ASPEN DECAY AND STAIN AND GENETIC FINGERPRINTING OF CLONES

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ABSTRACT

The primary objective of the study was to establish *in vitro* culture protocols for bud culture, suspension culture and micropropagation of *Populus tremuloides* (quaking aspen). Nodal bud cultures were initiated from 7 clonal populations of aspen by culturing buds, after removal of the outer scales, on agar-solidified MS medium (Murashige and Skoog, 1962) supplemented with 2 or 5 μM N^6-benzyladenine (BA) in petri plates. Buds formed shoots within 2-3 weeks of culture. Shoot elongation and multiplication were successful by transferring the microshoots onto MS medium with or without 1 μM BA. Rooting of the developing shoots occurred on transfer to half-strength basal MS medium ± 0.05% activated charcoal. The plantlets were hardened in covered plastic boxes or in a misting chamber and transferred to pots in the greenhouse. Leaves from the *in vitro* -developing shoots were excised and cultured onto MS medium + 10 μM 2,4-dichlorophenoxyacetic acid (2,4-D) + 5 μM BA to induce friable callus. Proliferation of callus was attained on half-strength MS medium supplemented with 5 μM 2,4-D + 5 μM BA. The callus pieces were then transferred to liquid MS medium with 5 μM 2,4-D and incubated on a shaker at 150 rpm to initiate suspension cultures. The suspension cultures were subcultured every 12-16 days depending on the growth rate of the genotype. After repeated subcultures, small white embryogenic cell clumps resembling globular-stage somatic embryos were seen. These cell clumps did not develop further on transfer to agar-solidified MS medium supplemented with either 0 to 10 μM 2,4-D or 0 to 5 μM thiadiazuron (TDZ). Efficient protocols for nodal bud culture, callus culture and suspension culture of aspen were developed.

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INTRODUCTION

Increasing demand for timber and paper pulp in turn requires fast growing trees of improved quality and disease resistance (Thorpe and Biondi, 1984). Aspens are important forest tree species due to their potential for establishment in new and widely different planting areas, short-rotation culture and faster growth rate (Ahuja, 1987; Chun et al., 1986; Evers et al., 1988; Hall et al., 1982; Park and Son, 1988). The aspen resource in Canada comprises about 10% of the forest inventory (Morley, 1986), and is mostly underutilized due to the poor grade of large tree stands. To enhance this potential, high-grading of stands is essential, and this can be accomplished by growing specific improved quality genotypes of aspen. Even though aspens can be vegetatively propagated by conventional methods, the commercial feasibility of the vegetative production of specific genotypes through these techniques is limited (Ahuja, 1987). Since most of the tree species which are amenable to vegetative propagation through conventional methods achieve results very slowly, it is highly imperative to devise methods by which large scale populations of characterized, high performance trees can be grown.

In vitro culture methods have been used extensively in micropropagation of a large number of tree species (Thorpe and Biondi, 1982; Bajaj, 1986; Thorpe et al., 1991). Among the various woody tree species, *Populus tremuloides* was the first successful species in which plants were produced through tissue culture via callus. A number of advanced applications of tissue culture technology with woody plants has been with different species of *Populus* (Ho and Raj, 1985; Wann and Einspahr, 1986; Russell and McCown, 1986, 1988; Fillatti et al., 1987; Schwartzenberg et al., 1994). In general, a basic disadvantage with most of the studies with woody plant species has often been the use of seeds, seedlings or juvenile parts (Mehra and Cheema, 1980; Thorpe and Biondi, 1982; Cheema, 1989; Thorpe and Harry, 1990). As clearly indicated by the last two references, most of the desirable characteristics in tree species are identifiable only after maturity and, therefore, studies with juvenile material may not have an immediate impact in tree improvement programs. Development of *in vitro* propagation protocols for mature tree explants is very limited and often encompasses a wide range of difficulties such as: i) inability to manipulate *in vitro* explants taken from mature trees, ii) episodic growth pattern observed in culture, iii) high amounts of phenolic exudates from older tissues, iv) systemic infection of tissue explants causing contamination in culture, and v) vitrification or hyperhydric malformation of shoots (Thorpe et al., 1991). The present study was designed to develop protocols for specific genotypically diverse clones of aspen which have been characterized to some extent. The specific objectives of the present study were: i) to develop optimum and efficient protocols for micropropagation of 7 clonal populations (involving 14 different genotypes) of poplar through nodal bud cultures, ii) to standardize techniques for successful transfer of *in vitro*-developed rooted-shoots to the greenhouse, iii) to induce callus cultures from leaves of *in vitro* /greenhouse grown poplars, iv) to develop a reliable protocol for suspension culture initiation from leaf-derived callus cultures, v) to

establish an optimum subculture schedule for cell suspension cultures in order to maximize growth rates, and vi) to induce somatic embryogenesis from cell suspension cultures. This project was a subcomponent of a larger project dealing with aspen decay and stain - a major problem in aspen, and genetic fingerprinting of clones.

MATERIALS AND METHODS

Plant materials

Aspen plants consisting of 35 different clones from 7 populations (CAR, I, BL, M, W, S, K) were obtained as a gift from Dr. Francis Yeh, Department of Renewable Resources, University of Alberta. These plants were maintained in pots in the greenhouse (Fig. 1 a). The pots were irrigated twice weekly and fertilized weekly with commercial fertilizer (Peters® 20-10-20). Insecticidal spraying was carried out every week in order to avoid infestation with spider mites. Dormant buds (Fig. 1 b) from these greenhouse-grown plants were used for initiation of nodal bud cultures, and leaves were utilized for initiation of callus cultures in some cases. Fourteen genotypes from the 7 clonal populations (CAR-1, 2, 3, 4; BL-1, 2, 6; W-1, 3; M-1, 4; I-2; Z-2; S-1) of aspen were selected, based on their growth patterns in the greenhouse, and were used for shoot initiation from nodal bud cultures. Based on the response of the different genotypes, further studies were carried out with a limited number of the selected genotypes, in view of the practical feasibility and resources available.

Micropropagation studies

Micropropagation of poplar was carried out in four distinct steps, which are, bud breaking and shoot initiation, microshoot multiplication and elongation, rooting of microshoot cultures, and establishment of *in vitro*-derived plants in the greenhouse, following established procedures for this technology (Thorpe and Patel, 1984; Dunstan and Thorpe, 1986).

Bud breaking and shoot initiation: Twigs containing a few buds in each were collected from trees of the 7 clonal populations (14 genotypes) maintained in the greenhouse. The twigs were cut into small pieces such that each contained a nodal bud. Buds were surface-sterilized by soaking them in 95% ethanol for 1 min, rinsing thrice with sterile-distilled water, soaking for 15 min in 20% commercial bleach (Javex™) with 2-3 drops of Tween-20, followed by rinsing thrice with sterile-distilled water. These sterile buds (intact with about 5-7 mm of stem) were either cultured directly, or dissected aseptically to remove the outer-most scales under a dissection microscope in a laminar flow hood and then cultured onto different media combinations, in petri-plates. The culture media used were either agar-solidified MS medium (Murashige and Skoog, 1962) or Aspen Culture Medium (ACM; Ahuja, 1983) supplemented with different concentrations of 6-benzylaminopurine (BA; 0-10 μM), or, 1-naphthaleneacetic acid (NAA; 0-10 μM). In order to minimize the detrimental effects of phenolic exudates from the explants, activated charcoal (0.05%) was also added to the medium in a few experiments. Similarly, the petri-plates were sealed with either Parafilm® or Stretch n' Seal® to compare their effects on bud breaking and shoot initiation. The cultures were incubated in a 26 ± 2 °C

growth chamber under light conditions for 2-4 weeks to induce bud breaking and shoot initiation. Optimum media combinations and culture methods were identified and used in the following experiments.

