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Research note

In vitro plant regeneration of *Alnus acuminata* H.B.K. ssp. *acuminata* and its root nodulation by *Frankia*

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Abstract

A procedure for *in vitro* plant regeneration of *Alnus acuminata* from epicotyls with cotyledonary buds was developed using different media formulations with different growth regulators and carbon sources. The development of multiple buds on explants at the initiation step was obtained with MS at 1/2 strength with either 1 or 2 μM of BAP but not without it. Multiplication gave up to 15 elongating shoots by explant, the best medium being MS supplemented with vitamins from B5 medium, 1 μM of BAP and 87 mM sucrose. Rooting of about 88% occurred in the medium MS with 83 mM sucrose and 1 μM IBA. *Alnus acuminata* did not developed well on WPM. Roots of *in vitro* propagated plants were nodulated by *Alnus*-infective *Frankia*. The root nodules show a typical alder root nodule anatomy and differentiation pattern and effectively fixed nitrogen. *Rhamnaceae*-infective *Frankia* did not nodulate *in vitro* cultivated *Alnus acuminata* suggesting that symbiotic recognition was not altered by *in vitro* regeneration of the plant.

Abbreviations: AH – Arnon and Hogland medium (1950); BAP – 6-benzylaminopurine; DM – dry matter; B5 – Gamborg et al., medium (1968); E – Evans et al., medium (1972); IBA – indolbutyric acid; MS – Murashige and Skoog medium (1962); WPM – Lloyd and McCown's woody plant medium (1981)

Alnus acuminata H.B.K. ssp. *acuminata*, Betulaceae (Santos Biloni, 1990) is an actinorhizal plant that fixes atmospheric nitrogen because of its symbiotic association with the actinomycete *Frankia* (Wall, 2000). This alder forms native deciduous forests between the 1350 and 2700 m.b.s. in the South American phyto-geographic region named Yungas (Kappelle and Brown, 2001), and is an important species for reforestation, land reclamation and soil improvement. *In vitro* propagation of different species of the genus *Alnus*, except for *A. acuminata*, succeeded in the eighties: *A. glutinosa*, *A. crispa*, *A. rubra*, *A. incana*, *A. japonica*, *A. sinuata*, *A. viridis*, *A. cremastogyne*, *A. cordata*, and interspecific hybrids from four progenitors: *A. glutinosa*, *A. cordata*, *A. incana* and *A. rubra* (see Perinet and Lalonde, 1983; Sbay et al., 1989; Tang et al., 1996 and references cited there).

Our aim was to *in vitro* propagate the Argentinean native species *A. acuminata*, which has not previously been reported, and to study its nodulation by *Frankia* from different cross inoculation groups (Wall, 2000).

Seeds of *Alnus acuminata* H.B.K. ssp. *acuminata* were collected in a natural forest in Tucumán, La Banderita (65.95° long. S, 27.42° lat. W). Seeds were germinated in hydroponia, in the medium

Table 1. Effect of basic media at different strengths and of BAP concentration on shoot and bud production of *Alnus acuminata* explants

Basic media	Dilution	[BAP] μM	Number of shoots/ explant	% explants with multiple buds[a]
MS	0	1	2.4 ± 0.9	56.8^{de}
MS	0	2	3.4 ± 0.9	65.9^{bcd}
MS	3/4	1	4.2 ± 0.4	71.7^{bc}
MS	3/4	2	2.9 ± 1.3	74.3^{b}
MS	1/2	1	4.2 ± 0.8	93.8^{a}
MS	1/2	2	4.2 ± 0.5	91.1^{a}
MS	1/4	1	2.1 ± 0.1	46.7^{e}
MS	1/4	2	2.6 ± 0.6	47.6^{e}
AH	0	1	3.7 ± 0.5	67.8^{bcd}
AH	0	2	3.0 ± 1.4	58.9^{cde}
AH	1/2	1	3.1 ± 0.3	16.7^{g}
AH	1/2	2	2.6 ± 0.2	31.7^{f}
E	0	1	0.8 ± 0.1	23.3^{fg}
E	0	2	0.8 ± 0.1	5.0^{h}
E	1/2	1	0.0 ± 0.0	0.0^{h}
E	1/2	2	0.0 ± 0.0	0.0^{h}
WPM	0	1	1.5 ± 1.0	23.3^{fg}
WPM	0	2	2.2 ± 0.6	27.8^{fg}

All media contain 0.5 μM IBA. 10 explants per treatment, 3 replications.

