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Effect of Cyclanilide on Auxin Activity

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Abstract Cyclanilide is a plant growth regulator that is registered for use in cotton at different stages of growth, to either suppress vegetative growth (in combination with mepiquat chloride) or accelerate senescence (enhance defoliation and boll opening, used in combination with ethephon). This research was conducted to study the mechanism of action of cyclanilide: its potential interaction with auxin (IAA) transport and signaling in plants. The activity of cyclanilide was compared with the activity of the auxin transport inhibitors NPA and TIBA. Movement of [^{3}H]IAA was inhibited in etiolated corn coleoptiles by 10 μM cyclanilide, NPA, and TIBA, which demonstrated that cyclanilide affected polar auxin transport. Although NPA inhibited [^{3}H]IAA efflux from cells in etiolated zucchini hypocotyls, cyclanilide had no effect. NPA did not inhibit the influx of IAA into cells in etiolated zucchini hypocotyls, whereas cyclanilide inhibited uptake 25 and 31% at 10 and 100 μM, respectively. Also, NPA inhibited the gravitropic response in tomato roots (85% at 1 μM) more than cyclanilide (30% at 1 μM). Although NPA inhibited tomato root growth (30% at 1 μM), cyclanilide stimulated root growth (165% of control at 5 μM). To further characterize cyclanilide action, plasma membrane fractions from etiolated zucchini hypocotyls were obtained and the binding of NPA, IAA, and cyclanilide studied. Cyclanilide inhibited the binding of [^{3}H]NPA and [^{3}H]IAA with an IC_{50} of 50 μM for both. NPA did not affect the binding of IAA, nor did IAA affect the binding of NPA. Kinetic analysis indicated that cyclanilide is a noncompetitive inhibitor of both NPA and IAA binding, with inhibition constants (K_i) 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. This research identifies a possible mechanism of action for cyclanilide when used as a plant growth regulator.

Keywords Cyclanilide · IAA · Auxin transport · Plant growth regulators

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Introduction

Cyclanilide (Fig. 1) is a plant growth regulator (PGR) that is registered for use in cotton, but only in combination with other PGRs. Cyclanilide is used for two different purposes at different growth stages: when combined with ethephon, it will enhance senescence processes (accelerate defoliation and boll opening); when combined with mepiquat chloride, it will reduce plant height during the vegetative growth phase (FINISH® and STANCE®, respectively). For both of these uses, cyclanilide appears to synergize or enhance the activity of the primary growth regulator, because ethephon or mepiquat chloride alone will initiate defoliation or reduce growth, 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.

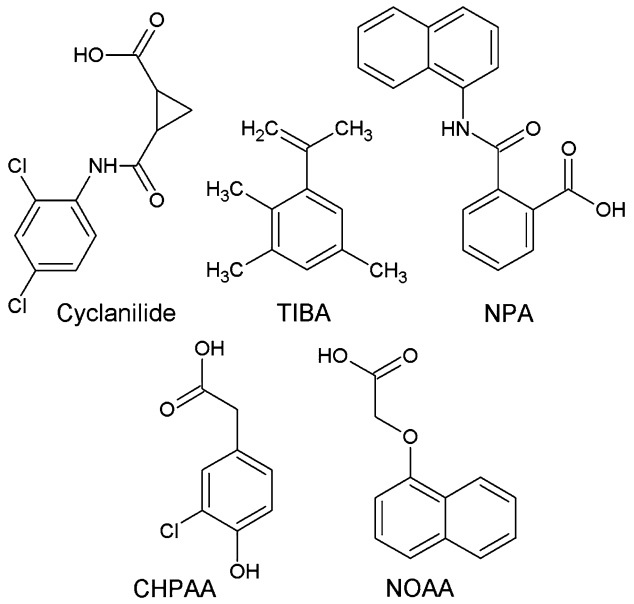

Fig. 1 Chemical structure of cyclanilide (2-[(2,4-dichlorophenyl) carbamoyl]cyclopropane-1-carboxylic acid), the auxin efflux inhibitors NPA (2-(naphthalen-1-ylcarbamoyl)benzoic acid) and TIBA (2,3,5-triiodobenzoic acid), and the auxin influx inhibitors CHPAA (2-(3-chloro-4-hydroxyphenyl)acetic acid) and NOAA (2-naphthalen-1-yloxyacetic acid)

Cyclanilide was first registered for use with ethephon to enhance defoliation (leaf abscission) in cotton to increase the yield and quality of the harvested crop. Leaf abscission is one aspect of senescence and is naturally induced in plants in response to morphologic development, environmental changes, and biotic or abiotic stress. Alternatively, abscission can be induced with the application of synthetic chemicals (Osborne 1989; Roberts and others 2002). At the molecular level, abscission is coordinately controlled by ethylene and auxin, an example of hormone crosstalk (Taylor and Whitelaw 2001; Roberts and others 2002). During leaf development and maturation, there is little ethylene present and auxin transport through the petiole suppresses leaf abscission. During senescence, auxin transport decreases, ethylene levels increase, and abscission is induced. Ethylene initiates abscission, in part, by stimulating the production of cell wall-degrading enzymes in the abscission zone, leading to petiole weakening and eventual shedding of the leaf (Sexton and Roberts 1982; Campillo and Lewis 1992). Studies using explants have demonstrated that auxin applied at a distal position with respect to the abscission zone can suppress abscission when the process has already been induced by ethylene (Wilson and others 1988). Synthetic auxins such as NAA and 2,4-D have commonly been used in fruit crops to prevent preharvest fruit drop and the eventual yield loss by inhibiting abscission (Cooper and others 1968).

