

# THE INFLUENCE OF METHYL JASMONATE AND SALICYLIC ACID ON SECONDARY METABOLITE PRODUCTION IN *REHMANNIA GLUTINOSA* LIBOSCH. HAIRY ROOT CULTURE

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*Rehmannia glutinosa* hairy roots were used to evaluate the effect of methyl jasmonate (MeJa) and salicylic acid (SA) on increase of root biomass and production of iridoids (catalpol, harpagide) and phenylethanoids (verbascoside and isoverbascoside). The elicitors were added to 23-day-old culture separately at concentrations between 50 and 200  $\mu\text{M}$  or in combinations at concentrations of 50 and 100  $\mu\text{M}$ . Roots were harvested 72 h and 120 h after elicitation. The type of elicitor, its concentration and exposure time were found to strongly affect the content of each analyzed compound. A 72-hour treatment with 200  $\mu\text{M}$  MeJa was the most effective in increase of verbascoside content (60.07  $\text{mg}\cdot\text{DW}^{-1}$  equivalent to 845.45  $\text{mg}\cdot\text{L}^{-1}$ ) and isoverbascoside (1.77  $\text{mg}\cdot\text{DW}^{-1}$  equivalent to 24.94  $\text{mg}\cdot\text{L}^{-1}$ ): these respective amounts were roughly 10- and 6.4-fold higher than the control values (unelicited roots). Exposure to 150  $\mu\text{M}$  MeJa provided optimal harpagide content after 72 hours (0.136  $\text{mg}\cdot\text{DW}^{-1}$ ; 7.5-fold increase compared to the control), and catalpol content after 120 hours (up to 2.145  $\text{mg}\cdot\text{DW}^{-1}$ ). The combination of MeJa and SA also resulted in higher levels of secondary metabolites compared to the control culture, although these levels were lower than those observed for MeJa alone at the optimal concentration and exposure time. SA alone was less efficient in enhancing metabolite production than MeJa.

**Keywords:** elicitation, catalpol, hairy roots, harpagide, verbascoside, isoverbascoside

## INTRODUCTION

*Rehmannia glutinosa* Libosch., a member of the Orobanchaceae family (Angiosperm Phylogeny Group III, 2009), is a perennial plant, which naturally occurs in China, Japan and Korea. It is one of the 50 fundamental herbs used in traditional Chinese medicine. Depending on the various processing methods described in the Chinese Pharmacopoeia (2000), *Rehmanniae radix* (Di Huang) is classified into three types: fresh root, dried root and steamed root. These types of roots are widely used as tonic, antianemic, antipyretic, antisenescence, antiulcer and antitumor agents (Kitagawa et al., 1991; Zhang et al., 2008). The pharmacological properties of the plant roots are generally attributed to the presence of such iridoid glycosides as catalpol, aucubin and harpagide (Zhang et al., 2008), which have been shown to have a range of biological activities. They have demonstrated antitumor activity by inhibiting the

enzyme topoisomerase I, involved in tumoral processes (Galvez et al., 2005), and possessing cytotoxic activity toward lung cancer cell lines (Hung et al., 2008). Additionally, catalpol possesses extensive ischemic neural protection (Li et al., 2005), increases brain angiogenesis (Zhu et al., 2010) and exhibits hypoglycemic and diuretic activities (Zhang et al., 2008). Harpagide expresses leishmanicidal activities (Tasdemir et al., 2008) and is known to suppress lipopolysaccharide-induced nitric oxide synthase and cyclooxygenase-2 (COX-2) expression through inhibition of nuclear factor  $\kappa\text{B}$  activation (Georgiev, 2013). Roots of *R. glutinosa* also accumulate phenylethanoid glycosides, including verbascoside and isoverbascoside.

Several studies describe production of metabolites characteristic for normal roots of *R. glutinosa*, using plant tissue culture such as callus cultures (Shoyama et al., 1986; Piątczak et al., 2015) and hairy root cultures obtained by *Agrobacterium rhizogenes* infection (Hwang, 2005; Hwang, 2009;

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Piåtczak et al., 2012). Hairy root cultures are particularly well suited for production of large amounts of biomass and bioactive compounds as they offer fast growth in hormone-free media, the ability to synthesize various chemical compounds and the possibility of growth in a bioreactor. In addition, accumulation of bioactive compounds in transformed roots can be increased, e.g., by elicitation (Rao and Ravishankar, 2002).

