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Organogensis in leafy spurge (*Euphorbia esula* L.)[1]

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Summary:

All parts of leafy spurge seedlings can be regenerated when isolated and placed onto B5 medium. One-centimeter isolated hypocotyl segments were tested successfully for their usefulness as a bioassay system by comparing the response of auxins, herbicides, and cytokinins. Indole-3-acetic acid (IAA) was the most effective auxin to stimulate root formation. IAA was effective whether the hypocotyl segments remained on the same medium up to 60 days, or the segments were transferred to basal media after 2 or 5 days (pulse treatment). Pulse treatments with the other auxins resulted in stimulation of root formation; continuous or 5-day pulses of higher concentrations of indole-3-butyric acid, α-naphthaleneacetic acid and especially 2,4-dichlorophenoxyacetic acid and picloram formed excessive callus instead of roots. Picloram did not stimulate root formation, whether the treatment was continuous or pulse-treated. No roots formed with continuous picloram at 0.1 mg/liter or greater, but transfer to basal media did result in root and shoot formation at about 50% of the number formed on the controls. Lesser picloram concentrations had no effect. Shoots formed readily on untreated (control) segments, but continuous treatment with all three cytokinins, kinetin, zeatin, and zeatin riboside, increased the numbers of shoots about equally. Root formation was inhibited by the cytokinins at the higher concentrations (0.1 to 0.2 mg/liter). With the exception of a 5-day pulse of 0.04 mg/liter IAA, the auxins did not stimulate shoot formation, but generally inhibited shoot formation, even in pulse-treated cultures.

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Introduction

Leafy spurge (*Euphorbia esula* L.) is a perennial plant of the family Euphorbiaceae, which includes economically important plants such as the Christmas ornamental Poinsettia (*Euphorbia pulcherrima* Willd.), and Cassava (*Manihot utilissima* Pohl), which is used as a major source of starch for human consumption in many tropical and subtropical countries (Kartha *et al*., 1974). Leafy spurge is also of economic importance as an invasive and persistent weed in non-cultivated areas in the temperate climates of North America (Watson, 1985). Leafy spurge persists and increases by seeds and vegetative propagation from buds at the base of established shoots and from buds formed on an extensive root system (Watson, 1985). The plant may be controlled, but seldom eradicated, by repeated treatments with herbicides such as picloram or 2-4-dichlorophenoxyacetic acid (2,4-D) (Lym and Messersmith, 1990). The present report is part of a research program to determine the organogenic potential of leafy spurge and to establish a bioassay for screening growth regulators or potential herbicides against this perennial weed.

Previous work in this laboratory resulted in regeneration of plants from one out of eight accessions with callus-derived cell suspension cultures of leafy spurge (Davis *et al*., 1988). Rhizogenesis could not be correlated with nitrate reductase activity (Evenson *et al*., 1988), roots were nearly always formed before shoots, and root formation was stimulated by removing the 2,4-D which was used to maintain the cell suspensions in an actively dividing and undifferentiated state. Although these cultures were potentially useful for in-depth studies of organogenesis, the cultures retained their regenerative capacity for less than 15 months and were slow growing. For these reasons, an alternative system (isolated hypocotyl segments) was chosen to determine the effects of exogenously applied growth regulators on organogenesis and as a bioassay to screen potential herbicides or growth regulators for their capacity to control organogenesis of leafy spurge.

Materials and methods

Seeds were field collected by hand in southeastern North Dakota and sterilized 2 minutes in 70% ethanol followed by 20 minutes in 30 or 60% bleach (2.75 or 5.5% Na hypochlorite, respectively) with 0.1% Tween 20 detergent. The seeds were germinated on sterile 0.7% agar/water (wt/vol) in 6-cm sterile plastic petri dishes, in continuous darkness at 30º and 20º C alternating temperatures (12 hours each), as described by Beasley (1964).

Initially, the organogenic potential of the apex, hypocotyl, and roots of etiolated seedlings was determined. These plant parts were isolated and placed onto basal B5 agar medium (Gamborg *et al*., 1968), with no growth regulators, for a total of 60 days. All three parts of the seedlings formed both roots and shoots (see Results). Because of the greater tissue mass of hypocotyls as compared to roots and the apex, hypocotyls were selected for subsequent experiments. Based on additional preliminary experiments, 1.0-cm segments of the hypocotyls were chosen as explants for consistency and convenience. Segments as short as 0.5 cm formed almost no roots or shoots in the absence of exogenous growth regulators.

