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Techniques in the Shoot Multiplication of the Leguminous Tree *Prosopis alba* Clone B_2V_{50}

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ABSTRACT

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Tissue culture techniques involving shoot multiplication through axillary branching of the *Prosopis alba* clone B_2V_{50} are described, using single lateral bud explants from 1–2 y old, greenhouse-grown trees. Benzylaminopurine (BA) at concentrations of 0.0, 4.4×10^{-8}, 4.4×10^{-7}, 4.4×10^{-6}, and 4.4×10^{-5} *M* and indole-3-acetic acid (IAA) at concentrations of 0.0, 5.7×10^{-9}, 5.7×10^{-8}, 5.7×10^{-7}, 5.7×10^{-6}, and 2.9×10^{-5} *M* showed significant interaction in the Murashige and Skoog (MS) basal salt medium. BA concentrations of 4.4×10^{-5} or greater are apparently required for consistent shoot production. Up to seven multiple shoots and a maximum shoot length of 10 cm have been produced in 90 days on full-strength MS with 6.7×10^{-5} BA and 2.9×10^{-5} IAA. Ammonium (NH_4) appears inhibitory to shoot development. Nitrate (NO_3) and glutamine are acceptable nitrogen sources for short incubations (28 days), but after longer periods (55 days) shoots on media containing glutamine remain green and healthy, while those on nitrate media are yellow and senescent.

INTRODUCTION

Tissue culture involves the isolation of cells or tissues and placing them under controlled, aseptic environments (Murashige, 1979; Bonga and Durzan, 1982; Locy et al., 1984). All of the environmental factors necessary for growth (heat, light, air, water, nutrients, and support) are provided artificially with the objective being to obtain rapid asexual multiplication of plant cells or plants. Any given tissue composed of cells with competent nuclei is a suitable explant for the initiation of a plant tissue culture (Sharp et al., 1979).

Multiplication of plant materials in vitro involves the manipulation of plant growth through modifications of the culture medium, culture environment, and the source and type of tissue taken for culture (Locy et al., 1984). Multiplication can be achieved by one of three different morphogenic processes:

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axillary bud enhancement, adventitious shoot formation, or somatic embryogenesis (Murashige, 1979; Locy et al., 1984).

Efforts with the *Prosopis alba* clone B_2V_{50} have dealt exclusively with the method of enhancing axillary branching. This is considered to be the 'safest' method because it will not stimulate a plant's tendencies to mutate (Zumwalt, 1976). Culture systems dependent on adventitious shoot development or somatic embryogenesis from either callus cultures or cell suspensions are usually considered to be inherently genetically unstable (Dodds, 1983). Plant material subjected to the axillary branching method can produce shoots at a greatly accelerated rate by stimulating bud development from the preexisting bud primordia (Zumwalt, 1976). Multiplication can be achieved by dissecting the new shoots into nodal segments which are then transferred individually to fresh medium. This shoot multiplication or Stage II process can be repeated several times (Zumwalt, 1976; Murashige, 1979; Locy et al., 1984).

Although the multiplication rate of this method is substantially slower than other tissue culture methods, it nevertheless enables yearly multiplication rates that are as much as a million times faster than traditional methods (Murashige, 1979). Tissue culture procedures may also result in pathogen elimination (Zumwalt, 1976; Murashige, 1979; Locy et al., 1984) and the production of pathogen-free stocks for plant breeders is an important use of in vitro techniques (Dodds, 1983). In vitro propagation may be cheaper and easier than some methods of plant propagation, however, it is generally more costly than propagation by seed or by simple vegetative means such as rooting of cuttings (Locy et al., 1984). Therefore, commercial applications of tissue culture techniques are usually based on the following criteria:

(1) the plants are elite because they are of superior genotype or are pathogen-indexed;

(2) the plants are expensive and difficult to propagate asexually, and seed propagation is unacceptable or undesirable (Locy et al., 1984), e.g., self-incompatibility in pollination, hybrid seed production, erratic seed production, and long periods until biological maturity.

