Phytochemistry 107 (2014) 50–60

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Effect of light, methyl jasmonate and cyclodextrin on production of phenolic compounds in hairy root cultures of *Scutellaria lateriflora*

Zachary Marsh [a,b], Tianhong Yang [a,b], Luis Nopo-Olazabal [a,b], Shuchi Wu [a], Taylor Ingle [a,1], Nirmal Joshee [c], Fabricio Medina-Bolivar [a,b,*]

[a] Arkansas Biosciences Institute, Arkansas State University, State University, AR 72467, USA
[b] Department of Biological Sciences, Arkansas State University, State University, AR 72467, USA
[c] Agricultural Research Station, Fort Valley State University, Fort Valley, GA 31030, USA

ARTICLE INFO

Article history:
Received 12 December 2013
Received in revised form 17 May 2014
Available online 15 September 2014

Keywords:
Scutellaria
Lamiaceae
Hairy roots
Verbascoside
Wogonin
Baicalein
Scutellarein
Phenolics
Methyl jasmonate
Cyclodextrin
Elicitation

ABSTRACT

Scutellaria lateriflora (American skullcap) has been used in traditional medicine to treat several medical conditions including nervous disorders and cancer. Previous studies have associated these medicinal properties to flavones present in roots and leaves of this species. In order to develop a production system and study the biosynthesis of these bioactive compounds, hairy root cultures of *S. lateriflora* were established and line 4 was selected for further studies based on its growth performance in a modified Murashige and Skoog's medium supplemented with 0.5 mg/l indole-3-butyric acid. Scanning electron microscopy of the hairy roots showed a high profusion of hairs along the root. Several phenolic compounds, including verbascoside, and the flavones wogonin, baicalein, scutellarein and their respective glucuronides were identified by high performance liquid chromatography–tandem mass spectrometry in the root tissue, but not in the culture medium. Among these compounds, verbascoside accumulated at the highest levels. Interestingly, cultures incubated under continuous light and treated with 15 mM methyl-β-cyclodextrin for 24 h produced significantly higher levels of the aglycones, baicalein and wogonin, but not scutellarein, compared to cultures incubated under continuous darkness. This work demonstrates that hairy root cultures of *S. lateriflora* have the biosynthetic capacity to produce known *Scutellaria* flavones and suggest that light may have a selected regulatory effect on the synthesis or accumulation of these phenolic compounds.

1. Introduction

Scutellaria, a member of the mint family, is an important perennial herb genus with approximately 360–400 species (Paton, 1990). This genus, commonly known as skullcap/scullcap, has been extensively studied due to its health benefits including, but not limited to, anti-allergic, anti-bacterial, anti-HIV, anti-hepatitis, antioxidant and anti-tumor activities (Patel et al., 2013b; Tiwari et al., 2008). Two of the most widely studied species of Scutellaria are *Scutellaria baicalensis* (Chinese skullcap) and *Scutellaria lateriflora* (American skullcap) due to their aforementioned medicinal properties (Zhang et al., 2009). *S. lateriflora* is endemic to the swampy woods and meadows of North America ranging from Canada to Florida and as far west as Oregon and British Columbia (Bergeron et al., 2005). This herb has been used as a nerve tonic, sedative, and anticonvulsant for centuries by Europeans and Native Americans (Zhang et al., 2009). Awad et al. (2003) have shown that aqueous crude extracts of *S. lateriflora* have anxiolytic properties with no negative side-effects which are thought to occur through either flavonoid or amino acid activity. The bioactivity of the different compounds present in these *S. lateriflora* extracts have not been studied in-depth as in *S. baicalensis*, yet the potential health benefits associated with the American skullcap encourage further investigation to elucidate their activity *in vitro* and *in vivo*.

Due to an increased demand for plants with anxiolytic activity, *S. lateriflora* is a prime candidate since its extracts contain numerous phenolic compounds of potential therapeutic effect

Abbreviations: β-CD, methyl-β-cyclodextrin; IBA, indole-3-butyric acid; MeJA, methyl jasmonate; MS, Murashige and Skoog's medium; mMS, modified MS medium; UHPLC, ultra high performance liquid chromatography.

* Corresponding author at: Arkansas Biosciences Institute, Arkansas State University, P.O. Box 639, State University, AR 72467, USA. Tel.: +1 870 680 4319; fax: +1 870 680 4348.
E-mail address: fmedinabolivar@astate.edu (F. Medina-Bolivar).
[1] Current address: National Center for Toxicological Research, Food and Drug Administration, Jefferson, AR 72079, USA.

http://dx.doi.org/10.1016/j.phytochem.2014.08.020
0031-9422/

(Awad et al., 2003). Indeed, these positive effects of consumption of *S. lateriflora* extracts on mood have been recently confirmed in a clinical study conducted in the United Kingdom (Brock et al., 2013). Of the well-known flavones, there are three compounds of elevated interest present in *S. lateriflora*, i.e., wogonin (**7**), baicalein (**6**) and its glucuronide, baicalin (**3**) (Fig. 1). An *in vitro* study has shown that all these flavones act on different brain receptors; wogonin (**7**) demonstrating potential to be used as a compound to treat anxiolytic conditions (Bergeron et al., 2005). Additionally, the biological activities of these three polyphenols are not only limited to their anxiolytic effects. Other researchers and our studies have demonstrated anti-cancer properties of *Scutellaria* flavonoids (Dandawate et al., 2012; Parajuli et al., 2009, 2011; Patel et al., 2013a). Gao et al. (2008) has attributed anti-inflammatory, anti-tumor, antioxidant and radical scavenging pharmacological properties to baicalin (**3**), baicalein (**6**) and wogonin (**7**). These promising results only further necessitate the development of efficient methods for obtaining large quantities of these polyphenols and other potential bioactive compounds from this species for potential therapeutic use and future studies.

It has been stated that over-exploitation of the natural plant population has led to an increased need to develop sustainable systems for natural products or specialized metabolites (Tiwari et al., 2008). *Agrobacterium rhizogenes*-induced hairy root cultures have received increasing attention as a bioproduction platform for diverse classes of specialized metabolites, because these cultures exhibit growth rates comparable to cell suspension cultures (Georgiev et al., 2007; Giri and Narasu, 2000). Further, in many cases, hairy root cultures can reproduce the biosynthetic capacity of the entire plant (Ono and Tian, 2011). *S. baicalensis* hairy roots

