Applied Biochemistry and Biotechnology (2019) 187:817–837
https://doi.org/10.1007/s12010-018-2851-y

Enhanced Production of Phenolic Compounds in Compact Callus Aggregate Suspension Cultures of *Rhodiola imbricata* Edgew.

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Received: 31 May 2018 / Accepted: 25 July 2018 /
Published online: 8 August 2018
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Abstract

Rhodiola imbricata is a rare medicinal plant of the trans-Himalayan region of Ladakh. It is used for the treatment of numerous health ailments. Compact callus aggregate (CCA) suspension cultures of *Rhodiola imbricata* were established to counter extinction threats and for production of therapeutically valuable phenolic compounds to meet their increasing industrial demands. The present study also investigated the effect of jasmonic acid (JA) on production of phenolic compounds and bioactivities in CCA suspension cultures. CCA suspension cultures established in an optimized Murashige and Skoog medium supplemented with 30 g/l sucrose, 3 mg/l NAA, and 3 mg/l BAP showed maximum biomass accumulation (8.43 g/l DW) and highest salidroside production (3.37 mg/g DW). Upon 100 μM JA treatment, salidroside production (5.25 mg/g DW), total phenolic content (14.69 mg CHA/g DW), total flavonoid content (4.95 mg RE/g DW), and ascorbic acid content (17.93 mg/g DW) were significantly increased in cultures. In addition, DPPH-scavenging activity (56.32%) and total antioxidant capacity (60.45 mg QE/g DW) were significantly enhanced upon JA treatment, and this was positively correlated with increased accumulation of phenolic compounds. JA-elicited cultures exhibited highest antimicrobial activity against *Escherichia coli*. This is the first report describing the enhanced production of phenolic compounds and bioactivities from JA-elicited CCA suspension cultures of *Rhodiola imbricata*.

Keywords *Rhodiola imbricata* · Compact callus aggregate · Phenolic compounds · Jasmonic acid · Antioxidant · Antimicrobial

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s12010-018-2851-y) contains supplementary material, which is available to authorized users.

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Abbreviations

AAC	Ascorbic acid content
BAP	6-Benzylaminopurine
CHA	Chlorogenic acid
DFRSA	DPPH free radical scavenging activity
DW	Dry weight
FW	Fresh weight
JA	Jasmonic acid
MBC	Minimal bactericidal concentration
MIC	Minimal inhibitory concentration
NAA	1-naphthaleneacetic acid
PGRs	Plant growth regulators
QE	Quercetin equivalent
RE	Rutin equivalent
TAC	Total antioxidant capacity
TFC	Total flavonoid content
TPC	Total phenolic content

Introduction

Rhodiola imbricata Edgew. (Shrolo) family Crassulaceae is a multipurpose medicinal plant with well-established adaptogenic and antioxidant properties [1–6]. The plant grows along the rocky slopes and stony crevices of high-altitude pass (Penzila (14,000 ft), Changla (17,586 ft), and Khardungla (18,380 ft)) in the trans-Himalayan region of Ladakh. Recently, *R. imbricata* has attracted considerable scientific and commercial interest due to its unique phytochemistry and pronounced physiological and pharmacological activities. *R. imbricata* is widely used by the traditional herbal practitioners of indigenous community for the treatment of various human health ailments such as cold, cough, fever, and central nervous system disorders [5, 7]. It is a well-known medicinal herb because of its high content of bioactive compounds, including polyphenol, phenolic acid, flavonol, and flavonoids [4]. *R. imbricata* root is used as an important constituent in several nutraceutical and pharmaceutical preparations [4, 8]. It is also the major constituent of an herbal antioxidant tea patented as a medical supplement in India [9]. *R. imbricata* root contains high amounts of health-promoting nutritional constituents like essential amino acids, fatty acids, and dietary mineral elements [10]. Recently, Tayade et al. [11, 12] have reported the presence of various volatile and semi-volatile phytochemicals, fat-soluble vitamins (vitamin B complex), and water-soluble vitamin (vitamin E) in the underground part of this plant. *Rhodiola* preparations have been used for treating disorders like cerebral ischemia, diabetes, hypoxia, neurodegenerative diseases, and cancer [13]. *Rhodiola* is also used as a dietary supplement to increase work performance, longevity and improve resistance to high-altitude sickness [14, 15]. A number of pharmacological investigations have demonstrated that *R. imbricata* preparations exhibit antiviral [16], radioprotective [17, 18], wound healing [19], immunomodulatory [20], adaptogenic [2], anti-cancer [6, 21], immunostimulatory [20, 22], hepatoprotective [23], cytoprotective, and antioxidant properties [1, 2, 4, 5]. The majority of these pharmacological properties have been attributed to its diverse array

of phenolic compounds [3, 4, 14, 23]. Phenolic compounds have been used for centuries in different medicinal applications [24]. Phenolic compounds exert a beneficial lifespan enhancing effect by acting as antioxidants [25]. These compounds are of great interest on account of their biological activities involved in human health-related issues [24]. Applications of phenolic compounds are also increasing rapidly in the food and pharmaceutical industry [26, 27]. Salidroside and Tyrosol (phenylethanoids) are considered to be the major bioactive compounds of *Rhodiola* [28–30]. Salidroside and tyrosol have received a considerable amount of attention due to their broad range of pharmacological properties, including anti-stress, antifatigue, adaptogenic, cardioprotective, antidepressant, hepatoprotective, and immunomodulatory properties [31–33].

The natural populations of *R. imbricata* are highly threatened and classified as rare in the red data list of Indian flora [34]. Field cultivation of this plant is a laborious and time-consuming process. These issues necessitate the development of alternate strategies for the production of medicinally important phenolic compounds of *R. imbricata*. Plant tissue culture, in particular compact callus aggregate (CCA) culture offers a promising system for the sustainable and economical production of secondary metabolites [35, 36]. The CCA shows some degree of differentiation that favors the increased production of secondary metabolites [35–38]. Several studies have demonstrated that the CCA culture is an efficient alternative source of secondary metabolites, such as, indole alkaloids of *Catharanthus roseus* [36, 38] and phenylethanoid glycosides of *Rhodiola sachalinensis* [35], but so far, the literature contains no reports on CCA suspension culture of *R. imbricata*.

