Effects of Ancymidol (a Growth Retardant) and Triarimol (a Fungicide) on the Growth, Sterols, and Gibberellins of *Phaseolus vulgaris* (L.)

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ABSTRACT

The effect of the two substituted pyrimidines, ancymidol (a growth retardant) and triarimol (a fungicide) on *Phaseolus vulgaris* was studied. Both compounds retarded shoot and root elongation as well as increases in fresh weight. Both compounds caused production of ethylene-like responses when given in high dosages or when applied shortly after germination. As growth retardation was shown to occur in the absence of net increase in sterol levels, neither ancymidol nor triarimol apparently retards growth by inhibiting sterol synthesis.

Both ancymidol and triarimol treatment drastically reduced the amount of extractable gibberellin-like activity in beans. Ancymidol also either induced or enhanced the production of a compound which gave a negative response in the bioassay plant *Oryza sativa* var. *Tan-ginbozu*. The addition of gibberellin completely relieved the dwarfing effects of both ancymidol and triarimol in dark-grown beans. It is concluded that ancymidol and triarimol affect a gibberellin-induced growth response, probably by inhibiting gibberellin biosynthesis.

Anfymidol, \(\alpha\)-cyclopregly-\(\alpha\)-(4-methoxyphenyl)-5-pyrimidinemethanol (EL-531), and triarimol, \(\alpha\)-(2,4-dichlorophenyl)-\(\alpha\)-phenyl-5-pyrimidinemethanol (EL-273) (Fig. 1) are synthetic pyrimidine analogues with growth retardant activity. Ancymidol is used as a growth retardant but is also weakly fungitoxic (18). Triarimol is a fungicide but also retards the growth of higher plants (5). Sherald et al. (18) found that two mutants of the fungus *Cladosporium cucumerium* selected for resistance to triarimol were also resistant to ancymidol.

Triarimol alters sterol metabolism in the fungus *Ustilago maydis* (17). The general effects of growth retardants on sterol metabolism are not clear. Barnes et al. (1) found that the growth retardants CCC and AMO-1618 do not affect sterol metabolism in either *Fusarium moniliforme* or barley. However, Douglas et al. (6, 7) found that AMO-1618 inhibits sterol biosynthesis in tobacco. It is generally accepted that most common growth retardants inhibit gibberellin biosynthesis as their primary mode of action (5, 13). It has also been shown that growth retardations caused by these compounds can generally be relieved by addition of gibberelin (13). We decided to study the effects of ancymidol and triarimol on sterol and gibberellin metabolism as related to their growth regulatory activity in a higher plant.

MATERIALS AND METHODS

**Plant Tissue.** Beans used in this study were *Phaseolus vulgaris* L. cv. *contender*. Seeds were planted in Perlite and inhibitors applied as an aqueous drench containing 0.2% (v/v) methanol. Plants grown under dark conditions were kept at 26 to 28°C. Plants grown under light conditions at 28 to 30°C were kept on a regime of 12 hr of about 400 ft-c of light from fluorescent bulbs and 12 hr darkness.

**Sterols.** Plant material was rinsed, freeze-dried, and the lipids were extracted (8). After the lipids were saponified they were separated by TLC using Silica Gel HF-254 + 366 (VWR) and hexane-diethyl ether-acetic acid (80:20:1, v/v/v). After drying, the portion of the silica gel containing sterols was scraped from the plate, and the sterols were eluted.

Sterols were analyzed by GLC using columns of Gas Chrom P (Applied Science Laboratories) coated with 3% SE-30, 3% QF-1 (Applied Science Laboratories), or 3% OV-17 (Supelco, Inc.). Cholesterol was used as an internal standard for quantitation and to determine relative retention times. Sterols were identified by comparing their relative retention times with standards on all three columns.

**Compound Application.** Compounds (10 µl) were applied to shoot meristems at the same time 10 µg/ml ancymidol or 50 µg/ml triarimol was applied to the roots (day 6).* GA\(_2\) at 2 µM was applied in 50% (v/v) methanol; ent-kaurone (Abbott Lot No. 2150-257-B1) at 4 µM in acetone; IAA at 10 µM in methanol; and sitosterol plus campesterol were applied in a saturated methanol solution. Light experiments were terminated on day 11 and dark experiments on day 9.

