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Somatic embryogenesis and plant regeneration of squash *Cucurbita pepo* L cv. YC 60

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Abstract

Plant regeneration from tissue cultures of summer squash, *Cucurbita pepo* L., cv. YC60, has been observed. Somatic embryos organized from shoot apex derived callus cultured on Murashige and Skoog (MS) medium supplemented with 1.2 mg/l 2,4,5-trichlorophenoxyacetic acid, 0.8 mg/l benzylaminopurine, and 0.1 mg/l kinetin. Embryos developed into plantlets by transfer of immature somatic embryos to MS medium with 0.05 mg/l NAA and 0.05 mg/l kinetin. Regenerated plants appeared morphologically normal and set fruits with seeds which could germinate normally.

Introduction

Squash (*Cucurbita pepo* L) is an important vegetable crop in the tropics, subtropics, and milder portions of the temperate zones of both hemispheres. The use of molecular and cellular genetic methods in breeding programs requires the development of tissue culture methods. Schroder (1968) reported the production of embryogenic tissue from pericarp of zucchini squash. Jelaska (1972, 1973, 1977, 1980) reported somatic embryogenesis in hypocotyl and cotyledon-derived callus of pumpkins and demonstrated that embryos could develop into normal plants. Pink and Walkey (1984) reported a rapid propagation method for pumpkin through apical meristem culture. There are no reports on whole plant regeneration of summer squash. This paper describes a method for induction of somatic embryos from shoot tips of summer squash, cv. YC60, and subsequent plant regeneration.

Materials and Methods

Tissue and medium preparation

Seeds of summer squash (*Cucurbita pepo* L cv. YC 60, Asgrow Seed Co, Kalamazoo) were soaked in tap water for approximately 15 min and the seed coats were removed manually. The de-coated seeds were treated with 70% alcohol for 2 minutes. The seeds were then sterilized for 25 min with a 25% (v/v) solution of Clorox (commercial bleach containing 5.25% sodium hypochlorite). The seeds were rinsed four times with sterile distilled water. Seeds were germinated in Magenta boxes with 50 ml of 1/2 x MS medium (Murashige and Skoog, 1962) containing 30 g/l sucrose, and 8 g/l Difco agar (Difco Laboratories). The seeds were germinated in darkness at 28°C. All tissue culture media were supplemented with 3% sucrose and solidified with 0.8% Phytagar (Gibco). The pH of media was adjusted to 5.8 prior to autoclaving at 121°C for 20 minutes.

Induction of somatic embryogenesis and plant regeneration

Shoot apices and some adjacent tissue were excised from in vitro grown seedlings 7 days after germination in darkness (Fig 1). The apices, approximately 2 mm in size were cut into longitudinal halves and cultured horizontally with cut surfaces facing up on MS + 1.2 mg/l 2,4,5-T + 0.8 mg/l BAP (Chee, 1990) and 0, 0.1, 0.2 or 0.5 mg/l KN for two months with one sub-culture after 4 weeks. Embryogenic callus tissue were propagated by successive subculturing of a slimy, translucent callus tissue onto fresh MS medium + 1.2 mg/l 2,4,5-T + 0.8 mg/l BAP + 0.1 mg/l KN every four weeks. All cultures were maintained in darkness at 28°C.

After 10 weeks of total culture, putative somatic embryos were transferred to conversion medium composed of MS medium + 0.05 mg/l NAA + 0.05 mg/l KN. These structures were maintained on this medium at 28°C under diffuse cool-white fluorescent lamps (4 klx) with a 16 hour photoperiod.

After 2 weeks on conversion medium, plantlets developed a root system, and were then transplanted to pots containing planting mix and covered with plastic bags for one week. Subsequently, the hardened plants were potted in a mixture (1:1, v/v) of soil and Perlite and grown in a greenhouse under standard conditions.

Histology

Embryogenic callus tissue containing putative somatic embryos were fixed in 10% neutral buffered formalin for 45 min, dehydrated in ethyl alcohol-tertiary butyl alcohol series and embedded in Paraplast (Monoject Scientific, St. Louis, MO) at 56°C. Embedded specimen were sectioned at 5 μm thickness and stained, sequentially, for 12 h with

Abbreviation: BAP, 6-benzylaminopurine; 2,4-D, 2,4-dichlorophenoxyacetic acid; IAA, indole-3-acetic acid; KN, kinetin; NAA, β-naphthyleneacetic acid; MS, Murashige and Skoog; 2,4,5-T, 2,4,5-trichlorophenoxyacetic acid

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1% (v/v) safranin O (Sigma Chemical Co.) dissolved in 50% alcohol, followed by a 20 sec exposure to 1% (v/v) fast green (Sigma Chemical Co.) dissolved in 95% alcohol.

