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Studies on soybean tissue culture and transformation

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Studies on soybean tissue culture and transformation

by

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TABLE OF CONTENTS

INTRODUCTION

The genus Glycine is a member of the family Leguminosae and is divided into two subgenera, Glycine and Soja (Hymowitz and Newell 1981). The subgenus Glycine includes seven wild perennial species, and the subgenus Soja comprises the cultigen G. max (L.) Merr. and its wild annual counterpart, G. Soja Sieb. and Zucc. Soybean [Glycine max (L.) Merr.] is a native of eastern Asia and has become the most important plant source of edible oils and protein in the world; for example, 40% of the world's edible oils come from soybeans (Sinclair 1982). The protein content of soybean is 24 to 54%, and the oil content is 8 to 27% (Hymowitz et al., 1974). The USA, Brazil, China, Argentina, Indonesia, and the USSR are the six most important nations in soybean production.

There are several major objectives in soybean breeding: improvement of seed protein and oil quality and production of stress tolerant, pest and disease resistant, and herbicide tolerant plants. Their objectives can be potentially achieved using biotechnology, including genetic engineering and tissue culture, because biotechnology should allow rapid incorporation of specific improvements within a short period, especially by the transfer of specific genes into plants.

The purpose of this study was to devise improved techniques for the regeneration in vitro of soybean plants and for their genetic transformation. Two specific objectives

were outlined. The first objective was to find an expedient method for regenerating whole soybean plants from excised parts of seeds or seedlings.

Dr. Sarobol regenerated roots from mature soybean cotyledons in some preliminary experiments when they were imbibed and cultured in vitro on a medium containing both auxin and cytokinin. It suggested that cotyledons from mature soybean seeds have a large capacity for regeneration, and these capacities were studied by me in detail. Although tissues from the "lamina" of the cotyledon showed three different developmental responses, as reported in Section I of this dissertation, shoot formation was not one of them. However, in the course of these studies, a very unusual developmental response, the formation of a reticulum consisting primarily of hypertrophied veins and of friable callus proliferating between them was observed. The tips of this reticulum resembled globular embryos and prompted my interest in its further study. This reticulum and its ontogeny at the anatomical level are described and interpreted in Section I.

Because mature soybean cotyledons did not regenerate shoots, I sought to regenerate them from various seedling parts, including leaves, hypocotyls, and especially seedling nodes, where shoots normally form and where earlier studies by others had suggested that ample regeneration could be expected. Early in this series of experiments, profuse shoot

formation from primary leaf nodes was observed, and my later efforts focused on this regeneration site. The protocol resulting from this particular focus is described in its soon-to-be published form in Section II. This represents the first stage in the development of a highly effective shoot-regeneration system, and much of its success derives from modifications of the Murashige and Skoog medium introduced here. These modifications consist of the addition of L-proline and an increase in the level of inorganic micronutrients.

The second stage in the development of this regeneration system consisted of an experimental analysis of the possible interaction of proline and inorganic micronutrients and of the effects of changed levels of individual elements in the MS inorganic micronutrient mixture. The results of this analysis and specific recommendations for a highly effective and much-improved shoot-regenerating medium for soybean are presented in Section III.

The second objective of this study was to improve the available but inefficient methods for soybean transformation using Agrobacterium tumefaciens. Although much of this work was done in the short interval available after the regeneration system had been fully devised, progress--including production of apparently transformed shoots of soybean--has been made and this is described in Section IV.

LITERATURE REVIEW

Soybean Plant Regeneration by Somatic Embryogenesis

One important route to plant regeneration is through somatic embryogenesis. Steward et al. (1958) and Reinert (1958) first reported the initiation and development of embryos from somatic tissues of carrot (Daucus carota) in culture. Adventive or asexual embryogenesis is the development of embryos from cells that are not the immediate product of gametic fusion. The most distinctive characteristic of somatic embryos is that they are not connected to the mother tissue and have an anatomically discrete radicle (Haccius, 1978).

Since Gamborg et al. (1968) first cultured soybean roots, the genus Glycine has been much studied in tissue culture, a chronological summary of those studies is shown in Table 1. Somatic embryogenesis has been frequently observed in immature embryo cultures of soybean. Beversdorf and Bingham (1977) observed somatic embryo-like structures. Although these structures superficially resembled somatic embryos, they were not competent because whole plants could not be obtained. These structures are neomorphs (Krikorian and Kann, 1981). Later, somatic embryos that had progressed as far as the torpedo stage were described by several research groups (Phillips and Collins, 1981; Gamborg et al., 1983b;

Table 1. Tissue culture research with the genus Glycine

Year	Species[a]	Source[b]	Cult. Type[c]	Final Product[d]	Reference
1968	G. max	Roots	S	Callus	Gamborg et al.
1970	G. max	Sus. cells	P	Callus	Kao et al.
1971	G. max	Sus. cells	P	Proto.	Miller et al.
1973	G. max	Hypocotyls	T	Plants	Kimball & Bingham
1977	Glycine (5 species)	Hypocotyls	T	Embryoids	Beversdorf and Bingham
1980	G. max	Cot. nodes	T	Plants	Cheng et al.
1980	G. max	Ste. nodes	T	Plants	Saka et al.
1980	G. max	Imm. pods	P	Callius	Zieg & Outka
1981	G. max	Sh. meri.	T	Plants	Kartha et al.
1981	G. max	Leaves	P	Proto.	Schwenk et al.
1981	G. max	Hypocotyls Epicotyls	S	Embryos	Phillips & Collins
1981	G. soja	Hypocotyls	T	Plants	Kameya & Widholm

[a] G. cane.: G. canescens
G. clan.: G. clandestina
G. argy.: G. argyrea

[b] Cot. nodes: cotyledonary nodes
Cul. cells: cultured cells
Imm. cot.: immature cotyledons
Imm. pods: immature pods
Mat. cot.: mature cotyledons
Sh. meri.: shoot meristems
Ste. nodes: stem nodes
Sus. cells: suspension-cultured cells

[c] P: protoplast cultures
S: suspension cultures
T: tissue cultures

[d] Proto: protoplasts
Emb.Res.: embryo rescue in vitro

Table 1 (Continued)

Year	Species[a]	Source[b]	Cult. Type[c]	Final Product[d]	Reference
1982	G. max	Roots	P	Callus	Xu et al.
1983a	G. soja	Cul. cells	P	Proto.	Gamborg et al.
1983b	G. max	Hypocotyls	T	Embryos	Gamborg et al.
	Glycine (6 species)				
1983	G. max	Imm. cot.	P	proto	Lu et al.
1983	G. max	Imm. cot.	S	Plantlets	Christianson et al.
1983	G. cane.	Hypocotyls Cotyledons	T+S	Shoots	Widholm & Rick
1984	G. max	Cot. nodes	T	Embryos	Lippmann & Lippmann
1984	G. max	Leaves	T	Plants	Yang et al.
1984	G. max	Imm. Cot.	T	Emb.Res.	Tilton & Russell
1984	G. cane.	cotyledons	T	Plants	Grant
1984	G. cane.	Imm. Cot.	T	Plants	Grant et al.
1985	G. max	Imm. cot.	T	Plants	Lazzeri et al.
1985	G. max	Imm. cot	T	Plants	Ranch et al.
1985	G. max	Imm. cot.	T	Plants	Li et al.
1985	G. max	Callus	P	Proto.	Bojsen & Wyndaele
1985	G. cane.	Hypocotyls	P	Plants	Newell & Luu
1986	G. max	Imm. cot.	S	Plants	Finer & Nagasawa
1986	G. max	Hypocotyls	S	Embryos	Kerns et al.
1986	G. max	Imm. cot.	T	Plants	Barwale et al.
1986	G. max	Imm. cot	T	Plants	Ghazi et al.
1986	G. max	Cot. nodes	T	Plants	Wright et al.
1986a	G. clan.	Mat. cot.	T	Plants	Hammatt et al.
1986	G. clan.	Leaves	T	Plants	Hymowitz et al.
1987a	G. max	Leaves	T	Plants	Wright et al.
1987b	G. max	Epicotyls	T	Plants	Wright et al.
1987a	G. max	Imm. cot.	T	Plants	Lazzeri et al.
1987	G. max	Imm. cot.	T	Plants	Hammatt & Davey
1987b	G. cane.	Mat. cot.	P	Plants	Hammatt et al.
1987a	G. cane. G. clan.	Cotyledons	P	Plants	Hammatt et al.

Table 1 (Continued)

Year	Species[a]	Source[b]	Cult. Type[c]	Final Product[d]	Reference
1988	G. max	Hypocotyls	P	Callus	Hammatt & Davey
1988	G. max	Imm. cot.	P	Plants	Wei and Xu
1988	G. max	Imm. cot.	S	Plants	Finer & Nagasawa
1988	G. max	Imm. cot	T	Embryos	Finer
1988	G. max	Imm. cot.	T	Embryos	Hartweck et al.
1989	G. max	Mat. cot.	T	Plants	Mante et al.
1989	G. max	Imm. cot.	T	Embryos	Parrott et al.
1990	G. max	Leaf nodes	T	Plants	Kim et al.

Lippmann and Lippmann, 1984), but these failed to develop further. There were no reports of successful soybean plant regeneration until 1985, even though Widholm and Rick (1983) and Grant (1984) had earlier obtained plant regeneration from tissue cultures of a perennial, wild Glycine species (Glycine canescens).

After the first reports of regeneration of soybean plants via somatic embryogenesis (Ranch et al., 1985; Lazzeri et al., 1985), several others followed (Li et al., 1985; Barwale et al., 1986; Ghazi et al., 1986; Hammatt and Davey, 1987). Effects of nutritional, physical and chemical factors (Lazzeri et al., 1987a), of hormones and culture manipulation (Lazzeri et al., 1987b), of auxins (Hartweck et al., 1988), and of genotype (Parrott et al., 1989) on soybean somatic embryogenesis were described in detail. Maturation of

immature somatic embryos, the most difficult step in plant regeneration, was discussed by Buchheim et al. (1989). Ranch et al. (1986) described a methodology for the initiation of somatic embryos, their serial proliferation, and plant regeneration in embryogenic tissue cultures derived from cotyledons of immature soybean embryos. Somatic embryo formation was also obtained in suspension cultures of callus derived from cotyledons of immature zygotic embryos. Formation of heart-shaped somatic embryos in cell suspension cultures of Glycine max and G. soja was first reported by Phillips and Collins (1981). Whole plants were regenerated by sequential transfers of the suspension cultures on solid media that did not contain hormones (Finer and Nagasawa, 1988).

Soybean Plant Regeneration by Organogenesis

Organogenesis in tissue culture refers to the formation of organs such as roots, leaves, stems, and shoots from plant parts during culture. Organogenesis is different from embryogenesis even though they may occur in the same tissue. Regeneration via organogenesis from parental tissue involves a vascular connection with that tissue, while somatic embryogenesis does not (Haccius, 1978). Plant regeneration via organogenesis is a frequently observed phenomenon in tissue culture.

In soybean, plant regeneration via organogenesis has usually been obtained from seedling parts: cotyledonary nodes

(Cheng et al., 1980; Kerns et al., 1986; Wright et al., 1986a), other undefined stem nodes (Saka et al., 1980), primary leaf nodes (Kim et al., 1990), epicotyls (Wright et al., 1987b), and primary leaf tissue (Wright et al., 1987a). Mante et al. (1989) regenerated plants from mature cotyledons of soybean seeds that had been soaked in water overnight. Wright et al. (1986b) described factors affecting plant regeneration from soybean cotyledonary nodes of seedlings and histology relating to this regeneration process.

Perennial Glycine species, the wild relatives of commercial soybean (Glycine max), are tolerant to cold, heat and drought (Marshall and Broue, 1981) and resistant to soybean rust (Burdon and Marshall, 1981) and yellow mosaic virus (Singh et al., 1974). This makes them attractive for transferring favorable traits to the cultivated soybean by hybridization. Because conventional hybridization has met difficulties, somatic hybridization mediated by protoplast fusion has been emphasized. This approach requires reproducible regeneration of plants from tissues and protoplasts. Most regeneration of Glycine species has involved organogenesis instead of embryogenesis.

Plants have been regenerated from hypocotyls of G. canescens and G. tomentella (Kameya and Widholm, 1981). Even though the regenerated plants are thought to be derived from pre-existing meristematic tissue of the hypocotyl, these findings represent considerable advances. Widholm and Rick

(1983) induced shoots from callus of G. canescens, but could not root the shoots. Subsequently, whole plants were regenerated from leaves (Hymowitz et al., 1986) and from seedling cotyledons, leaves and petioles (Hammatt et al., 1986) of G. clandestina, cotyledonary nodes of G. soja (Barwale et al., 1986), and seedling parts of G. canescens, G. tomentella, G. latrobeana and G. falcata (Hammatt et al., 1987b).

Gene Transfer by Nonsexual Methods

Plant breeders have transferred genes by using classical cross-fertilization methods. Hybridization is a simple method that does not require difficult techniques. Alhough they have contributed much to improvement of crop production, sexual methods have several limitations. First, sexual crossing can be used for gene transfer only between sexually compatible members of the same species of plants. Second, crop species do not always contain sufficient genetic diversity to allow the desired improvement. And third, even though successful, transfers of specific, single genes by sexual methods are laborious and time-consuming, compared with gene transfer by nonsexual gene-transfer methods. Even after six backcross generations in an intraspecific gene transfer, tightly linked genes may not separate (Goodman et al., 1987). Several nonsexual methods have been developed to overcome some of the limitations of gene transfer by sexual methods. These methods

include 1) cell (protoplast) fusion, 2) direct gene transfer, and 3) Agrobacterium-mediated gene transfer, and are further described below.

Cell fusion is a method for transfer of genetic material by fusion of two protoplasts. Power et al. (1970) and Power and Cocking (1971) fused somatic cells for the first time using protoplasts of maize and oat. The efficiency of protoplast fusion can be enhanced by addition of Ca^{++} ions (Keller and Melchers, 1973), polyethylene glycol (Kao et al., 1974) or electrical current (Senda et al., 1979). This fusion method may make it possible to combine the chromosomes of species that are not sexually compatible or to combine the nuclear genome of one species with the cytoplasm of another (Goodman et al., 1987). It is almost impossible to transfer a specific gene or genes by cell fusion, since gene transfer by this method, like sexual hybridization, results in transfer of many genes at the same time. Plant regeneration from fused protoplasts is also an obstacle to this method.

Plant protoplasts can take up nucleic acids directly from the culture medium. Treatments such as polyethylene glycol and high-voltage pulses (electroporation, Neumann et al., 1982) increase the permeability of membranes and result in increasing transformation. Low transformation frequency and difficulties of regeneration from protoplasts are defects of this gene-transfer method. Gene transfer by microinjection (Griesbach, 1983) has been developed as a method of mechanical

gene transfer into cells with microscopic pipettes. Recently, Klein et al. (1987) developed a new gene-transfer method using high-velocity microprojectiles. Small tungsten particles coated with DNA are accelerated to velocities that permit them to penetrate intact cells, and it cause transfer of DNA into the target cells.

The direct gene-transfer methods include direct DNA uptake, microinjection, and bombardment of cells with high-velocity microprojectiles. Transfer of DNA to plant cells by the bacterium Agrobacterium tumefaciens is another much-used non-sexual method of gene transfer, and it is described in the succeeding subsection.

Agrobacterium-mediated Gene Transfer

Agrobacterium tumefaciens is a soil bacterium that naturally infects many dicotyledonous and gymnospermous plants (Perani et al., 1986). Agrobacterium causes formation of a tumor, commonly called a crown gall, by introducing transferred DNA (T-DNA) into the plant cells at the wound site. The T-DNA is a part of the tumor-inducing (Ti) plasmid present in this bacterium. Three factors are required for DNA transfer by Agrobacterium: 1) cis-acting T-DNA border sequences, 25-base direct repeat sequences, 2) a trans-acting virulence region (Hille et al., 1984), and 3) bacterial chromosomal genes required for bacteria to attach to plant cells. T-DNA occupying a small region of the tumor-inducing

(Ti) plasmid is transferred into the plant cell and integrated into a plant chromosome (Chilton et al., 1977). The ability to cause crown gall disease can be removed by deletion of genes encoding enzymes that catalyze the synthesis of plant growth hormones (Zambryski et al., 1983). So-called "disarmed" Ti plasmids do not have the oncogenes within the T-DNA but retain the border regions for DNA transfer and integration. Foreign genes inserted within T-DNA are transferred from the plasmid to the chromosome of plant cells when gene expression of the virulence region of the Ti plasmid is stimulated by elicitors such as acetosyringone (Stachel et al., 1985; Bolton et al., 1986). The Ti-plasmid is so large that it is difficult to handle, and gene insertion based on homologous recombination with wild-type Ti plasmids is cumbersome. Ti-plasmid-based gene transfer was simplified by the development of binary vector systems, which consist of two plasmids, the binary vector plasmid and a helper plasmid (Hoekema et al., 1983). The binary vector plasmid is a small plasmid that has foreign genes between its T-DNA border sequences and a wide host-range replicon able to replicate in both E. coli and A. tumefaciens cells. The helper plasmid is an intact wild type or disarmed Ti plasmid that contains the virulence region.

