Elicitation of Salicylic Acid on Secondary Metabolite Production and Antioxidant Activity of *In Vitro Musa acuminata* L. cv. 'Gros Michel' Shoots

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Abstract

In vitro Musa acuminata L. cv. 'Gros Michel' is a potential alternative source of secondary metabolites but low yield was obtained. Elicitation is used to increase secondary metabolite production by many plant species. The objective of this study was to investigate the effect of salicylic acid (SA), an elicitor, on secondary metabolite accumulation and antioxidant activity in shoot cultures of *M. acuminata* cv. 'Gros Michel'. Shoots of one centimeter in length were cultured for four weeks on Murashige and Skoog (MS) medium supplemented with 22.19 µM 6-benzyladenine (BA) and 0, 100, 200, or 300 µM SA. The results showed that SA was effective in enhancing the secondary metabolite production and antioxidant activity of *M. acuminata* cv. 'Gros Michel' shoots. The contents of secondary metabolites and antioxidant activity were significantly different among the treatments. Shoots treated with 100 µM SA exhibited the greatest accumulation of total saponins (263.86±0.42 mg diosgenin/ g dry extract), total phenolics (124.44±8.39 mg GAE/g dry extract), and total flavonoids (105.26±6.43 mg CE/g dry extract). These were 1.27, 1.25, and 1.34 times that of the control. The strongest antioxidant radical scavenging was also observed from 100 µM SA treated shoots (DPPH EC_{50} value of 9.79±0.86 µg/ml and ABTS EC_{50} value of 86.66±1.50 µg/ml).

Keywords: antioxidant; elicitor; phenolics; salicylic acid; saponins
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1. Introduction

Musa acuminata belongs to family Musaceae. Its fruits are a rich source of secondary metabolites including vitamins, phenolics, carotenoids, biogenic amines and phytosterols. Some of these compounds perform as antioxidants and are useful for human health [1]. *Musa acuminata* is a wild species of banana distributed in tropical regions of South East Asia [2]. 'Gros Michel' is one of the triploid cultivars of *M. acuminata*, and has the genome AAA. It is a seedless cultivar and is usually eaten raw [1, 3]. Traditional doctors use *M. acuminata* fruit, peel, pseudostem, flower, leaf, and root for the treatment of cancer, allergies, fever, coughs, and diabetes mellitus [2]. Onyema *et al*. [4] reported that *M. acuminata* pseudostem had pharmaceutical value, containing flavonoids, phenolic compounds, saponins, tannin, alkaloids, oxalate, hemagglutinin, phytate, and cardiac glycoside. Saponins are secondary metabolites that normally accumulate in plants and are involved in plant defenses against pathogens, pests, and herbivores [5]. Biological and pharmacological properties of these compounds have been reported such as antiphlogistic, antihepatotoxic, hypoglycemic, and antiallergic [6]. Phenolic compounds and flavonoids are secondary metabolites with polyphenol structures and are also produced as defense mechanisms [7]. They possess several biological activities including antioxidant effects, anticancer [8, 9], anti-inflammatory, and cardio-protective effects [9].

Plant tissue culture has been used for mass propagation of several plant species of *Musa* [10-13]. This *in vitro* technique offers both plant multiplication and secondary metabolite production from cells and organs, as each cell maintains a complete genetic information [7, 14]. A single cell can develop into a new plantlet and is able to synthesize the same secondary metabolites as the parent plant grown under natural conditions [14]. Production of secondary metabolites from plant tissue culture offers shorter production cycles, a constant product supply, consistent quality and independence from climatic factors [7, 14]. Studies of medicinal plants have identified secondary metabolite contents with antioxidant properties, but production may be low [7, 15]. Elicitation is an effective technique for enhancing secondary metabolite production under aseptic conditions. Type and concentration of elicitor significantly affect elicitation. It is necessary to determine the optimum elicitor type and concentration for each plant species [16, 17]. Salicylic acid (SA) has been widely used as elicitor inducing defensive mechanisms in many plant species [16]. SA is a phenolic compound that is normally found as a phytohormone [18]. Enhancement of secondary metabolite accumulation by SA has been reported for *Taxus chinensis* [19], *Hypericum hirsutum* [20], *Jatropha curcas* [21], *Thevetia peruviana* [22], *Chlorophytum borivilianum* [23], *Fagonia indica* [24], *Centella asiatica* [25], and *Piper cumanense* [26]. However, SA has not yet been applied to *M. acuminata* cv. 'Gros Michel'. In this study, the effect of SA at different concentrations on enhancement of total saponins, total phenolic compounds, and total flavonoids including antioxidants was investigated using *in vitro* shoot cultures of *M. acuminata* cv. 'Gros Michel'.

