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Studies on Somatic Embryogenesis and Plant Regeneration of Squash (*Cucurbita pepo* L.)

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Abstract

An efficient and reproducible method for regeneration of squash (*Cucurbita pepo* L.) plants via somatic embryogenesis was developed. The influence of genotype, explant source and growth regulators concentrations on somatic embryogenesis induction was investigated. Embryogenic callus was induced from different organs of two squash genotypes. The embryogenic callus was developed within 13- 20 weeks incubation on MS medium containing different plant growth regulators combinations of auxin and cytokinin. Induction of embryogenesis in different explants ranged from 7 to 100 % depending on the organ and genotype used. Successful acclimatization of squash *in vitro* plants was achieved in the greenhouse and field. Regenerated plants appeared morphologically normal and set flowers and fruits with seeds that could germinated normally.

Keywords: Squash, *Cucurbita pepo* L., genotype, explant source, growth regulators somatic embryogenesis.

Introduction

Squash (*Cucurbita pepo* L.) is one of the important vegetables grown in Egypt (Mady, 2009). It is a member of the family *Cucurbitaceae* that consists of about 118 genera and 825 species, according to the last taxonomic treatment of Jeffery (1990). Cucurbits are among the largest and most diverse plant families that have a large range in plant and fruit characteristics including shape, size, colour, taste, aroma, sugar content, sex expression and parthenocarpy (Bates *et al*., 1990). Various genotypes of *Cucurbita pepo* are called summer squash, winter squash, pumpkin, vegetable marrow, zucchini, or spaghetti squash (Purseglove, 1968; Terrell *et al*., 1986).

Somatic embryogenesis is important to the genetic improvement of plants using biotechnology for the production of micropropagation, mutation breeding, cryopreservation and genetic transformation (Bhojwani and Razadan, 1996; Zavattieri *et al*., 2010; Rakha, *et al*., 2012). The somatic embryo is an independent bipolar structure and is not viscerally attached to the tissue origin (Ammirato, 1987). The developmental switching of somatic embryogenesis involves different gene expression conferring on the somatic cells the ability to manifest the embryogenic potential (Raghavan, 1997; Raghavan, 2000). Somatic embryogenesis thus involves many of molecular events encompassing not only differential gene expression, but also various signal transduction pathway for activating/repressing numerous gene sets, many of which are yet to be identified and characterized (Chugh and Khurana, 2002). The process of somatic embryogenesis is similar to zygotic embryogenesis; it can be divided into a morphogenic phase, characterized by pattern formation, morphogenesis and differentiation of the basic tissue, followed by a maturation phase (Debeaujon and Branchard, 1993; Luo *et al*., 2001).

Production of somatic embryos from cell, tissue and organ cultures may occur either directly or indirectly. The direct occurring somatic embryogenesis involves the formation of an asexual embryo from a single cell or a group of cells on a part of the explant tissue without an intervening callus phase. The indirect embryogenesis consists of establishing an explant in culture, subsequent proliferation of embryogenic callus or suspension, initiation of proembryos and inducing bipolar embryo from pro-embryo initials (Sharp, *et al.,* 1980; Jiménez 2005). The levels of embryo induction and plant regeneration from *in vitro* tissue cultures are basically influenced by genotype and physiological status of donor plant, plant organ used as an explant, the culture medium and the interaction between them (Lazer, *et al*., 1984; Mathias and Simpson, 1986; Bregitzer, 1992; Valdez-Melara, *et al*., 2009).

Several types and combinations of plant growth regulators and explant sources have been tested for induction of somatic embryogenesis from various *Cucurbitaceae* species (Jelaska *et al*., 1985; Chee, 1991; Chee, 1992; Kintzios *et al*., 2002; Valdez-Melara, *et al*., 2009). Auxins are absolutely required for the induction of somatic embryogenesis from different *Cucurbitaceae* species (Debeaujon and Branchard, 1993; Mihaljević *et al*., 2011)). In Many studies, somatic embryogenesis was obtained with a high concentration of an auxin source often in combination with other auxins or cytokinins (Jelaska *et al*., 1985; Juretic and Jelaska, 1991; Tabei *et al*., 1991; Kintzios *et al*., 2002; Valdez-Melara, *et al*., 2009). Maturation of squash somatic embryo was commonly conducted on medium without growth regulators (Kintzios *et al*., 2002).

Despite recent progress, still there is no general regeneration protocol available, which is robust

enough to be applied to different squash genotypes. Thus somatic embryogenesis is the best alternative or option to utilize biotechnology in propagation and breeding. The ultimate goal of this search is finding out new possibilities to improve the conditions for genetic engineering of squash, and identifying suitable squash genotypes or explant tissues susceptible to such modifications through seting up an *in vitro* regeneration procedure of somatic embryogenesis, as well as for the acclimatization of squash (*Cucurbita pepo* L.).

