In vitro organogenesis of zucchini squash cv. Caserta

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ABSTRACT

A protocol for the *in vitro* culture of *Cucurbita pepo* cv. Caserta was studied, using a cotyledon segment with an attached hypocotyl fragment as an explant. First, to determine the optimal seedling age, explants were collected from 4 to 6-day-old *in vitro* germinated seedlings and cultured in MS basal medium supplemented with benzylaminopurine (BAP, 4.5 µM), under a 16-h photoperiod at 27°C. Based on the results obtained, the explants collected from the 4-day-old seedlings were then cultured in MS basal medium supplemented with different concentrations of BAP (0, 1.1, 2.2, 3.3, 4.5, or 5.5 µM) and incubated under a 16-h photoperiod at 27°C. *In vitro* organogenesis was most efficient with explants collected from 4-day-old seedlings cultured in medium supplemented with 4.5 µM of BAP. After 4 weeks of incubation the development of adventitious buds at the cotyledon/hypocotyl junction could be observed. These buds were transferred to elongation and rooting medium and the developed plants were acclimatized to greenhouse conditions. The morphogenic process was characterized using light and scanning electron microscopy analyses to confirm the organogenesis. The results showed that this alternate explant is efficient for *in vitro* culture of zucchini squash cv. Caserta. The protocol will be further examined for future use in genetic transformation experiments in this species.

Keywords: adventitious buds, cotyledon attached to hypocotyl, *Cucurbita pepo, in vitro* culture.

RESUMO

Organogênese *in vitro* de abobrinha-de-moita cv. Caserta

O objetivo do trabalho foi estudar um protocolo para o cultivo *in vitro* de *Cucurbita pepo* cv. Caserta utilizando como explante um segmento de cotilédone associado a um fragmento do hipocótilo. Explantes foram coletados de plântulas germinadas *in vitro* com 4 a 6 dias de idade, cultivados em meio de cultura basal MS suplementado com benzilaminopurina (BAP, 4,5 µM), e incubados sob fotoperíodo de 16 h, a 27°C. Com base nos resultados obtidos, explantes coletados de plântulas germinadas *in vitro* com 4 dias de idade foram cultivados em meio de cultura MS, suplementados com diferentes concentrações de BAP (0; 1,1; 2,2; 3,3; 4,5 ou 5,5 µM), e incubados sob fotoperíodo de 16 h, a 27°C. Após 4 semanas de incubação, foi possível detectar o desenvolvimento de gemas adventícias na junção do cotilédone e hipocótilo. As gemas adventícias desenvolvidas foram transferidas para meio de cultura de alongamento e enraizamento e as plantas obtidas foram aclimatizadas para condições de casa-de-vegetação. O processo morfogênico foi caracterizado por análises de microscopia ótica e eletrônica de varredura, confirmando a organogênese. Os resultados obtidos mostram a eficiência do protocolo no cultivo *in vitro* de abobrinha-de-moita cv. Caserta utilizando este explante alternativo. O protocolo será testado, no futuro, para experimentos de transformação genética desta espécie.

Palavras-chave: *Cucurbita pepo*, cotilédone ligado ao hipocótilo, cultivo *in vitro*, gema adventícia.

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The Cucurbitaceae are an important plant family worldwide, comprising more than 120 genera (Teppner, 2004). In Brazil, four genera are cultivated: *Cucumis*, *Citrullus*, *Sechium*, and *Cucurbita* (Queiroz, 2004). The genus *Cucurbita* includes three economically important species: *C. moschata*, *C. maxima*, and *C. pepo* (Paris, 2005).

The producers and consumers in Brazil prefer *Cucurbita pepo* with a commercial production of 19,690,000 tons in 2010 (Ceagesp, 2011). This production is primarily of cv. Caserta and is concentrated in the Southeast region (Filgueira, 2003). The states of São Paulo and Minas Gerais are the leading producers (Camargo Filho *et al.*, 2003).

A wide range of pathogens hamper the cultivation of *C. pepo*, with more than eight different viruses infecting the crop (Kurozawa & Pavan, 1997). Among these viruses, *Papaya ringspot virus* (PRSV) and *Zucchini yellow mosaic virus* (ZYMV) are the most important due to their prevalence and the degree of damage caused, which reduces crop and fruit quality. The control of these viruses is difficult and conventional breeding has not succeeded in producing new varieties with resistance to more than one virus species (Gaba *et al.*, 2004).

