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# Effect of fluorophenylalanine on indole-3-acetic acid levels in *Avena* coleoptiles

ANDREW B. MAKSYMOWYCH[1], JOSEPH A.J. ORKWISZEWSKI[2]

[1] Department of Biology, Bryn Mawr College, Bryn Mawr, Pennsylvania 19010, U.S.A.
[2] Department of Biology, Villanova University, Villanova, Pennsylvania 19085, U.S.A.

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Abstract

The effect of the amino acid analogue D,L-p-fluorophenylalanine on indole-3-acetic acid levels in *Avena* has been examined. Previous studies have established that D,L-p-fluorophenylalanine promotes elongation, lowers phenolic levels and depresses auxin oxidase activity of etiolated *Avena* coleoptiles. This study employs an enzyme immunoassay to measure endogenous indole-3-acetic acid concentrations in coleoptile apices. These data demonstrate that treatment of *Avena* coleoptiles with D,L-p-fluorophenylalanine results in altered auxin levels and help clarify the mechanism of D,L-p-fluorophenylalanine action in *Avena*.

*Key words: Avena coleoptile, enzyme immunoassay, fluorophenylalanine, indole-3-acetic acid, phenolic metabolism*

## INTRODUCTION

The ability of D,L-p-fluorophenylalanine (p-FPA) to stimulate the elongation of etiolated *Avena* coleoptile apices was first reported by Hopkins and Bonnell (1969). Subsequently, the promotion of growth by this amino acid analogue was shown to be both isomer and substituent specific (Hopkins and Orkwiszewski 1971, Orkwiszewski et al. 1976, Maksymowych and Orkwiszewski 1983, 1987)

Further studies have shown that p-FPA stimulation of coleoptile growth was correlated with lowered L-phenylalanine ammonia-lyase (PAL, E.C.

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[2] To whom reprint requests should be sent.

4.3.1.5) activity, depressed phenolic levels and decreased auxin oxidase activity (Orkwiszewski and Hopkins 1974, Orkwiszewski et al. 1976, Maksymowych and Orkwiszewski 1983, 1987). These authors proposed that enhanced elongation in response to p-FPA treatment stems from a depression of PAL activity resulting in lower levels of potentially phytotoxic low molecular weight phenols which, directly or indirectly, lower auxin oxidase activity.

To date studies have not included measurements of the effect of p-FPA on endogenous indole-3-acetic acid (IAA) levels. The purpose of this paper is to determine whether or not his amino acid anlogue exerts an effect on IAA content as measured by the enzyme immunoassay technique.

## MATERIALS AND METHODS

Unhusked seeds of *Avena sativa* L. cv. Clintford (The Stanford Seed Co., Denver, Pennsylvania) were grown as described by Hopkins and Bonnell (1969). The etiolated shoots were harvested after 4 days and thoroughly washed with glass-redistilled water. Plant material used throughout this study was 8 mm apical coleoptile segments minus primary leaves which were excised by hand. The segments were collected, randomized in 20 mM phosphate buffer (pH 6.3) and distributed into 50 $cm^3$ beakers containing 5 $cm^3$ of the incubation medium. The incubation medium for control segments consisted of the above phosphate buffer plus 1.5% w/v sucrose (BSM). Treated segments were incubated on BSM containing 5 mM p-FPA or 5 mM p-FPA + 5 mM L-phenylalanine (Phe). Coleoptile segments were incubated for 18 hr at $26 \pm 1°C$ (Orkwiszewski et al. 1976). A group of segments was removed prior to incubation and considered to be the 0 hr time point. All studies involving living tissue were conducted either in darkness or under a dim green safelight (Hopkins and Bonnell 1969).