Biosynthesis of many secondary metabolites in plants usually begins as a result of a defense response. A number of elicitors, including jasmonic acid (Zhang and Guo, 2006), its derivative methyl jasmonate (MeJa), and salicylic acid (SA) act as important plant signal transduction molecules (Chetana and Ramawat, 2009; d'Onofrio et al., 2009); these are involved in stimulation of biochemical reactions which encourage the formation of low molecular weight defensive compounds in response to attack by pathogens (Ozawa et al., 2000; Schenk et al., 2000). The exogenous addition of such elicitors to plant cell culture or intact plants stimulates biosynthesis of a wide range of secondary metabolites including glucosinolates, terpenoids, phenylpropanoids, valepotriates, saponins and alkaloids (Van der Fits and Memelink, 2000; Memelink et al., 2001; de Costa et al., 2013; Russowski et al., 2013). However, elicitation is a highly complex process and depends on many factors such as elicitor concentration, growth stage of the culture, the time at which the elicitor is added and contact time with the elicitor (Namdeo, 2007). These parameters need to be optimized according to the plant species and type of bioactive compound.

The present study evaluates the influence of individually applied or combined elicitors: SA and MeJa on production of biomass, as well as iridoids (catalpol, harpagide, catalposide) and phenylethanoids (verbascoside, isoverbascoside), in hairy roots of *R. glutinosa*. The optimal concentrations and exposure times of the elicitors are also determined.

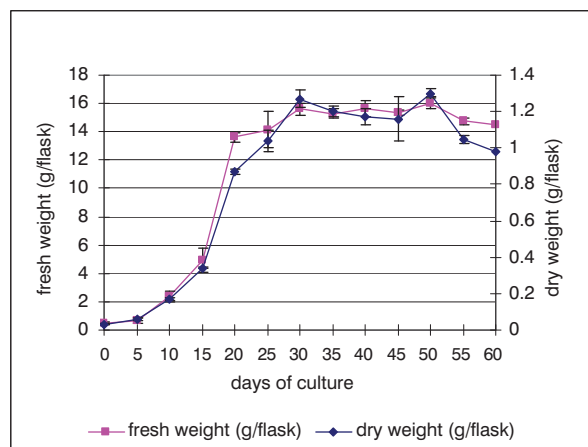
## MATERIALS AND METHODS

### CHARACTERIZATION OF GROWTH KINETICS

Hairy roots of *R. glutinosa* (RS-2 line) were obtained after transformation with *Agrobacterium rhizogenes* (strain A4) as described by Piåtczak et al. (2012). The cultures were grown in Erlenmeyer flasks (300 mL) containing 80 mL of liquid WPM (Woody Plant medium) (Lloyd and McCown 1980) without any growth regulators, supplemented with 3% of sucrose, in darkness at  $26^{\circ}\text{C} \pm 2^{\circ}\text{C}$  on a rotary shaker (70 rpm). The cultures were subcultured every 30 days. The average fresh weight

(FW) of inoculum was  $0.48 \pm 0.03$  g/flask and dry weight (DW) was  $0.03 \pm 0.006$  g/flask. For kinetics studies, *R. glutinosa* hairy root cultures after 25 subcultures were cultured for 60 days, with root growth being determined every 5 days on the basis of fresh and dry weights. At each time point, the growth of hairy roots was measured in terms of FW and DW (g per flask). FW was measured after separating of hairy roots growing in the Erlenmeyer flasks from the media and washing them with distilled water. Then they were dried on some absorbent paper and weighted on laboratory scales with accuracy of 0.01 g. For determination of DW, roots were divided into pieces of 300–600 mg, placed on aluminium caps and weighted with 0.0001 g accuracy. Then they were dried for an hour at  $100^{\circ}\text{C}$  and for 24 hours at  $80^{\circ}\text{C}$  in a laboratory oven and weighted. The experiments were repeated three times (3 flasks with roots for one experiment) for three consecutive subculture cycles.

The results are presented in Fig. 1. The specific growth rate ( $\mu$ ) and doubling time (dt) were also estimated from the exponential phase of growth, as indicated by Wodnicka et al. (2000).



**Fig. 1.** Time course study of *R. glutinosa* hairy roots. Fresh and dry weight of biomass was recorded for 60 days at 5-day intervals.

Data are represented as the mean  $\pm$  standard error (SE) from three independent experiments. For each experiment 3 Erlenmeyer flasks were taken at each time point.

### EXPERIMENTS WITH ELICITORS

Methyl jasmonate (MeJa) (95% purity, Sigma Aldrich) and salicylic acid (SA) (95% purity, Sigma Aldrich) solutions were prepared by dissolution in 96% ethanol. The solutions were sterilized by syringe filter (Merck Millipore Ltd., pore size  $\text{\O} 20 \mu\text{m}$ ) and added to flasks containing 80 mL of autoclaved liquid medium with hairy root cultures to reach final concentrations of 50, 100, 150 and 200  $\mu\text{M}$  (the total ethanol added was 80  $\mu\text{L}$  per

flask). In further experiments, MeJa and SA were also added in two combinations, i.e. 50  $\mu\text{M}$  MeJa + 50  $\mu\text{M}$  SA and 100  $\mu\text{M}$  MeJa + 100  $\mu\text{M}$  SA.