**Gibberellins.** Bean tissue was homogenized with four times the tissue fresh weight of 80% (v/v) acetone. The homogenate was allowed to stand overnight and filtered through sintered glass. The acetone was removed in vacuo at 40°C, and the pH was lowered to 2.5 with concentrated H\(_2\)PO\(_4\). This acidified solution was partitioned four times with ethyl acetate. The ethyl acetate was dried over anhydrous sodium sulfate, filtered, evaporated, and the gibberellins were taken up in acetone. This acetone was applied to TLC plates of Adsorbosil-1 silica gel (Applied Science Laboratories). The plates were developed to 15 cm using water, the silica gel from the upper 10 cm was scraped off, and the gibberellins were eluted with acetone.

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3. Abbreviations: CCC: (2-chloroethyl)trimethylammonium chloride; AMO-1618: 2'-isopropyl-4'-(trimethylammonium chloride)-5'-methylphenylpiperidine-1-carboxylate.

4. Throughout this text "day 0" refers to seeds, and the word day followed by a number refers to the number of days lapsed since planting, i.e. day 6 refers to seedlings planted 6 days previously.
ANCYMIDOL: MODE OF ACTION

In every experiment ancyclidol, triarimol, and GA₃ were added to plant extracts just prior to Adsorbosil-1, water chromatography. Ancymidol never migrated above 4.7 cm, triarimol never migrated above 3.3 cm, and GA₃ never migrated below 11.5 cm. We, therefore, believe we have retained the gibberellins in our preparation and removed any ancyclidol or triarimol which might be present.

The acetone solutions of gibberellins to be separated were applied to TLC plates of Silica Gel G (VWR). The plates were developed to 15 cm using chloroform-ethyl acetate-acetic acid (60:40:5, v/v/v). After drying, the plates were divided into 10 equal zones between origin and solvent front and each zone was scraped off. The silica gel was eluted with 100 ml of acetone, the acetone was evaporated in vacuo, and the residues were bioassayed in 25% (v/v) acetone. All bioassays were performed on Oryza sativa var. Tan-ginbozu (16).

RESULTS

Growth. Neither ancyclidol (10 µg/ml) nor triarimol (50 µg/ml) affected seed germination. In light experiments both compounds reduced leaf expansion, caused leaves to be darker green than normal, and occasionally caused raised areas between the veins and leaf epinasty. In both light and dark experiments, application of 50 µg/ml of triarimol to seeds at or shortly after germination (through day 2) resulted in drastically altered seedlings. Their hypocotyls had bulbous swellings just behind the hypocotyl arch. The seedlings were very susceptible to pathogen attack, and their hypocotyl arch usually failed to open. Ancymidol (10 µg/ml) applied in the same manner produced some of the same symptoms, but to a much lesser degree.

In order to test the effectiveness of ancyclidol and triarimol in retarding shoot and root development of beans, various concentrations of the two compounds were applied from day 3 through day 9 in a dark experiment (Figs. 2 and 3).

Ancymidol affected stem length and stem weight in nearly a linear fashion with increasing concentration (Fig. 2). Stem weight was affected less than stem length due to increased stem diameter. Increasing concentrations of ancyclidol appeared to affect stem and root lengths about equally. Roots treated with 1 µg/ml or above were noticeably thicker than normal, especially the lateral roots.

Neither stem length nor stem weight were affected until the concentration of 3.16 µg/ml triarimol was reached (Fig. 3). The root system, however, was affected at concentrations below 3.16 µg/ml. In fact, at most concentrations, whether measuring length or weight, the root systems were affected more than the stem systems. This might be related to a tendency of triarimol to concentrate more in the roots than in the stems. Again, the stem and root lengths appeared to be affected more than the weights.

Sterols. Experiments to determine effects on sterols were undertaken wherein both retardants were applied at 10 µg/ml from day 0 to day 4 under dark conditions. Ancymidol and triarimol reduced both hypocotyl and root elongation as well as increases in fresh weight. The sterols were extracted from seeds and seedlings. Sitosterol, stigmasterol, campesterol, isofucosterol, and cholesterol were found. The latter two sterols were relatively minor components and were not routinely quantitated.

