This is to certify that the

dissertation entitled

Biochemical and Physiological Aspects of the Plant Host Response in the Fusarium Dry Rot Disease of Potato

presented by

Yihua Zeng

has been accepted towards fulfillment of the requirements for

Ph.D. degree in Bot. & Plant Path

R Hammerschmidt
Major professor

Date 27 August 1993

BIOCHEMICAL AND PHYSIOLOGICAL ASPECTS OF THE PLANT HOST RESPONSE IN THE *FUSARIUM* DRY ROT DISEASE OF POTATO

By

Yihua Zeng

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Botany and Plant Pathology

1993

ABSTRACT

BIOCHEMICAL AND PHYSIOLOGICAL ASPECTS OF THE PLANT HOST RESPONSE IN THE *FUSARIUM* DRY ROT DISEASE OF POTATO

By

Yihua Zeng

Although the dry rot of potato caused by fungal pathogen *Fusarium sambucinum* Fuckel (Teleomorph, *Gibberella pulicaris* (Fries) Sacc.) has been known for more than half of a century, knowledge of the development of this pathogen in host tissue is lacking. Light and electron microscopy revealed that the pathogen was able to infect potato tuber tissue within 12 hr after inoculation, and the infection process involved enzymatic degradation of host middle lamella and cell walls. Toluidine blue staining and lignin-thioglycolate assay showed that deposition of lignin-like materials in the cell walls of infected cells was not detected until 24 hr after inoculation. Phenylalanine ammonia-lyase (PAL), cytoplasmic peroxidase (PO) and ionically bound cell wall peroxidase activity were induced after inoculation. The increase in PAL activity was preceded by accumulation of PAL mRNA. The production of chlorogenic acid (CGA) was suppressed by the pathogen.

Cladosporium cucumerinum, a non-pathogen of potato, was able to induce a localized resistance to infection by *F. sambucinum*. This resistance was evident within 24 hr after inoculation with *C. cucumerinum* where enhanced host general responses included induction of enzyme activity such as PAL, cytoplasmic and cell

wall bound PO, and coniferyl alcohol dehydrogenase (CAD). These increased enzyme activities were thought to contribute to the accumulation of lignin-like materials which may play an important role in host defense against *F. sambucinum*.

Two major potato steroid glycoalkaloids (SGA) α-solanine and α-chaconine inhibited spore germination and growth of *F. sambucinum in vitro*. α-Chaconine had a higher toxicity than α-solanine in every test. The toxicity of SGA was greater at pH 7 than at pH 6. The combination of α-chaconine and α-solanine showed a synergistic inhibitory effect on spore germination and growth of the fungus. In potato tuber discs, accumulation of SGAs was completely suppressed by pathogen *F. sambucinum* and partially suppressed by non-pathogen *C. cucumerinum*. The SGA accumulated in aged potato tuber discs may play a role in the general defense response against *F. sambucinum* infection.

ACKNOWLEDGMENTS

I would like to thank my major professor Dr. Ray Hammerschmidt for his guidance and support during the time of my Ph. D program at Michigan State University. I also thank the members of my graduate guidance committee Dr. Shauna Somerville, Dr. Art Cameron and Dr. David Douches for their helpful suggestions.

My thanks also go to Dr. Mike Zook for his generous help and valuable discussions with my research and to Dr. Barry Stein for his help with my EM work.

I am grateful to my mother, father and sister for their love and encouragement. I am deeply indebted to my wife, Susan Yuen-Zeng, for her loving support and encouragement.

TABLE OF CONTENTS

Page

Page

LIST OF TABLES

LIST OF FIGURES

Figure	Page

Figure Page

Figure Page

A REVIEW OF THE LITERATURE

A REVIEW OF THE LITERATURE

Potato Dry Rot Caused By *Fusarium sambucinum*

Fusarium dry rots of potato (*Solanum tuberosum* L.) occur world-wide but are particularly common in Europe and North America (Boyd, 1952). In Michigan, *Fusarium sambucinum* Fuckel (Teleomorph, *Gibberella pulicaris* (Fries) Sacc.) is the major causal agent of potato dry rot and causes significant economic losses in the field, during storage and in transit. This soil borne pathogen infects tubers only through wounds (Nielson, 1990). After germination on the wounded tubers or potato seed pieces, the fusoid-lanceolate-like macroconidia infect tubers and the hyphae first grow inter-cellularly and then intra-cellularly (Nielson, 1990). The infected tissue becomes macerated and in a few weeks a dry, brown rot develops. Under humid (70-95% relative humidity) and warmer (15-35 ^{0}C) conditions, the disease development can be accelerated and secondary infection by other pathogens such as *Erwinia spp.* may also occur (Nielson, 1990). This will cause more damage and loss of tuber tissues.

Although Fusarium dry rot disease was described many years ago (Small, 1944), only a limited number of resistant cultivars has been identified. Currently, the main and the most effective way to control this disease is the use of fungicides. However, because of both the increase in resistance of pathogens to fungicides (Desjardins *et al,* 1993; Staub, 1991) and the environment pollution due to the use of large quantities of fungicides, breeding potato varieties that are resistant to Fusarium dry rot is considered an important goal for some breeding programs. However, resistance to Fusarium is likely a multi-gene trait (Corsini and Pavek, 1986) and little is known about the mechanism of resistance in potato tuber to the disease. A study of biochemistry and physiology of host responses to infection by

F. sambucinum will provide useful information for breeding for resistance to this pathogen.

Host Responses To Wounding And Some Pathogen Infections In Potato Tuber And Other Plant Tissues

Wound responses

Wound healing is a common response of potato tuber tissues to injury (Artschwager, 1927). It occurs in two steps. Following wounding, the cell layer that is three to five layers away from the wound surface starts to divide in both inward and outward directions and forms brick-shaped cell layers that possess no intercellular spaces. This is called wound-periderm. During the time of wound-periderm formation, cell walls of the top several cells become suberized (Artschwager, 1927; Wigginton, 1974).

The wound healing process is mainly controlled by temperature and relative humidity (Artschwager, 1927; Wigginton, 1974). At room temperature and high humidity (70-95% RH), wound-periderm starts forming at 4-5 days after wounding while suberization starts at 2-3 days and is completed at 8-9 days (Barckhausen, 1978; Cunningham, 1953; Kolattukudy, 1981, Wigginton 1974). At temperatures lower than 10 ^{0}C, suberization and wound periderm formation are inhibited (Schippers, 1971). In water saturated atmospheres, wound periderm formation can also be retarded by the induction of cell proliferation at the wound surface (Wigginton, 1974). Recently, Morris *et al* (1989) have suggested that the humidity of the environment might be relatively more important in affecting the wound healing process than the temperature, because high relative humidity accelerates wound periderm formation and reduces the thickness of the layer of desiccated cells. In general, both temperature and relative humidity have very crucial effects on suberization and wound periderm formation in potato tubers.

Wound healing not only prevents water loss from the injured tissue, it also reduces pathogen infections (Barckhausen, 1978; Bostock and Stermer, 1989). The suberized wound-periderm is generally considered to be a physical barrier which restricts pathogen invasion (Bostock and Stermer, 1989). O'Brien and Leach (1983) have reported that suberized cell layers and wound-periderm together provide a physical barrier that prevents *F. sambucinum* from penetrating further into the potato tuber of resistant cultivars. Escande and Echandi (1988) have also shown that higher soil temperature accelerated the wound healing process in potato seed pieces and consequently reduced infections by different soil borne pathogens as well as infections by a combination of several fungal and bacterial pathogens.

Aging under optimal conditions can promote not only suberization but also stimulate the synthesis of resistance factors. Using the potato soft rot system, Zucker and Hankin (1970) were able to show that even the removal of 0.3 mm of the suberized layer from aged potato tuber slice could not convert the slice into complete maceration by purified pectate lyase from *Erwinia carotovora*, as occurred in the control. This indicated that the tissue deeper than 0.3 mm from the wound surface might contain other compounds such as chlorogenic acid-linked carbohydrate polymers (Riley and Kolattukudy, 1975) which prevent pectate lyase from reaching its substrate. It is also possible that the tissue might contain inhibitors of pectate lyase.

Wounding also stimulates steroid glycoalkaloids (SGA) accumulation. α-Solanine and α-chaconine, the main SGA in potato, are found normally in tuber peel, sprouts and leaves (Gull and Isenberg, 1960; Paseshnichenko, 1957). The concentration of SGA increases largely after injury and their accumulation is thought to be associated with the wound repair mechanism (Fitzpatrick *et al*, 1977;

Ishizaka and Tomiyama, 1972; Lisker and Kuć, 1977;). Also they are toxic to microorganisms. It has been demonstrated that α-chaconine is more toxic than α-solanine (Allen and Kuć, 1968; McKee, 1955; 1959; Sinden *et al*, 1973;). α-Chaconine contains a rhamnose-glucose-rhamnose sugar moiety and an aglycone called solanidine while α-solanine contains glucose-galactose-rhamnose moiety and solanidine. The differential fungitoxicity may therefore be determined by the sugar moiety (Roddick and Rijnenburg, 1987; 1988). In addition, the toxicity of both compounds increases as the pH level in the media increases (McKee, 1955; 1959). It has also been reported that α-solanine and α-chaconine may have synergistic effect on membrane stability (Roddick and Rijnenberg, 1987; 1988). This raises the possibility that SGA may interact with other chemicals to produce effects on invading microorganisms in plant tissues. Reports on the role of SGA in disease resistance have been inconclusive (1959; Allen and Kuć, 1968; Deah *et al*, 1973; Frank *et al*, 1975; McKee, 1955). As suggested by Kuć (Allen and Kuć, 1968; Locci and Kuć, 1967), SGA may be part of a general defense mechanism of potato tubers.

Accumulation of lignin and lignin-like materials

Lignins are phenolic polymers composed of p-coumaryl, coniferyl and sinapyl alcohols. In higher plants, lignin deposition in host cell walls after pathogen infection has been thought to be one of the mechanisms of disease resistance (Grisebach, 1981; Ride, 1983; Friend, 1981). The role of pathogen or non-pathogen induced lignin or lignin-like materials in disease resistance has been discussed for the last twenty years. As suggested by Ride (1983), induced lignification may (a) increase physical strength of cell walls which restricts further invasion by pathogens; (b) make the cell walls more resistant to cell wall degrading enzymes produced by pathogens; (c) provide lignin precursors that are toxic to pathogens; (d) restrain movement of nutrients and water from host to pathogen resulting in reduction of disease development; (e) reduce the plasticity of fungal cell wall by lignifying the growing hyphal tips. To date, some of these hypothetical events have been tested and some of the mechanisms have been demonstrated. Using ^{3}H-phenylalanine and ^{14}C-cinnamic acid labelling techniques, Ride was able to show that lignification occurred in the upper epidermal walls in wheat leaves within 12-19 hours after inoculation with non-pathogens (Ride, 1975; Ride and Pearce, 1979). The lignified epidermal walls were resistant to wall-degrading enzymes but not to delignifying chemicals. They were also resistant *in vitro* to fungal degradation by some wheat pathogens (Ride, 1980). Pathogen induced lignification has also been shown to occur in some resistant cultivars. In wheat carrying a resistance gene to stem rust, lignification occurred more rapidly than susceptible plants (Beardmore *et al*, 1983). Similar results were obtained with the cucurbit-*Cladosporium cucumerinum* system. Hammerschmidt *et al* (1984) confirmed the results of Hijwegen (1963) that lignin deposition was more rapid in resistant cucumber plants than in plants susceptible to *Cladosporium cucumerinum*. Furthermore, Hammerschmidt and Kuć (1982) have provided evidence that fungal hyphae could be lignified *in vitro*. The results from the above experiments strongly supports some of Ride's hypotheses.

In potato tuber tissues, rapid deposition of lignin-like materials has been shown by Friend and co-workers (1973) to occur in response to incompatible races of *Phytophthora infestans*. It was also demonstrated (Ampomah and Friend, 1988) that tuber discs from a resistant cultivar showed a higher and faster response to the infection by a complex race of *Phytophthora infestans* and to *Phoma exigua*. The host resistance to infection was correlated with the deposition of insoluble phenolic materials including both lignin and suberin. The inhibition of PAL by inhibitor amino-oxyacetic acid (AOA) before inoculation greatly reduced the deposition of lignin-like materials and resulted in further penetration of the tissue by the fungi. Similarly, Hammerschmidt (1984) has reported that *Cladosporium cucumerinum*, a non-pathogen of potato was also able to induce rapid deposition of lignin-like materials in cell walls of potato tuber tissues. The inhibition of accumulation of these induced lignin-like materials by a PAL inhibitor caused a state of "susceptibility" in the tissue to *C. cucumerinum*.

However, results showing that lignification was not a major factor in disease resistance have also been reported. As pointed out by Ride (1983), this difference may be due to the different host-parasite combinations, implying that pathogen-induced lignification may play a major role in host defense response in some host-parasite interaction, while in other cases the phytoalexins are playing a more important role in host resistance. Conceivably, pathogen-induced lignification and phytoalexin accumulation may also be complementary. First lignification may occur and restrict the further invasion by pathogens. Infection may then be further inhibited by phytoalexin accumulation or *vice versa*. More modern techniques are employed to researches of plant pathology, such as either suppressing the production of coniferyl alcohol dehydrogenase (CAD), the second to the last enzyme in lignin biosynthesis, by introducing antisense RNA gene of CAD or over-expressing CAD gene to enhance CAD production and subsequently reduce or enhance lignification in transgenic plant, which will allow us to address the role of pathogen-induced lignification in plant defense response more specifically in the future.

Induction of the activity of enzymes in phenylpropanoid biosynthesis

Many reports on changes in secondary metabolism in plant tissues following pathogen infection have been published in the past 50 years. Among them, many of the studies have focused on the increase in activity of some key enzymes in phenylpropanoid biosynthesis pathway in response to pathogen infection. Phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) is the first enzyme of general phenylpropanoid pathway and its regulatory role has been well studied (Jones, 1984). PAL deaminates L-phenylalanine to t-cinnamic acid. Through hydroxylation or methoxylation reactions the pathway provides a pool of substituted cinnamic acids that are subsequently activated to coenzyme A thioesters by specific CoA ligases. These coenzyme A thioesters are precursors for the synthesis of lignin, flavonoids, and other secondary metabolites (Grisebach, 1981).

PAL activity can be induced by pathogen infections and by fungal elicitors. Using protein synthesis inhibitors, Hadwiger *et al.* (1972) were able to show that the induced PAL activity might be due to *de novo* synthesis in both biotic and abiotic elicitor-treated pea tissue. Because of the non-specificity of protein synthesis inhibitors, the inhibition might be due to the inhibitory effects on the whole tissue metabolism. Using enzyme ^{2}H-labelling techniques, Lawton *et al* (1987) have provided convincing evidence that the increase in PAL activity in elicitor-treated bean cells was due to an increased rate of *de novo* protein synthesis. With antibodies specific for PAL, several researchers have conducted *in vivo* pulse-labelling and *in vitro* translation experiments to confirm the *de novo* synthesis of PAL in response to the elicitor treatments or fungal infections (Cramer *et al*, 1984; Hahlbrock *et al*, 1981; Lawton *et al*, 1983a; Lawton *et al*, 1983b). After PAL cDNA clones became available, various researchers were able to conduct Northern and nuclear run-off experiments which showed, at DNA level, that induction of PAL activity in the treatments resulted from new transcription of PAL (Chappell and Hahlbrock, 1984; Cramer *et al*, 1984; Cramer *et al*, 1985; Edwards *et al*, 1985; Frizemeier *et al*, 1987; Lawton *et al*, 1987). These studies also demonstrated that there is a differential expression of defense genes between the incompatible and compatible interaction (Bell and Ryderet, 1986). Since PAL activity can be induced by wounding, abiotic and biotic elicitors as well as pathogen infection, it is usually taken as a general marker to monitor changes of plant secondary metabolism.

Coniferyl alcohol dehydrogenase (CAD; E.C.1.1.1.2), the second to the last enzyme in the lignin biosynthesis pathway has also been studied in relation to pathogen-induced lignification. This enzyme reduces cinnamyl aldehydes to corresponding cinnamyl alcohols, which are later oxidized by peroxidase and polymerized into lignin. Because of its position in the lignin pathway, it is considered to be a specific marker for lignin biosynthesis. There have been some reports showing CAD activity can be induced by fungal infection and elicited by fungal elicitors and the increase in CAD activity is consistent with the accumulation of lignin (Moerschbacher *et al*, 1986, 1988, 1990). The studies of CAD activity in plant lignification have been mainly performed at the enzyme level for the past twenty years. To date, CAD enzyme protein from several plant species (tobacco, pine, soybean, poplar and spruce) has been purified into homogeneity and characterized (Halpin *et al*, 1992; Luderitz and Grisebach, 1981; O'Malley *et al*, 1992; Sarni *et al*, 1984; Wyrambick and Grisebach, 1975, 1979). Recently, CAD cDNA clones from loblolly pine and tobacco have been obtained (Knight *et al*, 1992; O'Malley *et al*, 1992). It is anticipated that the induction of CAD activity in plant defense responses will be investigated at molecular level in the near future.

Peroxidase (PO; E.C. 1.11.1.7) is the last enzyme in lignin biosynthesis. It oxidizes cinnamyl alcohols to radicals that polymerize non-enzymatically into lignin. A great number of lines of evidence from PO studies have shown that POs are associated with pathogen-induced lignification (van Loon, 1986). In cucumber plants, three acidic PO isozymes are associated with induced resistance (Smith and Hammerschmidt, 1988). Many similar studies on the correlation between PO isozyme activity and host defense response have been reported (Graham and Graham, 1991; Kerby and Somerville, 1989; Mohan and Kolattukudy, 1990; Reimers *et al*, 1992). Although there have been overwhelming numbers of reports on the study of PO, very few studies have addressed the role of specific PO isozymes in plant defense responses. Since there are many isozymes of PO in plant tissues and they are extremely sensitive to environmental stress, any change of isozyme activity may reflect an important alteration in host metabolism. Therefore, only by fully understanding the function of each PO isozyme can we elucidate the role of PO in plant defense responses.

Using molecular techniques, several researchers have been able to isolate cDNA clones of some PO isozymes associated with pathogen-induced lignification and suberization (Lagrimini, 1991; Roberts and Kolattukudy, 1988). Recently, following the availability of cDNA clones of PO isozyme, several researchers have investigated the function of specific PO isozyme using plant transformation system. Lagrimini (1991) reported that the activity of an anionic PO was over-expressed in transgenic tobacco plants containing the peroxidase gene. As a result, rapid browning and lignin deposition in response to wounding in the pith tissue of transgenic plants were observed. In addition, kinetic analysis of enzymatic reaction *in vitro* showed that the purified PO was able to polymerize phenolic acids in the presence of H_2O_2. This indicated that the anionic PO was fully capable of catalyzing polymerization of phenolics.

Another study on the function of PO isozymes using transgenic plant system came from Kolattukudy's laboratory (Sherf *et al*, 1993). A tomato gene sequence encoding an antisense transcript of an anionic PO, which was previously correlated to suberization, was introduced into tomato plants via *Agrobacterium*-mediated transformation. The introduction of the antisense gene successfully knocked out the production of this endogenous anionic PO. Unexpectedly, no significant changes in terms of appearance of the plants, suberization, content and composition of aliphatic components of suberin were detected. For this reason the authors speculated that it might be due to the substitution of other PO in the plant metabolism. From the results provided by these experiments, it is clear that more investigations of PO function using molecular approaches are needed in order to elucidate specific functions of PO isozyme in basic plant physiology as well as in plant defense responses.

Accumulation of phytoalexins

Phytoalexins are low molecular wight compounds that are toxic to a broad range of microorganisms. They are produced in plant tissues after infection by pathogens. Since Müller's (1949) first experiment on phytoalexin using the potato-*Phytophthora infestans* system, which led to his definition of phytoalexin concept, numerous reports on the study of phytoalexins have been published. In several cases, experimental evidence does support the idea that phytoalexins have a role in disease resistance (Hahn *et al*, 1985; Sato *et al*, 1971). The results of Van Etten and co-workers (Kistler and Van Etten, 1984; Tegtmeier and Van Etten, 1982) have demonstrated that highly virulent isolates of *Nectria haematococca* were able to detoxify pisatin, the phytoalexin produced by pea, while those isolates with low virulence were not able to detoxify pisatin and were sensitive to it. It was also shown by Van Etten *et al* (Shäfer *et al*, 1989) that transfer of the pisatin detoxification gene (demethylase gene, pda) into maize pathogen *Cocholiobolus heterostrophus* increased its virulence on pea. However, a saprophyte *Aspergillus nidulans* could not be converted into a pea pathogen by the same gene transfer. These studies provided evidence that pisatin plays an important role in disease resistance in pea.

Direct evidence that phytoalexins inhibit fungal growth due to their accumulation in plant tissue came from the work by Snyder and Nicholson (1990). Both susceptible and resistant young sorghum have the ability to produce 3-deoxyanthocyanidin flavonoids, which are phytoalexins with a red-orange color. After maturation, the susceptible cultivars lose the ability to produce the phytoalexins and become susceptible. When juvenile sorghum was inoculated with the fungus *Colletotrichum graminicola*, the phytoalexins were produced in inclusions in the cells under attack and later produced in the adjacent cells. The phytoalexins were released and deposited onto the fungal hyphae. Eventually the fungus and the infected cells died.

More direct evidence showing that disease resistance resulted from phytoalexin gene expression has been presented by Hain *et al* (1993). They transferred grapevine stilbene synthase genes into tobacco so that the tobacco produced the phytoalexin resveratrol due to over expression of the introduced genes. The transgenic tobacco was more resistant to *Botrytis cinerea*. This study demonstrated that plant resistance against pathogen infection can be enhanced by introducing a foreign gene for phytoalexin biosynthesis. The significance of this study may be questioned because of the use of *Botrytis cinerea*, which is not a strict tobacco pathogen, to challenge the transgenic tobacco plants. However, there is a positive correlation between the phytoalexin resveratrol concentration in grapevine and resistance to the pathogen.

Phytoalexins are also produced in potato tubers upon infection with pathogens. Among them, rishitin and lubimin are very commonly produced fungitoxic sesquiterpenes (Kuć, 1982). It has been known that rishitin inhibits growth of *Phytophthora infestans*, the potato late blight fungal pathogen (Kuć, 1982). However, in the potato-*Fusarium sambucinum* system, the fungus has been was able to tolerate rishitin and detoxify lubimin (Desjardine and Gardner, 1989b; Desjardine *et al*, 1989a). Genetic analysis demonstrated that the highly virulent strains of *Fusarium sambucinum* are tolerant of rishitin and the tolerance is inheritable (Desjardine *et al*, 1993).

In general, it seems that phytoalexins play an important role in disease resistance in plant-facultative parasite system. However, in the plant-obligate parasite system, the role of phytoalexins would be less important and the combination of gene-for-gene effect and phytoalexin inhibitory effect may be ultimate results of plant disease resistance.

Accumulation of chlorogenic acid (CGA)

Chlorogenic acid (CGA; 3-O-(caffeoyl) quinate) is one of the end products of phenylpropanoid metabolism. It is normally present in potato tuber peel in high quantity and it accumulates in tuber tissue after illumination (Lamb and Rubery, 1976), infection (Friend at al, 1973; Smith and Rubery, 1981) and wounding (Cottle and Kolattukudy, 1982). Wounding stimulates CGA accumulation with a gradually increasing rate within 12 days (Cottle and Kolattukudy, 1982). It is thought that wound-induced CGA accumulation may be one of the wound healing processes but its role in wound healing is still unknown.

Pathogen-induced CGA accumulation has been reported (Friend *et al,* 1973; Smith and Rubery, 1981). In potato tissue, CGA accumulated in the first 40 hr after infection by *Phytophthora infestans* and this accumulation was parallel to the induced PAL activity. After 50 hr, the increased accumulation of CGA fell and this was explained as a consequence of conversion of CGA into other metabolites (Smith and Rubery, 1981). In fact, in potato tuber discs the conversion of CGA into a caffeoyl moiety, which later is incorporated into "lignin-like" materials, has been demonstrated (Taylor and Zucker,1966). In addition, an enzyme that cleaves CGA, and in the presence of CoA, generates caffeoyl-CoA and quinic acid has been reported (Rhodes and Wooltorton, 1976). This clearly indicates that the conversion of CGA to other secondary metabolites could occur *in vivo.*

With regard to the role of CGA in disease resistance, CGA is generally considered less toxic to a broad range of microorganisms than its oxidation products, the quinones, and therefore the accumulation of CGA and its oxidation products may be part of plant general defence response (Kuć, 1972).

REFERENCES

Allen E, Kuć J 1968 α-Solanine and α-chaconine as fungitoxic compounds in extracts of Irish potato tubers. Phytopathology 58:776-781.

Ampomah YA, Friend J 1988 Insoluble phenolic compounds and resistance of potato tuber disc to *Phytophthora* and *Phoma*. Phytochemistry 27: 2533-2541.

Artschwager E 1927 Wound-periderm formation in the potato as affected by temperature and humidity. Journal of Agricultural Research 35:995-1000.

Barckhausen R 1978 Ultrastructural changes in wounded plant storage tissue cells. In: Kahl G ed. Biochemistry of wounded plant tissues. Walter de Gruyter & Co., Berlin. New York, 1-42.

Beardmore J, Ride JP, Granger JW 1983 Cellular lignification as a factor in the hypersensitive resistance of wheat to stem rust. Physiological Plant Pathology 22:209-220.

Bell JN, Ryder TB, Wingate VPM, Bailey JA, Lamb CJ 1986 Differential accumulation of plant defense gene transcripts in a compatible and an incompatible plant-pathogen interaction. Molecular and Cellular Biology 6:1615-1613.

Bostock RM, Stermer BA 1989 Perspectives of wound healing in resistance to pathogens. Annul Review of Phytopathology 27:343-471.

Boyd AEW 1952 Dry-rot disease of the potato. Annals of Applied Biology 39:322-357.

Chappell J, Hahlbrock K 1984 Transcription of plant defense genes in response to UV light or fungal elicitor. Nature 311: 76-78.

Corsini DL, Pavek JJ 1980 Phenylalanine ammonia lyase and fungitoxic metabolites produced by potato cultivars in response to *Fusarium* tuber rot. Physiological Plant Pathology 16:63-72.

Cottle W, Kolattukudy PE 1982 Biosynthesis, deposition, and partial characterization of potato suberin phenolics. Plant Physiology 96:393-399.

Cramer CL, Bell JN, Ryder TB, Bailey, JA, Schuch W, Bolwell GP, Rubbins MP, Dixon RA 1984 Co-ordinated synthesis of phytoalexin biosynthesis enzymes in biologically-stressed cells of bean (*Phaseolus vulgaris* L.). EMBO Journal 4:285-289.

Cramer CL, Ryder TB, Bell JN, Lamb CJ 1985 Rapid switching of plant gene expression induced by fungal elicitor. Science 227: 1240-1243.

Cunningham HS 1953 A histological study of the influence of sprout inhibitors on *Fusarium* infection of potato tubers. Phytopathology 43:95-98.

Deahl KL, Young RJ, Sinden SL 1973 A study of the relationship of late blight resistance to glycoalkaloid content in fifteen potato clones. American Potato Journal 50:248-253.

Desjardins AE, Gardner HW. 1989b Genetic analysis in: *Gibberella pulicaris* rishitin tolerance, rishitin metabolism and virulence on potato tuber. Molecular Plant-Microbe Interactions 2: 26-34.

Desjardins, AE, Gardner HW, Plattner RD 1989a Detoxification of the potato phytoalexin lubimin by *Gibberella pulicaris*. Phytochemistry 28: 431-437.

Desjardins AE, Christ-Harned EA, McCormis SP, Secor GA 1993 Population structure and genetic analysis of field resistance to thiabendazole in *Gibberella pulicaris* from potato tubers. Phytopathology 83:164-170.

Edwards K, Cramer CL, Bowell GP, Dixon RA, Shuch W, Lamb CJ 1985 Rapid transient inductions of phenylalanine ammonia lyase mRNA in elicitor-treated bean cells. Proceedings of The National Academy of Sciences (USA) 82:6731-6735.

Escande AR, Echandi E 1988 Wound-healing and the effect of soil temperature, cultivars and protective chemicals on wound healed potato seed pieces inoculated with seed piece decay fungi and bacteria. American Potato Journal 65:741-752.

Fitzpatrick TJ, Herb SF, Osman SF, McDermott JA 1977 Potato glycoalkaloids: Increases and variations of ratios in aged slices over prolonged storage. American Potato Journal 54:539-544.

