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Somatic embryogenesis and plant regeneration of pepper in liquid media

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Abstract

A protocol was developed for regeneration of pepper (*Capsicum annuum* var. Ace) through somatic embryogenesis in liquid media. For embryogenic callus formation, mature zygotic embryo explants were used on basal Murashige and Skoog medium with 9.05 μM 2,4-dichlorophenoxyacetic acid and 3% sucrose. Embryogenic callus was transferred to liquid basal Murashige and Skoog medium with 4.52 μM 2,4-dichlorophenoxyacetic acid and 3% sucrose in order to increase the mass of the embryogenic culture. After pretreatment with potassium citrate, cells were placed into embryo initiation medium with 6 g l^{-1} L-proline and a decreased (10 mM) ammonium concentration. Embryos were matured in 1.89 μM abscisic acid containing half-strength Murashige and Skoog medium and converted into plants both *in vivo* and *in vitro* at up to a 97% efficiency.

Abbreviations: ABA – abscisic acid; B5 – Gamborg et al. (1968); BA – 6-benzylaminopurine; 2,4-D – 2,4-dichlorophenoxyacetic acid; IAA – indole 3-acetic acid; MS – Murashige and Skoog (1962); NAA – naphthaleneacetic acid; pcv – packed cell volume; SH – Schenk and Hildebrandt (1972)

Introduction

Peppers (*Capsicum annuum* L.) are one of the major vegetable and spice crops grown world-wide. The conventional breeding of pepper hybrids for improved disease and pest resistance and desirable horticultural characteristics is a long process with problems of interspecific incompatibility and F1 hybrid sterility. *In vitro* somatic embryogenesis can offer solutions to these problems of conventional breeding. Furthermore, *in vitro* regeneration is one of the very few ways of recovering and propagating plants in large numbers from genetically transformed plant cells.

According to the literature, somatic embryogenesis for a variety of plants has been achieved using a variety of media ranging from relatively dilute White's medium (White, 1963) to the more concentrated formulations of Gamborg et al. (1968), Schenk and Hildebrandt (1972) and Murashige and Skoog (1962). Over 70% of the successful cases used Murashige and Skoog (1962) salts or its derivatives (Evans et al., 1981). Of the plant growth regulators, auxin is known to be essential for the induction of somatic embryogenesis in some plant species and although 2,4-D is the most commonly used auxin other auxins may be required for certain species (Ammirato, 1983). For example, only NAA was found to induce somatic embryogenesis in *Solanum melongena* (Gleddie et al., 1983). For the maturation of somatic embryos however, transfer to media containing a low concentration of or devoid of 2,4-D was essential (Ammirato, 1983; Cheema, 1989; van der Valk et al., 1989; Komamine et al., 1990). In some plant species, a combination of 2,4-D or NAA with cytokinin was reported to be essential for the induction of somatic embryos (Kao and Michayluk, 1981; Gingas and Lineberger, 1989; Schuller et al., 1989). ABA has been used successfully in a large number of somatic embryogenesis studies. It is an inhibitor for precocious embryo germination, whereby it allows embryo maturation to proceed in a more normal fashion, generally

increasing the uniformity of produced embryos and reducing the development of abnormal forms (Ammirato, 1983). Redenbaugh et al., (1991 a,b) suggested that maturation of alfalfa, carrot and celery somatic embryos with ABA was also critical for conversion of somatic embryos into plantlets. The source of nitrogen in the medium also affects *in vitro* embryogenesis in ways which depend on the plant species used (Reinert et al., 1967; Skokut et al., 1985; Rao et al., 1990; Chee et al., 1992). Ammonium or amino acids such as proline, alanine, arginine or glutamine stimulate prolific somatic embryo formation when supplied together with nitrate (Redenbaugh et al., 1991b; Stuart et al., 1985). Organic acids such as citric and malic acid which were applied during the last subculture of callus were found to increase the number of embryos formed, the conversion rates of the embryos and the amount of seed-specific storage proteins (Redenbaugh et al., 1991b).

