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High Frequency of Multiple Shoots and Plant Regeneration from Different Explants of *Cucumis melo* L. 'Flexuosus': Histological Study and Biochemical Analysis

Asma Ben Ghnaya* • **Najeh Ben Fadhel** • **Mohamed Boussaid**

Laboratoire de Biotechnologie Végétale, Institut National des Sciences Appliquées et de Technologie B.P.676, 1080 Tunis Cedex, Tunisia

Corresponding author: * **benghnaya_asma@yahoo.fr**

ABSTRACT

Within the framework of genetic improvement of a Tunisian Snake-melon (*Cucumis melo* L.) cultivar by biotechnological methods, we developed a method leading to the regeneration of whole plants by *in vitro* culturing of hypocotyl and cotyledon explants, seeds without one cotyledon or with quartile cotyledons, and the embryonic axis, on Murashige and Skoog (MS) medium with different combinations and concentrations of auxin and cytokinin. The percentage of caulogenesis varied with the source of the explant and the composition of the plant growth regulator. The highest percentage of caulogenesis (64.4%) was observed in embryonic axes cultivated on MS with 0.5 mg l^{-1} 2,4-D and 1 mg l^{-1} BAP. Rooting of buds occurred on MS containing 0.5 mg l^{-1} NAA. As soon as roots appeared, the plantlets were transferred into pots, and 80% survival was recorded. The origin of shoots was investigated by histological observation. In addition, the uniformity of regenerated plants was checked by polymorphism analysis of six isozymes by starch gel electrophoresis.

Keywords: caulogenesis, cucumber, *in vitro*, polymorphism, plant growth regulator, tissue culture
Abbreviations: BAP, 6-benzylamino purine, **2,4-D**, 2,4-dichlorophenoxyacetic acid, **NAA**, α-naphthaleneacetic acid; **MS**, Murashige and Skoog medium (1962); **PGR**, plant growth regulator

INTRODUCTION

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 (Trionfetti *et al.* 2002; Kuzuya *et al.* 2003). These pathogens affect the yield, and most of the resistance to these diseases is polygenic (Epinat *et al.* 1993). Gene resistance against these biotic stress factors can be found in wild species (Pitrat and Risser 1992).

However, the application of conventional plant breeding techniques is very slow and relatively ineffective, and is strongly limited because of interspecific incompatibility (Aegerter *et al.* 2000). Amelioration attempts by several institutions have been limited by constraints based mainly on classical selection methods (self-pollination of genotypes, etc.) and have addressed only a small number of characteristics such as resistance to cryptogrammic diseases. Artificial pollination techniques, however, are costly, although effective and interesting. This method of hybridization is limited by interspecific and intergeneric barriers (Deabeaujon and Branchard 1992; Gui *et al.* 2000). Actually for genetic transformation it is necessary an efficient *in vitro* protocol that permits plant regeneration. The protocol can be via direct or indirect organogenesis (Niedz *et al.* 1989; Chee 1991), or somatic embryogenesis (Homma *et al.* 1991; Kageyama *et al.* 1991) via cell and *in vitro* cultured tissues. The main point is to obtain plants.

In vitro regeneration of entire plants in *Cucumis melo* L. was achieved using cotyledons (Stipp *et al.* 2001), hypocotyls and some leaves (Debeaujon and Branchard 1992; Gray *et al.* 1993; Nakagawa *et al*. 2001; Hanana *et al.* 2002) or from protoplasts and suspended cells (Halit and Nebahat 2003). The *in vitro* regeneration of plants by caulogenesis or by somatic embryogenesis was dependent on the genotype, the explant's origin and the culture medium (Oridate *et al.* 1992; Gui *et al.* 2000; Nakagawa *et al*. 2001).

The Flexuosus Snake-Melon cultivar was selected by INRAT (Tunisian National Institute of Agronomic Research) for its yield and the possibility of including it as a catch crop for the intensification of crops grown under glass. However, this cultivar is 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 to obtain plants and in the second step to wards introducing interesting foreign genes via transgenesis by *Agrobacterium* or biolistic and mutagenesis. This technology will help to confer resistance to biotic (fungi and viruses) stresses or to induce various traits by somaclonal variation.

