Improving Sustainability of Perennial Ryegrass (*Lolium perenne)* Seed Production: Integrated crop management practices and the development of a novel metabolomics-assisted technique to select for resistance to rust (*Puccinia)* pathogens

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Eric J. Koeritz

Foreword

This dissertation consists of an overall introduction followed by three chapters with one chapter for each main project that I have worked on. The first two chapters have been written in the format of a Crop Science journal article. Chapter three is a manuscript in preparation that will be submitted for publication following thorough review. Each chapter includes its own overview, specific introduction, materials and methods, results, discussion, tables, and figures. A composite list of references is also included at the end of the document. The contents of this thesis shall not be made publically available until the date requested in Dissertation Hold Request Form signed by Eric J. Koeritz on 26 May 2014.

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INTRODUCTION

Perennial ryegrass seed, produced for sale as turf and forage grass around the world, is grown in rural agricultural areas of northern Minnesota, Oregon, and Canada. Currently there are around 8,000 ha of perennial ryegrass seed production in northern Minnesota as well as 42,500 (NASS, 2013) and 9,000 (MASC, 2012) ha in Oregon, and Canada, respectively. The perennial ryegrass seed industry in northern Minnesota has grown significantly over the past 10-15 years. Minnesota is an ideal climate for grass seed production and there are agronomic and economic benefits of having perennial ryegrass (effectively a biennial seed crop in MN) in a rotation with oilseed and wheat crops in this region (MN Turf Seed Council, personal communication, 2011).

Perennial ryegrass, as a seed crop in Minnesota, is typically planted in rows as an under-seeded crop in wheat in the spring or into wheat stubble following wheat harvest in the fall. Due to a vernalization requirement, perennial ryegrass remains in the field during the winter months and then produces a seed crop the following growing season (Najda, 2004). As a result, perennial ryegrass has many environmental benefits in a crop rotation including decreased soil erosion, reduced nitrate leaching, high organic matter production, and greater habitat for wildlife due to fewer tillage operations. For a farmer, perennial ryegrass is a desirable crop rotation option from an economic, labor, and weed management standpoint. Perennial ryegrass and its associated seed cleaning and distribution businesses have total economic value of $20 and $111 million in Minnesota and Oregon, respectively (NASS, 2013). Also, planting and harvest of perennial ryegrass

seed occur at different times than for annual crops so labor requirements are spread throughout the season.

Perennial ryegrass as a seed crop is relatively new to many farmers in northern Minnestota (Minnesota Turf Seed Council, personal communication). Despite the many benefits of growing perennial ryegrass in a rotation with wheat and oilseed crops, there are some challenges that threaten the profitability, sustainability, and quality of perennial ryegrass seed production and turfgrass systems. First, perennial ryegrass seed yields in Minnesota can be variable, commonly ranging between 500 and 2000 kg ha^{-1} (Koeritz et al., 2013; Ehlke et al., 2011; Kurcinka, 2009). Average seed yields are also far lower than the suggested yield potential of 3000 kg ha^{-1} given proper management and growing conditions (Rolston et al., 2010a). The impact of common management variables such as N fertilizer, growth regulators, seeding rate, and row spacing have not been extensively documented, particularly as interacting factors, in our region. Seed yield and yield parameters have been shown to vary significantly depending on the environment in which a perennial ryegrass seed crop is grown (Elgersma, 1990a), therefore, evaluating common management variables in multiple environments in our region is particularly important.

A second challenge to perennial ryegrass seed producers in Minnesota is weed and disease pests. Herbicides are a routinely sprayed on a perennial ryegrass crop in Minnesota following establishment and during the harvest year to control a variety of grass and broadleaf weeds. Spraying herbicides or fungicides to control weeds or disease pests is time consuming and costly. One major benefit of some cultivars, developed by the University of Minnesota and grown by farmers in Minnesota, is incorporated

resistance to the herbicide Assure II®, quizalofop P-ethyl, {ethyl (R)-2-[4-(6-chloroquinoxalin-2-yloxy)-phenoxy] propionate} which is useful for controlling annual and perennial grasses. Applications of Assure II can be costly, and intense use of acetyl coenzyme A carboxylase (ACC) inhibitors will likely lead to selection for resistance in some grass weed species (Delye, 2005). If weeds such as wild oats (*Avena fatua*) or quackgrass (*Elymus repens* (L.) Gould) were to develop resistance, contaminated perennial ryegrass seedlots would reduce profits. Growing public concern about the use of pesticides and the risk for the development of resistance to this useful herbicide mode of action has created a need for integrated management strategies which reduce weed pressure.

Minnesota farmers have indicated that rust pathogens, including stem rust (*Puccinia graminis* Pers. subsp. *graminicola* Urban) and sometimes crown rust (*Puccinia coronata* Corda f. sp. *lolii* Brown) are a severe issue in seed production fields (causing up to an 80% yield reduction) (MN Turf Seed Council, 2010). Fungicides (currently costing approximately $125 per acre per application) are routinely applied to obtain adequate seed yields suggesting that alternative cultural management options for disease control or rust resistant cultivars would improve sustainability of the crop (Pfender, 2009; Pfender, 2001). Seed from cultivars of perennial ryegrass that are more resistant to crown rust would be more a more marketable product for seed producers to grow.

We have identified three main areas of study which will have a significant impact on the perennial ryegrass seed industry and end users. They are: 1) the effects of alternative nitrogen and growth regulator management including their interaction on crop

agronomic characteristics and stem rust , 2) the effect of seeding rate and row spacing and their interaction with N rate on crop agronomic characteristics, competition with weeds, and stem rust and 3) the development of a novel breeding strategy (metabolomics-assisted selection) to help with faster and more accurate selection for crown rust resistance in perennial ryegrass.

Nitrogen and plant growth regulators

Key factors that influence a perennial ryegrass seed crop yield are N fertilization (Kurcinka, 2009; Cookson et al., 2000) and growth regulator applications to control lodging (Rolston et al., 2010a). Nitrogen directly affects seed yield and vegetative growth in perennial ryegrass seed production; therefore, timing of N fertilization is critical for maximizing yield and profitability (Cookson et al., 2000; Kurcinka, 2009). Nitrogen can also have indirect effects on seed yield and quality in perennial ryegrass seed production. Nitrogen availability has been positively correlated with floret sight utilization, seed weight, and seedling vigor in perennial ryegrass (Cookson et al., 2000; Young III et al., 1996). Nitrogen can also stimulate greater transcription of defense-related and photosynthetic genes (Ros et al., 2008). Nitrogen effects on plant defense-related gene transcripts and, ultimately, plant defense compounds, which may confer disease resistance, could be important in perennial ryegrass considering seed yields are often reduced by stem rust.

Perennial ryegrass seed farmers in northern MN currently apply up to half of the total N fertilizer as granular urea, in the late fall, following establishment. Typically, the

majority of the total N is applied in early spring of the harvest year, which is in line with research that suggests a yield benefit when a significant portion of the total N is applied in the spring of the harvest season (Young III et al., 1996; Kurcinka, 2009). Sandy soils and extremely wet conditions encountered during late fall and early spring in northern Minnesota make urea fertilizers prone to nitrate leaching, particularly since N demand from the perennial ryegrass crop is low at the time of a traditional N application (Agehara and Warncke, 2005; Kurcinka, 2009; Cookson et al., 2001; Bergstrom and Jokela, 2001). Significant N loss due to volatilization and de-nitrification can also occur when large amounts of N are applied as a single application (Burt et al., 1993; Cookson et al., 2001).

Applying N to match perennial ryegrass N demands during the growing season rather than single large applications could improve seed yields (Young III et al., 1996; Kurcinka, 2009), improve seed quality (Cookson et al., 2000; Young III et al., 1996), and potentially reduce environmental impacts of N fertilization by mitigating N loss (Cookson et al., 2000; Mullen et al., 2003). Research in Minnesota has suggested that polymer coated urea (a slow release N source) can be an unreliable N fertilizer source due to the need for regular rainfall to release N from the coated granules into the soil (Kurcinka, 2009). Split applications of fast release N may be a more reliable option for matching crop N demand in our region. Split applications of N in a perennial ryegrass seed crop during the harvest season in New Zealand resulted in greater apparent N recovery, greater N use efficiency, and higher quality seed (Cookson et al., 2000). Although better than slow release N sources, granular fast release N sources such as urea still require adequate rainfall and soil moisture to be available to the plant. Unlike soil

applied sources of urea N, which require rainfall to dissolve fertilizer granules before N uptake can occur, up to 69% of foliar applied N can be taken up by some turfgrasses within 1 hour of application (Stiegler et al., 2011). Several benefits of foliar N fertilization have been observed in small grains: reduced leaching and de-nitrification, greater plant N uptake in dry conditions, late season N uptake to increase grain N concentration, and the ability to apply N as a tank mix with other field operations such as growth regulator or fungicide applications (Gooding and Davies, 1992).

The application of a plant growth regulators, specifically gibberellic acid (GA) inhibitors, to prevent lodging is critical to obtaining good seed yields in perennial ryegrass given that the time to 50% lodging has been positively correlated with seed yield (Rolston et al., 2010a). In a situation where split N applications are made during the harvest season, it is likely that growth regulator timing will need to be adjusted to correspond to the split N application strategy. The half-life of common growth regulators used in perennial ryegrass seed production in MN can be extremely short (Rademacher, 2000) so it is possible that the single early season application, as is the current practice in MN, will not be as effective as a split application approach. Timing of application for GA inhibitors is critical in perennial ryegrass seed production and it is likely that a split application could result in a larger window of growth regulation (Borm and van den Berg, 2008; Rolston et al., 2010a). In turfgrass systems, split applications of a GA inhibitor are potentially more effective at providing season long vegetative growth suppression than single applications at higher rates (Kreuser and Soldat, 2011).

Plant growth regulators reduce plant height and help control lodging. These factors can have an effect on the microenvironment within a plant canopy. In barley (*Hordeum vulgare* L.), the plant canopy can affect nutrient status, light penetration, temperature, dew period, or humidity, some of which which may affect stem rust severity (Dill-Macky and Roelfs, 1999). Other research has shown that grasses treated with a GA inhibitor have greater chlorophyll content and cell density (Ervin and Koski, 2001). The stem rust fungus is an obligate biotroph and its success depends on the ability of an infection hyphae to penetrate the host and extract nutrients from the plant before fungal energy reserves are depleted or a host defense response is triggered (Leonard and Szabo, 2005). Perennial ryegrass plants with greater cell density as a result of a growth regulator treatment may limit the rate of infection and thus reduce disease development.

Seeding rate, row spacing, and N rate

Recommendations for optimal seeding rates of perennial ryegrass in Minnesota and other seed producing regions are variable and the impact of plant density in perennial ryegrass seed production in northern MN is not well documented. In Minnesota, typical perennial ryegrass seeding rates range from 4.5 to 7 kg seed ha^{-1}. In Saskatchewan, under irrigated conditions, seeding rates of 8 kg seed ha^{-1} are recommended (Najda, 2004). Increasing seeding rate is a strategy used in dry-seeded rice to suppress weeds by providing faster canopy cover and to compensate for poor establishment (Chauhan, 2012). Previous research in a greenhouse setting has shown that perennial ryegrass tiller density

converges toward a value determined by available light intensity, a value not affected by initial seed density, when seed densities between 320 and 10,000 seeds m^{-2} were evaluated (Kays and Harper, 1974). This suggests that slight variations in seeding rate may not critically affect final tiller density, however the effect of seeding rate on plant stand characteristics under field conditions should be determined. In *Festulolium* (a cross between *Festuca* L. and *Lolium* L.) seed production, seeding rates between 8 and 16 kg ha^{-1} did not affect seed yield (Deleuran et al., 2010). In annual ryegrass (*Lolium multiflorum* Lam.) seed production, there can be a seed yield increase as the result of higher seeding rates when environmental conditions are unfavorable for plant growth (Simic et al., 2000). Environmental factors such as soil type, drought, temperature, and winter-kill affect perennial ryegrass seed production in Minnesota. Seed yield and yield parameters have been shown to vary significantly depending on the environment in which a perennial ryegrass seed crop is grown (Elgersma, 1990a). Optimal perennial ryegrass seeding rates have not been determined in northern Minnesota and the effect of seeding rate on perennial ryegrass agronomic characteristics in multiple environments in northern Minnesota should be determined.

Row spacing in Minnesota and western Canada varies depending on soil moisture and farmers equipment. Row spacing can range from 15 to 30 cm, with 20 cm being most common in Minnesota. The effect of row spacing on yield and agronomic characteristics of perennial ryegrass in Minnesota has not been well characterized. At a given seeding rate, increasing row spacing increases the number of seeds planted within a row. This can increase within-row tiller density, may increase plant to plant competition

for light and nutrients, and could affect plant growth (Kays and Harper, 1974; Deleuran et al., 2009; Han et al., 2013). High planting density in a grasses can decreases the amount of tillering but high tiller density can cause plants to have longer leaves and shoots as a result of lower red/far-red light ratios in the plant canopy (Kays and Harper, 1974; Casal et al., 1985; Simic et al., 2009).

Plant spatial arrangement in grass seed production can affect weed and disease pressure. A study evaluating the effect of planting density in barley found that stem rust severity was greater at lower planting densities and suggested that differences in the microenvironment at different plant densities affected pathogen growth (Dill-Macky and Roelfs, 2000). That study also suggested that spatial arrangement of plants, in addition to the number of plants per unit area, could affect stem rust development. For organic seed production in Denmark, researchers have suggested that wider row spacing of 24 cm can be used (without affecting seed yield) to facilitate mechanical weed control (Deleuran et al., 2010). However, in various grass seed crops in China, weed growth is increased as row spacing increases (Han et al., 2013). Narrow row spacing, which promotes faster canopy closure and greater light interception, is suggested as a cultural practice to reduce weed pressure in dry-seeded rice and soybeans [*Glycine max* (L.) Merr.] (Chauhan, 2012; Yelverton and Coble, 1991). An evaluation of integrated management strategies that could affect yield, yield parameters, canopy formation, and microenvironments within the canopy is warranted.

Crown rust, resistance breeding challenges, and metabolomics-assisted selection

Puccinia coronata f. sp. *lolii* (phylum Basidiomycota, class Urediniomycetes, order Uredinales, family Pucciniaceae) is the causal agent of crown rust in perennial ryegrass (Dracatos et al., 2010). The asexual urediniospores produced by the fungus infect the leaf tissue of perennial ryegrass (Dracatos et al., 2010; Roderick and Thomas, 1997). Uredineospores land on the leaf surface of perennial ryegrass when the leaf blade is wet, germinate at an optimum temperature of 12 to 24°C, and then the germ tube from the spore grows perpendicularly to the ridges on the surface of the leaf until it reaches a stomatal opening (Roderick and Thomas, 1997). Once the germ tube reaches the stomatal opening, an appressorium is formed over the stomatal opening (Roderick and Thomas, 1997). A penetration peg is then formed and passes through the stomata where it forms a haustorium that penetrates mesophyll cells and extracts carbohydrates and nutrients from the plant (Roderick and Thomas, 1997; Dracatos et al., 2010; Potter, 1987). Once it begins deriving nutrients from the host, the fungus can clonally produce new spores; the latent period is generally between 6 and 25 d depending on the temperature (Roderick and Thomas, 1997). Crown rust spreads in the form of clonally propagated urediniospores which can travel in wind currents for thousands of miles where they can re-infect another susceptible grass host and propagate new urediniospores (Kolmer, 2005). Crown rust of perennial ryegrass (*Puccinia coronata* f. sp. *lolii*) is thought to be able to infect up to six other species including tall fescue (*Festuca arundinacea* Schreb.) and annual ryegrass (*Lolium multiflorum* Lam.).

Resistance to crown rust in perennial ryegrass (caused by *Puccinia coronata* f.sp. *lolii* Brown) is a major selection criteria for perennial ryegrass breeders developing both

turfgrass and forage varieties (Pauly et al., 2012; Dracatos et al., 2010; Kimbeng, 1999). In forage perennial ryegrass, crown rust can cause significant losses in dry matter yield (Price, 1987). Crown rust pustules can erupt through leaf surfaces (Roderick and Thomas, 1997) which can result in increased water loss and decreased photosynthetic ability in seed production, forage, and turfgrass systems (Dracatos et al., 2010). In perennial ryegrass used as a turfgrass, where visual appeal and turf quality is a high priority, a crown rust infection can significantly detract from visual appeal and turf quality (Stier, et al., 2008). Due to environmental concerns and costs, fungicides are generally not considered a viable option for controlling crown rust in perennial ryegrass and developing cultivars with genetic resistance is a high priority for breeders (Dracatos et al., 2010; Schejbel et al., 2007; Kimbeng, 1999).

Phenotypic screening for quantitative rust resistance in current cultivar development programs is time consuming, unreliable, and prone to human error (Nutter et al., 1993; Diaz-Lago et al., 2003). Progress in breeding for rust resistance depends on an accurate screening procedure. Phenotypic selection for rust resistance relies on the presence of natural inoculum which can be unreliable and variable (Aldaoud et al., 2004; Schejbel et al., 2007), or on the collection and maintenance of a range of isolates for artificial inoculation which can be costly and laborious (Kimbeng, 1999). Phenotypic screening can also be complicated by environmental effects which can cause plants to vary in expression of resistance or pathogens in expression of virulence resulting in inaccurate assessment of resistance levels (Schejbel et al., 2007; Kimbeng, 1999; Reheul and Ghesquiere, 1994).

There is evidence of both major and minor resistance genes conferring resistance to crown rust in perennial ryegrass (Kimbeng, 1999; Schejbel et al., 2007; Dracatos et al., 2010). There is evidence of different pathotypes among geographically distinct isolates of crown rust that affect perennial ryegrass (Aldaoud et al., 2004). Varieties of perennial ryegrass can vary in resistance phenotype depending on the isolates present in the locations where they are grown and there can be low correlation between field screening trials over multiple years (Potter et al., 1990; Aldaoud et al., 2004; Schejbel et al., 2007). The outcrossing nature of perennial ryegrass, the complexity of resistance to crown rust in perennial ryegrass, and the lack of knowledge of races of crown rust affecting perennial ryegrass make incorporating gene for gene resistance in perennial ryegrass a poor option (Aldaoud et al., 2004).

There is a wealth of knowledge regarding rust pathogens in wheat which may be applicable to breeding for crown rust resistance in perennial ryegrass. In wheat, there is evidence for both qualitative and quantitative resistance to stem rust (Kolmer, 2005; Singh et al., 2007). In the past, a common rust control strategy in wheat was to utilize qualitative resistance genes in new cultivars. This was a flawed strategy because mutations, sexual recombination on alternate hosts, and wind dissemination of urediniospores allowed for new virulent stem rust races to develop and rapidly increase over large areas (Kolmer et al. 2009, Kolmer, 2005; Singh et al., 2007). Resistance genes that had remained resistant to the prevalent stem rust races for many years, such as Sr32, are now being overcome by highly virulent races such as TTKS (Ug99) and vital wheat production areas are threatened (Pretarius et al., 2000; Singh, 2007). Attention has now

turned towards breeding wheat cultivars with durable, non-race-specific stem rust resistance which involves incorporating many minor resistance genes which have additive effects (Singh et al., 2007). Incorporating many minor resistance genes, a form of quantitative resistance, often results in delayed or slowed pathogen growth and is considered an important mechanism of durable resistance (Messmer et al., 2000; Krattinger et al., 2009; Poland et al., 2009). The Sr2-complex in wheat, for example, is a qualitative slow rusting gene in combination with a number of unknown minor genes which results in reduced pathogen growth providing durable resistance to all pathotypes (Singh et al., 2007; Pfender, 2009b). The leaf rust resistance gene Lr34 in wheat is an example of a single gene which confers partial durable resistance resulting from slow rust development which has been correlated with reduced intercellular hyphal growth possibly due to its involvement in the export of metabolites that affect fungal growth (Krattinger et al., 2009). In perennial ryegrass, incorporating quantitative rust resistance obtained by incorporating multiple minor or partial resistance genes will also provide a more stable form of rust resistance. Selecting for quantitative crown rust resistance in a recurrent selection program may be the most viable option for perennial ryegrass (Kimbeng, 1999).

There are a number of proposed mechanisms of quantitative disease resistance in plants. Such mechanisms include altered plant morphology and development, antimicrobial secondary metabolites such as phytoalexins or phytoanticipins, detoxification of pathogen-derived toxins and altered signal transduction (Poland et al., 2009; Parisy et al., 2007). Such mechanisms of quantitative resistance could provide

broad spectrum durable resistance and may be amenable to selection via alternative methods such as metabolomics-assisted selection.

The number of natural products, or metabolites, occurring in plants is estimated in the hundreds of thousands and there is a need to better understand their biosynthetic pathways or functions (LaCamera et al., 2004). Some metabolites present in plants are known to be highly involved in protection from biotic and abiotic stresses including involvement in quantitative disease resistance (Poland et al., 2009). For example, it is known that phenylpropanoids accumulate in large quantities in plants developing resistance to pathogens but many of these compounds still need to be identified (LaCamera et al., 2004). Phytoanticipins, antimicrobial compounds produced constitutively by healthy plants, as well as phytoalexins, antimicrobial compounds that accumulate in diseased plants and are responsible for induced resistance, are often phenylpropanoids (LaCamera et al., 2004; Poland et al., 2009). Other compounds found in plants stimulate pathogen growth or are highly correlated with disease symptoms. In oats, it has been suggested that certain phenolic compounds may exist at higher levels in genotypes that are more resistant to crown rust (*Puccinia coronata* f. sp. *avenae*). It was shown that avenanthramides (phenolic compounds in grains) and two unidentified compounds in leaves, were markers of rust resistance (Dimberg and Peterson, 2009). Searls and French (1964), determined that cinnamaldehyde, cinnamic acid and cinnamyl alcohol, all products of the phenyl propanoid pathway, can stimulate urediniospore germination of *Puccinia graminis* f. sp. *tritici*. Urediniospore germination of *Puccinia graminis* f. sp. *triticii* is also stimulated by nonanal and nonanol (French, 1992).

Numerous other plant volatile compounds were determined to have stimulatory effects on spore germination of other pathogens as well by French (1992). Crown rust severity on smooth bromegrass (*Bromus inermus*) has been negatively correlated with lignin and etherified ferulic content of leaf tissue (Delgado et al., 2002). Haustorial mother cells can be induced by trans-2-hexene-1-ol, a compound induced by pathogen attack (Wietholter et al., 2003). Such secondary metabolites, in perennial ryegrass or other grasses, which affect rust growth and development could serve as useful biomarkers for selecting for quantitative stem rust resistance.

Plant metabolomics is the study of the molecular metabolites present in a plant often with multiple goals including: discriminating between genotypes, understanding metabolic regulation and stress response, and selecting desirable traits as a component of a plant breeding program (Fernie and Schauer, 2009; Dettmer et al., 2007, Tohge and Fernie, 2009). Secondary metabolites are the end products of complex biochemical pathways that are regulated by genes and are often used directly as stress mediation or plant defense mechanisms making them closely associated to plant phenotypes (Dettmer et al., 2007; Harrigan et al., 2007). Metabolomics-assisted selection in plant breeding programs has recently emerged as a fast and accurate selection method for desirable traits which are difficult to select for using phenotyping or genotyping methods (Fernie and Schauer, 2009; Tohge and Fernie, 2009). Today there are abundant online resources for data processing and analysis as well as excellent protocols outlining procedures for plant metabolomics studies which may make metabolomics-assisted selection a reality in plant breeding programs (De Vos et al., 2007; Fernie and Schauer, 2009; Tohge and Fernie,

2009; Goodacre et al., 2007; Jansen et al., 2010). Additionally there are online databases where accurate mass HPLC-MS data can be searched against known metabolites (KEGG, HMDB, Metlin, etc.).

The potential for using secondary metabolites as biomarkers for selecting desired traits in plants has been demonstrated in Arabidopsis, grapes, wheat, and conifer (Meyer et al., 2007, Figueiredo et al., 2008, Hamzehzarghani et al., 2008; Robinson et al., 2009). For example, it was demonstrated that specific combinations of metabolites could be highly predictive of biomass of *Arabidopsis* using gas chromatography/mass spectrometry (GC/MS) along with partial least squares (PLS) regression analysis (Meyer et al., 2007). In grape (*Vitis vinifera*), cultivars varied in susceptibility to downy and powdery mildew, with the resistant cultivar having a higher constitutive abundance of inositol, glutamine, glutamate, alanine and caffeic acid (Figueiredo et al.,2008). A number of studies have also evaluated metabolic profiling as a potential selection tool for resistance to Fusarium head blight (FHB) (*Fusarium graminearum*) in wheat, which is a difficult disease on which to conduct phenotypic resistance screening (Browne and Brindle, 2007; Hamzehsarghani et al., 2008; Groth et al., 1999). Using NMR-based metabolic profiling to identify profiles associated with susceptibility and/or passive resistance to FHB in wheat, Browne and Brindle (2007) determined that genotypes demonstrating shorter disease latent periods (higher susceptibility) were associated with the presence of choline, betaine, glutamine, glutamate, alanine, and sucrose. They also found that glucose, various carbohydrates, and some aromatic compounds were associated with FHB resistance. Another study on FHB in wheat used an untargeted

GC/MS approach and identified 41 resistance related metabolites (Hamzehzarghani et al., 2008). Of the 41 resistance related metabolites, (which consisted of: amino acids, fatty acids, organic acids, phenolics, and sugars) 25 were produced constitutively in resistant cultivars, 23 were induced upon pathogen infection and seven were both constitutively produced and induced to higher concentrations upon infection. Hamzehzarghani et al. (2008) proposed that resistance related compounds could potentially be used as biomarkers for quantitative resistance screening. The discovery of such biomarkers for crown rust resistance in perennial ryegrass could be used to predict disease resistance levels and would thus be extremely beneficial for incorporating quantitative rust resistance in a breeding program.

CHAPTER 1.

A Split Application Approach to Nitrogen and Growth Regulator Management for Perennial Ryegrass Seed Production

Eric J. Koeritz, Eric Watkins, and Nancy J. Ehlke

OVERVIEW

Experiments were conducted during 2009 and 2010 in first year perennial ryegrass (*Lolium perenne* L.) fields under limiting or adequate spring nitrogen (N). Three N application methods (single, two-split, and three-split), five growth regulators (split and single applications of prohexadione Ca or trinexapac ethyl) and two spring N rates (56 or 100 kg N ha^{-1}) were evaluated in a split-split plot design. Under adequate spring N, all N application methods resulted in similar seed yields but when spring N was limiting a single application of N resulted in up to 14% greater yield vs. the three-split. The three-split N application improved relative chlorophyll index (RCI) 6-20% late in the harvest season and reduced stem rust (*Puccinia graminis* Pers. subsp. *graminicola* Urban) AUDPC by 18 to 39% vs. the single N application. Other effects of three-split N applications observed in 2010 were 8-19% less biomass, 2-3% greater harvest index, 4% greater seedling vigor and 25% less lodging. The split prohexadione Ca treatment gave the most consistent results and when vegetative growth was greatest it resulted in a 36% increase in seed yield, a 5.5% increase in harvest index, 14 cm shorter plants, 70% less lodging, 30% greater RCI, 13% lower stem rust incidence, and 3% lower stem rust severity. Development of a model to optimize rates and timings for split N and growth regulator applications based on growth stage, soil N availability, and GDD could improve efficacy of split applications.

Abbreviations: ANOVA, analysis of variance; AUDPC, area under the disease progress curve; FTN, fertile tiller number; GA, gibberellic acid; GDD, growing degree days; GRP, growth regulator program; HI, harvest index; NAM, nitrogen application method; RCI, relative chlorophyll index; SV, seedling vigor; TSW, thousand seed weight; WSC, water-soluble carbohydrate.

Perennial ryegrass seed, produced for sale as turf and forage grass around the world, is grown in rural agricultural areas of Minnesota, Oregon, and Canada. Currently there are around 8,000 hectares of perennial ryegrass seed production in northern Minnesota as well as 24,000 (OSCS, 2012) and 9,000 (MASC, 2012) hectares in Oregon, and Manitoba, respectively. Typical perennial ryegrass seed yields in Minnesota range from around 550 to 1800 kg ha^{-1} and it is likely that yields can be improved through better management practices suited for Minnesota's environmental conditions (Ehlke et al., 2011; Kurcinka, 2009). Perennial ryegrass research conducted in Oregon and New Zealand has suggested that seed yields of 1500 to 2000 kg ha^{-1} are common in those areas and that seed yields of 3000 kg ha^{-1} may be possible (Pfender, 2009; Rolston et al., 2010b). Key factors that influence a perennial ryegrass seed crops yield, which could be improved in northern Minnesota and other areas, are N fertilization (Kurcinka, 2009; Cookson et al., 2000), growth regulator applications to control lodging (Rolston et al., 2010a), and control of stem rust disease caused by *Puccinia graminis* Pers. subsp. *graminicola* Urban (Pfender, 2001; Pfender, 2009). Although significant research has been conducted in northern Minnesota to determine optimum N rates and sources (Kurcinka, 2009), growers have expressed concerns over N use efficiency, efficacy of growth regulators, and stem rust disease management (MN Turf Seed Council, personal communication, 2008).

Nitrogen directly affects seed yield and vegetative growth in perennial ryegrass seed production; therefore, timing of N fertilization is critical for maximizing yield and profitability of a perennial ryegrass seed crop (Cookson et al., 2000; Kurcinka, 2009). Nitrogen can also have indirect effects on seed yield and quality in perennial ryegrass

seed production. Nitrogen availability has been positively correlated with floret site utilization, seed weight, and seedling vigor in perennial ryegrass (Cookson et al., 2000; Young III et al., 1996). Nitrogen can also stimulate greater transcription of defense-related and photosynthetic genes (Ros et al., 2008). Nitrogen effects on plant defense-related gene transcripts and, ultimately, plant defense compounds, which may confer disease resistance, could be important in perennial ryegrass considering seed yields are often reduced by stem rust.

Perennial ryegrass seed farmers in northern MN currently apply up to half of the total N fertilizer as granular urea in the late fall following establishment and typically, the majority of the total N is applied in early spring of the harvest year, which is in line with research that suggests a yield benefit when a significant portion of the total N is applied in the spring of the harvest season (Young III et al., 1996; Kurcinka, 2009). Sandy soils and extremely wet conditions encountered during late fall and early spring in northern Minnesota make urea fertilizers prone to nitrate leaching, particularly since N demand from the perennial ryegrass crop is low at the time of a traditional N application (Agehara and Warncke, 2005; Kurcinka, 2009; Cookson et al., 2001; Bergstrom and Jokela, 2001). Significant N loss due to volatilization and de-nitrification can also occur when large amounts of N are applied as a single application (Burt et al., 1993; Cookson et al., 2001). Applying N to match perennial ryegrass N demands during the growing season rather than single large applications could improve seed yields (Young III et al., 1996; Kurcinka, 2009) , improve seed quality (Cookson et al., 2000; Young III et al., 1996), and potentially reduce environmental impacts of N fertilization by mitigating N loss (Cookson et al., 2000; Mullen et al., 2003).

Potential fertilization methods for matching perennial ryegrass crop demand include split granular applications during the growing season, slow release N products such as polymer coated urea, and split N applications using foliar applied urea. Research in Minnesota has suggested that polymer coated urea can be an unreliable N fertilizer source due to the need for regular rainfall to release N from the coated granules into the soil (Kurcinka, 2009). Split applications of fast release N could be the most reliable option for matching crop N demand in our region. In a study evaluating split applications of N on perennial ryegrass grown in Oregon, applying part of the total N as granular ammonium nitrate at the double ridge or spikelet initiation stage increased seed weight, number of seeds, and floret site utilization (Young III et al., 1996). Split applications of N in a perennial ryegrass seed crop during the harvest season in New Zealand resulted in greater apparent N recovery, greater N use efficiency, and higher quality seed (Cookson et al., 2000). Although better than slow release N sources, fast release N sources still require adequate rainfall and soil moisture to be available to the plant.

