Plant Cell Report (1997) 16: 628–631 © Springer-Verlag 1997

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Direct somatic embryogenesis and plantlet regeneration from seedling leaves of winged bean, *Psophocarpus tetragonolobus* (L.) DC.

Received: 5 April 1996 / Revised received: 1 October 1996 / Accepted: 9 October 1996

Abstract Excised seedling leaf segments of winged bean [*Psophocarpus tetragonolobus* (L.) DC.] underwent direct somatic embryogenesis under appropriate incubation conditions. Initiation and development of the somatic embryos occurred using a two-step culture method. The culture procedure involved incubation for 28 days on MS basal medium supplemented with 0.1–0.5 mg/l NAA and 1.0–2.0 mg/l BA (induction medium) before transfer to MS medium supplemented with 0.1 mg/l IAA and 2.0 mg/l BA (embryo development medium). The initial exposure to low levels of NAA coincident with high levels of BA in the induction medium was essential for embryogenic induction. Maximum embryogenesis (43.3%) was obtained with 0.2 mg/l NAA and 2.0 mg/l BA, and at least 14 days on induction medium were required prior to transfer to the embryo development medium. The conversion frequency of cotyledonary embryos was 53.3% upon culture on MS medium containing 0.1 mg/l ABA for 7 days followed by transfer to MS medium supplemented with 0.1 mg/l IBA and 0.2 mg/l BA. Following conversion, the regenerated plantlets were transferred to soil and showed normal morphological characteristics.

Key words *Psophocarpus tetragonolobus* · Somatic embryo induction · Embryo morphology · Plantlets

Abbreviations *MS* Murashige and Skoog (1962) medium · *2,4-D* 2,4-dichlorophenoxyacetic acid · *NAA* 1-naphthaleneacetic acid · *IAA* indole-3-acetic acid · *IBA* indole-3-butyric acid · *BA* 6-benzylaminopurine · *ABA* abscisic acid

Communicated by G. Phillips

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Introduction

There is an increasing interest in winged bean [*Psophocarpus tetragonolobus* (L.) DC.] tissue culture because of the potential of this species as a high protein crop for the tropics. Its protein content and oil quality are comparable to those of soybean (N.A.S. Report 1979). In vitro techniques can provide the means for obtaining somaclonal and induced variations or for introducing agronomically important genes through *Agrobacterium*-mediated transformation. However, the successful application of in vitro methods is largely dependent on a reliable regeneration system. Considerable progress has been made in plant regeneration systems for large-seeded legumes (Maheswaran and Williams 1984; Parrott et al. 1992). Unfortunately, winged bean is still difficult to manipulate because of its recalcitrance in culture. Among the modes of plant regeneration, organogenesis in winged bean has been achieved from callus cultures derived from epicotyls (Mehta and Mohan Ram 1981; Tran Thanh Van et al. 1986; Venketeswaran et al. 1992), leaves (Gregory et al. 1980) and protoplasts (Wilson et al. 1985), while somatic embryos have been induced from hypocotyl and epicotyl cultures (Venketswaren et al. 1992). However, in none of these investigations were plantlets regenerated from any of these structures. Recently, we documented somatic embryogenesis and plant regeneration from leaf-derived calli of winged bean (Ahmed et al. 1996).

Compared to indirect somatic embryogenesis, direct somatic embryogenesis from predetermined tissue appears to be associated with greater genetic and cytological uniformity (Maheswaren and Williams 1984). Direct somatic embryogenesis in grain legumes has been reported from zygotic embryos (Hazra et al. 1989; Ozias-Akins 1989; Sellers et al. 1990) and from embryo-derived leaflets of peanut (Baker and Wetzstein 1992; Chengalrayan et al. 1994), mature embryo axes (Suhasini et al. 1994) and leaflets of chickpea (Barna and Wakhlu 1995) and intact seedlings of *Phaseolus* sp. (Malik and Saxena 1992).

This paper reports direct somatic embryogenesis and plantlet regeneration from seedling leaf explants of winged bean.

Materials and methods

Plant material

Seeds of winged bean genotype EC-38825-2 were collected from NBPGR, Akola. The seedlings were raised for explants as described by Ahmed et al. (1996). Leaves 3- to 5-days old were excised from the seedlings, cut transversely (perpendicular to the mid-vein) into 1- to 2-mm-long sections and cultured for somatic embryo induction.