An efficient regeneration technique should take into account complex factors that are highly correlated such as the genotype, explant source, organ age, original source plants and the culture conditions.

In this article, we describe the optimization of *in vitro* regeneration of *Cucumis melo* L. cv. 'Flexuosus' by adventitious shoot regeneration from cotyledons, hypocotyls, other parts of seeds and some embryogenic axes. We also examined variation in the explants. For each explant, several hormonal concentrations and combinations were tested. The second part of this study consisted of the comparison between regenerated plants and others issued from seedlings, using a biochemical approach based on the polymorphism analysis of enzymatic systems.

MATERIALS AND METHODS

Plant material

Cucumber (*Cucumis melo* L.) cultivar was used to evaluate shoot regeneration. This cv is pure, genetically fixed lines, and was obtained by self-pollination.

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Table 1 Effect of explant source and growth regulator combinations on induction of caulogenesis.

Media	Growth regulators (mg. l^{-1})		Explants				
	2,4-D	BAP	A	B	C	D	E
							
M1	0.5	1	0.081ac	0.280 a	0.360 a	0.403 b	**0.644 a**
M2	0.5	2.5	0.180 a	0.360 ab	0.180 b	0.291 b	0.460 b
M3	1	1.5	0.306 b	0.122 c	0.122 b	0.307 ab	0.440 b
M4	1	2	0.122 ac	0.285 bc	0.080 b	0.375 ab	0.470 b
M5	2	0.5	0.117 ac	0.454 a	0.062 b	0.333 a	0.420 b
M6	2	1	0.036 c	0.140 c	0.113 b	0.241 b	0.166 c
M7	3	3	0.125 ac	0.146 c	0.113 b	0.217 a	0.107 c

Data (percentage of explants producing shoots and number of shoots per explant) were collected after three weeks of culture. The results were calculated from four replicate experiments, each with 100 explants per treatment. For each parameter, the values with different letters are significantly different (Duncan's multiple range test).
A = cotyledons, B = hypocotyls, C = entire seeds without a cotyledon, D = entire seeds retaining a quarter of their cotyledons, E = embryogenic axes.

Origin and preparation of explants

We used five types of explants: cotyledons (0.5/0.5 cm) (explant A), hypocotyls (0.5/0.5 cm) (explant B), entire seeds without a cotyledon (explant C), entire seeds retaining a quarter of their cotyledons (explant D) and some embryogenic axes (without cotyledons) (explant E). Cotyledons and hypocotyls were obtained from week-old seedlings. The other explants were from dry seeds.

Mature seeds of *Cucumis melo* L cv. 'Flexuosus' were surface-sterilized in 70% ethanol for 5 min, followed by immersion in calcium hypochlorite (5% w/v) containing two drops of Tween-20 for 15 min. The seeds were rinsed three times with sterile water following sterilization and sown in test tubes on MS (1962) medium (containing 30 g l^{-1} of sucrose and solidified with agar (Kalys, HP 696) at 7.5 g l^{-1}).

Culture medium

Explants were cultured on MS medium (comprising macronutrients, micronutrients and vitamins used by Murashige and Skoog 1962) supplemented with different combinations of BAP (1–3 mg l^{-1}), 2.4 D (0.5-3 mg l^{-1}) and sucrose (30 g l^{-1}). All media were solidified with agar (8 g l^{-1}), adjusted to pH 5.8 by 0.1 N NaOH and sterilized by autoclaving at 120°C and 1 kg cm^{-2} for 20 min (**Table 1**). Explants of 2-week-old seedlings were placed in contact with the medium (25 ml) in 90 mm plastic Petri dishes (25 per source). For each treatment the experiment was repeated three times (n = 3).

Culture conditions

The cultures were incubated under a photoperiod of 14 h (100 µmol m^{-2} s^{-1}) provided by cool white fluorescent lamps, with a 22/19°C thermoperiod (light/dark) and 80% humidity. After three weeks the cultures were transferred to new MS medium in which the concentration of growth regulators was reduced to half that in the induction period.