The combination of cyclanilide and mepiquat chloride was registered as a PGR in 2005 to reduce internode elongation during vegetative growth of cotton (Thomas and others 2007). Mepiquat chloride and other GA biosynthesis inhibitors have been used for height reduction in cotton for many years (Zhao and Oosterhuis 2000). Mepiquat chloride reduces cotton height by decreasing the GA concentration, which reduces the ability of cells to elongate (Reddy and others 1992). Although inhibition of GA biosynthesis and the resulting reduction of GA levels will decrease internode elongation, auxin also plays a role in mediating this process. Auxin will induce enzymes that metabolize GA_{20} to an active form, GA_1 (Ross and others 2003; Frigerio and others 2006). Thus, the addition of an auxin transport inhibitor, or interference with auxin signaling, may serve to enhance the efficacy of a GA biosynthesis inhibitor such as mepiquat chloride. This two-hormone based PGR activity is similar to the enhanced abscission activity when cyclanilide is combined with ethephon (Pedersen and others 2006).

Both NPA and TIBA, polar auxin transport (PAT) inhibitors (Fig. 1), enhanced the defoliation activity of ethylene (Morgan 1985). Also, cyclanilide, NPA, and TIBA enhanced the activity of ethephon (Pedersen and others 2006). Therefore, it is possible that cyclanilide also inhibits PAT. Auxin transport in plants is a coordinated process involving movement from sites of synthesis (apical regions) to sites of action, in a directional cell-to-cell transport process. In addition, the movement is directed toward sites of specific physiologic processes in different tissues (Friml 2003; Swarup and Bennet 2003). Auxin moves in a basipetal direction in shoots, from the shoot apex and developing leaves to the roots. In contrast, auxin movement in the roots can be either basipetal or acropetal. Basipetal auxin transport in the roots moves IAA from the root tip to the root elongation zone and is involved in the root gravitropic response (Boonshirichai and others 2003). Lateral root formation is regulated by acropetal transport (Casimiro and others 2001).

Three principal protein groups have been identified as key regulators of auxin transport: AUX1, PIN, and PGP (Blakeslee and others 2005; Sieberer and Leyser 2006; Teale and others 2006). These proteins perform different and specific functions in directing the flux of auxin, and the details of their individual and combined activities continue to be investigated. IAA movement into cells can occur via diffusion of the protonated form of IAA, but also via AUX1/LAX carrier proteins, proton symporters that facilitate the movement of IAA across the plasma membrane into the cytoplasm (Marchant and others 1999; Kramer and Bennett 2006; Yang and others 2006; Kerr and Bennett 2007). Auxin movement out of the cell is regulated by the PIN family of proteins (Zazímalová and others 2007),

which have tissue-specific localizations and function as quantitative and directional regulators of IAA efflux (Blakeslee and others 2005; Petrasek and others 2006; Teale and others 2006; Kerr and Bennett 2007; Zazímalová and others 2007). The third group of auxin transport proteins is P-glycoproteins (PGP), members of the ATP-binding cassette (ABC) membrane transporters, and is related to the multidrug resistance (MDR) transporters of animals (Noh and others 2001; Murphy and others 2002; Blakeslee and others 2005). Although the mechanism by which PGP proteins mediate auxin transport is not fully understood, PGPs can act independently or function interactively with PIN proteins (Blakeslee and others 2007). These three protein groups function to facilitate the polar or directional flux of auxin and to mediate the redirection of auxin flux in response to tropic events (Abas and others 2006).

Natural and synthetic chemical inhibitors, representing different chemical groups with sometimes distinct mechanisms of action, have been useful tools for elucidating polar auxin transport (Rubery 1990). Some flavonoids and fatty acids inhibit auxin efflux (Jacobs and Rubery 1988; Rubery 1990), and flavonoids have been suggested to be involved in the natural regulation of auxin transport and signaling (Murphy and others 2000; Brown and others 2001). Recent studies utilizing mutants, either with deficient or enhanced flavanoid levels, further demonstrated the likely role of these compounds in auxin flux (Buer and Muday 2004; Peer and others 2004).

Many synthetic auxin transport inhibitors have been developed and studied (Rubery 1990). Of those inhibitors, NPA and TIBA (Fig. 1) have been used extensively to study auxin flux. Although both compounds inhibit polar auxin transport, NPA is a phytotropin (inhibitor of gravitropic and phototropic responses), and TIBA is not (Rubery 1990).

Mechanistic studies demonstrated that both NPA and TIBA enhanced the accumulation of radiolabeled auxin in plant cells by inhibiting auxin efflux (Hertel 1987; Bernasconi 1996). Membrane-binding studies revealed other differences between these inhibitors: NPA and IAA did not compete for a common binding site (Hertel and others 1972), whereas TIBA did compete with IAA for binding (Katekar and Geissler 1977; Brunn and others 1992). Although both NPA and TIBA inhibit cellular auxin efflux and polar transport, there are differences in the mechanisms and effects of their activity in plants.

