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Shoot Proliferation of *Populus euramericana (Populus deltoides X P. nigra)* through *in vitro* Tissue Culture

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ABSTRACT

The efficiency of *in vitro* regeneration of four clones of *Populus euramericana*, Canada blanc, Eugenii, I-45/51, and Wisconsin #5, was examined. Cytokinins and the combinations with auxins affected the rate of regeneration from the explants of root segments, stem internodes, and leaf discs. Overall, BA and the combination with auxins were effective in root segments and leaf discs of the Canada blanc clone, whereas zeatin and the combination with auxins were important in stem internodes of the Wisconsin #5 clone. The highest number of shoots averaging 17.6 ± 0.47 from root segments in the Canada blanc clone, 18.2 ± 3.0 from stem internodes in the Wisconsin #5 clone, and 17.8 ± 1.92 from leaf discs in the Canada blanc clone were obtained with 2.0 mg/l BA, 2.0 mg/l zeatin combined with 0.2 mg/l IAA, and 0.5 mg/l BA combined with 0.05 mg/l 2,4-D, respectively. In particular, the addition of 2,4-D into cytokinin medium promoted shoot proliferation.

Key Words : *Populus euramericana, in vitro* clonal proliferation, root, stem, and leaf culture.

INTRODUCTION

In vitro shoot proliferation involves the culture of explants to produce shoots through axillary or adventitious shoot formation. Explant types, as well as the stages of development, are very important to maximize the efficiency of shoot formation.

Poplars can be propagated by stem cuttings and aspens can be vegetatively propagated by root suckers, graftings (Herrmann and Seuthe 1982) and green shoots (Behrens and Melchior 1978). However, vegetative reproduction by these methods is limited by expense, graft incompatibilities, and limited availability of a specific genotype as a planting material (Ahuja 1987). Nevertheless, these problems have been overcome for rapid and reliable clonal reproduction of selected aspen (Frohlich 1982, Ahuja 1983, 1984) and aspen hybrid genotypes. Micropropagation systems of aspen and the hybrids have been established by the culture of axillary bud, stem internode, leaf disc and root segments (Ahuja 1983). Although many crop and horticultural species and a few woody species have been extensively studied through organ or callus culture from stem, root, or leaf discs (Ahuja 1983, Fasolo et al. 1989, Kaul et al. 1990, McNicol et al. 1990), there are relatively few reports for *Populus deltoides* or their hybrids which are resistant to insects and diseases (Chalupa 1974, Whitehead and Giles 1977, Coleman and Ernst 1990, Kang and Hall 1996). So far, a genetic transformation system has been established for the most aspen species (Parsons et al. 1986, Fillatti et al. 1987, and Chun 1988) and one

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cottonwood hybrid (Heuchelin et al. 1990). The objective of this study is to develop *in vitro* clonal reproduction system from root segments, stem internodes, and leaf discs. The advance of this research will promote genetic transformation studies in the clones and provide an additional alternative for the commercial propagation of promising clones.

MATERIALS AND METHODS

Plant materials

Axillary buds of four clones (Canada blanc, Eugenii, I-45/51, and Wisconsin #5) of *Populus euramericana* were collected from actively growing greenhouse stock plants at Iowa State University. Stock plants were grown at 25℃ under natural day light and fluorescent night light with photosynthetically active radiation (PAR) and a 16h photoperiod by cool white fluorescent lamps. The stock plants were watered twice a day and treated with full strength nutrient solution (Peters Fertilite Special, 128:1) twice a week. The outer leaves were removed from the collected buds and the buds were immersed in water overnight at room temperature. The explants were dipped in 70% ethanol for 1 minute, sterilized in a solution of 2% sodium hypochlorite for 20 or 40 minutes and finally rinsed four times with sterilized deionized water.

Preparation of explants

Fresh cuts were made on the explants to remove the two end segments that were killed by the sterilization, and the explants were individually inoculated to test tubes (2.4 x 15 cm) containing 10 ml of WPM (Woody Plant Medium) without plant growth regulators. After developing shoots proliferated, the three or four elongated shoots were transferred to Magenta GA-7 vessels (7.6 x 7.6 x 10.2 cm, Magenta Corp. Chicago IL) with 50 ml of the same medium for shoot and root proliferation. After 2 months, the fully elongated shoots served as the source for experimental materials. The explants were separated into root segments, stem internodes, and leaf discs, and randomly assigned into plastic petri dishes (9 x 1.5 cm).