Frank JA, Wilson JM, Webb RE 1975 The relationship between glycoalkaloid and disease resistance in potatoes. Phytopathology 65:1045-1049.

Friend J. 1981. Plant phenolics, lignification and plant disease. Progress in Phytochemistry 7: 197-261.

Friend J, Reynolds SB, Aveyard MA 1973 Phenylalanine ammonia-lyase, chlorogenic acid and lignin in potato tuber tissue inoculated with *Phytophthora infestans*. Physiological Plant Pathology 3:495-507.

Fritzemeier K-H, Cretin C, Kombrink E, Rohwer F, Taylor J, Scheel D, Hahlbrock K 1987 Transient induction of phenylalanine ammonia-lyase and 4-coumarate:CoA ligase mRNAs in potato leaves infected with virulent or avirulent races of *Phytophthora infestans*. Plant Physiology 85: 34-41.

Graham MY, Graham TL 1991 Rapid accumulation of anionic peroxidases and phenolic polymers in soybean cotyledon tissues following treatment with *Phytophthora megasperma* f. sp. *glycinea* wall glucan. Plant Physiology 97:1445-1455.

Grisebach H. 1981 Lignins. In: Stumpf PK, Conn EE ed The Biochemistry of Plants. Academic Press, New York 457-478.

Gull D, Isenberg F 1960 Chlorophyll and solanine content and distribution in four varieties of potato tubers. Proceedings of American Society of Horticultural Science 75:545-556.

Hadwiger LA 1972 Induction of phenylalanine ammonia lyase and pisatin by photosensitive psoralen compounds. Plant Physiology 49:779-782.

Hahlbrock K, Lamb CL, Purswin C, Ebel J, Fautz E, Schafer E 1981 Rapid response of suspension-cultured parsley cells to the elicitor from *Phytophthora* *megasperma* var *sojae*. Plant Physiology 67:768-773.

Hahn MG, Bonhoff A, Grisebach H 1985 Quantitative localization of the phytoalexin glyceollin I in relation to fungal hyphae in soybean roots infected with *Phytophthora megasperma* f. sp. *glycinea*. Plant Physiology 77:591-601.

Hain R, Reif H-J, Krause E, Langebartels R, Kinde H, Vormam B, Wiese W, Schmelzer E, Schreier PH, Slöcker RH, Stenzel K 1993 Disease resistance results from foreign phytoalexin expression in a novel plant. Nature 361:153-156.

Halpin C, Knight ME, Grima-Pettenati J, Goffner D, Boudet A, Schuch W 1992 Purification and characterization of cinnamyl alcohol dehydrogenase from tobacco stems. Plant Physiology 98:12-16.

Hammerschmidt R, Lamport DTA, Muldoon EP 1984 Cell wall hydroxyproline enhancement and lignin deposition as an early event in the resistance of cucumber to *Cladosporium cucumerinum*. Physiological Plant Pathology 24:43-47.

Hammerschmidt R 1984 Rapid deposition of lignin in potato tuber tissue as a response to fungi non-pathogenic on potato. Physiological Plant Pathology 24: 33-42.

Hammerschmidt R, Kuć J 1982 Lignification as a mechanism for induced systemic resistance in cucumber. Physiological Plant Pathology 20: 61-71.

Hijwegen T 1963 Lignification, a possible mechanism of active disease resistance against pathogens. Netherlands Journal of Plant Pathology 69:314-317.

Ishizaka N, Tomiyama K 1972 Effect of wounding or infection by *Phytophthora infestans* of content of terpenoid in potato tubers. Plant Cell Physiology 13:1053-1063.

Jones DH 1984 Phenylalanine ammonia-lyase: regulation of its induction and its role in plant development. Phytochemistry 7: 1349-1359.

Kerby K, Somerville S 1989 Enhancement of specific intercellular peroxidase following inoculation of barley with *Erysiphe graminis* f. sp. *hordei*. Physiological and Molecular Plant Pathology 35:323-337.

Kistler HC, Van Etten HD 1984 Regulation of pisatin demethylation in *Nectria haematococca* and its influence on pisatin tolerance and virulence. Journal of General Microbiology 130:2605-2613.

Knight ME, Halpin C, Schuch W 1992 Identification and characterization of cDNA clones encoding cinnamyl alcohol dehydrogenase from tobacco. Plant Molecular Biology 19:793-801.

Kolattukudy PE 1981 Structure, biosynthesis, and biodegradation of cutin and suberin. Annual Review of Plant Physiology 32: 539-567.

Kuć J 1982 Phytoalexins from the solanaceae. In Bailey JA and Mansfield JW ed Phytoalexins. John Wiley and Sons, New York 81-105.

Kuć J 1972 Phytoalexins. Annual Review of Phytopathology 10:207-232.

Lagrimini LM 1991 Wound-induced deposition of polyphenols in transgenic plants overexpressing peroxidase. Plant Physiology 96:577-583.

Lamb CJ, Rubery PH 1976 Photocontrol of chlorogenic acid biosynthesis in potato tuber discs. Phytochemistry 15:665-668.

Lawton MA, Dixon RA. Hahlbrock H, Lamb C 1983b Rapid induction of the synthesis of phenylalanine ammonia-lyase and chalcone synthase in elicitor-treated plant cells. European Journal of Biochemistry 129:593-601.

Lawton MA, Lamb CJ 1987 Transcriptional activation of plant defense genes by fungal elicitor, wounding and infection. Molecular and Cell Biology 7:335-341.

Lawton MA, Dixon RA. Hahlbrock H, Lamb C 1983a Elicitor induction of mRNA activity. Rapid effects of elicitor on phenylalanine ammonia-lyase and chalcone synthase mRNA activities in bean cells. European Journal of Biochemistry 130:131-139.

Lisker N, Kuć J 1977 Elicitors of terpenoid accumulation in potato tuber slices. Phytopathology 67:1356-1359.

Locci R, Kuć J 1967 Steroid alkaloids as compounds produced by potato tuber under stress. Phytopathology 57:1272-1273.

Luderitz T, Grisebach H 1981 Enzymic synthesis of lignin precursors: comparison of cinnamoyl-CoA reductase and cinnamyl alcohol:$NADP^+$ dehydrogenase from spruce (*Picea abies* L.) and soybean (*Glycine max* L.). European Journal of Biochemistry 119:115-124.

McKee RK 1955 Host-parasite relationship in the dry-rot disease of potatoes. Annals of Applied Biology 43:147-148.

McKee RK 1959 Factors affecting the toxicity of solanine and related alkaloids to *Fusarium caeruleum*. Journal of General Microbiology 20:686-696.

Moerschbacher BM, Noll U, Ocampo CA, Flott BE, Gotthardt U, Wustefeld A, Reisener H-J 1990 Hypersensitive lignification response as the mechanism of non-host resistance of wheat against oat crown rust. Physiologia Plantarum 78: 609-615.

Moerschbacher BM, Heck B, Kogel KH, Obst O, Reisener HJ 1986 An elicitor of the hypersensitive lignification response in wheat leaves isolated from the rust fungus *Puccinia graminis* f. sp. *tritici*. II. Induction of enzymes correlated with the biosynthesis of lignin. Zeitschrift für Naturforschung 41c: 839-844.

Moerschbacher BM, Noll U, Flott BE, Reisener HJ 1988 Lignin biosynthetic enzymes in rust infected, resistant and susceptible near-isogenic wheat lines. Physiological and Molecular Plant Pathology 33:33-46.

Morhan R, Kolattukudy PE 1990 Differential activation of expression of a suberization-associated anionic peroxidase gene in near-isogenic resistant and susceptible tomato lines by elicitors of *Verticillium albo-atratrum*. Plant Physiology 92:276-280.

Morris SC, Forves-Smith MR, Scriven FM 1989 Determination of optimum conditions for suberization, wound periderm formation, cellular desiccation and pathogen resistance in wounded *Solanum tuberosum* tubers. Physiological and Molecular Plant Pathology 35:177-190.

Müller KO, Behr L 1949 Mechanism of *Phytophthora* resistance of potatoes. Nature 163:498-499.

Nielson LW 1990 Fusarium dry rots. In: Hooker WJ ed Compendium of Potato Diseases. APS Press 59-60.

O'Brien VJ, Leach SS 1983 Investigations into the mode of resistance of potato tubers to *Fusarium roseum* 'Sambucinum'. American Potato Journal 60: 227-233.

O'Malley DM, Porter S, Sederoff RR 1992 Purification, characterization, and cloning of cinnamyl alcohol dehydrogenase in loblolly pine (*Pinus taeda* L.). Plant Physiology 98:1364-1371.

Paseshnichenko VA 1957 Content of solanine and chaconine in the potato during the vegetation period. Biochemistry (USSR) 22:929-931.

Reimers P, Guo A, Leach JE 1992 Increased activity of a cationic peroxidase associated with a incompatible interaction between *Xanthomonas oryzae* pv. *oryzae* and rice (*Oryza sativa)*. Plant Physiology 99:1044-1050.

Rhods MJC, Wooltorton LSC 1976 The enzymic conversion of hydroxycinnamic acids to p-coumarylquinic acid and chlorogenic acid in tomato fruits. Phytochemistry 15:947-951.

Ride JP 1975 Ligninfication in wounded wheat leaves in response to fungi and its possible role in resistance. Physiological Plant Pathology 5: 125-134.

Ride JP 1980 The effect of induced lignification on the resistance of wheat cell walls to fungal degradation. Physiological Plant Pathology 16:187-196.

Ride JP 1983 Cell walls and other structural barriers in defence. In: Callow JA ed Biochemical Plant Pathology. John Wiley & Sons Ltd. 215-236.

Ride JP, Pearce RB 1979 Lignification and papilla formation at sites in attempted penetration of wheat leaves by non-pathogenic fungi. Physiological Plant Pathology 15:79-92.

Riley RG, Kolattukudy PE 1975 Evidence for covalently attached p-coumaric acid and ferulic acids in cutins and suberins. Plant Physiology 56:650-654.

Roberts E, Kolattukudy PE 1988 Cloning and sequencing of cDNA for a highly anionic peroxidase from potato and the induction of its mRNA in suberizing potato tubers and tomato fruits. Plant Molecular Biology 11:15-26.

Roddick JG, Rijnenberg AJ, Osman SF 1988 Synergistic interaction between potato glycoalkaloids α-solanine and α-chaconine in relation to destability of cell membranes: ecological implications. Journal of Chemical Ecology 14:889-902.

Roddick JG, Rijnenberg AJ 1987 Synergistic interaction between the potato glycoalkaloids α-solanine and α-chaconine in relation to lysis of phospholipid/sterol liposomes. Phytochemistry 26:1325-1328.

Sarni F, Grand C, Boudet AM 1984 Purification and properties of cinnamoyl-CoA reductase and cinnamyl-alcohol dehydrogenase from poplar stem (*Populus euramericana*). European Journal of Biochemistry 139:259-265.

Sato N, Kitazawa K, Tomiyama K 1971 The role of rishitin in localizing the invading hyphae of *Phytophthora infestans* in infection sites at the cut surfaces of potato tubers. Physiological Plant Pathology 1:289-295.

Schippers PA 1971 The influence of curing conditions on weight loss of potatoes during storage. American Potato Journal 48:278-286.

Schwochav ME, Hadwiger LA 1969 Regulation of gene expression by actinomycin D and other compounds which change the conformation of DNA. Archives of Biochemistry and Biophysics 1134:34-41.

Shäfer W, Straney D, Ciuffetti L, Van Etten HD, Yoder OC 1989 One enzyme makes a fungal pathogen, but not a saprophyte, virulent on a new host plant. Science 246:247-249.

Sherf BA, Bajar AM, Kolattukudy PE 1993 Abolition of an inducible highly anionic peroxidase activity in transgenic tomato. Plant Physiology 101:201-208.

Sinden SL, Gpth RW, O'Brien MJ 1973 Effect of potato alkaloids on the growth of *Alternaria solani* and their possible role as resistance factors in potatoes. Phytopathology 63:303-307.

Small T 1944 Dry rot of potato (*Fusarium caeruleum* (Lib.) Sacc.). Investigation of the sources and time of infection. Annals of Applied Biology 31: 291-295.

Smith BG, Rubery PH 1981 The effects of infection by *Phytophthora infestans* on the control of phenylpropanoid metabolism in wounded potato tissue. Planta 151: 535-540.

Smith JA, Hammerschmidt R 1988 Comparative study of acidic peroxidase associated with induced resistance in cucumber, muskmelon and watermelon. Physiological and Molecular Plant Pathology 33: 255-261.

Snyder BA, Nicholson RL 1990 Synthesis of phytoalexins in sorghum as a site-specific response to fungal ingress. Science 248:1637-1639.

Staub T 1991 Fungicide resistance: Practical experience with anti-resistance strategies and the role of integrated use. Annual Review of Phytopathology 29:421-442.

Taylor AO, Zucker M 1966 Turnover and metabolism of chlorogenic acid in *Xanthium* leaves and potato tubers. Plant Physiology 41:1350-1359.

Teftmeier K, Van Etten HD 1982 The role pisatin tolerance and degradation in the virulence of *Nectria haematococca* on peas: a genetic analysis. Phytopathology 72:608-612.

Van Loon LC 1986 The significance of changes in peroxidase in diseased plants. In: Greppin H, Penel C, Gaspar Th ed. Molecular and Physiological Aspects of Plant Peroxidases. University of Geneva, Switzerland, 405-418.

Wigginton MJ 1974 Effects of temperature, oxygen tension and relative humidity on the wound-healing process in the potato tuber. Potato Research 17:200-214.

Wyrambick D, Grisebach H 1979 Enzymic synthesis of lignin precursors. Further studies on cinnamyl-alcohol dehydrogenase from soybean suspension cultures. European Journal of Biochemistry 97:503-509.

Wyrambick D, Grisebach H 1975 Purification and properties of isoenzymes of cinnamyl-alcohol dehydrogenase from soybean-cell cultures. European Journal of Biochemistry 59:9-15.

Zucker M, Hankin L 1970 Physiological basis for cytoheximide-induced soft rot of potatoes by *Pseudomonas fluorescens*. Annals of Botany 34:1047-1062.

CHAPTER I

PATHOGENESIS OF *FUSARIUM SAMBUCINUM* AND HOST RESPONSES IN POTATO TUBER TISSUE

ABSTRACT

The pathogenesis of potato dry rot fungal pathogen *Fusarium sambucinum* Fuckel (Teleomorph, *Gibberella pulicaris* (Fries) Sacc.) was studied at the light microscopic (LM) and electron microscopic (EM) level. Light and electron microscopy revealed that the pathogen was able to infect potato tuber tissue within 12 hr after inoculation. The infection process involved enzymic degradation of host middle lamella and cell walls. Toluidine blue staining showed that very little amount of lignin-like materials were deposited in the cell walls of infected cells at 12 hr. However, at 24 hr after inoculation, deposition of some lignin-like materials was observed in the tissue already colonized. The results obtained with lignin-thioglycolate assay were in agreement with those with toluidine blue staining.

Phenylalanine ammonia-lyase (PAL), cytoplasmic peroxidase (PO) and ionically bound cell wall peroxidase activity were induced after inoculation with the spore suspension. The increase in PAL activity was preceded by accumulation of PAL mRNA. Cell wall PO activity increased at a higher rate than cytoplasmic PO. The production of chlorogenic acid (CGA) was suppressed by the pathogen. Plate assay for polygalacturonase (PG) and pectate lyase (PL) clearly showed *in vitro* production of these two cell wall degrading enzymes by the pathogen.

INTRODUCTION

Although Fusarium dry rot disease was described many years ago (Small, 1944) and pathogenesis of dry rot causing fungal pathogens, *Fusarium caeruleum* (Lib.) Sacc. and *F. avenaceum* (Fr.) Sacc.(McKee, 1954), has been described histologically, no information of the pathogenesis of *F. sambucinum* (Teleomorph: *Gibberella pulicaris*), the important dry rot causal agent in the North of America, was available at the ultrastructure level. In addition, knowledge of the host response to the infection at early stages was also lacking. Thus, the goal of this study was to investigate the infection process of Fusarium dry rot disease at the light and electron microscopic levels, to determine the *in vitro* production of pectolytic enzymes by the fungus and to examine certain host responses to the infection.

MATERIALS AND METHODS

Preparation of potato tuber discs

Tubers of potato (*Solanum tuberosum* L.) cultivar Russet Burbank stored for 2-6 months at 4^0C were used in all experiments. The tuber disc preparation procedure described by Hammerschmidt (1984) was followed. Briefly, the tubers were taken from the cold room and warmed up at room temperature (RT) overnight. The tubers were then washed with tap water and soaked in 5% of Chlorox for 5-10 min to disinfect tuber skin surface. Prior to slicing, the tubers were dipped into 95% ethanol (EtOH) and then flamed. The tubers were then sliced into 0.5-0.8 cm thick slices and discs were cut out from central parenchyma tissue with 2.0 cm diameter core borer. The discs were rinsed three times with sterile water and then placed in Petri dishes lined with moist Whatman 3 MM filter paper.

Fungal materials

Fusarium sambucinum was isolated from diseased tuber and single spore cultured on potato dextrose agar (PDA) plates. The stock culture was stored at 4 ^{0}C. *F. sambucinum* from 7-10 day old culture grown at RT was used for inoculation. The fungal spore suspension was made by flooding the plate with 10-15 ml of sterile distilled water. The spores then filtered through four layers of cheesecloth and adjusted to a concentration of 2×10^6 spores/ml for inoculation. The spore suspension (50 μl) was applied onto the discs. The discs were then incubated at RT in the dark.

Light microscopic observation of *F. sambucinum* infection in potato tuber tissue

At 12 and 24 hr following inoculation, the discs were soaked in 95% EtOH for at least 3- hr in order to clear out soluble phenols. Then the discs were sliced longitudinally into 1-2 cell layer thick sections with a Hooker microtome. The sections were first rinsed with water and stained with 0.1% toluidine blue O in 0.1 M Na-phosphate buffer, pH 6.5 for 2-3 min, then rinsed with water for 2-3 times. The stained tissue sections were mounted as a temporary slide and observed with a light microscope.

Electron microscopic observation of *F. sambucinum* infection in potato tuber tissue

The procedures described by Stein (1991) were used for EM sample preparation. Briefly, at different time points after inoculation with *F. sambucinum*, tissue samples were collected from the top 1 mm thick layer of potato tuber discs. The tissue was cut into approximately 1 mm^3 pieces and fixed in 2.7% glutaraldehyde in 0.1 M Na-phosphate buffer, pH 6.8 with four changes during overnight fixation at 4 ^{0}C. The fixed samples then were post-fixed in 1% OsO_4 in the same buffer at 4 ^{0}C for 90 min and rinsed twice with the buffer for 15 min each. Following post-fixation, the samples were dehydrated with series of EtOH (25, 50, 75, 95 and 100%) for 15 min each at RT.

For SEM sample preparation, the EtOH dehydrated samples were processed by critical point drying and mounted onto a aluminum stubs. After coated with gold for 3 min at 20 mA, the samples were viewed and photographed with a 35 CF JEOL scanning electron microscope.

For TEM sample preparation, the EtOH dehydrated samples were put into four changes of Spurr's (1969) epoxy embedding media and polymerized at 65 ^{0}C for 18 hr. Following polymerization, the samples were cut into silver-gold sections (approximately 90 nm thick) on a Reichert Ultracut E Ultramicrotome and placed on copper 300 mesh grids. Grids were stained in a saturated aqueous solution of urinal acetate for 30 min and then in lead citrate. Grids were viewed and photographed with a 201 Phillips transmission electron microscope.

Plate assay for pectolytic enzyme production of *F. sambucinum*

Production of pectolytic enzymes in culture by *F. sambucinum* was tested. For polygalacturonase (PG) activity test, the procedures described by Scott-Craig (1990) were followed. Briefly, the culture medium was made up with 5 g polygalacturonate, 2 g $(NH_4)_2SO_4$, 15 g agar and 1000 ml distilled water at pH 5.5. An agar plug (0.5 cm diameter) containing *F. sambucinum* spores and hyphae was placed in the plate. Incubation was carried out at RT in the dark for 2-4 days. After incubation, the plate was flooded with 5 ml of 0.4 N HCl for 10 min and then rinsed several times with water. The presence of a halo around the fungal colony indicated the ability to produce PG which breaks down polygalacturonate. For pectate lyase (PL) activity test, a plate assay was also used. The plate was made up with the same medium as that used for PG assay but contained 0.2 mM $CaCl_2$ and its pH was adjusted to 8.0 with Tris-HCl. After incubation, the plate was also flooded with 0.4 N HCl and the presence of halo around the fungal colony showed the production of PL by the fungus.

PAL and cytoplasmic PO extraction and assays

At intervals after inoculation with a *F. sambucinum* spore suspension, the top 0.5 mm layer of discs was peeled and extracted with acetone at -20^{0}C to make an acetone powder. One hundred mg of acetone powder was extracted in five ml of 0.1 M sodium borate buffer containing same amount of PVP in weight, pH 8.8 for one hr at 4 ^{0}C and filtered through two layers of cheesecloth and then centrifuged at 10,000 xg for 20 min at 4 ^{0}C. After centrifugation, the supernatant was used for PAL and cytoplasmic PO assays. PO activity was assayed by spectrophotometric procedure described by Borchert (1978). For PAL assay, procedure of Rahe et al (1970) was used. The protein content of enzyme extracts was determined by the method of Bradford (1976).

Extraction of ionically bound cell wall PO

A modified procedure from Grison and Pilet (1985) was used for ionically bound cell wall PO extraction. Briefly, 0.2 g of the acetone powder was stirred in 10 ml of 0.05 M sodium phosphate buffer (pH 6.0) for 2 hr at 4 ^{0}C and centrifuged at 1000 xg for 6 min. The pellet was washed 2 times with 2 ml of the buffer and once with water then centrifuged at 1000 xg for 6 min. The pellet was re-suspended in 1 ml of 1 M $CaCl_2$ and stirred for 0.5 hr at room temperature and then centrifuged at 13,000 xg for 20 min at 4 ^{0}C. The supernatant was used for assay of ionically bound cell wall PO. After being dialyzed against water and lyophilized to dryness, the samples were redissolved in water, centrifuged again at 13,000 xg for 5 min at 4 ^{0}C. The supernatant was used for IEF-PAGE analysis.

Electrophoresis of PO

Native activity PAGE (Hames, 1981) was used to separate the acidic cytoplasmic PO. After electrophoresis, PO isozymes were visualized by staining the gel with 200 ml of 0.05 M sodium acetate buffer solution (pH 5.5) containing 40 mg of 3-amino-9-ethylcarbazole, 10 ml of N,N, dimethylformamide and 63 μl of 30% H_2O_2 (Graham *et al*, 1965).

A commercially available, pre-made IEF gel (pH 3-10, 124 x 129 x 3.75 mm, ISOLAB, INC., Akron, OH, USA) was used for separating isozymes of ionically bound cell wall PO. The anode buffer was made up with 1 M H_3PO_4 and the cathode buffer contained 1 M ethanolamine, 30 mM arginine and 30 mM lysine. The gel was run at 10 ^{0}C at 5W for 10 min, 10W for 45 min, 15W for 15 min and finally at 20-25W for 5 min. The Protein Test Mixture 9 from Serva company was used as PI standards. Following electrophoresis, the gel was also stained with the acetate buffer to visualize the isozymes. Isoelectric points of PO isozymes were determined by running the protein standards with the samples.

Slot blot analysis of PAL mRNA

The procedure described by Yeh *et al* (1991) for total RNA extraction was followed. At intervals after inoculation with *F. sambucinum*, total RNA was isolated from the top 0.5 mm layers of the discs and 7.5 μg of each RNA sample was used for slot blot analysis. The procedure described by the manufacturer (Schleicher and Schuell) was followed. Briefly, the total RNA sample was mixed with water to a volume of 100 μl and then added to 300 μl of 6.15 M formaldehyde in 10 x SSC. After incubated at 65 ^0C for 15 min, the samples were loaded onto the slot blotter and house vacuum was applied. When the samples were drained out under vacuum, the wells were then washed with 400 μl of 10 x SSC under vacuum until it was completely drained out. After the samples were transferred onto the membrane, the membrane was baked at 80 ^0C for two hr in preparation for pre-hybridization. Two hour pre-hybridization and overnight hybridization were performed at 42 ^0C as described by Miniatis *et al* (1982). The ^{32}P-labelled PAL cDNA clone from potato was used as a probe. The clone was kindly provided by Dr. K. Hahlbrock (Fritzemeier *et al*, 1987). The procedures described by Feinberg and Vogelstein (1983) were used for random priming synthesis of ^{32}P-labeled probe. Following hybridization, the blot was finally washed in 0.1 x SSC at 65 ^0C for 10 min and exposed to X-ray film.

Measurements of lignin and chlorogenic acid content

The top 0.5 mm layers of discs were also used for the measurements of lignin-like materials and chlorogenic acid content. The thioglycolate procedure described by Hammerschmidt (1984) was used for lignin assay. The procedure from Sheen (1971) was used for chlorogenic acid content measurement.

Effect of aging of tuber discs on *F. sambucinum* infection

The discs were aged in the moist dishes at RT for different time of periods and then inoculated with 50 μl of the fungal spore suspension at concentration of 2 x 10^6 spore/ml. The disease development in the discs was recorded three days following inoculation.

Effect of wounding of aged tuber discs on *F. sambucinum* infection

At different time points of aging, a ca. 2 mm deep wedge was cut on the top tissue of each disc and 50 μl of the fungal spore suspension at the same concentration was applied immediately after cutting. Three days following inoculation, the disease development was recorded.

RESULTS

Light microscopic observation of *F. sambucinum* infection in potato tuber tissue

At 12 hr after *F. sambucinum* infection, the tissue stained with toluidine blue showed some purple-blue in the cell walls of cells adjacent to the cut surface (large arrow head), which indicated deposition of phenolics (Fig. 1-1 A and B). The fungal hyphae were stained as red to purple (small arrow head) (Fig. 1-1 A and B). At 24 hr, deposition of phenolics (purple-blue) and lignin-like materials (purple-green) was observed (large arrow head, Fig. 1-3 A and B) in almost the whole cell walls of cells next to the cut surface. In the control, the cell walls were stained purple-blue indicating that phenolics were deposited (Fig. 1-2). A large number of fungal hyphae were present in the cut surface and on both sides of lignified cell walls indicating either fungal penetration was completed before the cell walls were lignified or the hyphae were able to penetrate the partially lignified cell walls (Fig. 1-3 A and B).