As a result of the low frequency of transformation, an effective system for the selection of transformed cells is essential. Several selectable marker genes for plant

transformation have been developed; these confer resistance to antibiotics such as neomycin (Bevan et al., 1983; Herrera-Estrella et al., 1983), methotrexate (Eichholtz et al., 1987), or hygromycin (van den Elzen et al., 1985). Genes conferring resistance to herbicides are also useful as selectable markers. Examples are resistance to glyphosate (Shah et al., 1986; Fillatti et al.,1987) and chlorsulfuron (Chaleff and Ray, 1984). Reporter genes are useful for quick detection of transformation. Genes encoding β-galactosidase and chloramphenicol acetyltransferase have been frequently used as reporter genes. Currently, the most frequently used reporter gene is β-glucuronidase because of the extreme sensitivity and convenience with which it can be assayed (Jefferson et al., 1987). Other genes that can be used as reporter those include genes encoding NPT II and luciferase (Ow et al., 1986).

SECTION I. DICAMBA INDUCES FORMATION OF AN UNUSUAL TISSUE RETICULUM FROM MATURE SOYBEAN COTYLEDON SLICES IN VITRO

DICAMBA INDUCES FORMATION OF AN UNUSUAL TISSUE RETICULUM FROM MATURE SOYBEAN COTYLEDON SLICES IN VITRO

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SUMMARY

An unusual tissue reticulum is formed from slices of mature soybean (Glycine max cv. Minsoy) cotyledons cultured for 5 weeks on a Murashige and Skoog medium containing the synthetic auxin dicamba. This reticulum, revealed by removing the mantle of friable callus over it, consists of the original venation of the cotyledon--enlarged by proliferation of cells in and around each vein--and new vascular tissue extending centrifugally from the original venation into tissue formed by peripheral growth of the slice. Ground tissue between the veins proliferates as a friable callus, thus facilitating its easy separation from the reticulum. Bulbous structures form at the tips of the extended and hypertrophied veins that comprise the reticulum. Not themselves vascularized, the tips often protrude through the callus mantle and can be mistaken for globular embryos. The reticulum develops best from 1/4-cotyledon slices making contact at their abaxial surfaces with an MS agar medium containing 5 μM dicamba.

Key words: dicamba, Glycine max, tissue culture, reticulum formation

INTRODUCTION

While attempting to regenerate whole plants from slices of mature soybean cotyledons, we found globular structures resembling embryos at the surface of the callus produced after 5 weeks on a culture medium containing dicamba. On further examination we learned that these structures are part of an unusual tissue reticulum. We have explored the ontogeny of the reticulum using standard histological methods, and our findings are described and interpreted here. This is of interest for at least three reasons: (1) it illustrates in unexpected detail the complex pattern of venation in the soybean cotyledon, (2) it shows that auxin-induced growth of the slice is leaf-like in its peripheral expansion and centrifugal vascularization, and (3) it provides information about the histogenesis of the friable callus that is the basis for a well-known cytokinin bioassay (Miller, 1963).

Dicamba (3,6-dichloro-o-anisic acid) is a synthetic-auxin herbicide used to control both annual and perennial broadleaf weeds by foliage application (Boerboom and Wyse, 1988; Buhler and Mercurio, 1988; Herdi, 1986; Lym and Humburg, 1987). It is also used in tissue culture media to induce somatic embryogenesis (Gray and Conger, 1985; Nagasawa and Finer, 1988; Songstad and Conger, 1988; Trigiano and Conger, 1987), but we have found no reports of its use as an inducer of

vascular tissue formation, a role frequently attributed to auxins (Jacobs, 1954; LaMotte and Jacobs, 1963; Roberts, 1988).

MATERIALS AND METHODS

Tissue Culture Procedure

Mature dry seeds of soybean [Glycine max (L.) Merr.], cultivar Minsoy, were imbibed for 12 hours, surface-sterilized with 30% Clorox [a commercial bleach containing 5.25% (w/v) NaOCl] with 2-3 drops of Tween 80 for 12 min, and rinsed in sterile distilled water five times. Seed coats were removed, and cotyledons were excised and sliced transversely into two or four pieces. Explants (i.e., cotyledon slices) were cultured on the surface of a Murashige and Skoog (MS, Murashige and Skoog, 1962) medium containing dicamba (3,6-dichloro-o-anisic acid, Velsicol Chemical Corporation, Chicago, IL) and 7 $g \cdot l^{-1}$ Difco Bacto agar. The medium was adjusted to pH 5.8 after agar was added and autoclaved at 121°C for 15 min. After culture for four or more weeks at 26°C for 16 h/day in 5-10 $\mu mol \cdot m^{-2} \cdot sec^{-1}$ of cool-white fluorescent light, friable callus covering the reticulum was manually removed with a small artist's paintbrush (Richeson series 9000, No. 6).

In one experiment, five other auxins, IAA (indole-3-acetic acid), IBA (indole-3-butyric acid), 2,4-D (2,4-dichlorophenoxyacetic acid), NAA (α-naphthaleneacetic acid), and picloram (4-amino-3,5,6-trichloropicolinic acid), each at 7 concentrations varying from 0.1 to 20 μM, were compared with dicamba for their effects on root, callus, and reticulum

formation from cotyledon slices. In four other experiments, effects of explant size and orientation on the surface of the medium were examined. During weeks 4-6, macroscopic observations of the reticulum were made after the friable callus had been removed; fixation in FAA solution (4% formalin, 47.5% ethanol, and 5% glacial acetic acid in water) for one week increased contrast between the reticulum and intervening tissue.

Histochemical Methods

Anatomical observations were made on serial sections of cultured cotyledon explants fixed in FAA solution after 0 to 28 days in culture. After storage in fixative, samples were dehydrated in an ethanol series (50-100% ethanol), then transferred to xylene for imbedding with paraffin. All aqueous ethanol solutions were saturated with xylene before use in the series. Cast into paraffin blocks, the samples were sectioned at 10 μm-thickness using a rotary microtome. Ribbons of sections were mounted on glass slides using Haupt's adhesive (Jensen, 1962), allowed to dry for several days, and stained with 1% safranin in water and 1% fast green in 95% ethanol.

RESULTS AND DISCUSSION

Kind and Concentration of Auxin

The *in vitro* developmental responses of soybean cotyledon slices to six auxins are shown in Table 1. In preliminary experiments (data not shown), no development was observed from such slices in the absence of an auxin in the medium. The chlorinated auxins, 2,4-D, picloram, and dicamba, differed quantitatively from the other auxins in their effects on callus and root formation and qualitatively in their effects on reticulum formation. The chlorinated auxins caused callus formation at lower concentrations and produced much more callus at the high concentrations than did the other auxins.

The chlorinated auxins were, in contrast, much less effective in inducing root formation than the other auxins (Table 1) and completely inhibited it at 5 μM or higher concentration. The other auxins increased root formation more and more as concentration was raised from 0.5 μM all the way up to 10 or 20 μM.

Only the chlorinated auxins induced reticulum formation, and dicamba, which was by far the most effective in doing so, showed its effect at about the same threshold concentration as the other chlorinated auxins but continued to increase in effectiveness at higher levels than did the others. A complex reticulum was formed in response to dicamba (Fig. 11) but much simpler reticula were formed in

Table 1. In vitro developmental responses of cultured cotyledon slices to increasing concentrations of six different auxins[a]

Auxin	Conc. (μM)	Friable Callus	Roots	Reticulum
IAA	0.1	-	-	-
	0.5	-	-	-
	1.0	+	+	-
	2.5	+	++	-
	5.0	+	+++	-
	10.0	+	++++	-
	20.0	+	+++++	-
NAA or IBA	0.1	-	-	-
	0.5	+	+	-
	1.0	+	++	-
	2.5	+	+++	-
	5.0	+	++++	-
	10.0	+	+++++	-
	20.0	+	+++++	-
2,4-D	0.1	+	-	-
	0.5	+	++	-
	1.0	+++	+++	-
	2.5	++++	++	+
	5.0	+++++	-	+
	10.0	+++++	-	-
	20.0	++++	-	-
Picloram	0.1	+	-	-
	0.5	++	++	-
	1.0	+++	+++	-
	2.5	++++	++	-
	5.0	+++++	-	+
	10.0	+++++	-	-
	20.0	++++	-	-

[a]All explants were one-fourth cotyledon slices in contact with the medium at their abaxial surfaces. The number of plus signs represents relative size of callus, number of roots, and size and complexity of reticulum.

Table 1. (Continued)

Auxin	Conc. (μM)	Friable Callus	Roots	Reticulum
Dicamba	0.1	+	-	-
	0.5	+++	++	-
	1.0	+++	++++	-
	2.5	++++	++	++
	5.0	+++++	-	+++++
	10.0	+++++	-	++++
	20.0	++++	-	++

response to 2,4-D and picloram (Table 1). Even in the latter cases, protruding tips of the reticulum resembled globular embryos. Because of its greater effectiveness in reticulum formation, dicamba was used in all subsequent experiments.

Effects of Size and Orientation of Explants on Reticulum Formation

Whole cotyledons and transverse slices consisting of 1/2 or 1/4 of a cotyledon were tested by culturing them for 5 weeks on MS medium containing 5 μM dicamba. Orientation of these cotyledons, or slices of them, strongly affected the development of the dicamba-induced reticulum. Most extensive reticulum development was observed (Fig. 11) in cultures of 1/4 cotyledon slices which had been placed with their original abaxial surface in contact with the medium. About 80% of the slices cultured in this way produced a

reticulum, which could be revealed by physically removing the friable callus mantle. Larger explants consisting of whole or half cotyledons produced poor reticula when oriented in the same way on the culture medium. When the slices were cultured with their adaxial or cut surfaces in contact with the medium, even fewer formed a reticulum, and none of those was well-developed.

Histological Studies of Reticulum Development

Figure 1 shows a section through a cotyledon slice at the time of placing it on the medium. Each explant shows two cut and two uncut surfaces in the figure. Cell divisions and cell growth occur beneath both cut and uncut surfaces of the explant as early as 3 days after beginning culture (Figs. 2 and 3), and the explants begin to grow as a consequence. Explants increase in size up to 2 times over the first week, and this appears to determine the size of the reticulum. During the next four weeks, growth is mostly from proliferation of friable callus, thus achieving a final callus size 4 to 5 times that of the original explant. Between 1 to 6 days, cell divisions produce a pattern in or near the veins which differs from the pattern between veins. Cell divisions in and near the veins result in numerous small cells, which appear to be tightly joined to each other (Fig. 4). Cells between the veinal regions are larger and appear to separate more easily. We interpret this

demarcation of veinal from interveinal regions as the earliest visible stage of reticulum formation.

As a consequence of their distinctive cell wall characteristics, cells in the interveinal region begin to undergo an "erosion". We can not distinguish the separation caused by tissue handling and processing from that resulting from stresses induced by growth. The actual cell wall breakage seen in the sections (Figs. 4-8) probably results from the handling and processing. In any case, it is clear by 6 days after commencing culture that the interveinal or interfascicular region has become distinct from the veinal or fascicular region.

The early visual evidence (Figs. 4, 5) of friability of cells between the veins suggests strongly that this region represents the site of the histogenic origin of the friable callus so conspicuous at a later stage (Fig. 10). Friable callus begins to form from the cut surfaces first, then spreads to uncut surfaces so as to cover the explant like a mantle after 5 weeks of culture. Formation of the friable callus and dissolution of the explant occur simultaneously. When the explant has been cultured for 4 weeks or more, all cells between the rodlike structures--consisting of the veins and those small cells surrounding them--have become friable, and only the hypertrophied regions containing the veins remain hard and immune to forcible separation by a brush. The tearing and cell wall breakage seen in the

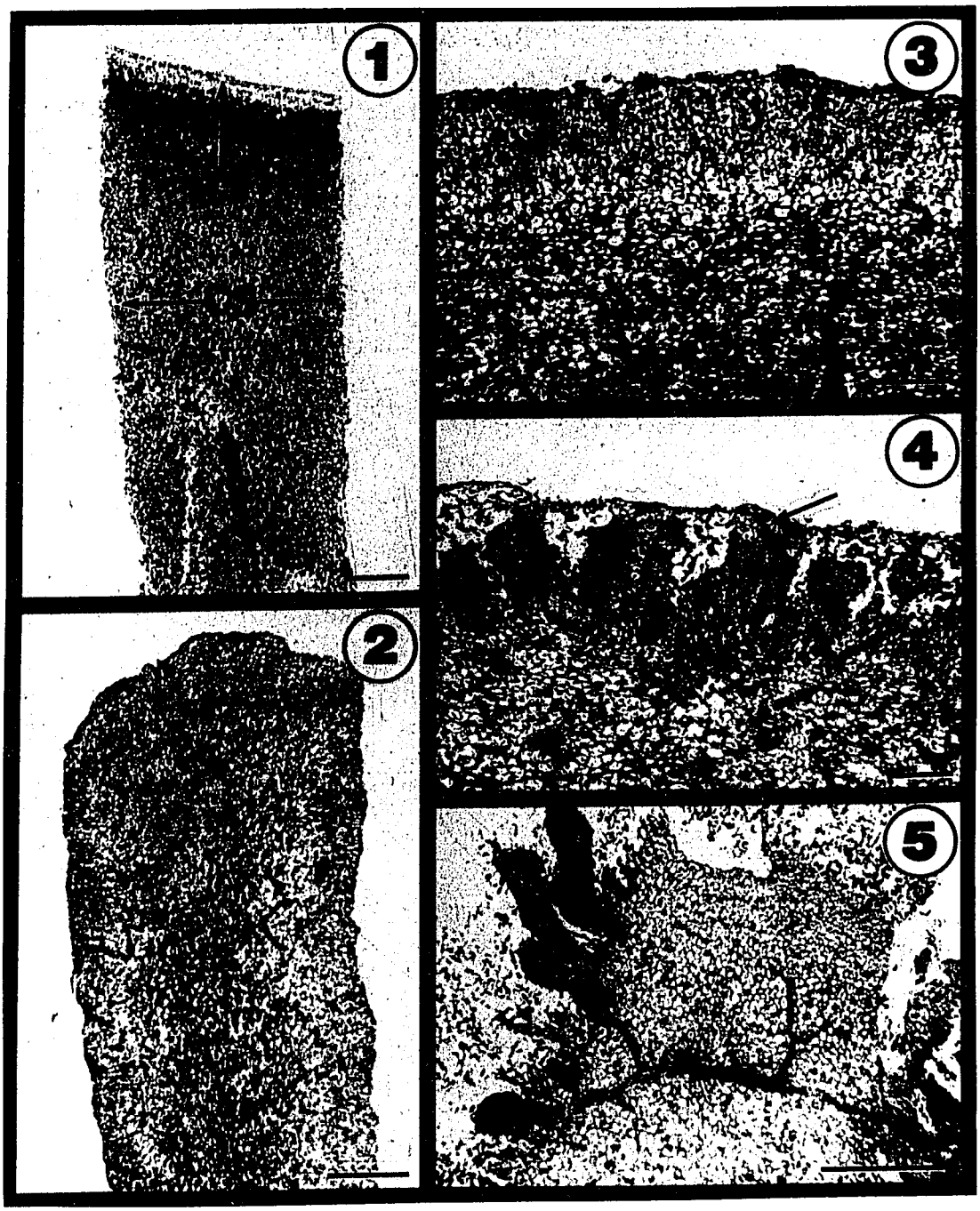
Fig. 1. A soybean cotyledon slice at the beginning of culture. It consists of about one-fourth of a cotyledon and is shown in a a section cut parallel to the long axis of the cotyledon. One uncut surface (US, the adaxial surface of original cotyledon) and two cut surfaces (CS) are shown. Bar = 0.5 mm

Fig. 2. Section from an explant after 3 days in culture. Black, mostly elongated objects are veins. Note the evidence of cell division activity at the periphery of the explant. Bar = 0.5 mm

Fig. 3. A section from the same explant in Fig. 2 is enlarged to show its cut surface and the small cells resulting from division. Bar = 0.1 mm

Fig. 4. Portion of an explant after 6 days in culture showing its cut surface where two distinctive regions have been demarcated beneath it. Note also the vein extending all the way to the cut surface of the explant (arrow), the cell separation, and the cell wall tearing (the latter probably an artifact of preparation). Bar = 0.1 mm

Fig. 5. Same as Fig. 4 except after 10 days in culture. Note the relation between the vein extending across the section and the dark-staining rodlike objects at the periphery of the explant. Bar = 0.5 mm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interveinal regions (Figs. 4, 5), and the lack thereof in the veinal regions, further indicate differences in the physical characteristics of the walls in those different regions.

Vascular differentiation in cells proliferated near the cut-vein endings serves to extend the veins centrifugally into the sheath formed early by growth at the periphery of the explant. Such vein extensions are observed all the way to the outer surface of this sheath (Figs. 2-4) and in their Hfinal form are seen as thin filaments extending from the thick ones in the interior of the reticulum. Cell proliferation and the divergence of changes serving to demarcate the interfascicular regions from the fascicular regions occur first near the periphery of the explant, centered around vein endings at the cut surfaces, and then progress to deeper levels in the explant. We interpret this progression in the following way. Separation of cells in the friable tissue between the veins exposes inner tissues of the explant to the medium (Fig. 5). This causes further changes like those occurring earlier at the periphery, and these changes result in further inward progress of the vein-centered hypertropy resulting in rod formation and further separation and disruption of cells between those rods (Figs. 5, 6).