Plant tissue culture systems are often constrained by low yield of secondary metabolites and genetic instability of cell lines [36, 38]. Elicitation has been widely used to increase the production of secondary metabolites in plant cell cultures [39, 40]. Plants have evolved a wide variety of inducible defense mechanisms that are triggered upon elicitation [41]. Numerous studies have demonstrated that the addition of an elicitor (biotic or abiotic) to the culture media significantly increase the production of secondary metabolites in vitro by triggering a metabolic cascade [40, 41]. Jasmonic acid has been recognized as an effective elicitor that triggers the biosynthesis of secondary defense compounds in plant suspension cultures by activating the genes of secondary metabolism [42]. Jasmonic acid (JA) is also considered as a global regulator of inducible defense gene expression in plants [43]. It has been reported that JA significantly increases the production of taxol and paclitaxel in *Taxus* sp. cell suspensions cultures [44], hypericin in *Hypericum perforatum* cell suspension cultures [45, 46], ginsenoside in *Panax ginseng* adventitious root cultures [47], triterpenes in *Jatropha curcas* cell suspension cultures [48] and terpenoid indole alkaloids in *Catharanthus roseus* cell suspension cultures [49]. Despite the proven role of JA in enhancing the production of secondary metabolites in in vitro cultures, such studies have never been applied to *R. imbricata* cell cultures.

Therefore, keeping in view the medicinal and commercial value of *R. imbricata*, the aim of the present study was to optimize the culture media components (basal media, carbon source, sucrose concentration and PGRs) for the sustainable production of medicinally important phenolic compounds by CCA suspension cultures of *R. imbricata*. Furthermore, the present study also investigated the time-course effect of Jasmonic acid (JA) on biomass accumulation, production of phenolic compounds (phenylethanoids (salidroside and tyrosol), total phenolics and total flavonoids), ascorbic acid, and antioxidant (DFRSA and TAC) and antimicrobial activity in CCA suspension cultures of *Rhodiola imbricata*.

Material and Methods

Plant Material and Chemicals

Rhodiola imbricata plants were collected from Khardungla pass of trans-Himalayan Ladakh region (18,380 ft., 34.282° N and 77.597° E) in July 2015. The plants were identified by the Botanical Survey of India, Dehradun (Accession number – 117062).

All authentic standards were obtained from Sigma-Aldrich. All solvents (methanol and acetonitrile) were of high-performance liquid chromatography (HPLC) grade and were obtained from Merck. MilliQ water was used for HPLC analysis. The other chemicals used in this work were of analytical grade purchased from Sigma-Aldrich.

Establishment of CCA Suspension Cultures

Callus cultures were established according to the previously developed method [50]. Briefly, callus was induced from the leaf explant and then propagated on modified Murashige and Skoog (MS) medium supplemented with 30 g/l sucrose, 8 g/l agar, 3 mg/l NAA and 3 mg/l BAP. After 3 weeks, the cellular clumps consisting of small compact callus aggregates (CCA) were carefully selected and excised.

To establish CCA suspension culture of *R. imbricata*, 20 g fresh weight of CCA was transferred to 500 ml Erlenmeyer flask containing 250 ml of modified MS medium [51] supplemented with 30 g/l sucrose, 3 mg/l NAA and 3 mg/l BAP. The culture flasks were placed on a gyratory shaker (110 rpm) at 25 ± 1 °C in continuous light. The pH of the medium was adjusted to 5.75 before autoclaving at 121 °C for 15 min. The cultures were maintained by regular sub-culturing at 10 days interval.

Effect of Culture Media Components

To investigate the effect of different strength of basal media on biomass (FW and DW) and phenylethanoids (Salidroside and Tyrosol) production, 1 g fresh weight of CCA was transferred to 100 ml Erlenmeyer flask containing 20 ml of different basal media (MS, 1/2 MS, B5 [52] and 1/2 B5) supplemented with 30 g/l sucrose, 3 mg/l NAA and 3 mg/l BAP. The culture flasks were placed on a gyratory shaker (110 rpm) at 25 ± 1 °C in continuous light. The biomass and phenylethanoids accumulation was determined after 10 days.

To investigate the effect of different carbon sources on biomass and phenylethanoids production, 1 g fresh weight of CCA was inoculated in modified MS medium containing 3 mg/l NAA, 3 mg/l BAP and 30 g/l of different carbon sources (sucrose, glucose, fructose and maltose). Further, the effect of different sucrose concentrations (10, 30, 50, 70 g/l) was investigated in the same media composition. The biomass and phenylethanoids accumulation was analyzed after 10 days.

To investigate the influence of different PGRs on biomass and phenylethanoids production, 1 g fresh weight of CCA was inoculated in modified MS medium containing 30 g/l sucrose and different concentrations and combinations of NAA (0.1, 0.5, 1, 1.5, 3 mg/l) and BAP (0.1, 0.5, 1, 1.5, 3 mg/l). The time course of biomass and phenylethanoids accumulation was accomplished with an interval of 2 days for 10 days period.

Effect of JA Treatment

For elicitation experiments, Jasmonic acid was dissolved in 95% (*v*/*v*) methanol and filter sterilized through 0.22 μm PVDF filter. Subsequently, 1 g fresh weight of CCA was transferred to 100 ml Erlenmeyer flask containing 20 ml of modified MS medium supplemented with 30 g/l sucrose, 3 mg/l NAA, 3 mg/l BAP and different concentrations of Jasmonic acid (5 and 100 μM). MS basal medium containing 3 mg/l of NAA and BAP without Jasmonic acid was used as control. The pH of the medium was adjusted to 5.75 before autoclaving at 121 °C for 15 min. The culture flasks were placed on a gyratory shaker (110 rpm) at 25 ± 1 °C in continuous light. The time course of biomass accumulation, phenolic compounds production, and antioxidant activity was done at an interval of 2 days for 6 days period.

