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Full Length Research Paper

Effect of salicylic acid application on biochemical changes in ginger (*Zingiber officinale* Roscoe)

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Salicylic acid (SA) belonging to plant phenolics group is found in some plant species and is capable of enhancing plant growth and yield. Effects of SA application (10^{-3} and 10^{-5} M) on synthesis of total soluble carbohydrate (TSC), total flavonoids (TF) and total phenolics (TP) were studied out in two ginger varieties (Halia Bentong and Halia Bara) under greenhouse conditions. In treated plants as the level of SA increased (from 10^{-5}M to 10^{-3}M) the production of TF increased while synthesis of TP decreased. SA induced production of TSC content in both varieties. Halia Bara exhibited a higher content of TSC (7.98 mg/g dry weight) compared to Halia Bentong (7.59 mg/g dry weight) when sprayed with low concentration (10^{-5}M) of SA. The result of high performance liquid chromatography (HPLC) analysis showed that concentration of the some majority flavonoids (quercetin, catechin and kaempferol) decreased significantly in plants when treated with different concentration of SA. Accordingly, high concentrations of these flavonoids were found in control plants. Furthermore, SA application stimulated synthesis of phenolic acids (cinnamic acid, vanillic acid, ferulic acid and gallic acid) in both varieties. These increases might be due to an increase in TSC content. The results implied that SA could be used for improving biochemical synthesis in young ginger.

Key words: Salicylic acid, ginger, total soluble carbohydrate, total phenolics, total flavonoids.

INTRODUCTION

Salicylic acid (SA) has been identified as one of the important phenolic compound in plants and also reported as allelopathic chemical (Chandra et al., 2007; Einhelling, 1986). The results of previous studies showed that production of soluble carbohydrates, sugars and secondary metabolites enhanced in plants exposed to SA. Shraiy and Hegazi (2005) reported positive effects of SA application were correlated with significant increase in total soluble proteins, phenol, total soluble carbohydrates and sugars in pea (*Pisum Sativum* L.) seeds. Flavonoids belong to a large family of polyphenolic components synthesized by plants (Ghasemzadeh et al., 2010). High contents of natural phenolic acids and flavonoids are found in green tea, fruits and vegetables, while some amounts of phenolics exist in red wine and coffee (Yao, 2004). Free radicals and single oxygen are recognized as major factors causing various chronic diseases such as cancer, diabetes, etc. The uptake of high levels of antioxidant supplements can reduce the risk of these diseases. Phenolic compounds have been implicated as natural antioxidants that may reduce oxidative damage to the human body. In addition phenolic acids and flavonoids are antioxidants with health benefits such as anti-inflammatory and antitumor effect (Ghasemzadeh and Jaafar, 2011; Chun et al., 2003). Recent work by Sung-jin et al. (2008) has shown that some flavonoid components in green tea are effective in inhibiting cancer or induce mechanisms that may kill cancer cells and inhibit tumor invasion. The shikimic acid pathway participates in the biosynthesis of most plant phenolics (Conn, 1986). In addition, Soluble carbohydrates are basic compounds required to produce phenolic component in the shikimic acid pathway. The shikimic acid pathway is able to convert simple carbohydrate precursors derived from glycolysis and the pentose phosphate pathway to the aromatic amino acids (Bryant et al., 1983). It was also noted that increase in phenolic concentration relates to the balance between carbohydrate source-sink, such that the greater the

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source:sink ratio, the greater the concentration of phenolic compounds (Shui-Yuan et al., 2009). Amin et al. (2007) found that salicylic acid regulates sugar contents (translocation from source to sink) and causes a significant increase in total soluble sugars. The plant hormones, salicylic acid (SA), ethylene and jasmonic acid (JA) have essential roles in mediating pathogen responses in plants (Navarro et al., 2008). Alonso-Ramirez et al. (2009) reported that giberellic acid stimulated in Arabidopsis may have an important role in SA biosynthesis and action, and that some of the physiological effects of this hormone may be mediated by SA. For instance, it has been proven that SA has an important role in heat and water stress responses (Singh and Usha, 2003; Clarke et al., 2004) or in the improved germination of *Arabidopsis thaliana* seeds under salt stress conditions (Rajjou et al., 2006).

The objective of this study was to examine the effect of foliar application of salicylic acid on synthesis of primary (total soluble carbohydrate) and secondary (flavonoids and phenolic acids) metabolites in two varieties of Malaysian ginger (*Zingiber officinale*) namely Halia Bentong and Halia Bara.

