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Research article

Elicitation enhancement of bioactive compound accumulation and antioxidant activity in shoot cultures of *Boesenbergia rotunda* L.

Yaowapha Jirakiattikul[a,*], Panumart Rithichai[a], Rattanaporn Prachai[a], Arunporn Itharat[b]

[a] *Department of Agricultural Technology, Faculty of Science and Technology, Thammasat University, Pathumthani 12120, Thailand*

[b] *Department of Applied Thai Traditional Medicine, Faculty of Medicine, Thammasat University, Pathumthani 12120, Thailand*

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Abstract

Boesenbergia rotunda L. is a tropical plant that yields several medicinal bioactive compounds. Using *in vitro* cultivation allows the quantity and quality of bioactive products to be controlled, but yields are typically lower than from field-grown plants. Elicitation techniques are used to increase these yields. The main goal of this study was to determine the bioactive compound yields and antioxidant activity from *in vitro B. rotunda* shoots following treatments with methyl jasmonate (MeJA) or salicylic acid (SA). The levels of pinostrobin, total phenolics and flavonoids, and the antioxidant activity of the *in vitro* shoots were determined and compared with those of the storage roots, shoot base and mature rhizome of field-grown plants. The *in vitro* shoots had lower levels of pinostrobin, total phenolics and flavonoids, and lower antioxidant activity than the field-grown extracts. To determine the optimal elicitation regime, the i*n vitro* shoots were elicited for 4 wk using MeJA at 200 µM or 400 µM or with SA at 100 µM or 200 µM. The results were compared with a non-elicited control. The shoots treated with 200 µM MeJA had the highest levels (mean ± SD) of pinostrobin (17.97±0.01 mg/g dry extract), total phenolics (86.01±1.33 mg gallic acid equivalents/g dry extract) and flavonoids (166.60±2.57 mg quercetin equivalents/g dry extract). These were respectively 2.44, 1.81 and 1.25 times greater than those of the control. In addition, the shoots treated with 200 µM MeJA had the strongest antioxidant activity. The study confirmed that application of MeJA to *Boesenbergia rotunda* shoot cultures enhanced the accumulation of pinostrobin, phenolic compounds and flavonoids, and strengthened the antioxidant activity.

Introduction

Boesenbergia rotunda L. or Fingerroot is a member of the family Zingiberaceae and is native to tropical areas, more specifically Southeast Asia and Indo-China (Ongwisespaiboon and Jiraungkoorskul, 2017). It is a perennial with a pseudostem that rises approximately 15–40 cm above the ground, while the underground parts comprise a small, globular, yellow rhizome with a diameter of 1.5–2 cm and several storage roots (Chong et al., 2012). The rhizome and storage roots are eaten in countries

* Corresponding author.
E-mail address: yjirakia@tu.ac.th (Y. Jirakiattikul)

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including Thailand, Malaysia and Indonesia, where the plant is valued for its aroma and slightly pungent taste (Jitvaropas et al., 2012; Chahyadi et al., 2014; Ongwisespaiboon and Jiraungkoorskul, 2017). In addition, the rhizome is a traditional medicine, used as an anticancer agent, for its anti-inflammatory, anti-allergic, antioxidant, antiulcer, anti-tumor and anti-HIV properties, and as an aphrodisiac (Ongwisespaiboon and Jiraungkoorskul, 2017). Kanjanasirirat et al. (2020) reported that the rhizome extract of *B. rotunda* was effective against SAR-CoV-2 (coronavirus-2) infection in Vero E6 cells, with a half maximal inhibitory concentration of 3.62 µg/mL. A series of studies have elucidated the bioactive compounds with flavonoids including pinostrobin, pinocembrin, alpinetin, boesenbergin A and cardamonin being found in the rhizome (Jaipetch et al., 1982; Tewtrakul et al., 2003; Ching et al., 2007), as well as polyphenols and essential oils (Sukari et al., 2008; Jing et al., 2010).

