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Dehydrozaluzanin C, a natural sesquiterpenolide, causes rapid plasma membrane leakage

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Abstract

Dehydrozaluzanin C, a natural sesquiterpene lactone, is a weak plant growth inhibitor with an I_{50} of about 0.5 mM for lettuce root growth. It also causes rapid plasma membrane leakage in cucumber cotyledon discs. Dehydrozaluzanin C is more active at 50 μM than the same concentration of the herbicide acifluorfen. Symptoms include plasmolysis and the disruption of membrane integrity is not light dependent. Reversal of its effects on root growth was obtained with treatment by various amino acids, with histidine and glycine providing ca. 40% reversion. The strong reversal effect obtained with reduced glutathione is due to cross-reactivity with DHZ and the formation of mono- and di-adducts. Photosynthetic, respiratory and mitotic processes, as well as NADH oxidase activity appear to be unaffected by this compound. Our results indicate that dehydrozaluzanin C exerts its effects on plants through two different mechanisms, only one of which is related to the disruption of plasma membrane function.

Keywords: Phytotoxins; Allelopathy; Dehydrozaluzanin C; Sesquiterpene lactones; Glutathione; Membrane leakage; Mode of action

1. Introduction

Isolation of natural phytotoxins is an important approach to the discovery of new herbicides, because these compounds tend to be structurally different from synthetic herbicides and often act on new target sites (Duke & Abbas, 1995; Duke, Dayan, Hernández, Duke & Abbas, 1997). Traditional approaches have focused on the isolation of natural phytotoxins of microbial origin (Abbas & Duke, 1995; Duke et al., 1996). Therefore, most of the natural compounds that have been leads for new herbicides belong to this group (Duke, Abbas, Amagasa & Tanaka, 1996). Isolation of plant natural products, particularly from allelopathic plants, would seem to be a good strategy for natural herbicide discovery. This approach has received relatively little attention, although it has apparently been successful with the commercial herbicide cinmethylin (Grayson et al., 1987) derived from the natural product 1,8-cineol (eucalyptol). Nevertheless, its mode of action remains unknown (DiTomaso & Duke, 1991).

Dehydrozaluzanin C (DHZ) is a sesquiterpenolide with a guaiane skeleton that has been isolated from the roots of many different Compositae families (Bohlmann, Brindöpke & Rastogi, 1978; Bohlmann & Le Van, 1977; Bohlmann, Jakupovic, Gupta, King & Robinson, 1981; Bohlmann, Müller, Gupta, King &

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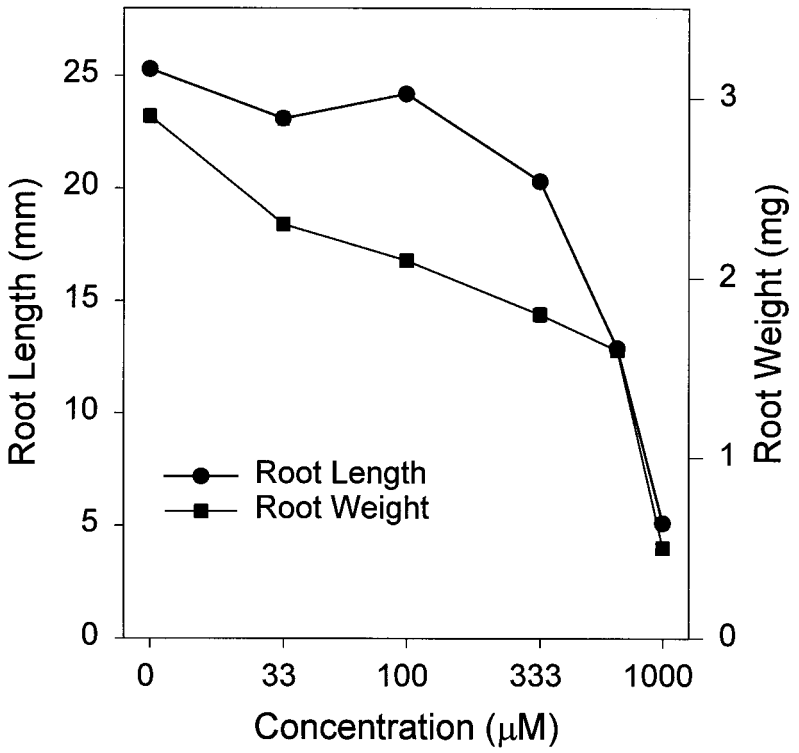

Fig. 1. Effects of different concentrations of DHZ on root length and root weight of seven-day-old lettuce seedlings. Error bars represent ±1 S.E.M. of three replicates with 20 observations each.

