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# Physiological and Biochemical Characterization of Quinclorac Resistance in a False Cleavers (*Galium spurium* L.) Biotype

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The physiological and biochemical basis for quinclorac resistance in a false cleavers (*Galium spurium* L.) biotype was investigated. There was no difference between herbicide resistant (R) and susceptible (S) false cleavers biotypes in response to 2,4-D, clopyralid, glyphosate, glufosinate-ammonium, or bentazon. On the basis of $GR_{50}$ (growth reduction of 50%) or $LD_{50}$ (lethal dose to 50% of tested plants) values, the R biotype was highly resistant to the acetolactate synthase (ALS) inhibitor, thifensulfuron-methyl ($GR_{50}$ resistance ratio R/S = 57), and quinolinecarboxylic acids (quinclorac R/S = 46), resistant to MCPA (R/S = 12), and moderately resistant to the auxinic herbicides picloram (R/S = 3), dicamba (R/S = 3), fluroxypyr (R/S = 3), and triclopyr (R/S = 2). The mechanism of quinclorac resistance was not due to differences in [$^{14}$C]quinclorac absorption, translocation, root exudation, or metabolism. Seventy-two hours after root application of quinclorac, ethylene increased ca. 3-fold in S but not R plants when compared to controls, while ABA increased ca. 14-fold in S as opposed to ca. 3-fold in R plants suggesting an alteration in the auxin signal transduction pathway, or altered target site causes resistance in false cleavers. The R false cleavers biotype may be an excellent model system to further examine the auxin signal transduction pathway and the mechanism of quinclorac and auxinic herbicide action.

**KEYWORDS:** ***Galium spurium*; quinolinecarboxylic acid; multiple resistance; auxinic herbicide; ALS inhibitor resistance; dose response; radiolabeled herbicide; ethylene; abscisic acid**

## INTRODUCTION

Quinclorac and quinmerac, members of the quinolinecarboxylic acid family of herbicides, are auxinic herbicides with monocot activity. Although the target site of the quinolinecarboxylic acids and the other chemical classes of auxinic herbicides is not known, these herbicides induce de novo synthesis of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and increases in ACC levels in susceptible monocot and dicot species (for review, see *1*, *2*). ACC oxidase catalyzes the oxidation of ACC resulting in the production of hydrogen cyanide and ethylene. In monocots, hydrogen cyanide levels accumulate to cytotoxic levels in susceptible but not in tolerant or resistant monocots (*3*) or susceptible dicot species (*4*). In sensitive dicots, auxin-induced ethylene biosynthesis stimulates de novo abscisic acid (ABA) synthesis (*2*, *5*) resulting in an overproduction of reactive oxygen species, particularly hydrogen peroxide ($H_2O_2$), which leads to tissue damage and cell death (*6*). In Canada, quinclorac is registered for pre- and postemergence control of cleavers (*Galium* spp.), volunteer flax (*Linum usitatissium* L.), green foxtail [*Setaria viridis* (L.) Beauv.], and barnyard grass [*Echinochloa crus-galli* (L.) P. Beauv.] in canary seed (*Phalaris canariensis* L.), barley (*Hordeum vulgare* L.), and various wheat (*Triticum aestivum* L.) varieties.

Most of the 23 plant species that are found worldwide with resistance to one auxinic herbicide are usually cross-resistant to some but not all auxinic herbicides (*7*). Absorption, translocation, root exudation, or metabolism were not responsible for auxinic herbicide resistance or reduced sensitivity in several weed biotypes including yellow starthistle (*Centaurea solstitialis* L.) to picloram (*8*) and clopyralid (*9*), kochia (*Kochia scoparia* L.) to dicamba (*10*), wild mustard (*Sinapis arvensis* L.) to dicamba and picloram (*11*), and *Echinochloa* spp. (*12*) to quinclorac. In the aforementioned species, a lack of differences between biotypes in auxinic herbicide absorption, translocation, and metabolism has led to the hypothesis that auxinic herbicide resistance is likely due to differences at the target site or differences along the signal transduction pathway.

A biotype of false cleavers (*Galium spurium* L.) was previously reported to express resistance to the acetolactate synthase (ALS) inhibitor herbicides, the sulfonylureas and imidazolinones, as well as to the auxinic herbicide quinclorac

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**Table 1.** Herbicides and the Field Dose (1×) Applied to R and S False Cleavers

| herbicide | dose[a] (g ai ha$^{-1}$) | formulated product | company | IUPAC chemical name |
|---|---|---|---|---|
| glyphosate | 450.0 | Roundup Transorb | Monsanto Canada | N-(phosphonomethyl)glycine |
| glufosinate-ammonium | 506.3 | Ignite | Syngenta Canada | ammonium DL-homoalanin-4-yl(methyl) phosphinate |
| thifensulfuron | 6.0 | Pinnacle | Dupont Canada | 3-(4-methoxy-6-methyl-1,3,5-triazin-2-yl-carbamoylsulfamoyl)thiophene-2-carboxylic acid |
| bentazon | 1100.0 | Basagran | BASF Corporation Canada | 3-isopropyl-1H-2,1,3-benzothiadiazin-4(3H)-one-2,2-dioxide |
| quinclorac | 125.0 | Accord | | 3,7-dichloroquinoline-8-carboxylic acid |
| quinmerac | 750.0 | Fiesta | | 7-chloro-3-methylquinoline-8-carboxylic acid |
| dicamba | 306.2 | Banvel | | 3,6-dichloro-*o*-anisic acid |
| triclopyr | 283.3 | Release | Dow AgroSciences Canada | 3,5,6-trichloro-2-pyridyloxyacetic acid |
| fluroxypyr | 144.1 | Vista | | 4-amino-3,5-dichloro-6-fluoro-2-pyridyloxyacetic acid |
| picloram | 273.8 | Tordon 22K | | 4-amino-3,5,6-trichloropicolinic acid |
| clopyralid | 306.2 | Stinger | | 3,6-dichloropyridine-2-carboxylic acid |
| MCPA | 212.5 | MCPA Amine 500 | United Agri Products | (4-chloro-2-methylphenoxy)acetate |
| 2,4-D amine | 563.5 | Amsol 500 | Rhone-Poulenc Canada | (2,4-dichlorophenoxy)acetic acid |

[a] The 1× dose was the recommended field dose for false cleavers control according to the Manitoba Guide to Crop Protection 2000: Weeds, Plant Diseases, Insects.

(*13*). This resistant biotype (designated R) was found in a field in central Alberta, Canada, in 1996. The discovery of quinclorac resistance in this R biotype was unexpected because although previously treated several times with ALS inhibitor herbicides, quinclorac had never been sprayed on this particular field and no quinolinecarboxylic acid herbicides were registered for use in Canada until 1997. In this R false cleavers biotype, the mechanism of ALS resistance is due to altered herbicide binding to the ALS target site (*13*), which resulted from a point mutation in the ALS gene that confers an amino acid alteration in the ALS enzyme (*14*). Previous research demonstrated that there are two distinct, nuclear resistance genes, one for quinclorac resistance and one for ALS inhibitor resistance in false cleavers (*15*).

