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Requirements for in vitro propagation of seven nitrogen-fixing *Alnus* species

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Abstract. Studies on the in vitro propagation of *Alnus crispa, A. glutinosa, A. incana, A. japonica, A. rubra, A. sinuata* and *A. viridis* indicated interspecific as well as intraspecific variations in their requirements for in vitro culture. The WPM and Blaydes media supported, respectively, growth of *A. glutinosa* and *A. crispa* but not that of both species, while the MS medium induced equal or significantly better growth than WPM and Blaydes media for both species. The optimum type and concentration of sugar to be used in the multiplication medium varied with species. Only *A. glutinosa* showed good growth on sucrose while glucose was optimum for all other species but at different concentrations. All species rooted in 3 weeks on half-strength MS medium including 1 μM IBA. All clones of *A. glutinosa* and *A. rubra* rooted 100%, whereas "easy-to-root" and "difficult-to-root" clones were observed in the other species. In the rooting medium, glucose promoted rooting of the "difficult-to-root" clones better than sucrose. Survival following transfer to an artificial substrate was 100% for all species. Nodulation tests using pure cultures of two *Frankia* strains showed 100% nodulation on all *Alnus* clones.

Introduction

The utilization of nitrogen-fixing species such as alder in forestry involves the selection of superior genotypes, which then need to be propagated through cloning [5, 19]. In vitro propagation offers the advantages of a fast multiplication rate of genetically identical and disease-free plants compared to conventional techniques [6, 12]. Applications of tissue culture in forestry have been reviewed including a list of the species of the different genera of trees propagated by in vitro culture [19].

In the genus *Alnus*, the micropropagation of *A. glutinosa* [4, 10, 16, 18], *A. crispa* [10, 11, 18, 21] and *A. rubra* [10, 11, 18] have been reported. We now report the in vitro propagation of four additional *Alnus* species together with the specific in vitro requirements of all seven species, and the nodulation of the micropropagated plantlets inoculated with pure cultures of *Frankia*.

Materials and methods

Seven *Alnus* species from different provenances were used: *A. glutinosa* (L.) Gaertn. (clones AG-3, AG-4, AG-5 and AG-8 from Penzenskoi Oblast, USSR),

A. crispa (Ait.) Pursh. (clones AC-1 and AC-15 from Chapleau, ON, Canada), *A. japonica* (Thumb.) Stand. (clones AJ-6, AJ-7 and AJ-8 from Perthshire, Scotland), *A. incana* (L.) Moench. (clones AI-2, AI-4 and AI-5 from Eganville, ON, Canada), *A. rubra* Bong. (clones ARb-8 and ARb-10 from Forbidden Plateau, BC, Canada), *A. viridis* (Villars) DC (clones AV-5, AV-7 and AV-8 from Perthshire, Scotland) and *A. sinuata* (Reg.) Rydb. (clone ASn-1 from Prince Rupert, BC, Canada).

Actively growing shoot tips were collected in May in the field on 2-year-old plants at the Canadian Forestry Service Station (Petawawa, ON) and immediately disinfected according to Tremblay and Lalonde [21]. Explants 4-6 mm long were placed on the initiation culture media following excision of leaves and petioles. The basal culture medium consisted of Murashige and Skoog's (MS, [13]) mineral salts and vitamins, 3% (W/V) sucrose and 0.7% (W/V) Difco-Bacto agar at pH 5.5, except that the NaFeEDTA stock solution was prepared according to Steiner and Van Winden [20]. This basal medium was used unless otherwise specified. Shoot tips of each species were initiated on I-1 and M-1 media which consisted of the basal medium including, for medium I-1, 1 μM benzylaminopurine (BAP) and 0.5 μM indolebutyric acid (IBA), and for the medium M-1, 2.5 μM BAP.

