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Enhanced Terpenoids Production of Elicited Hairy Root Cultures of *Scutellaria bornmuelleri*

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HIGHLIGHTS

- ½ MS medium containing 2.5% sucrose as the best medium for successful transformation and biomass production.
- The maximum accumulation of monoterpenes in hairy root cultures treated with 100 µM MeJA.
- The enhanced biosynthesis of sesquiterpenes in hairy root cultures elicited with 100 mg L^{-1} chitosan.

Abstract: *Scutellaria bornmuelleri* is recognized as a medicinal plant and a rich source of terpenoids. Hairy Root Cultures (HRCs) along with elicitation of *in vitro* cultures have been widely considered in order to produce important secondary metabolites such as terpenoids. In the present study, *S. bornmuelleri* HRCs were established by transformation of stem explants with *Agrobacterium rhizogenes* strain A4. The effect of different concentrations of sucrose on transgenic root induction and biomass production were investigated. The production and accumulation of terpenoids in HRCs elicited with chitosan (CS), methyl jasmonate (MeJA) and *β*-cyclodextrin (*β*-CD) was evaluated using Gas chromatography/mass spectrometry (GC/MS). Among various concentrations of sucrose, half-strength Murashige and Skoog (MS) medium supplemented with 2.5% sucrose resulted in significant increase in transformation frequency and maximum root biomass production. The highest sesquiterpene production was observed in HRCs elicited with 100 mg L^{-1} CS. However, the highest monoterpene accumulation was occurred in HRCs treated with 100 µM MeJA. Among the combined treatments, the highest sesquiterpene and monoterpene yield was achieved by 0.7 and 7 mM *β*-CD plus MeJA as well as 50 and 100 mg L^{-1} CS plus MeJA, respectively. To the best of our knowledge, the results of this study for the first time suggested that eliciting HRCs can be an effective method to accumulate sesquiterpenes in large scale cultures of *S. bornmuelleri* HRs in bioreactor.

Keywords: Chitosan; Genetically transformed root; Methyl jasmonate; *Agrobacterium rhizogenes*; *Scutellaria bornmuelleri*; Sesquiterpene.

INTRODUCTION

The genus *Scutellaria* (Lamiaceae family) comprises more than 360 species which are widely distributed in temperate regions and on tropical mountains [1]. 20 species and two hybrids of this genus are distributed in Iran. *Scutellaria bornmuelleri* is an important medicinal plant distributed in the northwest of Iran [2]. The anti-cancer activities of the *S. bornmuelleri*-derived methanolic extract have been reported on human colon cancerous cell line [3]. Flavones such as chrysin, wogonin, baicalin, wogonoside and baicalein are the major bioactive compounds extracted from the root and shoot parts of *S. bornmuelleri* [4, 5]. It has been widely used in Iran traditional medicine [6]. Previous study on phytochemical content of *S. bornmuelleri* essential oil revealed the presence of several volatile compounds such as steroids, sesquiterpenes, and monoterpenes [7]. In addition, phytochemical composition profiling of *S. bornmuelleri* methanol extract by GC/MS analysis, have been reported Phytol and neophytadiene diterpenoids from the aerial parts of plant [8].

Volatile compounds content of the plants depends mainly on plant genotype, developmental stage and some of the environmental factors such as plant age, sun exposure, plant collection method and the extraction process [9]. To date, a number of strategies have been used to increase secondary metabolites yield such as selection of high-yielding lines, changing the formulation of medium, culture conditions and the cultivation strategies i.e. cell culture, organ culture, HRs culture and elicitation [10-13]. Elicitation is an effective tool to improve secondary metabolites content in HRCs of medicinal plants. Stress-signaling molecules including elicitors are regularly used in elicitation experiments. They induce many physiological and morphological reactions and trigger the accumulation of secondary metabolite in plants. Therefore biotic and abiotic elicitors can be served as an effective tool for increasing the productivity of secondary metabolites in HRCs [14]. MeJA, *β*-CD and CS are used to improve terpene production in adventitious roots [15, 16]. Several studies have described the stimulated production of secondary metabolite in HRCs of medicinal plants using different elicitors [10, 17, 18]. Chung and coauthors (2016) used jasmonic acid (JA) and salicylic acid (SA) elicitors to enhance biomass accumulation and phenolic compound production in hairy root cultures of *Momordica charantia* L.. Their results revealed that cultures supplemented with 100 µM JA and SA can significantly enhance the production of phenolic compounds in compared to control cultures [19]. In recent years, there is a growing interest to biotechnological methods such as liquid and hairy root cultures through *A. rhizogenes* for enhancement of secondary metabolites production in medicinal plants [20-22].

Several studies have reported that *A. rhizogenes*–mediated transformation of plant cells, can effectively produce terpenoid compounds on a large scale [23]. To date, elicitors have been used to stimulate phenolic compounds production in *Scutellaria* hairy root culture [18, 24]. Marsh and co-authors (2014) reported that the cultures incubated under continuous light and treated with methyl-*β*-cyclodextrin produced significantly higher levels of baicalein and wogonin [18]. Our previous study reported that the combination of MeJA and CS can induce production of chrysin, wogonin and baicalein in *S. bornmuelleri* HRCs [24]. Various studies have revealed that CS could be a very effective elicitor to induce secondary metabolite production in plants [25]. CS, a modified natural carbohydrate polymer, exists in the shells of arthropods such as shrimps, crabs and insect exoskeletons [26] and is low-priced, non-toxic and safe. Addition of 100 mg L^{-1} CS enhanced the production of chrysin, wogonin and baicalein flavones compared to two other concentrations (50 and 200 mg L^{-1}). It increased the chrysin, wogonin and baicalein contents approximately 8.48, 7.56 and 7.04 fold compared to control, respectively [24]. The combination of CS and MeJA in *S. bornmuelleri* hairy root cultures demonstrated a significant increase in flavonoid content. In addition, the synergistic effect of combined elicitation of MeJA and 0.7 mM *β*-CD slightly increased the value of wogonin [24]. To date, there is no investigation on the alone and simultaneous elicitation of CS, MeJA, and *β*-CD for terpenoids production in *S. bornmuelleri* hairy roots. This is the first study to investigate proper elicitation using various combination strategies involving CS, MeJA, and *β*-CD for terpenoid compound induction in HRCs of *S. bornmuelleri*. Different concentrations of MeJA, *β*-CD and CS were used based on previous studies in our group. *β*-CD and CS were used at three concentrations and MeJA at one concentration in order to increase terpene production. Also, the combination effect of these elicitors was evaluated on terpene production in HRCs. The detection was performed by GC/MS technique.

MATERIAL AND METHODS

Chemicals

MeJA, HPLC-grade acetonitrile and methanol and CS were procured from Sigma-Aldrich Co. (Aldrich Division, Steinheim, Germany). *β*-CD was purchased from CAVASOL® W7M; Wacker Chemie AG.

