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Research Report

Production of Biomass and Bioactive Compounds from Shoot Cultures of *Rosa rugosa* using a Bioreactor Culture System

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Abstract. *Rosa rugosa* Thunb. is a popular ornamental and medicinal plant native to eastern Asia. In this study, a successful bioreactor culture system was established for the production of secondary metabolites of rugosa roses. We tested different concentrations and combinations of plant hormones in growth media for maximum shoot proliferation and production of bioactive compounds, different bioreactor systems for maximum biomass production and production of bioactive compounds, and different ratios of nitrogen sources for maximum shoot growth and accumulation of bioactive compounds. For multiple shoot proliferation, Murashige and Skoog (MS) medium was used, supplemented with different concentrations and combinations of plant hormones: 6-benzylaminopurine (BA; 0-13.2 μM), thidiazuron (TDZ; 0-13.5 μM), and indole butyric acid (IBA) at 2.5 μM, used alone or in a combination of IBA with BA or TDZ. Rapid micropropagation of multiple shoots of rugosa roses was successfully achieved using shoot tips explanted in semisolid MS medium supplemented with 4.4 μM BA. The average number of shoots grown was 15.6 per explant and the maximum shoot length was 2.7 cm at 8 weeks of culture. To investigate the effect of nitrogen sources on shoot growth and bioactive compound accumulation, shoots were treated with different ratios of nitrogen sources (NH_4^+:NO_3^-) for 1 week after 7 weeks of shoot culture. Next, to scale up biomass production for the generation of useful phytochemicals, multiple-shoot cultures were developed in large-scale bioreactors. Four bioreactor systems were used: continuous immersion bioreactor (CIB), continuous immersion bioreactor with net (CIB-N), temporary immersion bioreactor (TIB), and temporary immersion bioreactor with net (TIB-N). Solid and liquid media were used as controls. Of the different bioreactor types, the CIB system produced the highest biomass, followed by the TIB system. Multiple shoots grown in the CIB system resulted in the accumulation of 39.21 $mg \cdot g^{-1}$ dry weight (DW) of total phenolics and 13.28 $mg \cdot g^{-1}$ DW of total flavonoids. The productivity of total phenolics and flavonoids was highest in the shoots harvested from the CIB system. The results of this study suggest that multiple shoots of rugosa roses can be used in commercial-scale bioreactors to produce useful bioactive compounds for the pharmaceutical and cosmetic industries.

***Additional key words*:** air-lift bioreactor, flavonoids, NH_4^+:NO_3^- ratio, phenolics, rugosa rose

Introduction

The rugosa rose (*Rosa rugosa* Thunb.), also known as the sea rose, Japanese rose, Ramanas rose, or salt-spray rose, is a widely-grown shrub belonging to the family Rosaceae and has ornamental and medicinal values (Jung et al., 2005; Park and Paek, 2014; Yoshizawa et al., 2000). The rugosa rose is naturally distributed in sandy or gravelly beaches and in the sand dunes of eastern Asia in regions such as Korea, Japan, northeastern China, and southeastern Siberia (Krussmann, 1982). In East Asian countries, this species has been used in folk medicine to treat disorders such as diabetes, chronic inflammatory diseases, pain, and cancer (Ng et al., 2004; Park et al., 2005). The rugosa rose is also used as an astringent, to improve circulation to relieve blood stasis, to aid menstrual regulation, and to counteract toxins (Olech et al.,

2012; Youwei and Yonghong, 2007). Recent research has shown that this native rose is a good source of various secondary metabolites such as aromatics, phenolics, terpenoids, fatty acid derivatives, sugars, and other polar compounds (Hashidoko, 1996). These plants are also reported to have highly effective free-radical scavengers and are a good source of vitamin C (Czyzowska et al., 2015; Liangxiong et al., 2005; Olech et al., 2012). There is increasing interest in growing rugosa roses for commercial purposes, including its use in folk medicine and for the manufacture of rose oil and perfume. Its tomato-like fruits can alleviate muscular pain and crude extracts have an anti-parasite effect.

Rugosa roses are generally propagated by grafting or cuttings. This conventional method of propagation is very slow and time-consuming and is subject to seasonal variation. Moreover, disease and environmental hazards cause the cultivar to degenerate over time. Therefore, conventional cultivation methods are not satisfactory for multiplication. In vitro propagation of roses has received considerable attention in the last few years as an alternative to conventional propagation. The most important micropropagation technique is shoot proliferation, in which apical buds or nodal segments with an axillary bud are cultured to regenerate multiple shoots without any intervening callus phase (Ara et al., 1997; Carelli and Echeverrigaray, 2002; Xing et al., 2010). Despite the many benefits of this technique for the propagation of other rose cultivars, micropropagation or multiple shoot production of rugosa roses has been rarely reported.