2. Materials and Methods

2.1 Plant material and culture conditions

In vitro shoots of *M. acuminata* cv. 'Gros Michel' were subcultured at four week intervals on Murashige and Skoog (MS) medium supplemented with 22.19 µM 6-benzyladenine (BA) and 3% (w/v) sucrose until sufficient regenerated shoots were available. The medium pH was adjusted to 5.6-5.8 with 1 N NaOH prior to gelling with 0.8% (w/v) agar, followed by autoclaving at 121°C for 15 min. The cultures were incubated at 25±2° C with a photoperiod of 16 h under fluorescent lamps with light intensity of 3,000 lx.

2.2 Preparation of elicitors

A stock SA solution at a concentration of 0.1 M (BDH Prolabo, Belgium) was prepared following Wang *et al*. [19]. This was dissolved in absolute ethanol and diluted with distilled water. The solution was filter sterilized before adding to the culture medium through a 0.22 µm syringe filter.

2.3 Elicitation treatments

Four-week-old *in vitro* shoots of *M. acuminata* cv. 'Gros Michel' were used as the explants. Shoots were cut into samples approximately one centimeter in length and cultured on MS medium supplemented with 22.19 µM BA and 100, 200, or 300 µM SA for four weeks. MS medium supplemented with only 22.19 µM BA was used as a control. The experiment used a completely randomized design with four treatments and three replications. Shoots were harvested and the shoot fresh weight was recorded. The shoots were dried at 50ºC for 48 h and the dry weight was recorded. Extracts were prepared by powdering the dried sample with pestle and mortar. The ground samples were macerated with ethanol at the ratio of 1:3 (sample: ethanol) for three days. Extracts were filtered, evaporated in a hot air oven at 50ºC for 48 h, and kept at -20ºC until use.

2.4 Determination of total saponin content

Total saponin content was measured using a vanillin-sulfuric assay modified from Hiai *et al*. [27]. One milligram of plant extract was dissolved in 2 ml of absolute ethanol and sonicated for 1 min. A sample of 0.25 ml was mixed with 0.25 ml of 8% vanillin reagent and 2.5 ml of 72% H_2SO_4. The mixture was incubated in a water bath at 60ºC for 10 min then cooled to 25ºC for 10 min. Absorbance was measured at 544 nm using a microplate reader (PowerWave XS-BT-MQX200R). The total saponin content was reported as mg diosgenin/ g dry extract.

2.5 Determination of total phenolic content

Total phenolic content was measured using the Folin–Ciocalteu method, following Jirakiattikul *et al.* [28]. Plant extract solution at a concentration of 1 mg/ml was sonicated for 1 min. A sample of 20 µl was mixed with 100 µl of dilute Folin-Ciocalteu reagent and 80 µl of 7.5% (w/v) sodium carbonate solution. The solution was incubated at 25ºC for 30 min and the absorbance was measured at 765 nm using a microplate reader. The total phenolic content was reported as mg GAE/g dry extract (mg of gallic acid equivalents per g dry extract).

2.6 Determination of total flavonoid content

Total flavonoid content determined using a modified method of Zhu *et al*. [29]. Plant extract solution at a concentration of 1 mg/ml was prepared. A sample of 500 µl was mixed with 75 µl of 5% (w/v) $NaNO_2$ for 6 min, then 150 µl of 10% (w/v) $AlCl_3$ was added. After a 5-min reaction, 500 µl of 1M NaOH and 275 µl of distilled water were added. The solution was left for 15 min and the absorbance was measured at a wavelength of 510 nm. Flavonoid content was determined using a catechin standard calibration curve and the results were expressed as mg CE/g dry extract (mg catechin equivalents per g dry extract).

2.7 Determination of antioxidant activity

2.7.1 DPPH radical scavenging assay

The scavenging capacity was evaluated following Yamasaki *et al*. [30]. Plant extract solution was prepared at a concentration of 1 mg/ml, and 100 µl was mixed with 100 µl of $6x10^{-5}$ M DPPH. The solution was left in the dark for 30 min and absorbance was measured at a wavelength of 520 nm. Inhibition (%) was calculated as follows: % Inhibition = $[(Abs_{control} - Abs_{sample})/ Abs_{control}] \times 100$ where $Abs_{control}$ is the absorbance of the control and Abs_{sample} is the absorbance of the sample. EC_{50} values were calculated using a regression equation.

