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PRIMARY AND SECONDARY SOMATIC EMBRYOGENESIS FROM LEAF EXPLANTS OF ROSEMARY (*ROSMARINUS OFFICINALIS* L. - LAMIACEAE)

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Abstract

Somatic embryogenesis was obtained from young leaf explants of rosemary with 2, 4-Dichlorophenoxyaceti acid (2, 4-D) in different concentrations (0.45, 2.25, 4.52, 6.75, 9.04 µM) in combination with Benzyladenine (BA) using modified woody plant medium (WPM). The best frequencies of induction were obtained in a medium supplemented with 2.25 µM 2, 4-D and 0.45 µM BA, in which 100% of the calli produced somatic embryos. Subculture of primary somatic embryos on modified WPM without hormones gave rise to clusters with secondary somatic embryos and embryogenic calli. These clusters were sub-cultured every 4 weeks, and an average of 10% of the secondary somatic embryos developed into plantlets in each subculture. The same culture on WPM containing Abscisic acid (ABA) in different concentrations (3.48, 7.67, 11.35 µM) also yielded *de novo* secondary embryos and whitish embryogenic calli.

Introduction

Rosemary (*Rosmarinus officinalis* L.) is an economically important plant of family Lamiaceae. It is a woody perennial, aromatic herb, native to the Mediterranean region, but is now grown worldwide. It is a source of secondary metabolites, especially essential oils, which have antimicrobial, antiviral, antioxidant, antispasmodic, anticonvulsant, diuretic, hepato-protective, analgesic and anti-carcinogenic properties (Bozin *et al*., 2008, Kadri *et al*., 2011, Saltan & Ozaydin, 2013). It is also being used in food and cosmetic industry (Wang *et al*., 2008).

The chemical constituents of essential oil of rosemary include flavonoids such as carnasol, carnosic acid and rosmarinic acid, and volatile oils (Okamura *et al*., 1994). Carnosol and carnosic acid constitute 3.8-4.6% of the extracted material, and they are among the many constituents in rosemary that show pharmacological properties (Huang *et al*., 1994).

Due to the increase in the demand of this plant worldwide in pharmaceutical, cosmetic and food industry (Maistro *et al*., 2010), there is a need of propagation methods which can increase its production. At present, the propagation of rosemary is carried out essentially through cuttings as its seeds have poor germinating ability (Catizone *et al*., 1986). The field grown plants are exposed to variations of seasonal stresses which significantly alters the level and quantity of the essential oils (Dellacassa *et al*., 1999). Plant tissue culture has been used as a tool for the conservation and rapid micropropagation of medicinal plants and also for providing a source of secondary metabolites (Nagesh *et al*., 2010).

Among the plant tissue culture techniques, somatic embryogenesis offers an alternative and efficient protocol for plant regeneration (Xie *et al*., 2013). In this process, a single cell or clusters of cells, develop in to whole embryos capable of regenerating in to complete plants (Toonen *et al*., 1996). Furthermore, after the induction of embryogenesis *in vitro*, the primary somatic embryos produced can develop further not only to yield plantlets but also giving rise to clusters of *de novo* somatic embryos, a process called secondary somatic embryogenesis (Raemakers *et al*., 1995). Secondary somatic embryogenesis has certain advantages compared to primary somatic embryogenesis such as very high multiplication rate, independence of an explant source and repeatability (Te-chato & Hilae, 2007).

In rosemary, reports on callogenesis (Tawfik 1992; Caruso *et al*., 2000; Yesil-Celiktas *et al*., 2007; Fayaz *et al.,* 2008; Mondo, 2009, Dong *et al*., 2012), regeneration (Misra & Chaturvedi, 1991; Yang *et al*., 1997; Huang & Tsai, 2004) and micropropagation (Misra & Chaturvedi, 1984, Gabor-Potor & Pop, 2007) are available, however for commercial purposes, simple, reproducible and large scale vegetative propagation is required. To the best of our knowledge there are no reports on secondary somatic embryogenesis from leaf-derived callus of rosemary.