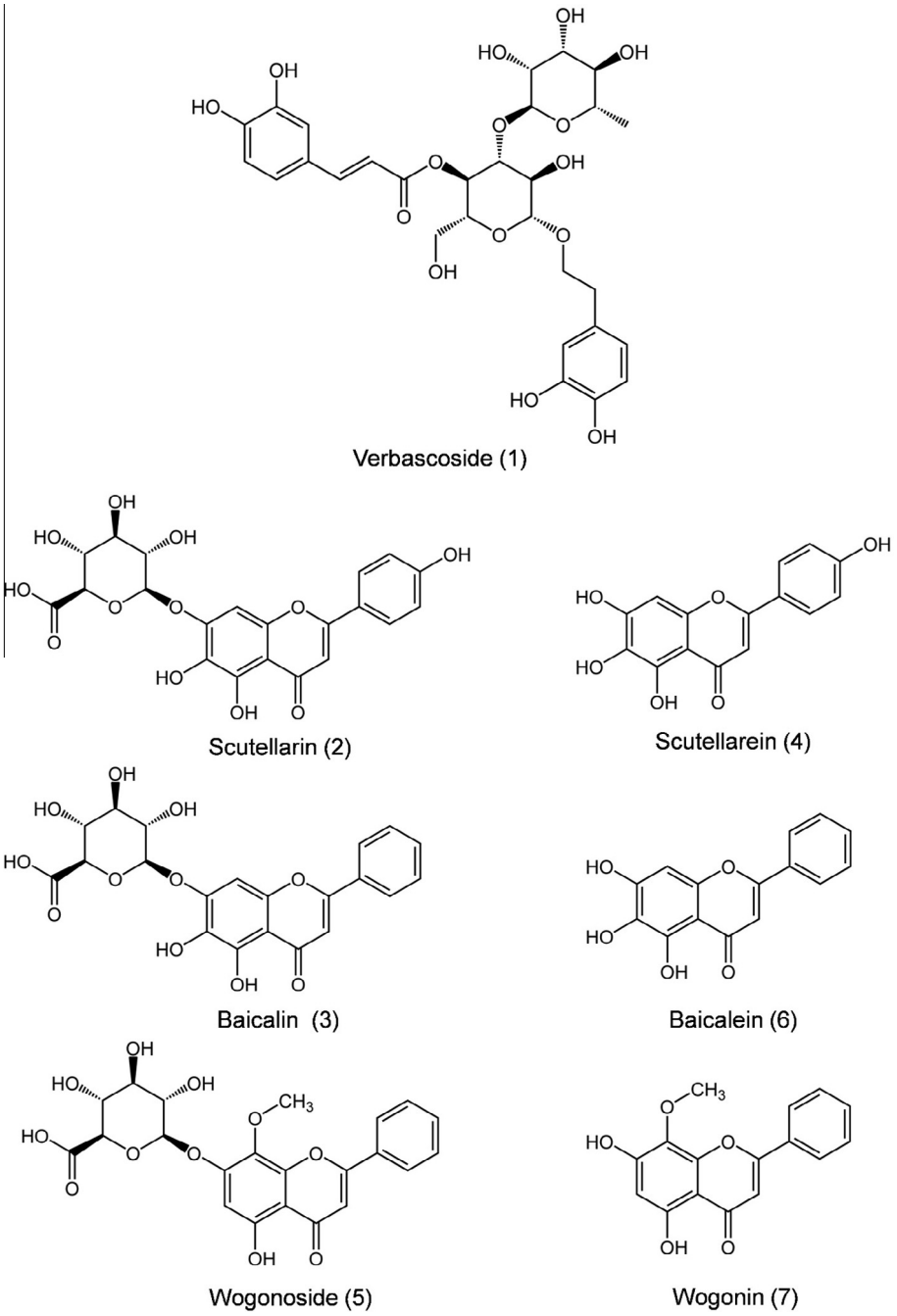

Fig. 1. Chemical structures of selected phenolic compounds identified in *Scutellaria lateriflora* hairy roots.

have been widely explored to study factors affecting production of certain bioactive flavones (Hirotani et al., 2000; Park et al., 2011); however, only limited studies have been conducted with *S. lateriflora* hairy roots. Recently, they were developed to study the effect of biotic elicitors on production of flavones (Wilczańska-Barska et al., 2012). Elicitors have been used to provoke an immune response in hairy root cultures in hopes of enhancing the levels of bioactive molecules (for a recent review see Wang and Wu, 2013). Yeast extract and bacterial suspensions were shown to increase the yields of wogonin (**7**) in *S. lateriflora* hairy roots (Wilczańska-Barska et al., 2012). In order to develop a sustainable and axenic production system of *S. lateriflora* bioactive metabolites, the effect of methyl jasmonate (MeJA) and cyclodextrin is reported. MeJA, a derivative of jasmonic acid, accumulates in high levels in abiotically or biotically damaged tissues of the plant and activates several defense biosynthetic pathways (Wasternack, 2007). Another recently studied compound is the cyclic oligosaccharide cyclodextrin, with methyl-β-cyclodextrin (β-CD), being the most commonly used form (Bru et al. (2006)). Lijavetzky et al. (2008) have shown that when MeJA and β-CD are used together in cell suspensions of grape, the two compounds operate synergistically yielding far greater levels of resveratrol, a highly researched health-beneficial stilbene which develops from the same phenylpropanoid pathway as the flavonoids. Furthermore, it was also demonstrated that this combined treatment was effective in the induction of stilbenoid levels in hairy roots of peanut (Medina-Bolivar and Yang, 2013). Therefore, this elicitation approach merits further investigation with hairy roots of other species, such as *S. lateriflora*, to produce a different class of phenolic compounds.

Chalcone synthase (CHS) catalyzes a key rate-limiting step in flavonoid biosynthesis (Dao et al., 2011). Previous studies have shown that CHS expression could be up-regulated by blue and UVB light (Kubasek et al., 1992). Consequently, the effect of light versus dark culture conditions on production of flavonoids was studied in *S. lateriflora* hairy roots. Herein, the effects of light and treatment with MeJA and β-CD on production of known *S. lateriflora* polyphenolics are presented. Furthermore, the advantages of the hairy root culture system are discussed to study the regulatory mechanisms affecting the biosynthesis and accumulation of these natural products.

2. Results and discussion

2.1. Establishment and molecular characterization of hairy root cultures

In order to establish hairy roots of *S. lateriflora*, internode sections were harvested from plantlets (Fig. 2A) and inoculated with *A. rhizogenes*. About 25% of internode sections developed roots after 2–3 weeks of inoculation. Between 1 and 5 hairy roots developed from each inoculated internode (Fig. 2B); however, only a few roots that started from the internode sections sustained growth in semi-solid medium, which contained cefotaxime to eliminate bacteria. After several subcultures, three lines were transferred to liquid medium. In the initial experiments, slow growth was observed in liquid medium; therefore, auxin supplementation was included to increase root biomass. Hairy roots grown in medium with 0.5 mg/l indole-3-butyric acid (IBA) showed increased biomass and, among the 3 lines, hairy root line 4 was selected for further studies based on its vigorous growth in this medium. In order to avoid clumping of the very thin *S. lateriflora* hairy roots in liquid medium, it was very important to disperse the root inoculum after its introduction into the culture medium.

Agropine type *A. rhizogenes* strains, such as ATCC 15834 used in this study, contain two transfer DNAs (T-DNAs) which are important for the establishment of hairy root cultures (reviewed by Sevon and Oksman-Caldentey, 2002). The T_L-DNA contains the *rol* genes which are essential for hairy root initiation, whereas the T_R-DNA contains the *aux* genes necessary for auxin biosynthesis (Camilleri and Jouanin, 1991; Schmulling et al., 1988; Slightom et al., 1986). Previous studies suggest that integration in the plant genome and expression of *rol* and *aux* genes are necessary for induction and establishment of hairy root cultures with the capacity to sustain growth in auxin-free medium. To address this issue, polymerase chain reaction (PCR) studies were conducted with genomic DNA from hairy root line 4. As shown in Fig. S1, positive PCR results were obtained for the *rolC* and *aux1* genes indicating that both left and right T-DNAs were integrated in the genome of these *S. lateriflora* hairy roots. Furthermore, the negative PCR result for the *virD2* gene (Fig. S1) confirmed that no *Agrobacteria* remained in the tissue. Interestingly, these results indicate that supplementation with auxin IBA was necessary for vigorous growth of hairy root cultures in the liquid medium in spite of the presence of the *rol* and *aux* genes. Possible explanations could be due to the positional genome integration effect of the T-DNA or an alternative functionality of either the *rol* and *aux* genes in *S. lateriflora*. Previous studies with *A. rhizogenes* in *S. baicalensis* (Kuzovkina et al., 2001) and the recent study with *S. lateriflora* by Wilczańska-Barska et al. (2012) have not addressed this issue.

After molecular characterization of hairy root line 4, a growth curve of 60 days (Fig. 3A and C) was performed with this line in order to identify the stages of development along the culture period. Three phases of growth were identified. The lag phase was identified between 0 and 4 days (Fig. 3B) and it was followed by a long exponential growth between days 4 and 50. As expected, an inverse correlation between the root biomass and conductivity of the medium was observed along the growth phases (Fig. 3B).

2.2. Light and scanning electron microscopy of hairy root morphology

The hairy roots induced on the internodal explants were white, slender, and branched (Fig. 2B and C). The growing tips of these roots became conspicuous by their white color. Scanning electron microscopy further established the detailed structure of hairy roots at various magnifications (Fig. 4A–C). It is clear that they exhibited frequent branching (Fig. 4A) and the distribution of single cell root hairs varied considerably from one section to another. Some of the hairs appeared as long unicellular tubes, while others were like short papillae. These could be two different stages in the growth of the root hair or structurally two different types of root hairs. Morphologically it is apparent that root hairs that populate the root surface are single cell extensions thereby potentially increasing biosynthetic surface area (Fig. 4C). The high degree of branching and the abundant root hairs are typical characteristics of *A. rhizogenes*-derived transformed roots reported in many genera (Flores and Medina-Bolivar, 1995).

