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Factors Influencing Adventitious Shoot Induction from Leaves of *Populus deltoides* Bartr

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Abstract

We have investigated several factors infulencing adventitious shoot induction from the *Populus deltodies* bartr. leaf segments. To obtain *in vitro* materials, at first, stem segments from *ex vitro* were tested for axillary bud breaking on the five macronutrients levels of two differenr media. WPM was better both in bud breaking and leaf expansion than MS medium. The amount of NH_4NO_3 added in the medium did not seem to affect axillary bud breaking, significantly and subsequent shoot elongation from the stem segments of *P. deltodies*. However, other components in WPM might have played important roles in axillary bud breaking and shoot elongation. Regenerability from three sections (the distal, the middle and the base) of leaves cultured on WPM supplemented with TDZ and NAA combinations appeared to be different; middle and basal sections of leaves produced more organogenic sites than those of top section on WPM and those sesctions produced the highest ogranogenic sites on the same medium supplemented with 0.01 mg/l TDZ and 0.02mg.l NAA. Among 3 carbon sources tested for adventitious shoot elongation, fructose seemed to be stimulating the elongation of adventitious shoots. Sucrose and glucose added in the medium resulted in the necrosis which caused dying of adventitious shoots, eventually.

Key words : *Populus deltoides, fructose, adventitious shoots, TDZ*

Introduction

Species and hybrids of the genus *Populus* are among the fastest growing trees and their ease in propagating via conventional asexual methods[1)] and cell and tissue culture methods has made them a model system for genetic improvement in tree species. Many biotechnological researches have been applied to *Populus* species and a considerable number of reports have been published, including regeneration of transgenic plants containing useful genes[2,3)]. The main reason for the focus on *Populus* species is the need for plant regeneration systems in successful biotechnological applications.

Eastern cottonwood, (*Populus deltoides*) a member of the *Aigeiros* section of the genus, is regarded as an excellent candidate for satisfying an increasing demand for wood and fiber products[4,5,6)]. As a member of the pure non-aspen poplars of the genus *Populus, P. deltoides* roots easily as stem cuttings. Therefore, *in vitro* micropropagation will not be economically competitive for the commercial scale up of desirable genotypes. However, *in vitro* manipulation can be an effective tool for better understanding of the differentiation of adventitiously derived organs and for gene transfer systems.

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This study was initiated to develop basic *in vitro* regeneration protocols using several factors, such as different types of plant materials, carbon sources and combinations of growth regulators (TDZ and NAA) to facilitate breeding programs of the species.

Materials and Methods

Dormant stem cuttings of *P. deltoides* were collected from clones maintained in stool beds. They were taken to a greenhouse, planted in pots containing peat : perlite (3 : 1) and were rooted under 16 hr photoperiod with a temperature of about 23℃. Stem segments were cut from succulent shoots collected from randomly chosen ramets of these greenhouse grown plants. The upper 15 −20 cm of actively growing shoots were removed, placed directly into a plastic bag and immediately transported to the laboratory. Shoots were then cut into 1.5 cm segments encompassing a subtending internode with an axillary bud. Stem segments containing axillary buds were surface disinfected and washed for 24 hrs in a solution containing 500 mg/l gentamicin and 300 mg/l streptomycin. For this step, as well as for all subsequent washes and rinses, stem segments were agitated using a gyro-rotary shaker (New Brunswick, G25) with 100 rpm, at room temperature. Then, they were washed for 20 min in a fresh solution containing 1 liter of 2 mg/l dry detergent (Alconox) and washed again with 1 liter of 10% bleach (v/v Clorox) for 20 min. Finally, the segments were rinsed three times with 1 liter sterile water for 10 min each. Following these treatments, the stem segments were transferred to glass tubes (15× 200 mm) containing different basal media.

