CHARACTERISTICS AND HOST RANGE OF A NOVEL *FUSARIUM* SPECIES CAUSING YELLOWING DECLINE OF SUGARBEET

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

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ABSTRACT

In 2008, a novel and distinct *Fusarium* species was reported in west central Minnesota causing early-season yellowing and severe decline of sugarbeet. This study was conducted to (i) establish optimum conditions for fungal growth and (ii) determine the host range of the novel *Fusarium*. The optimum temperature for fungal growth is 24°C and root injury is not needed to penetrate, infect, and cause disease of sugarbeet plants. Of the fifteen common crops and weeds tested for susceptibility to the new *Fusarium* sp. in field and greenhouse trials, disease symptoms were only observed in sugarbeet. Host range plants were tested for the presence of latent infection by root isolations and PCR. The pathogen was only present in canola and sugarbeet. The results suggest that canola has implications in the sugarbeet production system and management strategies for the novel *Fusarium* species. The name and description of the new *Fusarium* sp. is pending.

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Most importantly, my deepest gratitude and love go to my family and lovely husband for their constant encouragement and support during the most difficult times of this thesis. Thank you for helping me to keep moving forward.

DEDICATION

This manuscript is dedicated to my loved ones…

My parents: Margarita and Hermes

My sisters: Luz Elena and Leidy

Who have lived the absence of my kisses and hugs during these years.

My husband: Morgan

Who pushed me to the most challenging and wonderful moments of my life…

Thanks for making me feel so loved and lucky to have you all in my life, but much more for your tremendous support.

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INTRODUCTION

Sugarbeet (*Beta vulgaris* L.), which originated in north-west Europe (de Bock, 1985), has become an economically important crop because of its high sucrose content. In 2011, the world production of sugarbeet was 271.6 million tons. The United States is the third largest producer in the world producing 26.1 million tons from 490,930 ha (FAO, 2013).

In the United States, sugarbeet production is concentrated in Minnesota and North Dakota (Upper Midwest), California, Idaho, Oregon, and Washington (Far West), Colorado, Montana, Nebraska, and Wyoming (Great Plains region), and Michigan in the Great Lakes region (Harveson et al., 2002). From these states, Minnesota, Idaho, and North Dakota are the main producers. In 2012, Minnesota produced nearly 12.3 million tons of sugarbeets, compared to 6.4 million tons produced by Idaho and more than 6 million tons of sugarbeet in North Dakota (USDA-NASS, 2013).

In the Red River Valley (RRV) of Minnesota and North Dakota, the sugarbeet industry suffers significant losses annually, because of the different diseases that affect the crop. Among the more common diseases are Cercospora leaf spot, Rhizoctonia root and crown rot, Aphanomyces root rot, Rhizomania and Fusarium yellows (Bolton et al., 2012; Bolton et al., 2010; Dyer et al., 2004; Bradley et al., 2006; Windels et al., 2005).

Within this group, Fusarium yellows disease represents a constant constraint to sugarbeet production in the area. Seven *Fusarium* species have been associated with Fusarium yellows disease of sugarbeet, of which *Fusarium oxysporum* f. sp. *betae* is the main causal organism (Hanson and Hill 2004; Burlakoti et al., 2012). Fusarium yellows of sugarbeet was identified for the first time in United States in 1931 (Stewart, 1931), and in the RRV of Minnesota and North Dakota in 2002 (Windels et al., 2005). Affected plants develop interveinal yellowing of the leaves, chlorosis, scorching and wilting, which are accompanied by vascular discoloration of the taproot (Hanson and Hill, 2004). This disease

can cause substantial reduction in root yield, sucrose content, and juice quality (Hanson et al., 2009; Campbell et al., 2011). Economic losses due to this disease have been estimated to be 5 to 50 percent due to yield reduction (American Crystal Sugar Company, 2010).

In 2005, symptoms similar to those of Fusarium yellows disease were observed in American Crystal Fusarium screening trials near to Sabin, MN, as well as in a few fields in the Moorhead factory district in west-central Minnesota. However, this disease exhibited distinctive symptoms differing from yellows, including severe root rot, vascular discoloration of the petiole and earlier death of the plants 30 to 45 days after planting. Preliminary isolations from affected plants resulted in consistent recovery of an unknown *Fusarium* species (Burlakoti, 2007; Rivera et al., 2008). Cultures of the unidentified *Fusarium* in potato dextrose agar (PDA) medium, presented bright pink-orange color on the underside of the culture (Rivera et al., 2008); and production of abundant microconidia, but sparse macroconidia, which separated this new *Fusarium* from *F. oxysporum*.

Restriction fragment length polymorphism (RFLP) patterns of the internal transcribed spacer (ITS) regions of the rDNA of the uncharacterized *Fusarium*, using the restriction enzymes *Alu*1, *Fnu*4HI, *Hae*III and *Hha*I, revealed a distinct pattern that did not match any known *Fusarium* species (Rivera et al., 2008). In addition, the gene for the translation elongation factor 1-alpha (TEF-1α) from 12 single-spore isolates was partially sequenced. Comparisons of the TEF gene sequence data using the FUSARIUM-ID and GenBank online databases also did not match any described *Fusarium* species (Burlakoti, 2007; Rivera et al., 2008). Based on field symptoms, culture morphology, pathogenicity, RFLP patterns, and TEF sequence analysis the unknown *Fusarium* species was identified as a novel *Fusarium* species (Rivera et al., 2008). Recently, phylogenetic analysis of three *Fusarium* genes sequences (TEF-1α, Calmodulin, and mtSSU rDNA) showed *F. novum* to be distinct from all others *Fusarium* species, but most closely related to *F. acutatum* in the *F. fujikuroi* group (Secor et al., 2013 *in preparation*).

Since the novel *Fusarium* species is more aggressive and can affect sugarbeet plants very early in the season causing seedling death, petiole infection, and severe root rot, the disease produced by the new *Fusarium* has been named Fusarium yellowing decline (Rivera et al., 2008), to differentiate it from Fusarium yellows disease caused by *F. oxysporum* which affects the sugarbeet late in the season.

Monocyclic diseases produced by soil-borne pathogens can be managed by reducing the amount of initial inoculum by soil fumigation and crop rotation (Harveson and Rush, 1994). However, these management practices are not reliable for *Fusarium* species, because fumigants generally do not work and are expensive. Also *Fusarium* resistance structures such as chlamydospores, can survive in soil for many years (Khan, 2003). To date, the most effective and practical disease control strategy for soil-borne *Fusarium* pathogens is to use resistant or tolerant cultivars (Burlakoti, 2007). However, most of the resistance screening studies have been directed at *F. oxysporum*, the causal agent of Fusarium yellows, and not towards the novel *Fusarium* species which is more aggressive than *F. oxysporum*, and sugarbeet may have different resistance mechanisms to this new *Fusarium* (Burlakoti, 2007). Crop rotation can reduce fungal populations especially when rotated with crops not susceptible to the disease. In order to develop crop rotation schedules, it is important to know the susceptibility of rotational crops and weeds.

Thus, given that Fusarium yellowing decline on sugarbeet is a new disease in the Red River Valley of Minnesota and North Dakota, this study was conducted to determine the host range of the novel *Fusarium* species, referred in this paper as *Fusarium novum*, with the intention of understanding the biology of the pathogen as well as the possible implications on crop production system and management strategies.

LITERATURE REVIEW

Biomolecules, one of the main components of living organisms, are typically classified into proteins, carbohydrates, lipids, nucleic acids, and other small molecules. Of the carbohydrates, saccharides function in energy storage, and as structural components of the cell wall in plants and the exoskeleton of arthropods. One of the most common saccharides is sucrose. This molecule is a disaccharide composed of the monosaccharides glucose and fructose, which molecular formula is $C_{12}H_{22}O_{11}$. The structure is presented in Figure 1.

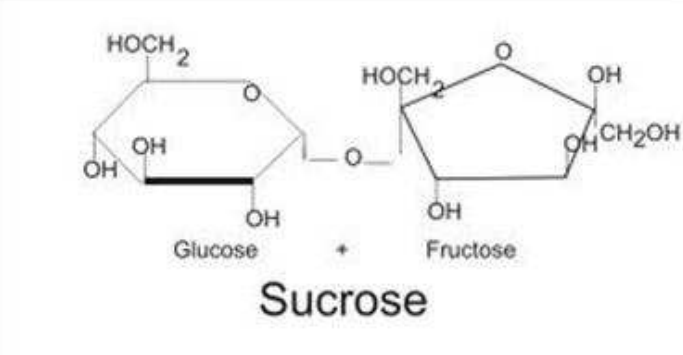

Figure 1. Structure of sucrose.

In plants, sucrose is produced in mesophyll cells of leaves and transported via the phloem to heterotrophic sink organs such as meristems, roots, flowers, and seeds, where it is finally stored. Thus, sucrose provides carbon skeletons to organ growth and carries energy at the same time (Lemoine, 2000; Künh and Grof, 2010).

Sucrose may be produced by many plants; however the world's commercial sucrose is primarily produced in two domesticated crops: sugarcane (*Saccharum officinarum* L.) and sugarbeet (*Beta vulgaris* L.). Although sugarcane provides about 80 percent of the world's sucrose, sugarbeet represents an alternative source of sucrose in cool temperate regions of

the world, whereas sugarcane grows mainly in tropical and subtropical regions of the world and does not tolerate frost.

Sugarbeet History and Significance

According to Winner (Campbell, 2010), fodder beets from Silesia, a region between Poland, Czech Republic, and Germany, were the ancestors of the first sugarbeet variety "White Silesian". The first sugar extraction from beets occurred in 1747, when the physics professor Andreas Marggraf discovered sucrose in beets (Rolph, 1917). Franz Karl Achard (Marggraf's student) developed processing methods to produce refined sugar from beets; it allowed him to establish in 1799 the first sucrose factory from beets at Cunern, Silesia, using the "White Silesian" beet, which had about 6% sucrose (de Bock, 1986).

Soon, Napoleon Bonaparte became interested in Achard's work and began to build factories in France. This was accompanied by laws allocating money to establish sugar schools and sugarbeet production, as well as restrictions to the importation of sugar from sugarcane in 1813. As a result, the sugarbeet industry rapidly developed in France (Rolph, 1917; de Bock, 1986). After the fall of Napoleon, excess sugar supplies caused prices to collapse and most of the sugarbeet factories were closed. However, the beet sugar industry arose again after decline of slavery in the West Indies and was successfully established in most of Europe (Rolph, 1917; Harveson et al., 2009).

In the early 1800s, beetroots brought by American colonists were already grown in gardens in United States, but efforts to develop beet sugar industry were unsuccessful. These attempts occurred during the mid-1800s in Philadelphia, Massachusetts, Wisconsin, Illinois, Michigan, and Utah (Harveson et al., 2009). Finally, during the 1870's, the commercial production of beet sugar was established in California. By 1879, E. H. Dyer built the first successful beet sugar factory in Alvarado (now Union City), California. Two other factories were established in California, one in Watsonville by Claus Spreckels (1888) and

the other in Chino by the Oxnard brothers (1891), who constructed other factories in 1890 in Grand Island and Norfolk, Nebraska (de Bock, 1986; Rolph, 1917; Harveson et al., 2009).

Soon after, sugarbeet production and factories expanded very quickly in United States. To date, the sugarbeet crop is concentrated in the Upper Midwest (Minnesota and North Dakota), the Far West (California, Idaho, Oregon, and Washington), the Great Plains region (Colorado, Montana, Nebraska, and Wyoming), and the Great Lakes region (Michigan) (Harveson et al., 2002). Of these areas, Minnesota and North Dakota leads the US in sugarbeet production in 2012, providing 52% of the total U.S. production (USDA-NASS, 2013).

Beet sugar production in the Red River Valley (RRV) of Minnesota and North Dakota began in 1926, when the first factory was established in East Grand Forks, MN. This factory, plus four others located in Drayton, Hillsboro, Crookston, and Moorhead are property of American Crystal Sugar Company, one of the three grower-owned cooperatives in the region. The other two cooperatives, Minn-Dak Farmers Cooperative and Southern Minnesota Beet Sugar Cooperative, began sugarbeet processing in 1974 and 1975 respectively, operating one factory each (Shoptaugh, 1997; Windels et al., 1998; Khan, 2005; Secor et al., 2010). In 2012, these cooperatives were responsible for 57% of the total U.S. area planted, which produced 18.3 million tons of sugarbeets (USDA-NASS, 2013).

Currently, sugarbeet is grown in about 50 countries, with the highest production in France, Russia, and United States. This crop, which is considered an important agricultural export commodity, had exports of 649,241 tons of sugar in 2010 with a value of more than $42.8 million. In addition, sugarbeet production in 2011 reached 271.6 million tons harvested from 5 million ha land (FAO, 2013).

Sugarbeet Diseases in the Red River Valley

Under favorable environmental conditions, pathogens that attack plants have a tremendous impact on food production, ecosystem stability, and worldwide economy. In the sugarbeet case, the crop is susceptible to many diseases caused by bacteria, fungi, viruses, nematodes, phytoplasmas, oomycetes, and parasitic plants.

