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Efficient plant regeneration from cotyledonary explants of Tunisian *Cucumis melo* L. cv. Maazoun and Beji

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Abstract

A procedure for the regeneration of melon *(Cucumis melo* L.) cv. Maazoun and Beji via shoot organogenesis from cotyledon explants is described. Cotyledons explants cultivated on the basal Murashige and Skoog (1962) medium, added with combinations of BAP, NAA and 2,4-D developed adventitious shoots after two weeks. The rates of caulogenesis varied according to the medium hormonal composition and the genotype. The best induction medium for a morphogenic response was MS containing 0.5 mgl^{-1} NAA and 2 mgl^{-1} BAP. The highest caulogenesis percentages were 79.16% and 58.33% for Maazoun and Beji cultivars respectively. The mean number of buds per callus was 2.92. Development phases were characterized by histological studies. Some phases of shoot organogenesis were observed in *Cucumis melo* L. Maazoun and Beji cultivars. The shoots were rooted in MS medium without growth regulators or added NAA (0.5 or 1 mgl^{-1}). The highest bud rooting of buds occurred on MS medium added with 1 mg/l NAA. The percentage of rooting varied from 16.66 to 75%. The highest percentage of survival plants after acclimatization were 41.73 and 57.63 for Beji and Maazoun respectively.

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Abbreviations: 22, 4-D: acide 2, 4-dichlorophenoxyacetic, NAA: acide aphtaleneacetic, BAP: 6-benzylaminopurine, cv: cultivar, MS: Murashige and Skoog medium.

Introduction

Cucumis melo L. is an important horticultural crop. In Tunisia it is grown over an area of 1691 hectares (DG/PDIA; Ministry of Agriculture of Tunisia, 1999). Increasing demand for these crops improvement of their agronomic characteristics, such as their resistance to disease, tolerance to climatic and edaphic factors and fruit sugar contents (Hernandez-Gomez *et al.*, 2005). Conventional plant breeding techniques (massal selection and line fixing) are very expensive and limited because of the interspecific and intergeneric reproductive barriers (Gui *et al.*, 2000).

Genetic engineering can be used to produce desirable agronomic characteristics rapidly and efficiently, and it has already been used to improve fruit quality and introduce disease resistance and environmental tolerance in melon. In vitro tissue culture and genetic transformation (e.g. via *Agrobacterium* strains) constitutes a rapid and reliable way of producing interesting varieties with desirable agronomic traits (disease resistance, habitat adaptation, fruit quality,...). However, it is very limited by the low production of regenerate plants.

Protocols using different explants (leaves, cotyledons, hypocotyls, protoplasts,...) to regenerate plantlets have been previously reported for some melon varieties (Halit and Nebahat, 2003). Plant regeneration occurred via caulogenesis or somatic embryogenesis. However, frequencies of bud and somatic embryo formation and their conversion into whole plants were low and varied significantly depending to genotypes, the source of explant and media tested (Hnana *et al.*, 2004).

Tunisian Maazoun (Eastern type) and Beji (Charentais) *Cucumis melo cultivars*, widely cultivated in Tunisia were selected by INRAT (Tunisian National Institute of Agronomic Research) (Jabberi, 1988) and very appreciated by the consumer. However, these *cultivars* are subject to climatic stresses (low temperature and drought) and pathogens (mottle mosaic virus, fusarial wilt). Therefore, the establishment of plant regeneration protocol for these *cultivars* constitutes a first step for propagation and the conservation of specific genotypes and genetic manipulations.

The purpose of this study is to develop and optimize an efficient methodology for rapid and reproducible *in vitro* plant regeneration from *C. melo* . cv. Maazoun and Beji cotyledonary explants. The induction of caulogenesis, within this specie, in addition to its interests for the propagation and the conservation of specific genotypes, might be crucial to the genetic improvement for commercial uses of Tunisian *Cucumis melo*.

Material and methods

Plant material

Mature seeds of *Cucumis melo* Maazoun (Eastern type) and Beji (Charentais) *cultivars* (Fig. 1A and B) were used for organogenesis induction in this study. For Maazoun cultivar the fruit is rough, light green at the base and dark at the flower stalk. Beji cultivar has oval shaped fruit. Its bark is golden yellow, smooth and slightly wrinkled. Both *cultivars* are grown in Tunisia. They are highly appreciated by consumers for their fruit.

The adventitious buds induction was tested on cotyledon segments. Cotyledon (0.5 x 0.5 cm^2) fragments were excised from 10 day old seedlings grown aseptically, *in vitro*. Cotyledonary explants were excised and inoculated horizontally onto the MS medium.

