In vitro organogenesis in watermelon cotyledons

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Abstract – The objective of this work was to study the in vitro organogenesis of *Citrullus lanatus*, by the induction of adventitious buds in cotyledon segments cultured in medium supplemented with cytokinin. Explants were collected from one, three and five-day-old in vitro germinated seedlings, considering the distal and proximal cotyledon regions. The data obtained showed that in vitro organogenesis of watermelon occurred with higher efficiency, when cotyledon segments from the proximal region collected from three-day-old seedlings were cultivated in medium MS, supplemented with BAP (1 mg L^{-1}) and coconut water (10%). The histological study showed that the organogenesis occurs directly, without callus formation, on epidermal and subepidermal layers of the explants. Adventitious shoots were characterized by the development of shoot apical meristem and leaf primordia. The formation of protuberances, that do not develop into adventitious buds, was also observed.

Index terms: *Citrullus lanatus*, histological study, adventitious bud.

Organogênese in vitro em cotilédones de melancia

Resumo – O objetivo do trabalho foi estudar a organogênese in vitro de *C. lanatus*, pela indução de gemas adventícias, em segmentos de cotilédones, cultivados em meio de cultura suplementado com citocinina. Os explantes consistiram de segmentos das regiões distal e proximal de cotilédones, coletados de plantas germinadas in vitro com um, três e cinco dias de idade. Os dados obtidos mostram que a organogênese de melancia, in vitro, ocorre com maior eficiência em segmentos da região proximal dos cotilédones, coletados de plântulas com três dias de idade e cultivados em meio de cultura MS, suplementado com a combinação BAP (1 mg L^{-1}) e água de coco (10%). Pelo estudo histológico, verificou-se que a organogênese ocorre diretamente, sem a formação de calo, na epiderme e subepiderme do explante. As gemas adventícias foram caracterizadas pela presença de meristema apical e primórdios foliares. Observou-se, também, o desenvolvimento de protuberâncias que não se desenvolvem em gemas adventícias.

Termos de indexação: *Citrullus lanatus*, estudo histológico, gema adventícia.

Introduction

Cucurbitaceae are an economically important family of plants, with species commercially cultivated in tropical and subtropical regions. Species such as watermelon, melon, cucumber and squash are widely cultivated in all regions of Brazil. Among cucurbit species watermelon is the most cultivated in Brazil, with a total of 1,450,324 t of fruits, in an area of 77,409 ha (FNP Consultoria e Agroinformativos, 2003). However, this production is hampered by serious disease problems, especially those caused by virus infections. In the State of São Paulo eight virus diseases were identified in the culture. The PRSV-W can be considered the most important virus that infects watermelon. The control of all these diseases can only be achieved by the use of tolerant or resistant varieties.

Cucurbits breeding programs can highly benefit of biotechnological tools, such as gene transfer technology, which allows the introduction of foreign genes into a germplasm, without modifying the genetic background of elite varieties. This method has been used to develop resistance to cucurbit potyviruses in different cucurbit species (Tricoli et al., 1995; Fuchs et al., 1998; Compton et al., 2004). However, a breeding program associated to biotechnological tools depends upon the development of an efficient in vitro plant regeneration system.

In vitro plant regeneration of watermelon has been reported using protocols for adventitious shoot regeneration from cotyledon segments (Srivastava et al.,

1989; Compton & Gray, 1993). The system is based on three culture steps (Dong & Jia, 1991): a bud induction phase, culturing the explants in medium supplemented with cytokinin (Compton & Gray, 1993); an elongation phase, transferring the shoot buds to medium with a lower concentration of cytokinin (Dong & Jia, 1991); and, a rooting phase, using a culture medium supplemented with auxin (Compton & Gray, 1994; Dabauza et al., 1997).

Besides growth regulators, seedling age at the time of explant preparation (Srivastava et al., 1989; Compton & Gray, 1994), explant dissection method (Compton & Gray, 1993; Compton, 2000), and culture conditions (Choi et al., 1994) also influence adventitious shoot development. The development of tissue culture systems, for commercially important Brazilian cucurbit species, is restricted to the analysis of in vitro morphogenesis of *Cucumis melo* var. *inodorus* (Stipp et al., 2001).