The number of explants with shoot buds was scored after two weeks and the adventitious shoots formed per explant were counted. Well-developed shoots were separated and transferred to MS medium with various doses of NAA (under the same conditions used for germination). The rooted shoots were acclimatized under a 16 h photoperiod at 50 mol. m^{-2} s^{-1} provided by cool white fluorescent lamps, with a 22/19°C thermoperiod (light/dark). The small plantlets were transferred to pots containing sterile compost, were watered daily and fertilized with Hoagland's solution twice a week in a mist chamber for 10 days prior to transfer to a naturally-lit greenhouse. The acclimatization experiments were repeated several times over two years to check the capacity for normal flowering and fertility.

Histological studies

To determine the ontogeny of the observed buds, explants at different stages of their development were fixed in acetic alcohol (3: 1, v/v) for 24 h. After dehydration in alcohol baths (from 70 to 100°), and substitution of alcohol by xylene, they were embedded in paraffin blocks. Seven-µm thick, longitudinal sections were made using a rotary microtome. Sections were stained with solutions of Haematoxylin 1% and Safranin (1 g dissolved in 100 ml of 50° alcohol).

Variability in regenerated plants

To check the conformity of regenerated plants to those issued from seeds, we analysed six isozymes by electrophoresis starch gel with 13% on the basis of histidine. The isozymes analysed were: phosphoglucoisomerase (PGI, EC 5.3.1.9); phosphoglucomutase (PGM, EC 2.7.5.1); isocitrate deshydrogenase (ICD, EC 1.1.1.4.2); malate deshydrogenase (MDH, EC 1.1.1.37); 6-phosphogluconate dehydrogenase (6-PGD, EC 1.1.1.44); and leucine amino-peptidase (LAP, EC 3.4.1.1). These isozymes are reputed to be codominate markers and are insensitive to environmental factors.

Extracts were obtained from young leaves of regenerated plants and six-day-old germinated seedlings. Seventy mg of fresh material was cold crushed in 200 µl of extraction buffer based on Tris HCL containing 0.5% β- mercaptoethanol added to 10% w/v sucrose and 2.5% (w/v) of polyvinylpyrolidone (PVP 40000).

The homogenate was centrifuged in the cold at 14000 rpm for 30 min. These extracts were placed in gel.

The horizontal migration at 4°C lasted 8 hours with an intensity of 40 mA. An ice-tray was placed on the gel to prevent the denaturation of proteins. The isoenzyme measurements were performed according to the procedure established by Wendel and Weeden (1989).

Data analysis

For each type of explant, we ran four replicate experiments, each with at least one hundred explants. The percentage of callogenesis was recorded 4 weeks after initiation of culture, and the percentage of calluses that formed shoots was recorded 8 weeks after initiation of culture. The values were compared by analysis of variance (ANOVA) and the differences among means (5% level of significance) were tested by the Duncan's Multiple Range Test using StatGraphics Plus 5.1.

RESULTS

Culture initiation and callus growth

In the absence of plant growth regulators (PGRs), callogenesis was null and the explants became brownish and necrotic within 5 to 6 days of culture. In the presence of PGRs, calli formation started after a week for all treatments and explants. However, the proliferation intensity and morphology (colour, texture, etc.) of the calli varied depending on the type of explant and culture medium.

Generally, callus proliferation was more vigorous in the cotyledonary explants than the hypocotylary ones. At the high concentration of 2,4-D, further enlargement of callus size occurred, up to five times the original size. Two types of calli were initiated from the culture:

- smooth, compact and greenish calli which became, in most cases, vitrified and necrotic (**Fig. 1A**);
- globular, friable and white to yellowish calli which seem to have a predisposition for embryogenesis (**Fig. 1B**).

Shoot regeneration

After 20 to 25 days of culture, some shoot regeneration occurred from explants and formed adventitious buds (**Fig. 2A, 2B**). These shoots were generally associated with intense rhizogenesis.

The results indicate that shoot regeneration ability is strongly influenced by the origin of the explants and by the culture medium.

