Effects of cold temperatures and high tunnel environments on symbiotic nitrogen fixation and growth of winter annual legume cover crops

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Abstract

We investigated the effect of Upper Midwest winter temperatures on nitrogen contributions of winter annual legume crops via productivity and symbiotic nitrogen fixation (SNF). Projects included: 1) a controlled environment study of growth, nodulation, and SNF in crimson clover (*Trifolium incarnatum*), Austrian winter pea (*Pisum sativum*), and hairy vetch (*Vicia villosa*), and 2) a high tunnel evaluation of four cover crop mixes for productivity, nodulation, and SNF. High tunnel treatments included: a) crimson clover monoculture (*T. incarnatum*), b) red clover monoculture (*Trifolium pratense*), c) Austrian winter pea/winter rye biculture (*Pisum sativum* and *Secale cereale*), and c) hairy vetch/tillage radish/winter rye mix (*V. villosa*, *Raphanus sativus*, and *S. cereale*). Legume biomass, nodulation, and SNF were limited below 10°C. In high tunnels, lowest biomass and nodulation occurred in mid-winter and lowest SNF in early spring. Delayed spring recovery of SNF suggested that later winter annual cover crop termination could increase nitrogen contributions.

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Chapter 1: Literature Review

Introduction

Winter-annual cover crops are an emerging set of tools to address complex problems in cash-crop cultivation, especially in organic systems. These cover crops are planted after harvest of the summer annual crop, establish in the fall, and protect the soil throughout the winter until the next growing season. Unlike a bare-fallow system, cover crops can reduce erosion, improve soil structure, prevent nutrient leaching and runoff, and add organic matter and nutrients to the soil (Dabney et al. 2001; Brandsaeter & Netland 1999; Blanco-Canqui et al. 2015; Barber & Navarro 1994; Wiesmeier et al. 2015). Developing winter-annual cover crops is especially pertinent in the cold zones of the upper Midwest, where the harsh winters are becoming more unpredictable with climate change and most farmers with annual cropping systems leave soils bare over the winter (Roesch-McNally et al. 2017). The same is true in increasing popular high tunnel growing environments, where season extension leaves an even shorter window for establishing a winter crop. In high tunnels, the added stress of temperature fluctuations, dry conditions, and no insulating snow cover makes winter growth especially challenging, but all the more important for safeguarding soil health (Pagliari et al. 2016; Carey et al. 2009; Drost et al. 2017; Rudisill et al. 2015). For growers in these regions, it is important to plant very hardy species of cover crops to ensure survival during harsh winter conditions and continue to offer the important services of a living crop on the soil (Rapacz et al. 2014; Wilke & Snapp 2008).

Lack of living winter cover on thousands of acres of Upper Midwest cropland has led to high levels of nutrient leaching and runoff (Drinkwater & Snapp 2007). These nutrients enter waterways and contribute substantially to far-reaching environmental issues like the hypoxic “dead zone” in the Gulf of Mexico at the delta of the Mississippi River, and both algal blooms and hypoxic conditions in local streams, rivers, and lakes (Syswerda et al. 2012; Vitousek et al. 1997). In covered high tunnel environments, intensive cultivation and fertility inputs, coupled with exposed soils and lack of adequate water movement lead to loss of soil structure and organic matter, as well as increasing salinity and nutrient imbalances that can reduce yields (Rudisill et al. 2015; Gluck & Hanson 2013; Morra et al. 2002; Knewtson et al. 2010).

Winter-annual cover crops are important to mitigating soil health issues in the field and in high tunnels because they can absorb excess nutrients and stabilize soil structure. In organic systems, winter annual legume cover crops can also become a critical source of fertility. Legumes fix nitrogen from the air in a symbiotic relationship with soil bacteria, thereby reducing the need for heavy fertilizer applications (Drury et al. 2014; Hubbard et al. 2013; Muchanga et al. 2017). Reducing fertilizer input helps to lower nitrate and phosphorus run-off and leaching, processes that lead to high levels of nutrients in waterways and the subsequent damaging downstream effects. In high tunnels, legumes can both reduce input costs for growers and provide fertility without adding excess salts to the soil (Sugihara et al. 2017).

Nitrogen fixation and soil improvements provided by winter annual legume cover crops in the cold but heavily agricultural regions of the Upper Midwest have led to research that explores legume cold-tolerance, their performance in protected cultivation systems like high tunnels, and their symbiotic nitrogen-fixation under cold and high tunnel conditions in order to maximize these potential benefits. Previous research on symbiotic nitrogen fixation (abbreviated SNF) at low temperatures has most often focused on cash or forage crop plants and little is known about the effect of cold temperatures on SNF in winter annual legume cover crops or their performance in high tunnels during the winter.

This literature review will assess published knowledge about the effects of cold temperatures and high tunnel conditions on productivity and nitrogen fixation in legumes, focusing on and relating findings to winter annual legume cover crops. By synthesizing published results on these interactions of temperature, growth, and nitrogen fixation, and how these results relate to farmer–identified goals for legume cover cropping in general, this review will inform this thesis research and future work on winter annual legume cover crops and their versatility in difficult conditions.

Winter annual legume cover crops

General practices and commonly used species

Winter-annual legume cover crops are winter hardy stands seeded in the late summer or early fall after the harvest of the summer annual crop. The crops establish in the fall as temperatures cool and light levels fall, grow dormant during the winter months, and begin to grow again in the spring with warmer temperatures and longer day lengths. During the “winter” season (which includes late fall and early spring) the cover crop provides a living root system and above-ground biomass to bind together and protect soil that would otherwise be bare or only covered by residue of harvested cash crops (Brandsaeter & Netland 1999).

In the Upper Midwest and other cold climates, commonly grown winter annual legumes include hairy vetch (*Vicia villosa*), red clover (*Trifolium pratense*), and field peas (*Pisum sativum*), with other species of clover and medics used less frequently (Brandsæter et al. 2008; Holderbaum et al. 1990; Silva & Delate 2017). Additionally, winter-annual legumes are often seeded in a mixture with winter hardy grasses and/or other cold-tolerant species. Winter rye (*Secale cereale)* is a common companion to vetch and peas in winter mixtures for several reasons. Rye, which grows more quickly and densely than these legumes and is very cold tolerant, can shelter the legume during the coldest parts of the winter and can also provide support for the vining legumes to climb in the spring as they begin to grow quickly again (Creamer et al. 1997; Ranells & Wagger 1997). In addition, research had shown that competition for soil mineral nitrogen from non-legume species like grasses can promote higher levels of nitrogen fixation in legume cover crops, resulting in greater potential nitrogen credits to a farmer's field when the legume is incorporated in the spring (Jensen 1996; Bedoussac et al. 2015). Growing cover crop mixtures can also help to achieve more than one desired ecosystem function at a time, depending on a grower's goals, planting time, and soil conditions (Blesh 2018). For example, a fall-planted mix of winter rye, hairy vetch and tillage radish (*Raphanus sativa)* can 1) scavenge excess nitrogen not used by the previous cash crop (rye) (Ranells & Wagger 1997), 2) fix more nitrogen to be released in the spring and fertilize the next cash crop (vetch) (Couëdel et al. 2018), and 3) break up soil compaction (radish) (Abdollahi & Munkholm 2014).

Symbiotic nitrogen fixation

Winter-annual legume cover crops obtain supplemental nitrogen, in addition to the mineral forms of N their roots take up from the soil, though symbiosis with nitrogen-fixing rhizobia bacteria. Rhizobia are free-living soil bacteria, but they also thrive as an endosymbiont of leguminous plants. The symbiosis is initiated when rhizobia infect root hairs of legumes and trigger the root cortical cells to form cyst-like structures called nodules (Somasegaran & Hoben 1994). Nodules are the site of nitrogen fixation in the plant, providing the space for rhizobia to differentiate from their free-living form to a host-dependent bacteriod. In the nodule, they are directly connected to the legume's vascular system, and exchange plant-available nitrogen in the form of ammonia they fix from soil air for carbohydrate compounds (carboxylic acids) metabolized by the plant (Arora et al. 2012). Atmospheric nitrogen (N_2) is converted to ammonia by the rhizobium nitrogenase enzyme. Nitrogenase is affected by many environmental factors and requires a substantial amount of energy in the form of ATP in order to perform nitrogen fixation, meaning that this is a costly process for both the plant, which is providing carbohydrates to the nodules, and for the rhizobia bacteria (Silsbury 1977). When soil inorganic (plant available) nitrogen is high or the legume is under environmental stress, SNF is often decreased due to this high energy requirement (Levai & Veres 2013; Arora et al. 2012; Voisin et al. 2002).

Through SNF, legume cover crops can potentially contribute enough nitrogen to reduce or eliminate the need for fertilization the next growing season (Parr et al. 2011; Boquet & Dabney 1991; Martin & Touchton 1983). This stored nitrogen is released into the soil when legume cover crops are terminated and incorporated at the end of their growing season, before the cash crop is planted. In this manner, they provide a slow-release, non-synthetic form of nitrogen that reduces the need for damaging levels of nutrient inputs into cropping systems (Malone et al. 2014; Dabney et al. 2001). Though there are many benefits of this cover cropping system, the challenges of very cold Upper Midwest winters remain a barrier to widespread adoption (Wayman et al. 2017; Snapp et al. 2005). Growers need further insight into managing winter-annual legumes in order to gain nitrogen credits, including a more detailed understanding of when nitrogen is fixed in plant tissues, at what temperatures legumes and rhizobia are active and able to fix this nitrogen, and what species of legume and their associated rhizobia will perform best in their particular climate.

Effects of low temperatures on legume productivity

Legume growth and productivity at low temperatures were assessed in numerous species of both horticultural and agronomic crops. Low temperatures ranged from 5°C to 15°C depending on the legume tested, and 20-25°C represented control or ideal conditions (Mahdavi et al. 2010; Dehaghi & Sanavy 2003; Robin et al. 2005). Results indicate that unless adapted to cold climates, most legumes have reduced growth at prolonged low temperatures. In controlled environment studies, researchers have found root zone temperatures of 5ºC reduce growth rate and metabolic processes in annual medic (a legume closely related to alfalfa), as well as dry matter content, stem and leaf biomass, and root length and biomass (Dehaghi & Sanavy 2003).

Exposing the whole plant to cold temperatures, defined as “chilling”, can negatively affect legume productivity. Chilling of legume food crops at temperatures between 5°C and 17°C at early stages of growth can reduce germination and lead to higher levels of fungal infection (Wery et al. 1994). Photosynthesis and CO^2 assimilation were also curtailed during chilling at 8ºC in soybean, at least partially due to stomatal closure (Van Heerden et al. 2003). Photoinhibition, or reduced capacity for photosynthesis, is also observed at chilling temperatures in legumes due to damage of photosystem II by excess light energy and subsequent slow rates of repair due to reduced enzymatic activity and the production of reactive oxygen species in response to cold stress (Takahashi & Murata 2008; Antolín et al. 2005).

Frost damage in legumes, assessed by whole-plant exposure to freezing temperatures, also can dramatically effect productivity and growth. Following acclimation, temperatures of -10°C for chickpeas and -15°C for peas can cause cell damage, especially in leaves and flowers (Wery et al. 1994). Cold hardy cultivars of these species and other legumes can minimize frost damage by osmotic adjustment in their cells or “supercooling” to prevent ice nucleation, as well as by increasing the unsaturated phospholipid composition of their cell membranes to offset rigidity and prevent lysis (Wery et al. 1994; Toker et al. 2007; Ruelland et al. 2009). Cold acclimation also is important to these processes; a study of forage clovers and medics in controlled environments showed lower LT50, greater root and shoot solute concentration (which indicates osmotic adjustment), and greater re-growth potential after freezing for plants exposed to cold temperatures before the freezing event than for control plants (Hekneby et al. 2006).