Understanding the mechanism of NPA inhibition of auxin transport was enhanced by the discovery of NPA-binding proteins (NBP). A high-affinity NBP was initially identified and proposed to be an accessory/regulatory protein to the true auxin efflux transporter (Lomax and others 1995; Bernasconi and others 1996). NPA was later shown to tightly and specifically bind with AtMDR1 (Multidrug Resistant) and AtPGP1 (P-Glycoprotein) proteins (Noh and others 2001). More recently, PIN proteins were also found to be sensitive to NPA inhibition (Petrasek and others 2006). Auxin efflux by individual PGP or PIN proteins expressed in a heterologous system was sensitive to NPA, and cells coexpressing PIN and PGP demonstrated elevated IAA efflux that was inhibited by NPA (Blakeslee and others 2007).

The overall purpose of this study was to develop a better understanding of the interaction between cyclanilide and auxin transport or signaling. In this article we compare the effect that cyclanilide has on polar auxin movement, auxin accumulation, and gravitropism. Additional insights into cyclanilide activity were pursued by conducting binding affinity assays with cyclanilide, IAA, and NPA utilizing enriched membrane fractions.

Materials and Methods

Polar Auxin Transport Assay

Plant Material Corn (*Zea mays*, c.v. Merit) seeds were germinated in the dark in moist vermiculite in a growth chamber at 27°C. Etiolated corn coleoptiles were harvested 3.5 days after planting when the average seedling length was 5 cm.

Transport Assay Auxin transport assays were conducted according to the methods of Jones (1990). For assay preparation, 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 potassium phosphate buffer (0.25 M sucrose, pH 6.0), containing 1 µM [^{3}H]IAA (specific activity = 26 Ci/mmol) alone or in combination with inhibitors. Inhibitor treatments included technical grade cyclanilide (98% pure), NPA, and TIBA. All solutions were prepared by dissolving the inhibitors in either acetone or dimethyl sulfoxide (DMSO) and diluting to the desired concentration with phosphate buffer so that the final concentration did not exceed 0.1% acetone or DMSO.

After a 10-min pulse in the radiolabeled solution, coleoptiles were rinsed and placed apical end down into a plate containing 1% solidified agar in 1× potassium phosphate buffer, pH 6.0. Small blocks of agar were placed at the base to collect any radiolabel moving from the apex to the base of the coleoptile. The coleoptiles were covered with a beaker lined with wet paper towels to maintain high humidity. Fresh agar blocks were replaced every 20 min throughout the assay. The entire assay was conducted in the dark under green fluorescent light for 3 h. The amount of radioactivity in each agar block was counted using liquid

scintillation spectrometry (LSS) in 5 ml scintillation cocktail. All experiments included three replications per treatment and were repeated three times. Data are presented as mean radioactivity collected (dpm) ± SE over time.

Efflux and Influx Studies

Plant Material Zucchini (*Curcurbita pepo*, c.v. black squash) seeds were germinated in the dark in moist vermiculite in a growth chamber 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.

Efflux and Influx Assay Efflux assays were conducted according to methods described by Bernasconi (1996). Ten hypocotyl segments were immersed in 4 ml uptake solution (0.25 M sucrose, 1 mM $MgCl_2$, and 20 mM citric acid, pH 5.5) containing 1 μM [^{3}H]IAA with or without inhibitors. Stock solutions of inhibitor treatment were prepared as described for auxin transport assays. Tissue segments were incubated for 2 h in uptake solution to allow [^{3}H]IAA uptake. Influx assays were terminated and analyzed at this point. For efflux, tissue was then rinsed and placed in 4 ml efflux solution (0.25 M sucrose, 1 mM $MgCl_2$, and 20 mM citric acid, pH 5.5) without [^{3}H]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. Each treatment consisted of three replications with ten hypocotyl segments per replicate, and data are presented as mean radioactivity (dpm) accumulated per gram tissue ± SE. Each experiment was repeated at least twice.

Gravitropic Studies

Plant Material Tomato (*Lycopersicon esculentum* Mill., cv Mountain Pride) seeds were surface sterilized in 20% commercial bleach and 0.01% Triton X-100 for 20 min and rinsed extensively. Seeds were germinated 2–3 days in the dark at 27°C on Whatman I filter papers moistened with water. Seeds were transferred to agar plates when roots emerged 1-3 mm from the seed.

Growth Media Agar plates were prepared with 1× Murashige and Skoog micronutrient solution (Sigma Co., St. Louis, MO) with 1 mg l^{-1} thiamine, 0.5 mg l^{-1} pyridoxine HCl and nicotinic acid, and 0.8% bacto-agar (Difco, Detroit, MI). Media were adjusted to pH 6.0 with HCl. Treatment stock solutions of IAA, NPA, and cyclanilide were prepared in DMSO and diluted to the desired concentration in agar media. After autoclaving media for 30 min and cooling to 65°C, the inhibitors were added to a given volume of media, mixed well, and poured so that each plate contained 30 ml media. Control plates contained 3% DMSO. Plates were allowed to solidify at room temperature and stored at 4°C until use.

Growth and Gravitropic Assay Gravitropic assays were performed according to methods from Muday and Haworth (1994). 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° 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. Data are expressed as mean percent control ± SE, with three plates per treatment, each containing ten roots. Experiments were repeated twice and results combined.

Binding Studies

Plant material was planted as described above for the efflux studies. About 150 g of the top 4 cm of etiolated zucchini hypocotyls was harvested 4 days after planting for extraction.