Analytical Methods

For fresh weight (FW) determination, the CCA suspension cultures were harvested, washed with distilled water and filtered through vacuum driven filter and then weighed. Subsequently, CCA suspension cultures were oven dried to constant weight at 40 °C for dry weight (DW) determination. The fresh weight and dry weight of CCA suspension cultures was expressed in grams per liter of medium (g/l).

The dried CCA suspension samples were extracted according to the previously established method [50]. Briefly, the samples were pulverized with a mortar and pestle and then suspended in methanol at a sample-to-solvent ratio of 1:15 (*w*/*v*). Subsequently, the samples were extracted by ultrasonication for 3 × 30 min at room temperature. After sonication, extracts were centrifuged at 5000*g* for 5 min at 4 °C, and the supernatant was collected and filtered through 0.22 μm PVDF filter. The filtered extracts were stored at − 20 °C till further analysis.

The quantification of phenylethanoids were performed according to the previously developed method [50]. Briefly, phenylethanoids (Salidroside and Tyrosol) were quantified by RP-HPLC using an Agilent 1260 infinity chromatographic system equipped with diode array detector. The separation of salidroside and tyrosol was achieved on a reverse phase column (Agilent Zorbax eclipse plus C18 (4.6 × 100 mm, 3.5 μm)). The mobile phase consisted of acetonitrile (A) and water (B) at a flow rate of 1 ml/min. The temperature of the column was maintained at 25 °C, sample injection volume was 5 μl and detection was done at 225 nm. The separation was done in an isocratic manner with 15% A/85% B for 6 min followed by a 4-min wash with 100% A and an equilibration period of 4 min with 15% A/85% B. The data was analyzed using Agilent OpenLAB CDS (EZChrom edition, version A.04.04). The absorption spectra and retention time of authentic standards were used to identify phenylethanoids in the extracts. The linear regression equation was used for the quantification of phenylethanoids and the results were expressed as mg/g DW.

The yield of phenylethanoids was calculated by using the following formula and the results were expressed as mg/l.

$$Phenylethanoids\ yield\ (mg/l) = Dry\ weight\ (g/l) \times Phenylethanoids\ content\ (mg/g)$$

The total phenolic content and ascorbic acid content were simultaneously determined by the method of Sánchez-Rangel et al. [53]. The absorbance of the reaction mixture was determined at 765 nm using a multi-mode microplate reader (SpectraMax M5, Molecular devices, USA) and the data was processed and analyzed by SoftMax Pro 6.1 software. Ascorbic acid (0.02–

0.1 mg/ml; (AA)) and Chlorogenic acid (0.02–0.125 mg/ml; (CHA)) were used for plotting standard calibration curve. The total phenolic content and ascorbic acid were calculated using the following equation based on the calibration curve ($y = 0.0012x + 0.0103$, $r^2 = 0.990$ (CHA) and ($y = 0.0006x - 0.0064$, $r^2 = 0.998$ (AA)). The total phenolic content of different methanolic extracts was expressed as mg CHA/g DW and the ascorbic acid content was expressed as mg/g DW.

The total phenolic production (TPP) was determined by using the following formula and the results were expressed as mg/l.

$$Total\ phenolic\ production\ (mg/l) = Dry\ weight\ (g/l) \times TPC\ (mgCHA/g)$$

The ascorbic acid production (AAP) was determined by using the following formula and the results were expressed as mg/l.

$$Ascorbic\ acid\ production\ (mg/l) = Dry\ weight\ (g/l) \times AAC\ (mg/g)$$

The total flavonoid content was determined according to the method of Zhishen et al. [54]. The absorbance of the reaction mixture was measured at 510 nm with a UV-Visible spectrophotometer (Genesys 10S UV-Vis, Thermo scientific). Rutin (0.025–0.125 mg/ml) was used for plotting standard calibration curve. The total flavonoid content was calculated using the following equation based on the calibration curve ($y = 0.0007x + 0.0011$, $r^2 = 0.998$) and the results were expressed as mg RE (rutin equivalent)/g DW.

The total flavonoid production (TFP) was determined by using the following formula and the results were expressed as mg/l.

$$Total\ flavonoid\ production\ (mg/l) = Dry\ wieght\ (g/l) \times TFC\ (mgRE/g)$$

The DPPH free radical scavenging activity was determined according to the method of Yesmin et al. [55], with slight modifications. Briefly in 0.2 ml of different methanolic extracts (0.125 mg/ml), 3 ml of methanolic DPPH solution (0.004% *w*/*v*) was added. The reaction mixture was left in the dark at room temperature for 30 min and the absorbance of the solution was measured at 517 nm with a UV-Visible spectrophotometer. The DPPH free radical scavenging activity (DFRSA (%)) of different methanolic extracts was calculated as follows:

$$DFRSA\ (\%) = [(Abs\ Control{-}Abs\ Sample) \div Abs\ Sample] \times 100$$

where $Abs_{\ Control}$ is the absorbance of DPPH radical and methanol and $Abs_{\ Sample}$ is the absorbance of DPPH radical with sample extract.

The total antioxidant capacity was determined by using phosphomolybdenum method as described by Prieto et al. [56]. The absorbance of the solution was measured at 695 nm with a UV-Visible spectrophotometer. Quercetin (0.025–0.125 mg/ml) was used for plotting standard calibration curve. The total antioxidant capacity was calculated using the following equation based on the calibration curve ($y = 0.0019x - 0.025$, $r^2 = 0.995$) and the results were expressed as mg QE (quercetin equivalent)/g DW.