Shoot-induction medium for explant culture

The shoot induction medium used was Woody Plant Medium. In addition, the medium was supplemented with the different types and various concentrations of plant growth regulators. Two major cytokinins, BA and zeatin were used at concentration of 0.1, 0.2, 0.5, 1.0, 2.0, and 5.0 mg/l and BA and zeatin combined with the auxins, NAA, IAA, or 2,4-D at concentration of 0.01, 0.02, 0.05, 0.1, 0.2, or 0.5 mg/l. Every plant growth regulator except zeatin was added into the medium before autoclaving, whereas zeatin was added into the sterilized medium through filters. The medium contained N and N vitamins, sucrose 30 g/l, and Difco Bacto agar 8 g/l. The medium was adjusted to pH 5.8 before the addition of agar and autoclaved at 1.05 kg cm^{-2} and 121℃ for 20 minutes. The prepared explants of root segments (4-5 cm), stem internodes (2-3 cm), and leaf discs (3-4 cm) were chosen at random from different plantlets after washing with sterilized deionized water to remove agar. Petioles were removed from their leaves. Each sample was inoculated onto the surface of 20 ml of medium in 15 x 100 mm plastic petri dishes.

Rooting test

Shoots propagated from the explants were tested for rooting ability in the same WPM medium supplemented with NAA and kinetin at concentration of 0.1, 0.2, 0.5, and 1.0 mg/l.

Culture conditions and statistical analysis

The explants were maintained at 26 ± 2℃ with a 16h photoperiod, and a photosynthetically active photon flux rate of 40-50 $u Em^{-2}S^{-1}$ from cool white fluorescent

tubes. Each treatment was composed of five replications. The number of shoots was observed after 4 weeks. A completely randomized design was used in the experiment and the data were analyzed by analysis of variance tests (ANOVA).

RESULTS AND DISCUSSION

In vitro establishment and shoot proliferation

Various combinations of plant growth regulators were tested. Axillary buds originating from four clones of greenhouse stock plants were disinfected with 2% sodium hypochlorite for 20 or 40 minutes. The percentage of contamination was much less after sterilization for 40 minutes and *in vitro* growth of every clone after 4 weeks of culture was relatively faster with disinfection for 40 minutes than for 20 minutes (Figure 1-A). The shoots induced from leaf, stem, and root segments initiated to elongate. The greatest number of shoots by explant type was obtained from root segments