The objective of the present work was to implement an efficient organogenesis protocol, followed by plant regeneration for watermelon cultivar Crimson Sweet, for further studies of watermelon genetic transformation via *Agrobacterium*.

Material and Methods

Citrullus lanatus cv. Crimson Sweet mature seeds were used as explant sources. Seeds were surface-sterilized for 30 min, in commercial bleach solution (2.5% NaClO), rinsed three times in distilled water, and soaked overnight in sterile distilled water, in the dark. Seed coats were then removed and the embryos surface-sterilized for 20 min, in 20% commercial bleach solution (2.5% NaClO), followed by three rinses in sterilized distilled water.

The embryos were placed in plastic boxes containing MS culture media, for germination at 27°C, in the dark. The cotyledons were then excised and 1 to 2 mm removed from the borders, so that the cotyledon central region was cut transversally into two halves, proximal and distal, and these were used as explants (Figure 1 a). To investigate the influence of seedling age on shoot organogenesis, explants were collected from one, three and five-day-old seedlings, and cultivated in MS medium supplemented with 6-benzylaminopurine – BAP (2.5 mg L^{-1}). In order to study the effect of different growth

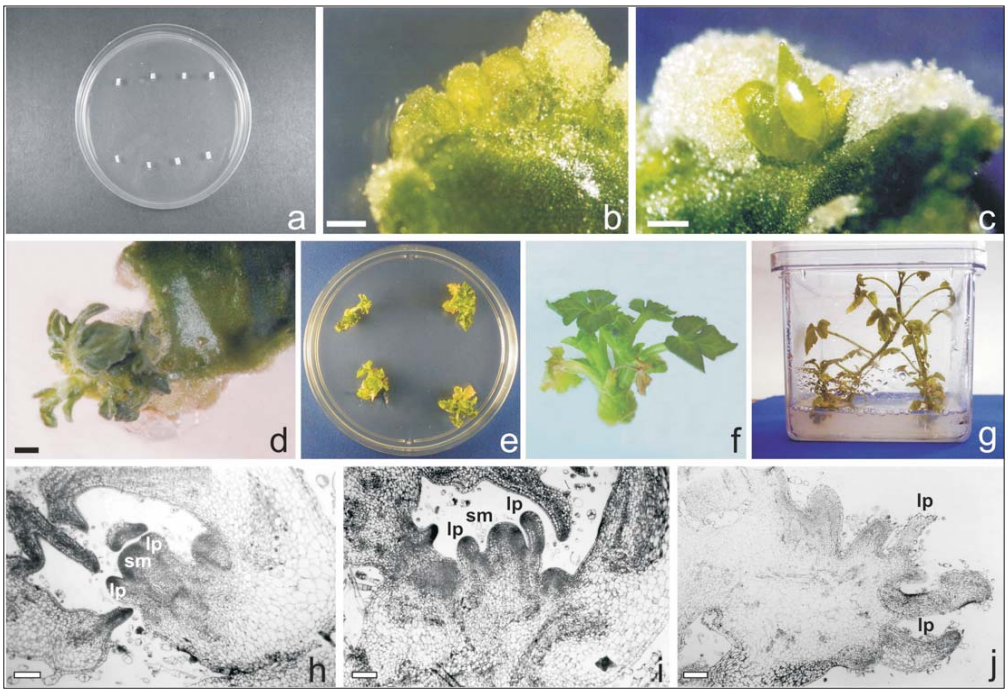

Figure 1. *Citrullus lanatus* cv. Crimson sweet in vitro organogenesis from cotyledonary segments, cultured in MS culture media supplemented with BAP (1 mg L^{-1}): a) initial explant; b) initial development of buds and leaf primordia; c-d) adventitious shoot on proximal explant; e) shoot bud elongation; f) plantlet; g) rooting. Histological sections after 21 days in culture showing: h) initial development of bud with apical meristem (sm) and leaf primordia (lp); i) bud; j) leaf primordia (lp). Bars = 100 µm (h-i); 200 µm (j); 1 mm (b-d).

regulators on shoot induction, cotyledon segments collected from three-day-old seedlings were cultured in Petri dishes (100 x 15 mm) containing MS basal medium, supplemented with BAP (0, 1.0, 2.0 or 3.0 mg L^{-1}), the combination of BAP (1 mg L^{-1}) and coconut water (10%), thidiazuron – TDZ (0, 0.25, 0.5, 0.75 or 1.0 mg L^{-1}), or the combination of BAP (0, 1.0, 2.5, 5.0 or 7.5 mg L^{-1}) and 1-naphthaleneacetic acid – NAA (0.1 mg L^{-1}).