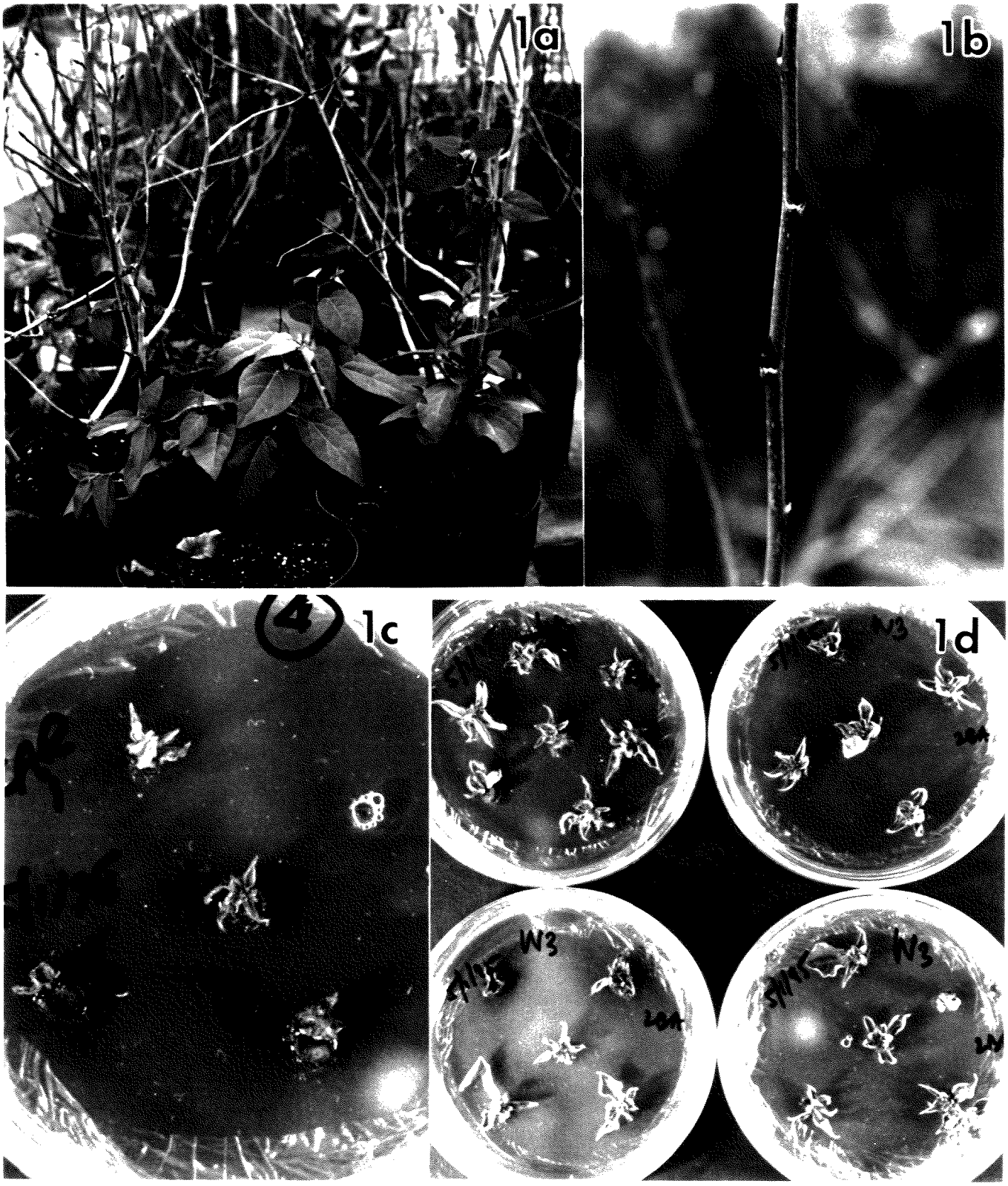
Shoot multiplication: Shoots initiated start to turn brown after prolonged culture in the same medium without transfer. In order to overcome this potential problem, shoots (or developing buds) were transferred every 2 weeks on to appropriate fresh medium. When the shoots were about 1 cm in length, they were transferred on to agar-solidified MS or ACM medium with or without different concentrations of BA (0-10 μM). The proliferating shoots were also transferred every 2-3 weeks on to fresh medium to enhance futher multiplication and maintain a higher growth rate. The effect of culture container on shoot elongation was also studied by using three different types of containers, viz., small glass jars (4 oz) sealed with either Parafilm® or Stretch n' Seal®, magenta vessels and test tubes.

Rooting of shoot cultures: After 8-12 weeks of culture, the proliferating shoots were transferred onto either full- or half- strength MS medium without any phytohormones. Activated charcoal (0.05 and 0.1%) was supplemented in a few replications to observe its effect on elongation and rooting of the shoots. Repeated subcultures of shoots onto similar media were made at 3 week intervals to enhance rooting.

Establishment of in vitro derived plants in the greenhouse: The shoots with developed root system were transferred from culture medium into covered transparent plastic boxes or seedling trays with sterile vermiculite. The transplanted seedling trays were maintained in a misting chamber for 4 weeks before transplanting the hardened plants into pots and transferring them to the greenhouse. The plants in the covered plastic boxes were sprayed with water every 12 hrs to maintain a very high relative humidity and were incubated under high light intensities in growth chambers for 4 weeks prior to transferring them to pots in the greenhouse.

Callus cultures from leaf explants

The leaves for inducing callus cultures were either collected from greenhouse grown plants or were excised from microshoots of *in vitro* grown bud cultures. The leaves from the greenhouse were surface-sterilized by swabbing with 70% ethanol, washed twice with sterile distilled water, soaked in 15% commercial bleach for 15 min, followed by rinsing thrice with sterile distilled water. The leaves from microshoots were excised and used as such. The leaves were either cut into small strips of 3-4 mm width or a 10 mm cork borer was used to obtain leaf discs for culture. The leaf explants were placed on appropriate medium with either the abaxial or the adaxial surface in contact with the medium. The media used for leaf-callus induction was agar-solidified MS medium supplemented with 2,4-D (1, 5, 10, 20 μM) or NAA (1, 5, 10, 20 μM) or BA (1-5 μM) or Kinetin (1-5 μM) or IAA/IBA (1, 5, 10 μM) alone or in different combinations. The leaf cultures were incubated


1a
1b
1c
1d
W3

under dark conditions in a 28°C growth chamber for 3-4 weeks. The initiated callus pieces were subcultured, every 2-3 weeks, onto the same medium with lower concentrations of appropriate growth regulators to induce callus multiplication.

Suspension culture initiation and subculture schedule

The freshly initiated, leaf-derived callus cultures of selected genotypes were multiplied for 2-3 months prior to utilizing them for initiating suspension cultures. The proliferating callus pieces were then transferred to Erlenmeyer flasks with 30 ml of liquid medium and were incubated in the dark on a shaker at 120-150 rpm. The liquid medium used was MS medium supplemented with 2,4-D (1, 2.5, 5, 10 μM concentrations). Subculturing of suspension cultures was carried out by two different methods depending on their growth rate. If the growth rate of the suspension culture was low, the flasks were left in the laminar flow for 2-3 hrs in order to facilitate settling of the cells. Then the top 20 ml of spent medium was pipetted out and fresh media was added to the flask. In the case of a faster growth rate, the suspensions were subcultured by transferring 10 ml of cell suspension into new flasks containing 20 ml of fresh medium. In either case subculturing was done at 2 week intervals. To obtain the cell growth rate in suspension cultures, the packed cell volume (PCV) was measured. PCV was measured after 2, 4, 6, 8, 10, 12, 14, 16 and 18 days of culture. The PCV was measured as ml of cells that formed a pellet after centrifugation at low speed (150-200 rpm for 5 min) in order to avoid damage to the cells.

Somatic embryogenesis from cell suspensions

During prolonged subculturing of the suspension cultures, cell clumps (about 1-3 mm in diameter) developed. These cell clusters were compact and resembled globular stage somatic embryos. The globular cell clusters were removed from the flasks and plated onto agar-solidified MS medium supplemented with either 2,4-D (0.5, 1 , 5 μM) or thiadiazuron (TDZ; 0.01, 0.05, 0.1, 0.5, 1.0, 5.0 μM) to induce further development. Some of the replicate plates were maintained under light conditions and some were incubated in the dark.

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RESULTS

Micropropagation of aspen

Shoot initiation from nodal buds

Shoot initiation from nodal bud explants of 14 genotypes of aspen cultured on different media and phytohormone combinations is shown in Table 1. MS and ACM media varied markedly in their ability to induce bud breaking and microshoot induction from nodal buds, and the former was found to be more suitable (Fig. 1 c, d). The response of nodal bud explants to media supplemented with BA was comparatively higher than NAA supplemented media. The average percent response with various levels of BA in both type of media was 77% compared to a 27% response with NAA. Among the various concentrations of BA tested, 2 µM and 5 µM levels gave the maximum response under both types of media. Maximum microshoot induction (96%) was seen in MS medium supplemented with 2 µM BA (Fig. 2 a-d), followed by ACM + 2 µM BA (89%) and MS + 5 µM BA (88%). Very high concentrations of NAA (10 µM) showed the lowest response in both MS (9%) and ACM (3%) media. Among the 14 different genotypes included in the study, CAR-5 showed the maximum response (100%) on both MS/ACM media + 2 or 5 µM BA combinations (Fig. 3 a, b). Similar high responses were also seen in genotypes, BL-6, BL-2, W-3 cultured in MS medium, and CAR-3, BL-2, BL-6 cultured in ACM medium. Reddish colouration of initiated shoots was seen in M-4 after 3 weeks of culture (Fig. 3 c, d).

Effect of activated charcoal on shoot initiation

The effect of activated charcoal, added to various media combinations, on shoot induction is given in Table 2. Activated charcoal (0.05%) added to MS + BA (1-10 µM) medium did not enhance bud breaking or microshoot initiation over its control. However, addition of activated charcoal to MS + NAA (0-10 µM) medium enhanced microshoot induction by 5.3%. Similar increases of 1.8% and 2.5% were seen by addition of activated charcoal to ACM medium supplemented with BA (1-10 µM) and NAA (1-10 µM), respectively. Based on overall response, MS medium with BA (2 µM) and without activated charcoal showed the maximum shoot induction response (96%, as shown in Table 1).

