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Metabolism Characteristics of Acifluorfen Tolerant in Somaclones of Eastern Black Nightshade (*Solanum ptycanthum* Dun.)

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ABSTRACT : Acifluorfen tolerance charateristics determined the involvement of absorption, translocation, and metabolism in acifluorfen tolerance. Less than 6% of the applied ^{14}C-acifluorfen was absorbed. There were no differences in acifluorfen absorption between susceptible and tolerant somaclones. More ^{14}C-acifluorfen was translocated in the susceptible than the tolerant somaclones. The susceptible somaclone did not metabolize acifluorfen while some somaclones (i.e., EBN-3A) metabolized ^{14}C-acifluorfen. Nomenclature: Acifluorfen, 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoic acid; eastern black nightshade, *Solanum ptycanthum* Dun., #[3] SOLPT.

Key words : herbicide tolerance, uptake, translocatioin, metabolism

INTRODUCTION

The development of herbicide-tolerant-plants has the potential to extend the number of herbicides available for use in crops. Plants differing in herbicide tolerance can be used to elucidate mechanisms of action and means of selectivity not fully understood for many herbicides (Ashton & Crafts, 1981). Herbicide resistance is an increasing problem, with over 90 weed species having resistant biotypes (Holt & Lebaron, 1990). An in vitro system was helpful to determine the potential of eastern black nightshade to detect herbicide resistance (Yu, 1991). Callus tolerant to the diphenyl ether herbicide acifluorfen was selected by increasing the herbicide concentration in the media (Yu, 1991). Wild-type eastern black nightshade were susceptible to 0.28 kg ai ha^{-1} of acifluorfen (Gorski *et al.*, 1987; Teasdale, 1987), while many of the somaclones regenerated from the selected callus tolerant to acifluorfen at 1.6 kg ha^{-1} (Yu, 1991). Selfing indicated that tolerance was inherited either as a semidominant or recessive trait (Yu, 1991).

Reduced uptake, changes in the site of action, and metabolism all can confer increased herbicide tolerance (Schulz *et al.*, 1990). Overproduction of the site of action due to gene amplification was reported to be the mechanism for glyphosate [*N*-(phosphonomethyl)glucine] (Smith *et al.*, 1986) and HOE-39866 [ammonium (3-amino-3-carboxypropyl) methylphosphinate] (Deak *et al.*, 1988; Donn *et al.*, 1984) resistance in selected somaclones. For example, alfalfa (*Medicago sativa* L.) somaclones isolated by stepwise selection were resistant to HOE-39866 because of an amplification of the gene coding for glutamine synthase. This amplification lead to a 5-fold higher enzyme concentration and caused a 60-to 100-fold increase in herbicide resistance (Donn *et al.*, 1984).

Diphenyl ether herbicides affect the tetrapyrrole pathway in the plastids. The enzymatic oxidation of protoporphyrinogen IX to protoporphyrin IX by protoporphyrinogen oxidase in the plastid is highly sensitive to acifluorfen and other diphenyl ether herbicides (Becerril & Duke, 1989; Witkowski & Halling, 1989). Accumulation of photodynamic protoporphyrin IX results in generation of toxic oxygen species, including singlet oxygen, superoxide, peroxide, and the hydroxyl radical (Halliwell & Gutteridge, 1984; Haworth & Hess, 1988). The toxic oxygen species trigger peroxidation of membrane lipids resulting in plant death (Haworth & Hess, 1988; Kunert & Borger, 1987). Changes in the site of action or in protective systems for toxic oxygen species could confer tolerance to diphenyl ethers.

Differential tolerance to diphenyl ethers can occur from differences in herbicide absorption, translocation, and metabolism. Frear *et al.* (1983) reported that from 85 to 95% of the absorbed ^{14}C-acifluorfen was metabolized in excised leaves of soybean [*Glycine max* (L.) Merr.] within 24 h. A rapid initial cleavage of the diphenyl ether bond resulted in the formation of a reactive phenolic intermediate (2-chloro-4-trifluoromethylphenol) and S-(3-carboxyl-4-nitrophenyl)-γ-glutamyl-cys-

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teinyl-β-alanine (Frear *et al.*, 1983). The initial reaction was catalyzed by glutathione-s-transferase (Frear & Swanson, 1973). Ritter and Coble (Ritter & Coble, 1981) reported slower metabolism, and greater penetration and translocation of acifluorfen in susceptible common ragweed (*Ambrosia artemissifolia* L.) and common cocklebur (*Xanthium strumarium* L.) than in tolerant soybean.