Establishment of *S. bornmuelleri* HRCs

S. bornmuelleri seeds were collected from East Azerbaijan, Iran and were identified by Dr. Talebpour, Tabriz University, Tabriz, Iran (No: 14748). The sterilization and germination of seeds was performed according to Gharari and co-authors [27, 28]. HR was induced through infection of stem explants with the wild-type strain of *A. rhizogenes* A4, as described by Gharari and coauthors [28] with modifications. The effect of different sucrose concentration (1.5, 2, 2.5 and 3%) was evaluated on HR induction. The stems of *in vitro* plantlets of *S. bornmuelleri* were cut into 1 cm pieces and were pre-cultured on half-strength MS medium containing 0.5 mg L^{-1} BAP for 24 h prior to infection. *A. rhizogenes* strain A4 was grown overnight on Luria-Bertani (LB) medium supplemented by 50 mg L^{-1} rifampicin, pH 7.2, at 28°C and 180 rpm under continues darkness. Bacterial cells were harvested by centrifugation at 3500 rpm at 4 °C for 15 min and then re-suspended in liquid MS medium (pH 5.5) until OD600 reached 0.6-0.8. The pre-cultured explants were inoculated with bacterial suspension containing acetosyringone 100 µM, and incubated for 35 min on shaker. After rinsing of explants with distilled water, they were blotted dry and transferred to co-cultivation medium (MS medium containing 100 µM acetosyringone, pH 5.5) and cultivated for 2 days. After this time, explants were transferred onto a hormone-free half-strength MS medium supplemented with 400 mg L^{-1} cefotaxime. After 10 days HRs appeared on wound sites of the explants and then the induced roots were sub-cultured every three weeks on fresh half-strength MS medium containing 400 mg L^{-1} cefotaxime.

Molecular confirmation of transgenic root lines

Genomic DNA was extracted from transformed and non-transformed roots of *S. bornmuelleri*. gDNA of samples (100 mg) was extracted by CTAB method. PCR mixture volume was 25 µL including PCR buffer (containing 1.5 mM $MgCl_2$), 100 ng of genomic DNA, 200 µM of each dNTPs, 10 µM of each primers and 2U *Taq* DNA. The PCR was performed to amplify *rol*B gene fragment (780 bp). The primer pair of *rol*B gene was 5′-ATG GAT CCC AAA TTG CTA TTC CTT CCA CGA-3′ and 5′- TTA GGC TTC TTT CTT CAG GTT TAC TGC AGC-3′. The PCR program comprised of an initial denaturing step of 5 min at 95°C followed by 30 cycles of 30 s at 95°C, 30 s at 57°C and 1 min at 72°C and a final extension step of 10 min at 72°C. Amplified products were visualized by ultraviolet light after electrophoresis on 1% agarose gel, stained with RedSafe™ DNA Stain.

Growth curve of HRs

Transformation frequency to each producing hairy root line is presented in Figure 1. Maximum biomass production by hairy roots was belonged to line 9 (Figure 1). Table 1 represents fresh and dry weights of different one-month-old hairy root lines (L_{1-9}) cultured in solid half-strength MS medium. Among nine different lines, line 9 was better respecting to its vigorous growth, fresh and dry weight on solid half-strength MS medium (Table 1). Therefore this line was selected for a time-course study of HR growths. After six successful sub-cultures of HRs at the same medium every two weeks, the suspension cultures were run. Thirty 1-cm root tips of this line were transferred to a 250 mL flask containing 50 mL of liquid half-strength MS medium supplemented with 0.5 mg L^{-1} Indole-3-butyric acid (IBA). Roots were cultured for 70 days under continues darkness at 28 ± 2°C on shaker at 120 rpm. The HRs from three culture flasks were collected every week until day 70 and their dry weight and the pH values of the media were recorded. A growth curve of HRs was plotted according to their dry weight.

Elicitation of HRCs

In order to assessment terpenoids accumulation in HR line 9 in response to elicitation, its liquid culture was established by inoculating root tips into 100 mL flasks, containing 40 mL of half-strength MS medium supplemented with 0.5 mg L^{-1} IBA. The cultures were incubated under continuous darkness on a rotary shaker at 28 °C and 120 rpm for 28 days. MeJA, CS and *β*-CD were prepared as described before [24]. HRs were exposed to three concentrations of *β*-CD (0.7, 7.0 and 14 mM); three concentrations of CS (50, 100 and 200 mg L^{-1}); one concentration of MeJA (100 µM) as well as mixed combination of two elicitors including CS plus MeJA (50, 100 and 200 mg L^{-1} plus 100 µM), *β*-CD plus MeJA (0.7, 7.0 and 14 mM plus 100 µM) and *β*-CD plus CS (0.7, 7.0 and 14 mM plus 100 mg L^{-1}). On the 28th day of culture, the elicitation experiments were performed by transferring roots to fresh liquid half strength MS media (30 mL), containing 0.5 mg L^{-1} IBA and specific elicitors. All cultures were grown at the same condition for 24 hours. Then, they were freeze-dried and subjected to terpenoid content analysis by GC/MS.

Preparation of extracts and determination of terpenoid content by GC/MS

Approximately 50 mg of each dried powdered HR samples were extracted three times using 3 mL MeOH. The extraction was carried out at room temperature using percolation method for 48 hours. The extract was filtered through a 0.22 µm membrane filter. 0.5 µL of the methanolic extract was used for GC-MS analysis in order to identify phytochemical compounds. Qualitative assessment of methanolic extracts was carried out by GC/MS method. GC (7890B, Agilent, USA) analysis was conducted in a Hewlett-Packard (HP5-MS, (60 m×0.25 mm i.d., 0.25 µm film thickness)) HP 5977A gas chromatograph system (Agilent, USA). Helium (99.999%) was utilized as the carrier gas, at the flow rate of 1 mL/min. The injection port temperature was set at 290 °C; column temperature was firstly held at 70 °C for 5 min, and next slowly risen to 290 °C at the rate of 8 °C /min. The relative quantity of the oil compounds of *S. bornmuelleri* was quantified based on GC peak areas produced in the chromatogram. Kovats indices of the components were calculated using a homologous series of C6-C24 n-alkanes injected at the same conditions.

Identification of chemical constituents

The compounds of the methanolic extracts were determined based on GC retention time on HP-5MS column and comparing their mass spectra with those of GC/MS library (WILEY 7n D. 04.00 and NIST).

This study was conducted based on a completely randomized design. The effect of different elicitors was compared by Duncan's multiple range tests ($p<0.01$). Data were subjected to the analysis of variance (ANOVA) and presented as mean ± SD, in three replicates. Statistical analysis and mean comparison were carried out using IBM SPSS Statistics V.22.0 software.