In Thailand, the rhizome is used as both a traditional medicine and in vegetative propagation. In field cultivation, the rhizome takes 8 mth to reach maturity, which may result in a shortage of plant material for medicinal use and as a raw material in the pharmaceutical sector. Soil-borne diseases may also degrade the quality of the plant material (Dohroo, 2007). Plant tissue culture offers rapid propagation and enhanced production of secondary metabolites. Plant tissue culturing of *B. rotunda* has been reported on somatic embryogenesis (Tan et al., 2005), plantlets (Yusuf et al., 2011) and root cultures (Yusuf et al., 2018). The accumulation of bioactive compounds in *in vitro* cell and organ culture have also been determined, with Yusuf et al. (2013) reporting the extraction of alpinetin, pinocembrin, cardamonin, panduratin A and pinostrobin from callus and cell suspension cultures, though the yields were lower than those of field-grown rhizomes. Pinostrobin, the dominant flavonoid, has been isolated from adventitious root cultures of *B. rotunda* (Yusuf et al., 2018). However, the isolation of pinostrobin and other bioactive compounds has not been reported and there has been no reported investigation of the antioxidant activity of material from *in vitro B. rotunda* shoot cultures.

Dias et al. (2016) suggested that plant material from *in vitro* cultures typically contained very low amounts of bioactive compounds; this was confirmed for *B. rotunda* (Yusuf et al., 2013). Elicitation is a method that is used to enhance the accumulation of target compounds by *in vitro* plant cultures (Vasconsuelo and Boland, 2007). Methyl jasmonate (MeJA) and salicylic acid (SA) have been applied widely as biotic elicitors in medicinal plant culturing; they have been used to enhance the accumulation of phenolic compounds, flavonoids and other bioactive compounds in *Phyllanthus pulcher* (Danaee et al., 2015), *Psoralea corylifolia* (Gajula et al., 2018), *Thevetia peruviana* (Mendoza et al., 2018) and *Talinum paniculatum* (Faizal and Sari, 2019). For the genus *Zingiber,* Ali et al. (2018) reported that the phenolic accumulation by callus of *Z. officinale* after 3 wk of elicitation using 50 mg/L (362 µM) SA was 34% higher than that of a control and 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging was 30% higher than for the control. Jalil (2018) reported that zerumbone accumulation by *Z. zerumbet* increased after 15 d of cultivation when the adventitious root cultures were treated with 800 µM MeJA. However, the use of MeJA and SA to enhance the accumulation of bioactive compounds by *B. rotunda* has not yet been investigated. Consequently, the current study compared the accumulation of bioactive compounds by *in vitro B. rotunda* shoots with accumulation by field-grown plants, determined after treatment with MeJA or SA at different concentrations. The antioxidant activity of the *in vitro B. rotunda* shoot material was also examined.

Materials and Methods

Plant material and explant preparation

Healthy *B. rotunda* rhizomes were bought from a local market in Pathum Thani province, Thailand. Sprouting shoots approximately 2.0–3.0 cm in length were excised and used as explants. The shoots were washed, then surface sterilized with sodium hypochlorite twice at concentrations of 20% and 10% (v/v) with a few drops of Tween 20 for 10 min in each cycle. The sterilized sprouting shoots were rinsed twice with sterilized distilled water before culturing on Murashige and Skoog (MS) medium supplemented with 8.87 µM 6-benzyladenine (BA) and 2.69 µM 1-naphthaleneacetic acid (NAA), 30 g/L sucrose and 8 g/L agar for shoot induction (Yusuf et al., 2011). The medium was adjusted to pH 5.6–5.8 and autoclaved at 121°C for 15 min. All cultures were kept under fluorescent lamps with a 16 hr photoperiod at 25±2°C. The regenerated shoots were subcultured for multiplication at 4 wk intervals.

Bioactive compound accumulation and antioxidant activity of in vitro shoots and field-grown plants

The *in vitro* shoots were harvested at 4 wk (Fig. 1A) and dried at 50°C for 48 hr. The powder was macerated for 3 d with 95% ethanol at a 1:3 ratio (sample powder:ethanol).

