Physiol Mol Biol Plants (October–December 2012) 18(4):387–392
DOI 10.1007/s12298-012-0130-x

SHORT COMMUNICATION

Induction of somatic embryogenesis in gum arabic tree [*Acacia senegal* (L.) Willd.]

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Published online: 17 August 2012

Abstract Factors affecting somatic embryogenesis from immature cotyledon of gum arabic tree [*Acacia senegal* (L.) Willd.] were investigated. Induction of somatic embryogenesis was influenced by plant growth regulator concentrations and addition of amino acids in medium. Best induction of somatic embryogenesis was obtained on MS medium supplemented with 0.45 μM 2, 4-D, 2.32 μM Kin and 15 mM L-glutamine. L-glutamine plays a significant role in the maturation of somatic embryos and most of embryos attained maturity only on L-glutamine (15 mM) containing medium. Maximum percent (75.0±2.5) germination of somatic embryos was recorded on medium containing 0.22 μM BAP.

Keywords *A. senegal* · L-glutamine · Plant growth regulators · Somatic embryo induction

Abbreviations

ABA	Abscisic acid
BAP	6-Benzylaminopurine
2, 4-D	2, 4 dichlorophenoxyacetic acid
Kin	Kinetin
MS	Murashige and Skoog (1962)
PEG	Poly ethylene glycol

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Introduction

Gum arabic tree (*Acacia senegal* (L.) Willd., family – Fabaceae) is an ecologically and economically important tree of arid and semiarid region of India, particularly of state Rajasthan. The plant is native to semi-desert regions of sub-Sahara Africa and mostly found in Sudano-Sahelian zone of Africa from Sudan to Senegal (Khalafalla and Daffalla 2008). It is a key component of Indian desert ecosystem and a well accepted agroforestry tree of Rajasthan because it checks soil erosion, protects agricultural land from animals, and increases soil fertility through symbiosis with *Rhizobium* and mycorrhiza (Badji et al. 1993; Singh and Pandey 1998). Seeds of plant are costly and used as dry vegetables (Singh and Pandey 1998). The stem yields a commercially important highly priced gum arabica, which is used in manufacture of chewing gum, as emulsifier in confectionary and binding agent in the pharmaceutical industries, and also has wide ranging applications in paint, ink and cosmetic industries. This plant is also well known for its medicinal values and tribes of state Rajasthan traditionally prescribe the gum as a part of food for diabetic patients (Singh and Pandey 1998).

Conventionally *A. senegal* is propagated through seeds, however poor germination and death of young seedlings in natural habitat limit the scope of seed propagation (Khalafalla and Daffalla 2008). Earlier, few studies also demonstrated the feasibility of cutting in vegetative propagation of this important tree (Badji et al. 1991; Danthu et al. 1992). However, success of cuttings in producing plants with well-developed roots was season dependent (Badji et al. 1991). Moreover, in majority of trees, propagation by cutting is often characterized by a rapid loss of rooting capacity of the cutting with increasing age of parent plant (Rai et al. 2010). Therefore, there is a great need to develop an efficient in vitro regeneration system, which not only fulfill the demand but also can apply in their improvement program. In last 3–4

decades, a number of papers have been published that reports the majority of trees are successful to propagate through organogenesis and somatic embryogenesis (Giri et al. 2004). As compared to organogenesis, regeneration of plants through somatic embryogenesis has more advantages because somatic embryogenesis leads to the formation of bipolar structures possessing both root and shoot meristem (Rai et al. 2007). Furthermore, somatic embryos are assumed to be of single cell origin and embryogenic cultures produce many embryos per volume of cell mass (Litz and Gray 1992).

Earlier, Badji et al. (1993) and Khalafalla and Daffalla (2008) described regeneration methods in *A. senegal* through organogenesis using explants obtained either from seedlings or mature trees, but both reports are not efficient as only 2–4 micropropagules were regenerated per explant. Another attempt has also been made to induce callus formation from cambium followed by the induction of suspension culture (Hustache et al. 1986). During past years, considerable efforts have been made for in vitro plant regeneration of other species of *Acacia* through somatic embryogenesis (Rout et al. 1995; Garg et al. 1996; Ortiz et al. 2000; Xie and Hong 2001; Vengadesan et al. 2002; Nanda and Rout 2003). In this paper, we report first time somatic embryogenesis in *A. senegal* using immature cotyledon explants. Efforts were also made to evaluate the effect of different factors on the induction of somatic embryogenesis in *A. senegal*.