For the multiplication step, different mineral salt solutions were compared on *A. crispa* and *A. glutinosa* previously micropropagated on two salt solutions (4, 10, 11, 16, 18, 21). The different salt formulations compared were MS at 0.5X, 1X, 2X and 4X, B5 [3], Nagata and Takebe (NT [14]), Blaydes [1] and Woody Plant Medium (WPM [9]), known tissue culture media, and the nitrogen-containing (KNO_3, 0.5 g/l) Crone's solution [8] used for the growth of *Alnus* seedlings. All media were supplemented with 2.5 μM BAP, 3% (W/V) sucrose, vitamins as in the MS medium and 0.7% (W/V) Difco-Bacto agar at pH 5.5 in 1.5 liter Mason jars at the rate of 200 ml by jar. Each jar was planted with 8 shoots 7-10 mm tall; 1 shoot from each culture was transferred twice to fresh medium every 3 weeks. After 9 weeks, each culture was checked for survival, number and length of shoots, callus production and leaf area. The callus production was evaluated following the rating scale 0:no callus, 1:light, 3:average, and 5:largest area obtained for a species. Leaf area was evaluated as 1: smallest area, 3: average and 5: largest area obtained for a species. The assay was a nested (jar (medium)) factorial design with 8 samples by replicate (Mason jar), with two replicates.

Sucrose, glucose and fructose and the sugar-alcohol mannitol were tested on one clone of each *Alnus* species at concentrations of 0 mM, 30 mM, 87 mM, 175 mM and 350 mM to determine their sugar requirements. Sucrose and glucose at 87 mM and 175 mM were then tested on the 18 clones of the seven *Alnus* species to verify possible clonal variations. Glucose and fructose were chosen as components of the sucrose molecule while mannitol was included to determine if it was metabolized by *Alnus* species.

The cytokinins bezylaminopurine (BAP), N6-(Y^2-isopentenyl) adenine

(2ip) and kinetin (kin) at 5 μM were compared on *A. incana* and *A. japonica* species, because the shoots of these species failed to elongate. Furthermore BAP was tried at 1, 2.5 and 5 μM and 2ip at 5, 50 and 500 μM on the same species. The MS basal medium modified by replacing sucrose by 87 μM glucose and poured at 15 ml in 20 x 150 mm culture tubes was used. The number and length of shoots taller than 0.5 cm as well as the leaf area were noted after 3 subcultures lasting 3 weeks each. Each species was assayed separately using a completely randomized block design with 5 replicates and 1 sample per replicate for each species.

The rooting media contained half strength MS salts with 1 μM IBA and 45 mM, 87 mM or 175 mM sucrose or 87 mM and 175 mM glucose with 15 ml distributed in 20 x 150 mm culture tubes. Thirty shoots of each clone were tested on each medium.

The pH of all media was adjusted with 1N NaOH to 5.5 after melting of agar and before autoclaving at 121 °C at 1 kg cm^{-2}, 12 min for tubes or 15 min for jars, including the media for the carbon source assay as preliminary tests showed no differences between autoclaved and filter-sterilized media (0.2 μm) on *Alnus*.

Cultures for the initiation and multiplication steps were grown in a growth chamber with a thermoperiod of 26/22 °C and a photoperiod of 16/8 day/night under 2.5 klux provided by Vita-Lite fluorescent tubes (Duro-Test Electric Ltd, ON, Canada) while the rooting and inoculation steps were performed under 7.5 klux provided by Cool-White:Gro-Lux WS fluorescent tubes (Sylvania, Drummondville, QUE, Canada) in the ratio 2 : 1.

After 21 days on the rooting media, the rooted plantlets were washed under tap water and transferred to PlantCon® containers (Flow Laboratory Inc., VI, USA) filled with a mixture of Turface (International Minerals and Chemicals Corp., Des Plaines, IL, USA) : vermiculite : peat moss (4 : 1 : 1) amended with N-free Crone's solution, with one plantlet per container. The plantlets were inoculated 21 days after their transfer to containers, the clones of *A. incana, A. sinuata* and *A. crispa* with the *Frankia* strain $ARgN22_d$ [15] and the clones of *A. japonica, A. viridis, A. rubra* and *A. glutinosa* with the *Frankia* strain $AGN1^{AG}_{exo}$ [7]. Growth of the *Frankia* strains, preparation of the inoculum and inoculation were as previously described [21]. Non-inoculated plantlets of each clone from the different *Alnus* species were used as controls. Nodulation, i.e. presence of nodules, was verified 3-4 weeks after inoculation and nitrogen fixation was confirmed by the appearance of green leaves on inoculated plantlets as compared to the yellow leaves on non-inoculated controls. At the end of the nodulation test, the plantlets were transplanted to 11 cm diameter pots containing the same artificial substrate without nitrogen. Following one week in a growth chamber under 70% relative humidity, plantlets were transferred to greenhouse with normal conditions.

