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Research Report

# Establishment of embryogenic cultures and determination of their bioactive properties in *Rosa rugosa*

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**Abstract.** Somatic embryogenesis represents a powerful in vitro plant culture system and provides high potential for the production of biomass in woody plants. *Rosa rugosa* is known not only for its ornamental value, but also as a source of antioxidants with the potential to be used as ingredients for industrial products such as cosmetics. The present study was conducted to investigate the effects of hormonal concentration on embryogenic callus (EC) induction from petal and leaf segments of this species. The effect of selected cell lines on the production of total phenolics and flavonoids in *R. rugosa* was also studied. The highest percentage of EC (14.1 ± 3.9%) and root formation (20.3 ± 6.9%) was observed in the presence of 45.0 μM of 2,4-D. Among the three studied lines, the highest total phenolics and flavonoid levels were observed in H-1 (17.71 $mg \cdot g^{-1}$ DW), while H-18 contained the lowest. Histological studies of the H-1 and H-18 cell lines revealed differences in polyphenol accumulation and polysaccharides. The effect of developmental stage on growth, shoot, and root regeneration, as well as on phenolic and flavonoid content, was also studied in H-18. The present study indicates that selection of the proper type and concentration of hormone is essential for EC induction from petals in *R. rugosa*. Moreover, the accumulation of bioactive compounds was found to greatly depend on the genotype. The results of this study are useful for the production of selected antioxidants by somatic embryos from *R. rugosa* petals.

***Additional key words*:** callus, flavonoid, petal, phenolics, somatic embryo

## Introduction

*Rosa rugosa* belongs to the family Rosaceae and is well known for its horticultural importance. The species naturally grows on sand or gravel beaches and in dune grassland, and it has been used in the breeding of other rose cultivars. The plant is an astringent and a stomachic, as well as traditionally used for increasing blood circulation to relieve blood stasis, aiding menstrual regulation, and counteracting toxins (Olech, 2012). Several studies have shown it to be a good source of phenolics, terpenoids, sugars, aromatics (Youwei and Yonghong, 2007), and vitamin C (Czyzowska et al., 2015). Recent investigations have revealed the presence of free radical scavenging activity, which has been attributed to the presence of polyphenolics (Cho et al., 2003a, b; Liangxiong et al., 2005). Helsper et al. (2003) reported on the production of antioxidants in *Rosa hybrida* after exposure to UV radiation.

Plant tissue culture techniques have recently become an attractive option for the large-scale production of plant metabolites, because tissue culture requires less space and is independent of environmental effects and seasonal changes (Fiuk and Rybczyński, 2008). Somatic embryogenesis is considered to be an efficient method for in vitro multiplication of plants (Junaid et al., 2006) and it has also been used for secondary metabolite production in many plant species (Park et al., 2005). Somatic embryogenesis in *R. rugosa* from immature seeds (Kunitake et al., 1993) as well as from mature seeds (Kim et al., 2009) has been reported, but the regeneration efficiency has been low. A reproducible protocol for the mass production of somatic embryos (SEs) from this valuable plant, to use as a source of bioactive compounds, has not been developed to date. In the present

study, an attempt has been made to establish a high efficiency protocol for somatic embryo production in *R. rugosa.* In the present study, the effect of genotype on growth and secondary metabolite production was also examined, because the genotype has been previously shown to influence secondary metabolite production (Scalzo et al., 2005). Phenolic compounds are efficient oxygen radical scavengers that protect plants against the damaging effects of UV and pathogens among other things (Liangxiong et al., 2005). They are therefore considered to play an important role in controlling oxidative damage in the human body and exhibit anti-carcinogenic and anti-atherogenic activity (Tulipani et al., 2008). Flavonoids also play a protective role in the human body due to their strong antioxidant activity (Lotito and Frei, 2006). Flavonoids are widely used food additives with various functional roles, many of which are based on oxidation-reduction properties, and they are important co-enzymes for internal hydroxylation reactions (Khan et al., 2006). Thus, in the present study, the total phenolic and flavonoid contents were examined to study the effect of different cell line genotypes on antioxidant metabolite production. Histological studies were conducted on selected cell lines to observe cellular differences in the genotypes that produce the highest and lowest amounts of selected secondary metabolites. The developmental stage of the seed is an important factor in the growth of SEs as well as in regeneration, and it can also influence secondary metabolite content (Misra et al., 2005). Recently, *R. rugosa* was selected as a potential natural ingredient in the cosmetics industry in Korea. However, none of the published studies focused on exploiting somatic embryogenesis using selected cell lines, to provide a rich source of bioactive compounds for industrial purposes from *R. rugosa*.

