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SYSTEMIC RESISTANCE AND PEROXIDASE ACTIVITY IN CUCUMBER IN RESPONSE TO SELECTED AGENTS

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ABSTRACT

SYSTEMIC RESISTANCE AND PEROXIDASE ACTIVITY IN CUCUMBER IN RESPONSE TO SELECTED AGENTS

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Resistance was induced in cucumber plants by treatment with selected agents. The following were applied, in one application, to different plants, one or three days before a challenging inoculation: D-alanine, DL-amino-n-butyric acid, 6-benzylaminopurine, silicic acid, acetylsalicylic acid, 2,6-dichloro-isonicotinic acid (INA), or *Pseudomonas syringae* pv. *syringae* (Pss). The first five of these induced local resistance; INA and Pss induced both local and systemic resistance. INA and Pss treatment led to an increase in soluble, ionically wall-bound and covalently wall-bound peroxidase activity. Particularly noteworthy were increases in activity of two fast moving isozymes of soluble peroxidase (A_2, A_3) and two slow moving isozymes of ionically wall-bound peroxidase (C_1, C_2). This expression of different peroxidase species in different cellular locations suggests that individual peroxidase isozymes may perform specific functions, such as H_2O_2 production and free radical formation during lignin biosynthesis.

For my husband Tim and son David

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GENERAL INTRODUCTION

The mechanisms by which plants defend themselves against pathogen infections have been studied since the turn of the century. Resistance development in response to pathogen infection is a natural phenomenon in plants first recognized in 1901 by Ray and Beauverie, both of whom worked with *Botrytis cinerea* (Beauverie 1901, cited in Kesssmann 1994). This phenomenon has since been studied using different plant-pathogen interactions. Plants which are normally susceptible to a particular pathogen can often be made resistant by a pre-infection with a pathogen which causes localized necrosis. In 1933, Chester reviewed research related to this phenomenon and determined that it " may play an important role in the preservation of plants in nature" (Chester 1933, cited in Kessmann 1994). Resistance so induced showed a systemic effect against subsequent infections by pathogens, including fungi, bacteria, and viruses. Resistance that develops in the infected leaf was termed localized induced resistance (Ross 1961a) and resistance in distal, uninfected parts of the plant was termed systemic induced resistance (Ross 1961b).

Induced resistance has been very well studied in some plants. Tobacco reacting hypersensitively to tobacco mosaic virus (TMV) became resistant to various other viruses in untreated leaves (Ross 1961a ,1961b) and also showed systemic resistance to

blue mold (Ye 1992). *Arabidopsis thaliana* showed systemic resistance to a virulent race of *Peronospora parasitica* after a predisposing infection with a pathogenic isolate of *Fusarium oxysporum* (Mauch-Mani and Slusarenko 1994). The necrotizing pathogen turnip crinkle virus (TCV) can also induce systemic resistance in *Arabidopsis* against itself, and against bacterial *Pseudomonas syringae* (Ukness 1993).

In the 1970's, Kuc' and others presented the first paper on induced resistance in cucumber (Hammerschmidt 1997). They demonstrated that inoculation of cucumber leaves with *Colletotrichum lagenarium* resulted in protection of distal, uninfected parts of cucumber against subsequent challenge by *C. lagenarium* within one week. This resistance was characterized by a decrease in the size and number of lesions (Kuc' 1975). In a subsequent study, Kuc' and Richmond (1977) reported that prior inoculation of cucumber with tobacco necrosis virus (TNV) on cotyledons or true leaves can also induce systemic resistance against TNV or *C. lagenarium*. Caruso and Kuc'(1979) showed that inoculation with *C. lagenarium* induces systemic resistance against the bacterium *Pseudomonas syringae* pv. *lachrymans*. Gessler and Kuc' (1982) provided evidence for induced resistance to the root pathogen *Fusarium oxysporum* f. sp. *cucumerina* after inoculation of one leaf with *C. lagenarium*. This work showed that the resistance inducing signal(s) moved to the tissues below the inoculated leaf as well as above. Prior inoculation with C. lagenarium or TNV on the first two leaves of cucumber plants induced resistance against cucumber mosaic virus; the resistance was observed as a decrease in the number of chlorotic cucumber mosaic virus lesions on the challenged leaves and the delay of mosaic symptoms (Bergstrom *et al.* 1982). Induced resistance to the powdery mildew

(*Pseudopenonospora cubense*) has also been reported (Okuno *et al.* 1991). Induced resistance in cucumber to powdery mildew (*Sphaerotheca fuliginea)* occured after cucumber leaves were inoculated with TNV; the level of resistance was dependent on the number of TNV lesions on the leaves of inoculation (Conti *et al.* 1990). From these studies, it is apparent that the induced resistance response in cucumber is a non-specific response with respect to the agent of induction as well as the type of pathogen protected against.

In addition to biological agents, certain chemical compounds can also induce systemic resistance. A chemical is considered a resistance inducer if: 1. It induces resistance to the same pathogens as biological inducers; 2. The chemical induces biochemical and molecular changes in the plant that are similar to those induced biologically; 3. The chemical has no direct antimicrobial activity (Kessmann *et al.* 1994). Many authors have claimed resistance-inducing activity for certain chemicals (Kessmann *et al.* 1994, Metraux *et al.* 1991, Schneider *et al.* 1994). Salicylic acid has been reported to induce resistance to tobacco mosaic virus in tobacco (White 1979, Vernooij *et al.* 1994). 2,2-dichloro-3,3-dimethylcyclopropane carboxylic acid (DCP) which has weak antifungal activity and can induce peroxidase activity in treated rice plants has been reported to inhibit the growth of *Pyricularia oryzae* (rice blast) in rice plants (Langcake and Wickins 1975). Schneider and Ullrich (1994) reported that silicic acid (0.008%, w/v) and aspirin (acetylsalicylic acid--0.008%, w/v), when applied 1-3 days before a challenge inoculation, more effectively inhibited disease development in cucumber and tobacco than bacterial or fungal cultures. These chemicals did not reduce disease development by directly

inhibiting the growth of pathogens. Mills (1986) reported that 6-benzylaminopurine (6-BAP), sprayed on the first true leaves of cucumber, substantially decreased development of lesions from subsequent inoculation with *C. lagenarium*. DL-3-amino-n butanoic acid is an inducer of resistance to *Phytophthora infestans* in potato and tomato (Cohen 1994). Arimoto *et al.* (1991) reported that after rice seeds were soaked in a 500 ppm solution of DL-alanine dodecylester HCl, rice plants had resistance to blast disease for several generations. Metraux (1991) reported that 2,6-dichloro-isonicotinic acid (INA, CGA41396) and its ester derivative (CGA 41397) induced local and systemic resistance in cucumber against *C. lagenarium,* in rice to *Pyricularia oryzae*,and in tobacco to *Peronospora tabacina*. Uknes *et al.* (1992) reported that *Arabidopsis* develops induced resistance to *Peronospora parasitica* and *Pseudomonas syringae* pv tomato DC3000 following treatment with 2,6-dichloroisonicotinic acid. Changes in gene expression were also associated with induced resistance: PR-1 mRNA was induced more than 50-fold, and PR-2 and PR-5 mRNAs were induced approximately 20-fold after INA treatment. In another study, Uknes *et al.* (1993) found that INA treatment can induce resistance to turnip crinkle virus (TCV) in *Arabidopsis*.