Previous research with *Prosopis* spp. has encountered extreme variability between and within species in characteristics such as biomass production, cold tolerance, pod production, and salt tolerance (Felker et al., 1981; 1982; 1983; 1984). This large variability has been attributed to the self-incompatibility of *Prosopis* spp. resulting in the propagation of seed that is not true-to-type (Simpson, 1977). Numerous years of research have led to the development of the high-biomass producing *Prosopis alba* clone B_2V_{50} (referred to as $V_{50}B_2$ in Felker et al., 1983)

First year dry biomass productions of 3460 kg/ha^{-1} have been measured on a 3×3 m spacing (Felker et al., unpublished) after nine months when receiving 470 mm rainfall and 17,000 kg ha^{-1} at the end of the second growing season. The subsequent tissue culture techniques concerning *Prosopis* deal exclusively

with this thornless, high-biomass producing *Prosopis alba* clone B_2V_{50} originating in Big River, California (Felker et al., 1983).

The real key to economic tissue culture propagation is in the Stage II, or shoot multiplication process. Production of one explant all the way through the process of shoot initiation, root production, and hardening off is of little consequence for operational production. Thus, we have concentrated on the development of techniques to enhance shoot multiplication in the *Prosopis alba* clone B_2V_{50}.

As this was the first report of propagation of this clone, it was necessary to investigate deinfestation procedures for woody plants, to examine the influences of various media, to establish the basic environmental requirements, and to analyze the traditional hormonal influences of auxins and cytokinins on shoot production.

MATERIALS AND METHODS

Lateral buds from the vegetative shoots of 1–2 y old, 2–3 m tall, greenhouse-grown stock plants of *Prosopis alba* clone B_2V_{50} were employed. The shoots were cut into nodal segments containing the axillary bud and a small portion of the internode on either side. The leaf was excised from the stem, leaving only a small portion of the petiole attached to it.

The explants were then surface sterilized by passing them through the following series of solutions:

(1) a 3 s dip in ethyl alcohol,
(2) a 5 min dip in 20% v/v solution of a sodium hypochlorite bleach, and
(3) a 5 min dip in 0.1% mercuric chloride ($HgCl_2$).

After each stage, the explants were rinsed in sterile distilled water.

The explants were placed vertically in 25 × 150 mm culture tubes containing 18–25 ml of medium and 0.8% agar. Various media and medium concentrations were examined, including auxins (napthaleneacetic acid [NAA], indole-3-acetic acid [IAA], and indole-3-butyric acid [IBA] and cytokinins (kinetin [K] and benzylaminopurine [BA]). The medium was adjusted to pH 5.8 and the tubes plugged with cotton and autoclaved at 100 kPa for 15 min before use.

The cultures were maintained at 26–29 °C with 12 hours of light at an intensity of 80 $\mu E\ m^{-2}\ s^{-1}$ from cool white fluorescent tubes. The explants were subcultured every three to four weeks onto fresh medium. Observations were recorded prior to each subculture. Results excluded cultures that were contaminated or that dried up within several days of culture initiation.

A rating scheme was devised to rank explant color and leaf size. Color was rated as 0 for brown, 1 for yellow, 2 for green, and 3 for dark green. Leaf size class was estimated as follows: 0 = 0 mm, 1 = 1–10 mm, 2 = 11–20 mm, 3 = 21–30 mm, and 4 = > 31 mm.

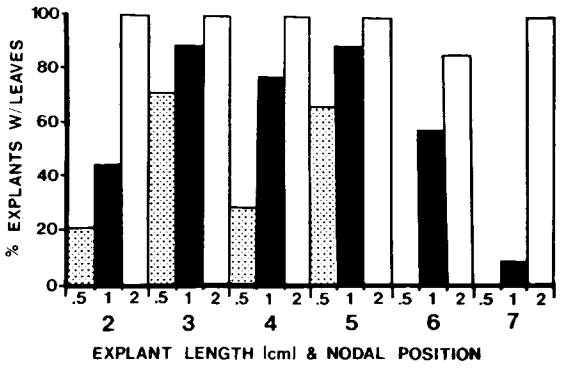

Fig. 1. Influence of nodal position and explant length on development of *Prosopis alba* clone B_2V_{50} after nine weeks. The basal salt medium was MS full strength. The hormone concentration was kinetin 0.015 mg/l, IAA 0.5 mg/l.