The present study investigated factors that influence embryogenic callus induction and regeneration from *R. rugosa* petals, with the purpose of obtaining antioxidant secondary metabolites from the plant to use as potential ingredients for industrial products.

## Materials and Methods

### Plant Material and Embryogenic Callus (EC) Induction

Five-year-old *Rosa rugosa* plants were obtained at a local market in 2012 and planted in an experimental field of Chungbuk National University. Immature flower buds (approximately 2.0-2.5 cm in length) and green shoots (5 cm) were collected and rinsed with running water before surface sterilization. The material was sterilized using 70% Et-OH for 1 min, followed by soaking in 0.2% $HgCl_2$ for 15 min, and in 2% NaOCl containing one or two drops of Tween 20 for 30 min. After sterilization, the material was rinsed three times with sterilized water. The petal and leaf segments (0.5 × 0.5 cm) were placed upside down on Murashige and Skoog (MS) medium containing 0-45.0 μM 2,4-D. The petal segments (0.5 × 0.5 cm) were cultured on MS medium containing 0.0-45.0 μM 2,4-D and 2iP (9.8, 19.6 μM) and zeatin (9.1, 18.4 μM).

The experiments were repeated twice, and each treatment had 5 replications with 10 explants per petri dish. The culture period for EC induction was eight weeks, and the cultures were transferred to fresh medium every four weeks. After induction of ECs, all explants were transferred to hormone-free MS medium to develop somatic embryos (SEs) from the explants. After four weeks, the survival rate, browning rate, number of ECs, and shoot regeneration were analyzed. The number of ECs and the EC induction rate were calculated after SEs had developed.

### Characteristics of Embryogenic Callus Lines and the Development of Somatic Embryos

Five lines of embryogenic callus (H-1, H-3, H-11, H-18, H-31) were placed on MS medium containing 22.5 or 45.0 μM 2,4-D and cultured for 3 weeks. To determine the properties of each embryogenic cell line, EC and SE production rates were determined, and fresh weight and dry weight were measured. Three from the five lines (H-1, H-3, H-18) were maintained and proliferated on MS medium containing 45.0 μM 2,4-D. After 6 months of proliferation, SE clumps were harvested and their total phenolic and flavonoid contents were determined.

To investigate changes in antioxidant content during the developmental stages of somatic embryos, embryogenic calli (H-18) were transferred to hormone-free, half-strength MS medium containing 3% sucrose and 0.9% phyto agar (Duchefa, The Netherlands) for development into plantlets. Four masses of embryogenic calli (diameter 1 cm) were inoculated in 100-mL Erlenmeyer flasks. The experiments were conducted at 24 ± 1°C under continuous light from cool-white fluorescent lamps (PPFD 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for 8 weeks. The developed propagules were sampled every two weeks and analyzed for total phenolics and flavonoids. The survival rate, number of somatic embryos and plantlets, and the number of roots were also counted.

### Light Microscopy

Embryogenic calli (H-1 and H-18) with the highest and lowest phenolic content were collected from cultures and fixed in a solution containing 2.5% glutaraldehyde and 1.6% paraformaldehyde (in 0.05M phosphate buffer) for 24 h at room temperature. Samples were dehydrated in an alcohol series and then embedded in Technovit 7100 (Kulzer, Germany) according to the procedure of Yeung (1999). Serial 3-μm sections were cut with disposable tungsten knives on an

Autocut rotary microtome (RM 2165, Leica, Wetzlar, Germany). The sections were stained with toluidine blue O (Sigma T0394) for 5 min. The sections were examined using a Leica DMR light microscope and images were recorded with a digital camera (Leica DC 300F) and IM 50 software.