The present investigation reports the regeneration of *Rosmarinus officinalis* through somatic embryogenesis using leaf as an explant. Further establishment of cultures with a high frequency of secondary somatic embryogenesis is also reported.

Materials and Methods

Plant material: Fresh young leaves of *R. officinalis* were collected from plants taken from the local nursery and were rinsed with tap water for 10 minutes. The explants were washed with water and liquid detergent and later treated with 2.5% sodium hypochlorite for 10 minutes. Finally after rinsing with autoclaved distilled water for three times, the leaves were cut in to 2 cm discs with the help of sterilized scalpel blade.

Culture media: These leaf explants were cultured on modified Llyod & McCown, (1980) woody plant medium (WPM) containing KNO_3 (400 mg/l) in place of NH_4NO_3 and Myo-inositol (100 mg/l), 30 gm/l sucrose, 7 gm/l agar (Bacto agar, Difco) and supplemented with different concentrations of 2, 4- D (0.45, 2.25, 4.52, 6.75, 9.04 µM) alone and with BA (0.45 µM) for callus initiation and somatic embryogenesis. For the production of secondary somatic embryos and regeneration, hormone free

modified WPM and different concentrations of ABA (3.48, 7.57 and 11.35 µM) were tried.

Culture conditions: Each treatment was applied to 20 explants and the results were scored after 8 weeks of culture. All media had the pH adjusted to 5.6-5.8 and autoclaved at 121°C (108kpa) for 20 min. The temperature of the culture room was adjusted to 25±1°C with 16 h photoperiod supplied by cool white fluorescent tube having 30 µmol m^{-2} s^{-1} light intensity. After the formation of somatic embryos, they were shifted to hormone free and ABA containing media for the production of secondary somatic embryos and regeneration.

Results and Discussion

After one week of culturing of leaf explants of *R. officinalis*, callus formation was initiated from cut ends in modified WPM medium supplemented with different concentrations of 2,4-D. An increase in the percentage of callus formation was observed with an increase in the concentration of 2,4-D (Table 1). Maximum callus formation (75%) was observed at 9.4 µM concentration of 2,4-D (Fig. 1a). No callus formation was detected on hormone free modified WPM. The texture of the callus varied from compact to friable and granular and color varied from whitish, light green to yellowish green. Somatic embryogenesis was not observed in this medium.

Callogenesis from different explants of *R. officinalis* has been reported using different growth regulators. Tawfik, (1992) obtained callogenic response from stem and leaf explants with 2.0 mg/l TDZ and 0.5 mg/l IAA. Caruso *et al*., (2000) used MS medium with Zeatin (1.0 mg/l) and IAA (0.1 mg/l) for callogenesis from leaf explants and nodal stem segments. Yesil-Celiktas *et al*., (2007) observed that MS medium having 1.0 mg/l NAA was the best medium for callus formation. Fayaz *et al*., (2008) demonstrated that when MS medium was augmented either with BA (200 µM) and IAA (100 µM) or NAA (150 µM) and IAA (100 µM), it resulted in a green, friable callus in both nodal and shoot tip explants. According to Mondo, (2009), the medium containing 10.0 mg/l IAA, 4.0 mg/l BA and 1.0 mg/l of Kinetin or 0.05 mg/l 2, 4-D with 1.5 mg/l BA proved to be the best medium for developing and maintaining rosemary callus cultures.

In the present investigation embryogenic callus was only obtained when a combination of 2,4-D with BA was tried (Table 1). The initial primary embryogenic callus was greenish yellow, nodular and friable. All the media tested were able to support somatic embryogenesis over the entire range of concentrations of 2,4-D (0.45, 2.25, 4.52, 6.75, 9.04 µM) with BA (0.45 µM). However, the highest frequencies of induction were obtained with 2.25µM 2,4-D and 0.45µM BA in modified WPM (Table 1, Fig. 1b). Globular somatic embryos were first observed after 4-5 weeks of culture from the callus produced at the cut ends of cultured leaf explants (Fig. 1c, d). At the end of eight weeks embryos at different morphological stages ranging from heart shaped stage to torpedo stage could be seen (Fig. 1e, f).