RESULTS

Results of a comparison of the influence of nodal position and explant length on leaf development are presented in Fig. 1. The nodes were numbered starting with 1 for the terminal bud. Therefore, the greater the number, the older the node. It is clear that the length of the explant had a profound influence on the percent of explants bearing leaves, with the 2 cm explant length giving the highest percentage. Although less conclusive, nodes 2 through 5 seemed to have the greatest developmental potential. However in other experiments, up to node 11 gave good results for those nodes which were not contaminated.

Results of a comparison of three of the better known basal salt media for woody plants; Murashige–Skoog (1962), Gamborg B5 (1968), and McCowen's (Lloyd and McCowen, 1980) Woody Plant medium are presented in Table 1. Ten replicates were used per treatment. Overall, the full-strength media yielded a greater response than the quarter or half-strength media. While the Gamborg B5 medium was initially quite good, nearly all of the leaves dropped off after 55 days. Considerable leaf drop also occurred in the Woody Plant medium, however the higher concentrations continued to show good development. The Murashige–Skoog medium yielded the best results overall and was adopted for routine use.

Environmental conditions were studied in an experiment consisting of four air temperatures and four light intensities. Four growth chambers were used with air temperatures of 25, 28, 31, and 34 °C, respectively. Shade cloth was used to reduce light intensities within each chamber to 180, 90, 44, and 22 μE m^{-2} s^{-1}. Ten replicates were used per treatment. The number of leaves per explant and the leaf length were reduced at all temperatures by the lowest light intensity (22 μE m^{-2} s^{-1}). The 90 μE m^{-2} s^{-1} light treatment had the greatest number of leaves per explant and the 180 μE m^{-2} s^{-1} treatment had the largest

TABLE 1

Effect of basal nutrient salt media on development of *Prosopis alba* clone B_2V_{50} after 55 days

Type of basal medium	Strength	*n*	Percent of ex- w/leaves	Number of leaves per explant[a]	Leaf size class[a]	Leaf color[a]
Murashige-Skoog	Quarter	12	25	1.3±0.5	4.0±0.0	2.0±0.0
	Half	11	75	1.3±0.5	2.9±0.8	1.9±0.8
	Full strength	12	42	2.0±0.7	3.0±0.7	1.8±0.4
Gamborg B5	Quarter	12	8	1	3	3
	Half	12	8	2	4	3
	Full strength	12	8	2	6	3
Woody plant medium	Quarter	12	0	–	–	–
	Half	12	33	1.3±0.5	2.3±1.0	1.8±0.5
	Full strength	12	42	1.4±0.5	3.0±0.0	3.0±0.0

Conditions and ratings as described in text.
[a]Mean ± standard deviation.

leaf length. The leaf color rating was highest at the 44 and 90 μE m^{-2} s^{-1} light intensities when the temperature was 28°C or less. Although far from conclusive, the 28°C air temperature with a light intensity of 90 μE m^{-2} s^{-1} seemed to yield the best results when considering the overall effects on leaf number, leaf size, and leaf color.

In spite of the fact that these earlier experiments only resulted in leaf production, they set the foundation concerning culture medium, culture environment, and tissue source for subsequent experiments.

The first real breakthrough in shoot production came from a factorial experiment consisting of five concentrations of BA (0.0, 4.4×10^{-8}, 4.4×10^{-7}, 4.4×10^{-6}, and $4.4\times10^{-5}M$) and six concentrations of IAA (0.0, 5.7×10^{-9}, 5.7×10^{-8}, 5.7×10^{-7}, 5.7×10^{-6}, and 2.9×10^{-5} M). A comparison of these concentrations for their effect on shoot number and shoot length is shown in Figs. 2 and 3, respectively. All treatments with 4.4×10^{-7} M BA and below had leaves but no shoots. In contrast, all treatments with 4.4×10^{-5} M BA had only shoot production. Thus, the BA concentration seemed to be the key in stimulating the explants to produce shoots rather than leaves. An analysis of variance indicated a significant interaction between BA and IAA for both shoot number and shoot length ($P=0.0007$ and $P=0.0001$, respectively). Therefore, BA and IAA concentrations could not be optimized independently.

