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Auxin Activity of Phenylacetic Acid in Tissue Culture

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Abstract. The ability of phenylacetic acid (PAA), a naturally occurring auxin, to initiate and support growth of callus and suspension cultures of several species is reported. Callus tissue of tobacco (*Nicotiana tabacum* L. var. WI-38), initiated and maintained on a medium with 2,4-dichlorophenoxyacetic acid (2,4-D), was transferred to and maintained on media supplemented with 25–500 μM PAA as the only plant growth regulator (PGR). Optimal concentrations of PAA were determined for tobacco callus proliferation in the dark (250 μM PAA) and with a 16-h light/8-h dark photoperiod (500 μM PAA). Tobacco suspension cultures were maintained for over 28 transfers in media containing 20–40 μM PAA as the sole PGR. When tobacco callus tissue maintained on PAA-supplemented media for over 18 months was transferred to liquid media containing kinetin, plantlets were regenerated. Callus of sunflower (*Helianthus annuus* L. var. Russian Mammoth) proliferated on media containing PAA at 5–250 μM as the sole PGR. Similar PAA concentrations inhibited normal development and promoted callus formation in tobacco and pea (*Pisum sativum* L. vars. common, Frogel, and Frimas) epicotyl tissue. PAA as the sole PGR did not support the growth of soybean (*Glycine max* (L.) Merrill var. Fiskeby) callus or suspension cultures. Chickpea (*Cicer arietinum* L. var. UC-5) and lentil (*Lens culinaris* Medic. var. Laird) callus cultures proliferated on media containing 25–500 μM PAA, but habituation of the cultures was common. PAA was not toxic to tobacco, chickpea, and lentil tissues at levels as high as 500 μM.

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The auxin-like activity of phenylacetic acid (PAA) was first recognized in the mid-1930s (Haagen-Smit and Went 1935, Zimmerman and Wilcoxon 1935). The reported responses of plants to PAA have varied from anesthesia of growing parts to initiation and stimulation of adventitious roots. PAA showed activity in indoleacetic acid (IAA) bioassays but was active only at much higher concentrations than IAA. PAA possessed only weak auxin activity when compared to IAA, IPA (indolepropionic acid), IBA (indolebutyric acid), and NAA (naphthaleneacetic acid) (Thimann and Schneider 1939). Interest in PAA waned, and research with PAA and its derivatives was primarily limited to studies on the structural requirements for compounds with auxin activity (Chamberlain and Wain 1971, Pybus et al. 1959).

Interest in PAA as a plant growth regulator (PGR) was stimulated by its discovery in algae (Abe et al. 1974) and higher plants (Wightman and Rauthan 1974). Wightman and Lighty (1982) further identified PAA in plant extracts from six different plants and demonstrated auxin activity of the extracted PAA in the *Avena* coleoptile bioassay. In the investigated plants, PAA occurred at concentrations four to six times higher than IAA. PAA has since been shown to influence morphoregulation (Procházka et al. 1983, Suttle and Mansager 1986), and its effects on enzymatic activity and expression have been investigated (Machackova et al. 1981, Scherer 1981). Very recent work has demonstrated that PAA may be involved in the regulation of auxin transport (Johnson and Morris 1987).

To our knowledge only one study on the effects of PAA in a tissue culture system has been published (Milborrow et al. 1983). These investigators demonstrated that PAA plus kinetin in a solid medium would support tobacco callus proliferation. We have recently shown that PAA is stable in tissue culture media (Leuba et al. 1989). The purpose of

the current study was to determine the auxin activity of PAA in culture establishment and maintenance, and to demonstrate the regenerative potential of tobacco callus maintained on a PAA-containing medium.

Materials and Methods

Preparation of Media

All media were prepared from stock solutions. Final concentrations of inorganic salts and organic supplements (vitamins) and sucrose were as given in Dodds and Roberts (1985) except, unless otherwise indicated, all MS media were prepared with the B5 vitamin mix. Additions of PGRs are noted as they occur. Media were prepared with Millipore-filtered deionized water and solidified, when necessary, by the addition of 0.8% Bacto-agar after pH adjustment. All B5 media were adjusted to a pH of 5.5–5.6, and all MS media were adjusted to a pH of 5.7–5.8. Complete media were autoclaved for 15 min at 1.05 kg/cm^2.