2.3. Effect of light and dark conditions on hairy root culture growth

The conductivity and pH of each of the cultures used for the elicitation studies were checked to ensure that all cultures were within the same growth stage at the time of elicitation (Fig. S2). In previous studies, these two parameters were used to confirm the growth stage of hairy root cultures of peanut (Condori et al., 2010) and muscadine grape (Nopo-Olazabal et al., 2014). Having a uniform stage is important because the elicitation response may vary depending on developmental stage of the hairy root culture (Medina-Bolivar et al., 2007). To this end, hairy root cultures of *S. lateriflora* line 4 were cultured under either continuous light or darkness for 30 days. On this time point, the culture medium (spent medium) was removed, the conductivity and pH measured

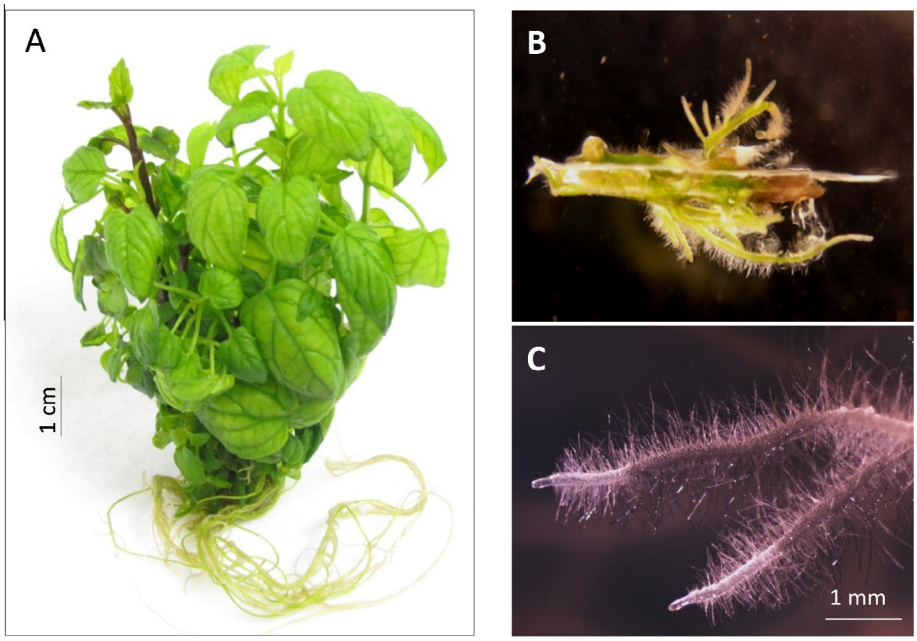

Fig. 2. (A) *Scutellaria lateriflora* plant grown *in vitro* for 4 months. (B) Stem explant showing development of hairy roots after inoculation with *Agrobacterium rhizogenes*. (C) Close-up of hairy roots showing a profusion of root hairs.

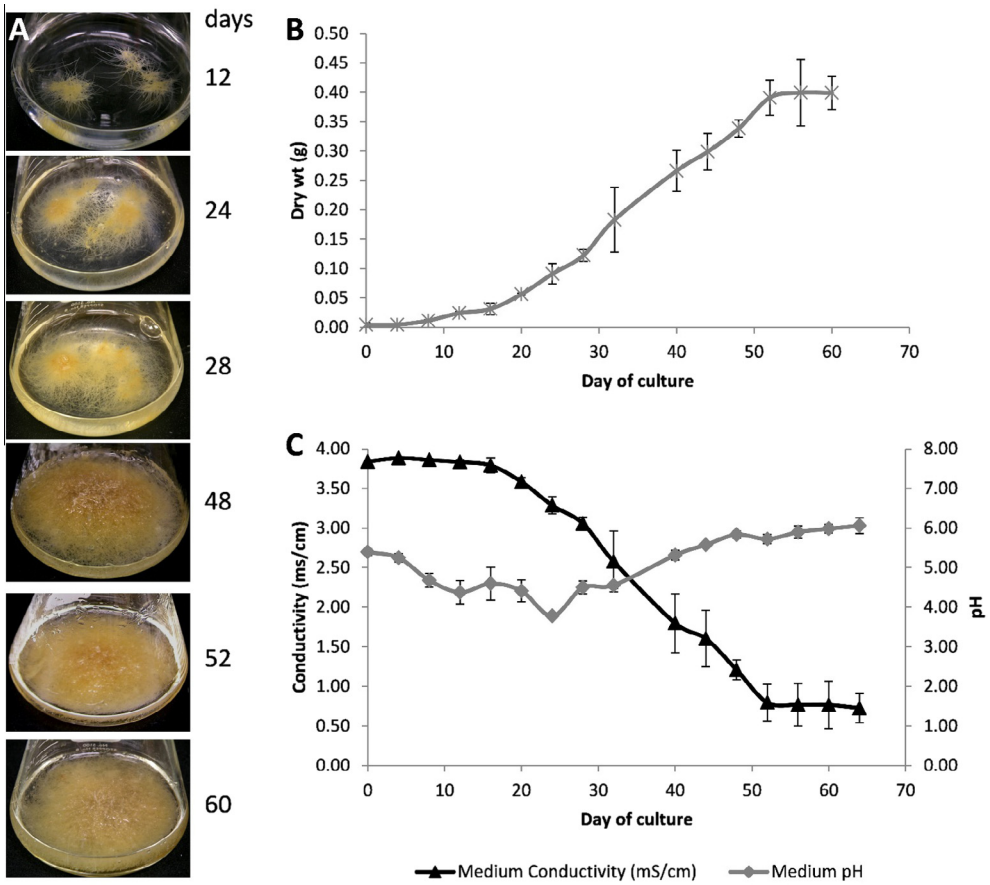

Fig. 3. Growth curve analysis of hairy roots of *S. lateriflora* line 4 grown under continuous darkness. Hairy roots were grown in modified MS medium (MSV) supplemented with 0.5 mg/l IBA. (A) Phenotype of hairy roots at different stages of growth. (B) Biomass (dry wt) of the root tissue at different stages of the culture period. (C) Conductivity and pH values of the medium during the culture period. Each value represents the average of three biological replicates ± standard deviation.

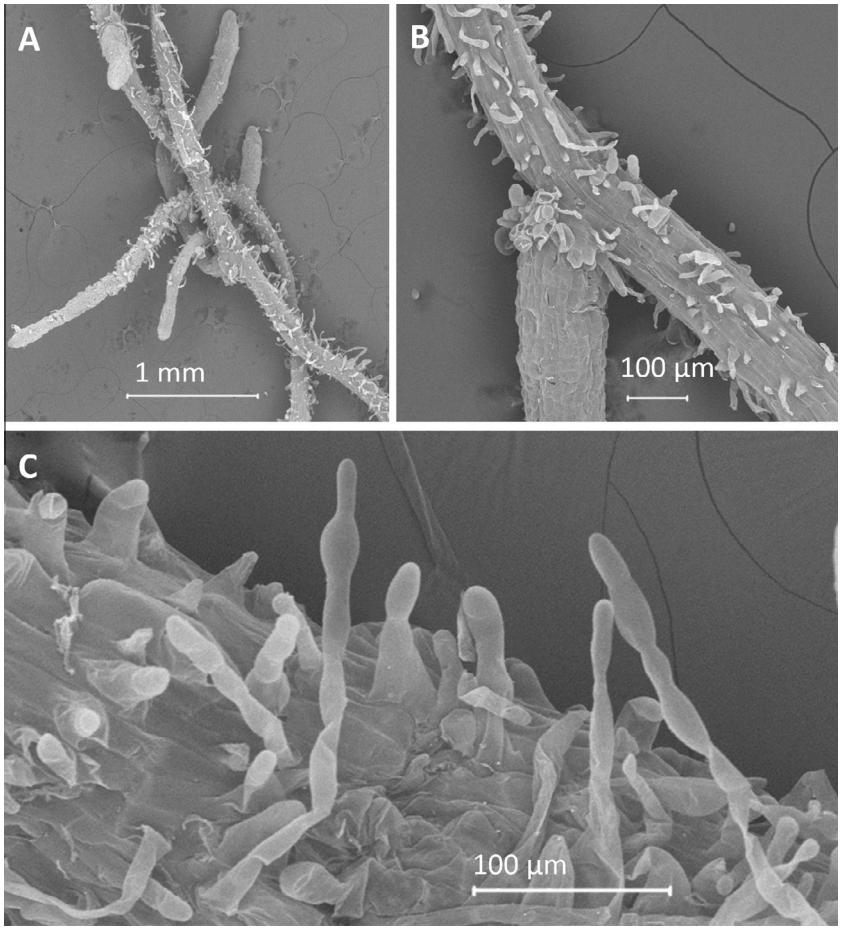

Fig. 4. Scanning electron microscopy analysis of hairy roots of *S. lateriflo*a line 4. Observations under different magnification (A, B, and C) show an abundance of root hairs.

and cultures were subjected to fresh medium containing the elicitors for 24 h.

The conductivity values of the spent medium from all cultures grown under dark were similar. In the same way, all conductivity values were similar among cultures grown in the light. These parameters confirmed that the cultures were at approximately the same developmental stage before the elicitation treatment. Overall, the conductivity of the spent medium was higher in cultures grown in the dark than in the light (Fig. S2A). This observation seemed to correlate with the fact that the dark grown cultures had a lower pH consistently (Fig. S2B). Conductivity parameters of the medium are inversely correlated with the dry mass of roots; therefore, parameters suggested that cultures grown under light might have a slightly higher biomass at the 30th day of growth. It is worth noting that there were no consistent differences between the light and dark grown cultures with regard to root quality, density, and color. Occasionally, some hairy roots grown in the light exhibited green coloration, a potential indication of photosynthetic capacity. Figure S3 shows the growth index of the roots grown in the light and the dark. The growth index was calculated by dividing the final dry weight (DW) of the hairy root tissue by the initial root inoculum weight (which averaged 6 mg DW). The average growth index among all cultures was 65, and no apparent differences in growth index were observed among the cultures grown in the dark versus light, with the exception of the culture treated with 15 mM β-CD (Fig. S3). As mentioned above, cultures grown under light had a lower conductivity and the expectation was that these had a higher biomass. However, this was only noticed for the 15 mM β-CD treatment. Recent experiments conducted by our group using the same combination of elicitors with peanut hairy roots have shown that longer elicitation periods than the 24 h used in this study, could have more impact on root growth (Medina-Bolivar, unpublished). Gharechahi et al. (2013) have shown that hairy root cultures of *Silybum marianum* treated with MeJA for 96 h exhibited significantly lower biomass than non-treated hairy root cultures, indicating the diversion of energy resources to defense compounds rather than growth upon MeJA treatment.

