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PHYSIOLOGICAL AND BIOCHEMICAL ASPECTS OF POTATO SCAB DISEASE CAUSED BY *STREPTOMYCES* SPECIES

By

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ABSTRACT

PHYSIOLOGICAL AND BIOCHEMICAL ASPECTS OF POTATO SCAB DISEASE CAUSED BY *STREPTOMYCES* SPECIES

By

Frank Richard Spooner, Jr.

Physiological and biochemical aspects of the potato scab disease and pathogens were studied. A Michigan deep-pitting *Streptomyces* designated D.P. was isolated from an infected potato tuber. In culture, D.P. produced reflexuous spore chains and lacked melanin production. In culture the *S. scabies* isolate RL 232 produced spiral spore chains and melanin. D.P. caused deep pitted lesions on the potato cultivar Atlantic, and RL 232 produced common scab lesions on the cultivar Atlantic. D.P., RL 232, and the nonpathogen RL 95 produced extracellular pectinases when grown on polygalacturonic acid or pectin. The culture filtrates of all the isolates exhibited lyase activity in the range of pH 7-9 on pectin and 8-10 on polygalacturonic acid. The culture filtrates from all isolates were found to macerate potato tuber tissue. Ultraviolet light generated PL-deficient mutants of D.P. produced scab lesions on Atlantic tubers. No correlation was shown between the loss of pectinase production and the ability to produce disease. The deep-pitting isolate D.P. produced the thaxtomin-like compounds that elicited a browning response from potato tuber discs within 18 hours after application. Spores of RL 232, RL 95, and D.P. elicited the production of chlorogenic acid (CGA) and lignin from Russet Burbank and Atlantic tuber discs. D.P. elicited higher amounts of CGA between 48 and 72 hours after inoculation and nearly identical amounts of lignin within 24

hours after inoculation on both Russet Burbank and Atlantic tubers, relative to uninoculated controls. Both RL 232 and RL 95 elicited lower amounts of CGA and higher levels of lignin throughout the time course relative to the uninoculated controls. The physiological and biochemical differences between the pathogenic isolates D.P. and *S. scabies* (RL 232) suggested that D.P. was a different species of potato scab-causing *Streptomyces*.

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CHAPTER I

INTRODUCTION TO DISSERTATION: RESEARCH OBJECTIVES

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INTRODUCTION TO DISSERTATION: RESEARCH OBJECTIVES

The potato scab disease is caused by *Streptomyces scabies*, *S. acidiscabies*, and several other *Streptomyces* species. These bacteria cause corky lesions to form in the tuber periderm of several cultivars of *Solanum tuberosum* L. The type of lesions formed range in appearance from superficial corky lesions to deep pitted furrows. All types of lesions lower the quality of potato tubers.

Potato scab research has focused primarily on finding effective methods to control the pathogen. Control of potato scab has been achieved through manipulation of soil moisture, soil pH, soil mineral composition, and soil microorganisms, which are antagonistic to *S. scabies*. Additionally, control of the disease has been partially achieved through crop rotation and use of resistant cultivars of potato. Several studies also have been conducted to determine the periods at which the susceptibility of potato to infection is greatest and the means by which the pathogen gains access to susceptible tubers.

Establishing the cultural, morphological, and pathological characteristics that are associated with potato scab-causing bacteria has been the subject of much research. Several diagnostic methods have been developed to categorize *S. scabies*. However, the only criterion which has been accepted is the ability of an isolate to cause scab lesions on potato tubers. Traditionally, if an isolate of *Streptomyces* caused potato scab, it was grouped with *S. scabies* regardless of whether the isolate fit the given descriptions for the group. Recent studies using molecular methods have shown that several *Streptomyces* spp. grouped with *S. scabies*, on the basis of pathogenicity, standard cultural, and

physiological test used by *Streptomyces* researchers were not related to *S. scabies* on the basis of DNA homology.

Although more is now known about the taxonomy of *S. scabies* and other potato scab-causing *Streptomyces* spp., relatively little is known about the sequence of physiological events that occur during the infection process and how the disease progresses to the expression of symptoms. In addition, very little is known at the physiological level regarding the response of potato tissue to infection by *Streptomyces*. *S. scabies* and *S. acidiscabies* are known to produce phytotoxic compounds in potato tissue and in culture that are capable of reproducing common scab lesions on microtubers. However, whether this characteristic is shared by streptomycetes other than *S. scabies* and *S. acidiscabies* has yet to be reported. The mode of action and the nature of cellular destruction caused by the toxin, called thaxtomin, has also yet to be reported.

S. scabies, *S. ipomoea*, and several other *Streptomyces* species are known to produce cell-wall degrading enzymes of which the pectinase have been implicated in the infection process. As histological studies have revealed, the hyphal filaments penetrate the middle lamellae which is primarily composed of pectic polymers. However, whether there is a correlation between the ability to synthesize pectolytic enzymes in culture and the ability to cause disease is not known. With regard to the host defense responses, histochemical work shows that potato tissue infected by *S. scabies* deposits suberized barriers to prevent infection. However there is not a physiological study to support whether the accumulation of phenolic compounds, lignin, or suberin occurs specifically in response to *S. scabies*.

In this dissertation, I have attempted to examine potato scab disease from a physiological perspective. In the initial phases of my research on potato scab, I was able to isolate several strains of *Streptomyces* which were culturally dissimilar to *S. scabies*. One isolate, in particular, causes deep-pitted scab, lacks the ability to produce melanin or colored pigments in culture, and bears its spores in reflexuous spore chains. This isolate was designated D.P. and was used in several of my studies because it was culturally and morphologically different from *S. scabies* and produced very deep lesions. Further, I wanted to determine what physiological and biochemical differences or similarities existed between the D.P. isolate and *S. scabies*.

In my study of the physiology of potato scab, I reexamined whether pectolytic enzymes were produced by pathogenic and nonpathogenic streptomycetes in culture to determine whether different classes of enzymes were produced by nonpathogens and pathogens. I also attempted to determine whether specific pectic substrates caused the induction of pectolytic enzymes. It was also important to determine whether pectolytic enzymes from *Streptomyces* species caused plant tissue damage and if this was a characteristic of exclusive to pathogens. I also attempted to generate pectinase deficient mutants to determine whether the loss of this function correlated with a subsequent loss in pathogenicity or virulence.

Streptomyces scabies has been reported to produce a toxin called thaxtomin. I wanted to determine whether this phytotoxic compound was synthesized by the deep-pitting scab isolate D.P. Such a finding would suggest that divergent forms of pathogenic *Streptomyces* may posses similar or identical pathogenic mechanisms. I also attempted to determine how potato tissues responded to *Streptomyces* infection by

monitoring the accumulation of two compounds which have been implicated in the defense response of potato to several fungal and bacterial pathogens. The compounds monitored were chlorogenic acid and lignin, both of which may have antimicrobial activity and/or prophylactic properties which prevent infection by streptomycetes.

The ultimate objective of my research was to develop starting points for future investigations into the host-parasite physiology of potato scab disease. Understanding the physiological dynamics and details of infection and symptom formation should allow for the design of effective measures to control potato scab.

REVIEW OF LITERATURE

After cereals, potatoes (*Solanum tuberosum* L.) are the most important food crop in the world (Bajaj, 1987). Potato has been a staple crop around the world for at least 400 years (Burton, 1989). Potatoes are a versatile food product and are sold as fresh produce, potato chips, frozen french fries, and potato starch. The potato provides an excellent source of protein and contains nearly a full complement of essential amino acids (Bajaj, 1987). Potatoes are also used in producing ethanol (Burton, 1989). The multiple uses of potatoes as food, fodder and other purposes account for its dominance among the "root" crops currently used around the world.

Over the past several hundred years as the potato was introduced around the world it has encountered a variety of environmental conditions and has been exposed to numerous pest and pathogens to which it may not have natural resistances. These include Colorado potato beetle, potato viruses X and Y, late blight, field and post harvest tuber rots, and several environmental conditions (Agrios, 1978; Hooker, 1981; Burton 1989). The potato has been important in our understanding of several fundamental concepts of plant pathology (Agrios, 1978). Potato scab is a "classic" plant pathological condition which first appeared in the plant pathology literature during the late 1800's (Bolley, 1890; and Thaxter, 1891).

Proper identification of the causal agent responsible for potato scab has proven to be difficult for most researchers (Lambert and Loria, 1989 a). Bolley correctly identified the causal agent as a bacterium (Bolley, 1890). Thaxter (1891), however, was able to pure culture the organism, confirm its pathogenicity, and concluded that the

causal agent of the disease was a fungus. It was surprising, considering the detail of Thaxter's study of *Oospora scabies*, that he failed to recognize the organism was a bacterium. Cunningham (1912) reported that *Oospora scabies* was a bacterium and speculated that it was related to the *Actinomycetes* (Cunningham, 1912; and Lutman and Cunningham, 1914). Gussow (1914) redesignated the species *Actinomyces scabies*. The genus *Streptomyces* later replaced the genus *Actinomyces* (Waksman and Henrici, 1948). *Streptomyces scabies* was the new name designated for the potato scab-causing streptomycetes (Waksman, 1961).

Potato scab has not been considered an extremely destructive disease of potato. Potato scab primarily reduces the quality of tubers by producing disfiguring lesions in the periderm which may range from superficial pimples or flecks too pits 0.5 cm to 1.0 cm deep in harvested tubers (Millard and Burr, 1926; and Hammerschmidt and Lacy, 1990).

Potato scab occurs in nearly all major potato-growing regions of the world (Keinath and Loria, 1989). However, there are few accurate assessments of the world-wide loss that this disease causes potato crops. Potato scab does not usually cause significant yield reductions. However, it is not uncommon to find whole fields of potatoes deemed unacceptable to be sold in markets (Manzer et al, 1977; and Hammerschmidt and Lacy, 1990). This point becomes apparent when one considers a situation where pitted scab of potato is prevalent and which causes deformation of tubers and destruction of immature tubers under soil conditions ideal for the pathogen.

Descriptions of Potato Scab Symptoms and Isolates of Bacteria.

"Scab" a generic term, describes any superficial roughness produced on the periderm of a potato tuber. Powdery scab, for example, caused by the slime mold *Spongospora subterranea*, produces lesions occasionally mistaken for those of *Streptomyces scabies* (Agrios, 1978). Various symptomatic lesions have been identified as potato scab (Thaxter, 1891; Lutman and Cunningham, 1916; Millard and Burr, 1926; and Hammerschmidt and Lacy, 1990), the reported types of scab are common, pitted, russet, and tumulus or upraised scab.

Common scab is identified by superficial, corky lesions on the tuber surface and lacks evidence of the pathogen penetrating beneath the periderm (Thaxter, 1891; Lutman and Cunningham, 1914; Millard and Burr, 1925; Jones, 1931; Lutman , 1941; Lapwood, 1973; Lambert and Loria, 1989 a; and Hammerschmidt and Lacy, 1990). *Streptomyces scabies* is usually identified as the causal agent of common scab (Lambert and Loria, 1989 a). However the acid tolerant scab pathogen, *S. acidiscabies*, also causes common scab lesion identical to those of *S. scabies* (Manzer et al, 1977; and Lambert and Loria, 1989 b). It has also been, reported that several infective isolates differing in morphology from *S. scabies* and *S. acidiscabies* can be isolated from common scab lesions (Faucher et al, 1992).

The minimal phenotypic characters used to identify *S. scabies* are: the ability to cause common scab on potato; a grey colored spore mass with spores borne in spiral spore chains; and use of all the International *Streptomyces* Project (ISP) sugars (Lambert and Loria, 1989 a). *S. scabies* also produces black or deep brown melanoid pigments in culture, especially on peptone yeast extract iron agar (PYI) and yeast extract malt extract agar (YEME) (Shirling and Gottlieb, 1966; and Lambert and Loria, 1989 a).

There has been considerable revision of the taxonomy of common scab-producing streptomycetes. This was partly due to the loss of the original type species and misclassification of subsequent isolates pathogenic on *S. tuberosum* (Thaxter, 1891; and Lambert and Loria, 1989 a). Lambert and Loria (1989 a) proposed that the name *Streptomyces scabies* be revived and that Thaxter's original description be used for the type species.

S. acidiscabies, an acid-tolerant form of common scab producing bacterium, has the ability to cause disease in acid soils with pH as low 4.5 (Manzer et al, 1977; and Lambert and Loria, 1989 b). The ability to cause disease in acid soils differentiates *S. acidiscabies* from *S. scabies* which typically causes disease in neutral to alkaline soils (Lambert and Loria, 1989 b). In culture, *S. acidiscabies* produces flexuous spore chains, a pH sensitive diffusible pigment instead of melanoid pigments, can grow on acidified medium, and has a spore mass color ranging from white to orange-red and a red or yellow, and does not use raffinose as a sole carbon source (Lambert and Loria, 1989 b). To date, *S. scabies* and *S. acidiscabies* are the only proper taxonomic designations for scab-causing *Streptomyces* species pathogenic on *S. tuberosum* L.

Deep-pitted scab, unlike common scab, is identified by semi-circular concave lesions which may be up to a 1 cm in diameter and 0.5 cm deep in tuber surfaces (Jones, 1931; Archuleta and Easton 1981; Hammerschmidt and Lacy, 1990). In several cases, the lesions coalesce to form furrows which disfigure tubers (Millard and Burr, 1926; and Jones, 1931). The problem with deep-pitted scab is the depth which the pathogen penetrates into a tuber; under severe conditions, tubers may be unsuitable for processing (Archuleta and Easton, 1981). The pitted symptom has been reported numerous times

(Lutman and Cunningham, 1914; and Millard and Burr, 1926), but it is not clear whether *S. scabies* is responsible for the symptom (Lutman and Cunningham 1914; Millard and Burr, 1926; Jones, 1931; and Archuleta and Easton, 1981). Deep-pitted scab may be caused by several species of *Streptomyces* (Archuleta and Easton, 1981; and Faucher et al, 1992). Archuleta and Easton (1981) isolated *S. diastatochromogenes, S. atroolivaceus, S. lydicus,* and *S. resistomycificus* from deep-pitted lesions and found that these isolates caused deep-pitted scab both in field and greenhouse experiments. One exception was *S. cinerochromogenes* that produced common scab lesions in the field and deep-pitted scab in the greenhouse. Greenhouse symptoms may vary from those observed in the field, and this can further complicate proper identification of an isolate.

Identification on the basis of phenotypic characteristics has not always been accurate. Unfortunately, Archuleta and Easton (1981) did not provide information on cultural morphology in their report however their isolates secreted melanin into culture medium. Faucher et al (1992) isolated a deep-pitting isolate which produces light gold to brown colonies and white spores borne in flexuous chains, does not produce melanoid pigments in culture, and only utilizes raffinose as a sole carbon source. A deep-pitting isolate was discovered in Michigan in 1987 that produces a grey aerial spore mass in culture, has a golden reverse colony color, does not produce melanoid pigments, bears spore in flexuous spore chains, uses all ISP sugars, will only grow in media with pH 6 and above, and produces deep pits in the cultivars Atlantic, Shepody, and Conestoga, but not in Russet Burbank (Spooner and Hammerschmidt, 1989, 1992; and Spooner, *this dissertation*). The relatedness of deep-pitting isolates will remain uncertain until

molecular techniques can be applied to developing profiles on group relatedness of all isolates.

Russet scab or "scurf" is another form of superficial scab lesion which may cover the tuber surface and is identified by browning or roughening of the skin (Harrison, 1962; Lapwood, 1973; Bang, 1979). It is also called "superficial scab" (Millard and Burr, 1926). Russet scab is not as severe as common or pitted scab, yet it can reduce tuber quality (Harrison, 1962). Russet scab develops in moist field conditions which do not favor development of common scab (Harrison, 1962). The descriptions of this scab isolate are some what vague. Millard and Burr (1926) described the growth habit of a superficial scab isolate on numerous media. They reported color of the spore mass as grey (depending on the medium), a lack of melanin production in culture, and spores borne in straight or erect chains. Harrison's isolates were very similar to Millard and Burr's isolate. They were slow growing, produced spores in chains, produced white-grey aerial mycelium on synthetic sucrose agar, and did not produce melanin in culture (Harrison, 1962). The russet scab isolate differs from *S. scabies* by not producing aerial mycelium on gelatin and being negative for tyrosinase and soluble pigments on egg albumin and nutrient potato agar (Harrison, 1962). Bang (1979) found three grouping of russet scab isolates in northern Sweden, all of which infected only the Bintje cultivar, caused disease in soil moisture and pH conditions that do not typically favor scab development. Bang's isolates were also fast growing. Groups 1 and 3 of the isolates produced heavy aerial mycelium white or grey in color and group 2 lacked aerial mycelium. All groups were tyrosinase negative (Bang, 1979).

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Tumulus (also called upraised or pimple scab) represents a form of lesion which does not penetrate the periderm but results in swellings over the tuber surface and arises as a result of hyperplastic cell growth of infected tissue within lenticels (Millard and Burr, 1926; Jones, 1931; and Hammerschmidt and Lacy, 1990). Millard and Burr (1926) described, two isolates the tumulus form designated *Actinomyces flavus and* the pimple form *A. wedmorensis*. Both isolates had simple branched spore chains. The tumulus isolate produced a grey spore mass on potato agar and the pimple isolated did not. Both isolates were tyrosinase negative (Millard and Burr, 1926). Jones (1931) histological observations on tumulus scab indicated that cells of the infected lenticel proliferated causing a swelling of the surrounding tissue which gives rise to the tumulus lesion, *S. scabies* however causes cell proliferation in the meristem below the point of infection. Scab lesion often appear on roots and stolons which give rise to tubers (Hooker, 1949). *S. scabies* can cause lesions or necrotic flecks on fibrous roots of potato, pea, beets, and cucumber roots reducing weight of the roots (Hooker, 1949).

It is apparent that several *Streptomyces* species are able to infect potato. In the past the criteria used to classify scab-producing bacteria were superficial and reveal the lack of interaction between plant pathologist and taxonomist which has caused the development of our current state of miscategorized scab-causing isolates. Millard and Burr (1926) recognized that without the use of standardized cultural techniques the "task of defining any species by its characters on artificial media is practically hopeless."

Also, this brings into question whether there is more than one species of potato scab-causing organism or merely several variant forms. It could also be the case that genetic material encoding pathogenicity is being transferred among *Streptomyces spp.*

Plasmid transfers among the *Streptomyces* are possible in soil environments (Rafii and Crawford, 1988; and Wellington et al, 1990).

Researchers have begun to investigate the question of relatedness among plant pathogenic *Streptomyces* using DNA homology techniques (Tashiro et al, 1990; Healy and Lambert, 1991; and Labeda and Lyons, 1992). *S. scabies* and *S. acidiscabies* are 39% and 17% respectively related to *S. ipomoea* causal organism of soil rot or pox of sweet potato *Ipomoea batatas* (L.) Lam. (Labeda and Lyons, 1992). Tashiro et al (1990) placed potato and beet scab isolates into two groups on the basis of spore chain morphology and observed that these groups did not differ significantly on the basis of physiology and biochemistry. Utilizing DNA homology, however, they distinguished four groups of scab-producing bacteria. Healy and Lambert (1991), also utilizing DNA homology, found that there was high degree of genetic diversity among strains phenotypically related to *S. scabies*. Examination of scab-producing isolates revealed the following groups of species: *S. scabies, S. acidiscabies, S. albidoflavus* and *S. diastatochromogenes*. They found that the level of DNA relatedness of each grouping of isolates to the type isolate *S. scabies* ACTCC 49173 was 74% for *S. scabies*, 17.6% for *S. acidiscabies*, 5.5% for *S. albidoflavus* group and 28% for *S. diastatochromogenes* group (Healy and Lambert, 1991). Interestingly, it was observed that DNA relatedness test did not always differentiate between nonpathogenic and pathogenic strains of *S. scabies* which were grouped together on the basis of phenotypic characteristics (Healy and Lambert, 1991). Doering-Saad et al (1992), using a DNA probe from the rRNA operon of *S. coelicolor*, detected restriction fragments length polymorphisms (RFLPs) among 40 pathogenic and nonpathogenic *Streptomyces* isolates. The resultant southern

blots revealed a low degree of relatedness among the pathogenic strains tested, with no significant correlation between the phenotypic tests and RFLP groupings of the strains tested (Doering-Saad et al, 1992). The low level of relatedness or high degree of genetic diversity among these isolates suggested to this research group that the genes for coding for pathogenicity were being transferred among different species by plasmids (Doering-Saad et al, 1992).

Influence of population density on the incidence potato scab.

Lutman was believed that there was only one species of potato scab- producing bacterium (*Actinomyces scabies*) with variable pathogenicity (Lutman, 1941). The problem with his conclusion is determining what he considered to constitute a species or population. It is not unusual to isolate numerous pathogenic and nonpathogenic types of *Streptomyces* from scab lesions (Archuleta and Easton, 1981; and Faucher et al, 1992). However, only pathogenic *Streptomyces* are considered *S. scabies*.

Noncultivated soil (sod) generally has been shown to contain higher *Actinomycetes* populations than cultivated soil as determined by colony counting (Conn, 1916). Conn (1916) found that the ratio of actinomycetes populations in sod versus cultivated soil on average was 2.15 : 1 colony forming units (cfu) per gram of soil. In an exceptional case the ratio was 6.4 : 1 cfu/g soil. Conn (1916) estimated the average population of actinomycetes in sod that was stored in the laboratory for several weeks (ie. allowed to desiccate) was 9.8×10^6 cfu/g soil, in freshly unearthed sod 6.6×10^6 cfu/g soil, and in cultivated soil 2.9×10^6 cfu/g. Conn's work suggested that cultivation of soil with agronomic crops may reduce the resident actinomycete population of the soil. Both Conn (1916) and Lutman (1945) were under the impression that "Actinomycetes" are normally

active in the decomposition of grass roots or, in general, plant roots. However, what happens to the populations of pathogenic *Streptomyces*?

Hooker (1956), monitoring the influence of crop rotations on the incidence of potato scab in peat soils, determined that the incidence of potato scab was lower in fallow soil than in soil that was planted with onion, soybean, corn, or potatoes. Peat soils planted with potatoes had a similar incidence of potato scab as soil planted with a cover crop of corn. The incidence of potato scab was found to increase over a three year period in plots planted to potato. There was no consistent difference in the incidence of potato scab on plots that had been planted with onion, corn, soybean, or left fallow. These findings suggested that crop rotations or leaving fields fallow reduces the population of infective *Streptomyces* over time as revealed by the lower incidence of potato scab (Hooker, 1956). This implies that *Streptomyces* pathogenic on potato may have the capacity to survive in the rhizosphere of crops other than potato. However, this capacity is limited as the incidence of potato scab steadily declines in the absence of potatoes.

Lutman (1923), found that potato scab could occur in soil never planted with potatoes. In these studies a pine wood lot was cleared and planted with surface disinfected potatoes in isolated hills to prevent inoculum from being transported throughout the area. The implications were that the resident *Streptomyces* species, which may not have been *S. scabies*, were able to shift from a saprophytic form of subsistence to a pathogenic mode of subsistence. This may also imply that *S. scabies* has a very wide host range and is able to live on many nonagronomic plants.

There appears to be some effect on *S. scabies* populations size associated with a potato cultivars resistance to potato scab. Keinath and Loria (1988; 1989 a and b), found that the rhizosphere and tuber surface populations of *Streptomyces* isolates that were morphologically and physiologically similar to *S. scabies* were greater on the scab-susceptible cultivar Chippewa than the scab-resistant cultivar Superior. The population of scab-producing bacteria increases during the growing season, and declines between crops (Keinath and Loria, 1989 a and b). Thus, an important factor for control may be the inoculum density at the time of planting. Keinath and Loria (1991) were able to monitor populations rifampin-resistant strains of *S. scabies* in soil and observed that the higher the initial level of infective inoculum, the greater the population of bacteria in the rhizsophere, rhizsoplane, and on tuber surfaces. Increased severity and incidence of potato scab were positively correlated with high initial levels of inoculum (Keinath and Loria, 1991). Keinath and Loria (1989) suggest that knowledge of the population density of scab-producing streptomycetes may be useful for predicting disease severity. However, since melanin production is an unstable characteristic in several streptomyces species and antibiotic resistance genes may be exchanged among various *Streptomyces* species by plasmids in soil environments, finding suitable markers for monitoring the populations of *S. scabies* in the soil may be difficult (Hollis, 1952; and Hopwood et al, 1986; Rafii and Crawford, 1988; and Wellington et al, 1990).

Conditions favoring the development of potato scab and their control.

It is not clear what effect specific cultural practices have on populations of potato scab-causing organisms in the soil. The build up of inoculum or increased virulence of pathogenic streptomycetes may be favored by improper cultural practices such as

improper management of soil pH and moisture, or use of susceptible cultivars of potato. Investigations into the effects of cultural practices on the populations of potato scab-causing streptomycetes have apparently been overlooked and yet, it is this type of research which may explain how certain cultural control methods succeed.

The effect of soil moisture.

The development of potato scab is favored by several environmental factors. The most notable of these factors are soil moisture content and soil pH. Increased incidence of potato scab has been reported to occur during brief periods of low soil moisture content (Lapwood and Hering, 1968). Thaxter (1891) noted that potato scab proliferates on light "dry" soil and did not agree with the popular notion that excessive soil moisture favored the development of the disease. It was thought excessive soil moisture stimulated protuberance or swelling of lenticels and infections could occur through these lenticels. Thus, it could be reasoned that excessive soil moisture increased the number of susceptible lenticels (Lutman and Cunningham, 1914). Common and pitted scab, however, are favored by a low soil moisture content and the severity and incidence of disease increases in warm and relatively dry soils (Hammerschmidt and Lacy, 1990). Russet scab of potato, however, is favored by moist soil conditions (Harrison, 1962). Apparently, the critical period in which a tuber is most susceptible to infection is the first 5 weeks after tuber initiation, if soil moisture is adequate during this time, the incidence of disease will be lowered (Lewis, 1970). Sanford (1923) thought the critical time for the tuber to be in moist soil was the first month of tuber initiation, in order to avoid infection. Tuber susceptibility to infection appears to be temporal, restricted to a few internodes close to the tuber apex, and related to the environmental conditions under

which tubers form. Lapwood and Hering (1968 and 1970) suggest a tuber is most susceptible to infection during the third week of tuber initiation when the first internodes are formed. Infection and disease severity may be suppressed by maintaining sufficient moisture over the first month during tuber initiation (Lapwood and Hering, 1970). Davis et al (1976) confirmed the work of Lapwood and reported that a mean moisture depletion to -0.65 bars approximates the minimum soil moisture required for scab control and that in their studies this level of soil moisture provided for the highest yield of potato.

Why infection occurs under low soil moisture is not clear? The anatomy of the lenticel is influenced by the amount of soil moisture (Adams and Lapwood, 1978). It has been suggested that in dry soils the tuber internodes bearing lenticels are heavily colonized by actinomycete hyphae and in wet soils the actinomycetes are unable to proliferate on susceptible tissue (Adams and Lapwood, 1978). This supports the findings which indicated that actinomycetes tend to proliferate on periderm under conditions of low soil moisture (Lewis, 1970). Adams and Lapwood (1978) suggest that microbial antagonism through competition for sites on the tuber or microbial antibiosis is the responsible for this effect. Lewis (1970) thought the effect of irrigation was to lower the population of pathogenic streptomycetes.

The effect of soil pH.

The incidence of common scab increases as the soil pH increases (Schaal, 1940; Lambert and Loria, 1989 b). Soils pH 6 or above are conducive to the development of common scab caused by *S. scabies* and several other the infective potato scab-causing species. *S. acidiscabies*, however, may cause potato scab in soils with pH 5.2 or lower (Manzer et al, 1977; Loria et al, 1986; and Lambert and Loria, 1989 b). Schaal (1940)

demonstrated isolates causing acid scab were tolerant of low pH by growth on acidified potato dextrose agar. The pH effect has been studied primarily from the aspect of its potential as a control measure (Schaal, 1940; Houghland and Cash, 1956; and Goto, 1985). In regard to controlling potato scab, proper management of soil pH is important and it seems to follow that knowing the disease history of an area would go along ways to prevent favoring the build up of potato scab bacteria that prefer alkaline or acid soils.

The effect of liming and calcium.

Calcium-containing compounds, when applied to acidic soils, improve yield and increases the incidence of potato scab (Blodgett and Cowan, 1935; Odland and Allbritten, 1949; Horsefall et al, 1954; Doyle and MacLean, 1960; and Goto, 1985). The effect of calcium may be to increase soil pH (Blodgett and Cowan, 1935; Odland and Allbritten, 1949; and Doyle and MacLean, 1960). However, the effect of calcium may work independently of the pH effect (Horsefall et al, 1954). The reasoning for this is as follows: lime reduces soil acidity, and this appears to increase the pathogen's ability to penetrate tubers and induce scab lesions. Horsefall et al (1954) observed that calcium levels in soil influence accumulation of calcium in tubers.

The level of exchangeable calcium in soil may be a more reliable indicator for anticipating the incidence of potato scab, associated with liming, than soil pH (Goto, 1985). When the level of a CaO (exchangeable calcium) in the soil exceeds 150 mg/100 g soil, the incidence of scab was shown to exceed 20% of the scab index. A scab index of 30% is critical for market quality tubers (Goto, 1985).

There appears to be a physiological basis for the effect of calcium on increasing the incidence of potato scab. Calcium has been shown to enhance the germination of

S. viridochromogenes spores and regulate the formation of aerial mycelium (Eaton and Ensign, 1980; and Natsume et al, 1989). Elevated calcium levels in the tuber periderm or the rhizosphere could possibly trigger germination of dormant spore of pathogenic *Streptomyces* in the soil. Arteca (1982) suggested that calcium accumulates in the tuber periderm. In this study ^{45}Ca was infiltrated into whole tubers, it was observed that most of the ^{45}Ca sampled in the tuber was initially found between 0-5mm from the tuber surface. After several days of storage the amount of radioactive calcium in the surface region decreased and the amount of calcium in the middle section of the tuber increased (Arteca, 1982). Therefore it may be that a tuber is most susceptible to infection when the amount of calcium in the periderm was elevated, which could account for increased incidence of potato scab after lime is applied to fields. To test this idea, it may be necessary to lime the soil at various times during the development of the tubers, and then determine whether high calcium levels in periderm favors susceptibility to potato scab. The role of calcium in potato scab development may be to initiate a series of physiological events occurring in or near the tuber periderm that result in increased rates of infection.

