Plant Cell Reports (1998) 17: 313–317

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Plant regeneration from callus protoplasts of the forage legume *Astragalus adsurgens* Pall.

Received: 25 April 1997 / Revision received: August 1997 / Accepted: 2 September 1997

Abstract A reproducible release of viable protoplasts was obtained from friable calli of *Astragalus adsurgens*. Protoplasts underwent sustained divisions and formed cell colonies when cultured in either liquid or agarose-solidified Kao and Michayluk (1975) protoplast medium (KM8P) supplemented with 1.5 mg/l 2,4-D, 0.5 mg/l BA and 0.5 *M* glucose. Compared to liquid culture, agarose bead culture improved division frequency effectively, the two culture systems showing a plating efficiency of 0.8±0.5% and 6.5±0.7%, respectively. Upon transfer to Murashige and Skoog (1962) medium (MS) with 1–2 mg/l BA, alone or in combination with NAA or 2,4-D at 0.1 mg/l, the protoplast-derived calli produced complete plantlets through somatic embryogenesis. The maximum percentage of calli producing somatic embryos (52.5± 2.2%) occurred on MS medium containing 0.1 mg/l NAA and 1 mg/l BA, whereas the maximum number of calli regenerating plantlets (64.7±6.2) was obtained on MS medium with 0.1 mg/l NAA and 2 mg/l BA.

Key words *Astragalus adsurgens* · Protoplast culture · Plant regeneration · Embryogenesis

Abbreviations *2,4-D* 2,4-Dichlorophenoxyacetic acid · *NAA* α-naphthaleneacetic acid · *BA* 6-benzylaminopurine · *MS* Murashige and Skoog (1962) medium · *KM8P* Kao and Michayluk (1975) protoplast medium

Introduction

Astragalus adsurgens Pall. (2n=16) is distributed throughout China and Mongolia and also in some regions of Korea, Russia and Japan (Fu et al. 1993). It is a drought- and sandstorm-tolerant legume and widely cultivated in arid areas of north China as a highly palatable forage and green manure rotation crop (Fu et al. 1993). However, its non-synchronized flowering with different sequences of flowering and long growth periods have resulted in poor seed production, which have limited its application. Plant genetic manipulation, including somatic hybridization and direct genetic transformation, would provide the means for improving this species, and the development of a procedure for regenerating plants from isolated protoplasts is one of the approaches to this goal. It is important, therefore, that the optimum conditions for protoplast isolation and culture be established and that plant regeneration from protoplast-derived calli be repeated routinely. Although plants have been obtained from hypocotyl-derived calli (An et al. 1992), there has been no report of complete regeneration from protoplast-derived calli in *A. adsurgens*. We describe here a procedure for high-frequency plant regeneration of *A. adsurgens* from isolated callus protoplasts through somatic embryogenesis.

Communicated by F. Constabel

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Materials and methods

Callus induction and subculture

Mature seeds of *Astragalus adsurgens* Pall. were obtained from the Grassland Ecological Research Institute of Gansu province, China. They were surface-sterilized in 0.1% $HgCl_2$ for 8 min, followed by three rinses with sterile distilled water. Sterilized seeds were germinated on agar-solidified (0.8%), half-strength MS (Murashige and Skoog 1962) medium without growth regulators and incubated at 24°±2°C under fluorescent light (30 μmol m^{-2} s^{-1}, 16-h photoperiod). Callus was induced from hypocotyl segments (5 mm in length) excised from 10-day-old seedlings on agar-solidified (0.8%) MS medium containing 2.0 mg/l 2,4-D and 0.5 mg/l BA. After 21 days in culture, the friable, yellowish calli were detached from explants and subcultured every 21 days on the same medium as described for callus induction . Callus induction and subculture were carried out in the dark at 24°±2°C.