The experimental design was completely randomized with 10 replications, each one consisting of a Petri dish with eight explants. Cultures were maintained at 27°C, under a 16 hour photoperiod (63 μM m^{-2} s^{-1}). After 4 weeks, the explants were scored with the aid of a stereomicroscope, for the number of explants forming multiple shoot buds, and for the number of shoots per responsive explants. The statistical analysis was performed by ANOVA on $(x + 0.5)^{1/2}$ transformed data. Tukey's multiple range test was used for means comparison.

Developed buds were isolated and transferred to MS basal medium supplemented with kinetin (0.2 mg L^{-1}). Three cm long shoots were transferred to rooting medium consisting of MS basal medium, supplemented with indole-3-butyric acid – IBA (1.0 mg L^{-1}).

All media were supplemented with sucrose (2.5%), agar (0.8%) and the pH was adjusted to 5.8, before autoclaving (121°C, 20 min).

Explants cultivated in organogenesis induction medium, containing BAP (1.0 mg L^{-1}) and coconut water (10%), were sampled for histological study after 21 days in culture, according to Rodriguez & Wetzstein (1998). Samples were fixed in paraformaldehyde (3%) and glutaraldehyde (2%), in cacodylate buffer (0.2 M, pH 7.2) under refrigeration, for five days. Dehydration was done at room temperature in a series of 100% methyl cellosolve, ethanol, propanol and butanol, followed by infiltration, in a series of butanol:infiltration medium, and 100% infiltration medium at 4°C, overnight. Polymerization was done at room temperature, for 24–48 hours in Historesin (Leica, Heildelberg). Serial sections (5 μM) were prepared in a rotary microtome (Leica RM2155) with a steel carbide knife. The sections, floated in water drops, were dried on a hot plate (40°C) stained with acid fuchsin (0.1%), and then rinsed in distilled water and counterstained with toluidine blue (0.05%), for general observations and documentation under an inverted Zeiss Axiovert 35 microscope.

Results and Discussion

Cotyledon segments collected from three-day-old seedlings responded with adventitious bud formation at a much higher percentage (Table 1) than explants collected from either one or five-day-old seedlings (Figure 1 b-d). Seedling age is reported to be an important factor for obtaining high frequency adventitious shoot regeneration in watermelon (Choi et al., 1994; Compton & Gray, 1994). For watermelon organogenesis, explants were collected from seedlings younger than five-day-old (Choi et al., 1994; Compton, 2000), as young cotyledons are physiologically very active and respond efficiently to exogenous hormones (Dong & Jia, 1991).

Together with seedling age, explant type has shown to be important for morphogenesis induction, since competent cells for adventitious shoot formation in cucurbits seem to be restricted to specific cotyledon regions (Choi et al., 1994; Compton, 2000; Ananthakrishman et al., 2003). For watermelon, it has been shown that organogenic competent cells concentrated at the proximal region of the cotyledon (Compton & Gray, 1993), since most of the adventitious buds develop at the explant basal region (Compton, 2000). Regardless of the seedling age, a higher percentage of explants forming adventitious buds was obtained in explants collected from the cotyledon proximal region (Table 1) (Figure 1 c-d). Efficient response from the distal portions of the watermelon cotyledons was reported only by Choi et al. (1994), with a F_1 hybrid watermelon (cvs. Sweet Gem and Gold Metal).

The composition of induction media is also important for adventitious bud development; the presence of cytokinin is critical for shoot induction and for differentiation from cotyledonary explants of watermelon (Dong & Jia, 1991). BAP has been reported as highly

Table 1. Effect of seedling age on adventitious shoot formation on proximal and distal halves of cotiledonary explants of *Citrullus lanatus* cv. Crimson sweet.