Further proliferation of cells at outer ends of the vein extensions, and enlargement of cells within them,

result in the bulbous tips seen at the edge of the reticulum after about 4 weeks in culture (Figs. 7, 10, 11). The rods (Figs. 5-8) continue to develop centripetally over the next few weeks, and their bulbous ends may then protrude (Fig. 10) from the mantle of friable callus forming around them and become light green. The cylindrical filaments or rods of the reticulum are branched (Fig. 5) and may occur in closed loops (Fig. 11). After 6 weeks in culture, the well-developed reticulum structure (Fig. 11) is easily seen upon removing the overlying friable callus (Fig. 10). According to our interpretation, the pattern of the reticulum is simply the pattern of the original venation in the cotyledon slice, which in its late stage of development consists almost exclusively of the hypertropied veins after the friable callus between them has been removed. This interpretation was first suggested to us by the closed loops in the reticulum (Fig. 11), so reminiscent of the areoles in the venation of dicot leaves (Hickey, 1973) in general and of soybean cotyledons in particular (Fig. 9).

Reticulum development mimics leaf growth (Postek and Tucker, 1982) in that it involves peripheral expansion of the explant at its edges and centrifugal extension of the veins into the newly formed tissue at those edges. This is not surprising as the cotyledon is itself a leaf.

In conclusion, an unusual reticulum develops from slices cut from mature soybean cotyledons when those are

Fig. 6. Enlargement of a structure like those shown at the left edge of Fig. 5. After 14 days in culture. Bar = 0.2 mm

Fig. 7. A bulbous structure of the kind found at the edge of the reticulum is shown in longitudinal section. After 21 days in culture. Bar = 0.2 mm

Fig. 8. Fascicular rod-like structural units of the reticulum in transverse section. After 21 days in culture. Bar = 0.5 mm

Fig. 9. Portion of a soybean cotyledon showing veins, an areole consisting of a closed vein loop (A) and a vein ending (B). The section was cut parallel to the long axis of the cotyledon. Bar = 0.1 mm

Fig. 10. Callus developed from a mature cotyledon slice after 6 weeks on a medium containing 5 μM dicamba. Arrow indicates a bulbous tip that has protruded through the friable callus mantle. Bar = 5 mm

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7
8
9
A
V
10

Fig. 11. A tissue reticulum that had developed from a cotyledon explant after six weeks in culture is shown after removal of the friable callus with a brush. Arrows at A indicate areoles and arrows at V indicate bulbous tips developed from vein endings within the areoles. Note too, the bulbous tips at the bottom edge of the reticulum. Bar = 2 mm


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cultured on a MS medium containing 5 to 10 μM dicamba by placing their abaxial surfaces in contact with the medium. Coincident in its early stages of development with growth in vitro of the cotyledon explant, the reticulum is produced by centrifugal extension of the vein endings into the newly formed peripheral tissues by fascicular cell divisions and cell growth in and near the pre-existing and newly formed vein endings. This series of processes is paralleled in time by the centripetal development of an interfascicular friable callus by hypertrophy of ground tissue cells between the original veins of the cotyledon. It is the friable nature of this callus that permits its physical removal so as to reveal the underlying reticulum that has developed in juxtaposition.

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SECTION II. PLANT REGENERATION *IN VITRO* FROM PRIMARY LEAF NODES OF SOYBEAN (*GLYCINE MAX*) SEEDLINGS

PLANT REGENERATION *IN VITRO* FROM PRIMARY LEAF NODES OF SOYBEAN (*GLYCINE MAX*) SEEDLINGS

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SUMMARY

A simple and reproducible protocol for regenerating soybean plants from explants derived from 7-day-old seedlings has been developed. Explants, each consisting of the primary leaf node (the unifoliate leaf node), the cotyledonary node, the internode between them, and one cotyledon, were cultured in vitro on a modified Murashige and Skoog (MS) agar medium containing 3% sucrose, four times the MS inorganic micronutrient level, the vitamins of B5 medium, 2 $g \cdot l^{-1}$ L-proline, 2 $mg \cdot l^{-1}$ N^6-benzyladenine (BA), and 0.02 $mg \cdot l^{-1}$ α-naphthalene acetic acid (NAA) to induce the formation of adventitious shoots at the primary leaf node. In inducing shoot formation, this medium was superior to MS and B5 media even when all were supplemented with the same BA, NAA and proline levels. Explants from seedlings of the cultivar Peking produced an average of ca. 20 shoots per explant after 4-5 weeks on this medium. Shoot formation was greater when a cotyledon remained attached to the explant. Shoot formation was also influenced by medium composition, explant orientation, age of donor seedling, and cultivar. Exogenous cytokinin (BA) was essential for shoot formation; proline and the fourfold-raised level of MS inorganic micronutrients caused a large increase in shoot number.

Key words: Glycine max, micronutrients, plant regeneration, primary leaf node culture, proline, soybean

Abbreviations: BA, N^6-benzyladenine; conc., concentration; NAA, α-naphthaleneacetic acid; MMS, modified Murashige and Skoog medium; SE, standard error

INTRODUCTION

Several parts from seeds and seedlings have proved to be effective explants for regenerating soybean (Glycine max) plants in vitro. Parts that have been successfully used are cotyledons from immature seeds (Barwale et al., 1986; Lazzeri et al., 1985; Lippmann and Lippmann, 1984; Ranch et al., 1985), cotyledons from mature seeds (Mante et al., 1989), and several seedling parts, including epicotyl segments (Wright et al., 1987b), cotyledonary nodes (Cheng et al., 1980; Wright et al., 1986), other nodes (Saka et al., 1980), young primary leaves (Wright et al., 1987a), and cotyledons (Hinchee et al., 1988). Kimball and Bingham (1973) reported that they had regenerated shoots from seedling hypocotyl segments, but their evidence suggests that these shoots developed from axillary bud primordia on residual cotyledonary-node tissue present on some segments.

Cheng et al. (1980) and Wright et al. (1986) have increased the effectiveness of seedlings as sources of shoot-regenerating explants by pretreating with the cytokinin BA during germination and seedling development. The protocol of Wright et al. (1986) using cotyledonary node explants gives the highest number of shoots per seedling node explant so far reported, and countable shoots are formed by five weeks after starting seed imbibition.

We report here that we have obtained efficient regeneration of fertile plants from explants of primary leaf nodes prepared from 7-day-old seedlings. Explants from such young seedlings require an attached cotyledon for effective regeneration, but no BA pretreatment is necessary. At least as many shoots are regenerated and as quickly by these primary leaf node explants as by the cotyledonary node explants from seedlings pretreated with BA (Wright et al., 1986). Many of the shoots at the primary leaf node continue to develop. Several additional factors affecting the formation of shoots from primary leaf node explants are described, in particular, proline and inorganic micronutrients have substantial effects on the efficiency of shoot formation.

MATERIALS AND METHODS

Sterilization and Selection of Seeds

Dry seeds of soybean [Glycine max (L.) Merr.] are soaked in 75% (v/v) ethanol for 1 min, sterilized in 30% Clorox [commercial bleach containing 5.25% (w/v) sodium hypochlorite] with 2-3 drops of Tween 80 for 12 min, rinsed in sterile distilled water five times, and cultured aseptically on water agar (0.7% Difco Bacto agar) at 26°C under 16 h/day of 45 μmol m^{-2} s^{-1} of cool-white fluorescent light.

Seeds are selected both before and after sterilization to obtain smooth, crack-free seeds that do not wrinkle during sterilization. The cultivar Peking was used except in one experiment comparing cultivars.

Preparation of Explants

In the routine protocol, explants are excised from 7-day-old seedlings. Two transverse cuts are made, one at the top of the primary leaf node and the other at the base of the cotyledonary node. A third cut removes one of the two cotyledons. Each explant consists of the primary leaf node, the cotyledonary node, the internode between them, and one cotyledon (Fig. 1 Inset). The terminal bud and primary (unifoliate) leaves, severed by the first cut, are discarded.

In establishing this procedure, explants with no or two attached cotyledons, or with a portion of a single cotyledon, were also tried.

Shoot-induction Medium for Explant Culture

The shoot-induction medium consists of a modified Murashige and Skoog (MS; 1962) basal medium, designated as MMS, to which is added 2 $mg \cdot l^{-1}$ BA and 0.02 $mg \cdot l^{-1}$ NAA. The MMS medium contains MS macronutrient salts (including Fe), four times the MS micronutrient salts, B5 vitamins (Gamborg et al., 1968), 30 $g \cdot l^{-1}$ sucrose, 2 $g \cdot l^{-1}$ L-proline, and 7 $g \cdot l^{-1}$ Difco Bacto agar; this medium differs from the MMS medium of Saka et al. (1980). All ingredients of all test media were added before autoclaving. Each medium was then adjusted to pH 5.8 and autoclaved at 121°C for 15 min.

Because Difco Bacto agar has become unavailable since the experiments reported here were performed, we have tried several substitutes, each in a concentration giving similar gel hardness. Agar (0.6%, Sigma) and Phytoagar (0.6%, Gibco) are satisfactory, although Bacto agar appears preferable to either. When the agar substitute Phytagel (0.2%, Sigma) was used, almost all the regenerated shoots became vitrified (*i.e.*, translucent and hyperhydrated, according to Pierik, 1987).

In the routine procedure, sets of 4 explants are inoculated onto the surface of 30 ml of medium in 15 mm x 100

mm plastic petri dishes and cultured for 5 weeks under 16 h/day of cool-white fluorescent light (40-50 μmol m^{-2} s^{-1}) at 26°C. After 10 days of culture, axillary buds at the cotyledonary node have grown out and are excised. Improperly cut explants, discernible at this time, are discarded.

Each treatment reported here involved at least 16 explants. Counts of shoots were made 30 days after placing explants on the test media.

Shoot and Plant Development

Primary leaf nodes with regenerated shoots are cut off at their bases and transferred to Magenta boxes, type GA7 (7.6 cm X 7.6 cm X 10.2 cm; Magenta Corp., Chicago, IL, USA). Each box receives four nodes, placed on 30 ml of B5 medium (Gamborg et al., 1968) with 2% sucrose, 0.7% agar, but no growth regulators (the shoot-development medium). Cultures are maintained on this medium for about 2 weeks under conditions identical to those for shoot induction except for lighting, which consists of about 60 μmol m^{-2} s^{-1} of cool-white fluorescent light supplemented by daylight from a north-facing window. When shoots have produced 2 to 3 leaves and are at least 3 cm in length, they are separated, and 5 to 6 such shoots are transferred to a Magenta box containing 30 ml of half-strength B5 medium with 2% sucrose, 0.7% agar, and no growth regulators (the plant-development medium). Other conditions remain the same. After about 2 weeks, plants

having shoots at least 10 cm long and a well-developed root system are selected, washed to remove agar from their roots, and transferred to steam-treated potting soil (40% peat moss, 40% perlite, and 20% loam soil by volume) in 16 cm X 20 cm X 28 cm plastic containers with transparent lids, and grown with the same lighting as before. After a few days, lids are removed for a gradually increasing number of hours per day. After about a week of such hardening, plants are transplanted to clay pots in a mist chamber in a ggreenhouse and finally moved to a dry bench. Exact timing for the various stages in the hardening process depends on the condition of each plant.

RESULTS AND DISCUSSION

Selection of a Shoot-induction Medium

Preliminary experiments showed that MMS medium was an effective basal medium for the regeneration of shoots from primary leaf node explants. To define as precisely as possible the best conditions for shoot formation, a series of experiments was conducted in which three basal media were tested, and concentrations of inorganic micronutrients, proline, and plant growth regulators were varied.

On the basis of studies cited in the Introduction, BA was chosen as cytokinin, and its concentration was varied in the MMS medium supplemented with 0.02 $mg \cdot l^{-1}$ NAA. BA at 2 $mg \cdot l^{-1}$ induced the most shoot formation, and BA at 3 or 4 $mg \cdot l^{-1}$ was more effective than BA at 0.5 or 1 $mg \cdot l^{-1}$ (Table 1). Elongation of explant stems was least at the highest BA concentration and increased as the BA concentration was decreased. After 2 weeks of culture, stems on the lowest BA concentration (0.5 $mg \cdot l^{-1}$) became long (5 to 8 cm) and thin whereas those on the highest BA concentration (4 $mg \cdot l^{-1}$) became short (3 to 4 cm) and thick.

NAA was tested at concentrations of 0, 0.02, 0.04, 0.08, and 0.16 $mg \cdot l^{-1}$ in the MMS medium supplemented with 2 $mg \cdot l^{-1}$ BA. NAA had no effect on number of shoots formed except for a 55% inhibition at 0.16 $mg \cdot l^{-1}$. Although NAA did not increase the number of regenerated shoots, shoots formed on medium

Table 1. Effect of BA concentration on shoot regeneration from the primary leaf node[1]

BA conc. ($mg \cdot l^{-1}$)	Shoots per explant[2]
0.5	4.8 a
1.0	8.8 ab
2.0	18.9 c
3.0	15.1 c
4.0	13.7 bc

[1]MMS medium plus 0.02 $mg \cdot l^{-1}$ NAA.

[2]Means followed by the same letter are not significantly different at the 5% level in the LSD test.

containing 0.02 $mg \cdot l^{-1}$ NAA became more robust than those on medium without it. For this reason, 0.02 $mg \cdot l^{-1}$ NAA was included in the shoot-induction medium.

After NAA and BA concentrations had been selected, the choice of basal medium was re-examined. Three media, MS, B5, and MMS, were tested; all three contained 2 $mg \cdot l^{-1}$ BA and 0.02 $mg \cdot l^{-1}$ NAA. In agreement with our preliminary results, the MMS medium was most effective in inducing shoot formation, followed in order by B5 and MS media (Table 2). The effectiveness of the MMS medium must in large part be due to its higher level of inorganic micronutrients and the addition of proline. In a separate experiment, increasing the level of micronutrients fourfold above the MS level (as in the MMS medium) increased shoot formation four- to fivefold whereas increasing the level eightfold had a slightly smaller effect (Table 3). Proline also had a striking effect on shoot formation, increasing shoot number five- to sixfold when 1-3 $g \cdot l^{-1}$ was supplied in the MMS medium supplemented with 0.02 $mg \cdot l^{-1}$ NAA and 2 $mg \cdot l^{-1}$ BA (Table 4). In contrast, Barwale et al. (1986) reported that proline did not enhance <u>in vitro</u> regeneration of plants from immature soybean cotyledons via somatic embryogenesis. The same workers added proline to their medium for shoot formation via organogenesis from immature cotyledons, but have no evidence for its effectiveness (J.M. Widholm, personal communication, 1989, University of Illinois, Urbana, Ill). In the absence of

Table 2. Effect of basal medium[1] on shoot regeneration from the primary leaf node

Basal medium	Shoots per explant[2]
MS	6.7 a
B5	10.0 b
MMS	18.9 c

[1]All media contained 0.02 $mg \cdot l^{-1}$ NAA, 2 $mg \cdot l^{-1}$ BA, and 7 $g \cdot l^{-1}$ agar.

[2]Means followed by the same letter are not significantly different at the 5% level in the LSD test.

Table 3. Effect of the level of MS inorganic micronutrients on shoot regeneration from the primary leaf node[1]

Relative level	Shoots per explant[2]
1X	3.8 a
2X	6.6 a
4X	18.8 c
8X	13.7 b

[1]MMS medium (except for MS inorganic micronutrients) with 0.02 $mg \cdot l^{-1}$ NAA and 2 $mg \cdot l^{-1}$ BA.

[2]Means followed by the same letter are not significantly different at the 5% level in the LSD test.

Table 4. Effects of L-proline on shoot regeneration from the primary leaf node[1]

Proline conc. ($g \cdot l^{-1}$)	Shoots per explant[2]
0	3.2 a
1	15.8 b
2	19.7 c
3	18.3 bc

[1]MMS medium (except for proline) with 0.02 $mg \cdot l^{-1}$ NAA and 2 $mg \cdot l^{-1}$ BA.

[2]Means followed by the same letter are not significantly different at the 5% level in the LSD test.

proline, friable callus was produced at the primary leaf node of our explants. This result is consistent with the report of Saka et al. (1980) that callus grew on explants under conditions that yielded few shoots.

Effects of Cotyledons and Age of Seedlings

The number of attached cotyledons and removal of a portion of a single attached cotyledon both strongly affected shoot formation by explants (Table 5). More adventitious shoots were produced by explants with one or one-half cotyledon than by those with one-fourth cotyledon or two cotyledons. Few shoots were regenerated on explants without a cotyledon. Instead, friable callus was produced, as in the absence of proline.