The antimicrobial activities of JA-treated (5 and 100 μM) and untreated (control) CCA suspension cultures of *R. imbricata* were tested against Gram-positive methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300 and Gram-negative *Escherichia coli* ATCC 25922 using agar well diffusion method [57]. Briefly, 100 μl of test bacterial suspension containing 10^6-10^8 cells/ml was poured and uniformly spread over the entire surface of sterile nutrient agar plates. Then, the wells (5 mm in diameter) were cut from

the agar using a sterile-cork borer, and 50 μl of 40 mg/ml extract solution was loaded in the wells. Gentamicin (50 μg/ml) was used as a positive control. Methanol was used as a negative control. The extract solution and control were allowed to diffuse for 1–2 h at room temperature and subsequently, the plates were incubated at 37 °C for 18–24 h. After incubation for 18–24 h, the antimicrobial activity was assessed by measuring the diameter of the growth-inhibition zone in millimeters. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined by CLSI guidelines [58, 59]. For determination of MIC, a twofold dilution (50 μl) of CCA suspension cultures extracts (0.15, 0.31, 0.62, 1.25, 2.50, 5, 10, 20, 40 mg/ml) were prepared in brain heart infusion (BHI) liquid growth medium and dispensed in a sterile 96-well microtiter plate. Then, each well was inoculated with 50 μl of test bacterial suspension (10^6-10^8 cells/ml). The microtiter plates were then incubated at 37 °C for 18–24 h. After incubation for 18–24 h, the microtiter plates were examined for turbidity which corresponds to the growth of bacterial cells. The MIC was determined as the lowest concentration of the compound that completely inhibit the visible bacterial growth. The MBC was determined by inoculation of test samples (100 μl) from the 96-well microtiter plates on sterile nutrient agar plates. The plates were incubated overnight at 37 °C. After overnight incubation, the plates were observed for cell viability. The MBC was defined as the lowest concentration of the compound that kill 99.5% of bacteria in the original inoculum.

Statistical Analysis

The experiments were conducted in a completely randomized design (CRD). All analyses were performed in triplicate and the results were presented as mean ± standard error (SE). One-way ANOVA was used to evaluate the difference between group means and pair-wise comparison among group means was assessed using the Bonferroni post hoc test at $p \leq 0.05$. Pearson's correlation coefficient was used for correlation analyses at $p \leq 0.01$. All statistical analyses were performed using SPSS software (SPSS version 21.0, USA). Figures were generated using Microsoft office (Windows version 2016, USA).

Results and Discussion

Effect of Basal Media

The present study investigated the effect of different strength of basal media on biomass and phenylethanoids accumulation in CCA suspension cultures of *R. imbricata*. As shown in Fig. 1a, CCA suspension cultures showed maximum biomass accumulation (FW: 221.59 ± 0.71 g/l, DW: 8.43 ± 0.01 g/l) and highest salidroside production (3.37 ± 0.03 mg/g DW) in full strength MS media, followed by 1/2 MS, B5 and 1/2 B5 media. These findings demonstrated the superior role of full strength MS medium on biomass accumulation as well as salidroside production in CCA suspension cultures of *R. imbricata.* This increase might be attributed to the higher ionic strength and nitrogen content of the MS medium [60]. Similar to our results, full strength MS medium was found to be most suitable for biomass accumulation and secondary metabolite production in cell suspension cultures of *Withania somnifera* [61] and *Gymnema sylvestre* [62].

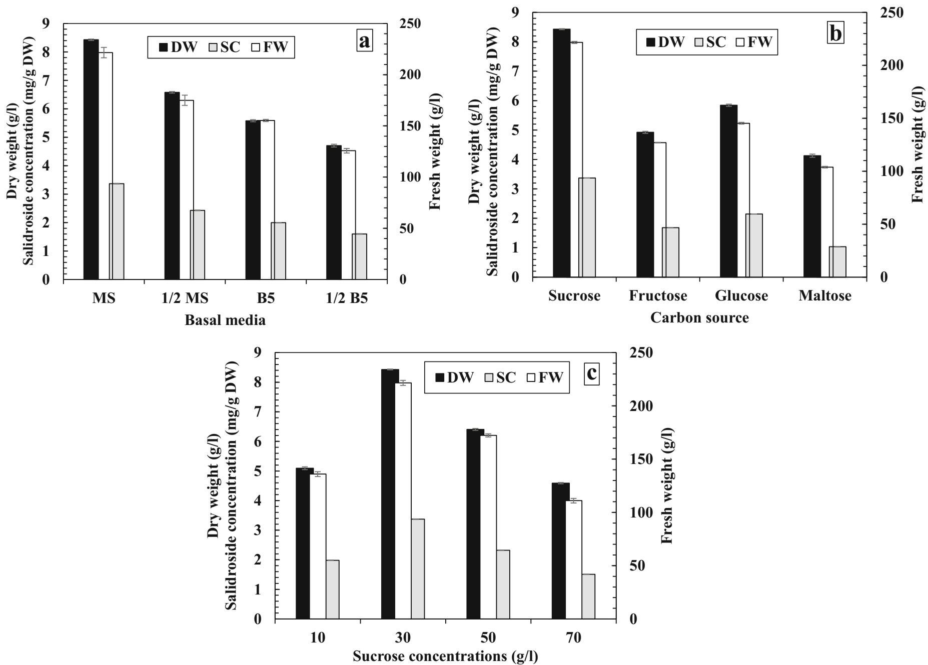

Fig. 1 Effect of different **a** strength of basal media, **b** carbon sources, and **c** sucrose concentrations on dry weight (DW), fresh weight (FW), and salidroside concentration (SC) in CCA suspension cultures of *Rhodiola imbricata* after 10 days of culture. Values are mean ± standard error (vertical error bars) of three replicates

Effect of Carbon Source and Sucrose Concentration

Sugar acts as an important energy source as well as signaling molecule that affects growth and metabolism of cultured cells [63, 64]. Therefore, the present study investigated the effect of carbon source on biomass and phenylethanoids accumulation in CCA suspension cultures of *R. imbricata.* As shown in Fig. 1b, the CCA suspension cultures showed maximum biomass accumulation (FW: 221.59 ± 0.71 g/l, DW: 8.43 ± 0.01 g/l) and highest production of salidroside (3.37 ± 0.03 mg/g DW) in MS medium supplemented with sucrose, followed by glucose, fructose and maltose. The variations in the effect of different sugar might be due to the differential signaling levels of sugar [61]. Similar to our results, sucrose-induced enhanced biomass accumulation and secondary metabolites production in cell suspension cultures of *Withania somnifera* [61] and *Artemisia absinthium* [65]. This might be attributed to the efficient uptake of sucrose across the plasma membrane [66]. These findings demonstrated the superior role of sucrose on biomass accumulation as well as salidroside production in CCA suspension cultures of *R. imbricata.*

