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Embryogenesis and plant regeneration of spinach (*Spinacia oleracea* L.) from hypocotyl segments

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Abstract

A system for somatic embryogenesis and plant regeneration of spinach from hypocotyl segments has been established. Callus was induced on solid media supplemented with 8.5-15.0 mg.l^{-1} of indole-3-acetic acid and 3.46-34.64 mg.l^{-1} gibberellic acid. Callus was then subcultured on different media (solid or liquid) with or without IAA, or continuously maintained on the initiating media. Somatic embryos were obtained in subcultures on IAA-containing media as well as in long-term cultures on initiating media. The best results were achieved in liquid subcultures. About 60% of plantlets survived after transplanting in pots.

Abbreviations: 2,4-D: 2,4-dichlorophenoxyacetic acid; GA3: gibberellic acid; IAA: indole-3-acetic acid; MS: Murashige and Skoog's medium (1962); NAA: 1-naphthaleneacetic acid

Key Words: hypocotyl, plant regeneration, spinach (*Spinacia oleracea* L), somatic embryogenesis

Introduction

In vitro somatic embryogenesis offers great potential in crop improvement since it allows for the coupling of efficient cloning and genetic modification (Sharp *et al.* 1980). In many plant species, including important crops, for instance, rice, maize, wheat, melon etc., somatic embryogenesis and plant regeneration have been achieved. In spinach, a dioecious vegetable crop, although shoot-bud formation was reported as long ago as 1973 (Neskovic and Radojevic 1973), plant regeneration was realized only recently by organogenesis from leaf callus (Al-Khayri *et al.* 1991), hypocotyl and root segments (Xiao and Branchard, unpublished). As far as we know, no information about somatic embryogenesis and plant regeneration is available.

In this paper, we report a protocol for *in vitro* embryogenesis and plant regeneration of spinach from hypocotyl segments.

Materials and Methods

Seeds of spinach (*Spinacia oleracea* L. cv. Carpo) were surface-sterilized in 70% (v/v) ethanol for 10 seconds, followed by immersion in 0.1% (w/v) mercuric chloride for 10 min, then in 5% (w/v) calcium hypochlorite (70% active chlorine) for 30 min, and rinsed 4 times in sterile water. The seeds were germinated on medium containing 1/2-strength MS salts (Murashige and Skoog 1962), 2% (w/v) sucrose and 0.7% (w/v) Difco agar in 750 ml glass jars in the dark at 24 ± 1° C. Sub-apical hypocotyl segments of 2-3 mm in length were aseptically excised from one-week-old seedlings and cultured on MS medium (Murashige and Skoog 1962) supplemented with 0.01 mg.l^{-1} biotin, 250 mg.l^{-1} glutamine, 2% sucrose, 0.7% Difco agar (medium designated as BM) and various concentrations and combinations of indole-3-acetic acid (IAA) and gibberellic acid (GA3) (Table 1) in Petri dishes (diameter 100 mm; 10 explants, 20 ml medium per dish). Cultures were held at 22 ± 1° C in the dark for one week, then exposed to a 10-h photoperiod of a 1:1 mixture of Sylvania Gro-lux and Philips cool white fluorescent light ca. 250 μmol.s^{-1}.m^{-2} at 22/15° C day/night for two weeks, and finally maintained at 24 ±1° C under continuous light. After a total of 7 weeks of culture, each callus was cut into three pieces. One third of the pieces were kept on their original media, one third were subcultured on a fresh BM supplemented with different concentrations of IAA and GA3 (Table 1) and the last third were subcultured in liquid BM containing 0.50 mg.l^{-1} IAA and 3.46 mg.l^{-1} GA3. The liquid cultures (about 1 g callus and 40 ml medium per 250 ml Erlenmeyer flask) were held on a platform shaker at 125 *rpm* for one week, then at 95 *rpm* for two weeks at 22 ± 1° C under continuous light ca. 50 μmol. s^{-1}.m^{-2}. The embryos and plantlets were transferred onto BM supplemented with 0.50 mg.l^{-1} IAA in 250 ml flasks at the photoperiod described above. Two or three weaks

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later, the plantlets were transplanted into a 1:3 mixture of vermiculite and nutritive soil in pots, watered with 1/2-strength MS salts for the first week, then with tap water, and placed in a greenhouse.

All media were sterilized in an autoclave at 120 ° C for 20 min. The pH of the media was adjusted to 5.8 before sterilization.

The experiments were performed at least twice, and more than 60 explants were used in each treatment.