With a focus on the elucidation of the mechanism of quinclorac resistance in false cleavers, the research objectives in this sudy were to (i) characterize phytotoxic responses of R and S plants to the quinolinecarboxylic acids, auxinic herbicides, and other herbicides with different modes of action, (ii) compare the pattern of absorption, translocation, root exudation, and metabolism of [$^{14}$C]quinclorac in R and S plants, and (iii) quantify endogenous ethylene and ABA in R and S plants to assess their role in the mechanism of action of quinclorac phytotoxicity.

## MATERIALS AND METHODS

**Plant Material and Growth Conditions.** R false cleavers seeds were collected from plants that survived triasulfuron treatment in a field in central Alberta while S seeds were collected from a field outside of Edmonton, Alberta (*13*), not previously treated with quinclorac. The seed was propagated by L. M. Hall, Department of Agricultural, Food and Nutritional Science University of Alberta, Edmonton, AB, and sent to the University of Guelph. Approximately 10 plants of each biotype were grown in separate growth rooms and allowed to set seed. This seed stock was used in all experiments.

Plants were grown in a controlled environment growth room maintained at 24/16 ± 1 °C day/night temperature with a 16 h photoperiod and an average relative humidity of 65%. The irradiance level was constant at 400 $\mu$Einstein m$^{-2}$ s$^{-1}$. Plants were irrigated as required with water and fertilizer containing 20:20:20 N:P:K (20 g L$^{-1}$) and micronutrients.

**Dose−Response Experiments.** False cleavers were grown from seed, one plant per pot (450 mL), in Premier Promix (Premier Horticulture Inc., Red Hill, PA), a peat moss-based potting medium. Plants were sprayed at the three-whorl stage of foliar development. The commercial formulations of 13 herbicides were individually tested using at least six doses ranging from 1/16× to 32×, depending on plant response and relative to the recommended field dose (1×) required for false cleavers control in western Canada (**Table 1**). Quinclorac and thifensulfuron were applied with their recommended adjuvant, 1% (v/v) Merge (BASF Corporation Canada, London, ON, Canada), a 1:1 surfactant and petroleum hydrocarbon solvents blend and the nonionic surfactant Agral 90 (Dupont Canada Inc. Missisauga, ON, Canada) at 0.1% (v/v), respectively. All herbicides were applied with a motorized hood sprayer equipped with a 80015E flat-fan nozzle (TeeJet Spraying Systems Co., Wheaton, Il) calibrated to deliver 110 L ha$^{-1}$ of spray solution at 250 kPa. Visual ratings of phytotoxic symptoms were determined 14 days after treatment (DAT), and plants were harvested by severing the shoot from the root at the soil level 14 DAT with quinclorac-treated plants and 21 DAT with all other herbicides. Mortality and shoot dry weights were determined.

Quinclorac dose−response experiments were conducted three times; glyphosate, glufosinate-ammonium, bentazon, quinmerac, and MCPA dose−response experiments were conducted twice; and all other dose−response experiments were conducted once, although preliminary screens with 0, 1/4×, 1×, and 4× doses were previously conducted (*16*). Dose−response experiments consisted of at least three replications per dose.

**Radiolabeled Quinclorac Experiments.** Radiolabeled and technical quinclorac (3,7-dichloroquinoline-8-carboxylic acid) were obtained from BASF Aktiengesellschaft (Ludwigshafen, Germany). The specific activity of [$^{14}$C]quinclorac was 361.97 MBq mmol$^{-1}$ with a radiochemical purity of >96%. All chemicals and reagents used were of reagent quality or better.

*Treatment of Plants.* Seeds were sown in vermiculite (Therm-o-Rock East INC, New Engle, PA). False cleavers plants at the three-whorl stage of foliar development were removed from vermiculite by rinsing with water and placed in a 22 mL glass scintillation vial containing 15 mL of hydroponic solution, which consisted of 75% (v/v) strength fertilizer solution as previously described. Plants were foliar treated with [$^{14}$C]quinclorac ca. 12 h after being moved to the hydroponic solution.

[$^{14}$C]Quinclorac was dissolved in 0.1 M phosphate-buffered saline (pH 7.5) containing 10% (v/v) ethanol and 0.5% (v/v) Tween 20 (oxysorbic 20-polyoxyethylene sorbitan monolaurate). Ten microliters of this solution containing ca. 3.3 kBq (ca. 200000 DPM; 2.2 $\mu$g ai plant$^{-1}$) was applied as ca. 40, 0.25 $\mu$L droplets with a 10 $\mu$L Wiretrol micropipet (Drummond Scientific Company, Broomall, PA) to the adaxial surface of all leaflets (4−5) of the second whorl of false cleavers plants. Plants were harvested 0, 24, 48, and 96 h after treatment (HAT).

*Absorption, Translocation, and Root Exudation Experiments.* At each harvest time, plants were dissected into roots, treated whorl, and nontreated foliage above and below the treated whorl. At the time of harvest, the amount of [$^{14}$C]quinclorac present on the surface of all the treated leaflets (4−5 per whorl) was determined using a foliar rinse treatment, which involved directing 10 mL of aqueous 10% (v/v) ethanol containing 0.5% (v/v) Tween 20 over the surface of each treated leaflet. The rinse solution from all leaflets was collected in two 22 mL scintillation vials containing Ecolite (+) (ICN Biomedicals Inc., Irvine, CA) scintillation cocktail. Radioactivity was quantified by liquid scintillation spectrometry (LSS) using a Beckman LS6K-SC scintillation counter (Beckman Instruments Inc., Fullerton, CA). Each plant part was wrapped in KimWipe paper tissue (Kimberley Clark Inc., Roswell, GA) and dried, and the quantity of radioactivity was determined by oxidative combustion of the samples as described below. Radioactivity in the nutrient solution was quantified as previously described for the leaf rinse solution.

*Metabolism Experiments.* Plants were grown and treated with [$^{14}$C]-quinclorac, and foliar leaf rinse was conducted as previously described. At harvest, roots were wrapped in KimWipe tissue, dried, and oxidized as described below. After a foliar leaf rinse, individual shoots were stored in a glass test tube at −20 °C until [$^{14}$C]quinclorac and its metabolites were extracted.