We further investigated the effect of different concentrations of sucrose on biomass accumulation and phenylethanoids production in CCA suspension cultures of *R. imbricata.* As shown in Fig. 1c, the fresh weight, dry weight and salidroside content decreased when the initial sucrose concentration was increased from 30 g/l to 50 g/l and 70 g/l, respectively. The maximum fresh weight of 221.59 ± 0.71 g/l, dry weight of 8.43 ± 0.01 g/l and salidroside content of 3.37 ± 0.03 mg/g DW were obtained at an initial sucrose concentration of 30 g/l (Fig. 1c). These findings suggest that the higher concentrations of sucrose suppressed the

biomass accumulation as well as salidroside production in *R. imbricata* CCA suspension cultures, probably due to high osmotic stress and substrate inhibition. Similarly, Xu et al. [35] also reported that the higher concentrations of sucrose reduced the salidroside production in CCA suspension cultures of *Rhodiola sachalinensis*. Zhao et al. [38] found that the sucrose regime affected the degree of compaction and differentiation level of the compact callus cluster cultures of *Catharanthus roseus* and therefore influenced alkaloid production. Our data supports the proposition that the effects of carbohydrate concentration were different according to the plant species and type of secondary metabolites [67, 68]. The results suggest that 30 g/l of sucrose is optimum for growth and salidroside accumulation in CCA suspension cultures of *R. imbricata*.

Effect of Plant Growth Regulators

The effect of different concentrations and combinations of PGRs on biomass and phenylethanoids accumulation in CCA suspension cultures of *R. imbricata* is shown in Fig. 2a–d. Among different concentrations and combinations of PGRs tested, MS medium supplemented with 3 mg/l NAA + 3 mg/l BAP showed maximum accumulation of biomass (FW: 221.59 ± 0.71 g/l, DW: 8.43 ± 0.01 g/l) and highest production of salidroside (3.37 ± 0.03 mg/g DW) and salidroside yield (28.45 ± 0.04 mg/l). The fresh weight, dry weight, salidroside content and salidroside yield reached their respective peak after 6 days of culture (Fig. 2a–d). NAA and BAP at lower concentrations resulted in significant reduction in the

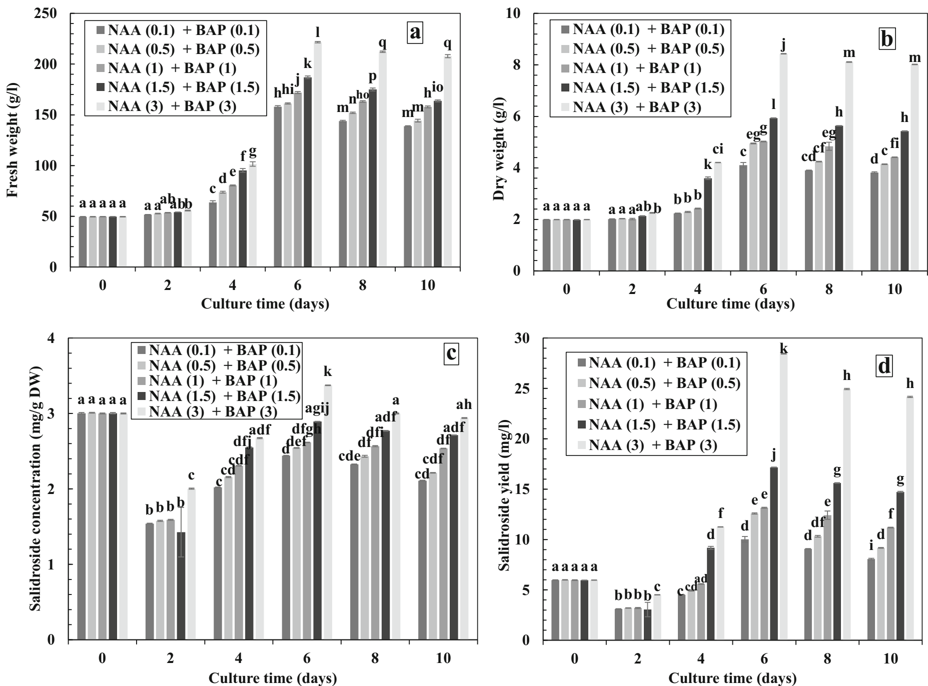

Fig. 2 Effect of different concentrations and combinations of plant growth regulators on **a** fresh weight, **b** dry weight, **c** salidroside concentration, and **d** salidroside yield in CCA suspension cultures of *Rhodiola imbricata*. Values are mean ± standard error (vertical error bars) of three replicates. Means with similar letters are not significantly different at $p \leq 0.05$ according to Bonferroni post hoc test

biomass accumulation and salidroside production, as compared to higher concentrations ($p \leq 0.05$, Fig. 2a–c). It is noteworthy that none of the treatments of NAA and BAP showed the presence of tyrosol in CCA suspension cultures (Supplementary Fig. 1). This might be attributed to the conversion of tyrosol into salidroside or some other unknown intermediates. The pH of the medium declined during the first 6 days and then increased gradually (data not shown). The time course analysis revealed that the accumulation of salidroside was growth-dependent, which might be attributed to the differentiated structure of CCA [69]. A similar growth-associated pattern for secondary metabolites accumulation has been observed in other plant cell cultures [70, 71]. Our findings, together with the previous reports, suggest that the synergistic combinations of auxin and cytokinin can significantly alter the production of secondary metabolites depending on plant species [72, 73]. Plant cell cultures are required to be maintained in empirically optimized auxin/cytokinin composition to obtain high productivity [74]. In this study, we have optimized that the full-strength MS medium supplemented with 3 mg/l NAA + 3 mg/l BAP is suitable for the obtention of maximum biomass accumulation and salidroside production in CCA suspension cultures of *R. imbricata*.