Foliar applications of N in wheat, sometimes applied as split applications during the growing season, are thought to be an excellent way to improve N use efficiency although the effects on seed yield can be variable (Otteson et al., 2007). Several benefits of foliar N fertilization have been observed in small grains: reduced leaching and de-nitrification, greater plant N uptake in dry conditions, late season N uptake to increase grain N concentration, and the ability to apply N as a tank mix with other field operations such as growth regulator or fungicide applications (Bly and Woodard, 2003; Gooding and Davies, 1992). Unlike soil applied sources of N, which require rainfall to dissolve fertilizer granules before N uptake can occur, up to 69% of foliar applied N can be taken

up by some turfgrasses within 1 hour of application (Stiegler et al., 2011). Despite potential benefits, the effects of split, foliar applications of N applied during the harvest season in MN are unknown and the potential positive agronomic impacts of using foliar fertilization to match perennial ryegrass N demand should be explored.

Integrated management studies, which evaluate important management components in addition to N fertilization, are essential. The application of a plant growth regulator to prevent lodging is critical to obtaining good seed yields in perennial ryegrass given that the time to 50% lodging has been positively correlated with seed yield (Rolston et al., 2010a). The half-life of common growth regulators used in perennial ryegrass seed production in MN can be extremely short (Rademacher, 2000) so it is possible that the single early season application, as is the current practice in MN, will not be as effective as a split application approach. In a potential situation where split N applications are made during the harvest season, it is likely that growth regulator timing will need to be adjusted to correspond to the split N application strategy.

Growers have expressed interest in assessing the effects of alternative management strategies in contrast to their traditional management practices in MN. Research evaluating split N applications, applied as foliar urea N during the harvest season, and the interaction with growth regulators and N rate in MN is non-existent. Therefore, the objectives of this study were to: 1) Evaluate the effect of split applications of foliar applied N compared to a standard single application of granular N on seed yield, agronomic characteristics, and seed quality for perennial ryegrass seed production in northern Minnesota, 2) Determine how management variables including N rates and alternative plant growth regulator programs (GRP) interact with nitrogen application

methods (NAM) and/or affect yield and agronomic characteristics of the perennial ryegrass seed crop, and 3) Quantify the effect of alternative, split applications of N and GRPs on crop resistance to stem rust disease and to observe plant agronomic characteristics associated with resistance.

MATERIALS AND METHODS

Two similar experiments (differing only in total N applied post-planting and location) were conducted under non-irrigated conditions during the 2009 and 2010 harvest seasons to test the effects of GRPs, N rate, and NAMs on seed yield, yield components, and stem rust disease in a first year perennial ryegrass seed production field. In August 2008 and 2009, 'Arctic Green' perennial ryegrass was seeded at a rate of 5.6 kg ha^{-1} into wheat stubble shortly after wheat harvest for both experiments and no fertilizer was applied at the time of establishment to keep in line with common practices in northern Minnesota. Experiment 1 received no fertilizer during fall of each establishment year. For Exp.1, located near Roseau, MN in both years, N rates applied in the spring of the harvest year were 56 or 100 kg ha^{-1} depending on treatment. The soil type for the Exp. 1 2009 site was a Wabinica silt loam and the soil type for the Exp. 1 2010 site was a Zippel very fine sandy loam. Perennial ryegrass seed production near Roosevelt, MN, the site of Experiment 2 for both years, requires greater N inputs to achieve acceptable seed yields due to sandy soils. Experiment 2 received an N, P, and K fertilizer application, broadcast across the entire study, in November of the establishment year at a rate of 56 kg N ha^{-1}, 10 kg P ha^{-1}, and 20 kg K ha^{-1} and spring N rates were either 56 or 100 kg ha^{-1}. This resulted in total N rates of either 112 or 156 kg ha^{-1}. The

soil type for the Exp. 2 2009 site was a Zippel very fine sandy loam and for the 2010 site, soil type was a Percy fine sandy loam. The locations for each experiment were chosen because they were representative of the different soil conditions in the growing region for perennial ryegrass seed production in northern MN. Growing degree days for the Roseau and Roosevelt region were calculated using a base temperature of 0° C from a University of Minnesota sponsored weather station located at the GPS coordinates 48.655 N and 95.734 W.

Weeds were controlled according to standard protocol for the cultivar and region in May of each harvest year with applications of 0.07 kg a.i. ha^{-1} quizalofop P-ethyl, {ethyl (R)-2-[4-(6-chloroquinoxalin-2-yloxy)-phenoxy] propionate} (Assure II, Dupont, Wilmington, DE) ('Arctic Green' is tolerant to Assure II), 0.4 kg a.i. ha^{-1} 2, 4-D, (2, 4-dichlorphenoxyacetic acid), and 0.4 kg a.i. ha^{-1} diglycolamine salt of dicamba, (3,6-dichloro-o-aniscic acid) (Clarity, BASF Corp., Research Triangle Park, NC). Fungicides were not applied to allow for evaluation of treatment effects on stem rust disease.

Treatments were initiated at 243 and 251 growing degree days (GDD) in the spring of the 2009 and 2010 harvest years respectively. Experiments were set up as a randomized complete block design with a split-split plot treatment arrangement and GRPs as whole plot treatments, N rate as sub-plot treatments, and NAMs as sub-sub-plot treatments. Each experiment consisted of four replications and each experimental unit (sub-sub plot) measured 3.05 m wide by 3.70 m long with a 0.6 m untreated border around each experimental unit to minimize edge effects due to spray drift.

Five GRPs were evaluated and total application rates, as well as the use of non-ionic surfactants, were based on label recommendations for grass seed production (Table

1). Prohexadione calcium and trinexapac-ethyl are the most common growth regulators used in perennial ryegrass seed production in northern MN, hence, they were chosen for this study. Growth regulator applications were initiated when plants reached the two node stage and timing was kept consistent (weather permitting) from year to year based on GDD records (Table 1). Growth regulators were applied using a CO_2 powered bicycle sprayer equipped with 1002 TurboTeeJet® (TeeJet, Springfield, IL) nozzles operating at 186 kPa in water equivalent to 116 L ha^{-1}.

Spring N applications consisted of two N rates (56 or 100 kg ha^{-1}) and three NAMs (standard single, two- split, and three-split) with the split NAMs being applied in equal portions via foliar fertilization using urea and the standard single NAMs being applied as a conventional granular urea application (Table 1). Granular N applications were broadcast evenly across the entire experimental unit by hand. Foliar applications were applied using urea dissolved in water and sprayed through a CO_2 powered backpack sprayer equipped with 8002 XR TeeJet® (TeeJet, Springfield, IL) nozzles operating at 234 kPa in water equivalent to 467 L ha^{-1}. The first portion of N for the split NAMs was applied at the same time as the single standard NAM (Table 1). Timing of second or third foliar N applications was chosen to correspond with recommended timing for applications of growth regulators and the typical time of initial fungicide applications in perennial ryegrass seed production fields in northern MN based on crop stage and GDD (Table 1).

Data were collected on relative chlorophyll index (RCI), stem rust incidence and severity, lodging, harvest height, fertile tiller number (FTN), vegetative biomass, seed yield, thousand seed weight (TSW), percent germination of seed, and seedling vigor

(SV). Relative chlorophyll index was measured using a Field Scout CM1000 chlorophyll meter (Spectrum Technologies, Inc., Plainfield, IL); five readings were taken from each plot at a height of 0.83 M and then averaged for analysis. Relative chlorophyll index was measured as a plant N status indicator going into the seed development period as well as prior the predicted time of first stem rust incidence. Relative chlorophyll index readings were taken in all plots following each N application date with the measurement timed to be just before the next scheduled N application timing for the split NAM treatments. The final RCI reading, for all treatments, occurred about two weeks after the final scheduled N application date for the three split NAM. Stem rust incidence and severity due to natural infection was recorded when stem rust pustules were first observed in the study (around 1110 GDD) and again at 1459 and 1303 GDD in 2009 and 2010 respectively, before senescence of the crop. The pathogen was identified as stem rust based on disease symptoms and spore morphology. For stem rust ratings, twenty random tillers were collected from each plot and visually inspected in the field. In 2009, stem rust disease was not particularly severe; therefore, the number of erumpent pustules per tiller was counted. In 2010, stem rust disease was much more severe at the Exp. 2 site so the percent coverage of stem rust on each the 20 tillers was visually estimated using a reference key (James, 1971). Stem rust severity data was converted to area under the disease progress curve (AUDPC) using the formula from Shaner and Finney (1977):

$$\text{AUDPC} = \sum_{i=1}^{n-1}\left[\left(X_{i+1} + X_i\right)/2\right]\left[t_{1+1} - t_i\right]$$

where

X_i = the rust severity at the i^{th} day

t_i = the time in days after the initial rust rating at the i^{th} day

n = the total number of observations

Lodging was rated visually on a 0-100% scale where 0 = no lodging and all plants are upright, and 100 = all plants lying flat on the ground. Plant height was evaluated just prior to harvest by taking two measurements, each at two random locations throughout the plot, and recording the average of the two measurements to the nearest centimeter. Fertile tiller number, a measure of seed-producing tillers per unit area, was evaluated at the time of harvest by collecting all of the above ground plant material in a 15.24 cm wide by 70 cm long section in each experimental unit and counting the number of tillers in the sample that appeared to hold viable seed. At harvest, all above ground plant biomass was harvested from a 1 m^2 section and dried for 48 h at 90° C in a forced air drier. Once dry, the biomass was weighed and then passed through a grass seed thresher to separate the seed from non-seed biomass. Following initial threshing the seed was cleaned further by passing through a 1.625 by 9.525 mm oblong sieve two times, passing through a Almaco Seed Cleaner (Allan Machine Co., Ames, IA), then passing through a Superior® Fractionating Aspirator (Carter-Day Int. Inc., Minneapolis, MN). Finally, the clean seed was passed through 1.190 by 9.525 mm sieve to remove any remaining large inert material or un-separated seed. The clean seed was weighed and the weight subtracted from the above ground biomass weight to get a vegetative biomass weight. Harvest index (HI) was calculated by dividing the weight of the seed by the total above ground biomass weight. Thousand seed weight was determined by counting 300

randomly selected seeds from each treatment and weighing to the nearest 10^{-5}g then converting to weight 1000 seeds^{-1}. Percent germination and SV assays were conducted on 100 random seeds in 10 cm by 10 cm clear plastic germination containers (Tri-State Plastics, Inc., Dixon, KY) with seed placed on wetted 332 white blotter paper (Anchor Paper Co., St. Paul, MN) according to Association of Official Seed Analysts (AOSA) standards for perennial ryegrass (AOSA, 2010). Seedling vigor was determined by harvesting the vegetative portion of all germinated seedlings after 10 d in the germination chamber. Seedlings were counted, dried, and weighed, and then SV was reported as weight per 100 seedlings.

Statistical Analysis

Analysis of variance (ANOVA) of the data from each experiment was evaluated using the PROC MIXED procedure of SAS (SAS Institute, Cary, NC). Replication nested within year and all interactions with this factor were considered random effects while all other factors were considered fixed effects. The test of fixed effects used the Satterthwaite approximation for the denominator degrees of freedom. The CONTRAST statement was used to compare among GRPs as well as NAMs of particular interest. Otherwise, treatment effects were considered significant at $P < 0.05$ and pair-wise comparisons were performed using the Tukey-Kramer method. Post hoc analysis of treatment means, to obtain letter groupings based on the Tukey-Kramer HSD test, was conducted using the PDMIX800 SAS macro (Saxton, 1998). Pearson correlations were calculated on an individual plot basis between all measured variables using the PROC CORR procedure in SAS.

Data from Exp. 1 and 2 were analyzed separately due to known site soil differences and different total N application rates with the goal of understanding treatment effects under the unique experimental conditions. Due to significant effects of year and year by treatment interactions in the initial analysis of variance using combined data from both years ($P<0.05$), as well as known differences in crop growth patterns and weather between years, it was decided that data from each year within an experiment would be analyzed separately.

RESULTS

Yield, Agronomic Parameters, and Stem Rust

Experiment 1 (Roseau)

Seed yield was significantly affected by GRP, N rate, and NAM in 2009 and 2010 (Table 2). Despite treatment differences, mean seed yields for all NAMs were greater than 1100 kg ha^{-1} (in northern Minnesota seed yields typically range from around 550 to 1300 kg ha^{-1} (Ehlke et al., 2011). Applying N as a single standard application early in the harvest season resulted in greater seed yields than the split NAMs in each year at this location (Table 3). There were no significant interactions between NAM and N rate or GRP, however, main effects of N rate and GRP were significant in both years (Table 2). The high N rate resulted in 231 kg ha^{-1} greater seed yield than the low N rate in 2009 but 71 kg ha^{-1} less seed yield in 2010 which may be attributed to excess lodging in high N rate plots in 2010 (Table 3). Plots that received a growth regulator resulted in greater seed yields than those that did not in both years of the study (Table 3). In 2009, all GRPs resulted in similar seed yields and split GRPs demonstrated no seed yield benefit compared to single GRPs (Table 3). In the 2010 harvest season, single GRPs resulted in

91 kg ha^{-1} greater seed yield than split GRPs on average which was mostly due to the split trinexapac-ethyl GRP resulting in lower seed yields than the other GRPs (Table 3).

Overall, biomass production in 2010 was greater than 2009 ($P<0.05$) (Table 3). Above ground vegetative biomass production was not affected by NAM in 2009 and as a result of seed yields being greater with the single granular NAM, this treatment resulted in 3.8% greater HI than the three-split NAM (Table 2, 3). In 2010, the standard-single NAM resulted in 970 kg ha^{-1} more biomass vs. the three-split NAM and a 1.5% lower HI (Table 3). Analysis of variance for the 2010 season (Table 2) indicated that the effect of NAM on HI was more pronounced at the low N rate where the three-split NAM resulted in a HI of 26.6 % and the standard-single NAM resulted in an HI of 23.8% ($P<0.01$, data not shown). Nitrogen rate significantly affected biomass production and HI with the high N rate resulting in 925 and 1704 kg ha^{-1} greater biomass production as well as 1.6 and 7.4 % lower HI in 2009 and 2010 respectively (Table 3). The only GRP to significantly reduce biomass production in either year was the split prohexadione Ca GRP (Table 3) and there were no interactions of GRP with any other treatments (Table 2). Overall, applying growth regulator increased HI by 2.7 and 2.4 % in 2009 and 2010, respectively, however the split trinexapac-ethyl GRP was no different than the no GRP treatment in 2009.

In 2010, a year characterized by overall earlier and greater vegetative biomass production, the three-split NAM resulted in 4 cm shorter plant height, 25% less lodging, and 563 fewer fertile tillers m^{-2} than the standard single NAM (Table 3). Plant height, lodging, and FTN were greater at the high N rate than the low N rate in both years (Table 3). There was a significant main effect of GRP on plant height and lodging during both

years of this study (Table 2). Applying a GRP reduced plant height by 5 and 7 cm in 2009 and 2010, respectively, and reduced lodging 11 and 33% in 2009 and 2010 respectively (Table 3). A split GRP was consistently more effective than a single GRP for reducing plant height in 2009 and 2010 (4 and 2 cm difference in each year respectively). The efficacy of a split GRP on height was particularly noticeable when the active ingredient was prohexadione Ca (Table 3). Along with a greater reduction in plant height, the split GRP reduced lodging 19 % in 2009 (Table3).

Relative chlorophyll index was significantly affected by NAM on all rating dates in 2009 and 2010. Plots treated with the standard single NAM demonstrated greater RCI than the split NAMs at the first RCI rating date, after all of the total N had been applied to the standard single NAM plots (Fig. 2a,b). By the second RCI rating date in 2009, all of the total N had been applied to the two-split NAM plots and RCI was similar between the two-split and standard single NAMs (Fig. 2a). At the final RCI rating date in 2009 and 2010, 100% of the total N had been applied to all NAMs and the plots treated with the three-split NAM tended to have a greater RCI than the two-split and standard single NAMs (Fig. 2a,b).

Natural stem rust infection was observed and rated at the Exp. 1 site in 2009, however, disease ratings were not taken at the Exp. 1 site in 2010 due to non-uniform rust incidence and extremely light infection. In 2009, the high N rate generally resulted in a lower AUDPC value indicating less severe stem rust disease but the reduction in AUDPC was greatest for the three-split and single standard NAMs at the high N rate (Table 4). Rust severity was also significantly higher in the split prohexadione Ca GRP at the low N rate than for any other GRP.

Experiment 2 (Roosevelt)

Nitrogen application method (NAM) in the spring of the harvest year did not significantly affect seed yield in either year in this experiment and seed yields were near average historic levels for this location (Table 2 and 5) (Ehlke et al., 2011). In 2009, a contrast comparing the standard single NAM to the three-split NAM indicated that the three-split NAM resulted in 189 kg ha^{-1} greater vegetative biomass production (Table 5). The difference due to NAM was only observed at the high N rate, where the three-split NAM resulted in significantly greater biomass production than the single standard NAM (2510 vs. 2114 kg biomass ha^{-1}) (Table 2). The main effect of NAM on biomass production for the 2010 growing season mirrored the results observed in Exp. 1 where the two and three-split NAMs resulted in less vegetative biomass (Table 5). In 2009, the standard single NAM resulted in 2.4% greater HI but in 2010, 1.7% lower HI than the three-split NAM, which is similar to the trend observed in Exp. 1 (Table 5). In both years the main effect of GRP had no effect on biomass production and the high N rate resulted in greater biomass production (Table 5). Applying a GRP improved HI by 2.4 and 3.8% in 2009 and 2010 respectively (Table 5). In 2010, the prohexadione Ca GRPs resulted in greater HI than the trinexapac-ethyl GRPs and applying GRP as a split application resulted in 1.0% greater HI than the single GRP treatments (Table 5). Analysis of variance indicated a GRP by N rate interaction for HI in both years (Table 2). Further examination of the data revealed that in 2009 the single trinexapac-ethyl GRP and in 2010 the single prohexadione Ca GRP resulted in significantly lower HI at the

high N rate than the low N rate (25.4 vs. 29.1 and 13.6 vs. 14.6% in each year respectively).

Nitrogen application method did not affect plant height in 2009 or 2010 in Exp. 2 but did have a significant effect on lodging and FTN in 2009 (Table 2). Plots receiving the three-split NAM demonstrated 1% more lodging than the standard single NAM (Table 5) and the difference was greater at the high N rate (4.3 vs. 1.3% lodging for the three split and standard single NAMs, respectively). Fertile tiller number was greatest in plots treated with the single standard NAM in 2009 but there was no significant difference between the single standard and three-split NAM in either year. Applying a GRP as a split application resulted in plants that were 4 and 2 cm shorter than applying GRP as a single application in 2009 and 2010 respectively, but the benefit was observed mainly in the split prohexadione Ca GRPs (Table 5). In 2010, plots that did not receive a GRP were 90% lodged, which is likely a contributing factor to yield loss, while the split prohexadione Ca GRP resulted in the least amount of lodging (20%) (Table 5). It should be noted that plots treated with the trinexapac-ethyl GRPs were over 50% lodged at harvest time (Table 5), a threshold for yield loss in perennial ryegrass (Rolston et al., 2010a). Analysis of variance indicated that the high N rate promoted lodging but the effect of N rate depended on GRP in some cases (Table 2 and 5). In 2009 the high N rate only resulted in significantly more lodging when no GRP was applied (7.7 vs. 2.8 % lodging at the high and low N rate respectively). In 2010, lodging at harvest time was 6 and 38 % in plots treated with the split prohexadione Ca and single trinexapac-ethyl GRPs at the low N rate, respectively. At the high N rate, percent lodging was 33 and 72% for those same GRPs, the only GRPs in which N rate had a significant effect. In

2010 the split prohexadione Ca GRP resulted in lower FTN (2948 m^{-2}) than all other GRPs but resulted in the greatest seed yield and shortest plant height while not affecting total vegetative biomass (Table 5). This data supports visual observations which indicated that there was more seed produced per fertile tiller and that the vegetative parts of plants may have been more robust and/or had more photosynthetic area. All other GRPs in 2010 resulted in equal or greater FTN compared to the no GRP treatment.

Relative chlorophyll index in Exp. 2 was significantly affected by NAM on five of six rating dates in 2009 and 2010. In 2009, the effect of NAM had a similar trend to what was observed in Exp. 1. Relative chlorophyll index was initially greatest in plots treated with the single standard NAM but at the final rating date the plots treated with the three-split NAM had the greatest RCI (Fig. 3a). In 2010, although ANOVA indicated no differences among the three NAM treatments at the final rating date, a contrast between the standard single and three-split NAMs indicated significantly greater RCI with the three-split NAM (280 vs. 263). Analysis of variance of RCI data in 2010 indicated that GRP also had a significant effect on RCI at the final two rating dates. At the second rating date (30 June), the split prohexadione Ca GRP resulted in greater RCI than the no GRP treatment (811 vs. 736 respectively) while all other GRPs were similar to the no GRP treatment (data not shown). At the third and final rating date in 2010 (18 July), RCI was significantly greater (284 vs. 216) for treatments that received a GRP than the no GRP treatment. The split prohexadione Ca GRP resulted in an RCI of 307 which was significantly greater than both of the trinexapac-ethyl GRPs and the no GRP treatment.

Stem rust was very severe at the Exp. 2 site in 2010, however, due to non-uniform rust incidence and extremely light infection, ratings were not possible in 2009. In 2010, a

contrast comparing the single standard NAM to the three-split NAM indicated that the three-split NAM resulted in an 18% reduction in AUDPC compared to the single standard NAM (Table 6). This was a result of both lower incidence at the first stem rust rating date as well as reduced severity at the second rating date (Table 6). Application of a GRP significantly reduced AUDPC compared to the no GRP treatment, particularly if the GRP was applied as a split application (Table 6). Incidence and severity were not affected by GRP at the first rating date, however, the split prohexadione Ca GRP resulted in 13% lower incidence and about 3% lower disease severity at the second rating date than the no GRP treatment indicating that this GRP may help limit the spread of stem rust within the plant (Table 6).

Seed Quality Characteristics

Experiment 1 (Roseau)

Germination averaged 94% for all treatments in 2009 and 96% for all treatments in 2010 (data not shown). In 2009, the split GRP treatments, in combination with the high N rate, always resulted in TSW and SV statistically equal to the top treatment, however, the single GRP treatments at the high N rate always resulted in TSW and SV that was significantly lower than the top treatment (Table 7). In 2010, GRP by N rate interactions did not affect TSW or SV but N rate and NAM main effects did significantly affect both parameters. The three-split NAM resulted in 3.5 and 4% higher TSW and SV, respectively, than the standard single NAM (Table 8).

Experiment 2 (Roosevelt)

The average germination rate of ryegrass seed harvested from all treatments in this experiment was 93% and 96% in 2009 and 2010 respectively (data not shown). Seedling vigor was unaffected by the treatments imposed in 2009 and 2010 (Table 2). In 2009 the three-split NAM resulted in about 3% greater TSW than the single standard NAM (Table 9). A GRP by NAM interaction was significant in 2009 where the single prohexadione Ca GRP treated with the single standard NAM resulted in 7.4% lower TSW than the two-split NAM (Table 9). All other GRPs resulted in statistically similar TSW regardless of NAM. In 2010, analysis of variance indicated an N rate by NAM interaction as well as a three way interaction for TSW (Table 2). The standard single NAM at the high N rate resulted in lower TSW than the standard single NAM at the low N rate (1.4379 vs. 1.5187 g respectively) while all other NAMs resulted in similar TSW regardless of N rate. In plots treated with the single trinexapac-ethyl GRP, the reduction of TSW at the high N rate vs. the low N rate was more dramatic when combined with the single standard NAM (1.3502 vs. 1.5314 g for the high and low N rate respectively). The difference was not as great for the other GRPs.

DISCUSSION

One of the objectives of this study was to evaluate the effects of foliar-applied split spring N applications on seed yield, agronomic characteristics, and stem rust disease in a perennial ryegrass seed production field in northern Minnesota. The three-split NAM led to differences in these parameters compared to the single standard NAM, however the effect of NAM varied between the two experiments and the years in some cases. Many of the varying NAM effects observed between the two experiments were likely due to differences in soil N availability at each experimental location or varying

weather conditions between years (Fig 1). Although we did not directly measure available soil N in our experiments, it can be assumed that total available soil N was greater in Exp. 2 than in Exp. 1 as a result of a Fall N application and greater total N rates (112 and 156 kg N ha^{-1} for Exp. 2 vs. 56 and 100 kg N ha^{-1} for Exp. 1) applied at the Exp. 2 location. Nitrogen may have been more limiting to plant growth early in the growing season in Exp. 1 due to these imposed fertility differences. Adequate spring N availability in Exp. 2 could have resulted in spring NAM treatments having less of an impact on plant growth (Cookson et al., 2000; Cookson et al., 2001).

Vegetative biomass production was significantly greater in 2010 compared to 2009 for each experiment (4614 vs. 3509 kg ha^{-1} for Exp. 1 and 5094 vs. 2058 for Exp. 2 on average in each year respectively). Vegetative biomass production was significantly and positively correlated with seed yield in each experiment and year except for Exp. 2 in 2010 where lodging was severe (r = 0.60, P<0.001; r = 0.34, P<0.001; r = 0.72, P<0.001; and r = 0.16, P = 0.09 for Exp. 1 in 2009, Exp.1 in 2010, Exp. 2 in 2009, and Exp. 2 in 2010 respectively). In 2010, plots left untreated with a growth regulator were on average 58 and 90 % lodged in Exp. 1 and 2 respectively and lodging was negatively correlated with yield (r=-0.35, P<0.001) in Exp. 2. The 2009 growing season was characterized by cool temperatures and slow growth rates early in spring while GDD accumulation and vegetative growth began earlier at the onset of the 2010 growing season leading to significantly greater and more rapid vegetative biomass accumulation (Fig 1; Table 2, 3; personal observation). In 2010, RCI and vegetative biomass were significantly correlated at the earliest RCI reading for Exp. 1 and Exp. 2 (r=0.60, P<0.001 and r=0.28, P=.003 respectively) but by final RCI reading date, the correlation was weaker for Exp. 1 and

non-significant for Exp. 2 (r=0.21, P=0.022 and r=-0.06, P=0.538 respectively). The opposite trend was observed in 2009 for Exp. 1 and 2 (r=0.71, P<0.0001 and r=0.56, P<0.0001 respectively at the last RCI reading; r=0.29, P=0.002 and r=0.14, P=0.137 respectively at the first RCI reading). This suggests that early season N availability is more important for driving biomass production when biomass production begins early but late season N availability drives biomass production when biomass production is more vigorous later in the growing season. Soil N may have been more available early in the 2010 growing season vs. the 2009 growing season as a result of warmer soil temperatures or a longer duration of warm soil temperatures causing greater mineralization of organic matter to NH_4^+ explaining the greater biomass production (Agehara and Warncke, 2005). Previous research on annual ryegrass (*Lolium multiflorum* Lam.), which is closely related to perennial ryegrass, indicated that N accumulation begins between 333 and 614 GDD (Griffith et al., 1997b). The first N application to perennial ryegrass in this study was made around 243 and 251 GDD in 2009 and 2010 respectively. Soil temperatures at this time as indicated by a local weather station were approximately 7.2 and 12.2°C in 2009 and 2010 respectively. Warmer soil temperatures at or around the time of the first N application in 2010 could have also resulted in more rapid hydrolysis of urea to NH_4^+ and subsequent nitrification to NO_3^- leading to greater soil N availability (MacLean and McRae, 1987).

We hypothesized that using a foliar applied split N management approach during the harvest season could better match N demand of perennial ryegrass for seed production. Through matching the crop N demand, we could possibly improve plant agronomic characteristics which could in turn result in better seed yields and/or reduced

stem rust disease severity. In this study RCI readings were taken as an estimate of relative plant N status and potential photosynthetic activity (Bunderson et al., 2009; Ros et al., 2008). Our RCI data suggests greater N availability in single standard NAM treatments vs. the three split NAMs early in the growing season but greater N content and photosynthetic activity from the three split NAMs later in the growing season at the time of seed development and the predicted time of first stem rust infection (Fig. 2a,b). A study evaluating plant N accumulation in annual ryegrass, found that maximum plant N accumulation occurred around mid-head emergence (Griffith et al., 1997b). The final foliar N application for the three-split NAM in our studies occurred just prior to mid-head emergence, a time of significant N accumulation, possibly explaining the greater RCI that we observed in the three-split NAM later in the growing season following the final N application. It appears a split NAM approach may be effective in preventing excess N availability and uptake early in the growing season during vegetative stages of growth while providing adequate N during periods of high N demand for seed development.

Although all NAMs resulted in average or better seed yields compared to historical averages, split NAMs resulted in lower seed yields than the single standard NAM in Exp. 1 but equivalent seed yields in Exp. 2 (Table 3 and 5). The positive yield response as a result of the single standard NAM in Exp. 1 may have occurred because no N was applied to the site in the fall following seeding resulting in N deficient soils in the spring of the harvest year. When N is limiting at an early growth stage of a perennial ryegrass seed crop, a timely N application has been shown to significantly increase seed yields (Cookson et al., 2000). Seed yield in our Exp. 1 was positively correlated with the first RCI readings (an indicator of N status) in both years (r = 0.56 and 0.31, $P<0.01$).

The plots treated with the standard single NAM in Exp. 1 may have benefited from having more N available at an early growth stage compared to the split NAMs. Greater N availability early in spring with the single standard NAM vs. the three-split NAM may have resulted in increased early season tillering and growth leading to greater seed yield and vegetative biomass production, and lower 2010 HI (Table 3) (Cookson et al., 2000; Boelt and Studer, 2010). In Exp. 2, it is likely that N availability was sufficient in early spring following a fall N application to support N demands of the plant so the first N application had less of an effect on plant growth compared to Exp. 1. In Exp. 2, the final RCI readings were more highly correlated with seed yield than the first RCI readings (r = 0.34 and 0.40, $P<0.001$ for Exp. 2 2009 and 2010 final RCI readings respectively) suggesting that early harvest season N availability was less critical for obtaining a seed yield response and that split N applications may be more appropriate when a fall N application has been made.

Biomass production generally increases and HI generally decreases with greater N availability in perennial ryegrass as was observed in our study and is often observed in small grain crops (Table 3 and 5) (Chen et al., 2011; Cookson et al., 2000). The effect of NAM on biomass and HI in our study may have been caused by differences in N availability during periods of vigorous vegetative growth however the effects were drastically different in each year, likely due to weather, plant growth patterns, and timing of N application. Our results indicated that a three-split NAM significantly reduced vegetative biomass production and increased HI in 2010 but the opposite trend was observed in 2009 in both experiments (Table 3 and 5). The time to maximum shoot production in annual ryegrass can vary by at least 800 GDD and it is likely that maximum

biomass production in our study occurred at greater GDD in 2009 than in 2010 based on weather and plant growth patterns discussed above (Griffith et al., 1997a). Maximum biomass production at later GDD in 2009 would have coincided with the second and third N applications in the split NAMs while maximum biomass production occurred earlier in 2010 and coincided with the first N application in the standard single NAM (Cookson et al., 2001; Griffith et al., 1997a; Simon and Lemaire, 1987).

