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ORIGINAL PAPER

Elicitation of antioxidant secondary metabolites with jasmonates and gibberellic acid in cell suspension cultures of *Artemisia absinthium* L.

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Abstract *Artemisia absinthium* L. is a very important species with worldwide traditional medicinal uses. In this study, effects of different concentrations of two important elicitors, methyl jasmonate (MeJA) and jasmonic acid (JA), and a phytohormone gibberellic acid (GA) on growth kinetics, secondary metabolites accumulation and antioxidant activity in cell suspension cultures of *Artemisia absinthium* L. were investigated. The results showed inhibition in dry biomass accumulation and shorter log phases of cultures growth in response to most treatments, compared to control. Further, we observed enhanced accumulation of total phenolic content (TPC), total flavonoid content (TFC) and highest radical scavenging activity (RSA) in suspension cultures treated with 1.0 mg/l of MeJA, JA and GA, each. The correlation studies between secondary metabolites and antioxidant activity showed TPC-dependent RSA in most cultures. MeJA and JA treated cultures showed no significant variation in the context of total flavonoid contents and 1.0 mg/l of both displayed significantly comparable maximum RSA profiles on day-24 of culture. GA-treated cultures showed minimum accumulation of TPC, but TFC and RSA were found to be significantly comparable to those in MeJA and JA treated cultures. The results showed elicitors-induced enhancement in phenolic and flavonoid accumulation and antioxidant activity in suspension cultures of *Artemisia absinthium* L.

Keywords *Artemisia* · Phytohormone · Growth kinetics · Phenolics · Flavonoids

Abbreviations

GA	Gibberellic acid
JA	Jasmonic acid
MeJA	Methyl jasmonate
MS	Murashige and skoog
TFC	Total flavonoid content
TPC	Total phenolic content
RSA	Radical scavenging activity

Introduction

The genus *Artemisia* is one of the largest and most widely distributed genera of the family Asteraceae. Most of its species have been used worldwide in folk medicine since ancient times (Nalbantsoy et al. 2013). *Artemisia absinthium* L., commonly known as Wormwood, has been used as herbal medicine throughout Europe, the Middle East, North Africa, and Asia (Sharopov et al. 2012). The plant has traditionally been used as anthelmintic, choleretic, antiseptic, balsamic, depurative, digestive, diuretic, emmenagogue and in treating leukaemia and sclerosis (Canadanovic-Brunet et al. 2005). Furthermore, it has been reported as a rich source of terpenes, antioxidant phenolics, flavonoids and other biologically active compounds (Singh et al. 2012).

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Antioxidant compounds can delay or inhibit oxidation of lipids and other molecules by inhibiting the initiation or propagation of oxidizing chain reactions (Ferreira et al. 2010). Plant-derived polyphenols provide a prolonged and balanced dose of antioxidants beneficial to human health (Canadanovic-Brunet et al. 2005). These compounds are known to provide protection against a wide range of diseases such as cardiovascular diseases, cancers, neurodegenerative diseases, diabetes and osteoporosis (Scalbert et al. 2005). Flavonoids are well known for their antioxidant capacity due to their redox properties, and it has been assumed that a diet rich in flavonoids is inversely correlated with cell aging, lipid peroxidation, cancer, etc. (Ferreira et al. 2010).

The biotechnological production of secondary metabolites in plant cell and organ cultures is an attractive alternative to the extraction of the whole plant material (Skrzypczak-Pietraszek et al. 2014). Especially, plant cell and organ culture systems are promising technologies for the production of valuable plant-specific metabolites (Verpoorte et al. 2002). Elicitation has been one of the most efficient strategies to enhance the biosynthesis of these metabolites in plant cell cultures (Lu et al. 2001; Wang et al. 2004; Zhang et al. 2000). Jasmonates, including jasmonic acid (JA) and methyl jasmonate (MeJA), are a family of cyclopentanone compounds that modulate a wide range of plant responses (Creelman and Mullet 1997; Sembdner and Parthier 1993) and act as effective elicitors to enhance secondary metabolites in in vitro cultures. Gibberellin (GA), a phytohormone, is also well known as an effective elicitor for production of secondary metabolites (Liang et al. 2013).