Recently, genetic engineering has been presented as an alternative for obtaining plants that are resistant to viral diseases; this approach has been widely applied in cucurbit breeding (Tricoli *et al.*, 1995; Compton *et al.*, 2000; Klas *et al.*, 2006). However, the use of this biotechnological tool requires an efficient *in vitro* culture system that allows plant regeneration.

An *in vitro* culture system for *C. pepo* was first described using hypocotyl segments as explants, with plant regeneration occurring via somatic embryogenesis (Jelaska *et al.*, 1985). Due to the difficulties in obtaining and culturing somatic embryos, alternative protocols for plant regeneration via organogenesis have been studied. These protocols used cotyledon or hypocotyl segments collected from *in vitro* germinated seedlings, explants of high organogenic competence. Although these protocols succeeded in plant regeneration, the efficiency was low, and the development of structures with multi-fold leaves and no shoot apex was common (Kim *et al.*, 2000).

To improve adventitious bud

formation and plant development Ananthakrishnan *et al.* (2003) suggested the use of a novel explant consisting of a cotyledon segment with an attached hypocotyl fragment. These explants were collected from *in vitro* germinated seedlings, and the shoot apices were carefully removed.

The present work is intended to examine the efficiency of the use of an explant cotyledon with an attached hypocotyl fragment in the *in vitro* organogenesis of the Brazilian *C. pepo* cv. Caserta (zucchini squash). The age of the seedlings used for the explants and the cytokinin culture medium supplementation were evaluated. The morphogenesis was characterized through histological analyses.

MATERIAL AND METHODS

The mature seeds of *C. pepo* cv. Caserta (CAC Melhorada; Sakata, Brazil) were peeled and surface sterilized in a hypochlorite solution (0.6%) for 15 minutes, followed by 3 rinses with sterilized distilled water. The seeds were germinated on MS basal medium (Murashige & Skoog, 1962) incubated under a 16-h photoperiod (63 µmol $m^{-2}s^{-1}$) at 27°C. The explant preparation was performed under a laminar flow hood using a stereo microscope. Each explant consisted of a cotyledon segment with an attached hypocotyl fragment. The explants were collected from seedlings germinated for 4-6 days and cultured in MS basal medium supplemented with benzylaminopurine (BAP 4.5 µM).

Based on the results obtained by experimentally varying the age of the seedlings, explants collected from 4-day-old seedlings were cultivated in Petri dishes (100 x 15 mm) containing MS basal medium supplemented with BAP (0, 1.1, 2.2, 3.3, 4.5, or 5.5 µM). An evaluation was performed after 4 weeks of incubation and the mean number of responsive explants per treatment was determined. The experimental design was completely randomized with 7 replicates. Each replicate consisted of one Petri dish with 6 explants, totaling 42 explants per treatment. The material was incubated at 27°C under a 16-h photoperiod (63 µmol $m^{-2}s^{-1}$). The experiments were repeated twice, and the data were subjected to regression analyses. The adventitious buds developed were transferred to MS basal medium supplemented with BAP (0.44 µM) and gibberellic acid (GA_3 2.88 µM) for shoot elongation. Shoots grown to 1 cm in length were transferred to MS basal medium supplemented with indole-butyric acid (IBA-4.9 µM) for rooting. Plantlets grown to 5 cm in length were acclimatized to greenhouse conditions.

The explants cultivated in induction medium containing 4.5 µM BAP were sampled after 20 days in culture. For light microscopy (LM), the samples were fixed in paraformaldehyde (4%) under refrigeration, for 5 days. Dehydration was conducted at room temperature in a series consisting of 100% methylcellosolve, ethanol, propanol, and butanol, followed by infiltration in a butanol:infiltration medium (1:1) mixture (Historesin, Leica, Heidelberg, Germany) at 4°C overnight. The infiltration was completed in 100% infiltration medium for 48 h. Polymerization was conducted at room temperature for 24-48 h. Serial sections (5 µm) were prepared using a rotary microtome (Leica RM2155) with a steel carbide knife. The sections were floated on water droplets and dried on a hot plate (40°C). The material was stained with toluidine blue (0.05%) and rinsed in distilled water for general observation. For scanning electronic microscopy (SEM), the samples were fixed in a glutaraldehyde (2%) sodium cacodylate buffer (0.1 M, pH 7.2). After fixation, the samples were dehydrated in an ethanol series and critical point-dried using carbon dioxide. These samples were mounted on aluminum stubs, sputter coated with gold (30-40 nm), and observed under a LEO 0P435 electronic microscope (Carl Zeiss, Jena, Germany) at 20 kV. For both light and scanning microscopy five explants were sampled and analyzed.

