In Vitro Cellular & Developmental Biology - Plant (2022) 58:181–187
https://doi.org/10.1007/s11627-021-10195-z

RESEARCH REPORT

Methyl jasmonate elicits enhancement of bioactive compound synthesis in adventitious root co-culture of *Echinacea purpurea* and *Echinacea pallida*

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Received: 17 April 2021 / Accepted: 2 May 2021 / Published online: 11August2021 / Editor: Jayasankar Subramanian

Abstract

Co-culture of adventitious roots (ARs) of *Echinacea purpurea* (L.) Moench and *E. pallida* is a novel method for the production of *Echinacea* bioactive compounds. In the co-culture system, implementation of an elicitation strategy can likely promote bioactive compound accumulation in ARs. Therefore, in this work, methyl jasmonate (MeJA) was tested as an elicitor to treat 30-d-old bioreactor cultured ARs and the effect of MeJA concentrations on metabolite accumulation to select an optimal concentration of this elicitor. Furthermore, the antioxidant enzyme activities of ARs were also determined for understanding the mechanism of MeJA elicitation. Results showed that the 25 μM MeJA treatment increased metabolite accumulation in ARs with maximum production of phenolics (728.2 mg L^{-1}), flavonoids (622.2 mg L^{-1}), and caffeic acid derivatives (255.3 mg L^{-1} cichoric acid and 143.9 mg L^{-1} echinacoside); however, the highest polysaccharide production (approximately 440 mg L^{-1}) was determined at 50 to 200 μM MeJA. The activities of antioxidant enzymes (superoxide dismutase, peroxidase, ascorbate peroxidase, and catalase) reached maximum levels with 25 μM MeJA, demonstrating a close relationship between antioxidant enzymes and secondary metabolite synthesis of co-cultured *Echinacea* ARs. In addition, the bioactive compound content in MeJA-treated ARs was compared with that in natural plant roots. The result indicated that the contents of phenolics, flavonoids, and caffeic acid derivatives (cichoric acid and echinocoside) were higher in the co-cultured ARs than in plant roots of *E. purpurea* and *E. palida*.

Introduction

Adventitious root (AR) cultures of plants are good biological materials for the large-scale production of useful metabolites Cui *et al.* 2010; Jiang *et al.* 2015; Lee *et al.* 2015; (Wu *et al.* 2017). At present, *Echinacea* ARs of several species, including *E. pallida*, *E. purpurea*, and *E. angustifolia*, have been successfully cultured in bioreactors for the mass production of caffeic acid derivatives (CADs), phenolics, and flavonoids (Jeong *et al.* 2009; Cui *et al.* 2013; Wu *et al.* 2013). In particular, studies have indicated that co-culture of *E. pallida* and *E. purpurea* ARs can improve biomass and bioactive compound yield, such as caffeic acid (Wu *et al.* 2017; Wu *et al.* 2018).

During plant cell or organ culture, the bioactive compound synthesis is controlled by several parameters, such as medium components, culture environment, and elicitation, among which elicitation is the most effective strategy. Several mechanisms of elicitation action have been identified, one of which is the effect on the antioxidant system. Specifically, elicitation enhances metabolite synthesis through triggering antioxidant enzyme activities (Murthy *et al.* 2014). As a volatile methyl ester of the plant hormone jasmonic acid, methyl jasmonate (MeJA) has been widely used as an effective elicitor in the culture of several plant organs, such as the ARs of *Panax ginseng* (Kim *et al.* 2004) and *Eleuthrococcus kpreanum* (Lee *et al.* 2015), and protocorm-like bodies of *Dendrobium candidum* (Wang *et al.* 2016). MeJA has also been used in *Echinacea* AR culture of a single species, in which 12.3 mg g^{-1} DW echinacoside of *E. angustifolia* was produced after MeJA treatment (100 μM) in a 500-L pilot-scale bioreactor culture system (Cui *et al.* 2013). This content of echinacoside is more than twofold that of MeJA-untreated control group. However, an elicitation strategy has not been applied to the

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AR culture system of other *Echinacea* species including AR co-culture system of *Echinacea purpurea* and *E. pallida* to date.