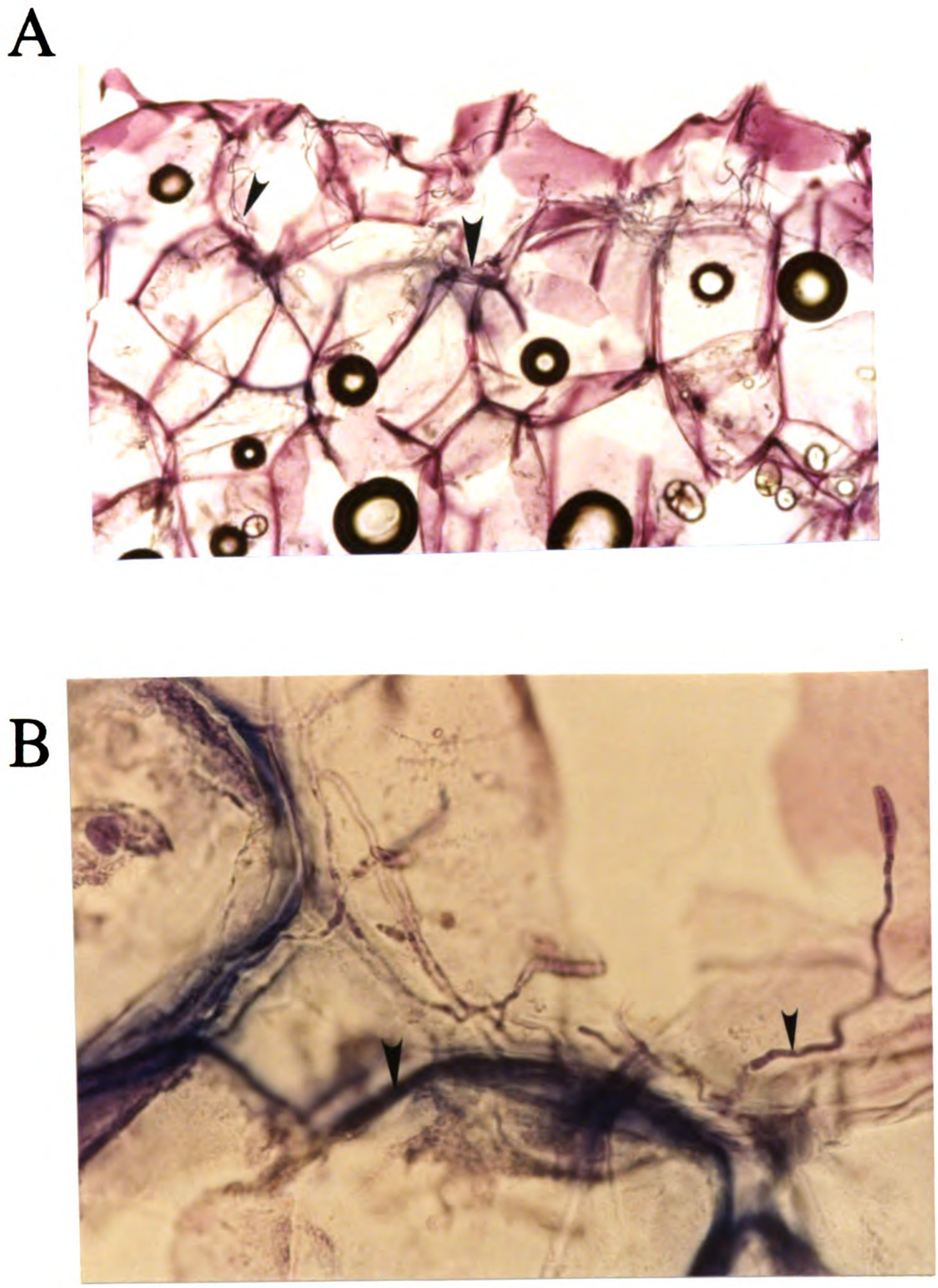


Fig. 1-1. Light micrograph of toluidine blue stained longitudinal sections of potato tuber disc at 12 hr after inoculation with *F. sambucinum*. Note the fungal hypha (small arrow head) and purple-blue cell walls (large arrow head) indicating deposition of phenolics in both panels A and B. A, 100x; B, 400x magnification.

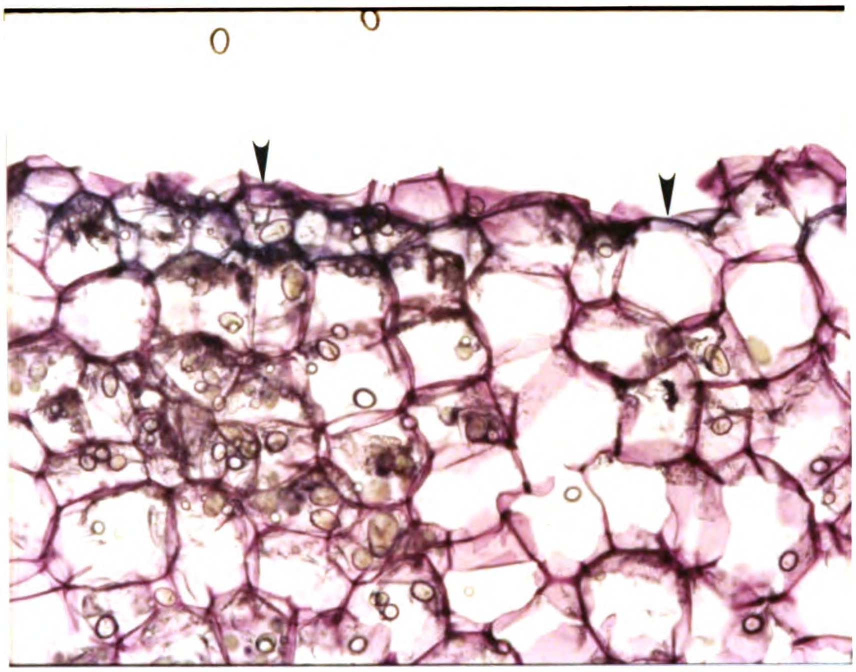


Fig. 1-2. Light micrograph of longitudinal section of un-inoculated potato tuber discs 24 hr after slicing. Note purple blue cell walls (arrow head) indicating deposition of phenolics. 50x magnification.

A

B

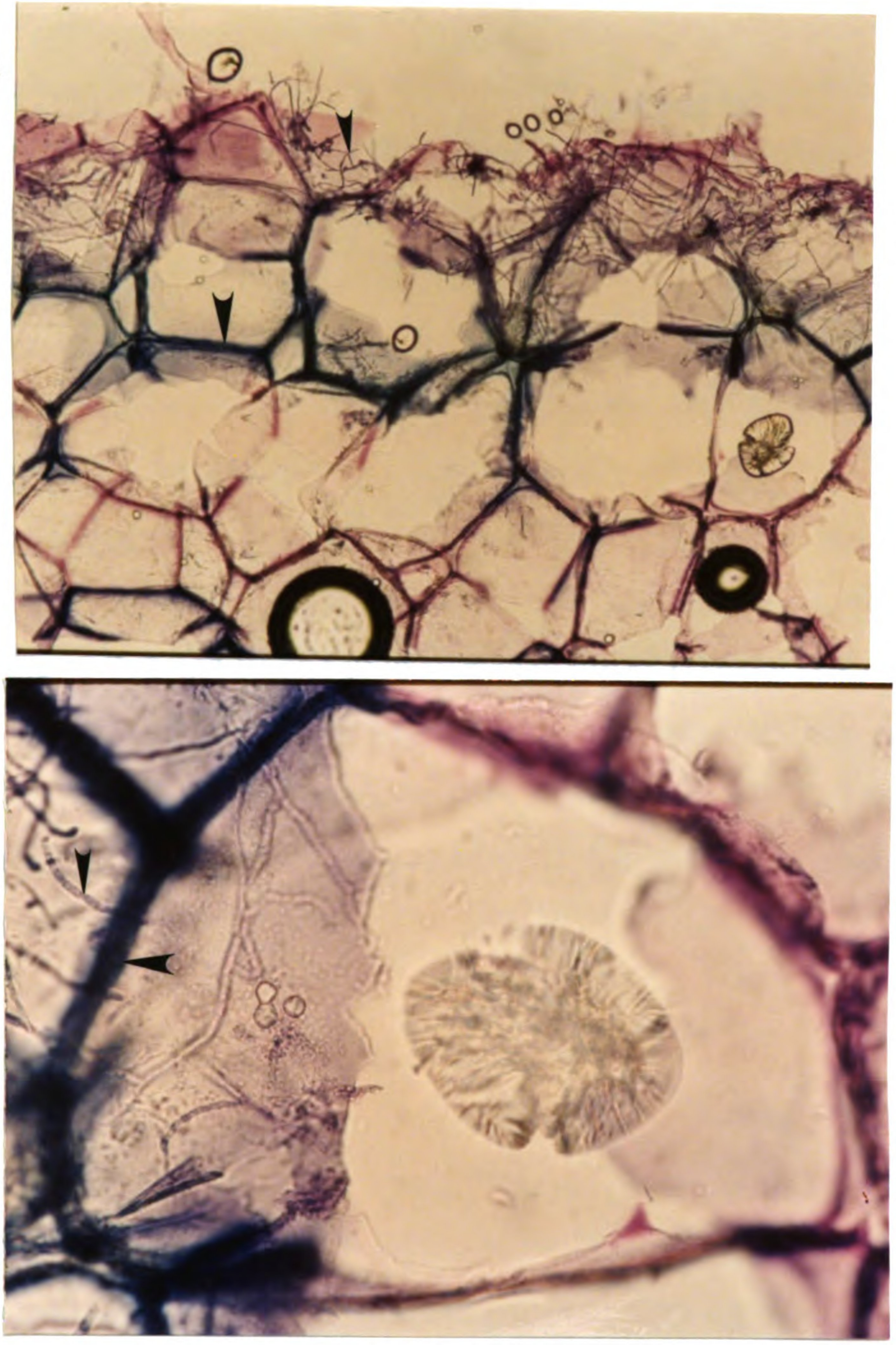


Fig. 1-3. Light micrograph of longitudinal sections of potato tuber discs 24 hr after inoculation with *F. sambucinum*. Note the fungal hyphae (small arrow head) and host cell walls (large arrow head) indicating deposition of phenolics (purple-blue in A and B) and some lignin-like materials (purple-green in A). A, 100x; B, 400x magnification.

Electron microscopic observation of *F. sambucinum* infection in potato tuber tissue

Scanning electron microscopic observation of *F. sambucinum* infected tuber tissue revealed that the fungus had germinated at 6 hr after inoculation (Fig. 1-4 A and B). At 12 hr, a large numbers of hyphae could be seen in the infected tissue (Fig. 1-5 A and B). At 24 hr, more hyphae were present (Fig. 1-6 A) and penetration of the cell wall by some hyphae was also observed (Fig. 1-6 B and C). The cell wall materials around penetrating hyphae seemed to be dissolved indicating enzymatic degradation (Fig. 1-6 B and C).

Transmission electron microscopic observation of infected tissue at 24 hr after inoculation showed the fungal hyphae (H) were present on and in the host cell wall (W) (Fig. 1-7 A and B) and in intercellular spaces between cell walls (Fig. 1-8 A and B), indicating enzymatic degradation of host cell wall (Fig. 1-7 A and B, arrow heads) and of the middle lamella region (Fig. 1-8 A and B, ML) surrounding the fungal hyphae. This suggested that pectolytic enzymes were involved in the infection process.

Production of pectolytic enzyme by *F. sambucinum*

To confirm production of polygalacturonase (PG) and pectate lyase (PL) by *F. sambucinum*, a plate assay was used. At lower pH (5.5) in the presence of polygalacturonate, the fungus produced PG, which breaks down polygalacturonate into acid soluble oligomers. After acidifying the medium, the areas surrounding fungal colonies became clear while the rest of the medium precipitated (Fig. 1-9 A). At high pH (8.0) in the presence of polygalacturonate, PL production by the fungus was also detected (Fig. 1-9 B).

A

B

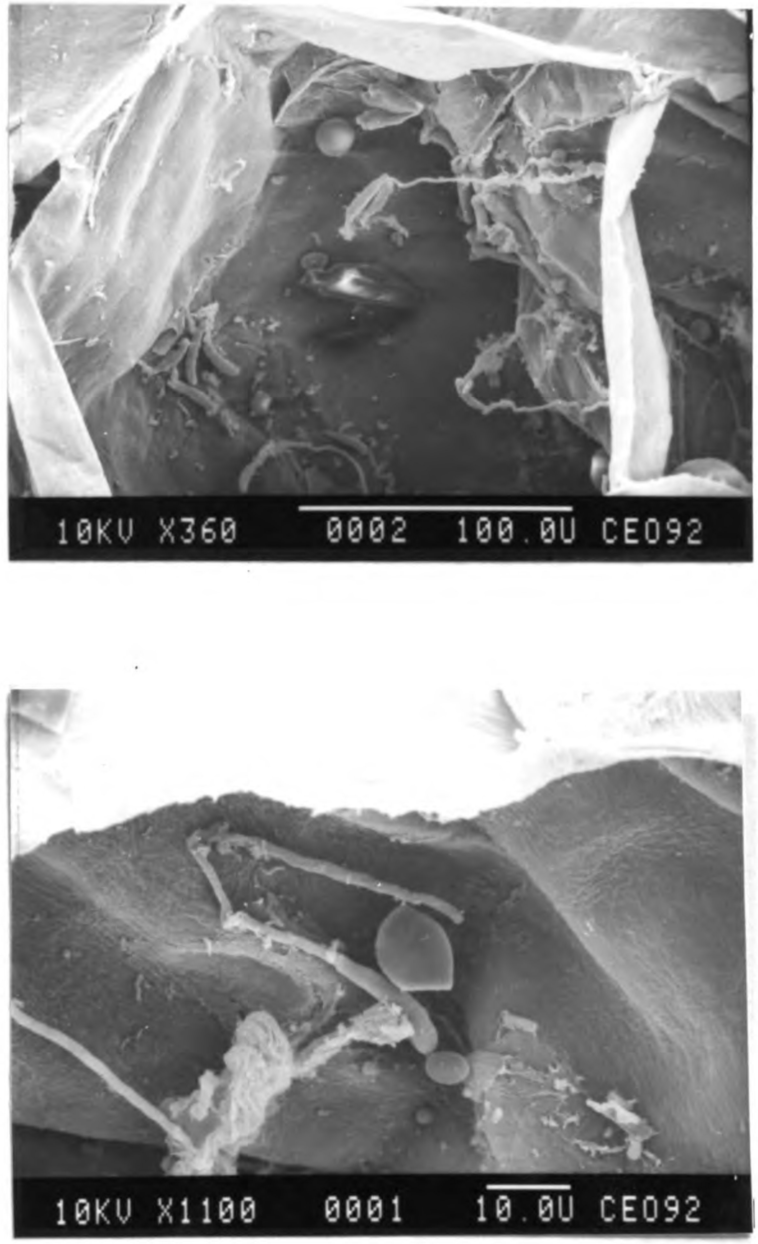
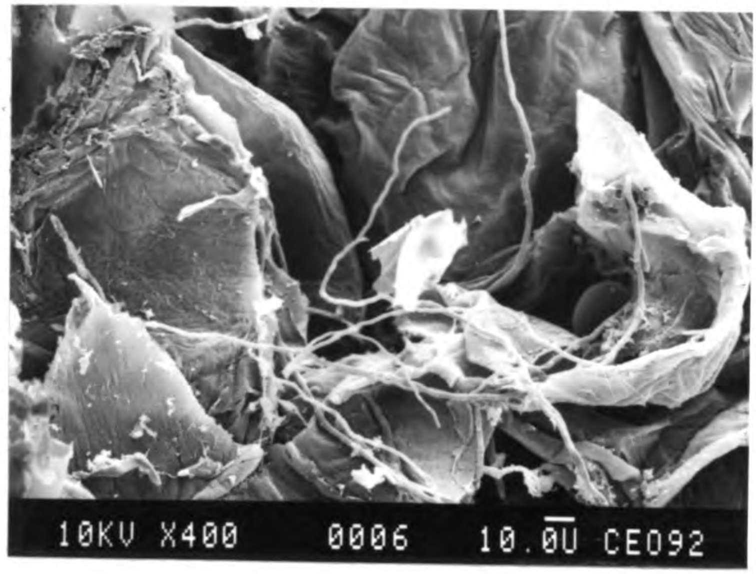


Fig. 1-4. SEM micrograph of surface of potato tuber disc 6 hr after inoculation with *F. sambucinum* showing germinating spores. A, 360x; B, 1,100x magnification.

A



B

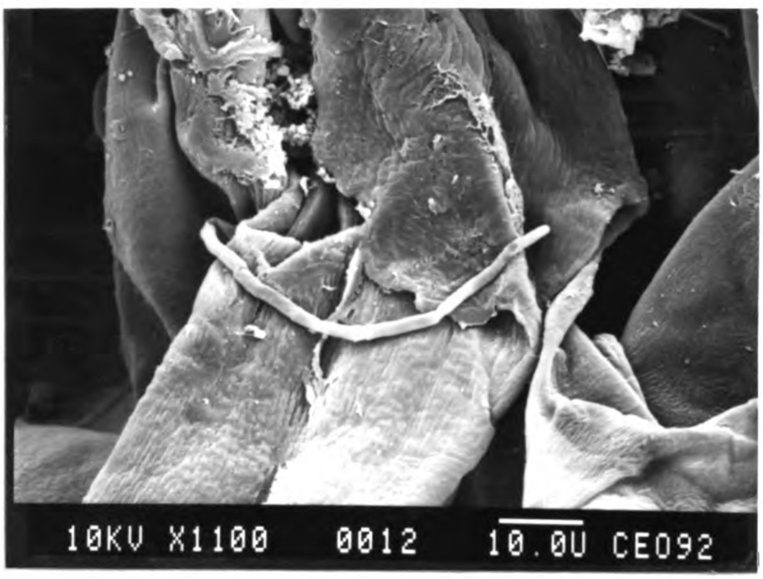


Fig. 1-5. SEM micrograph of surface of potato tuber disc at 12 hr after inoculation with *F. sambucinum* showing the fungal hyphae. A, 400x; B, 1,100x magnification.

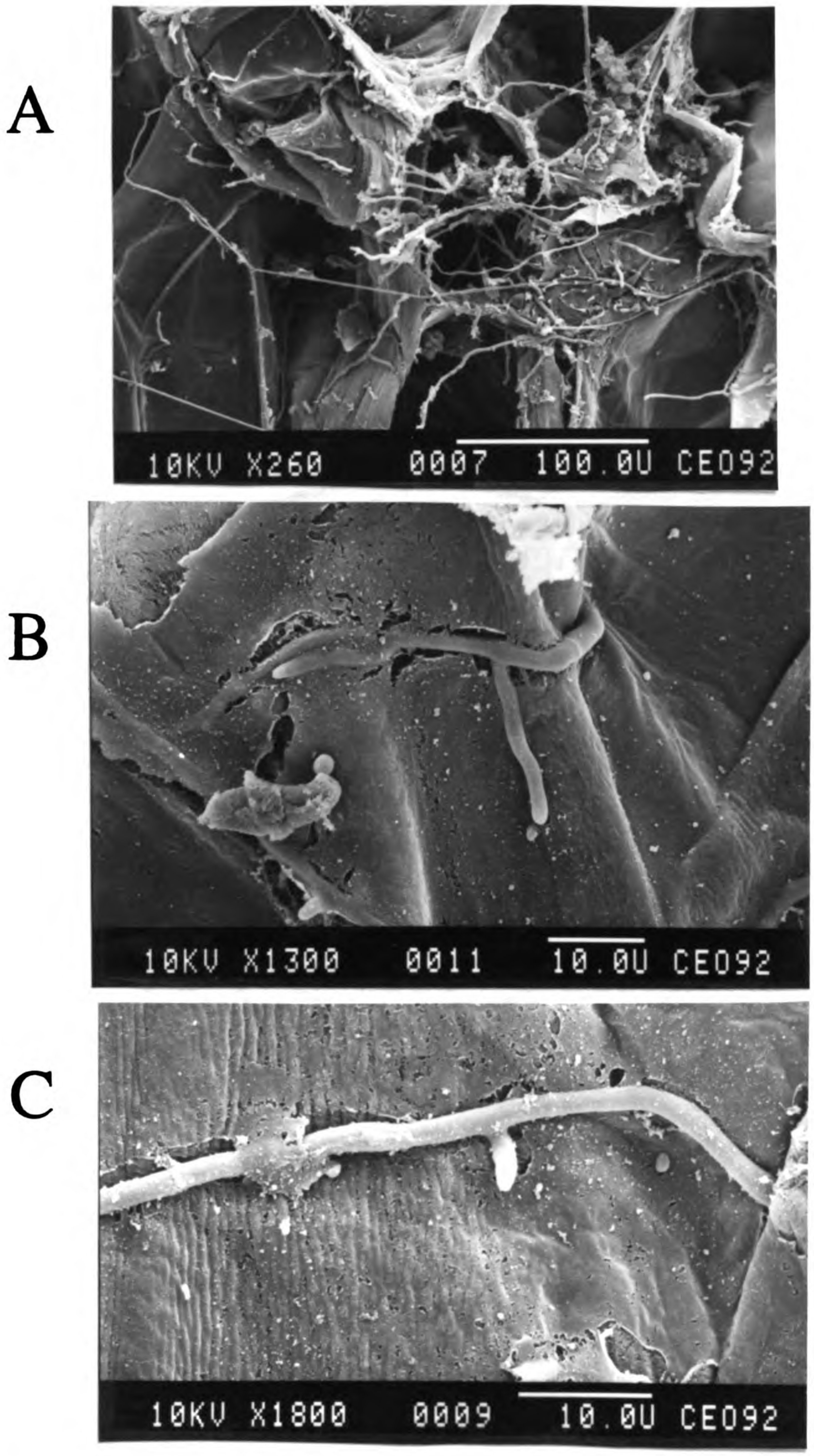
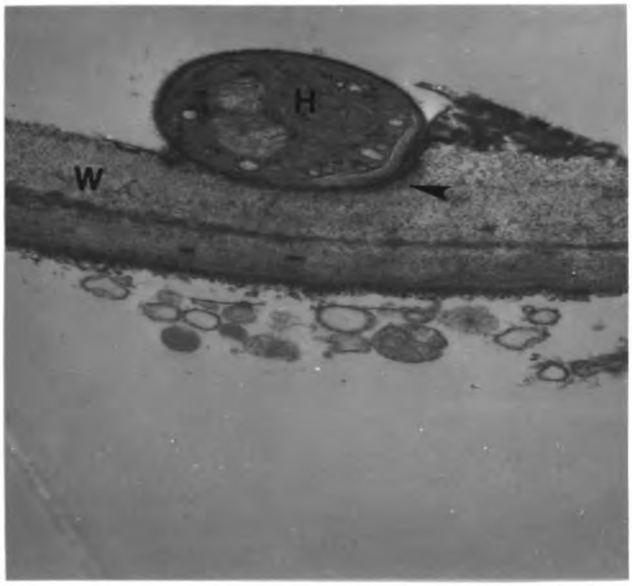


Fig. 1-6. SEM micrograph of surface of potato tuber disc 24 hr after inoculation with *F. sambucinum* showing the growing fungal hyphae (A) and penetration and degradation of host cell wall by the hyphae (B and C). A, 260x; B, 1,300x; C, 1,800 magnification.

A



B

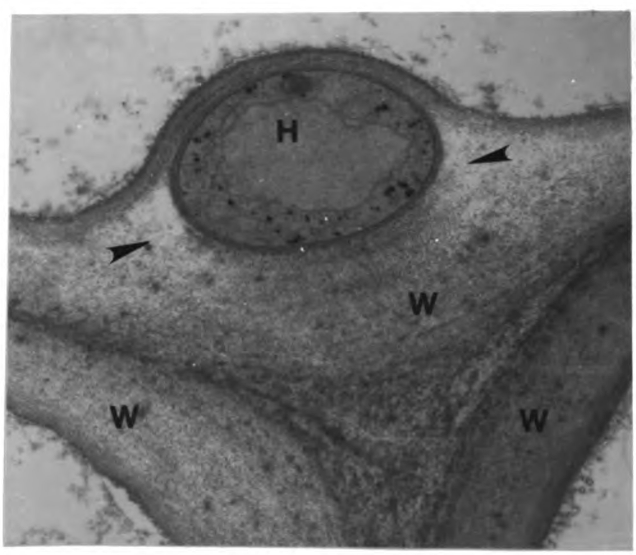
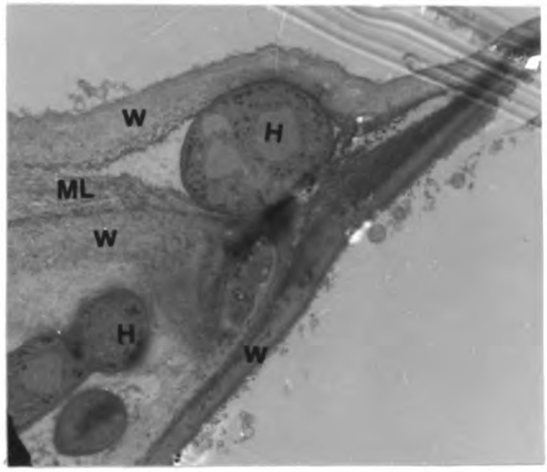


Fig. 1-7. TEM micrograph of longitudinal sections of potato tuber discs 24 hr after inoculation with *F. sambucinum* showing the fungal hypha (H) on and in the host cell wall (W) (panel A and B) indicating enzymatic degradation of the host cell wall (arrow heads). A, 15,000x; B, 11,500x magnification.

A



B

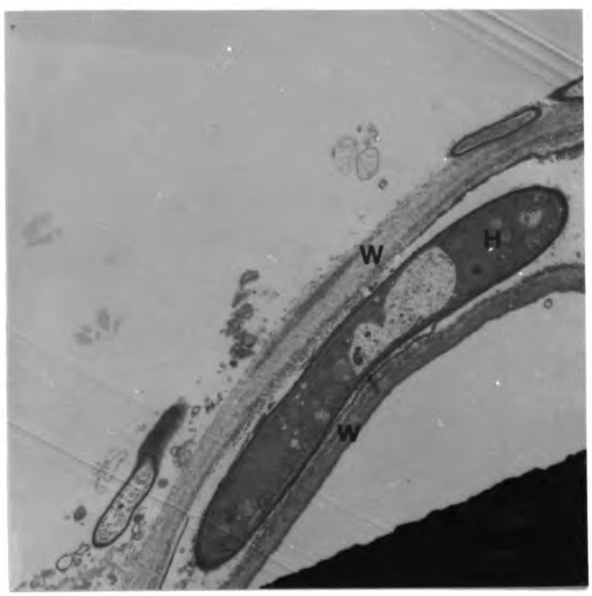


Fig. 1-8. TEM micrograph of longitudinal sections of potato tuber discs 24 hr after inoculation with *F. sambucinum* showing fungal hypha (H) growing in the middle lamella (ML) between host cell walls (W) (panel A and B), indicating enzymatic degradation of middle lamella. A, 3,900x; B, 3,900x magnification.

A

B

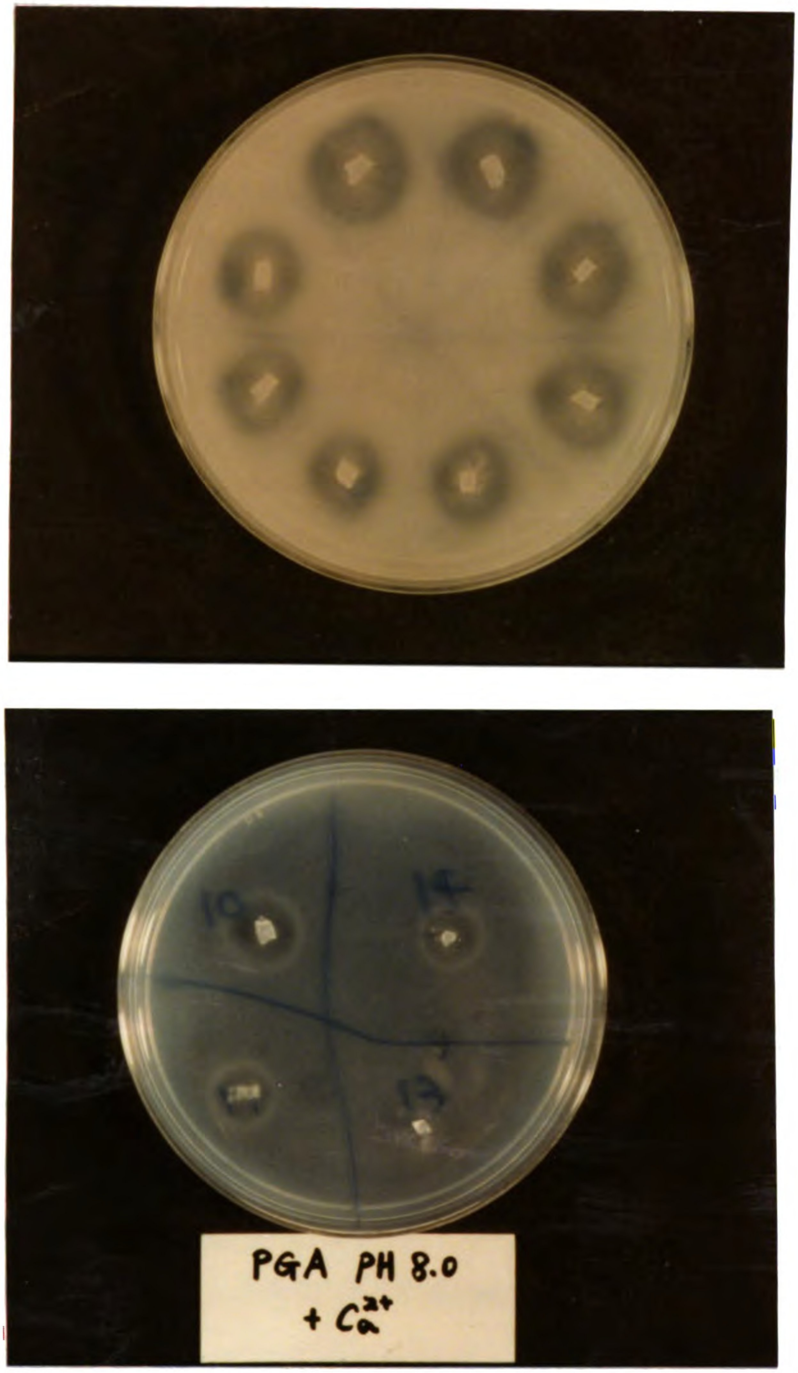


Fig. 1-9. Plate assay of polygalacturonase (PG) and pectate lyase (PL) activity produced by *F. sambucinum*. The clear halos indicate presence of PG and PL activity in panel A and B, respectively.

