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Multiple Shoot Induction from Ex Vitro and In Vitro Derived Stem Node Culture of *Populus alba* L. × *P. grandidentata* Michx.

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줄기 절간조직 배양에 의한 교잡종 사시나무의 대량증식

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Physiologically modified stem nodes derived from ex vitro and in vitro explants of hybrid aspen (*Populus alba* L. X *P. grandidentata* Michx. 'Crandon') were tested for their multiple shoot regeneration capacity using a broad spectrum dosage of cytokinins. Ex vitro derived stem nodes with excised axillary buds at the time of culture produced 11 to 13 multiple shoots on 20 to 30 μM zeatin containing Woody Plant Medium (WPM) after 6 weeks. Excision of axillary bud sprouts after 2 weeks of culture and re-culture of the remaining stem nodes on WPM with 1.0 to 2.0 μM BA or 10 to 30 μM zeatin produced 13 to 15 and 7 to 8 shoots per explant, respectively. Multiple tiny shoots were produced when in vitro derived stem nodes (on whichall leaves were removed) were cultured on WPM with 30 to 50 μM 2iP or 20 to 50 μM zeatin. The greatest number of multiple tiny shoot proliferation (32 to 50 shoots per explant) were obtained when the explants were cultured on media containing 20 μM zeatin. Successful transplanting of these multiple shoots into the greenhouse and/or nursery was achieved.

Key words: nodal explants, physiological modification, poplar, tissue culture

In vitro culture systems are now well-established as a useful tool for solving practical problems of current breeding programs (Chalupa, 1977: Wann et al., 1988) and mass cloning of superior genotypes of diverse woody plants (Ahuja, 1987: Lloyd and McCown, 1981: Son and Hall, 1990a: Wann and Einspahr, 1986). Commercially adoptable clonal propagation using tissue culture method was extensively studied on *Populus* species (Ahuja, 1984: Barocka et al., 1985: Christie, 1978: Park and Son, 1988: Son and Hall, 1990b: Whitehead and Giles, 1977).

To obtain true-to-type micropropagules, the axillary branching method was commonly used (Chun et al., 1986: Coleman and Ernst, 1990). Although this method seems to be reliable for cloning plus trees in vitro, the number of proliferations per given explant is highly limited compare with that of the adventitious shoot regeneration methods (Ahuja, 1987: Son and Hall, 1989a: Son and Hall, 1989b). To overcome these obstacles for large scale propagation, adventitious shoot regeneration systems from cell suspension, callus, and leaf mid-vein cultures have been alternatively suggested (Douglas, 1984: Lee-Stadelmann et al., 1989: Park and Son, 1988a: Park and Son, 1988b).

Due to the distinct advantages of poplar stem node explants such as: (1) easy collection, (2) simple process for in vitro establishment, (3) nature of shoot break ability, (4) high degree of reproducibility, and (5) easy of rooting in vitro and ex vitro, etc., these explants were extensively employed for clonal propagation of *Populus* species (Ahuja, 1983:

Chalupa, 1983: Douglas, 1984). Even though ex vitro and in vitro derived stem node explants were frequently used to establish aseptic shoot cultures as starting source material and/or direct shoot proliferation studies, there are still relatively few reports on the commercial scale multiple shoot proliferation from stem node explants.

Previously published reports of poplar tissue culture systems focused on the type of the culture media (Ahuja, 1983: Kohlenbach and Wernicke, 1978) and micro-environment (Monette, 1983). However, the importance of physiological modification of explants for improving multiple shoot regeneration capacity has been rarely investigated. The objective of this study was to develop a stem node culture system which could be used to increase the total number of shoots by modifying the physiological conditions of explants using a broad spectrum dosage of plant growth regulator (PGR).

MATERIALS AND METHODS

Plant Materials

Stem nodes were collected from upper parts of actively growing branches of 1-year-old greenhouse grown stock plants (*Populus alba* L. × *P. grandidentata* Michx. 'Crandon' clone). After removal of all leaves, the stem nodes were surface sterilized in 5% NaOCl solution with two drops of wetting agent (Tween-20) for 5 min and rinsed 5 times using sterile distilled water (Park and Son, 1986). The stem nodes having a small piece of petiole were cut into pieces (ca. 3-4 cm in length) and used as ex vitro source material.