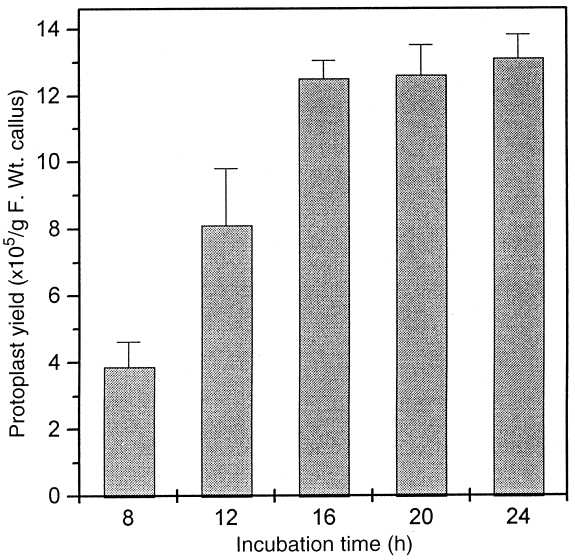

Fig. 1 Effect of enzymatic incubation period on protoplast yield

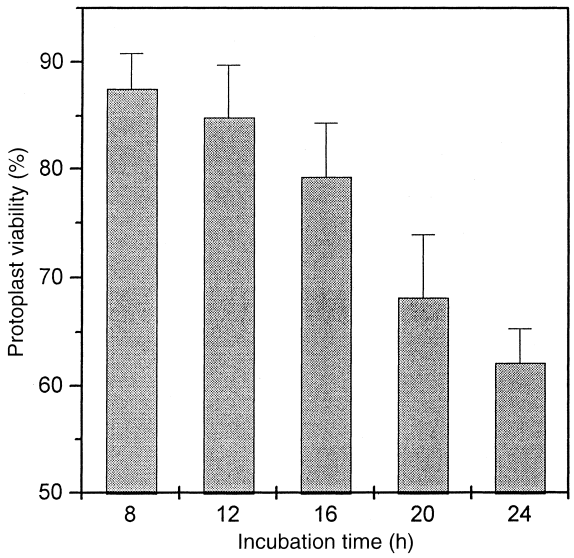

Fig. 2 Effect of enzymatic incubation period on protoplast variability

Protoplast isolation

Protoplasts were isolated from 10-day-old friable, yellowish callus. One gram of fresh callus was transferred to 10 ml of enzyme solution and incubated at 24°±2°C for 8, 12, 16, 20 or 24 h on a rotary shaker (30 rpm) in the dark. The enzyme solution contained 1.5% (w/v) Cellulase Onozuka R 10 (Yakult Pharmaceutical Industry Co, Tokyo, Japan), 0.5% (w/v) Cellulase Onozuka RS (Yakult Honsha Co, Tokyo, Japan), 1.0% (w/v) Pectinase (Serva) and 0.3% (w/v) Hemicellulase H2125 (Sigma Co, St. Louis, USA) in CPW salt solution (Frearson et al. 1973) with 9% (w/v) mannitol at pH 5.8. The protoplasts were filtered through a stainless steel sieve (74 μm pore size) and centrifuged (70 *g*, 5 min). The pelleted protoplasts were resuspended in 5 ml of protoplast liquid medium (see below). The protoplast suspension was loaded gently onto the top of a 0.52 *M* sucrose solution containing CPW salts at pH 5.8 and then centrifuged at 70 *g* for 20 min. A protoplast band was withdrawn with a pipette, diluted and then washed three times by centrifugation (70 *g*, 5 min) with protoplast liquid medium. Protoplast yield was calculated as the mean ±standard error of five isolations. Protoplast viability was assessed by FDA staining following the procedure of Widholm (1972). For each protoplast isolation, 20 microscopic fields were scored, and the viability (%) was expressed as the mean ±standard error of five isolations.

Protoplast culture

Protoplasts were cultured at a final concentration of 2×10^5/ml either in liquid or in agarose-solidified KM8P medium (Kao and Michayluk 1975) containing 1.5 mg/l 2,4-D, 0.5 mg/l BA and 0.5 *M* glucose. In the case of liquid culture, protoplasts were dispersed in a 5 cm petri dish containing 2.0 ml of KM8P medium. For culture in agarose-solidified medium, protoplasts were embedded in 100–200 μl agarose beads (0.6%) in a shallow layer of liquid medium (1.5 ml) in a 5 cm petri dish. The osmotic pressure of the liquid medium was gradually reduced at 10 day intervals: in the agarose bead cultures, 0.5 ml of spent medium was replaced with a similar volume of a 1:1 and 0:1 (v/v) mixture of KM8P and KM8 (Kao and Michayluk 1975) medium containing 1.5 mg/l 2,4-D and 0.5 mg/l BA; for liquid cultures, 0.3 ml of the above protoplast medium mixtures was added to each dish. The division frequency (number of dividing protoplasts/total protoplasts×100) and the plating efficiency (percentage of the plated protoplasts forming cell colonies after 21

