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Morphogenetic effects of 2,4-dichlorophenoxyacetic acid on pinto bean (*Phaseolus vulgaris* L.) leaf explants in vitro*

J. W. Saunders, G. L. Hosfield, and Amnon Levi

USDA-ARS Crops and Entomology Research Unit, P.O. Box 1633, East Lansing, MI 48823-6633, USA

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ABSTRACT

Roots, callus and/or globular structures were produced on primary leaf and distal cotyledon explants of pinto bean (*Phaseolus vulgaris* L. cv. *UI 114*) cultured on semisolid MS medium with a wide range of 2,4-D concentrations (0.01 to 80 mg/L) with either 0 or 1.0 mg/L kinetin. Explants rooted at lower 2,4-D concentrations than at those favoring globule formation on callus, although roots, callus and globules often developed from the same explant. Isolated opaque green globular structures developed when callus initiated on media with 3 or more mg/L 2,4-D was subcultured in liquid MS + 30 mg/L 2,4-D. These structures multiplied with a fresh weight doubling time of 8-9 days in MS + 30 mg/L 2,4-D. Although this multiplicative behavior and opaque color were reminiscent of embryoids reported for other species, no cotyledons or roots were seen.

ABBREVIATIONS

2,4-D, 2,4-dichlorophenoxyacetic acid; KIN, kinetin; MS, Murashige-Skoog medium

INTRODUCTION

Progress has been made in recent years in manipulating morphogenesis in cultured legume tissues, but consistent shoot or embryoid regeneration in the large seeded annual legume *Phaseolus vulgaris* L., the common bean, has been difficult to achieve. The only report of plant regeneration from tissues lacking initial meristematic activity involved production of only two plantlets on medium containing bean seed extract (Crocomo, Sharp and Peters, 1976b). Early stages of somatic embryogenesis were obtained by Martins and Sondahl (1984) from callus induced on shoot apices, but no further development was achieved. Allavena and Rossetti (1983) induced somatic embryos with cotyledon-like structures from leaf explants and immature embryos, but no further development occurred. All three efforts used seedling explants, and the latter two reports included use of 2,4-D.

The common use of 2,4-D in these preliminary reports of somatic embryogenesis in common bean as well as in soybean tissue cultures (Beversdorf and Bingham, 1977; Christianson et al, 1983; Lippmann and Lippmann, 1984), and the knowledge of its effects on alfalfa morphogenesis (Saunders and Bingham, 1975; Walker et al, 1978), led us to assess the effects of 2,4-D in a tissue culture system of the common bean.

MATERIALS AND METHODS

The basal medium consisted of Murashige-Skoog (MS) mineral salts (1962) with 3% sucrose, 100 mg/L *myo*-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine·HCl, and 0.1 mg/L thiamine·HCl. The growth regulators 2,4-D and kinetin in combinations of 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, 10, 20, 30, 40, or 80 mg/L 2,4-D with 0 or 1.0 mg/L kinetin were added prior to autoclaving. The pH was adjusted to 5.95 with KOH prior to autoclaving. Semisolid medium containing 0.9% Difco Bacto agar was dispensed into disposable plastic 100 x 20 mm petri plates after autoclaving. Liquid medium was dispensed into 125 ml erlenmeyer flasks before autoclaving. Both plates and flasks contained 35 ml of medium. Media were sterilized by autoclaving at 121 C for 15 minutes. Seeds of the pinto bean cultivar UI 114 were obtained from Dr. J. D. Kelly (East Lansing, Michigan) and were disinfected prior to dissection to obtain cotyledon explants. Explants were 1 cm^2 leaf discs (29±6 mg fresh weight, 4±1 mg dry weight) without the midvein from just unfolded but not fully expanded primary leaves from seedlings grown in the greenhouse in September-November, or the distal one-third portions (114±13 mg fresh weight, 64±6 mg dry weight) of mature unimbibed cotyledons. Surface sterilization was achieved with two 20 minute soaks in 15% commercial hypochlorite bleach with 0.01% sodium laurylsulfate, followed by six rinses in sterile distilled water. There was one explant per plate and plates were wrapped in parafilm strips. Plate cultures were kept in stacks at 26 ± 1 C under a 16/8 hour light/dark cycle with a light intensity of 10-65 $\mu Em^{-2}s^{-1}$ from cool white fluorescent bulbs. The light intensity at the tissue level depended on the plate position in the stack and on the shelf. Liquid cultures received 45-55 $\mu Em^{-2}s^{-1}$ continuous light from cool

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Offprint requests to: J. W. Saunders

white fluorescent bulbs and were kept on a gyrotory shaker at 150 rmp.

