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Indirect Organogenesis in Summer Squash (*Cucurbita pepo* L.)

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Abstract: Efficient plant regeneration via organogenesis was established for 2 summer squash (*Cucurbita pepo* L.) cultivars, viz. Bulum and Rumbo, using hypocotyl and cotyledon derived calli. Seeds were surface sterilized in 0.1% $HgCl_2$ for 5 min, and germinated in vitro in plant growth regulator free MS media. The maximum morphogenic callus induction rate (86%) was observed from a hypocotyl explant by culturing in MS medium supplemented with 2.5 mg l^{-1} 2,4-D. Calli size and fresh weight increased substantially through subculturing. The highest percentage of shoot regeneration (85%) and highest mean number of shoots (6.89) per culture were obtained with 0.5 mg l^{-1} thidiazuron. Initiation of multiple shoots through organogenesis from the calli was histologically proven. Hypocotyl explants were more responsive than cotyledon explants in terms of callus induction and subsequent plant regeneration. Regenerated shoots were rooted in MS medium supplemented with 1.0 mg l^{-1} IBA. About 70% of regenerated plantlets survived and showed new branch development under ex vitro conditions.

Key Words: *Cucurbita pepo*, in vitro, organogenesis, summer squash, thidiazuron

Abbreviations: 2,4-D: 2,4-Dichlorophenoxy acetic acid · BAP: 6-Benzylaminopurine · GA_3: Gibberellic acid ·IBA: Indole-3-butyric acid · NAA: Naphthalene acetic acid · TDZ: Thidiazuron

Introduction

Cucurbita pepo L. includes a diverse range of vegetable crops including pumpkins, winter squash and various kinds of summer squash (Paris, 1989). In 2005, 21.47 million US tons of squash production was noted worldwide (FAOSTAT, 2006). China contributes about 30% of the world's production, making it the leading producer. Considering its tremendous importance in the world's vegetable basket, both conventional breeding and transgenic approaches (Fuchs and Gonsalves, 1995; Tricoli et al., 1995; Paris and Cohen, 2000; Fuchs et al., 2004) have been used in summer squash in order to confer disease resistance. Despite these advances and considering its economic importance, effort should be made to utilize biotechnology for further improvement of this valuable crop.

Establishment of transgenic plants can be useful in basic research or in studies on applied purposes to improve the nutritional value or characteristics of plants to confront biotic or abiotic stress (Arnholdt-Schmitt, 1996). In any case, crucial for the application of any transformation technology is the development of regeneration systems. However, the key technology for regeneration that starts from tissues or callus cultures, i.e. the induction of organogenesis, is still insufficient in summer squash. Application of in vitro regeneration techniques in cucurbits has been reported from cotyledonary nodes (Gambley and Dodd, 1991), leaf (Mishra and Bhatnagar, 1995), leaf and cotyledon (Stripp et al., 2001), anther (Kumar et al., 2003), and cotyledon (Kathiravan et al., 2006). Somatic embryogenesis is achieved in several cucurbits (Chee, 1991; Kintzios and Taravira, 1997; Leljak-Levanic et al., 2004; Urbanek et al., 2004). On the other hand, the organogenic route of regeneration from callus culture is also reported in *C. pepo* (Jelaska et al., 1985). Organogenic pathway bypassing calli have also been reported from seedling-derived cotyledon (Rakoczy-Trojanowska and Malepszy,

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1989; Ananthakrishnan et al., 2003; da Silva et al., 2006) and internode segments (Rahman et al., 1993).

Because of genotypic dependence a limited range of transformable genotypes would necessitate much backcrossing to introgress a transgene into different genotypes of *C. pepo*. Therefore, it is necessary to develop an effective regeneration protocol by a range of different techniques to widen the possibilities for the development of transgenic lines or somaclonal variants of different cultivars. We developed a reproducible organogenic regeneration protocol for 2 commercial summer squash cultivars (*C. pepo*) from cotyledon and hypocotyl via indirect organogenesis.

