

Induction of caulogenesis and somatic embryogenesis in *Cucumis melo* (var. *Flexuosus*)

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Abstract

Within the framework of genetic improvement of Tunisian Snake-melon (*Cucumis melo* L.) cultivar by biotechnological methods, we have determined a method leading to the regeneration of whole plants by culturing *in vitro* cotyledon and hypocotyl explants on MS medium with different combinations and concentrations of auxin and cytokinin. Adventitious buds were initiated from hypocotyls grown on medium with 1.5 mg l⁻¹ 2,4-D and 0.5 mg l⁻¹ BAP. A Maximum percentage of embryogenesis (20%) was obtained for cotyledons grown in MS medium containing 0.5 mg l⁻¹ 2,4-D and 1 mg l⁻¹ KIN. For stimulating the development of adventitious buds and the embryo's germination and their conversion into plants, MS medium diluted twenty times and supplemented with 1.5 % sucrose was used. Histological studies showed that adventitious buds were initiated from the peripheral zones of the organogenic calli by aggregation of meristematic cell masses which organized into a typical shoot meristem. Embryoids resulted from the division of cells with a dense cytoplasm, a large nuclei and a thick wall which are differentiated on the callus periphery.

Key words: Melon, organogenesis, plant regeneration, somatic embryos, tissue culture.

Abbreviations: cv – cultivar, var – variety, CMV - Cucumber Mosaic Virus, MS - Murashige and Skoog, GA₃ - gibberellic acid, NAA - 1-naphtaleneacetic acid, 2,4-D -2,4-dichlorophenoxyacetic acid, KIN – kinetin, BAP - 6-benzylaminopurine.

Introduction

In general, *Cucumis melo* var. *Flexuosus* is susceptible to several viral and fungal diseases that severely limit yield and for which adequate levels of native resistance are not available. Gene resistance against these biotic stress factors can be found essentially in wild species (Pitrat and Risser, 1992). However, application of conventional plant breeding techniques is very slow and little or none efficacious and is strongly limited because of interspecific incompatibility.

Plant biotechnology strategies could help overcoming these problems, but their use generally requires reliable procedures for plant regeneration from *in vitro* culture.

Several studies showed that melon could regenerate both via caulogenesis or somatic embryogenesis (Dirks & Van Buggenum, 1989; Kageyama *et al.*, 1990; Homma *et al.*, 1991; Gray *et al.*, 1993). Diverse types of explants are able to regenerate plantlets, but seedling material, such as cotyledon or hypocotyl, is especially successful (Moreno *et al.*, 1985; Jelaska, 1986; Tabei *et al.*, 1991; Debeaujon and Branchard, 1993).

Mornagui Snake-Melon cultivar is selected by INRAT (Tunisian National Institute of Agronomic Research) for its interesting yield and its possibility to be included as a catch-crop for the intensification of crops grown under greenhouse. However, this cultivar was sensitive to viruses, particularly to CMV at early stages of growth (Mnari *et al.*, 1989).

Establishing a plant regeneration protocol for this species is the first step for applying plant genetic engineering such as

introducing interesting foreign genes via *Agrobacterium* or biolistic. This technology will help to lead conferring resistance to biotic (fungi and viruses) stresses or to induce some traits by somaclonal variations.

The present paper describes a procedure for *in vitro* regeneration of whole plants from cotyledon and hypocotyl tissues.

Materials and methods

Plant material: Mature seeds of Snake-Melon cv. Mornagui were soaked in tap water for ten minutes. The seeds were manually de-coated and then were surface disinfected by soaking for one minute in 70 % alcohol solution, followed by sterilization in 5% calcium hypochlorite solution for ten minutes and washed three times with sterilized distilled water. For germination, seeds were placed on KNOP medium (Gautheret, 1959), solidified with 0.8 % agar in Petri dishes. Cotyledons and hypocotyls from one to thirteen days-old seedlings grown aseptically, were excised and 1mm from both the basal and the apical end was discarded to expose cut surfaces. Explants were then inoculated on different MS (Murashige and Skoog, 1962) media.

