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Application of the Disk Method: Responses to Growth Regulators

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Abstract. The paper disk method of screening several plant growth regulators was evaluated. Leaf explants of *Vigna unguiculata* (L) Walp. were placed on solidified Murashige and Skoog's minimal organics medium containing 0.5 mg/l nicotinic acid. Hormones were tested, singly and in combinations, on paper disks in large Petri plates (150 × 20 mm). Hormones tested were 2,4-D (2,4-dichlorophenoxyacetic acid), 2,4,5-T (2,4,5-trichlorophenoxyacetic acid), IAA (indole-3-acetic acid), IBA (indole-3-butyric acid), picloram (4-amino-3,5,6-trichloropicolinic acid), dicamba (3,6-dichloro-2-methoxybenzoic acid), BA (6-benzyladenine), 2iP (2-isopentenyl adenine), and kinetin [6-(furfurylamino)-purine]. Root formation was stimulated by IAA and IBA; dicamba, picloram, 2,4-D, and 2,4,5-T stimulated callus formation. All cytokinins tested suppressed root formation. Dicamba in combination with either 2iP or kinetin induced the greatest callus formation. Root formation was optimal with kinetin and either IAA or IBA. The disk method provided a rapid, nonquantitative evaluation of callus and root formation from leaf disks.

The disk method was originally devised to test microbial susceptibility to antibiotics (Barry et al. 1970, Bauer et al. 1966). In this method a disk carrying a specific drug concentration is placed on the surface of a solidified medium inoculated with viable cells. As the drug diffuses out from the disk, an inhibition zone forms which is related to toxicity and sensitivity of the organism (Cooper 1963). The method has been used with yeast (Saubolle and Howprich 1978), algae (Wright 1975), and higher plant cells (Strauss and King 1984, Strauss et al. 1984). Both inhibition (Strauss and King 1984) and exhibition zones (Strauss et al. 1984) have been observed with plant cells.

In vitro culture of plant tissue and cells may require testing of several growth hormones (auxins, cytokinins, etc.) in various combinations and concentra-

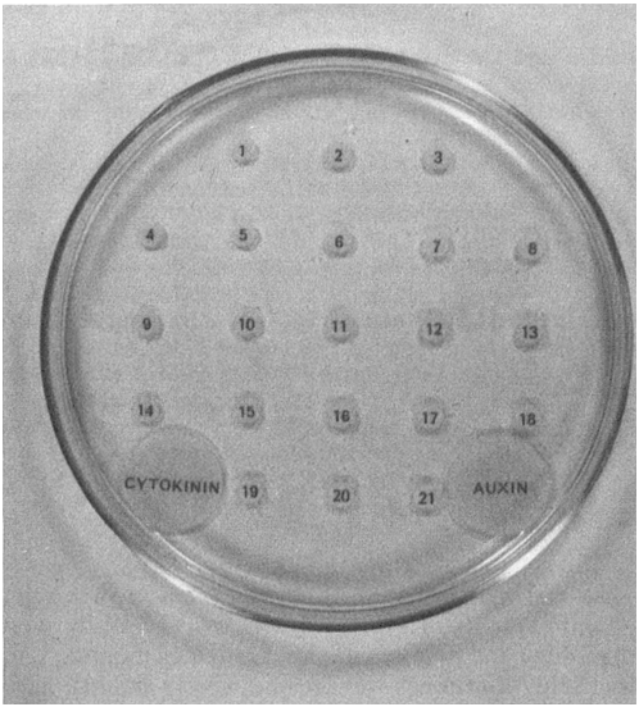

Fig. 1. Placement of filter paper disks and leaf disk explants on solidified medium.

tions. This is especially true if a new species or variety is to be cultured. The growth hormone diffuses from the disk through the medium as a function of time and establishes a concentration gradient. The disk method is rapid and provides good, qualitative indications of the tissue response. The objective of this study was to examine the application of the disk method for evaluating qualitative growth regulator responses; that is, morphological and growth behavior.

Materials and Methods

Plant Material

Seeds of *Vigna unguiculata* L. var. Magnolia Blackeye were planted in plastic or clay pots containing potting soil (5 seeds per pot) and placed in a growth room (27 ± 3°C). The first true, fully expanded leaf from 11-day-old plants was excised. The surface was then sterilized by immersion in 70% ethanol for 5 min, followed by 20% Clorox with 1 to 2 drops of Tween 20 for 5 min. Leaves were then rinsed twice in sterile distilled water. Explants (9.0 mm) were cut with a cork borer and placed on the surface of the solidified medium.