Changes in absorption, translocation, and metabolism may result in cross-tolerance both to diphenyl ethers and to herbicides in other chemical families. Comparing plant tolerance to herbicides with similar modes of action is also useful in determining mechanisms of selectivity (Vaughn *et al.*, 1989). A biotype of annual ryegrass (*Lolium rigidium* Gaudin.) was reported to be resistant to a number of herbicides, based on its ability to metabolize them (Shimabukuro & Hoffer, 1991). The objectives of this study were to determine if differential metabolism, uptake, or translocation could explain tolerance of eastern black nightshade somaclones to acifluorfen.

MATERIALS AND METHODS

Uptake and translocation

Rooted cuttings, from somaclones EBN-3A, EBN-12,EBN-20E, EBN-23, and EBN-24A regenrated from acifluorfen-tolerant callus and from a control eastern black nightshade (EBN)[5] which had not been selected for acifluorfen tolerance, were planted into a greenhouse soil mixture(1:1:1 by vol loam soil:peat:perlite) contained in 700-cm^3 plastic pots. When the plants were 15 to 20 cm tall, they were placed in a growth chamber. The environmental conditions were a constant 22°C temperature, 18/6 h day/night, approximately 70% humidity, and 150 μmol s^{-1} m^{-2} of light.

After 48 h to acclimate, 3.7×10^3 Bq ^{14}C-acifluorfen (chlorophenyl ring labeled, specific activity - 1.7×10^4 Bq mM^{-1}) was applied as five 1-μℓ droplets to the middle fully expanded leaf using the procedure of Ricotta and Masiunas (Ricotta & Masiunas, 1992). Plants were harvested 24 and 48 h and 1 wk after treatment. Plants were separated into the area treated on the leaf, the remainder of the treated leaf, shoots above the treated leaf, shoots below the treated leaf, and roots. The treated leaf was shaken for 1 min in 15 mℓ of distilled water to remove unabsorbed herbicide, and then shaken for 1 min in 15 mℓ of chloroform to extract any herbicide present in the epicuticular wax. A 1-mℓ aliquot was taken from both the leaf wash and the chloroform extract, and the radioactivity quantified using a liquid scintillation analyzer (LSA).

Following the water and chloroform rinses, each plant section was homogenized in 10 mℓ of methanol for 1 min, then centrifuged at 15,000 g for 15 min. The suspernatant was decanted into glass scintillation vials (24 by 58 mm) and photobleached under fluorescent lights for 48 h to remove pigmentation and decrease quenching. A 200-μℓ aliquot was removed from each photobleached sample and quantified by LSA. Recovery was greater than 96% for all treatments. There were three replcations. The experiment was repeated.

Metabolism

Plants of each callus line were treated as previously described. After 24 and 48 h and 1 wk, the treated leaf was harvested, homogenized, and centrifuged. The extracts were evaporated to dryness and resuspended in 200 μℓ methanol. Thin-layer chromatography (TLC)[5] was used to separate metabolites (Ricotta & Masiunas 1992). The entire 200-μℓ sample was spotted 5 cm from the bottom of a 0.25-mm silica gel TLC plate[6]. There were duplicate plates for each somaclone. A plate[6]. There were duplicate plates for each somaclone. A plate with a sample of parent ^{14}C-acifluorfen was included as a standard. The plates were chromatographed in a solvent system containing ethyl acetate: propanol: water (65:25:10 by vol). Radioactivity was detected using an image scanner[7].

Extraction and determination of tetrapyrroles. Extraction of tetrapyrroles from plant tissues was achieved using the following procedures. 8 micromolar acifluorfen was sprayed on to the plant., using an air sprayer, and plants were then grown in a growth room illuminated by nine 1000-watt metal halide lamps (30.7 mW cm^{-1}) for 8 h. Two grams of plant material was homogenized in 14 mℓ of acetone: 0.1 M NH_4OH (9:1, v/v) under green safelight. The homogenate was cleared of cell debris by centrifuged lipoprotein pellet was used for protein analysis.

The acetone supernatant containing the pigments was extracted with one-third volume of hexane, and the total quantities of Protoporphyrine, MPE and Pchlide were determined in the hexane-extracted fraction by room temperature spectrofluorometry (Lee & Duke, 1994).

Protein determination. Protein was determined according to the method of Smith *et al.* (1986). The acetone-insoluble residue that resulted from centrifugation of the tissue homogenate was dissolved in distilled water, and diluted before adding trichloroacetic acid (TCA). Total proteins were determined using an aliquot of this suspension after delipidation using bicinchoninic acid.

RESULTS AND DISCUSSION

Uptake and translocation

The majority of ^{14}C-acifluorfen remained on the leaf surface.