In vitro studies on pepper aimed at plant regeneration have been reviewed by Fari (1986) and Morrison et al., (1986). Although some reports have been published on the morphogenetic response of pepper tissues (Fari and Czako, 1981; Philips and Hustenberger, 1985) and some authors have claimed direct or indirect plant regeneration from cells and tissues of pepper (Gunay and Rao, 1978; Agrawal and Chandra, 1983; Ge et al., 1989; Arroya and Revilla, 1991; Valera-Montero and Ochao-Alejo, 1992), so far there is only one report in the literature on direct somatic embryogenesis on solid medium (Harini and Sita, 1993). If most stages of somatic embryogenesis could be carried out in liquid media, then the process could be scaled-up using bioreactors which in turn, could reduce labour costs and space requirements. There is no report in the literature on the indirect somatic embryogenesis of pepper in liquid medium. This article therefore, presents for the first time a detailed protocol for regenerating pepper plants from mature-embryo explants through somatic embryogenesis in liquid culture. In this study, the effects of explant type, basal media formulation, growth regulators, various organic and amino acids on embryogenic callus formation, somatic embryo induction, frequency of embryogenesis and maturation were investigated.

Materials and methods

General conditions relevant to all experiments

Unless otherwise indicated, the following conditions apply to all cultures in this study. Prior to sterilization, pH was adjusted to 5.8 in all media with 1M NaOH or 1M HCl. Media were autoclaved for 18 min at 110 KPa at 121 °C in either one litre or 50 ml batches, in 1.51 or 250 ml Erlenmeyer flasks, respectively. When used, ABA was filter sterilized and added to autoclaved medium. Liquid cultures were placed on a rotary shaker and maintained at 100 rpm. Cultures were kept at 25 °C under continuous illumination provided by white fluorescent lamps (15 μmol m^{-2} s^{-1}). For the calculation of "% embryogenesis", 3 ml samples were taken in triplicate and a drop of the sample was placed on a microscope slide. The number of cells, cell clusters and embryos were counted under a light microscope. This was repeated for five microscope fields for each drop sample and the average numbers were calculated for three drops from each sample. "% embryogenesis" - 100 x (Number of embryos) / (Number of cells+cell clusters+embryos)