In vitro shoot cultures were obtained by subculturing bud sprouts on PGR free WPM. To obtain multiple shoots, apices were removed from cultured shoots, then 10 bi- or tri- nodal shoots were cultured in Magenta GA-7 vessels (7.6 × 7.6 × 10.2 cm: Magenta Co., Chicago, IL) containing 50 ml of medium with 0.88 μM BA. After 5 subcultures with a 4-week-interval on the same medium, each shoot culture excised from multiplied axillary branches was maintained on shoot elongation media (PGR free WPM) for more than 6 weeks. Plants of 8 to 10 cm in height with fully expand leaves were used as in vitro source materials. To test the ability for shoot proliferation, the preparation of ex vitro derived explants was as follows: (1) stem node containing one intact axillary bud, (2) excision of the axillary bud from the stem node at the time of inoculation, and (3) removal of axillary bud sprout which was obtained after 2 weeks of stem node culture.

Culture Media

In vitro explants were obtained by cutting 3 to 4 cm of aseptic shoot cultures which were maintained at least 6 weeks on shoot elongation media. For in vitro explants, stem nodes with and without leaves were cultured. To determine optimal type and concentrations of PGRs for multiple shoot induction from differently prepared explants, media were formulated by incorporating four types of cytokinin (BA, 2iP, zeatin, and kinetin) at different levels. The concentrations of each cytokinin were as follow: 1, 2, 3, 4, 5, 10, 20, 30, 40, and 50 μM. All media used in this experiment were WPM (Lloyd and McCown, 1981). The pH of medium was adjusted to 5.8 before the addition of 0.75% (w/v) Difco Bacto agar and autoclaved at 1.05 kg cm^{-2} and 121°C for 15 min. The PGRs incorporated into the tested media were filter sterilized using a 0.2 μm pore size membrane filter and added into the medium at 45°C.

Culture Condition

Each stem node explant was cultured in a plastic Petri dish (10 × 1.5 cm: Fisher Sci. Co. Canada) containing 20 ml of tested media. The vessels were double sealed with Nesco film (Bando Chemical Ind. Ltd. Kobe, Japan) and incubated in a growth chamber at 25 ± 2°C with 60 + 10% relative humidity, a 16 h photoperiod, and a photosynthetically active photon flux rate of 40-60 μmE m^{-2} s^{-1} from cool-white fluorescent tubes.

Data Collection

Stem nodes from ex vitro and in vitro were inoculated on culture vessels with 4 and 8 explants, respectively. The vessels were randomly placed on the culture shelves to minimize the effect possibly caused by different micro-conditions. Each experiment consisted of five replications. Number of multiple shoots (higher than 0.5 cm) was counted after 6 weeks of culture. Shoots smaller than 0.5 cm in size were described as multiple tiny shoots and counted with a dissecting-microscope.

RESULTS

Multiple Shoot Induction from Ex Vitro Derived Stem Node Cultures

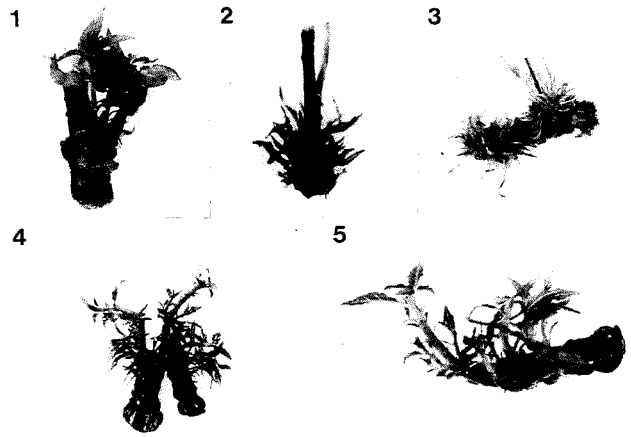

Figure 1. Multiple shoot induction from ex vitro derived stem node culture of hybrid aspen (*Populus alba* L. × *P. grandidentata* Michx.) 'Crandon'. (1-1) stem node whichexcised axillary bud., (1-2) and (1-3) stem node excised axillary bud at the time of culture, stem node which excised axillary bud sprout after 2 weeks of culture on media containing zeatin (1-4) and BA (1-5).