During most of the 1980's and 1990's, Aphanomyces root rot was the predominant disease in the Red River Valley (RRV) of Minnesota and North Dakota (American Crystal Sugar Company, 2011a). Aphanomyces root rot is caused by *Aphanomyces cochlioides* Drechsler. Plants can be infected by the fungi during the seedling stage as well as older roots. Visible symptoms are seedlings with yellowed leaves that wilt during hot, sunny days and appear to recover overnight. The roots develop water-soaked lesions with tan-yellow color. The pathogen survives as oospores in plant debris and soil, and is stimulated to germinate by root exudates when wet soils provide favorable conditions for infection (Harveson et al., 2009; Dyer et al., 2004).

Rhizomania appeared late in the 1990s affecting several fields in the region, and now most field are infected. The disease is caused by the virus Beet Necrotic Yellow Vein Virus (BNYVV), which is transmitted by the protozoan *Polymyxa betae* Keskin by zoospores. Root symptoms are present as an extended mass of fine secondary roots that prevent water uptake by the plant; consequently, leaves exhibit chlorosis and a fluorescent yellow appearance (Bradley et al., 2006; Harveson et al., 2009; American Crystal Sugar Company, 2011a). Resistance to the virus is used to manage the disease, but resistance-breaking strains of the virus have appeared.

This decade, the greatest concern has been *Rhizoctonia*, a pathogen that although has always been present in the region, seldom was a problem (American Crystal Sugar Company, 2011a; American Crystal Sugar Company, 2013). Rhizoctonia root and crown rot

is produced by *Rhizoctonia solani* Kühn. Most of the infections are initiated in the crown; typical symptoms are wilting of the leaves and black lesions of petioles at the point of attachment to the crown, which spread quickly to the root tissue. *Rhizoctonia solani* survives as mycelium or sclerotia in plant debris and soil. The pathogen becomes active at soil temperatures of 12 to 35°C and 26.7°C is the optimum for infection (Khan et al., 2009). The disease is favored by poorly-drained soil (Harveson et al., 2009; Bolton et al., 2010).

Other diseases such as Cercospora leaf spot and Fusarium yellows have been detected during the last years affecting commercial fields and causing significant economic losses (American Crystal Sugar Company, 2010; American Crystal Sugar Company, 2011a; American Crystal Sugar Company, 2011b; Burlakoti et al. 2010).

Cercospora leaf spot, caused by the fungus *Cercospora* beticola (Sacc.), is favored by warm weather, frequent rains, and high humidity. Leaf spots about 3-to 4-mm in diameter with ash-gray center and dark-brown borders are typical symptoms of the disease. The fungus survives over winter on plant debris, and when moisture is adequate, conidia are formed again to infect new plants via splashing water (Harveson et al., 2009; Secor et al., 2010, Bolton et al., 2012). Fungicide resistance management is critical for disease control.

Fusarium yellows is a disease primary induced by *F. oxysporum* f. sp. *betae*. It is characterized by interveinal chlorosis and wilting of the older leaves, as well as vascular discoloration of the taproot without external root symptoms. The pathogen survives as macroconidia, chlamydospores, and mycelium in soil and plant material; temperatures over 25 to 28°C are favorable to optimal symptoms development (Windels et al., 2005; Hanson and Hill, 2004; Harveson et al., 2009).

Fusarium Species Causing Disease in Sugarbeet

Fungal species belonging to the genus *Fusarium* are worldwide and can be found in a wide range of crops causing significant yield and quality reductions. The sugarbeet crop is not an exception. At the moment there are more than seven species of the genus *Fusarium* associated with foliar yellowing, wilting, and root rot in sugarbeet plants. The diseases induced by these *Fusarium* species cause significant reduction in yield and quality of the roots, resulting in low sucrose content and juice impurity.

In United States, foliar symptoms are characterized by interveinal chlorosis and wilting, as well as vascular discoloration of the taproot, which were described for first time in sugarbeet in 1931 (Stewart, 1931). Stewart identified the causal agent of this disease, Fusarium yellows, as *F. conglutinans* var. *betae*, however the pathogen was reclassified later as *F. oxysporum* f. sp. *betae* (Ruppel, 1991).

By 1991, Ruppel reported that isolates of *F. acuminatum*, *F. avenaceum*, *F. sambucinum*, *F. equiseti*, *F. proliferatum*, *F. solani*, and *F. oxysporum* were isolated from diseased sugarbeet plants on seven states in the U.S. and Canada. However, only *F. acuminatum* and *F. oxysporum* infected 3-month old sugarbeet plants and produced symptoms typical of Fusarium yellows, while *F. avenaceum* just affected seedlings. Ruppel also reported that *F. solani* produced necrosis in the taproot and secondary roots of seedlings without visible wilting or foliar chlorosis (Ruppel, 1991).

In a subsequent study, Harveson and Rush (1998) established that *F. oxysporum* isolates producing root-rot symptoms on sugarbeet seedling in Texas, were in fact a *formae speciales* different to that producing yellows disease, and were designated as *F. oxysporum* f. sp. *radicis-betae* Harv. & Rush. Furthermore, from isolates collected in the western U.S. during 2001, four species, *F. acuminatum, F. avenaceum, F. solani,* and *F. moniliforme*,

induced symptoms identical to those caused by *F. oxysporum* f. sp. *betae* on sugarbeet in greenhouse tests (Hanson and Hill, 2004)

Fusarium oxysporum f. sp. *betae*, the cause of yellows disease, was identified for first time in the RRV of Minnesota and North Dakota in 2002 (Windels et al., 2005). By 2004, eight *F. graminearum* isolates were associated with yellowing in sugarbeet fields from Minnesota; however, only three of those isolates were pathogenic to sugarbeet after pathogenicity tests (Hanson, 2005). In 2005, three species of *Fusarium*, *F. oxysporum*, *F. graminearum* and what appeared to be *F. sambucinum* were isolated from sugarbeet affected with yellows disease in Minnesota (Secor and Rivera, 2005). After further examination, *F. sambucinum* first found in 2005 was actually a previously non-characterized *Fusarium* species. Subsequently, this novel *Fusarium* species was tentatively called *F. novum* and documented as the cause of yellow decline of sugarbeet (Rivera et al., 2008).

Of these *Fusarium* species reported to infect sugarbeet, only *Fusarium oxysporum* f. sp. *betae* and recently *F. novum*, have been recognized as the main causal organisms of Fusarium yellows disease and Fusarium yellowing decline on sugarbeet respectively (Ruppel, 1991; Rivera et al., 2008). Although both species have been implicated to induce yellowing symptoms in the field, little is still known about the biology of *F. novum*. However, it is known that *F. oxysporum* can survive in soil and plant residues as spores, chlamydospores, and mycelium between growing seasons. At the beginning of the growing season, the fungus penetrates susceptible sugarbeet roots forming new spores and colonizing the vascular system. Once the fungus has become established, it moves through the vascular system producing the typical foliar symptoms of the disease. In addition, colonization of the plant tissue accelerates wilting of the plant, followed by necrosis of the roots and petiole vascular elements which ultimately lead to death of the plant. Thus, the fungus returns to the soil as macroconidia to overwinter as chlamydospores and infect new plants the next season (Khan et al., 2003; Harveson et al., 2009).

Taxonomy of the Genus *Fusarium*

Within the ascomycetous fungi, the genus *Fusarium* is highlighted as one of the most important groups due to it is wide distribution around the world and the many species associated with destructive diseases in several economically important crops. Since the genus *Fusarium* was described in 1809 by Link, based only in the morphological character of its banana-shaped maconidia (Leslie and Summerell, 2006), almost a thousand species have been assigned to this genus.

Several taxonomic systems have been proposed during the last decades, most of them supported by Wollenweber and Reinking's work in *Die Fusarien*. They proposed a system based on 16 sections of *Fusarium* containing 65 species, 55 varieties, and 22 forms (Moretti, 2009). This system was replaced by the system of Snyder and Hansen in 1940, which regrouped the genus into just nine species using single conidia cultures. One of these was *F. oxysporum*, which clustered species in section Elegans (Snyder and Hansen, 1940). By 1983, Nelson and collaborators published an identification manual with photographs of structures such as macroconidia, microconidia, conidiophores, and chlamydospores, which were produced on carnation leaf agar for proper identification (Nelson et al., 1983). This manual basically was a bridge between the most important taxonomic systems previously developed. *Fusarium oxysporum* and *F. solani* were the only species that continued unchanged from Snyder and Hansen taxonomy (Nelson, 1991). The last identification manual available for the *Fusarium* genus was developed by Leslie and Summerell (2006), in which they present 70 species whose classification is based on morphological and phylogenetic information.

At the moment there are reported more than 80 species in the genus *Fusarium*, from which *F. oxysporum* f. sp. *betae*, *F. graminearum*, *F. sambucinum*, *F. acuminatum*, *F. avenaceum*, *F. solani*, and *F. moniliforme* have been reported as pathogenic on sugarbeet

(Ruppel, 1991; Harveson et al., 2002; Hanson and Hill, 2004; Hanson 2005; Secor and Rivera, 2005; Burlakoti, 2007). In 2005, a previously non-characterized *Fusarium* was implicated as the etiological agent of yellowing decline disease on sugarbeets (Rivera et al., 2008). For this pathogen, the temporary taxonomy is:

Kingdom: Fungi

Phylum: Ascomycota

Class: Sordariomycetes

Order: Hypocreales

Family: Nectriaceae

Genus: *Fusarium*

Species: *Fusarium novum* (Formal name pending final approval)

Source: G. Secor (*personal communication*)

History of Fusarium Yellowing Decline

Fusarium yellows disease, produced by *F. oxysporum* f. sp. *betae*, is one of the most significant diseases of sugarbeet in the Red River Valley of Minnesota and North Dakota because of the monetary losses that it produces (Windels et al., 2005). Nevertheless, in 2005, a serious Fusarium yellows-like disease was noticed for first time in American Crystal Fusarium screening trials near to Sabin, MN, as well as in a few fields in the Moorhead factory district in west-central Minnesota. Even though symptoms of this disease resembled those of Fusarium yellows disease, affected sugarbeet plants were noticed very early in the season with vascular discoloration of the petiole vascular elements and a severe root rot

that resulted in death of seedling just 30 to 40 days after planting (Burlakoti, 2007; Rivera et al., 2008; G. Secor, *personal communication*).

Morphological characterization of the pathogen linked to the disease on sugarbeets was possible from isolates recovered from infected fields, indicating that it belongs to the genus *Fusarium*. Because the symptoms observed in sugarbeet were more aggressive and similar but different to those normally observed in Fusarium yellows, the disease was referred as Fusarium yellowing decline, to differentiate it from the disease caused by *F. oxysporum* (Rivera et al., 2008).

Symptoms of Fusarium yellowing decline disease have been observed in commercial fields in west-central and southern Minnesota, including several cultivars with reported resistance to yellows (Rivera et al., 2008). In 2013, several fields with yellowing decline were documented in the Crookston factory district. This indicates that the disease has spread to other sugarbeet production areas in the Red River Valley of Minnesota and North Dakota since it was found in 2005. However, the complete distribution and range of the disease are not yet known (G. Secor, *personal communication*).

This unknown *Fusarium* species has been tentatively reported as *F. novum*, based on restriction fragment length polymorphism (RFLP) of the internal transcribed spacer (ITS) region and phylogenetic analysis of three gene sequences, TEF-1α, Calmodulin, and mtSSU rDNA, that did not match any previously reported *Fusarium* spp from *Fusarium* ID (Geiser et al., 2004). This species appears to be most closely related to *F. acutatum* in the *F. fujikuroi* complex with 98% homology (Figure 2) (Rivera et al., 2008; Secor et al., 2013 *in preparation*). *Fusarium acutatum*, is a pathogen recently found in chickpeas (*Cicer arietinum* L.) and potato (*Solanum tuberosum* L.) in India, Pakistan, and Egypt, and it is not known to be a pathogen of sugarbeet or found in the U.S. (Gopalakrishnan and Strange, 2005; Gopalakrishnan et al., 2005; Abo-Elnaga et al., 2013). Plants affected by the novel

Fusarium species typically develop interveinal chlorosis and yellowing of the leaves early in the season, as soon as 4 to 6 weeks after emergence or even as seedlings (G. Secor, *personal communication*). Because the pathogen and any associated toxins are transported via vascular system (xylem), at the beginning of the disease is very common to see affected only one side of the older leaves, which show the characteristic yellowing moving distally while the other side still remains green. Eventually, the older leaves wilt and scorched with each passing day, and plants may die early. Vascular discolorations of the taproot and petiole vascular elements, as well as death of seedlings are strong evidence of Fusarium yellows decline (Figure 3) (Rivera et al., 2008).

