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BRIEF COMMUNICATION

Regeneration *via* organogenesis in callus cultures of *Argyrolobium roseum*

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Abstract

A reproducible protocol has been developed for high frequency plant regeneration from immature embryos of *Argyrolobium roseum* Jaub & Spach, an important medicinal legume. Green nodular calli were initiated from immature embryos excised from 10-d-old pods in 70 % of cultures within 3 weeks when grown on Murashige and Skoog (MS) medium supplemented with 0.5 mg dm^{-3} benzylaminopurine (BAP) + 0.25 mg dm^{-3} indole-3-acetic acid (IAA). Subsequent transfer of 5 mm^2 callus pieces to MS medium supplemented with BAP (0.5 mg dm^{-3}) alone or in combination with IAA (0.25 mg dm^{-3}) facilitated regeneration of multiple shoots. Organogenic calli bearing multiple shoots when transferred to MS medium supplemented with BAP (0.5 mg dm^{-3}) + IAA (0.25 mg dm^{-3}) supported rapid shoot elongation. Shoot propagules subcultured to Gamborg's medium (B_5) with 0.5 mg dm^{-3} indole-3-butyric acid (IBA) rooted with 80 % frequency and developed into phenotypically normal plants. Plantlets were successfully acclimatized in a sterile mixture of sand and garden soil (1:1) under greenhouse and thereafter transferred to field beds.

Additional key words: BAP, 2,4-D, IAA, IBA, immature embryo, KIN, legume, medicinal plant, NAA.

Argyrolobium roseum is a sexually reproducing rare annual herb restricted to open, dry and coarse silty to sandy habitats. It is distributed in tropical and temperate tracts of Northwest India. Stem weak, ascending, branched, with digitately trifoliate leaves having obovate to cuneate end leaflets. Flowers yellow with rosy tinge, bilipped, upper lip exceeding limb. Pods glabrous, 10 - 15 seeded. Seeds exhibit poor germination (Hooker 1973). It is an important medicinal legume reported to possess antidiabetic, antibacterial, hypoglycemic and anti-inflammatory activities (Gupta *et al*. 2003). Scarce availability of planting material, slow regeneration in nature and lack of cultivation practices might be the possible reasons for multiplication of this plant species by shoot organogenesis from callus cultures, an effective method for multiplication of medicinal plants (Grewal and Atal 1976, Rout *et al.* 2000). Legumes are often recalcitrant to *in vitro* regeneration from callus cultures (Nagl *et al*. 1997). Callus cultures capable of organogenesis are possible utilizing mature and immature embryos as explants (Halámková *et al*. 2004, Zapata *et al*. 2004). The present study describes callus mediated shoot organogenesis as a method for shoot multiplication to achieve high frequency plant regeneration in *A. roseum.* Reports on regeneration *in vitro* are elusive in the species and this is the first report of *in vitro* regeneration in this medicinally important legume.

Green pods of *Argyrolobim roseum* Jaub & Spach were collected during November from the fields of Regional Research Laboratory, Jammu, India (32.43°N, 74.54°E). Immature embryos (15 - 20 mm) were excised from green pods (2.5 - 2.9 cm) and inoculated aseptically on Murashige and Skoog (1962; MS) medium supplemented with various concentrations and/or combinations of plant growth regulators (PGRs) 2,4-dichlorophenoxyacetic acid (2,4-D), benzylaminopurine (BAP), indole-3-acetic acid (IAA), kinetin (KIN) and naphthalene acetic acid (NAA), and with 3 % sucrose (m/v) and 0.8 % agar (m/v), pH 5.8. Data were recorded after 4 weeks from 5 experiments with 4 replicates each. All the cultures were maintained at 12-h photoperiod, irradiance of 40 μmol m^{-2} s^{-1} (white fluorescent lamps)

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Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; BAP - benzylaminopurine; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; KIN - kinetin; NAA - α-napthalene acetic acid, PGRs - plant growth regulators.

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and temperature of 25 ± 2 °C. Cultures were transferred to fresh proliferation medium and subculturing was carried out regularly at 4 week intervals. For rooting, *in vitro* derived multiple shoots were transferred to MS or B_5 (Gamborg *et al*. 1968) media supplemented with either of IAA, indole-3-butyric acid (IBA) or NAA. Completely regenerated plants were acclimatized in a sterile mixture of sand and garden soil (1:1) in polythene bags in diffused light for 3 to 4 weeks and then transferred to pots containing soil and grown in greenhouse under high relative humidity (85 - 90 %) for further eight weeks before transfer to field beds.