This procedure was repeated three times. The shoot extract was filtered, evaporated at 50°C and stored at -20°C until use. Accumulation of pinostrobin, total phenolics and flavonoids and DPPH radical scavenging were determined and compared with those of 6 mth shoot base, 8 mth storage roots and the rhizome of field-grown plants (Figs. 1B–D). The experiment was carried out using a completely randomized design (CRD) with four treatments and three replicates.

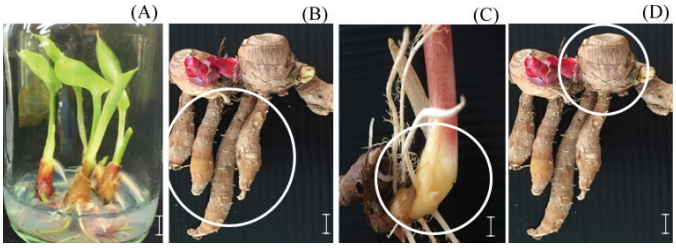

Fig. 1 Plant material: (A*) in vitro* shoots cultured on Murashige and Skoog medium supplemented with 8.87 µM and 2.69 µM; (B) field-grown storage roots; (C) field-grown shoot base; (D) field-grown rhizome of *B. rotunda* (scale bar = 0.5 cm)

Elicitation treatments

The in vitro shoot explants (1 cm long) were cultured for 4 wk on MS medium supplemented with 8.87 µM BA and 2.69 µM NAA in combination with 0, 200 µM or 400 µM MeJA or with 100 µM or 200 µM SA. The fresh weight before and after elicitation was recorded. The growth index (GI) was calculated as GI = shoot fresh weight after / before elicitation. Shoots were dried at 50°C for 48 hr then ground. The powder was macerated, filtered and evaporated as described above. The bioactive compound contents were determined and the antioxidant activity was investigated. The experiment was carried out using a CRD with five treatments and three replicates.

Analysis of pinostrobin content

The pinostrobin content was investigated using a modified method from Yusuf et al. (2013). A sample extract at a concentration of 1 mg/mL was sonicated for 10 min then passed through a 0.22 µm filter membrane. Ultra-high performance liquid chromatography (UHPLC, Nexera LC-30 A, Shimadzu) was performed using a Nova-Pak C18 column (150 mm × 3.9 mm I.D., 4 µm) with a guard column. Gradient elution mode was used and a mobile phase of phosphoric acid (A)-to-acetonitrile (B) at a volume ratio of 5 min, 80% A, 20% B; 4.5 min, 65% A, 35% B; 5.0 min, 40% A, 60% B; and 4.0 min, 100% B was delivered at a flow rate of 1.5 mL/ min, over a total run time of 14 min. A sample of 20 µL was injected into the system. Peak detection was at 285 nm (Fig. 2). The pinostrobin content was reported in milligrams per gram of dry extract.