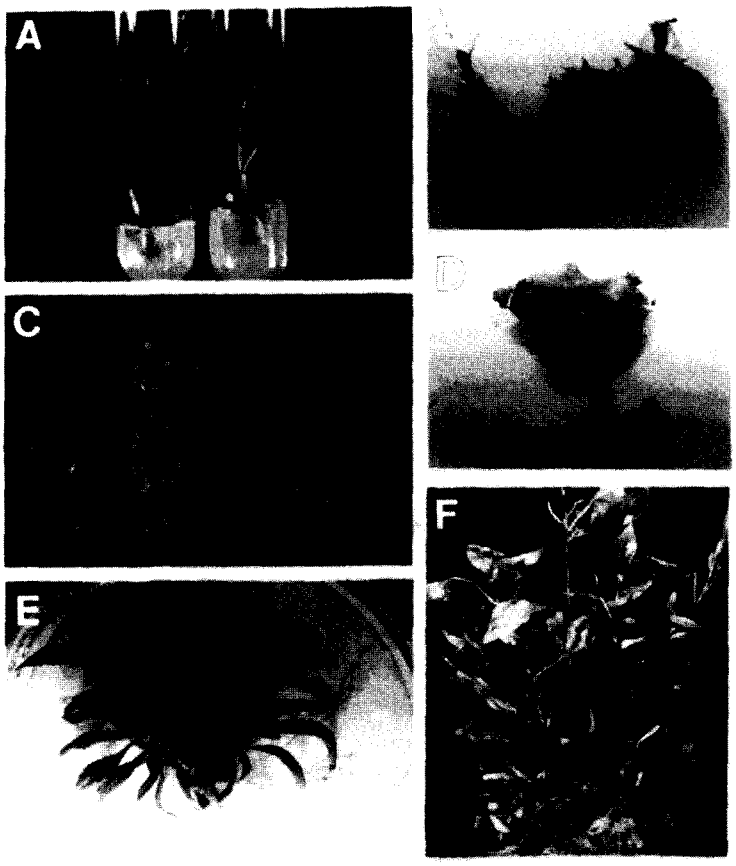

Fig. 1. Clonal proliferation of *Populus euramericana (Populus deltoides* x *P. nigra)* from three different explants of four clones. A: The induced axillary buds from the Eugenii clone after 4 weeks culture on WPM medium without plant growth regulators. B: Shoots from root segment culture of the Canada blanc clone on WPM medium containing 0.2 mg/l BA. C: Shoots from stem internode culture of the Wisconsin #5 clone on WPM medium supplied with 2.0 mg/l zeatin and 0.2 mg/l IAA. D: Shoots from leaf disc culture of the I-45/51 clone on WPM medium with 0.1 mg/l BA and 0.01 mg/l NAA. E: Shoot elongation from stem internodes of the Wisconsin #5 clone on WPM medium with 0.2 mg/l NAA. F: Rooting from the multiplied shoots of the Eugenii clone on WPM medium with 0.2 mg/l kinetin.

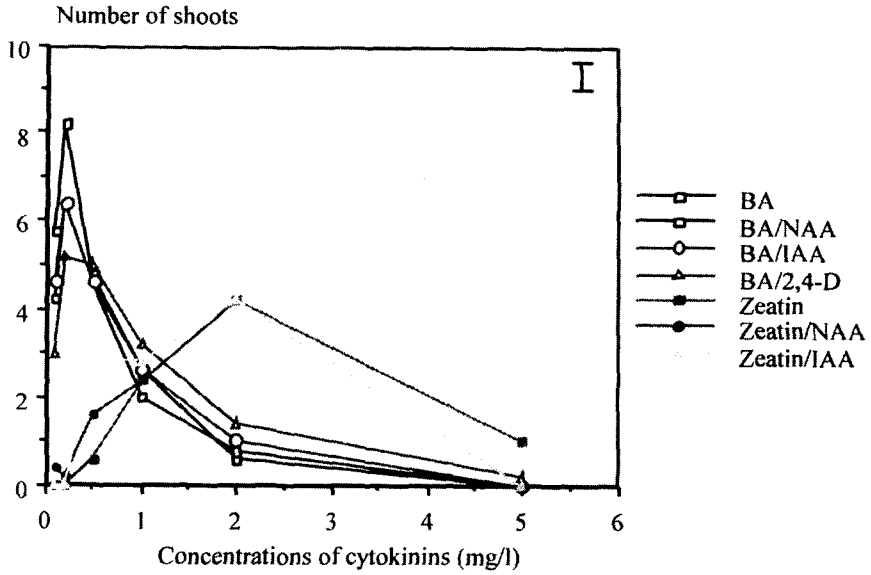

Fig. 2. The average effect of plant growth regulators on shoot proliferation after 4 weeks culture from root segment explants for the Eugenii clone. The graph shows a general response that was also observed for most of the other explants/clones the vertical bar on the graph shows the standard error.

of the Canada blanc clone in medium containing 2.0 mg/l BA, from stem internodes of the Wisconsin #5 clone in combination with 2.0 mg/l zeatin and 0.2 mg/l IAA, and leaf discs of the Canada blanc clone with 0.5 mg/l BA and 0.05 mg/l 2,4-D (Figure 1-B, C and D). The efficiency of clonal shoot regeneration was strongly affected by the types and concentrations of plant growth regulators ($F < 0.001$) and among four different clones ($F < 0.002$) and three different tissue types ($F < 0.001$). In general, BA was more effective than zeatin in stimulating shoot multiplication from root explants. Stem internodes produced more shoots in the presence of BA for the Canada blanc and Wisconsin #5 clones and in the presence of zeatin for the Eugenii and I-45/51 clones. Leaf explants worked best with BA for the Canada blanc and I-45/51 clones and with zeatin for the Eugenii and Wisconsin #5 clones. Spontaneous shoot elongation was promoted in a medium containing 2,4-D.