Plant cell and tissue cultures are considered promising technologies for the production of valuable plant-specific metabolites (Verpoorte et al., 2002) as these technologies promote a higher rate of metabolism than that found in field-grown plants. Bioreactor systems have been used for the efficient, large-scale culture of horticultural and medicinal plants. This automated micropropagation system has been promoted as a possible method to reduce the production cost and labor of conventional propagation. Bioreactor technology can potentially be used to produce a large number of plants efficiently and economically (Paek et al., 2005). The immersion culture system, in which explants are submerged in the supplied medium, is commonly used in bioreactors. Temporary immersion may also have potential benefits including greater biological efficiency and productivity (Sajc et al., 2000; Alvard et al., 1993; Park et al., 2004). In addition to the immersion culture system, the modified balloon-type bubble bioreactor (BTBB) is widely used for plant culture (Paek et al. 2005). This technology has been used for large-scale cultivation of medicinal plants through shoot multiplication. Many researchers used bioreactors for the mass proliferation of different plants, including chrysanthemum (Hahn and Paek, 2005), coffee (Ducos et al., 2008), cacao (Niemenak et al., 2008), and wild grape (Park et al., 2015).

Bioactive compounds are low-molecular-weight, natural products, which are the most abundant secondary metabolites produced in plants (Ciesla et al., 2012; Dai and Mumper, 2010; Joo et al., 2010). Of the different groups of bioactive compounds, plant phenolics are the largest class of natural antioxidants with more than 8,000 phenolic structures currently known (Dai and Mumper, 2010). Plant phenolics are generally involved in defense against stressful conditions such as pathogen aggressions in nature. Phenolics include phenolics acids, flavonoids, and tannins and are considered more potent antioxidants than vitamin C or carotenoids (Ibrahim et al., 2012). Among phenolics, flavonoids are the most abundant polyphenols (Dai and Mumper, 2010). A strong relationship exists between the antioxidant activities of different plants and the phenolic compounds produced by their in vitro cultures (Amid et al., 2013; Al Khateeb et al., 2012; Diwan et al., 2012).

In the present study, we report successful production of multiple shoots in bioreactors and the production of total phenolics and flavonoids from multiple shoots of *Rosa rugosa* in vitro.

Materials and Methods

Plant Materials

Five-year-old plants of *Rosa rugosa* were purchased from a local market in 2012 and planted in the experimental field of Chungbuk National University. Green shoots (5 cm in length) were collected and rinsed with running tap water. For surface sterilization, the shoots were treated with 70% ethanol for 30 sec, then soaked in 0.2% $HgCl_2$ for 15 min followed by 2% NaOCl containing one or two drops of Tween 20 for 30 min. After completion of surface sterilization, the shoots were rinsed three times with sterilized water. Two-cm length shoot tips were cultured and multiplied in MS medium containing 4.4 μM BA and 3% sucrose. The pH of the media was adjusted to 5.8 using 1 N NaOH and the media were then autoclaved at 121°C/1.2 atm for 25 min. Cultures were maintained at 25 ± 1°C at 60 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PPFD light intensity and a 16/8 h (day/night) photoperiod.

Shoot Multiplication and Bioreactor Systems

Shoot tips (approximately 2 cm long) from plantlets grown in vitro were dissected and placed on semisolid MS (Murashige and Skoog, 1962) medium supplemented with different concentrations and combinations of plant hormones: 6-benzylaminopurine (BA; 0-13.2 μM), thidiazuron (TDZ; 0-13.5 μM), and indole butyric acid (IBA) at 2.5 μM, used alone or in a combination of IBA with BA or TDZ. The medium was supplemented with 3% sucrose (w/v) and the pH was adjusted to 5.8 before autoclaving at 121°C. This medium was then

used in experiments to investigate the effect of different growth regulators for multiple shoot production. Additionally, the growth of plantlets was measured and the plantlets were harvested every week for the entire culture period to investigate changes in phenolics.

For bioreactor culture, a 3 L balloon-type air-lift bubble bioreactor (BTBB) was used in this study. About 100 single-node shoots of *R. rugosa* were inoculated into different types of 3 L BTBB using 1 L of MS liquid medium. Four different types of bioreactors were tested in this experiment: (1) continuous immersion bioreactor (CIB), (2) continuous immersion bioreactor with net (CIB-N), (3) temporary immersion bioreactor (TIB), and (4) temporary immersion bioreactor with net (TIB-N). All cultures were incubated under a 16-h photoperiod provided by cool-white fluorescent light (60 $\mu mol \cdot m^{-2} \cdot s^{-1}$) with temperature maintained at 24 ± 1°C.