Similar combination of hormones i.e., BA and 2,4-D has resulted in embryo formation from primary culture stage in *Pelargonium x domesticum* (Haencsh, 2007). Likewise, same combination of growth regulators induced embryogenic callus in *Acacia arabica* (Nanda & Rout, 2003) and in *Ocimum basilicum* (Gopi & Ponmurugan, 2006). Arnaud *et al*., (2006) reported formation of somatic embryos from zygotic embryos from *R. officinalis* using BSAA (Benzo-selenienyl acetic acid) or 2,4-D, but failed to obtain mature embryos and confronted to a high level of vitrification.

Auxin 2,4-D alone has been employed by many workers for obtaining somatic embryogenesis from different explants including mature zygotic embryos of *Myrtus communis* (Lopes *et al.,* 1995), hypocotyls sections of *Sesamum indicum* (Mary & Jayabalan, 1997) and leaf and internode explants of *Ceropegia candelabrum* (Beena & Martin, 2003) and leaf explants of *Cucumis sativus* (Usman *et al*., 2011). However, in the present investigation we were unable to get somatic embryos even after maintaining the callus for up to six months through four-week transfer to fresh medium of similar composition.

Table 1. Effect of 2,4-D and BA on induction of somatic embryos from leaf explants of *Rosmarinus officinalis*.

WPM + Growth regulators (µM)		Explants forming callus (% ± SE)	% Callus producing somatic embryos
2,4-D			
0.0		0.0	0
0.45		40 ± 5.77^{b}	0
2.25		65 ± 5.77^{a}	0
4.52		65 ± 2.88^{a}	0
6.75		60 ± 7.63^{a}	0
9.04		75 ± 5.77^{a}	0
2,4-D	**BAP**		
0.0	0.0	0.0	0
0.45	0.45	70 ± 5.77^{b}	14.27 ± 4.11^{c}
2.25	0.45	90 ± 2.88^{a}	100 ± 0.00^{a}
4.52	0.45	90 ± 0.00^{a}	66.66 ± 6.41^{b}
6.75	0.45	90 ± 5.77^{a}	9.26 ± 1.85^{cd}
9.04	0.45	95 ± 0.00^{a}	5.26 ± 0.00^{cd}

Mean ± standard error of three repeated experiments means having the same letter in columns are not significantly different by Duncan's Multiple Range Test ($p \leq 0.05$)