2.4. Yield of phenolic compounds in the hairy root tissue

The production of seven polyphenolic compounds was studied in hairy root cultures of *S. lateriflora* line 4. These include three flavones, i.e., scutellarein (**4**), baicalein (**6**) and wogonin (**7**), and their respective glucuronides, scutellarin (**2**), baicalin (**3**) and wogonoside (**5**) (Fig. 1). In addition, the phenolic compound verbascoside (**1**) (also referred as acteoside) was included in the study. After HPLC (Fig. 5), the identity of these phenolics was confirmed by comparing the UV spectra of the separated compounds to authentic standards. As expected, similar UV spectra were observed for the flavone aglycones and their respective glucuronides (Fig. S4). For instance, the UV spectra of scutellarein (**4**) and scutellarin (**2**) show two UV absorption maxima, one at 282 nm and a second one at 333–335 nm (Fig. S4). A second confirmation was done by tandem mass spectrometry. The MS fragmentation pattern of the seven compounds matched the one observed for the reference standards (Figs. S5–S7). As far as we know, this is the first report of the MS fragmentation pattern for verbascoside (**1**) in the positive ion mode (Fig. S7). Recently, verbascoside (**1**) was detected in ash

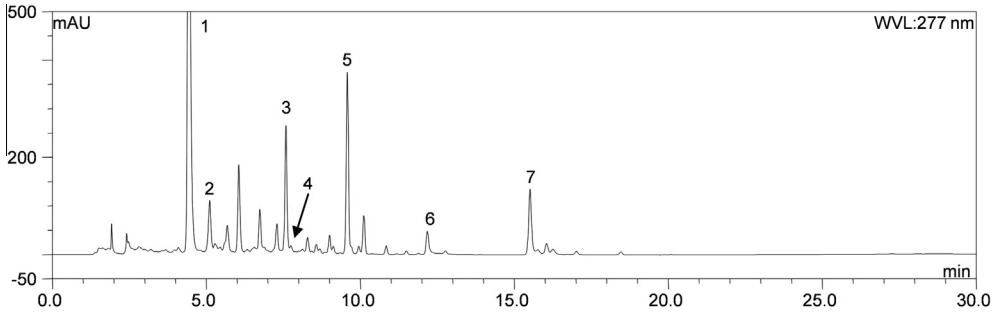

Fig. 5. HPLC chromatogram (UV 277 nm) of methanol extract from the tissue of hairy root cultures of *Scutellaria lateriflora* incubated under continuous darkness. **1**, Verbascoside; **2**, scutellarin; **3**, baicalin; **4**, scutellarein; **5**, wogonoside; **6**, baicalein; **7**, wogonin.

heartwood and its MS fragmentation pattern in the negative ion mode was provided (Sanz et al., 2012).

Different studies have shown the presence of flavones in different species of *Scutellaria*. Recently, Wilczańska-Barska et al. (2012) described the production of scutellarin (**4**), baicalin (**3**), wogonin (**7**) and wogonoside (**5**) in hairy roots of *S. lateriflora*, whereas *S. baicalensis* hairy roots have been shown to accumulate baicalin (**3**), baicalein (**6**), wogonin (**7**), and wogonoside (**5**) (Kuzovkina et al., 2005). Herein, it was found that *S. lateriflora* has the capacity to synthesize/accumulate at least six flavones, i.e., scutellarein (**4**), scutellarin (**2**), baicalein (**6**), baicalin (**3**), wogonin (**7**), and wogonoside (**5**). These flavonoids exhibit biological activities, and an increasing number of *in vitro* and *in vivo* studies have proven anti-cancer properties for these compounds (Dandawate et al., 2012; Kim et al., 2013). Nonetheless, the biosynthetic pathways of these compounds have not been elucidated in *Scutellaria*. Hairy root cultures of *S. lateriflora* could therefore provide an important biological system for studying the regulatory mechanisms affecting the biosynthesis and accumulation of these six valuable compounds.

Verbascoside (**1**) or acteoside is a phenylethanoid and caffeic acid sugar ester. It has exhibited important bioactivities including anti-inflammatory and antiproliferative properties in cancer cell lines (Lee et al., 2007). Among the seven phenolic compounds studied in the tissue extracts, verbascoside (**1**) was the most abundant.

In order to study the effect of elicitors on the production of these phenolic compounds, the hairy root cultures were treated with MeJA alone or in combination with β-CD. Light versus dark incubation was also studied as a potential way to affect the accumulation of these compounds. In all treatments, the above seven compounds were found in the tissue extracts (Figs. S8–S11) but not detected in the medium of the hairy root cultures. For comparative studies and to better understand the molar contribution of these compounds, all yields of the compounds are expressed in nmol/g dry wt of root. Yields of these compounds in mg/g dry wt are also reported in the Supplementary Tables S1 and S2.

2.4.1. Verbascoside (*1*)

The treatments with β-CD alone or MeJA combined with β-CD did not significantly affect the levels of verbascoside (**1**) when compared to the control cultures under either continuous light or dark conditions. However, a statistically significant increase was observed in the dark grown cultures treated with 15 mM β-CD when compared to the light grown cultures (Fig. 6). Overall, the levels of verbascoside (**1**) varied between 12,899 ± 4849 and 26,356 ± 4984 nmol/g dry wt (0.95–1.65% DW) depending on the growing conditions. Wilczańska-Barska et al. (2012) reported levels of 18.5 mg/g DW of acteoside (herein referred as verbascoside (**1**)) in hairy root cultures of *S. lateriflora* grown under darkness. However, the age of the culture was not detailed in this previous work. In the study herein, the levels of verbascoside (**1**) were approximately between 10 and 14 mg/g DW root after 30 days of culture conditions (Table S2). In a previous study by Saimaru and Orihara (2010), cell cultures of *Olea europaea* were reported to produce verbascoside (**1**) at 1.7% dry wt. Interestingly, these studies show that hairy root cultures of *S. lateriflora* and cell cultures of *O. europaea* produce similar levels of verbascoside (**1**).

2.4.2. Scutellarin (*2*) and scutellarein (*4*)

Overall, the molar levels of the glucuronide scutellarin (**2**) were at least 4-fold and 20-fold higher than the aglycone scutellarein (**4**) in control light and dark grown cultures, respectively (Fig. 7). In fact the levels of the aglycone, scutellarein (**4**), were very low. These experiments consistently show that most of the scutellarein (**4**) is accumulated in its glucuronide form, scutellarin (**2**). When compared between dark and light grown cultures, statistically significant differences in the levels of scutellarin (**2**) were observed in dark grown hairy root cultures treated with 15 mM β-CD. No significant differences were observed among the other treatments. The levels of scutellarin (**2**) (average 0.3 mg/g dry wt; Tables S1 and S2) in our study were about half of those reported by Wilczańska-Barska et al. (2012). These differences could be due to the age of the cultures. Previous studies with hairy root cultures of related species such as *S. baicalensis* (Stojakowska and Malarz, 2000; Kuzovkina et al., 2005) and wild-type root cultures of *S. barbata* (Wilczańska-Barska et al., 2011) have not reported the presence of scutellarein (**4**) or scutellarin (**2**). Nonetheless, the study herein is the first to report the production of scutellarein (**4**) in hairy root cultures of *S. lateriflora*.

