CHARACTERIZATION OF PECTOBACTERIUM CAROTOVORUM SUBSP.

BRASILIENSE AS A CAUSAL AGENT OF SUGARBEET SOFT ROT

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Title

Characterization of *Pectobacterium carotovorum* subsp. *brasiliense*
as a Causal Agent of Sugarbeet Soft Rot

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The Supervisory Committee certifies that this ***disquisition*** complies with North

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DOCTOR OF PHILOSOPHY

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ABSTRACT

A soft rot decay of sugarbeet was observed in commercial fields in North Dakota and Minnesota from 2012 to 2016. Symptoms reported are similar to those for bacterial vascular necrosis and rot caused by *Pectobacterium betavasculorum* including soft decay of internal root tissues, reddening of affected tissue after cutting, blackening of petiole vascular bundles, half-leaf yellowing, and root frothing. The disease can cause serious yield losses in the field, and additional economic losses in storage and during processing due to accumulation of invert sugars that reduce sugarbeet quality. Sap from the margin of diseased root tissue was streaked on pectate agar medium and incubated. Single pectolytic colonies were selected and transferred to nutrient broth for bacterial identification and completion of Koch's postulates. Pathogenicity of isolates was assessed by inoculating greenhouse-grown sugarbeet roots. Symptoms characteristic of the disease were observed at 30 days after inoculation included all of the aforementioned, previously stated symptomology. Bacterial DNA was extracted from 46 pathogenic isolates and analyzed by restriction-associated DNA genotype-by-sequencing (RAD-GBS). Ion-torrent sequencing reads (n = 8.54 million) were assembled *de novo* producing ~6,000 sequence tags representing approximately 21% of each bacterial genome analyzed. Partial sequences of five of the seven genes previously used in *Pectobacterium* subspecies phylogenetic analysis were represented in the RAD-GBS isolate sequences. Gene sequences were aligned using Workbench 8.0.3 software to the corresponding reference gene sequences of *P. carotovorum* subsp. *carotovorum*, *P. atrosepticum*, *P. betavasculorum*, *P. carotovorum* subsp. *odoriferum*, and *P. wasabiae*. The alignments showed 99.76% nucleotide sequence identity on average across all five genes to the *P. carotovorum* subsp. *brasiliense* reference sequences. The alignments to *P. cartovoroum* subsp. *carotovorum*, *P. atrosepticum*, *P. betavasculorum*, *P. carotovorum* subsp.

odoriferum and *P. wasabiae* reference sequences showed 96, 95.4, 94.3, 97 and 94.4% identity, respectively, on average across the five genes. The nearly 100% identity across all five genes previously utilized in multi-locus sequencing and divergence from the closely related subspecies strongly suggests that the isolates are *P. carotovorum* subsp. *brasiliense*. To our knowledge, this is the first report of this pathogen causing field decay of sugarbeet in North America.

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DEDICATION

To Jessica, my amazing wife, whose sacrificial care for me and for our children made it possible

for me to complete this work, and to our three beautiful children, Claire, Benjamin and Ryan;

may the effort put forth into this doctoral degree be an example for you to never give up on

something that you desire, no matter how difficult the task. Without the unconditional support,

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OVERVIEW OF DISSERTATION

This dissertation presents and analyzes data concerning the molecular identification and characterization of *Pectobacterium carotovorum* subsp. *brasiliense* as a causal agent of soft rot decay of sugarbeet in North America. This data is derived from isolates collected from multiple commercially-grown sugarbeet fields in North Dakota and Minnesota over a five-year period, and within a laboratory and greenhouse located at North Dakota State University, Fargo, ND. The dissertation is divided into a Literature Review and single manuscript. The Literature Review introduces the soft rot decay of sugarbeet and the biology and control methods for its causal agent, the pectolytic organism *P. carotovorum* subsp. *brasiliense*. The focus of the manuscript is an explanation of the applied and molecular techniques utilized to isolate, characterize, and identify *P. carotovorum* subsp. *brasiliense* as a causal agent of soft rot decay of sugarbeet in North America.

Study objectives included the determination of the pathogen's geographic distribution within the Red River Valley of Minnesota and North Dakota, the identification of the pathogen itself, and the impact and severity of bacterial soft rot both within the field and factory processing facilities.

LITERATURE REVIEW

The Sugarbeet

The sugarbeet is an industrial crop grown commercially as a hybrid, with sucrose refined from the root as the plant constituent of interest. Domesticated from a wild sea beet (*B. vulgaris* ssp. *maritima*), sugarbeets are widely recognized as a halophyte (Glenn et. al. 1999) and taxonomically are ordered as Dicotyledoneae, Caryophyllales (Centrospermae), Amarantheaceae (formerly Chenopodiaceae), *Beta vulgaris* L. subsp. *vulgaris* (McGrath et. al. 2011). Sugarbeets were first commercially grown and processed in 1802 in Northern Europe (Draycott 2008) and production has since expanded throughout Europe, North and South America, Asia and, Africa (Cooke and Scott 1993). Today, global sugarbeet production encompasses over 4,610,000 million hectares grown across 56 different countries (Food and Agriculture Organization of the United Nations 2017) with the top three sugarbeet-producing countries (by annual crop volume) being France, Russia and the United States (37,600, 35,300 and 33,400 million metric tons, respectively) (Statista 2017).

Introduction to the United States

After cultivation in Europe, numerous attempts were made to introduce sugarbeet into the United States. The first effort to grow sugarbeets took place in 1830 near Philadelphia, Pennsylvania, but no factory was ever built, and the idea was eventually abandoned. The first sugarbeet factory built in the U.S. was at Northampton, Massachusetts, in 1838, but ceased operating after 1840 (Kaufman 2009). Other unsuccessful attempts were made to establish factories in Wisconsin, Illinois, Michigan, and later in Utah by the Mormon pioneers (Harveson 2016). The first successful commercial production of beet sugar in the U.S. was in central California in 1870 and by the year 1890, two factories were operating in Alvarado (now known

as Union City) and Watsonville, California (Souder 1970). This success lead to the rapid expansion of the U.S. sugarbeet industry and by 1917 there were 91 factories operating in 18 states, matching the production of its European counterparts (Magnuson 1918).

During the next several decades, many sugar factories were built within the United States, but most only remained in operation for a short time. Regardless of where sugarbeets are grown, climate and soil type are the two major factors for successful production (Draycott 2008). These early start-up efforts often were done on a trial-and-error basis, moving around frequently from place to place, searching for the right combination of factors that would result in greater long-term success (Harveson 2016).

Today, the sugarbeet industry in the United States consists of 23 factories operating within four diverse regions in eleven states. These areas include the intermountain region (California, Idaho, Oregon and Washington), the Northern Great Plains (Colorado, Nebraska, Montana and Wyoming), the Great Lakes (Michigan) and the Red River Valley (Minnesota and North Dakota). In 2016, these areas produced a combined total of 33,458,000 metric tons of sugarbeets grown on 453,000 hectares (U.S. Department of Agriculture, National Agricultural Statistics Service 2017). The total sugar production from this hectarage (4.9 million metric tons) represents nearly 60% of the domestic sugar production within the United States (Statista 2017).

Red River Valley of Minnesota and North Dakota

Sugarbeet has been grown in the Red River Valley (RRV) region of eastern North Dakota and western Minnesota since 1918, when the first sugarbeet crop was planted on a farm near Crookston, Minnesota. The sugarbeet raised on this small hectarage was grown under contract from the Minnesota Sugar Company and was sent by railcar to Chaska, Minnesota, for processing (Shoptaugh 1997). Over the next few years, the area under cultivation grew steadily

until there was enough sugarbeet production in the RRV to justify building a processing facility near East Grand Forks, Minnesota, that was completed in 1926. Processing plants in Crookston and Moorhead, Minnesota, soon followed in 1948 and 1954, respectively, as sugarbeet hectarage continued to expand rapidly, and another factory was built in 1965 near Drayton, North Dakota (Strand 1998).

Since the mid-1970s, when three independent grower-owned sugar cooperatives (American Crystal Sugar Company, which purchased the cooperative at Hillsboro after one year of operation; Minn-Dak Farmers Cooperative; and Southern Minnesota Beet Sugar Cooperative) were formed, the RRV has become the largest production area of sugarbeet within the United States. This particular region represented 32% of the U.S. area cultivated to sugarbeet in 1978 (170,500 hectares) and production area has increased steadily over the past three decades to 263,000 hectares, which comprised 57% of U.S. area sown to the crop during the 2016 growing season (USDA-NASS 2017).

One of the main reasons for the increased production and continued popularity of this specialty crop is the vital role that sugarbeet production plays in the regional economy. The total economic activity (direct and secondary impacts) generated by the sugar industry of the RRV was nearly $4.9 billion USD in 2011 alone; or, expressed alternatively, each hectare of sugarbeet planted generated about $18,550 in total local business activity (Bangsund et al. 2012). Expansion of acreage also has resulted from the development of long-term storage technology that allows these factories to have the longest processing campaigns in the world – sometimes more than 280 days in length.

Production Challenges of the Red River Valley

Weed Control

Annual sugarbeet production surveys conducted by North Dakota State University from 1983 to 2007 consistently listed weed control as the top response by growers as their "worst production issue" (Carlson et al. 2008). Multiple species of pigweeds (*Amaranthus* spp.), lambsquarters (*Chenopodium album* L.), and Kochia [*Bassia scoparia* (L.) A.J. Scott] were among the weed species reported as causing the largest problem, costing the cooperatives millions of dollars in lost revenue each year. As a result, row crop cultivation and hand labor for weed control was a common practice throughout the growing season. Row crop cultivation was used by 99% of the respondents for each year from 1996 to 2007, but hand labor has steadily declined since the mid-1990s (62% of respondents used hand labor to thin the crop in 1996, 32% in 2002, and 20% in 2007) (Carlson et al. 2008). The addition of several new herbicide chemistries and new application methodology (i.e., the Micro-Rate Program) (Rothe et al. 2004) were the main causes for the decline in hand weeding.

In 2008, the sugarbeet industry in the RRV transitioned into the use of glyphosate-resistant sugarbeet. Each cooperative held its grower membership to a maximum of 50% of their total allocated hectarage during the first season and allowed unlimited use for the second season (2009) and thereafter. The adoption of this new technology by the growers was a nearly 98% use rate by the second season. When comparing commercial yields, the average yield per hectare of sugarbeet grown in this region from 1988 to 2007 was 43.43 metric tons per hectare. This average increased by almost 14 metric tons per hectare after the introduction of glyphosate-resistant sugarbeet (56.50 average 2008–2016). Sugarbeet quality also benefited as recoverable

white sugar per hectare increased from 6,563 kilograms per hectare in 1988–2007 to 8,357 kilograms per hectare in 2008–2016.

Foliar Diseases

Two foliar pathogens of sugarbeet are endemic to the Red River Valley of Minnesota and North Dakota: Bacterial Leaf Spot and Cercospora Leaf Spot. Although Bacterial Leaf Spot (*Pseudomonas syringae* pv. *aptata* Stevens) commonly occurs throughout the middle to latter part of the growing season, it rarely causes economically significant losses (Windels et. al. 2009).

Cercospora Leaf Spot (CLS), caused by the fungus *Cercospora beticola* Saccardo, is the most important, widespread, and destructive foliar disease of sugarbeet and occurs wherever the crop is grown causing crop losses that can exceed 50% (Whitney and Duffus 1986). In commercial fields, losses are expressed by the reduction of both the harvested root weight and the percent sucrose content in the roots. Long-term storage losses result from increased root decay, and factory processing efficiencies are dramatically impacted by the greater levels of non-sugars and impurities, both of which result in increased sugar loss to molasses (Smith and Ruppel 1971).

Root Diseases

Root diseases are one of the primary yield-limiting factors affecting sugarbeet production in the Red River Valley. The diseases caused and/or vectored by soilborne, root-rotting pathogens often cause more devastating losses than foliar pathogens because they are difficult to detect before serious damages occurs, and control measures are often ineffective or impractical (Harveson 2000). Three root pathogens are the most important cause of economic losses within the commercial fields sown in the Red River Valley.

Aphanomyces Root Rot

Aphanomyces Root Rot is caused by the oomycete *Aphanomyces cochlioides* Drechsler. Capable of persisting in the soil for years, the disease has two distinct phases in the Red River Valley including an acute seedling blight and a chronic root rot, most commonly occurring anytime during the season from June until harvest (Whitney and Duffus 1986). The disease develops in light-textured soils but development is favored in heavy-textured soils and portions of fields that tend to remain wet, such as near drainage ditches, hillsides, low spots and compacted areas (Harveson 2009).

While there are no commercially available cultivars with specific disease resistance to *A. cochlioides*, there are several available that appear to have a tolerance to the pathogen (Wilson 2001). Other management strategies include treating the sugarbeet seed with hymexazol (IUPAC: 5-methyl-1,2-oxazol-3-ol) (Claudis-Petit 2002) that will protect a sugarbeet seedling for approximately three to four weeks when applied at the 45-gram level and five to six weeks at the 70-gram level (Khan 2002). The field application of factory spent lime also reduces this disease (Windels et. al. 2007). A combination of both chemical and cultural farming practices are required in order to achieve desired levels of disease control. Due to the complications experienced with both storage and processing, commercial fields with severe infestations can be declared total losses and released from their contractual obligation with the cooperatives.

Fusarium Yellows and Fusarium Decline

Fusarium yellows of sugarbeet was identified in the Red River Valley in several fields between Moorhead, Minnesota, and Drayton, North Dakota, in 2002 (Khan et. al. 2009). By 2004, roughly 5% of the fields in the RRV had symptomatic plants (Windels et. al. 2005), and incidence has continued to spread throughout the last decade. Caused by *Fusarium oxysporum* f.

sp. *betae*, (Stewart) Snyder and Hansen, plants infected with Fusarium yellows exhibit necrotic wilt symptomology. In many cases, only one-half of the leaf exhibits yellowing and eventually necrosis. Entire leaves eventually die but remain attached to the plant (Draycott 2008). Although the external root symptoms are absent, internal tissues reveal a greyish brown or reddish-brown discoloration that appear in a 'bullseye' pattern when a transverse section of the root is cut. Even though mature plants rarely die, the disease can cause significant reductions in root yield and recoverable sucrose as well as storage and processing complications (Khan et. al. 2009).

From 2005 to 2007, isolations were made from sugarbeets collected in commercial fields and from a Fusarium screening nursery field showing symptoms of yellowing, interveinal chlorosis, scorching, stunting, vascular discoloration of the taproot, and early death of plants (Rivera et. al. 2008). The pathogen isolated from the infected beets revealed a new species of Fusarium (*Fusarium secorum* Secor) responsible for Fusarium yellowing decline of sugarbeet. Similar to *F. oxysporum*, *F. secorum* isolates are able to induce half- and full-leaf yellowing foliar symptoms and vascular necrosis in sugarbeet roots and petioles, but occur earlier in the season than Fusarium yellows (Secor et. al. 2014).

There are no fungicides currently registered for control of either Fusarium yellows or Fusarium yellowing decline. Weed control is essential as several species can serve as alternate hosts to the pathogen (Postic et. al. 2012). Commercially available cultivars with resistance to Fusarium spp. have been released but are only effective in limited areas due to the variability and geographic distribution of the pathogen. Since the pathogen can be carried in soil, the transfer and spreading of tare soil into areas where the disease is not known to occur is closely monitored by all three RRV cooperatives.

Rhizoctonia Root Rot

Rhizoctonia root rot is the most common and most serious root disease of sugarbeet in the United States; it is endemic and of economic importance in most areas where the crop is grown, including the Red River Valley (Whitney and Duffus 1986). Rhizoctonia infects both the seedling (root) and adult (crown) stages of the plant (Harveson et. al. 2009). Rhizoctonia root and crown rot are caused by the fungus *Rhizoctonia solani* Kühn and, in both cases, symptoms may range from scattered brown to black lesions on the root surface to rotting of the entire root. This fungus is divided into a number of anastomosis groups based on vegetative compatibility reactions that occur when hyphae of two similar isolates fuse and genetic material is exchanged (Khan and Bolton 2016). The anastomosis group causing Rhizoctonia root and crown rot of sugarbeet are *R. solani* AG 2-2 and AG 2-4 (Ogoshi 1987). Within the Red River Valley, *R. solani* AG 2-2 is further divided into two intraspecific groups: AG 2-2 IIIB and AG 2-2 IV (Windels and Brantner 2007). Rhizoctonia root and crown rot of sugarbeet can be caused by both intraspecific groups of *R. solani* AG 2-2 (IIIB and IV), but *R. solani* AG 2-2 IIIB is the most aggressive on sugarbeet, and it also causes disease in corn and soybean (Panella 2005). Within the Red River Valley, the pathogen is present in all soil types and becomes problematic in fields where the pathogen population is high because of the frequent use of susceptible host crops in the rotation (Brantner and Windels 2007).