Nitrogen application method significantly affected plant height, lodging, and FTN in the perennial ryegrass seed crop, particularly in Exp. 1 where N may have been limiting (Table 3). In Exp.1, greater N in spring with the single standard NAM vs. the three-split NAM may have resulted in increased early season tillering, a trend documented in other studies (Simon and Lemaire, 1987; Cookson et al., 2000). In Exp. 1 there was a positive correlation between the earliest RCI reading and FTN in both years (r = 0.19 and 0.39, $P<0.05$ and 0.001 in 2009 and 2010 respectively). Greater tillering early in the growing season could lead to more fertile tillers, greater vegetative biomass and lower HI which was observed during the 2010 growing season in our study (Table 3) (Cookson et al., 2000; Boelt and Studer, 2010). Shorter plant height and less lodging in the plots treated with the 3-split NAM vs. the standard single NAM could be attributed to N being applied in multiple smaller quantities and later in the season when N demand for reproductive growth is high and vegetative growth has slowed. Shorter plant height and less lodging could also result from the application of a growth regulator which would limit cell elongation caused by N fertilization (Cookson et al., 2000; Griffith et al., 1997b). The decrease in lodging as a result of the three-split NAM is consistent with a study in New Zealand where N application was delayed until heading resulting in lower

severity of lodging in perennial ryegrass (Hebblethwaite and Ivins, 1978). Our data suggest that N applied as a foliar 3-split program could be used to improve HI and limit excess vegetative growth if timed properly according to GDD and plant growth stage.

The second objective of our study was to determine how common management variables such as GRP or N rates interact with NAM's or affect yield and agronomic characteristics of a perennial ryegrass seed crop in northern Minnesota. Interactions between GRP's and NAM's were generally insignificant however the effects of GRP's on yield and other agronomic characteristics were important due to GRP control of excess vegetative biomass production and GRP reduction of lodging in each experiment (Table 3 and 5). In both experiments, applying a growth regulator generally resulted in shorter plant heights and less lodging (well below the 50% threshold for significant yield loss) than if no growth regulator was applied (Table 3 and 5) (Rademacher, 2000). Applying a growth regulator improved seed yields when the risk for lodging was severe which is consistent with other studies (Borm and van den Berg, 2008; Rolston et al., 2010a). Seed yield in perennial ryegrass is dependent on the amount of carbohydrates transported to the seed (Trethewey and Rolston, 2009), so any GRP treatment, which results in an increase in water-soluble carbohydrate (WSC) concentration or greater ability to produce or transport WSC, should improve seed yields. Total vegetative biomass was rarely affected by the application of a growth regulator yet plant height was reduced, HI was improved and seed yields were generally higher in treated vs. untreated plots (Table 3 and 5). These results, based on field observations and previous research, suggest that GRP treated perennial ryegrass plants in our study had greater leaf area, had more robust stem and shoot tissue, and diverted more photosynthetic assimilates to seed production

than non-treated plants which tended to be tall, thin, and prone to lodging. Upright and robust stems, leaves, and seedheads with high chlorophyll content are an important source of photosynthetic assimilates during seed fill and may maintain photosynthetic activity and transport WSC longer resulting in greater seed yields and HI (Trethewey and Rolston, 2009).

The split GRPs were generally more effective at regulating plant height than the single GRPs. Timing of application for gibberellic acid (GA) inhibitors is critical in perennial ryegrass seed production and it is likely that the split application resulted in a larger window of growth regulation (Borm and van den Berg, 2008; Rolston et al., 2010a). Consistent and significant reduction in plant height was most often observed in plots treated with the split prohexadione Ca treatment in both of our experiments (Table 3 and 5). The split prohexadine Ca treatment also proved to be most effective at reducing lodging, limiting seed yield loss, and improving HI in Exp. 2 during the 2010 growing season when the risk of lodging was most severe and lodging was negatively correlated with seed yield (r= -0.35, P<0.001). It should be noted that the trinexapac-ethyl treatments did not reduce the amount of lodging to below the yield loss threshold (Rolston et al., 2010a) (Table 5). A study evaluating the timing of trinexapac-ethyl application on perennial ryegrass in the Netherlands found no benefit of a split application of this growth regulator and suggested that trinexapac-ethyl has inconsistent effects on seed yield (Borm and van den Berg, 2008), which corresponds with historical and current observations at our experimental locations (Vellekson, 2010; Ehlke et al., 2011). Farmers may benefit from additional studies evaluating a wider range prohexadione Ca application rates and split application timings. The development of a

GDD based model for predicting plant growth regulator application timing that takes into account weather, crop stage, and plant N status would be beneficial.

Seedling vigor was positively correlated with TSW in Exp. 1 in 2009 and Exp. 2 in 2010 (r = 0.32 and 0.29 respectively, P<0.01). Seedling vigor was positively correlated with the final RCI readings in Exp. 1 in 2010 and Exp. 2 in 2009 (r = 0.34 and 0.27 respectively, P<0.01). The results from our study suggest that TSW and N availability may be important for SV and that under N limiting conditions, TSW can be increased by applying N as a split foliar application. We observed an increase in TSW and SV in plots treated with a three-split NAM vs. a single standard NAM in Exp. 1 during the 2010 growing season and an increase in TSW in Exp. 2 conducted during the 2009 growing season. In Exp. 1, for the 2010 growing season, an increase in TSW and SV at the higher N rate was also observed indicating that higher N availability may promote seed fill and seedling vigor under N limiting conditions. In another study conducted in New Zealand, TSW increased with greater N availability and SV increased in a linear relationship with TSW (Cookson et al., 2000). Our data, as evidenced by RCI and plant growth characteristics discussed above, N may have been more available later in the growing season in plots treated with the three-split NAM. Greater N availability could have delayed leaf senescence, improved photosynthetic capacity to produce WSC for seed fill, or increased N content in harvested seed (Chen et al. 2011; Trethewey and Rolston, 2009) resulting in greater TSW or SV.

In Exp. 2 during the 2010 growing season, where N was likely not limiting, our results show that using a single standard NAM in combination with the high N rate resulted in lower TSW than the low N rate but this effect was observed in plots treated

with the single trinexapac-ethyl GRP. The single trinexapac-ethyl GRP did not adequately reduce lodging in 2010 so it is likely that the high N rate applied as a single standard NAM early in the growing season resulted in excess lodging which could limit photosynthesis or restrict WSC flow through stem tissue during seed fill (Trethewey and Rolston, 2009; Rolston et al., 2010a).

Data from Exp. 1 in 2009 also indicate that the effects of N rate on TSW and SV may depend on GRP. A split application of a GRP generally results in a positive TSW and SV response to N rate while plots treated with single application of a GRP demonstrated a negative TSW and SV response to N rate (Table 7). The split GRPs were generally more effective at reducing plant height and lodging (Table 3). Previous studies have shown that GA inhibitors can increase WSC within grass plants following application so the positive TSW and SV response to N rate could be due to a combination of increased WSC production and WSC transport during seed fill as a result of greater efficacy of the split GRP application (Han et al., 2004, Rolston et al., 2010b). Vegetative biomass accumulation in Exp. 1 occurred relatively late during the 2009 growing season and it is possible that the lack of efficacy with the single GRP applications was due to metabolism of the active ingredient by the plant before vegetative growth had ceased. Split applications of a GRP may be more effective at providing season long vegetative growth suppression than single applications at higher rates (Kreuser and Soldat, 2011), which could potentially result in higher quality seed in a seed production field. It is possible that the negative TSW and SV response to N rate in plots treated with a single GRP could be a result of a flush of vegetative growth often observed after a GA inhibitor has been metabolized by the plant (Ervin and Zhang, 2008; Fagerness and Yelverton,

2000). Vegetative growth enhancement just prior to seed fill could potentially divert WSC away from the seed head during grain fill. Further research on longevity of GA inhibitors in a perennial ryegrass seed crop and proper application intervals is warranted.

Stem rust severity level, and management conditions for Exp. 2 during 2010, were most representative of disease severity and management during a typical yield-limiting stem rust outbreak in northern Minnesota. Where total N rates were at least 100 kg ha^{-1} the three-split NAM resulted in the lowest stem rust severity (Table 4 and 6), especially in Exp. 2 during the 2010 harvest season. The lower stem rust incidence levels in plots treated with the three-split NAM at the first stem rust rating date in Exp. 2 indicate that the three-split NAM could help the ryegrass crop resist initial infection and in turn delay or reduce stem rust disease affecting the crop. Our results indicate greater RCI later in the growing season in the three-split NAM treatments around the time of first stem rust incidence. The final RCI reading in Exp. 2 was negatively correlated with AUDPC (r = -0.20, $P<0.05$). Greater availability of N can result in greater expression of photosynthetic genes as well as plant defense related genes and may result in plants investing more energy into a plant defense response (Ros et al., 2008; Walls et al., 2005). These results suggest potential for further improvements in stem rust disease management using cultural practices, which may be important considering the complexity of the stem rust pathogen and a current lack of genetic resistance to stem rust pathogens in perennial ryegrass seed crops.

Additionally, in Exp. 2, during the 2010 harvest season, the split prohexadione Ca GRP resulted in 13% lower incidence and about 3% lower disease severity at the second rating date than the no GRP treatment indicating that the split prohexadione Ca GRP may

help limit the spread of stem rust within the plant (Table 6). The positive benefit of the split prohexadione Ca GRP in Exp. 2 during 2010 in terms of stem rust severity is in contrast to what was seen in Exp. 1 in 2009. We believe higher N rates in Exp. 2 in 2010 were more favorable for seed production, biomass production and the potential production of plant defense compounds, which may explain the contrasting results between experiments. In Exp. 1, the greater stem rust AUDPC in plots treated with the split prohexadione Ca GRP at the low N rate also could have been partially due to more favorable microclimate effects for stem rust growth or greater pathogen movement within the canopy as a result of a more sparse plant canopy with significantly less biomass. A study evaluating the effect of barley stand density on stem rust (*Puccinia graminis* f. sp. *tritici*) determined that rust severity increased as plant stand density decreased (Dill-Macky and Roelfs, 2000) which is consistent with our observations in Exp. 1. In Exp. 2 during 2010 the split prohexadione Ca GRP resulted in greater RCI, fewer fertile tillers, and shorter plant height than all other GRPs while maintaining the same level of vegetative biomass indicating that plants may have been more robust (Table 5). A more robust plant with a higher RCI may have greater photosynthetic capacity, leaf N content, and ability to produce plant defense compounds (Bingham and Newton, 2009; Ros et al., 2008; Walls et al., 2005).

Other research has shown that grasses treated with a GA inhibitor have greater chlorophyll content and cell density (Ervin and Koski, 2001). Greater cell density, and potential cell wall thickening as a result of less cell elongation caused by the application of a GA inhibitor, could make it more difficult for the stem rust infection hyphae to penetrate plant cells. This may limit the infection area from a single stem rust

urediniospore. In beans (*Phaseolus vulgaris* L.), application of active GA results in decreased silica deposition and possibly fewer phenolic compounds in mesophyll cells along with an increase in tissue susceptibility to rust haustorium formation (Li and Heath, 1990). If GA has similar effects on perennial ryegrass and its interaction with stem rust, significant inhibition of active GA in our Exp. 2 with the split prohexadione Ca GRP may have resulted in increased silica and phenolic compound deposition potentially explaining the decrease in tissue susceptibility to rust fungi (Li and Heath, 1990).

Antifungal compounds which are produced constitutively by plants or that are induced by disease are often products of the phenylpropanoid pathway which may have been impacted by side activities of the growth regulator application or resulting metabolic effects (La Camera et al., 2004; Poland et al., 2009; Rademacher, 2000). Another possible scenario would be that the split application of prohexadione Ca directly affected growth or sterol metabolism of the stem rust urediniospores (Rademacher, 2000). However, the last growth regulator application consisted of a relatively low rate (134.5 g a.i. ha^{-1}) and was applied 305 GDD prior the second stem rust rating date making it unlikely that the active ingredient would be present in sufficient concentrations to affect fungal metabolism considering that the half life of GA inhibitors in turfgrass is thought to be 100 GDD (Beasley and Branham, 2005).

CONCLUSION

In perennial ryegrass seed production fields, split applications of foliar applied N can be used to increase N availability and uptake later in the harvest season and minimize excess N availability early in the harvest season during vegetative growth stages resulting

in improved plant growth and seed quality characteristics. Where N is not limiting in the spring, the split N application approach resulted in equivalent seed yields to the single standard application approach and provided many added benefits to crop agronomic characteristics and seed quality. Some of the benefits of using a three-split application approach to N fertilization in a perennial ryegrass seed crop included: an 18-39% reduction in stem rust AUDPC, 6-20% greater RCI later in the harvest season, 8-19% less vegetative biomass production during years with early and vigorous vegetative growth, greater TSW and SV, and a more consistent and positive response to N rate and GRPs in terms of seed quality. In cases where no fall N has been applied to a perennial ryegrass seed production field and N is limiting in the spring of the harvest season, a single application of granular urea may still provide up to 14% greater seed yields than the split application approach. Additional research regarding split N applications should be conducted as it is likely that N rates and timing could be adjusted based on crop growth stage, plant N status, and weather conditions to improve seed yields in fields that are N limited. Cost effectiveness of split applications of foliar N and GA inhibitors will need to be evaluated based on the variable cost of inputs as well as the value of each individual ryegrass crop.

This research indicates that growth regulators generally did not impact the effects of split N applications, however, the results confirm that the application of a growth regulator is necessary to limit lodging as well as to maximize HI and seed yields in a perennial ryegrass seed crop in northern Minnesota. Split applications of a growth regulator, particularly prohexadione Ca, appear to be more effective than single applications (of the same overall rate) at controlling excess vegetative growth, preventing

lodging, reducing severity of stem rust and improving seed quality characteristics. Split applications of growth regulators appear to have significant positive benefits for crop agronomic characteristics, resistance to stem rust pathogens, and seed quality. Farmers may benefit from additional research and development of a model to suggest optimum rates and timings for growth regulator applications based on the crop growth stage, plant N status, soil N availability and GDD in the different perennial ryegrass seed growing regions.

Table 1. Spring treatment application rates and application timing for nitrogen application methods (NAM) and growth regulator programs (GRP), for Experiment 1 (Roseau, MN) and Experiment 2 (Roosevelt, MN), 2009 and 2010.

	Spring Application Rates†		2009		2010	
Treatment	**Low N Rate**	**High N Rate**	**Application Dates**	**Growing Degree Days**	**Application Dates**	**Growing Degree Days**
Nitrogen Application Method (NAM)‡	kg N ha^{-1}					
Standard Single	56	100	14 May	243	24 April	251
Two-Split	28 + 28	50 + 50	14 May + 14 June	243 + 572	24 April + 3 June	251 + 727
Three-Split	18.7 + 18.7 + 18.7	33.3 + 33.3 + 33.3	14 May + 14 June + 1 July	243 + 572 + 889	24 April + 3 June + 21 June	251 + 727 + 998
Growth Regulator Program (GRP)	g a.i. ha^{-1}					
Prohexadione Calcium (Split)	134.5 + 134.5		14 June + 1 July	572 + 889	3 June + 21 June	727 + 998
Trinexapac-ethyl (Split)	105 + 105		14 June + 1 July	572 + 889	3 June + 21 June	727 + 998
Prohexadione Calcium (Single)	269		14 June	572	3 June	727
Trinexapac-ethyl (Single)	210		14 June	572	3 June	727

† All spring application rates were identical for similar treatments in Exp. 1 and 2.

‡The standard single NAM was applied as granular urea and the split NAM treatments were applied as foliar N using urea dissolved in water and sprayed with a backpack sprayer.

Table 2. Analysis of variance for seed yield, vegetative biomass, harvest index (HI), plant height (PH), lodging, fertile tiller number (FTN), one thousand seed weight (TSW), seedling vigor (SV), and area under the disease progress curve (AUDPC) calculated from stem rust severity ratings for Experiment 1 (Roseau, MN) and Experiment 2 (Roosevelt, MN), 2009 and 2010.

Source of variation	Yield	Biomass	HI	PH	Lodging	FTN	TSW	SV	Rust
Experiment 1					2009				
Growth regulator program (GRP)	*	**	**	**	**	NS†	NS	NS	**
N Rate (R)	**	**	**	**	*	**	NS	NS	**
GRP x R	NS	NS	NS	NS	NS	NS	*	*	**
N application method (NAM)	**	NS	**	NS	NS	NS	NS	NS	NS
GRP x NAM	NS	NS	NS	NS	NS	NS	NS	NS	NS
R x NAM	NS	NS	NS	NS	NS	NS	NS	NS	*
GRP x R x NAM	NS	NS	NS	NS	NS	NS	NS	NS	NS
					2010				
Growth regulator program (GRP)	**	NS	**	**	**	NS	NS	NS	--
N rate (R)	*	**	**	**	**	**	**	*	--
GRP x R	NS	NS	NS	NS	NS	NS	NS	NS	--
N application method (NAM)	**	**	**	**	**	**	*	*	--
GRP x NAM	NS	NS	NS	NS	NS	NS	NS	NS	--
R x NAM	NS	NS	**	NS	NS	NS	NS	NS	--
GRP x R x NAM	NS	NS	NS	NS	NS	NS	NS	NS	--
Experiment 2					2009				
Growth regulator program (GRP)	NS	NS	**	**	**	NS	NS	NS	--
N rate (R)	**	**	**	**	**	NS	NS	NS	--
GRP x R	NS	NS	*	NS	*	NS	NS	NS	--
N application method (NAM)	NS	NS	**	NS	*	*	**	NS	--
GRP x NAM	NS	NS	NS	NS	NS	NS	*	NS	--
R x NAM	NS	*	NS	NS	*	NS	NS	NS	--
GRP x R x NAM	NS	NS	NS	NS	NS	NS	NS	NS	--
					2010				
Growth regulator program (GRP)	**	NS	**	**	**	*	NS	NS	**
N rate (R)	NS	**	NS	NS	**	NS	**	NS	NS
GRP x R	NS	NS	*	*	**	NS	NS	NS	NS
N application method (NAM)	NS	**	**	NS	NS	NS	NS	NS	NS
GRP x NAM	NS	NS	NS	NS	NS	NS	NS	NS	NS
R x NAM	NS	NS	NS	NS	NS	NS	*	NS	NS
GRP x R x NAM	NS	NS	NS	NS	NS	NS	**	NS	NS

*Significantly different at the 0.05 probability level.

** Significantly different at the 0.01 probability level.

† NS indicates not significant P<0.05.

Table 3. Main effects of growth regulator program (GRP), spring N rate, and N application method (NAM) on means for yield, biomass, harvest index (HI), plant height (PH), lodging, and fertile tiller number (FTN). Experiment 1 (Roseau, MN), 2009 and 2010.

	Yield		Biomass		HI		PH		Lodging		FTN	
	2009	2010	2009	2010	2009	2010	2009	2010	2009	2010	2009	2010
	----------kg ha^{-1}----------				---------%---------		-------cm-------		-------%-------		----no. m^{-2}----	
Growth Regulator Program (GRP)												
Prohexadione Ca (Split)	1204 a†	1217 ab	3047 b	4566 a	28.2 a	21.8 a	54 d	55 c	4 b	25 bc	2481 a	2813 a
Trinexapac-ethyl (Split)	1284 a	1149 b	3836 a	4456 a	25.3 bc	21.4 a	62 bc	59 b	14 ab	32 b	2328 a	2696 a
Trinexapac-ethyl (Single)	1299 a	1302 a	3482 ab	4573 a	27.0 ab	22.5 a	61 c	59 b	24 a	16 c	2451 a	2748 a
Prohexadione Ca (Single)	1272 a	1246 a	3537 a	4632 a	26.7 ab	21.6 a	64 ab	59 b	33 a	28 b	2304 a	2561 a
No GRP	1140 b	1105 b	3640 a	4844 a	24.1 c	19.4 b	65 a	65 a	29 a	58 a	2442 a	2520 a
Mean	1240	1204	3508	4614	26.3	21.3	61	59	21	32	2401	2668
Contrast: no GRP minus all others§	-125 **	-123 **	165 NS	288 NS	-2.7 **	-2.4 **	5 **	7 **	11 NS	33 **	51 NS	-185 NS
Contrast: split minus single	-42 NS‡	-91 *	-68 NS	-91 NS	0.0 NS	0.0 NS	-4 **	-2 **	-19 **	6 NS	26 NS	100 NS
Spring N Rate (kg ha^{-1})												
56	1125 b	1239 a	3046 b	3762 b	27.1 a	25.1 a	59 b	57 b	15 b	14 b	2242 b	2476 b
100	1356 a	1168 b	3971 a	5466 a	25.5 b	17.7 b	63 a	61 a	26 a	49 a	2560 a	2859 a
N Application Method												
Standard single	1337 a	1312 a	3452 a	5153 a	28.0 a	20.6 b	61 a	61 a	19 a	43 a	2543 a	2986 a
Two-split	1224 b	1175 b	3410 a	4507 b	26.6 b	21.3 ab	61 a	60 a	20 a	35 a	2307 a	2593 b
Three-split	1160 b	1126 b	3664 a	4183 b	24.2 c	22.1 a	62 a	57 b	23 a	18 b	2353 a	2423 b
Contrast: single minus three-split	177 **	186 **	-212 NS	970 **	3.8 **	-1.5 **	-1 NS	4 **	-3 NS	25 **	191 NS	563 **

*Significantly different at the 0.05 probability level.
** Significantly different at the 0.01 probability level.
† Means followed by the same letter within a column and section are not statistically different from one another at P<0.05.
‡ NS indicates not significant at P<0.05.
§ Values shown for each contrast are the difference between the sum of the means of the first group minus the sum of the means of the second group.

Table 4. Interaction of growth regulator program (GRP) or N application method (NAM) with spring N rate and the effect on area under the disease progress curve (AUDPC) calculated from stem rust severity data. Experiment 1 (Roseau, MN), 2009.

	Spring nitrogen rate (kg N ha^{-1})	
Source of Variation	**56**	**100**
Growth Regulator Program (GRP)	----Rust severity, AUDPC----	
Prohexadione Ca (Split)	45 a†	21 b
Trinexapac-ethyl (Split)	21 b	20 b
Trinexapac-ethyl (Single)	19 b	13 b
Prohexadione Ca (Single)	12 b	22 b
No GRP	27 b	15 b
N Application Method (NAM)		
Single standard	27 ab	17 b
Two-split	19 ab	21 ab
Three-split	28 a	17 b
Mean	25*	18

* Indicates the mean main effect of spring N rate is significant at the 0.05 probability level.
† Means followed by the same letter within GRP or NAM are not statistically different from one another at $P < 0.05$.

Table 5. Main effects of growth regulator program (GRP), spring N rate, and N application method (NAM) on seed yield, vegetative biomass, harvest index (HI), plant height (PH), lodging, and fertile tiller number (FTN). Experiment 2 (Roosevelt, MN), 2009 and 2010.

	Yield		Biomass		HI		PH		Lodging		FTN	
	2009	2010	2009	2010	2009	2010	2009	2010	2009	2010	2009	2010
	----------kg ha^{-1}----------				---------%---------		---------cm---------		--------%---------		------no. m^{-2}------	
Growth Regulator Program (GRP)												
Prohexadione Ca (Split)	714 a†	980 a	1922 a	5037 a	27.5 a	16.4 a	42 c	56 c	0 b	20 d	2236 a	2948 c
Trinexapac-ethyl (Split)	744 a	841 b	2053 a	5147 a	26.8 a	14.1 b	46 b	61 b	0 b	78 a	2085 a	3383 a
Trinexapac-ethyl (Single)	779 a	812 b	2085 a	5078 a	27.3 a	13.8 b	47 b	61 b	1 b	55 b	2065 a	3012 b
Prohexadione Ca (Single)	749 a	872 ab	2056 a	5046 a	26.8 a	14.6 ab	48 b	61 b	1 b	35 c	2095 a	3350 ab
No GRP	696 a	623 c	2171 a	5158 a	24.7 b	10.9 c	54 a	70 a	5 a	90 a	2002 a	3017 b
Mean	736	826	2057	5093	26.6	14.0	47	62	1.4	56	2097	3142
Contrast: no GRP minus all others§	-50 NS‡	-253 **	142 NS	81 NS	-2.4 **	-3.8 **	8 **	10 **	4.7 **	44 **	-118 NS	-156 NS
Contrast: split minus single	-36 NS	68 *	-84 NS	30 NS	0.0 NS	1.0 *	-4 **	-2 **	-0.9 NS	3 NS	80 NS	-15 NS
Spring N Rate (kg ha^{-1})												
56	700 b	819 a	1849 b	4945 b	27.5 a	14.1 a	45 b	62 a	1 b	47 b	2075 a	3064 a
100	774 a	832 a	2266 a	5242 a	25.7 b	13.8 a	49 a	62 a	2 a	64 a	2118 a	3220 a
N Application Method												
Standard single	752 a	814 a	1973 a	5378 a	27.8 a	13.1 b	47 a	61 a	1 b	54 a	2215 a	3176 a
Two-split	729 a	804 a	2038 a	4956 b	26.5 b	14.0 ab	48 a	62 a	1 b	60 a	1985 b	3080 a
Three-split	728 a	857 a	2161 a	4945 b	25.5 b	14.8 a	48 a	62 a	2 a	53 a	2090 ab	3171 a
Contrast: single minus three-split	25 NS	-43 NS	-189 *	433 **	2.4 **	-1.7 **	-1 NS	1 NS	-1 *	1 NS	124 NS	5 NS

*Significantly different at the 0.05 probability level.
** Significantly different at the 0.01 probability level.
† Means followed by the same letter within a column and section are not statistically different from one another at P<0.05.
‡ NS indicates not significant at P<0.05.
§ Values shown for each contrast are the difference between the sum of the means of the first group minus the sum of the means of the second group.

Table 6. Rust incidence, severity, and area under the disease progress cure (AUDPC) as affected by main effects of growth regulator program (GRP), N rate, and nitrogen application method. Experiment 2 (Roosevelt, MN), 2010.

	25 June		7 July		
	Incidence	**Severity**	**Incidence**	**Severity**	**AUDPC**
	----------%----------				
Growth Regulator Program (GRP)					
Prohexadione Ca (Split)	26 a†	1.0 a	73 b	5.1 b	37 c
Trinexapac-ethyl (Split)	22 a	1.2 a	79 ab	6.0 ab	42 bc
Trinexapac-ethyl (Single)	22 a	0.8 a	82 ab	6.6 ab	45 abc
Prohexadione Ca (Single)	23 a	1.1 a	88 a	7.6 a	52 ab
No GRP	28 a	1.4 a	86 a	8.0 a	57 a
Mean	24	1.1	82	6.7	47
Contrast: no GRP minus all others§	5 NS‡	0.4 NS	6 NS	1.7 **	13 **
Contrast: split minus single	2 NS	0.1 NS	-9 **	-1.6 **	-9 *
Spring N Rate (kg ha^{-1})					
56	23 NS	1.0 NS	82 NS	6.7 NS	46 NS
100	26 NS	1.2 NS	81 NS	6.6 NS	47 NS
N Application Method					
Standard single	31 a	1.3 a	85 a	7.3 a	51 a
Two-split	23 b	1.1 a	79 a	6.6 a	46 a
Three-split	19 b	1.0 a	80 a	6.1 a	42 a
Contrast: single minus three-split	12 **	0.3 NS	5 NS	1.2 *	9 *

*Significantly different at the 0.05 probability level.
** Significantly different at the 0.01 probability level.
† Means followed by the same letter within a column and section are not statistically different from one another at P<0.05.
‡ NS indicates not significant at P<0.05.

Table 7. Interaction of growth regulator program with spring N rate and its effect on thousand seed weight (TSW) and seedling vigor (SV). Experiment 1 (Roseau, MN), 2009.

	TSW		SV	
	Low N Rate†	High N Rate	Low N rate	High N Rate
Growth Regulator Program (GRP)	g 1000 seeds^{-1}		mg 100 seedlings^{-1}	
Prohexadione Ca (Split)	1.6215 ab‡	1.5858 ab	51.171 b	54.220 ab
Trinexapac-ethyl (Split)	1.5750 ab	1.6729 a	51.856 ab	54.006 ab
Trinexapac-ethyl (Single)	1.5672 b	1.5587 b	53.912 ab	50.781 b
Prohexadione Ca (Single)	1.5873 ab	1.5598 b	55.261 a	50.704 b
No GRP	1.5709 ab	1.5764 ab	53.273 ab	53.302 ab
Mean	1.5844	1.5907	53.095	52.603

† The total spring N rates were 56 or 100 kg ha^{-1}.

‡ Means followed by the same letter within TSW or SV are not statistically different from one another at $P < 0.05$.

Table 8. Main effect of N rate and nitrogen application method (NAM) on thousand seed weight (TSW) and seedling vigor (SV). Experiment 1 (Roseau, MN), 2010.

Source of variation	TSW	SV
Spring N Rate (Kg ha^{-1})	g 1000 $seeds^{-1}$	mg 100 $seedlings^{-1}$
56	1.6044 b†	56.352 b
100	1.6551 a	58.648 a
N Application Method (NAM)		
Standard single	1.6019 b	56.847 ab
Two-split	1.6276 ab	56.433 b
Three-split	1.6597 a	59.220 a
Contrast: single minus three-split‡	-0.0578 **	-2.372*

*Significantly different at the 0.05 probability level.
** Significantly different at the 0.01 probability level.
† Means followed by the same letter within each column and source of variation are not statistically different from one another at $P < 0.05$.
‡ Values shown for each contrast are the difference between the sum of the means of the first group minus the sum of the means of the second group.

Table 9. Thousand seed weight (TSW) as affected by the interaction between growth regulator program (GRP) and N application method (NAM). Experiment 2 (Roosevelt, MN), 2009.

	Nitrogen application method		
	Single standard	**Two-split**	**Three-split**
	----------g----------		
Growth Regulator Program (GRP)			
Prohexadione Ca (Split)	1.4679 ab†	1.5363 a	1.5354 a
Trinexapac-ethyl (Split)	1.4754 ab	1.5061 ab	1.5629 a
Trinexapac-ethyl (Single)	1.5201 ab	1.5510 a	1.4945 ab
Prohexadione Ca (Single)	1.4303 b	1.5449 a	1.5176 ab
No GRP	1.5107 ab	1.4950 ab	1.5390 a
Mean	1.4809	1.5267	1.5299
Contrast: single standard mean minus three-split mean‡	-0.0490 **		

*Significantly different at the 0.05 probability level.
** Significantly different at the 0.01 probability level.
† Means followed by the same letter not statistically different from one another at $P < 0.05$.
‡ Values shown for the contrast are the difference between the mean of the first group minus the mean of the second group.

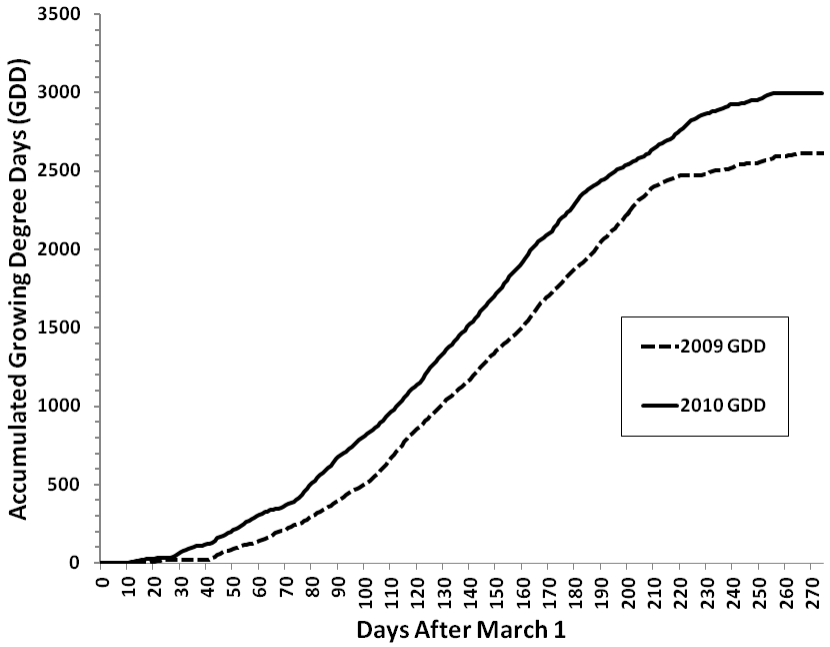

Figure 1. Accumulated growing degree days (GDD) for the Experiment 1 and Experiment 2 region calculated using a base temperature of 0° C starting March 1 in 2009 and 2010. The weather station to provide all GDD data was located at the GPS coordinates 48.655 N and 95.734 W.