Material and methods

Explant, surface sterilization, culture medium and culture condition

The green unripe pods (6–8 weeks of anthesis) were collected from 15 to 20 year old tree growing in Botanical Garden, Jai Narain Vyas University, Jodhpur, Rajasthan during the months of September and October. The pods were thoroughly washed with tap water and were split open to remove immature seeds. Seeds were surface sterilized with 0.1 % mercuric chloride (disinfectant; Merck, India) for 3–5 min under aseptic conditions. It was finally rinsed 3–4 times with sterile distilled water. The seed coat was removed using a scalpel and forceps and immature cotyledons were dissected out and cultured on MS (Murashige and Skoog 1962) medium containing 3 % sucrose, additives (0.13 mM adenine sulfate, 0.14 mM arginine, 0.28 mM ascorbic acid, 0.13 mM citric acid) and different plant growth regulators for the induction of somatic embryogenesis. Cotyledons isolated from mature seeds were also evaluated for somatic embryo induction. The pH of the medium was adjusted to 5.8±0.02 prior to autoclaving for 15 min at 121 °C temperature and 1.1 kg cm^{-2} pressure. Cultures were maintained at 28±2 °C with 16/8 (light/dark) photoperiod at a photon flux density (PFD) of 30–40 μmol m^{-2} s^{-1} from white fluorescent tubes (Phillips, India).

Effect of PGRs on the induction of somatic embryogenesis

For the induction of somatic embryogenesis, immature cotyledons were cultured on MS medium containing 2, 4-D (0–9.05 μM) either alone or combination of optimized concentrations (0.45 or 2.26 μM) of 2, 4-D with Kin (2.32 μM).

Effect of selected amino acids on the induction of somatic embryogenesis

To evaluate the effect of amino acids on the induction of somatic embryogenesis, immature cotyledons were cultured on optimized induction medium (MS + 0.45 μM 2, 4-D + 2.32 μM Kin + additives) supplemented with different concentrations (0, 5, 10, 15 or 20 mM) of L-glutamine, L-asparagine or L-arginine. Amino acids were added to the medium prior to adjustment of pH of medium. All cultures were maintained under the same culture conditions as previously described.

Development, maturation and germination of somatic embryos

For the development and maturation, globular stage somatic embryos along with cotyledon explants were transferred to fresh induction medium with or without L-glutamine (15 mM). When most of the embryos attained maturity, somatic embryos were transferred to MS medium containing different concentrations (0, 0.22, 0.44, 2.22, 4.44 or 8.88 μM) of BAP for their germination.

Experimental design, data collection and statistical analysis

For the above experiments, 20 explants were used for each treatment and each experiment was repeated thrice. The frequency of embryogenesis was calculated as the percentage of cultures showing at least one somatic embryo. The results are expressed as mean ± S.E. of three experiments. The data was analyzed statistically using SPSS ver. 17 (SPSS Inc., Chicago, USA) and the significance of differences among mean values was carried out using Duncan's multiple range test (DMRT) and paired sample T- test at $P<0.05$.

Results and discussion

Induction of somatic embryogenesis

In the present investigation, cotyledons isolated from immature seeds collected during the months of September and

