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Effect of stress factors on somatic embryogenesis of rose

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Abstract

In the present study, somatic embryogenesis was optimized in three cut-flower roses including *Rosa hybrida* 'Full House', 'Ocean Song' and 'Maroussia'. Stress factors including ABA, NaCl, reduction in sucrose concentration, different concentrations of NAA or 2,4-D were applied. In order to detect ploidy changes in the regenerated somatic embryos, the ploidy level of the regenerants and their donor plants were assessed by flow cytometry. The results indicated that somatic embryogenesis was highly genotype dependant. The highest induction rate of embryogenic calli (46.66%) and number of primary and secondary embryo (7.33 and 116.66) were obtained in 'Ocean song' in MS medium supplemented with NAA (3 mg l⁻¹) in combination with 300 mg l⁻¹ proline. Moreover, application of 100 mg l⁻¹ NaCl as stress factor along with 2 mg l⁻¹ 2, 4-D resulted in high rate of somatic embryogenesis (40.0%) in 'Ocean song'. The highest rate of somatic embryogenesis (26.66%) in *Rosa hybrida* cv. Maroussia in MS medium with 1.5 mg l⁻¹ 2, 4-D and 0.1 mg l⁻¹ ABA and Full House with 0.5 mg l⁻¹ 2, 4-D along with half sucrose concentration were observed. In all experiments, hormone-free MS medium were used for production and proliferation of secondary somatic embryos from primary embryos. Our results suggest that stress agents have an important role in the cellular mechanisms conducting somatic embryogenesis induction in rose, and that the ploidy level of the regenerated plants remains unchanged.

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Introduction

Roses are one of the most popular cultivated ornamental plants. Many efforts have been carried out to improve the interested criteria such as color, morphology, fragrance and length of vase life of cut flowers. Being perennial ornamental and sterile caused by abnormal chromosome number, traditional breeding programs encounter many problems in rose improvement. Nowadays, biotechnology has emerged as an appropriate alternative to conventional rose propagation and breeding systems (Sondahl, 1996; Canli and Kazaz, 2009). Somatic embryogenesis is valuable in plant breeding using modern techniques such as gene transformation. Somatic embryogenesis is a stress-inducible process in which somatic cells re-differentiate and give rise to cells that can form somatic embryos, with the occurrence of cell–cell interactions (Zavattieri *et al.*, 2010). Several induced stresses are applied for inducing somatic embryogenesis including osmotic stress using sucrose, culture medium dehydration, heavy metal ions, pH of induction medium, heat or cold shock, hypoxia, antibiotics, mutagenic agents, plant growth regulators (PGR) or stress hormones i.e. abscisic acid (ABA) and jasmonic acid (Fehér, 2005; Karami *et al.*, 2006; Potters *et al.*, 2007; Lincy *et al.*, 2009; Ahamdi *et al.*, 2014).

Absciscic acid as stress hormone plays an important role during somatic embryogenesis and enhances somatic embryo quality by increasing desiccation tolerance and preventing precocious germination in tissue culture systems (Rai *et al.*, 2011). Carbohydrates also have a significant role in different aspects of somatic embryogenesis (Gerdakaneh *et al.*, 2009). Sucrose, the standard carbohydrate in the majority of plant tissue culture media, acts both as a carbon source and an osmotic regulator (Last and Brettell, 1990). Changes in sucrose concentration as an osmoticum may result in the induction of stress in a culture medium.

Salinity, as a major abiotic stress, causes physiological, biochemical, and genetic changes in plants (Dajic, 2006). There are several reports on the

selection of salt tolerant plants in several species such as *Triticum aestivum* (Arzani and Mirodjagh, 1999), *T. durum* (Zair *et al.*, 2003), and sugarcane (Gandonou *et al.*, 2005).