The elicitor was added on the 23<sup>rd</sup> day of culture without changing the medium, when the cells were in the late growth phase. The control hairy roots were treated with 80  $\mu\text{L}$  of 96% ethanol at the same time. The hairy roots were allowed to grow in the presence of the elicitor for further 72 or 120 hours before being harvested. Their FW and DW were then measured and their secondary metabolite contents were analyzed by UHPLC.

#### EVALUATION OF IRIDOID AND PHENYLETHANOID GLYCOSIDE CONTENTS

The plant material was lyophilized, powdered and extracted as described by Piątczak et al. (2012). The UHPLC analyses were carried out on Agilent Technologies 1290 Infinity apparatus on a Zorbax Eclipse Plus C18 column (100  $\times$  3.1 mm id; 1.8  $\mu\text{m}$  Agilent Technologies) (Piątczak et al., 2015). The compounds were identified according to their retention times and UV spectra by comparison with authentic standards (Piątczak et al., 2015). The contents of iridoid (catalpol, harpagide, catalposide) and phenylethanoid (verbascoside, isoverbascoside) glycosides in the samples were expressed as mg of the compound per gram of dry weight ( $\text{mg} \times \text{g}^{-1}$  DW). The results are presented in Figs. 3 and 4. Finally, metabolite production ( $\text{mg} \cdot \text{L}^{-1}$ ) after MeJa treat-

ment of hairy root culture was also determined according to the formula: mean DW ( $\text{g} \cdot \text{L}^{-1}$ )  $\times$  mean content of each metabolite ( $\text{mg} \cdot \text{g}^{-1}$  DW) (Table 1).

#### STATISTICAL ANALYSIS

All the presented results are means from three independent experiments  $\pm$  standard error (SE). The results presented in Table 1 and Figs. 2–4 were analyzed by the Kruskal-Wallis test, with  $p \leq 0.05$  as the significance level. STATISTICA 10 (STATSoft) Software was used for all the analyses.

## RESULTS AND DISCUSSION

#### GROWTH KINETICS IN SHAKE FLASKS

The growth curve of *R. glutinosa* hairy roots grown in WPM liquid medium was determined based on FW and DW accumulation over a period of 60 days (Fig. 1). With regard to FW accumulation, an initial 5-day lag phase was first observed, followed by an exponential phase from day 5 to day 20, during which the maximum growth rate was obtained and the culture reached 85% of the final fresh weight. From the exponential growth phase, a maximum specific growth rate of  $\mu = 0.354 \text{ day}^{-1}$  (corresponding to  $dt = 1.96$  days) was calculated. The values were similar to those for cultures of other plant species, for example *Atropa belladon-*

TABLE 1. Production of iridoid and phenylethanoid glycosides in hairy root culture of *R. glutinosa* after MeJa elicitation for 72 and 120 hours

MeJa concentration ( $\mu\text{M}$ )	Exposure time (hours)	Metabolite production ( $\text{mg} \cdot \text{L}^{-1}$ ) $\pm$ SE*			
		Catalpol	Harpagide	Verbascoside	Isoverbascoside
0 (control)	72	15.71 <sup>ad</sup> $\pm$ 3.57	0.29 <sup>a</sup> $\pm$ 0.01	100.14 <sup>a</sup> $\pm$ 6.67	4.58 <sup>a</sup> $\pm$ 0.79
50		3.64 <sup>b</sup> $\pm$ 1.45	0.70 <sup>b</sup> $\pm$ 0.34	679.04 <sup>bc</sup> $\pm$ 50.58	8.31 <sup>b</sup> $\pm$ 0.90
100		3.09 <sup>b</sup> $\pm$ 0.33	1.22 <sup>c</sup> $\pm$ 0.01	712.70 <sup>b</sup> $\pm$ 41.88	10.25 <sup>c</sup> $\pm$ 0.79
150		4.89 <sup>c</sup> $\pm$ 0.48	1.86 <sup>d</sup> $\pm$ 0.01	677.07 <sup>c</sup> $\pm$ 28.92	10.35 <sup>c</sup> $\pm$ 0.23
200		3.85 <sup>b</sup> $\pm$ 0.54	1.83 <sup>d</sup> $\pm$ 0.07	845.45 <sup>d</sup> $\pm$ 5.64	24.94 <sup>d</sup> $\pm$ 7.38
0 (control)	120	17.15 <sup>a</sup> $\pm$ 3.57	0.30 <sup>a</sup> $\pm$ 0.06	104.17 <sup>a</sup> $\pm$ 11.08	7.34 <sup>bg</sup> $\pm$ 1.15
50		14.17 <sup>ad</sup> $\pm$ 2.15	0.63 <sup>b</sup> $\pm$ 0.17	465.92 <sup>e</sup> $\pm$ 84.88	18.36 <sup>e</sup> $\pm$ 2.51
100		22.51 <sup>c</sup> $\pm$ 0.87	0.80 <sup>b</sup> $\pm$ 0.39	348.07 <sup>f</sup> $\pm$ 65.64	4.77 <sup>a</sup> $\pm$ 2.07
150		29.68 <sup>f</sup> $\pm$ 3.57	1.30 <sup>c</sup> $\pm$ 0.06	412.95 <sup>ef</sup> $\pm$ 5.59	12.95 <sup>f</sup> $\pm$ 2.18
200		12.69 <sup>d</sup> $\pm$ 2.12	0.92 <sup>cb</sup> $\pm$ 0.12	280.40 <sup>h</sup> $\pm$ 32.87	6.65 <sup>g</sup> $\pm$ 0.74