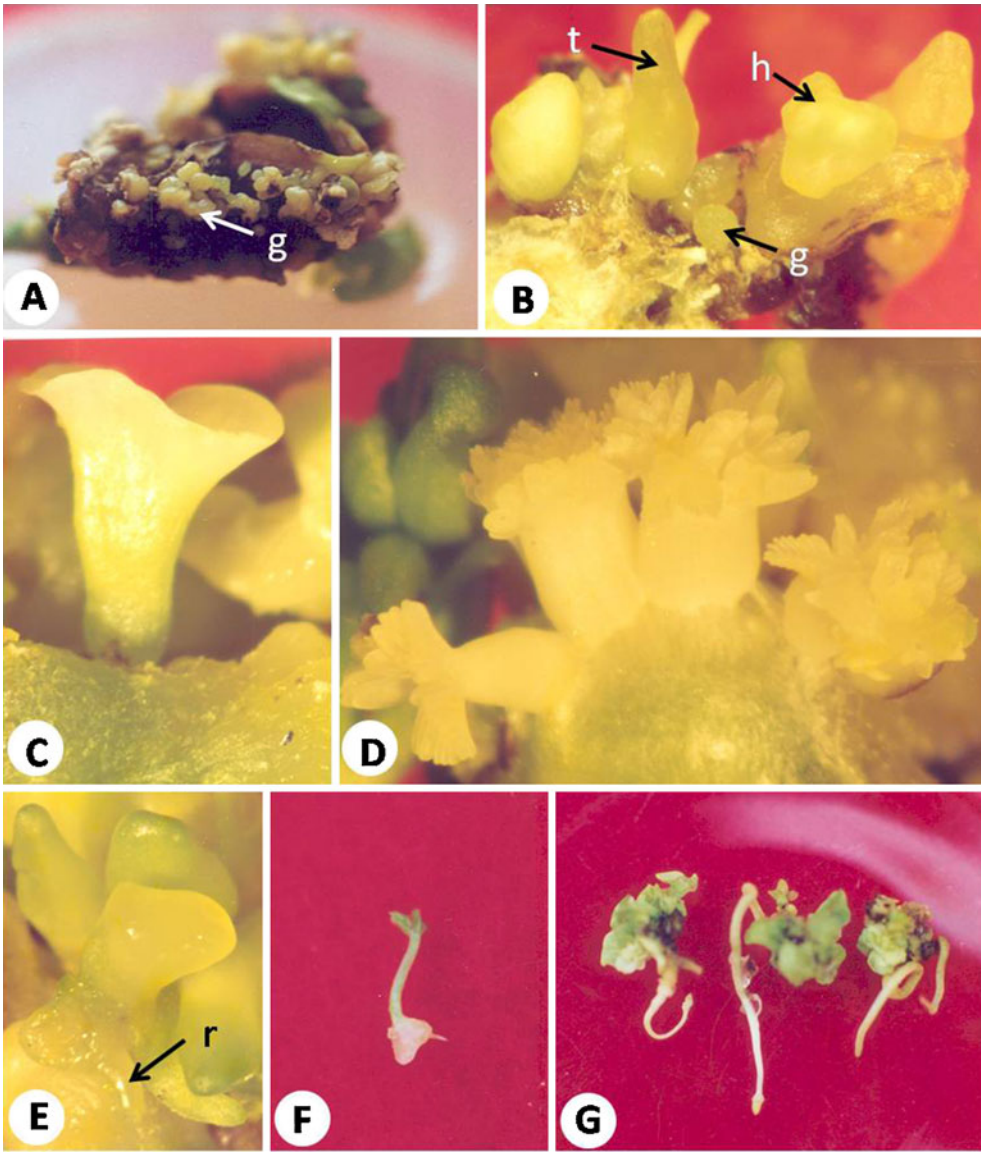

Fig. 1 Induction of somatic embryogenesis in *Acacia senegal* (L.). (**a**) Induction of somatic embryogenesis and the development of globular (g) stage somatic embryos on the surface of explants on MS medium containing 2, 4-D (0.45 μM) and Kin (2.32 μM) + 15 mM L-glutamine. (**b**) Development of different stages of somatic embryos (g-globular, h-heart and t-torpedo stage) on MS medium containing 2, 4-D (0.45 μM) and Kin (2.32 μM) + 15 mM L-glutamine. (**c**) Cup-shaped somatic embryo. (**d**) Maturation of somatic embryos and formation of leaf-like structure. (**e**) Initiation of germination of somatic embryo (r-root). (**f**) Germinating somatic embryo on MS medium + BAP (0.22 μM). (**g**) Profuse rooting in somatic embryos

October were able to produce somatic embryos on induction medium, whereas cotyledons obtained from mature seed failed to induce somatic embryo. Induction of somatic embryogenesis is usually restricted to certain responsive cells of explants and largely determined by a specific developmental stage of the tissue (von Arnold et al. 2002; Rai et al. 2007). The result of present study shows that cotyledon isolated only from immature seeds have the ability to produce somatic embryos, which probably may be owing to their favorable physiological make-up at the stage of development. Similar to our observation, somatic embryos were also induced from cotyledons or zygotic embryos obtained from immature seeds in some other species of *Acacia* (Rout et al. 1995; Ortiz et al. 2000; Xie and Hong 2001; Nanda and Rout 2003).