Robinson, 1981; Bohlmann, Sing & Jakupovic, 1982; Bohlmann & Zdero, 1982; Bohlmann, Zdero, King & Robinson, 1980). It inhibits root growth and germination of plants (Asakawa & Takemoto, 1979; Macías, Galindo, Molinillo & Castellano, 1999) and antiprotozoal activity has recently been reported (Fournet et al., 1993). Our previous results (Macías et al., 1999) indicate that the levels of activity obtained with DHZ and other sesquiterpene lactones (Dayan et al., 1999; Duke, Vaughn, Croom & Elsohly, 1987) make these structures candidates as lead compounds in the development of new herbicides. Despite the increasing amount of data about the phytotoxicity of several sesquiterpene lactones, little attention has been paid to the elucidation of their mode(s) of action. Artemisinin, an endoperoxide containing sesquiterpene lactone, is one of the few examples where such studies have been attempted (Dayan et al., 1999; Duke et al., 1987).

In this paper we explore the mechanism of action of DHZ. Membrane integrity is rapidly and strongly affected; some amino acid biosynthetic pathways appear to also be affected, but results suggest these effects are secondary. No major effects on mitotic, respiratory, or photosynthetic processes were observed. Discussion of the relationship between the chemical structure and potential mechanism of action of DHZ is also presented.

2. Results and discussion

2.1. Phytotoxicity of DHZ

Preliminary results (Macías et al., 1999) demonstrated phytotoxic effects of DHZ on several plant species (lettuce, tomato, carrot, cress, onion, wheat and barley) in a range between 0.1 and 1 mM. DHZ caused a decrease of root length and a slight decrease in germination. Current results of microbioassays (100 μM DHZ) tested on lettuce, *Arabidopsis*, onion and *Agrostis* confirm this report, showing fresh weight reduction, especially in *Agrostis*, but no effect on chlorophyll or carotenoid contents (data not shown). Dose–response experiments (10–1000 μM) performed with lettuce indicated an IC_{50} value for inhibition of longitudinal root growth of about 500 μM (Fig. 1).

Parthenin, a sesquiterpene lactone similar to DHZ, has already been associated with the allelopathic activity of *Parthenium hysterophorus* (Batish, Kohli, Saxena & Sing, 1997; Kanchan & Jayachandra, 1979, 1980). Its growth regulatory activity has been described recently (Kohli & Batish, 1994). Parthenin was shown to reduce seedling length of *Phaseolus aureus* at 50 mg l^{-1} (ca. 200 μM), which is similar to the concentration required with DHZ (Fig. 1).

Another potent sesquiterpene lactone, artemisinin,

Table 1
Mean effects of 100 μM DHZ on oxygen consumption of five- and seven-day-old lettuce and onion roots, respectively ($n = 4 \pm 1$ S.E.M.)

	nmol O_2/root tip (nmol s^{-1})	nmol O_2/dry weight (nmol g^{-1} s^{-1})	nmol O_2/fresh weight (nmol g^{-1} s^{-1})
Lettuce			
Control	0.42 ± 0.03	8.52 ± 0.69	0.66 ± 0.06
100 μM DHZ	0.58 ± 0.06	11.15 ± 0.98	0.89 ± 0.04
Onion			
Control	0.84 ± 0.09	6.36 ± 0.79	0.55 ± 0.07
100 μM DHZ	1.12 ± 0.08	7.02 ± 1.68	0.61 ± 0.12

inhibits mitosis (Dayan et al., 1999). DHZ appears to act differently since no major effects of DHZ were observed on the different mitotic phases (data not shown), nor were any aberrant mitotic phases detected. Nevertheless, similar to artemisinin (Duke et al., 1987), root oxygen consumption is slightly enhanced by DHZ in lettuce and, to a lesser extent, in onion (Table 1). This could be a result of secondary stress caused by the presence of the phytotoxin, rather than an indication of the primary site of action of DHZ.

2.2. Amino acid complementation experiments

Effects of phytotoxins that act through inhibition of amino acid biosynthesis can be reversed with exogenous supplies of amino acids (e.g. Amagasa, Paul, Heitholt & Duke, 1994). Attempts to reverse the phytotoxic effects of 500 μM DHZ were made by supplying 200 μM essential amino acids. Results were expressed on the basis of germination, root/shoot and length/weight parameters. Since phytotoxic effects were more pronounced on roots than shoots, discussion of the reversal studies are limited to the effects on root growth.

Amino acids caused a time-dependent recovery of the germination rate of DHZ-treated seeds, compared with the control. This implies that either the plant is able to overcome the effect of the herbicide on germination, or that DHZ is metabolically deactivated in plants. The strongest reversal effects on germination rates were observed with a mixture of aspartate, glutamate and alanine, and single treatments of histidine, asparagine and glycine. Aspartate, glutamate and alanine are ineffective when tested alone. Therefore, the effect of the combination treatment may not be due to a true complementation effect, but may be associated with enhanced growth stimulated by the amino acid mixtures.