RESULTS

Establishment of HRCs

The growth of *S. bornmuelleri* HRCs is shown in Figure 2. HR cultures were established from stem explants derived from one month old *in vitro* cultured plants using *A. rhizogenes* A4 strain on half-strength MS medium as co-cultivation. To achieve the maximum rate of HR induction, the effect of various sucrose concentrations were studied. HRs appeared from wounded sites of explants seven days after injection (Figure 2). The maximum frequency of transformation (100%) was observed in half-strength MS medium supplemented with 2.5% sucrose, whereas the media containing 1.5, 2 and 3% sucrose resulted in 80%, 85% and 86.6% HR induction frequency, respectively (Figure 3). The uninfected explants on the culture medium maintained without any damage during culture and after two subculture at every four weeks interval they were discarded. After establishment of HRs, the HR lines were subjected to DNA isolation and PCR amplification of *rol*B gene (Figure 4). The DNA of transformed roots showed the expected size of PCR product for *rol*B gene that revealed the integration of *rol*B gene into the genome of *S. bornmuelleri*.

Establishment of growth curve of *S. bornmuelleri* HR liquid cultures

In order to determine the growth curve of HRs, liquid culture of HR line 9 was initiated in half-strength MS medium. The growth rate was calculated by dividing the final dry weight of HRs at the end of subculture by the inoculum weight (~4.9 mg dry weight).

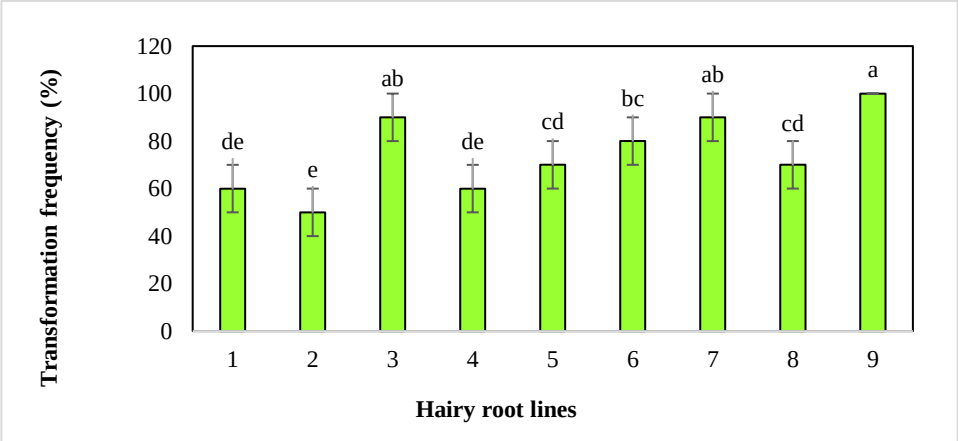


Figure 1. Transformation frequency of producing hairy root lines. Different letters indicate significant differences at p≤ 0.01

Table 1. Fresh and dry weight of different one-month-old hairy root lines (L_{1-9}) in solid half-strength MS medium.

	Lines								
Weight (g)	**L_1**	**L_2**	**L_3**	**L_4**	**L_5**	**L_6**	**L_7**	**L_8**	**L_9**
Fresh	7.83 ± 2.44^{f}	7.14 ± 2.56^{h}	9.41 ± 4.02^{b}	7.56 ± 3.06^{g}	8.35 ± 2.18^{e}	9.15 ± 3.65^{c}	9.27 ± 2.78^{bc}	8.62 ± 1.46^{d}	11.45 ± 3.43^{a}
Dry	0.64 ± 0.12^{f}	0.59 ± 0.08^{h}	0.76 ± 0.13^{b}	0.61 ± 0.09^{g}	0.688 ± 0.05^{e}	0.735 ± 0.06^{c}	0.75 ± 0.07^{b}	0.708 ± 0.04^{d}	0.92 ± 0.09^{a}

Data points are mean ± SE of three determinations. Different letters indicate significant differences at p≤ 0.01

Figure 2. (A) *S. bornmuelleri* HRs initiated from stem explants inoculated with *A. rhizogenes* A4 strain after 2 weeks of inoculation, (B) after 4 weeks, (C) *S. bornmuelleri* HRs cultured in half strength liquid MS medium. Scale bar; 5 mm.

The initial establishment of root cultures on half-strength MS liquid media was most suitable for biomass accumulation. The stages of HRs development during the culture period of fastest growing line (HR line 9), is demonstrated in Figure 5. During the first seven weeks, the biomass of the HRs was increased (4.9-238.9 mg), while from the 7th week onwards, the growth of the roots was constant and then decreased (Figure 5A). The highest biomass (~48.8- fold) was obtained in half-strength MS media at the end of 7th week, while normal root showed a slight increase in dry weight (Data not show).

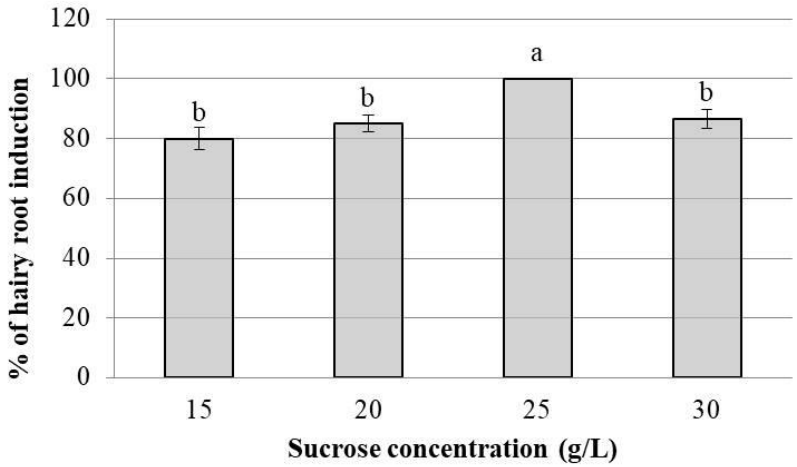


Figure 3. Frequency of HR induction using different concentrations of sucrose. Different letters indicate significant differences at p≤ 0.05.

Figure 4. Molecular confirmation of *S. bornmuelleri* HRs; Lane M: marker; Lane No. 1–9: transformed HR lines; Lane N: negative control; Lane P: positive control (Plasmid from *A. rhizogenes* A4 strain).

A medium pH change of growing culture was measured every week until the 70th day (Figure 5B). Half-strength MS medium provided a relatively stable medium pH during culture period relative to starting pH value (5.8) despite the presence of explants (HRs) in the medium. The pH value was fluctuated between 4.23 and 5.8 during the culture period, indicating the buffering capacity of the medium.

