Cyclanilide [1-(2,4-dichlorophenylaminocarbonyl)-cyclopropane carboxylic acid] is a plant growth regulator that is registered for use in cotton at different stages of growth. It can be used to suppress vegetative growth when used in combination with mepiquat chloride; or it can enhance defoliation and boll opening when used in combination with ethephon. To understand how cyclanilide affects these different processes, the mechanism of action of cyclanilide was studied. We tested the hypothesis that cyclanilide acts by inhibiting auxin transport, or in some way alters auxin signaling. Auxin and ethylene are known to regulate abscission, so the interaction of cyclanilide and ethephon was studied on bean leaf defoliation. Ethephon induced bean leaf defoliation, but ethephon plus cyclanilide induced greater defoliation. The induction of defoliation by cyclanilide and ethephon was dose dependent. Combining auxin transport inhibitors (TIBA or NPA) or cyclanilide with a low, ineffective rate of ethephon induced 50% defoliation. In addition, movement of [3H]IAA in etiolated corn coleoptiles was inhibited by 10 µM cyclanilide, NPA, and TIBA. These results demonstrated that cyclanilide affected polar auxin transport. However, NPA is a known inhibitor of auxin efflux, but cyclanilide did not affect efflux. Though NPA is a known inhibitor of the gravitropic response in tomato roots (85% at 1 µM) cyclanilide inhibition of gravitropism was 30% at 1 µM. Although NPA inhibited tomato root growth (30% at 1 µM), cyclanilide stimulated root growth (165% of control at 5 µM). Using plasma membrane fractions, cyclanilide inhibited the binding of [3H]NPA and [3H]IAA with an IC50 of 50 µM for both. NPA did not affect the binding of IAA, nor did IAA affect the binding of NPA. Cyclanilide is a noncompetitive inhibitor of both NPA and IAA binding, with inhibition constants (Ki) of 40 and 2.3 µM, respectively. These data demonstrated that cyclanilide interacts with auxin-regulated processes via a mechanism that is distinct from other auxin transport inhibitors, and identify a possible mechanism of action for cyclanilide when used as a plant growth regulator.

Introduction.

Cyclanilide is a plant growth regulator that is currently registered for use in cotton. It is used for two different purposes, and is only used in combination with other PGRs. When cyclanilide is combined with mepiquat chloride, it is used for early season growth management, to prevent excessive vegetative growth, and produce a uniform stand. Alternatively, cyclanilide is combined with ethephon and utilized at the end of the season as harvest aid, primarily for defoliation, boll opening and prevent vegetative re-growth.

Cyclanilide, mepiquat chloride, and ethephon each have a distinct mechanism of action. Mepiquat chloride is an inhibitor of gibberellic acid (GA) biosynthesis, and was first registered in the 1980's to manage vegetative growth. In 2006, mepiquat chloride combined with
cyclanilide was registered for the same use. Ethephon is an ethylene releasing agent and was first registered as a harvest aid in cotton in the 1980s. Cyclanilide appears to synergize or enhance the activity of the primary growth regulator, because mepiquat chloride or ethephon alone will reduce growth or initiate defoliation, respectively. Cyclanilide used alone will block apical dominance and enhance lateral branching in apple nursery stock (Elfving and Visser 2005) and in kidney beans (Pedersen and others 2006). The mechanism of action of cyclanilide is not known, and the purpose of this research was to study cyclanilide’s physiologic activity.

Materials and Methods

Whole plant defoliation and temperature studies were conducted according the methods in Pedersen et al., (2006). Kidney bean seeds were germinated in sand, and transferred to 10.2-cm pots containing greenhouse soil and watered as needed. Plants were grown in the greenhouse. Treatments were applied 3 wk after planting when the third trifoliate was fully expanded, and an average of 14 leaves per.

Chemicals (cyclanilide, TIBA, and NPA) were applied either alone or in combination with ethephon. Control treatment consisted of water alone. Spray treatments were applied using a spray chamber.