Initiation and Maintenance of Tobacco Callus and Suspension Cultures

Two separate tobacco callus cultures were used in long-term callus proliferation experiments. One callus culture of *Nicotiana tabacum* L. var. WI-38 (obtained from Dr. D. Oliver, Department of Bacteriology and Biochemistry, University of Idaho) was maintained on a MS medium supplemented with B5 vitamins and 5 μM 2,4-dichlorophenoxyacetic acid (2,4-D). Seeds of *Nicotiana tabacum* L. var. WI-38 (obtained from Dr. D. Oliver) were used to initiate a second callus culture. Seeds were surface sterilized, placed in 60-ml jars containing 25 ml of solid B5 medium without PGRs, and germinated at 25°C with a 16-h light/8-h dark photoperiod of 1200 lux. After 5 days, epicotyl tissue from 8–10 seedlings was placed in 50 ml of liquid B5 medium supplemented with 5 μM 2,4-D in 250-ml Erlenmeyer flasks. Flasks were agitated on a gyratory shaker at 125 rpm in the dark at ambient (22–27°C) temperature. After 2 weeks, 10 ml of the suspension was transferred to 50 ml of fresh B5 medium containing the same 2,4-D supplement. The resulting culture was subsequently subcultured (10 ml of suspension to 50 ml fresh medium) every 7–10 days. After the fourth subculture, 1 ml of the suspension culture was transferred to 25 ml of the same medium solidified with 0.8% agar in 60-ml jars. The jars were loosely capped and incubated at 26°C in the dark for 31 days. Light, friable callus was obtained and subcultured to solid medium approximately monthly for three more months prior to use in the long-term proliferation experiments.

Epicotyl tissue of aseptically germinated WI-38 seedlings was cultured directly onto B5 media containing 5, 20, and 40 μM PAA to test for callus induction. Controls contained no exogenous auxin.

A liquid culture of WI-38 that had been maintained for several years on MS medium supplemented with 2,4-D was subcultured to MS media containing 0, 20, or 40 μM PAA. The suspension cultures were transferred every 7–10 days.

Long-term Tobacco Callus Proliferation on PAA

The healthiest callus material from both the newly established tobacco callus and the older tobacco callus was multiplied to supply sufficient material (~20 g) to inoculate 40 jars of media. Each jar was inoculated with about 0.5 g callus tissue, equivalent to a dry weight of roughly 50 mg. Eight exogenous auxin levels were tested for callus maintenance: 0, 2.5, and 5.0 μM 2,4-D; and 25, 50, 100, 250, and 500 μM PAA. The media tested contained no exogenous cytokinin. In one experiment, callus was allowed to grow at 26°C in the dark. In a second experiment, callus was grown at 26°C with a 16-h light/8-h dark photoperiod with a light intensity of 1600 lux. The initial transfer of callus growing on 2,4-D-containing medium to the eight experimental media was considered generation 1. Subsequent transfers were made to the same experimental medium and are referred to as generation 2, 3, etc., respectively. The growth period of each generation was approximately 1 month. Five jars of each medium type were inoculated for each generation. If sufficient inoculum could not be obtained from a single jar of callus, two or more jars of callus were combined to provide inoculum. At the end of the growth period, all calli not needed for inoculation of a subsequent generation were carefully lifted from the medium and individually weighed in preweighed dishes. The tissue was dried to constant weight at 50°C. Data obtained from both the dark- and photoperiod-incubated experiments were subjected to a global statistical analysis performed by the general linear model (GLM) of SAS (SAS Institute, Inc. 1985). For each experiment, a least significant difference (LSD) was computed by Fisher's least significance procedure (Ott 1977).

Tobacco Plantlet Regeneration

Liquid B5 media without PGRs or supplemented with NAA or PAA, both with and without 2.2 μM kinetin (conditioning media), were inoculated with tobacco callus established and grown for 18–22 months on 40 μM PAA in B5 medium. Liquid cultures were maintained on a gyratory shaker at 125 rpm in the dark at ambient temperature (20–27°C). After 14–24 days, a portion of the parent culture was transferred to solid B5 medium containing no PGRs. Cultures on solid media were incubated at 26°C in a continuously lighted incubator and scored for plantlet development after 14–21 days.

