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PLANT DEFENSIVE PROTEINS AND DISEASE RESISTANCE IN CITRUS

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Abstract. **Tissue cultures of sweet orange embryogenic callus and suspension cells can be induced to produce high levels of chitinase and β-1,3-glucanase by altering the carbon source in the culture medium. To determine if these enzymes inhibit disease-causing fungi, *Penicillium digitatum*, a postharvest pathogen, was inoculated onto Petri dishes with sweet orange callus that had been grown on media containing either sucrose or glycerol as the carbon source. Medium that contained sucrose induced high levels of chitinase and β-1,3-glucanase in the callus, which remained undifferentiated. Medium that contained glycerol repressed the production of these enzymes in the callus, which differentiated into embryos. Fungal growth was significantly inhibited by the callus producing high levels of enzyme for up to 6 weeks. Chitinases and β-1,3-glucanases have been identified in the roots, leaves, and flavedo of 'Marsh' grapefruit, and in the leaves and blossoms of navel orange. These enzymes might be useful in developing new economical and environmentally friendly disease control measures.**

Plants produce a wide variety of chemicals that are noxious or inhibitory to pathogens, insects, nematodes, and herbivores. These chemicals include secondary products, alkaloids, terpenes, phytoalexins, lignins, tannins, phenols, and melanins, as well as proteins, peptides, and lectins. Some of these defensive compounds are found constitutively in the plant's tissues or may be induced in response to pathogen or insect attack. Except for the proteins, these compounds are the end-products of multiple biochemical reactions involving many enzymes. Proteins are more amenable to genetic manipulation and study because they are the direct products of gene expression.

Proteins induced in the plant by pathogen attack are called pathogenesis-related proteins (PR) (Antoniw et al., 1980) and have been grouped into 11 families by Van Loon et al. (1994). PR proteins are classified by DNA or amino acid sequence similarity, immunological relationships, and biological activity. Current PR protein families include chitinases, β-1,3-glucanases, peroxidases, proteinase inhibitors, ribonuclease-like proteins, and thaumatin-like proteins.

The chitinases and β-1,3-glucanases exhibit antifungal activity by attacking the chitin and β-1,3-glucan components in fungal cell walls. Roberts and Selitrennikoff (1986) isolated a chitinase from barley and found that it inhibited hyphal growth of the fungi *Trichoderma reesei, Alternaria alternaria, Phycomyces blakesleeanus,* and *Neurospora crassa.* Mauch et al. (1988) isolated a chitinase and β-1,3-glucanase from pea and tested these enzymes individually and in combination against eight fungi. The pea chitinase inhibited hyphal growth of only *T. viride* while the β-1,3-glucanase inhibited only *Fusarium solani* f.sp. *pisi.* However, when they were combined the growth of all eight fungi were inhibited. These experiments demonstrated that chitinases and β-1,3glucanases act synergistically and are more effective fungal inhibitors together than either enzyme individually. Zhu et al. (1994) also demonstrated the synergistic action of chitinases and β-1,3-glucanases, but used tobacco plants transgenic for both enzymes individually and together. Plants transgenic for both genes exhibited greater resistance to frogeye disease (*Cercospora nicotianae*) than plants transgenic for either gene individually.

Chitinases, chitosanases, and β-1,3-glucanases have been isolated and partially characterized in citrus callus cultures (Osswald et al., 1993, 1994). Multiple forms of each enzyme were identified. It is not known which forms, if any, are induced by pathogen activities. The purpose of this study was to determine if chitinases and β-1,3-glucanases produced by citrus callus cultures exhibit antifungal activity.

Material and Methods

Citrus cell lines

A 4-year-old embryogenic callus line (H89) of *Citrus sinensis* (L.) Osbeck 'Hamlin' was initiated and maintained as a callus and suspension culture as previously described (Niedz, 1993). For enzyme analysis and fungal inhibition studies H89 was grown in a suspension culture in the same medium as described (Niedz, 1993), but with 2% glycerol substituted for the 5% sucrose to initiate embryogenesis.

Protein extraction from plant tissues

Ten-day-old suspension cultured cells were first frozen in liquid nitrogen and powdered in an Omni-mixer cell homogenizer (Omni International, Gainsville, Va). The powdered cells were resuspended at 10 g/100 ml of ice-cold homogenizing buffer (0.1 M phosphate, pH 7.4, 15 mM β-mercaptoethanol, 1 mM EDTA, 1 mM dithiothreitol). The suspension was homogenized for 1 min with a Polytron (Kinematica GmbH, Switzerland) equipped with a PTG 36-50 generator. The resulting homogenate was centrifuged at 15,000xg for 20 min to remove cell debris. The supernatant was gently decanted and recentrifuged at 100,000xg at 4 C for 1 hr. This supernatant was stored at -20 C until β-1,3-glucanase, chitinase, and chitosanase activities were measured.

