Systemic resistance induction by Bacillus mycoides isolate Bac J : the mode of action on Beta vulgaris (sugar beet)
by Rebecca Lynn Bargabus

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Plant Sciences
Montana State University
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Abstract:
Bacillus mycoides isolate Bac J, a non-pathogenic, phyllosphere-inhabiting biological control agent, reduced Cercospora leaf spot of sugar beet by 60-80% in glasshouse experiments, even when spatially separated from the causal agent, Cercospora beticola Sacc. Disease control was attributed to the ability of the bacterium to induce systemic resistance in the host, which was demonstrated through classical induced resistance challenge assays. Additionally, in glasshouse and field experiments three pathogenesis-related proteins, chitinase, β-glucanase and peroxidase, that are accepted molecular markers of systemic induced resistance, were increased by nearly 2-fold in distal, untreated sugar beet leaves following treatment with Bacillus mycoides isolate Bac J and acibenzolar-S-methyl, a chemical inducer of systemic resistance. The increased activity in all cases was a result of the production of unique isoforms of the enzymes not found in the water treated control. The Bacillus mycoides isolate Bac J-induced systemic defense response was preceded by a biphasic oxidative burst. The hydrogen peroxide production pattern was similar in timing, but not intensity to that elicited by avirulent bacterial pathogens of sugar beet, Erwinia carotovora pv. betavasculorum isolates 1 and 6. Although normally coupled with programmed cell death, the oxidative burst elicited by Bacillus mycoides isolate Bac J was independent of the hypersensitive response. Observations made during the oxidative burst experiments provided keys for understanding the signaling in Bacillus mycoides isolate Bac J-sugar beet interactions, including signal delivery not being reliant upon stomatal conductance and sugar beet receptor location being cytosolic or plasma membrane bound. Additionally, the biochemical and oxidative changes observed in sugar beet following Bacillus mycoides isolate Bac J treatment were consistent with changes seen in other Bacilli-sugar beet interactions in which systemic resistance was induced. These chemical consistencies provided a framework with which to establish a host response-based high throughput screen for the systematic identification of novel, putative Bacilli biological control agents, the first such method of its kind.

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APPROVAL

of a dissertation submitted by

Rebecca Lynn Bargabus

This dissertation has been read by each member of the dissertation committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

Bacillus mycoides isolate Bac J, a non-pathogenic, phyllosphere-inhabiting biological control agent, reduced Cercospora leaf spot of sugar beet by 60-80% in glasshouse experiments, even when spatially separated from the causal agent, *Cercospora beticola* Sacc. Disease control was attributed to the ability of the bacterium to induce systemic resistance in the host, which was demonstrated through classical induced resistance challenge assays. Additionally, in glasshouse and field experiments three pathogenesis-related proteins, chitinase, β-glucanase and peroxidase, that are accepted molecular markers of systemic induced resistance, were increased by nearly 2-fold in distal, untreated sugar beet leaves following treatment with *Bacillus mycoides* isolate Bac J and acibenzolar-S-methyl, a chemical inducer of systemic resistance. The increased activity in all cases was a result of the production of unique isoforms of the enzymes not found in the water treated control. The *Bacillus mycoides* isolate Bac J-induced systemic defense response was preceded by a biphasic oxidative burst. The hydrogen peroxide production pattern was similar in timing, but not intensity to that elicited by avirulent bacterial pathogens of sugar beet, *Erwinia carotovora* pv. *betavasculorum* isolates 1 and 6. Although normally coupled with programmed cell death, the oxidative burst elicited by *Bacillus mycoides* isolate Bac J was independent of the hypersensitive response. Observations made during the oxidative burst experiments provided keys for understanding the signaling in *Bacillus mycoides* isolate Bac J-sugar beet interactions, including signal delivery not being reliant upon stomatal conductance and sugar beet receptor location being cytosolic or plasma membrane bound. Additionally, the biochemical and oxidative changes observed in sugar beet following *Bacillus mycoides* isolate Bac J treatment were consistent with changes seen in other Bacilli-sugar beet interactions in which systemic resistance was induced. These chemical consistencies provided a framework with which to establish a host response-based high throughput screen for the systematic identification of novel, putative Bacilli biological control agents, the first such method of its kind.

CHAPTER 1

INTRODUCTION

Sugar Beet, A Montana Crop

History

Although historical records of sugar use date back to 750 B.C., the "sweet spice" was primarily a luxury reserved for the wealthy (Austin, 1928). Sugar was expensive since it was purified from a single source, the sugar cane. However, sugar has now gone from luxury to necessity, due in large part to the discovery of the sugar beet. Achard, a German chemist, invented the first viable beet sugar extraction method in 1799 and the first sugar beet processing plant opened shortly thereafter in 1801 (Austin, 1928). These and several of the other early attempts at processing failed shortly after opening, due to the low sugar content of the cultivated beets. However, following 10 years of research and breeding, the French increased the sugar content of the beet taproot to nearly 15%. This was a tremendous improvement over the original 5% and nearly double that found in sugar cane (Jackson and McRae, 2001). Soon after, Napoleon demanded 90,000 acres of France's land to be devoted to the production of sugar beets. He also appropriated funds for the development of two beet-processing plants. Within the two years that followed, 334 small factories had popped up all over France. Prior to World War I, 1,200 large factories were speckled across the whole of Europe, producing nearly half the world's supply (Palmer, 1918). The sugar beet had made its mark.

Classification and Description

Sugar beet (*Beta vulgaris* L.) belongs to family *Chenopodiaceae*. The origin for all the genera *Beta* was in the Middle East. Speciation of the wild beets occurred when those that grew throughout the Mediterranean became isolated in mountainous regions of Turkey, Iran, Russia and the Canary Islands (Cooke and Scott, 1993). The sugar beet of today (subspecies *vulgaris*) was evolved through the subspecies *provulgaris*, a descendent of the ancestral *maritime* variety. *Beta vulgaris* ssp. *maritime* was a desirable breeding candidate due to its high level of resistance to *Cercospora beticola*, the causal agent of Cercospora leaf spot and most destructive fungal pathogen of sugar beet (Winner, 1993). There are also many weedy plants in the *Beta* genus including pigweed, winged pigweed, lambsquarter, mallow, wild buckwheat, and common unicorn flower (Viard et al, 2002). Sugar beet is a poor competitor with all of these. Additionally, these weeds often serve as alternate hosts to many sugar beet pathogens, therefore special care must be taken to rid beet fields of these weeds (Schweizer and May, 1993).

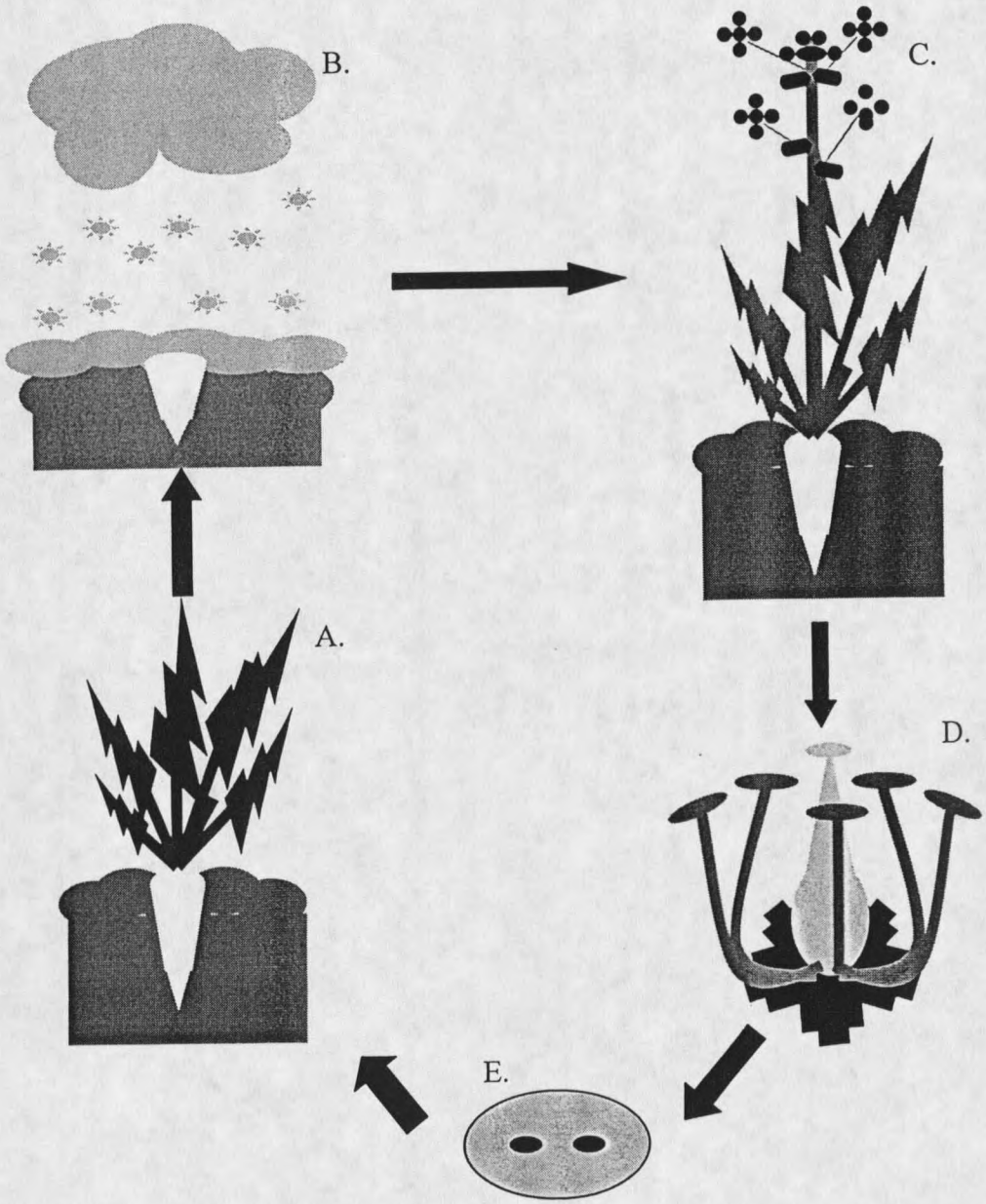
The beet family is broken down into four agricultural groups. The first is the leaf beets, also known as foliage beets. The leaves of this group are harvested for human consumption, such as spinach or swiss chard. The second group contains garden beets, including the red and table beets. In this case the root material is harvested for human consumption. Mangolds are part of the third group known as fodder beets. These beets are exclusively used for stock feed. Sugar beets comprise the last group. The roots, in

this case, contain multiple types of sugar to be extracted. The leftover leaves and pulp are then used for feed (Winner, 1993).

Sugar beet is a biennial, herbaceous, dicotyledonous crop that completes its life cycle over a two-year period (Smith, 1987) (Figure 1-1). However, as a crop it is treated like an annual and the taproot is harvested the first year (Viard et al, 2002). Vernalization signals for production of perfect, incomplete flowers. Since flower production is the result of exposure to the cold, it occasionally occurs during the first year of planting, known as bolting (Hohmann et al, 2003). The green flowers, perched along a 1.2-1.5 meter tall stalk contain five sepals, but lack petals (Smith, 1987). They contain a centrally located compound pistil (3 styles) encompassed by five stamens (Forster et al, 1997). The tricarpelate pistil contains a single receptacle that once fertilized produces a single fruit containing one round to kidney-shaped seed (Artschwager, 1926). The entire fruit is planted as seed for the next growing season.

Sugar beets are generally self-incompatible (Shaw, 1914) and are fertilized by out-crossing. Although primarily wind pollinated (Stewart, 1946), some insect pollination can also occur (Poole, 1937). Thrips (Shaw, 1914) and syrphids (Treherne, 1923) are considered the main insect pollinators of beet flowers. Bees are also known to pollinate sugar beets (Sharma and Sharma, 1968), however some reports state this is only the case when other nectar sources are not available (Mikitenko, 1969; Arcimowitsch, 1949).

Figure 1-1. The life cycle of the biennial crop, *Beta vulgaris* L. (sugar beet). The first year, a fleshy tap root grows that may be harvested for sugar refining (A). Following vernalization (B), the sugar beet will bolt (C). The flowers produced (D) are perfect, but incomplete, with a main tricarpelate pistil surrounded by 5 stamens, 5 sepals and no petals. Once fertilized, each flower produces one fruit containing one or more seeds (E). The entire fruit is planted for crop production the next year.



Economic Importance

Minnesota and North Dakota lead the production of sugar beets in the United States. Idaho, California, Michigan, Nebraska, Wyoming, Montana, Colorado and Texas are the other major producers in the U.S. (Cattanach et al, 1991). Montana beet production is primarily limited to irrigated valleys even though the crop is extremely drought tolerant (Dunham, 1993) and the range of production is approximately 60,000 acres. Although sugar beet production has been on the decline since the 1970's, the amount of sugar refined each year in the United States greatly exceeds demand for consumption, which is around 12.0 million short tons of sugar per year (Angelo and Barry, 1984).

Today sugar beet is cultivated for four main purposes. It is used to process crystalline sugar (Schneider et al, 2002), extract molasses (Ulber et al, 2000), process pulp feed (Scipioni and Martelli, 2001) and produce seed (Paspisil et al, 2000). Eighty percent of the sugar found in beets is sucrose under optimal conditions, which is sold for human consumption. The other twenty percent is molasses (saccharose) and other sugar impurities that cannot be affordably recrystalized into sucrose (Bichsel, 1987). Since sucrose is worth more per kilogram than molasses, studies have been conducted to determine fertilization regimes and harvesting methods to increase sugar yield and purity. One example includes the processing of the crown with the root, which has been shown to increase sucrose yield by six percent (Jaggard et al, 1999). However, molasses is used as a constituent of cattle feed (Scipioni and Martelli, 2001), a carbon source for yeast

culture (Atiyeh and Duvnjak, 2002), and in the production of chemicals and pharmaceuticals (Faurie and Fries, 1999). Once the sucrose and molasses have been removed, the beet pulp that remains is sold in a wet or dried, pelleted form as cattle and sheep feed (Fiems et al, 2002). Sugar beet seed production in the United States is done by the direct seed method, which integrates receptive females with cytoplasmically sterile males in alternating rows of the field. The seed, produced mostly in Oregon, is sold worldwide (Hampton et al, 1998).

Genetics

Sugar beets are naturally diploid with 18 chromosomes (Elliot and Weston, 1993). Autotetraploids were first produced in the 1930s. Researchers proposed the increase in genetic material would increase sugar yield (Zeven, 1979). Since then triploid beets have been developed by crossing the tetraploids with diploids progenitors. However, the triploids were unstable and yielded both diploids and tetraploids upon seeding. Therefore, diploids are the most widely used for crop seed today (Bosemark, 1993).

Under normal conditions, multigerm seed is produced once the flower is fertilized (Smith, 1987). The fruit, containing up to eight seeds, is the result of the fusion of multiple flowers growing at the same node. Once the multigerm seeds were sown, multiple seedlings would sprout at each location negatively affecting the health of the stand. However, in 1950, a monogerm seed was successfully developed by selecting for the production of a single flower at each node, eliminating the need for hand removal of excess seedlings (Bornscheuer et al, 1993).

The Cercospora Leaf Spot Pathogen, *Cercospora beticola*

Classification and Description

Cercospora beticola Sacc. (Sacchardo) is the fungal pathogen that causes Cercospora leaf spot of *Beta vulgaris* L. (sugar beet), the most destructive of all sugar beet pathogens worldwide (Smith and Ruppel, 1974). This fungus is part of the taxonomic phylum *Deuteromycota*, class *Deuteromycetes*, order *Hyphomycetales* (formerly *Moniliales*), and family *Dematiaceae*. Unlike other members of the *Cercospora* genus, such as the pathogen causing Sigatoka disease of bananas, no sexual (teleomorphic) stage has been defined for *C. beticola*. The other *Cercospora* sp. have been assigned the teleomorphic classification of genus *Mycosphaerella* and class *Loculoascomycetes* (Agrios, 1997).

Cercospora beticola produces acicular, septate (3-14 septa/spore) conidia that are straight to slightly curved. The long, slender spores are tapered on one end and blunt-ended at the point of conidiophore attachment. The unbranched conidiophores are dark brown in color, in contrast to the hyaline conidia. Under appropriately humid conditions (>90% relative humidity), stromatic cells enteroblastically give rise to conidiophores (Pons et al, 1985). Holoblastic conidial ontogeny follows (Minter et al, 1982), in which each conidium is delimited by a transverse, uniperforate septum produced at the conidiogeneous locus (Burnett, 1976). The succession of conidial delimitation is schizolytic, meaning that the cell wall separating the conidium from the conidiogeneous cell breaks along with the delimiting septum (Hughes, 1971).

Symptoms and Signs

The Cercospora leaf spot infection manifests into lesions primarily covering leaves, but occasionally the petiole as well (Ruppel, 1986). The leaf spots are rather small, usually less than 5 millimeters in diameter. The shape of the spot is circular when on the leaf surface and angular when on the petiole. Localized conidia production gives the center of the lesion a fuzzy, ashen gray, papery appearance. The black speckles intermixed with the conidia are the stromata (pseudo-sclerotia) of the fungus. Although similar in appearance to bacterial leaf spots, the two aforementioned fungal signs help to visually distinguish between the two plant symptoms. The lesion is surrounded with a reddish-purple border, the result of the host anthocyanin production, a symptom lacking with bacterial infection (Ruppel, 1986). Following a severe infection, the leaves of the beet collapse following chlorosis and necrosis (Steinkamp et al, 1979), however the petioles remain attached to the crown (Duffus and Ruppel, 1993).

Disease and Life Cycle

The inoculum for Cercospora leaf spot disease arises primarily from infected sugar beet residue harboring both spores and stromata. The latter serve as overwintering structures. Spores germinate on leaf surfaces of newly emergent beets, with appropriate temperatures and humidity (Ruppel, 1986). With or without the formation of appressoria, the germinated spore can penetrate into leaves through the stomata (Pool and McKay, 1916) (Figure 1-2). Once inside the sugar beet, the fungus grows intercellularly. *C. beticola* is a hemibiotroph and produces two toxins, beticolin and cercosporin

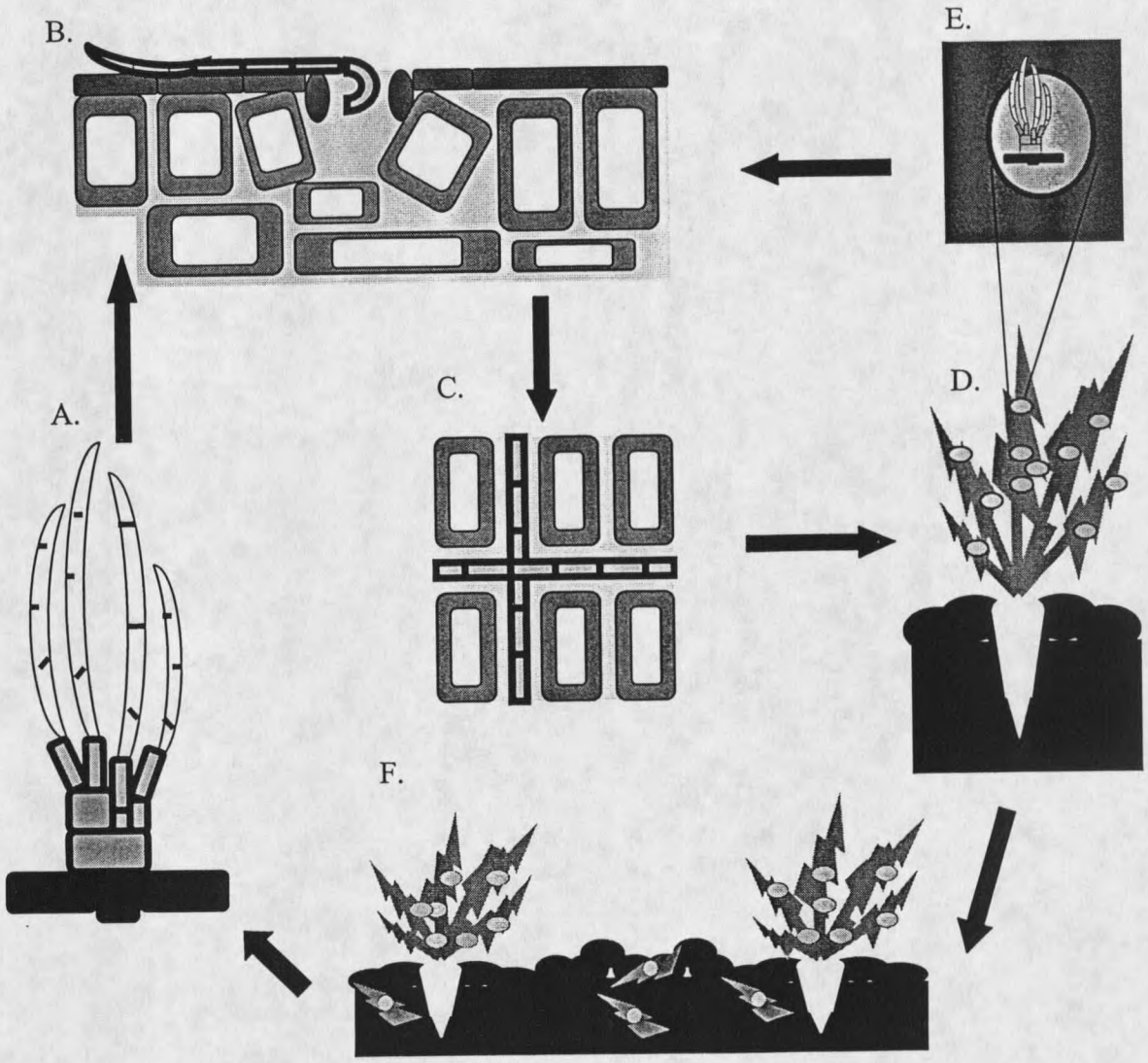


Figure 1-2. *Cercospora beticola* life cycle on sugar beet. Hyaline spores produced on brown conidiophores (A) are spread by wind and rain. Once the spores have landed on host leaf tissue, they germinate and penetrate into the leaf through the stomata (B) with or without the formation of appresoria. The mycelia grow intercellularly once inside the plant (C). New conidiophores and conidia are produced in each lesion (D and E) that serves as inoculum for secondary infection cycles. Beet residue serves as an overwintering site (F) for *Cercospora beticola* pseudo-sclerotia and spores that are able to reinfect the crop the following year.

Cercosporin. The toxins cause electrolytes and other nutrients, that serve as a food source for the fungus, to leak from the plant cells (Sollod et al, 1992). The disease cycle is microcyclic and, therefore, many reinfections take place during the growing season (Chupp, 1953).

Fungal Toxins

Cercospora beticola produces two toxins: cercosporin and beticolin. Cercosporin, a lipid-soluble perylenequinone, is produced via the polyketide pathway (Okubo et al, 1975). This photoreactive compound produces singlet oxygen when exposed to light (optimally between 490-510 nm) in a two-step process. First, cercosporin is excited into a triplet state following exposure to light (Daub, 1982a). Then, the light-activated cercosporin reacts with dioxygen to form singlet oxygen (Bilski et al, 2002). Scavengers for this active oxygen species are scarce in biological systems (Bellus, 1979), and singlet oxygen reacts readily with cellular components, including fatty acids and other lipids (Knox and Dodge, 1985). The lipid peroxidation forms holes in the membrane causing electrolyte leakage and cell death (Daub, 1982b). Cercosporin is also effective against other fungi (Sollod et al, 1992), which may help it with niche occupation by eliminating potential competitors. However, some fungi such as *Botrytis cinerea* are able to actively efflux the toxin through the use of major facilitator superfamily (MFS) and ATP-binding cassette transporters and are therefore immune to the toxin (Hayashi et al, 2002). On the other hand, *Cercospora* sp. utilize MFS transporters for secretion of the endogenously produced toxins. This exportation of the toxin creates a self-protection mechanism (Hayashi et al, 2002). Additionally,

Cercospora sp. are able to avoid the effects of cercosporin by keeping the toxin in a reduced, inactive state (Sollod et al, 1992; Daub et al, 2000). Although some information is known about the transcription factors involved in regulation of the transcription of the enzymes responsible for the reduction (Chung et al, 2003), the identity of the enzymes still remains unknown (Chung et al, 2002).

Beticolins have demonstrated a variety of functions, both *in planta* and *in vitro*. *In planta*, beticolins are able to chelate Mg^{++} (Mikes et al, 1994). Following the chelation, a dimeric complex is formed with an increased affinity for membranes. The dimeric complexes then aggregate into weakly selective cationic channels that are able to increase membrane conductance (Goudet et al, 1998). These channels cause a massive loss of solutes (Macri and Vianello, 1979) that leads to membrane depolarization (Gapillout et al, 1996) and an inhibition of ATP-dependent H^+ transport (Macri et al, 1983). These functions are both antimicrobial and deleterious to plant cell survival, once again eliminating competition and sensitizing the host. *In vitro* analyses also suggested an active oxygen-scavenging role for beticolins (Rusterucci et al, 1996), however this mode of action has never been demonstrated *in vivo*.

Diagnosis and Treatment

Diagnosis of Cercospora leaf spot in the field can be achieved by observing the symptoms that develop on the leaves. The possibility for disease can also be predicted using software analysis based on estimated inoculum levels and weather conditions (Windels et al, 1998). Lastly, molecular methods for detection have been developed based on a unique 1.1 kB band amplified from the ITS region of the fungal rRNA gene,

which distinguishes *C. beticola* from other prevalent fungal pathogens of sugar beet (Weiland and Sundsbak, 2000).

Several cultural methods have been effective for controlling *C. beticola* on sugar beet. These include using clean seed. Additionally, using seed that is over 3 years old guarantees that the fungus will not have survived since *C. beticola* can only survive in seed for a defined, limited amount of time. Furthermore, crop rotation helps to reduce inoculum levels in the field, assuming all alternative hosts are removed from the perimeter as well. The weed hosts to be removed include winged pigweed, lambsquarter, pigweed, mallow, wild buckwheat and common unicorn flower (Viard et al, 2002).