Orientation of explants on the shoot-induction medium was an important factor in shoot formation. The highest number of shoots was produced when explants were oriented so that the adaxial side of the cotyledon was in contact with the medium. This orientation placed the rest of the explant in good contact as well. In contrast, no shoots were produced when the abaxial side of the cotyledon was placed in contact with the medium. Contact was inevitably poor when explants had two cotyledons; this may explain their low shoot production. Direct contact of the explant stem with the medium may be needed for active cytokinin to reach the bud-forming sites at the node, since exogenous cytokinins seem generally to be

Table 5. Effect of attached cotyledons on shoot regeneration from the primary leaf node[1]

Cotyledons/explant	Shoots per explant[2]
0	1.3 a
0.25	6.9 b
0.5	18.6 c
1	18.4 c
2	7.3 b

[1]MMS medium with 0.02 $mg \cdot l^{-1}$ NAA and 2 $mg \cdot l^{-1}$ BA.

[2]Means followed by the same letter are not significantly different at the 5% level in the LSD test.

immobile unless they or their metabolites (Fox et al., 1972) are taken up by vascular tissues and translocated to an already active growing point (Van Staden et al., 1988).

Explants from 9-day-old seedlings produced fewer shoots (an average of 13 per explant) than explants from 7-day-old seedlings (19 per explant). Explants from seedlings less than 6 days old could not be easily and reproducibly prepared because of the small size of the epicotyls.

Explant Development on Shoot-induction Medium

Fig. 1 shows a seedling shoot from which an explant (Fig. 1 Inset) was prepared as described in the Materials and Methods. After one week of culture, the primary leaf node and any internode tissue remaining attached at the distal end of the explant had enlarged to form a dome- or fan-shaped structure (Fig. 2). After 2 weeks on the shoot-induction medium, adventitious shoots became evident on the dome-shaped structure. Sometimes, shoots also developed from the axillary buds; if these were larger (Fig. 3: arrow) than the adventitious shoots, they were removed (Fig. 3 Inset). Adventitious shoots sometimes formed at the cotyledonary node of the explants, but these shoots were less numerous than reported by Cheng et al. (1980) and Wright et al. (1986), who removed the primary leaf node from their explants.

Wright et al. (1986) reported that adventitious shoots that formed on cotyledonary-node explants originated from

Fig. 1. A seedling shoot cultured for 6 days on water agar. One cotyledon has been excised. x 2.

Fig. 1 Inset. An explant ready for culturing. The side shown is placed down on the agar surface. Arrow indicates cut made just above the primary leaf node. x 2.

Fig. 2. Explant after 7 days of culture on the shoot induction medium. Arrow indicates enlarged primary leaf node. Note the small shoots formed at the node. x 2.

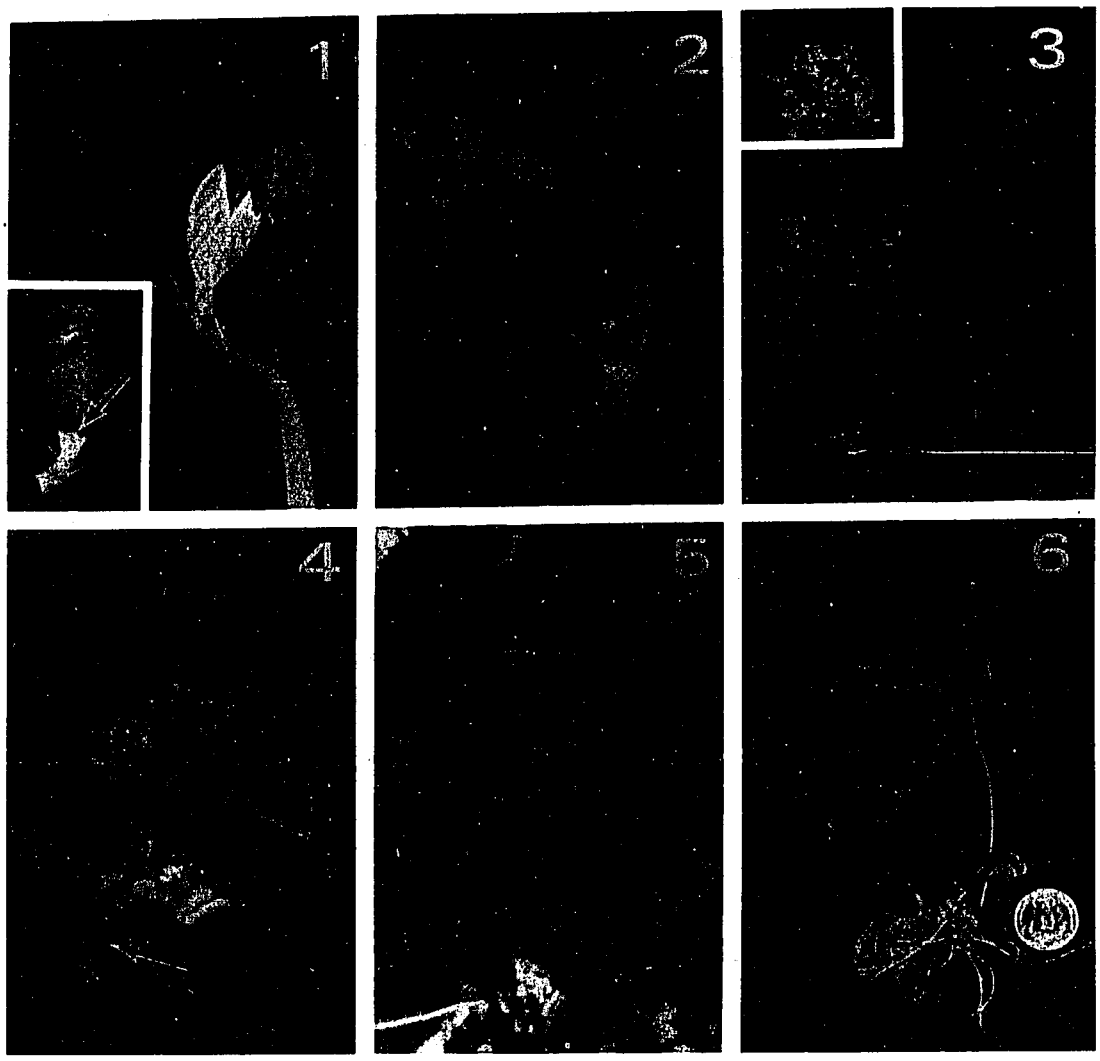
Fig. 3. Explant after 14 days of culture on shoot-induction medium showing axillary (arrow) and adventitious shoots formed at the primary leaf node. x 2.

Fig. 3 Inset. Same as Fig. 3 except viewed obliquely from above after axillary buds had been removed. Note the large number of small shoots. x 3.

Fig. 4. Explant after 28 days of culture on the shoot-induction medium. Arrow indicates a half-cotyledon at the basal end of the explant. x 1.5.

Fig. 5. Primary leaf node region of the explant shown in Fig. 4. Note the numerous leafy shoots, all of similar size. x 5.

Fig. 6. A Ransom soybean plant after 9 weeks of culture <u>in vitro</u> and just before transfer to soil. Arrow indicates one of three inflorescences formed on it. x 0.5.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clusters of small, actively dividing cells in and just under the epidermis. These clusters formed on the petiole side and stem side of the cotyledonary axil and on the two sides of the cotyledonary node not bearing cotyledons. Our observations of the positions of newly formed shoots on primary leaf nodes are consistent with their having similar sites of origin, but confirmation by histological examination is needed.

Comparison of Protocols for Shoot Regeneration from Seedling Nodes

There have been several reports of shoot regeneration from seedling nodes. The number of shoots per explant is greater and the total time for seed imbibition, seedling development, and node culture shorter with the protocol described here than with those reported previously, with the exception of a protocol developed by Wright et al. (1986). For cotyledonary node explants, Cheng et al. (1980) and Wright et al. (1986) found that growing seedlings in the presence of BA was essential for shoot regeneration. Cheng et al. (1980) counted an average of eleven shoots per explant twelve weeks after starting seed imbibition; on their two most effective culture media, Wright et al. (1986) counted an average of seventeen shoots per explant five weeks after starting imbibition.

Saka et al. (1980) and Wright et al. (1987b) investigated the regeneration capacity of explants bearing primary leaf

nodes. In both cases the explants were derived from 2-week-old seedlings, and neither group retained cotyledons on their explants. Saka et al. (1980) found that maximal regeneration required a long pretreatment: explants were first cultured on growth-regulator-free medium for one to two weeks to reduce endogenous auxin levels, and only then transferred to BA-containing medium for shoot regeneration. Saka et al. (1980) and Wright et al. (1987b) counted shoots at 7 or 8 weeks after starting seed imbibition; we counted shoots at 5.3 weeks. Exact comparisons of shoot numbers are difficult because Wright et al. (1987b) used segments of epicotyls, some bearing primary leaf nodes and others not, and Saka et al. (1980) did not make clear whether their explants included other nodes besides the primary leaf node. Nevertheless, the number of shoots per initial explant obtained by Saka et al. (1980) and Wright et al. (1987b) was substantially smaller than reported here.

Comparison of Cultivars

The performance of eight cultivars, representing seven maturity groups, was tested in the culture system described here. The percentage of seeds producing healthy seedlings on water agar varied widely among cultivars. Less than 30% of Ransom, Minsoy, Williams 82, and Star seeds produced healthy seedlings. In contrast, more than 80% of Peking, Corsoy, and

Table 6. Varietal responses in shoot formation from the primary leaf node[1]

Cultivar	Maturity group	Shoots per explant[2]
Flambeau	00	13.4 bc
Minsoy	0	11.6 ab
Star	I	18.7 c
Corsoy 79	II	18.2 c
Williams 82	III	7.9 a
Peking	IV	19.7 c
Union	IV	15.4 bc
Ransom	VII	8.0 a

[1]Explants, prepared from 7-day-old seedlings, were cultured on MMS medium supplemented with 0.02 $mg \cdot l^{-1}$ NAA and 2 $mg \cdot l^{-1}$ BA. Shoots were counted 30 days after culture was commenced.

[2]Means followed by the same letter are not significantly different at the 5% level in the LSD test.

Flambeau seeds produced healthy seedlings. All tested cultivars were capable of shoot regeneration, but the average number of regenerated shoots varied from 8 to 20 (Table 6). Neither maturity group (Table 6) nor the ability of seeds to germinate and produce healthy seedlings was related to the capacity of explants to regenerate shoots.

It is possible that optimizing the levels of crucial ingredients of the medium, such as BA, for individual cultivars can increase their responsiveness. C.Y. Hu (William Paterson College of New Jersey; personal communication, 1988) found that different soybean cultivars do exhibit different BA optima for shoot formation from cotyledonary nodes. Our experience with Peking seeds from various sources of supply (data not shown) indicates that seed quality is another important determinant of shoot-forming ability. Thus, the variation observed among cultivars may not be entirely genetic.

Selection of Shoot- and Plant-development Media

After explants had been cultured for about 4 weeks on the shoot-induction medium, the enlarged, dome-shaped nodal structures with regenerated shoots (Figs. 4 and 5) were cut off at the base of the node and transferred to four different media without growth regulators to test for shoot development. The tested media were MS and half-strength MS, both with 3% sucrose, and B5 and half-strength B5, both with 2% sucrose.

The B5 medium resulted in the most shoot growth as determined by visual inspection, and it was adopted as the shoot-development medium. After 2 weeks on this medium, about half the regenerated shoots had grown to a length of 2 to 3 cm and developed 3 to 4 small leaves. Some shoots, however, grew more slowly or failed to grow at all.

The same four media were tested for their effects on root formation and later plant development. The 2- to 3-cm-long shoots described above were excised and transferred to Magenta boxes containing the test media. Roots developed better on the half-strength B5 medium than on the other three media. On this medium, almost all of the shoots formed roots. Therefore, it was adopted as the plant-development medium.

After transfer of such rooted plants to potting soil in a greenhouse (see Materials and Methods), all cultivars listed in Table 6 except Ransom exhibited normal plant development.

Many of plantlets regenerated from Ransom produced flowers <u>in vitro</u> (Fig. 6), and these plants later set 1-2 pods, each containing 1-2 seeds. The mature dry seeds produced by these cultures were similar in size and color to those produced by greenhouse-grown plants. One of these seeds was tested and found to be able to germinate.

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SECTION III. EFFECTS OF PROLINE AND INORGANIC MICRONUTRIENTS ON SHOOT REGENERATION FROM SOYBEAN (*GLYCINE MAX*) EXPLANTS *IN VITRO*

EFFECTS OF PROLINE AND INORGANIC MICRONUTRIENTS ON SHOOT REGENERATION FROM SOYBEAN (*GLYCINE MAX*) EXPLANTS *IN VITRO*

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SUMMARY

We reported earlier that adding 2 $g \cdot l^{-1}$ L-proline and raising fourfold the level of inorganic micronutrients in Murashige and Skoog medium enhances soybean shoot regeneration from primary leaf node explants of 7-day-old seedlings. We have confirmed these findings and extended them to cotyledon explants cut from the same seedlings so as to document a synergistic, threefold stimulation of shoot regeneration from both kinds of explants caused by these two supplements. Proline increased number but decreased length of regenerated shoots, whereas raising micronutrient level increased both shoot number and length and, thus, partly overcame the effect of proline on length. Examining separately the effects of singly omitting, including, raising, and lowering the levels of the seven different micronutrient elements of the original MS medium has provided evidence that zinc is the most limiting element for regeneration from both kinds of explants and that the other six elements, too, are all in less than optimal supply.

Key words: boron, bud formation, cobalt, copper, Glycine max, inorganic micronutrients, iodine, leaf node culture, manganese, metals, molybdenum, proline, shoot regeneration, soybean, zinc

Abbreviations: BA, N^6-benzyladenine; NAA, α-naphthaleneacetic acid; MSR, medium for shoot regeneration

INTRODUCTION

In a previous report (Kim et al., 1990), we showed that the levels of proline and inorganic micronutrients have large effects on shoot regeneration from primary leaf nodes of 7-day-old soybean seedlings. Because we found only one report (Barwale et al., 1986) in which the effects of several levels of inorganic micronutrients on shoot regeneration from soybean tissue cultures had been described, we examined closely their individual effects and, collectively, their interaction with added proline.

The inorganic micronutrients used in plant tissue culture media usually include B, Cl, Co, Cu, I, Mn, Mo, and Zn. Chlorine, present in the MS medium in more than an adequate supply as a counterion for calcium, and iron, required in an intermediate amount by tissue cultures (Murashige and Skoog, 1962; Ohira et al., 1975; Schenk and Hildebrandt, 1972) and thus not categorized as a micronutrient, were maintained at the level in Murashige and Skoog (MS, 1962) medium throughout this study while the other elements were varied in concentration.

Studies of the effects of tissue culture micronutrients (e.g., Murashige and Skoog, 1962) have mostly concerned growth rather than the ability to form differentiated structures, assuming--probably correctly--that what is good nutrition for one is good nutrition for the other. Barwale et al. (1986)

showed that raising the level of inorganic micronutrients caused a decrease in shoot regeneration from immature soybean cotyledon explants. Also, when they tested individual micronutrient elements, none seemed to have clear-cut effects on regeneration.

In this paper, we describe *in vitro* studies of the interaction between proline and inorganic micronutrients in their effects on soybean shoot regeneration. In addition, we describe our attempts to analyze the separate contributions of the individual elements comprising the inorganic micronutrients of MS medium to shoot regeneration by these explants.

MATERIALS AND METHODS

Seedling growth and explant culture procedures are identical to those described earlier (Kim et al., 1990). The agar medium, designated here as MSR, is identical to the basal MMS medium of our earlier paper except that BA and NAA are always at concentrations of 2 and 0.02 $mg \cdot l^{-1}$, respectively, Difco Bitek agar, instead of Difco Bacto agar, is used, and proline and inorganic micronutrients are varied. As before, shoots are counted 30 days after commencing culture.

Concentrations of the whole micronutrient mixture and of each element in it are expressed relative to those concentrations in the Murashige and Skoog (MS; 1962) medium. Concentrations used are the same as in the MS medium (normal level, 1X) and two, four, and eight times higher than in it (2X, 4X, and 8X, respectively).

Two kinds of explants are prepared from 7-day-old seedlings of Peking soybean (Fig. 1A, left). The hypocotyl, root, and parts above the primary leaf node, including primary leaves and shoot tip, are removed from the seedling and discarded. One cotyledon is excised at its base. This explant (Fig. 1A, right) is called the "cotyledon explant" or simply the "cotyledon." The other cotyledon remains attached to its node, where together with the primary leaf node and the stem that connects the two nodes, it comprises the explant

(Fig. 1A, middle) hereafter called the "primary leaf node explant" or simply the "primary leaf node."

After 4 days of culture, axillary buds that have grown out at the cotyledonary node are excised. When measuring regenerated shoot lengths, the shoots larger than 2 mm in length are removed individually from the explant, and their base to shoot-apex distance is determined with a ruler and recorded.