Basal media were tested by assessing the growth of axillary shoots from stem segments. The basal media we tested included half-strength (0.5x) and full- strength (1x) MS (Murashige and Skoog, 1962), 1x and 2x WPM (Lloyd and McCown, 1982), and WPM modified by increasing the level of NH_4NO_3 to that of 1x MS, denoted MWPM. All of those media contained 1 mg/l thiamine, 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine, 2 mg/l glycine, and 100 mg/l myo-inositol. We used explants from leaves harvested from axillary shoots. Leaf segments were dissected and classified according to their origin on the leaf (the distal, the middle, and the basal), and then transferred to agar-solidified WPM containing 20 mg/l of sucrose and 4 levels of TDZ (Thidiazuron) (0.01, 0.02, 0.05, and 0.10 mg/l) in combination with 4 levels of NAA (0.0, 0.01, 0.1, and 0.2 mg/l). Each of the 16 combinations was represented by 2 petri dishes each containing 6 segments (2 leaves, each representing the distal, the middle, and the basal segment).

To determine the best condidtion for the elongation of induced adventitious shoots, various components were tested. For the shoots induced from newly expanded leaves (i.e. elongated *in vitro*), various growth regulators and carbon sources, including : 0.01 to 0.05 mg/l of BA with or without 1 mg/l kinetin or 1 mg/l GA_3 ; no growth regulators ; and either 20 mg/l glucose or fructose substituted for 20 mg/l sucrose as a carbon source supplemented in WPM were tested. To elongate the shoots induced from greenhouse-grown leaves, we used WPM containing 10 g/l fructose supplemented with 0. 05 mg/l BA and 1 mg/l kinetin. Roots were induced from the elongated shoots using WPM containing 10 mg/l fructose supplemented with 1 mg/l IBA. The pH was adjusted to 5.6 after adding all components except agar. Cultures were grown under the 16 hr photoperiod using cool white fluorescent tubes (60 μmoles. m^{-2}. S^{-1}) at 25℃.

Results

The tendency of breaking dormancy in axillary buds on stem segments was similar in all of the media tested (Table 1). However, more shoots subsequently elongated when they were grown on WPM and its derivatives

Table 1. Effect of basal medium on growth of greenwood cuttings from *Populus deltoides*. Cuttings were stem segments with an axillary bud form of Populus deltoides. Observations were made after 5 weeks

Basal medium[a]	No. of stem segment	No. of Contaminated[b]	Bud Break(%)	Elongated Shoots(%)	Leaves/ Shoots(%1cm)
1×WPm	18	2	100	70	2.4
MWPM	16	1	100	60	2.0
2×WPM	16	1	100	60	2.0
1×MS	15	0	100	10	0.9
1/2MS	14	0	93	40	1.1

[a]1×WPM - basal woody plant medium

MWPM - WPM modified by addition of NH_4NO_3 to the level in 1×MS

2×WPM - WPM with 2x macro-nutrients

1×MS - Murashige and Skoog medium

1/2x MS - Half-strength MS

[b]Values are based on uncontaminated stem segments with axillary buds

than on MS and its derivatives (Table 1). Leaf expansion was also better on the shoots grown on WPM than those on shoots grown on MS medium. Consequently, we used WPM as the basal medium for all subsequent experiments.

Initial efforts to induce *in vitro* organogenesis were made using leaves expanded from axillary buds. We reasoned that the regenerative capacity of newly-expanded leaves of *in vitro* cultures would be higher than would any other type of leaf tissues. After three weeks in culture, distinctive organogenic sites were developed (Fig. 1). Some of these sites were near wound (i.e. cut) surfaces, while others were on or near the portions of the leaf segments in contact with the medium. Small and compact adventitious shoots were apparent at all of the organogenic sites. Of the three types of leaf segments, those originating from the middle or the basal portions of the leaves had more organogenic sites than did those originating from distal portions (i.e. nearer the leaf tip). Of the 16 combinations of growth regulators tested, the combination of 0.01 mg/l TDZ and 0.2 mg/l NAA induced the highest number of organogenic sites per leaf segement (i.e. an average of 3.6 organogenic sites per leaf segment : Table 2). It is noteworthy that we did not observe any roots induced. To elongate the adventitious shoots induced, leaf segments having several organogenic sites were transferred to the fresh WPM containing 20 g/l sucrose and 0.01 mg/l BA (without auxin). However, only few adventitious shoots began to elongate. And shortly after elongation, most of the shoots became necrotic. In an attempt to rescue these adventitious shoots, we employed several modified WPM, including lowering the concentration of BA from 0.05 to 0.01 mg/l with or without 1 mg/l of kinetin and GA_3 ; no growth regulators ; and replacing the 20 g/l sucrose with 20 g/l fructose or 20 g/l glucose with 0.01 mg/l BA. Adventitious shoots elongated and remained healthy only when they were cultured on the medium supplemented with 20 g/l fructose and 0.01 mg/l BA (Fig. 2). Some adventitious shoots also elongated on other media, but they were not healthy, and became necrotic and died eventually (Fig. 3A). To test the effect of fructose as a carbon source, several unhealthy leaf segments with adventitious shoots were removed from the medium containing sucrose and placed onto the medium containing fructose. Within a week, shoots on