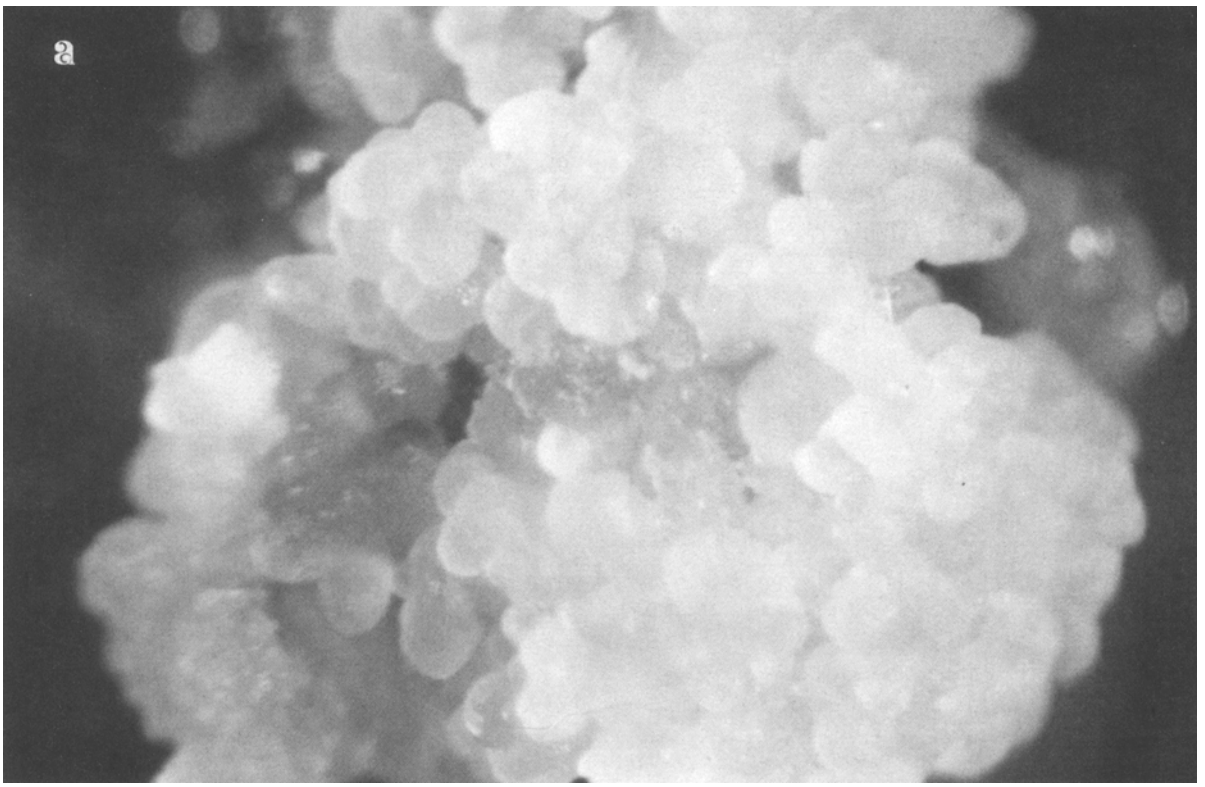
The statistical analysis of the data was based on Tukey's test (Compton, 1994; Lentner and Bishop, 1986).

Plant material

Seeds of *Capsicum annuum* var. Ace (Unwins Seeds Ltd, UK) were surface sterilized by soaking in 70% (v/v) ethanol solution for 2 min, then were immersed in 10% (v/v) sodium hypochlorite for 20 min and finally rinsed three times with sterile distilled water. They were then soaked in sterile distilled water for 24 h. Sterilized seeds were either aseptically dissected to remove the embryo explant or germinated on 20 ml solid MS medium with 2% (w/v) sucrose in presterilized 30x70 mm plastic bottles (Sterilin Ltd, UK) at 25 °C with a 16-h photoperiod. Mature zygotic embryos, hypocotyl (1 cm in length), cotyledon (fully expanded without petiole), petiole (0.8-1 cm in length) and root (1-1.2 cm in length) sections from 3 to 4-week-old seedlings were used as explants.

Callus culture

The factors examined for their effects on the frequency of embryogenic callus induction and somatic embryo formation included MS, SH and B5 media containing



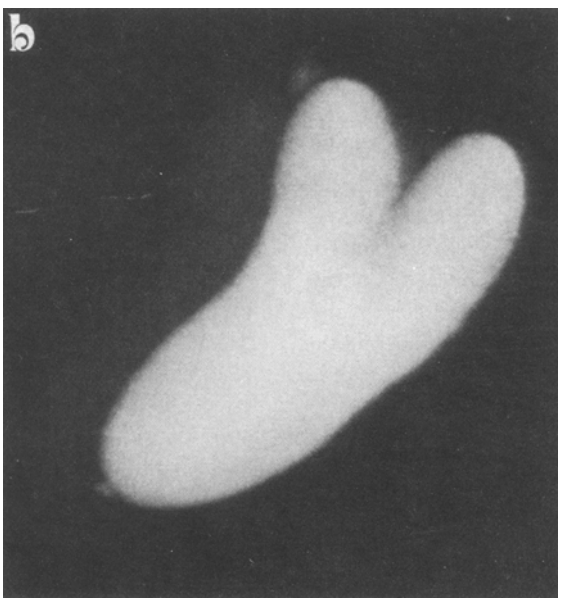


Figure 1. (*a*) Embryogenic callus of *C. annuum* var. Ace. (*b*) Somatic embryo of *C. annuum* var. Ace.