EXPERIMENTAL

Plant material and maintenance

Rhizomes of ginger varieties, Halia Bentong and Halia Bara (*Z. officinale*), were sprouted for two weeks in 10 cm diameter pots which were filled with peat. They were then transferred to polyethylene bags, which were filled with soilless mixture media including burnt rice husk and coco peat (ratio 1:1). The plants were grown in glasshouse at the glasshouse complex of University Putra Malaysia (UPM). The seedlings were raised in specially constructed growth houses receiving 12 h photoperiod and average photosynthetic photon flux density of 310 µmol/m^2/s. Day and night temperatures were recorded at 30 ± 1.0 and 20 ± 1.5°C, respectively, and relative humidity at about 70 to 80%. When the ginger seedlings were at the second leaf stage, they were sprayed with two concentrations (10^{-3} and 10^{-5} M) of salicylic acid solution (SA; 2-hydroxybenzoic acid + 100 µl dimethyl sulfoxide + 0.02% Polyoxyethylenesorbitan monolaurate, Tween 20, Sigma Chemicals; pH 6.5). The control plants were sprayed with same solution but without SA. Plants were sprayed once on the leaves early in the morning and every week until one month.

Extract preparation for total flavonoids and total phenolics

Leaves were dried (Freeze dry) to constant weights and powdered using mortar. Powdered sample (1 g) extracted using methanol (50 ml). The solutions were shaken for 1 h at room temperature using an orbital shaker. Extracts were filtered under suction and stored at −20°C for further use.

Determination of total soluble carbohydrate

A few drops of ethanol (80%) were added onto 0.1 g of freeze dried samples. Then 25 ml aqueous ethanol was added and mixed with shaking. Solutions were centrifuged at 5000 rpm. About 1 ml of supernatant was placed into test tubes and 10 ml of anthrone solution (0.15%) were added and finally the samples were heated. Tubes cooled down to room temperature. Absorption of the samples was recorded at 625 nm using a spectrophotometer (U-2001, Hitachi Instruments Inc., Tokyo, Japan) (Sivaci, 2006). Total soluble carbohydrate concentrations of the samples were calculated using the calibration curve drawn for glucose standard solutions. The soluble carbohydrate in the sample was expressed as mg glucose/g dry sample.

Determination of total phenolic content

The total phenolic content was determined using the method of Kim et al. (2003). Briefly, 1 ml of extract was added to deionized water (10 ml) and Folin–Ciocalteu phenol reagents 10% (1.0 ml). After 5 min, 20% sodium carbonate solution (2.0 ml) was added to the mixture. Solution was kept in total darkness and after that the absorbance was measured at 750 nm using a spectrophotometer (U-2001, Hitachi Instruments Inc., Tokyo, Japan).

Determination of total flavonoid Contrnt

The TF were measured following a previously reported spectrophotometric method (Bushra et al. 2009). Briefly, extracts of each plant material (1 ml) were diluted with 4 ml water in a 10 ml volumetric flask. Initially, 5% $NaNO_2$ solution (0.3 ml) was added to each volumetric flask; at 5 min, $AlCl_3$ added total content of flask's 10%; and at 6 min, 1.0 M NaOH (2 ml) was added. Absorbance of the reaction mixture was read at 430 nm.

High performance liquid chromatography (HPLC)

Analysis of flavonoids composition by HPLC

Reversed-phase HPLC was used to assay flavonoid compositions. The Agilent HPLC system used consist of a Model 1100 pump equipped with a multi-solvent delivery system and an L-7400 ultraviolet (UV) detector. The column was an Agilent C18 (5 µm, 4.6 × 250 mm). The mobile phase composed of: (A) 2% acetic acid (CH_3COOH) and (B) 0.5% acetic acid-100% acetonitrile (CH_3CN), (50:50 v/v), and gradient elution was performed as follows: 0 min, 95:5; 10 min, 90:10; 40 min, 60:40, 55 min, 45:55; 60 min, 20:80; and 65 min, 0:100. The mobile phase was filtered under vacuum through a 0.45 µm membrane filter before use. The flow rate was 1 ml/min and UV absorbance was measured at 280 to 365 nm. The operating temperature was maintained at room temperature. Identification of the flavonoids was achieved by comparison with retention times of standards, UV spectra and calculation of UV absorbance ratios after co-injection of samples and standards (Wang et al., 2007). Commercial standards were purchased from Sigma–Aldrich (St Louis, MO, USA).