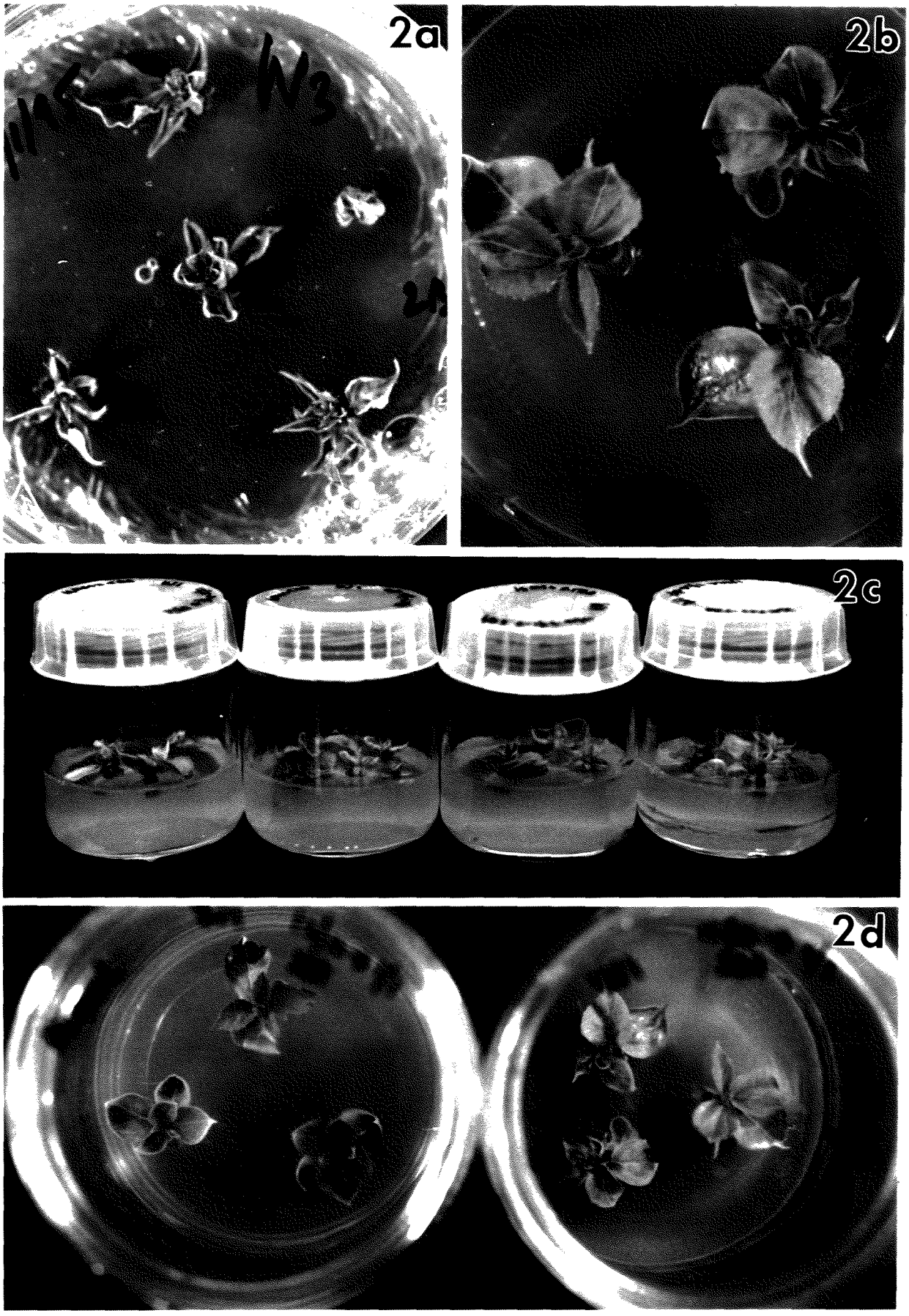
Bud culture method & petri plate sealant effects on shoot initiation

Buds cultured with intact without removal of outer scales responded poorly (37.5%) compared to culture after removal of these scales (98.75%), as shown in Table 3. Phenolic exudates from intact buds was very high and most of the buds did not

Table 1. Shoot initiation from nodal bud explants of 7 aspen clonal populations on various medium combinations*.

Medium and phytohormone combinations	Genotypes														Total Average (in %)
	CAR				BL			W		M		I	Z	S	
	1	2	3	5	1	2	6	1	3	1	4	-- 2	-- 2	-- 1	
MS medium + BA															
1 µM	32	29	36	38	32	31	37	30	30	29	33	27	31	25	79
2 µM	39	38	39	40	36	39	40	36	39	37	40	39	39	34	96
5 µM	31	34	28	40	39	40	40	37	40	34	36	32	36	28	88
10 µM	21	18	17	25	29	27	32	26	31	28	27	28	17	15	61
MS medium + NAA															
1 µM	18	19	21	25	13	17	22	19	19	10	17	15	18	18	45
2 µM	25	23	23	31	19	21	26	17	29	9	13	19	22	17	53
5 µM	12	14	7	19	8	12	17	5	7	0	1	7	12	8	23
10 µM	1	0	2	14	0	12	8	0	11	2	0	0	1	0	9
ACM medium + BA															
1 µM	28	27	32	31	22	36	31	35	26	15	21	32	28	22	69
2 µM	34	36	39	40	31	40	39	32	37	29	38	34	36	33	89
5 µM	36	33	37	40	32	36	37	24	34	23	29	36	28	21	80
10 µM	19	22	25	27	25	32	32	26	18	12	20	27	16	13	56
ACM medium + NAA															
1 µM	13	17	15	11	15	12	10	9	12	7	15	14	17	13	32
2 µM	16	21	20	17	18	11	16	12	15	6	11	16	18	11	37
5 µM	7	5	2	12	1	6	8	7	11	8	4	2	14	6	17
10 µM	0	0	0	5	0	3	0	0	1	4	2	0	0	0	3

* Data presented are observations on 40 buds cultured / treatment in a single experiment. The experiments were repeated > 4 times.


2a
2b
2c
2d

3c
3d
3a
3b

Table 2. Morphogenetic responses of nodal buds from 7 clonal populations of aspen cultured on various medium combinations with or without activated charcoal (AC).

Treatments (Media combinations ± activated charcoal)			% of buds developing	Morphogenetic response
Medium	Hormone (μM)	Supplements		
MS	+ BA (1-10)	0% AC	81.0	MS medium ± 2 or 5 μM BA without any AC was optimum for shoot initiation from nodal bud cultures. Some cultures showed callus formation in the bud-stem interface, but callus was not calcitrant.
MS	+ NAA (1-10)	0% AC	32.5	NAA induced rooting in some genotypes, but was not effective for shoot initiation. Callusing seen at interface with 5 μM NAA. Necrosis of apex at higher NAA levels after 2-3 weeks.
MS	+ BA (1-10)	0.05% AC	80.3	No significant difference in shoot initiation seen by addition of AC.
MS	+ NAA (1-10)	0.05% AC	37.8	2 and 5 μM NAA with AC increased shoot initiation. No callusing seen at interface and necrosis of apex was delayed.
ACM	+ BA (1-10)	0% AC	73.5	Similar response as seen in MS medium.
ACM	+ NAA (1-10)	0% AC	22.3	Shoot apex turned necrotic at higher NAA levels. Leaves were crinkled. Shoots were small and did not elongate further.
ACM	+ BA (1-10)	0.05% AC	75.3	No significant effect of AC.
ACM	+ NAA (1-10)	0.05% AC	24.8	AC alleviated some of the effects, but shoot initiation low.

Table 3. Morphogenetic responses of aspen genotypes, CAR-5, BL-2, BL-6, and W-3, nodal buds cultured intact or after excision of outer scales on agar-solidified MS medium supplemented with 2 μM BA in petri plates sealed with Parafilm® or Stretch 'n' Seal® in separate experiments.

Treatments (Bud culture method/Sealant used for plates)	% of buds developing	Morphogenetic response
Bud culture method		
Buds cultured with scales intact	37.50	Bud breaking did not occur (not efficient), high amounts of phenolic exudates seen in media, developing shoots were not healthy, browning of shoots initiated within 2 weeks.
Buds cultured with outer scales removed	98.75*	Shoots start developing from buds within 1 week of culture, shoots are healthy and leaves start to unfurl, microshoots do not elongate and the apex starts browning if not subcultured within 3 weeks of initiation.
Sealant of Petri plates		
Parafilm®	95.00	No significant difference in shoot initiation over Stretch 'n' Seal®
Stretch 'n' Seal®	98.75*	Stretch 'n' Seal used in further experiments due to its cost effectiveness.