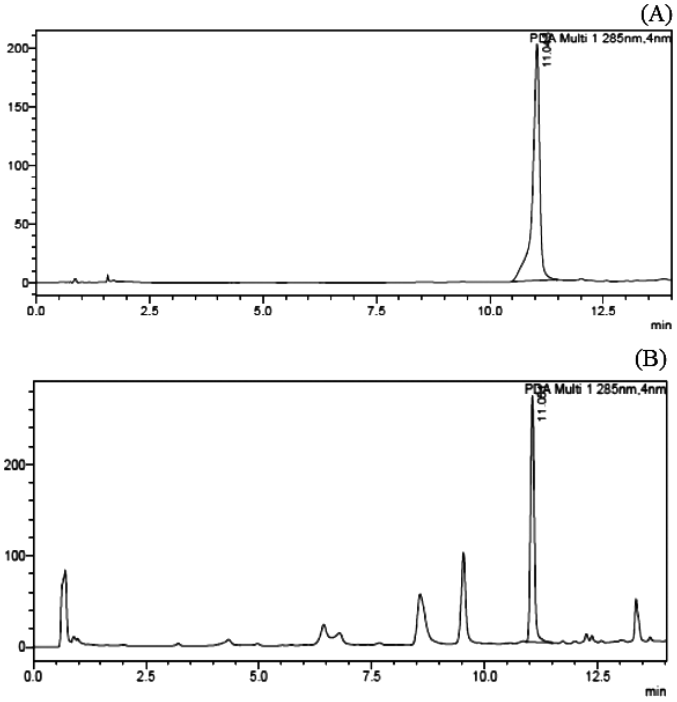

Fig. 2 HPLC chromatograms of pinostrobin (A) a standard; (B) *in vitro* shoots of *B. rotunda* treated with 200 µM methyl jasmonate

Analysis of total phenolic and flavonoid contents

The total phenolic content was determined using the Folin-Ciocalteu method modified from Jirakiattikul et al. (2016). The total flavonoid content was measured using the aluminum chloride colorimetric method (Zhu et al., 2010). The absorbance values of samples were read from a microplate reader (PowerWave XS-BT-MQX200R) at 765 nm for the total phenolic compounds and at 510 nm for the total flavonoids. The total phenolic content was reported in milligrams of gallic acid equivalents per gram of dry extract (mg GAE/g dry extract) and the total flavonoid content was reported in milligrams of quercetin equivalents per gram of dry extract (mg QE/g dry extract).

Determination of antioxidant activity

The antioxidant activity was assessed using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assays, following Jirakiattikul et al. (2016). Absorbance values of 520 nm were measured using the microplate reader. The effective concentration required to obtain a 50% antioxidant effect (EC_{50}) was then calculated using a regression equation.

Statistical analysis

Data were subjected to analysis of variance. The means were compared using Tukey's test to identify significant differences ($p < 0.05$). Results were presented as mean ± SD.

Results

Bioactive compound accumulation and antioxidant activity of in vitro shoots and field-grown plants

The extraction yield of the *in vitro B. rotunda* shoots was 19.20 ± 0.60% while yields of 11.41 ± 0.15%, 11.27 ± 0.86% and 11.43 ± 0.04% were obtained from the field-grown storage roots, shoot base and rhizome, respectively. The accumulations of pinostrobin, total phenolics and flavonoids varied significantly between trials (Fig. 3). The *in vitro B. rotunda* shoots yielded a pinostrobin content of 5.15 ± 0.04 mg/g dry extract, which was below those for the field-grown storage roots, shoot base and rhizome (Fig. 3A). The greatest pinostrobin yield, (151.65 ± 0.07 mg/g dry extract) was obtained from the rhizome. The storage roots yielded 139.59 ± 0.25 mg/g of dry extract and the shoot base 23.70 ± 0.22 mg/g dry extract.

The *in vitro* shoots yielded the lowest amount of total phenolic compounds (35.10 ± 1.35 mg GAE/g dry extract), as shown in Fig. 3B, while the shoot base and rhizome had the higher accumulations (168.98 ± 6.20 mg GAE/g dry extract and 172.21 ± 8.56 mg GAE/g dry extract).

The shoot base had the highest concentration of total flavonoids, yielding 220.03 ± 7.32 mg QE/g dry extract, compared with 174.98 ± 3.01 mg QE/g dry extract for the storage roots and 169.65 ± 2.43 mg QE/g dry extract for the rhizome. The *in vitro* shoots had the lowest concentration of total flavonoids at 121.56 ± 1.84 mg QE/g dry extract (Fig. 3C).

The antioxidant activity also varied significantly between trials. The shoot base had the strongest antioxidant activity, with an EC_{50} value of 142.57 ± 1.15 µg/mL (Fig. 3D). The storage roots had an EC_{50} of 159.71 ± 3.42 µg/mL and the rhizome 172.35 ± 4 .33 µg/mL. The *in vitro* shoot had the weakest antioxidant activity, with an EC_{50} value of 213.76 ± 8.41 µg/mL.