For temperature studies, Kidney bean plants were grown in controlled environmental chambers. Three-week-old kidney bean plants were sprayed with ethephon alone, or in combination with cyclanilide. Water was used as a control. After spray treatments, plants were placed in chambers set at the desired temperature.

Polar auxin transport, efflux, gravitropic response, and binding assays were conducted according to methods described by Burton et al., (2008). For polar auxin transport assay, etiolated corn coleoptiles were harvested 3.5 days after planting. The coleoptiles were cut 1 cm below the apical end and 1 cm below the first node so that the average length of the coleoptiles used for assaying was 3 cm. The apical end of the cut coleoptile was immediately immersed in 200 µl of a 50-mM KH2PO4 buffer (0.25 M sucrose, pH 6.0), containing 1 µM [3H]IAA alone or in combination with inhibitors. Inhibitor treatments included cyclanilide, NPA, and TIBA. After a 10-min pulse in the radiolabeled solution, coleoptiles were rinsed and placed apical end down into an agar plate. Small blocks of agar were placed at the base to collect any radiolabel moving from the apex to the base of the coleoptile. Fresh agar blocks were replaced every 20 min throughout the assay. The 3 h assay was conducted under green fluorescent light. The amount of radioactivity in each agar block was counted using LSS.

Efflux studies were conducted with zucchini (Curcurbita pepo, c.v. black squash) seeds germinated in the dark at 27 C. Zucchini hypocotyls were harvested 3.5 days after planting. After removing the apical hook, 0.5-cm segments of ten zucchini hypocotyls were cut and used for each treatment. Hypocotyl segments were pre-loaded 1 µM [3H]IAA with or without inhibitors. Tissue segments were incubated for 2 h in uptake solution to allow [3H]IAA uptake. For efflux, tissue was then rinsed and placed in 4 ml efflux solution without [3H]IAA, but containing inhibitors, and incubated for another 2 h to allow efflux. At the end of the elution period, tissue was rinsed and placed in a scintillation vial with 5 ml scintillation cocktail. After soaking tissue overnight, radioactivity was assayed via LSS.

For gravitropic studies, tomato seeds were germinated 2–3 days in the dark at 27 C on filter papers moistened with water. Seeds were transferred to agar plates when roots emerged 1-3 mm. Agar plates were prepared with a modified Murashige and Skoog micronutrient solution,
adjusted to pH 6.0. Ten emerged tomato roots were placed in a straight line on an agar plate and allowed to grow for 24 h parallel to the field of gravity under fluorescent light with a 16 h photoperiod. After 24 h of growth, a straight reference line was drawn on the plate perpendicular to the tip of the grown roots. Plates were then either turned 90 degrees so the roots would be oriented perpendicular to the field of gravity to stimulate a gravitropic response, or allowed to continue to grow parallel to the field of gravity to stimulate further growth. After 24 h, the angle of root curvature was measured in reference to the drawn line with a protractor in gravistimulated roots, and root length was measured on roots grown parallel to the field of gravity in reference to the drawn line.

Competitive binding studies utilized enriched plasma membrane fractions from etiolated zucchini hypocotyls, harvested 4 days after planting. Tissue was homogenized in a blender with Tris buffer (pH 7.2), filtered through cheesecloth and a nylon membrane, and then centrifuged at 7,000g for 10 min. The supernatant was subjected to ultracentrifugation at 100,000g for 30 min to obtain the total membrane fraction (microsomes). The microsomal pellet was resuspended in buffer and mixed in a prepared phase separation solution. Phase separation was performed by centrifuging at 1,000g for 10 min. The plasma membrane fraction was combined with 1:3 (v/v) resuspension buffer and centrifuged at 100,000g for 30 min to precipitate plasma membranes. The final pellet was resuspended in a citric acid buffer (pH 5.5) 0.25 M sucrose and 1 mM MgCl₂, and stored at -80°C. The plasma membrane fractions were free of mitochondrial membranes. Binding assays were conducted in microtiter plates with 0.2 mg/ml protein, 13 nM [³H]NPA or 10 nM [³H]IAA. Bound radiolabel was separated from free radiolabel and the radioactivity was determined using LSS.