Plant growth regulators (PGRs), one of the effectiveness factors on somatic embryogenesis, in particular auxins act as central signals to reprogram somatic cells towards embryogenic pathways (Pasternak *et al.*, 2002; Gaj, 2004; Fehér, 2005; Jiménez and Thomas, 2005), however, endogenous content and the application of exogenous auxins are both determining factors during the induction phase (Jiménez and Thomas, 2005). Auxins also cause stress in plant cells. It is suggested that a high concentration of auxin may be perceived as a stress condition (Kitamiya *et al.*, 2000; Estabrooks *et al.*, 2007).

An efficient regeneration system is the major limiting factor in genetic transformation of Rose. Despite several studies performed on somatic embryogenesis in *Rosa hybrida*, the effects of stress factors on somatic embryogenesis in rose are not well explored. In this study, the stress effect of different concentrations of ABA, NaCl, NAA, 2, 4-D and sucrose were assessed on somatic embryogenesis in *Rosa hybrida* 'Full house', 'Ocean song' and 'Maroussia' and a reliable protocol for regeneration of these three cultivars were introduced.

Materials and methods

Plant material and general procedures

Stem nodal segments of three rose cultivars (*Rosa hybrida* 'Full house', 'Ocean song' and 'Maroussia') were collected from the Agricultural Biotechnology Research Institute of Iran (ABRII). Nodal explants were washed thoroughly with running tap water for 30 min and surface sterilized for 30 seconds with ethanol (70%, v/v), followed by a 15 min soak in 2.5% (v/v) sodium hypochlorite solution with a few drops of Tween-20 as a wetting agent and then rinsed three times with sterile distilled water. The nodal explants were placed into Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with

benzylaminopurin (BAP) (0.45 mg l^{-1}), sucrose (30 g l^{-1}) and plant agar (7 g l^{-1}), with a pH adjusted to 5.7 with 1 N NaOH and 1 N HCl, followed by incubation at $22 \pm 2^\circ \text{C}$ for two weeks under a 16-h photoperiod with cool-white fluorescent light and a PPFD of $60 \mu\text{mol M}^{-2}\text{s}^{-1}$. Auxiliary shoots of Full house and Ocean song were transferred to VS proliferation medium (van der Salm *et al.*, 1994), while shoots of 'Maroussia' were placed on MS proliferation medium. Proliferation media were supplemented with BAP (0.45 mg l^{-1}), sucrose (30 g l^{-1}) and plant agar (7 g l^{-1}), with a pH adjusted to 5.7 with 1 N NaOH and 1 N HCl. These shoots were sub-cultured on the same fresh medium once every 6 weeks.

Induction of somatic embryogenesis

Four different experimental approaches were applied to investigate somatic embryogenesis in three rose cultivars ('Full house', 'Ocean song' and 'Maroussia'). In all experiments, the leaflets on third and fourth vigorously growing leaves were excised from *in vitro* shoots, scraped on midrib by scalpel blade and placed with their adaxial sides down onto the embryo induction media. Frequency (%) of regenerated somatic embryos, number of somatic embryos and secondary embryos were recorded.

Experiment 1. MS medium with different concentrations of ABA (0, 0.1 and 0.2 mg l^{-1}) along with 2, 4-D (0, 1.5 and 2 mg l^{-1}) and sucrose (30 g l^{-1}) was used and solidified with plant agar (7 g l^{-1}). Calli were sub-cultured every 4 weeks in the same medium for 16 weeks at $22 \pm 2^\circ \text{C}$ under a 16/8 hrs photoperiod until the formation of primary embryos.

Experiment 2. This experiment included an inducing medium based on MS medium supplemented with various concentrations of NAA or 2, 4-D (0.5, 1 and 1.5 mg l^{-1}) and sucrose (30 g l^{-1}), and solidified with plant agar (7 g l^{-1}). After 6 weeks, calli were transferred to the same medium with 1.5% sucrose to reduce osmotic potential. Cultures were incubated for 32 weeks at $22 \pm 2^\circ \text{C}$ under a 16/8 hrs photoperiod until the formation of primary embryos.