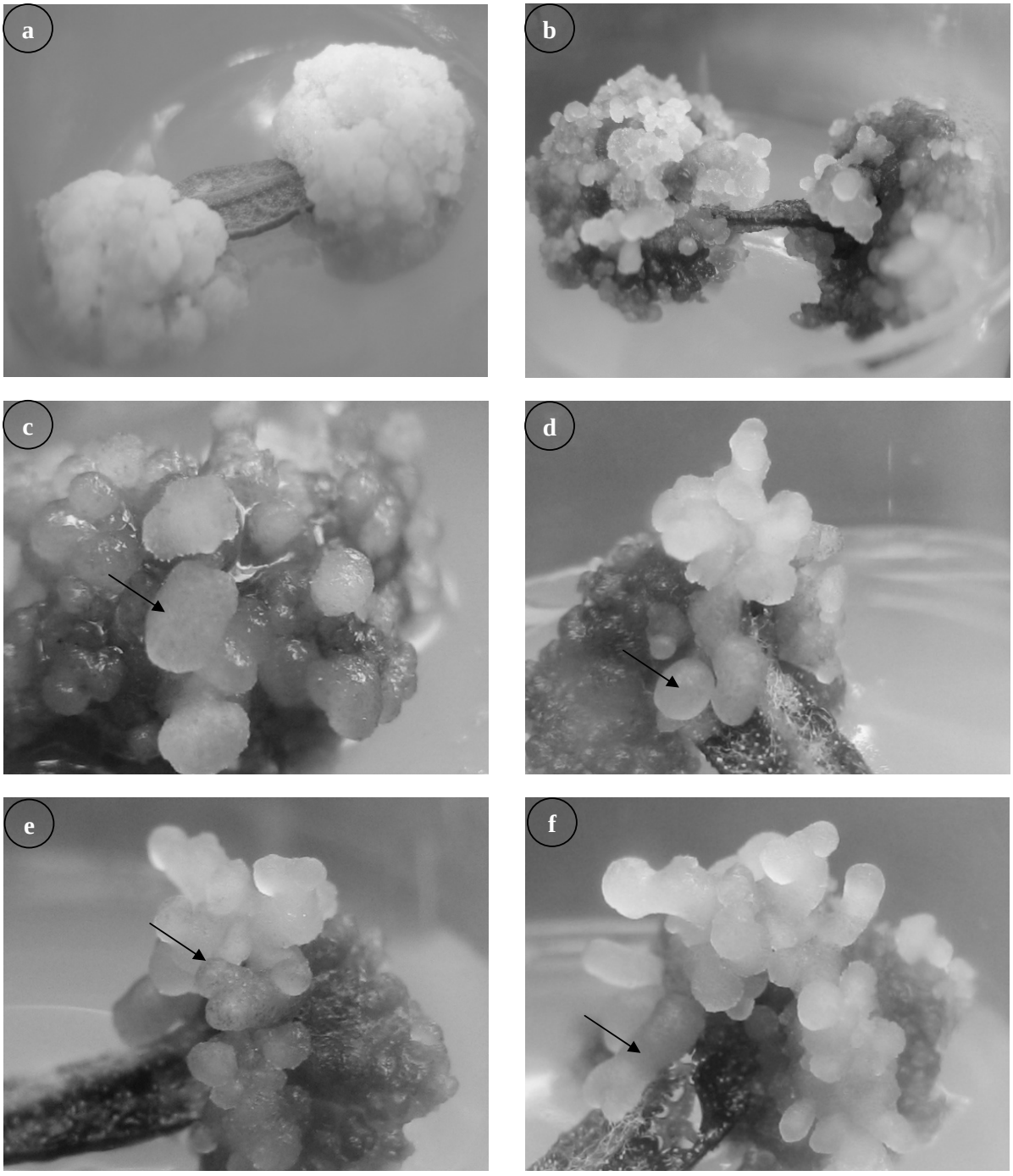

Fig. 1. Callogenesis and somatic embryogenesis from leaf explants of *Rosmarinus officinalis*. **a** Maximum callogenic response from leaf explants in modified WPM with 9.4uM 2,4-D. **b** Friable embryogenic callus obtained in modified WPM with 2.2 uM 2,4-D and 4.45 uM BA. **c,d** Globular embryos. **e** Heart-shaped embryos. **f** Torpedo shaped embryos.

It is generally believed that somatic embryogenesis is mediated by a signal transduction pathway triggered by an external auxin like 2,4-D (Zimmerman, 1993; Mordhorst *et al*., 1997; Schmidt *et al*., 1997). However, very little is known about the molecular mechanism involved in the transition of a vegetative cell to an embryogenic cell capable of regenerating into a complete plant.

The somatic embryos of *R. officinalis* did not germinate on the induction medium. In order to achieve germination, the somatic embryos were removed from the induction medium and transferred to hormone free WPM or containing Abscisic acid (ABA) in varying concentrations (3.48, 7.57 and 11.35 µM). It was observed that when embryogenic callus or embryos shifted singly on hormone free medium, resulted in

both secondary somatic embryos and de novo embryogenic callus formation (Table 2, Fig. 2a, b, c). Similarly, embryogenic callus when shifted to media containing different concentrations of ABA also resulted in the formation of secondary somatic embryos with the maximum percentage at 7.57 µM. In addition the callus turned white when shifted on ABA containing medium (Fig. 2d). Secondary somatic embryos germinated 4 weeks after being cultured on hormone free modified WPM (Fig. 2e, f), but the percentage germination was low (about 10%).

