

Citrus rootstock responses to herbivory by larvae of the sugarcane rootstock borer weevil (*Diaprepes abbreviatus*)

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The responses of roots to feeding by larvae of a citrus root weevil (*Diaprepes abbreviatus*) were investigated in *Citrus grandis* (L.) Osb. × *Poncirus trifoliata* (2N) (L.) Raf.; *C. grandis* × *P. trifoliata* (4N); *P. trifoliata* × *C. grandis* (Flying Dragon × Nakon); *C. paradisi* Macf. × *P. trifoliata* (Swingle citrumelo); *C. aurantifolia* (Christm.) Swingle (*Citrus macrophylla*); *C. reticulata* Blanco (Cleopatra mandarin); *C. sinensis* (L.) Osb. × *P. trifoliata* (Carrizo citrange); *C. aurantium* (L.) (sour orange). Chitinase, chitosanase, β -1,3-glucanase, peroxidase and lysozyme activities were measured and significant differences were observed for some of the cultivars between infested and uninfested rootstocks. Generally, increased activities were observed for chitinases and decreased activities were observed for the other enzymes measured. Numerous significant differences in hydrolase and peroxidase activities were observed between cultivars. Immunological detection revealed that new protein bands occurred in root protein extracts for six of the eight cultivars infested with larvae when an antibody to a class I potato leaf chitinase was used. Antibodies generated against two citrus chitinases of M_r 24 000 (basic chitinase cv. Valencia (*C. sinensis*) callus, BCVC) and M_r 28 000 (basic chitinase/lysozyme cv. Valencia callus, BCLVC) indicated that chitinases in Carrizo were induced in infested roots when the BCVC antibody was employed. These findings justify calling these proteins pathogenesis-related proteins. The chitinase that BCLVC was prepared from exhibited high lysozyme activities, and the results of western blots showed the presence of proteins at M_r 24 000 and 27 000 which are presumed to be lysozymes. Similar tests using antibodies against β -1,3-glucanases and peroxidases indicated a diminution of protein bands that cross-reacted with infested root protein extracts compared with what occurred in controls. All of the root extracts were tested against chitosans with various percentages of acetylation; activities were linearly dependent on the amount of chitosan acetylation; i.e. the larger the amount of acetylation, the greater the activity. Significant differences in hydrolase activities were observed between infested and uninfested roots for the rootstocks using the variously acetylated substrates. All of the root protein extracts were capable of degrading peritrophic membranes removed from larvae of *D. abbreviatus*. This suggests that citrus chitinases may play a role in disrupting the peritrophic membrane such that ingested substances that pose a hazard to the insect may penetrate the membrane more easily.

Key words – Chitinase, chitosanase, citrus, defense, β -1,3-glucanase, insect, larval feeding, lysozyme, pathogenesis-related proteins, peroxidase, roots.

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Introduction

The sugarcane rootstock borer weevil (*Diaprepes abbreviatus* L.) is a pest of citrus rootstocks in the Caribbean (Marshall 1974). It is reported in central and South America (Marshall 1979) have shown the preferred host plant for the weevil is subtropical citrus. The most damage to citrus rootstocks of citrus and other plants will kill the plant root.

There are several studies on citrus rootstock weevils (Marshall 1974, Beavers & Wald 1995). In citrus root systems are examined. Which were highly susceptible to larvae, some authors and Hutchings. None of these studies or resistance morphological

Over the last few years chitinases and chitinase (et al. 1993, 1994) activities of citrus chitinases, β -1,3-glucanases, β -1,3-glucosyltransferases (PR) proteins have been induced in plants under conditions can be used to detect sitic attacks by herbivores (van der Meer & Dreyer 1994) attacks by *D. abbreviatus* proteins mentioned above.

Abbreviations – callus; BCVC, basic chitinase cv. Valencia; BCLVC, basic chitinase/lysozyme cv. Valencia; β -1,3-glucosyltransferase; pathogenesis-related protein.

Materials and Methods

Chemicals

Shrimp shell chitinase, IR spectroscopy, chitinase, Chemetals Co., Chitosans used

Introduction

The sugarcane rootstalk borer weevil (*Diaprepes abbreviatus* L.) is a plant pest of economic importance in the Caribbean (Martorell 1976). The weevil was first reported in central Florida in 1964 and has since continued to spread to other parts of the state. Schroeder et al. (1979) have shown that *Citrus* spp. appear to be one of the preferred hosts of *D. abbreviatus*. Citrus production losses attributed to the weevil in Florida in 1993 were estimated by the Florida *Diaprepes* Task Force to be more than \$70 million annually. The larval stage of the weevil is subterranean and it is this stage that does the most damage to plants. Larvae feed on bark of the roots of citrus and other plants. Given enough time the larvae will kill the plant by girdling the tree at the crown of the root.

There are several reports on the susceptibility of various citrus rootstocks to *D. abbreviatus* (Norman et al. 1974, Beavers and Hutchison 1985, Shapiro and Gottwald 1995). In these reports the overall damage to the root systems and the numbers of surviving larvae was examined. While the majority of the rootstocks evaluated were highly susceptible to damage by *D. abbreviatus* larvae, some appeared to exhibit some resistance (Beavers and Hutchinson 1985, Shapiro and Gottwald 1995). None of these studies attempted to link either susceptibility or resistance to any biochemical, physiological or morphological characteristic.