Results

All species of *Alnus* were successfully initiated i.e. aseptic and viable (Figure 1), on I-1 and M-1 media. However, the medium I-1 induced the production of basal and leaf callus on all species and transfer to the medium M-1 without auxin was necessary within 1 month. After 8-10 weeks, the explants developed as shoot clumps ready to divide. Only the cultures of *A. glutinosa* showed good development when multiplied on the M-1 medium. The maintenance of cultures of other species on M-1 led to the production of callus and leaf abnormalities on *A. crispa, A. viridis* and *A. sinuata* while basal necrosis and inhibition of shoot multiplication were observed on *A. crispa, A. viridis, A. sinuata, A. incana, A. japonica* and *A. rubra.*

The use of 2X and 4X MS salts induced the complete necrosis of *A. crispa* and *A. glutinosa* cultures within 3 weeks. Half strength MS salts supported growth of cultures for the first 3 weeks, but after that time, cultures showed

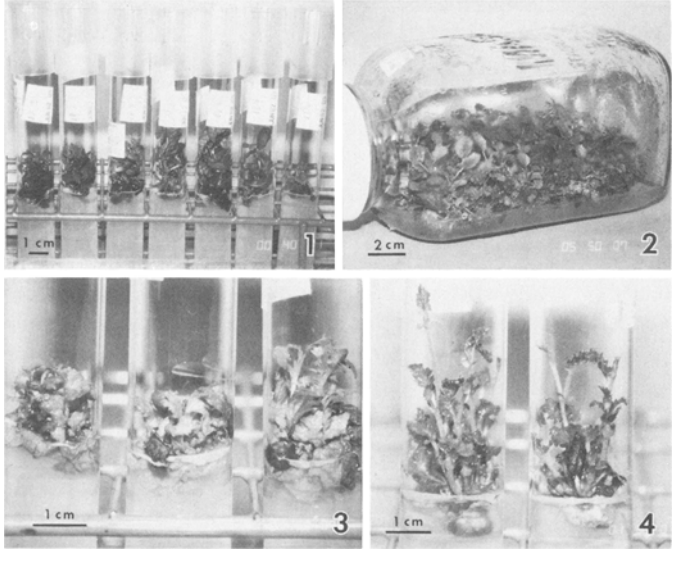

Figure 1. *Alnus* species initiated on I-1 medium. From left to right: *A. japonica, A. rugosa* (not discussed here), *A. rubra, A. viridis, A. glutinosa, A. crispa* and *A. incana* (*A. sinuata* not represented).

Figure 2. *A. sinuata,* clone ASn-1, on the MS basal medium including 2.5 μM BAP and glucose at 175 *mM*.

Figure 3. Culture of *A. crispa* showing leaf abnormalities (large and curling leaves) on the M-1 medium including 2.5 μM BAP and 87 *mM* sucrose.

Figure 4. Culture of *A. crispa* after growth on the basal medium as in Figure 3 but supplemented with 175 *mM* glucose.

Table 1. Comparative effects of the MS, WPM and Blaydes media on the multiplication of *A. crispa* (AC) and *A. glutinosa* (AG)

Medium	Shoot number			Shoot length (cm)			Callus (0-5)			Leaf area (1-5)		
	AC	AG	$\bar{x}c$	AC	AG	$\bar{x}c$	AC	AG	$\bar{x}c$	AC	AG	$\bar{x}c$
MS	11.4	2.3	7.0a	1.4	1.5	1.5a	0	2.1	1.1a	2.7	2.4	2.5a
WPM	4.2a	3.0	4.0b	1.0	1.3	1.3a	0	2.0	1.0a	2.7	2.0	2.4a
Blaydes	11.7	1.0b	6.7a	1.0	0.8	0.9b	0	0.5	0.2b	1.7	1.7	1.7b

[a]Cultures showing important necrosis on leaves and shoots.
[b]Means established with 10/16 cultures 6 were dead.
[c]Means ($\bar{x}$) not affected by the same letter are significantly different at 5% with Tukey's Studentized Range (HSD).

numerous deficiency symptoms. On B5 medium and Crone's solution, there was little and no growth, respectively, of *A. crispa* and *A. glutinosa* while growth on the NT medium was good although less than on the MS medium. MS medium was found to promote the growth of the two species tested contrary to WPM and Blaydes media (Table 1). The WPM medium resulted in good growth of *A. glutinosa* but poor growth of *A. crispa,* while Blaydes medium promoted growth of *A. crispa* but not of *A. glutinosa.* The MS medium was equal or significantly better than WPM and Blaydes media for all the observed growth characteristics of the two species.