To control the level of potato scab which results from increased liming it seems reasonable to monitor the rate of liming. Also use of sources of calcium that do not elevate the level of exchangeable calcium in the soil.

Control measures: the effect of mineral amendments.

The chemicals that are used to control potato scab either lower soil pH or may be toxic to the pathogenic streptomycetes. Manganese-containing compounds have been successful in control of common scab and function by lowering soil pH (Mortvedt, et al,

1961; and McGregor and Wilson, 1964). McGregor and Wilson (1964) observed that acid soils generally have higher levels of soluble manganese than alkaline soils, and this could explain the effect acid soils have on common scab. Sulfur was observed to reduce the incidence of potato scab by lowering the soil pH, but not all sulfur-containing compounds were effective at controlling potato scab (Hooker and Kent, 1950). Aluminum compounds were also effective against potato scab and worked by lowering soil pH (Houghland and Cash, 1956). Bordeaux mixture, a copper-containing fungicide, also reduced the incidence of scab on potatoes. However, the reason Bordeaux mixture is effective against potato scab is not clear (Mader and Mader, 1937).

Additional methods of controlling potato scab.

Crop rotation has been effective at managing potato scab (Hooker, 1949 and 1956). Hooker's work demonstrated that pathogenic *Streptomyces* spp. survive in the rhizosphere of crops other than potato but to a limited extent. Rotations of three or more years between potato crops was shown to reduce the incidence of potato scab in peat soils (Hooker, 1956).

Menzies (1959) found that fields in Washington state exerted a suppressive effect on potato scab-causing bacteria. The suppressive activity of this soil was biological in nature and could be deactivated through autoclaving. Soil containing pathogenic *Streptomyces* when mixed with equal amounts of suppressive soil did not yield scabby tubers. The effect of suppressive soil on potato scab was enhanced by additions of alfalfa meal. The use of green manures have been recommended over the years to control potato scab but this biological control has inconsistent results in the field and although

it may have future uses it requires more study (Oswald and Lorenz, 1956; and Hanson, 1990).

Foliar applications of 3,5-dichlorophenoxyacetic acid have been shown to reduce the incidence of potato scab, and this compound was readily translocated to tubers after foliar application (Burrell, 1982 and 1984). It was suggested that 3,5-dichlorophenoxyacetic acid modified potato metabolism by inhibiting the synthesis of certain phenolic compounds (Burrell, 1984). McIntosh et al (1988) found that foliar sprays of certain benzoic and picolinic acids also reduced the severity of common scab. Finally, a lipoglycoprotein elicitor from *Phytopthora infestans* was shown to induced resistance to a wide variety of potato pathogens including *S. scabies* (Metlitskii et al, 1978).

Resistance of potato to *S. scabies*.

The basis for resistance of potatoes to *S. scabies* and other pathogenic *Streptomyces* is not known. Some researchers suggest that resistance to potato scab resides in some special properties of the periderm (Cooper et al, 1954; Emilsson and Heiken, 1956; Hooker and Page, 1960). It has been observed that *S. scabies* can only infect newly formed lenticels of the apical end of a tuber (Fellows, 1926). Cooper et al (1954) found that resistance or susceptibility to potato scab correlated with the presence or absence of nuclei in the cells of the outer cell layer of the periderm, respectively. This correlation was uniform for the different cultivars of potato examined. Emilsson and Heiken (1956) confirmed the findings of Cooper et al (1954). However, some cultivars of potato did not correspond to either of Cooper's periderm categorizes. Emilsson and Heiken (1956) therefore concluded that periderm structure was not the only

factor regulating resistance to potato scab. Hooker and Page (1960) could not obtain growth of *Streptomyces* on intact periderm surfaces, nor could the bacterium grow on autoclaved or propylene-oxide treated periderm. The periderm therefore, primarily functions as a barrier to infection, but this view of the periderm is too simplistic and some other factors must be involved. Richardson (1952) demonstrated that there was a noncharacterized type of resistance in potato tubers, regardless of cultivar resistance, that is related to the growth rate of the tuber.

Resistance to potato scab has been correlated with greater amounts of chlorogenic acid (CGA) in the periderm and outer layers of resistant tubers when compared to susceptible tubers (Johnson and Schaal, 1952). Chlorogenic acid is thought inhibit or poison pathogenic organisms (Nicholson and Hammerschmidt, 1992).

Jones (1931) observed that suberized barriers are deposited in response to infection of potato tissue. Spooner and Hammershcmidt (1992) observed the rate of lignification is greater on tuber discs inoculated with *Streptomyces* spores, than uninoculated controls. The initial lignification response to a deep-pitting streptomycetes was much lower than *S. scabies* or the nonpathogenic isolates of *Streptomyces*. Thus there is an interaction at the physiological level between potato tissue and pathogenic *Streptomyces* the contribution of phenolic compounds and lignification in resistance is not clearly defined.

The infection process.

Infection has been thought to occur primarily through lenticels (Adams and Lapwood, 1978). The lenticels are the sites of gas exchange in the periderm and arise from stomata of growing tubers as the periderm forms (Adams, 1975). According to

several investigators, the infection of potato tubers by many soil- borne pathogens proceeds through the developing lenticel at the time in which the stomata are differentiating into lenticels (Lutman, 1913 and 1941; Fellows, 1926; Jones, 1931; Smith and Ramsey, 1947; Adams, 1975; and Stein et al, 1994). This is also true of potato scab-causing streptomycetes (Lutman, 1913 and 1941; Jones, 1931; Adams, 1975; and Adams and Lapwood, 1978). Light and electron microscopy indicate that the bacterium may also enter through stomata or penetrate directly through lignified surfaces (Lutman, 1941; and Stein et al, 1994). It is interesting to note that mature lenticels are resistant to infections and the period of susceptibility to infection via lenticels is only 5-10 days (Smith and Ramsey, 1947; Lapwood and Adams, 1973; Adams, 1975; and Adams and Lapwood; 1978). Scab lesions appear to form in the region of the tuber where the most rapid growth is occurring (the apical end of the tuber where stomata are rapidly differentiating into lenticels) (Fellows, 1926). As the tissues beneath the stomata that develop into the lenticel pushes through the stomata, the exposed tissue may become susceptible to infection (Fellows, 1926).

The series of events following penetration of *S. scabies* into susceptible tissue are not too clear. It has been observed that the bacterial hyphal filaments grow intercelluarly, (i.e., in the pectic materials of the middle lamella) (Lutman, 1941; Richards, 1943; Shoemaker and Riddell, 1953; and Stein et al, 1994). *S. ipomoea*, the causal organism of soil rot or pox of sweet potato, was observed using scanning and transmission electron microscopy growing both intracellularly and intercelluarly in infected root tissues (Clark and Matthews, 1987). Clark and Matthews (1987) observations suggested the hyphal filaments penetrated cell walls by an enzymatic

process, as there was not evidence that mechanical force was used to penetrate cell walls. Stein et al (1994) reported that filaments of *S. scabies* penetrate cell walls of potato by what appeared to be enzymatic action.

Once the bacterium gains access to the host tissue it stimulates meristematic tissues to undergo hyperplasia and the newly divided cells then undergo hypertrophy (Lutman, 1913; and Jones, 1931). The invaded tissue eventually collapses and turns to a brown color characteristic of a newly formed scab lesion (Jones, 1931). Beneath the disrupted tissue the healthy meristem tissue is induced to deposit a layer of suberin. The suberin may be six cell layers thick (Jones, 1931). At this point the pathogen may be completely localized or its hyphal filaments may penetrate through incompletely suberized cells in the wound cork barrier. Jones (1931) observed that in deep-pitted scabs the wound cork barrier was penetrated by hyphal filaments, which induced another round of cell division and enlargement followed by the formation of another wound cork barrier. No more than three wound barriers were ever observed in pitted lesions. Jones (1931) speculated that deposition of more wound cork was possible but was terminated as active growth of the tuber ceased. Lutman (1913) never observed hyphal filaments growing within cells of infected tissues and also noticed that starch grains underlying a scab spot were remained unaffected. *S. scabies* has been observed to only infect and cause scab lesions on actively growing tubers within the actively growing apical region (Fellows, 1926; and Jones, 1931). This correlates with the observation that small slowly growing tubers and nongrowing mature tubers are practically immune to disease development (Fellows, 1926).

Histological studies have determined where the bacterium grows in the host tissue and that its presence elicits a wound response in the healthy meristem tissue. What has been lacking, however, was physiological studies to determine the effect of the pathogen on the host tissues. Lutman (1941 and 1945) speculated that the organism was deriving nutrition from the pectic materials in the middle lamella. Jones (1931) thought *S. scabies* created alkaline conditions in the tissue which resulted in hyperplasia and hypertrophy of the infected tissues. Fellows (1926) could not detect hyphal filaments in infected host tissues but speculated that hyperplasia and hypertrophy were caused by diffusible enzymes or toxin of bacterial origin. Electron microscopy supports a role for the involvement of enzymes in the infection process and does not suggest that *S. scabies* utilizes mechanical pressure to invade host tissues (Clark and Matthews, 1987 and Stein et al, 1994).

The production of extracellular enzymes and/or toxins is currently thought to explain how *S. scabies* and other pathogenic *Streptomyces* invade and colonize potato tissue. The ability of *Streptomyces* species to produce degradative enzymes is well known, and several streptomycetes degrade substrates such as lignin, cellulose, and pectin which are often inaccessible to many bacteria (Knosel, 1970; Sato and Kaji, 1973,1975,1977 a and b; Antai and Crawford, 1981; Borgmeyer and Crawford, 1985; Pometto and Crawford, 1986; and MacKenzie et al, 1987; Korn-Wendisch and Kutzner, 1992). Potato scab-causing bacteria are thought to utilize pectolytic enzymes to grow within the middle lamella of plant tissues (Richard, 1943; Shoemaker and Riddell, 1953; and Knosel, 1970; Spooner and Hammerschmidt, 1992). Knosel (1970) found that *S. scabies* and some nonpathogenic *Streptomyces* produced pectate lyase when induced on

a medium consisting pectin and cellulose. The nonpathogens *S. fradiae* and *S. nitrosporeus* produce pectate lyases in culture filtrate that cause tissue maceration (Sato and Kaji, 1973, 1975, 1977 a and b). Knosel (1970) found that there was no correlation between synthesis of pectolytic enzymes by streptomyces and virulence. Considering that the enzymes were bioassayed in pea leaf, we cannot be certain of the validity of his conclusion. To date no one has ever investigated the possible involvement of lignin degrading enzymes in potato scab disease, however, some studies indicate lignin is degraded by streptomycetes (Jones, 1931; and Pometto and Crawford, 1986).

Production of toxin by *S. scabies* has been investigated as a pathogenicity factor. The phytotoxin produced by *S. scabies* and *S. acidiscabies* in scabby tissues, culture filtrates and agar medium was named thaxtomin (King et al, 1989 and 1991; Lawrence et al, 1989; and Eckwall et al, 1992). Thaxtomin occurs in two major forms (A and B) and is characterized as unique 4-nitroindol-3-yl containing 2,5-dioxopiperazines. The A form occurs at 20 fold higher concentration than the B form (Lawerence et al, 1990). Thaxtomin induces scab-like lesions on minitubers. Thaxtomin also elicits rapid browning of tuber discs within 24 hours after applications (Spooner, *in this dissertation*). Doering-Saad et al (1992) suggests that genes coding for thaxtomin production be used to determine whether *S. scabies* pathogenicity genes occur on plasmids and to develop DNA probes for detection of potato scab-causing streptomycetes, without having to resort to traditional pathogenicity screening methods. However, the validity of using such a DNA probe depends on whether thaxtomin is necessary for *Streptomyces* to cause potato scab.

Many of the fundamental questions related to pathogenicity of *S. scabies* and other potato scab-causing streptomycetes may be addressed by obtaining a greater understanding of the physiology of the infection process. Obtaining physiologically based information on the factors involved with spore germination, infection, and spread of the pathogen within the host tissue, as well as the physiology of the host responses to infection, would tie together, in a meaningful fashion, the numerous observations made on this disease over the past 103 years since Thaxter's (1891) initial report on potato scab.

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CHAPTER II

THE CHARACTERIZATION OF CULTURAL, MORPHOLOGICAL, PHYSIOLOGICAL, BIOCHEMICAL, AND PATHOLOGICAL PROPERTIES OF POTATO SCAB CAUSING ISOLATES OF *STREPTOMYCES*

INTRODUCTION

Potato scab, a superficial roughening of potato periderm reduces tuber quality and marketability (Lapwood, 1973). Potato scab lesions may appear as superficial flecking, russeting, corky surface lesions, deep-pitted lesions, or upraised lesions on tubers of several cultivars of potato (Millard and Burr, 1926; Lapwood, 1973; and Hammerschmidt and Lacy, 1990). All varieties of potato scab lesions or symptoms were thought to be caused by a single species of streptomycetes, namely *Streptomyces scabies* (Waksman, 1961; Kutzner, 1981; Lambert and Loria, 1989 a; and Healy and Lambert, 1991). Lutman (1913) thought the causal agent of common scab, was exclusively responsible for all forms of potato scab lesions and that the symptom variation associated with the disease was due to variation in the degree of pathogenic virulence of *S. scabies*. This was a reasonable conclusion, when one considers that the primary characteristic used to confirm the identify *S. scabies* was lesion formation on any susceptible cultivar of potato. The *S. scabies* type-isolate discovered by Thaxter (1891), produced dark pigments when grown in agar culture (the pigment color varied according to the culture media used), produced spiral spore chains or filaments consisting of rod-like bodies of various lengths and the filaments gave rise to the grey colored spore mass. The description given by Thaxter for *Oospora scabies* (*S. scabies*) became the standard by which all other potato scab-causing streptomycetes were compared (Lambert and Loria, 1989 a; Healy and Lambert, 1991). The taxonomy of the potato scab-causing *Streptomyces* became uncertain for two reasons. First, the Thaxter type-isolate was not maintained and became unavailable for the purpose of comparison (Lambert and Loria,

1989 a). Finally, there has not been an attempt to standardize the methods used to study potato scab disease or potato scab-causing *Streptomyces* until quite recently (Kutzner, 1981; Lambert and Loria, 1989 a; and Korn-Wendisch and Kutzner, 1992). This has resulted in improper classification of several potato scab-causing *Streptomyces*.

Millard and Burr (1926) analyzed 24 strains of potato scab-causing actinomycetes, and found several isolates that were morphologically and physiologically different from *A. scabies* (*S. scabies*). Subsequent reports have suggested that potato scab could be caused by several species of *Streptomyces* other than *S. scabies* (Jones 1931; Harrison, 1962; Manzer, et al, 1977; Bang, 1979; Archuleta and Easton, 1981; Loria et al, 1986; Lambert and Loria, 1989 b; and Faucher et al, 1992).

One of the most enterprising attempts to standardize the study of *Streptomyces* was the International Streptomyces Project (ISP) (Shirling and Gottlieb, 1966, 1968 a and b, 1969, and 1972). The criteria used for the ISP were: spore chain morphology; spore ornamentation; color of the mature sporulating aerial mycelium; production of melanoid pigments; color of the vegetative mycelium as seen from the reverse side of growth; color in agar medium as caused by diffusible pigments; the effect of pH on pigment color; and utilization of ISP carbon sources (Shirling and Gottlieb, 1966 and 1976). However, researchers have not relied entirely upon the criteria set forth by the ISP. Several other techniques have been developed that have proven useful to study *Streptomyces*. Paper chromatography of whole cell hydrolysates has been used to distinguish between species of *Nocardia* and *Streptomyces* (Becker, et al, 1964). Polyacrylamide gel electrophoresis (PAGE) and SDS-PAGE of culture filtrates and whole-cell proteins have been used to supplement the taxonomic study of streptomycetes

(Gottlieb and Hepden, 1966; Hughes et al, 1971; Valu et al, 1984; Gardes and Lalonde, 1987; and Dietz, 1988). Molecular techniques have also been used to study the taxonomy of *Streptomyces* (Tashiro et al, 1990; Healy and Lambert, 1991; Doering-Saad et al, 1992; and Labeda and Lyons, 1992). On the basis of DNA homology, the potato scab-causing *Streptomyces* have been found to represent a genetically diverse group (Tashiro et al, 1990; Healy and Lambert, 1991; and Doering-Saad et al, 1992). Species grouped with *S. scabies* on the basis of phenotypic similarities, were found to posses low relatedness to the *S. scabies* group on the basis of DNA homology (Healy and Lambert, 1991).

The taxonomy of *S. scabies* has been complicated by the seemingly ubiquitous distribution of the pathogen. Potato scab-causing bacteria have been reported to occur in most potato-producing regions around the world (Keinath and Loria, 1989). Potato scab-causing bacteria have been reported to survive in the rhizospheres of several agronomic crops other than potato (Hooker, 1949 and 1956). Also, potato scab-causing bacteria have been reported to survive for several years in fallow fields usually planted with potatoes (Hooker, 1945). Potato scab has been reported to occur in soils which have never been planted to potato or any other agronomic crop (Conn, 1916; and Lutman, 1923). Researchers have suggested that *Streptomyces,* as a group and potato scab-causing streptomycetes in particular, survive in the absence of a suitable host plant by degrading complex polymers in plant debris (Lutman and Cunningham, 1914; Conn, 1916; and Lutman, 1945).

We must expand our current definition of *S. scabies* or develop an alternative system of classification for the potato scab-causing streptomycetes. To control potato

scab we must understand more about the potato scab-causing streptomycetes interactions with agronomic crops and alternate hosts, their distribution and survival, as well as their interaction with nonpathogenic streptomycetes. To properly identify scab-causing species, standard methods of identification must be developed.

The purpose of this study was to characterize the isolates of *Streptomyces*, especially the Michigan isolates, that would be used in the other aspects of my research. My purpose was not to determine whether the isolates were *S. scabies*, but to develop a profile of morphological, biochemical, physiological, and pathogenic characteristics for each isolate. Between the summers of 1987 and 1988, isolations were made from potatoes with scab lesions. Some of the resultant *Streptomyces* cultures appeared to be morphological and culturally distinct from *S. scabies*. It appeared to be necessary to establish the similarity of Michigan isolates to *S. scabies*, because variation in the patterns of infection could possibly be related to variations in the physiology and biochemistry of the individual isolates.

MATERIALS AND METHODS

Reference isolates. Reference isolates were provided by Dr. Rose Loria of Cornell University, New York. The isolates RL 84-01-232 (RL 232) and RL 83-01-03 (RL 03) pathogenic strains of *S. scabies*. RL 232 and RL 03 cause common scab lesions on several different cultivars of potato (Fig. 2.1) The nonpathogenic isolates RL 84-01-95 (RL 95) and RL 84-01-108 (RL 108) saprophytic forms of *Streptomyces* appeared morphologically similar to *S. scabies*. The isolates RL 95 and 108 were virtually indistinguishable from one another on the basis of cultural morphology. RL 232 and

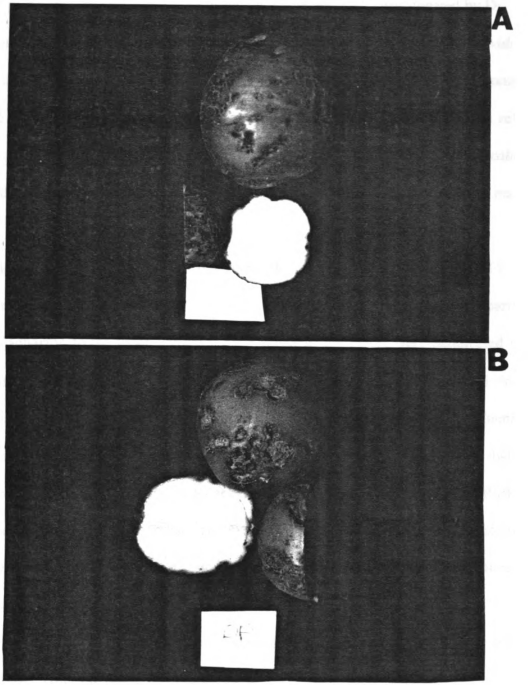

Fig. 2.1. Types of potato scab on Atlantic tubers. **A**, Common scab caused by RL 232; **B**, Deep pitted scab caused by D.P.

RL 95 were selected as isolates to be studied in the majority of the physiological studies and pathogenicity screening. The isolate of *S. lividans* (S.l.) was donated by Dr. Wendy Champness (Department of Microbiology, Michigan State University). *S. lividans* has been studied by antibiotic researchers and geneticists and was selected for its comparison to *S. scabies*. The Wallace and Hammerschmidt isolate 3S was selected as a reference isolate. 3S, was isolated from potato and was similar in appearance to *S. scabies*. 3S produced a deep blue pigment on agar medium, had a blue-grey colored spore mass, and was not pathogenic on any cultivars of potato.

Isolation of *Streptomyces* from potatoes. Infected field tubers were rinsed for several minutes in tap water and scrubbed with a soft bristle brush to remove heavy particles of soil adhering to the tuber surface. After washing, the tubers were disinfected in 10% commercial Chlorox solution for 10 minutes. The lesions were excised from the periderm and soaked in sterile distilled water to leach out excess disinfectant. Alternatively, infected tissue was excised from the tuber after washing and disinfected directly for 5 minutes followed by several rinses of the tissue in sterile distilled water. When heavily infected tubers were available, only small amounts of periderm were required for isolations. With smaller tubers or tubers with sparse infection it was necessary to remove all of the infected periderm for adequate isolations.

Disinfected lesion tissue was ground in a mortar and pestle flamed with 95% ethanol. After the tissue was macerated, 2 ml to 3 ml of sterile distilled water was added to produce a suspension of tissue. The suspension was used to inoculate 2% water agar. The inoculated plates were incubated in the dark at 27 C. Small, isolated, powdery grey colonies formed within 10 days. Water agar provided the minimal nutrients required for

sporulation, but growth of mycelium was not detected. It was usually necessary to subculture the isolated bacteria on water agar for two or three rounds until fungal and bacterial contaminants were no longer present in culture. Within 30 and 60 days the streptomycetes were pure cultured on yeast malt extract agar (YEME) (Shirling and Gottlieb, 1966). The Michigan isolates were pure cultured during the summer of 1987 and 1988. The summers were particularly hot, dry and favored the development of potato scab (Table 2.1).

Maintenance of pure cultured Streptomyces isolates. The pure cultured *Streptomyces* isolates were grown on YEME agar. Cultures were maintained in the incubator for at least three weeks prior to subculture. A culture was not transferred more than three times. Short-term cultures were maintained at 4 C on YEME slants and plates for several months. Several methods were used for long-term storage of inoculum: desiccated cultures stored at 4 C and -20 C, the spore were rehydrated to start new cultures. Cultures were also maintained at -20 C on YEME slants overlaid with 20% glycerol. These methods kept the inoculum viable for several months to years. The preferred method of inoculum storage was freezing spores at -20 C in 20% glycerol/water solution (Hopwood et al, 1985). Frozen inoculum remained viable for several years. YEME agar was inoculated with stocks of frozen spores and incubated 10 to 14 days at 27 C. The mature spore could be subcultured or used to inoculate other substrates for physiological testing.

Naming of the isolates. The isolates were named on the basis of the type of lesions it produced on the sample tuber, the cultivar of the sample tuber, or according to the label

TABLE 2.1. Designation and initial characterization of Michigan isolates

Designation	Abbr.[a]	Origin[b]	Year[c]	Lesion[d]	Cultivar
Conestoga 1	Con. 1	Samaria[e]	87	common	Conestoga
Conestoga 2	Con. 2	Samaria	87	common	Conestoga
Conestoga 3	Con. 3	Samaria	87	common	Conestoga
Plant residue[f]	P.R. 1	Samaria	87	NA[g]	NA
Farmer's Stuff	F.S.	MI	87	?[h]	?[i]
Red Pontiac 1	R.P. 1	MI	87	common/deep	Red Pontiac
Red Pontiac 2	R.P. 2	MI	87	common/deep	Red Pontiac
Onaway 9/25	same	MI	87	?	Onaway
Field II	Fi II	MI	87	common	?
Atlantic 9/25	AT	MI	87	deep	Atlantic
Deep Pit 1	D.P.	MI	87	deep	?
Deep Pit 2	D.P(W)	MI	87	deep	?
Onaway Large Lesion	same	Montcalm	87	deep	Onaway
Onaway Little Lesion	same	Montcalm	87	common	Onaway
F945	same	MI	87	deep	F945
88-001	same	MI	87	common	Onaway
88-002[j]	same	MI	88	NA	NA
88-003	same	Newbury	88	deep	Atlantic
88-004	same	Newbury	88	deep	Atlantic

[a] Abbreviated designation within text.
[b] Site where sample was collected.
[c] Year sample was collected.
[d] Type of Lesion.
[e] City. If unknown MI (Michigan).
[f] Isolated from plant debris in potato field.
[g] Not applicable
[h] Type of lesion on the sample was not recorded.
[i] Cultivar of sample unknown.
[j] A contaminant on an *Erwinia* culture.

on the bags of tubers brought into the laboratory for inspection (Table 2.1). The 1988 isolates were assigned a numerical code designation.

Propagation of plants. The isolates pathogenicity were confirmed with greenhouse cultivated potatoes. Tubers of scab-susceptible Atlantic and scab-resistant Russet Burbank were propagated for pathogenicity screenings. Single-eye cuttings from mature tubers were usually used to propagate whole plants.

Preparation of inoculum for pathogenicity screening. Inoculum was produced from mature sporulating cultures grown on YEME agar or from mycelium cultured in nutrient solution 20 g sucrose, 10 g yeast extract, 1.2 g L-asparagine, 0.6 g K_2HPO_4 diluted to 1 L with water, adjusted to pH 7.5 and autoclaved for 20 minutes (Loria and Davis, 1988).

Design and preparation of pots for greenhouse screening of isolates for pathogenicity. The inoculum was mixed by hand with Bacto-mix potting mixture. Twelve to twenty sporulating YEME culture plates were mixed with the approximately 7-10 full pots of Bacto-mix. When the nutrient solution was used to produce inoculum, 1 L of the solution was mixed with Bacto-mix until the consistency was similar to ordinary Bacto-mix (Loria and Davis, 1988). Bacto-mix/inoculum mixture was applied to the upper 6 inches of the pot since tubers were usually formed in this region of a pot. This method of soil preparation allowed for high inoculum densities in a confined area. An additional layer of potting soil was applied over the inoculum-zone to prevent dispersal of bacteria by splashing from over head watering.

The plants were watered heavily twice a week and additional water was provided on hot days. To favor disease development the soil was kept relatively dry, while

preventing the plants from wilting. Normal sized tubers and mini-tubers were usually produced within 8 to 12 weeks after planting.

Initial characterization of pure culture isolates. Cultures of the isolates were examined for aerial spore-mass color, reverse color of mycelium, production of diffusible colored pigments and melanin in culture on YEME and oatmeal agar (OA). Peptone yeast extract iron agar (PYI) was used to detect melanin production (Shirling and Gottlieb, 1966). These methods of isolate characterization were adapted from the ISP methods for examination of *Streptomyces* (Shirling and Gottlieb, 1966).

Cultural, physiological and biochemical characterization of *Streptomyces* isolates. Various physiological and biochemical tests were used to characterize the Michigan isolates and determine their similarity to RL 232 (S. scabies) and the other reference isolates.

The biochemical and physiological test used were a combination of the ISP criteria (Shirling and Gottlieb, 1966), and methods recommended by Kutzner (1981), also tests of my own design. Kutzner's methods of characterization were developed by various *Streptomyces* researchers and also included some ISP criteria. The Kutzner (1981) tests originally came from the following sources: 1) Resistance to lysozyme (Kutzner et al, 1978); 2) Melanin formation on PYI (Shirling and Gottlieb, 1966); 3) Utilization of ISP carbohydrates D-glucose, fructose, L-arabinose, D-xylose, rhamnose, sucrose, raffinose, D-mannitol, and i-inositol (Shirling and Gottlieb, 1966); 4) Hydrolysis of esculin (Gordon and Horan, 1968); 5) Utilization of organic acids gluconate, citrate, malate, lactate, malonate and, oxalate (Nitsch and Kutzner, 1969 a); 6) Formation of organic acids (Cochrane, 1947 and Ziegler and Kutzner, 1973); 7)

Hydrolysis of urea (Nitsch and Kutzner, 1969 b); 8) Hydrolysis of hippuric acid (Ziegler and Kutzner, 1973 b); 9) Lecithovitellin-reaction on egg yolk agar (Nitsch and Kutzner, 1969 c); 10) Resistance toward sodium chloride (Tresner et al, 1969). Spore chain morphology, spore color, and growth at different temperatures (25 C and 37 C) were also determined as recommended by the ISP (Shirling and Gottlieb, 1966).

Tolerance of low pH was determined by growing isolates in acidified YEME broth, dispensed in 5 ml aliquots to test tubes. The cultures were incubated at room temperature on a utilization rotary shaker. Pectin and polygalacturonic acid was determined on a modified Czapek-Dox medium: 5 g of pectin or polygalacturonate, 2 g $NaNO_3$, 1 g K_2HPO_4, 0.5 g $MgSO_4.7H_20$, 0.5 g KCL, 0.01 g $FeSO_4$, 0.15 g $CaCl_2$, and 15 g agar adjusted to pH 8.5 with NaOH and diluted to 1 liter (Mann, 1962; and Tuite, 1969). The cultures were incubated at 25 C and growth was assessed weekly. The growth ratings were based on two and three week old cultures.

The ability of the streptomyces isolates to grow in the presence of antibiotics was also determined. Filter sterilized antibiotic solutions were incorporated into 300 ml of YEME agar in the following formulations (Maniatis, 1982): ampicillin 45 μg/ml, chloramphenicol 10 μg/ml, kanamycin 50 μg/ml, streptomycin 25 μg/ml, and tetracycline 14 μg/ml. Penicillin was also used at the concentration of 28 μg/ml. The medium was dispensed in 25 ml aliquots to sterile plastic petri plates. Five replicates were used for each treatment for each bacterial isolate tested. The plates were inoculated with mature spores and incubated at 25 C for 3 weeks. Growth was assessed weekly and growth ratings were based on two and three old week cultures.