The rate of caulogenesis in cotyledon explants (explant A) was 30.6% in M3 medium (1 mg.l^{-1} 2.4-D + 1.5 mg. l^{-1} BAP) (**Table 1**). Caulogenesis was higher in the hypocotyls (explant B) than the cotyledons. In M5 medium (2 mg. l^{-1} 2.4 D + 0.5 mg.l^{-1} BAP), the percentage of caulogenesis reached 45.4% in hypocotyls. This same medium produced a low rate of caulogenesis of 6.2% for the seeds deprived of cotyledons (explant C). The rate of caulogenesis in seeds retaining a quarter of the cotyledons (explant D) was 42.3% in M3, 40.3% in M1 and 40% in M2.

The embryogenic axes (explant E) showed relatively high rates of caulogenesis in M1 (61.4%), M2 (46 %) and M4 (47%) (**Table 1**).

The highest percentage of caulogenesis was observed in M1 (64.4%) for the embryogenic axes, and for seeds that retained a quarter of their cotyledons in M1 (40.3%) and M3 (42.3%). M1, M2, M3 and M4 were the most favourable media for caulogenesis; whatever the nature of the explants these media produced the highest percentages (64.4, 46 and 44% for M1, M2 and M3 respectively) except for the isolated cotyledons in M1 (8.1% caulogenesis) (**Table 1**).

Histological observations

After a week of culture, organogenic callus tissues were composed of an unorganized cell mass without any recognizable structure. These aggregated cells that lacked a specialized form are a sign of disorganized growth.

The formation of adventitious shoot buds started after twenty days at the periphery (**Fig. 3A**) of the callus that is composed of meristematic cell masses. These structures did not have a precise ultimate size. These cells have a central nucleus with a large nuclear and dense cytoplasm with a large vacuole. They are organized in the typical structure of the shoot meristem, with a meristematic dome, primordial leaf and subapical zone (**Fig. 3B, 3C**).

Vascular formations connect the meristems with the profound tissues of the callus. The meristems of the auxiliary buds originate at the armpit of the foliar primordia (**Fig. 3D**). Shoot buds developed small leaves after 3 weeks of culture.