Extraction Procedure Plasma membrane extraction and assays were performed according to Hodges and Leonard (1974) and Hodges and Mills (1986). The entire extraction was done at 4°C. Tissue was homogenized in a blender with 1:1 (w/v) Tris buffer, pH 7.2 (25 mM Tris base, 3 mM EDTA, 0.25 M sucrose, 0.2 mM PMSF, 0.15 mg/l DTT). The homogenate was squeezed through two layers of cheesecloth and one layer of nylon membrane and the filtered solution was centrifuged at 7,000*g* for 10 min. The pellet was discarded and the supernatant was subjected to ultracentrifugation at 100,000*g* for 30 min to obtain the total membrane fraction (microsomes). The resulting supernatant was discarded and the microsomal pellet was resuspended in resuspension buffer (5 mM potassium phosphate, pH 7.8, 0.25 M sucrose). Phase separation medium was prepared by layering the following components in order: 12.6 g 20% Dextran (mw = 519,000), 6.3 g 40% polyethylene glycol (mw = 3350), 7.5 ml phase buffer (20 mM potassium phosphate, pH 7.8, 1 M sucrose), and 1.0 ml 770 mM NaCl. The resuspended microsomal pellet was layered on top of the phase separation medium and resuspension buffer was added until the final weight of

the solution was 40 g. Tubes were inverted to mix the contents, and separation was performed by centrifuging at 1,000*g* for 10 min. The top white layer containing plasma membranes was transferred to a new tube with fresh phase separation medium, prepared as described above, mixed, and centrifuged at 1,000*g* for 10 min. The top white layer was combined with 1:3 (v/v) resuspension buffer and centrifuged at 100,000*g* for 30 min to precipitate plasma membranes. The final pellet was resuspended in a citric acid buffer (pH 5.5) containing 0.25 M sucrose and 1 mM $MgCl_2$. The resuspended plasma membranes were stored at −80°C until used for assaying.

Protein quantification was performed with BCA protein reagent using methods from Pierce Chemical Co. (Rockford, IL) and BSA as the standard. Typically, 24 mg plasma membrane protein was obtained from 150 g tissue. ATPase activity was assayed as a marker enzyme for plasma membrane purity using Fiske and Subbarrow reducer and acid molybdate solution from Sigma Co. (St. Louis, MO) to detect inorganic phosphorus released from the enzyme-catalyzed reaction (Peterson 1978). The plant plasma membrane ATPase activity can be distinguished by its sensitivity to vanadate at pH 6.4 and insensitivity to azide (Gallagher and Leonard 1982). ATPase activity in mitochondria, on the other hand, is sensitive to azide at pH 8.5. Vanadate inhibited ATPase activity by 97 and 100% in the microsomal and plasma membrane pellets, respectively. Azide inhibited ATPase activity by 31% in the microsomal pellet, but no inhibition was detected in the plasma membrane fraction. These results indicate that the plasma membrane fraction is free of mitochondrial membranes that are present in the microsomal pellet.

Binding Assay Competition binding assays were conducted according to the methods of Muday and others (1993) in microtiter plates. The assay was performed in citric acid buffer (pH 5.5) (total volume = 200 µl) with 0.2 mg/ml protein, 13 nM [^{3}H]NPA (specific activity = 58 µCi/mmol), or 10 nM [^{3}H]IAA (specific activity = 26 µCi/mmol) and increasing concentrations of unlabeled NPA, IAA, or cyclanilide. Samples were incubated for 1 h at 4°C on an orbital rotary shaker at 100 rpm. Bound radiolabel was separated from free radiolabel by vacuum filtration using 2.4-cm Baxter glass fiber filters presoaked in 0.3% polyethylenimine. Filters were rinsed with 5 ml ice-cold citric acid buffer (pH 5.5) and placed in 5 ml scintillation cocktail. After soaking overnight, the amount of radioactivity was determined using LSS.

Binding experiments were conducted at least twice, with three replications per treatment. Data are presented as mean ± SE specific bound [^{3}H]NPA or [^{3}H]IAA. Specific binding is defined as the difference between total radioactivity detected from samples incubated with radioligand alone (total bound) and samples containing radioligand plus unlabeled ligand (nonspecific and specific bound). Kinetic analysis of the data was conducted according to Segel (1976) and Hulme and Birdsall (1992).

Results

Polar Auxin Transport

Polar auxin transport was measured to determine if cyclanilide inhibits auxin movement in a manner similar to known auxin transport inhibitors. Polar auxin movement can be detected experimentally by placing radiolabeled auxin at the apical end of a corn coleoptile and then collecting auxin at the basal end of the coleoptile via a receiver agar block. Movement of a pulse of radiolabeled IAA from the apex to the base of a corn coleoptile in control treatments increased over time, peaked at approximately 2 h after pulsing, and then decreased (Fig. 2). This is typical of an auxin transport profile where a peak followed by a decline in auxin movement is observed over time as the auxin pulse moves through the coleoptile (Leopold and Lam 1962). However, 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. In addition, when coleoptiles were first pulse-treated with cyclanilide, followed by a [^{3}H]IAA pulse, auxin transport was also inhibited (data not shown). These results indicate that cyclanilide, like NPA and TIBA, inhibits polar auxin transport in corn coleoptiles.