*1. Extraction of [$^{14}$C]Quinclorac and Its Metabolites.* The frozen shoot from one plant (ca. 1 g) was minced and homogenized in 5−8 mL of acetonitrile/water (7:3, v/v) using a tissue grinder (Pyrex 7727-7, Corning Inc., Wilmington, NC) and centrifuged (10000*g* for 10 min). The supernatant was decanted, and the pellet was resuspended in acetonitrile/water (7:3, v/v) and extracted twice more. After the final extraction, the resulting pellet was dried and combusted to determine the amount of unextractable radioactivity. Supernatants were pooled, and an aliquot (100 $\mu$L) was removed to determine the percent recovery of radioactivity by LSS. Pooled supernatants were concentrated at 35 °C to ca. 0.5 mL under a stream of $N_2$. The supernatant was further purified, prior to high-performance liquid chromatography (HPLC) analysis, by passing the radioactive plant solution through a preparative $C_{18}$ cartridge ($C_{18}$ Sep-Pak Plus; Waters Associates, Milford, MA). Radioactivity adsorbed to the $C_{18}$ matrix was eluted with 3 mL of acetonitrile/water (7:3, v/v). Samples were stored at −20 °C until HPLC analysis. To ensure that herbicide degradation did not occur during storage or extraction, appropriate controls were conducted as described in Van Eerd and Hall (*17*).

*2. HPLC Analysis.* Following preparative $C_{18}$ chromatography, plant extract was filtered through a 3 mm diameter, 0.22 $\mu$m nylon syringe filter (MSI Cameo, MSI Micron Separations Inc., Honeoye Falls, NY). The filtered plant extract (100 $\mu$L) was analyzed by HPLC on a Shimadzu model LC-6A chromatograph equipped with a Waters $\mu$Bondapak $C_{18}$ column ($C_{18}$ 5 $\mu$m particle size, 300 mm × 3.9 mm; Waters) using the modified method of Lamoureux and Rusness (*18*). The solvents used were acetonitrile (B) and Nanopure water (Diamond water system, Barnstead International Inc., Dubuque, IA) containing 0.1% (v/v) acetic acid (A) at a flow rate of 1.5 mL min$^{-1}$. Chromatographic conditions consisted of a linear gradient of 10−40% B over 30 min for elution, followed by a 2 min linear gradient to 99% B, after which the concentration of B (99%) was held for 10 min before equilibration at 10% B over 3 min. The column temperature was maintained at ambient room temperature (21 ± 3 °C). [$^{14}$C]Quinclorac and its radiolabeled metabolites were detected and quantified using a Radiomatic Flo-One\Beta A-250 radioactivity flow detector (Radiomatic Instruments and Chemical Co. Inc., Tampa, FL).

*Oxidative Combustion of Samples.* The quantity of radioactivity in false cleavers shoots, roots, and pellets was determined by oxidative combustion of tissue samples to $^{14}CO_2$ using a biological oxidizer. $^{14}CO_2$ was trapped in carbon-14 scintillation cocktail (model OX-300, R. J. Harvey Instrument Corp., Hillsdale, NJ). $^{14}CO_2$ recovery was >96% as determined by combusting known quantities of D-mannitol-1-$^{14}$C (Sigma Aldrich Co., St. Louis, MO).

[$^{14}$C]Quinclorac metabolism experiments with R and S plants were conducted three times, with three replications per treatment. Data were expressed as a percentage of total recovered radioactivity for each plant prior to statistical analysis.

**Exposure to Exogenous Ethylene.** Exogenous ethylene exposure experiments were conducted on dark-grown germinating R and S false cleavers. These experiments were conducted to determine if there were differences in the ethylene responsive pathway between R and S plants which may explain the mechanism of quinclorac resistance (*19*, *20*). Twelve seeds were sown in ca. 15 cm$^3$ of Premier Promix contained in a 15 mL wax-coated paper cup. The soil was moistened, and each cup was placed in a wide mouth 1 L Mason jar, which could be sealed with a metal lid fitted with a rubber septum to allow gas sampling. Jars (not sealed) were placed in a cardboard box and covered with a thick black cotton sheet to prevent light exposure. The box was placed underneath benches in the growth room. Five days after planting and shortly after emergence, jars were sealed and 1 or 10 $\mu$L of ethylene (Sigma Aldrich Co., Milwaukee, WI) was injected into the sealed jars with a gastight syringe (1700 Series, Hamilton Company Inc., Reno, NV), resulting in a final ethylene concentration of 1 or 10 $\mu$L L$^{-1}$. Nontreated control jars were sealed, but no ethylene was injected. At 12 h intervals, all jars were opened, flushed with ethylene-free air, and sealed, and the appropriate quantity of ethylene was injected into each jar. Dark-grown seedlings were exposed to ethylene for 36 h prior to harvesting. The effect of ethylene on dark-grown seedlings was determined by measuring hypocotyl length from the radicle to the tip of the cotyledons. The dark-grown exogenous ethylene seedling experiment consisted of 12 seedlings per jar, two jars per treatment, and the experiment was conducted twice.

The exogenous ethylene exposure experiment was also conducted on light-grown plants at the two-whorl stage of development. One plant, grown in a 15 mL wax-coated paper cup to the two-whorl stage of development, was sealed in a 1 L wide mouth Mason jar. Experimental procedures were the same as for dark-grown seedlings, except the following ethylene doses were used, 0, 100, and 1000 $\mu$L L$^{-1}$. Jars were placed in the growth room under the same light and environmental conditions as previously described. Thirty-six hours after ethylene exposure, the plant response was assessed by visual rating of epinasty (downward curving of leaf) and/or hyponasty (upward curving of leaf). Light-grown exogenous ethylene plant experiments consisted of one plant per jar, two replications per treatment, and the experiment was conducted twice.

**Endogenous Ethylene Biosynthesis.** For endogenous ethylene biosynthesis experiments, false cleavers plants were grown to the two-whorl stage of development in vermiculite. The vermiculite was removed from the roots by rinsing with water. To minimize microbial ethylene production, roots were dipped for ca. 30 s in a household bleach solution diluted to 12% (v/v) with water [0.063% (v/v) sodium hypochlorite]. The roots were rinsed with at least 250 mL of water to remove the bleach solution. One plant was placed in a 30 mL sealable jar containing 2 mL of 50% (v/v) strength nutrient solution prepared as previously described. Each jar also contained a 2 mL gas chromatography vial filled with the contents of two carbonate−bicarbonate capsules (i.e., ca. 1 g; Sigma Aldrich Co.). The open carbonate−bicarbonate vial within the sealed jar provided plants with $CO_2$.

Approximately 2 h after transplanting, quinclorac or 2,4-D stock solution was added to the nutrient solution producing a final herbicide concentration of $10^{-4}$ M (ca. 1/50 field dose). After the addition of herbicide, all jars were sealed and placed in the growth room under light. A 300 $\mu$L gas sample was removed through the rubber septum with a gastight syringe 0, 6, 12, 24, 48, and 72 HAT. The quantity of ethylene was determined using a gas chromatograph (CP-3800 Varian, Walnut Creek, CA) equipped with a fused silica capillary column (0.53 mm i.d., Supelco, Oakville, ON, Canada) and a flame ionization detector. To quantify ethylene, known quantities of ethylene were used to create standard curves. The endogenous ethylene biosynthesis experiment was conducted three times, with five replications (jars) per treatment and one plant per jar.