Analysis of phenolics acids composition by HPLC

An Agilent HPLC system consisting of a Model 1100 pump equipped with a multi-solvent delivery system and a L-7400 ultraviolet (UV) detector was used. The column was an Agilent C18 (5 µm, 4.6 × 250 mm). The mobile phase was composed of (A) phosphoric acid (aqueous) and (B) acetonitrile and gradient elution,

Table 1. Effect of SA application (10^{-3} and 10^{-5} M) on total soluble carbohydrate (TSC), total phenolic (TP) and total flavonoids (TF) content in ginger (*Zingiber officinale*) varieties.

Characteristics	Halia Bentong			Halia Bara		
	Control	SA 10^{-5}	SA 10^{-3}	Control	SA 10^{-5}	SA 10^{-3}
TSC	5.95 ± 0.46^{b}	7.59 ± 0.69^{ab}	7.41 ± 0.69^{ab}	6.3 ± 0.97^{ab}	7.98 ± 0.97^{a}	7.72 ± 1.32^{a}
TF	9.3 ± 0.88^{ab}	7.98 ± 0.76^{b}	8.21 ± 0.92^{b}	10.87 ± 1.04^{a}	8.97 ± 0.78^{b}	9.35 ± 0.28^{ab}
TP	39.6 ± 2.91^{c}	49.5 ± 0.72^{ab}	46.9 ± 3.01^{ab}	44.06 ± 3.85^{bc}	53.23 ± 5.4^{a}	50.1 ± 2.78^{ab}

All analyses are the mean of triplicate measurements ± standard deviation; All of results expressed in mg/g dry weight; Means not sharing a common single letter were significantly different at $P \leq 0.05$.

was performed as follows: 0 min, 85:15; 12 min, 75:25; 20 min 75:25; 22 min, 85:15 and 30 min, 85:15. The mobile phase was filtered under vacuum through a 0.45 µm membrane filter before use. The flow rate and injection volume were 1 ml/min and 20 µl. UV absorbance was measured at 220 to 365 nm. The operating temperature was maintained at room temperature. Identification of the phenolic acids was achieved by comparison with retention times of standards, UV spectra and calculation of UV absorbance ratios after co-injection of samples and standards (Standard Operating Protocol, 2001). Commercial standards were purchased from Sigma-Aldrich.

Statistical analysis

The experimental results were expressed as mean ± standard deviation of three replicates. Data were analyzed using analysis of variance by Statistical Analysis System (SAS, system 9.0, 2002). Mean separation test between treatments was performed using Duncan multiple range test and P-value of < 0.05 was regarded as significant.

RESULTS AND DISCUSSION

Effect of foliar application of salicylic acid on total soluble carbohydrate (TSC), total flavonoids (TF) and total phenolics (TP)

SA treatments significantly ($P \leq 0.05$) affected TSC content (Table 1). Treated varieties by SA showed higher TSC content in comparison to control plants. Halia Bara had a higher content of TSC (7.98 mg/g dry weight) compared to Halia Bentong (7.59 mg/g dry weight) when the varieties were sprayed with 10^{-5} M SA. This concentration of SA enhanced production of TSC at about 27.5% in Halia Bentong and 26.6% in Halia Bara. SA treatment might also be assumed to inhibit polysaccharide-hydrolyzing enzyme system on one hand and accelerate the incorporation of soluble sugars into polysaccharides. In this regard, soluble sugar content was also increased in tomato plants in relation to salt stress (Maria et al., 2000). Our assumption could be supported by the result that SA increased polysaccharide level that is related to soluble sugars (Jeyakumar et al., 2008). In this context, Sharma and Lakhvir (1998) postulated that foliar spray of SA to ray plants resulted in decreasing their soluble sugar level. Khodary (2004) pointed out that SA application increased TSC content in maize. These results are in agreement with Amin et al. (2007) who reported that salicylic acid regulates sugar contents (translocation from source to sink) and causes a significant increase in total soluble sugars.

From Table 1, it is apparent that TF and TP accumulation and partitioning in the plant were significantly affected by the SA application ($P \leq 0.05$). High content of TF was observed in control plants (Halia Bentong: 9.3 mg/g dry weight; Halia Bara 10.87 mg/g dry weight). In treated plants, TF content increased from 7.98 to 8.21 mg/g dry weight in Halia Bentong and from 8.97 to 9.35 mg/g dry weight in Halia Bara with increasing in SA concentration from 10-5 to 10-3M. Conversely to TF, high content of TP was observed in treated plants (Halia Bentong: 49.5 mg/g dry weight; Halia Bara 53.23 mg/g dry weight) compared to control plants. However, with decreased SA concentration from 10-3 to10-5M, this resulted in the highest values in TP and lowest value in TF. In addition, TP content decreased from 49.5 to 46.9 mg/g dry weight in Halia Bentong and from 53.23 to 50.1 mg/g dry weight in Halia Bara when treated with higher concentration of SA (10-3M). Our results suggest the ability of SA application to alter or modify both the concentration and profiling of flavonoids and phenolic acids in ginger. According to our result, the increase in TP content might be due to increase in TSC production in the leaves. A positive and significant correlation between soluble carbohydrate and total phenolics was observed in previous studies (Ibrahim and Jaafar, 2011; Ghasemzadeh et al., 2010).