Sucrose, glucose and fructose, compared to mannitol, were tested on the seven *Alnus* species. Controls without sugar as well as culture grown with mannitol alone died within 6 weeks, indicating that mannitol is not metabolized by any *Alnus* species. Cultures grown with fructose remained alive for the duration of the experiment although leaf development was completely inhibited. Sucrose and glucose in the range of 87 *mM* to 350 *mM* supported growth of the seven species of *Alnus* tested, with an optimum at 87 to 175 *mM* depending on the species (Table 2). Sucrose was optimum only for *A. glutinosa* whereas glucose gave a better growth for all other *Alnus* species in culture. All species when exposed to a sugar other than the optimal one, i.e. glucose or sucrose (Figure 3, 4), developed brownish calluses, basal necrosis and leaf abnormalities. Furthermore, in these suboptimal conditions, little multiplication if not death of cultures, was observed.

The cytokinins BAP, 2ip and kin were compared at 5 μM on the clones AI-2 and AJ-6 to check on a possible inhibition of shoot elongation by BAP (Table 3). At 5 μM, 2ip enhanced significantly (alpha level: 0.05) shoot length and leaf area of the clone AI-2, while the type of cytokinin did not affect these parameters for the clone AJ-6. Increasing the concentration of 2ip to 50 or 500 μM induced necrosis of cultures instead of increasing the number of shoots longer than 0.5 cm. Lowering the concentration of BAP to 1 μM permitted both the formation and the elongation of numerous shoots.

Table 2. Sugar requirements of *Alnus* species and clones during the multiplication stage on the MS medium including 2.5 μM BAP

Alnus species	Clone number	Sugar			Multiplication rate[b]
		Type[a]	Concentration (mM)	%(W/V)	
A. glutinosa	3,4,5,8	S	87	3	3–5
A. crispa	1,15	G	175	3	10–12
A. rubra	8,10	G	87	1.5	3–5
A. incana[c]	2,4,5	G	87	1.5	3–5
A. japonica[c]	6,7,8	G	87	1.5	3–5
A. viridis	5,6,7	G	175	3	5–7
A. sinuata	1	G	175	3	5–7

[a]S = Sucrose, G = Glucose
[b]Number of shoots taller than 0.5 cm produced with 3 weeks from 1 subcultured apex
[c]Medium including 1 μM BAP

Table 3. Effect of BAP, kin and 2ip at 5 μM on the number of shoots longer than 0.5 cm, leaf area and shoot length on clones AI-2 and AJ-6[a]

Clone	Number of shoots (> 0.5 cm)			Leaf area (1–5)			Shoot length (cm)		
	BAP[b]	kin	2ip	BAP	kin	2ip	BAP	kin	2ip
AI-2	1.0a	1.2a	1.0a	1.0a	1.4a	3.4b	0.8a	1.0ab	1.3b
AJ-6	1.2a	1.4a	1.0a	1.0a	1.0a	1.8a	1.3a	1.2a	1.1a

[a]Each clone was tested separately with 5 replicates, 1 culture per replicate
[b]Numerous shoots shorter than 0.5 cm not counted. For each clone, values not affected by the same letter are significantly different at 5% with Duncan's test

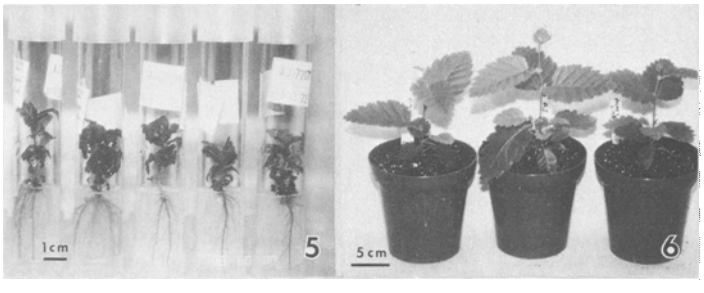

Figure 5. Rooted plantlets of *A. japonica* after 3 weeks on the rooting medium and showing well developed root sytems.