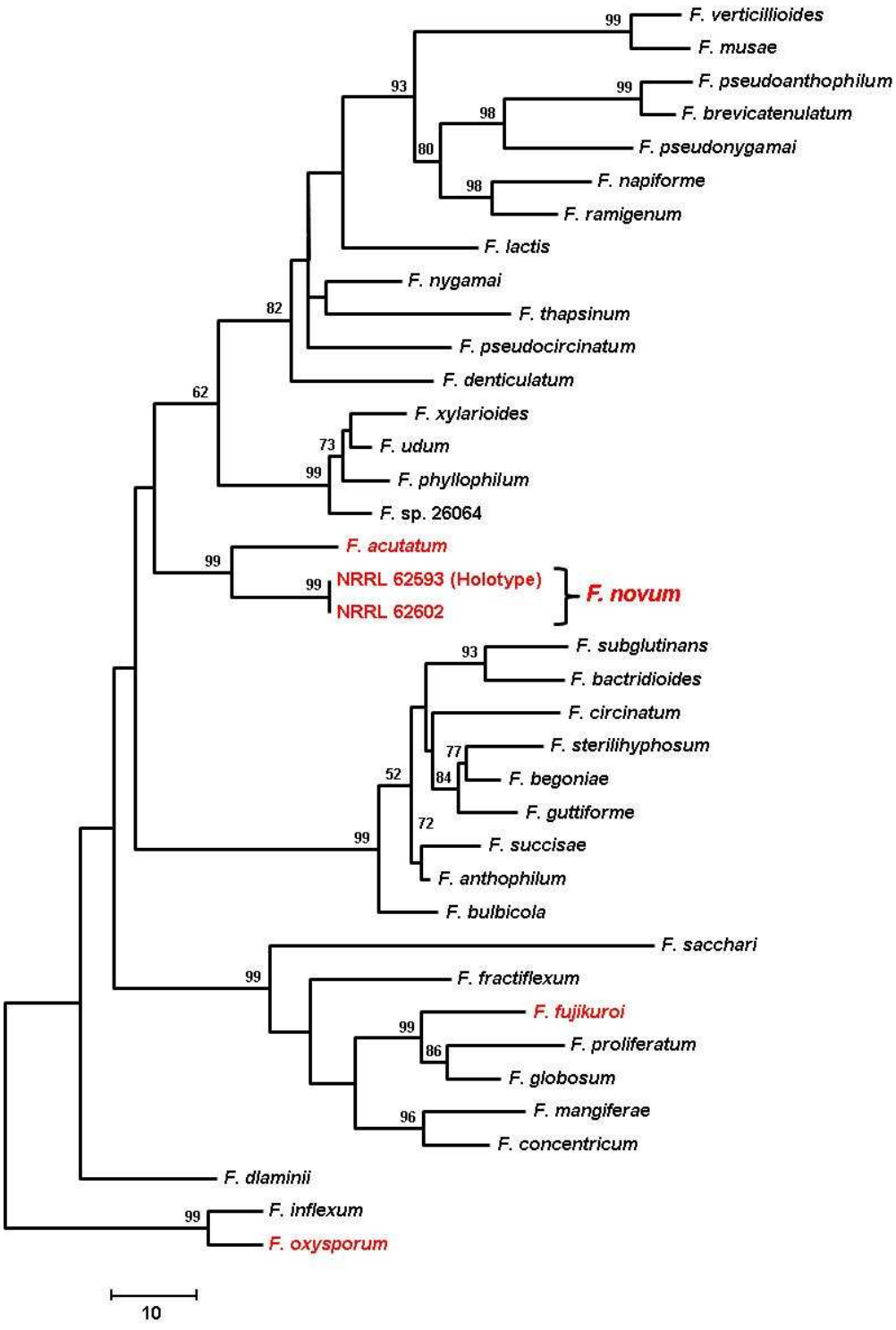

Figure 2. Phylogenetic analysis inferred from the partial EF-1α, CAL, and mtSSU rDNA sequence datasets. *Fusarium novum* groups next to *F. acutatum* in trees inferred from the combined datasets. *Fusarium oxysporum* was used as outgroup to root the tree.

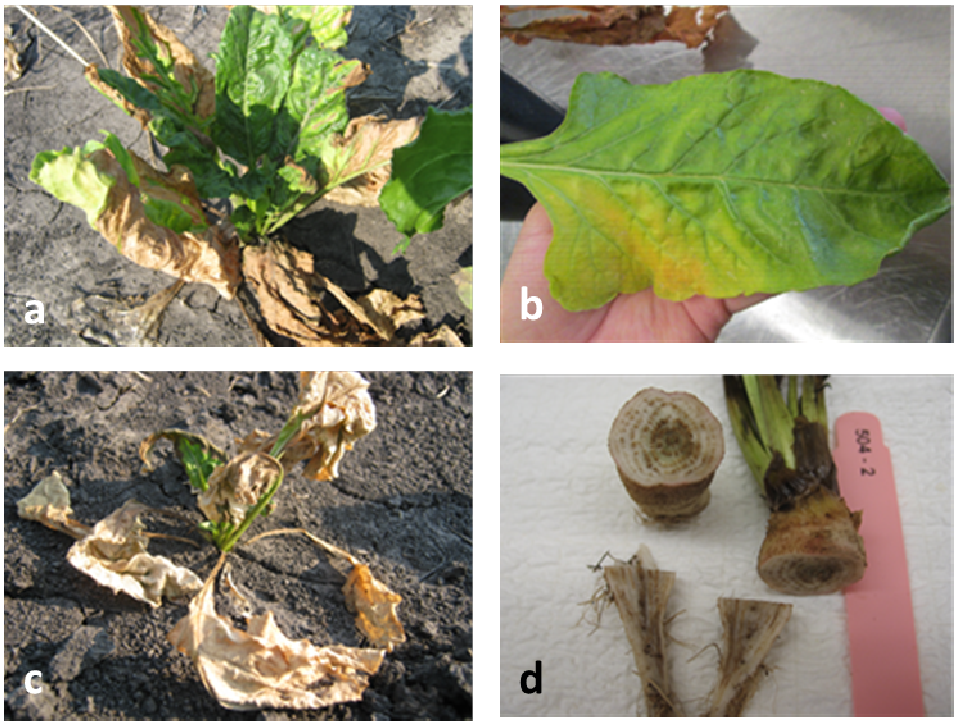

Figure 3. Fusarium yellows decline symptoms on sugarbeet. Interveinal chlorosis and yellowing of leaves (A and B), scorching of leaves (C), and vascular discoloration of the root in cross and longitudinal sections (D).

Morphology and Development

Cultures of *F. novum* on potato dextrose agar (PDA), present fluffy aerial mycelium with light-pink color on the surface and bright orange-pink color on the underside of the culture dish (Rivera et al., 2008). On both PDA and Carnation Leaf Agar (CLA), the pathogen produces microconidia, macroconidia, chlamydospores, and coiled hyphae (Figure 4). Microconidia have two cells and ovoid shape that measure 14.4-18.2 x 2.9-3.6 µm on average, and are produced abundantly. Macroconidia are produced sparsely within the first two weeks of culture. These typical sickle shaped spores are produced in sporodochia-like structures in older cultures, and are composed of two to four cells measuring in total 60.7-68.0 x 3.5-4.2 µm on average. In addition, chlamydospores have been found in older

mycelium as a round, think-walled spores that may be either terminal or intercalary measuring 7-14.5 x 5-12.5 µm (Secor et al., 2013 *in preparation*)

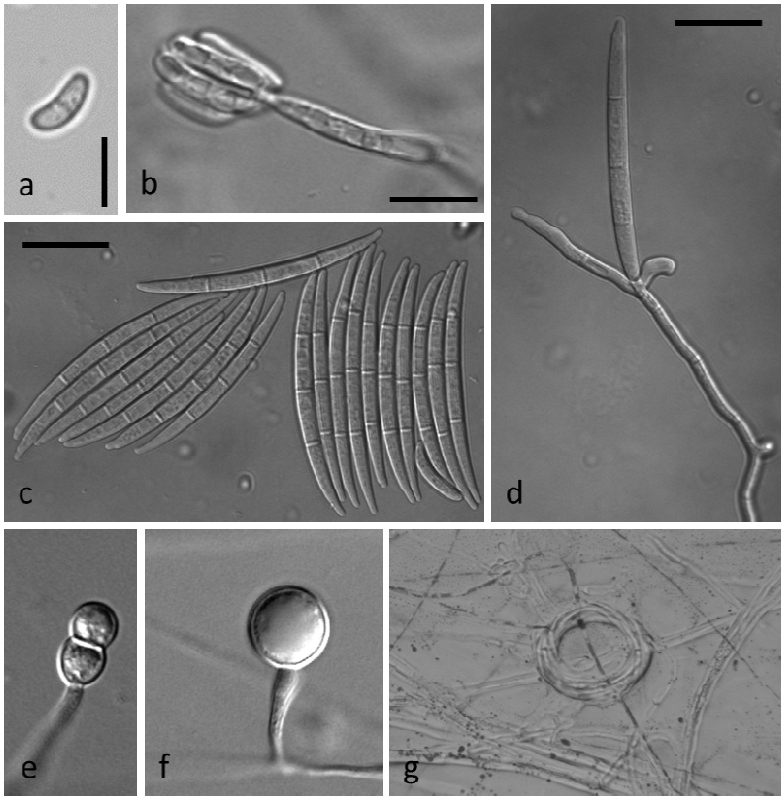

Figure 4. *Fusarium novum* conidia. Microconidia (a-b =10 micros), macroconidia (c-d =20 microns), chlamydospores (e-f) and coiled hyphae (g).

Disease Management

Management of soil-borne pathogen such as *F. oxysporum* and other soilborne *Fusarium* spp. has been mainly dependent on variety resistance, because cultural practices such as crop rotation and fungicides have had limited success. Fungicides to control Fusarium yellows disease have only been tested *in vitro* (Burlakoti, 2007; Burlakoti et al., 2010), and field studies are necessary to determine field efficacy. Soil fumigation does not appear to reduce soil populations of *Fusarium* (Sinclair et al., 1975). Generally for *Fusarium*

spp. crop rotation is unreliable because of alternate hosts which increase inoculum and inoculum longevity in the soil.

Although the pathogen associated with Fusarium yellowing decline on sugarbeet has been successfully characterized as a new species in the genus *Fusarium*, little information is available about alternate hosts that can be a reservoir of inoculum. The identification of alternate hosts may have significant implications for disease management strategies based on inoculum reduction, particularly by crop rotation. Understanding the range of symptomatic and asymptomatic plants able to support the survival and reproduction of the novel *Fusarium* species is critical to managing this disease.

The most effective and practical disease management strategy for soil-borne pathogens such as *Fusarium*, and potentially *F. novum*, is tolerant or resistant cultivars (Burlakoti, 2007). In sugarbeet most of the resistance screening studies have been directed at *F. oxysporum* and towards *F. novum* only in recent years. Resistance to Fusarium yellowing decline has been scored in the foliage of sugarbeet cultivars in the Red River Valley region of Minnesota and North Dakota, but root resistance has not been documented (Niehaus, 2011). Continuing work will be necessary to produce new sugarbeet varieties with good resistance to *F. novum*.

OBJECTIVES

General Objective

The overall objective of this study was to determine the host range of *F. novum*, in order to understand the biology and possible implications on crop production system and disease management.

Specific Objectives

1. Determine optimum conditions for growth of *F. novum*
    - ✓ Determine *in vitro* the optimum temperature for growth of *F. novum* in growth chambers
    - ✓ Evaluate the effects of wounding on infection of sugarbeet in greenhouse trials.
2. Determine the host range of *F. novum* from crops commonly grown in rotation with sugarbeet as well as weeds commonly found in sugarbeet fields by field and greenhouse trials.

MATERIALS AND METHODS

Locations

This research was carried out at North Dakota State University in Fargo, North Dakota, between 2012 and 2013. Temperature assessment, molecular characterization, and pathogenicity studies were conducted on the research laboratories of the Department of Plant Pathology and AES-Research Greenhouse Complex. Field experiments were conducted at American Crystal Sugar Company *Fusarium* screening nursery near Moorhead, Minnesota.

Isolates of *Fusarium* Used in this Study

The isolates used for this research were *F. novum* 670-10, 670-48 and 757-1, and *F. oxysporum* 742-23 (Table 1). All were single spore isolates from the collection of Dr. Gary Secor, NDSU, which originated from symptomatic sugarbeet collected in the American Crystal Sugar Company *Fusarium* screening nursery in Moorhead, MN, in 2005 and 2006. Identity was confirmed by morphological characteristics and RFLP patterns of the internal transcribed spacer (ITS) regions of the rDNA (Rivera et al., 2008). These isolates were selected for this study based on aggressiveness showed in previous pathogenicity assays. Isolate 670-10 is the holotype for *F. novum* which has been designated *Fusarium secorum* nom. prov. and reported in the US National Fungus Collection with the BPI number 892692: *Fusarium secorum* 2006, USA NRRL 62593. Because *F. secorum* acceptance is provisional, *F. novum* will be used in this thesis.

Table 1. *Fusarium* species isolates used in this study.