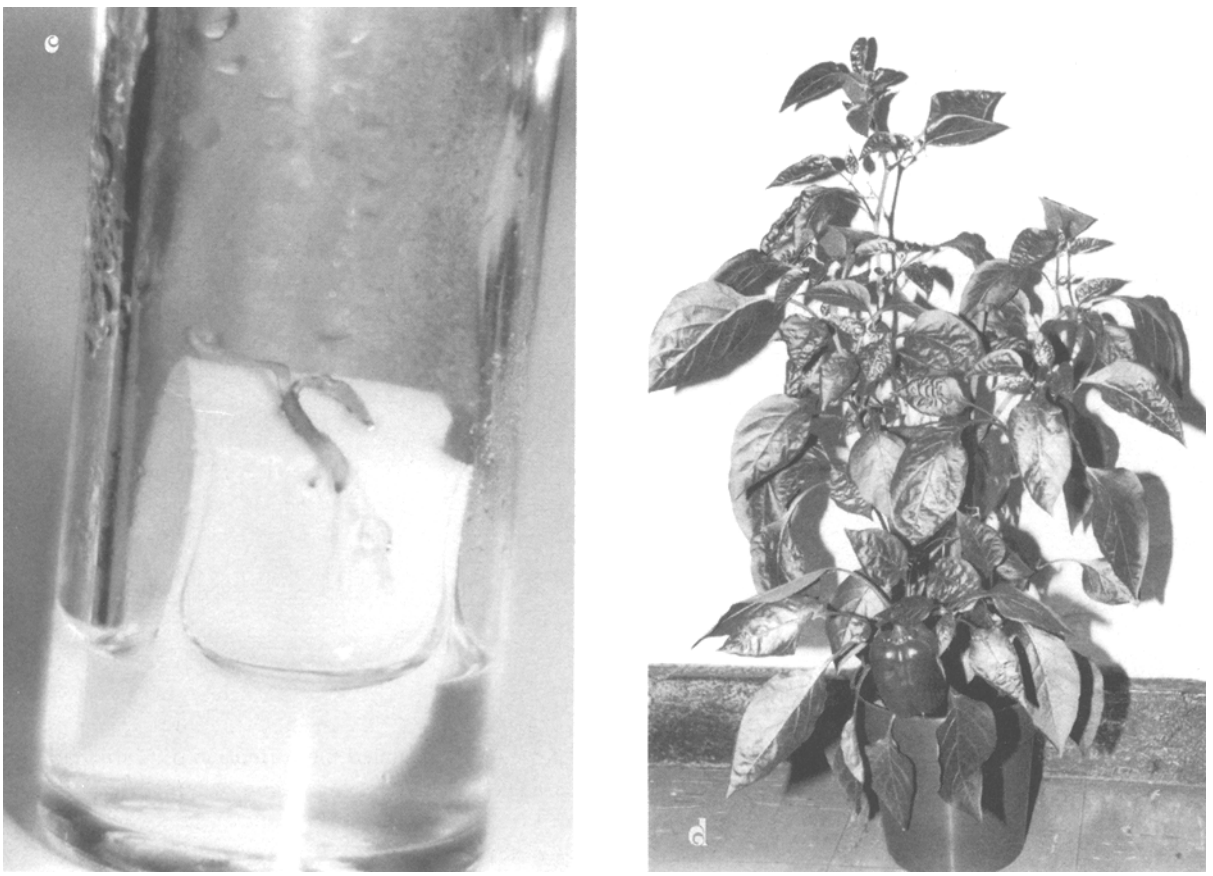


Figure 1. (*c*) Germination of a somatic embryo on paper bridge. (*d*) A pepper plant produced from a somatic embryo.