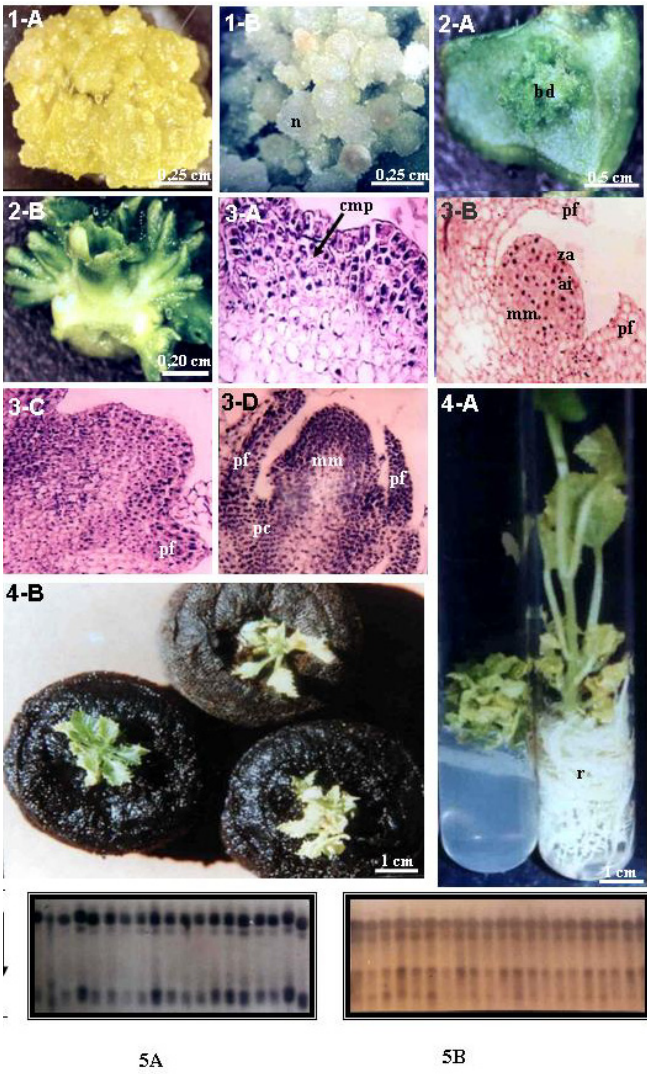

Fig. 1 Different types of calli derived from cotyledon explants. (**A**) Smooth callus. (**B**) Globular callus (n: nodule). **Fig. 2** Shoot regeneration from *Cucumis melo* var. 'Flexuosus' after three weeks of culture on MS + 1 mg.l^{-1} 2.4-D + 1.5 mg. l^{-1} BAP. (**A**) Multiple shoots formed on cotyledon explant. (**B**) Multiple shoots formed on hypocotyl explant. **Fig. 3** Histological study of shoot regeneration from organogenesis calli showed meristematic regions in the peripheral tissues of these calli (cmp) (**A**). (**B, C**) Numerous periclinal divisions form a dome that emerges from the calli and produces a foliar primordium (pf); az: apical zone, mm medullar meristem, ai: initial anneau. (**D**) Meristems of auxiliary buds with foliar primordia (pf) and procambium (pc). **Fig. 4** (**A**) Plants regenerated from cotyledon callus and transferred to test tubes containing MS + 0.5 mg. l^{-1} NAA for development and rooting. R: roots. (**B**) Rooted plants regenerated from hypocotyl explant of *C. melo* var. 'Flexuosus' transplanted into pots for one week. **Fig. 5** isozymes system of phosphoglucomutase (PGM). (**A**) isozyme regenerated plants. (**B**) isozyme of plants derived from seeds. (**B**) Profile.

Rooting and transfer to pots

Elongated shoots were excised from all treatments and rooted as detailed below. Shoots collected *in vitro* (> 3 cm long) were transferred to MS medium containing 20 g.l^{-1} of sucrose and 8 g.l^{-1} of phytaglel (Sigma, USA). The medium was further fortified with different concentrations of NAA. Cultures were incubated as described previously. An optimal rooting rate of 66% from a total of 48 buds was obtained in MS containing 0.5 mg.l^{-1} of NAA (**Fig. 4A**). In the other media, rhizogenesis was quite weak. After the initiation of roots, we witnessed an increase in the formation of leaves comparable to those from plants issued from seedlings.

Plantlets with well-developed roots were removed from the tubes, washed to remove agar and then transferred to

jars (7 cm diameter) containing autoclaved compost and sand (**Fig. 4B**). The potted plants were maintained under the same controlled environmental conditions for 2 weeks. They were watered every 2 days for 15 days. Subsequently they were transferred to earthenware pots (12 cm diameter) containing coarse sand and compost in the greenhouse. The acclimatization rate of severed *in vitro* plants was relatively feeble.

Variability of regenerated plants

To analyse the variability of newly formed plants, we analysed the polymorphism of six isozymes: phosphoglucoisomerase (PGI, EC 5.3.1.9); phosphoglucomutase (PGM, EC 2.7.5.1); isocitrate deshydrogenase (ICD, EC 1.1.1.4.2); malate deshydrogenase (MDH, EC 1.1.1.37); 6-phosphogluconate deshydrogenase (6-PGD, EC 1.1.1.44); and leucine amino-peptidase (LAP, EC 3.4.1.1). The profiles obtained for regenerated plants and those issued from seedlings were practically identical in terms of the number of bands and the level of their migration (**Fig. 5A-C**).