RESULTS AND DISCUSSION

The highest number of responsive zucchini squash explants was obtained with those collected from 4-day-old seedlings (50%). The percentage of responsive explants decreased to 27.8 and 11.1% with the use of 5 and 6-day-old seedlings, respectively. The age of the seedlings used for the explants is an important factor in cucurbit *in vitro* regeneration. The use of seedlings older than 4-5 days for collecting the explants, resulted in a decrease in the rate of shoot induction for winter squash (*C. maxima*) (Lee *et al.*, 2003), watermelon (*Citrullus lanatus*) (Compton, 2000), *C. moschata* x *C. maxima* (Sarowar *et al.*, 2003), and *Cucumis sativus* (Kim *et al.*, 2000). This result was also confirmed by our data using *C. pepo* cv. Caserta, showing that the explant age has a profound effect on *in vitro* organogenesis.

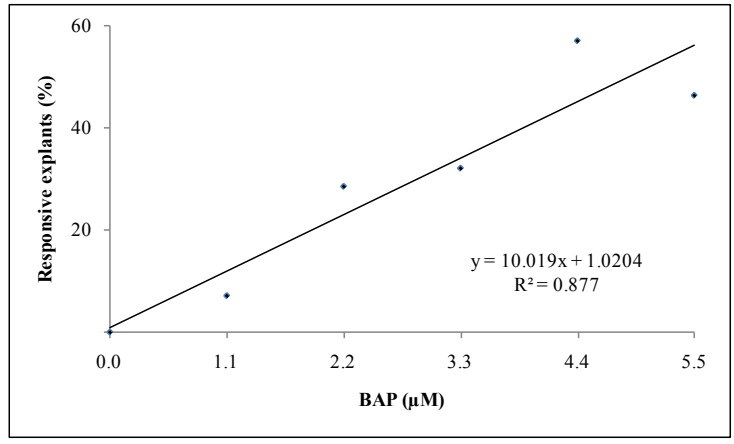

Figure 1. Efficiency of *in vitro* organogenesis of *Cucurbita pepo* cv. Caserta considering culture media supplementation with different concentrations of benzylaminopurine (BAP) (eficiência da organogênese *in vitro* de *Cucurbita pepo* cv. Caserta considerando-se a suplementação do meio de cultura com diferentes concentrações de benzilaminopurina (BAP)). Piracicaba, CENA-USP, 2009.