2.4.3. Baicalin (*3*) and baicalein (*6*)

The yields of the glucuronide baicalin (**3**) and its aglycone baicalein (**6**) are shown in Fig. 8 and Tables S1 and S2. Interestingly, light has a strong effect on the production of the baicalein (**6**). Levels were approximately 12-fold higher in control cultures grown under continuous light versus dark suggesting that light might have some influence on the biosynthesis of baicalein (**6**) or possibly metabolism of baicalin (**3**) to baicalein (**6**). Baicalin (**3**), the glucuronide, did not show any major differences in yields between light and dark grown cultures. The levels of baicalein (**6**), the aglycone, were significantly higher in control cultures and certain elicitation treatments when cultured under continuous light. In particular, treatment with 7.5 mM β-CD and 15 mM β-CD alone or in combination with 100 μM MeJA led to significantly higher levels when the cultures were incubated under constant light versus darkness. Overall, the levels of baicalein (**6**) were highest among all six flavones studied. Regarding control and elicited light grown cultures, these yields varied from 1.96 to 5.71 mg/g dry wt (0.196–0.571% dry wt, Table S1). In the previous study involving hairy roots of *S. lateriflora*, baicalein (**6**) levels were not reported (Wilczańska-Barska et al., 2012), whereas their study reported higher levels of baicalin (**3**) than those found here. Interestingly, both the presence

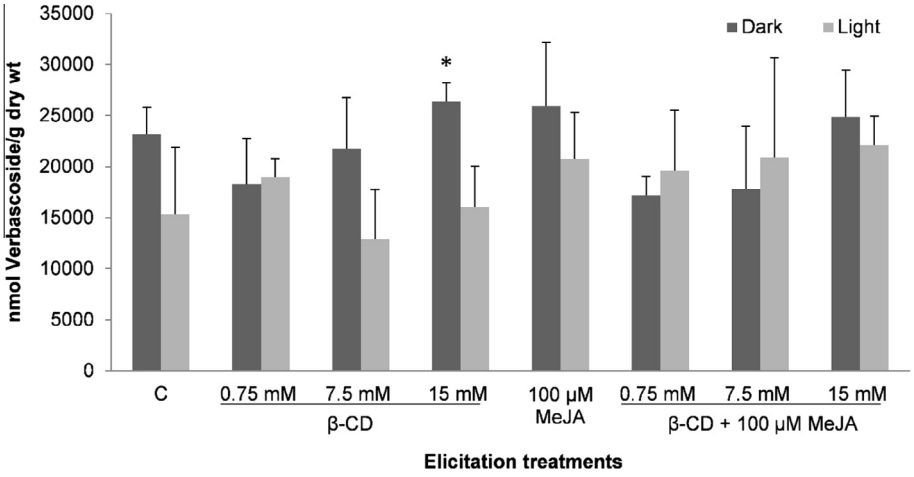

Fig. 6. Yield of verbascoside (**1**) in the tissue of hairy root cultures of *Scutellaria lateriflora* line 4 grown under light and dark conditions and treated with various elicitors. Each value represents the average + standard deviation of three biological replicates. (*) Denotes significant difference ($p < 0.05$) between light and dark culture conditions. C, control; β-CD, methyl-β-cyclodextrin; MeJA, methyl jasmonate.

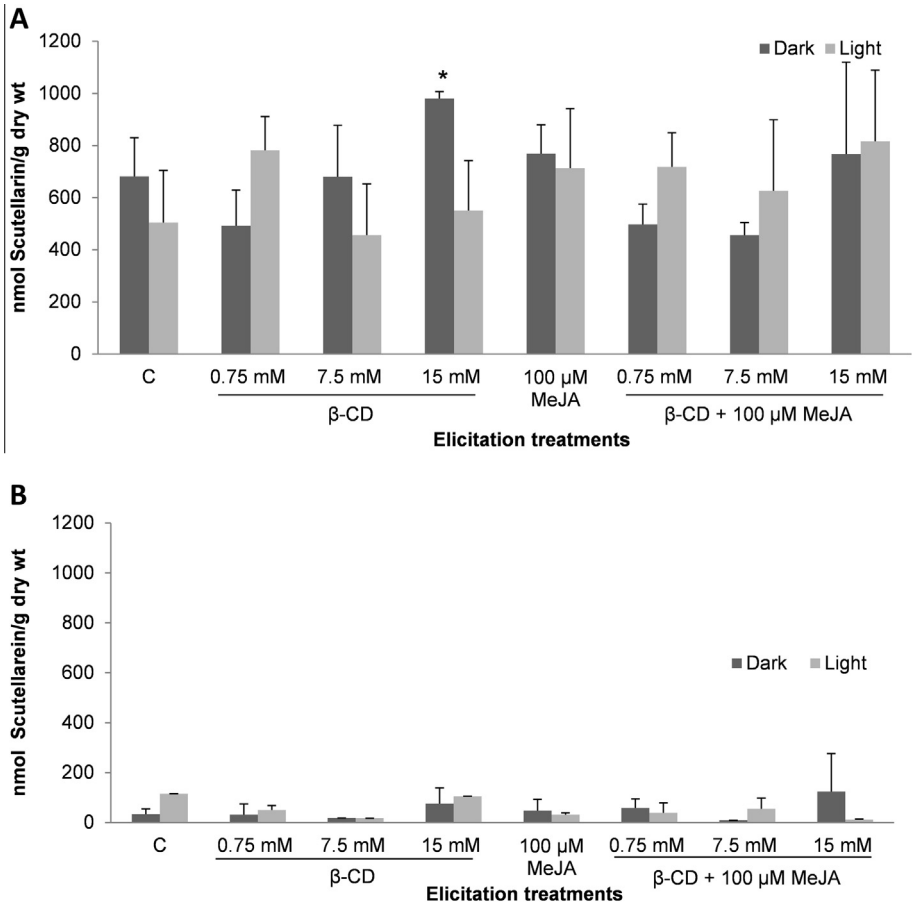

Fig. 7. Yield of (A) scutellarin (**2**) and (B) scutellarein (**4**) in the tissue of hairy root cultures of *Scutellaria lateriflora* line 4 grown in light and dark conditions and treated with various elicitors. Each value represents the average + standard deviation of three biological replicates. (*) Denotes significant difference ($p < 0.05$) between light and dark culture conditions. C, control; β-CD, methyl-β-cyclodextrin; MeJA, methyl jasmonate.

baicalein (**6**) and baicalin (**3**) in hairy root culture of this species are reported herein, suggesting a possible role of light on their metabolism.

*2.4.4. Wogonoside (**5**) and wogonin (**7**)*

The levels of wogonin (**7**) were slightly higher than its glucuronide wogonoside (**5**) under either dark or light culture conditions (Fig. 9). Interestingly, the 15 mM β-CD treatment induced accumulation of a significantly higher ($p < 0.05$) amount of wogonoside (**5**) in cultures grown in darkness, whereas the same treatment led to a significantly higher ($p < 0.05$) wogonin (**7**) level in cultures grown under light conditions. Wogonin (**7**) and wogonoside (**5**) both have been previously reported in hairy root cultures of *S. lateriflora* grown under dark conditions. In that previous study, the levels of

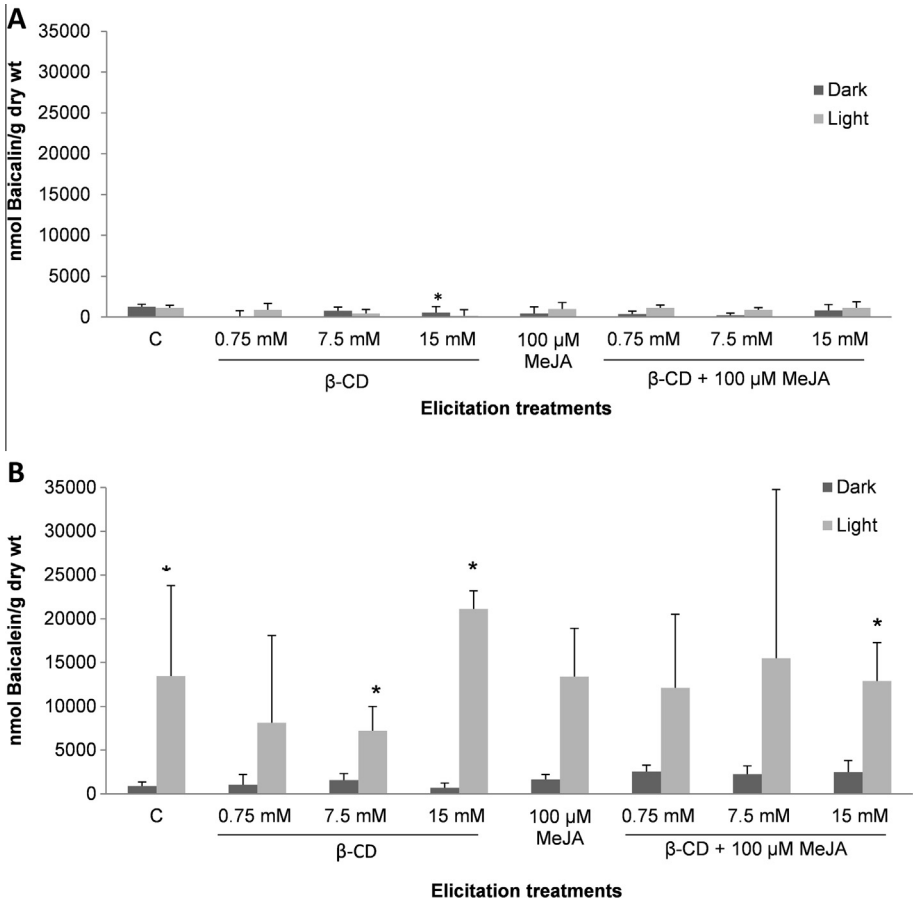

Fig. 8. Yield of (A) baicalin (**3**) and (B) baicalein (**6**) in the tissue of hairy root cultures of *Scutellaria lateriflora* line 4 grown in light and dark conditions and treated with various elicitors. Each value represents the average + standard deviation of three biological replicates. (*) Denotes significant difference ($p < 0.05$) between light and dark culture conditions. C, control; β-CD, methyl-β-cyclodextrin; MeJA, methyl jasmonate.

these flavones were 11.5 and 12 mg/g dry wt for wogonin (**7**) and wogonoside (**5**), respectively (Wilczańska-Barska et al., 2012). In comparison, the study here shows lower levels of these flavones in control and elicitor treated cultures; these differences could be due to age of roots used in these studies. More importantly, it was observed that light had a positive effect on the production of the aglycone, wogonin (**7**). When the levels of these flavones were interpreted in mg/g dry wt, there was an eightfold increase in wogonin (**7**) levels when cultures were grown under continuous light and treated with 15 mM β-CD (Table S1).