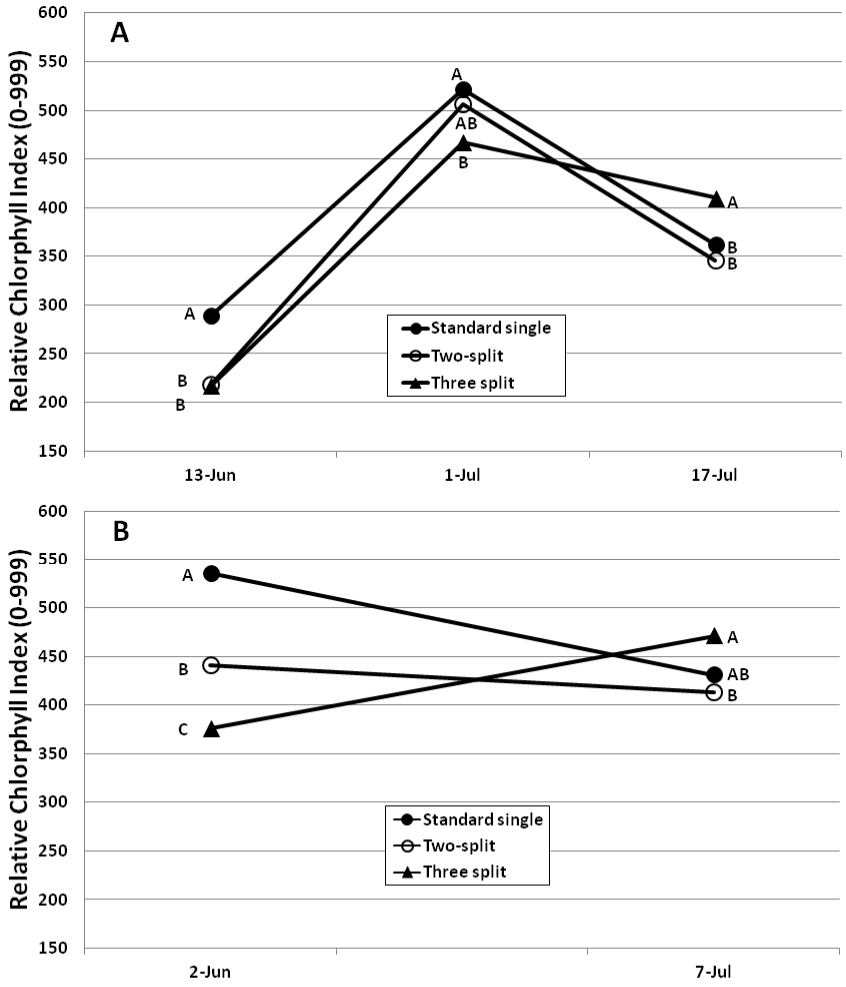

Figure 2. Relative chlorophyll index (RCI) for the three nitrogen application methods (NAM) on three rating dates in 2009 (A) and 2010 (B) for Experiment 1 in Roseau, MN. Symbols noted with the same letter on not significantly different according to Tukey's HSD at $P < 0.05$.

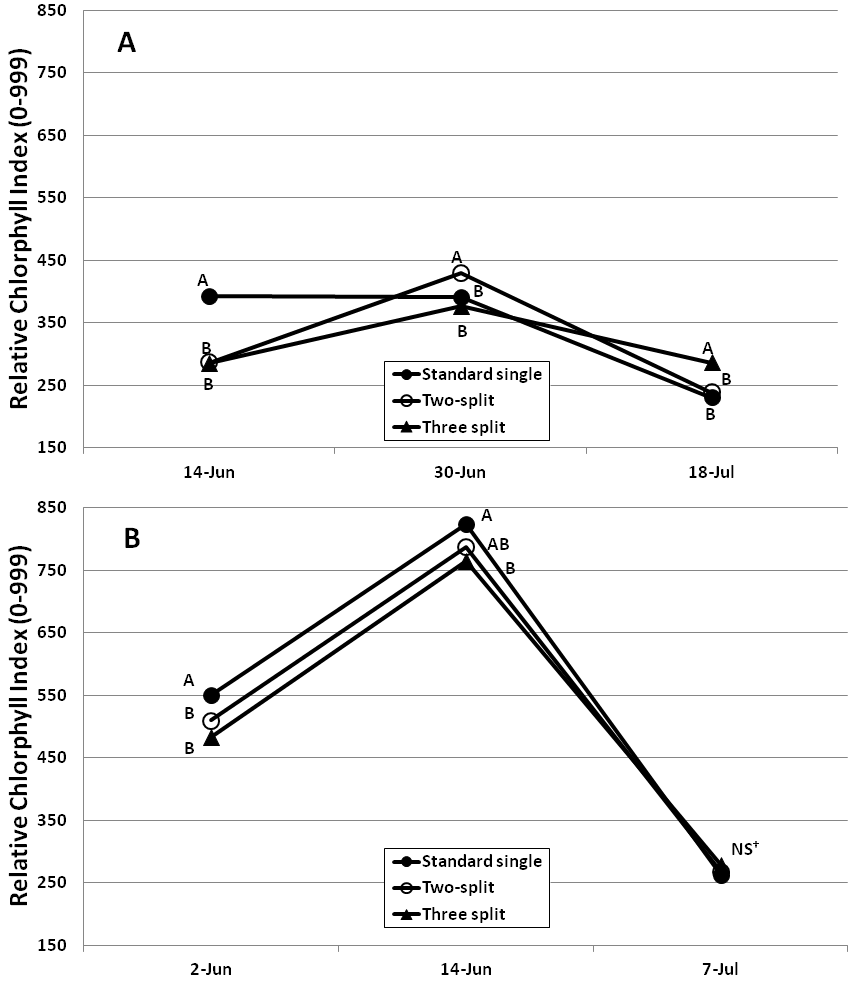

Figure 3. Relative chlorophyll index (RCI) for the three nitrogen application methods (NAM) on three rating dates in 2009 (A) and 2010 (B) for Experiment 2 in Roosevelt, MN. Symbols noted with the same letter on not significantly different according to Tukey's HSD at $P < 0.05$. † Indicates no significant difference among treatments according to analysis of variance however contrasts indicate a significant difference between the standard single and three-split NAM.

CHAPTER 2.

Seeding Rate, Row Spacing, and Nitrogen Rate Effects on Perennial Ryegrass Seed Production

Eric J. Koeritz*, Eric Watkins, and Nancy J. Ehlke

OVERVIEW

Seeding rates (SRs) and row spacing widths (RSWs) in perennial ryegrass seed production are variable and the impacts of these factors on perennial ryegrass seed yield and plant growth characteristics are not well characterized. Experiments were conducted during 2010 and 2011 in first year seed production (*Lolium perenne* L.) fields near Roseau and Roosevelt, MN where five SRs [1.3, 2.6, 5.2, 7.8, and 10.4 kg PLS (pure live seed) seed ha^{-1}], three RSWs (10, 20, and 30 cm), and three N rates (67, 112, and 157 kg N ha^{-1}) were evaluated in a split-split plot design under non-irrigated conditions. The 30 cm RSW resulted in 45% greater stem rust incidence, 8% fewer fertile tillers and up to 2% greater thousand seed weight vs. the 10 cm RSW. For every unit of N applied, N increased biomass by 4 to 25 kg ha^{-1}, fertile tillers by 3.7 tillers m^{-2}, and seed yield by up to 4.7 kg seed ha^{-1} depending on environment. The 7.8 and 10.4 kg PLS ha^{-1} SRs combined with the 10 cm RSW, 157 ha^{-1} N rate, and other management factors, virtually eliminated weeds (<2% weed cover) compared to wider RSWs and lower SRs. Weed cover was always inversely correlated with early season vegetative cover (EVC). Seeding rates of 2.6 kg PLS ha-1 and greater resulted in similar seed yields suggesting that early season plant density is not as important for seed yield as other management factors such as N rate which significantly improved seed yield in three environments.

Abbreviations: RSW, row spacing width; SR, seeding rate; PLS, pure live seed; TSW, thousand seed weight; HI, harvest index; FTN, fertile tiller number; EVC, early-season vegetative cover; SR, seeding rate; RSW, row spacing width; GDD, growing degree days; AUDPC, area under the disease progress curve.

Perennial ryegrass (*Lolium perenne* L.) is a cool-season grass species that is used as a turfgrass and forage grass around the world. The main perennial ryegrass seed production areas in North America are in Minnesota, the Pacific Northwest, and western Canada. The perennial ryegrass seed industry in northern Minnesota has grown significantly over the past 10-15 years due to the presence of an ideal climate for grass seed production as well as agronomic and economic benefits of having perennial ryegrass (effectively a biennial seed crop in MN) in a rotation with oilseed and wheat crops in this region (MN Turf Seed Council, personal communication, 2011). Minnesota currently has around 8000 ha^{-1} of perennial ryegrass seed production with seed yields between 1000 and 2000 kg ha^{-1} being common (Koeritz et al., 2013; Ehlke et al., 2011; Kurcinka, 2009). Research has indicated that seed yields of 3000 kg ha^{-1} may be possible with proper management and growing conditions (Rolston et al., 2010a). In recent years, studies evaluating proper nitrogen (N) and growth regulator management on perennial ryegrass seed production in northern Minnesota have been conducted (Koeritz et al., 2013, Kurcinka, 2009), but it is likely that additional management factors such as seeding rate and row spacing could affect seed yield, agronomic characteristics, and crop sustainability.

Recommendations for optimal seeding rates of perennial ryegrass in Minnesota and other seed producing regions are variable and the impact of plant density on perennial ryegrass seed production in northern MN is not well documented. In Minnesota, typical perennial ryegrass seeding rates range from 4.5 to 7 kg seed ha^{-1} and in Saskatchewan, under irrigated conditions, seeding rates of 8 kg seed ha^{-1} are

recommended (Najda, 2004). Previous research in a greenhouse setting has shown that perennial ryegrass tiller density converges toward a value determined by available light intensity, a value not affected by initial seed density, when densities between 320 and 10,000 seeds m^{-2} were evaluated (Kays and Harper, 1974). This suggests that slight variations in seeding rate may not critically affect final tiller density, however the effect of seeding rate on plant stand characteristics under field conditions should be determined. In *Festulolium loliaceum* (Hudson) seed production, seeding rates between 8 and 16 kg ha^{-1} did not affect seed yield (Deleuran et al., 2010), but in annual ryegrass (*Lolium multiflorum* Lam.) seed production, there can be a seed yield increase as a result of a higher seeding rate when environmental conditions are unfavorable for plant growth (Simic et al., 2009). Environmental factors such as soil type, drought, temperature, and winter-kill could affect perennial ryegrass seed production in Minnesota as seed yield and yield parameters have been shown to vary significantly depending on the environment (Elgersma, 1990). Increasing seeding rate is a strategy used, in dry-seeded rice (*Oryza sativa* L.), to suppress weeds by providing faster canopy cover and to compensate for poor establishment (Chauhan, 2012). The influence of different environmental factors on the effect of seeding rate in Minnesota is unclear, and the effect of plant density should be evaluated in combination with various row spacing widths and N rates.

Perennial ryegrass, as a seed crop in Minnesota, is typically planted in rows as an under-seeded crop in wheat or into wheat stubble following wheat harvest. Row spacing in Minnesota and western Canada varies depending on equipment used and soil moisture and can range from 15 to 30 cm, with 20 cm being most common in MN. The effect of row spacing on yield and agronomic characteristics of perennial ryegrass in Minnesota

has not been well characterized. At a given seeding rate, increasing row spacing increases the number of seeds planted within a row. This can increase within-row tiller density, and the resulting increased plant-to-plant competition for light and nutrients in more densely planted rows could have an effect on plant growth (Kays and Harper, 1974; Deleuran et al., 2009; Han et al., 2013). High planting density in grass decreases tillering but high tiller density can cause plants to have longer leaves and shoots as a result of lower red/far-red light ratios in the plant canopy (Kays and Harper, 1974; Casal et al., 1985; Simic et al., 2009). Researchers in Denmark found that row spacings ranging from 12-48 cm did not affect yield in first year perennial ryegrass seed production however the number of fertile tillers produced per unit area was reduced as spacing increased (Deleuran et al., 2009). Studies evaluating the effect of row spacing in various grass seed crops in China indicated that the number of seeds per spikelet, an important seed yield component (Young III et al., 1996), increased with row spacing and that thousand seed weight (TSW) for Chinese sheepgrass (*Leymus chinensis* Trin. Tzvel.) and smooth bromegrass (*Bromus inermis* Leyss.) was lower at row spacings of 30 and 50 cm vs. 70 and 90 cm (Han et al., 2013).

Plant spatial arrangement in grass seed production can also have an effect on weed and disease pressure. A study evaluating the effect of planting density in barley found that stem rust severity was greater at lower planting densities and suggested that differences in microenvironment at different plant densities could affect pathogen growth (Dill-Macky and Roelfs, 2000). That study also suggested that spatial arrangement of plants, in addition to the number of plants per unit area, could affect stem rust development. For organic seed production in Denmark, researchers have suggested that

wider row spacing of 24 cm can be used, without affecting seed yield, to facilitate mechanical weed control (Deleuran et al., 2010). However, in various grass seed crops in China, weed growth is increased as row spacing increases (Han et al., 2013). Narrow row spacing, which promotes faster canopy closure and greater light interception, is suggested as a cultural practice to reduce weed pressure in dry-seeded rice and soybeans [*Glycine max* (L.) Merr.] (Chauhan, 2012; Yelverton and Coble, 1991). Considering growing concerns about herbicide resistance, especially to acetyl coenzyme A carboxylase (ACC) inhibitors (Delye, 2005) which are important herbicides in our region, it is important to find integrated management solutions to control weeds. In northern Minnesota, spring nitrogen rates of 100 vs. 56 kg N ha^{-1} can increase vegetative biomass production by up to 45 % and fertile tiller number by up to 15%, both canopy characteristics that could affect light interception (Koeritz et al., 2013). An evaluation of integrated management strategies that could affect yield, yield parameters, canopy formation, and microenvironments within the canopy is warranted. Therefore, the objectives of this study were to evaluate the effect of seeding rate, row spacing, and nitrogen rate on pre-harvest plant stand characteristics, yield parameters, and seed quality in multiple environments in northern Minnesota.

MATERIALS AND METHODS

Field experiments were conducted under non-irrigated conditions during the 2010 and 2011 harvest seasons to test the effects of seeding rate (SR), row spacing width (RSW), and N rate on seed yield, yield components, and overall crop agronomic performance in a first year perennial ryegrass seed production field. In each year the

study was conducted at two locations including the University of Minnesota Magnusson Research Farm in Roseau, MN, and a grower site in Roosevelt, MN. The locations were chosen to be representative of the major perennial ryegrass growing region in northern MN. Each year by location combination was considered a different environment with Roseau 2010, Roosevelt 2010, Roseau 2011, and Roosevelt 2011 designated environments E1, E2, E3, and E4 respectively. The soil type for E1 and E3 was a Zippel very fine sandy loam. For E2 and E4 the soil type was a Garnes fine sandy loam.

The experimental design was a randomized complete block with a split-split plot treatment arrangement. Seeding rate was the whole plot factor, RSW was the split-plot factor, and N rate was the split-split plot factor. The experiment consisted of four replications and each experimental unit (split-split plot) measured 2.43 m wide by 3.35 m long. A 0.91 m wide untreated perennial ryegrass border seeded at a rate of 5.6 kg seed ha^{-1} with an RSW of 20 cm surrounded each plot to minimize edge effects.

In early June of 2009, and mid May of 2010, 'Arctic Green' perennial ryegrass was seeded according to treatment specifications into tilled ground, on which a soybean crop had previously been grown, at each location. A total of five SRs, including 1.3, 2.6, 5.2, 7.8, and 10.4 kg PLS ha^{-1}, were evaluated and represented a range of standard and non-standard seeding rates for perennial ryegrass seed production fields. Three RSWs (10, 20, and 30 cm) were evaluated. The 20 cm RSW represented the average RSW used for perennial ryegrass production in northern MN while the 10 cm RSW was more similar to a broadcast seeding of perennial ryegrass which is sometimes practiced. The 30 cm RSW was included for evaluation of wide RSWs and to fully understand the effect of spatial arrangement of perennial ryegrass vegetation. The entire study was seeded

using a single-row hand planter to eliminate variation due to planter type and to accommodate the experimental design. Immediately following seeding, the entire study area was overseeded with hard red spring wheat (*Triticum aestivum* L.) at a rate of 89 or 67 kg seed ha^{-1} at E1 and E3 (Roseau) or E2 and E4 (Roosevelt) respectively in each year to keep in line with the common practice the region. At the time of wheat planting, N was applied at a rate of 67 kg ha^{-1} in each year for E1 and E3 (Roseau) as well as a rate of 110 and 67 kg ha^{-1} for E2 and E4 respectively (Roosevelt). Phosphorus (P) and potassium (K) were applied at a rate of 15 kg P ha^{-1} and 37 kg K ha^{-1} and incorporated into the soil prior to planting at each environment. Wheat was harvested according to standard practices during late summer of each perennial ryegrass establishment year and the wheat chaff was returned to the field.

Three N rates (67, 112, and 157 kg N ha^{-1}) were evaluated for their effect on the perennial ryegrass seed crop and N treatment applications were split so half was applied in the fall of the perennial ryegrass establishment year and half was applied in the spring of the harvest year. Fall N applications were made on 18 October 2009 for E1 and E2 (2009 seeding) and on 28 October 2010 for E3 and E4 (2010 seeding). Spring N applications were made on 14 May 2010 for E1 and E2 (2009 seeding) and on 20 May 2011 for E3 and E4 (2010 seeding). Nitrogen treatments in the form of granular urea (46-0-0) were applied uniformly to each experimental unit using shaker jars. At the time of the fall N treatment applications, P and K were broadcast uniformly across the entire study area at a rate of 15 and 37 kg ha^{-1} respectively.

Other management practices including weed control and application of plant growth regulators were applied to the entire study area when needed. Vertical growth of

the perennial ryegrass seed crop was controlled with an application of prohexadione calcium (calcium 3-oxido-5-oxo-4-propionylcyclohex-3-enecarboxylate), applied at the two-node growth stage, at a rate of 115 and 192 g a.i. ha^{-1} in each environment in 2010 and 2011 respectively. During the spring of the ryegrass harvest year, monocot weeds were controlled with quizalofop P-ethyl, {ethyl (R)-2-[4-(6-chloroquinoxalin-2-yloxy)-phenoxy] propionate} (Assure II, Dupont, Wilmington, DE) ('Arctic Green' is tolerant to quizalofop P-ethyl) applied at a rate of 71 g a.i. ha^{-1} in each environment. Environment 1 received an application of 70 g a.i. ha^{-1} fenoxaprop-p-ethyl ({+}-ethyl 2-{4-[(6-chloro-2-benzoxazolyl)oxy]phenoxy}propanoate and 107 g a.i. ha^{-1} clopyralid (3, 6-dichloro-2-pyridinecarboxylic acid, monoethanolamine salt) on 18 July 2009. Environment 2 received applications of 421 g a.i. ha^{-1} dicamba (3, 6-dichloro-o-anisic acid) on 16 September 2010 and 9 June 2011 as well as applications of 400 g a.i. ha^{-1} 2, 4-D (2, 4-dichlorophenoxyacetic acid) on 26 June and 16 Sept. 2010 and 9 June 2011. Environment 3 received an application of a tank mix of 70.2 g a.i. ha^{-1} fenoxaprop-p-ethyl, 107 g a.i. ha^{-1} clopyralid, and 562 g a.i. ha^{-1} 2, 4-D on 18 July 2009 as well as an application of a tank mix of 400 g a.i. ha^{-1} 2, 4-D and 421 g a.i. ha^{-1} dicamba on 1 June 2010. Environment 4 received an application of a tank mix of 400 g a.i. ha^{-1} 2, 4-D and 421 g a.i. ha^{-1} dicamba on 10 June 2011 as well as an application of 351 g a.i. ha^{-1} of the octanoic and heptanoic esters of bromoxynil (3, 5-dibromo-4-hydrozybenzonitrile) and 351 g a.i. ha^{-1} of the isooctyl ester of MCPA [(4-chloro-2-methylphenoxy) acetic acid] on 30 June 2010. Herbicides were applied only after weed cover ratings were taken. Fungicides were not applied to allow for evaluation of treatment effects on stem rust disease.

Data were collected on seed yield, vegetative biomass, harvest index (HI), thousand seed weight (TSW), fertile tiller number (FTN), plant height, lodging, percent early season vegetative cover (EVC), percent weed cover and stem rust incidence and severity. At harvest, all above-ground plant biomass was harvested from a 1 m by 1 m section and dried for 48 h at 90° C in a forced air drier. Once dry, the biomass was weighed and then passed through a grass seed thresher to separate the seed from non-seed biomass. Following initial threshing the seed was further cleaned by passing through a 1.625 by 9.525 mm oblong sieve two times, next passing through an Almaco Seed Cleaner (Allan Machine Co., Ames, IA), and then passing through a Superior® Fractionating Aspirator (Carter-Day Int. Inc., Minneapolis, MN). Finally, the clean seed was passed through 1.190 by 9.525 mm sieve to remove any remaining large inert material or un-separated seed. The clean seed was weighed and the weight subtracted from the above-ground biomass weight to get a vegetative biomass weight. Harvest index was calculated by dividing the weight of the seed by the total above-ground biomass weight. Thousand seed weight was determined by counting 300 randomly selected seeds from each treatment and weighing to the nearest 10^{-5}g then converting to weight per 1000 seeds. Fertile tiller number, a measure of seed-producing tillers per unit area, was evaluated at the time of harvest by collecting all of the above-ground plant material in a 15.24 cm wide by 70.0 cm long section in each experimental unit and counting the number of tillers in the sample that appeared to hold viable seed. Plant height was evaluated at the time of pollination by taking two measurements, each at two random locations throughout the plot, and recording the average of the two measurements to the nearest centimeter. Lodging was rated visually just prior to harvest on a 0-100%

scale where 0 = no lodging and all plants are upright, and 100 = all plants lying flat on the ground. Percent EVC was evaluated at 402 growing degree days (GDD) for E 1 and 2 (2010) as well as 349 GDD for E 3 and 4 (2011). To calculate EVC, percent row coverage was first measured on two 0.94 m long row sections by using a measuring tool placed next to the row, which was divided into 5.08 cm sections, and counting the number of sections adjacent to vegetative cover and then dividing by the number of total sections possible. The average width of each row was calculated based on the average of two measurements then using percent row coverage, row width, row length, and the number of rows per plot, the total percent EVC was calculated for each experimental unit. Growing degree days for the general region were calculated using a base temperature of 0° C from a University of Minnesota sponsored weather station located at the GPS coordinates 48.655 N and 95.734 W. The total percentage of weed cover in each experimental unit was visually estimated on a scale from 0 to 100% where weeds were present. Stem rust incidence and severity due to natural infection was recorded when stem rust pustules were first observed in E3 (Roseau 2011) (around 1504 GDD) and again at 1689 GDD. The pathogen was identified as stem rust based on disease symptoms and spore morphology. For stem rust ratings, twenty random tillers were collected from each plot and visually inspected in the field. The percent coverage of stem rust on each the 20 tillers was visually estimated using a reference key (James, 1971). Stem rust incidence data was converted to area under the disease progress curve (AUDPC) using the formula from Shaner and Finney (1977):

$$\text{AUDPC} = \sum_{i=1}^{n-1}\left[\left(X_{i+1} + X_i\right)/2\right]\left[t_{1+1} - t_i\right]$$

where

X_i = the rust incidence at the i^{th} day

t_i = the time in days after the initial rust rating at the i^{th} day

n = the total number of observations

Statistical Analysis

Each location and year combination was considered a unique environment. Data were analyzed and type III tests of fixed effects were calculated (considered significant at $P<0.05$) using the PROC MIXED procedure of SAS (SAS Institute, Cary, NC). Replication nested within environment and all interactions with this factor were considered random effects while all other factors were considered fixed effects. The test of fixed effects used the Satterthwaite approximation for the denominator degrees of freedom. Treatment effects were considered significant at $P < 0.05$ and pair-wise comparisons were performed using the Tukey-Kramer method. Post hoc analysis of treatment means, to obtain letter groupings based on the Tukey-Kramer honestly significant difference (HSD) test, was conducted using the PDMIX800 SAS macro (Saxton, 1998). Where treatment effects were significant, single degree of freedom linear or quadratic contrasts were used to evaluate trends. Pearson correlations were calculated on an individual plot basis between all measured variables using the PROC CORR procedure in SAS.

RESULTS AND DISCUSSION

All four environments were treated identically during the harvest season. Therefore, our analysis initially tested treatment effects across all environments along with the effect of environment (Table 1). Type III tests of significance for the measured parameters indicated that some of the variables tested often had different effects depending on the environment in which they were tested (Table 1). Growing degree day accumulation, an important influence on agronomic characteristics in this region (Koeritz et al., 2013), began earlier during the 2010 harvest season (E1 and E2) than in the 2011 harvest season (E3 and E4) (Figure 1). The soil type for E1 and E3 (Roseau) was also different from the soil type for E2 and E4 (Roosevelt). Previous observations have indicated that more N may be required for optimum perennial ryegrass seed yield at the site of E3 and E4 in this study (Kurcinka, 2009; Koeritz et al., 2013). As a result of interactions with environment in the initial analysis and known environmental differences, data for this study were ultimately analyzed within each environment where appropriate.

Early Season Vegetative Cover (EVC)

The EVC measurements were largely a measure of how SR and RSW affected the vegetative growth of the perennial ryegrass crop from establishment, through its period of growth as an under-seeded crop in wheat, and finally its survival through winter and the spring green-up period. At the time of evaluation of EVC (349-390 GDD), vegetative tillers were in approximately the four leaf stage and the vegetation in each individual row covered a width of around 10.2 cm on average. Nitrogen did not have an effect on EVC (Table 1) because the fall N application (half of the total N) had been applied after most

vegetative growth had stopped and the spring N application had not been applied at the time of evaluation. Mean EVC for the 10 cm RSW was 77, 71, 81, and 86% for E1, E2, E3, and E4 respectively and the rank order of percent EVC for the three RSW treatments was always 10 cm > 20 cm > 30 cm (Figure 2). Increased RSW results in fewer rows per unit area, and as a result, more seeds will be planted within a row. Other studies, evaluating RSW effects in perennial ryegrass and *Festulolium*, found that at a given SR, wider RSW resulted in greater within row plant density in early spring and the authors suggested the potential for within row plant competition at higher within row plant densities (Deleuran et al., 2009; Deleuran et al., 2010). If narrow RSW in this study provided more rows per unit area and less within row competition among plants, it would explain the greater percent EVC at narrow RSW in this study. Narrower RSW (12 cm) in *Festulolium* also resulted in greater reproductive tiller number than 24 or 36 cm RSW which also suggests less competition in these grasses at narrower RSW (Deleuran et al., 2010).

There was a significant effect of the interaction between SR and RSW that differed depending on the environment (Table 1). Percent EVC increased linearly as SR increased at E1, E2, and E4, however, the rate of increase was greatest in combination with the 10 cm RSW where EVC increased 2.4, 4.2, and 2.3 % for each unit increase in SR at each environment respectively. All SRs, when seeded with a 20 or 30 cm RSW, resulted in statistically similar EVC at E1, E3, and E4 ($P<0.05$; Figure 2). Early season vegetative cover, at the 10 cm RSW, even at the 2.5 kg PLS ha^{-1} SR, was 23, 20, 35 and 35 percent greater than the percent EVC at the 10.4 kg PLS ha^{-1} SR planted at the 20 cm RSW at E1, E2, E3, and E4 respectively ($P<0.05$; Figure 2). As SR increased to 10.4 kg

PLS ha^{-1} at the 10 cm RSW, percent EVC increased to a mean of 87 % EVC (Figure 2). At E3, EVC increased with SR at the 10 cm RSW but the trend was quadratic and maximum EVC was achieved with the 7.8 kg PLS ha^{-1} SR (Figure 2c).

The results of this study indicated that the effect of increasing SR on percent EVC at the 20 and 30 cm RSWs was negligible. This suggests that within-row competition may be limiting any benefit of higher SRs at the 20 and 30 cm RSW in the environments evaluated in this study. In this study, at all four environments, plant establishment and winter survival were excellent compared to what has previously been observed in seed production fields (personal observation) and it is possible that SR may have had more of an effect on percent EVC if winter injury of the ryegrass crop had been greater or early season moisture had been limiting. A study evaluating SR and RSW effects on annual ryegrass (*Lolium multiflorum*) indicated that SR can have significant positive effects on yield during growing seasons with unfavorable weather conditions that result in less biomass growth (Simic et al., 2009). When determining SRs which provide optimal plant density (or vegetative cover) it is important to consider loss of seedlings during establishment (Fairey and Lefkovitch, 1999b) so the effect of SR on EVC at the 20 and 30 cm RSW should be evaluated following more harsh winters. The overall greater EVC and greater response to SR at the 10 cm RSW suggests that the 10 cm RSW is optimal for promoting greater EVC in the environments tested here, which may be due to less within row plant competition (Deleuran et al., 2009; Deleuran et al., 2010). Maximizing EVC could be an important integrated management strategy to minimize nitrate leaching and help reduce weed competition since a perennial ryegrass cover crop can reduce nitrate leaching by up to 50% (Bergstrom and Jokela, 2001) and it has been suggested that early

season shading by a grass crop canopy could substantially reduce weed growth (Chauhan, 2012; Han et al., 2013).

Weeds and Disease

Percent weed cover was always inversely correlated with EVC (r = -0.45, -0.20, and -0.33 for E2, E3, and E4 respectively; $P<0.05$; Table 2) but N also had an effect of decreasing percent weed cover (Table 2), which may have been a result of N increasing vegetative biomass production, causing increased competition with weeds following the EVC ratings (Table 3). In E2, where there was severe competition from volunteer wheat (29% wheat cover at the lowest seeding rate), the 7.8 and 10.4 kg PLS ha^{-1} SRs drastically reduced the amount of volunteer wheat (Table 2) but the10.4 kg PLS ha^{-1} SR at the 10 cm RSW, fertilized with 112 or 157 kg ha^{-1} N rate virtually eliminated competition from volunteer wheat (wheat cover was < 2%) compared to the three lowest seeding rates with the same row spacing widths and nitrogen rates (data not shown; $P<0.05$). At E4, where multiple grass and broadleaf weeds were present, 7.8 and 10.4 kg PLS ha^{-1} SR in combination with the 10 cm RSW and 157 ha^{-1} N rate resulted in one percent or less total weed cover while the 1.3 kg PLS ha^{-1} SR in combination with the 20 or 30 cm RSW and the 67 kg ha^{-1} N rate resulted in greater than 14 % total weed cover (data not shown; $P<0.05$).

The higher SRs and narrower RSWs are suggested as strategies to improve crop competition with weeds in dry-seeded rice (Chauhan, 2012). Higher SRs, RSWs, and higher N rates in this study all likely promoted faster canopy closure and interception of photosynthetically active radiation, which is important for suppressing weeds (Yelverton

and Coble, 1991). Other research, evaluating the effect of SR and RSW on *Festulolium* seed production in Denmark, has suggested that a 24 or 36 cm RSW would be ideal to allow mechanical weed control in organic seed production systems (Deleuran et al., 2010). Mechanical weed control however, requires additional labor, fossil fuels, and dry soils to avoid compaction from tractor tires. In contrast, the results of our study suggest that narrow RSW in combination with higher SRs and optimal nitrogen rates may be a good integrated management strategy for nearly eliminating weeds. In general, the percentage of volunteer wheat observed decreased linearly by 2.7% with each unit increase in SR and total weed cover in E4 decreased linearly by 0.8 % with each unit increase in SR (Table 2). The 10 cm RSW on average resulted in three percent less cover of volunteer wheat than the 30 cm RSW (Table 2), however, the greatest difference between the 10 and 30 cm RSW treatments in terms of percent volunteer wheat (9 vs. 20 % cover for the 10 and 30 cm RSW respectively) was observed when the perennial ryegrass crop was seeded at a rate of 5.2 kg PLS ha^{-1} and fertilized at the 67 kg ha^{-1} N rate (Data not shown; $P<0.05$).