Culture media and culture conditions

Seeds germination media

Seed coats were removed and seeds were sterilized with calcium hypochlorite (5%) for 10 min, followed by repeated washings with sterile distilled water. Sterilized decoated seeds were germinated on MS (Murashige and Skoog 1962) basal medium added with 3 mgl^{-1} BAP and 0.5 mgl^{-1} NAA, supplemented

with 2% (w/v) sucrose, solidified by 7 gl^{-1} agar (Ben Ghnaya and Boussaid, 2002).

Culture media

Shoot bud induction was tested on MS basal medium supplemented with 3% (w/v) sucrose, solidified by 7 gl^{-1} agar and added with different concentrations and combinations of 2,4-dichlorophenoxyacetic acid (2,4-D) (0.25, 0.5 and 1 mgl^{-1}), naphthaleneacetic acid (NAA) (0.25, 0.5 and 1 mgl^{-1}) and 6-benzylaminopurine (BAP) (0.25, 0.5, 1 and 2 mgl^{-1}) (Table 1). Explants consisting of intact cotyledons were inoculated on petri dishes (8 x 8 x 2cm) containing 25 ml of the desired medium. For each experiment, 48 explants were used.

Well developed shoots (4 to 6 leaves) were isolated from the initial culture and transferred onto rooting MS medium without growth regulators or added with NAA (0.5 or 1 mgl^{-1}) and 3% sucrose.

The pH of all media was adjusted to 5.8 with NaOH (0.1N) or HCl (0.1N), and autoclaved at 121°C, 100 KPa for 20 min.

Culture conditions

Seed germination and culture of explants performed aseptically under a laminar flow hood. Cultures were placed in petri dishes containing each 25 ml of medium without or added with growth regulators. Cultures were incubated in a growth room at 25 ± 1°C with a 16 hours/day photoperiod under 40 $\mu moles.m^{-2}.s^{-1}$ cool white fluorescent light. The humidity is 80%.

Histological analysis

In order to define the early histological events occurring during the regeneration process, investigations were done at the level of the cut end of the fragments tissue remaining connected to the cotyledon (Fig. 1). This explant gave a best regeneration response in our experiment.

Observations were carried out on explants before culturing and after 1, 2, 3, 4, 5, 6, 8, 10, 12 and 14 days of culture. Samples (four per harvest date) were fixed in AA (glacial acetic acid, absolute alcohol 100°: 1:2, v/v). After 24 h fixation at 4°C the specimens were rinsed in distilled water for 24h and stored in 70% alcohol until required. Tissues were then dehydrated in graded series of ethanol solutions (50, 70, 95 and 100%) for 45 min each bath and embedded in paraffin. Serial sections of 7μm were made using a rotary microtome and slides containing the sections were deparaffinized through graded series of xylen-ethanol solutions, hydrated with decreasing ethanol solutions (100, 95, 70, 50%) and stained with hematoxylin (Panreac Quimica, SA) and safranin (RAL Reactifs). Nuclear material is stained in blue with hematoxylin and cell walls are red in presence of safranin. Slide observations were performed under a photonic microscope (Leica DMLB2).

Data analysis

The percentages of caulogenesis and the mean number of buds by explant for each genotype and for each medium tested were calculated after one week of culture. The analyses of variance (ANOVA) with one effect (medium effect) were performed to compare explant responses of the two *cultivars* in the different media. It was carried out for each measured parameter with medium effect. The following model was used: X_{ik}-m = a_i + d_{jk}. (Dagnelie, 1994), where X_{ik} is measured variable, m average value of the variable for the two *cultivars*, a_i medium effect, d_{jk} the residual effect (Dagnelie, 1994). All calculations were made by the SAS program (version 6, 1994, procedure ANOVA).

Results

Organogenesis induction

Sixteen media (M_1 to M_{16}) which differed by the level and the combination of growth regulators were tested for their ability to induce organogenesis for the cotyledonary explant of the two *cultivars* (Table 1). The results obtained with the different NAA, 2,4-D and BAP combinations for the two *cultivars* were shown in table1.

Explants which developed on free MS hormone medium (Rhimi and Boussaid, 2000, Rhimi *et al.*, 2006) didn't exhibit any growth. They became brown and necrosed after a few days of culture. On the other media, where growth regulators were added, the explants formed callus developed on the cut surface of all explants cultured on all treatments after a week. However, calli proliferation intensity and morphology (colour, texture...) were different according to culture medium. Globally, callus proliferation was vigorous for the explants cultured on media with relatively high concentration of BAP (0.5 to 2 mgl^{-1}). Some other calluses were compact and green and showed organogenic structures at the edge of the explants (Fig 2). These structures developed adventitious shoots (Fig 3). The level of the caulogenesis and the mean number of buds by callus varied according to the media and genotypes.