Culture procedure: Ten cotyledonary and fifteen hypocotylary explants were placed separately in each sterile Petridish containing 25 ml medium supplemented with a combination of auxin-cytokinin, 3% sucrose and solidified with 0.8% agar.

As growth regulators, 2,4-D, NAA, BAP and KIN were chosen and a great number of combinations of one auxin plus one cytokinin was tested.

For each treatment, three replications of four Petri dishes containing 10 to 15 explants each were made. Explants were transferred after 20, 30 or 40 days of culture on a new MS medium without growth regulators.

In order to stimulate bud development and embryo germination and transformation into plants, we cultured these structures in different media : KNOP diluted at a half (KNOP/2), MS (3 % sucrose) diluted at a half (MS/2), MS/2 added with GA₃ (0.25 mg l⁻¹) or NAA (0.5 mg l⁻¹), and MS diluted twenty times (MS/20) with 0, 1, 1.5, or 2 % sucrose. The pH of media was adjusted to 5.7 with KOH (0.1 N) or HCl (0.1 N) before autoclaving at 120°C for 20 minutes. All cultures were incubated at 25°C under fluorescent light (5000 lux) with a 16 hours photoperiod.

Histological studies : To precise the ontogeny of the observed embryoids and buds, explants at different stages of their development were fixed in acetic alcohol (3v/1v) for 24h. After dehydration in alcohol baths, and substitution of alcohol by xylene, they were embeded in paraffin blocks. Seven µm thick, longitudinal sections were made using a rotary microtome. Sections were stained with solutions of Hematoxylin and Safranin.

Results

Culture initiation and callus growth: Calli formation started after a week for all treatments and explants. However, calli proliferation intensity and morphology (colour, texture...) were different depending on explant nature and culture medium.

Generally, callus proliferation was more vigorous for the cotyledonary explants than the hypocotylary ones. For the high concentration of 2,4-D, an additional enlargement of callus size occurred, up to five times than the original size.

Two types of calli were initiated from the culture: globular, friable and white to yellowish calli which seem to have predispositions for embryogenesis, and smooth, compact and greenish calli which became, in most of the case, vitrified.

Organogenesis: Growth regulators combinations including NAA didn't perform very well in caulogenesis, except in low concentrations. Adventitious shoot and root neoformations appeared after calli transfer in free or poor growth regulators medium. Maximal percentage of caulogene fragments (75%) was obtained for the hypocotyles grew in MS medium added with 1.5 mg l⁻¹ 2,4-D and 0.5 mg l⁻¹ BAP (average number of buds was 24)

(Table 1). It could also reach 60 and 65.5% for the cotyledons, respectively grew in MS medium added with 0.5 mg l⁻¹ 2,4-D and 1.5 mg l⁻¹ KIN, or with 0.5 mg l⁻¹ 2,4-D and 1 mg l⁻¹ KIN. In the same way, these treatments proved most responsive for root neoformation. They reached respectively 16.16 and 14% of rhizogenesis. Nevertheless, the treatment showing 75% of caulogenesis didn't exceed 7% of rhizogenesis (Table 1).

Furthermore, the neoformed roots didn't always form a continuous axis with regenerated shoots. That's why we didn't obtain the best ability to regenerate plantlets for these organogenic calli, but for the cotyledons grew in MS medium added with 0.5 mg l⁻¹ 2,4-D and 0.25 mg l⁻¹ BAP, or 0.1 mg l⁻¹ NAA and 1 mg l⁻¹ BAP which proved respectively 21 and 24.28% of regenerated plantlet per organogenic callus (Table 1). Shoot buds formed by the callus were dark green and developed into 0.5-2 cm long shoots.

Initially, the roots differentiated on callus were glossy and translucent, and then they became opaque and white, and developed into 1-4 cm long roots, possibly with secondary roots formation.

Embryogenesis: Embryogenic calli were generally friable, nodular and yellow to lightgreen in colour. Contrary to organogenesis, embryogenesis phenomenon began to appear after 2 months of culture in medium without growth regulators. The optimal production of somatic embryos requires 5 to 6 months of culture. For embryogenesis induction, the optimal age of explant seedlings was 4 to 7 days. We could observe embryogenesis at low level, for culture made from 13 days old seedlings.

