

# Optimization of shoot cultures and bioactive compound accumulation in *Rosa rugosa* during acclimatization

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**Abstract** *Rosarugosa* is a medicinal, ornamental, and edible plant native to Eastern Asian countries, including Korea, Japan, and China. The aim of this study was to establish a system for biomass production and secondary metabolite accumulation during *in vitro* culture and acclimatization of *Rosa rugosa*. The highest rate of multiple shoot proliferation was achieved with 8.8  $\mu$ M benzyladenine (BA) (83.3%). However, the number of shoots (14.4 per explant) at 4.4  $\mu$ M BA was higher than that at 8.8  $\mu$ M BA. Compared to BA, a combination of thidiazuron (TDZ) and indole butyric acid (IBA) exhibited significantly lower shoot induction, with only 50.0 ~ 79.2% and 4.2 ~ 16.7% relative shoot formation, respectively. During acclimatization, shoots were sampled every week and their total phenolic contents were analyzed. Among various growth factors, fresh weight showed the most dramatic increase from the 3rd week (88.0 mg/plant) to 4th week (132.7 mg/plant). Total phenolics and flavonoids contents were the highest at 1<sup>st</sup> week of acclimatization. Depending on developmental stages, total phenolics and flavonoids contents were higher in 1-yr-old shoots grown *ex vitro* than in those of older field-grown or *in vitro*-grown plants. Amongst different ages of field grown plants, 6-year-old plants, the oldest in this study, showed the lowest content in total phenolics.

**Keywords** Total phenolics, Flavonoids, Acclimatization, Thidiazuron, Multiple shoot

## Introduction

Micropropagation offers the potential to produce a large number of cloned individuals from tissue culture using various plant tissues and organs. Since the first *Begonia* bioreactor culture reported by Takayama and Misawa (1981), the culture method has been proven applicable to many species and various explant types, including shoots, bulbs, microtubers, roots, and somatic embryos (Paek et al. 2005). Amongst the explants, shoots are the most suitable in terms of secondary metabolite accumulation and biomass. In *Eleutherococcus koreanum*, the shoots were found to contain more eleutheroside E than that in adventitious roots and somatic embryos (Park et al. 2005). As expected, shoot cultures produce secondary metabolites similar to that of plants. For large-scale cultures such as bioreactors, the primary shoot culture is usually initiated in solid medium gelled with agar or gelrite. Three main factors are needed for successful multiple-shoot formation: (1) selection of responsive explants, (2) suitable basal medium, and (3) optimum concentrations and combinations of auxins and cytokinins. Numerous factors are reported to influence the success of *in vitro* propagation of shoot cultures in various medicinal plants (Khan et al. 2009; Debnath 2009; Jain et al. 2012). The effects of auxins and cytokinins on shoot multiplication of various medicinal plants have been reported. Benjamin et al. (1987) showed that 6-benzylaminopurine (BAP), at high concentrations (1.0 ~ 5.0 mg L<sup>-1</sup>) stimulates the development of axillary shoots of *Atropa belladonna*. Lal et al. (1996) observed a rapid proliferation rate in *Picrorhiza kurroa* using kinetin at 1.0 ~ 5.0 mg L<sup>-1</sup>. In *Nothapodytes*, the highest shoot multiplication was achieved on 2.2  $\mu$ M thidiazuron-containing medium. Regeneration capacity and specific response to exogenous hormones in plant tissue cultures is related to the

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physiological status of the plant and its endogenous hormone levels, and is known to be a species-specific characteristic (Neelakandan et al. 2012). Earlier, it was reported that the regeneration capacity varies even among the genotypes of a single species and the endogenous cytokinin/abscisic acid (ABA) ratio may have an influence over the regeneration of *Kalopanax septemlobus* individuals (Park et al. 2011).

Plant secondary metabolites have various functions throughout the life cycle of a plant. It is clear that some secondary metabolites play important roles in defense mechanisms and signaling in plants (Dai and Mumper 2010). In the past decade, their importance has increased rapidly, as these molecules also determine important aspects of human food quality and have pharmaceutical effects. Thus, it is important to increase the production of these useful bioactive compounds and to exploit plant materials through the large-scale culture of medicinal plants. During the culture period of a medicinal plant, the content of bioactive compounds changes with the physiological changes in the propagules, such as proliferation and development (Park et al. 2005). As a result, it is important to know the relationship between bioactive compounds and plant development or tissue type, in order to determine the ideal tissue (or explant) type and harvesting time. Secondary metabolites often accumulate in special types of cells or organs, as their biosynthesis is often coupled with certain morphological differentiations (Alfermann and Petersen 1995).