The *Cucumis melo* L. cv. Maazoun and Beji revealed organogenic potentialities in our conditions. Growth regulators combinations including NAA and BAP were perform to stimulate caulogenesis (Table 1). The analysis of variance performed on medium effect showed high significant (at $P< 0.001$) differences of the measured parameters (Table 2) for the two cultivars. Cotyledons formed organogenetic calli with various percentages. Media M_9, M_{11}, M_{12}, M_{13}, M_{15} and M_{16} performed the better organogenesis induction. The responses of Maazoun cotyledon were better than those observed for Beji *cultivar* (Table 2). 58.33 and 79.16% of cotyledons cultivated on M_{15} and M_{13} were organogenic respectively for Beji and Maazoun. The means of buds number / callus were respectively 2.50 and 2.91.

Table 1. Organogenic callus percentage of cotyledonary explants in two cultivars.

Media	Growth regulators (mgl-1)			Maazoun		Beji	
	BAP	2.4-D	NAA	CC(%)	NB	CC(%)	NB
M_1	0.25	0.00	0.25	12.50	2.33	0.00	0.00
M_2	0.25	0.25	0.00	12.50	2.66	2.08	2.00
M_3	0.25	0.00	1.00	4.16	2.5	16.66	1.75
M_4	0.25	1.00	0.00	16.66	2.5	8.33	1.75
M_5	0.50	0.00	0.50	8.33	2.75	0.00	0.00
M_6	0.50	0.50	0.00	20.83	2.6	8.33	2.00
M_7	0.50	0.00	1.00	16.66	2.75	27.08	2.00
M_8	0.50	1.00	0.00	25.00	2.66	18.75	2.33
M_9	1.00	0.00	0.50	58.33	2.60	4.16	2.50
M_{10}	1.00	0.50	0.00	29.16	2.50	12.50	2.33
M_{11}	1.00	0.00	1.00	37.50	2.61	56.25	2.07
M_{12}	1.00	1.00	0.00	20.83	2.60	54.16	1.96
M_{13}	2.00	0.00	0.50	79.16	2.92	8.33	1.75
M_{14}	2.00	0.50	0.00	37.50	2.61	14.58	2.00
M_{15}	2.00	0.00	1.00	66.66	1.15	58.33	2.50
M_{16}	2.00	1.00	0.00	58.33	2.85	37.50	2.11

Percentages are calculated on 48 explants; M: MS media; CC: Caulogenesis (%); NB: Mean number of buds by callus.

Plant regeneration

For rooting, the elongated shoots (4 to 6 leaves) were separated from the parental callus and transferred onto rooting MS medium without growth regulators or added NAA (0.5 or $1mgl^{-1}$) and 3% sucrose (Fig 3). The first root emerges ten days after culture. The rooting capacity is relatively high both in the presence and absence of NAA (Table 3). On media without NAA, the rooting percentage does not exceed 34.72% (cv. Maazoun) (Table 3). A maximum of rooted shoots (76.19 and 56.07 % respectively for Maazoun and Beji) were obtained in MS medium with 1 mgl^{-1} NAA after 3 weeks culture period (Fig 3). The effectiveness of NAA in rooting has been

reported in *Cucurbita pepo* (Kathiravan *et al.*, 2006), *Cucumis melo* (Rhimi *et al.*, 2012). The rooted plants were washed for 7 min in distilled water to remove adhered agar and traces of the medium to avoid contamination. They were then cultured in pots containing sterile soil (2/3 peat and 1/3 sand) and acclimatized to greenhouse conditions for two weeks (Fig 4).

We had a 57.63 and 41.73 % survival rates respectively for Maazoun and Beji of plants derived from cotyledonary explants when rooted plantlets were transferred from pots to field conditions. Regenerated plants transferred to the field became fully established and grew well and were similar to the parental plants in their morphology.

Table 2. Analysis of variance single factor classification (medium effect) performed on the mean number of organogenic explants and the average number of shoots per cotyledonary explants in both *cultivars*.

Cultivar	Variable	ddl	CM	F
Maazoun	Number of caulogenic callus	15	2.542	14.94**
	Number of buds by callus	15	19.352	14.46**
Beji	Number of caulogenic callus	15	1.970	15.62**
	Number of buds by callus	15	8.190	13.10**

CM: mean square, ddl: degree of freedom, F: Snedecor F and (**) high significant ($P < 0.001$).