Effect of PGRs on the induction of somatic embryogenesis

Cotyledon explants cultured on MS medium containing 2, 4-D either alone or combination with Kin showed swelling after 10–12 d of inoculation and followed by somatic embryo induction on the edge of explants after 25–30 d (Fig. 1a). Somatic embryos were induced only on medium containing low concentrations (0.45–4.52 μM) of 2, 4-D. Cotyledon explants were unable to induce somatic embryo on comparatively higher concentrations of 2, 4-D (9.05 μM). The frequency and intensity of somatic embryogenesis were significantly ($P<0.05$) higher when explants cultured on medium supplemented with 2, 4-D and Kin than those cultured on 2, 4-D alone (Table 1). Among all treatments, MS medium containing 2, 4-D (0.45 μM) and Kin (2.32 μM) was found to be best for the induction of somatic

Table 1 Effect of 2, 4-D and Kin on the induction of somatic embryogenesis

PGRs (μM)	Frequency of embryogenesis (%)	Number of somatic embryos
2, 4-D		
0.45	30.5±2.6c	18.4±1.3c
2.26	21.8±1.9d	10.2±1.1d
4.52	14.6±1.5e	6.6±0.8d
9.05	0.0±0.0f	0.0±0.0e
2, 4-D + Kin		
0.45+2.32	60.0±3.5a	45.0±3.4a
2.26+2.32	41.1±2.8b	30.6±2.3b

Medium: MS + additives (0.13 mM adenine sulfate, 0.14 mM arginine, 0.28 mM ascorbic acid, 0.13 mM citric acid). Means in each column followed by different letters are significantly different according to DMRT at $P<0.05$

embryogenesis. In most cases, auxins particularly 2, 4-D is required for the induction of somatic embryogenesis and 2, 4-D alone usually plays a key role in the induction of either unorganized callus growth or polarized growth leading to somatic embryogenesis (von Arnold et al. 2002; Rai et al. 2007). On the other hand, it was also reported that 2, 4-D alone could not produce sufficient number of somatic embryos in some other plants and addition of cytokinin in 2, 4-D containing medium was beneficial for somatic embryogenesis (Jimenez 2005). This type of various responses to different combination of PGRs may be due to differences in genetic make-up among different plant species (Xie and Hong 2001).

Table 2 Effect of amino acids (A, L-glutamine; B, L-asparagine; C, L-arginine) on the induction of somatic embryogenesis

Concentrations of amino acids (mM)	Frequency of embryogenesis (%)	Number of somatic embryos
(A) L-glutamine		
0	60.0±3.5c	45.0±3.4d
5	66.6±2.8bc	48.4±2.6cd
10	72.8±3.2b	56.5±2.8c
15	80.5±3.5a	94.4±4.2a
20	60.0±3.5c	67.2±2.7b
(B) L-asparagine		
0	60.0±3.5a	45.0±3.4a
5	45.5±2.4b	20.6±1.8b
10	36.8±2.6c	16.6±1.5b
15	33.3±1.8c	15.2±1.2bc
20	25.0±1.5d	10.5±1.4c
(C) L-arginine		
0	60.0±3.5a	45.0±3.4a
5	40.8±3.2b	22.5±2.1b
10	39.6±2.4b	16.8±1.8bc
15	25.0±1.8c	11.4±1.1c
20	11.4±1.2d	10.2±0.9c

Induction medium: MS + additives (0.13 mM adenine sulfate, 0.14 mM arginine, 0.28 mM ascorbic acid, 0.13 mM citric acid) + 2, 4-D (0.45 μM) + Kin (2.32 μM). Means in each column followed by different letters are significantly different according to DMRT at $P<0.05$

Table 3 Effect of L-glutamine on development of somatic embryos

Concentrations of L-glutamine (mM)	Number of normal somatic embryos	Number of abnormal somaic embryos
0	9.2±2.4b	85.8±4.2a
15	56.4±5.6a	34.2±2.8b

Medium: MS + additives (0.13 mM adenine sulfate, 0.14 mM arginine, 0.28 mM ascorbic acid, 0.13 mM citric acid) + 2, 4-D (0.45 μM) + Kin (2.32 μM). Means in each column followed by different letters are significantly different according to paired sample T- test at $P<0.05$

For further development, globular stage somatic embryos along with cotyledon explants were transferred to fresh medium with or without L-glutamine (15 mM) after 4 weeks of induction

Effect of amino acids on the induction of somatic embryogenesis

The frequency and intensity of somatic embryogenesis enhanced significantly by the addition of L- glutamine in optimized induction medium (MS + 0.45 μM 2, 4-D + 2.32 μM Kin). Medium supplemented with 15 mM L-glutamine was most favorable for the induction of somatic embryogenesis (Table 2A). L-asparagine and L-arginine did not have a positive effect on the induction of somatic embryogenesis (Table 2B & C). L-glutamine is frequently used as source of organic nitrogen in plant tissue culture which provides reduced nitrogen to plant tissues (Barrett et al.