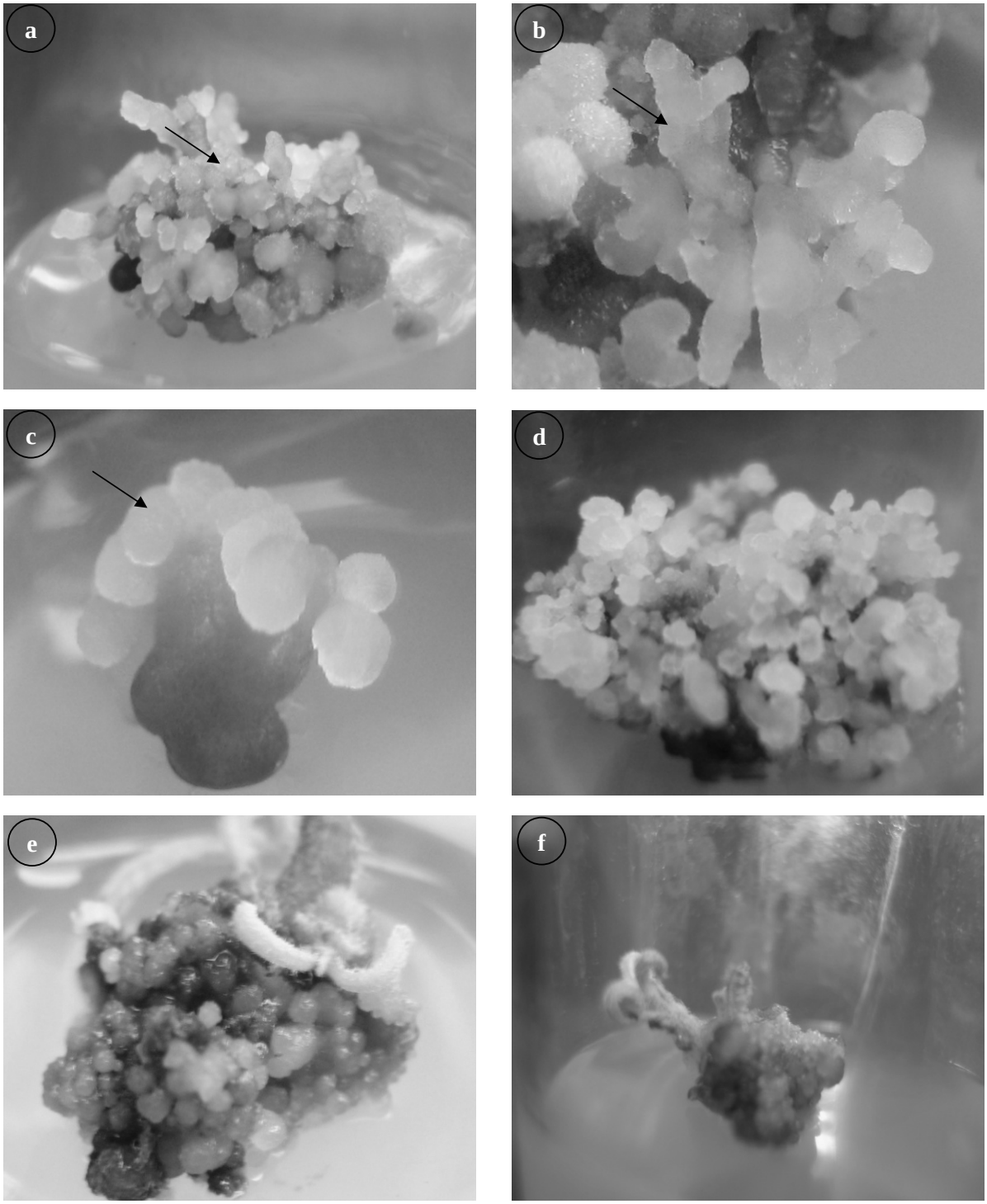

Fig. 2. Seondary somatic embryogenesis and plant regeneration of *Rosmarinus officinalis*. **a,b** Secondary somatic embryos formed on hormone free modified WPM. **c** Secondary somatic embryos formed from a single primary embryo. **d** Whitish emryogenic callus formed on ABA containing medium. **e,f** Germination of plantlets from secondary embryos.