2,4-D (0.45, 4.52, 13.58 μM), IAA (0.57, 5.71, 17.12 1.μM), NAA (0.54, 5.37, 16.11 μM) alone or in combination with Kinetin (4.65 μM) and BA (4.44 μM) as growth regulators. The first set of experiments consisted of the interaction of the different types of explants on three basal media with these growth regulators at different concentrations. In order to compare the results, the size of the explant within a particular type and the amount of callus on all different media formulations were kept constant. The effect of medium type was investigated from the callus induction stage to globular embryo stage. Further experiments which are not reported here showed that sucrose at 3% (w/v) was the best carbohydrate source compared with fructose and glucose.

For callus initiation, three explants were cultured in 15 ml medium solidified with 0.8% agar (Sigma, UK); in a 90x15 mm sterile plastic Petri dish which was sealed with parafilm and incubated at 25 °C under continuous illumination. Five replicate Petri dishes were used for each explant type and, in all cases, the experiments were repeated at least twice. Once initiated, embryogenic callus was either transferred to liquid medium or maintained as stock culture by subculturing two pieces of 0.5 g callus onto fresh medium every four weeks.

Suspension culture

After the preliminary investigation of the effects of different media type on embryogenic callus initiation and embryo development until the globular stage, the culture media for the rest of the investigation were based on MS medium containing 3% sucrose hereafter referred to as the basal medium. Suspension cultures were initiated by transferring one-month-old 1-1.2 g embryogenic callus to 50 ml MS liquid basal medium containing 4.52 μM 2,4-D in a 250 ml Erlenmeyer

flask. Suspension cultures were subcultured every two weeks for two months after which unused cultures were discarded.

Pretreatment

Suspension cultures were transferred to liquid KNO_3-free MS basal medium containing 9.05 μM 2,4-D and either K-citrate or K-malate at the concentration of either 6 or 10 g l^{-1} and kept in these media for three weeks.

Embryo initiation

The liquid basal MS medium was modified by reducing NH_4NO_3 concentration from 20 mM to 10 mM and was used with one of the four amino acids, L-proline, L-alanine, L-arginine or L-glutamine at a time at 0, 2, 4, 6, 8 and 10 g l^{-1}. The cultures were kept on a rotary shaker at 70 rpm.

Maturation

Somatic embryos at late torpedo stage (3-5 mm in length) were transferred to full- and half-strength 50 ml MS liquid basal media containing 1.89, 3.78 and 7.57 μM of *cis-trans* isomer ABA (Sigma) and placed on a rotary shaker at 40 rpm in the dark for 20 days.

Conversion into plantlets

Somatic embryos from the maturation medium were germinated either *in vitro* or *in vivo*. The *in vitro*-conversion medium consisted of half-strength of MS major and minor salts and full-strength MS vitamins. A paper bridge in 15 ml medium was used in a 70x15 mm glass bottle for *in vitro* germination. After placing each embryo on the paper bridge, glass bottles were incubated at 25 °C with a 16-h photoperiod. Sterilized compost and sand mixture (1:2) in 4-cm pots was used without any supplement for *in vivo* germination. Pots were covered with clear plastic in order to prevent desiccation.

Results and discussion

Embryogenic callus formation

As mentioned in the introduction, the most important factors affecting the induction of embryogenic callus and plant regeneration through somatic embryogenesis include the explant type, media formulation and growth regulators. All these factors were investigated in this study because there is no previous report in the literature on the somatic embryogenesis of pepper. Depending on these factors, four different types of callus were obtained: white-watery, brown-friable which were both nonembryogenic; green-compact with low embryogenic frequency and yellowish-nodular-friable with high embryogenic frequency (Figure 1a).