Stem rust was observed in E3 where a significant SR by RSW interaction and a significant effect of N rate on stem rust incidence were observed (Table 4). Stem rust incidence AUDPC was 22% greater when 157 kg N ha^{-1} was applied vs. 67 kg N ha^{-1} (Table 4). Another effect of increasing N rate in E3 was a 5 cm increase in plant height and 24% more lodging at the highest N rate vs. the lowest N rate (Table 3) despite the application of a growth regulator earlier in the growing season suggesting that the perennial ryegrass stems at the high N rate were thin and weak. A study evaluating N and growth regulator effects on stem rust in perennial ryegrass also indicated that stem

rust incidence was less severe in plants that were shorter with more robust stems, which may have been due to greater cell density, cell wall thickening, or concentration of plant protective compounds (Koeritz et al., 2013).

On average, across all SRs, stem rust AUDPC increased linearly as RSW increased (Table 4). The effect of RSW on stem rust incidence was best explained by the linear trend when the perennial ryegrass crop was seeded at a SR of 1.3 kg PLS ha^{-1} (Table 4). At this SR the 30 cm RSW had almost 45% greater stem rust incidence than the 10 cm RSW ($P<0.05$; Table 4). Stem rust in barley develops more rapidly in plant stands with lower density (Dill-Macky and Roelfs, 1999) which is similar to what was observed in our study. In another perennial ryegrass study, greater stem rust severity was also observed in plant stands with a more open canopy (Koeritz et al., 2013). In our study, wider RSW created a more open plant canopy but also, the number of seed producing tillers (FTN) was eight percent lower when RSW was 30 cm vs. 10 cm (Table 3). One explanation for the greater stem rust incidence at wider row spacing in our study is that there was less of a physical barrier to spread of uredineospores within the plant canopy at wider row spacing. Alternatively, in the barley study, the authors suggested that the increased stem rust disease development at lower stand density could be due to differences in plant nutrient status, light penetration, temperature, dew period, or humidity (Dill-Macky and Roelfs, 1999), all potentially important factors in the stem rust infection process (Leonard and Szabo, 2005). The effect of plant density, spatial arrangement, and nutrient status on stem rust disease and within canopy microenvironments in perennial ryegrass should be further evaluated under more severe

disease conditions in multiple environments. In addition, the influence of N and plant growth on the stem rust infection process should be evaluated in more detail.

Vegetative Biomass, Fertile Tiller Number (FTN), and Lodging

The effect of N rate on above ground vegetative biomass production was significant in all four environments (Table 1 and 4). Vegetative biomass always generally increased with increasing N rate and linear contrasts indicated a significant positive trend where biomass increased 25, 4, 15, and 8 kg ha^{-1} for each unit increase in N rate at E1, E2, E3, and E4 respectively (Table 3). In E2, the 1.3 kg PLS ha^{-1} SR resulted in significantly lower vegetative biomass production than all other SRs and there was an overall linear trend of increasing vegetative biomass production as SR increased (Table 3). The 10 cm RSW at E2 resulted in almost seven percent greater biomass production than the 30 cm RSW (Table 3). Correlations between vegetative biomass production and seed yield ranged between 0.32 and 0.68 and were always highly significant (Table 5). Correlations between biomass production and lodging ranged between 0.42 and 0.80 and were always highly significant suggesting that increased biomass production may cause more severe lodging (Table 5).

The number of fertile tillers producing seeds per unit area was evaluated at each location and a significant effect of RSW and N rate was observed across all environments (Table 3). On average E1 and E3 (Roseau) produced 35% more fertile tillers than E2 and E4 (Roosevelt) (data not shown; $P<0.05$). The 30 cm RSW significantly decreased FTN compared to a 10 or 20 cm RSW, where tiller counts were 2613 vs. 2827 or 2823 tillers m^{-2} respectively (Table 3). There was a significant linear trend of 10.7 fewer tillers m^{-2}

for every cm increase in RSW across all environments, SRs and N rates (Table 3). The trend of decreasing FTN as RSW increases, that was observed in this study, is similar to what was observed in other studies in perennial ryegrass and other grass seed crops (Deleuran et al., 2010; Deleuran et al., 2009; Fairey and Lefkovitch, 1999a; Han et al., 2013; Kusvuran and Tansi, 2011). When RSW is greater, there are more seeds planted in each row resulting in greater within-row density (Han et al., 2013) which can result in greater competition for nutrient or light resources then, lack of tiller formation or tiller death during reproductive stages (Kays and Harper, 1974).

The 157 and 112 kg ha^{-1} N rate produced significantly greater FTN than the 67 kg ha^{-1} N rate (2904 and 2790 vs. 2570 tillers m^{-2} respectively) and there was a significant linear trend of 3.7 more fertile tillers m^{-2} for each additional unit of N applied (Table 3). This trend is similar to what other studies on perennial ryegrass seed production in Minnesota and New Zealand have found where higher spring N availability resulted in greater FTN (Cookson et al., 2000; Koeritz et al., 2013).

Fertile tiller number was not significantly correlated with EVC in E1, E3, or E4 while only being slightly correlated with EVC in E2 (Table 5). This, and the fact that SR did not affect FTN, indicates that early season plant density (at least densities provided by the treatments in this study) may not be as important as RSW and N rate for determining FTN in perennial ryegrass seed production fields. This observation is similar to those of a study on perennial ryegrass which showed that perennial ryegrass tiller density converges on an optimum density regardless of seeding rate and that the final tiller density may be determined by the amount of shading in the plant canopy (Kays and Harper 1974).

Fertile tiller number and the amount of vegetative biomass were important plant stand characteristics that were significantly and positively correlated with the amount of lodging of the perennial ryegrass seed crop at each environment (*r* for FTN = 0.28, 0.30, 0.17, and 0.21; *r* for biomass = 0.80, 0.65, 0.60, and 0.42 for E1, E2, E3, and E4 respectively) (Table 5). Factors that affect lodging are extremely important as lodging in perennial ryegrass can negatively impact seed yield as percent lodging reaches 50% (Rolston et al., 2010b).

Lodging at harvest was greatest in E2, E3, and E4 ($P<0.05$; Table 3). Lodging was always affected by N rate while SR only significantly affected lodging in E2 and E4 (Roosevelt) (Table 3). In E2 and E4, lodging increased linearly as SR increased and lodging was greater than 75% for the 7.8 and 10.4 kg PLS ha^{-1} SRs (Table 3). Lodging was always greater at the 157 kg ha^{-1} N rate than the 67 kg ha^{-1} N rate and the effect of N rate on lodging could be explained by a linear trend where lodging increased between 0.18 and 0.26% for each additional unit of nitrogen applied (Table 3). Lodging was also positively correlated with plant height in most environments, and plant height increased as N rate increased (Table 3 and 5). All N rates in E2, E3, and E4 resulted in lodging greater than 50% (Table 3). In E2, there was a linear trend towards greater lodging at narrower RSW and the crop was 65% lodged at the widest RSW (Table 3). These results, and correlations with FTN, indicate that increased plant density may have partially contributed to lodging as lodging was moderately but significantly and positively correlated with FTN at all environments (Table 5).

Lodging, in this study and another on perennial ryegrass seed production in northern Minnesota, reached levels of greater than 70% when fertilized with total N rates

around 157 kg N ha^{-1} and treated with a single application of a growth regulator (Table 3) (Koeritz et al., 2013). In this study, the dry weight of vegetative biomass was 6 to 54% greater at the 157 kg ha^{-1} N rate than at the 67 kg ha^{-1} N rate and it is likely that the increased weight of above-ground vegetative biomass contributed to lodging (Table 3). Tillering and additional biomass production in a grass stand can cause intense shading in the plant canopy which Simon and Lemaire (1987) suggested can lead to plants allocating carbohydrate to leaf elongation. Increases in SR, a variable that also led to increases in EVC and canopy closure, also led to increases in vegetative biomass and lodging at times in this study (Table 3). Additional leaf biomass near the top of the plant canopy, as a result of greater leaf elongation, in plots with greater amounts of within canopy shading caused by increases in biomass or FTN could in part explain the correlation between biomass or FTN and lodging.

Yield Parameters and Seed Quality

Average seed yields were greatest in E1 and E2 (1338 and 1364 kg PLS ha^{-1} respectively), the two sites harvested during the 2010 harvest season (Table 6). Seed yield was affected by SR and N rate as well as an interaction between seeding rate and N rate in some environments (Table 6). On average, SRs of 2.6 kg PLS ha^{-1} and above resulted in similar seed yields ($P<0.05$), a trend also observed in *Festulolium* and in annual ryegrass under good weather conditions (Deleuran et al., 2010; Simic et al., 2009). The 1.3 kg PLS ha^{-1} SR resulted in significantly lower seed yield than the top yielding SR on average ($P<0.05$), especially in E2 where it resulted in significantly lower seed yield than all other SRs which was likely a result of result of significant competition from

volunteer wheat (25% volunteer wheat cover) at this SR (Table 1 and 6). This suggests that perennial ryegrass should be planted at SRs of 2.6 kg PLS ha^{-1} and greater when weed competition is possible. Greater SRs may also be beneficial where the perennial ryegrass stand is stressed by weather conditions (i.e. winterkill or drought), however, additional research should be conducted in this area. There was a slight correlation between EVC and seed yield in E2 which was also likely a result of early season competition between the ryegrass crop and volunteer wheat (Table 5). In other environments there was no correlation between EVC and seed yield (Table 5). This and the results showing equivalent seed yields at SRs above 1.3 kg PLS ha^{-1} suggest that early season plant density is not as important for seed yield as other management factors such as N rate.

In E2, linear and quadratic contrasts, within the 157 and 112 kg ha^{-1} N rates respectively, indicated a trend of increasing seed yield as SR increased, however linear or quadratic contrasts were not significant at the lowest N rate ($P<0.05$; Table 6). At E2, within the 112 kg ha^{-1} N rate, maximum seed yields were obtained at the 5.2 and 7.8 kg PLS ha^{-1} SRs (Table 6). In E1, E3, and E4, across all seeding rates, the 157 kg ha^{-1} N rate resulted in 18, 22, and 10% greater seed yield than the 67 kg ha^{-1} N rate in each environment respectively (Table 6). Within environments however, N rate had differing effects on seed yield depending on the SR (Table 1 and 6). In E1, N did not improve seed yield when the SR was 1.3 kg PLS ha^{-1} (Table 6). At all other SRs in E1, seed yield increased with higher N rates according to a linear trend with the greatest increase being 4.7 and 4.0 kg seed ha^{-1} per unit N increase at the 2.6 and 5.2 kg PLS ha^{-1} SR (Table 6). At E3, the regression slope was greatest at the 10.4 kg PLS ha^{-1} SR ($b=4.2$) while the

other two significant regressions (1.3 and 2.6 kg PLS ha^{-1} SRs) were the same (b=2.7 and 3.0, respectively) (Table 6). At E4, N did not improve seed yield at SRs above 5.2 kg PLS ha^{-1} (Table 6). Although the effect of N rate on seed yield sometimes depended on the SR, applying N at a rate of 157 kg ha^{-1} (even in combination with the lowest SRs) always resulted in seed yields equivalent to the top yielding treatments ($P<0.05$, Table 6). Seeding rate had an effect on weed cover, lodging and EVC at the 10 cm RSW but the results suggest that adequate yields can be obtained, regardless of these other factors, with proper N management. Previous research has shown that increased N availability can improve the number of seeds set per spike in perennial ryegrass, which is a primary factor driving seed yield (Young III et al., 1996).

Seed weight is a factor that also drives seed yield in perennial ryegrass, in addition to the number of seeds per spike, both of which tend to increase at lower plant or reproductive tiller densities (Young III et al., 1996; Han et al., 2013; Fairey and Lefkovitch, 1999b). The TSW data (Table 3), suggests that this compensational effect also occurred in our study. The results indicated 8% fewer fertile tillers m^{-2} at the 30 cm RSW vs. the 10 cm RSW (Table 3), however, seed harvested from plants seeded at the 30 cm RSW had 1.7 to 2.0% greater TSW than seed harvested from plants seeded at the 10 cm RSW and seed yields were not affected by RSW (Table 1). The number of seeds per spike were not measured in this study, however, it has been suggested that the amount of seed per spikelet can increase as RSW increases in grass seed production (Han et al., 2013). The correlation between FTN and seed yield in this study was inconsistent and in E2 and E4 (Roosevelt) was not existent. The results from Young III et al. (1996), suggested that the number of seeds set per spike was more important than the number of

fertile tillers per unit area for driving seed yield and that it is possible for the number of seeds set per spike to increase in stands with lower tiller numbers. In cases where FTN is lower, the 30 cm RSW for example, seed yields were not affected (Table 1) which was also observed in *Festulolium* (Deleuran et al., 2010; Deleuran el al., 2009). Equivalent seed yields where FTN is lower in our study suggests that an increased number of seeds set per spike may be responsible for maintaining yields, however, further research in this area should be conducted.

On average over all environments, HI was significantly affected by N rate (Table 1) and the 67 kg ha^{-1} N rate resulted in significantly higher HI than the 157 kg ha^{-1} N rate (18.6 vs. 17.4 % respectively; data not shown; $P<0.05$) which was likely a result of N increasing biomass production. The effect was greatest in E1 where HI decreased 0.04% for each unit increase in N rate (Table 3).

CONCLUSION

Although important for certain plant stand characteristics, SR and RSW are not as important as N availability for improving seed yields in perennial ryegrass seed production in northern Minnesota. The number of fertile tillers produced in a perennial ryegrass seed production field appears to be largely independent of initial planting density or early season vegetative cover which is in line with results of previous greenhouse experiments (Kays and Harper, 1974). A compensation effect occurred, where seed weight and possibly seed number per tiller increased, to make up for lower FTN at observed at the 30 cm RSW. Although SRs of 1.3 and 2.6 kg ha^{-1} (lower than a typical SR in perennial ryegrass seed production in Minnesota) provided good seed yields

in the optimum environments tested here, further research should be conducted when winter injury or moisture stress is more severe.

Other factors such as vegetative cover, weed competition, lodging, stem rust incidence, and vegetative biomass were more significantly affected by SR and RSW. The 10 cm RSW provided the greatest amount of EVC and SR had a greater impact on EVC at this narrow row spacing. Early season vegetative cover was important for minimizing weed cover which was virtually eliminated at SRs of 7.8 or 10.4 kg PLS ha^{-1} planted at the 10 cm RSW and fertilized at the 157 kg ha^{-1} N rate. Narrow rows (10 cm), particularly when SRs were low, significantly reduced stem rust incidence, suggesting that spatial arrangement of plant or tiller density can affect stem rust disease. The effect of plant spatial arrangement, tiller density, or plant biomass on stem rust should be evaluated in more detail under more severe disease conditions and where the canopy microenvironment can be monitored. Higher SRs (7.8 or 10.4 kg PLS ha^{-1}) seemed to pose a greater risk of lodging however it is possible that growth regulator management could be adjusted to reduce this risk.

Optimal SRs, RSW, and N rates could vary depending on the type of production system (organic or conventional), cost of inputs, risk for disease or weeds, weather, etc., however, this study indicated that these management variables can be used as part of an integrated management program to provide good seed yield, reduce the need for herbicides and fungicides, and potentially reduce input costs. In perennial ryegrass seed production systems where weed management is an issue or herbicide usage is not feasible, SRs of 7.8 or 10.4 kg PLS ha^{-1}, RSW of 10 cm, and N rates of 157 kg ha^{-1} would be optimal. Where weed management or herbicide usage is less of a concern, and there

are good growing conditions, SRs of 2.6 to 10.4 kg PLS ha^{-1} should yield well. Minimizing RSW should help to improve EVC, FTN, stem rust resistance, and competition with weeds.

Table 1. Type III test of significance for the effect of fixed sources of variation on early season vegetative cover (EVC), plant height, lodging, fertile tiller number (FTN), vegetative biomass at harvest, seed yield, harvest index, and one thousand seed weight (TSW) across all environments including Roseau 2010 (E1), Roosevelt 2010 (E2), Roseau 2011 (E3), and Roosevelt 2011 (E4).

Source of variation	EVC	Plant Height	Lodging	FTN	Biomass	Yield	Harvest Index	TSW
Environment (E)	***	***	***	***	***	**	***	***
Seeding Rate (SR)	***	NS	***	NS	***	*	NS	NS
E * SR	***	***	***	NS	***	**	NS	**
Row Spacing Width (RSW)	***	NS	NS	**	**	NS	NS	NS
E * RSW	***	NS	NS	NS	NS	NS	***	**
SR * RSW	***	NS	NS	NS	NS	NS	NS	NS
E * SR * RSW	***	NS	NS	NS	NS	NS	NS	NS
Nitrogen Rate (NR)	NS	***	***	***	***	***	***	NS
SR * NR	NS	NS	NS	NS	NS	NS	NS	NS
RSW * NR	NS	NS	NS	NS	NS	NS	NS	NS
SR * RSW * NR	NS	NS	NS	NS	NS	NS	NS	NS
E * NR	NS	***	NS	NS	**	***	***	*
E * SR * NR	NS	NS	NS	NS	NS	***	NS	NS
E * RSW * NR	NS	NS	NS	NS	NS	NS	**	NS
E * SR * RSW * NR	NS	NS	NS	NS	NS	NS	NS	NS

*Effect significant at the 0.05 probability level.

** Effect significant at the 0.01 probability level.

*** Effect significant at the 0.001 probability level.

† NS indicates not significant $P<0.05$.

Table 2. Type III test of significance and means for the effect of fixed sources of variation on percent weed cover including volunteer wheat in Roosevelt 2010 (E2), total weed cover in Roseau 2011 (E3), and total weed cover in Roosevelt 2011 (E4). Correlation coefficients and corresponding P values are also indicated for the correlation between volunteer wheat or weed cover at each site and early season vegetative cover (EVC).

Source of variation	Volunteer Wheat (E2)	Total Weed Cover (E3)	Total Weed Cover (E4)
Seeding Rate (SR)	***	NS	**
Row SpacingWidth (RSW)	***	NS	**
SR * RSW	NS†	NS	NS
Nitrogen Rate (NR)	NS	NS	*
SR * NR	NS	NS	NS
RSW * NR	NS	NS	NS
SR * RSW * NR	***	NS	*
Weed cover correlation with EVC	-0.45***	-0.20*	-0.33***
Seeding rate			
---kg seed ha^{-1}---	--%--	--%--	--%--
1.3	29 a‡	3 a§	11 a
2.6	21 ab	2 a	7 ab
5.2	14 b	2 a	4 b
7.8	5 c	1 a	3 b
10.4	4 c	2 a	4 b
Linear R^2	0.94***	NS	0.72***
Equation (y=)	-2.7x + 29.5	NS	-0.8x + 10.3
Quadratic R^2	0.99*	NS	0.98*
Equation (y=)	$0.3x^2 – 5.8x + 35.3$	NS	$0.2x^2$ -2.9x + 14.5
Row spacing width			
---cm---			
10	13 b	2 a	5 b
20	14 ab	2 a	6 ab
30	16 a	3 a	8 a
Linear R^2	0.96***	NS	0.96**
Equation (y=)	0.2x + 11.3	NS	0.2x + 3.3
Nitrogen rate			
---kgN ha^{-1}---			
67	15 a	2 a	7 a
112	15 a	3 a	6 ab
157	15 a	2 a	5 b
Linear R^2	NS	NS	0.92**
Equation	NS	NS	-0.02x + 8.0

*Effect, linear contrast, or quadratic contrast is significant at the 0.05 probability level.
** Effect, linear contrast, or quadratic contrast is significant at the 0.01 probability level.
*** Effect, linear contrast, or quadratic contrast is significant at the 0.001 probability level.
† NS indicates not significant P<0.05.
‡ Means followed by the same letter within a source of variation and column are not significantly different at P<0.05.
§ Total weed cover in E3 and E4 included: volunteer wheat, foxtail barley (*Hordeum jubatum* L.), broadleaf plantain (*Plantago major* L.), redroot pigweed (*Amaranthus retroflexus* L.), Pennsylvania smartweed (*Polygonum pensylvanicum* L.), field pennycress (*Thlaspi arvense* L.), Canada thistle (*Cirsium arvense* L.), and dandelion (*Taraxacum officinale* F.H. Wigg.).

Table 3. Main effect of seeding rate, row spacing width (RSW) and nitrogen rate on fertile tiller number (FTN) and vegetative biomass data. Data shown is the mean over four environments including Roseau 2010 (E1), Roosevelt 2010 (E2), Roseau 2011 (E3), and Roosevelt 2011 (E4).

Treatment factors	Plant Height				Lodging				Harvest Index			
and regression type	E 1	E 2	E 3	E 4	E 1	E 2	E 3	E 4	E 1	E 2	E 3	E 4
Seeding rate			----------cm----------				----------%----------				----------%----------	
---kg seed ha^{-1}---												
1.3	53 a†	59 a	56 a	58 a	19 a	40 c	71 a	62 b	22.7 a	18.0 a	18.9 a	15.0 a
2.6	55 a	60 a	56 a	58 a	30 a	65 b	69 a	72 ab	20.9 ab	18.7 a	17.3 a	15.5 a
5.2	54 a	60 a	52 ab	58 a	26 a	74 ab	75 a	72 ab	20.2 b	18.0 a	19.2 a	15.4 a
7.8	54 a	60 a	53 ab	59 a	28 a	79 ab	75 a	77 ab	20.4 b	18.2 a	17.7 a	15.2 a
10.4	55 a	61 a	51 b	59 a	29 a	81 a	62 a	84 a	20.5 b	17.1 a	18.3 a	14.9 a
Linear R^2	--	--	0.81**	--	--	0.74***	--	0.86**	0.50**	--	--	--
Equation (y=)	--	--	-0.50x + 56.6	--	--	3.8x + 47.0	--	1.9947x + 62.387	-0.002x + 0.22	--	--	--
Quadratic R^2	--	--	ns	--	--	0.92***	--	ns	0.87*	--	--	--
Equation (y=)	--	--	ns	--	--	-0.75x^2 + 12.5x + 30.2	--	ns	0.001x^2 – 0.010x + 0.24	--	--	--
Row spacing width												
---cm---												
10	54 ab	60 a	54 a	59 a	25 a	70 a	70 a	70 a	22.0 a	17.7 a	18.6 a	14.8 a
20	55 a	60 a	54 a	59 a	26 a	68 ab	70 a	75 a	20.4 b	17.7 a	17.8 a	15.3 a
30	53 b	60 a	54 a	58 a	27 a	65 b	71 a	75 a	20.4 b	18.6 a	18.4 a	15.4 a
Linear R^2	ns	--	--	--	--	0.97**	--	--	0.72**	--	--	--
Equation (y=)	ns	--	--	--	--	-0.28x + 73.3	--	--	-0.001x + 0.23	--	--	--
Nitrogen rate												
---kgN ha^{-1}---												
67	51 c	59 a	51 b	58 a	14 c	59 c	58 c	61 b	22.7 a	18.2 a	18.1 ab	15.3 a
112	54 b	60 a	54 a	58 a	27 b	70 b	71 b	77 a	21.3 b	18.0 a	18.9 a	15.0 a
157	56 a	60 a	56 a	59 a	37 a	74 a	82 a	82 a	18.8 c	17.9 a	17.8 b	15.3 a
Linear R^2	0.99***	--	0.97***	--	0.99***	0.95***	0.99***	0.90***	0.97***	--	ns	--
Equation (y=)	0.06x + 47.4	--	0.05x + 48.4	--	0.25x – 2.2	0.18x + 47.4	0.26x + 41.2	0.24x + 46.8	-0.043x + 25.79	--	ns	--

*Significant at the 0.05 probability level.
**Significant at the 0.01 probability level.
***Significant at the 0.001 probability level.
† Means followed by the same letter within each column and source of variation are not statistically different from one another at P < 0.05.

Table 3 continued. Main effect of seeding rate, row spacing width (RSW) and nitrogen rate on fertile tiller number (FTN) and vegetative biomass data. Data shown is the mean over four environments including Roseau 2010 (E1), Roosevelt 2010 (E2), Roseau 2011 (E3), and Roosevelt 2011 (E4).

Treatment factors	Thousand Seed Weight				Vegetative Biomass				FTN
and regression type	E 1	E 2	E 3	E 4	E1	E2	E3	E4	All Environments
Seeding rate		----------g 1000 seeds^{-1}----------				----------kg ha^{-1}----------			--tillers m^{-2}--
---kg seed ha^{-1}---									
1.3	1.6883 a	1.7590 a	1.5764 a	1.6519 a	4694 a	4917 b	4840 a	6105 a	2753 a
2.6	1.6919 a	1.6445 b	1.6077 a	1.6630 a	5365 a	6197 a	4732 a	6360 a	2742 a
5.2	1.6940 a	1.6977 ab	1.5587 a	1.6572 a	5539 a	6397 a	4521 a	6371 a	2698 a
7.8	1.6902 a	1.6720 b	1.5759 a	1.6673 a	5261 a	6632 a	4952 a	6302 a	2771 a
10.4	1.6956 a	1.6972 ab	1.5806 a	1.6576 a	5311 a	6921 a	4446 a	6449 a	2807 a
Linear R^2	--	ns	--	--	--	0.74***	--	--	--
Equation (y=)	--	ns	--	--	--	178.13x + 5240	--	--	--
Quadratic R^2	--	0.31*	--	--	--	0.85**	--	--	--
Equation (y=)	--	$0.0022x^2$ – 0.029x + 1.7607	--	--	--	$-27.67x^2$ + 499.3x + 4618	--	--	--
Row spacing width									
---cm---									
10	1.6723 b	1.7031 a	1.5596 b	1.6497 b	5035 a	6333 a	4583 a	6313 a	2827 a
20	1.6974 ab	1.6987 a	1.5944 a	1.6482 b	5493 a	6409 a	4814 a	6432 a	2823 a
30	1.7063 a	1.6805 a	1.5856 ab	1.6803 a	5174 a	5897 b	4698 a	6208 a	2613 b
Linear R^2	0.93*	--	ns	0.72**	--	0.62**	--	--	0.76**
Equation (y=)	0.0017x + 1.658	--	ns	0.0015x + 1.629	--	-21.80x + 6649	--	--	-10.70x + 2969
Nitrogen rate									
---kgN ha^{-1}---									
67	1.6856 a	1.6915 a	1.6107 a	1.6580 a	4164 c	5895 b	3992 c	5971 b	2570 b
112	1.6976 a	1.7063 a	1.5677 b	1.6561 a	5119 b	6501 a	4757 b	6333 ab	2790 a
157	1.6929 a	1.6844 a	1.5612 b	1.6642 a	6419 a	6242 ab	5345 a	6649 a	2904 a
Linear R^2	--	--	0.85***	--	0.99***	0.33*	0.99***	0.99***	0.97***
Equation (y=)	--	--	-0.0005x + 1.6415	--	25.05x + 2427	3.85x + 5781	15.03x + 3014	7.53x + 5475	3.71x + 2339

*Significant at the 0.05 probability level.
**Significant at the 0.01 probability level.
***Significant at the 0.001 probability level.
† Means followed by the same letter within each column and source of variation are not statistically different from one another at $P < 0.05$.

Table 4. Least square means for the main effect of seeding rate and the effect of the interaction of seeding rate and row spacing width on stem rust incidence presented as area under the disease progress curve (AUDPC) at Roseau 2011 (E3).

Seeding Rate	Stem Rust Incidence	Row Spacing Width (cm)			S.E.	Linear R^2	Equation (y =)
		10	20	30			
--kg PLS† seed ha^{-1}--	--AUDPC--		-----AUDPC-----				
1.3	324 a‡	213	375	385	39	0.79**	8.6x + 152
2.6	304 a	278	360	275	65	NS	NS
5.2	381 a	355	350	437	34	NS	NS
7.8	290 a	285	278	307	40	NS	NS
10.4	324 a	278	325	370	54	NS	NS
Mean	325 a	288 B§	344 A	342 A	21	0.73*	2.7x + 271
Nitrogen rate							
--kg N ha^{-1}--							
67	290 b						
112	322 ab						
157	355 a						
Linear R^2	0.99*						
Equation (y =)	0.6x + 254						

Type III test of significance for stem rust incidence AUDPC at E3

Source of variation	
Seeding Rate (SR)	NS
Row Spacing Width (RSW)	*
SR * RSW	*
Nitrogen Rate (NR)	*
SR * NR	NS
RSW * NR	NS
SR * RSW * NR	NS

*Linear contrast is significant at the 0.05 probability level.
**Linear contrast is significant at the 0.01 probability level.
*** Linear contrast is significant at the 0.001 probability level.
† PLS stands for pure live seed.
‡ Means followed by the same lowercase letter within a row and main effect are not statistically different from one another at $P < 0.05$.
§ Means followed by the same uppercase letter are not statistically different from one another at $P < 0.05$.

Table 5. Correlation among measured variables including seed yield, above-ground vegetative biomass, harvest index (HI), percent lodging prior to harvest, thousand seed weight (TSW), plant height at harvest (PH), fertile tiller number (FTN), and early season vegetative cover (EVC) at four environments including Roseau 2010 (E1), Roosevelt 2010 (E2), Roseau 2011 (E3), and Roosevelt 2011 (E4).

Trait		Yield	Biomass	HI	Lodging	TSW	PH	FTN	EVC
		----------r†							
Yield	E1	1	0.66***	-0.2 NS	0.54 ***	0.24 **	0.55***	0.33***	0.01 NS
	E2	1	0.60***	0.66***	0.47***	-0.25***	0.04 NS	0.12 NS	0.15*
	E3	1	0.68***	0.38***	0.58***	-0.28**	0.27**	0.27**	-0.06 NS
	E4	1	0.32***	0.48***	0.35***	-0.09 NS	-0.02 NS	-0.05 NS	-0.05 NS
Biomass	E1		1	-0.74***	0.80***	0.19**	0.68***	0.27***	-0.10 NS
	E2		1	-0.18*	0.65***	-0.20**	0.23**	0.28***	0.33***
	E3		1	-0.41***	0.60***	-0.09 NS	0.45***	0.19*	-0.10 NS
	E4		1	-0.65***	0.42***	-0.14 NS	0.04 NS	0.08 NS	0.04 NS
HI	E1			1	-0.60***	-0.05 NS	-0.47 ***	-0.10 NS	0.16 NS
	E2			1	-0.01 NS	-0.13 NS	-0.18*	-0.11 NS	-0.10 NS
	E3			1	-0.02 NS	-0.24**	-0.22*	0.11 NS	0.06 NS
	E4			1	-0.10 NS	0.04 NS	-0.06 NS	-0.13 NS	-0.09 NS
Lodging	E1				1	0.14 NS	0.68 ***	0.28***	-0.06 NS
	E2				1	-0.27***	0.16*	0.30***	0.37***
	E3				1	-0.32***	0.35***	0.17*	-0.01 NS
	E4				1	-0.15 NS	-0.00 NS	0.21 **	0.01 NS
TSW	E1					1	0.23**	0.00 NS	-0.21 *
	E2					1	0.06 NS	0.01 NS	-0.00 NS
	E3					1	0.22*	-0.01 NS	-0.16 NS
	E4					1	0.06 NS	-0.07 NS	-0.17*
PH	E1						1	0.29***	-0.01 NS
	E2						1	0.09 NS	0.12 NS
	E3						1	0.17 NS	0.03 NS
	E4						1	-0.18*	0.09 NS
FTN	E1							1	0.07 NS
	E2							1	0.17*
	E3							1	0.12 NS
	E4							1	0.03 NS
EVC	E1								1
	E2								1
	E3								1
	E4								1

*Significant at the 0.05 probability level.
**Significant at the 0.01 probability level.
***Significant at the 0.001 probability level.
†*r*, Pearson correlation coefficient calculated on an individual sub-sub-plot basis.
‡ NS, not significant at the 0.05 probability level.

