Antagonism by Clopyralid of Picloram-Induced Ethylene Biosynthesis in Rapeseed Plants

J. Christopher Hall and Mira Soni

Department of Environmental Biology, University of Guelph, Guelph, Ontario, N1G 2W1, Canada

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The effect of clopyralid pretreatment (500 g/ha) on picloram-induced ethylene, ACC (1-aminocyclopropane-1-carboxylic acid), and MACC [1-(malonylamino)-cyclopropane-1-carboxylic acid] was measured in rapeseed plants that were treated with 50 or 100 g/ha of picloram. In contrast to plants that did not receive a clopyralid pretreatment, ethylene biosynthesis was significantly reduced in plants pretreated with clopyralid prior to picloram. Picloram-induced levels of ACC also were significantly reduced in plants receiving pretreatment with clopyralid. In contrast, there was no difference between the levels of MACC in plants that were and were not pretreated with clopyralid. Therefore, the mechanism by which clopyralid pretreatment interferes with picloram-induced synthesis of both ACC and ethylene may be manifested through the blocking of *de novo* synthesis of ACC synthase normally stimulated by picloram. The lack of significant difference in MACC levels between plants that were and were not pretreated with clopyralid precludes the stimulation of enhanced ACC conversion to MACC as an exclusive mechanism of clopyralid's antidoting activity. It is likely that the rate of picloram-induced ACC synthesis by plants receiving pretreatment is within their capacity to convert ACC to MACC, thereby limiting the substrate available for conversion to ethylene. In contrast, it appears that the extent of ACC synthesis by plants receiving no pretreatment supersedes their capacity for conversion to MACC, thereby resulting in greatly enhanced rates of ethylene evolution and subsequent development of injury symptoms.

Introduction

Clopyralid (3,6-dichloro-2-pyridinecarboxylic acid) and picloram (4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid) are members of the pyridine class of herbicides. They exhibit auxin-like activity, including the induction of severe epinasty, hypertrophy, fasciation of the crown and leaf petioles and premature abscission of leaves [1]. Clopyralid is used to selectively control weeds belonging to the Compositae and Polygonaceae families while members of the Cruciferae family, particularly rapeseed (*Brassica napus*, L. *cv.* Altex), are quite tolerant, being able to withstand doses in excess of 5 kg a.i./ha. Clopyralid is used to control *Cirsium arvense* and *Polygonum convolvulus* which are severe weed problems in western Canada [1, 2]. Picloram provides a wider spectrum of weed control than clopyralid with woody plants and most broadleaf species, including rapeseed, being susceptible to picloram.

Based upon the information above, Hall and Vanden Born [2] conducted detailed time course experiments to determine whether differences in selectivity between clopyralid and picloram in rapeseed could be attributed to differences in uptake, translocation, and/or metabolism of these herbicides. They found that sensitivity differences of rapeseed to clopyralid and picloram could not be accounted for by differences in their extent of absorption, translocation or metabolism. Based upon these results, those of other researchers [3–5], and the similarity in structure between these two herbicides, Hall and Vanden Born [2] concluded that within rapeseed, selectivity differences to picloram and clopyralid result from differences in sensitivity at the targeted site of action for these two compounds.

With these results in mind, Hall and Soni [6] predicted that pretreatment of rapeseed with clopyralid may reduce or completely eliminate the herbicidal effects of picloram. Indeed, they found that clopyralid reduced or completely eliminated the phytotoxic symptoms of picloram in rapeseed; the most effective regime being 500 g/ha clopyralid applied 4 days and again 1 day before application of up to 250 g/ha of picloram. The insecticide, chlorpyrifos (O,O-diethyl O-(3,5,6-trichloro-2-pyridyl)phosphorothioate), was the only other

Reprint requests to Dr. J. Christopher Hall.