Over the last few years we have identified several chitinases and chitinases/chitosanases in *Citrus* (Osswald et al. 1993, 1994). Here we examine changes in the activities of citrus rootstock chitinases, chitosanases, peroxidases, β -1,3-glucanases and lysozymes in response to attacks by *D. abbreviatus* larvae. Pathogenesis-related (PR) proteins have been defined as plant proteins that are induced in pathological or related situations, and those conditions can include infections by pathogens and parasitic attacks by nematodes and phytophagous insects and herbivores (van Loon et al. 1994). The plant responses to attacks by *D. abbreviatus* observed here for some of the proteins mentioned previously would qualify them as PR proteins.

Abbreviations – BCLVC, Basic chitinase/lysozyme cv. Valencia callus; BCVC, basic chitinase cv. Valencia callus; BGVC, basic glucanase cv. Valencia callus; Glc, glucose; GlcNAc, N-acetylglucosamine; PODVC, peroxidase cv. Valencia callus; PR, pathogenesis-related.

Materials and methods

Chemicals

Shrimp shell chitosan (18% acetylated as determined by IR spectroscopy; Osswald et al. 1992) routinely used for chitosanase assays was purchased from Atomergics Chemetals Corp. (Farmingdale, NY, USA; Lot L.O729). Chitosans used for substrate specificity were either as

described in Osswald et al. (1992) or Aiba (1992a, 1993); the latter samples were moderately deacetylated (K-30), highly deacetylated (4C-5) or prepared from highly deacetylated (2C-5-49) chitosans. Fluorescamine (Floram) was obtained from Fluka (Buchs, Switzerland). Bovine serum albumin (BSA, fraction V), 5-bromo-4-chloro-3-indolyl phosphate, laminarin, aminoantipyrine, and nitroblue tetrazolium were purchased from Sigma and US Biochemicals (Cleveland, OH, USA).

Insects

Diaprepes abbreviatus larvae were obtained from a colony maintained at this laboratory for over 5 years. Rearing was according to Beavers (1982). Newly hatched larvae (ca 10) were placed in rearing cups (30 ml) containing 20 ml artificial diet (Bio-Serve, Frenchtown, NJ, USA) and allowed to develop at room temperature until they reached 15–20 mg (29–32 days).

pH measurements of larval midguts

Larvae (ca 0.5 g) were dissected and the midguts removed. The pH of the midguts was measured by inserting a pH microelectrode (Lazar Research Labs, Los Angeles, CA, USA) into the lumen of the midgut. The pH meter had been previously calibrated using standardized buffers.

Rootstocks and larval infestations

The eight rootstocks used in these experiments were *Citrus grandis* (L.) Osb. \times *Poncirus trifoliata* (2N) (L.) Raf. (pummelo 2N); *C. grandis* \times *P. trifoliata* (4N) (pummelo 4N); *P. trifoliata* \times *C. grandis* (Flying Dragon \times Nakon); *C. paradisi* Macf. \times *P. trifoliata* (Swingle citrumelo); *C. aurantifolia* (Christm.) Swingle (*C. macrophylla*); *C. reticulata* Blanco (Cleopatra mandarin); *C. sinensis* (L.) Osb. \times *P. trifoliata* (Carrizo citrange); *C. aurantium* (L.) (sour orange). Root samples were taken from trees grown, maintained and infested with weevil larvae as described by Shapiro and Gottwald (1995). Briefly, 13½-month-old rootstocks were each infested with 10 weevil larvae ($n = 7$ for each cultivar; however, larval feeding reduced the amount of tissue and consequently some analyses were performed on $n < 7$); all controls were $n = 7$. Larvae were allowed to feed on the roots for 44 days and then removed and the roots processed for protein and enzyme analyses.

Root enzyme preparation

Roots (5 g fresh weight when tissue was available) were first freeze-dried, then ground in liquid N_2 using an Omni-mixer (OCI Instruments, Waterbury, CT, USA). The resulting powder was suspended in 15 ml of 0.1 M sodium phosphate buffer (pH 7.4) and homogenized for 1 min in a Polytron homogenizer (Brinkman Instruments,

Westbury, NY, USA) equipped with a PTG 36/50 generator. The homogenate was filtered through four layers of cheesecloth and the filtrate was centrifuged at 15 000 g for 15 min. The supernatant was passed through one layer of Miracloth (Calbiochem, La Jolla, CA, USA). Three ml of the filtrate were subjected to desalting (Econo-Pac 10 DG, BioRad, Hercules, CA, USA) to remove possible interfering carbohydrates. Desalted samples were freeze-dried, resuspended in 0.2 ml distilled water and used for protein and enzyme assays. The remaining portions of 15 000 g supernatants were stored at -20°C until used.

Protein assay

Protein contents were determined following the dye binding method of Bradford (1976). BSA (fraction V) was used as the standard.

Enzyme assays

Chitinase activity was measured as previously reported using colloidal [^3H] chitin from shrimp shell chitosan (Osswald et al. 1993). The specific activity of the prepared [^3H] chitin was 16.35 MBq per mol N-acetylglucosamine (GlcNAc). Reaction times were 30 min at 45°C , unless specified otherwise.

The chitosanase assay followed Osswald et al. (1992, 1993) using solubilized shrimp shell chitosan. Reactions were conducted at 45°C for 45 min unless specified otherwise. Enzyme activities were reported as nmol GlcN mg^{-1} protein min^{-1} .