Histological studies of induced resistance to plant pathogens help to identify some possible mechanisms. Richmond *et al.* (1979) reported a light microscopy study on the induced resistance in cucumber to *C. lagenarium*. The major difference observed between induced and control plants was a lack of penetration by the pathogen into induced host tissues (cited in Hammerschmidt *et al.* 1997). Uknes *et al.* (1992) treated *Arabidopsis* plants with different concentrations of INA. At higher INA concentrations, *Arabidopsis*

reacted to infection by *Peronospora parasitica* with single cell necroses at sites of attempted penetration. Usually, hyphae did not penetrate beyond these sites; but in some cases, if hyphae penetrated, they became surrounded by a cluster of necrotic cells, and growth ceased. Cells of INA-treated plants became necrotic; this was never observed in control plants. Hyphae from INA-treated plants were also thinner than control plants. Using light microscopy, Ye (1992) showed that blue mold development in tobacco plant cells was severely restricted 2 days after challenging with TMV. Using histochemical techniques, Hammerschmidt and Kuc'(1982) found that there was a direct relationship between a failure of *C. lagenarium* penetration into cucumber cells and the deposition of a lignin-like material in the epidermal cell wall under none-penetrating appressoria. Stein *et al.* (1993)confirmed these results at the ultrastructural level and found lignification of both the host cell walls and fungal hyphae as part of the cucumber induced resistance response for lignin. Specific inhibitors of lignification, such as α-aminooxyacetate (AOA) and α-aminooxy-β-phenylpropionic acid (AOPP), can decrease resistance of wheat to stem rust. This result suggests that "there is a causal relationship between the formation of lignin precursors and the resistance" (Moerschbacher *et al.* 1990).

Lignification involves the polymerization of hydroxycinnamyl alcohols on a cellulose matrix after the thickening of secondary cell wall. This process is thought to be catalyzed by peroxidase in the presence of hydrogen peroxide (Hammerschmidt *et al.* 1982). Hammerschmidt and Kuc' reported that peroxidase activity increases systemically in cucumber tissue, after pathogen-induced necrotic lesions appear on the

preinoculated leaves; they associated the increased peroxidase activity with an anodic triple band on a negative acrylamide gel (Hammerschmidt *et al.* 1982). Data from Vance *et al* (1976) indicated that peroxidase activity increased in the cell wall area around the penetration site. Conti *et al.* (1990) presented a detailed examination of the induced resistance of cucumber to the powdery mildew fungus, *S. fuliginea.* They found that induced plants exhibited increased peroxidase activity in cell walls, but this was not observed in the control plants. A marked change in peroxidase isozymes of bean leaves resulted from inoculation with southern bean mosaic virus (Farkas *et al.* 1966). Tobacco leaf tissue responded to infection by *Colletotrichum destructivum* with the accumulation of peroxidase (Yu 1964). Injection of commercial purified peroxidase at a concentration of 50μg/ml to the tobacco leaves produced protection against disease symptom development caused by *Psuedomonas tabaci.* Injection of boiled peroxidase yield no protection against *P. tabaci* in tobacco (Lovrekovich *et al.* 1968a, cited in Stahmann 1973). This suggests that increased resistance may be the result of direct peroxidase action or other peroxidase-mediated activities such as generation of free radicals (Mader 1980), production of antimicrobial levels of hydrogen peroxide (Fridorich 1976), and cross-linking of hydroxy-proline-rich glycoproteins (Lamport 1986).

The activity of soluble, ionically wall-bound and covalently wall-bound peroxidases increases during induced resistance (Chibbar *et al.* 1984, Hammerschmidt *et al.* 1982, Vance *et al.* 1976). In both tobacco and cucurbits, the increases in peroxidase activity appears to be associated with enhanced activity of a specific group of peroxidase

isoforms. In reed canarygrass, the increase was attributable to increases in activity of three cationic isozymes of peroxidase and to the appearance of a new cationic isozyme (Vance *et al.* 1976). Bruce and West (1989) reported that treating castor bean cultures with elicitor caused substantial changes in the activity of four isozymes of peroxidase (one anionic, three cationic); elicitor treatment also resulted in the appearance of three new cationic isozymes that were not detectable in healthy cultures (Bruce *et al.* 1989). Increases of two anionic peroxidases, present in intercellular spaces and ionically bound to cell walls, were associated with induced resistance to blue mold in tobacco (Ye *et al.* 1992).

The study described here had three purposes. The first was to further determine the effect of certain chemicals, reported to induce resistance in other plant-pathogen systems, on induced systemic resistance in cucumber. The second purpose was to determine whether these induced resistances are associated with soluble, ionically wall-bound, and/or covalently wall-bound peroxidase activity in cucumber, since in some plant-pathogen systems these three kinds of peroxidase increased in activity during induced resistance. The third purpose was to determine whether cationic isozymes of peroxidase are associated with this process; this has never been examined in cucumber before.

CHAPTER I.
CHEMICAL INDUCTION OF RESISTANCE TO *COLLETOTRICHUM LAGENARIUM* IN CUCUMBER

Introduction

Application of chemical pesticides and fungicides is an important disease control option for growers. Many chemicals are used because they have direct antibiotic activity (Kessmann *et al.* 1994). Over the last 30 years, a number of compounds that do not have direct antimicrobial activity have been shown to increase resistance or at least to decrease symptoms in some host-pathogen interactions (Hammerschmidt and Becker 1997; Kessman *et al.* 1994).

Six groups of chemical inducers of resistance are recognized. These include amino acid analogs, such as derivatives of aminobutyric acid. Tomato plants sprayed with DL-3-amino-n-butanoic acid were protected against isolates of *Phytophthora infestans* in seven cultivars of tomato, which had different levels of susceptibility to this pathogen; A single foliar spray provided about 95% control of the disease compared with unsprayed challenged plants (Cohen *et al.* 1994a). Phosphates and oxalates have been reported as effective inducers of systemic resistance in potato against late blight (Stromberg *et al.* 1991, cited in Hammerschmidt 1997). Fatty acid derivatives, e.g., jasmonic acid, have been shown to protect potato plants against *P. infestans* (Cohen *et al.* 1994). Siegrist *et al.* (1994) reported that the phenolic compounds salicylic acid and its 5-chloroderivative induced resistance to *C. lagenarium* in hypocotyl segments but not intact hypocotyls of cucumber. The resistance seems to be due to inhibition of *C. lagenarium* penetration into epidermal cells; the increased deposition of phenolics were seen by autofluorescence.