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compound tested with a structure similar to clopyralid that produced antidoting activity. They [6] also found that clopyralid pretreatment did not reduce the uptake and translocation of [^{14}C]picloram 24, 48, and 72 h after application of the radiolabeled herbicide. Significantly more [^{14}C]picloram was metabolized 24, 48, 72, and 120 h after application to rapeseed plants pretreated with clopyralid. However, 12 h after picloram was applied, Hall and Soni [6] found no difference in picloram metabolism, regardless of pretreatment with clopyralid. They [6] concluded that the increased rate of picloram metabolism after pretreatment with clopyralid is probably not a factor involved in the antidoting activity of clopyralid since injury by picloram was evident as soon as 6 h after its application, whereas little or no injury occurred in pretreated plants as much as 2 weeks after treatment with picloram. Two distinct metabolite peaks were separated by chromatography, but at no time was there any significant difference in the metabolite profiles other than the greater extent of metabolism by pretreated plants. Taken together, the results of Hall and Soni [6] appear to support their hypothesis that sensitivity differences of rapeseed to picloram and clopyralid are the result of differential sensitivity at the targeted sites of action of these two herbicides. Furthermore, it is possible that the antidoting activity of clopyralid involves the interaction of these two herbicides at these sites of action.

In an earlier study, Hall *et al.* [1] examined the levels of ethylene evolution after application of picloram or clopyralid to rapeseed. They found that picloram increased ethylene evolution in rapeseed, whereas clopyralid did not. Picloram-induced ethylene synthesis was implicated in much of the herbicidal activity of picloram by both temporal [1] and activity-related evidence. However, since rapeseed plants receiving clopyralid pretreatment as an antidote to picloram lack the symptoms associated with either ethylene or picloram, it is possible that pretreatment interferes with picloram-induced ethylene biosynthesis. This would occur if pretreated plants either do not respond to picloram at all, or if the specific events leading to the production of picloram-induced ethylene are blocked by clopyralid. Conversely, clopyralid may not act by interfering with the ethylene response, but rather by interfering with ethylene activity itself. Therefore, the purpose of the research presented in this paper was to examine the effects of clopyralid on picloram-induced ethylene production and action in rapeseed plants.

Materials and Methods

General procedures

Rapeseed (*Brassica napus* L. *cv.* Altex) was sown in 350 ml styrofoam cups containing potting soil. Plants were watered daily and fertilized weekly with nutrient solution containing 3 g/l of 20:20:20 (N:P:K) fertilizer and grown in a controlled environment growth room maintained at 26/21 ± 1 °C day/night with a 16 h photoperiod, relative humidity of 65%, and a light intensity of 450 μE m^{-2} sec^{-1}.

In all experiments, commercial formulations of picloram (Tordon 22K as the K^+ salt, 240 g a.e./l) and clopyralid (Lontrel 360 as the acid, 50 g a.e./l) were used. In all experiments, chemicals were applied with a motorized-hood sprayer equipped with a flat-fan nozzle (8002E) and calibrated to deliver 100 l/ha at 276 kPa. Clopyralid, when used as a safener, was applied at 500 g a.i./ha 4 days before, and again 1 day before, rapeseed plants were treated with 50, 100, or 200 g a.i./ha of picloram at the five-leaf stage of development. Controls consisted of plants treated with either water, antidote alone, or picloram alone.

Effects on ethylene production

Rapeseed plants were treated with various concentrations of commercial formulations of clopyralid and picloram as described above. Immediately after application of picloram, the third leaf of a treated plant was removed and its petiole placed in a 22 ml vial containing water. The vial was then placed in a 1 l glass jar. Each jar was flushed for 1 min with ethylene-free air and sealed with a metal lid containing a small hole in which a serum stopper was placed. The jars were placed in the controlled environment growth room and allowed to incubate for 3 h, at which time the first 3 ml sample of headspace gas was removed with a hypodermic syringe.

Ethylene was quantified using a Hewlett-Packard gas chromatograph equipped with a 1.8 m × 3 mm Poropak Q column (80–100 mesh) and flame ionization detector. The helium carrier gas flow rate was 27 ml/min and the injector, col-

umn, and detector temperatures were 60, 90, and 300 °C, respectively. Under these conditions, the limit of sensitivity was 5 pmol ethylene. Ethylene samples were taken every 8 h and the experiment was terminated 72 h after the first gas sample was taken.

