanced seed germination (Clouse, 1996; Clouse and Sasse, 1998), therefore the anti-autotoxicity probably occurred faster via a rapid growth and development of embryos as well.

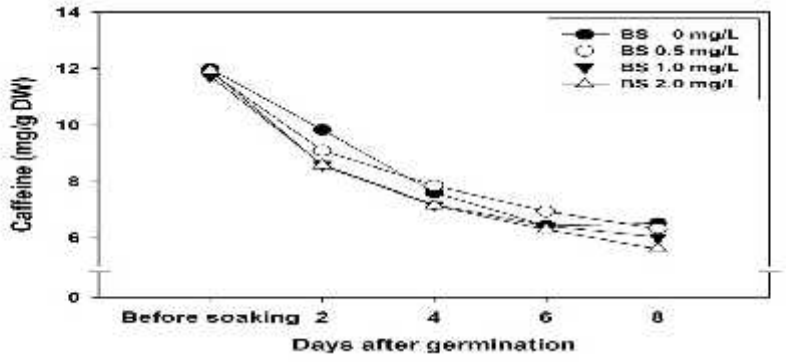

Figure -1: Changes of caffeine content during *Coffea arabica* seed germination after soaking in BS at different concentrations

Table- 1: Effect of brassin-like substance on caffeine contents of early germinated *Coffea arabica* seeds

BS	Caffeine content (mg/gDW)					
	Days after soaking (DAS)					
	Before Soaking	2	4	6	8	Mean
0 mg/L	*11.99*	*9.84*	*7.61*	*6.43*	*6.53*	8.48^{A}
0.5 mg/L	*11.87*	*9.10*	*7.86*	*6.94*	*6.33*	8.42^{A}
1.0 mg/L	*12.04*	*8.60*	*7.17*	*6.43*	*6.02*	7.99^{B}
2.0 mg/L	*11.94*	*8.57*	*7.14*	*6.30*	*5.61*	7.91^{B}
Mean	11.88^{a}	9.03^{b}	7.45^{c}	6.52^{d}	6.12^{d}	
	Statistical results					
	Caffeine content					
BS	*					
DAS	*					
BS×DAS	NS					
C.V. (%)	9.77					

Means within the column followed by different capital letters, means within the row followed by different lowercase letters showed significant difference by Duncan's multiple range test (DMRT) at $p<0.05$.
" * " Denotes significant difference between treatments (BS)/days after soaking (DAS).
NS means no significant difference.

3.2 Chlorogenic acid content: Chlorogenic acid is a group of secondary phenolic metabolites formed by coffee (*Coffea* spp). (Lallemand et al., 2012). At early germination stage, chlorogenic acid did not changed much after 2 days from soaking, however, it sharply rose during 4 days after germination.Then, the contents declined rapidly afterward (Figure-2). BS treatment significantly affected the afore mentioned scenario. Coffee seeds in the control and BS concentration of 0.5 mg/L had higher chlorogenic acid content than BS 1.0 and 2.0 mg/L. Likewise caffeine content, the certain acid might be translocated and/or catalyzed slightly slower than seeds treated by high concentration of BS (Friedman and Waller, 1983).

An interaction effect among BS concentration and germination time was noted in this compound. In which, coffee seeds soaked in BS 2.0mg/L showed the highest amount of chlorogenic acid at day 4 54.86 mg/g DW (Table 2). While the minimum contents was found in seed treated by using the same BS concentration, however at day 8 of germination. The results corresponded to Aerts and Baumann (1994) who studied the physiological role of chlorogenic acid during development of coffee seedlings and discovered that over 98% of chlorogenic acid was found abundant in cotyledons and disappeared thereafter. This phenomenon suggested that the acid was mostly translocated to other organs for reasons, such as self defense and/or other compounds' forming. Furthermore, the authors also suggested that a rapid diminishing of chlorogenic acid during germination might coincide with the presence of cell-wall bound phenolic polymers in developing cotyledons. In addition to, Steck (1968), Rhodes and Wooltorton (1976) and Aerts and Baumann (1994) suggested that during Arabica coffee seed germination, chlorogenic acid could be rapidly metabolized to ferulic acid in order to form lignin molecules in cotyledons.