In present study, we evaluated effects of different concentrations of MeJA, JA and GA on growth kinetics and antioxidant phenolic and flavonoid accumulation in cell suspension cultures of *Artemisia absinthium* L.

Materials and methods

Cell suspension culture establishment and elicitation

Callus was induced from leaf explants of in vitro seed germinated plantlets, as described previously (Ali et al. 2013). To develop inoculum culture, 35-day old proliferated calli were cultured in liquid MS medium supplemented with 1.0 mg/l of TDZ and 1.0 mg/l of NAA and placed on gyratory shaker (25 °C, 120 rpm) in dark. 14 days old inoculum culture was used for further experiments. Subsequent experiments were carried out in 250 ml Erlenmeyer flasks containing 50 ml MS media with 30 g l^{-1} sucrose, 1.0 mg l^{-1} TDZ and 1.0 mg l^{-1} NAA in combination and 1.5 g fresh cell suspension was inoculated in each flask.

For elicitation experiments, methyl jasmonate (MJ) and jasmonic acid (JA) were added to culture media in different concentrations (0.5, 1.0, 2.0 mg/l). Gibberellic acid (GA) was also tested as a signaling molecule in the same concentrations. Murashige and skoog basal medium (MS0) containing 1.0 mg l^{-1} TDZ and 1.0 mg l^{-1} NAA without elicitors was used as control. Observations and data recording of the growth kinetics and secondary metabolites accumulation were performed with an interval of 3 days for 39 days period. Triplicate flasks were used in all experiments.

Before autoclaving (121 °C, 20 min, Systec VX 100, Germany), pH of all media was adjusted to 5.8 (Eutech Instruments pH 510, Singapore). All cultures were placed in gyratory shaker (25 °C, 120 rpm) under continuous light and temperature was maintained at 25 ± 1 °C.

Analytical methods

For dry biomass (DBM) determination, each suspension culture obtained was filtered through 0.45 μm stainless steel sieve (Sigma), washed with distilled water, pressed gently on filter paper to remove excess water and oven dried (60 °C, 24 h).

Dried cell suspension cultures were extracted as described previously (Ali et al. 2013). Briefly, each finely ground dried sample (100 mg) was mixed with 80 % (v/v) methanol (10 ml). The mixtures were sonicated (10 min; Toshiba, Japan) three times with a resting period of 30 min in between and centrifuged (8,000 rpm, 10 min). The supernatants were collected and either immediately used for analysis or stored at 4 °C.

Total phenolic content was determined by using Folin-Ciocalteu reagent according to the protocol of (Velioglu et al. 1998). Absorbance was measured at 725 nm by using UV/VIS-DAD spectrophotometer (Halo DR-20, UV–VIS spectrophotometer, Dynamica Ltd, Victoria, Australia). The calibration curve (0–50 μg/ml, $R^2 = 0.968$) was plotted by using gallic acid as standard and the TPC was expressed as gallic acid equivalents (GAE)/g of dry weight.

Total flavonoid content was determined by using aluminum chloride colorimetric method as described by (Chang et al. 2002). Absorbance of the reaction mixtures was measured at 415 nm by using UV/VIS-DAD spectrophotometer. The calibration curve (0–40 μg/ml, $R^2 = 0.998$) was plotted by using quercetin as standard. The TFC was expressed as quercetin equivalents (QE)/g of dry weight.

For antioxidant activity determination, the DPPH free radical scavenging assay (FRSA) as described by (Abbasi et al. 2010) was used. Absorbance of the mixtures was recorded at 517 nm by spectrophotometer. For background correction, a methanolic solution of DPPH that had decayed with no resultant purple color (2 mg of butylated hydroxyanisole (BHA) dissolved in 4 ml of methanol with 0.5 ml of DPPH solution added) was used instead of pure methanol. The radical scavenging activity was calculated by the following formula and expressed as % of DPPH discoloration:

$$\% \text{ scavenging DPPH free radical} = 100 \times (1 - \mathrm{AE}/\mathrm{AD})$$

where AE is absorbance of the solution when an extract was added at a particular concentration and AD is the absorbance of the DPPH solution with nothing added.