Resistant varieties of sugar beet that are available also aid in reducing Cercospora leaf spot disease incidence. Quantitative trait loci have been described for resistance to *C. beticola* (Setiawan et al, 2000). It was generally accepted that there were four or five major genes involved in sugar beet recognition of the pathogen (Smith and Gaskill, 1970). However, single gene resistance has also been described (Lewellen and Whitney, 1976; Whitney and Lewellen, 1976; Hunger et al, 2003). Using resistant varieties in the presence of *C. beticola* inoculum not only increases the sugar yield by 45% (Setiawan et al, 2000), but also yields higher quality, more pure sugar as well (Koch, 1972). However, there are some disadvantages that occur with the use of resistant varieties. The overall yield and purity of the sugar beet sugar is greater from susceptible varieties when grown in the absence of *C. beticola* (Smith and Campbell, 1996). Therefore, if fields are kept clean and effective predictions are made with computer software for preventative

fungicide sprays, the farmer will see a financial benefit with planting susceptible instead of resistant plants.

There are several fungicidal treatments for Cercospora leaf spot of sugar beet, the most popular of which are the strobilurins, benzimidazoles and triazoles, which include trifloxy strobin, thiophanate methyl (Weiland and Halloin, 2001) and tetraconazole and triphenyltin hydroxide (Griffiths and Howlett, 2002) respectively. These treatments must be applied four to five times during the growing season at an estimated cost of ten to eighteen dollars per acre. The treatments are only effective if applied as a preventative measure, as they are no longer effective when applied 48-72 hours after infection. Another problem with fungicide treatments for Cercospora leaf spot is the growing tolerance to triphenyltin hydroxide (Windels et al, 1998) and the resistance of the fungus to benzimidazoles (Brantner et al, 1998). This demonstrated the need for alternative means of control, including biological means of controlling disease.

Bacillus mycoides isolate Bac J, Biological Control Agent

Description and Classification

Bacillus mycoides isolate Bac J (BmJ) is a gram-positive, saprophytic bacterium. Being a member of the family *Bacilliaceae* designates it is rod-shaped and grows in the presence of air. All 65 members of the *Bacillus* genus are able to form dormant structures known as endospores that protect the genetic material under unfavorable environmental conditions (Priest, 1993). *Bacillus mycoides* has at times been referred to as a subspecies of *Bacillus cereus*. However, in light of morphological and genetic

differences, it has been afforded species status (Nakamura and Jackson, 1995), although it remains closely related to *B. cereus* group members *B. anthracis* and *B. thuringiensis* (von Wintzingerode et al, 1997). This relationship between *B. mycoides* and *B. cereus* is demonstrated by the high degree of similarity of 16S rDNA sequence (Ash et al, 1991), RFLP of rRNA genes (Priest et al, 1994) and of rDNA ITS regions (Bourque et al, 1995). However, considerable molecular variation exists within the plasmids, especially those encoding for toxins (Henderson et al, 1994). On a phenotypic level *Bacillus mycoides* is quite different from the other Bacilli. It lacks motility and has a rhizoidal colony phenotype in plate cultures. *B. mycoides* also grows in long chains of bacterium, due to the lack of complete septation following transverse fission (Di Franco et al, 2002).

Ecological Role

Bacillus mycoides is a saprophyte (Sorheim et al, 1989). It is considered a ubiquitous organism and has been found in the rhizosphere and phyllosphere of plants (Daffonchio et al, 1999). *Bacillus* spp. are a popular option for biological control agents (Mathre et al, 1999; Sutton, 1995; Sutton and Peng, 1993) and growth promoting rhizobacteria (Peterson et al, 1995; Murphy et al, 2000; Zhang et al, 2001). Occasionally they are preyed upon by protozoa (Casida Jr, 1989).

Biological Control

Biological Control: An Overview

Biological control can be defined as the application of beneficial organisms that are natural enemies to perceived pests as a means of controlling their population size and reducing their overall effects. For various reasons, biological control agent use in commercial settings has been fairly limited, however with an increased environmental awareness and a dramatic increase in pathogen resistance to agrochemicals, the use of biological control agents has become more widespread in recent years (Wedge and Nagle, 2000). Some major issues still exist that prevent the overall acceptance of biological control as a viable alternative to chemical treatments. Although extensive lab work has shown biological control agents are effective against certain plant pathogens, the effects they have on the complex array of non-pathogenic microorganisms in the soil environment are not well understood (Dunne et al, 2000). Additionally, the level of disease control achieved using biological control agents is much more variable than that achieved using chemical agents. This in part is tied to initial lack of population establishment (Smith et al, 1997), therefore improving biological control agent efficacy (Guestsky et al, 2001) and colonization (Walker et al, 2002) under greenhouse and field conditions are merited investigations. Lastly, biological control agents require special preparation and application, making the transition from researcher to farmer one of great concern. Packaging depends most often on the type of agent being used: fungi as spores (de Vriji et al, 2001) or mycelia on dead seed (Zriba et al, 1999), gram-positive bacteria

as dormant endospores (Nicholson, 2002), and gram-negative bacteria on "peat carriers" (Georgakopoulos et al, 2002). The latter keeps *Pseudomonas corrugata* and *Pseudomonas fluorescens* stable at ambient temperatures for up to 24 months (Georgakopoulos et al, 2002).

Modes of Action

The mode of action of biological control agents is diverse and can be the result of parasitism or predation (de Vriji et al, 2001), antibiosis (Dunne et al, 2000; Krechel et al, 2002), niche exclusion (Bacon et al, 2001), or induction of plant defense responses (Murphy et al, 2000; Raupach and Kloepper, 2000). All these modes of action have been effective under both field and glasshouse conditions.

Parasitism or predation of one organism by another is an employable method of biological control. The introduction of novel insects into an environment has been effective at controlling insect pests (Liu and Jiang, 2003) and invasive weeds (Barratt and Johnstone, 2001). Additionally, *Pythium oligandrum* can parasitize the oomycotal pathogen *Phytophthora parasitica* (Picard et al, 2000). One major concern with this method, and biological control in general, is the undetermined effect the introduced organisms may have on non-target native species (Henneman and Memmott, 2001).

Antibiosis is characterized by the production of hydrolytic enzymes or antibiotics by the biological control agents that are effective against the target pathogen. This mode of action can be very limited (i.e. to one particular race of a plant pathogen) or fairly general (i.e. several major plant fungal pathogens), depending primarily on the compound(s) being produce (Mohapatra et al, 2002). Improvements in the efficacy of

hydrolytic enzyme-producing microorganisms can be achieved through genetic modification and some have been modified to overproduce enzymes such as proteases (Dunne et al, 2000) and glucanases (Rey et al, 2001).

Niche exclusion can be explained as the competition for physical location or nutrient acquisition. This method of biological control is based on the premise that if two species overlap significantly with regards to spatial occupation or nutritional demands, one of the two will eventually be ousted from the niche. An example of this method put into practice is the preemptive application of saprophytic organisms that compete for similar carbon sources with target pathogens thereby reducing disease (Ji and Wilson, 2002). Recently it was proposed that this type of competition promotes species coexistence as opposed to one or the other dominating the environment. Since both organisms have alternative food sources to take advantage of under competitive situations (Vandermeer et al, 2002). This further exemplifies the fact that biological control serves primarily to keep pathogens in check, as opposed to eliminating them from the system all together, like their chemical counterparts.

Evidence for the effectiveness of plant-induced resistance has been observed for well over 100 years, however it has only been within the past 15 years that this phenomenon has been employed as a means of biological control (van Peer et al, 1991). Several pseudomonads and the occasional *Bacillus* spp. are able to induce broad-spectrum plant defense responses (Pal et al, 2001; van Loon and Pieterse, 2002) and have been classified as plant-growth promoting rhizobacteria (Pieterse et al, 1998). One of the major concerns with this approach of biological control is the potential negative effect the

plant antimicrobial compounds may have on beneficial microorganisms such as mycorrhizae and rhizobium.

Identification of Potential Biological Control Agents

Historically, the use of brute force methods, including *in planta* disease challenge assays or *in vitro* antibiosis studies, for the identification of biological control agents has been time and labor intensive. However, replacing mature plants with seedlings (Handelsman et al, 1991) and leaf disks (Fiddaman et al, 2000) can reduce the length of time required for *in planta* assays. Nevertheless, potential pitfalls exist with these replacements since differences in maturation state or tissue specificities could lead to false positive and negative identifications. *In vitro* methods, such as gel diffusion assays, allow for fairly rapid screening of potential biological control agents (McAfee et al, 2001). However the results are usually not representative of what is found *in planta* (May et al, 1997; Ji and Wilson, 2002), due to differences between natural substrata and media (Schoeman et al, 1994).

Several novel approaches for the discovery of potential biological control agents have been developed. For identification of new antifungal compounds, 2D-TLC bioautography can be employed. The two-dimensional separation reliably resolves both hydrophobic and hydrophilic compounds and the antifungal activity can be determined right on the solid matrix. Unfortunately, the metabolite identification and extraction that follow are intensive and may never yield enough product to be effective on a whole plant level (Wedge and Nagle, 2000). Recent work has shown that molecular and biochemical characteristics of a microorganism can be used for identification purposes. Of the

Trichoderma spp., only specific strains of the fungus are effective at controlling disease. Visually indistinguishable, on a molecular level the differences are vast and ITS sequencing, natural polymorphic regions and sequence characterized amplified regions have been effective tools for identifying effective biological control agent strains (Hermosa et al, 2000; Hermosa et al, 2001).

Bacilli as Biological Control Agents

Various *Bacillus* spp. have been used for biological control of plant diseases (Emmert and Handelsman, 1999), displaying both antagonistic qualities (Walker et al, 2002) and systemic resistance induction capabilities (Murphy et al, 2000; Guetsky et al, 2002; Bargabus et al, 2002). Of the antagonistic Bacilli, *Bacillus thuringiensis*, producer of Bt toxin, has been the most widely used to control insect pathogens (Jyoti and Brewer, 1999; Porter et al, 1997). The toxin is so effective that transgenic crops containing the gene encoding for Bt toxin have been developed (Moffat, 1991). *Bacillus subtilis* has also been widely used since it is effective against a wide array of phytopathogenic fungi (Foldes et al, 2000). Other Bacilli capable of antibiosis include, *Bacillus brevis* and *Bacillus mycoides*. *Bacillus brevis* produces a novel antibiotic effective against *Fusarium oxysporum* f.sp. *udum*, the casual agent of fusarium wilt of pigeon pea (Bapat and Shah, 2000) and *Bacillus mycoides* is effective against *Botrytis cinerea* (Guetsky et al, 2002).

Several Bacilli can elicit systemic resistance in plants as a means of controlling disease. When induced systemic resistance was first described, pseudomonads were thought to be the only elicitors (Pieterse et al, 1996). However, recently several *Bacillus* spp. have also been described as plant growth-promoting rhizobacteria (Sturtz et al,

2001). *Bacillus pumulis* induces resistance in both pea and Indian mustard (Belimov et al, 2001). For use with tobacco, *Bacillus amyloliquefaciens* confers resistance to pepper mild mottle virus (Ahn et al, 2002). *Bacillus mycoides* is used on strawberry to induce resistance to *Botrytis cinerea* (Guetsky et al, 2002). Lastly, other unnamed species promote growth of Norway spruce stands (Elo et al, 2000).

Systemic Induced Resistance

An Overview

Plant resistance to pathogens can be broken down into four broad categories: physical barriers, non-host resistance, local resistance, and systemic resistance. The primary physical barrier to pathogen ingress is the thick cuticular wax on the surfaces of leaves (Beattie, 2002). In addition to being structurally sound and therefore hard to penetrate, the wax also repels water needed by many pathogens to penetrate into the leaf (Carver et al, 1994). The highly lignified cell walls serve as a secondary physical line of defense (Friend, 1985). During host and non-host defense reactions the thickness of the cell walls are often altered, creating an even larger obstacle towards pathogenesis (Brisson et al, 1994).

Thousands of phytopathogenic microorganisms exist in nature, yet plants only succumb to disease caused by a very specific subset of plant pathogens. This phenomenon is primarily the result of constitutive lines of defense, namely non-host resistance (Heath, 2000). Disease prevention can be the result of the production of pathogen-specific toxins (Carter et al, 1999) or phytoanticipins (Osbourn, 1999). The

latter is exuded to inhibit spore germination and germ tube elongation (Grayer and Kokubun, 2001). Additionally, the lack of substrates necessary for pathogen growth, infection and proliferation within the host can prevent infection from taking place (Hammond-Kosack and Jones, 2000). Recently, inducible responses have been tied to non-host resistance as well (Peart et al, 2002). As more information is gathered, several points of overlap between host and non-host resistance have been found, suggesting more similarities within the mechanisms required to fend off pathogens of all types than previously accepted (van Wees and Glazebrook, 2003). In fact, certain schools of thought suggest it is simply a difference in timing that distinguished host from non-host resistance (Dangl and Jones, 2001).

Inducible lines of defense also aid in the prevention of disease symptoms. Local resistance, or the hypersensitive response, is marked by the production of large quantities of small molecular weight antimicrobial compounds called phytoalexins. Between plant species, these compounds vary in structure but not function (Grayer and Kokubun, 2001). Most of the phytoalexins are localized in the region of production, however in a few instances, these compounds have been shown to move systemically. Local resistance also results in rapid programmed cell death of one or a few plant cell(s). Several morphological changes are seen in the cell during programmed cell death including cessation of cytosolic streaming (Mittler et al, 1997), breakdown of mitochondria following the release of cytochrome *c* (Balk et al, 1999), DNA laddering (Mittler and Lam, 1995; Stein and Hansen, 1999) and the formation of apoptotic-like bodies (Wang et al, 1996).

Systemic resistance can be broken down into two main defensive pathways: systemic acquired resistance (SAR) (Delaney, 1997) and induced systemic resistance (ISR) (Pieterse et al, 1998). Differences between the two lies in the secondary signal molecules employed during signal transduction and defensive compounds/proteins ultimately induced. However, there are points of convergence between the two pathways, such as employment of the no expression of pathogenesis-related proteins-1(NPR1) gene product (Feys and Parker, 2000). Induction of SAR has been demonstrated in tobacco (Brederode et al, 1991; Heitz et al, 1994), Arabidopsis (Dempsey et al, 1993; Mauch-Mani and Slusarenko, 1994), tomato (Christ and Mosinger, 1989), potato (Schroder et al, 1992), cucumber (Dalisay and Kuc, 1995; Hammerschmidt et al, 1982), melon (Roby et al, 1988), rice (Nishizawa and Hibi, 1991; Smith and Metraux, 1991), corn (Nasser and Bukard, 1988), and wheat (Rebmann et al, 1991). The secondary signaling molecule salicylic acid (SA) elicits the production of the defensive proteins (pathogenesis-related proteins, PR-proteins) and enzymes: PR-1, β-1,3-glucanase (PR-2), Thaumatin-like proteins (PR-5), chitinase (PR-8), peroxidase (PR-9) and lipid transfer proteins (PR-14) (van Loon and van Strein, 1999). The secondary signaling molecules employed for the establishment of ISR are jasmonates (Ryan, 1992) and ethylene (Boller, 1990; Grosskopf et al, 1991). Ethylene and jasmonates can be elicited together, or up or downstream of each other (van Wees et al, 2000; Genoud and Metraux, 1999; Clarke et al, 2000). The PR-proteins induced following elicitation include chitinase (PR-3 and PR-4), proteinase-inhibitors (PR-6), defensins (PR-12), and thionins (PR-13) (van Loon and van Strein, 1999). Another group of defensive compounds, known as systemic wound-

inducible proteins are also induced with jasmonate signaling (Farmer and Ryan, 1992). Following induction with plant growth-promoting rhizobacteria (PGPR), which include Pseudomonads (Pieterse et al, 1996) and Bacilli (Zhang et al, 2001), no PR-protein production is observable.

Activators of Systemic Resistance

Effectors used in the priming of a plant for enhanced disease resistance (Conrath et al, 2002) are defined by the following attributes, according to Sticher et al (1997). They must: 1) not have direct anti-microbial activity *in vitro* or *in planta*, 2) elicit a phenotypical incompatible interaction response from the plant, and 3) protect the plant against pathogens. The effector molecules fall into several categories: inorganic and organic compounds, synthetic compounds and avirulence gene products.

Several organic and inorganic compounds have systemic resistance induction capabilities. Phosphate salts, such as dipotassium phosphate, through the sequestration of Ca^{++}, generate endogenous production of SA, the signaling molecule of systemic resistance (Gottstein and Kuc, 1989; Reuveni et al, 1994). Furthermore exogenous application of SA elicits downstream defense responses (Stotz et al, 2002) as do several amino acids (Van Andel, 1958). Some generic bacterial constituents serve as organic inducers of resistance including the lipopolysaccharides (Newman et al, 2002) and exopolysaccharides (Jahr et al, 1999) found on bacterial cell walls. Similarly generic components of fungi such as glucan (Anderson, 1980), chitin and chitosan (Vander et al, 1998) are active inducers of plant defense as well. Most recently it was discovered that the phytohormone DL-3-aminobutrytic acid (BABA), a phloem-mobile systemic signal

(Cohen and Gisi, 1994), provides effective reduction of disease caused by fungi, oomycetes, viruses, bacteria and nematodes in a wide array of plant systems through the induction of resistance (Cohen, 2002).

Increased understanding of systemic resistance component interactions has opened up an avenue for the development of synthetic inducers of resistance. Some have limited use, such as 2,2-Dichloro-3,3-dimethylcyclopropane that only works in rice that relies upon the production of momilactone phytoalexins (Cartwright et al, 1977). Conversely, compounds like acibenozolar-S-methyl (ASM) and 2,3-dichloroisonicotinic acid (INA) have a broader range of efficacy and induce resistance in most monocots and dicots. Both compounds act through functional mimicry of SA (Delaney et al, 1995; Friedrich et al, 1996) which imparts the broad-range efficacy. The defense responses following treatment differ in that INA induces peroxidase and lipoxygenase (Seguchi et al, 1992), while ASM induces chitinases, β-1,3-glucanases and PR-1 (Friedrich et al, 1996). Therefore, the signaling methods must differ at some level.

AVR proteins produced by viral, fungal, and bacterial pathogens are perceived through gene-for-gene recognition that initiates downstream systemic resistance responses (Yuan et al, 2002; Tuzun, 2001; Ellingboe, 2001). The function of AVR proteins is not limited to recognition and they increase the fitness of the pathogen on the plant (Hammond-Kosack and Jones, 2000; White et al, 2000). Viral AVR proteins are often coat proteins, replicases or polymerases, or movement proteins, all of which are required for disease through viral proliferation (Karasawa et al, 1999). Bacterial *avr* genes encode for small soluble, hydrophilic proteins with unknown functions. They have

little homology to other cloned genes, but are highly homologous to other bacterial *avr* genes (Leach and White, 1996), often containing varying numbers of tandem repeats that affect recognition (Marois et al, 2002). Much like bacterial AVR proteins, fungal AVR protein function is unknown. The fungal *avr* genes have little homology to one another or other cloned genes (Hammond-Kosack and Jones, 2000), albeit most encode for small, soluble peptides. Although it was proposed that AVR products directly interact with plant receptors, emerging evidence does not support this theory (Luderer et al, 2001).

Delivery of the avirulence gene products by bacteria varies depending on the nature of the microorganism. Plant bacterial pathogens live in the apoplast of plants and remain extracellular throughout the disease progression (Staskawicz et al, 2001). Most plant bacterial pathogens are gram (-) bacteria that are capable of forming type III secretion systems for the delivery of AVR products (Jin et al, 2001). The long, slender appendage is able to penetrate through the intricate cell wall matrix to make physical contact with the plant cell plasma membrane (Jin et al, 2001). Several proteins are relayed through the secretion system affecting various aspects of induced resistance such as the oxidative burst and hypersensitive response (Kariola et al, 2003) and downstream immunity (Petnicki-Ocwieja et al, 2002). Gram-positive plant pathogenic bacteria, which lack the type III secretion machinery, must rely on local diffusion of effectors through the plant cell wall (Staskawicz et al, 2001).

Recognition in Plant-Microbe Interactions

Perception of plant pathogens in order to mount a defense response is two-sided. The pathogen produces an effector molecule (*avr* gene product) that is recognized by a

specific receptor (*R* gene product) produced by the plant. The receptor will recognize, either directly or indirectly, a specific isolate of a particular species of pathogen. This interaction was first described by Flor (1971) and named the 'gene-for-gene interaction'. It is the furthest upstream event in the establishment of enhanced disease resistance (Hammond-Kosack et al, 1996).

A high degree of structural similarity exists between *R* genes (Dangl and Jones, 2001). The conserved sequences allow for easy isolation of receptors by polymerase chain reaction amplification of the conserved regions (Xiao et al, 2001). The high degree of similarity also allows for the recombination and evolution of new *R* gene products, therefore providing a greater expanse of recognition (Michelmore and Meyers, 1998; Bergelson et al, 2001).

The structural motifs, leucine rich repeats (LRR) (Kobe and Deisenhofer, 1994), toll interleukin-like receptors (TIR), transmembrane regions (TM), leucine zippers (LZ), serine-threonine protein kinases (ser/thr kinase), and nucleotide binding sites (NBS) (Traut, 1994), are grouped into five main structural classes (Baker et al, 1997; Dangl and Jones, 2001). Class one consists of LRR-NBS proteins with either a LZ of TIR domain on the N-terminus (Zhu et al, 2002). It is the hypervariable region of the LRR domain that is hypothesized to recognize the AVR proteins and other effectors (Wulff et al, 2001). However, one instance has shown TIR domains to be involved in recognition as well (Ronni et al, 2003). Some pathogens, such as fungi that feed with haustoria, cause disease without ever penetrating the plant cell. This creates the need for extracellular perception of invading pathogens. Class two consists of receptors composed of an

extracellularly exposed LRR anchored to the membrane by a TM region (Dangl and Jones, 2001). The third class contains cytosolic ser/thr kinases. The classical example of a class three *R* gene is *Pto*, used to recognize *Pseudomonas syringae* in tomato (Bogdanove, 2000). The fourth class contains LRR-TM-Ser/Thr kinase domains. Finally, the last class carries a putative signal anchor at the N-terminus of a LZ (Dangl and Jones, 2001).

Plant Recognition of Systemic Resistance-Inducing Biological Control Agents

Although the recognition between plants and pathogens has been fairly well explored, the dissection of perception of biological control agents by plants has begun just recently (Smith et al, 1999; Simon et al, 2001). Based on the observed difference in responsiveness to biological control agents between cultivars, it was hypothesized that a genetic basis for the interaction between plants and disease suppressive bacteria exists (Liu et al, 1995, van Wees et al, 1997). Three quantitative trait loci that confer resistance to *Pythium torulosum* by *Bacillus cereus* have been characterized in tomato (Smith et al, 1999). This knowledge may help breeders more effectively select for traits conferring high levels of biological control agent efficacy in plants.

Even less is known about effectors released from biological control agents to activate the systemic resistance response. Recently PGPRs have been found to produce salicylic acid, which may impart limited disease resistance (van Loon et al, 1998). Several bacilli used for biological control produce a wide array of volatile compounds thought to be involved in defense induction (Guetsky et al, 2002; Ryu et al, 2003).

Salicylic Acid Signaling in Plant-Microbe Interactions

Salicylic acid (SA) plays a central role in systemic acquired resistance. Several key experiments set the stage for the hypothesized SA-signaling. White (1979) was the first to discover that exogenous SA application could make tobacco plants more tolerant to TMV infection, thereby reducing the overall levels of disease. Several years later van Loon (1983) hypothesized SA was a signal in plant defense since he found endogenous production both locally and distally from the primary TMV inoculation site. Three years later (Dean and Kuc 1986 a and 1986 b), SA was said to be the primary signal for defense response establishment. This was the accepted theory for many years and further supported when the levels and timing of endogenous production were found to correlate with defense response induction (Yalpani et al, 1991). However Rassumussen et al (1991) provided key evidence contradicting this theory by showing the detachment of a leaf prior to salicylic acid production still conferred resistance to distal parts of the plant. SA is still considered an essential component of systemic resistance as shown through the use of SA compromised mutants (*nahG* plants) that are unable to mount a defense response (Takahashi et al, 2002; Cordelier et al, 2003). However it has a firmly established role as a secondary signal. To date, the chemical nature of the primary signal has yet to be elucidated.

As a signal molecule, SA has been shown to "cross-talk" with other components of induced resistance, most notably hydrogen peroxide. SA has an affinity for and binds to catalase (Du and Klessig, 1997). The binding inhibits the enzymes normal catalysis of

hydrogen peroxide, thereby increasing the cytosolic concentrations of the active oxygen species. The excess hydrogen peroxide potentiates the production of SA, through the stimulation of two enzymes: phenylalanine ammonia lyase (Desikan et al, 1998) and benzoic acid 2-hydroxylase (Leon et al, 1995). These are the first and last enzymes in the biosynthesis of SA respectively (Ribnicky et al, 1998). Cytotoxicity occurs when SA levels reach a certain threshold, however, whether cell death is the cause or consequence of SA production has not yet been determined (Alvarez, 2000). SA is also implicated in the production of pathogenesis-related proteins, but the specific mode of action behind the production is unknown.

Pathogenesis-Related Proteins and Their Role in Plant Defense

Pathogenesis-related (PR) proteins were first discovered in 1970 (van Loon and van Kammen, 1970) when their association with localized hypersensitive response to pathogens was observed. It was not until 1982 that a relationship was drawn between systemic defense responses and PR-protein production (van Loon, 1982). What started with a select group of hydrolytic enzymes has grown into a diverse group of 14 different families of proteins (Table 1). Van Loon et al (1994) have developed a

Table 1-1. Families of Pathogenesis-related proteins induced in plants during pathological and related situations.