* CAR-5 nodal buds showed 100% response when cultured with outer scales removed and the plates were sealed with Stretch 'n Seal®.

show bud breaking ability. Removal of outer scales from buds reduced the amount of phenolic exudates and also enhanced bud breaking. In some cases where most of the bud scales were removed, percent bud breaking was very high. However, these buds did not show a higher growth rate and showed very high levels of leaf senescence. Culturing nodal buds with just the outer scales removed was found to be the efficient culture method for microshoot induction. The effect of two different types of sealant used to wrap petri plates is also shown in Table 3. There was no significant difference in microshoot induction response due to the type of sealant used. Both, Parafilm® (95%) and Stretch n' Seal® (98.75%) were found to enhance microshoot induction. The aspen genotype, CAR-5 showed a 100% response when the outer scales excised-nodal buds were cultured on MS + 2 μM BA medium in petri plates sealed with Stretch n' Seal®.

Microshoot multiplication

Shoot multiplication from microshoots of CAR-5 genotype of aspen cultured on various concentrations of BA supplemented to MS or ACM medium is given in Table 4. Basal MS or ACM medium, showed similar effects on microshoots of CAR-5. The shoot multiplication rate was very low as is evident from the average shoots formed per microshoot explant of 2.13 and 2.73 under MS and ACM medium, respectively. However, these shoots were very healthy and showed the maximum shoot elongation and leaf expansion. The number of shoots formed per microshoots were as high as 12.5, 10.7, 10.47 and 10.3 in ACM + 1 μM BA, MS + 10 μM BA, MS + 1 μM BA and ACM + 5 μM BA, respectively. Although such high shoot multiplication rates were observed in these treatments, the quality of the shoots produced were poor. Most of the shoots were axillary shoots with lengths varying from 0.3-1.5 cm. Strikingly, the quality of shoots decreased and had a bushy apperance as the concentration of BA increased in the medium. Among the various treatments tested, MS medium with or without 1 μM BA was found to be optimum for inducing higher number of both axillary and adventitious shoots, as well as shoot and leaf expansion.

Microshoot elongation

The effect of three types of culture containers on CAR-5 aspen microshoot elongation is shown in Table 5. Among the three types of containers used, 4 oz. glass jars sealed with either Parafilm® or Stretch n' Seal® was found to show the maximum shoot elongation (1.46 cm increase in shoot length). The shoots cultured in larger glass jars also gave a better response than the 4 oz. glass jars (data not shown). Comparatively, the shoots were healthier, shoot elongation was high (average was > 3 cm) and leaves were green and wider (Fig. 4 a, b). Similar to the small glass jars, sealing the jars with Parafilm® or Stretch n' Seal® did not show significant effect on shoot elongation in the larger glass jars. The microshoots cultured in magenta containers did not elongate (0.63 cm increase) comparably. The leaves turned chlorotic with the shoot apex turning black and drying (Fig. 4 c, d).

Table 4. Effect of BA (0-10 μM) on shoot multiplication from microshoot cultures of CAR-5 aspen genotype.

Media + BA (μM)	Microshoots cultured	Number of shoots formed	Average shoots/explant	Remarks Visual observations
MS + 0	30	64	2.13	Shoot elongation (up to 2.5 cm) and leaf expansion (up to 2 cm long) seen. Rooting of some shoots also occurs.
MS + 1	30	314	10.47	Shoot elongation (up to 2.0 cm) and leaf expansion (up to 1 cm long) seen. No. of shoots high but leaves small. Both axillary and adventitious shoots.
MS + 5	30	279	9.30	Shoot elongation (up to 1.5 cm) and showing a bushy appearance. High no. of axillary shoots.
MS + 10	30	321	10.70	Highest no. of shoots formed, but shoots and leaves are very small. The leaves are necrotic. Shoots are mostly axillary.
ACM + 0	30	82	2.73	Similar to MS medium without BA, with shoot and leaf expansion highest.
ACM + 1	30	375	12.50	No. of shoots higher than MS media, but shoot elongation (up to 1 cm) and leaf expansion very low. Bushy in appearance.
ACM + 5	30	309	10.30	Mostly axillary shoots, very small and compact on each other. Callusing seen. Shoots originate from the hardened stem or the callus.
ACM + 10	30	287	9.57	Generally short and compact axillary shoots (up to 0.8 cm long) with small leaves.

Table 5. Effect of different types of culture containers on shoot elongation in nodal bud-derived microshoots of CAR-5 aspen genotype.

Type of culture container	Number of shoots cultured@	Average increase in shoot length* (cm)	Morphological observations
Magenta containers	38 (2)	0.63	Shoots stayed short, some large leaves were seen. Chlorosis of leaves occurs in most cases.
4 oz. glass jars with petri plate covers sealed with Parafilm®/ Stretch n' Seal®	30 (2)	1.46	Shoots were larger compared to other types of containers. However, they were smaller compared to larger glass jars. Shoots were healthy with dark green leaves. Sealing the jars with Parafilm® or Stretch n' Seal® did not show any significant difference in the shoot elongation response.
Test tubes with clear caps	30 (1)	1.31	Most shoots elongated but the leaves were curved and damaged due to crowding in the test tube.

* Based on average shoot explant of 0.5 cm used for culturing in all cases. Measurements were made after 4 weeks of culture in appropriate containers containing agar-solidified MS medium without any hormones.

@ Numbers in parentheses indicate the number of explant shoots cultured per culture container.

4b
4d
4a
4c

Microshoots cultured in test tubes showed a better response, but the leaves were crowded in the test tube and after 4 weeks of culture, they were curved and damaged.

Rooting of in vitro-derived microshoots

The rooting response of *in vitro*-derived microshoots cultured in different media combinations is shown in Table 6. Full-strength MS medium with or without charcoal induced rooting in 60-89% of the microshoots of CAR-5 aspen. However, modifying the rooting media to half-strength MS and with or without activated charcoal addition increased the rooting response to 100%. The growth response of the rooted-shoots, after 6 months in the greenhouse, suggested that rooting of shoots on half-strength MS with or without 0.05% activated charcoal was the best treatment. These plants showed prolific production of buds and leaves compared to other rooting treatments used. All the rooted-shoots were transplanted successfully to the greenhouse, either after transferring them into plastic boxes with high relative humidity (100% success) or by transferring them into seedling trays and maintaining them in a mist chamber (95% success). The *in vitro*-derived plants were established and are maintained in the greenhouse (Fig. 5 a, b).

Callus induction from leaves of greenhouse-grown trees

Callus induction from *in vitro*-developed leaves of 4 selected genotypes of aspen under different media and phytohormone combinations is given in Table 7. Preliminary studies on callus induction from greenhouse-grown trees were not successful due to two possible reasons. First, the insect infestation in greenhouse-grown trees was high, which caused a considerable level of contamination in cultures. Secondly, the greenhouse trees were also sprayed with insecticides to control the insect problem. These insecticides might have some detrimental effects on callus production as is evident from the absence of any response in cultured non-contaminated leaves of many genotypes. In order to avoid this potential problem, leaves from *in vitro*-grown shoot cultures were used to induce callus.

Callus induction from leaves of in vitro-derived microshoots

Among the various treatments, MS + 5-20 μM 2,4-D + 5 μM BA was found to be the best callus induction medium for leaf explants (Table 7). This media combination produced friable yellowish-white callus and was later found to be highly suitable for initiating cell suspension cultures (Fig. 6 a-c). All the genotypes studied performed well on this media, with the prolific callusing seen in the order of BL-2 > BL-6 > W-3 > CAR-5. Supplementing ACM medium with the same levels of phytohormones produced yellowish nodular callus and prolific rooting. Addition of IAA or IBA (1-10 μM) to either MS or ACM medium, did not produce any callus but showed high frequency of root initiation. Similarly, no callus production was observed from leaves cultured on MS or ACM medium supplemented with either BA (1-5 μM) or

Table 6. Effect of culture media and activated charcoal (AC) on rooting response of *in vitro*- derived shoots of CAR-5 aspen genotype.

Media combination	Number of shoots cultured	Number of shoots rooting	Transplantation success (%)*	Plantlet quality@
MS + 0% AC	18	12 (67%)	100	+
MS + 0.05% AC	15	9 (60%)	100	++
MS + 0.1% AC	18	16 (89%)	100	++
1/2MS + 0% AC	18	18 (100%)	100	+++
1/2MS + 0.05% AC	15	15 (100%)	100	+++
1/2MS + 0.1% AC	14	14 (100%)	100	++

* Transplantation success is expressed as the percentage of shoots which rooted. Transplantation of the shoots was done by transferring the rooted-shoots into transparent plastic boxes. The shoots were sprayed frequently with water in order to maintain a high humidity inside the box. Rooted-shoots maintained in a misting chamber on planting trays had a 95% success rate.