In some cases it was desirable to determine and compare chitinase and chitosanase activities using chitosan substrates that were acetylated to different extents (0–100%). These comparisons were made using a modified Schales method (Imoto and Yagishita 1971) which measured reducing ends of hydrolyzed carbohydrates. Generally enzyme assay reactions were performed in glass test tubes containing 5 μg root protein, 280 μl 0.2 M sodium acetate buffer (pH 5) and 5 μg substrate. Reactions were incubated for 1 h at 45°C in a gyratory shaker-water bath and terminated by transferring the tubes to a boiling water bath for 5 min. Subsequently, an additional 450 μl of 0.2 M sodium acetate buffer and 1 ml of 1.5 mM potassium ferricyanide in 0.5 M sodium carbonate were added to the tubes, and the tubes were capped with aluminum foil. The contents of the tubes were mixed, and then the tubes were incubated for 15 min in a boiling water bath. After the tubes had been removed and cooled to room temperature, the absorbance was measured at 420 nm with a spectrophotometer. Activity was based on a standard curve generated from GlcNAc and reported as GlcNAc reducing equivalents mg^{-1} protein min^{-1} .

Digestion of peritrophic membranes by root extracts was also assayed using the modified Schales method (Imoto and Yagishita 1971). Larval *D. abbreviatus* weighing 0.3–0.5 g were the source of peritrophic mem-

branes. Larvae were placed in Petri dishes containing distilled water and ice and the intestinal tracts were removed with the aid of a dissecting microscope using microdissection scissors. The intestine was opened and the peritrophic membrane (usually containing a food bolus) was separated from intestinal tissue. The peritrophic membrane was opened and the food was washed away by rinsing the membrane several times in distilled water. Usually 9–12 peritrophic membranes were homogenized using a 0.1-ml glass homogenizer containing distilled water. Homogenized membranes were transferred to microcentrifuge tubes and centrifuged for 7 min at 10 000 g in a microcentrifuge. The supernatant was removed and the peritrophic membranes were resuspended in 100 μl distilled water, recentrifuged and rehomogenized as before. The recovered pellet was freeze-dried, weighed and resuspended in 0.2 M sodium acetate buffer, pH 5, at 2 mg membranes per ml. Twenty micrograms of peritrophic membrane were used for each enzyme assay.

β -1,3-Glucanase activity was assayed by the method of Abeles and Forrence (1970), which determines the increase in reducing groups resulting from the hydrolysis of laminarin. Typically, 125 μl of plant extract diluted in 100 mM sodium acetate buffer (pH 5) were added to 125 μl of laminarin (20 mg ml^{-1}) in acetate buffer. The mixtures were incubated at 50°C for 15 to 90 min, depending on the amount of activity. Reactions were terminated by addition of 0.25 ml copper reagent (24 mM CuSO_4 and 0.91 M Na_2SO_4) and then boiled for 20 min. An aliquot (0.25 ml) of arsenomolybdate solution (450 ml of 45 mM ammonium molybdate, 21 ml of concentrated H_2SO_4 and 25 ml of 134 mM Na_2HAsO_4) was added to develop color and the absorbance at 540 nm was measured following dilution with 3 ml of water. Standard curves were prepared using Glc. Activity was expressed as μmol Glc equivalents released mg^{-1} protein min^{-1} .

The assay of peroxidase activity followed the method outlined in the Worthington Enzyme Manual (Worthington 1978). Reactions contained plant extract in 10 mM potassium phosphate buffer (pH 7), 0.85 mM H_2O_2 , 1.2 mM aminoantipyrine and 79 mM phenol in a total volume of 200 μl . Reactions were conducted for 3 min at room temperature during which absorbance at 510 nm was monitored every 15 s. Activity was expressed as ΔA_{510} mg^{-1} protein min^{-1} .

Lysozyme activity was determined following the method provided by Sigma. Briefly, this method uses *Micrococcus lysodeikticus* (Sigma) suspensions (0.28 mg bacteria per ml of 30 mM sodium phosphate buffer) as the substrate. Assays (2 ml) were conducted at pH 5 in a cuvette (1 cm pathlength) contained in a thermostatted (25°C) cuvette holder of a spectrophotometer. The reaction was initiated by addition of enzyme and the decrease in turbidity absorbance at 645 nm was recorded. Root extract protein concentrations in the reaction ranged from 0.1–0.2 mg per assay.

Tab. 1. Summary of results. No significant differences; *, significant; **, highly significant.

Rootstock cultivar
Cleopatra
Swingle
Sour orange
Pummelo 2N
Pummelo 4N
Citrus macrophylla
Flying Dragon × Carrizo
Treatment (TRT)
Uninfested
Infested
Source
CV
TRT
CV × TRT
Immunodetection
Proteins (5–10 μg) separated on 12.5% SDS-PAGE; 12% gels transferred onto nitrocellulose membrane and probed with anti- β -1,3-glucanase antibodies. Primary antibodies: BVCV (BioVet, CA, USA) and BVCV (BioVet, CA, USA). Secondary antibodies: BVCV (BioVet, CA, USA) and BVCV (BioVet, CA, USA). Molecular weight markers (MW) were 24 000, 28 000, 32 000, 36 000, 40 000, 44 000, 48 000, 52 000, 56 000, 60 000, 64 000, 68 000, 72 000, 76 000, 80 000, 84 000, 88 000, 92 000, 96 000, 100 000. Additional details are available upon request.