Results and Discussion.

Whole plant experiments in the greenhouse were conducted to compare the effect of ethephon alone or in combination with cyclanilide and auxin transport inhibitors such as TIBA and NPA. Without ethephon, cyclanilide alone induced a proliferation of lateral shoot growth and inhibited apical growth. These observations are similar to the findings of Elfving and Visser (2005), which demonstrated the application of cyclanilide caused the induction of lateral branching in apple trees.

Defoliation is dependent on three factors: time, type of chemical, and rate of application. Combining ethephon with cyclanilide enhanced defoliation compared with ethephon alone (Fig. 1). Ethephon applied at the highest tested rate (0.140 kg a.i./ha) induced 75% defoliation 7 DAT. Ethephon alone at half this rate (0.067 kg a.i./ha) did not induce any defoliation 7 DAT. Combining 0.140 kg a.i./ha cyclanilide with 0.067 kg a.i./ha ethephon induced 100% defoliation by 6 DAT. Combining cyclanilide with a low rate of ethephon resulted in greater defoliation in less time than the application of a high rate of ethephon alone.

At 5 DAT, increasing rates of ethephon alone induced a maximum of 50% defoliation at the highest rate (Fig. 2). While 0.067 kg a.i./ha ethephon alone did not induce any defoliation 5 DAT, combining this low rate of ethephon with increasing rates of cyclanilide resulted in 63 to 80% defoliation. In fact, cyclanilide applied at 0.017 to 0.034 kg a.i./ha combined with 0.067 kg a.i./ha ethephon resulted in 55 to 70% defoliation. These results demonstrated that low rates of cyclanilide combined with inactive rates of ethephon will enhance defoliation.
Whole plant experiments were also conducted to determine if the cyclanilide treatment affected ethephon-induced defoliation at varying temperatures. Combining 0.067 kg a.i./ha cyclanilide with an equal rate of ethephon consistently induced >75 to 85% defoliation, 5 DAT at all tested temperatures except at 16/14 C (Fig. 3). In contrast, ethephon alone was more temperature sensitive. At the highest temperature tested (30/26 C), ethephon alone induced only 23% defoliation, and did not induce any defoliation at other temperatures. These results demonstrated that cyclanilide combined with ethephon enhanced defoliation at all temperatures.

Similar to cyclanilide, NPA and TIBA also enhanced defoliation when combined with ethephon (Fig. 4). As shown earlier, ethephon alone at 0.067 kg a.i./ha did not induce any defoliation 5 DAT. However, combining this rate of ethephon with 0.140 kg a.i./ha NPA or TIBA resulted in 50 and 52% defoliation, respectively. Although cyclanilide enhances defoliation more than NPA and TIBA, these results demonstrate that auxin transport inhibitors can also enhance defoliation when combined with ethephon. These results suggest that cyclanilide may be acting as an auxin antagonist or transport inhibitor, thereby enhancing ethephon efficacy.

Polar auxin transport was measured to determine if cyclanilide inhibits auxin movement in a manner similar to known auxin transport inhibitors. Movement of IAA from the apex to the base of a corn coleoptile increased over time, peaked at approximately 2 h after pulsing, and then decreased (Fig. 5). When 10 µM NPA, TIBA, or cyclanilide was included in the radiolabel pulse, auxin transport was inhibited. No further increase in auxin transport was observed after 3 h in the presence of these inhibitors. These results indicate that cyclanilide, like NPA and TIBA, inhibits polar auxin transport.

The potential effect of cyclanilide on cellular auxin efflux and influx was studied by incubating zucchini hypocotyls in [³H]IAA uptake solution for 2 h, either alone or with either NPA or cyclanilide, and then allowing efflux for 2 h in the absence of IAA. When NPA was present in the uptake and efflux solutions, the IAA concentration was 56% higher than in the control (data not shown). In contrast, increasing concentrations of cyclanilide did not result in an increase in IAA accumulation. Thus, cyclanilide did not affect net [³H]IAA efflux indicating it affects auxin transport differently than NPA or TIBA.