RESULTS

Effects of Proline and Micronutrient Levels on Shoot Regeneration

The numbers of shoots formed from both primary leaf node (Fig. 1B,C) and cotyledon (Fig. 1D,E) explants depend on proline and micronutrient concentrations. Tables 1 and 2 show that from both types of explant the number of regenerated shoots was generally greatest at 2 to 4 $g \cdot l^{-1}$ proline, irrespective of micronutrient level and, except in the absence of proline, was greatest at 4X the MS micronutrient concentration, thus confirming our earlier findings (Kim et al., 1990). The only exception to the positive effect of raising micronutrient levels above 1X was seen with cotyledons in the absence of proline, in which case raising the micronutrient level actually decreased the number of shoots formed.

Proline and micronutrients have a synergistic effect: the increase due to both is greater than the sum of their individual effects (Tables 1, 2). This is supported by analysis of variance, in which their interaction was become to be highly significant ($P = 0.001$). As an example, the number of shoots regenerated from primary leaf nodes was 54% greater with 2 $g \cdot l^{-1}$ proline than without it in the presence of 1X micronutrients, but 146% greater in the presence of 4X micronutrients. Similarly, the number of shoots was 23%

Fig. 1. Seedling shoots, the explants prepared from them, and shoots regenerated from the explants after 30 days of culture. Bars are equal to 1 cm.

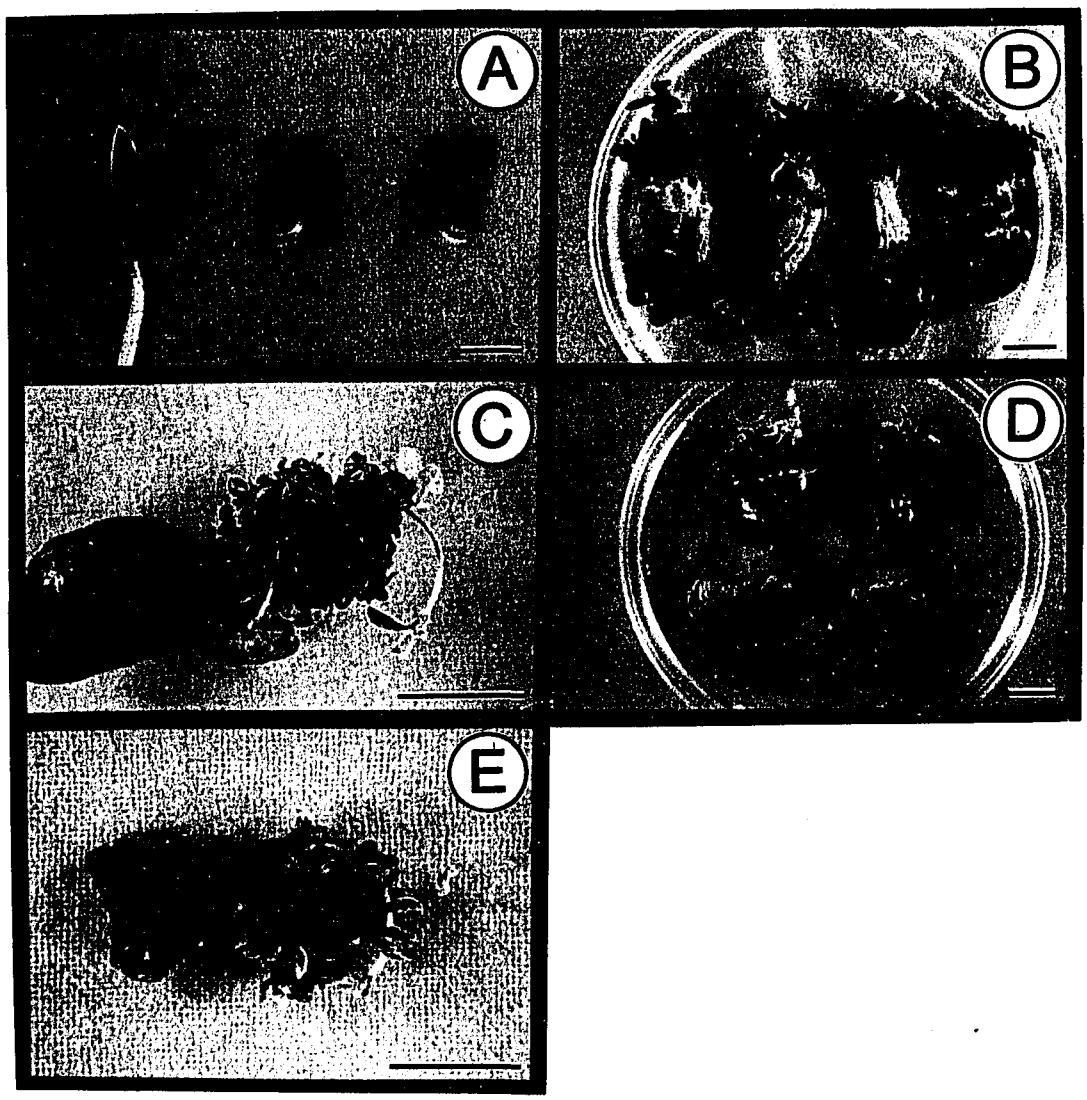
A. A shoot of a seven-day-old seedling (left), a primary leaf node explant (middle), and a cotyledon explant (right).

B. Primary leaf node explants showing regenerated shoots after culture on MSR medium containing 2 $g \cdot l^{-1}$ proline and 4X the level of MS inorganic micronutrients. Note the thickened and elongated stem of each explant and the uniformity of the shoot-forming response.

C. Enlarged view of a cultured explant from Fig. 1B showing numerous shoots formed at the nodal region.

D. Cotyledon explants showing the regenerated shoots formed at their proximal ends after culture on MSR medium containing 2 $g \cdot l^{-1}$ proline and 4X the level of MS inorganic micronutrients. Note the uniformity of their development.

E. Enlarged view of a cultured cotyledon explant from Fig. 1D showing the large number of shoots formed at its proximal end.


A
B
C
D
E

greater with 4X micronutrients than with 1X micronutrients in the absence of proline, but 97% greater in the presence of 2 $g \cdot l^{-1}$ proline. For both types of explant, 2 $g \cdot l^{-1}$ proline and 4X micronutrients gave the highest number of shoots, an average of 34 shoots per primary leaf node and 30 shoots per cotyledon.

Although proline increased the number of shoots formed, it reduced the rate of shoot elongation (Fig. 2), so that the average shoot length 30 days after placing primary leaf nodes on medium containing 4 $g \cdot l^{-1}$ proline was only about one-third the average length on medium containing no added proline. In contrast, increasing the level of micronutrients generally increased shoot lengths. Despite this increase, shoots regenerated in the presence of 8X micronutrients started to die after about 4 weeks, whereas those regenerated on other micronutrient levels survived for at least 6 weeks. This result suggests that micronutrients provided at the highest level become toxic to the regenerated shoots from both explants when allowed to accumulate in them over a period of a few weeks. The same high level of micronutrients also reduced the number of shoots formed by the explants (Tables 1, 2).

Effects of Individual Inorganic Micronutrient Elements

It seemed desirable to determine whether the effect of raising, collectively, the inorganic micronutrients on regeneration was due to a specific element, or group of

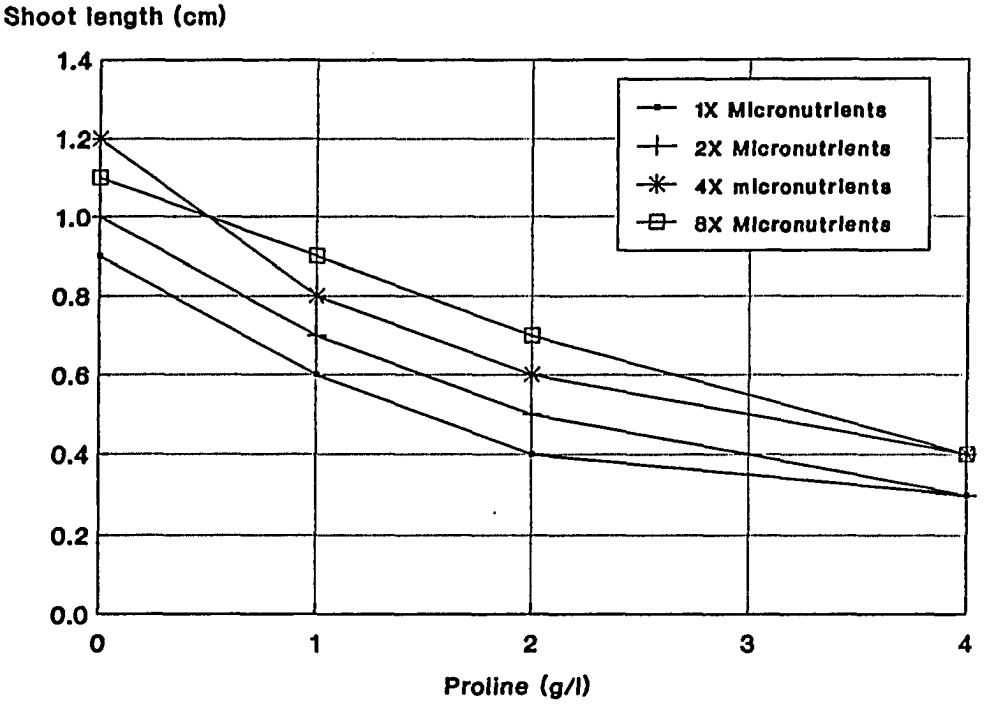


Fig. 2. Effects of proline and inorganic micronutrients on length of shoots regenerated from primary leaf node explants cultured for 30 days on MSR medium

Table 1. Analysis of effects of proline and micronutrients on the number of shoots regenerated from primary leaf node explants[1]

(Average number of shoots per explant)[2]

Proline (g/l)	Micronutrients (X)			
	1	2	4	8
0	11.4 a	11.6 a	14.0 b	15.3 bc
1	15.5 bc	21.7 de	24.4 e	24.3 e
2	17.5 c	24.0 e	34.5 h	27.6 f
4	20.3 d	24.3 e	30.9 gh	27.5 f

[1]Explants were cultured on MSR medium. Shoots were counted one month after culture was commenced.

[2]Numbers followed by the same letter are not significantly different according to the LSD test (P = 0.05).

Table 2. Analysis of effects of proline and micronutrients on the number of shoots regenerated from excised cotyledon explants[1]

(Average number of shoots per explant)[2]

Proline (g/l)	Micronutrients (X)			
	1	2	4	8
0	10.6 bc	11.8 bc	9.0 ab	6.7 a
1	14.7 cde	17.9 ef	25.2 g	12.1 bcd
2	12.2 bcd	19.2 f	29.5 h	18.9 ef
4	15.9 def	17.3 ef	26.0 g	17.7 ef

[1]Explants were cultured on MSR medium. Shoots were counted one month after culture was commenced.

[2]Numbers followed by the same letter are not significantly different according to the LSD test (P = 0.05).

elements, and whether individual elements might have adverse effects at high concentration. Based on our earlier report (Kim et al., 1990), a concentration of micronutrients four times higher than in the original MS medium was used to examine the effect of individual micronutrients on shoot regeneration from both types of explant. When each element was tested at the 4X level while the other elements were held at the MS level (1X), all elements enhanced shoot regeneration from primary leaf nodes, with Zn, B, I, Mo, and Cu having the greatest effects (Fig. 3A) and Co and Mn having the least. Mn, Zn, and Cu, but not B, I, Mo, or Co significantly enhanced shoot regeneration from cotyledons (Fig. 3B).

When each micronutrient element was individually lowered to the MS level (1X) while the others were held at the 4X level, only Zn, I, and Co significantly changed numbers of shoots regenerated from the primary leaf nodes: reduction of Zn or I concentration decreased and reduction of Co increased shoot regeneration (Fig. 4A). With excised cotyledons, reduction of Zn concentration decreased shoot number while reduction of the other elements had no significant effect (Fig. 4B). It should be noted that, though it failed to have a statistically significant effect, decreasing the level of Co to 1X resulted in the largest average level of shoot formation (35.0 shoots per explant) by excised cotyledons (Fig. 4B) observed in these experiments. We believe, therefore,

Fig. 3. Effects on shoot regeneration from primary leaf node (A) and cotyledon (B) explants of singly raising the concentration of each micronutrient element to 4X the normal level while maintaining the MS concentrations of the other elements The same letter on the top of any two or more bars indicates that they are not significantly different in the LSD test (P = 0.05)

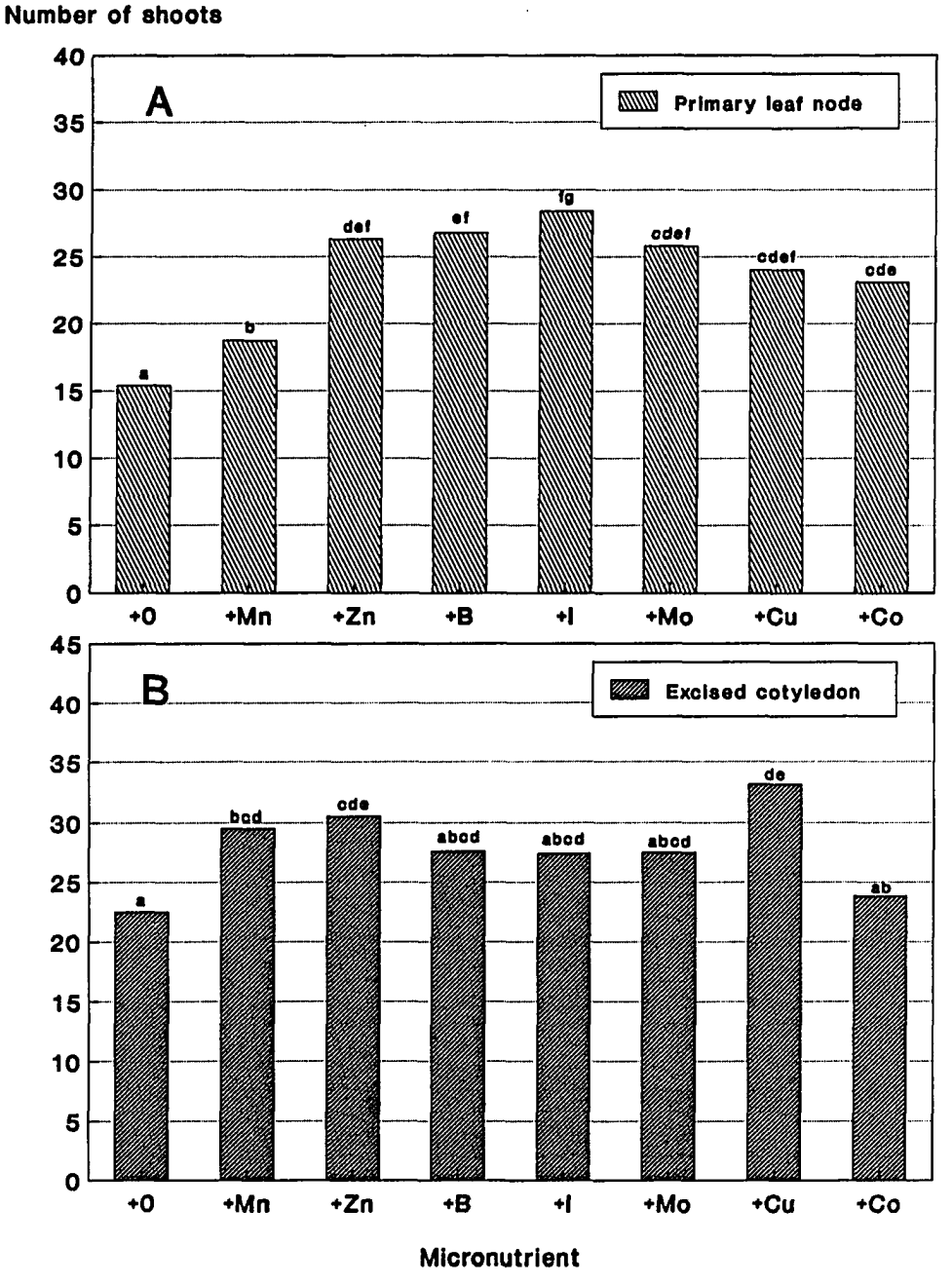
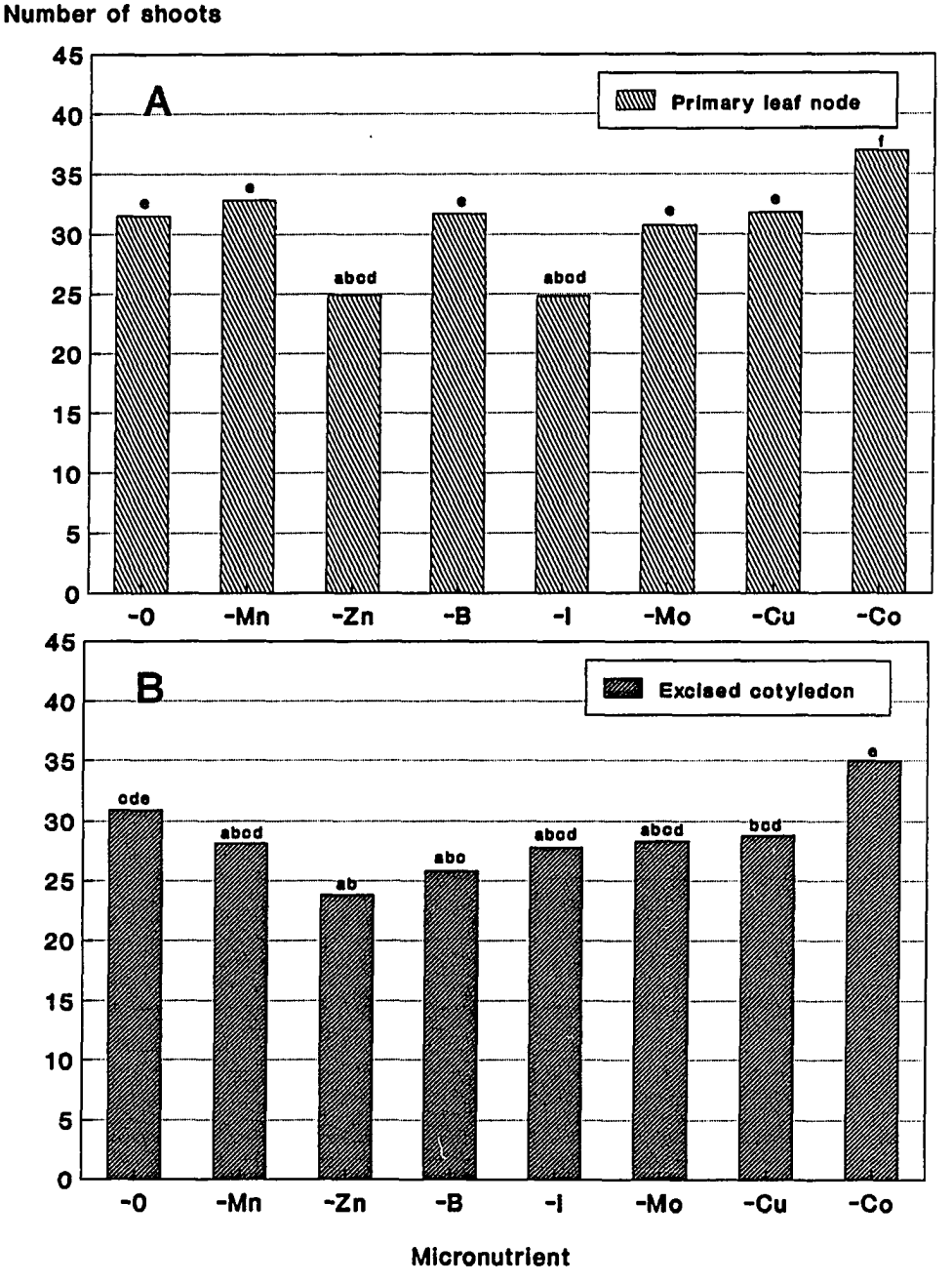

