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DIRECT SOMATIC EMBRYOGENESIS AND PLANT REGENERATION IN STRAWBERRY (*FRAGARIA ANANSSA*)

OMID KARAMI[1]*, GONA KARIMI KORDESTANI[2] AND MOHMAD MOHAMADI[3]

[1]Department of Biotechnology, Faculty of Agriculture, Bu-Ali Sina University, Hamadan, Iran
[2]Department of Microbiology, Zanjan Islamic Azad University, Zanjan, Iran
[3]Department of Irrigation, Faculty of Agriculture, Bu-Ali Sina University, Hamadan, Iran

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SUMMARY

This is the first report for optimal culture conditions to induce direct somatic embryogenesis in two strawberry cultivars (Selva and Comarosa). Somatic embryos were directly formed from leaf explants plated on Murashige and Skoog (MS) medium containing picloram. Maximum embryogenesis was obtained with 2 mg/l picloram. Globular shape embryos were developed into cotyledonary-shaped embryos when they were transferred to hormone-free media containing different concentrations of sucrose. Increasing sucrose concentrations in culture media enhanced somatic embryos development. Cotyledonary somatic embryos were converted into plantlets when they were transferred on MS medium containing GA_3. Maximum germination was obtained with 1 and 2 mg/l GA_3. Plantlets were also continued to grow under greenhouse condition.

***Key words*: Direct somatic embryo, embryogenesis, regeneration, strawberry**

INTRODUCTION

Application of biotechnology in plant breeding programs requires efficient *in vitro* regeneration procedures. Somatic embryogenesis is a desirable method of plant regeneration (Williams and Maheswaran 1986). In addition, to the fastest method of plant micropropagation, somatic embryos may also be encapsulated in various gelling systems to form artificial seed that can be easily stored and transported to long distances (Ghosh and Sen 1994). Due to the presence of well-developed root and shoot primordia, somatic embryos simply germinate to produce plantlets without additional step of rooting (Laux and Jugens 1997). Although plant regeneration with direct (Rugini and Orlando 1992, Sorvari *et al.* 1993, Jemmali *et al.* 1994, Monticelli *et al*. 1995, Boxus 1999) and indirect organogenesis (Liu and Sanford 1988, Nehra *et al.* 1990, Jones *et al*. 1998) have already been reported in strawberry, only one study on somatic embryo induction from this plant has been conducted (Wang *et al.* 1984).

Induction of somatic embryos directly from plant tissue is the most desirable approach because it appears to be associated with the genetic stability of regenerated plantlets (Vasil 1988, Pedroso and Pais 1995). Although direct somatic embryogenesis have been developed for numerous species, plant regeneration through direct somatic embryogenesis has not been reported in strawberry. The present study accomplishes plant regeneration through direct somatic embryogenesis from leaf explants of strawberry.

MATERIALS AND METHODS

Plant material and culture condition: Strawberry cultivars Selva and Comarosa were used in study. Runner tissues were washed in tap water for 30 min and

*Corresponding author, E-mail: okarami215@gmail.com

surface disinfected in 70% (v/v) ethanol for 10 s, 0.1% (w/v) $HgCl_2$ for 8 min followed by three washes for 5 min with sterile distilled water. Regenerated shoots were obtained by culturing shoot-tips on Murashige and Skoog (MS) medium supplemented with 6-benzylaminopurine (BA 0.5 mg/l), gibberellic acid (GA_3 0.1 mg/l), indole-3-butyric acid (IBA 0.1 mg/l) as described by Boxus (1999). Explants from leaf segments (approximately 4×4 mm) were excised from 4-week old plantlets and placed abaxial side down on different embryo induction medium. All cultures were incubated at 24 C° and 16 h photoperiod under 35 µmol m^{-2} s^{-1} illumination provided by cool white fluorescent lamps in growth room. The pH of all culture media were adjusted to 5.8 using NaOH (1N) before adding gelling agent (Agar-Agar, Merck). All culture media were sterilized by wet autoclave at 121 C° for 15 min.

Induction and development of somatic embryo: Explants were placed on Murashige and Skoog (MS) basal medium containing 3% sucrose supplemented with 0.5, 1, 2, 4 and 6 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid (picloram), α-naphthalene acetic acid (NAA) and indole-3-butyric acid (IBA) for the induction of direct somatic embryos. Data were collected on percentage of explants exhibiting direct somatic embryogenesis and number of somatic embryos per responding explant after 4 weeks of culture. Twenty explants were considered for each treatment and the experiments were conducted in six replicates. Differences between means were scored with Duncan's multiple range test. For developing embryos, globular–shape embryos were excised from the explants and transferred onto a series of culture media containing MS basal slat mixture supplemented with different concentrations of sucrose (2, 4, 6, 8 and 10 %) without growth regulators. One-handed globular embryos were considered for each treatment and the experiments were conducted in three replicates. Differences between means were scored with Duncan's multiple range test.