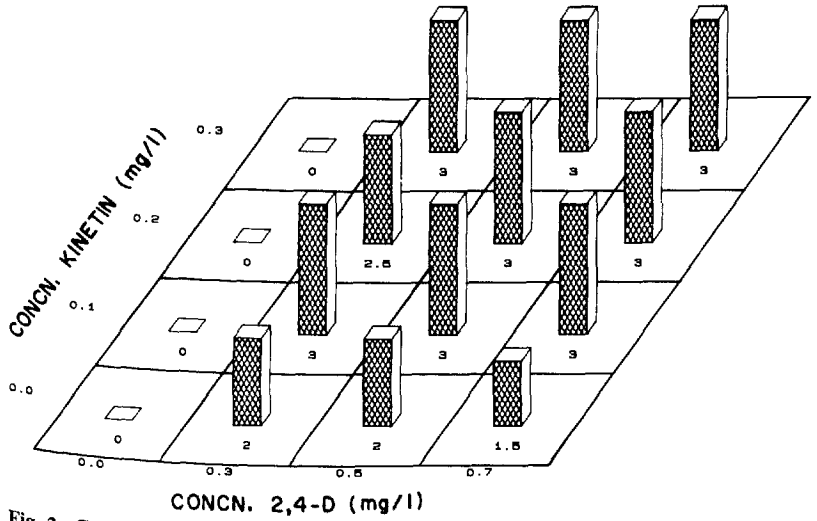

Fig. 2. Callus proliferation from *Vigna unguiculata* leaf disks in response to various levels of 2,4-D and kinetin. Callus production rated as follows and shown as numbers in each block: 0, no callus; 1, small amount of callus; 2, moderate; and 3, abundant.

Media

Medium used in all experiments was Murashige's minimal organics medium (Murashige and Skoog 1962) supplemented with 0.5 mg/l nicotinic acid and solidified with 8 g/l Bacto agar. The pH was adjusted to 5.8 and the medium autoclaved at 20 psi (1.05 kg/cm^2) and 121°C for 15 min. It was dispersed (50 ml) into 150 × 20 mm sterile Petri dishes for leaf disk tests. Medium tests with specific hormone concentrations used 25 × 95 mm culture vials (10 ml per vial) covered with Bellco Kaput® caps.

Growth regulators tested were NAA (naphthylene acetic acid) from KC Biological Inc.; IAA (indole-3-acetic acid), 2iP (2-isopentenyladenine), kinetin [6-(furfurylamino)-purine], and BA (benzyladenine) from Carolina Biological Supply Co; IBA (indole-3-butyric acid) and 2,4-D (2,4-dichlorophenoxyacetic acid) from Sigma; dicamba (3,6-dichloro-2-methoxybenzoic acid) from Velsicol Chemical Corp.; and picloram (4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid) from Dow Chemical Co. Stock solutions of all but kinetin were prepared in ethanol. Kinetin was dissolved in 1 N HCl. Final volumes were adjusted with distilled, deionized water. Media with specific regulator concentrations contained 2,4-D (0, 0.3, 0.5, and 0.7 mg/l) and kinetin (0, 0.1, 0.2, and 0.3 mg/l). Tests consisted of 2.5 cm disks of Whatman No. 40 chromatography paper saturated with a 1000 mg/l solution of the hormone. Saturated disks were autoclaved as described above. Ethanol control disks were used; no effect of ethanol-treated disks was detected.