Number of shoots
A
Primary leaf node
40
35
30
25
20
15
10
5
0
a
b
def
ef
fg
cdef
cdef
cde
+0
+Mn
+Zn
+B
+I
+Mo
+Cu
+Co
B
Excised cotyledon
45
40
35
30
25
20
15
10
5
0
a
bcd
cde
abcd
abcd
abcd
de
ab
+0
+Mn
+Zn
+B
+I
+Mo
+Cu
+Co
Micronutrient

Fig. 4. Effects on shoot regeneration from primary leaf node (A) and cotyledon (B) explants resulting from a decrease in the concentration of each micronutrient element to the MS level (1X) while maintaining the other elements at the higher level (4X) The same letter on the top of any two or more bars indicates that they are not significantly different in the LSD test (P = 0.05)


Number of shoots
A
Primary leaf node
45
40
35
30
25
20
15
10
5
0
e
e
abcd
e
abcd
e
e
f
-0
-Mn
-Zn
-B
-I
-Mo
-Cu
-Co
B
Excised cotyledon
cde
abcd
ab
abc
abcd
abcd
bcd
a
Micronutrient

that a 4X level of Co is supraoptimal whereas a 1X level may be suboptimal.

We did two experiments to examine the effects of single-element supply and single-element deprivation on both kinds of explants. When a single element was supplied at 4X concentration and the other six elements were completely omitted, shoot regeneration from the primary leaf node explants was stimulated significantly by each element except Co, and dramatically by Zn and B (Fig. 5A). When supplied singly to cotyledons, Zn, B, Cu, and Co had a stimulatory effect, which was most pronounced with Zn (Fig. 5B).

When each element was individually omitted while the other elements were held at 4X level, Zn or B omission significantly lowered shoot regeneration from primary leaf nodes (Fig. 6A). Only omission of Zn significantly lowered regeneration from cotyledons (Fig. 6B). Omission of B had no effect on cotyledon regeneration, even though B was required for optimal regeneration from the primary leaf nodes.

Fig. 5. Effects on shoot regeneration from primary leaf node (A) and cotyledon (B) explants of individual micronutrient elements raised to 4X the MS level while omitting the other elements completely The same letter on the top of two or more bars indicates that they are not significantly different in the LSD test (P = 0.05)

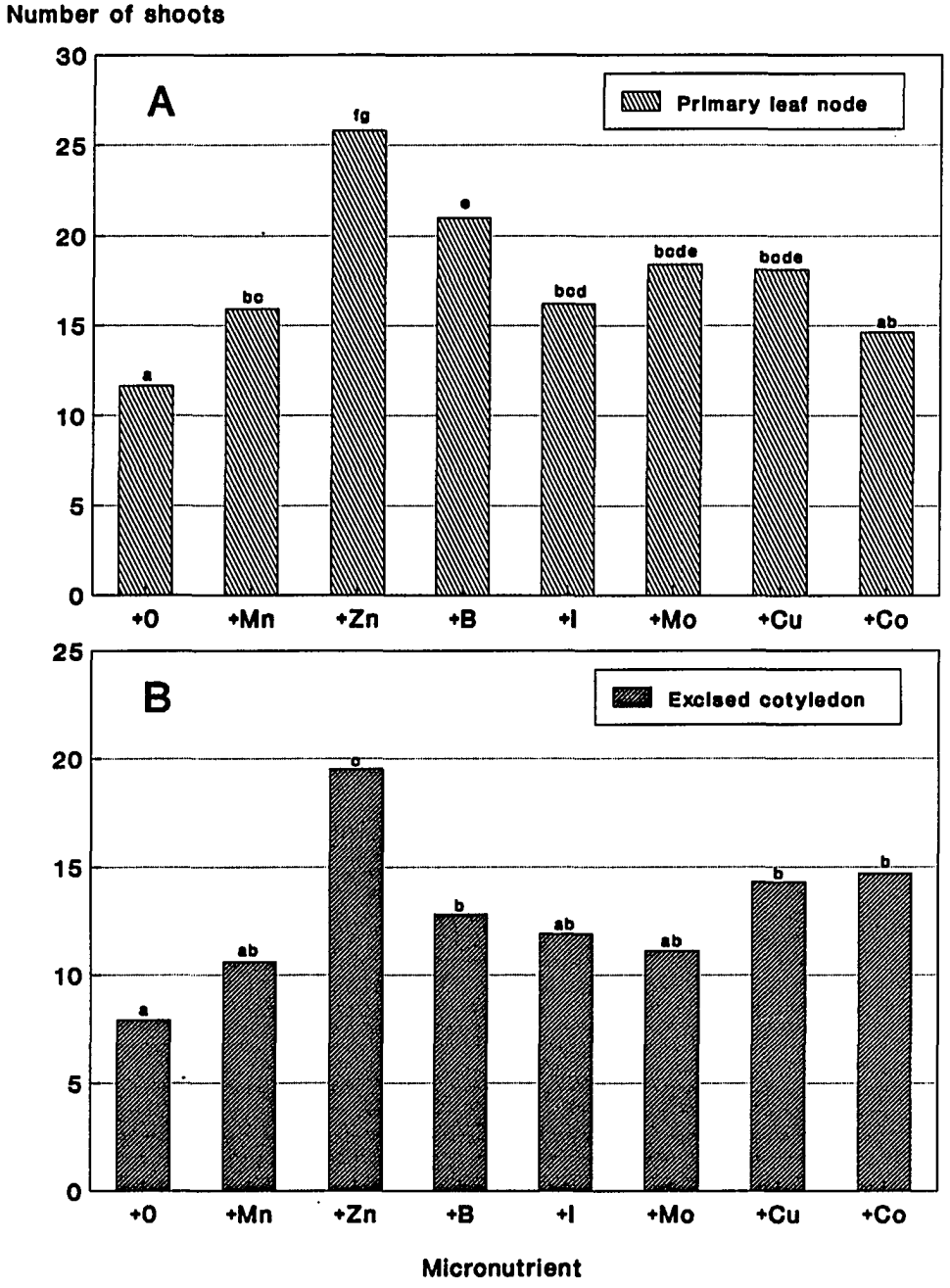
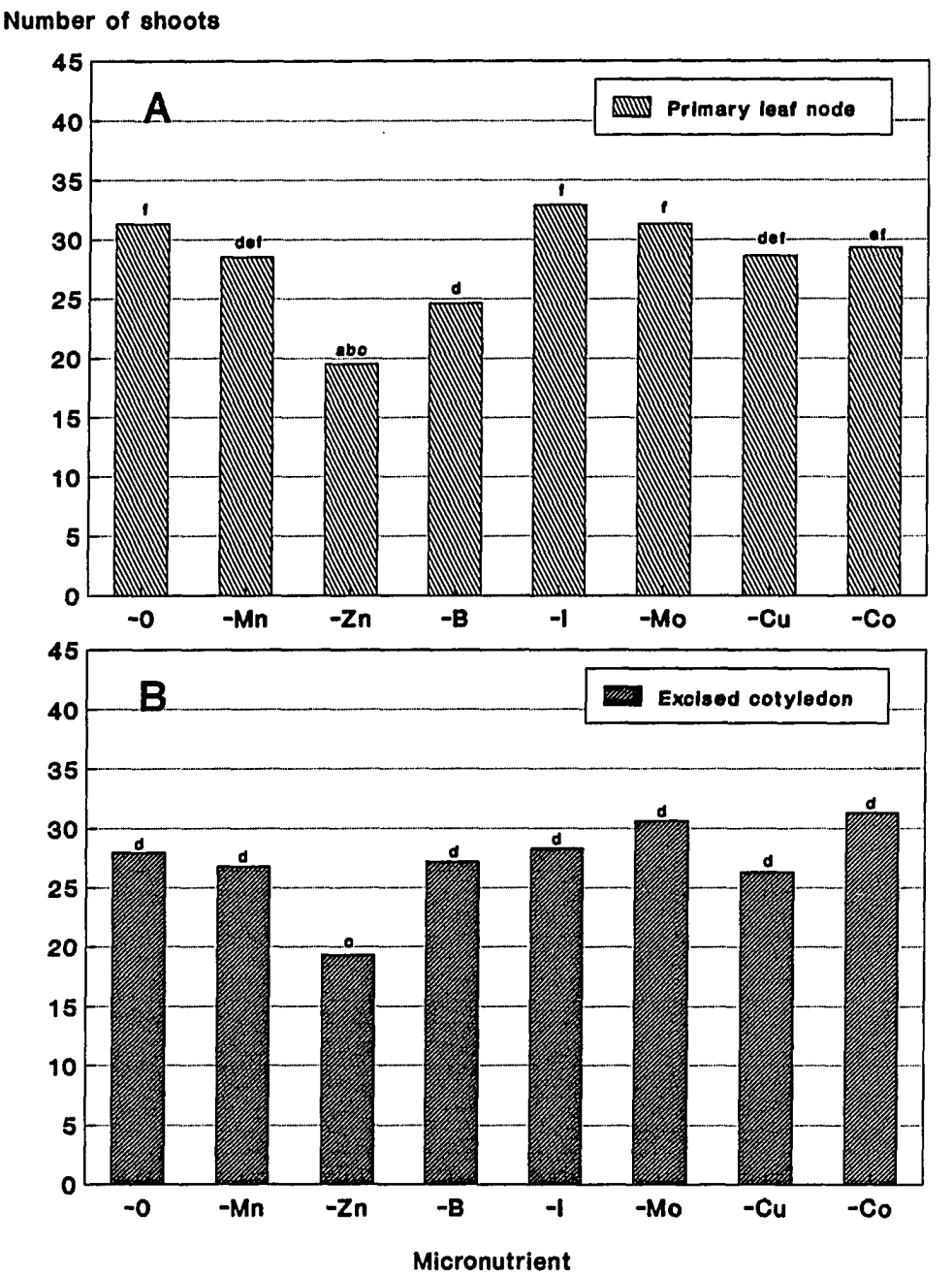

Number of shoots
A
Primary leaf node
30
25
20
15
10
5
0
a
bc
fg
e
bcd
bcde
bcde
ab
+0
+Mn
+Zn
+B
+I
+Mo
+Cu
+Co
B
Excised cotyledon
25
20
15
10
5
0
a
ab
c
b
ab
ab
b
b
+0
+Mn
+Zn
+B
+I
+Mo
+Cu
+Co
Micronutrient

Fig. 6. Effects on shoot regeneration from primary leaf node (A) and cotyledon (B) explants of omitting each micronutrient element while maintaining others at 4X the MS level The same letter on the top of two or more bars indicates that they are not significantly different in the LSD test (P = 0.05)


Number of shoots
A
Primary leaf node
45
40
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15
10
5
0
f
def
abc
d
f
f
def
ef
-0
-Mn
-Zn
-B
-I
-Mo
-Cu
-Co
B
Excised cotyledon
d
d
c
d
d
d
d
d
Micronutrient

DISCUSSION

Using the same proline- and micronutrient-supplemented medium, an average of 34.5 shoots per explant was recorded in this study (Table 1), whereas a highest average of 19.7 shoots per explant was reported earlier (Kim et al., 1990). An even higher average of 37 shoots was obtained in the present study when the levels of all inorganic micronutrients except cobalt were four times the level in MS medium (Fig. 4). Two changes may explain this improvement in shoot regeneration from primary leaf nodes. First, the seed source was changed from a field at Stoneville, Mississippi, to a greenhouse at Ames, Iowa, and second, the time in culture before axillary buds that had grown out at the cotyledonary node of explants were removed was reduced from 10 days to 4 days. The latter change probably reduced competition from the cotyledonary axillary buds for growth-limiting nutrients being supplied by the cotyledons to the shoot-forming sites at the primary leaf nodes.

Proline has been reported to stimulate somatic embryogenesis in several plant species (Stuart and Strickland, 1984a; Stuart and Strickland, 1984b; Ronchi et al., 1984; Trigiano and Conger, 1987; Armstrong and Green, 1985), but not in soybeans (Barwale et al., 1986). We have previously reported that proline also stimulates shoot formation in an organogenic rether than embryogenic system, soybean primary

leaf node cultures (Kim et al., 1990). Here we have extended this observation of a stimulation effect of proline to a second organogenic system, cotyledonary node cultures. We have also shown a synergistic effect of proline and micronutrients (Tables 1, 2). The effect of 4X micronutrients and 2 $g \cdot l^{-1}$ proline (relative to 1X micronutrients and no added proline) is much greater (+23 shoots in primary leaf nodes and +19 shoots in cotyledons) than the sum of their individual effects (+9 shoots in primary leaf nodes and 0 shoots in cotyledons). It should also be noted that neither we nor Barwale et al. (1986) observed an increase in shoot or embryo formation when inorganic micronutrients were increased but proline was not added. Judging from their Table 7, Barwale et al. (1986) did not test both supplements together, and thus did not observe any stimulation by added micronutrients.

The effects of varying levels of individual micronutrients are complex. For most micronutrients other than zinc, lowering a single element from 4X to 1X (Fig. 4), or even omitting it altogether (Fig. 6), did not have much effect. Thus, no single micronutrient must absolutely be supplied to obtain regeneration, but clearly zinc must be supplied to obtain high regeneration. This is not surprising because the explant itself is likely to serve as a reservoir of micronutrients; the zinc reservoir is probably smallest relative to the need for it.

In contrast, including a single element when the others were omitted (Fig. 5), or raising its level from 1X to 4X (Fig. 3) while others were held at 1X, usually caused a significant increase in regeneration and in every case resulted in average numbers of shoots higher than control averages (Figs. 3, 5). The contrast between the effects of including and removing a single element, especially evident in the effects of Mn, Mo, and Cu on primary leaf nodes (Figs. 3A, 5A versus 4A, 6A), indicates that the effects of the different nutrients are not additive. We would predict that in an experiment where micronutrients are added one-by-one to the medium until all are present, each additional component will have a progressively smaller effect if zinc is added first.

There are several possible explanations for the observed non-additive effects. Some other factor is limiting at the high level of micronutrients, and this other factor is still limiting except when Zn (and maybe B in the case of primary leaf nodes) is removed.

However, the larger is the number of elements added at a raised level the greater is the likelihood that other-element contaminants in the added chemicals may suffice to attain a stimulatory level of an element not intentionally added. Also, relieving one nutrient limitation may serve to raise the optima for others as the general rate of development becomes incrementally increased.