There was no dramatic reversal of the root growth inhibition caused by DHZ for any of the compounds tested. The effects of DHZ differed, however, for root length and weight, although there were similar relative effects on both parameters (around 50 % inhibition). The most dramatic reversal effects were observed with root weight using histidine and glycine (Fig. 2). There was no similar effect on root length, where minimal reversal can be observed with (aspartate + glutamate + alanine) > proline, (phenylalanine + tyrosine + tryptophane), (cysteine + methionine), glycine and alanine. Bioassays performed with histidine, glycine,

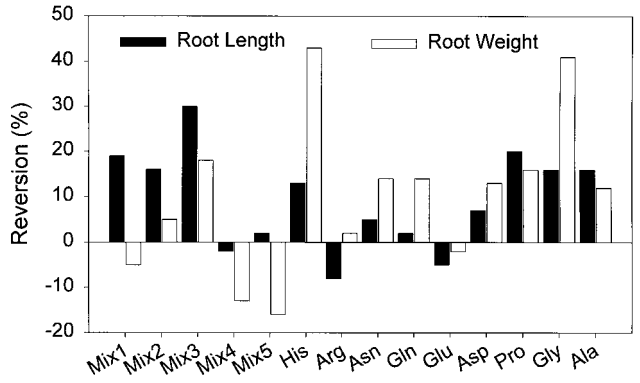

Fig. 2. Reversion study of the inhibitory effect of 500 μM DHZ on root growth of seven-day-old lettuce seedlings with various amino acids treatments. Mix 1 = phenylalanine + tyrosine + tryptophan; mix 2 = cysteine + methionine; mix 3 = aspartate + glutamate + alanine; mix 4 = lysine + threonine + methionine; mix 5 = leucine + valine + isoleucine.

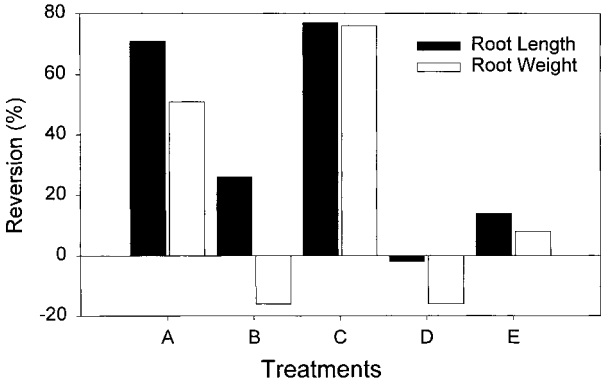

Fig. 3. Effects of reduced and oxidized forms of glutathione in reversing the inhibitory effect of 500 μM DHZ on root growth of seven-day-old lettuce and onion seedlings. Treatment A = 1 mM GSH on lettuce; treatment B = 0. 1 mM GSH on lettuce; treatment C = 1 mM GSH on onion; treatment D = 0.5 mM GSH on onion; treatment E = 1 mM GSSG on lettuce.

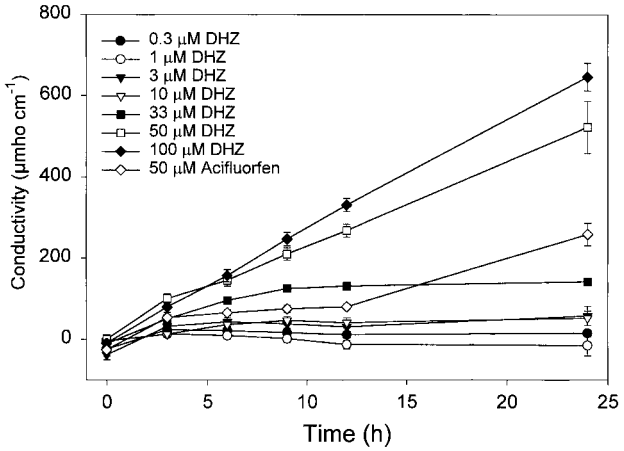

Fig. 4. Dose–response for cellular leakage as determined by changes in conductivity of treatments minus control conductivity changes of cucumber cotyledons as affected by exposure to different DHZ concentrations. Error bars are ±1 S.E.M. of six plates; 50 μM acifluorfen was used as positive control.

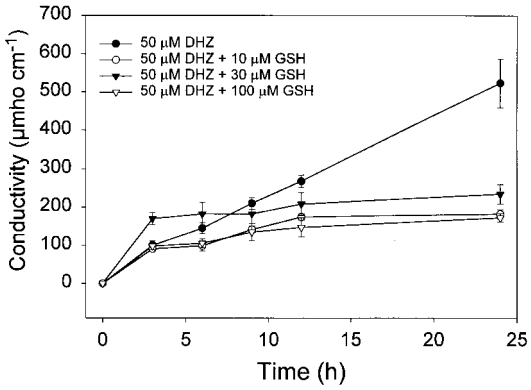

Fig. 5. Dose–response reversal effect for cellular leakage as determined by changes in conductivity of treatments minus control conductivity changes of cucumber cotyledons as affected by exposure to 50 μM DHZ and different concentrations of GSH. Error bars are ±1 S.E.M. of six plates.

proline and alanine (100, 200, 400 and 800 μM) resulted in atypical dose–response curves, suggesting that DHZ does not affect amino acid biosynthesis directly.