SDS-PAGE of water soluble extracts from whole cells. The purpose of this experiment was to detect whether discernable differences exist between the electrophoretic patterns of water soluble protein from the various bacterial isolates.

The isolates were grown in glucose-asparagine (GA) medium: 1 g asparagine, 4 g $K_2HPO_4.3H_2O$, 0.7 g KH_2PO_4, 2 g $MgSO_4.7H_2O$, 10 g glucose, 5 g glycine, 0.1 g yeast extract, 1000 ml dH_2O adjusted to pH 7 (Gottlieb and Hepden, 1966). This medium was modified with yeast extract to provide additional growth factors and nutrients, and glycine to produce mycelium predisposed to easy extraction. The medium was dispensed in 25 ml aliquots in 250 ml erlenmeyer flask and autoclaved for 20 minutes. The flasks of the medium were inoculated with mature spores and incubated at room temperature on a rotary shaker for 10 days. The mycelium was recovered and rinsed with sterile distilled water. The mycelium was frozen with liquid nitrogen, and ground with sterile sand in a mortar until a powder was produced. The powdered mycelium was rehydrated with sterile water. The suspension was centrifuged and the supernatant containing water soluble proteins was retained and concentrated by lyophilization. Lyophilized extracts were dissolved in 2 ml of sterile distilled water and the protein content of each sample was determined by the Bradford method (Bradford, 1976).

Electrophoresis was carried out on slab gels (Studier, 1973). The formulations of the stock solutions used for casting SDS-polyacrylamide gels were adapted Keleti and Lederer (1974). The formulations for the gel buffer and electrode buffers were adapted from Suelter (1985). Formulations for the gel stock solutions were: acrylamide solution 22.2 g acrylamide and 0.6 g methylene-bisacrylamide diluted to 100 ml with water; APS

solution, 1.5 g ammoniumpersulfate diluted to 100 ml with water. The gel buffer contained 18.3 g Tris, 6 ml concentrated HCl, 4 ml of 10% SDS in water, the solution diluted with water to 100 ml.

A single 12.5% acrlyamide slab gel of 1.5 mm thickness was cast with solutions combined as follows: 22.81 ml acrylamide solution, 2.56 ml APS solution, 14.82 ml gel buffer, and 30 ul of N,N,N',N'-tetramethylethylenediamine (TEMED). The solution was degassed for approximately 2 minutes and the gel was cast without a stacking gel. The electrode buffer (pH 8.3 Tris-glycine-SDS) consisted of 3 g Tris, 14.4 g glycine, 10 ml of 10% SDS in water, with the entire solution diluted with water to 1 L.

The protein samples were denatured and diluted to the desire concentration with solutions recommended by Suelter (1985). The denaturing solution consisted of: 3 ml 0.5 M Tris-Hcl buffer (pH 6.8), 5 ml of a 10% SDS in water, 10 ml glycerol, and 2 ml 2-mercaptoethanol. Protein samples were mixed with the denaturing solution in a test tube, boiled for 2 minutes, and removed an immediately placed in a ice-bath. The protein samples were diluted to a concentration of 15 μg/sample with the diluting solution which consisted of 1.5 ml of 0.5 M Tris-HCl buffer (pH 6.8), 2.5 ml 10% SDS in water, 5 ml glycerol, 4 ml of 0.005% bromophenol blue, and 7 ml water. Electrophoresis was carried out at 120 V for approximately 7 hours. Following electrophoresis the gels were fixed and silver stained by the Bonnen (1988) method, which was modified from Morrissey (1981).

RESULTS

Explanation of the origin of selected isolates. Twenty isolates were pure cultured including the 3S isolate (Wallace and Hammerschmidt, *unpublished data*) (Tables 2.1

and 2.2). All the isolates came from potato, except for (P.R.1) plant residue and 88-002. P.R.1 came from plant debris in a potato field in Samaria, Michigan and 88-002 was found growing as a contaminant of an *Erwinia* culture. P.R.1 and 88-002 were PYI negative and produced no diffusible pigments on YEME or OA. 88-002 to slightly darken YEME medium which suggested melanin production, but this was not confirmed by the PYI test (Table 2.2). The Conestoga isolates (Con.1, Con.2, and Con.3) were isolated from tubers with common scab lesions, from Samaria, MI (Table 2.1). Con.1 and Con.3 appeared to be similar on the basis of cultural morphology, however the reverse color of the culture on YEME was slightly different for Con.2 (Table 2.2). The Red Pontiac isolates (R.P.1 and R.P.2) were isolated from a single Red Pontiac tuber with common and deep pitted lesions (Table 2.1). When grown on YEME R.P.1 and R.P.2 produced melanin of slightly different shades of brown. 88-003 and 88-004 were isolated from an Atlantic tuber with deep-pitted lesions from Newbury, Michigan (Table 2.1). The pure cultured 88-003 was PYI negative and 88-004 was PYI positive (Table 2.2). Isolations from other single tubers never produced streptomycetes isolates that were PYI and positive and negative. The Onaway large lesion and Onaway little lesion isolates were isolated from Onaway tubers grown in Montcalm County, Michigan (Table 2.1). The large lesion isolate came from a tuber with deep pitted lesions and the little lesion isolate came from a tuber with common scab lesions (Table 2.1). Neither isolate produced pigment on PYI, YEME, or OA (Table 2.2). Onaway little lesion was the only common scab isolate that was PYI negative (Table 2.2). The deep pitting isolates (D.P.1 and D.P.(W)) were isolated from tubers of an unidentified cultivar (Table 2.1). D.P.1 and D.P.(W) were PYI negative (Table 2.2). D.P (D.P.1) was used in other phases of

TABLE 2.2. Apperance of pure cultured isolates on media and pathogencity

Isolate[a]	Pigments[b] PYI	YEME	OA	Spore Color on YEME[c]	Reverse Color on YEME[d]
Con. 1	+	-	-	Grey	Golden Brown
Con. 2	+	+/-[e]	-	Grey	Light Brown
Con. 3	+	-	-	Light Grey	Golden Brown
P.R. 1	-	-	-	Yellowish White	Golden Brown
F.S.	+	+	-	Grey	Light Brown
R.P. 1	+	+	+	Grey	Light Brown
R.P. 2	+	+	-	Grey	Chocolate Brown
Onaway 9/25	+	+	-	Grey	Golden Brown
Fi II	+	+	-	Grey	Brown
A.T.	+	+	-	Grey	Brown
D.P.	-	-	-	Grey	Brown
D.P.(W)	-	-	-	Grey	Golden Brown
Onaway Large Lesion	-	-	-	Grey	Golden Brown
Onaway Little Lesion	+	N.R.[f]	-	Grey	Golden Brown
F945	+	+	-	Grey	Brown
88-001	+	+	-	Grey	Light Brown
88-002	-	+/-	-	Greenish White	Brown
88-003	+	+	-	Grey	Brown
88-004	-	-	-	Grey	Golden Yellow
3S[g]	-	+[h]	+	Grey Blue[i]	Deep Blue
RL isolates[j]					
84-01-232	+	+	-	Grey	Brown
83-01-03	+	+	-	Grey	Yellow Brown
84-01-95	-/+	+	-	Olive Grey	Black Brown
84-01-108	-/+	+	-	Olive Grey	Black Brown
Champness isolate[k]					
S. lividans(S.l.)	-	+/-[l]	-	Whitish Grey	Golden Brown

[a] Abbreviated form of names (Table 1).
[b] Melanin (chromogenic) and diffusible (colored) pigment formation in media.
PYI (peptone yeast iron agar): Melanin.
YEME (yeast extract malt extract agar): Melanin and/or colored pigment.
OA (oatmeal agar): Melanin and/or colored pigment.
[c] Color of the spore mass.
[d] Color of culture as seen from the botton of the petri plate in full light.
[e] +/- The ability to produce pigment is variable.
[f] N.R. No result was obtained.
[g] Wallace and Hammerschmidt isolate from potato.
[h] 3S produced a deep blue pigment on YEME and OA. No melanin observed.
[i] The blue pigment diffused into the medium and spore mass.
[j] Rose Loria's isolates also written as RL 232, RL 03, RL 95, RL 108.
[k] Wendy Champness' isolate *S. lividans*.
[l] Exhibited weak to moderate production of diffusible pigment on YEME. No melanin observed.

TABLE

Isolate	
Con. 1	
Con. 2	
Con. 3	
P.R. 1	
F.S.	
R.P. 1	
R.P.(F)	
Onaway	
Fi ll	
A.T.	
D.P.	
D.P.(W	
Onawa	
Lesion	
Onaw	
Lesion	
F945	
88-00	
88-00	
88-00	
88-00	
3S[b]	
RL	
84-0	
83-0	
84-0	
84-0	
Cha	
S.	

[a] P
w
[b] T

TABLE 2.2. (*continued from the preceding page*)

Isolate	Spore Color on OA[c]	Reverse Color on OA[d]	Pathogenicity on potato[o]
Con. 1	Light Grey	Light Grey	+
Con. 2	Grey	White	+
Con. 3	Grey	White	+
P.R. 1	Greenish White[p]	Greenish Yellow	-
F.S.	Dark Grey	Grey	N.R.[f]
R.P. 1	Grey	Brown	+
R.P.(F)	Grey	Grey	+
Onaway 9/25	Grey	Light Grey	N.R.
Fi II	Grey	Light Grey	+
A.T.	Grey	Light Grey	+
D.P.	Grey	Light Grey	+
D.P.(W)	Grey	Light Grey	+
Onaway Large Lesion	Grey	Light Grey	+
Onaway Little Lesion	Grey	Light Grey	+
F945	Grey	Light Grey	+
88-001	Grey	Light Grey	+
88-002	Greenish White	Greenish White	N.R.
88-003	Grey	Light Grey	+
88-004	Grey	Grey	N.R.
3S[h]	Purple Grey[i]	Blue Purple[h]	-
RL isolates			
84-01-232	Grey	Light Grey	+
83-01-03	Grey	Light Grey	+
84-01-95	Grey	Light Grey	-
84-01-108	Grey	Light Grey	-
Champaca isolate			
S. lividans(S.l.)	Grey	White[i]	-

[o] Pathogenecity as exhibited on potatoes when cultivated in potting soil inoculated with the pure cultured isolate.

[p] The greenish cast was not due to the production of diffusible pigments.

my research because this isolate was PYI negative, culturally different from *S. scabies* and because it caused deep pitted scab (Fig 2.1 B). Fi II (Field II) was isolated from a tuber with common scab lesion. Pure cultured Fi II had the characteristic appearance of *S. scabies* (Table 2.2).

Growth of cultures on PYI, YEME, and OA and pathogenicity testing. Most of the pure cultured streptomycetes were PYI positive (Table 2.2). Several of the PYI negative isolates were nonpathogenic on potato (Table 2.2). RL 95 and RL 108 produced a weak PYI reaction after a minimum of 14 days of growth. On YEME RL 95 and RL 108 produced melanin (Table 2.2).

Except for the Conestoga isolates, most PYI positive isolates produced melanin on YEME (Table 2.2). *S. lividans* (S.l.) and 3S were PYI negative, but produced diffusible or colored pigments in YEME (Table 2.2). 3S produced a deep blue to purplish pigment in YEME and OA. S.l. to produced colored pigment in YEME but was unable to produce pigments in OA. Shirling and Gottlieb (1966) recommend comparing the characteristics of cultures grown on YEME to cultures grown on OA. Several isolates failed to produce melanin or colored pigments when grown on OA. However, R.P.1 produced a brown melanin-like pigment on OA and 3S produced a blue pigment on OA (Table 2.2).

Spore mass color varied from whitish grey or very light grey to dark grey for most isolates grown on OA. However, 3S produced blue-grey spore, P.R.1 produced light yellow spores, and 88-002 produced greenish spores on oatmeal agar (Table 2.2). Isolates generally produced the typical grey *S. scabies* type spore mass when grown on YEME. The spore masses of RL 95 and RL 108 were grey with an olive cast (Table

2.2). On OA the spore mass color of RL 95 and RL 108 were light grey to dark grey. The spore mass color of P.R.1 and 88-002 on OA were light green or a greenish-white (Table 2.2). The spore mass color of 3S on OA was blue or purple grey spore (Table 2.2).

The reverse color of the cultures, as viewed from through bottom of a petri dish, was also used as a cultural characteristic (Shirling and Gottlieb, 1966). This method of characterizing cultures was very subjective. The reverse colors were usually yellowish, golden brown, brown, or chocolate brown for cultures grown on YEME (Table 2.2).

In greenhouse screenings most of the isolates were pathogenic on potato except for P.R.1, 3S, RL 95, RL 108, and *S. lividans*. In greenhouse screenings the cultivar Russet Burbank was resistant to infection by all of the streptomyces isolates.

Physiological and biochemical testing of isolates and further pathogenicity screening.

Physiological and biochemical characterization were performed on RL 232, RL 95, D.P., Fi II, and to a lesser extent 3S and S.1. (Table 2.2). The pathogenic D.P. and Fi II were compared to RL 232 and RL 95. All the isolates had typical spiral spore chain morphology, except for the D.P. which had reflexuous spore chains. RL 232 and Fi II were PYI positive, RL 95 gave a weak positive PYI reaction, and D.P., 3S, and S.1. were PYI negative. Colored pigments were produced by 3S on YEME and OA. S.1. weakly produced colored pigments on YEME.

RL 232 and Fi II produced common scab on Atlantic and D.P. produced deep pitted scab on Atlantic (Table 2.3). On the basis of the above mentioned characteristics RL 232 and Fi II appeared *S. scabies*. D.P. lacked several *S. scabies* characteristics, but most notably it produced deep-pitted scab. The isolate RL 95 was culturally similar to

TABLE 2.3. Characteristics of *S. scabies* and other *Streptomyces* isolates

Characteristics[a]	Isolates					
	RL 232	D.P.	Fi II	RL 95	3S	*S.l.*
Spore color[b]	G	G	G	G	BG	G
Chain morphology[c]	S	F	S	S	S	S
Pigment on PYI	+[d]	-	+	+/-[e]	-	-
Colored pigments on [f]:						
YEME	-	-	-	-	+[g]	+\-[h]
OA	-	-	-	-	+[g]	-
Carbon usage						
Glucose	+	+	+	+	+	N.R.[i]
Fructose	+	+	+	+	+	N.R.
Sucrose	+	+	+	+	+	N.R.
Mannitol	+	+	+	+	+	N.R.
Arabinose	+	+	+	+	+	N.R.
Xylose	+	+	+	+	+	N.R.
Raffinose	+	+	+	+	+	N.R.
Rhamnose	+	+	+	+	+	N.R.
myo-Inositol	+	+	+	+	+	N.R.
Degradation of:						
Pectin	+	+	+	+	+	+
Polygalacturonate	+	+	+	+	+	+
Minimum growth pH[k]	5.0	6.0	4.5	4.5	6.0	6.0
Salt tolerance[l]						
0% NaCl	+	+	+	+	+	+
4% NaCl	+	+	+	+	+	+
7% NaCl	-	-	-	-	+	+
10% NaCl	-	-	-	-	+	-
13% NaCl	-	-	-	-	-	-
Lysozyme resistance[m]						
0 μg/ml	+	+	+	+	+	+
10 μg/ml	+\-[n]	+\-	+\-	+	+	-
50 μg/ml	-	+\-	+\-	+\-	+	-
100 μg/ml	-	-	-	-	-	-
Hydrolysis of:						
Esculin	+	+	+	+	+	+
Urea	+	+	+	+	+	+
Hippuric acid	-	-	-	-	+	+
LV reaction	-	-	-	-	+	+
Antibiotic resistance [o]:						
Ampicillin 45 μg/ml	+	+	+	+	N.R.	N.R.
Chloramphenicol 10 μg/ml	-	-	-	+	N.R.	N.R.
Kanamycin 50 μg/ml	-	-	-	-	N.R.	N.R.
Penicillin[p] 28 μg/ml	+	+	+	-	N.R.	N.R.
Streptomycin 25 μg/ml	-	-	+	-	N.R.	N.R.
Tetracycline 14 μg/ml	-	-	+	+	N.R.	N.R.

[a] Test were taken from ISP methods (Shirling and Gottlieb, 1966) and Kutzner (1981) and our own assays.
[b] G, Grey and BG, Blue-Grey.
[c] S, Spiral and F, Flexuous as determined by electronmicroscopy.
[d] Positive(+) and Negative(-).

TABLE 2.3. (*continued from the preceding page*)

Characteristics	Isolates					
	RL 232	D.P.	Fi II	RL 95	3S	*S.l.*
Growth at [q]:						
25 C	+	+	+	+	+	+
37 C	-	-	-	+\-	+	+
Utilization of organic acids						
Citrate	+	+	+	+	+	+
Malate	+	+	+	+	+	+
Malonate	+	+	+	+	+	+
Lactate	+	+	+	+	+	+
Succinate	+	+	+	+	N.R.	N.R.
Gluconate	+	+	+	+	+	+
Oxalate	+	+	+	+	+	+\-
Production of organic acids	-	-	+\-	+\-	+	+
Pathogenicity on potato [r]:						
Russet Burbank	-	-	-	-	-	-
Atlantic	CM[s]	DP	CM	-	-	-

[e] +/- Variable reaction quite often this was a weak reaction.
[f] Diffusible colored pigment on Yeast Extract Malt Extract(YEME) and Oatmeal Agar(OA).
[g] 3S produced a blue colored pigment on both YEME and OA.
[h] Weak reaction on YEME.
[i] N.R. no result.
[j] Czapek medium with 0.5% pectin or polygalacturonic acid as the sole carbon source in 1.5% agar.
[k] Acidified YEME solution.
[l] NaCl incorporated into YEME agar at 40 g/L, 70 g/L, 100 g/L, and 130 g/L.
[m] Liquid broth with lysozyme. Determines the ability of the organism to grow in presence of lysozyme.
[n] Either no growth or very weak growth.
[o] Antibiotic mixed with YEME agar. Rate at which antibiotics are incorporated into the medium are from Maniatis (1982).
[p] This concentration of antibiotic was of our own design.
[q] The effect of growing bacteria at two temperatures.
[r] Infection on greenhouse grown tubers. The lesion type was noted.
[s] CM, common scab; DP, deep pitted scab.

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RL 232 but was not pathogenic and therefore not *S. scabies*. Isolates 3S and S.1., appeared similar in regard to basic cultural appearance although not identical. S.1. tended to produce less pigment in culture.

The six isolates appeared to fit into four groups. RL 232 and Fi II formed the *S. scabies* group, RL 95 formed a nonpathogenic group which bore superficial similarities to *S. scabies*, D.P. formed a pathogenic group that lacks many cultural characteristics ascribed to *S. scabies*, and 3S and S.1. formed an nonpathogenic group that formed colored pigments in culture.

The utilization of ISP carbon sources, pectic substrates, and organic acids was similar for all isolates studied (Table 2.2). The hydrolysis of esculin, urea, hippuric acid, and lecithovitellin (LV) reaction, tolerance toward sodium chloride, and resistance to lysozyme was identical for RL 232, RL 95, D.P. and Fi II. The isolates 3S and S.1. were both positive for hydrolysis of hippuric acid and LV reaction, both isolates exhibited high tolerance for sodium chloride. The 3S isolate was more resistant to lysozyme than S.1., The D.P., 3S, and S.1. would only grow at pH 6 and above, and RL 232; RL 95 and Fi II were able to grow in YEME broth pH 4.5 which was uncharacteristic of *S. scabies* (Lambert and Loria, 1989 a and b). RL 232 and D.P. had similar resistance to antibiotics, and RL 95 and Fi II had additional antibiotic resistance lacked by RL 232 and D.P. (Table 2.2).

SDS-PAGE of soluble proteins extracted from *Streptomyces* isolates.

The electrophoretic patterns of water soluble proteins, extracted from mycelium, were compared between isolates cultured under identical conditions (Fig. 2.2 and 2.3). From experiment to experiment this method of characterization lacked consistent results. The

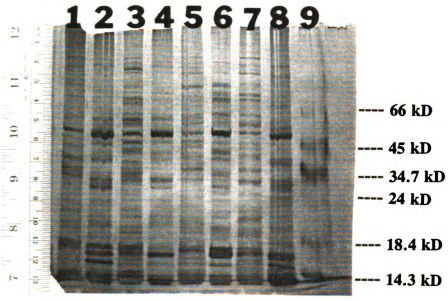

Fig. 2.2. SDS-PAGE of soluble proteins extracted from 10 day old *Streptomyces* cultures. Lane 1, RL 232; lane 2, D.P.; lane 3, B_1; lane 4, 3C; lane 5, RL 95; lane 6, 2A; lane 7, 3S; lane 8, Fi II; lane 9, molecular weight markers in kiloDaltons (kD): bovine albumin (66 kD), egg albumin (45 kD), pepsin (34.7 kD), trypsinogen (24 kD), ß-lactoglobin (18.4 kD), lysozyme (14.3 kD).

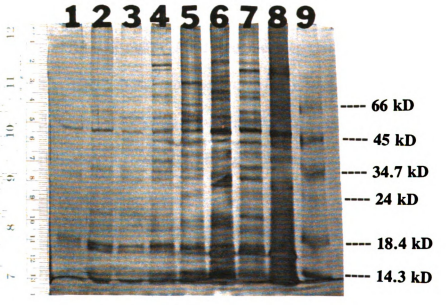

Fig. 2.3. SDS-PAGE of soluble proteins extracted from 10 day old *Streptomyces* cultures. Lane 1, RL 232; lane 2, D.P.; lane 3, B_1; lane 4, 3C; lane 5, RL 95; lane 6, 2A; lane 7, *S. lividans*; lane 8, Fi II; lane 9, molecular weight markers in kiloDaltons (kD): bovine albumin (66 kD), egg albumin (45 kD), pepsin (34.7 kD), trypsinogen (24 kD), ß-lactoglobin (18.4 kD), lysozyme (14.3 kD).

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variation in the bands were probably due to the highly variable growth conditions. This form of characterization would best be used for streptomycetes that grow faster than *S. scabies*.

Heavy staining bands of protein appeared with the approximate MW of 45 kD and in between 18.4 kD and 14.3 kD for the isolates RL 232, D.P., Fi II, RL 95, 3S, and S.l. (Fig. 2.2 and 2.3). The banding patterns for RL 232, DP, and Fi II were similar (Fig. 2.2 and 2.3). However, D.P. had a triplet of protein bands between 18.4 kD and 14.3 kD, where as RL 232 and Fi II had a doublet of protein bands (Fig. 2.2). Both 3S (Fig. 2.2) and S.l. (Fig. 2.3) had protein patterns that were similar. The protein patterns from 3S and S.l. appeared different from RL 232, D.P., and Fi II. The protein pattern from RL 95 was similar to 3S and S.l., but also resembled the protein patterns from the pathogens (Fig. 2.2 and 2.3).

DISCUSSION

The purpose of the work in this chapter was to catalog the morphological, cultural, and physiological characteristics of the *Streptomyces* isolates used in these studies. Nearly all of the isolates that came from potato scab lesions were pathogenic on potato. Deep-pitted and common scab lesions were most frequently observed on the field grown tuber samples from which isolations were made (Fig 2.1). The common scab isolates RL 232 and Fi II produced melanin and spiral spore chains, had similar physiological and biochemical characteristics. The deep-pitting isolate D.P., did not produce melanin, had reflexuous spore chains, and thus on the basis of commonly used characteristics could not be a strain of *S. scabies*. However, the physiological and biochemical characteristics of D.P. suggested similarity to RL 232 and Fi II (*S. scabies*).

The physiological and biochemical methods used to categorize the *S. scabies* isolates was not entirely conclusive. On the basis of culture morphology, physiology, and biochemical tests RL 95 could have been placed in the *S. scabies* group, but was unable to infect potato. Since it was not my purpose to identify the isolates to the species level, the physiological tests served to determine similarity of the potato scab-causing *Streptomyces* used in these studies were to one another. The value of using electrophoresis to differentiate between streptomyces species was not specific. However, electrophoresis clearly showed differences between isolates that had major physiological and biochemical differences from the potato scab-causing bacteria, namely the nonpathogenic isolates 3S and S.1.

Finding easily detectable characters that are correlatable with pathogenicity would help to overcome our reliance upon pathogenicity testing to confirm the identity of *S. scabies* and other potato-scab causing streptomycetes. Doering-Saad et al (1992) suggested developing a DNA probe for thaxtomin genes, the production of thaxtomin has been positively correlated with pathogenicity. Traditionally, melanin production was thought to be correlated with pathogenicity for potato scab-causing streptomycetes (Hollis, 1952). However, my research and Faucher et al (1992) demonstrated that melanin-negative pathogenic *Streptomyces* occur. Molecular techniques such as DNA homology may overcome the limitations of the physiological tests used in taxonomic studies of *S. scabies* and other potato scab-causing streptomycetes.

A finding of major importance in this study was the deep-pitting isolate D.P. The characteristics which distinguished it from *S. scabies,* ultimately made this isolate idea to study in comparison to the *S. scabies* isolate RL 232. D.P. was similar to the deep

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pitting isolate found in Canada by Faucher et al (1992). This isolate was characterized by gold to light brown colonies, white spores borne in flexuous chains, lack of melanoid pigment production, resistance to streptomycin, and utilization of raffinose as sole carbon source (Faucher et al, 1992). D.P. had to have a grey colored spore mass, streptomycin sensitivity, and utilized all ISP carbon sources. The Faucher et al (1992) isolate and the D.P. (Spooner and Hammerschmidt) isolates differ from the deep pitting isolates of Archuleta and Easton (1981) by being unable to produce melanin agar media.

Considering the powerful molecular tools currently available for the study of streptomyces taxonomy it would be worth while to determine how related the Michigan isolates, particularly D.P., are to *S. scabies* and other deep-pitting isolates around the world. This could be useful in determining the distribution of *S. scabies* versus deep-pitting isolates in the United States.

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CHAPTER III

THE ROLE OF EXTRACELLULAR PECTINASES PRODUCED BY *STREPTOMYCES* SPECIES PATHOGENIC ON POTATO IN THE DISEASE PROCESS: STUDIES ON EXTRACELLULAR PECTINASES PRODUCED *in VITRO* BY PATHOGENIC AND NONPATHOGENIC *STREPTOMYCES* SPECIES

INTRODUCTION

Streptomycetes produce a wide array of extracellular enzymes which degrade several polymers found in plant and animal debris (Korn-Wendisch and Kutzner, 1992). Many *Streptomyces* species enzymatically degrade persistent plant polymers such as lignin, cellulose, and xylan (Antai and Crawford, 1981; Crawford et al, 1982 and 1983; McCarthy and Broda, 1984; Brogmeyer and Crawford, 1985; Pometto and Crawford, 1986; and MacKenzie et al, 1987). Chitin (N-acetyl-D-glucosamine residues linked by ß 1→4 glycosidic linkages), a polymer of fungal and animal origin, can be degraded by some *Streptomyces* species (Lingappa and Lockwood, 1961 and 1962; Hsu and Lockwood, 1975; Trudel and Asselin, 1989). Most *Streptomyces* are relatively slow-growing soil inhabitants and have evolved the life strategy of degrading complex biopolymers which are inaccessible to many rapidly-growing soil bacteria and fungi. Lutman (1945) thought that *S. scabies* survived on plant debris, in fallow potato fields. This may explain why the potato scab-causing streptomycetes are able to degrade complex biopolymers.

The mode of pathogenesis used by *S. scabies* and other *Streptomyces* species pathogenic on *Solanum tuberosum* has been a subject of much speculation. How the infection occurs has not been established. The presence of hyphal filaments within infected potato tissues suggests that the bacterium possesses means to actively degrade tissues. Light microscopy has revealed hyphal filaments of *S. scabies* growing in the middle lamellae of potato tissue (Richards, 1943; and Shoemaker and Riddell, 1954). Electron microscopy has revealed hyphae growing between cells and penetrating cell

walls directly (Stein et al, 1994). The pattern of infection demonstrated by *S. scabies* and other plant pathogenic streptomycetes that suggests enzymatic degradation of the middle lamella and cell walls occurs during the infection process (Jones, 1931; Lutman, 1941; and Clark and Matthews, 1987; and Stein et al, 1994). Lutman (1923, 1941, and 1945) has speculated that *S. scabies* utilizes pectin as a primary carbon source both in the pathogenic and saprophytic phases of its life cycle.

The major plant cell wall components are cellulose, hemicellulose, pectic polysaccharide, structural proteins, and lignin (Talmadge et al, 1973). Plant cells are held together by polymeric carbohydrates, of which pectin is a major component (Rombouts and Pilnik, 1980). Pectin occurs in most plant species and functions to maintain the structural integrity of plant tissues. Pectin is a complex mixture of acidic and neutral polymers which are characterized by chains of $\alpha 1 \rightarrow 4$ linked galacturonosyl residues in which 2-linked rhamnosyl residues are interspersed (Talmadge et al, 1973). The carboxyl groups of some galacturonosyl residues are methyl esterified and the pectic fraction usually possess a neutral polysaccharide which may or may not be covalently attached to the acidic rhamnogalacturonan main chain (Talmadge et al, 1973).

The pectolytic enzymes can be divided into two general classes: the desterifying enzymes, also called pectinesterases, and chain-splitting enzymes, the depolymerases (Rombouts and Pilnik, 1980). The depolymerases cause the greatest damage to plant tissue (Bateman and Millar, 1966; Collmer and Keen, 1986). There are two categories of depolymerases, the hydrolases and lyases, which respectively degrade pectin by hydrolysis and ß-elimination (Rombouts and Pilnik, 1980). These two classes of enzymes are particularly destructive and usually cleave the pectic substrate in a random

fashion. In plant tissues, this results in rapid loss of tissue integrity and eventual death of plant tissue (Garibaldi and Bateman, 1971; Quantick et al, 1983; Bashan et al, 1985; Keon, 1985; and Movahedi and Heale, 1990).