### Analysis of Bioactive Compounds

***Preparation of plant 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 Rosihi Kaisha Ltd., Japan). The final volume of the solution was set at 15 mL using 80% ethanol.

***Determination of total phenolics*:** Total phenolics were analyzed by the Folin-Ciocalteu colorimetric method (Folin and Ciocalteu 1927). The ethanol extracts from the explants (0.1 mL) were mixed with 2.5 mL distilled water, and 0.1 mL (2N) Folin-Ciocalteu reagent was added. Subsequently, 0.5 mL of 20% $Na_2CO_3$ solution was added after 5 min and mixed well. The color was allowed to develop for 30 min in the dark at room temperature and the absorbance was detected at 760 nm on a 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.

***Determination of total flavonoids*:** The total flavonoid content was determined colorimetrically using the method described by Park et al. (2015). The ethanol extracts from the explants and the standard (0.25 mL) were mixed with 1.475 mL distilled water, followed by the addition of 0.075 mL 5% $NaNO_2$ solution and the mixture was vigorously shaken. After a 6-min incubation, 0.15 mL 10% $AlCl_3$ solution was added. After 5 min, the absorbance was measured at 510 nm using a spectrophotometer. The results were expressed as mg of (+)-catechin (Sigma, St. Louis, MO, USA) equivalents per gram of dry explant. The total phenolic and flavonoid 'content' was calculated to indicate the accumulation of each metabolite per gram dry mass, the 'productivity' was calculated as 'content' produced by amount of dry mass per liter medium.

### Statistical Analysis

The results shown are the mean values of the experiments. One-way analysis of variance (ANOVA) was used to determine whether the groups differed significantly. Statistical assessments of the differences between mean values were compared by Duncan's Multiple Range Test (DMRT). 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, Cary, NC, USA).

## Results and Discussion

### Induction of Embryogenic Calli from Different Explants

Embryogenic calli were induced from two types of explants: petal and leaf segments. The results of embryogenic callus formation from *R. rugosa* petals under different concentrations and combinations of plant growth regulators (PGRs) are shown in Tables 1 and 2. In the absence of other

**Table 1.** Effects of different concentrations of 2,4-D on embryogenic callus formation from leaf and petal segments of *R.rugosa*after eight weeks of culture

| Explant type | 2,4-D (μM) | Survival rate (%) | Callus formation (%) | Embryogenic callus (%) | Root formation (%) |
|---|---|---|---|---|---|
| Petal | 0.0 | 0.0 c[z] | 0.0 c | 0.0 d | 0.0 b |
| | 2.3 | 76.3 a | 77.8 a | 0.0 d | 15.4 ab |
| | 4.5 | 68.9 ab | 75.6 a | 0.0 d | 20.0 a |
| | 13.5 | 74.4 a | 76.0 a | 1.7 b | 12.9 ab |
| | 22.5 | 64.2 ab | 64.2 ab | 1.7 b | 19.9 a |
| | 45.0 | 62.5 ab | 70.3 a | 14.1 a | 20.3 a |
| Leaf | 0.0 | 0.0 e | 0.0 e | 0.0 d | 0.0 b |
| | 2.3 | 1.2 e | 1.2 e | 0.0 d | 1.2 b |
| | 4.5 | 32.5 d | 28.3 c | 0.0 d | 5.0 ab |
| | 13.5 | 46.3 c | 30.3 c | 0.7 b | 15.0 a |
| | 22.5 | 47.2 c | 43.7 b | 1.3 b | 4.9 ab |
| | 45.0 | 47.2 c | 19.3 d | 0.3 c | 0.0 b |