Experiment 3. This treatment was performed based

on a modified method of Rout *et al.*, (1991) using MS medium supplemented with different concentrations of NAA (0, 1, 2, 3 and 4 mg l^{-1}) with proline (0 or 300 mg l^{-1}) as an induction medium. Leaf explants were incubated in this medium for six weeks in the dark to induce callogenesis. Friable calli were transferred to the same culture medium and incubated at $22 \pm 2^\circ \text{C}$ for 12 weeks under a 16/8 hrs photoperiod to the point of embryogenesis and the production of cotyledonary-stage embryos were observed.

Experiment 4. MS medium with different concentrations of NaCl (0, 10, 50, 100 and 150 mg l^{-1}) and 2, 4-D (0, 1, 2, 3 and 4 mg l^{-1}) was studied. The explants were incubated in this medium in the dark for 6 weeks to induce friable calli. Friable calli were transferred to the same culture medium and incubated at $22 \pm 2^\circ \text{C}$ for 12 weeks under a 16/8 hrs photoperiod until embryogenesis and the production of cotyledonary-stage embryos were observed.

In all experiments, primary embryogenic calli were transferred to hormone-free MS medium and maintained for several months. In this medium, germination of somatic embryos and proliferation of secondary somatic embryos were observed. After 8 weeks, data on the number of secondary somatic embryos were recorded. Following 8 weeks after the formation of secondary somatic embryos, the frequency of regenerating shoots from embryogenic calli was also recorded.

The proliferation of somatic embryos and regenerating shoots continued at a constant rate for a period of more than 16 months (data not shown).

Histological studies

Embryogenic calli at different developmental stages were fixed in FAA (formalin, acetic acid, ethanol: 1,2,17 v/v) for 24 h, dehydrated by serial grades of ethanol-xylol and embedded in paraffin, and serial sections of $8 \mu\text{M}$ thick were cut and stained with hematoxylin and eosin. The sections were then visualized under a light microscope (Zeiss, AxioStar Plus, Germany).

Determination of the ploidy level

In order to detect ploidy changes in the regenerated somatic embryos, the ploidy level of the regenerants and their donor plants were assessed by flow cytometry (PAI, Partec, Germany) according to Yokoya *et al.*, (2000). *Petroselinum crispum* cv. Champion Moss Curled ($2n = 2x = 22$; $2C$ DNA amount = 4.46 pg) was used as an internal calibration standard and 4', 6-diamidino-2-phenylindole (DAPI) was used as the fluorochrome. Leaves (50 mm^2) of rose were excised and chopped separately to the size of parsley leaves, using a sharp razor blade, in $400 \mu\text{l}$ nuclear extraction buffer (Partec, Germany). Then $1,600 \mu\text{l}$ of DAPI stain (Partec, Germany) was added

and the solution was filtered through a $50 \mu\text{m}$ filter. Estimates of the ratio of fluorescence intensities of each rose to parsley (p_1/p_2) were based on the mean of three samples (from one plant), each with a minimum of 10,000 nuclei, giving peaks with a coefficient of variation of less than 5%.

Results and discussion

Callogenesis was initiated from cut surfaces of explants after two weeks for all three cultivars. Calli had different sizes depending on the type of cultivar (data not shown). Callus morphology was different depending on the treatment. However, all calli formed in the dark were soft and yellow in color.

Table 1. Effect of plant growth regulators supplemented to the culture medium for embryogenesis from *in vitro* leaf explants of *Rosa hybrida* cv. Maroussia.

Cultivar	2,4-D (mg l^{-1})	ABA (mg l^{-1})	Number of explant	Callogenesis (%)	Embryogenic calli (%)	Number of embryos after 16 weeks	Number of primary secondary embryos	Number of normal plantlet
	1.5	0	30	100	0	0	0	0
	1.5	0.1	30	100	26.66±6.66	5.0±1.0	180±23.09	3±0.57
'Maroussia'	1.5	0.2	30	100	0	0	0	0
	2	0	30	100	0	0	0	0

Table 2. Effect of plant growth regulators supplemented to the culture medium along with half concentration of sucrose for embryogenesis from *in vitro* leaf explants of *Rosa hybrida* cv. Full house.