Suspension culture medium was freeze-dried, resuspended in buffer (50 ml buffer/1000 ml medium), dialyzed against water in a 15,000 Dalton cutoff membrane, freeze-dried, and stored at -80 C until used.

Roots and leaves were collected from 4-month-old, greenhouse-grown *C. paradisi* Macfayden 'Marsh' grapefruit plants. The flavedo was obtained from fruit collected from field-grown trees. Leaves and blossoms were also collected from field-grown navel orange trees (*C. sinensis* (L.) Osbeck). These tissues were prepared for hydrolytic enzyme analysis as previously described (Mayer et al., 1994).

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Protein determination followed the method of Bradford (1976). A standard curve in the range of 0-20 µg was constructed using bovine serum albumin as the standard.

Chitinase assay

Chitinase activity was measured as previously reported using blue chitin (Saborowski et al., 1993). Reaction times were 10 min at 45 C. Reaction mixtures contained 100 µl of blue chitin suspension, 0.1-0.5 µg protein and enough 0.2 M sodium acetate buffer (pH 5) to bring the volume to 0.5 ml in microcentrifuge tubes. Reactions were terminated using 100 µl of chilled 1 N HCl. The reactions mixtures were centrifuged at 10,000 g for 2 min and the absorbance of the supernatants recorded at 550 nm. Activities are reported as ΔA_{550}·min^{-1}·mg protein^{-1}.

β-1,3-Glucanase assay

β-1,3-Glucanase activity was determined by the method of Abeles and Forrence (1970) using laminarin as the substrate and a dinitrosalicylic acid reagent to measure the reducing sugars. β-1,3-glucanase activity is reported as the release of µg glucose(Glc)·min^{-1}·mg protein^{-1}.

Fungal growth and inoculation

A monosporic culture of *P. digitatum* was maintained in the dark at 25 C on potato dextrose agar (Dhingra and Sinclair, 1985). The fungus was grown in 60 × 15 mm Petri dishes until the surface of the plate was covered with mycelium. Agar plugs (9 mm) were extracted aseptically with a #5 cork borer and used to inoculate culture plates containing citrus callus.

About 1 g of sucrose- or glycerol-grown suspension cells were collected and inoculated onto culture medium supplemented with 2% glycerol. The cells were placed near one side of the culture dish. A single agar plug containing fungal mycelium was placed on the opposite side of each dish. Plates containing both citrus cells and *P. digitatum* were incubated in the dark at 27 C for 6 weeks.

Results and Discussion

H89 cells were grown in 2 suspension culture media that varied only in the carbon source used, i.e. either glycerol or sucrose. The H89 cells grown in the glycerol-based medium differentiated into globular-stage embryos in 6 weeks. Differentiation occurred rapidly as embryo primordia were visible within 6 days upon transfer to the glycerol-based medium The type and concentration of carbohydrate used as a carbon source has a significant influence on initiating somatic embryogenesis in citrus nucellar-derived tissue cultures (Kochba et al., 1982), with glycerol being the most effective (Ben-Hayyim and Neumann, 1983). No embryogenesis was observed in the sucrose-based medium that was used to maintain the H89 cell line. This resulted in 2 cell lines - an embryo suspension culture maintained in a glycerol-based medium and an undifferentiated cell suspension culture maintained in a sucrose-based medium.

Chitinase and β-1,3-glucanase specific activity were measured in the callus cells and embryos and the culture medium of each (Table 1), and in the roots, leaves, and flavedo of 'Marsh' grapefruit and new and old leaves and blossoms of navel orange (Table 2). High levels of both enzymes (251 ΔA_{550}·min^{-1}·mg protein^{-1} for chitinase and 699 µg glc·min^{-1}·mg protein^{-1} of β-1,3-glucanase) were measured in the medium of the sucrose grown cells. Conversely, low levels of both enzymes (14 ΔA_{550}·min^{-1}·mg protein^{-1} of chitinase and 4 µg glc·min^{-1}·mg protein^{-1} of β-1,3-glucanase) were measured in the medium of the glycerol grown embryos. There was no difference in β-1,3-glucanase activity between the sucrose grown cells or the glycerol grown embryos. Chitinase activity was significantly higher (694 ΔA_{550}·min^{-1}·mg protein^{-1}) in the glycerol grown embryos than in sucrose grown cells (47 ΔA_{550}·min^{-1}·mg protein^{-1}).