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

**Table 2.** Effects of 2,4-D, 2ip, and Zeatin on embryogenic callus formation from petal segments of *Rosa rugosa* after eight weeks of culture

| PGRs (μM) | | | Survival rate (%) | Frequency of callus formation (%) | Frequency of EC formation (%) | Frequency of root formation (%) |
|---|---|---|---|---|---|---|
| 2,4-D | 2ip | Zeatin | | | | |
| 0.0 | 0.0 | 0.0 | 0.0 c[z] | 0.0 c | 0.0 c | 0.0 c |
| 22.5 | 9.8 | 0.0 | 65.0 ab | 65.0 ab | 0.0 c | 5.3 a |
| 22.5 | 19.6 | 0.0 | 51.8 b | 51.8 b | 1.0 b | 2.0 b |
| 45.0 | 9.8 | 0.0 | 74.0 ab | 75.6 ab | 1.2 b | 0.0 c |
| 45.0 | 19.6 | 0.0 | 74.2 ab | 74.2 ab | 0.4 c | 0.0 c |
| 22.5 | 0.0 | 9.1 | 60.6 ab | 60.6 ab | 0.0 c | 2.7 b |
| 22.5 | 0.0 | 18.4 | 84.2 a | 84.2 a | 0.0 c | 0.0 c |
| 45.0 | 0.0 | 9.1 | 79.7 a | 81.3 a | 6.2 a | 0.0 c |
| 45.0 | 0.0 | 18.4 | 66.7 ab | 66.7 ab | 0.7 ab | 0.0 c |

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

PGRs, embryogenic callus formed only when high concentrations of 2,4-D were present in the medium (Table 1). The highest percentage of EC formation (14.1 ± 3.9%) was observed from petal segments when 45.0 μM of 2,4-D was added to the medium. In some medicinal plants, such as *Mucuna pruriens* and *Taxus mairei*, the beneficial effect of 2,4-D on callus formation for L-DOPA production and taxol production, respectively, has been reported (Vanisree et al., 2004). The influence of exogenously applied auxins, specifically 2,4-D, on somatic embryogenesis in several plant species is well known (Feher et al., 2003). In alfalfa protoplasts, endogenous IAA levels increased considerably after exposure to 2,4-D (Pasternak et al. 2002). High concentrations of 2,4-D in plant cultures not only act as an auxin but also as a stressor (Feher et al., 2001, 2002). Several lines of experimental evidence point to an interaction between auxin-induced stress and ABA signaling (Feher et al., 2003).

To study the effect of a combination of cytokinin and 2,4-D on embryogenic callus formation from petals, the 2,4-D medium was supplemented with the cytokinins 2iP and zeatin (ZA) (Table 2). Embryogenic calli were induced only when the petal was treated with high concentrations of 2,4-D; at lower concentrations, even when supplemented with cytokinins, 2,4-D failed to induce embryos. However, maximum callus formation (84.2 ± 3.2%) was observed when 22.5 μM 2,4-D was supplemented with 18.4 μM zeatin (Table 2). Exogenous auxin and cytokinin work indirectly by modifying endogenous hormone status, which directly controls the formation and proliferation of callus. Chen et al. (2014) reported that low concentrations of cytokinin increased the frequency of embryogenic callus induction and embryo production. However, in this experiment, a combination of cytokinin and 2,4-D was not effective for inducing embryogenic callus in *R. rugosa*. Since Hill (1967) reported the first successful rose regeneration, the presence of 2,4-D alone or in combination with other PGRs, has been reported to be important for induction of somatic embryogenesis in rose. However its effect on somatic embryogenesis was variable (Li et al., 2002). Hsia and Korban (1996) induced 6.6% somatic embryogenesis in leaf sections of 'Carefree Beauty' on a medium containing 23 μmol TDZ and 3 μmol $GA_3$, and similarly, Li et al. (2002) obtained somatic embryos from leaf segments on lower concentrations of TDZ and $GA_3$.

## Characterization of Embryogenic Callus Proliferation and Bioactive Compound Accumulation in Different Cell Lines

Growth measurements of various cell lines on MS medium containing 22.5 and 45.0 μM of 2,4-D are shown in Fig. 1. The growth ratio was determined by cell line, with maximum values (3.1 ± 0.4) being observed in H-18, the cell line with the highest fresh weight. The lowest growth ratio (1.5 ± 0.3) was observed in cell line H-3 (Table 3). Measurements of the bioactive compounds in the three genotypes (H-1, H-3, and H-18) after four weeks of culture on the medium are shown in Fig. 2. The highest total phenolic content (45.0 $mg \cdot g^{-1}$ DW) and productivity were observed in genotype H-1. The highest flavonoid content (24.3 $mg \cdot g^{-1}$ DW) and productivity were also observed in cell line H-1.