Materials and Methods

Plant materials

Two summer squash cultivars of Bangladesh, viz. Rumbo and Bulum (Seminis Inc., Korea), were used in the experiments. Seeds were collected from a local market. After soaking in water for 10 min, the pilled seeds were treated in 70% ethanol for 1 min followed by 5-min immersion in 0.1% $HgCl_2$ solution containing 2-3 drops of Tween-20 per 100 ml with gentle shaking. Sterilized seeds were rinsed 4-5 times with sterile distilled water and then allowed to dry in a running laminar air flow cabinet. After that they were placed on MS medium (Murashige and Skoog, 1962) supplemented with 3% sucrose, vitamins and 8.0 g l^{-1} agar for germination in a growth chamber maintained at 25 ± 1 °C under a 16/8-h (light/dark) photoperiod with a light intensity of 28-30 mol m^{-2} s^{-1} (supplied by cool-white fluorescent lamps, Philips). Similar cultural conditions were maintained in the subsequent experiments.

Callus induction, plant regeneration and rooting

Collected cotyledons and hypocotyls from germinating seeds (8-9 days old) were cut into pieces and were placed on MS medium supplemented with different concentrations of 2,4-D for callus induction. Induced calli were subcultured into fresh media after a 21-28-day interval for developing an organogenic nature. Watery, spongy, very compact, brown and dead portions of calli were discarded during every subculture. Friable, nodular calli were assumed potentially organogenic and were selected for maintenance and regeneration. For adventitious shoot induction, calli were cultured in MS medium supplemented with various concentrations of TDZ and BAP in combination with GA_3 or NAA (Table 1). When regenerated shoots attained a height of 1.0-1.3 cm they were excised and transferred to MS medium fortified with different concentrations of IBA for root induction.

Table 1. Effect of different types and concentrations of plant growth regulators in MS media on organogenic regeneration from calli. Data were recorded after 7 weeks of subculturing in regeneration medium. Experiments were carried out 3 times.

Growth regulator (mg l^{-1})	Regeneration frequency (%)		Shoots/Callus ($\bar{x}$ ± SE)	
	Bulum	Rumbo	Bulum	Rumbo
TDZ				
0.1	50.33	48.70	2.30 ± 0.32	1.46 ± 0.29
0.5	85.36	72.65	6.89 ± 0.25	4.75 ± 0.42
1.0	68.20	59.42	4.26 ± 0.15	3.10 ± 0.16
2.0	46.50	39.30	2.36 ± 0.45	1.70 ± 0.33
BAP + GA_3				
1.0 + 0.1	70.33	62.46	4.56 ± 1.05	3.20 ± 0.18
1.0 + 0.5	58.91	50.20	2.10 ± 0.32	1.82 ± 0.45
2.0 + 0.1	64.25	54.46	2.56 ± 0.60	2.15 ± 0.20
2.0 + 0.5	43.33	38.55	1.60 ± 0.35	1.20 ± 0.41
BAP + NAA				
1.0 + 0.1	65.30	56.70	3.10 ± 0.50	2.25 ± 0.35
1.0 + 0.5	44.56	35.20	1.58 ± 0.22	1.10 ± 0.08
2.0 + 0.1	50.45	43.33	2.70 ± 0.35	2.11 ± 0.30
2.0 + 0.5	38.21	30.40	1.45 ± 0.17	1.30 ± 0.28

As the carbon source 3% sucrose was used in all media. After adjusting the pH to 5.7 ± 0.01 prior to gelling with 0.8% agar (w/v) (BHD, UK), the medium was sterilized by autoclaving at 121 °C for 20 min (1.06 kg cm^{-2}). Sterilized medium was poured into either 25 × 150 mm test tubes (12 ml of medium) or a 250-ml flask (40 ml of medium) for use.

Histological study of callus

Histological study of organogenic calli was undertaken to determine the nature of shoot formation on sections obtained with a microtome (Esau, 1965). Putative organogenic calli were fixed in FAA (5% formalin, 5% acetic acid, 45% alcohol) solution following Johansen (1940). Dehydration of the fixed materials was accomplished by placing them in a grade series of ethyl alcohol (50%, 60%, 70%, 80%, 90% and 100%) and absolute alcohol with chloroform (2:1; 1:1 and 1:2). Then the dehydrated materials were embedded in paraffin blocks and sections were prepared serially at 12-µm thickness using a rotatory microtome (The Cambridge Rocker). After staining with safranin-orange-G and tannic acid, the sections were mounted on glass slides using Canada balsam. Microphotographs were taken at × 100 magnification.

Acclimatization and Transfer to Soil

Regenerated plantlets with a well-developed root system were washed carefully to remove agar and then transferred to pots containing sterile vermiculite. Each pot was enclosed in a polyethylene bag after watering, and maintained in a growth chamber at 25 ± 1 °C under 16-h illumination (45 mol m^{-2} s^{-1}) with fluorescent lamps. Bags were progressively opened weekly. After 3 weeks of acclimatization, plantlets were transferred to large pots for further growth.

