BIOLOGIA PLANTARUM 49 (2): 169-173, 2005

Direct differentiation of somatic embryos on cotyledons of *Azadirachta indica*

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Abstract

Somatic embryos regenerated in high-frequency (up to 100 %) on immature cotyledons of *Azadirachta indica* at a low concentration of thidiazuron (TDZ; 0.5 μM). Regeneration occurred exclusively from abaxial surface. Frequency of regeneration declined with the age of the cotyledons: on semimature cotyledons regeneration occurred only from some regions of the abaxial surface and on mature cotyledons it was confined to corners. However, an increase in concentration of TDZ to 1.0 μM improved the regeneration response. Higher regenerative capacity of immature cotyledons was due to presence of milk in immature fruits because regeneration response of immature cotyledons declined on washing of cotyledons for 24 h in liquid medium, and milk from immature fruits augmented the regeneration response of mature cotyledons. Somatic embryos regenerated readily on hormone-free medium and plantlets derived were able to survive after transfer to soil.

Additional key words: azadirachta milk, coconut milk, immature and mature cotyledons, thidiazuron.

Introduction

Somatic embryogenesis can follow two routes; indirect and direct. In indirect embryogenesis the somatic cells, in response to an auxin divide and form a tissue which differentiates into somatic embryos. Contrary to it in direct somatic embryogenesis, the embryos differentiate directly on the explant; without a callus interphase. Indirect somatic embryogenesis is recorded in tissue cultures of a large number of genera (*e.g.* D′Onofrio and Morini 2003/4), whereas direct embryogenesis is relatively rare (Raghavan 1997, Bobák *et al*. 2003/4). Direct embryogenesis is advantageous for true cloning of plants, because the chances of a change in genotype are minimized (Peshke and Phillips 1992).

Direct embryogenesis was observed on cotyledons of *Juglans* spp. (Tulecke and McGrahanan 1985, Neuman *et al*. 1993), *Carya illinoensis* (Merkle *et al*. 1987), *Fraxinus americana* (Preece *et al*. 1987, Bates *et al*. 1992), *Citrullus lanatus* (Compton and Gray 1992), *Arachis hypogaea* (Gill and Saxena 1992, Baker and Wetzstein 1994), *Azadirachta indica* (Murthy and Saxena 1998) and *Panax ginseng* (Choi *et al*. 1999). Of these, cotyledons of *P. ginseng* are exceptional in embryogenesis on hormone-free medium. For embryogenesis in *Fraxinus* cytokinin benzylaminopurine (BAP) and 2,4-dichlorophenoxyacetic acid (2,4-D) were required (Preece *et al*. 1987), later it was shown that thidiazuron (TDZ) could substitute BAP (Bates *et al*. 1992). Also in *J. nigra* TDZ and 2,4-D were required (Neuman *et al*. 1993) whereas in *Arachis hypogaea* 2,4-D or naphthalene acetic acid (NAA) was effective (Baker and Wetzstein 1994). As for TDZ, it was alone effective for somatic embryogenesis in *Arachis* (Gill and Saxena 1992) and *Azadirachta indica* (Murthy and Saxena 1998) and BAP supported shoot formation (Murthy and Saxena 1998, Salvi *et al*. 2001). Another outcome of these investigations is high regenerative potential of immature cotyledons of *Juglans*, *Carya, Citrullus, Arachis* and *Panax*, as compared to mature cotyledons.

Employing TDZ as the sole stimulus for direct somatic embryogenesis on cotyledons of *Azadirachta* the questions addressed in this communication are: *1*) Is there dependency of regeneration response on age of cotyledon

Received 5 July 2003, *accepted* 23 September 2004.

Abbreviations: AzM - azadirachta milk; BAP - 6-benzylaminopurine; CM - coconut milk; 2,4-D - 2,4-dichlorophenoxyacetic acid; MS medium - Murashige and Skoog medium; NAA - naphthalene acetic acid; TDZ - thidiazuron.

Acknowledgements: One of authors (AG) is thankful to CSIR for the award of a JRF.