Since the highest BA concentration tested in the previous experiment gave the most shoot production, another factorial experiment consisting of four concentrations of BA (5.3, 6.2, 7.1, and $8.0\times10^{-5}M$) and five concentrations of IAA (2.3, 3.4, 4.6, 5.7, and $6.9\times10^{-5}M$) was conducted to examine higher

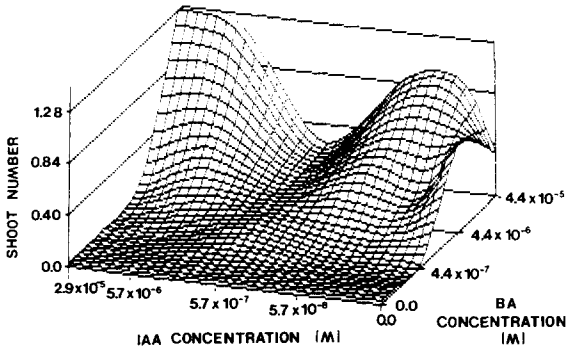

Fig. 2. Influence of benzylaminopurine (BA) and indoleacetic acid (IAA) on shoot number of *Prosopis alba* clone B_2V_{50} after 45 days. Values are the mean of those explants which remained green in culture. The basal salt medium was MS full strength.

BA concentrations in more detail. A comparison of these concentrations for their effect on shoot number and length is presented in Figs. 4 and 5, respectively. Fifteen replicates were used per treatment. The greatest number of shoots was produced at $7.1\text{–}8.0\times10^{-5}$ *M* BA and $3.4\text{–}4.6\times10^{-5}$ *M* IAA. However, no significant difference ($\alpha=0.05$) in shoot number per explant resulted for any of the BA and IAA combinations. Since the explants became excessively lignified at these higher concentrations, a slightly lower concentration of 6.7×10^{-5} *M* BA and 2.9×10^{-5} *M* IAA was adopted for further nonhormonal studies. The greatest absolute shoot length and number of shoots per explant found at this combination were 10 cm and 7 shoots per explant, respectively.

A major problem has been that after about five or six weeks, the shoots became yellow and then would die after about eight weeks. Earlier studies also found significant differences in leaf development between various mineral nutrient media (MS, Gamborg B5, and Woody Plant). One of the major dif-

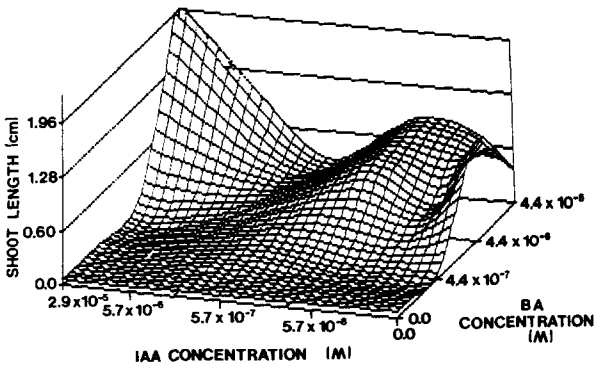

Fig. 3. Influence of benzylaminopurine (BA) and indoleacetic acid (IAA) on shoot length of *Prosopis alba* clone B_2V_{50} after 45 days. Values are the mean of those explants which remained green in culture. The basal salt medium was MS full strength.