Somatic embryo induction and development

Leaf segments were placed abaxially in contact with the induction medium. The induction medium was a MS-based medium supplemented with either 2,4-D or NAA at various concentrations (0.1, 0.2, 0.5, 1.0, 2.0 and 4.0 mg/l) in combination with BA (0.0, 0.5, 1.0 and 2.0 mg/l) and 3% (W/V) sucrose. All media were solidified with 0.8% agar (Hi-media, Bombay), and the pH of the medium was adjusted to 5.8 before autoclaving at 121 °C (106 kPa) for 15 min. Fifty explants were cultured for each treatment; these were incubated at 25° ± 1 °C under a 16-h photoperiod regime of cool white fluorescent light set at an intensity of 15 μmol $m^{-2} s^{-1}$. After an incubation period of 28 days, the explants were transferred to embryo development medium, which was MS medium supplemented with 0.1 mg/l IAA, 2.0 mg/l BA and 3% sucrose. This combination was found to be optimal for inducing embryo development in callus-mediated somatic embryogenesis of winged bean (Ahmed et al. 1996). To determine the effect of incubation duration on embryo induction we cultured leaf explants for 7, 14, 21 and 28 days on an induction medium consisting of MS with 0.2 mg/l NAA, 2.0 mg/l BA and 3% sucrose before transfer to the embryo development medium. Embryo development treatments consisted of 15 replications (culture tubes, 25 mm × 100 mm) of 2 explants in each treatment. Cultures were incubated for 3 weeks under the conditions mentioned above. Data were collected on percentage of explants exhibiting somatic embryogenesis and number of somatic embryos per responding explant after 3 weeks of culture on the embryo development medium since no somatic embryos could be detected on the induction medium. Data on percentage embryogenesis were subjected to arcsin transformation before analysis and were converted back to percentages for presentation. Means were separated using Duncan's multiple range test.

Plantlet development

Cotyledonary-stage somatic embryos (hereafter referred to as cotyledonary embryos) were isolated from the explants and cultured individually on MS medium supplemented with 0.1 mg/l IBA and 0.2 mg/l BA, (conversion medium) in test tubes (25 mm × 100 mm). In earlier studies, optimum conversion was obtained with this combination (Ahmed et al. 1996). In the second set, cotyledonary embryos were incubated on MS medium containing 0.1 mg/l abscisic acid (maturation medium) for 7 days and then transferred to conversion medium. A total of 60 cotyledonary embryos were tested in each set with three repeats. Cultures were incubated at 25 ° ± 1 °C under a light intensity of 50 μmol $m^{-2} s^{-1}$ and a 16-h photoperiod regime. Somatic embryo conversion was evaluated after 3 weeks of culture. Regenerated plantlets were successfully transferred to glasshouse conditions as previously described (Ahmed et al. 1996).

Results and discussion

Somatic embryogenesis

Swelling of the leaf explants was apparent within 7 days of incubation on embryo induction medium. Upon transfer to the embryo development medium somatic embryos developed directly from the edge of the cut ends. Sequential development of somatic embryos from the globular stage (Fig. 1a) to the heart-shaped stage (Fig. 1b) and finally to the cotyledonary stage (Fig. 1c) were observed on embryo development medium. No somatic embryo was detected on embryo induction medium. This concurs with the report in peanut (Baker and Wetzstein 1992). Percentage somatic embryogenesis and the number of somatic embryos per responding explant were determined 21 days after transfer to the embryo development medium. The ratio of NAA and BA in the induction medium clearly affected the frequency of somatic embryogenesis. Only relatively narrow range of NAA and BA concentrations, from 0.1 to 0.5 mg/l NAA in combination with 1.0–2.0 mg/l BA, was capable of inducing the formation of somatic embryos (Table 1). The best response was obtained with 0.2 mg/l NAA and 2.0 mg/l BA. Leaf explants precultured under this regime showed embryogenesis in 43.3% of the explants, with an average of 5.6 embryos per responding explant. Percentage somatic embryogenesis was significantly and negatively affected by increasing the level of NAA from 0.2 to 0.5 mg/l. Significant variation was also noted with changes in BA concentration, particularly in combination with higher levels of NAA. However, no clear trend could be observed with respect to mean number of somatic embryos per responding explant for different induction media. The substitution of NAA and BA with 2,4-D alone or in combination with BA in the induction medium failed to elicit embryogenesis. Similarly, neither NAA nor BA alone induced embryogenesis. High concentrations of NAA (1–4 mg/l) with low concentrations of BA (0.1–0.5 mg/l) yielded roots with little callus formation (data not shown).