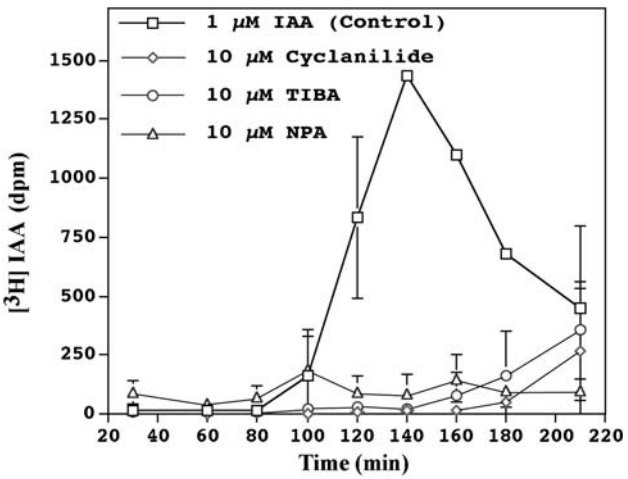

Fig. 2 Time course of auxin transport in etiolated corn coleoptiles. Movement of a 1-µM [^{3}H]IAA pulse, alone or combined with 10 µM cyclanilide, NPA, or TIBA, through a 3-cm etiolated corn coleoptile over time. Each point represents mean ± SE, $n = 3$

Efflux and Influx Studies

The potential effect of cyclanilide on cellular auxin efflux and influx was studied by incubating zucchini hypocotyls in [^{3}H]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 (Fig. 3). In contrast, increasing concentrations of cyclanilide did not result in an increase in IAA accumulation. Considering these results, the effect of cyclanilide on influx was studied. Cyclanilide caused a moderate inhibition of IAA (25 and 32% at 10 and 100 μM, respectively) and NPA had no effect (Table 1). These results demonstrated that cyclanilide did not affect net [^{3}H]IAA efflux and had a minor but significant effect on auxin influx, suggesting that it affects auxin transport differently than NPA or TIBA.

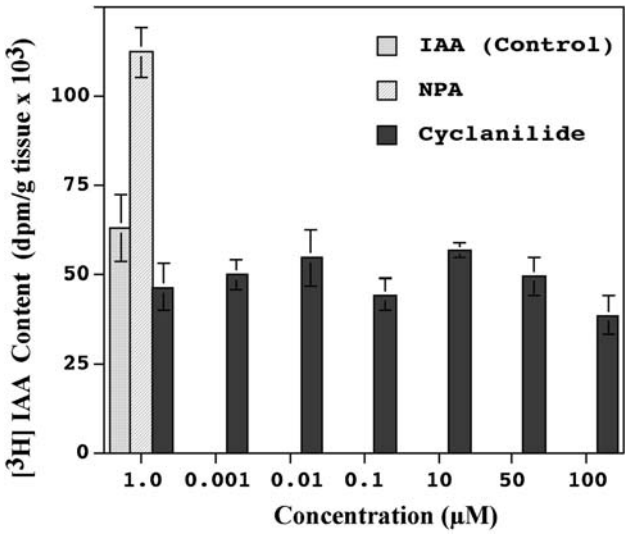

Fig. 3 Accumulation of [^{3}H]IAA into zucchini hypocotyl segments with or without inhibitors in uptake and efflux solution. Comparing accumulation of [^{3}H]IAA with 1 μM NPA or increasing concentrations of cyclanilide in uptake and efflux solutions. Each bar represents mean ± SE, $n = 3$, with ten hypocotyl segments per replicate

Table 1 Inhibition of IAA uptake by cyclanilide

Treatment	DPM/g tissue (±SE)
IAA (1 μM, Control)	196 (±17)
IAA + 1 μM NPA	205 (±2)
IAA + 1 μM Cyclanilide	207 (±9)
IAA + 10 μM Cyclanilide	146 (±1)
IAA + 100 μM Cyclanilide	134 (±4)

Effect of NPA (1 μM) and cyclanilide (1, 10, and 100 μM) on IAA uptake by zucchini hypocotyls. Uptake time = 2 h

Growth and Gravitropic Studies

Changes in root length and curvature were measured in tomato roots grown on media with NPA or cyclanilide to determine if there were effects on the gravitropic response. Root length decreased by 25–70% when grown on increasing concentrations (0.1–10 μM) of NPA (Fig. 4). Root gravitropic response (curvature) was also inhibited by NPA, and the gravitropic response was more sensitive than root growth. For example, whereas root length was reduced 30% when grown on 1 μM NPA, root curvature was inhibited by 85%. These results confirm that NPA is a phytotropin (Rubery 1990).

Root phytotropic response was inhibited by 25% at 0.1 μM cyclanilide concentration (Fig. 4). However, 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. 4). Thus, cyclanilide at low concentrations stimulated root growth and had a relatively low inhibitory effect on gravitropism at concentrations below 100 μM, very different effects than observed with NPA.

Binding Studies

To further study the effect of cyclanilide on auxin activity, competition-binding studies were conducted using microsomal (crude membrane fraction) and 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