PAL activity and slot blot analysis of PAL mRNA

PAL activity was enhanced through the duration of experiment (Fig. 1-10). The induction of PAL activity occurred within several hours after inoculation with *F. sambucinum* and continued to increase at approximately three-fold rate over the control till 24 hr.

Slot blot analysis (Fig. 1-11) showed that in the inoculated tissue PAL mRNA accumulated to a higher level over the control by three hr after inoculation with *F. sambucinum* and continued to accumulate through the following 24 hr. PAL mRNA also accumulated at lower rate in the control tissue starting at 3 hr and reached its highest point at 6 hr. Then the PAL mRNA level declined almost to zero.

Activity of soluble cytoplasmic and ionically bound cell wall PO and their isozyme patterns

The activity of soluble cytoplasmic PO in both control and inoculated tissues increased after 12 hr (Fig 1-12 A). In the inoculated tissue, the induced PO activity increased at higher rate and it was 3-fold over the control by 24 hr.

Ionically bound cell wall PO showed a faster increase in activity in the inoculated tissue (Fig. 1-12 B). This induced PO activity was seen at 12 hr and continued through the rest of experiment. The ionically bound PO level was increased 3-fold at 12 hr and 9-fold at 24 hr over the control. In contrary, the increased PO activity in the control was kept at a extremely low rate through out the time of experiment.

Both acidic and basic cytoplasmic PO isozyme profiles are shown in Fig. 1-13 A. On IEF polyacrylamide gel (pH 3-10), both PO from control and

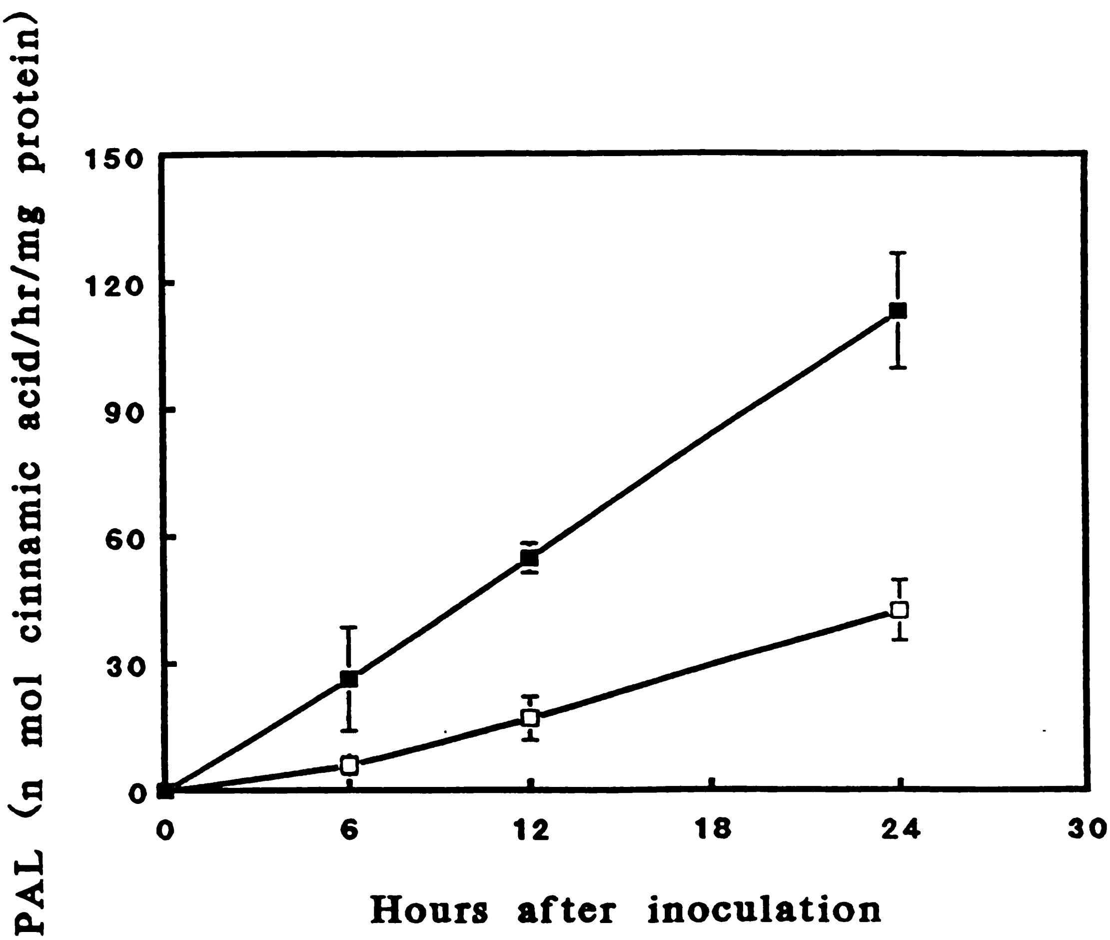


Fig. 1-10. Time course of PAL activity in potato tuber discs after inoculation with *F. sambucinum*. Control (-□-), inoculated (-■-).

In this study, every experiment was repeated at least twice and each data point contained three replicates. A representing experimental data were shown. Bars=SD.

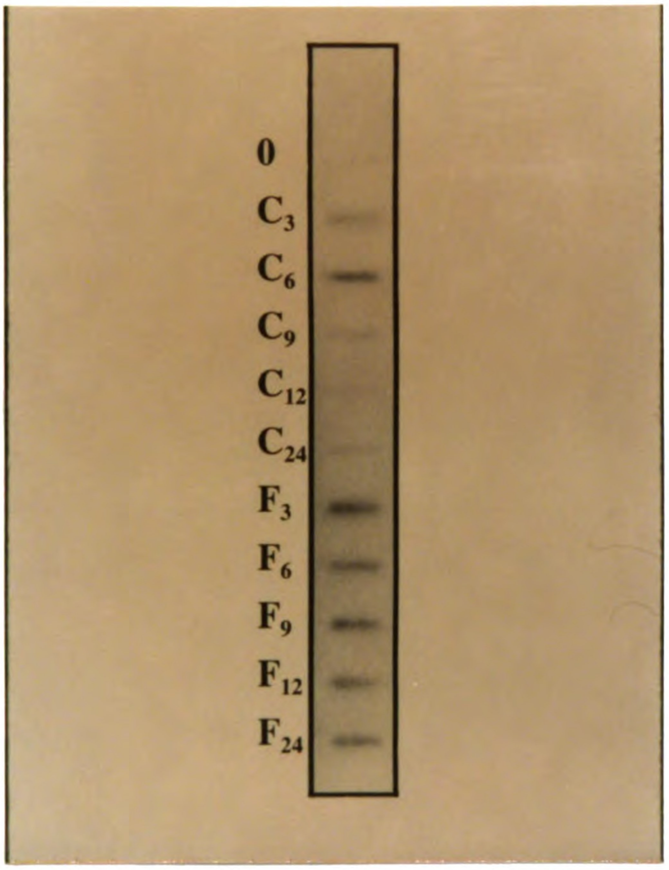


Fig. 1-11. Slot blot analysis of total RNA from potato tuber discs at different time after inoculation with *F. sambucinum*. In order to insure a uniform amount of RNA was loaded in each slot, the RNA samples were electrophoresed in agarose gel and stained with ethidium bromide. The approximately equal intensity of ribosomal bands was observed. Total RNA (7.5 μg) treated with RNAse-free DNAse was used for each sample and the blot was probed with PAL cDNA from potato. Each sample contained two replicates. C, control; F, there inoculated. The numbers represent hours after slicing or inoculation.

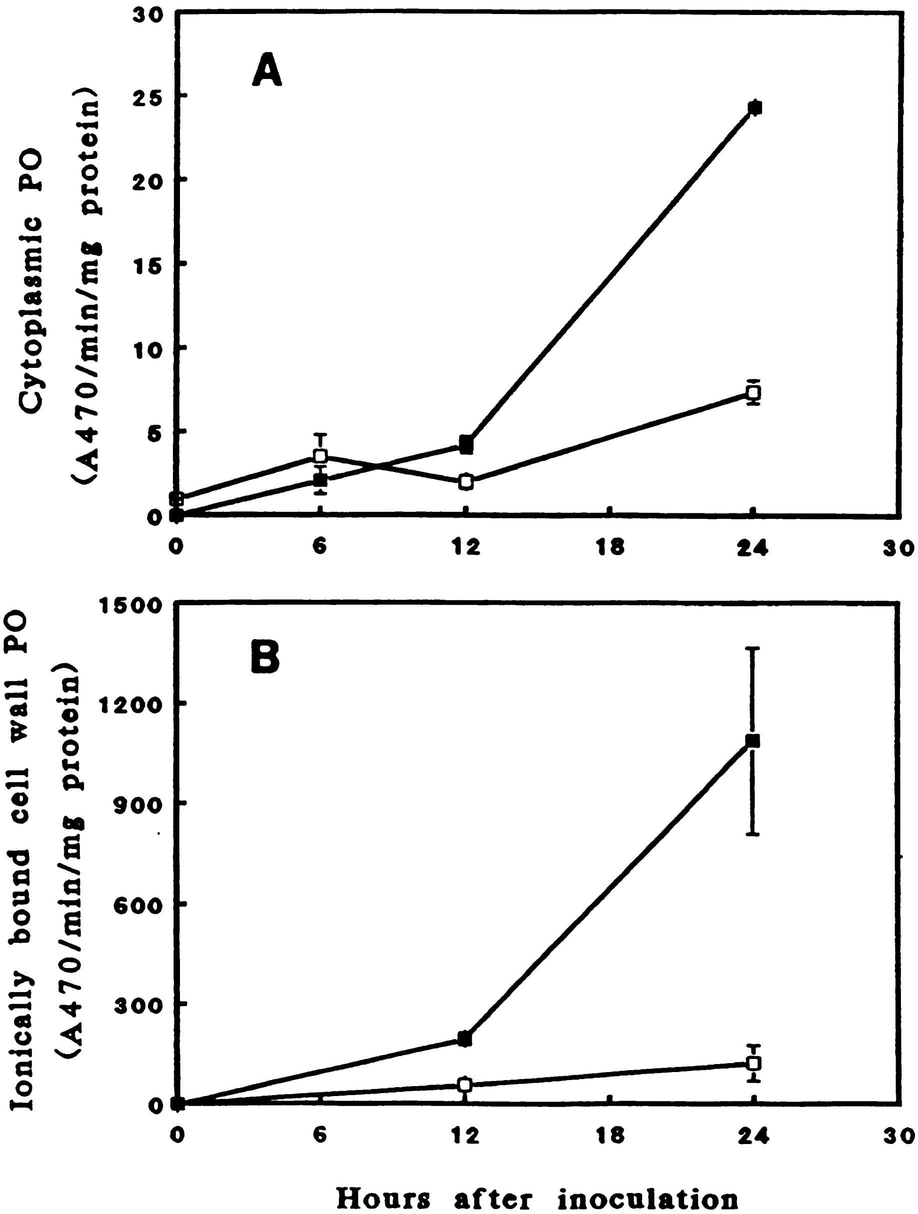


Fig. 1-12. Time course of (A) soluble cytoplasmic and (B) ionically bound cell wall PO from potato tuber discs after inoculation with *F. sambucinum*. Control (-□-), inoculated (-■-).

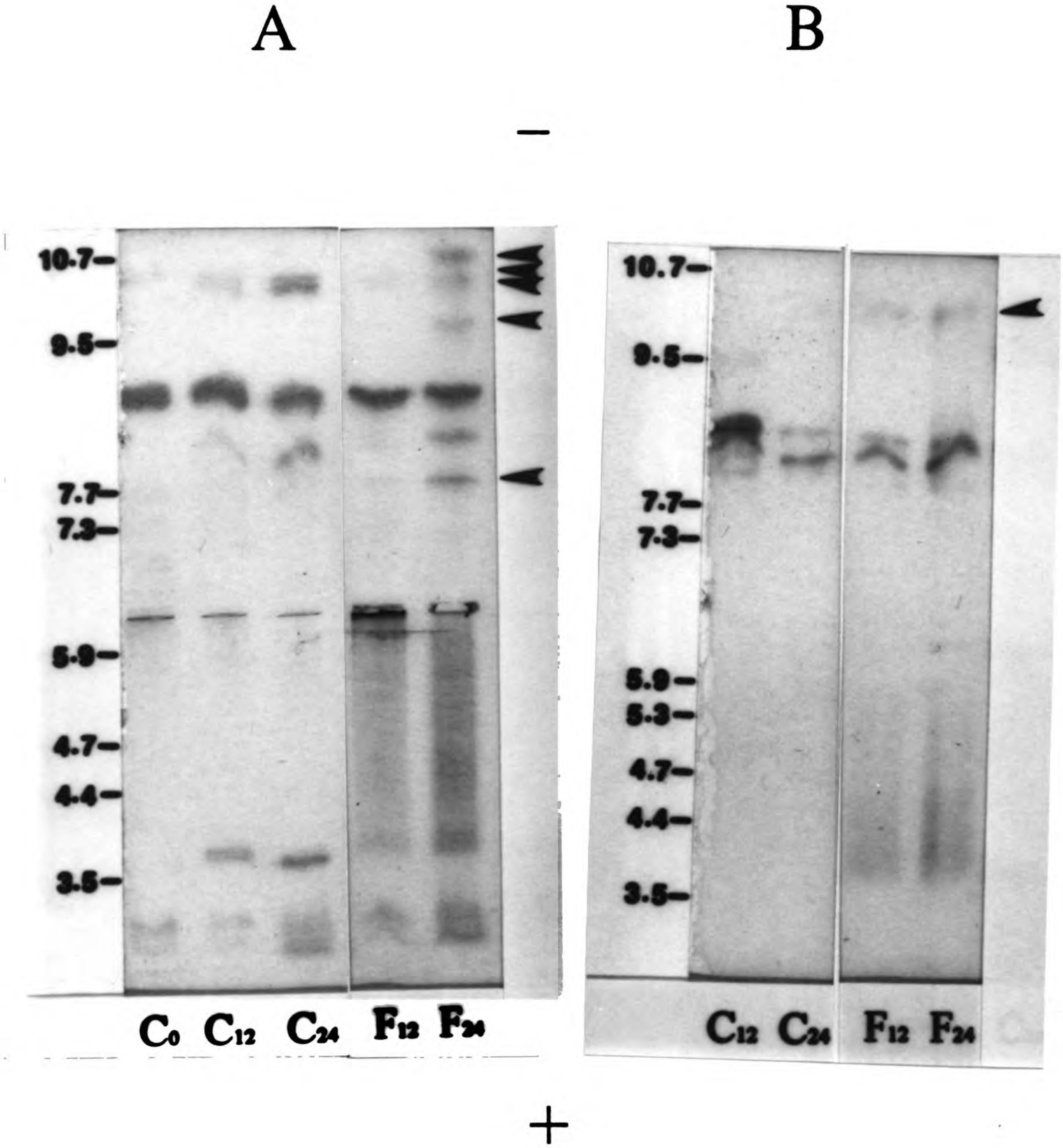


Fig. 1-13. IEF-PAGE (pH 3-10) of cytoplasmic (A) and ionically bound cell wall PO (B) isozymes from potato tuber discs inoculated with *F. sambucinum*. In A, 3 μg of sample protein was loaded in each lane. The arrow heads indicate the PO isozymes with increased or decreased activity. In B, 0.1 μg of sample protein was loaded in each lane. The arrow head indicates the isozyme with increased activity. Control (C), inoculated (F). The numbers represent the hours after inoculation.

inoculated tissue showed a similar isozyme banding pattern. However, at 24 hr there were at least three basic isozymes with approximate PI of 10.6, 9.7 and 7.7 (the first, fourth and the last arrow heads from the top of the gel) showing an increase in activity in inoculated tissue and two basic isozymes with approximate PI of 10.1 and 9.9 (the second and the third arrow heads from the top) showed decreased intensity. Acidic PO isozymes in inoculated tissue gave a smearing appearance on IEF gel.

IEF-PAGE of ionically bound cell wall PO from both control and the inoculated tissue also showed a similar banding pattern (Fig. 1-13 B). However, in the inoculated tissue an isozyme with a PI of 10 (arrow head) was slightly increased in its intensity on the gel and there also was smearing of the acidic isozyme. No acidic isozyme activity was detected in the control.

Accumulation of chlorogenic acid (CGA) and lignin-like materials

CGA accumulated in both control and inoculated tissue (Fig. 1-14 A). In control tissue, CGA accumulated after slicing. In contrast, CGA level did not change in the inoculated tissue through out the experimental period. In the first 12 hr, there were no detectable lignin-like materials accumulated in the control (Fig. 1-14 B), while in the inoculated tissue a low level of lignin-materials had been deposited. After 12 hr, both the control and the inoculated tissues accumulated lignin-like materials at a similar rate through 24 hr.

Effects of aging and wounding of aged tuber discs on the fungal infection

Table I shows the effects of aging and wounding of aged tuber discs on *F. sambucinum* infection. Aging of the tuber discs for two days greatly reduced the fungal infection. After three days of aging, no infection occurred in the discs.

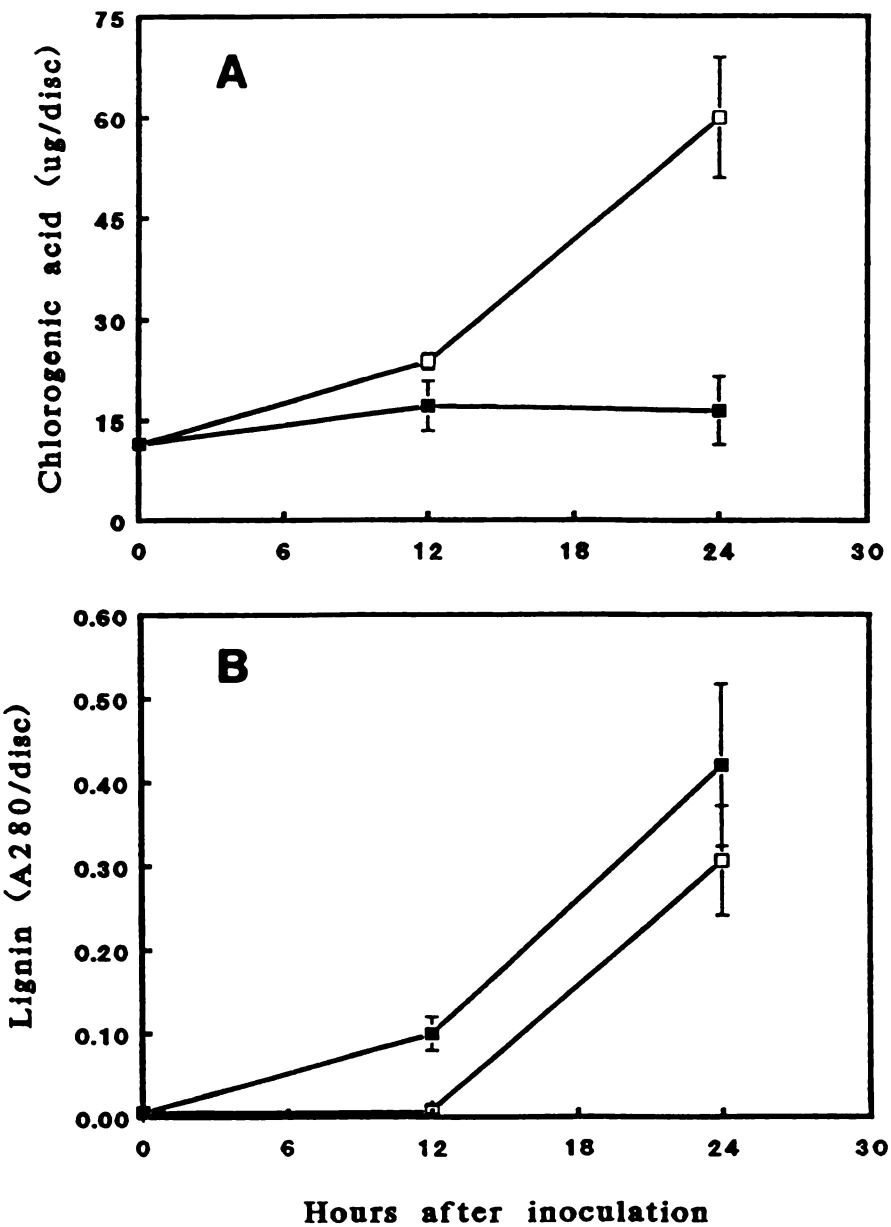


Fig. 1-14. Time course of (A) chlorogenic acid (CGA) and (B) lignin-like materials accumulated in potato tuber discs inoculated with *F. sambucinum*. Control (-□-), inoculated (-■-).

Table I.

Effects of aging and wounding of aged tuber discs on F. sambucinum *infection*

Time of aging (hr)	Disease ratings[a]	
	Aging only[b]	Aging plus wounding[c]
0	+++	+++
24	+	+
48	—	—
72	—	—

[a]Disease ratings were recorded at three days after inoculation. +++ =Top layer tissue of the discs was macerated and became dark brown. + =Slightly browning. — =No maceration nor browning.
[b]The discs were incubated in the moist Petri dishes at RT.
[c]Ca. 2 mm wedge was cut at intervals of aging immediately prior to inoculation.

Wounding of aged tuber discs by cutting a 2 mm deep wedge on the surface of the discs could not accelerate infection by the fungus (Table I). The reduction rate of infection was similar to that of aged tuber discs without wounding. There was no infection detected after three days of aging.

DISCUSSION

LM and SEM observations of potato tuber tissue inoculated with *F. sambucinum* revealed the early establishment of disease infection. Most fungal spores inoculated to the tuber discs germinated by 6 hr (Fig. 1-4 A and B) and the fungal hyphae penetrated into host tissue after 12 hr (Fig. 1-1 A and B). By 24 hr, the fungus had penetrated into 2-3 layer of cells (Fig. 1-3 A). During the first 12 hr of inoculation, no obvious changes were seen in the infected cells as compared to the control. At 12 hr, very little lignin-like materials (blue-green) but phenolics (purple-blue) were detected in the wall of cells adjacent to the cut surface. By this time the fungus had entered into 1-2 layers of host cells (Fig. 1-1 A). The slow host response may indicate that the pathogen somehow escaped host defense mechanisms. It was also noticed that there were fungal hyphae in both sides of some cell walls with some deposited lignin, indicating either the penetration was completed before deposition of lignin or the hyphae were able to penetrate the partially lignified cell walls (Fig. 1-3 A and B).

Cell wall degradation by cell wall degrading enzymes produced by pathogens is found in a wide range of diseases, especially those caused by nectrophic pathogens. Among the cell wall degrading enzymes, endo-polygalacturonase (PG) and pectate lyase (PL) are found to be most responsible for degradation of cell middle lamella resulting in tissue maceration, which is commonly seen in diseases caused by nectrophic fungal pathogens (Cooper, 1983). The plate assay performed in the present study clearly demonstrated that *F. sambucinum*, which is nectrophic fungal pathogen, produced PG and PL in the presence of polygalacturonate *in vitro* (Fig. 1-9 A and B). Although this is not a direct evidence that the pathogen produces the enzymes *in vivo*, the tissue maceration and the degradation of cell wall (Fig. 1-6 B and C, 1-7 A and B) and the middle lamella (Fig. 1-8 A and B) during the disease development strongly suggested that the cell wall degrading enzymes, especially endo-PG was highly involved in pathogenesis of this disease. Further study is needed to determine how many cell wall degrading enzymes are produced during infection and their roles in disease pathogenesis.

At physiology and biochemistry level, the host cells did show some responses to the fungal infection at early stages. PAL, the first enzyme in phenylpropanoid metabolism, showed a 2-3 fold increase in the inoculated tissue at as early as 6 hr (Fig. 1-10). The increased PAL activity was preceded by the accumulation of PAL mRNA level (Fig. 1-11). Since PAL plays a important role in phenylpropanoid metabolism (Jones, 1984), the increase in PAL activity clearly indicated a change in the metabolism of the host in response to the fungal infection. The host response was also evident by the increase in synthesis of phenolic compounds including lignin-like materials shown in Fig. 1-14 B. Although this accumulation was detected between 6-12 hr after inoculation, and it was not detected in the control tissue, the amount of lignin-like materials either did not reach the point at which the lignified cell wall could function as physical barrier against the fungal invasion, or the lignin deposition occurred after the penetration by the fungus.

It was also observed that the production of chlorogenic acid (CGA), which normally accumulates at a high rate after wounding (Kuć, 1982), was suppressed (Fig. 1-14 A) by the pathogen. Generally, it is considered that CGA and its oxidation products such as quinones which have anti-microbial activity may have a role in plant general defence response (Friend, 1981; Kuć, 1972). The suppression of CGA production indicated that the fungus somehow escaped from host defense responses by suppressing host defense mechanism(s).

The host response to the pathogen infection also involved in the increase in activity of both soluble cytoplasmic peroxidase (PO) and ionically bound cell wall PO (Fig. 1-12 A and B). Compared to the control, ionically bound cell wall PO activity increased after 12 hr following infection and reached a 9-fold level at 24 hr. Since cell wall is the place where polyphenolics including lignin are polymerized and deposited (Friend, 1981; Grisebach, 1981; Whitmore, 1976), the increase in cell wall PO activity may significantly reflect more about the rate of lignin and other polyphenolics deposition in the cell wall than that of cytoplasmic PO. In this study, the activity of cell wall PO was 40 times (absolute values) greater than that of cytoplasmic PO at 24 hr after inoculation. On IEF-PAGE gel both POs showed that basic PO isozymes with high PI changed their activity after infection (Fig. 1-13 A and B). This also indicated that basic isozymes may be more sensitive to pathogen infection than the acidic PO isozymes, which are usually thought to be associated with lignification in plants (Lagrimini, 1991; Espelie *et al*, 1986; Espelie and Kolattukudy, 1985). It is not known why the acidic isozyme from infected tissues smeared on the gels. It is possible that some acidic isozymes released from the middle lamella were partially degraded by pathogen cell wall degrading enzymes and contained different sizes of sugar chains resulting in no distinct molecular weight. Alternatively, the acidic PO isozymes were partially degraded in macerated tissue by cytoplasmic enzymes. From the results of PO activity in this study, it is obvious that only by fully understanding the function of each PO isozyme can we elucidate the role of PO in plant defense responses.

Aging of wounded potato tuber slices has been known to reduce pathogen infections. This reduction is thought to be due to two main factors: (a). Wound healing in which the suberized wound-periderm not only prevents water loss but also functions as a physical barrier which restricts pathogen invasion (Bostock and Stermer, 1989; Escande and Echandi, 1988; O'Brien and Leach, 1983); (b). Synthesis of resistance factors beneath the wounded surface which inhibit either cell wall degrading enzymes produced by pathogens (Zucker and Hankin, 1970) or are toxic to pathogens. The two main steroid glycoalkaloids (SGA), α-solanine and α-chaconine produced in potato tuber tissue after wounding may belong to the resistance factors. The toxicity of this two SGA to microorganisms and insects and even animals makes it very possible that they play an important role in general chemical defense response of host (Allen and Kuć, 1968; Lisker and Kuć, 1977; McKee, 1955; 1959; Sinden *et al*, 1973). In present study, two days of aging of potato tuber discs at RT greatly reduced infection by the fungus (Table I). There was no detectable disease caused by the fungus in the discs aged for three or more days. This may be due to the suberized cell wall of wounded surface tissue, which functions as physical barrier to reduce the fungal invasion. However, the fact that wounding of the surface of aged discs could not enhance the fungal infection strongly indicated that there must be chemical defense mechanism(s) functioning beneath the wounded surface. Further study is needed to elucidate this defense mechanism(s).

REFERENCES

Allen E, Kuć J 1968 α-Solanine and α-chaconine as fungitoxic compounds in extracts of Irish potato tubers. Phytopathology 58:776-781.

Borchert R 1978 Time course and spatial distribution of phenylalanine ammonia-lyase and peroxidase activity in wounded potato tuber tissue. Plant Physiology 62: 789-793.

Bostock RM, Stermer BA 1989 Perspectives of wound healing in resistance to pathogens. Annul Review of Phytopathology 27:343-471.

Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72: 248-254.

Cooper, RM 1983 The mechanisms and significance of enzymic degradation of host cell walls by parasites. In Callow JA ed. Biochemical Plant Pathology pp. 101-136.

Escande AR, Echandi E 1988 Wound-healing and the effect of soil temperature, cultivars and protective chemicals on wound healed potato seed pieces inoculated with seed piece decay fungi and bacteria. American Potato Journal 65:741-752.

Espelie KE, Kolattukudy PE 1985 Purification and characterization of an abscisic acid-inducible anionic peroxidase associated with suberization in potato (*Solanum tuberosum*). Archives of Biochemistry and Biophysics 24: 539-545.

Espelie KE, Frenceschi VR, Kolattukudy PE 1986 Immunocytochemical localization and time course of appearance of an anionic peroxidase associated with suberization in wound-healing potato tuber tissue. Plant Physiology 81: 487-492.

Feinberg AP, Vogelstein B 1983 A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Analytical Biochemistry 132: 6-13.

Friend J 1981 Plant phenolics, lignification and plant disease. Progress in Phytochemistry 7: 197-261.

Graham RC, Lundholm U, Karnovsky MJ 1965 Cytochemical determination of peroxidase activity with 3-amino-9-ethylcarbazole. Journal of Histochemistry and

Cytochemistry 13: 150-152.

Grisebach H. 1981 Lignins. In: Stumpf PK, Conn EE ed The Biochemistry of Plants. Academic Press, New York 457-478.

Grison R, Pilet PE 1985 Cytoplasmic and cell wall isoperoxidases in growing maize roots. J. Plant Physiology 118: 189-199.

Gross GG 1980 The biochemistry of lignification. In: Woolhouse HW ed. Advances in Botanical Research. Academic Press, London, 25-63.

Hames BD 1981 An introduction to polyacrylamide gel electrophoresis. In: Hames BD, Rickwood D ed. Gel Electrophoresis of Proteins. IRL Press, 1-86.

Hammerschmidt R 1984 Rapid deposition of lignin in potato tuber tissue as a response to fungi non-pathogenic on potato. Physiological Plant Pathology 24: 33-42.

Jones DH 1984 Phenylalanine ammonia-lyase: regulation of its induction and its role in plant development. Phytochemistry 7: 1349-1359.

Kuć J 1972 Phytoalexins. Annual Review of Phytopathology 10:207-232.

Kuć J 1982 Phytoalexins from the Solanaceae. In Bailey JA and Mansfield JW ed Phytoalexins. John Wiley and Sons, New York 81-105.

Lagrimini LM 1991 Wound-induced deposition of polyphenols in transgenic plants overexpressing peroxidase. Plant Physiology 96:577-583.

Lisker N, Kuć J 1977 Elicitors of terpenoid accumulation in potato tuber slices. Phytopathology 67:1356-1359.

Maniatis T, Fritsch EF, Sambrook J 1982 Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory.

McKee, RK 1954 Dry-rot disease of the potato. VIII. A study of the pathogenicity of *Fusarium caeruleum* (Lib.) Sacc. and *Fusarium avenaceum* (Fr.) Sacc. Annals of Applied Biology 41: 417-434.

McKee RK 1955 Host-parasite relationship in the dry-rot disease of potatoes. Annals of Applied Biology 43:147-148.

McKee RK 1959 Factors affecting the toxicity of solanine and related alkaloids to *Fusarium caeruleum*. Journal of General Microbiology 20:686-696.

O'Brien VJ, Leach SS 1983 Investigations into the mode of resistance of potato tubers to *Fusarium roseum* 'Sambucinum'. American Potato Journal 60: 227-233.

Rahe JE, Kuc J, Chung CM 1970 Cinnamic acid production as a method of assay of phenylalanine ammonia-lyase in acetone powder of *Phaseolus vulgaris*. Phytochemistry 15: 947-951.

Ride JP 1983 Cell walls and other structural barriers in defence. In: Callow JA ed Biochemical Plant Pathology. John Wiley & Sons Ltd. 215-236.

Scott-Craig LS, Panaccione DG, Cervone F, Walton JD 1990 Endopolygalacturonase is not required for pathogenicity of *Cochliobolus carbonum* on maize. The Plant Cell 2: 1191-1200.

Sheen SJ 1971 The colorimetric determination of chlorogenic acid and rutin in tobacco leaves. Tobacco Science 15: 116-120.

Sinden SL, Gpth RW, O'Brien MJ 1973 Effect of potato alkaloids on the growth of *Alternaria solani* and their possible role as resistance factors in potatoes. Phytopathology 63:303-307.

Small T 1944 Dry rot of potato (*Fusarium caeruleum* (Lib.) Sacc.). Investigation of the sources and time of infection. Annals of Applied Biology 31: 291-295.

Spurr, AR 1969 A low-viscosity epoxy resin embedding medium for electron microscopy. Journal of Ultrastructure Research. 26: 31-43.

Stein BD 1991 The ultrastructure and histochemistry of infection by *Colletotrichum lagenarium* in cucumbers induced for resistance. Ph.D. Dissertation, Michigan State University.

Whitmore FW 1976 Binding of ferulic acid to cell walls by peroxidases of *Pinus elliottii*. Phytochemistry 15: 375-378.

Yeh KW, Juang RH, Su JC 1991 A rapid and efficient method for RNA isolation from plants with high carbohydrate content. Focus 13: 102-103.

Zucker M, Hankin L 1970 Physiological basis for cytoheximide-induced soft rot of potatoes by *Pseudomonas fluorescens*. Annals of Botany 34:1047-1062.

CHAPTER II

INDUCTION OF LOCALIZED RESISTANCE TO *FUSARIUM* DRY ROT DISEASE IN POTATO TUBER TISSUE BY NON-PATHOGEN *CLADOSPORIUM CUCUMERINUM*

ABSTRACT

The induction of local resistance of potato tuber tissue to *Fusarium sambucinum* by *Cladosporium cucumerinum* was studied. Resistance to infection by Fusarium was evident within 24 hours after inoculation with *C. cucumerinum*. Previous work has suggested that lignin synthesis is involved in the induced resistance response to *C. cucumerinum*. Tissue inoculated with *C. cucumerinum* developed levels of phenylalanine ammonia lyase (PAL) that were 5 fold greater than controls at 24 hours after inoculation. This induced PAL activity was associated with an increase in PAL mRNA. Northern blot analysis showed that PAL mRNA accumulated to a higher level than the control by three hours after inoculation with *C. cucumerinum* and continued to accumulate over the following 24 hours. Peroxidase (PO) isozymes, especially acidic cytoplasmic and basic ionically bound cell wall isozymes, were induced to a greater extent by *C. cucumerinum* than by wounding alone. At 24 and 48 hours, the activity of ionically bound cell wall PO was 5 and 8-fold higher in the inoculated tissue, respectively. IEF-PAGE analysis of these activities revealed that at least two acidic cytoplasmic PO and three basic cell wall PO isozymes were induced. Coniferyl alcohol dehydrogenase (CAD) activity was induced at the same rate in both control and inoculated tissue at 12 hours and then declined until 36 hours. After that time, CAD activity in the inoculated tissue increased and reached a small peak at 48 hours and then declined to the same level as the control by 60 hours. These increased enzyme activities are thought to contribute to the accumulation of lignin-like materials, which are deposited in the cell wall and restrict fungal invasion. following inoculation with a non-host pathogen.

INTRODUCTION

Fusarium sambucinum Fuckel (Teleomorph, *Gibberella pulicaris* (Fries) Sacc.) is a major causal agent of Fusarium dry rot of potato (*Solanum tuberosum* L.) tubers causing significant economic losses in the field, storage and transit in North America and Europe (Boyd, 1952, 1972; Seppanen, 1989). This pathogen infects tubers through wounds and results in a dry, brown rot. Under humid conditions secondary infection caused by bacteria may also occur. Although Fusarium dry rot disease was described many years ago (Small, 1944), only a limited number of resistant cultivars has been identified (Leach and Webb, 1981) and the mechanism of disease resistance is unknown.

Physical barriers against pathogen invasion in potato tuber tissue include natural and wound-induced suberization of periderm cell walls (Kolattukudy, 1981), as well as pathogen and non-pathogen induced lignin-like materials deposited in cell walls (Vance *et al*, 1980; Hammerschmidt, 1984). Suberin, a polymer composed of aliphatic and aromatic domains is deposited quickly and forms a continuous layer in resistant cultivars of potato tubers infected with *F. sambucinum* (O'Brien and Leach, 1983). Hammerschmidt (1984) has shown that a rapid deposition of lignin-like materials in potato tuber cell walls occurred after inoculation with *Cladosporium cucumerinum*, a cucumber pathogen. Inhibition of the accumulation of these induced lignin-like materials by a PAL inhibitor caused a state of "susceptibility" in the tissue to *C. cucumerinum*. Therefore, the induced suberin and lignin must have a role in resistance to *F. sambucinum*.

This study reports biochemical and physiological studies of *C. cucumerinum* induced lignification in potato tuber tissue and the relationship of lignification to resistance to *F. sambucinum*. In order to understand the early events of the lignification, the activity of three enzymes, phenylalanine ammonia-lyase (PAL), peroxidase (PO) and coniferyl alcohol dehydrogenase (CAD) involving lignin biosynthesis and the contents of lignin and chlorogenic acid in the tissues after inoculation with *C. cucumerinum* were examined.

MATERIALS AND METHODS

Fungal materials

Fusarium sambucinum and *Cladosporium cucumerinum* cultures were maintained as described by Hammerschmidt (1982, 1984). The concentration of spore suspension for inoculation of *C. cucumerinum* was $1x10^6$ spores/ml. For inoculum of *F. sambucinum*, a 5 mm diameter agar plug taken from the edge of growing fungal colony was used for each disc.

Preparation and inoculation of tuber discs

Tubers of potato (*Solanum tuberosum* L.) cv. Russet Burbank stored for 2-6 months at 4 ^{0}C were used in all experiments. For preparation of tuber discs and inoculation of *C. cucumerinum*, the procedures described by Hammerschmidt (1984) were followed.

Measurement of *Fusarium* dry rot disease development

At different time intervals after inoculation with a *C. cucumerinum* spore suspension, each tuber disc was inoculated with a 5 mm diameter agar plug containing *F. sambucinum* culture. For the control, the discs were inoculated with *F. sambucinum* without prior inoculation with *C. cucumerinum*. Following inoculation with *F. sambucinum*, the control and inoculated discs were incubated at room temperature in the dark for two days. After incubation, the depth and diameter of diseased tissue were measured.

Enzyme extraction and assays

At intervals after inoculation with a *C. cucumerinum* spore suspension, the top 0.5 mm layer of each disc was peeled and extracted with acetone at -20^{0}C to make acetone powder. One hundred mg of acetone powder was extracted in five ml of 0.1 M sodium borate buffer containing same amount (in weight) of insoluble PVP, pH 8.8 for one hr at 4 ^{0}C and filtered through two layers of cheesecloth and then centrifuged at 10,000 xg for 20 min at 4 ^{0}C. After centrifugation, the supernatant was used for PAL, PO and CAD assays. Spectrophotometric methods described by Borchert (1978) and Moerschbacher *et al* (1986), were used for PO and CAD assays, respectively. For the PAL assay, procedures from Rahe *et al* (1970) were used. The protein content of enzyme extracts was determined by the method of Bradford (1976).

Extraction of ionically bound cell wall PO

A modified procedure from Grison and Pilet (1985) was used for extraction of ionically bound cell wall PO. Briefly, 0.2 g of acetone powder was stirred in 10 ml of 0.05 M sodium phosphate buffer (pH 6.0) for 2 hr at 4 ^{0}C and centrifuged at 1000 xg for 6 min. The pellet was washed 2 times with 2 ml of the buffer, and once with water and then centrifuged at 1000 xg for 6 min. The pellet was re-suspended in 1 ml of 1 M $CaCl_2$ and stirred for 0.5 hr at room temperature and then centrifuged at 13,000 xg for 20 min at 4 ^{0}C. The supernatant was used for assay of ionically bound cell wall PO. After being dialyzed against water and lyophilized to dryness, the residues were re-dissolved in water, centrifuged again at 13,000 xg for 5 min at 4 ^{0}C. The supernatant was used for IEF-PAGE analysis.

Electrophoresis of PO

Native PAGE (Hames, 1981) was used to separate the acidic cytoplasmic PO. After electrophoresis, PO isozymes were visualized by staining the gel with 200 ml of 0.05 M sodium acetate buffer solution (pH 5.5) containing 40 mg of 3-amino-9-ethylcarbazole, 10 ml of N,N, dimethylformamide and 63 μl of 30% H_2O_2 (Graham *et al*, 1965).

The commercially available, pre-made IEF gel (pH 3-10, 124 x 129 x 3.75 mm, ISOLAB, INC., Akron, OH, USA) was used for separating isozymes of ionically bound cell wall PO. The anode buffer was made up with 1 M H_3PO_4 and the cathode buffer contained 1 M ethanolamine, 30 mM arginine and 30 mM lysine. The gel was run at 10 ^{0}C at 5W for 10 min, 10W for 45 min, 15W for 15 min and finally at 20-25W for 5 min. The Protein Test Mixture 9 from Serva company was used as PI standards. Following electrophoresis, the gel was also stained with the acetate buffer to visualize the isozymes. Isoelectric points of PO isozymes were determined by running the protein standards with the samples.

Mini IEF gel (pH 9-11, 125 x 65 x 0.4 mm), self-cast according to procedures from BioRad, was also used to separate basic PO isozymes. The electrophoresis was conducted in a mini IEF cell (model 111, BioRad) according to manufacturer's manual (catalog # 170-2975 and 170-2976). The running conditions were at 100V for 15 min, 200V for 15 min and finally at 450V for 60 min. After electrophoresis, the gel was also stained with the acetate buffer to visualize the isozymes.

Northern blot analysis of PAL mRNA

The procedure described by Yeh *et al* (1991) for total RNA extraction was followed. At intervals after inoculation with *C. cucumerinum*, total RNA was isolated from the top 0.5 mm layers of discs and 30 μg of the RNA was fractionated in 0.8% agarose formamide gel. The RNA was transferred onto Nylon membrane and probed with ^{32}P labelled PAL cDNA clone from potato. The clone was kindly provided by Dr. K. Hahlbrock (Fritzemeier *et al*, 1987). The procedures described by Feinberg and Vogelstein (1983) were used for random priming synthesis of ^{32}P-labeled probe. Following hybridization, the blot was finally washed in 0.1 x SSC at 65 ^{0}C for 10 min and exposed to X-ray film. As a positive control, ^{32}P-labeled cDNA clone of ATPase gene (ß-10) from tobacco provided by Boutry and Chua (1985) was probed to the same blot. The intensity of the signals from the positive control on X-ray film was used to correct corresponding PAL signals from the samples.

Measurements of lignin and chlorogenic acid content

The top 0.5 mm layers of discs were used for measurements of lignin and chlorogenic acid contents. The thioglycolic procedure described by Hammerschmidt (1984) was used for lignin assay. The procedure from Sheen (1971) was used for chlorogenic acid content measurement. Briefly, the top layers of discs were extracted with MeOH for three times in two days and the extracts were pooled and evaporated to near dryness under low pressure. The samples were then redissolved in 2.1 ml of 50% MeOH. For chlorogenic acid assay, 100 μl of each sample was mixed with 500 μl of 0.5 N HCl, 500 μl of Arnow's reagent and 500 μl of 1 N NaOH in the order. Arnow's reagent was made up of 10 g of sodium nitrite, 10 g of sodium molybdate and 100 ml of H_2O. A series of concentration of chlorogenic acid from 25 to 250 μg/ml was used as standard. OD_{510} of samples was measured spectrophotometrically and the readings were converted into concentrations according to the standard.

RESULTS

Reduction of *F. sambucinum* infection by *C. cucumerinum*

Pre-inoculation with *C. cucumerinum* reduced infection by *F. sambucinum* in potato tuber discs (Fig. 2-1 A and B). The reduction of the infection was evident by 24 hr in both diameter and depth of diseased tissues. This reduction was more obvious in the depth of diseased tissue (Fig. 2-1 B). At 24 hr, the depth of decay was only ca. 50 % of that in the control. By 72 hr it was only 1/3 of that of the control.

PAL activity and Northern blot analysis of PAL mRNA

PAL activity was elicited through the time of experiment. The induction of PAL activity occurred within several hours after inoculation with *C. cucumerinum* and PAL activity reached a 5-fold increase at 24 hr (Fig. 2-2). The induced activity remained at this level until 48 hr and then declined to a level similar to that of the control.

Northern blot analysis (Fig. 2-3 A) showed that PAL mRNA accumulated to a higher level than the control by three hr after inoculation with *C. cucumerinum* and continued to accumulate through the following 24 hr. PAL mRNA also accumulated in the control tissue but at a very low level. With normalization of densitometry analysis of the Northern blot, the accumulated PAL

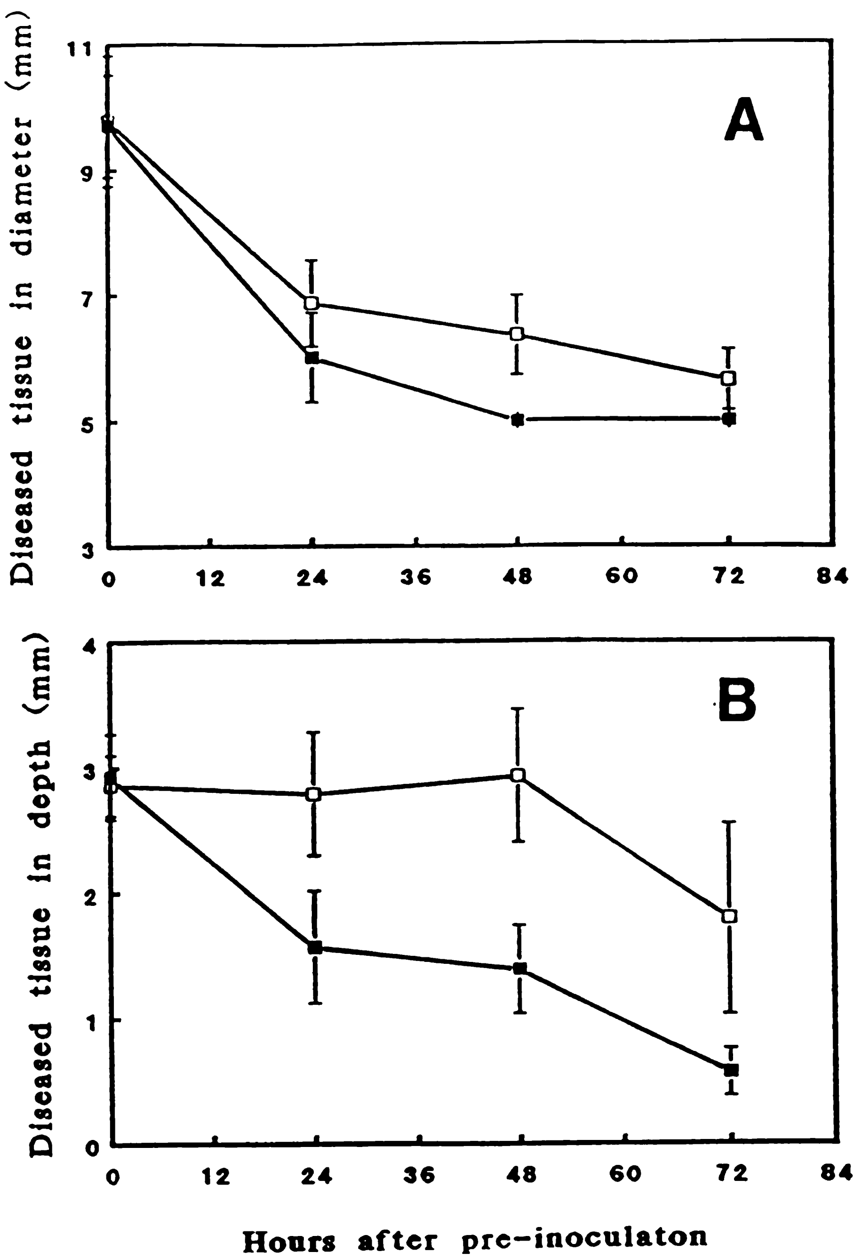


Fig. 2-1. Infection of *F. sambucinum* in potato tuber discs pre-inoculated with non-potato pathogen *C. cucumerinum* spore suspension at concentration of 1x10^6 spore/ml. Degree of the infection was determined two days after inoculation with *F. sambucinum*. A. Diseased tissue in diameter. B. Diseased tissue in depth. Control (-□-), pre-inoculated (-■-).

The data of this study were averages of the results from three experiments. Bars=S.D.

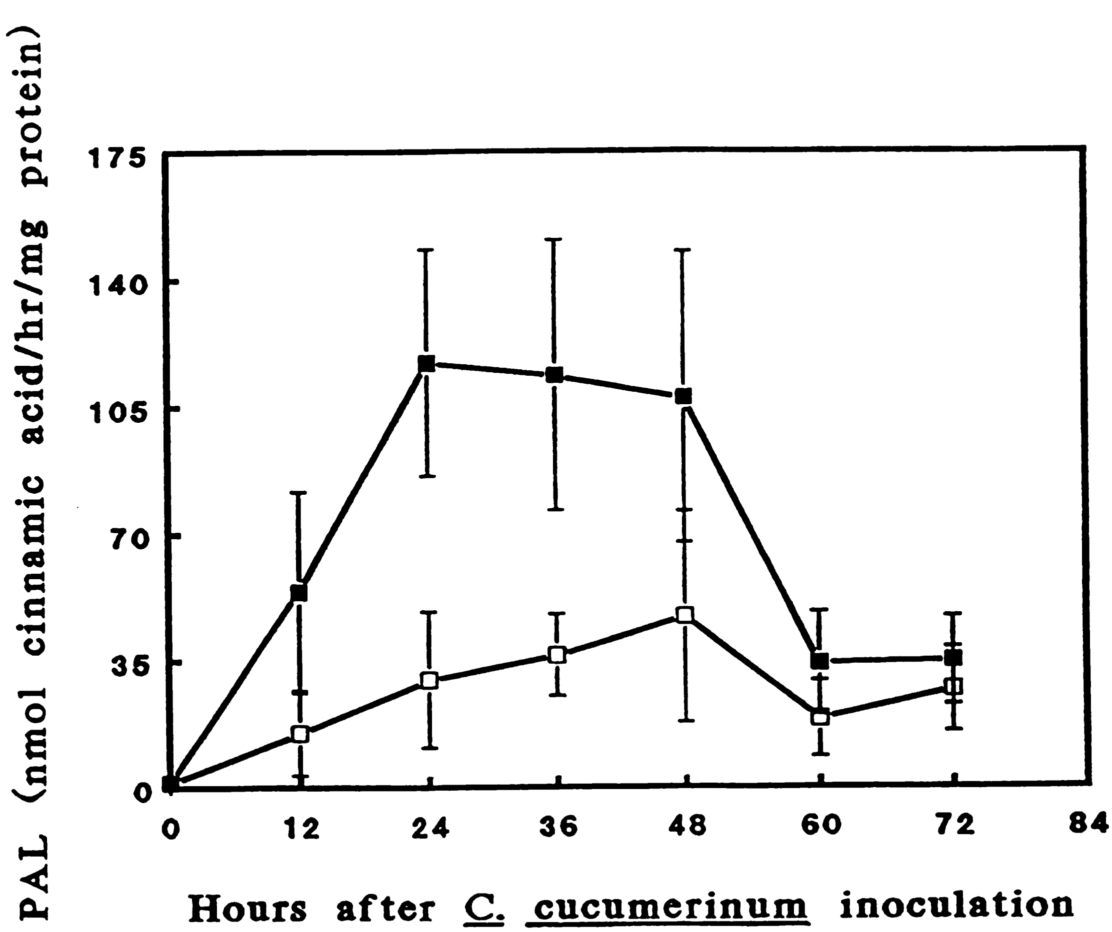


Fig. 2-2. Time course of PAL activity in potato tuber discs after inoculation of *C. cucumerinum*. Control (-□-), inoculated (-■-).

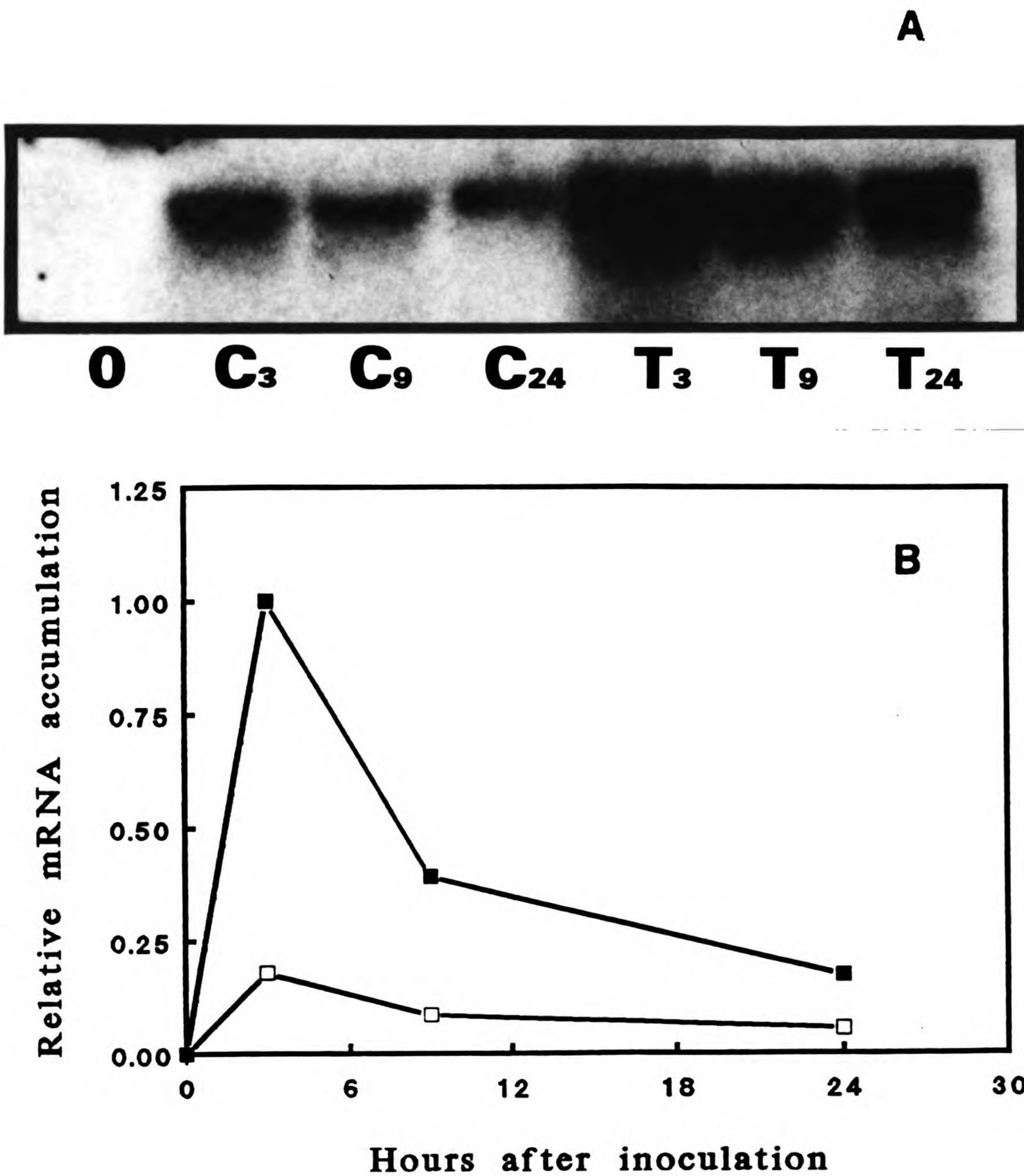


Fig. 2-3. Panel A: Northern blot analysis of PAL mRNA from potato tuber discs after inoculation of *C. cucumerinum*. Thirty μg of total RNA was used for each sample and the blot was probed with PAL cDNA clone from potato. C, control. T, inoculated. The numbers represent the hours after inoculation with *C. cucumerinum*. Panel B: Normalized densitometry analysis of Northern blot of PAL mRNA from potato tuber discs inoculated with *C. cucumerinum*. The normalization was done by calculating the ratio of densitometry readings of PAL signals and ß-10 ATPase signals. Control (-□-), inoculated (-■-).

mRNA level in the inoculated tissue was 5-fold at three hr and 4-fold at 9 hr over the control (Fig. 2-3 B).