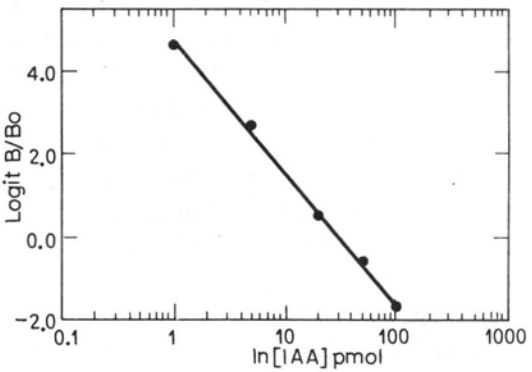
**IAA purification**. Extraction and purification of endogenous IAA for enzyme immunoassay (EIA) was based on established procedures (Bandurski and Schulze 1974, Weiler et al. 1981, Konig 1983, Rivera et al. 1986). During extraction and analysis, all samples and fractions were protected from overhead fluorescent lights to minimize photooxidation. Coleoptile apices were grown and treated as described above. After the appropriate 18 hr incubation, coleoptiles were drained on a strainer and rinsed with glass-redistilled water. Coleoptile apices (15 per treatment group) were blotted and dried for 5 min on filter paper to remove excess moisture and transferred to preweighed vials containing 3.0 $cm^3$ of 80% HPLC grade MeOH:H20 (v/v) with 10 $\mu g\ cm^{-3}$ BHT and 0.1 mM ammonium acetate. The tissue was homogenized with a Brinkmann (11 mm probe) Polytron at full speed, 45 sec, ice bath. Sample vials were wiped dry and weighed to determine gram fresh weight of sample,

then placed in darkness and extracted at 3°C for 12 hr. Subsequently, the extracted samples were centrifuged at 25,000 × g, 3°C, for 30 min. The supernatant was decanted and held at 3°C in darkness. Pellets were washed in 3 $cm^3$ of extraction media and recentrifuged. Second supernatants were combined with the first and the sample was evaporated to aqueous volume under vacuum in a Brinkmann rotary flash-evaporator. Three $cm^3$ of glass-redistilled water were added to the aqueous fraction to facilitate handling. Samples were acidified to pH 2.5 with 1 N $H_2SO_4$ and partitioned against 3 × 1 volumes of ether. The organic phases were pooled, evaporated to original volume and partitioned against 1 N $NaHCO_3$. The aqueous phase was washed with 3 × 1 volumes of ether, slowly acidified to pH 2.5 (conc. $H_2SO_4$) and partitioned against 3 × 1 volumes of ether. Ether phases were pooled and washed with glass-redistilled water. The organic fraction was evaporated to the water dissolved in the ether and 3.0 $cm^3$ of HPLC grade MeOH were added to the sample. Samples were methylated by treatment (5 min) with an excess of freshly prepared ethereal diazomethane, 0°C. Excess diazomethane was destroyed by dropwise addition of 1 N acetic acid and the samples were evaporated do dryness. Residues were taken sequentially into 3.0 $cm^3$ MeOH and 0.7 $cm^3$ Tris-buffered saline (TBS), pH 7.5. Samples were held at 3°C for EIA.

**Enzyme immunoassay**. IAA recrystallized from MeOH was methylated as described above and used for the EIA standard curve. Commercially prepared IAA-methylester (K&K Labs – ICN Biomedicals, Inc.) was used as a comparative standard with the laboratory prepared IAA-methylester. Samples were analyzed for IAA content by a commercially available EIA for IAA (Phytodetek-IAA, IDETEK, Inc., San Bruno, California) according to established procedures (Weiler 1981, Weiler et al. 1981, Mertens et al. 1985). Stock solutions (1 mM) were prepared in absolute MeOH from the IAA-methylester prepared in the laboratory and from commercial IAA-methylester. Stocks were further diluted in 25 mM TBS, pH 2.5. A minimum of 5 points at 1, 5, 20, 50 and 100 pmol per assay along with Bo (100% binding) and a non-specific binding standard (NSB; 500 pmol per assay) were prepared for the standard curves. Appropriate numbers of microplate reaction strips were placed in a strip holder and 100 $mm^3$ of standard or sample were added to each well. All standards and samples were assayed in triplicate. 100 $mm^3$ of tracer (enzyme coupled IAA) were added to each well mixed, covered with a plate sealer and incubated at 3°C for 3 hr. After incubation, solutions were decanted and wells rinsed with 3 × 200 $mm^3$ each of wash solution. Reaction wells were patted dry on paper towels, 200 $mm^3$ of substrate (PNPP) were added to each well, the plates were covered with plate sealer and incubated at 37°C for 60 min in a Titertek microplate incubator. Following incubation, one drop of stopping reagent (Phytodetek) was added to each well and allowed to react for 5 min. Color absorbance was determined at 405 nm on a Titertek

miniscan microstrip spectrophotometer. Absorbance readings of replicates were averaged before further analysis.