Therefore, this study used different concentrations of MeJA to treat 30-d-old co-cultured ARs of *E. purpurea* and *E. pallida* and investigated the accumulation of bioactive compounds (i.e., phenolics, flavonoids, polysaccharides, and CADs) for selecting a suitable MeJA concentration. Furthermore, antioxidant enzyme activities at different MeJA concentrations were evaluated to understand the relationship between antioxidant enzymes and metabolite synthesis of co-cultured ARs after MeJA treatment. Finally, bioactive compound contents between MeJA-elicited ARs and natural plant roots were compared for the production of *Echinacea*-related products.

Materials and Methods

Plant materials ARs of *Echinacea purpurea* (L.) Moench or *Echinacea pallida* (Nutt.) Nutt. were cultured in an airlift balloon-type bioreactor according to the method of Wu *et al.* (2007a). In brief, approximately 1-cm-long ARs were inoculated in a three-quarter strength Murashige and Skoog (MS; Murashige and Skoog 1962) medium supplemented with 1 mg L^{-1} indolebutyric acid (IBA; Beijing Solarbio Science and Technology Co., Ltd., Beijing, China), and 50 g L^{-1} sucrose (Tianjin Kemiou Chemical Reagent Co., Ltd., Tianjin, China). The bioreactor was aerated at 400 mL min^{-1} and maintained in the dark at 25 ± 2°C. After 30 d of culture, the ARs were harvested and used in the experiment. Dry natural plant roots were obtained from the 3-y-old plants of *E. purpurea* and *E. pallida*, which were kindly provided by Professor Kee-Youp Paek from Chungbuk National University, Korea.

MeJA treatment Four liters of three-quarter strength MS medium supplemented with 1 mg L^{-1} IBA and 50 g L^{-1} sucrose was added to the 5-L bioreactor, and 12 g (fresh weight) *E. purpurea* ARs and 16 g *E. pallida* ARs were simultaneously inoculated according to Wu *et al.* (2018). After 30 d of co-culture, 0, 10, 25, 50, 100, or 200 μM of filter-sterilized MeJA (Beijing Solarbio Science and Technology Co., Ltd.,) was added to the bioreactors. The bioreactors were aerated at 400 mL min^{-1} and maintained at 25 ± 1°C in the dark. After 5 d of MeJA treatment, AR biomass (dry weight), contents of bioactive compounds (i.e., total phenolics, flavonoids, polysaccharides, and CADs), and activities of antioxidant enzymes (i.e., superoxide dismutase [SOD, EC1.15.1.1], peroxidase [POD, EC1.11.1.7], ascorbate peroxidase [APX, E.C. 1.11.1.11], and catalase [CAT, EC 1.11.1.6]) were determined. Three independent experiments were repeated in the same conditions.

Determination of AR dry weight The harvested ARs were rinsed with tap water to remove the medium. After the surface water was blotted, the ARs were dried at 60°C for 1 d until a consistent weight was achieved, and their dry weight (DW) was recorded.

Determination of total phenolic, flavonoid, and polysaccharide contents The method of Wu *et al.* (2007a) was used to extract for the determination of phenolic and flavonoid contents. In brief, the powdered root sample (0.1 g) was soaked in 10 mL 70% (v/v) ethanol at 60°C for 3 h and centrifuged, and the supernatant was used for the spectrophotometric determination of total phenolic content, using the Folin-Ciocalteu colorimetric method (Folin and Ciocalteau 1927). Absorbance at 760 nm (UV- T6, Beijing Purkinje General Instrument Co., Ltd., Beijing, China) was recorded, and the results were expressed as milligram gallic acid (Sigma-Aldrich, St. Louis, MO) equivalent g^{-1} DW sample. Total flavonoid content was determined using the aluminum nitrate colorimetric method (Cui *et al.* 2010). Absorbance at 510 nm (UV- T6, Beijing Purkinje General Instrument Co., Ltd.) was recorded, and the result was expressed as rutin (Shanghai Yuanye Bio-Technology Co., Ltd., Shanghai, China) equivalents per g^{-1} DW sample. Each sample was tested three times and the mean value was determined.