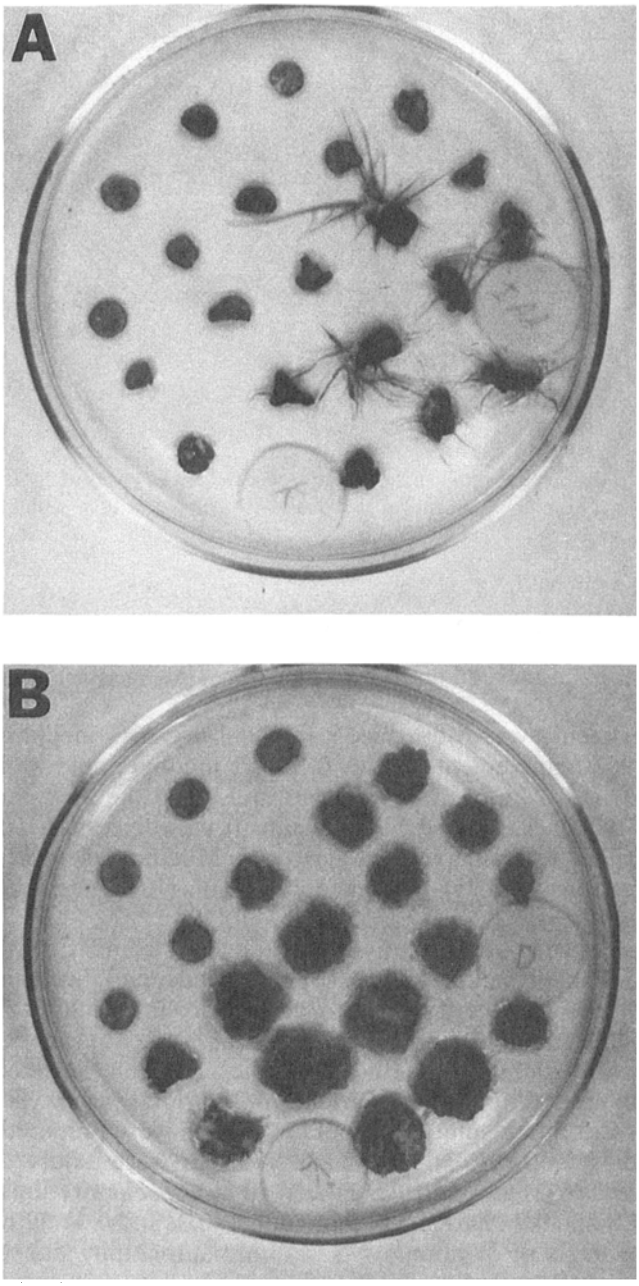

Fig. 3. Morphological response of *Vigna unguiculata* leaf disks to IAA and kinetin (A), and dicamba and kinetin (B). Each hormone disk originally contained 1000 mg/l.

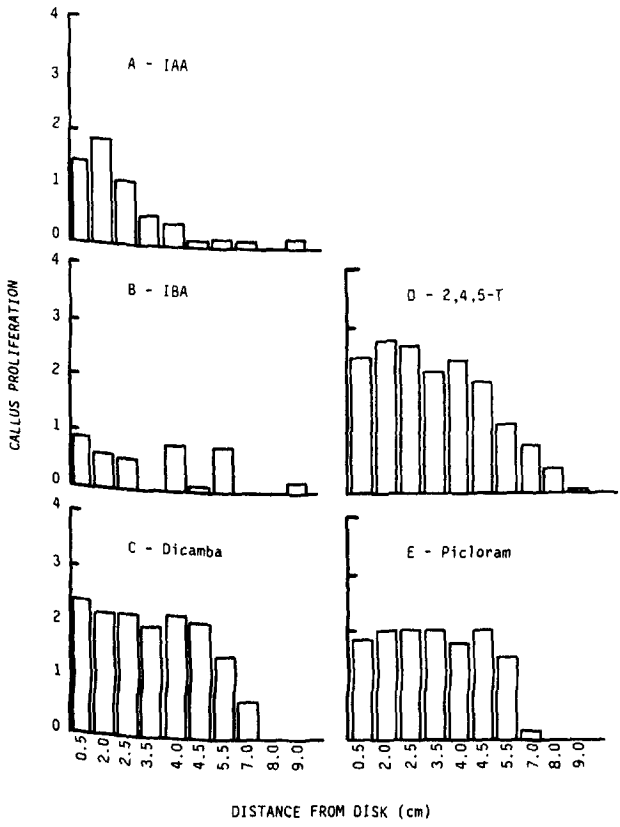

Fig. 4. Callus proliferation from *Vigna unguiculata* leaf disks in response to various auxins. Data presented as callus production (1, small amount of brown callus; 2, small amount of light brown or green callus; 3, moderate; and 4, abundant) as a function of distance from the impregnated filter paper disk.

Test Conditions

Optimum auxin (2,4-D) and cytokinin (kinetin) concentrations necessary for callus induction and maintenance were determined by testing the concentrations listed above. Leaf explants were placed with the abaxial surface in contact with the medium and incubated in the dark (28°C). After 4 weeks the tissue was scored visually for callus production, color, and root formation. The experiment contained five replicates and was repeated once.