Materials and Methods

Plant material and explant preparation

Mature seeds of two summer squash genotypes were used. Seeds of the local genotype Eskandarani and Squash-white Bush Marrow were obtained from the Preservation Germplasm Laboratory of the Dpartment of Horticulture, Faculty of Agriculture, Benha University.

Seeds were surface sterilized according to method of Chee (1992). After removal of the seed coat, de-coated seeds were washed in 70% ethanol for one minute and rinsed three times for five minutes each with sterile water followed by 4.5% Sodium hypochlorite supplemented with eight drops of tween-20 for 20 minutes. Finally, seeds were washed three times with sterile distilled water. Sterilized seeds were cut transversely into two unequal sections; one section consisted of the embryonic axis and one-third of the cotyledon and the remaining section contain two-thirds of cotyledons (Figure 1a). Explants including the embryonic axis and one-third of the cotyledon were cultured facing up and the explants containing only the cotyledons were cultured horizontally. The two sections were cultured together in perty dishes on MS medium (Murashige and Skoog, 1962) with 30 g/l sucrose and 8 g/l agar. The MS media were adjusted to 5.8 pH prior autoclaving at 121°C for 21 minutes. Explants were cultured with 16 h light photoperiod at 25°C.

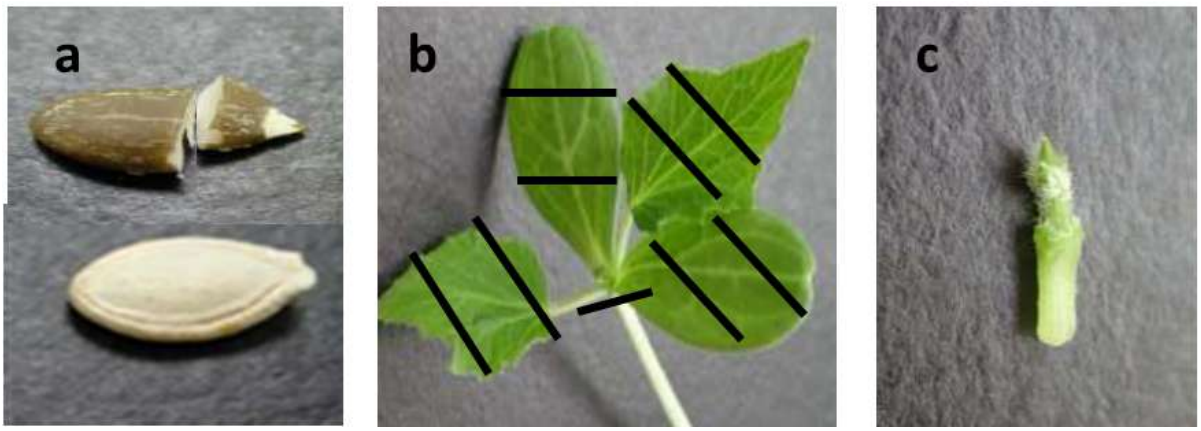

Figure 1. Preparation of explants; a) mature seed, b) cotyledons and leaves, c) shoot tips.

Induction of somatic embryogensis and plant regeneration

Cotyledons and young leaves were removed, divided into 1-cm pieces (Figure 1b), and cultured horizontally on the MS medium. Shoot apices consisting of the apical domes and some supporting tissues (Figure 1c) were cut into longitudinal halves and cultured horizontally on the cut side on MS induction medium. Cultures were maintained in the dark at 25°C. Explants induction media for all experiments contained 8 g/l agar and 30 g/l sucrose. Explants were sub-cultured every 4 weeks. The MS media were adjusted to 5.8 pH prior autoclaving at 121°C for 25 minutes. All the previous explants were incubated on MS medium containing different plant growth regulators as the following: 2, 5, or 10 mg/l 2, 4- D as auxin alone or in combination with 1.5 or 3 mg/l kinetin as cytokinin. Explants were sub-cultured every 4 weeks on a fresh medium.

Somatic embryos induced on hormone medium were transferred into propagation medium contained 5 mg/l of 2, 4-D and 3 mg/l of kinetin for eight weeks then they were transferred into regeneration medium MS hormone free for 2 weeks and subcultured on MS medium supplemented with activated charcoal at 5 g/l for 4 weeks. Afterwards, embryos were subcultured again on fresh medium and moved to light/dark growth chamber 16/8 h for another 4 weeks. The experiment was carried out with four plates each contains four callus clusters; each callus cluster weight was about 500 mg.