For the determination of total polysaccharide content, the dry root sample was extracted according to the method described by Wu *et al.* (2018). In brief, the powdered root sample (0.1 g) was soaked in 90% ethanol for 6 h to adequately remove the interfering compounds. After centrifugation, the precipitate was added to 20 mL distilled water for 30 min of ultrasonic treatment, and centrifuged again. The polysaccharide content in the supernatant was quantitatively determined at 490 nm (UV- T6, Beijing Purkinje General Instrument Co., Ltd.) with a phenol-sulfuric acid assay (Dubois *et al.* 1955), and glucose (Tianjin Kemiou Chemical Reagent Co., Ltd., Tianjin, China) was used as the reference standard. Each sample was tested three times and the mean value was determined.

Determination of CAD contents The extract for the determination of phenolic and flavonoid contents was used to determine the contents of CADs (cichoric acid and echinacoside) according to the method of Wu *et al.* (2007a) through high-performance liquid chromatography (HPLC; Waters Corporation, Milford, MA) equipped with a C_{18} column (250 × 4.6 mm, 5.0 μm). The mobile phases were water (A) and 100% acetonitrile (B). Gradient elution was modified as follows: initial 10% B for 40 min, 25% B for 11 min, 50% B for 1 min, and returning to the initial condition for 8 min at a

flow rate of 1 mL min^{-1} CADs were detected at 330 nm. Standard cichoric acid and echinacoside were purchased from Sigma-Aldrich. Each sample was tested three times and the mean value was determined.

Determination of SOD, POD, APX, and CAT activities The activities of four enzymes were determined by using the method of Ali *et al.* (2005a) with some modifications. In brief, the fresh ARs (1 g) were ground under liquid nitrogen and extracted in 5 mL 50 mM phosphate buffer saline (PBS; pH 7.8) for determination of SOD, 2 mL PBS (pH 6.0) for POD, 3.0 mL PBS (pH 7.0) for APX, and 2.5 mL PBS (pH 7.8) for CAT. The homogenate was centrifuged at 12,000 rpm for 15 min at 4°C, and the supernatant was used to determine the antioxidant enzyme activities.

For SOD activity, 0.1 mL of the supernatant or buffer (as the control) was mixed with 1.7 mL PBS, 0.3 mL 0.75 mM nitro blue tetrazolium (NBT), 0.3 mL 0.02 mM riboflavin, and 0.3 mL 130 mM methionine. After 20-min incubation under 50 μmol m^{-2} s^{-1} light intensity, the absorbance of the reaction solution was determined at 530 nm (UV- T6, Beijing Purkinje General Instrument Co., Ltd.). One unit of SOD activity was defined as the amount of enzyme required to cause the 50% inhibition of NBT reduction. For the measurement of POD activity, 1 mL supernatant was mixed with 2 mL cold extract buffer, 0.1 mL 0.3% H_2O_2, and 0.1 mL 1% guaiacol. POD activity was determined following the rate of formation of tetraguaiacol at 470 nm (UV- T6, Beijing Purkinje General Instrument Co., Ltd.). For the determination of CAT activity, 0.2 mL supernatant was mixed with 2.5 mL cold extract buffer and 0.1 mL 100 mM H_2O_2, and decomposition in H_2O_2 was followed at 240 nm (UV- T6, Beijing Purkinje General Instrument Co., Ltd.). For the determination of APX activity, the reaction mixture contained 1 mL supernatant, 3.0 mL cold extract buffer, 50 μL 0.1 mM ascorbic acid, and 50 μL 300 mM H_2O_2. The reaction was started with the addition of H_2O_2 and the rate of oxidation of ascorbic acid was followed at 290 nm (UV- T6, Beijing Purkinje General Instrument Co., Ltd.). One unit of POD (or APX or CAT) was defined as the 0.01 decrease in absorbance within 1 min. Antioxidant activity was expressed as U g^{-1} FW (g fresh weight). Each sample was tested three times and the mean value was determined. The reagents used in above analyses were purchased from Beijing Solarbio Science and Technology Co., Ltd. for NBT, riboflavin, methionine, guaiacol, and ascorbic acid and that from Sinopharm Chemical Reagent CO., Ltd. (Shanghai, China) for H_2O_2. Each sample was tested three times and the mean value was determined.

Statistical analysis The results are expressed as the mean ± standard deviation of three replicates. The mean values were subjected to Duncan's multiple range test. A probability of $p < 0.05$ was considered significant.