Comparison of clones

Root segments

Root segments were cultured in WPM medium containing seven combinations of cytokinin (BA and zeatin) with auxin (NAA, IAA, and 2,4-D). After 4 weeks, the proliferated shoots from the explant were counted (Figure 1-B). The highest average number of shoots (17.6 ± 0.47) was induced from the explants of Canada blanc. The proliferation patterns of shoots showed that the clones of Eugenii, I-45/51, and Wisconsin #5 responded similarly to produce a greater number of shoots in low concentrations of BA or the combinations with the auxins and in high concentrations of zeatin or the combinations with the auxins (Figure 2). This difference in response can be explained by the function of these two cytokinins because they have similar molecular weights and therefore the differences in concentrations by weights are approximately equal to differences in molar

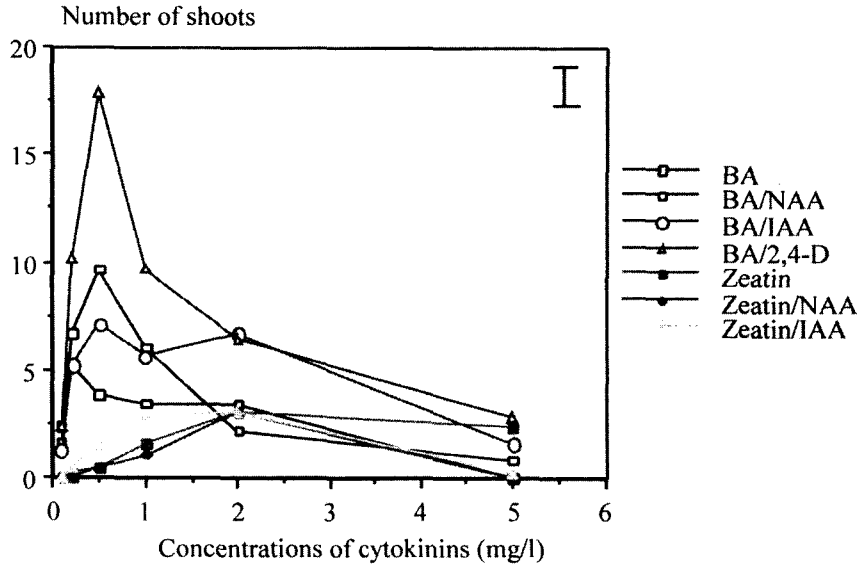

Fig. 3. The average effect of plant growth regulators on shoot proliferation after 4 weeks culture from leaf disc explants for the Canada blanc clone. The graph shows a different pattern compared with general response. The vertical bar on the graph represents the standard error

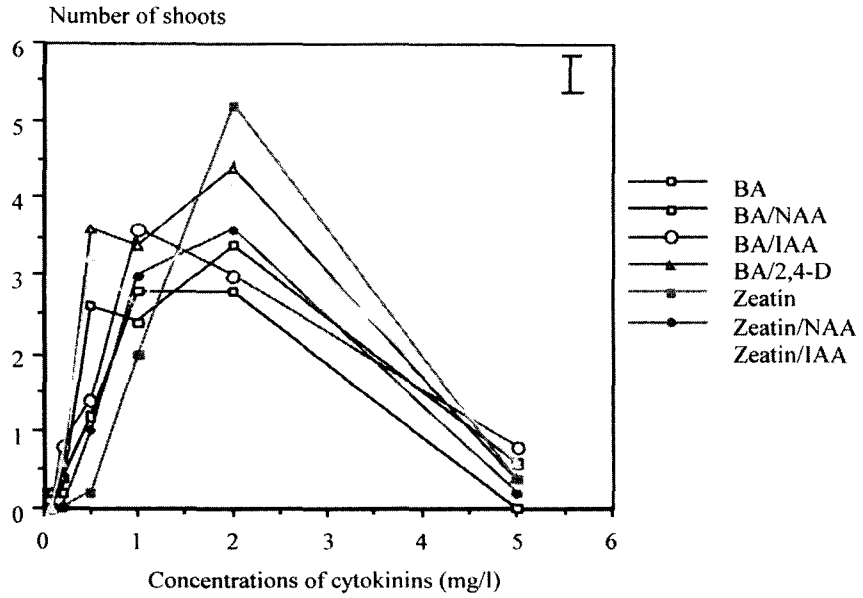

Fig. 4. The average effect of plant growth regulators on shoot proliferation after 4 weeks culture from leaf disc explants for the Wisconsin #5 clone. The graph shows a different pattern compared with the general response. The vertical bar on the graph represents the standard error

