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The Effects of Carbohydrate Source and Concentration on Somatic Embryogenesis of Strawberry (*Fragaria* x *ananassa* Duch.)

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Abstract: This experiment was performed to evaluate the effects of different types and concentrations of carbohydrate source on callus fresh weight, number of somatic embryos per embryonic explant and percentage of globular embryos developing into cotyledonary embryos from the leaf explants of three strawberry cultivars (Kurdistan, Paros and Camarosa). MS medium containing 1.0 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 mg/l 6-benzyladenine (BA) supplemented with three types of carbohydrates (sucrose, glucose and fructose) at concentration of 1.5%, 3%, 6%, 9% and 12% separately were tested. Among the different sugars tested, 6% sucrose was found superior not only for giving optimum embryo induction of embryonic culture but also a uniform embryo developmental stages. Maximum number of 11.68, 12.68 and 13.35 globular embryos per explant, 81.53, 87.65 and 86.35% of globular embryos developing into cotyledonary embryos obtained for the cultivars of Kurdistan, Paros and Camarosa, respectively. Sucrose also was the best for proliferation of embryonic tissue with an optimal concentration of 3%.

Key words: Strawberry · Carbohydrates · Somatic embryogenesis

INTRODUCTION

Cultivated strawberry (*Fragaria* × *ananassa* Duch.) as an octoploid species ($2n = 8x = 56$) belonging to the genus *Fragaria* of the family *Rosaceae*, is the most important soft fruit worldwide [1]. Its delicious flavor and taste, attractive appearance and seasonal availability make this fruit an excellent crop. Even more, strawberries are rich in phytochemical compounds with potential antioxidant compounds and also they are mainly rich in ellagic acid and flavonoids, which can lower the risk of cardiovascular events and tumorogenesis [2]. According to FAO [3], the global production of strawberry averages nearly 3.8 million metric tones from almost 256 thousand hectare.

Somatic embryogenesis is the process by which somatic cells, under induction conditions, generate embryogenic cells, which go through a series of morphological and biochemical changes that result in the formation of a somatic embryo [4]. It plays an important role in clonal propagation. When integrated with conventional breeding programs and molecular and cell biological techniques, somatic embryogenesis provides a valuable tool to enhance the pace of genetic improvement of commercial crop species [5].

Although, the regeneration of Strawberry via somatic embryogenesis has previously been reported [6,7] but Somatic embryogenesis research with strawberry is still in a preliminary stage and some more efforts would be required to develop the technology [8,9].

Carbohydrate type and concentration have been found to play important roles in different stages of the somatic embryogenesis process. Although the majority of media used in plant tissue culture contain sucrose as the standard carbon and energy source, in several plant species including *Dianthus caryophyllus* [10], *Acacia sinuata* [11], significance of different source and concentration of carbohydrate is proved for induction of embryonic callus, embryo development and regeneration, but there is no report on somatic embryogenesis using various source and concentration of carbohydrate in strawberry. In this study, an efficient procedure of somatic embryogenesis and plant regeneration in strawberry is described.

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MATERIALS AND METHODS

Plant Material: Runner tips and immature flower buds of three strawberry cultivars (Camarosa, Paros and Kurdistan) were taken from greenhouse and were washed in running tap and then explants were surface-sterilized by a 10 sec immersion in 70% (v/v) ethanol and for 15 min in aqueous solution of 1% (v/v) sodium hypochlorite. After three washes in sterile double distilled water, sterilized runner tips were cultured on MS medium supplemented with 0.5 mg/l 6-benzyladenine (BA), 0.1 mg/l gibberellic acid (GA3) and 0.1 mg/l indole–3-butyric acid (IBA), as described by Boxus [12]. The pH of medium was adjusted to 5.8 using 0.1N NaOH or HCl and medium was solidified with 0.8% agar before autoclaving. The cultures were incubated in a 16-h photoperiod under 35 μmol $m^{-2}{}_{s}{}^{-1}$ illumination in cool, white fluorescent light at 25 ± 1°C. Pieces from fully expended young leaves and petioles of 4-5 week-old plantlets from auxiliary shoots of *in vitro* cultured runner tips and sepal, receptacle and petal segments (each approximately 4×3 mm) were excised from immature flower buds were used as explants.

Callus Induction and Somatic Embryogenesis: Explants were cultured on MS medium supplemented with 4 mg/l NAA for callus induction. 6-8 explants were cultured in each petri dish (100 X 20 mm) containing 30 ml of medium. During callus induction period all the cultures were incubated at dark in 25 ± 1°C.