Rugosa rose (*Rosa rugosa* Thunb.; syn. rugosa rose, Japanese rose, or Ramanas rose) is a deciduous shrub belonging to the Rosaceae family. This species grows on the sea coast and in sand dunes in Eastern Asia, including Northeastern China, Korea, Japan, and Southeastern Siberia. In Asia, the plant of *R. rugosa* has been used as a medicinal plant and food in tea (Youwei and Yonghong 2007). Several researchers have systematically investigated the chemical components of the flowers, fruits, leaves, roots, and galls of this plant and it has been shown that this native rose is a good source of aromatics, phenolics, terpenoids, fatty acid derivatives, sugars, and other polar compounds (Hashidoko 1996).

In the present study, culture conditions were optimized for shoot multiplication and bioactive compounds were analyzed during acclimatization in *Rosa rugosa*. To this end, the effects of various cytokinins were investigated, and bioactive compounds such as total phenolics and flavonoids were analyzed.

## Materials and Methods

### Plant material

Mature seeds of *Rosa rugosa* were collected from their natural

habitat in Cheonnam. They were soaked in 70% ethanol for 1 min, rinsed with sterile deionized water, and placed in 2% (v/v) sodium hypochlorite solution supplemented with three to four drops of Tween-20 for 20 min. The seeds were rinsed three times in sterile deionized water before removal of the zygotic embryos with a scalpel and forceps. Zygotic embryos were placed on full-length MS medium containing  $3.0 \text{ mg L}^{-1}$  2,4-D at 25°C in the dark. After 8 weeks of culture, clumps of embryogenic calli and somatic embryos were isolated and subcultured on MS medium containing  $10.0 \text{ mg L}^{-1}$  2,4-D. The embryogenic callus clumps were proliferated on the same fresh medium for embryogenic cultures. For shoot cultures, somatic embryos were transferred onto half strength MS medium without plant growth regulators, and developed plants from somatic embryos were used as culture material for multiple shoot formation.

### Effect of IBA, BA, and thidiazuron on adventitious shoot formation from nodal culture

Around 1.5 ~ 2 cm length of node was used as an explant. For inducing shoot multiplication, MS medium supplemented with  $2.5 \text{ }\mu\text{M}$  IBA, 0 ~  $13.5 \text{ }\mu\text{M}$  BA or TDZ alone, or combination of  $2.5 \text{ }\mu\text{M}$  IBA,  $4.4 \text{ }\mu\text{M}$  BA or  $4.5 \text{ }\mu\text{M}$  TDZ were used. All media contained 3% sucrose, and were solidified with 0.3% gelrite. The cultures were performed in 100 mL Erlenmeyer flasks containing 30 mL of medium. The experiments were conducted with 5 times replication. The cultures were maintained for 8 weeks. After completing the experiment, shoot proliferation rate, number of shoots per explant, number of nodes per explant, number of leaves, and shoot length were investigated.

### Changes of growth and bioactive compound contents during *ex vitro* acclimatization periods

For rooting, terminal 3 ~ 4 cm long portions from 8-week-old *in vitro* differentiated shoots were excised and transferred to MS basal medium containing  $2.5 \text{ }\mu\text{M}$  IBA, 3% sucrose, and 0.3% gelrite. The pH of the medium was adjusted to 5.8 before autoclaving at  $1.06 \text{ kg cm}^{-2}$  and 121°C for 15 minutes. The cultures were maintained at  $25 \pm 2^\circ\text{C}$  temperature and a 16/8 h (light/dark) photoperiod provided with fluorescent lamps ( $50 \sim 70 \text{ }\mu\text{mol m}^{-2} \text{ s}^{-1}$  PPFD).

After 8 weeks, rooted shoots were washed with water to remove the agar and were transferred to plastic pots (5 cm) containing a mixture of autoclaved vermiculite, perlite and garden soil (1:1:1) and maintained at greenhouse conditions. The plants were acclimatized by covering the pots with a polythene bag to maintain high humidity for 6 ~ 7 days. After acclimatization, ten plants were sampled every one week for 4

weeks for the analysis of bioactive compounds.