These phenolics compounds were shown to be either lignin-like polymers, or cell wall bound 4-OH-benzaldehyde or 4-coumaric acid. Treatment of hydroponically grown cucumber plants with soluble silicates was reported to induce resistance to infection by *Pythium,* a root pathogen. Treating cucumber plants with soluble Si resulted in a stimulation of chitinase activity, rapid increase of peroxidase activity and accumulation of polymerized phenolics after inoculation with *Pythium* spp. These have important functions in plant disease resistance. (Cherif *et al.* 1994). In tobacco, INA , a derivartive of nicotinic acid, induces resistance to several different pathogens and also induces genes that are associated with expression of the systemic induced resistance (Kessmann *et al.* 1994, Uknes 1992).

The purpose of the research reported in this chapter is to describe the resistance inducing ability of selected chemicals on cucumber plants.

Materials and Methods

Pathogen, chemicals and culture of host plant

Inoculum of *Colletotrichum lagenarium* for challenging induced cucumber plants was prepared from 7 to 10-day-old cultures grown on PDA as described by Hammerschmidt *et al.* (1982).

The following chemicals to be tested as inducers were dissolved in distilled water and tested for resistance-inducing activity: D-alanine (100 ppm and 2000 ppm), DL-β-amino-n-butyric acid (100 ppm and 2000 ppm), 6-benzylaminopurine(100 ppm), Silicic acid (0.008%), acetylsalicylic acid (0.008%), and 2,6-dichloro-isonicotinic acid (INA, 25

ppm). These were sprayed on the leaves of cucumber SMR 58, which was grown in a greenhouse under artificial light.

Inoculation

A spore concentration of 5×10^5 spores/ml was used for challenging. Three days after resistance-inducing treatment, leaves were collected for challenging and split along their mid ribs. Half of each leaf was treated; the other half of the leaf was frozen immediately for later use in peroxidase assays. Treated halves received a 10-µl drop of inoculum at each of five locations. The amount of resistance was determined by counting the number of necrotic lesions that appeared and measuring the diameter of each lesion.

Soluble peroxidase extraction and assay

Soluble peroxidase was extracted and assayed as described by Hammerschmidt *et al.* (1982), with some modifications. Leaves were homogenized in 0.01M sodium phosphate buffer, pH 6.0 (1 mg/ml). The extract was centrifuged at 10,000 G for 15 min. at 4°C, and the resulting supernatant used as a source of soluble peroxidase. Soluble peroxidase activity was assayed using guaiacol as the hydrogen donor. The reaction mixture consisted of 0.25 ml guaiacol, 1.1 ml 30% H_2O_2 and 98.9 ml 0.01 M phosphate buffer, pH 6.0. Enzyme extract (10 µl) was added to 1 ml of reaction mixture to initiate the reaction, which was followed colorimetrically at 470 nm. Activity was expressed as the change in absorbance at 470 nm, per min. per mg protein.

Results

Effects of selected chemicals on induction of resistance

Table 1 reports the effects of the chemicals tested on local lesion size in leaves #1 and #2 of cucumber after challenging with *C. lagenarium*. Local resistance was induced in the # 1 leaf by D-alanine (D), DL-amino-n-butyric acid (DL), 6-bebzylaminopurine (6-BAP) and INA. Compared to control plants, treatment with D-alanine (100 ppm) decreased local lesion size to 71% of the control by the fifth day (LSD = 0.0276, mean dif. = 0.05, P-value= 0.000 < 0.05); treatment with DL-amino-n-butyric acid (100 ppm) decreased local lesion size to 65% (LSD= 0.0276, mean dif.= 0.062, P-value= 0.000 < 0.05); and 6-benzylaminopurine (100 ppm) decreased local lesion size to 43% (LSD= 0.0169, mean dif.= 0.1, P-value = 0.000 < 0.05). INA treatment did not develop any local lesion on the 5th day (LSD= 0.0179, mean dif. = 0.175, P-value = 0.000 < 0.05). The size of local lesions on the second true leaves, however, were as much as 92% of the control for D-alanine (LSD= 0.2011, mean dif.= 0.023, P-value = 0.754 > 0.05), 104% for DL-amino-n-butyric acid (LSD = 0.2835, mean dif.= –0.007, P-value = 0.944 > 0.05), and 96% for 6-benzylaminopurine (LSD= 0.1509, mean = 0.01, P-value= 0.859 > 0.05). Systemic induced resistance after one application of D, DL or 6-BAP was not significantly different from the control, at the 5% level; but there was a significant difference between the INA treatment and the control (LSD= 0.1043, mean dif. = 0.21, P-value = 0.000 < 0.05). INA showed the greatest effect on local and systemic induced

resistance. Even after increasing the concentration to 2000 ppm, D-alanine and DL-amino-n-butyric acid did not increase resistance relative to the control (Table 2). The plants treated with D-alanine developed more local lesion on the #2 leaf than the control plants on the 5th day (LSD= 0.1857, mean dif.= –0.17, P-value = 0.02 < 0.05). The plants treated with DL- amino-n-butyric acid had the same lesion development as the control plants (LSD= 0.1659, mean dif.= –0.06, P-value = 0.338 > 0.05). None of these chemicals have a visible effect before challenging--such as chlorosis, phytotoxicity or stunting--at the lower application concentrations. INA showed some chlorosis and stunting after its concentration was increased to 50 ppm.

Silicic acid (SA), acetylsalicylic acid (AA) and INA, when applied to leaf 1, altered local lesion size or soluble peroxidase activity of leaf 2 (Table 2). The plants treated with SA or AA had less lesion development than the control plants (LSD= 0.1639, mean dif. = 0.14, P-value = 0.038 < 0.05 for SA and LSD = 0.1986, mean dif. = 0.18, P-value = 0.028 < 0.05 for AA). Leaves treated with INA developed smaller or no lesions after challenged as compared to control plant leaves (LSD = 0.1325, mean dif.= 0.34, P-value = 0.000 < 0.05). There were statistically significant differences between each of three inducers and the control at 5% level (Table 3). There were also significant differences between SA and INA (P-value = 0.001 < 0.05)and between AA and INA (P-value= 0.024 < 0.05). Systemic resistance was induced by SA and AA but not as strongly as by INA. A similar relationship holds for peroxidase activity.