There was no measurable change in sterol content during the 4 days of treatment. As growth was retarded in the absence of net sterol synthesis, it seems unlikely that this retardation is due to a lack of sterols. In view of the insignificant biosynthesis of sterols during early stages of germination and the presence of sterols in dried seed, Grunwald (9) concluded: “that sufficient quantities of sterols are on hand to satisfy the initial demands made during germination.”

Gibberellins. Leopold (14) showed that GA₃ is capable of “relieving” the dwarfing effects of ancyclidol on corn. The amount of “relief” obtained is, however, difficult to assess because the control of minus ancyclidol/plus GA₃ was not included. An experiment under light conditions was undertaken with beans to determine reversibility of the ancyclidol and triarimol effects by GA₃. The addition of 10 µl of 2 mm GA₃ greatly increased the epicotyl elongation of all treatments (Table I). Although epicotyl elongation in ancyclidol plus GA₃ and triarimol plus GA₃ treatments exceeded the control minus GA₃, retardation has not been fully relieved, as is evident if we compare the ancyclidol plus GA₃ with the control plus GA₃.

It is known that some plants are more sensitive to certain gibberellins in the dark as compared to the light (11). An
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Fig. 3. Dose-response curve for triarimol. Beans were grown under dark conditions and treated with several concentrations of triarimol on day 3. Four parameters of growth were measured on day 9: hypocotyl length was used as a measure of stem length; everywhere above the crown minus the seed coat was used as stem weight; the main root was used as root length; and everything below the crown was used as root weight. The vertical bars represent standard deviations at selected points. Standard deviations were determined for all points; however, most have been omitted for visual clarity. Each point is the average of two experiments.

Table I. Retardation Relief by GA$_3$

Anymidol at 10 µg/ml and triarimol at 50 µg/ml were applied to the roots of beans. At the same time (day 6) 10 µl of 2 mM GA$_3$ was applied to the shoot meristems of the plants in one-half of the pots. The mean epicotyl elongation from day 6 was determined on day 11 under light conditions (see “Materials and Methods”). The mean hypocotyl elongation from day 6 was determined on day 9 under dark conditions. Numbers followed by no letters in common, within one condition, are significantly different ($P = 0.05$).

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Treatment</th>
<th>Control</th>
<th>Anymidol</th>
<th>Triarimol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light</td>
<td>$-$(GA$_3$)</td>
<td>6.9a</td>
<td>2.5b</td>
<td>1.7c</td>
</tr>
<tr>
<td>Dark</td>
<td>$+$(GA$_3$)</td>
<td>12.7a</td>
<td>19.3b</td>
<td>4.7c</td>
</tr>
</tbody>
</table>

An attempt to overcome completely the dwarfing effects of anymidol and triarimol was made under dark conditions using dark-grown plants.

The hypocotyl elongation of all plants receiving GA$_3$ was essentially the same whether or not anymidol or triarimol was applied (Table I). We have therefore completely relieved the dwarfing effects of anymidol and triarimol by the addition of a gibberelin.

Ent-kaurene at 4 mM was tested under dark conditions for its ability to relieve growth retardations. It had no effect upon hypocotyl elongation. IAA at 10 µM and a methanol solution saturated with sitosterol and campesterol were tested under light conditions for their ability to relieve growth retardations. Neither solution altered epicotyl elongation.

An experiment was performed to determine whether or not anymidol or triarimol inhibited gibberellin biosynthesis by Gibberella fujikuroi (Sawada) Wollenweber. The procedures of Kende et al. (12) were followed except for the gibberellin purification and bioassay. They were as described under “Materials and Methods” except that the culture filtrate was directly acidified and partitioned against ethyl acetate.

As both anymidol and triarimol are fungitoxic (18), no concentration was found where the dry weight of the cultures was not affected and the gibberellin-like activity produced was affected. However, the inhibition of gibberellin production did exceed inhibition of dry weight. For example at 100 µg/ml anymidol, gibberellin production was inhibited 97% and dry weight increase by only 23%. At 1 µg/ml triarimol, gibberellin production was inhibited 92% and dry weight increase only 30%.

Gibberellins were extracted and bioassayed from dark-grown bean seedlings treated from day 0 to day 4 with 10 µg/ml anymidol or 10 µg/ml triarimol. The quantities of gibberellin-like activity extracted were quite variable from one experiment to another (Table II). However, in every case the activity was much less in treated tissue, whether treated with anymidol or triarimol. Both treatments were significantly different ($P = 0.05$) from the control but not from each other.