Family Name	Molecular Weight Range (kDa)	Biochemical Properties	Literature Cited[1]
PR-1[sa]	14-15	Anti-Oomycotal	Niderman et al, 1995
PR-2[sa]	31-35	Beta-1,3-glucanase	Parent and Asselin, 1984
PR-3[ja]	27-34	Chitinase	Hogue and Asselin, 1987
PR-4[ja]	13-20	Chitinase	Kauffmann et al, 1990
PR-5[sa]	24-26	Thaumatin-like	Trudel et al, 1998
PR-6[ja]	6	Proteinase-inhibitor	Stintzi et al, 1993
PR-7	69	Endoproteinase	Vera and Conejero, 1988
PR-8[sa]	28	Chitinase/Lysozyme	Niderman et al, 1995
PR-9[sa]	39-40	Peroxidase	Stintzi et al, 1993
PR-10	18	Ribonuclease-like	Somssich et al, 1986
PR-11	41-43	Chitinase	Melchers et al, 1994
PR-12[ja]	5-6	Defensin	Terras et al, 1992
PR-13[ja]	6	Thionin	Epple et al, 1995
PR-14[sa]	9-10	Lipid-Transfer Protein	Garcia-Olmedo et al, 1995

[1] Protein or enzyme functions exemplified by but not limited to authors work cited.
[sa] Denotes PR-protein families induced through SA-dependent signaling
[ja] Denotes PR-protein families induced through JA-dependent signaling

unique nomenclature with which to name these select antimicrobial proteins and enzymes based on three criteria: 1) shared amino acid sequence, 2) serological relatedness and 3) enzymatic or biological activity. Strict designations have been made between proteins shown to occur only under pathological and related situations as "PR-proteins" (Antoniw et al, 1980), and those that simply show sequence homology that have been designated "PR-like proteins" until induction studies are completed (van Loon et al, 1994).

PR-1 is strongly correlated with SA-dependent resistance, and therefore is the most widely used molecular marker of systemic acquired resistance induction (van Loon and van Strein, 1999). Both acidic and basic isoforms are induced during incompatible interactions (Kim et al, 2001). Pathogenesis-related protein 1 (PR-1) has anti-oomycotal activity *in vitro* as demonstrated by the inhibition of *Phytophthora infestans* and *Peronospora tabacina* (Niderman et al, 1995). Additionally *in vivo* analysis has shown numerous PR-1 isoforms accumulate around oomycete cell walls during incompatible interactions (Hong and Hwang, 2002).

Family PR-2 contains β-1,3-glucanases (Parent and Asselin, 1984). β-glucan is found in higher plants, in the form of callose (Kudlicka and Brown Jr, 1997; Fridborg et al, 2003) and β-glucanase serves a minor role in plant development (Punja, 2001). However, a main constituent of both fungal and oomycete cell walls is β-glucan (Liu and Balasubramanian, 2001; Inglis and Kawchuk, 2002), therefore the β-glucanases (PR-2) induced during defense response have been assigned an antifungal role. Support for this hypothesis was obtained through numerous *in vitro* inhibition assays (Sela-Buurlage et al, 1993) and from the results with transgenic plants overexpressing (Nishizawa et al, 2003) and with silenced β-glucanase activity (Neuhaus et al, 1992). The sequence and primary structure of sugar beet β-1,3-glucanase have been deduced. Histological analysis showed high concentrations of the enzymes were found in apoplast surrounding the initial site of *Cercospora beticola* infection with lower levels detected in the distal tissues (Gottschalk et al, 1998).

Pathogenesis-related protein families 3,4, 8 and 11 all display chitinolytic activity. This hydrolytic enzyme is another popular choice for transformation studies, since it is present in high concentrations within higher plants with no obvious substrate in the plant itself (Mauch et al, 1988). These enzymes are hypothesized to work synergistically with β-glucanases displaying antifungal activity, acting on chitin and glucan, the primary constituents of fungal cell walls (Bernard and Latge, 2001). Although all the chitinases degrade chitin, their relative affinities for chitin substrates vary 100-fold (Brunner et al, 1998).

Thaumatin-like proteins belong to PR family 5. These compounds were named for the plant they were originally isolated from, *Thaumatococcus danielle* (Cornelissen et al, 1986). Some small thaumatin-like proteins have various binding specificities for fungal β-glucans (Trudel et al, 1998; Zareie et al, 2002). By binding to the β-glucan thaumatins attach to fungal cell walls (Osmond et al, 2001). Following attachment, they can be inserted into the plasma membrane to form a transmembrane pore (van Loon and van Strein, 1999). Pore formation causes membrane permeabilization and electrolyte leakage leading to the ultimate demise of fungi as well as oomycetes (Vigers et al, 1992). Other isoforms can hydrolyze fungal β-glucans, which is also deleterious to fungal survival (Grenier et al, 1999).

Proteinase inhibitors induced during plant defense response in reaction to herbivore or nematode attack belong to family PR-6 (Stintzi et al, 1993). Insects, other herbivores and nematodes deploy serine, cysteine or aspartate proteases to aid in digestion of plant proteins (Koiwa et al, 1997). The plant recognition of oligosaccharide

fragments released during insect and nematode attack activates *de novo* production of protease inhibitors (Koiwa et al, 1997). Over-expression studies using transgenic plants determined serine protease inhibitors are most effective in fending off insects (Urwin et al, 1995) while cysteine protease inhibitors are effective against nematodes (Wolfson and Murdock, 1990; Urwin et al, 1995; Cowgill et al, 2002).

The only characterized member of family PR-7 was isolated from tomato (Goldman and Goldman, 1998). It is a proteolytic enzyme that degrades the protein constituent of fungal cell walls, leading to the conclusion that the enzyme is antifungal. The degradation of cell wall proteins makes the oligosaccharide constituent more susceptible to degradation (van Loon and van Strein, 1999).

Peroxidases comprise family PR-9 (Stintzi et al, 1993). Cytoplasmic and vacuolar peroxidases serve housekeeping functions. Those involved in defense responses tend to be extracellular or cell wall conjugated (Jackson et al, 2001). Peroxidases, through the use of hydrogen peroxide, are capable of oxidizing and cross-linking a variety of organic compounds (Chittoor et al, 1999). This action leads to lignin deposition and the physical strengthening of cell wall that prevents pathogen ingress (Brisson et al, 1994).

Ribonuclease-like proteins belong to family PR-10. Originally ribonucleases were thought to be involved solely in nutrient remobilization, however the observed systemic production of these enzymes in incompatible interactions suggests they play an additional role in defense (LeBrasseur et al, 2002). The defensive roles include the

digestion of viral RNA (Moiseyev et al, 1997) and inhibition of fungal hyphal elongation (Hugot et al, 2002).

PR-12 (Defensins) and PR-13 (Thionins) are both induced through jasmonate signaling (van Loon and van Strein, 1999). They act in similar fashions to inhibit both bacterial and fungal pathogens (Terras et al, 1992; Epple et al, 1995). Both are small, cysteine-rich proteins that target the pathogen plasma membrane (Vernon, 1992). Thionins bind phospholipids and accumulate around bacteria and around fungal penetration pegs in incompatible interactions (Epple et al, 1995 and 1997). Defensins bind to and form pores in the plasma membrane that changes the ion flux (Thomma et al, 2003). In sugar beet following infection with avirulent *Cercospora beticola*, globular bodies of cysteine-rich proteins can be found around lesions therefore they are thought to be involved in controlling the fungal infection (Kragh et al, 1995).

PR-14 (Lipid-Transfer Proteins) is induced via the SA signaling pathway (van Loon and van Strein, 1999). They are hypothesized to deposit extracellular lipophilic materials such as cutin or wax that forms a defensive barrier against pathogen ingress (Garcia-Olmedo et al, 1995). They are induced following treatment with fungal elicitors (Gomes et al, 2003), viruses (Park et al, 2002), and bacteria (Molina and Garcia-Olmedo, 1997), helping to confer resistance to all the phytopathogens.

It is important to mention that although the PR-proteins appear to be antimicrobial in many facets, it has never been shown *in vivo* that these proteins and enzymes are responsible for the reduction in disease noted with systemic resistance induction. In fact, currently multiple lines of defense may exist that have avoided characterization. For now

this particular group of defense related proteins is best suited as markers for systemic resistance induction.

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CHAPTER 2

CHARACTERIZATION OF SYSTEMIC RESISTANCE IN SUGAR BEET ELICITED BY A NONPATHOGENIC, PHYLLOSPHERE-COLONIZING *BACILLUS MYCOIDES,* BIOLOGICAL CONTROL AGENT

Introduction

Effective biological control of plant diseases with epiphytic microbes has been documented for numerous phyllosphere- and rhizosphere-inhabiting organisms. Foliar biological control agents include yeast and filamentous fungi (Hofstein and Chapple, 1999; Sutton and Peng, 1993) as well as bacteria; including both gram (-) species such as *Erwinia* and *Pseudomonas* (Andrews, 1992), and gram (+) organisms such as *Bacillus* (Kokalis-Burelle et al, 1992). Biological control agents applied to the rhizosphere include Psuedomonads (Alstrom, 1991; van Peer et al, 1991; van Loon and Pieterse, 2002) as well as *Bacillus* sp. (Zhang et al, 2001; Murphy et al, 2000) that are classically referred to as plant growth-promoting rhizobacteria (PGPR). For the most part, biological disease control is attributed to direct antagonism against the pathogen via production of antibiotics or hydrolytic enzymes, or through competition for nutrients (Weller, 1988). However, PGPRs and rhizosphere-inhabiting fungi are also capable of stimulating the induction of systemic resistance mechanisms within the plant (van Peer at al, 1991; Wei et al, 1991; van Loon and Pieterse, 2002).

Systemic induced resistance (SIR) has been described in many plant systems, most notably tobacco, bean, tomato, cucumber, and *Arabidopsis thaliana* (Ross, 1961a; Kuc, 1982; Ryals et al, 1996, van Loon and Pieterse, 2002). The broad-spectrum resistance makes an otherwise susceptible plant resistant to a wide array of subsequent pathogen attacks (Kuc, 1982; Hutcheson, 1998). According to Pieterse et al (1996), elicitation of systemic disease resistance in plants can be achieved through treatment by three types of stimuli: necrotizing pathogens (Ross, 1961a and 1961b; Kuc, 1982), secondary signal molecules (i.e. salicylic acid, SA) (White, 1979) and their functional analogs (e.g. 2,6 dichloroisonicotinic acid, INA [Metraux et al, 1991] and acibenzolar-S-methyl, ASM [Tally et al, 1999]), and PGPR introduction into the rhizosphere (Alstrom, 1991; van Loon and Pieterse, 2002; Wei et al, 1991;Zhang et al, 2001;Murphy et al, 2000). Additionally, oomycete and fungal hyphal wall fragments (Doke, 1983; Anderson, 1980), bacterial cell wall fractions (lipopolysaccharides) (Sequera, 1983), and phytohormones (Cohen et al, 1999; Oka et al, 1999, Cohen, 2002) have displayed induction capability. Two systemic resistance pathways have been described: 1) systemic acquired resistance (SAR), which utilizes salicylic acid as a secondary signal molecule and leads to the production of pathogenesis-related (PR) proteins (Delaney, 1997) and 2) induced systemic resistance (ISR), which utilizes jasmonates and ethylene as secondary signal molecules and controls disease independently of PR-protein production (Pieterse et al, 1998).

Cercospora beticola (CB), the causal agent of Cercospora leaf spot, is a fungal pathogen found throughout the United States that reduces both the yield and quality of the sugar derived from sugar beets (Duffus and Ruppel, 1993, Windels et al, 1998). Control of the disease has become increasingly difficult because the fungus has become tolerant to triphenyltin hydroxide (TPTH) (Bugbee, 1995, Windels et al, 1998, Jacobsen et al, 2001) and resistant to the benzimidazole fungicides (e.g. Benlate, Topsin M), two common fungicidal treatments. In the current study, biological control is being investigated as an alternative disease control option.

Bacillus mycoides isolate Bac J (BmJ) was isolated from sugar beet leaves for use as a biological control agent because its innate ability to form endospores, which makes commercial treatments easier to formulate; and it provided the best control of isolates tested in early glasshouse trials (Jacobsen, unpublished work). In preliminary studies, a spontaneous Rifampicin (Rif) mutant of BmJ, that did not differ in growth rate or disease control capabilities, was utilized in repeated attempts to isolate BmJ from sugar beet at 3, 6, 9, and 18 days post treatment from distal untreated and treated leaves and petioles (Jacobsen, unpublished work). Due to the low level of BmJ populations on treated leaf surfaces and the lack of Rif mutant isolation from distal untreated leaves, it was concluded the level of disease control from BmJ treatment could not be due to direct effects of BmJ on CB.

The objectives of this study were to confirm the ability of BmJ to control disease in both glasshouse experiments, and to determine if systemic resistance in sugar beet was

induced by BmJ as the mode of action for disease control. The latter objective was addressed by monitoring the activity of three families of pathogenesis-related proteins (β-1,3-glucanase, chitinase, and peroxidase) that are accepted markers of systemic induced resistance (van Loon and van Strien, 1999).

Materials and Methods

Plant Culture

Sugar beet varieties Holly Hybrid (HH) 88 (hybrid) and Seedex 920002 (inbred) were seeded into flats for germination, transplanted into 4" pots after 1 week, and grown in the glasshouse for 6 weeks in MSU mix (1/3 sand, 1/3 peat and 1/3 topsoil plus the wetting agent Aquagrow 2000). Plants were maintained at 24°C ± 2°C and were watered daily and fertilized twice a week to maintain vigorous growth. Photoperiod was 16 h light and 8 hours dark.

Bacterial Culture

Bacillus mycoides isolate Bac J (BmJ) cells, originally isolated from sugar beet leaves from Sidney, Montana in 1994, were stored at –80 °C in 10% glycerol and 1% tryptic soy broth (TSB) (Difco). For fresh cell preparations, BmJ was cultured in TSB for 48 h (28°C). Cells were centrifuged 15 min at 10,000 x g (4°C), washed with sterile water (2x), then resuspended in distilled water. The optical density was adjusted to OD_{600} 1.0, then diluted 1:2 based on optical density curves confirmed by dilution plating. This optical density and dilution provided for approximately 1 x 10^8 cfu/ml. The precise number of cells was not determined due to the chain-forming nature of the organism. For

experiments testing dead cells, BmJ was autoclaved in water for 30 min following washing. Autoclaved cells were tested for lack of viability by plating 100 µl onto 3 plates of 50% Tryptic soy agar (TSA).

Fungal Culture

C. beticola (CB) (wild type isolate EC3, isolated in Sidney, Montana in 1996) was grown on V-8 agar for a minimum of two weeks with exposure to fluorescent or natural light for at least one week to promote sporulation. Spores were harvested at approximately 30 days after plating in 0.1% carboxymethyl cellulose by scraping with a cotton swab, counted with a haemocytometer and adjusted to 1 x 10^4 spores/ml.

Growth Chamber Plant Treatment

For growth chamber experiments, the leaf penultimate to the oldest two true leaves of sugar beet plants, in replicates of 10, was treated with BmJ, Acibenzolar-S-methyl (ASM, 50 ppm a.i.; Actigard™50WG, Syngenta, Greensboro, N.C.), or dead BmJ in β-glucan with an aerosol sprayer. After drying, the treated leaf was covered with a plastic bag to ensure spatial separation from CB. The susceptible sugar beets were incubated for three days which was previously determined to be the timing that provided the best level of disease control (Braun-Kiewnick et al, 1998), at which time the remainder of the leaves were challenged with the fungal pathogen CB (10^4 spores/ml), which was applied to near run-off using an aerosol sprayer. After treatment, plants were transferred to a 28°C growth chamber equipped with plastic tents and humidifiers. Plants were kept at 100% humidity for 72 h following inoculation with CB. Disease severity

was calculated according to the KWS scale (Kleinwanzler, 1970) and disease reduction was determined for the various inducing agents at 14 and 21 days post inoculation.

Protein Extractions

For protein extractions, leaves distal to the treated leaves were collected from plants at 6 days post treatment with the live and dead BmJ, ASM, or water. One leaf per replicate was collected for each treatment and immediately placed in buffer (150mM NaCl, 25 mM MES, pH 6.2). Apoplast extractions were collected as described by Klement (1965) having substituted buffer (150 mM NaCl, 25 mM MES, pH 6.2) for water.

Western Analysis of Apoplastic Proteins

Apoplast samples were acetone precipitated (3:1 v/v), boiled in SDS sample buffer for 2 min, and resolved (1.5 µg/lane) (12% SDS-polyacrylamide gel electrophoresis (PAGE) gel) for 45 min (200 volts) at pH 8.3 using midrange molecular standards (Sigma) for molecular weight determination. Proteins were then transferred to polyvinylidene fluoride membranes (Millipore) for 1 h (100 volts) in 25mM Tris, 192 mM glycine, and 20% (v/v) methanol (pH 8.3) using a BioRad mini-blot apparatus (Gallagher et al, 1997). Membranes were blocked with 3% BSA for 1h, incubated in primary antibody (anti-chitinase, diluted 1:5000) (Syngeta, Greensboro, NC) in 1% BSA for 1 h, followed by incubation in secondary antibody (peroxidase conjugated, diluted 1:10,000) (Sigma). Colorimetric detection was performed using the 3-amino-9-

ethylcarbazole (AEC) staining kit (Sigma). Loading equality was demonstrated with silver staining (Maniatis, 1982).

Determination of Chitinase Activity Following Non-reducing PAGE

Apoplastic protein samples (1.5 μg/lane) were resolved on a 12% polyacrylamide gel containing 0.01% glycol chitin. Following electrophoresis, the gel was gently shaken for 2 hours (37°C) in 100 mM sodium acetate buffer, pH 5.0 containing 1% (v/v) triton x-100. The gel was then stained with 0.01% calcofluor white M2R in 500 mM Tris-HCL, pH 8.9 for 5 minutes. The gel was quickly rinsed 3x with distilled water, then soaked overnight in the dark in distilled water. Chitinase isoforms were visualized as lytic bands under a UV light source (Trudel and Asselin, 1989). Size comparisons were made between active isoforms and isoforms detected by western analysis using mid-range molecular markers (Sigma).

Chitinase Specific Activity Determination by Plate Assay

Sodium phosphate buffer (pH 5.0, 0.01 M) containing 1% agarose and 0.1% glycol chitin was added to a 9 cm diameter glass petri dish. Wells, 3 mm diameter, were excised from the agarose (3/sample for each of the three replicates per treatment). Dilutions of apoplastic protein (0.7, 0.35, and 0.23 μg) and chitinase standards (Chitinase from *Streptomyces griseus* Sigma) were loaded into the wells. The plate was incubated at 37°C for 24 hours. Following the incubation, 50 ml of 500 mM Tris-HCl (pH 8.9) containing 0.01% fluorescent brightener 28 was added to the plate and incubated for 10 minutes. The plate was then quickly rinsed 3x with water, flooded with water, and

destained overnight in the dark. Non-fluorescent lytic regions on a fluorescent background were measured while the plate was on a UV light source. Specific activity (mg of N-acetyl-D-glucosamine released/hr/mg of apoplastic protein) was determined by comparison of the diameters of the lytic regions for the standards and the lytic regions for the apoplastic protein samples (personal communication, Drs. Ray Hammerschmidt and Luis Velasquez).

Detection of β-1,3-Glucanase Activity

Apoplastic proteins for each treatment (3.0 μg) were separated using acidic PAGE coniditons as described by Reisfeld et al (1962), with the following modification. In the running buffer, L-alanine was substituted for β-alanine with a final pH of 3.8 rather than the prescribed 4.3, allowing for better separation of the isoforms. Following separation, the gels were incubated in 0.1 M citrate buffer (pH 4.8) containing 250 mg laminarin per 100 ml of buffer at room temperature for 20 min. The gels were then transferred to 0.1% Congo red and incubated overnight with constant shaking at room temperature. The gels were then transferred to destaining solution (1 M NaOH) and incubated overnight at room temperature with constant shaking. β-glucanase activity was visualized as yellow-orange bands on a reddish-purple background (Velasquez, 2002).

Determination of β-1,3-Glucanase Specific Activity

The specific activity of sugar beet apoplastic β-1,3-glucanase was determined by measuring the release of glucose units from laminarin. Sodium acetate buffer (100 μl, pH 5.0, 100 mM) containing 0.5% laminarin and 0.5 μg – 2.0 μg apoplastic protein (3 plants

per treatment replicated 3 times) was incubated at 37°C for 30-60 minutes. Following incubation, 900 µl of water and 1 ml of alkaline copper reagent (Somogyi, 1952) was added to each reaction. The tubes were then placed into a boiling water bath for 10 minutes. After cooling on ice, 1 ml arsenomolybdate color reagent (Nelson, 1944) was added to each reaction. Once the bubbling had subsided, 10 ml of water were added to each tube before reading the absorbance at 660 nm. A standard curve was established by adding 5 µg- 25 µg glucose to 1 ml total reactions that did not contain laminarin.

Peroxidase Assay

Apoplastic peroxidase activity from three plants per ASM, BmJ and water treatment (3 replicates of each) was measured using guaiacol reagent according to Hammerschmidt et al (1982). The concentration of protein was adjusted to give a change in absorbance units greater than 0.100, but less than 0.200, per minute. Specific activity was expressed as the increase in absorbance (470 nm) over time (2 min) per mg of protein, as determined using Bradford reagent (BioRAD).

Determination of Peroxidase Activity Following Native-PAGE

Polyacrylamide gel electrophoresis was performed according to Reisfeld et al. (1962). Following electrophoresis, the gels were stained using an AEC staining kit (Sigma) for 16 hours while gently shaking in the dark. The gels were then rinsed with distilled water 3x for a total of 15 minutes to stop the reactions.

Results

Cercospora Leaf Spot Disease Reduction in Glasshouse Experiments

To determine the effectiveness of BmJ at reducing disease severity of Cercospora leaf spot on sugar beet while spatially separated from CB, the distal untreated leaves of BmJ-treated plants were challenged with CB 3 d post treatment. ASM and dead BmJ in β-glucan were also used as treatments before fungal challenge as positive and negative controls, respectively. All plants were rated using the KWS (1-9) scale at 14 and 21 d post challenge. The more susceptible of the two cultivars of sugar beet tested (HH88, a hybrid), resulted in the greatest systemic reduction in disease severity (~80% reduction) following priming with BmJ (Table 2-1). The decreased occurrence of leaf spot symptoms was statistically significant in comparison to the negative control (dead BmJ treatment), but not statistically different from the 63.6% reduction resulting from ASM treatment (Table 2-1). Priming HH88 sugar beets with virulent CB did not statistically reduce disease symptoms (Table 2-1). The inbred sugar beet cultivar (Seedex 920002) was less susceptible than its hybrid counterpart, and the overall disease severity was lower. With the inbred cultivar, BmJ was less effective than ASM-pretreatment, however the approximate 2% difference was not statistically significant (Table 2-1). The 66.7% reduction in disease severity noted with the inbred variety following BmJ treatment was similar to that observed with the hybrid sugar beet and was statistically higher when compared to the negative (dead BmJ) control pretreatment (Table 2-1). Dead BmJ cells in

β-glucan were not effective at controlling disease when applied to either cultivar when compared to untreated controls (data not shown) and the plants not challenged with CB showed no sign of infection.

Production of Chitinase in Response to *B. mycoides* Treatment.

Analysis of PR-protein production was used to help evaluate our hypothesis that BmJ induced resistance to CB. A polyclonal antibody to tobacco chitinase (provided by Syngenta, Greensboro, NC) bound to several putative chitinases in sugar beet following treatment with BmJ, ASM and water (Figure 2-1A). As a means of determining which isoforms were potentially involved in sugar beet defense responses, the activity of the isoforms was observed following non-reducing PAGE (Figure 2-1A). Certain isoforms showed increased activity while others appeared to have reduced activity in sugar beet following BmJ-treatment (Figure 2-1B). There was equal loading in the PAGE analyses, as demonstrated when the apoplastic protein samples were also run on a separate

Table 2-1. Systemic disease control of Cercospora leaf spot on two different cultivars of sugar beet using *Bacillus mycoides* isolate Bac J and acibenzolar-S-methyl in glasshouse experiments.

	% Disease Severity				% Reduction at 21 DPC[w]	
	HH88[v]		Seedex[v]			
Treatment[x]	14 DPC	21 DPC	14 DPC	21 DPC	HH88	Seedex
Control[y]	5.76	14.34	0.32	0.48	n.r.	n.r.
Cercospora beticola	5.24	14.10	n.d.	n.d.	n.r.	n.r.
Acibenzolar-S-methyl	0.76	5.26	0.16	0.17	63.6	64.6
Bacillus mycoides	1.03	2.94	0.13	0.16	79.5	66.7
LSD (0.05)[z]	2.87	3.78	0.09	0.12	n.d.	n.d.

[v]Holly Hybrid 88 (HH88, hybrid) and Seedex 920002 (Seedex, inbred) were the two sugar beet cultivars used in glasshouse experiments.
[w]DPC= days post challenge with *Cercospora beticola.*
[x]Plants were treated with dead *Bacillus mycoides* isolate Bac J in β-glucan (control), *Cercospora beticola* (virulent on HH88 and Seedex), acibenzolar-S-methyl, or live *Bacillus mycoides* isolate Bac J on one leaf, then challenged 3 days later with *Cercospora beticola*, the fungal pathogen, on the distal untreated leaves.
[y]Control = dead *Bacillus mycoides* isolate Bac J cells applied with β-glucan.
[z]LSD = least significant difference (probability = 0.05)
Plants treated with dead or live *Bacillus mycoides* isolate Bac J or acibenzolar-S-methyl without challenging with *Cercospora beticola* showed no disease symptoms.
n.r. = no reduction in disease symptoms.
n.d. = no data

polyacrylamide gel, then silver stained (Figure 2-1C). One of the isoforms produced in response to BmJ-treatment, but lacking in the water-treated plants, was also found following ASM treatment, which is known to induce plant systemic resistance responses. The overall changes in specific activity of chitinase in sugar beet following treatment with ASM, live and dead BmJ and water were determined. Even though ASM-treatment

resulted in fewer active isoforms being produced than the BmJ-treatment, the specific activity level was statistically equal (Table 2-2). Both live BmJ and ASM treatments resulted in statistically higher chitinase specific activity that was nearly twice that observed with water or dead BmJ treatment (Table 2-2).