Results and Discussion

Effect of MeJA concentrations on ARs biomass and bioactive compound accumulation After 5 d of MeJA treatment, AR biomass did not significantly change with increase in MeJA concentration up to 25 μM, but decreased when MeJA concentrations were higher than 25 μM (Fig. 1). MeJA promoted phenolic, flavonoid, and polysaccharide synthesis. Total phenolic content increased with MeJA concentrations and reached its maximum at 25 μM MeJA, at which, 728.15 mg L^{-1} phenolics were produced and the value was approximately 144 mg L^{-1} higher than that in the 0 μM MeJA control. Then, phenolic synthesis decreased at MeJA concentrations higher than 25 μM, but phenolic content was still higher than that of the control (Fig. 2*A*).

Total flavonoid content was enhanced at MeJA concentrations higher than 25 μM but did not show significant differences at MeJA concentrations from 25 to 200 μM. Furthermore, high flavonoid production was found at 25

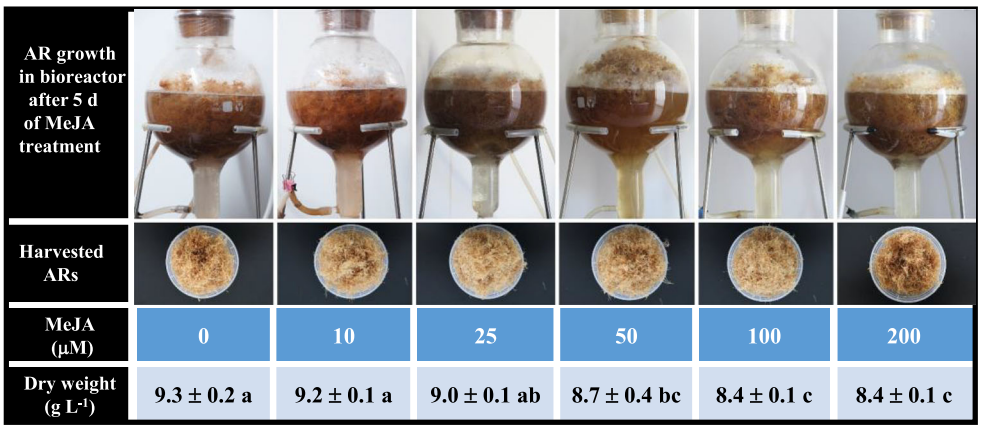

Figure 1. Effect of methyl jasmonate (MeJA) concentrations on biomass of co-cultured adventitious roots (AR) of *Echinacea purpurea* (L.) Moench and *Echinacea pallida* (Nutt.) Nutt. after 5 d of elicitation. Data represents the means of three replicates ± standard deviation. Different *letters* within the same *column* indicate significant difference by Duncan's multiple range test at $p<0.05$.