Effect of JA on Growth

As shown in Fig. 3a, b, the growth of CCA suspension cultures was inhibited by the addition of JA to the culture medium. Supplementation of 5 μM JA to *R. imbricata* CCA suspension cultures showed a significant decrease in biomass accumulation (FW: 191.71 ± 0.79 g/l, DW: 7.30 ± 0.01 g/l), as compared to control cultures (FW: 221.59 ± 0.70 g/l, DW: 8.43 ± 0.01 g/l). Moreover, this effect was accentuated with the increase of JA concentration (Fig. 3a, b). An addition of 100 μM JA to the medium led to a substantial decrease in biomass accumulation (FW: 177.95 ± 1.51 g/l, DW: 7.05 ± 0.02 g/l), as compared to 5 μM JA-treated cultures and control ($p \leq 0.05$, Fig. 3a, b). The results suggest that the fresh weight and dry weight of the CCA suspension cultures of *R. imbricata* decreased with increasing jasmonic acid concentration. Similar phenomenon has also been observed in cell cultures of *Panax ginseng* [47] and *Hevea brasiliensis* [75]. JA has long been associated with the inhibition of plant growth [76]. JA affects the cell proliferation and cell cycle progression by downregulating the expression of the CYCB1;1, cyclin-dependent kinase CDK-B and mitotic phase genes, largely in a COI1-dependent manner [77–79]. A recent study by Yang et al. [80] demonstrated that JA induced growth inhibition by interfering with gibberellin signaling cascade via COI1–JAZ–DELLA–PIF signaling module.

Effect of JA on Production of Phenylethanoids

JA is a well-established signal transducer in plant defense responses and an effective inducer of secondary metabolite accumulation in plant cell cultures [42, 81]. It has been reported that JA signaling triggers genome-wide alterations in gene expression [82]. Therefore, the present study investigated the effect of JA on the production of phenylethanoids in CCA suspension cultures of *R. imbricata.* As shown in Fig. 3c, the JA-treated CCA suspension cultures showed an exponential increase in accumulation of salidroside during the first 4 days and then a gradual decrease until day 6. After 4 days of treatment with 100 μM JA, the CCA suspension cultures showed maximum accumulation of salidroside (5.25 ± 0.01 mg/g DW), which was 1.11-fold and 1.55-fold higher than 5 μM JA-treated cultures (4.72 ± 0.01 mg/g DW) and control (3.37 ± 0.03 mg/g DW), respectively (Fig. 3c). As shown in Fig. 3d, 100 μM JA-

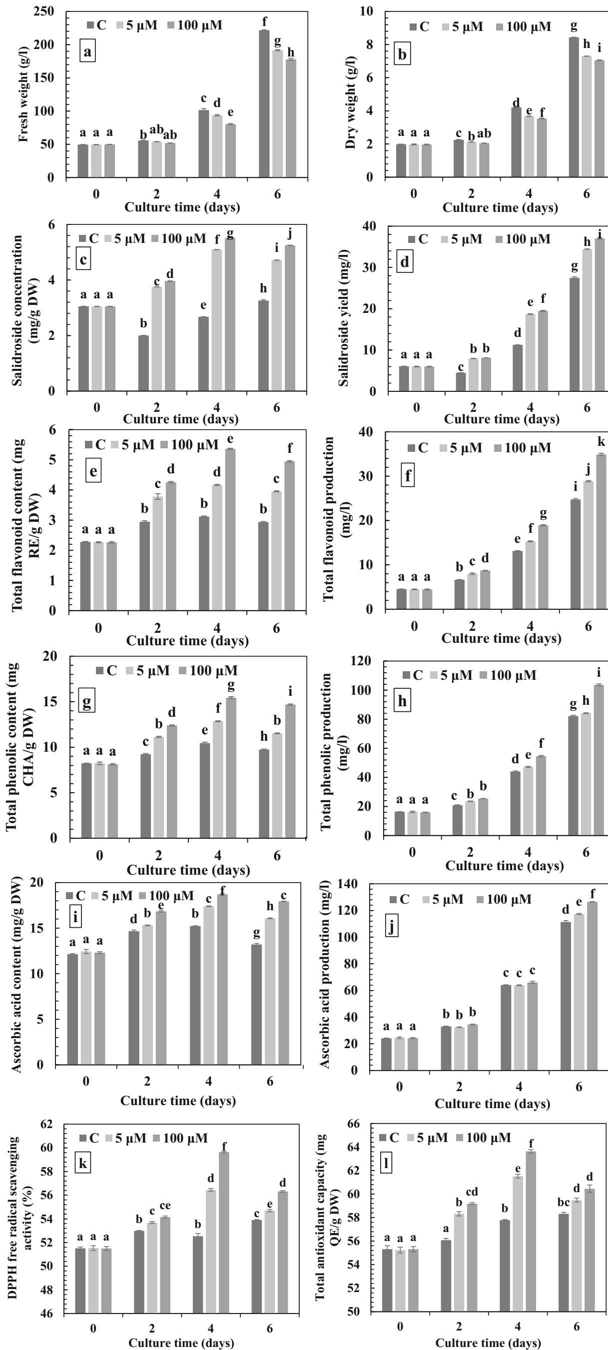

Fig. 3 Effect of different concentrations of jasmonic acid on **a** fresh weight, **b** dry weight, **c** salidroside concentration, **d** salidroside yield, **e** total flavonoid content, **f** total flavonoid production, **g** total phenolic content, **h** total phenolic production, **i** ascorbic acid content, **j** ascorbic acid production, **k** DPPH free radical scavenging activity, and **l** total antioxidant capacity in CCA suspension cultures of *Rhodiola imbricata.* Values are mean ± standard error (vertical error bars) of three replicates. Means with similar letters are not significantly different at $p \leq 0.05$ according to Bonferroni post hoc test

treated cultures demonstrated maximum salidroside yield (37 ± 0.15 mg/l) after 6 days of treatment, which was 1.34-fold higher than the control (27.49 ± 0.25 mg/l). It is noteworthy that the tyrosol was absent in all the cultures (Supplementary Fig. 2). These findings suggest that CCA suspension cultures of *R. imbricata* were amenable for elicitation with JA as evident by increased accumulation of salidroside. It is plausible that this stimulation is mediated by enhanced transcription of JA-responsive genes encoding key enzymes involved in salidroside biosynthesis. Yu et al. [83] found that methyl jasmonate (MJ; 250 μM) treatment upregulated the expression pattern of UDP-glycosyltransferases (UGTs) involved in salidroside biosynthesis in cell cultures of *Rhodiola sachalinensis*. Similarly, Bhat et al. [84] also observed that MJ treatment (100 μM) induced a rapid increase in the transcript level of UGTs in *Picrorhiza kurrooa*. These findings indicate that the addition of JA to the culture medium increased the accumulation of salidroside in a concentration and time-dependent manner. The results suggest that the treatment with 100 μM JA is an effective way to enhance the biosynthesis of salidroside in CCA suspension cultures of *R. imbricata*.