Six to eight segments cut from the central portion of each hypocotyl were placed onto agar medium containing the compounds to be tested. In some experiments the hypocotyls were left on the same medium for the duration of the experiments. In other experiments, the hypocotyls were transferred after 2 or 5 days to fresh basal B5 medium (without growth regulators). Two- and five-day exposures with 2,4-D were determined by preliminary experiments to give the best root response without the formation of massive amounts of callus (Davis and Olson, 1987).

Kinetin and 2,4-D solutions were added to the B5 medium before autoclaving at 120º C and 1.5 atm pressure for 20 minutes. Picloram, indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) solutions were filter sterilized and added to the liquified medium shortly after autoclaving. Zeatin and zeatin riboside were added as methanol solutions after autoclaving, before solidification of the media. The concentration of methanol in the medium was 0.04% (wt/vol) in both treated and control cultures. Concentrations of methanol of 0.5% or less have little or no effect on the growth of cell suspension cultures (Davis *et al*., 1978) and had no effect on the organogenesis of leafy spurge (Davis, unpublished). The hypocotyls were grown at 28º C in darkness for 4 to 8 weeks. Roots and shoots were counted using a dissecting microscope.

Chemicals used in this study were: picloram (analytical 99.6%) from Dow Chemical Co., Midland, MI; 2,4-D from Eastman Kodak Co., Rochester, NY; α-naphthaleneacetic acid (NAA) from Mann Research Laboratories, New York, NY; and kinetin from Aldrich Chemical Co., Inc., Milwaukee, WI. All other chemicals were from Sigma Chemical Co., St. Louis, MO, and were plant tissue culture tested.

Experiments were repeated 2 or more times. Comparisons of treatments were done using a two-sample test of the computer program Statistix (NH Analytical Software, St. Paul, MN).

Results and discussion

Organogenic potential of leafy spurge. The organogenic potentials of the three plant parts isolated from etiolated leafy spurge seedlings are shown in Table 1. All three parts of the seedlings used formed approximately 2 to 4 roots or shoots per organ, except for the intact hypocotyls (which averaged 10.8 cm in length) with an average of 12.7 shoots. Although considerable variation occurred with some tissues between the two experiments, in all cases, more than 50% of the explants were organogenic.

One-centimeter segments of the hypocotyls formed both roots and shoots on basal media without growth regulators (Fig. 1, 0.0 mg/liter 2,4-D or IAA), and the percent of the segments forming shoots was about twice the percent that formed roots (Fig. 1 A,C). Also, on the controls, the number of shoots was more than twice the number of roots formed per segment (Fig. 1 B, D). The results shown in Fig. 1 are the combined results of four experiments that were run for 29 to 32 days and are typical of controls for numerous experiments run over several years.

Table 1. Organogenesis of leafy spurge seedling parts, grown 60 days on B5 medium with no growth regulators[a].

	Explants					
	Entire Hypocotyls		Entire Roots		Apical Part of Shoots	
	Organs Formed					
	Shoots	Roots	Shoots	Roots	Shoots	Roots
	Number per explant					
	12.7 + 1.1	3.1 ± 0.8	3.3 ± 0.6	4.0 ± 0.8	1.8 ± 0.6	2.0 ± 0.4
	Percent of total seedling parts that formed organs					
Exp 1	100	61	94	59	56	75
Exp 2	100	100	100	100	40	80

[a]Data combined from 2 experiments. $n = 23$. Values are means ± standard error.

The hypocotyl segments responded differently from the cell suspension cultures used previously in this laboratory (Davis *et al*., 1988). Hypocotyl segments formed roots without exogenous growth regulators, followed by numerous shoots. In cell suspension cultures, roots were the initial organs formed, followed by only a few shoots. The only requirement for root formation in the cell suspension cultures was that the 2,4-D (used to maintain cell division and retard organogenesis or somatic embryogenesis) be removed thoroughly from the medium.