2.3. Other complementation experiments

Attempts to reverse the phytotoxic effect of 500 μM DHZ on root and shoot growth with a mixture of purines and pyrimidines (adenine, thymine, cytosine, methylcytosine, uracil and hypoxanthine, 100 μM each) failed (data not shown). Similarly, TCA cycle intermediates, α-ketoglutarate, oxalacetate, malate, citrate and succinate in their sodium salts forms, were tested at 1 mM with negative results (data not shown). Finally, the antioxidants glutathione (GSH), pyruvate and ascorbate sodium salts, as well as sodium acetate were also tested at the same concentration. Treatment with 1 mM GSH caused 71 and 77% reversal of DHZ-induced growth inhibition on lettuce and onion, respectively (Fig. 3). Further experiments with GSH (0.5 and 0.1 mM) showed that this effect only occurred when GSH was at a much greater concentration with respect to the phytotoxin. Reversion was not obtained with these two lower concentrations, nor when 1 mM oxidized glutathione (GSSG) was tested.

2.4. Electrolyte leakage

Increased conductivity of media in which leaf or cotyledonary tissues are treated with a herbicide is a good indication of possible damage to plasma membranes (Duke & Kenyon, 1993). When cucumber cotyledon discs were treated with DHZ, a dramatic and rapid increase in cellular leakage was detected (Fig. 4). After 24 h, the activity threshold is about 3 μM DHZ and activity increases with an almost linear relationship to log concentration. The effect of DHZ is greater than that caused by the same concentration of acifluorfen, a commercial herbicide known to cause plasma membrane damage. Moreover, the membrane peroxidative damage associated with the mode of action of acifluorfen is light dependent (Dayan & Duke, 1997), whereas the effect of DHZ is not, with loss of membrane integrity occurring during the dark incubation phase (Fig. 4). Thus, the mode of action of this compound is clearly different from that of protoporphyrinogen oxidase inhibitors (Dayan & Duke, 1997). Since the inhibiting effect of DHZ is reflected primarily in the reduction of root growth, the same conductivity experiment was repeated under dark conditions using excised six-day-old lettuce roots. In this experiment, acifluorfen and DHZ had similar effects. Acifluorfen and related compounds are known to have secondary sites of action which are not dependent on light (Kunert, Sandmann & Böger, 1987).

Antimycin A, an inhibitor of respiration, can reduce the electrolyte leakage associated with the effect of acifluorfen (Duke, Vaughn & Meeusen, 1984), apparently because the action of acifluorfen depends on biosynthetic processes. Attempts to reverse the damage of 30 μM DHZ on membrane integrity with different concentrations of antimycin (10, 30 and 100 μM) were unsuccessful (data not shown). In fact, all antimycin treatments also enhanced the electrolyte leakage effect caused by DHZ on treated cucumber discs.

α-Tocopherol, a radical scavenger also known to partially reverse the effect of acifluorfen (Duke et al., 1984), was tested at 1, 10 and 100 μM. None of the treatments reversed the electrolyte leakage caused by 30 μM DHZ (data not shown), indicating that the loss

Table 2
Mean effects of 50 and 500 μM DHZ on plasma membrane NADH oxidase activity isolated from seven-day-old ethiolated soybean hypocotyls ($n = 3 \pm 1$ S.E.M.)

	Control	50 μM DHZ	500 μM DHZ
nmol min^{-1} mg $(protein)^{-1}$	7.28 ± 0.97	8.89 ± 0.39	10.32 ± 1.09

of membrane integrity may not be due to peroxidative damage.

A complete reversion of the ion leakage effect was obtained when DHZ-treated cucumber cotyledon discs were also treated with 10 to 100 μM GSH (Fig. 5). Reduced glutathione at 1 and 3 μM failed to produce any reversion under the same conditions.

2.5. Effects on photosynthetic processes

The effect of 33 and 100 μM DHZ on in vivo variable chlorophyll fluorescence was tested. No significant effect was produced on photosynthetic electron transport, since neither q_P, nor q_N were affected by the herbicide. A slight inhibitory effect was observed on F_v/F_m with 100 μM DHZ after 20 h, but the change was within experimental error (data not shown).