Table 6. Least square means for the main effect of seeding rate and the effect of the interaction of seeding rate and nitrogen rate on seed yield in four environments including Roseau 2010 (E1), Roosevelt 2010 (E2), Roseau 2011 (E3), and Roosevelt 2011 (E4).

			Nitrogen Rate (kg N ha^{-1})					
Environment	**Seeding Rate**	**Mean Seed Yield**	**67**	**112**	**157**	**S.E.**	**Linear R^2**	**Equation (y =)**
	--kg PLS† seed ha^{-1}--	--(kg seed ha^{-1})--	--Seed Yield (kg seed ha^{-1})--					
E1	1.3	1329 a	1246	1431	1313	78	0.13 NS	NS
	2.6	1387 a	1151	1446	1573	110	0.95*	4.7x + 864.9
	5.2	1356 a	1191	1324	1547	74	0.98***	4.0x + 910.9
	7.8	1292 a	1185	1330	1364	90	0.89*	2.0x + 1070.2
	10.4	1328 a	1167	1327	1490	90	0.99*	3.6x + 925.6
	Mean	1338 AB‡	1189 bβ	1371 a	1456 a			
E2	1.3	1089 b	1003	1140	1107	113	NS	NS
	2.6	1421 a	1549	1292	1422	108	NS	NS
	5.2	1412 a	1325	1540	1380	106	NS	NS
	7.8	1472 a	1384	1543	1488	126	NS	NS
	10.4	1425 a	1308	1537	1432	156	NS	NS
	Mean	1364 A	1313 a	1412 a	1366 a			
E3	1.3	1104 a	994	1073	1241	70	0.96*	2.7x + 795.8
	2.6	999 a	824	1090	1098	54	0.77*	3.0x + 662.4
	5.2	1070 a	934	1180	1094	67	NS	NS
	7.8	1061 a	931	1109	1144	80	NS	NS
	10.4	992 a	774	1050	1150	35	0.93***	4.2x + 523.5
	Mean	1045 C	892 b	1101 a	1142 a			
E4	1.3	1052 a	941	1075	1140	49	0.96**	2.2x + 804.9
	2.6	1146 a	1082	1090	1266	41	0.78**	2.0x +917.1
	5.2	1140 a	1045	1140	1240	43	0.99**	2.2x + 900.2
	7.8	1126 a	1128	1100	1151	54	NS	NS
	10.4	1114 a	1131	1097	1121	66	NS	NS
	Mean	1116 BC	1064 b	1101 b	1181 a			

*Linear contrast is significant at the 0.05 probability level.
**Linear contrast is significant at the 0.01 probability level.
*** Linear contrast is significant at the 0.001 probability level.
† PLS stands for pure live seed.
‡Environment means followed by the same uppercase letter are not statistically different from one another at $P < 0.05$.
β Means followed by the same lowercase letter within a row or column are not statistically different from one another at $P < 0.05$.

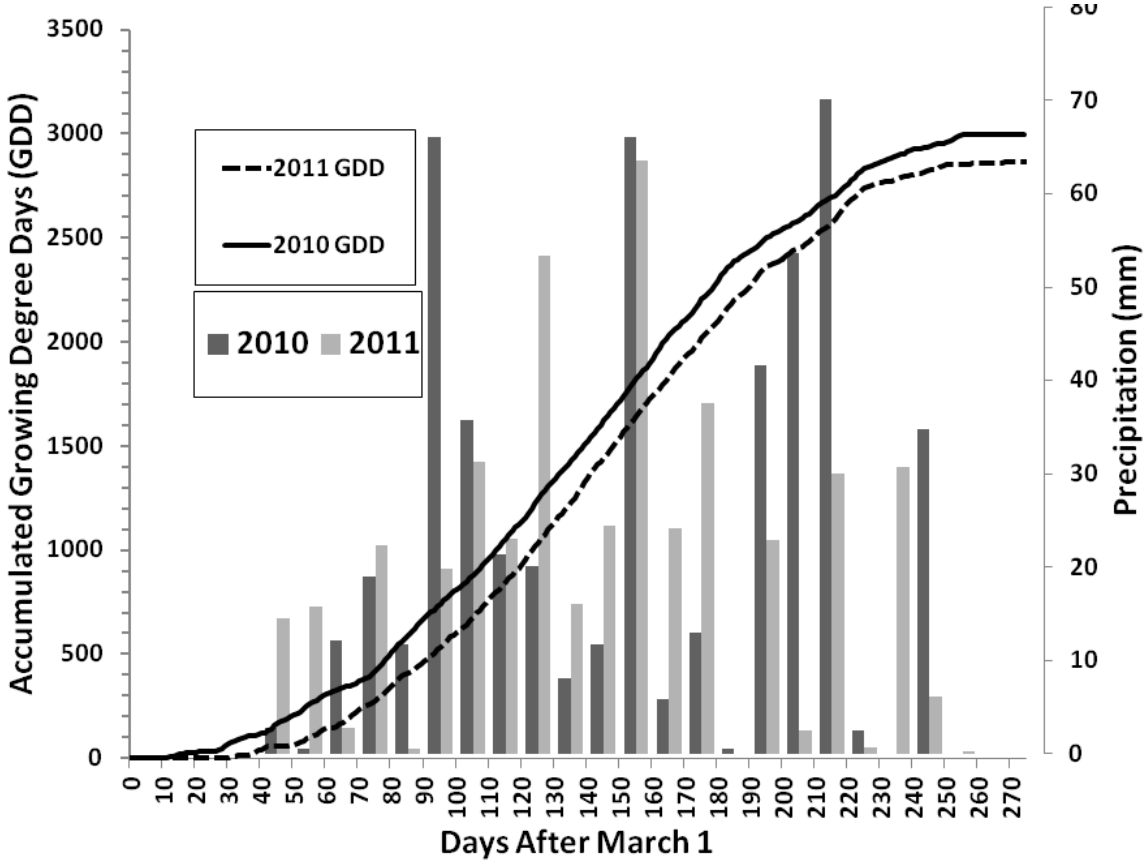

Figure 1. Accumulated growing degree days (GDD) and total precipitation for 10 day intervals following March 1 for the Roseau and Roosevelt, MN region. Growing degree days were calculated using a base temperature of 0° C starting March 1 in 2010 and 2011. The weather station to provide all GDD data was located at the coordinates 48.655 N and 95.734 W. Precipitation data for the first four10 day intervals was not available.

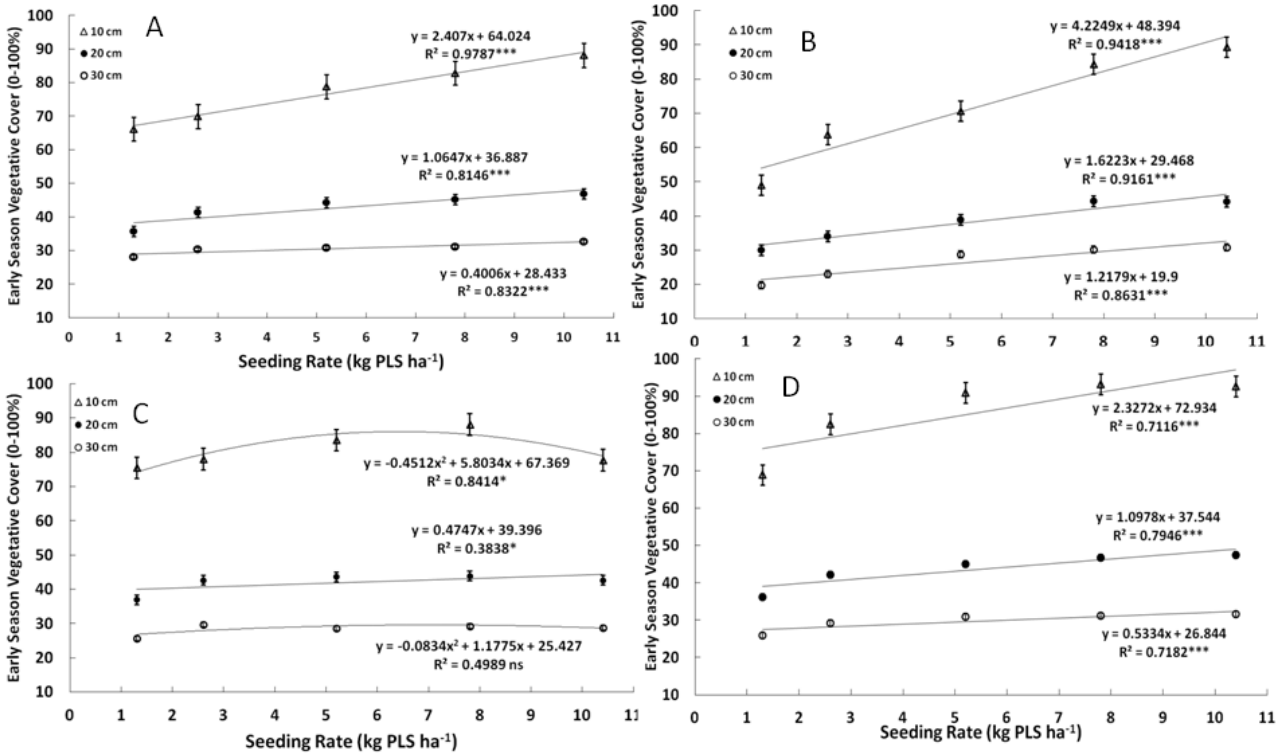

Figure 2. Interaction of seeding rate (SR) and row spacing width (RSW) and the effect on early season vegetative cover (EVC) at: A) Environment 1, Roseau, 2010; B) Environment 2, Roosevelt, 2010; C) Environment 3, Roseau, 2011; D) Environment 4, Roosevelt, 2011.

CHAPTER 3.

Developing Metabolomics-Assisted Selection Techniques for Crown Rust Resistant Cultivar Development in Perennial Ryegrass

INTRODUCTION

Perennial ryegrass is grown worldwide as a turf and forage grass. The perennial ryegrass seed production industry is extremely important in the United States and Canada where there are currently around 32,000 and 9,000 ha of seed production annually, respectively. Resistance to crown rust in perennial ryegrass (caused by *Puccinia coronate* Corda f.sp. *lolii* Brown) is a major selection criteria for perennial ryegrass breeders developing both turfgrass and forage varieties (Pauly et al., 2012; Dracatos et al., 2010; Kimbeng, 1999). In forage perennial ryegrass, crown rust can cause significant losses in dry matter yield (Price, 1987). Crown rust pustules can erupt through leaf surfaces (Roderick and Thomas, 1997) which results in increased water loss and decreased photosynthetic ability in seed production, forage, and turfgrass systems (Dracatos et al., 2010). Additionally in turfgrass, where visual appeal and turf quality are a high priority, a crown rust infection can significantly detract from these parameters (Stier, et al., 2008). Due to environmental concerns and costs, fungicides are generally not considered a viable option for controlling crown rust in perennial ryegrass and developing varieties with genetic resistance is a high priority for breeders (Dracatos et al., 2010; Schejbel et al., 2007; Kimbeng, 1999).

In small grains ,incorporating many minor resistance genes, a form of quantitative resistance, results in delayed or slowed pathogen growth and is considered an important mechanism of durable resistance (Messmer et al., 2000; Krattinger et al., 2009; Poland et al., 2009). Phenotypic screening for durable, quantitative rust resistance in current cultivar development programs is time consuming, unreliable, and prone to human error (Nutter et al., 1993; Diaz-Lago et al., 2003). Progress in breeding for rust resistance depends on an accurate screening procedure. Phenotypic selection for rust resistance relies on the presence of natural inoculum which can be unreliable and variable (Aldaoud et al., 2004; Schejbel et al., 2007), or on the collection and maintenance of a range of

isolates for artificial inoculation which can be costly and laborious (Kimbeng, 1999). Often, when depending on natural inoculation in the field, crown rust symptoms are not observed in perennial ryegrass selection nurseries until after outcrossing has occurred which complicates the selection process. Phenotypic screening can also be complicated by environmental effects which cause plants to vary in expression of resistance or pathogens in expression of virulence resulting in inaccurate assessment of resistance levels (Schejbel et al., 2007; Kimbeng, 1999; Reheul and Ghesquiere, 1994). There is a need to develop a method to select for rust resistance in the absence of the actual pathogen and prior to outcrossing in order to be able to select for other traits simultaneously and speed cultivar development.

There are a number of proposed mechanisms of quantitative disease resistance in plants. Such mechanisms include altered plant morphology and development, antimicrobial secondary metabolites such as phytoalexins or phytoanticipins, detoxification of pathogen derived toxins and altered signal transduction (Poland et al., 2009; Parisy et al., 2007). Some metabolites present in plants are known to be highly involved in protection from biotic and abiotic stresses, and metabolites are undoubtedly involved in in quantitative disease resistance (Poland et al., 2009). For example, it is known that phenylpropanoids accumulate in large quantities in plants developing resistance to pathogens but many of these compounds still need to be identified (LaCamera et al., 2004). Phytoanticipins, antimicrobial compounds produced constitutively by healthy plants, as well as phytoalexins, antimicrobial compounds that accumulate in diseased plants that are responsible for induced resistance, are often phenylpropanoids (LaCamera et al., 2004; Poland et al., 2009). Such mechanisms of quantitative resistance could provide broad spectrum durable resistance and may be amenable to selection via alternative methods such as metabolomics-assisted selection.

Metabolomics-assisted selection has recently emerged as a technique to select for desired traits, based on chemical biomarkers that have been previously associated with a trait of interest (Fernie and Schauer, 2009). In the medical field, metabolomics is well established as a technique to identify chemical biomarkers associated with diseases or gene function (Patti et al., 2012). Metabolomics is now considered an important technique for improving the speed and precision of phenotyping in forage species including perennial ryegrass (Rasmussen et al., 2012). There have been several studies using metabolomics to identify chemical biomarkers for predicting plant phenotypes as it is thought that metabolite biomarkers have the potential to predict phenotypes before the phenotype becomes apparent (Steinfath et al., 2010).

In potato (*Solanum tuberosum* L.) tubers, gas chromatography and mass spectrometry (GC-MS) was used to identify biomarkers to predict chip quality and susceptibility to black spot bruising (Steinfath, et al., 2010). In loblolly pine (*Pinus taeda* L.), a GC-MS approach was used to develop a 47 metabolite model which accurately predicted (r = 0.98) regenerative capacity of somatic embryogenic cultures (Robinson et al., 2009). Green tea quality from the plant *Camellia sinensis* L., was analyzed using a ultra performance liquid chromatography (UPLC-MS) approach that detected 301 metabolites (many of which were phenolic compounds) that were associated with the amount of shade that the plants were exposed to (Lee et al, 2012). In grape (*Vitis vinifera* L.), a UPLC-MS approach was used to detect biomarkers (many of which were flavenoids) that were associated with growing conditions and grape variety (Tarr et al., 2013). Another grape study analyzed cultivars that varied in susceptibility to downy and powdery mildew and found that the resistant cultivars had a higher constitutive abundance of inositol, glutamine, glutamate, alanine and caffeic acid (Figueiredo et al., 2008). In oats, it has been suggested that certain phenolic compounds may exist at higher levels genotypes that are more resistant to crown rust (*P. coronata* f. sp. *avenae*) and it

was shown that avenanthramides (phenolic compounds) in grains and two unidentified compounds in leaves were markers associated with crown rust resistance (Dimberg and Peterson, 2009). Searls and French (1964), determined that cinnamaldehyde, cinnamic acid and cinnamyl alcohol, all products of the phenyl propanoid pathway, can stimulate urediniospore germination of *Puccinia graminis* f. sp. *tritici* Urban. Urediniospore germination of *P. graminis* f. sp. *triticii* is also stimulated by nonanal and nonanol (French, 1992). In Italian ryegrass (*Lolium multiflorum* Lam.), a species closely related to perennial ryegrass, 23 phenolic compounds (thought to be important in resistance to pathogens) were isolated, some of which were constitutively produced, and some of which production varied depending on the presence or absence of endophytes (Ponce et al., 2009). Such secondary metabolites, if detected in perennial ryegrass and associated with rust resistance and susceptibility, could serve as useful biomarkers for selecting for quantitative crown rust resistance.

Detecting secondary metabolites, which are biomarkers associated with particular phenotypes, is useful because secondary metabolites are the end products of complex biochemical pathways. These pathways are regulated by genes and secondary metabolites are often used directly as stress mediation, or plant defense, mechanisms making them closely associated to plant phenotypes (Patti et al., 2012; Dettmer et al., 2007; Harrigan et al., 2007). A single gene can affect a number of metabolic pathways complicating the association of genes with phenotypes, however, secondary metabolites are the end products of the "omics cascade" making them closely associated with plant phenotypes (Patti et al., 2012). An untargeted metabolomics approach to detecting secondary metabolites in plants attempts to detect as many metabolites as possible in a single extract (De Vos et al., 2007; Patti et al., 2012). Detecting a large number of secondary metabolites in a single extract can improve the speed of the screening process and increase the chances of detecting important metabolite biomarkers. In addition, an

approach that uses one instrument platform and one extraction protocol with minimal processing will be most useful for future high-throughput analysis of any potential biomarkers of interest to a plant breeding program.

Liquid chromatography (LC) coupled to mass spectrometry (MS) enables the detection of more metebolites in a single run than other techniques (Patti et al., 2012). Some relavant types of metabolites detectable by LC-MS include alkaloids, phenolic acids, phenylpropanoids, and flavenoids, which all could be important in plant defense (De Vos et al., 2007). Sample preparation when using LC-MS is also faster and less complicated than when using other analysis techniques such as gas chromatography (Berg et al., 2013; De Vos et al., 2007). No instrument platform will be able to analyze all classes of metabolites in a single pass quickly; however, reversed phase LC-MS using electrospray ionization (ESI) performed in positive and negative mode will allow detection of a very broad range of semi-polar and non-polar metabolites (Dettmer et al., 2007; De Vos et al., 2007). Using aqueous methanol as an extraction solvent for extracting the metabolites to be analyzed via LC-MS can help maximize the number of detectable metabolites (De Vos et al., 2007; Maier et al., 2010). Another method which can help maximize the number of metabolites detected and improve data quality is the use of ultra performance liquid chromatography (UPLC) which uses columns with small particle size resulting in better separation of compounds prior to MS analysis (Dettmer et al., 2007). In combination with UPLC, modern mass spectrometers with high resolution, high mass accuracy, and the ability to quickly switch between positive and negative ESI allow the detection of hundreds to thousands of potential secondary metabolites in a single complex plant extract (Tarr et al., 2013).

When setting up an experiment to detect secondary metabolites as biomarkers for predicting a plant phenotype, it is important to remember that the predictive ability of the potential biomarkers is only as good as the data used as the training data set for

developing the predictive model. Therefore, great care must be taken to obtain accurate phenotypic data used for associating with the presence or quantity of secondary metabolites in the plants being phenotyped. Additionally, sample processing and environmental conditions can affect the detection of metabolites, so great attention to minimizing variation these areas is warranted (Maier et al., 2010).

The goal of this research was to detect a set of metabolic biomarkers to be used for fast and accurate selection of crown rust resistant perennial ryegrass genotypes. These chemical biomarkers will serve as a "metabolic fingerprint" associated with crown rust resistance in perennial ryegrass. Termed metabolomics-assisted breeding, this technique will lead to faster cultivar development and ultimately reduce fungicide use, making perennial ryegrass seed production a more profitable, marketable and sustainable option for farmers in rural communities in northern Minnesota as well as the Pacific Northwest and Canada.

Our approach was to first identify a set of perennial ryegrass lines differing in stable resistance levels to crown rust in multiple environments and to insure that resistance levels were consistently observed against multiple isolates of the crown rust pathogen. Then, extracts from the set of perennial ryegrass lines were analyzed via UPLC-MS to detect as many constitutively produced secondary metabolite features as possible in a single run. These features were then associated with crown resistance levels using multivariate and univariate statistical techniques. A set of metabolite biomarkers for crown rust resistance was determined and then evaluated for the ability to predict the crown rust resistance phenotype.

MATERIALS AND METHODS

Plant germplasm and field crown rust resistance screening

Fifty-nine perennial ryegrass lines (the lines were half sib families) were evaluated for crown rust resistance. The lines evaluated were previously developed by repeatedly backcrossing a spreading type perennial ryegrass from the University of Minnesota perennial ryegrass breeding program to high quality germplasm from Rutgers University. The 59 lines had previously been selected for horizontal growth, good turf quality and winterhardiness. All lines contained endophyte as determined using an Agrinostics (Agrinostics Ltd. Co., Watkinsville, GA) Phytoscreen© test kit. Initial crown rust resistance screening of the population of 59 perennial ryegrass lines took place in the University of Minnesota's agricultural experiment fields in St. Paul, MN. To summarize, beginning in the fall of 2008, 59 perennial ryegrass lines were planted into soybean stubble with four replications for each line and 20 genetically distinct plants representing each line in each replication. The 59 lines were rated for crown rust disease severity resulting from natural infection on 6 July, 13 July, and 4 August during the summer of 2009. Crown rust coverage on leaf surfaces for each of the plants within a line was visually estimated using a disease area rating key (James, 1971) and a modified Horsefall-Barret rating scale where 1= no rust, 2 = <10%, 3 = 11-25%, 4 = 26-40%, 5 = 41-60%, 6 = 61-70%, 7 = 71-80% 8 = 81-90% 9 = >90%, and 10 = 100% coverage of rust pustules (Helgeson et al., 1998). Using mean crown rust data from each line within a replication, area under the disease progress curve (AUDPC) was calculated for each line and the 12 most resistant, 12 moderately resistance and 12 most susceptible were selected for further evaluation (Table 1). Within each selected line the most resistant (from the 12 selected resistant plants), or most susceptible (from the 12 selected susceptible plants) plant was selected from each replication and clonally propagated in the greenhouse for use in later crown rust resistance verification screening. For the 12 moderately resistant lines the plant having rust resistance closest to the median for the genotype was selected.

In the fall of 2009, remnant seed, which was the seed used to plant the nursery in 2008, was used to establish a second nursery in St. Paul, MN similar in design to the one planted in 2009 with a main difference being that it included six of the most resistant lines, seven of the most susceptible, and 12 lines identified as having medium resistance in summer of 2009. All selected lines could not be re-evaluated due to a lack of seed. The nursery planted in fall of 2009 contained a total of 25 of the original 59 perennial ryegrass lines and each line was represented by 6 plants in each of the four replications. Crown rust disease severity caused by natural infection was rated for this nursery three times during the summer of 2010 using the same modified Horsefall-Barret scale (Helgeson et al., 1998) as in 2009. Mean crown rust severity ratings from the rating date at peak severity were used to make selections. A total of 14 lines ranked consistently in the resistant (30% lowest scores), medium resistance, or susceptible categories (30% highest scores) (4, 5, and 5 lines, respectively) (Table 1). From these 14 lines, the most resistant plant (the plant with the greatest severity rating), most susceptible plant (the plant with the lowest severity rating, or median plant (the plant with the severity rating closest to the median for the line) was selected from each line within each replication and each plant was clonally propagated in the greenhouse for future screening.

In the fall of 2010, clones from each of the selected 14 lines demonstrating consistent crown rust resistance over two growing seasons, were planted into a field nursery in St. Paul, MN to verify resistance rankings and obtain a more robust data set. The 14 lines were screened in four replications using a randomized complete block design. Each line was represented by eight clones which included the four clones selected from the 2009 screening experiment for each line, and the four clones selected from the 2010 screening experiment for each line. Each replication of a line in the nursery planted in 2010 contained an identical set of plants (genotypes) obtained through clonal propagation. The nursery was rated for crown rust disease severity in the summer

of 2011 using the modified Horsefall-Barret scale (Figure 1). A set of plants, identical to and clonally propagated from those found in the 2011 field nursery, were maintained in the greenhouse. Beginning in the summer of 2012 this material was screened for crown rust resistance in the growth chambers via artificial inoculation using a diverse crown rust urediniospore collection.

Crown rust inoculum

Collections of crown rust urediniospores from multiple locations were made between 2009 and 2012. Crown rust isolates used for artificial inoculation in growth chamber experiments were collected from the environments listed in Table 2. All urediniospores were collected off of perennial ryegrass leaf tissue and identified by spore morphology. Perennial ryegrass material, from which urediniospores were collected at the field locations, included 48 accessions from a total of 23 countries that were obtained from the United States Department of Agriculture National Plant Germplasm System (USDA-NPGS) (Cisneros and Watkins, 2010). The 48 accessions were planted at each location in a randomized complete block with a split-plot treatment arrangement where each accession received a mowed and un-mowed treatment to create variable environments for crown rust development (Cisneros and Watkins, 2010). Crown rust urediniospores were collected by scraping spores off the leaf surface with the edge of a gelatin capsule and letting spores fall into the capsule. Gelatin capsules containing spores were then placed in a sealed plastic bag and chilled on ice until they were placed in a desiccator with the plastic bag un-sealed for four d. Following desiccation the gelatin capsules containing urediniospores were place inside a sealed air free plastic bag and stored at -80° C until needed.

Crown rust resistance ranking verification

Fourteen perennial ryegrass lines from the University of Minnesota breeding program that demonstrated consistent crown rust resistance rankings over three years of field screening trials (Table 1; Figure 1) were further screened under controlled conditions in a growth camber to verify resistance rankings and develop a robust data set for use in further development of a metablomics-assisted selection model. Clones (described in the "Plant Germplasm and Crown Rust Resistance Screening" section above) from all plants used in the 2011 field crown rust screening nursery were maintained in 6.4 x 6.4 x 8.9 cm plastic form pots in the greenhouse with a single pot containing one single clone. The pots containing plants from the 14 lines were arranged in a randomized complete block design with four replications and individual plants from a line were grouped into whole plots. Leaf tissue was sampled for metabolomics analysis (see below) prior to inoculation, and then each line was inoculated with a bulked sample of six previously collected crown rust isolates. For inoculation, urediniospores were first heat shocked inside plastic zip top bags at 45° C for 15 minutes, then re-hydrated at ambient temperature (25° C) and humidity for 3 hours. Spores were then mixed with Soltrol 170 isoparaffin in a gelatin capsule and sprayed onto the perennial ryegrass plants with a siphon feed spray nozzle at a rate of 0.0268 mg of urediniospores per pot. Following application of spores, the Soltrol 170 isoparaffin was allowed to evaporate for 1 hour and then plants were placed in a dark dew chamber, consisting of a closed box with three humidifiers, where they were continually misted for 1 hour. Following the initial 1 hour misting period the plants were then misted for two minutes every hour for 14 h under dark conditions. After 14 h the mist was stopped and the dew chamber door was opened for four h to facilitate drying of the leaf surface. The plants were then transferred into a growth chamber where they were maintained at 25° C daytime temperature and 20° C nighttime temperature with a photoperiod of 14 hours. Relative humidity in the growth

chamber was maintained near 80%. Potted plants were placed in a tray which was filled with water daily to maintain adequate moisture without disturbing crown rust infections.

Crown rust severity was rated after 21 d using the same rating scale that was used in the field rust screening nursery. One plant was selected that had disease severity representative of each number of the rating scale and all ratings were based on comparisons with the selected representative plants. Means for each line were computed and then the growth chamber and field 2011 data were subject to the PROC CORR procedure in SAS to determine the Spearman rank-order correlation between the field and growth chamber data.

Plant tissue extraction and sample preparation for UPLC-MS analysis

Leaf tissue was sampled from all lines that were screened for crown rust resistance in the growth chamber just prior to artificial inoculation with the intent of obtaining a metabolic fingerprint at the time of disease infection. The first fully expanded leaf was harvested from each plant representing a line within a replication. Tissue from each line within a replication was bulked, placed in a 1.5 ml microcentrifuge tube, sealed and immediately quenched in liquid N until frozen. Samples were then stored at -80° C until further processing. After 1 month, samples were lyophilized and then again stored at -80° C until needed.

To prepare extracts for metabolomics analysis, the lyophilized tissue samples were ground in 1.5 ml Eppendorf tubes with two tungsten beads using a bead mill (Spex Sample Prep 2010 GenoGrinder) set to 1100 revolutions per minute, for 15 minutes. Approximately 16.82 mg (+/- 5%) of ground leaf tissue was weighed into a new low-retention 1.5 ml Eppendorf tube for each sample (this is approximately equivalent to 40 mg fresh weight for the perennial ryegrass leaf tissue samples), and the exact weight recorded. Two clean tungsten carbide beads were then added to the tube along with 800

µl of a 90% methanol (Chromasolv®, Sigma-Aldrich) and 10% double distilled water solution. The samples were then placed in the bead mill for 30 min. at a speed of 800 revolutions $min.^{-1}$ for extraction. Samples were then centrifuged at 14,000 revolutions $min.^{-1}$ for one minute. Using low retention pipette tips, 400 µl of the supernatant was removed and placed in another low-retention 1.5 ml Eppendorf tube where it was evaporated using a speed-vac. The remaining supernatant was stored at -80° C for future analysis. Once dried, the sample was reconstituted in 100 µl of 15% acetonitrile (Chromasolv® Plus, Sigma-Aldrich) and 85% double distilled water by shaking in the bead mill with one tungsten bead for 10 min.. The tube containing the sample was then sonicated for 5 min.. To prepare for HPLC analysis, the sample was centrifuged for 20 min. at 14,000 revolutions $min.^{-1}$, then 50 µl of the supernatant was pipetted into a glass HPLC vial insert. A composite sample of all lines was also created by bulking 20 µl of each extract. The remaining sample was stored at -80° C for future analysis.

Ultra performance liquid chromatography (UPLC)

Chromatography was performed using a Thermo Scientific Dionex UltiMate 3000 HPLC. To start analysis, 2 µl of each sample was injected onto a Acquity UPLC BEH C18 column (2.1 x 100 mm, 1.7 µm particle size, Phenomenex SecurityGuard Ultra guard column), equilibrated in 90% solvent A (0.1% aqueous solution of formic acid), 10% solvent B (Chromasolv® Plus acetonitrile). Prior to injection, samples were stored in the autosampler at 10° C. The column temperature was maintained at 40° C. Compounds were eluted from the column using a constant flow rate of 400 µl per minute and linearly increasing the concentration of solvent B beginning one minute after injection from 10% to 98% over 26.5 minutes. The concentration of solvent B was maintained at 98% for five minutes and then re-equilibrated in 90% solvent A and 10% solvent B for two min. for a total run time of 34.5 min..

Mass spectrometry

A Thermo Scientific Q Exactive™ hybrid quadrupole-Orbitrap mass spectrometer for high resolution and accurate mass was operated using electrospray ionization in positive and negative polarity switching mode. The resolving power was 35,000. Other parameters were IT = 200 ms, AGC Target = 1,000,000, and full scan mode was used with a mass range of 215 m/z to 2000 m/z. Conditions at the source for both ionization polarities were: spray voltage = 3.80 kV, sheath gas = 50 arbitrary units, auxillary gas = 20 arbitrary units, sweep gas = 0 arbitrary units, heater temperature = 300° C, and capillary temperature = 350° C. With positive electrospray ionization, a lock mass was used to ensure accuracy and reproducibility. Solvent blanks were run before each set of biological replicates as well as after every fourth unknown sample. A quality control standard of known composition and accurate mass (LCMS QC Reference Standard, Waters) was run before each biological replicate, and once at the end of all data collection to ensure consistent and accurate performance of the mass spectrometer over time. A composite sample of supernatant taken from each line was evaluated before analysis of each set of biological replications and at the end of all analysis to evaluate any differences in performance over time.

Data processing

Chromatographic alignment, background subtraction, and component detection were conducted using Thermo SIEVE™ 2.1 software. This software performed data reduction by grouping isotopes and adducts into main components. Components with greater than 40% covariance in the quality control composite sample of all lines were eliminated from analysis. Components that were not detected in the composite sample were also removed

from further analysis. Integrated intensity of each detected component within a sample was normalized based on sample dry weight.