The production of somatic embryos persisted to 20 months with subculturing and transferring explants in new MS medium, but at low levels.

The best medium for somatic embryos expression and maintenance was MS free growth regulators, especially if cotyledonary calli were taken from MS medium added with 0.5 mg l⁻¹ 2,4-D and 1 mg l⁻¹ KIN. The percentage of embryogenesis observed was 20 %, the average number of embryos per callus was thirteen. This medium succeeded to produce a maximum of 63 embryos per callus, but it doesn't induce any embryogenesis on explants derived from hypocotyls. Otherwise, hypocotyls grew in MS medium added with 1.5 mg l⁻¹ 2,4-D and 0.5 mg l⁻¹ BAP could produce somatic embryos with lower percentage (5.23%).

Table 1. Effects of growth regulator combinations on induction of somatic embryogenesis and organogenesis^z

Results in best treatment							
Growth regulators (mg l ⁻¹)				Explant	Rhizogenesis (%)	Caulogenesis (%)	Embryogenesis (%)
2,4-D	NAA	BAP	KIN				
1.5	0	0.5	0	Hypocotyl	6.4 (±1.1)	74.92(±10.8)	5.23 (±0.18)
1	0	0.25	0	Cotyledon	7.7 (±0.9)	40.7 (±4.04)	0.00
0.5	0	0	1		14.1 (±1.1)	65.5 (±4.54)	20.3 (±2.16)
0.5	0	0	1.5		16.16 (±2.1)	60.27 (±5.1)	0.00
0	0.1	1	0		3 (±1.04)	39.55 (±2.8)	3.1 (±0.86)
0	2.5	1	0		3.1 (±2.3)	18.18 (±9.7)	11.37 (±6.65)
2	0.5	0.5	0		10.5 (±4.2)	5.22 (±3.43)	7.19 (±5.36)
1.1	0.28	0.23	0		0.95 (±1.07)	3.95 (±1.34)	2.63 (±1.9)

Table 2. Effects of the medium composition on the stage of development of the somatic embryos²

Medium	Morphology of embryos (%)			Embryo necrosis(%)	Germinated embryos(%)
	Globular	Cordiform	Cotyledonary		
KNOP/2	53.33 b	17.78 d	28.89 b	16.35 d	56.73 ab
MS/2 (3% sucrose)	57.14 b	21.43 bc	21.43 c	77.5 a	29.62 cd
MS/2 (3% sucrose) + GA ₃ (0.25 mg l ⁻¹)	74.48 a	15.63 d	9.89 e	26.38 c	29.41 cd
MS/2 (3% sucrose) + NAA (0.5 mg l ⁻¹)	74.58 a	18.64 dc	6.78 e	41.46 b	31.1 c
MS/20 (2% sucrose)	70.1 a	22.43 b	7.48 e	45.75 b	20.36 e
MS/20 (1.5% sucrose)	41.1 c	10.96 e	47.95 a	20.28 cd	61.15 a
MS/20 (1% sucrose)	42.62 c	31.15 a	26.23 b	9.62 e	54.81 b
MS/20 (0% sucrose)	53.15 b	33.57 a	13.29 d	3.33 e	25.06 de

²Data are means of 80 to 100 somatic embryos grown in each medium. Mean separation within columns by Duncan's multiple range test at $p=0.05$.

These embryos were in several stages, such as globular, heart-shaped, torpedo-shaped and cotyledonary (Fig. 1). Some morphological abnormalities such as polycotyledony, missing or fused cotyledons, cotyledonary dimorphism, fusion of embryos, absence of pigmentation and embryos with two root poles, occurred notably for explants which have been cultured 40 days on medium containing high concentrations of hormones.

The number of somatic embryos from each callus varied depending on explant nature and hormonal composition in medium.