Effect of JA on Total Phenolic Content and Total Flavonoid Content

Jasmonic acid is considered to be involved in the signal transduction pathway that induces the production of defense compounds in plants, such as alkaloids, terpenoids and polyphenols [85]. Therefore, the present study investigated the influence of JA on TPC, TFC, TPP and TFP in CCA suspension cultures of *R. imbricata*. The time course analysis of JA-treated CCA suspension cultures showed an exponential increase in TPC and TFC during the first 4 days and then a gradual decrease until day 6 (Fig. 3 e, g). After 4 days of addition of 100 μM JA, the CCA suspension cultures displayed maximum TPC (14.69 ± 0.06 mg CHA/g DW) and TFC (4.95 ± 0.02 mg RE/g DW), which was 1.50-fold and 1.68-fold higher than the control (TPC: 9.74 ± 0.06 mg CHA/g DW and TFC: 2.93 ± 0.02 mg RE/g DW). In contrast, 5 μM JA induced low accumulation of TPC (11.53 ± 0.04 mg CHA/g DW) and TFC (3.95 ± 0.02 mg RE/g DW), as compared to 100 μM JA-treated cultures ($p \leq 0.05$, Fig. 3 e, g). As shown in Fig. 3 f, h, 100 μM JA-treated cultures showed maximum TPP (103.59 ± 0.67 mg/l) and TFP (34.89 ± 0.25 mg/l) after 6 days of treatment, which was 1.26-fold and 1.49-fold higher than the control (TPP: 82.14 ± 0.52 mg/l and TFP: 24.74 ± 0.19 mg/l). These findings clearly demonstrated a concentration and time-dependent response to JA treatment. Similar responses have been previously reported in other plant cell cultures [86]. Gadzovska et al. [46] reported a 6-fold increase in phenolic compounds and flavonols in *Hypericum perforatum* suspensions cultures after addition of JA to the culture medium. This enhancement in phenolic compounds is usually associated with a transient increase in activities of key enzymes of the phenylpropanoid pathway such as phenylalanine ammonia lyase and chalcone isomerase [42, 87]. Jasmonates are considered as conserved elicitors of plant secondary metabolism [88]. Several researchers have reported that in different plant species, such as, *Arabidopsis*, tobacco and periwinkle, JA trigger a transcriptional cascade, consisting of primary inductive wave, which regulate the primary members of JA signalling (COI1 (CORONATINE INSENSITIVE 1), MYC2 (bHLH transcription factor) and Jasmonate ZIM-domain (JAZ) repressor proteins), followed by several other waves that include members of other transcription factor family (AP2/ERF), which eventually regulate the species-specific secondary metabolic pathways [89–91]. In primary inductive wave, JA-Ile complex stimulates the recruitment of JAZ repressor proteins (negative regulator of JA-responsive genes) by SCF^{COI1} complex for ubiquitination and subsequent degradation by 26S proteasome. Subsequently, MYC2 can

activate transcription of early JA-responsive genes [92]. Therefore, in case of CCA suspension cultures of *R. imbricata*, genome-wide transcript profiling studies are warranted to elucidate the JA-induced biosynthesis of phenolic compounds. In this study, the phenolic compounds production profile indicated that 4th day is the optimum time for the harvest of the CCA suspension cultures to obtain maximum phenolic compounds production. The results demonstrated the suitability of JA-elicited CCA suspension cultures of *R. imbricata* for the biotechnological production of phenolic compounds, which have high medicinal and commercial value [27]. The JA-elicited CCA suspension cultures of *R. imbricata* could be used as a potential model system to expand and enhance our understanding on the biosynthesis of phenolic compounds.

Effect of JA on Ascorbic Acid Content

As shown in Fig. 3 i, the JA-treated CCA suspension cultures of *R. imbricata* showed an exponential increase in AAC during the first 4 days and then a gradual decrease until day 6. After 4 days of treatment with 100 μM JA, the CCA suspension culture showed maximum AAC (17.93 ± 0.03 mg/g DW), which was 1.12-fold and 1.35-fold higher than the 5 μM JA-treated cultures (16.08 ± 0.04 mg/g DW) and control (13.20 ± 0.13 mg/g DW), respectively (Fig. 3 i). As shown in Fig. 3 j, 100 μM JA-treated CCA suspension cultures demonstrated maximum AAP (126.47 ± 0.19 mg/l) after 6 days of treatment, which was 1.13-fold higher than the control (111.35 ± 1.10 mg/l). A similar increase in de novo synthesis of ascorbic acid has been observed in jasmonate-elicited BY-2 cells of tobacco, which involves the induction of late-jasmonate responsive GDP-Man 3″,5″-epimerase and L-gulono-1,4-lactone dehydrogenase genes [93, 94]. A rapid synthesis and regeneration of ascorbate in response to JA treatment has been associated with the protection of tissue against increased ROS levels [95]. Nishikawa et al. [96] found that the expression of key enzymes responsible for ascorbate regeneration, MDAR and DHAR was increased upon methyl jasmonate treatment. The results suggest that the JA-treated *R. imbricata* CCA suspension cultures could be considered as a promising alternative source of ascorbic acid, which is a powerful water-soluble antioxidant with multiple health benefits [97, 98].