Genotype appears to be one of the important factors that determine the morphogenetic reaction in vitro. Genotypic differences can be observed between species, cultivars, and individuals (Fiuk and Rybczyński, 2008). In our study, cell lines were induced from petal segments of different individual plants with different genotypes. Tulipani et al. (2008) reported that dry matter content varies among different genotypes of strawberry, and that these different genotypes also exhibited differences in flavonoid content. Similarly, cell lines derived

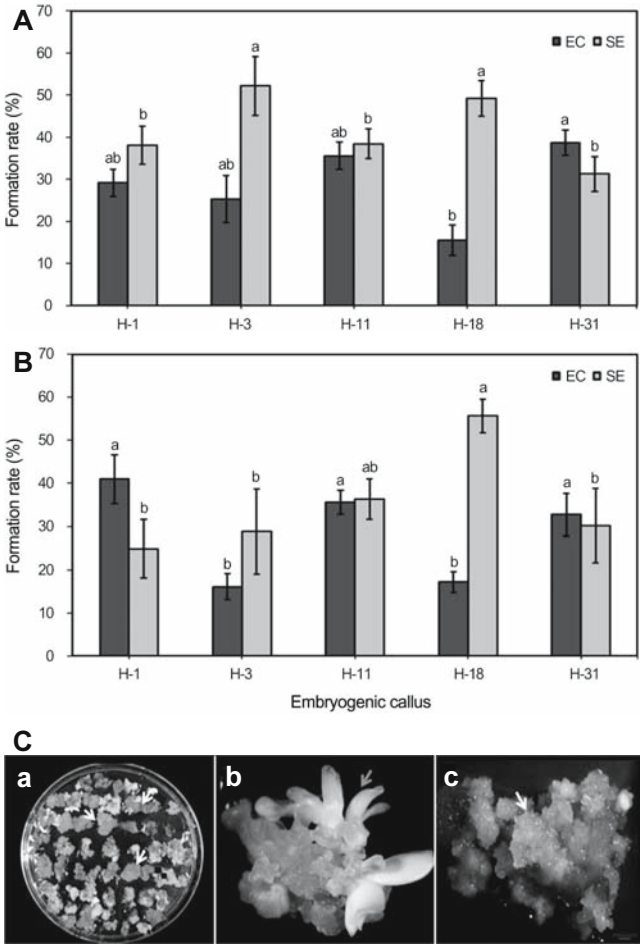


**Fig. 1.** Effects of 2,4-D concentration on embryogenic callus (EC) and somatic embryos (SE) proliferation from different cell lines (genotypes) of *R. rugosa* after four weeks of culture. (A) MS medium containing 22.5 μM 2,4-D, (B) MS medium containing 45.0 μM 2,4-D, (C) EC and SE proliferation in H-1. Blue arrows indicate somatic embryo, and white arrows indicate embryogenic cells (a-b. 22.5 μM 2,4-D; c. 45.0 μM 2,4-D). Bars represent means ± SE (n = 5).