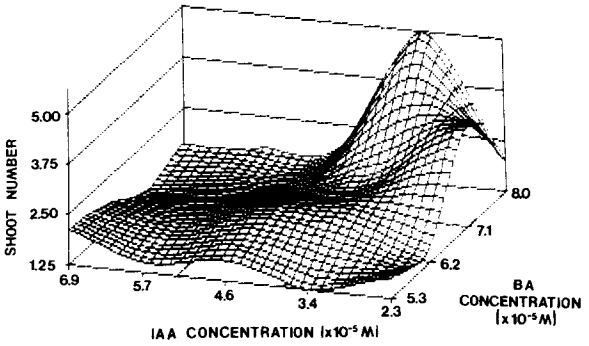

Fig. 4. Influence of benzylaminopurine (BA) and indoleacetic acid (IAA) on shoot formation of *Prosopis alba* clone B_2V_{50} after 45 days. Values are the mean of those explants which remained green in culture. The basal salt medium was MS full strength.

ferences between these media is the amount and kind of the nitrogen source. A 3×3 factorial experiment was conducted to evaluate the interaction between Stock A (NH_4NO_3) and Stock B (KNO_3) of the Murashige–Skoog basal medium. The concentrations used for both of the stocks were 5, 10, and 20 m*M*. No significant interaction between the two was observed.

Since Stock A (NH_4NO_3) contains both ammonium and nitrate it was difficult to differentiate between the effect the ammonium was having and that of the nitrate. An experiment was conducted using KCl at a concentration of 20 m*M* as the primary potassium source and three nitrogen sources (NH_4Cl, $NaNO_3$, glutamine) at concentrations of 20, 30, 40, 50, and 60 (full-strength MS) m*M* nitrogen. Since one mole of glutamine contains two moles of nitrogen; 10, 15, 20, 25, and 30 m*M* glutamine were used to achieve the appropriate nitrogen levels. Ammonium and nitrate contain only one nitrogen molecule, thus their nitrogen content is the same as the molar concentration of the com-

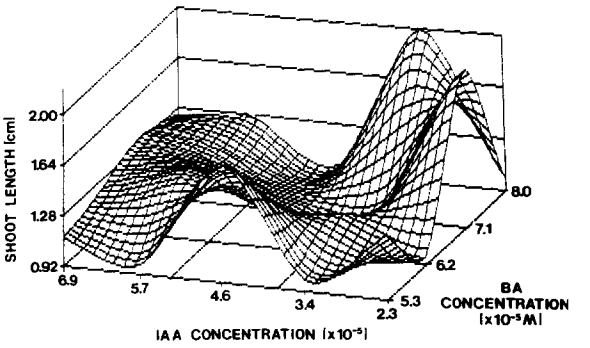

Fig. 5. Influence of benzylaminopurine (BA) and indoleacetic acid (IAA) on shoot length of *Prosopis alba* clone B_2V_{50} after 45 days. Values are the mean of those explants which remained green in culture. The basal salt medium was MS full strength.

TABLE 2

Effect of nitrogen source and concentration on development *Prosopis alba* clone B_2V_{50}

Nitrogen source		Concentration (m*M*) Mean ± SD				
		20	30	40	50	60
28-day evaluation						
NH_4Cl	Shoot number	0.6 ± 0.51	0.5 ± 0.52	0.3 ± 0.46	0.2 ± 0.41	0.1 ± 0.35
	Length (cm)	0.6 ± 0.51	0.5 ± 0.52	0.3 ± 0.46	0.2 ± 0.41	0.1 ± 0.35
$NaNO_3$	Shoot number	0.9 ± 0.26	1.1 ± 0.35	1.5 ± 0.52	1.1 ± 0.52	1.3 ± 0.49
	Length (cm)	1.1 ± 0.52	1.3 ± 0.49	1.3 ± 0.62	1.1 ± 0.46	1.3 ± 0.62
Glutamine	Shoot number	1.0 ± 0.53	1.1 ± 0.35	1.1 ± 0.35	1.3 ± 0.46	1.2 ± 0.41
	Length (cm)	0.9 ± 0.46	1.4 ± 0.51	1.5 ± 0.74	1.7 ± 0.70	1.5 ± 0.64
55-Day evaluation						
NH_4Cl	Shoot number	0.2 ± 0.41	0.2 ± 0.43	0.1 ± 0.26	0	0
	Length (cm)	0.3 ± 0.59	0.2 ± 0.43	0.1 ± 0.26	0	0
$NaNO_3$	Shoot number	0.9 ± 0.83	0.9 ± 0.51	0.9 ± 0.95	1.4 ± 0.76	1.0 ± 0.96
	Length (cm)	0.8 ± 0.75	0.9 ± 0.51	0.9 ± 1.12	1.1 ± 0.73	0.7 ± 0.61
Glutamine	Shoot number	1.8 ± 0.67	1.5 ± 0.52	1.8 ± 0.73	1.9 ± 0.33	1.6 ± 0.52
	Length (cm)	1.6 ± 0.73	2.4 ± 1.33	2.3 ± 0.95	2.2 ± 1.30	2.1 ± 0.64