Data analysis

The results from SIEVE™ 2.1 (m/z values, retention time, and integrated intensity for each component) were then imported into GeneData Analyst™ (Genedata, Basel, Switzerland) for the remaining data analysis. The corresponding crown rust severity score data was also imported and used for annotation. For annotation, the rust severity mean from each replication within a line was matched with UPLC-MS data from the same sample. Data were visualized using partial least squares discriminant analysis (PLS-DA) using rust severity score (Table 3) as supervisory data set. Components with a variable importance in the projection (VIP) value of greater than 0.5 (high relevance for explaining differences in rust severity) were selected for further evaluation. Components were then subject to correlation analysis where the integrated intensity was correlated with rust severity values and the corresponding p-value was calculated. Components that had VIP > 0.5 were then subject to partial least squares regression analysis using correlation as ranking data to calculate the optimal set of components that best explain differences in crown rust severity. Leave one out cross validation was then performed to determine the performance of the model. To further characterize components discriminating between crown rust susceptible groups, fold change in integrated intensity from the resistant to the susceptible group was calculated and a corresponding T-test was then performed. Fold change in integrated intensity of features was considered significantly different at $p<0.05$.

RESULTS AND DISCUSSION

It is known that the crown rust resistance phenotypes of perennial ryegrass lines or accessions can vary depending on the year and location in which they are grown and on the crown rust isolates (Aldaoud et al., 2004; Schejbel et al., 2007). Therefore, it was necessary to screen the perennial ryegrass lines used in this study in multiple environments and against a wide range of potential isolates to observe a durable resistance phenotype and to get reliable data for use in identifying potential biomarkers that are predictive of crown rust resistant phenotypes.

Initial field crown rust screening

During the 2009 growing season, 59 perennial ryegrass lines were screened for crown rust resistance levels in the field in St. Paul MN (Table 1.). Fifteen percent of the lines with the lowest AUDPC were considered resistant (R) and fifteen percent of the lines with the highest AUDPC were considered susceptible (S) (Table 1). The fifteen percent of lines around the median were considered moderately resistant (M) (Table 1). These 36 lines that were designated R, M, or S were selected for further screening to verify rust resistance levels in the field in 2010. In 2010, 21 of the 36 selected lines, plus four additional lines which had available remnant seed were screened for resistance to naturally occurring crown rust and 14 of the lines ranked into R, M, or S groups similarly to the 2009 ranking (Table 1). These initial screening studies provided knowledge on which lines would react consistently to crown rust disease pressure. Due to the outcrossing nature of perennial ryegrass, the plants representing a line in each year of the screening trials were genetically different. In order to have more robust data and to develop a set of plants that could be accurately phenotyped and sampled for metabolomics analysis, clones from the most representative plant from the stable lines identified in the initial field screening (Table 1) were used for the final resistance verification experiments (see methods).

Final field and growth chamber crown rust resistance verification

For the final rust resistance screening experiments each line was represented by eight plants including a single clone selected from each of replications of field screening in 2009 and 2010. These eight plants per line were clonally propagated and evaluated in a randomized complete block with four replications in both the field and growth chamber (Figure 1). This replicated trial that was screened in the field and gave good rust resistance phenotypic data, however it was decided that plant tissue harvested from the field trial was not acceptable for metabolomics analysis due to field variability, small plant size, and potential stress on the plants due to water shortage or other pathogens. Average standard error within a plant line was 0.32 in the field screening trial in 2011 (Figure 1). Environment or variability in sampling in the field can affect the metabolic profile of a plant (Maier et al., 2010) therefore it was decided that the replicated field experiment would be repeated with identical plant material under the controlled conditions of a growth chamber (Figure 1). Identifying metabolic biomarkers under more controlled conditions should improve the predictive power of the biomarkers by potentially avoiding selecting environmentally induced metabolites as biomarkers (Steinfath et al., 2010). Figure 1 shows the crown rust severity and relative growth chamber based ranking of crown rust resistance levels for the 14 lines evaluated in the rust resistance verification experiments. The average standard error for crown rust severity ratings within a line in the field experiment was 0.32 vs. 0.16 in the growth chamber experiment indicating that there is less variability in the growth chamber (Figure 1). This is to be expected as the growth chamber is a controlled environment with uniformly applied rust inoculum while the field can have variable soil, variable moisture, non-uniform rust inoculum, and variable fertility. Field and greenhouse rankings for crown rust severity were highly correlated however, so it is likely that crown rust

resistance phenotypes observed in the growth chamber will also be predictive of field crown rust resistance phenotypes.

The growth chamber experiment was exposed to a bulked sample of crown rust urediniospore isolates that were representative of multiple years and locations within Minnesota and collected from highly variable perennial ryegrass germplasm (Table 2). The stability in final rust resistance rankings between the field and growth chamber (Figure 1; Table 3) may indicate that the crown rust resistance phenotypes that were designated are accurate and that the crown rust resistance observed in the growth chamber are at least somewhat durable given that the perennial ryegrass lines were exposed to a wide range of crown rust isolates in the growth chamber (Table 2). Additionally, the high correlation between field and growth chamber data suggest that biomarkers for rust resistance identified in the growth chamber may also be predictive of the phenotype in the field which is the ultimate goal. It should be mentioned that while a diverse set of crown rust isolates were screened against, the crown rust isolates used in this study only represent those found in Minnesota and they may not represent the broad range of crown rust isolates found throughout the United States or the world. Further work could be done to assess the robustness of the detected biomarkers against additional crown rust isolates.

For the purposes of data analysis, the 14 perennial ryegrass lines were placed into final resistance group designations of Resistant (R), Moderately Resistant (MR), Moderately Susceptible (MS), and Susceptible (S) based on standard deviation from the mean and stability of ranking between the 2010 field and growth chamber experiment (Table 3; Figure 1). The resistant group was highly resistant with plant phenotypes showing little to no rust while the susceptible phenotypes received rust severity scores of up to 4.5 which indicated over 40% coverage of rust pustules on the leaf surfaces (Table 3). Due to the low average standard error detected within a plant line, and the relatively

large variation in crown rust severity score among the plant lines, the growth chamber crown rust severity score was selected as the training data set to be used for identifying potential metabolic features as biomarkers. The average crown rust severity score given to a line within a replication was used as the training phenotypic data set for a robust set of 56 data points (Table 3).

Metabolome analysis of perennial ryegrass extracts

In order to obtain a snapshot of the metabolic profile for each line just prior to exposure to the rust pathogen, samples were harvested from each line prior to inoculation with the pathogen for the growth chamber rust screening experiment. Extreme care was taken to reduce variability due to sampling and metabolism was quenched with liquid nitrogen to avoid further variation in metabolism with the harvested tissue (Berg et al., 2013). To further reduce potential variation, samples were stored at -80°C which minimizes further changes in the metabolic profile (Berg et al., 2013).

To facilitate comparing samples semi-quantitatively, based on equivalent sample weight, it was necessary to lyophilize samples to obtain moisture free leaf tissue. Accurately weighing dry samples prior to extraction allowed for adjusting for minor differences in sample weight during subsequent analysis, further reducing variation in detected features due to sample processing and potentially maximizing the predictive power of the any potential biomarkers (Berg et al., 2013; Steinfath et al., 2010). For UPLC-MS analysis, an appropriate randomization of samples was chosen to minimize the effects of changes in instrument performance over time and quality control samples were analyzed along with each biological replicate insuring consistent and accurate instrument performance (data not shown) (Berg et al., 2013). In addition, a composite sample, made of equal portions of all 56 sample extracts, was analyzed at regular intervals throughout

the UPLC-MS analysis to insure consistent instrument performance and identify which metabolites are not detected consistently throughout the run.

After background subtraction, choromatographic alignment, and peak detection, the component detection function of SIEVE 2.1 ^{TM} was used to perform grouping of isotopes, adducts, fragments, and multimers under single root features which is an effective means of reducing UPLC-MS data (Tarr et al., 2013). A total of 506 metabolic features, detected compounds having a unique mass-to-charge ratio (m/z) and retention time, were detected from positive ESI UPLC-MS and 604 features from negative ESI analysis by Sieve 2.1^{TM}. The metabolite profiles of the perennial ryegrass lines were subject to PLS-DA, using the rust resistance score by replication (Table 3) as the training data. This was done to visualize how well the detected features explain separation between rust resistance groupings and to calculate VIP for evaluating a metabolic features relative importance in discriminating based on rust severity data (Figure 2 and 3; Table 4 and 5). When discrimination between samples is the goal and a training data set is available, partial least squares (PLS) analysis has been shown to be more appropriate than principle component analysis (Barker et al., 2003).

Scores plots from PLS-DA analysis on the positive ESI UPLC-MS data indicate that 8.4, 36.6 and 6.6% of the variation in rust score between samples could be explained by the features contributing to components 1, 2, and 3 respectively (Figure 2). For the positive ESI data, the R and S resistance groups could clearly be distinguished on the PLS-DA scores plot by component 1 (Figure 2). Components 1 and 3 (8.4 and 6.6%) separated the MS and MR groups from the R and S groups and also distinguished between the MS and MR groups (Figure 2B). The distinction between the MS and MR groups was less clear but is to be expected because the crown rust severity ratings, which the PLS-DA scores plot is based on, are more similar among these groups than among the R and S groups (Figure 2). For the negative ESI UPLC-MS data, components 1, 2 and 3

in the scores plots accounted for 44.6, 18.4 and 7.0% of the variation in the crown rust severity score (Figure 3). For the negative ESI UPLC-MS data, components 2 and 3 (18.4 and 7.0% variation respectively) separated the R and S groups well and also showed relatively good separation of the MS and MR groups from one another (Figure 3C). Components 1 and 3 separated the R and S groups well but demonstrated poor separation among other groups (Figure 3B).

To begin the process of detecting biomarkers for predicting crown rust resistance or severity, the relative contribution of the metabolic features for discrimination between crown rust severity phenotypes was first calculated. Calculating the relative contribution of a metabolic features for plant phenotype discrimination using the variable importance in projection (VIP) method has been successfully demonstrated previously in potatoes as well as tea (Steinfath et al., 2010; Lee et al., 2013). In the potato study, features with calculated VIP > 2.0 were considered potential biomarkers however in the tea study, features with VIP > 0.5 were considered as potential biomarkers (Steinfath et al., 2010; Lee et al., 2013). Variable importance in prediction values are relative however, and tend to be higher when the number of features included in the analysis are smaller. Due to the large number of detected features in our study, VIP values greater than 0.5 were considered potential biomarkers for predicting crown rust resistance and selected for further analysis.

A total of 91 features detected using positive ESI UPLC-MS analysis had VIP values > 0.5 and were considered potential biomarkers for crown rust severity (Table 4). For the positive ESI data, potential biomarkers had m/z values that ranged from 245.0808 to 885.3421 and retention times that ranged from 1.00 minutes to 27.39 minutes indicating good representation of the detectable range of features selected as potential biomarkers (Table 4). For the negative ESI data, 93 features were determined to have VIP > 0.5 and the range of m/z values was from 191.0187 to 1109.2997 (Table 5). The

range of retention times for features detected for the negative ESI data was smaller with a maximum retention time of 11.42 (Table 5). This suggests that future negative ESI analysis of potential biomarkers of crown rust resistance could focus on compounds with shorter retention times (more polar compounds) on a reverse phase C18 column and potentially eliminate the need for longer runs. The data indicate that different and potentially important features for discriminating crown rust severity score phenotypes were detected from positive and negative ESI analysis; however; the relationship between the detected features and the phenotype needed to be investigated in greater detail.

The features that had VIP values > 0.5 were subject to correlation analysis where integrated intensity of each feature detected in the MS analysis was correlated to crown rust severity score from the growth chamber screening experiment (Table 5). Of the 91 features from the positive ESI data with VIP values > 0.5, 31 unique features were significantly correlated with rust severity ($P<0.05$; Table 6). Five features were negatively correlated with rust severity score while the rest were positively correlated with rust severity with correlation values ranging from -0.33 to 0.65 (Table 5). Many of the most highly correlated components had VIP values > 1.0 indicating that they are highly relevant for discriminating between crown rust severity phenotypes (Table 6). For the negative ESI data, 38 of the 93 features that had VIP values > 0.05 showed significant correlation of integrated intensity and rust severity score (Table 7). From the negative ESI data, more negative significant correlations with rust score were found (11 in total) (Table 7). Features that have a negative correlation with rust score may be important because they are potential compounds that inhibit crown rust infection or are compounds that could be involved in the defense response to pathogen attack. Positive correlations indicate that a compound may promote pathogen growth or that the compound is involved in altering the plant in some way that makes it more susceptible to crown rust.

From the negative ESI data, a total of 27 features, of the 93 detected with VIP values > 0.5, were determined to have significant positive correlation of integrated intensity with crown rust severity score (Table 7). Correlations for the negative ESI data ranged from -0.61 to 0.69 (Table 7). Correlations between abundance of metabolites regarded as good biomarkers and regenerative capacity of loblolly pine ranged from -0.44 to 0.62 (Robinson et al., 2009). The high correlations between integrated intensity of features and crown rust score in our study seem to be in line with correlations observed in the Robinson et al. (2009) biomarker discovery study. Both positive and negative correlations of metabolic biomarkers with crown rust severity phenotypes are not unprecedented. In oat, where nine genotypes were analyzed and rust severity ranged from 0 to 85%, correlations of chromatographic peak area of four phenolic compounds with crown rust severity ranged from -0.629 to 0.765 (Dimberg and Peterson, 2009). The compounds found that were negatively correlated with rust score in the oat study were not identified. Although significant correlations show relevance for predicting a phenotype, further analysis was required to determine the set of features that, when analyzed together, are most predictive of crown rust score.

Figures 4 and 5 show fold change in integrated intensity of the detected components in the susceptible group compared to the resistant group for the positive and negative ESI data respectively. For fold change evaluation and the corresponding T-test, the perennial ryegrass lines were evaluated using their resistance grouping (Table 3) and only the resistant and susceptible groups were compared. Simply evaluating fold change in integrated intensity between two groups can be misleading because metabolites that are not related to crown rust resistance may also be changing between the two groups. However, the features shown in Figures 4 and 5 had integrated intensity that was determined to be correlated with crown rust severity score and had VIP values > 0.5 prior to conducting the fold change analysis. For the positive ESI data, three of the features

that were negatively correlated with the rust severity data had a significant fold change in integrated intensity between the R and S groupings (a 1.5 to 57 fold difference) while 23 of the positively correlated components had a significant fold change between the R and S groupings (Figure 4). Seven of the positively correlated features for the positive ESI data demonstrated a fold change of five or greater between the resistant and susceptible groups (Figure 4). For the negative ESI data, six of the features that were negatively correlated with crown rust resistance score had a significant fold change of integrated intensity (fold change ranged between 1.8 and 5) between the R and S groupings (Figure 5). For the negative ESI data, 20 of the features that were positively correlated with rust severity score demonstrated significant fold change in the susceptible group compared to the resistant group (Figure 5).

Partial least squares regression models were developed and cross validated to determine the optimal set of features for predicting crown rust severity for each MS data set. Correlation values were used for ranking of features to be used in the partial least squares regression models (Tables 6 and 7). Using this method, features are included in the model based on correlation of integrated intensity of the feature with rust severity score, with priority going to those features demonstrating the highest correlation. Additional features are added to the model until the best set of features for predicting crown rust severity is found. To determine the optimal set of biomarkers, actual crown rust severity values are plotted against predicted crown rust severity values for each possible set of features included in the prediction model. A regression line is then fit to the data which minimizes deviation of the data points from the regression line and root mean squared error (RMSE) and correlation are calculated. An optimal set of biomarkers will minimize RMSE and maximize correlation.

For the positive ESI data, including the top 20 positively correlated features in the prediction model resulted in RMSE of 0.695 and correlation between actual and predicted

crown rust severity values of 0.677 (Figure 6A). Including the top 31 features resulted in the lowest root mean squared error (RMSE) and highest correlation (r = 0.764) between actual and predicted crown rust severity values in cross validation (Table 8; Figure 6B). It should be noted that including the top 31 most highly correlated feature included the 5 negatively correlated features (Table 6) indicating that these features contribute significantly to prediction. Including all 91 components with VIP values > 0.5 resulted in a relatively high RMSE of 0.895 and poorer prediction (Table 8). For the negative ESI data, the most optimal biomarkers for predicting crown rust severity were the top four most highly correlated features (Table 8; Table 7; Figure 6C). Correlation between actual crown rust severity values and predicted values was 0.764 and RMSE was minimized (Figure 6C). The top four biomarkers included three which were positively correlated with rust severity and one that was the most inversely-correlated feature with crown rust severity indicating that it is of high importance for predicting crown rust resistance in perennial ryegrass (Table 7). For the negative ESI data, including all 38 features that were significantly correlated with crown rust severity score in the predictive model did not improve the predictive ability of the model over the four biomarker model (Figure 6D). Correlations between actual phenotypic values for black spot bruising and predicted values in another biomarker study on potato ranged from 0.53 to 0.82 which is in line with correlations observed our study (Steinfath et al., 2010). In our study, the partial least squares regression plots from model cross validation (Figure 6) demonstrate excellent ability to distinguish between R and S groups when the optimal set of biomarkers is chosen (Figure 6B and 6C).

The goal of using metabolic biomarkers to screen for crown rust resistance in a perennial ryegrass breeding program would be to eliminate the most crown rust susceptible genotypes from a population. It appears that the biomarkers detected in this study would likely be useful for accomplishing accurate classification of resistance level

based on the high correlation between actual and predicted values observed in this study and the ability to easily distinguish between R and S perennial ryegrass lines. Figure 6C indicates that reliable prediction of crown rust resistance levels can be achieved using only four features as biomarkers when negative ESI is used. The four highly predictive biomarkers had retention times ranging between 1.43 and 8.01 minutes (Table 7). In a breeding program, large numbers of plants must be phenotyped accurately for crown rust resistance in a short period of time. The biomarkers detected via negative ESI may be very useful for metabolomics-assisted selection because they are both accurate and are detectable using short instrument run times. The benefit of the biomarkers detected via positive ESI is that the group of 31 biomarkers together is slightly more accurate in predicting crown rust severity score (Figure 6B).

CONCLUSIONS

This research was successful in finding a set of secondary metabolite biomarkers in perennial ryegrass that are predictive of crown rust resistance levels. The biomarkers detected in this study should be tested for their utility in classifying other perennial ryegrass lines into R or S groups to determine which set of biomarkers accomplishes the objectives of the breeding program with the shortest and most straight forward analysis. Using the training data set developed here, and biomarkers determined in this study, other perennial ryegrass plants can be analyzed in a similar fashion and a prediction of their resistance levels can be made using GeneData Analyst. In addition, integrated intensity of specific features known to contribute to rust resistance could be compared among the perennial ryegrass genotypes of interest. Although correlations between actual and predicted crown rust severity scores were good in this study, there is room for improvement. An effort was made in this study to ensure that the perennial ryegrass lines screened had broad durable resistance to a wide range of crown rust isolates. Screening

perennial ryegrass lines against crown rust isolates collected from a broader geographical region may help improve the selection of robust metabolic biomarkers as there is evidence of varying levels of pathogenicity in crown rust isolates collected from different geographical regions (Aldaoud et al., 2004). The set of perennial ryegrass lines evaluated in this study were from a single breeding population. It is likely that a set of biomarkers for predicting rust severity could be made more robust if a larger number of genotypes are screened over a greater number of environments (Steinfath et al., 2010). However, the biomarkers found in our study are useful in that they can predict differences in rust resistance in otherwise similar genotypes.

Outcomes from this study

There were a number of short-term outcomes from this study. We performed thorough analysis and verification of rust resistance variability of important perennial ryegrass germplasm in our breeding program. Also we demonstrated the potential for using metabolomics-assisted selection for high-throughput, accurate, and dependable crown rust resistance screening in a perennial ryegrass breeding program. This research was critical in making steps toward understanding the biological basis for perennial ryegrass resistance to rust pathogens.

A number of more intermediate term outcomes were also realized. Crown rust resistant perennial ryegrass lines identified in this study were made available to be incorporated into our breeding program. This research resulted in our breeding program having a faster, more reliable method for rust resistance screening to supplement often unpredictable phenotypic screening methods leading to faster delivery of rust resistant cultivars to farmers. Finally, the crown rust resistance selection model developed in our study could be adapted for stem rust resistance selection.

This research will also have some more long-term impacts on perennial ryegrass seed producers, consumers, the environment, and the plant breeding community. New crown rust resistant cultivars will make perennial ryegrass seed crops a more profitable and environmentally sustainable crop rotation option for farmers in northern Minnesota. Ultimately, seed from rust resistant cultivars could be more marketable to end users and will result in more environmentally sustainable turfgrasses. Finally, the metabolomics-assisted selection methods developed in this study could be used as a model for metabolomics-assisted selection in other important agricultural crops or for other traits in perennial ryegrass.

Future directions for research

Identification of the predictive secondary metabolite biomarkers detected in this study would give a better biological understanding for the mechanisms of rust resistance in perennial ryegrass. Identification of the biomarkers could eventually lead to a better understanding of the metabolic pathways and genes which are involved in their production and in crown rust resistance (Schauer et al., 2006). Although UPLC-MS allows detection of more metabolites than other instrument platforms, identification of secondary metabolites detected via LC-MS can be a challenging and time consuming task (Patti et al., 2012). The first step in metabolite identification is typically to search the accurate mass of the metabolite of interest in metabolite databases. Typically database matches are only a putative identification and often, if there is even a match, often only metabolite class can be determined (Patti et al., 2012). If the metabolite returns matches in the database, the MS/MS spectrum of the biomarker of interest can be compared to that of available standards of that compound if they exist. Many of the metabolites detected in complex biological samples, such as the perennial ryegrass extracts in this study, do not return matches in data bases and/or do not have reference standards available making

identification more difficult (Patti et al., 2012). In this case, metabolites of interest could be purified, collected and subject to nuclear magnetic resonance spectroscopy for structure identification. In light of the challenges, it is recognized that positive identification of the biomarkers involved in predicting crown rust resistance could be a very time consuming and labor intensive process however it would be an area of research worth pursuing in the future.

Another direction that should be followed in regards to this research is to evaluate the effect of endophytes on metabolites that are involved in crown rust resistance. It is well known that perennial ryegrass is often infected with endophytes including *Neotyphodium* and *Epichloe* species. Metabolomics studies have indicated that some secondary metabolites including those often involved in pathogen interactions, are affected by endophytes (Rasmussen et al., 2012). It has been determined that the presence of endophytes can increase the quantity and variety of phenolic compounds in annual ryegrass (*L. multiflorum*) (Ponce et al., 2009). In our study, all plant lines were infected with endophytes and the goal was to develop a metabolomics-assisted selection strategy to select for crown rust resistance in endophyte-infected plants. The influence of endyphytes on the quantity and quality of biomarkers associated with rust resistance should be evaluated further as it is possible that there are perennial ryegrass genotypes that have different metabolic reactions to endophyte infection (Ponce et al., 2009).

Table 1. Initial field crown rust resistance screening at St. Paul, MN during the 2009 and 2010 growing season.

2009 Field				2010 Field			
Rank	Line Number	Rust Severity -AUDPC†-	Resistance Grouping	2009 Ranking	Line Number	Rust Severity -1 to 10‡-	Resistance Grouping
1	34	66	S	39	8	2.9	S
2	12	65	S*	7	73	2.7	S*
3	10	64	S*	3	10	2.6	S*
4	19	63	S	2	12	2.5	S*
5	61	63	S	6	14	2.5	S*
6	14	62	S*	41	42	2.4	S
7	73	62	S*	20	22	2.4	S
8	44	61	S*	8	44	2.4	S*
9	26	60	S	49	54	2.3	M
10	20	58	S	26	75	2.3	M*
11	4	56	S	14	67	2.2	M*
12	6	56	S	42	5	2.2	M
13	18	56		55	89	2.2	M
14	67	55		34	88	2.1	M*
15	92	54		11	4	2.0	M
16	36	54		32	9	2.0	M*
17	3	54		37	51	2.0	M*
18	32	53		50	56	2.0	R*
19	33	51		57	49	1.9	R*
20	22	50		54	38	1.9	R*
21	27	49		25	57	1.9	R
22	2	48		31	74	1.8	R
23	76	48		5	61	1.7	R
24	50	48	M	27	11	1.7	R
25	57	48	M	53	25	1.5	R*
26	75	48	M*				
27	11	48	M				
28	16	48	M				
29	37	48	M				
30	47	47	M				
31	74	47	M				
32	9	47	M*				
33	7	46	M				
34	88	46	M*				
35	87	46	M				
36	30	46					
37	51	46					
38	23	45					
39	8	44					
40	86	43					
41	42	43					
42	5	43					
43	13	43					
44	93	43					
45	91	42					
46	45	42					
47	58	41					
48	41	41	R				
49	54	41	R				
50	56	41	R*				
51	1	39	R				
52	39	38	R				
53	25	38	R*				
54	38	37	R*				
55	89	37	R				
56	52	37	R				
57	49	37	R*				
58	48	35	R				
59	68	33	R				

†AUDPC, area under the disease progress curve for crown rust severity data collected on 6 July, 13 July, and 4 August.
‡Crown rust severity was rated on a modified Horsefall-Barret rating scale in both years where 1= no rust, 2 = <10%, 3 = 11-25%, 4 = 26-40%, 5 = 41-60%, 6 = 61-70%, 7 = 71-80% 8 = 81-90% 9 = >90%, and 10 = 100% coverage of rust pustules (Helgeson et al., 1998).

Table 2. Years, locations, and germplasm from which crown rust urediniospores were collected for final growth chamber rust resistance verification using artificial inoculation. Isolates collected from each year and location were checked for germination and bulked.

Year	Location	Plant Material
2009	Becker, MN	NPGS (USDA National Plant Germplasm System) accessions†
2009	St. Paul, MN	NPGS accessions
2011	St. Paul, MN	NPGS accessions (in greenhouse flats placed near alternate host)
2011	St. Paul, MN	NPGS accessions
2012	St. Paul, MN	'Arctic Green' perennial ryegrass and parent material to our lines
2012	Greenhouse	'Ragnar II' perennial ryegrass

†NPGS accessions included 48 accessions from a total of 23 countries. The 48 accessions were planted at Becker and St. Paul, MN in a randomized complete block with a split-plot treatment arrangement where each accession received a mowed and un-mowed treatment to screen for crown rust resistance under variable conditions (Cisneros and Watkins, 2010).

Table 3. Rust severity data for each perennial ryegrass line selected for metabolomics analysis. Crown rust severity score is listed by replication and final resistance groupings for each line determined from final growth chamber crown rust resistance verification are given.

Line	Rust Severity by Replication†	Mean Rust Severity	Final Resistance Group‡
25	1.1, 1.0, 1.0, 1.1	1.1	Resistant
49	1.1, 1.1, 1.0, 1.0	1.1	Resistant
56	1.0, 1.0, 1.4, 1.1	1.1	Resistant
51	1.5, 1.9, 1.6, 1.9	1.7	Moderately Resistant
38	1.6, 1.9,1.4, 2.1	1.8	Moderately Resistant
67	1.6, 1.8, 2.3, 1.6	1.8	Moderately Resistant
44	2.0, 2.0, 2.0, 2.0	2.0	Moderately Resistant
88	1.6, 1.9, 2.4, 2.3	2.0	Moderately Resistant
9	2.8, 2.5, 2.0, 2.0	2.3	Moderately Susceptible
75	2.0, 2.0, 2.1, 3.5	2.4	Moderately Susceptible
10	2.4, 2.3, 2.5, 3.0	2.5	Moderately Susceptible
73	2.3, 2.6, 3.4, 2.3	2.6	Moderately Susceptible
12	3.3, 3.5, 3.4, 4.1	3.6	Susceptible
14	3.1, 4.3, 3.8, 4.5	3.9	Susceptible
	Mean	2.1	
	Standard Dev.	0.82	

†Crown rust severity values for replications 1, 2, 3, and 4 respectively from the growth chamber rust resistance verification experiment. Crown rust severity was rated on modified Horsefall-Barret rating scale in both years where 1= no rust, 2 = <10%, 3 = 11-25%, 4 = 26-40%, 5 = 41-60%, 6 = 61-70%, 7 = 71-80% 8 = 81-90% 9 = >90%, and 10 = 100% coverage of rust pustules (Helgeson et al., 1998). Each value within a replication and line is the mean value for crown rust severity ratings from eight genetically distinct clones within a line that were collected from the 2009 and 2010 field screening experiments. The set of genetically distinct clones within a line was clonally propagated to get an identical set to use for each of the 4 replications.

‡Lines were given their final resistance group designation based on standard deviation (S.D.) from the mean of mean growth chamber rust severity data and stability in resistance ranking between the final field and growth chamber resistance verification experiments. Lines with severity more than one S.D. below the mean were designated resistant, those within one S.D. below the mean designated moderately resistant, those within one S.D. above the mean designated moderately susceptible, and those with mean severity greater than one S.D. above the mean were designated susceptible.

Table 4. Ninety-one unique features detected from UPLC-MS positive ionization analysis, along with their retention time and mass-to-charge ratio (m/z), that showed variable importance in projection scores (VIP) > 0.05 in partial least squares discriminant analysis (PLS-DA).

Feature ID	Retention Time	m/z	VIP Scores†	Feature ID	Retention Time	m/z	VIP Scores
930	3.97	727.1712	8.18	42	4.90	245.0808	0.91
511	2.95	406.1642	8.07	997	8.11	795.3454	0.87
734	6.36	535.1081	5.22	884	2.85	642.1733	0.85
767	6.56	565.1190	4.90	62	4.90	263.0904	0.85
46	1.72	248.1504	4.88	854	9.39	615.2458	0.85
862	8.01	619.2767	4.67	693	9.62	509.2364	0.82
561	5.06	435.2214	4.63	1029	4.17	434.7205	0.80
623	5.35	457.1159	4.15	940	7.91	737.3391	0.78
448	4.56	360.2536	3.77	905	5.76	681.1669	0.77
948	9.06	749.3027	3.64	616	1.34	452.2283	0.76
649	1.06	474.1732	3.13	529	2.45	419.1524	0.76
545	12.51	431.1202	3.08	371	11.85	334.2353	0.74
770	25.82	568.4280	2.99	575	3.85	441.1196	0.72
727	5.62	531.2437	2.97	25	1.72	231.1242	0.69
167	5.98	287.0546	2.95	1035	11.42	885.2421	0.68
1023	6.29	859.1923	2.89	798	8.96	593.3168	0.67
841	2.15	611.1610	2.76	445	1.88	356.1057	0.65
985	3.39	787.2288	2.63	507	4.03	401.1597	0.65
989	3.60	788.3128	2.46	657	6.23	479.1176	0.64
942	4.67	741.2239	2.10	826	25.48	600.4174	0.64
1006	5.06	813.1718	2.10	633	5.07	465.1030	0.63
1025	5.35	859.3027	1.88	106	12.51	275.0664	0.63
360	9.39	333.1227	1.82	723	4.18	527.1726	0.62
422	1.14	345.1309	1.73	346	6.03	327.2275	0.61
913	8.79	691.2985	1.64	1010	1.00	816.4094	0.61
539	5.35	425.1787	1.64	936	7.59	735.2864	0.60
715	1.96	522.3065	1.58	780	6.39	579.1342	0.59
961	4.72	758.3236	1.46	793	8.99	591.2054	0.58
762	11.42	557.1656	1.45	825	25.32	600.4177	0.58
482	13.30	376.2601	1.43	99	4.34	272.0580	0.57
919	3.73	698.1644	1.38	527	1.06	419.0954	0.57
1063	5.30	561.2929	1.35	348	6.85	328.1468	0.55
1067	4.14	667.8528	1.35	859	10.52	617.3215	0.55
210	12.79	294.2429	1.25	714	6.86	520.2321	0.55
926	2.57	713.1564	1.21	921	12.37	699.2947	0.54
497	3.85	387.2013	1.20	300	6.45	311.2329	0.54
887	4.49	642.2755	1.19	720	7.05	523.2163	0.53
628	7.30	459.2228	1.19	95	7.89	271.1906	0.53
606	5.95	449.1078	1.18	551	7.89	433.2441	0.53
86	6.08	271.1324	1.16	753	5.53	551.1032	0.53
1065	3.43	562.2828	1.07	654	2.83	479.1190	0.52
689	4.17	505.2325	1.03	1037	6.38	889.2011	0.52
322	6.52	317.0652	1.02	502	2.94	396.2017	0.52
876	8.12	633.2913	0.96	726	4.05	531.2053	0.51
880	6.70	639.1559	0.96	1055	27.39	497.2565	0.51
408	3.41	369.1170	0.95				

†PLS-DA analysis was conducted using crown rust severity score by replication as the supervised grouping data. Only features with VIP scores greater than 0.5 were considered as potential biomarkers and used for further analysis.