Changes in root length and curvature were measured in tomato roots with NPA or cyclanilide to determine if there were effects on the phytotropic response. Root length decreased by 25–70% when grown on increasing concentrations (0.1–10 µM) of NPA (Fig. 6). Root phytotropic response (curvature) was also inhibited by NPA (85% at 1 µM), and the gravitropic response was more sensitive than root growth. Cyclanilide inhibited root phytotropic response 25% at 0.1 µM; but increasing concentrations did not inhibit root curvature any further except at concentrations above 100 µM, when both gravitropic response and growth were severely inhibited. Opposite to NPA, cyclanilide increased in root growth as length increased 20% at 0.1 µM and 65% at 5 µM (Fig. 6).

To further study the effect of cyclanilide on auxin activity, competition-binding studies were conducted using plasma membrane (PM)-enriched fractions extracted from zucchini hypocotyls. The membrane isolation procedure resulted in an enriched PM fraction and substantially removed ER, nuclear, and other membrane fractions (data not shown). The binding of [³H]IAA and [³H]NPA in the zucchini PM protein fraction was saturable, and Scatchard plot analysis estimated an apparent affinity constant (Kd) for IAA binding of 0.098 µM. Competition-binding studies were performed between [³H]IAA and unlabeled cyclanilide, NPA, and IAA. NPA did not inhibit [³H]IAA binding, consistent with previous reports (Hertel and others 1972; Rubery 1990). However, unlabeled cyclanilide inhibited the binding of [³H]IAA in the PM fraction (Fig.
7). The estimated IC$_{50}$ value for cyclanilide inhibition of IAA binding was 50 µM, whereas the estimated IC$_{50}$ value for IAA binding was 0.1 µM. Dixon plots suggest that cyclanilide is a noncompetitive inhibitor of [³H]IAA binding, with a calculated inhibition rate constant (K$_i$) for cyclanilide of 2.3 µM. IAA binding was reversible, and unlabeled cyclanilide also displaced prebound [³H]IAA. Thus, cyclanilide was both an inhibitor of [³H]IAA binding (association) and dissociation. Membrane-binding studies were also conducted to assess the competitive interactions between [³H]NPA and unlabeled NPA or cyclanilide. Unlabeled NPA inhibited [³H]NPA binding at low concentrations (IC$_{50}$ = 0.01 µM), confirming previous work (Muday and others 1993). Cyclanilide also inhibited [³H]NPA binding in plasma membranes, with an estimated IC$_{50}$ value of 50 µM. These results demonstrated that cyclanilide competes with NPA binding, but with a much lower binding affinity. Dixon plot analysis revealed that cyclanilide is a noncompetitive inhibitor of [³H]NPA binding, with an estimated K$_i$ of 40 µM (data not shown). In addition, NPA binding was reversible, as unlabeled NPA displaced prebound [³H]NPA. Unlike the interaction with IAA, cyclanilide was unable to displace prebound [³H]NPA. Cyclanilide is a noncompetitive inhibitor of [³H]NPA binding, but does not affect the dissociation.

Cyclanilide is registered for use only in combination with other PGRs (ethephon and mepiquat chloride), with activity at different phases of plant growth (abscission and boll opening versus vegetative growth reduction, respectively). Cyclanilide is similar but distinct from other auxin transport inhibitors. The experimental results suggest cyclanilide interferes with the regulation of auxin flux. Disruption of directed auxin movement could account, in part or completely, for the observed plant growth regulatory properties of cyclanilide.

**LITERATURE CITED**

Burton et al., 2008, J. Plant Growth Reg. 27:342–352


Hertel et al., 1972, Planta 107:325–340

Muday et al., 1993, Plant Physiol. 103:449–456

Pedersen et al., 2006, Crop Sci. 46:1666–1672