CAD activity

CAD activity in both inoculated and the control tissues was induced at early hours (Fig. 2-4). At 12 hr, CAD activity in both tissues reached a similar peak and later declined to a lower level at 36 hr. The decreasing degree of CAD activity was higher in the inoculated tissue, but by 36 hr CAD activity increased again and reached a second peak at 48 hr. In contrast, CAD activity of the control was at the same level from 36 to 72 hr.

Activity of soluble cytoplasmic and ionically bound cell wall PO and their isozyme patterns

Soluble cytoplasmic PO increased slowly in both inoculated and control tissues in the first 48 hr. The increase in activity was first detected at 12 hr (Fig. 2-5 A). At 48 hr, PO activity in the inoculated tissue increased at a higher rate and it was 2.5-fold higher over the control at 72 hr.

The activity of ionically bound cell wall PO increased rapidly in the inoculated tissue (Fig. 2-5 B). This induced PO activity was seen between 12-18 hr and continued through the rest of experiment. The PO level was 5-fold at 24 hr and 8-fold at 48 hr over the control.

Acidic isozyme banding pattern of soluble cytoplasmic PO is shown in Fig. 2-6. On the activity polyacrylamide gel, there were at least four isozymes showing an increase in activity. Among the isozymes, there were two new isozymes that were not present in the control (arrows). These two new isozymes were detected at 36 hr and band intensity increased through 96 hr.

IEF-PAGE revealed that at least two isozymes of ionically bound PO were

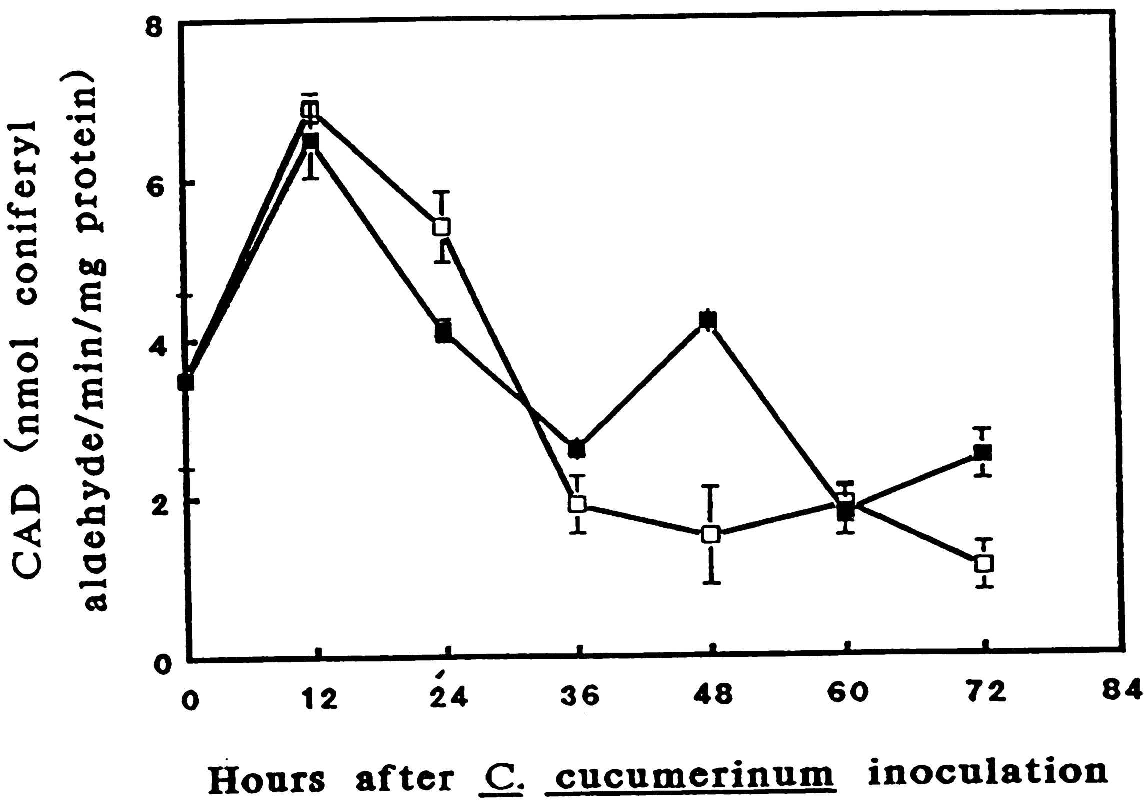


Fig. 2-4. Time course of CAD activity in potato tuber discs after inoculation with *C. cucumerinum*. Control (-□-), inoculated (-■-).

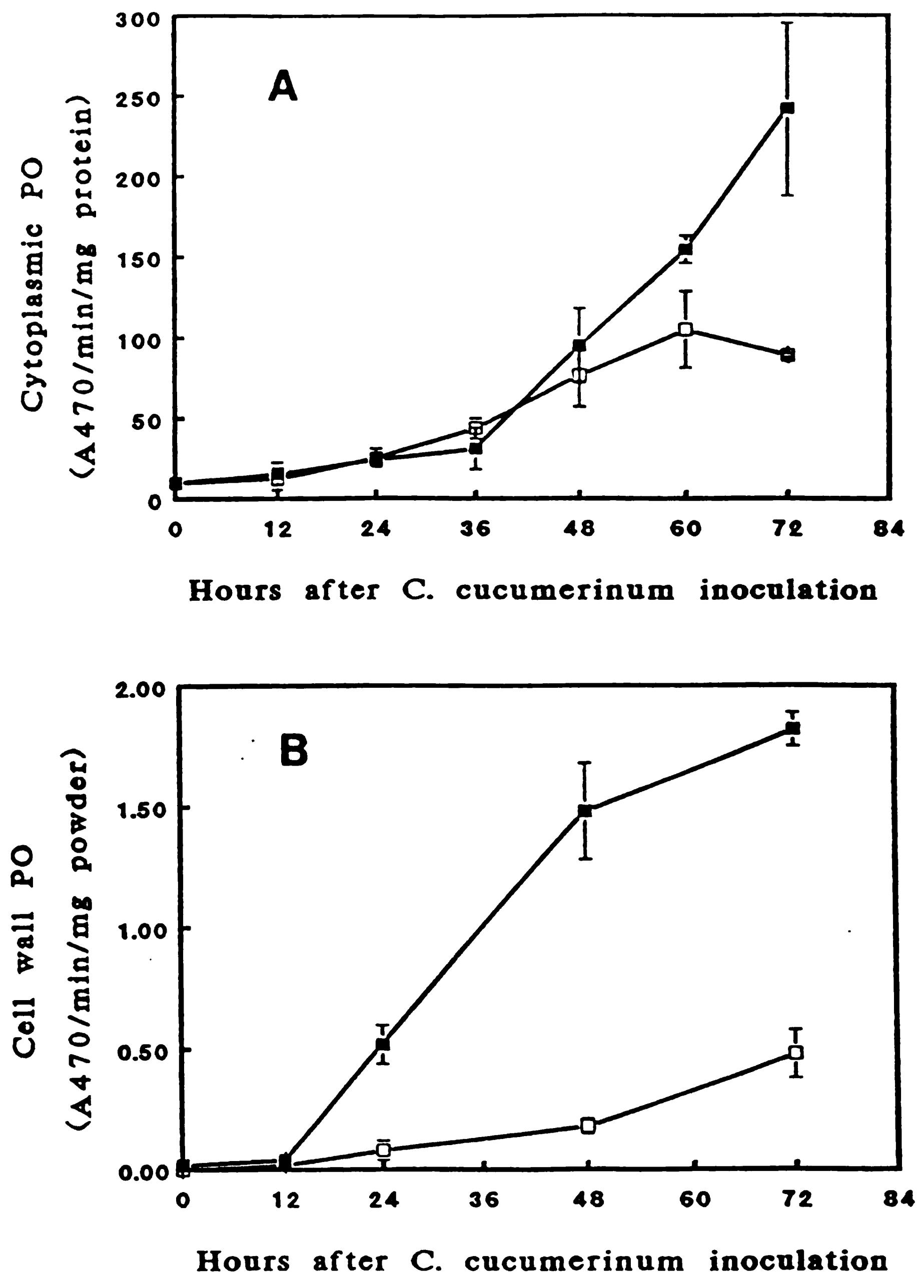


Fig. 2-5. Time course of soluble cytoplasmic (A) and ionically bound cell wall (B) PO activity in potato tuber discs after inoculation with *C. cucumerinum*. Control (-□-), inoculated (-■-).

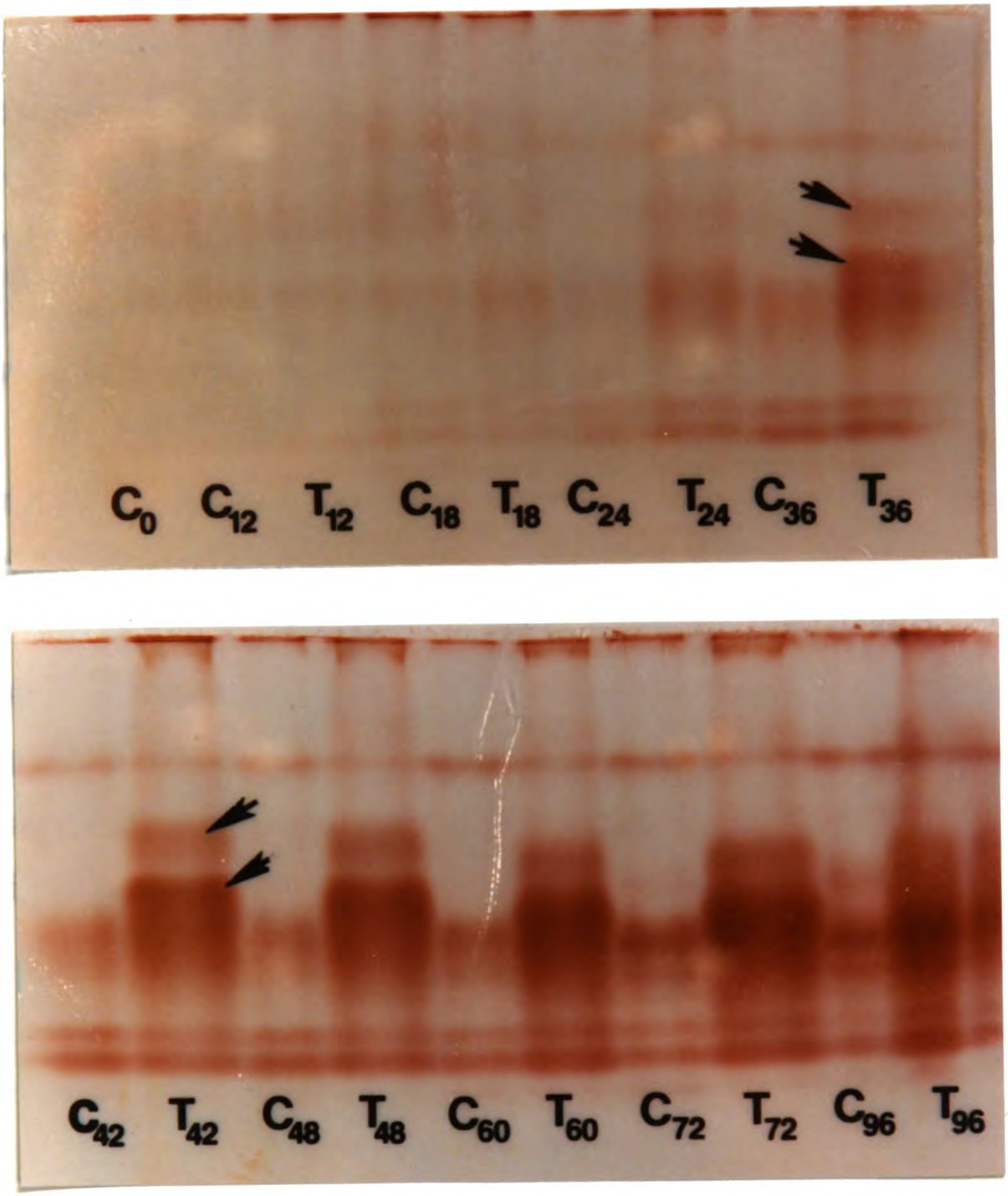


Fig. 2-6. 7.5% PAGE of acidic cytoplasmic PO isozyme. Equal volume of enzyme extract was loaded in each lane. C, control; T, inoculated. The numbers represent the hours after inoculation with *C. cucumerinum*. Arrows indicate the isozymes with induced activity.

induced as early as 24 hr (Fig. 2-7 A and B arrows). The PIs of these isozymes are above 9 (Fig. 2-7 A). It seemed that one of the two isozymes was a new isozyme which was not present in the control. By 72 hr, an isozyme with approximate PI of 8.5 also showed a large increase in intensity (arrow on the left side of the gel). In addition, the cationic isozymes with PI lower than 4 also showed an increase in activity.

Accumulation of chlorogenic acid and lignin-like materials

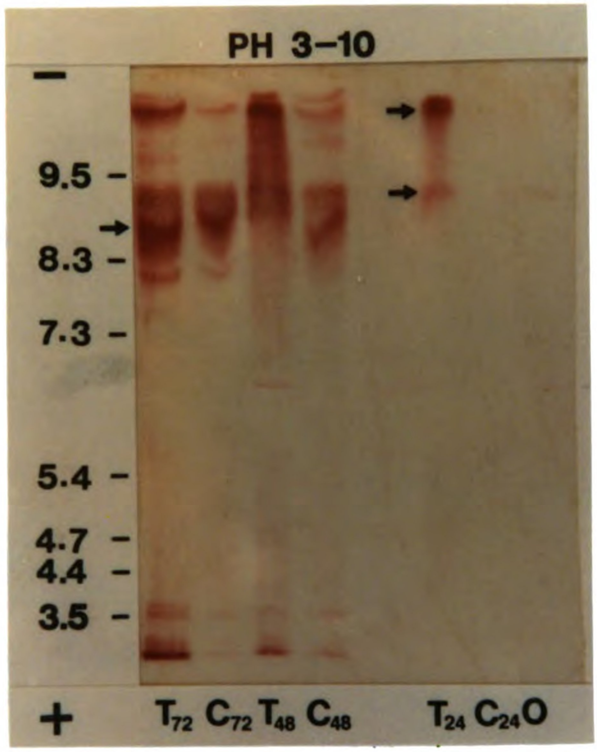
The content of chlorogenic acid in both tissues increased in a similar pattern in first 36 hr. After that time chlorogenic acid content dropped slightly in inoculated tissue through 60 hr (Fig. 2-8 A).

The increased accumulation of lignin-like materials was detected between 12 and 24 hr after inoculation with *C. cucumerinum* and continued to accumulate through 72 hr (Fig. 2-8 B). The increased levels of lignin were about 5-fold above the control at 24 to 36 hr. At later time points, the amount of lignin in the inoculated tissue was 2-3 times higher than the control.

DISCUSSION

Previous work by Hammerschmidt (1984) showed that *Cladosporium cucumerinum*, a non-pathogen of potato induced a rapid deposition of lignin in potato tuber cell walls. It was suggested that this induced lignin may inhibit *C. cucumerinum* penetration into the tissue. The results from this study further demonstrate the induction of lignification also reduced infection by potato pathogen *Fusarium sambucinum* (Fig. 2-1 A and B). The induced lignin-like materials

A



B

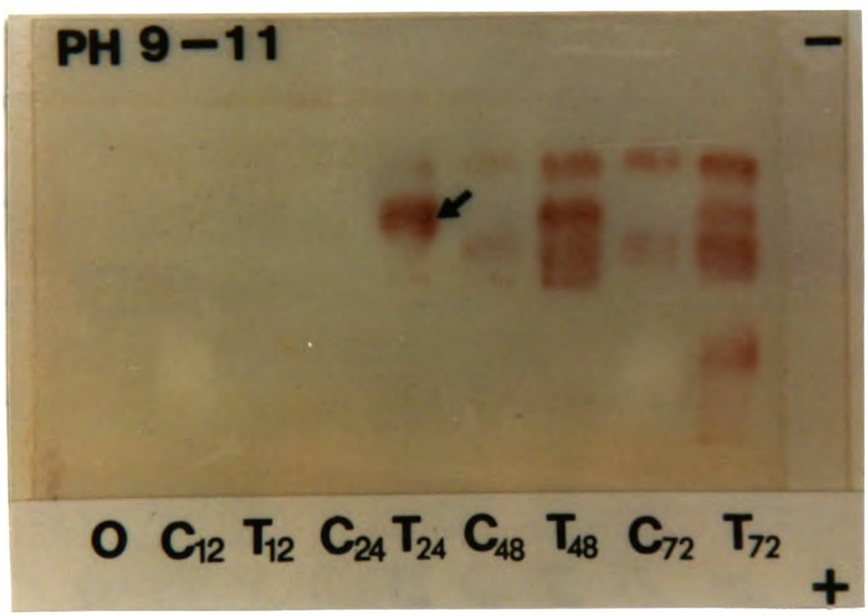


Fig. 2-7. IEF-PAGE of ionically bound cell wall PO isozymes from potato tuber discs. Panel A: IEF-PAGE of pre-made polyacrylamide gel (pH 3-10). The arrows indicate the three most induced PO isozymes. Panel B: IEF-PAGE of self-cast polyacrylamide gel (pH 9-11). The arrow indicates the new isozyme. C, control; T, inoculated. The numbers represent the hours after inoculation with *C. cucumerinum*. Equal volume of sample extract was loaded in each lane.

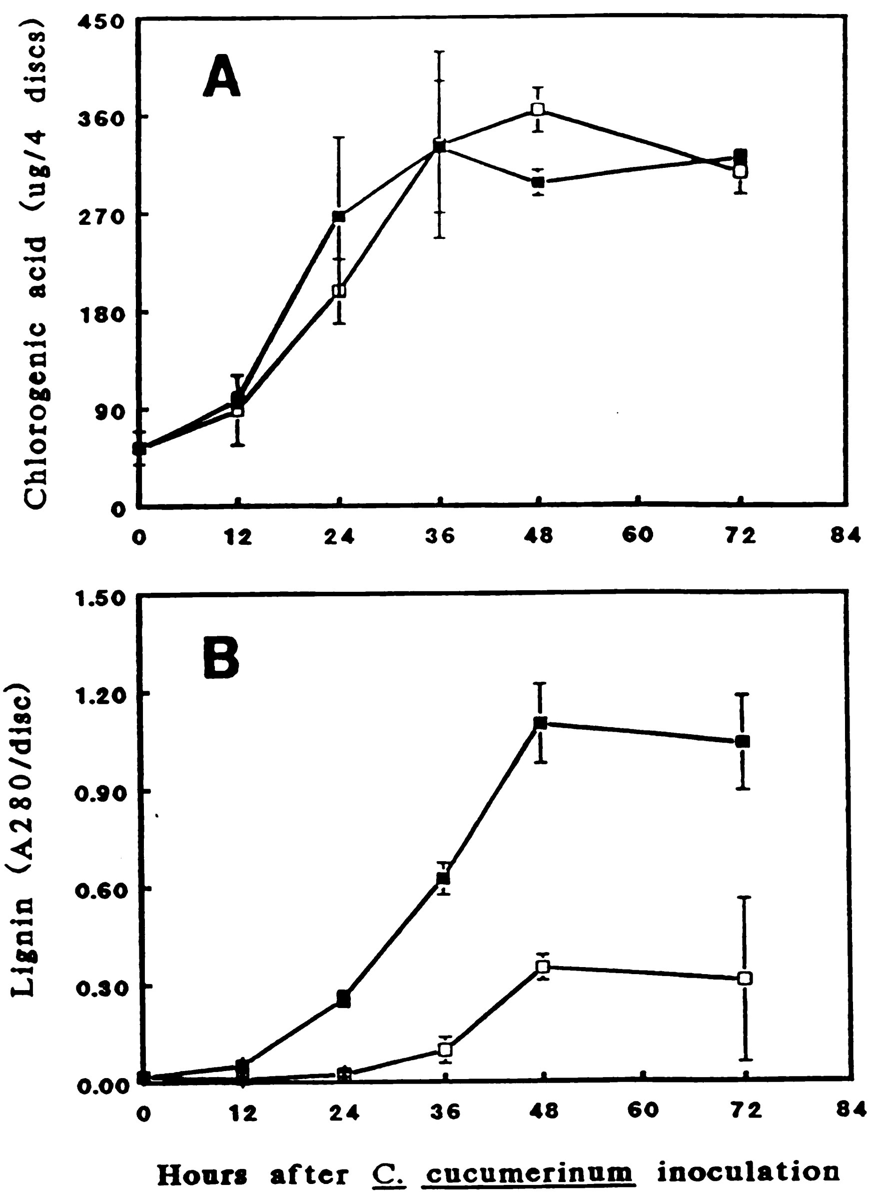


Fig. 2-8. A, Time course of lignin accumulation in potato tuber discs after inoculation with *C. cucumerinum*. B, Time course of chlorogenic acid accumulation in potato tuber discs after inoculation with *C. cucumerinum*. Control (-□-), inoculated (-■-).

started accumulating at 12 hr after inoculation with *C. cucumerinum* (Fig. 2-8 B) and continued to accumulate at a higher rate though out the rest of time of the experiment. In the control, although the lignin also increased, the process of lignification did not start until after 36 hr. The 5-fold higher accumulation of lignin in the tissue pre-inoculated with *C. cucumerinum* in the time between 24-36 hr may account for the 50% reduction of *F. sambucinum* infection in depth of diseased tissue (Fig. 2-1 B).

The induction of the three enzymes associated with lignin biosynthesis correlated well with the induced accumulation of lignin by *C. cucumerinum*. PAL is the first enzyme and its regulatory role in phenylpropanoid metabolism has been well documented (Jones, 1984). In potato-*Phytophthora* system, PAL activity has been shown to increase faster in the resistant cultivar during incompatible interaction (Fritzemeier *et al*, 1987; Smith and Rubery, 1981). The increase in PAL activity was consistent with an increased lignin accumulation. In this study, PAL activity was also induced by 12 hr after inoculation with *C. cucumerinum*. This increase in PAL activity was due to the accumulation of PAL messenger which was detected as early as 3 hr following inoculation (Fig. 2-3 A and B). Although in the control PAL activity and mRNA accumulation also showed an increase, the inductions were much lower than that in the inoculated tissue. This may be due to a wounding response. As pointed out by Cramer, *et al* (1985) and Hahlbrock (Chappell and Hahlbrock, 1984; Fritzemeier *et al*, 1987), the increase in PAL activity is either due to the increase in the rate of PAL mRNA synthesis or the reduction in its rate of turn-over. Whether or not the induction of PAL gene expression was due to elicitation by fungal elicitor from *C. cucumerinum*, which stimulated PAL transcript synthesis, could not be determined in this study.

CAD, the second to the last enzyme in lignin biosynthesis pathway was induced in both inoculated and control tissues in the first 12 hr (Fig. 2-4). This early induction may be due to wounding response to the cutting. However, following a drop in both cases after 36 hr, CAD activity in the inoculated tissue rose again and reached a second peak at 48 hr. This was consistent with the accumulation of lignin which continued to increase in the following hours (Fig. 2-8 B). The results also are in agreement with those from the studies by Moerschbacher (1986, 1990), in which an increase in CAD activity was accompanied by the accumulation of lignin.

It is interesting to note that the activity of soluble cytoplasmic PO in both inoculated and control tissues did not show much difference in the first 48 hr following inoculation (Fig. 2-5 A). However, PAGE of acidic PO (Fig. 2-6) showed that four isozymes were induced at 24-36 hr and among them there were two new isozymes (arrows) that were not present in the control. These two isozymes seemed to be different from the one identified and purified by Espelie and Kolattukudy (1985) who claimed that the isozyme was responsible for suberization during wound healing process. The difference between the PO isozyme patterns in both wounding alone and wounding plus inoculation of *C. cucumerinum* indicated that the later involves certain active defense mechanism(s). The study of function of these isozymes would allow us to understand more about the mechanism(s) in plant active defense response.

Deposition of insoluble phenolics including lignin to the cell wall has been known to be involved with POs, especially cell wall bound POs (Catesson *et al*, 1986; Goldberg *et al*, 1983; Harkin and Obst, 1973; Whitmore, 1976). Whitmore (1976) demonstrated that ionically bound and covalently bound cell wall peroxidase from cambial cell walls of pine (*Pinus elliottii*) bark tissue had higher capacity than that of cytoplasmic peroxidase to catalyze bond formation between cell wall carbohydrate and ferulic acid, and its condensation products in both cell walls of the bark tissue and microcrystalline cellulose. The strongest binding strength of ferulic acid to cell wall carbohydrates was also catalyzed by the cell wall bound peroxidases. Whitmore (1978) also showed that incubation of cell walls isolated from *Pinus elliottii* callus, which contained cell wall bound PO, with H_2O_2 and coniferyl alcohol generated polymers which had physical and chemical properties of lignin. The lignin was found to be bound to cell wall carbohydrates and proteins. Thus, in this study it is important to know whether cell wall bound PO activity would change during accumulation of lignin-like materials. One M $CaCl_2$ was used to extract ionically bound PO from cell walls of potato tuber tissue. Since it has been reported that cationic PO isozymes can be activated by low concentration of Ca^{2+} during PO assay (Penel, 1986), I have tested that the ionically bound PO extracted from the cell walls with 1 M NaCl. Both extraction methods showed the same increase in activity of PO (data not shown), thus indicating that the induction of ionically bound cell wall PO activity was due to fungal infection but not by Ca^{2+} activation. The activity of ionically bound cell wall PO showed a greater increase at 24 hr following inoculation with *c. cucumerinum* (Fig. 2-5 B). At 24 and 48 hr, its activity was 5-fold and 8-fold greater than the control. At least two basic ionically bound cell wall PO were induced by *C. cucumerinum* in the first 24 hr (Fig. 2-7 A and B). By 72 hr an isozyme with PI of approximately 8.5 (arrow on the left of the gel) also showed an increase in activity. These induced PO isozymes were likely to account for the increase in cell wall PO activity in the inoculated tissue. The induction of both acidic cytoplasmic and ionically bound cell wall PO in the first 48 hr after inoculation were correlated to the induction of lignin accumulation by *C. cucumerinum*. It has been reported in many cases that the increase in PO activity of some cytoplasmic isozymes is associated with plant defense response against pathogen infection (Reimers *et al*, 1992; van Loon, 1986). However, very few reports have addressed the role of specific PO isozymes in plant defense response. There are many isozymes of PO in different organs of plant tissues and they have different functions or affinity to different substrate under various conditions, eg. H_2O_2 generation, oxidation of lignin precursors into free radicals (Friend, 1981; Gross, 1980). Therefore, only fully understanding the function of each PO isozyme can help us to elucidate this non-pathogen induced resistance.

Finally, accumulation of chlorogenic acid, which is one of the final products of phenylpropanoid pathway, did not differ between inoculated and control tissues during the first 36 hr (Fig. 2-9 A). Starting at 36 hr after inoculation with *C. cucumerinum*, the chlorogenic acid content began to decline through 60 hr. As suggested by Friend (1981), the decrease in accumulation of chlorogenic acid in potato tuber tissue inoculated with incompatible race of pathogen could be due to (a) conversion of chlorogenic acid into insoluble phenolic compounds that are bound to cell wall protein; (b) direct diversion of cinnamoyl-CoA units to lignin like materials but not chlorogenic acid. These two factors may explain the result of chlorogenic acid accumulation in this study. In the first 36 hr, the increase in chlorogenic acid in both inoculated and control tissues reflected a general stimulation of phenolic biosynthesis.

It may be argued that some antimicrobial compounds, such as rishitin and lubimin, that may inhibit *F. sambucinum* and thus contribute to the overall resistance to the fungus. I do not think it is likely to be the case since it has been shown by Henfling *et al* (Henfling *et al*, 1980) that *C. cucumerinum* did not induce phytoalexin production in potato tuber tissue. Even if rishitin and lubimin were produced, *F. sambucinum* would detoxify them (Desjardins *et al*, 1989; Desjardins and Gardner, 1989). Thus, the results from this study strongly suggest that the induced lignin-like materials by *C. cucumerinum*, which deposited to the cell wall, play an important role in restricting further invasion by the pathogen in potato tuber tissue.

Acknowledgement

I would like to thank Dr. K. Hahlbrock for kindly providing the potato PAL cDNA clone.