Effect of MeJA and SA on bioactive compound accumulation and antioxidant activity

The shoots treated with MeJA and SA had significantly lower GI values than the control (Fig. 4). The highest GI (3.24 ± 0.18) was registered for the control. The shoots treated with 200 µM MeJA had a GI value of 2.59 ± 0.05 and the shoots treated with 400 µM MeJA had a GI value of 2.33±0.27, representing a 20–28% reduction compared to the control. The shoots treated with 100 µM SA had a GI value of 2.14 ± 0.12 and the shoots treated with 200 µM SA had a GI value of 1.15 ± 0.10, representing a 33–64% reduction compared to the control.

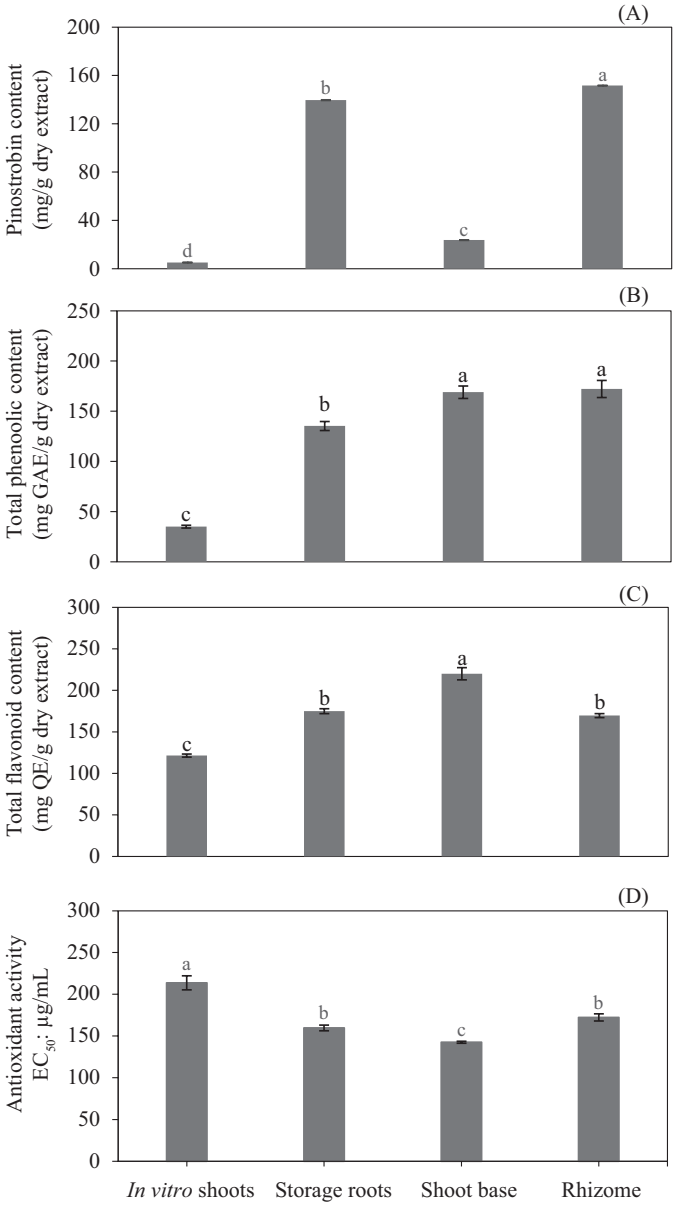

Fig. 3 Contents of (A) pinostrobin; (B) total phenolic compounds; (C) total flavonoids; and (D) 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity of *in vitro* shoots, field-grown storage roots, field-grown shoot base and field-grown rhizome of *B. rotunda*, where EC_{50} is 50% antioxidant effect, QE is quercetin equivalents, GAE is gallic acid equivalents, different lowercase letters above columns indicate a significant ($p < 0.05$) difference and error bars indicate ± SD.