Effect of Nitrogen Sources

To investigate the effect of nitrogen sources on shoot growth and bioactive compound accumulation, fresh 5 g shoots (around 100 shoot tips) were cultured in 1 L liquid MS medium containing 4.4 μM BA and 3% sucrose for 7 weeks for growth and multiplication of shoots. At 7 weeks in culture the medium was removed and the modified MS media with different ratios of NH_4^+:NO_3^- (60:0, 40:20, 30:30, 20:40, 0:60) was supplied in the BTBB and maintained for 1 week to increase bioactive compounds from shoots. After the culture, fresh and dry weights were measured and the percentage dry weight was calculated. Before and after culture, 10 mL of residual media samples were collected and filtered through filter paper. The electrical conductivity (EC) and pH values were determined using a conductivity meter (Model LF-54, WTW GmbH, Weilheim, Germany) and a pH meter (Corning, Ciba, USA).

Quantification of the Bioactive Compound Contents

***Preparation of explant extracts*:** The dried explants (0.1-0.2 g) were refluxed (LS-2050-S10, LS-TECH, Korea) with 20 mL of 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 brought to 15 mL using 80% ethanol.

***Analysis of total phenolics*:** Total phenolics were determined using 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. After 5 minutes, 0.5 mL 20% Na_2CO_3 solution was added 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 equivalent per gram of dry explant.

***Analysis of flavonoids*:** The total flavonoid content was 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. Then, 0.075 mL 5% $NaNO_2$ solution was added and the mixture was vigorously shaken. After a 6 min reaction time, 0.15 mL 10% $AlCl_3$ solution was added. After 5 min, the absorbance was measured at 510 nm using a spectrophotometer (Sigma chemical Co., St. Louis, MO, USA). The results were expressed as mg of (+)-catechin equivalent per gram of dry explant.

Statistical Data Analysis

Solid cultures were designed in quintuplicate with ten explants and bioreactor cultures were conducted in triplicate with one hundred explants for each treatment. Average values of all data were subjected to ANOVA and assessed by Duncan's multiple range test using the SAS program (SA 9.3; SAS Institute, Inc., Cary, NC, USA). Mean values with different letters differed significantly at $p < 0.05$.

Results and Discussion

Multiple Shoot Cultures and Bioactive Compound Accumulation

The effects of different growth regulators on shoot bud explants of *R. rugosa* cultured in semisolid medium are shown in Table 1. Among the various concentrations of 6-benzylaminopurine (BA; 4.4, 8.8, and 13.2 μM) and thidiazuron (TDZ; 4.5, 9.0, and 13.5 μM) tested to initiate multiple shoots from the shoot bud explants, the highest proliferation of multiple shoots (83.3%) was obtained with 8.8 μM BA followed by 4.5 μM TDZ (79.2%). The highest total number of shoots per explant (15.6) was obtained with 4.4 μM BA followed by 8.8 μM BA (10.1). The number of leaves per explant was highest with 4.5 μM BA (101.2) (Table 1). Shoot buds differentiated from the explants without any callus formation in medium supplied with BA (data not shown). Maximum shoot numbers per explant were obtained with 4.4 μM BA. Shoot numbers decreased with increasing BA concentration. Shoot proliferation was also observed with different concentrations of TDZ in the culture medium, with maximum shoot proliferation (79.0%) in the medium supplemented with 4.5 μM TDZ.

Table 1. The effects of BA, thidiazuron (TDZ), and IBA on multiple shoot formation of R. rugosa node culture on semisolid MS medium

PGRs (μM)			Shoot proliferation rate (%)	No. of shoots/ explant	No. of nodes/ explant	No. of leaves/explant	Shoot length (cm)
BA	TDZ	IBA					
0	0	0	0.0 d [z]	0.0 d	5.3 a	11.8 d	3.4 a
4.4	0	0	75.0 ab	15.6 a	2.2 b	101.2 a	2.7 ab
8.8	0	0	83.3 a	10.1 b	2.1 b	63.7 b	1.9 bcd
13.2	0	0	75.0 ab	5.9 c	1.6 b	41.8 c	2.1 bc
0	4.5	0	79.2 ab	3.8 cd	2.4 b	47.2 bc	2.3 b
0	9	0	58.3 bc	2.3 cd	1.7 b	29.5 c	2.2 bc
0	13.5	0	50.0 c	1.8 d	2.0 b	42.8 c	2.2 b
0	0	2.5	4.2 d	0.0 d	2.2 b	5.1 d	2.9 ab
4.4	0	2.5	16.7 d	0.2 d	1.3 b	7.1 d	1.2 ab
0	4.5	2.5	8.3 d	0.3 d	0.9 b	5.3 d	0.8 b

[z]Mean separation within columns by Duncan's multiple range test at 5% level.