Isolate	*Fusarium* Species	Origin	Year collected
670-10	*F. novum*	Moorhead, MN	2005
670-48	*F. novum*	Moorhead, MN	2005
742-23	*F. oxysporum*	Moorhead, MN	2006
757-1	*F. novum*	Moorhead, MN	2006

Determining Optimum Conditions for Growth of *F. novum*

Temperature Evaluation

Single spore *F. novum* isolates 670-10, 670-48, and 757-1 and *F. oxysporum* isolate 742-23 were prepared and used to evaluate optimum temperature for growth. Each isolate was grown on three plates containing PDA medium (Appendix A) and incubated under continuous light at room temperature for seven days. After incubation, each replicate plate was processed for each isolate by removing 3 mm diameter discs, at 10 mm from the colony edge at two day intervals. Discs were placed at the center of a 10 cm diameter plastic petri dish (BD Falcon ™, San Jose, California) containing PDA medium. Petri dishes were incubated in growth chambers (Percival, Perry, IA) at seven different temperatures (5, 10, 15, 20, 25, 30, and 35±1°C) in the dark. The temperatures were evaluated by two separated experiments, first experiment evaluated temperatures range from 5 to 25°C and second experiment temperatures from 15 to 35°C. Both experiments were conducted in a randomized complete block design (RCBD) with three replicates, and three petri plates per treatment. Colony diameters were recorded at two day intervals for each replicate at all temperatures for nine days. Each colony was measured four times and then discarded. An analysis of variance was conducted to establish temperature and isolate effects on fungal growth using SAS program version 9.3 (Statistical Analysis System, Cary, NC). The rate of

growth for each isolate, dy/dt, was calculated for each temperature as the product between the colony area at the end of the experiment and the number of days needed to reach the area (cm^2 d^{-1}). A regression equation was developed for each isolate to describe the effects of temperature on fungal growth using Excel program (Microsoft Excel 2010). The minimum temperature (T_{min}) and optimum temperature (T_{opt}) required for fungal growth were estimated from the quadratic-order polynomial temperature-response function per isolate.

Inoculation Methods

Three greenhouse inoculation methods were compared for infection efficacy using *F. novum* isolates 670-10, 670-48, and 757-1, and *F. oxysporum* 742-23. Sugarbeet seeds of the *Fusarium* susceptible variety 4010RR (Syngenta Seeds, Minnetonka, MN) were used for these trials. For all experiments, seeds were sown in cylindrical plastic pots, 20 cm diameter pots (National Polymers Inc., Lakeville, MN), filled with growing medium Sunshine® Mix 1 (Sun Gro Horticulture, Bellevue, WA). Fertilization was performed with 10 g of Osmocote 15-9-12 (Scotts-Sierra Horticultural Products Co., Marysville, OH) per pot. Each experiment was conducted twice in a randomized complete block design (RCBD) with five replicates, each with five plants. Plants were maintained into the greenhouse at a constant temperature room of 24°C supplemented with 16 h light and at 16°C with 8 h without supplemental light.

Inoculation method 1: pre-plant infested soil

Sugarbeet seeds were sown in Sunshine® Mix 1 growing medium infested with *F. novum* inoculum prior to planting. Inoculum to infest growing medium was prepared for each isolate by mixing mycelium scraped from a sporulating 10-day-old culture with 200 g of a sterile mix of sand (80%) and corn meal (20%) (Quaker Oats Company ©, Chicago, IL) previously moistened with 150 mL of distilled water. This inoculum mix was incubated under

continuous light at room temperature for ten days. The fungal concentration of sand/corn meal was calculated by the MPN (Most Probable Number) serial dilutions method (Fernández et al., 2006) using water agar medium, and was adjusted with sterile sand/corn meal mix to $7x10^4$ colony-forming units (CFU).

Previously moistened Sunshine® Mix 1 was infested with 25 g of inoculum in each pot, which was lightly mixed to a depth of 1 cm. For negative control treatment, sterile sand/corn meal mix alone was used as inoculum. Sugarbeet seeds cv. 4010RR were placed on infested Sunshine® Mix 1 surface, covered with 100 g of non-infested Sunshine® Mix 1 and gently moistened. Pots were covered with plastic film during 10 days to maintain humidity into the growing medium. Plants were grown for four weeks. The experiment was conducted twice.

Inoculation method 2: post-emergence inoculation without injury

Five weeks old (4-leaf stage) greenhouse grown sugarbeet plants cv. 4010RR, were inoculated with a *F. novum* spore suspension at $4x10^4$ conidia mL^{-1} from 10-day-old cultures grown under continuous light at room temperature. Inoculation was performed by applying 2 mL of inoculum directly onto the crown of the plants using a pipette. For negative control treatment, distilled water was used as inoculum. Plants were grown for four months. Re-isolations from plant roots were conducted at the end of the experiment on PDA-medium to confirm infection by *F. novum* and detect symptomless infections. The experiment was conducted twice.

Inoculation method 3: root-dip of injured seedlings

Inoculation was done based on the procedure of Hanson and Hill (2004). Briefly, 5-week-old plants (4-leaf stage) were removed from pots by pulling them from the soil to produce wounds in the roots. Growing mix residues were brushed from the roots by hand.

Roots were washed with distilled water, and soaked in a conidial suspension from 10-days-old cultures of *F. novum* at $4x10^4$ conidia mL^{-1} for 8 min under constant agitation. Distilled water was used as inoculum for the negative control treatment. Plants were quickly re-planted in clean and moistened growing mix, and gently sprayed with water to minimizer transplanting shock. Plants were grown for 4.5 months. Re-isolations were conducted at the end of the experiment on PDA-medium to confirm infection by *F. novum* and to detect asymptomatic infections. The experiment was conducted twice.

Data analysis of infection methods

Disease severity for pre-plant infested soil method was determined by counting the emergence and death of seedlings. Data were recorded every two days after germination for three weeks. Results were analyzed by ANOVA with the GLM procedure in SAS program.

For post-emergence and root-dip inoculation methods, disease severity was evaluated by weekly assessment of all plants for foliar symptoms. Assessment began two weeks after inoculation for post-emergence method and five weeks after inoculation for root-dip method, and continued for nine weeks. Disease severity was rated using the rating system of Burlakoti (2007) for Fusarium yellows disease in percentage, where: 0= healthy plants, 1= plants stunted and wilted with few yellowing leaves (1-24%), 2= chlorosis and necrosis of the leaves (25-59%), 3= crown becoming dried and brown to black in color, leaves dying (60-89%), and 4= death of entire plant (90-100%).

Disease severities rating in percentage were used to calculate area under disease progress curve (AUDPC) of each isolate inoculated as:

$$\text{AUDPC} = \sum_{i=1}^{n-1} \left[\frac{X_i + X_{i+1}}{2}\right](t_{i+1} - ti) \qquad \text{(Shaner and Finney, 1977)}$$

where X_i = Disease severity in percentage at the i^{th} observation, t_i = time (weeks between ratings) at the i^{th} observation, and n = total number of observations. Levene's test for homogeneity of variances was conducted to determine if data from the two trials could be combined for analysis. Data was evaluated by analysis of variance using SAS macro program version 9.3 (Statistical Analysis System, Cary, NC).

Host Range Study

Crop plants grown in rotation with sugarbeet, and weeds commonly found in sugarbeet fields were tested for susceptibility to *F. novum* in field and greenhouse studies.

Host Range Plants Tested

Fifteen common crops and weeds were tested in field and greenhouse (Table 2) for susceptibility to *F. novum*. Seeds of the different plant species were not treated with a fungicide, insecticide, or other compound prior to planting.

Table 2. Crops and weeds tested for susceptibility to *F. novum*.

Crops	Scientific name	Variety	
		Field	Greenhouse
Dry bean	*Phaseolus vulgaris* L.	Pinto (147)	Pinto (147)
Potato	*Solanum tuberosum* L.	Red La Soda	Russet Burbank
Wheat	*Triticum aestivum* L.	Select	Select
Barley	*Hordeum vulgare* L.	Tradition	Tradition
Corn	*Zea mays* L.	DKC 35-43VT3	DKC 35-43VT3
Soybean	*Glycine max* L.	Peterson 12RR05	Peterson 12RR05
Canola	*Brassica napus* L.	InVigor 8440	InVigor 8440
Sunflower	*Helianthus annuus* L.	Mycogen 8N270	Mycogen 8N270
Flax	*Linum usitatissimum* L.	Omega	Omega
Oat	*Avena sativa* L.	Souris	Souris
Sugarbeet	*Beta vulgaris* L.	4010RR	4195RR
Redroot pigweed	*Amaranthus retroflexus* L.	Unspecified	Unspecified
Lambsquarters	*Chenopodium album* L.	Unspecified	Unspecified
Wild oat	*Avena fatua* L.	Unspecified	Unspecified
Green foxtail	*Setaria viridis* L.	Unspecified	Unspecified

Field Trial

A preliminary trial was conducted at two American Crystal Sugar Company *Fusarium* screening nursery sites in 2011, but was partially lost due to flooding. The trial of crops and weeds was repeated in the American Crystal Sugar Company's *Fusarium* screening nursery, a field localized in Moorhead, MN, in 2012. The site is naturally infested with *F. novum* based on previous trials (Secor unpublished). The experiment was conducted in a randomized complete block design (RCBD). A total of eleven treatments (crops and weeds) organized in four replicates were planted in the field. Each treatment (plot) was 3.3 x 7.5 m. Crops were planted individually in each plot, while weeds (redroot pigweed,

lambsquaters, wild oat, and green foxtail) were planted together in the same plot. Separation between plots and repetitions was approximately 1 m and 7.5 m respectively. Crop plants and weeds were evaluated every two weeks for foliar symptoms for a total of four times after emergence. A total of five plants were collected randomly per treatment in each repetition at each observation date (20 plants x four times = 80 total plants/species). The lower stem was separated from whole plant and thoroughly washed with tap water to remove soil, disinfected by immersion in 10% sodium hypochlorite (Clorox®, Pleasanton, CA) plus 0.06% Tween 20 (Agdia, San Marcos, CA) for eight minutes and rinsed with deionized water twice. A single small subsample was removed from each lower stem piece and placed on PDA to determine the presence of *F. novum*.

Molecular confirmation of *Fusarium* spp. isolates from field trial

Eleven *Fusarium* isolates from dry bean, corn, wheat, canola, soybean plants and 42 sugarbeet plants with yellowing decline were used to determine the identity of *Fusarium* in the field trial in 2012. Approximately 0.5 g of mycelial tissue was obtained by filtering 5-day-old cultures grown on half strength PDB-medium. DNA was extracted from each culture using cetyltrimethylammonium bromide (CTAB) procedure with modifications (Nicholson et al., 1997). Briefly, mycelium was disrupted in a Fast Prep Machine (FP120, Bio101, Thermo Electron Corporation, Carlsbad, CA) for 4.5 sec at 6.5 m/sec of speed. Samples were centrifuged and incubated at 60°C after addition of β-mercaptoethanol. Phenol/chloroform/isoamyl alcohol mixture (25:24:1) and chloroform:isoamyl alcohol mixture (24:1) were used to denaturalize proteins and lipids. Precipitation of DNA was performed with isopropanol and washed with 70% ethanol. DNA was finally resuspended in 50 µL of sterile distilled water (Appendix C).

For these isolates the ITS region was amplified using the primers ITS-1F and ITS-4 designed by Gardes and Bruns (1993) and White et al. (1990) respectively, cited by

Harrington et al. (2000) (Table 3). PCR amplifications were carried out in 25 µL reactions containing 30 ng of genomic DNA. A total of 22 µl master mix, which contained 1X PCR Gold Buffer (Applied Biosystems, Foster City, CA), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of primer, and 0.02 unit of AmpliTaq Gold ® DNA polymerase (Applied Biosystems, Foster City, CA) were added to the reaction mixture.

Table 3. Nucleotide sequences of primers used for internal transcribed spacer region (ITS) amplification.

Primer	Sequence 5' to 3'
ITS4	TCCTCCGCTTATTGATATGC
ITS1F	CTTGGTCATTTAGAGGAAGTAA

Reactions were amplified in a PTC-200 thermocycler (MJ. Research, Inc., Watertown, MA) through a predenaturation at 95°C for 10 min, denaturation at 95°C for 30 sec, 40 sec at 52°C (Annealing), 72°C for 2 min (elongation), followed by 34 cycles since denaturation, and a final elongation during 10 min at 72°C. As a control, sterile water was used instead of fungal DNA in order to test for primer dimerization and contamination. To confirm DNA amplification, PCR products were separated by electrophoresis on 1% agarose gel stained with GelRed (Biotium, Hayward, CA), and visualized using the UV AlphaImager® HP System (ProteinSimple, Santa Clara, CA).

The RFLP-ITS characterization was performed with the restriction enzyme *Hha*I (New England Biolabs, Beverly, MA) in 20 µL reactions. PCR-amplified products (10 µL) were digested with 0.5 units of *Hha*I in the presence of 1X BSA (New England Biolabs, Beverly, MA), 1X NE Buffer No. 2 (New England Biolabs, Beverly, MA), and 0.365X TE buffer (10 mM Tris-HCl pH 7.6 and 0.1 mM EDTA). Digestions were performed for 12 hours at 37 °C. Restriction patterns of the ITS amplification products were obtained in 5% acrylamide gels

stained with ethidium bromide (Appendix D) and digitalized. RFLP profiles were analyzed to identify polymorphisms specific to *Fusarium novum*.

Data analysis

Sugarbeet disease severity was evaluated by rating the foliar symptoms according to Burlakoti (2007) in percentage. Data were fitted to the monomolecular model using the linearized equation:

$$\ln [1/(1-y)] = \ln [1/(1-y_0)] + r_M t \qquad \text{(Campbell and Madden, 1990)}$$

where 1 = Maximum disease severity, y = Observed disease severity, y_0 = Epidemic at disease onset, r_M = Rate of change for the monomolecular model, and t = Time in weeks after inoculation. Data were evaluated using SAS macro program version 9.3 (Statistical Analysis System, Cary, NC).

Greenhouse Trial

In this trial, seeds were sown in 12.5 x 25 cm plastic liner box pots (Belden Plastics, St. Paul, MN), filled with Sunshine® Mix 1 (Sun Gro Horticulture, Bellevue, WA). Fertilization was performed with 20 g of Osmocote 15-9-12 (Scotts-Sierra Horticultural Products Co., Marysville, OH) per pot. Plants were kept in a constant temperature room at 24°C (16 h light) and 16°C (8 h darkness).