Data Recording

To test the efficiency of callus induction medium, callus induction rates were calculated. For the determination of callus growth in terms of mean weight, only actively growing calli having organogenic potential were selected, with watery, spongy, very compact, brown or dead calli being discarded. Plant regeneration efficiency was considered in terms of regeneration frequency and number of shoots per callus. The same parameters were also used for calculating rooting frequency. Each experiment contained at least 15 replications and the whole experiment was repeated 3 times.

Results and Discussion

Callus was induced on medium containing 2,4-D from both explants, but not on 2,4-D-free medium. The highest frequency of callus induction was observed in MS medium containing 2.5 mg l^{-1} 2,4-D from both the explants in both cultivars (Figure 1) and hypocotyl was more responsive in cv. Bulum than in Rumbo. Levels of 2,4-D above or below this gradually decreased the frequency of callus induction. Both the color and texture of the callus derived from the 2 explants varied. Calli derived from hypocotyls were mostly friable and creamy in color (Figure 2A), with very few brownish exceptions. Cotyledon derived calli were mostly watery and pale brown and had less potential for further organogenesis. Calli size and fresh weight increased substantially through subculturing. The fresh weight of effective calli induced from hypocotyl increased from 0.236 to 2.29 g after 21 weeks of culturing (3-4-week interval) in cv. Bulum (Figure 3). Growth of most of the calli was found to plateau after the fifth subculture. The predominance of Bulum over Rumbo proved genotypic variation for this trait. It was further observed that the presence of light affected callus induction and proliferation.

2,4-D is among the most widely used auxins for in vitro callus induction in a wide range of plant species. In our study, successful induction of potentially organogenic callus from hypocotyl and cotyledons was achieved using 2,4-D. Similar results were reported previously in this species (Leljak-Levanic et al., 2004). Moreover, Srivastava et al. (1989) obtained organogenic calli using a combination of BAP and NAA. Callus morphology was also in agreement with Thomas and Sreejesh (2004) as in *B. hispida*, and with Branchard and Chateau (1988) in melon calli, as we obtained. We showed that friable and creamy calli derived from hypocotyl can be used to initiate organogenic calli and shoot-bud induction with subsequent regeneration of *C. pepo* plant. Calli induced from hypocotyl explants were larger in size than those from the cotyledon, and when transferred to a basal medium at low levels of PGRs (in particular TDZ) promoted only shoot organogenesis.

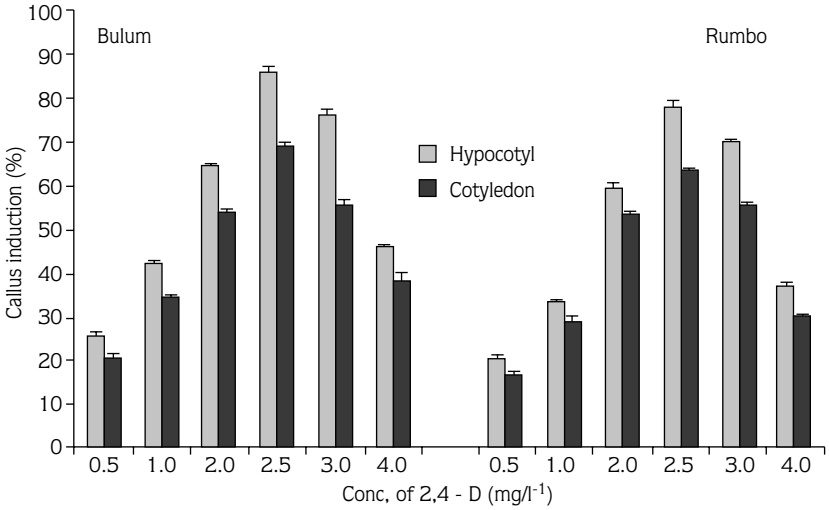

Figure 1. Effect of different concentrations of 2,4-D on callus induction in cv. Bulum and cv. Rumbo. Data were recorded 4 weeks after inoculation. Vertical lines indicate standard error.