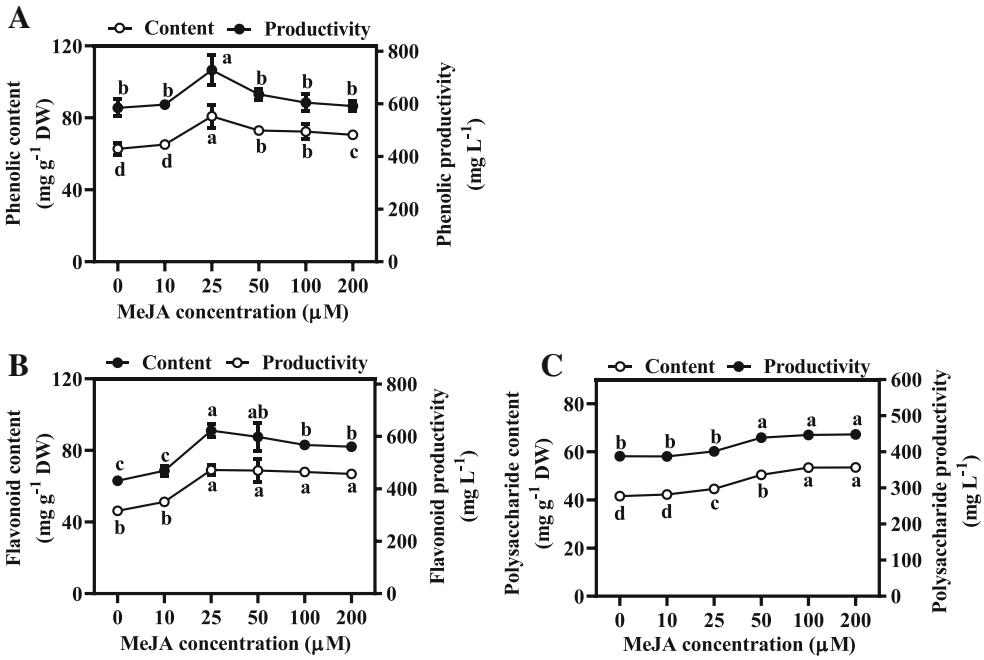

Figure 2. Effect of methyl jasmonate (MeJA) on phenolic (*A*), flavonoid (*B*), and polysaccharide (*C*) accumulation of co-cultured adventitious roots of *Echinacea purpurea* (L.) Moench and *Echinacea pallida* (Nutt.) Nutt. after 5 d of elicitation. Productivity = dry weight × content. Data represents the means of three replicates ± standard deviation. Different *letters* within the same *line* indicate significant difference by Duncan's multiple range test at $p<0.05$.

and 50 μM MeJA, which were approximately 1.4-fold the flavonoid productivity of the control. However, no statistical difference was observed between both concentrations (Fig. 2*B*).

MeJA promoted polysaccharide synthesis after treatment with MeJA concentrations higher than 25 μM in a concentration-dependent manner and highest polysaccharide contents equally obtained with 100 and 200 μM MeJA (Fig. 2*C*). Polysaccharide production reached maximum values at 50, 100, and 200 μM MeJA and did not result in significant difference among these MeJA concentrations.

The effect of MeJA concentrations on the contents of the two main CADs (cichoric acid and echinacoside) had similar patterns, that is, both increased after MeJA treatment, peaked at 25 μM MeJA, and remained stable when MeJA concentrations were higher than 25 μM (Fig. 3*A* and *B*). The highest production of cichoric acid and echinacoside was obtained at 25 μM MeJA, and the values were 2.2- and 1.4-fold the levels of the control, respectively. At this MeJA concentration, the total CAD content reached the maximum value (44.3 mg g^{-1} DW) and 399.5 mg L^{-1} of CADs was produced (Fig. 3*C*).