Acclimatization and field transfer

Regenrated shoots (approximately 3 cm long) with well developed leaves and roots were transferred to pots containing peat moss, covered with plastic bags with high relative humidity and maintained in the growth room under 12 h light photoperiod at 25ºC. Plantlets were watered twice a week with water. Then, after two week, plantlets were transferred into greenhouse, then to the field conditions.

Experimental design and Statistical analysis

Experiments were arranged in a completely randomized design with four replications. Data were estimated as the percentage of the different traits. The Calculations were done using Microsoft Excel 2010 program.

Results

Induction of somatic embryogenesis in squash

The current study was carried out on two squash genotypes to obtain embryogenic callus which are a good source for *Agrobacterium* transformation. Consequently, it enables us to introduce virus resistance to squash plant. Different plant growth regulators concentrations were examined using four explant types.

Development of somatic embryogenesis and plant regeneration

Mature seed, cotyledon, leaf and shoot tip explants were enlarged on the induction medium at day 7-10, the green colour of the cotyledon and leaf explants became light green or light brown after 4 weeks, and green colour disappeared within 8 to 10 weeks. All explants became brownish after 8 weeks. Formation of somatic embryos were observed on mature seed explants derived from genotype Squash-white Bush Marrow after 17 weeks, and for genotype Eskandarani after 20 weeks. Somatic embryos were observed on the shoot tip explants derived from Squash-white Bush Marrow after 15 weeks and after 13 weeks for Eskandarani. Leaf and cotyledon explants of Eskandarani produced somatic embryos after 13 and 18 weeks, respectively (Figure 2). Somatic embryos in globular stage were well matured in dark on plant growth regulator free medium containing activated charcoal after four weeks. Somatic embryos converted into plantlets within two to three weeks under light/dark condition (16/8 h) and developed into entire plants within three to four weeks for Squash-white Bush Marrow and Eskandarani, respectively. Seventy embryos were accounted from embryogenic callus (500 mg) derived from Squash-white Bush Marrow; 50 embryos were normally developed into entire plants and 20 embryos showed abnormal development. From Eskandarani, 60 embryos were accounted from one embryogenic callus (500 mg), 30 embryos developed normally into whole plants and 30 embryos showed abnormal developments. All regenerated plants were successful acclimated and developed into adult plants with normal flowers and fruits (Figure 2).

Figure 2. Development of somatic embryos and plant regeneration from *Cucurbita pepo* cv. Eskandarani. (a) Formation of somatic embryos on cotyledons explants cultured on MS medium supplemented with 5 mg /l 2,4-D. (b) Germination of somatic embryos after exposing to light/dark 16/8 h for two weeks. (c) Regenerated squash plantlets from somatic embryos after 3 weeks of culture on basal MS medium. (d) Acclimatization of regenerated squash plantlet using peat moss. (e) Normal regenrated squash plants in the field.

Effect of different growth regulator regimes

In mature seed explants derived from Eskandarani, different 2,4-D concentrations were tested alone or in combination with kinetin. Results in Table 1 indicated that 5 mg/l 2,4-D was the best concentration giving the highest percentage of embryogenesis (54 %). No effect was observed when kinetin was combined with 5 mg/l 2,4-D. Reducing 2,4-D concentration to 2 mg/l in the presence or in the absence of kinetin resulted in decreasing of embryogenesis induction. No embryogenic calli were observed with the highest 2,4-D concentration (10 mg/l) in the presence or in the absence of kinetin. In regard to seed explants derived from Squash-white Bush Marrow (Table 2), the highest percentage of embryogenesis was obtained with a combination of 5 mg/l 2,4-D and 3 mg/l kinetin. Reducing 2,4-D concentration in the presence or in the absence of kinetin resulted in low percentage of embryogenesis.

In shoot tip explants derived from Eskandarani (Table 1), it was found that the highest percentage of embryogenesis (100 %) was obtained with 2 mg/l 2,4-D. Adding of kinetin in combination with 2 mg/l or 10 mg/l 2,4-D led to decreasing the percentage of embryogenesis induction. Hundred percent of embryogenesis was also obtained with 2 mg/l of 2,4-D from shoot tip explants derived from Squash-white Bush Marrow (Table 2).

In cotyledon explants derived from Eskandarani (Table 1), the increase of 2, 4-D concentration from 5 to 10 mg/l did not effect the embryogenesis induction. Thirty four percent embryogenesis was

observed in both mentioned concentrations in the cotyledon explants. 5 mg/l 2,4-D was the best concentration giving the highest percentage of embryogenesis (30 %) of cotyledon explants derived from Squash-white Bush Marrow (Table 2).