Isolation of flavonoid and phenolic compounds using HPLC

High performance liquid chromatography (HPLC) analysis of some flavonoids and phenolic acids are present in Table 2. Malaysian ginger especially Halia Bara contained considerably ($P \leq 0.05$) high amount of quercetin (1.13 mg/g dry weight), catechin (0.553 mg/g dry weight) and kaempferol (0.06 mg/g dry weight) in control plants. According to the data obtained from Table 2, the concentration of the majority flavonoids(quercetin, catechin and kaempferol) decreased significantly

Table 2. High performance liquid chromatography analysis of some flavonoid and phenolic constituents of SA-treated ginger (*Zingiber officinale*) varieties.

Compounds	Halia Bentong			Halia Bara		
	Control	SA 10^{-5}	SA 10^{-3}	Control	SA 10^{-5}	SA 10^{-3}
Quercetin	0.893 ± 0.03^{bc}	0.736 ± 0.09 ^{c}	0.79 ± 0.06^{c}	1.13 ± 0.12 ^{a}	0.883 ± 0.07^{bc}	0.993 ± 0.07^{ab}
Catechin	0.384 ± 0.049^{c}	0.276 ± 0.08 ^{d}	0.305 ± 0.09 ^{d}	0.553 ± 0.06^{a}	0.45 ± 0.06 ^{b}	0.55 ± 0.04 ^{a}
Kaempferol	0.04 ± 0.009^{b}	0.038 ± 0.009 ^{b}	0.029 ± 0.018 ^{b}	0.06 ± 0.001^{a}	0.048 ± 0.004^{ab}	0.044 ± 0.006^{ab}
Gallic acid	0.227 ± 0.049^{a}	0.29 ± 0.1^{a}	0.259 ± 0.045 ^{a}	0.259 ± 0.033^{a}	0.303 ± 0.097^{a}	0.304 ± 0.02^{a}
Vanillic acid	nd	0.037 ± 0.017^{bc}	0.028 ± 0.03 ^{bc}	0.077 ± 0.011^{b}	0.139 ± 0.046^{a}	0.134 ± 0.01^{a}
Ferulic acid	0.117 ± 0.02^{ab}	0.173 ± 0.055^{ab}	0.158 ± 0.042^{ab}	0.102 ± 0.042^{b}	0.193 ± 0.03 ^{a}	0.182 ± 0.017^{a}
Tannic acid	0.429 ± 0.049^{a}	0.332 ± 0.041 ^{a}	0.376 ± 0.122 ^{a}	0.354 ± 0.141^{a}	0.281 ± 0.05 ^{a}	0.269 ± 0.053^{a}
Cinnamic acid	nd	0.193 ± 0.045 ^{b}	0.184 ± 0.08 ^{b}	nd	0.242 ± 0.046^{a}	0.231 ± 0.014^{a}

All analyses are the mean of triplicate measurements ± standard deviation; All of results expressed in mg/g dry weight; Means not sharing a common single letter were significantly different at $P \leq 0.05$.; n.d: not detected.

in plants when treated with different concentration of SA. Conversely, amount of phenolic acids (gallic acid, cinnamic acid, ferulic acid and vanillic acid) increased significantly in both varieties when treated with different concentration of SA and only tannic acid did not show the highest content in this treatment. Furthermore, in both varieties of ginger, amount of vanillic acid, ferulic acid and cinnamic acid were observed to be higher at low concentration (10-5 M) of SA.

Among phenolics acids, tannic acid and gallic acid had more content in both varieties followed by ferulic and vanillic acids. The interesting finding was that the application of SA in both varieties induced synthesis of vanillic acid and cinnamic acid. These compounds were not detected from the control plants. According to HPLC analysis results, it could be concluded that application of SA induce synthesis of phenolic acids and conversely inhibit flavonoids synthesis in ginger.