Seedling age (days)	Number of explants with shoots[(1)]	
	Proximal	Distal
1	0.50±0.92Aa	0.0Ba
3	3.25± 2.37Ab	1.00±1.0Bb
5	0.75±0.88Aa	0.25±0.46Ba

[(1)]Each value represents the mean of 10 replications, a total of 80 explants per treatment; means followed by the same letter do not differ significantly (Tukey, p = 0.01) (lower-case in columns and upper-case in line).

effective for cucurbit organogenesis (Srisvastava et al., 1989; Compton & Gray, 1993), inducing multiple adventitious shoot bud differentiation. Chatuverdi & Bhatnagar (2001) stated that 2-isopentenyladenine (2iP) acts synergistically with BAP, improving organogenesis rates in watermelon cv. Sugar Baby. The combination of BAP (1 mg L^{-1}) with 10% coconut water favored the morphogenesis, resulting in a higher number of explants with shoots and of plantlets developed (Table 2). Although no significant differences were detected between this treatment and 2 mg L^{-1} BAP, the use of a lower growth regulator concentration (1 mg L^{-1} BAP + coconut water) seems to be more adequate, in order to avoid somaclonal variation.

The use of either TDZ or the combination of BAP and NAA caused a drastic decrease in the frequency of explants with shoot buds. Dong & Jia (1991) reported an improvement in shoot bud development in watermelon cotyledons, when combining cytokinin and auxin in the induction media, but both Compton & Gray (1993) and Srisvastava et al. (1989) detected an inhibition of shoot organogenesis, when NAA or indole-3-acetic acid (IAA) was added to the induction medium. TDZ has been reported to stimulate organogenesis in several crops (Zhang et al., 2001; Fratini & Ruiz, 2002), but this does not seem to be the case for watermelon, as Compton & Gray (1993) also reported a lower number of explants with shoot buds, using culture media supplemented with this growth regulator.

An accurate determination of the number of buds developed per explant is very difficult, because the organogenesis occurs in clusters and not as individual buds (Figure 1 d-f). Transferring the explants with multiple shoots to elongation and rooting media allowed the development of complete plants ready for acclimatization (Figure 1 e-g, Table 2).

Table 2. Effect of BAP concentrations and association with coconut water (10%) on the number of explants with shoots, the number of shoots per responsive explant, and the number of developed plantlets of *Citrullus lanatus* cv. Crimson sweet.

BAP (mg L^{-1})	Explants with shoots[(1)]	Shoots per responsive explant[(1)]	Plantlets/ shoots
0.0	0.0a	0.0a	0/0
1.0	0.50±0.7bc	3.37±2.28b	25/27
1.0 + coconut water	2.20±1.23d	4.39±2.68b	51/94
2.0	1.67±1.5cd	3.46±2.68b	42/50
3.0	1.00±1.41bc	4.60±2.70b	43/69

[(1)]Each value represents the mean of 10 replications per treatment, a total of 80 explants per treatment; means followed by the same letter do not differ significantly (Tukey, p = 0.01).

Although a high number of multiple shoot buds apparently formed on the explants, very few buds were found when a histological study of cultures was done (Figure 1 h-i), in a similar way to the one reported for other cucurbit species (Gaba et al., 1999; Stipp et al., 2001). Adventitious shoots developed directly from the explant epidermal and subepidermal layers, without callus formation. These adventitious shoots were characterized by the development of shoot apical meristem and leaf primordia with trichomes (Figure 1 h-i). Protuberances with trichomes (Figure 1 j) which do not develop into buds were formed, indicating that these structures could be leaf primordia. Structures from which only leaves are developed have been described in cucurbit organogenesis (Gaba et al., 1999; Stipp et al., 2001; Yalcin-Mendi et al., 2003). This explains the apparently large number of buds observed during induction, resulting in a low number of plants after transfer to elongation medium.

Conclusion

In vitro morphogenesis of watermelon cotyledon segments can be obtained from explants collected from the proximal region of three-day-old cotyledons of in vitro germinated seedlings, on a culture media supplemented with BAP and coconut water.

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