After 3-4 weeks of culture on regeneration medium, 2 morphologically different types of calli were observed: organogenic and non-differentiated. Organogenesis was predominantly observed on calli previously induced from hypocotyls. In this case, green spots (Figure 2B) and development of several shoots were observed gradually from a single callus mass (Figure 2E). Generally, shoot initiation was observed 5 weeks after inoculation on regeneration medium. The shoot-like structures could be distinguished by the presence of green, opaque, and compact nodules. Distinct shoot tip-like structures were observed as shown in Figure 2C, indicating a monopolar structure characteristic of shoots. The histological studies carried out with the regenerating callus at different stages of growth revealed the indirect organogenic regeneration pathway. Anatomical sections of regenerating callus showed the emergence of shoot primordia or meristemoids having vascular connection with the callus (Figure 2D). Such structures have been described for organogenesis in watermelon (Yalcin-Mendi et al., 2003; Krug et al., 2005).

The optimum response in terms of regeneration frequency and the number of shoots per callus was recorded in MS medium supplemented with 0.5 mg l^{-1} TDZ. In this medium, 85.36% and 72.65% of calli induced multiple shoots with an average of 6.89 and 4.75 shoots per callus in cvs. Bulum and Rumbo, respectively (Table 1). The regenerated shoots attained a height of about 2 cm in 8 weeks of culture. Our results indicated that TDZ at low concentrations (0.5 mg l^{-1}) effectively increased shoot differentiation and was more effective than BAP in combination with GA_3 or NAA. The use of high doses of TDZ (≥1.0 mg l^{-1}) had negative effects on organogenesis (Table 1). Furthermore, TDZ had a marked effect on the quality of regenerated plants, especially on those from hypocotyls; TDZ reduced shoot length and leaves were greener than those produced in other treatments.

Higher TDZ levels lead to the development of undifferentiated hard green callus instead. TDZ is thought to be involved in the regulation of purine cytokinin metabolism and may act directly as a cytokinin or in combination with cytokinins. It has also been suggested that the biological activity of TDZ is higher than or comparable to that of the most active adenine type of cytokinins (Mok et al., 2000; Khawar et al., 2004). A significant advancement in the development of protocols for induction of somatic embryogenesis was the discovery of the efficiency of the plant growth regulator TDZ for induction of regeneration in a wide range of species. The substitution of TDZ for the BAP-NAA requirement for organogenesis demonstrated that TDZ may possess an auxin-like property or may impinge upon the endogenous auxins by modifying their biosynthesis or metabolism. Exposing explants to either TDZ or the combination of auxin and cytokinin, it was concluded that TDZ stimulated