2.7.2 ABTS radical scavenging assay

The assay procedure was modified from Re *et al*. [31]. Twenty µl of 10 mg/ml sample solution was combined with 180 µl of ABTS reagent. The solution was left for 6 min in the dark and absorbance was measured at a wavelength of 734 nm. Inhibition (%) and EC_{50} values were calculated as mentioned above.

2.8 Statistical analysis

All tests were performed in triplicate. Data was analyzed using one-way ANOVA followed by Duncan's new multiple range test for mean comparison at $p \leq 0.05$.

3. Results and Discussion

3.1 Fresh and dry weight of shoots

SA at all concentrations affected shoot fresh and dry weight (Figure 1). The lowest fresh weight of 53.85±7.50 mg/shoot was recorded for 300 µM SA, while the 100 and 200 µM SA treated shoots had fresh weights of 77.12±8.76 and 74.96±12.07 mg/shoot, respectively. The control treatment had the greatest shoot fresh weight of 153.91±12.40 mg/shoot (Figure 1 A).

The dry weight of SA treated shoots was significantly lower than that of control (Figure 1 B). The dry weights of the 100-300 µM SA treated shoots were 6.82±1.04-9.11±1.47 mg/shoot, compared with 11.82±0.86 mg/shoot for control.

The growth response to SA is different in each plant species. At low concentrations, it promotes plant growth, as SA is a growth regulator. Sakhanokho and Kelley [32] reported that the shoot biomass of *Hibiscus acetosella* and *H. moscheutos* (cv. '*Luna Red*') was improved by low concentrations of SA (0.5 mM). In our experiment, however, SA had a negative effect on shoot biomass. The concentration used may have been insufficiently low to act as a plant hormone. This may have generated an imbalance condition [33] or have reduced primary metabolism [34]. This was in agreement with previous studies, which reported decreased growth of plant cells or organs such as the callus of root cultures of *Panax ginseng* [35], cell suspension of *Gardenia jasminoides* [36], root cultures of *Talinum paniculatum* [37], and shoot cultures of *Dioscorea membranacea* [38].

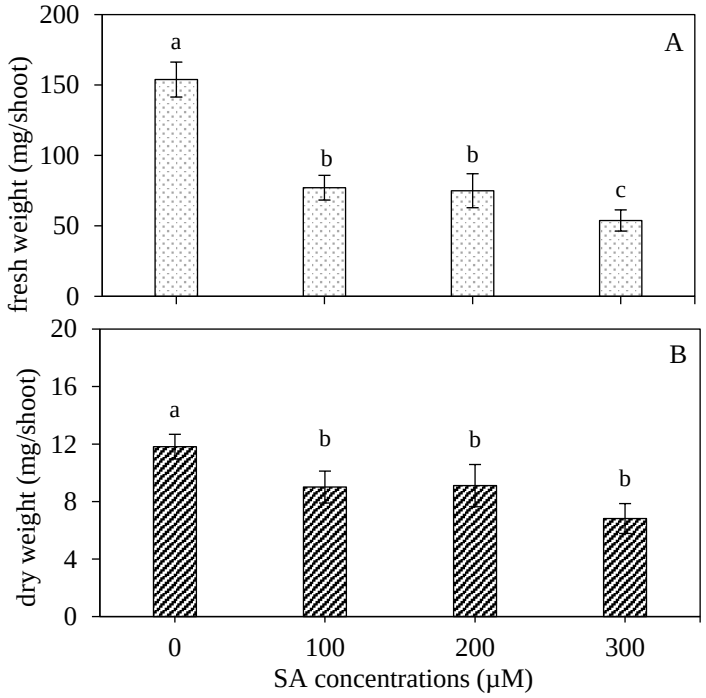

Figure 1. A) fresh weight, and B) dry weight of *Musa acuminata* cv. 'Gros Michel' shoots after treatment with salicylic acid at 0-300 µM for four weeks

3.2 Secondary metabolites and antioxidant activity

As can be seen from Figure 2 A, the total saponin contents of the 100 µM SA treated shoots (263.86±0.42 mg diosgenin/g dry extract) were not significantly different from that of the 200 µM SA treated shoots (261.81±2.61 mg diosgenin/g dry extract). These were 1.27 and 1.26 times that of control (208.31±0.42 mg diosgenin/g dry extract). The lowest total saponin content of 201.91±3.37 mg diosgenin/g dry extract was recorded in the 300 µM SA treated shoots.