Methods for controlling Rhizoctonia crown and root rot on sugarbeet include rotating to non-host crops (i.e. small grains), planting tolerant varieties, and avoiding the movement of infected soil. The crown rot infection is often initiated when infected soil is placed against the side of the crowns (hilling) during cultivation, by rain splash of infected soil into the crowns or where the petiole attachment to the crown is covered with infected soil (Khan and Bolton 2016).

Weed control is essential as the pathogen infects numerous weed species including pigweed, lambsquaters and kochia (Harveson et. al. 2009). Seed treatment fungicides and the post emergence applications of the fungicide azoxystrobin (IUPAC: methyl (E)-2-[2-[6-(2-cyanophenoxy) pyrimidin-4-yl]oxyphenyl]-3-methoxyprop-2-enoate) may also aid in the delay of the onset and severity of this disease (Chanda et. al. 2016).

Losses in Storage and Factory Processing

The primary objective of sugarbeet storage operations is to maintain a high level of the sugar accumulated during the growing season and to prevent the formation of compounds that interfere with sugar extraction (Draycott 2008). In the Red River Valley of Minnesota and North Dakota, the annual volume of harvested sugarbeets (nearly 18.2 million metric tons) are placed into long-term storage in piles comprised of 127,000 metric tons and on average are 77 meters wide, 7.5 meters tall and over 430 meters in length. These piles can remain frozen for processing campaigns exceeding 280 days. Although there are many environmental and biological factors that can influence the amount of sucrose lost during sugarbeet storage, the rate of respiration and invert sugar accumulation are of greatest concern to the sugar cooperatives. These two losses incurred during storage represent a substantial decrease in revenue for the three RRV cooperatives and, when multiplied over the volume of roots harvested and placed into storage, even small reductions in storage loss can have a significant economic impact (Campbell and Klotz 2007).

Sugar loss within sugarbeet roots begins at harvest and rapidly increases while the crop is maintained in long-term storage. Stored sugarbeets metabolize their own sucrose for life support through an oxidative process where cellular organic compounds are converted to carbon dioxide and water to generate metabolic substrates and energy (Cooke and Scott 1993; Campbell and

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Klotz 2006a). It has been estimated that 60-80% of the sucrose loss during storage is attributed to this metabolic process alone (Harveson et. al. 2009). The rate of respiration is strongly influenced by temperature (McGinnis 1982) and the respiration rate of stored roots generally declines with decreasing pile temperatures until the roots freeze and respiration stops (Campbell and Klotz 2006b). Within the Red River Valley, the average sugar loss per day is estimated to be 0.23 kg per metric ton under ideal storage conditions (Cooke and Scott 1993). However, diseases present at the time of harvest may exert an equal or greater impact on respiration rate than does temperature (Harveson et. al. 2009). The respiration rates of roots with severe symptoms of Aphanomyces Root Rot were shown to be five times those observed in healthy roots 18 days after harvest and remained higher during the 138 days of storage (Campbell and Klotz 2003a). Consequently, the volume of extractable sugar ranged from more than 150 kg/metric ton for the healthy roots to 90 kilograms per metric ton for the most diseased (Campbell and Klotz 2003b). The higher respiration rates of Aphanomyces-infected roots can also increase storage pile temperatures and increase sugar loss in adjacent healthy roots (Campbell and Klotz 2003a).

Similar trends were found with Fusarium yellows in that postharvest respiration rates of roots with high incidence of disease ranged from 0.85 to 2.28 mg CO_2 kg^{-1} h^{-1} higher than roots with low disease only 30 days after harvest and 1.36 to 3.35 mg CO_2 kg^{-1} h^{-1} higher after 90 days in storage (Campbell et. al. 2011). Rhizoctonia-infected roots rated on a 0-7 scale (0 = no rot to 7 = 100% rotted) with ratings of three, four, and five exhibited respiration rates that were 22, 92, and 213%, respectively, greater than roots with a two rating after 30 days in storage (Campbell et. al. 2014). After 90 days in storage, respiration rate increases of 17, 84, and 201% were associated with ratings of three, four, and five for the same study.

In conjunction with respiration, invert sugar accumulation is highly influenced by the amount of root disease present when the sugarbeets enter long-term storage. The invert sugars glucose and fructose are produced as a result of the enzymatic breakdown of sucrose. Sucrolytic enzymes originating from root pathogens are responsible for most of the increase in glucose and fructose concentration within infested roots (Klotz and Finger 2004). Although this breakdown of sucrose naturally results in yield loss, the greatest economic impact results in the complications associated with factory processing. High levels in invert sugars increase the sugar loss to molasses, increase color formation during juice purification, impede sugar crystallization and filtration, and slow down factory operations; all of which significantly increase the cost to produce a unit of sugar (Harveson et. al. 2009).

Roots with severe Aphanomyces root rot were shown to contain 250% more fructose and 190% more glucose than uninfected roots a few days after harvest, and also accumulated invert sugars more rapidly during storage (Campbell and Klotz 2006a). Rhizoctonia-infected roots rated on a 0-7 scale (0 = no rot to 7 = 100% rotted) with ratings of four and five exhibited invert sugar concentrations that were 3.3 and 10.8 times the concentration of roots with a three rating at 30 days post-harvest (Campbell et. al. 2014). Roots with ratings of four and five had 6.6 and 26.1 times the invert sugar of roots with a three rating at 90 days post-harvest for the same study. Research studies conducted by the USDA-ARS in Fargo, ND showed that the invert sugar concentration of roots exhibiting high levels of Fusarium yellows were 2.1-, 4.3-, and 2.7-fold greater than the invert sugar concentration of those with the lowest disease incidence ratings at Sabin, Minnesota, in 2007, Moorhead, Minnesota, in 2007, and Moorhead, Minnesota, in 2008, respectively (Campbell et. al 2011).

Bacterial Soft Rot

Bacterial soft rot of sugarbeet, also known as bacterial vascular necrosis or bacterial soft rot, is a potentially devastating disease to sugarbeet crops. Colloquially known as 'Erwinia,' the Red River Valley of Minnesota and North Dakota has been gradually impacted by this disease over the past several seasons. Agriculturists from all three regional beet sugar cooperatives observed multiple incidences of this disease in their commercial fields between 2012 and 2016. American Crystal Sugar Company reported that of the 190,000 hectares planted in 2013, 5,700 (3% of the total) were affected by bacterial soft rot (T. Grove, American Crystal Sugar Company, pers. comm.). Minn-Dak Farmers Cooperative estimated that of the 46,500 total hectares of sugarbeets planted annually, 6,975 (15% of the total) were affected, over one-half of which had in-field infestations as high as 20% (personal observation). Southern Minnesota Beet Sugar Cooperative estimated affected hectares closer to 10% on an annual basis (4,900 hectares affected of the 49,000 total; M. Bloomquist, Southern Minnesota Beet Sugar Cooperative, pers. comm.).

Although disease incidence is typically lower on an annual basis than other root rot pathogens endemic to the region, sugarbeets infested with bacterial soft rot can drastically reduce on-farm profit to the growers by reducing the overall tonnage and quality of the crop. Due to the contamination by invert sugars associated with this disease, major economic losses also occur for the sugar cooperatives from complications arising during both storage and processing (Strausbaugh and Gillen 2008).

Geographic Distribution

Bacterial soft rot of sugarbeet is readily found in nearly all of the sugarbeet-producing regions of the United States. The disease was first reported in the San Joaquin Valley of

California in 1972 (Thomson and Schroth 1972), and has since been discovered in Texas, Arizona, Washington, Idaho, and Montana (Zidack and Jacobson 2001). Globally, reports of the disease have also been documented in Europe (Campbell 2005), Iran (Zohour 2003), Egypt (Saleh et. al. 1996) and Croatia (Đermić 2010).

Within the Red River Valley of Minnesota and North Dakota, bacterial soft rot of sugarbeet was first reported in 2016, with symptomology characteristic of the pathogen occurring as early as 2012 (Secor et. al. 2016). It is more frequently found in the southern portion of the valley, but recently has been increasing in prevalence and severity in central and northern areas of the valley, extending as far north as Pembina and Kittson Counties along the Canadian border (T. Grove, American Crystal Sugar Company, pers. comm.).

Origin and Taxonomy

Presently, bacterial soft rot of sugarbeet is reported to be caused by *Pectobacterium betavasculorum* (Thomson) Gardan (syns. *Pectobacterium carotovorum* subsp. *betavasculorum* (Thomson) Hauben and *Erwina carotovora* subsp. *betavasculorum* Thomson) (Bull et. al. 2010; Harveson et. al. 2009).

In 1901, L.R. Jones reported soft rot of stored cabbage, celery, and carrots, and named the pathogen *Bacillus carotovorus* (Kado 2010). Jones' finding was confirmed in 1910 by Erwin Smith. In 1927, the soft rot bacterium identified by Jones was renamed *Bacterium carotovorum*, and in 1945, it was again renamed to *Pectobacterium carotovorum* (Kado 2010). In 1969, genetic evidence suggested that the pathogen be reclassified into its own unique group, that being the genus *Erwinia* (Dye 1969) resulting in another renaming to *Erwinia carotovora*. During this time period, the subspecies of *E. carotovora* were historically described as distinct species or subspecies on the basis of pathogenicity and host-plant origin (Gardan et. al. 2003).

Thus, when the pathogen was discovered on sugarbeet in 1972, it was officially classified as *Erwinia carotovora* (Jones) Bergey et. al. subsp. *betavasculorum* Thomson et. al (Thomson et. al. 1981b).

Based on a comparative analysis of the sequences of the 16S rRNA genes of 16 known *Erwinia* species, Kwon et. al. (1997) showed that *Erwinia* species could be divided into four distinct phyletic lines and that Clade III comprised *E. chrysanthemi* and three subspecies of *E. carotovora* (*carotovora*, *betavasculorum* and *wasabiae*) clustered together. Subsequently, all soft rot-causing members of the genus *Erwina* were reclassified to the genus *Pectobacterium* and divided *P. carotovorum* into five subspecies: *atrosepticum*, *betavasculorum*, *carotovorum*, *odoriferum* and *wasabiae* (Hauben et. al. 1998). Utilizing analyses including DNA–DNA hybridization, numerical taxonomy of phenotypic characteristics, serology and new phylogenetic analysis of previously reported sequences from a database of aligned 16S rDNA sequences were conducted by Gardan et. al. (2003) resulting in the elevation of several subgroups of *P. carotovora* to species status including *Pectobacterium betavasculorum*.

Taxonomically, *P. betavasculorum* is categorized as a part of the Kingdom Bacteria, Phylum Proteobacteria, Class Gamma Proteobacteria, Order Enterobacteriales and Family Enterobacteriaceae (Bull et. al. 2010).

Organism

Pectobacterium betavasculorum (Thomson) Gardan is a single-celled, gram-negative, non-spore-forming straight rod (0.5-1.0 x 1.0-3.0 μm) and is motile by lateral peritrichous flagella (Whitney and Duffus 1986). The organism favors a warm environment and can grow at temperatures exceeding 36 °C (Harveson et. al. 2009). The pathogen is facultatively anaerobic, negative for cytochrome oxidase, positive for catalase and acid-producing when cultured on

fructose, glucose, galactose, lactose, methyl alpha-glucoside, inulin, xylose, and raffinose (Kado 2010; Thomson et. al. 1977). It produces reducing sugars from sucrose and does not produce acid from sorbitol or $_D(+)$-arabitol (Harveson et. al. 2009). It assimilates palatinose and $_L$-alanine but not meso-tartrate, $_D(-)$-tartrate, galacturonate, malonate, melezitose, melibiose or citrate (Harveson et. al. 2009; Thomson et. al. 1977). The organism is capable of surviving in culture medium sodium levels of up to 7-9% and the G+C content of the DNA ranges from 54.1 to 54.6 mol% (Harveson et. al. 2009).

When cultured on rich agar medium, colonies are white with a yellow to orange center and wavy coralloid margins, often resembling a 'fried egg' (Draycott 2008). Since *P. betavasculorum* produces pectinase and protease, colonies are strongly pectolytic on pectate medium causing characteristically deep pits or cavities within the medium aiding in genera identification (Charkowski 2007).

Host Range

Pectobacterium spp. are considered broad-host range pathogens in part because they have been isolated from so many plant species and in part because single strains are pathogens of numerous plant species under experimental conditions (Ma et. al. 2007). *P. betavasculorum* is an exception to the broad-host-range nature of *Pectobacterium* spp. as it is reported to naturally occur almost exclusively on sugarbeet. Artificially inoculated hosts have been demonstrated as suitable in multiple studies and, in addition to sugarbeet, include fodder beet and sugarbeet-fodder crosses, *Beta maritima*, *B. macrocarpa*, *B. corolliflora*, sweet potato, radish, squash, cucumber, carrot, sunflower, artichoke, chrysanthemum, tomato, and potato (Thomson et. al. 1977; Saleh et. al. 1996; Harveson et. al. 2009; Draycott 2008). Research conducted by Nedaienia and Fassihiani (2011) also confirmed melon, maize and eggplant as artificial hosts.

This host range may not be complete since only a limited number of plant species have been tested for susceptibility (Harveson et. al. 2009).

The role of weed species in the natural host range of bacterial soft rot of sugarbeet is largely untested to date. *Pectobacterium atrosepticum* is a bacterial soft rot pathogen that is responsible for the disease blackleg of potato (*Solanum tuberosum* L.), and variants of this bacterium can cause root rot in sugarbeet (De Mendonça and Stanghellini 1979; Gardan et. al. 2003). This subspecies has been detected in the rhizosphere of several weed species including *Amaranthus palmeri* (Palmer Amaranth) (De Mendonça and Stanghellini 1979). The weed species *Amaranthus retroflexus* (Red Root Pigweed) and *Chenopodium album* (Common Lambsquarter) among several others were found to also harbor known soft rot causing bacterium including *Pectobacterium* spp. (Zimdahl 2013). These findings may be significant considering the populations of these weed species in most cultivated regions where beets are commercially grown is relatively high due to the increasing prevalence of glyphosate tolerance, including in the Red River Valley of Minnesota and North Dakota.

Epidemiology and Disease Cycle

Bacterial soft rot infects sugarbeets in both the seedling and adult stages. It is somewhat unusual, because of the many bacterial diseases that occur on sugarbeet, it is one of a few capable of causing extensive damage (Campbell 2005). Conditions that must take place simultaneously for the disease to occur are that 1) *P. betavasculorum* must be present within the field, 2) a susceptible crop must be grown, and 3) favorable environmental conditions must be present. The onset of the seedling disease is favored by warm, wet soils, and stand establishment is improved when seed is sown early in cooler soils. Young plants (less than eight weeks old) are considered to be more susceptible (Thomson et. al. 1977). Soft rot in adult plants typically occurs

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in July and August in Minnesota and North Dakota, following extended warm periods. Adult root rot has been particularly severe in recent years in this production region due to unusually warm and wet summers.

In order for infection to occur, injury to the leaves, petioles or crown is mandatory for the pathogen to gain entry to the host tissue (Harveson et. al. 2009; Wilson et. al. 2001). This often occurs mechanically during routine agronomic practices associated with commercial sugarbeet production such as cultivation, mechanical-thinning, spraying, irrigation and defoliation. Natural means of injury include insects, foliar damage from gusting winds, animals, etc. Hail damage has also been correlated with a higher degree of disease outbreak (Zidack and Jacobson 2001).

Temperature and free moisture are key factors in the overall rate of disease development. Like most plant-pathogenic bacteria, water promotes disease development by providing a more optimal environment for the pathogen (Kado 2010), and has been a key factor in augmenting disease outbreak in fields with sprinkler irrigation since 'splashing' has been documented as a means of disease transmission (Whitney et. al 1986). While symptoms can occur when temperatures are as low as 18 degrees Celsius, warm temperatures between 24-30 °C promote rapid disease development and can result in acute symptoms (Thomsom et. al. 1977; Harveson et. al. 2009).

Infection often starts at the crown near the base of the petioles and then moves downward into the root. The invading bacteria target pectin, a major component of the middle lamella that helps bind cells together. Based upon the uronic acid content found in polysaccharides, sugarbeets have been shown to have a very high volume of pectin compared to other crops (Müller-Maatsch et. al. 2016). Sugarbeet pectin contains acetylated galacturonic acid in addition to methyl esters which increases the stabilizing effects of pectin within the plant (Draycott 2008).

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In order to induce maceration of the tissue, the organism produces plant cell wall degrading enzymes via type-II secretion systems including pectin acetyl esterase, pectin methyl esterase, polygalactronases and celluases (Kim et. al. 2011; Kado 2010). These pectinases result in the loss of the skeletal components of the host's cells and the eventual release of the cellular fluids for uptake by the pathogen. This loss of cellular integrity is what eventually causes the tissue maceration and characteristic symptomology of this pathogen. The bacteria will continue to reproduce and infect as long as host resources are available (Thomson et. al. 1977).