Greenhouse host range testing of crops and mix of weeds was conducted using the root inoculation procedure with injury, because it caused the most severe and consistent disease of sugarbeet in preliminary trials. For this trial, *Fusarium novum* isolate 670-10 was used as inoculum. A parallel experiment was conducted, using distilled water as inoculum. The water only treatment was conducted as a negative control, in order to evaluate any yellowing due to plant stress during the inoculation process.

Inoculum was prepared by washing 10-day-old fungal colonies with 1mL of sterile distilled water containing 0.06% Tween 20 and 0.02% Ampicillin (Sigma-Aldrich Co., St. Louis, MO). Plates were gently scraped with a sterile glass rod and the resulting spore suspension was filtered through a 150 mesh 6.0 cm diameter stainless steel sieve to remove the hyphal fragments. Spore concentration was counted with haemocytometer and adjusted to $4x10^4$ conidia mL^{-1}.

Plants were inoculated when the second true leaf was unfolded at the second node. Root inoculation was performed as previously mentioned by removing plants from the pots, washing the roots with distilled water, soaking in the inoculum for eight minutes, and replanting in the original pot.

The experiment was conducted twice in randomized complete block design (RCBD). Twelve treatments (eleven crops plus a mix of weeds) of eight plants each/replicate were organized in five replicates. Susceptibility was evaluated by monitoring all plants for foliar symptoms weekly for four weeks. Lower stems were separated from whole plant and thoroughly washed with tap water to remove soil. Main lower stem samples were disinfected by immersion in 10% sodium hypochlorite (Clorox®, Pleasanton, CA) plus 0.06% Tween 20 (Agdia, San Marcos, CA) for 8 min and rinsed with deionized water twice. Isolates from all plants were done on PDA-medium to confirm infection by *Fusarium novum* and detect root infections without foliar symptom expression.

Data analysis

Sugarbeet disease severity was evaluated by rating the foliar symptoms according to Burlakoti (2007) in percentage. Levene's test for homogeneity of variances was conducted to determine if data from the two trials could be combined for analysis. Data were fitted to the monomolecular model using the linearized equation:

$$\ln [\ 1/(1-y)] = \ln [1/(1-y_0)] + r_M t \qquad \text{(Campbell and Madden, 1990)}$$

where 1 = Maximum disease severity, y = Observed disease severity, y_0 = Epidemic at disease onset, r_M = Rate of change for the monomolecular model, and t = Time in weeks after inoculation. Data were evaluated using SAS macro program version 9.3 (Statistical Analysis System, Cary, NC).

RESULTS

Determining Optimum Conditions for Growth of *F. novum*

Growth Temperature Evaluation

The analysis of variance (Table 4) showed highly significant differences on fungal growth (cm^2) among the main effect of isolates and temperatures. After nine days of incubation, the *F. oxysporum* isolate showed significantly faster growth and higher growth rate compared with *F. novum* isolates, but growth rate among the three *F. novum* isolates was not different (Table 5).

Table 4. Mean squares for fungal growth as influenced by temperature.

Sources of variation	d.f.	Fungal growth	F value	Pr > F
Temp	6	3115	134.69	<.0001*
Isolate	3	130	5.63	0.002*
Isolate Temp	18	33	1.45	0.1483
Rep	2	293	12.7	<.0001*
Residual	54	23		

* Significant at $P<0.05$

Table 5. Fungal mean growth rate after nine days of incubation for four *Fusarium* isolates.

Isolate	Species	Mean growth rate (cm^2 day^{-1})	Letter group
670-10	*F. novum*	1.35	B
670-48	*F. novum*	1.36	B
757-1	*F. novum*	1.41	B
742-23	*F. oxysporum*	1.92	A

* Significant at $P<0.05$

Fungal growth rates of each isolate per temperature were used to determine the trend lines that best fitted the data. The predicted growth equation and r^2 value were established for each isolate (Figure 5).Correlation values fluctuated between 0.48 and 0.51 for *F. novum* isolates and 0.63 for *F. oxysporum*. The quadratic-order polynomial regressions allowed establishing the minimum and optimum temperature required by each isolate to grow. The minimum temperature established were 7.8 to 7.9°C for *F. novum* isolates and 7.3°C for *F. oxysporum*. Optimum growth temperature required were 24±0.1°C and 22.5°C for *F. novum* isolates and *F. oxysporum* respectively.

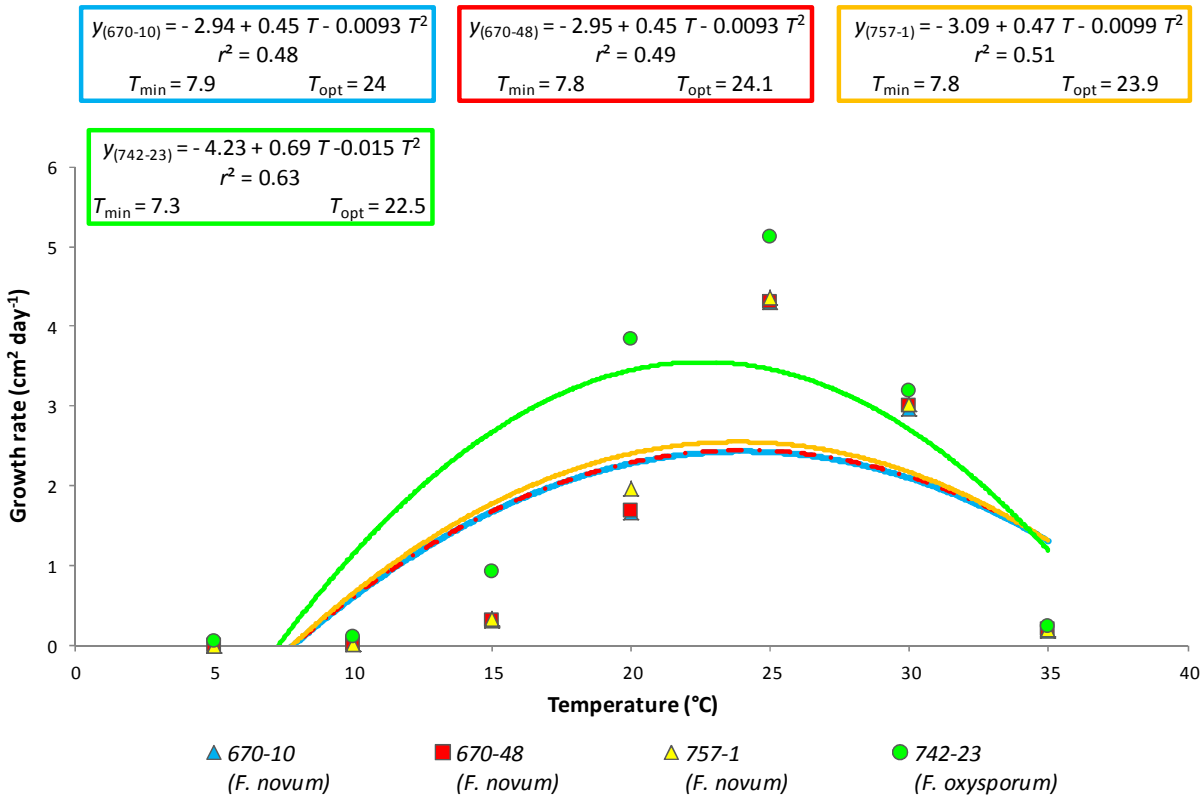

Figure 5. Estimated values for growth rate on a temperature gradient, predicted regression curves of quadratic-order polynomial regression equations, r^2 values, and minimum and optimum temperatures of *Fusarium* isolates.

Inoculation Methods

Inoculation method 1: pre-plant infested soil

Levene's test for homogeneity of variances was performed before comparing the data from the trials of the pre-plant infested soil method. Variances among trials did not show significant differences (Table 6), so data from both trials was combined for analysis. Significant differences were observed in disease severity, expressed as percentage of dead plants, among isolates, trials, and for the interaction trial x isolate (Table 7). Percentage of dead plants least square means of the interaction trial x isolate ranged from 86.7 to 8.5 in trial 1 and from 82.5 to 4.4 in trial 2 (Figure 6). In addition, mean disease for the isolates showed that *F. novum* isolates were different from *F. oxysporum* at $P< 0.05$ (Figure 7).

Table 6. Levene's test of homogeneity of variances for percentage of dead sugarbeet plants due to inoculation with the pre-plant infested soil method.

Source	DF	Mean Square	F value	Pr > F
Trial	1	318284	0.21	0.6436^{NS}
Error	448	1484877		

^{NS} Non Significant at $P<0.05$

Table 7. Sources of variation, degrees of freedom, and mean squares for percentage of dead sugarbeet plants due to inoculation with the pre-plant infested soil method.

Source	DF	Mean Square	F value	Pr > F
Trial	1	2923	7.33	0.0071*
Rep (Trial)	8	686	1.72	0.0913
Isolate	4	130669	327.47	<.0001*
Trial x Isolate	4	4761	11.93	<.0001*

* Significant at $P<0.05$
Coefficient of variation = 51.91
R-Square = 0.76

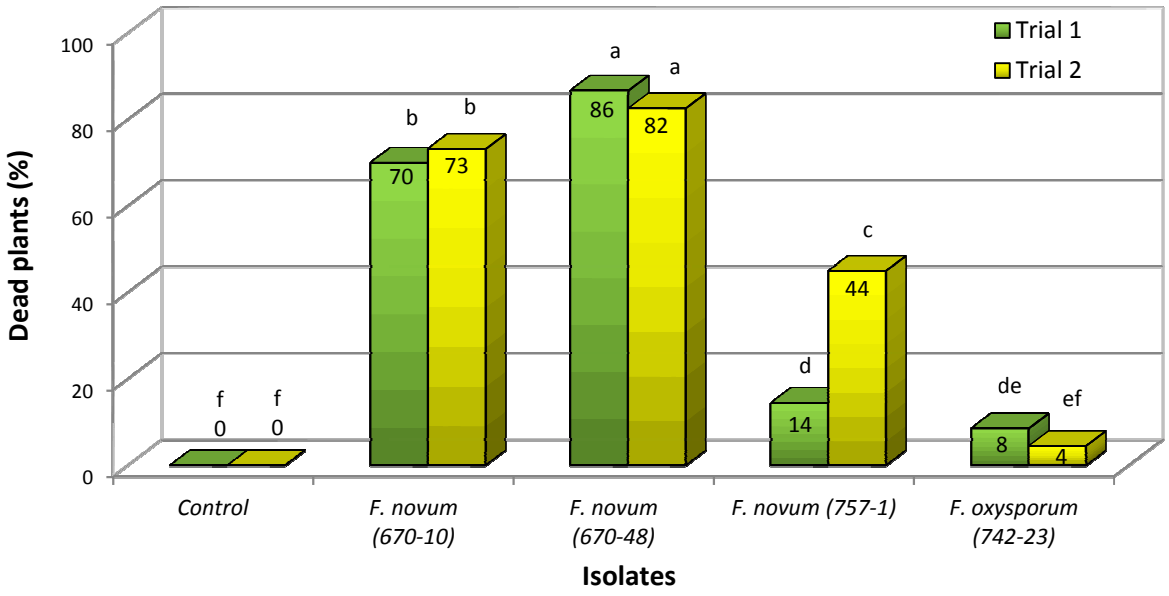

Figure 6. Percentage of dead sugarbeet plants for the interaction trial per isolate in the pre-plant infested soil method. Means followed by the same letter are not significantly different at $P<0.05$.

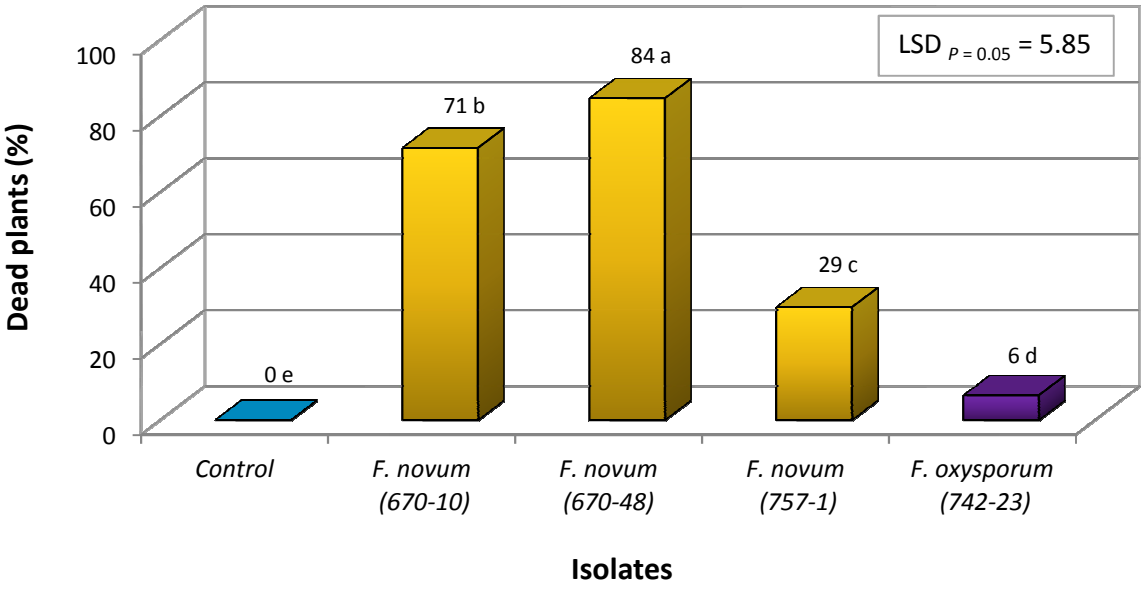

Figure 7. Mean percentage of dead plants due to *Fusarium* isolates in the pre-plant infested soil method. Means followed by the same letter are not significantly different at $P<0.05$.

