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PICLORAM-INDUCED SOMATIC EMBRYOGENESIS IN LEAVES OF STRAWBERRY (*FRAGARIA ANANASSA* L.)

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This is the first report of somatic embryogenesis from leaves of two strawberry cultivars (Selva and Comarosa) cultured on MS medium containing picloram. Maximum embryogenesis was induced using 2 mg/l picloram. Globular stage embryos developed into cotyledonary ones after transfer to hormone-free media containing 2%, 4%, 6%, 8% and 10% (w/v) sucrose. Increasing sucrose concentrations in culture media enhanced somatic embryo development. Cotyledonary somatic embryos were converted to plantlets after transfer on MS medium containing GA_3, and maximum conversion was achieved with 1 mg/l and 2 mg/l GA_3. Plantlets were capable of continuous growth under greenhouse conditions.

Key words: Strawberry, somatic embryogenesis, leaves, picloram, conversion.

INTRODUCTION

The application of biotechnology in plant breeding requires efficient in vitro regeneration procedures. Somatic embryogenesis is a desirable method of plant regeneration (Williams and Maheswaran, 1986). Somatic embryos can be encapsulated in various gelling systems to form artificial seeds which are easily stored and transported long distances (Ghosh and Sen, 1994). Due to the presence of well-developed root and shoot primordia, somatic embryos germinate easily to produce plantlets without an additional step of rooting (Laux and Jurgens, 1997).

Although plant regeneration through direct and indirect organogenesis has been reported in strawberry (Monticelli et al., 1995; Boxus, 1999; Jones et al., 1988; Kauahal et al., 2004), only a few studies on somatic embryo induction in this plant have been conducted (Wang et al., 1984; Biswas et al., 2007).

Picloram has been successfully used for somatic embryogenesis in wheat (Mendoza and Kaeppler, 2002), barley (Castillo et al., 1998), kodo millet (Preeti and Kothari, 2004) and *Lilium longiflorum* (Tribulato et al., 1997), but there is no report on somatic embryogenesis using picloram in strawberry. Here we report a protocol using picloram for somatic embryogenesis in strawberry.

MATERIALS AND METHODS

PLANT MATERIAL AND CULTURE CONDITIONS

Strawberry cultivars Selva and Comarosa were used in this study. Runner fragments 3–4 cm long were washed in tap water for 30 min and surface-sterilized in 70% (v/v) ethanol for 10 sec and 0.1% (w/v) $HgCl_2$ for 8 min followed by three washes with sterile distilled water for 5 min. Plantlets were obtained by culturing shoot tips on Murashige and Skoog (MS) medium supplemented with 0.5 mg/l 6-benzylaminopurine (BA), 0.1 mg/l gibberellic acid (GA_3) and 0.1 mg/l indole–3-butyric acid (IBA), as described by Boxus (1999). The upper leaves of 4-week-old plantlets were used as explants. They were cut into 4×4 mm pieces and placed upside down on embryo induction media.

All cultures were incubated at 24°C with a 16 h photoperiod under 35 μmol m^{-2} s^{-1} illumination provided by cool-white fluorescent lamps in a growth room. The pH of the culture media was adjusted to 5.8 using NaOH (1N) before adding gelling agent (Agar-Agar, Merck). All culture media were sterilized in a wet autoclave at 121°C for 15 min.

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INDUCTION AND DEVELOPMENT OF SOMATIC EMBRYO

In order to induce somatic embryos, explants were placed on MS basal medium containing 3% sucrose and different concentrations (0.5, 1, 2, 3, 4 and 6 mg/l) of 2,4-Dichlorophenoxyacetic acid (2,4-D), 4-amino–3,5,6-trichloro-2-pyridinecarboxylic acid (picloram), α-naphthalene acetic acid (NAA) and 1H-indole-3-butanoic acid (IBA). The data collected were the percentage of explants exhibiting somatic embryogenesis as well as the number of somatic embryos per responding explant 4 weeks after initiation of culture. Twenty explants were used in each treatment and the experiments were done in six replicates. Differences between means were scored with Duncan's multiple range test. To study embryo development, globular stage embryos were transferred to a series of culture media containing MS supplemented with 2%, 4%, 6%, 8% and 10% (w/v) sucrose without growth regulator. A hundred globular stage embryos were used in each treatment and the experiments were done in three replicates. Differences between means were scored with Duncan's multiple range test.