\* Production was expressed in mg of each metabolite per liter of medium. Presented data are the means from three replicates  $\pm$  standard error (SE). Presented values followed by the same letter within each column are not statistically different at  $p \leq 0.05$  according to the Kruskal-Wallis test.

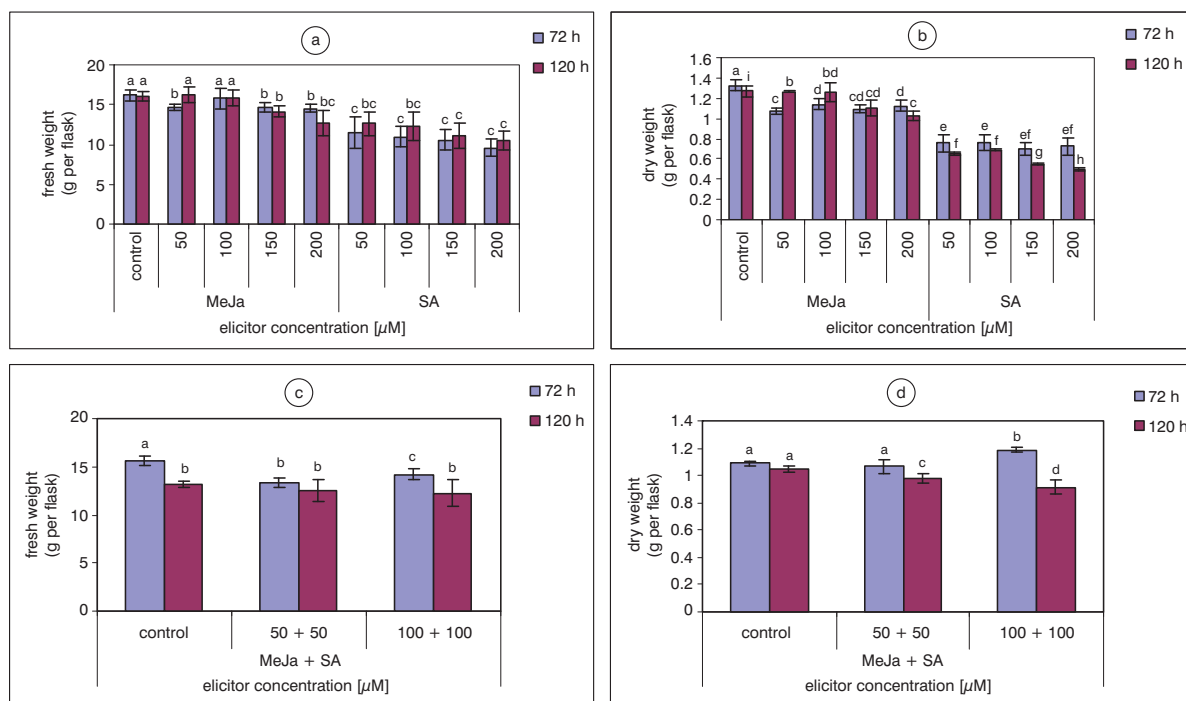
na (Kanokwaree and Doran, 1997) and *Nicotiana tabacum* (Wongsamuth and Doran, 1997). Beyond day 20, the rate of hairy root growth was reduced. A stationary phase was identified between days 30 and 50, in which biomass remained generally unchanged. However, this began to decrease after day 50, probably due to the depletion of nutrients in the culture medium. A similar pattern was shown by DW (Fig. 1). The highest values for biomass obtained in shake flasks were 16.04 g/flask (FW) and 1.30 g/flask (DW) at day 50 of culture.