Statistical analysis

All experiments were conducted in a completely randomized design and were repeated twice. Each treatment was consisted of three replicates. Mean values of various treatments were subjected to analysis of variance (ANOVA) using GraphPad Prism 5.01. Significant differences between treatments at $P < 0.05$ were performed using Satistix 8.1. Figures were generated using Origin 8.5.

Results and discussion

Growth kinetics of suspension cultures

In preliminary experiments, dry biomass accumulation in response to several concentrations of MeJA, JA and GA was determined and compared with control cultures. Overall, a decrease in biomass with shorter log phases was observed in response to most of the treatments, compared to control. When treated with MeJA, 2.6-fold, 2.9-fold and threefold increase in dry biomass was observed in cultures treated with 0.5, 1.0 and 2.0 mg/l, respectively (Control: fourfold) (Fig. 1a). When treated with Jasmonic Acid, 2.7-fold, 2.8-fold and 2.7-fold increase in dry biomass was observed in response to 0.5, 1.0 and 2.0 mg/l, respectively (Fig. 1b). No significant variation in the effect of MeJA and JA in the context of biomass accumulation was observed. Among all treatments, MeJA 1.0 mg/l resulted in maximum biomass (7.64 g/l) on day 30 of culture (control: 8.88 g/l). Furthermore, higher concentrations (2.0 mg/l) of MeJA and JA reduced the peak of log phases by 3 and 6 days, respectively, while the same concentration of GA had no effect on the length of log phase, compared to control. However, 1.0 mg/l of MeJA, JA and GA resulted into maximum respective biomass profiles of 7.64, 7.61 and 7.21 g/l.

GA resulted in dry biomass profiles, with 2.5-fold, 2.6-fold and 2.4-fold maximum increase in response to 0.5, 1.0 and 2.0 mg/l, respectively (Fig. 1c). The effect of GA on biomass accumulation in cell suspension cultures is rarely available in literature; however, it is well demonstrated for hairy root cultures of different plants. Previously, GA containing treatments have resulted in highest dry weights in hydroponics of *Artemisia annua* intact plants (Jehnsen 2010). This could be due to the differential response of suspension cultures and intact plants to the same phytohormone. On the other hand, Abbasi et al. (2012) observed GA-concentration dependent response in accumulation behavior of dry biomass in hairy roots of *Echinacea pupurea*.

Total phenolic content (TPC) and total flavonoid content (TFC)

Plants develop a variety of defense responses against biotic and abiotic stress, including elicitor exposure (Tan et al. 2004) which leads to the production of several secondary metabolites such as phenolics, flavonoids and other low molecular weight metabolites (Ali et al. 2006). The role of jasmonic acid (JA) and its volatile methyl ester, methyl jasmonate (MeJA) as signaling molecules is well known in the form of morphological and physiological changes (Sembdner and Parthier 1993). Recently, jasmonates-induced enhanced phenolics and flavonoids accumulation in cell suspension culture of *Taxus baccata* was reported (Jalalpour et al. 2014). In present study, we evaluated the