Table 2. Growth of *R. rugosa* shoot cultures in semi-solid MS medium with 4.4 μM BA for 8 weeks.

Time (weeks)	Fresh weight/explant (mg)	% dry weight	Shoot length (cm)	Shoot proliferation rate (%)
1	29.7 e[z]	17.9 c	1.7 d	0.0 d
2	45.7 d	21.5 bc	2.3 cd	37.0 b
3	85.0 c	22.8 b	3.6 c	31.0 c
4	93.7 c	22.6 b	4.0 c	48.4 a
5	120.6 bc	24.9 a	3.6 c	48.1 a
6	125.5 bc	24.9 a	3.8 c	42.0 ab
7	145.9 b	24.3 a	5.5 b	40.5 ab
8	202.0 a	23.7 ab	7.0 a	48.7 a

[z]Mean separation within columns by Duncan's multiple range test at 5% level.

Our results revealed that 4.4 μM BA induced a greater number of shoots per explant than other concentrations of BA or TDZ. The relative benefits of BA in micropropagation have been well documented in many species (Krishnan et al., 1995). Among the cytokinins tested in *Trichopus zeylanicus*, only BAP induced callus-free multiple shoot bud formation (Krishnan et al., 1995). Similar positive results of BA were also obtained from in vitro culture of *Rehmannia glutinosa* (Piątczak et al., 2014).

In our study, no significant results were obtained when IBA was used in the culture medium (Table 1). IBA induced callus formation at the basal part of rugosa rose explants without forming shoots properly, but producing adventitious buds. Mark and Simpson (1994) reported that this callus formation could be related to increased auxin accumulation in the explants because of exogenous auxin. Among the different tissues and organs used for the production of secondary metabolites, shoots are found most suitable for production of biomass and useful secondary metabolites (Park et al., 2014).

Table 2 shows the changes in shoot growth cultured in MS medium containing 4.4 μM BA during the 8-week in vitro culture period. Among the various growth factors, fresh weight showed the most dramatic increase from the 1st week (29.7 ± 5.4) to the 8th week (202.0 ± 17.4). However, percent dry matter decreased at 8 weeks of culture. In vitro shoots were harvested every week and their total phenolics were analyzed. Production of total phenolics and total flavonoids is presented in Fig. 1. The profile of total phenolics increased linearly over time and leveled off after 8 weeks of culture. On the other hand, total flavonoid content reached its maximum level at 6 weeks of culture and then decreased gradually (Fig. 1).

Shoot cultures have been established in many medicinal plants and can promote higher secondary metabolite accumulation than that of naturally grown plants. Recently, many reports have shown the potential for using shoots for the production of industrial product ingredients. For example, shoot cultures were established in *Bacopa monnieri* for the production of bacoside A and regenerated shoots possessed three-fold higher bacoside A than field grown plants (Praveen et al., 2009). Similarly, the shoots of *Nothapodytes*