Several individual micronutrients did have characteristic effects. Lowering iodine, as iodide ion, from 4X to 1X had a small but significant adverse effect on regeneration from primary leaf nodes (Fig. 4A), but no adverse effect was observed when it was omitted altogether (Fig. 6). At present, we have no explanation for this difference. Iodine clearly enhanced growth *in vitro* of excised tomato roots (White, 1938) and tobacco callus tissues (Hildebrandt et al., 1946), and Miller (1963) includes iodine in his medium for soybean callus culture.

Complete omission of boron had a significant adverse effect on regeneration from primary leaf nodes (Fig. 6A), consistent with the major positive effect of including it individually (Fig. 5A). In the case of primary leaf nodes (Fig. 5A), for example, it almost doubled the number of shoots formed when added singly. Omission of B from the medium for rice (Ohira et al., 1975) and carrot (Heller, 1953) suspension cultures diminished their growth.

The only element that showed evidence of being present in excess at the 4X level is cobalt: reduction of cobalt from the 4X to the 1X level (while the other elements remained at the higher level) significantly enhanced shoot regeneration from primary leaf nodes (Fig. 4A), and in both explants the highest averages for shoot formation (37.1 shoots per primary leaf node explant and 35.0 shoots per cotyledon explant) were recorded when Co was at the original MS level (Fig. 4).

Complete omission of cobalt did not, however, increase shoot number significantly. This suggests that the higher level is harmful to explants, but the presence of some Co is beneficial. Murashige and Skoog (1962) found that a concentration higher than 10.4 $mg \cdot l^{-1}$ of $CoCl_2$ caused toxic effects on growth of tobacco callus. Shoot regeneration from primary leaf nodes was inhibited by as low a concentration as 0.055 $mg \cdot l^{-1}$ $CoCl_2$, the 4X level.

Somewhat paradoxically, raising Co individually from the 1X level to the 4X level even seems advantageous to shoot formation in soybean primary leaf nodes (Fig. 3A) and is not inhibitory in cotyledons (Fig. 3B). Most tissue culture media include cobalt because Cobalt promotes auxin-dependent cell extension growth in bean leaf disks, pea epicotyls, and oat coleoptiles (Miller, 1952; Miller, 1954; Thimann, 1953), and is likely to be involved in flowering (Salisbury, 1959).

Mn, Mo, and Cu were similar in their effects on soybean explants. All three consistently stimulated shoot formation in primary leaf node explants when supplied singly to them (Fig. 5A) or when individually raised from the 1X to the 4X level (Fig. 3A) and, though not always statistically significant in their effects on cotyledon explants, appeared to have effects on them parallel to those on primary leaf nodes (Figs. 3B, 5B). In contrast, when omitted completely (Fig. 6) or lowered from the 4X to the 1X level (Fig. 4), they consistently failed to have an effect. Two explanations for

these results seem plausible. First, cross-contamination of the micronutrient chemicals supplied may serve to alleviate deficiencies; in our design other micronutrients are at the higher level when the one being considered is lowered or omitted and thus contaminants too would be at a higher level. Also, some "sparing action" on one metal ion by the others supplied at a high level may be involved, especially, perhaps, by release from metabolically inactive anionic binding sites in the cell walls and elsewhere in the explants.

Ohira et al. (1975) found that the omission of Mn, Mo, or Cu decreased growth of rice suspension cultures with Mo omission having the smallest effect. Deficiency effects resulting from Mn and Cu omissions were reported for growth of carrot suspension cultures by Heller (1953). Heller (1953) did not, however, include Mo as a micronutrient, and Murashige and Skoog (1962), who did include it, failed to observe any difference in growth of tobacco callus when they varied Mo concentration. Molybdenum is a component of nitrate reductase and other enzymes in plants (Clarkson and Hanson, 1980) and is an essential nutrient for whole plants. Thus, it is generally included as an inorganic micronutrient in plant tissue culture media, an addition justified for soybean by our findings reported here (Figs. 3A, 5A).

The individual element with the strongest effects on shoot regeneration from soybean explants is Zn. Complete omission of Zn (Fig. 6), or reducing it from the 4X to the 1X

level (Fig. 4), had a significant adverse effect on shoot regeneration from both types of explant, and conversely, inclusion of Zn had a particularly large positive effect (Fig. 5) when no other micronutrients were included.

Zinc is an important micronutrient for plants (Mengel and Kirkby, 1987; Clarkson and Hanson, 1980); it is present in many enzymes, including the ubiquitous carbonic anhydrase, and is known to be essential for the synthesis of the major auxin of higher plants, indole-3-acetic acid (Takaki and Kushizaki, 1970). In citrus and other trees, a deficiency of zinc results in a disease called "little leaf" (Meyer et al., 1973, Salisbury and Ross, 1985). The leaf symptom is believed to be caused in major part by the diminished auxin synthesis in those leaves. Supplying zinc alleviates this symptom.

It seems, however, that auxin synthesis is not the zinc-limited step in soybean regeneration, as a synthetic auxin is amply supplied in the medium. A strong effect of Zn deficiency has previously been noted in tissue cultures of other species of plants. For example, Ohira et al. (1975) reported that of all the micronutrient elements, omission of Zn from the medium reduced growth of rice suspension cultures most dramatically, and Heller (1953) observed adverse effects of Zn deficiency on growth of carrot suspension cultures.

In general, the soybean primary leaf node explants showed a greater sensitivity to varying micronutrient levels than did the excised cotyledon explants (Figs. 3-6), even though both

types of explant include a cotyledon. This difference in response is likely to derive from a difference in surface contact with the medium by the two kinds of explants during the early part of the 30-day culture period. Because the primary leaf node explants each include a young stem internode, which during the first week grows and curves so as to diminish contact of the explant with the agar medium, we would expect that a concentration of a limiting micronutrient in the medium that is optimal for shoot regeneration from cotyledon explants, which retain their firm contact with the medium, would be suboptimal for a primary leaf node explant.

While it is possible that further investigations of the micronutrient balance might identify small additional effects, our results include that no major improvements are likely to come from their further scrutiny--at least for the stages of development examined here. For efficient shoot regeneration from cotyledon and primary leaf node explants of 7-day-old seedlings, we recommend the following additions to the standard MS medium: a fourfold raised level of the MS micronutrients except cobalt, and 2 $g \cdot l^{-1}$ of L-proline. These improvements in the nutrition of soybean explants for shoot regeneration, which modify a medium specifically designed for tobacco callus (Murashige and Skoog, 1962), may be more widely applicable to the culture of soybean parts, including organs, tissues, cells, and perhaps even protoplasts, and may possibly

serve to improve culture nutrition for other legume species too.

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SECTION IV. TRANSFORMATION OF SOYBEAN (*GLYCINE MAX*) SHOOTS BY *AGROBACTERIUM TUMEFACIENS*

SUMMARY

Transformed shoots have been produced using Agrobacterium-mediated gene transfer. The procedure relies on our productive regeneration system in which shoots are regenerated from primary leaf node explants of 7-day-old seedlings. Agrobacterium tumefaciens strains A208, A281, and LBA4404, all carrying the binary vector pBI121 containing neomycin phosphotransferase type II and β-glucuronidase genes, were tested for their ability to transform. Transformation efficiency was highest with strain LBA4404, followed by A208 and A281. Tests of the effects of kanamycin levels on callus or other explants suggested that large tissue pieces tend to be more resistant to kanamycin than small ones. Callus, smallest in size among the tested explants, was the most sensitive to kanamycin, and cotyledons or primary leaf nodes of seedlings, largest in explant size, were the most resistant. Several other factors affected transformation of primary leaf node explants. The highest efficiency of transformation was achieved with 6 days of explant pre-culture, a 100-fold dilution of the Agrobacterium suspension produced by overnight culture, 40-60 μmol $m^{-2}s^{-1}$ of white light during pre-culture, one-tenth of that light intensity during co-culture, and about 0.5-mm deep punctures with an array of needles dipped in Agrobacterium suspension.

INTRODUCTION

With the development of tissue culture techniques for plant regeneration, soybean improvement can be approached in two novel ways. One involves the direct use of tissue culture for the selection of desirable traits such as disease or herbicide resistance and the other involves the use of soybean tissue culture methods to aid in the transfer of foreign genes into soybean using genetic engineering. Several methods have been developed for soybean shoot regeneration from tissues such as cotyledons of mature seeds (Mante et al., 1989) and seedling parts including cotyledonary nodes (Cheng et al., 1980; Wright et al., 1986), primary leaf nodes (Kim et al., 1990), young primary leaves (Wright et al., 1987) and cotyledons (Hinchee et al., 1988).

Genetic engineering techniques would provide a way for the rapid development of varieties with useful new traits in a manner unobtainable by traditional breeding methods. The Agrobacterium-mediated gene transfer system has some advantages over other approaches in that it is simpler than other gene-transfer systems in which special instruments or complicated techniques are required (Griesbach, 1983; Klein et al., 1987). Successful infection of target plants by Agrobacterium requires a compatible interaction between target plant components and bacterial components. Target-plant components involved in the reaction may include endogenous

plant hormone levels, cell wall fragments (Yamazaki et al., 1983), quantity and type of phytoalexins (Davis et al., 1984) and other factors involved in plant defense strategies (Byrne et al., 1987). A region of the bacterial chromosome involved in bacterial attachment to the plant cell wall has also been identified (Douglas et al., 1985).

Agrobacterium-mediated gene transfer is now a well established technique for transferring foreign genes to certain plants such as tobacco, tomato, potato, and petunia (An et al., 1986), all members of the Solanaceae. Soybean is also susceptible to Agrobacterium infection. Agrobacterium-mediated transformation of soybean tissue was first reported by Pedersen et al. (1983). However, not all Agrobacterium strains can readily infect soybean tissues, at least as assayed by tumor formation (Facciotti et al., 1985; Owens and Cress, 1985; Byrne et al., 1987). Coinfection of Agrobacterium strain LBA4404 with strain A281 increased transformation of soybean cotyledons, whereas inoculation of LBA4404 alone failed to give transformation (Owens and Smigocki, 1988). Strain LBA4404 contains a helper plasmid ineffective for soybean cotyledon transformation.

One reason for difficulty in regenerating transgenic soybean plants is the necessity of having transformation and the potential for regeneration occur in the same cells. Hinchee et al. (1988) first reported regeneration of transgenic plants from cotyledons of seedlings inoculated with

Agrobacterium. Transgenic soybean plants were also regenerated without tissue culture by infecting germinating seeds with Agrobacterium tumefaciens (Chee et al., 1989).

As alternatives to the use of Agrobacterium, gene transfer by microprojectiles or protoplast culture with DNA can be used for transformation of soybean. There have been reports that stable transformation was achieved by using soybean protoplasts (Blades et al., 1987; Cress, 1982; Lin et al., 1987). Until now, there have been no reports on regeneration of transgenic soybean plants from transformed protoplasts, even though Wei and Xu (1988) reported successful plant regeneration from soybean protoplasts. McCabe et al. (1988) have, however, produced transgenic soybean plants using bombardment with DNA-coated microprojectiles.

Even though there have been three reports on regeneration of transgenic soybean plants, the efficiencies obtained are relatively low. Our objective was to increase the frequency of transgenic plant production by combining Agrobacterium-mediated transformation with our productive tissue culture system for soybean plant regeneration (Kim et al., 1990). We have succeeded in producing transformed shoots, based on selection by kanamycin and assay of β-glucuronidase activity, and have solved some technical problems in transformation with Agrobacterium. We report here some investigations of transformation using primary leaf nodes of soybean seedlings. Our approach involved: (1) use of primary leaf nodes of Peking

seedlings as a target site for transformation, (2) selection of transformed shoots by kanamycin, (3) use of the GUS gene as a histochemical marker for transformed cells, (4) examination of factors affecting co-culture with Agrobacterium, and finally, (5) comparison of shoot regeneration and transformation methods for tobacco leaves with those for soybean nodes.

MATERIALS AND METHODS

Regeneration of Soybean Plants

Conditions for germination of seeds, development of seedlings, preparation of primary leaf node explants, and *in vitro* culture were the same as those described in Materials and Methods of Section II. Also, the composition of media for shoot induction, shoot development, and root induction were the same as those in Materials and Methods of Section II except for a change from Difco Bacto agar tto Difco Bitek agar.

Effects of Kanamycin Concentration

The effects of kanamycin on three soybean developmental events were assessed 30 days after beginning culture: (1) growth of callus derived from mature cotyledon slices, (2) callus formation from hypocotyl segments and primary leaves of 7-day-old seedlings, and (3) shoot formation from cotyledons and primary leaf nodes of 7-day-old seedlings. Calli 5 mm in length were transferred to MS medium containing 1 $mg \cdot l^{-1}$ N^6-benzyladenine (BA), 3 $mg \cdot l^{-1}$ α-naphthalene acetic acid (NAA), and kanamycin, and growth was measured by size. Hypocotyl segments 5 mm in length and primary leaves 10 mm in length were cultured on MS medium containing kanamycin, 2 $mg \cdot l^{-1}$ kinetin, and 5 $mg \cdot l^{-1}$ NAA, and callus growth was visually estimated. The number of shoots formed from cotyledon explants and primary leaf node explants was counted after

growth on the kanamycin-containing medium. Composition of that medium is described in detail in Section II. Callus growth, callus formation, and shoot formation were measured one month after starting cultures.

Bacterial Strains

Three Agrobacterium strains, A208, A281, and LBA4404, were tested for soybean transformation (Table 1); all of them contain a binary plasmid vector, pBI121. Strains LBA4404 have a disarmed helper plasmid and the others have a wild-type helper plasmid. The binary vector pBI121 contains two chimeric genes, a selectable marker gene and a reporter gene, between the T-DNA border sequences. The selectable marker codes for a neomycin phosphotransferase (NPTII) with a nopaline synthase (NOS) promoter; the reporter gene codes for β-glucuronidase (GUS) with a cauliflower mosaic virus (CaMV) 35S promoter. Both have a nopaline synthase termination sequence.

Agrobacterium was inoculated in liquid YEP medium (10 $g \cdot l^{-1}$ Bacto-peptone + 10 $g \cdot l^{-1}$ Bacto-yeast extract + 5 $g \cdot l^{-1}$ NaCl) containing 50 $mg \cdot l^{-1}$ kanamycin and 10 $mg \cdot l^{-1}$ acetosyringone (Stachel et al., 1985) and cultured at 28°C overnight (for ca. 12 h) with rotary shaking at 100 rpm. Agrobacterium cultured overnight was diluted with liquid Murashige and Skoog (MS: 1962) basal medium before inoculation into explants as described below.

Table 1. Agrobacterium strains and plasmids used for soybean transformation

Agrobacterium strain	Helper plasmid	Chromosomal background	Reference
A208	pTiT37	C58	A[a] and B[b]
A281	pTiBo542	C58	A and B
LBA4404	pAL4404	Ach5	A and B

[a]A: Sciaky et al., 1978.

[b]B: Hood et al., 1987.

Co-culture of Explants with Agrobacterium

Primary leaf node explants were cultured on shoot-induction medium for 0, 2, 4, 6 or 8 days under cool-white fluorescent light (40-60 μmol $m^{-2}s^{-1}$) at 26°C before being co-cultured with Agrobacterium. Such cultures are called "pre-cultures". Explants were taken out of culture for inoculation with Agrobacterium, and then replaced for co-culture. Agrobacterium cultured in YEP medium overnight was diluted 10, 50, 100, or 200 times with liquid MS medium before being inoculated into explants. Agrobacterium suspensions diluted with liquid MS medium were inoculated into primary leaf nodes of explants using punctures about 0.5 mm deep made by five small sewing needles taped together and dipped in Agrobacterium suspension. After the explants had been co-cultured for 48 hours, they were transferred to selection medium, which consists of shoot-induction medium supplemented with 250 $mg \cdot l^{-1}$ kanamycin and 250 $mg \cdot l^{-1}$ cefotaxime, and

cultured under the same conditions as pre-cultures. Analysis of GUS gene activity was carried out after the explants had been cultured on the selection medium for 1 to 2 weeks.

Histochemical Assays of β-glucuronidase

GUS activity was assayed histochemically as described by Jefferson (1987). Small shoots formed on selection medium were sliced into small pieces ca. 0.5 mm thick, which were transferred to a solution of X-Gluc (5-bromo-4-chloro-3-indolyl-β-glucuronide) and incubated at 37°C for 12 hours in small wells. After the histochemical reaction was complete, the sections were fixed in FAA [10% (v/v) formaldehyde, 42.5% (v/v) ethanol, 5% (v/v) glacial acetic acid] for 12 hours to remove pigments masking the blue color produced by GUS activity. The sections were examined with a dissecting microscope.

Transformation of Tobacco

Tobacco leaf pieces cultured in vitro were transformed by the Agrobacterium strain LBA4404 containing pBI121. Agrobacterium was inoculated into tobacco leaf pieces without dilution of the overnight-cultured Agrobacterium suspension by puncturing them with small needles, as in the soybean procedure, or by dipping them in the suspension. After 2 days of co-culture, the leaf pieces were transferred to MS medium containing 0.5 $mg \cdot l^{-1}$ BA, 250 $mg \cdot l^{-1}$ cefotaxime, and 200

$mg \cdot l^{-1}$ kanamycin. The co-cultured leaf pieces were inoculated onto the medium with the abaxial or adaxial side down.