Cultivar	2,4-D (mg l^{-1})	NAA (mg l^{-1})	Sucrose (1.5%)	Number of explant	Callogenesis (%)	Embryogen ic calli (%)	Number of embryos after 32 weeks in culture	Number of primary secondary embryos	Number of normal plantlet
	0	0	+	30	0	0	0	0	0
	0.5	0	+	30	100	26.66±6.66	5.0±0.57	83.33±8.81	4.0±0.57
'Full House'	1	0	+	30	100	0	0	0	0
	1.5	0	+	30	100	0	0	0	0
	0	0	+	30	0	0	0	0	0
	0	0.5	+	30	100	0	0	0	0
	0	1	+	30	100	20.0±0	3.67±0.88	90±17.32	2.67±0.66
	0	1.5	+	30	100	0	0	0	0

Mean ± standard error of three repeated experiments.

According to our results, response to embryogenesis differed significantly with respect to cultivars. In experiment 1 (see Materials and Methods), the highest frequency of somatic embryogenesis (26.66%) was only observed in 'Maroussia' after 16 weeks in the MS medium supplemented with 2, 4-D (1.5 mg l^{-1}) and ABA (0.1 mg l^{-1}) (Table 1). Since embryogenesis did not occur in the remaining cultivars, data are not shown. ABA has been used in plant tissue culture systems to promote somatic embryo maturation and

synthesis of storage reserves during embryo maturation (Marchant *et al.*, 1996; ZakiZadeh *et al.*, 2008; Rai *et al.*, 2011; Azadi *et al.*, 2013). However, our results indicated that ABA as a stress promoter could be used in the induction medium to promote somatic embryogenesis in *Rosa hybrida*. Effectiveness of ABA treatment on the promotion of somatic embryogenesis has also been reported in *Camellia sinensis* (Akula *et al.*, 2000).

Table 3. Effect of different concentrations of NAA alone in combination with proline on the induction of embryogenic calli of *Rosa hybrida* cv. Ocean song.

Cultivar	NAA (mg l ⁻¹)	Proline (mg l ⁻¹)	Number of explant	Callogenesis (%)	Embryogeni c calli (%)	Number of primary embryos after 12 weeks in culture	Number of secondary embryo	Number of normal plantlet
	0	-	30	0	0	0	0	0
	1	-	30	100	0	0	0	0
	2	-	30	100	0	0	0	0
	3	-	30	100	0	0	0	0
'Ocean sung'	4	-	30	100	0	0	0	0
	0	300	30	0	0	0	0	0
	1	300	30	100	0	0	0	0
	2	300	30	100	0	0	0	0
	3	300	30	100	46.66±0.33	7.33±1.20	116.66±14.53	9.67±1.45
	4	300	30	100	16.67±3.33	3.67±0.33	70±1.52	9.33±0.66

Mean ± standard error of three repeated experiments.

Table 4. Effect of different concentrations of 2, 4-D in combination with NaCl on the induction of embryogenic calli of *Rosa hybrida* cv. Ocean song.

Cultivar	2,4-D (mg l ⁻¹)	NaCl (mg l ⁻¹)	Number of explant	Callogenesis (%)	Embryogen ic calli (%)	Number of primary embryos after 12 weeks in culture	Number of secondary embryo	Number of normal plantlet
	0	0	30	0	0	0	0	0
	1	0	30	100	0	0	0	0
	2	0	30	100	0	0	0	0
	3	0	30	100	0	0	0	0
	4	0	30	100	0	0	0	0
	0	50	30	0	0	0	0	0
'Ocean sung'	1	50	30	100	0	0	0	0
	2	50	30	100	13.33±3.33	2.33±0.33	56.66±20.81	2.66±0.33
	3	50	30	100	0	0	0	0
	4	50	30	100	0	0	0	0
	0	100	30	0	0	0	0	0
	1	100	30	100	0	0	0	0
	2	100	30	100	40.0±5.77	6.33±1.85	126.66±17.63	10.33±0.33
	3	100	30	100	22.33±3.33	3.33±0.88	66.66±12.01	4.0±0.57
	4	100	30	100	0	0	0	0

Mean ± standard error of three repeated experiments.