Initiation and Maintenance of Other Callus and Suspension Cultures

After sunflower (*Helianthus annuus* L. var. Russian Mammoth) seeds of local origin were aseptically germinated on moist filter paper in petri plates, the cotyledons were roughly chopped for explant material. The chopped cotyledons from one seed were placed on 25 ml of solidified B5 medium supplemented with 5 μM 2,4-D in 60-ml jars and incubated in the dark at ambient temperature for 30–45 days. After at least six subcultures on 2,4-D-containing medium, approximately 0.5 g portions of callus were transferred to experimental media.

To test the potency of PAA for callus induction, pea seeds (*Pisum sativum* L. vars. Frogel, Frimas, and common; obtained from Dr. D. Auld, Department of Plant, Soil, and Entomological Science, University of Idaho) were aseptically germinated as described for sunflower, and epicotyl tissue was explanted to solidified B5 media supplemented with PAA. Levels of PAA and controls were the same as those described for callus induction from tobacco seedlings.

Soybean (*Glycine max* (L.) Merrill var. Fiskeby), lentil (*Lens culinaris* Medic. var. Laird), and chickpea (*Cicer arietinum* L.

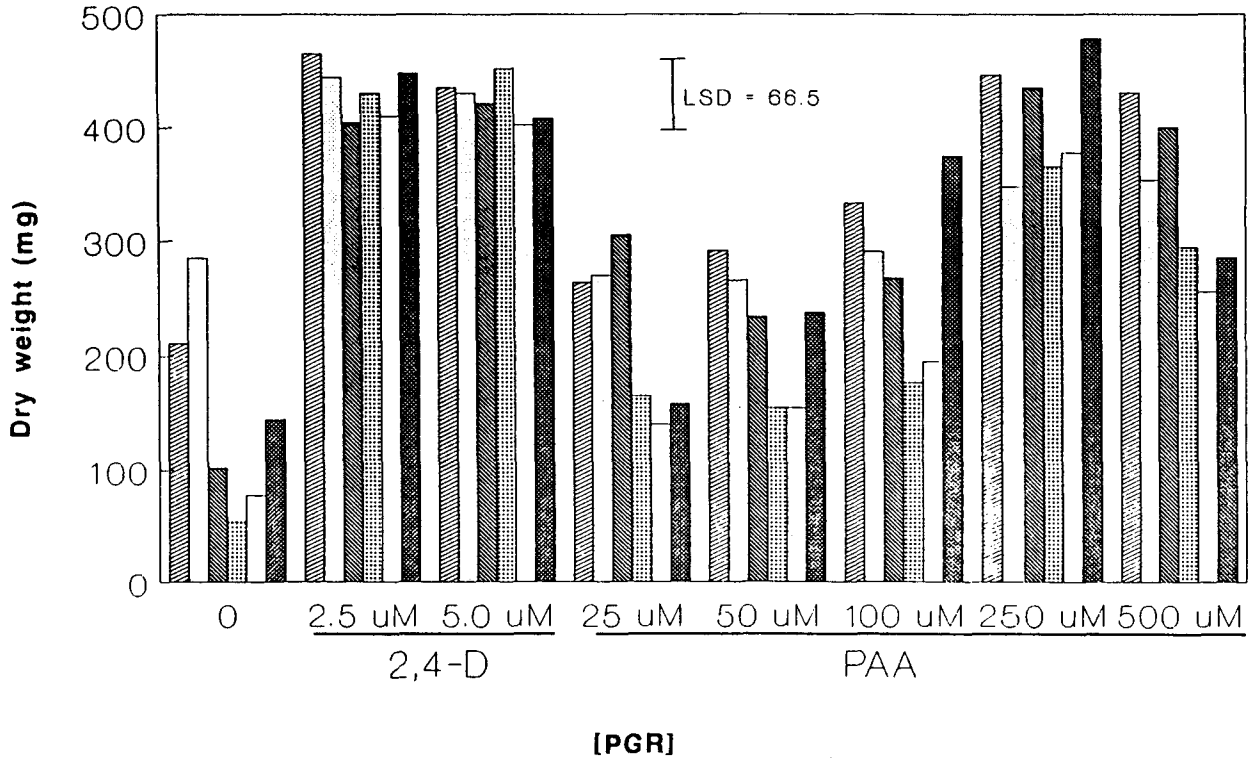

Fig. 1. Dry weight of tobacco callus (WI-38) grown in the dark on MS medium supplemented with various concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) and phenylacetic acid (PAA). Generation: 1, ▨; 2, □; 3, ▧; 4, ▤; 5, □; 6, ▩.

var. UC-5) callus maintained for several years on 5 μM 2,4-D on MS or B5 media were used to determine if PAA could replace 2,4-D as the auxin.