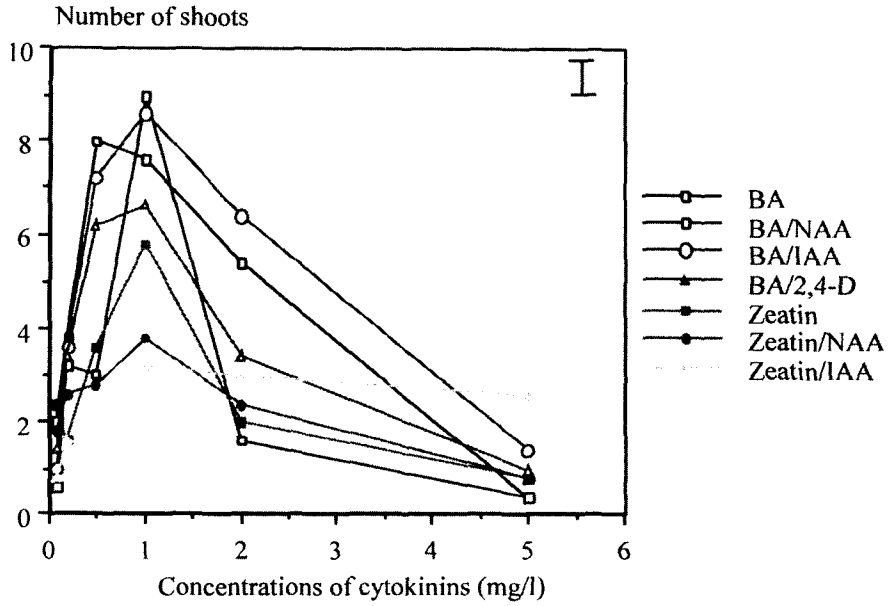

Fig. 5. The average effect of plant growth regulators on shoot proliferation after 4 weeks culture from stem internode explants for the Canada blanc clone. The graph shows a different pattern compared with the general response. The vertical bar on the graph represents the standard error.

concentrations. However, the explants of the Canada blanc clone responded to either high concentrations of BA (2.0 mg/l) or in high concentrations of zeatin (5.0 mg/l).

Leaf discs

Four different clones and three different explants from them were tested. Each type of explant was cultured for 4 weeks and transferred to the medium supplemented with NAA or kinetin for a root initiation and elongation test (Figure 1-D). In Eugenii and I-45/51 clones, the responses of shoot proliferation from leaf discs seemed to follow a general pattern like that of root explants for the Eugenii clone (Figure 2). The highest average number of shoots (17.8 $\pm$ 1.92) was obtained from leaf discs of Canada blanc clone (Figure 3). The greatest average number of shoots was obtained in low concentrations of BA or the combinations with auxin (NAA, IAA, or 2,4-D) and in high concentrations of zeatin or the combinations with auxin (NAA or IAA). The leaf disc explants of Canada blanc and Wisconsin #5 clones appeared as the variants in their response patterns to plant growth regulators. Around 0.5 mg/l of BA or the combinations with auxin highly affected to produce greater average number of shoots in the Canada blanc clone. However, zeatin produced relatively few shoots with leaf discs of Canada blanc (Figure 3), whereas the effects of BA and zeatin appeared to be similar for shoot production from the explants of Wisconsin #5 (Figure 4).

Stem internodes

Eugenii, I-45/51, and Wisconsin #5 showed similar responses to the treatments with the greatest number of shoots induced at low concentrations of BA or at high concentrations of zeatin. The highest average number of shoots (18.2 $\pm$ 3.0) was produced in the combination of zeatin and IAA in stem internode