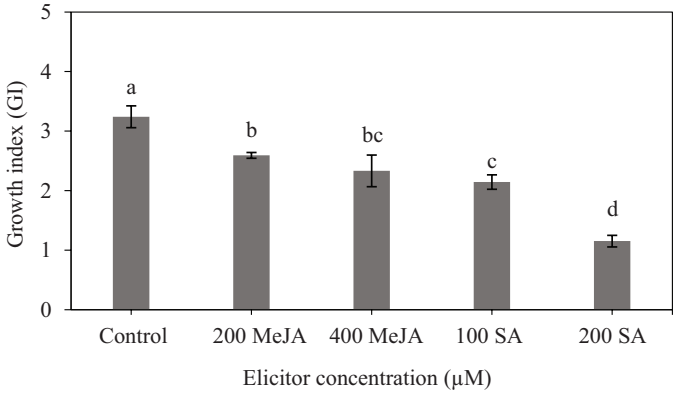

Fig. 4 Growth index of *in vitro* shoots of *B. rotunda* cultured on Murashige and Skoog medium supplemented with 8.87 µM BA and 2.69 µM NAA in combinations with different levels of methyl jasmonate (MeJA) or salicylic acid (SA) for 4 wk, where different lowercase letters above columns indicate a significant ($p < 0.05$) difference and error bars indicate ± SD.

There were significant differences in the bioactive compound yields and antioxidant potency among treatments (Fig. 5). The highest pinostrobin concentration (17.97 ± 0.01 mg/g dry extract) was in the shoots treated with 200 µM MeJA. This was 2.44 times that of the control (7.35 ± 0.04 mg/g dry extract), as shown in Fig. 5A. The shoots treated with 400 µM MeJA had a pinostrobin content of 17.01 ± 0.16 mg/g dry extract, compared with 12.11 ± 0.01 mg/g dry extract for the shoots treated with 100 µM SA. These values equated to 2.31 and 1.64 times that of the control, respectively. However, the pionostrobin content of the shoots treated with 200 µM SA was lower than that of the control.

Fig. 5B shows that the shoots treated with 200 µM MeJA had the greatest content of total phenolic compounds (86.01 ± 1.33 mg GAE/g dry extract) or 1.81 times that of the control (47.50 ± 1.32 mg/g dry extract). The total phenolic content of the shoots treated with 400 µM MeJA was 58.96 ± 0.85 mg GAE/g dry extract, compared with 57.93 ± 3.97 for the shoots treated with 100 µM SA. These values were 1.24 and 1.21 times that of the control, respectively. The shoots treated with 200 µM SA had the lowest accumulation of total phenolic compounds (43.78 ± 1.24 mg GAE/g dry extract).

The shoots treated with 200 µM MeJA had the highest total flavonoid content (166.60 ± 2.57 mg QE/g dry extract) or 1.25 times that of the control (Fig. 5C). The shoots treated with 400 µM MeJA had a flavonoid content of 133.36 ± 1.90 mg QE/g dry extract and the shoots treated with 100 µM SA had a content of 132.50 ± 4.83 mg QE/g dry extract. These were not significantly different from the control which accumulated 133.18 ± 3.05 mg QE/g dry extract. The shoots treated with 200 µM SA had the lowest total flavonoid content (57.71 ± 4.28 mg QE/g dry extract).

Finally, the shoots treated with 200 µM MeJA had the greatest antioxidant activity, with an EC_{50} value of 138.27 ± 3.37 µg/mL, as shown in Fig. 5D. The shoots treated with 200 µM SA had an EC_{50} value of 312.89 ± 11.92 µg/mL, indicating that their antioxidant activity was below that of the control (194.97 ± 2.27 µg/mL).