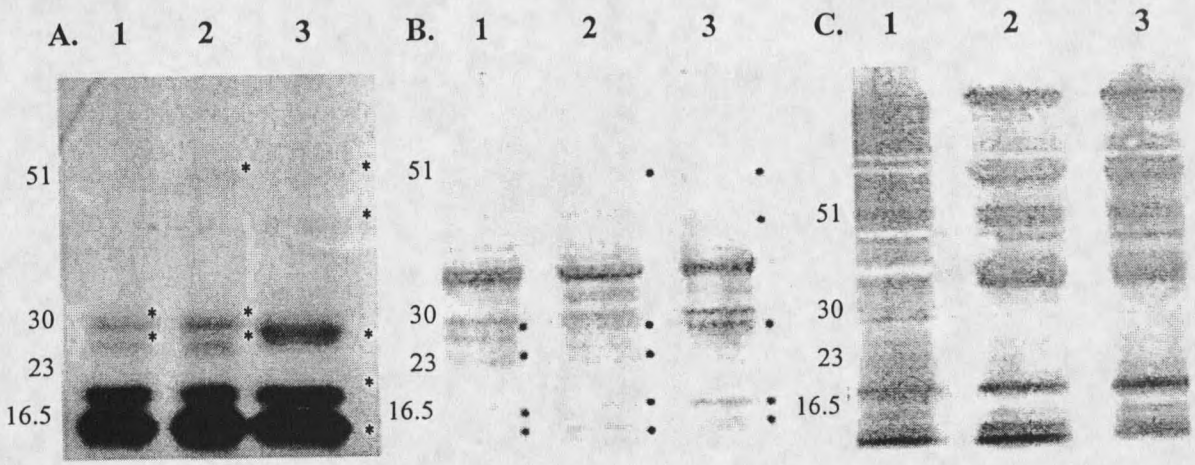


Figure 2-1. Analysis of systemically produced apoplastic chtinases extracted from sugar beet 6 days post treatment. Treatments: 1: water; 2: acibenzolar S-methyl; 3: *Bacillus mycoides* isolate Bac J. Molecular weights are expressed in kilodaltons. (A) Identification of active chitinase isoforms following Non-reducing polyacrylamide gel electrophoresis. (B) Identification of chitinase using polyclonal anti-tobacco chitinase antibody. Isoforms able to degrade glycol chitin following electrophoresis (A) designated with an asterisk (*). (C) Silver stain shows loading equality of total apoplastic proteins.

Table 2-2. Systemic sugar beet apoplastic pathogenesis-related protein activity six days post treatment with live and dead *Bacillus mycoides* isolate Bac J, acibenzolar-S-methyl and water.

	Specific Activity		
Treatment	Chitinase[w]	Beta-glucanase[x]	Peroxidase[y]
Water	0.46	36.1	42.8
Live *Bacillus mycoides*	1.02	77.9	61.6
Acibenzolar-S-methyl	1.26	198.6	71.4
Dead *Bacillus mycoides*	0.45	37.7	26.8
LSD (0.05)[z]	0.21	36.1	12.2

[w]Chitinase specific activity expressed as milligrams of N-acetyl-D-glucosamine released per hour per milligram of apoplastic protein and is the mean of the data from 3 plants per treatment replicated 3 times.
[x]β-glucanase specific activity expressed as micrograms of glucose released per minute per milligram of apoplastic protein and is the mean of 3 plants per treatment replicated 3 times.
[y]Peroxidase specific activity expressed as the changes in absorbance (470 nm) per minute per milligram of apoplastic protein and is the mean of 3 plants per treatment replicated 3 times.
[z]LSD = least significant difference (probability = 0.05).

β-1,3-Glucanase Production in Response to BmJ Treatment.

Native polyacrylamide gel electrophoresis (PAGE) was run under acidic conditions to examine basic β-1,3-glucanases produced in response to ASM, BmJ or water treatment. Two active isoforms were produced in sugar beet following BmJ treament (Figure 2-2). One of the two isoforms was also present and active in sugar beet following ASM-treatment, however both were lacking in water-treated plants (Figure 2-2). To determine the total activity of β-1,3-glucanase in sugarbeet, colorimetric assays

were performed. BmJ-treated plants had a specific activity that was approximately twice

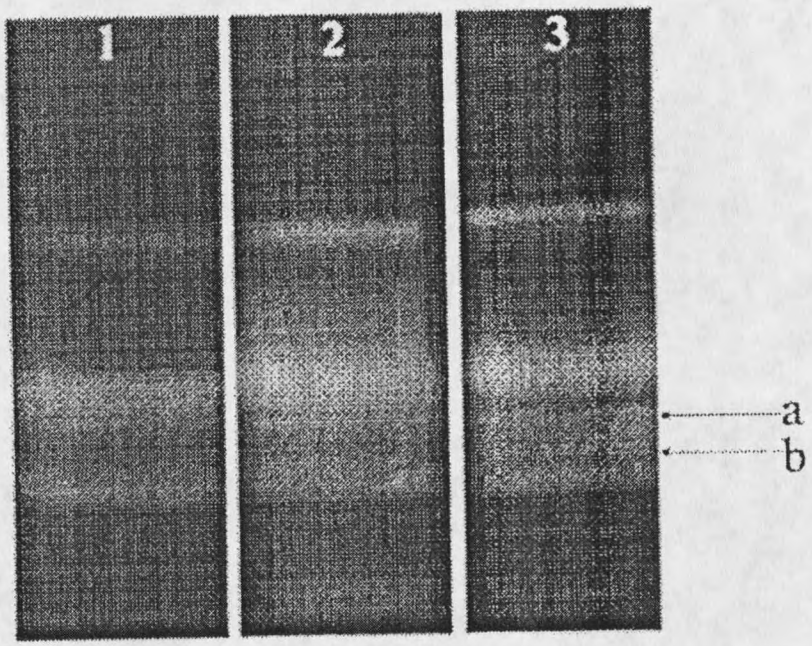


Figure 2-2. Analysis of systemically produced glucanases extracted from sugar beet 6 days post treatment. Treatments: 1: water; 2: acibenzolar-S-methyl; 3: *Bacillus mycoides* isolate Bac J. Results of the glucanase gel activity assay showed differential production of glucanase isoforms. Isoform "a" appears following acibenzolar-S-methyl treatment. Isoforms "a" and "b" are produced following *Bacillus mycoides* isolate Bac J treatment. Both acibenzolar-S-methyl and *Bacillus mycoides* isolate Bac J treated plants contained the other three isoforms found in the water-treated sugar beets.

that of the activity in water-treated plants, but approximately one-third the ASM-induced activity; both were statistically significant increases when compared to the water-treated and dead BmJ-controls (Table 2-2). Dead BmJ-treated plants had specific activities statistically equivalent to the water-treated controls (Table 2-2).

Peroxidase Activity in the Apoplast of Sugar Beet.

Peroxidase is a PR-protein that can easily be measured using activity assays. BmJ treatment elicited significantly greater peroxidase activity in the apoplast of distal sugar beet leaves than water or dead BmJ treatment (Table 2-2). Peroxidase activity following BmJ treatment was also statistically equivalent to that elicited by ASM (positive control) treatment (Table 2-2). To determine if the increased activity noted in the chemical SIR-inducer and bacterial treatments was due to plant production of new peroxidase isoforms, in-gel activity assays were performed. Both the ASM- and BmJ-treated plants had two additional active isoforms not detected in the negative (water) control (Figure 2-3). There were several other minor isoforms that were not discussed here due to their occasional appearance in the water controls.

Discussion

As hypothesized, *Bacillus mycoides* isolate Bac J provided consistent disease control of Cercospora leaf spot of sugar beet. This was evident from glasshouse experiments where it worked just as well as ASM, a known inducer of systemic resistance (Table 2-1) and from the field where control comparable to standard fungicide treatments was observed (Bargabus et al, 2002). One of the major pitfalls with biological control is maintaining a consistent level of disease control (Stack, 2002). However, the disease control elicited by BmJ was not reliant upon low amounts of inoculum and was as

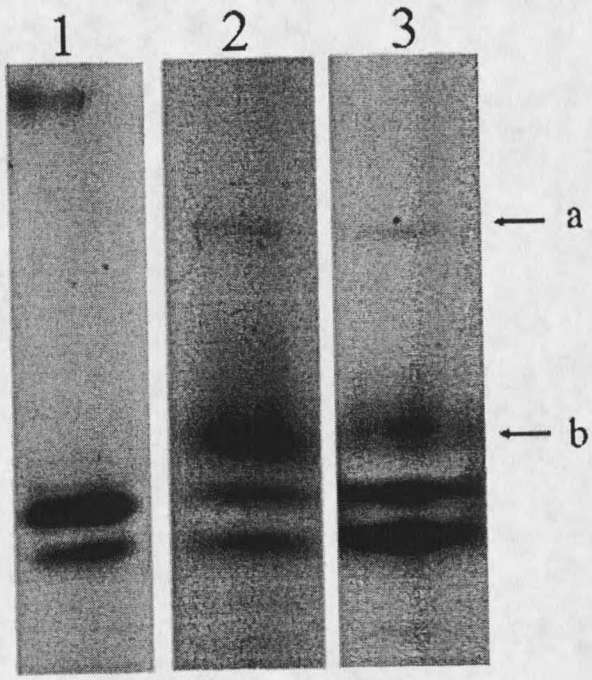


Figure 2-3. Analysis of systemically produced apoplastic peroxidases extracted from sugar beet 6 days post treatment. Treatments: 1: water; 2: acibenzolar-S-methyl; 3: *Bacillus mycoides* isolate Bac J. Results of the peroxidase in-gel activity assay showed the increase in the peroxidase acitivity following acibenzolar-S-methyl and *Bacillus mycoides* isolate Bac J treatment was due to the production of the two isoforms ("a" and "b") lacking in the water-treated control.

effective in years with extreme levels of disease, such as 1996, as in years with lower levels of disease (Bargabus et al, 2002). It was also noted that dead BmJ cells were not effective at reducing Cercospora leaf spot symptoms, this suggests live BmJ is either producing compounds that are: 1) antagonistic to CB or 2) perceived by sugar beet that elicit plant resistance mechanisms. If the latter were true, the slight differences in disease reduction seen in the field is more likely due to varience in varietal perception of BmJ than in susceptibility to CB, since all varieties used have the same KWS rating

(BetaSeed, Shakopee, MN) as is true for other biological control agent-plant interactions (Smith et al, 1997). During the disease control assays, it was observed that BmJ was able to control disease in glasshouse experiments even when spatially separated from CB. In addition, it was not possible to isolate BmJ Rif mutants from distal tissues, indicating that direct interaction between BmJ and CB was not necessary for disease control. This suggests that plant defenses, namely SIR, are involved as the mode of action for BmJ on sugar beet.

To test the hypothesis that SIR was involved in disease control production of 3 of the 14 families of PR-proteins was monitored. These proteins have become accepted markers of plant disease resistance and are differentially induced at the onset of various plant defense reactions (van Loon, 1999). Since BmJ produces chitinases (Thamthiankul et al, 2001), β-glucanases (Navarro et al, 1995), and peroxidases (Moerschbacher, 1992), protein samples were only collected from untreated leaves distal to the bacterial-treated leaf. Chitinases belong to PR families 3, 4, 8, and 11 and have been characterized in tobacco (Legrand et al, 1987), cucumber (Metraux and Boller, 1986) and numerous other plant systems. The presence and activity of chitinases in the negative (water and dead BmJ) control-treated sugar beets was not unusual (Figure 2-1A), since the detection of these proteins in healthy, non-induced plant tissue is not uncommon (Punja, 2001). What was more significant was the production of unique chitinase isoforms following treatment with BmJ and ASM (Figure 2-1A), since it shows participation of proteins not associated with potential housekeeping roles. The use of ASM helped to positively identify defense-related proteins in all the studies, since the mode of action for this compound has already

been established as systemic induced resistance (Tally, 1999). In all of the plant treatments, several chitinase isoforms identified by western analysis were not active (Figure 2-1A). Although chitinase amino acid sequences are highly conserved between plant genera, some binding may be attributed to non-specific interactions between the tobacco antibody and other sugar beet proteins. Alternatively, some of the smaller isoforms may simply be due to antibody binding to chitinases proteolytic degradation products.

β-1,3-glucanases (PR-2) produced in sugar beet during systemic resistance responses were first isolated and characterized by Gottschalk et al (1998). They reported the production of two distinct isoforms, 33 and 29 kDa in size with pIs of 9.4 and 9.5, respectively. In part, this was consistent with the findings in sugar beet treated with BmJ. The exact isoelectric point of the two sugar beet isoforms was not determined, although electrophoresis in an acidic environment, as done here, separates proteins that are basic under those conditions. Once again, the presence of active isoforms in the negative control (Figure 2-2) was not unexpected since the presence β-glucanase in healthy plant tissue is common (van Loon and van Strien, 1999, Punja, 2001) due to the numerous roles fulfilled during reproduction and development (Lotan et al, 1989). More importantly, unique β-glucanase isoforms were consistently detected between BmJ- and ASM-treated sugar beets in comparison to water treatment that were reflective of an increase in specific activity (Table 2-2).

BmJ-elicited production of both β-1,3-glucanase and chitinase was significant since these PR-proteins have a synergistic association leading to fungal pathogen control

not evident when the two occur independently (Jongedijk et al, 1995; Zu et al, 1994). It was hypothesized that together, these enzymes may be involved in reducing fungal disease severity by degrading the chitin and β-glucan components of fungal cell walls (Abeles et al, 1971; Mauch et al, 1988). Although these enzymes may not be directly acting on *C. beticola,* the induction of both enzymes by BmJ- and ASM-treatment of sugar beet (Table 2-2) is correlative with significant disease control of Cercospora leaf spot, agreeing with findings in tobacco using *Cercospora nicotianeae* (Neuhaus et al, 1992). Additionally, disease control was increased markedly when the BmJ was applied in combination with propiconazole (Tilt) or Eminent, presumably due to the multiple lines of defense activated in sugar beet following BmJ treatment. Preliminary results also suggested that BmJ used in combination with fungicides to which CB is prone to the develop resistance (i.e. benzimidazoles, triazoles and strobilurins) reduced the level of resistance that developed in the field (Jacobsen et al, 2001). The latter data suggested that in addition to using BmJ alone for biological control of Cercospora leaf spot, combining the bacterium with the few available fungicides could prolong their effectiveness over time.

Defense-related peroxidases (PR family 9) (van Loon and van Strien, 1999), are capable of cross-linking, through the oxidation of hydrogen peroxide, a variety of organic compounds, thereby strengthening physical barriers that prevent pathogen ingress (Chittoor et al, 1999). Apoplastic peroxidases were measured to discriminate between those involved in lignification (apoplastic and cell wall conjugated) from the housekeeping (cytoplasmic and vacuolar) isoforms, which are less likely to be involved

in plant defense. The unique peroxidase isoforms produced following BmJ treatment lead to a transient increase in specific activity, which peaked at six days and diminished around 11 days. The reduction in activity has been found to correlate with a loss of bacterial disease (*Erwinia carotovora* pv. *betavasculorum*) control (Jacobsen, unpublished work). On the other hand, the PR-proteins with putative antifungal activity have greater longevity in this particular system and fungal disease control is more prolonged and effective.

Through the exclusion of direct antibiosis, maintanence of spatial separation between BmJ and CB in glasshouse experiments, the demonstration of the need for live BmJ cells for disease control, and the production of three families of PR-proteins by live BmJ consistent with ASM controls, we have concluded BmJ is a systemic resistance-inducing biological control agent that is capable of controlling Cercospora leaf spot of sugar beet. The characterization of systemic induced resistance as a mode of action for BmJ has opened up several new avenues of research possibilities. Work begun in our lab includes investigation of the oxidative burst elicited in sugar beet by BmJ as well as an extension of the classical systemic resistance challenge experiments in the field, in which we are monitoring the production of PR-proteins and systemic disease reduction. Preliminary results from the field have been consistent with glasshouse experiments described here (Jacobsen, unpublished work). Since it is known that the infection is less severe after priming with BmJ, we plan to examine the infection process microscopically to determine where the pathogen ingress is halted relative to the stage of systemic resistance induction. In addition, using BmJ as a model, we are developing a high throughput

screening technique for identifying *Bacillus* biological control agents that induce systemic resistance in sugar beet.

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CHAPTER 3

OXIDATIVE BURST ELICITED BY *BACILLUS MYCOIDES* ISOLATE BAC J, BIOLOGICAL CONTROL AGENT, OCCURS INDEPENDENTLY OF HYPERSENSITIVE CELL DEATH IN SUGAR BEET

Introduction

The oxidative burst is one of the earliest events in plant defense responses (Costet et al, 2002). It is marked by the production of active oxygen species (AOS) through four sequential one-electron reductions of dioxygen to water (Hippeli et al, 1999). The AOS include, in order of least to most reactive and longest to shortest lived, hydrogen peroxide, superoxide anion, and hydroperoxyl and hydroxyl radicals (Boveris, 1998).

Active oxygen species are produced in both compatible and incompatible plant-pathogen interactions (Baker and Orlandi, 1995; Glazener et al, 1996; Jabs et al, 1997; Wolfe et al, 2000). The production of hydrogen peroxide and superoxide anion has also been observed in rhizobium-plant interactions (Santos et al, 2001). However, the role of AOS in elicitation of induced resistance by biological control agents has not yet been elucidated. In a compatible plant-pathogen interaction, a single, rapid burst of hydrogen peroxide is observed in cell suspension cultures (Grant and Loake, 2000). This response is believed to be due to the perception by the host of generic pathogen constituents, such as fungal glucans, chitins or chitosans (Boller, 1995), the conserved N-terminal region of bacterial flagella (Felix et al, 1999) or viral coat proteins (Allan et al, 2001). The transient

primary burst is non-specific and has no effect on disease progression (van Breusegem et al, 2001). During incompatible interactions a second more prolonged peak of hydrogen peroxide production quickly follows the initial burst as a result of specific gene-for-gene recognition (Levine et al, 1994; Baker and Orlandi, 1995).

Biphasic production of AOS at the attempted sites of avirulent pathogen infection is significant for several reasons. Hydrogen peroxide is needed in the physical strengthening of plant cell walls through peroxidase-catalyzed lignification (Hammerschmidt and Kuc, 1982) and cell wall protein crosslinking (Bradley et al, 1992; Lamb and Dixon, 1997). This bolstering of the physical barrier makes it harder for pathogens to penetrate plant cells by way of enzymatic digestion. AOS are also directly toxic to microorganisms (Levine et al, 1996; Gilchrist, 1998; Imlay, 2002) and inhibitory towards fungal spore germination (Peng and Kuc, 1992). Additionally, plant cell death can result from AOS-mediated lipid peroxidation of cell membranes (Grant and Loake, 2000; Bradley et al, 1992; Jalloul et al, 2002). This localized programmed cell death serves to prevent nutrient acquisition and pathogen spread (Parker and Coleman, 1997). Hydrogen peroxide can freely diffuse across cell membranes and therefore, has been implicated in the signaling for the establishment of downstream plant immunity events (Levine et al, 1994). Often, in conjunction with nitric oxide, hydrogen peroxide can stimulate production of salicylic acid biosynthetic enzymes (Chen et al, 1993a and Chen et al, 1993b) and pathogenesis-related proteins (Bi et al, 1995; Alvarez, 1998; van Camp et al, 1998; van Breusegem et al, 2001; Mackerness et al, 2001), two key players in plant systemic resistance (van Loon and van Strein, 1999). Previously, biphasic hydrogen

peroxide production was thought to orchestrate hypersensitive cell death (Alvarez, 1998, Levine at al, 1994 and 1996). However, several recent examples have shown the oxidative burst and hypersensitive response can occur independently of one another (Glazener et al, 1996; Dorey et al, 1999).

In previous work, we have shown *Bacillus mycoides* isolate Bac J (BmJ) is capable of eliciting systemic induced resistance in sugar beet. We had speculated that an oxidative burst preceeds induction due to the downstream involvement of pathogenesis-related proteins (Bargabus et al, 2002) and other preliminary results (Bargabus et al, 2001). The objectives of the research reported here were to further characterize the oxidative burst elicited in sugar beet by the biological control agent BmJ. To determine if the oxidative burst was consistent in timing and intensity to that elicited by avirulent *Erwinia carotavora* pv. *betavasculorum* (Ecb) and to determine if the oxidative burst was accompanied by sugar beet cell death.

Materials and Methods

Plant Cultures

Sugar beet varieties USH11 and C40 (provided by Robert Lewellen, USDA-ARS, Salinas, CA) were seeded into flats for germination, transplanted into 10 cm pots after one week, and grown in the glasshouse for 6 weeks in MSU mix (1/3 sand, 1/3 peat and 1/3 topsoil plus the wetting agent Aquagrow 2000). Plants were maintained at 24±2°C and were watered and fertilized twice a week to maintain vigorous growth. The photoperiod was 16 h light and 8 h dark.

Bacterial Cultures

Bacillus mycoides isolate Bac J (BmJ) cells, originally isolated from sugar beet leaves from Sidney, MT in 1994, were prepared as previously described (Bargabus et al, 2002). For experiments testing dead cells, BmJ suspensions were diluted to 1 x 10^8 cfu/ml and autoclaved for 30 min. Autoclaved cells were tested for lack of viability by plating 100 µl onto each of 3 plates of tryptic soy agar.

Erwinia carotavora pv. *betavasculorum* isolates 1 and 6 (Ecb 1 and Ecb 6) were obtained from Dr. Robert Lewellen (USDA-ARS, Salinas, CA). Ecb1 and Ecb 6 are avirulent on C40 and USH11, respectively. Fresh cell preparations were grown on tryptic soy agar for 24-48 h. Cells were harvested into 10 ml distilled water and diluted to 1 x 10^6 cfu/ml.

Fungal Culture

Cercospora beticola (wild type isolate EC3, isolated in Sidney, Montana in 1996) was grown on potato dextrose agar (Difco) for a minimum of two weeks at 22±2°C with exposure to fluorescent or natural light for at least one week to promote sporulation. Spores were harvested at approximately 30 days after plating in 0.1% carboxymethyl cellulose by scraping with a cotton swab, counted with a hemocytometer and adjusted to 1 x 10^4 spores/ml.

Disease Control Assay

Typically, treatment of sugar beet with *Erwinia* sp. is carried out by stab inoculation into the petiole while in previous studies BmJ was applied to leaves by spraying (Bargabus et al, 2002). Results from the experiments described below indicated that syringe infiltration was effective for all cultures as a means of standardizing the treatment method for disease control, hydrogen peroxide and cell death assays. BmJ (1×10^2, 1×10^4, 1×10^6, 1×10^8 cfu/ml), Ecb 1 (1×10^2, 1×10^4, 1×10^6, 1×10^8 cfu/ml), Ecb 6 (1×10^2, 1×10^4, 1×10^6, 1×10^8 cfu/ml) and water were syringe infiltrated into the leaf surface of the leaf penultimate to the oldest first true leaf, which was then immediately bagged. Three days post treatment, the remaining untreated leaves were inoculated with 1×10^4 spores/ml of *Cercospora beticola.* Disease severity was determined using the KWS scale (Kleinwanzler et al, 1970) at 21 days post challenge. Three independent disease control assays were performed containing 5 replicates per bacterial or water treatment. The data were pooled and the mean disease severity rating was determined for each treatment. Percent disease reduction was calculated as follows: (KWS rating for water control-KWS rating for treated plant)/KWS rating for water control x 100.

Pathogenesis-Related Protein Analysis

To evaluate elicitation of systemic resistance using stab, syringe or spray inoculation methods for BmJ (1×10^8 cfu/ml), Ecb 1 (1×10^6 cfu/ml) and Ecb6 (1×10^6 cfu/ml), the production of glucanase and chitinase was determined as previously described (Bargabus et al, 2002). These enzymes are accepted molecular markers of

systemic resistance induction (van Loon and van Strein, 1999). Stab, syringe and spray treatments were replicated 3 independent times for each bacterium, with three subsamples for each treatment. The data was pooled and the mean was determined for each treatment method with BmJ, Ecb 1 and Ecb 6.

Sugar Beet Protoplast Generation

Protoplasts were isolated from sugar beet leaves to provide one medium to measure hydrogen peroxide production. Sugar beet leaves were gently brushed on the abaxial and adaxial surfaces with a soft bristle brush to create small abrasions. The leaves were then cut into1 cm strips and vacuum infiltrated for 5 minutes with 0.7M sucrose containing 3.8% $CaCl_2$, CPW salts (Frearson et al, 1973), 1.2% cellulase (Sigma) and 0.4% macerozyme (ICN Biomedicals Inc.). The infiltrated leaves were incubated in the 0.7M sucrose-salt and enzyme solution for 24-48 hours in the dark. Following incubation, the enzyme solution was gently removed and the protoplasts were released into 0.7M sucrose containing 3.8% Ca Cl_2 and CPW salts by gently shaking the leaf strips in the solution.

Assay for Hydrogen Peroxide Production

To examine the oxidative response of sugar beet to BmJ, Ecb 1 and Ecb 6, hydrogen peroxide production was assayed by monitoring the oxidation of phenol red (Pick and Keisari, 1980). Whole leaf disks (12-7cm disks/reaction) were utilized to study the response and native sugar beet peroxidases carried out the oxidation reaction. Phenol red oxidation levels by bacteria and leaf disks alone were subtracted from the amount of

oxidation in reactions containing both bacterial cells and leaf disks. For the determination of hydrogen peroxide production using protoplasts, an external source of peroxidase (type II horseradish peroxidase, Sigma) had to be added to the reaction mix. The amount of hydrogen peroxide produced from protoplasts (250 protoplasts/reaction) in response to BmJ treatment was calculated as follows: amount of hydrogen peroxide produced in BmJ-protoplast reaction- (amount of hydrogen peroxide produced in protoplast only reaction + amount of hydrogen peroxide produced in BmJ only reaction). Three replications, containing two subsamples each, were conducted for each reaction. The concentration of hydrogen peroxide [mM] was calculated by establishing a linear standard curve with 6.2 µg/ml type II horseradish peroxidase (E.C. 1.11.1.7, Sigma) and 1-40 mM hydrogen peroxide. Three replications for each plant-bacterial combination were carried out with two subsamples each. The data was pooled and the mean hydrogen peroxide concentration was determined for each time point.