EMBRYO CONVERSION, PLANTLET FORMATION AND PLANT ACCLIMATIZATION

For embryo conversion, cotyledonary somatic embryos were placed on media containing 3% (w/v) sucrose either without growth regulator or supplemented with different concentrations of GA_3 and NAA (Tab. 3). Twenty embryos were used in each treatment and the experiments were done in six replicates. Somatic embryos converted to entire plantlets were transferred to plastic pots containing an autoclaved mixture of garden soil, sand and compost (1:1:1 v/v) and kept for 2 weeks, then transferred to garden soil and allowed to grow in a growth room (18±2°C, 16 h photoperiod, 30 μmol m^{-2} s^{-1} illumination). Finally the plants were acclimatized in a greenhouse at 28°C for 3 weeks and then field-cultivated.

HISTOLOGICAL EXAMINATION

For histological studies, explants with globular somatic embryos were fixed in formaldehyde/glacial acetic acid/ethanol (FAA, 5:5:90, v/v/v) for 24 h, dehydrated through a graded alcohol series for 24 h at each concentration and embedded in saturated paraffin wax. Embedded materials were sectioned 7 μm thick with a rotary microtome. Paraffin wax was removed by xylene prior to rehydration of the sections in a graded ethanol series and staining with 1.0% (w/v) safranin. Sections were washed briefly in water to remove excess stain, then dehydrated in a graded ethanol series and stained with hematoxylin.

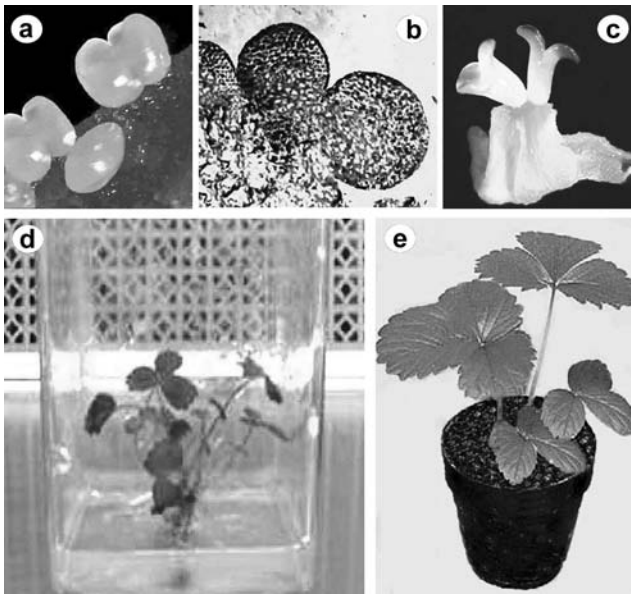

Fig. 1. Somatic embryogenesis and plant regeneration in strawberry. (**a**) Induction of globular stage embryos from leaf explant on MS medium containing 2 mg/l picloram after 4 weeks of culture, (**b**) Somatic embryos at globular stage, (**c**) Somatic embryos at cotyledonary stages on MS medium containing 6% sucrose after 3 weeks of culture, (**d**) Plantlet converted from a somatic embryo cultured on MS medium containing 1 mg/l GA_3, after 5 weeks of culture, (**e**) Potted plant in greenhouse.

RESULTS

INDUCTION OF SOMATIC EMBRYOS

Leaf explants were initially cultured on MS medium supplemented with 2,4-D, NAA, IBA, and picloram alone. Initiation of somatic embryos was begun on explants within 3–4 weeks of inoculation on media supplemented with various concentrations of picloram (Fig. 1a). Histological analysis revealed somatic embryos on explants (Fig. 1b). Development of somatic embryos from the globular to cotyledonary stage was not observed on the induction media. Prolonging the culture of globular stage embryos caused them to be converted to nonembryogenic callus.

Among the different treatments used for somatic embryo induction, somatic embryos were induced only on media supplemented with picloram. The effects of different concentrations of picloram on the percentage of responding explants and the number of somatic embryos are shown in Table 1. Medium containing 2 mg/l picloram gave the maximum embryogenic response (42%) and the maximum number of somatic embryos per responding explant (13). Both higher and lower picloram concentrations reduced the frequency of embryogenesis.