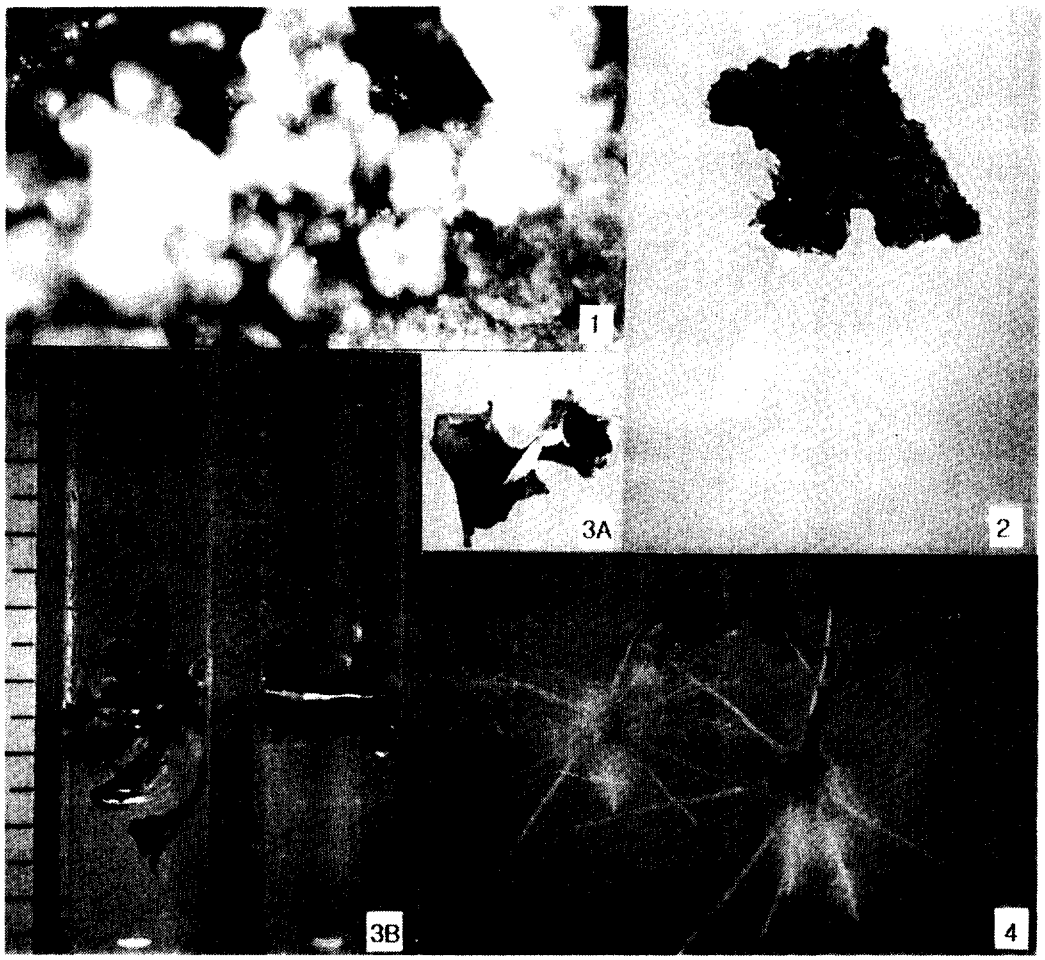

Fig. 1. 1. Development of the structures resembling somatic embryos on leaf explants from *P. deltoides*. These structures developed 2 weeks after culture under the light condition.