DHZ was also tested on photosynthetic PSII oxygen evolution from isolated spinach thylakoids. No effects were observed (data not shown). Cytochrome *f* photooxidation/dark reduction provides another estimation of the integrity of electron transport between photosystems II and I (Kenyon, Duke & Vaughn, 1985). Since DHZ seemed to cause plasma membrane damage, some effects would be expected in the systems located in chloroplast membranes. Nevertheless, no significant activity could be detected in the cytochrome *f* photooxidation/dark reduction in cucumber discs treated with 50 μM DHZ (data not shown), indicating no primary effects on the thylakoid membrane.

2.5.1. Plasma membrane NADH oxidase assays

Plasma membrane (PM) NADH oxidase, a membrane-localized enzyme, was tested as a possible molecular site of action of DHZ because some diterpene lactone natural products such as the quassinoids have been found to be potent inhibitors of this enzyme (Morré, Frederick, Barr, Penel & Wu, 1988). The increased electrolyte leakage resulting from the presence of DHZ suggested that this sesquiterpene lactone may inhibit this site as well. Activity of PM NADH oxidase isolated from etiolated soybean hypocotyls was not significantly affected by 50 and 500 μM DHZ (Table 2).

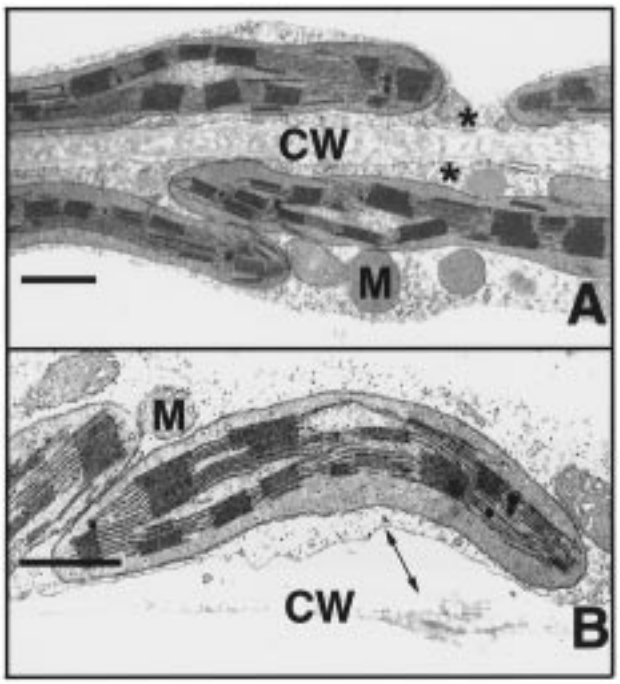

Fig. 6. Transmission electron micrographs of control (a) and 50 μM DHZ-treated (b) cucumber cotyledon cells 24 h after treatment. Arrows show plasmalemma separation of the cell wall of a treated cell. Bars = 1 μm.

2.5.2. Electron microscopy

Light and transmission electron micrographs were made of seven-day-old cucumber cotyledons floated on buffer solution with and without 50 μM DHZ for up to 24 h. The only clearly observable effect was an increase in separation of the plasma membrane from the cell wall in the cells of DHZ-treated tissue (Fig. 6), a symptom of plasmolysis. Not every cell of treated tissue was plasmolyzed at 24 h, but the symptoms were apparent in a large proportion of the cells. There were no ultrastructural effects on the chloroplast or any other organellar structures, other than slightly distended cristae of mitochondria.

2.5.3. Reactivity between DHZ and glutathione

Most of the phytotoxic effects caused by sesquiterpene lactones may be due to the Michael addition that eventually can take place between the exocyclic methylene group of the lactone moiety and the sulfhydryl groups of proteins, amino acids and other thiol-containing biomolecules (Hall et al., 1980). Nevertheless, the binding site between a sesquiterpene lactone and a protein or DNA fragment through this mechanism has never been determined. Among the few reports of such an interaction, the binding between the guaianolide-type sesquiterpene lactone thapsivillosin A and the Ca^{2+}-ATPase of skeletal muscle sarcoplasmic reticulum has been reported (Khan, Witcome, East & Lee, 1995). This lactone does not possess the exomethylene

Table 3
HPLC analysis of the reaction between DHZ and GSH with different molar ratios. Absorbance was monitored at $\lambda = 218$ nm

Reaction time	Peak	Retention time	Absorbance
	100 μM DHZ + 20 μM GSH		
3 h 40 min	DHZ	6.28	0.47
	DHZ-SG adduct	3.80	0.13
24 h	DHZ	6.28	0.47
	DHZ-GSH adduct	3.80	0.21
	100 μM DHZ + 200 μM GSH		
30 min	DHZ	6.68	0.0017
	DHZ-GSH adduct	3.27	0.09
24 h	DHZ	6.59	0.0005
	DHZ-$(SG)_2$ adduct	2.88	0.105

moiety in the lactone ring, but has three α,β-unsaturated carbonyl-containing side chains. Another example is the germacrane α,β-unsaturated-γ-lactone parthenolide supression of lipopolysaccharide-stimulated protein tyrosine phosphorylation, which has been correlated with the inhibition of expression of the cyclooxygenase COX-2 and proinflammatory cytokines (Hwang et al., 1996).