Embryo germination, plantlet formation and plants acclimatization: For plant regeneration, cotyledonary somatic embryos were placed onto media containing 3% sucrose without growth regulators or supplemented with different concentrations of GA_3 and NAA (Table 3). Twenty embryos were considered for each treatment and the experiments were conducted in six replicates. Germinated somatic embryos were transferred into plastic pots containing an autoclaved mixture of soil, sand, and compost (1:1:1 v/v) and were kept for 2 weeks, then were transplanted into plastic pots containing garden soil and allowed to be grown in the growth room (18 ± 2 C°, 16 h photoperiod under 30 µmol m^{-2} s^{-1} illumination). Acclimatization of plants were finally carried out for 3 weeks in a greenhouse at 28 C° followed by transferring them to a greenhouse.

RESULT

Induction of somatic embryos: Initiation of direct somatic embryos was started on explants within 3-4 weeks from inoculation (Fig. 1 A). Development of somatic embryos from the globular-stage to the torpedo and cotyledonary stage was not observed on the induction media. With prolonging culture globular-shaped

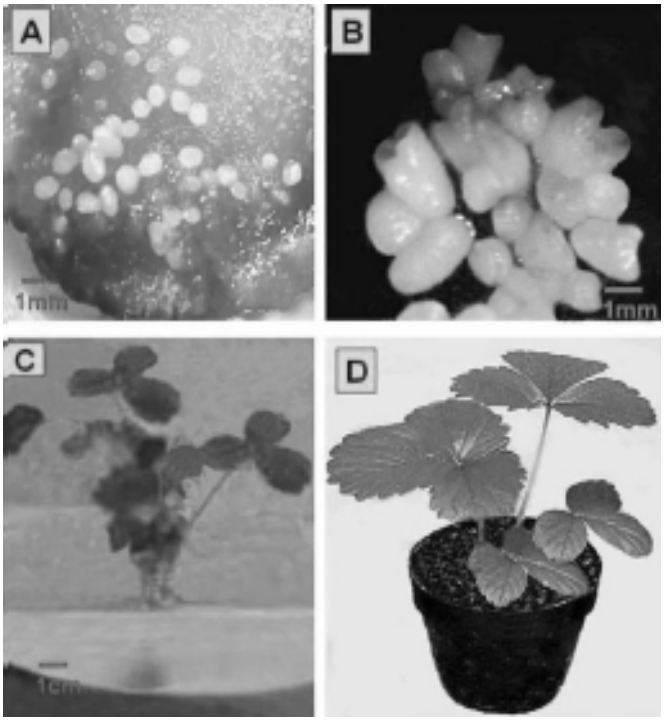

Fig. 1. Direct somatic embryogenesis and plant regeneration in strawberry. (A) Induction of globular embryos from leaf explant on MS medium containing 2 mg/l picloram, 4 weeks after culture. (B) Somatic embryos at different developmental stages on MS medium containing 6% sucrose, 3 weeks after culture. (C) Plantlet regenerated from a somatic embryo cultured on MS medium containing 1mg/l GA_3, 5 weeks after culture. (D) A potted plant in greenhouse.

embryos converted to succulent calli (Non-embryogenic callus). Among different treatments used for somatic embryo induction, direct somatic embryos were induced only at media supplemented with picloram. Significant differences between the concentrations of picloram and their effects on the percentage of responding explants and also the mean number of somatic embryos formed on each explant are shown in table 1. Maximum embryogenic response (42 %) with maximum number of somatic embryos per responding explant (13) was observed in 2 mg/l picloram. Increase or decrease of the picloram concentration reduced the embryogenic frequency.

Development of somatic embryos: Globular shape embryos were developed into torpedo and cotyledonary-shaped embryos (Fig. 1B) within 1-2 weeks when they were transferred to hormone-free media containing different concentrations of sucrose. Significant differences between the concentrations of sucrose and their effects on mean number of the globular embryos developed into cotyledonary embryos are shown in table 2. Increasing sucrose concentrations in culture media enhanced globular embryo development into cotyledonary embryos.