In experiment 2, somatic embryogenesis occurred only in the 'Full house' (Table 2), while other cultivars did not respond to this treatment (data are not shown) (Table 2). The highest frequency of somatic embryogenesis (26.6%) occurred in the MS medium containing 2, 4-D (0.5 mg l⁻¹) and NAA (1.0 mg l⁻¹) after 32 weeks of culture when the concentration of sucrose was reduced to half. However, the somatic embryogenesis did not occur when 3% sucrose was used (data not shown). In roses, the most commonly used source of carbon (sucrose) is applied at a concentration of 3%. Carbohydrates serve as energy

and osmotic agents to support the growth of plant tissue (Lipavska and Konradová, 2004). Earlier findings in roses showed that sucrose is an important factor for somatic embryo maturation (Castillon *et al.*, 2002; Kunitake *et al.*, 1993). A number of studies have reported the enhancing effect of sucrose on somatic embryogenesis in several species (Korbes and Droste, 2005; Karami *et al.*, 2006; Moon *et al.*, 2013; Gholami *et al.*, 2013). However, our results indicated that in some cultivars somatic embryogenesis is initiated when sucrose levels are reduced.



Fig. 1. Somatic embryogenesis, secondary somatic embryogenesis and plant development in *Rosa hybrida*. **a–b:** Primary somatic embryos (bar = 0.5 mm); **c:** Secondary somatic embryos of 'Full house' from the calli cultured on MS medium supplemented with 0.5 mg l^{-1} 2, 4-D (bar = 1 mm); **d:** Primary somatic embryos at the heart shape and cotyledonary stages (bar= 1 mm); **e:** Secondary somatic embryogenesis in 'Ocean song' from the calli cultured on MS medium supplemented with 3 mg l^{-1} NAA (bar = 2 mm); **f:** Elongation of cotyledons in embryos of 'Ocean song' (bar = 2 mm); **g:** Plantlet regenerated from somatic embryos in 'Ocean song' (bar= 3cm); **h:** Green house acclimated plants regenerated from somatic embryos in 'Ocean song' (bar= 4cm).

In experiment 3, the highest frequency of embryogenesis (46.66%) was observed in 'Ocean song', when the induction culture medium was supplemented with NAA (3 mg l^{-1}) and proline (300 mg l^{-1}) (Table 3). However, this medium did not

induce somatic embryogenesis in other cultivars (data not shown). Stimulation of auxin- induced somatic embryogenesis by proline has been well established by several studies (Rout *et al.*, 1991; Shetty and Mckersi, 1993; Marchant *et al.*, 1996). In the present

study, NAA at 3 mg l⁻¹ and 4 mg l⁻¹ along with proline (300 mg l⁻¹) induced embryogenesis, however, some reports have declared that proline combined with 2, 4-D alone could represent a conclusive factor in somatic embryogenesis (Eguchi *et al.*, 1997). According to Das (2010), proline acts as a nitrogen

pool and a source of NADP⁺ which is necessary for rapidly growing embryos. In fact, exogenous auxin in culture medium acts as a stress chemical agent and proline as an antioxidant can help in ameliorating the stress (Pasternak *et al.*, 2002).