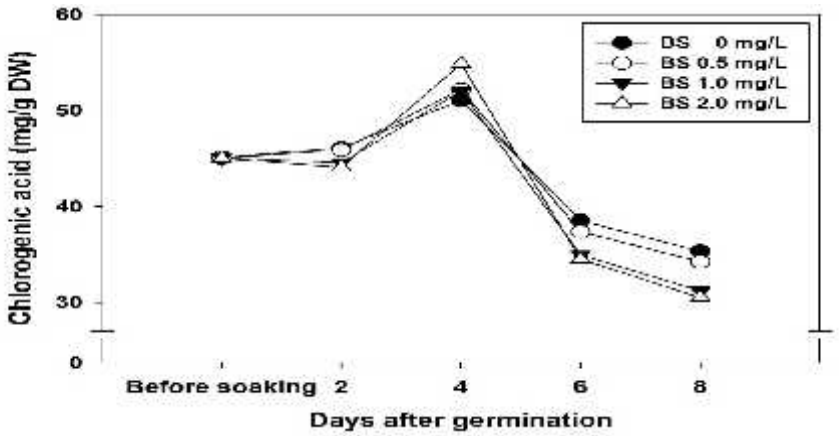

Figure -2: Changes of chlorogenic acid content during *Coffea arabica* seed germination after soaking in BS at different concentrations

Table -2: Effect of brassin-like substance on chlorogenic acid contents of early germinated *Coffea arabica* seeds

BS	Chlorogenic acid content (mg/gDW)					
	Days after soaking (DAS)					
	Before soaking	2	4	6	8	Mean
0 mg/L	*45.11 C*	*46.11 C*	*51.06 B*	*38.51 D*	*35.34 EF*	43.23 A
0.5 mg/L	*45.08 C*	*45.89 C*	*52.20 B*	*37.36 DE*	*34.24 F*	42.95 A
1.0 mg/L	*45.07 C*	*44.55 C*	*52.03 B*	*34.95 F*	*31.22 G*	41.56 B
2.0 mg/L	*45.01 C*	*44.11 C*	*54.86 A*	*34.50 F*	*30.51 G*	41.80 B
Mean	45.07 b	45.17 b	52.54 a	36.33 c	32.83 d	
	Statistical results					
	Chlorogenic acid content					
BS	*					
DAS	*					
BS×DAS	*					
C.V. (%)	4.65					

Means within the column followed by different capital letters, means within the row followed by different lowercase letters and interaction effects means followed by different italic capital letters showed significant difference by Duncan's multiple range test (DMRT) at $p<0.05$.
" * " Denotes significant difference between treatments (BS)/days after soaking (DAS).

3.3 Total phenolic contents: Early germinated coffee seeds treated by all BS concentrations had lower total phenolic content than BS-untreated seeds (Table 3). Application of BS could enhanced afast germination and resulted in a rapid loss of phenolic compound (Clouse, 1996; Clouse and Sasse, 1998) since the compounds were oxidized by activated polyphenol oxidase (PPO) during early germination (Rao and Deosthale, 1982; Saxena et al., 2003). Figure 3 clearly illustrates gradual losses of phenolic content in all treatments within 8 days of germination. Khandelwal et al., (2010) suggested that most of total phenolic content measured from the crude extract was a free and/or water soluble phenolic acids. During germination, small phenolic molecules were typically polymerized and formed to the insoluble structure; binding with organic substances such as carbohydrate and protein. Thus, the declining scenario of phenolic content probably occurred due to the polymerization of insoluble phenolic compounds.

There was a significant interaction among BS concentration and germination time. Total phenolic content in all treatments significantly lost about 50% of initial value. Aerts and Baumann (1994) indicated that phenolic acids occurred (from degradation chlorogenic acids) and deposited in cotyledonary cell walls prior to form phenolic polymers. Similar scenarios also found in several plant seeds, for examples, soy bean, kidney bean, mung bean (Rasha et al., 2011), cowpea (Giami et al., 2001) and Indian pulses (Khandelwal et al., 2010). Those potential literatures revealed that phenolic compounds gradually lost during germination, and the presence of polymerization of hydrophobic phenols was associated.