Table 4 Effect of BAP on germination of somatic embryos

Concentrations of BAP (μM)	Number of germinating somatic embryos
0	19.6±2.7d
0.22	42.0±3.6a
0.44	32.8±2.5b
2.22	25.6±2.8c
4.44	0.0±0.0e
8.88	0.0±0.0e

Medium: MS + additives (0.13 mM adenine sulfate, 0.14 mM arginine, 0.28 mM ascorbic acid, 0.13 mM citric acid). Means in each column followed by different letters are significantly different according to DMRT at $P<0.05$

1997) and enhances the synthesis of certain metabolites (Deo et al. 2010). Earlier, the beneficial role of L-glutamine in the induction of somatic embryogenesis has also been reported in a number of studies (Garin et al. 2000; Robichaud et al. 2004; Zouine and Hadrami 2007; Deo et al. 2010). In contrast to our observations, insignificant role of L-glutamine in somatic embryogenesis was also reported in *Acacia catechu* (Rout et al. 1995), *Prunus incise* (Cheong and Pooler 2004), *Psidium guajava* (Rai et al. 2009).

Development and maturation of somatic embryos

Development of globular stage somatic embryos was observed in 3–4 week-old cultures. Earlier formed globular stage somatic embryos proceeded towards the development of heart, torpedo and cotyledonary stage somatic embryos after 4–5 weeks. However, development of somatic embryos was asynchronous and different stages of somatic embryos were seen at the same explant at the same time (Fig. 1b). About 60 % somatic embryos were found to be cotyledonary and torpedo staged types on L-glutamine (15 mM) containing medium whereas only 10 % somatic embryos underwent further development when explants were subcultured to L-glutamine free medium. Several abnormalities such as mono or polycotyledonary, fused hypocotyls and plantlets etc. were also observed during embryo development when they could not attain maturity. As compared to medium containing L-glutamine, percent embryo abnormality was significantly higher in L-glutamine free medium (Table 3). On L-glutamine free medium, 8–10 % somatic embryos were converted into typical cup-shaped structures (Fig. 1c). Maturation is an essential phase between embryo development and germination and mature embryos of normal morphology that accumulated sufficient amount of storage proteins develop into normal plants (von Arnold et al. 2002; Rai et al. 2008). In the present investigation, the beneficial role of L-glutamine in maturation of somatic embryos is not clear but it may probably due to their involvement in synthesis of certain metabolites and storage proteins.

Germination of somatic embryos

Most of embryos lost their germination potential and died if they continued to be on the same development and maturation medium for longer duration. Therefore, they had to be removed from this medium and transferred to growth regulator free or BAP containing medium for their germination. The appearance of leaf like structure (Fig. 1d) and root initiation (Fig. 1e) were observed in somatic embryos after 2–3 weeks of transfer on medium containing BAP. Somatic embryos on higher concentration (4.44–8.88 μM) of BAP showed inhibition in rooting whereas callus formation on the base of plantlet and the development of non-functional root on medium containing 2.22 μM BAP. Maximum percent germination of somatic embryos was recorded on medium containing 0.22 μM BAP (Table 4; Fig. 1f). Similar to our observation, germination of somatic embryos on BAP containing medium has also been reported in *Arachis hypogaea* (Venkatachalam et al. 1999), *Quassia amara* (Martin and Madassery 2005) and *Catharanthus roseus* (Junaid et al. 2006). In the present study, precocious germination of somatic embryos and profuse rooting was observed during germination of embryos (Fig. 1g). Low germination rate, profuse rooting and precocious germination are some major hurdles, which limit the application of somatic embryogenesis in a number of plant species (Rizvi et al. 2012). However, several investigations have also indicated that the addition of some adjuvants like ABA, PEG, amino acids etc. to the medium can help to overcome these problems and improve the maturation and germination of somatic embryos (Rai et al. 2009, 2011).

In conclusion, this is the first report of somatic embryogenesis in *A. senegal*. We were able to induce somatic embryo from cotyledons isolated from immature seeds and also successful in their germination. Although, similar to a number of tree species, our protocol also exhibited some morphological abnormalities such as mono or polycotyledony, fused hypocotyls and plantlets etc. during embryo development. Therefore, for large-scale application of the process, further experiments are needed to achieve a higher percent mature somatic embryos along with germination and plantlet development and minimize the proportion of abnormal somatic embryos.

Acknowledgements The financial supports provided to the Department of Botany by University Grants Commissions (UGC), Department of Biotechnology (DBT) and the Department of Science and Technology (DST), Govt. of India are gratefully acknowledged. Author (M.K. Rai) wishes to acknowledge to University Grants Commission (UGC), New Delhi for the award of Dr. D.S. Kothari Post Doctoral Fellowship.

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