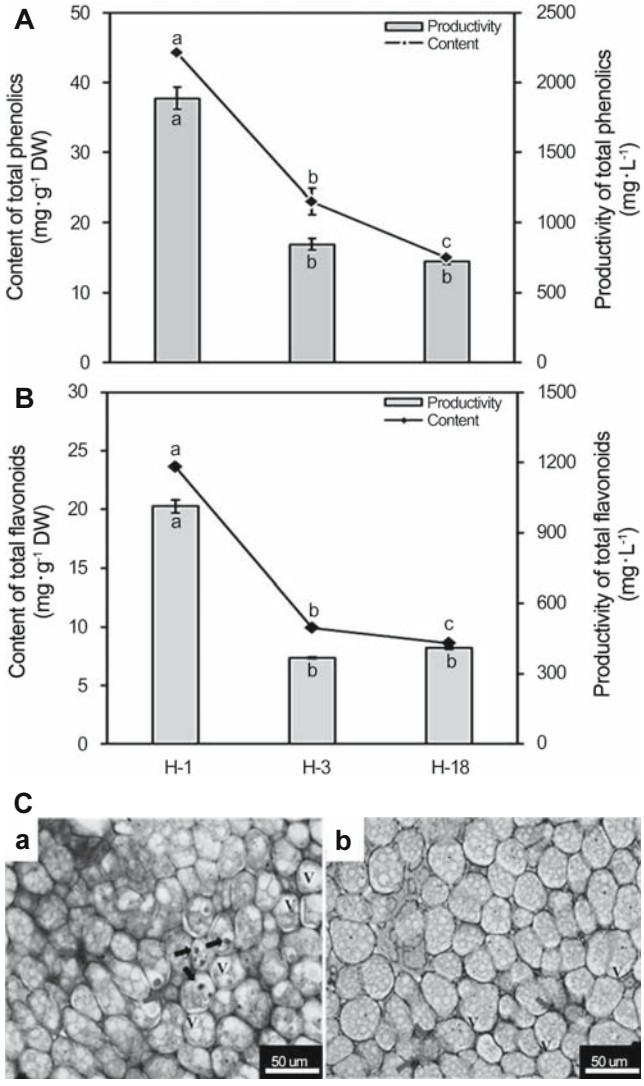


**Fig. 2.** Content and productivity of total phenolics and flavonoids from different cell lines (genotypes) on MS medium containing 45.2 μM 2,4-D in *R. rugosa* after four weeks of culture. (A) Total phenolics, (B) Total flavonoids, (C) Histological analyses of embryogenic callus in H-1 (a) and H-18 (b) (polyphenols *red arrows,* polysaccharide *black arrows,* vacuoles *V*); Scale bar = 50 μm.

from different genotypes are known to produce different concentrations and compositions of essential oils in several medicinal plants. Changes in gene expression and functional enzymatic diversity have been implicated in the evolution of these genotypes, with natural selection being the determining factor (Tripathi and Tripathi, 2003).

Histological analyses of the embryogenic calli of cell lines H-1 and H-18 are shown in Fig. 2. Cells of the callus derived from H-1 show high accumulation of polyphenols in the intercellular spaces. Cells of this genotype have several vacuoles and contain polysaccharide granules. By contrast, callus cells derived from H-18 contain polyphenols, whereas polysaccharides are absent. Cells from this cell line have small and dense vacuoles.

Polyphenolics were observed in sections from both H-1 and H-18 cell lines. During SE formation in *Feijoa sellowiana*, embryogenic cells contained phenolic compounds and starch grains (Cangahuala-Inocente et al., 2004). Polyphenols are considered to inhibit hyperhydricity and also serve as precursors of lignin biosynthesis in *Eucalyptus nitens* cultures (Bandyopadhyay and Hamill, 2000). However, in the present study the H-18 cell line showed an absence of polysaccharides, whereas H-1 showed an abundance of polysaccharides. Further studies are required to establish the correlation between absence of polysaccharides and secondary metabolite production in this genus.

**Table 3.** Growth of different cell lines (genotypes) on MS medium containing 45.2 μM 2,4-D in *R. rugosa* after four weeks of culture

| Line | Fresh weight (g) | Dry weight (g) | % dry weight | Growth ratio[z] |
|---|---|---|---|---|
| H-1 | 1.50 ab[y] | 0.24 a | 16.10 a | 2.0 bc |
| H-3 | 1.25 b | 0.19 b | 15.82 ab | 1.5 c |
| H-18 | 2.05 a | 0.27 a | 13.31 c | 3.1 a |

[z]Growth ratio = [Harvested fry weight (g) - inoculated fry weight (g)] / inoculated fry weight (g)
[y]Mean separation within columns by Duncan's multiple range test at 5% level

**Table 4.** Effects of developmentalstage on shoot and root regeneration in H-18 somatic embryos

| Time (week) | Shoot formation (%) | No. of shoots (per EC clump) | Rooting (%) | No. of roots (per EC clump) |
|---|---|---|---|---|
| 0 | 0.0 b[z] | 0.0 c | 0.0 b | 0.0 c |
| 2 | 0.0 b | 0.0 c | 0.0 b | 0.0 c |
| 4 | 79.5 a | 2.1 b | 80.4 a | 2.7 b |
| 6 | 80.8 a | 3.4 ab | 93.8 a | 3.7 ab |
| 8 | 81.3 a | 4.4 a | 96.9 a | 5.0 a |