Response of the explants was determined in part by measuring fresh weights after 28 days. Explant and explant callus were weighed together, as in many cases the callus arose internally. Roots, root swellings and root callus were weighed together as a second group. Additional parameters measured were proportion of explants forming roots or globular structures. Ten to thirteen explants were used per treatment, and each experiment was repeated once with similar results. Data from one experiment is presented.

RESULTS AND DISCUSSION

When leaf discs and cotyledon pieces were placed on semisolid MS medium with a wide range of 2,4-D concentrations, a diversity of responses was obtained. In the presence of 1.0 mg/L KIN (Fig. 1), a small proportion of leaf discs at 0.01 and 0.03 mg/L 2,4-D produced small single roots, whereas a larger proportion produced a small dark callus. About half the explants did not respond at these growth regulator combinations. Maximum number and mass of roots occurred on the leaf discs at 0.1 and 0.3 mg/L 2,4-D, with considerable white or green callus formation on the explant as well as on some of the roots. At 1.0 mg/L 2,4-D in this series, callus production was maximized (Fig. 1). Much of the callus surface was 'frosty', with several short (up to 1 cm) roots on each. At 3.0 mg/L 2,4-D, a small proportion of the calli had a few short roots. Some of the callus surface was frosty, the rest translucent or friable. With 2,4-D at 10 or 30 mg/L, most calli had at least several smooth green or translucent globular structures up to 4 mm wide (Fig. 2). At 80 mg/L 2,4-D, some explants failed to respond, possibly because of toxicity, and the respondents developed only a few very small globules on the explant surface.