Fresh weight, dry weight, shoot length, stem diameter, root length, and leaf areas were measured at every sampling time, and the number of leaves, shoots, and nodes were counted.

#### Analysis of the content of bioactive compounds

##### Preparation of explant extracts

The dried explants (0.1 ~ 0.2 g) were refluxed (LS-2050-S10, LS-TECH, Korea) with 20 ml 80% ethanol at 80°C for 1 h and filtered through filter paper (Advantec 110 mm, Toyo Roshi Kaisha Ltd., Japan). The final volume of the solution was set at 15 ml using 80% ethanol.

##### Analysis of total phenolics

Total phenolics were analyzed by the Folin–Ciocalteu colorimetric method (Folin & Ciocalteu 1927). The ethanolic explant extracts (0.1 ml) were mixed with 2.5 ml distilled water, followed by the addition of 0.1 ml (2N) Folin–Ciocalteu reagent. A 0.5 ml solution of 20% Na<sub>2</sub>CO<sub>3</sub> was added after 5 min and mixed well. The color was developed after 30 min in the dark at room temperature and the absorbance was detected at 760 nm on a visible spectrophotometer (UV-1650 PC, Shimadzu, Japan). These measurements were compared to a standard curve for gallic acid (Sigma Chemical Co., St. Louis, MO, USA) and were expressed as mg of gallic acid equivalents per gram of dry explant.

##### Analysis of total flavonoids

The contents of total flavonoids were determined colorimetrically,

using the method described by Wu et al. (2006). The ethanolic explant extracts and standard (0.25 ml) were mixed with 1.475 ml distilled water. Subsequently, 0.075 ml 5% NaNO<sub>2</sub> solution was added and the mixture was shaken vigorously. After a 6-min reaction time, 0.15 ml 10% AlCl<sub>3</sub> solution was added. After waiting for 5 min, the absorbance was measured immediately at 510 nm using a spectrophotometer. The results were expressed as mg of (+)-catechin (Sigma Chemical Co., St. Louis, MO, USA) equivalents per gram of dry explant.

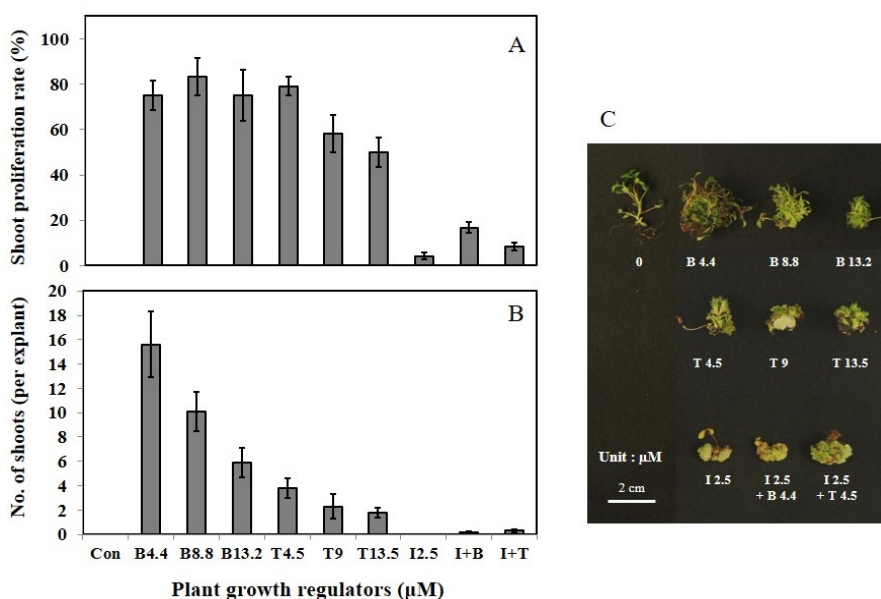
#### Statistical analysis

The results shown are the mean values of three independent experiments. One-way analysis of variance (ANOVA) was used to determine if the groups differed significantly. Statistical assessments of the difference between mean values were then assessed by the least significant difference (LSD) test. A P-value of <0.05 was considered to indicate statistical significance and all data were analyzed using the SAS program (SA 9.3; SAS Institute, Inc., Cary, NC, USA).