Reduction in growth and slow development at cold temperatures as well as the previously mentioned mechanisms for frost survival are often assumed to be similar in cold hardy cover crop legumes. However, Power and Zachariassen (1993) showed large variation in growth rate among species that are commonly used as winter annual cover crops, including hairy vetch, crimson clover (*Trifolium incarnatum*), and field pea. Similarly, when a set of winter annual and biennial legumes were screened for freezing tolerance at different growth stages, hairy vetch and crimson clover showed decreasing freezing tolerance with plant age, while white clover (*Trifolium repens*) and sweet clover (*Melilotus officinalis*) behaved the opposite (Brandsaeter et al. 2002). This indicates that though there are general trends towards low temperatures limiting cover crop growth, and certain growth stages and process being highly sensitive in legumes, it is important to consider species differences -even among cold hardy legumes- in order to select ideal cover crops for cold regions.

These discrepancies and many considerations demonstrate the under-developed body of research surrounding these cold-hardy legumes. Importantly, little recent literature assesses cold hardy winter cover crop legumes in harsh environments like the Upper Midwest (Liebman 2018; Wiering et al. 2018). Although recent breeding efforts have selected winter hardy lines of cover crop legumes such as hairy vetch based on survival and agronomic productivity in the field, more study is needed to adequately evaluate these cover crops for farm-scale productivity and utility in this climate (Maul et al., 2011; Wiering et al., 2018). Additionally, in order to gain the full benefits of growing legume cover crops, cold tolerance of their nitrogen-fixing symbionts and the process of SNF itself also must be assessed.

Effects of low temperatures on rhizobia, nodulation, and SNF

Rhizobia

Rhizobia bacteria performance and survival at low temperatures has been evaluated both in controlled environments and in the field. With most temperate legume crops, rhizobia survive, successfully infect and nodulate roots, and fix nitrogen optimally at temperatures between 20 and 30°C (Alexandre & Oliveira 2013). Cold stress responses are sparsely characterized in rhizobia, however it is known that similar to other bacteria, they produce cold shock proteins (CSP) in response to cold temperatures. CSP may help to stabilize single-stranded DNA and RNA, which become unstable at the translation phase during cold stress (Alexandre & Oliveira 2013). Membrane fluidity and permeability also decreases at cold temperatures and can cause rhizobial cell wall fragility while also hindering solute, water and ion transport. Rhizobia can to respond to this stress by shifting the fatty acid composition of their cell walls (Drouin et al. 2000). These physiological responses to cold stress may help rhizobia associated with cold-hardy legume cover crops survive the harsh winter conditions in the Upper Midwest and continue to perform SNF at low temperatures.

There is a wide range of cold tolerance observed in rhizobia, depending on the strain, its origin, and its host legume. Among alfalfa rhizobia strains, some were observed to grow in culture on media at 10°C, and also nodulate and perform SNF in alfalfa at 10 -

12°C (Rice et al. 1995). Clover-associated *Rhizobia trifolii* from different latitudes in Finland grew at similar temperatures in media cultures (10, 15, or 18°C), but showed no significant growth at 5°C. The strains also performed differently when days to visible growth at the three temperatures were measured, showing a significant variation in cold tolerance (Lipsanen & Lindstrom 1986). Rhizobia associated with annual medics, adapted to survive cool winters in the Mediterranean, were able to successfully grow and nodulate these legumes at both 7 and 9°C (Robin et al. 2005). Studies of arctic rhizobia strains demonstrated that these cold-adapted rhizobia could still grow at temperatures as low as 0°C (Prévost & Bromfield 1991). This wide range of temperature tolerance for rhizobia offers much potential for improved SNF under the very cold winter conditions of the Upper Midwest.

Notably, several studies have also found that selected strains with greater cold tolerance in controlled environment and media studies also have higher rates of nodulation, nodule residency, and plant productivity under cool temperature conditions in the field compared to commercial or local strains of rhizobia. Increased plant productivity with these strains may result from greater rates of SNF (Prévost et al. 2003; Rice et al. 1995; Zhang et al. 2002). Commercial inoculants are often optimized for high productivity and greater SNF under ideal conditions, and may not be as competitive or productive at marginal temperatures in the field (Zimmer et al. 2016). Due to the results of these studies, research interest has grown surrounding the development of more effective strains for agricultural production in cold climates or other challenging conditions (Sultan et al. 2001; Schmidt et al. 2015; Htwe & Yamakawa 2018). Though some of these interactions with more cold tolerant strains have been observed and tested in forage and food legumes, however, there is little literature characterizing or evaluating the rhizobia strains that form symbioses with reliably winter-hardy legume cover crops for the Upper Midwest.

Nodulation

Nodulation, defined as the formation of nodules following rhizobia infection of the root, is also curtailed by low temperatures. In a study of pea, bean, and lentil, nodulation was delayed for all species at root zone temperatures below 15°C, and nodule number was correspondingly lower at these cooler temperatures. However, species differences were observable, with no nodules forming on bean at 10°C, but pea and lentil both forming nodules (Lira Junior et al. 2005). In the previously mentioned *Rhizobia trifolii* study in Finland, red clover (*Trifolium pratense*) was also inoculated with different rhizobia strains, and days to nodulation and nitrogenase activity were measured in a cold or warm (4°C higher) temperature regime in a growth chamber. Nodulation time was longer and nitrogenase activity was lower overall in the cold climate, but there were only significant differences in nodulation time between strains at the colder temperature regime. Additionally, there was a stronger relationship between nodulation time and nitrogenase activity, which was measured by acetylene reduction, after 10-12 weeks in the cold climate than the warm climate, suggesting that cold stress over time continues to effect rhizobia function, even after they have successfully nodulated (Lipsanen & Lindstrom 1986).

This delay in nodulation and subsequent effect on nodule function is initially due to lower production of signaling molecules at low temperatures. Plant roots synthesize fewer flavonoids, such as genistein in soybean, at low temperatures (Poustini et al. 2005). These flavonoids are required to induce the synthesis of bacterial signaling molecules called nod-factors in rhizobia that are responsible for beginning the process of infection and nodulation in legume roots. Nod-factor production in rhizobia is also curtailed at cold temperatures (Duzan et al. 2006). The lower levels of plant and rhizobia signaling molecules reduces nodulation rate and can eventually result in slow nodule growth, plus fewer and smaller nodules (Abd-alla & Issa 2014; Poustini et al. 2005). The mechanisms that govern nodulation and the observed responses of different legume and rhizobia species and strains to cold conditions indicate that nodulation, measured by nodule number, size, and mass, can be used to assess cold tolerance in both legumes and their associated rhizobia. Nodulation success, in turn, is expected to dictate the amount of symbiotic nitrogen fixation in legumes.

Symbiotic nitrogen fixation: rhizobia

Whole-plant SNF at cold temperatures has been studied in many legume cash and forage crops (Prévost & Bromfield 1991; Tsialtas et al. 2004; Rennie & Kemp 1981; van Heerden & Kruger 2004). Overall, experiments with temperate-zone plants have shown that many legumes have reduced ability to form symbioses with rhizobia, as seen before with nodulation, or perform SNF at temperatures below 10ºC (Mahdavi et al. 2010). Experiments with cold root zone temperatures in soybean revealed that SNF was significantly delayed at temperatures below 17°C and that root infection and nodule development were very sensitive to these low temperatures (Zhang et al. 1995; Zhang et al. 1997). In beans (*Phaseolus vulgaris L.*), cold temperature regimes in a controlled environment, beginning at 10°C, also reduced SNF in mg of N fixed per plant compared to a 5°C warmer temperature regime. Cultivars differed in SNF capacity at the colder temperatures, with one in particular, the semi-vining 'Aurora', outperforming the others. The higher SNF capacity in this cultivar was attributed in part to greater root growth at cold temperatures, allowing for more plant-to-rhizobia signaling and nodulation to occur (Rennie & Kemp 1981). Similarly, in forage species such as annual medics (*Medicago aculeata, Medicago rigidula*), field studies showed differences in SNF between cultivars at cold winter temperatures. Since the cultivars were from the same region and climate, this study concluded that cultivar genotype could also contribute to variability in SNF at cold temperatures, regardless of regional adaptation (Sultan et al. 2001).

Differences among cultivars for cold tolerance and SNF at cold temperatures are also seen in legumes that can be used as winter annual cover crops in the Upper Midwest, however these studies do not necessarily reveal the same trends as seen in previous studies of food legumes or less cold-tolerant forages (Wilke & Snapp 2008; Brandsæter et al. 2008). For example, at cool (10-18°C) instead of very cold temperatures (5°C), winter annual legumes such as hairy vetch, field pea and red clover may have greater SNF capacity than at 20 to 30ºC (Power & Zachariassen 1993; Ofosu-Budu et al. 1992; Lipsanen & Lindstrom 1986). Capacity for SNF at low temperatures is an ideal trait for winter annual cover crops, and may help provide supplemental nitrogen for cash crops even when cover crops are terminated early in the spring after a season of growth.

Despite a fairly robust understanding of the rhizobia-legume symbiosis at cold temperatures in soybean and a few other legumes, there are few recent studies specifically exploring winter annual legume cover crop species, their rhizobia symbioses, and SNF in these environments.

Upper Midwest climate and development of high tunnel systems

The challenges of winter legume cover crop productivity and nitrogen fixation in cold climates demonstrates the potential difficulty of successfully overwintering these cover crops in harsh Upper Midwest conditions, especially in parts of both northern Minnesota and Wisconsin. Notably, winter temperatures in this region are some of the coldest in the contiguous United States; the area includes plant hardiness zones 5b to 2a (USDA-ARS 2012). This is not only a challenge for year-round cover cropping, but also limiting to cash crop production on farms. The growing season and the period of frost-free days is relatively short, spanning 90 to 150 days from late April/early May to late September/early October (NOAA-NESDIS 2000). Due to this short season, many crops may not have the time or Growing Degree Days (heat units) to produce optimal yields and may have trouble with establishment or cold damage at the beginning and end of the season. To extend their season beyond this shortened period and maintain yields, vegetable and fruit producers in the Upper Midwest have increasingly turned to high tunnels as critical farming tools (Carey et al. 2009; Orzolek 2013). High tunnels create a generally warmer micro-climate and allow greater control in water management, helping growers to continue to produce high quality fruit and vegetables earlier in the spring and later in the fall (Lamont 2005). High tunnels are not a new technology, but the increasing use and value of tunnels for growers makes them an important subject of research in

Upper Midwest agriculture (NSAC 2014). Legume cover crops in high tunnels are one way to improve both soil health and soil nitrogen status, but research in high tunnel soil health and cover cropping in high tunnel environments is minimally represented in scientific literature and deserving of further study (Perkus 2018). A brief introduction to high tunnels and their use is required in order to focus on the important gaps in this body of research.

High tunnels: definition and uses

High tunnels are important season extension tools in the United States and worldwide. They are semi-permanent structures, usually constructed using heavy polyethylene plastic that is stretched over a metal frame. High tunnels are sometimes called "hoop houses", but the frames for tunnels come in many styles and sizes beyond the commonly-seen half-circle (Orzolek 2013). Unlike a greenhouse, high tunnels are usually unheated, not built on a permanent foundation, and plants are seeded or transplanted directly into the ground rather than into raised beds or pots/flats on tables.

As previously introduced, high tunnels are increasingly popular in temperate growing regions because even without a heat-source, they offer warmer air and soil temperatures (especially during sunny days) that can allow growers to start crops earlier in the spring and maintain them later in the fall, thereby extending the growing season (Wien & Pritts 2009; Jett 2000). In warmer regions, like the Southern and Pacific Western United States, high tunnels allow growers to produce high quality fruit and vegetables year-round. Tunnels also offer protection from precipitation and control of soil moisture, which can lead to lower disease prevalence and higher produce yields and marketability (Rogers & Wszelaki 2012; Lamont 2005; O’Connell et al. 2012). Due to these advantages, high tunnels are often used for very intensive production of high value crops that prefer warmer air and soil temperatures and benefit from dry foliage. In cold regions during spring and fall, or during the winter in warmer regions, growers may also plant cool-season greens and other species that need minimal extra protection from the elements to survive (Drost et al. 2017). Tomatoes, cucumbers, peppers, salad mix, spinach, flowers and berries are common crops for high tunnels in the northern U.S., and there has also been successful experimentation with tree-fruit production in high tunnels as well (Carey et al. 2009; Demchak 2009; Lang 2009; Santos & Salame-Donoso 2012; H. C. Wien 2009).