Table 1. Specific activity of chitinase and β-1,3-glucanase in 'Hamlin' sweet orange cell and embryo cultures (H89). Data are the mean ± SE.

Samples	Chitinase (ΔA_{550}·min^{-1}·mg protein^{-1})		β-1,3-Glucanase (µg glc·min^{-1}·mg protein^{-1})	
	Glycerol	Sucrose	Glycerol	Sucrose
Medium	14 ± 3	251 ± 31[A]	4 ± 3	699 ± 365[A]
Cells/Embryos	694 ± 108**	47 ± 14	84 ± 26	93 ± 8

[A]P<0.01 by Student's *t*-test.

The extremely low levels of chitinase and β-1,3-glucanase in the medium of glycerol-grown embryos compared to levels in the medium of sucrose-grown cells made it possible to test the possible antifungal activity of these 2 enzymes. A significant inhibition of fungal mycelial growth would be expected to occur on culture medium containing high levels of chitinase and β-1,3-glucanase. *P. digitatum*, a fungal pathogen that attacks fruit in postharvest situations, was therefore grown in the presence of the 2 sweet orange cell lines that differed markedly in the amounts of chitinase and β-1,3-glucanase released into their respective culture media. Agar plugs containing *P. digitatum* mycelium were placed onto culture medium (MS salts and vitamins + 2% glycerol) with either low chitinase and low β-1,3-glucanase producing, glycerol-grown globular embryos or high chitinase and high β-1,3-glucanase producing sucrose-grown cells. After the plates were incubated in the dark for 6 weeks the growth of the fungus was evaluated. A significant reduction in hyphal growth was observed in the plates where the fungus was co-cultured in the presence of the high chitinase and high β-1,3-glucanase producing cells (Figure 1B). No reduction in growth was observed when the fungus was co-cultured with the low chitinase and low β-1,3-glucanaseproducing embryos (Figure 1A). Fungal growth remained inhihited for up to 6 weeks, after which the plates were discarded.

Table 2. Specific activity of chitinase and b-1,3-glucanase in 'Marsh' grapefruit roots, leaves, and flavedo. Data are the mean ± SE.

Samples	Chitinase (ΔA_{550}·min^{-1}·mg protein^{-1})	β-1,3-Glucanase (µg glc·min^{-1}·mg protein^{-1})
'Marsh' grapefruit		
roots	8.4 ± 2.0	901.5 ± 69.5
leaves	16.2 ± 0.6	14,820 ± 67.0
flavedo	14.9 ± 1.8	9,294 ± 1,765
Navel orange		
old leaves	4.4 ± 2.1	28.6 ± 20.7
new leaves	1.9 ± 0.9	1.0 ± 0.3
blossom	1.7 ± 0.9	1.4 ± 0.6

Figure 1. Six week old cultures of *P. digitatum* on MS salts and vitamins + 2% glycerol culture medium with H89 cells or embryos. **A)** Low chitinase and β-1,3-glucanase producing H89 cell line. Cells grown on glycerol medium for 6 weeks to induce embryo formation before transfer to the same medium (i.e. MS salts and vitamins + 2% glycerol). **B)** High chitinase and β-1,3-glucanase producing H89 cell line. Cells grown on sucrose before transfer to MS salts and vitamins + 2% glycerol.

The growth inhibition observed in the presence of the high chitinase and β-1,3-glucanase producing cells is consistent with the known effects of these enzymes on fungal growth (Mauch et al., 1988). However, this experiment does not eliminate the possibility that another factor(s) was responsible for the inhibition of fungal growth. There may be significant differences between H89 cells grown on sucrose and H89 embryos grown on glycerol other than in levels of chitinase and β-1,3-glucanase activity. For example, the production of volatiles, other secondary products, or pH gradient differences might also inhibit fungal growth. Fungal inhibition assays using the purified hydrolases would be necessary to demonstrate the specific contribution of these enzymes.

These results strongly suggest the possibility of improving disease resistance by raising the endogenous chitinase and β-1,3-glucanase levels in citrus plants. We examined chitinase and β-1,3-glucanase levels in plant parts of 'Marsh' grapefruit and navel orange to establish that the enzymes were present in a commercial citrus variety (Table 2). β-1,3-Glucanase activity was 14,820 and 9,294,μg glc·min^{-1}·mg^{-1} protein as measured in the leaves and flavedo of 'Marsh' grapefruit, respectively. Unlike β-1,3-glucanase, chitinase activity was very low (< 20 ΔA_{550}·min^{-1}·mg protein^{-1}) in the three tissues sampled from 'Marsh' grapefruit. Chitinase and β-1,3-glucanase levels were also very low in the old and new leaves and blossoms of navel orange.