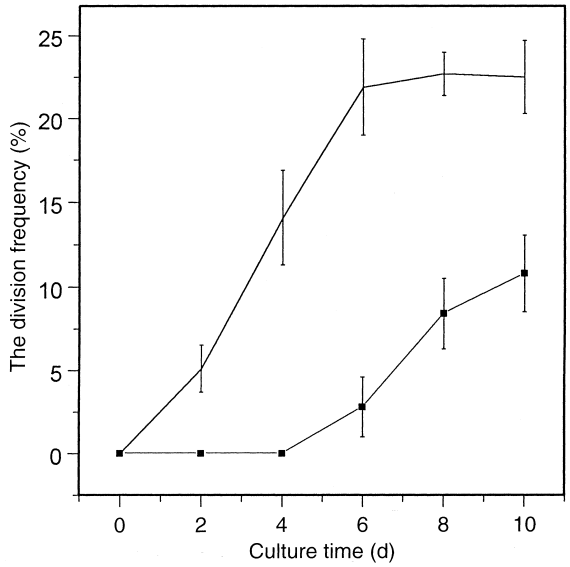

Fig. 3 Time course of division frequency of protoplasts. –■– Cultured in liquid medium, —— cultured in agarose beads

Fig. 4A–L Plant regeneration from callus protoplasts of *Astragalus adsurgens* Pall. **A** Freshly isolated callus protoplasts (*bar*: 25 μm), **B** first division of protoplast-derived cell (*bar*: 25 μm), **C** second division of protoplast-derived cell (*bar*: 25 μm), **D** a protoplast-derived cell colony (*bar*: 40 μm), **E** microcalli in agarose beads (*bar*: 10 mm), **F** small calli on agarose-solidified KM8 medium (*bar*: 10 mm), **G** somatic embryos formed on MS medium with 0.1 mg/l NAA and 1.0 mg/l BA (*bar*: 5 mm), **H** a somatic embryo at the late cotyledonary stage (*bar*: 3 mm), **I** regenerated plantlets from development of somatic embryos (*bar*: 10 mm), **J** plantlet with well-developed roots (*bar*: 15 mm), **K** 6-week-old potted plantlet (*bar*: 20 mm), **L** somatic metaphase chromosomes (2n=16) (*bar*: 10 μm)