The *Pectobacterium* spp. responsible for sugarbeet infection continues disease progression beyond harvest. Within the Red River Valley of North Dakota and Minnesota, sugarbeet roots are stored before processing in large outdoor piles that are subject to high relative humidity and warm climate conditions which likely help facilitate and promote the incidence of bacterial soft rot in early storage. Since the onset of bacterial soft rot does not usually occur until the latter parts of July to early August when ambient air temperatures stay within ranges optimal for disease development, there is generally a wide-range of disease severity at the beginning of commercial harvest. Bacterial soft rot of sugarbeet is unique in that plants with severe levels of infection (> 90% of tissue macerated) are still able to remain both physically intact and in place during the aggressive nature of the harvest process. Unlike other root pathogens endemic to the region that attack the host plant from the 'outside-in,' bacterial soft rot of sugarbeet works 'inside-out,' thereby leaving the lateral and tap roots secured in the ground. These root structures help keep the infected beet within the harvested row during the aggressive defoliation process as opposed to displacing the entire root in between the harvested row as is common with severe infections from *A. cochlioides* or *R. solani*. Roots infected with these pathogens are easily dislodged or are too small to be harvested (Windels and Lamey 1998). Therefore, sugarbeets

infected with bacterial soft rot, regardless of severity, are more apt to be collected by the harvester and delivered for storage and processing. Infected plants that are harvested not only have reduced yield and sugar content, but also have higher levels of impurities (invert sugars) which makes sugar extraction less efficient and more costly.

Although soft rot enterobacteria have been studied for decades, very little currently is known about their survival strategies between growing seasons. Upon the death of the host or harvest of the field, the pathogen appears to survive in living plant tissue such as beet roots, volunteer beets (Wilson et. al. 2001) and several weed species (De Mendonça and Stanghellini 1979; Zimdahl 2013). To date, the organism has not been found to survive in or on sugarbeet seeds and is only capable of survival in the soil for a period of two months post-harvest (Harveson et. al. 2009). Due to the ability of soft rot bacteria to colonize plants latently without symptoms, weed species related to known hosts may be important in the epidemiology of this pathogen (Davidsson 2013). Further, dispersal of the bacteria could also happen via usage of surface water for irrigation, via aerosols generated by rain, via movement of the bacteria in soil water or mechanically via contaminated agricultural equipment (Perombelon and Kelman 1980).

Nadarasah and Stavrinides (2011) demonstrated that insects can act as vectors for many plant pathogenic bacteria, including *Pectobacterium* spp. Soft rot enterobacteria have been found associated with insects and transmission via insects has been suggested (Perombelon and Kelman, 1980; Davidsson 2013). Several isolates of *P. carotovorum* have been documented as persistent in the gut of the fly genera Drosophila (Basset et. al., 2003). In addition to insect vectors, Nykyri *et. al.* (2014) demonstrated that soft rot enterobacteria were able to withstand nematode grazing, colonize the gut of *Caenorhabditis elegans* (var Bristol) and subsequently disperse to plant material while remaining virulent. The existence of bacterial genes promoting

interactions with insects and nematodes suggests that adaptation to these organisms as vectors or as alternative hosts may have played an important role in the evolution of these plant-pathogenic bacteria (Davidsson 2013).

Symptoms

Pectobacterium spp. is the only bacteria known to cause severe damage of sugarbeet (Campbell 2005); symptoms can be found on the foliage or roots of infected plants. Many times affected plants are difficult to detect until the rot is well advanced (Dunning and Byford 1982). Foliar symptoms include dark to black-colored longitudinal lesions along one-half of the petiole. This is eventually followed with vascular necrosis of the leaf in either a clockwise or counter-clockwise direction dependent upon which petiole exhibits symptoms. Much like the petiole, only one side of the leaf is affected first. The foliage of adult plants infected with bacterial soft rot will often wilt during the afternoons of hot, sunny days and appear to recover overnight and on cooler, cloudy days. The plant canopy will ultimately become dry and brittle before being found heaped around the crown (Harveson et. al. 2009). Foliar symptoms are most often detected in sugarbeet fields exhibiting higher infection levels but are difficult to detect in fields exhibiting moderate to lower levels of infection.

Infection in the petioles can continue systemically down into the crown and eventually the root. Severe rot occurring in the root is often depicted by viscous biofilm deposits on the center or side of the crown, which are formed as a result of escaping gases emitted as a by-product of bacterial metabolism (Thomson et. al. 1977; Harveson et. al. 2009). Root symptoms vary from soft rot to dry rot and vascular bundles become necrotic. When the root is cut to expose the vascular bundles, surrounding areas turn pink to reddish within 20-30 seconds (Wilson et. al. 2001). Occasionally, the organism invades the lower portion of the taproot,

inducing the same wet rot on the root tip. Degradation of the root tip severely stunts the plant and, in severe cases, leads to both rotting and eventual death of the plant. In many cases, the root does not rot completely and only exhibits circular, necrotic areas that become blackened, hollowed-out cavities (Thomson et. al. 1977; Harveson et. al. 2009). Affected roots may become almost completely hollow without dying (Dunning and Byford 1982). Significant variability in root symptoms can vary from plant to plant as multitude of additional microorganisms that may colonize the damaged tissue (Perombelon and Kelman 1980).

Within a commercial sugarbeet field, bacterial soft rot most often occurs from mid-season through harvest during periods of warm temperatures and above-normal precipitation. Plants that were infected as seedlings or as those with healthy, more mature sugarbeet roots may become infected at this time. Sugarbeets infected with bacterial soft rot occur in patches ranging in size from a few meters in diameter to extreme cases where greater than 70% of an entire field is infested (Zidack and Jacobsen 2001). Because of the ability of this bacterium to move with soil moisture, fields that generally remain wet and saturated are prime candidates for bacterial soft rot (Whitney and Lewellen 1985). Some of these areas may be impacted by the proximity of drainage ditches, hillsides, low spots, and areas of heavily compacted soil.

Disease Control

Although modern technology has changed the face of agriculture over the past several decades, sugarbeet producers remain heavily dependent upon simple cultural practices to manage bacterial soft rot. Such cultural practices include early spring planting, improved tillage and drainage techniques, specific crop rotations, specialized cultivars, and timely weed control.

Since infectivity of sugarbeets by bacterial soft rot is poor at temperatures below 18 ℃ and young plants less than eight weeks old are considered to be more susceptible (Thomson et.

al. 1977), infection can be avoided by planting into cooler soils as early as possible in the growing season. Normal planting dates for most sugarbeet producers in the Red River Valley of Minnesota and North Dakota are between April 20 and May 1. This practice enables sugarbeet seedlings to advance beyond the stage where they are most susceptible to soft rot before soil temperatures rise and pathogen activity increases.

Planting seeds at shallower depths, 1.27 to 1.91 centimeters deep (2.54 to 3.18 centimeters is recommended for maximum germination and emergence), encourages maximum emergence and vigorous growth (Khan 2017). Since *Erwinia* spp. exhibit a distinct vertical pattern of distribution in soil and can consistently be detected at soil depths exceeding 12.7 centimeters deep (De Mendonça and Stanghellini 1979), rapid plant emergence becomes a key factor in disease control.

Achieving optimal stands also reduces favorable conditions for disease development (Harveson et. al 2009). Within the Red River Valley, getting a good, even stand established is one of the most critical factors in optimizing sugar production. Due to losses from planter seed drop, germination, emergence, plant competition and environmental factors, it is common for only 60-80% of the seeds sown to make it to the final harvested plant stand. As such, sugarbeets sown in 56-centimeter rows are planted at a targeted spacing of 11.43 cm between each individual seed (156,566 seeds per hectare) and growers planting sugarbeets into 76-centimeter rows target a seed spacing of 8.9 cm (147,619 seeds per hectare). In order to achieve the optimal balance between high root yield and high sugar content, plant populations at harvest should be near 103,800 evenly-spaced plants per hectare on 56-centimeter rows and 89,000 plants per hectare for 76-centimeter rows (Yonts and Smith 1997). This equates to 180 and 210 plants per 30-meter of row, respectively.

Since soil moisture plays a significant role in the onset and spread of bacterial soft rot, tillage practices that promote soil drying are highly favored. Many producers favor row crop tillage equipment, such as triple- or single-shank cultivators, that provide very thorough soil perturbation on the upper 5 to 7.6 centimeters of the soil profile. Equipped with either tunnel or rolling shields to protect the crop, each machine is able to work safely between rows and cultivate soil without "hilling" infected soil next to or into the plant's crown or physically damaging the plant itself, which could open a potential site of infection. Multiple trips across an infested beet field are sometimes necessary after periods of rain. In some cases, growers have implemented deep tillage practices using plows and other implements in hopes of burying the pathogen. This is not commonly practiced in the Red River Valley of Minnesota and North Dakota since it surfaces heavier clays and buries fertile topsoil.

Another tactic implemented by sugarbeet producers in their farming practice to manage soil moisture is field drainage. Many growers utilize heavy scrapers and earthmovers combined with laser-guided measuring devices for engineering precise elevation drops within a field. Other producers discovered that plastic drain tile strategically placed throughout wet areas of a field aids in reducing excess moisture (Windels and Brantner 2001). By promoting drainage, growers are not only able to drain wet areas of fields much faster, limiting the spread and onset of the disease, but also protect the crop by confining affected areas to ditch bottoms and low areas.

Choosing and managing specific crop rotations can also help producers reduce the effects and impact of bacterial soft rot within their farming operation. However, this becomes difficult for managing pathogens such as *Pectobacterium* spp. given their wide host range. Within the Red River Valley of Minnesota and North Dakota, crop rotations with summer fallow may help

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reduce bacterial soft rot as *Pectobacterium* spp. do not survive well in a field that is fallow and repeatedly tilled (Mohler and Johnson 2009).

Heritable tolerance is available to bacterial soft rot and presently is being incorporated into commercial sugarbeet varieties and should be utilized wherever the disease is endemic (Cooke and Scott 1993). However, the yield of sugarbeet varieties characterized by tolerance to bacterial soft rot currently is lower in comparison to top producing varieties under disease-free conditions (SESVanderHave USA 2016), thereby decreasing their attractiveness to growers. Heritable tolerance is apparently of two types: tolerance to bacterial soft rot is monogenic and dominant, whereas tolerance governing the rate of development of soft rot within the root may be quantitative (Draycott 2008). Although moderate levels of tolerance can be obtained through selective breeding, no sugarbeet variety is immune to infection by this pathogen (S. Kaffka, University of California-Davis, pers. comm.). Many growers must rely on their crop records and field history to evaluate the use of tolerant varieties within their fields.

Weed control may be an important factor in the control of bacterial soft rot that is overlooked. Both red root pigweed (*Amaranthus retroflexus*) and common lambsquarters (*Chenopodium album*) are hosts of soft rot causing bacterium including *Pectobacterium* spp. and *Pectobacterium atrosepticum* (De Mendonça and Stanghellini 1979). *P. atrosepticum* has also been found in the rhizosphere of *Amaranthus palmeri* (Palmer Amaranth) (Zimdahl 2013), an emerging threat to the sugarbeet production region of the Red River Valley (Peters and Jenks 2018; Gunsolus et. al. 2018). The presence of these three weeds in sugarbeet fields can cause serious yield reductions, not only through competition with beet plants, but also could potentially serve as reservoirs for bacterial soft rot. Thus, control of these weeds during the sugarbeet

season, as well as during the production of other crops, could help reduce build up and preservation of these pathogens within fields.

Biological control of plant diseases is an exciting strategy that has generated a considerable amount of interest over the past quarter-century. This interest has been generated due to the increasing regulations and restrictions associated with pesticide use, unsuccessful control attempts by other means (Maloy 1993), and the potential for biocontrol agents to protect a crop throughout the growing season. Several bacteriophages have been identified as effective controls of *Pectobacterium carotovorum* subsp. *betavasculorum* including the strains ΦEcc2, ΦEcc3, ΦEcc9 and ΦEcc14 (Ravensdale et. al. 2007). When ΦEcc3 was applied to inoculated calla lily tubers in greenhouse conditions, it reduced the amount of diseased tissue from 30% to 5% in one study and from 50% to 15% in a second experiment (Ravensdale et. al. 2007).

The role of nitrogen fertility has been investigated as a factor contributing to this disease because high rates of nitrogen stimulate rapid sugarbeet crown growth, resulting in more growth cracks at the bases of old petioles and thus more sites for bacterial infection. Field experiments near Davis, California, showed that incidence and severity of *E. carotovora* subsp. *betavasculorum* was increased proportionally with respect to the rate of nitrogen fertilizer applied (Thomson et. al 1981a). Under inoculated conditions, the amount of rot per sugarbeet root increased from 11% without any nitrogen fertilizer applied to 36% when nitrogen in the form of ammonium nitrate was applied. There was no corresponding increase in root or sugar yield in contrast to the significant increases that were obtained with higher nitrogen fertilizer rate in the uninoculated plots (Thomson et. al 1981a). Thus, the benefits of higher nitrogen fertilizer rates expressed in healthy sugarbeets may not be obtained from beets infected with bacterial soft rot (Thomson et. al 1981a).

This is problematic for sugarbeet growing regions of the Red River Valley in Minnesota and North Dakota, because nitrogen is the single-most important nutrient when planning a fertilizer program for sugarbeet production. The highest quality sugarbeet is produced when nitrogen deficiency occurs late in the growing season, thus the amount of total nitrogen available to the plant (based upon the nitrogen applied plus the residual nitrate-N from a soil test) needs to be carefully analyzed. Excess nitrogen at or near the end of the growing season reduces sugarbeet quality by reducing sucrose concentration and increasing impurity concentration (Lamb et. al. 2001).

While nitrogen fertilizer equivalents exceeding 135 kilograms per hectare are required to achieve the optimal balance between high root yield and high sugar content (Khan 2017), the nitrogen fertilizer guideline will depend on the location that the sugarbeet is grown. For the American Crystal Sugar growing areas, a total of 135 kilograms per hectare as soil test NO_3^- nitrogen in the upper 1.22-meter of the soil profile plus supplemental nitrogen fertilizer is needed (Kaiser 2011). Due to differences in quality parameters utilized to determine the company's per tonne payment to the producer, Minn-Dak Farmers Cooperative targets a total nitrogen availability of 157 kilograms per hectare (soil test NO_3^- nitrogen in the upper 1.22-meter of the soil profile plus supplemental nitrogen fertilizer). Due to the high availability of residual nitrogen and elevated levels of organic matter present within their geography, Southern Minnesota Beet Sugar Cooperative recommends the amount of NO_3^- nitrogen for a 1.22-meter soil test and supplemental fertilizer should be 112 kilograms per hectare and 90 kilograms per hectare for a 0.61-meter soil test, respectively (Kaiser 2011). Nitrogen fertilizer rates in all three Red River Valley cooperatives may have to be reduced if bacterial soft rot is present within commercial fields, although this remains untested to date.

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CHARACTERIZATION OF PECTOBACTERIUM CAROTOVORUM SUBSP. BRASILIENSE AS A CAUSAL AGENT OF SUGARBEET SOFT ROT

Introduction

The Red River Valley (RRV) region of eastern North Dakota and western Minnesota is the largest production area of sugarbeet within the United States. Over 263,000 hectares are sown in non-irrigated fields on an annual basis, all of which are grown under contract to one of three independent grower-owned sugar cooperatives (American Crystal Sugar Company, Moorhead, MN; Minn-Dak Farmers Cooperative, Wahpeton, ND; and Southern Minnesota Beet Sugar Cooperative, Renville, MN). Representing 57% of U.S. sugarbeet hectarage during the 2016 growing season (USDA-NASS 2017), the crop plays a vital role in the regional economy. The total economic activity (direct and secondary impacts) generated by the sugar industry of the RRV was nearly $4.9 billion USD in 2011 alone; or expressed alternatively, each hectare of sugarbeet planted generated about $18,550 in total local business activity (Bangsund et al. 2012).

A soft rot decay of sugarbeet was observed in commercial fields within the RRV from 2012 to 2016. Symptoms reported were similar to those for bacterial vascular necrosis and rot caused by *Pectobacterium betavasculorum* reported in other sugarbeet-producing regions of the United States including California, Texas, Arizona, Washington, Idaho, Nebraska and Montana (Thomson and Schroth 1972; Zidack and Jacobson 2001; Harveson et. al. 2009). Symptoms included soft decay of internal root tissues, reddening of affected tissue after cutting, blackening of petiole vascular bundles, half-leaf yellowing, and frothing (Thomson et. al. 1977; Wilson et. al. 2001; Harveson et. al. 2009). Symptomology is often not outwardly expressed until the rot is well advanced, rendering it difficult to detect in many commercial fields (Dunning and Byford 1982). The disease can cause serious yield losses in the field and economic losses while in

storage and during processing due to contamination by invert sugars reducing sugarbeet quality (Strausbaugh and Gillen 2008). In 2013, estimates of infested fields within the combined sugarbeet hectarage of American Crystal Sugar Company (Moorhead, MN), Minn-Dak Farmers Cooperative (Wahpeton, ND) and Southern Minnesota Beet Sugar Cooperative (Renville, MN) exceeded 16,200 hectares with increasing incidence each subsequent season (T. Grove, American Crystal Sugar Company and M. Bloomquist, Southern Minnesota Beet Sugar Cooperative, pers. comm.). In-field disease has been documented as high as 20% incidence.