The results with all the explants and all the growth regulators on each media are summarized in Table 1; the results indicate that MS basal medium yielded the highest amount of embryogenic callus while B5 induced none. The conversion frequency of embryogenic callus to globular-shaped embryos was also the highest in MS medium. High concentrations of major salts in MS medium compared with others seem to be the reason for this result. The most important factor that affected the callus type was the growth regulator. The general observation in this study was that IAA stimulated non-embryogenic callus; NAA, that is, stimulated the formation of low-frequency embryogenic callus and 2,4-D stimulated the formation of high-frequency embryogenic callus formation. Therefore, in the rest of the experiments, MS medium was used as the basal medium and 2,4-D as the growth regulator. By this stage of the investigation, it was also observed that the root explant did not produce any embryogenic callus under any condition. Consequently, it was excluded from the rest of this study.

Since all the results in our study indicated that 2,4-D was the most effective growth regulator for embryogenic callus induction, further experiments were performed in order to determine the optimum 2,4-D concentration. Table 2 presents the effect of 2,4-D on the percentage of number of explants that formed embryogenic callus (% C) and the percentage of the number of cells that differentiated into embryos (% E) calculated as described above. Depending on the explant type, percentage of number of explants that formed embryogenic callus increased 5-6 fold and the percentage of the number of cells that differentiated into embryos increased 7-14 fold when 2,4-D concentration was increased from 0.45 to 9.05 μM. Although % C remained constant, % E decreased by as much as 50% when the concentration of 2,4-D was doubled from 9.05 to 18.1 μM. Results in Table 2 indicated that the best explant source was the mature zygotic embryo and the optimum 2,4-D concentration was 9.05 μM.

Table 1. The effect of media formulation on the formation of callus and the frequency of embryogenesis.

Media formulation	Callus fresh weight (g/explant)			(%)
	Embryogenic	Low-frequency embryogenic	Non-embryogenic	Embryogenesis
MS	1.89 a	1.01 a	1.15 a	51 a
SH	0.76 b	0.82 b	1.09 a	11 b
B5	0 c	0.31 c	1.11 a	0 c
D %5	0.317	0.155	0.209	4.5737

Values followed by different letters are significantly different at the 5% level in Tukey's test.

Table 2. The effect of 2,4-D concentration on the formation of embryogenic callus and the frequency of embryogenesis.

2,4-D (μM)	Explant							
	Hypocotyl		Cotyledon		Petiole		Embryo	
	% C	% E	% C	% E	% C	% E	% C	% E
0.45	20 cd	8 l	16 cd	5 lm	21 c	10 kl	12 cd	5 lm
2.26	75 b	35 fg	70 b	27 h	70 b	31 gh	65 b	40 ef
4.52	90 a	40 ef	90 a	30 gh	95 a	51 bc	95 a	55 b
9.05	95 a	47 cd	90 a	35 fg	100 a	56 b	100 a	62 a
13.58	95 a	32 gh	90 a	20 i	97 a	45 de	100 a	35 fg
18.1	90 a	17 ij	90 a	14 jk	95 a	20 i	95 a	27 h
45.25	10 d	0 m	15 cd	0 m	10 d	0 m	20 cd	0 m

D % 5 (for % C data) = 10.13
D % 5 (for % E data) = 5.72
% C: The percentage of number of explants that formed embryogenic callus.
% E: Percent embryogenesis.
Values followed by different letters are significantly different at the 5% level in Tukey's test.

Suspension culture

After embryogenic callus induction, the proliferation of cells were carried out on both solid and liquid MS basal medium containing 9.05 μM 2,4-D. Liquid medium yielded almost double the cells compared to the solid medium over the sample growth period. After two months of subculturing the viability, dry weight and embryogenic frequency of the suspension culture started to decrease. The experiments performed to remedy this deterioration indicated that a decrease in the 2,4-D concentration to 4.52 μM was satisfactory to obtain healthy growth with unaffected embryogenic capacity for more than 6 months.