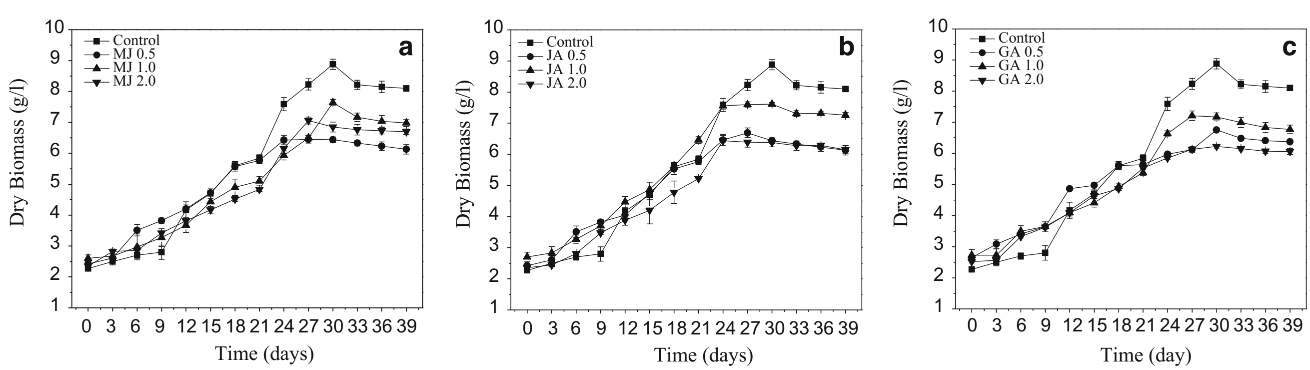

Fig. 1 Growth kinetics of cell suspension cultures of *Artemisia absinthium* L. on MS medium supplemented with 0.5, 1.0 and 2.0 mg/l of methyl jasmonate (**a**), jasmonic acid (**b**) and gibberellic acid (**c**). Values are mean ± standard error of three replicates

effect of three different concentrations (0.5, 1.0, 2.0 mg/l) of MeJA, JA and GA on phenolics and flavonoids accumulation. When treated with 0.5 and 1.0 mg/l of MeJA, suspension cultures induced maximum corresponding TPC levels of 5.6 mg GAE/g DW and 6.7 mg GAE/g DW on day-24 and day-21, respectively, while 2.0 mg/l MeJA resulted into inhibited levels with the value 5.29 mg GAE/g DW on day-21 of culture (Fig. 2a). The results suggest that lower concentrations of MeJA have enhancing effect on phenolics accumulation compared to control (5.32 mg GAE/g DW). Similar results were obtained when the cultures were treated with different concentrations of JA. In response to 0.5 and 1.0 mg/l, the maximum levels of TPC observed were 5.71 mg GAE/g DW and 6.7 mg GAE/g DW on day-24 and day-21, respectively while 2.0 mg/l JA resulted into 5.29 mg GAE/g DW (Control: 5.32 mg GAE/g DW, day-24) (Fig. 2b).

Unlike the enhancing effect of lower concentrations of jasmonates on total phenolics induction, 0.5 mg/l of GA resulted into 4.9 mg GAE/g DW of TPC on day-24, while 1.0 and 2.0 mg/l of GA treated cultures showed 5.9 mg GAE/g DW and 5.36 mg GAE/g DW on day-24 and day-21, respectively (Control: 5.32 mg GAE/g DW, day-24) (Fig. 2c).

Like TPC, significantly higher levels of TFC were recorded in suspension cultures in response to lower concentrations of MeJA and JA. Maximum levels of TFC recorded in 0.5 and 1.0 mg/l of MeJA treated cultures were 1.84 mg QE/g DW and 2.19 mg QE/g DW, on day-27 and day-30, respectively, while 2.0 mg/l MeJA resulted into 1.56 mg QE/g DW on day-27 of culture, which was comparable to control (1.57 mg QE/g DW, day-27) (Fig. 3a). Similarly, enhanced levels of TFC in response to 0.5 and 1.0 mg/l of JA found were 1.68 and 1.89 mg QE/g DW on day-27 of culture while 2.0 mg/l of JA resulted into inhibited maximum TFC level of 1.38 mg QE/g DW on day-24 of culture (Fig. 3b).

Unlike the inhibitory effect of 0.5 mg/l GA on TPC, enhancement in maximum TFC level (1.98 mg QE/g DW) was observed in response to the same treatment, in comparison to control cultures (1.57 mg QE/g DW). Furthermore, maximum TFC levels in suspension cultures treated with 1.0 and 2.0 mg/l of GA were found to be 1.71 mg QE/g DW and 1.57 mg QE/g DW, respectively (Fig. 3c), which clearly shows the inhibitory effect of higher concentration of GA on TFC accumulation.