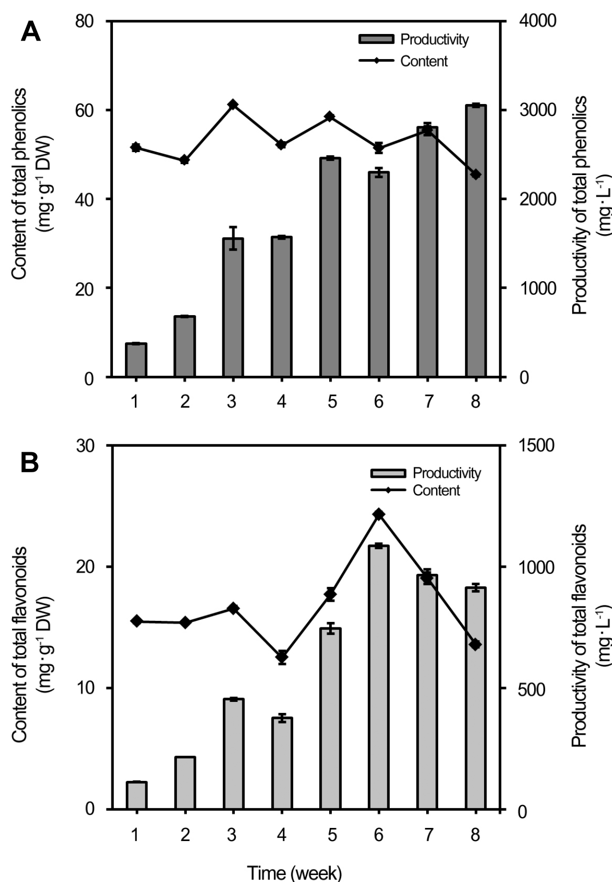

Fig. 1. Effect of culture time on bioactive compound accumulation in *R. rugosa* shoots grown in semi-solid MS medium with 4.4 μM BA for 8 weeks.

nimmoniana, which were regenerated in semisolid and liquid medium, had several fold higher camptothecin compared to the mother plants (Dandin and Murthy, 2012).

Shoot Biomass and Bioactive Compound Accumulation in Various Bioreactor Systems

In this experiment, different types of bioreactor culture systems were used to attain maximum shoot biomass. The results of biomass production using different culture systems are presented in Table 3. Maximum biomass was recorded in the continuous immersion bioreactor [CI; 91.9 $g \cdot L^{-1}$ fresh weight (FW) and 12.1 $g \cdot L^{-1}$ DW] followed by the continuous immersion with net bioreactor (CIN; 61 $g \cdot L^{-1}$ FW and 7.1 $g \cdot L^{-1}$ DW). We found that the temporary immersion bioreactor and the temporary immersion bioreactor with net (TIB & TIB-N) were not suitable for developing multiple shoots of *R. rugosa*. The factors that showed significant differences between the biomass production systems (solid culture, liquid culture, continuous immersion, and temporary immersion) were agitation and air supply. Aeration volume is one of the most important factors that may affect the growth of propagules in liquid culture (Praveen et al., 2009). The type of vessel and culture system affects the gaseous composition inside the vessel and dissolved oxygen content in the medium, which affects the growth of cells and tissues (Park and Paek, 2014). In the present study, the CIB bioreactor was supplied with 0.1 vvm (volume per volume per minute) of aeration, which helped to achieve maximum biomass accumulation over the solid and liquid cultures in flasks (Figs. 2 and 3). Studies performed on several other plant species cultured in liquid media, in various bioreactor types, have also shown that shoot growth and multiplication were more efficient than those achieved on solid media (Konstas and Kintzios, 2003). The immersion bioreactor was successfully used in our laboratory for the proliferation of *Chrysanthemum* shoots (Hahn and Paek, 2005) and also in other studies in cacao (Niemenak et al., 2008), coffee (Ducos et al., 2008), and *Vitis* (Park et al., 2015). The data obtained in the present study demonstrated the application of the continuous immersion bioreactor for mass multiplication of *R. rugosa*.

We also compared the total phenolic and flavonoid contents of shoots harvested from different bioreactor systems (Fig. 2). The total phenolic and flavonoid content

Table 3. Effect of bioreactor culture methods on shoot growth in *R. rugosa* after 8 weeks of culture

Culture method[z]	Biomass ($g \cdot L^{-1}$)		% dry weight	Growth ratio[y]
	Fresh weight	Dry weight		
Solid	43.0 c[x]	3.0 c	7.0 c	7.7 c
Liquid	67.0 b	7.8 b	11.6 ab	12.5 b
CIB	91.9 a	12.1 a	13.2 a	17.5 a
CIB-N	61.0 b	7.1 b	11.6 ab	11.3 b
TIB	44.1 c	3.7 c	8.4 b	7.9 c
TIB-N	14.5 d	1.0 d	6.9 c	1.9 d

[z]Solid (solid medium culture in bottle), Liquid (liquid medium culture in flask), CIB (continuous immersion bioreactor), CIB-N (continuous immersion bioreactor with net), TIB (temporary immersion bioreactor), TIB-N (temporary immersion bioreactor with net).
[y]Growth ratio = [harvested dry weight (g) – *initial* dry weight (g)]/*initial* dry weight (g)]
[x]Mean separation within columns by Duncan's multiple range test at 5% level.

