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

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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).

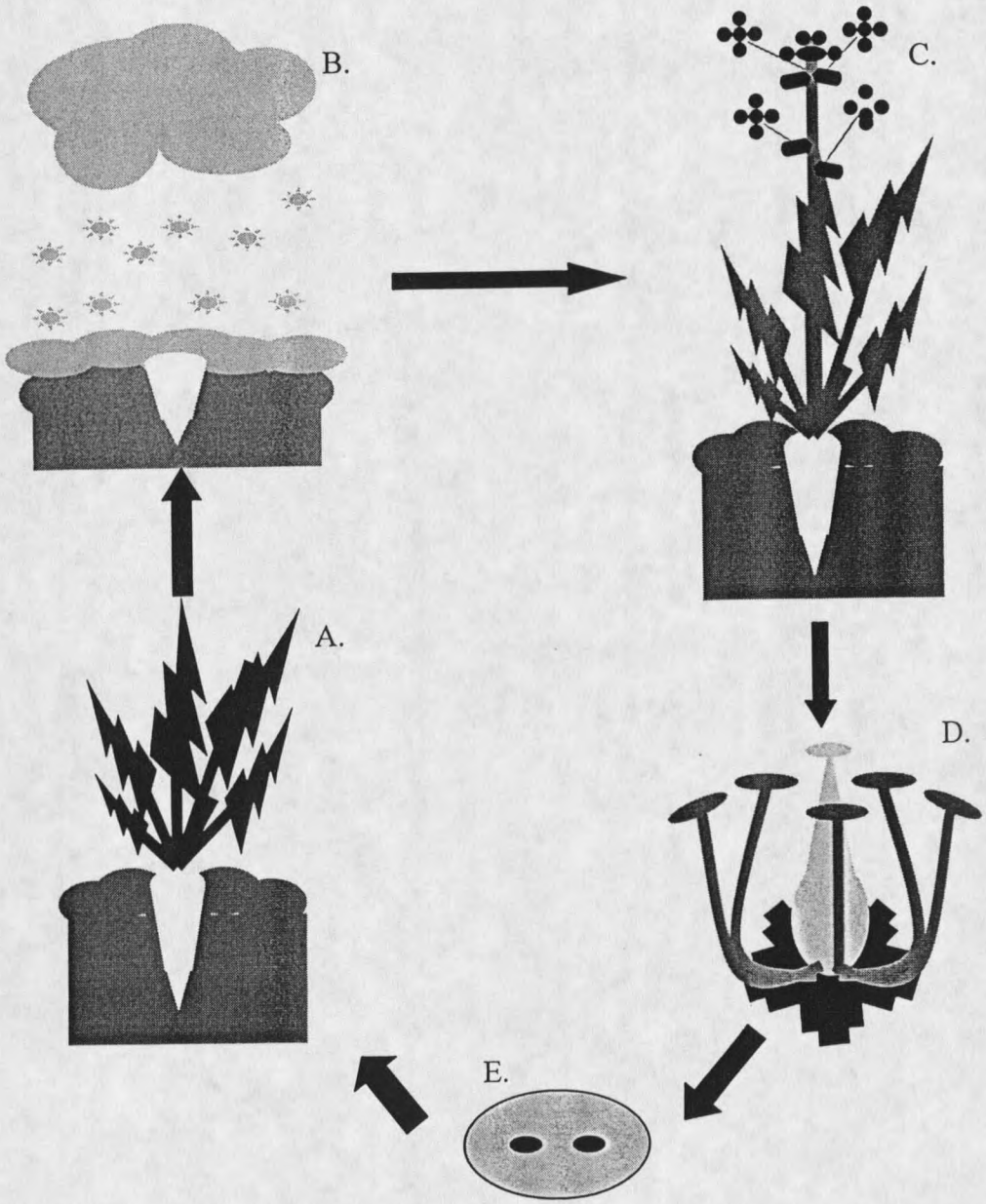
The beet family is broken down into four agricultural groups. The first is the leaf beets, also known as foliage beets. The leaves of this group are harvested for human consumption, such as spinach or swiss chard. The second group contains garden beets, including the red and table beets. In this case 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