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and concentration of stimulus? *2*) Is there cell- or tissue-specificity for somatic embryogenesis? *3*) Is there loss of regeneration, in mature cotyledons and if so can it be retrieved? *4*) Why immature cotyledons are more regenerative than mature cotyledons? *5*) What is the role of milk from immature fruits in regeneration? None of these aspects is covered in an earlier investigation on *Azadirachta* (Murthy and Saxena 1998) because mature cotyledons from seeds were employed for regeneration. Also in other plants worked out, cited above, one or more of these aspects are lacking. In particular, there is no explanation for higher frequency of regeneration by immature cotyledons.

Materials and methods

Seeds of neem (*Azadirachta indica* L.), freely-growing tree in the rural landscape of India, were washed with Teepol (1:1 v/v) for 10 min and then dipped in 70 % ethanol for 30 s. After rinsing with sterile distilled water seeds were dissected and intact cotyledons cultured aseptically on MS (Murashige and Skoog 1962) mineral medium with 0.8 % *Difco* agar, 100.00 mg dm^{-3} inositol, 2.0 mg dm^{-3} glycine, 0.4 mg dm^{-3} thiamine-HCl, 0.5 mg dm^{-3} pyridoxine-HCl, 0.5 mg dm^{-3} nicotinic acid, 2 % sucrose, and different concentrations of thidiazuron (TDZ, N-phenyl-N'-1,2,3 thidiazol-5-yl urea). The pH of the medium was adjusted to 5.8, prior to autoclaving. Other additives included milk from immature fruits of *Azadirachta* or milk from immature fruits of *Cocos nucifera* (CM). The treatments to cotyledons, prior to culture, included immersion of *a*) immature cotyledons into mineral medium and *b*) mature cotyledons in sterile 1 M sucrose solution for 24 h.

Cultures were raised in screw cap vials of 15 cm^3 capacity, each having 2 cm^3 of nutrient medium, and maintained in a culture room at 25 ± 2 °C and continuous light (fluorescent tubes 36 W/54, 6500 K; irradiance of 15 μmol $m^{-2}s^{-1}$). For every treatment 50 cultures, each with one cotyledon, were raised.

Results

Immature cotyledons from stage I of fruit development, characterized by its milky contents, formed somatic embryos within a week in 90 - 100 % cultures at 0.5 μM TDZ (Fig. 1). Embryogenesis was, however, dependent on the placement of cotyledons on the medium. When cotyledons were cultured with their abaxial surface in contact with the medium, the entire lower surface responded to form numerous embryoids, without callusing. These structures (Fig. 2*A*), passing through globular-shape (Fig. 2*B*) to heart-shape (Fig. 2*C*) stages progressed into well-developed somatic embryos (Fig. 2*D*). At times there was regeneration of secondary somatic embryos and regeneration of somatic embryos into shoots. At a lower concentration of TDZ (0.1μM) there was reduced response; 10 - 30 % cultures responded to form a lower number of embryoids from the abaxial surface of cotyledons. When cotyledons were cultured with their adaxial surface facing the medium the response was reduced to 5 % of cultures forming a few embryoids, again, from their abaxial surface.

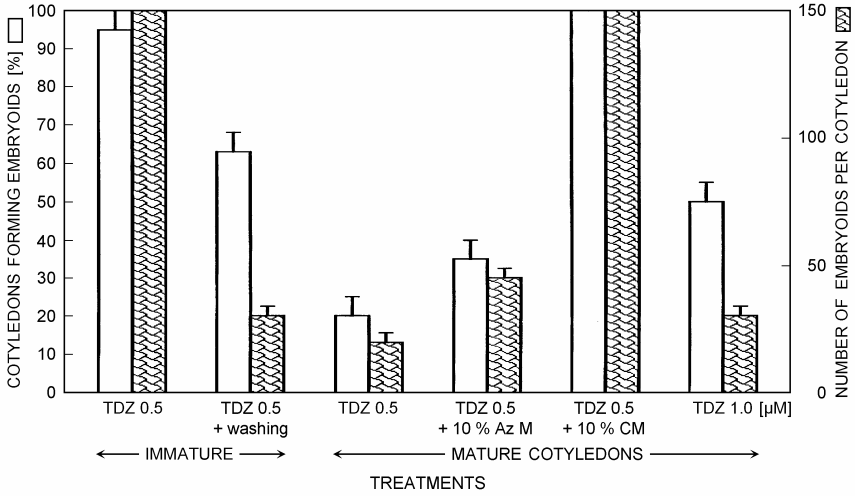

Fig. 1. *Azadirachta indica,* regeneration response of immature and mature cotyledons under different cultural conditions (*error bars* denote SE, *n* = 50).