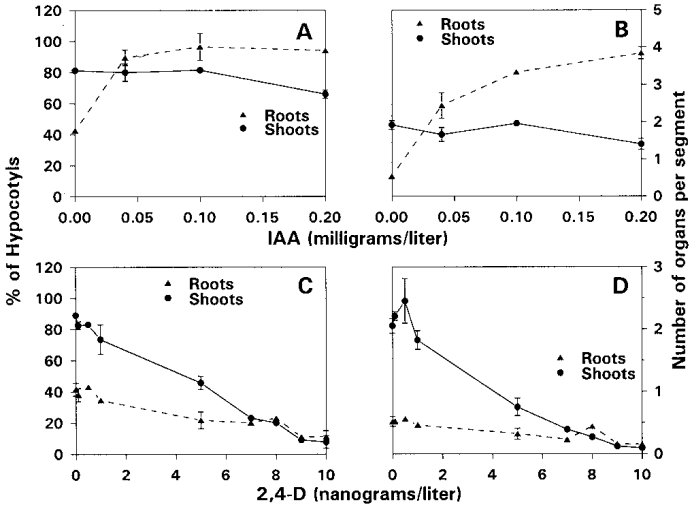

Fig. 1. Effects of continuous exposure to IAA and 2,4-D on root and shoot formation in isolated, etiolated 1-cm hypocotyl segments of leafy spurge. Combined results of four experiments. Values are means + SE. $n = 229$. A and C = % of hypocotyl segments forming organs; B and D = number of organs per hypocotyl segment.

Auxin effects on root formation. Isolated leafy spurge hypocotyl segments responded, as expected, to exogenously applied growth regulators: i.e. cytokinins stimulated shoot formation and inhibited root formation, whereas auxins generally reversed that trend. Also, the responses were dependent on the concentrations and chemistry of the growth regulator and to the duration of exposure. IAA, IBA, and NAA greatly enhanced root formation. IAA effects ranged from little or none to slight inhibition of shoot forma-

tion, and nearly always stimulated root formation; 0.04 mg/liter IAA seems to be a threshold concentration for the percent of segments that formed roots (Fig. 1 A) and higher concentrations of IAA resulted in more roots per segment (Fig. 1 B).

The mechanisms of IAA action are still not clear, and none of the synthetic auxins stimulated rhizogenesis as much as IAA. IAA gave more consistent results than the synthetic auxins. Root formation was much greater when IAA was used, whether as a pulse treatment or applied continuously. In contrast, 2,4-D stimulated root formation primarily if the hypocotyl segments were pulse treated with the compound. IBA was slightly more inhibitory to shoot formation and somewhat less stimulatory to root formation than was IAA. IBA has been detected in some plant tissues (Epstein *et al*., 1991) and it was converted to IAA in grapevine and olive (Epstein and Lavee, 1984). Epstein and Lavee (1984) indicated that IBA is a good root promoter and its action may be due to slow translocation and conversion to IAA.

Nissen and Foley (1987) reported on IAA and NAA effects on growth of 2-cm excised root segments of leafy spurge containing preformed buds. Partial inhibition of bud growth occurred within 9 days at 10^{-7} *M* NAA and at 10^{-5} *M* IAA, with complete inhibition at 10^{-5} *M* (1.9 mg/liter) NAA and 10^{-3} *M* (241 mg/liter) IAA. The effects of NAA can be compared to the present study, because 1 to 4 mg/liter NAA inhibited shoot formation in hypocotyl segments treated for only 5 days. In our experiments, IAA was not used at the high concentration used by Nissen and Foley (1987), and IAA was not inhibitory up to 4 mg/liter. Nissen and Foley (1987) obtained some stimulation of (shoot) growth with IAA at 10^{-11} and 10^{-9} *M* concentrations perhaps analogous to the stimulation shown in Fig. 2 B in the present report.