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

**Table 5.** Effects of developmental stage on growth of H-18 somatic embryos

| Time (week) | Fresh weight (g per bottle) | Dry weight (g per bottle) | % dry weight | Growth ratio[z] |
|---|---|---|---|---|
| 0 | 0.5 c[y] | 0.0 c | 13.4 c | 0.0 d |
| 2 | 0.8 c | 0.1 c | 13.6 c | 0.0 d |
| 4 | 1.2 b | 0.2 bc | 15.8 a | 0.8 c |
| 6 | 1.2 b | 0.3 b | 14.9 b | 1.8 b |
| 8 | 1.9 a | 0.5 a | 13.2 c | 3.6 a |

[z]Growth ratio = [harvested dry weight (g) – inoculated DW (g)] / inoculated DW (g)
[y]Mean separation within columns by Duncan's multiple range test at 5% level.

## Accumulation of Bioactive Compounds Depending on the Developmental Stage during Conversion of SE

The effect of developmental stage on growth, regeneration, and bioactive compound formation in the SEs derived from cell line H-18 was studied (Table 4 and 5). Table 4 indicates that the developmental stage had a significant effect on the growth of somatic embryos of *R. rugosa*. At the end of eight weeks, the SEs developed a maximum number of roots (5.0 per EC clump) and shoots (4.4 per EC clump) with a maximum percentage of shoot development (81.3%) and root development (96.9%). The highest growth ratio (3.6 ± 0.1) was observed after eight weeks of growth (Table 5).

The effect of developmental stage on the accumulation of phenolics and flavonoids in the SEs of H-18 was also studied (Fig. 3). The results indicate that the maximum total phenolic content (50 $mg \cdot g^{-1}$ DW) was observed after six weeks of growth, after which it declined. The maximum total flavonoid content was also recorded after the same time period, with a similar subsequent decline. The maximum productivity of total phenolics (600 $mg \cdot L^{-1}$) and total flavonoids (300 $mg \cdot L^{-1}$) was achieved by the eighth week.

These studies on the effect of the developmental stage on growth, regeneration of shoot and root, and secondary metabolite content in H-18 cell lines indicate that maximum productivity is achieved for both phenolics and flavonoids by the eighth week, which coincides with the period during which the growth ratio is maximal. After the sixth week, however, secondary metabolite content declines, which may be the result from the release of metabolites into the medium.

In conclusion, the above studies help to establish a high efficiency protocol for EC formation from *R. rugosa* petals (Fig. 4). The present study also indicates that selection of a proper genotype is crucial for the production of selected metabolites from SEs derived from *R. rugosa* petals. In addition, the results indicate the potential for a useful system for *R. rugosa* mass micropropagation and for future genetic transformation.