Table 2. Effect of ABA and hormone free WPM on secondary somatic embryo formation and regeneration

Medium used	No. of primary embryogenic calli	% primary callus showing secondary somatic embryos	% of regenerated shoots per embryogenic calli
WPM	10	90 ± 5.77^{a}	10 ± 0.00
WPM + ABA 3.48µM	10	60 ± 11.54^{bc}	0.0 ± 0.00
WPM + ABA 7.57µM	10	80 ± 5.77^{ab}	0.0 ± 0.00
WPM + ABA 11.35µM	10	40 ± 0.00^{c}	0.0 ± 0.00

Means having the same letter in columns are not significantly different by Duncan's Multiple Range Test ($p \leq 0.05$)

Secondary somatic embryogenesis has been also reported in many plants including *Myrtus communis* (Parra & Amo-Marco, 1998) *Feijoa sellowiana* (Cruz *et al.*, 1990), *Eucalyptus citriodora* (Muralidharan *et al*., 1989), banana (Khalil *et al*., 2002) oil palm (Rajesh *et al*., 2003) and carnation (Karami *et al*., 2008). According to Carman, (1990) some "memory" effects, like changes in the DNA methylation pattern, can operate in somatic embryos making their tissues more capable of undergoing secondary somatic embryogenesis.

The effect of ABA on secondary somatic embryogenesis has been demonstrated by many workers (Sagare *et al*., 2000; Kuo *et al*., 2002). According to Smiskova *et al*., (2005) in *Schisandra chinensi*s, ABA significantly influenced the synchronization of development of the somatic embryos to the globular stage, following culture on a medium without growth regulators resulted in fully developed cotyledonary stage somatic embryos. While Goebel-Tourand *et al*., (1993) observed that addition of ABA inhibited the precocious germination of somatic embryos and resulted in the formation of normal shaped plants in grapevine. Our results indicated that ABA does not promote secondary somatic embryogenesis and germination of somatic embryos in rosemary.

In our experiments secondary somatic embryogenesis was induced on a medium devoid of growth regulators, and the same medium yielded both secondary somatic embryos and germinated plantlets. Smith & Krikorian, (1991) suggested that cultures that undergo the whole embryogenic cycle without the addition of exogenous plant growth regulators are appropriate model systems for studying the physiological basis of somatic embryogenesis. According to Raemakers *et al*., (1995) clusters of embryogenic cells may result in loss of control when high level of auxin is present in the medium. Some cells being excluded from medium may result in starting a new cycle of somatic embryogenesis (Williams & Maheswaran, 1986).

In modified WPM which was used in the present investigation, nitrogen was supplied in form of KNO_3. Generally it is believed that considerable amount of nitrogen in the form of ammonium salts is needed for initiation and maturation of embryos (Merkle *et al*., 1995). Potassium is thought to involve in the proton pump and also indirectly in the transport of solutes (Briskin & Hanson, 1992; Shetty & McKersie, 1993). In wild carrot suspension cultures high potassium concentrations enhanced the number of embryos (Brown *et al*., 1976).

Conclusion

In the present study, plant regeneration system has been described from somatic embryos obtained from young leaf explants of *R. officinalis*. Somatic embryos from leaf-derived explants were obtained in modified WPM containing 2,4-D and BA. Moreover, secondary somatic embryogenesis and regeneration was observed in modified WPM without hormones. Further work is needed to increase the regeneration potential of the somatic embryos of rosemary.

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