Leaf explants were placed in a regular pattern with the abaxial surface in contact with the medium (Fig. 1). After all explants were in place, the growth regulator impregnated paper disks were placed on the medium (Fig. 1). The dishes were sealed and placed in a growth chamber (16-h photoperiod under cool white fluorescent lights, 150 ftc, 28°C) for 2 weeks. After 2 weeks the plates were scored for callus and root production (three replicates per experiment, repeated once).

Results and Discussion

Callus growth was evaluated in culture vials of media containing varying con-

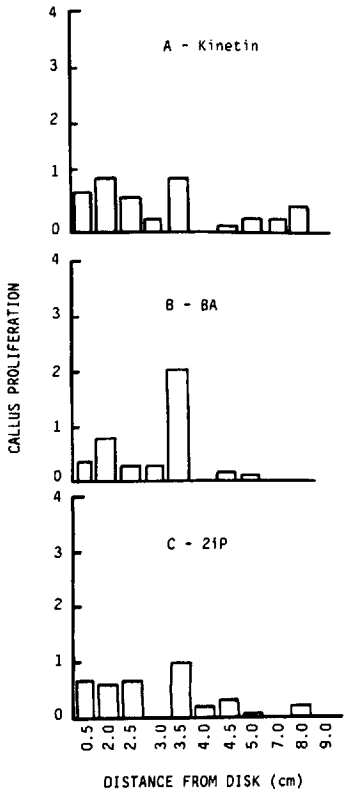

Fig. 5. Callus proliferation from *Vigna unguiculata* leaf disks in response to various cytokinins. Data presented as callus production (1, small amount of brown callus; 2, small amount of light brown or green callus; 3, moderate; and 4, abundant) as a function of distance from the impregnated filter paper disk.

centrations of 2,4-D and kinetin (Fig. 2). A small amount of callus was formed as early as 1 week after inoculation on some treatments. No callus formed in the absence of 2,4-D. Callus proliferation increased as the amount of 2,4-D increased, with the optimum callus production at 0.5 mg/l. Kinetin alone did not induce callus formation, but in combination with 2,4-D, kinetin increased callus proliferation. Callus formed on all treatments was light in color and friable. The hormone concentration that produced the most callus tissue was 0.5 mg/l 2,4-D with 0.1 mg/l kinetin. This combination was selected for callus initiation and maintenance and is similar to that used previously in callus-producing systems for cowpea (Jha and Roy 1982; Matsubara 1975). The morphological and growth response observed served as a reference for evaluating the leaf disk responses.

Representative disk responses are shown in Fig. 3. The plates shown contained two disks, each impregnated with a different regulator. Root formation was evident on leaf explants in response to IAA and kinetin (Fig. 3A). The growth response to kinetin and dicamba was primarily callus formation (Fig. 3B). In the absence of a cytokinin, 2,4,5-T stimulated the most callus growth (Fig. 4). Cytokinins alone did not cause significant callus production (Fig. 5). IAA and IBA promoted root formation; 2,4,5-T promoted limited root forma-

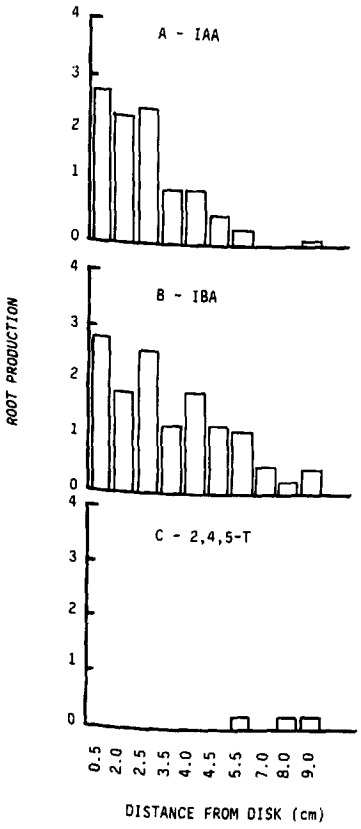

Fig. 6. Root formation from *Vigna unguiculata* leaf disks in response to various auxins. Data presented as rooting responses (1, 1 to 4 roots less than 0.5 cm; 2, 1 to 4 roots 0.5 cm or longer; 3, more than 4 roots less than 0.5 cm; 4, more than 4 roots 0.5 cm or longer) as a function of distance from the impregnated filter paper disk.