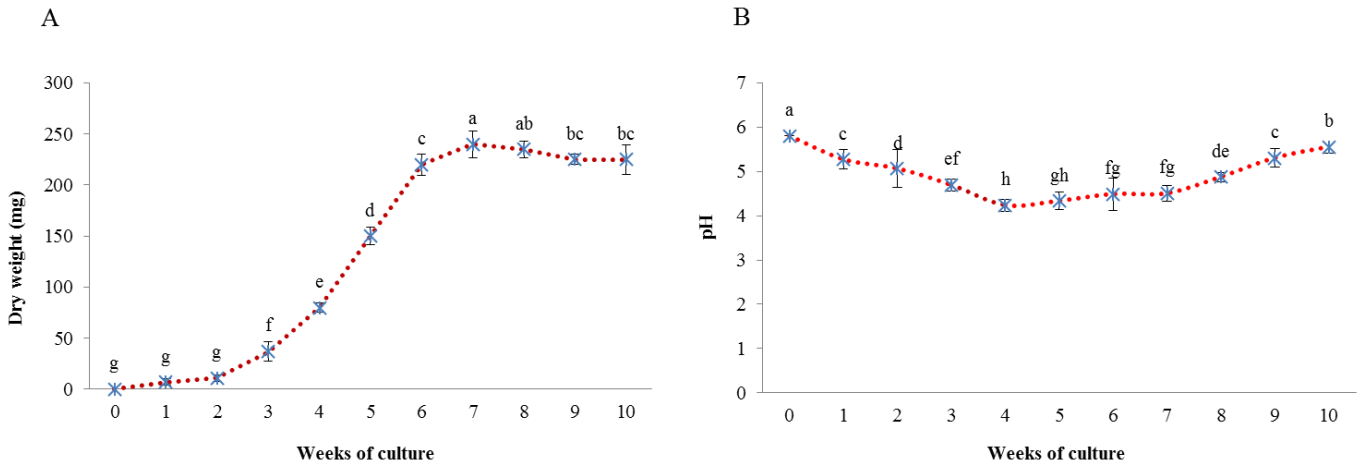


Figure 5. (A) The growth course of HRs of *S. bornmuelleri* in liquid half-strength MS medium, (B) pH value of the medium during the culture period, each value represents the average of three biological replicates ± standard deviation. Different letters indicate significant differences at p≤ 0.01.

GC/MS analysis of wild roots of *S. bornmuelleri*

Various terpenes identified in methanol extract of elicited HRCs, control and wild root are presented in Table 2. Methanolic extract of *S. bornmuelleri* wild roots showed a total of 12 terpenes (18.52%) including 7 sesquiterpene hydrocarbons (12.66%), 4 oxygenated sesquiterpenes (4.8%) and 1 oxygenated monoterpene (0.61%). Sesquiterpenes were the main components, with sesquiterpene hydrocarbons as the major sesquiterpene and caryophyllene (5.49%) as the major sesquiterpene hydrocarbon (Table 2). No common compound was found between wild roots and controls.

GC/MS analysis of non-elicited HRs of *S. bornmuelleri*

GC/MS analysis of non-elicited *S. bornmuelleri* HRs detected the presence of 9 compounds (7.38%) including 2 oxygenated sesquiterpenes, 4 oxygenated monoterpenes, 1 sesquiterpene hydrocarbon and 2 monoterpene hydrocarbons (Figure 6, Table 2). Oxygenated terpenes were the most abundant in comparison with terpene hydrocarbons. B-cymene; Artemisia alcohol and geranic acid were observed only in control HRCs, but were absent in elicited HRs and wild roots (Table 2). *α*-curcumene, spatulenol, caryophyllene oxide, citral, eucalyptol and D-limonene were common compounds found between elicited HRs and control cultures (Table 2).

GC/MS analysis of elicited HRs of *S. bornmuelleri*

Three elicitors including MeJA, CS and *β*-CD were used individually and in combinations in order to induce terpenes production in *S. bornmuelleri* HRs (Figure 6). GC/MS analysis revealed the presence of 72 elicitor specific terpenes in elicited HRCs (Table 2).

Effect of different elicitors on sesquiterpene hydrocarbons production

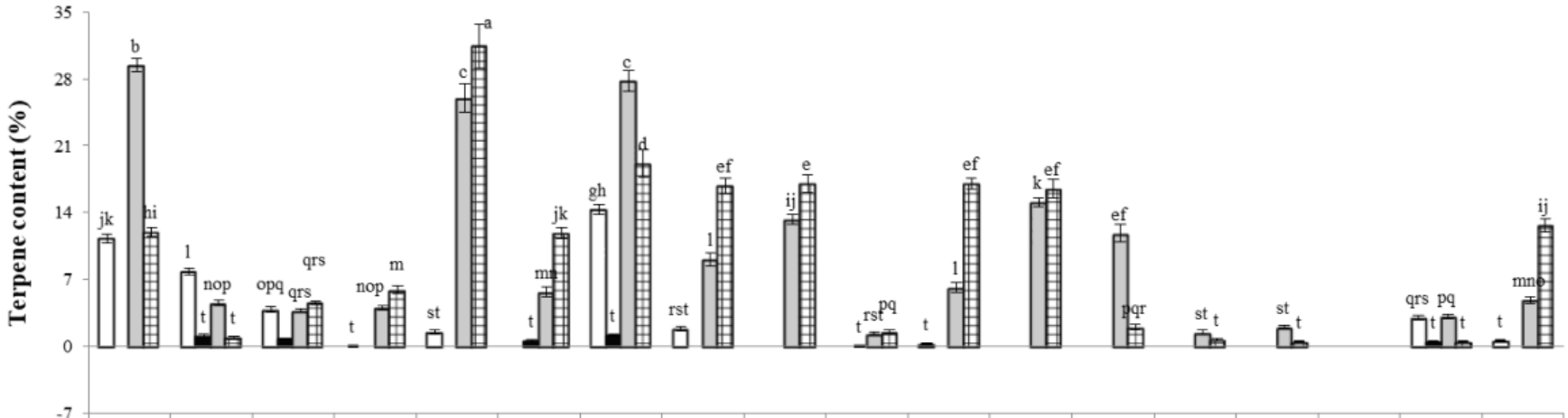
A total of 42 different sesquiterpene hydrocarbons were identified in HRCs (control and elicited HRCs; Table 2). In comparison to other terpenes, sesquiterpene hydrocarbons were the most accumulated terpenes in HRCs (Figure 6). Among different elicitor treatments, 100 mg L^{-1} CS was the most efficient concentration to accumulate sesquiterpene hydrocarbons. The highest sesquiterpene hydrocarbons content (31.39%) was observed in HRCs, elicited with 100 mg L^{-1} CS (Figure 6). The sesquiterpene hydrocarbons content of the cultures treated with the combination of 100 mg L^{-1} CS plus 100 µM MeJA was less than (16.5%) those treated with 100 mg L^{-1} CS, alone (Figure 6). In addition, the accumulation of sesquiterpene hydrocarbons was increased in HRCs elicited with 100 µM MeJA, when applied alone (19.14%). Among combined elicitor treatments, the highest amount of sesquiterpene hydrocarbons with the rate of 17.05% and 17.01% was obtained in cultures treated with 50 mg L^{-1} CS plus 100 µM MeJA and 7.0 mM *β*-CD plus 100 µM MeJA, respectively (Figure 6). Among various sesquiterpene hydrocarbons detected in HRCs, some of them such as aromadendrane (4.72%) and isocaryophyllene (4.84%) were elicitor-specific being individually present in HRCs, elicited with 100 µM MeJA and 100 mg L^{-1} CS, respectively (Table 2). 1,4,7,-cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z-, selina-3,7(11)-diene, caryophyllene, *α*-curcumene, (E)-*β*-famesene and valencene were the most common sesquiterpene hydrocarbons between several elicited HRCs, among which 1,4,7,-cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z-, selina-3,7(11)-diene and caryophyllene were found common between wild root and elicited HRCs. *α*-curcumene was found in low amount (0.52%) in control cultures (Table 2), while its content was increased to 9.43% in HRCs elicited with 0.7 mM *β*-CD (Table 2). The highest sesquiterpene hydrocarbons content in elicited HRs belonged to caryophyllene with 10.36% content in culture treated with 7 mM *β*-CD plus 100 µM MeJA. Curcumene and longipinene were two different sesquiterpene hydrocarbons harboring *α* and *β* anomers found in elicited HRCs. Unlike *β* anomer (0.59%), *α* anomer of curcumene was found in high values (26.88%) in elicited root cultures (Table 2). However, for longipinene, no significant difference was observed in *α* (2.13%) and *β* (1.27%) anomers content (Table 2).