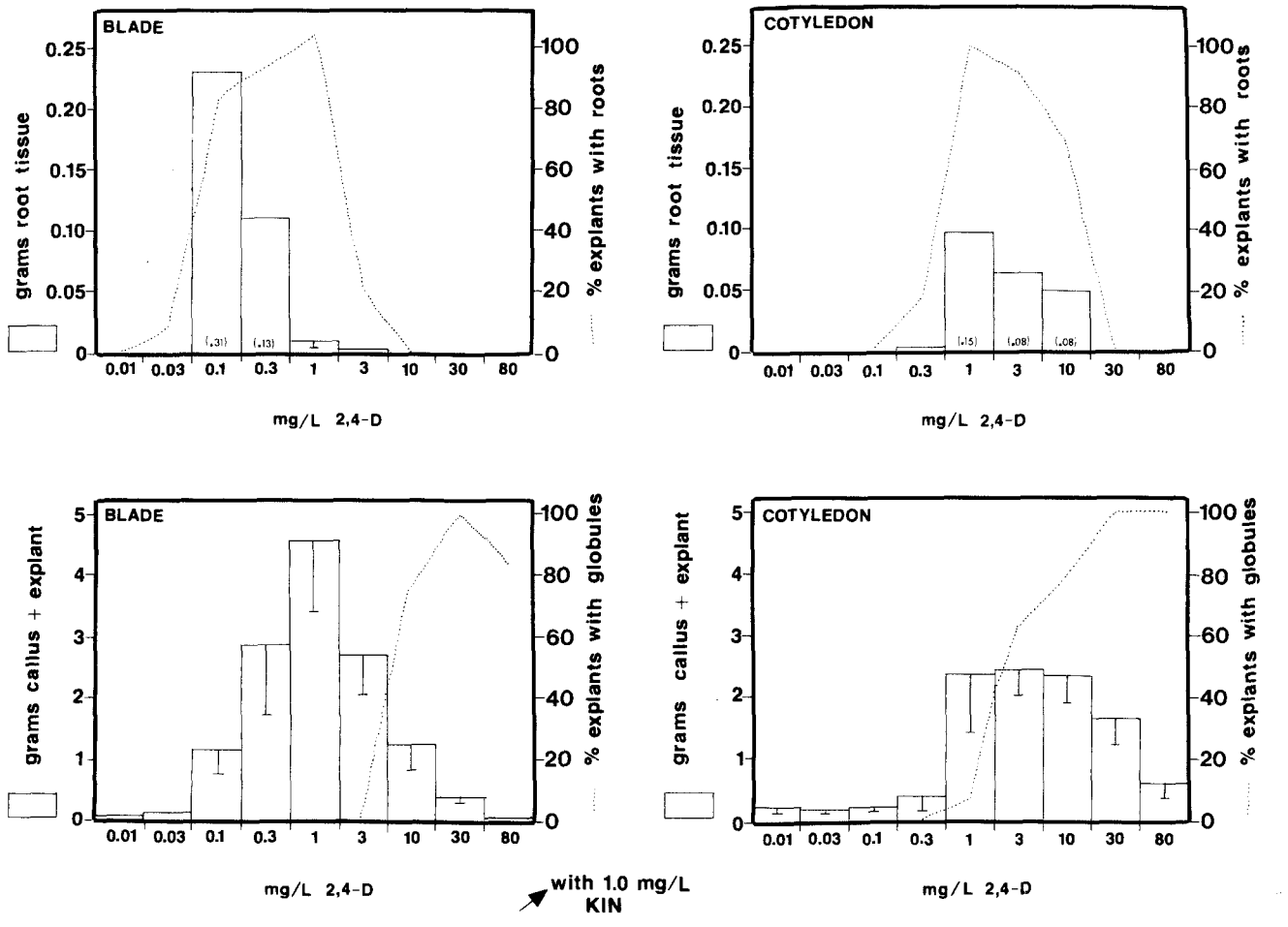

Figure 1. Effect of 2,4-D concentration on amount of root tissue and % explants with roots (upper graphs) and on amount of explant callus and % explants with globules (lower graphs) for primary leaf blade explants (left) and distal cotyledon pieces (right). Numbers in parentheses indicate standard deviations that exceed the corresponding means.

Cotyledon explants on media with 1.0 mg/L KIN responded in a similar way, except that the 2,4-D concentrations corresponding to particular responses were 3-10 fold higher. This might have been due to the greater dry weight of the cotyledon explant compared with the leaf disc. Thus, there was no response of cotyledon pieces at 0.01 and 0.03 mg/L

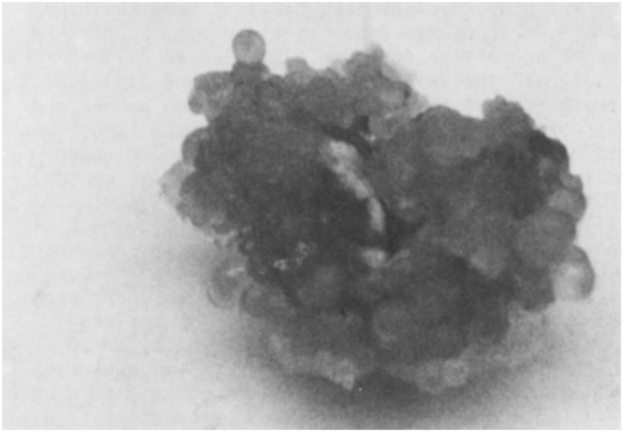

Figure 2. Globular structures on leaf disc callus after six weeks on MS + 20 mg/L 2,4-D + 1.0 mg/L KIN. Callus is 2 cm wide.

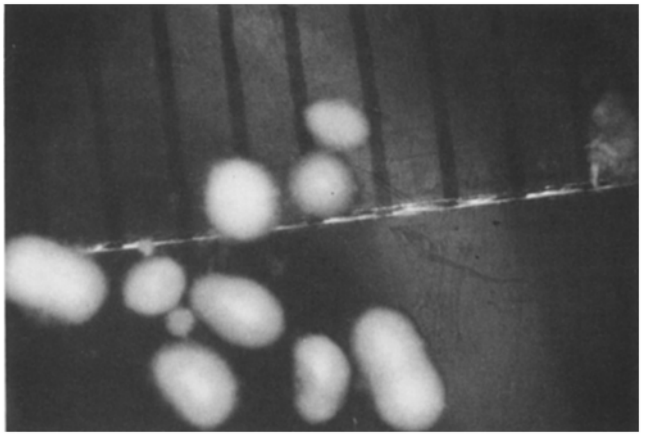

Figure 3. Globular structures from liquid culture in MS+30 mg/L 2,4-D. Linear eradations are 1 mm apart.