A statistically significant difference in total phenolic contents was found among treatments (Figure 2 B). The highest total phenolic content (124.44±8.39 mg GAE/g dry extract) was recorded from the 100 µM SA treated shoots. This was 1.25 times that of control. The total phenolic content of the 200 µM SA treated shoots was 104.96±8.14 mg GAE/g dry extract, which was not significantly different from that of the control (99.66±7.65 mg GAE/g dry extract). The total phenolic content was lower when shoots were treated with SA at the highest concentration of 300 µM.

The effect of SA on total flavonoid content was similar to that on total phenolic content. The greatest content of 105.26±6.43 mg CE/g dry extract was observed in the 100 µM SA treated shoots. This was 1.34 times that of control (Figure 2 C). The total flavonoid content of the 200 µM SA treated shoots (81.84±3.68 mg CE/g dry extract) was not significantly different from that of the control (78.08±5.46 mg CE/g dry extract). The 300 µM SA concentration produced the lowest total flavonoid accumulation (54.49±2.68 mg CE/g dry extract).

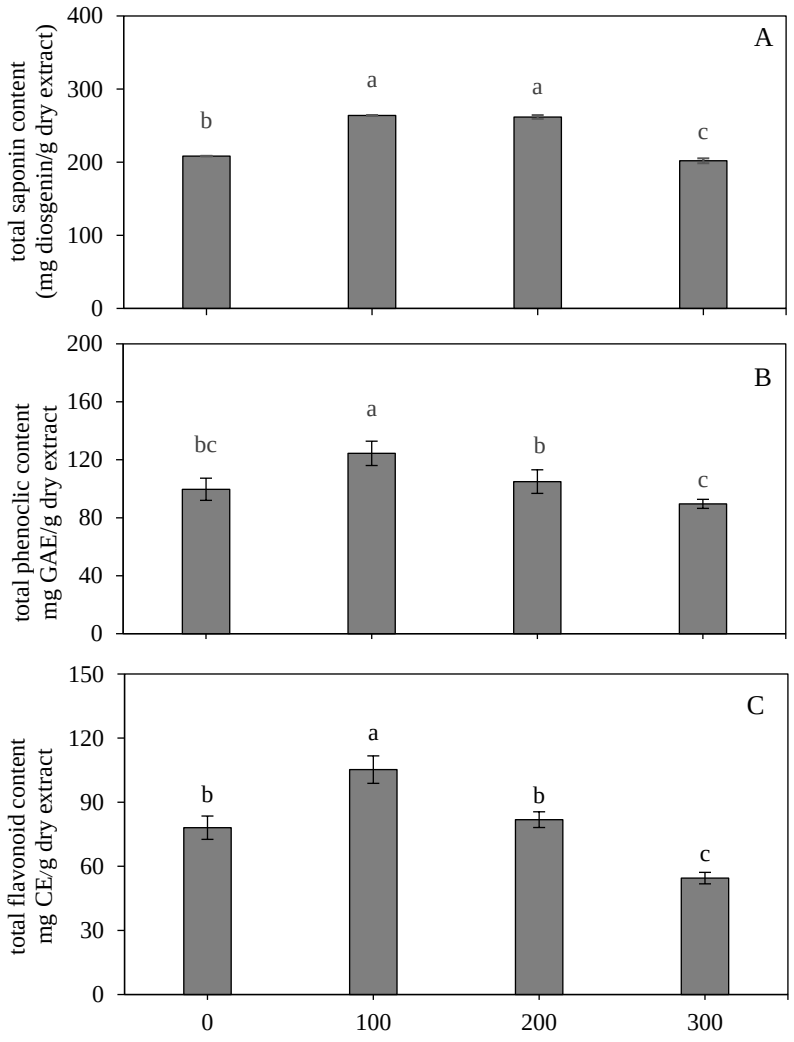

Figure 2. A) total saponin content, B) total phenolic content, and C) total flavonoid content after shoots were treated with salicylic acid at 0-300 µM for four weeks

The strongest DPPH radical scavenging was recorded for the 100 µM SA treated shoots, with an EC_{50} of 9.79±0.86 µg/ml (Figure 3 A). DPPH radical scavenging by the 200 and 300 µM SA shoots (EC_{50} values of 13.90±0.42 µg/ml and 17.44±0.96 µg/ml, respectively) was below that of control (EC_{50} of 11.99±0.73 µg/ml).