Carbohydrates like sucrose, fructose and glucose were tested at this experiment. After four weeks, in order to induce somatic embryos, the induced calli were transferred to MS media with 1.0 mg/l 2,4-D + 0.5 mg/l BAP as described by Biswas *et al.* [9], supplemented with 1.5%, 3%, 6%, 9% and 12% concentrations of sucrose, fructose and glucose. The data collected were the percentage of explants exhibiting somatic embryogenesis, the percentages of globular stage embryos developing into cotyledonary ones as well as the number of somatic embryos per responding explant 10-12 weeks after subculture. Fresh weight of callus was determined every 4 weeks prior to subculturing. The data was subjected to statistical analysis. Thirty six 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.

RESULTS AND DISCUSSION

This experiment was performed to evaluate the effects of the type and concentration of carbohydrate on callus fresh weight, mean number of somatic embryos per embryonic explant and percentage of globular embryos developing into cotyledonary embryos from the leaf explants of strawberry. In order to induce embryonic callus formation, the leaf explants of three strawberry cultivars were cultured on MS medium supplemented with 4.0 mg/l NAA.

In this study the effect of sucrose, glucose and fructose at various concentrations were tested for their potential on callus fresh weight in leaf explants of strawberry. At low sucrose concentration (3%), was much more effective than high levels on callus fresh weight. The rise of sucrose concentration decreased callus fresh weight (Table 1). The reduction in the callus fresh weight capacity by the increase of sucrose concentration, could be attributed to an osmotic effect. To identify the best carbohydrate source and concentration for maintenance and proliferation of embryonic tissue, sucrose was substituted with similar concentration (3%) of fructose and glucose in repetitive embryogenesis medium. Comparing the growth in fresh weight with three different carbohydrates, it was observed that sucrose was the most effective carbohydrate source followed by glucose. Fructose was less effective in proliferation of embryonic tissue (Table 2). Sucrose is assumed to be the best carbon source in cell and tissue culture media, because it is the main sugar translocated in the phloem of many plants [13]. The data obtained in this study indicate that the callus fresh weight response could be greatly modified with the concentrations as well as the kind of carbon source. The use of different types and concentrations of carbohydrate on callus fresh weight has been reported for some plant species [11, 14, 15]. Table 1 and 2 shows the callus fresh weight on the media containing different Carbohydrates type and concentration, which have significant differences ($P < 0.05$) among cultivars. The highest callus fresh weight was observed for cv. Kurdistan followed by Paros and Camarosa. Embryonic calli (Fig. 1a) were cultured on MS medium supplemented with 1.0 mg/l 2, 4-D, 0.5 mg/l BA and sucrose, at 1.5%, 3%, 6%, 9% or 12% sugar concentrations.

Table1: Effect of different sucrose concentrations on proliferation of embryonic culture on the leaf explant of strawberry cultivars

	Callus fresh Weight (mg) after 4 weeks				Growth rate mg/ day		
% Sucrose	Initial	Kurdistan	Paros	Comarosa	Kurdistan	Paros	Comarosa
1.5	103	283.30de	296.60d	265.25e	6.44	6.91	5.79
3.0	99	437.53a	417.40b	403.43b	12.09	11.37	10.88
6.0	102	355.08c	342.20c	281.68de	9.04	8.58	6.42
9.0	100	198.93f	196.20f	175.15g	3.58	3.44	2.68
12.0	101	159.08g	138.43h	159.58g	2.07	1.34	2.09

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

Table 2: Effect of various carbohydrates on proliferation of embryonic culture on the leaf explant of strawberry cultivars

	Callus fresh Weight (mg) after 4 weeks				Growth rate mg/ day		
3 % Carbohydrate	Initial	Kurdistan	Paros	Comarosa	Kurdistan	Paros	Comarosa
Sucrose	99	437.53a	417.40ab	403.43b	12.09	11.37	10.88
Glucose	100	273.58cd	285.70c	261.48d	6.20	6.63	5.77
Fructose	103	200.08e	201.25e	188.05e	3.47	3.51	3.04