Exopolygalacturonase and endopolygalacturonase are two examples of hydrolases. The endopolygalacturonases have been frequently cited for their ability to macerate plant tissue (Collmer and Keen, 1986; Rombouts and Pilnik, 1980; and Talmadge et al, 1973). Several plant pathogenic fungi and bacteria have been shown to produce polygalacturonases in culture (Collmer and Keen, 1986). Similarly, pectolytic activity has been noted for saprophytic bacteria and fungi (Rombouts and Pilnik, 1980). The purified polygalacturonases (PG) exhibit high affinity for polygalacturonic acid and exhibit optimal activity between pH 4 and pH 7 (Rombouts and Pilnik, 1980). The endoPGs do not require calcium for optimal activity (Rombouts and Pilnik, 1980). Endopolygalacturonases cleave endopolygalacturonic acid internally or randomly to create large fragments of galacturonides, whereas the exopolygalacturonases cleave the pectic polymer terminally releasing only the D-galacturonide residue (Rombouts and Pilnik, 1980).

Lyases or transeliminases are the most extensively studied pectic enzymes implicated in plant disease (Keon, 1985; and Collmer and Keen, 1986). Three classes of lyases have been reported: endopectin lyases (which show preference for methylated pectin substrate), exopectate lyases and endopectate lyase (both of which prefer polygalacturonic acid as a substrate) (Rombouts and Pilnik, 1980). The lyases attack the pectic polymer by introducing a double bond between carbons 4 and 5 of the galacturonic acid residue, in transeliminative fashion, which results in the formation of a free reducing

group. Lyases activity is stimulated by millimolar concentrations of calcium or other divalent ions (Rombouts and Pilnik, 1980). The endolytic lyases rapidly degrade pectic polymers in plant tissues which results in tissue maceration. Several *Streptomyces* species produce extracellular pectolytic enzymes in culture. *S. fradiae* and *S. nitrosporeus* produce pectate lyase in shake culture (Sato and Kaji 1973, 1975, and 1977 a and b). The culture filtrate from *S. fradiae* was observed to macerate plant tissue (Sato and Kaji 1973). Neither *S. fradiae* or *S. nitrosporeus* are pathogenic toward any plant species. Knosel (1970) observed that *S. scabies* produced endopectate lyase and cellulase in shake culture when supplied a medium consisting of citrus pectin and cellulose. Knosel (1970) and Sato and Kaji (1973, 1975, 1977 a and b) demonstrated that purified transeliminases produced by streptomyces in shake culture exhibit optimal activity at pH 9 or higher. Knosel (1970) demonstrated that *S. scabies* produced pectic enzymes that macerate plant tissue. However, no correlation was found between the ability to produce pectolytic enzymes in culture and the virulence of the isolates studied.

My objective was to determine whether there was a correlation between pathogenicity of potato scab-causing streptomycetes and their ability to degrade pectic substrates. It was necessary to determine whether pathogenic and nonpathogenic streptomycetes grew on purified pectic substrates, as a sole carbon source and produced extracellular pectinase in culture. Secondly, I wanted to establish which classes of pectolytic enzymes were induced by pectin or polygalacturonic acid in shake culture and establish pH optimums for the enzyme preparation from crude culture filtrates on pectin and polygalacturonic acid substrates. This would indicate the types of pectolytic enzymes that were being induced. Thirdly, I wanted to determine whether pectolytic

enzymes from pathogenic and nonpathogenic streptomyces were capable of causing damage to potato tuber tissue. Finally, an attempt was made to determine whether the loss of pectin degrading ability would affect the pathogenecity of isolate D.P. This was examined with ultraviolet light mutants of D.P., the deep-pitting scab isolate.

MATERIALS AND METHODS

Bacterial isolates. The isolates RL 232, RL 95, D.P., 3S, and Fi II were used in the experiments.

Maintenance of the bacteria and cultivation of inoculum. The bacterial stocks were maintained as spores at -20 C in 20% glycerol/water solution (Hopwood et al, 1985). Spores were used to inoculate plates of yeast extract malt extract agar (YEME) (Shirling and Gottlieb, 1966). The cultures were incubated at 25 C for 14 days or until they produced mature spores which served as the inoculum for other media.

Plant materials. The potato cultivars Russet Burbank and Atlantic were used for greenhouse pathogenicity screening. Tissue maceration assays were performed on tuber Russet Burbank tubers discs.

Induction of pectolytic enzymes in shake cultures. Bacteria were cultured in a modified Czapek-Dox medium (Mann, 1962; and Tuite, 1969) containing: 5 g pectin or polygalacturonic acid, 2 g $NaNO_3$, 1 g K_2HPO_4, 0.5 g $MgSO_4.7H_20$, 0.5 g KCl, 0.01 g $FeSO_4$, 0.15 g $CaCl_2$ /L adjusted with 0.5 M NaOH to pH 8.5. Since pectin has low solubility in water, it was mixed into 500 ml boiling distilled water with a Waring blender. The pectin solution was diluted with 400 ml of salts solution. Polygalacturonic acid (PGA) is very soluble in water but forms an insoluble precipitate when $CaCl_2$ is added to solution. Thus, $CaCl_2$ was slowly added as an aqueous solution to the PGA

medium. The pectin or PGA media was dispensed into 1 L erlenmeyer flasks in 250 ml aliquots and autoclaved for 30 minutes. The pH of the pectin medium occasionally dropped as low as pH 7 after autoclaving, without adverse effects on bacterial growth. Spores scraped from the surface of mature cultures were used to inoculated the liquid pectin medium. The cultures were incubated on a rotary shaker at 23 C for 3 to 5 weeks.

Preparation of the culture filtrates. After the culture period the medium was filtered through two layer cheesecloth, centrifuged at 4000 rpm for 45 minutes at 4 C, and dialyzed against distilled water for 48 hours at 4 C. The culture filtrates were concentrated by freeze drying to powder form. The powders were maintained at -20 C until assayed.

Preparation of concentrated culture filtrates for enzyme assays. In general, 200 ml of culture filtrate produced less than 0.5 g of dry powdered material. The powders were suspended in 1 to 2 ml of distilled water. The undegraded substrate in the powders gelled and the insoluble material was removed by centrifugation. The protein content of the enzyme preparations was determined by the method of Bradford (1976). Enzyme preparations containing little protein were reconcentrated by lyophilization, rehydrated in less water, and measured for protein concentration. Culture filtrates stored at -20 C for long-term usage or maintained at 4 C for 14 days.

Enzyme assays. Characterization of the pectolytic activities in the concentrated culture filtrates was conducted on pectin and PGA substrates. The pH of the pectic substrates were maintained with the following buffering systems: (pH 4 - 5.5) 0.05 M acetate

buffer; (pH 6 - 7) 0.05 M citrate phosphate buffer; (pH 7.5 - 9) 0.05 M Tris-HCl buffer; and (pH 9.5 - 10.5) 0.05 M glycine-NaOH buffer.

Hydrolase and lyase activity were detected by Nelson's (1944) assay for reducing groups, viscometry (Cooper and Wood, 1975), and the cup plate assay (Dingle et al, 1953; Mann, 1962; and Movahedi and Heale, 1990). Lyase activity was detected by the thiobarbituric acid assay (TBA) (Weissbach and Hurwitz, 1959; and Bateman, 1966) and measuring the change in absorbance of a reaction mixture after 2 minutes at A_{235} (Edstrom and Phaff, 1964; and Nasuno and Starr, 1966). Lyase activity was detected with pectin and PGA agarose gels overlaid onto polyacrylamide gels containing electrophoresed pectolytic enzymes (MacKenzie and Williams, 1984; Ried and Collmer, 1985 and 1986).

For the Nelson's (1944) assay, the volume of the reaction mixture was initially 1.25 ml but was later scaled down by half the volume to conserve the enzyme, therefore the total volume was 0.625 ml. A 1.25 ml reaction mixture contained 0.75 ml of buffered 1.2% pectin or PGA substrate, 0.25 ml enzyme preparation or 0.25 ml buffer for controls, and 0.25 ml of 0.05 M $CaCl_2$ to stimulate lyase activity. The reaction proceeded for 30 minutes at 27 C after which time the reaction was stopped by the addition of 1 ml Nelson's reagent (copper solution) and boiled for 20 minutes. The reaction was stopped by immersion of test tubes in a cold water bath, and 1 ml of aresenomolybdate solution (Nelson, 1944) was added with shaking, and the reaction was diluted to 7 ml with distilled water. The absorbance of the solution was read at A_{540}. Estimates of the amount of reducing groups per reaction were based on a standard curve developed from D-galacturonic acid. The results were expressed as micrograms of

galacturonic acid units released per minute per microgram of protein in each reaction mixture.

The thiobarbituric acid assay reaction mixture consisted of 0.5 ml of 1.2% buffered pectic substrate (0.05 M Tris-HCl or 0.05 M glycine-NaOH), 0.17 ml of 0.05 M $CaCl_2$, 0.17 ml distilled water, 0.17 ml of the enzyme preparation or buffer for controls. The reaction proceeded for 30 minutes until stopped with the addition of 3.33 ml of 0.01 M TBA reagent and 0.83 ml 1 N HCL, then was boiled for 30 minutes and cooled to room temperature. The absorbance of the solution was read at A_{548}. The molar extinction coefficient 1.55 X 10^5 M^{-1} was used to determine the molar concentration of product formed per reaction. The results were expressed in terms of millimicromoles (mμ moles) of product formed per minute per microgram of protein.

Lyase activity was detected by the measuring the change in absorbance (A_{235}) of pectic substrate over time. The reaction contained 1 ml of 0.5% buffered substrate (0.05 M Tris-HCl or 0.05 M glycine-NaOH), and a volume of enzyme preparation which provided 2.5 μg of protein. To stimulate lyase activity the substrate was formulated with $CaCl_2$ to a concentration of 0.002 M. The change in absorbance of the substrate was measured while the reaction proceeded for 2 minutes at 27 C.

IRG 200 viscometers (International Research Glass Ware, Kenilworth, NJ) were used for viscometry assays which were performed by the Hancock et al (1964) method. The reaction mixture consisted of 2 ml enzyme preparation and 4 ml of 1.2% buffered pectic substrate, and 1 ml of 0.05 M $CaCl_2$ to stimulate lyase activity. The reduction in viscosity of the substrate was measured at 28 C. Rapid loss of viscosity was usually indicative of endolyase or endopolygalacturonase activity.

Cup plate assays were utilized for detection of hydrolase and lyase activity. The cup plate medium consisted of 50 ml of the desired buffer solution (0.05 M buffer solution mentioned above), 0.25 g of pectic substrate, 1.5 g agar, 5 ml of 0.02 M $CaCl_2$ to stimulate lyase activity. The medium was adjusted to the desired pH and diluted to 100 ml. The solution was steamed until the agar liquified and approximately 20 ml of the medium was poured into sterile plastic petri dishes. Wells were cut out of the solidified medium with a cork borer. The enzyme preparations were adjusted to similar protein concentrations and pipetted into the wells. The cup plates were incubated for 20 hours at 27 C. After incubation the cups were rinsed with distilled water. The cup plates containing PGA were flooded with 1 N HCl (Dingle, et al 1953; and Puhalla and Howell, 1975). The cup plates containing pectin were stained with 0.03% ruthenium red (Sterling, 1970; Ried and Collmer, 1985; and Movahedi and Heale, 1990). Evidence of enzyme activity was detected by zones of clearing around the wells.

Polyacrylamide gel electrophoresis of concentrated culture filtrates. Polyacrylamide gel electrophoresis was performed under native conditions at pH 9.3 (anionic gel system) using the Keleti and Lederer (1974) method for preparation of tube gels, which was modified for slab gel electrophoresis (Studier, 1973). A gel was typically 1.5 mm thick and consisted of a 7.5% acrylamide resolving gel and a 2.5% acrylamide stacking gel. Enzyme samples to be loaded for electrophoresis contained 20 μg to 60 μg of protein in a 40 μl aliquot, 20 μl of glycerol, and 10 μl of bromophenol blue tracking dye. Electrophoresis was conducted at room temperature under constant voltage using 60 V through the stacking gel for 1 hour and 120 V through the resolving gel for approximately 6 to 7 hours. After electrophoresis, the gels were fixed for silver staining

as described by Bonnen (1988), and silver staining was carried out according to Bonnen (1988) which was a modification of Morrissey (1981).

SDS-PAGE of pectic enzymes. The SDS-polyacrylamide gels were cast according to the methods outlined in Chapter 2 of this dissertation. The formulations for stock solutions and procedures for casting gels were adapted from Keleti and Lederer (1974) and Seulter (1985). The SDS polyacrylamide gel contained of 12.5% acrylamide, was 1.5 mm thick and cast without a stacking gel. Samples for electrophoresis were prepared according to the method of Suelter (1985) as outlined in Chapter 2 of this dissertation. The final concentration of the sample loaded in each well was 12 μg of protein. The enzymes (Sigma) polygalacturonase EC 3.2.1.15 from *Aspergillus niger* and pectin lyase EC 4.2.2.10 from *Aspergillus japonicus* were electrophoresed for comparison with the electrophoretic patterns of *Streptomyces* culture filtrates. The samples were electrophoresed under constant voltage (120 V) for approximately 7 hours. After electrophoresis the gels were fixed and silver stained by the Bonnen (1988) method, which was a modification of the Morrissey (1981) method.

Agarose pectin/pectate overlay of polyacrylamide gels for detection of pectolytic activity. The pectic enzymes electrophoresed in native polyacrylamide gels were detected with pectic substrate gels (Ried and Collmer, 1985 and 1986; and Collmer et al, 1988). The pectin agarose overlays were cast in a Studier electrophoresis apparatus to a thickness of 1.5 mm. The glass plates were heated with boiling water prior to casting a gel. Initially the overlays were formulated as two solutions: solution (1) consisted of 100 ml of 0.2 M Tris-HCl, 20 ml of a 1% pectin or PGA solution, and 10 ml of 0.02 M $CaCl_2$ solution; solution (2) consisted of 100 ml of 2% agarose. Both

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solutions heated by steaming and 75 ml of each solution was mixed together. The resultant solution was pipetted into the gel casting apparatus. After the gel solidified it was wrapped in plastic film and stored at 4 C. A gel was adjusted to room temperature prior to use.

Enzyme samples for PAGE were loaded so that each half of a gel contained a duplicate sample. After the polyacrylamide gel was electrophoresed it was cut in half. Half of the gel was silver stained and the other half was overlaid to detect pectic enzymes. A polyacrylamide gel to be assayed for pectic enzymes was rinsed in distilled water, and soaked in three changes of buffer, to adjust the gel pH. The agarose overlay was placed on a glass plate and the polyacrylamide gel was affixed on top of the agarose overlay. The two gels were wrapped in plastic film to minimize loss of moisture and incubated for 4 to 7 hours at 28 C. After incubation the overlay was trimmed to the size of the polyacrylamide gel and the two gels were separated. The agarose overlay was stained with 0.03% ruthenium red and destained by soaking in distilled water. Enzymatic activity was indicated by clear bands in the pink or red stained pectin agarose overlay.

Tissue maceration assay. Potato tuber discs were used to assess the tissue macerating ability of the enzyme preparations. Tuber discs were prepared according to Hammerschmidt (1984). A cork borer was used to remove a single cylinder of tissue from the internal medullary region of a tuber, which was sliced into individual tuber discs 2 cm in diameter and 0.5 cm in thickness. The discs were placed into a 1% sodium chloride solution to prevent browning and rinsed twice with sterile distilled water. The discs were transferred to sterile plastic petri dishes containing sterile distilled

water or sterilized 0.05 M Tris-HCl buffer pH 8.8. The tuber discs were treated with a volume enzyme preparation which contained 5 μg of protein, and the controls were treated with sterile distilled water. Tuber discs were incubated in 0.1 M Tris-HCl pH 8.8 or water to determine the effect of pH on the maceration. The discs were incubated at 27 C in darkness and observed every 8 hours for 48 hours. The extent of the tissue damage was visually assessed and expressed qualitatively in terms of tissue browning or blackness and loss of tissue hardness.

Mutagenesis of D.P. with ultraviolet light. Mutagenesis by ultraviolet light has been used to create mutants of fungi and *Streptomyces* (Mann, 1962; Puhalla, 1973; Puhalla and Howell, 1975; Hopwood et al, 1985; and Baltz, 1986). Attempts were made to produce pectinase deficient streptomyces mutants by ultraviolet light mutagenesis to determine whether pathogenicity and production of pectinases were correlated. The D.P. isolate was chosen for this experiment because of its marked virulence and ability to produce deep pitted lesions on potato tubers.

The method of mutagenesis was a modification of Hopwood et al (1985). D.P. spores were maintained at -20 C as a stock in 20% glycerol/ water solution (v/v) at a concentration of 2.8 X 10^5 cfu/ml. One ml of the stock was diluted to 100 ml with sterile distilled water and this solution was transferred in 20 ml aliquots to sterile petri dishes. An ultraviolet lamp (General Electric germicidal lamp 254 nm) was used to irradiate the spore suspension. To reduce contamination prior to carrying out the mutagenesis, the work area was irradiated with U.V. light for 40 minutes. The U.V. light source was placed approximately 22.5 cm from the surface of the spore suspension. The spore suspension was exposed to the U.V. light for approximately 40 seconds. The

spore suspension was gently agitated by a bench top rotary shaker or a sterile magnetic stir bar placed in the solution, while being exposed to the U.V. light. One ml of the irradiated spores were plated to a modified Czapek-Dox medium containing 0.5% pectin or polygalacturonic acid medium adjusted to pH 8.5 (Mann, 1962; and Tuite, 1969) and to YEME agar (Shirling and Gottlieb, 1966). To verify that the U.V. light was killing the spores 1 ml of untreated spore suspension was also plated on pectin media and YEME agar. The inoculated plates were wrapped in aluminum foil to avoid photo repair of the DNA and incubated at 25 C for 14 days. Plates with untreated spores usually produced 200 or more colonies. The U.V. dose on average resulted in the recovery of 10 to 40 colonies per plate.

Sporulating colonies, resulting from U.V. irradiated spores, were transferred from pectin or PGA medium to YEME agar. The original pectin or PGA plate from which the spores were selected was then flooded with 1 N HCl (Puhalla and Howell, 1975). Colonies lacking distinct zones of clearing around them were selected, as this indicated that the colony was unable to degrade the surrounding pectic substrate. Colonies were not selected that had zones of clearing around them as this indicated the substrate was enzymatically degraded. This screening method worked best when PGA was used as a substrate, thus PGA deficient mutants were selected for in my experiments. The screening medium (PGA) was adjusted to a pH between pH 8 and 9.5, to favor selection of PGA lyase-deficient mutants. The isolates selected were physiological and biochemically characterized and compared to the wild-type D.P. isolate.

RESULTS

Growth of *Streptomyces* on pectic substrates. The isolates studied were capable of utilizing pectin or polygalacturonic acid (PGA), as a sole carbon source in agar culture or shake culture. The size of streptomyces grown colonies on pectin agar medium were usually smaller than colonies grown on YEME agar. The colonies grown on pectic substrates would not always sporulate and bald (nonsporulating) colonies occurred frequently. Most isolates grew well in shake culture and produced bands of sporulating mycelium. Growth was not usually visible for at least one week, but heavy growth normally occurred after 2 to 3 weeks. The 3S isolate (a nonpathogen) grew more rapidly than the isolates RL 232, D.P., and RL 95. The pectolytic activity found in the 3S culture filtrates were compared to RL 232, D.P., and RL 95 enzyme preparations in enzyme assays.

Detection of hydrolase activity in concentrated culture filtrates. The reducing groups assay detected a peak of activity for pectin and PGA induced enzyme preparations from RL 232, D.P., and RL 95, when assayed on PGA substrate at pH 5 (Fig. 3.1 A and B). Viscometry suggested that the enzyme activity was exolytic, as PGA and pectin induced enzyme preparations caused minimal reduction in the viscosity of PGA substrate at pH 4.5, 4.8, 5, and 5.2. Zones of clearing were not detected in PGA substrate (pH 5) for pectin or PGA induced enzyme preparations assayed by the cup plate method. This finding suggested that at low pH on PGA substrate the enzyme preparations exhibited exolytic activity. An example of an assayed PGA cup plate after flooding with 1 N HCL is shown in figure 3.7 A.

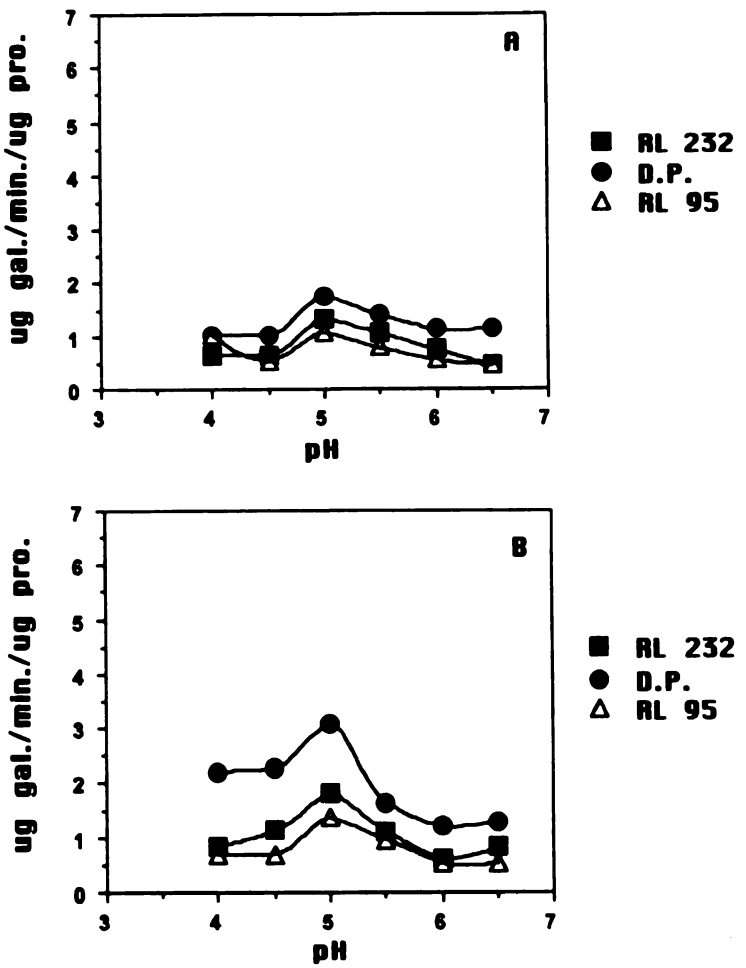

Fig. 3.1. The influence of pH on the activity of pectolytic enzymes from culture filtrates of RL 232, D.P., and RL 95 induced on **A**, **PGA**; or **B**, **pectin**. The reducing groups assay was used to detect hydrolase activity on PGA substrate, activity was measured on the basis of micrograms (μg) galacturonic acid units (gal.) produced per minute (min.) per microgram (μg) protein (pro.) at 540 nm.

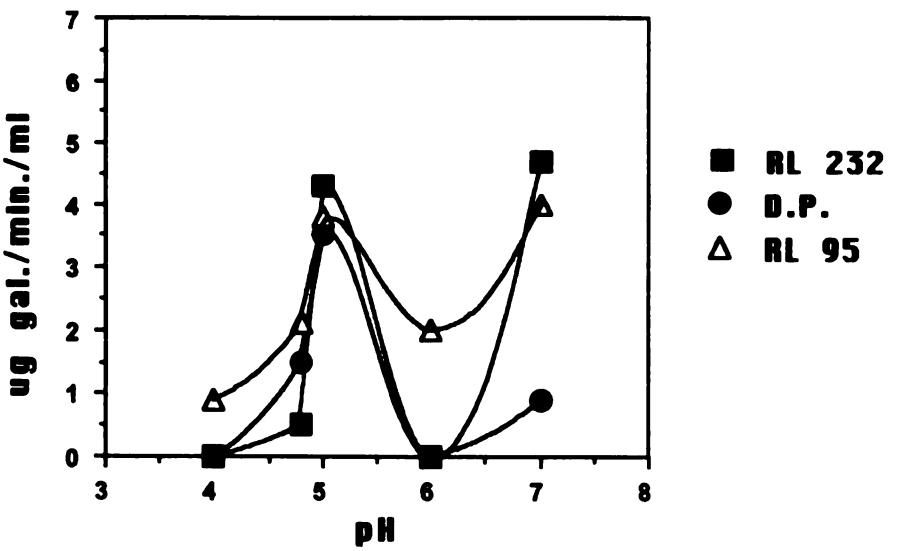

Fig. 3.2. The influence of pH on the activity of pectolytic enzymes from culture filtrates of RL 232, D.P., and RL 95 induced on pectin. The reducing groups assay was used to detect hydrolase activity on pectin substrate, activity was measured on the basis of micrograms (μg) galacturonic acid units (gal.) produced per minute (min.) per milliliters (ml) reaction mixture at 540 nm.

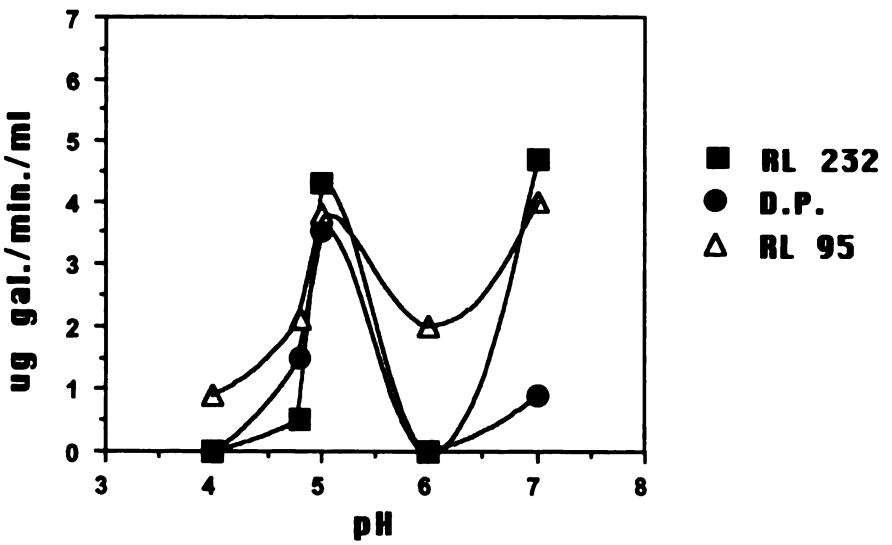

Fig. 3.2. The influence of pH on the activity of pectolytic enzymes from culture filtrates of RL 232, D.P., and RL 95 induced on pectin. The reducing groups assay was used to detect hydrolase activity on pectin substrate, activity was measured on the basis of micrograms (μg) galacturonic acid units (gal.) produced per minute (min.) per milliliters (ml) reaction mixture at 540 nm.

Zones of clearing were detected in pectin substrate (pH 5) for pectin and PGA induced enzyme preparations from RL 232, D.P., and RL 95 by the cup plate assay. An example of an assayed pectin cup plate after flooding with ruthenium red solution is shown in figure 3.7 B. Hydrolases (polygalacturonases) generally show higher affinity for PGA substrate. However, the reducing groups assay detected peaks of activity for pectin induced enzyme preparations, from RL 232, D.P., and RL 95, when assayed on pectin substrate at pH 4.8 and 7 (Fig. 3.2). Viscometry suggested that the enzyme activity was exolytic as the pectin induced enzyme preparations caused minimal reduction in the viscosity of pectin substrate at pH 4.8. These results suggested that RL 232, D.P. and RL 95 produced exopolygalacturonase when grown on pectin or PGA substrate.

Detection of lyase activity in culture filtrates assayed on pectin substrate. Pectin induced enzyme preparations, from RL 232, D.P., and RL 95 caused peak increases in the absorbance (235 nm) of pectin substrate at pH 6.5, 7.5, and 8.5 (Fig. 3.3 A). This suggested that the isolates produced pectin lyase when grown on pectin. The reducing groups assay detected peaks of activity for pectin induced enzyme preparation, from RL 232, D.P., and RL 95, when assayed on pectin assayed on pectin substrate at pH 8.5, and 9.5 (Fig. 3.3 B). This also suggested lyase activity, but the activity occurred at a pH much higher than reported for most pectin lyases to function (Rombouts and Pilnik, 1980). The TBA assay detected low activity for the enzyme preparations which were assayed on pectin substrate, however enzyme activity increased at pH 9.5 (Fig. 3.3 C). Cup plate assays confirmed that pectin induced enzymes, from RL 232, D.P., and RL 95, degraded pectin substrate at pH 7, 8.5, 9, and 9.5. This suggested that Streptomyces produce pectin lyases when grown on pectin substrate. The cup plate assay

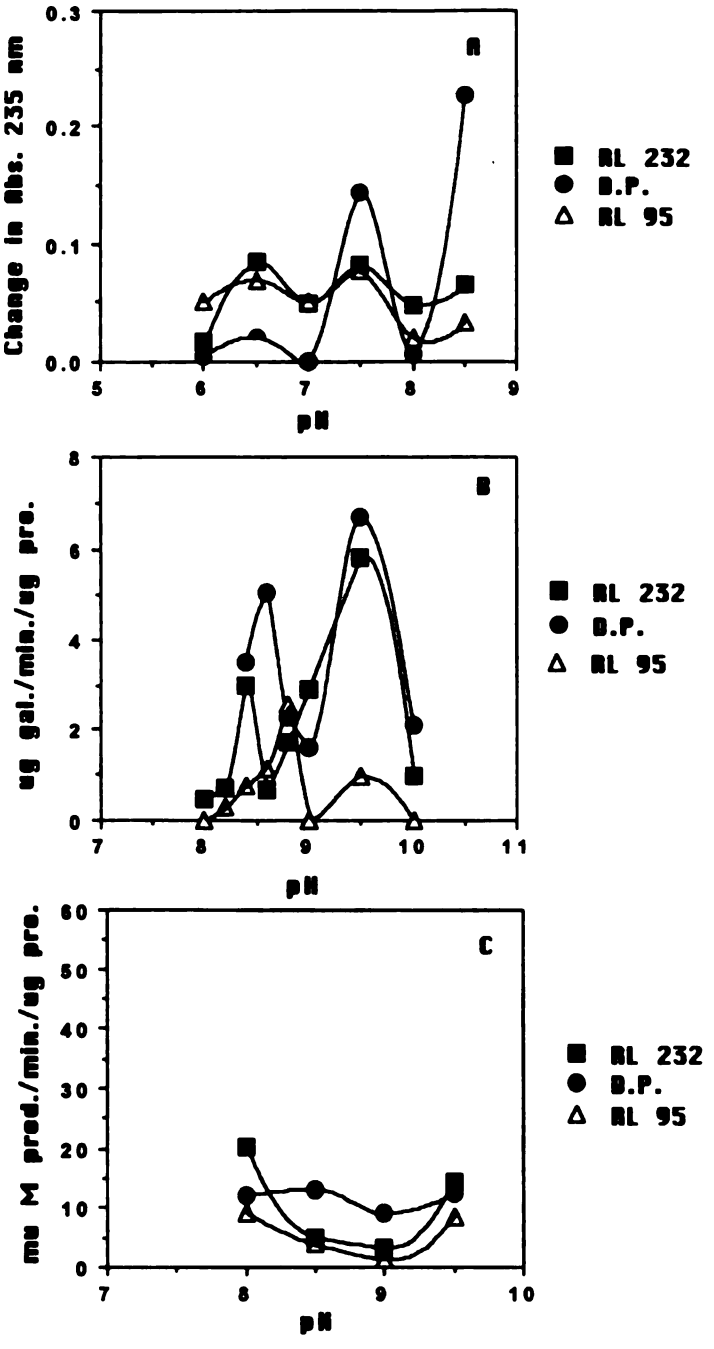

Fig. 3.3. The influence of pH on the activity of pectolytic enzymes from culture filtrates of RL 232, D.P., and RL 95 induced on pectin. Lyase activity was detected on pectin substrate by **A**, measuring the change in absorbance of a reaction after 2 minutes (min.) at 235 nm; **B**, the reducing groups assay, where activity was measured on the basis of micrograms (μg) of galacturonic acid units (gal.) produced per minute (min.) per microgram (μg) protein (pro.) at 540 nm; **C**, thiobarbituric acid assay, where activity was measured on the basis of the concentration in millimicro moles (mμ M) of colored product (prod.) formed per minute (min.) per microgram (μg) protein (pro.) at 548 nm.