Table 1. Size of lesions after local induction with selected chemicals and challenging with *C. lagenarium.*

Challenged leaf	Control[a]	D-alanine 100ppm [a]	DL-amino-n-butyric acid 100ppm [a]	6-benzylaminopurine 100 ppm [a]	INA 25 ppm [a]
#1 3rd day	0.013±0.01[b]	0.013±0.01	0	0	0
#1 5th day	0.175±0.02	0.125±0.03	0.113±0.03	0.075±0.01	0
#2 3rd day	0.023±0.02	0.025±0.01	0.013±0.02	0.025±0.02	0
#2 5th day	0.248±0.12	0.225±0.22	0.255±0.32	0.238±0.15	0.038±0.01

[a] Mean diameter (cm) of 60 lesions, 3 or 5 days after challenging. Twelve half-leaves were challenged in five places each with 10-µl of *C. lagenarium* suspension (5×10^5 spores/ml).
[b] Mean and standard deviation.

Table 2. Size of local lesions after induction by D-alanine, DL-amino-n-butyric acid or INA and challenging with *C. lagenarium*.

Challenged leaf	Control[a]	D-alanine 2000 ppm[a]	DL-amino-n-butyric acid 2000 ppm[a]	INA 25 ppm[a]	INA 50 ppm[a]
#1 3rd day	0.02±0.01[b]	0.13±0.16	0.03±0.01	0	0
#1 5th day	0.22±0.05	0.48±0.15	0.21±0.11	0	0
#2 3rd day	0.08±0.02	0.1±0.05	0.06±0.02	0	0
#2 5th day	0.23±0.15	0.4±0.18	0.29±0.15	0.04±0.01	0.02±0.01

[a] Mean diameter (cm) of 60 lesions, 3 or 5 days after challenging. Twelve half-leaves were challenged in five places each with 10-µl of *C. lagenarium* suspension (5×10^5 spores/ml).
[b] Mean and standard deviation.

Table 3. Size of local lesions and soluble peroxidase activity for leaf 2 after induction of leaf 1 with silicic acid, acetylsalicylic acid, or INA.

	Control	Silicic acid (0.008%)	Acetylsalicylic acid (0.008%)	INA 25 ppm
SPA [a]	0.24±0.029 [c]	0.34±0.016 [c]	0.71±0.022 [c]	1.37±0.041 [c]
Local lesion size[b] (cm)	0.54±0.16	0.4±0.15	0.36±0.21	0.2±0.03

[a] Soluble peroxidase activity expressed as change in absorbance at 470 nm per min. per mg protein using guaiacol as the hydrogen donor.

[b] Mean diameter (cm) of 60 lesions, 7 days after challenging. Twelve half-leaves were challenged in five places each with 10-µl of *C. lagenarium* suspension (5×10^5 spores/ml).

[c] Means and standard deviation.

Discussion

D-alanine, DL-amino-n-butyric acid and 6-benzylaminopurine were inducers of local resistance but not systemic resistance. These results contrast with other reports (Arimoto *et al.* 1991, Cohen *et al.* 1994, Schneider *et al.* 1994) where these chemicals induced systemic resistance. Differences were probably due to the different treatment methods, different application technique, different plant materials and/or different pathogens. Silicic acid and acetylsalicylic acid are better systemic inducers than the three chemicals above, but are not as effective as INA. INA was a much better inducer of both local and systemic resistance than any of the other chemicals. Although the resistance-inducing effects of INA are evident, the mechanism remains obscure. In tobacco, preinoculation with TMV and treatment with INA induces the same set of nine gene families (Ward 1991). In rice, lipoxygenase activity increases within two days of INA application, but an inactive analog did not show the inducing effect (Hofmann 1993, cited in Kessmann 1994). INA-induced resistance in cucumber was identical to that seen in biologically induced resistance (Hammerschmidt 1982). My data indicate that INA may trigger the same defense gene as the biological inducer during the resistance response. More research is needed comparing INA and biological inducers with respect to histology and biochemistry. Results reported here justify intensified field-testing of INA. In order to test other chemical inducers, INA could be used as a reference inducer.

CHAPTER II.
PEROXIDASE DISTRIBUTION IN CUCUMBER AFTER RESISTANCE INDUCTION BY PSEUDOMONAS SYRINGAE PV. SYRINGAE OR 2,6-DICHLORO-ISONICOTINIC ACID

Introduction

Peroxidases have been frequently used as a biochemical marker for infection and various types of plant stress (Gaspar *et al.* 1982, Kim *et al.* 1988). Peroxidases respond to infections of bacteria, viruses or fungi by increasing activity, which is usually measured in crude extracts of the plant tissues (e.g., Hammerschmidt *et al.* 1982). Enhanced peroxidase activity and increased resistance have been reported in tomato after inoculation with *Fusarium oxysporum* f.sp. *lycopersic;* in bean after infection with alfalfa mosaic virus; and in several other plants as well (Van Loon 1986). The systemic accumulation of acidic peroxidase isozymes is closely associated with the onset of systemic resistance in cucumber (Hammerschmidt *et al.* 1982; Smith and Hammerschmidt 1988; Smith *et al.* 1991). In addition, the appearance of the peroxidase is preceded by the systemic expression of the mRNA for the peroxidases (Rasmussen *et al.* 1995).

Rapid increases in peroxidase activity may be part of the plant's defense against pathogenic organisms through lignification or in the production of activated oxygen species (Hammerschmidt and Becker 1997). Peroxidase exists in both anionic and cationic isozymes. Some research has shown peroxidase isozyme changes during pathogenesis. These changes may have different functions in different plants (Vance *et al.* 1976, Bruce *et al.* 1989). In tobacco plants reacting with localized or systemic necrosis due to TMV or TNV, a barely detectable isozyme band became moderately strong and a new, very

active isozyme appeared (Van Loon 1986). The soluble peroxidases have provided a convenient marker for induced resistance because increases in activity of these isozymes are closely correlated to the expression of induced resistance in cucumber; however, the function of the cationic forms in cucumber has not been studied in relation to indued resistance. Since *Pseudomonas syringae* pv. *syringae* (Pss) can rapidly induce both systemic resistance and peroxidase activityin cucumber (Smith *et al.* 1991), the cucumber-Pss interaction is an attractive model for studying the function and induction of different peroxidases. This model was used to evaluate the distribution of peroxidase in cellular fractions of leaves from systemically induced cucumbers. Pss was also compared to INA as an inducer, since INA gave the greatest resistance in previous experiments (Chapter I).