#### EFFECT OF MEJA AND SA ON ROOT BIOMASS AND METABOLITE PRODUCTION

Figures 2a, b and 3 show the individual effects of MeJa and SA on biomass accumulation and level of secondary metabolites (catalpol, harpagide, verbascoside, isoverbascoside) in hairy root culture of *R. glutinosa*. MeJa significantly decreased fresh and dry weight of roots compared to the controls (untreated roots), especially at higher concentrations (150 and 200  $\mu\text{M}$ ). Maximum reduction in root biomass was observed at the highest MeJa concentration (200  $\mu\text{M}$ ) during the 120 h exposure to the elicitor. In the culture, fresh and dry weights

of roots decreased to 12.70 and 1.02 g/flask, respectively, which were 20% lower than those in the control roots (16.05 g FW/flask and 1.27 g DW/flask). At a shorter exposure time (72 hours) to 200  $\mu\text{M}$  MeJa, the fresh (14.49 g/flask) and dry weights (1.13 g/flask) of root biomass were only 10% and 15% lower than the control roots (16.15 g FW/flask and 1.33 g DW/flask) (Fig. 2a, b).

The application of SA (50–200  $\mu\text{M}$ ) also inhibited the growth of *R. glutinosa* hairy roots in respect to both fresh and dry biomass (Fig. 2a, b). In general, biomass accumulation in root cultures treated with SA was lower than that at the same MeJa concentrations. For example, the fresh root biomass (9.60 g/flask) after 72 h incubation with 200  $\mu\text{M}$  SA was 40.6% lower than that in the control (16.15 g/flask) and 33.8% lower compared to that of treatment with 200  $\mu\text{M}$  MeJa over the same time. In our study, SA (50–200  $\mu\text{M}$ ) also caused browning and necrosis of *R. glutinosa* root tissues, whereas there was no apparent change in the color and morphology of the roots treated with MeJa. It may be due to direct toxic effect of SA and loss of viability of the culture. Such an effect of SA was also observed in *Salvia miltiorrhiza* cell culture (Dong et al., 2010) and *Taxus baccata* cell culture (Rezaei et



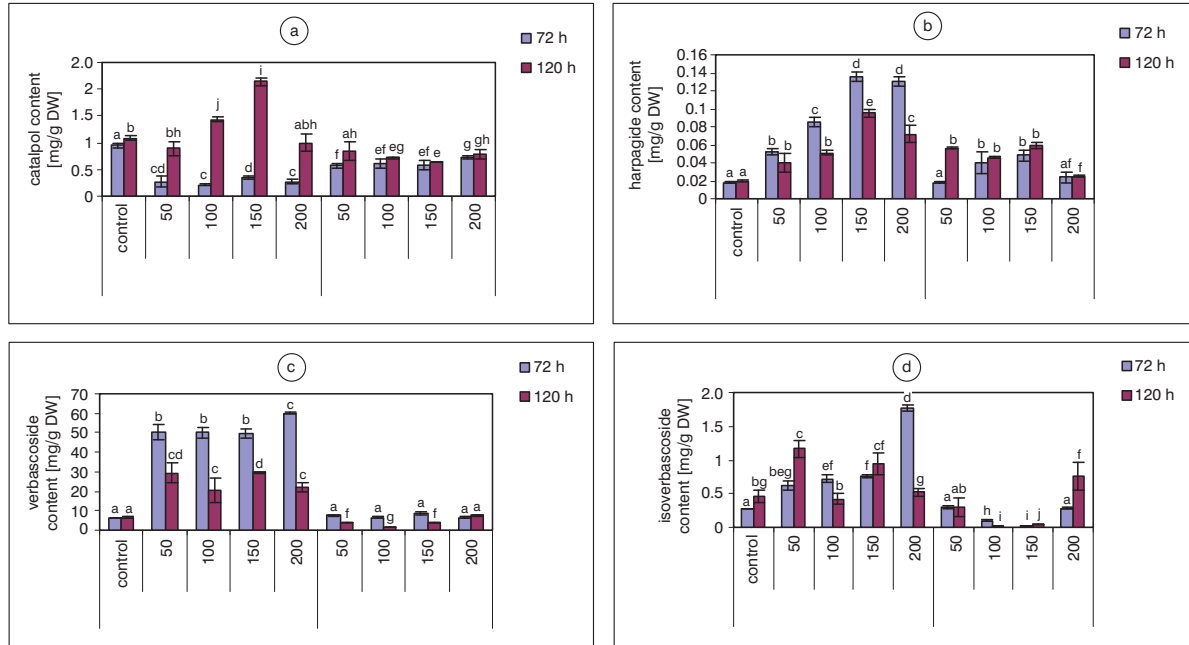
**Fig. 2.** Effect of different MeJa and SA concentrations alone (a, b) or combined (c, d) on accumulation of (a, c) fresh, (b, d) dry weights in g/flask in *R. glutinosa* hairy root cultures at two different times of exposure to elicitor (72 and 120 hours). The elicitor was added to 23-day-old culture (average FW was  $15.3 \pm 1.2$  g/flask and average DW was  $1.3 \pm 0.002$ ). Data are represented as means from three independent experiments (3 flasks per single experiment)  $\pm$  standard error (SE). Presented values followed by the same letter are not statistically different at  $p \leq 0.05$  according to the Kruskal-Wallis test.