The result demonstrates that the initiation and development of somatic embryos occurs in two steps, one involving embryo induction in the presence of low levels of NAA along with high levels of BA and the second consisting of embryo development in the presence of IAA and BA. Such a two-step process of direct somatic embryogenesis has

Table 1 Effect of NAA and BA combinations in induction medium on percentage embryogenesis and mean number of somatic embryos from leaves of winged bean. Leaves were cultured on induction medium for 28 days. The percentage embryogenesis and mean number of somatic embryos were recorded 21 days after transfer to embryo development medium

Growth regulator (mg/l)		% Embryogenesis[a]	Total number of somatic embryos	Mean no. of somatic embryos per responding explant (±SD[a])
NAA	BA			
0.1	1.0	30[b]	40	4.4 ± 1.3[b]
0.1	2.0	33.3[b]	46	4.6 ± 1.1[b]
0.2	1.0	30[b]	37	4.1 ± 1.4[bc]
0.2	2.0	43.3[a]	74	5.6 ± 1.3[a]
0.5	1.0	13.3[d]	15	3.7 ± 1.2[c]
0.5	2.0	20.0[c]	30	4.5 ± 1.4[b]

[a] Means within columns followed by the same letter are not significantly different at $P = 0.05$ according to the Duncan Multiple Range test

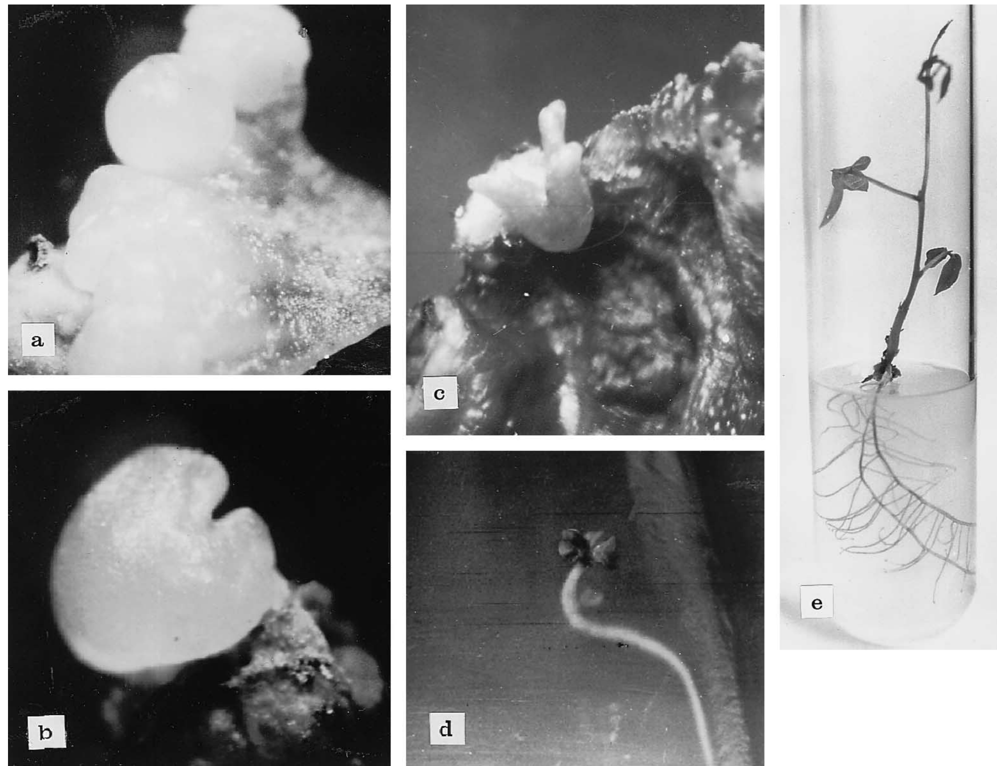

Fig. 1a–e Stages of somatic embryogenesis from leaf explants of winged bean. **a** Globular embryos induced directly from leaves of winged bean, **b** a heart-shaped embryo arising from the leaf surface, **c** cotyledonary-stage somatic embryo, **d** germination of somatic embryo with defined cotyledon and root, **e** plantlet with well-established shoot and root system developed from somatic embryo

also been observed in peanut (Baker and Wetzstein 1992, 1994; Chengalrayan et al. 1994) and chickpea (Suhasini et al. 1994; Barna and Wakhlu 1995). However, in our studies simultaneous exposure to low levels of NAA and high levels of BA was essential for the induction of embryogenesis. This observation is very striking since in most of the reported examples of direct somatic embryogenesis in grain legumes, high auxin favoured embryo induction and the gradual omission of auxin resulted in embryo development, although, there are divergent results on the effect of the type and concentration of auxin used for the induction of somatic embryos (McKently 1991; Hazra et al. 1989; Baker and Wetzstein 1994). Cytokinin-stimulated direct somatic embryogenesis has also been reported in *Phaseolus* (Malik and Saxena 1992). Thus, it is difficult to formulate a generalized protocol for somatic embryogenesis in grain legumes as the growth regulator requirements appear to be very species and tissue specific.