High tunnel use has also increased in the United States due to government cost-share programs that lower the barrier to purchasing and constructing a high tunnel. The Natural Resource Conservation Service (NRCS) provides this cost-sharing benefit for farmers through a program called the Environmental Quality Incentives Program (EQIP), and, as of 2014, has helped to fund construction of over 13,000 new tunnels on farms since its launch in 2009 (NSAC 2014).

Temperature fluctuations

Understanding temperature dynamics within a high tunnel is key to understanding the advantages and disadvantages of high tunnel growing conditions. High tunnel temperatures are generally warmer than those of the open field. On days with ample solar radiation, high tunnels frequently reach temperatures 10°C warmer than the ambient air, especially if they are not vented. At night, the high tunnel plastic covering offers little insulation, and temperatures usually drop to just above the ambient air temperature (Jett 2000; H.C. Wien 2009). The protected environment and warmer air temperatures can allow growers to overwinter crops that would not reliably survive the winter in the open field (Yao & Rosen 2011). Despite the advantage of prolonged warm days, the large daily temperature fluctuations, especially during the winter months, require plants to tolerate a wide range of growing conditions in a short time-period and can potentially cause unusual physiological stresses. Some growers will further insulate crops in high tunnels with a floating fabric row cover to provide extra buffer against fluctuating temperature and also provide some protection from desiccation and sun damage (Drost et al. 2017; Ward & Bomford 2013). Though temperature dynamics in high tunnels have been shown to be both useful (Yao & Rosen 2011) and challenging (Ward & Bomford 2013) to growers and have motivated them to find ways to mitigate these temperature changes, the actual effects of temperature fluctuations in tunnels on crop productivity and physiology are largely unstudied (Drost et al. 2017; Wien & Pritts 2009).

Soil health and production challenges

In addition to the challenges of high tunnel temperature management, problems and uncertainties surrounding soil health management have emerged as more growers begin to use and familiarize themselves with high tunnel environments. Some of these soil health issues are similar to concerns for open-field intensive vegetable production environments such as the loss of organic matter and degradation of soil structure, but some are unique (Pagliari et al. 2016; Lecompte 2012). High tunnels face potential intensification of pest issues (Ingwell et al. 2017; Zambon et al. 2013), soil-surface salt accumulation (Gluck & Hanson 2013), and nutrient and pH imbalances (Knewtson et al. 2012). Salinity and nutrient imbalances are mostly due to lack of rain inside the tunnel, coupled with continuous application of fertilizer, manure, or compost to maintain fertility. In tunnels where drip irrigation is primarily used, there is rarely enough water movement through the soil profile to wash salts and mobile nutrients not used by plants into deeper soil layers. Instead they remain in the top six inches of soil, and can begin to negatively affect crop growth after years of continuous production (Morra et al. 2017). One solution to the lack of rain in high tunnels is to remove the plastic covering during a fallow season (often winter) and allow precipitation to wash accumulated salts and soil amendments out of the topsoil. This method has been shown to decrease electrical conductivity of high tunnel soils (EC is a measure of salt concentration) to acceptable levels for plant growth where the primary ions contributing to salt levels were calcium, potassium and magnesium (Gluck & Hanson 2013). However, for some growers, frequently removing and replacing plastic on high tunnels is not an option, and it does not necessarily address other high tunnel challenges such as soil structure and weed and pest/disease pressure (Montri & Biernbaum 2009).

Nutrient management is also a unique challenge in high tunnels soils. With continuous and intensive production there are high nutrient demands from crop plants, however, the limited water movement in high tunnel soils can result both in shortages of nutrients that plants use quickly, and surpluses of others that plants do not take up readily. Conventional growers commonly use mineral fertilizers or drip fertigation coupled with drip irrigation to meet crop needs. Organic growers rely on plant-based compost, composted animal manures, or organic liquid fertilizers such as liquid fish emulsion or liquid kelp (Everhart et al. 2010). Animal manures in particular have been shown to add excess phosphorus, potassium, and soluble salts to high tunnel soils that can in time create difficulties for plant growth and yield (Reeve & Drost 2012). Adding legume cover crops into a high tunnel rotation could provide a potential alternative source of nitrogen fertility for future cash crops without adding excess salinity or abundant phosphorus to the system. Cover cropping is still an uncommon technique for high tunnel maintenance, but merits development as a tool to increase high tunnel sustainability. The aforementioned soil health benefits of winter annual cover crops in the open field could be applicable in high tunnel systems to address unique tunnel management challenges; however, published research on high tunnel cover cropping is limited and little has been done in the Upper Midwest.

Cover crops in high tunnels

Cover crops are not a logistically or temporally simple tool for high tunnel soil health, as growers opt to fit in as many cash crop rotations as possible during a season, leaving few windows for cover cropping (Lecompte 2012). A survey of high tunnel growers in the central U.S. revealed that, on a Likert-type scale, about 16% regularly grow cover crops in their high tunnels, either during the summer or during the winter, and 21% more occasionally grow cover crops (Knewtson et al. 2010). These low numbers of cover crop users in high tunnels is also reflected in the lack of research on cover crops and their potential benefits for high tunnel soils, weed control, and fertility. The few studies on high tunnel cover cropping that do exist have mixed results. Cover cropping with sorghum-sudangrass (*Sorghum bicolor X S. bicolor var. sudanese*) in high tunnel vegetable crop rotations did not increase soil organic carbon relative to a bare-ground control (Morra et al. 2017). However, hairy vetch used as a green manure in tomato production provided increased soil nitrogen for increased tomato yields and slightly increased soil carbon (Muchanga et al. 2017), and in another study showed potential to provide long-term soil nitrogen fertility (Sugihara et al. 2017). Though some results contradict one another, the recent literature on legume cover crops in high tunnels indicates that there is potential for cover crops to become a productive part of high tunnel rotations and that more research is needed in more locations to understand cover crop contributions and viability in high tunnel systems.

Nitrogen contributions of winter annual legume cover crops

In addition to cover crops' contributions to soil health, cover crop legumes are important in their provisioning of nitrogen through SNF. In studies of multiple cash crops from sweet corn to cotton, the nitrogen contributions of a preceding legume cover crop have been well documented (Perdigão et al. 2012; Fageria 2007; Hanly & Gregg 2004; Rochester & Peoples 2005; Cherr et al. 2007; Martin & Touchton 1983). In some of the systems studied, legume cover crops have contributed enough nitrogen to the system to replace synthetic fertilizers or compost as a fertility source for the cash crop, with vetch species consistently supplying the most available N (Boquet & Dabney 1991; Parr et al. 2011). However, many of these studies were performed in significantly warmer climates than the Upper Midwest (Louisiana, North Carolina, Southern Europe), and little knowledge exists about the potential for nitrogen delivery to cash crops from these legumes grown under very cold conditions, or in the variable conditions of a high tunnel in northern regions. The important potential of legume cover crops as an alternate source of fertility both in the open field and in high tunnels makes this knowledge gap particularly glaring and crucial to be addressed, especially when farmer priorities are considered.

Farmer interest and goals for winter annual legumes

As demonstrated earlier, SNF in winter annual legume cover crops can offset the need for fertilization for a following cash crop. Reducing fertilizer inputs is important to environmental health and important to farmers both economically and as stewards of their land. In high tunnel environments, reducing fertilizer and manure additions can lower salt accumulation and nutrient imbalances in the soil, maintaining the health and productivity of these growing environments. In addition to recognizing these services of cover crop SNF, farmers make the connection between improving SNF in cover crop legumes and the nitrogen credits they may receive from growing these crops. In a survey of U.S. farmers in 2016, the most important trait for three legume species of winter annual cover crops (hairy vetch, crimson clover, Austrian winter pea) was nitrogen fixation, followed by winter hardiness. In same survey, organic farmers identified nitrogen fixation as one of top three reasons they grow cover crops, following improved soil health and increased organic matter (Wayman et al. 2017). Organic producers cannot use synthetic nitrogen in their systems and are required to include cover crops as part of their farm rotation, so SNF is an important possible source of nitrogen fertility (USDA 2015).

Despite the recognized benefits of cover crops and the identification of specific traits of interest, less than 2% of overall cropland in the United States includes cover crops in the crop rotation (USDA ARS 2012). This low level of adoption can potentially relate to the requirements and preferences farmers identified: there is little research and literature on legume cover crop varieties that fix adequate nitrogen and are reliably winter hardy across many systems. To support this, a Conservation Technology Information Center survey of U.S. farmers ranked identified challenges and barriers to cover cropping. After time/labor requirements, establishment of cover crops and choosing the right species (for their region) were the two major challenges to farmer adoption (CTIC 2017). For the Upper Midwest, these challenges are very pertinent. In a climate with early frosts, cool fall temperatures, and cold winters, plants often establish poorly, and those with poor establishment struggle to survive the season. Additionally, unadapted and untested cover crop varieties may not be cold tolerant and will fail to perform the ecosystem services farmers prioritize (Snapp et al. 2005). The tension between farmer interests and priorities for cover crops and the low rate of adoption in the U.S. drives recent research on cover crop traits, species and performance (Maul et al. 2011; Perdigão et al. 2012; Parr et al. 2014; Young-Mathews 2017). In the particular environment of the Upper Midwest and the context of legume cover crops more research is required to understand plant productivity and nitrogen fixation at cold temperatures or in high tunnel environments.

Conclusions

Research has thoroughly characterized many aspects of SNF, plant productivity, and cold tolerance in legume crops over the years, however, there are gaps in the literature surrounding SNF in winter-hardy cover crop legumes at cold temperatures or in protected growth environments like high tunnels. Important questions still unanswered are:

1. How do prolonged cold temperatures affect plant productivity, symbiotic initiation, nodulation, and SNF in commonly-used, winter annual cover crop legumes in the Upper Midwest?

2. How do high tunnel temperatures over the winter season in the Upper Midwest affect plant productivity, survival, nodulation, and SNF in commonly used, winter annual cover crop legumes and cover crop mixtures?

These questions, shaped by knowledge of farmer priorities for cover crop legumes and the existing gaps in recent literature, form the topics for this thesis and inform future research in the area of winter annual cover crop legumes and SNF.

Chapter 2- Effects of cold temperatures on symbiotic nitrogen fixation in winter annual legume cover crops

Introduction

Farmers worldwide use cover crops and green manures to maintain soil fertility, increase soil organic matter, break pest and disease cycles, and protect soil structure and function during fallow periods in their production cycles (Scholberg et al. 2010; Perdigão et al. 2012; Blanco-Canqui et al. 2015). Organic farmers in the U.S. are particularly invested in cover crops to maintain soil health, as they are required to include cover crops and crop rotation in their farm planning as part of the National Organic Program standards (USDA-AMS 2018).

In the Upper Midwest, where average temperatures from December to March range from -4 to -12°C, most agricultural production consists of large-scale annual crops like corn and soybean, planted in the late spring and harvested in late summer/early fall (NOAA-NCEI 2018; USDA-NASS 2012). Smaller-scale vegetable and fruit growers also produce most of their crops in the summer months, with some carry-over into the spring and fall using season extension techniques such as high tunnels (see Chapter 3). In this region, summer annual cash-crop production is followed by a winter fallow period, usually from September to April, where crop residues have been removed and bare soil may remain. This winter fallow period can be vulnerable to erosion, nutrient leaching, and structural degradation (Kladivko et al. 2014), and could be an important window of opportunity for growing cover crops. Winter hardy cover crop species can be seeded in the fall after the removal of the summer cash crop, remain dormant but alive during the winter months, and be terminated and incorporated in the late spring, when they have developed sufficient biomass. Winter annual cover crop use reduces nutrient loss and soil degradation, and terminated spring biomass can provide fertility, weed suppression, and increased soil water for the following cash crop (Baraibar et al. 2018; Mendes et al. 1999; Abdollahi & Munkholm 2014; Malone et al. 2014).