RESULTS AND DISCUSSION

Effects of Kanamycin Concentration

The effects of kanamycin concentration on the growth of callus derived from mature cotyledon slices, on callus formation from hypocotyl segments and primary leaves, and on shoot formation from cotyledons and primary leaf nodes of seedlings are summarized in Table 2. Callus grew threefold in

Table 2: Responses of soybean culture sources to various concentrations of kanamycin sulfate

Observation:		Growth	Callus formation		Shoot formation	
Source:		Callus	Hypocotyl	Leaf	Cotyledon	P.L. Node
Kana-mycin ($mg \cdot l^{-1}$)	0:	++++	++++	++++	++++	++++
	10:	++	+++	++++	++++	++++
	25:	+	++	+++	+++	+++
	50:	-	+	++	+++	+++
	75:	-	+	++	++	++
	100:	-	-	+	++	++
	150:	-	-	-	+	+
	200:	-	-	-	+	+
	250:	-	-	-	-	-

P.L. Node: Primary leaf nodes

++++: no detectable reduction in response (90 - 100%)

+++: a small reduction in response (60 - 90%)

++: a large reduction in response (30 - 60%)

+: a severe reduction in response (10 - 30%)

-: no detectable response (less than 10%)

size in the absence of kanamycin. The callus is so sensitive to kanamycin that it stops growing on medium containing 50 $mg \cdot l^{-1}$ kanamycin. Callus was not formed from hypocotyl segments on medium containing 100 $mg \cdot l^{-1}$ kanamycin or from primary leaves on medium containing 150 $mg \cdot l^{-1}$ kanamycin. In the absence of kanamycin, about 30 shoots were regenerated from primary leaf nodes or cotyledons of seedlings on the shoot-induction medium. No shoots were regenerated from either in the presence of 250 $mg \cdot l^{-1}$ kanamycin.

Usually large tissues are more resistant to kanamycin than small ones; callus is the most sensitive to kanamycin, and cotyledons and primary leaf nodes of seedlings are the most resistant to it. Probably kanamycin cannot be easily transferred over a long distance through the tissues.

Factors Affecting Transformation

There are several factors affecting transformation of primary leaf node explants, such as days of pre-culture, dilution of suspension-cultured Agrobacterium, light intensity during pre-culture and co-culture, and depth of needle puncture for inoculation of Agrobacterium.

Primary leaf nodes of 7-day-old seedlings are so small and sensitive that it is difficult to co-culture them with Agrobacterium without damage. Agrobacterium was inoculated into primary leaf nodes of explants after they had been pre-cultured for 0, 2, 4, 6 or 8 days on the shoot-induction

medium under the same conditions as for shoot regeneration. During pre-culture of explants for 2 days, stems of explants elongated and primary leaf nodes grew to dome-shaped structures from which shoots were subsequently regenerated. After pre-culture for 4 days, the stems had elongated almost fully but no shoots had regenerated from primary leaf nodes. Pre-culture for 8 days was not satisfactory for transformation since small shoots had already formed from primary leaf nodes. Pre-culture for 6 days was chosen for routine transformation because explants were large and strong enough for inoculation of Agrobacterium with needles.

Soybean tissues are highly sensitive to Agrobacterium. When Agrobacterium was cultured overnight and inoculated into primary leaf nodes without dilution of the suspension, bacteria grew so rapidly in the co-culture medium that they mechanically damaged the wounded surfaces of explants and caused them to brown. This death of cells around the wound was associated with a low transformation rate. This kind of damage by Agrobacterium could not be detected with tobacco leaves. We tested five Agrobacterium densities and three different diluents: the original overnight-cultured suspension and suspensions diluted 10, 50, 100, and 200 times with liquid YEP medium, liquid MS medium, or distilled water. Browning around wound tissue decreased sharply with a 50- or 100-fold dilution of Agrobacterium with liquid MS medium. A 50- or 100-fold dilution of the Agrobacterium suspension was

therefore chosen for routine transformation of primary leaf node explants.

Light was required for normal regeneration of shoots from primary leaf nodes. When Agrobacterium was inoculated into primary leaf nodes pre-cultured for 6 days in the absence of light, it caused more damage on the cut surface of primary leaf nodes and transformation efficiency decreased in comparison with explants pre-cultured in light of 40 to 60 μmol $m^{-2}s^{-1}$. However, a dim light (4 to 6 μmol $m^{-2}s^{-1}$) or no light was more favorable than light of 40 to 60 μmol $m^{-2}s^{-1}$ for co-culture. Therefore, primary leaf node explants were routinely pre-cultured under light of 40 to 60 μmol $m^{-2}s^{-1}$ and co-cultured under light of 4-6 μmol $m^{-2}s^{-1}$.

The cut surfaces of primary leaf nodes, which had healed during pre-culture, were intentionally wounded again when Agrobacterium was inoculated by needle punctures.

Interaction of Agrobacterium Strains with Soybean

Mixed-strain Agrobacterium infections with wild-type strain 208 and engineered strain LBA4404 carrying pBin6 containing the NPTII gene caused production of tumors from cotyledon slices of mature Peking soybean seeds, whereas inoculation of LBA4404 alone failed to give rise to tumors (Owens and Smigocki, 1988). Peking proved to be an excellent host for infection with Agrobacterium when the bacteria were applied to cotyledonary nodes of 3-week-old plants; 100% of

the explants formed tumors with strain A208, but no tumors were formed with A281 or C58 (Byrne et al., 1987). In our experiments, A281 and LBA4404 as well as A208 transformed primary leaf nodes of seedlings when transformation was tested by kanamycin selection and the GUS test. Therefore, it seems that Agrobacterium strains A281 and LBA4404 can transform primary leaf nodes of seedlings but do not readily transform cotyledon slices of imbibed seeds or cotyledonary nodes of young plants.

Formation of Transformed Soybean Shoots

Transformation of tissue and shoots (Figs. 1A and 1B) was tested by examining GUS activity. Transformed shoots were formed from primary leaf node explants after 6 days of pre-culture and 2 days of co-culture with Agrobacterium. The intensity of the blue color in tests of GUS activity was less in soybean shoots than in tobacco shoots. This difference may be explained in at least two ways. First, the expression of the GUS gene may be stronger in transformed tobacco shoots than in soybean shoots. Second, X-Gluc, the substrate in the GUS assays, may penetrate into tobacco cells more easily than into soybean cells. A darker blue color developed in the cells around vascular bundles than in other cells in transformed soybean shoots. This phenomenon suggests that X-Gluc cannot easily be transferred into cells of a compact tissue but easily moves through vascular bundles.

Transformation of Tobacco

Several important things were found in tobacco leaf transformation tests which were useful upon comparison with soybean transformation. Tobacco leaves are not damaged by Agrobacterium suspension and are more resistant than soybean to co-culture with Agrobacterium. More transformed tobacco shoots were produced when adaxial surfaces of leaves were placed in contact with selection medium than when abaxial surfaces were so placed. Transformed tobacco shhoots were regenerated from callus forming around leaf wounds, whereas transformed soybean shoots probably regenerated from new cells that proliferated during culture but were not of callus origin. According to GUS activity tests, not all tobacco shoots that had regenerated on medium containing 200 $mg \cdot l^{-1}$ kanamycin were transformed: approximately 40% of the shoots were transformed completely, 30% of them contained both transformed and untransformed cells, and the rest had no GUS-positive cells even though they were from apparently kanamycin-resistant shoots.

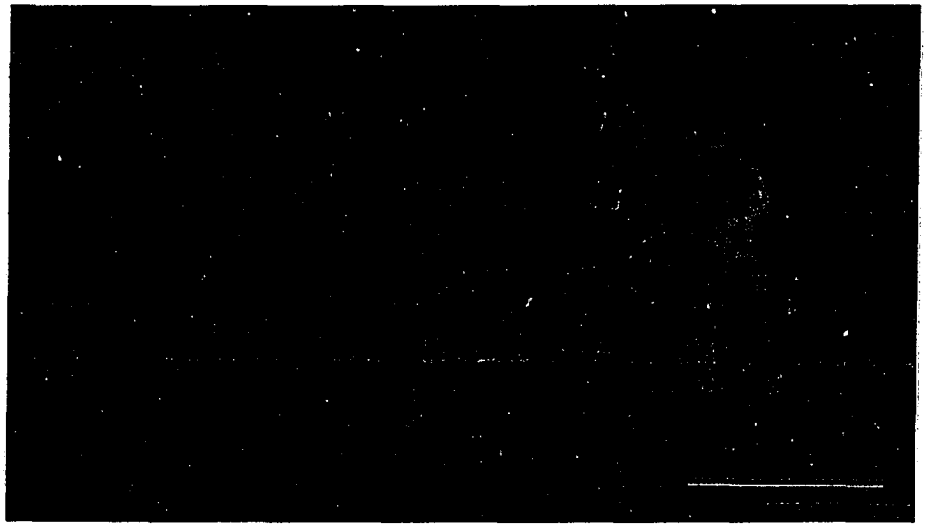

Fig. 1A. Transformed tissue (left) was formed from primary leaf node explant cultured on shoot-induction medium containing 250 $mg \cdot l^{-1}$ kanamycin and 250 $mg \cdot l^{-1}$ cefotaxime for 7 days. Non-transformed tissue (right) from control does not have blue color. Bar = 5 mm

Fig. 1B. Transformed shoot was formed from primary leaf node explant cultured on shoot-induction medium containing 250 $mg \cdot l^{-1}$ kanamycin and 250 $mg \cdot l^{-1}$ cefotaxime for 14 days. Bar = 1 mm

Future Work

Transgenic soybean plants have not been produced in spite of the success in transgenic shoot formation because of the long time required to do so. We expect that transgenic plants can be produced and transformation efficiency can be improved in the near future using our productive regeneration system. After production of putative transgenic plants, several things should be tested to make sure they are truly transformed. Included are (1) selection of transformants again using kanamycin during root formation, (2) performance of GUS assays again using fully developed leaves, (3) Southern blotting, and (4) checking of segregation ratios in the progeny. When transformation has been accomplished, verified, and made routine, it will be possible to introduce genes that improve seed quality and quantity and increase resistance of the plants to stresses and pests.

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OVERALL SUMMARY AND DISCUSSION

Several phenomena related to soybean tissue culture and transformation were examined in this study: (1) developmental events in the formation of an unusual tissue reticulum from mature soybean cotyledon slices cultured *in vitro*, a phenomenon leading to the formation of structures that can be easily mistaken for globular embryos, (2) effects of proline, inorganic micronutrients, and other factors on shoot regeneration from primary leaf nodes and cotyledons of seedlings, (3) regeneration of whole soybean plants *in vitro* from primary leaf nodes of seedlings via organogenesis, and (4) production of transgenic soybean shoots by *Agrobacterium* acting on the primary leaf node regeneration system developed in this study.

An unusual reticulum develops from 1/4-cotyledon slices cut from mature soybean cotyledons when those are cultured on an MS medium containing 1.2 $mg \cdot l^{-1}$ of the synthetic auxin dicamba by placing their abaxial surfaces in contact with the medium. About 80% of the slices cultured in this way produced a reticulum. Among six tested auxins, IAA, IBA, NAA, dicamba, 2,4-D, and picloram, only the chlorinated auxins, dicamba, 2,4-D, and picloram, induced reticulum formation, and the latter two only weakly so. Dicamba was by far the most effective, and only it induced a complex reticulum. 2,4-D and picloram induced a reticulum of smaller size and of much

simpler pattern. Many roots, instead of a reticulum and friable callus, were produced by IAA, IBA and NAA in the tested concentrations.

The reticulum is covered with friable callus and consists of the original venation of the cotyledon, which was enlarged by proliferation of cells in and around each vein and by new vascular tissue extending centrifugally from the original venation into new tissue formed by peripheral growth of the slice. Bulbous structures form at the tips of the extended and hypertrophied veins that comprise the reticulum. These events are paralleled in time by the centripetal development of an interfascicular friable callus by hypertrophy of ground tissue cells between the original veins of the cotyledon. Exploration of ontogeny of the reticulum showed three interesting things: (1) the reticulum illustrates the complex pattern of venation in the soybean cotyledon, (2) auxin-induced growth of the slice is leaf-like in its peripheral expansion and centrifugal vascularization, and (3) reticulum development parallels and complements the histogenesis of a friable callus used to assay cytokinins.

Factors involved in shoot regeneration from primary leaf nodes of soybean seedlings were investigated; these factors included concentrations of proline, BA and NAA, the level of inorganic micronutrients, and the size of cotyledon explants. The best regeneration of shoots occurred when primary leaf nodes were cultured <u>in vitro</u> on a modified MS agar medium

containing 3% sucrose, four times the MS inorganic micronutrient level, the vitamins of B5 medium, 2 $g \cdot l^{-1}$ proline, 2 $mg \cdot l^{-1}$ BA, and 0.02 $mg \cdot l^{-1}$ NAA. Shoot formation was greater when a cotyledon remained attached to the explant and its adaxial side was in contact with the medium. The B5 basal medium served best for development of regenerated shoots and the B5 medium in half strength served best for development of roots and for later plant development.

Added proline and increased inorganic micronutrients were particularly effective in stimulating shoot formation from primary leaf node and cotyledon explants from 7-day-old seedlings. These two supplements interacted synergistically in this role. Proline increased number but decreased length of regenerated shoots, whereas raising micronutrient level increased both shoot number and length and, thus, partly overcame the effect of proline on length.

Based on these findings, it seemed desirable to determine whether the effect of raising, collectively, the inorganic micronutrients on regeneration was due to a specific element, or group of elements, and whether individual elements might have adverse effects at high concentration. Examining separately the effects of singly omitting, including, raising, and lowering the levels of the seven different micronutrient elements of the original MS medium provided evidence that zinc is the most limiting element for regeneration from both kinds of explants and that the other six elements, too, are all in

less than optimal supply. The only element that showed evidence of being present in excess at the 4X level is cobalt: reduction of cobalt from the 4X to the 1X level while the others remained at the 4X level significantly enhanced shoot regeneration from primary leaf nodes. Complete omission of cobalt did not, however, increase shoot number significantly. This suggests that the higher level is harmful to explants, but the presence of Co at the 1X or slightly higher level may be beneficial.

Change of the seed source from a field at Stoneville, Mississippi, to a greenhouse at Ames, Iowa, and reduction of time from 10 days to 4 days before removing axillary buds during culture probably improved shoot regeneration from primary leaf nodes. An average of 34.5 shoots per explant was regenerated after adopting these changes but a highest average of only 19.7 shoots was recorded before the changes (Section II).

Small transformed shoots have been produced using the Agrobacterium-mediated gene transfer and the productive regeneration system involving shoot regeneration by primary leaf node explants from 7-day-old seedlings. Agrobacterium strain A208 can readily infect cotyledonary nodes of Peking soybean seedlings, when infection is assayed by tumor formation, but A281 cannot (Byrne et al., 1987). Coinfection of Agrobacterium strain LBA4404 with strain A281 increased transformation of soybean cotyledons, whereas inoculation of

LBA4404 alone failed to give transformation (Owens and Smigocki, 1988). In our experiments, A281 and LBA4404 as well as A208 transformed primary leaf nodes of seedlings when transformation was tested by kanamycin selection and the GUS histochemical assay. Possible, Agrobacterium strains A281 and LBA4404 can transform primary leaf nodes of seedlings but not so readily transform cotyledon slices of imbibed seeds or cotyledonary nodes of young plants.

Tests of the effects of kanamycin levels on callus or seedling explants suggested that large tissue pieces tend to be more resistant to kanamycin than small pieces. Callus, smallest in size among the tested explant sources, was the most sensitive to kanamycin, and cotyledons or primary leaf nodes of seedlings, largest of the explants, were the most resistant.

Transformation of primary leaf node explants by Agrobacterium was affected by several factors, pre- and co-culture time, density of Agrobacterium, method of Agrobacterium inoculation, and light intensity during culture. The highest efficiency of transformation was achieved by 6 days of pre-culture of explants under 40-60 μmol $m^{-2}s^{-1}$ light intensity, by use of a 50- to 100-fold diluted Agrobacterium suspension cultured overnight, by 2 days of co-culture in darkness or under dim light of 4-6 μmol $m^{-2}s^{-1}$, and by about 0.5-mm deep punctures with needles dipped into Agrobacterium suspension. Primary leaf nodes of soybean seedlings are so

sensitive to Agrobacterium that cells around wounded tissue are killed by suspensions that do not harm tobacco cells. Damage by Agrobacterium can be reduced by the pre-culture and bacterial dilution described here.

Transgenic soybean plants have not been produced in spite of the success in transgenic shoot formation confirmed by GUS activity because of the long time required to do so. We expect that transgenic plants can be produced, and transformation efficiency can be improved, using our productive regeneration system. When this process of developing a highly efficient transformation system involving Agrobacterium, now well under way, has been completed, the methods of modern molecular biology can more easily be applied to improving the soybean crop for an increasing number of human uses. It is expected that the improvements described here for shoot regeneration may also serve to benefit soybean tissue culture as it pertains to other uses.

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