Signaling by *Bacillus mycoides* Isolate Bac J

To test the hypothesis that signaling between BmJ and sugar beet involved stomatal conductance of signal(s), hydrogen peroxide secondary peak production patterns were examined by phenol red oxidation while the stomata were closed. To confirm stomatal closure following 15 minute adjustment to green light conditions (Roscolux filter #90; Dark Yellow Green; 13% transmission; 480-580nm), leaf surfaces were painted with clear nail polish, allowed to dry, then the nail polish was peeled off the leaf surface and examined under the microscope. These peels were compared to peels conducted on plants kept in the light, where the stomata were open. Approximately 100

stomata were observed per leaf. Plants were kept under green light or complete darkness for the entire experiment. As a positive control for the oxidative burst studies, 1 x 10^8 BmJ cells/ml were syringe infiltrated into plants kept under the same green light conditions. Secondary peaks from experiments conducted in the dark were compared to secondary peaks elicited from BmJ syringe and spray infiltration in the light. In both sets of experiments the background phenol red oxidation, determined using plants infiltrated or sprayed with water, was subtracted from the BmJ infiltrated and sprayed plants respectively. Three independent replications of each treatment were conducted and the mean was determined for each time point.

Observation of Cell Death

Sugar beet leaves were syringe infiltrated with Ecb 1 (1 x 10^6 cfu/ml), Ecb 6 (1 x 10^6 cfu/ml) and BmJ (1 x 10^8 cfu/ml). Observations of disease symptom progression were made daily for one month. The infiltration site and those areas surrounded the infiltration site were evaluated and photographed. This experiment was repeated on three independent occasions.

Results

Disease Control

Varying cell concentrations of *Erwinia carotovora* pv. *betavasculorum* isolate 1 (Ecb 1) and isolate 6 (Ecb 6) were syringe infiltrated into sugar beet leaves to determine the concentrations that would reduce *Cercospora beticola* disease symptoms to a statistically equivalent level as 1 x 10^8 BmJ cells/ml. This allowed for a better

comparison of the oxidative burst results obtained for BmJ and the two *Erwinia* isolates. Only the incompatible interactions of Ecb 1 and Ecb 6 with C40 and USH11, respectively, resulted in statistically significant *C. beticola* disease reduction (Figure 3-1A and B). Of the four *Erwinia carotovora* pv. *betavasculorum* cell concentrations tested, 1 x 10^6 cells/ml provided the greatest level of *C. beticola* symptom reduction on systemic, untreated leaves (Figure 3-1A and B). This reduction in disease symptoms, approximately seventy percent, was statistically equivalent to the systemic disease control afforded by syringe infiltration of 1 x 10^8 cfu/ml BmJ (Figure 3-1A and B). The incompatible interactions also reduced *C. beticola* symptoms to a statistically significant level when 1 x 10^8 and 1 x 10^4 cells/ml were syringe infiltrated, however this reduction was statistically lower than that achieved with 1 x 10^8 BmJ cells/ml. Syringe infiltration of 1 x 10^6 BmJ cells/ml reduced *C. beticola* symptoms approximately twenty five percent. Compatible interactions did not reduce *C. beticola* disease symptoms at any cell concentration tested (Figure 3-1A and B).

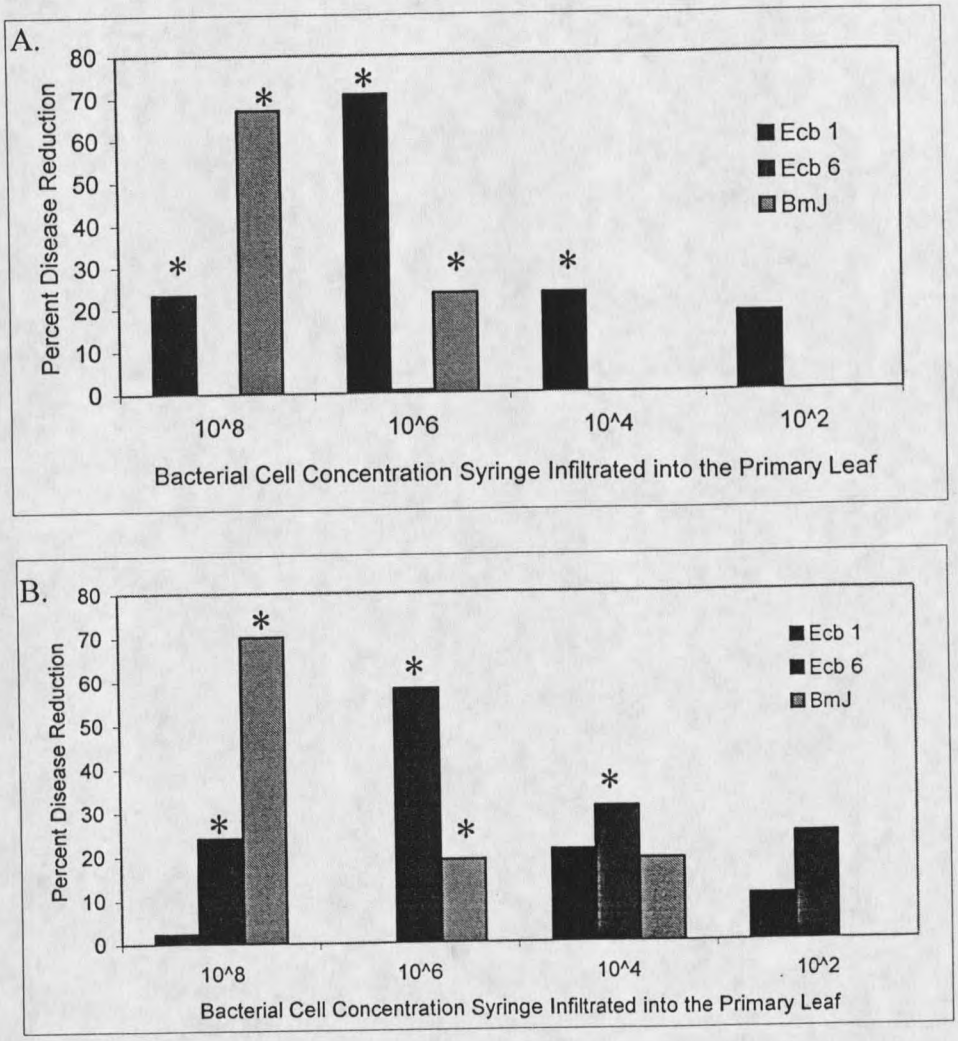


Figure 3-1. Systemic reduction of Cercospora leaf spot symptoms on sugar beet compared to a water-treated control following priming with *Erwinia carotovora* pv. *betavasculorum* isolates 1 (Ecb 1) and 6 (Ecb 6) or *Bacillus mycoides* isolate Bac J (BmJ) on two sugar beet cultivars C40 (A) and USH11 (B). Data are the mean of three independent experiments containing five replicates per treatment. Treatments demonstrating a statistically significant decrease in disease severity compared to the water-treated control are designated with an asterisk (*). Ecb1 and Ecb 6 are avirulent on C40 and USH11 respectively. The KWS rating for disease severity was 9.5±0.6 and 9.0± 0.5 for water-treated C40 and USH11 controls respectively.

Induction of Pathogenesis-Related Proteins in Sugar Beet

Three inoculation methods were examined to determine which treatment means provided the most consistent level of induction of pathogenesis-related proteins by BmJ and the two *Erwinia* isolates. Of the three treatment methods employed, syringe infiltration provided the most consistent induction of the sugar beet pathogenesis-related proteins chitinase and β-glucanase following treatment with the avirulent Ecb isolates and BmJ. Although the chitinase activity was slightly higher following BmJ treatment when compared to the incompatible interactions, the resultant β-glucanase activity was statistically equivalent. With both pathogenesis-related proteins the activities were approximately 2-fold greater than found in the water-treated controls (Table 3-1). The petiole stab treatment was effective at inducing both pathogenesis-related proteins in the incompatible interactions (Table 3-1). BmJ was moderately effective with stab inoculation and led to a slight induction of chitinase activity when compared to the water control, however on both cultivars the induction level was statistically lower than that elicited by the incompatible interactions (Table 3-1). BmJ stab inoculation did not induce β-glucanase in either cultivar (Table 3-1). Spray application of BmJ elicited a statistically significant induction of both pathogenesis-related proteins. However, that treatment was ineffective at inducing β-glucanase using Ecb 1 on C40 (Table 3-1). Ecb 6, when spray applied to USH11, elicited a statistically significant increase in β-glucanase activity when compared to the water infiltrated control, although it was statistically lower than the level induced by BmJ (Table 3-1). Both incompatible

interactions led to an induction of chitinase activity. However, in both instances the induction level was lower than that observed following BmJ infiltration. Treatment with water and the virulent pathogens, regardless of the method, rarely resulted in a statistically significant increase in chitinase or β-glucanase specific activity (Table 3-1).

The Oxidative Burst of Sugar Beet.

Induction of an oxidative burst was examined using phenol red oxidation. Biphasic hydrogen peroxide production was observed in both incompatible interactions (Figure 3-2A and B). In both instances, the primary burst was observed within 15 minutes and peaked at approximately 2.5 mM H_2O_2 (Figure 3-2A and B). Statistically equivalent primary peaks were observed with both compatible interactions and live and dead BmJ (Figure 3-2A and B). Both incompatible interactions elicited a secondary burst, absent in the compatible interactions and with dead BmJ infiltration. This burst occurred at approximately 2 hours post syringe infiltration and peaked at approximately 4 mM H_2O_2 (Figure 3-2A and B). BmJ elicited a secondary burst from both sugar beet cultivars as well. Although the timing was identical to that observed in the incompatible interactions, the H_2O_2 concentration was statistically lower at approximately 2.5 mM (Figure 3-2A and B).

To determine in the putative sugar beet receptor responsible for the recognition of BmJ used in the elicitation of systemic resistance was cytosolic or plasma membrane bound, phenol red oxidation was monitored in protoplasts as well as leaf disks. There was no statistical difference between the biphasic hydrogen peroxide production curve produced by sugar beet protoplasts and leaf disks (Figure 3-3).

Table 3-1. Systemic specific activities of 2 pathogenesis-related proteins following various treatments with *Erwinia carotovora* pv. *betavasculorum* isolates 1 and 6, water, and *Bacillus mycoides* isolate Bac J.

	Chitinase Specific Activity[1]					
	Stab		Syringe		Spray	
	C40[2]	USH11[2]	C40	USH11	C40	USH11
Water	1.56	1.52	1.22	1.53	1.22	1.53
Ecb 1[3]	2.35	1.66	2.90	1.66	1.78	1.59
Ecb 6[3]	2.06	2.30	1.40	2.70	1.35	2.79
BmJ[4]	2.05	1.91	3.33	3.17	2.70	3.56
LSD (0.05)	0.25	0.26	0.31	0.28	0.21	0.37

	Beta-Glucanase Specific Activity[5]					
	Stab		Syringe		Spray	
	C40	USH11	C40	USH11	C40	USH11
Water	39.3	58.2	41.9	64.3	37.4	49.8
Ecb 1	47.7	68.0	85.4	74.9	25.7	59.7
Ecb 6	45.5	75.8	42.1	114.3	34.6	93.7
BmJ	36.1	66.8	88.9	103.2	49.5	121.5
LSD (0.05)	6.0	11.6	20.3	13.0	11.9	26.1

[1] Chitinase specific activity as determined by chitinase plate assay is expressed as mg of N-acetyl-D-glucosamine released per hour per mg of apoplastic protein. The data are the mean of three independent experiments containing three replicates for each treatment.
[2] C40 and USH11 were the two cultivars of sugar beet tested for reactivity to both Ecb isolates and BmJ.
[3] *Erwinia carotovora* pv. *betavasculorum* isolates 1 (Ecb 1) and 6 (Ecb 6) were applied at 1×10^6 cells/ml. Ecb 1 and Ecb 6 are avirulent on C40 and USH11 respectively.
[4] *Bacillus mycoides* isolate Bac J (BmJ) was applied at 1×10^8 cells/ml.
[5] β-glucanase specific acivity as determined by laminarin digestion with the Nelson method is expressed as μg of glucose released per minute per mg of apoplastic protein. The data are the mean of three independent experiments containing three replicates for each treatment.
LSD=Least significant difference (probability = 0.05)

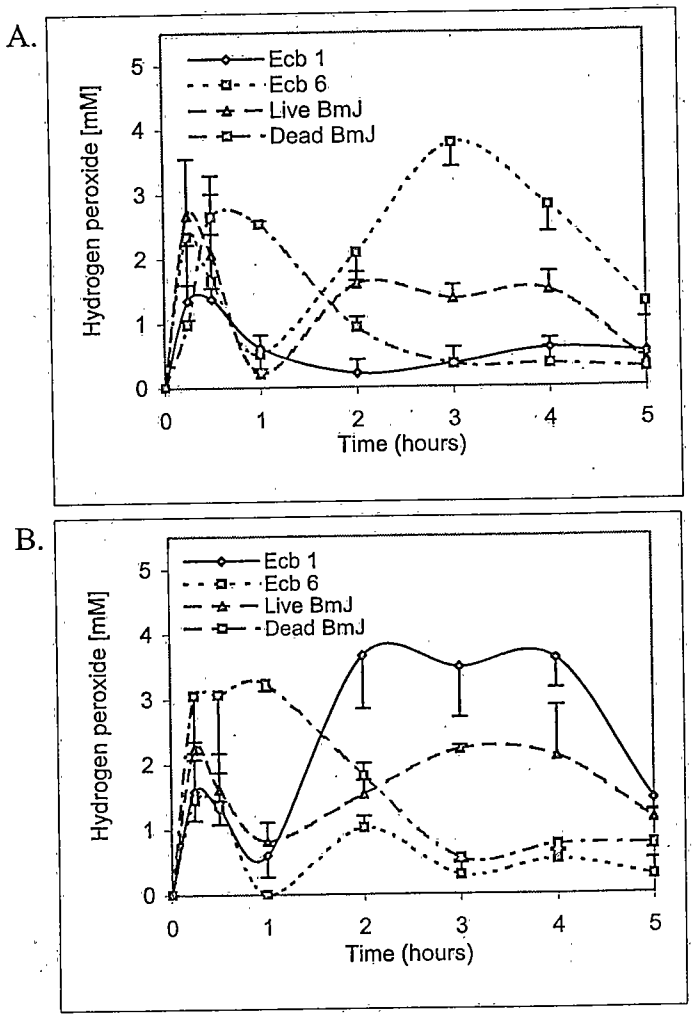


Figure 3-2. Hydrogen peroxide produced by two cultivars of sugar beet as determined by phenol red oxidation. (A) Amount of hydrogen peroxide produced by 12 USH11 leaf disks following syringe infiltration with 1 x 10^6 *Erwinia carotovora* isolates 1 (Ecb1; virulent) and 6 (Ecb 6; avirulent), and 1 x 10^8 live and dead *Bacillus mycoides* isolate Bac J (BmJ) after subtracting the background phenol red oxidation produced in plants syringe infiltrated with water only. The data are the mean of three independent experiments containing two replicates for each treatment. (B) Amount of hydrogen peroxide produced by 12 C40 leaf disks following syringe infiltration with 1 x 10^6 *Erwinia carotovora* pv *betavasculorum* isolates 1 (Ecb 1; avirulent) and 6 (Ecb 6; virulent), and 1 x 10^8 live and dead *Bacillus mycoides* isolate Bac J (BmJ) after subtracting the background phenol red oxidation produced in plants syringe infiltrated with water only. The data are the mean of three independent experiments containing two replicates for each treatment.

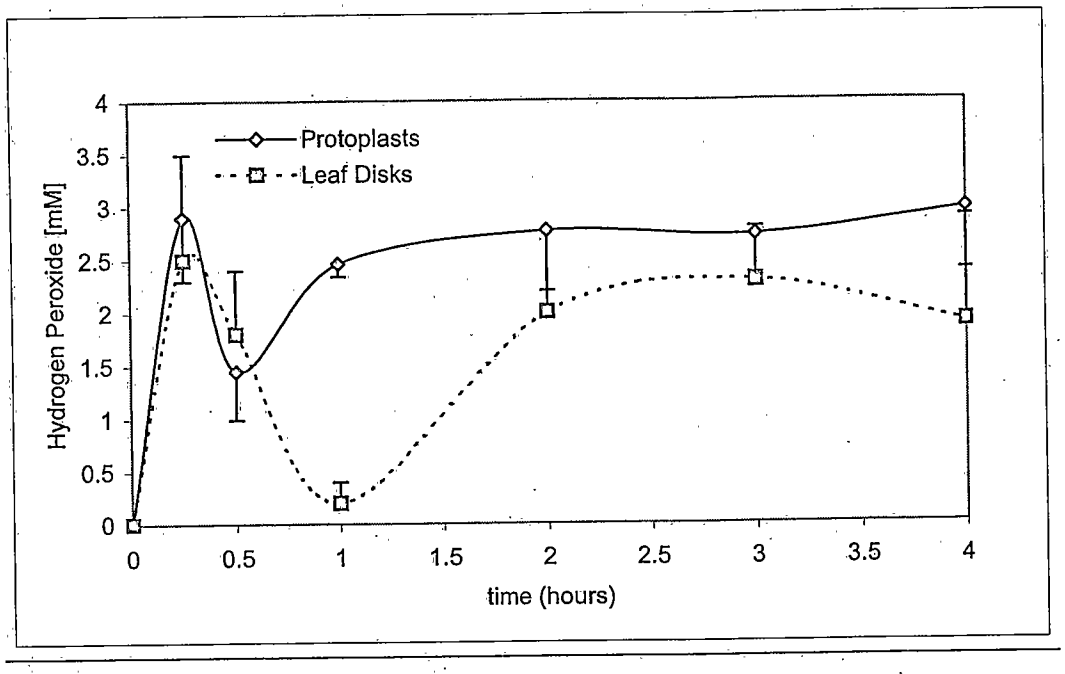


Figure 3-3. *Bacillus mycoides* isolate Bac J (1 x 10^8 cells/ml) treatment resulted in biphasic hydrogen peroxide production, as determined by phenol red oxidation, regardless of the medium used. There was no statistical difference between the phenol red oxidation using protoplasts (250/reaction) or leaf disks (12/reaction), following the subtraction of background phenol red oxidation as determined by the amount of phenol red oxidized by protoplasts, leaf disks and BmJ alone. Data are the mean of three independent experiments containing two replicates per treatment.

Signaling in BmJ-Sugar Beet Interactions

It was previously determined that BmJ is an epiphyte on sugar beet (Bargabus et al, 2002), therefore signals for the establishment of systemic resistance must be able to enter in through the plant leaf. To examine the possible role of stomata in the relaying of signals, the oxidative burst following spray application of BmJ in the dark was observed. Since treatment of sugar beet with any microorganism, regardless of systemic resistance induction capability, resulted in a primary burst of hydrogen peroxide, in this case observations were limited to the secondary hydrogen peroxide burst (Figure 3-4B). The

secondary peak elicited in sugar beet by BmJ under green light conditions was statistically equivalent to that elicited by BmJ in the light (Figure 3-4A). Prior to analysis of phenol red oxidation, the stomata were observed. Under green light conditions the stomata were closed within fifteen minutes (Figure 3-4C); in the light the stomata remained open (Figure 3-4D).

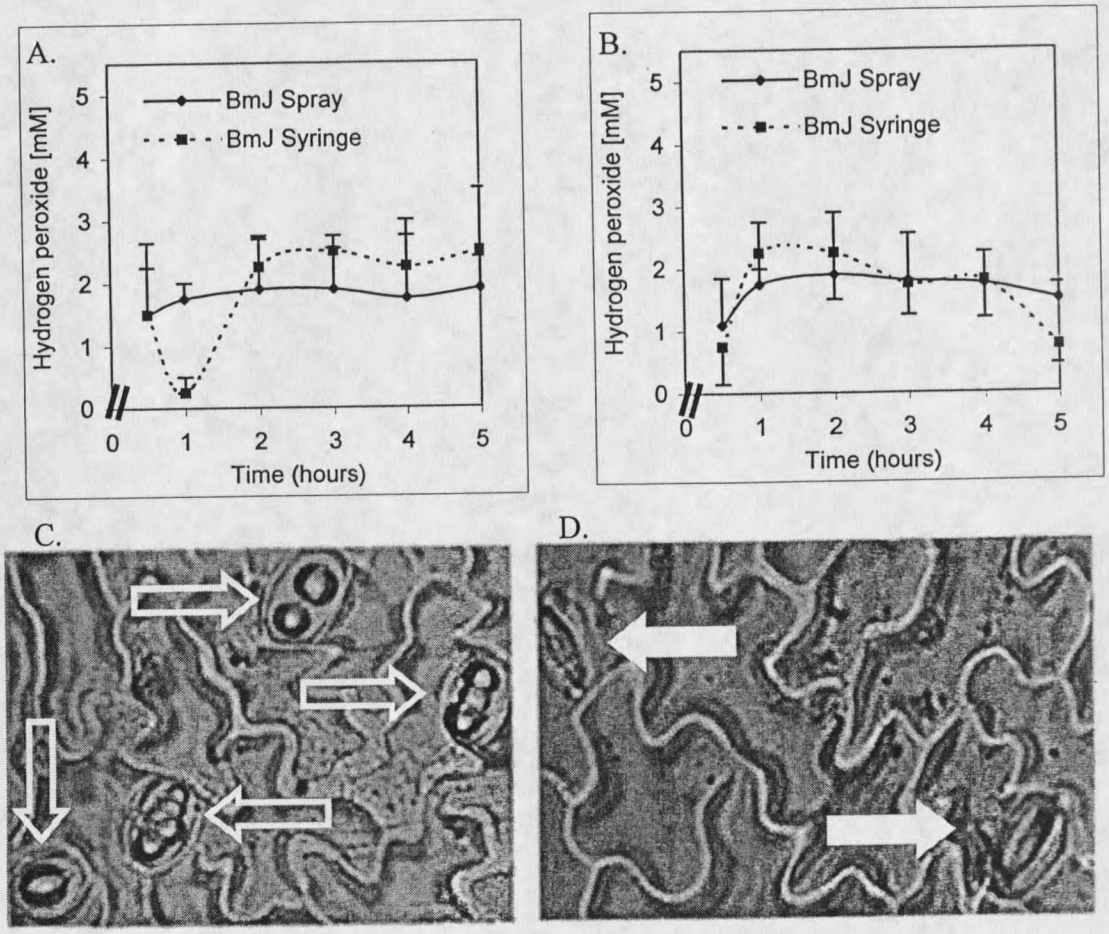


Figure 3-4. Secondary peak of hydrogen peroxide produced by sugar beet, as measured using phenol red oxidation, following spray application and syringe infiltration of *Bacillus mycoides* isolate Bac J (BmJ) in the light (A) and under green light conditions (B) after subtracting the background phenol red oxidation produced in plants with water spray applied and syringe infiltrated respectively. In the light stomata (open arrows) were open (C) and in the dark stomata (filled arrows) were closed (D).

Cell Death in Sugar Beet

Both the compatible and the incompatible interactions resulted in sugar beet cell death. In the former case, necrotic death of the infiltrated tissue was observed within one week following treatment, followed by systemic spread of the pathogen (data not shown). In the latter case, a discrete hypersensitive-like lesion formed in the immediate area in and around the infiltration site within one week following treatment that appeared to prevent the systemic spread of the pathogen (Figure 3-5 A-F). Regardless of the incubation time, BmJ infiltration failed to result in any cell death beyond that observed with the water control (Figure 3-5 A-F).

Discussion

Induction of systemic plant defense responses has been observed in several plants following treatment with pathogens (Hutchenson, 1998; Kuc, 1992), biological control agents (Pal et al, 2001; van Loon and Pieterse, 2002; Bargabus et al, 2002), phytohormones (Cohen, 2002), and chemicals (Tally et al, 1999). The typical means of demonstrating induction of these responses is through challenge assays in which distal, untreated leaves are challenged with a pathogen following a short priming period with an inducing agent on a primary, spatially separated leaf or root system (Conrath et al, 2002). These experiments are often followed by characterization of pathogenesis-related protein production that are molecular markers of systemic resistance induction (van Loon and van Strein, 1999). In the current report, treatment with BmJ or avirulent *Erwinia*

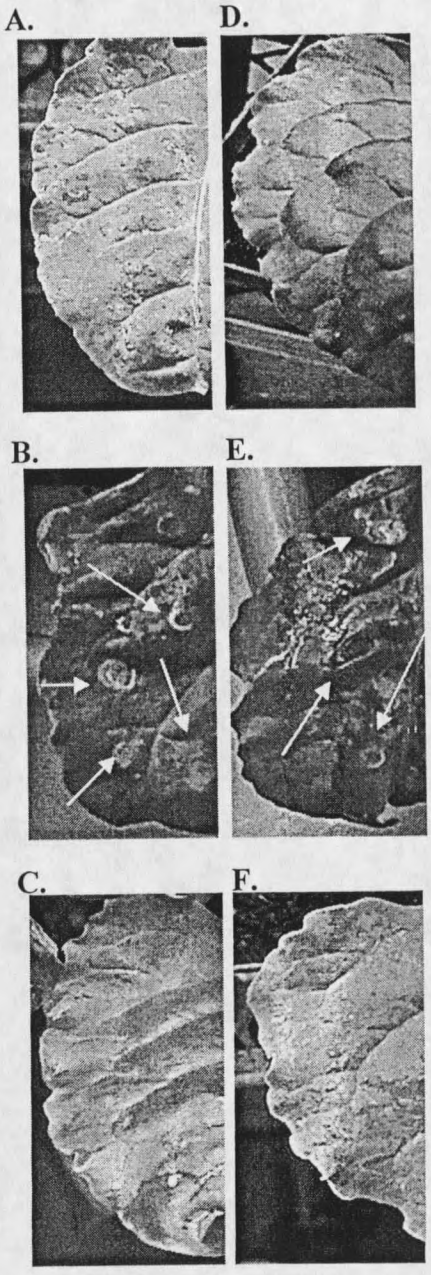


Figure 3-5. Response of sugar beet leaves following syringe infiltration with water (A and D), *Erwinia carotovora* pv *betavasculorum* isolates 1 (B) and 6 (E), and *Bacillus mycoides* isolate Bac J (C and F) on sugar beet cultivars C40 (A-C) and USH11 (D-F). Incompatible interactions (B and E) resulted in localized hypersensitive-like cell death (indicated with arrows). Compatible interactions (data not shown) resulted in necrosis and eventual systemic spread of the pathogen. Biological control agent (C and F) and water infiltration (A and D) resulted in no macro- or microscopic cell death.

isolates resulted in significant disease control of *C. beticola* on sugar beet (Figure 3-1). Additionally, BmJ and avirulent Ecb treatment resulted in a 2-fold increase in chitinase and β-glucanase specific activity, indicative of systemic resistance induction (Table 3-1). Other reports have shown *Erwinia* spp. can also induce systemic resistance in *Arabidopsis* (Dong et al, 1999; Norman-Setterblad et al, 2000; Brader et al, 2001; Kariola et al, 2003).