Antioxidant activity and its relation with total phenolic content (TPC) and total flavonoid content (TFC)

The antioxidant potential of *A. absinthium* L. has been reported several times in literature (Asghar et al. 2011; Canadanovic-Brunet et al. 2005; Craciunescu et al. 2012; Lopes-Lutz et al. 2008; Mahmoudi et al. 2009). In our previous studies, we have investigated phenolic and flavonoid contents and their inter-relationship with antioxidant activity in callus cultures (Ali and Abbasi 2014; Tariq et al. 2014) and cell suspension cultures of *A. absinthium* (Ali et al. 2013). Most of the in vitro cultures tested, have shown a positive correlation of phenolics and antioxidant activity. Furthermore, reports are available; suggesting a strong relationship between the phenolic compounds produced by in vitro cultures of different plants and their antioxidant activities (Al Khateeb et al. 2012; Ali et al. 2007; Amid et al. 2013; Diwan et al. 2012). These studies suggest the involvement of phenolic metabolites in antioxidant activities of some medicinal plants and their in vitro cultures.

In present study, antioxidant activity was determined as % DPPH radical scavenging activity (RSA). Overall, highest radical scavenging activity (80.9 %) was found in suspension cultures treated with 1.0 mg/l MeJA. Suspension cultures treated with 0.5 and 2.0 mg/l of MeJA showed RSA with the respective levels of 72.4 and 65.9 %, while 1.0 mg/l resulted into highest RSA 80.9 %. The same pattern was followed by suspension cultures treated with different concentrations of JA and highest RSA of

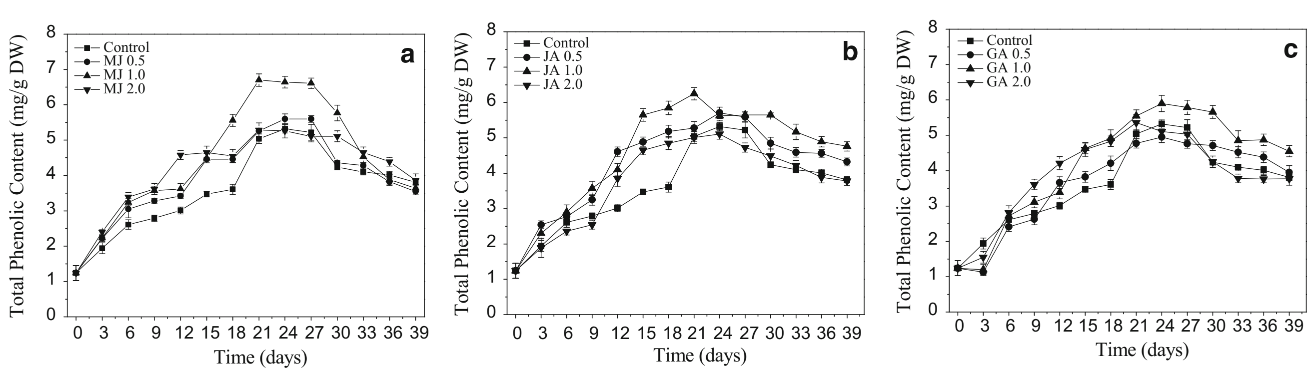

Fig. 2 Total phenolic content (mg Gallic acid/g DW) in cell suspension cultures of *Artemisia absinthium* L. on MS medium supplemented with 0.5, 1.0 and 2.0 mg/l of methyl jasmonate (**a**), jasmonic acid (**b**) and gibberellic acid (**c**). Values are mean ± standard error of three replicates