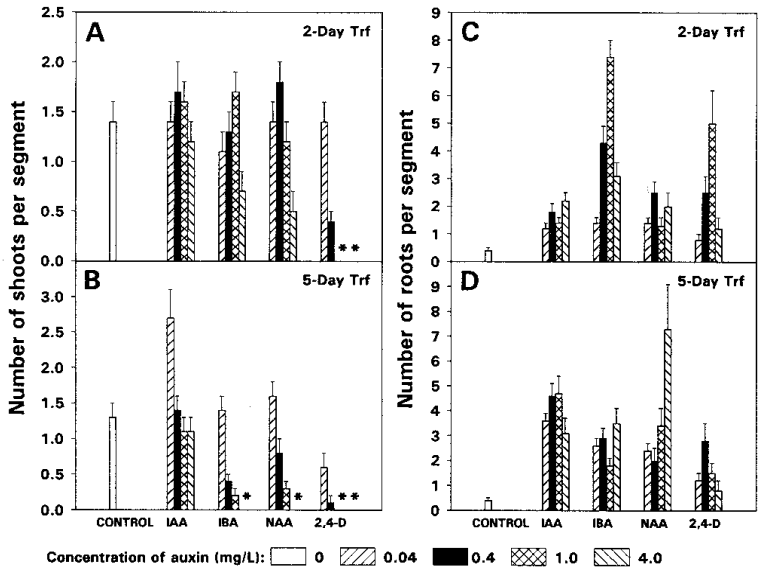

Fig. 2. Root and shoot formation in isolated leafy spurge hypocotyl segments pulse-treated 2 or 5 days with four auxins. Left, number of shoots formed per hypocotyl segment transferred after 2 days (A) or 5 days (B) to basal B5 medium. Right, number of roots formed per hypocotyl segment transferred after 2 days (C) or 5 days (D). Organs were counted after 30 days (total) of culture. *Asterisk*, no shoots observed at these concentrations. *Trf* = transferred. Combined results of two experiments. Values are means ± SE. *n* = 40.

The effects of 2,4-D on root and shoot formation ranged from no significant effects at low concentrations to inhibition at higher concentrations when 2,4-D remained in the same medium the entire time (Fig. 1 C,D). The lack of stimulation of root formation by continuous exposure to 2,4-D and the inhibition at nanograms per liter 2,4-D contrasts to the stimulation of root formation by IAA, with no indication of inhibition by IAA at any concentration tested.

Figure 2 compares the effects of IAA, IBA, NAA, and 2,4-D on hypocotyl segments pulse treated for either 2 or 5 days, followed by growth on basal media without exogenous plant growth regulators for a total of 60 days. Organ formation was usually maximal by about 28 days, but in some treatments roots and shoots continued to be formed for longer times, similar to that shown in Fig. 3.

Root formation was stimulated by all four auxins in hypocotyl segments pulse treated for either 2 or 5 days (Fig. 2 C, D). IAA exposure for 5 days resulted in greater root formation than a 2-day exposure, whereas the results for IBA were generally reversed. NAA increased root formation slightly in the 5-day treatment compared to the 2-day treatment, except at 4 mg/liter where the increase (and the sample variation) was much greater with a 5-day than with a 2-day exposure. 2,4-D gave similar results with either 2 or 5-day treatments, except at 1 mg/liter where a 2-day exposure resulted in greatly stimulated root formation (Fig. 2 D).

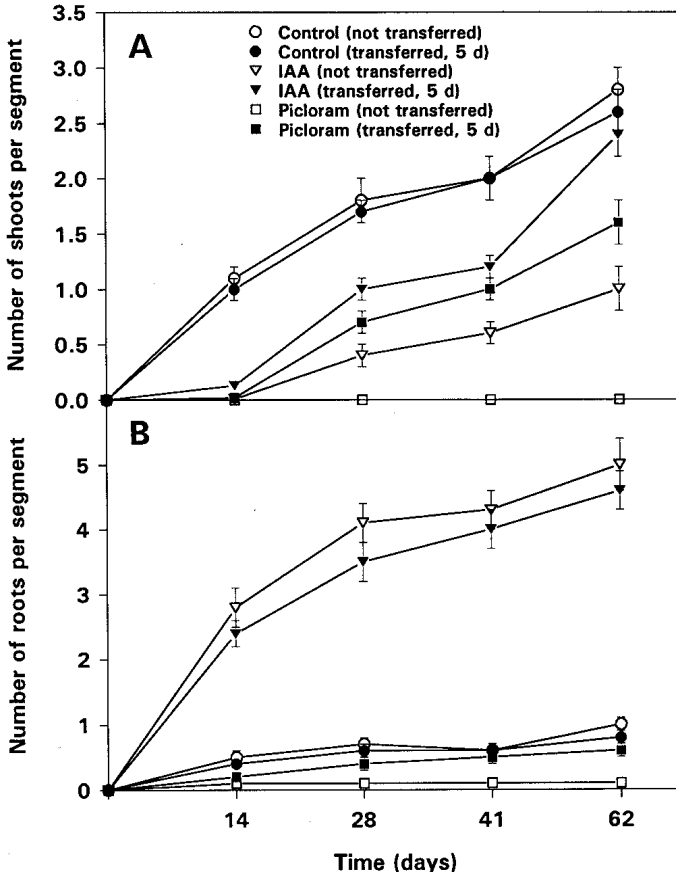

Fig. 3. A comparison of IAA and picloram (0.4 mg/liter each) as they affect shoot (A) or root (B) formation on leafy spurge hypocotyl segments. Tissue was either left on the same medium for the duration or they were transferred at 5 days to fresh medium without growth regulators for 55 days. Values are means ± SE. n = 64.