Histological studies

Anatomical study, carried out during a period of two culture weeks, allowed us to describe the following events occurring during the regeneration process. During the first four days, no clear histological changes were detectable. The first visible changes thereafter consisted of the setting of a cell dedifferentiation process in the epidermal and subepidermal layers of the cut edge of the explant. The proximal cotyledon explants increased in size, exhibited swelling, and produced calli on the adaxial surface in contact with the medium in 7-day-old tissues.

Table 3. Rooting and survival of buds formed from cotyledonary explants.

Media	*Cultivar*			
	Maazoun		Beji	
	% rooting	% survival	% rooting	% survival
MS + 0 mgl^{-1} NAA	34.72	26.66	18.69	25.00
MS + 0.50 mgl^{-1} NAA	65.74	49.29	32.71	31.42
MS + 1 mgl^{-1} NAA	76.19	76.19	56.07	53.33
% means	74.88	57.63	35.82	41.73

Organogenic callus observation shows that they are constituted essentially of parenchymatous tissues and have many nodules Issus from localized cell divisions (Fig 5A). In the deep zone of callus, cambial type cell divisions appear (Fig 5B). These cell proliferations taking place nearby conductors beam. The initiated cells outwards of the cambial zone differentiate into parenchyma, those formed inwards become tracheids separated by parenchymal cells (Fig 5C). Premeristematic cells at the origin of a Organogenic meristem are visible in the epidermal layer or in callus depth (Fig 5D, E). The formation of early shoot apical meristems was observed in 10-dayold tissues (Fig 5F), and meristematic structures were seen in 12-day-old tissues. The first shoot was found after 14 days. By this time, the surface was covered with protrusions and leaves, mostly without accompanying buds.

Discussion and conclusions

The cotyledons exhibited shoot regeneration in *Cucumis melo* cv. Maazoun and Beji. Other researchers also observed that the cotyledon showed a higher frequency of adventitious shoot regeneration in comparison to other explants (leaves, hypocotyls, protoplasts) (Halit and Nebahat, 2003,

Ntui *et al.*, 2010**).** Moreover, the cotyledon being used as an explant in regeneration studies such as those for melon (Zang *et al.*, 2011), watermelon (Compton and Gray, 1993), winter squash (Lee *et al.*, 2003), cucumber (Yalçın, 2003), summer squash (Ananthakrishnan *et al.*, 2003), and bottle gourd (Han *et al.*, 2004) was motivated by the cotyledonary explant's greater vigor. Most of the reports on cotyledon culture in cucurbits describe the direct shoot regeneration without any callus phase as in the case of cucumber (*Cucumis sativus* L.) (Gambley and Dodd, 1991) and muskmelon (*Cucumis melo*) (Niedz *et al.*, 1989). But in the present study we obtained multiple shoots through callus regeneration from cotyledons. Shoot regeneration from the calli was determined by the type of growth regulator, its concentration and combination. The regeneration of explants treated with medium containing 2 mgl^{-1} BAP and 0.5 mgl^{-1} NAA was the highest (79.16%) for Maazoun. The result for Beji cotyledon explants was much lower (58.33%) on 2 mgl^{-1} BAP and 1 mgl^{-1} NAA. Maintaining the same levels of phytohormones and changing the ANA by 2,4-D reduced regeneration to 37.5% for Maazoun and Beji, but not for the number of buds by callus. The culture medium containing BAP and NAA promoted a significant increase on organogenic index. As the concentrations in both combinations (BAP/NAA and BAP/2,4-D) were the same in different culture media, these suggested that, for the tow cultivars, BAP/NAA combination was more effective than BAP/2,4-D on organogenic induction. The combination of these 2 hormones (BAP and NAA) also caused the best shoot formation in another cucurbit (Chengalrayan *et al.*, 2001).

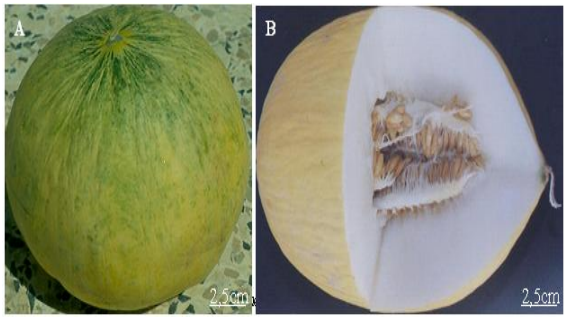

Fig. 1. Fruits of *Cucumis melo* L. A: c.v., Maazoun, B: c.v., Beji.