3. Concluding remarks

Hairy root cultures of *S. lateriflora* have the capacity to produce verbascoside (**1**) and the flavones scutellarein (**4**), baicalein (**6**), and wogonin (**7**) and their respective glucuronides scutellarin (**2**), baicalin (**3**) and wogonoside (**5**). The effect of light on the accumulation of these compounds was demonstrated for the first time in the current study. Cultures incubated under continuous light and treated with 15 mM methyl-β-cyclodextrin (β-CD) for 24 h produced significantly higher levels of the aglycones baicalein (**6**) and wogonin (**7**), but not scutellarein (**4**), than cultures incubated under continuous darkness.

4. Experimental

4.1. Micropropagation of S. lateriflora

In vitro established plantlets of *S. lateriflora* were provided by Dr. Nirmal Joshee (Fort Valley State University, GA) and subcultured using single node explants. Explants were cultured in Phytatray™ (Sigma) vessels containing Murashige and Skoog's medium (MS medium, Murashige and Skoog, 1962) supplemented with 0.04% $MgSO_4 \cdot 7H_2O$, 1% D-sorbitol, 2% sucrose, and 0.4% Phytagar (Gibco). Cultures were maintained at 20 °C under a 16 h photoperiod.

4.2. Establishment of hairy root cultures

Hairy roots were induced by infecting *S. lateriflora* stem explants harvested from *in vitro* cultures with *A. rhizogenes* strain ATCC 15834 as described before (Medina-Bolivar and Cramer, 2004). Roots that developed at the infection site were harvested and placed on semi-solid modified Murashige & Skoog's medium (MSV medium, Condori et al., 2010) containing 600 mg/l cefotaxime. After several weekly subcultures in this medium, root tips were transferred to antibiotic-free medium. Selected hairy root lines were established in liquid cultures by inoculating 1-cm root tips in 250 ml Erlenmeyer flasks containing 50 ml MSV medium (Condori et al., 2010) supplemented with 0, 0.1, 0.5 or 1 mg/l of indole-3-butyric acid (IBA). Hairy root line 4 was selected based on its vigorous growth performance in MSV medium supplemented with 0.5 mg/l IBA (2.45 μM). This hairy root line and medium were used in all subsequent experiments. All cultures were maintained under continuous darkness at 90 rpm and 28 °C.

4.3. PCR analysis of hairy roots

Molecular characterization of hairy roots was done by PCR targeting the transferred *rolC* and *aux1* genes from *A. rhizogenes* as

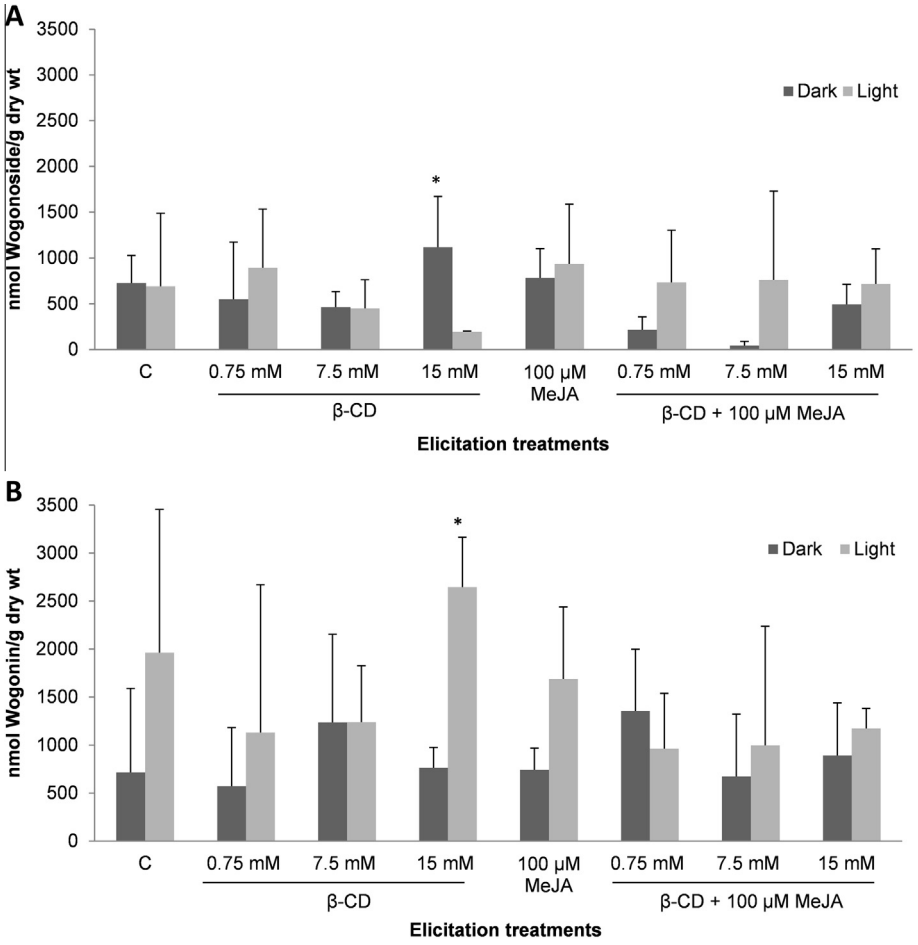

Fig. 9. Yield of (A) wogonoside (**5**) and (B) wogonin (**7**) in the tissue of hairy root cultures of *Scutellaria lateriflora* line 4 grown in light and dark conditions and treated with various elicitors. Each value represents the average + standard deviation of three biological replicates. (*) Denotes significant difference ($p < 0.05$) between light and dark culture conditions. C, control; β-CD, methyl-β-cyclodextrin; MeJA, methyl jasmonate.

described in Medina-Bolivar et al. (2007). PCR of the *virD2* gene was done to discard hairy root lines containing any remaining *A. rhizogenes* in their tissue.

4.4. Growth curve of hairy roots

To establish the growth curve of *S. lateriflora* hairy root line 4, thirty 1-cm long root tips were inoculated into 250 ml flasks containing 50 ml of MSV liquid medium (Condori et al., 2010) supplemented with 0.5 mg/l IBA. The flasks were incubated in the dark at 28 °C on an orbital shaker at 90 rpm. Three culture flasks were collected every 4 days until day 60. The pH and conductivity of the medium was recorded. The harvested roots were rinsed with distilled H_2O, dried with a paper towel and weighed to register its fresh weight. The roots were frozen and then dried in a lyophilizer (Labconco) to record the dry weight.

4.5. Scanning electron microscopy

All scanning electron microscopy work was conducted at the Center for Ultrastructural Research, University of Georgia, Athens, GA, USA. Hairy roots were collected from actively growing cultures, and fixed in 2% glutaraldehyde in phosphate-buffered saline (PBS) for 1 h at room temperature in the fume hood. Fixed roots were washed with PBS for 10 min three times. Secondary fixation was achieved by exposing the tissues to 1% OsO_4 in PBS for 1 h at room temperature in the hood. After secondary fixation, the tissues were washed with H_2O for 15 min three times. Then the hairy roots were processed for dehydration through a series of EtOH washes starting with 25% EtOH in H_2O. EtOH concentrations used were 25, 50, 70, 90, and 100%. Tissues were incubated for 10 min at each step, and at 100% at least two times to ensure all H_2O was removed. Once dehydration was complete, the tissues were processed in a critical point dryer (Tousimis, Rockville, MD). At this stage, the tissues were mounted on aluminum scanning electron microscope (SEM) stubs with double stick tape (preferably conductive carbon). Stubs with mounted tissues were placed in a sputter coater machine (SPI Supplies, West Chester, PA) to be coated with gold. Coated material was viewed under scanning electron microscope (Zeiss 1450EP, Zeiss SMT, Inc. Peabody, MA). The SEM was run at high vacuum at 20 kV and a spot size of 500 nA.