The glycerol-grown embryo cell line may be useful for the *in vitro* screening of compounds that elicit the production of these antifungal compounds in citrus. Tissue culture-derived embryos would be expected to respond more like the whole plant than nondifferentiated cells. Such screening would be both more cost effective and efficient than field screening. Also, this strategy of improving a tree's disease resistance should be environmentally friendly as eliciting compounds are generally nontoxic.

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EFFECTS OF SEAWEED SPRAYS ON CITRUS FRUIT PRODUCTION[1]

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Abstract. **The effect of seaweed sprays were evaluated on orange and grapefruit trees. Trees sprayed with seaweed extracts increased fruit production 10-25% over that of the control. A combination of prebloom, postbloom, and summer sprays appeared to give greater response in fruit production than other timing treatments. Trees sprayed with seaweed extracts had fewer premature fruit drop than the control. Fruit size from seaweed treated trees were equal or larger than fruit from the control trees, despite the fact that higher fruit production is usually accompanied by smaller sized fruit. In general, rind color break occurred earlier in fruit from the seaweed treated trees than fruit from the control trees.**

Seaweed-based products have been used on agricultural crops in the United States as an organic fertilizer supplement. Increased yield of poato and corn with seaweed sprays have been reported (Chapman and Chapman, 1980). Increased shelf life of peaches have been reported from trees sprayed with seaweed extracts (Skelton and Senn, 1966). In Florida trials, seaweed sprays increased total yield as well as early maturity of tomatoes (Csizinszky, 1984).

In citrus, under controlled greenhouse conditions, seaweed extract sprayed at a dilution of 1:25 parts of extract and water corrected Mg, Mn, Zn, and B deficiency symptoms (Aitken and Senn, 1964). A 3-yr field study of seaweed extract on young Sunburst tangerines showed a 30% increase in fruit production (Koo, 1988). Studies were initiated in 1991 to study the effects of seaweed sprays on oranges and grapefruit. This paper summarizes the data from 3 experiments over a 2- to 3-yr period.

Materials and Methods

Three experiments were initiated in central (Polk County), east coast (Martin County) and southwest (Hendry County) regions of Florida to study the effects of seaweed sprays on oranges and grapefruit growing under different production conditions. Experiment 1 was conducted on Washington Navel orange [*Citrus sinensis* (L) Osbeck] and Ruby Red grapefruit (*Citrus paradisi* Macf) on sour orange (*Citrus aurantium*) rootstock near Frostproof, Florida. The trees were planted in 1980 on single beds at a 17½ x 30 foot spacing. Experiment 2 was located near LaBelle, Florida using Ruby Red grapefruit (*Citrus paradisi* Macf) on Carrizo citrange [*Poncirus trifoliata* (L) Raf x *C. sinensis*] rootstock planted on double beds. The trees were planted in 1987 at 15 x 20 foot spacing. Experiment 3 was located near Indiantown, Florida using Hamlin and Pineapple oranges. Hamlin orange [*Citrus sinensis* (L) Osbeck] on Cleopatra mandarin (*C. reticulata* Blanco) rootstock were planted in 1980 on double beds with a tree spacing of 15 x 22½ feet. Pineapple orange [*Citrus sinensis* (L) Osbeck] on rough lemon (*Citrus jambhiri*) rootstock were planted on single beds in 1962 using a 20 x 25 foot spacing. Treatments of all 3 experiments are listed in Table 1 together with the mineral compositions of the seaweed extracts. Experimental design in all 3 experiments was complete randomized block. Each treatment was replicated 4 times using 6-tree plots. Experiments 1 and 2 were designed to obtain information on the best timing to apply seaweed extracts. Experiment 3 was a comparison of seaweed sprays and gibberellic acid as foliar sprays.

Fruit production data were collected at the time of fruit harvest. Fruit samples were collected 1-2 weeks before fruit was harvested to measure quality. Premature fruit drops were counted 3-4 times a year at 5-6 week intervals. Only the freshly dropped fruit were counted. Fruit size and fruit color were measured both in the laboratory and in the field. Leaf samples were collected to evaluate the ability of seaweed extracts to supply plant nutrients. Four- to five-month-old spring flush leaves from nonfruiting terminals were collected in 1991 and 1992 for this purpose. Data from all 3 experiments were analyzed by analysis of variance on an annual basis. The multiyear average data were not analyzed. They are listed only to show trends if any.

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