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

Picloram (mg/l)	Somatic embryogenesis on leaf explants (mean ± S.E)			
	Selva		Comarosa	
	% of responding explants	Number of embryos/ explant	% of responding explants	Number of embryos/ explant
0.5	20.3 ± 1.25 d	5.0 ± 0.30 d	17.0 ± 1.50 c	6.0 ± 0.39 cd
1.0	24.0 ± 2.00 c	5.3 ± 0.33 cd	26.0 ± 1.75 b	10.3 ± 0.8 b
2.0	42.0 ± 3.75 a	10.0 ± 1.00 a	33.0 ± 3.25 a	13.6 ± 1.00 a
4.0	31.0 ± 2.50 b	7.0 ± 0.50 bc	28.0 ± 2.00 b	7.3 ± 0.53 c
6.0	10.0 ± 0.60 e	8.0 ± 0.60 ab	12.0 ± 1.25 d	4.6 ± 0.33 d

a–e – Means with the same letter in columns do not significantly differ by Duncan's multiple range test ($p < 0.05$)

TABLE 2. Effect of different sucrose concentrations on somatic embryo development of strawberry after 4 weeks of culture

% Sucrose	Mean % of globular embryos developing into cotyledonary embryos	
	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 with the same letter in columns do not significantly differ by Duncan's multiple range test ($p < 0.05$)

TABLE 3. Effect of different NAA and GA_3 concentrations on somatic embryo conversion of strawberry after 4 weeks of culture

NAA (mg/l)	GA_3 (mg/l)	% of embryos converted into entire plantlet	
		Selva	Comarosa
0	0	42.6 d	51.3 d
0.2	0	55.66 c	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 with the same letter in columns do not significantly differ by Duncan's multiple range test ($p < 0.05$)

DEVELOPMENT OF SOMATIC EMBRYOS

Globular stage embryos developed into cotyledonary ones (Fig. 1c) within 1–2 weeks after transfer to hormone-free media containing different concentrations of sucrose.

The percentages of globular stage embryos developing into cotyledonary ones at different concentrations of sucrose in culture media are shown in Table 2. Increasing sucrose concentrations enhanced the development of globular stage embryos into cotyledonary ones.

EMBRYO CONVERSION

Cotyledonary somatic embryos transferred to MS medium containing GA_3 and NAA were converted to entire plantlets within 4–5 weeks (Fig. 1d). The effects of GA_3 and NAA on the frequency of embryo conversion are shown in Table 3. The best response was with media containing 1 and 2 mg/l GA_3.

A high percentage (~80%) of rooted plantlets were successfully transferred to soil (Fig. 1e), where they developed into fully formed plants in the greenhouse with 95% survival. All acclimatized plants were transferred to the field and grown normally outdoors.

DISCUSSION

Somatic embryo induction is usually promoted by auxins (Williams and Maheswaran, 1986). Among the different auxins, 2,4-D has been the one most commonly applied for somatic embryo induction. Induction of somatic embryos on medium containing picloram has also been reported in many species (Castillo et al., 1998; Mendoza and Kaeppler, 2002; Preeti and Kothari, 2004). In this work, somatic embryos formed on media containing picloram alone. Tribulato and Remotti (1997) reported similar results in *Lilium longiflorum*. In our experiments, 2,4-D did not effectively induce embryo formation from leaf explants of Selva and Comarosa cultivars of strawberry. Biswas et al. (2007) reported induction of somatic embryos on MS medium supplemented with 2,4-D from leaf explants of the pbgel-2000 strawberry cultivar. Genotypes within a given species vary greatly in embryogenic capacity (Merkele et al., 1995). Such differences might reflect

differences in the ability to activate key elements of the embryogenic pathway.

Plant regeneration through indirect somatic embryogenesis has been achieved in strawberry (Wang et al., 1984; Biswas et al., 2007), but direct somatic embryogenesis has not yet been reported. In this research we did not observe callus formation prior to embryo production on leaf explants, so direct embryogenesis apparently occurred.

This study showed that somatic embryo development was halted at the globular stage on induction media containing picloram. They developed to cotyledonary stage when transferred to hormone-free media. In general, further differentiation of the embryo beyond the globular stage and its subsequent maturation require the removal of growth regulators from the medium or reduction of the concentrations (Merkele et al., 1995).

In this study, increasing sucrose concentrations in the medium improved the development of globular somatic embryos. Similar results have been reported in other plant species (Ricci et al., 2002; Karami et al., 2006). Higher sucrose concentrations may cause osmotic stress, but promote somatic embryogenesis. It is known that the application of high sugar concentrations in media for somatic embryogenesis may affect cell osmolarity. This suggests that the osmotic effect of sucrose is what triggers the development of somatic embryos. This positive effect could mimic the changes in osmolarity that occur in the environment surrounding the zygote embryo within the seed (Merkle et al., 1995).

Our results confirmed the usefulness of this protocol for induction of somatic embryogenesis in strawberry, defined the conditions for induction and development of somatic embryos from strawberry leaves, and demonstrated that somatic embryos could successfully be converted to fully formed plants. This work should facilitate genetic transformation and artificial seed production in strawberry.

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