**ABA Biosynthesis.** For studying the effects of short-term exposures to quinclorac (<24 h), plants at the three-whorl stage of development were removed from the vermiculite as previously described and the roots of three intact plants were placed in a 250 mL jar containing nutrient solution as previously described. Twenty-four hours after transplanting, a quinclorac stock solution was added to the nutrient

**Table 2.** $LD_{50}$ and $GR_{50}$ Values[a] with CIs[b] in Parentheses and Resistance Ratios[c] for R and S False Cleavers Treated with the Formulated Product of Various Herbicides and Harvested 21 DAT

| | | $LD_{50}$ | | | $GR_{50}$ | | |
|---|---|---|---|---|---|---|---|
| herbicide | dose (g ai $ha^{-1}$) (1×) | resistant | susceptible | resistance ratio (R/S) | resistant | susceptible | resistance ratio[e] (R/S) |
| glyphosate | 450 | 0.51[e] (0.45, 0.68) | 0.51[e] (0.45, 0.68) | 1[g] | 0.22 (0.20, 0.25) | 0.24 (0.21, 0.26) | 0.9[g] |
| glufosinate | 506 | 0.52[e] (0.48, 0.72) | 0.48 (0.38, 0.62) | 1.1[g] | 0.27 (0.25, 0.30) | 0.24 (0.22, 0.26) | 1.1[g] |
| bentazon | 1100 | 0.14 (0.11, 0.19) | 0.13 (0.10, 0.18) | 1.1[g] | 0.04 (0.04, 0.05) | 0.06 (0.05, 0.06) | 0.8[g] |
| thifensulfuron | 6 | >16[d] | 2.58 (1.87, 3.71) | >6 | 5.71 (–2.90, 14.33) | 0.10 (0.06, 0.14) | 57.1 |
| quinclorac[f] | 125 | >12 | 0.39 (0.30, 0.50) | >31 | 2.13 (1.39, 2.88) | 0.05 (0.03, 0.07) | 42.6 |
| quinmerac | 750 | >16 | 0.96 (0.70, 1.36) | >16 | >16[d] | 0.03 (–0.01, 0.06) | >533 |
| MCPA | 212 | >16 | 7.94 (6.08, 10.90) | >2 | 3.35 (1.00, 5.69) | 0.29 (0.06, 0.52) | 11.6 |
| 2,4-D | 563 | 15.73[e] (12.11, 20.58) | 26.63 (19.10, 42.89) | 0.6[g] | 1.74 (1.26, 2.23) | 0.80 (0.47, 1.14) | 2.2 |
| clopyralid | 306 | 4.82 (3.96, 6.06) | 4.16 (3.43, 5.19) | 1.2[g] | 0.82 (0.42, 1.23) | 0.79 (0.40, 1.18) | 1.0[g] |
| triclopyr | 283 | 3.32 (2.72, 4.13) | 1.76 (1.26, 2.43) | 1.9 | 0.27 (0.19, 0.35) | 0.18 (0.19, 0.23) | 1.5[g] |
| fluroxypyr | 144 | 0.62 (0.48, 0.80) | 0.20 (0.14, 0.27) | 3.1 | 0.04 (0.02, 0.06) | 0.02 (0.01, 0.03) | 2.0[g] |
| picloram | 274 | 0.23[e] (0.17, 0.24) | 0.08 (0.04, 0.13) | 2.9 | 0.03 (0.03, 0.04) | 0.01 (0.01, 0.02) | 3.0 |
| dicamba | 290 | 0.80 (0.63, 1.04) | 0.27 (0.20, 0.36) | 3.0 | 0.08 (0.06, 0.10) | 0.05 (0.04, 0.06) | 1.6 |

[a] $LD_{50}$ and $GR_{50}$ values were expressed as × of field dose. $LD_{50}$ [the lethal dose (g ai $ha^{-1}$) to 50% of the plants tested] values were calculated using the GLM at the 95% level. $GR_{50}$ [dose (g ai $ha^{-1}$) that reduced shoot growth by 50% as compared to the nontreated control] values were calculated using SAS 8.0 software (SAS Institute Inc. Cary, NC) using PROC NLIN model at the 95% level. Quinclorac dose–response experiments were conducted three times ($n = 12$); glyphosate, glufosinate-ammonium, bentazon, quinmerac, and MCPA dose–response experiments were conducted twice ($n = 10$); and all other dose–response experiments were conducted once ($n = 5$), although preliminary screens with 0, 1/4×, 1×, and 4× were previously conducted ($n = 11$) (*17*). Data from all experiments were pooled. [b] CIs, in parentheses, were calculated at the 95% level. [c] Resistance ratios were calculated using $LD_{50}$ (or $GR_{50}$) values [e.g., resistance ratio = $LD_{50}$(R) ÷ $LD_{50}$(S)]. [d] No death at the doses tested or no reduction in shoot dry weight at the doses tested. Therefore, $LD_{50}$ values, $GR_{50}$ values, or resistance ratios could not be accurately determined. [e] Data were very underdispersed; thus, $LD_{50}$ estimates were not significant based on $t$ values. [f] Shoot dry weight and % survival were determined 14 DAT for quinclorac-treated plants. [g] Indicates no difference between the $LD_{50}$ (or $GR_{50}$) values of the R and S biotypes based on 95% confidence limits.

solution to give a final concentration of $10^{-5}$ M (ca. 0.25 g ai $ha^{-1}$ or 1/500 field dose). The stock solution was made by dissolving technical grade quinclorac in 95% (v/v) ethanol containing ca. 150 $\mu$L of NaOH (5 N). The final nutrient solution (5.8 pH) contained <0.01% (v/v) ethanol. Plants were harvested 0, 6, and 12 h after quinclorac treatment. For studying the effects of long-term exposures to quinclorac (≥24 h), plants were grown in vermiculite to the three-whorl stage of development, and the entire container including vermiculite was placed in the same nutrient solution as previously described. This saturated the vermiculite with the quinclorac solution. Plants were harvested 0, 24, 48, and 72 HAT for ABA analysis and at 48 HAT for shoot fresh and dry weights.

At harvest, shoots from six to nine plants were immediately frozen in liquid nitrogen, wrapped in tin foil, and stored at −80 °C. For harvest times of 0, 6, and 12 HAT, frozen shoots were homogenized with a pestle in a mortar containing liquid nitrogen. For samples harvested 0, 24, 48, and 72 HAT, shoots were lyophilized and dried shoot material was ground to a fine powder using a Polytron probe (Kinematica GmbH, Switzerland).