Figure 6. Nodulated in vitro-propagated plantlets of *A. sinuata*, clone ASn-1, 2 months after inoculation with *Frankia* strain ARgN22$_d$.

Table 4. Percentages[a] of rooting in relation with *Alnus* species, clones and type and concentration of sugar used in the rooting medium after 3 weeks

Alnus species	Clone number	Sucrose		Glucose	
		87 *mM*	175 *mM*	87 *mM*	175 *mM*
A. glutinosa	3,4,5,8	100	100	100	100
A. rubra	8,10	80	100	100	100
A. crispa	1	30	10	60	40
	15	90	30	100	100
A. incana	2,5	100	100	90	100
	4	40	50	50	50
A. japonica	6,7	100	100	100	100
	8	50	30	60	80
A. viridis	5	0	0	0	10
	7	60	30	100	60
A. sinuata	1	30	30	50	40

[a]Determined with 30 shoots on each medium

Following the multiplication of every clone of each species on its optimal multiplication medium (Table 2, Figure 2), shoots were excised and placed on rooting media including sucrose or glucose at different concentrations. The rooting percentage after 3 weeks (Figure 5) varied with species, clone, type of sugar and sugar concentration used in the rooting medium (Table 4). *A. glutinosa* and *A. rubra* rooted 100% independently of clone and medium while the species *A. crispa, A. japonica, A. incana* and *A. viridis* showed clonal variation in their rooting potential. The clones considered "easy-to-root" were AC-15, AI-2, AI-5, AJ-7 and AV-7 while the "difficult-to-root" clones were AC-1, AI-4, AJ-8 and AV-5. The type and concentration of sugar used in the rooting medium was critical for the "difficult-to-root" clones. Glucose enhanced rooting at the two concentrations tested compared to sucrose, the optimal concentration varying with the clone and the species.

The plantlets survival following the transfer to an artificial substrate was 100% for all clones, whatever the multiplication or rooting media previously used.

Within 3-4 weeks, 100% of the *Frankia*-inoculated plantlets of all clones of the seven species tested were nodulated while no nodulation occurred on the non-inoculated controls (Table 5). The development of green leaves on the nodulated plantlets as compared to the yellow leaves of the non-nodulated controls indicated that *Frankia* was effective in fixing nitrogen. This observation confirmed the effectiveness of the numerous root nodules on all inoculated plantlets. Following the transfer to the greenhouse, the nodulated plantlets showed homogenous and rapid growth (Figure 6).

Table 5. Percentages of nodulation of the different species of *Alnus* inoculated with pure cultures of *Frankia*

Alnus sp.	*Frankia* strain	Number of nodulated/ inoculated plantlets[a]	Number of nodulated/ controls[b]
A. crispa	$ARgN22_d$	20/20	0/4
A. glutinosa	$AGN1^{AG}_{exo}$	40/40	0/8
A. incana	$ARgN22_d$	30/30	0/6
A. japonica	$AGN1^{AG}_{exo}$	30/30	0/6
A. rubra	$AGN1^{AG}_{exo}$	20/20	0/4
A. sinuata	$ARgN22_d$	10/10	0/2
A. viridis	$AGN1^{AG}_{exo}$	10/10	0/2
Total		160/160	0/32

[a]Ten plantlets per clone were inoculated
[b]Two plantlets per clone were not inoculated as controls

Discussion

Eighteen clones of seven *Alnus* species can be successfully propagated by in vitro techniques and have now been maintained in culture in a good physiological state for periods of 1.5 to 4 years.

The initiation step was not critical for any species of *Alnus* tested. However presence of auxin in the medium was deleterious, inducing callus development on all species as reported during the multiplication step for *A. glutinosa* [16, 18]. Dividing and transferring the cultures to the same basal medium led to callus production, leaf abnormality, basal necrosis, inhibition of shoot multiplication and often death of cultures. This was the case with *A. crispa, A. incana, A. japonica, A. rubra, A. sinuata* and *A. viridis* while the cultures of *A. glutinosa* developed normally on the basal medium and under the same growth conditions. This variation indicates that *A. glutinosa* and the other species of *Alnus* differ in their requirements.