tion (Fig. 6). Leaf disks did not form roots in the presence of picloram and dicamba, and the rooting response to the cytokinins tested was similar to the level observed with 2,4,5-T. When the regulators were tested in combinations, 2iP or kinetin with dicamba induced the greatest callus formation. Kinetin or 2iP with IAA or IBA induced the greatest root formation. Fig. 7 presents data obtained with kinetin and 2,4,5-T and is an example of tissue response to regulator combinations. The variety Magnolia Blackeye responded similarly in vitro to previously tested cultivars (Jha and Roy 1982, Matsubara 1975). Approximately the same regulator ratios and concentrations were optimal for callus production. Slight variations were observed which can be attributed to cultivar responses (Malmberg 1979, Sebastian 1983). In each experiment, the leaf disk responses were the same. This indicated similar gradients were established each time.

The disk method provided a way to quickly screen several concentrations and combinations of growth regulators. However, the actual concentrations of the growth regulators tested cannot be determined by the disk method. The purpose of this method was to identify favorable growth regulator combinations for further study. An approximation of the optimal concentrations of the regulators used in the disk method may be possible by comparison with a fac-

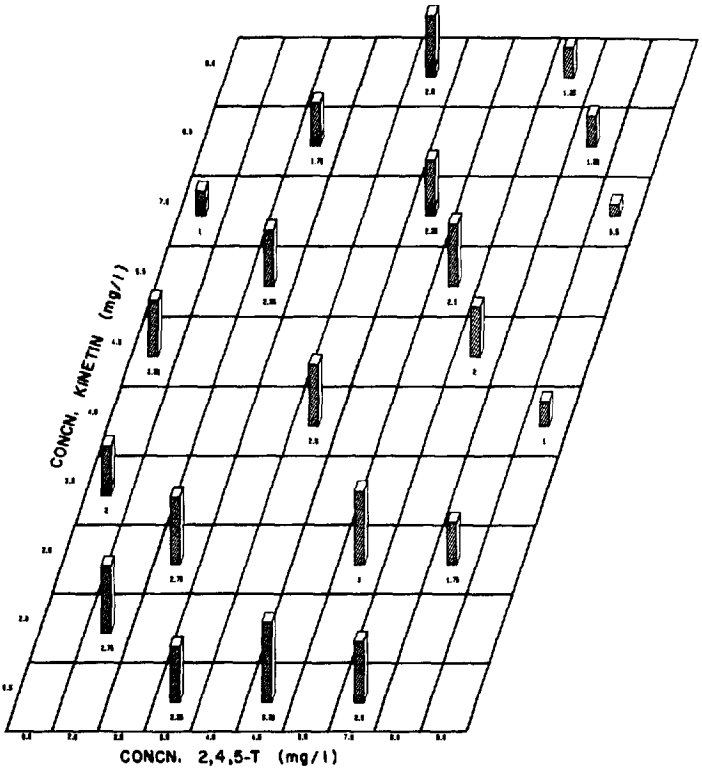

Fig. 7. Callus proliferation from *Vigna unguiculata* leaf disks in response to 2,4,5-T and kinetin. Numbers in each block reflect response as described for Fig. 4. Double cross hatching is score for brown callus.

torial test of various concentrations of hormones. Optimal concentrations of appropriate regulators and combinations for the desired growth response must be determined quantitatively.

Several factors could influence the results obtained with the disk method. The paper used for the disks should not contain soluble foreign substances. The size, diameter, or thickness of the paper disk could be increased or decreased depending upon the amount of regulator to be tested. The solubility and rate of diffusion of the regulator may affect the time during which a concentration gradient will exist in the medium.

Leaf explants were chosen for this experiment because of the uniformity of size, and both differentiated and meristematic tissues could be tested; that is, mesophyll and vascular cambium, respectively. Other modifications of this technique may include impregnating the paper disk with a known quantity of growth regulator, varying the position of the paper disks, initially incorporating other regulators into the medium, varying the number of explants per plate, and testing different types of explants.

The method described herein has several potential uses in plant tissue and

cell culture. It could identify appropriate regulators and their combinations for plants previously not cultured in vitro. A rapid screening of untested growth regulators could be accomplished. An aliquot from a cell suspension could be placed on the medium allowing growth regulator sensitivity testing or identification of cell colonies resistant to an inhibitory compound.

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