Materials and methods

Preparation of cultures and chemicals

Pseudomonas syringae pv. *syringae* (Pss) was used for induction. Pss was grown on LB culture for 2-3 days, then transferred to LB liquid medium culture and incubated at room temperature on a shaker overnight. Before inoculation of the cucumber leaf, the LB liquid medium culture containing the Pss was diluted with distilled water until the concentration was $A_{600} = 0.1$. *Colletotrichum lagenarium* (*C. lagenarium*) was prepared for use as a challenging inoculum from 7- to 10-day-old cultures grown on PDA, as described by Hammerschmidt *et al.* (1982). INA (25 ppm) solution was made with distilled water. Cucumber plants (SMR 58) were grown in a greenhouse under artificial light (14 hour photoperiod).

Inoculations

Procedures used for resistance-induction and for the challenge inoculation were the same as those described in Chapter I. Injection of Pss suspensions (5 to 10 µl) into the intercellular space of leaves was achieved with a 0.5 ml tuberculin syringe (Hammerschmidt 1982). A spore concentration of 5×10^5 spores/ml of *C. lagenarium* was used for challenging. Challenge inoculations were performed on half-leaves. One day or three days after the resistance-inducing inoculation, leaves for challenging were collected and split along the mid rib. Ten µl of inoculum was placed at each of five locations on half of each leaf; the other halves were frozen immediately for later use in peroxidase assays.

INA treatments

Experiments were done to determine the extent of induction of systemic resistance by INA. A solution of INA was sprayed on both cotyledons of cucumber seedlings. Leaves 1 through 4 were sequentially harvested as they became fully expanded, and analysed for soluble peroxidase activity. Other experiments were done to measure peroxidase activity for comparison with Pss inoculations. INA solution was sprayed on the first leaf, and the second leaf was harvested for analysis after 3 days.

Extraction of soluble, ionically wall-bound, covalently wall-bound and tightly wall-bound peroxidase

Peroxidases were extracted and assayed as previously described (Hammerschmidt 1982) with some modifications. Leaves were homogenized in 0.01M sodium phosphate buffer, pH 6.0 (1 mg/ml). The extract was centrifuged at 10,000 g for 15 min. at 4°C, and

the resulting supernatant used as a source of soluble peroxidase. The pellet was washed repeatedly with 1 ml buffer repeatedly by resuspension and centrifugation until peroxidase activity could not be detected in the supernatant (3-4 times). To release ionically wall-bound peroxidase, the pellet was suspended in 1 ml 0.01 M sodium phosphate buffer, pH 6.0, containing 0.5 M $CaCl_2$. The suspension was stirred occasionally for 1 hr on ice and then centrifuged at 10,000 g for 15 min. at 4°C. The supernatant was used as a source of ionically wall-bound peroxidase. The pellet was then washed repeatedly with 1ml volumes of the $CaCl_2$ buffer until no peroxidase activity could be detected in the supernatant (3-4 times). The pellet was then washed with 0.01 M sodium phosphate buffer, pH 6.0, and finally with distilled water to remove salts. The cell wall residue was homogenized in 5 ml cold acetone (-20^{0}C), washed and centrifuged 3-4 times, then transferred to a vacuum dryer to form an acetone powder. The powder was stored at -20°C in a capped vial until assayed for covalently wall-bound peroxidase and tightly wall-bound peroxidase. The ionically wall-bound peroxidase preparations were dialyzed against several changes of 0.01 M sodium phosphate buffer, pH 6.0 at 4^{0}C, prior to storage at -20^{0}C.

Peroxidases assay

Assays of soluble and ionically wall-bound peroxidase activity were carried out using guaiacol as the hydrogen donor. The method was based on that of Hammerschmidt *et al.* 1982. The reaction mixture consisted of 0.25 ml guaiacol, 1.1 ml 30% H_2O_2 and 98.9 ml 0.01 M phosphate buffer, pH 6.0. Enzyme extract (10 µl) was added to 1 ml

reaction mixture to initiate the reaction, which was followed colorimetrically at 470 nm. Activity was expressed as the increase in absorbance at 470 nm, per min. per mg protein. Total covalently wall-bound peroxidases were assayed by suspending half of the acetone powder in 1 ml of 0.01 M sodium phosphate buffer, pH 6.0. To this slurry, 1 ml of double strength guaiacol-based peroxidase reagent was added, with stirring. After 60 s, the slurry was filtered under reduced pressure through Whatman filter paper (no. 1). The absorbance of the clear solution was read at 470 nm. Tightly wall-bound peroxidases (i.e., peroxidases that cannot be removed from the cell walls by enzymatic treatment) were assayed by suspending the rest of the acetone powder in 2 ml 0.5% cellurase and 2 ml 0.5% pectinase for 24 hr. and centrifuged at 5000g. The pellet was suspended in 1ml of 0.01 sodium phosphate buffer, pH 6.0. To this slurry, 1 ml of double strength guaiacol-based peroxidase reagent was added, with stirring. After 60 s, the slurry was filtered under reduced pressure through Whatman filter paper; the absorbance of the clear solution was read at 470 nm.

Soluble and ionically wall-bound peroxidase activity under different pH

The effect of pH on soluble and ionically wall-bound peroxidase activity was determined by preparing a substrate of 0.25 ml guaiacol, 1.1 ml 30% H_2O_2 and 98.9 ml 0.01 M phosphate buffer with the pH adjusted to 3.88, 5.48, 6.56, 7.7 or 9.35 respectively. Peroxidase assays were the same as those described above.

Electrophoretic analysis

The peroxidase isozymes from control and induced plants were examined by native polyacrylamide gel electrophoresis (Bruce and West 1989). Slab gels 1 mm thick

with a 9-cm resolving gel (7.5%) and a 2.5-cm stacking gel (3.75%) were prepared with a 30:0.8(w/w) and 10:0.8(w/w) ratio of acrylamide to bisacrylamide, respectively. Acidic isoperoxidases were run in the buffer system (pH 9.3) of Reisfeld *et al.* (1962) and basic isoperoxidases were run in the buffer system (pH 3.8) of Davis (1964). Bromphenol blue tracking dye was used for acidic isoperoxidase and methyl green tracking dye for basic isoperoxidase. Peroxidases were visualized by soaking the gel in the buffer containing 40 ml methanol, 120 mg 4-chloro-naphthol, 140 ml water, 20 ml 10×PBS Buffer, and 40 µl 30% H_2O_2. After development and visualization of the peroxidase, the gels were photographed. Some photographs were digitized and enhanced by computer to improve contrast (Figures 3 and 4).

Results

Enhancement of soluble peroxidase activity by spraying INA on the cotyledon

For cotyledon-treatment with INA, all tested leaves (i.e., #1, #2 and #3) exhibited enhanced soluble peroxidase activity (P-value are 0.002, 0.002 and 0.000 respectively, all less than 0.05). #4 leaves of the treated plant showed the similar peroxidase activity as the control leaves (P-value=0.054 >0.05). These results confirmed that INA can systemically increase soluble peroxidase activity (Table 4).