During the multiplication stage, B5 medium and Crone's solution cannot support growth of *A. crispa* or *A. glutinosa* while the NT medium yields less growth than MS medium for the same species. Modifying the MS salt strength resulted in deficiency or toxicity symptoms on the cultures. The compared growth of *A. crispa* and *A. glutinosa* on MS, WPM and Blaydes media indicated that these 2 species had different salt requirements, both being only fullfiled by the MS medium. Indeed, growth of *A. glutinosa* is similar on WPM and MS media but inhibited on Blaydes medium while growth of *A. crispa* is good on Blaydes and MS media but not on WPM medium. Therefore the MS medium was used for the propagation of the other *Alnus* species since it appeared to be a superior and generally well-accepted medium by *Alnus*. These results differ with those of Read et al. [18] which considered that the

MS medium "tended" to stimulate more callus and fewer microshoots than the WPM medium on growth of both *A. crispa, A. glutinosa* and *A. rubra,* without mention of any difference between these two salt formulations in regard to the species tested.

With respect to the sugars tested, sucrose and glucose gave good results depending on the *Alnus* species or clone tested, while the various *Alnus* species can metabolize fructose to some extent but not mannitol. Mannitol should therefore constitute a good osmoticum for these *Alnus* species when necessary. Our results indicated differences in the sugar requirements for the various *Alnus* species tested. For example, *A. glutinosa* was the only species tested that grew on sucrose whereas all other species tested required glucose at 87 or 175 *mM*. Comparisons of different clones of *A. glutinosa* from two geographic provenances, clones AG-1 and AG-2 from the Federal Republic of Germany [16] and clones AG-3, AG-4, AG-5 and AG-8 from the USSR, indicated genetic stability for medium requirements and growth characteristics contrary to a previous report on *A. rubra* (18). However, the species *A. crispa* showed clonal variation in its sugar requirement: the optimal sugar for the multiplication of *A. crispa* clone AC-2 [21] was sucrose while all other clones of *A. crispa* needed glucose.

The cytokinin BAP at 2.5 μM gave good shoot multiplication for *A. crispa, A. glutinosa, A. rubra, A. sinuata* and *A. viridis,* while a BAP concentration of 1 μM was better for *A. incana* and *A. japonica.* With these two species, BAP gave better results than kin and 2ip, as previously reported on *A. glutinosa* [17], *A. crispa* and *A. rubra* [11].

In vitro rooting of all species has been achieved in 3 weeks on the MS medium at half strength including 1 μM of IBA. In vitro rooting of *A. glutinosa* was previously reported (16, 17), while *A. crispa* (10, 11, 18), *A. glutinosa* (10, 16, 18) and *A. rubra* (11, 18) were rooted as microcuttings in a mist bed. Our results showed an important variation in the rooting potential of *Alnus* species. All clones of *A. glutinosa* (AG-3, AG-4, AG-5 and AG-8) and of *A. rubra* (ARb-8 and ARb-10) rooted 100%. However, we can consider two groups of *Alnus* clones in the other species: the "easy-to-root" and the "difficult-to-root" clones. This variable rooting potential of clones of the same species was independent of the geographic provenance, for example, the *A. viridis* clones, clones AV-5 and AV-7, were from the same provenance. This clonal variation appeared to be related to individual characteristics of the seedlings used as mother-plants. Replacing sucrose with glucose in the rooting medium enhanced rooting of the "difficult-to-root" clones.

Survival of the plantlets following soil transfer was not affected by the clone, neither by the rooting medium nor by the species. The in vitro growth of *Alnus* on the high salts MS medium results in vigorous fast-growing plantlets with a well developed root system. As previously reported (17), the in vitro propagation technique did not affect the ability of *Alnus* roots to form actinorhizae with *Frankia* as all inoculated plantlets were nodulated.

The development of green leaves on the nodulated plantlets as compared to the yellow leaves of the non-nodulated controls indicated that *Frankia* was effective in fixing nitrogen. This observation confirmed the effectiveness of the numerous root nodules on all inoculated plantlets.