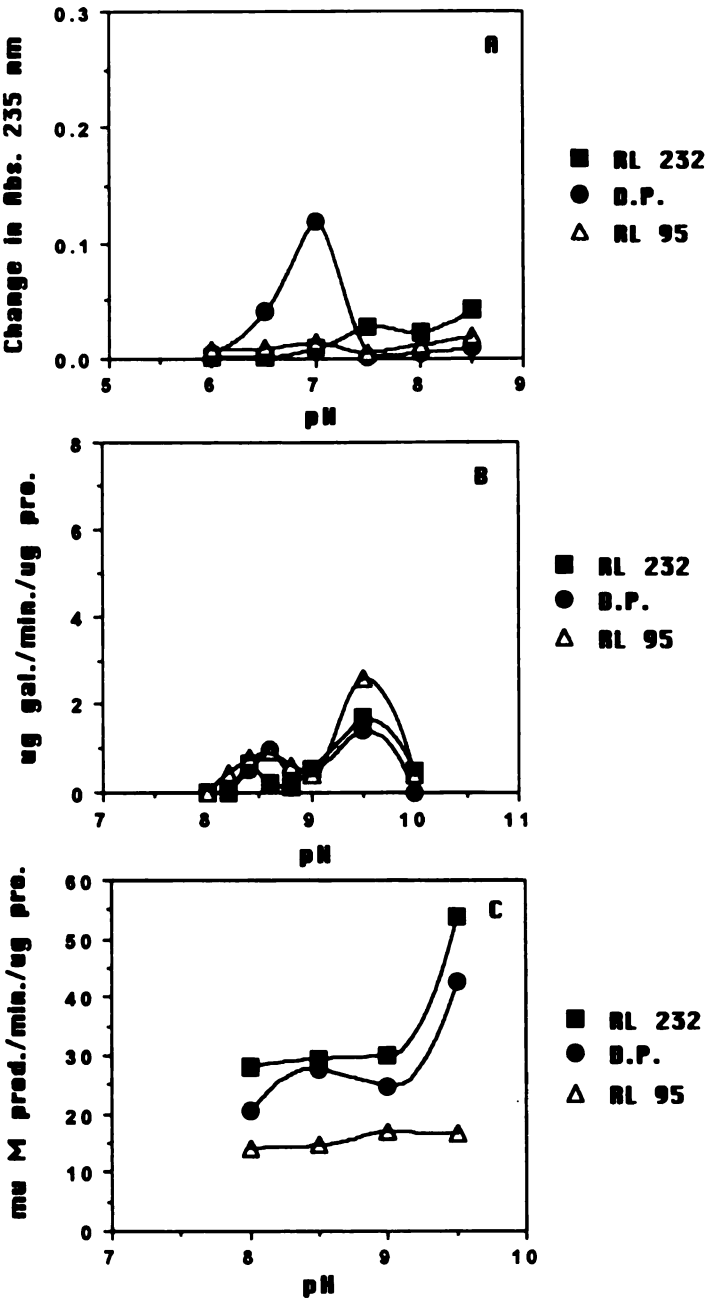

Fig. 3.4. The influence of pH on the activity of pectolytic enzymes from culture filtrates of RL 232, D.P., and RL 95 induced on PGA. Lyase activity was detected on pectin substrate by **A**, measuring the change in absorbance of a reaction after 2 minutes (min.) at 235 nm; **B**, the reducing groups assay, where activity was measured on the basis of micrograms (μg) of galacturonic acid units (gal.) produced per minute (min.) per microgram (μg) protein (pro.) at 540 nm; **C**, thiobarbituric acid assay, where activity was measured on the basis of the concentration in millimicro moles (mμ M) of colored product (prod.) formed per minute (min.) per microgram (μg) protein (pro.) at 548 nm.

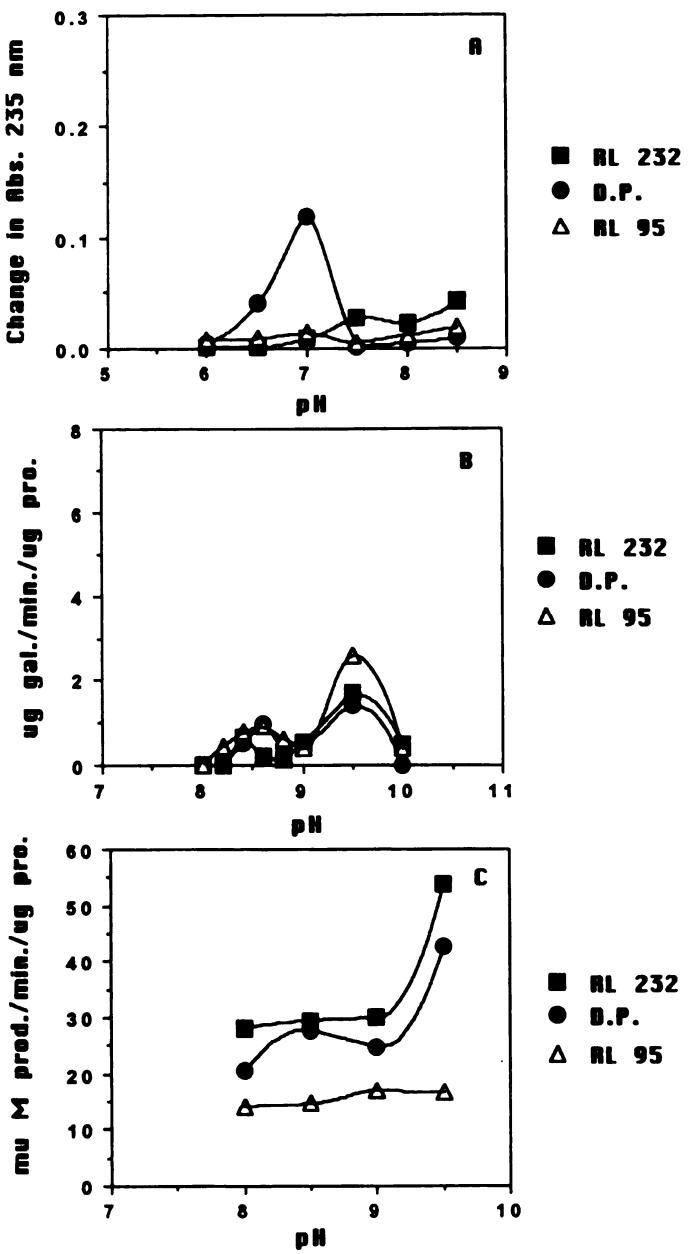

Fig. 3.4. The influence of pH on the activity of pectolytic enzymes from culture filtrates of RL 232, D.P., and RL 95 induced on PGA. Lyase activity was detected on pectin substrate by **A**, measuring the change in absorbance of a reaction after 2 minutes (min.) at 235 nm; **B**, the reducing groups assay, where activity was measured on the basis of micrograms (μg) of galacturonic acid units (gal.) produced per minute (min.) per microgram (μg) protein (pro.) at 540 nm; **C**, thiobarbituric acid assay, where activity was measured on the basis of the concentration in millimicro moles (mμ M) of colored product (prod.) formed per minute (min.) per microgram (μg) protein (pro.) at 548 nm.

could not detect activity for pectin induced enzyme preparation when assayed on pectin at pH 10.5. An example of an assayed pectin cup plate is shown in figure 3.7 B.

PGA induced enzyme preparations, from D.P. caused an increase in absorbance (235 nm) of pectin substrate at pH 7. However, no increase in absorbance of pectin substrate (pH 7) was observed when PGA induced enzyme preparations from RL 232 and RL 95 were assayed (Fig. 3.4 A) The reducing groups assay detected peaks of activity for PGA induced enzyme preparations, from RL 232, D.P., and RL 95, when assayed on pectin substrate at pH 8.5 and 9.5 (Fig. 3.4 B). The TBA assay detected peak activity for PGA induced enzyme preparations, from RL 232 and D.P., when assayed on pectin substrate at pH 9.5. However, no peak of activity was observed at pH 9.5 for the RL 95 enzyme preparation when assayed on pectin substrate (Fig. 3.4 C). These findings suggested that the PGA induced enzyme preparations contained pectin lyase. Cup plate assays confirmed that PGA induced enzymes, from RL 232, D.P., and RL 95, degraded pectin substrate at pH 7, 8.5, 9, and 9.5 (Fig. 3.7 B). Viscometry suggested that the PGA induced enzyme preparations contained endolyase as the enzyme preparations from RL 232, D.P., and RL 95 reduced the viscosity of pectin substrate at pH 8.4.

Detection of lyase activity in culture filtrates assayed on polygalacturonic acid substrate. The reducing groups and TBA assays detected peak activity for the PGA induced enzyme preparations, from RL 232, D.P., and RL 95, when assayed on PGA substrate at pH 8.5 and 9.5 (Fig 3.5 A and B). These findings suggested that streptomyces produce lyases when supplied PGA as a substrate. The PGA lyases generally exhibit optimal activity at pH 8 or higher. PGA induced enzyme preparation,

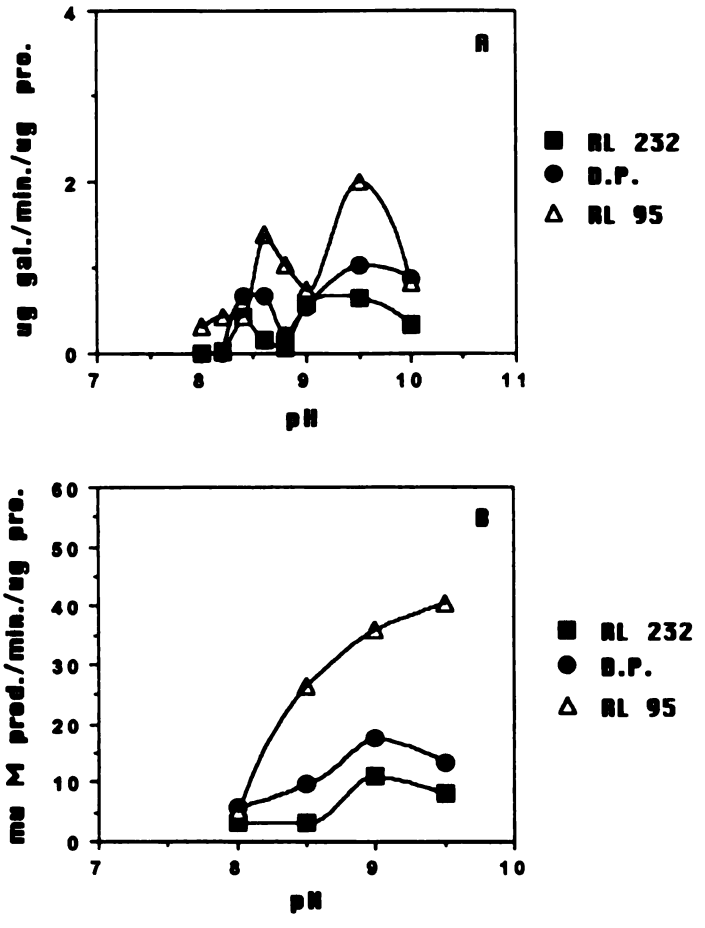

Fig. 3.5. The influence of pH on the activity of pectolytic enzymes from culture filtrates of RL 232, D.P., and RL 95 induced on PGA. Lyase activity was detected on PGA substrate by **A**, the reducing groups assay, where activity was measured on the basis of micrograms (μg) of galacturonic acid units (gal.) produced per minute (min.) per microgram (μg) protein (pro.) at 540 nm; **B**, thiobarbituric acid assay, where activity was measured on the basis of the concentration in millimicro moles (mμ M) of colored product (prod.) formed per minute (min.) per microgram (μg) protein (pro.) at 548 nm.

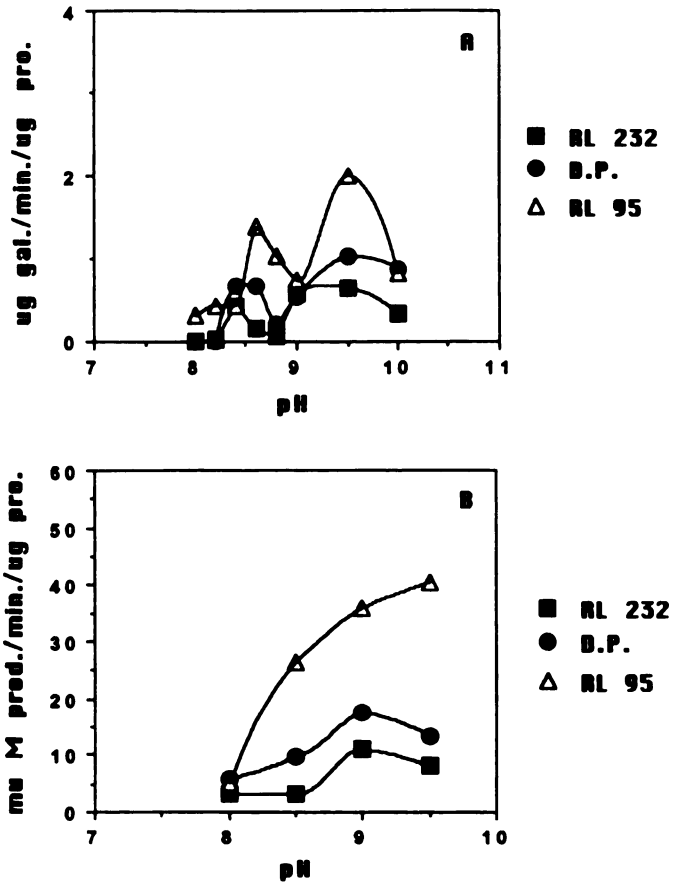

Fig. 3.5. The influence of pH on the activity of pectolytic enzymes from culture filtrates of RL 232, D.P., and RL 95 induced on PGA. Lyase activity was detected on PGA substrate by **A**, the reducing groups assay, where activity was measured on the basis of micrograms (μg) of galacturonic acid units (gal.) produced per minute (min.) per microgram (μg) protein (pro.) at 540 nm; **B**, thiobarbituric acid assay, where activity was measured on the basis of the concentration in millimicro moles (mμ M) of colored product (prod.) formed per minute (min.) per microgram (μg) protein (pro.) at 548 nm.

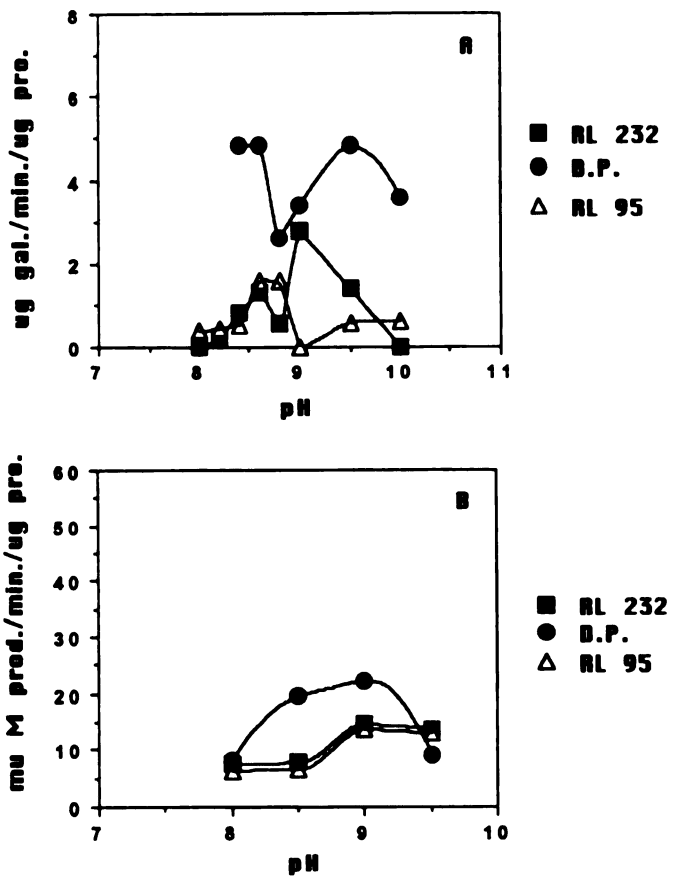

Fig. 3.6. The influence of pH on the activity of pectolytic enzymes from culture filtrates of RL 232, D.P., and RL 95 induced on pectin. Lyase activity was detected on PGA substrate by **A**, the reducing groups assay, where activity was measured on the basis of micrograms (μg) of galacturonic acid units (gal.) produced per minute (min.) per microgram (μg) protein (pro.) at 540 nm; **B**, thiobarbituric acid assay, where activity was measured on the basis of the concentration in millimicro moles (mμ M) of colored product (prod.) formed per minute (min.) per microgram (μg) protein (pro.) at 548 nm.

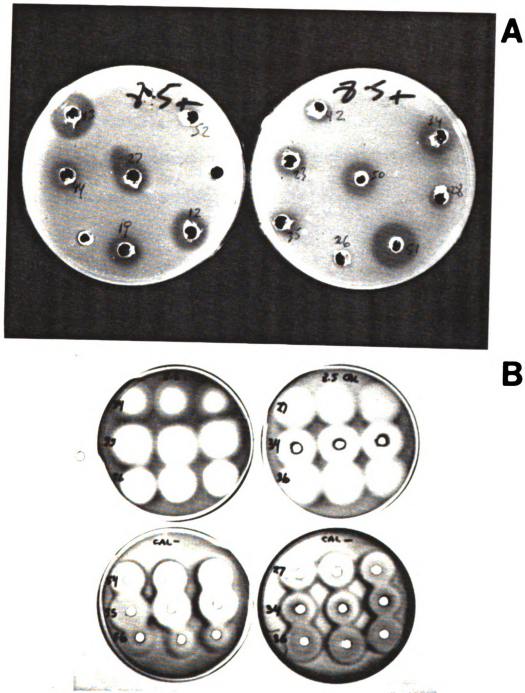

Fig. 3.7. Cup plates illustrating zones of clearing corresponding to lyase activity. **A**, **P**GA substrate (pH 8.5) was treated with 1 N HCl to precipitate the undegraded **s**ubstrate, enzyme activity was indicated by the clear halos which formed around the **w**ells. **B**, pectin substrate (pH 8.5) stained with 0.03% ruthenium red solution, **u**ndegraded substrate was stained pink or red and enzyme activity was indicated by the **h**alos (unstained pectin) which formed around the wells.

from RL 232, D.P., and RL 95 reduced the viscosity of PGA substrate at pH 8.4, which suggest that the enzyme preparations contained endolyases. Zones of clearing were detected in PGA substrate at pH 7, 8, 8.5, for PGA induced enzyme preparations assayed by the cup plate method (Fig. 3.7 A). The PGA induced enzyme preparation could not degrade PGA substrate buffered to pH 10.5. The above findings suggested that streptomyces produced lyases when grown on PGA substrate.

The reducing groups and TBA assays detected peak activity for the pectin induced enzyme preparations, from RL 232, D.P., and RL 95, when assayed on PGA substrate between pH 8 and 9.5 (Fig. 3.6 A and B). Pectin induced enzyme preparations degraded PGA substrate at pH 7, 8, and 8.5, as detected by the cup plate method (Fig. 3.7 A). Viscometry also suggested that this activity was endolyase as the pectin induced enzyme preparations reduced the viscosity of PGA substrate at pH 8.4. All these findings suggested that the streptomycetes produced PGA lyase when grown on pectin substrate.

Detection of pectolytic enzyme activity with pectic agarose overlays. Preliminary studies revealed that the 3S isolate produced lyases in shake culture when grown on pectin or PGA substrates. When the 3S enzyme preparations were analyzed using the pectin agarose overlay method, highly visible bands of clearing appeared, which corresponded to the electrophoresed enzymes in the overlaid polyacrylamide gel. The bands of clearing produced by electrophoresed 3S enzymes preparations were usually more intense than those produced by enzyme preparations from the other isolates studied. Therefore the 3S isolate was used in all PAGE-overlay work.

Lanes 1 and 2 of the PAGE-overlay gel (Fig. 3.8) showed that PGA induced enzymes from 3S and RL 232 produced dissimilar patterns of enzyme degradation at pH

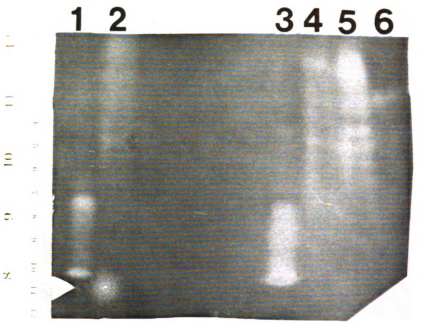

Fig. 3.8. Detection of pectic enzymes produced by *Streptomyces* with 0.1% pectin agarose overlay stained with 0.03% ruthenium red solution. Each lane shows the pattern of substrate degradation characteristic to each enzyme preparation, which came from culture filtrates of the bacteria induced on pectin or PGA. Lane 1, 3S PGA; lane 2, RL 232 PGA; lane 3, 3S pectin; lane 4, RL 232 pectin; lane 5, D.P. pectin; lane 6, RL 95 pectin.

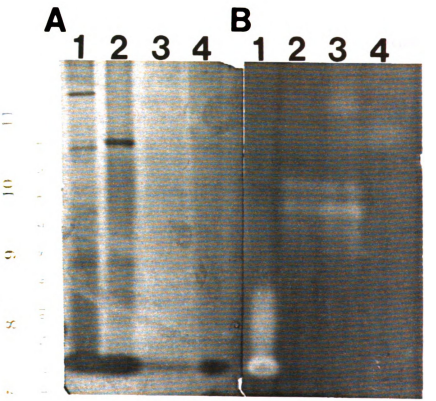

Fig. 3.9. A, Silver stained polyacrylamide gel containing electrophoresed pectolytic enzymes from *Streptomyces* induced on pectin. Lane 1, 3S; lane 2, RL 232; lane 3, D.P.; and lane 4, RL 95. **B,** The pectin agarose overlay stained with ruthenium red shown in figure 3.8 (**lanes 3-6**). Lane 1, 3S; lane 2, RL 232; lane 3, D.P.; lane 4, RL 95.

8.4. The pectin induced enzymes from RL 232, and D.P. appeared to produce similar types of pectic enzymes in culture, and caused similar type clearing on the overlay (Fig. 3.8, lanes 4-5). Pectin induced enzymes from RL 95 did not produce bands of clearing in the overlay similar to RL 232, D.P., or 3S isolates (Fig 3.8, lane 6).

Pectin and PGA induced enzymes from 3S culture filtrates were predominately acidic in nature, whereas pectin induced enzymes from RL 232 and D.P. culture filtrates were basic in nature (Fig. 3.9 A and B, lane 1-4). In general, pectin substrate gels gave the best evidence of clearing with sharp bands of activity at pH 8.4 for the isolates tested. PGA substrate gels also gave sharp bands of clearing for the 3S enzyme preparations induced on pectin or PGA substrates. The enzyme preparations for RL 232, D.P., and RL 95 induced on pectin or PGA, usually gave very faint and diffused zones of clearing at pH 8.4 and 9.1 on PGA substrate gels (not shown).

Recovery and screening of pectinase deficient mutants of D.P. The U.V. light dosage killed approximately 99.9% of the irradiated D.P. spores, between 10 to 40 colonies per plate were recovered on the PGA medium. Three isolates were selected to study, which appeared to have loss the ability to degrade PGA agar medium. The three isolates designated B_1, 2A, and 3C were able to grow on pectin and PGA medium in shake culture. Accurate screening for mutants lacking the ability to produce pectinases was difficult, the D.P. isolate would not pit PGA medium, as do many *Erwinia* species. Also, the Puhalla and Howell (1975) method of screening for pectinase deficient mutants on agar gave unreliable results with streptomyces. This may indicate that streptomyces fail to export large amounts of enzyme into the surrounding agar medium. Additionally the colonies, resulting from irradiated spores, on PGA medium were slow-growing and

Fig. 3.10. Reverse culture color of the U.V. mutants of D.P., viewed from the bottom side of the petri dish. **A**, 2A; **B**, 3C; **C**, D.P. (wild type); **D**, B_1.

TABLE 3.1. Characteristics of D.P. wild type and U.V. generated isolates of D.P.

Characteristics[b]	Isolates[a] D.P.	B_1	3C	2A
Spore color[c]	G	G	G	G
Chain morphology[d]	F	F	F	F
Pigment on PYI	-	-	-	-
Colored pigments on [e]:				
YEME	-	-	-	-
OA	-	-	-	-
Carbon usage				
Glucose	+	+	+	+
Fructose	+	+	+	+
Sucrose	+	+	+	+
Mannitol	+	+	+	+
Arabinose	+	+	+	+
Xylose	+	+	-	+
Raffinose	+	+	-	+
Rhamnose	+	+	+	+
myo-Inositol	+	+	-	+
Degradation of [f]:				
Pectin	+	+	+	+
Polygalacturonate	+	+	+	+
Salt tolerance[g]				
0% NaCl	+	+	+	+
4% NaCl	+	+	+	+
7% NaCl	-	-	-	-
10% NaCl	-	-	-	-
13% NaCl	-	-	-	-
Lysozyme resistance[h]				
0 μg/ml	+	+	+	+
10 μg/ml	+\-[i]	-	-	-
50 μg/ml	+\-	-	-	-
100 μg/ml	-	-	-	-
Hydrolysis of:				
Esculin	+	+	+	+
Urea	+	+	+	+
Hippuric acid	-	-	-	-
LV reaction	-	-	+\-	-
Antibiotic resistance [j]:				
Ampicillin 45 μg/ml	+	+	+	+
Chloramphenicol 10 μg/ml	-	-	-	-
Kanamycin 50 μg/ml	-	-	-	-
Penicillin[k] 28 μg/ml	+	+	+	+
Streptomycin 25 μg/ml	-	-	-	-
Tetracycline 14 μg/ml	-	-	+	-

[a] Isolates from D.P. spores irradiated with U.V. 254 nm light, recovered on PGA medium.
[b] Test were taken from ISP methods (Shirling and Gottlieb, 1966) and Kutzner (1981) and our own assays.
[c] G, Grey
[d] F, Flexuous

TABLE 3.1. (*continued from the preceding page*)

Characteristics	Isolates			
	D.P.	B_1	3C	2A
Growth at [l]:				
25 C	+	+	+	+
37 C	-	+	-	+
Utilization of organic acids				
Citrate	+	+	+	+
Malate	+	+	-	+
Malonate	+	+	-	+
Lactate	+	+	-	+
Succinate	+	+	+	+
Gluconate	+	N.R.[m]	N.R.	N.R.
Oxalate	+	+	-	+
Production of organic acids	-	-	-	+\-
Pathogenicity on potato [n]:				
Russet Burbank	-	-	-	-
Atlantic	DP[o]	DP	DP	DP

[e] Diffusible colored pigment on Yeast Extract Malt Extract (YEME).
[f] Czapek medium with 0.5% pectin or polygalacturonic acid as the sole carbon source in 1.5% agar.
[g] NaCl incorporated into YEME agar at 40 g/L, 70 g/L, 100 g/L, and 130 g/L.
[h] Liquid broth with lysozyme. Determines the ability of the organism to grow in presence of lysozyme.
[i] +\-, either no growth or very weak growth.
[j] Antibiotic mixed with YEME agar. Rate at which antibiotics are incorporated into the medium are from Maniatis (1982).
[k] This concentration of antibiotic was of our own design.
[l] The effect of growing bacteria at two temperatures.
[m] N.R., no result
[n] Infection on greenhouse grown tubers. The lesion type was noted.
[o] DP, deep pitted lesions, the isolates D.P. and 3C were more virulent than the isolates B_1 and 2A.