Pretreatment medium

Prior to transfer into embryogenesis medium, suspension cultures were treated with organic acids as shown in Table 3. Without this treatment, even with L-proline in the embryogenesis medium as explained below, it was not possible to obtain mature embryos. Although cultures grew only slightly during the three-week pretreatment in liquid media, packed cell density increased, especially with the inclusion of 6 g l^{-1} potassium citrate in the medium; it also had the most pronounced effect on the concentration of heart-shaped embryos in the medium. There are few reports in the literature on the need of a pretreatment as a prerequisite for somatic embryogenesis (Nichol et al., 1991). Such pretreatment may be a selection for more embryogenic cells or cause an alteration in cellular metabolism towards cell differentiation and away from cell proliferation (Redenbaugh et al., 1991b). The pretreatment agents in our study were citric and malic acids. Since potassium salts of these acids were used there could have been an effect of the potassium ion as it is the major and the most common cation involved in main-

taining the ionic balance of the medium and the osmotic potential of the cytoplasm. The effect of potassium in sweetpotato somatic embryogenesis is studied by Chee et al. (1992).

Embryo initiation

In the preliminary attempts of embryo initiation, embryogenic suspension cultures were transferred to liquid growth regulator free MS basal medium after pretreatment. In this medium, cell clusters developed through globular-shaped into heart-shaped embryos but no further. Since the amino acids are reported to be effective on embryo development (Stuart and Strickland, 1984; Redenbaugh et al., 1991; Shetty and McKersie, 1993; Nichol et al., 1991), L-proline, L-alanine, L-arginine and L-glutamine were tested after a three-week period at all the stages of embryogenesis at various concentrations in liquid MS basal medium containing 10 mM NH_4NO_3.

In addition to the standard MS medium which contains nitrate and ammonium ions at the molar ratio of 40:20, modified MS media with other ratios of nitrate to ammonium at 40:0, 20:20, and 20:40 were tested. Although the basal MS medium gave the best results, when the level of NH_4^+ was high, somatic embryo formation was reduced and severe tissue browning occurred. On media without or with a very low level of NH_4^+, the number of embryos produced decreased considerably. Therefore, liquid MS basal medium containing 10 mM NH_4NO_3 was chosen for embryo initiation. The same concentration of NH_4NO_3 was found to be the best in a detailed study of the media optimization for sweetpotato embryogenesis by Chee et al., (1992).

As shown in Figure 2, L-proline at 6 g l^{-1} was the most effective amino acid and it yielded the highest number of torpedo-shaped embryos. This result is in agreement with that of Stuart and Strickland (1984) who tested nine amino acids in alfalfa somatic embryogenesis and found that L-proline was the most effective one. Kamada and Harada (1979) and Wetherell and Dougall (1976) did not find L-proline effective in carrot somatic embryogenesis. They had however, excluded NH_4^+ from their medium thinking that L-proline, being an amino acid, could be used as the sole nitrogen source. In the work reported here, NH_4NO_3 was not excluded from the medium but only reduced in concentration.

Table 3. The effect of organic acids during pretreatment stage on cell fresh weight per cm^3 packed cell volume and subsequent embryogenesis.

Treatment	Fresh cell density (gfwt/cm^3 pcv)	Number of heart shaped embryos/ ml of media
(Control)[1]	0.406 d	61 b
6 g l^{-1} K-citrate[2]	0.728 a	74 a
10 g l^{-1} K-citrate[2]	0.630 b	54 b
6 g l^{-1} K-malate[2]	0.520 c	24 c
10 g l^{-1} K-malate[2]	0.542 c	21 c
D % 5	0.078	9.15

1: MS + 30% (w/v) Sucrose + 9.05 μM 2,4-D
2: MS - KNO_3 + 30% (w/v) Sucrose + 9.05 μM 2,4-D + organic acid.
Values followed by different letters are significantly different at the 5% level in Tukey's test.