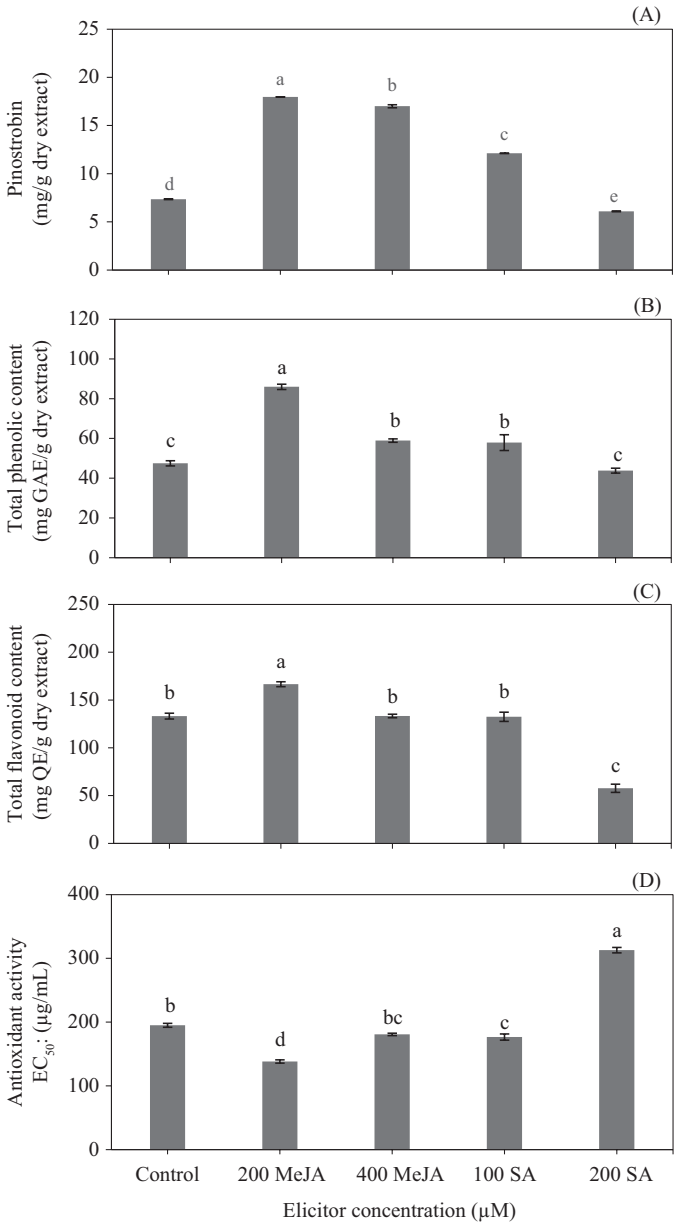

Fig. 5 Contents of (A) pinostrobin; (B) total phenolic compounds; (C) total flavonoids; (D) 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity of *in vitro* shoots treated in combinations with different levels of methyl jasmonate (MeJA) or salicylic acid (SA), where different lowercase letters above columns indicate a significant ($p < 0.05$) difference and error bars indicate ± SD.

Discussion

When bioactive compounds are produced under aseptic conditions, consistent quality and quantity can be achieved. Unfortunately, the micro-rhizome of *B. rotunda* could not be induced using the plant tissue culture technique. Bioactive compounds from the callus, cell suspension and adventitious root of *B. rotunda* have been reported by Yusuf et al. (2013, 2018), but the same investigation has not been made of *in vitro* shoots. The current study was the first to identify i*n vitro* shoot cultures of *B. rotunda* as an alternative source of high-value bioactive plant compounds.

In vitro shoots of *B. rotunda* can synthesize and accumulate pinostrobin, phenolics and flavonoids in the same way as field-grown plants. Rao and Ravishankar (2002) reported that the plant cells preserve their genetic information and can therefore express the bioactive compounds of the parent plants; however, yields have been low. Muhitch and Fletcher (1985) found that mature plants continued to synthesize complex bioactive compounds during a stationary growth phase. As that phase was shorter for *in vitro* plants, the accumulation of enzymes and bioactive compounds was reduced. Similar results have been found across a range of plants, with low bioactive compound yields having been observed from *in vitro* cells and organs of *B. rotunda* (Yusuf et al., 2013), *Asparagus officinalis* (Esmaeili et al., 2016), *Dioscorea birmanica* (Jirakiattikul et al., 2016) and *Z. officinale* (Ali et al., 2018). The rhizome and storage root material used in the current study yielded higher levels of pinostrobin than the shoot base, though the highest total phenolic and flavonoid contents and also the strongest antioxidant activity were observed in material from the shoot base. This contradicted the results of Tan et al. (2015), who reported the highest pinostrobin yield from the shoot base of field-grown plants. Verma and Shukla (2015) reported that secondary metabolite accumulation by plants was influenced by genetic, ontogenetic, morphogenetic and environmental factors. This was confirmed for *Hypericum*, whose secondary metabolite content reflected differences among species, organs, developmental stages and climatic conditions (Cirak et al., 2013).