@ Evaluation carried out 6 months after transfer to the greenhouse: +++, plants healthy with many leaves and produce many buds; ++, plants healthy, fewer leaves and buds; + plants with leaves showing chlorotic symptoms on leaves, very poor growth.

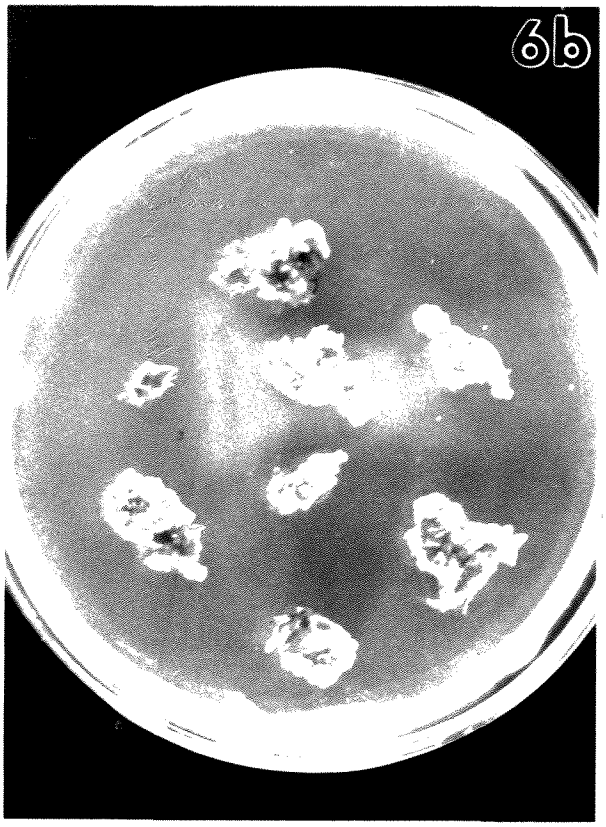
5b

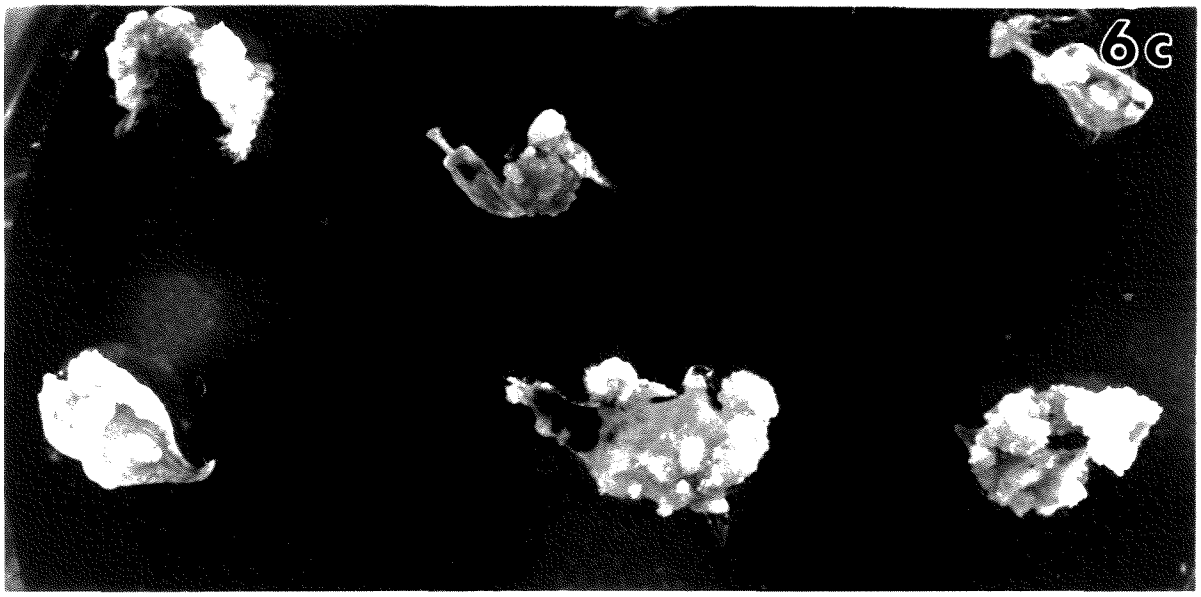
5a

Table 7. Effect of MS or ACM medium supplemented with various phytohormones on callus initiation from leaf explants of CAR-5, BL-2, BL-6 and W-3 genotypes of aspen.

Media combinations Media	Phytohormones	Morphogenetic response
MS	+ 1 μM 2,4-D + 5 μM BA	The leaf discs turned brown and died after 2 weeks, no callus formation observed.
MS	+5/10 μM 2,4-D + 5 μM BA	Prolific callus formation at 10 μM 2,4-D and both roots and callus formed at 5 μM 2,4-D. MS + 10 μM 2,4-D + 5 μM BA was found to be optimum for callus induction from all four genotypes. Callus formation was: BL-2 > BL-6 > W-3 > CAR-5. The abaxial surface in contact with medium produced more callus than the adaxial surface facing medium. Callus was friable and yellowish -white. Used for initiating suspension cultures.
ACM	+ 1 μM 2,4-D + 5 μM BA	No response seen. Leaves remained green for an extended ? period than MS medium.
ACM	+ 5/10 μM 2,4-D + 5 μM BA	Number of roots formed was high at both concentrations. Callus formed was nodular and yellowish. Not suited for initiation of suspension cultures.
MS/ACM	+ 20 μM 2,4-D + 5 μM BA	Root formation limited, but prolific callusing seen. MS medium produced friable and compact callus, while ACM medium induced nodular callus only. Callus induced on MS medium was also used to initiate suspension cultures. Abaxial surface facing medium gave better response than adaxial facing media.
MS	+ 1/5/10 μM IAA or IBA	Prolific rooting but no callus formation.
MS/ACM	+ 1/5/10 μM NAA + 5 μM BA	Some callus formed but more rooting and elongation of roots observed. Callus formed was compact and nodular.
MS/ACM	+ 1-5 μM BA (or) 1-5 μM Kn	No callusing response, leaves became chlorotic in few cases, turned brown and died.


6a


6b


6c

←Kinetin (1-5 µM). These leaves turned chlorotic and dried during the culture period. Addition of NAA (1-10 µM) with BA (5 µM) to MS or ACM medium, induced some nodular compact callus but produced more roots, which elongated on prolonged culture. In all the successful callus induction treatments, leaves cultured with the abaxial surface in contact with the medium showed better response than leaves with adaxial surface in contact with the medium. Callus initiated was mainly from the midrib and veins of leaves (Fig. 6 c), and roots formed either from the callus or directly from the cut edges without an intervening callus (Fig. 6 a, c). Based on the various experiments, the best callus induction treatment was culturing leaf discs from *in vitro*-grown microshoots with abaxial surface facing media on MS media supplemented with 5 or 10 µM 2,4-D and 5 µM BA (Fig. 7 a).