Results and Discussion

Long-term Tobacco Callus Proliferation on PAA

Tobacco callus in culture for several years and tobacco callus of relatively recent origin were tested for callus maintenance on eight different PGR treatments. Data presented are from the callus previously cultured for several years. Results from the more recently established callus were essentially identical.

In the dark, 250 μM PAA promoted growth comparable to 2,4-D at 2.5 and 5 μM. Figure 1 presents the data graphically with all generations of each treatment grouped together for comparison. The highest concentration of PAA tested (500 μM) appeared to inhibit tobacco callus proliferation after three generations. The dry weight increase, which occurred at generation 2 on a medium without PGRs, may have been due to a supraoptimal concentration of 2,4-D in the medium of the parent culture. By generation 3 most carry-over effects of 2,4-D were diluted out of the tissue, and an accurate representation of the callus proliferation activity of PAA obtained. Calli on medium without PGRs and with the lower PAA concentrations did not proliferate well and formed hard, compact masses of beige to dark brown. All other calli on 2,4-D and higher (≥100 μM) PAA concentrations formed friable, beige masses. By generation 6 the tissue had begun to adapt to the new PGR, and the increase in proliferation on 50, 100, and 250 μM PAA may have been due to an increased sensitivity of the tissue for the new auxin.

Under photoperiod conditions, 250 and 500 μM PAA promoted growth as well as, if not better than, 2.5 and 5 μM 2,4-D. Figure 2 shows results from all generations of each treatment grouped together for comparison. Under photoperiod conditions the initial increase at generation 2 on medium without PGRs was missing. Calli on lower PAA concentrations did not proliferate well and formed hard, compact masses with intense green areas. Calli on higher (≥100 μM) PAA concentrations formed friable, pale green masses, whereas calli on 2,4-D treatments formed a friable, beige mass.

During the course of the experiments, auxin habituation was tested by subculturing callus growing on 500 μM PAA to B5 medium containing no PGRs. This callus did not proliferate. Therefore, PAA acted as an auxin but was not sequestered or syn-