Effect of different elicitors on oxygenated sesquiterpenes production

A total of 34 different oxygenated sesquiterpenes were found in control and elicited HRCs (Table 2). The GC-MS results revealed that CS (100 and 200 mg L^{-1}) significantly ($p<0.01$) improves oxygenated sesquiterpenes accumulation as compared with control when applied individually or in combination with 100 µM MeJA, however *β*-CD improves that significantly only in 1 concentration (0.7 mM *β*-CD) when is used individually or in combination with 100 µM MeJA. The highest oxygenated sesquiterpenes content (29.4%) was observed in HRCs elicited with 0.7 mM *β*-CD (Figure 6). Among different combined treatments, combination of 100 mg L^{-1} CS plus 100 µM MeJA showed the highest oxygenated sesquiterpenes accumulation rate (15.07%), that was approximately 4.9 fold increase as compared to control (Figure 6). Among various oxygenated sesquiterpenes, Epi-Cubebol (2.23%), was elicitor specific being abundantly and individually present in HRCs elicited with 0.7 mM *β*-CD (Table 2). *α*-Bisabolol, spatulenol, caryophyllene oxide, *β*-Eudesmol, γ-eudesmol, guaiol and bulnesol were the most common oxygenated sesquiterpenes among several elicited HRCs, with *α*-bisabolol and guaiol being common between wild roots and elicited HRs. The highest oxygenated sesquiterpene content in elicited HRs was belonged to spatulenol, with the rate of 9.19% in culture treated with 0.7 mM *β*-CD (Table 2).

Effect of different elicitors on oxygenated monoterpenes production

The GC-MS results showed that unlike sesquiterpenes which are accumulated by most treatments, monoterpenes are produced only in a few treatments (Figure 6). A total of 11 different oxygenated monoterpenes were identified in HRCs (Table 2). The maximum level of oxygenated monoterpenes (14.32%) was 4.8 fold increased as compared with non-elicited cultures (2.96%), when experimental samples were treated with 100 µM MeJA. *β*-CD in two concentration levels (0.7 and 7 mM) significantly induced oxygenated monoterpenes accumulation as compared with control cultures (Figure 6).



	b-CD 0.7 mM	b-CD 7 mM	b-CD 14 mM	Chi 50 mg/L	Chi 100 mg/L	Chi 200 mg/L	MeJa 100µM	b-CD 0.7 mM MeJa 100µM	b-CD 7 mM MeJa 100µM	b-CD 14 mM MeJa 100µM	Chi 50 mg/L MeJa 100µM	Chi 100 mg/L MeJa 100µM	Chi 200 mg/L MeJa 100µM	b-CD 0.7 mM Chi 100 mg/L	b-CD 7 mM Chi 100 mg/L	b-CD 14 mM Chi 100 mg/L	Control	Wild root
□Oxygenated monoterpenes	11.26	7.82	3.82	0	1.47	0	14.32	1.82	0	0	0.27	0	0	0	0	0	2.96	0.61
■Monoterpene hydrocarbons	0	1.1	0.84	0	0	0.57	1.12	0	0	0	0	0	0	0	0	0	0.49	0
▨Oxygenated sesquiterpenes	29.4	4.54	3.75	4	25.96	5.7	27.76	9.1	13.26	1.34	6.13	15.07	11.81	1.46	2.03	0	3.1	4.8
▨Sesquiterpene hydrocarbons	11.97	0.89	4.59	5.89	31.39	11.84	19.14	16.82	17.01	1.52	17.05	16.5	1.99	0.73	0.52	0	0.52	12.66

Elicitor treatments

Figure 6. Effects of different elicitor treatments on terpenoids content in HR culture of *S. bornmuelleri*. Mean values marked with different letters are statistically significant, according to Duncan's multiple range tests (P ≤ 0.01). Error bars for standard errors (SE), n = 3.

Citral was the most common oxygenated monoterpene identified in control and elicited HRCs (Table 2). The highest Citral level (12.99%) was obtained when HRs were treated 100 µM MeJA, resulting in an approximately eight-fold rise as compared to corresponding control. Among various oxygenated monoterpenes detected in HRCs, Thymol (3.01%) was found exclusively in HRCs elicited with 100 µM MeJA, as one of the elicitor-specific compounds (Table 2).

Effect of different elicitors on monoterpene hydrocarbons production

A total of 5 different monoterpene hydrocarbons were identified in HRCs, a value which was comparatively less than other identified terpenes (Table 2). Unlike other terpenes, monoterpene hydrocarbons were not present in wild root of *S. bornmuelleri* (Figure 6). The most common monoterpene hydrocarbon between different cultures was limonene that was found with maximum level of 1.1% in HRCs treated with 7 mM *β*-CD (Table 2). In addition, no obviously improved monoterpene hydrocarbons accumulation was observed as compared to control cultures, after HRs were treated with elicitors (Figure 6). Our results showed that other terpenes including diterpene and tetraterpenes did not find in elicited HRCs, control and wild roots of *S. bornmuelleri*. On the other hand, squalene (0.69%) was observed as the only triterpene in HRCs treated with 7 mM *β*-CD.

DISCUSSION

The main objective of the current study was to determine the potential of *S. bornmuelleri* HRs for production of terpenoids. HRCs were established by infection of stem explants with *A. rhizogenes* strain A4 with 100% frequency of HR induction.