Table-3: Effect of brassin-like substance on total phenolic content of early germinated *Coffea ara bica* seeds crude extract

BS	Total phenolic content (mgGAE/gDW)					
	Days after soaking (DAS)					
	Before soaking	2	4	6	8	Mean
0 mg/L	*72.66 A*	*67.90 B*	*53.28 C*	*45.28 E*	*38.51 F*	55.52 A
0.5 mg/L	*72.77 A*	*52.30 CD*	*46.88 E*	*38.48 F*	*37.03 FG*	49.49 B
1.0 mg/L	*72.79 A*	*51.14 D*	*46.37 E*	*37.81 F*	*35.19 G*	48.65 B
2.0 mg/L	*72.74 A*	*52.03 CD*	*46.43 E*	*37.88 F*	*35.57 G*	48.93 B
Mean	72.74 a	55.84 b	48.24 c	39.86 d	36.58 d	
	Statistical results					
	Total phenolic content					
BS	*					
DAS	*					
BS×DAS	*					
C.V. (%)	2.45					

Means within the column followed by different capital letters, means within the row followed by different lowercase letters and interaction effects means followed by different italic capital letters showed significant difference by Duncan's multiple range test (DMRT) at $p<0.05$.
" * " Denotes significant difference between treatments (BS)/days after soaking (DAS).

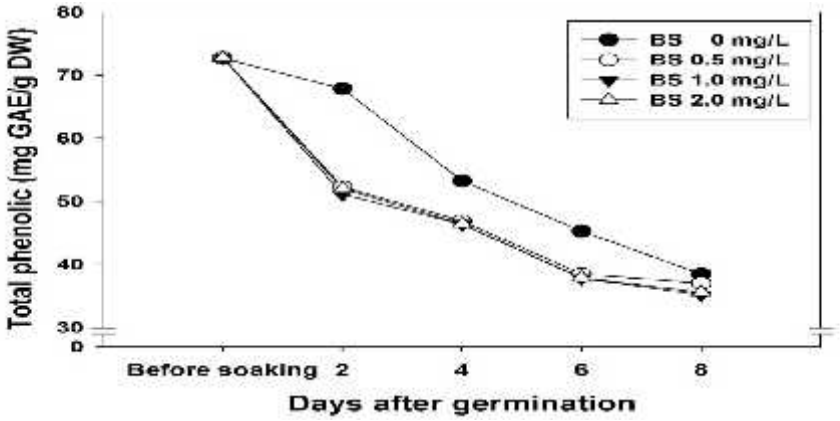

Figure -3: Changes of total phenolic content during *Coffea arabica* seed germination after soaking in BS at different concentrations.

3.4 Antioxidant activity: Coffee seeds soaked with BS concentrations of 1.0 and 2.0 mg/L had higher antioxidant activity greater than ones soaked with BS 0.5 mg/L and the control (Table 4). Early germinated coffee seed crude extracts exhibited high DPPH radical scavenging activity which up to 786.29 µMTrolox/gDW. The activities increased up to 10% from initial values in seeds treated by BS 1.0 and 2.0 mg/L concentrations. Figure 4 clearly showedthe sharp increases of antioxidant activities during germination. Nonetheless, there was no significant interaction among BS concentration and germination time. Generally, level of reactive oxygen species (ROS); free radicals that induce plant cellular damages upon cell membrane lipid peroxidation (Kelly, et al. 1998; Amin et al., 2015), increases together with coordinated changes of antioxidant enzymes in degraded endosperm during seed germination and development (Rogozhin et al., 2001; Bailly et al., 2002). Coffee seed typically enriches in several antioxidant compounds such as phenolic acids and chlorogenic acids which exhibited high level of radical scavenging activity (Natella et al., 2002). However, our obtained results showed negative relations between antioxidant activity and caffeine, chlorogenic acid and total phenolic contents (Figure 1, Figure 2 and Figure 3). Antioxidant activity from early germinated coffee seed crude extract gradually escalated while antioxidant compounds considerably declined. In such case, antioxidant activity might parallel with the increases of antioxidant enzymes rather than bioactive compounds during germination. Moreover, chlorogenic and phenolic acids were possibly metabolized, formed and/or conjugated with other compounds as previous discussion. Cai et al., (2011) studied lipid peroxidation and antioxidant responses during seed germination of physic nut (*Jatropha curcas*) and found that three major antioxidant enzymes were activated during early germination (within 10 days). In order to control excessive ROS from germination process, superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) were established and coherently functioned.