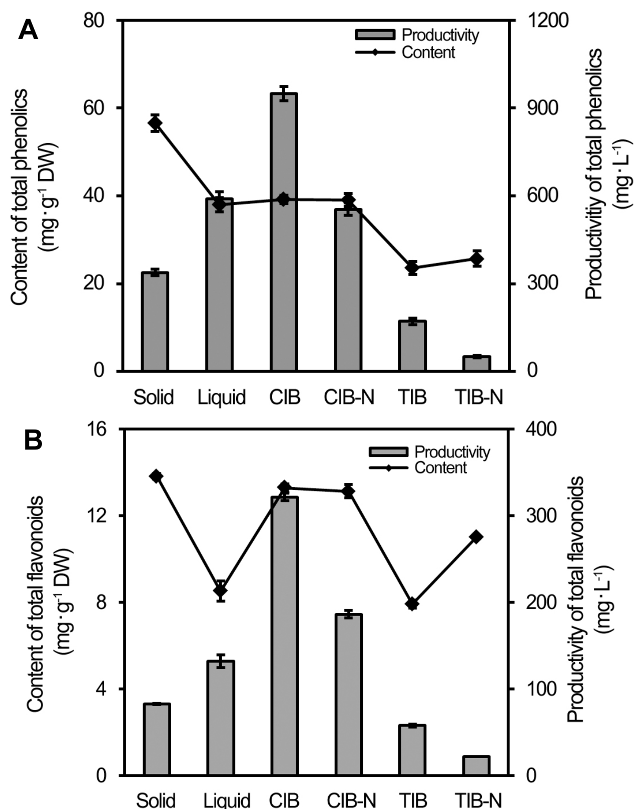

Fig. 2. Effect of culture methods on bioactive compounds in shoots of *R. rugosa* after 8 weeks of culture. A. Total phenolics. B. Total flavonoids. Bars represent means ± SE (n = 5).

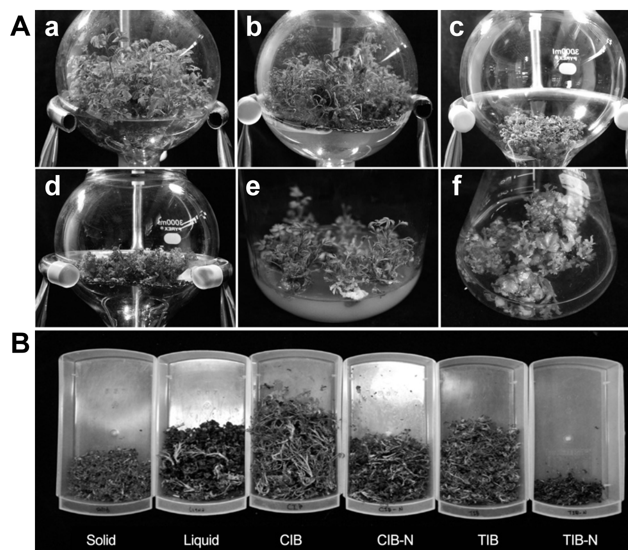

Fig. 3. Shoot cultures of *R. rugosa* from various bioreactor systems after 8 weeks of culture. A, Shoot growth in different bioreactor cultures and controls. [a, continuous immersion bioreactor (CIB); b,. continuous immersion bioreactor with net (CIB-N); c, temporary immersion bioreactor (TIB); d, temporary immersion bioreactor with net (TIB-N); e, solid culture (control 1); f, liquid culture (control 2); B, Harvested shoots of *R. rugosa* from bioreactor cultures, solid, and liquid cultures.

per g dry weight was higher in solid culture, while the content of these compounds was comparatively lower in shoots harvested from liquid cultures, including bioreactor cultures. However, the productivity (mg per L medium) of these compounds was the highest in the CIB system. It can be postulated that since biomass per L of medium was the highest in CIB with 12.1 $g \cdot L^{-1}$ medium, the production of total phenolics and flavonoids could be the highest in this CIB system as well. In comparison with CIB, the production of these antioxidant compounds was lower in TIB and TIB-N, in which shoots were temporarily immersed in liquid medium (Fig. 2). It is also speculated that this low productivity was due to low biomass in these TIB systems.

Phenolics obtained from plants are one of the major groups of compounds acting as primary antioxidants or free radical terminators (Miliauskas et al., 2004; Sakanaka et al., 2005). These plant phenolics include flavonoids along with other compounds such as phenolic acid, tannins, and stilbenes. Flavonoids are divided into six groups according to the oxidation state: anthocyanins, flavanones, flavones, flavanols, flavonols, and isoflavones (Dai et al., 2010). Herein, total phenolic compounds were analyzed by colorimetric determination, a rapid and simple assay to determine the whole complex of phenolic compounds possessing a capacity to neutralize free radicals (Piatczak et al., 2014). Taking into account the results obtained in this study, as shown in Table 3 and Fig. 2, the highest phenolic productivity was achieved in the CIB system, which indicated that this system can be used for commercial scale production of rugosa rose biomass containing high antioxidant compounds.