ABA extraction from frozen shoot tissue was carried out according to the method of Hansen and Grossmann (*5*). Powdered shoot material (100 mg dry weight or 1 g fresh weight) was extracted with 80% (v/v) aqueous methanol, and the extracts were passed through a $C_{18}$ reversed phase prepacked column ($C_{18}$ Sep-Pak Plus; Waters Associates). The effluent was concentrated in vacuo, dissolved in 3 mL of double-distilled water, acidified to pH 2.5 with 1 M HCl, and partitioned three times into ethyl acetate (3 mL). The organic solvent was evaporated to dryness under a $N_2$ stream, and samples were dissolved in 2 mL of 5% (v/v) methanol in 0.1 M acetic acid. Separation of ABA in the extract was performed by HPLC on a reverse phase Nucleosil 120−5 $\mu$m $C_{18}$ column (250 mm × 10 mm, Machery-Nagel, Düren, Germany) using a linear gradient from 5% (v/v) methanol in 0.1 M acetic acid to 95% (v/v) methanol. The fractions containing ABA were collected, and the quantitative determination was performed by enzyme immunoassay according to ref *5*. Monoclonal antibodies for ABA (kindly provided by Professor E. W. Weiler, University of Bochum, Germany) were used for analyses. The endogenous ABA experiment was conducted three times, with three replications per treatment.

**Statistical Analysis.** All experiments were conducted as completely randomized designs. All data were expressed as a percentage of the mean of the nontreated control R or S plants, except for [$^{14}$C]quinclorac experiments, which were expressed as percent recovered $^{14}$C. All data except dose−response data were transformed by subjecting the data to the quadratic root prior to analysis of variance (ANOVA) with the mixed procedure (PROC MIXED) or the general linear model (PROC GLM) using SAS 8.02 software (SAS Institute Inc., Cary, NC). This transformation was done to meet the criteria of ANOVA. Data from repeated experiments were pooled, and differences between treatment means were analyzed using Tukey−Kramer multiple comparison procedure. The type I error rate was set at 0.05 for all statistical tests.

*Dose−Response Statistical Analysis.* Shoot dry weight data were expressed as a nonlinear model, and $GR_{50}$ values were calculated with the nonlinear model (PROC NLIN) using SAS 8.02 software (SAS Institute Inc.). For each herbicide, plant mortality data were expressed as a generalized linear model (GLM) with a binomial response distribution and a probit link function (*21*). Lethal concentration estimates ($LD_{50}$) were obtained by solving the generated model for herbicide dose. Ninety-five percent confidence intervals (CI) were obtained by the profile deviance approach using the GLM function of S-Plus software (S-Plus 2000 Professional Release 2, MathSoft, Cambridge, MA).

## RESULTS AND DISCUSSION

**Dose−Response Experiments.** The R biotype was highly resistant to both quinolinecarboxylic acid herbicides, quinclorac, and quinmerac (**Table 2**), which differ at position 3 (Cl vs $CH_3$, respectively). Hall et al. (*13*) reported similar results for quinclorac-treated R and S false cleavers biotypes (the same biotypes described here). Similarly, the quinclorac resistant grass species, smooth crabgrass (*22*) and *Echinochloa* spp. (*23*), have resistance ratios of 9 or greater. To our knowledge, quinclorac resistant monocots have not been tested for resistance to other auxinic herbicide families or quinmerac.

There was no difference between the R and the S biotypes in response to 2,4-D or clopyralid. The R as opposed to the S biotype was highly resistant to MCPA and moderately resistant to picloram, fluroxypyr, triclopyr, or dicamba (**Table 2**). The variable sensitivity to auxinic herbicides of R false cleavers is similar to other auxinic R biotypes (*8*, *11*). Moreover, each

**Table 3.** Distribution of Radioactivity, Expressed as Percent of Recovered $^{14}C$, in R and S False Cleavers after Treatment with [$^{14}C$]Quinclorac[a]

| | | distribution of [$^{14}C$] (% of recovered) | | | |
|---|---|---|---|---|---|
| | | harvest time (h)[b] | | | |
| plant part | biotype | 0 | 24 | 48 | 96 |
| leaf rinse (% not absorbed) | R | 92.5 (1.0) | 57.5 (4.8) | 43.2 (5.0) | 37.6 (6.7) |
| | S | 88.7 (1.6) | 50.7 (5.6) | 46.1 (5.0) | 32.3 (3.5) |
| treated whorl | R | 6.9 (1.0) | 8.0 (2.5) | 6.0 (0.9) | 5.6 (0.8) |
| | S | 9.7 (1.5) | 10.2 (1.3) | 5.1 (0.5) | 5.5 (1.2) |
| total translocated | R | 0.6 (0.1) | 34.4 (4.2) | 50.9 (5.4) | 56.8 (7.2) |
| | S | 1.6 (0.8) | 39.1 (5.0) | 48.8 (5.1) | 62.2 (3.8) |
| shoots above treated whorl | R | 0.2 (0.04) | 13.4 (1.5) | 22.2 (2.4) | 21.3 (2.3) |
| | S | 0.2 (0.07) | 20.2 (3.0) | 24.8 (3.2) | 27.2 (2.4) |
| shoots below treated whorl | R | 0.3 (0.04) | 17.4 (2.4) | 23.1 (2.9) | 28.6 (4.2) |
| | S | 1.2 (0.7) | 15.4 (2.5) | 15.6 (2.4) | 20.0 (2.2) |
| roots | R | 0.1 (0.1) | 1.5 (0.3) | 1.9 (0.4) | 2.0 (0.6) |
| | S | 0.1 (0.02) | 1.2 (0.2) | 2.4 (0.7) | 3.5 (0.9) |
| root exudate | R | 0.1 (0.1) | 2.2 (0.7) | 3.7 (1.1) | 4.9 (1.1)[d] |
| | S | 0.1 (0.01) | 2.2 (0.5) | 6.1 (1.5) | 11.5 (2.2)[d] |
| total percent $^{14}C$ recovered[c] | R | 86.2 (1.7) | 90.6 (2.2) | 93.6 (2.2) | 93.1 (3.8) |
| | S | 86.3 (2.3) | 90.7 (1.4) | 94.3 (0.8) | 93.3 (1.9) |

[a] Data from three experiments were pooled ($n = 10$) and presented as treatment means with SE in parentheses. [b] Plants were treated with [$^{14}C$]quinclorac at the three-whorl stage of development and harvested 0, 24, 48, and 96 h after treatment of the second whorl. [c] Total percent $^{14}C$ recovered was expressed as percent of applied [$^{14}C$]quinclorac. [d] Indicates a difference between R and S within a plant part at a harvest time, based on *t*-test at the 95% confidence level.

auxinic herbicide induced different symptoms in S false cleavers (data not shown) suggesting that each auxinic herbicide tested caused slightly different physiological responses in false cleavers. Differences in perception of each auxinic herbicide may be analogous to altering auxin concentrations to stimulate different cellular responses. Alternatively, each auxinic herbicide may be acting at different auxin responsive elements. Regardless, the cross-resistance of false cleavers to the auxinic herbicides including quinclorac suggests a common mode of action.