Plant regeneration: Cotyledonary somatic embryos transferred on MS medium containing GA_3 and NAA were germinated into shoot and root initials within 4–5 weeks producing entire plantlets (Fig. 1C). The effect of GA_3 and NAA on the frequency of embryos germination is showed in Table 3. The best response was obtained with 1 and 2 mg/l GA_3. A high percentage (approximately 80%) of rooted plantlets were successfully transferred to soil (Fig.1D) and developed to entire normal plants in the greenhouse with 95% survival. All acclimatized plants were transferred to field conditions and were grown normally in the natural environment.

DISCUSSION

The induction of somatic embryos has been reported in several species, such as wheat and tritordeum (Barro *et al*. 1998), myrtle (Canhoto *et al*. 1999), durum wheat (He and Lazzeri 2001**),** cashew (Vinitha and Sousa 2002) and kodo millet (Preeti and Kothari 2004) in media containing picloram. Wang *et al* (1984) have reported the induction of indirect somatic embryos on medium contaning 2,4-D. In this paper, direct somatic embryo formation on media containing picloram is shown. This study showed that development of somatic embryo was halted at globular stages in induction media and also when they were transferred to hormone-free media, they developed into cotyledonary-shaped embryos. Although exogenous auxins are essential for somatic embryo induction, in many of plants showed that maintenance of globular somatic embryos on medium containing auxin inhibited their development (Merkle *et al*. 1995).

Table 1. Effect of different concentrations of picloram on the percentage of responding explants and also the mean number of embryos formed on each explant of the two cultivars of strawberry, 4 weeks after culture.

	Direct embryogenesis on leaves explants			
	Selva		Comarosa	
Picloram (mg/l)	Per cent of responding explants	Mean of embryos per explant	Per cent of responding explants	Mean of embryos per explant
0.5	20.3 d	5.0 d	17.0 c	6.0 cd
1.0	24.0 c	5.3 cd	26.0 b	10.3 b
2.0	42.0 a	10.0 a	33.0 a	13.6 a
4.0	31.0 b	7.0 bc	28.0 b	7.3 c
8.0	10.0 e	8.0 ab	12.0 d	4.6 d

a-e: Means having the same letter in columns are not significantly different by Duncan's multiple range test ($P< 0.05$)

Table 2. Effect of different sucrose concentrations on somatic embryos development in two cultivars of strawberry, 4 weeks after culture.

	Number of globular embryos developed into cotyledonary embryos	
Sucrose (%)	Selva	Comarosa
2.0	64.0 c	54.6 c
4.0	86.6 b	70.0 b
6.0	89.0 a	90.3 a
8.0	92.3 a	92.6 a
10.0	91.6 a	93.0 a

a-c: Means having the same letter in columns are not significantly different by Duncan's multiple range test (P< 0.05)

Table 3. Effect NAA and GA_3 on somatic embryos germination in two cultivars of strawberry, 4 weeks after culture.

		Per cent embryos germinated into entire plantlet	
NAA (mg/l)	GA_3 (mg/l)	Selva	Comarosa
0	0	42.6 d	51.3 d
0.2	0	55.66c	59.0 c
0.5	0	54.3 c	60.3 c
0	0.5	66.3 b	70.3 b
0	1.0	80.3 a	76.0 a
0	2.0	79.3 a	79.3 a
0.5	1.0	64.6 b	67. 3 b

a-d: Means having the same letter in columns are not significantly different by Duncan's multiple range test (P< 0.05)

The result of this study showed that increasing sucrose concentration improved the development of globular somatic embryos. Similar result has been reported for other plant species (Themblay and Themblay 1991, Ricci *et al.* 2002, Karami *et al.* 2006). Increasing sucrose concentration in the medium may create the osmotic stress, but helps in development of somatic embryogenesis. However, it is of common knowledge that high sugar concentration in somatic embryogenesis may impact the cell osmolarity. Therefore, it could be suggested that osmotic effect of sucrose may cause development of somatic embryos. The positive effect of high osmolarity may mimic the osmolarity alterations that occur surrounding the embryo in nature (Merkle *et al*. 1995).

In conclusion, results clearly showed that this protocol is applicable to induce direct somatic embryogenesis in strawberry. The optimal condition to induce and develop direct somatic embryos for strawberry and that somatic embryos could successfully be regenerated into entire normal plants were shown. In addition, establishment of conditions required for the regeneration via somatic embryogenesis would facilitate somatic hybridization, genetic transformation and artificial seed production in strawberry.

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