Effect of 2 mg/l 2,4-D was almost similar to the effect of 10 mg /l 2,4-D in leaf explants derived from cv. Eskandarani; 47 and 50 %, respectively (Table 1). The highest percentage (35%) was also obtained with 2 mg/l of 2,4-D from leaf explants derived from Squash-white Bush Marrow (Table 2).

Table 1. Effect of plant growth regulators on somatic embryo induction in different types of explants of squash genotype Eskandarani.

Growth regulator (mg/l)		Mature seed			Shoot tip			Cotyledon			Leaf		
2,4-D	Kinetin	No. of explants	No. of embryogenic callus	%	No. of explants	No. of embryogenic callus	%	No. of explants	No. of embryogenic callus	%	No. of explants	No. of embryogenic callus	%
2	0	50	20	40	50	50	100	75	15	20	60	28	47
5	0	50	27	54	30	15	50	60	20	34	30	12	40
10	0	50	0	0	20	10	50	60	20	34	40	20	50
2	1.5	30	2	7	20	2	10	30	3	10	40	10	25
5	1.5	30	14	47	20	5	25	30	3	10	40	12	30
10	1.5	30	0	0	20	10	50	30	4	14	30	5	17
2	3	20	0	0	15	0	0	30	2	7	30	0	0
5	3	20	4	20	20	1	5	40	2	5	30	2	7
10	3	20	0	0	15	1	7	30	3	10	30	3	10

Table 2. Effect of plant growth regulators on somatic embryo induction in different types of explants of genotype Squash-white Bush Marrow.

Growth regulator (mg/l)		Mature seed			Shoot tip			Cotyledon			Leaf		
2,4-D	Kinetin	No. of explants	No. of embryogenic callus	%	No. of explants	No. of embryogenic callus	%	No. of explants	No. of embryogenic callus	%	No. of explants	No. of embryogenic callus	%
2	0	20	2	10	15	15	100	20	3	15	20	7	35
5	0	15	3	20	15	6	40	20	6	30	15	4	27
10	0	10	0	0	10	3	30	20	5	25	15	5	33
2	1.5	15	1	7	15	3	20	20	2	10	20	3	15
5	1.5	15	4	27	10	1	10	20	3	15	20	4	20
10	1.5	15	0	0	10	2	20	20	4	20	15	2	13
2	3	10	0	0	10	0	0	20	1	5	15	0	0
5	3	20	8	40	15	4	27	20	1	5	20	2	10
10	3	10	3	30	10	3	10	15	1	7	15	2	13

Discussion

The first report on somatic embryogenesis was published by Steward *et al.* (1958). Regeneration of plants *in vitro* via somatic embryogenesis has some distinct features such as single-cell origin, the consequent low frequency of chimeras and the production of high number of regenerates (Ammirato, 1983; Sato *et al*., 1993). For these reasons, we decided to choose somatic embryogenesis as a regeneration method in the current study.

Using explants derived from mature seeds, cotyledons, shoot tips and leaves of three summer squash genotypes, efficient plant regeneration via somatic embryogenesis was established. The length of induction period of somatic embryogenesis ranged from 13 to 20 weeks depending on the explant type and genotype used. Formation of somatic embryos was noted on leaf explants 13 weeks, on mature seed explants 17 to 20 weeks and on cotyledon explants 18 weeks after starting of the induction. In a study by Kintzios *et al*. (2002), somatic embryogenesis was observed 4 weeks after initiation of induction from leaf explant derived from squash and melon. However, it has taken only 9 weeks by a method described by Chee (1992) who used mature seeds as a source of explants in squash (*Cucurbita pepo* L. cv.YC60), whereas in another study on Styrin pumpkin by the method of Urbaek *et al.* (2004) it lasts 20 weeks. These results may be referred to the different genotypes and explants used.

For plant regeneration, only three reports have been published on regeneration of summer squash through somatic embryogenesis (Chee, 1991, Chee, 1992; Gonsalves *et al*., 1995). In this study, 120 and 140 embryos were converted into plantlets per gram of embryogenic callus from Eskandarani and Squash-white Bush Marrow genotypes, respectively. About 50 % and 72 % of the converted embryos were normal looking plants from Eskandarani and Squash-white Bush Marrow genotypes, respectively. The mentioned (72 %) normal looking plants are nearly similar to results obtained by Juretic and Jelaska (1991) who obtained 77 % normal plants from pumpkin and the 50 % are in agreement with results obtained by Chee (1992) in squash.