All NAA/KIN combinations were unsuccessful to develop embryos and plantlets either from cotyledons or from hypocotyls. That shows the significant influence of the explant and of the medium's hormonal composition on inducing embryogenesis.

Influence of the medium on the buds' development and the maturation and germination of the embryos: For all the media tested, there was an embryogenesis recovery by 20% at least. It seems to be a generalized phenomenon which becomes pronounced with the presence of growth regulators and high concentration of mineral elements in the medium.

We could observe secondary embryogenesis in different intermediate stages; so all the daughter embryos didn't develop simultaneously.

The MS/2 medium display as the most important embryogenesis recovery (60.82%) but also the strongest rates of embryos' necrosis (77.5%), which is synonymous of a low rate of maturation for this last medium as for those containing growth regulators and strong concentrations of sucrose (MS/20 + 2% sucrose). Although, KNOP/2 and MS/20 (1.5 and 2 % sucrose) present the weakest rates of resumption of the embryogenesis, they are most favourable to the maturation of embryos. Indeed, on MS/20 (1.5% sucrose) and KNOP/2, respectively, 47.95 and 28.89% of the globular embryos evolve in cotyledonary embryos, with weak rates of necrosis (20.28 and 16.35%, Table 2).

Concentrated medium and the presence of growth regulators encourage certainly the resumption of the embryogenesis and permits to get an elevated percentage of globular embryos, but these latter have difficulty evolving to cotyledonary stadium.

On the other hand, the reduction of the quantity of sucrose in the medium MS/20 accompanied a very meaningful reduction of the rate of global necrosis and different embryonic stadium percentages.

This discrimination preserves itself concerning the germination potential (Fig. 2). The cultivated embryos on MS/20 (1.5 % sucrose) and KNOP/2 medium recorded the high germination percentage: 61.15 and 56.73 % (Table 2).

The reduction of the quantity of sucrose in the medium MS/20 also permits to limit the percentage of germinated embryo necrosis. However, this tendency is reversed for the rhizogenesis. Thus, the elevated contents of sucrose in the MS/20 medium encourage a better rate of rhizogenesis.

Percentages of vitrification and necrosis of roots were raised in KNOP/2 medium and those containing hormones, contrary to MS/20 media containing the weak quantities of sucrose. Latter displayed the weakest rates of root necrosis.

The KNOP/2 medium seems most favourable for buds development (numbers of leaves and inter-nodes number) with a percentage of caulogenesis of 98.61%.

The MS/20 (1.5 % sucrose) medium displays positive results of caulogenesis, it is the one where one observes the less developmental anomalies. It is also most effective of the plants regeneration (Fig. 3) from somatic embryos and adventitious buds (30.43%). The absence of growth regulators and the weak concentration of the mineral composition of the medium seem to be some of the essential elements for the maturation of embryos.

Rates of vitrification and necrosis of plantlets were very high for media containing growth regulators or those containing a strong mineral concentration, but they decrease considerably when quantities of sucrose are reduced.

Histological studies: After a week of culture, organogenic callus tissues were composed from an unorganized cell masses without any recognizable structure. These aggregated cells which didn't develop a specialized form are a sign of an unorganized growth.

The formation of adventitious shoot buds started after twenty days on the peripheric zone of the callus composed by meristematic cell masses. These structures didn't have a precisely ultimate size. These cells have a central nuclei with a big nucleole, and a dense cytoplasm with a large vacuole. Finally, they organized a typical structure of shoot meristem with, meristematic dome, primordial leaf and subapical zone (Fig. 5). Shoot buds seems to have a pluricellular origin.