Results and Discussion

Of the media formulations evaluated, only MS with 1.2 mg/l 2,4,5-T, 0.8 mg/l BAP and 0.1 mg/l KN (TBK medium) was effective in the induction of somatic embryogenesis (Table 1). The difference in response between all media formulations from two independent experiments was significant at 1%. A shoot tip explant is shown in Fig. 1. After two weeks of culture on TBK medium, the shoot tip explants became swollen. The tissue lost its green color and became amber (Fig. 2). After 6 weeks, the explants formed different tissue morphologies. One particular type of tissue was shiny, translucent and slimy (Fig. 3). Following 8 week incubation, putative somatic embryos were observed to organize from sectors on the surface of the slimy callus (Fig. 4). Longitudinal cross near-median section of somatic embryos before transfer to conversion medium is shown in Fig. 5.

To promote further development of the putative somatic embryos, they were transferred to conversion medium, which is MS medium + 0.05 mg/l NAA + 0.05 mg/l KN. Within three weeks, shoot-tips of the embryos became green and plantlets were obtained 3 weeks later (Fig. 6). Fifty percent of the somatic embryos developed into whole plants. Visual evaluation on one hundred regenerated plants revealed that they displayed no gross phenotypic abnormality (Fig 7). All regenerated plants flowered and set fruits with seeds which could germinate normally. Other growth regulator combinations (2,4-D + BAP; 2,4-D + KN; IAA + KN; NAA + BAP; NAA + KN; 2,4-D + NAA + KN; 2,4-D + NAA + BAP + KN; IAA + BAP) were also tested. However, none of these formulations favored the formation of embryogenic callus tissues.

Regeneration of plants from embryogenic tissue cultures has been achieved in several genera of the family *Cucurbitaceae*. Schroder (1968) reported that somatic embryos of zucchini squash were generated from callus tissue, but plantlet recovery was not claimed. Jelaska (1980) reported that only one pumpkin plant was obtained from a hypocotyl-derived embryogenic callus. However, other than this current report, there is no documentation of plant regeneration in the genus *Cucurbita pepo* L. Based on the above described method and observations, squash plantlets may be obtained within 4 months of culture initiation. Because YC60 was the only cultivar tested, further studies are required to determine if this protocol is genotype-specific.

This procedure was carried out with shoot tips of young seedlings where somatic embryos developed from shoot apices. Putative embryogenic tissue that developed on the surface of the explant was easily identified by a characteristic translucent appearance. This tissue morphology was similar to embryogenic tissue derived from primary leaves, hypocotyl, and cotyledons of cucumber (Malepszy and Nadolska-Orczyk, 1983, Chee, 1990) that belongs to the same family, *Cucurbitaceae*. The embryogenic potential of the calli is not lost with successive subculturing on induction medium and can be maintained for up to 18 months. Similar results have been observed in cucumber (Chee, 1990), and pumpkin (Jelaska, 1985). The procedure described in this report has been applied in gene transfer experiments *via* agrobacterium or microprojectile bombardment (Chee, unpublished data).

Table 1. Embryogenic responses of shoot tips from *in vitro* grown seedlings after 8 weeks in culture on MS basal medium supplementing with growth regulators.

Growth regulators	Number of explants with embryogenic response		Pooled frequency(%) of embryogenic tissue induction
	I[a]	II	
1.2 mg/l 2,4,5-T 0.8 mg/l BAP	0(100)[b]	0(85)	0
1.2 mg/l 2,4,5-T 0.8 mg/l BAP 0.1 mg/l kinetin	8(96)	17(144)	8
1.2 mg/l 2,4,5-T 0.8 mg/l BAP 0.2 mg/l kinetin	0(100)	0(100)	0
1.2 mg/l 2,4,5-T 0.8 mg/l BAP 0.5 mg/l kinetin	0(100)	0(110)	0
	[c]χ^2 = 24.49**	χ^2 = 36.23**	

[a]Experiment number.
[b]Number of explants.
[c]Data were entered into a contingency table and χ^2 values were computed.
** indicated significance at 1% level of probability.

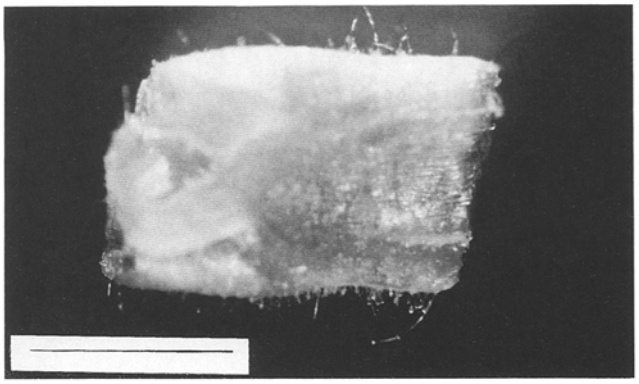

Figure 1. A fresh shoot tip explant from squash seedling. Bar = 1.0 mm.