## Results and Discussion

### Effect of BA, TDZ and IBA on adventitious shoot formation from node culture

In *R. rugosa*, multiple shoot formation varied significantly depending on the types and concentrations of plant growth

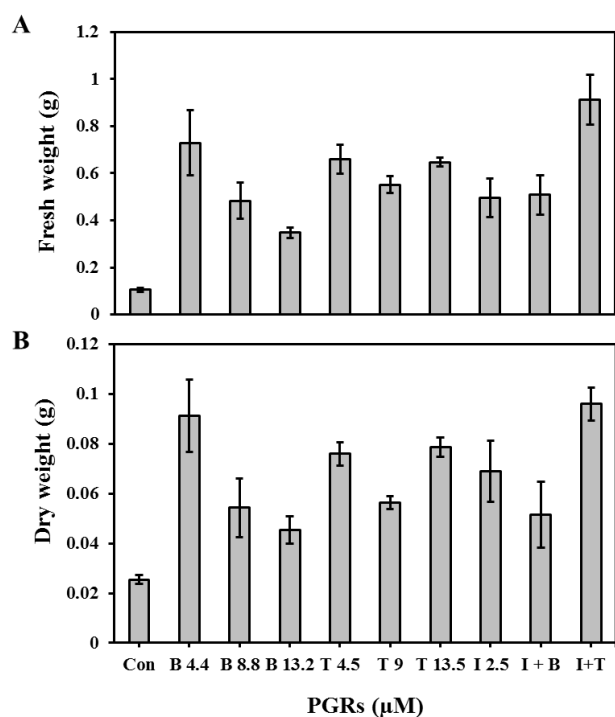


**Fig. 1** Effect of different concentrations of plant growth regulators on adventitious shoot formation from node cultures of *Rosa rugosa*. A. Shoot proliferation rate, B. No. of shoot, C. Regenera [Con: non-treated control, B: BA, I: IBA, T: thidiazuron]. Bars represent means  $\pm$  SE (n = 6)

regulator used (Fig. 1). Nodal explants showed the earliest signs of multiple shoot formation after 2 weeks of culture in BA-containing medium, and the maximum shoot numbers were achieved with 4.4  $\mu\text{M}$  BA. These explants showed 15.6 shoots per explant after 8 weeks of culture. However, maximum shoot formation was achieved with 8.8  $\mu\text{M}$  BA (83.3%). In comparison with BA, a combination of TDZ and IBA exhibited significantly lower shoot induction, with 50.0 ~ 79.2% and 4.2 ~ 16.7% shoot formation respectively (Fig. 1). Overall, BA was the best cytokinin for shoot multiplication, this result being different from a previous report on other species of rose

(Ibrahim and Debergh 2001). In *Rosa hybrida*, shoots were regenerated in a 6.8  $\mu\text{M}$  TDZ + 0.49  $\mu\text{M}$  IBA-containing medium, and shoots were well developed when they were transferred to a medium containing BA and IBA (Ibrahim and Debergh 2001). In this experiment, the highest biomass (fresh and dry weight) was obtained in a combination of TDZ and IBA while shoot numbers were higher in BA treatment (Fig. 2).

BA and TDZ showed a synergistic effect on shoot proliferation in many plants such as rose (Ibrahim and Debergh 2001) and Eastern redbud (Distabanjong and Geneve 1997). Nodal segments produced the highest number of shoots in medium containing a combination of BA and TDZ in Eastern redbud (Distabanjong and Geneve 1997). The highest number of shoots was obtained when explants were treated with a combination of 10 ~ 15  $\mu\text{M}$  BA and 0.5 ~ 1.0  $\mu\text{M}$  TDZ before being transferred to the same medium without TDZ (Distabanjong and Geneve 1997). A rapid and reproducible plant regeneration protocol was successfully developed for *Cassia angustifolia* using nodal explants, and in the plant TDZ was also more effective than BA in inducing multiple shoots (Siddique and Anis 2007).