Somatic embryos (Fig. 4) originated from cells which are characteristically meristematic and differentiated on callus

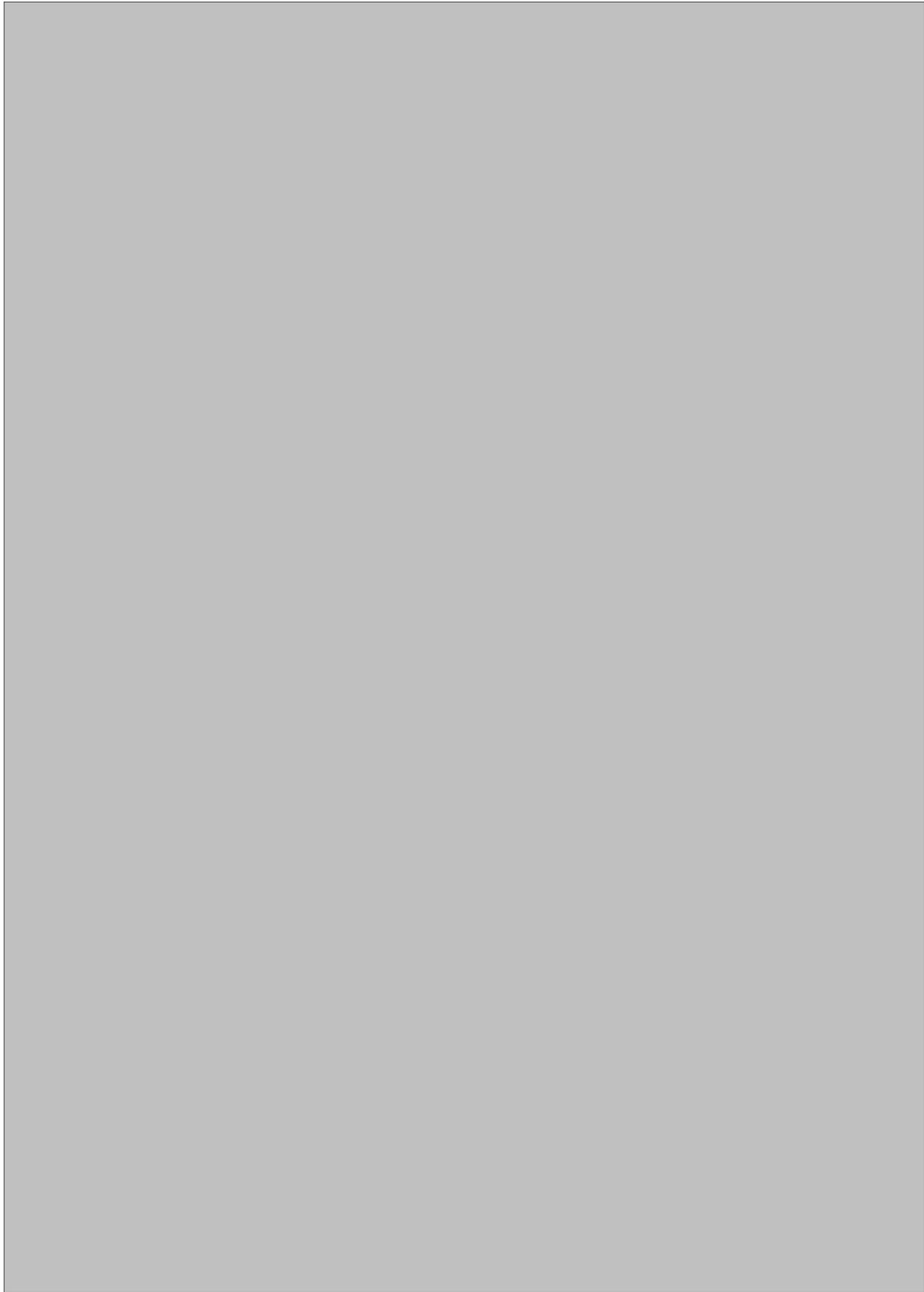


Fig. 1. Somatic embryos: (A) neoformation of adventitious embryos. (B) cotyledonary embryo. Fig. 2. Germination of somatic embryo: (A) hypocotyl emission. (B) germinated embryo Fig. 3. Plant regeneration: (A) *in vitro* plantlet. (B) plantlet before its transfer in pot. Fig. 4. Different stages of somatic embryo: (A) (B) first globular stages. (C) torpedo shape. (D) heart shape. (E) cotyledonary stage. Fig. 5. Shoot meristem initiation.

periphery. These cells have a dense cytoplasm, a large nuclei, a thick wall and contained many starch grains.