Tab. 1. Summary of rootstock enzyme responses to herbivory by *Diaprepes abbreviatus* larvae. Like letters within columns indicate no significant difference in activities. Mean separation within columns by Duncan's multiple range test at $P = 0.05$. $n = 3-7$. NS, not significant; *, significant at $P < 0.05$; **, significant at $P < 0.01$.

Rootstock cultivar (CV)	Enzyme activity					
	Chitinase (nmol GlcNAc mg ⁻¹ protein min ⁻¹)	Chitosanase (nmol GlcN mg ⁻¹ protein min ⁻¹)	Peroxidase (ΔA_{510} mg ⁻¹ protein min ⁻¹)	β -1,3-Glucanase (μ mol Glc mg ⁻¹ protein min ⁻¹)	Lysozyme (ΔA_{645} mg ⁻¹ protein min ⁻¹)	
	Uninfested	Infested				
Cleopatra	20.8a	40.3a	93de	61d	27	65
Swingle	9.2ab	13.2bc	158cde	64cd	34	83
Sour orange	10.6ab	15.1bc	219bcd	92cd	45	57
Pummelo 2N	11.9ab	10.7c	374ab	120ab	27	84
Pummelo 4N	23.6a	20.2b	549a	247a	60	116
<i>Citrus macrophylla</i>	8.0b	34.9a	355bc	150b	24	90
Flying Dragon \times Nakon	8.4ab	10.1c	193cde	112cd	28	48
Carrizo	13.9ab	10.8c	97e	78d	32	32
Treatment (TRT)						
Uninfested	–	298	NS	41	88	
Infested	–	212	NS	28	55	
Source						
CV	**	**	**	NS	NS	
TRT	*	**	NS	*	**	
CV \times TRT	*	NS	NS	NS	NS	

Immunodetection

Proteins (5–10 μ g per lane; amounts were constant for each enzyme analyzed) were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 12% gels) according to Laemmli (1970) and blotted onto nitrocellulose (pore size 0.2 μ m, BioRad) using a semidry electroblotting system according to the manufacturers instructions (BioRad). Several antibodies for β -1,3-glucanases, peroxidases and chitinases were used. Primary antibodies were raised in rabbits (BAbCo, Richmond, CA, USA) against citrus chitinase, β -1,3-glucanase and peroxidase antigens. The two citrus chitinase (BCVC and BCLVC) and the β -1,3-glucanase (BGVC) antibodies were prepared using purified proteins of ca M_r 24 000, 28 000 and 32 000, respectively, from cv. Valencia (*C. sinensis*) nonembryogenic callus; all had basic pIs (unpublished data). The peroxidase antigen (PODVC) was also prepared from cv. Valencia nonembryogenic callus using a mixture of three peroxidases (one acidic and two basic) of M_r 40 000. The enzymes (chitinases, β -1,3-glucanase, peroxidases) were purified from 4-week-old callus (3 kg) that had been extracted into a neutral sodium phosphate buffer. The proteins were initially separated on a CM52 (Whatman, Maidstone, UK) packed column. Active fractions were further resolved by application to a Superdex-75 size exclusion followed by application to a Resource S column (Pharmacia). Purity was determined via SDS-PAGE and isoelectric focusing. Additional details on the purification and characterization

of these proteins will be given elsewhere. All of the antibodies exhibited high specificity towards the citrus proteins used for their preparation with no cross-reactivity to other purified proteins. An antibody raised in rabbits against a basic (class I) potato leaf chitinase was obtained from Dr E. Kombrink (Max Planck Institute, Cologne, Germany). Information on the cross-reactivity and preparation of the antibody has been reported (B. Witte 1991. Thesis, Univ. of Cologne, Germany; Osswald et al. 1993, 1994).

Results

Effects of herbivory on citrus root enzyme activities

Statistical analyses for interaction between cultivar and treatment indicated that the interaction was significant for chitinase only (Tab. 1). Therefore only for chitinase are uninfested and infested activities given separately in Tab. 1. Because there was no significant interaction for the remaining rootstock cultivars, the uninfested and infested values were analysed as a single group. Chitinase activities increased in several rootstock varieties after larval feeding; however, the effect of larval feeding was significant only in Swingle, sour orange and *C. macrophylla*. There were significant differences in chitinase activities between rootstock cultivars as well; the cultivar differences were not always the same when uninfested and infested groups were compared (Tab. 1). For example, chitinase activity between Cleopatra and pummelo 4N is