DISCUSSION AND CONCLUSION

This is the first report describing the *in vitro* selection of Tunisian Snake-melon: *Cucumis melo* L cv. 'Flexuosus' from cotyledon (explant A) and hypocotyl (explant B) explants, as well as entire seeds without a cotyledon (explant C), entire seeds that retained a quarter of their cotyledons (explant D) and some embryogenic axes (without cotyledons) (explant E), and its effect on regeneration and micropropagation. One other report on regeneration via caulogenesis and somatic embryogenesis of *Cucumis melo* L cv. 'Flexuosus' exists (Hanana *et al.* 2002) in which the authors developed a classic method to regenerate whole plants by *in vitro* culture using only cotyledon and hypocotyl as the explant on MS medium with different combinations and concentrations of auxin and cytokinine. Unfortunately chromosome anomalies were present throughout the culture, which led to difficulty in transforming whole plants (Burza and Malepszy 1995).

The experimental protocol used in our work differs from that used by other researchers to induce regeneration in cucumber (Dirks and Van Buggenum 1989; Nakagawa *et al.* 2001). We varied the source of explants and tried to achieve quick caulogenesis starting from diverse organs without particular effects on plant variability.

Hypocotyl and cotyledon explants were used to test the potential of these organs in the presence of other internal factors that could influence caulogenesis.

The rate of caulogenesis in different explants depends on the nature of the original explants in a given culture medium. Variations in these factors influence the degree of cellular differentiation of competent cells, depending on the physiological state of the organs, the nature of the tissues and messages they can receive during the seed germination process.

The medium was also a critical factor in our experiment: for cotyledons, a decrease in BAP and the rate of increase of 2,4-D in the medium seemed to favour the percentage of caulogenesis. Bud formation on the cultivated shoots in the presence of grain organs was weak. The presence of embryo axes seemed to inhibit caulogenesis in cotyledon tissues.

Caulogenesis in excised hypocotyls cultivated with embryo axes and a single cotyledon decreased in M1 and M3. Ablation of three quarters of the cotyledons stimulated the regeneration of buds on the hypocotyls; a notable amelioration of caulogenesis was noticed in all culture media. Thus the cotyledons seem to inhibit the organogenic potential of hypocotyls. The complete deletion of the two cotyledons favoured the organogenesis of the explant's hypocotyl.

Our results show the importance of internal correlations in the expression of cultivated tissues *in vitro*. The cotyledons seem to inhibit the regeneration of axes buds through the normal morphogenesis of the plant (Champagnat 1965; Neville 1970).

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.

The monomorphism observed in most of the isozyme analysis in both the regenerated plants and those issued from seeds could be interpreted as follows:

- *Cucumis melo* is a cultivated species in which polymorphisms are scarce;
- Isoenzymatic analysis shows only 30% of the genetic variability. We can also use other techniques of analysis such as PCR (Polymerase Chain Reaction).
- This monomorphism in the regenerated plants shows the conformity of these with the original plants.
- *In vitro* culture allowed the regeneration of plants identical to their mothers, reflecting the importance of *in vitro* culture.

Our study of plant conformity opens up the opportunity to establish molecular transformation of cultivated varieties. Improvement in the acclimatization conditions of *in vitro* plants and research on other hormonal combinations to allow the induction of somatic embryogenesis will also contribute to the establishment of a quick and efficient method of micropropagation in these plants.

REFERENCES

Aegerter BJ, Gordon TR, Davis RM (2000) Occurrence and pathogenicity of fungi associated with melon root rot and vine decline in California. *Plant Disease* **84**, 224-230

Burza W, Malepszy S (1995) *In vitro* culture of *Cucumis sativus* L. XVIIL. Plants from protoplasts through direct somatic embryogenesis. *Plant Cell, Tissue and Organ Culture* **41**, 259-266

Champagnat P (1965) Rameaux courts et rameaux longs: Problèmes physiologiques. *Pflanzenphysiologie* **15 (1)**, 1165-1171

Chee PP (1991) Plant regeneration from cotyledons of *Cucumis melo*. *Topmark*. *Scientia Horticulturae* **26**, 908-910

Debeaujon I, Branchard M (1992) Induction of somatic embryogenesis and caulogenesis from cotyledon and leaf protoplast derived colonies of melon (*Cucumis melo* L.). *Plant Cell Reports* **12**, 37-40

Dirks R, van Buggenum M (1989) *In vitro* plant regeneration from leaf and cotyledon explants of *Cucumis melo* L. *Plant Cell Reports* **7**, 626-627