Cell suspension cultures from leaf-derived callus

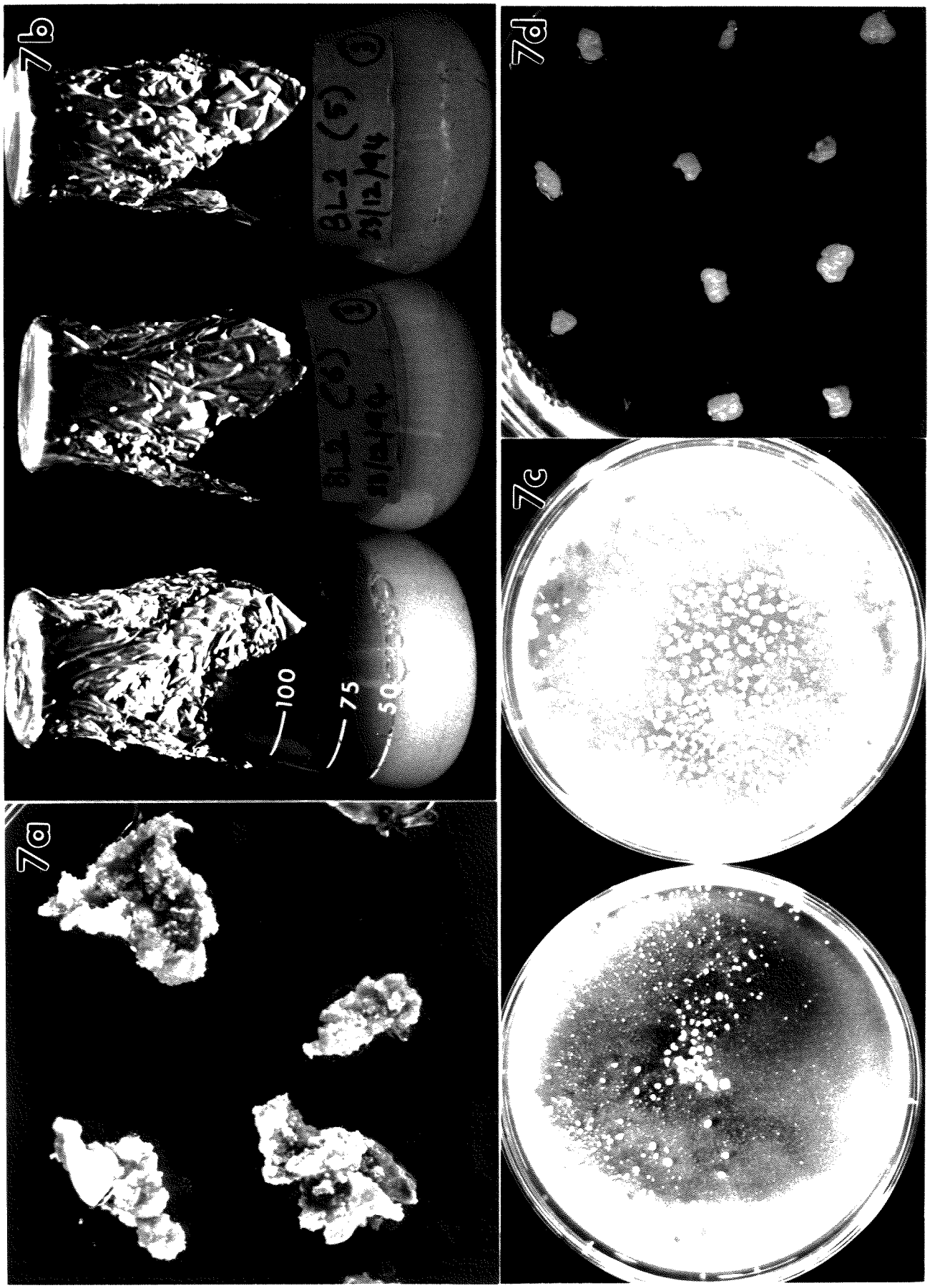
The effect of liquid MS medium supplemented with 1-10 µM 2,4-D on growth of cell suspension cultures of 4 aspen genotypes is shown in Fig. 7-9. The packed cell volume (PCV) measured over 14 days of culture growth on appropriate medium suggested varying growth rates between the genotypes with increasing 2,4-D concentration. Among the genotypes, BL-2 showed the maximum growth of cell suspensions at all levels of 2,4-D tested, and the maximum growth rate was observed at 5 µM, 2,4-D (Fig. 7 b, c). The optimum 2,4-D concentration was found to be 5 µM beyond which growth of cell suspensions either reached a plateau or decreased, except for BL-6 genotype where the optimum was 10 µM 2,4-D.

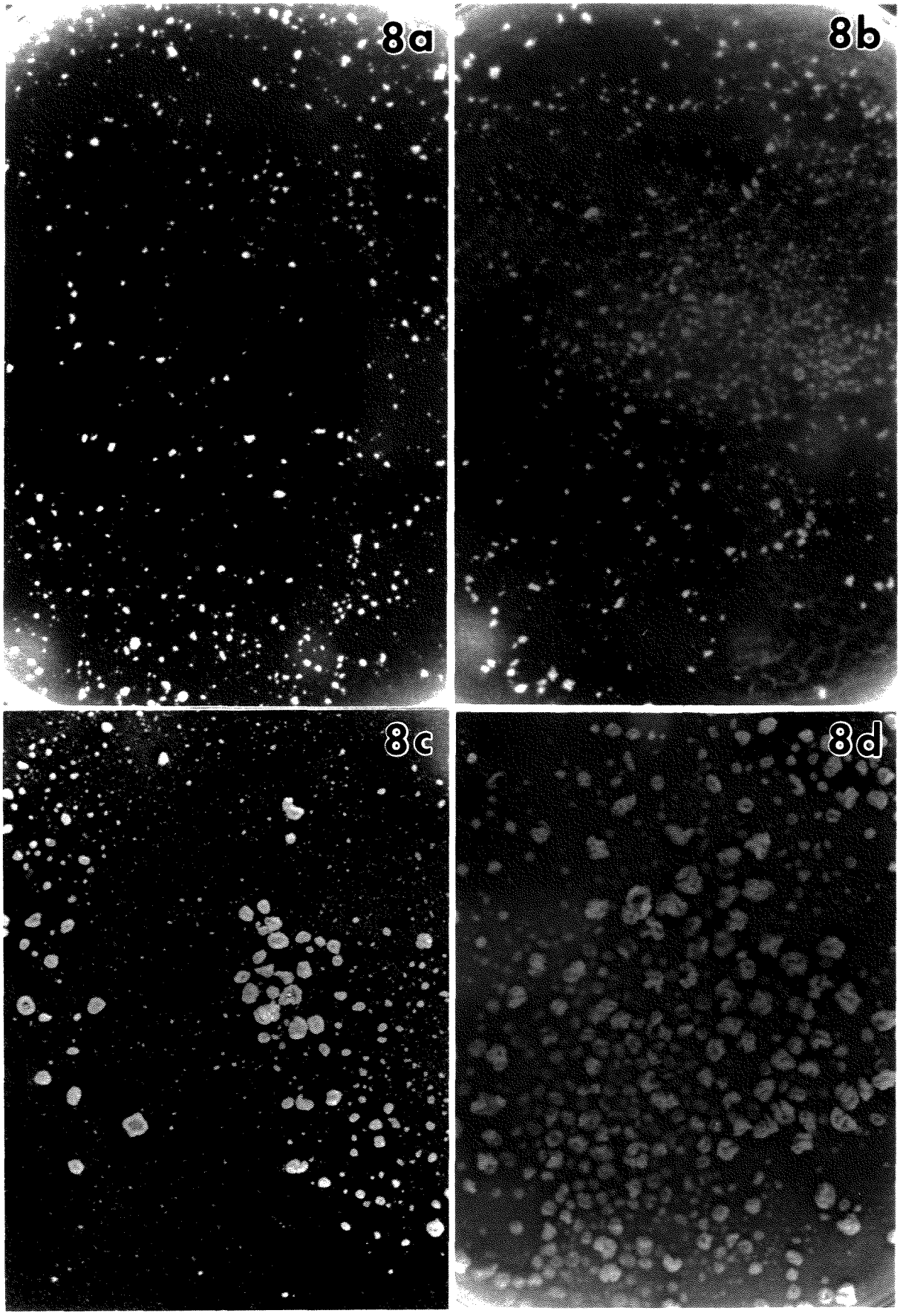
Optimum subculture period for cell suspensions

The growth rate, as indicated by PCV, of cell suspension cultures of the 4 aspen genotypes over 18 days of culture in liquid MS + 5 µM BA is shown in Fig. 10. The growth rates of all the genotypes were almost similar up to 10 days of culture, beyond which the growth rates differed significantly. The genotype BL-2 showed the maximum growth rate over the entire culture period (Fig. 8 c, d), followed by W-3, CAR-5 (Fig. 8 a, b) and BL-6. The optimum period for subculturing ranged from 12 – 16 to 16 days, depending on the genotype. In the case of BL-2, the maximum growth was found to be by day 16, while it was day 14 for CAR-5 and BL-6 and day 12 for W-3. The maximum cell multiplication occurred between day 6 and day 12 in all the genotypes.

Somatic embryogenesis from cell suspensions

The cell clusters resembling globular embryos did not develop further in any of the treatments used (Fig. 7 d). The cell clusters turned corky after 4 weeks of plating on agar-solidified MS supplemented with 0 to 5 µM Thiadiazuron. However, on low levels of 2,4-D (0.5 and 1 µM) supplemented medium, the cell clusters proliferated but did not form any organized structures. Higher levels of 2,4-D (5 µM) in the medium showed similar response to TDZ treatments.