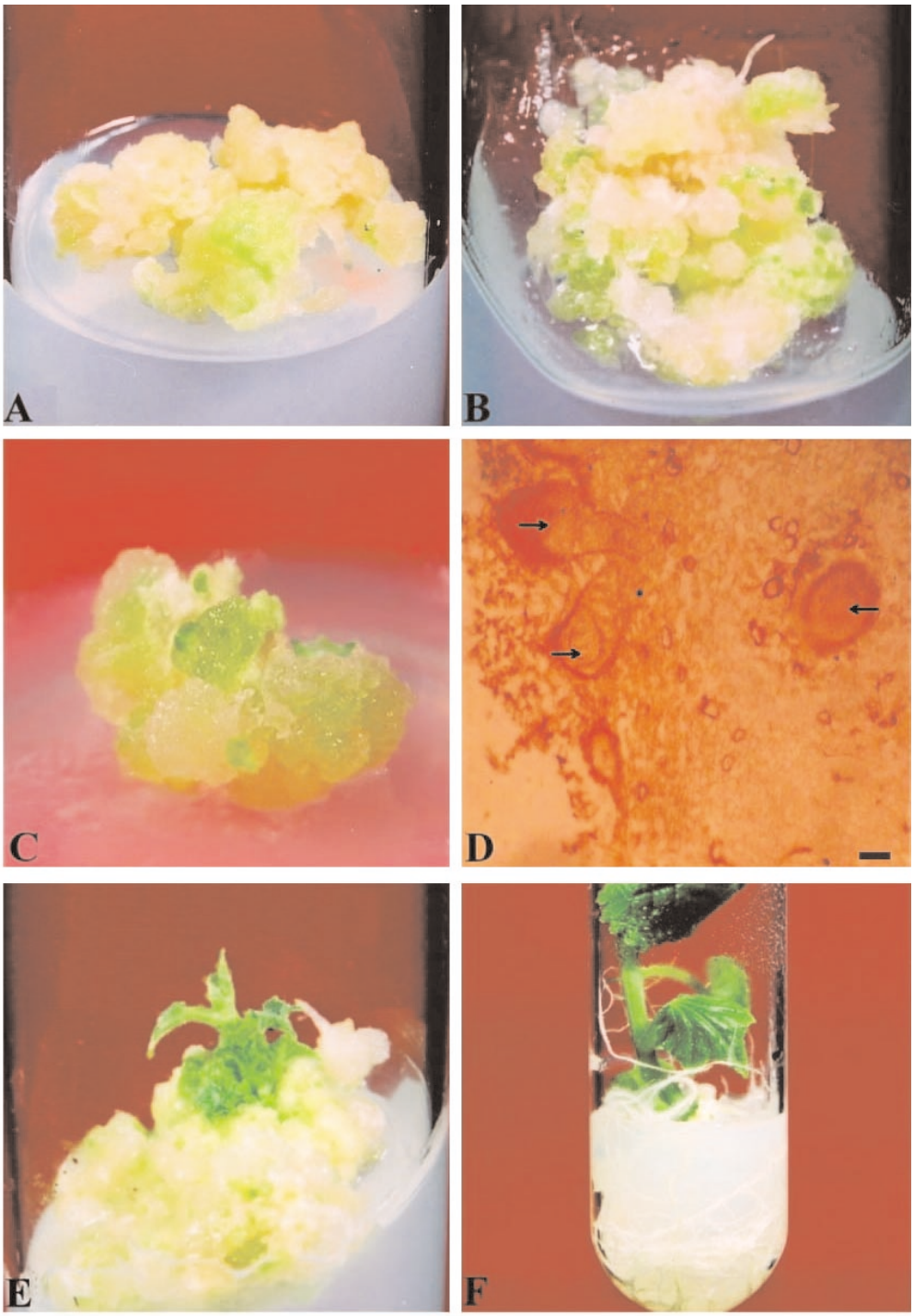

Figure 2. Different stages of shoot differentiation from callus via organogenesis in *Cucurbita pepo*
A: Proliferating callus induced from hypocotyl explant.
B: Organogenic calli showing green points.
C: Primary shoot primodium emerged from organogenic mass.
D: Histological section of regenerating callus showing initial development of shoot buds (indicated by arrows). Bars = 100 µm.
E: Development of plantlets from callus.
F: Regenerated plantlets with well-developed roots.

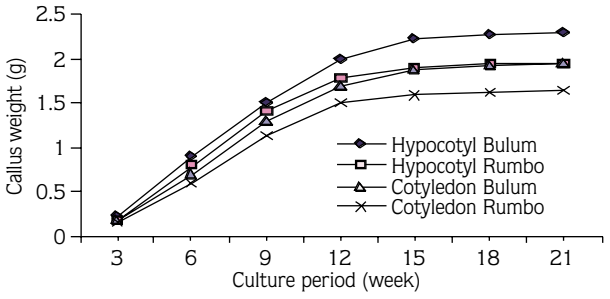

Figure 3. Effect of culture period on effective callus growth in cv. Bulum and cv. Rumbo.

an accumulation of endogenous auxins and cytokinins in the explants. This increases the number of meristematic centers, leading to the formation of significantly more somatic embryos than with auxin-cytokinin treatments (Hutchinson and Saxena, 1996), although the superiority of BAP over TDZ was reported for this family (Ficcadenti and Rotino, 1995).

Shoot initiation was not observed when calli were transferred to MS medium supplemented with different levels of BAP; instead only green spots were visible even after 12 weeks of culturing for regeneration. On the other hand, BAP along with a low concentration of GA_3 or NAA initiates shoot differentiation. Among various combinations, 1.0 mg l^{-1} BAP with 0.1 mg l^{-1} GA_3 was most effective (Table 1). Moreover, 1.0 mg l^{-1} BAP plus 0.1 mg l^{-1} NAA was also impressive. These plantlets continued to develop and root formation was seen after transfer to rooting medium.

In vitro induction of organogenesis depends on the endogenous concentration of plant growth regulator, their distribution in the cultured tissue and interaction with exogenously supplied growth regulator. Good (70%) shoot organogenesis was observed using BAP in combination with GA_3 from hypocotyl derived calli. A similar effect of this combination was noted previously by Ananthakrishnan et al. (2003) in *C. pepo*. It is suggested that GA_3 along with a cytokinin stimulates the development of shoot primordia induced by 2,4-D (Molvig and Rose, 1994). Adding GA_3 to BAP containing media at any level has been reported to improve the frequency of somatic embryogenesis (Li et al., 2002).