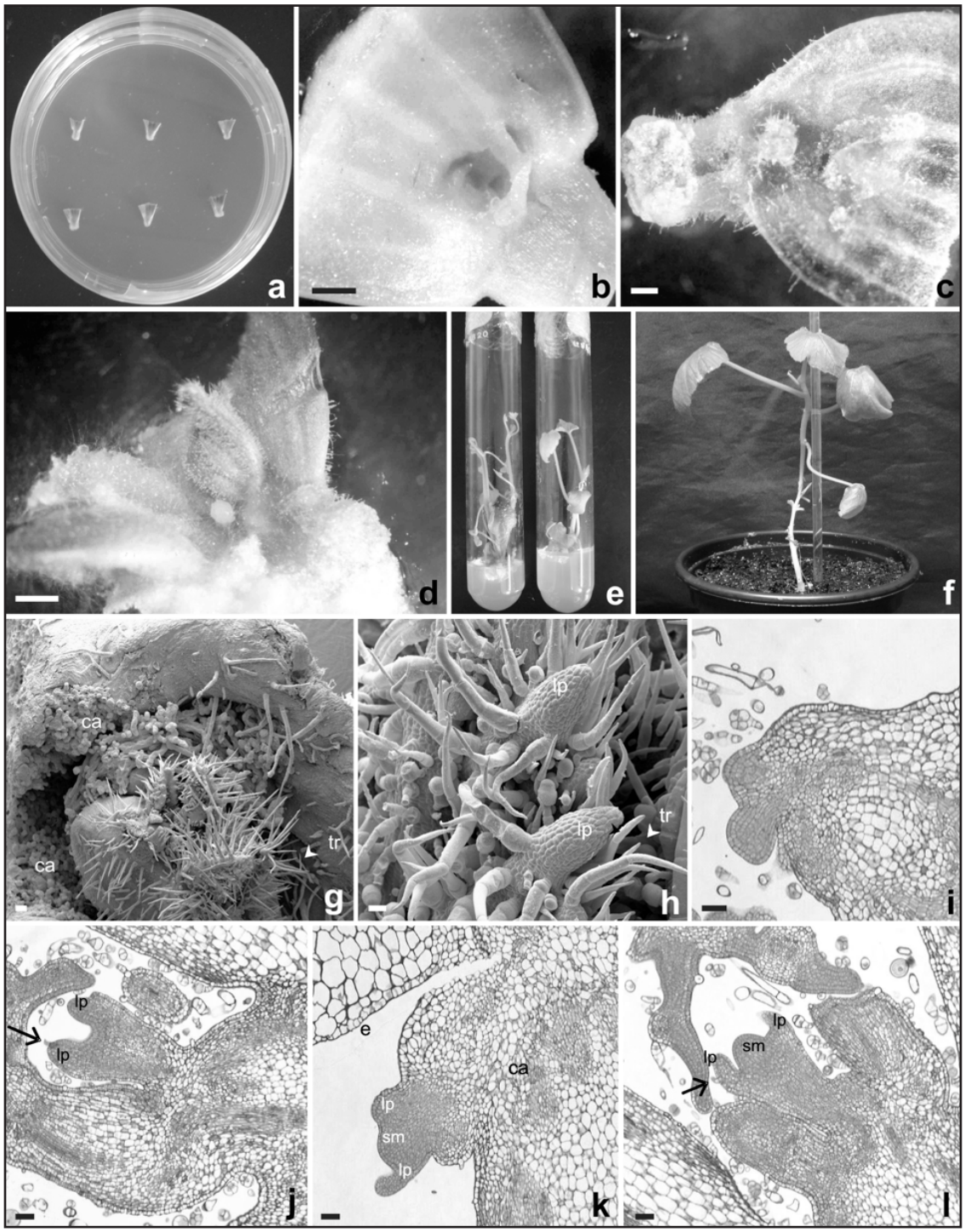

Figure 2. Stages of *in vitro* organogenesis of *Cucurbita pepo* cv. Caserta (estágios da organogênese *in vitro* de *C. pepo* cv. Caserta); a-b) initial explant consisted of cotyledon segment with a hypocotyl fragment attached (explante inicial, segmento de cotilédone associado a fragmento do hipocótilo); c) callus development after 7-10 days in culture (desenvolvimento de calo após 7-10 dias de cultura); d) adventitious bud developed after four weeks in culture (desenvolvimento de gema adventícia após 4 semanas de cultura); e) plantlet developed in elongation and rooting culture medium (plântula desenvolvida em meio de cultura para alongamento e enraizamento); f) acclimatized plant (planta aclimatizada); g-h) scanning electron micrographs showing adventitious bud development with tricomes (microscopia eletrônica de varredura mostrando o desenvolvimento de gemas adventícias com tricomas); i–l) histological sections of adventitious buds showing explants (e) leaf primordial (lp), shoot apical meristem (sm), callus (ca) development (i-l) corte histológico de gemas adventícias mostrando explante (e), primórdio foliar (lp), meristema apical (sm) e desenvolvimento de calo (ca)). Bars (barras) = 30 µm (h); 50 µm (i-l); 100 µm (g); 200 µm (c); 1 mm (b, d). Piracicaba, CENA-USP, 2009.

The supplementation of the culture medium with cytokinin was essential for adventitious bud development in this work. In the absence of BAP, the explants did not exhibit any shoot growth, and the number of responsive explants increased with BAP concentrations up to 4.5 µM (Figure 1).