Despite the available seasonal window for cover crop use on farms in the Upper Midwest, adoption of winter annual cover cropping practices is still fairly low among farmers (Wade et al. 2015). Non-adopting farmers frequently report concerns about cash crop yield to explain why they do not grow cover crops, however, there is potential nutrient provision for following cash crops by winter cover crops (SARE & CTIC 2015). In most soils, nitrogen is the main limiting nutrient for crop production, which has led to increased interest in legumes as cover crops (Rütting et al. 2018). Legume cover crops provide soil health benefits and ecosystem services during the non-production season and, in symbiosis with rhizobia soil bacteria, can potentially fix sufficient nitrogen through Symbiotic Nitrogen Fixation (SNF) to reduce the need for fertilization in agricultural systems (Parr et al. 2011; Martin & Touchton 1983; Liebman et al. 2018; Perdigão et al. 2012). Organic farmers have higher rates of cover crop adoption, due in part to NOP crop rotation requirements, but also because these growers are prohibited from using synthetic chemical fertilizers on their farms and may rely on cover crops as a fertility input (Wayman et al. 2017). Nitrogen provision by legume cover crops is especially important to organic farmers. These farmers cite nitrogen fixation as one of the top three reasons they grow cover crops, and identify nitrogen fixation as a major priority when agronomists are breeding improved varieties of legume cover crops (Wayman et al. 2017).

Though organic farmers prioritize legume cover crops and their SNF services and current users report increasing acreage of on-farm legume cover crops (CTIC 2017), barriers to successful use of legume cover crops exist in cold climates. In the Upper Midwest particularly, cold transitional fall and spring temperatures potentially limit SNF in winter hardy cover crop legumes, reducing nitrogen accumulation and eventual soil fertility contributions. Furthermore, published cold-temperature thresholds for legume performance are often based on non-cover crop agronomic species, such as soybean, chickpea, and dry beans (Bhandari et al. 2017; Lira Junior et al. 2005; Zhang et al. 1995). Additionally, despite studies of cover crop legume freezing tolerance and winter hardiness (Brandsaeter et al. 2002; Wiering et al. 2018; Brandsæter et al. 2008) both in the field and in controlled environments, specific effects of cold temperatures on nodulation and SNF in many of these species are not well characterized (Wilke & Snapp 2008; Lipsanen & Lindstrom 1986).

The objective of this study was to examine the effects of steady cold temperatures on the growth and symbiotic nitrogen fixation of winter annual legume cover crops in hopes of better understanding the potential responses of these cover crops to cold climates. To accomplish this objective, we quantified 1) plant vigor, 2) initiation and productivity of nodulation, and 3) SNF for three widely used species of winter annual cover crop legumes at cold temperatures under controlled conditions. We expected that colder temperature treatments would reduce plant growth, nodulation, and SNF, but we also expected to see more cold tolerance in these cover crop species at mid-range temperatures (10-15°C) than other, better studied annual legumes like soybean. We additionally expected differences in cold tolerance for growth, nodulation, and SNF among the species tested. The final aim of this research was to synthesize new findings surrounding winter annual legume cover crop growth and SNF and relate this to outcomes for farmers in the Upper Midwest.

Methods

Experimental Design and Treatments

This study was conducted in four growth chambers at the University of Minnesota Plant Growth Facility in the Department of Horticultural Science. The study was a split plot design with temperature as whole plot treatment and cover crop species as split plot treatment. Each growth chamber was held at one of four steady temperature treatments, 20°C, 15°C, 10°C or 5°C, for the duration of the experiment. Three cover crop legume treatments were evaluated including hairy vetch (*Vicia villosa*), Austrian winter pea (*Pisum sativum*), and crimson clover (*Trifolium incarnatum*). To account for variability across different growth chambers, plants were grown in two identical growth chamber runs for six weeks each, including four blocked replications in each run for a total of eight replications. To allow for measurement of Symbiotic Nitrogen Fixation, each species treatment included a negative control, which were non-inoculated plants supplied with only N-free solution, and a positive control, non-inoculated plants supplied with a full nutrient, or N-plus, Hoagland's solution (Hoagland & Arnon 1950).

Chamber environment and seed source

Chamber conditions were 12hr day/12hr night with 200 umol of fluorescent light and 65% RH. Temperature and light levels (Lux) were logged in each chamber at half hour intervals using four Labjack Digit temperature and light sensors per chamber (Labjack Corporation, Lakewood, CO) in order to monitor chamber consistency.

The legume species were sourced based on availability as organic cover crop seed from cold-climate sources. Hairy vetch was a cold hardy accession selected at the University of Minnesota, St. Paul, MN (UMN23) (Wiering et al. 2018), crimson clover from Johnny's Selected Seeds in Winslow, ME, and Austrian winter pea from Albert Lea Seeds in Albert Lea, MN.

Plant germination, inoculation, and maintenance

Legume species treatment seeds were surface sterilized with 1.5 M NaHOCl (bleach) solution and germinated in parafilm-sealed sterile petri dishes on germination paper moistened with sterile double deionized (DDI) water. The petri dishes were germinated at 20°C growth chamber for five days. Chamber conditions were 65% RH, 200 umol fluorescent light and 14 hr day/10 hr night, to mimic early fall conditions in Minnesota.

After five days of germination, seedlings were transplanted into sterile plastic growth-pouches lined with germination paper (CYG Germination Pouches, Mega International), and 10 mL of N-free solution was added to each treatment pouch. Positive-N controls were supplied instead with Hoagland's solution (Hoagland and

Arnon, 1950) containing 210 ppm N. Treatment seedlings of each species were inoculated by pipette with 0.5 mL commercial rhizobia inoculant suspended in 0.85% NaCl solution. Crimson clover was inoculated with *Rhizobium leguminosarum* biovar *trifolii,* Austrian winter pea and hairy vetch were inoculated with *Rhizobium leguminosarum* biovar *viciae* (N-dure Peat Inoculant, Verdesian Life Sciences, Cary, NC). Growth pouches were sealed around the base of the seedling stem with laboratory tape to prevent contamination of non-inoculated plants with rhizobia, as well as excess water loss via evaporation. In a previous pilot study, temperatures within the growth pouches, or root-zone temperatures, were recorded alongside air temperatures. No differences were found between air and root zone temperature, therefore root zone temperature was not recorded during this study. The whole plant was assumed to be experiencing the same temperature within the growth chambers.

Nutrient solution was replenished every 7 days by pipetting 10 mL of the appropriate solution into each growth pouch. Solutions were sterilized and adjusted to the temperature of each chamber before watering.

Measurements

Nodulation observations

Inoculated plant roots were observed daily in all chambers to establish average time to nodulation at the four temperatures. The number of individuals of each species with visible nodules was recorded after daily observations.

Biomass, nodule number, and nodule mass collection

After six weeks, all plants were harvested from the four chambers. Nodule number and number of pink nodules was counted on the roots of inoculated plants. Pink nodule interior is an indicator of active Symbiotic Nitrogen Fixation (SNF) by rhizobia (Pommeresche & Hansen 2017; Dakora 1995). Nodules were removed and dried for 48 hours at 60°C, then weighed to obtain a dry mass. Shoots were separated from roots at the cotyledonary node, both were dried at 60°C for 48 hours, then weighed to obtain plant biomass.

Nitrogen fixation measurement and calculation

Dried shoot biomass was ground to 2 mm particle size using a hammer mill, then to a fine powder (> 0.5 mm) with a Geno/Grinder tissue homogenizer (SPEX Sample Prep, Metuchen, NJ). Dried ground plant material from each treatment in each chamber was weighed on a microscale into 10 mg subsamples and analyzed using a Pyrocube elemental combustion analyzer (Elementar, Mt Laurel, NJ) to obtain C:N ratio and percent N. Total plant N was calculated by multiplying percent N by the plant tissue mass, then calibrated for differences in sample size. To quantify SNF, the N-Difference approach was used (Unkovich et al. 2008), relying on differences in plant tissue N in inoculated versus non-inoculated controls. To calculate, N of biomass from the negative controls (un-inoculated plants) was subtracted from the total N of the inoculated plants to estimate quantity of nitrogen fixed.

Data analysis

The effects of temperature and species on plant vigor, nodulation, and SNF were measured with ANOVA utilizing a split-plot linear model. Means were separated using Tukey's HSD tests with an α-value of 0.05. Data were tested for normalcy and homogeneity of variance by evaluating residual plots. When data failed to meet assumptions of normality, variables were transformed before ANOVA. Reported means are of un-transformed data. Statistical analysis was performed using R statistical software (R Core Team, 2018).

Results

Legume species response to temperature

Growth and vigor were quantified using legume biomass (g dry mass of individual plant tissue). There was an interaction between temperature and species for this variable ($p<0.001$, Table 2.1).

Shoot biomass across species increased with increasing temperature. Species were analyzed separately and performance at each temperature compared within species (Table 2.2). All species had lower biomass at 5°C than at 15°C and 20°C. Austrian winter pea and hairy vetch had greater biomass at 10°C compared to 5°C, while crimson clover biomass did not differ between 10 and 5°C (Table 2.2).

Nodulation response to temperature and species treatments

Days to nodulation onset increased as temperature decreased. Plants at 20°C initiated nodulation as early as 6 days after inoculation (DAI), while plants at 5°C did not initiate nodules until 15 DAI. Over six weeks, plants at 20°C, 15°C, and 10°C initiated nodules at a greater rate than those at 5°C (Figure 2.1).

Effect of temperature and species on nodule number are reported in Figure 2.2. Austrian winter pea and hairy vetch both had a negative nodulation response to temperature only at 5°C, while crimson clover had fewer nodules per plant at 5°C and 10°C than at 20°C.

Main effects of treatments on pink nodule number are reported in Table 2.3, there was no interaction effect for this variable (Table 2.1). There were no pink nodules at 5°C, and pink nodule number did not differ among the warmer temperatures.

Nodulation efficiency, calculated as g nodule mass per g shoot biomass, demonstrates the ability of a legume to successfully establish nodules (Dobert & Blevins 1993; Mothapo et al. 2013). Higher nodulation efficiency is correlated to greater SNF, or more efficient nodule function. Effects of temperature on nodulation efficiency are reported in Figure 2.3. Nodulation efficiency did not differ within each species between 15- 20°C, supported by no measured differences in overall nodulation efficiency at these higher temperatures. Negative effects of low temperature on nodulation efficiency were observed in all species at 5°C, where very few or no nodules formed. When species were examined across temperature, vetch had greater nodulation efficiency than clover and pea overall ($p < 0.01$).

Shoot nitrogen and symbiotic nitrogen fixation

Shoot nitrogen, measured as percent N, did not respond linearly to temperature. The main effects of temperature and species on shoot N are reported in Table 2.3; there was no interaction effect for this variable (Table 2.1). Shoot N was higher at 5°C and 15°C than at 10°C, and also differed among species, with the greatest overall percent N in hairy vetch (4.8 %) and lower percent N in winter pea and crimson clover (4.0 % and 3.4 %, respectively).

Estimated symbiotic nitrogen fixation (SNF) was calculated as g of N per treatment plant tissue relative to non-inoculated control plants. Only 4% of 5°C plants initiated nodules, therefore the 5°C treatment was omitted from SNF analysis. When species were combined, plants grown at 20°C had similar levels of SNF to those at 15°C, and greater SNF than those at 10°C ($p < 0.05$, Table 2.3). However, due to interaction of species and temperature, species were analyzed separately and SNF response to temperature within each species reported in Figure 2.4. Colder temperatures reduced SNF in hairy vetch and Austrian winter pea, but for crimson clover SNF was indistinguishable among the three temperature treatments. Analysis within each temperature revealed differences between species at 10°C, 15°C, and 20°C; crimson clover had lower SNF values at 15°C and 20°C.

Relationship between SNF, biomass, and nodulation

Predictors in the experiment other than temperature possibly contributed to patterns of SNF. A Pearson's pairwise test examined linear correlations between SNF and variables of biomass, percent shoot N, and nodulation. While percent N, nodule number, and pink nodule number did not strongly correlate to SNF, there were stronger positive correlations between shoot biomass and SNF, as well as nodule mass and SNF (r= 0.78 and 0.72, respectively).

Adjusted R^2 values for the relationship between SNF, biomass, and nodule mass are reported in Figure 2.5. For both nodule mass and biomass, slopes of linear regression to SNF were significantly higher at 15°C and 20°C than 10°C.