GA_3 stimulates the production of numerous enzymes, notably α-amylase, in germinating cereal grains (Davies, 1995). In addition, GAs (gibberellic acids) promote seed germination in some species that otherwise require cold stratification and/or light for inducing seed germination (Davies, 1995). Experimental results suggest that the Gibberellin Responsive Gene (*HvGR*) (localized to pockets of subepidermal cells from where shoot primordia originate) is responsible for GA induced shoot induction. *HvGR* showed the highest level of expression in the regeneration stage of the initiated shoot where the signal was localized primarily in the developing shoot primordia (Seong et al., 2004).

No substantial difference in regeneration rates was found when calli were taken from the first 4 subcultures. It was observed that from the fifth subculture and onwards regeneration rates showed marked differences (data not shown). Although the callus maintains its regeneration capacity for a longer period, prolonged subculturing may lead to a higher frequency of mutants, especially in higher concentrations of 2,4-D. The incubation period of callus induced on hypocotyl appears to be critical for induction of organogenesis. Most of the transformation procedures have relied on inducing long-term morphogenic potential after repeated subculturing, and this is critical for successful transformation. With this goal in mind, the selected callus (having regeneration potential) was multiplied on 2.5 mg l^{-1} 2,4-D and maintained a high regeneration rate from 1-6 subcultures on this medium. Longer incubation periods (9 weeks) reduced shoot organogenesis, whereas BAP+GA_3 was able to regenerate shoots from older callus compared with TDZ induced organogenesis. An adverse effect of prolonged in vitro culture is reported (Jureti and Jelaska, 1991; Choi et al., 2001).

Roots were observed as early as 2 weeks after placing the microshoots (2-3 cm) on rooting medium. Most of the shoots had developed roots by week 4 (Figure 2F). Overall, Bulum had a better rooting response (88%) than Rumbo (80%). The highest number of roots was induced in MS supplemented with 1.0 mg l^{-1} IBA (Table 2). Shoots exposed to higher concentrations of IBA (2.0 mg l^{-1} or more) became necrotic and lost leaves and the shoot tips died gradually.

IBA is a widely used plant growth regulator for root induction in cucurbits (Sarowar et al., 2003; Thomas and Sreejesh, 2004; Krug et al., 2005), while NAA is also used (Kathiravan et al., 2006). In some cases, rooting in PGR free medium during organogenesis has been reported in *C. pepo* (Ananthakrishnan et al., 2003) and

Table 2. Effect of different types and concentrations of plant growth regulators in MS media on root induction. Data were recorded 4 weeks after transfer in rooting media.

Growth regulator (mg l^{-1})	Rooting frequency (%)		Root/shoot ($\bar{x}$ ± SE)	
	Bulum	Rumbo	Bulum	Rumbo
IBA				
0.5	59.33	51.36	6.50 ± 0.30	5.15 ± 0.47
1.0	88.30	80.43	9.80 ± 0.13	7.33 ± 0.50
1.5	67.32	62.28	7.56 ± 0.33	6.28 ± 0.18
2.0	47.83	42.62	4.80 ± 0.15	4.12 ± 0.36
NAA				
0.5	50.50	46.70	4.60 ± 0.31	3.30 ± 0.32
1.0	75.61	69.34	7.90 ± 0.13	6.35 ± 0.43
1.5	58.45	50.34	5.86 ± 0.20	4.10 ± 0.22
2.0	40.92	36.28	4.16 ± 0.20	3.59 ± 0.33

C. maxima (Lee et al., 2003). Rooting response variation may be affected by different conditions of the shoots used for root induction, variations in the medium used for multiplication before root induction, the number of subcultures before root induction and the culture period on multiplication medium before transfer to root induction medium. The differences in rooting response may be a result of genotype or cultural conditions.

The potential of in vitro propagated *C. pepo* plantlets to be used for ex vitro establishment was investigated with plantlets transferred to soil pots after 2 weeks of initial hardening under culture-room conditions. Almost 70% of these regenerants survived and showed new branch development. These may be useful for the production of somaclonal variants for breeding programs.

Of the 2 cultivars used, better organogenesis was observed in cv. Bulum. Genotypic differences in response to exogenous PGRs and their undergoing of different cellular differentiation pathways are well established (Ferriol et al., 2003; Kathiravan et al., 2006). In conclusion, the reproducible culture system described here could be useful for developing transgenic lines and somaclonal variants.

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