Elicitation is a technique for enhancing the accumulation of bioactive compounds by medicinal plants. In a preliminary study, treatments with MeJA and SA at concentrations of 400 µM and 800 µM were tested. However, the 800 µM MeJA and 400 µM and 800 µM SA treatments induced shoot necrosis and death (data not shown), Consequently, MeJA treatments at 200 µM or 400 µM and the SA treatments at 100 µM or 200 µM, were used in the study. At all concentrations, these elicitors had a negative effect on the GI of the *in vitro* shoots (Fig. 4). Under elicitation, the primary metabolism of the plant cell decreases, while secondary metabolite synthesis increases (Chen and Chen, 2000). This has been attributed to the delivery of substrates (Norman et al., 1994) or energy (Broeckling et al., 2005) to the secondary metabolite process. MeJA or SA or both have been reported to influence the growth and biomass accumulation of *in vitro* shoot cultures of plant species including *H. maculatum* (Coste et al., 2011), *Musa acuminata* (Mahmood et al., 2012) and *D. membranacea* (Jirakiattikul et al., 2020).

The choices of elicitor type and the concentration are key decisions in elicitation (Vasconsuelo and Boland, 2007). The current results suggested that the optimum elicitor type and concentration for *B. rotunda* shoot culture was MeJA at 200 µM. SA had a deleterious effect on the GI and on the bioactive yield, particularly at a concentration of 200 µM. This may have been due to toxicity to plant cells (Wang et al., 2001). Treatment with 100 µM SA enhanced the accumulation of pinostrobin and total phenolics, but the shoot GI was lower than under the MeJA treatment. The lowest MeJA concentration used in this experiment was 200 µM. However, the results suggested that even lower concentrations, used over shorter or longer elicitation periods, might be optimal for the accumulation of bioactive compounds. Therefore, MeJA was applied at concentrations of 0 µM, 50 µM, 100 µM, 150 µM or 200 µM, with elicitation periods of 3, 4 and 5 wk. The optimum conditions were a MeJA loading of 200 µM with culture for 4 wk (data not shown). In bioactive compound synthesis, MeJA plays a role in signal transduction and enzyme induction (Mizukami et al., 1993). Wang et al. (2015) reported the activity of phenylalanine ammonia lyase (the first enzyme in the phenylpropanoid pathway) increased under MeJA elicitation and this enhanced flavonoid production. In addition, Zhao et al. (2005) reported that the JA signaling pathway stimulates the accumulation of bioactive compounds including terpenoids, alkaloids and phenylpropanoids in a range of plants. MeJA is widely used and has been found to outperform SA in enhancement of bioactive compounds in species including *Scrophularia kakudensis* (Manivannan et al., 2016), *Eleutherococcus koreanum* (Lee et al., 2015) and *Thevetia peruviana* (Mendoza et al., 2018).

The current study produced lower yields of bioactive compounds elicited from the *in vitro* shoots of *B. rotunda* than those from the storage roots, shoot base or rhizome of field-grown plants. However, the *in vitro* growth cycle was much shorter. Elicitation with MeJA at a concentration of 200 µM

was optimal for the accumulation of pinostrobin, total phenolic compounds and total flavonoids by *in vitro B. rotunda* shoots. This regime also maximized the antioxidant activity. The protocol developed in the current study may support increased extraction of high-value bioactive compounds from *B. rotunda*, for use in the pharmaceutical sector.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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