Inoculation method 2: post-emergence inoculation

Levene's test for homogeneity of variances was performed before comparing the data from the trials of the post-emergence inoculation method. Variances among trials had significant differences (Table 8). However, observed differences were due to magnitude which means the mean AUDPC was higher in one trial compared with the other, mainly due to experimental error such as variations in greenhouse temperature or water supplied (Table 9). Thus, the data from both trials was combined for further analysis (Table 10). Analysis of variances showed significant differences in disease severity, expressed by area under disease progress curve, for the interaction trial x isolate, which is due to the observed differences in magnitude between trials. AUDPC least square means of the interaction trial x isolate ranged from 379.5 to 43.2 in trial 1 and from 207.8 to 81.5 in trial 2. In addition, mean disease AUDPC for the isolates showed that *F. novum* isolates were different from *F. oxysporum* at $P< 0.05$ (Figure 8).

Table 8. Levene's test of homogeneity of variances for AUDPC of sugarbeet plants inoculated with the post-emergence inoculation method.

Source	DF	Mean Square	F value	Pr > F
Trial	1	7.69×10^{9} *	18.07	<.0001*
Error	48	4.25×10^{8}		

* Significant at $P<0.05$

Table 9. Mean AUDPC per trial for post-emergence inoculation method.

Trial	N	AUDPC	
		Mean	Std Dev
1	25	203.59	183
2	25	127.93	87

Table 10. Sources of variation, degrees of freedom, and mean squares for AUDPC of sugarbeet plants inoculated by post-emergence inoculation method.

Source	DF	Mean Square	F value	Pr > F
Trial	1	71574	15.39	0.0004*
Rep (Trial)	8	5507	1.18	0.3387
Isolate	4	175025	37.64	<.0001*
Trial x Isolate	4	23739	5.11	0.0027*

* Significant at $P<0.05$
Coefficient of variation = 41.13
R-Square = 0.85

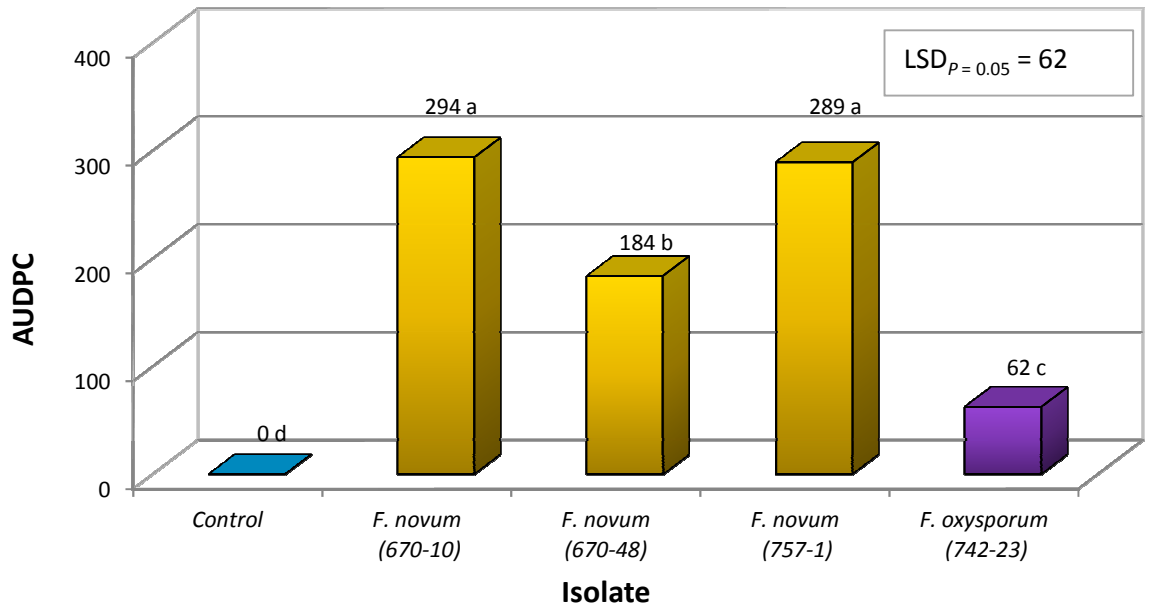

Figure 8. Mean AUDPC for *Fusarium* isolates after post-emergence inoculation. Means followed by the same letter are not significantly different at $P<0.05$.

Inoculation method 3: root dip of injured seedlings

Levene's test for homogeneity of variances was performed before comparing both trials with the root dip of injured seedlings method. Variances among trials had significant

differences (Table 11). However, observed differences were due to magnitude which means the mean AUDPC was higher in one trial compare with the other, mainly due to experimental error such as variations in greenhouse temperature or water supplied (Table 12). Thus, the data from both trials was combined for further analysis (Table 13). Analysis of variances showed significant differences in disease severity, expressed by area under disease progress curve, for the interaction trial x isolate, which is due to the observed differences in magnitude between trials. AUDPC least square means of the interaction trial x isolate ranged from 459.8 to 137.3 in trial 1 and from 307 to 112.1 in trial 2. In addition, mean disease AUDPC for the isolates showed that *F. novum* isolates were different from *F. oxysporum* at $P< 0.05$ (Figure 9).

Table 11. Levene's test of homogeneity of variances for AUDPC of injured sugarbeet plants inoculated with the root-dip method.

Source	DF	Mean Square	F value	Pr > F
Trial	1	$7.84x10^{9}$ *	10.54	0.0021*
Error	48	$7.44x10^{8}$		

* Significant at $P<0.05$

Table 12. Mean AUDPC per trial of injured sugarbeet plants inoculated with the root-dip method.

Trial	N	AUDPC	
		Mean	Std Dev
1	25	295.53	204
2	25	185.16	124

Table 13. Sources of variation, degrees of freedom, and mean squares for AUDPC of injured sugarbeet plants inoculated by root-dip method.

Source	DF	Mean Square	F value	Pr > F
Trial	1	152288	35.75	<.0001*
Rep (Trial)	8	1674	0.39	0.9162
Isolate	4	284968	66.89	<.0001*
Trial x Isolate	4	21365.05	5.02	0.0030*

* Significant at *P*<0.05
Coefficient of variation = 27.16
R-Square = 0.91

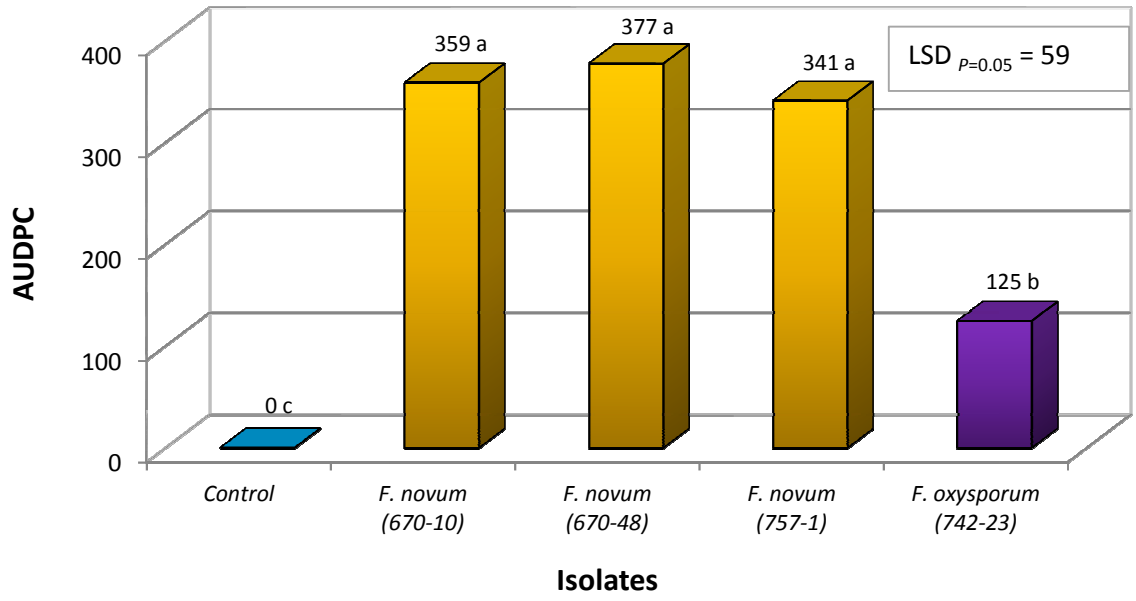

Figure 9. Mean AUDPC for *Fusarium* inoculated by root-dip method. Means followed by the same letter are not significantly different at *P*<0.05.

Host Range Study

Field Trial

Yellowing symptoms were observed in sugarbeet plants two weeks after emergence, which increased over time killing the plants in the field (Figure 10). Epidemic on sugarbeet fitted the monomolecular model with an r^2 = 0.97, MSE = 0.00506, and r_M = 0.113 (Figure 11). Disease symptoms were not observed in any other crop or weed (Figure 12). A total of 53 *Fusarium* isolates were recovered from the crops and weeds. These isolates were identified by morphology as *F. novum*, *F. oxysporum*, and other *Fusarium* species not pathogenic to sugarbeet (Figure 13).

Figure 10. (a) Sugarbeet plot infected with yellowing decline disease, (b) sugarbeet plant with yellowing and scorching of the leaves, (C) dead sugarbeet plant due to yellowing decline disease.

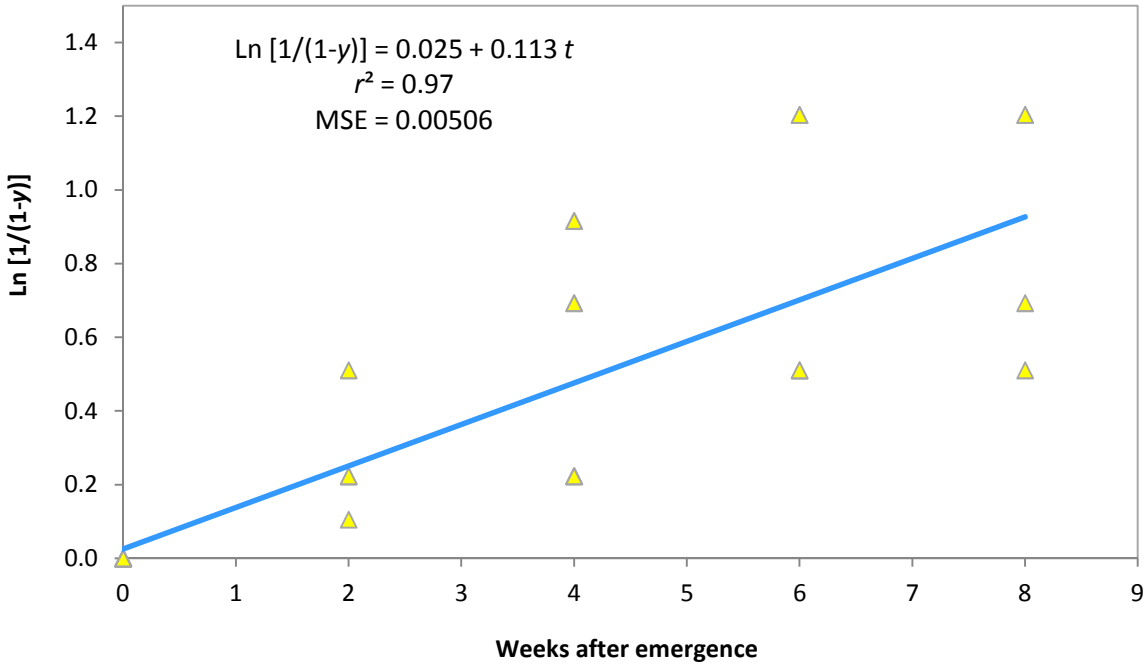

Figure 11. Yellowing decline disease severity on sugarbeet plants in the field over time.