The reversion of DHZ effects by GSH on growth development and electrolyte leakage suggests that there is an interaction between the compounds. HPLC analysis of a DHZ and GSH mixture showed evidence of mono- and di-adduct formation. A new peak with a higher polarity than DHZ appeared within the first 4 h of incubation, becoming more intense over time (Table 3). The maximum absorbance of this new peak falls below 200 nm, suggesting the loss or modification of one of the chromophore groups. Moreover, when 100 μM DHZ is incubated with 200 μM GSH, a similar peak with the same retention time appears within the first 30 min of reaction. This peak is replaced by another with a lower retention time when the reaction is monitored 24 h later (Table 3). The peak with the higher retention time (3.80 min) is the 1,4-mono adduct of the reaction at the cyclopentanone ring and the second more polar peak (2.88 min) is the bis-1,4-adduct from the reaction at both sites.

Picman, Rodriquez and Towers (1979) reported a reaction similar to that observed in our experiment, between parthenin and cysteine or glutathione. Sulfhydryl-containing important biomolecules, such as cysteine and glutathione, are known to react with the cyclopentenone and the exomethylene-γ-lactone groups of the pseudoguaianolide helenalin (Schmidt, 1997a). The two reactions have different kinetics, with cyclopentenone ring reaction estimated to be 10 times faster than the addition at the C-13 exomethylene position (Schmidt, 1997b). Our experimental data indicates that the formation of the monoadduct product occurs very rapidly (Table 3) and can be clearly identified by mass

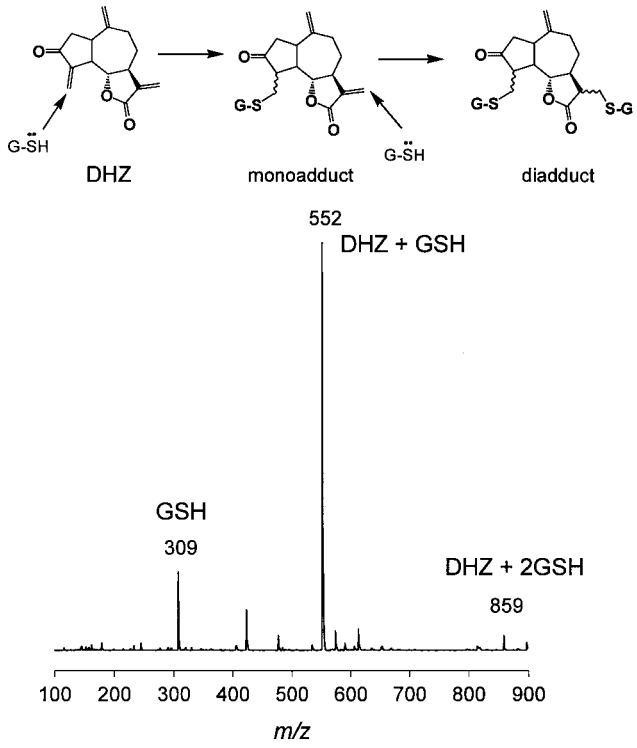

Fig. 7. Putative mechanism of detoxification of DHZ via Michael addition of glutathione (GSH) to the Michael acceptors centers of these molecules. The mechanism was derived from the HPLC and mass spectrometry data presented in the text.

spectrometry analysis as a signal at 552 (Fig. 7). HPLC analysis shows that most of the DHZ exists as a diadduct when incubated with GSH for 24h, as confirmed by the signal at 859 in the mass spectrum of the reaction product.

*Iso*zaluzanin C, the 3α-hydroxy derivative of DHZ, also reduces root growth at 500 μM which can be fully reversed with 1 mM GSH and partially with 0.5 mM GSH (data not shown). The molar ratios for DHZ and *iso*zaluzanin C reactions with GSH are 1:2 and 1:1, respectively, since there are two α,β-unsaturated carbonyl groups in DHZ and only one in *iso*zaluzanin C. Based on this consideration, a 1:1 molar relationship in the DHZ bioassay would not be expected to cause appreciable reversion since there are still free reactive groups on DHZ. However, the same molar relationship is still able to exert reversion of the phytotoxicity of *iso*zaluzanin C (data not shown). Since 50 μM *iso*zaluzanin C does not cause membrane leakage, it is hypothesized that the plasma membrane damage caused by DHZ is related with the second Michael acceptor group. This hypothesis is supported by the kinetics of the reversion: inactivation of DHZ by GSH takes place at the onset of the experiment, which is in accordance with the higher reactivity of the cyclopentenone group (Schmidt, 1997b). Thus, the first reacting α,β-unsaturated carbonyl group should be that of the

cyclopentenone ring and this reaction is believed to be responsible for the leakage effect (Fig. 7).