7a
7b
100
75
50
7c
7d


8a
8b
8c
8d

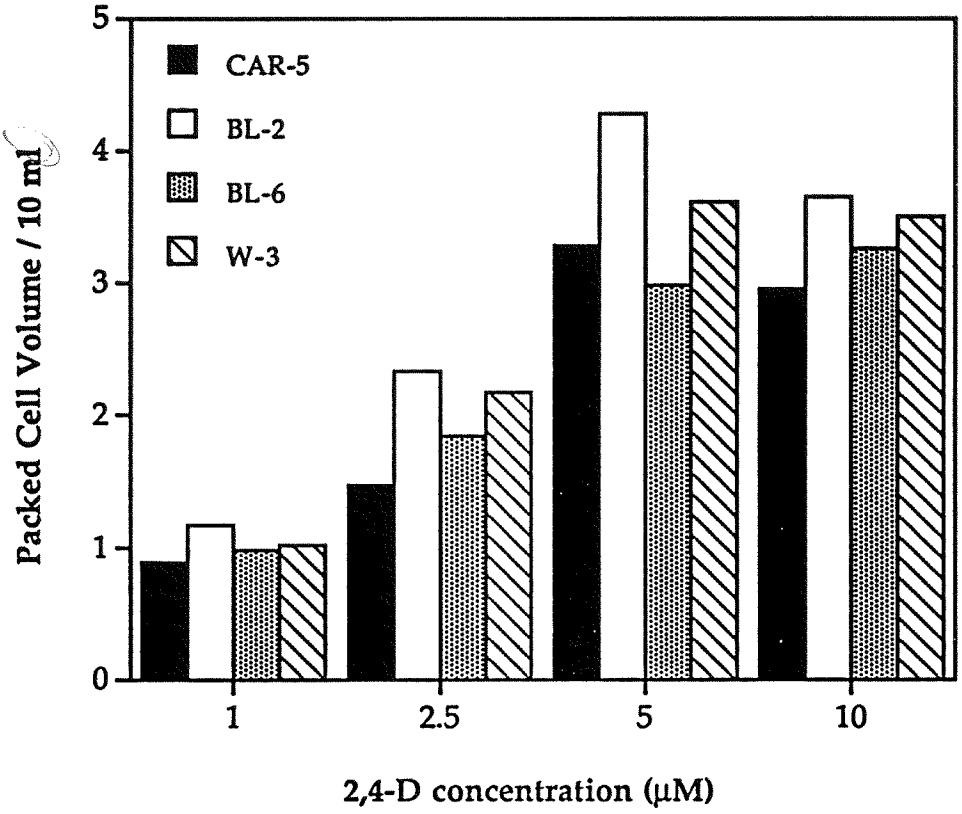


Figure 9. Effect of different concentrations of 2,4-D supplemented to MS medium on growth of cell suspensions of CAR-5, BL-2, BL-6 and W-3 genotypes of aspen. [10 ml of cell suspension subcultured into 20 ml of appropriate fresh medium and packed cell volume measured after 14 days of culture.]

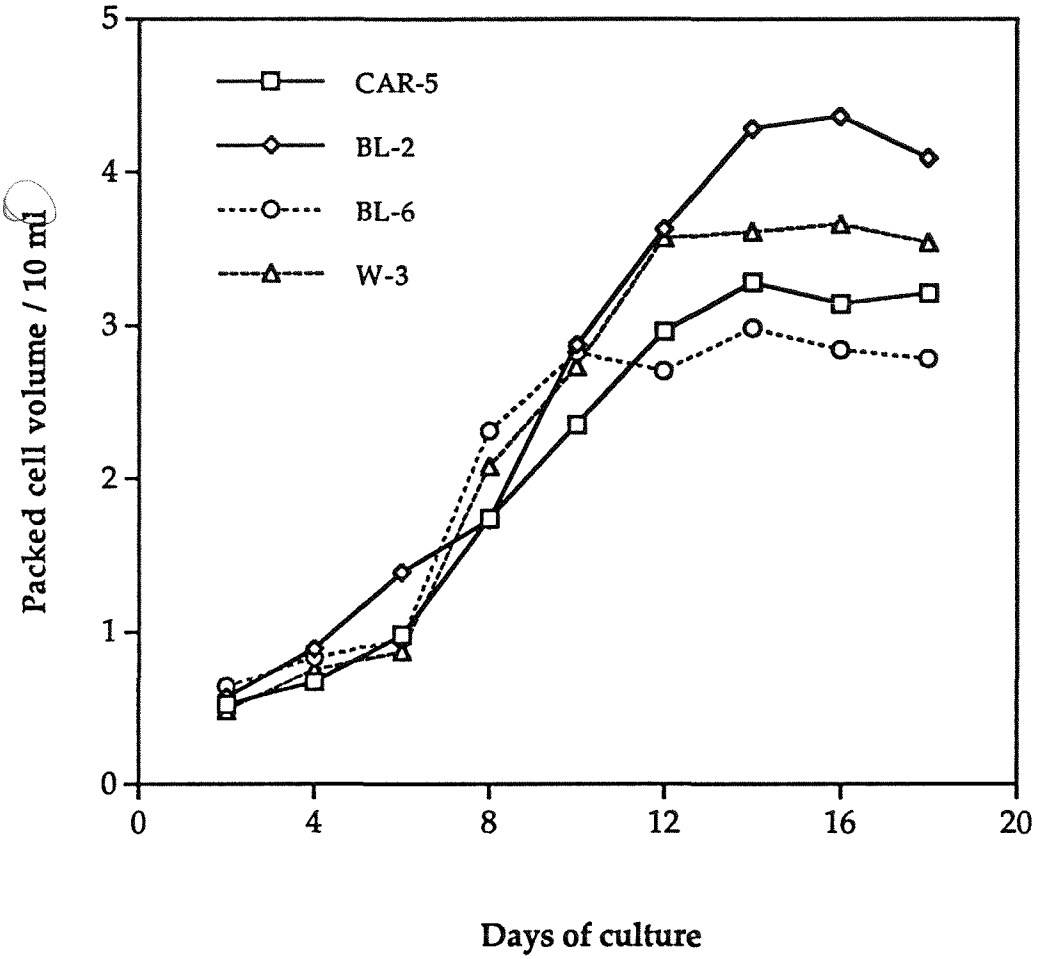


Figure 10. Growth rate curves of cell suspension cultures of CAR-5, BL-2, BL-6 and W-3 genotypes of aspen cultured in MS + 5 µM 2,4-D over an 18-day culture period. [10 ml of cell suspension subcultured into 20 ml of appropriate fresh medium and packed cell volume measured over 18 days of culture.]

on liquid MS-medium wi µM 2,4-D.

DISCUSSION

The potential use of micropropagation and increasing interest in *Populus* as a test organism in biotechnology has in recent years generated a wide interest in defining culture systems for diverse poplar germplasm (Sellmer et al., 1989). Various explant tissues such as axillary/apical bud, stem, leaf, bud meristem, root and anthers of many species belonging to the *Populus* genera have been cultured *in vitro* with varying degrees of success (Lubrano, 1992 and references therein). The growth responses in most studies often range from a callus to a whole plant, depending on the genotype used. Genotypic variations between trees have often compounded the problems and restricted efforts at attaining optimum culture systems. As suggested by Lubrano (1992), each species or in fact each clonal genotype might require a different set of basal media, thereby making it harder in standardizing an optimum media or culture condition. The present study was hence designed to develop *in vitro* culture methods for specific but diverse genotypes of aspen, belonging to 7 clonal populations.

Nodal bud explants were found to be the suitable material for initiating microshoots from all the clonal populations of aspen studied. Among the various media combinations, MS medium supplemented with 2 µM BA was found to be optimum in inducing bud breaking and shoot organogenesis. Whitehead and Giles (1977) have also reported that, the best media composition for axillary bud cultures was MS medium + 0.9 µM BA, and on this media bud breaking normally occurred within 4 weeks of culture. However, with the present set of genotypes and a higher level of BA, bud breaking was attained within 2-3 weeks. The added advantage to the present study was due to the bud preparation method employed, wherein, the outer scales of the buds were removed prior to culture. This in turn could be the reason for the reduced time requirement for bud breaking and shoot initiation. Woody plants, especially angiosperms, secrete brown/black pigments into the medium in response to wounding on excision. These pigments, mainly oxidized polyphenols and tannins, have been shown to have inhibitory effects on growth and development of the explants and can even cause necrosis (Thorpe and Harry, 1990). Addition of activated charcoal to the culture medium, to absorb these phenolic exudates, did not enhance the percent shoot initiation in the optimum media combination, but was able to alleviate to some extent the ill-effects, such as necrosis of shoot apex and crinkling of leaves, observed in the other medium combinations.

Shoot multiplication was successfully carried out by culturing microshoots on either MS or ACM medium, with resultant shoots differing in quality depending on media used. The optimum media combination for shoot multiplication and elongation was found to be MS + 1 µM BA, even though maximum shoots were obtained on ACM medium + 1 µM BA. On ACM medium, the microshoots did not elongate and had a bushy appearance, while those on MS medium elongated up to 2.5 cm in length. The shoot multiplication rates were found to be 10.47 and 12.50 on MS and ACM medium supplemented with same level of BA (1 µM). Christie (1978) has also observed similar rates of shoot multiplication (10 shoots per culture) in a number of

poplars and aspens (*P. alba*, *P. canescens*, *P. alba* x *P. grandulosa*, *P. alba* x *P. tremula*, *P. tremula*, and *P. tremuloides*). Similar multiplication rates, of approximately 10 shoots per explant, were also observed in five genotypically diverse clones of *Populus* (*P. alba* x *P. grandidentata* 'Crandon', *P. nigra* 'Betulifolia' x *P. trichocarpa*, *P. nigra* x *P. laurifolia* 'Strathglass', *P. maximowiczii* x *P. trichocarpa* 'Andorscoggin', and *P. deltoides* x *P. nigra* 'Eugenei') (Sellmer et al., 1989) when microshoots were cultured on either MS or WPM (Woody Plant Medium, Lloyd and McCown, 1981) supplemented with 0.4 to 1.0 μM BA.