Effect of JA on Antioxidant Activity

The DFRSA and TAC antioxidant assays were performed to assess the therapeutic potential of JA-treated CCA suspension culture of *R. imbricata*. As shown in Fig. 3 k, l, the JA-treated CCA suspension cultures of *R. imbricata* showed an exponential increase in DFRSA and TAC during the first 4 days of culture. After 4 days of addition of 100 μM JA, the CCA suspension cultures displayed maximum DFRSA (56.32 ± 0.06%) and TAC (60.45 ± 0.31 mg QE/g DW), which was 1.04-fold and 1.03-fold higher than the control (DFRSA: 53.90 ± 0.04% and TAC: 58.31 ± 0.13 mg QE/g DW). In contrast, 5 μM JA-treated CCA suspension cultures showed low DFRSA (54.70 ± 0.10%) and TAC (59.49 ± 0.13 mg QE/g DW), as compared to 100 μM JA-treated cultures ($p \leq 0.05$, Fig. 3 k, l). The correlation analysis showed a positive correlation between the phenolic compounds (Salidroside, TPC, TFC), AAC and antioxidant activity of JA-treated CCA suspension cultures of *R. imbricata* (Supplementary Table 1). Similar correlation pattern has also been observed in other plant cell cultures [99, 100]. The difference in the antioxidant activities at different time intervals could be due to the variations in the quantity of phenolic compounds as shown in Fig. 3c–g. The results suggest that the JA-elicited

Table 1 Antimicrobial activity of jasmonic acid-treated CCA suspension cultures of *Rhodiola imbricata*

Treatments	Zone of inhibition (mm)*		Minimal inhibitory concentration (mg/l)		Minimal bactericidal concentration (mg/l)	
	Escherichia coli	*Staphylococcus aureus*	*Escherichia coli*	*Staphylococcus aureus*	*Escherichia coli*	*Staphylococcus aureus*
100 μM JA-treated cultures	12 ± 1[a]	7 ± 1[a]	2.5	10	5	20
5 μM JA-treated cultures	11 ± 1[a]	7 ± 1[a]	2.5	10	5	20
Control (untreated cultures)	8 ± 1[b]	7 ± 1[a]	5	10	10	20

*Diameter of inhibition zone (including diameter of well 5 mm). Values are mean ± standard error. Mean values followed by the similar letters within a column are not significantly different at $p \leq 0.05$ according to Bonferroni post hoc test

R. imbricata CCA suspension culture is an effective alternative source of natural antioxidants for pharmacological preparations. These natural antioxidants have protective effects against oxidative stress-related disorders like cancer and coronary heart disease [101].

Effect of JA on Antimicrobial Activity

Jasmonic acid has been implicated in the production of multifarious array of plant chemical defenses, such as pathogenesis-related molecules, including antimicrobial secondary metabolites, proteinase inhibitors and toxins [95]. Therefore, the present study investigated the effect of JA-treated CCA suspension cultures of *R. imbricata* against the Gram-negative *Escherichia coli* and Gram-positive *Staphylococcus aureus.* Well diffusion assay postulates a potent antimicrobial activity of JA-treated CCA suspension cultures (Table 1**)**. As shown in Table 1, the JA-treated (5 and 100 μM) CCA suspension cultures exhibited highest antimicrobial activity against Gram-negative *E. coli* with MIC – 2.5 mg/ml and MBC – 5 mg/ml, which was 2-fold lower than the control ($p \leq 0.05$). However, the JA-treated and control cultures showed moderate antimicrobial activity against Gram-positive *S. aureus* (MIC – 10 mg/ml, MBC - 20 mg/ml; Table 1). These variations in the susceptibility of test microorganisms might be attributed to the differences in their cell wall structures [102]. The high antimicrobial activity of JA-treated CCA suspension cultures of *R. imbricata* against *E. coli* could be due to the presence of high amount of phenolic compounds (Fig. 3c–g). A similar positive correlation between the phenolic compounds and antimicrobial activity has been previously reported in cell cultures of several medicinal plants [103, 104]. The antimicrobial activity of phenolic compounds could be due to its ability to deform the structure and functionality of membrane proteins [105]. The phenolic compounds can also interfere with the membrane functions (nutrient uptake and electron transport) and alter the microbial cell permeability [105]. Banasiuk et al. [106] have also proposed that the secondary compounds have a substantial effect on antimicrobial properties. The results suggest that the JA-treated

CCA suspension cultures of *R. imbricata* could be used as an effective antimicrobial agent against potent pathogenic bacterial strains.

Conclusion

In this study, we have successfully optimized the culture media components (MS medium supplemented with 30 g/l sucrose, 3 mg/l NAA and 3 mg/l BAP) for sustainable, eco-friendly and economical production of medicinally important phenolic compounds by CCA suspension cultures of *R. imbricata*. Elicitation of CCA suspension cultures with 100 μM JA further increases the yield of phenolic compounds (Salidroside (5.25 mg/g DW), TPC (14.69 mg CHA/g DW) and TFC (4.95 mg RE/g DW)) with concomitant enhancement in antioxidant (DFRSA: 56.32% and TAC: 60.45 mg QE/g DW) and antimicrobial activities. Thus, the JA-elicited CCA suspension culture of *R. imbricata* could be considered as a promising alternative source of phenolic compounds and natural antioxidants with potential applications in pharmaceutical and nutraceutical industry. The principal findings of this study could be highly beneficial for bioprocess engineers for potential large-scale production of bioactive compounds for commercial applications. Based on our findings, future studies can be prospectively directed towards industrial-level production of phenolic compounds of *Rhodiola imbricata* in a bioreactor.

Acknowledgements The authors wish to acknowledge Defence Research & Development Organization (DRDO), Ministry of Defence, Government of India, for financial support. Authors also wish to acknowledge Dr. Somen Acharya and Sc. 'C' Rajkumar for providing necessary facilities. The authors are also grateful to Rashmi Gupta for reviewing and copy-editing of the manuscript.

Authors' Contribution Experimental design, analysis and interpretation of data, and writing of the manuscript: SK, AS, HS, SS and OPC; HPLC analysis: SK and PB; Critical revision of the manuscript: HS.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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