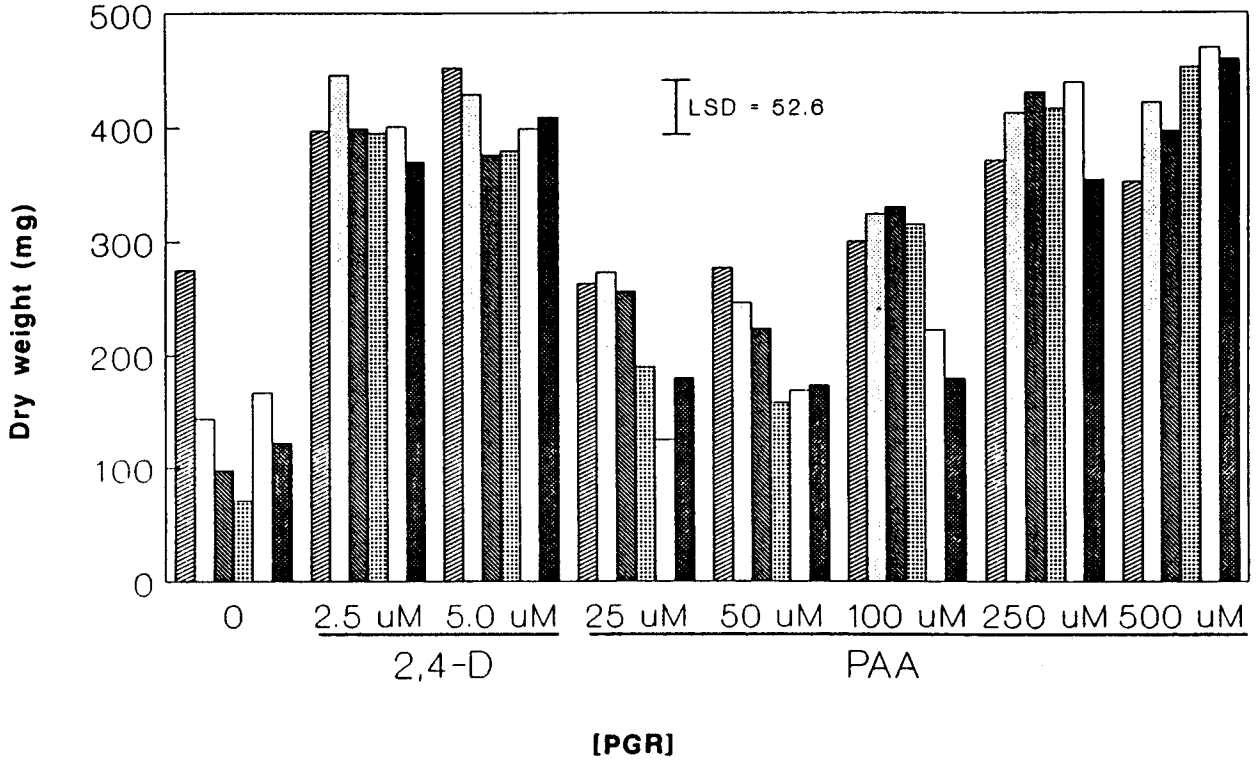

Fig. 2. Dry weight of tobacco callus (WI-38) grown in a 16-h light/8-h dark photoperiod on MS medium supplemented with various concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) and phenylacetic acid (PAA). Generation: 1, ▨; 2, ▩; 3, ▧; 4, □; 5, ⬚; 6, ▩.

thesized in tobacco callus at sufficient levels to support callus growth. In contrast, callus subcultured from B5 medium containing 2.5 or 5 μM 2,4-D to B5 medium containing no PGRs had to be subcultured a second and often a third time before the callus ceased to proliferate. This was attributed to a carryover effect of 2,4-D. Callus could be transferred from 2,4-D medium to media containing the higher PAA concentrations and vice versa with no observable decrease in the proliferation of callus tissue. Under both photoperiod and dark conditions, transfer beyond six generations of callus grown on medium containing no PGRs or lower PAA concentrations was hindered by the lack of inoculum.

Both Figs. 1 and 2 include the LSD. Inspection of the graphs shows there is no significant difference under dark conditions between 2.5 μM 2,4-D treatment and 250 μM PAA treatment in generations 1, 3, 5, and 6. Under the specified photoperiod conditions, there is no significant difference between the 2.5 μM 2,4-D treatment and 250 μM PAA treatment in any generation. This result varies from the optimum concentration of PAA for tobacco callus growth previously determined (Milborrow et al. 1975). They found PAA to be 30% as effective as IAA in stimulating tobacco callus proliferation at optimum PAA (73 μM) and IAA (20 μM) concentrations in the presence of 0.01–0.04 mg/L kinetin. Milborrow et al. (1975) suggested inhibitory metabolites may accumulate at PAA concentrations greater than 73 μM. Our data does not support such an interpretation; concentrations of 250 μM PAA, in both the photoperiod and the dark, stimulated callus proliferation. At 500 μM PAA under photoperiod conditions, PAA was more effective than 2,4-D at stimulating tobacco callus proliferation.

The global statistical analysis performed by the general linear model (GLM) of SAS (SAS Institute, Inc. 1985) showed a significant interaction between treatment and generation for all PAA treatments under both photoperiod and dark conditions. The 2,4-D treatments did not have a generation/treatment interaction; this was probably due to growth of starting material on a 2,4-D containing medium.

Plantlet Regeneration After Sustained Growth on PAA

Plantlets regenerated when tobacco callus, cultured for 18–22 months on solidified B5 medium supplemented with 40 μM PAA, was transferred to a liq-

Table 1. Plantlet regeneration from WI-38 callus maintained on B5 medium containing phenylacetic acid (PAA) after incubation in a liquid conditioning medium containing kinetin and various concentrations of PAA or naphthaleneacetic acid (NAA) and subsequent transfer to solidified B5 without PGRs.