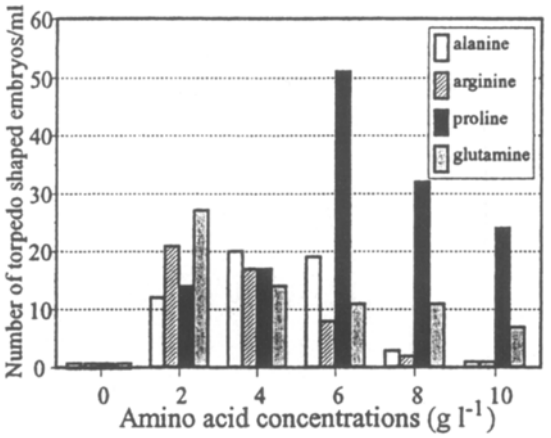


Figure 2. The effect of the types and concentration of amino acids on the number of torpedo shaped embryos. For all the values SD = 11.12.

Maturation of somatic embryos

Embryos from the embryo initiation medium were transferred to conversion medium as explained below but they did not convert into plantlets. Since ABA is well known as a growth regulator of many developmental stages in zygotic embryos and similarly found to favourably affect maturation of somatic embryos (Fuji et al., 1990), embryos of pepper were placed in full- and half-strength MS basal medium containing various concentrations of ABA (Table 4). Embryo maturation was observed under a light microscope (x 10 magnification) as somatic embryos became firmer, larger and smoother (Figure 1b). In all the media test-

Table 4. Effect of ABA concentration on maturation and conversion of somatic embryos.

Medium	Maturation (%)	Conversion (%)
MS	0 d	0 d
MS+1.89 μM ABA	0 d	0 d
MS+3.78 μM ABA	0 d	0 d
MS+7.57 μM ABA	0 d	0 d
1/2 MS	0 d	0 d
1/2 MS+1.89 μM ABA	55 a	97 a
1/2 MS+3.78 μM ABA	39 b	71 b
1/2 MS+7.57 μM ABA	25 c	58 c

D % 5 (Maturation) : 5.41
D % 5 (Conversion) : 7.45
Values followed by different letters are significantly different at the 5% level in Tukey's test.

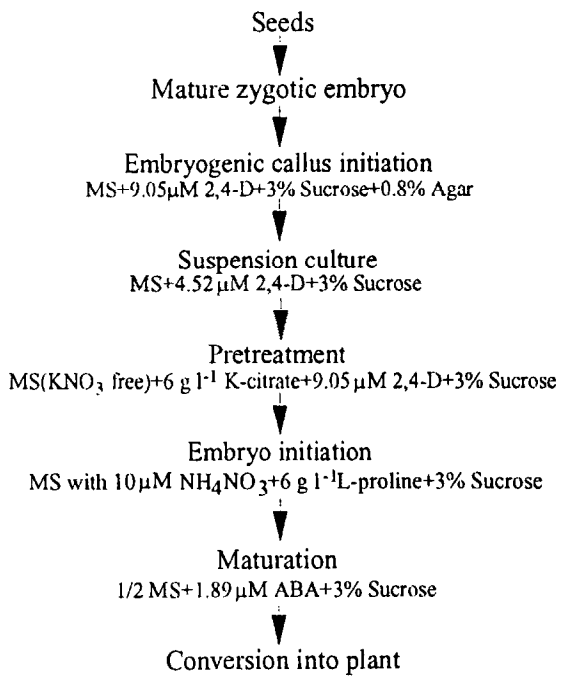


Figure 3. Procedure developed for the regeneration of pepper plants through somatic embryogenesis.

ed in continuous light and full-strength MS medium in dark, embryos became brown and eventually died. The highest maturation and conversion frequencies were obtained in darkness in half-strength MS medium with a concentration of 1.89 μM ABA.

Conversion into plantlets

On germination media, the somatic embryos enlarged slightly before a gradual greening of the entire embryo. Germination (Figure 1c) generally occurred 10-15 days after transfer. Figure 1d is the photograph of a pepper plant produced from a somatic embryo. The conversion frequency was 97% for *in vitro* germination and 48% for *in vivo* germination. In conclusion, this study investigated various factors effecting the pepper plant regeneration through somatic embryogenesis. The best procedure based on the results obtained is given in Figure 3.

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