The recent discovery, unknown cause, source and lack of effective and economic control measures for this disease prompted initiation of this study. Isolates collected from commercial fields within the growing geographies of all three sugar cooperatives in the RRV were compared, both chemically and molecularly, to known isolates causing symptoms of a similar nature on sugarbeet as well as other root crops. A comparative description of this disease, geographic distribution and results of several tests to identify the pathogen are presented in this manuscript. A preliminary report of this novel bacterium of sugarbeet has been published (Secor et. al. 2016).

Materials and Methods

Field Sampling

From 2012 to 2015, sugarbeets with visual symptoms of bacterial soft rot were collected from commercial sugarbeet fields (Figures 1, 2 and 3). Twenty symptomatic roots were collected from each field (800 roots in total). Twenty-five fields were located in Minnesota and fifteen in North Dakota. Approximately one-half were Minn-Dak Farmers Cooperative fields and one-quarter from fields under contract with Southern Minnesota Beet Sugar Cooperative and American Crystal Sugar Company, respectively (Figure 4). Samples were dug by hand to

Figure 1. Commercial sugarbeets exhibiting foliar symptoms of bacterial soft rot within a low area of the field.

Figure 2. Half-leaf necrosis associated with bacterial soft rot on mature sugarbeet leaf collected from a plant in a commercial field. Note the dark-brown- to black-colored longitudinal lesion along one-half of the petioles.

Figure 3. Advanced field soft rot decay of sugarbeet showing root frothing symptoms resulting from bacterial infection.
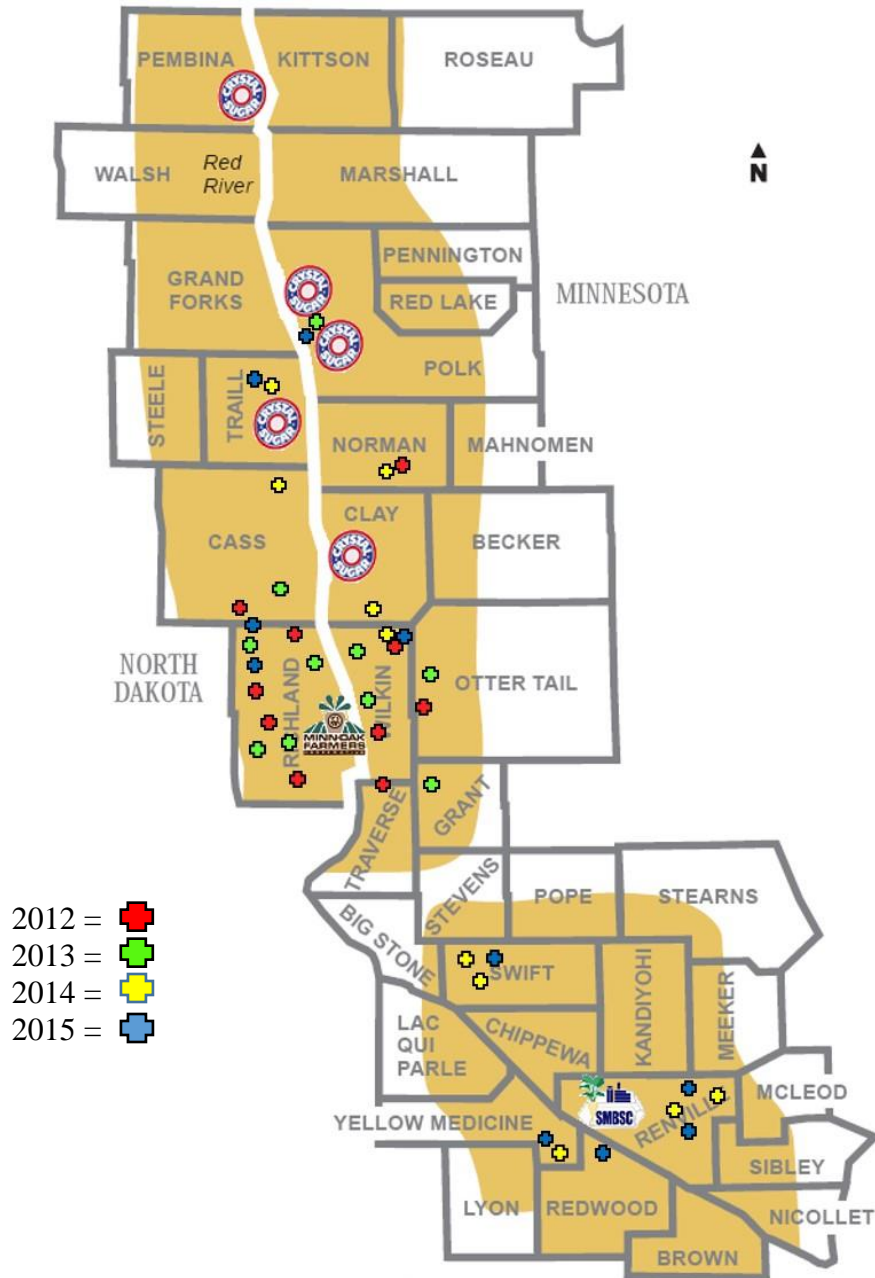
Figure 4. Locations of fields where bacterial soft rot samples were collected in the Red River Valley of Minnesota and North Dakota. Individual crosses are color coded by year and represent the sampled field locations. Sugar cooperative logos represent the seven factory locations throughout the region.

maintain intact roots, leaves were harvested and both subsequently stored at 4 °C and ~95%

relative humidity until used for analysis.

Sample Processing and Pathogen Isolation

The root from each plant sampled was hand washed, latitudinally cut in half and a small

section (8.2 – 12.2 cubic centimeters) of diseased tissue collected from each individual half

(Figure 5). The diseased samples (n=40) were combined into one composite sample representing

each respective commercial field and placed in a 3.78-liter capacity zip-lock bag, and stored at 5

°C until used for analysis.

Figure 5. Infected tissue extraction for pathogen isolation. After being cut latitudinally, small
portions of diseased tissue were removed (8.2 – 12.2 cubic centimeters) from each root half.

Leaves and petioles collected from the same samples were removed from storage and hand washed. The petioles were separated from the leaves by cutting with a knife 2.54 cm below the base of each leaf. All petioles from an entire field sample (n=20) were combined into a 3.78-liter zip-lock bag and stored at 5 °C until used for analysis.

Root and petiole samples were processed separately within 24 hours for bacterial recovery. Each infected tissue piece was removed from the zip-lock bag within a laminar flow hood (Environmental Air Control, Inc., Model 6467, Hagerstown, MD) and placed in a Petri dish. Using standard pliers that were flame-sterilized between each infected piece, each sample was squeezed to produce droplets of sap that were allowed to fall into an empty Petri dish. Approximately a 10 µl subsample of sap was transferred with a sterile bacterial loop to a Petri dish containing Crystal Violet Pectate (CVP) medium (Cuppels and Kelman 1974), spread utilizing standard triple-streak methodology (Cappuccino and Welsh 2017) and secured with Parafilm (Bemis NA, Neenah, WI). Plates from roots (1,600 in total) and petioles (800 in total) were labeled as a secondary subsample of the original field sample and stored in total darkness for 48 hours at 22 ℃.

A sterile loop was used to recover up to five single colonies causing pitting on CVP medium (Figure 6) that were individually transferred to 9-cm Petri dishes containing nutrient agar (Difco BD, VWR, Randnor, PA). Labeled as tertiary samples, the inoculated plates were incubated at 30 ℃ in total darkness for 48 hours. Based on visual depth of pitting, a single isolate was selected from samples representing each field, 32 from root extractions and 8 from petiole extractions, and used for further evaluation (Table 1). A loop of each isolate was collected and transferred to a 2-ml microcentrifuge tube (VWR Model 16466, Randnor, PA) containing 0.75 ml of sterile distilled water (SDW) and 0.5 ml of 15% sterile glycerol. All tubes were vortexed
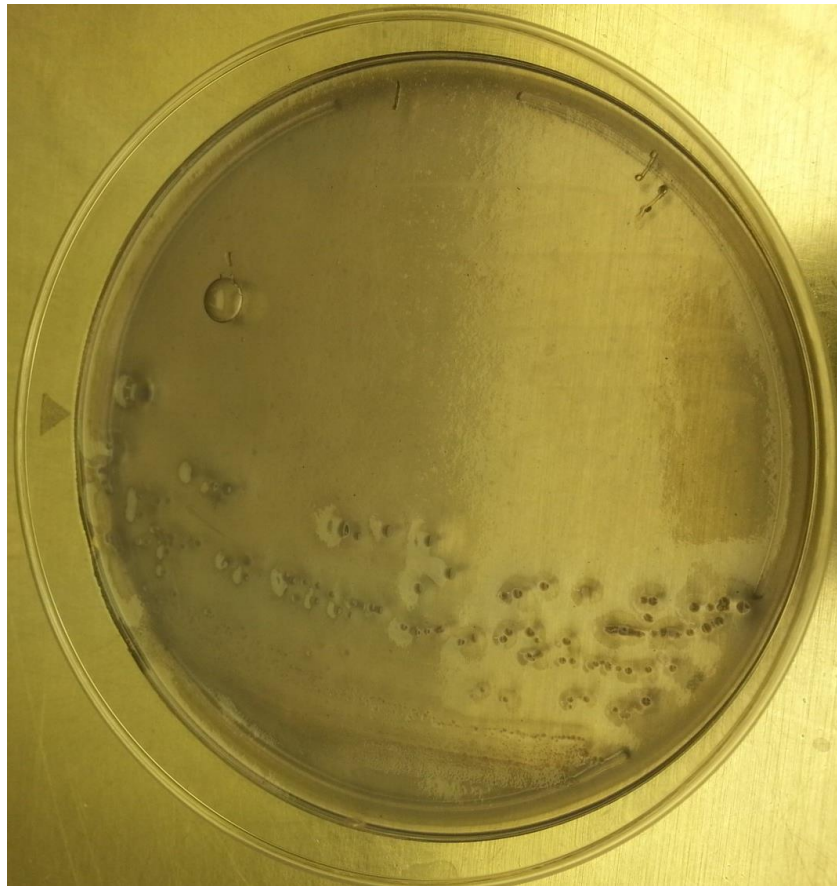
Figure 6. Crystal Violet Pectate (CVP) agar showing pits caused by pectinolytic bacteria from sugarbeets exhibiting soft rot symptoms.

Table 1. Isolates selected for analysis recovered from 2012-2015 sugarbeet fields.

Isolate ID	Sugar Cooperative	Year Sampled	Quarter Section	Section Number	Township	County	State
MD-1	Minn-Dak	2012	NE	31	Walcott East	Richland	ND
MD-2	Minn-Dak	2012	NH	3	Taylor	Traverse	MN
MD-3	Minn-Dak	2012	SE	15	Brandrup	Wilkin	MN
MD-4	Minn-Dak	2012	NW	6	Nansen	Richland	ND
MD-5	Minn-Dak	2012	SE	5	Danton	Richland	ND
MD-6	Minn-Dak	2012	NE	17	Orwell	Otter Tail	MN
MD-7	Minn-Dak	2012	SE	5	LaMars	Richland	ND
MD-9	Minn-Dak	2012	EH	24	Prairie View	Wilkin	MN
AC-1	American Crystal	2012	SE	27	Lockhart	Norman	MN
AC-5	American Crystal	2012	NE	34	Highland	Cass	ND
MD-8	Minn-Dak	2013	SW	36	Viking	Richland	ND
MD-10	Minn-Dak	2013	NW	10	Barney	Richland	ND

Table 1. Isolates selected for analysis recovered from 2012-2015 sugarbeet fields (continued).

Isolate ID	Sugar Cooperative	Year Sampled	Quarter Section	Section Number	Township	County	State
MD-11	Minn-Dak	2013	NE	4	Mooreton	Richland	ND
MD-13	Minn-Dak	2013	SH	21	Mitchell	Wilkin	MN
MD-14	Minn-Dak	2013	WH	20	Meadows	Wilkin	MN
MD-15	Minn-Dak	2013	WH	3	Dwight	Richland	ND
MD-19	Minn-Dak	2013	SW	31	Trondhjem	Otter Tail	MN
MD-20	Minn-Dak	2013	NH	33	Lawrence	Grant	MN
AC-4	American Crystal	2013	SH	33	Huntsville	Polk	MN
AC-8	American Crystal	2013	SW	28	Warren	Cass	ND
MD-12	Minn-Dak	2014	NW	9	Prairie View	Wilkin	MN
AC-2	American Crystal	2014	NE	18	Ervin	Traill	ND
AC-3	American Crystal	2014	NH	33	Gardner	Cass	ND
AC-6	American Crystal	2014	EH	11	Humboldt	Clay	MN
AC-7	American Crystal	2014	SW	22	Pleasant View	Norman	MN
SM-1	Southern-Minn	2014	SW	22	Boon Lake	Renville	MN
SM-3	Southern-Minn	2014	NW	21	Bird Island	Renville	MN
SM-4	Southern-Minn	2014	EH	28	Moyer	Swift	MN
SM-5	Southern-Minn	2014	SW	15	Edison	Swift	MN
SM-9	Southern-Minn	2014	SH	5	Echo	Yellow Medicine	MN
MD-16	Minn-Dak	2015	SE	13	Garborg	Richland	ND
MD-17	Minn-Dak	2015	NW	1	Barrie	Richland	ND
MD-18	Minn-Dak	2015	SE	14	Prairie View	Wilkin	MN
AC-9	American Crystal	2015	EH	11	Wold	Traill	ND
AC-10	American Crystal	2015	SE	27	Bygland	Polk	MN
SM-2	Southern-Minn	2015	NW	20	Martinsburg	Renville	MN
SM-6	Southern-Minn	2015	SE	36	Redwood Falls	Redwood	MN
SM-7	Southern-Minn	2015	NE	20	Brookfield	Renville	MN
SM-8	Southern-Minn	2015	NE	19	Wood Lake	Yellow Medicine	MN
SM-10	Southern-Minn	2015	SW	5	Tara	Swift	MN

s

(20 s) at 7,000 RPM before being placed into cryo-storage at -80 ℃ for future greenhouse

inoculations, biochemical analysis and molecular assays.

Reference Isolates

Isolates of *Pectobacterium betavasculorum* courtesy of Dr. Carolee Bull (USDA, Salinas,

CA) served as reference isolates and comparative positive controls this study (Table 2).

Table 2. Reference *Pectobacterium carotovorum* subsp. *betavasculorum* isolates used in this study.

Bacterial Strain	USDA Id[a]	Other Designation[b]
Pectobacterium carotovorum subsp. *betavasculorum*	BS0109	ATCC 43762
Pectobacterium carotovorum subsp. *betavasculorum*	BS0110	CFBP 1539
Pectobacterium carotovorum subsp. *betavasculorum*	BS0111	ICMP 4226
Pectobacterium carotovorum subsp. *betavasculorum*	BS0350	LMG 2464
Pectobacterium carotovorum subsp. *betavasculorum*	BS0969	LMG 2466
Pectobacterium carotovorum subsp. *betavasculorum*	BS0970	NCPPB 2795

[a]Courtesy of Dr. Carolee Bull (USDA, Salinas, CA)

[b]Other Designation	Collection Name	Collection Location
ATCC	American Type Culture Collection	Manassas, Virginia
CFBP	Collection Française de Bactéries Phytopathogènes	Angers, France
ICMP	International Collection of Microorganisms from Plants	Auckland, New Zealand
LMG	Baceria Collection - Laboratorium voor Microbiologie	Ghent, Belgium
NCPPB	National Collection of Plant-pathogentic Bacteria	York, United Kingdom

Greenhouse Inoculation

Sugarbeet Planting

       The sugarbeet cultivar Hilleshög 4062 (Lot Number 2734295, Syngenta Seeds, Longmount, CO), a commercially approved bacterial soft rot-susceptible variety was selected for pathogenicity testing. Seed commercially pelleted with Apron® (metalaxyl), Thiram® (Thiram), Tachigaren® (hymexazol) and Kabina® (penthiopyrad) were sown in 35 x 66 centimeter trays, each containing 98 individual cones measuring 2.5 cm in diameter and 18 cm in length (Stuwe and Sons, Corvallis, OR).

       Cones were filled with Sunshine Mix Number One (Sungro Horticulture, Seba Beach, Canada). Three individual seeds were sown within each cone in a triangular pattern two centimeters deep, watered, and transferred to a greenhouse at 29 ºC and a 12-hour light/dark cycle for optimum plant growth. Three weeks post-planting, each individual cone-tainer was each cone-tainer was transferred to 15-cm diameter pot at six weeks post-planting and allowed to progress for ten weeks before inoculation.