We are now able to propagate by in vitro techniques eighteen clones of seven *Alnus* species from different geographic locations. This opens up the possibility of large scale production of interesting *Alnus* clones. The development of these techniques can be a powerful genetic tool, for example the in vitro propagation of a non-nodulating clone of *A. crispa* [21]. Furthermore, the in vitro propagated clones can be used to compare the effects of *Frankia* isolates on the growth of genetically defined host-plants, as done by Dawson and Sun [2] with clones obtained from cuttings. Genetic studies of cloned actinorhizal plants without the interference of other microorganisms [17], as present on cuttings, is now possible.

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References

1. Conger BV (1981) Agronomic crops. In: Conger, BV (ed), Cloning agricultural plants *via* in vitro techniques. CRC Press, Boca-Raton, 165–215
2. Dawson JO, Sun SH (1981) The effect of *Frankia* isolates from *Comptonia peregrina* and *Alnus crispa* on the growth of *Alnus glutinosa, A. cordata* and *A. incana* clones. Can J For Res 11:758–762
3. Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158
4. Garton S, Hosier MA, Read PE, Farnham RS (1981) In vitro propagation of *Alnus glutinosa* Gaertn. Hort Science 16:758–759
5. Hall RB, Maynard CA (1979) Considerations in the genetic improvement of alder. In: Gordon JC, Wheeler CT, Perry DA (eds), Symbiotic nitrogen fixation in the management of temperate forests. Corvallis: Oregon State University, pp 322–344
6. Hughes KW (1981) Ornamental species. In: Conger, BV (ed.) Cloning agricultural plants *via* in vitro techniques. CRC Press, Boca Raton, 5–50
7. Lalonde M (1979a) A simple and rapid method for the isolation, cultivation in vitro and characterization of *Frankia* strains from *Alnus* root nodules. In: Gordon JC, Wheeler CT, Perry DA (eds), Symbiotic nitrogen fixation in the management of temperate forests. Corvallis, OR.: Oregon State University, p 480
8. Lalonde M (1979b) Techniques and observations of the nitrogen-fixing *Alnus* root nodules symbiosis. In: Subba Rao NS (ed.), Recent advances in biological nitrogen fixation. Oxford and IBH Publ., New Delhi: 421–434
9. Lloyd G, McCown B (1980) Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. Proc Intl Plant Prop Soc 30: 421–427

10. Louis K, Garton S, Read PE (1982) Factors influencing the micropropagation of *Alnus* species. (Abstract) Hort Science 17:533
11. Louis K Read PE (1983) The effect of 2ip, BA and GA3 on in vitro recultured *Alnus*. (Abstract) Hort Science 18:617
12. Mott RL (1981) Trees. In: Conger BV (ed.), Cloning agricultural plants *via* in vitro techniques. CRC Press, Boca Raton, 217–254
13. Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497
14. Nagata T, Takebe I (1971) Plating of isolated tobacco mesophyll protoplasts on agar medium. Planta 99:12–20
15. Normand P, Lalonde M (1982) Evaluation of *Frankia* strains isolated from provenances of two *Alnus* species. Can J Microbiol 28:1133–1142
16. Périnet P, Lalonde M (1983) In vitro propagation and nodulation of the actinorhizal host plant *Alnus glutinosa* (L.) Gaertn. Plant Sci Lett 29:9–17
17. Périnet P, Lalonde M (1983) Axenic nodulation of in vitro propagated *Alnus glutinosa* plantlets by *Frankia* strains. Can J Bot 61:2883–2888
18. Read PE, Garton S, Louis K, Zimmerman ES (1982) In vitro propagation of species for bioenergy plantations. In: Akio Fujiwara (ed) Proc 5th Intern Congr Plant Tissue and Cell Culture, pp 757–758
19. Sommer HE, Brown CL (1979) Application of tissue culture to forest tree improvement. In: Sharp WR, Larsen PO, Paddock EF, Raghavan V, (Eds), Plant cell and tissue culture, principles and applications. Colombus: Ohio State University, pp 461–491
20. Steiner AA, Van Winden H (1970) Recipe for ferric salts of ethylenediaminetetraacetic acid. Plant Physiol 47:862–863
21. Tremblay FM, Nesme X, Lalonde M (1984) Selection and micropropagation of nodulating and non-nodulating clones of *Alnus crispa* (Ait.). Pursh. Plant & Soil 78: 171–180