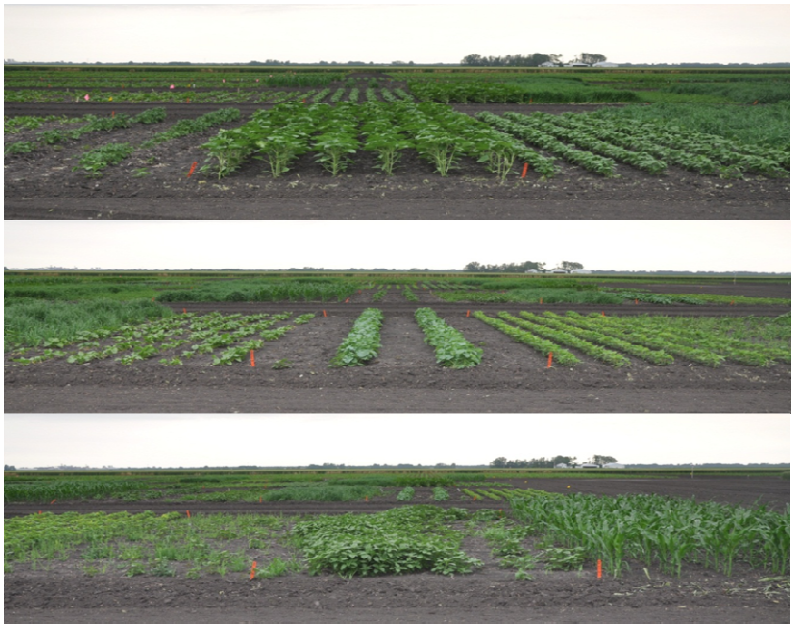

Figure 12. Crops and weeds tested for susceptibility to *F. novum* in the American Crystal Sugar Company's *Fusarium* screening nursery in Moorhead, MN.

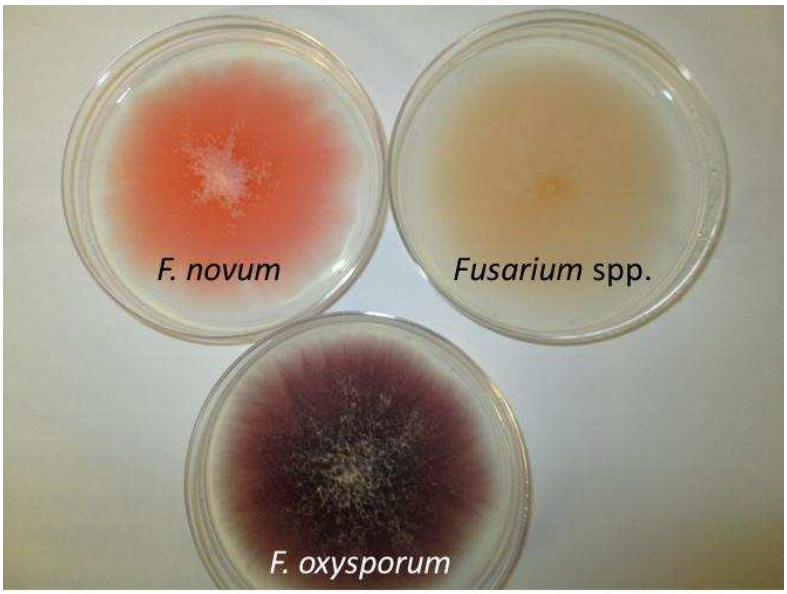

Figure 13. *Fusarium* spp. isolates recovered from crops and weeds in the field in 2012.

Molecular identification of the 53 *Fusarium* spp. isolates recovered from the field trial was confirmed through amplification of the ITS region with the primers ITS-1 and ITS-4 and RFLP analysis with the restriction enzyme *Hha*I. RFLP products visualized on 5% acrylamide gels, showed the characteristic ITS profile of *F. novum* for only one isolate recovered from canola and for all isolates from sugarbeet (Figure 14 and Table 14)

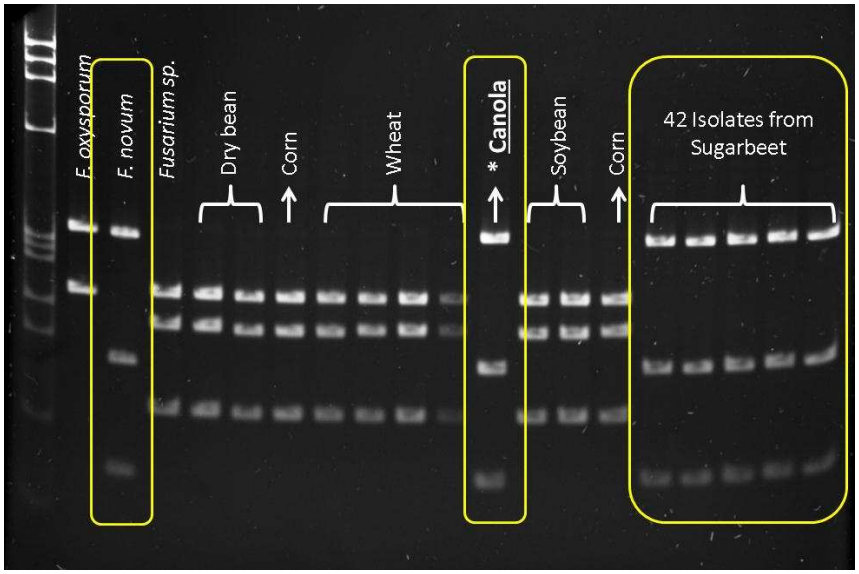

Figure 14. Restriction patterns of amplified DNA from the ITS region of *F. novum* isolates restricted with *Hha*I and visualized in 5% acrylamide gels.

Table 14. Host reaction to field infection by *F. novum* and recovery of *F. novum* from plant samples.

Crop	Scientific name	Host Reaction	# *F. novum* isolates recovered
Dry bean	*Phaseolus vulgaris* L.	A*	0
Potato	*Solanum tuberosum* L.	A	0
Wheat	*Triticum aestivum* L.	A	0
Corn	*Zea mays* L.	A	0
Soybean	*Glycine max* L.	A	0
Canola	*Brassica napus* L.	A	1
Sunflower	*Helianthus annuus* L.	A	0
Flax	*Linum usitatissimum* L.	A	0
Oat	*Avena sativa* L.	A	0
Sugarbeet	*Beta vulgaris* L.	S**	42
Red root pigweed	*Amaranthus retroflexus* L.	A	0
Lambsquarters	*Chenopodium album* L.	A	0
Wild oat	*Avena fatua* L.	A	0
Green foxtail	*Setaria viridis* L.	A	0

* Asymptomatic ** Symptomatic

Greenhouse Trial

Yellowing symptoms were observed in sugarbeet plants one week after inoculation, which increased over time killing the plants in the greenhouse (Figure 15). Levene's test for homogeneity of variances was performed before comparing the data from greenhouse trials. Variances among trials did not show significant differences (Table 15), so data from both trials was combined for analysis. Epidemic on sugarbeet fitted the monomolecular model with an r^2 = 0.89, MSE = 0.44572, and r_M = 1.015 (Figure 16). Disease symptoms were not observed in any other crop or weed. A total of 254 *F. novum* isolates, identified by morphology, were recovered from eleven plant species in both greenhouse trials. From those isolates, a total of 174 were recovered from nine asymptomatic crops and one asymptomatic weed (Table 16). All sugarbeet plants inoculated with *F. novum* in the trial were infected by *F. novum*, followed by canola (63%), sunflower (49%), dry bean (30%), flax (24%), potato (21%), soybean (20%), wheat (6%), oat (3%), corn and green foxtail (1%).

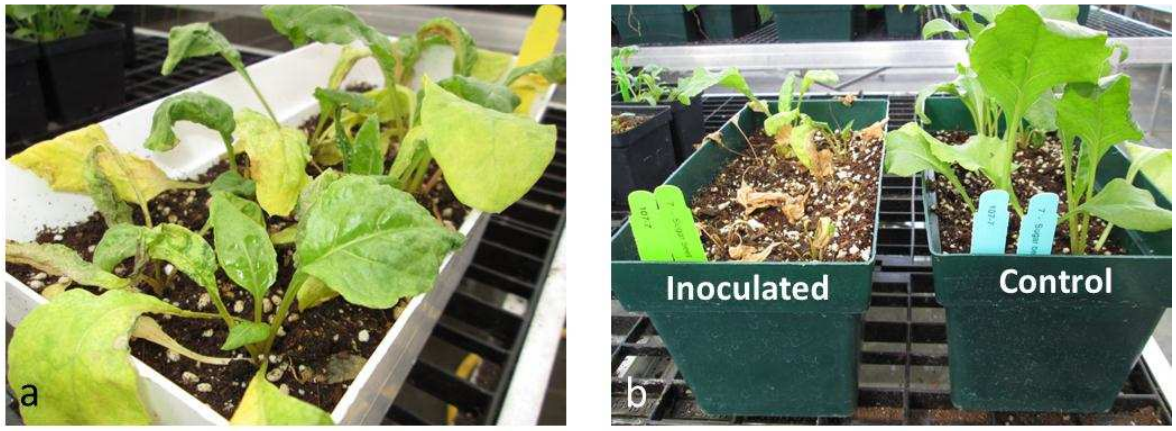

Figure 15. (a) Fusarium yellowing decline symptoms in sugarbeet plants inoculated with *F. novum* (isolate 670-10). (b) Comparison between sugarbeet inoculated with *F. novum* and negative control inoculated with distilled water.

Table 15. Levene's test of homogeneity of variances for percentage of dead sugarbeet plants due to inoculation with the pre-plant infested soil method.

Source	DF	Mean Square	F value	Pr > F
Trial	1	3516670	1.88	0.1743^{NS}
Error	78	1870898		

^{NS} Non Significant at $P \leq 0.05$

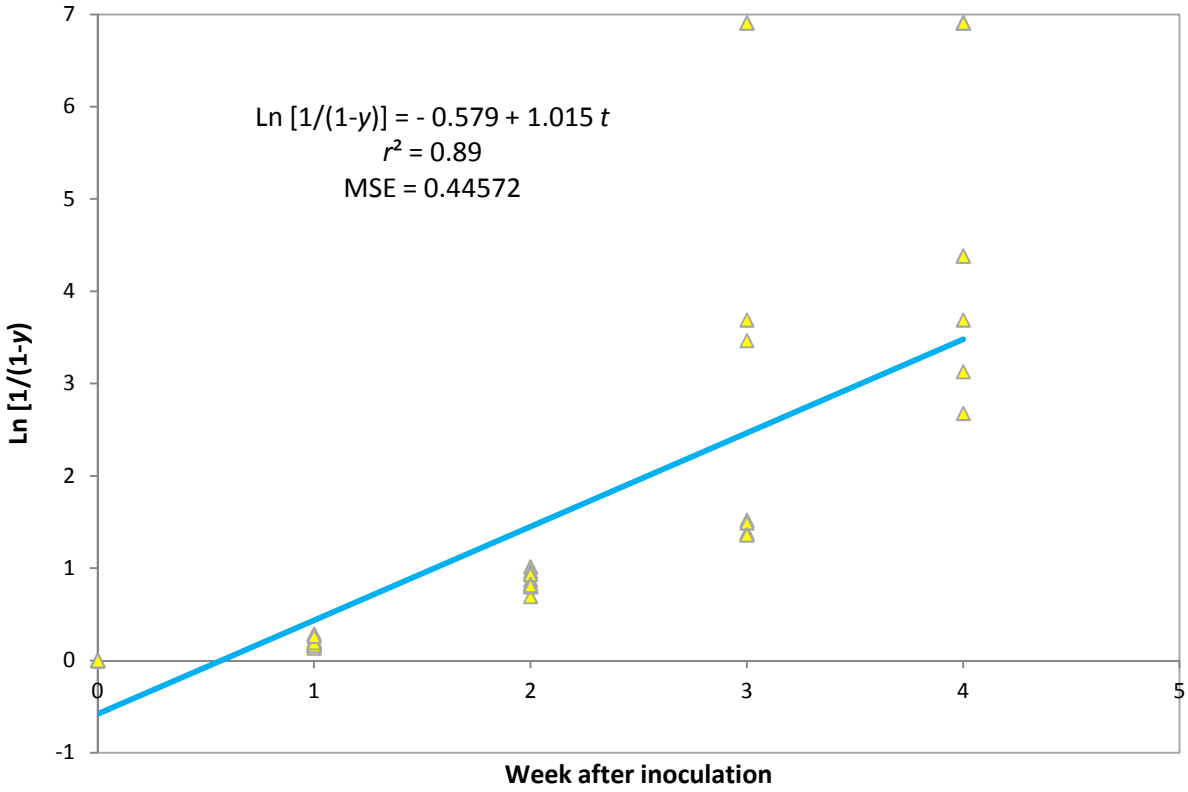

Figure 16. Yellowing decline disease severity on sugarbeet plants inoculated with *F. novum* isolate 670-10.

Table 16. Host reaction to *F. novum* and recovery of *F. novum* from plants in greenhouse inoculation trials.