The 100 µM SA treated shoots exhibited the strongest ABTS radical scavenging, with an EC_{50} of 86.66±1.50 µg/ml (Figure 3 B). The weakest ABTS antioxidant (EC_{50} of 111.08±4.39 µg/ml) was exhibited by the 300 µM SA shoots, and was not significantly different from control (EC_{50} of 105.40±2.42 µg/ml).

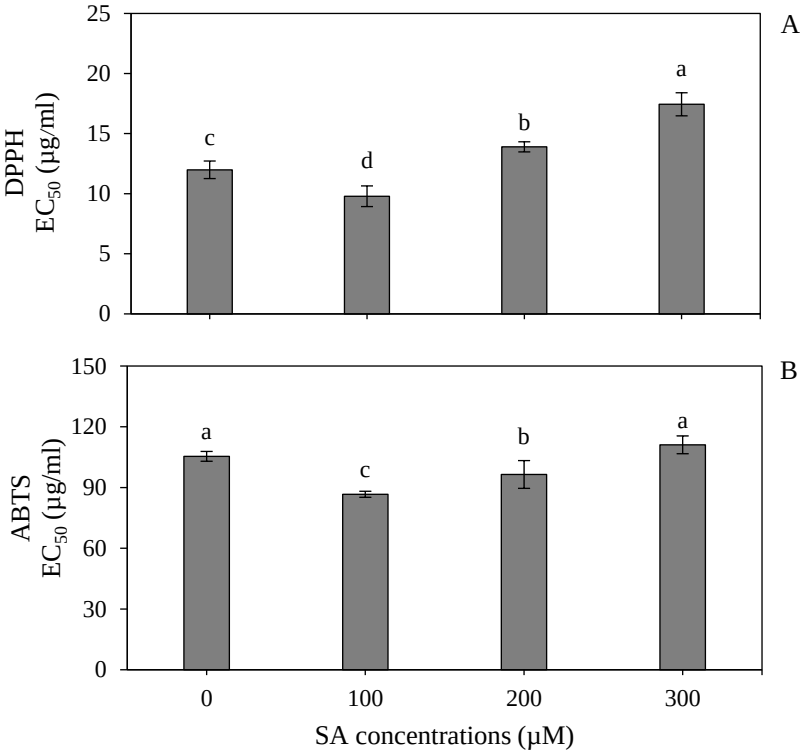

Figure 3. A) DPPH, and B) ABTS antioxidant scavenging activity after shoots were treated with salicylic acid at 0-300 µM for four weeks

From the results, SA at a concentration of 100 µM was most effective for enhancement of total saponin, total phenolics, and total flavonoids from *in vitro* shoots of *M. acuminata* cv. 'Gros Michel'. DPPH and ABTS radical scavenging assays were performed to analyze antioxidant activity. In both assays, the greatest antioxidant activity was observed from 100 µM SA treated shoots. This may reflect the greater accumulation of total phenolic compounds and flavonoids. These compounds have antioxidant properties, as their polyphenol structures scavenge free radicals [9]. SA at a concentration of 300 µM adversely affected secondary metabolite accumulation and antioxidant activity, perhaps through a toxic effect on plant cells. This result was consistent with that of Coste *et al*. [20] for *H. hirsutum*, and of Cai *et al*. [39] for *Changium smyrnioides*. In both cases, high concentrations of elicitor reduced accumulation of secondary metabolites. Therefore, elicitor concentration is a factor that must be considered for each specific case [16, 17, 40].

SA is a signaling molecule that triggers a defensive mechanism or systemic acquired resistance (SAR) when under pathogen attack [40-42]. It also induces expression of genes involved in biosynthesis of some secondary metabolites [40]. Many studies have successfully used SA to increase production of secondary metabolites including hypericin and pseudohypericin by *H. hirsutum* [20], phenolics, flavonoids, and antioxidants by *T. peruviana* [22], and polysaccharides, phenolics, and flavonoids by *Orostachys cartilaginous* [43].

4. Conclusions

Total saponin, total phenolic, total flavonoid accumulation, and antioxidant activity of *in vitro M. acuminate* cv. 'Gros Michel' shoots were enhanced by SA at a concentration of 100 μM. The results of this investigation will support further pharmaceutical study of *in vitro M. acuminate* cv.'Gros Michel' shoots.

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