Inoculum Preparation

       From each of the six reference isolates and 40 bacterial isolates originally isolated from commercial fields, 250 µl of solution was removed from individual storage vials and deposited into the center of a Petri dish containing nutrient agar (Difco BD, VWR, Randnor, PA). The drop was spread across the entirety of each dish utilizing a flame-sterilized loop. Dishes were then labeled, sealed with parafilm and incubated at 30 ºC in total darkness for 48 hours. Five milliliters of distilled water was added to each Petri dish before bacterial colonies were dislodged from the surface of the media with a flame-sterilized loop. Inoculum densities were calculated with an eosinophil hemocytometer (Hausser Scientific, Horsham, PA) and the subsequent

bacterial suspensions were prepared by serial dilution to a concentration of 10^7 CFU ml^{-1} in sterile test tubes for sugarbeet inoculations.

Pathogenicity Testing by Root and Petiole Inoculation

Two sugarbeet plants per bacterial isolate (one plant for petiole inoculation and one for root inoculation) were grown in a greenhouse for 70 days for each of the three experimental replications. Plants placed into the greenhouse after inoculation were arranged in random order.

Petiole Inoculation. A 1.5 ml solution of the prepared bacterial inoculum was placed at the base of the healthy petioles along each side of the sugarbeet (3 ml in total) in the concave base of the petiole just above its attachment to the root crown (Figure 7). After placing the inoculum, turgid sugarbeet leaves were physically bent downward until an audible 'snap' noise could be heard at the base of the petiole to establish the necessary wound for bacterial infection.

Root Inoculation. Two holes 6.4-mm in diameter and 13-mm deep were created on opposite sides of the root using a flame-sterilized drill bit in a cordless drill (Figure 8). A sterile dental pick was inserted into each hole and manipulated to cause additional injury to the smooth tissue surface left by the rotation of the drill bit (Figure 9). Inoculum (1.5 ml) was placed inside each of the drill holes. After inoculation, the hole was hand-sealed with petroleum jelly (Vaseline®) to prevent evaporation. Inoculated plants were maintained in a greenhouse at 30-32 ºC for 28 days prior to evaluation.

Evaluations of Inoculated Plants

Plants were examined visually and rated for the presence of foliar, crown and root symptoms of bacterial soft rot. Foliar symptoms evaluated were necrosis of the petiole bases (Figure 10) and a black-colored longitudinal lesion along one-half of the petioles (Figure 11). For crown symptoms, each plant was latitudinally cut just below the base of the petioles leaving

Figure 7. Bacterial inoculum placement in petioles of greenhouse grown sugarbeet for pathogenicity testing.

Figure 8. Preparation of greenhouse-grown sugarbeet roots for bacterial inoculation using a cordless drill and bit.

Figure 9. Subsequent inoculation hole left by the drill bit being 'roughed up' by the use of a sterile dental pick.

Figure 10. Necrosis at petiole bases resulting from petiole inoculation.

Figure 11. Longitudinal necrosis extending from petiole base to the leaf from a bacterial inoculation of a greenhouse-grown sugarbeet.

approximately a five-centimeter diameter cross-section of the crown exposed for evaluation

(Figure 12). Symptoms evaluated were discoloration and decay of the beet core (Figure 12). The

remaining root was cut down the center and split into two halves to expose a longitudinal section

of the entire root. Root symptoms evaluated included the discoloration and exterior softening of

the root (Figure 13), maceration of the internal root tissues (Figure 14), and pink to reddish

discoloration of the vascular bundles within 20-30 seconds after exposure to ambient air (Figure

15).

Figure 12. Inoculated sugarbeet with top removed exhibiting root symptoms of bacterial soft rot resulting from petiole inoculation during greenhouse screening. Note macerated tissue in center of the root surrounded by healthy, white vascular tissue.

Figure 13. External root symptoms of bacterial soft rot as a result of a petiole inoculation during a greenhouse screening.
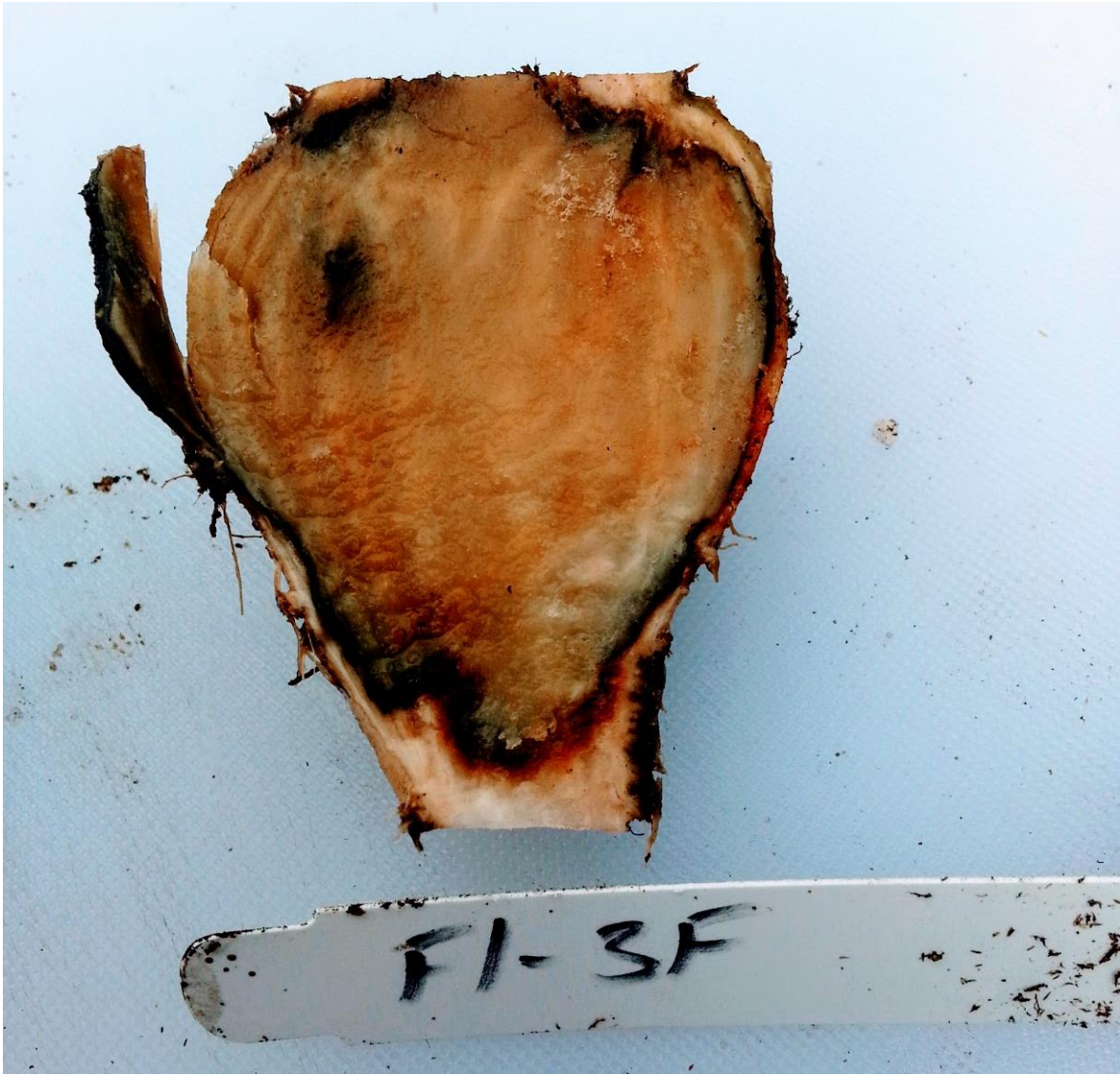
Figure 14. Internal root symptoms of bacterial soft rot as a result of a petiole inoculation during greenhouse screening.

Figure 15. Latitudinal section of a sugarbeet 21 days after bacterial root inoculation exhibiting the pink to reddish discoloration of the root tissue 20-30 seconds after cutting.

Data was recorded in a binary manner and categorized by petiole, crown and root symptomology for each inoculation. Data were subjected to a logistic regression model for binary outcomes using Minitab 18 (State College, Pennsylvania) at a 0.05 level of significance for mean separation of the main effects of inoculation method, symptoms, and isolates. Root and petiole tissue was collected from each of the greenhouse-infected plants as previously described

to confirm cause of disease. The subsequent bacterial colonies were re-isolated and placed into cryo-storage for further analysis and comparison to the original field isolates.

Biochemical Testing

Bacterial isolates of the 40 field isolates (Table 2) were removed from long-term storage and thawed on ice. Six reference isolates of *P. carotovorum* subsp. *betavasculorum* and single isolates of *P. carotovorum* subsp. *carotovorum*, *P. carotovorum* subsp. *atrosepticum* and *Dickeya dadantii* served as controls. A 250-µl aliquot of each isolate was deposited into the center of a Petri dish containing nutrient agar. The aliquot drop was spread with a flame-sterilized loop and the dish sealed with parafilm and incubated at 30 ºC for 48 hours in total darkness. Five biochemical tests were conducted on these bacterial isolates.

Gram Stain

Using a sterile loop, bacterial colonies were smeared across the face of a clean, glass microscope slide and the underside was passed through the flame of a Bunsen Burner twice to fix the bacteria to the slide. The face of the slide was then flooded with crystal violet solution for one minute, rinsed with water and lightly blotted with a paper towel to aid in drying. The same flood, rinse and dry procedure was used for iodine. The bacterial smear was decolorized with ethyl alcohol for 30 seconds, allowed to dry and then counterstained for ten seconds with safranin solution. After being rinsed with water and allowed to dry, slides were examined microscopically for either a red (gram-negative) or purple (gram-positive) coloration.

Growth at 37 ºC

Under sterile conditions, bacterial colonies were transferred via a sterile loop to a Petri dish containing nutrient agar and streaked across the entirety of each plate. Dishes were labeled, sealed with parafilm, and incubated at 30ºC for 48 hours in total darkness.

A 1-ml aliquot of nutrient agar was placed into 2-ml cryotubes. Each tube was allowed to harden so the medium surface would be at a 45-degree angle to the tube wall when cool. Bacterial colonies were aseptically transferred from the nutrient agar plates to the cryotubes and incubated at 37ºC for 24 hours in total darkness. After incubation, growth was assessed by holding a light emitting diode flashlight to the side of the tube to visually observe bacterial growth.

Oxidase

Bacterial colonies were transferred via a sterile loop to a Petri dish containing nutrient agar, spread across each plate, labeled, sealed with Parafilm, and incubated at 30 ºC for 24 hours for bacterial growth. After incubation, three drops of OxiDrops liquid oxidase reagent (Hardy Diagnostics, Santa Maria, CA) were added to each plate. After five seconds, the plate was tipped vertically forcing the reagent to collect at the bottom of the plate to allow observation of the colonies. Colonies that turned purple after ten seconds were considered positive reactions and colonies that did not change color were considered negative.

Catalase

Bacterial colonies were transferred via a sterile loop to a Petri dish containing nutrient agar, spread across the plate, individually labeled, sealed with Parafilm, and incubated at 30 ºC for 24 hours. After incubation, 10 ml of 3% hydrogen peroxide solution was poured onto the newly formed bacterial lawn. Rapid elaboration of oxygen bubbles indicated that the bacterial colonies produced the catalase enzyme and were therefore recorded as a positive reaction.

Alpha-Methyl Glucoside

Bacterial colonies were transferred via a sterile loop to a Petri dish containing nutrient agar, spread across the plate, individually labeled, sealed with parafilm and were incubated at 30

ºC for 24 hours. Colonies were then transferred to Petri dishes containing medium as described

by Perombelon and van der Wolf (2002) and incubated at 20 ºC for 48 hours. Colonies that

exhibited a pinkish to red appearance, due to the bromocresol purple serving as a pH indicator of

acid production, were considered positive and blue to purplish colonies were considered

negative.

DNA Extraction and Analysis

      To help clarify species and/or sub-species designations, DNA sequence analysis was

undertaken. Bacterial colonies were transferred via a sterile loop to a 2-ml centrifuge tube

containing nutrient broth, labeled, sealed, and incubated at 30 ºC for 24 hours for bacterial

growth. DNA was isolated using the CTAB method (Stewart 1993), re-suspended in 500 µl of

0.1-µm filtered water and quantified using a NanoDrop-1000 spectrophotometer (Thermo Fisher

Scientific, Wilmington, Delaware). Concentrations higher than 1 µg/ml were diluted with 0.1 µm

filtered water and DNA was stored at -80 ºC until PCR testing.

      Initial PCR detection of the bacterial isolates was conducted using the universal bacterial

primers p8FPL (5'-AGTTTGATCCTGGCTCAG-3') and p806R (5'-

GGACTACCAGGGTATCTAAT-3') that amplify the ITS of the 16S-23S region of ribosomal

RNA (Angert et al. 1993 and Relman et al. 1992). PCR was conducted with a 25-µl reaction

consisting of 5 µl of 5× GoTaq Reaction buffer (Promega Corp., Madison, WI), 1.5 µl of 25 mM

MgCl2, 0.5 µl containing 10 mM each dNTP, 0.125 µl of Taq polymerase, 1.25 µl each of a 10-

µM concentration of primers p8FPL and p806R, 2 µl of DNA at 1:10 dilution (one part DNA in

Qiagen buffer AE to nine parts deionized filter sterilized water), and 13.375 µl of de-ionized,

filter-sterilized water.

PCR parameters consisted of 40 cycles of 94 °C for 1 minute, 55 °C for 1 minute, and 72 °C for 2 minutes, and a final extension at 72 °C for 10 minutes using an automated PTC-200 Peltier Thermal Cycle PCR system (GMI, Inc., Ramsey, MN). Amplicons were resolved by gel electrophoresis (100 volts/1 hour) on a 1.5% agarose gel in 1xTBE buffer containing 0.5 µl ml^{-1} GelRed (Biotium, Freemont, CA). PCR products were purified using the Quiaquick PCR purification kit (Qiagen) and sequenced (MCLabs, South, San Francisco, CA). The DNA sequence data were compared with those found in GenBank (National Center for Biotechnology Information, Bethesda, MD) using the nucleotide-nucleotide Basic Local Alignment Search Tool (BLASTn) for sequence lengths, e-values, maximum identity match, and query coverage (Altschul et al. 1990).

Due to the high interspecies similarity values within the genus *Pectobacterium*, species- and sub-species-specific PCR detection was completed on *Dickeya* spp., *Pectobacterium* spp., *Pectobacterium carotovorum* subsp. *carotovorum* and *Pectobacterium carotovorum* subsp *atrosepticum* for use in comparison to the unknown and reference isolates as there are no known published primers for *Pectobacterium carotovorum* subsp. *betavasculorum*. Following Czajkowski et. al (2009), identification of *Dickeya* spp. was conducted using ADE1 (5'-ATCAGAAAGCCCGCAGCCAGAT-3') and ADE2 (5'-CTGTGGCCGATCAGGATGGTTTTGTCGTGC-3') primers (Nassar et. al. 1996) with an expected fragment length of 420 base pairs, and *Pectobacterium* spp. was identified using Y1 (5'-TTACCGGACGCCGAGCTGTGGCGT-3') and Y2 (5'-CAGGAAGATGTCGTTATCGCGAGT-3') primers (Hélias et. al. 1998) with an expected amplicon size of 434 base pairs. Identification of *Pectobacterium carotovorum* subsp. *carotovorum* was conducted using EXPCCF (5'-GAACTTCGCACCGCCGACCTTCTA-3') and

EXPCCR (5'-GCCGTAATTGCCTACCTGCTTAAG-3') oligonucleotide primers (Kang et. al. 2003) yielding an expected fragment length of 550 base pairs. *Pectobacterium carotovorum* subsp *atrosepticum* using Y45 (5'-TCACCGGACGCCGAACTGTGGCGT-3') and Y46 (5'-TCGCCAACGTTCAGCAGAACAAGT-3') primers (Czajkowski et. al 2009) with an expected fragment length of the amplicons being 439 base pairs. For nested PCR of *Pectobacterium carotovorum* subsp. *carotovorum*, primers INPCCR (5'-GGCCAAGCAGTGCCTGTATATCC-3') and INPCCF (5'-TTCGATCACGCAACCTGCATTACT-3') were selected from the sequence bases downstream 3′-ends of primers EXPCCF and EXPCCR for amplification of an expected 0.4 kb (Kang et. al. 2003). In all cases, amplified DNA was detected by gel electrophoresis in a 1.5 % agarose gel in 1.0×TAE buffer and stained with 5 µl per 100 ml of GelRed (Biotium, Freemont, CA). Results confirmed the genera and species with subspecies remaining unknown.