al., 2011). In general, elicitation by chemical elicitors such as MeJa or SA has a negative effect on biomass accumulation leading to low overall productivity in *in vitro* cultures. Decrease in biomass accumulation in the presence of MeJa and SA has been earlier reported in *Panax ginseng* adventitious roots (Kim et al., 2004), *Scopolia parviflora* adventitious roots (Kang et al., 2004) and *Eleuterococcus koreanum* adventitious roots (Lee et al., 2015). Moreover, Lee et al. (2015) also reported that adventitious roots of *E. koreanum* treated with high MeJa concentration (400  $\mu$ M) showed better growth than at high SA concentration (400  $\mu$ M). To minimize the negative effect of elicitors on biomass accumulation in the present study, we added the elicitor (MeJa or SA) on the 23<sup>rd</sup> day of cultivation, when the root culture was found to be at late growth stage (Fig. 1). Earlier, a strategy of using the elicitor after the end of intensive culture growth or at the stationary phase was employed by Bonfill et al. (2011) for increased production of centelloside in *Centella asiatica* cell culture. Also, Cui et al. (2012) showed that addition of the elicitor at the late exponential growth phase could increase the valtrate content in *Valeriana amurensis* adventitious root culture without significantly affecting the biomass. According to James et al. (2008), when intensive growth had ended

secondary metabolites rather than primary ones, were synthesized.

Our study showed that metabolite profile in hairy roots of *R. glutinosa* elicited with MeJa or SA remained the same as in the unelicited roots. However, the quantity of the phytochemicals was affected by the elicitation procedure. We found that the content of each metabolite tested was considerably higher compared to the control (untreated roots), when the root culture was treated with MeJa (Fig. 3). The efficiency of the elicitor varies depending on its concentration (50–200  $\mu$ M) and exposure time (72 h or 120 h). Both parameters need to be optimized for each individual compound. This conclusion agrees with these reached for several other metabolites and *in vitro* culture systems (Namdeo, 2007; Sivanandhan et al., 2013). Among concentrations tested, 150  $\mu$ M MeJa was the best for biosynthesis of iridoids (catalpol, harpagide) (Fig. 3a, b), whereas for maximum improvement of phenylethanoid (verbascoside and isoverbascoside) content 200  $\mu$ M MeJa was required (Fig. 3c, d).

The maximum level of catalpol (2.15 mg·g<sup>-1</sup> DW) was achieved in the roots treated with 150  $\mu$ M MeJa for 120 h. The amount was 2 times higher compared to the control. Treatment of roots with the same concentration of MeJa (150  $\mu$ M) but for



**Fig. 3.** Effect of different MeJa and SA concentrations on accumulation of catalpol (a), harpagide (b), verbascoside (c) and isoverbascoside (d) in *R. glutinosa* hairy root culture. The elicitor was added to 23-day-old culture and roots were harvested after 72 and 120 hours. Data are represented as means from three replicates  $\pm$  standard error (SE). Presented values followed by the same letter are not statistically different at  $p \leq 0.05$  according to the Kruskal-Wallis test.

72 h reduced the catalpol content below the control (Fig. 3a). 150  $\mu\text{M}$  MeJa and 120 h exposure time were also found to be favorable for production of catalposide (6-O-*p*-hydroxybenzoyl catalpol), whose carbon skeleton is closely related to catalpol. Its amount increases from traces (in control roots) to 0.04, 0.07 and 0.093  $\text{mg}\cdot\text{g}^{-1}$  DW at the concentration of 50, 100 and 150  $\mu\text{M}$  MeJa, respectively. The greatest level of harpagide was detected in the roots treated with 150  $\mu\text{M}$  MeJa within short treatment duration (72 h). The roots produced 0.136  $\text{mg}\cdot\text{g}^{-1}$  DW of harpagide, i.e. 7.5-fold more than in the control culture without the elicitor (Fig. 3b). The amount of the iridoid declined by about 30% when the exposure time to the elicitor was prolonged to 120 h. It might be a result of either secondary metabolite degradation *in situ* or its leakage into the medium. Kang et al. (2004) reported release of tropane alkaloids from roots of *Scopolia parviflora* into the culture medium as a result of treatment with MeJa.

Treatment with the highest concentration of MeJa (200  $\mu\text{M}$ ) in a short period (72 h) was needed to maximize accumulation of phenylethanoid glycosides in hairy root culture of *R. glutinosa*. Under these conditions verbascoside and isoverbascoside levels of 60.07  $\text{mg}\cdot\text{g}^{-1}$  DW and 1.77  $\text{mg}\cdot\text{g}^{-1}$  DW were obtained, which were, respectively, 10 and 6.4 times higher than those in the control.