The R biotype was highly resistant to thifensulfuron based on $LD_{50}$ and $GR_{50}$ values (**Table 2**). There are at least four other weed biotypes with resistance to both auxinic and ALS inhibitor herbicides; corn poppy (*Papaver rhoeas* L.) in Spain (*24*), kochia in Montana (*10*), and in Malaysia yellow bur-head [*Limnocharis flava* (L.) Buchenau] and marshweed (*Limnophila erecta* Benth.) (*7*). In these regions, the extensive use of ALS inhibitors and auxinic herbicides likely contributed to the independent selection of these resistant biotypes (*7*).

Both false cleavers biotypes were equally susceptible to glyphosate, glufosinate-ammonium, and bentazon (**Table 2**). On the basis of the lack of cross-resistance to these herbicides, i.e., each has a unique mode of action that differs from that of either the auxinic or the ALS inhibitor herbicides, it can be concluded that a common resistance mechanism, such as enhanced metabolism or sequestration, is unlikely to impart resistance to both auxinic and ALS inhibitor herbicides in R false cleavers. Furthermore, this conclusion is supported by the work of Horsman and Devine (*14*) who showed that ALS inhibitor resistance in the R false cleavers biotype was due to a mutation in the ALS gene resulting in an amino acid substitution. Finally, classical genetic analysis by Van Eerd et al. (*15*) showed that quinclorac and ALS inhibitor resistances are due to two distinct nuclear genes in R false cleavers.

**Radiolabeled Quinclorac Experiments.** [$^{14}C$]Quinclorac was foliar applied at ca. 3.6 $\mu$g ai plant$^{-1}$, which is approximately the registered field dose of 125 g ai ha$^{-1}$. During the first 24 h, the S biotype displayed symptoms of quinclorac phytotoxicity, particularly epinasty of new leaflets and by 96 HAT symptoms also included elongated internodes. No phytotoxic symptoms were observed in the R plants at any time after treatment. There were no differences in the recovery of applied [$^{14}C$]quinclorac between the R and the S biotypes. For both biotypes, the average total recovery of $^{14}C$ at 0, 24, 48, and 96 HAT was 86.2 $\pm$ 1.4, 90.7 $\pm$ 1.2, 94.0 $\pm$ 1.1, and 93.2 $\pm$ 1.9%, respectively (**Table 3**). Regardless of the harvest time, there was no difference in [$^{14}C$]quinclorac absorption between the two biotypes (**Table 3**). An average of 65.2 $\pm$ 3.6% of recovered radioactivity was absorbed by both biotypes 96 HAT. Furthermore, there were no statistical differences between R and S plants in terms of the quantity of $^{14}C$ remaining in the treated whorl (no more than 10.2 $\pm$ 1.3%), and the amount of $^{14}C$ translocated to nontreated shoots above (24.4 $\pm$ 1.8%) or below (24.1 $\pm$ 2.4%) the treated whorl or to the roots (<4%) 96 HAT (**Table 3**). Only at 96 HAT was there more $^{14}C$ in the nutrient solution of the S (11.5 $\pm$ 2.2%) than the R biotype (4.9 $\pm$ 1.1%) (**Table 3**). This difference in $^{14}C$ root exudation does not explain differences in observed phytotoxicity because only S plants displayed phytotoxic symptoms but S plants exuded more $^{14}C$ than R plants. The increase in root exudation in S plants may be due to root damage, which may have resulted in cell leakage. Root exudation was also observed in other susceptible monocot and dicot species treated with quinclorac (*18*, *25*). Although the identity of the $^{14}C$ compound in the root exudate is not known, it is hypothesized to be quinclorac and not a metabolite because there was little or no quinclorac metabolism in false cleavers. Furthermore, root exudates from leafy spurge were identified by HPLC analysis as [$^{14}C$]quinclorac 2 DAT (*18*).

HPLC analysis indicated there were no quantitative or qualitative differences in quinclorac metabolism between the R and the S plants. Prior to 48 HAT, there were no detectable quantities of [$^{14}C$]quinclorac metabolites (data not shown). Only 2.9 $\pm$ 0.9% of recovered [$^{14}C$]quinclorac was metabolized in either biotypes 48 HAT. Furthermore, 96 HAT, 94.6 $\pm$ 1.7 and 90.0 $\pm$ 1.3% of the recovered radioactivity remained as [$^{14}C$]-quinclorac in the R and S biotype, respectively. Furthermore, all samples were also analyzed using the HLPC method described by Grossmann and Kwiatkowski (*25*) (data not shown), which further validated the lack of extensive quinclorac metabolism in false cleavers. Previously, Lamoureux and Rusness (*18*) identified a malonylglucose ester of quinclorac

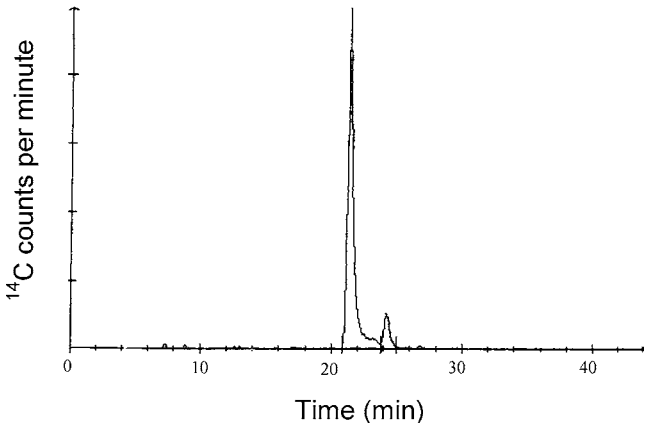


**Figure 1.** Typical HPLC chromatograph of extract obtained 96 h after treatment of false cleavers with [$^{14}$C]quinclorac at the three-whorl stage of development. Retention times for quinclorac and metabolite A were 21.33 and 24.17 min, respectively. See text for chromatographic conditions.