2. Adventitious shoots developing on a leaf segment of *P. deltoides* on WPM supplemented with TDZ (0.5 mg/l) and NAA (0.05 mg/l). The explant was originated from fully expanded, greenhouse-grown leaves. Note that shoots are developing from red, compact calli.

3. Elongation of an adventitious shoot on a leaf segment derived from an axillary shoot of a stem segment from *P. deltoides*. Leaf segment had begun to senescence on medium containing sucrose (A), but they revived after being transferred to the same medium containing fructose. Adventitious shoots began to elongate within a week (B).

4. Development of adventitious roots from elongated shoots on rooting medium (WPM containing 1 mg/l IBA and 10 g/l fructose).

Table 2. *In vitro* induction of organogenic sites from *Populus deltoides* leaf segments originating from partially expanding leaves grown *in vitro*. Data represent the total number of organogenic sites counted after three weeks in culture (n=12)[a]

Growth regulators and conc.(mg/l)		Origin of Leaf segment[b]			Site of Regeneration[c]		Total Number of	Avg. per Leaf
TDZ	NAA	D	M	B	wound	nonwound	Organogenic site[d]	segment
0.01	0.00	1	1	4	6	0	6	0.5
	0.01	4	5	5	14	0	14	1.2
	0.10	2	15	13	22	8	30	2.5
	0.20	15	18	10	31	12	43	3.6
0.02	0.00	0	2	1	3	0	3	0.3
	0.01	1	3	4	8	0	8	0.7
	0.10	3	7	11	19	2	21	1.8
	0.20	2	3	5	9	1	10	0.8
0.05	0.00	1	4	0	4	1	5	0.4
	0.01	0	0	5	5	0	5	0.4
	0.10	2	2	3	5	2	7	0.6
	0.20	1	4	4	9	0	9	0.8
0.10	0.00	0	2	3	5	0	5	0.4
	0.01	1	0	0	0	0	1	0.1
	0.10	0	1	4	5	0	1	0.4
	0.20	0	1	3	4	0	4	0.3

[a]Total number of leaf segments for each combination of growth regulators
[b]distal (D), middle (M), and base (B)
[c]Indicates whether or not the organogenic sites were located near a wounded or unwounded portion of the leaf segments.
[d]Total number of organogenic sites observed on 12 leaf segments. These were located on segments with distal, middle, or basal origins, or near wounded or unwounded sites as indicated.

these segments began to elongate (Fig. 3B). This indicates that fructose has a promoting effect on the elongation of *P. deltoides* shoots *in vitro*. Once they elongated to about 3 cm in height, 15 shoots were transferred to the rooting medium (WPM containing 10 g/l fructose and 1 mg/l IBA) where they all rooted well. Within 2 weeks of culture on the rooting medium, each shoot produced several healthy roots (Fig. 4).

Discussion

Ammonium nitrate, one of the most important ingredients of the macro-elements in the medium, is selectively uptaken as either NH_4^+ or NO_3^-, or both. However, the amount needed is considerably different, depending on plants or types of tissues of the same plants. Ammonium nitrate can be toxic to some plants that make establishing *in vitro* culture difficult. Flinn *et al*[7] reported that high concentration of ammonium nitarte in the medium markedly decreased the number of adventitious buds from the mature embryos of white pine (*Pinus strobus*). When compare the concentration of ammonium nitrate in two media, that in MS medium is almost as twice as that of WPM. In *P. deltoides* stem segments used in this experiment, WPM was much better in bud breaking and shoot elongation from axillary buds

MS (Table 1). However, MWPM in which NH_4NO_3 concentration is increased upto that of MS also showed high rates (Table 1). Therefore, other components in the medium might be more critical for breaking bud and elongating shoots from *P. deltoides* stem segments.