To obtain high terpenoid-producing root lines, systematic selection based on dry weight was used, because the induced HRs lines exhibited different growth rates. The majority of HRs showed a vigorous growth, however, the line 9 was selected as the fastest growing line based on its fresh and dry weights (Table 1). Therefore the effect of CS, MeJA and *β*-CD elicitors on terpenoids production by *S. bornmuelleri* HR line 9 was investigated. This study is the first report that revealed the synthesis of 93 different terpenoids in HRs of *S. bornmuelleri* (Table 2). GC/MS analysis of untreated HRs showed the presence of 9 terpenes (Table 2). Results revealed the occurrence of new occurring metabolites in HRCs that normally are not found in the wild root. GC/MS analysis of methanolic extracts from control and elicited HRs revealed the presence of 79 new compounds (Table 2). Among new occurring terpenoids in *S. bornmuelleri* HRCs, 30 oxygenated sesquiterpenes, 35 sesquiterpene hydrocarbons, 10 oxygenated monoterpenes and 5 monoterpene hydrocarbons were identified (Table 2). Similar result was observed in *Artemisia annua* HRCs producing new sesquiterpene compounds [29].

Table 2. Terpenoids detected in control, elicited hairy root cultures and wild roots of *S. bornmuelleri*.

o	Terpenes	*RI	Elicitor treatments/Relative Quantity (%)																*Total %
	Sesquiterpene hydrocarbons		*W	*C	*MJ	*CD_1	*CD_2	*CD_3	*CS_1	*CS_2	*CS_3	*CD_1 MJ	*CD_2 MJ	*CD_3 MJ	*CS_1 MJ	*CS_2 MJ	*CS_3 MJ	*CD_1 CS_2	*CD_2 CS_2	
1	(+)-epi-Bicyclo sesquiphellandrene	1482	0.21	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	0.21
2	Eudesma-4(14),7(11)-diene	1544	1.14	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	1.14
3	Caryophyllene	1415	5.49	–	–	–	–	–	–	–	5.98	9.37	10.36	–	9.84	7.19	–	0.73	–	48.96
4	*cis*-*α*-Bergamotene	1415	0.63	–	–	–	–	–	–	–	–	–	0.29	–	–	–	–	–	–	0.92
5	2-Isopropyl-5-methyl-9-methylene[4.4.0]dec-1-ene	1503	–	–	0.5	–	–	–	0.52	–	–	–	–	–	–	–	–	–	–	1.02
6	1,4,7,Cycloundecatriene, 1,5,9,9-tetramethyl-	1552	2.21	–	–	–	–	–	–	4.13	1.89	2.12	2.38	–	1.54	2.5	0.71	–	–	17.48
7	γ-Gurjunene	1473	1.46	–	–	–	–	–	–	–	–	–	–	–	1.63	–	–	–	–	3.09
8	Selina-3,7(11)-diene	1542	1.52	–	–	–	–	–	–	3.44	2.45	1.75	1.8	–	2	1.88	0.65	–	–	15.49
9	Aromadendrene oxide	1648	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.63	–	–	0.63
10	*α*-Curcumene	1487	–	0.52	8.82	9.43	0.89	2.25	–	3.95	–	–	–	–	–	1.02	–	–	–	26.88
11	*α*-Patchoulene	1457	–	–	–	–	–	–	0.65	–	–	–	–	–	–	–	–	–	–	0.65
12	*α*-Cubebene	1352	–	–	0.32	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.32
13	*α*-Longipinene	1353	–	–	0.8	–	–	–	–	1.33	–	–	–	–	–	–	–	–	–	2.13
14	*β*-Bourbonene	1385	–	–	0.32	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.32
15	Germacrene D	1477	–	–	0.3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.3

Cont Table 2

16	**4-epi-*α*-Acoradiene**	**1463**	**–**	**–**	**1.01**	**–**	**–**	**–**	**–**	**–**	**–**	**–**	**–**	**1.52**	**–**	**–**	**–**	**–**	**–**	**2.53**
17	*α*-Curcumene, dihydro-	1445	–	–	2.35	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**2.35**
18	7-epi-Sesquithujene	1387	–	–	–	–	–	–	–	–	0.96	–	–	–	–	–	–	–	–	**0.96**
19	*α*-Guaiene	1457	–	–	–	–	–	–	–	0.3	–	–	–	–	–	–	–	–	–	**0.3**
20	*β*-Famesene	1452	–	–	–	1.03	–	–	–	4	0.56	–	–	–	–	0.47	–	–	–	**6.06**
21	Humulene	1455	–	–	–	–	–	–	–	1.76	–	0.73	–	–	–	–	–	–	–	**2.49**
22	Prezizaene	1391	–	–	–	–	–	–	–	0.7	–	–	–	–	–	–	–	–	–	**0.7**
23	Isoitalicene	1397	–	–	–	–	–	–	–	0.37	–	–	–	–	–	–	–	–	–	**0.37**
24	7-Epi-*α*-Selinene	1518	–	–	–	–	–	0.61	–	0.4	–	–	–	–	–	–	–	–	–	**1.01**
25	Isocaryophyllene	1417	–	–	–	–	–	–	–	4.84	–	–	–	–	–	–	–	–	–	**4.84**
26	*γ*-Selinene	1498	–	–	–	–	–	–	–	0.6	–	–	–	–	–	0.49	–	–	–	**1.09**
27	*trans*-*α*-Bergamotene	1434	–	–	–	–	–	–	–	–	–	–	–	–	–	0.69	–	–	–	**0.69**
28	*α*-Bisabolene	1450	–	–	–	–	–	–	–	–	–	–	0.68	–	0.52	0.66	–	–	–	**1.86**
29	Camphor	1150	–	–	–	–	–	–	–	0.14	–	–	–	–	–	–	–	–	–	**0.14**
30	Valerena-4,7(11)-diene	1453	–	–	–	–	–	–	–	–	–	–	–	–	1.18	–	–	–	–	**1.18**
31	Guaia-10(14),11-diene	1488	–	–	–	1.21	–	–	–	–	–	–	–	–	–	–	–	–	–	**1.21**
32	*β*-Barbatene	1452	–	–	–	0.3	–	–	–	–	–	–	–	–	–	–	–	–	–	**0.3**
33	*α*-Cedrene	1409	–	–	–	–	–	–	–	–		1.12	–	–	–	–	–	–	–	**1.12**
34	*β*-Himachalene	1499	–	–	–	–	–	–	–	–		0.27	–	–	–	–	–	–	–	**0.27**
35	*β* -Longipinene	1412	–	–	–	–	–	–	–	1.27	–	–	–	–	–	–	–	–	–	**1.27**
36	*β*-Maaliene	1414	–	–	–	–	–	0.52	–	–	–	–	–	–	–	–	–	–	–	**0.52**