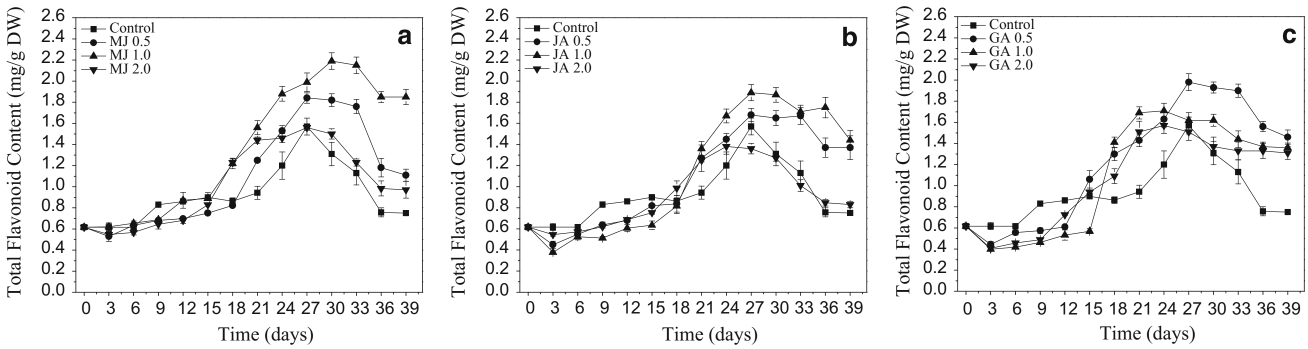

Fig. 3 Total flavonoid content (mg Quercetin/g DW) in cell suspension cultures of *Artemisia absinthium* L. on MS medium supplemented with 0.5, 1.0 and 2.0 mg/l of Methyl Jasmonate **a** Jasmonic Acid **b** and Gibberellic Acid **c**. Values are mean ± standard error of three replicates

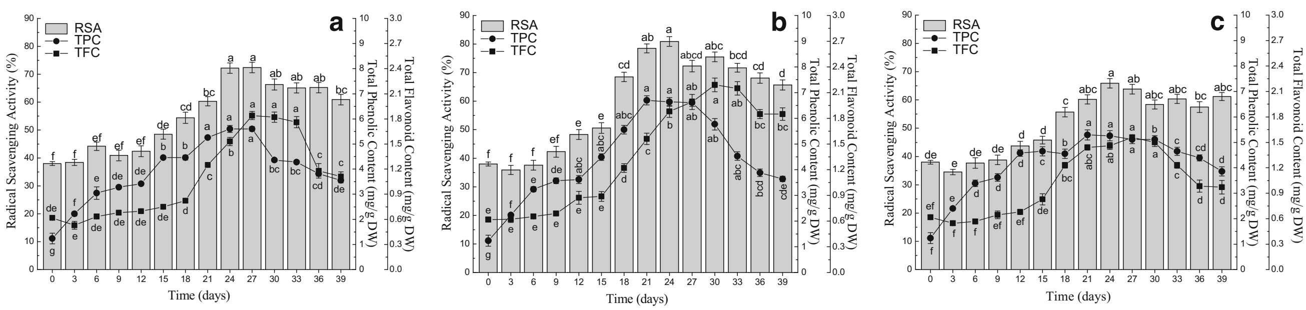

Fig. 4 DPPH radical scavenging activity (%) with respect to total phenolic content and total flavonoid content in cell suspension cultures of *Artemisia absinthium* L. on MS medium supplemented with methyl jasmonate 0.5 mg/l (**a**), 1.0 mg/l (**b**) and 2.0 mg/l (**c**). Values are mean ± standard error of three replicates. Columns with similar alphabets are not significantly different at $P < 0.05$

78.2 % was observed in cultures treated with 1.0 mg/l, followed by 75.7 and 72.3 % in response to 0.5 and 2.0 mg/l, respectively. The results show that lower (0.5 mg/l) and higher concentrations (2.0 mg/l) of jasmonates resulted into inhibition of RSA in suspension cultures of *A. absinthium*, compared to the optimum concentration (1.0 mg/l) used in present study. Similar pattern of RSA was observed for GA, where highest activity 74.1 % was found in 1.0 mg/l GA treated cultures, followed by 62.4 % in response to 0.5 mg/l GA. However, lowest RSA of 57.6 % was observed in response to 2.0 mg/l GA.