**Fig. 2** Effect of different concentrations of plant growth regulators (PGRs) on multiple shoot formation from node cultures of *Rosa rugosa* after 8 weeks of culturing. Fresh weight (A) and dry weight (B) of plants. Con: non-treated control; B: BA; I: IBA; T: thidiazuron. Bars represent means  $\pm$  SE (n = 6)

Changes in growth and bioactive compounds during the *ex vitro* acclimatization period

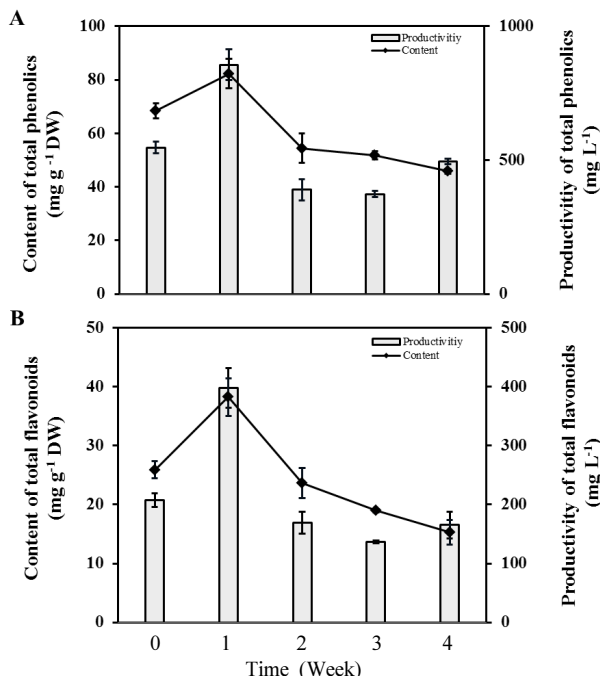
After the transfer of shoots to the greenhouse, ten shoots were sampled and were analyzed for growth and bioactive compound production (Table 1). Amongst various growth aspects, the highest increase in fresh weight, shoot length, and leaf number was achieved at 4 weeks of acclimatization (Table 1). Dry matter (%) was the highest at 0 weeks, whereas it was the lowest at 4 weeks, which showed dramatic growth increase. Number of shoots and stem diameter were not significantly changed during the acclimatization period. On the other hand, the number of nodes increased with acclimatization time. Leaf area did not increase at 0 ~ 3 weeks after acclimatization, but significantly increased around 2 times at 4 weeks. Number of

**Table 1** Effect of acclimatization periods on plant growth of *Rosa rugosa*

Time (week)	Fresh weight (mg)	% dry weight	No. of shoots	No. of nodes	No. of leaves	Shoot length (cm)	Root length (cm)	Stem diameter (mm)	Leaf area (cm <sup>2</sup> )
0	40.7 $\pm$ 2.6 <sup>z</sup>	35.0 $\pm$ 2.3	1.0 $\pm$ 0.0	5.0 $\pm$ 0.4	29.5 $\pm$ 3.1	3.2 $\pm$ 0.1 c <sup>y</sup>	0.6 $\pm$ 0.1	0.8 $\pm$ 0.1	0.2 $\pm$ 0.0b
1	77.2 $\pm$ 5.8	20.5 $\pm$ 0.8	1.2 $\pm$ 0.2	4.8 $\pm$ 0.8	23.2 $\pm$ 3.1 b	3.9 $\pm$ 0.2 c	1.1 $\pm$ 0.1	0.9 $\pm$ 0.1	0.3 $\pm$ 0.0b
2	77.5 $\pm$ 6.6	21.4 $\pm$ 1.3	1.2 $\pm$ 0.2	7.0 $\pm$ 0.4	28.5 $\pm$ 2.2 b	5.1 $\pm$ 0.3 b	1.0 $\pm$ 0.1	0.8 $\pm$ 0.1	0.3 $\pm$ 0.0b
3	88.0 $\pm$ 8.8	20.4 $\pm$ 1.6	1.2 $\pm$ 0.2	6.8 $\pm$ 0.6	25.3 $\pm$ 3.2 b	5.5 $\pm$ 0.3 b	1.3 $\pm$ 0.2	0.8 $\pm$ 0.1	0.3 $\pm$ 0.0b
4	132.7 $\pm$ 8.9	18.9 $\pm$ 0.4	1.2 $\pm$ 0.0	7.8 $\pm$ 0.7	34.7 $\pm$ 1.9 a	7.5 $\pm$ 0.2 a	1.3 $\pm$ 0.1	0.8 $\pm$ 0.0	0.6 $\pm$ 0.0a

<sup>z</sup>Values represent means  $\pm$  SE (n = 5)