Genome Sequencing

The use of a two-enzyme restriction-associated DNA genotype-by-sequencing (RAD-GBS) method adapted for Ion Torrent sequencing technology provided a reproducible high-density genotyping of the unknown bacterium (LeBoldus et. al. 2015). Bacterial genomic DNA was sequentially digested with the restriction enzymes *HhaI* then *Ape*KI. The ABC1 adaptor containing the sequencing primer site, the Ion Torrent key site, barcodes and *Ape*KI sticky ends, as well as the P1 adaptor containing the Ion Sphere Particle attachment nucleotides and *HhaI* sticky ends were ligated to the restriction-digested genomic DNA fragment. The barcoded fragments were pooled and subsequently size-selected using the Pippin Prep (Sage Sciences) to select for ~275 bp fragments and eliminate unligated adapters. The ~275 bp fraction from the pooled libraries was amplified using the Ion Torrent sequencing primers and Ion Sphere Particle

(ISP) primer, without sphere particles attached. The PCR products were quantified and emulsion PCR was performed to add monoclonal DNA templates to a single ISP. The ISPs with DNA templates were enriched on the Ion One Touch 2 bead enrichment station and loaded into single wells on the Ion Torrent PGM 318 chip and sequenced on the Ion Torrent PGM Sequencer (Thermo Fisher Scientific Inc., Waltham, MA).

The 8.54 million ion torrent sequencing reads, ∼89,000 sequences at 169 bp on average per isolate, were assembled *de novo*, producing ∼6,000 sequence tags on average. This represents approximately 21% of each bacterial genome analyzed. Partial sequences of five of the seven genes previously used in *Pectobacterium* subspecies phylogenetic analysis (malate dehydrogenase partial CDS:KX159481, mannitol-1-phosphate-5-dehydogenase partial CDS:KX159482, glyceralhehyde-3-phosphate dehydrogenase partial CDS:KX159483, aconitrate hydratase partial CDS:KX159484, glucose-6-phosphate isomerase partial CDS:KX159485) were represented in the RAD-GBS isolate sequences. Gene sequences from each isolate were aligned using Genomics Workbench 8.0.3 software (CLC Bio, Aarhus, Denmark) to the corresponding reference gene sequences of *P. carotovorum* subsp. *carotovorum*, *P. atrosepticum*, *P. betavasculorum*, *P. carotovorum* subsp. *odoriferum*, and *P. wasabiae* (Glasner et al. 2008). Determination of species within the *Pectobacterium* genus was conducted by calculating the percent identity of individual reference gene alignments for each isolate.

After the bacterial genomes were analyzed, subspecies identification was confirmed for *Pectobacterium carotovorum* subsp. *brasiliensis* using BR1f (5'-GCGTGCCGGGTTTATGCACT-3') and L1r (5'-CAAGGCATCCACCGT-3') primers yielding an expected fragment length of the amplicons at 322 base pairs (Duarte et. al. 2004).

Host Specificity Screening and Pathogenicity Testing

The broad host range of the *Pectobacterium* species and subspecies prompted host-specificity studies conducted on sugarbeet and potato, the two major root tuber crops grown in the Red River Valley of Minnesota and North Dakota. Tubers from a susceptible potato cultivar (Russet Burbank) (Black Gold Farms, Grand Forks, ND) and sugarbeet roots from the susceptible cultivar Hilleshög 4062 (Syngenta Seeds, Longmount, CO) were collected from commercial production fields in the Red River Valley. Sugarbeet roots were also collected from commercial fields in the Imperial Valley of California sown to SES RR602TT (SESVanderHave, Fargo, ND), a cultivar known to have high levels of resistance to bacterial soft rot.

Potato tubers and sugarbeet roots were hand washed with a mild detergent and allowed to air dry. Latitudinal slices (2-3 cm thickness) were collected from beet roots and potato tubers ('pucks'). Pucks were individually cored with a No. 6 handheld T-core sampler (VWR, Radnor, PA). A 10-mm diameter depression approximately 1.5 cm deep was made in the freshly cut plane. The cored pucks were transferred into plastic containers placed depression-side up on top of a plastic mesh and moist paper towels.

A 50-µl aliquot was collected from storage vials of inoculum from each of the forty field isolates, transferred into tubes containing nutrient broth, labeled, sealed with Parafilm, and incubated on a rotary shaker at 30 ºC for 48 hours for colony multiplication. After inoculum densities were calculated with an eosinophil hemocytometer (Hausser Scientific, Horsham, PA) and the subsequent bacterial suspensions were prepared by serial dilution to a concentration of 10^6 CFU ml^{-1} (Wolf et.al. 2017), one milliliter of solution was transferred via pipette from the test tube to the cored depression in the puck. Nine pucks per bacterial isolate were inoculated; three potato, three susceptible sugarbeet and three resistant sugarbeet. Non-inoculated nutrient

broth and the six reference isolates served as controls for the experiment. An air-tight lid was placed onto the container before the pucks were incubated at 30 ℃ without light for 48 hours. Data for the sugarbeet and potato pucks were rated with a binary assessment for the presence/absence of bacterial soft rot (Figure 16).

Data were subjected to analysis of variance using Minitab 18 (State College, Pennsylvania) software using Fisher's protected least significant difference (LSD) test at a 0.05 level of significance for main effects of puck type and individual isolate as well as all possible interactions.

Figure 16. Typical potato and sugarbeet 'puck' sample reaction to inoculation with sugarbeet bacterial isolate. Sugarbeets (left) show the commercial importance of genetic resistance where the top puck (resistant cultivar) remains healthy while the bottom puck (susceptible cultivar) develops symptoms 48 hours after inoculation with the same bacterial strain. Potato pucks (right) show the comparison between the control (top) and the reference isolate (bottom).

Sugar Factory Quality Assay Losses Due to Bacterial Soft Rot

When harvested sugarbeets first enter a factory for processing, they are washed in a flume to remove rocks, soil and other debris before being gravity-fed through a hopper leading to a slicing machine. In order to offer the maximum surface area for sugar extraction, the roots are cut into 'cossettes,' corrugated V-shaped strips, generally one centimeter by five to eight centimeters, prior to being placed onto a conveyor for elevation into the diffusion tower. The 'cossette belt' is an industry standard sampling point for laboratory analysis of the quality of raw sugarbeets entering the factory for processing.

Sugarbeet cossettes with symptoms of bacterial soft rot were collected at random across six different time periods from the cossette belt during the 2015 and 2016 commercial sugarbeet processing campaign at Minn-Dak Farmers Cooperative in Wahpeton, ND (Figure 17). Non-symptomatic cossettes were collected at the same time to serve as controls. Cossettes (400 grams) were blended in a kitchen food processor, and 52 grams was transferred to a second blending pitcher and deionized water was added to reach a final weight of 407 grams. The mixture was blended at high speed for five minutes, filtered through 11-µm filter paper and the filtrate collected and cooled to 20 °C. Ten grams of the sample was then placed into a volumetric flask and brought to a remaining volume of 100 ml. After mixing, a 10-ml syringe filled with the diluted sample was filtered through a Target PVDF 0.45-µl syringe filter (Thermo Fisher Scientific Inc., Waltham, MA) into two separate vials (0.5 ml each) for independent evaluations. The contents of one vial was analyzed by using a Dionex AP-AS50 autosampler (Thermo Fisher Scientific Inc., Waltham, MA) for ion chromatography analysis of fructose, glucose and sucrose, and the second vial analyzed for dextrose and lactate with high performance liquid chromatography by using a 2700 Select Biochemistry Analyzer (YSI Incorporated, Yellow

Springs, OH). Data from these procedures were subjected to analysis of variance using Minitab

18 (State College, Pennsylvania) software using Fisher's protected least significant difference

(LSD) test at a 0.05 level of significance.

Figure 17. Cossettes sampled during a commercial processing campaign. Sampling targeted sugarbeet root material that expressed symptomology of bacterial soft rot including pink to reddish discoloration of the vascular bundles after exposure to ambient air (left) and soft, macerated root tissue (right).

Quality Analysis Study

Sugarbeets were sampled from a commercial field planted with Hilleshög 4062

(Syngenta Seeds, Longmont, CO) infected with bacterial soft rot. Healthy and infected beets

were collected by hand, being careful that the plant roots remained intact. Infected beets were

usually identified by foliar and root symptoms, notably by the presence of frothing or residue on

the side of the roots, due to escaping gases emitted as a by-product of bacterial metabolism,

indicating severe rot. Healthy plants were selected by their absence of any disease symptoms.

Immediately after harvest, leaves were removed and beets were segregated into separate 76-liter tubs (Newell-Rubbermaid, Atlanta, GA) and labeled accordingly for transportation and subsequent quality analysis. A total of 360 individual roots were collected; 180 healthy roots and 180 roots with bacterial soft rot symptoms.

A completely random design with six replications was utilized to assess quality analysis. Harvested sugarbeets were assembled into groups of ten sugarbeets. The groups consisted of differing ratios of healthy/diseased roots: ten healthy roots/zero infected roots, eight healthy/two infected, six healthy/four infected, four healthy/six infected, two healthy/eight infected and zero healthy/ten infected. All sugarbeets in each respective pile were selected to be of comparable size and mass. Roots were cut in half longitudinally from the crown to the root tip with a beet knife to confirm internal infection of the roots (Figure 18). Each group of ten roots was placed into a commercial tare bag and labeled with a bar-coded ticket assigning a corresponding sample number to each individual bag. Bagged roots were transported within four hours of harvest to the Minn-Dak Farmers Cooperative Tare Lab (Wahpeton, ND) for standard quality and purity analysis. Samples were washed and each composite sample was ground into brei (mash) for relative dry substance (purity) and polarimeter (sucrose) analysis. After a 1:1 (weight to weight) dilution of the brei with reverse-osmosis-treated water was blended, the sample was equally split by volume. The first half-portion of this extract was measured for relative dry substance utilizing a Bellingham Stanley RFM 300 (Model RFM34M, Suwanee, GA) refractometer. The second-half portion of the extract was further diluted with 0.6% aluminum sulfate and filtered before sugar content was quantified by using a polarimeter (Rudolph Research Analytical, Model Autopol 880, Hackettstown, NJ). Data were analyzed for significant differences using Fisher's protected least significant difference (LSD) test in percent purity, sugar content and recoverable

sugar per ton of sugarbeets at the p = 0.05 level utilizing Minitab 18 statistical software (State

College, PA).

Figure 18. Typical internal decay of sugarbeet roots infected with bacterial soft rot.

Results and Discussion

Pathogen Identification and Geographic Distribution in the Red River Valley of Minnesota

and North Dakota

Greenhouse Evaluations of Inoculated Plants

Characteristic symptoms of the disease were observed in the greenhouse trials 30 days

after inoculation including root decay, reddening of root tissue after cutting, vascular blackening

of the petioles, half-leaf yellowing, and frothing. All isolates recovered caused symptoms in at least one replication in the petioles, crown or root by either root or petiole inoculation (Tables 3 and 4). Bacteria with similar characteristics as those artificially inoculated were reisolated from the macerated tissue. The strains recovered were biochemically and molecularly identified as being the same as the original isolates, thus completing Koch's postulates. Root inoculation was more effective for overall symptom development than petiole inoculation. Root inoculations resulted in 75% (90/120) symptomatic plants compared to 62% (74/120) expressing symptoms of bacterial soft rot from petiole inoculations (Tables 3 and 4). Regardless of inoculation method, the infection rates were greatest near the point of inoculation and spread to subsequent areas of the plant. Petiole inoculations resulted in 62/120 (51.6%) plants expressing petiole symptoms which was significantly greater than root inoculations expressing symptoms in the petioles (33/120 (27.5%) (Table 5). Similarly, root inoculations resulted in 71/120 (59.2%) plants with root symptoms, whereas petiole inoculations expressed significantly less root symptoms (12/120 (10%) in the evaluated plants (Table 5). Symptoms of bacterial soft rot expressed in the crown did not significantly differ by inoculation method resulting in 37/120 (31%) and 25/120 (21%) for root and petiole inoculations, respectively.

Table 3. Number of sugarbeet plants expressing symptoms in greenhouse trials 30-days after inoculation of roots or petioles with bacterial isolates recovered from sugarbeets with soft rot decay from commercial Minn-Dak Farmers Cooperative sugarbeet fields.

Isolate	Root Inoculation[a]			Petiole Inoculation[a]		
ID	Petiole	Crown	Root	Petiole	Crown	Root
MD-1	0	+	+	+	+	0
MD-2	++	0	+	+	+	0
MD-3	0	++	+++	++	0	0
MD-4	0	+	+++	++	+	0
MD-5	++	+	+	+	0	0
MD-6	+	0	0	+	0	0
MD-7	+	+	++	++	+	0

(continues)

Table 3. Number of sugarbeet plants expressing symptoms in greenhouse trials 30-days after inoculation of roots or petioles with bacterial isolates recovered from sugarbeets with soft rot decay from commercial Minn-Dak Farmers Cooperative sugarbeet fields (continued).

Isolate	Root Inoculation[a]			Petiole Inoculation[a]		
ID	Petiole	Crown	Root	Petiole	Crown	Root
MD-8	0	+	++	+++	0	0
MD-9	+	0	+	+	+	0
MD-10	0	+	++	++	0	0
MD-11	+	0	+	+	+	0
MD-12	+	0	++	+	0	0
MD-13	0	+	++	++	+	0
MD-14	+	+	+	+	0	0
MD-15	0	+	++	++	0	0
MD-16	+	+	++	++	0	0
MD-17	+	0	++	++	+	0
MD-18	+	+	++	+	0	0
MD-19	+	0	+	++	0	0
MD-20	0	++	++	++	0	0

[a] Number of sympomatic plants by replication: 0 = no symptoms expressed, + = one plant,
++ = two plants, +++ = three plants

Table 4. Number of sugarbeet plants expressing symptoms in greenhouse trials 30-days after inoculation of roots or petioles with bacterial isolates recovered from sugarbeets with soft rot decay from commercial American Crystal Sugar Company and Southern Minnesota Beet Sugar Cooperative sugarbeet fields.

Isolate	Root Inoculation[a]			Petiole Inoculation[a]		
ID	Petiole	Crown	Root	Petiole	Crown	Root
AC-1	0	+	++	++	0	0
AC-2	+	++	+++	++	+	0
AC-3	+	++	+++	+	0	0
AC-4	+	+	+	+	0	0
AC-5	++	+	++	+	+++	0
AC-6	+	+	++	+	0	++
AC-7	++	+	++	+++	+++	+++
AC-8	+	+	+	+	0	0
AC-9	+	++	++	++	+	0
AC-10	+	+	++	+	0	0
SM-1	++	+	++	+	0	0
SM-2	+	0	++	++	0	0

Table 4. Number of sugarbeet plants expressing symptoms in greenhouse trials 30-days after inoculation of roots or petioles with bacterial isolates recovered from sugarbeets with soft rot decay from commercial American Crystal Sugar Company and Southern Minnesota Beet Sugar Cooperative sugarbeet fields (continued).

Isolate	Root Inoculation[a]			Petiole Inoculation[a]		
ID	Petiole	Crown	Root	Petiole	Crown	Root
SM-3	+	+	+	+++	+	0
SM-4	+	+	+	+	++	++
SM-5	0	0	++	0	+	++
SM-6	+	+	+	+	0	0
SM-7	+	0	++	+	+	0
SM-8	+	++	+	++	++	+
SM-9	0	++	+++	++	+	+
SM-10	0	+	+++	++	+	+

[a] Number of sympomatic plants by replication: 0 = no symptoms expressed, + = one plant,

  ++ = two plants, +++ = three plants

Table 5. Number of sugarbeet plants expressing symptoms in greenhouse trials 30 days after inoculation of roots or petioles with bacterial isolates recovered from sugarbeets with soft rot decay from commercial sugarbeet fields.

Inoculation	Symptoms		
Location	Petiole	Crown	Root
	(n=120)	*(n=120)*	*(n=120)*
Root	33	37	71
Petiole	62	25	12
P-Value$_{(0.05)}$[a]:	*0.0001*	*0.0797*	*0.0001*

[a] Logistic regression model for binary outcomes

Similar trends were also noted in the reference isolates. Root inoculation with reference samples resulted in 2/18 (11.1%) plants with petiole symptoms, 4/18 (22%) plants with crown symptoms and 7/18 (38.9% plants with root symptoms (Table 6). Petiole inoculation with reference samples caused 13/18 (72.2%) plants with petiole symptoms and 3/18 (16.7%) plants expressing crown and root symptomology, respectively. The reference isolates also exhibited the

highest infection rates for petiole symptomology for petiole-based inoculations at 72%. Root

inoculations of reference isolates BS0969 and BS0109 failed to cause any symptoms in the

petioles, crown or roots, but did cause petiole symptoms from petiole-based inoculations, both at

rates of 22%.

Table 6. Number of sugarbeet plants expressing symptoms in greenhouse trials 30-days after inoculation of roots or petioles with bacterial isolates recovered from sugarbeets with soft rot decay from the reference isolates used in this study.