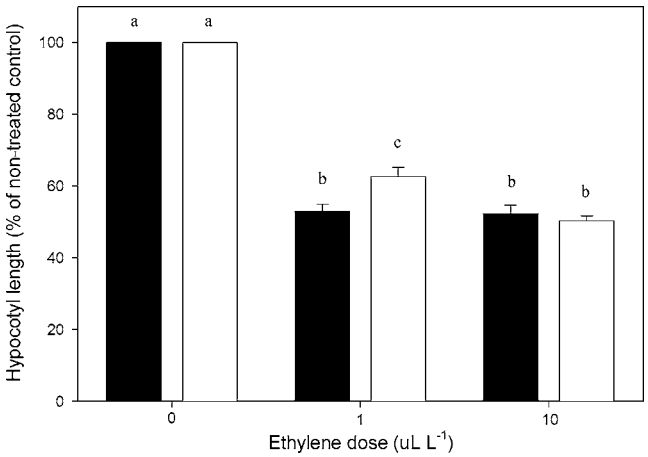


**Figure 2.** Hypocotyl length expressed as percent of the nontreated control of dark-grown herbicide resistant (R; dark bars) and susceptible (S; open bars) false cleavers seedlings exposed to exogenous ethylene (1 or 10 $\mu$L L$^{-1}$) for 36 h. Data from two experiments were pooled, and bars represent treatment means with standard error bars. Bars with a common letter are not different according to the Tukey–Kramer test at $P = 0.05$.

in leafy spurge. This metabolite and the quinclorac metabolite found in false cleavers (**Figure 1**) had the same HLPC retention time when chromatographic conditions of Lamoureux and Rusness (*18*) were used, indicating that the radiolabeled metabolite found in false cleavers was likely the malonylglucose ester of quinclorac. However, there was considerably more metabolism of quinclorac in leafy spurge (41.6%) than in false cleavers (≤10%), 96 HAT. On the basis of the above results, the mechanism of quinclorac resistance was not due to differences in [$^{14}$C]quinclorac absorption, translocation, root exudation, or metabolism in R false cleavers and is supported by the result of other researchers who studied the selectivity of quinclorac susceptible, tolerant, or resistant monocot and dicot species (*18, 25*).

**Exposure to Exogenous Ethylene.** Nontreated, dark-grown R seedlings had longer hypocotyls than did the S biotype, with lengths of 7.2 ± 0.2 and 5.3 ± 0.2 cm, respectively. Regardless, both R and S etiolated seedlings were responsive to 1 and 10 $\mu$L L$^{-1}$ of exogenous ethylene, having both shorter hypocotyls (**Figure 2**) and exaggerated curvatures of the hypocotyl hook (data not shown) than the nontreated controls. These responses resembled the "triple response" of dicot seedlings grown in the

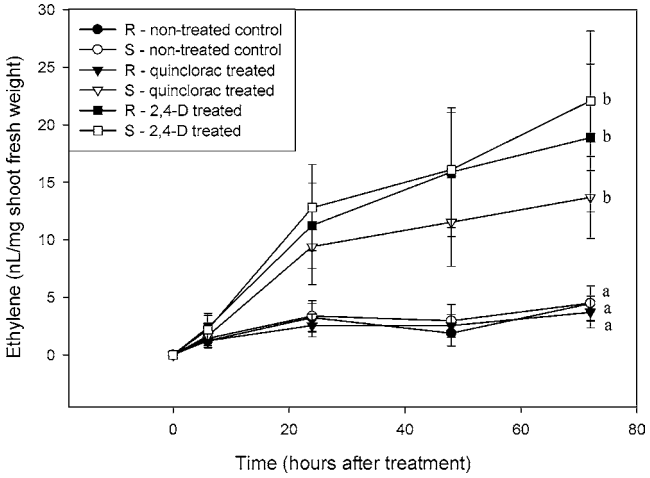


**Figure 3.** Ethylene biosynthesis, over 72 h, in herbicide resistant (R; dark symbols) and susceptible (S; open symbols) false cleavers plants following root treatment with water (control), quinclorac ($10^{-4}$ M), or 2,4-D ($10^{-4}$ M) at the two-whorl stage of foliar development. Symbols are treatment means of pooled data from three experiments with standard error (SE) bars. Where no bars are shown, the SE was smaller than the symbol. Over the time course (72 HAT), biotype treatment responses followed by a common letter were not different according to the Tukey–Kramer test at $P = 0.05$.

dark (*19*). Likewise, in light, both R and S seedlings at the two-whorl stage of foliar development were equally responsive to 100 and 1000 $\mu$L L$^{-1}$ exogenous ethylene as indicated by epinasty of new leaflets and elongation of internodes (data not shown). The response of both false cleavers to exogenous ethylene was similar to that observed in auxinic R and S wild mustard (*20*). On the basis of these results, one can conclude that both R and S false cleavers biotypes have the same physiological response to exogenous ethylene. Therefore, there are no major differences in ethylene responsive pathway that confer resistance to quinclorac in false cleavers.

**Endogenous Ethylene Biosynthesis.** Regardless of sampling time, there was no difference in ethylene biosynthesis among quinclorac-treated R and nontreated R and S plants, <4.5 nL of ethylene per mg shoot fresh weight (**Figure 3**). Conversely, ca. >3-fold increase in ethylene was induced in quinclorac-treated S plants 24, 48, and 72 HAT as compared to the quinclorac-treated R biotype and the nontreated R and S plants (**Figure 3**). Ethylene biosynthesis was induced in both 2,4-D-treated ($10^{-4}$ M) R and S plants (**Figure 3**). Both biotypes displayed phytotoxic symptoms <24 h after 2,4-D was applied to the roots ($10^{-4}$ M) or foliage (see dose−response experiments). The most obvious symptom was epinasty of new leaflets in the quinclorac-treated S biotype and in both R and S plants treated with 2,4-D. These symptoms were similar to those displayed by hydroponically grown S false cleavers treated with quinclorac ($10^{-4}$ or $10^{-5}$ M) in ethylene and ABA experiments described below. Furthermore, these results are similar to those seen in auxinic R wild mustard, where ethylene remained at basal levels following treatment with picloram or dicamba (*20*). In contrast, ethylene appears not to be involved in yellow starthistle resistance to clopyralid (*26*). On the basis of our endogenous and exogenous ethylene experiments, we conclude that false cleavers resistance to quinclorac is likely triggered by some mechanism upstream of the ethylene biosynthetic pathway.

**ABA Biosynthesis.** At the time of treatment (0 HAT), basal ABA levels of nontreated R plants were lower than those of

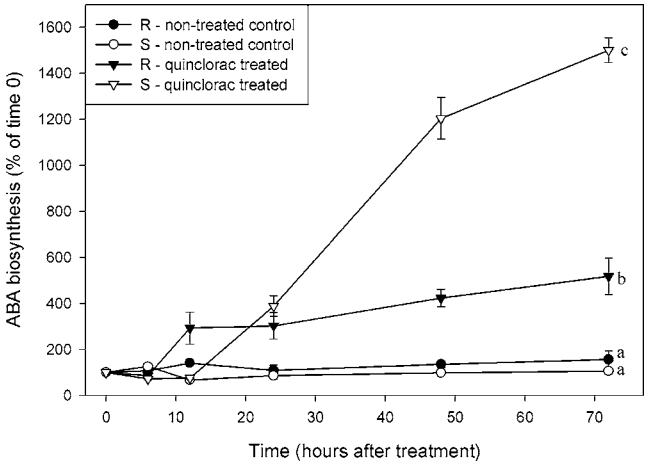


**Figure 4.** ABA concentration, over 72 h, in herbicide resistant (R; dark symbols) and susceptible (S; open symbols) false cleavers plants following root treatment with water (control) or quinclorac ($10^{-5}$ M) at the three-whorl stage of foliar development. Data are expressed as a percentage of the ABA concentration from the nontreated controls at the time of treatment (0 HAT) in fresh (6 and 12 HAT) and lyophilized (24, 48, and 72 HAT) shoot tissue. Symbols are treatment means of pooled data from three experiments with SE bars. Where no bars are shown, the SE was smaller than the symbol. Over the time course (72 HAT), biotype treatment responses followed by a common letter were not different according to the Tukey–Kramer test at $P = 0.05$.