One hundred times more BmJ cells were required to elicit statistically equivalent disease control as the incompatible Ecb-sugar beet interactions. Furthermore, the differences in responsiveness to BmJ and Ecb by sugar beet were exemplified by the different responses induced by the treatment methods as measured by chitinase and β-glucanase activity. Stab inoculation, which limits the cell concentration, was not an effective means to inoculate BmJ as no induction of systemic resistance was observed, while it was a very effective inoculation method for the induction of systemic resistance markers in incompatible interactions. On the other hand, syringe infiltration and spray application of BmJ, both of which deliver high cell titers, were effective methods for the induction of chitinase and β-glucanase (Table 3-1).

The large discrepancy in BmJ and avirulent Ecb cell concentration requirement for systemic resistance elicitation may be due to differences in pathogen signal delivery and plant signal recognition. *Erwinia* spp. are gram-negative bacteria that assemble type III secretion systems for the intracellular delivery of signals, including *avr* gene products (Venisse et al, 2002; Kariola et al, 2003). Certain constituents found in the apoplast stimulate *hrp* gene activation needed for type III secretion system formation (Rahme et

al, 1992), without which certain pathogenesis-related proteins cannot be produced (Kariola et al, 2003). Interestingly, spray application of avirulent Ecb isolates was the only approach that was ineffective at inducing resistance in sugar beet with the incompatible interactions, presumably since cues for type III secretion system development were not available through external application. BmJ, a gram-positive, epiphytic bacterium, lacks the capability to form such specialized delivery machinery. However, we have hypothesized that the sugar beet receptor(s) for BmJ perception are either cytosolic or plasma membrane bound, since the oxidative burst, the earliest response in the establishment of plant immunity (Grant and Loake, 2000), is identical when using protoplasts or leaf disks (Figure 3-3). Therefore our hypothesized means of entry for the BmJ signal is either acquisition through the stomata or diffusion through the cuticle and cell wall. The former has been eliminated as a possible means of entry due to the observed oxidative burst with BmJ treatment under green light conditions where a clear secondary burst occurred even when the stomata were closed (Figure 3-4). An alternate explanation for inefficient signaling in BmJ-sugar beet interactions could be due to imperfect recognition of the signal by sugar beet. In this case, both cultivars responded to treatment with BmJ equally as well, suggesting that if a recpetor for BmJ exists, it is conserved in C40 and USH11. These hypotheses are based on one major assumption: sugar beet has an innate means of detecting the signal produced by BmJ.

BmJ is non-pathogenic and therefore poses no real threat to sugar beet, yet sugar beet exhibits biochemical changes that are characteristic of avirulent pathogen-plant interactions. There is one published account of *Bacillus mycoides* causing bacteriosis of

sugar beet at varying developmental stages (Stankiewicz and Krezel, 1984). It is possible these observations were made using a different *B. mycoides* isolate or sugar beet cultivar. Since BmJ treatment has not resulted in disease on cultivars C40, USH11, Seedex or Holly Hybrid 88, the former seems more likely. Additionally, there are no references to *Bacillus mycoides*-related disease in the Compendium of Beet Diseases and Insects (Whitney and Duffus, 1991). Plants contain several recognition mechanisms for other non-pathogenic microorganisms, such as mycorrhizal fungi and rhizobia. In fact, the formation of mycorrhizal associations has also been shown to induce systemic resistance (Feugey et al, 1999; Pozo et al, 2002). The fact that BmJ naturally establishes itself and proliferates on the sugar beet leaf could be an indication that it is involved in a mutualistic association with the plant, as seen in other Bacilli-plant interactions (Beattie, 2002). Recently the genetic basis controlling biological control agent-plant interactions has begun to emerge (Smith et al, 1999; Simon et al, 2001). In tomato, a quantitative trait locus has been identified that is linked to suppression of disease by *Bacillus cereus* (Smith et al, 1999), a close relative of BmJ (von Wintzingerode et al, 1997). It is possible that similar genetic recognition of BmJ exists within sugar beet.

The oxidative burst, following BmJ recognition, is another example of sugar beet responding to BmJ as though it were an avirulent pathogen. Pathogen-induced oxidative burst prior to systemic resistance elicitation has been extensively studied and characterized as biphasic production of hydrogen peroxide (Baker and Orlandi, 1995; Glazener et al, 1996; Jabs et al, 1997; Rajasekhar et al, 1999; Cessna et al, 2000; Martinez et al, 2000; Wolfe et al, 2000). Several biological control agents have been

shown to induce plant defense responses (Pal et al, 2001; van Loon and Pieterse, 2002), but to date it has not been determined if the elicitation of systemic resistance in these cases is proceeded by an oxidative burst. To study hydrogen peroxide concentrations in intact leaf tissue, the phenol red oxidation method was substituted for the more widely used luminol-dependent chemiluminescence approach that requires the use of protoplasts or cell suspension cultures (Glazener et al, 1991). BmJ elicited an oxidative burst in sugar beet similar in timing, but not intensity, to that elicited during the incompatible interactions (Figure 3-2). With BmJ there was an approximate 2-fold reduction in the overall concentration of hydrogen peroxide being produced in the secondary peak (Figure 3-2A and B). That difference becomes even more pronounced when it is taken into account that 100-fold fewer Ecb cells elicited a greater oxidative response. These data demonstrate the oxidative burst preceding the induction of systemic resistance is not limited to plant-pathogen interactions as shown by other's work with symbionts (Santos et al, 2001) and now our contribution with Bacilli biological control agent induction. Additionally, the oxidative response was only seen following live cell treatment, a requirement for effective disease control as well (Bargabus et al, 2002), suggesting active production and secretion of a metabolite that is recognized by sugar beet.

In many instances, the biphasic hydrogen peroxide production during the oxidative burst has been tied to the orchestration of hypersensitive cell death (de Pinto et al, 2002; Dat et al, 2003). However several reports have now indicated the oxidative burst and hypersensitive response occur independently (Baker and Orlandi, 1995; Glazener, 1996; Dorey et al, 1999; Sasabe et al, 2000). The observation that BmJ can

elicit biphasic production of hydrogen peroxide in the absence of hypersensitive cell death provides more evidence to the newly emergent idea that hydrogen peroxide is not sufficient in and of itself to cause cell death. Recently, the role of hydrogen peroxide in plant defense has been shown to vary in a concentration-dependent manner. Low to moderate concentrations stimulate the production of protective antioxidant enzymes and activate systemic resistance signal transduction cascades (Solomon et al, 1999). In contrast, moderately high to extremely high levels lead to programmed cell death and necrosis respectively (Fleury et al, 2002). The BmJ-Ecb comparison works well to demonstrate these points since they both effectively induce resistance to the same extent, but differentially induce cell death. Other recent reports provide evidence that hydrogen peroxide is not the single, key player in cell death orchestration. Concomitant production of nitric oxide and hydrogen peroxide leads to cell death, even at concentrations that are ineffective when they occur independently of one another, further supporting synergistic interactions as a requirement for hypersensitive cell death (de Pinto et al, 2002). Nevertheless, these two chemical species act independently of one another to induce various defensive compounds (Delledonne et al, 1998). Therefore, differential production of hydrogen peroxide and nitric oxide may be the chemical basis for having different cell death responses following BmJ and avirulent *Erwinia carotovora* pv. *betavasculorum* treatment while having no effect on systemic resistance induction. Mitochondria have also been recently shown to play a central role in plant programmed cell death (Lam et al, 2001) through the translocation of cytochrome *c* into the cytosol that ultimately leads to the procaspase cleavage and activation of a caspase cascade (Balk

et al, 1999). The latter chemical changes in the host may be occurring following priming with the avirulent Ecb isolates, however it is unlikely that mitochondrial signaling plays a role in BmJ-elicited systemic resistance since there is an absence of cell death.

Future work could include the elucidation of the underlying chemical differences leading to and preventing cell death in sugar beet following treatment with the avirulent *Erwinia* isolates and BmJ respectively. Examination of the localized production of antioxidant enzymes, caspase activation, and nitric oxide production would be included in these studies. Lastly, the stage has been set for future isolation and characterization of putative BmJ effector molecules and sugar beet receptors.

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CHAPTER 4

HOST RESPONSE-BASED SCREENING FOR THE IDENTIFICATION OF POTENTIAL BIOLOGICAL CONTROL AGENTS

Introduction

Screening thousands of microorganisms to find potential biological control agents has typically involved processes that are time consuming, labor intensive, error prone, non-specific and serendipitous. Brute force methods like *in vitro* gel diffusion assays make it possible to examine the production of enzymes or antibiotics by microorganisms that are deleterious to the target pathogen (Jacobsen and Backman, 1993; Dunne et al, 2000; Krechel et al, 2002). However, when applied *in vivo* these antagonists often prove ineffective (Ji and Wilson, 2002). The conflicting results between *in vitro* and *in vivo* studies may be a result of differences in media and the nutrients available in the environment that affect enzyme or antibiotic production (Shoeman et al, 1994) or spatial constraints between the antagonist and the target pathogen often found *in planta* (Zhou et al, 2002). Additionally, pH (Ownley et al, 2003), water availability (Hase et al, 2001) and potential competitive interactions with native microflora (Brimecombe et al, 2000) affect the antagonist's ability to establish a suitable cell density, a critical requirement for biological control agent effectiveness (Bull et al, 1991; Parke, 1990; Paulitz and Baker, 1987). To examine efficacy and population establishment simultaneously, *in planta* disease challenge experiments have been developed (May et al, 1997). These assays

usually take months for completion for each organism being tested. However, replacing mature plants with seedlings (Han et al, 2000; Handelsman et al, 1991) or leaf disks (Fiddaman et al, 2000) has reduced the time needed to 17 or 18 days.

Reliance on antibiosis as the mode of action to control plant disease places limits on the range of effectiveness of the biological control agent. The enzyme(s) and/or antibiotics produced by the biological control agent tend to target a specific type of pathogen (i.e. all fungal phytopathogens) or a subset there within (i.e. only *Fusarium* spp.) (Mohapatra et al, 2002). To address this point, broad-spectrum modes of action, like the induction of plant defense responses by plant growth-promoting rhizobacteria (PGPR) (Alstrom, 1991; van Peer et al, 1991; Murphy et al, 2000; Zhang et al, 2001; van Loon and Pieterse, 2002), are being employed. However, PGPR-induced systemic resistance (ISR) is much less effective than other forms of plant immunity such as systemic acquired resistance (SAR) (Hammond-Kosack and Jones, 2000). Additionally, SAR is associated with pathogenesis-related (PR) proteins, known molecular markers (van Loon and van Strein, 1999) that could be exploited for identification of inducers. ISR lacks known molecular markers (Pieterse et al, 1998) therefore identification of PGPRs is largely serendipitous. To date, no one has devised a reliable, systematic approach for the discovery of novel SAR-eliciting biological control agents. Therefore it is likely that many optimal biological control agents with this mode of action have not yet been identified.

We have previously shown that biological control agent, *Bacillus mycoides* isolate Bac J (BmJ), is capable of inducing SAR in sugar beet (Bargabus et al, 2002). We found

two molecular markers associated with pathogen-induced SAR, β-glucanase and chitinase, induced during BmJ-plant interactions (Bargabus et al, 2002). In the past, identification of SAR-inducing biological control agents using biochemical markers has had limited success (Siegrist et al, 1998) largely due to the choice of markers for use in the screen. Therefore lengthy disease challenge assays, followed by mode of action studies has been the main method for identification. We propose monitoring specific activity levels of the two aforementioned enzymes, and active oxygen species (AOS) production prior to *in planta* disease challenge assays could provide a reliable, high-throughput method of screening for biological control agents.

The objectives of this research were to use a pool of *Bacillus pumulis* isolates, containing both SAR-inducing and non-inducing isolates, to determine if the host oxidative and biochemical changes observed during BmJ-sugar beet interactions also occurred with other Bacilli-sugar beet interactions following SAR induction. An additional objective was to determine which assay method(s) was most reproducible and correlated best with systemic disease control of *Cercospora beticola*. These assays could be used as a primary screen to reduce the total number of isolates used in subsequent disease challenge assays, thereby reducing the total amount of time needed for SAR-inducing biological control agent identification.

Materials and Methods

Plant Culture

Sugar beet varieties Holly Hybrid 88 and Seedex 920002 were seeded into flats for germination, transplanted into 10.2 cm pots after one week, and grown in the glasshouse for 6 weeks in MSU mix (1/3 sand, 1/3 peat and 1/3 topsoil plus the wetting agent Aquagrow 2000). Plants were maintained at 24°C±2°C and watered daily and fertilized twice a week to maintain vigorous growth. The photoperiod was 16 h light and 8 h dark.

Bacterial Cultures

Bacillus mycoides isolate Bac J (BmJ) was originally isolated from the phylloplane of sugar beet grown in Sidney, Montana in 1994. *Bacillus pumulis* isolates 203-11, 341-21-15, 203-6, 341-20-14, 241-20-1, 203-3, 203-4, 341-16-5, and 203-7 were isolated from embryos of germinating sugar beet seeds in 1997. *Bacillus pumulis* isolates BMH5E-33 and BMH5E-40 were isolated from the sugar beet rhizosphere in Sidney, Montana in 1997. All isolates were stored at -80°C in 10 % glycerol and 1% tryptic soy broth (Difco). For fresh cell preparations, the bacilli were cultured in tryptic soy broth for 48 h (28°C). Cells were centrifuged 15 minutes at 10,000 x g (4°C), washed with sterile water (2x), and resuspended in distilled water. The optical density was adjusted to A600=1.0, and diluted 1:2 to obtain approximately 1 x 10^8 cfu/ml.

Fungal Culture

Cercospora beticola (CB) isolate EC3 (isolated in Sidney, Montana in 1996) was grown on V-8 agar for a minimum of two weeks with exposure to fluorescent or natural light for at least one week to promote sporulation. Spores were harvested approximately 30 days after plating in 0.1% carboxymethylcellulose by scraping with a cotton swab, counted with a hemocytometer and adjusted to 1×10^4 spores/ml.

Disease Control Assays

Sugar beet cultivars Seedex 900012 and Holly Hybrid 88, in replicates of 10, were treated with one of the Bacilli biological control agent candidates, BmJ, Acibenzolar-S-methyl (ASM), or distilled water with an aerosol sprayer to the leaf penultimate to the oldest true leaf. This leaf was then immediately bagged to ensure spatial separation from *C. beticola*, which was applied 3 days later at a rate of 1×10^5 spores/ml to near run-off on the remaining leaves using an aerosol sprayer. The sugar beets were kept at 28±2°C and 100% relative humidity for 48-72 hours. Plants were kept at 28±2°C until disease symptoms developed and were rated for disease at 21 days post inoculation using the KWS scale (Kleinwanzler, 1970).

Apoplastic Protein Extractions

The leaf penultimate to the oldest true leaf was treated with one of the Bacilli biological control agent candidates, BmJ, ASM, or distilled water, in replicates of 3, with an aerosol sprayer, and immediately bagged. The plants were kept at 28±2°C for 6 days

at which time the apoplastic proteins were collected as described by Klement (1965), with the following modification: a buffer containing 150 mM NaCl and 25 mM MES, pH 6.2 was substituted for distilled water. The proteins were quantified using Bradford reagent (BioRAD) using bovine serum albumin standards and frozen at -80°C until analyzed.

The Nelson Method for β-Glucanase Activity

The specific activity of β-glucanase was determined by measuring the rate of release of glucose from laminarin. Apoplastic protein (1.0 μg per triplicate sample) was added to 60 μl of 100 mM sodium acetate buffer (pH 5.0) and 20 μl of laminarin (Sigma), and was incubated for 60 minutes at 37°C. Immediately following incubation, 900 μl of water was added to each reaction to bring the final volume to 1 ml. After adding 1 ml of alkaline copper reagent (Somogyi, 1952) to each reaction, the tubes were boiled for ten minutes. The tubes were then cooled on ice and 1 ml of arsenomolybdate color reagent (Nelson, 1944) was added to each reaction, followed by 10 ml water. The OD_{660} was determined for each against a blank that contained laminarin. A standard curve was established using glucose (5-25 μg). The specific activity was expressed as μg of glucose released/minute/mg of apoplastic protein.

Aniline Blue Plate Assay for β-glucanase Activity

Apoplastic protein (0.75, 0.50, 0.25 μg for each sample in replicates of 3) was added to 3-mm diameter wells in a 0.5% agarose gel containing 0.005% aniline blue (MCB) and 0.5 mg/ml laminarin (from *Laminaria digitata*, Sigma) in a 14 cm diameter

glass petri plate, along with laminarinase standards (*Penicillium* spp., Sigma). The plates were incubated at 30°C for 18-24 hours. Following incubation, the pink lytic zones on a blue background were measured while the plate was on a white light source. Specific activity (μg of glucose released/min/mg of apoplastic protein) was determined by comparison of the lytic zone diameter of the standards and the apoplastic samples.

Glycol Chitin Plate Assay for Chitinase Activity

Apoplastic protein (0.009, 0.006, 0.005 μg for each sample in replicates of 3) was added to wells in a 1% agarose gel containing 0.01% glycol chitin in a 14 cm diameter glass petri plate, along with chitinase standards (*Streptomyces griseus*, Sigma). The plates were incubated at 37°C for 24 hours. Following incubation, 50 ml of 500 mM Tris-HCl (pH 8.9) containing 0.01% fluorescent brightener 28 was added to the plate for 10 minutes. The plate was then rinsed three times with distilled water, flooded with water and allowed to destain in the dark for 2-24 hours. The non-fluorescent lytic zones on a fluorescent background were measured while the plate was on a UV light source. Specific activity (mg of N-acetyl-D-glucosamine released/hour/mg of apoplastic protein) was determined by comparison of the lytic zone diameter of the standards and the apoplastic samples (Velasquez, 2002).

Sugar Beet Protoplast Generation

Sugar beet protoplasts were isolated from sugar beet leaves to provide a medium to measure hydrogen peroxide production. Sugar beet leaves were gently brushed on the adaxial and abaxial surfaces with a soft bristle brush to create small abrasions. The

leaves were then cut into 1 cm strips and vacuum infiltrated for 5 minutes with 0.7M sucrose containing 3.8% $CaCl_2$, CPW salts (Frearson et al, 1973), 1.2 % cellulase (Sigma) and 0.4% macerozyme (ICN Biomedicals Inc). The infiltrated leaves were incubated in the 0.7M sucrose-salt and enzyme solution for 24-48 hours in the dark. Following incubation, the enzyme solution was gently removed and the protoplasts were released into 0.7M sucrose containing 3.8% $CaCl_2$ and CPW salts by gently shaking the leaf strips in the solution.

Phenol Red Oxidation for Hydrogen Peroxide Production

To determine if the Bacilli isolates elicited biphasic hydrogen peroxide production in sugar beet, phenol red oxidation assays were performed according to Pick and Keisari (1980). Both protoplasts (250 protoplasts/reaction) and whole leaf disks (12 disks/reaction) were used to study the plant response. When using protoplasts, an external source of peroxidase (type II horseradish peroxidase, Sigma) was added to each reaction while in the latter case, the peroxidase contained within the added leaf disks was sufficient for the oxidation reaction. Phenol red reactions were run with each Bacillus isolate alone, protoplasts alone, leaf disks alone, Bacillus-treated leaf disks and combinations of Bacillus isolates and protoplasts. Bacillus-treated leaves were washed thoroughly before adding to each reaction to ensure that all bacteria had been removed and therefore were not contributing to the oxidation of phenol red. To calculate the amount of hydrogen peroxide produced in each instance, the A470 was compared to a standard curve established using 0-40mM hydrogen peroxide and 6.2 μg/ml type II horseradish peroxidase. The amount of hydrogen peroxide produced from protoplasts in

response to Bacillus treatment was calculated as follows: amount of hydrogen peroxide produced in Bacillus-protoplasts reaction – (amount of hydrogen peroxide produced in protoplast only reaction + amount of hydrogen peroxide produced in Bacillus only reaction). The amount of hydrogen peroxide produced by the leaf disks in response to Bacillus treatment was calculated as follows: amount of hydrogen peroxide produced by Bacillus-treated leaf disks – amount of hydrogen peroxide produced by leaf disks only reaction.

Results

Disease Controlling Organisms

Of the fourteen different treatments applied to sugar beet cultivars Seedex 900012 and Holly Hybrid 88, four resulted in economically significant disease control (economically significant disease control = > 50% disease reduction). The disease-controlling Bacilli included BmJ and *Bacillus pumulis* isolates 203-6 and 203-7 while the chemical inducer of systemic acquired resistance, ASM, also controlled disease. *Bacillus pumulis* isolates 241-20-1 and BMH5E-40 reduced Cercospora leaf spot symptoms to a statistically significant level, but <50 % reduction presumably would not make the bacteria commercially desirable candidates (Table 4-1).

Table 4-1. Disease reduction capabilities of the pool of *Bacillus pumulis* isolates, *Bacillus mycoides* isolate Bac J, Acibenzolar-S-methyl and water used in development of the host response-based high-throughput screen method for the identification of biological control agents.

	Percent Disease Reduction at 21 days post challenge with *Cercospora beticola*[1]	
Treatment[2]	HH88[3]	Seedex[3]
Water	0.0%	0.0%
203-3	0.0%	0.0%
203-4	0.0%	0.0%
203-6	73.7%	80.5%
203-7	70.3%	68.4%
203-11	0.0%	0.0%
BMH5E-33	0.0%	0.0%
BMH5E-40	35.7%	29.4%
341-20-14	0.0%	0.0%
341-20-15	0.0%	0.0%
241-20-1	34.0%	39.4%
341-16-5	0.0%	0.0%
BmJ[4]	65.9%	66.7%
ASM[5]	63.3%	64.9%

[1] Percentage represents the level of systemic disease reduction as compared to water-primed 21 days post challenge with *Cercospora beticola*. Data are the mean of 10 plant treatments.
[2] Numbers in the treatment column (203-3 → 341-16-5) represent the isolate number of the *Bacillus pumulis* included in the pool. All treatments were applied to the leaf penultimate to the oldest true leaf and immediately bagged. The primed plants were allowed a three day incubation period before challenging with *Cercospora beticola.*
[3] HH88 and Seedex were the two cultivars of sugar beet used in the systemic disease challenge assays.
[4] BmJ = *Bacillus mycoides* isolate Bac J, a known systemic acquired resistance-inducing biological control agent on sugar beet.
[5] ASM = Acibenzolar-S-methyl, a known chemical inducer of systemic acquired resistance.

β-Glucanase as a Predictor of Disease Control

The Nelson method was an inferior assay when compared to aniline blue plates for the determination of β-glucanase activity in apoplast protein extracts. With the Nelson method, the standard deviation was twenty-two percent between replications of one plant treatment and twenty-eight percent between plants within a treatment (Table 4-2). Additionally, the precision was extremely low, having achieved a different result between runs fifty percent of the time (Table 4-3). The accuracy with the Nelson method was less than desirable. Within each individual experiment there was anywhere from twenty to eighty percent false positive and twenty-five to fifty percent false negative identification respectively (Table 4-3). Completion of five independent experiments resulted in 6 false positive identifications (Table 4-4). Using the aniline blue plate assay resulted in standard deviations of eleven percent between replications of one plant treatment and twenty-one percent between plants within a treatment (Table 4-2). In comparison to the Nelson method, the precision was greatly improved, receiving the same result between experiments ninety-six percent of the time (Table 4-3). Additionally, an improvement was seen in the precision using the aniline blue plate assay with few false positive identifications (0-4% between runs) and no false negative identifications (Table 4-3). The cumulative results of two independent experiments yielded only one false positive identification (Table 4-4).

Table 4-2. Precision and method analysis of the biochemical and oxidative assays to be used as potential indicators of biological control agent systemic acquired resistance induction.

	Nelson Method[1]	Aniline Blue Plant Assay[1]	Glycol Chitin Plate Assay[2]	Phenol Red Oxidation[3]
Average Deviation Between Replicates with One Plant Treatment[4]	22%	11%	10%	13%
Average Deviation Between Plants within One Treatment[4]	28%	21%	17%	25%
Level (μg) of Sensitivity[5]	2.5-4.0	0.1-1.0	0.005-0.01	12 leaf disks/ 250 protoplasts
Time (h) Required to Run 1000 Samples[6]	23.3	10	10	200

[1] The Nelson method and aniline blue plate assays are both methods for the detection of β-glucanase activity in apoplastic protein samples from *Bacillus pumulis*-, *Bacillus mycoides*-, acibenzolar-S-methyl- and water-treated plants.
[2] Glycol chitin plate assay was the method used for the detection of chitinase activity in apoplastic protein samples from *Bacillus pumulis*-, *Bacillus mycoides*-, acibenzolar-S-methyl- and water-treated plants.
[3] Phenol red oxidation was the method used for examining hydrogen peroxide production patterns in sugar beet protoplasts and leaf disks following treatment with the *Bacillus pumulis* isolates, *Bacillus mycoides* isolate Bac J, and water.
[4] The percent deviation is the mean of 5 independent experiments for the Nelson method and glycol chitin plate assays, the mean of two independent experiments for the aniline blue assay and the mean of three independent experiments for the phenol red oxidation.
[5] Level of sensitivity is the lowest amount of apoplastic protein in μg that can be detected in the Nelson method and aniline blue and glycol chitin plate assays.
[6] Represents average time in hours required to perform 1000 1 ml reactions of the Nelson method, aniline blue and glycol chitin plate assays and phenol red oxidation.

Table 4-3. Statistical results of the host biochemical and oxidative response based high-throughput screening method for the identification of potential systemic acquired resistance-inducing biological control agents.