We also investigated the effect of incubation duration on somatic embryogenesis. Maximum embryogenesis (43.3%) was observed following a 28-day exposure to the embryo induction medium (Table 2). There were no significant differences between 21 and 28 days on induction medium either with respect to percentage embryogenesis or number of somatic embryos per responding explant. However, a low percentage of explants (6.6%) with somatic embryos was also recorded when culture in the induction medium was for only 14 days. The results indicate that maximum embryogenic induction is attained during a culture period of 14–21 days in the presence of NAA and BA and that the duration of primary culture has a decisive influence on embryogenic response. In contrast, no clear trend for the effect of incubation time on the induction medium was seen with peanut leaflets for percent embryogenesis or mean number of somatic embryos (Baker and Wetzstein 1992). However, the effect of incubation duration on embryogenesis has been well-documented in alfalfa (Finstad et al. 1993) and chickpea (Barna and Wakhlu 1995). The embryogenic response is comprises three distinct stages: competence, induction and differentiation (Christianson and Warnick 1985). The present data are inadequate to characterize the competence phase and distinguish it from the induction phase in terms of time requirements.

Table 2 Effect of length of exposure to induction medium on direct somatic embryogenesis. Induction medium included 0.2 mg/l NAA and 2.0 mg/l BA. Percentage embryogenesis and mean number of somatic embryos were recorded 21 days after transfer to embryo development medium

Days on induction medium	% Embryogenesis	Mean no. of somatic embryos per responding explant (±SD[a])
7	0	0
14	6.6	2.55 ± 0.7[a]
21	40	5.16 ± 1.26[b]
28	43.3	5.6 ± 1.3[b]

[a] Means within columns followed by the same letters are not significantly different at $P = 0.05$ according to the Duncan Multiple Range test

Plantlet development

Germination (Fig. 1d) and conversion of cotyledonary embryos into plantlets (Fig. 1e) occurred within 3 weeks of culture on MS medium supplemented with 0.1 mg/l IBA and 0.2 mg/l BA. The conversion percentage was approximately 42% and comparable to that reported by Wetzstein and Baker (1993) in peanut. In another report, Wetzstein and Baker (1992) recorded only 2% conversion. A comparable poor response was also noted in chickpea (Suhasini et al. 1994; Barna and Wakhlu 1995) and could be due to the high proportion of abnormal somatic embryos formed and their use in conversion studies. It has been asserted that the morphology of the somatic embryo affects the conversion rate (Buchheim et al. 1989; Lazzeri et al. 1987; Wetzstein and Baker 1993). Interestingly, in our findings cotyledonary embryos appeared to be normal and were similar to class-2 embryos described in peanut by Wetzstein and Baker (1993). Presumably, the normal appearance of cotyledonary embryos and their selective use in germination/conversion studies may account for the higher conversion rate over the lower rates noticed in peanut and chickpea. However, the conversion rate could be enhanced from 41.6% to 53.3% by preculturing the cotyledonary embryos for 7 days on maturation medium. A similar promotive effect of ABA on embryo maturation has been well-documented (Barna and Wakhlu 1993; Suhasini et al. 1994). The regenerated plantlets grew into well-developed single-stemed plants with normal leaf growth under greenhouse conditions, similar to our earlier findings (Ahmed et al. 1996).

In conclusion, we achieved the successful development of a somatic embryogenesis system and plantlet regeneration from seedling leaf explants of winged bean. The use of seedling tissues excludes the need to maintain plants under greenhouse conditions or to obtain plant material throughout the year at specific developmental stages. Further improvement in the efficiency of somatic embryogenesis and conversion may make the system suitable for research efforts toward gene transfer and somatic hybridization.

Acknowledgements The award of a Research Associateship to R.A. by the CSIR, New Delhi is gratefully acknowledged.