Effects on ACC and MACC levels

Rapeseed plants were treated with commercial formulations of clopyralid and picloram as described previously. At each harvest time, the third leaf of a treated plant was detached and the fresh weight recorded. Individual detached leaves were then placed in zip-lock freezer bags, frozen in liquid nitrogen, and stored at −20 °C. To extract ACC, each frozen leaf was broken up and placed in a 50 ml polyethylene round-bottom centrifuge tube containing 10 ml of 75% ethanol (v/v in water) and homogenized using a Polytron (Brinkmann Instruments, Westbury, N.Y.) equipped with a 2 cm wide probe. The residue was removed from the probe with two 10 ml rinses of 75% ethanol and the combined 30 ml homogenate centrifuged at 3000 rpm for 5 min. The supernatant was poured through a glass funnel containing glass wool (to remove any particulate matter) and collected in a 150 ml round bottomed flask. The pellet was then washed twice with 10 ml of 75% ethanol and recentrifuged for 5 min. The supernatant from each washing was added to the previous amount and the collective volume of supernatant reduced to dryness under vacuum at 38 °C. To this residue, 10 ml of 95% ethanol was added. This solution was swirled over the residue for a minimum of 10 min after which it was removed under vacuum as described above. The water-soluble portion of the dried residue was dissolved in 1 ml of water and quantitatively transferred to a 10 ml calibrated test tube. This was repeated 3 times to obtain a total 4 ml volume of plant extract. This solution was stored over night in a refrigerator.

The ACC in the leaf extracts was assayed by chemical conversion to ethylene in the presence of mercury chloride. The extract to be assayed (0.1 to 0.3 ml) and 0.1 ml of 10 mM $HgCl_2$ were added to a 16 × 125 mm test tube and the volume adjusted to 0.5 ml with distilled water. The reaction tube was sealed with a serum cap, agitated on a vortex mixer and kept on ice. Conversion of ACC to ethylene was initiated by injecting 0.1 ml of a cold mixture of 5% NaOCl and 50% saturated NaOH into the reaction mixture through the serum cap with a 1 ml syringe. The appropriate NaOCl solution was obtained by the mixing the following ingredients in the described proportions: 57% commercial bleach:35% NaOH:8% distilled H_2O. Immediately after NaOCl addition, the reaction tube was briefly agitated and held at 0 °C for 1.0 min. The reaction mixture was then exposed to strong light for 25 sec, and incubated at 38 °C for an additional 1 min. The reaction vessel was then briefly agitated and returned to 0 °C for 1 min. A 3 ml gas sample was withdrawn and analyzed for ethylene as previously described.

The ACC was quantified using internal standards. For each extract tested, two equal volumes were taken and one spiked with 10 nmol ACC. From the differences in ethylene produced, the yield of ethylene from the added ACC was calculated, and this amount used to determine the efficiency of ethylene production from ACC in the unspiked sample. The efficiency was 85 ± 10%.

The MACC content was determined by acid hydrolysis of MACC to ACC followed by the conversion of the total ACC to ethylene. A 0.5 to 1.0 ml aliquot of leaf extract was placed in a calibrated test tube containing 0.1 ml of 12 N HCl and was boiled for 2 h to digest MACC to ACC. To this mixture 0.6 ml of 2 N NaOH was added and the volume brought up to 2.0 ml with distilled water. This mixture was then agitated, centrifuged and the supernatant removed and transferred to a test tube. The total ACC content of this solution was then analyzed as described above. Ethylene produced from the free ACC was subtracted from that produced from the total ACC (free ACC plus MACC converted to ACC) to yield the amount of ethylene produced solely from the converted MACC. The efficiency of ACC conversion was determined using internal standards as described above, the efficiency being 75 ± 10%. The efficiency of ACC conversion to ethylene in this extract mixture was reduced by the presence of salts from the addition of HCl and NaOH.

Statistics

All experiments were of a randomized design with at least three replications per treatment and two subsamples per replication were quantified for ACC and MACC levels. Experiments were repeat-

ed at least once and the data subjected to analysis of variance. Means were separated using a Fisher's protected LSD ($P < 0.05$).

Results and Discussion

The effect of clopyralid pretreatment (500 g/ha) on picloram-induced ethylene evolution and/or biosynthesis by the third leaf of rapeseed plants was measured in plants that were treated with 50 or 100 g/ha of picloram. In all cases, ethylene evolution and/or biosynthesis was significantly reduced in plants pretreated with clopyralid prior to picloram treatment (Fig. 1). For example, ethylene evolution was at least 2- to 3-fold greater when plants were not pretreated with clopyralid prior to picloram. When compared to the control, application of 500 g/ha of clopyralid did not elevate ethylene evolution.