	Glucanase Specific Activity[1]							Chitinase Specific Activity[2]					Biphasic Active Oxygen[3]		
	The Nelson Method					Aniline Blue Plate		Glycol Chitin Plate					Phenol Red Oxidation		
Treatment	**Rep 1**	**Rep 2**	**Rep 3**	**Rep 4**	**Rep 5**	**Rep 1**	**Rep 2**	**Rep 1**	**Rep 2**	**Rep 3**	**Rep 4**	**Rep 5**	**Protos 1**	**Protos 2**	**Disks**
Water	47.2e	40.7f	189.8fg	77.3cd	64.7ef	2.3f	3.2de	1.0c	1.4hi	1.0b	2.3h	2.1e	no	no	no
203-3	*124.3ab*	*133.4c*	206.6def	65.4de	73.2cdef	2.8ef	3.6cd	*1.8ab*	*3.2bc*	*3.3a*	2.3gh	2.1e	no	no	no
203-4	*96.7bcd*	96.7def	189.4f	86.3cd	82.6cdef	2.7ef	4.0cd	*1.8ab*	1.6hi	1.5b	3.1efgh	2.1e	no	no	no
203-6	*110.3abc*	*123.1cd*	132.2g	96.4bc	*88.0bcd*	*4.5c*	*5.3b*	*2.2b*	*2.7def*	*2.7a*	*4.4abcd*	*2.8cd*	yes	yes	yes
203-7	*159.6abc*	*166.1b*	*216.9de*	97.1bc	*113.2a*	*5.1bc*	*6.4a*	*3.4a*	*4.2a*	*4.4a*	*4.9ab*	*3.2a*	yes	yes	yes
203-11	71.4de	*62.7e*	*292.5ab*	*113.9ab*	*88.1bcd*	*4.8c*	3.2de	1.1c	2.0gh	1.4b	3.6defgh	2.1e	no	no	no
BMH5E-33	73.5cde	*170.7b*	*259.5bc*	78.4cd	67.4def	2.6ef	3.0de	1.7c	1.7hi	1.5b	*3.9cdef*	2.1e	no	no	no
BMH5E-40	39.1e	*181.3b*	202.4ef	51.4e	*89.0bc*	1.3g	3.4de	1.8c	*2.4fg*	1.2b	3.0fgh	2.7de	no	no	no
341-20-14	*148.7a*	*104.0de*	*239.3cd*	*118.1ab*	62.9f	4.3cdef	3.4cde	*2.1ab*	*2.6ef*	*2.7a*	*3.8cdefg*	2.3e	yes	yes	yes
341-20-15	58.0e	*80.8e*	196.5f	83.0cd	78.2cdef	2.1fg	3.3de	1.8c	1.6hi	1.1b	*4.0bcde*	2.2e	yes	yes	no
214-20-1	*147.8a*	*104.2cde*	192.3f	68.1de	84.3cde	2.2fg	2.9e	*1.8ab*	*3.4b*	*3.3a*	2.9gh	2.3e	no	no	no
341-16-5	*139.0a*	*138.9bcd*	*261.9bc*	88.5cd	78.3cdef	3.4def	2.9e	*3.4a*	*3.1bcd*	*3.6a*	*3.9cdef*	*3.0bc*	no	no	no
BmJ	*120.6ab*	*79.2e*	*234.5cde*	*128.4a*	*107.8ab*	*6.4a*	*5.8ab*	*3.6a*	*2.9cde*	*2.5a*	*5.0a*	*3.0ab*	yes	yes	yes
ASM	*421.8f*	*277.3a*	*302.0a*	*118.7ab*	*93.8abc*	*5.8ab*	*5.6b*	*2.6a*	*2.6ef*	*2.5a*	*4.7abc*	*3.1ab*	n	no	no

[1] β-glucanase activity expressed as μg of glucose released/min/mg of apoplastic protein. Data in each replication is the mean of three independent treatments.
[2] Chitinase activity expressed as mg of N-acetyl-D-glucosamine released/hr/mg of apoplastic protein. Data is each replication is the mean of three independent experiments.
[3] "no" means there was monophasic production of hydrogen peroxide; "yes" means there was biphasic hydrogen peroxide production.
Treatments numbers in boldface are those that reduced disease symptoms on sugar beet greater than 50%.
Data in italics are statistically significantly higher than the water-treated control. Numbers followed by the same letter are not statistically different from one another as determined by least significant difference (probability = 0.05).

Table 4-4. Cumulative results of the methods for host-response based high-throughput screening for the identification of Bacilli biological control agents.

Treatment	Nelson Method[1]	Aniline Blue Plate[1]	Glycol Chitin Plate[2]	Nelson + Glycol Chitin[3]	Aniline Blue + Glycol Chitin[3]
Water	80.5g	2.75cd	1.56g		
203-3	*109.5def*	3.20cd	*2.55def*	+	
203-4	*134.1cd*	3.35bcd	2.03fg		
203-6	*133.4cd*	*4.90ab*	*2.97bcd*	✓	✓
203-7	*166.5b*	*5.75a*	*4.00a*	✓	✓
203-11	*134.7cd*	*4.00b*	2.04fg		
BMH5E-33	*117.7de*	2.80cd	2.19efg		
BMH5E-40	89.5fg	2.35d	2.22efg		
341-20-14	*121.6cde*	3.85bcd	*2.68def*	+	
341-20-15	100.0efg	2.70cd	2.14efg		
214-20-1	106.3efg	2.55cd	*2.74cde*		
341-16-5	*147.9bc*	3.15cd	*3.44ab*	+	
BmJ	*172.3b*	*6.10a*	*3.41abc*	✓	✓
ASM	*220.1a*	*5.70a*	*3.09bcd*	✓	✓

[1] The Nelson method and aniline blue plates both measure β-glucanase activity. Specific activity expressed as μg of glucose released per minute per mg of apoplastic protein. Data are the mean of five independent experiments for the Nelson method and two independent experiments for the aniline blue plate assays. Specific activities in italics are statistically significantly higher than the water control.

[2] The glycol chitin plates measure chitinase activity. Specific activity expressed as mg of N-acetyl-D-glucosamine released per hour per mg of apoplastic protein. Data is the mean of five independent experiments. Specific activities in italics are statistically significantly higher than the water control.

[3] Accuracy of the combined results of β-glucanase and chitinase assays. Treatments were determined to be systemic acquired resistance inducers if a statistical increase in specific activity was found in both methods. The Nelson method and glycol chitin plates combined identified all of the inducers correctly (check mark) along with three false positive identifications (plus sign). The aniline blue and glycol chitin plate assays combined identified all of the inducers correctly (check mark).

Treatments in boldface are those that reduced *Cercospora beticola* disease severity greater than 50%.

Chitinase as a Predictor of Disease Control

The glycol chitin plate assays had a reasonable level of precision with indiscrepencies having occurred only nine percent of the time (Table 4-3). The standard deviation between replicates of one plant treatment was approximately ten percent and approximately seventeen percent between plants within one treatment (Table 4-2). There were still problems with false positive identifications, with anywhere from forty to fifty percent of the treatments having been identified incorrectly as being capable if inducing systemic resistance responses between replications (Table 4-3). However, there were no false negative identifications with this method (Table 4-3). Cumulative results of five independent experiments correctly identified all four SAR-inducers as such and yielded four false positive and no false negative identifications (Table 4-4).

Biphasic Hydrogen Peroxide Production Curves as Predictors of Disease Control

Regardless of the plant material used to analyze the production pattern of hydrogen peroxide the reproducibility was quite high, with only seven percent disagreement between the results obtained with protoplasts and leaf disks (Table 4-3). All biphasic curves elicited by the Bacillus isolates was using protoplasts were statistically similar in timing and intensity (Figure 4-1). In all cases the primary peak (~3mM) occurred at 15 minutes post treatment and the secondary peak (~2-4 mM) occurred at approximately 2 hours post treatment (Figure 4-1). The isolate 341-20-15 elicited biphasic production of hydrogen peroxide when assayed using protoplasts, but

failed to elicit the same production pattern when using leaf disks as the medium (data not shown), however isolate 341-20-15 did not

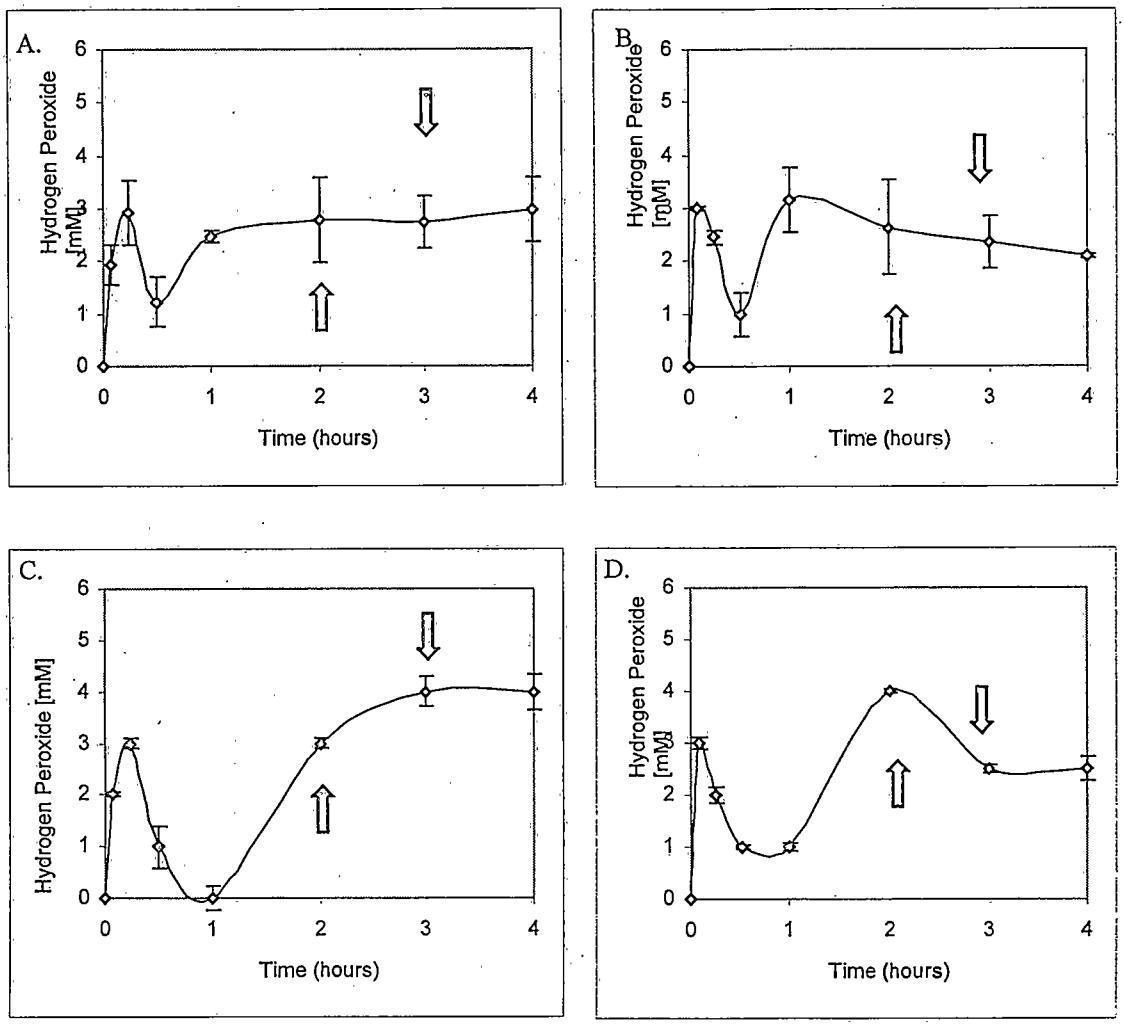


Figure 4-1. Hydrogen peroxide production curves for HH88 sugar beet protoplasts following treatment with *Bacillus mycoides* isolate Bac J (A), and *Bacillus pumulis* isolates 203-6 (B), 203-7 (C) and 341-20-15 (D) as measured by phenol red oxidation. Data is the mean of two replicates using protoplasts. All the Bacilli treatments resulted in similar production curves. The first peak occurred at approximately 30 minutes and the second peak occurred at approximately 2 hours post treatment. Both bursts produced a transient accumulation of hydrogen peroxide to approximately 2-4 mM. Arrows designate points of consistency that should be used to determine secondary peak production.

control disease to any statistically significant extent (Table 4-1). False positive identification using this method occurred eight to fifteen percent of the time between replications (Table 4-3). ASM did not induce biphasic hydrogen peroxide production (data not shown).

Discussion

Current methods for the identification of novel biological control agents primarily focus on isolating antagonistic microorganisms that have a limited range of efficacy (Mohapatra et al, 2002). Additionally, the widely-used time and labor intensive *in vitro* and *in planta* identification methods tend to be error prone (May et al, 1997; Ji and Wilson, 2002). Plant systemic resistance-inducing biological control agents elicit a broader spectrum resistance, however the isolation of effective inducers tends to be serendipitous. To date no one has developed a reliable method for the identification of this particular type of biological control agent. We propose using changes in host biochemistry and oxidative state for high-throughput identification of novel bacilli systemic resistance-inducing biological control agents. This proposal is based on previous work characterizing the production of β-glucanase, chitinase, and biphasic hydrogen peroxide in sugar beet following treatment with *Bacillus mycoides* isolate Bac J, a systemic acquired resistance inducing biological control agent (Bargabus et al, 2002).

Bacillus pumulis isolates 203-6 and 203-7, *Bacillus mycoides* isolate Bac J and ASM, a chemical inducer of systemic resistance, all reduced *Cercospora beticola*

symptoms by greater than 50 percent (Table 4-1), which was suspected to lead to economically significant disease control. The fact that isolates 203-6 and 203-7 also induced chitinase, β-glucanase and biphasic AOS showed the oxidative and biochemical changes observed in the BmJ-sugar beet interactions (Bargabus et al, 2002) are consistent with other Bacilli SAR inducer-sugar beet interactions. The two families of PR-proteins, chitinase and β-glucanase, were selected for specific activity determination due to their synergistic association that leads to fungal disease control (Zu et al, 1994; Mauch et al, 1988). Previous work with the BmJ-sugar beet system showed the results from the specific activity colorimetric and in-plate assays were consistent with the more time and labor intensive in-gel activity assays (Bargabus et al, 2002). Since the goal with this line of investigation is to screen hundreds of isolates, the simpler, faster approaches were employed.

When trying to quickly and efficiently identify biological control agents in a systematic fashion, it is important that the results are both accurate and precise. The Nelson method was problematic in this respect. The accuracy was poor with this approach with thirty percent false positive identifications (Table 4-4). Falsely identifying an isolate as a SAR-inducer counteracts one of the main purposes of a high-throughput screening method, which is to reduce the number of isolates included in subsequent time and labor intensive *in planta* disease challenge assays. Additionally, the precision with this approach was poor since over fifty percent of the results differed between replicates (Table 4-3). A critical analysis of the standard deviations between replications (~22%) and between plants in one treatment (~28%) shows that much of the error lies in the

method itself (Table 4-2). This high level of variability is common with this method, especially when working with low enzyme concentrations and large blank values (Dygert et al, 1965). The error is introduced through interference between reagents (Miller, 1959), human error introduced during the multi-step process, non-specific binding of the reagents to non-target reducing products and destruction of the glucose by oxidation prior to detection by the reagents (Miller, 1959).

The use of aniline blue plate assays proved to be a superior method for β-glucanase activity determination based on the increased precision and accuracy in the results. Furthermore, the aniline blue plate approach is at least twice as sensitive as the Nelson method approach. The aniline blue dye is specific for the detection of polysaccharides containing β-1,3 linkages, such as the laminarin substrate (Nakanishi et al, 1976; Evans et al, 1984). By detecting a specific component of the enzyme reaction the overall error is reduced between replicates. There was a vast improvement in precision and accuracy over the Nelson method since the results were ninety-six percent identical and included only one false positive (Table 4-4). This method would theoretically reduce the pool of isolates entering further testing by nearly seventy percent, while at the same time eliminating ninety percent of the ineffective isolates. Glycol chitin plate assays were used for detection of chitinase activity. The increased precision with the chitinase gel diffusion assay in comparison to the Nelson colorimetric method (Table 4-3) prompted our development of the highly analogous aniline blue plate assay. The less variable aniline blue plate method (Table 4-2) was developed based on work by Mahasneh and Stewart (1980) and Cote et al (1989) using glycol chitin plates as a model.

The precision of the glycol chitin plate assay was no indication of accuracy, since several false positive identifications were part of the results (Table 4-3). The high incidence of consistent chitinase induction in the absence of disease control is not unfounded since these enzymes are frequently encountered in both resistant and susceptible cultivars following various treatments (Punja, 2001). The glycol chitin plate assay is not able to detect slight differences in the hydrolytic enzymes, such as substrate binding specificities and pH optimum that could provide some insight into the role, if any, different isoforms play in disease reduction. In previous work, sugar beet has numerous active chitinase isoforms induced following BmJ and ASM treatment in addition to several active isoforms found in the water-treated control (Bargabus et al, 2002).

Phenol red oxidation assays for the detection of biphasic hydrogen peroxide production served as a good predictor of SAR-induction capability. There was a high degree of precision, with only 7% disagreement between the results using leaf disks and protoplasts (Table 4-3). The latter requires an additional 24-48 hours for the generation of protoplasts using expensive cell wall digesting enzymes and an external source of peroxidase included in the phenol red oxidation assay. These add extra time and expense to the approach, therefore leaf disks would be selected for additional screening efforts. Although the hydrogen peroxide profiles provided a fairly accurate prediction of systemic resistance induction capacity, this method is the most complex and time consuming of those tested (Table 4-2). The timing and production level was highly conserved in all instances of biphasic hydrogen peroxide production, therefore the proposed abridged sampling schedule at 2 and 3 hours post treatment would suffice. Since all treatments

induced the primary peak, the abridged sampling scheme would detect the differentially induced secondary burst of 2-4 mM hydrogen peroxide (Figure 4-1). However, relying on these two time points allows for the data to be more easily corrupted by common occurring outliers that in more frequent sampling schemes can be eliminated as such. The chemical elicitor of SAR, ASM, did not induce biphasic hydrogen peroxide in sugar beet protoplasts or leaf disks. The enzymatic methods for identification worked equally as well with chemical and biological treatments, this difference could be a potential pitfall when relying on phenol red oxidation for identification.

Analysis of the production of a single PR-protein was a poor predictor of SAR-induction capability. However, the occurrence of chitinase and β-glucanase alone is not correlated with disease control (Punja, 2001), especially with fungal pathogens, since the enzymes function synergistically (Melchers and Stuiver, 2000). Therefore examination of the PR-proteins together should provide a more accurate prediction method for biological control agent identification. Using the most popular method for the determination of β-glucanase activity (the Nelson method) in combination with the results from glycol chitin plate assays, correctly identified all of the SAR-inducers, however the results also yielded three false positive identifications (Table 4-4). A combined positive result from the glycol chitin and aniline blue plate assays correctly identified all SAR-inducing isolates (Table 4-4). Furthermore, relying on statistically significant increases in specific activity for both assays eliminated all non-inducers from the pool, making this combination the most accurate approach for identification.

Ideally, in addition to being accurate and reproducible, high throughput screening methods should encompass rapid setup, run-time and interpretation. The glycol chitin/aniline blue plate assay combination fits all these criteria, requiring minimal time for completion (Table 4-2). Additionally, the analysis requires no special equipment and is relatively cost effective. Furthermore, the two plate assay methods are highly analogous in relation to execution and analysis, therefore it would be easy to transfer screening duties to any untrained technician.

Selection of SAR-inducers using rapid biochemical screens (i.e. glycol chitin and aniline blue plate assays) creates a systematic means of searching for novel biological control agents; the first method of its kind. Performing the less time consuming biochemical assays prior to *in planta* disease challenge assays has the potential to reduce the total workload by over 70%. The application of the high-throughput screening method allows for increased numbers of microorganisms to be tested in a shorter period of time, which potentially could lead to the unveiling of numerous novel, more effective SAR-inducing biological control agents. In this subset of samples, 2 new potentially useful systemic resistance-inducing organisms for use against *Cercospora beticola* on sugar beet were identified: *Bacillus pumulis* isolates 203-6 and 203-7. These particular isolates were previously overlooked since they were originally isolated from germinating sugar beet seed, which made foliar-treatment seem unlikely to work. Since the high-throughput screen method was shown to be effective for Bacilli inducers of sugar beet systemic resistance only, future work includes applying the same method for selection of

SAR-inducing biological control agents, other than Bacilli, and Bacilli that could potentially work on other crop plants.

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CHAPTER 5

SUMMARY

Cercospora beticola, the causal agent of Cercospora leaf spot of sugar beet, is one of the most destructive fungal pathogens worldwide (Smith and Ruppel, 1974). Biological control was investigated as an alternative means to chemical control currently employed for two reasons. First, *C. beticola* has become increasingly tolerant to triazoles (Windels et al, 1998) and almost completely resistant to benzimidazoles (Brantner et al, 1998), the two fungicides most widely used for control of Cercospora leaf spot. Second, humanity as a whole has become increasingly environmentally aware (Moffat, 1991). This awareness includes being conscious of the detrimental effects chemical pesticide treatments have on ground water quality, residue buildup in plant material and human health for farmers and others living in close proximity to areas of widespread pesticide usage (Wedge and Nagel, 2000).

Bacillus mycoides isolate Bac J (BmJ) was selected for use against *C. beticola* from a larger pool of microorganisms isolated from the phyllosphere, rhizosphere, and seed of sugar beet grown in Sidney, Montana during a brute force screen for organisms displaying disease control capabilities. The selection, in part, showed BmJ was effective in field and glasshouse experimentation. Bacilli have become popular options for biological control agents based on their ability to form endospores. Endospores allow for easy formulation of commercial products and increase the shelf life of a biological control agent much longer than would otherwise be possible (Nicholson, 2002). The fact

that BmJ was isolated from the environment to which it is ultimately going to be reintroduced, provides two potential benefits for biological control. First, being in its native environment increases the likelihood that BmJ will establish a suitable population size, which has been shown to be critical for effective disease control (Smith et al, 1997; Walker et al, 2002). Second, one of the major fears associated with biological control includes the unforeseen effects the introduced organism may have on other native, non-pathogenic microflora. Presumably, since BmJ naturally coexisted with these organisms, albeit at a lesser cell concentration, this negative effect would be minimized.

Several contributions were made in the current investigation that further the understanding of BmJ-sugar beet interactions and how it relates to systemic resistance induction by other Bacilli. Although BmJ was known to be effective in both glasshouse and field experiments for the control of Cercospora leaf spot on sugar beet, its mode of action was previously unknown. The discovery of systemic resistance induction by BmJ, as demonstrated through classical disease challenge assays, pathogenesis-related protein production assays (Bargabus et al, 2002) and oxidative burst characterization was significant since it is a broad-spectrum response (Delaney, 1997). Subsequent investigations showed BmJ treatment controlled bacterial pathogens of sugar beet as well (Jacobsen, unpublished data). It was further noted the host changes observed in BmJ-sugar beet interactions were consistent with other Bacilli-sugar beet interactions in which systemic resistance was induced. The consistency of host response allowed the oxidative and biochemical changes to be used to identify effective biological control agents in a

systematic, high throughput fashion. This screen could eventually replace the current serendipitous, error prone, non-specific selection methods for biological control agents.

The characterization of the BmJ-sugar beet interaction provided a framework for understanding how the host response to systemic resistance inducing biological control agents relates to what is known of host responses to avirulent pathogens during the establishment of plant immunity. Systemic resistance induction has been shown following treatment with numerous pathogens (Hammond-Kosack and Jones, 2000). The elicitation is a result of specific recognition, known as gene-for-gene interactions (Flor, 1971). More recently, a genetic component within the host for recognition of biological control agent that elicit disease suppression has been shown (Smith et al, 1999; Simon et al, 2001). The investigations with BmJ have provided evidence for recognition on behalf of both the plant and biological control agent.

Although exopolysaccharides have been implicated in systemic resistance induction in other instances (Jahr et al, 1999), during the mode of action characterization it became apparent that BmJ was actively producing and secreting a signal recognized by sugar beet (Figure 5-1). This conclusion was drawn based on the evidence that disease control, pathogenesis-related protein production (Bargabus et al, 2002), and biphasic hydrogen peroxide production were not induced following treatment with dead BmJ cells. Furthermore, the filter-sterilized spent growth media also showed limited effectiveness at inducing the former two aforementioned host responses (Jacobsen, unpublished data). During the host response-based high throughput screen method development it was noted that only certain isolates of *Bacillus pumulis* elicited systemic resistance (Table 4-1),

suggesting three possible locations within the biological control agent DNA for the gene encoding for the signal. The gene may be contained on a plasmid present only in isolates

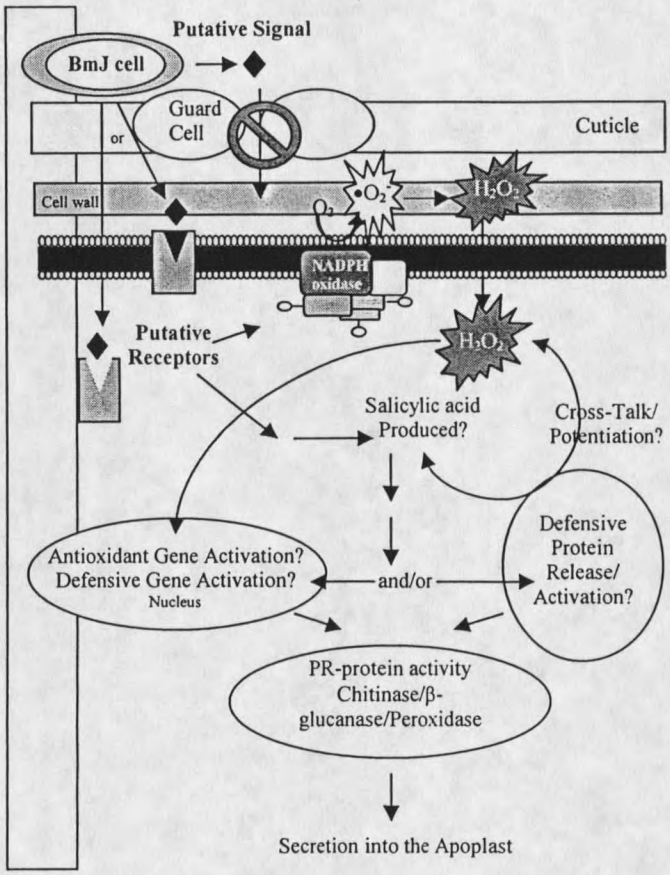


Figure 5-1. Schematic representation of *Bacillus mycoides* isolate Bac J (BmJ) interaction with sugar beet during systemic resistance induction. BmJ, an epiphyte, secretes a putative signal (Black diamond) that enters into sugar beet through the culticular waxes. Once inside the plant, the signal interacts with a putative receptor that is either cytosolic or plasma membrane bound. This interaction leads to the production of superoxide anion and biphasic hydrogen peroxide. The lack of cell death normally associated with the oxidative burst may be a result of hydrogen peroxide induced activation of antioxidant enzymes. Presumably a systemic signal is produced leading to systemic disease control. The production of hydrogen peroxide and the induction of pathogenesis-related proteins (PR-proteins) suggests salicylic acid is employed as a secondary signal molecule. The PR-proteins, chitinase, β-glucanase, and peroxidase, are produced and secreted into the apoplast.

capable of inducing systemic resistance. Alternatively, the gene may have been acquired from another systemic resistance inducing bacterium through horizontal transfer, as is the case with avirulence genes contained within pathogenicity islands (Buttner and Bonas, 2002). Lastly, all *Bacillus pumulis* isolates may have originally contained the signal coding sequence, however through time certain isolates may have had mutations occur within the coding sequence thereby preventing recognition by sugar beet. The latter is a common occurrence in the race between plants and pathogens.