Effect of Nitrogen Sources

Shoots were pre-cultured in liquid MS medium containing 4.4 μM BA in the CIB system for 7 weeks, and then were re-cultured for 1 week in media containing various ratios of NH_4^+:NO_3^-. After 1 week, dry weight and shoot length were not significantly different depending on the nitrogen source. However, fresh weight and stem diameter were higher in the plants grown in a 20:40 NH_4^+:NO_3^- ratio than other ratios of ammonium and nitrate. The 20:40 NH_4^+:NO_3^- ratio is the same ratio as that of the standard MS medium. Stem diameter in the media with 20:40 NH_4^+:NO_3^- was significantly thicker than in other nitrogen treatments. Shoot numbers decreased when the NH_4^+ ratio increased. Ivanova and Van Staden (2009) reported that the use of ammonium as the sole source of nitrogen appeared to have a negative effect on the growth and regeneration of new shoots in *Aloe polyphylla*. Zhong and Wang (1998) demonstrated that high ammonium concentrations had an inhibitory effect on cell growth in the culture of *Panax quinquefolius.* Kaul and Hoffman (1993) also reported that ammonium as the sole nitrogen source

Table 4. Effect of NH_4^+:NO_3^- on shoot growth of *R. rugosa* after 1 week of culture

NH_4^+:NO_3^- (mM)	Fresh weight ($g \cdot L^{-1}$)	% dry weight	No. of shoots	No. of nodes	Shoot length (cm)	Stem diameter (mm)
60:0	17.3 b[z]	9.3	4.8 b	11.1	8.2 a	2.6 b
40:20	16.8 c	9.9	4.9 b	10.2	7.6 b	2.9 b
30:30	17.7 b	9.5	5.6 ab	10.7	8.2 a	2.7 b
20:40	18.3 a	9.2	6.3 a	10.8	8.7 a	6.7 a
0:60	16.6 c	9.6	6.2 a	10.8	7.1 b	2.7 b
		ns[y]		ns		

[z]Mean separation within columns by Duncan's multiple range test at 5% level.
[y]Non-significant

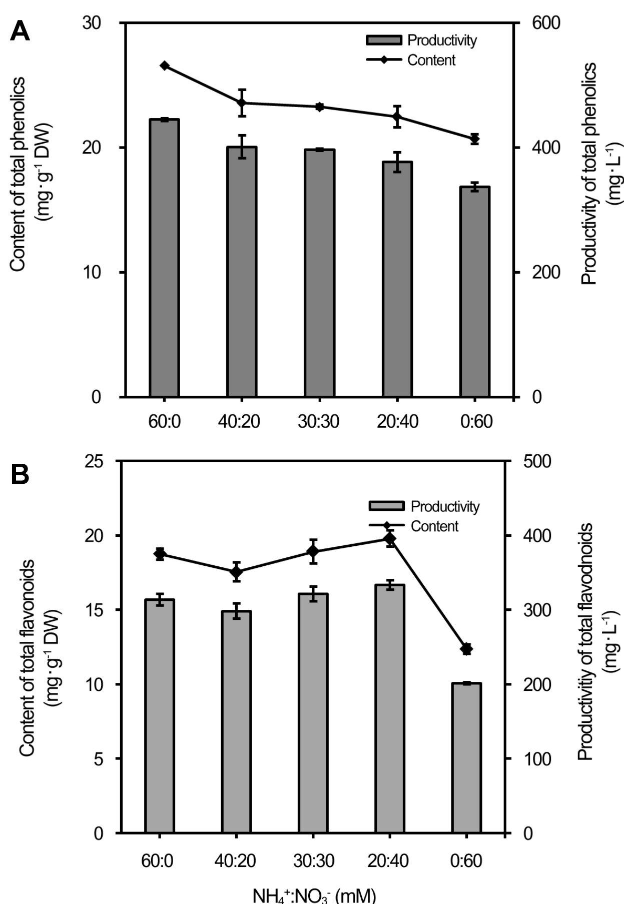

Fig. 4. Effect of NH_4^+:NO_3^- on bioactive compounds in shoots of *R. rugosa* after 1 week of culture. A, Total phenolics; B, Total flavonoids. Bars represent means ± SE (n = 5).

inhibited the growth of callus of *Pinus strobus*. However, bioactive compounds increased when the NH_4^+ ratio increased, while biomass decreased (Fig. 4). The content of total flavonoids was the lowest in the medium lacking ammonium. Yin et al. (2013) reported that ammonium and nitrate as nitrogen sources have different effects on cell and tissue growth and active component production.