Crop	Scientific name	Host Reaction	# *F. novum* isolates recovered	% *F. novum* isolates recovered
Dry bean	*Phaseolus vulgaris* L.	A*	24	30
Potato	*Solanum tuberosum* L.	A	17	21
Wheat	*Triticum aestivum* L.	A	5	6
Barley	*Hordeum vulgare* L.	A	0	0
Corn	*Zea mays* L.	A	1	1
Soybean	*Glycine max* L.	A	16	20
Canola	*Brassica napus* L.	A	50	63
Sunflower	*Helianthus annuus* L.	A	39	49
Flax	*Linum usitatissimum* L.	A	19	24
Oat	*Avena sativa* L.	A	2	3
Sugarbeet	*Beta vulgaris* L.	S**	80	100
Red root pigweed	*Amaranthus retroflexus* L.	A	0	0
Lambsquarters	*Chenopodium album* L.	A	0	0
Wild oat	*Avena fatua* L.	A	0	0
Green foxtail	*Setaria viridis* L.	A	1	1

* Asymptomatic ** Symptomatic

DISCUSSION

In 2005 a novel and distinct *Fusarium* species, referred as *F. novum*, was reported in Minnesota causing early season yellowing, seedling death and severe decline of sugarbeet plants (Rivera et al., 2008). The disease, Fusarium yellowing decline, caused by this new pathogen has subsequently been found in the sugar production of west central Minnesota. In 2013 this disease was surprisingly found for the first time in multiple fields in the Crookston factory district of north central MN. The mechanism of spread is unknown. In this study, we determined optimum infection conditions of sugarbeet root by *F. novum*, as well as its host range from crops commonly grown in rotation with sugarbeet and weeds commonly found in sugarbeet fields. The results show that (a) *F. novum* grows slower than *F. oxysporum* and its temperatures required to grow are minimum 7.8°C and optimum 24 ±0.1°C, (b) *F. novum* does not need wounds to penetrate, infect the host, and cause disease, and (c) canola appears to be an asymptomatic host for *F. novum*.

The first finding that *F. novum* grows much slower than *F. oxysporum* and requires 7.8°C and 24 ±0.1°C as temperatures minimal and optimal to grow respectively, is supported by the observations in growth chambers where the mean growth for *F. novum* was null at 5°C. Growth of *F. oxysporum* at 10°C was ten times higher than *F. novum*. The equation predicted that *F. novum* isolates (670-10, 670-48, and 757-1) have a maximum growth rate at 24 ±0.1°C, in contrast with the equation prediction for *F. oxysporum*, which at 22.5°C may reach its maximum growth rate.

Although the results in this experiment indicate that *F. oxysporum* grows at lower temperature and faster than *F. novum*, symptom expression of both diseases in the field differs in time. *Fusarium novum* symptoms are observed earlier in the field during the growing season than *F. oxysporum*. This suggests that *F. novum* may be more aggressive than *F. oxysporum*. Evidence of differences in aggressiveness between *F. novum* and *F.*

oxysporum were reported by Burlakoti (2007). This author evaluated the pathogenicity, virulence, and aggressiveness of both *Fusarium* species and found that *F. novum* was highly aggressive, inducing first disease symptoms 18 days after inoculation (DAI), and high disease severity expressed as AUDPC in 38 day-old sugarbeet plants. Sugarbeet in the Red River Valley is planted usually at the third or four week of May, when soil temperature is around 10 to 15°C and overwintering structures for both species have initiated the germination process on crop residues. In this scenario, sugarbeet seedlings are susceptible and do not have enough energy to defend itself against the pathogen. Thus, sugarbeet seedlings may be more susceptible to infection by *F. novum* because of its aggressiveness, which may explain why Fusarium yellowing decline is observed earlier in the season.

The second finding that *F. novum* does not need wounds to penetrate, infect the host, and cause disease is supported by the two first inoculation methods, infested soil and post-emergence inoculation, both without root injury. Four weeks after planting sugarbeet seeds in soil infested with *F. novum*, the percentage of dead plants for ranged from 29.6% to 84.6%, significantly higher than the percentage of dead plants for *F. oxysporum* which was 6.4%. The post-emergence inoculation method also showed that disease severity caused for *F. novum* was higher (AUDPC from183.6 to 293.7) compared with *F. oxysporum* (AUDPC = 62.4). The plant symptoms observed with these two non-injury methods are the same symptoms observed with the root dip inoculation of injured seedlings. However, the root dip inoculation method of injured seedlings showed the highest disease severity levels of the three methods (AUDPC = 124.7 to 377.3), and although disease severity on sugarbeet plants were higher with *F. novum* isolates (AUDPC = 340.9 to 377.3) than with *F. oxysporum* (AUDPC = 124.7), no differences could be observed between the three *F. novum* isolates.

Other studies have similarly shown that an injury is not needed for *Fusarium* infection of roots. Smith and Walker (1930) showed that *F. conglutinans* Wollenw enters the cabbage roots through the intercellular spaces of the root cap. van Peer and Schippers (1992) showed that natural infection of the carnation roots by *F. oxysporum* f. sp. *dianthi* may occur either by direct penetration of the epidermis or through wounds. The results obtained in this study not only suggest that *F. novum* does not need an injury to penetrate the sugarbeet roots and cause disease, but also show that injury presence in the host may increase the disease severity caused by *F. novum*. This finding is consistent with the results obtained by Estrada (2007) who reported that the primary dry rot pathogen *F. sambucinum* was able to produce disease in the non-injured-inoculated potatoes, but disease severity was higher in the injured-inoculated potatoes regardless of the injury size.

Thus, the results in this study suggest that injury facilitates the pathogen entry and infection of the host, which is especially an advantage when plant material is being tested for resistance. One limitation of this study is that the infested soil inoculation method was evaluated only at one concentration ($7x10^4$ conidia g^{-1}) and the seedlings died fast and the role of physiological development of the plants could not be evaluated. However, at $7x10^4$ conidia mL^{-1} the percentage of dead plants by *F. novum* was almost four times greater than by *F. oxysporum*, which suggests one more time that *F. novum* is more aggressive than *F. oxysporum*. Another possibility is that the fungal concentration used in the greenhouse is much higher than the real fungal concentration in the field in 2012, which also may explain why disease severity in the RRV is still low despite that *F. novum* is a new pathogen in the area.

The third finding that canola appears to be an asymptomatic host for *F. novum* is supported by the host range study in field and greenhouse. In both experiments, sugarbeet had high disease incidence, 57.5% in field and 98.4% in greenhouse, and *F. novum* was

consistently isolated from sugarbeet plants with symptoms of interveinal chlorosis, yellowing, stunting and scorching of the leaves, as well as vascular discoloration of the root. Among those symptoms, vascular discoloration of the petiole elements and early death of plants were observed, which are reported as distinct to Fusarium yellowing decline disease. In those trials, none of the other crops or weeds presented yellowing/scorching of the leaves, vascular discoloration/necrosis of the roots, stunting or any other symptom related with Fusarium yellowing decline. However, in the field only canola was found as an asymptomatic host to *F. novum*, as confirmed by both morphology and RFLP patterns of the ITS region. In addition, *F. novum* was isolated in greenhouse trials from one asymptomatic weed and nine asymptomatic crops besides sugarbeet, including canola, sunflower, and dry bean from which a high percentage of *F. novum* was isolated.

As in the present study, previous studies have found that some *Fusarium* species may infect other crops different than its primary host, without symptom expression. Kolander et al. (2012) reported that *F. virguliforme* O'Donnell & T. Aoki, causal agent of the Sudden Death Syndrome in soybean, was able to infect fifteen plant species other than soybean, and five species (corn, wheat, ryegrass, pigweed, and lambsquarters) did not show any disease symptoms after inoculate them at planting with a layer of infested sorghum seeds. In this study, however, host range plants were tested in a naturally infected field and artificially in the greenhouse using both high inoculum concentration and root injury. As a result, *F. novum* was isolated only from one canola plant in the field, but in greenhouse was isolated from 50 canola plants and also from other eight crop species and one weed. The discrepancy on the number of species that were infected by *F. novum* between field and greenhouse evaluations may be due to the inoculation method used in the greenhouse which involved root injury to guarantee pathogen penetration on the different crop plants. Thus, due to the magnitude of the injury it is suggested that some cultural practices (including mechanical weed control, irrigation systems, tillage operations, and

others) may help that *F. novum* expand its host range due to possible injuries performed during those practices, as showed by Estrada (2007) with *F. graminearum*.

So far, canola appears to be consistently a potential host to *F. novum*. This may have serious implications on sugarbeet production system and Fusarium yellowing decline management strategies, because canola may increase the amount of inoculum present in a field. In North Dakota, canola may be in rotation with sugarbeet, especially in the northeast region near to the Canadian border (Raymer, 2002; Nepal, 2013) where there is also substantial sugarbeet hectarage grown. Although the most effective disease management strategy for soil-borne pathogens such as *F. novum* is tolerant or resistant cultivars, in 2010 and 2012 this pathogen was isolated respectively from 67% (80/120) and 89% (82/92) of the cultivars in the American Crystal Fusarium resistance screening trial near Moorhead, MN (*unpublished data*). These observations suggest that at least most of the sugarbeet varieties with resistance to *F. oxysporum* may be susceptible to *F. novum*. Further studies are necessary to compare resistance of sugarbeet varieties to *F. oxysporum* and *F. novum*.

Aggressiveness of *F. novum* is demonstrated by both findings that it does not need an open injury to penetrate the sugarbeet plants, and is able to reproduce at high temperatures, indicates that this species is successfully adapting to the new cropping conditions of the region. For this reason, new crop system strategies such as early planting and development of resistance varieties must be implemented, in order to avoid the increase of the pathogen population in the soil and disease incidence in the field, as well as the quick spread in the region and to other crops such as canola.

Since *F. novum* has been reported as a new species closely related to *F. acutatum*, which is not known to be a pathogen of sugarbeet or found in the United States, it is suggested that *F. novum* may have emerged as a result of gene transfer between two or more *Fusarium* species in the RRV region. Mobile pathogenicity gene transfer has been

documented in others *Fusarium* species (Ma et al., 2010) and could account for the development of *F. novum*. This gene transfer could further involve asexual recombination or parasexualism between species that could be or not be pathogenic to sugarbeet. As result of this, the new genetic arrangements in the novel *Fusarium* species may be involving complex interactions that are yet unknown, which also may lead to *F. novum* be more aggressive during the infection process to sugarbeet. In this scenario, it is also suggested that high temperatures during the cropping season, such as the registered during the last years in the RRV, may also be acting as a selection factor for *F. novum* which explain also why this pathogen is spreading in the region.

CONCLUSION

This study shows that *F. novum* growth is favored by high temperatures and sugarbeet infection may occur without injury presence. Also, since canola appears to be the only symptomless host for this pathogen, this crop must be considered in crop rotation strategies to avoid inoculum increase and severity of Fusarium yellowing decline disease.

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APPENDIX A. GROWTH MEDIA

Water Agar (WA)

Agar (Agar Moorhead and Company, Moorhead, MN)	15 g L^{-1}
Deionized water	1 L

Autoclave at 121 °C for 20 minutes. When medium reaches room temperature, add 0.2 g of ampicillin in laminar flow cabinet.

Potato Dextrose Agar (PDA)

Extract of 200 g slice potatoes	0.5 L
Dextrose	10 g L^{-1}
Agar (Agar Moorhead and Company, Moorhead, MN)	15 g L^{-1}
Deionized water	1 L

Autoclave at 121 °C for 20 minutes. When medium reaches room temperature, add 0.2 g of ampicillin in laminar flow cabinet.

Carnation Leaf Agar (CLA)

Wash carnation leaves (cv. Improved White Sim) under running water for 15 min. Cut into pieces (5x7 mm), sterilizing the surface with 70% ethanol for 5 min, rinse three times with sterile distilled water, and dry. Add five or six carnation leaves pieces onto a 2% water agar-medium.

Potato Dextrose Broth (PDB)

Potato Dextrose Extract (Himedia Laboratories Ltd., Mumbal, India)	24 g L^{-1}
Deionized water	1 L

Autoclave at 121 °C for 20 minutes. In laminar flow cabinet and when medium reaches room temperature, add 0.2 g of ampicillin. Dispense 75 mL of medium in 125 mL Erlenmeyer flasks autoclaved and capped with foil.

APPENDIX B. FUNGAL GENOMIC DNA EXTRACTION

1. Recover the mycelia (approximately 0.5 g) from half strength PDB-medium and put in the lysing-matrix-A tube
2. Add 1 mL of 2% CTAB buffer
3. Mix in Fast Prep Machine at 6.5 speed for 4.5 sec
4. Put in ice for 3 min and centrifuge for 10 min at 14.000 rpm
5. Transfer 700 µL of supernatant to 2 mL micro-centrifuge tubes
6. Add 2 µL of β-mercaptoethanol
7. Incubate for 30 min at 60°C
8. Add 500 µL of phenol:chloroform:isoamyl alcohol mixture (25:24:1) vol/vol, and mix for 10 min on flat vortex
9. Centrifuge for 10 min at 14.000 rpm and transfer 600 µl of supernatant to 2 mL micro-centrifuge tubes
10. Add 500 µl of chloroform:isoamyl alcohol mixture (24:1) vol/vol, and mix for 10 min on flat vortex
11. Centrifuge for 10 min at 14.000 rpm and transfer 500 µL of supernatant to 1.5 micro-centrifuge tubes
12. Add 500 µL of isopropanol and incubate for 30 min at -20°C
13. Centrifuge for 10 min at 14.000 rpm
14. Wash the pellet with 500 µL of 70% ethanol
15. Dry the pellet in hood for 15 min
16. Resuspend the DNA pellet in 50 µL of sterile distilled water
17. Add 2 µL of RNAse and incubate for 30 min at 37 °C

APPENDIX C. ACRYLAMIDE GEL (5%)

Acrylamide/Bis (29:1) Sln 30% (Bio-Rad Laboratories, Hercules, CA)	20 mL
Deionized water	75 mL
TBE 5X	24 mL
Ammonium persulfate 10% (w/v)	840 µL
TEMED	160 µL

The polymerization process takes approximately 1h, run samples for 2:30 h at 200 volts in 1X Tris borate-EDTA (TBE) buffer.