Immature embryo explants excised from 10 d-old-pods of *A. roseum* initiated calli after 3 weeks, which gradually extended over the surface of media. The entire embryo explants transformed into calli at various frequencies on different combinations of MS medium although the morphology varied with the nature of plant growth regulator used in the culture medium (Table 1). Soft or hard white callus was initiated with low frequency in presence of NAA (1.0 mg dm^{-3}) and KIN (1.0 mg dm^{-3}). The treatment containing 2,4-D (1.0 mg dm^{-3}) singly resulted in nodular yellowish green callus. Among the various combinations tested, the treatment containing BAP (0.5 mg dm^{-3}) and IAA (0.25 mg dm^{-3}) was most effective for initiation of callus. Green compact hard nodular callus was formed with high frequency in this treatment with 70 % of the cultures producing

Figs. 1 - 4. Regeneration of *Argyrolobium roseum via* organogenesis. Fig. 1. Callus and shoot bud differentiation from cultured immature embryo explants after 4 weeks of incubation on MS medium supplemented with BAP (0.5 mg dm^{-3}) + IAA (0.25 mg dm^{-3}). Fig. 2. Shoot proliferation after 4 weeks of incubation on MS medium supplemented with BAP (0.5 mg dm^{-3}). Fig. 3. Proliferation of subcultured shoots on MS medium supplemented with BAP (0.5 mg dm^{-3}) + IAA (0.25 mg dm^{-3}). Fig. 4. Rooting of *in vitro* regenerated shoots on B_5 medium supplemented with IBA (0.5 mg dm^{-3}).

Table 1. Morphogenetic response of immature embryo explants of *Argyrolobium roseum* cultured on MS medium supplemented with various PGR concentrations (data recorded after 4 weeks from 5 experiments with 10 replicates each).

PGR [mg dm^{-3}]	Responsive embryos [%]	Nature of callus
0	-	-
IAA (1.0)	20	hard nodular brown
IBA (1.0)	-	-
2,4-D (1.0)	60	soft nodular yellow
BAP (0.5)	30	hard nodular brown
BAP (1.0)	20	hard nodular green
KIN (0.5)	30	hard compact brown
KIN (1.0)	20	hard compact brown
BAP (0.5) + IAA (0.25)	70	hard nodular yellow with shoot primordia
BAP (1.0) + NAA (1.0)	60	soft friable green
KIN (0.5) + IAA (1.0)	30	hard compact brown
KIN (1.0) + NAA (1.0)	40	hard nodular brown

organogenetic callus. The treatment containing 2,4-D induced callus relatively quickly and callus tended to be soft and yellowish, while, those containing NAA required more time to initiate callus growth. Regeneration competence of calli was influenced by subculture passage and declined after 5th subcultures (data not shown). Subsequently, to induce organogenesis, the green nodular callus was transferred to regeneration media with various PGR treatments.

Shoot bud regeneration through callus was influenced by the interaction between growth regulators after three weeks of transfer to MS medium containing BAP (0.5 mg dm^{-3}) whereby deep green patches were observed on the callus surface. At the end of 4 weeks multiple shoot bud and meristemoid like structures were observed on the surface of green patches (Fig. 1). Subsequently after additional subculture for 2 weeks highly organized shoot proliferation was observed in treatment with BAP (0.5 mg dm^{-3}) with IAA (0.25 mg dm^{-3}) (Fig. 2, 3). The combination of NAA (0.5 mg dm^{-3}) and BAP (0.5 mg dm^{-3}) was ineffective in eliciting shoot proliferation and further development (Table 2).

Multiple shoot regeneration potential was better obtained when MS medium was supplemented with BAP (0.5 mg dm^{-3}) with a maximum of 35 ± 3.1 shoots exhibiting an average length of 6 ± 1.2 cm (Table 2). On the other hand, shoot production in KIN (0.5 mg dm^{-3}) containing medium was two fold lesser with a maximum of 18 ± 1.9 shoots exhibiting mean length of 4.8 ± 0.9 cm. Interestingly, the cultures maintained their growth ability, though the growth was characterized by difference from sub-culture to sub-culture, where the length of regenerated shoots decreased with increase in subculture passage (data not shown).

Table 2. Shoot differentiation *via* organogenesis from immature embryo derived callus cultures of *Argyrolobium roseum* on MS medium supplemented with various PGR concentrations (data recorded after 4 weeks; mean ± SE, n = 20).