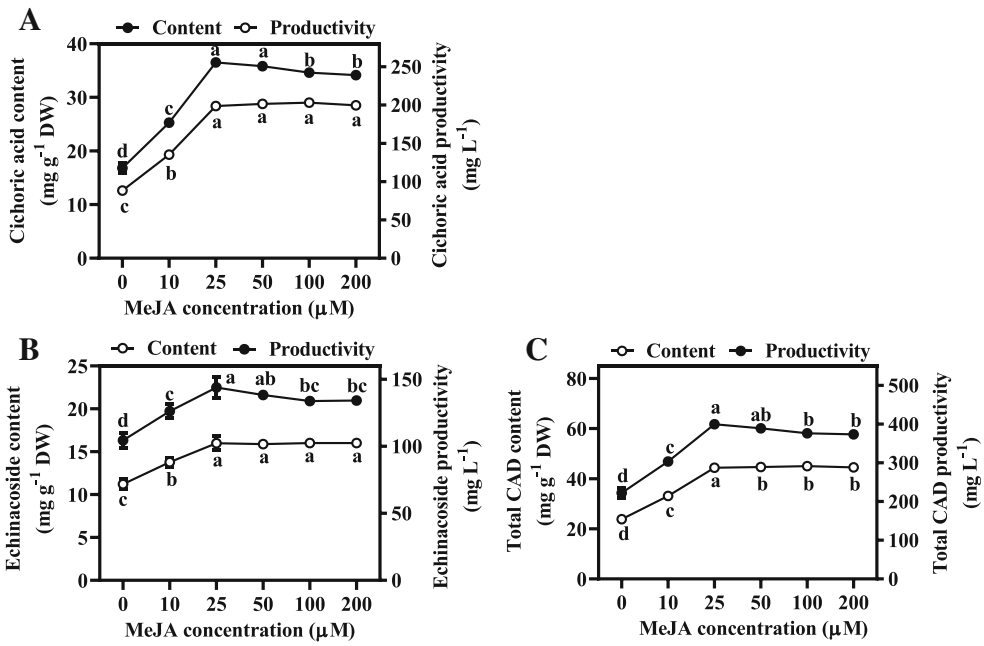

Figure 3. Effect of MeJA on contents and productivities of cichoric acid (*A*), echinacoside (*B*), and total caffeic acid derivatives (CADs) (*C*) of co-cultured adventitious roots of *Echinacea purpurea* (L.) Moench and *Echinacea pallida* (Nutt.) Nutt. after 5 d of elicitation. Total CAD content = cichoric acid + echinacoside. Productivity = dry weight × content. Data represents the means of three replicates ± standard deviation. Different *letters* within the same *line* indicate significant difference by Duncan's multiple range test at $p<0.05$.

During elicitation in plant cell or organ culture, various defense mechanisms are induced when the concentration of an elicitor reaches a level where metabolite synthesis is promoted. However, elicitors at high concentrations inhibit AR biomass and thereby affect final metabolite production yields. In accordance with the present study, biomass decreases after MeJA treatment was performed in various plant organ culture systems, such as *Eleutherosides koreanum* ARs (Lee *et al.* 2015), *Panax ginseng* ARs (Wang *et al.* 2013), and *Eleutherococcus sessiliflorus* embryos (Shohael *et al.* 2008). This phenomenon was probably due to the fact that MeJA activates proteinase inhibitor II promoter genes (Kim *et al.* 1992) or to its direct toxic effect. Thus, MeJA concentration not inhibiting culture's biomass is prerequisite of an elicitation strategy.

As an elicitor molecule, MeJA is a signal transducer in intracellular signal cascades, initially interacts on plant cell surfaces, and ultimately results in the accumulation of secondary compounds (Gundlach *et al.* 1992). The role of MeJA in the enhancement of metabolite accumulation in plant organ cultures has been investigated (Lee *et al.* 2015; Andi *et al.* 2019; Gai *et al.* 2019). MeJA elicitation critically affects metabolite accumulation and the concentrations of MeJA treatment for obtaining the maximum yield of metabolites are various according to plant species, culture types, and target metabolites. For example, Wang *et al*. (2016) demonstrated that the suitable MeJA concentrations are 75 μM for the production of flavonoids or polysaccharides and 100 μM for the phenolic production in the protocorm-like body culture of *Dendrobium candidum*. Shohael *et al.* (2008) indicated that the optimal eleutherosides and cholrogenic acid contents are obtained with 200 μM MeJA during the somatic embryo culture of *E. senticosus*. Lee *et al.* (2015) found that 100 μM MeJA is beneficial for increasing phenolic, flavonoid, eleutheroside E, and chlorogenic contents in *E. koreanum* AR cultures. By contrast, a relatively low MeJA concentration (25 μM) is optimal for phenolic, flavonoid, and CAD accumulation in the present study. This result showed that co-cultured *Echinacea* ARs are sensitive to the response of MeJA elicitation. In addition, the optimal MeJA concentration (50 μM) for polysaccharide content was higher than that of other assayed compounds. Therefore, screening a suitable concentration of an elicitor is necessary for the maximal production of useful metabolites.

Effect of MeJA concentrations on antioxidant enzyme activity

Antioxidant enzymes play a crucial role in reactive oxygen species (ROS) scavenging during plant metabolism. Thus, the activities of major ROS-scavenging enzymes (SOD, CAT, APX, and POD) of co-cultured ARs by the treatment of MeJA with different concentrations were measured. The activities of the four antioxidant enzymes increased after the MeJA treatments at all concentrations (Fig. 4). However, the effect of MeJA concentrations varied with enzymes. Specifically, the enzyme activities reached the highest values at 25 and 50 μM MeJA for SOD (Fig. 4*A*), 25 μM MeJA for POD and APX (Fig. 4*B* and C), and 25 to 200 μM MeJA for CAT (Fig. 4*D*).