In summary, because DHZ causes rapid and pronounced cellular leakage and its 3-deoxy-hydroxy-derivative *iso*zaluzanin C does not (data not shown), we propose that the phytotoxicity of DHZ is due to two separate mechanisms. The first is common to all exomethylene-γ-lactone containing guaianolides and works through this moiety. A second mechanism caused by the reaction of the α,β-unsaturated carbonyl group of the cyclopentanone is responsible of the leakage effect. To our knowledge, this is the first report of such a mode of action for a sesquiterpene lactone. Previously, only the alteration in cellular permeability for encelin, a eudesmanolide with a cross-conjugated enone function, has been reported (Trejo, Gaytan, Mendoza & Sabanero, 1996). Further work is necessary to determine the primary molecular site(s) of action of DHZ.

3. Experimental

3.1. Preparation of DHZ

Dehydrozaluzanin was synthesized from dehydrocostuslacone as previously described (Kalsi et al., 1984; Macías et al., 1999). Purification was made by CC with hexane:EtOAC (7:3), followed by recrystallization from hexane:EtOAc. Dehydrocostuslactone was obtained from crude Costus Resin Oil (Pierre Chauvet) by CC purification and recrystallization from hexane:EtOAC mixt. Structures of all starting and final compounds were verified by comparison of spectroscopic data (MS, IR and ^{1}H-NMR) with those reported in the literature.

3.2. Effects on growth and photosynthetic pigments concentrations

Phytotoxicity tests were performed in 24 well plates. Each treatment consisted of two replicates and two controls. A 10 mM stock soln of DHZ was prepd in DMSO and diluted with water to a final conc of 100 μM DHZ in 1% DMSO aq soln. Control treatments received 1% DMSO without the test compound. Seeds were grown on moistened Whatman No. 1 filter paper. Five seeds per plate well were used for lettuce (*Lactuca sativa* var. Iceberg) and onion (*Allium cepa* L. cv. Evergreen bunching), while 15 mg of seeds per plate well were weighed for *Arabidopsis thaliana* and *Agrostis sp*. A 200 μl volume of the test soln was applied to each well. Plates were incubated at 25 ± 2°C under fluorescent lights maintaining a 16 h photoperiod (400 μmol m^{-2} s^{-1} PAR). Germination and growth rates, as well as chlorophyll and carotenoid contents, were measured on seven-day-old plants for lettuce, onion and *Arabidopsis* and eight-day-old plants for *Agrostis*. Chlorophyll was extracted from five pairs of lettuce cotyledons per treatment, two replicates each, in 3.5 ml dimethyl sulfoxide (Hiscox & Israelstam, 1979) and concentrations determined spectrophotometrically according to Arnon (1949).

In other experiments, 21 lettuce seeds were germinated in 6-cm diam polystyrene disposable Petri dishes lined with No. 1 Whatman filter paper moistened with 1.5 ml of water with or without test compounds. Dishes were maintained in darkness for 24 h before placing under light (400 μmol m^{-2} s^{-1} PAR) in a growth chamber at 25°C for seven days. Readings of root/shoot length/weight were made.

3.3. Effect on root cell division (mitotic index)

Onion seeds were germinated as previously described in the presence of 100 μM DHZ at 25°C under a 14 h photoperiod. Root tips were prepd according to Armbruster, Molin and Bugg (1991) and mitotic analysis was performed on 1000 cells per slide (4000 cells per treatment).

3.4. Oxygen consumption

Twenty lettuce or onion root tips (6 mm), five- and seven-day-old, respectively, pretreated with 100 μM DHZ were cut and then placed into a thermostabilized electrode chamber (25°C) with 2 ml of O_2-saturated water. Oxygen uptake was measured polarographically for 10 min using a computer-controlled Hansatech DWI oxygen probe. Results were expressed as nmol O_2 consumed and expressed per fresh weight and dry weight of root tips.

3.5. Complementation experiments

Attempts to reverse the phytotoxic effects of 500 μM DHZ by complementation studies were performed with amino acids, purines, pyrimidines, reductants and organic acids. All purines and pyrimidines (adenine, hypoxanthine, uracil, methylcytosine, thymine and cytosine) were tested at 100 μM. Krebs cycle intermediates (α-ketoglutarate, oxalacetate, malate, citrate and succinate), as well as glutathione (GSSG and GSH forms), pyruvate, ascorbate and acetate (all of them in their sodium salts forms) were tested at 1 mM.

3.6. Dose–response complementation experiments with GSH

All 20 amino acids were tested at 200 μM either individually or in groups, except methionine, tyrosine and tryptophan, which were tested at 40 μM because

of phytotoxicity at higher concentrations. Dose–response complementation experiments were performed with those amino acids with positive reversion responses.