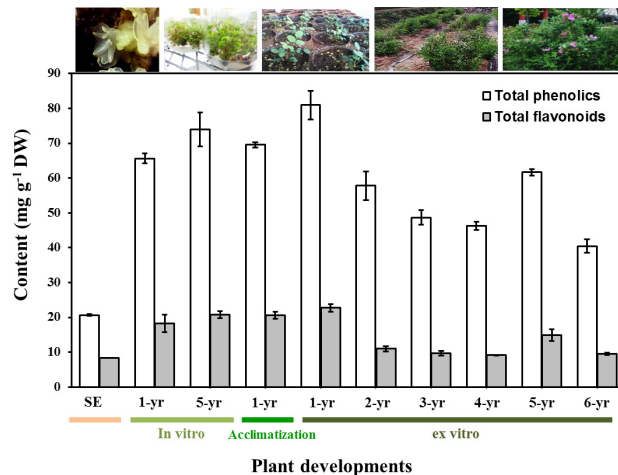
<sup>y</sup>Within each column, different letters indicate mean separation using Duncan's multiple range test at 5 % level of significance



**Fig. 3** Changes in bioactive compound content during acclimatization periods of *Rosa rugosa*. A: Total phenolics, B: Total flavonoids. Bars represent means  $\pm$  SE (n = 3)

leaves decreased at the 1<sup>st</sup> week because of acclimatization stress, and it increased again in 4 weeks of acclimatization. Total phenolics and flavonoids were the highest at 1<sup>st</sup> week of acclimatization (Fig. 3). The results of total phenolics and flavonoids can be interpreted as *in vitro* grown plantlets were exposed to natural conditions during transplanting, and it made stressful conditions for plantlets. In general, the plants produce more antioxidant compounds such as polyphenols and flavonoids when exposed to stressful conditions, because of defense mechanisms that are in place. In this experiment, *in vitro* *R. rugosa* plantlets were transplanted; therefore, the dry atmosphere and different light conditions may influence the increase in bioactive compounds.

Figure 4 shows that the content of total phenolics and flavonoids depends on the developmental stage and age of *in vitro* and field-grown plants. The content of total phenolics was the highest in 1-year-old plants grown in *ex vitro* conditions. Before the analysis of phenolics, it was assumed that older samples contain more secondary metabolites, and that *ex vitro*-grown shoots contain more secondary metabolites than *in vitro*-grown shoots. However, in contrast to our assumption, total phenolic and flavonoid content was higher in *in vitro*-grown shoots than in field-grown plants. Amongst various *in vitro* propagules, somatic embryos showed the lowest content of total phenolics. Amongst different ages of field grown plants, the 6-year-old plants, which were the oldest, had the



**Fig. 4** Changes in contents of total phenolics and flavonoids during the developmental process of *Rosa rugosa*. SE: somatic embryo, In-1: *in vitro* 1-yr-old, In-5: *in vitro* 5-yr-old, Acclimatization: 0-day, Ex-1: *ex vitro* 1-yr-old, Ex-2: *ex vitro* 2-yr-old, Ex-3: *ex vitro* 3-yr old, Ex-4: *ex vitro* 4-yr-old, Ex-5: *ex vitro* 5-yr-old, Ex-6: *ex vitro* 6-yr-old. Bars represent means  $\pm$  SE (n = 3)

lowest content of total phenolics (Fig. 4).

Numerous reports indicate that it is difficult to select an ideal stage of plant source, which would ensure good production of the selected secondary metabolites and simultaneously, fast and continuous growth of propagules that are capable of consistently producing bioactive compounds. One reason is that the primary and secondary metabolism pathways often compete for nutrients and precursors, and are often mutually exclusive (Hagendoorn et al. 1999; Luczkiewicz and Cisowski 2005).

Shoot cultures have been established for many medicinal plants; these can accumulate a higher amount of secondary metabolites than natural plants. Recently, many reports have shown the possibility of shoots acting as a source of industrial product ingredients. For example, shoot cultures were established for *Bacopa monnieri* for the production of bacoside A and regenerated shoots possessed threefold higher bacoside A than field grown plants (Paraveen et al. 2009). Similarly, the shoots of *Nothapodytes nimmoniana*, which were regenerated in semisolid and liquid medium, had several-fold higher camptothecin content compared to the mother plants (Dandin and Murthy 2012). Santos-Gomes et al. (2002) reported that the increase in concentration of kinetin decreased the accumulation of most of the phenolic diterpenes in sage shoots.

Our study showed that young plants such as *in vitro* grown-, acclimatized-, and 1-yr-old *R. rugosa* produce higher levels of secondary metabolites than field grown old plants, thereby establishing a system that can be used for large-scale production of secondary metabolites.

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