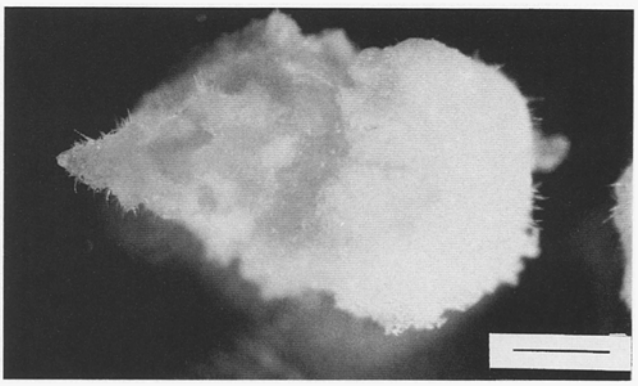

Figure 2. Callus tissue formed after two weeks on MS basal medium supplemented with 1.2 mg/l 2,4,5-T, 0.8 mg/l BAP, 0.1 mg/l kinetin. Bar = 1.0 mm.

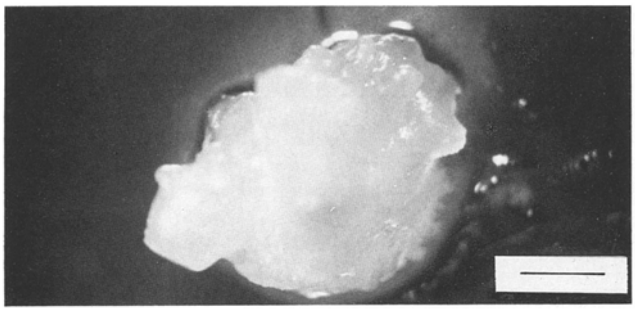

Figure 3. Embryonic callus formed after six weeks on MS basal medium supplemented with 1.2 mg/l 2,4,5-T, 0.8 mg/l BAP, 0.1 mg/l kinetin. Bar = 1.0 mm.

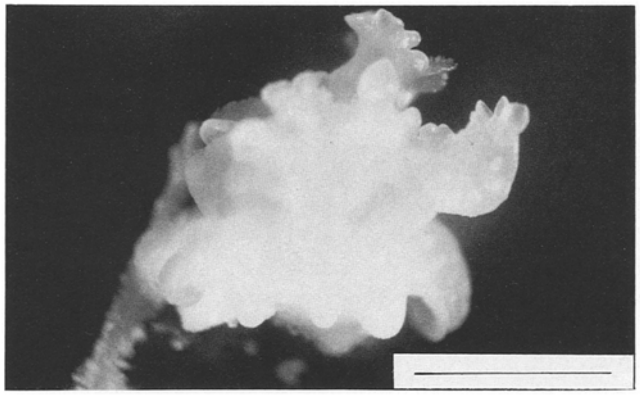

Figure 4. Somatic embryos emerging from callus derived from shoot tips. Bar = 1.0 mm.

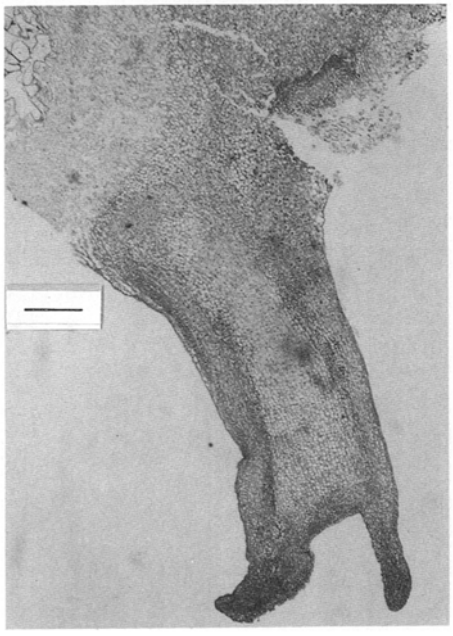

Figure 5. Longitudinal section of a mature embryo with bipolar structure. Bar = 0.1 mm.

Figure 6. Regenerated squash plantlet from a somatic embryo.

Figure 7. Regenerated mature squash plant in an eight inch pot in the greenhouse.

Acknowledgements

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