Data were processed for both standards and experimental samples in the same manner. Percent Binding was calculated for each standard point or sample. Plotting % Binding versus Concentrations (pmol IAA) on 4 cycle semi-log paper produces a sigmoid curve from which sample concentrations can be extrapolated. Concentrations can be calculated by transforming data through a Log-Logit function. Linear standard graphs can be drawn on 3 cycle semi-log paper by plotting the Logit versus IAA concentration ($y = a+b \ln x$). Concentrations for experimental samples were calculated from the standard curve (Fig. 1).




Fif. 1. Standard curve for IAA enzyme immunoassay. Known concentrations of IAA (1 to 100 pmol) were methylated with an excess of ethereal diazomethane and subjected to a commercially prepared enzyme immunoassay for IAA-methylester. Percent binding data were calculated and transformed by the Log-Logit-function. The Logit values were plotted on 3 cycle semi-log paper vs the IAA concentration in pmol. A standard line for IAA concentrations was fitted. Concentrations for experimental samples were calculated from the standard curve

## RESULTS

Table 1 summarizes data from a series of (a minimum of 6) experiments conducted to determine the endogenous content of IAA in coleoptile tissues from untreated and treated (BSM, p-FPA, p-FPA+Phe) *Avena*. Data are expressed in terms of nmol IAA present as IAA methylester per gram fresh weight tissue extract. Time 0 hr (To) values were determined immediately after excision. Concentrations for the remaining groups were determined after their respective 18 hr incubations. IAA levels are compared as percents of To normalized to 100%. Data indicate that p-FPA pretreated coleoptiles contain higher IAA concentrations (53%) after the 18 hr incubation than controls (BSM, 43%). The inclusion of Phe with p-FPA in the incubation reversed the effect of p-FPA. Concentrations of IAA from p-FPA + Phe pretreated coleoptiles were 35% of To levels.

Table 1

Endogenous IAA concentration in *Avena* coleoptiles

| Treatment [a,b] | nmoles per g FW | %[c] |
|---|---|---|
| $T_o$ | 6.83 | 100 |
| BSM | 2.94 | 43 |
| p-FPA | 3.65 | 53 |
| p-FPA + Phe | 2.38 | 35 |

[a] Initial values ($T_o$) were determined at time excision. The remainder of the segments were analyzed after an 18 hr dark incubation at 26±1°C on BSM with and without 5 mM p-FPA or 5mM p-FPA + 5mM Phe.

[b] $T_o$ n = 7; BSM n = 8; p-FPA n = 7; p-FPA + Phe n = 6.

[c] Data expressed as % $T_o$ concentration after the 18 hr incubation.

## DISCUSSION

Pretreatment of coleoptile apices with p-FPA increases coleoptile elongation, depresses extractable PAL activity, decreases in vivo chlorogenic acid levels and lowers auxin oxidase activity as compared to controls (Hopkins and Bonnell 1969, Hopkins and Orkwiszewski 1971, Orkwiszewski and Hopkins 1974, Orkwiszewski et al. 1976, Maksymowych and Orkwiszewski 1983, 1987). These authors suggested that p-FPA promotes elongation by lowering the levels of potentially phytotoxic phenols.

Suggestions that a decrease of low molecular weight phenols lowers auxin oxidase activity have appeared in the literature (Galston 1967, Maksymowych and Orkwiszewski 1987). Reduced oxidation may result in an increase of the endogenous pool of free IAA which, in turn, may contribute to enhanced coleoptile elongation.

A series of experiments were conducted to attempt to quantify the levels of endogenous IAA in coleoptiles pretreated with BSM, p-FPA and p-FPA + Phe. Combined p-FPA and Phe treatment was employed, for previous studies had demonstrated that pretreatment of coleoptiles with p-FPA + Phe reverses the effects of p-FPA on coleoptile elongation and phenolic metabolism (Hopkins and Orkwiszewski 1971, Orkwiszewski et al. 1976).

The data of Table 1 indicate that p-FPA pretreated coleoptiles may contain higher levels of IAA than the BSM or p-FPA + Phe pretreatment groups. Incubation of coleoptiles with p-FPA + Phe appears to reverse the effects of p-FPA on endogenous IAA levels.