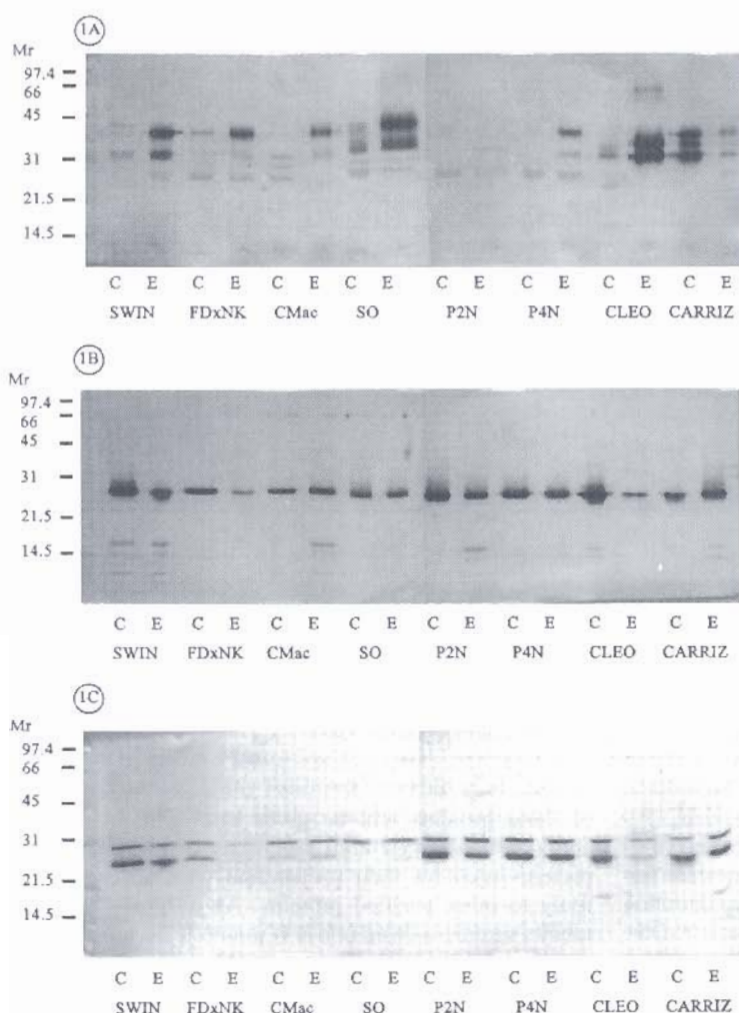


Fig. 1. Immunostained western blots of root extracts from citrus rootstock cultivars using antibodies from: A, Basic class I potato leaf chitinase; B, a basic citrus chitinase (BCVC) of M_r 24 000; C, a basic citrus chitinase (BCLVC) of M_r 28 000 that exhibits high lysozyme activity. The left-hand column indicates M_r ($\times 1000$). Legends: C, control; E, experimental; SWIN, Swingle; FDxNK, Flying Dragon \times Nakon; CMac, *Citrus macrophylla*; SO, sour orange; P2N, pummelo 2N; P4N, pummelo 4N; CLEO, Cleopatra; CARRIZ, Carrizzo.

not significantly different in the uninfested plants but is significantly different in the infested group. The opposite is true with Cleopatra and *C. macrophylla*. Pummelo 4N exhibited the highest activity in the control group while that of Cleopatra was highest in the experimental group. Induction of chitinase was indicated for *C. macrophylla* (4.4-fold), Swingle (1.4-fold) and sour orange (1.4-fold). Although not statistically significant, Flying Dragon \times Nakon and Cleopatra also showed increases in chitinase activities at 1.2- and 1.9-fold, respectively.

The interaction between treatment and cultivar was not significant for chitosanase (Tab. 1). Herbivory (infested) was highly significant and is shown as a decrease in chitosanase activity in Tab. 1. There were highly significant differences in chitosanase activity between cultivars, with pummelo 4N and 2N having the highest activities.

Neither treatment nor the interaction between treatment and cultivar was found to be significant for rootstock peroxidases (Tab. 1). However, differences in peroxidase activities between cultivars were highly signif-

icant. The activities ranged from 247 (pummelo 4N) down to 61 (Cleopatra) $\Delta A_{510} \text{ mg}^{-1} \text{ protein min}^{-1}$.

β -1,3-Glucanase and lysozyme activities were significantly affected by larval feeding, but there was neither significant difference among cultivars nor any significant interaction observed between treatment and cultivar (Tab. 1). Herbivory appears to reduce β -1,3-glucanase and lysozyme activities in citrus rootstocks by approximately 32 and 38%, respectively.

Immunological detection

Immunological detection was useful in demonstrating either the induction or decrease of enzymes in the various rootstocks tested. Figure 1 gives the results of western blots using three different antisera for chitinases. Figure 1A is a blot made utilizing an antibody to a basic class I chitinase from potato leaf. This antibody cross-reacts with citrus chitinases in the 26 000 to 42 000 M_r range (Osswald et al. 1993, 1994). Chitinases were induced in six of the eight rootstock cultivars tested (Swingle, Flying

Dragon \times Nakon, P4N and Cleopatra). The M_r s from ca 26 000 to 42 000 in the western blots of citrus rootstocks using the BCLVC preparation (Fig. 1B) are the bands seen in the western blots shown in Fig. 1A. Some cultivars (Carrizzo using the BCLVC preparation) showed different levels of chitinase activity (Fig. 1A, B) than those mentioned above.

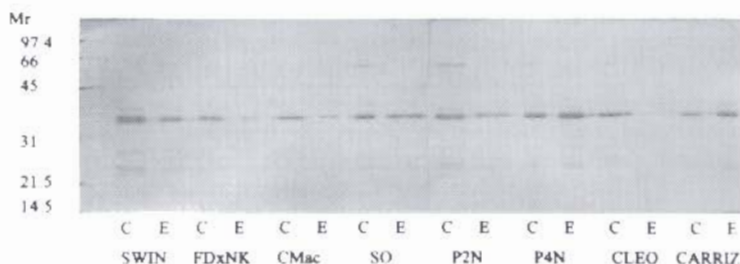
Immunostaining of citrus rootstocks against a β -1,3-glucanase in citrus are given in Tab. 1. There were few or no differences in activity in infested rootstocks.