In the present study, nitrate was more necessary than ammonium for shoot growth and metabolite accumulation in in vitro cultured *R. rugosa*. In *Echinacea angustifolia,* NO_3^- rather than NH_4^+ was essential for better root growth and the accumulation of secondary metabolites (Wu et al., 2006). Additionally, work on cultures of *Bacopa monnieri* (L.) showed that shoot cultures, the number of adventitious shoots, and bacoside content were optimum at a lower NH_4^+:NO_3^- ratio (Naik et al., 2011). When the concentration of ammonium is high in the medium, the plant tissue releases hydrogen, and subsequently the pH of the medium is lowered (Behrend and Mateles, 1975). Acidified medium inhibits the absorbance of nutrients and eventually prohibits nitrogen anabolism in plants (Behrend and Mateles, 1975; Sathyanarayana and Blake, 1994; Yin et al., 2013). When exposed to very low pH, plant cells convert inorganic polyphosphate into organic polyphosphate, which inhibits ATP synthesis (Ivanova and Van Staden, 2009). These physiological changes have a negative effect on plant growth, and plant growth can be prohibited (Ivanova and Van Staden, 2009; Mimura et al., 2000). Therefore, the pH of the medium was low at the 60:0 NH_4^+:NO_3^- ratio, and the shoot number was less than in other treatments for *R. rugosa* (Fig. 5). Electrical conductivity (EC) usually increased when the ion concentration was high. Fig. 5 showed that EC was high with the ammonium treatment of 60:0. It can be postulated that the medium was acidified because of the high ammonium concentration and that this inhibited the uptake of nutrients, especially cations. Subsequently, the plants cultured in the medium containing ammonium 60:0 were stressed, and this influenced the increase of total flavonoids in *R. rugosa*.

Bioactive compounds, which are secondary metabolites of plants, are currently being obtained commercially by extraction from whole plants or tissues. For large-scale production of useful plants, bioreactors are an effective but underexploited tool for the production of biomass and bioactive compounds. Easy manipulation and harvest are advantages of growing multiple shoots in bioreactors for large-scale production. The present study describes the

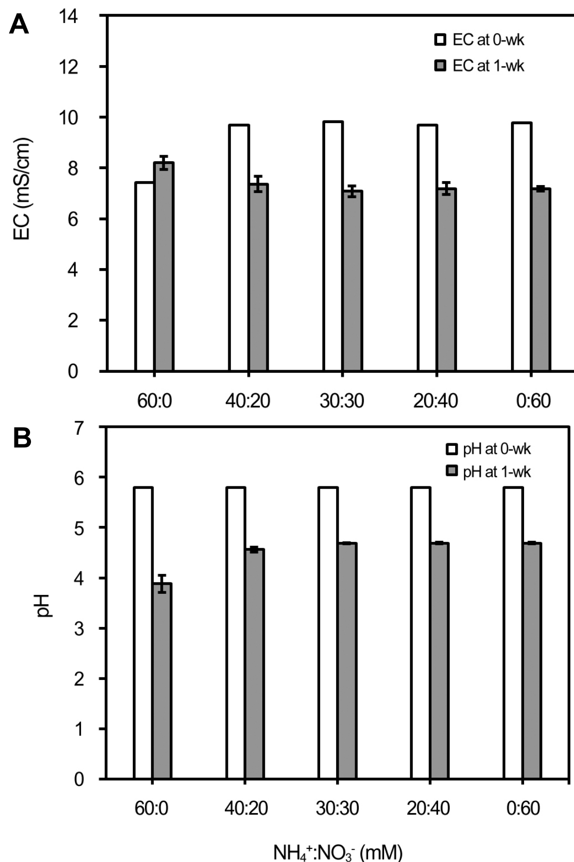

Fig. 5. Electrical conductivity (EC) and pH of the medium as affected by the NH_4^+:NO_3^- ratio after 1 week of culture. Bars represent means ± SE (n = 5). A, Electrical conductivity (EC); B, pH.

establishment of a multiple-shoot culture method for rugosa rose and its production in bioreactors for the accumulation of total phenolics and flavonoids. It can be concluded that in vitro cultures, particularly multiple-shoot cultures of *R. rugosa,* have the potential for scaled-up studies on a commercial level by pharmaceutical and cosmetic industries to further enhance the production of important phenolic and flavonoid compounds by the introduction of foreign genes for the production of valuable recombinant proteins.

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