Epinat C, Pitrat M, Bertrand F (1993) Genetic analysis of resistance of five melon lines to powdery mildews. *Euphytica* **65**, 135-144

Gray DJ, McColley DW, Compton ME (1993) High frequency somatic embryogenesis from quiescent seed cotyledons of *Cucumis melo* cultivars. *Journal of the American Society for Horticultural Science* **118**, 425-432

Gui M, Ben Amor M, Latche A, Pech JC, Roustan P (2000) A reliable system for the transformation of cantaloupe charentais melon (*Cucumis melo* L., var. Cantalupensis) leading to a majority of diploid regenerants. *Scientia Horticulturae* **84**, 91-99

Halit Y, Nebahat S (2003) A new method for haploid muskmelon (*Cucumis melo* L.) diploidization. *Scientia Horticulturae* **98**, 277-283

Hanana M, Harrabi M, Boussaïd M (2002) Induction of caulogenesis and somatic embryogenesis in *Cucumis melo* var. 'Flexuosus'. *Journal of Applied Horticulture* **4 (2)**, 77-82

Homma Y, Sugiyama K, Oosawa K (1991) Improvement in production and regeneration of somatic embryos from mature seed of melon (*Cucumis melo* L.) on solid media. *Ikushugaku Zasshi* **41**, 543-551

Kageyama K, Yabe K, Miyajima S (1991) Somatic embryogenesis in suspension culture of mature seed of *Cucumis melo* L. *Japanese Journal of Breeding* **41**, 273-278

Kuzuya M, Hosoya K, Yashiro K, Tomita K, Ezura H (2003) Powdery mildew (*Sphaerotheca fuliginea*) resistance in melon is selectable at the haploid level. *Journal of Experimental Botany* **54**, 1069-1074

Mnari M, Jebari H, Cherif C (1989) Etude de la résistance du melon (*Cucumis melo* L.) au virus de la mosaïque du concombre (CMV) : Réponse de certaines variétés au virus et contrôle génétique. *Annales de l'Institut National de la Recherche Agronomique de Tunisie* **12**, 1-16

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15**, 473-497

Nakagawa H, Sajyo T, Yamauchi N, Shigyo M, Kako S, Ito A (2001) Effects of sugars and abscisic acid on somatic embryogenesis from melon (*Cucumis melo* L.) expanded cotyledon. *Scientia Horticulturae* **90**, 85-95

Neville P (1970) Morphogénèse chez *Gleditisia triacanthos.* VI. Corrélations contrôlant la forme de premières feuilles des bourgeons axillaires. *Revue Générale de Botanique* **77**, 409-428

Niedz RP, Smith SS, Dunbar KB, Stephen CT, Murakishi HH (1989) Factors influencing shoot regeneration from cotyledonary explants of *Cucumis melo* L. *Plant Cell, Tissue and Organ Culture* **18**, 313-319

Oridate T, Atsumi H, Ito S, Araki H (1992) Genetic difference in somatic embryogenesis from seeds in melon (*Cucumis melo* L). *Plant Cell, Tissue and Organ Culture* **29**, 27-30

Pitrat M, Risser G (1992) Le melon. In: Gallais A, Bannerot H (Eds) *Amélioration des Espèces Végétales Cultivées (Objectif et Critères de Sélection)*, I.N.R.A. (Paris), 762 pp

Stipp LCL, Mendes BMJ, Piedade SMDS, Rodriguez APM (2001) *In vitro* morphogenesis of *Cucumis melo* var. *inodorus*. *Plant Cell, Tissue and Organ Culture* **65**, 81-89

Trionfetti NP, Colla G, Granati E, Temperini O, Crino P, Saccardo F (2002) Rootstock resistance to *fusarium* wilt and effect on fruit yield and quality of two muskmelon cultivars. *Scientia Horticulturae* **93**, 281-288

Wendel JF, Weeden NF (1989) Visualization and interpretation of plant isozymes. In: Soltis DE, Soltis PS (Eds) *Isozymes in Plant Biology*, Discorids Press, Portland, Oregan, USA, pp 5-45