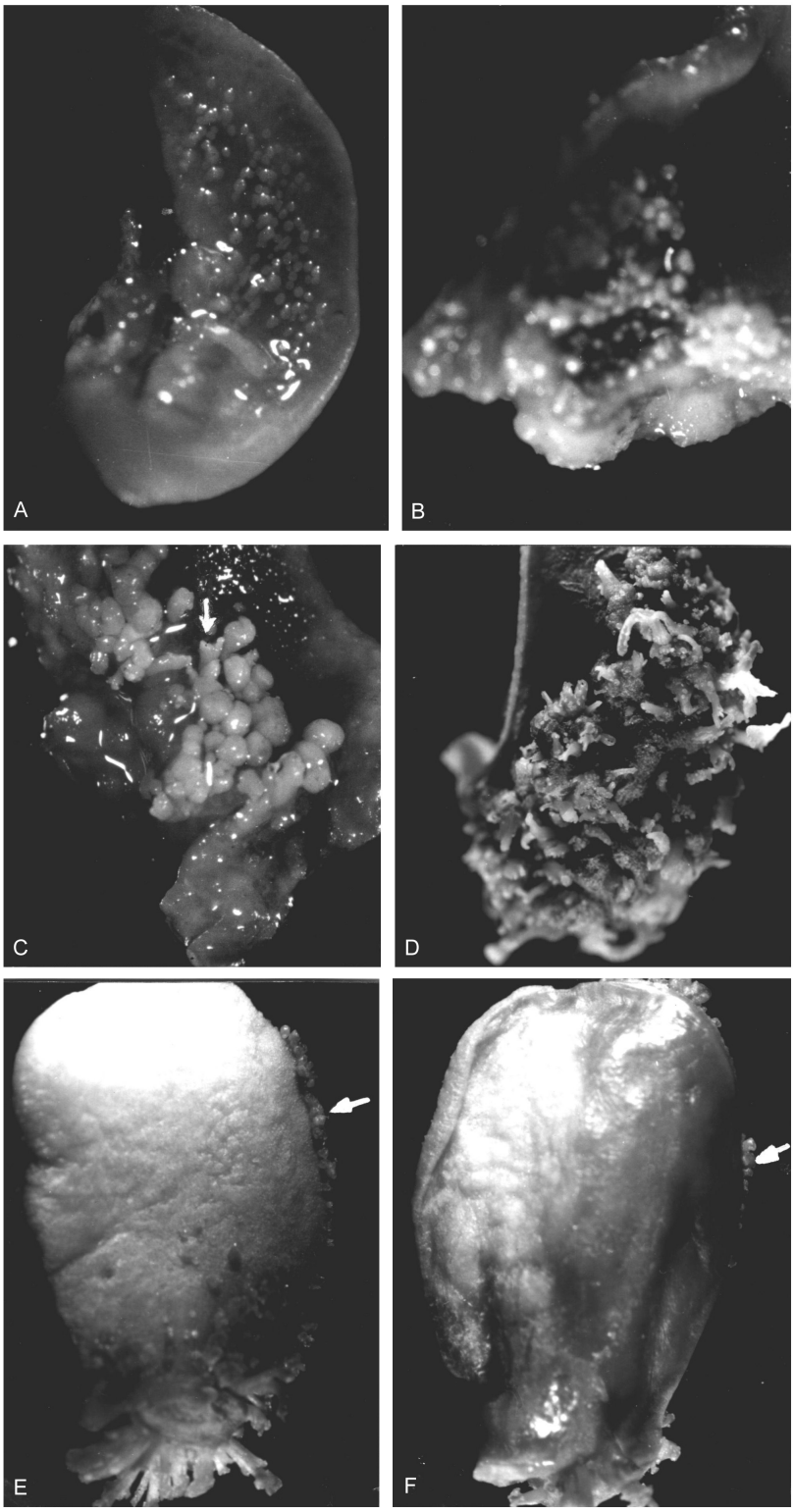

Fig. 2. Induction of somatic embryos on cotyledons at 0.5 μM TDZ. *A* - immature cotyledon, from 5-d-old culture, showing the beginning of somatic embryogenesis on the abaxial surface; *B* - the same, from 7-d-old culture, with globular stage somatic embryos; *C* - the same, from 10-d-old culture, with somatic embryos; in globular and heart-shape (*arrow*) stages; *D* - the same, from 15-d-old culture, with mature somatic embryos; *E* - semimature cotyledon, showing regeneration of somatic embryos from basal end and margin (*arrow*, this photograph is taken from dorsal surface); *F* - mature cotyledon, showing poor regeneration from basal end and margin (*arrow*, this photograph is taken from dorsal surface).

Semimature cotyledons from stage II of fruit development, characterized by the stony seed coat, formed somatic embryos in 30 to 50 % cultures at 0.5 μM TDZ. Instead of entire abaxial surface regenerating to form embryoids, these cotyledons regenerated from their basal region (Fig. 2*E*), which remains attached to the embryonal axis.

Mature cotyledons from stage III of fruit development, when it drops from the tree, were least responsive; 10 - 30 % cultures responded to form embryoids, characteristically from the corners (Fig. 2*F*), at 0.1 and 0.5μM of TDZ. However at 1.0 μM TDZ, a higher frequency of cotyledons (up to 50 %) responded to form embryoids (Fig. 1).

The presumption that high-frequency and high-density of regeneration from immature cotyledons from milky stage fruits was possibly due to milk from immature fruits turned out to be true when immature cotyledons were washed, by keeping these in liquid medium for 24 h, to remove the adhering and absorbed milk. On culture of these cotyledons a considerable reduction in frequency and propensity of regeneration was recorded (Fig. 1). Also mature cotyledons on culture to medium with a low TDZ (0.5 μM) and 10 % milk, obtained from immature fruits, regenerated at a higher frequency (Fig. 1). The regeneration of mature cotyledons at low TDZ (0.5 μM) could be further increased on medium fortified with 10 % CM.