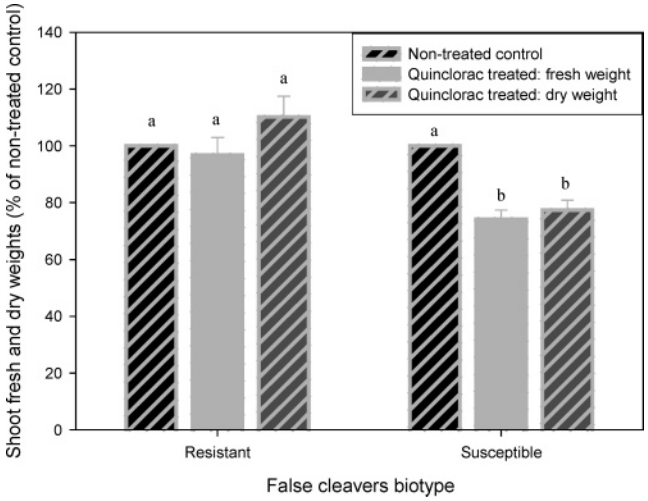


**Figure 5.** Growth response in terms of fresh and dry weight in herbicide resistant (R) and susceptible (S) false cleavers following root treatment with water (control) or quinclorac ($10^{-5}$ M) at the three-whorl stage of foliar development and harvested 48 h after treatment. Data were expressed as percent of the nontreated control. Symbols are treatment means of pooled data from two experiments with SE bars. Bars with a common letter are not different according to the Tukey–Kramer test at $P = 0.05$.

nontreated S plants when both lyophilized (data not shown) and fresh shoot tissue were used ($60.1 \pm 6.4$ and $131.3 \pm 25.8$ pmol ABA $g^{-1}$ fresh weight for R and S, respectively). However, basal ABA concentrations in nontreated false cleavers shoots were similar to those found in other broadleaved species; for example, basal ABA levels in common cleavers (*Galium aparine* L.) are approximately 100 pmol $g^{-1}$ fresh weight (*5*). For both nontreated R and S biotypes, there was no increase in basal ABA concentrations during the 72 h experiment (**Figure 4**). As compared to nontreated R and S plants, ABA biosynthesis was ca. 14-fold higher in quinclorac-treated S plants, 72 HAT (**Figure 4**). In the quinclorac-treated R biotype, there was ca. 3-fold increase in ABA when compared to the nontreated R and S plants, but the increase was less than in quinclorac-treated S plants (**Figure 4**). As compared to nontreated plants, root-applied quinclorac ($10^{-5}$ M) caused leaflet epinasty as well as a reduction in fresh and dry shoot weights in S plants but not in R plants (**Figure 5**).

Similar to our results with false cleavers response to quinclorac, ABA levels in quinmerac-treated common cleavers increased ca. 10−18-fold (*5*, *6*). In common cleavers, increases in ABA concentration correlated with decreases in shoot fresh weight in quinmerac susceptible plants treated with this herbicide (*5*). In susceptible dicots, quinmerac induction of ABA closely correlates with stomata closure, reduced water consumption, and lower $CO_2$ fixation, accompanied by $H_2O_2$ accumulation. This leads to growth inhibition, tissue damage, and cell death (*2*, *6*). Evidence presented by several researchers suggests that the mechanism of auxinic R in different weed species (*9*, *11*, *27*) including quinclorac resistant monocots (*1*, *22*) may result from altered target site and/or altered auxin signal transduction.

In the false cleavers S biotype, we suspect that quinclorac binds to a putative target site and elicits a cascade of effects including ethylene and ABA biosynthesis, which lead to $H_2O_2$ accumulation and ultimate plant death. Because ethylene biosynthesis was not stimulated in the quinclorac-treated R biotype, the increased ABA in R plants relative to nontreated control plants may not be high enough to generate a physiological response. On the basis of these results, we propose the following two models to explain quinclorac action in R false cleavers biotypes. In the R biotype, quinclorac may bind to the putative target site, similar to the S biotype, but a lesion upstream of ACC synthase and ACC oxidase reduces or prevents the production of ACC and ethylene, respectively, while allowing low level ABA biosynthesis. Alternatively, in the R false cleavers biotype, a mutation in the putative target site may prevent quinclorac binding but quinclorac binds instead to other auxin responsive elements causing low level ABA biosynthesis but no ethylene biosynthesis. Regardless of the model, the observed ABA levels in quinclorac-treated R false cleavers did not result in phytotoxic effects. To fully characterize quinclorac resistance in false cleavers, further experimentation comparing R and S plants is required including: quantification of ACC and $H_2O_2$ following application of various doses of quinclorac, determination of ethylene and ABA biosynthesis after wounding and/or water stress, quantification of ABA after exposure to exogenous ethylene, and characterization of plant responses to exogenous ABA.

In conclusion, this is the first study to physiologically and biochemically characterize the link between quinclorac resistance and resistance to other auxinic herbicides. Furthermore, this is the first examination of the mechanism of quinclorac resistance in a dicot species. Quinclorac resistance in false cleavers cannot be attributed to differences in [$^{14}$C]quinclorac absorption, translocation, root exudation, and/or metabolism. Furthermore, the concentrations of the phytohormones ethylene and ABA were larger in the S as compared to the R biotype following root application of quinclorac. These results indicate that an alteration in the cascade of biochemical events in the auxin signal transduction pathway or a mutation at the putative target site may result in quinclorac resistance. Moreover, the R false cleavers biotype clearly provides an excellent model system for the future study of the cellular and molecular mechanisms of resistance not only to quinclorac but to other auxinic herbicides.

## ABBREVIATIONS USED

ABA, abscisic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; ALS, acetolactate synthase; ai, active ingredient; CI, confidence interval; DAT, days after treatment; $GR_{50}$, dose (g ai $ha^{-1}$) that reduced shoot growth by 50% as compared to the nontreated control; $H_2O_2$, hydrogen peroxide; HAT, hours after treatment; HLPC, high-performance liquid chromatography; $LD_{50}$, the lethal dose (g ai $ha^{-1}$) to 50% of the plants tested; LSS, liquid scintillation spectrometry; P, statistical probability; R, herbicide resistant false cleavers biotype; S, herbicide susceptible false cleavers biotype.

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