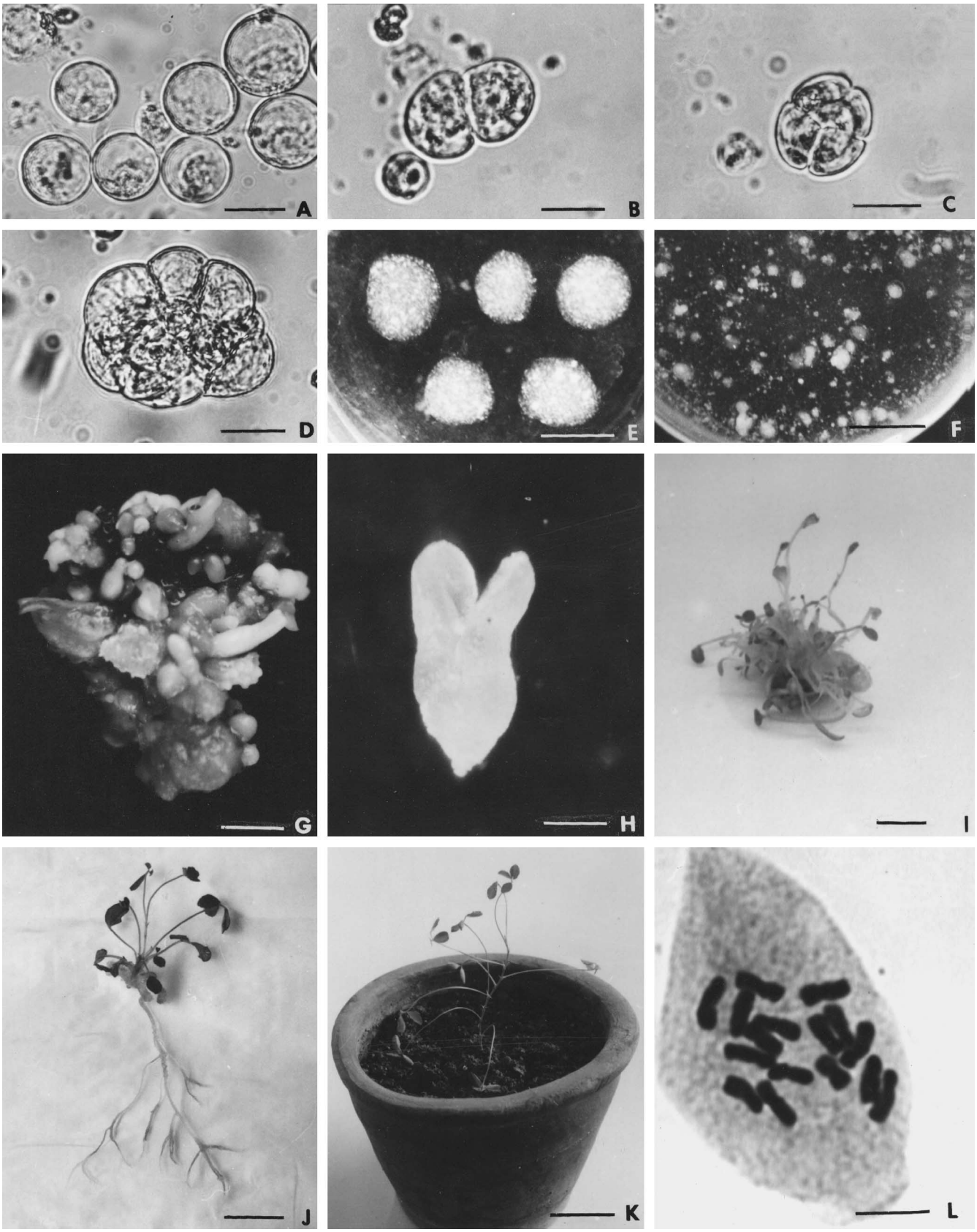
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Table 1 Effects of different combinations of 2,4-D, NAA and BA on somatic embryogenesis after 40 days in culture

Concentration (mg/l)			Percentage (%) of calli producing somatic embryos	Number of plantlets per gram callus
2,4-D	NAA	BA		
0.0	0.0	0.0	0.0	0.0
1.0	0.0	0.0	0.0	0.0
0.0	1.0	0.0	0.0	0.0
0.0	0.0	1.0	30.1 ± 1.8	46.5 ± 3.9
0.0	0.0	2.0	18.3 ± 3.2	42.7 ± 5.1
0.0	0.1	1.0	52.5 ± 2.2	58.1 ± 3.9
0.0	0.1	2.0	35.1 ± 2.9	64.7 ± 6.2
0.1	0.0	1.0	23.0 ± 1.2	38.4 ± 4.8
0.1	0.0	2.0	21.2 ± 1.6	48.3 ± 3.9

days) were expressed as mean ± standard error. Each culture system was repeated three times with 3–5 replicates. After 30 days of culture, the cell colonies from liquid cultures or agarose beads were transferred onto agarose-solidified KM8 (0.3%) medium for continued growth. Small calli, 2–3 mm in diameter, were isolated and cultured on MS medium as described for callus induction and subcultured every 21 days. All cultures were incubated at 24°±2°C in the dark.