Phenotypic abnormality of embryos was observed on cucumber (Ziv and Gadasi, 1986) on pumpkin (Juretic and Jelaska, 1991), and on squash (Chee, 1992). These abnormalities may refer to somaclonal variation occurred in tissue culture in response to the effect of components of culture media. Somaclonal variation events generated through tissue culture may induce unwanted mutations (Venkatachalam *et al*., 2000). On the other hand, somaclonal variation can produce desirable agronomic characteristics such as increases in salt tolerance, resistance to herbicides, diseases, extreme temperatures, desiccation, or can yield interesting biochemical (Maluszynsky *et al*., 1995). For example, cell selection and regeneration via somatic embryogenesis have been used to improve salt tolerance and disease resistance in Citrus (Litz *et al*., 1985), virus resistance in sugarcane (Oropeza and de Gracia, 1996), germination at low temperature in melon (Ezura *et al*., 1995) and resistance to phytotoxins in coffee (Nyange *et al*., 1995).

The presented results showed that the highest number of embryos obtained from various explant types and different genotypes were induced on medium supplemented with 2,4-D alone. These results indicated that stress induction through high auxin concentration, especially 2,4-D, is required for induction of somatic embryogenesis in squash. The effect of auxin on induction of somatic embryogenesis was investigated in several cucurbitaceous species. Previous investigations have demonstrated that the induction of somatic embryogenesis among cucurbitaceous species can be controlled by the ratio of auxin and cytokinin in the culture medium. Oridate and Oosawa (1986) found that 0.26 μM BA was the most suitable concentration for inducing embryo formation in melon. The induction of somatic embryogenesis in the same species was favored at high levels (105-525 μM) of indole-3- acetic acid (IAA) but at relatively low concentration of 2,4-D (4.5-9 μM) (Tabei *et al.,* 1991). Using 2,4-D alone or in combination with other auxins is usually required for the induction of somatic embryogenesis (Debeaujon and Branchard 1993; Nadolska-Orczyk and Malepszy, 1987; Rajasekaran *et al*., 1983; Tabei *et al*., 1991). Somatic embryogenesis was achieved in squash on medium contained 2,4-D alone (Chee, 1992; Gonsalves *et al*., 1995; Valdez-Melara, *et al*., 2009, Rakha, *et al*., 2012) or in combination with kinetin (Kintzios *et al*., 2002). Somatic embryogenesis in pumpkin can be induced on auxin-containing medium and also on hormone-free medium containing 1mM ammonium (NH_4^+) as the sole source of nitrogen (Mihaljević *et al*., 2011).

The maturation of somatic embryos was commonly conducted on media without growth regulators and in some cases by the addition of activated charcoal. Its addition to culture medium may promote or inhibit *in vitro* growth depending on the species and tissue used. The effect of activated charcoal may attributed to establishing a darkened environment, adsorption of undesirable/inhibitory substances, adsorption of growth regulators and other organic compounds or the release of growth promoting substances present in or adsorbed by activated charcoal (Pan and Staden, 1998). In cucumber, differentiation of embryos was enhanced by washing the suspension culture cells with MS medium containing 0.5 % activated charcoal; 60 to 70 % of the embryos pre-washed with activated charcoal germinated into plantlets with normal morphology (Chee and Tricoli, 1988).

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دراسات على الأجنة الجسمية و اعادة تجديد النمو فى الكوسة

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قسم البساتين، كلية الزراعة، جامعة بنها، جمهورية مصر العربية

الملخص العربى

تم تجديد النمو فى الكوسة بطريقة كفء من خلال تكوين الأجنة الجسمية و قد تم دراسة تأثير الصنف والجزء النباتى ومنظمات النمو على اعادة تجديد النمو و ذلك من خلال تكوين الأجنة الجسمية. لقد تم انتاج الكالوس المحتوى على أجنة جسمية من صنفين من الكوسة فى خلال 13–20 أسبوع باستخدام بيئة موراشيج و سكوج المضاف اليها تركيزات مختلفة من منظمات النمو (الأوكسينات و السيتوكينينات). تم الحصول على أجنة جسمية من الأجزاء النباتية المختلفة بمعدل 7–100% تبعا للجزء النباتى و الصنف المستخدم. تم اجراء عملية الأقلمة لنباتات الكوسة الناتجة معمليا بنجاح فى المعمل والصوبة ثم تم نقلها الى الحقل المستديم. النباتات الناتجة كانت طبيعية من الناحية الظاهرية حيث عقدت الأزهار و تكونت الثمار المحتوية على البذور و التى يمكن أن تنبت طبيعيا.