The responses of stem node explants on different types and levels of PGRs are summarized in Table 1. When ex vitro derived stem nodes having one axillary bud were cultured on the tested media, most of the explants produced 1 to 2 shoots with some exceptions. Although 5 to 6 shoots were rarely obtained on low level of BA (1.0 μM) and high levels of zeatin (10-30 μM), percentages of multiple shoot producing explants were below 10% (data not shown).

Ex vitro originated stem nodes excised axillary buds at the time of culture produced 11 to 13 multiple shoots on the zeatin contained media at the levels of 20 to 30 μM. However, this type of explant also were considered as a poor shoot-forming source material.

Excision of axillary bud sprouts after 2 weeks of culture led to increased numbers of multiple shoots. In the present of BA at 1.0 to 2.0 μM, rapidly growing multiple shoots (13 to 15 shoots per explant) were initiated (Figure 1-5). Similar results were observed on media supplemented with 10-30 μM zeatin, but the size of the shoots was smaller than that of BA derived plants under the same culture periods. The rate of explants forming shoots using this source material was more than 80%.

Multiple Shoot Induction from In Vitro Derived Stem Node Cultures

Using stem nodes with leaves (same terminology described as single shoot culture removed from their shoot apex: Son and Hall, 1990a: 1990b), highly reproducible results were obtained (Table 1). The number of shoot per stem node using BA (1.0-2.0 μM) and zeatin (5-10 μM) was 14 to 19

Table 1. Multiple shoot regeneration from ex vitro and in vitro derived stem node culture of hybrid poplar (*Populus alba* L. × *P. grandidentata* Michx.) 'Crandon'.