Table 4. The soluble peroxidase activity of leaves 1-4 with respect to pre-treatment of cotyledon

	Control[a]	INA[a] 25 ppm	INA/control[c]
#1 leaf	2.3±0.22[b]	23.9±1.45[b]	10.39
#2 leaf	2.23±0.07	6.18±0.31	2.78
#3 leaf	3.08±0.15	6.68±0.26	2.17
#4 leaf	3.3±0.68	4.55±0.42	1.38

[a] Soluble peroxidase activity expressed as change in absorbance at 470 nm per min. per mg protein using guaiacol as the hydrogen donor.
[b] Means and standard deviation.
[c] Ratio of activity of INA induced plants/control plants

Soluble, ionically wall-bound, covalently wall-bound and tightly wall-bound peroxidase activity after different treatments.

Soluble, ionically wall-bound and covalently wall-bound peroxidase activity of the second leaves were increased (relative to the control) four days after treatment with either Pss or INA on #1 leaves (Figure 1). Peroxidase activity increased significantly in the challenged leaves, measured 3 days after challenging with *C. lagenarium*. Pss or INA treatment increased peroxidase activity in the challenged leaves more than four fold. Peroxidase activity was increased 24 h after Pss or INA treatment (for soluble peroxidase, LSD=1.995, mean dif. are –4.85 and –9.77 for Pss and INA respectively, P-value are 0.000 and 0.000, both less than 0.05; for ionically wall-bound peroxidase, LSD =7.828, mean dif. are –22.9 and –25.54 for Pss and INA respectively, P-value are 0.000 and 0.000, both less than 0.05), and local lesion size decreased (LSD = 0.145, mean dif. = 0.15 P-value= 0.021 for Pss and LSD = 0.143, mean dif. = 0.27. P-value = 0.000 for INA), showing that both treatments can induce systemic resistance significantly at 5% level

(Table 5). About 88.3% of total peroxidase activity is located in the soluble fraction, while the ionically wall-bound, covalently wall-bound and tightly wall-bound fractions comprised 10.2%, 0.38% and 1.12%, respectively.

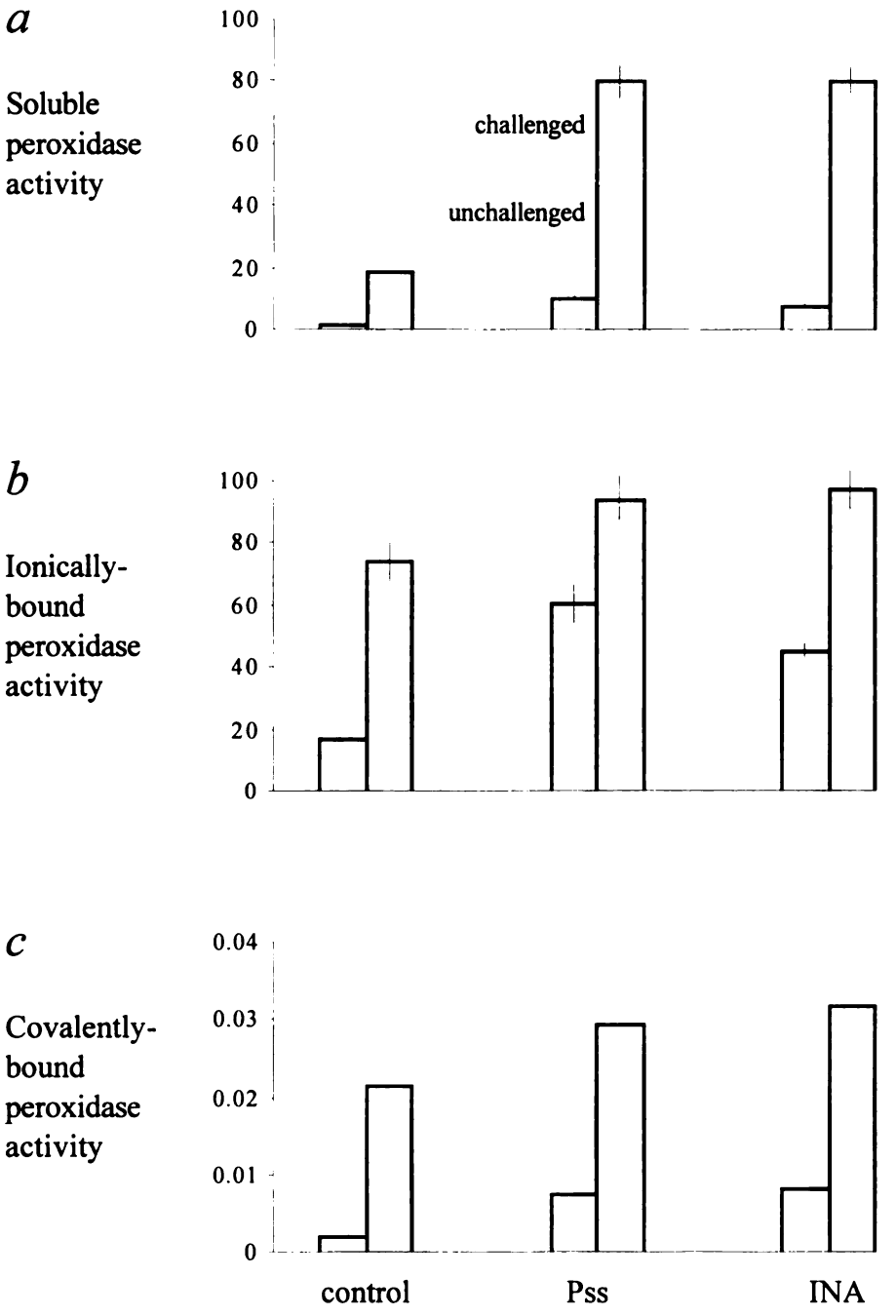

Figure 1. Peroxidase activities and standard errors of #2 leaves of "SMR 58" cucumber after different treatments. Left bars of each pair represent unchallenged leaves; right bars, leaves three days after challenging with *C. lagenarium*. Peroxidase activity is expressed as change in absorbance per min. per mg protein, at 470 nm, for soluble and ionically wall-bound peroxidase and as change in absorbance per min. per mg dry weight for covalently wall-bound peroxidase.

Table 5. Size of local lesions on #2 leaves and peroxidase activities under various treatments

	Local lesion size [a] (cm)	SPA [b]	IBPA [b]	CBPA [b]	TBPA [b]
Control	0.303±0.19[c]	13.37±0.57 [c]	94.67±2.79 [c]	0.0103	0.0235
P.S.S	0.153±0.03	18.22±0.79	117.57±3.65	0.0232	0.0324
INA	0.033±0.015	23.14±1.04	120.21±3.19	0.0219	0.0603

[a] Mean diameter of 60 lesions, 4 days after challenging. Twelve half-leaves were challenged in five places each with 10-ul of *C. lagenarium* suspension (5×10^5 spores/ml).