The in *vitro* organogenesis of cucurbits has been performed using cotyledon segments as explants. Although shoot development occurs quite frequently, shoot elongation and rooting are difficult (Stipp *et al.*, 2001; Yalcin-Mendi *et al.*, 2003; Krug *et al.*, 2005). Ananthakrishnan *et al.* (2003) reported the successful use of a new type of explant for the *in vitro* organogenesis of *C. pepo*. The data presented in our work confirm the efficacy of this new explant (cotyledon segment with an attached hypocotyl fragment) in the organogenesis of *C. pepo* cv. Caserta, the most commercially important zucchini squash cultivar in Brazil. Figures 2a-b illustrate the use of a cotyledon segment with an attached hypocotyl fragment as an explant in these experiments. After 3-4 days in culture, the explants had become enlarged and turned green. After 7-10 days, callus formation could be detected at the junction between the hypocotyl and cotyledon (Figure 2c). Adventitious bud development could be detected after 4 weeks in culture at the cotyledon/hypocotyl junction (Figure 2d). The efficacy of this new cucurbit explant could also be associated with the presence of the proximal part of the cotyledon. This region has shown a high competence for shoot bud development in *C. maxima* (Lee *et al.*, 2003), *C. pepo* (Ananthakrishnan *et al.*, 2003), and *Citrullus lanatus* (Compton & Gray, 1993).

The efficiency of explants regeneration also depends on the genotype, as not all cultivars of a genus or species respond equally well to a specific procedure. Kathiravan *et al.* (2006) conducted experiments with 15 *C. pepo* cultivars and reported an explant responsiveness ranging from 22-94%, with a rate of shoot regeneration per responding explant of 1.2-3.9. Our data with cv. Caserta showed that an explant responsiveness of 57% is a good proportion considering that most of the developed shoots elongated and generated complete plants with a mean of 3.6 plants per responsive explants. The plants obtained after transferring the shoots to elongation and rooting media (Figure 2e) were acclimatized with success (Figure 2f).

The characterization of the process using microscopical analyses confirmed the *de novo* regeneration through the organization of meristematic regions and further adventitious bud development, with an apical meristem, leaf primordia and vascular connections in the explant tissue (Figures 2i-l). The analyses using scanning electron microscopy showed bud development through the callus phase from the junction between the hypocotyl and cotyledon with numerous trichomes (Figures 2g-h).

The histological analyses revealed the development of monopolar structures with vascular connections in the original explant tissue, which is characteristic of the organogenic process. Notably, the development of protuberances and multi-fold leaves with no development of the shoot apex was not observed, as has been reported for cucurbit species in previous studies (Kim *et al.*, 2000; Stipp *et al.*, 2001; Yalcin-Mendi *et al.*, 2003). The search for explants that are more adequate for *in vitro* organogenesis was also reported for passion fruit (Fernando *et al.*, 2007). In this crop, the low frequency of *in vitro* bud elongation was likely a consequence of the regeneration of leaf structures on the surfaces of explants, which can be erroneously interpreted as buds that do not elongate (Fernando *et al.*, 2007).

The *in vitro* organogenesis of zucchini squash cv. Caserta was obtained using a novel explant comprising a cotyledon segment with an attached hypocotyl fragment. The *in vitro* regeneration protocol presented in this work for zucchini squash cv. Caserta is potentially useful for the genetic transformation of this species, to obtain transgenic plants resistant to viral diseases.

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REFERENCES

ANANTHAKRISHNAN G; XIA X; ELMAN C; SINGER S; GAL-ON A; GABA V. 2003. Shoot production in squash (*Cucurbita pepo*) by *in vitro* organogenesis. *Plant Cell Reports* 21: 739-746.

CAMARGO FILHO WPD; MAZZEI AR; ALVES HS. 2003. Mercado de abóboras nas cidades de São Paulo e Buenos Aires: oportunidades de expansão. *Informações Econômicas* 33: 61-65.

CEAGESP - Companhia de Entrepostos e Armazéns Gerais de São Paulo, 2008. Disponível em www.ceagesp.gov.br, 17/01/2011.

COMPTON ME. 2000. Interaction between explant size and cultivar impacts shoot organogenic competence of watermelon cotyledons. *Hortscience* 35: 749-750.

COMPTON ME; GRAY DJ. 1993. Shoot organogenesis and plant regeneration from cotyledons of diploid, triploid, and tetraploid watermelon. *Journal of the American Society for Horticultural Science* 118: 151-157.

COMPTON ME; GRAY DJ; GABA VP. 2000. Use of tissue culture and biotechnology for the genetic improvement of watermelon. 2004. *Plant Cell, Tissue and Organ Culture* 77: 231-243.