PGR [mg dm^{-3}]	Number of shoots [flask^{-1}]	Shoot length [cm]	Number of nodes	Shoot morphology
BAP (0.5)	35 ± 3.1	6.0 ± 1.2	7	well elongated multiple shoots with long internodes
KIN (0.5)	18 ± 1.9	4.8 ± 0.9	3	well elongated, long internodes, multiplication from shoot buds
BAP (0.5) + IAA (0.25)	25 ± 2.1	4.0 ± 0.1	6	stunted shoots, multiplication from shoot buds
BAP (0.5) + IAA (0.5)	20 ± 2.1	2.5 ± 0.5	3	inhibition of shoot proliferation
BAP (0.5) + NAA (0.25)	15 ± 1.6	3.8 ± 0.5	3	stunted multiple shoot proliferation with callus
BAP (0.5) + NAA (0.5)	17 ± 1.9	2.0 ± 0.1	4	short shoot clumps, multiplication from shoot buds
KIN (0.5) + IAA (0.25)	13 ± 2.1	1.7 ± 0.1	2	green multiple shoot clumps, inhibition of shoot elongation
KIN (0.5) + IAA (0.5)	15 ±1.2	2.0 ± 0.9	2	short shoot clumps, multiplication from shoot buds
KIN (0.5) + NAA (0.25)	18 ± 1.5	3.8 ± 0.6	3	green stunted shoot clumps
KIN (0.5) + NAA (0.5)	12 ± 1.2	5.3 ± 1.1	3	well elongated, long internodes, multiplication from shoot buds

Table 3. Effect of B_5 medium with different auxins on *in vitro* rooting of regenerated shoots of *Argyrolobium roseum* (data recorded after 4 weeks; mean ± SE, n = 24).

Auxin [mg dm^{-3}]	Rooting frequency [%]	Number of roots [shoot^{-1}]	Root length [cm]	Root colour
IAA (0.1)	30	15 ± 1.8	0.5 ± 0.1	whitish
IAA (0.5)	40	18 ± 1.8	0.5 ± 0.1	whitish
IBA (0.1)	65	35 ± 2.1	1.5 ± 0.6	brownish
IBA (0.5)	80	41 ± 9.0	1.8 ± 0.6	brownish
NAA (0.1)	50	18 ± 2.1	0.8 ± 0.2	creamish white
NAA (0.5)	60	23 ± 2.1	1.0 ± 0.2	creamish white

Rhizogenesis from shoots was difficult on MS medium with or without exogenous addition of hormones; however, Gamborg's B_5 nutrient medium supplemented with IBA/NAA at different concentrations was found to be effective. Rhizogenesis could effectively be induced in well-elongated shoots (> 4 - 6 cm). The medium supplemented with IBA (0.5 mg dm^{-3}) was most

effective for rooting resulting in highest frequency (80 %) of rooting, followed by NAA (0.1 or 0.5 mg dm^{-3}) or IAA (0.1 or 0.5 mg dm^{-3}). Incidence of rooting was comparatively higher when auxins were used at a concentration of 0.5 mg dm^{-3} (Table 3). This is supported by earlier observations, which state that elongation has been a preparatory stage for rooting during which the carry over effect of PGRs is reduced (Druart *et al*. 1986, Gulati and Jaiwal 1994). Phenotypically normal plants were transferred to a 1:1 sterile mixture of sand-garden soil in polythene bags for acclimatization and hardening in greenhouse and later transferred to field beds with 60 % survival. The duration from explanting up to recovery of transplantable plantlets took about five and half months. The acclimatized plants exhibited normal development and no gross morphological variations were observed in the regenerants.

The present work registers for the first time that it is possible to regenerate *A. roseum*, a medicinally important recalcitrant legume by culturing young immature embryo explants and callus derived thereof on a sequence of media. Callus formation could be improved by manipulating the concentration and combination of auxins (IAA, 2,4-D or NAA) and cytokinins (BAP or KIN) in *A. roseum*. Appropriate concentrations of auxins and cytokinins [NAA (1.0 mg dm^{-3}, KIN (1.0 mg dm^{-3}) or BAP (0.5 mg dm^{-3}) with or without IAA (0.25 mg dm^{-3})] are probably the critical factors responsible for plant regeneration through their synergistic activity. Similar effect of auxins like 2,4-D, NAA and cytokinins like BAP, KIN in the formation of callus and morphogenesis observed in the present work is in agreement with available reports on legume regeneration *in vitro* (Bajaj and Dhanju 1979, Malik and Saxena 1992, Dillen *et al*. 1996, McClean and Grafton 1989). Further, differentiation could be induced following 3 - 4 subcultures on MS medium with BAP alone or in combination with NAA or IAA. Though 2,4-D favored callus formation, its absence in the aforesaid media is a prerequisite for differentiation. The potential of the presently described regeneration system could be appropriately employed for mass propagation, induction of somaclonal variants and genetic transformation of *A. roseum*, which augment further studies.

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