REFERENCES

Bell JN, Ryder TB, Wingate VPM, Bailey JA, Lamb CJ 1986 Differential accumulation of plant defense gene transcripts in a compatible and an incompatible plant-pathogen interactions. Molecular and Cellular Biology 6: 1615-1623.

Borchert R 1978 Time course and spatial distribution of phenylalanine ammonia-lyase and peroxidase activity in wounded potato tuber tissue. Plant Physiology 62: 789-793.

Boutry M, Chua N-H 1985 A nuclear gene encoding the beta subunit of the mitochondrial ATP synthesis in *Nicotiana plumbaginifolia*. The EMBO Journal 4: 2159-2165.

Boyd AEW 1952 Dry-rot disease of the potato. Annals of Applied Biology 39: 322-357.

Boyd AEW 1972 Potato storage disease. Review of Plant Pathology 51: 297-321.

Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72: 248-254.

Catesson AM, Imberty A, Goldberg R, Czaninski Y 1986 Nature, localization and specificity of peroxidases involved in lignification processes. In: Greppin H, Penel

C Gaspar Th. ed. Molecular and Physiological Aspects of Plant Peroxidases. University of Geneva, Switzerland, pp. 189-198.

Chappell J, Hahlbrock K 1984 Transcription of plant defense genes in response to UV light or fungal elicitor. Nature 311: 76-78.

Cramer CL, Ryder TB, Bell JN, Lamb CJ 1985 Rapid switching of plant gene expression induced by fungal elicitor. Science 227: 1240-1243.

Desjardins, AE, Gardner HW, Plattner RD 1989 Detoxification of the potato phytoalexin lubimin by *Gibberella pulicaris*. Phytochemistry 28: 431-437.

Desjardins AE, Gardner HW 1989 Genetic analysis in: *Gibberella pulicaris* rishitin tolerance, rishitin metabolism and virulence on potato tuber. Molecular Plant-Microbe Interactions 2: 26-34.

Espelie KE, Kolattukudy PE 1985 Purification and characterization of an abscisic acid-inducible anionic peroxidase associated with suberization in potato (*Solanum tuberosum*). Archives of Biochemistry and Biophysics 24: 539-545.

Espelie KE, Frenceschi VR, Kolattukudy PE 1986 Immunocytochemical localization and time course of appearance of an anionic peroxidase associated with suberization in wound-healing potato tuber tissue. Plant Physiology 81: 487-492.

Feinberg AP, Vogelstein B 1983 A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Analytical Biochemistry 132: 6-13.

Fink W, Haug M, Deising H, Mendgen K 1991 Early defense response of cowpea (*Vigna sinesis* L.) induced by non-pathogenic rust fungi. Planta 185: 246-254.

Friend J 1981 Plant phenolics, lignification and plant disease. Progress in Phytochemistry 7: 197-261.

Fritzemeier K-H, Cretin C, Kombrink E, Rohwer F, Taylor J, Scheel D, Hahlbrock K 1987 Transient induction of phenylalanine ammonia-lyase and 4-coumarate:CoA ligase mRNAs in potato leaves infected with virulent of avirulent races of *Phytophthora infestans*. Plant Physiology 85: 34-41.

Goldberg R, Catessom AM, Czaninski Y 1983 Some properties of syringaldazine oxidase specifically involved in the lignification process. Zeitschrift für Pflanzenphysiologie 110:267-279.

Graham RC, Lundholm U, Karnovsky MJ 1965 Cytochemical determination of peroxidase activity with 3-amino-9-ethylcarbazole. Journal of Histochemistry and Cytochemistry 13: 150-152.

Grison R, Pilet PE 1985 Cytoplasmic and cell wall isoperoxidases in growing maize roots. J. Plant Physiology 118: 189-199.

Gross GG 1980 The biochemistry of lignification. In: Woolhouse HW ed. Advances in Botanical Research. Academic Press, London, 25-63.

Hames BD 1981 An introduction to polyacrylamide gel electrophoresis. In: Hames BD, Rickwood D ed. Gel Electrophoresis of Proteins. IRL Press, 1-86.

Hammerschmidt R 1984 Rapid deposition of lignin in potato tuber tissue as a response to fungi non-pathogenic on potato. Physiological Plant Pathology 24: 33-42.

Hammerschmidt R, Kuć J 1982 Lignification as a mechanism for induced systemic resistance in cucumber. Physiological Plant Pathology 20: 61-71.

Harkin JM, Obst JR 1973 Lignification in trees: Indication of exclusive peroxidase participation. Science 180: 296-298.

Henfling JWDM, Bostock R, Kuć J 1980 Effects of abscisic acid on rishitin and lubimin accumulation and resistance to *Phytophthora infestans* and *Cladosporium cucumerinum* in potato tuber tissue. Phytopathology 69: 609-612.

Jones DH 1984 Phenylalanine ammonia-lyase: regulation of its induction and its role in plant development. Phytochemistry 7: 1349-1359.

Kolattukudy PE 1981 Structure, biosynthesis, and biodegradation of cutin and suberin. Annual Review of Plant Physiology 32: 539-567.

Leach SS, Webb RE 1981 Resistance of selected potato cultivars and clones to Fusarium dry rot. Phytopathology 71: 623-629.

Maniatis T, Fritsch EF, Sambrook J 1982 Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory

Moerschbacher BM, Noll U, Ocampo CA, Flott BE, Gotthardt U, Wustefeld A, Reisener H-J 1990 Hypersensitive lignification response as the mechanism of non-host resistance of wheat against oat crown rust. Physiologia Plantarum 78: 609-

615.

Moerschbacher BM, Heck B, Kogel KH, Obst O, Reisener HJ 1986 An elicitor of the hypersensitive lignification response in wheat leaves isolated from the rust fungus *Puccinia gramminis* f. sp. *triticis*. II. Induction of enzymes correlated with the biosynthesis of lignin. Zeitschrift für Naturforschung 41c: 839-844.

O'Brien VJ, Leach SS 1983 Investigations into the mode of resistance of potato tubers to *Fusarium roseum* 'Sambucinum'. American Potato Journal 60: 227-233.

Penel C 1986 The role of calcium in the control of PO activity. In: Greppin H, Penel C, Gaspar Th. ed. Molecular and Physiological Aspects of Plant Peroxidases. University of Geneva, Switzerland, 155-164.

Rahe JE, Kuc J, Chung CM 1970 Cinnamic acid production as a method of assay of phenylalanine ammonia-lyase in acetone powder of *Phaseolus vulgaris*. Phytochemistry 15: 947-951.

Reimers P, Guo A, Leach JE 1992 Increased activity of a cationic peroxidase associated with a incompatible interaction between *Xanthomonas oryzae* pv *oryzae* and rice (*Oryza sativa*). Plant Physiology 99: 1044-1050.

Seppanen E 1989 *Fusarium* as pathogens of potato tubers and their pathogenicity. In: Chelkonski J ed. *Fusarium* Mycotoxin, Taxonomy and Pathogenicity. Elsevier, New York, 421-433.

Sheen SJ 1971 The colorimetric determination of chlorogenic acid and rutin in tobacco leaves. Tobacco Science 15: 116-120.

Small T 1944 Dry rot of potato (*Fusarium caeruleum* (Lib.) Sacc.). Investigation of the sources and time of infection. Annals of Applied Biology 31: 291-295.

Smith BG, Rubery PH 1981 The effects of infection by *Phytophthora infestans* on the control of phenylpropanoid metabolism in wounded potato tissue. Planta 151: 535-540.

van Loon LC 1986 The significance of changes in peroxidase in diseased plants. In: Greppin H, Penel C, Gaspar Th ed. Molecular and Physiological Aspects of Plant Peroxidases. University of Geneva, Switzerland, 405-418.

Vance CP, Kirk TK, Sherwood RT 1980 Lignification as a mechanism of disease resistance. Annual Review of Phytopathology 18: 259-288.

Whitmore FW 1976 Binding of ferulic acid to cell walls by peroxidases of *Pinus elliottii*. Phytochemistry 15: 375-378.

Whitmore FW 1978 Lignin-carbohydrate complex formation in isolated cell walls of callus. Phytochemistry 17: 421-425.

Yeh KW, Juang RH, Su JC 1991 A rapid and efficient method for RNA isolation from plants with high carbohydrate content. Focus 13: 102-103.

CHAPTER III

THE ROLE OF POTATO STEROID GLYCOALKALOIDS, α-SOLANINE AND α-CHACONINE IN DISEASE RESISTANCE TO THE FUNGAL PATHOGEN *FUSARIUM SAMBUCINUM*

ABSTRACT

Toxicity of two potato steroid glycoalkaloids (SGA), α-chaconine and α-solanine was tested for their effect on spore germination and growth of the fungal pathogen, *Fusarium sambucinum* Fuckel (Teleomorph, *Gibberella pulicaris* (Fries) Sacc.), a causal agent of potato dry rot. α-Chaconine had a greater inhibitory effect on the fungal spore germination and growth than α-solanine. The toxicity of each SGA was greater at pH 7 than at pH 6. The combination of α-chaconine and α-solanine showed a synergistic inhibitory effect on spore germination and growth of the fungus. In potato tuber discs, accumulation of SGA was completely suppressed by the pathogen *F. sambucinum* and partially suppressed by the non-pathogen *Cladosporium cucumerinum*. The SGA accumulated in aged potato tuber discs may play a role in the general defense response against *F. sambucinum* infection.

INTRODUCTION

α-Solanine and α-chaconine are the two major steroid glycoalkaloids (SGA) produced in potato tuber tissues after wounding (Kuć and Lisker, 1977). The accumulation rate of these two compounds in top 2 mm layer of tissue increases continuously up to 5 days following wounding (Kuć, 1982; Ishizaka and Tomiyama, 1972; Shih *et al*, 1973). This accumulation is thought to be associated with the wound repair mechanism (Kuć and Lisker, 1977; Ishizaka and Tomiyama, 1972; Fitzpatrick *et al*, 1977). In addition, because of their toxicity to insects (Tingey, 1984) and fungi (Allen and Kuć, 1968; Sinden *et al*, 1973; McKee, 1955, 1955), these two compounds may have a role in chemical defense of potato against invasion by other organisms. Moreover, the fact that *F. sambucinum* could not cause disease in potato tuber discs aged at room temperature two or more days even with a 2 mm deep wedge cut on the aged discs (Chapter I data) indicates that some chemical defense mechanism(s) may be functioning in tissue beneath the wounded surface. Therefore, the purpose of this study was to test whether α-solanine and α-chaconine were toxic to *F. sambucinum in vitro* and whether there was a synergistic effect of these two compounds on *F. sambucinum* spore germination and growth. Finally, the correlation between production *in vivo* and toxic concentration of these two compounds *in vitro* was investigated.

MATERIALS AND METHODS

Effect of SGA on spore germination and growth of *F. sambucinum*

The effect of commercial α-solanine and α-chaconine (Sigma) on *F. sambucinum* spore germination was tested. α-Solanine and α-chaconine dissolved in solvent containing tetrahydrofuran: acetonitrile: water (2:1:1. v/v/v) were placed in the wells of spot plate. After evaporation of the solvent, 10 μl of 0.01 N HCl was added to redissolve SGA and 90 μl of the fungal spore suspension at concentration of 1×10^6 spores/ml was then mixed with the SGA solution. The fungal spore suspension was made with 0.22 μm Millipore membrane filtered 0.05 M Na-phosphate buffer, pH 6.0 or 7.0 containing 1/10 x potato dextrose broth (PDB). The control contained everything except SGA. The plate was incubated at 25 ^{0}C in the dark for 6 hr. The germinated and non-germinated spores were counted using hemocytometer. For each sample, the spores in three 1x1 mm squares of hemocytometer were counted and averaged. The inhibition rate of spore germination by SGA was calculated as follows:

$$\text{Inhibition of spore germination (\%)} = \frac{\text{Avg. germt'n rate of cont.} - \text{Germt'n rate of treated}}{\text{Average germination rate of control}} \times 100\%$$

The effect of α-solanine and α-chaconine on *F. sambucinum* growth was tested using plate assay. α-Solanine and α-chaconine were placed in glass petri dishes (5 cm in diameter). After evaporation of the solvent, 50 μl of 0.01 N HCl was added to dissolve SGA and 1 ml of PDA in 0.05 N Na-phosphate buffer, pH 6.0 or 7.0 was added to each plate and mixed well. Following solidification of the medium, a 0.5 cm agar plug taken from growing edge of 6-8 day old culture of the fungus was placed in center of the plate. The plates were then incubated at RT for 4 days, and the diameter of fungal colony was measured.

Synergistic effect of α-solanine and α-chaconine on *F. sambucinum* spore germination and fungal growth was also tested. The procedures were the same as those described in the above except that individual SGA was replaced with mixture of α-solanine and α-chaconine at different ratio of both compounds. The inhibition of fungal colony growth was calculated as follows:

$$\text{Inhibition of fungal colony growth (\%)} = \frac{\text{Avg. growth of control (cm)} - \text{Growth of treated (cm)}}{\text{Average growth of control (cm)}} \times 100\%$$

Extraction of SGA from aged or *F. sambucinum* or *C. cucumerinum* inoculated potato tuber discs

The tuber disc preparation was the same as described in Chapter I. The procedures described by Zook and Kuć (1991) were used for SGA extraction. Briefly, 20 slices of top 0.5 mm layer of tissue from aged or inoculated tuber discs with 2 cm in diameter were collected and homogenized in about 100 ml of solvent containing $CHCl_3$, MeOH and HOAc (10:9:1, v/v/v) at 1/2 speed for 30 second. The homogenate was stored at RT for 24 hr and then filtered through Whatman #1 filter paper. The filtered homogenate was dried near dryness with rotary evaporator and mixed with 15 ml of 2% of HOAc. The sample mixtures were partitioned with the same volume of $CHCl_3$ for two times. After centrifugation at 12,000 rpm with SS-34 roter (Sorvall) for 10 min, the aqueous phase was collected and combined. Approximately 10 ml of concentrated NH_4OH then was added to bring the pH to 10. The samples were heated at 80 ^{0}C for 30 min. After cooling to RT, the samples were stored at 4 ^{0}C overnight and then centrifuged at 12,000 rpm for 10 min. The residue was dried in a desiccator over P_2O_5 and NaOH pellets for 2-3 days. The dried SGA residues were re-suspended in 3.5 ml of solvent containing tetrahydrofuran and water (6: 1, v/v) and then applied to a 3 ml amino column (Supelco, LC-NH_2) which had been conditioned with 5 ml of the same solvent. The SGA were eluted from the column with 2x 750 μl of solvent containing tetrahydrofuran, acetonitrile and water (2:1:1, v/v/v) and the eluted volume was adjusted to 2 ml with the same solvent. The eluted SGA samples were ready for HPLC analysis.

HPLC analysis of SGA from the tuber discs

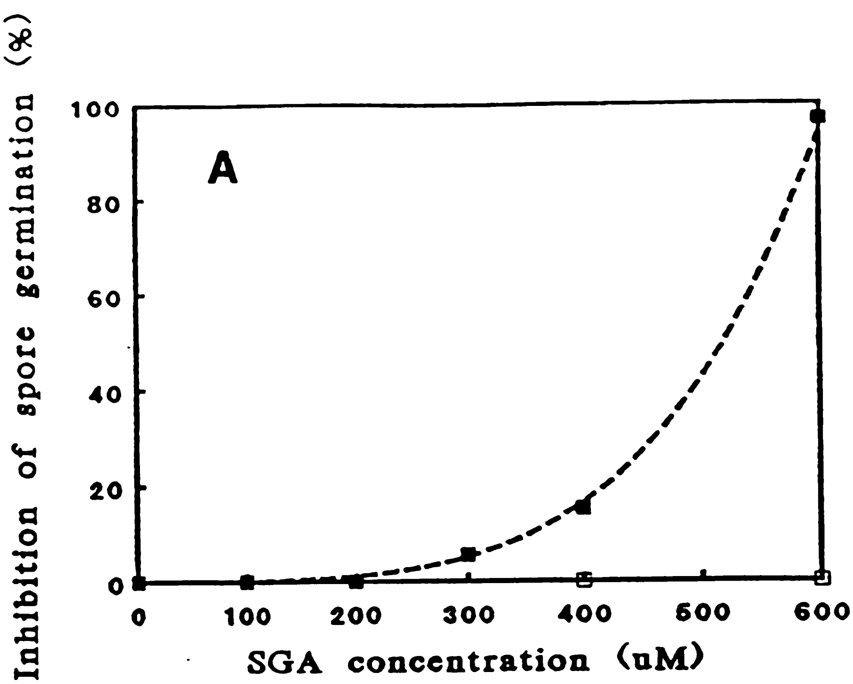
HPLC separation for SGA was performed at RT with a 4.6x 250 mm amino column (Alltech, Dearfield, IL), according to the procedures described by Zook and Kuć (1991). The mobile phase (same as the sample solvent) was pumped through at a flow rate of 1.0 ml/min. The absorbance wavelength of detector was set up at 215 nm. The detection sensitivity was 0.1 and chart speed was 1 cm/min. 15 μl of aliquot of each sample was injected into HPLC to analyze the content of SGA. Commercial α-solanine and α-chaconine dissolved in the same solvent as sample solvent were used as standards to quantify SGA in the samples.

RESULTS

Inhibitory effect of SGA on spore germination and growth of *F. sambucinum* at pH 6.0 and pH 7.0

At pH 6.0 α-Chaconine had an inhibitory effect on *F. sambucinum* spore germination in 1/10 x PDB at concentrations higher than 300 μM (Fig. 3-1 A). At concentration ca. 500 μM, α-chaconine caused 50% inhibition of fungal spore germination. In contrary, α-solanine did not show any inhibitory effect on the fungal spore germination at all tested concentrations (Fig. 3-1 A).

Both α-chaconine and α-solanine inhibited the fungal growth in PDA at concentration higher than 300 μM at pH 6.0 (Fig. 3-1 B). The inhibitory effect



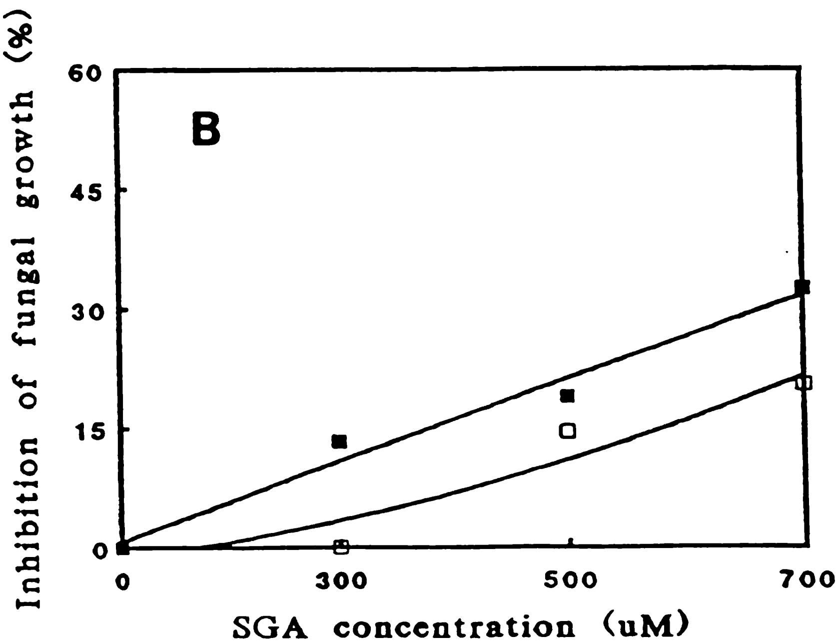


Fig. 3-1. Inhibitory effect of α-solanine (-□-) and α-chaconine (-■-) on spore germination (A) and colony growth (B) of *F. sambucinum* at pH 6.0. Standard deviations of the data points varied from 0.45 to 4.8%.

caused by α-chaconine could be detected at concentration of 150 μM, while the effect caused by α-solanine did not occur at concentrations less than 300 μM. Overall, α-chaconine was more toxic than α-solanine at the same concentrations.

At pH 7.0 the inhibitory effect caused by α-chaconine on the fungal spore germination was detected at concentration higher than 200 μM and was higher than that at pH 6.0 at concentrations between 300 and 500 μM (Fig. 3-2 A). At concentration of ca. 450 μM, α-chaconine inhibited 50% of spore germination. However, unlike that at pH 6.0 α-chaconine could not completely inhibit spore germination at 600 μM at pH 7.0. α-Solanine showed a some inhibitory effect on the spore germination at pH 7. At concentration of 600 μM, it inhibited 15% of the spore germination.

Both compounds showed similar higher levels (2-3 folds) of inhibitory effect on the fungal growth on PDA at pH 7.0 (Fig. 3-2 B) than those at pH 6.0.

Synergistic inhibitory effect of α-solanine and α-chaconine on spore germination and growth of *F. sambucinum*

At pH 6.0 the combination of α-solanine and α-chaconine at certain ratio showed greater inhibitory effect on both spore germination and growth of *F. sambucinum* than that caused by either one alone. Figure 3-3 shows that inhibition of spore germination by combination of the two compounds (dashed line) occurred at ratio of 2/4 (200 μM α-chaconine/400 μM α-solanine) and there was no inhibition with either one alone at the concentrations (Fig. 3-1 A and 3-3, solid lines, α-chaconine alone). Combination of α-chaconine and α-solanine at ratio of 5/1 (500 μM α-chaconine/100 μM α-solanine) gave a complete inhibition on the fungal spore germination. Combination of the two compounds also showed higher levels of inhibition on the fungal growth on PDA compared to those at the same

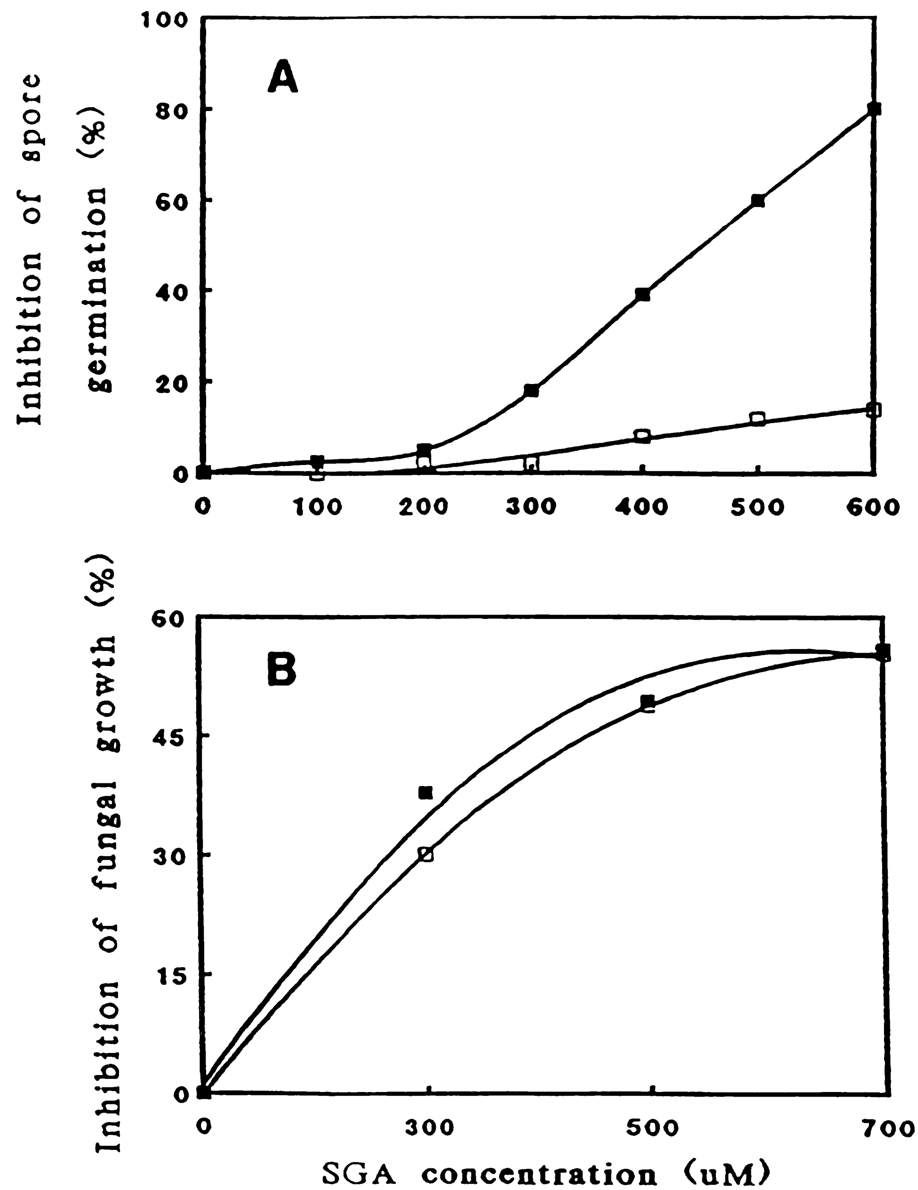


Fig. 3-2. Inhibitory effect of α-solanine (-□-) and α-chaconine (-■-) on spore germination (A) and colony growth (B) of *F. sambucinum* at pH 7.0. Standard deviations of the data points varied from 1.2 to 4.3%.

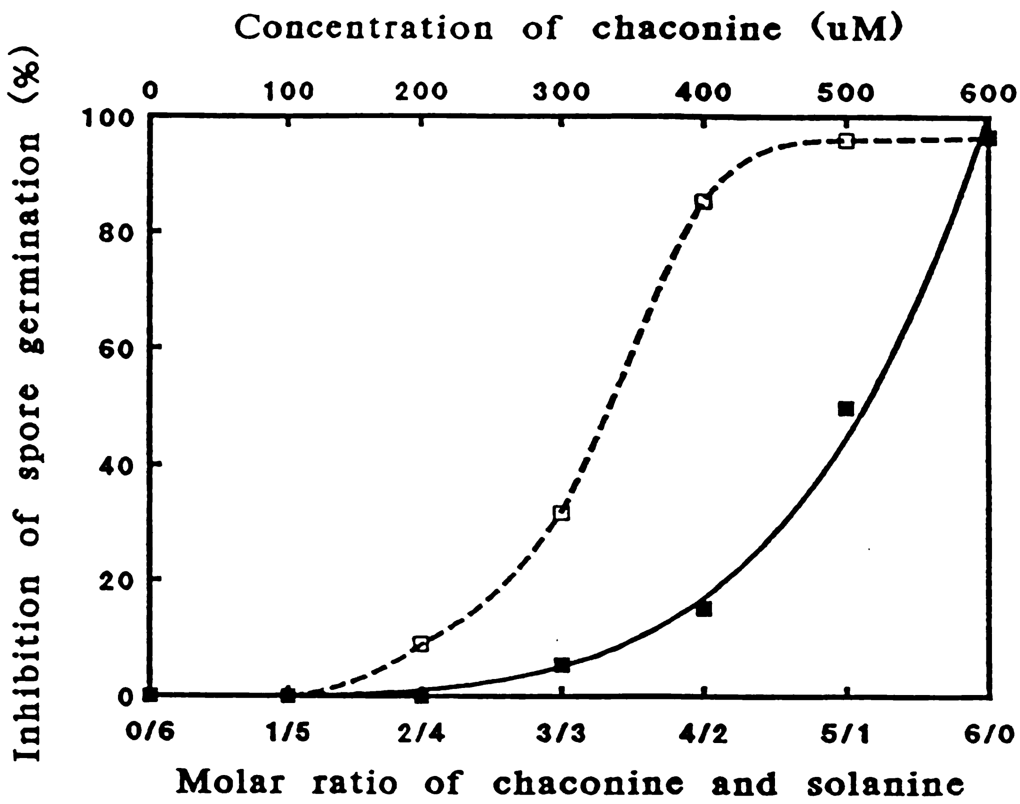


Fig. 3-3. Synergistic inhibitory effect of α-chaconine and α-solanine on spore germination of *F. sambucinum* at pH 6.0. Mixture of α-solanine and α-chaconine (-□-) or α-chaconine (-■-) only. Total concentration of each SGA mixture was 600 μM. Standard deviations of the data points varied from 1.6 to 3.6%.