Clues to the location of a putative receptor in sugar beet for the recognition of BmJ were provided in the oxidative response experimentation. Biphasic hydrogen peroxide production was the same whether tested using intact leaf disks or protoplasts, suggesting the putative sugar beet receptor for recognition of BmJ is cytosolic or plasma membrane bound (Figure 5-1), which is consistent with other receptors for the perception of phytopathogens (Dangl and Jones, 2001). The biphasic oxidative burst under green light conditions demonstrated stomatal conductance does not play a role in relaying BmJ derived signal into sugar beet (Figure 5-1), the signal must therefore not only penetrate through the cell wall, but also make it past the cuticular waxes. Other Bacilli have been shown to burrow into the leaf surface by way of cutinase activity (Beattie, 2002). This burrowing action would eliminate a portion of the physical barriers, however it is not known if BmJ contains cutinase. If BmJ could digest cutin, the digestion would have to be completed 45 minutes post treatment to account for the induction of the secondary burst of hydrogen peroxide (Figure 4-1) which is the result of specific gene-for-gene

recognition (Levine et al, 1994; Baker and Orlandi, 1995). Once inside the plant, the signal would still have to contend with the cell wall that are intricate matrices. However, other components usually less than 6-8 micrometers in size are capable of penetrating through these physical barriers (Buttner and Bonas, 2002).

The fact that sugar beet responds to BmJ as though it were an avirulent pathogen is further supported by the fact that following recognition, an oxidative burst and pathogenesis-related protein production occurs. The involvement of hydrogen peroxide and the ultimate production of pathogenesis-related proteins suggested salicylic acid, a secondary signal molecule, plays a role in the establishment of BmJ-elicited systemic resistance. Salicylic acid leads to the potentiated production of hydrogen peroxide through the inhibition of catalase (Chen et al, 1993; Durner and Klessig, 1996) and ascorbate peroxidase (Durner and Kelssig, 1995), two antioxidant enzymes. Alternatively, hydrogen peroxide leads to the increased production of salicylic acid through the stimulation of phenylalanine ammonia lyase and benzoic acid 2-hydroxylase (Martinez et al, 2000), the first and last enzymes, respectively in salicylic acid biosynthesis (Ribnicky et al, 1998). Salicylic acid, in turn, has been shown to be a necessary player in the induction of many families of pathogenesis-related proteins (van Loon and van Strein, 1999), such as those produced following BmJ-treatment.

The previously accepted dogma of biphasic hydrogen peroxide orchestrating hypersensitive cell death has recently fallen by the wayside, being replaced with several alternative roles for hydrogen peroxide in the establishment of systemic resistance (Solomon et al, 1999; Fleury et al, 2002; de Pinto et al, 2002; Delledonne et al, 1998).

One newly emergent idea states that at low to moderate levels hydrogen peroxide protects the cell from programmed cell death through the activation of antioxidant enzymes (Solomon et al, 1999), which fits into our hypothesized model for BmJ-sugar beet interaction (Figure 5-1). Additionally, at these low levels, hydrogen peroxide is still able to contribute to the activation of downstream defense responses, such as pathogenesis-related protein production.

Although extensive knowledge has been gained related to BmJ induced systemic resistance responses of sugar beet, several areas of importance have yet to be investigated. The stage has been set for future work isolating and characterizing the sugar beet receptor and BmJ effector involved in recognition and establishment of systemic resistance. The discovery of an oxidative burst in sugar beet following treatment with BmJ was an interesting find that can be built upon in the future. Examination of the localized production of antioxidant enzymes, caspase activity assays, and nitric oxide production studies could be used to determine why there is a lack of programmed cell death following the oxidative burst.

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APPENDIX A

PRELIMINARY DEMONSTRATION *BACILLUS MYCOIDES* ISOLATE BAC J-INDUCED SYSTEMIC RESISTANCE IS EFFECTIVE IN THE FIELD

PRELIMINARY DEMONSTRATION THAT *BACILLUS MYCOIDES* ISOLATE BAC J-INDUCED SYSTEMIC RESISTANCE IS EFFECTIVE IN THE FIELD

Introduction

Induction of systemic resistance has been widely studied under glasshouse conditions using a wide range of inducing agents including pathogens (Kuc, 1992), chemicals (Tally 1999; Cohen, 2002), biological control agents (Wei et al, 1991; Murphy et al, 2000) and transgenic plants (Wretblad et al, 2003). In contrast, there are few accounts of systemic resistance induction in the field. Field studies that have been conducted are primarily limited to the use of transgenic crops (Germundsson et al, 2002) or cultivars that naturally contain resistance (Chen et al, 2001).

Due to a number of confounding factors, biological control agents that employ systemic resistance induction as their mode of action are rarely tested under field conditions (Tuzun et al, 1992; Srinivas and Danielson, 2001). Biological control agent efficacy is very dynamic under field condition (Stack, 2002) due in large part to the variable field environment including fluctuations in temperature, relative humidity, surface humidity, gasses, light exposure, ultraviolet light intensities and air movement (Burrage, 1971; Hebber et al, 1992; Hannusch and Boland, 1996; Beattie, 2002). All of these factors effect biological control agent survival and population establishment, and without proper colonization efficient disease control is not obtained (Smith et al, 1997; Guestsky et al, 2001; Walker et al, 2002; Bull et al, 1991; Paulitz and Baker, 1987; Parke,

1990). Conversely, glasshouse conditions can be manipulated to create an environment best suited for biological agent survival and proliferation (Guetsky et al, 2001). Additionally, when introducing biological control agents in the field, there are a multitude of unpredictable interactions with the native microflora (Blakeman and Fokkema, 1982) that are lacking in the highly sterile glasshouse environment. Naturally occurring saprophytes may form antagonistic associations with the introduced biological control agent (Casida Jr, 1989), elicit metabolic changes within the plant (Landa et al, 2002), or outcompete the biological control agent for niche occupation of nutrient acquisition (St Leger et al, 1997).

Bacillus mycoides isolate Bac J (BmJ) induces systemic resistance as its mode of action as demonstrated in glasshouse experiments, where a reduction in Cercospora leaf spot disease severity was seen even when BmJ was spatially separated from the causal agent *Cercospora beticola* (Bargabus et al, 2002). In field studies, where BmJ was applied to the entire phylloplane, effective control of *C. beticola* symptoms was obtained (Bargabus et al, 2002). However, since BmJ is antagonistic toward *C. beticola* (Jacobsen, unpublished data), those field studies did not discern between disease control attributed to antibiosis versus systemic resistance induction. The primary focus of the current study was to develop parameters for BmJ treatment and apoplastic protein collection in the field allowing for the determination of BmJ-induced production of pathogenesis-related proteins, β-glucanase and chitinase under field conditions. The last objective was to determine if β-glucanase and chitinase accumulation in systemic tissues correlated with disease reduction of Cercospora leaf spot.

Materials and Methods

Bacterial Culture

Bacillus mycoides isolate Bac J cells, originally isolated from sugar beet leaves in Sidney, Montana in 1994, were stored at -80°C in 10% glycerol and 1% tryptic soy broth (Difco). For fresh cell preparations, BmJ was cultured in tryptic soy broth for 48 hours (28°C). Cells were centrifuged 10 minutes at 10,000 x g (4°C), washed with sterile water 2 x, then resuspended in distilled water. The optical density was adjusted to OD_{600} 1.0, then diluted 1:2 that resulted in approximately 1 x 10^8 cfu/ml. The precise number of cells cannot be determined due to the chain forming nature of the bacterium.

Plant Culture and Treatment

Three rows (10 sugar beets each) of sugar beet variety HH111 were used per treatment. Plants were transected with a sheet of Plexiglas at approximately the 12-14 leaf stage. The leaves on one half of the Plexiglas divider were marked with orange twist ties then treated with 1 x 10^8 cfu/ml BmJ, 100 ppm acibenzolar-S-methyl (ASM, 50 ppm a.i.; Actigard™50WG, Syngenta, Greensboro, N.C.) or water using a CO_2 backpack sprayer with a single nozzle boom. The leaves on the other half of the Plexiglas barrier were left untreated and used for subsequent protein extraction and disease rating.

Protein Collection

Three sugar beet leaves per row for each of the three rows per treatment located on the side of the Plexiglas barrier that remained untreated were collected, bagged and placed on ice for 6 hours prior to vacuum infiltration with apoplast buffer (150 mM NaCl, 25 mM MES, pH 6.2). Apoplast extractions were collected according to Klement (1965) having substituted the apoplast buffer (Bargabus et al, 2002) for distilled water. The proteins were quantified using Bradford reagent (BioRAD) with bovine serum albumin (Sigma) for standards. Proteins were frozen at -80°C until the time of analysis.

Determination of β-Glucanase Specific Activity

The specific activity of β-glucanase from the sugar beet apoplastic proteins collected from the field was determined by measuring the release of free glucose from laminarin. Sodium acetate buffer (100 µl, pH 5.0, 100 mM) containing 0.5% laminarin (Sigma) and 2.0-8.0 µg of apoplastic protein (3 replicates for each of the three samples per row for the three rows per treatment) was incubated at 37°C for 60 minutes. Following the incubation, 900 µl of water and 1 ml of alkaline copper reagent (Somogyi, 1952) was added to each reaction. The reactions were then boiled for 10 minutes and returned to room temperature by cooling on ice. After cooling, 1 ml of aresenomolybdate color reagent (Nelson, 1944) was added to each reaction. Once the bubbling subsided, 10 ml of distilled water was added to each tube and the OD_{660} was determined for each. A blank containing laminarin was used to subtract the background from each reaction. A

standard curve was established by adding 5-25µg glucose to 11 ml total reactions that did not contain laminarin.

Chitinase Specific Activity Determination by Plate Assay

Sodium phosphate buffer (pH 5.0, 0.01 M) containing 1% agarose and 0.1% glycol chitin was added to a 9 cm diameter glass petri dish. Wells, 3 mm in diameter, were excised from the agarose (3 per sample for each of the three replicates per row for all three rows per treatment). Dilutions of apoplastic proteins (0.009, 0.006 and 0.005 µg) and chitinase standards (Chitinase from *Streptomyces griseus*, Sigma) were loaded into the wells. The plates were incubated at 37°C for 48 hours. Following the incubation, 50 ml of 500 mM Tris-HCl (pH 8.9) containing 0.01% fluorescent brightener 28 was added to each plate and incubated for 10 minutes. The plates were rinsed 3 x with distilled water, flooded with 50 ml distilled water, and destained in the dark for 24 hours. The diameters of the non-fluorescent lytic regions on a fluorescent background were measured while the plate was on a UV light source. Specific activity of the chitinase (mg of N-acetyl-D-glucosamine released/ hour/mg of apoplastic protein) was determined through comparison of lytic regions for the standards and apoplastic proteins (Velasquez, 2002).

Disease Control Assessment in the Field

Disease ratings were made of the entire plant surface utilizing the KWS scale (Kleinwanzler, 1970) for consistency with previous studies (Bargabus et al, 2002). Disease ratings were made starting at disease onset, approximately 4 weeks after the

initial treatment. Disease ratings were taken every two weeks until the time of harvest at ten weeks post planting. The disease severity was calculated and expressed as Area Under the Disease Progress Curve.

Results

Parameters For Studying Systemic Resistance in the Field

β-glucanase activity was used to determine the appropriate timing for apoplast extractions from leaves collected in the field. There was no statistical difference between the specific activities of apoplastic proteins when collected immediately after removal from the plant (at USDA-ARS facility in Sidney, Montana) or after transferring the plant material on ice to Bozeman, Montana. The latter extractions occurred approximately 8 hours after removal from the sugar beets in the field (Table A-1).

Table A-1. β-glucanase specific activity levels when extracted in Sidney versus Bozeman, Montana.

Sample Location	Specific Activity[A]
Field	15.17 ± 3.56
Lab	16.73 ± 8.58

[A] β-glucanase specific activity expressed as μg of glucose released/minute/mg of apoplastic protein.

Incubating the leaves at 4°C for up to 48 hours did not have a statistical effect on the β-glucanase specific activity levels (Table A-2).

Table A-2. β-glucanase specific activity level stability at 4°C over time.

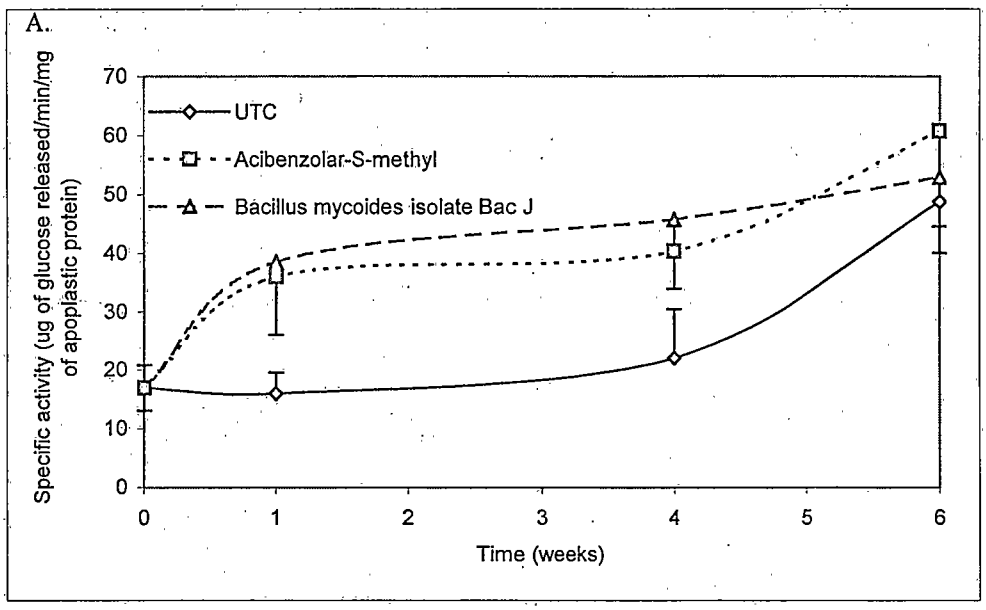
Time (hours)	Specific Activity[A]
6	21.11 ± 5.74
24	16.69 ± 3.29
48	21.37 ± 2.28

[A] β-glucanase specific activity expressed as μg of glucose released/minute/mg of apoplastic protein.

Sugar Beet Pathogenesis-Related Protein Specific Activity in the Field

The basal β-glucanase specific activity detected in the field was approximately 17 μg of glucose released per minute per mg of apoplastic protein (Figure A-1A). By day seven, BmJ and ASM treatments had resulted in a 2-fold statistically significant increase in specific activity over the untreated control (Figure A-1A). The differences between the β-glucanase specific activity found in ASM and BmJ treated plants versus water-treated plants were statistically significant prior to disease onset (Figure A-1A). Following *C. beticola* symptom development, all specific β-glucanase specific activities were statistically equivalent (Figure A-1A).

The basal level of chitinase specific activity found in the field was approximately 0.17 mg of N-acetyl-D-glucosamine released per hour per mg of apoplastic protein. By seven days post treatment there were statistically significant differences detected between the chemical positive control, ASM, and BmJ when compared to water with an approximately a 6-7 fold increase in specific activity (Figure A-1B). The chitinase



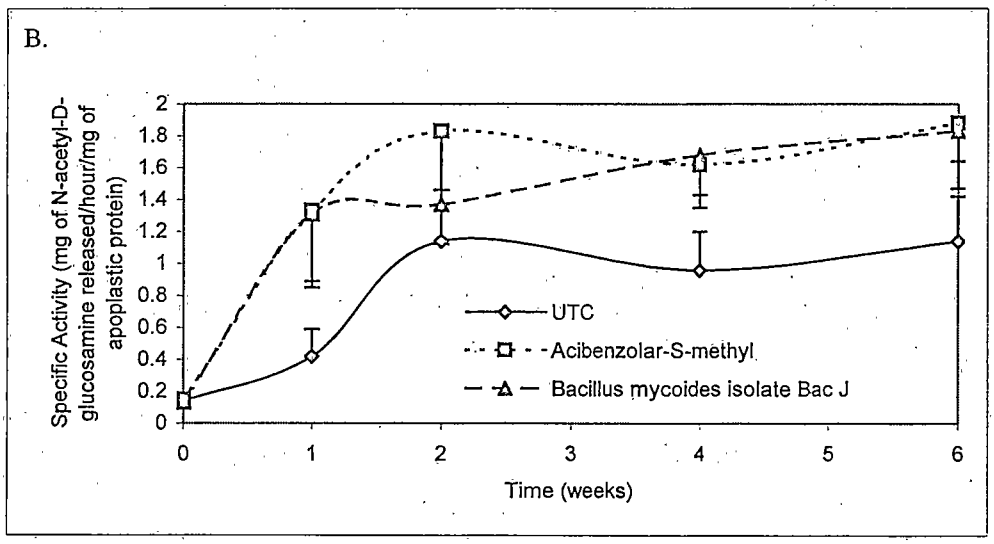


Figure A-1. Changes in systemic specific activity levels of two pathogenesis-related proteins under field conditions (2002). (A) β-glucanase activity of sugar beet following treatment with 100 ppm Acibenzolar-S-methyl, *Bacillus mycoides* isolate Bac J, or the untreated control (UTC) as determined by laminarin digestion. Data is the mean of three independent samples replicated three times. (B) Chitinase specific activity of sugar beet following treatment with 100 ppm Acibenzolar-S-methyl, *Bacillus mycoides* isolate Bac J, or the untreated control (UTC) as determined by chitinase plate assay. Data is the mean of three independent samples replicated three times.

specific activity found in sugar beets treated with ASM and BmJ was statistically higher than that found in water treated plants for the duration of the sampling (Figure A-1B). At all time points, no statistical difference was detected between ASM- or BmJ-elicited chitinase specific activities.

Disease Control of *C. beticola* by BmJ under Field Conditions

BmJ and ASM delayed disease onset on the entire plant by approximately 1 to 2 weeks. There was a trend in disease reduction by BmJ by 6-10 weeks. ASM failed to reduce disease symptoms in comparison to water-treated controls (Figure A-2).

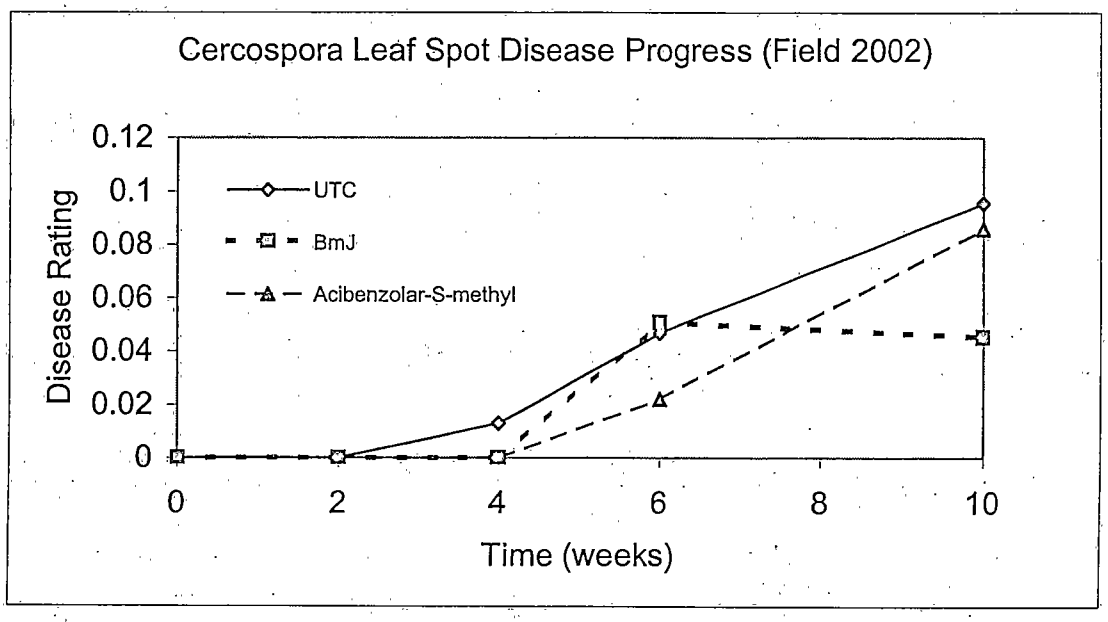


Figure A-2. Preliminary results suggest systemic resistance induction by BmJ is effective at controlling Cercospora leaf spot in the field. Disease Rating is expressed as area under the disease progress curve (higher number = more disease).

Discussion

Bacillus mycoides isolate Bac J, a biological control agent, induces systemic resistance which reduces sugar beet Cercospora leaf spot disease severity (Bargabus et al, 2002). This mode of action has been well elucidated in glasshouse experiments and through systemic resistance molecular marker characterization (Bargabus et al, 2002). Unlike glasshouse experiments, previous field studies have not introduced spatial constraints between the biological control agent and the pathogen. Since BmJ is antagonistic towards *Cercospora beticola*, the casual agent of Cercospora lead spot, the disease reduction noted in the field may be a result of combined modes of action. Therefore a BmJ-treatment and apoplastic protein collection method needed to be developed to determine the overall impact of systemic resistance induction on the reduction of disease severity in the field.

Developing a treatment method for studying sugar beet systemic resistance in the field was difficult for several reasons. First, sugar beets grow in rosettes making it difficult to keep the biological control agent spatially separated from *C. beticola.* To address this issue, a Plexiglas barrier was devised to ensure separation during the treatment. Previous fieldwork determined multiple applications throughout the growing season are required for effective control of *C. beticola* with BmJ (find a good ref). However, strong winds frequently sweep through the fields of Sidney, Montana disallowing permanent installation of the barrier, therefore the treated leaves were further marked with orange twist ties to aid in the replacement of the barrier with each

subsequent spray. It is possible that following treatment, the untreated leaves could be contaminated with BmJ through wind and rain dispersal of the microorganism. It was assumed that transfer of this kind has a minimal impact on the overall disease control. Nevertheless at a later date it would be critical to quantify BmJ levels on untreated leaves, to rule out antibiosis as a contributing factor to disease reduction. Additionally, future work should include devising a mechanism for supporting the barriers against strong crosswinds so they could become permanent installments. Another concern when developing the systemic resistance field study was the location of the field plots in relation to the lab where subsequent experimentation would take place. Wounding can induce pathogenesis-related proteins (van Loon and van Strein, 1999) and therefore there was concern about the effect travel time between collecting the leaves in Sidney, Montana and extracting the proteins in the lab in Bozeman, Montana would have on specific activities. However, when the leaves were kept on ice the 8-hour lag time did not significantly alter β-glucanase activity in the untreated controls (Table A-1). It was later discovered the leaves could be kept at 4°C for up to 48 hours without having a statistically significant impact on β-glucanase specific activity (Table A-2).

BmJ and ASM induced pathogenesis-related proteins in sugar beet in the field, consistent with observations made in glasshouse experiments (Bargabus et al, 2002). The timing of induction (7 days post treatment) was consistent with previous observations under glasshouse conditions (Figure A-1A and A-1B). The absolute level of chitinase specific activity in the field was similar to previous results, but the absolute β-glucanase activity was two-fold lower than previously observed (Bargabus et al, 2002). However,

both field and glasshouse treatments with BmJ and ASM resulted in a two-fold increase in specific activity over the water control (Bargabus et al, 2002). Following disease onset at approximately week four to five of the field trial, statistical differences in chitinase and β-glucanase specific activity were decreased and no longer discernable respectively between the ASM-, BmJ- and water-treated beets (Figure A-1A and A-1B). This coincides with previous observations that *Cercospora beticola* induces chitinase (Nielsen et al, 1993) and β-glucanase (Gottschalk et al, 1998) in sugar beet. Because the Nelson method was used to determine β-glucanase specific activity, there may have been some statistical differences between the two inducers, BmJ and ASM, and water that were simply masked by large standard deviations often present when using this methodology. Since more accurate and reproducible methods for the determination of β-glucanase have been developed (aniline blue plate assay, Chapter 4), they should be employed in future experimentation.

Disease severity ratings were not limited to the untreated sugar beet leaves in this initial set of experiments, however several interesting results can be gleaned from the information. First, disease symptom manifestation was slowed in the ASM- and BmJ-treated plants. Water-treated controls showed symptom development one week prior to symptom development on ASM- or BmJ-treated plants (Figure A-2). Secondly, even though only one half of the leaf surfaces of the sugar beet were treated, overall disease reduction (approximately 50%) was comparable to that observed in previous years experiments where the entire plant was treated (Bargabus et al, 2002). Lastly, the *C. beticola* symptoms appeared to plateau on BmJ-treated plants at 6 weeks post disease

onset. Cercospora leaf spot infections are microcyclic, with multiple disease cycles occur in one growing season. Preliminary results showed that BmJ treatment not only reduced the number and size of lesions being produced by *C. beticola*, but also the number of spores contained in each lesion. However, ASM has been shown to reduce lesion size and spore production as well. The discrepancies between the disease control achieved between the two treatments may ultimately be a result of the small sample size used in this preliminary investigation. Even though no conclusive evidence exists from this preliminary research as to the correlation between pathogenesis-related protein production and systemic disease control, the initial observations warrant further investigation into the matter.

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