Chitinase/chitosanase

Because previous studies have shown that chitinases and chitosanase inhibit lysozyme activity, we determined if chitinase activity with Schales colorimetric assay was utilized to obtain results of these assays. Ivory resulted in all substrates and activity for all cultivars. Significant differences between cultivars were only. The la-

Fig. 3. Immunostained western blots of root extracts from citrus rootstock cultivars using antibody against a basic citrus chitinase (BCLVC) of M_r 28 000. The left-hand column indicates M_r ($\times 1000$). Legend: C, control; E, experimental; SWIN, Swingle; FDxNK, Flying Dragon \times Nakon; SO, sour orange; P2N, pummelo 2N; P4N, pummelo 4N; CLEO, Cleopatra; CARRIZ, Carrizzo.

Fig. 2. Immunostained western blots of root extracts from citrus rootstock cultivars using an antibody (BGVC) against a basic citrus β -1,3-glucanase of M_r 32 000. The left-hand column indicates M_r ($\times 1000$). Legends: C, control; E, experimental; SWIN, Swingle; FDxNK, Flying Dragon \times Nakon; CMac, *Citrus macrophylla*; SO, sour orange; P2N, pummelo 2N; P4N, pummelo 4N; CLEO, Cleopatra; CARRIZ, Carrizo.



Dragon \times Nakon, *C. macrophylla*, sour orange, pummelo 4N and Cleopatra). The chitinases that were induced had M_r s from ca 26 000 to 41 700. Figure 1B and C show western blots probed with antibodies (BCVC and BCLVC) prepared against citrus chitinases. The protein bands seen in Fig. 1B were about M_r 25 000 and the two bands shown in Fig. 1C were about M_r 24 000 and 27 000. Some induction of chitinases was indicated for Carrizo using the BCVC antibody. The remaining western blots indicated either no induction or diminished levels of chitinases present in infested roots. Comparison of Fig. 1A, B and C indicates that the antibodies used here differentiate chitinases in the various M_r ranges mentioned above.

Immunostaining of western blots with antibodies against a β -1,3-glucanase and three peroxidases from citrus are given in Figs 2 and 3, respectively. There were few or no differences between controls and weevil-infested rootstocks.

Chitinase/chitosanase substrate specificities

Because previous studies (Osswald et al. 1993, 1994) have shown that citrus tissues contain a large number of chitinases and chitinase/chitosanases which may also exhibit lysozyme activities, we felt that it would be useful to determine if the rootstocks exhibited differences in hydrolysis with differentially acetylated substrates. The Schales colorimetric method for reducing end groups was utilized to obtain results that were comparable. The results of these experiments are presented in Tab. 2. Herbivory resulted in statistically significant differences for all substrates tested. Larval feeding increased hydrolytic activity for all substrates except the 59% acetylated chitosan. Significant differences in hydrolytic activity among cultivars were observed for the 59% acetylated chitosan only. The largest amount of activity observed was for

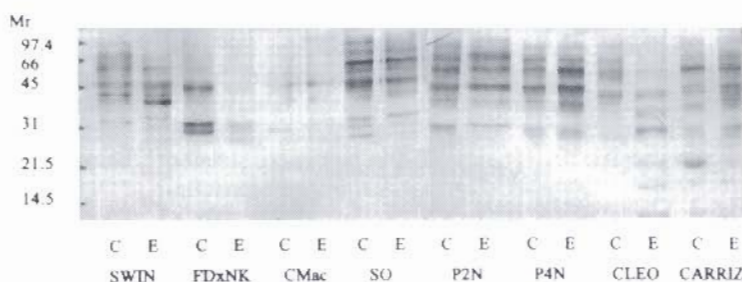
Swingle and the lowest was that of Carrizo using the 59% acetylated chitosan.

Overall, the highest activities were observed with 100% acetylated chitosan. The activities decreased with decreasing substrate acetylation. All of the rootstock enzyme preparations, whether from infested or uninfested roots, were capable of hydrolyzing 0% acetylated chitosan (Tab. 2). Activities ranged from 8 (Carrizo) to 143 (sour orange) nmol reducing equivalents GlcNAc mg^{-1} protein min^{-1} . This is an activity range of approximately 18-fold. Chitinase/chitosanase activities were plotted against the acetylation content of the chitosans tested in Fig. 4. There is a linear relationship between activity and the degree of acetylation of the substrate. Linear regression analyses yielded correlation coefficients of 0.995 for the uninfested and 0.979 for the infested rootstocks.

Activity against *Diaprepes abbreviatus* peritrophic membranes

Protein extracts from all of the cultivars were capable of digesting *D. abbreviatus* peritrophic membrane suspensions (Tab. 3). Significant differences in the ability to digest peritrophic membranes were observed between rootstocks as observed above for other substrates. Treatment alone was not significant; however, the interaction between treatment and cultivar was. In the uninfested group there was an apparent 2.5-fold difference between the cultivars with the highest activities (Cleopatra and Swingle) and the lowest (Carrizo). In the infested group, Cleopatra again had the highest activity closely followed by pummelo 4N and sour orange. There was an apparent 2.8-fold difference in activity between the highest and lowest activity cultivars in the experimental group. Only pummelo 4N and Swingle exhibited significant differences between uninfested and infested groups; the former had an increase in activity, while the latter had a decrease.

Fig. 3. Immunostained western blots of root extracts from citrus rootstock cultivars using an antibody against three basic citrus peroxidase of M_r 40 000. The left-hand column indicates M_r ($\times 1000$). Legends: C, control; E, experimental; SWIN, Swingle; FDxNK, Flying Dragon \times Nakon; CMac, *Citrus macrophylla*; SO, sour orange; P2N, pummelo 2N; P4N, pummelo 4N; CLEO, Cleopatra; CARRIZ, Carrizo.