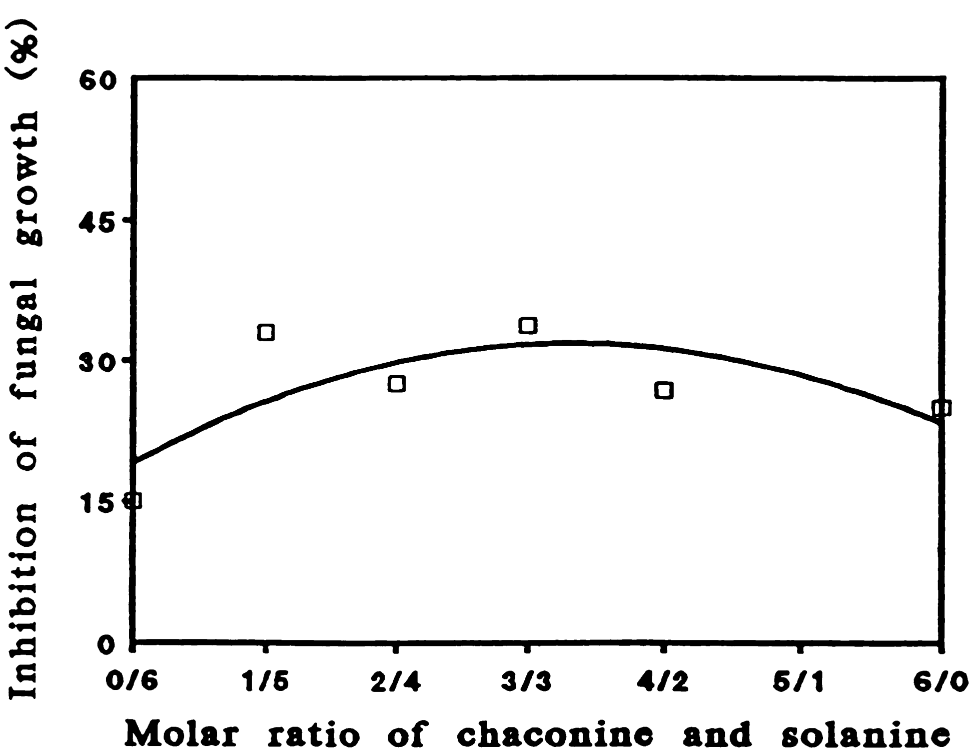


Fig. 3-4. Synergistic inhibitory effect of α-solanine and α-chaconine on colony growth of *F. sambucinum* at pH 6.0. Total concentration of each SGA mixture was 600 μM. Standard deviations of the data points varied from 0.4 to 4.4%.

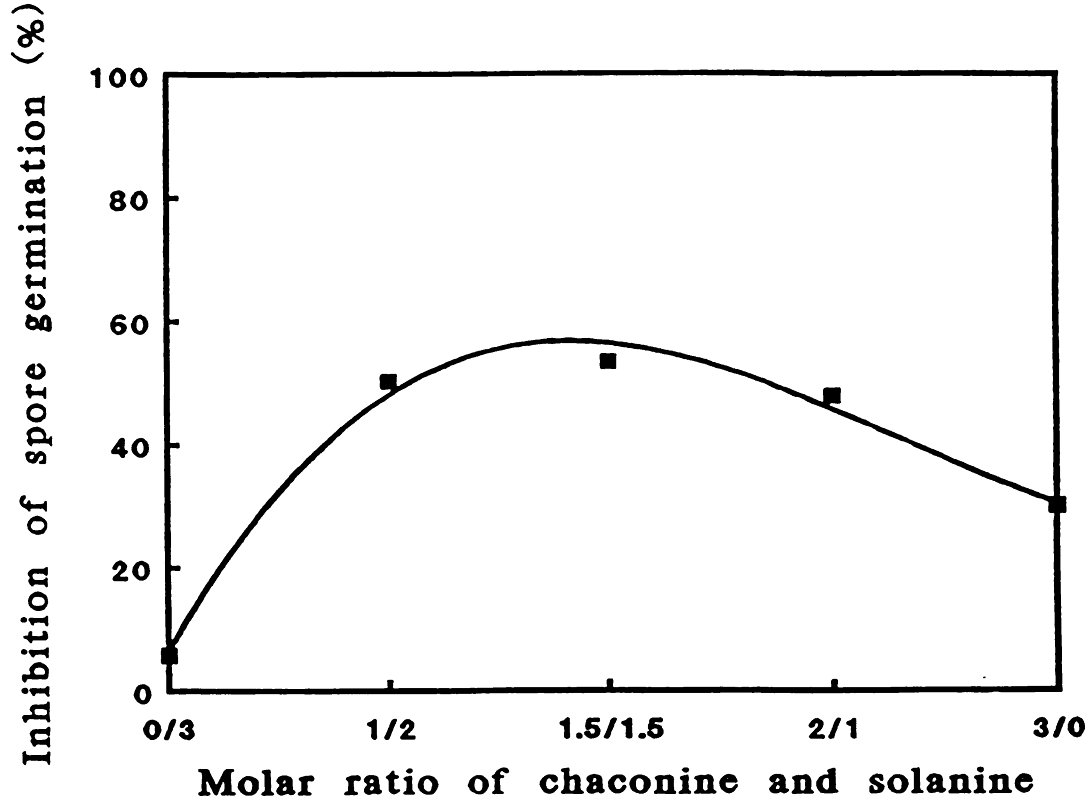


Fig. 3-5. Synergistic inhibitory effect of α-chaconine and α-solanine on spore germination of *F. sambucinum* at pH 7.0. Total concentration of each SGA mixture was 300 μM. Standard deviations of the data points varied from 0.9 to 5.9%.

concentrations of single compound (Fig. 3-4). This clearly indicated that there was a inhibitory synergism between these two compounds.

At pH 7.0, combination of the two compounds at lower concentration ($\leq$300 μM) gave a higher inhibitory effect on the fungal spore germination (Fig. 3-5) compared to that at pH 6.0. The inhibition reached its maximum at approximate 55% at 1.5/1.5 (150 μM/150 μM) ratio of α-chaconine and α-solanine. This indicates that pH condition also influences toxicity of these two compounds.

Accumulation of SGA in aged tuber discs

HPLC analysis of the samples showed that SGA accumulated in aged discs. After 24 hr of aging, both α-solanine and α-chaconine accumulated in a similar pattern, but rate of α-solanine accumulation was faster (Fig. 3-7 A and B). And both reached their peaks at 72 hr and stayed at the peaks through 96 hr during aging.

Suppression of SGA accumulation in tuber discs by *F. sambucinum* and by *C. cucumerinum*

HPLC analysis showed that inoculation with *F. sambucinum* almost completely suppressed accumulation of both α-solanine and α-chaconine (Fig. 3-6 A and B) during 36 hr incubation after inoculation.

C. cucumerinum also suppressed accumulation of both α-solanine and α-chaconine but to a lesser degree (Fig. 3-7 A and B). SGA in the inoculated tissue accumulated slowly during 96 hr incubation after inoculation with *C. cucumerinum*.

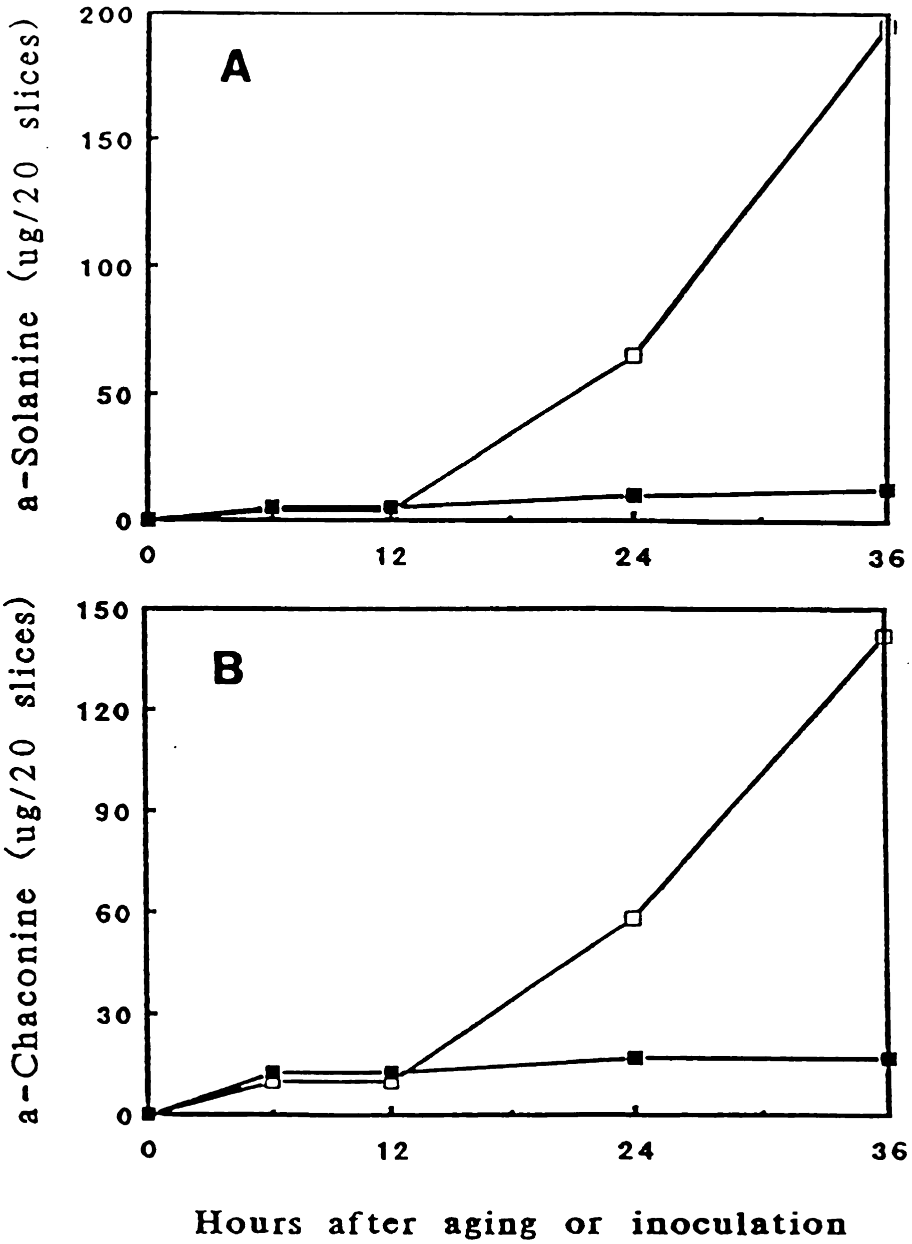


Fig. 3-6. Time course of α-solanine (A) and α-chaconine (B) accumulation in aged (-□-) and *F. sambucinum*-inoculated (-■-) tuber discs.

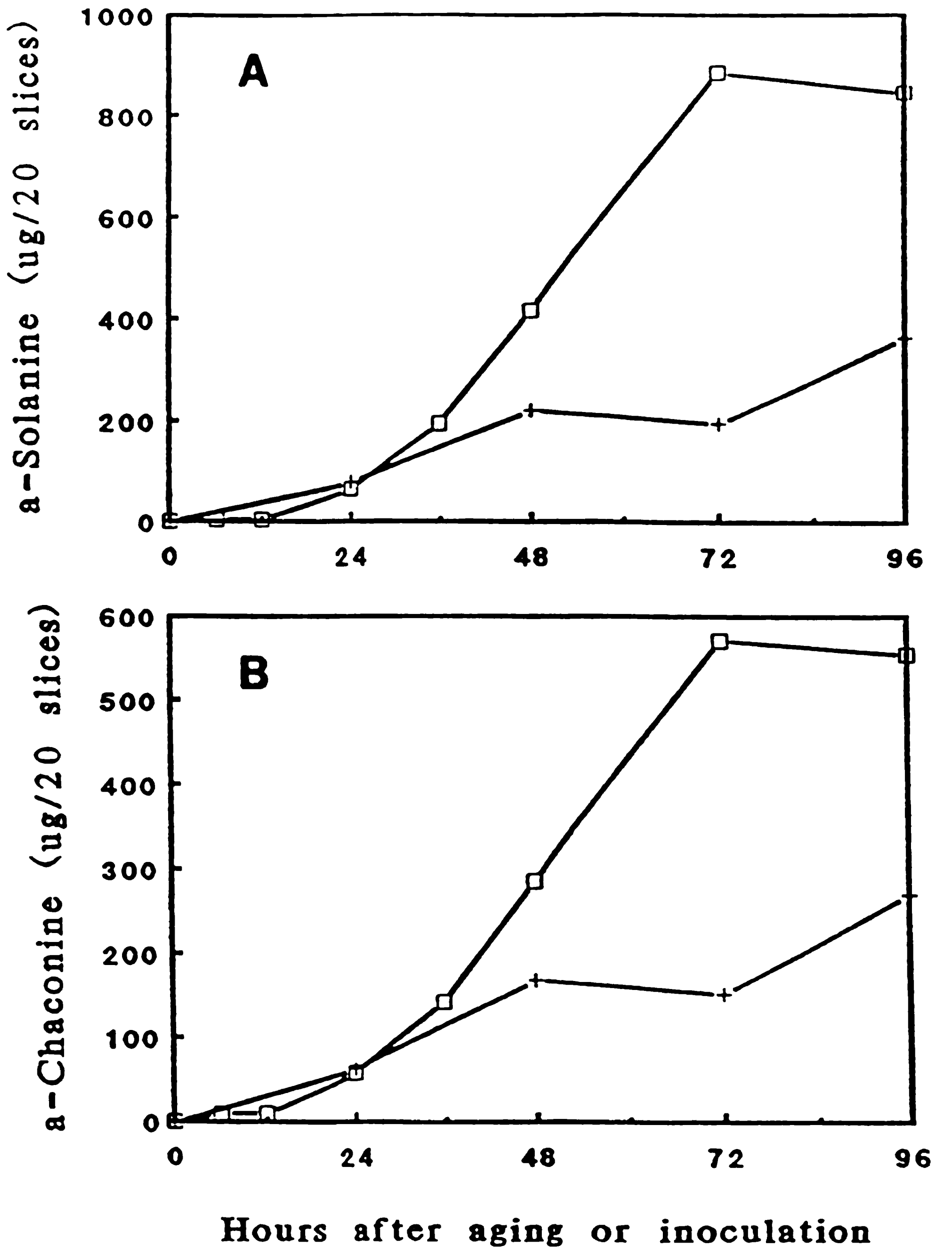


Fig. 3-7. Time course of α-solanine (A) and α-chaconine (B) accumulation in aged (-□-) and *C. cucumerinum*-inoculated (+) tuber discs.

Inhibition of SGA from aged tuber discs on spore germination and colony growth of *F. sambucinum*

Based on the results from Fig. 3-7, SGA concentrations in top 0.5 mm layer of the aged discs were calculated (Table II). The inhibition on spore germination and colony growth of the fungus by SGA at calculated concentrations also deduced, according to the results from *in vitro* SGA toxicity tests.

DISCUSSION

It has been known that potato SGA, mainly α-chaconine and α-solanine are toxic to insects (Tingey, 1984) and fungi (Allen and Kuc, 1968; Sinden *et al*, 1973; McKee, 1955, 1959). However, the toxicity of potato SGA to potato dry rot causing fungal pathogen *F. sambucinum* has not been reported. The present study demonstrated that α-chaconine and α-solanine have an inhibitory effect on spore germination and colony growth of *F. sambucinum* (Fig. 3-1 A and B and 3-2 A and B). In the experiments conducted in this study, α-chaconine showed a higher toxicity than α-solanine at both pH 6 and 7. The results of this investigation were in agreement with reports presented by Sinden *et al* (1973) and McKee (1955, 1959) on SGA inhibitory effects on growth of *Alternaria solani* and spore germination of *Fusarium caeruleum* (Sinden *et al*, 1973; McKee, 1955, 1959).

The toxicity of both α-chaconine and α-solanine to spore germination and colony growth of *F. sambucinum* was also greater at higher pH levels, as reported by Sinden *et al* (1973) and McKee (1955, 1959). At pH 6.0 α-chaconine at concentration of 500 μM inhibited 50% of the fungal spore germination (Fig. 3-1 A), while at pH 7.0 the same concentration gave a 60% inhibition (Fig. 3-2 A). Similarly, at pH 6.0 α-solanine did not have any inhibitory effect on the spore

Table II

Inhibition on spore germination and colony growth of F. sambucinum *by SGA in aged tuber discs at pH 6 and 7*

Aging time(hr)	SGA conc.[a] S/C[b] (μM)	Inhibition on spore germt'n (%) at pH 6 S/C	Total S+C[c]	pH 7 S/C	Total S+C	Inhibition on colony growth (%) at pH 6 S/C	Total S+C	pH 7 S/C	Total S+C
24	23/22	0/0	0	0/0	0	0/0	0	0/0	0
48	153/107	0/0	0	0/2	2	0/6	6	15/15	30
72	324/214	0/2	2	2/6	8	5/12	17	30/30	60
96	310/207	0/2	2	2/6	8	5/12	17	30/30	60

[a]SGA concentration in the top 0.5 mm layer of aged potato tuber discs.
[b]S=α-Solanine, C=α-chaconine.
[c]S+C=Sum of inhibition (%) caused by α-solanine and α-chaconine alone.

germination at concentration up to 600 μM, while at pH 7.0 the same concentration gave a 15% inhibition of the spore germination. In the cases of fungal colony growth on PDA, the two compounds also showed a higher toxicity at pH 7.0. At pH 6.0 α-chaconine at concentration of 700 μM inhibited 30% of the fungal colony growth, while at pH 7.0 α-chaconine gave a 55% inhibition. On the contrary, α-chaconine and α-solanine showed a similar inhibitory effect on the fungal colony growth at pH 7.0 compared to that at pH 6.0 where α-solanine showed a slightly lower toxicity. In addition, at pH 7.0 α-chaconine at concentration of 600 μM only gave a 80% of inhibition on spore germination, unlike that at pH 6.0 where α-solanine at the same concentration completely inhibited the fungal spore germination. The reason for this is not known. Nevertheless, the results of this study clearly demonstrated that these two compounds are more toxic at higher pH level and this may be due to, as suggested by McKee (1959), that the toxicity is contributed by molecules of un-dissociated base whose concentration must reach a certain level to affect membrane stability. However, it is not clear why the fungal mycelia were more sensitive to these two compounds than the fungal spores at concentrations lower than 300 μM but less sensitive at concentrations higher than 500 μM (Fig 3-1 A and B; Fig 3-2 A and B).

It has been reported that combination of potato alkaloids had higher inhibitory effects than single alkaloid alone on fungal growth (Sinden, 1973). Recently, Roddick and Rijnenberg (1987, 1988) have demonstrated that combination of α-solanine and α-chaconine gave a synergistic effect on disruption of phosphotidylcholine/cholesterol liposomes and on the lysis of rabbit erythrocytes, red beet cells, and *Penicillium notatum* protoplasts. The results obtained from this study also showed that a combination of α-chaconine and α-

solanine gave a higher inhibition than either one compound alone on spore germination and growth of *F. sambucinum* (Fig. 3-3, 3-4 and 3-5). The mechanism of synergism between these two compounds in relation to their membrane disruption is not clear. However, it is known that binding to 3ß-hydroxy sterols in membranes *in vitro* by α-solanine and α-chaconine is the cause of membrane disruption, although α-solanine has a less binding capacity which may be due to the difference in sugar moiety between the two compounds (Roddick and Rijnenberg, 1986). Therefore, as suggested by Roddick *et al* (1988), the synergism between these two compounds may be an "analog" type effect where the activity of α-chaconine is enhanced by α-solanine or vise versa, or both.

Of production of potato SGA (primarily α-solanine and α-chaconine) in tuber tissues, the rate of SGA accumulation increased largely after slicing (Fig. 3-7 A and B). This is somehow related to wound repair process (Lisker and Kuć, 1977). In contrast, inoculation with non-pathogen of potato *C. cucumerinum* or pathogen *F. sambucinum* on tuber discs suppressed SGA production (Fig. 3-6 A and B; 3-7 A and B) and the suppression by *F. sambucinum* was much severe than *C. cucumerinum*. This is different from that reported by Shih *et al* (1973) where the incompatible race of *Phytophthora infestans* or *Helminthosporium carbonum* had a greater suppression than compatible race of *P. infestans* on SGA accumulation. This may be due to tissue damage caused by *F. sambucinum* which completely macerated the top layer of discs in 48 hr and *C. cucumerinum* has little effect on eliciting production of terpenoid phytoalexins.

There have been reports of study on the role of potato SGA in disease resistance. Frank *et al* (1975) have shown that there was no correlation between SGA contents in tubers or leaves or stems and disease resistance against *A. solani, P. infestans, Streptomyces scabies* and *Verticillium albo-atrum* after invasion of potato tubers by theses pathogens. Two years earlier, Deahl *et al* (1973) also reported that there was no relationship between SGA content and resistance to *P. infestans* in fifteen potato clones. However, Sinden *et al* (1973) also reported that according to the test of SGA toxicity on *A. solani in vitro*, SGA in young potato leaves showed a correlation between their contents and reduction of lesion development caused by the pathogen. They proposed that SGA in potato leaves may be an important factor of temporary resistance. In this study, the role of SGA in disease resistance against *F. sambucinum* in aged potato tuber discs was investigated and the results indicated that the accumulation of α-chaconine and α-solanine may be part of chemical defense mechanism(s). With the known diameter (2 cm) of each tuber disc, the concentration of α-chaconine and α-solanine accumulated in top 0.5 mm layer of discs can be calculated (Table II). At 48 and 72 hr after aging at RT, α-chaconine concentration had reached the levels which could only inhibit 0 and 2% of the fungal spore germination at pH 6 *in vitro*, respectively. These concentrations could also inhibit the fungal colony growth at levels of 6 and 12%, respectively. With the same calculation, the concentrations of α-solanine at 72 hr after aging could have only reached 5% inhibition level on the fungal colony growth. However, at pH 7.0, the concentrations of both α-chaconine and α-solanine at 48 and 72 hr had reached the levels of 15 and 30% inhibition on the fungal growth. If the inhibitory effects were added, the total inhibition would be 30 and 60%, respectively. Although it is assumed that pH in the cells is 6.0 since pH of tuber tissue extract is ca. 6, it can not rule out that the and local change of pH value in the cells adjacent to wounded surface. If this change increases local cytoplasmic pH, the SGA toxicity would be expected to be higher. In addition to these deductions, the antifungal activity of SGA accumulated in aged tuber discs was also evident by the results from TLC bioassay of potato

SGA toxicity to *C. cucumerinum* (Data not shown). The inhibition of the fungus resulted in clear zone on TLC plate and the zone size corresponding to α-chaconine was larger than that of α-solanine. In addition, the sizes of clear zones increased along with time of aging indicating that there was higher content of SGA in discs aged for longer period of time.

If a synergistic inhibitory effect caused by combination of these two compounds is taken into account, the effect on spore germination and growth of *F. sambucinum* would be higher for both compounds than either compound alone. The antifungal activity of potato SGA and their concentration in aged potato tissues near the wounded surface may partially account for the findings noted by Meyer (1940) that infection of *P. infestans* was largely reduced if the inoculation was performed at 3 days after wounding. It may also provide some explanation to the phenomenon reported by Lansade (1950) that exposure of light to tuber tissues would make it resistant to infection by *F. caeruleum*. Since it is known that SGA accumulate largely in tuber tissues under light (Conner, 1937). The accumulation of SGA in the top layer of aged discs at toxic concentration to *F. sambucinum* may also be part of the reason that there is no disease development in tuber discs aged for 72 hr even with a fresh cut wedge after inoculation with the fungal spore suspension at concentration of 10^6 spore/ml and there is only a little disease development when inoculated with a 5-mm agar plug containing large density of inoculum (Chapter I). However, it can not be ruled out that other antifungal compound(s) are present in the top layer tissue of aged tuber discs which may contribute to inhibition of *F. sambucinum* invasion.

REFERENCES

Allen E, Kuć J 1968 α-Solanine and α-chaconine as fungitoxic compounds in extracts of Irish potato tubers. Phytopathology 58: 776-781.

Conner HW 1937 The effect of light on solanine synthesis in the potato tuber. Plant Physiology 12: 79-98.

Deahl KL, Young RJ, Sinden SL 1973 A study of the relationship of late blight resistance to glycoalkaloid content in fifteen potato clones. American Potato Journal. 50: 248-253.

Fitzpatrick TJ, Herb SF, Osman SF, McDermott JA 1977 Potato glycoalkaloids: Increases and variations of ratios in aged slices over prolonged storage. American Potato Journal 54: 539-544.

Frank JA, Wilson JM, Webb RE 1975 The relationship between glycoalkaloid and disease resistance in potatoes. Phytopathology 65: 1045-1049.

Ishizaka N, Tomiyama K 1972 Effect of wounding or infection by *Phytophthora infestans* of content of terpenoid in potato tubers. Plant and Cell Physiology. 13: 1053-1063.

Kuć J 1982 Phytoalexins from the Solanaceae. In Bailey JA and Mansfield JW ed Phytoalexins. John Wiley and Sons, New York 81-105.

Lansade, M 1950 Recherches sur la Fusariose ou pourriture séche de la pomme deterre. Ann. Inst. nat. Rech. agron., Paris, Sér. C, (Ann. Epiphyt.), I, 157.

Lisker N, Kuć J 1977 Elicitors of terpenoid accumulation in potato tuber slices. Phytopathology 67: 1356-1359.

McKee RK 1955 Host-parasite relationship in the dry-rot disease of potatoes. Annals of Applied Biology 43: 147-148.

McKee RK 1959 Factors affecting the toxicity of solanine and related alkaloids to *Fusarium caeruleum*. Journal of General Microbiology 20: 686-696.

Meyer, G 1940 Zellphysiologische und anatomische Untersuchungen über dié Reaktion der Kartoffelknolle auf den Angriffder *Phytophthora infestans* bei Sorten verschiedener Resistenz. Arb. biol. Abt. (Anst.-Reichsanst.)Berl. 23: 97.

Roddick JG, Rijnenberg AL 1986 Effect of steroidal glycoalkaloids of the potato on the permeability of liposome membranes. Physiologia Plantarum. 68: 436-440.

Roddick JG, Rijnenberg AL 1987 Synergistic interaction between the potato glycoalkaloids α-solanine and α-chaconine in relation to lysis of phospholipid/sterol liposomes. Phytochemistry 26: 1325-1328.

Roddick JG, Rijnenberg AL, Osman SF 1988 Synergistic interaction between potato glycoalkaloids α-solanine and α-chaconine in relation to destabilization of cell membranes: ecological implications. Journal of Chemical Ecology 14: 889-902.

Shih M, Kuć J, Williams EB 1973 Suppression of steroid glycoalkaloid accumulation as related to rishitin accumulation in potato tubers. Phytopathology 63: 821-826.

Sinden SL, Gpth RW, O'Brien MJ 1973 Effect of potato alkaloids on the growth of *Alternaria solani* and their possible role as resistance factors in potatoes. Phytopathology 63:303-307.

Tingey WM 1984 Glycoalkaloids as pest resistance factors. American Potato Journal 61: 157-167.

Zook MN, Kuć, JA 1991 The use of a sterol inhibitor to investigate changes in the rate of synthesis of 2,3-oxidosqualene in elicitor-treated potato tuber tissue. Physiological and Molecular Plant Pathology 39: 391-401.

CONCLUSIONS AND FUTURE DIRECTIONS

The study of this dissertation demonstrates that enhancement of general host defense responses can result in improved resistance to *F. sambucinum* of potato tuber tissues. In the host defense responses, two mechanisms seem to have important roles in resistance against *F. sambucinum* infection. Firstly, rapid and higher accumulation of lignin-like material may be functioning as physical barrier to the fungal invasion. This may be due to the lignified host cell walls are more resistant to fungal cell wall degrading enzymes. The induction of host enzyme activity involving in lignification correlates the increased rate of lignin accumulation. Secondly, compounds with antifungal activity such as SGA, chlorogenic acid and its oxidation products may also contribute to the induced general resistance.

For future study, the following investigations may be needed:

1. Further characterization of ionically bound cell wall PO will help to elucidate the role of cell wall PO isozymes in lignification.

2. Effect of different concentration of *C. cucumerinum* on the elicitation of host defense response. It would be very interesting to investigate what elicits the host defense response, cell wall component or any other compounds.

3. The relation of accumulation of SGA to the wound healing process, as well as phytoalexin accumulation.

4. *In situ* localization of PAL mRNA accumulated in tuber discs inoculated with *C. cucumerinum*.

5. Since there is no cultivar resistant to *F. sambucinum* available, the most effective way to reduce dry rot disease would be to try to find biotic or abiotic compounds to elicit host general defense and to enhance the rate of wound healing.

6. Fast rate of wound healing and lignin deposition, as well as the rapid accumulation of SGA in the wounded tissue may be used as markers to select germplasm for breeding for resistance to *F. sambucinum*.