Isolate ID	Root Inoculation[a]			Petiole Inoculation[a]		
	Petiole	Crown	Root	Petiole	Crown	Root
BS0109	0	0	0	++	0	0
BS0110	0	+	++	+++	+	+
BS0111	0	+	+	+	0	0
BS0350	++	+	+	+++	+	+
BS0969	0	0	0	++	0	0
BS0970	0	+	+++	++	+	+

[a] Number of sympomatic plants by replication: 0 = no symptoms expressed, + = one plant,
  ++ = two plants, +++ = three plants

There was no significant difference found between sugar cooperatives when comparing

the symptoms caused by the root inoculation. Root inoculations of Minn-Dak Farmers

Cooperative (MDFC) field isolates resulted in 14/60 (23.3%) plants with petiole symptoms,

15/60 (25%) plants with crown symptoms and 33/60 (55%) plants with root symptoms (Table 7).

Southern-Minnesota Beet Sugar Cooperative (SMBSC) isolates resulted in 8/30 (26.7%) plants

with petiole symptoms, 9/30 (30%) plants with crown symptoms and 18/30 (60%) plants with

root symptoms for root-based inoculations (Table 7). American Crystal Sugar Company (ACSC)

had the most aggressive isolates observed during root inoculations with 11/30 (36.6%) plants

showing petiole symptoms, 13/30 (43.3%) plants exhibiting crown symptoms and 20/30 (66.7%)

plants with root symptoms.

Table 7. Percent of sugarbeet plants expressing symptoms in greenhouse trials 30-days after inoculation of roots or petioles with bacterial isolates recovered from sugarbeets with soft rot decay from commercial sugarbeet fields compared across the geographies of the three Red River Valley sugar cooperatives.

Isolate Source[a]	Root Inoculation			Petiole Inoculation		
	Petiole	Crown	Root	Petiole	Crown	Root
ACSC[b]	36.6	43.3	66.7	50	26.7	16.7 a
MDFC[c]	23.3	25	55	53.3	13.3	0.0 b
SMBSC[b]	26.7	30	60	50	30	23.3 a
P-Value$_{(0.05)}$[d,e]:	*NS*	*NS*	*NS*	*NS*	*NS*	*0.028*

[a] ACSC = American Crystal Sugar Company (Moorhead, MN), MDFC = Minn-Dak Farmers Cooperative (Wahpeton, ND), SMBSC = Southern Minnesota Beet Sugar Cooperative (Renville, MN)

[b] n = 30

[c] n = 60

[d] Logistic regression model for binary outcomes

[e] Values within a column sharing a letter are not significantly different

MDFC isolate petiole inoculations resulted in 32/60 (53.3%) plants with petiole symptoms and 8/60 (13.3%) plants with crown symptoms. Petiole inoculation of ACSC isolates resulted in 15/30 (50%) plants with petiole symptoms and 8/30 (26.7%) plants with crown symptoms. SMBSC isolate petiole inoculations resulted in 15/30 (50%) plants with petiole symptoms and 9/30 (30%) plants with crown symptoms (Table 7). Significant differences were found between the isolates collected at each sugar cooperative for root symptoms expressed as a result of petiole-based inoculations. SMBSC isolates were the most aggressive resulting in 7/30 (23.3%) plants expressing root symptoms, followed by ACSC isolates showing root symptoms on 5/30 (16.7%) plants, while MDFC isolates failed to produce any root symptomology (Table 7).

Although greenhouse infection proved successful, there may be some refinement to the inoculation process that could improve future experiments, such as a smaller diameter infection hole, higher concentration of inoculum, and/or higher volume of inoculation aliquot dispensed. It is of note to mention that aside from the petiole symptoms for petiole-based inoculations, MDFC had the lowest percentage of infected plants in the remaining five categories. It is known that *Pectobacterium* species lose pathogenicity while in laboratory storage (Dr. Carolee Bull, Penn State University, personal communication). Given that a majority of the MDFC isolates were collected from 2012-2013 and the ACSC and SMBSC isolates collected during 2014-2015 (Table 2), the extended length of storage may have had an impact on the separation expressed in isolate aggressiveness observed between isolates of the respective cooperatives.

Host Specificity Screening and Pathogenicity Testing

Symptoms of bacterial soft rot were observed 48 hours after inoculation of pucks. Of the isolates recovered, 28% were found to be only pathogenic on sugarbeet with the remaining 72% pathogenic on both sugarbeet and potato (Tables 8 and 9). Across all isolates, infection rates

Table 8. Host specificity screening of bacterial soft rot on Minn-Dak isolates using potato and sugarbeet pucks.

Isolate	Potato - Russet Burbank				Sugarbeet - Hilleshög 4062				Sugarbeet - SES RR602TT			
ID	Trial 1[a]	Trial 2[a]	Trial 3[a]	Total[b]	Trial 1[a]	Trial 2[a]	Trial 3[a]	Total[b]	Trial 1[a]	Trial 2[a]	Trial 3[a]	Total[b]
MD-1	0	0	0	0.0%	1	2	2	55.6%	2	2	1	55.6%
MD-2	0	0	1	11.1%	2	3	2	77.8%	2	2	2	66.7%
MD-3	1	0	1	22.2%	1	2	1	44.4%	1	1	0	22.2%
MD-4	0	0	0	0.0%	2	2	2	66.7%	2	2	1	55.6%
MD-5	0	0	0	0.0%	2	3	2	77.8%	2	2	2	66.7%
MD-6	3	2	2	77.8%	3	3	2	88.9%	2	3	2	77.8%
MD-7	0	0	0	0.0%	2	2	1	55.6%	2	2	2	66.7%
MD-8	3	3	3	100.0%	3	3	3	100.0%	2	3	3	88.9%
MD-9	0	0	0	0.0%	3	3	2	88.9%	3	2	2	77.8%
MD-10	0	0	1	11.1%	1	2	1	44.4%	1	0	1	22.2%
MD-11	0	0	0	0.0%	2	2	3	77.8%	2	3	2	77.8%
MD-12	2	0	0	22.2%	2	3	2	77.8%	1	1	2	44.4%
MD-13	1	2	2	55.6%	3	2	2	77.8%	2	3	3	88.9%
MD-14	0	0	0	0.0%	2	1	2	55.6%	2	2	2	66.7%
MD-15	0	0	1	11.1%	2	2	2	66.7%	2	2	2	66.7%
MD-16	1	0	0	11.1%	3	2	3	88.9%	3	2	2	77.8%
MD-17	2	2	2	66.7%	3	3	3	100.0%	3	3	3	100.0%
MD-18	3	0	2	55.6%	3	3	3	100.0%	3	3	3	100.0%
MD-19	2	3	2	77.8%	3	2	3	88.9%	2	3	3	88.9%
MD-20	2	0	1	33.3%	2	3	2	77.8%	2	2	1	55.6%
NBA	0	0	0	0.0%	0	0	0	0.0%	0	0	0	0.0%
BS0350	0	0	0	0.0%	3	3	3	100.0%	0	0	0	0.0%
BS0969	0	0	0	0.0%	0	0	0	0.0%	3	3	3	100.0%
BS0970	0	0	0	0.0%	1	2	1	44.4%	3	3	3	100.0%

[a] Sum of infected pucks for each replication (n=3)
[b] Total percent infected pucks for experiment (n=9)

75

Table 9. Host specificity screening of bacterial soft rot on American Crystal and Southern-Minn isolates using potato and sugarbeet pucks.

Isolate ID	Potato - Russet Burbank				Sugarbeet - Hilleshög 4062				Sugarbeet - SES RR602TT			
	Trial 1[a]	Trial 2[a]	Trial 3[a]	Total[b]	Trial 1[a]	Trial 2[a]	Trial 3[a]	Total[b]	Trial 1[a]	Trial 2[a]	Trial 3[a]	Total[b]
AC-1	0	0	0	0.0%	2	2	1	55.6%	1	2	1	44.4%
AC-2	0	0	0	0.0%	0	2	1	33.3%	0	1	2	33.3%
AC-3	3	1	2	66.7%	3	2	3	88.9%	2	3	2	77.8%
AC-4	1	0	1	22.2%	2	1	3	66.7%	2	2	3	77.8%
AC-5	3	3	3	100.0%	3	2	3	88.9%	1	3	2	66.7%
AC-6	0	0	1	11.1%	1	2	1	44.4%	2	0	2	44.4%
AC-7	3	3	3	100.0%	3	3	3	100.0%	3	2	1	66.7%
AC-8	1	3	2	66.7%	2	3	2	77.8%	2	3	2	77.8%
AC-9	3	3	3	100.0%	3	3	3	100.0%	3	2	2	77.8%
AC-10	3	3	3	100.0%	3	3	3	100.0%	2	1	3	66.7%
SM-1	3	3	3	100.0%	3	2	3	88.9%	2	2	2	66.7%
SM-2	3	3	3	100.0%	3	3	3	100.0%	3	2	0	55.6%
SM-3	0	0	0	0.0%	1	2	2	55.6%	1	2	2	55.6%
SM-4	2	3	2	77.8%	3	3	3	100.0%	2	1	2	55.6%
SM-5	3	3	3	100.0%	3	2	3	88.9%	3	3	3	100.0%
SM-6	0	0	0	0.0%	2	2	2	66.7%	2	2	2	66.7%
SM-7	3	3	3	100.0%	2	3	3	88.9%	2	2	2	66.7%
SM-8	3	3	3	100.0%	3	3	3	100.0%	2	2	2	66.7%
SM-9	3	3	3	100.0%	2	3	2	77.8%	1	3	2	66.7%
SM-10	3	3	3	100.0%	3	2	3	88.9%	3	2	3	88.9%
BS0109	0	0	0	0.0%	0	0	0	0.0%	3	3	3	100.0%
BS0110	0	0	0	0.0%	2	2	1	55.6%	3	2	3	88.9%
BS0111	0	0	0	0.0%	3	2	2	77.8%	3	3	2	88.9%

[a] Sum of infected pucks for each replication (n=3)

[b] Total percent infected pucks for experiment (n=9)

were found to be significantly different between each type of puck evaluated resulting in 47.7% infection for Russet Burbank potato, 67.3% infection for the resistant sugarbeet cultivar SES RR602TT and 78% infection for the susceptible sugarbeet cultivar Hilleshög 4062 (Table 10).

Table 10. Average number of potato and sugarbeet pucks expressing symptoms per trial in host specificity and pathogenicity trials with bacterial isolates recovered from sugarbeets with soft rot decay from commercial sugarbeet fields.

Puck Type[a]	Average number of symptoms observed per 3 pucks
Potato (Russet Burbank)	1.43 c
Sugarbeet (Hilleshög 4062)	2.34 a
Sugarbeet (SES RR602TT)	2.02 b
P-Value$_{(0.05)}$[b,c]:	*0.14*

[a] n = 360

[b] Fisher's Protected Least Significant Difference

[c] Values sharing a letter are not significantly different

Although infection rates varied between replications, over one-third of the isolates caused symptoms on sugarbeet at a rate greater than 80%, and nine out of ten isolates resulted in symptoms at a rate of greater that 50% for the experiment, demonstrating the aggressive pathogenicity of the Red River Valley isolates. The isolates recovered from SMBSC were found to cause the greatest frequency of infection per trial for all three of the different types of pucks. Isolates recovered from SMBSC expressed symptoms on 2.33 out of three potato pucks, which was significantly greater than that of ACSC isolates (1.70/3) and MDFC isolates (0.83/3) (Table 11). There were no significant differences found between sugar cooperative isolates when evaluating either the resistant (SES RR602TT) or susceptible (Hilleshög 4062) cultivar.

Table 11. Average number of potato and sugarbeet pucks expressing symptoms per trial in host specificity and pathogenicity trials with bacterial isolates recovered from sugarbeets with soft rot decay from commercial sugarbeet fields compared across the geographies of the three Red River Valley sugar cooperatives.

Isolate Source[a]	Potato (Russet Burbank)	Sugarbeet (Hilleshög 4062)	Sugarbeet (SES RR602TT)
ACSC[b]	1.70 b	2.27	1.90
MDFC[c]	0.83 c	2.27	2.05
SMBSC[b]	2.33 a	2.57	2.07
P-Value$_{(0.05)}$[d,e]:	*0.46*	*NS*	*NS*

[a] ACSC = American Crystal Sugar Company (Moorhead, MN), MDFC = Minn-Dak Farmers Cooperative (Wahpeton, ND), SMBSC = Southern Minnesota Beet Sugar Cooperative (Renville, MN)

[b] n = 90

[c] n = 180

[d] Fisher's Protected Least Significant Difference

[e] Values within a column sharing a letter are not significantly different

Reference isolates of *Pectobacterium carotovorum* subsp. *betavasculorum* (Table 2) collected from sugarbeet-growing regions of California, Idaho, Washington, Nebraska and Montana served as comparative positive controls that did not cause any symptoms on potato and showed a high correlation in infection to the frequency of symptoms observed during the greenhouse evaluation of inoculated plants. All but one of the reference isolates (BS0350) were found to cause symptoms on only the SES RR602TT cultivar (Table 8 and 9). However, it should be noted that these select isolates and commercial cultivar were both sourced from the Imperial Valley in California where *Pectobacterium carotovorum* subsp. *betavasculorum* is known to be used in resistance screening trials. Reference isolate BS0350 only caused symptoms on the susceptible commercially-available Red River Valley cultivar (Hilleshög 4062), whereas

isolates BS0109 and BS0969 were found only to produce symptomology of bacterial soft rot on the resistant cultivar. Reference isolates BS0110, BS0111 and BS0970 caused disease on both cultivars evaluated. These results are not surprising given the heterogenous nature and mediocre definition of *Pectobacterium carotovorum* strains to date, and may suggest that the causal agent of bacterial soft rot in the Red River Valley of Minnesota and North Dakota differs from the sugarbeet host-specific *Pectobacterium carotovorum* subsp. *betavasculorum* found in other sugarbeet-producing regions of the United States. Subsequent to this work, the reference isolates were sent to SESVanderHave (Tienen, Belgium) for use in their breeding program. Upon receipt, they were analyzed for species identification and two isolates were found to be incorrectly identified as *Pectobacterium* sp. (E. De Bruyne, SESVanderHave, personal communication). These finding are in agreement with the work of this study as two of the reference isolates utilized did not function as previously documented in prior studies.

Biochemical Testing

Forty isolates causing soft rot on either sugarbeet only (11 isolates) or both potato and sugarbeet (29 isolates) were tested (Tables 8 and 9). All field and reference isolates were found to be both gram-negative and oxidase-negative. *P. carotovorum* subsp. *atrosepticum* was the only isolate tested that did exhibit growth *in vitro* at 37 ºC and *P. carotovorum* subsp. *carotovorum* and *Dickeya dadantii* were incapable of acid production from alpha-methyl glucoside (Table 12). Isolates that infected both sugarbeet and potato were found to have biochemical profiles similar to the reference isolates of *P. carotovorum* subsp. *betavasculorum*, even though these particular strains were previously known to be host-specific to sugarbeet. Isolates that infected only sugarbeet were found to be catalase positive, a characteristic shared only by *Dickeya dadantii* (Table 12).

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Table 12. Biochemical comparisons of unknown Red River Valley isolates. Four known isolates including *P. cartovoroum* subsp. *betavasculorum*, *P. cartovoroum* subsp. *carotovorum*, *P. atrosepticum*, *Dickeya dadantii* served as negative control references for the study.

Characteristic	Sugarbeet Only Isolates	Sugarbeet and Potato Isolates	Pc-b[a]	Pc-c[b]	P-a[c]	*Dickeya dadantii*
	(n=11)	(n=29)	(n = 6)	(n=1)	(n=1)	(n=1)
Gram Stain	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-
Catalase	+	-	-	-	-	+
α-Methyl Glucoside	+	+	+	-	+	-
Growth at 37 ºC	+	+	+	+	-	+

[a] = *P. carotovora* subsp. *betavasculorum*

[b] = *P. carotovora* subsp. *carotovorum*

[c] = *P. atrosepticum*

In addition to biochemical characteristics, DNA sequences were also used to identify all isolates to species and subspecies. The internal transcribed spacer (ITS) sequence is a widely used DNA region within the microbiological community and it has been utilized to study the phylogenetic relationships among different soft-rot bacteria (Toth et. al. 2001). However, because of the large number of insertions and deletions in the ITS, accurate identification can be hindered below the genus level (Toth et. al. 2001). In addition, formally described species that are evolutionarily closely related may have similar sequences and thus leading to erroneous identifications (Janda and Abbott 2007). This reinforces the importance of our utilizing both biochemical and DNA sequence data to identify the unknown Red River Valley isolates.

DNA Analysis and Gene Sequencing

Amplification and sequencing of DNA extracted by a standard CTAB procedure followed by a nucleotide BLASTn search via GenBank confirmed all field isolates as

Pectobacterium carotovorum with greater than 96% homology. RAD-GBS testing showed

99.76% nucleotide sequence identity on average across all five genes to the *P. carotovorum*

subsp. *brasiliense* reference sequences. The alignments to *P. cartovoroum* subsp. *carotovorum*,

P. atrosepticum, *P. betavasculorum*, *P. carotovorum* subsp. *odoriferum*, and *P. wasabiae*

reference sequences averaged 96.0%, 95.4%, 94.3%, 97.0% and 94.4% identity, on average,

across the five genes, respectively. The nearly 100% identity across all five genes previously

utilized in multi-locus sequencing and divergence from the closely related subspecies indicates

that the isolates are *Pectobacterium carotovorum* subsp. *brasiliense*.