[b] Peroxidase activity expressed as change in absorbance at 470 nm/min. mg protein for SPA and IBPA; change in absorbance at 470 nm/min mg dry weight for CBPA and TBPA. SPA: soluble peroxidase activity, CBPA: covalently wall-bound peroxidase activity, IBPA: ionically wall-bound peroxidase activity, TBPA: tightly wall-bound peroxidase activity.

[c] Means and standard deviation.

Effect of pH on soluble and ionically wall-bound peroxidase activity

Soluble peroxidase activity was highest at pH about 6.56 (Fig 2a). Ionically wall-bound peroxidase activity was highest at pH about 7.7 (Fig 2b). The three treatments (control, INA and Pss) showed the similar results. This indicates that soluble peroxidase activity and ionically wall-bound peroxidase activity have different pH optima.

Electrophoretic analysis of peroxidase isozymes

Cationic (C) and anionic (A) isozymes can be separated by electrophoresis, ion exchange chromatography and isoelectric focusing of enzyme extracts (Gaspar *et al.* 1982, cited in Kim *et al.* 1988). Tissue induced by Pss, compared to control tissue, had increased activity of the fast moving anionic peroxidase isozymes (A1, A2, A3; Figure 3); this agrees with the results of others (Hammerschmidt *et al.*1982, Smith *et al.* 1988,1991). INA-induced tissue had results similar to Pss-induced tissue (Figure 3). Tissue induced by either Pss or INA had increased activity of the slow-moving cationic peroxidase isozymes (C1,C2,C3; Figure 4).

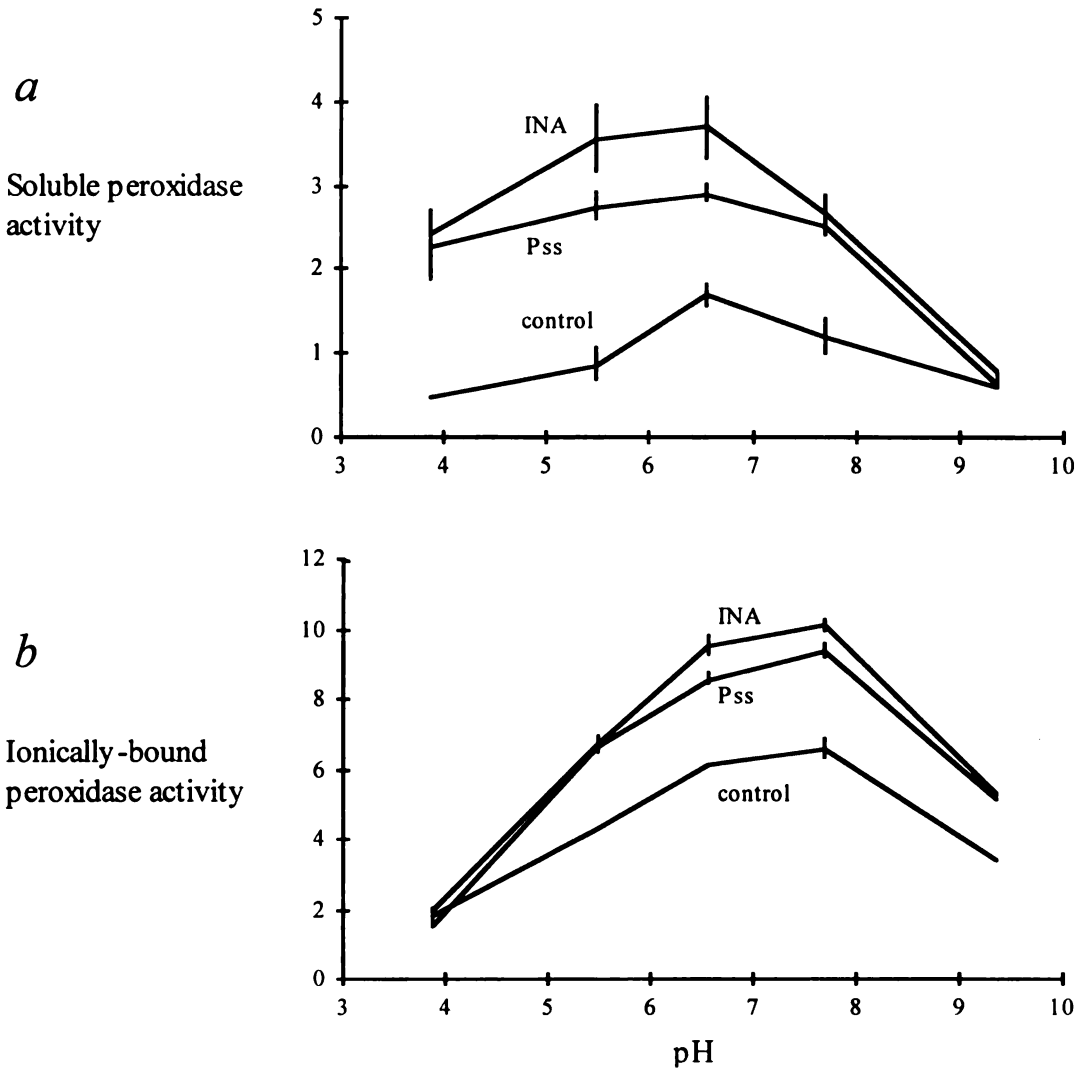

Figure 2. Soluble (a) and ionically wall-bound (b) peroxidase activity under different pH. Peroxidase activity expressed as change in absorbance per min. per mg protein at 470 nm using guaiacol as the hydrogen donor. Each point represents the mean of three replicates.

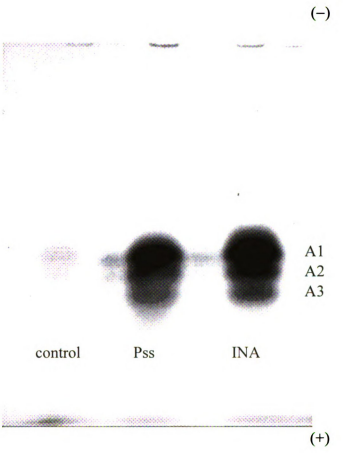

Figure 3: Soluble peroxidase isozymes from control and induced leaf tissue of "SMR 58" cucumber. 50 mg equivalent of protein was loaded on each gel. Pss: isozymes from tissue with induced resistance by *Pseudomonas syringae pv. syringae*; INA: isozymes from tissue with induced resistance by 2,6-dichloro-isonicotinic acid; A: anionic isozymes. A1, A2 and A3 refer to the three acidic peroxidase isoforms that show enhanced activity in induced plants.