[a] Different letters indicate significantly different means at the level $\alpha \leq 0.05$, using Duncan's multiple range test.

defined by Arnon and Hoagland (AH) (1950) diluted to 1/4. The epicotyl part of 20-days-old-seedlings with the cotyledons and 1–2 mm of hypocotyl, was surface sterilized as described elsewhere (Mroginski et al., 1997). Explants were cultivated in tubes with agar-media at pH 5.8. The number and length of new shoots of at least 0.5 cm long were considered for data analysis. Each treatment included 3 explants in each of 10 different containers. For germination and cultivation a photoperiod of 14/24 h of light (116 μmol m^{-2} s^{-1}, provided by cool white fluorescent tubes) and constant temperature (27 ± 2 °C) was used. Different media for *in vitro* culture were tested at the different steps of propagation.

At the initiation step, MS, AH, E and WPM culture media were used at different dilution rates plus 87 mM of sucrose, 0.5 μM IBA, and 1 or 2 μM BAP (Table 1). Thirty five days after starting the culture, 92% of the explants developed multiple buds in MS half strength medium while only 62% developed multiple buds in full strength AH (Table 1). Less than 23% of the explants formed multiple buds in E or WPM media at different dilution rates (Table 1). BAP, either at 1 or 2 μM, but not its absence, induced the development of multiple buds in a rosette (Figure 1A). Shoots also grew from adventitious buds on the cotyledonary node and/or from those of the rosette. Bud development occurred without the need of subculture, as was also the case with *Alnus crematosgyne* (Tang et al., 1996). The dilution of the nutrients increased the production of multiple buds in *A. acuminata*, but there were no adventitious buds at the leaf margin, as was the case in cultures of other *Alnus* species (Pèrinet and Lalonde, 1983).

The explants with shoots were transferred to either MS supplemented with vitamins taken from B5 medium, or AH medium for multiplication. The media were supplemented with 1 μM of BAP, and either 87 mM sucrose or 43.5 mM glucose. Subcultured shoots initially cultured on 1/2 MS, plus 1 or 2 μM BAP, produced the highest number of axillary buds. The shoots reached up to 3 cm in length in 15 days. The multiplication rate was 7.2

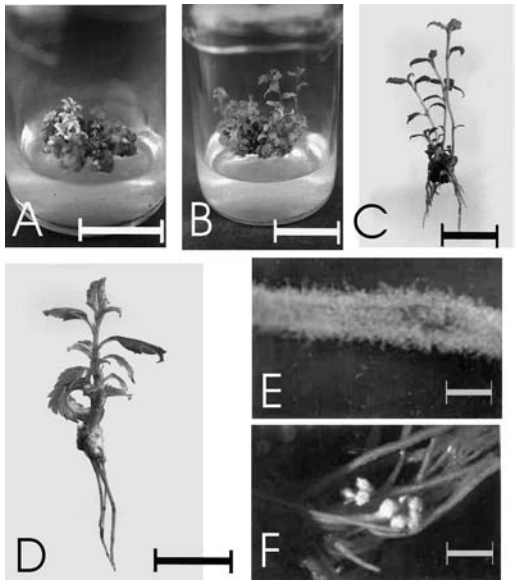

Figure 1. Alnus acuminata in vitro. (A) Initiation of shoots in 4 week-old cultures; bar = 2 cm. (B) Multiplication; bar = 2 cm. (C) Rooted shoots after 4 weeks on rooting medium, bar = 2 cm. (D) A single rooted shoot, bar = 2 cm. (E) Nodulation of a root on a *in vitro* propagated plantlet root 3 weeks after inoculation, bar = 1 mm, and (F) 5 weeks after inoculation, bar = 4 mm.

± 1.3 elongated shoots per explant (Figure 1B) in 3 weeks. The sucrose and glucose showed no differences in effect at this second step.

Thirty days after starting multiplication, the new shoots were separated and subcultured in single tubes for rooting. The following media were used:

– half strength MS medium supplemented with B5's vitamins, 87 μM sucrose or glucose and 1 or 2 μM of IBA,
– half strength AH medium with 87 μM of sucrose and 1 or 2 μM of IBA (Table 2).

Although the first roots appeared in the media with glucose at day 10, the best result, evaluated 30 days after transferring to rooting medium, was obtained in MS with 87 μM sucrose and 1 μM IBA: 88% of shoots rooted with 4.6 roots per shoot and 1.5 cm average root length (Table 2). Independent of the sugar source, 1 or 2 μM IBA produced a significant increase in the numbers of roots per shoot, but not in their lengths, compared to the zero IBA control ($p < 0.05$ Duncan's multiple range test) (Table 2). A further increase in the IBA dose led to excessive callus production. The rooting of the multiplied shoots of *A. acuminata* was easier than observed by Tremblay and Lalonde (1984) for some clones of other *Alnus* species.