FERNANDO JA; VIEIRA MLC; MACHADO SR; APPEZZATO-DA-GLÓRIA B. 2007. New insights into the *in vitro* organogenesis process the case of *Passiflora*. *Plant Cell, Tissue and Organ Culture* 91: 37-44.

FILGUEIRA FAR. 2003. *Novo manual de Olericultura: agrotecnologia moderna na produção e comercialização de hortaliças.* 2ª ed. Viçosa:UFV, 412p.

GABA V; ZELCER A; GAL-ON A. 2004. Cucurbit biotechnology - the importance of virus resistance. *In Vitro Cellular and Developmental Biology* 40: 346-358.

JELASKA S; MAGNUS V; SERENTIN M; LACAN G. 1985. Induction of embryogenic callus in *Cucurbita pepo* hypocotyl explants by indole-3-ethanol and sugar conjugates. *Physiologia Plantarum* 64: 237-242.

KATHIRAVAN K; VENGEDESAN G; SINGER S; STEINITZ B; PARIS HS; GABA V. 2006. Adventitious regeneration in vitro occur across a wide spectrum of squash (*Cucurbita pepo*) genotypes. *Plant Cell, Tissue and Organ Culture* 85: 285-295.

KIM JW; HAN SK; KNON SY; LEE HS; LIM YP; LIU JW; KWAK SS. 2000. High frequency shoot induction and plant regeneration from cotyledonary hypocotyl explants of cucumber (*Cucumis sativus* L.) seedlings. *Journal Plant Physiology* 157: 136-139.

KLAS FE; FUCHS M; GONSALVES D. 2006. Comparative spatial spread overtime of *Zucchini yellow mosaic virus* (ZYMV) and *Watermelon mosaic virus* (WMV) in fields of transgenic squash expressing the coat protein genes of ZYMV and WMV, and in fields of

nontransgenic squash. *Transgenic Research* 15: 527-541.

KRUG MGZ; STIPP LCL; RODRIGUEZ APM; MENDES BMJ. 2005. *In vitro* organogenesis of *Citrullus lanatus*. *Pesquisa Agropecuária Brasileira* 40: 861-865.

KUROZAWA C; PAVAN MA. 1997. Doenças das cucurbitáceas. In: KIMATI H; AMORIM L; BERGAMIN FILHO A; CAMARGO LEA; REZENDE JAM (eds). *Manual de Fitopatologia: doenças das plantas cultivadas*. 3ed. São Paulo: Editora Agronômica Ceres. 2: 325-337.

LEE YK; CHUNG WI; EZURA H. 2003. Efficient plant regeneration via organogenesis in winter squash (*Cucurbita maxima* Dush.). *Plant Science* 164: 413-418.

MURASHIGE T; SKOOG F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* 15: 473-497.

PARIS HS.2005. The genes of pumpkin and squash. *Hortscience* 40:1620-1630.

QUEIROZ MA. 2004. Germplasm of Cucurbitaceae in Brazil. *Crop Breeding and Applied Biotechnology* 4: 377-383.

SAROWAR S; OH HY; HYUNG NI; MIN BW; HARN CH; YANG SK; OK SH; SHIN JS. 2003. *In vitro* micropropagation of a *Cucurbita* interspecific hybrid cultivar - a root stock plant. *Plant Cell, Tissue, Organ and Culture* 75: 179-182.

STIPP LCL; MENDES BMJ; PIEDADE S; RODRIGUEZ APM. 2001. *In vitro* morphogenesis of *Cucumis melo* var. *inodorus*. *Plant Cell, Tissue, Organ and Culture* 65: 81-89.

TEPPNER H. 2004. Notes on Lagenaria and Cucurbita (*Cucurbitaceae*) - Review and new contributions. *Phyton* 44: 245-308.

TRICOLI DM; CARNEY KJ; RUSSEL PF; MCMASTER JR; GROFF DW; HADDEN KC; HIMMEL PT; HABBARD JP; BOESHORE ML; REYNOLDS JF; QUEMADA HD. 1995. Field evaluation of transgenic squash containing single or multiple virus coat protein gene constructs for resistence to *cucumber mosaic virus*, *watermelon mosaic virus*. *Nature Biotechnology* 13: 1458-1465.

YALCIN-MENDI NY; IPEK M; KACAN H; CURUK S; SARI N; CETINER S; GABA V. 2003. A histological analysis of regeneration of watermelon. *Journal Plant Biochemistry and Biotechnology* 12: 147-150.