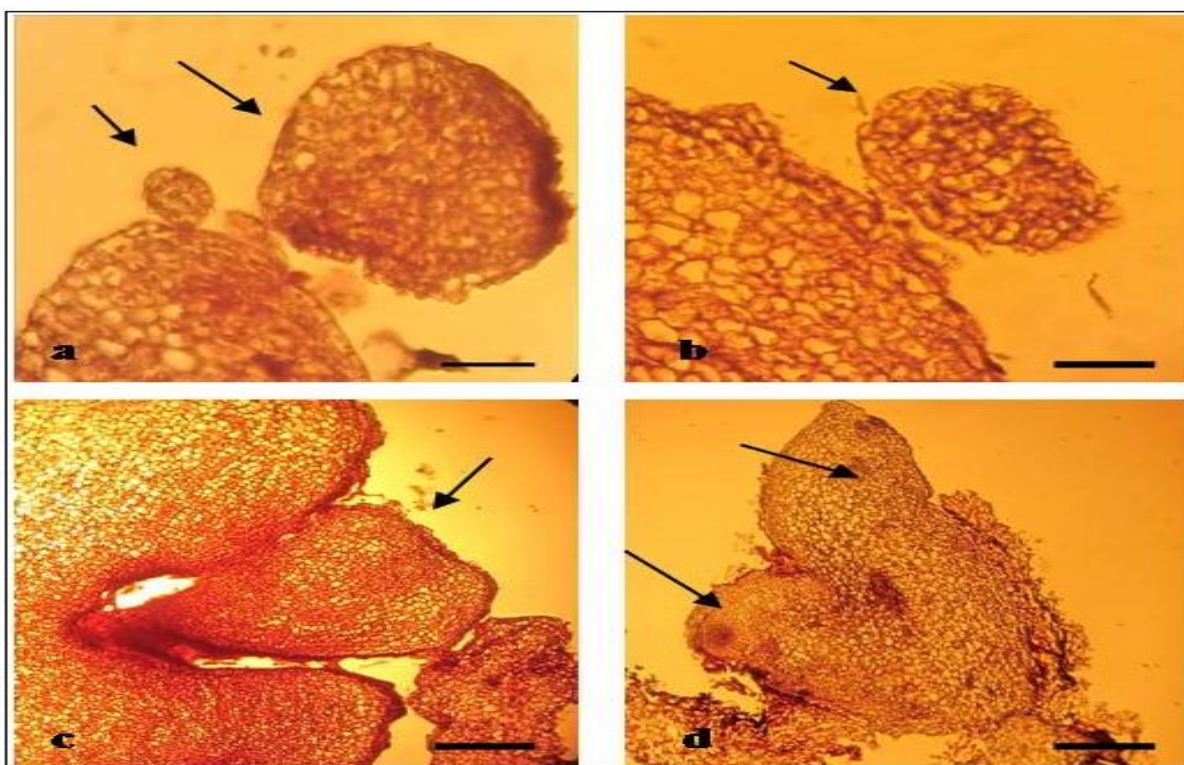


Fig. 2. Histology of somatic embryogenesis in *R. hybrida* cv. Ocean song; a: Longitudinal section of the globular embryo development on the surface of embryogenic callus (bar = 100 μ m); b: Longitudinal section of transient stage to heart-shaped embryo (bar = 100 μ m); c: Longitudinal section of heart-shaped embryo (bar = 50 μ m); d: Longitudinal section of somatic embryo (cotyledonary stage) showing two distinct cotyledons (bar = 50 μ m).

In experiment 4, different concentrations of 2, 4-D and NaCl were used (Table 4). A 2, 4-D (2 mg l⁻¹) and NaCl (100 mg l⁻¹) treatment resulted in a high percentage of embryogenesis in 'Ocean song'. Lower and higher levels of 2, 4-D and NaCl were not effective on somatic embryogenesis induction. 2, 4-D is known as a stress factor activating the expression of different stress related genes (Das, 2010; Shinoyama *et al.*, 2004). NaCl which is applied in culture medium as an osmoticum, also results in generating abiotic stress. These results indicated that abiotic stress plays an important role in embryogenesis induction.

Histological studies

The different stages of 'Full house' embryo growth are shown in Figure 1. The most somatic embryos appeared to have a normal morphology with two cotyledons (Fig. 1 a and b). Primary and secondary embryos were observed on the top surface of the callus on MS medium supplemented with 0.5 mg l⁻¹ 2, 4-D (Fig. 1 c). Asynchronous development of embryogenic callus was observed in 'Ocean song' from the calli cultured on MS medium supplemented with 3 mg l⁻¹ NAA (Fig. 1d, e). The different stages of embryo were also observed in the histological examinations (Fig. 2), a finding that is consistent with that of Li *et al.*, (2002) reporting the asynchronous state of embryo development.