Cont Table 2

37	***cis*-muurola-4(14),5-diene**	**1445**	**–**	**–**	**–**	**–**	**–**	**0.69**	**–**	**–**	**–**	**–**	**–**	**–**	**–**	**–**	**–**	**–**	**0.69**
38	1H-Indene, octahydro-2,2,4,4,7,7-hexamethyl-, *trans*-	1428	–	–	–	–	–	0.52	–	–	–	–	–	–	–	–	–	–	**0.52**
39	Valencene	1492	–	–	–	–	–	–	–	2.78	–	1.46	1.5	–	–	1.6	–	–	–	**7.34**
40	*β*-Curcumene	1514	–	–	–	–	–	–	–	0.59	–	–	–	–	–	–	–	–	–	**0.59**
41	*β*-Sesquisabinene	1462	–	–	–	–	–	–	4.72	0.79	–	–	–	–	0.34	–	–	–	–	**5.85**
42	Aromadendrane	1437	–	–	4.72	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**4.72**
	Total % of Sesquiterpene hydrocarbons	–	**12.66**	**0.52**	**19.14**	**11.97**	**0.89**	**4.59**	**5.89**	**31.39**	**11.84**	**16.82**	**17.01**	**1.52**	**17.05**	**16.5**	**1.99**	**0.73**	–	
	Oxygenated sesquiterpenes	–																		
43	*γ*-Eudesmol	1631	0.69	–	–	–	–	–	0.42	1.82	0.79	1.2	1.42	–	0.69	0.92	0.79	–	–	**8.74**
44	*α*- Eudesmol	1652	–	–	–	–	–	–	–	0.47	–	–	–	–	–	–	–	–	0.52	**0.99**
45	Guaiol	1597	1.28	–	–	–	–	–	0.76	2.92	1.2	1.32	1.58	–	1.59	1.85	1.65	–	–	**14.15**
46	*δ*-Cadinene	1523	–	–	–	–	–	–	–	–	–	–	0.23	–	0.3	–	–	–	–	**0.52**
47	Spatulenol	1578	–	1.92	8.74	9.19	1.14	2.32	–	3.21	–	0.95	0.83	–	–	0.85	–	–	0.45	**29.6**
48	Eudesm-7(11)-en-4-ol	1693	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.86	–	–	**0.86**
49	*β*-Eudesmol	1650	–	–	–	–	–	–	–	5.2	1.16	–	2.36	1.34	1.79	2.51	–	0.7	–	**15.06**
50	3-Eudesmen-11-ol	1641	–	–	–	–	–	–	–	–	–	2.23	0.37	–	–	4.34	–	–	–	**6.94**
51	Bulnesol	1666	–	–	–	–	–	–	–	2.09	–	0.88	1	–	–	1.93	3.19	–	–	**9.09**
52	Caryophyllene oxide	1580	–	1.18	1.77	5.38	1.68	0.84	–	0.36	–	–	1.84	–	–	–	–	–	–	**13.05**
53	*α*-Humulene epoxide II	1605	–	–	–	–	–	–	–	0.6	–	–	–	–	–	–	–	–	–	**0.6**

Cont Table 2

54	**α-Caryophylladienol**	**2323**	**–**	**–**	**–**	**4.25**	**–**	**–**	**–**	**0.43**	**–**	**–**	**–**	**–**	**–**	**–**	**–**	**–**	**4.68**
55	*cis*-Nuciferol	1728	–	–	–	1.93	–	–	–	–	–	–	–	–	–	–	–	–	**1.93**
56	Elemol	1548	1.3	–	–	–	–	–	1.6	–	0.96	–	–	–	–	–	–	–	**3.86**
57	Zizanol	1743	–	–	–	0.95	–	–	–	–	–	–	–	–	–	–	–	–	**0.95**
58	Limonen-6-ol, pivalate	1748	–	–	–	–	0.75	–	–	–	–	–	–	–	–	–	–	–	**0.75**
59	Nerolidol	1535	–	–	1.19	1.45	0.19	–	–	1.86	–	–	0.51	–	–	0.43	0.9	–	–	**6.53**
60	ar-Turmerol	1583	–	–	–	1.96	0.16	–	–	0.21	–	–	–	–	–	–	–	–	–	**2.33**
61	*α* -Bergamotenol	1702	–	–	0.95	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**0.95**
62	*β*-Santalol	1709	–	–	–	0.59	0.3	–	–	–	–	–	–	–	–	–	–	–	–	**0.89**
63	Aristolene epoxide	1896	–	–	0.71	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**0.71**
64	*β*-Funebrene	1411	–	–	1.59	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**1.59**
65	*cis*-Calamenene	1523	–	–	0.71	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**0.71**
66	Cedrol	1600	–	–	1.62	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**1.62**
67	Carotol	1596	–	–	0.53	0.55	–	–	–	–	–	–	–	–	–	–	–	–	–	**1.08**
68	*τ*-Cadinol	1640	–	–	3.83	0.92	0.32	0.59	–	–	–	–	–	–	–	–	–	–	–	**5.66**
69	*trans*-Longipinocarveol	1624	–	–	1.62	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**1.62**
70	ent-Germacra-4(15),5,10(14)-trien-1*β*-ol	1684	–	–	1.75	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**1.75**
71	1,4-*trans*-1,7-*cis*-Acorenone	1634	–	–	0.71	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**0.71**
72	*α*-Bisabolol	1683	1.53	–	–	–	–	–	–	6.79	1.59	2.52	3.12	–	1.76	2.24	4.42	0.76	1.06	**25.79**
73	*α*-Muurolene-14-ol	1771	–	–	0.29	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**0.29**

Cont Table 2

74	**ent-Germacra-4(15),5,10 (14)-trien-1b-ol**	**1680**	**–**	**–**	**1.75**	**–**	**–**	**–**	**–**	**–**	**–**	**–**	**–**	**–**	**–**	**–**	**–**	**–**	**1.75**
75	7-epi-*trans*-sesquisabinene hydrate	1582	–	–	–	–	–	–	1.22	–	–	–	–	–	–	–	–	–	–	**1.22**
76	Epi-Cubebol	1491	–	–	–	2.23	–	–	–	–	–	–	–	–	–	–	–	–	–	**2.23**
	Total % of Oxygenated sesquiterpenes	–	**4.8**	**3.1**	**27.76**	**29.4**	**4.54**	**3.75**	**4**	**25.96**	**5.7**	**9.1**	**13.26**	**1.34**	**6.13**	**15.07**	**11.81**	**1.46**	**2.03**	
	Oxygenated monoterpenes	–																		
77	1,2,4-Metheno-1H-cyclobuta[cd]pentalene-3,5-diol, octahydro-	1545	0.61	–	–	–	–	–	–	–	–	1.82	–	–	0.27	–	–	–	–	**2.7**
78	Eucalyptol	1030	–	0.44	0.25	0.3	0.92	–	–	–	–	–	–	–	–	–	–	–	–	**1.91**
79	*Artemisia* alcohol	1083	–	0.23	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**0.23**
80	Citral	1269	–	1.5	12.99	10.58	6.1	0.81	–	–	–	–	–	–	–	–	–	–	–	**31.98**
81	Geranic acid	1353	–	0.79	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**0.79**
82	cis-Carveol	1227	–	–	1.08	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**1.08**
83	Thymol	1308	–	–	–	–	–	3.01	–	–	–	–	–	–	–	–	–	–	–	**3.01**
84	*α*-Terpineol	1189	–	–	–	–	0.36	–	–	–	–	–	–	–	–	–	–	–	–	**0.36**
85	Isomenthol	1178	–	–	–	–	–	–	–	1.33	–	–	–	–	–	–	–	–	–	**1.33**
86	Isocitral	1184	–	–	–	–	0.44	–	–	–	–	–	–	–	–	–	–	–	–	**0.44**
87	*trans*-Ocimenol	1145	–	–	–	0.38	–	–	–	0.14	–	–	–	–	–	–	–	–	–	**0.52**