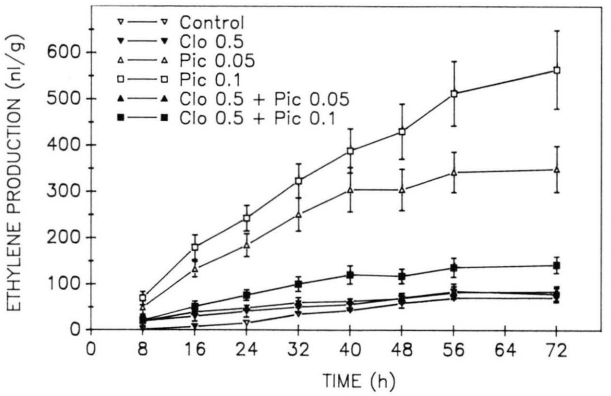

Fig. 1. The effect of clopyralid (Clo), picloram (Pic), or clopyralid/picloram (Clo + Pic) treatments on ethylene production by the third leaf of rapeseed plants. Doses in the legend are in kg a.i./ha.

These results support the direct involvement of ethylene in the activity of picloram. They also indicate that clopyralid pretreatment acts, at least in part, by inhibiting the picloram-induced ethylene response in rapeseed plants.

Although the primary mechanism of auxin-induced ethylene biosynthesis remains unresolved, it appears that auxin enhances the conversion of S-adenosylmethionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC) *via* the induction of *de novo* ACC synthase synthesis [7]. ACC is the immediate precursor of ethylene, but can also be enzymatically and irreversibly converted to 1-(malonylamino)-cyclopropane-1-carboxylic acid (MACC). This latter conversion represents an important mechanism for the regulation of ethylene biosynthesis [8].

With the ethylene biosynthesis pathway in mind, clopyralid pretreatment may limit the rate of picloram-induced ethylene evolution by one of two mechanisms. In the first case, clopyralid could act by blocking picloram's ability to stimulate *de novo* synthesis of ACC synthase, thereby limiting the supply of ACC available for subsequent conversion to ethylene. Alternatively, clopyralid pretreatment could stimulate the enzyme responsible for conjugating ACC to malonate. In the second case, the conversion of ACC to MACC is probably irreversible thereby limiting the substrate available for conversion to ethylene. The effect of clopyralid pretreatment on the level of picloram-induced ACC and MACC synthesis by the third leaf of rapeseed plants was measured in plants that were or not pretreated with 500 g/ha of clopyralid prior to application of 100 g/ha of picloram. The corresponding levels of ACC and MACC were measured in plants receiving no herbicidal treatments as controls.

As with picloram-induced ethylene evolution, picloram-induced levels of ACC were significantly reduced in plants receiving pretreatment with clopyralid 6, 12, 24, 36, 48, and 72 h after picloram application (Table I). However, the level of ACC

Table I. The effect of clopyralid pretreatment on the level of ACC in the third leaf of rapeseed plants*.

Time [h]	ACC content (nmol/g FW) Control	Pretreated**	Not pretreated**
6	571a	896a	2502b
12	158a	787b	3759c
24	50a	504b	3599c
36	178a	464a	8713b
48	274a	438a	4336b
72	167a	82a	3186b

* Means followed by the same letter(s) are not significantly different at the 5% value according to a protected LSD range test.

** Plants were sprayed with 100 g/ha picloram with those plants receiving pretreatment being sprayed with 500 g/ha clopyralid 4 days and again 1 day before picloram was applied. At various times after picloram application, the third leaf was harvested, weighed, and analyzed for ACC content.

in pretreated plants was not significantly higher than in control plants with the exception of those plants harvested 12 and 24 h after picloram application. Although the level of ACC was significantly greater than in pretreated plants, the difference in the level of ACC between plants that were and were not pretreated can not be expected to be limitless. The maximum attainable level of ACC found in plants receiving picloram alone is limited by the continual removal of ACC from the newly-synthesized pool by the active conversion of ACC to both ethylene and MACC.

In contrast, pretreatment with clopyralid did not significantly reduce the levels of MACC with the exception of those plants harvested 36 h after picloram treatment (Table II). When compared with control plants, both pretreated and non-pretreated plants contained significantly higher levels of MACC 6, 12, 48, and 72 h after picloram treatment. It is not clear whether the significantly higher level of MACC found in plants receiving picloram alone at the 36 h harvest is real or just an anomaly.

Table II. The effect of clopyralid pretreatment on the level of MACC in the third leaf of rapeseed plants*.