4.6. Treatment with methyl jasmonate and cyclodextrin

Hairy roots of *S. lateriflora* line 4 were cultured in 250 ml flasks containing 50 ml of MSV liquid medium (Condori et al., 2010) supplemented with 0.5 mg/l IBA as described above. Each flask was inoculated with a 1-cm × 1-cm section of root tips (~6 mg dry wt) that were separated to prevent the natural clumping exhibited by the fine *S. lateriflora* root tips. Cultures were incubated for 30 days under either continuous light or darkness at 90 rpm and 28 °C. At day 30, the spent medium was collected and replaced with fresh MSV containing the elicitor treatments: 0.75 mM methyl-β-cyclodextrin (β-CD; CAVASOL® W7M; Wacker Chemie

AG), 7.5 mM β-CD, 15 mM β-CD, 100 μM methyl jasmonate (MeJA), 0.75 mM β-CD and 100 μM MeJA, 7.5 mM β-CD and 100 μM MeJA, and 15 mM β-CD and 100 μM MeJA. In control cultures, at day 30 the spent medium was collected and replaced with fresh MSV medium containing EtOH (solvent of MeJA) at a final concentration of 0.1%. All treatments were done for 24 h in the same conditions used for growth. Each treatment included three biological replicates.

4.7. Extraction of phenolic compounds from the root tissue and culture medium

After 24 h of elicitor treatment, the roots and medium from each flask were collected. The medium was transferred to 50 ml falcon tubes and the pH and conductivity measured. The roots were rinsed three times with distilled H_2O, dried on paper towels, placed in pre-weighed aluminum foil packets, and weighed to obtain their FW. The root packets were then frozen in liq N_2 for two min and dried in a lyophilizer (Labconco) for 48 h. Upon completion of the drying cycle, the root tissue packets were individually weighed to obtain the dry weight. Each tissue was ground to a fine powder using a mortar and pestle. Powdered tissue (50 mg each) was placed in an Eppendorf microcentrifuge tube (1.5 ml), and MeOH (1 ml) was added to the tube. Each sample was sonicated for 1 min, centrifuged at 15,682xg for 7 min, and the supernatant collected. Each supernatant was individually added to an HPLC vial and dried under N_2. Once dried, the sample was resuspended in MeOH (300 μl), filtered through a 0.2 μm filter, and placed in a 250 μl glass adapter within the HPLC vial. To ensure that aglycones were not produced as artifacts during extraction, a known amount of wogonoside (**5**) was spiked to the dry root tissue and extraction was followed as described above. After HPLC analysis, the expected increase in wogonoside (**5**) levels was observed, whereas wogonin (**7**) levels were not altered confirming that the extraction procedure did not produce aglycone artifacts.

Each medium was extracted twice with EtOAc (40 ml) and dried in a Rotavapor (Büchi; Rotavapor R-200). Each dried sample was resuspended in MeOH (1.5 ml) and placed in an amber HPLC vial. The vial was placed under N_2 to dry the sample. The sample was finally re-suspended in MeOH (300 μl), filtered through a 0.2 μm filter, and placed in a 250 μl glass adapter within the HPLC vial.

4.8. HPLC analysis

Samples were analyzed by reversed-phase HPLC in a Dionex Summit system, using a SunFire™ C18, 5 μm, 4.6 × 250 mm (Waters) column at 40 °C and a flow rate at 1.5 ml/min. The system was equipped with photodiode array (PDA) and fluorescence detectors controlled by Chromeleon® software (Dionex). The mobile phase consisted of 0.1% methanesulfonic acid in H_2O (A) and CH_3CN (B). The column was initially equilibrated with 80% A and 20% B for 30 min. Then a linear gradient was performed from 80% A and 20% B to 20% A and 80% B (0–25 min), followed by isocratic elution for 5 min (25–30 min). Verbascoside (**1**) (ChromaDex), baicalin (**3**) (Sigma), baicalein (**6**) (Sigma), scutellarin (**2**) (Sigma), scutellarein (**4**) (Toronto Research Chemicals), wogonoside (**5**) (Stanford Material Corporation) and wogonin (**7**) (Wako) were used as standards. Standard solutions for HPLC were prepared by dissolving the reference compounds in methanol. Dilutions were made in the range of 7.8125–500 ppm to obtain calibration curves for quantitative analysis.

4.9. Mass spectrometry analysis

The UltiMate 3000 ultra high performance liquid chromatography (UHPLC) system (Dionex, Thermo Scientific) was used for chromatography. The chromatographic separation method followed the same HPLC conditions as described above. Mass spectrometry was performed on a LTQ XL linear ion trap (Thermo Scientific) with an electrospray ionization (ESI) source. Ultrahigh pure helium (He) was used as the collision gas and high purity nitrogen (N_2) as the sheath and auxiliary gas. All mass spectra were performed in the positive ion mode with ion spray voltage at 4 kV, sheath gas at 45 arbitrary units (AU), auxiliary gas at 15 AU, capillary voltage at 9 V, capillary temperature at 300 °C, and tube lens offset at 45 V. Full mass scan was recorded in the range m/z 100–2000. Collision-induced dissociation (CID) was used for breakage of the molecular ion into smaller fragments. The relative collision energy was set at 35% of the maximum to produce optimum yields of fragment ions. The data were analyzed using the Xcalibur software (Thermo Scientific).

4.10. Statistical analysis

Statistical analyses were conducted using SAS System version 8 (SAS Institute Inc.). Comparisons between the means of each elicitor treatment under light and the dark conditions were analyzed by a two way ANOVA followed by Tukey posthoc analysis test with an alpha value of 0.05. Comparisons between elicitor treated samples versus control were done by the Dunnett test. Each treatment included three biological replicates.

Acknowledgements

We would like to thank Maria I. Ferrand-Malatesta for her assistance with the establishment of the hairy root cultures of *Scutellaria lateriflora*. This work was funded by the United States Department of Agriculture 1890 Capacity Building Grant Awards N° 2008-38814-04737 (PI: N. Joshee and Co-PI: F. Medina-Bolivar). The UHPLC and LTQ-XL mass spectrometer were acquired with funds from National Science Foundation-EPSCoR (grant # EPS-0701890; Center for Plant-Powered Production-P3), Arkansas ASSET Initiative and the Arkansas Science & Technology Authority.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2014.08.020.

References

Awad, R., Arnason, J.T., Trudeau, V., Bergeron, C., Budzinski, J.W., Foster, B.C., Merali, Z., 2003. Phytochemical and biological analysis of skullcap (*Scutellaria lateriflora* L.): a medicinal plant with anxiolytic properties. Phytomedicine 10, 640–649.

Bergeron, C., Gafner, S., Clausen, E., Carrier, D.J., 2005. Comparison of the chemical composition of extracts from *Scutellaria lateriflora* using accelerated solvent extraction and supercritical fluid extraction versus standard hot water or 70% ethanol extraction. J. Agric. Food Chem. 53, 3076–3080.

Brock, C., Whitehouse, J., Tewfik, I., Towell, T., 2013. American skullcap (*Scutellaria lateriflora*): a randomised, double-blind placebo-controlled crossover study of its effects on mood in healthy volunteers. Phytother. Res. 28, 692–698.

Bru, R., Sellés, S., Casado-Vela, J., Belchí-Navarro, S., Pedreño, M.A., 2006. Modified cyclodextrins are chemically defined glucan inducers of defense responses in grapevine cell cultures. J. Agric. Food Chem. 54, 65–71.

Camilleri, C., Jouanin, L., 1991. The TR-DNA region carrying the auxin synthesis genes of the *Agrobacterium rhizogenes* agropine-type plasmid pRiA4: nucleotide sequence analysis and introduction into tobacco plants. Mol. Plant Microbe Interact. 4, 155–162.

Condori, J., Sivakumar, G., Hubstenberger, J., Dolan, M.C., Sobolev, V.S., Medina-Bolivar, F., 2010. Induced biosynthesis of resveratrol and the prenylated stilbenoids arachidin-1 and arachidin-3 in hairy root cultures of peanut: effects of culture medium and growth stage. Plant Physiol. Biochem. 48, 310–318.

Dandawate, S., Williams, L., Joshee, N., Rimando, A.M., Mittal, S., Thakur, A., Lum, L.G., Parajuli, P., 2012. Scutellaria extract and wogonin inhibit tumor-mediated induction of T(reg) cells via inhibition of TGF-β1 activity. Cancer Immunol. Immunother. 61, 701–711.