Pairwise correlations also revealed strong correlation between nodule mass and shoot biomass (r= 0.90), where nodule mass increased with shoot mass. Adjusted R^2 values for the relationships between nodule mass and shoot biomass for three species and four temperatures are reported in Figure 2.6. Vetch and pea display a stronger relationship between nodule mass and shoot biomass than clover. Temperatures of 10°C, 15°C, and 20°C show similar trends of increasing nodule mass with increasing shoot biomass, however, at 5°C, there is no relationship and nodule mass and biomass are both low.

Discussion

Legume growth

We found that cold temperatures negatively affected legume cover crop growth and vigor in this study. Biomass across all species was lowest at the coldest temperature treatment, though responses to intermediate cold temperatures differed between species. Decreased biomass accumulation at low temperatures is well-established in plant physiology and more specifically, legume physiology, especially if plants are exposed to low temperatures at early growth stages (Wery et al. 1994; Dehaghi & Sanavy 2003). At the species level, crimson clover biomass production was more sensitive to low temperature than Austrian winter pea and hairy vetch, possibly due to lower cold hardiness. This species is known to be sensitive to cold; crimson clover is recommended as a winter annual for USDA hardiness zones 6-9 due to poor cold tolerance (Young-Mathews 2013). In these zones, fall frost dates may occur within the range of late-October to early January, well after the September range in zones where the USDA endorses winter pea and hairy vetch as winter annuals (NOAA-NESDIS 2000; USDA-NRCS 2002; Pavek 2012; Young-Mathews 2013). In the Upper Midwest, where cool fall temperatures begin early in the season, concurrent with planting and early vegetative growth of winter annual cover crops (NOAA-NCEI 2018), fall-planted hairy vetch and winter pea may be able to accumulate greater biomass than crimson clover before winter. However, our results also indicate that temperatures below 10°C may limit early biomass in vetch and pea, and suggest that for greater cover crop biomass production these legumes should be sown before temperatures are consistently at or below this range. These results are in contrast to studies that examined hairy vetch, field pea, chickpea, and lentil, and found 15°C to be the low-temperature threshold in the field for early growing conditions in these legume species (Teasdale et al. 2004; Bhandari et al. 2017). However, conditions in the field are highly heterogeneous and include potentially stressful variables of wind, soil moisture, nutrient levels, and daily temperature and light fluctuation not present in our study (Takahashi & Murata 2008; Chen et al. 2006; Wery et al. 1994).

Biomass was positively correlated to SNF in our study, supporting that biomass quantification may be able to be used to predict cover crop N accumulation and potential

N contributions to agricultural systems (Scholberg et al. 2010). Early vigor and biomass of cover crops is also vital for on-farm outcomes like fall weed suppression and organic matter contributions (Brandsaeter & Netland 1999; Alonso-Ayuso et al. 2018; Gieske et al. 2016). Furthermore, a recent study of 30 accessions of hairy vetch indicated that fall vigor correlates to winter survival (Wiering et al. 2018). However, the same study also showed poor correlation of fall vigor to following spring biomass, suggesting that some hairy vetch genotypes may grow well in the spring and fulfill farmer goals such as organic matter additions and SNF despite less vigorous fall growth (Wiering et al. 2018). Low biomass production in the six-week period of our experiment may not indicate that these legumes are unsuitable for winter annual cover cropping over a full season.

Nodulation

Nodule initiation across all three species in our study was increasingly delayed at colder temperatures. Nodulation lag was most pronounced at 5°C compared to all warmer temperatures, where nodules were not initiated on average until 36 days after inoculation, and ultimately formed on fewer than five plants. Studies of lentil, peanut, and soybean also found nodule initiation to be delayed at cold (10°C) temperature extremes (Lira Junior et al. 2005; Zhang et al. 1995; Alexandre & Oliveira 2013). Nodule initiation at 5°C may be delayed due to reduced production of Nod factor rhizobia-to-plant signaling molecules at low temperatures (Duzan et al. 2006). With lower Nod-factor concentration, fewer legume root hairs become receptive to potentially infective rhizobia, and the rhizobia-legume symbiosis is less likely to be initiated (Lira et al. 2015).

Nodule number across species decreased in response to decreasing temperatures, but species responded differently to low temperatures. Crimson clover was more sensitive to mid-range low temperatures than Austrian winter pea and hairy vetch, which both produced the same amount of nodules at all but the lowest temperature. Pea and vetch both have larger seeds than clover, and therefore more initial nutritional resources. Due to reserve seed resources, these species possibly used stored carbohydrates to develop a greater number of nodules at low temperatures, even when biomass was not developed enough to support early nodule formation with photosynthesis (Dobert & Blevins 1993). Crimson clover has also been shown to respond with greater crown nodulation if rhizobia density is increased during early growth, but sites for nodulation may become inactive if symbiosis is not initiated very soon after inoculation (Nazih & Weaver 1994). In our experiment, delayed nodulation due to slow plant growth and low production of Nod factors at colder temperatures may have resulted in fewer sites for nodulation and lower nodule numbers, even if adequate rhizobia were present.

Nodule mass and nodulation efficiency were negatively affected by decreasing temperatures. We observed both reduced nodule mass and nodulation efficiency at 5°C compared to the warmer temperatures, suggesting a threshold temperature between 5°C and 10°C at which winter annual cover crop legumes do not increase nodule mass, which may be related to seedling biomass production. This 5-10°C threshold that limits nodule mass, and therefore nodulation efficiency, is similar to 9°C limiting temperatures found in forage legumes (Rice et al. 1995), and colder than limiting temperatures ranging from 10°C to 19°C found for soybean (Zhang et al. 1997; Lira Junior et al. 2005; Zhang et al.

1995). However, it is also important to note that differences in nodule mass and biomass varied by plant cultivar in other studies, a metric not tested in our experiment (Zhang et al. 1997).

Nodule mass and plant biomass were correlated in this study. This correlation was especially strong for vetch and pea, and at all temperatures but 5°C, which is consistent with studies of soybean under temperature stress; in young plants, nodule mass is closely related to the limited biomass available to fix carbon through photosynthesis (Lynch & Smith 1993). Furthermore, without a source of N besides SNF, plants must invest photosynthates in nodules as biomass is produced and needs more N to develop normally (Dobert & Blevins 1993; Bethlenfalvay et al. 1978). At chilling temperatures plants experience photoinhibition, where photosynthesis is curtailed due to production of reactive oxygen species, damage and necessary repair of photosystem II, and reduced enzymatic activity (Ruelland et al. 2009; Takahashi & Murata 2008). Therefore, at the prolonged colder temperatures in this experiment, it is likely that photosynthesis was limited, thereby limiting photosynthates to the roots (Antolín et al. 2005) and legume investment in nodules. In plants at 5°C, where temperatures severely limit initiation of nodules, and photoinhibition is probable, the positive relationship between biomass and nodule mass is not seen. We found that nodule number is less strongly correlated to shoot biomass than nodule mass (r= 0.57), indicating that initial nodule formation is not as closely linked to shoot resources as nodule mass (Ferguson et al. 2018; Mothapo 2017; Dobert & Blevins 1993). Additionally, not all nodules formed may be active (Denison 2015; Pate & Dart 1961; Puppo et al. 2005). These relationships suggest that cold temperatures have a limiting effect on nodulation in cover crop legumes similar to the effect seen in biomass, although adequate numbers of nodules may develop even on plants with limited biomass at cool temperatures.

Symbiotic nitrogen fixation

Our overall results suggested that the optimum temperature for Symbiotic nitrogen fixation (SNF) in the observed legumes was 15-20°C. This agrees with other findings that cold-hardy legumes may have equal SNF capacity at cool temperatures (10-17°C) as at warmer temperatures (25-30°C) (Power & Zachariassen 1993; Ofosu-Budu et al. 1992; Lipsanen & Lindstrom 1986). At the species level, we observed levels of SNF at cool temperatures in winter pea and hairy vetch that were the same as warm temperatures, which may be attributed to legume and rhizobia adaptation to cool environmental conditions. Cold tolerance in hairy vetch has been documented in the upper Midwest and Northern Europe, but can be inconsistent among cultivars (Wilke & Snapp 2008; Brandsaeter & Netland 1999; Wiering et al. 2018). The seed used in this experiment was obtained following a screening of hairy vetch for winter survival in Minnesota, meaning the plants were likely very cold tolerant (Wiering et al. 2018). Indeed, we found vetch had greater SNF than the other species at the lowest temperature. Our results show less SNF capacity overall in crimson clover than vetch and pea, similar to the results for nodule number and biomass, suggesting that low SNF in clover may have been partially driven by reduced plant cold hardiness. Indeed, other studies found crimson clover had less freezing resistance and cold tolerance than hairy vetch in the field (Brandsæter et al. 2000; Magdoff 1993). Our results suggest that hairy vetch and winter pea may be better able to jump-start SNF at early growth stages under temperature stress and accumulate more fixed N following fall planting in cold climates than crimson clover.

Lack of nodulation at 5°C indicated no symbioses with inoculant rhizobia or nitrogen fixation and thus plants in this treatment were omitted from SNF analysis. Reduced biomass at 5°C indicated low legume tolerance of this temperature at early growth stages. It is important to recognize that fall temperatures in temperate climates dip to 5°C early in the season (NOAA-NCEI 2018) and winter annual legumes could have greater nodulation and SNF capacity with inoculant rhizobia tolerant of these cold temperatures during the establishment phase. Rhizobia strains for cold conditions have been well studied in soybean (Lynch & Smith 1993; Zhang et al. 2002; Schmidt et al. 2015) and utilized in inoculant production (Htwe & Yamakawa 2018). However, rhizobia community studies for pasture, forage, and cover crop legumes like vetch, pea, and clover have found wide variation in cold tolerance and symbiotic effectiveness (Lipsanen & Lindstrom 1986; Drouin et al. 2000; Rice et al. 1995; Roughley 1970; Mothapo et al. 2013). Future study is needed to potentially increase cold-temperature SNF in these legumes through well-characterized cold-tolerant rhizobia strains.

Biomass and nodulation as predictors of SNF

We observed positive correlation between SNF and shoot biomass as well as between SNF and nodule mass, consistent with other legume studies that suggest that nodulation or dry matter/dry biomass can be used to predict SNF capacity (Zhang et al. 1995; Sato 2014; Lira et al. 2015; Divito & Sadras 2014; Unkovich et al. 2010). These relationships may be particularly strong in this study due to nitrogen limitation; SNF was the only source of nitrogen after seed reserves were depleted, forcing legumes to invest more resources in nodules and subsequent SNF in order to produce healthy biomass. At 10°C, these correlations were not as robust as at the warmer temperatures, which aligns with other research finding that at low temperatures, photosynthesis may be curtailed, and therefore even plants with greater biomass may have low SNF due to the close relationship between the processes of nitrogen fixation and carbon fixation in legumes (Denison 2015; Bethlenfalvay et al. 1978). Overall, our observations of these relationships between biomass, nodule mass, and SNF indicate that the legumes in our study behaved similarly to plants in field environments (Unkovich et al. 2010), and therefore our results on SNF at cold temperatures for these legumes may have relevance for environments other than the growth chamber.

Conclusions

Results from this study reinforce that low temperatures negatively affect biomass and nodulation of winter annual cover crop legumes, but also offer new evidence that these legumes can still grow and form symbiotic relationships at temperatures as cold as 10°C. At six weeks of growth, hairy vetch, Austrian winter pea, and crimson clover nodule numbers, nodule mass, nodulation efficiency, and biomass at 10°C in most cases was greater than that at 5°C, and often was not reduced from higher temperatures of 15°C or 20°C. These plants also performed as much SNF at 15°C as at 20°C, the warmest temperature in this study. This indicates greater plant and rhizobia tolerance of cool temperatures for these species than for species such as soybean, common bean, or lentil (Bhandari et al. 2017; Schmidt et al. 2015; Lindemann & Ham 1979). Results also show greater cold tolerance at early growth stages for evaluated species than previously published literature on these and other legumes (Young-Mathews 2013; Pavek 2012; Mahdavi et al. 2010; Lira Junior et al. 2005; Rice et al. 1995). However, though low temperature thresholds for plant growth and nodulation appear to occur between 5°C and 10°C for the legumes in this study, values for SNF were not determined below 10°C due to lack of nodulation and pink nodules for all species that suggested SNF was not occurring. Vetch and pea had increasing SNF capacity above 10°C, but crimson clover had low SNF capacity even under mild conditions, despite increasing biomass and nodule mass at the warmer temperatures. The differences in SNF response to temperature suggested that crimson clover is not an ideal cover crop for establishment or early nitrogen fixation and accumulation under cool fall conditions, but that hairy vetch and Austrian winter pea may be more suitable. Additionally, though we observed low nodulation and lack of SNF at 5°C, legume biomass was still produced, suggesting there may be an opportunity for functional symbiotic relationships in this temperature range with more cold-tolerant rhizobia strains, and that future study is warranted.