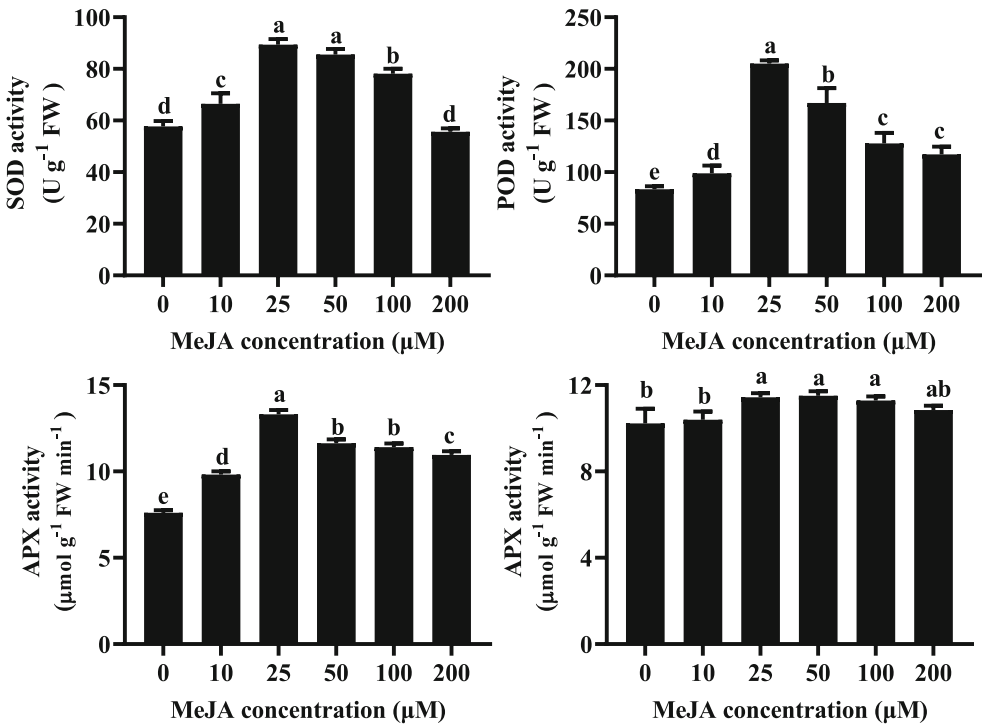

Figure 4. Effect of MeJA concentrations on superoxide dismutase (SOD) (*A*), peroxidase (POD) (*B*), ascorbate peroxidase (APX) (*C*), and catalase (CAT) (*D*) activity of co-cultured adventitious roots of *Echinacea purpurea* (L.) Moench and *Echinacea pallida* (Nutt.) Nutt. after 5 d of MeJA elicitation. Data represents the means of three replicates ± standard deviation. Different *letters* within the same *column* indicate significant difference by Duncan's multiple range test at $p<0.05$.

Table 1 Comparison of bioactive compound contents among co-cultured adventitious roots and natural plant roots of *Echinacea purpurea* (L.) Moench and *Echinacea pallida* (Nutt.) Nutt.

Species	Root type	TPN content (mg g^{-1} DW)	TFL content (mg/g DW)	CAD content (mg g^{-1} DW)		TPL content (mg g^{-1} DW)
				CiA	EcS	
Epu + Epa	ARs	80.9 ± 6.3	69.1 ± 2.8	28.4 ± 0.3	15.9 ± 0.9	44.6 ± 0.9
Epu	NRs	23.9 ± 1.1	21.1 ± 0.4	6.2 ± 0.5	ND	61.2 ± 3.2
Epa	NRs	21.2 ± 0.5	14.2 ± 0.2	0.7 ± 0.1	5.9 ± 0.4	34.1 ± 4.3

TPN, total phenolics; *TFL*, total flavonoids; *CADs*, caffeic acid derivatives; *CiA*, cichoric acid; *EcS*, echinacoside; *TPL*, total polysaccharides; *Epu + Epa*, *Echinacea purpurea* (L.) Moench + *Echinacea pallida* (Nutt.) Nutt.; *Epu*, *Echinacea purpurea* (L.) Moench; *Epa*, *Echinacea pallida* (Nutt.) Nutt.; *ARs,* co-cultured adventitious roots; *NRs*, naturally produced roots. Data represents the means of three replicates ± standard deviation. *ND*, not determined