TDZ, which is one of the cytokinins, has been used to induce axillary and adventitious shoot proliferation from various woody perennial species[8,9]. It also has shown to be suitable for inducing adventitious shoots from *P. deltoides* leaf segments as demonstrated in this study. In general, adequate combination of cytokinin and auxin is necessary for inducing adventitious shoots, usually the level of cytokinin is higher than that of auxin. However, the results observed in this study were unusual because the combination of higher auxin concentration and lower cytokinin concentration showed better inducibility (Table 2). It may be due to a strong activity of TDZ that can induce adventitious shoots, even used at low level.

Most adventitious shoots on leaf explants were induced at or near wound sites. Structures resembling somatic embryoids were observed in the some organogenic sites (Fig. 1). However, we did not find shoots and roots connected together (i.e. connected by vascular tissues) after they were transferred and grown on the fresh media.

Although *in vitro* grown leaves produced many adventitious shoots on WPM containing sucrose and low concentrations of BA, their subsequent elongation was not successful. Elongation of these shoots was obtained only when they were grown on the medium containing fructose with a low concentration of BA (0.05 mg/l). Since adventitious shoots died on all media containing either sucrose or glucose, it seems that these compounds may have a inhibitory effect on the growth of adventitious shoots originated from the leaves of *P. deltoides*. After shoots were transferred to the fresh medium containing sucrose, they became dark and senescenced. Even though sucrose is most frequently used carbon source for *in vitro* cultures[10,11,12,13], it does not seem that it is suitable for elongation and further development as shown in this experiment.

Reports indicated that other carbon sources than sucrose are needed for successful *in vitro* culture. Ohyama and Oka[14] found that fructose was needed to proliferate shoots from winter buds and shoot tips of mulberry. Viss and Ruzin[15] reported that calli induced from several poplars developed shoots with a high frequency on the media containing maltose or glucose rather than sucrose. Although Prakash and Thielges[17] induced adventitious shoots from leaf segments of *P. deltoides* using glucose as a carbon source, they did not induce adventitious shoots directly from leaves, but from calli. Coleman and Ernst[11] observed consistent browning and necrosis and suggested that cytokinins, especially BA, might be toxic to *P. deltoides* or its hybrids (*P. deltoides*×*P. nigra*) grown *in vitro*. In an effort to minimize cytokinin toxicity, Coleman and Ernst[11] used zeatin in the media they used. In contrast to the previous reports, we found that TDZ in combination with NAA stimulated development of adventitious shoots, and these shoots could be readily elongated on the media containing fructose and a low concentration of BA. *In vitro* elongation of *P. deltoides* shoots may be inhibited by sucrose alone, or perhaps by sucrose in combination with certain concentration of BA. Different carbon sources may be needed for successful culture of explants from different organs or from different species, or even from explants from the different organs but from same plants[14]. Our direct regeneration system based on the use of greenhouse-grown mature leaves may be suitable for rejuvenating forest plantations with minimizing somaclonal variation and for production of transgenic plants using *Agrobacterium* or biolistic mediated transformation systems.

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초록 : 미류나무 잎절편체 조직배양을 통한 植物體 再分化에 미치는 요인

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미류나무 잎절편 조직배양에 의한 식물체 再分化에 關한 여러 가지 要因을 調査한 結果를 要約하면 다음과 같다.

1. 5가지 각기 다른 濃度를 가진 培地에 줄기절편을 치상하여 側牙의 發達을 유기시킨 결과, WPM이 MS 배지 보다 더 좋은 反應을 보였고 또한 배지내의 NH_4NO_3의 양은 측아 增殖에 큰 영향을 미치지 않는 것으로 觀察되었다.
2. 기내에서 增殖된 잎을 3부분으로(윗, 중간 그리고 밑) 切斷하여 2종류의 植物成長 調節物質(TDZ와 NAA)의 16가지의 濃度配合을 使用하여 培養한 結果 TDZ 0.01 mg/l 와 NAA 0.02 mg/l에서 가장 높은 부정줄기 形成體들이 生成되었다. 또한 잎의 윗 부분 보다는 중간과 밑부분에서 높은 부정줄기 形成體들이 生成되었는 것을 觀察하였다.
3. 3가지의 炭水貨物을 사용하여 부정줄기의 유기를 觀察한 바, sucrose 나 glucose보다 fructose를 添加한 배지에서 좋은 反應을 보여 주었다.