Overall, results of this study suggest that growers may be able to plant these winter annual species at cool temperatures later in the fall and still see growth, nodule development, and SNF prior to winter. Furthermore, at a cooler temperature of 15°C, these legumes had SNF capacity equal to SNF at the warmest temperature in our study. Therefore, farmers could see adequate nitrogen contributions from these species even without ideal planting temperatures. Hairy vetch and Austrian winter pea were overall more cold tolerant than crimson clover and may be the more ideal cover crop legumes for planting under cool fall conditions. Our results from this controlled-environment study require verification by field testing in Upper Midwest fall conditions in order to offer responsible recommendations to farmers.

Tables

Table 2.1 Analysis of variance of biomass, nodulation, shoot percent N, and SNF due to treatments.

Fixed Effects	**Biomass**	**Nodule Number**	**Pink Nodule number**	**Nodule mass**	**Percent N**	**SNF**
	--p values	---				
Temp	< 0.001***	< 0.001 ***	0.007 **	< 0.001 ***	0.007 **	0.04 *
Species	< 0.001 ***	0.004 **	0.01 *	< 0.001 ***	0.001 **	< 0.001 ***
Temp x Species	0.001 **	0.04 *	0.07ns	< 0.001 ***	0.2 ns	0.02 *

*, **, and *** represents significance of corresponding p values at α = 0.05, 0.01, 0.001 respectively.

Table 2.2 Interaction effects of species and temperature on biomass, nodule number, nodule mass and estimated SNF.

Species		**Biomass (mg)**	**Nodule number**	**Nodule mass (mg)**	**SNF (mg/plant)**
	Temperature	-----------------------Mean (within species)	------------------------		
Crimson Clover	5	6.2 ± 0.30 c[1]	0.01 ± 0.08 c	7.8e-5 ± 5.0e-3 c	NA
	10	8.9 ± 0.80 c	3.9 ± 1.3 b	0.2 ± 8.9e-2 b	0.02 ± 0.02 a
	15	19.5 ± 8.3 b	8.1 ± 1.4 ab	0.7 ± 0.2 ab	0.9 ± 0.4 a
	20	46.4 ± 32.2 a	13.9 ± 3.3 a	2.2 ± 0.6 a	1.8 ± 1.3 a
Austrian Winter Pea	5	9.5 ± 1.9 c	0 ± 0 b	0 ± 0 c	NA
	10	71.9 ± 12.4 b	8.3 ± 2.1 a	1.5 ± 0.5 b	0.1 ± 0.3 b
	15	117.4 ± 23.5 b	11.9 ± 2.0 a	6.3 ± 1.1 a	3.7 ± 1.1 a
	20	223.5 ± 31.7 a	11.3 ± 1.5 a	10.2 ± 1.4 a	7.1 ± 1.1 a
Hairy Vetch	5	7.2 ± 0.61 c	0.02 ± 0.05 b	3.03e-4 ± 4.2e-3 c	NA
	10	42.0 ± 14.9 b	13.1 ± 3.9 a	2.5 ± 0.9 b	1.3 ± 0.5 b
	15	92.7 ± 13.2 ab	16.8 ± 3.3 a	6.8 ± 0.7 ab	5.3 ± 0.9 ab
	20	167.7 ± 16.8 a	26.1 ± 3.1 a	9.4 ± 0.6 a	7.7 ± 0.8 a

[1] Letters indicate significant differences between means within species. Means were separated by Tukey's HSD. Errors displayed are 1 standard error.

Table 2.3 Main effects of species and temperature on percent nitrogen and pink nodule number.

		Percent N	**Pink Nodule Number**
Main Effect Treatments		------------------------Mean	-------------------------------
Temperature	5	4.9 ± 0.4 a[1]	0.0 ± 0.0 b
	10	2.9 ± 0.2 b	5.9 ± 1.5 ab
	15	4.6 ± 0.2 a	10.7 ± 1.6 a
	20	4.0 ± 0.2 ab	14.4 ± 1.7 a
Species	Crimson Clover	3.4 ± 0.2 b	5.4 ± 1.3 b
	Austrian Winter Pea	4.0 ± 0.3 b	6.3 ± 1.1 b
	Hairy Vetch	4.8 ± 0.2 a	11.6 ± 1.9 a

[1] Letters indicate significant differences between means within species. Means were separated by Tukey's HSD.

Figures

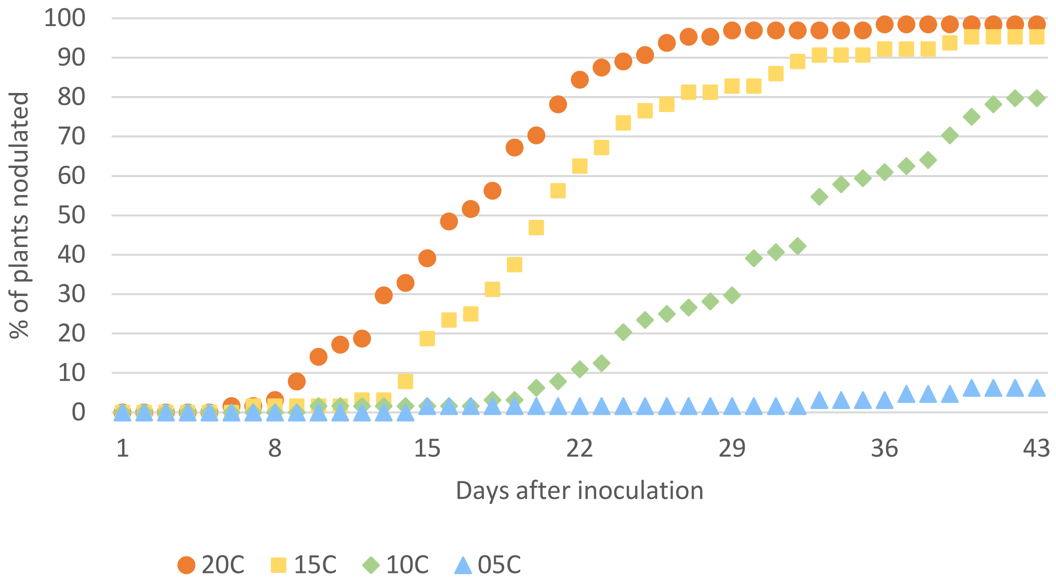

Figure 2.1. Percent of nodulated plants in each growth chamber per day at four temperatures.

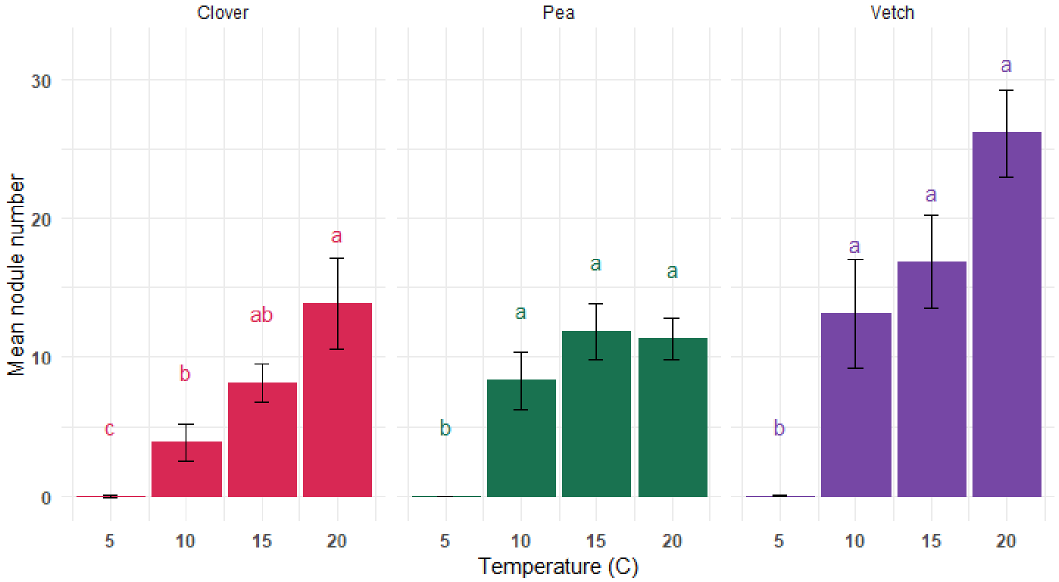

Figure 2.2. Nodule number per plant across four temperatures. Bars indicate treatment means. Error bars indicate +/- one standard error. Means sharing a letter are not significantly different within species (Tukey's HSD comparison, p<0.05).

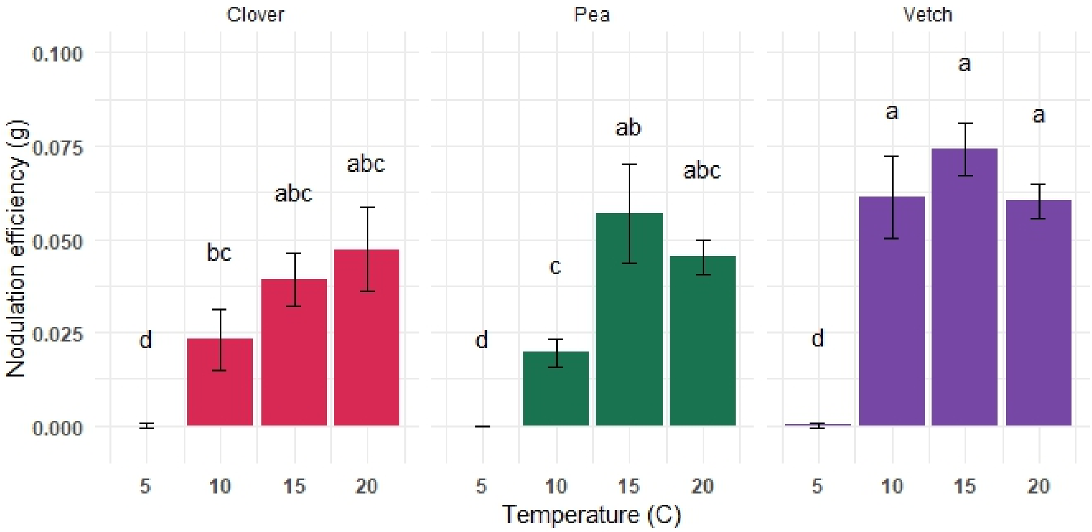

Figure 2.3. Nodulation efficiency (g nodule mass per g shoot biomass) compared among three species at four temperatures. Bars indicate treatment means. Error bars indicate +/- one standard error. Means sharing a letter are not significantly different (Tukey's HSD, p<0.05).