PGRs(μM)[a]		Mean number of shoots[b] ± SD			
		Ex vitro- Ⅰ[c]	Ex Vitro- Ⅱ[d]	In vitro- Ⅰ[e]	In vitro- Ⅱ[f]
BA	1.0	1.3 ± 1.2	15.2 ± 3.9	18.7 ± 3.4	2.4 ± 0.8
	2.0	1.7 ± 0.5	13.3 ± 3.2	14.3 ± 2.5	5.3 ± 1.6
	3.0	0.7 ± 0.5	6.3 ± 0.7	8.0 ± 1.6	9.7 ± 1.3
	4.0	1.0 ± 0.0	3.3 ± 3.3	9.3 ± 1.4	14.5 ± 3.2
	5.0	1.6 ± 1.2	3.0 ± 0.6	7.6 ± 3.6	6.3 ± 1.2
	10.0	0.7 ± 0.5	1.3 ± 0.5	2.4 ± 1.4	23.3 ± 13.2[g]
	20.0	0.3 ± 0.2	2.3 ± 1.2	1.3 ± 0.6	22.6 ± 6.2[g]
	30.0	0.4 ± 0.4	2.4 ± 1.1	1.3 ± 0.3	12.3 ± 4.1[g]
	40.0	0.0 ± 0.0	1.2 ± 0.6	1.0 ± 0.5	11.3 ± 11.9[g]
	50.0	0.0 ± 0.0	1.0 ± 0.2	1.6 ± 0.8	13.2 ± 7.4[g]
2iP	1.0	0.0 ± 0.0	1.7 ± 0.5	1.3 ± 0.4	3.3 ± 0.7
	2.0	0.3 ± 0.5	2.3 ± 1.2	1.2 ± 0.2	4.3 ± 1.8
	3.0	0.0 ± 0.0	1.4 ± 0.5	1.8 ± 0.2	3.3 ± 1.2
	4.0	0.7 ± 0.9	1.9 ± 1.1	2.1 ± 0.1	3.3 ± 0.7
	5.0	0.6 ± 0.4	1.3 ± 0.5	4.6 ± 1.1	7.2 ± 1.8
	10.0	5.7 ± 2.8	2.3 ± 1.2	9.3 ± 2.5	26.3 ± 12.6[g]
	20.0	8.0 ± 2.1	3.0 ± 1.6	5.7 ± 1.3	33.3 ± 15.3[g]
	30.0	8.0 ± 3.5	4.3 ± 2.5	11.5 ± 5.2[g]	22.4 ± 8.8[g]
	40.0	2.3 ± 2.1	1.0 ± 0.0	23.3 ± 12.2[g]	23.5 ± 12.3[g]
	50.0	1.0 ± 0.9	1.0 ± 0.1	12.5 ± 8.6[g]	12.1 ± 3.3[g]
Kn	1.0	0.0 ± 0.0	1.0 ± 0.2	1.5 ± 0.5	3.0 ± 1.8
	2.0	0.0 ± 0.0	1.3 ± 0.8	3.4 ± 2.6	2.3 ± 0.4
	3.0	0.0 ± 0.0	1.3 ± 0.5	4.3 ± 1.3	2.7 ± 2.1
	4.0	0.0 ± 0.0	1.0 ± 0.8	5.3 ± 1.3	3.0 ± 1.9
	5.0	1.0 ± 0.4	1.4 ± 0.3	6.8 ± 2.4	2.8 ± 2.6
	10.0	2.1 ± 1.8	1.2 ± 0.8	7.0 ± 3.2	2.3 ± 0.5
	20.0	2.0 ± 1.6	2.7 ± 1.1	3.2 ± 0.6	1.7 ± 0.2
	30.0	1.3 ± 1.2	2.4 ± 1.5	4.7 ± 1.2	1.0 ± 0.8
	40.0	3.1 ± 2.6	3.2 ± 2.5	3.2 ± 2.6	2.0 ± 1.0
	50.0	0.7 ± 0.5	0.5 ± 0.8	1.8 ± 0.2	1.0 ± 0.2
Zn	1.0	3.0 ± 1.8	1.0 ± 0.0	4.3 ± 1.0	3.7 ± 0.5
	2.0	2.7 ± 0.5	2.7 ± 1.2	6.3 ± 1.2	4.6 ± 0.8
	3.0	2.7 ± 2.1	3.2 ± 1.1	4.3 ± 0.5	5.9 ± 1.0
	4.0	3.2 ± 0.8	3.4 ± 1.0	7.2 ± 2.3	8.9 ± 1.2
	5.0	7.2 ± 2.5	4.5 ± 2.4	18.6 ± 3.8	6.1 ± 2.1[g]
	10.0	6.3 ± 1.6	7.3 ± 2.2	10.5 ± 4.1	23.2 ± 8.6[g]
	20.0	13.2 ± 5.6	8.7 ± 1.3	22.5 ± 5.4[g]	51.7 ± 11.3[g]
	30.0	11.0 ± 4.5	8.3 ± 3.1	24.9 ± 17.6[g]	47.3 ± 13.5[g]
	40.0	25.2 ± 12.6[g]	21.0 ± 7.2[g]	21.8 ± 10.3[g]	43.5 ± 15.4[g]
	50.0	21.8 ± 13.7[g]	13.4 ± 4.6[g]	12.9 ± 6.6[g]	32.1 ± 11.6[g]

[a] Abbreviations: PGR: Plant Growth Regulator: BA: 6-benzylamino purine: 2iP: N^6-isopentenyladenine: Kn: kinetin: Zn: zeatin.
[b] Each numerical value (mean ± standard deviation) was determined after 4 weeks of culture from 5 replications each in 2 experiment.
[c] Ex vitro derived stem nodes with axillary bud excised at the time of culture.
[d] Ex vitro derived stem nodes with axillary bud sprout excised after 2 weeks of culture.
[e] In vitro stem nodes containing leaves.
[f] In vitro stem nodes with leaves excised at the time of culture.
[g] Represent number of multiple tiny shoots (shoot size shorter than 5 mm).