When correlated with TPC and TFC, different trends were followed by RSA in response to three different concentrations (0.5, 1.0, 2.0 mg/l) of MeJA, JA and GA. A positive correlation between RSA and secondary metabolites was displayed by suspension cultures treated with 0.5 mg/l MeJA; where the highest levels of RSA (72.4 %), TPC (5.6 mg GAE/g DW) and TFC (1.84 mg QE/g DW) were observed on day-27 of culture (Fig. 4a). Furthermore, maximum biomass accumulation (6.45 g/l) was also observed on the same day. However, cultures treated with 1.0 mg/l MeJA showed TPC dependent but TFC independent correlation with the maximum RSA; where significantly higher levels of TPC (6.64 mg GAE/g DW) and RSA (80.9 %) were observed on day-24 of culture (Fig. 4b). Cultures treated with 2.0 mg/l also showed a positive correlation among the maximum levels of RSA (65.9 %), TPC (5.26 mg GAE/g DW) and TFC (1.46 mg QE/g DW) on day-24 of culture (Fig. 4c). The results indicate the involvement of TPC as a common class of antioxidants in suspension cultures of *A. absinthium*, when treated with different concentrations of MeJA.

Radical scavenging activity was found to be mainly TPC dependent in suspension cultures treated with different concentrations of JA. As indicated (Fig. 5a), the significantly comparable RSA levels 75.6 and 76.5 % on day-24 and day-27, respectively, are in positive correlation with their corresponding TPC and TFC profiles. Further, highest RSA (78.2 %) was found in positive correlation with significantly higher level of TPC (6.25 mg GAE/g DW) on day-21 of culture while TFC was found to fluctuate during the culture growth (Fig. 5b). Additionally, suspension cultures treated with 2.0 mg/l JA displayed positively correlated profile of RSA (72.3, 72, 68.4 %), TPC (5.01 mg GAE/g DW, 5.11 mg GAE/g DW, 4.73 mg GAE/g DW) and TFC (1.25 mg QE/g DW, 1.38 mg QE/g DW, 1.36 mg QE/g DW) on day-21, day-24 and day-27, respectively (Fig. 5c).

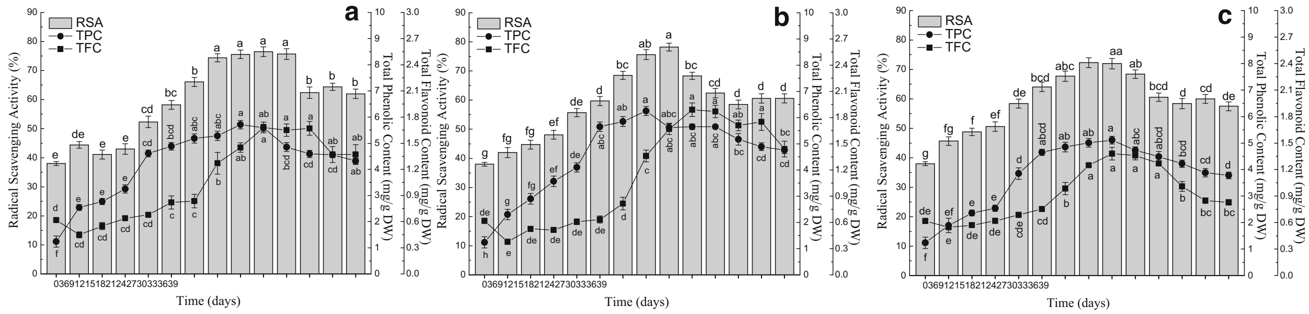

Fig. 5 DPPH radical scavenging activity (%) with respect to total phenolic content and total flavonoid content in cell suspension cultures of *Artemisia absinthium* L. on MS medium supplemented with jasmonic acid 0.5 mg/l (**a**), 1.0 mg/l (**b**) and 2.0 mg/l (**c**). Values are mean ± standard error of three replicates. Columns with similar alphabets are not significantly different at $P < 0.05$