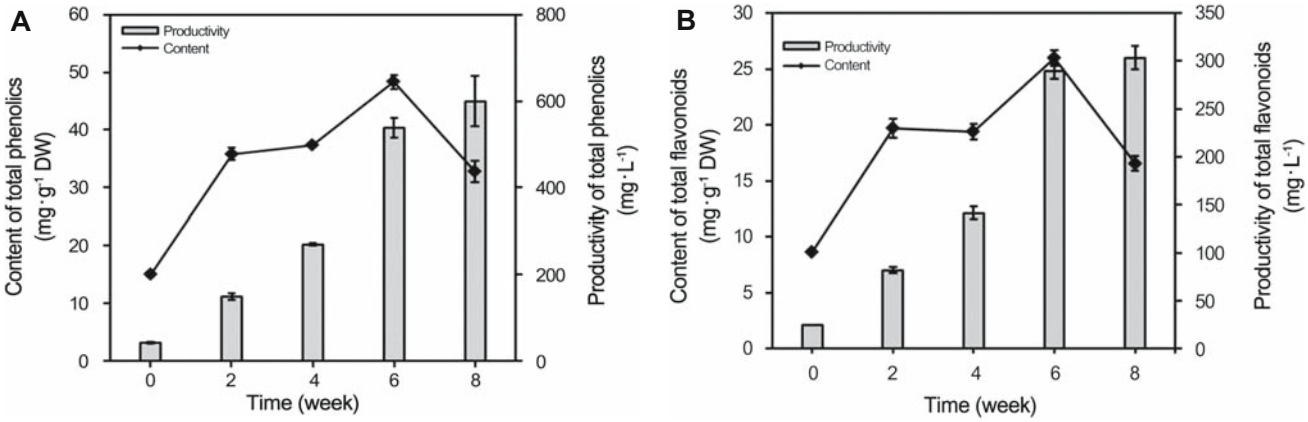


**Fig. 3.** Effects of developmental stage on the accumulation of bioactive compounds in somatic embryos (H-18) of *R. rugosa.* (A) Total phenolics, (B) Total flavonoids. Bars represent means ± SE (n = 5).

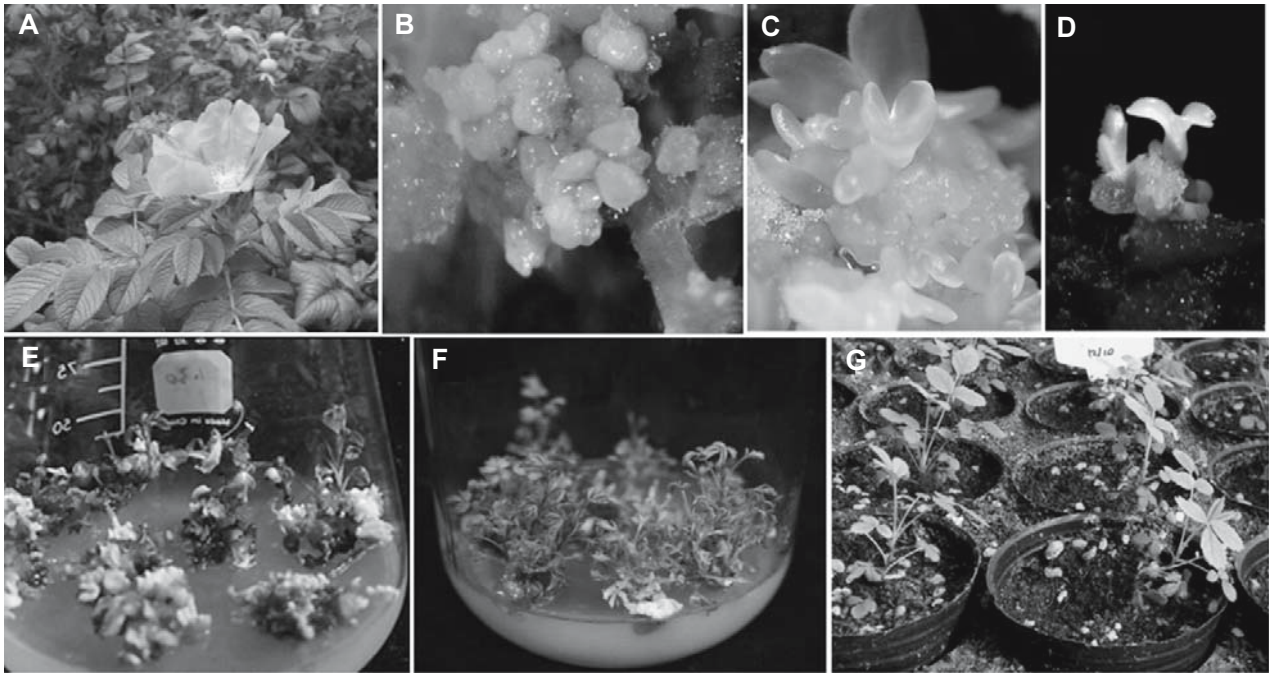


**Fig. 4.** Plant regeneration through somatic embryogenesis in *Rosa rugosa.* (A) Flower of *R. rugosa*, (B) Induction of embryogenic callus from leaf segments on MS medium containing 45.2 μM 2,4-D, (C-D) Developed somatic embryos (SEs), (E) Conversion of plantlets on hormone-free 1/2 MS medium, (F) Growth of SE-derived plantlets, (G) Acclimatization to greenhouse conditions.

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