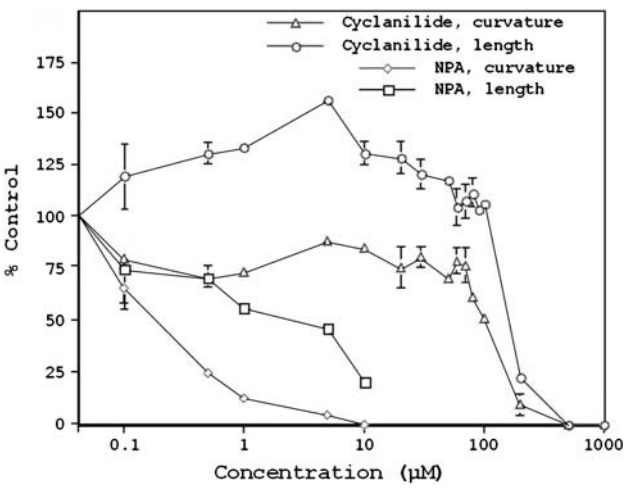

Fig. 4 Gravitropic response of tomato seedlings in the presence of NPA or cyclanilide. Tomato root length and curvature inhibition with increasing concentrations of cyclanilide or NPA. Each point represents mean ± SE, $n = 6$, with ten roots per replicate plate

other membrane fractions (data not shown). Binding of [^{3}H]IAA and [^{3}H]NPA was determined in microsomal and PM extractions (Table 2). Separating the total membrane fraction (microsomal) from the crude extract substantially increased the specific binding of IAA and NPA (Table 2). Specific IAA binding decreased by half when separating PM from the total microsomal preparation; however, specific binding of NPA increased. The binding of IAA and NPA in the zucchini PM protein fraction was saturable (data not shown). With Scatchard plot analysis an apparent affinity constant (k_d) for IAA binding of 0.098 μM was estimated at a concentration of 9.768 pmol/mg protein.

Competition-binding studies were performed between [^{3}H]IAA and unlabeled cyclanilide, NPA, and IAA. NPA did not inhibit [^{3}H]IAA binding (data not shown), indicating that these two compounds act at separate and distinct binding sites in these membrane fractions. This is consistent with previous reports that also found that NPA does not inhibit IAA binding (Hertel and others 1972; Rubery 1990). However, unlabeled cyclanilide inhibited the binding of [^{3}H]IAA in the PM fraction (Fig. 5). 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 (Table 3).

Plotting the data as a Dixon plot can determine the category of inhibition (Segel 1976). Dixon plots suggest that cyclanilide is a noncompetitive inhibitor of [^{3}H]IAA binding (Fig. 6), with a calculated inhibition rate constant (K_i) for cyclanilide of 2.3 μM.

IAA binding was reversible, as evidenced by the displacement of previously bound [^{3}H]IAA with unlabeled IAA (Fig. 7). Unlabled cyclanilide also displaced prebound [^{3}H]IAA. Thus, cyclanilide was both an inhibitor of [^{3}H]IAA binding (association) and dissociation.

Binding studies conducted with [^{3}H]IAA in competition with cyclanilide analogs and TIBA also demonstrated similar patterns of inhibition as cyclanilide (data not shown). The estimated IC_{50} value for the competition between TIBA and [^{3}H]IAA was 54 μM, similar to the estimated IC_{50} value for cyclanilide (Table 2). Three chemical analogs of cyclanilide, each with similar levels of plant growth regulator activity in vivo, also inhibited

Table 2 Differences in NPA- and IAA-specific binding in the extracted fractions for binding assays

Extracted fraction	^{3}H-NPA bound (pmol/mg)	^{3}H-IAA bound (pmol/mg)
SI (crude)	0.006 ± 0.002	0.224 ± 0.04
SII (microsomal supernatant)	0.004 ± 0.001	0.04 ± 0.01
PIII (microsomal pellet)	0.226 ± 0.021	0.93 ± 0.12
SIII (plasma membrane wash)	0 ± 0	0.01 ± 0.02
PIII (plasma membrane pellet)	0.299 ± 0.036	0.50 ± 0.05

Each value represents average specific bound ± SE, $n = 3$

Table 3 Concentration values for 50% binding inhibition of different chemicals in the zucchini plasma membrane fractions

Chemical	IC_{50} for NPA binding site (μM)	IC_{50} for IAA binding site (μM)
NPA	0.01	N/A[a]
IAA	N/A	0.1
TIBA	ND[b]	50
Cyclanilide	50	50

[a] N/A indicates not applicable because of no competition for binding

[b] ND indicates not determined. Muday and others (1993) determined IC_{50} of 54 μM

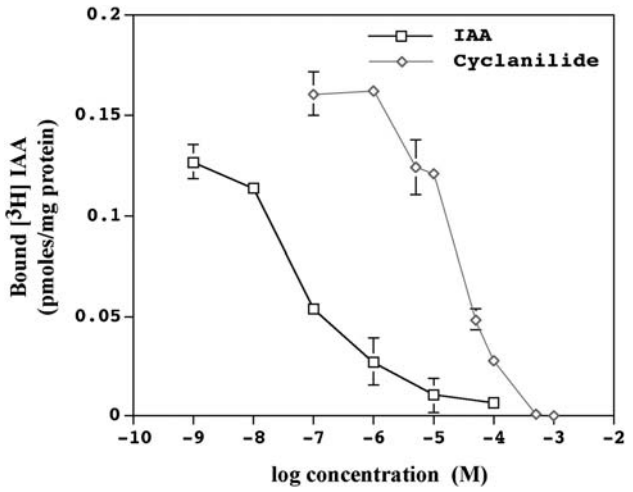

Fig. 5 Competition binding between IAA and cyclanilide in zucchini PM fractions. Competition binding between 10 nM [^{3}H]IAA and increasing concentrations of unlabeled IAA and cyclanilide. Each point represents mean ± SE, $n = 3$