Despite the fact that MeJa fed to the hairy root culture suppressed the biomass, increment in secondary metabolite production (calculated as mg per liter of medium) in the root culture treated with MeJa was observed; at optimal conditions of elicitation production of verbascoside, isoverbascoside, catalpol and harpagide in hairy roots of *R. glutinosa* increased up to 8.4-, 5.4-, 1.7- and 6.4-fold, respectively, as compared to the control (Table 1).

Our results clearly suggested that phenylethanoids, especially verbascoside were more responsive to MeJa treatment than iridoids. It is known that verbascoside production is often enhanced by both biotic and abiotic stress conditions, e.g., elicitation (Chen et al., 2007; Marsh et al., 2014). Matsumoto et al. (1987) suggested that verbascoside and its derivatives play an important role in the defense mechanism of plants and may act as a resistant component or a protector against attacks by fungi or viruses. No reports have documented catalpol-type or harpagide-type iridoid production in *in vitro* cultures by employing a chemical elicitor. The compounds which belong to terpenoid group use different metabolic pathways than those used by phenylethanoid glycosides, as verbascoside and isoverbascoside. Therefore, the effect of MeJa on both groups of compounds may be different. Hayashi et al. (2003) showed that stimulated effects of MeJa on production of various secondary metabolites are at least partially due to activation

of key enzymes, which catalyze their biosynthesis. The time required for reaching maximal levels of the enzyme activity is different for each of biosynthetic pathways. Therefore, the amounts of metabolites varied with duration of incubation period with the elicitor.

Fig. 3 also shows the effects of different concentrations of SA on accumulation of iridoids and phenylethanoids in hairy root culture of *R. glutinosa*. It was found that SA elicitation was less effective than MeJa elicitation in producing secondary metabolites. When compared to the control culture, addition of SA led to significant decrease of catalpol content at all the concentrations tested and both used times of exposure (72 and 120 h). Treatment with different concentrations of SA increased harpagide content in almost all root cultures as compared with the untreated roots but the increase was not as high as maximum level obtained by MeJa elicitation (Fig. 3b). A similar trend was observed in respect to phenylethanoid biosynthesis. At optimal conditions of SA elicitation, i.e. 150  $\mu\text{M}$  of the elicitor concentration and 72h exposure time verbascoside level (8.45  $\text{mg}\cdot\text{g}^{-1}$  DW) was only 1.4-fold higher in comparison to the control (6.04  $\text{mg}\cdot\text{g}^{-1}$  DW) and up to 5.8 times lower than that in the roots treated with the same concentration of MeJa at the same exposure time. Likewise, Gueven and Knorr (2011) reported that MeJa was more effective than SA for production of isoflavones in soybean cell suspension culture.

The inability of SA to increase the content of metabolites in hairy root culture of *R. glutinosa* to the magnitude of that caused by MeJa could be attributed to the difference in the modes of action of the two kinds of signaling transducers. While both compounds regulate pathogen-induced plant defense responses, the concentration of MeJa usually increases with insect and/or animal invasions and wounding responses. SA has been shown to be essential for systemic acquired resistance to microbial pathogens (Dong, 1998). However, it is not a universal inducer of phytoalexins (Zhao et al., 2005).

#### THE EFFECT OF MEJA AND SA COMBINATION ON ROOT BIOMASS AND METABOLITE CONTENT

As elicitors often display a synergistic effect on the production of secondary metabolites when applied in combination (Sahu et al., 2013), the present study also investigates the effects of combined MeJa and SA application on biomass accumulation and the content of iridoids and phenylethanoids in *R. glutinosa* hairy root cultures (Figs. 2c, d and 4). Two combinations of elicitors (i.e. 50  $\mu\text{M}$  SA + 50  $\mu\text{M}$  MeJa and 100  $\mu\text{M}$  SA + 100  $\mu\text{M}$  MeJa) were administered using the same procedure for MeJa