Tab. 2. Chitin/chitosan specificity of rootstock extracts in regard to percentage acetylation. Assays were performed using the Schales procedure described in Materials and methods. Like letters within columns indicate no significant difference in activities. Mean separation within columns by Duncan's multiple range test at $P = 0.05$, $n = 3-7$. NS, not significant; **, significant at $P < 0.01$.

Rootstock cultivar (CV)	Substrate acetylation						
	100%	59%	30%	18%	13.8%	5%	0%
	Activity (nmol GlcNAc reducing equiv. mg ⁻¹ protein min ⁻¹)						
Carrizo	115	48d	45	14	11	12	8
Cleopatra	522	176bc	124	111	102	89	55
Pummelo 4N	234	156c	138	68	80	70	40
Pummelo 2N	251	205abc	193	192	131	126	102
<i>Citrus macrophylla</i>	203	169c	68	64	102	72	60
Sour orange	339	188bc	122	109	155	152	143
Flying Dragon × Nakon	148	218ab	202	146	119	111	111
Swingle	330	251a	193	165	140	136	133
Treatment (TRT)							
Uninfested	254	188	121	104	101	94	77
Infested	282	164	149	113	108	97	86
Source							
CV	NS	**	NS	NS	NS	NS	NS
TRT	**	**	**	**	**	**	**
CV × TRT	NS	NS	NS	NS	NS	NS	NS

The pH of larval midguts ranged from 4.5 to 5. This is well within the optimal pH range of citrus chitinases (Osswald et al. 1994).

Discussion

It is well known that plants respond to herbivore and disease stresses in many ways. Known responses include production of defensive chemicals and proteins (Harborne 1979, Ryan 1987, Carr and Klessig 1989, Fischer

et al. 1990, Flach et al. 1992, Gershenozon 1994). Ligands, flavonoids, isoflavonoids, alkaloids, terpenoids and

Tab. 3. Digestion of peritrophic membranes from *Diaprepes abbreviatus* larvae by citrus root extracts. Like letters within columns indicate no significant difference in activities. Mean separation within columns by Duncan's multiple range test at $P = 0.05$. Shaded areas indicate significant differences between columns ($P < 0.05$). $n = 3-7$. NS, not significant; **, significant at $P < 0.01$.

Rootstock cultivar (CV)	Enzyme activity (nmol GlcNAc mg ⁻¹ protein min ⁻¹)	
	Uninfested	Infested
Cleopatra	102a	114a
Swingle	104a	79.8ab
Sour orange	66.8bc	103.5ab
Pummelo 2N	68.8bc	85.3ab
Pummelo 4N	63.8bc	105.3ab
<i>Citrus macrophylla</i>	79.7ab	38c
Flying Dragon × Nakon	72.3abc	71.8bc
Carrizo	40.8c	39.5c
Treatment (TRT)		
Uninfested	NS	
Infested	NS	
Source		
CV	**	
TRT	NS	
CV × TRT	**	

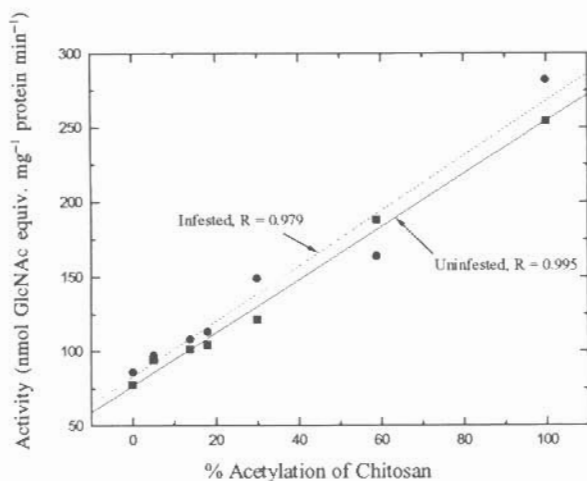


Fig. 4. Chitinase/chitosanase activities of uninfested and infested citrus rootstocks plotted against the percent acetylation content of chitosan substrates. Linear regression for the uninfested (solid) and infested (dash) analyses are shown.

coumarins are chitinases, chitinases are exar responses can wounding and curic chloride and cell-free e

Our studies induced in the *abbreviatus* larvae with immunoblotting PAGE. In particular, an antibody that reacted with a protein of 42 000 M_r to 26 000 M_r (Fig. 1A). Blots made with chitinase of *abbreviatus* (Fig. 1B). With 4N, the induction of stress, measured with increased protein did not show surprising the immunoblots (Fig. 1C). et al. (1994) have shown acidic chitinase callus, and other chitinases (uninfested) to total protein. Certainly, the related protein since they are

Chitosanase enzyme activity feeding had a 1). None of them were as decreases with β -1,3-glucanase indicate a downfed rootstock

Significant differences are evident in different rootstocks have, for example, which significant differences may have resulted in enzyme levels. The result would be different in also be true

Plant chitinase activities as shown by Oswald et al.

coumarins are examples of defensive chemicals, while chitinases, chitosanases, peroxidases and β -1,3-glucanases are examples of defensive proteins. Many of the responses can be triggered in a variety of plants by wounding and by applications of chemicals such as mercuric chloride, ethylene, salicylic acid, oligosaccharides and cell-free extracts of pathogens.