References

Ahmed R, Dutta Gupta S, De DN (1996) Somatic embryogenesis and plant regeneration from leaf derived callus of winged bean [*Psophocarpus tetragonolobus* (L.) Dc.] Plant Cell Rep 15:531–535

Baker CM, Wetzstein HY (1992) Somatic embryogenesis and plant regeneration from leaflets of Peanut *Arachis hypogaea*. Plant Cell Rep 11:71–75

Baker CM, Wetzstein HY (1994) Influence of auxin type and concentrations on peanut somatic embryogenesis. Plant Cell Tiss Org Cult 36:361–368

Barna KS, Wakhlu AK (1993) Somatic embryogenesis and plant regeneration from callus cultures of chickpea (*Cicer arietinum* L.). Plant Cell Rep 12:521–524

Barna KS, Wakhlu AK (1995) Direct somatic embryogenesis and plantlet regeneration from immature leaflets in chickpea. In Vitro Cell Dev Biol 31P:137–139

Buchheim JA, Colburn SM, Ranch SP (1989) Maturation of soybean somatic embryos and the transition to plantlet growth. Plant Physiol 89:768–775

Chengalrayan K, Sathaye SS, Hazra S (1994) Somatic embryogenesis from mature embryo-derived leaflets of peanut (*Arachis hypogea* L.). Plant Cell Rep 13:578

Christianson ML, Warnick DA (1985) Temporal requirement for phytohormone balance in the control of organogenesis. In Vitro Dev Biol 112:494–497

Finstad K, Brown DCB, Joy K (1993) Characterization of competence during induction of somatic embryogenesis in alfalfa tissue culture. Plant Cell Tis Org Cult 34:125–132

Gregory HM, Haq N, Evans PK (1980) Regeneration of plantlets from leaf callus of the winged bean *Psophocarpus tetragonolobus* (L) DC. Plant Sci Lett 18:395–400

Hazara S, Sathaye SS, Mascarenhas AF (1989) Somatic embryogenesis in peanut (*Arachis hypogaea*). Biotechnology 7:949–951

Lazzeri PA, Hildebrand DF, Collins GB (1987) Soybean somatic embryogenesis. Effects of hormones and culture manipulations. Plant Cell Tiss Org Cult 10:197–208

Maheswaren G, Williams EG (1984) Direct somatic embryoid formation on immature embryos of *Trifolium repens, T. pratense*, and *Medicago sativa*, and rapid clonal propagation of *T. repens*. Ann Bot 54:201–211

Malik KA, Saxena PK (1992) Somatic embryogenesis and shoot regeneration from intact seedlings of *Phaseolus acutifolius. P. aures* (L) Wilczek, *P. coccineus* L., and *P. wrightii* L. Plant Cell Rep 11:163–168

McKently A (1991) Direct somatic embryogenesis from axes of mature peanut embryos. In Vitro Cell Dev Biol 27P:197–200

Mehta U, Mohan Ram HY (1981) Tissue culture and whole plant regeneration in the winged bean (*Psophocarpus tetragonolobus* L.). Ann Bot 47:163–166

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

NAS Report (1979) The winged bean. A high protein crop for the tropics. National Academy of Sciences, Washington, D.C.

Ozias-Akins P (1989) Plant regeneration from immature embryos of peanut. Plant Cell Rep 8:217–218

Parrott WA, Bailey MA, Durham RE, Mathews HV (1992) Tissue culture and regeneration in legumes. In: Moss JP (eds) Biotechnology and crop improvement in Asia. ICRISAT, Patancheru, India, pp 115–148

Sellers RM, Southward GM, Phillips GC (1990) Adventitious somatic embryogenesis from cultured immature zygotic embryos of peanut using soybean as a model system. Crop Sci 30:408–414

Suhasini K, Sagare AP, Krishnamurthy KV (1994) Direct somatic embryogenesis from mature embryo axes in chickpea (*Cicer arietinum* L.) Plant Sci 102:189–194

Tran Thanh Van K, Lie-Schricke H, Marcotte JL, Trinh TH (1986) Winged bean [*Psophocarpus tetragonolobus* (L.) DC.]. In: Bajaj YPS (eds) Biotechnology in agriculture and forestry,vol 2: crop 1. Springer, Berlin Heidelberg New York, pp 556–567

Venketeswaran S, Dias MADL, Weyers UV (1992) Organogenesis and somatic embryogenesis from callus of winged bean [*Psophocarpus tetragonolobus* (L.) DC.] Acta Hortic 280:202–206

Wetzstein HY, Baker CM (1993) The influence of somatic embryo morphology and histology on germination and conversion in peanut. Plant Sci 92:81–89

Wilson VM, Haq N, Evans PK (1985) Protoplast isolation culture and plant regeneration in the winged bean. *Psophocarpus tetragonolobus* (L.) DC. Plant Sci 41:61–68