Auxin effects on shoot formation. Shoot formation was generally inhibited by the higher concentrations of all of the auxins except IAA. At 0.4 mg/liter, IAA did inhibit shoot formation in hypocotyls segments not transferred, but a 5-day exposure had little effect by 60 days (Figs. 2 B and 3 A). Treatment for only 5-days with 0.04 mg/liter IAA stimulated shoot formation (Fig. 2 B) and at the higher IAA concentrations the results were similar to the controls for either the 2- or 5-day exposures (Fig. 2 A, B). NAA or IBA at 1 mg/liter or

less for only 2 days (Fig. 2 A) had little effect on shoot formation, but both compounds were inhibitory if the exposure was for 5 days (Fig. 2 B). At 4 mg/liter, NAA and IBA inhibited shoot formation in hypocotyl segments treated for 2 days, and at either 1 or 4 mg/liter with a 5-day treatment. 2,4-D at 1 or 4 mg/liter totally inhibited shoot formation in all treatments (Fig. 2 A, B).

Both NAA and 2,4-D are probably persistent within the tissues and not catabolized readily. Conjugation of plant growth regulators may be a common mechanism to regulate the availability of plant growth regulators (Bandurski *et al*., 1987; Cohen and Bandurski, 1982). NAA conjugates readily with aspartic acid (Andreae, 1967; Goren and Bukovac, 1973) and can conjugate with glucose in tobacco explants within a few hours and to other metabolites over time (Smulders *et al*., 1990). The conjugates may be storage forms of the compound, releasing NAA over time to maintain the tissue in a non-differentiated state. Similarly, 2,4-D forms amino acid conjugates in stem segments of Alaska peas (Hangarter and Good, 1981) which may serve as a reserve form of 2,4-D to continually release the free acid to the tissue, such as occurs in soybean callus (Davidonis *et al*., 1980). However, the role of conjugation in plant growth regulator action has not been determined unequivocally.

Picloram effects. Picloram and 2,4-D are often considered growth regulators as well as herbicides. Both are commercial herbicides used effectively in the control of leafy spurge (Lym and Messersmith, 1990). Ashton and Crafts (1981) place picloram in a chapter on "unclassified" herbicides, yet indicate that picloram behaves like "other auxin-type growth regulators" (p. 414). Some auxinic properties of picloram were reported by Kefford and Caso (1966), and picloram was marketed earlier as a herbicide, under the trade name Tordon (Hamaker *et al*., 1963). Both 2,4-D and picloram enhance the frequency of embryogenesis of *Glycine* species (Gamborg *et al*., 1983). Collins *et al*. (1978) compared picloram to other auxins, including 2,4-D, and indicated that a kinetin-picloram combination might be useful for regeneration of wheat.

Picloram is a more powerful inhibitor of organogenesis than the other compounds used, and did not stimulate organogenesis in leafy spurge as did the other compounds. 2,4-D usually prevented root growth when applied continuously to hypocotyl segments, but root formation was stimulated when the segments were transferred to basal medium. Although root formation occurred in hypocotyls transferred away from picloram at 5 days, there was no stimulation of root formation above the controls, as occurred for the other auxins used.

Continuous treatment with picloram 0.1 mg/liter or greater inhibited both organs, but 0.01 mg/liter or less had no significant effect on root or shoot formation up to 28 days (Table 2).Total inhibition of root formation by 0.4 mg/liter picloram occurred unless the hypocotyl segments were transferred to picloram-free media at 5 days. Transfer resulted in root formation nearly equal to controls, and shoot formation to about 50% that of the controls at 60 days (Fig. 3). The results of a single experiment (not shown) with 0.4 mg/liter picloram and 2-day transfers were very similar to those for 5-day transfers shown in Fig. 3. All of the other auxins tested showed some stimulation of roots at this concentration if the hypocotyl segments were transferred, but picloram did not.