	Kinetin	
Auxin	0 μM	2.2 μM
0	ϕ	+ + +
5 μM NAA	○	○
5 μM PAA	○	○
10 μM PAA	○	○
50 μM PAA	○	+ + +
100 μM PAA	○	+ + +
250 μM PAA	○	+ +
500 μM PAA	○	+

ϕ, No growth or regeneration; ○, cell proliferation, no clumping or regeneration; +, cell clumping, subsequent regeneration—slow and low frequency; + +, higher frequency than +; + + +, highest frequency of regeneration.

uid conditioning media containing a cytokinin. The media that conditioned plantlet regeneration among the matrix of media tested is shown in Table 1. After 14–21 days in conditioning media, cell clumps were transferred to solidified B5 medium without PGRs. If the liquid conditioning medium contained PAA in a sufficient concentration, normal plantlets developed. Plantlets appeared watery and abnormal when the conditioning medium lacked PAA. In the presence of 5 μM NAA, suspension cultures established, but cells did not proliferate nor did plantlets form when aliquots of the suspension were transferred to solidified B5 medium without PGRs. Other concentrations of NAA were not tested.

These findings support the theory proposed by Skoog and Miller (1957) concerning control of organogenesis by the auxin to cytokinin ratio. In addition, plantlet development may be influenced favorably by transfer from solid media to liquid media (White 1939, as found in Thorpe 1980).

Callus Induction and Maintenance of Callus and Suspension Cultures on PAA

Tobacco epicotyls incubated in the dark on B5 media supplemented with PAA responded in a concentration-dependent manner. In the absence of PGRs and at 5 μM PAA, tobacco stems elongated and produced normal shoots. At 20 μM PAA, development was altered: shoots were shorter and thickened and some callus was produced. Normal development was completely inhibited and callus formed prolifically at 40 μM PAA.

Epicotyl sections of pea on PAA-supplemented media responded in a variety-specific manner. Epicotyls of common pea explanted to medium containing no PGRs or to media containing PAA in concentrations of 25 and 50 μM did not form callus and shoots elongated. At 100 μM PAA, shoot elongation was inhibited and callus tissue formed. Callus formation was predominant above 250 μM PAA. There were no toxic effects even at 500 μM PAA, the highest concentration tested. Epicotyls from Frogel and Frimas peas formed callus at 5 μM PAA. This callus tissue was habituated and grew equally well on a medium containing no PGR. A slight inhibition of proliferation, however, was observed on 2,4-D-containing media. Callus cultures of Frogel and Frimas peas initiated on PAA-supplemented media were subcultured and maintained on a 5 μM PAA medium for over 2 years. Previously, Ponappa (1987) found the addition of 5 μM BA to the medium reduced the minimum concentration of PAA required to initiate callus induction of lentil from 50–0.5 μM.

Tobacco suspension cultures transferred from MS medium containing 5 μM 2,4-D were maintained on MS media with either 20 or 40 μM PAA as the only PGR for over 24 months.

Sunflower callus was maintained on media with PAA as the sole PGR for over 6 months and was not habituated. PAA levels of 5–500 μM in B5 media supported callus tissue proliferation. If not subcultured, the sunflower callus grown on 5 μM 2,4-D in B5 medium browned and died after approximately 21 days. All tissue grown on media containing PAA remained friable and creamy for up to 6 weeks. No attempts to regenerate this material were made.

Soybean callus cultures did not proliferate on media containing PAA as the sole PGR. After initial transfer to PAA-containing media, cells turned brown and died. Similar results were obtained with a soybean suspension culture, but 250 μM PAA in combination with 5 μM 2,4-D did not appear to be toxic. In contrast, both lentil and chickpea callus cultures grew well on all levels of PAA tested; they also grew in the absence of any PGR and were habituated. Thus, while the tissue did not need an auxin, PAA was not toxic to lentil and chickpea calli at PAA concentrations up to 500 μM.

In these studies PAA acted as an auxin in a number of tissue culture systems. The effective concentration of PAA for auxin activity in most systems was quite high compared to other auxins used in tissue culture. The mode of action of auxins is not

understood, but it seems doubtful that the mode of action of PAA is identical to the mode of action of other auxins. Normal plantlets readily regenerated from suspension cultures with PAA and with a high frequency. This may indicate that PAA does not interfere with the regenerative potential of the parent culture.

Because of its presence in plants at significant concentrations, its ability to serve as an auxin in several tissue culture systems, and its involvement in auxin transport regulation, PAA warrants continued investigation. Determining the role of PAA in plants should help elucidate the function and interactions of other PGRs.

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