Gibberellin extracts of these same 0 to 4 day dark-grown beans were separated on TLC as described. As seen from the histogram (Fig. 4), two general zones of gibberellin activity were found; one with an $R_F$ less than 0.1 and one with an $R_F$ approximating 0.85.

$R_F$ values for the following compounds, run at the same time as bean extracts but not within these bean extracts, were obtained: GA$_3$, $R_F$ = 0.14; GA$_7$, $R_F$ = 0.54 and 0.60; anymidol, $R_F$ = 0.35; triarimol, $R_F$ = 0.47; IAA, $R_F$ = 0.45; kinetin, $R_F$ = 0.19; and abscisic acid, $R_F$ = 0.43.

A tabIndexl- and triarimol-treated tissue extracts contained much less gibberellin-like activity in both activity zones (Fig. 4). Though the possible presence of an inhibitor in these zones is not ruled out, these results are taken to indicate a lack of gibberellin-like activity produced by anymidol and triarimol treated tissues.

Gibberellin extracts showed considerably less total activity when separated on TLC than when the gibberellin extract was used directly in the bioassay. For example, the quantity of

Table II. Gibberellin-like Activity in Bean Extracts

Beans were treated with 10 µg/ml anymidol and 10 µg/ml triarimol from day 0 to day 4 in the dark. Gibberellins were extracted and bioassayed using the Tan-ginbozu bioassay.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatment</th>
<th>ng GA$_3$ Equivalents/Plant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Anymidol</td>
<td>2.8</td>
</tr>
<tr>
<td>1</td>
<td>Triarimol</td>
<td>0.1</td>
</tr>
<tr>
<td>Control</td>
<td>Anymidol</td>
<td>0.4</td>
</tr>
<tr>
<td>2</td>
<td>Triarimol</td>
<td>1.1</td>
</tr>
<tr>
<td>Control</td>
<td>Anymidol</td>
<td>1.7</td>
</tr>
<tr>
<td>3</td>
<td>Triarimol</td>
<td>0.8</td>
</tr>
<tr>
<td>Control</td>
<td>Anymidol</td>
<td>0.0</td>
</tr>
<tr>
<td>4*</td>
<td>Triarimol</td>
<td>0.0</td>
</tr>
</tbody>
</table>

*In this experiment a longer extraction procedure was used wherein the extract was partitioned from ethyl acetate into sodium bicarbonate and back into ethyl acetate.
ANCYMIDOL: MODE OF ACTION

Fig. 4. Histogram of gibberellin extracts from bean seedlings. Beans were treated from day 0 to day 4 with 10 μg/ml ancymidol and 10 μg/ml triarimol. Gibberellins were extracted and chromatographed on Silica Gel G plates using chloroform-ethyl acetate-acetic acid (60:40:5, v/v/v) as solvent. The plates were divided into 10 equal zones, the silica gel scraped off, and the gibberellins eluted and bioassayed using Tan-ginbozu as the bioassay plant. Darkened areas indicate significant difference from the controls (P = 0.01).

extract applied to the experiment 1 control TLC of Figure 4 (if bioassayed just before gibberellin separation TLC) was determined to be 189 ng of GA3 equivalents. Obviously much less total activity was recovered after the separation. Possibly, the effects of the different gibberellins are multiplicative when combined.

The histograms also indicate the presence of a compound (or compounds) which retards Tan-ginbozu leaf sheath elongation. This compound appears to be present in all treatment extracts; however, the ancymidol treated tissue extracts appear to have much more of this compound(s).

We do not believe that these inhibitory zones were due to the presence of ancymidol, triarimol, or abscisic acid. Ancymidol and triarimol were removed during the extraction procedures as described under "Materials and Methods." Abscisic acid when applied to the bioassay at 40 μg/ml had no effect upon the leaf sheath elongation.

We concluded from this experiment that ancymidol and triarimol in bean seedlings somehow reduced the production of gibberellin-like activity and that ancymidol in addition induced or enhanced the formation of a compound which retarded leaf sheath elongation.