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

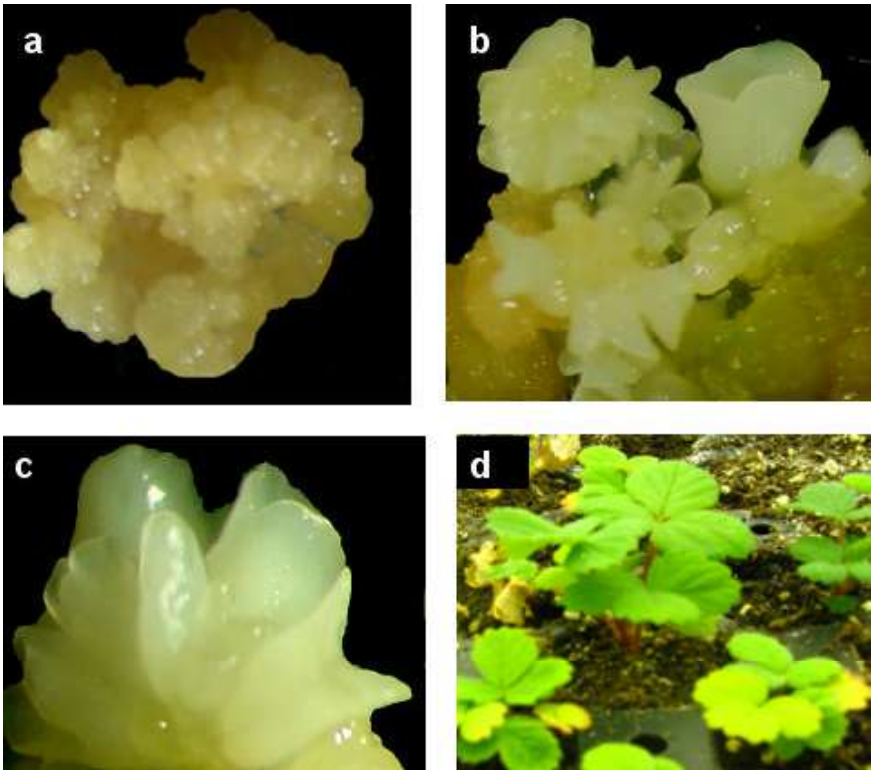

Fig. 1a: Embryonic calli on MS medium containing 4 mg/l NAA. (b) Cotyledonary embryo on MS medium containing 3% sucrose. (c) Cotyledonary embryo on MS medium containing 6% sucrose. (d) Well developed regenerated plants after transferred to soil.

Table 3 and 4 indicates that development of somatic embryos from the globular to cotyledonary stage was strictly dependent on the concentration of sugar in the medium. Sucrose, stimulated embryo development, the frequency of developing embryos decreased on media containing lower (1.5 and 3%) or higher (9 and 12%)

Table 3: Effect of different sucrose concentrations on the number of somatic embryos per explant and developing into cotyledonary embryos on the leaf explant of strawberry cultivars

	Number of somatic embryos per explant			% globular embryos developinginto cotyledonary embryos		
% Sucrose	Kordistan	Paros	Comarosa	Kordistan	Paros	Comarosa
1.5	2.85g	3.38g	3.05g	37.78de	42.83d	30.48e
3.0	5.60f	7.28de	6.18ef	61.10c	71.98b	59.35c
6.0	11.68b	12.68ab	13.35a	81.53ab	87.65a	86.35a
9.0	7.98cd	8.63c	8.55c	72.53b	80.35ab	81.15ab
12.0	5.78f	7.35de	7.20de	61.83c	72.25b	62.90c

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

Table 4: Effect of various sugars on the number of somatic embryos per explant and developing into cotyledonary embryos on the leaf explant of strawberry cultivars

	Number of somatic embryos per explant			% globular embryos developing into cotyledonary embryos		
Carbohydrate (6%)	Kordistan	Paros	Comarosa	Kordistan	Paros	Comarosa
Sucrose	11.68bc	12.68ab	13.35a	81.53 a	87.65 a	86.35 a
Glucose	10.20d	10.55cd	9.80d	67.55 b	64.35 bc	59.63 bc
Fructose	5.58e	6.40e	6.35e	57.23 c	61.60 bc	57.63 c

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

concentrations of sugar. The highest percentage of stimulated embryos and normal development occurred on media with 6% sucrose (Fig. 1c). MS medium supplemented with 6% sucrose were found superior not only for giving optimum growth rate of embryonic culture (Table 3, 4) but also a uniform embryo developmental stages (Fig 1c). Increasing sucrose concentration in the medium improved the development of globular somatic embryos. Similar results have been reported in other plant species [16,10]. 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 sugar 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 [17]. Our study demonstrated that the type and concentration of sugar have important effects on the embryo development. The best carbon source for embryo culture was sucrose, followed by glucose and fructose. The differences in frequencies of developing embryos between sugars were statistically significant ($P < 0.05$).

Table 3 and 4 shows significant difference among the type and concentration of sugars on embryo induction. In three cultivars, the number of embryos per explant enhanced as the concentration of sucrose increased from 1.5% to 6%. In the present study, sucrose was superior for induction of embryos to glucose or fructose (Table 3, 4). The highest numbers of embryos were induced on medium containing 6% sucrose in all cultivars. The number of embryos per explant were decreased as the concentration of sucrose increased from 6% to 12%. Using different Carbohydrate source and concentration on number of embryos per explant has been reported for some plant species [18,10].

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