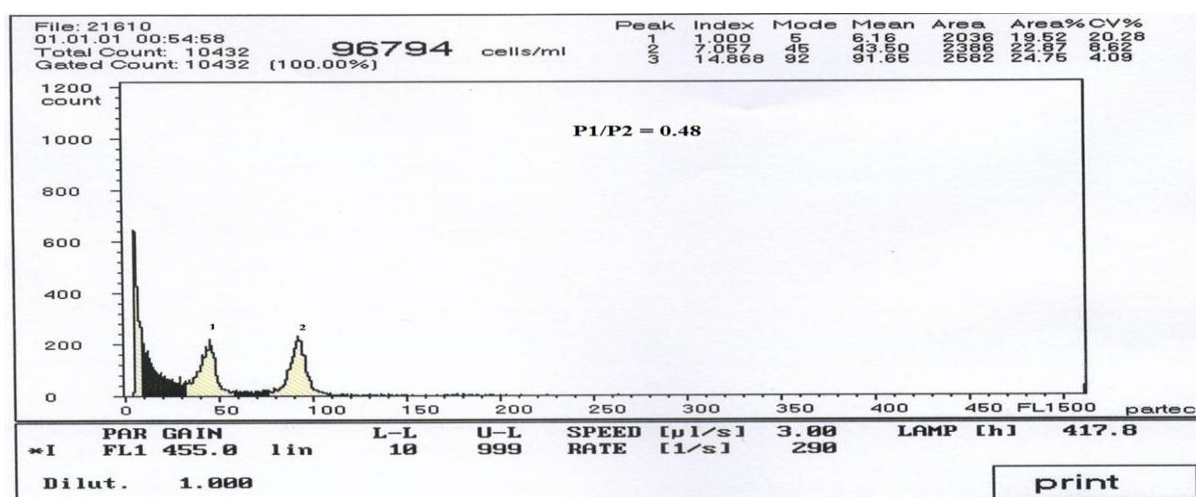


Fig. 3. Sample graphs of flow cytometry related to plants regenerated from somatic embryogenesis in *R. hybrida* cv. Ocean song (from the calli cultured on MS medium supplemented with 3 mg l⁻¹ NAA); (P1/P2): the mean relative ratio of the rose peak/parsley peak.

The proliferation and maturation of secondary somatic embryos occurred in PGR-free MS medium. Figure 1f shows the elongation of cotyledons in embryos of Ocean song in PGR-free MS medium. The formation of leaf and root, and regeneration of plantlet were detected throughout the incubation period in PGR-free medium. Continuous subculturing resulted in the development of most somatic embryos in to bipolar plantlet. Rooted plantlets were transferred to small pots containing peat: perlite (1:1) in the green house (Fig. 1g and h).

Determination of ploidy level

Numerous tetraploid rose cultivars had produced a mean relative ratio of rose peak to the internal standard (parsley) peak of 0.47 (Fig. 3). Our results showed that all the tested shoots were tetraploid, indicating stability of the ploidy level throughout the somatic embryogenesis process in rose.

Conclusion

The highest frequency of somatic embryogenesis (46.66%) was detected in Ocean song and in the culture medium supplemented with NAA (3 mg l⁻¹) and proline (300 mg l⁻¹). The best medium for somatic embryogenesis for 'Maroussia' was MS medium supplemented with 2, 4-D (1.5 mg l⁻¹) and ABA (0.1 mg l⁻¹). The maximum rate of somatic embryogenesis in 'Full house' (26.6%) was in the MS

medium containing 2, 4-D (0.5 mg l⁻¹) along with a reduction in sucrose concentration. These results indicated that the production capacity of somatic embryogenesis was highly cultivar dependant. The results confirmed that auxin plus ABA, auxin along with a reduction in sucrose concentration, auxin plus proline concentration, and auxin plus NaCl concentration all act as stress factors and play important roles in embryogenesis. This is the first report on the use of NaCl in inducing embryogenesis in *Rosa hybrida*, which not only demonstrates NaCl being useful for the selection of tolerant cultivars, but also is a suitable agent to promote the process of somatic embryogenesis in recalcitrant species.

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