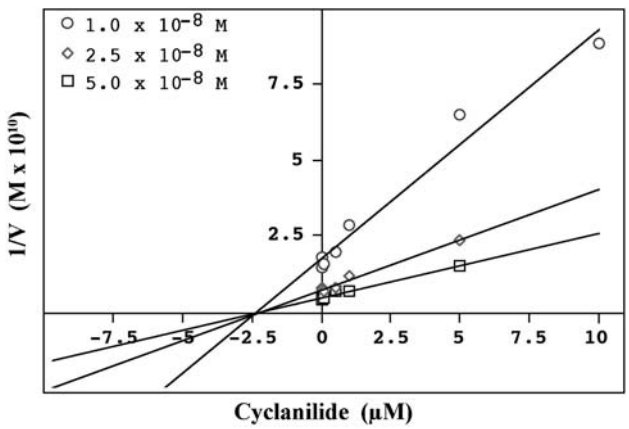

Fig. 6 Dixon plot of cyclanilide competition with [^{3}H]IAA in zucchini PM fractions. Lines were fit with linear regression. The graph illustrates noncompetitive inhibition by cyclanilide and the x intercept of all lines represents the inhibition rate constant (K_i) for cyclanilide. Each point represents mean, $n = 3$

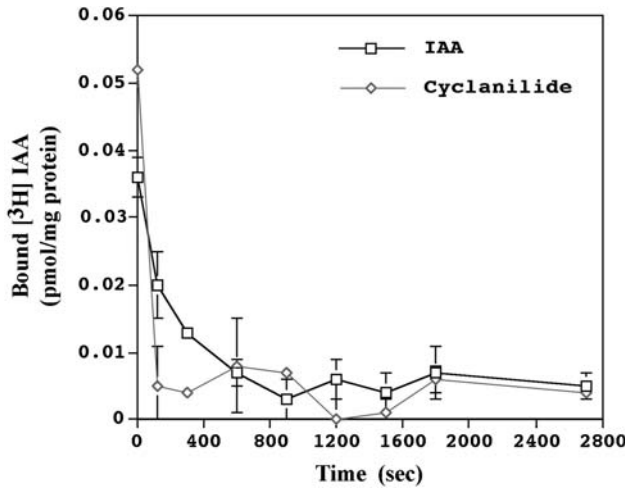

Fig. 7 Reversible [^{3}H]IAA binding over time. Dissociation of prelabeled zucchini PM fractions with 10 nM [^{3}H]IAA in the presence of 1.6 μM unlabeled IAA or cyclanilide over time. Each point represents mean ± SE, $n = 3$

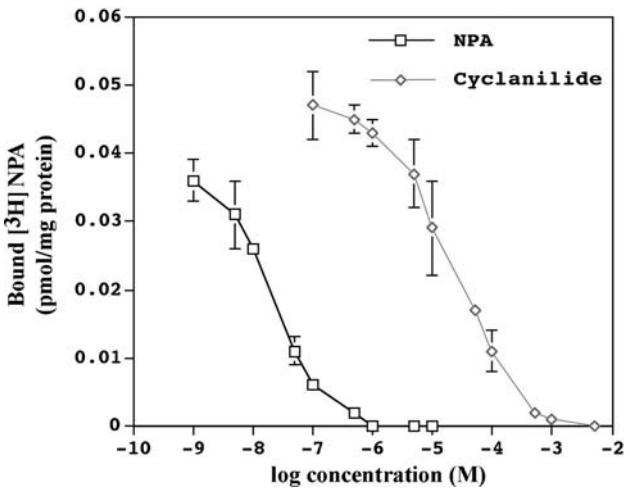

Fig. 8 Competition binding between NPA and cyclanilide in zucchini PM fractions. Competition binding between 10 nM [^{3}H]NPA and increasing concentrations of unlabeled NPA and cyclanilide. Each point represents mean ± SE, $n = 3$

[^{3}H]IAA binding with apparent affinities (IC_{50}) similar to cyclanilide (data not shown).

Membrane-binding studies were also conducted to assess the competitive interactions between [^{3}H]NPA and unlabeled NPA or cyclanilide. Unlabeled NPA inhibited [^{3}H]NPA binding at low concentrations ($IC_{50} = 0.01$ μM; Table 2, Fig. 8), confirming previous work (Muday and others 1993). Increasing concentrations of unlabeled cyclanilide also inhibited [^{3}H]NPA binding in plasma membranes, with an estimated IC_{50} value of 50 μM. These results demonstrated that cyclanilide competes with NPA binding, but the binding affinity of cyclanilide ($IC_{50} = 50$ μM) was much lower than the binding affinity of NPA ($IC_{50} = 0.01$ μM; Table 2).

Dixon plot analysis revealed that cyclanilide is a noncompetitive inhibitor of [^{3}H]NPA binding, with an estimated K_i of 40 μM (data not shown). In addition, NPA binding was reversible, as unlabeled NPA displaced prebound [^{3}H]NPA. Unlike the interaction with IAA, cyclanilide was unable to displace prebound [^{3}H]NPA. Cyclanilide is a noncompetitive inhibitor of [^{3}H]NPA binding, but does not affect the dissociation.

Discussion

Cyclanilide is a PGR that is utilized for different agricultural purposes, yet little is known about its mechanism of action. Because published research suggested that cyclanilide interacted with auxin-related signaling in plants (Pedersen and others 2006), experiments were conducted to characterize the effect that cyclanilide has on auxin movement, cellular efflux, gravitropism, and binding.