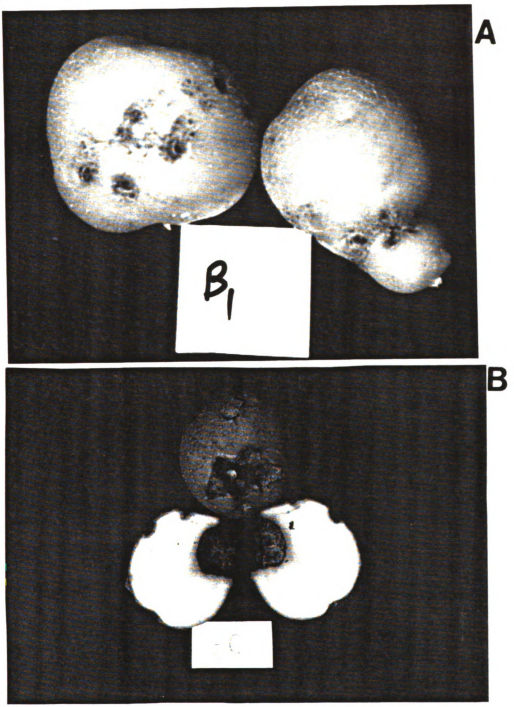

Fig. 3.11. Deep-pitted scab caused by the U.V. mutants of the D.P. isolate. **A**, B_1; and **B**, 3C.

nonsporulating which made replica plating of the colonies difficult. Therefore, screening of D.P. mutants lacking the ability to degrade PGA substrate was discontinued and the three isolates were further characterized and studied.

Characterization of the U.V. irradiated isolates of D.P. The isolates B_1, 2A, and 3C were physiologically characterized and compared to D.P. (Table 3.1). When grown on YEME agar the U.V. generated isolates were nearly indistinguishable from D.P., except the 2A culture had a darker reverse color (Fig. 3.10 A). The 3C isolate was unable to utilize several substrates (xylose, raffinose, myo-inositol, malate, malonate, lactate, and oxalate) utilized by D.P. 3C also acquired the ability to grow in presence of tetracycline. However, the level of growth was very poor. The isolate 3C's utilization of pectin and PGA and its ability to cause deep-pitted scab lesions were unaffected by mutagenesis (Table 3.1; and Fig. 3.11 B). The isolates B_1 and 2A grew poorly at 37 C and formed bald colonies, however, this may not be the result of a mutation. The isolates B_1 and 2A produced deep pitted scab on Atlantic tubers, but the lesions appeared to be shallower than those caused by D.P. This lowered virulence was not correlated with a subsequent loss of pectinase degrading ability.

Detection of pectolytic activity in culture filtrates of the U.V.-irradiated isolates of D.P. The reducing groups assay detected activity for pectin and PGA induced enzyme preparations from B_1, 2A, and 3C, when assayed on PGA and pectin substrate (between pH 5-6) (Fig. 3.12 A and B). This suggested that the isolates produced hydrolase in culture as was observed for RL 232, D.P., and RL 95. However, the activity was not confirmed by cup plate assays on PGA substrate at pH 5. Viscometry suggested that the

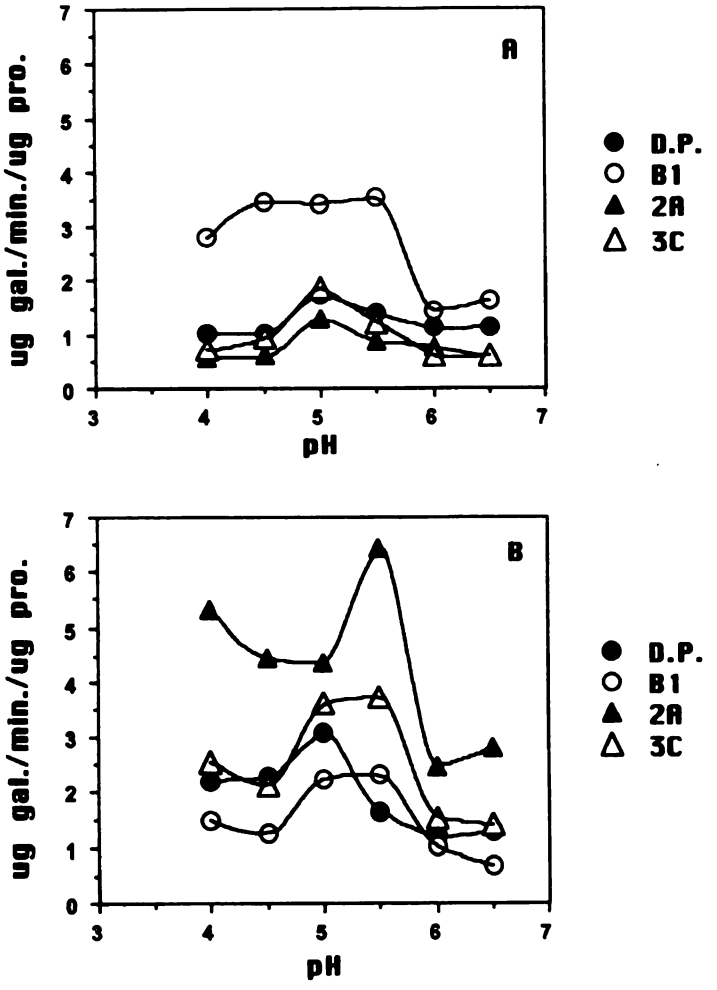

Fig. 3.12. The influence of pH on the activity of pectolytic enzymes from culture filtrates of D.P., B_1, 2A, and 3C induced on **A, PGA**; or **B, pectin**. The reducing groups assay was used to detect hydrolase activity on PGA substrate, activity was measured on the basis of micrograms (μg) galacturonic acid units (gal.) produced per minute (min.) per microgram (μg) protein (pro.) at 540 nm.

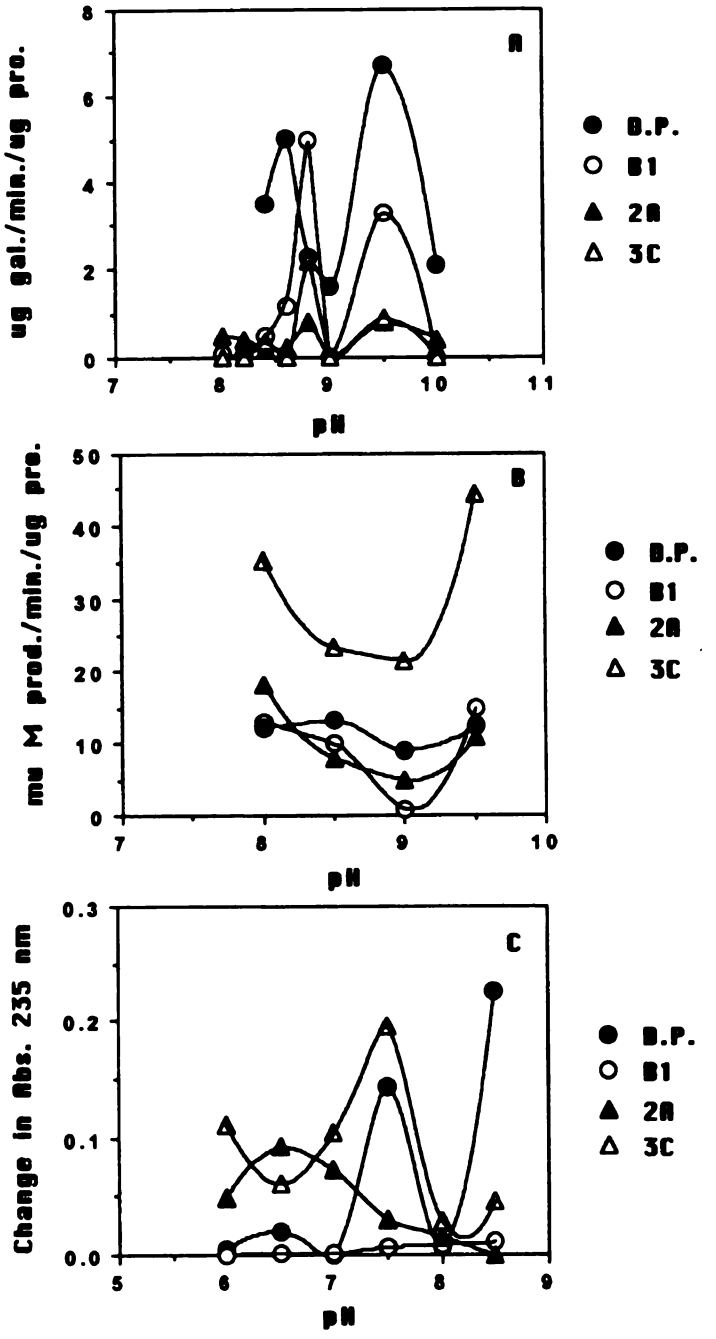

Fig. 3.13. The influence of pH on the activity of pectolytic enzymes from culture filtrates of D.P., B_1, 2A, and 3C induced on pectin. Lyase activity was detected on pectin substrate by **A**, the reducing groups assay, where activity was measured on the basis of micrograms (μg) of galacturonic acid units (gal.) produced per minute (min.) per microgram (μg) protein (pro.) at 540 nm; **B**, thiobarbituric acid assay, where activity was measured on the basis of the concentration in millimicro moles (mμ M) of colored product (prod.) formed per minute (min.) per microgram (μg) protein (pro.) at 548 nm; **C**, measuring the change in absorbance of a reaction after 2 minutes (min.) at 235 nm.

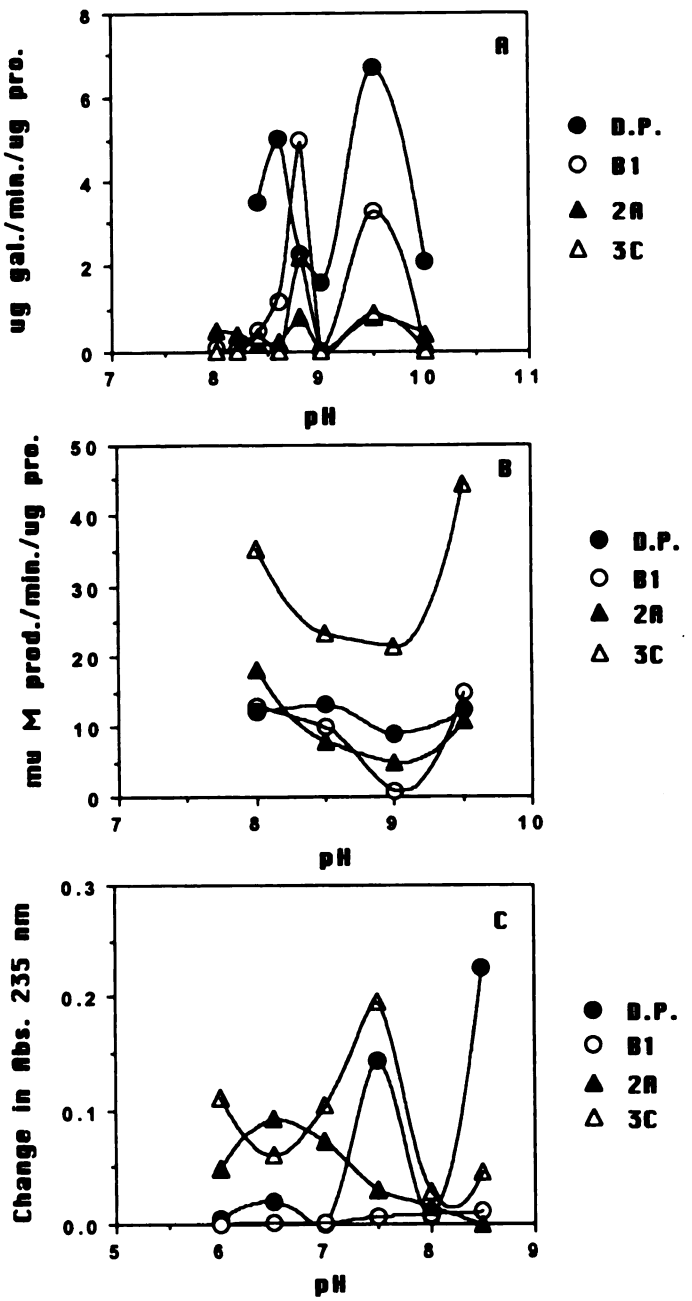

Fig. 3.13. The influence of pH on the activity of pectolytic enzymes from culture filtrates of D.P., B_1, 2A, and 3C induced on pectin. Lyase activity was detected on pectin substrate by **A**, the reducing groups assay, where activity was measured on the basis of micrograms (μg) of galacturonic acid units (gal.) produced per minute (min.) per microgram (μg) protein (pro.) at 540 nm; **B**, thiobarbituric acid assay, where activity was measured on the basis of the concentration in millimicro moles (mμ M) of colored product (prod.) formed per minute (min.) per microgram (μg) protein (pro.) at 548 nm; **C**, measuring the change in absorbance of a reaction after 2 minutes (min.) at 235 nm.

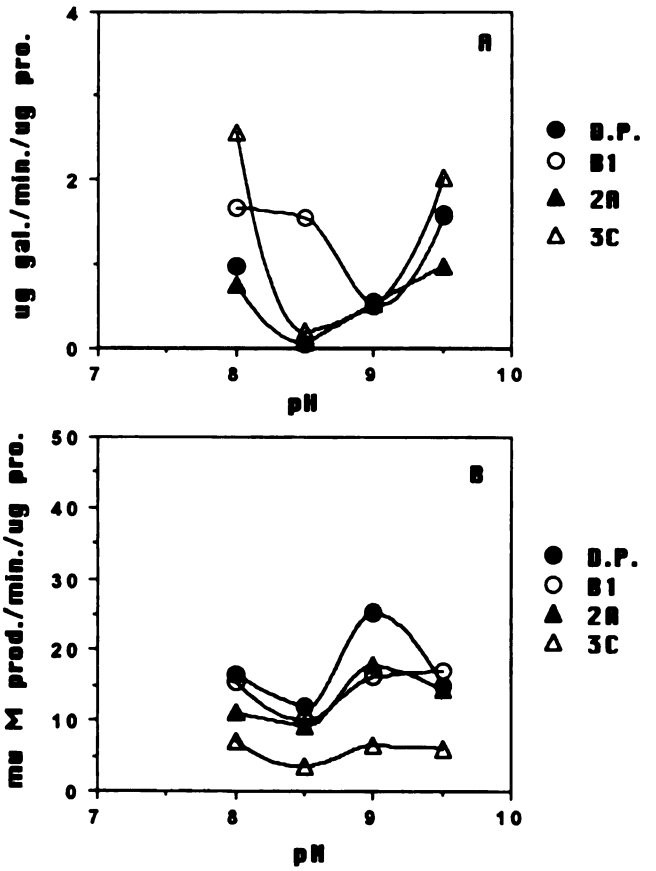

Fig. 3.14. The influence of pH on the activity of pectolytic enzymes from culture filtrates of D.P., B_1, 2A, and 3C induced on PGA. Lyase activity was detected on PGA substrate by **A**, the reducing groups assay, where activity was measured on the basis of micrograms (μg) of galacturonic acid units (gal.) produced per minute (min.) per microgram (μg) protein (pro.) at 540 nm; **B**, thiobarbituric acid assay, where activity was measured on the basis of the concentration in millimicro moles (mμ M) of colored product (prod.) formed per minute (min.) per microgram (μg) protein (pro.) at 548 nm.

enzyme activity was exolytic as the PGA induced enzyme preparation from 2A caused minimal reduction in the viscosity of PGA substrate at pH 5.

The pectin induced enzyme preparations from B_1, 2A, and 3C generally exhibited the lyase activity that was associated with enzyme preparations from D.P., when assayed on pectin substrate (Fig. 3.13 A,B, and C). Pectin induced enzymes from B_1, 2A, and 3C when assayed by the cup plate method, exhibited activity on pectin substrate at pH 7, 8, 8.5, 9, and 9.5. This suggested that the mutants were still producing lyases in culture when supplied pectin as a substrate. The PGA induced enzyme preparations from B_1, 2A, and 3C also exhibited lyase activity which was associated with enzyme preparations from D.P., when assayed on PGA substrate (Fig. 3.14 A and B). The PGA induced enzyme preparations from B_1, 2A, and 3C when assayed by the cup plate method on PGA substrate exhibited activity at pH 7, 8, and 8.5. Viscometry suggested that endopectate lyase was produced in culture, as the PGA induced enzyme preparation from B_1 rapidly reduced the viscosity of PGA substrate at pH 8.4.

SDS-PAGE of enzyme preparations from *Streptomyces* isolates. SDS polyacrylamide gel electrophoresis of culture filtrates has been used to determine the similarity of different strains of actinomycetes (Dietz, 1988). The electrophoretic patterns of the proteins in the enzyme preparations from the streptomyces shake cultures induced on pectin and PGA were compared for similarity. The electrophoretic patterns of proteins in from PGA induced cultures (Fig. 3.15 A) were different for each isolate tested, which was also the case for enzyme preparations from pectin induced culture (Fig. 3.15 B). Electrophoretic protein patterns of the enzyme preparations differed for a single isolate when grown on pectin or PGA substrates. For example, the protein pattern for RL 232

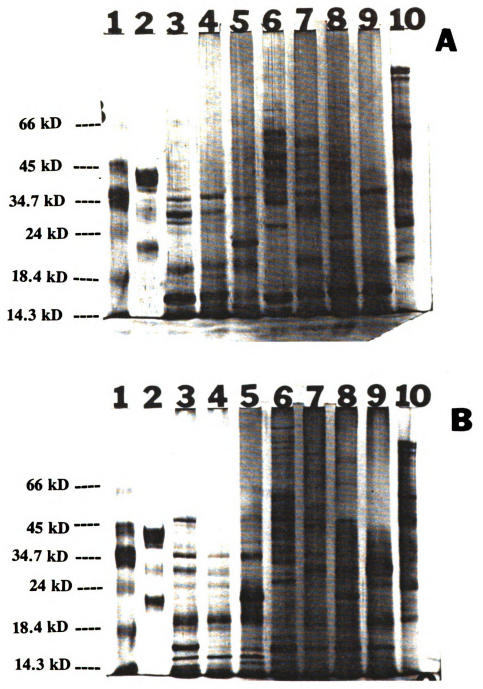

Fig. 3.15. SDS-PAGE of extracellular pectinases from culture filtrates of *Streptomyces* induced on **A,** PGA or **B,** pectin substrates. Lane 1, molecular weight markers in kiloDaltons (kD): bovine albumin (66 kD), egg albumin (45 kD), pepsin (34.7 kD), trypsinogen (24 kD), ß-lactoglobin (18.4 kD), lysozyme (14.3 kD); lane 2, pectin lyase (EC 4.2.2.10) from *Aspergillus japonicus*; lane 3, RL 232; lane 4, D.P.; lane 5, RL 95; lane 6, 3S; lane 7, B_1; lane 8, 2A; lane 9, 3C; and lane 10, polygalacturonase (EC 3.2.1.15) from *Aspergillus niger*.

Fig. 3.16. Rating scale for tissue maceration: **0,** no browning or maceration (comparable to controls); **1,** tissue slightly discolored without maceration; **2,** tissue slightly discolored with minimal visible maceration; **3,** brown tissue with moderate maceration; **4,** brown or blackened tissue, at least 90% maceration (tissue was very soft).

TABLE 3.2. Potato tissue macerating capability of enzyme preparations from *Streptomyces* species cultured on pectin and polygalacturonic acid[a]

	Enzyme induced on[b]:			
	PGA		Pectin	
Enzyme Prep.[d]	Tris[c]	H_2O[c]	Tris	H_2O
RL 232	3[e]	3	3	3
RL 95	1	0	2	2
D.P.	2	2	3	2
B_1[f]	2	2	3	2
2A[f]	1	0	1	2
3C[f]	2	2	2	2
3S	3	2	2	2
PG[g]	3	3	-	-
PL[g]	4	2	-	-

[a] Rating scale: 0, no browning or maceration; 1, slight browning and no maceration; 2, slight browning and slight maceration; 3, moderate browning and moderate maceration; 4, tissue is brown or black with 90% or more maceration.
[b] *Streptomyces* isolates were induced to produce enzymes on polygalacturonic acid (PGA) or pectin substrates.
[c] Tuber discs were incubated in sterile Tris-HCl buffer pH 8.8 and distilled H_2O.
[d] The isolate of *Streptomyces* from which the enzyme preparation was obtained.
[e] Numerical values corresponding to the level of tissue maceration.
[f] U.V. generated isolates of the D.P. (Chapter 3, this dissertation).
[g] Commercial enzyme preparations. PG, polygalacturonase from *Aspergillus niger*; PL, pectin lyase from *Aspergillus japonicus*.

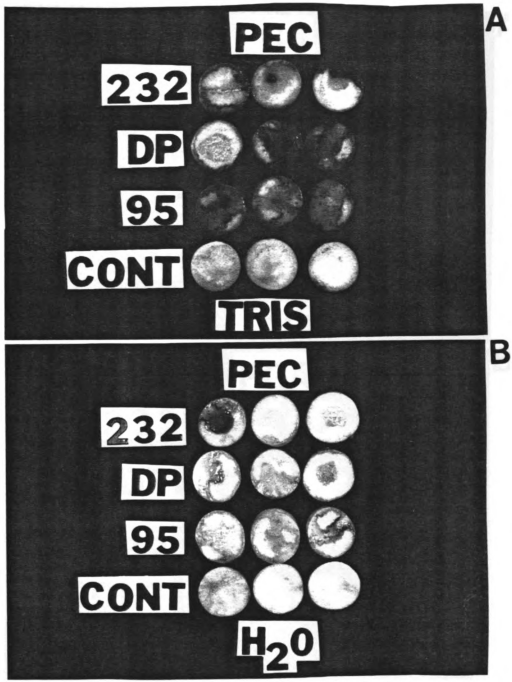

Fig. 3.17. Tuber discs 36 hours after treatment with pectin induced enzyme preparations from *Streptomyces* isolates RL 232, D.P., and RL 95. **A,** Tissue incubated in Tris-HCl buffer (pH 8.8); **B,** Tissue incubated in water.

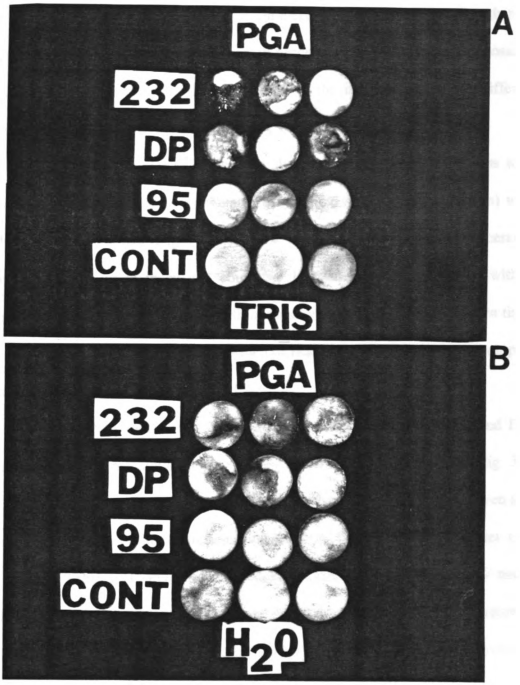

Fig. 3.18. Tuber discs 36 hours after treatment with PGA induced enzyme preparations from *Streptomyces* isolates RL 232, D.P., and RL 95. **A,** Tissue incubated in Tris-HCl buffer (pH 8.8); **B,** Tissue incubated in water.

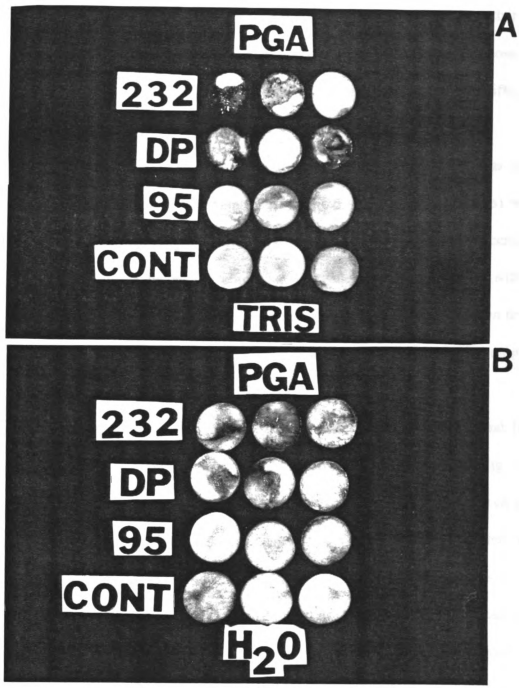

Fig. 3.18. Tuber discs 36 hours after treatment with PGA induced enzyme preparations from *Streptomyces* isolates RL 232, D.P., and RL 95. **A,** Tissue incubated in Tris-HCl buffer (pH 8.8); **B,** Tissue incubated in water.

induced on PGA was different from that of RL 232 enzyme preparation induced on pectin. The wild type isolate D.P. had different electrophoretic protein patterns than its U.V. generated mutants B_1, 2A, and 3C, which suggested that the isolates were possibly mutated in their ability to synthesize pectinases, or the isolates synthesized different extracellular proteins than D.P., while growing on pectin and PGA.

Tissue maceration assay. Tissue macerating ability of the enzyme preparations were observed on potato tuber discs. Browning and loss of tissue integrity (hardness) were visually rated. The ratings ranged from 0 to 4 where: 0, no browning or maceration (comparable to the water inoculated controls); 1, tissue slightly discolored without maceration; 2, tissue slight discolored with minimal visible maceration; 3, brown tissue with a moderate level of maceration; 4, brown or blackened tissue with nearly complete maceration, tissue was very soft (Fig. 3.16).

Pectin and PGA induced enzyme preparations from RL 232, RL 95, and D.P. cultures caused tissue maceration approximately 24-36 hours after treatment (Fig. 3.17 and 3.18). Controls browned slightly after 36 hours without loss of hardness, even after 48 hours. Maceration occurred on tissue incubated in Tris-HCl buffer or water (Fig. 3.17, 3.18 and Table 3.2). PGA induced culture filtrates of RL 95 and 2A (U.V mutant of D.P.) did not cause maceration of tissue incubated in water (Table 3.2). Commercial preparations of polygalacturonase EC 3.2.1.15 from *A. niger* and pectin lyase EC 4.2.2.10 from *A. japonicus* caused maceration of tuber discs incubated in Tris-HCl buffer or water (Table 3.2).

DISCUSSION

The isolates RL 232, D.P., RL 95, and 3S were induced on citrus pectin or

sodium polypectate (PGA) to produce pectolytic enzymes in shake culture. Additionally, the isolates RL 232, D.P., and RL 95 were not found to produce pectolytic enzymes when supplied glucose as a sole carbon source. The pectin and PGA enzyme preparations, from the culture filtrates of each isolate, exhibited hydrolase and lyase activity when assayed on pectin or PGA substrates. Using the reducing groups assay hydrolase activity was detected in enzyme preparations from the various streptomyces isolates used in this study. Hydrolases exhibited activity on PGA substrate at pH 5. However, hydrolase activity was not detected with viscometry or the cup plate assay on PGA substrate at pH 5, which suggested that possibly exopolygalacturonases were induced in culture. The reducing groups assay detected hydrolase activity in pectin induced enzyme preparations assayed on pectin substrate at pH 4.8 (Fig. 3.2). Pectin and PGA induced enzyme preparations, however, exhibited activity on pectin substrate (pH 5) when assayed by the cup plate method. This activity however, was not confirmed by viscometry on pectin substrate at pH 5. This also suggested that exopolygalacturonases were induced in culture. The production of hydrolases by *Streptomyces* species in culture has not been reported. However, Seguin and Lalonde (1989) reported that culture filtrates from *Frankia* species grown on pectin showed activity on pectin substrate at pH 5.2. *Frankia* species are symbionts of *Alnus* and are mildly pathogenic to the many *Alnus* species (Lalonde and Knowles, 1975).

All the streptomyces isolates examined produced lyases in shake culture when grown on pectin or PGA substrates. Pectin and PGA induced enzyme preparations exhibited activity on pectin and PGA substrates at the characteristic pH optimums for endopectin lyase and endopolygalacturonate lyase. Lyases activity was detected readily

with all the enzyme assays used in this study. Lyase activity was usually optimal near pH 9.5 for both pectin and PGA induced enzyme preparations, assayed on PGA substrate. Pectin and PGA induced enzyme preparations, assayed on pectin substrate were active between pH 6-8, but also exhibited activity between pH 8-9.5. The production of lyases by *Streptomyces* species in culture was reported by Knosel (1970) for *S. scabies* and *S. ipomoea*, and by Sato and Kaji (1973, 1975, 1977 a and b) for *S. nitrosporeus* and *S. fradiae*. Knosel purified pectate lyases, which exhibited optimal activity at pH 9, from culture filtrates of streptomycetes grown on a medium consisting of pectin and cellulose. Sato and Kaji (1973) detected pectate lyase in the crude culture filtrate of *S. fradiae*, which exhibited optimal activity on PGA substrate at pH 9.5. Upon purification the enzyme was determined to be an endopectate lyase which exhibited optimal activity between pH 9-9.2 (Sato and Kaji, 1975). *S. nitrosporeus* produced a pectate lyase in culture, which exhibited optimal activity at pH 9.3 and 9.5 on pectin substrate, however, upon purification the enzyme was found to be more active on PGA substrate at pH 9.5 (Sato and Kaji, 1977 b). The concentrated culture filtrates of RL 232, D.P., and RL 95 also exhibited activity on pectin and PGA substrate at pH 9.5.

Thus, streptomycetes produce several types of pectic enzymes that may be induced by pectin or PGA substrates. Further purification of the culture filtrates from RL 232, D.P., and RL 95 induced on pectin and PGA substrates, would help to confirm which types lyases were produced in shake culture.