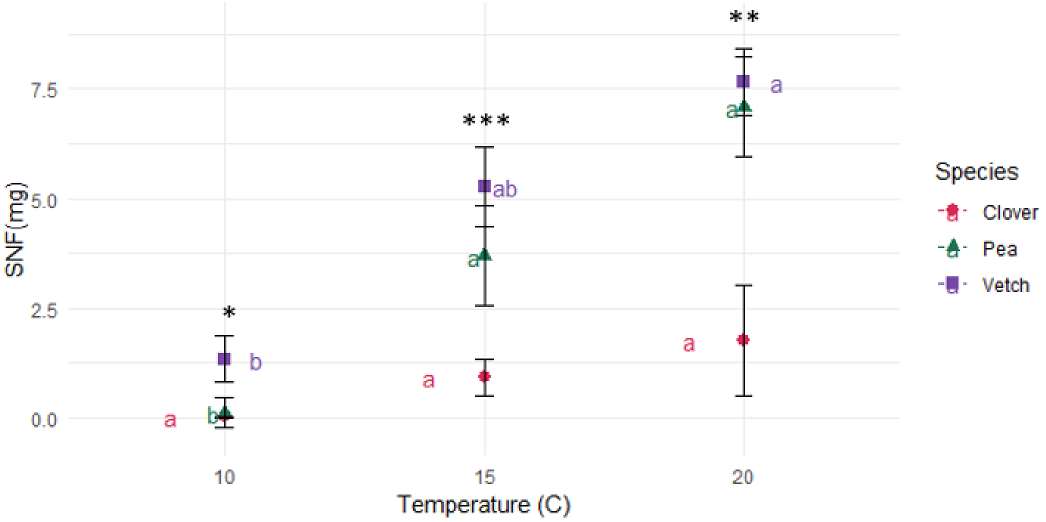

Figure 2.4. Mean estimated SNF in mg N for each of three species at three temperatures. Error bars indicate +/- one standard error. Means sharing a letter are not significantly different within species (Tukey's HSD comparison, p<0.05). Stars indicate significant differences between species within temperature treatment (Tukey's HSD, p<0.05).

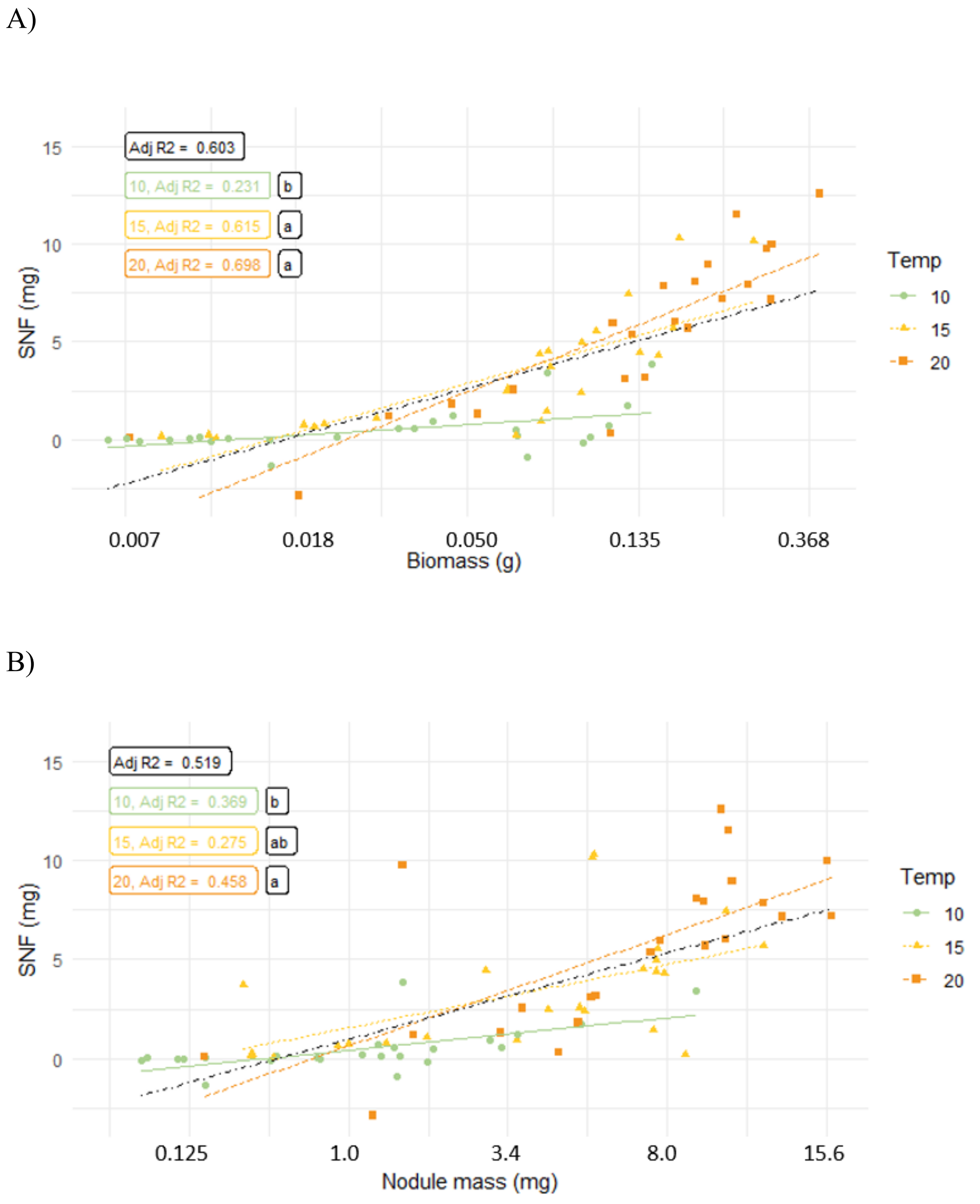

Figure 2.5. Relationships of SNF to shoot biomass and to nodule mass. Plots A shows linear regressions between SNF and shoot biomass overall (dot-dash line) and for three temperatures. Plot B shows linear regressions between SNF and nodule mass overall (dot-dashed line) and for three temperatures. Both shoot biomass and nodule mass were transformed to meet assumptions of normalcy. Adjusted R^2 values are reported for each regression, letters represent significant differences between slopes, α = 0.05.

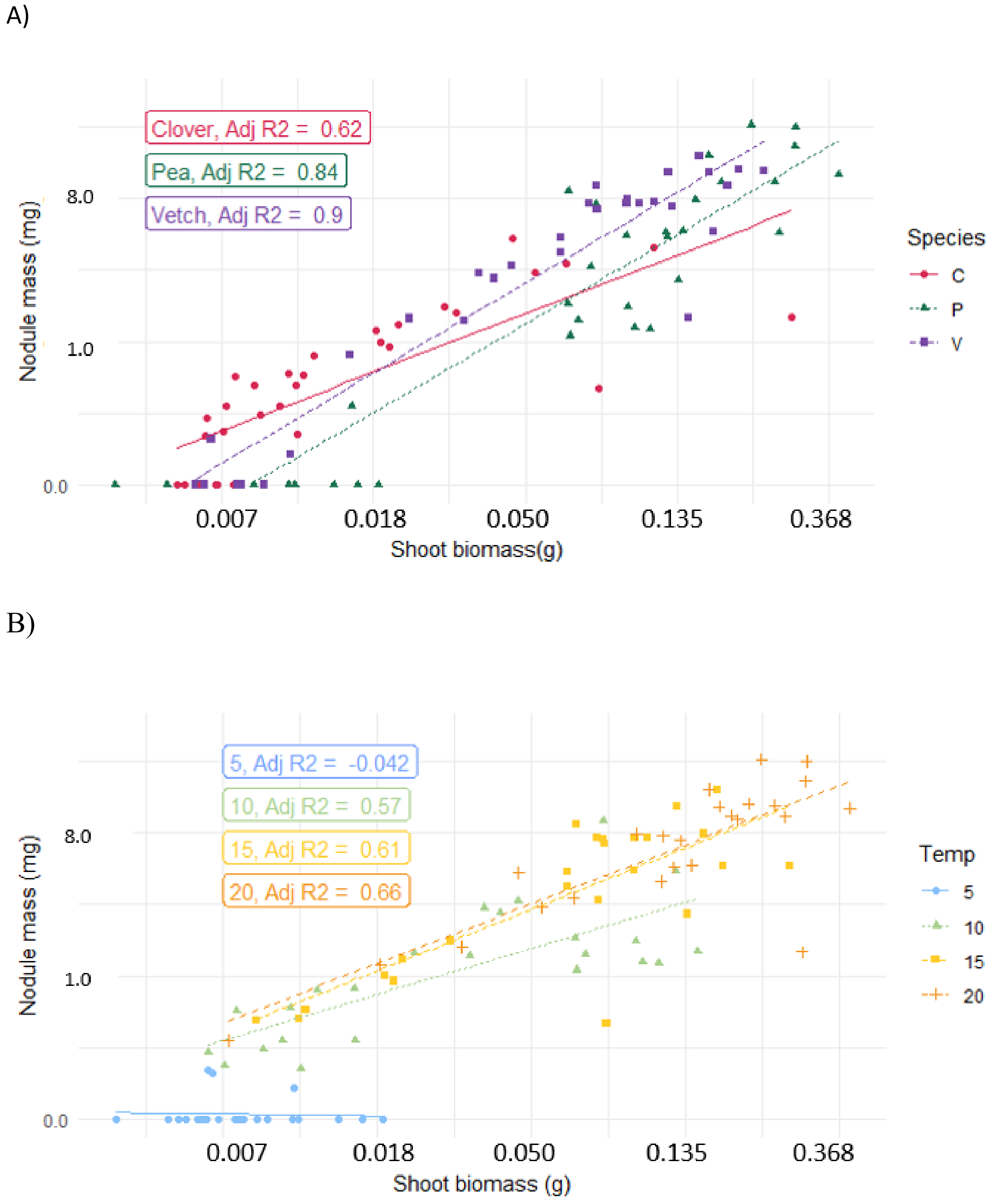

Figure 2.6. Relationship of nodule mass (g) to shoot biomass (g). Linear regressions of this relationship are shown for three species (A), and four temperatures (B). Variables are transformed to meet assumptions of normality for linear regression. Adjusted R^2 values are reported for each regression line.

Chapter 3: Symbiotic nitrogen fixation and productivity of winter annual legume cover crops in high tunnel environments

Introduction

High tunnels are semi-permanent, protective, walk-in structures commonly used on farms for season extension. Though they may serve a purpose similar to greenhouses, high tunnel crops are grown directly in the ground and heated only by passive solar radiation (Lamont 2005). Usually composed of clear plastic covering a rigid frame, high tunnels provide crops warmer day temperatures, slightly warmer night temperatures, and protection from wind during sensitive periods of the year when outdoor conditions may prohibit growth (Everhart et al. 2010). In cold climatic zones such as the Upper Midwest, farmers may especially benefit from high tunnel season extension potential (Yao & Rosen 2011).

Due to large initial costs for high tunnel construction, they are generally used for intensive high value crop production in order to maximize the use of the protected space (Everhart et al. 2010; Bruce & Farmer 2017). In the U.S., small-acreage organic fruit and vegetable farmers are a major demographic who build and use high tunnels, as they are more likely to rely on income from small-scale specialty crops and benefit from premium prices for off-season produce (Dorais & Cull 2017; Carey et al. 2009).

Prolonged intensive cultivation in high tunnels may lead to depletion of soil nutrients and reduced yields (Lecompte 2012). To maintain productivity, organic farmers often add nutrient-dense inputs, including manure and compost (Reeve & Drost 2012). While these inputs can replace some lost nutrients, they may also lead to excess phosphorus and salts in tunnel soils due to lack of rain to flush ions through the soil profile (Knewtson et al. 2012). Nutrient imbalances and excesses may exacerbate plant and soil health problems and further reduce crop yields (Gluck & Hanson 2013). Similarly, soil structural issues like compaction are not alleviated by these inputs (Montri & Biernbaum 2009). Legume cover crops can be an *in situ* source of soil fertility; replacing nitrogen fertilizers in high tunnel systems through symbiotic nitrogen fixation (SNF) without adding to nutrient imbalance (Muchanga et al. 2017). Recent studies have shown that overwintered legumes can contribute nitrogen to summer cash crops in high tunnels, as well as offer other soil health improvements like increased soil carbon and microbial activity (Perkus 2018; Rudisill et al. 2015). More studies on overwintered cover crops are needed, as many high tunnel cover cropping studies in temperate climates only utilize cover crops planted in the early spring (Araki et al. 2009; Muchanga et al. 2017; Nair et al. 2014).