Somatic embryos resulted from the asymmetric division of this single cell. But in some cases, it could be from a cellular aggregation as polyembryony. Other somatic embryos could appear from superficial cells of somatic embryos suspensor or cotyledons. Most of these daughter embryos were attached to the parent embryos at only a small point but without a vascular connection and they lacked a suspensor. Embryos have a bipolar structure, both a shoot and a root pole, a shoot axis and two cotyledons but without connection with the underlying parental tissue (Fig. 4E).

Sometimes adventitious shoots are thought to have arisen by caulogenesis, when in reality they have developed from somatic embryos. It can occur that pro-embryos structure cultured in a medium containing inappropriate growth regulators, produce shoots lacking roots. Proembryo cell cluster and early globular stage were also observed (Fig. 4A, B).

Discussion

In this study, we showed that adventitious buds and somatic embryos can be induced from both hypocotyls and cotyledons grown onto MS medium added with specific combination and concentration of cytokinin and auxin. The combination of 0.5 mg l⁻¹ 2,4-D and 1 mg l⁻¹ BAP was efficient to induce caulogenesis and somatic embryogenesis in melon calli.

We observed that tissues required 3 weeks of culture before an organogenic response and at least 3 months of culture before an embryogenic response. Many investigations show that time for obtaining somatic embryos was variable depending on medium and explant nature from five weeks (Oridate and Oosawa, 1986; Branchard and Chateau, 1988) to a longer time period of 18 months (Schroeder, 1968).

MS medium, free from growth regulators seems to be very efficacious for somatic embryos production and maintenance.

The phenomenon of embryogenesis recovery was also observed by Jia and Chua (1992) who showed that when primary embryooids were individually transferred onto MS medium containing 0.5 mg l⁻¹ NAA, multiple secondary embryooids formed on the root pole of the primary embryooid at a frequency of 90%.

In our study, we showed that high concentrations of plant growth regulators and sucrose in the medium inhibits maturation and germination of embryos, and provokes abnormality development. This phenomenon has also been described by Debeaujon & Branchard (1993). Homma *et al.* (1991) observed that melon embryo abnormalities were related to 2,4-D concentration in induction medium.

The rate of embryo germination decreased by 30% when 2,4-D was added to the medium, and by nearly 80% if 60g l⁻¹ sucrose was supplemented. Also, rhizogenesis was reduced by including 2,4-D and high concentration of sucrose in the medium (Kim and Janick, 1989). So, somatic embryo's maturation and germination was commonly accomplished with growth regulator free media (Chee, 1991; Kageyama *et al.*, 1990; Trulson and Shahin, 1986). But maturation of somatic embryos could be effected on

MS medium containing NAA and KIN (Chee, 1992) or gibberellins (Jelaska 1980; Tabei *et al.*, 1991).

The thesis that meristem clearly originates from one cell is subject of controversy. There are many papers which describe how such meristems can arise from one or a few daughter cells that originate from a single cell (Mikkelsen and Sink, 1978; Broertjes and Keen, 1980; Jansson and Borman, 1981; Flinn *et al.*, 1988). Nevertheless, this view is not shared by others authors Ross *et al.* (1973), Attfield and Evans (1991), Falavigna *et al.* (1980), Julliard *et al.* (1992), and Pieron *et al.* (1993), which defend the pluricellular origin of meristems.

It is generally agreed that somatic embryos arises from a single cell (Nomura and Komamine, 1986; Mehra and Jaidka, 1985; Miura and Tabata, 1986; Jones and Rost, 1989). However, Cade *et al.* (1990) showed that embryos obtained from cotyledon-derived calli were probably multicellular in origin. In our study, the earliest stages of embryogenesis haven't been perfectly observed and cellular origin of adventitious buds couldn't be exactly specified. So we have to determine the exact origin of the structures, it is important to ensure the genetic manipulations.

Somatic embryos could be an interesting material for Melon improvement. They may be used for screening resistance to pathogens, salts stress, or also to regenerate genetic transformed plants via *Agrobacterium* mediated transfer.

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