Figure 2. Multiple shoot induction from in vitro derived stem node culture of hybrid aspen (*Populus alba* L. × *P. grandidentata* Michx.) 'Crandon'. Shoot proliferation from stem node with leaves cultures on BA (2-1 to 2-2), kinetin (2-3), and zeatin (2-4) supplemented media. Stem node without leaves cultured on media containing kinetin (2-5), 2iP (2-6), and zeatin (2-7).

and 10 to 19, respectively. The rate of multiple shoot forming explants were almost 100%. Multiple tiny shoots were observed using the explants on the media containing high concentrations of 2iP (30-50 μM) or zeatin (20-50 μM). Most of these multiple tiny shoots were induced on basal portions of the stem nodes with the mean shoot number of 11 to 25.

Cultures of stem nodes with all leaves removed at the time of inoculation gave slightly lower numbers of rapidly growing shoots compared with that of in vitro stem nodes having leaves. However, these explants showed highly increased number of multiple tiny shoots when cultured on zeatin-containing (20-50 μM) media. Numbers of multiplied shoots was 32 to 52 per explant.

DISCUSSION

In vitro bud and stem node cultures of various species of *Populus* have previously been reported (Ahuja, 1984: Kim et al., 1981). However, each explant normally gave 2 to 10 shoots when supplied with basal medium and relatively low levels of BA (0.88 μM) or zeatin (below 5 μM). To increase the number of shoots per given cell and tissue, various studies were undertaken on poplar micropropagation. Examples include the use of liquid media, selection of suitable vessel size, and shoot density of explants (Chun et al., 1986). This study suggests that there may be a strong relationships between modified physiological conditions of explants and their multiple shoot production habits and abilities. The selection of suitable explants will possibly be linked to commercial scale propagation programs of this genotype of hybrid aspen.

One of the significant results from our observation with axillary bud sprouts excised ex vitro suggests a high possibility of application of this two-step method (Ahuja, 1987: Son and Hall, 1990a). The importance of reducing tissue cultural steps for commercial application has been well understood (Ahuja, 1987).

Coleman and Ernst (1990) reported shoot proliferation rates of 6 per stem node culture of *Populus deltoides*, using 4.56 μM zeatin. Even though the species tested differ, the data from this study where 5 μM zeatin was incorporated into the medium revealed very similar results to that of their observations. By increasing the level of zeatin from 2 to 10 times as much as Coleman and Ernst's dosage, it was possible to obtain multiple tiny shoots (30-50 shoots per explant). Without excised individual shoots, transferred multiple tiny shoots containing stem node explants or pieces of the explants (containing at least 10 tiny shoots) on PGR free media usually elongated 3 to 5 shoots. Because of the problem related to multiple tiny shoots was overcome by a rescue treatment (Son and Hall, 1995), this system may be considered to be employed for commercial scale micropropagation if genetic stability of the propagules are guaranteed.

적 요

잡종 사기나무의 기내 대량증식을 목적으로 줄기절간 유래의 기내 및 기외시료를 이용하여 다경줄기 유도에 관여하는 식물생장 조절물질의 효과에 대하여 조사 하였다. 기외시료의 측아를 제거한 다음 20에서 30 μM의 zeatin이 함유된 WPM 배지에 6주간 배양하였을때 각각 11개와 13개의 다경줄기가 유도되었다. 그러나 기외시료의 측아가 붙어있는 상태에서 기내배양하여 2주후에 측아를 제거하고 동일 배지에 배양하였을 경우에는 BA가 1.0에서 2.0 μM 함유된 배지에서도 13개와 15개의 다경줄기를 유도할 수 있었으며, 특히 이때 생산된 줄기의 상태는 아주 건전한 것으로 나타났다. 기내줄기를 사용하였을때는 20 μM의 zeatin을 처리하였을때 가장 많은 줄기를 생산할 수 있었으며, 생산된 조직배양묘는 일정기간 순화를 시킨 후 온실이나 포지에 식재가 가능하였다.

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