Table -4: Effect of brassin-like substance on antioxidant activity of early germinated *Coffea arabica* seeds crude extract

BS	Antioxidant activity(µMTrolox/gDW)					
	Days after soaking (DAS)					
	Before soaking	**2**	**4**	**6**	**8**	**Mean**
0 mg/L	*705.78*	*711.22*	*731.63*	*743.20*	*751.36*	728.33^{B}
0.5 mg/L	*703.06*	*711.90*	*722.11*	*747.28*	*754.76*	728.06^{B}
1.0 mg/L	*705.10*	*784.69*	*788.10*	*806.46*	*826.19*	781.94^{A}
2.0 mg/L	*703.06*	*790.14*	*797.62*	*811.90*	*827.55*	786.29^{A}
Mean	704.25^{d}	749.49^{c}	759.86^{bc}	777.21^{ab}	789.97^{a}	
	Statistical results					
	Antioxidant activity					
BS	*					
DAS	*					
BS×DAS	NS					
C.V. (%)	3.59					

Means within the column followed by different capital letters, means within the row followed by different lowercase letters showed significant difference by Duncan's multiple range test (DMRT) at $p<0.05$.

" * " Denotes significant difference between treatments (BS)/days after soaking (DAS).

NS means no significant difference.

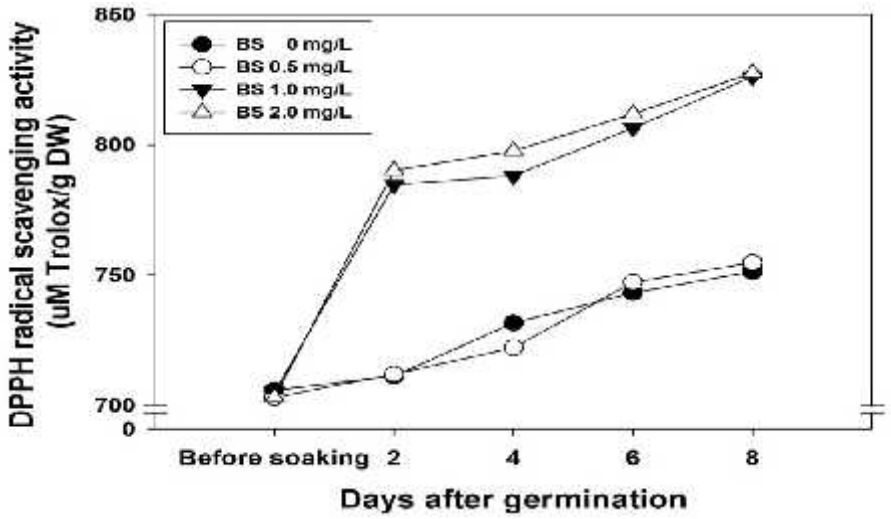

Figure -4: Changes of antioxidant activity during *Coffea arabica* seed germination after soaking in BS at different concentrations

4. CONCLUSIONS

The present finding suggested that BS application significantly influenced on bioactive compounds in Arabica coffee seeds during early germination. The use of high BS concentration exhibited a great presence of chlorogenic acid at day 4 of germination. Caffeine and total phenolic contents gradually declined upon germination. Changes of antioxidant activity might be regulated by antioxidant enzymes rather than bioactive compounds in degrading coffee seeds.

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