Of the other factor affecting the response, a disturbance of cell-communication on preplasmolysis of mature cotyledons in 1 M sucrose solution and their culture on TDZ (0.5 μM) medium did not result in release of regeneration.

Somatic embryos formed on cotyledons readily regenerated to form plantlets which survived on transfer to soil.

Discussion

Direct differentiation of somatic embryos on cotyledons of *Azadirachta*, is dependent on age and surface of explants. Regeneration was possible only from the abaxial surface of cotyledons. This brings in cell-specificity for somatic embryogenesis and it remains to be resolved as to why cells from the abaxial surface alone are responsive to regeneration. Immature cotyledons from milky stage fruits were highly responsive and the regeneration response declined with the maturation of fruits.

Decline in regeneration response was marked by its restriction to basal region and some select patches and final confinement to corners of cotyledons, at a lower concentration of TDZ (0.5 μM). An increase in concentration of TDZ to 1.0 μM, not only reduced the time but also increased space for regeneration.

Present results, in terms of higher regeneration of immature cotyledons and refraction of mature cotyledons are in conformity with the results obtained with cotyledons of *Juglans* spp. (Tulecke and McGrahanan 1985, Neuman *et al*. 1993), *Carya illinoensis* (Merkle *et al*. 1987), *Citrullus lanatus* (Compton and Gray 1992), *Arachis hypogaea* (Gill and Saxena 1992, Baker and Wetzstein 1994) and *Panax ginseng* (Choi *et al*. 1999). However, in studies on somatic embryogenesis from immature cotyledons of the plants, cited above, there is no explanation for high regenerative capacity of immature cotyledons.

Loss of regeneration from mature cotyledons was possibly due to absence of milk in mature fruits. Supply of milk from immature fruits of *Azadirachta* or a growth-supporting substance such as coconut milk not only augmented the frequency of regeneration but also increased the propensity of regeneration. These results explain the high-frequency regeneration response of immature cotyledons and account for loss of regeneration from mature cotyledons. A reduced frequency of regeneration by immature cotyledons, on washing for 24 h in liquid medium, indirectly confirmed these results.