2,4-D, but root mass and number were maximal at 1-10 mg/L 2,4-D (Fig. 1). Smooth green or translucent globular structures were present on some calli at 1.0 mg/L 2,4-D, and the proportion of calli with these globules reached 100% at higher 2,4-D concentrations. Callus production at 80 mg/L, 2,4-D was great enough to suggest that some higher concentrations would support callus proliferation as well.

Leaf blade discs and cotyledon pieces tested against the same range of 2,4-D concentrations (0.01-80 mg/L) in the absence of KIN responded in a similar manner to those tested with 1.0 mg/L KIN. The main difference was that on average only about half as much callus was produced without KIN compared to with 1.0 mg/L KIN. Also, this callus was much less friable and was smoother than callus produced with KIN.

In these experiments the formation of roots and globules was stimulated within the range of 2,4-D concentrations employed regardless of the presence of KIN. Roots generally appeared on lower 2,4-D concentrations than globules, although at 1.0, 3.0 and 10 mg/L 2,4-D both roots and globules commonly appeared, especially on cotyledon explants. At low 2,4-D concentrations where callus production was limited, roots were few in number (Fig. 1) and were seen to arise from vascular areas on both leaf discs and cotyledons. At somewhat higher concentrations of 2,4-D permissive to rooting there were more but shorter roots, and it was difficult to ascertain whether the roots arose from the explant or from the prolific callus. Globular structures did not appear to be merely teratoid roots. Weight of roots and root callus, which sometimes formed where the longer roots contacted the agar surface, was quite variable when only few roots formed. This was reflected in the standard deviations for most of the root tissue weights being higher than the corresponding means.

Callus arose from leaf blade explants largely from the interior and not from the cut margins. Globules appeared over the callus surface without regard to proximity of the agar surface. At 40 or 80 mg/L 2,4-D some globules arose in the absence of any visible callus.

When four week old callus containing the globular structures was placed in liquid MS + 30 mg/L 2,4-D in the light, within two weeks opaque green globular or discoid structures up to four mm across were found free in the medium (Fig. 3) or embedded in the remaining callus clump. Upon transfer to fresh liquid MS + 30 mg/L 2,4-D, these globular structures proliferated with a fresh weight doubling time of 8-9 days with very little growth of the remnant callus.

Several subcultures were made at three week intervals without these cultures losing their character.

The smooth globular structures were also seen on callus growing from seedling hypocotyl explants on MS + 30 mg/L 2,4-D + 1.0 mg/L KIN, and on cotyledon callus of the white bean cultivar Fleetwood on the same medium. Globular structures taken from liquid MS + 30 mg/L 2,4-D and placed on semisolid MS with no supplements, MS + 1.0 g/L yeast extract, or MS + 30 mg/L 2,4-D did not develop into somatic embryos.

Because these globules are self-replicating in the manner of embryoids, yet lacking either root or shoot apices, they probably can be considered neomorphs in the sense of Krikorian and Kann (1981) and Christianson et al (1983). We feel that they are aberrant forms of somatic embryos and as such warrant further research. The globule cultures can grow in a sustainable manner without added cytokinin. This may be related to the cytokinin autonomy reported for some bean genotypes (Mok et al, 1980).

Rhizogenesis from bean leaf sections had been explored previously by Peters et al (1976), Crocomo, Peters and Sharp (1976), and Tonin et al (1981). Bevan and Northcote (1979) examined the loss of morphogenetic potential in bean suspension cultures maintained on 2,4-D. Mok and Mok (1977) and Kim et al (1982) examined the effect of a range of 2,4-D concentrations on subcultured hypocotyl callus, but no roots or globular structures were reported. This paper is the first to report the relationship of 2,4-D to in vitro morphogenesis in bean.

Induction of competent somatic embryogenesis has been elusive with *Phaseolus vulgaris*. This study has identified 2,4-D as an agent that induces the formation of green globular structures appearing to be aberrant forms of somatic embryos. Further effort is needed to identify factors that will interact with 2,4-D to promote the induction of viable somatic embryos in common bean.

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