Subspecies identification was confirmed using BR1f and L1r primers (yielding an

expected fragment length of the amplicons at 322 base pairs) utilizing *P. parmentieri*, *P.

cartovoroum* subsp. *carotovorum*, *P. atrosepticum*, *Dickeya dadantii*, *D. dadanthicola*, D.

chrysanthemi and *Cercospora beticola* as negative reference isolates (Figure 19).

The taxonomy of the *P. cartovoroum* subspecies remains unclearly defined to date. Despite the

lack of valid publication, the *Pectobacterium carotovorum* subsp. *brasiliense* name has been

used in more than ten publications since 2004 as *Erwinia carotovora* subsp. *brasiliense* (Nabhan

et. al. 2012). Although Zhang et. al. (2016) proposed the elevation of *P. carotovorum* subsp.

brasiliense to the species level, this distinction has yet to be accepted within the greater plant

pathology community (Secor et. al. 2016; Fujimoto et. al. 2017; Gillis et. al. 2017; Jaramillo et.

al. 2017; Meng et. al. 2017; Wolf et. al. 2017; Naas et. al. 2018; Zhao et. al. 2018).
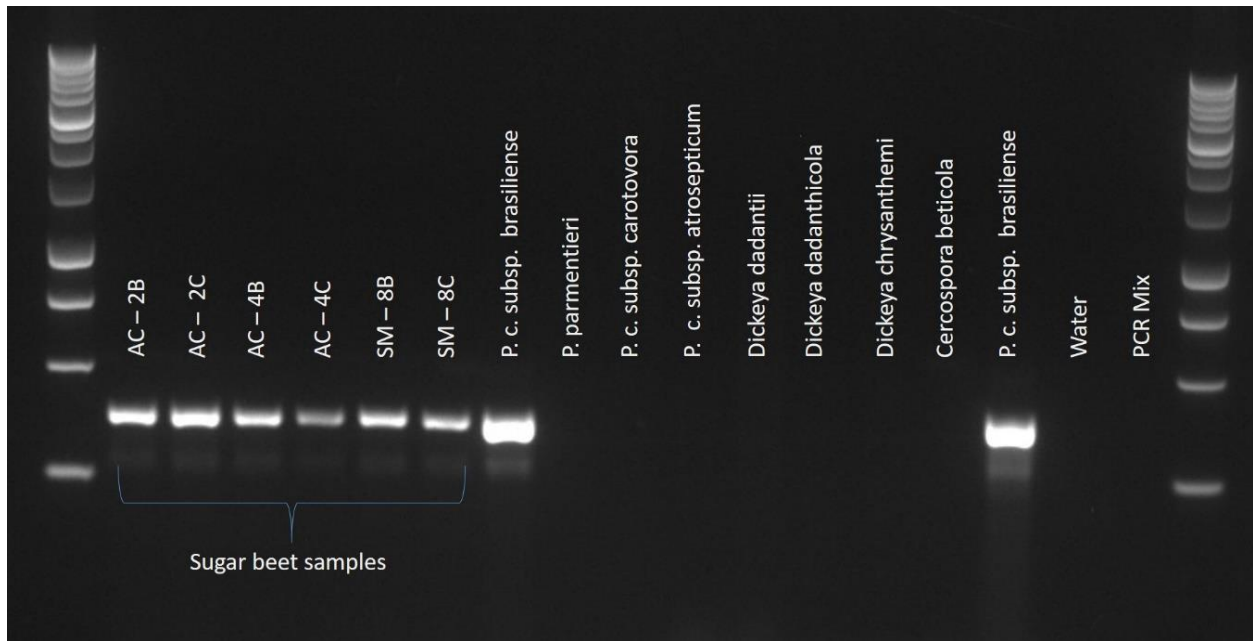
Figure 19: Sub-species confirmation of unknown Red River Valley isolates to *Pectobacterium carotovorum* subsp. *brasiliense*. Nine known isolates including *P. parmentieri*, *P. cartovoroum* subsp. *carotovorum*, *P. atrosepticum*, *Dickeya dadantii*, *D. dadanthicola*, *D. chrysanthemi* and *Cercospora beticola* served as negative control references for the study.

Pathogen Impact and Severity on Commercial Fields and Factory Processing

Quality Analysis Study

Results determined that a very low sample ratio of infected to healthy sugarbeet roots can have a significant impact on both sugar content and purity. Samples consisting of ten healthy roots and zero infected roots served as the baseline for the experiment averaging values of 17.78% sugar content and 87.14% purity, yielding 144.99 kg/tonne of sucrose (Table 13). It is of note that these quality parameters were very similar to that of the sugar cooperative's averages for the same year in which they were sampled (17.00% sugar content and 89.41% purity, yielding 143.51 kg per metric ton of sucrose). The highest sample ratio of infected roots (ten infected to zero healthy roots) had 6.31% sucrose content, 66.01% purity, and yielded only 24.00 kilograms per metric ton of sucrose, indicating a 67% reduction in percent sucrose and a 25%

increase in the amount of impurities on a fresh-weight basis compared to the baseline.

Furthermore, for every increase in two infected beets per quality sample, there was an average

decrease of 2.29 percentage points of sugar, a 4.23% decrease in purity, and a 24.20 kilogram

reduction in sucrose content per metric ton of roots which were all statistically significant (Table

13). Because sugar cooperatives utilize the data from the quality samples collected during

harvest to calculate the beet payment to the grower, this documented reduction in sugar content

and increase in impurities would result in portentous financial losses to both the grower, and

ultimately, the sugar cooperative.

Table 13. Quality analysis of harvested sugarbeets by varying sample ratios of healthy
and bacterial soft rot infected roots.

Sample Ratio	Sucrose (%)	Purity (%)	Sucrose (kg / M Ton)
10 Healthy - 0 Infected	17.78 a	87.14 a	144.99 a
8 Healthy - 2 Infected	15.93 b	84.41 b	123.30 b
6 Healthy - 4 Infected	13.17 c	81.49 c	95.10 c
4 Healthy - 6 Infected	11.55 c	78.27 d	76.71 d
2 Healthy - 8 Infected	9.19 d	69.98 e	44.97 e
0 Healthy - 10 Infected	6.31 e	66.01 f	24.00 f
P-Value$_{(0.05)}$[a,b]:	*1.76*	*2.11*	*9.72*

[a] Fisher's Protected Least Significant Difference
[b] Values within a column sharing a letter are not significantly different

Unlike other root pathogens endemic to the region that attack the host plant from the

'outside-in,' bacterial soft rot of sugarbeet works 'inside-out,' thereby leaving the lateral and tap

roots secured in the ground. These structures help keep infected beets within the harvested row

during the aggressive defoliation process as opposed to displacing the entire root in between the

harvested row. Even plants with severe levels of infection (> 90% of tissue macerated) are still

able to remain both physically intact and in place during harvest. Therefore, sugarbeets infected

with bacterial soft rot, regardless of severity, are highly likely to be collected by the harvester, delivered for processing, and be selected for harvest quality sampling.

In addition to grower payment, sugar cooperatives utilize the data gleaned from the quality samples to make a logistical assessment of sugarbeet pile storage. The effects of bacterial soft rot on post-harvest sugarbeets is largely untested to date. However, the manner in which sugarbeets are stored before processing creates large outdoor piles that are subject to high relative humidity and warm climate conditions that likely help facilitate and promote the incidence of bacterial soft rot in storage. Since the onset of bacterial soft rot does not usually occur until the latter part of July to early August when the ambient air temperatures stay within ranges optimal for disease development, there is generally a wide-range of disease severity at the beginning of commercial harvest. Previous studies have shown that a healthy sugarbeet in storage utilizes nearly 253 grams of sucrose per tonne per day. This value is increased nearly five-fold with moderate levels of other root pathogens including *Rhizomania*, *Aphanomyces*, *Fusarium*, and *Rhizoctonia* (Campbell and Klotz 2006; Campbell et. al 2008; Campbell et. al 2011; Campbell et. al 2014) causing significant financial losses to the cooperatives in addition to other storage and processing complications associated with infected roots.

In order to deliver clean beets for processing, sugar cooperatives and their producers subject the harvested roots to several mechanical cleaning methodologies during the harvest campaign. Occurring both in the field and at receiving stations, the cleaning processes utilized subject harvested roots to falling, tumbling and rubbing in order to remove as much foreign material as possible before they are placed into long-term storage. Although efficient, these methodologies often break, rupture and pierce areas of the roots thereby opening potential sites

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for both bacterial pathogen escape and subsequent infection sites of healthy roots as they are transferred and placed into long-term storage.

Since affected plants are difficult to detect until the rot is well advanced, the 'hidden nature' of this disease would have enabled it to remain virtually undetected within commercial sugarbeet fields for years prior to the initiation of this study. In addition, the novelty of this disease may have been misinterpreted by producers and agronomists alike. Sugarbeet root symptoms caused by *P. carotovorum* subsp. *brasiliense* can be similar in nature to the wet, necrotic lesions caused by *Rhizoctonia* spp., while the half-leaf necrosis symptomology could have easily been previously misdiagnosed as being caused by *Fusarium* spp. As such, it is likely that this disease may have had more of a negative economic impact to the regional sugarbeet industry than previously estimated by skewing efforts for commercial control, research endeavors and complications associated with sugarbeet sampling and storage.

Sugar Factory Sampling and Cossette Lab Analysis

For comparative purposes, the carbohydrate profiles were calculated as a weight-to-weight percentage (the weight of dissolved solid in question per the total weight of beet sample). Healthy sugarbeet tissue had relative dry substance (RDS) values of 16.32%, 20.33% and 2.85% for sucrose, glucose, and fructose, respectively (Table 14). Tissues consistently symptomatic of bacterial soft rot yielded a significantly lower RDS value for sucrose (4.42%) and significantly higher values for glucose and fructose (45.15% and 12.62%, respectively) (Table 14). The difference between the sampled tissue equates to nearly a four-fold decrease in sucrose content and a 25% and 10% increase in glucose and fructose content, respectively, both of which are recognized during the factory process as 'invert sugars.' It is of note that nearly half of all dissolved solids in the macerated tissue is glucose.

Table 14. Carbohydrate analysis of sugar factory sampled cossettes summarized as a percentage of relative dry substance.

Cossette Sample[a]	Ion Chromatography			HPLC[b]		Percent Solids
	Sucrose	Glucose	Fructose	Lactate	Dextrose	
Healthy Tissue	1.26	1.57	0.22	0.08	0.02	7.72
Macerated Tissue	0.62	6.33	1.77	0.5	2.75	14.02
P-Value$_{(0.05)}$[c]:	*0.0001*	*0.0008*	*0.0001*	*0.0016*	*0.0001*	*0.0104*

[a] Data combined from 2014-2015 & 2015-2016 processing campaigns

[b] High-Perfomance Liquid Chromatography

[c] Fisher's Protected Least Significant Difference

Similar trends can also be seen when analyzing the sampled levels of lactate and dextrose (D-glucose). The healthy sugarbeet tissue exhibited RDS values of 0.08 (1.04% weight to weight) for lactate and 0.02 (0.26%) for dextrose. Macerated tissue values were significantly higher, increasing over three-fold for lactate (3.57%) and seventy-five times greater for dextrose (19.61%).

Sugar factories routinely monitor lactate concentrations within the factory as an indicator of microbial activity. Microbial infections result in increased sugar losses and in severe cases, can lead to other processing problems such as poor settling and filtration during carbonation. Since lactate is not removed during juice purification, lactic acid is converted to calcium lactate in the carbonation process. Calcium lactate is soluble in the juice, so it passes through the process all the way into molasses. This increases the amount of sugar lost to molasses as every 500 grams of non-sugars will take approximately 750 grams of sucrose into molasses with it.

Invert sugar concentrations within the factory are just as critical as lactate, if not more so. Invert sugars are also indicators of microbial infection or beet degradation and are detrimental to

sugar extraction and overall processing efficiency. Under normal operation, destruction of invert sugars occurs during the carbonation process. Within the liming vessel, elevated temperatures and pH in the presence of both calcium carbonate and carbon dioxide break down invert sugars. Sugarbeet roots with elevated levels of invert sugars can overwhelm the carbonation system so excess invert sugars pass through without being destroyed. These invert sugars eventually break down in the evaporation process, causing elevated color generation and a significant drop in pH of the thick juice as the invert sugars convert to acids. This increased coloration and acidic pH causes complications in granulation and juice storage, both of which pose significant financial losses to the sugar company.

The elevated levels of both lactate and invert sugars present within the cossette samples and decreased levels of sucrose concentration clearly demonstrate that sugarbeet roots infected with bacterial soft rot would be detrimental to the normal operation of a sugar factory. The increased molasses production alone would remove more sugar than what an individual root would bring into the process.

General Conclusions

The recent outbreaks, unknown origin, and lack of effective and economic control measures for bacterial soft rot within the Red River Valley of Minnesota and North Dakota prompted initiation of this study. Forty isolates collected from commercial fields within the growing geographies of all three sugar cooperatives in the Red River Valley were shown to be pathogenic to sugarbeet and potato. The isolates were tested and compared, both chemically and molecularly, to known isolates causing similar symptoms. The cause was found to be distinct from previous reports of *P. betavasculorum* (*Erwinia betavasculorum*) that caused similar symptoms on commercially grown sugarbeets in other sugarbeet production areas of the United

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States. The causal organism of the disease was identified as *P. carotovorum* subsp. *brasiliense*. To our knowledge, this is the first report of this pathogen causing field decay of sugarbeet in North America. *Pectobacterium carotovorum* subsp. *brasiliense* has been previously reported in Poland as causing soft rot decay of sugarbeet *in vitro* (Waleron, et. al. 2015), but this being the first report on isolates identified from a commercial sugarbeet field.

These experiments show that the Red River Valley isolates causing bacterial soft rot in sugarbeet are significantly different from those causing the same or similar symptoms in other sugarbeet growing regions of the United States. Distribution suggests that *P. carotovorum* subsp. *brasiliense* is present in the northcentral United States and *P. betavasculorum* is present in the western United States, however *P. betavasculorum* is poorly defined and may be improperly described. *Pectobacterium. carotovorum* subsp. *brasiliense* also appears to be an emerging bacterial pathogen causing soft rot of numerous vegetables, notably potato soft rot in the European Union and North America (Naas et. al. 2018).

Recommendation for Future Work

The source of infection and mechanisms of spread are not yet known and should be part of ongoing research to further characterize this newly problematic disease. Management strategies for this disease can be further developed when the source of infection is determined. Potential candidates could include surface water, nematodes, insects, weeds or even other hosts. The results of this study may serve as a platform for the investigation of these candidates and others as vectors or alternative hosts playing an important role in the source of infection, mechanism of spread and evolution of this disease.

The genome sequencing of *Erwinia carotovora* subsp. *atroseptica* indicated that this species of bacteria is capable of nitrogen fixation (Bell et. al. 2004). Similarly, it has been found

that *Erwinia carotova* subsp. *betavasculorum* could be moderately controlled by limiting the amount of nitrogen applied to a commercial field before sugarbeets are sown (Thomson et. al 1981). Due to differences in grower payment calculations, nitrogen rates applied per acre at Minn-Dak Farmers Cooperative average 151-168 kg/ha, whereas the rates applied at American Crystal rarely exceed 134 kg/ha. This may help explain the low incidence of this new disease in the American Crystal growing area and relatively high levels of disease in the Minn-Dak region, and may serve as a conventional-means of control for the southern areas of the Red River Valley.

Once the complete sequencing of *P. carotovorum* subsp. *brasiliense* has been completed, the hope is that primers can be developed/established to help further classify and/or identify this destructive pathogen. Preliminary studies have shown differences among sugar beet varieties in response to *P. carotovorum* subsp. *brasiliense* and *P. betavasculorum* suggesting that both pathogens be used in resistance screening trials. These molecular markers will also aid in determining if the pathogen is seed-borne, which given the uniform incidence across the infested fields, merits further investigation. Although the yield of sugarbeet varieties characterized by heritable tolerance to bacterial soft rot currently is lower in comparison to top producing varieties under disease-free conditions (SESVanderHave USA 2016), thereby decreasing their attractiveness to growers, advancements in this area of study may provide the most rapid and effective method of bacterial soft rot control for growers.

The post-harvest disease progression of the pathogen within the long-term ventilated storage systems utilized in Minnesota and North Dakota remains unanswered, and given the processing complications caused by this pathogen, is of great interest to the sugarbeet companies.

Many questions about the control of this disease remain unanswered. The present work

hopefully provides a foundation to further investigate *P. carotovorum* subsp. *brasiliense* in future

research aimed at controlling bacterial soft rot of sugarbeet. Finally, it is only through careful

trials and basic investigation that this disease caused by *P. carotovorum* subsp. *brasiliense* will

be better understood. It is hoped that researchers who follow in this line of investigation will,

ultimately, fulfill the potential of this work.

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