The production of phenolic compounds is catalyzed by phenylalanine ammonia-lyase (PAL), (Figure 1). The results of our study are consistent with other studies and suggest that high content of some phenolic components such as cinamic acid can inhibit flavonoid biosynthesis with inhibition of PAL enzyme activity (Shui-Yuan et al., 2009). In this study, cinnamic acid was not detected in the control plants where instead high content of flavonoids was registered; but foliar application of SA induced synthesis of cinnamic acid in both varieties and following that, amount of flavonoids decreased in these plants. At higher SA concentration (10^{-3} M), the level of soluble phenolics in ginger leaves decreased and this reduction was already reported by Dixon and Paiva (1995). PAL is a key gateway enzyme in the secondary metabolic pathway leading to the synthesis of phenolic compounds (Keski-Saari, 2005).

SA inhibits the activity of PAL, a key enzyme in the synthesis of phenolic compounds (Nicholson and Hammerschmidt, 1992) and stimulates activity of chalcone synthase (CS) a key enzyme in the synthesis of flavonoids. In this study at low concentrations (10^{-5} M), SA stimulated the accumulation of soluble phenolic compounds in ginger leaves. It has been suggested that SA inhibits catalase activity leading to increased levels of H_2O_2 (Chen et al., 1993), which in turn induces PAL gene expression (Desikan et al., 1998) and synthesis of phenolic compounds (Dorey et al., 1997; Dihazi et al., 2003). In addition, at low concentration of SA, PAL catalyses the deamination of L-phenylalanine, and the product, trans-cinnamate, is converted in plants to various phenylpropanoid compounds such as chlorogenic acid, lignin monomers and flavonoids (Dihazi et al., 2003). This improvement in plant secondary metabolites might be due to increased total soluble content

Conclusions

Our assumption could be supported by the results that SA increased polysaccharide level related to soluble sugars. Furthermore, treatment with SA greatly increased synthesis of phenolic acids in ginger leaves whereas flavonoids synthesis decreased in both varieties. The results indicate that increasing in TP might be contributed by the increase in TSC content. Accordingly, synthesis of some phenolic acids like as vanillic acid and cinnamic acid induced by SA application whereas these compounds were not detected from control plants.

The results of our study are consistent with other studies and suggest that high content of some phenolic components such as cinamic acid can inhibit flavonoid biosynthesis with inhibition of phenylalanine ammonia lyase (PAL) enzyme activity.

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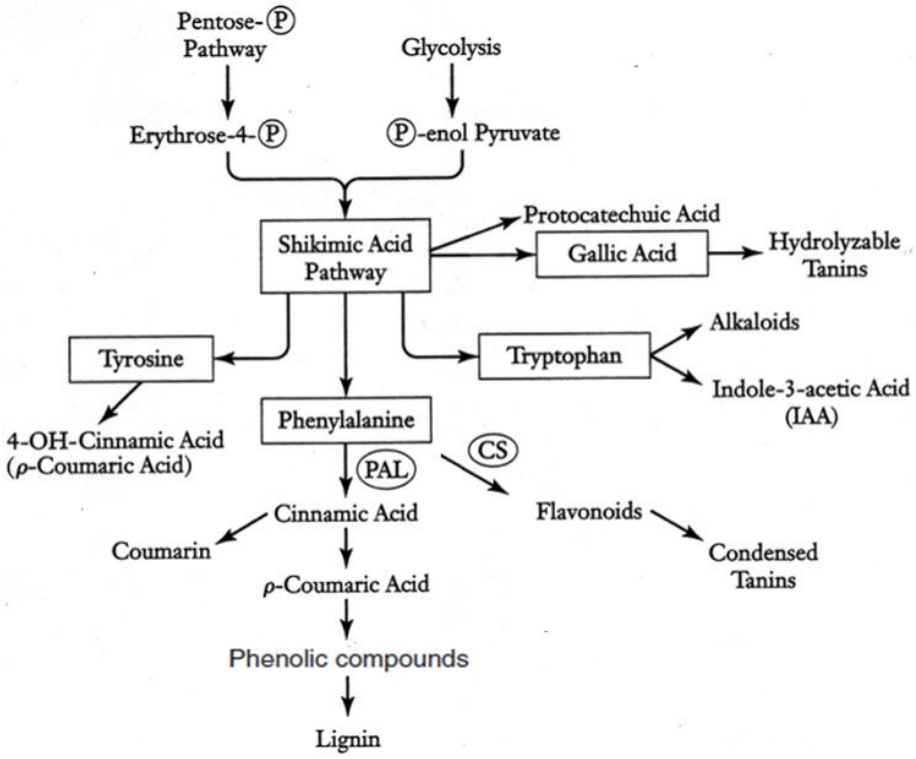

Figure 1. Shicimic acid pathway. Enzyme abbreviations: PAL, phenylalanine ammonia-lyase; CS, chalcone synthase.

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