Table 1. Initiating and subculture solid media used in the experiment

Initiating media		Subculture media	
BM* + (mg.l^{-1}) IAA + GA3	Code	BM + (mg.l^{-1}) IAA + GA3	Code
15.00 + 34.64	M1a	5.00 + 3.46	M2a
15.00 + 3.46	M1b	2.00 + 3.46	M2b
8.50 + 34.64	M1c	0.00 + 3.46	M2c
8.50 + 3.46	M1d		

*: BM: MS medium supplemented with 0.01 mg.l^{-1} biotin, 250 mg.l^{-1} glutamine, 2% sucrose and 0.7% Difco agar

Results and Discussion

Callus induction

All explants on the 4 initiating media formed callus after about 4 weeks of culture. The size of the callus was smaller (ø ≤ 4 mm) on media M1a and M1b than on media M1c and M1d. Three weeks later, some callus formed adventitious shoots and/or roots (on media M1a, M1b, M1c and M1d), some produced embryogenic callus or embryo-like structures (on media M1a and M1b).

Callus continuously grown on the initiating medium

When calluses were kept on their original media for another 4 weeks, 26.7 and 6.3% of them produced visible somatic embryos on media M1a and M1b respectively, whereas no embryos were observed on media M1c and M1d. Embryos, in general, looked like red granules at the globular stage, then became single or multi-torpedo-shaped (3 or 4), as observed by Prioli *et al.* (1990) in the culture of *Zea mays* . In these long-term initiating media, no typical heart-shaped embryos could be detected (Fig.1).

Callus subcultured on solid medium

When the callus from the initiating treatments with higher concentrations of IAA (≥8.5 mg.l^{-1}) was transferred to the lower-IAA-containing media (M2a and M2b), embryos (Fig.2) were obtained 4 weeks later (Table 2). However, when subcultured on the auxin-free medium (M2c), most of the callus browned and died, and neither embryo nor embryo-like structures could be observed. The frequency of embryo-forming callus was dependent on both initiating and subculture media. On the same subculture media (M2a and M2b), the callus originating from the treatment of higher concentrations of IAA and GA3 (M1a) most frequently formed embryos (18.8-33.3%), whereas those from the lower one (M1d) produced embryos at lower frequency (5.3-10.0%) (Table 2).

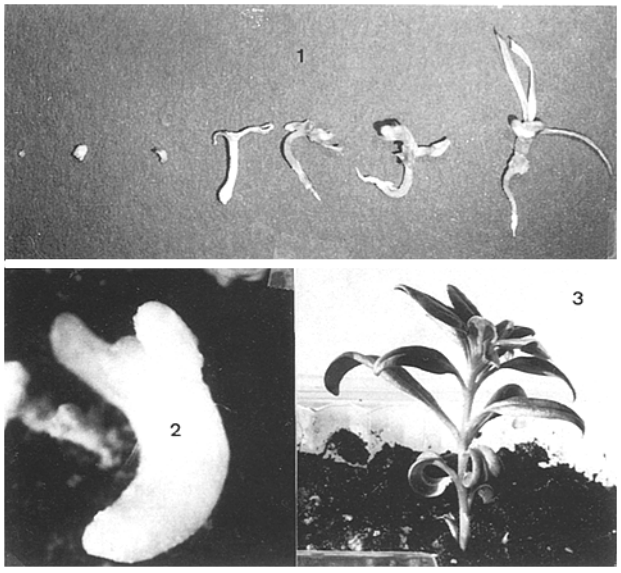

Fig. 1. Different developmental stages of somatic embryo from the hypocotyl segments of spinach after about 9 weeks of culture on the initiating solid medium: lack of typical heart-shaped embryo.

Fig. 2. A somatic embryo from the hypocotyl segment of spinach after about 4 weeks of subculture on the solid medium.

Fig. 3. A well developed plantlet from a somatic embryo of the hypocotyl segment of spinach

Table 2. Influence of the initiating and subculture solid media on somatic embryogenesis and plant regeneration of spinach hypocotyl segments after 4 weeks of subculture (2 repetitions)

Initiating medium	Subculture medium	No. calluses* subcultured	% of embryo-forming calluses**
M1a	M2a	48	18.8 ± 0.4
	M2b	51	33.3 ± 7.0
	M2c	60	0.0
M1b	M2a	50	16.0 ± 0.4
	M2b	52	15.4 ± 0.7
	M2c	55	0.0
M1c	M2a	56	17.9 ± 2.8
	M2b	32	15.5 ± 2.7
	M2c	58	0.0
M1d	M2a	38	5.3 ± 3.0
	M2b	40	10.0 ± 0.5
	M2c	43	0.0

*: Those contaminated were not included. **: Mean ± SE.