Dao, T.T., Linthorst, H.J., Verpoorte, R., 2011. Chalcone synthase and its functions in plant resistance. Phytochem. Rev. 10, 397–412.
Flores, H., Medina-Bolivar, F., 1995. Root culture and plant natural products: "unearthing" the hidden half of plant metabolism. Plant Tissue Cult. Biotechnol. 1, 59–73.
Gao, J., Sanchez-Medina, A., Pendry, B.A., Hughes, M.J., Webb, G.P., Corcoran, O., 2008. Validation of HPLC method for flavonoid biomarkers in skullcap (*Scutellaria*) and its use to illustrate wide variability in the quality of commercial tinctures. J. Pharm. Pharm. Sci. 11, 77–87.
Georgiev, M.I., Pavlov, A.I., Bley, T., 2007. Hairy root type plant in vitro systems as sources of bioactive substances. Appl. Microbiol. Biotechnol. 74, 1175–1185.
Gharechahi, J., Masumeh, K., Tahereh, H., Ghasem, H.S., 2013. An integrated proteomic approach to decipher the effect of methyl jasmonate elicitation on the proteome of *Silybum marianum* L. hairy roots. Plant Physiol. Biochem. 70, 115–122.
Giri, A., Narasu, M.L., 2000. Transgenic hairy roots: recent trends and applications. Biotechnol. Adv. 18, 1–22.
Hirotani, M., Kuroda, R., Suzuki, H., Yoshikawa, T., 2000. Cloning and expression of UDP-glucose: flavonoid 7-O-glucosyltransferase from hairy root cultures of *Scutellaria baicalensis*. Planta 210, 1006–1013.
Joshee, N., Tascan, A., Medina-Bolivar, F., Parajuli, P., Rimando, A.M., Shannon, D.A., Adelberg, J.W., 2013. *Scutellaria*: biotechnology, phytochemistry and its potential as a commercial medicinal crop. In: Chandra, S., Lata, H., Varma, A. (Eds.), Biotechnology for Medicinal Plants: Micropropagation and Improvement. Springer-Verlag, Heidelberg, pp. 69–99.
Kim, D.H., Hossain, M.A., Kang, Y.J., Jang, J.Y., Lee, Y.J., Im, E., Yoon, J.H., Kim, H.S., Chung, H.Y., Kim, N.D., 2013. Baicalein, an active component of *Scutellaria baicalensis* Georgi, induces apoptosis in human colon cancer cells and prevents AOM/DSS-induced colon cancer in mice. Int. J. Oncol.. http://dx.doi.org/10.3892/ijo.2013.2086.
Kubasek, W.L., Shirley, B.W., McKillop, A., Goodman, H.M., Briggs, W., Ausubel, F.M., 1992. Regulation of flavonoid biosynthetic genes in germinating *Arabidopsis* seedlings. Plant Cell 4, 1229–1236.
Kuzovkina, I.N., Guseva, A.V., Alterman, I.E., Karnachuk, R.A., 2001. Flavonoid production in transformed *Scutellaria baicalensis* roots and way of its regulation. Russ. J. Plant Physiol. 48, 448–452.
Kuzovkina, I.N., Guseva, A.V., Kovács, D., Szöke, É., Vdovitchenko, M.Y., 2005. Flavones in genetically transformed *Scutellaria baicalensis* roots and induction of their synthesis by elicitation with methyl jasmonate. Russ. J. Plant Physiol. 52, 77–82.
Lee, K.W., Kim, H.J., Lee, Y.S., Park, H.J., Choi, J.W., Ha, J., Lee, K.T., 2007. Acteoside inhibits human promyelocytic HL-60 leukemia cell proliferation via inducing cell cycle arrest at G0/G1 phase and differentiation into monocyte. Carcinogenesis 28, 1928–1936.
Lijavetzky, D., Almagro, L., Belchi-Navarro, S., Martínez-Zapater, J.M., Bru, R., Pedreño, M.A., 2008. Synergistic effect of methyl jasmonate and cyclodextrin on stilbene biosynthesis pathway gene expression and resveratrol production in Monastrell grapevine cell cultures. BMC Res. Notes 1, 132. http://dx.doi.org/10.1186/1756-0500-1-132.
Medina-Bolivar, F., Condori, J., Rimando, A., Hubstenberger, J., Shelton, K., Bennett, S., Dolan, M., 2007. Production and secretion of resveratrol in hairy root cultures of peanut. Phytochemistry 68, 1992–2003.
Medina-Bolivar, F., Cramer, C., 2004. Production of recombinant proteins by hairy roots cultured in plastic sleeve bioreactors. Methods Mol. Biol. 267, 351–363.
Medina-Bolivar, F., Yang, T., 2013. Method to increase the yield of products in plant material. US Patent Application 13784877.
Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. 15, 473–497.
Nopo-Olazabal, C., Condori, J., Nopo-Olazabal, L., Medina-Bolivar, F., 2014. Differential induction of antioxidant stilbenoids in hairy roots of *Vitis rotundifolia* treated with methyl jasmonate and hydrogen peroxide. Plant Physiol. Biochem. 74, 50–69.
Ono, N.N., Tian, L., 2011. The multiplicity of hairy root cultures: prolific possibilities. Plant Sci. 180, 439–446.
Parajuli, P., Joshee, N., Chinni, S.R., Rimando, A.M., Mittal, S., Sethi, S., Yadav, A.K., 2011. Delayed growth of glioma by *Scutellaria* flavonoids involve inhibition of Akt, GSK-3 and NF-κB signaling. J. Neurooncol. 101, 15–24.
Parajuli, P., Joshee, N., Rimando, A.M., Mittal, S., Yadav, A.K., 2009. In vitro antitumor mechanisms of various *Scutellaria* extracts and constituent flavonoids. Planta Med. 75, 41–48.
Park, N.I., Xu, H., Li, X., Kim, S.J., Park, S.U., 2011. Enhancement of flavone levels through overexpression of chalcone isomerase in hairy root cultures of *Scutellaria baicalensis*. Funct. Integr. Genomics 11, 491–496.
Patel, P., Joshee, N., Rimando, A., Parajuli, P., 2013a. Anti-cancer scopes and associated mechanisms of *Scutellaria* extract and flavonoid wogonin. Curr. Cancer Ther. Rev. 9, 34–42.
Patel, K., Singh, R.B., Patel, D.K., 2013b. Medicinal significance, pharmacological activities, and analytical aspects of solasodine: a concise report of current scientific literature. J. Acute Dis. 2, 92–98.
Paton, A., 1990. A global taxonomic investigation of *Scutellaria* (Labiatae). Kew. Bull. 45, 399–450.
Saimaru, H., Orihara, Y., 2010. Biosynthesis of acteoside in cultured cells of *Olea europaea*. J. Nat. Med. 64, 139–145.
Sanz, M., Fernandez de Simon, B., Cadahia, E., Esteruelas, E., Munoz, A., Hernandez, T., Estrella, I., Pinto, E., 2012. LC-DAD/ESI-MS/MS study of phenolic compounds in ash (*Fraxinus excelsior* L. and *F. americana*) heartwood. Effect of toasting intensity at cooperage. J. Mass Spectrom. 47, 905–918.
Schmulling, T., Schell, J., Spena, A., 1988. Single genes from *Agrobacterium rhizogenes* influence plant development. EMBO J. 7, 2621–2629.
Sevon, N., Oksman-Caldentey, K.M., 2002. *Agrobacterium rhizogenes*-mediated transformation: root cultures as a source of alkaloids. Planta Med. 68, 859–868.
Slightom, J.L., Durand-Tardif, M., Jouanin, L., Tepfer, D., 1986. Nucleotide sequence analysis of TL-DNA of *Agrobacterium rhizogenes* agropine type plasmid. Identification of open reading frames. J. Biol. Chem. 261, 108–121.
Stojakowska, A., Malarz, J., 2000. Flavonoid production in transformed root cultures of *Scutellaria baicalensis*. J. Plant Physiol. 156, 121–125.
Tiwari, R.K., Trivedi, M., Guang, Z.-C., Guo, G.-Q., Zheng, G.-C., 2008. *Agrobacterium rhizogenes* mediated transformation of *Scutellaria baicalensis* and production of flavonoids in hairy roots. Biol. Plant. 52, 26–35.
Wang, J.W., Wu, J.Y., 2013. Effective elicitors and process strategies for enhancement of secondary metabolite production in hairy root cultures. Adv. Biochem. Eng. Biotechnol. 134, 55–89.
Wasternack, C., 2007. Jasmonates: an update on biosynthesis, signal transduction and action in plant stress response, growth and development. Ann. Bot. 100, 681–697.
Wilczańska-Barska, A., Krauze-Baranowska, M., Majdan, M., Głód, D., 2011. Wild type root cultures of *Scutellaria barbata*. J. Biotechnol. Comput. Biol. Biotechnol. 92, 369–377.
Wilczańska-Barska, A., Królicka, A., Głód, D., Majdan, M., Kawiak, A., Krauze-Baranowska, M., 2012. Enhanced accumulation of secondary metabolites in hairy root cultures of *Scutellaria lateriflora* following elicitation. Biotechnol. Lett. 9, 1757–1763.
Zhang, Z., Lian, X.Y., Li, S., Stringer, J.L., 2009. Characterization of chemical ingredients and anticonvulsant activity of American skullcap (*Scutellaria lateriflora*). Phytomedicine 16, 485–493.