Sato and Kaji (1973) found that pectic enzymes from *S. fradiae* macerated potato and several other types of plant tissues when incubated in Tris-HCl buffer (pH 8). Pectin and PGA induced enzymes, from RL 232, D.P., and RL 95 macerated potato tuber discs

incubated in Tris-HCl buffer at pH 8.8 or water pH 7 (Figs. 3.17 and 3.18). The pH at which the tissue was incubated had no effect on the ability of the enzyme preparations to cause tissue maceration. Neither *S. fradiae* or *S. nitrosporeus* are plant pathogens and yet produce enzymes that degrade plant tissue. RL 95 and 3S were not pathogenic on potato, but both isolates produced pectolytic enzymes in culture that macerated potato tuber discs (Table 3.2). This finding suggested the ability to produce pectolytic enzymes was not exclusively restricted to *S. scabies* or other potato scab-causing streptomycetes. However, this finding does not negatively implicate the involvement of pectolytic enzymes in the infection process.

Production of pectinase deficient *S. scabies* mutants would determine the importance of pectolytic enzymes for infection. Attempts to produce endopectate lyase deficient mutants from the D.P. isolate were not successful. Screening for mutants was not specific enough to determine whether certain pectinase functions were inactivated. U.V. mutagenesis causes nonspecific base shifts and does not inactivate specific genes as does transposon mutagenesis, and therefore has limited uses in the study of pectinases. Transposon mutagenesis has been used to specifically inactivate pel genes in *Erwinia* species (Collmer and Keen, 1986). The U.V. generated isolates of D.P. (B_1, 2A, 3C) which were screened for the loss of pectinases grew on both PGA and pectin as sole carbon sources. The pectin and PGA induced enzymes from the mutants had hydrolase and lyase activity similar to the wild-type D.P. (Figs. 3.12, 3.13, 3.14) and caused tissue maceration (Table 3.2). The isolates B_1, 2A, and 3C remained pathogenic on Atlantic. U.V. mutagenesis appeared to alter the isolates physiology (Table 3.1 and Fig. 3.10), apparently without affecting utilization of pectic substrates. Pel genes in *Erwinia* species

occur in multiple copies and inactivating one gene does not prevent the bacterium for producing pectolytic enzymes (Collmer and Keen, 1986). Therefore it is possible that *Streptomyces* species have a similar type of arrangement of pectinase genes in multiple forms. This would account for the apparent loss of pectinase production by B_1, 2A, and 3C during the mutant screening process on agar plates, and allow for the production of pectinases in shake culture.

The pectin agarose overlay experiments (Figs. 3.8 and 3.9) suggested that RL 232 and D.P. produced similar types of pectinases, which were different from the nonpathogen 3S. The PGA and pectin induced enzyme preparations from 3S produced identical bands of clearing on the pectic substrate overlays (Fig. 3.8), which suggested PGA and pectin substrates induced production of similar forms pectolytic enzymes from streptomyces in shake culture. It was not possible to distinguish pectolytic enzymes from other proteins by SDS-PAGE of culture filtrates (Fig. 3.15 A and B). However the varied protein patterns on the SDS-PAGE gels suggested that PGA and pectin substrates induced different proteins in culture for the streptomyces used in these studies. Also the SDS-PAGE gels demonstrated that U.V. light generated isolates were physiologically altered, as none of the isolates produced a protein pattern to identical to the D.P. isolate (Fig. 3.15 A and B). The pattern of proteins extracted from whole cells of B_1, 2A, and 3C grown on glucose-asparagine medium (Gottlieb and Hepden, 1966), were different from those of D.P. This also suggested that the U.V. generated D.P. isolates were altered by the mutagenic agent (Fig. 2.2 and 2.3).

The next series of studies should begin with the purification of the pectolytic enzymes induced on potato tissue. As the isolates RL 232, D.P., RL 95, and 3S grew

on a medium which consisted of potato tissue. Using the cup plate assay, it was possible to detect pectolytic activity on pectin substrate (pH 5 and 7) and PGA substrate (pH 7 and 9) for all isolates induced on potato tissue. This finding was significant because it demonstrated that the streptomycetes produced pectolytic enzymes even in the presence of the more accessible carbon sources which should have been provided by the potato tissue. Using the potato based medium should provide a more comprehensive view of the array of cell wall degrading enzymes produced by potato scab-causing streptomycetes during the infection process. My studies indicate that *Streptomyces* species have the capacity to degrade pectic substrates and tend produce lyases in shake culture. As endopectate lyases are known to cause tissue maceration, production of these enzymes by streptomycetes would facilitate invasion of potato tissue by streptomyces. However, *S. scabies* and other potato scab-causing streptomyces do not produce any type of tissue rot. Therefore pectinases cannot be considered a pathogenicity factor in potato scab disease, and should be considered enhancers of the pathogens virulence. However, to test this idea mutants need to be produced which definitely lack the ability to synthesize pectinases. Also these mutants would have to be tested for the loss of virulence on potato. Conversely it may be possible to create hypovirulent mutants of *S. scabies* and assess whether the ability to produce various cell wall degrading enzymes is subsequently loss.

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CHAPTER IV

THE PRODUCTION OF THAXTOMIN BY A DEEP PITTING FORM OF *STREPTOMYCES*

INTRODUCTION

Many species of *Streptomyces* synthesize secondary metabolites which allow them to survive in soil environments (Kutzner, 1981). The streptomycetes are well known for their ability to synthesize antibiotics which inhibit the growth of other microorganisms (Korn-Wendisch and Kutzner, 1992).

S. scabies and *S. acidiscabies* (Lambert and Loria, 1989 a and b), the causal agents of common scab and acid scab of potato (*Solanum tuberosum* L.), synthesize the phytotoxic secondary metabolites designated as thaxtomins (Lawrence et al, 1990 and King et al, 1991). Thaxtomins constitute a unique group of 2,5-dioxopiperazines that have a 4-nitroindol group attached to the 3rd carbon of a 2,5- dioxopiperazine structure (King et al, 1992). Two forms of thaxtomin have been isolated: Form A, contains a hydroxyl group at carbon 20 of the 2,5-dioxopiperazine ring; and Form B has a hydrogen group at carbon 20 of the 2,5-dioxopiperazine ring (King et al, 1989, 1991, and 1992; and Lawrence et al, 1991). The production of thaxtomin A normally exceeds the B form by the ratio of 20:1 in potato tissue (King et al, 1991).

Thaxtomin reproduces the symptoms of common scab on mini-potato tubers (Lawrence et al, 1990). Since *S. scabies* and *S. acidiscabies* both synthesize thaxtomin it is possible that other species of potato scab-causing bacteria produce thaxtomin. The purpose of this study was to determine whether D.P., a deep-pitting scab isolate, produces thaxtomin or thaxtomin-like compounds.

MATERIALS AND METHODS

Streptomyces isolates. The *Streptomyces* isolates RL 232, D.P., and 3S (Tables 2.1 and 2.3) were selected for their contrasting morphologies and differing pathogenic abilities.

Production of inoculum. The bacteria were grown for inoculum production on yeast extract malt extract agar (YEME) (Shirling and Gottlieb, 1966). YEME was inoculated with spores from stocks stored frozen in 20 % glycerol/water at -20 C (Hopwood et al, 1985). The cultures were usually incubated at 25 C for at least 14 days. The spores of mature cultures were used to inoculate all other media and plant tissue.

Induction of thaxtomin in culture. Oatmeal agar and broth (Shirling and Gottlieb, 1966) both support the production of thaxtomin A and B by *S. scabies* (Eckwall et al, 1992). Oatmeal agar was inoculated with mature spores and incubated at 25 C for up to 22 days. Thaxtomin was, however, detected in 9 day old oatmeal agar cultures and the induction period was reduced to 9 days.

Oatmeal broth was dispensed in 25 ml aliquots to 125 ml flask and autoclaved under standard conditions. The flasks were inoculated with a suspension of mature spores and placed on a rotary shaker at 23 C. Twelve and 18 day incubation periods were initially used to induces thaxtomin in oatmeal broth culture. However, thaxtomin was detected in 7 day old oatmeal broth cultures and the induction time was reduced to 7 days. Uninoculated media served as controls.

Plant materials for induction of thaxtomin and bioassay of toxin. Katahdin and Onaway tubers were used to induce thaxtomin. Onaway was selected for its marked susceptibility to the D.P. isolate in greenhouse pathogenicity screenings. Katahdin has been reported to be susceptible D.P. and *S. scabies* (Ludlam, 1991). Russet Burbank

tuber discs were used to bioassay the toxin extracted from tissue and artificial media. Russet Burbank was resistant to infection by RL 232, D.P., and 3S in greenhouse pathogenicity tests.

Induction of toxin on tuber disks. Thaxtomin was induced on tuber discs. A single cylinder of internal medullary tissue was removed from each tuber, sliced into individual discs 2 cm in diameter and 0.5 cm thick (Hammerschmidt, 1984). After slicing the discs were placed into 1% sodium chloride solution to prevent browning. The discs were rinsed in several changes of sterile distilled water prior to inoculation. Inoculum was applied to the discs by pressing them directly onto the surface of a sporulating culture. Only RL 232 and D.P. were used to induce toxin in tissue. The discs were transferred to sterile petri dishes lined with moist filter paper and incubated in darkness at 25 C for 9 days. Uninoculated discs were used as controls.

Initial acetone extraction of agar and culture filtrate. The extraction of toxin was performed according to the procedure of Lawrence et al (1990) and King et al (1991). Six sporulating cultures, grown on oatmeal agar, were combined to form single sample per treatment in the first experiment. For subsequent experiments, four plates were used per sample per treatment. Two flasks of oatmeal broth culture filtrate were combined to form a single sample per treatment in the first experiment. For subsequent experiments the sample size was increased to four flasks of oat meal broth culture filtrate per treatment.

A sample of oatmeal agar culture was extracted with 200 ml of cold acetone by high speed blending with a homogenizer. The resultant slurry was filtered through Whatman No. 1 filter paper and the acetone extract was retained for further extraction.

A sample of broth culture was filtered through cheesecloth followed by high speed centrifugation. The filtrate was extracted with an equal volume of acetone and filtered through Whatman No. 1 filter paper.

Initial acetone extraction of tuber discs. Inoculated tuber discs were necrotic and showed sporulation on all surfaces. Seven tuber discs were extracted by homogenization in cold acetone. The resultant slurry was filtered through Whatman No. 1 filter paper and the acetone extract was retained for further extraction.

Extraction of the media and tissue acetone extracts. Acetone extracts from artificial media or tissue were treated identically. The extracts were evaporated under reduced pressure at 35 C and the remaining residue was dissolved in 25 ml of distilled water. The residue was extracted twice with equal volumes of chloroform. The chloroform fractions were combined and concentrated under reduced pressure at 35 C. The resultant residue was dissolved in 1 ml of acetone and concentrated by evaporation to approximately 0.25 ml, and stored at -20 C or immediately assayed for the presence of toxin by thin layer chromatography (TLC).

Thin layer chromatography (TLC) of crude extracts. The crude extracts were applied to silica gel G thin layer plates in three 5 μl aliquots. The plates were chromatographed in a chloroform/methanol 9:1 (v/v) solvent system (Lawrence et al, 1990 and King et al, 1991). A sample containing toxin generally resolved two bright yellow bands.

Pooling of the crude extracts to concentrate toxin for bioassaying. To maximize the bioassay efficiency of the toxin, the final acetone extracts (from each type of induction media or tissue) for each streptomyces isolate (crude extract) were pooled together.

These final acetone extracts were reextracted with chloroform as previously mentioned and the concentrated residue was solubilized in 0.5 ml acetone.

The concentrated acetone extracts from tissue, agar medium, or culture filtrate for RL 232, D.P., and 3S were banded onto TLC plates in 100 μl aliquots. The plates were chromatographed in a chloroform/methanol 9:1 (v/v) solvent system. The bands that resolved (visibly corresponding to the yellow spots associated with thaxtomin), were scraped directly from the plates. The silica powders for each spot were extracted with ethyl-acetate. The ethyl-acetate was evaporated under vacuum at 35 C and the remaining residue was redissolved in acetone. The concentrated extracts were maintained at -20 C until bioassayed.

Bioassay of the concentrated extracts for toxicity. The acetone was evaporated from the extracts and the residue was suspended in 200 μl of distilled water. Twenty microliters of the toxin suspension was applied to the upper surface of tubers discs, which were maintained in a petri dishes containing moist filter paper. Six tuber discs were bioassayed per treatment. The bioassay was repeated twice for each toxin sample to verify the result.

RESULTS

Induction of thaxtomin in agar culture. In the first experiment, 22 day old OA cultures of RL 232 and D.P. were extracted. The final acetone extract was yellowish-green for both samples. When chromatographed, each sample produced a single yellow band with a R_f of 0.34 for the RL 232 sample and a R_f of 0.17 for the D.P. sample. Control samples did not reveal any yellow bands. When these samples were reextracted and concentrated, the RL 232 sample resolved into two bands with R_fs of 0.32 and 0.98.

The D.P. sample resolved into two bands with R_fs of 0.40 and 0.44, the second band appeared very faint. In each case the concentrated samples retained their yellow color through the second round of extractions.

The RL 232 acetone extracts were yellowish-green in color, the D.P. samples were faintly yellowish. The 3S acetone extract was pink. When the samples were chromatographed, only the RL 232 extracts resolved into single yellow bands in each lane. The average R_f for all the RL 232 samples was 0.30. The D.P., 3S, and control samples produced no visible bands.

The pooled extracts of RL 232 and D.P. were yellowish-green when concentrated. The concentrated extract of 3S was a dark pink color. When chromatographed the RL 232 sample produced a single yellow band with an R_f of 0.27. The D.P. sample produced a single yellow band with an R_f of 0.32. The 3S and control samples produced no bands when chromatographed.

Induction of thaxtomin in culture filtrates. In the first experiment, oatmeal broth cultures were allowed to incubate for 12 or 18 days. Two separate batches of oatmeal broth were prepared for this experiment and RL 232 and D.P. were used to inoculate the medium. Two flasks containing 25 ml of culture medium (50 ml total) were sampled for extraction. When the 12-day and 18-day old D.P. cultures were extracted, one batch of the extracted medium lacked the characteristic yellow-green tint associated with toxin production. After chromatography, only those extracts of yellowish-green color produced yellow bands characteristic of thaxtomin. The 12 day D.P. extracts which produced a single yellow band upon being chromatographed had an average R_f of 0.24, and the 18 day extracts had an average R_f of 0.29. RL 232 produced thaxtomin in both

batches of oatmeal agar broth as indicated by the yellowish-green acetone extracts for each sample. The majority of the acetone extracts for the 12 day cultures revealed two yellow bands after chromatography. The average R_f of the first resolving band was R_f 0.35, the second resolving band had an average R_f of 0.83. After chromatography the extracts of 18 day cultures of RL 232 resolved into single yellow bands for each sample. The average R_f for the extracts of 18 day cultures of RL 232 was R_f 0.23.

The extracts from 12 day and 18 day cultures of RL 232 or D.P. were pooled to form a sample for each of isolate. The sample was extracted and concentrated under vacuum, the intensity of the yellow-green color in each acetone extract increased. After chromatography the RL 232 sample yielded a single yellow band with a R_f of 0.29, the D.P. sample produced a single yellow band with an R_f of 0.32.

Oatmeal broth cultures were incubated for 7 days in a second experiment, which included the isolate 3S in addition to RL 232 and D.P. Approximately 100 ml of culture filtrate (four flask containing 25 ml of culture medium) was extracted for toxin. Only one D.P. sample extract resolved a single yellow band after chromatography with a R_f of 0.19. All RL 232 sample extracts produced single yellow bands after chromatography, with an average R_f of 0.27 for all samples. Bands corresponding to thaxtomin were not detected for 3S and control treatments.

Pooling the extracts intensified the yellow-green color of the final acetone extracts for both RL 232 and D.P. The final acetone extract for 3S was a dark pink color. After chromatography, the RL 232 extract resolved a single yellow band with a R_f of 0.30 and the D.P. extract resolved a single yellow band with a R_f of 0.24. Bands were not detected for 3S or control pooled extracts after chromatography.

Induction of thaxtomin in potato tuber discs. A seven tuber disc sample was extracted for toxin. The acetone extracts from D.P. infected Onaway tuber discs produced no detectable bands corresponding to thaxtomin after chromatography. The acetone extracts from D.P. infected Katahdin tuber discs produced detectable bands corresponding to thaxtomin after chromatography. The average R_f of the material from crude extracts was R_f 0.28. The acetone extracts RL 232 infected Onaway tuber discs resolved into single yellow bands for each sample after chromatography. The average R_f for all the acetone extracts was R_f 0.23. The acetone extracts of RL 232 infected Katahdin tuber discs resolved a single yellow band for each sample tested after chromatography. The average R_f for all the acetone extracts was R_f 0.20. Bands corresponding to thaxtomin were not detected in the acetone extracts of uninoculated tuber discs after chromatography.

The acetone extracts from both Katahdin and Onaway samples for either RL 232 and D.P. were concentrated by pooling and reextracted. The concentrated extracts for both RL 232 and D.P. were yellow-green in color. The pooled D.P. extract after chromatography, resolved into a single yellow band with a R_f of 0.38. The pooled RL 232 extract, after chromatography resolved into a single yellow band with a R_f of 0.29. Bands were not detected in the pooled extracts of uninoculated tuber discs after chromatography.

Bioassay of the RL 232 and D.P extracts. The bioassays were conducted with the concentrated, pooled extracts from the tissue, oatmeal agar cultures, or oatmeal broth cultures. When applied to tuber discs the aqueous toxin suspension resulted in rapid browning (by 18 hours) over most of the discs surface. The compound extracted from artificial media or tissue for RL 232 and D.P. produced the phytotoxic compound

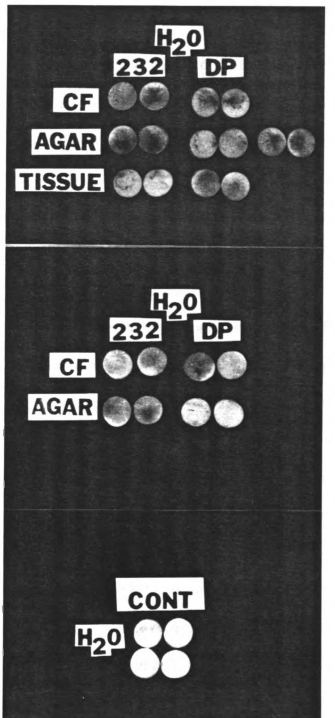

Fig. 4.1. Bioassay of thaxtomin from RL 232 and D.P. extracts on Russet Burbank tuber discs. Sources of toxin **A,** Culture filtrate from oatmeal broth (**CF**), oatmeal agar (**agar**), and inoculated tuber discs (**tissue**). **B,** Culture filtrate from oatmeal broth (**CF**), and oatmeal agar (**agar**). **C,** Tissue treated with water (H_2O).

thaxtomin (Fig. 4.1 A). RL 232 and D.P. produced thaxtomin in oatmeal broth and oatmeal agar (Fig. 4.1 B). The browning caused by the toxin extracted from liquid culture was more rapid and intense than toxin extracted from agar culture (Fig. 4.1 B). Browning was not detected on discs treated with water (Fig. 4.1 C). The toxin caused browning response on tuber discs approximately 18 hours after application.

DISCUSSION

This investigation demonstrated, that the deep pitting *Streptomyces* isolate D.P. produced a thaxtomin-like compound, and confirmed that RL 232 (*S. scabies*) and D.P. produced the phytotoxin under similar cultural conditions. The nonpathogenic *Streptomyces* 3S, produced no detectable thaxtomin-like compounds in culture, which suggested that synthesis of thaxtomin was correlated with pathogenicity. This also demonstrated that tuber discs could be used to simply confirm the phytotoxicity of thaxtomin without growing potato tubers.

Concentrating the toxin by pooling acetone extracts made it possible to approximate which forms of thaxtomin were being synthesized (Table 4.1). Based upon the R_f values for crude acetone extracts, it appears that RL 232 produced thaxtomin A (Table 4.1). D.P. possibly produced both thaxtomin A and B or an intermediate of the two forms of toxin (Table 4.1). The averaged R_f values from experiments 1 and 2, showed that RL 232 produced thaxtomin A R_f 0.27 (Tables 4.1 and 4.2). The reported R_fs for thaxtomin A and B are 0.27 and 0.41 respectively (Lawrence et al, 1990; and King et al, 1991). The predominance of form A over B of the toxin may explain the inability to detect the B form in any of the RL 232 extracts. All the concentrated extracts from RL 232 and D.P. exhibited phytotoxicity on tuber discs regardless of the

TABLE 4.1. The R_fs for the concentrated acetone extracts as determined by thin layer chromatography in chloroform/methanol 9:1 (v/v) solvent system

	Isolates[a]			
Source of toxin	RL 232[b]	D.P.[c]	3S[d]	Control[e]
Experiment 1[f]				
Tuber discs[g]	0.29	0.38	-[h]	-
Agar[i]	0.32	0.40	-	-
Culture filtrates[j]	0.29	0.32	-	-
Experiment 2[f]				
Agar	0.27	0.32	-	-
Culture filtrates	0.30	0.24	-	-

[a] The characteristics of these isolates are listed in chapter 2 tables 2.2 and 2.3 this dissertation.
[b] RL 232 *S. scabies* from R. Loria, causes common scab.
[c] D.P. deep pitting isolate from Michigan.
[d] 3S nonpathogen Wallace and Hammerschmidt.
[e] Extracts from uninoculated tuber discs or media.
[f] Two experiments performed. Induction of thaxtomin on tuber discs was examined once.
[g] Discs from Russet Burbank tubers.
[h] 3S was not used in the first experiment.
[i] Extracts from oatmeal agar (Eckwall et al, 1992).
[j] Culture filtrate from oatmeal broth (Eckwall et al, 1992).

Table 4.2. Averaged R_f values for extracts from experiments 1 and 2[a]

Source of toxin	Isolates[b] RL 232	D.P.
Tuber discs[c]	0.29	0.38
Agar	0.29	0.36
Culture filtrates	0.29	0.28

[a] The data from table 4.1 averaged together.
[b] Chromatography from the extracts of 3S and the controls did not resolve into discernable spots.
[c] The R_f values are from one experiment.

form of thaxtomin produced in culture. The extracts from RL 232 and D.P. were toxic to Russet Burbank tuber discs. In greenhouse tests, RL 232 and D.P. were incapable of infecting this cultivar. However, thaxtomin produced by RL 232 induced lesions on tissue cultured mini-tubers of Russet Burbank (Stein and Hammerschmidt, unpublished data).

Doering-Saad et al (1992) suggests that a plasmid may actually encode thaxtomin synthesis by streptomycetes and this characteristic may be common to several species of potato scab-causing *Streptomyces*. One implication of this hypothesis is a nonpathogenic streptomyces species may acquire the plasmid and the ability to produce thaxtomin, thus potentially become pathogenic. Such a phenomenon would further complicate proper identification of the streptomycete species which cause potato scab.

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CHAPTER V

THE EFFECTS OF INFECTION BY *STREPTOMYCES* SPECIES ON THE BIOSYNTHESIS OF CHLOROGENIC ACID AND LIGNIN IN TUBER DISCS OF A SCAB-RESISTANT AND SCAB-SUSCEPTIBLE CULTIVAR OF POTATO

INTRODUCTION

Little is known about the infection process or symptom development in potato scab disease. Potato scab-causing bacteria apparently infect through stomata and through stomata differentiating into lenticels (Jones, 1931; Lutman, 1941; Cooper et al,1954; and Adams, 1975). The host's responses following infection by streptomycetes are not known. Light microscopic observations reveal that cells several layers beneath the advancing bacterial filaments, respond by undergoing cell division and becoming suberized (Jones, 1931). The suberized cells function as a barrier and are thought to prevent the bacterium from invading the unaffected tissue beneath the suberized zone of cells (Jones, 1931). In the case of deep-pitted scab, the suberized barrier is either breached or develops cracks as the tuber expands. These openings may allow the hyphal filaments to continue through to the healthy tissue. Tuber expansion combined with repeated formation and breaching of suberized cell layers may ultimately lead to the formation of deep-pitted scab lesions (Jones, 1931). The formation of suberized barriers in response to infection may constitute all or part of the defense responses to streptomycetes.

The physiological basis for resistance of potato to *S. scabies* has not been determined. Resistance was thought to be a function of the physical and chemical properties inherent to the periderm of some cultivars of potato (Fellows, 1926; Johnson and Schaal, 1952; Cooper et al, 1954; Emilsson and Heiken, 1956; and Johnson and Schaal, 1957). Hooker and Page (1960) observed that *S. scabies* could not grow on living periderm, or grow on heat or chemically-killed periderm. This demonstrated that periderm was possibly a barrier to infection. Cooper et al (1954) observed that the

periderm of scab-resistant potato cultivars possess a greater number of living nucleated cells than scab-susceptible potato cultivars. However, Emilsson and Heiken (1956) observed that not all cultivars of potato fit the categories for resistant and susceptible types of periderm described by Cooper et al (1954).

It seems that periderm is not solely responsible for resistance of potato to infection by streptomycetes. Hooker and Page (1960) observed that *S. scabies* grew poorly on living tuber discs. When tuber discs were killed by autoclaving or chemical treatment prior to inoculation, the bacterium was able to grow. This suggested that freshly wounded potato tissue had active wound responses that may have inhibited the growth of *S. scabies*. Emilsson and Heiken (1956) indicated that the physical barrier provided by periderm in combination with active host responses possibly constitutes the sources of potato scab resistance.

Wounded and infected plant tissues have been shown to synthesize phenolic compounds, which are thought to play an integral role in resistance responses that include the synthesis of chlorogenic acid, lignin, or suberin (Hammerschmidt, 1985; and Nicholson and Hammerschmidt, 1992). Plant phenolics appear to possess antimicrobial activity (Schaal and Johnson, 1955; Friend et al, 1973; Ride, 1978; and Nicholson and Hammerschmidt, 1992). Wounded and infected potato tissue has been reported to accumulate chlorogenic acid (CGA) which inhibits growth of several fungal pathogens of potato and *S. scabies* (Johnson and Schaal, 1952; Schaal and Johnson, 1955; Patil et al, 1966; Friend et al, 1973; and Nicholson and Hammerschmidt, 1992). Johnson and Schaal (1952 and 1957) found that highly scab-resistant cultivars of potato possessed high levels of CGA in the peripheral layers of their tubers. CGA was hypothesized to either

lower the host tissue pH thus creating an unfavorable environment for growth of the pathogen, or contributed to the formation of suberized periderm (Johnson and Schaal, 1952).

Accumulation of phenolics as a result of infection in plant tissues not only functions to inhibit pathogen growth, but phenolics also polymerize to form lignin which functions as a barrier to penetration by plant pathogens (Ride, 1978; Vance et al, 1980). Ride (1978) proposed several functions for lignification. Lignin may reinforce cell walls, making them resistant to mechanical penetration. Lignification may make cell walls resistant to degradation by pathogen enzymes. A lignified barrier may prevent diffusion of pathogen enzymes or toxins into host tissue and also prevent nutrients and water from reaching the pathogen. Lignification of host tissue, may encase the pathogen and prevent its growth. Low molecular weight phenolic precursors of lignin and free radicals produced during polymerization may render pathogen membranes, enzymes, toxins, and elicitors non-functional.

Lignification constitutes a measurable resistance response in potato (Hammerschmidt 1984). Several studies suggest that suberin prevents the invasion of host tissues by *S. scabies* (Lutman, 1913 and 1941; and Jones, 1931). Suberin is a complex heteropolymer consisting of aliphatic and phenolic domains in association with waxes (Hammerschmidt, 1985). The structure of the phenolic domains of suberin and lignin are similar (Cottle and Kolattukudy, 1982). Wounded plant tissues accumulate precursors of lignin which eventually contribute to the formation of suberin, which in turn prevents infection and loss of moisture from tissues (Kolattukudy and Agrawal,

1974). Fellows (1926) suggested that hyphal filaments of *S. scabies* gain access to host tissues through the unsuberized lenticels.

The objective of this study was to assess the physiological responses of potato tuber discs to inoculation with pathogenic and nonpathogenic streptomycetes. I attempted to measure whether differential patterns of CGA and lignin accumulation could be detected in resistant and susceptible potato cultivars in response to inoculation with *Streptomyces*. Attempts were also made to determine whether chlorogenic acid functions as an inhibitor of *Streptomyces* growth.

MATERIALS AND METHODS

Streptomyces isolates. The isolates used were RL 232 and RL 95 (R. Loria) and the deep-pitting Michigan isolate D.P. RL 232 (*S. scabies*) caused common scab and D.P. (unclassified) caused deep-pitted scab on the cultivar Atlantic. RL 232, D.P., and RL 95 were not pathogenic on Russet Burbank. RL 95 was not pathogenic on Atlantic.

Plant materials. Tubers of the cultivars Russet Burbank and Atlantic were used in these studies and stored at 4 C. Russet Burbank exhibited resistance to several isolates of potato scab-causing bacteria in greenhouse pathogenicity screenings. The cultivar Atlantic was found to be susceptible to several isolates of potato scab-causing bacteria. Tubers were held at room temperature for at least 3 days prior to use. The tubers were washed, surface sterilized in 10 % commercial Chlorox solution, and flamed with 95 % ethanol before preparation of the tuber discs.

Production of inoculum. Spore of the isolates were frozen in 20 % glycerol/water solution (v/v) at -20 C (Hopwood et al, 1985). The spores were used to inoculate yeast

extract malt extract agar (YEME) (Shirling and Gottlieb, 1966). The cultures were incubated at 25 C for 14 days or until the cultures were sporulating and mature.

Two to three inoculating loops of spores were dislodged from the surface of a sporulating culture and suspended in approximately 5 ml of sterile distilled water, and 100 μl of the spore suspension was used to inoculate single tuber discs.

Preparation and inoculation of tuber discs. Tuber discs were prepared according to Hammerschmidt (1984). A cork borer was used to remove a single cylinder of tuber tissue from the internal medullary region and sliced into discs 2 cm in diameter and 0.5 cm thick. The discs were placed 1 % sodium chloride solution to prevent tissue browning and rinsed twice with sterile distilled water prior to being placed in petri dishes. Five to six tuber discs were placed in sterile petri dishes lined with moistened filter paper and each disc was inoculated with 100 μl of spore suspension. Tuber discs treated with sterile distilled water served as controls. The inoculated discs were incubated at 25 C in complete darkness and sampled daily for 3 days.