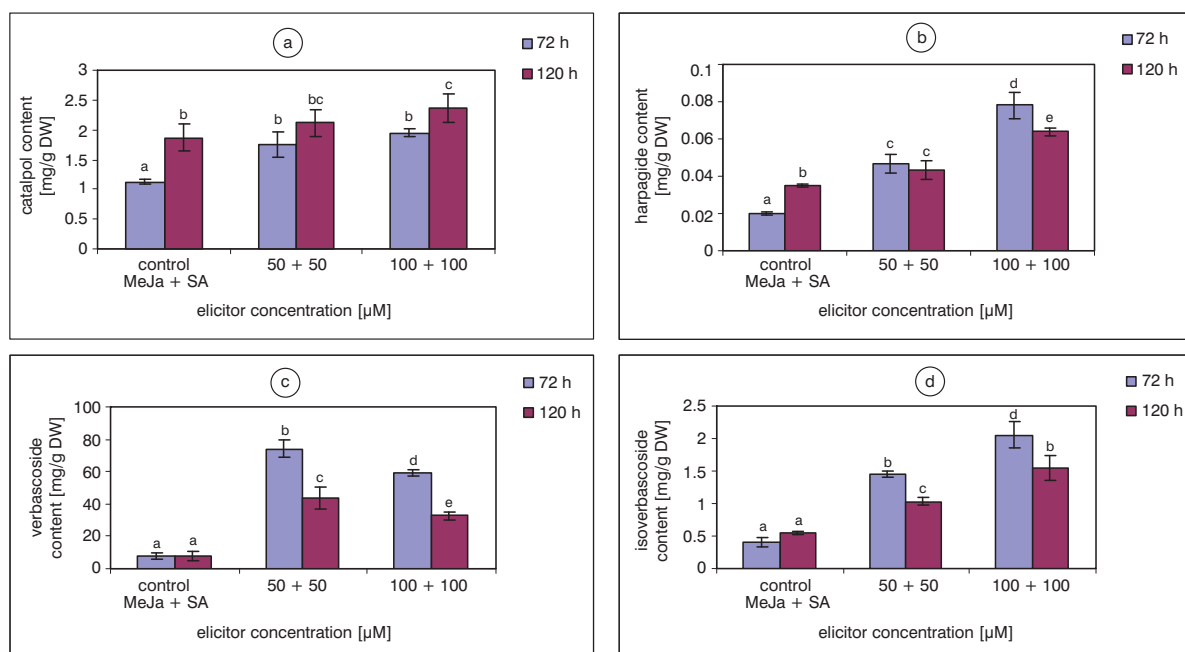
or SA alone, i.e. elicitors were added to a 23-day-old culture and the roots were harvested after 72 and 120 hours. Both elicitor combinations slightly decreased root biomass accumulation, expect for the results of dry weight at the combination of 100  $\mu\text{M}$  SA + 100  $\mu\text{M}$  MeJa and 72-hour period of exposure (Fig. 2c, d). In addition, combined elicitation of MeJa and SA resulted in elevated catalpol, harpagide, verbascoside and isoverbascoside content, compared to the control culture, but these values were not as high as the maximum levels obtained following elicitation by MeJa alone.

Likewise, elicitation of metabolite production by single elicitors was found to be dependent on the elicitor concentration and contact period. The highest verbascoside content (9.7 times higher than in the control) was detected in the roots elicited by 72-hour treatment with 50  $\mu\text{M}$  SA + 50  $\mu\text{M}$  MeJa (Fig. 4c). However, the greatest level of isoverbascoside and harpagide was observed after treatment with a combination of higher levels of SA and MeJa (100  $\mu\text{M}$  SA + 100  $\mu\text{M}$  MeJa) for the same exposure time: production being almost 4–5 times higher than observed in the untreated roots. The highest quantities of catalpol (1.7 times more than in the control) were observed in the roots treated with the same combination of elicitors for 120 hours. In

addition, the combination of MeJa and SA stimulated catalpol accumulation at an earlier stage of elicitation (72 hours) than MeJa alone, where increased production was observed only after a longer period of exposure (120 hours).

## CONCLUSIONS

Our results showed that iridoid (catalpol, harpagide, catalposide) and phenylethanoid (verbascoside, isoverbascoside) glycoside content was substantially enhanced when MeJa at optimal concentration (150 and 200  $\mu\text{M}$ ) was added to the 23-day-old hairy root culture of *R. glutinosa*. Among the compounds verbascoside was accumulated at the highest amount (60.07  $\text{mg}\cdot\text{g}^{-1}$  DW; up to 10 times more than in the control). The concentration of secondary metabolites was also increased in response to MeJa combined with SA. However, SA alone showed no substantial increase in their levels. The results suggest that MeJa and SA have different models of action on transformed root culture of *R. glutinosa*. This is the first report on the use of elicitation strategy on enhancement in bioactive metabolite production in hairy root culture of *R. glutinosa*.



**Fig. 4.** Effect of combined MeJa and SA treatment on accumulation of catalpol (a), harpagid (b), verbascoside (c) and isoverbascoside (d) in *R. glutinosa* hairy roots cultures at two different times of exposure to elicitor. The elicitors were added to 23-day-old culture and roots were harvested after 72 and 120 hours. Presented data are the means of three replicates  $\pm$  standard error (SE). Values followed by the same letter are not statistically different at  $p \leq 0.05$  according to the Kruskal-Wallis test.

## AUTHORS' CONTRIBUTIONS

EP designed and carried out all the elicitation experiments, prepared the plant extracts, analyzed the data and wrote the manuscript, ŁK was responsible for UHPLC analyses of the plant material, HW was responsible for verification of the paper. All Authors declare that they have no conflicts of interest.

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