At this time, it is not possible to determine whether or not the relatively small change in IAA between BSM and p-FPA is sufficient to significantly contribute to the elongation response. Nevertheless, using the relative direction of the changes presented in Table 1 as an indication of IAA levels, one might

anticipate that p-FPA treated coleoptiles would elongate more than those incubated on BSM. These data are consistent with the previously reported effects of p-FPA on PAL activity, phenolic metabolism and auxin oxidase.

Based on the reported effects of p-FPA on *Avena* coleoptiles, a summary model is proposed. Figure 2 illustrates a cascade or possible series of pathways by wich p-FPA induced physiological changes may occur. The amino acid analogue may compete with Phe or alter PAL itself in some fashion resulting in decreased cinnamic acid levels. Lowering of cinnamic acid is reflected by a decline in the in vivo levels of chlorogenic acid and possibly other, as yet unspecified, low molecular weight phenolics.

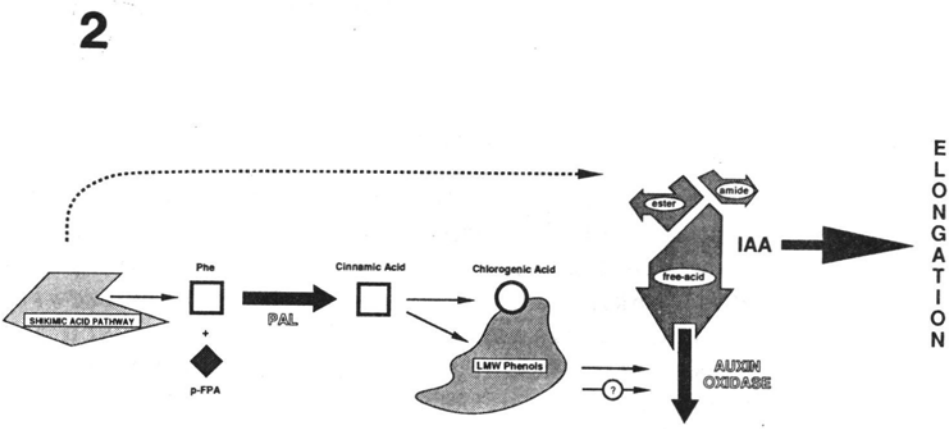



Fig. 2. Summary model for p-FPA induced *Avena* coleoptile elongation. The cascade illustrated in Figure 2 presents several alternative mechanisms by which p-FPA may stimulate *Avena* coleoptile elongation. The amino acid analogue may directly or indirectly lower PAL activity ultimately resulting in lowered auxin oxidase activity which spares endogenous IAA. Alternatively, p-FPA may inhibit the metabolism of Phe precursors and lead directly to enhanced production of IAA by intermediates of the shikimic acid pathway

Phenols, acting as cofactors, may alter auxin oxidase levels and thus decrease auxin oxidase specific activity which, in turn, spares endogenous IAA and leads to growth enhancement. Alternatively, phenolics may act by another mechanism which depresses auxin oxidase activity and protects IAA. Lastly, the levels of Phe precursors, intermediates of the shikimic acid pathway, may accumulate due to inhibition by p-FPA and lead to a direct synthesis of endogenous IAA resulting in enhanced coleoptile elongation. The data to date do not permit a distinction to be made among these alternative mechanisms.

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## *Wpływ fluorofenyloalaniny na poziom kwasu indolo-3-octowego w koleoptylach owsa*

### Streszczenie

Badano wpływ D,L-p-fluorofenyloalaniny, analogu aminokwasowego, na poziom kwasu indolo-3-octowego u owsa. We wcześniejszych badaniach wykazano, że D,L-p-fluorofenyloalanina stymuluje wydłużanie, obniża poziom fenoli i zmniejsza aktywność oksydazy auksynowej etiolowanych koleoptyli owsa. W niniejszej pracy zmierzono metodą immunoenzymatyczną stężenie endogennego kwasu indolo-3-octowego w wierzchołkach koleoptyli. Wykazano, że D,L-p-fluorofenyloalanina zmienia poziom auksyn w koleoptylach owsa. Ponadto doświadczenie to pomaga wyjaśnić działanie D,L-p-fluorofenyloalaniny u owsa.