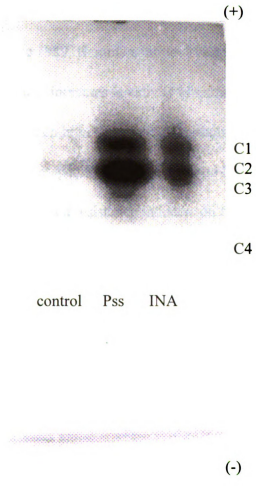

Figure 4: Ionically wall-bound peroxidase isozymes from control and induced leaf tissue of "SMR 58" cucumber. 35 mg equivalent of protein was loaded on each gel. Pss: isozymes from tissue with induced resistance by *Pseudomonas syringae* pv. *syringae*; INA: isozymes from tissue with resistance induced by 2,6-dichloro-isonicotinic acid. C1-C4: cationic peroxidase isozymes.

Discussion

Increases in peroxidase activity that is associated with induced resistance has been reported for many plants (Bruce 1989, Hammerschmidt 1982, Hammerschmidt and Becker 1997 and Hu 1989). Large increases in activity of extractable peroxidases are frequently associated with pathogen infection in plants, although the increases do not always correlate with race/cultivar specificity (Stahmann and Demorest 1973). This study evaluated the effects of selected resistance inducers on three types of peroxidase activity in cucumber.

Inoculation of the #1 leaf of cucumber with Pss elicited a systemic resistance against *C. lagenarium* and enhanced peroxidase activity. This result is in agreement with results of Smith *et al.*(1991). Spraying INA (25 ppm) on the #1 leaf produced results similar to Pss inoculation. INA treatment is unlikely to have a direct antibiotic effect, but it may affect other defense reactions, such as increased peroxidase activity and associated lignification in induced plants.

Soluble peroxidases induced in cucumber migrate as three bands on native polyacrylamide gels (pH 9.3) (Figure 3, "A" indicates "anionic"). These three bands are likely the 30 kD, 31 kD and 33 kD peroxidases identified by Smith and Hammerschmidt (1989). Ionically wall-bound peroxidase induced in cucumber migrate as 4 bands (C1,C2,C3 and C4) on native polyacrylamide gels (pH 3.8) (Figure 4; C4 is visible in the original photograph, "C" indicates "cationic"). Pss and INA treatment induced C1 and C2 strongly, suggesting that both inducing agents act similarly with respect to these peroxidases. It is possible that elicited isozymes of peroxidase in this study have a

function in induced protection of cucumber plants. Pss and INA treatment have very similar effect on three types of peroxidase activity and induce the same set of bands as seen on native polyacrylamide gels (Figures 3 and 4). Bands corresponding to peroxidase were excised with a razor blade and extracted with 0.05 M Tris-glycine pH 4.3 as the extract buffer, then the SDS system was used with a 10% acrylamide gel to determined the molecular weight of basic isozymes of peroxidase, but not successfully. Monoclonal antibodies have been developed that are specific for cell wall peroxidases (Kim *et al.* 1988). By using radiolabeling and immunoprecipitation techniques, Hu *et al* (1989) compared the biosynthesis of cationic and anionic peroxidase and their subcellular localization in peanut cell culture. They reported that no differences were found between the biosynthesis of these two isozymes, but more cationic isozymes were released into the culture medium. Both cationic and anionic peroxidases were associated with golgi bodies and starch granules of amyloplasts; anionic isozymes were also associated with plasmalemma and cationic isozymes were located on the cell wall (Hu *et al* 1989). The immunoprecipitation technique may be able to distinguish between the soluble and ionically-wall bound peroxidase isozymes and to advance the study of the structure and function of the wall-localized peroxidases related to the cell wall metabolism in cucumber.

The experimental techniques which have been used for the separation and detection of plant peroxidase usually do not provide a complete and clear picture of the isozymes. It is difficult to distingish the three kinds of peroxidase. This is probably due to the extreme range of isoelectric pH values and the location of isozymes in the cell. Peroxidase isozymes from horseradish have been reported to migrate off both ends of

wide pH range isoelectric focusing gels and can escape detection (Hoyle 1977, cited in Bruce 1989). Cationic and anionic peroxidases from peanut cell cultures differed with respect to their structure and molecular weight, but not in their catalytic properties (Chibbar *et al.* 1984).

Peroxidase catalyzes the conversion of cinnamyl alcohols into their corresponding free-radicals, which will polymerize into lignin (Bruce and West 1989). Peroxidase is also likely to be responsible for the generation of H_2O_2 required during this step from molecular oxygen and extracellular NADH (Kuc' and Richmond 1977, Mader *et al.* 1980). Some other biochemical processes associated with plant disease resistance may also need peroxidases. They are "(a) covalent insolubilization of hydroxyproline-rich glycoproteins within the cell wall, (b) crosslinking of wall-esterified p-coumaric or ferulic acids, (c) membrane lipid peroxidation, (d) oxidative degradation of auxin and (e) generation of H_2O_2 required for these events" (Bruce and West 1989).

The expression of different peroxidase species in induced plants suggest that individual isozymes of peroxidase may perform specific functions such as H_2O_2 production and free radical formation during the biosynthesis of lignin. Therefore, individual isozymes of peroxidase may have a special role in plant induced resistance. However, the specific role cannot be elucidated from the results presented in this thesis. My results further demonstrate the association of isoperoxidase in plant defense responses against pathogen attack.

SUMMARY

Systemic induced resistance is an important component of disease control that contributes to cucumber plant health. This study further determined the effect of some chemicals which were reported to induce resistance in cucumber and other plants. INA strongly induces resistance in cucumber and could be a reference inducer in tests of other chemicals. The induced resistances are associated with aionic and cationic peroxidase isozymes. Both *Pseudomonas syringae* pv. *syringae* and INA induced cationic 1 and cationic 2 strongly; this result had never been reported in cucumber before. It is possible that these elicited isozymes may have some function in induced protection of cucumber plants.

Despite many studies, information on the defense mechanisms used in induced resistance is still very limited. More research is needed to determine the function of different types of peroxidases during systemic induced resistances, to determine the genes involved in the expression of the induced resistance, to compare the mechanisms for chemical induction and biological induction, to determine the signal transduction pathway for systemic induced resistances, and to further test INA induction in other plants in the field.

Understanding the biochemical and molecular basis for systemic induced resistance may lead to the development of methods for l disease control through host plant resistance. Systemic induced resistance results in a natural defense response against diseases that should be relatively difficult for pathogens to overcome because of the multiple defense mechanisms used by the plant (Hammerschmidt and Becker 1997).

Systemic resistance inducer, either chemical or biological, will provide the farmer and researcher with an additional way to protect plants against disease in the future.

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