Of the two concentrations of IAA used in the subculture medium, 2.0 mg.l^{-1} seemed more effective

than 5.0 mg.l^{-1}, but this difference was not marked for the callus originating from media M1b and M1c (Table 2). Compared with long-term-used initiating media, the fresh subculture media containing lower levels of IAA favoured further complete differentiation and development of embryos. The callus, which did not form embryos in the initiating media (M1c and M1d), produced embryos on the subculture media at the frequencies of 5.3-17.9%.

Callus subcultured in liquid medium

One week of high-speed (125 *rpm*) shaking culture separated the embryos or embryo-like structures from their mother-callus and most of them were dark-red and at the single globular stage. During another 2 weeks of culture at 95 *rpm*, the embryos developed into different stages: globular-, heart-, torpedo-, cotyledonary-shaped and complete plantlets. At the same time, about 10% of them became abnormal: plantlets without visible hypocotyl, with more than one embryonic root or embryos with only shoot or root etc.. The callus originating from higher concentrations of IAA and GA3 (medium M1a) produced the greatest number of embryos (more than 100 embryos per flask), followed by those from media M1b, M1c and M1d, approximately 75, 50 and 25 embryos per flask, respectively. High concentrations of IAA and GA3 in the initiating phase stimulated also the formation of secondary embryos on the cotyledons and hypocotyl of the first embryo in liquid subculture medium. Compared with the solid subculture media and long-term initiating media used, liquid subculture medium containing 0.5 mg.l^{-1} IAA was more favourable to the completion of differentiation and development of the embryos: more embryos and plantlets were obtained regardless of the callus origin. On the other hand, liquid subculture resulted in more abnormal embryos.

When the cotyledonary embryos and plantlets obtained in both solid and liquid media were transferred onto solid medium containing 0.5 mg.l^{-1} of IAA, they grew and developed normally. However, only about 60% of them survived after transplanting in pots in a greenhouse (Fig.3).

Transfer of the cultures from relatively high concentrations of 2,4-D + kinetin medium to auxin-free medium is a routine method for *in vitro* somatic embryogenesis in various plant species (Sharp *et al.* 1980). However, in our preliminary experiments, 8 weeks of culture of spinach hypocotyl segments on MS basal medium complemented with kinetin (0.0, 0.05, 0.1, 0.5, 1.0, 2.0, 5.0 mg.l^{-1}) + 2,4-D (0.0, 0.05, 0.1, 0.5, 1.0, 2.0 mg.l^{-1}) did not induce embryogenic callus, and transfer of this non-embryogenic callus to fresh medium containing kinetin (0.0, 0.5, 1.0, 2.0, 5.0 mg.l^{-1}) + IAA or NAA (0.0, 0.5, 1.0, 2.0 mg.l^{-1}) did not produce any embryo or embryo-like structures (data not presented). In the present study, subculture of the callus induced on the initiating phase on auxin-free solid medium produced neither embryo nor embryo-like structures. These results suggested that spinach tissues would need high concentrations of IAA and GA3, but neither cytokinin nor 2,4-D for somatic embryogenesis, and need a certain level of auxin for embryo development. The necessity of high concentrations of IAA and GA3 for organogenesis in spinach has been observed by Sasaki (1989) and ourselves (Xiao and Branchard, unpublished). Nitsch and Nitsch (1969) demonstrated that GA3 affected the rate of embryo development, but not the frequency of embryogenesis. This kind of permissive effect of external application of growth regulators on somatic embryo differentiation occurred only in the case where pre-embryogenic determinated cells (PEDC) and the possibility of direct embryogenesis existed (Sharp *et al.* 1980). Under our experimental conditions, only induced embryo determinated cells (IEDC) and indirect embryogenesis were obtained, and the frequency of somatic embryogenesis was affected by the GA3 together with IAA. This means that the external application of appropriate concentrations of IAA and GA3 would be indispensable, which was proved by the fact that the initiating medium had an important influence on the further embryogenesis of the callus and on the embryo development in subculture media.

From our results and discussion, we conclude:

a). the initiating phase was more important than the subculture phase in the induction of somatic embryogenesis, and its influence continued at the subculture phase;

b). the best combination for the initiating phase was 15.0 mg.l^{-1} IAA + 34.64 mg.l^{-1} GA3 (M1a);

c). subculture of the induced callus onto fresh medium containing a lower concentration of IAA was imperative and favourable to the development of embryos and the completion of embryogenesis;

d). liquid subculture seemed superior to solid one although the IAA level was different in the two types of subculture systems used;

e). problems of abnormal embryo control, transplanting of the plantlets into pots and histological studies of the origin of the callus and somatic embryos are in progress.

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