Cyclanilide inhibited movement of [^{3}H]IAA in etiolated corn coleoptiles in a manner similar to that of NPA and TIBA, demonstrating that cyclanilide is an auxin transport inhibitor. There are many natural and synthetic compounds that inhibit auxin transport. These inhibitors can act via distinct mechanisms of action and display varied phenotypic responses (Rubery 1990). Auxin transport in plants is mediated by several proteins, and the regulation and direction of transport varies with tissue localization, age, and environmental factors (Leyser 2006). Thus, the category of auxin transport inhibitor does not define a specific mechanism of action, but rather a targeted process. This does not rule out the possibility that cyclanilide could affect some other component of auxin signaling. Nevertheless, knowledge that the process of auxin transport is targeted by cyclanilide enables further efforts toward a definition of a more precise mechanism of action.

NPA and TIBA inhibition of polar auxin transport is based on the inhibition of auxin efflux from the cell (Rubery 1990; Bernasconi and others 1996). Recent studies have confirmed that NPA inhibits PGP and PIN transporter proteins that play a role in IAA efflux (Blakeslee and others 2005, 2007). In the studies reported herein, NPA caused the accumulation of radiolabel auxin in zucchini hypocotyl tissue segments, confirming the inhibition of auxin efflux. In contrast to NPA, cyclanilide did not enhance IAA accumulation at any tested concentration, demonstrating that it did not inhibit auxin efflux. However, cyclanilide moderately inhibited IAA influx, whereas NPA had no effect. Therefore, cyclanilide is an auxin transport inhibitor but does not inhibit cellular auxin efflux, suggesting a distinct mechanism of action, different than NPA and TIBA.

NPA and TIBA differ in their effect on root gravitropism, as NPA is a phytotropin (inhibits tropic responses)

and TIBA is not (Rubery 1990; Lomax and others 1995; Ruegger and others 1997). In these studies, NPA completely inhibited gravitropic response in tomato roots, whereas cyclanilide inhibition of gravitropism remained at only 20–25% from concentrations of 0.1–75 μM.

It has long been known that auxin rapidly increases shoot growth and decreases root growth (see Christian and others 2006). Recent studies have shown that IAA (and TIBA) inhibited root growth by reducing the length of the root growth zone, and NPA inhibited growth by reducing cell production rate (Rahman and others 2007). Because IAA will inhibit root growth, the fact that cyclanilide is an auxin transport inhibitor offers a mechanism for cyclanilide-induced stimulation of root growth. However, TIBA and NPA are also auxin transport inhibitors, yet they inhibit root growth. This indicates that cyclanilide action may involve an interaction with auxin activity at a level beyond inhibition of auxin transport, or that the specificity of cyclanilide inhibition of auxin transport is fundamentally distinct from the inhibition by NPA and TIBA.

It is possible that cyclanilide interaction with the auxin influx proteins (AUX) could be part of the mechanism of action, given that a moderate level of uptake inhibition was observed. However, these results demonstrated that cyclanilide activity is substantially different from the reported affects of auxin influx inhibitors 1-NOA and CHPAA (Parry and others 2001). Those compounds specifically inhibit the auxin influx proteins (AUX), with inhibition values (IC_{50}) in the low μM range, and their effects are sometimes distinct from the effects of auxin efflux inhibitors. Neither 1-NOA nor CHPAA inhibited the basipetal transport of IAA, but both compounds inhibited the gravitropic response in *Arabidopsis* roots (Parry and others 2001). Considering that cyclanilide inhibited IAA transport, had a moderate effect on the gravitropic response, and moderately inhibited influx, it seems unlikely that cyclanilide's mechanism is based primarily on an interaction with the influx proteins.

The membrane-binding assays provided evidence for cyclanilide interaction with IAA- and NPA-binding proteins at the plasma membrane. There may be cyclanilide-protein interactions in other cellular compartments (nucleus, ER, tonoplast), but these were not considered in this research. NPA has known affinities for PGP and PIN proteins which are localized at the PM (Geisler and Murphy 2006; Petrasek and others 2006; Schlicht and others 2006; Blakeslee and others 2007). IAA also accumulates at the PM, as demonstrated with the use of a specific IAA antibody (Schlicht and others 2006). Although cyclanilide affected binding of both compounds, it was a relatively low-affinity inhibitor of both IAA and NPA. Dixon plots demonstrated that cyclanilide was a noncompetitive inhibitor of both IAA and NPA, but the calculated K_i values suggest a somewhat higher affinity for IAA binding. The fact that cyclanilide did not displace prebound NPA (data not shown) but displaced prebound IAA demonstrated differences in the activity of NPA and cyclanilide. The results of the binding assays and kinetics should be interpreted conservatively, as it is known that there are several protein families that interact with IAA at the plasma membrane. Also, auxin transport inhibitors may not be as specific as previously thought, and recently they were shown to inhibit vesicle motility and actin cytoskeleton dynamics in plants and other eukaryotes (Dhonukshea and others 2008).

Cyclanilide is unique as a plant growth regulator, as it is currently registered for use only in combination with other PGRs (ethephon and mepiquat chloride), and its activity involves different phases of plant growth (abscission and boll opening versus vegetative growth reduction, respectively). The research presented herein demonstrated that cyclanilide is unique in its physiologic effects, especially when compared with other auxin transport inhibitors. The observed inhibition of polar auxin transport, absence of inhibition of cellular efflux, and absence of effect on the gravitropic response could result from cyclanilide's interaction with IAA transport proteins that are involved in 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. Further research is needed to understand the mechanism of cyclanilide's activity as a plant growth regulator. Alternatively, understanding cyclanilide's mechanism of action may provide further insight into the transport and signaling of auxin.

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