Shoot elongation and rooting occurred in microshoots when they were cultured on full-strength MS medium without any growth regulators. However, the percent rooting was enhanced when half-strength MS medium was used. In both cases, activated charcoal supplemented to the media did not enhance the rooting response. Lubrano (1981, 1989) has also reported basal medium to be sufficient for shoot elongation in *P.* x *euramericana* and *P. deltoides* x *P. maximowiczii* 'Eridano' clones. Similar to the present findings, Ahuja (1983) has reported that basal ACM medium was sufficient to induce rooting from microshoots, with or without a callus interface, while in contrast Nkanka (1981) has shown that, ACM medium supplemented with lower concentrations of NAA was essential to induce maximum rooting response (greater than 95%). The transplantation protocol developed in the present study had a 100% success rate for establishing *in vitro*-developed plants in the greenhouse. The closed-transparent plastic box method, with sterile vermiculite, was also found to be better than maintaining the plants in seedling trays in a misting chamber. This was in fact due to the better hardening of plants, by simultaneous exposure to high relative humidities and high light intensities, under controlled conditions, by incubating the covered plastic boxes in growth chambers under a 16:8, light:dark regime and spraying the plants with water every 12 hrs over a 4 week period. The plants were also successfully transferred into pots and are currently being maintained in the greenhouse. The above results are consistent with those obtained with other angiosperm species following the systematic approach outlined by Thorpe and Patel (1984). The present study demonstrated that optimum culture conditions for genotypically diverse clones of aspen can be established. It is also likely that the present approach would be suitable for any other genotype of poplar or aspen. Similar results were obtained previously in this laboratory with *Eucalyptus globulus* (Oka et al., 1982) and mulberry (Sharma and Thorpe, 1990).

Callus-based systems from different explants, provides us with a wide array of starting material for tissue culture studies of woody plants species (Son and Hall, 1990). Ever since Mathes (1964) established callus cultures from cultured stem explants of *P. tremuloides*, there have been numerous successful reports of callus induction from various explants (leaves, anthers, ovaries, internodal stem segments, roots, etc.) of different species of poplars and aspens. In the present study, we were able to successfully initiate friable callus from leaves of *in vitro*-derived microshoots of aspen on MS medium supplemented with 10 μM 2,4-D and 5 μM BA. Earlier, Son and Hall, (1990) have also shown that a combination of 2,4-D (2.5

μM) and BA (0.5 μM) were essential for callus initiation from leaf discs of *P. alba* x *P. grandidentata*. The difference in phytohormone concentrations could be due to the differences in clonal genotypes studied. Although various other media and phytohormone combinations also induced callus (such as ACM + 5 or 10 μM 2,4-D + 5 μM BA), they were often nodular and yellowish. These nodular callus had a very poor multiplication rate and turned corky after subculture onto half-strength MS medium + 5 μM 2,4-D + 5 μM BA. The friable callus on the other hand, showed profuse proliferation on half-strength MS + 5 μM 2,4-D + 5 μM BA medium. Root organogenesis occurred spontaneously in all the treatments used in the present study, either directly from the leaf midrib-callus interface or from the initiated callus. In contrast to the present findings, Winton (1968) observed callus initiation from root-sprout segments of aspen cultured on WS medium (Wolter and Skoog, 1966) supplemented with 2.2 μM 2,4-D and rooting occurred only at lower concentrations of 2,4-D (0.2 μM).

Cell suspension cultures of aspen were initiated successfully from *in vitro*-grown leaf derived callus cultures. Liquid MS medium with 5 μM 2,4-D was found to be optimum for suspension initiation in 3 of the 4 genotypes studied, while MS + 10 μM 2,4-D was required for the other genotype. As mentioned earlier, genotypic differences between clonal populations often require different media combinations (Lubrano, 1992) for optimal response. Similar to the present findings, Ahuja (1986) has reported that 2,4-D (4.5 μM) supplemented to ACM medium was optimum for establishing cell suspension cultures of aspen. Park and Son (1988) have also shown that MS medium supplemented with 4.52 μM 2,4-D and 0.44 μM BAP were sufficient to establish cell suspensions of stem-derived callus of *P. alba*. The optimum subculture interval was also found to be genotype-dependant. The intervals varied from 12 days to 16 days depending on genotype based on maximum increase of packed cell volume measured over 18 days. The lowest growth rate in BL-6 over the culture period might be due to the differences in the optimum levels of 2,4-D required for this genotype compared to the other three. During prolonged subculture the cell suspensions formed cell clusters which resembled globular-stage somatic embryos. However, these embryo-like structures did not develop further on any of the media combinations tried. In contrast to the present study, Cheema (1989) was able to obtain somatic embryogenesis in cell suspension cultures of *P. ciliata*. Cheema (1989) showed that embryogenic suspensions formed somatic embryos on MS + 0.91 μM 2,4-D and these embyros developed further and formed plantlets on MS medium with low levels of BAP and NAA.

In the present investigation, we have demonstrated an efficient four-step method of micropropagation of aspen, similar to that of Ahuja, (1983), based on the 14 genotypes involved in the study. The four distinct steps are: i) bud breaking and microshoot initiation from nodal bud explants; ii) microshoot multiplication and elongation; iii) rooting of microshoots; and iv) hardening of *in vitro*-derived plants and establishing them in the greenhouse. We were also successful in initiating and proliferating *in vitro*-grown leaf-derived callus cultures, as well as cell suspension cultures from these callus cultures. The production of viable cell suspension

cultures of various genotypes allows this material to possibly be used as an experimental bioassay tool in aspen stain and decay studies, thus fulfilling one of the aims of this project.

RECOMMENDATIONS

The following recommendations are made for initiation and maintenance of efficient micropropagation of nodal bud cultures, *in vitro*-grown leaf derived callus cultures and leaf callus-derived cell suspension cultures. These conclusions are based on the wide range of media, phytohormone, media additives and culture conditions tested on some or all of the 14 genotypes of aspen (from 7 clonal populations), depending on the experiment.

Shoot initiation from nodal bud cultures:

Media : MS medium + 2 μM BA ± 0.05% activated charcoal.

Culture method : Excision of outer scales of buds and culturing on petri plates sealed with Parafilm® or Stretch n' Seal®.

Subculture : At 2 to 3 week intervals.

Microshoot multiplication/elongation:

Media : MS medium ± 1 μM BA (multiplication & elongation).
ACM medium + 1 μM BA (multiplication).

Culture method : Culturing in 4 oz glass jars or bigger and sealing with Parafilm® or Stretch n' Seal®.

Suculture : At 3 to 4 week intervals.

Rooting of microshoots:

Media : Half-strength MS medium ± 0.05% activated charcoal.

Transplanting of *in vitro*-derived plants:

Method : Transfer plants to transparent plastic boxes with sterile

vermiculite and maintain high humidity by spraying water every 12 hrs.

Maintain plastic boxes (plants) under high light in a growth chamber for 4 weeks.

Callus induction from leaves of *in vitro*-derived microshoots:

Media : MS medium + 10 μM 2,4-D + 5 μM BA (friable callus).
ACM medium + 5/10 μM 2,4-D + 5 μM BA (nodular callus).

Culture method : Culture abaxial surface of leaf facing medium.

Subculture : At 4 week intervals on half-strength MS medium with lower concentrations of 2,4-D (5 μM).

Cell suspension cultures from *in vitro*-grown leaves:

Media : MS medium* + 5 μM 2,4-D (for CAR-5, BL-2, W-3).
MS medium* + 10 μM 2,4-D (for BL-6).

Subculture : At 12 to 16 days interval, onto same medium.

* liquid medium (without agar).

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APPENDIX

Comparative media constituents of MS (Murashige and Skoog, 1962) and ACM (Ahuja, 1983) media employed throughout the project. (from Ahuja, 1983).

	Concentrations in mg l^{-1}	
	MS media	ACM media
Macronutrients		
NH_4NO_3	1650	400
KNO_3	1900	--
$CaNO_3.4H_2O$	--	556
K_2SO_4	--	990
$CaCl_2.2H_2O$	440	96
$MgSO_4.7H_2O$	370	370
KH_2PO_4	170	170
Micronutrients		
$MnSO_4.H_2O$	22.3	22.3
$ZnSO_4.7H_2O$	8.6	8.6
H_3BO_3	6.2	6.2
KI	0.83	0.83
$Na_2MOO_4.2H_2O$	0.25	0.25
$CuSO_4.5H_2O$	0.025	0.025
$CoCl_2.6H_2O$	0.025	0.025
Iron		
Na_2. EDTA	37.25	--
$FeSO_4.7H_2O$	27.85	--
Sodium Ferric EDTA	--	30.00
Vitamins		
Thiamine. HCl	0.1	0.1
Nicotinic acid	0.5	0.5
Pyridoxine HCl	0.5	0.5
Glycine	2.0	--
Lysine	--	100
Sugars		
Myo-inositol	100	100
Sucrose	30,000	20,000
pH	5.7-5.8	5.5-5.6