Values are the mean of those explants which were not contaminated in culture. The basal medium was MS full strength. The 55-day cultures were recultured 2 times.

pound. The preliminary results, summarized in Table 2, indicate that ammonium was inhibitory to shoot production. Both the nitrate and the glutamine produced healthy shoots and no noticeable difference was present between the two after the four week evaluation. The results from an eight week evaluation, also summarized in Table 2, indicate that glutamine is superior to either inorganic nitrogen source.

DISCUSSION

The application of in vitro techniques to trees had a rather late beginning, but is now becoming a very active area of research. Tissue culture of semiarid tree legumes has recently been reported by Goyal (1982) and Goyal and Arya (1981, 1984) on *Prosopis cineraria*. This is the first report concerning the propagation of the *Prosopis alba* clone B_2V_{50}.

The fact that explant size is crucial to development implies that some nutrient reservoir is important that is not being supplied in the culture media. Although the full-strength Murashige–Skoog medium was the best overall (after 55 days), the Gamborg B5 medium initially (after 19 days) was quite effective in promoting leaf development. The major differences between the Murashige–Skoog (MS) medium, the Gamborg B5 medium, and the Woody Plant

medium (Lloyd and McLowen) are the amount and source of inorganic nitrogen. MS has approximately 20 m*M* NH_4NO_3 and 20 m*M* KNO_3; the Woody Plant medium has approximately 5 m*M* NH_4NO_3 and 2.5 m*M* Ca $(NO_3)_2$; and the B5 has approximately 25 m*M* KNO_3 and 1 m*M* $(NH_4)_2SO_4$. Normally, plants use nitrate as the source of nitrogen and cultured tissues would be expected to utilize nitrate for growth. Organic nitrogen has been shown to be important in promoting shoot development in some tree species (R. Mott and D. Durzan, personal communication, 1983). Glutamine is the first carbon compound to accept reduced nitrogen from nitrogen fixation (Meeks et al., 1978) and is the transport form of organic nitrogen in many legumes (Reynolds et al., 1982). Gamborg (1970) noted that glutamine or asparagine, but not other amino acids, could replace ammonium [$(NH_4)_2SO_4$] in soybean cell cultures and that the addition of glutamine would enable the cells to maintain a high growth rate for a longer period.

The auxin IAA and the cytokinin BA have proven superior to the other hormones tested. Goyal and Arya (1984) reported a positive effect from IAA in inducing shoot formation in *Prosopis cineraria*, with K (0.05 mg/ml) being more effective than BA. The concentrations of both BA and IAA had a profound effect upon shoot differentiation in the *Prosopis alba* clone B_2V_{50}. However, it appears that a BA concentration of 4.4×10^{-5} or greater is the key to shoot stimulation.

Tissue culture of forest trees has lagged behind that of more economically important horticultural and agricultural crops (Bonga and Durzan, 1982). As a group, the semiarid woody legumes lag even further behind with few having been successfully tissue cultured. This research has concentrated on the initial stages of the tissue culture process. Significant progress has been made and well-defined techniques have been developed concerning the explant size and position, the culture environment, and the nutritive and hormonal requirements for shoot multiplication. Additional work is needed to maintain shoot viability, to increase shoot multiplication, and to produce roots and successfully transfer the plants back to the soil.

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