Plant regeneration

Protoplast-derived calli were cultured on MS medium supplemented with various combinations of BA, 2,4-D and NAA (see Table 1) in order to induce somatic embryogenesis and plantlet regeneration. After 40 days of culture, the percentages of calli producing somatic embryos and the number of plantlets per gram callus were recorded. Each treatment consisted of 30–40 replicates and was repeated three times. The results shown represent the mean ± standard error. The regenerated plantlets were transferred to hormone-free half-strength MS medium for further development. Plantlets with well-developed roots were transplanted to pots with soil and maintained in a growth cabinet at 24°±2°C with 30 μmol m^{-2} s^{-1} illumination from cool-white fluorescent lamps and a 16-h photoperiod. The chromosome number of the plantlets was determined in the root tips (3–4 mm long). They were treated with saturated parachlorobenzene for 90 min, fixed in ethanol - acetic acid (3:1, v/v) overnight, then hydrolyzed in 1*N* HCl at 60°C for 4 min and stained with 1% acetocarmin.

Results and discussion

Protoplast isolation

The availability of a large number of protoplasts with high viability is the first step for successful protoplast culture. It was observed that protoplast yields improved with longer enzymatic digestion periods (Fig. 1), while protoplast viabilities showed a tendency to decline (Fig. 2). These results suggested that in *A. adsurgens* the optimum incubation time for obtaining of protoplasts with a high yield and viability is between 12 and 16 h (Fig. 4A).

Protoplast culture

Protoplasts grown in liquid medium changed their spherical shape and became oval within 3 days. The first division of the protoplasts occurred within 5–6 days (Fig. 4B) and the second division 3–4 days later (Fig. 4C). In agarose bead culture, the first division was observed as early as 2 days after isolation, and the second division took place after 5–6 days in culture. In this latter case, the frequency of dividing protoplasts increased linearly up to 22% from 2 to 6 days. However, the division frequency in liquid culture increased slowly to about 10% after 10 days of isolation (Fig. 3). In both culture systems, a great number of protoplasts did not divide further. Reducing the osmotic pressure promoted some dividing cells to form cell colonies (Fig. 4D). In agarose bead cultures, colonies became visible after 2 weeks of culture and the plating efficiency was 6.5±1.7%, while in liquid culture, colonies were present after 3 weeks and the plating efficiency was only 0.8±0.5%. After 4 weeks of culture, the surfaces of the agarose beads were covered with large quantities of microcalli (Fig. 4E), which further developed into small calli when transferred to agarose-solidified KM8 medium (Fig. 4F). The results clearly show that agarose embedding improved protoplast survival and division more effectively than liquid culture for protoplast culture in *A. adsurgens*. A similar improvement has also been found in other legumes (Gilmour et al. 1987; Lörz et al. 1983; Puonti-Kaerlas and Eriksson 1988; Shillito et al. 1983; Webb et al. 1987). It has been suggested that agarose stabilizes the membranes of protoplasts through the inhibition of lipid peroxidation (Schnabl and Youngman 1985) or prevents cell-wall precursors and other metabolites from leaking from protoplasts (Shillito et al. 1983; Thompson et al. 1986).

Plant regeneration

A high cytokinin/auxin-supplemented medium was crucial for inducing somatic embryos of *A. adsurgens*. Somatic embryos were observed within 3 weeks after protoplast-derived calli were cultured on MS medium with 1–2 mg/l of BA, singly or in combination with 2,4-D or NAA at 0.1 mg/l (Fig. 4G). Continuous culture resulted in somatic embryos at the cotyledonary stage (Fig. 4H), which then developed into plantlets after 40 days of culture (Fig. 4I). If somatic embryos were left on the callus surface and transferred onto MS medium without growth regulators, they failed to develop further. The percentage of calli producing somatic embryos was the highest in medium containing 0.1 mg/l NAA plus 1.0 mg/l BA, whereas the optimum medium for the development of somatic embryos into plantlets contained 0.1 mg/l NAA and 2.0 mg/l BA (Table 1). Although most plants require an exogenous auxin for the induction of somatic embryos, a similar need for cytokinin during embryo development has also been reported in a few plants (Hatanaka et al. 1991; Raghav Ram and Nabors 1984; Sharma and Kumar 1994). Plantlets could be readily isolated from calli and grew quickly on half-strength MS medium without growth regulators (Fig. 4J). The plantlets with well-developed roots could be transplanted to soil (Fig. 4K), and their survival was

around 80%. Fifty plantlets were examined for chromosome number in root tips, and all were found to be diploid (2n=16) (Fig. 4L).

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