3.7. Electrolyte leakage

Fifty 4-mm cotyledon discs of seven- to ten-day-old cucumber plants were placed on a 2% sucrose/1 mM MES-NaOH buffer (pH 6.5) containing the appropriate concentration of each compound (Duke & Kenyon, 1993). Acifluorfen (50 μM) was used as a positive control. Plates were incubated in darkness for 18 h prior to exposure to light (400 μmmol m^{-2} s^{-1}). Measurements were made using a Markson 1054 EC Meter every 3 h during the following 12 h. A final 24-h reading was made the following day. A second set of experiments was carried out in complete darkness, without any incubation time and measures made in the same way as previously described. Each experiment consisted of six replicates. Dose–response curves with DHZ over a range from 0.3 to 100 μM were performed. In another set of experiments, fifty 20-mm long cut root tips of five-day-old lettuce seedlings were treated in the same conditions as described above for cucumber cotyledon discs and incubated in darkness. Observations were made every 2 h during the first 12 h and a final 24-h measurement was made. Maximum conductivity was measured by boiling three samples of each treatment for 20 min.

3.8. Complementation using the electrolyte leakage experiments

Attempts to reverse the leakage induced by DHZ using complementation studies were carried out with α-tocopherol, antimycin and GSH. The concentration of DHZ was 50 μM in all cases. Dose–response curves with 1, 10 and 100 μM for α-tocopherol, and 10, 30 and 100 μM antimycin, were performed. GSH was tested from 1 to 100 μM. The methods were the same as described previously.

3.9. Fluorescence experiments

In vivo chlorophyll fluorescence transients were determined with a Model OS5-FL fluorimeter on 6-mm diam seven- to ten-day-old cucumber cotyledons discs (Vaughn & Duke, 1983). The cotyledons were placed into 7.5 ml of 2% sucrose/1mM MES-NaOH buffer (pH 6.5) with or without the test compounds and incubated in the dark. Acifluorfen (50 μM) was used as positive control and DHZ was tested at 33 and 100 μM. Measurements were made at 2, 5 and 8 h and then exposed to continuous light before 10 h. A final reading was made at 20 h. Parameters measured were F_v/F_m and F_o.

3.10. Cytochrome f measurements

In vivo cytochrome *f* oxidation/reduction activity measurements were performed in 6 mm diameter seven- to ten-day-old cucumber cotyledons discs incubated in the same media used for leakage and fluorescence experiments, using a DW-2000 SLM-AMINO dual-wavelength spectrophotometer (Duke et al., 1996). Cytochrome *f* measurements were repeated three times, with four measurements at each point per treatment during each repetition.

3.11. NADH Oxidase activity

Plasma membranes (PM) containing NADH oxidase were obtained from eight-day-old soybean hypocotyls as described by Morré et al. (1988). The assay was carried out in medium containing 25 mM Tris–HCl, 0.75 mM Na_2HPO_4, 150 mM NaCl and 5 mM KCl adjusted to a final pH of 7.0. 50 μg of protein were used for each assay with a final concentration of 150 μM NADH and 1 mM KCN (used to inhibit any mitochondrial NADH oxidases contaminating the PM). Samples were incubated for 10 min prior to adding NADH. The rate of the reaction was determined by measuring the decrease in absorbance at $\lambda = 340$ using a Shimadzu UV-3101 PC spectrophotometer. The change in A_{340} was recorded as a function of time and the specific activity of the plasma membrane was calculated using an extinction coefficient of 6.21 mM^{-1} cm^{-1} and expressed as nmol min^{-1} mg^{-1} protein. Assays were initiated by addition of NADH and measured over 10 min after an equilibration period with NADH of 2 min to achieve an initial steady-state rate. Experiments were made in triplicate with 50 and 500 μM DHZ.

3.12. Electron microscopy

Tissue was fixed in 4% (v/v) glutaraldehyde in 0.1 M cacodylic acid (pH 7.0) for 2 h, rinsed in distilled water, then post-fixed in 1% (w/v) OsO_4 for 1 h. After dehydration in acetone, the tissues were embedded in Spurr's medium. Thin sections (70 nm) were obtained with a diamond knife on an ultramicrotome (Ultracut E, Cambridge Instruments). Thin sections were stained with uranyl acetate and lead citrate and then observed and photographed in a TEM (EM10 CR, Carl Zeiss).

3.13. Characterization of glutathione adducts

100 μM DHZ soln was incubated with 20 or 200 μM GSH. The analyses were performed using a

3.9 × 300 μBondapak C-18 column, with MeOH:H_2O (55:45) as eluant and a flow rate of 1 ml min^{-1}. The reaction was monitored at $\lambda = 218$ nm and parallel controls with 100 μM DHZ and GSH at the same concentrations as the reaction were performed. Samples were also analyzed by direct injection in MS.

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