Table 5. Ninety-three unique features detected from UPLC-MS negative electrospray ionization analysis, along with their retention time and mass-to-charge ratio (m/z), that showed variable importance in projection scores (VIP) > 0.05 in partial least squares discriminant analysis (PLS-DA).

Feature ID	Retention Time	m/z	VIP Scores†	Feature ID	Retention Time	m/z	VIP Scores
980	1.80	707.1827	15.26	644	8.99	523.2189	1.09
355	5.35	401.1823	7.48	894	5.44	651.2537	1.05
1021	3.01	755.2044	5.51	663	5.60	529.2322	1.05
665	9.18	529.2302	3.99	417	4.49	431.1924	1.04
453	6.24	443.1562	3.98	165	0.97	315.0726	1.03
1000	3.98	725.1584	3.80	418	4.65	431.1930	1.03
953	3.95	695.1500	3.80	1034	4.98	769.2211	1.02
950	3.71	695.1469	3.71	739	8.86	565.3236	1.01
168	4.99	319.0856	3.70	128	4.53	283.0860	0.93
275	4.59	385.1141	3.48	1089	6.29	858.1841	0.87
816	2.87	609.1465	3.09	1117	3.00	1109.2997	0.86
471	1.68	449.2037	3.05	583	7.49	497.1350	0.84
966	1.83	705.1714	2.87	337	1.82	395.1571	0.84
452	5.98	443.1587	2.63	228	2.93	349.0602	0.83
562	6.36	489.1044	2.52	542	9.73	481.1386	0.81
427	3.49	433.2080	2.51	216	3.76	341.0881	0.81
539	4.50	478.2085	2.50	711	3.58	547.1682	0.81
31	0.70	191.0187	2.46	924	5.73	679.1530	0.76
949	3.27	695.1466	2.39	600	5.67	501.1622	0.74
791	3.37	639.1569	2.28	667	6.36	533.0938	0.73
832	8.01	617.2637	2.27	41	5.13	193.0498	0.73
540	5.00	478.2086	2.20	760	5.23	575.1997	0.73
312	2.86	385.1142	2.20	869	2.83	639.1575	0.71
271	2.30	367.1039	2.20	166	1.43	315.0729	0.71
375	5.51	413.1462	2.03	813	5.00	607.1315	0.70
124	0.69	275.0230	2.03	602	4.18	503.1785	0.70
1025	3.02	756.2083	2.02	861	5.72	635.1640	0.70
1013	4.63	739.2119	2.02	279	7.82	367.1401	0.69
274	3.80	367.1036	2.01	820	8.98	609.3130	0.69
1085	6.28	857.1783	2.00	198	0.66	337.0778	0.68
1020	2.29	755.2061	1.80	1043	3.36	785.2177	0.66
174	9.18	321.1707	1.76	541	5.00	479.2119	0.64
888	3.71	651.1575	1.75	1046	8.91	787.3062	0.64
420	3.86	432.1965	1.69	157	5.13	309.0617	0.64
636	3.37	519.1722	1.61	112	1.79	258.0980	0.62
634	6.61	519.1159	1.51	1092	11.42	861.2462	0.60
798	1.90	595.2607	1.47	313	3.39	385.1146	0.58
290	4.20	371.0995	1.47	716	3.19	549.1838	0.58
886	3.27	651.1576	1.43	531	5.21	473.2033	0.55
794	5.62	593.1533	1.34	553	7.73	485.1675	0.54
414	3.45	431.1928	1.32	373	6.89	411.1349	0.54
234	3.55	351.1298	1.21	335	9.25	395.1383	0.53
1017	9.05	747.2908	1.16	707	4.45	547.1460	0.51
700	6.06	545.2247	1.13	359	2.91	404.1500	0.51
589	6.07	499.2191	1.13	621	1.18	515.1413	0.51
493	3.35	459.1514	1.12	317	5.70	385.1157	0.51
645	9.12	523.2191	1.09				

†PLS-DA analysis was conducted using crown rust severity score by replication as the supervised grouping data. Only features with VIP scores greater than 0.5 were considered as potential biomarkers and used for further analysis.

Table 6. Correlation of integrated intensity of detected features from positive ESI with crown rust severity score along with the corresponding P-Value, overall rank for partial least squares regression analysis, and variable importance in projection (VIP) value from initial partial least squares discriminant analysis (PLS-DA). Only features with significant correlation (P≤0.05) and VIP > 0.5 are shown.

Feature ID	RT	m/z	Correlation with Rust Severity†	P-Value‡	Overall Rank§	VIP Score
921	12.37	699.2947	-0.33	1.39E-02	22	0.54
539	5.35	425.1787	-0.31	1.81E-02	23	1.64
1025	5.35	859.3027	-0.31	1.98E-02	24	1.88
348	6.85	328.1468	-0.27	4.22E-02	28	0.55
623	5.35	457.1159	-0.27	4.84E-02	30	4.15
948	9.06	749.3027	0.26	4.90E-02	31	3.64
616	1.34	452.2283	0.27	4.43E-02	29	0.76
753	5.53	551.1032	0.27	4.11E-02	27	0.53
997	8.11	795.3454	0.27	4.09E-02	26	0.87
507	4.03	401.1597	0.28	3.73E-02	25	0.65
989	3.60	788.3128	0.33	1.39E-02	21	2.46
657	6.23	479.1176	0.33	1.39E-02	20	0.64
793	8.99	591.2054	0.33	1.39E-02	19	0.58
913	8.79	691.2985	0.33	1.23E-02	18	1.64
723	4.18	527.1726	0.34	1.07E-02	17	0.62
1006	5.06	813.1718	0.36	7.11E-03	16	2.10
654	2.83	479.1190	0.36	6.68E-03	15	0.52
1010	1.00	816.4094	0.37	5.52E-03	14	0.61
408	3.41	369.1170	0.37	4.61E-03	13	0.95
884	2.85	642.1733	0.37	4.46E-03	12	0.85
767	6.56	565.1190	0.40	2.55E-03	11	4.90
930	3.97	727.1712	0.41	1.75E-03	10	8.18
649	1.06	474.1732	0.46	3.58E-04	9	3.13
322	6.52	317.0652	0.48	1.86E-04	8	1.02
1067	4.14	667.8528	0.50	9.11E-05	7	1.35
693	9.62	509.2364	0.54	1.90E-05	6	0.82
862	8.01	619.2767	0.57	3.64E-06	5	4.67
854	9.39	615.2458	0.58	2.67E-06	4	0.85
727	5.62	531.2437	0.61	7.40E-07	3	2.97
497	3.85	387.2013	0.64	8.30E-08	2	1.20
575	3.85	441.1196	0.65	5.72E-08	1	0.72

†Average Pearson correlation of the integrated intensity for each feature detected with crown rust severity data from the final growth chamber crown rust resistance verification experiment. Integrated intensity for each feature detected from a biological replicate was correlated with the crown rust score from that particular biological replicate for each line.

‡Correlation coefficients for all 91 unique features having a VIP of 0.5 or greater were calculated using GeneData Analyst. The components listed were significantly correlated (P≤0.05) with rust severity data.

§Ranking for partial least squares regression analysis using correlation as a ranking method. Partial least squares regression was conducted on all 91 features and the ranking for the features most significantly correlated with rust score is shown.

Table 7. Correlation of integrated intensity of detected features from negative ESI with crown rust severity score along with the corresponding P-Value, overall rank for partial least squares regression analysis, and variable importance in projection (VIP) value from initial partial least squares discriminant analysis (PLS-DA). Only features with significant correlation (P≤0.05) and VIP > 0.5 are shown.

Feature ID	RT	m/z	Correlation with Rust Severity†	P-Value‡	Overall Rank§	VIP Score
166	1.43	315.0729	-0.61	7.12E-07	2	0.71
583	7.49	497.1350	-0.40	2.19E-03	12	0.84
760	5.23	575.1997	-0.39	2.69E-03	13	0.73
553	7.73	485.1675	-0.35	8.14E-03	17	0.54
373	6.89	411.1349	-0.33	1.38E-02	21	0.54
1013	4.63	739.2119	-0.29	3.09E-02	30	2.02
794	5.62	593.1533	-0.29	3.11E-02	31	1.34
1034	4.98	769.2211	-0.28	3.49E-02	33	1.02
375	5.51	413.1462	-0.28	3.58E-02	34	2.03
739	8.86	565.3236	-0.27	4.30E-02	36	1.01
820	8.98	609.3130	-0.27	4.67E-02	37	0.69
335	9.25	395.1383	0.26	4.97E-02	38	0.53
791	3.37	639.1569	0.28	3.81E-02	35	2.28
636	3.37	519.1722	0.29	3.23E-02	32	1.61
602	4.18	503.1785	0.29	3.01E-02	29	0.70
869	2.83	639.1575	0.29	2.77E-02	28	0.71
417	4.49	431.1924	0.30	2.31E-02	27	1.04
1000	3.98	725.1584	0.31	2.19E-02	26	3.80
711	3.58	547.1682	0.31	2.13E-02	25	0.81
644	8.99	523.2189	0.32	1.65E-02	24	1.09
645	9.12	523.2191	0.32	1.54E-02	23	1.09
531	5.21	473.2033	0.33	1.42E-02	22	0.55
716	3.19	549.1838	0.33	1.33E-02	20	0.58
667	6.36	533.0938	0.34	1.02E-02	19	0.73
493	3.35	459.1514	0.34	9.38E-03	18	1.12
562	6.36	489.1044	0.36	6.21E-03	16	2.52
275	4.59	385.1141	0.37	4.72E-03	15	3.48
312	2.86	385.1142	0.39	2.83E-03	14	2.20
665	9.18	529.2302	0.40	2.18E-03	11	3.99
634	6.61	519.1159	0.40	2.15E-03	10	1.51
313	3.39	385.1146	0.40	2.09E-03	9	0.58
707	4.45	547.1460	0.42	1.40E-03	8	0.51
174	9.18	321.1707	0.42	1.34E-03	7	1.76
234	3.55	351.1298	0.43	8.70E-04	6	1.21
894	5.44	651.2537	0.44	7.45E-04	5	1.05
663	5.60	529.2322	0.53	2.99E-05	4	1.05
832	8.01	617.2637	0.57	5.63E-06	3	2.27
420	3.86	432.1965	0.69	4.77E-09	1	1.69

†Average Pearson correlation of the integrated intensity for each feature detected with crown rust severity data from the final growth chamber crown rust resistance verification experiment. Integrated intensity for each feature detected from a biological replicate was correlated with the crown rust score from that particular biological replicate for each line.

‡Correlation coefficients for all 93 unique features having a VIP of 0.5 or greater were calculated using GeneData Analyst. The features listed were significantly correlated (P≤0.05) with rust severity data.

§Ranking for partial least squares regression analysis using correlation as a ranking method. Partial least squares regression was conducted on all 93 features and only the ranking for the features most significantly correlated with rust score is shown.

Table 8. The root mean squared error (RMSE) from the regression between actual and predicted crown rust severity values calculated using between 2 and 95 features (biomarkers) identified in UPLC-MS analysis that had variable importance in projection scores (VIP) > 0.5. Analysis from both positive and negative electrospray ionization (ESI) is shown.

Positive Ionization		Negative Ionization	
Number of features	RMSE†	Number of features	RMSE
2	0.708	2	0.585
3	0.726	3	0.584
4	0.746	4‡	0.576
6	0.733	6	0.635
8	0.763	8	0.709
11	0.715	11	0.761
16	0.700	16	0.724
23	0.635	23	0.702
31‡	0.547	32	0.675
45	0.710	45	0.646
64	0.734	64	0.760
91	0.749	95	0.766

†Low RMSE values indicate a better set of biomarkers and a more accurate prediction of crown rust severity.

‡Root mean squared error was minimized by using the 31 features having the greatest and most significant correlation of integrated intensity with crown rust severity score for features detected using positive ESI. For negative ESI, the top 4 features that had integrated intensity most highly correlated with rust severity score provided the best fitting predictive model.

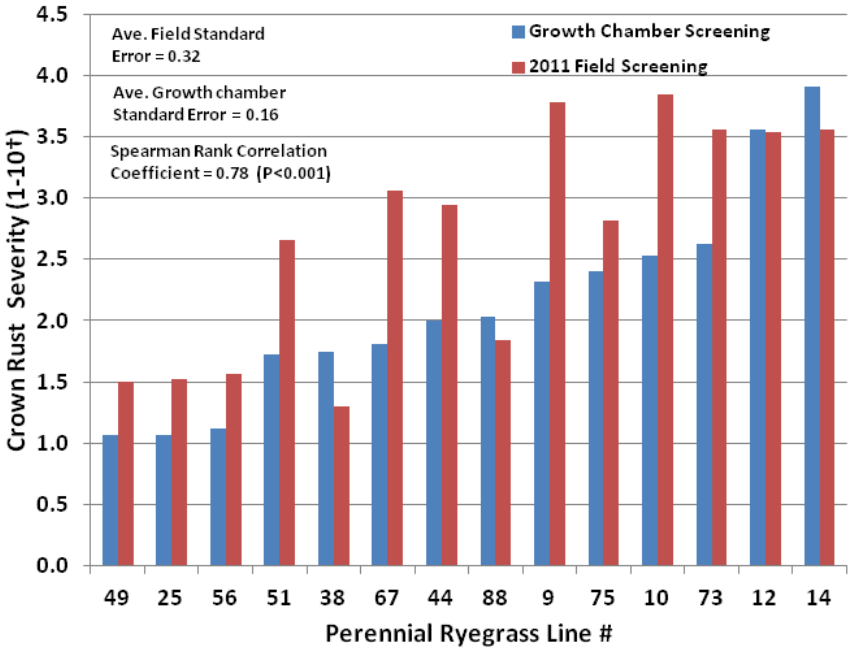

Figure 1. Crown rust severity of 14 perennial ryegrass lines (represented by eight previously selected distinct plants from each line) in growth chamber screening via artificial inoculation (blue bars) and field screening via natural inoculation during the 2011 growing season (red bars). Lines are ranked in order of growth chamber rust severity. †Crown rust severity was rated on a modified Horsefall-Barret rating scale where 1= no rust, 2 = <10%, 3 = 11-25%, 4 = 26-40%, 5 = 41-60%, 6 = 61-70%, 7 = 71-80% 8 = 81-90% 9 = >90%, and 10 = 100% coverage of rust pustules (Helgeson et al., 1998).

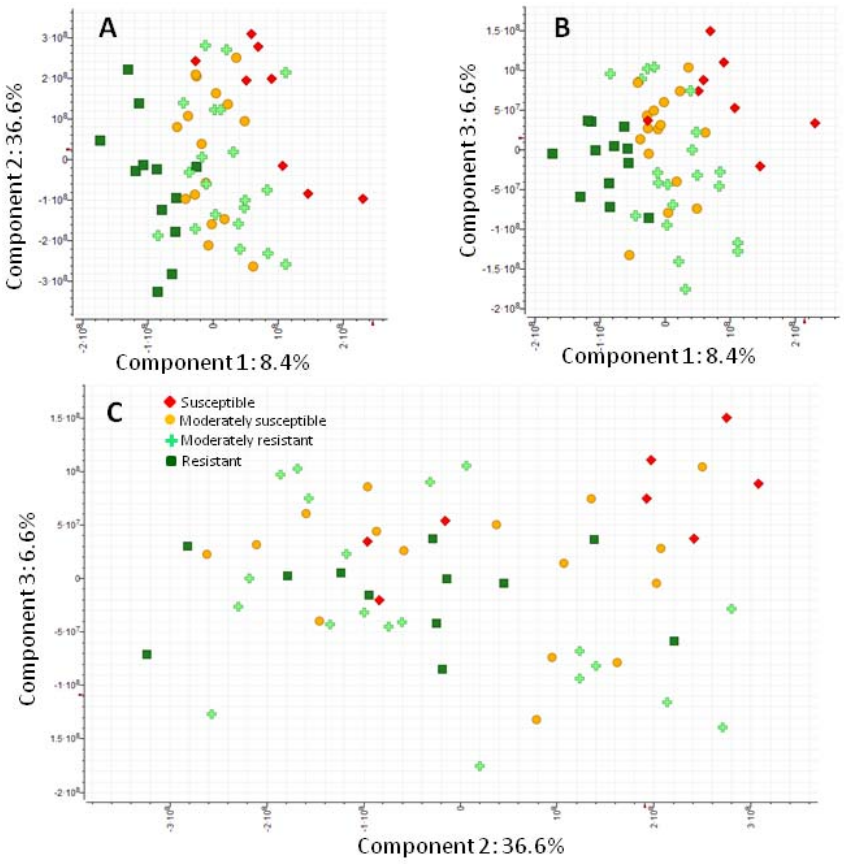

Figure 2. Scores plots from three principle component partial least squares discriminant analysis (PLSDA) of positive ESI data. Analysis used all features detected from 56 samples previously analyzed via UPLC-MS (positive electrospray ionization) analysis and rust severity score from the growth chamber screening experiment as the supervisory data. A) Component 1 and 2 accounted for 8.4 and 36.6 % of the variation, B) components 1 and 3 together explained 8.4 and 6.6% of the overall variation, and C) components 2 and 3 explained 36.6 and 6.6% of the overall variation. Samples are grouped according to resistance grouping, according to Table 3, where dark green squares = resistant, light green crosses = moderately resistant, orange circles = moderately susceptible, and red diamonds = susceptible.

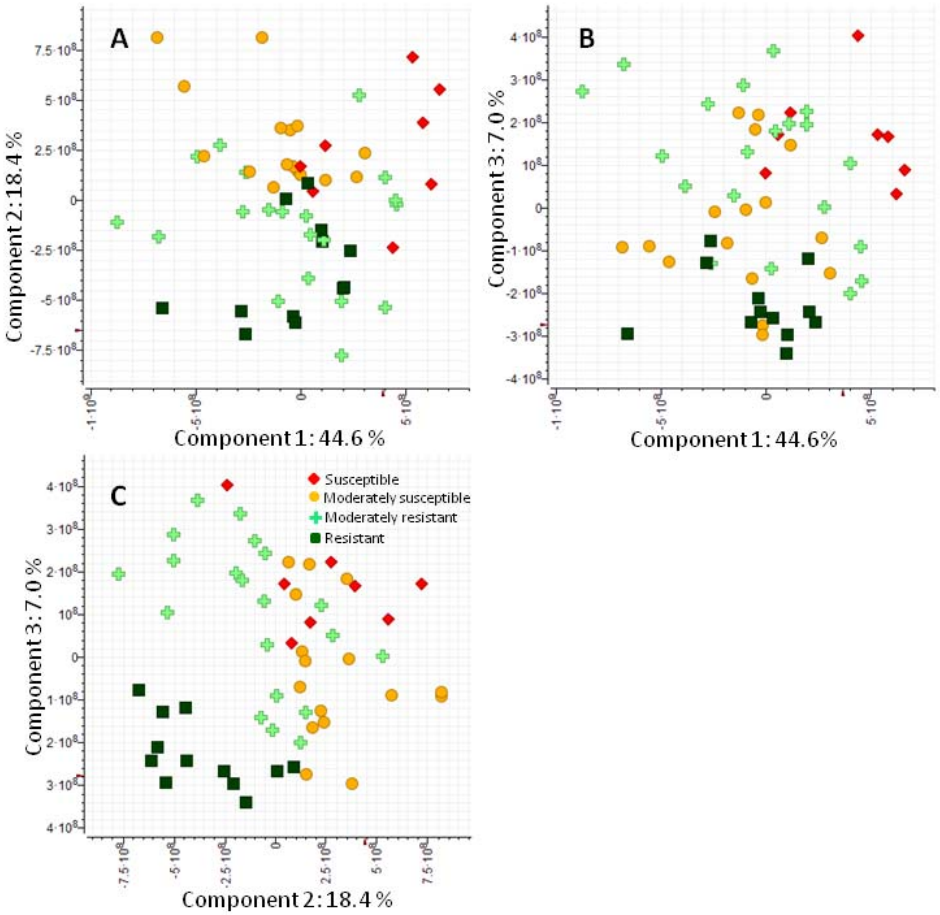

Figure 3. Scores plots from three principle component partial least squares discriminant analysis (PLSDA) for the negative ESI data. Analysis used all features detected from 56 samples previously analyzed via UPLC-MS (positive electrospray ionization) analysis and rust severity score from the growth chamber screening experiment as the supervisory data. A) Component 1 and 2 accounted for 8.4 and 36.6 % of the variation, B) components 1 and 3 together explained 8.4 and 6.6% of the overall variation, and C) components 2 and 3 explained 36.6 and 6.6% of the overall variation. Samples are grouped according to resistance grouping, according to Table 3, where dark green squares = resistant, light green crosses = moderately resistant, orange circles = moderately susceptible, and red diamonds = susceptible.

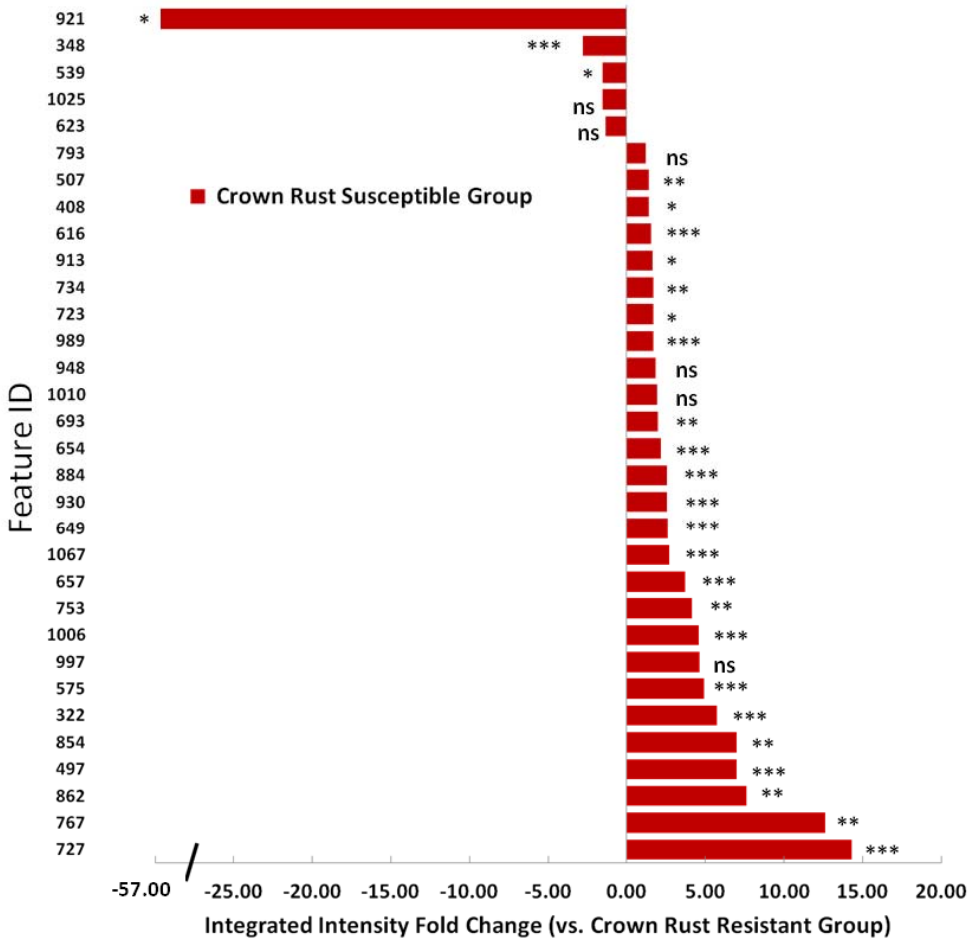

Figure 4. Fold change of metabolic features that were detected in positive ESI analysis and were significantly correlated with rust severity score. Quantitative fold change was calculated for the susceptible group against the resistant group. * Indicates that fold change that was significant at P<0.05, ** indicates fold change that was significant at P<0.01, and *** indicates fold change that was significant at P<0.001.

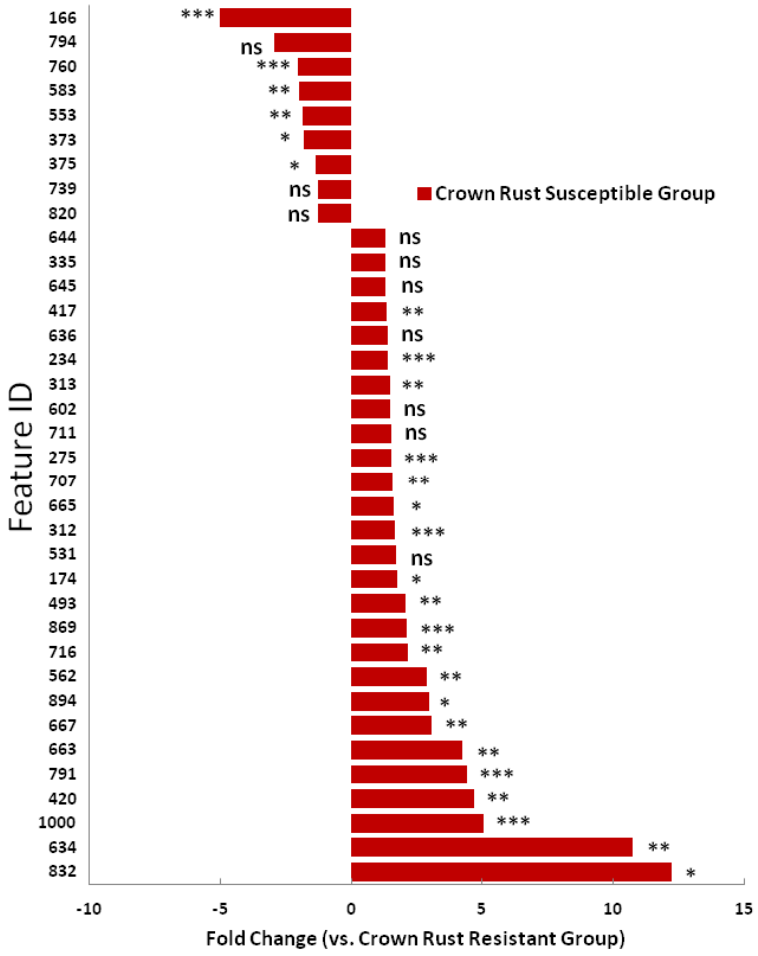

Figure 5. Fold change of metabolic features that were detected in negative ESI analysis and were significantly correlated with rust severity score. Quantitative fold change was calculated for the susceptible group against the resistant group. * Indicates that fold change that was significant at P<0.05, ** indicates fold change that was significant at P<0.01, and *** indicates fold change that was significant at P<0.001.

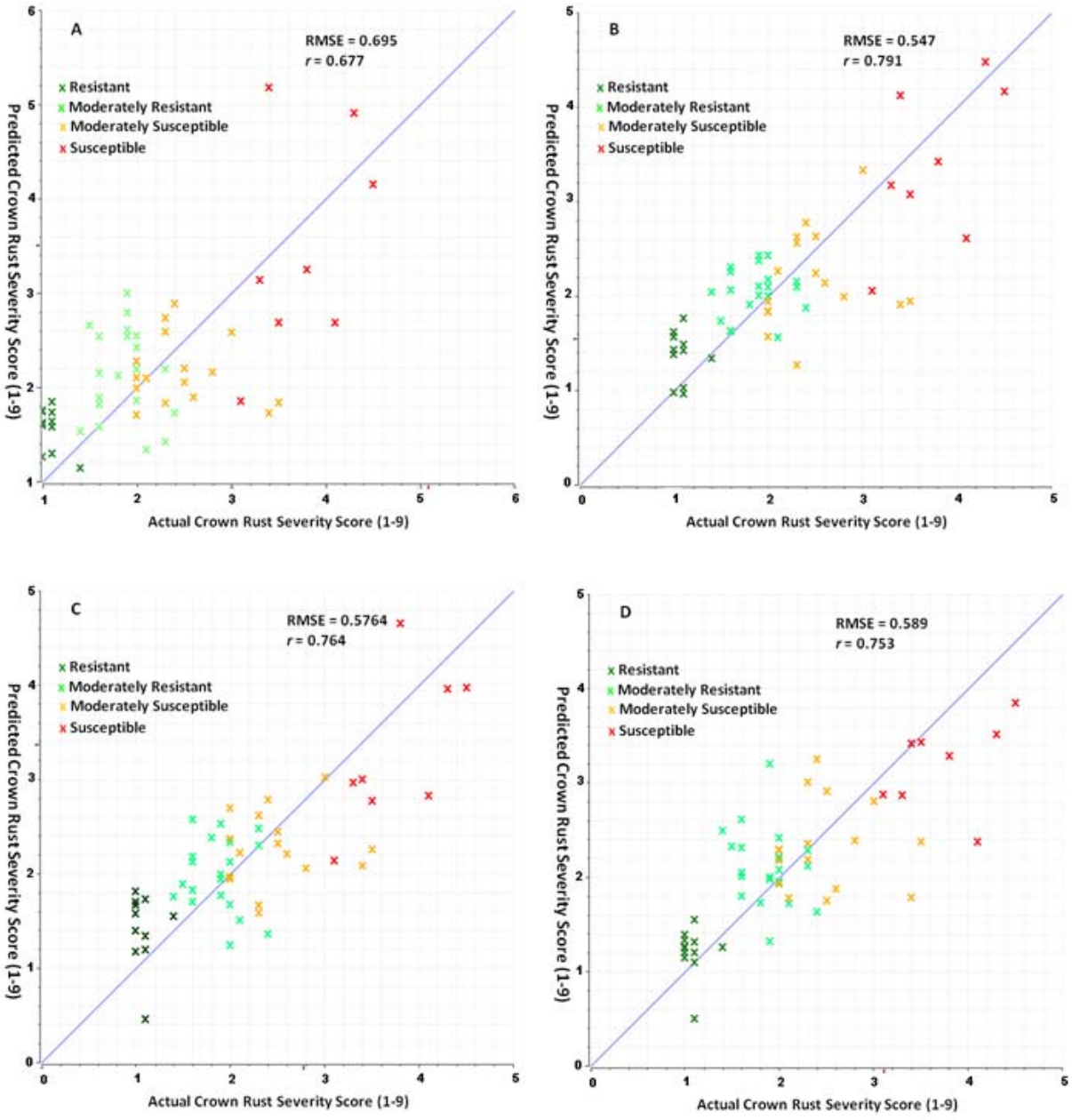

Figure 6. Partial least squares regression of actual versus predicted crown rust severity scores determined from leave one out cross validation of metabolic features which were correlated with crown rust severity. A) Cross validation of positive ESI data using the top 20 most correlated features. B) Cross validation of positive ESI data using the top 31 most correlated features. C) Cross validation of negative ESI data using the top 4 most correlated features. D) Cross validation of positive ESI data using the top 38 most correlated features. Root mean squared error (RMSE) and correlation (*r*) between actual and predicted crown rust severity scores for each sample are shown.

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