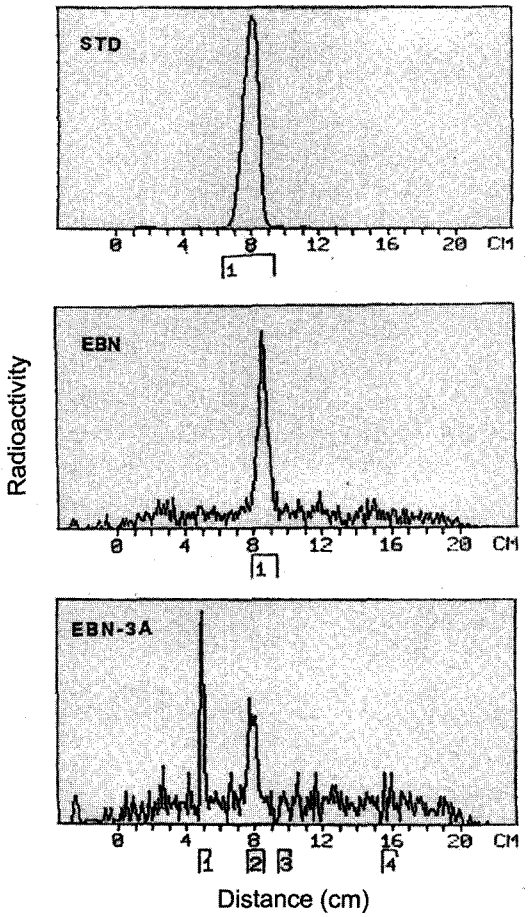

Fig. 1. A scan of the radioactivity on thin-layer chromatography plates. The parent ^{14}C-acifluorfen (STD) along with ^{14}C-acifluorfen and its metabolites from treated leaflet of unselected control (EBN) and a selected somaclone (EBN-3A)

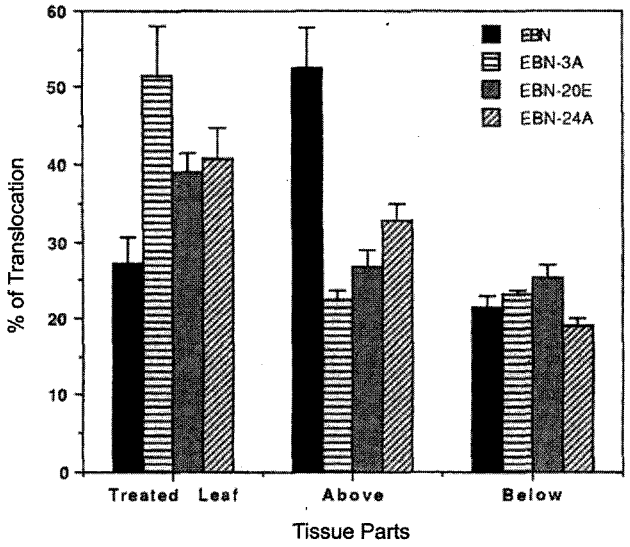

Fig. 2. The translocation of absorbed ^{14}C-acifluorfen in unselected (EBN) and selected (EBN-3A, EBN-20E, and EBN-24A) *Solanum ptycanthum* lines 1 week after treatment

Less than 6% of the acifluofen was absorbed by either the susceptible control (EBN) or tolerant somaclones (EBN-3A, EBN-20E, and EBN-24A). There were no differences in acifluorfen absorption between somaclones at any treatment time. Ricotta and Masiunas (Ricotta & Masiunas, 1992) also reported that less than 6% of the applied acifluorfen was absorbed by eastern black nightshade. They also found that the amounts of acifluorfen absorbed did not correlate with tolerance or susceptibility of *Solanum* and *Lycopersicon* genotypes (Ricotta & Masiunas, 1992).

One week after treatment, a greater percentage of the absorbed ^{14}C-acifluorfen was translocated from the treated leaf of the susceptible control than the tolerant somaclones (Fig. 1). The susceptible control retained 27% of absorbed acifluorfen in the treated leaf while the tolerant somaclones retained over 39% in the treated leaf. The susceptible control translocated 52% of absorbed acifluorfen to leaves above the treated leaf compared to less than 33% for the tolerant somaclones (Fig. 1). The amount of basipetal translocation was similar between the somaclones. These differences in acifluorfen translocation could be a partial explanation for the differential tolerance.