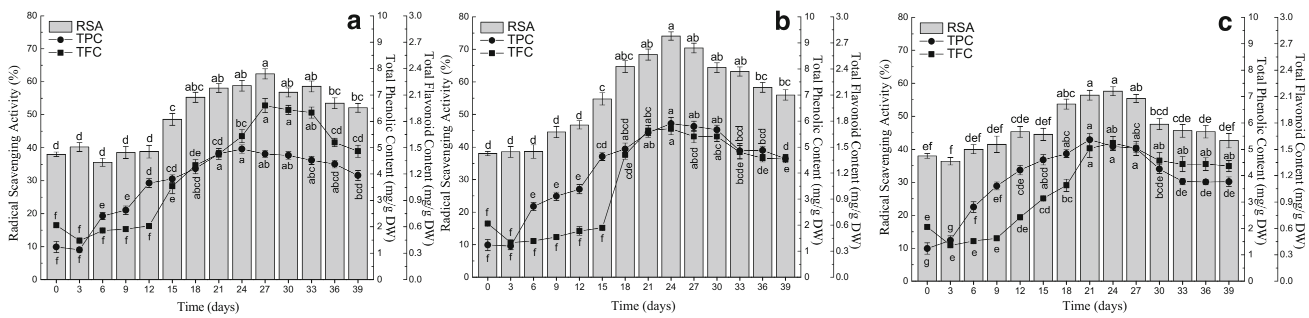

Fig. 6 DPPH radical scavenging activity (%) with respect to total phenolic content and total flavonoid content in cell suspension cultures of *Artemisia absinthium* L. on MS medium supplemented with gibberellic acid 0.5 mg/l (**a**), 1.0 mg/l (**b**) and 2.0 mg/l (**c**). Values are mean ± standard error of three replicates. Columns with similar alphabets are not significantly different at $P < 0.05$

Phytohormones, a group of crucial signal molecules, are actively involved in plant secondary metabolism along with regulating plant growth and development (Zhao et al. 2005). GA has been used as efficient elicitor to stimulate production of tanshinones in *S. miltiorrhiza* hairy roots (Yuan et al. 2008) and caffeic acid derivatives (CADs) in *Echinacea pupurea* hairy roots (Abbasi et al. 2012). GA-induced enhanced Artemisinin content in *Artemisia annua* was also reported (Banyai et al. 2011; Smith et al. 1997). Further, GA was reported to improve phenolics production and PAL activity in *S. miltiorrhiza* hairy roots (Liang et al. 2013). In the present study, we used three concentrations of GA (0.5, 1.0, 2.0 mg/l) to determine their effect on secondary metabolites accumulation and RSA in suspension cultures of *A. absinthium* L. We found a positive correlation between RSA and TPC in GA treated cultures. Significantly comparable RSA levels in 18–33 day old suspension cultures were found to be TPC dependent; however, RSA and TFC showed positive correlation only in 27–33 day old suspension cultures. In response to this treatment, 27-day old suspension cultures showed maximum levels of RSA (62.4 %) and TFC (1.98 mg QE/g DW) (Fig. 6a). In response to 1.0 mg/l GA, the comparable highest RSA levels in 18–30 day old cultures were found to be TPC dependent while RSA and TFC inter-relationship was observed in 21–30 day old cultures. However, 30-day old suspension cultures displayed maximum RSA (74.1 %), TPC (5.9 mg GAE/g DW) and TFC (1.71 mg QE/g DW) (Fig. 6b). When treated with 2.0 mg/l GA, significantly comparable RSA in 21 to 27-day old cultures was found to be in positive correlation with its corresponding TPC and TFC. Further, highest levels of RSA (57.6 %), TFC (1.57 mg QE/g DW) and comparable level of TPC (5.11 mg GAE/g DW) to its maximum level were displayed by 24-day old suspension cultures (Fig. 6c).

Conclusions

The results showed enhanced accumulation of phenolics and flavonoids in suspension cultures of *A. absinthium* L. when treated with MeJA, JA and GA. Interestingly, most of the suspension cultures displayed positive correlation of phenolics and antioxidant activity. Furthermore, MeJA and JA treated cultured showed no significant variation in the context of total flavonoid contents and antioxidant activities. It will be helpful to use other elicitors alone and in different combinations to investigate the biosynthetic potential of medicinally important secondary metabolites in suspension cultures of *A. absinthium* L.

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