DISCUSSION

Several phenomena have been observed in bean plants when triarimol or ancymidol were applied at high dosages or early after germination. These phenomena include inhibition of stem and root growth, root swelling, stem swelling of a bulbous nature, inhibition of hypocotyl hook opening, leaf epinasty, and abscission of leaves and flowers. All of these processes have been induced by the addition of ethylene to plant tissues (2, 15).

In other experiments, we have shown that when the retardants are incorporated into the agar upon which the Tan-ginbozu seedlings are grown (from day 2 through day 8) they inhibit root and leaf sheath elongation. The root length of seedlings treated with 10 μg/ml ancymidol were 43% and the second leaf sheath length 25% of the controls. The root length of seedlings treated with 50 μg/ml triarimol were 5% and the second leaf sheath length 3% of the controls. Tan-ginbozu is reported to contain no detectable gibberellin-like activity (19). Therefore either of two explanations seem possible for the growth retardations of ancymidol and triarimol in Tan-ginbozu: (a) Tan-ginbozu seedlings require an undetectable amount of gibberelin, and these growth retardants inhibit the biosynthesis or action of this gibberellin, or (b) ancymidol and triarimol facilitate growth retardation through some means other than inhibition of gibberellin biosynthesis or action, possibly by inducing ethylene synthesis.

It is tempting to speculate that many of our findings are due to increased ethylene production. We believe this to be partially the case where high dosages or early application are concerned, but not necessarily in all cases. Evidence does exist that ethylene alone cannot explain the effects of ancymidol or triarimol. Most of the observable ethylene effects are missing in beans treated with dosages of less than 50 μg/ml ancymidol or triarimol after day 5. Lieberman and Kunishi (15) have shown that, although gibberelin and ethylene are mutually antagonistic as regards pea epicotyl elongation, gibberelin can only partially reverse ethylene growth inhibition. We have shown that gibberellin can totally reverse ancymidol or triarimol inhibition when applied on day 6 to dark-grown plants.

It is of some note that Lieberman and Kunishi's photograph (15) of peas treated on day 4 with 4 μl/l ethylene appears surprisingly like our Tan-ginbozu, day 2 treated seedlings as well as those bioassay seedlings treated with inhibitory substances in the histogram. One might therefore hypothesize that control,
riarimol, and especially ancymidol-treated beans contain a compound which induces ethylene formation in Tan-ginbozu, and that ancymidol and triarimol are themselves capable of this induction.

Douglas et al. (6) have ascribed sterol synthesis inhibition as a possible mode of action for other growth retardants. We have found that ancymidol and triarimol growth retardations take place in tissues where there is no net sterol synthesis. Therefore, the mode of action of ancymidol and triarimol, as regards these growth retardations, cannot be ascribed to a deficiency of sterols.

We have shown that both ancymidol and triarimol treatments drastically reduce the gibberellin-like activity in bean extracts. We have likewise shown that application of gibberelin can completely relieve the dwarfing effects of ancymidol and triarimol. It appears that the primary mode of action of ancymidol and triarimol as regards growth retardation is the inhibition of a gibberellin-induced response(s). The most likely site of this interference is in the biosynthesis of gibberellins. Coolbaugh and Hamilton (4) have reported that ancymidol blocks the conversion of kaurene to kaurenol in the gibberellin biosynthetic pathway.

One final topic deserves to be discussed. The fungicide, triarimol, alters sterol metabolism in a fungus (17) and reduces germ tube extension while causing swelling (18). Ancymidol, the growth retardant (and triarimol), reduces extractable gibberellin-like activity and reduces bean elongation while causing swelling. These facts introduce two feasible correlations between the mode of action in fungi and higher plants. First, sterols are known to be membrane components. Gibberellins have been shown to increase the fluidity and permeability of synthetic membranes (21) and inhibit the activity of a membrane-bound arabinosyl transferase (10). Therefore both sterols and gibberellins are apparently involved in membrane phenomena, and the growth retardant and fungicide modes of action may be linked somehow in maintenance of membrane structure and function. Second, ancymidol (and triarimol) is now known to have an effect(s) on a higher plant growth hormone(s). Hormones affecting cell expansion in fungi are currently being found by Japanese workers (20). Therefore, the ancymidol and triarimol modes of action in fungi and higher plants may be linked through the control of growth hormones.

LITERATURE CITED