Table 2. Effects of picloram on organogenesis of leafy spurge hypocotyl segments.[a]

Picloram mg/liter	Percent of Hypocotyl Segments Forming:		Number per Segment	
	Shoots	Roots	Shoots	Roots
0	89	58	2.20 ± 0.20	0.78 ± 0.10
0.0001	88	44	2.58 ± 0.23	0.58 ± 0.10
0.001	91	72	2.67 ± 0.22	1.03 ± 0.12
0.01	81	58	1.72 ± 0.18	0.81 ± 0.11
0.1	3	42	0.03 ± 0.02[b]	0.47 ± 0.08[b]
1.0	0	3	0.00[b]	0.03 ± 0.02[b]

[a] B5 medium, 28 days without transfer. Values are average number of organs per 1-cm segment ± SE. $n = 120$.
[b] Differs from controls ($P < 0.05$).

Callus formation. Callus formation was not quantified because the emphasis of this study was on organogenesis. Picloram, NAA, and 2,4-D induced substantial amounts of callus even in hypocotyls exposed to the auxinic compounds for 2 to 5 days. Callus was also formed on many of the control and IAA-treated leafy spurge hypocotyls, but the amount was negligible compared to that induced by NAA, 2,4-D, or picloram. Picloram has been used to establish and maintain callus and cell suspension cultures of banana (Huang and Chi, 1988) and picloram was very effective in callus induction of several plant species (Collins *et al*., 1978). The herbicidal action of 2,4-D and picloram may be due to their inducement of callus formation at the expense of organogenesis.

Cytokinin effects on organogenesis. Because leafy spurge hypocotyl segments usually produced shoots readily, it was assumed there would be little or no effects of exogenous cytokinins. However, all three cytokinins stimulated the number of shoots at concentrations of 0.01 to 0.2 mg/liter (Fig. 4). Root formation was not stimulated by cytokinins, even at the lower concentrations, but all three cytokinins inhibited root formation at 0.1 and 0.2 mg/liter.

Hypocotyl segments as a bioassay. The hypocotyl system used in this research can be used to screen for compounds that alter organogenesis. The data require 4 weeks or longer for full expression of organ formation. The requirement for sterile conditions increases the cost, but the system has the advantage that the undesirable effects of other organisms are eliminated. Variation in the results was larger than desired, but attempts to use intact plants of leafy spurge in this laboratory frequently have resulted in even larger variations between replicates. Also, considerable greenhouse or growth chamber space is required for suitable replicated experiments using whole plants. The major advantages of the hypocotyl segment system are the minimal space requirements and easily obtained materials, the elimination of other organisms that may metabolize the applied chemicals, the requirement for relatively small amounts of test chemicals, and, perhaps most important, greater reproducibility than with cell suspension cultures or whole plants.

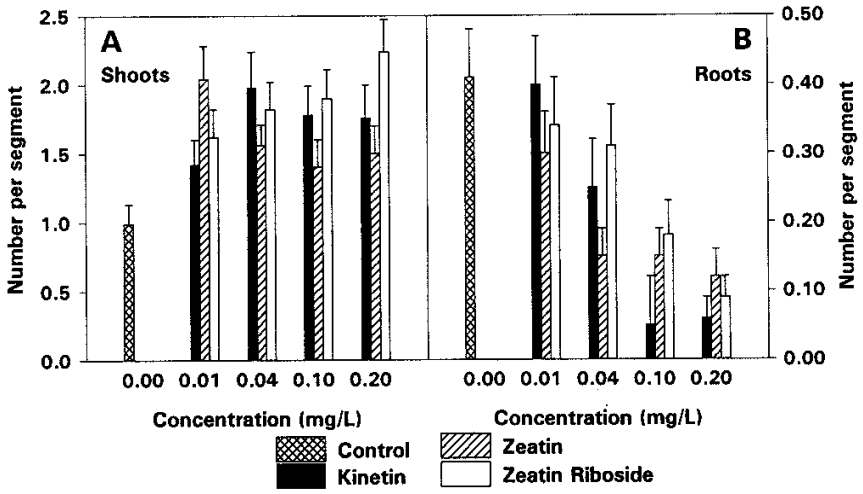

Fig 4. Effects of cytokinins on the organogenesis of leafy spurge hypocotyls left on the same medium for 28 to 32 days, depending on the experiment. Combined results of two experiments. Values are means ± SE. *n* = 80.

Acknowledgements

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