Cont Table 2

	Total % of Oxygenated monoterpenes	**–**	**0.61**	**2.96**	**14.32**	**11.26**	**7.82**	**3.82**	**–**	**1.47**	**–**	**1.82**	**–**	**–**	**0.27**	**–**	**–**	**–**	**–**	
	Monoterpene hydrocarbons	–																		
88	*β*-Cymene	1025	–	0.26	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**0.26**
89	D-Limonene	1030	–	0.23	0.21	–	1.1	–	–	–	–	–	–	–	–	–	–	–	–	**1.54**
90	*p*-Cymenene	1088	–	–	0.34	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**0.34**
91	*β*-Ocimene	1048	–	–	–	–	–	–	–	–	0.57	–	–	–	–	–	–	–	–	**0.57**
92	Santolina triene	908	–	–	–	–	–	0.84	–	–	–	–	–	–	–	–	–	–	–	**0.84**
	Total % of Monoterpene hydrocarbons	–	**–**	**0.49**	**0.55**	**–**	**1.1**	**0.84**	**–**	**–**	**0.57**	**–**	**–**	**–**	**–**	**–**	**–**	**–**	**–**	
	Triterpene	–																		
93	Squalene	2832	–	–	–	–	0.69	–	–	–	–	–	–	–	–	–	–	–	–	**0.69**
	**Total %	–	**18.52**	**7.38**	**44.88**	**54.03**	**15.23**	**13.92**	**9.86**	**59.4**	**18.11**	**27.93**	**30.78**	**2.86**	**24.8**	**31.57**	**13.8**	**2.19**	**2.03**	

*RI: Retention Index; *W: Wild root; C: Control (non-elicited hairy root); MJ: MeJA 100 µM; CD_1, CD_2 and CD_3: *β*-CD (0.7, 7.0 and 14 mM); CS_1, CS_2 and CS_3: CS (50, 100 and 200 mg L^{-1}); CD_1MJ, CD_2MJ and CD_3MJ: *β*-CD (0.7, 7.0 and 14 mM) plus MeJA 100 µM; CS_1MJ, CS_2MJ and CS_3MJ: CS (50, 100 and 200 mg L^{-1}) plus MeJA 100 µM; CD_1CS_2 and CD_2CS_2: *β*-CD (0.7 and 7 mM) plus CS 100 mg L^{-1}; *Total %: total % of specific terpene under all of treatments; **Total %: total % of terpenoids accumulated under specific treatment.

Terpenoids, also called isoprenoids, represent the most numerous and diverse class of plant floral volatiles, approximately 60% of known innumerable compounds found in many plants [30]. Generally, in plants monoterpenes are more frequent than sesquiterpenes, accounting for approximately 53% and 28% of the total floral terpenoids, respectively [31]. Nevertheless, the findings of this study showed the opposite results indicating significantly lower monoterpene content than sesquiterpenes in elicited HRCs. The main reason for the lower monoterpenes content as compared to sesquiterpenes is probably the growth conditions of HRCs i.e. dark condition. Given that HRCs were incubated in the dark to grow, the absence of light for photosynthesis caused less monoterpene production, whereas, because of light-independent biosynthesis of sesquiterpenes, their content was increased in HRCs treated with elicitors, even in dark condition.

In the last years, a number of studies have been conducted to evaluate the effect of different elicitors on secondary metabolites production by medicinal plants. Among different biotechnological techniques, application of elicitors on HRCs has been more common. Present study revealed that CS, MeJA and *β*-CD can act as stimulants. There are limited reports on enhanced sesquiterene production in CS elicited HRCs. Artemisinin is an antimalarial sesquiterpene endoperoxide isolated from *Artemisia* plant [32]. Putalun and coauthors (2007) reported increased artemisinin production in *Artemisia annua* L. HRCs by 6-fold over 6 days [33]. Similarly, our results showed that CS is the most effective elicitor for enhanced sesquiterpene production in *S. bornmuelleri* HRCs (Figure 5). Adding 100 mg L^{-1} CS, the production of sesquiterpenes including sesquiterpene hydrocarbons and oxygenated sesquiterpenes in elicited HRCs was increased 60 fold and 8.3 fold, respectively, as compared to controls. HRs elicitation with 100 µM MeJA, combined with CS (50 and 100 mg L^{-1}) increased sesquiterpene hydrocarbon and oxygenated sesquiterpenes production by 32.8 fold, 31.7 fold, 2 fold and 4.86 fold, respectively (Figure 5). In addition, of the elicitors used for treatment of HRCs, 100 µM MeJA had the greatest positive effect on oxygenated monoterpene production, besides its role in promoting sesquiterpenes production. MeJA is an important signaling compound in elicitation process, leading to secondary metabolite production in medicinal plants [34].

Enhanced sesquiterpene production in elicited HRCs was reported in *Solanum tuberosum* by adding *β*-CD individually or combined with MeJA [15]. Their study cleared that the production of sesquiterpenes was increased in *A. rhizogenes*-mediated transformed *Scutellaria* HRCs, when elicited with *β*-CD plus MeJA. Similarly, our research showed that the combination of *β*-CD and MeJA has a strong synergistic effect on sesquiterpene production. Treatment of cultures with *β*-CD individually (0.7 mM *β*-CD and 7 mM *β*-CD) or in combination with other elicitors (0.7 mM *β*-CD and 7 mM *β*-CD plus 100 µM MeJA) increased oxygenated sesquiterpene content by 2.9 fold and 4.3 fold, respectively, (Figure 5), while sesquiterpene hydrocarbon production was increased 32.3 fold and 32.7 fold, respectively, by adding 0.7 and 7 mM *β*-CD plus 100 µM MeJA and 0.7 mM *β*-CD when applied individually (Figure 5). GC-MS results revealed that among different terpenoids accumulated in elicited HRCs, caryophyllene with 43.47% (total content rate in elicited HRCs), spatulenol with 29.6%, *α*-curcumene with 26.88% and citral with 31.98% were the most abundant terpenes (Table 2). These terpenoids are found in various medicinal plants and are known for their potential biological activities in human diseases [35-37].

CONCLUSION

In conclusion, the maximized terpenoid production in *A. rhizogenes* mediated transformed *Scutellaria* HRCs using elicitors was reported here. Among accumulated terpenes, sesquiterpene production was significantly higher than monoterpene production. From the biotechnological point of view, hyper-production of terpenes following elicitation is of appreciable value. The present study could be recommended as an effective method to accumulate new and pharmaceutically useful sesquiterpenes in large scale cultures of *S. bornmuelleri* HRCs in bioreactor.

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