Regeneration from immature cotyledons of *Panax ginseng* (Choi *et al*. 1999) is restricted to the basal region. A pre-plasmolysis of these cotyledons extended the regeneration to all over the surface probably due to disturbance of cell-communication induced by such treatment (Choi *et al*. 1999). Plasmolysis of mature cotyledons of *Azadirachta* was, however, of no help in increasing either the frequency or propensity of regeneration. One of the possible reasons for this might be either ineffective plasmolysis due to fleshy nature of cotyledons or toxicity of plasmolytic agent, 1.0 M autoclaved sucrose. Sucrose on autoclaving results in formation of an inhibitory substance, such as 5-hydro-xymethyl furfural (Wetherhead *et al.* 1978).

References

Baker, C.M., Wetzstein, H.Y.: Influence of auxin type and concentration on peanut somatic embryogenesis. - Plant Cell Tissue Organ Cult. **36**: 361-368, 1994.

Bates, S., Preece, J.E., Navarrete, N.E., Sambeek, J.W. Van Gaffney, G.R.: Thidiazuron stimulates shoot organogenesis and somatic embryogenesis in white ash (*Fraxinus americana* L.). - Plant Cell Tissue Organ Cult. **31**: 21-29, 1992.

Bobák, M., Šamaj, J., Hlinková, E., Hlavačka, A., Ovečka, M.: Extracellular matrix in early stages of direct somatic embryogenesis in leaves of *Drosera spathulata*. - Biol. Plant. **47**: 161-166, 2003/4.

Choi, Y.E., Yang, D.C., Yoon, E.S., Choi, K.T.: High-efficiency plant production via direct somatic single embryogenesis from preplasmolysed cotyledons of *Panax ginseng* and possible dormancy of somatic embryos. - Plant Cell Rep. **18**: 493-499, 1999.

Compton, M.E., Gray, D.J.: Somatic embryogenesis on immature cotyledons of watermelon. - *In Vitro* cell dev. Biol. Plant **28**: 98A, 1992.

D´Onofrio, C., Morini, S.: Simultaneous regeneration of different morphogenic structures from quince leaves as affected by growth regulator combination and treatment length. - Biol. Plant. **47**: 321-325, 2003/4.

Gill, R., Saxena, P.K.: Direct somatic embryogenesis and regeneration of plants from seedlings explants of peanut *(Arachis hypogaea)*: promotive role of thidiazuron. - Can. J. Bot. **70**: 1186-1192, 1992.

Merkle, S.A., Wetzstein, H.Y., Sommer, H.E.: Somatic embryogenesis in tissue cultures of pecan. - Hort. Sci. **22**: 128-130, 1987.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue culture. - Physiol. Plant. **15**: 473-497, 1962.

Murthy, B.N.S., Saxena, P.K.: Somatic embryogenesis and plant regeneration of neem (*Azadirachta indica* A. Juss.). - Plant Cell Rep. **17**: 469-475, 1998.

Neuman, M.C., Preece, J.E., Sambeek, J.W., Van Gaffney, G.R.: Somatic embryogenesis and callus production from cotyledon explants of eastern black walnut. - Plant Cell Tissue Organ Cult. **32**: 9-18, 1993.

Peshke, V.N., Phillips, R.L.: Genetic implications of somaclonal variation in plants. - Adv. Genet. **30**: 41-75, 1992.

Preece, J.E., Christ, P.H., Ensenberger, L., Zhao, J.: Micropropagation of ash (*Fraxinus*). - Combined Proc. int. Plant Propag. Soc. **37**: 366-372, 1987.

Raghavan, V.: Somatic embryogenesis. - In: Raghavan, V. (ed.): Molecular Embryology of Flowering Plants. Pp. 467-499. Cambridge University Press, Cambridge - New York 1997.

Salvi, N.D., Singh, H., Tivarekar, S., Eapen, S.: Plant regeneration from different explants of neem. - Plant Cell Tissue Organ Cult. **65**: 159-162, 2001.

Tulecke, W., McGrahanan, G.: Somatic embryogenesis and plant regeneration from cotyledons of walnut. - Plant Sci. **40**: 57-63, 1985.

Wetherhead, M.A., Burdon, J., Henshaw, G.G.: Some effects of activated charcoal as an additive to plant tissue culture media. - Z. Pflanzenphysiol. **89**: 141-147, 1978.