Time [h]	MACC content (nmol/g FW) Control	Pretreated**	Not pretreated**
6	414a	4039b	3452b
12	1137a	5523b	2558b
24	1036a	5415b	5468b
36	1830a	3370a	11480b
48	484a	3353b	4345b
72	1079a	3732b	7108b

* Means followed by the same letter(s) are not significantly different at the 5% value according to a protected LSD range test.

** Plants were sprayed with 100 g/ha picloram with those plants receiving pretreatment being sprayed with 500 g/ha clopyralid 4 days and again 1 day before picloram was applied. At various times after picloram application, the third leaf was harvested, weighed, and analyzed for MACC content.

The reduction of ACC levels in pretreated plants parallels the reduction of ethylene evolution previously observed from these plants. It can, therefore, be concluded that clopyralid pretreatment interferes with the picloram-induced synthesis of both ACC and ethylene, presumably by blocking *de novo* synthesis of ACC synthase. The lack of significant difference in MACC levels between plants that were and were not pretreated precludes the stimulation of enhanced ACC conversion to MACC as an exclusive mechanism of clopyralid's antidoting activity. However, the significantly enhanced levels of MACC in pretreated plants compared with control plants coupled with the corresponding lack of significant difference in ACC levels between these two groups, indicate that *de novo* synthesis of ACC synthase occurs in both plants that were and were not pretreated with clopyralid; albeit at much lower rates by pretreated plants. It is likely that the rate of picloram-induced ACC synthesis by plants receiving pretreatment is within their capacity to convert ACC to MACC, thereby limiting the substrate available for conversion to ethylene. In contrast, it appears that the extent of ACC synthesis by plants receiving no pretreatment supersedes their capacity for conversion to MACC, thereby resulting in greatly enhanced rates of ethylene evolution and subsequent development of injury symptoms.

It is well established that auxins, including pyridines, stimulate ethylene biosynthesis in susceptible plants [9]. However, due to the similarity of injury symptoms produced by auxins and ethylene, the distinction between ethylene-induced and auxin-induced responses remains controversial. The results presented in this paper clearly show that pretreatment with clopyralid significantly reduced the extent of picloram-induced ethylene biosynthesis. This, together with the earlier work of Hall *et al.* [1] which correlates the onset of picloram symptoms with the stimulation of ethylene production implies that the inhibition of ethylene biosynthesis must be involved in the overall antidoting activity of clopyralid [6]. The observation that pretreatment significantly reduced the extent of picloram-induced ACC synthesis further implies that this inhibition results from the inhibition of picloram-induced synthesis of ACC synthase. Although *de novo* synthesis of ACC synthase was not proven in this work, it has been previously established as being a specific auxin-induced response by Yoshii and Imaseki [7].

The question now arises whether the antidoting of picloram in rapeseed is complete. There was no

difference in the amount of ethylene evolution (Fig. 1) or in the levels of ACC and MACC (data not shown) between plants receiving either water or clopyralid alone. In contrast, plants receiving clopyralid followed by picloram contained significantly more MACC than both water and clopyralid controls, but without significant differences in the respective levels of ACC. This indicates that picloram stimulated some degree of *de novo* synthesis of ACC synthase in pretreated plants, but that this excess ACC was converted to MACC. Therefore, it appears that clopyralid pretreatment did not produce complete inhibition of picloram activity.

It is possible that the enhanced rate of picloram metabolism stimulated by pretreatment with clopyralid [6] was great enough to reduce picloram to sublethal concentrations. These sublethal concentrations of picloram may have stimulated the synthesis of ACC, but to a degree that was within the capacity of conversion to MACC by rapeseed plants. Consequently, pretreatment eliminated the development of injury symptoms produced by ethylene. This would also explain why doses of picloram in excess of 250 g/ha could not be antidoted with clopyralid pretreatment (data not shown).

Although ethylene undoubtably plays a critical role in picloram activity, the extent of its contribution to phytotoxicity remains unclear. Continued study of the relationship between clopyralid antidoting activity and the inhibition of picloram-induced ethylene synthesis will help define the role of ethylene in the expression of auxin activity.

The results presented here document a clear blockage of the toxic effects of picloram, an auxinic herbicide, with a less toxic, but structurally-related synthetic auxin. In rapeseed plants, picloram caused a rapid and marked increase in ethylene evolution and it is possible that picloram injury in rapeseed is largely caused by ethylene. Pretreatment with clopyralid clearly blocked the increased production of ethylene in picloram-treated plants. This system could therefore, prove to be very useful in further studies to elucidate the biochemical mechanisms of auxin action.

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