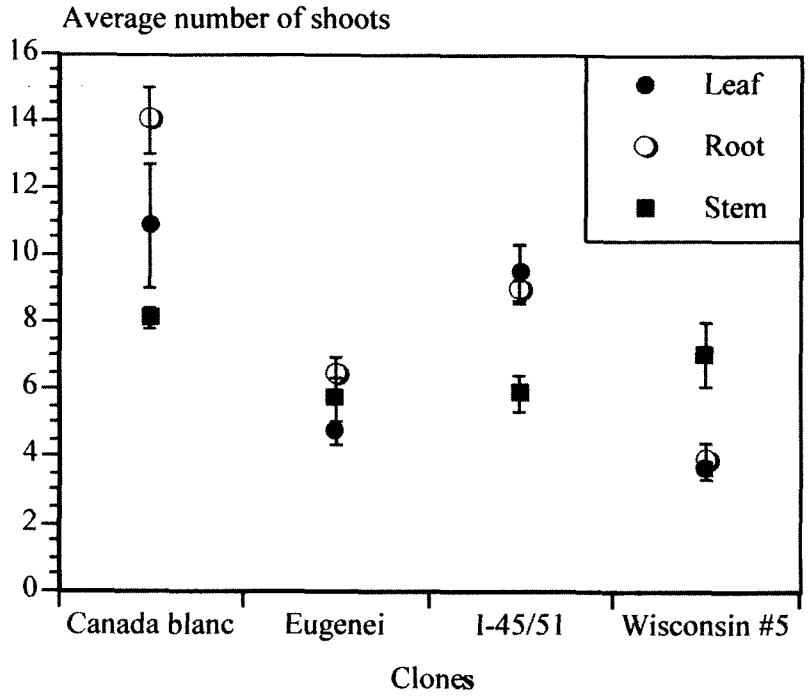

Fig. 6. Overall effect of BA on shoot proliferation after 4 weeks incubation of root segment, stem internode, and leaf disc explants of 4 clones. The vertical bars on the graph represents the standard error.

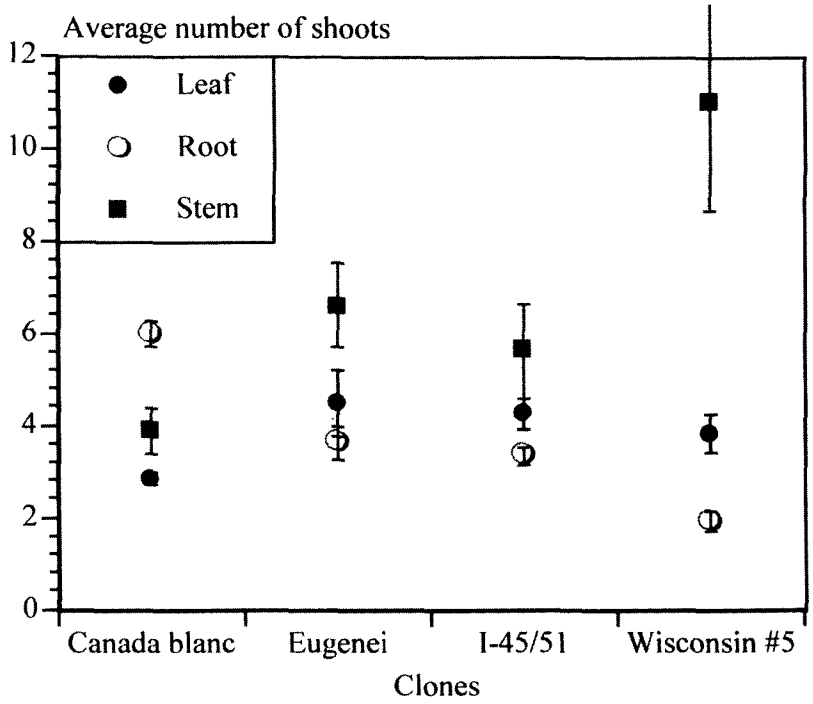

Fig. 7. Overall effect of zeatin on shoot proliferation after 4 weeks incubation of root segment, stem internode, and leaf disc explants of 4 clones. The vertical bars on the graph represents the standard error.

explants of the Wisconsin #5. Canada blanc showed a different pattern from the other clones (Figure 5). Both BA and zeatin in the middle zone of concentration (1.0mg/l) produced the maximum number of shoots. However, for this clone, leaf discs and root segments responded better to BA or the combinations with the auxins.

The effect of BA and zeatin for shoot proliferation

The main variables in producing shoots were BA and zeatin as the cytokinin rather than NAA, IAA, and 2,4-D as the auxin. Both leaf discs and root segments as explants were effective in producing shoots with BA in Canada blanc clone, whereas the explants produced relatively few shoots in Eugenii and Wisconsin #5 clones (Figure 6). The responses of stem internodes were very constant among the four clones. Overall, the Canada blanc clone worked best with the three different explants, which had the greatest average number of shoots (root; 13.96, leaf; 10.84, and stem; 8.08 $\pm$ 0.47 in each explant).