Metabolism

Metabolism of ^{14}C-acifluorfen varied depending on the somaclone (Fig. 2). The susceptible control did not metabolize a significant amount of the acifluorfen 1 wk after treatment. Less than 2% of the ^{14}C was in areas other than the peak for the parent acifluorfen. Two tolerant somaclones, EBN-3A and EBN-20E, metabolized 52.6% and 28.3% of the acifluorfen within 24 h after treatment, respectively. Frear *et al.* (1983) reported that in soybean 85 to 95% of ^{14}C-acifluorfen within 24 h after treatment. We found no metabolism in one of the tolerant somaclones, EBN-24A, indicating differences in tolerance mechanisms between the somaclones. EBN-24A could be tolerant to acifluorfen because of reduced accumulation of photodynamic tetrapyrroles (Matringe & Scalla, 1987; Sherman *et al.*, 1991; Vaughn *et al.*, 1989) or enhanced free radical protective systems (Fuerst & Vaughan, 1990; Halliwell, 1982). Sherman et al. (Sherman *et al.*, 1991) also reported that there could be several different mechanisms of tolerance to acifluorfen.

Two major methbolites of acifluorfen were detected. One major metabolite with an R_f value of 0.25 was found in somaclone EBN-3A, whereas a metabolite with an R_f value of 0.47 was detected in EBN-20E (Fig. 2). This suggests that in eastern black nightshade somaclones there are two different

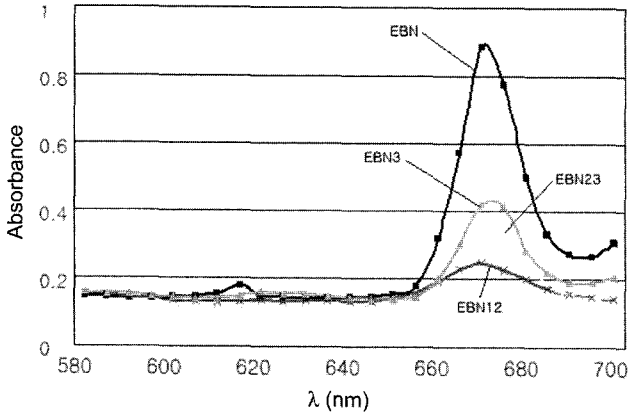

Fig. 3. Room temperature absorption spectra of the 80% acetone extracts of unselected (EBN) and selected (EBN-3, EBN-12, and EBN-23) *Solanum ptycanthum* somaclone 1 week after treatment. The absorption spectra of the acetone extracts were recorded on an Aminco spectrophotometer model DW-2, at an absorbance scale of 0 to 1.0 times the factors indicated on the spectra

metabolites of the chlorophenyl ring of acifluorfen, one with less and one with greater mobility on TLC plates than parent acifluorfen. Higgins *et al.* (Higgins *et al.*, 1988) reported that 24 h after treatment, four metabolites of acifluorfen were found in ivyleaf morningglory and metabolite was found in pitted morningglory. Ritter and Coble (Ritter & Coble, 1981) found four metabolites of acifluorfen in soybean and five in common cocklebur. They suggested that the rapid metabolism of acifluorfen may explain tolerance of some eastern black nightshade somaclones.

Tetrapyrrole accumulation in *in vitro* selected somaclones. To determine the difference of tetrapyrrole accumulation between acifluorfen-susceptible (seed-grown) and acifluorfen-tolerant plant, 8 ㎛ acifluorfen was applied and plants were illuminated for 8 h at 30.7 ㎽ cm^{-1}.

Acifluofen spray accumulated at higher concentrations in susceptible PPIX plants than in acifluorfen-tolerant somaclones. There was 2~35 fold increase in accumulation of PPIX in susceptible control plants than in tolerant somaclones. Protoporphyrin IX content differed depending on the somaclones, ranging from 2.0 to 43.5 ㎚ole per 100 ㎎ protein. Somaclone EBN 3 accumulated the large amount of protophyrin IX. As the concentration of acifluorfen increased, the amount of PPIX accumulation increased (data not shown). These results indicate that the possible site of action of the acifluorfen is protoporphyrinogen oxidase as reported by other researchers (Witkowski & Halling, 1989; Choi *et al.*, 1998). When protoporphyrinogen oxidase is inhibited, PPIX accumulates. Protoporphyrinogen IX leaks into the cytosol and/or stroma where it is nonenzymatically converted into PPIX (Lee *et al.*, 1993; Lermontova *et al.*, 1997; Horikoshi *et al.*, 1999; Horikoshi & Hirooka, 1999; Lermontova & Grimm, 2000). The PPIX, which cannot readily be processed by either magnesium or ferro-chelatase, accumulates to toxic concentrations. Illumination caused singlet oxygen and lipid peroxiadation, which resulted in a loss of membrane integrity and cell death.

This study indicates that tolerance to acifluorfen can occur in eastern black nightshade. It suggests that multiple mechanisms, including differntial translocation and metabolism, can cause acifluorfen tolerance. Tolerance to acifluorfen in some cases can confer tolerance to other diphenyl ether and bipyridylium herbicides. These studies suggest that eastern black nightshade tolerance to diphenyl ether herbicides could be a problem in field situations.

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