Our studies indicate that at least three chitinases are induced in the roots of citrus rootstock cultivars by *D. abbreviatus* larval feeding. The bulk of the evidence lies with immunological detection of chitinases after SDS-PAGE. In particular, the class I potato leaf chitinase antibody that cross-reacts with citrus chitinases of 26 000 to 42 000 M_r showed the induction of chitinases from ca M_r 26 000 to 41 700 in Cleopatra, Swingle, pummelo 4N, *C. macrophylla* and Flying Dragon \times Nakon (Fig. 1A). Blots made with the BCVC antibody indicated that a chitinase of about M_r 24 000 in Carrizo was also induced (Fig. 1B). With the exception of the results with pummelo 4N, the induction of chitinases after the larval feeding stress, measured immunochemically, roughly correlated with increased chitinase activity (Tab. 1). Those cultivars not showing any induction of antigenically similar protein did not show any increase in chitinase activity. It was surprising that there was any correlation between the immunoblots and activity measurements since Osswald et al. (1994) have reported that there are as many as eight acidic chitinases present in cv. Valencia sweet orange callus, and other studies indicate at least that many basic chitinases (unpublished data). At this point we do not know what the percent contribution of individual chitinases to total activity is for any of the cultivars examined. Certainly, these enzymes can be called pathogenesis-related proteins as defined by van Loon et al. (1994), since they are induced in pathological conditions.

Chitosanase, peroxidase, β -1,3-glucanase and lysozyme activities were also measured to determine if insect feeding had any effect on these citrus root enzymes (Tab. 1). None of these enzymes showed any induction when they were assayed by immunoblots; however, significant decreases were observed in activities for chitosanase, β -1,3-glucanase and lysozyme. Generally, the data indicate a down regulation of these enzymes for the infested rootstocks.

Significant differences in enzyme activities among cultivars are evident from the data given in Tab. 1. Significant differences in chitinase activities change after the rootstocks have been infested with weevil larvae. For example, where two cultivars may not have exhibited a significant difference in the uninfested group one of them may have responded to insect feeding by elevating the enzyme level while the other showed no change. The result would be that the two cultivars are significantly different in the experimental groups. The reverse would also be true.

Plant chitinases often have chitosanase and lysozyme activities associated with them (Flach et al. 1992, Osswald et al. 1993, 1994). Figure 1C shows proteins de-

tected using the BCLVC antibody. The basic chitinase from which this antibody was prepared is a chitinase of approximately M_r 28 000 that possesses a large amount of lysozyme activity; thus far, only one of the chitinases purified from cv. Valencia callus tissues has large amounts of lysozyme activity and this antibody is specific for this protein. Therefore, the two bands shown for each of the cultivars in Fig. 1C are chitinase/lysozymes of ca M_r 24 000 and 27 000; the lysozyme activities have been confirmed by purification of the M_r 24 000 enzyme (unpublished data).

Aiba (1992a,b, 1993) has shown that microbial lysozyme and chitinase activities are affected by the amount and distribution of the acetyl groups occurring along the polysaccharide chain. He has shown that microbial chitinases have low activity towards chitosans with 10–30% acetyl content and suggested that this is due to the fact that chitinases recognize only the GlcNAc residues in the chitosan chain. The data presented here (Fig. 4) and previously (Osswald 1993, 1994), using substrates with varying degrees of acetylation, corroborate Aiba's conclusions. Further, the results presented in Tab. 2 demonstrate that citrus root hydrolases have the ability to digest and/or defend the plant against a wide range of pests that may have varying amounts of chitin/chitosan.

Although numerous reports are available concerning the relationships of insects and pathogens to plant defensive chemicals and pathogens to defensive proteins, few of these relate insects to plant defensive proteins. There is one report of a chitinase from seeds of Job's tears that affected insects, but the effect was an inhibition of insect α -amylase (Ary et al. 1989). There is little possibility that plant chitinases would affect the exoskeleton of insects since this structure is covered with a waxy layer that prevents penetration of water soluble materials. However, there is good evidence to suggest that ingested plant enzymes interact with the peritrophic membrane that lines the midgut region of insects. The membrane is composed of chitin, proteins and lipids and is permeable to materials less than 7–8 nm in diameter and to proteins less than M_r 100 000 (Richards and Richards 1977, Appel 1994). The data presented here indicate that citrus root chitinases are capable of digesting the peritrophic membrane in larvae of *D. abbreviatus* (Tab. 3) and that the pH of the midgut is within a pH range for maximal activity. Shapiro and Gottwald (1995) report that the rootstocks yielding the least *D. abbreviatus* larval weight gains were Cleopatra < Swingle < *C. macrophylla*. These three cultivars also exhibit the highest activities for peritrophic membrane digestion in the control groups. Based on damage indexes, Swingle and the two pummelo \times *P. trifoliata* crosses (diploid and tetraploid) have the greatest potential for resistance. At this time it is not possible to say that resistance and larval weight gains are influenced by chitinases.

With the exception of a single report on gut chitin synthase (Ward et al. 1991), there is no literature available that relates to the peritrophic membrane in *D. abbre-*

of plants to give them elevated defensive protein levels. Of course, one could also genetically engineer the entomopathogen to produce chitinases to facilitate infection; this has been accomplished using insect chitinases (Gopalakrishnan et al. 1994).

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