Sampling of tuber discs. Disc samples for each treatment were selected from petri dishes at random. The upper 1 to 2 mm of the inoculated disc surface was excised and placed into a vial containing 5 ml of absolute methanol. A single disc sample or replication consisted of two excised discs. The samples were stored in this fashion for 2 to 6 weeks.

Chlorogenic acid (CGA) assay. Prior to measuring the CGA content, the methanol extract for each sample was diluted to 10 ml with absolute methanol. The absorbance of the diluted methanol extract was measured at A_{328}. The concentration of CGA per 2 discs sample was calculated using the molar extinction coefficient (19,200 M^{-1}). Each

treatment was replicated a minimum of 3 times, and the experiment was repeated three times.

Lignin assay. After the methanol extract was removed, the tissue sample remaining in each vial was dried under vacuum. Lignin was extracted from the samples by the thioglycolic procedure (Hammerschmidt, 1984). The resultant ligninthioglycolic acid (LGTA) precipitate was solubilized in 5 ml of 0.5 N NaOH. High yields of the LGTA precipitate were diluted to a volume of 10 ml to 15 ml with 0.5 N NaOH. The absorbance of the solution was read at A_{280}. The concentration of lignin was estimated from a standard curve for lignin extracted from potato tuber periderm and expressed in terms of μg of lignin per area of disc. Each treatment was replicated a minimum of 3 times and the experiment was repeated three times.

Inhibitory activity of chlorogenic acid (CGA) on the growth of Streptomyces. The inhibitory effect of CGA on the growth of streptomycetes was tested by incorporating CGA into YEME agar. The CGA was dissolved in water, filtered sterilized, and poured into cool YEME to achieve the final concentrations of 0.005, 0.01, 0.1, 0.5, and 1 mg CGA/ml medium. The bacterial isolates were streaked into quadrants on a single dish of media such that 4 isolates were grown in the same dish. Each treatment was replicated a minimum of 5 times per isolate. The cultures were incubated 14 days at 25 C and growth was checked weekly.

RESULTS

Accumulation of CGA in response to inoculation. The amounts of extractable CGA increased in inoculated Russet Burbank and Atlantic tuber discs and uninoculated controls over the three day time course (Figs. 5.1 A-C and 5.2 A-C). The general patterns of

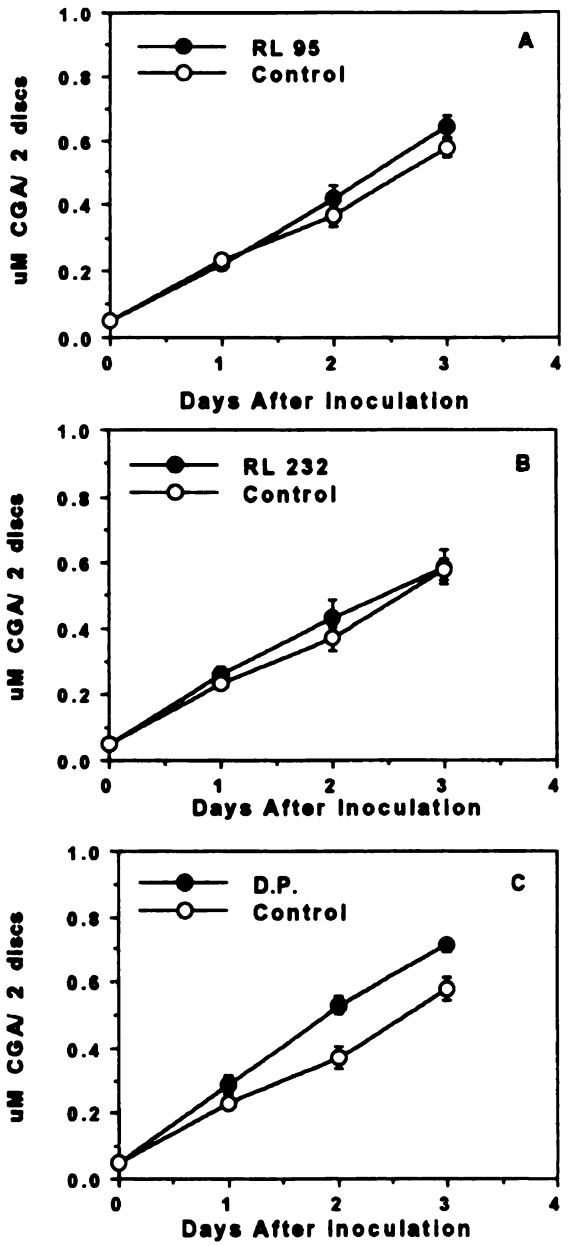

Fig. 5.1. Three day time course of chlorogenic acid (CGA) accumulation in tuber discs (cv. **Russet Burbank**), where the concentration of CGA was measured in micro moles (μM) per two tuber discs sample at 328 nm. Tuber discs were inoculated with **A**, RL 95 (nonpathogen); **B**, RL 232 (common scab pathogen); and **C**, D.P. (deep pitting pathogen).

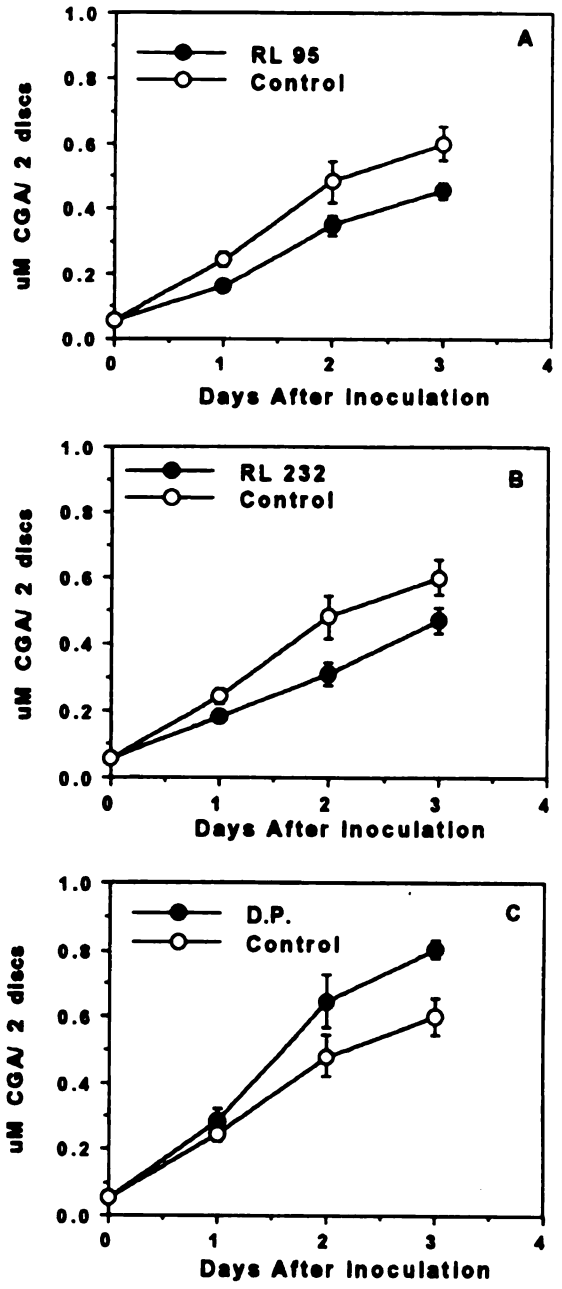

Fig. 5.2. Three day time course of chlorogenic acid (CGA) accumulation in tuber discs (**cv. Atlantic**), where the concentration of CGA was measured in micro moles (μM) per two tuber discs sample at 328 nm. Tuber discs were inoculated with **A,** RL 95 (nonpathogen); **B,** RL 232 (common scab pathogen); and **C,** D.P. (deep pitting pathogen).

CGA accumulation were not specific for either cultivar of potato. After 3 days, a two disc sample of Atlantic tissue on average accumulated 0.572 μ moles of extractable CGA and uninoculated controls 0.576 μ moles of extractable CGA. After 3 days, a two disc sample of Russet Burbank tissue on average accumulated 0.640 μ moles of extractable CGA and the uninoculated controls 0.578 μ moles of extractable CGA.

The amounts of CGA extracted from Russet Burbank tuber discs were similar for all isolates over the 3 day time course (Fig 5.1 A-C). The amounts of CGA extracted from Russet Burbank tuber discs inoculated with RL 95 and RL 232 were not significantly different from the uninoculated controls (Fig. 5.1 A and B). Higher amounts of CGA were extracted from Russet Burbank tuber discs inoculated with D.P., than uninoculated controls at day 2 and 3 of the time course (Fig. 5.1 C). The amounts of CGA extracted from Atlantic tuber discs inoculated with RL 232 and RL 95 were lower than the uninoculated controls over the 3 day time course (Fig. 5.2 A and B). The amounts of CGA extracted from Atlantic tuber discs inoculated with D.P. were generally higher than the uninoculated controls (Fig 5.2 A), and higher than the amounts of CGA extracted from RL 232 and RL 95 inoculated tuber discs at day 2 and 3 of the time course (Fig. 5.2 A-C).

Deposition of lignin in response to inoculation. Lignin deposition increased steadily for 3 days after inoculation of Russet Burbank and Atlantic tuber discs (Figs. 5.3 A-C and 5.4 A-C). The amounts of lignin deposited on Russet Burbank discs inoculated with RL 232, RL 95, and D.P. were similar to uninoculated controls during the first 2 days of the time course (Fig. 5.3 A-C). Uninoculated Russet Burbank controls produced nearly identical amounts of extractable lignin as tuber discs inoculated with D.P., and

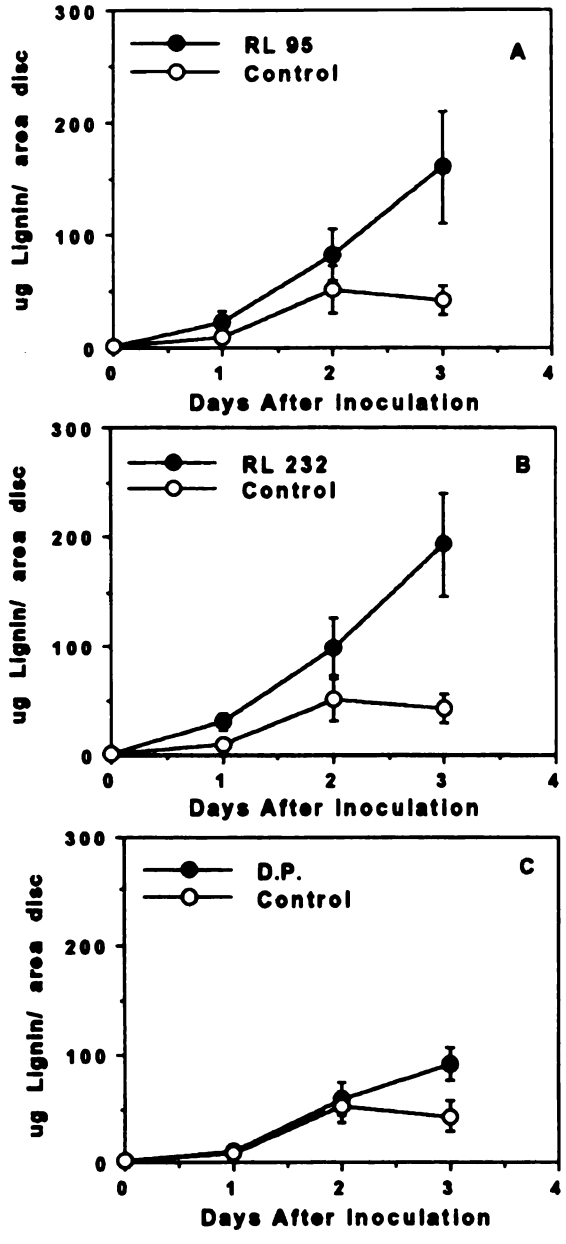

Fig. 5.3. Three day time course of lignin accumulation in tuber discs (**cv. Russet Burbank**), where the concentration of lignin was measured in micrograms (μg) per unit area discs at 280 nm. Tuber discs were inoculated with **A**, RL 95 (nonpathogen); **B**, RL 232 (common scab pathogen); and **C**, D.P. (deep pitting pathogen).

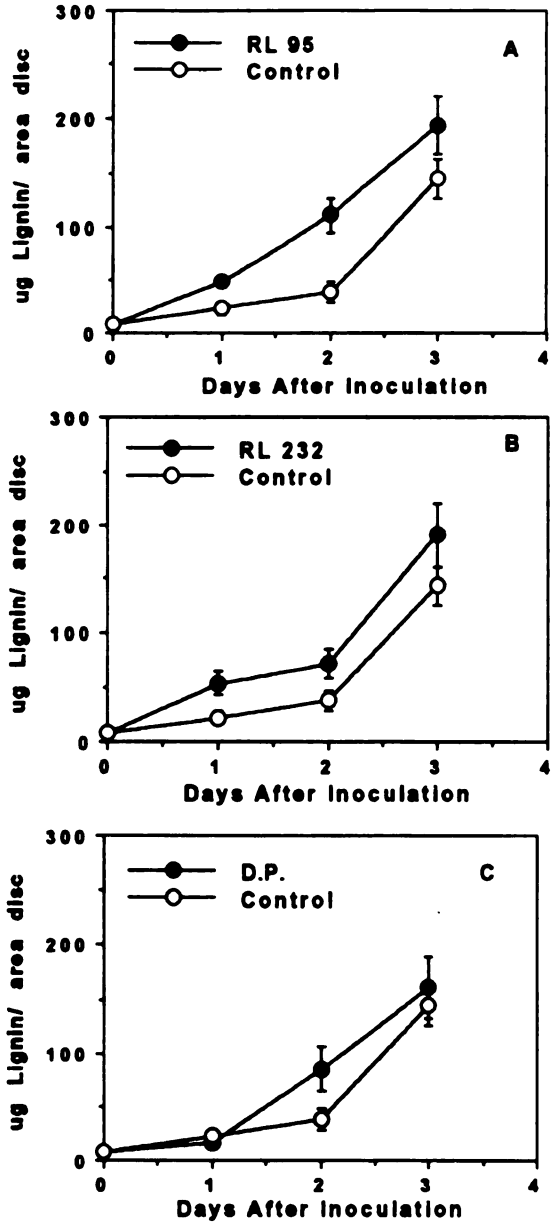

Fig. 5.4. Three day time course of lignin accumulation in tuber discs (**cv. Atlantic**), where the concentration of lignin was measured in micrograms (μg) per unit area discs at 280 nm. Tuber discs were inoculated with **A,** RL 95 (nonpathogen); **B,** RL 232 (common scab pathogen); and **C,** D.P. (deep pitting pathogen).

less lignin was extracted from the D.P. inoculated discs than Russet Burbank tissue inoculated with RL 232 and RL 95 after three days (Fig. 5.3 A-C).

Generally, less lignin was deposited on uninoculated Atlantic controls, than on Atlantic tuber discs inoculated with RL 232, RL 95, and D.P. (Fig. 5.4 A-C). After, 24 hours nearly identical amounts of lignin were extracted from uninoculated Atlantic tuber discs and discs inoculated with D.P. (Fig. 5.4 A-C). However, less lignin was extracted from Atlantic tuber discs inoculated with D.P. than from Atlantic tuber discs inoculated with RL 232 and RL 95 (Fig. 5.4 A-C).

Inhibitory effect of CGA on *Streptomyces* growth. The isolates RL 232, RL 95, and D.P. were able to grow normally in the presence of CGA at all concentrations (Table 5.1). The isolate RL 232 was slightly inhibited at 1 mg/ml CGA. The nonpathogenic isolates 3S (Wallace and Hammerschmidt) and *Streptomyces lividans* (donated by W. Champness) were able to grow in the presence of CGA, but at 1 mg CGA/ml the isolates produced scant sporeless mycelium (Table 5.1).

DISCUSSION

The physiological basis for resistance of potato to *S. scabies* and other potato scab-causing streptomycetes is not understood. The periderm appears to facilitate resistance by acting as a physical barrier to infection (Cooper et al, 1954; Emilsson and Heiken, 1956; and Hooker and Page, 1960). However, Hooker and Page (1960) illustrated that periderm was not entirely responsible for resistance to infection by showing that spores were unable to grow on living potato tuber discs. It was difficult to detect *Streptomyces* growth on Russet Burbank or Atlantic tuber discs in my experiments, even after 3 days. However, electron microscopy has revealed that RL

TABLE 5.1. The effect of chlorogenic acid on the growth of *Streptomyces*

Concentration[b] of CGA (mg/ml)	Isolates[a]				
	RL 232	D.P.	RL 95	3S	*S.l.*
0.005	4[c]	4	4	4	4
0.01	4	4	4	4	4
0.1	4	4	4	4	4
0.5	4	4	4	3	3
1.0	3	4	4	1	1

a Characteristics of the isolates shown in Tables 2.2 and 2.3 this dissertation.
b Yeast extract malt extract agar (YEME) supplemented with varying concentrations (mg/ml) of chlorogenic acid (CGA).
c Visual rating of growth based on comparison to growth of bacteria on unsupplemented (YEME), Best growth = 4 and no growth = 0.

232, D.P. and RL 95 spores germinate and grow over the surface of tubers (Stein and Hammerschmidt, *unpublished data*). It may be assumed that after spore germination mycelia spreads over the disc surface (this is true for nonpathogenic strains as well), but only pathogenic strains infect the tissue. By using very high concentrations of spores, I have induced visible growth of aerial mycelium on living tuber discs.

Hooker and Page's (1960) study suggests that resistance to potato scab is coupled to the natural wound healing responses. Phenolic compounds such as CGA and precursors of lignin and suberin accumulate in wounded and infected potato tissues (Vance et al, 1980; and Hammerschmidt, 1984 and 1985). Johnson and Schaal (1952) suggest that CGA may be a source of resistance to *S. scabies* infection. Russet Burbank a particularly scab-resistant cultivar was found to have much higher amounts of CGA in the peel than the scab-susceptible cultivar Triumph (Johnson and Schaal, 1957). Johnson and Schaal (1957) observed that CGA levels in potato periderm were highest when tubers undergo their most rapid growth, which oddly coincides with the period in which stomata differentiate into the unsuberized lenticels that appear to provide the natural portals for infections to occur.

My study showed the amounts of extractable CGA in freshly cut tuber discs of Atlantic and Russet Burbank were roughly similar (Figs. 5.1 and 5.2). Therefore the constitutive levels of CGA in the medullary tissues for both cultivars were similar. Also similar amounts of CGA were extracted from uninoculated controls of Russet Burbank and Atlantic at day 3 of the time course (Figs. 5.1 and 5.2). The amounts of CGA extracted from Russet Burbank tuber discs inoculated with RL 232 or RL 95 were nearly identical to the amounts of CGA extracted from the uninoculated controls over the course

of three days. However, D.P. inoculated discs accumulated significantly higher levels of extractable CGA than the controls two days after inoculation (Fig. 5.1 A-C). The amounts of CGA extracted from Atlantic tuber discs inoculated with RL 232 and RL 95 were generally lower than the uninoculated controls (Fig 5.2 A and B). However, the amounts of CGA extracted from D.P. inoculated Atlantic tuber discs were generally higher than the uninoculated controls at days 2 and 3 of the time course (Fig 5.2 C).

When incorporated in YEME medium, chlorogenic acid appeared to exert minimal inhibition on the growth of RL 232, RL 95, and D.P. (Table 5.1). CGA at the concentration of 1 mg/ml approximately corresponds to 2.8 μ mole CGA/ml of media. This concentration (1 mg/ml) approximated the average amount of CGA/ml extracted from a single inoculated tuber disc, which was roughly 3 μ mole CGA/ml/ disc of Russet Burbank or Atlantic. The isolates 3S and S.1. were inhibited by the highest concentration of CGA incorporated into YEME. Schaal and Johnson (1955) demonstrated that CGA and other phenolic compounds inhibited growth of pathogenic streptomycetes. When chlorogenic acid was applied to filter discs it exerted the greatest inhibitory effect at concentrations of 4 mg/disc and 6 mg/disc at pH 8.5 (Schaal and Johnson, 1955). However, my experimental design was such that the CGA was incorporated directly into the medium thus effectively lowering the concentration of CGA in any region. Therefore, the inhibitory effect demonstrated by Schaal and Johnson's study appears to result from the CGA concentration being much higher in the region immediately surrounding a filter disc. As the photographs accompanying their article illustrated, inhibition of growth occurred only a few millimeters away from the CGA saturated disc

(Schaal and Johnson, 1955). From my experiments it appears that CGA has minimal effect on inhibiting the growth of pathogenic *Streptomyces*.

Lignification of tissues is thought to be part of the host defense responses (Vance et al, 1980). Hammerschmidt (1984) demonstrated that the rate of lignification of potato tuber discs was higher in response to a nonpathogenic fungal species than to a pathogenic fungal species. The pattern of lignin deposition in response to streptomyces infection was similar to that reported by Hammerschmidt (1984). Tuber discs of Atlantic and Russet Burbank inoculated with RL 232 and RL 95 accumulated greater amounts of extractable lignin than discs inoculated with D.P., 24 after inoculation (Figs. 5.3 and 5.4). The response of tuber discs to D.P. was similar to that of potato toward the pathogen *Fusarium sambucinum*, within 1 day (Fig. 5.4) and 2 days (Fig. 5.3). It appears as though *F. sambucinum* was suppressing the lignification response or inhibiting the conversion of phenolics into lignin (Hammerschmidt, 1984). The findings of my study were consistent with those of Hammerschmidt (1984), because less lignin was extracted at days 1 and 2 from Russet Burbank discs (Fig. 5.3 B and C) and at day 1 from Atlantic discs (Fig. 5.4 B and C) inoculated with D.P., the most virulent isolate, than tuber discs inoculated with RL 232. This could possibly explain how deep-pitted lesions are produced by D.P. It may be possible that D.P. prevents the conversion of phenolics into lignin thus becomes able to penetrate much further into susceptible tissues than RL 232 and RL 95.

Inoculation of tuber discs with pathogenic streptomycetes may serve as a rapid and simple method to screen for potato scab resistance. The isolates RL 232 and D.P. grew on tuber discs of Atlantic and produced superficial damage 7 days after inoculation.

None of the tested isolates were able to infect Russet Burbank or cause observable damage even after 7 days of growth. Thus a screening program may be developed in which scab resistant potato cultivars are selected on the basis of their ability to rapidly synthesize high levels of lignin in response to inoculation with highly virulent streptomyces isolates. Also potential resistance to potato scab could be indicated by the inability of streptomyces to grow on potato tuber discs.

The contribution of chlorogenic acid and lignin synthesis in resistance of potato toward *S. scabies* infection was not clearly demonstrated by these experiments. However, chlorogenic acid and lignin apparently accumulated in tuber discs inoculated with *Streptomyces*. My experiments suggested that the patterns of CGA and lignin accumulation in response to *Streptomyces* were analogous to the types of CGA and lignin responses elicited by fungal pathogens of potato. Based on these observations, D.P. expressed a compatible-type interaction with the susceptible Atlantic cultivar, where the pathogen grew without eliciting a hypersensitive response from the host. While, RL 232 and RL 95 possibly induced incompatible responses and were recognized as pathogens and caused rapid synthesis of the previously mentioned resistance related compounds.

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CONCLUSIONS

From my research it has been possible to draw several conclusions about the physiology of potato scab disease. The isolate D.P., a strain of *Streptomyces* with the ability to produce deep-pitted scab on potato, bears many physiological similarities to the isolate RL 232 which causes common scab of potato. Both isolates degraded several of the same substrates, had similar tolerances for antibiotics, produced thaxtomin in culture, and the electrophoretic patterns of the soluble proteins extracted from whole cells were very similar although not identical. Inspite of their physiological similarities D.P. and RL 232 should not be considered to belong to a single species of *Streptomyces*. The facts that D.P. does not produce melanoid pigments in culture, bears its spores in flexuous chains, and causes deep-pitted scab constitutes significant variations from the typical physiology associated with *S. scabies*. The D.P. isolate also differed from the deep-pitting isolates of Archuleta and Easton (1981) which produced melanoid pigments in culture, and from the Faucher et al (1992) deep-pitting isolate which only used raffinose as a sole carbon source. Physiological characterization of the potato scab-causing streptomycetes has not established the phylogeny of this group. Therefore, the relatedness of D.P. to *S. scabies* and other potato scab-causing streptomycetes should be verified by DNA homology or finger printing by the polymerase chain reaction method.

The ability to degrade pectin has been cited as a possible means by which *S. scabies* and other pathogenic streptomycetes invade potato tissues or survive as saprophytes on plant debris (Lutman, 1923 and 1945). From my work, it appears that the ability to degrade pectic substrates was not restricted to the pathogenic streptomycetes, as several nonpathogens produced pectic enzymes in culture. Pectolytic enzymes were induced in culture by both pectin and sodium polypectate for pathogenic

and nonpathogenic streptomyces. The enzyme activities detected in culture filtrates suggested that endopectate or endopectin lyases were induced in culture, as well as an enzyme which could degrade pectin between pH 5 and 6, which could have been an exopectin lyase or an exopolygalacturonase. The literature however does not indicate that polygalacturonases (PG) degrade pectin (Rombouts and Pilnik, 1985). Other researchers have found that *Streptomyces* species produce endopectate lyases which exhibit activity on pectin substrate (Sato and Kaji, 1973).

Knosel (1970) could not establish a correlation between the ability to produce pectinase in culture and virulence of *S. scabies* toward potato. My work concurred with Knosel's, as I was unable to find any correlation between the ability to produce pectinases in culture and pathogenicity. This was illustrated by the fact that pectinase preparations from nonpathogenic streptomyces which were capable of degrading pure pectic substrates also caused maceration of potato tuber discs. Sato and Kaji (1973) demonstrated that culture filtrates from *S. fradiae* were able to cause extensive tissue maceration of potato and several other plants. However, *S. fradie* has not been reported to be pathogenic toward potato. Thus, I regard the ability of streptomycetes to degrade pectin as a factor that enhances the infection process, rather than constituting the primary factor in pathogenicity. To test this hypothesis it would be necessary to generate pectinase deficient mutants from a pathogenic streptomyces isolate and determine whether the loss of pectin degrading ability results in the loss of pathogenicity or lowered virulence. The method of mutagenesis used in my research did not produce true pectinase deficient mutants, the mutants apparently remained able to degraded pectic

substrates. Interestingly, a mutant was recovered that loss the ability to degrade several sugars and organic acids without loss of pathogenicity.

Preliminary research showed that nonpathogenic and pathogenic isolates of *Streptomyces*, when grown on a dilute slurry of potato tissue, produced extracellular pectolytic enzymes capable of degrading both pectin and sodium polypectate. I was able to detect pectolytic activity in the concentrated culture filtrates of RL 232, RL 95, D.P., and 3S grown on the potato slurry medium, using the cup plate assay (Dingle et al, 1953). The culture filtrates of these isolates exhibited activity on polygalacturonic acid at pH 7 and 9 but not at pH 5, and on pectin at pH 5, 7, 8.5, and 9. Therefore similar types of pectolytic enzymes were produced by both pathogenic and nonpathogenic streptomycetes grown on potato tissue, citrus pectin or sodium polypectate. This finding suggests that pathogenic *Streptomyces* species, synthesize pectolytic enzymes while invading living potato tissue, however it must noted that the potato slurry medium was autoclaved and the tissue was killed. It was amazing that pectolytic enzymes were induced at all, the cells of the potato tissue should have been disrupted by autoclaving making available several substrates more easily utilized than pectin for bacterial growth. The potato tissue would have supplied numerous sugars, starches, celluloses and other readily accessible substrates. Bearing this in mind, it would be very interesting to determine whether other cell wall degrading enzymes and thaxtomin were induced on potato slurry medium as well.

Synthesis of thaxtomin appears to be a characteristic unique to pathogenic *Streptomyces* species (King et al, 1992). Production of thaxtomin by D.P. was significant because this represented a pathogenic factor which could possibly be identified

as a common characteristic among all potato scab-causing streptomycetes. However, further research needs to done in this area to establish whether species other than *S. scabies, S. acidiscabies,* and the deep-pitting isolate D.P. synthesize the phytotoxin. It would be interesting to determine whether *Streptomyces* species that infect beets, carrots, and other root crops produce thaxtomin as well.

The deep-pitting isolate D.P., elicited a different pattern of host defense responses from potato tuber discs than RL 232 and RL 95. My findings supports those of Jones (1931) which suggested that deep-pitting streptomycetes affect the lignification response in potato tissue in a manner that differs from the common scab-producing streptomyces. Lowered lignin accumulation in potato tissues infected with deep-pitting streptomyces, may possibly explain how the D.P. isolate is able to penetrate further into potato periderm than common scab streptomyces, thus producing deep lesions. High level lignin deposition in response to infection by common scab-producing streptomyces, may explain why these streptomyces are confined to the outer layers of the periderm, thus producing in superficial lesions. However, further work must be done to establish how early in the infection process the potato tissue recognizes the bacterium and responds to the infection. Dr. Barry Stein has observed streptomyces spores germinating as early as 6 hours following inoculation of *in vitro* grown potato tubers and producing an extensive hyphal network on the tuber surface within 48 hours (Stein et al, 1994). It may be reasonable then to assume that the pathogenic interactions between streptomycetes and potatoes are established within 24 hours.

Ultimately the significance of my research is found in that it examines both the infection process and host responses in potato scab disease from a physiological

perspective. In this regard, we can see that potato scab-causing streptomycetes synthesize cell wall degrading enzymes and phytotoxin, which may facilitate the infection process. Furthermore, potato scab-causing streptomycetes may in fact be necrotrophs or facultative parasites. Their ability to synthesize cell wall degrading enzymes and phytotoxin appear to be necessary for them to produce the necrotic tissue required to establish an infection. This hypothesis appears to be consistent with two observations. First, the streptomycetes are hardly ever observed to growing in living tissue, and second streptomycetes seem to cause potato scab in an opportunistic fashion, which appears to be dependent on the occurrence of several environmental factors. To ultimately develop effective control measures for potato scab disease the physiological impact of the interactions between the environment, potato, and the species of streptomyces must be understood.

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