In an antioxidant system, metalloenzyme SOD converts O_2^- to H_2O_2 and subsequently triggers CAT, POD, and APX activities, which in turn convert H_2O_2 to non-toxic H_2O, where the toxic effects of H_2O_2 can be mitigated. In the present study, the activities of the four enzymes increased in response to MeJA treatment, and this result was probably attributed to ROS generated by the MeJA elicitation. ROS generation is one of the earliest responses of plant cells or organs during elicitation (Pietrowska *et al.* 2014; Lu *et al.* 2019). ROS induce the expression of various defense genes responsible for secondary metabolism; additionally, the synthesis of signaling molecules is triggered, and secondary metabolite accumulation is enhanced (Hu *et al.* 2003; Zhao *et al.* 2005). Therefore, antioxidant enzymes are closely related to secondary metabolite synthesis. Wu *et al.* (2007b) reported that SOD and APX in *Echinacea purpurea* AR cultures are upregulated by 100 μM sodium nitroprusside, and phenolics, flavonoids, and caffeic acid derivative accumulation is promoted. Ali *et al.* (2006) indicated that MeJA treatment increases the SOD, POD, and APX activities of *Panax ginseng* ARs and ginsenoside contents are enhanced. In the present study, similar to the secondary metabolite contents, antioxidant enzyme activities obviously increased after MeJA treatment in a concentration-dependent manner, indicating a close relationship between antioxidant enzymes and secondary metabolite synthesis of co-cultured *Echinacea* ARs.

Comparison of bioactive compound contents The contents of phenolics, flavonoids, CADs (cichoric acid and echinacoside), and polysaccharides in the MeJA-treated (25 μM) co-cultured ARs of *E. purpurea* and *E. pallida* were compared with those in the 3-y-old field-grown natural plant roots of *E. purpurea* and *E. pallida*. Table 1 shows that phenolic, flavonoid, cichoric acid, and echinacoside contents in the ARs were considerably higher than those in the natural plant roots. In comparison with natural roots of *E. purpurea*, the contents of total phenolic, flavonoid, and cichoric acid in ARs were 3.4-, 3.3-, and 4.6-fold higher, respectively, while echinacoside was not tested in natural roots. Furthermore, when comparing with natural roots of *E. pallida*, contents of total phenolic, flavonoid, cichoric acid, and echinacoside in ARs were 3.6-, 4.9-, 40.6-, and 2.7-fold higher, respectively. By contrast, the total polysaccharide content of ARs was lower than that of the *E. purpurea* roots, and was slightly higher than that of the *E. pallida* roots.

During plant cell or organ culture, the goal is to achieve bioactive compound levels higher than or at least equal to those of the mother plants (Ali *et al*. 2005b; Wang *et al.* 2013; Lee *et al.* 2015;). The present study implemented the elicitation strategy with MeJA during AR co-culture of *E. purpurea* and *E. pallida* and obtained high amounts of phenolics, flavonoids, and CADs, which were significantly higher ($p<0.05$) than those in the natural roots of *E. purpurea* and *E. pallida*, indicating that a successful culture system was established.

Conclusions

MeJA concentration positively affected bioactive compound accumulation and antioxidant enzyme activities in the AR co-cultures of *E. purpurea* and *E. pallida*. The maximum contents of phenolics, flavonoids, CADs, and polysaccharides were obtained by using 25 μM MeJA without decrease in biomass. At this MeJA concentration, 728.2 mg L^{-1} phenolics, 622.2 mg L^{-1} flavonoids, 255.3 mg L^{-1} cichoric acid, and 143.9 mg L^{-1} echinacoside were produced. MeJA, at 50 to 200 μM, were equally beneficial for increasing polysaccharide production to approximately 440 mg L^{-1}. The SOD, POD, AXP, and CAT activities reached their highest values at 25 μM MeJA. The comparison result indicated that the contents of the secondary metabolites (phenolics, flavonoids, cichoric acid, and echinocoside) in the co-cultured ARs of *E. purpurea* and *E. pallida* were higher than those in the natural roots of each species. However, the polysaccharide content did not considerably increase and was even lower than that in roots of *E. pallida* and *E. purpurea*. The present study

suggests that MeJA is an effective approach for improvement of bioactive compound accumulation during AR co-culture of *E. pallida* and *E. purpurea*.

Author contribution DA and CHW designed and conducted experiments, MW maintained plant materials, MW conducted data analysis, GNC and XJC detected bioactive compound contents, and MLL wrote the paper.

Funding This research was funded by National Natural Science Foundation of China (31370388).

Declarations

Conflict of interest The authors declare no competing interests.

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