In conclusion, the best basal medium for culture of *A. acuminata* was MS while WPM has been reported to be the best for the culture of other *Alnus* species like *A. glutinosa* (Tremblay and Lalonde, 1984), *A. cremastogyne* (Tang et al., 1996) and interspecific hybrids from four progenitors: *A. glutinosa*, *A. cordata*, *A. incana* and *A. rubra* (Sbay et al., 1989). MS was also better than AH for elongation, multiplication and rooting of *A. acuminata*.

Gradual acclimation of the plantlets was achieved by growing them in pouches (Mega International, Minniapolis USA) in which the humidity was changed from near 100 to 65% in 1 week. This resulted in 90% survival of the plants.

The *in vitro* propagated plants were initially assayed for nodulation by inoculation with a crushed nodule suspension, from *A. acuminata* nodules collected from a natural alder forest in Tucumán province, Argentina (see above). Nodulation started 20–30 days after inoculation with the crushed nodule suspension (Figure 1E and F).

In order to further characterize nodulation by *Frankia* of *in vitro* propagated plants, the nodulation kinetic was measured after inoculation with *Frankia* ArI3. Eight pouches were used per treatment, each of them with three *in vitro* propagated plants after agar medium was carefully removed from the roots. The *in vitro* propagated plants had 6–8 leaves at the time of inoculation, 100 days after initiation of the cultures (40 days of initiation, 30 days of multiplication and 30 days of rooting). To test the effect of plant acclimatization, a similar group of micropropagated plants were kept in pouches for one month before inoculation. Non-inoculated plants were used as negative controls. The pouches were weekly watered and the number of nodules was weekly recorded, multilobed nodules were considered as one nodule. Plants were harvested 15 weeks after inoculation and the dry weights of shoot, root and nodules

Table 2. Effect of the basic media, the carbon source and IBA concentration on root production of *in vitro* propagated *Alnus acuminata* shoots

Basic media	Carbon source	[IBA] μM	% of rooting	Number of roots /shoot	Roots length (cm) /explant
MS	Sucrose	0	32.3^{d}	2.3^{d}	1.34^{a}
MS	Glucose	0	33.2^{d}	2.9^{c}	1.47^{a}
MS	Sucrose	1	88.1^{a}	4.6^{ab}	1.49^{a}
MS	Glucose	1	72.4^{bc}	4.0^{b}	1.48^{a}
MS	Sucrose	2	65.3^{c}	4.8^{a}	1.47^{a}
MS	Glucose	2	76.9^{b}	4.4^{ab}	1.36^{a}
AH	Sucrose	0	67.3^{bc}	1.6^{e}	1.23^{a}
AH	Sucrose	1	73.1^{bc}	2.8^{cd}	0.79^{b}
AH	Sucrose	2	75.3^{bc}	2.2^{d}	0.54^{b}

15 explants per treatment.
Different letters indicate significantly different means at the level $\alpha \leq 0.05$, using Duncan's multiple range test.

were measured. Shoot dry weight was higher in nodulated plants than in non-inoculated controls, mainly due to an increment in the foliar area. (data not shown).The number of nodules per plant was higher in the acclimatized *in vitro* propagated plants than in the non acclimatized plants, 39.7 ± 3.6 and 24.8 ± 2.3 nodules per plant, respectively. This difference disappeared when the effect of plant growth on nodulation was taken into account by expressing nodulation on the basis of plant dry weight, 241 ± 39 nodules per g of acclimatized *in vitro* propagated plant, and 286 ± 44 nodules per g of non-acclimatized plant. The efficiency of the nodulation was evidenced by the absence of symptoms of nitrogen deficiency (chlorosis), after a month of growth without any nitrogen addition in the watering solution. In comparison, the non-inoculated controls were chlorotic. Dry weight of nodulated plants was significantly ($p < 0.05$) higher than that of non-inoculated plants ($\bar{x}$ ± SE: 0.115 ± 0.015 and 0.057 ± 0.006 g respectively). Microscopy of the nodules on the *in vitro* propagated plants revealed a typical nodule anatomy and normal development of *Frankia* with hyphae and vesicles (Wall, 2000). Pure *Frankia* cultures were used as inoculum to test for symbiotic specificity of *in vitro* propagated plants. *Frankia* ArI3 (D. Baker, Panlabs Inc., Bothell, WA, USA) and *Frankia* CpI1 (K. Huss-Danell, SLU, Sweden), both of them *Alnus* infective strains, effectively nodulated the *in vitro* propagated plants. Meanwhile *Frankia* BCU110501, isolated from *Discaria trinervis* (E. Chaia, CRUB, University of Comahue, Argentina), belonging to a different cross inoculation group (Wall, 2000) did not. These results suggest that symbiotic recognition was not altered by *in vitro* regeneration of *Alnus acuminata*.

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