The different responses of shoot proliferation with zeatin are shown in Figure 7. The explants of leaf and root responded consistently in each of the four clones. However, stem internodes did sprout effectively in the Wisconsin #5 clone. BA and the combinations with the auxin were very effective in leaf and root explants of the Canada blanc clone, whereas zeatin or the combinations with the auxins worked well in stem internodes of the Wisconsin #5 clones. In summary, when the results are averaged across the three explant types and four clones, BA produced the greatest number of shoots at the concentrations from 0.2 to 0.5 mg/l, whereas zeatin induced a response around 2.0 mg/l (Figure 8).

Rooting test of *in vitro* formed shoots

Shoots induced from each explant type were tested for rooting capacity with NAA and kinetin. The shoots produced one or two main roots in WPM medium without plant growth regulators. However, NAA and kinetin stimulated the production of roots (no data presented). In the presence of NAA and kinetin, shoots formed 20-30 roots, including secondary roots, after 4 weeks of transfer. Roots elongated to a length of 4-5 cm and appeared to mature. The optimum concentration of NAA and kinetin was 0.2 mg/l in both plant growth regulators and the response was better in kinetin than in NAA (Figure 1-F). After root induction, both shoots and roots continued to grow until complete plantlets were formed in the vessels.

In this study, BA was found to be best for shoot multiplication from root or leaf explants in Canada blanc clone, whereas zeatin was best for stimulating shoot proliferation with stem internodes in Wisconsin #5 clone. Similar studies have been done from the explants of aspen (Ahuja 1984, 1987) and *Populus alba* x *P. grandidentata* (Chun 1986). However, these studies used BA alone or the combination of auxins instead of zeatin. Even though many shoots were produced from stem internodes in the media containing zeatin, the greatest number of shoots was obtained from root and leaf explants supplemented with 2.0 mg/l BA alone or 0.5 mg/l BA combined with 0.05 mg/l 2,4-D. This result with BA is similar to those reported on propagation studies of aspen. The studies of aspen by Ahuja showed that 0.5 mg/l BA in combination with 0.02 mg/l NAA produced vigorous shoots from leaf discs and root explants of aspen. In addition to leaf discs and root segments, meristem produced many shoots on ACM (aspen culture medium) containing 0.5 mg/l BA and 0.02 mg/l NAA.

Genetic variation was shown among different clones by measuring morphogenetic responses. Root, stem and leaf explants from four different clones showed different phenotypic responses such as shoot elongation or rooting patterns. The Wisconsin #5 clone had lower activity in rooting than the other three clones though

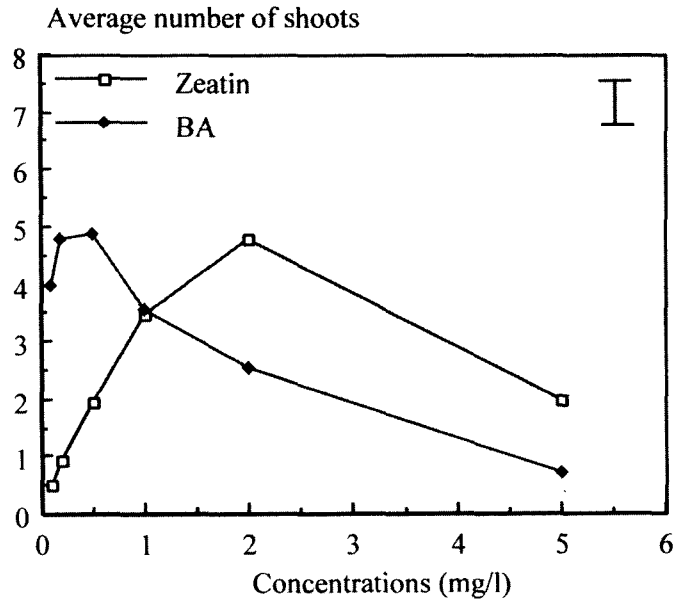

Fig. 8. Overall effect of BA and zeatin on shoot proliferation across all explant types for all four clones. The vertical bar on the graph shows the standard error

shoot growth was best among the clones. The explants from root, stem, and leaf can be manipulated under *in vitro* conditions. Clonal propagation systems using different plant parts as explant sources have been established for these four clones of *Populus euramericana*. The morphogenetic responses among different clones depended on the types of plant growth regulators and on the sources of explants.

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