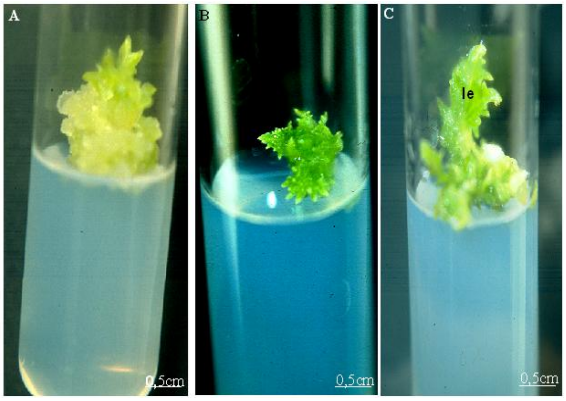

Fig. 2. A- Organogenic callus with adventitious bus Maazoun cotyledon. B and C- Shoots regenerated from organogenic callus after 21 days of culture on MS + (le: leaves).

Organogenic response in Cucurbitaceae is highly genotype dependent. Ficcadenti and Rotini (1995) reported shoot production in 11 melon *cultivars* using BA as growth regulator. In addition, Dabaúza (1995) obtained an expressive organogenic response in cotyledon explants from *Citrullus colocynthis*, using only BA as growth regulator. In our study, the organogenic response of explants from Maazoun and Beji cultivars in sixteen culture media was different. The organogenic callus and the number of shoots per callus were higher in the Maazoun *cultivar* when BAP and NAA combination or BAP and 2,4-D combination were used in the culture medium, while for Beji cultivar was less efficient.

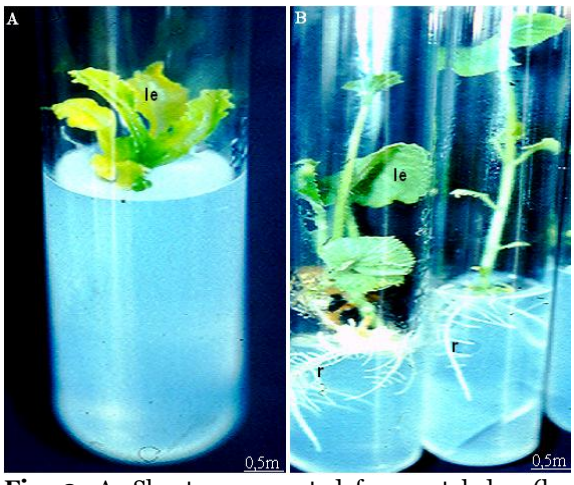

Fig. 3. A: Shoots regenerated from cotyledon (le: leaves). B: Entire plant regenerated from cotyledon (le: leaves; r: root).

Fig. 4. Entire plant regenerated from cotyledon.

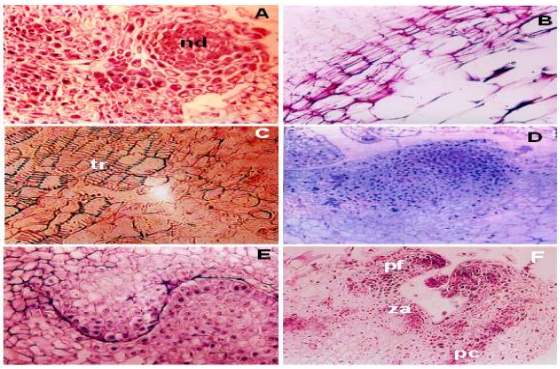

Fig. 5. Histological analysis of organogenic callus of *Cucumis melo*.

A- Parenchymal nodules (nd) in the depth of the callus

B- Differentiated cambial zone in parenchymal tissue of the callus

C- Tracheid (tr) differentiated in the depth of the callus

D- Differentiated meristematic cell zone at the periphery of the callus

E- Differentiated meristematic cell zone at the depth of the callus

F- Longisection of early shoot development showing the typical dimorphic meristem
(apical zone (za), medullary meristem (mm), leaf primordium (pf)).

In histological analysis, epidermal cells were the source of organogenesis. Epidermal cells were also found to be the starting point of regeneration by other scientists, such as for melon (Chovelon *et al.*, 2008), sunn hemp (Daimon *et al.*, 2002), watermelon (Yalçın, 2003), and pepper (Mezghani *et al.*, 2007). Protuberances and some meristematic bulges were observed after 10 days. Similar results were obtained by Gaba *et al.* (1999) with melon and Yalçın (2003) with watermelon. The first shoot was found after 21 days. In watermelon, premeristematic and meristematic tissues were seen in 7- and 12-day-old tissues. The first regenerated shoot buds and shoots were observed in 10- and 14- day-old tissues, respectively, in melon. This indicates that the formation of meristematic structures and shoots in melon might be later than in watermelon.

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