Understanding the seasonal change in nodulation and SNF of overwintered high tunnel legumes may help to predict fertility contributions the following spring. In zones like the Upper Midwest, average ambient winter temperatures typically remain below freezing. Even in the warmer conditions of a high tunnel, overwintering plants are challenged by extreme diurnal temperature fluctuations within the semi-insulated environment (Ward & Bomford 2013; NOAA 2019). Maximum daily temperatures of 20°C (68°F) and minimums of -24°C (-11°F) during mid-winter have little-known effects on plant growth, and may halt or reduce SNF, leading to lower N contributions to following cash crops (Robin et al. 2005; Ofosu-Budu et al. 1992). Studies of common cover crop legumes in Upper Midwest high tunnels during the winter annual season may lead to informed recommendations of ideal legume species for high tunnel production, as well as a greater understanding of how SNF in these species responds to extreme temperatures. However, no studies have closely examined the performance of nitrogen fixing legumes throughout the season in these environments.

The aim of this study was to build further knowledge about cover cropping practices, winter-annual legumes, and SNF in high tunnel environments. We expected that warm day temperatures in high tunnels over the winter would allow legume cover crops a longer active period during the winter-annual season and increase SNF capacity, leading to greater accumulation of fixed N in tunnels, despite the potential stress of high diurnal temperature fluctuation. We also expected that less cold-tolerant legumes might survive the winter in high tunnels in regions where they would not be expected to survive in the field. Our objectives were to quantify 1) legume cover crop productivity, 2) nodulation, and 3) SNF throughout the winter-annual season in Upper Midwest high tunnels to better understand legume cover crop performance and potential nitrogen delivery in these protected environments and build knowledge of high tunnel cover cropping for widespread use by growers.

Materials and Methods

Experimental design

This study was conducted in high tunnels located at five sites including Grand Rapids, Saint Paul, and Rosemount in Minnesota, and Turtle Lake and Osceola in Wisconsin. The sites spanned three plant-hardiness zones, from 4a to 3a. The study was designed as a Mother and Baby trial, an approach used to foster greater efficacy of on-farm participatory research (Snapp, 1999). Two mother sites were replicated in a randomized complete block design, including the North Central Research and Outreach Center in Grand Rapids, MN and the Horst-Rechlebacher farm in Osceola, WI. Three additional sites were unreplicated baby sites, each located on working farms in high tunnels (Table 3.1).

Site preparation

In late August and early September 2017 high tunnels were either tilled with a walk-behind rototiller (Horst, HAFA, Cala), or hoed and raked (GR, PF) to create a uniform seed bed. At baby sites, plots were arranged according to tunnel size and farmer space availability (Table 3.1). Baseline soil samples were collected prior to cover crop planting using hand-held soil probes. Ten cores were collected to 15 cm soil depth per plot in each tunnel. Aggregate samples of these soils were sent to the soil testing lab at the University of Minnesota for analysis. Un-aggregated soil samples from fall sampling at GR and Horst were also analyzed for total Extractable N using 1M KCl extraction.

Cover crop treatments

The two mother trials had 3 (GR) or 4 (Horst) replications of four cover crop treatments: a monoculture of crimson clover (*Trifolium incaratum,* CC), a monoculture of red clover (*Trifolium pratense,* RC), a mix of tillage radish, winter rye and hairy vetch (*Raphanus sativus, Secale cereale, Vicia villosa,* VM), and a mix of winter rye and

Austrian winter pea (*S. cereale, Pisum sativum,* PM). The three baby trials had a single replication of three cover crop treatments: RC, VM, and PM.

Inoculation and seeding

Prior to planting, legume seeds were inoculated with species-appropriate peat inoculant containing compatible rhizobia (N-dure, Verdesian Life Sciences, Cary, NC). Seeds were lightly coated in a 25% sucrose solution, inoculant added according to manufacturer specifications (0.011oz inoculant per 100g seed), mixed thoroughly with inoculant, and air-dried on paper-lined trays for 24 hours. Mixes were weighed and combined pre-inoculation. See Table 3.2 for seeding rates, sources, and rhizobia inoculant species. All seeds purchased were certified organic.

Seeds were broadcast at recommended seeding rates and plots raked to ensure seed-to-soil contact and burial. Plots were initially watered with overhead irrigation or with a gentle hose nozzle stream to at least 15 cm damp soil depth. Seeds were watered daily until emergence, and then as-needed until late October/early November. Plots were heavily watered immediately prior to consistent freezing temperatures, or until farmers turned off water sources. Beds were covered in floating row-cover (Agribon, J & M Industries Inc., Ponchatoula, LA) supported by low wire hoops, and tunnel sides and doors closed. Heavy watering, row cover, and tunnel closure were efforts to prevent desiccation and frost damage to cover crops.

Legume biomass and nodule sampling

Stand counts were taken in 0.10 m^2 quadrats at all sites at two to three weeks after the seeding date in September 2017. Early stand establishment and weed pressure were assessed by counting individual cover crop and weed seedlings within quadrats. Cover crop seedlings were differentiated by species, weeds seedlings were counted collectively.

Destructive sampling for roots, shoots, and nodules was performed at four seasonal time-points to determine impact of winter high tunnel conditions on stand persistence and SNF parameters. The first sampling took place in November 2017, after approximately two months of cover crop growth, and was followed by subsequent samplings in January 2018, March 2018, and at cover crop termination in late April or early May 2018. Four legume plants from each plot were randomly harvested at each time-point, data was collected on individual plants and the mean used for analysis. Roots were dug using weeding knives, loosening roots 10 cm from the plant stem and to 10 cm depth and carefully excavating with surrounding soil. During January and March sampling times, deeper soil layers were frozen. Roots were collected just down to the frozen layer. Only the living, green tissue of legume shoots was collected for sampling. Legume roots were separated from shoots and placed in plastic bags in a 4°C cooler until nodules could be counted and removed. Shoots were dried in a 120°C oven for 48 hours to constant weight, measured, and then recorded. Roots were removed from cold storage and washed; nodules were then counted, removed, and dried in a 60°C oven for 48 hours. Nodule dry mass was measured with a fine scale (Sartorius Cubis MSE, Goettingen, Germany) and recorded. Fresh nodules were observed for signs of active SNF, and were scored as “pink” (actively fixing N) with a binary score of yes (1) or no (0). Following nodule removal, roots were also dried for 48 hours in a 120°C oven and mass measured and recorded.

Sampling and preparation for SNF quantification

Legume biomass collected at each sampling time-point for biomass quantification was also used in SNF analysis. Simultaneous to legume sampling, above-ground tissue of adjacent winter rye was collected as reference plant material, also for SNF analysis. Winter rye was selected as a reference because it was a non-legume present at all sites and persisting throughout the winter months. Like legume biomass, reference plant biomass was dried in paper bags at 120°C for 48 hours and weighed. Dried shoot biomass of legumes and references was ground to a fine powder (< 0.5 mm) with a Geno/Grinder tissue homogenizer (SPEX Sample Prep, Metuchen, NJ). Ground plant material from each sampling time-point and each plot at both mother sites was weighed on a microscale into 10 mg subsamples and analyzed using an Isotope Ratio Mass Spectrometer (IRMS) to obtain delta ^{15}N values and N content. IRMS analysis was performed in the UC Davis Stable Isotope Facility (UC Davis Department of Plant Sciences, Davis, CA).

Stand measurement at termination

In addition to collecting legume roots and shoots, two measurements of stand biomass per plot were collected in 0.10 m^2 quadrats at the final sampling time-point in April/May 2018 prior to termination. Above-ground biomass of all plants within the quadrat was cut at ~1 cm above the soil surface and separated into weeds and cover crop species as required for each treatment. Biomass was dried for 48 hours in a 120°C oven, weighed, and mass recorded in order to quantify relative cover crop species and weed abundance at termination.

Environmental data

Air temperature (Celsius) and light (Lux) were collected every 30 minutes by four LabJack Digit temperature sensors (LabJack Corp, Lakewood, CO), hung from hoops to 25-30 cm from the soil surface at different points within the high tunnels. At Grand Rapids, an additional Decagon temperature sensor was also used to collect both air and soil temperature (Celsius) at 15 cm depth (Decagon Devices Inc., Pullman, WA). The Decagon sensor was un-replicated. Labjack temperature data was used to calculate site Growing Degree Days (GDD), with 4°C used as a base temperature.

Quantifying symbiotic nitrogen fixation

Symbiotic nitrogen fixation (SNF) was estimated using the Natural Abundance method (Unkovich et al. 2008), referring to the natural abundance of two nitrogen isotopes in the environment. The calculated amount of atmospheric nitrogen fixed by a legume is defined as nitrogen derived from the atmosphere (Ndfa). Percent Ndfa was calculated in this experiment using the following equation:

$$\mathrm{Ndfa}\ (\%) = \frac{\delta^{15}\mathrm{N}_{\mathrm{ref}} - \delta^{15}\mathrm{N}_{\mathrm{leg}}}{\delta^{15}\mathrm{N}_{\mathrm{ref}} - B} \times 100 \qquad [1]$$

Where the delta ^{15}N refers to the difference in ^{15}N atoms in the plant tissue in relation to the atmosphere, and the B value represents the difference in ^{15}N in just the shoots of the legume being measured. The B value accounts for isotopic fractionation within the plant, adjusting %Ndfa to account for the use of solely shoot tissue to make these calculations. B values are determined by growing a legume with only atmospheric N as an N source (through SNF) and then measuring the isotopic ratio of that plant. B values used here were species specific and drawn from established SNF literature as well as experiments performed at the University of Minnesota (Table 3.4).

Statistical analysis

Analysis was performed using R statistical software (R Core Team, 2018). Mother sites were analyzed separately from baby sites using a *lmer* mixed effects model with sampling time-point and cover crop species as fixed effects, and site and block (replication within site) as random effects. Dependent variables were tested for normality and square root or log transformed where appropriate. Means were back-transformed for tables and reported results. Interactions between sampling time-point, high tunnel site, and species indicated that sites and species could not always be pooled and compared across time-points, so analyses were separated by site and by cover crop species to compare the response of single treatments over time (response of variables to seasonal change). Means were separated using least squared means and comparisons were adjusted using Tukey's HSD ($p < 0.05$). Baby sites were analyzed separately for trends and patterns of seasonal change. Due to site-wise interactions and lack of within-site replication, these sites could not be analyzed with the mother sites and differences could not be determined as statistically significant. Temperature was a replicated measurement and was compared among baby sites, but other variables lacking replication were only compared within site. Due to late fall planting and subsequently no cover crop growth until late spring, data from the Cala farm site (Turtle Lake, WI) is reported as observational data only.

Results

Replicated mother sites

The mother sites, at the North Central Research and Outreach Center in Grand Rapids, MN and at the Horst-Rechelbacher Farm in Osceola, WI, were analyzed together. These two sites were pooled for all statistical tests unless analysis showed them to be different and warrant separate analyses. Legume biomass and percent Ndfa differed across sites and thus were analyzed separately for these variables. Results from baseline soil tests for all sites are reported in Table 3.3, while results of fall 2017 KCl Extractable N analysis for mother sites soils are reported in Table 3.5. Of these analyses, Extractable N is highlighted due to its importance in driving overall SNF, where Horst had greater overall Extractable N than GR.

Seasonal temperature fluctuations in high tunnels

Temperature fluctuation in high tunnels at both sites was large, even during the shortest days of winter. During the month of December at Grand Rapids, daily high temperatures were consistently 14°C greater than low temperatures. The overall highest temperature in the tunnel at Grand Rapids was 58°C in late April, the overall low was -24°C in early January. The greatest daily temperature fluctuations occurred during April, where diurnal highs were 48°C warmer than lows. Growing Degree Days (GDD) accumulated most from late August to mid-October, and then again in late March to termination in May (Figure 3.1). See Table 3.6 for Cumulative GDD at each sampling time-point.

Mean daily temperatures at Grand Rapids were lower than those at Horst. However, there was an interaction between month and site (Figure 3.3). When separated by site and month, the two tunnels only differed in temperature in November, December, January, and February, where Horst had a warmer mean daily temperature than GR (Figure 3.3).

Temperature differences were also large at Horst. The overall highest temperature in the tunnel at Horst was 43°C in late September; the overall low was -19°C in early January. However, contrary to GR, February was the month with the greatest diurnal temperature fluctuations at Horst, with highs 32°C greater than lows. GDDs accumulated most from late August to mid-October, and then again in April until termination in early May (Figure 3.2). See Table 3.6 for Cumulative GDD at each sampling time-point.

Seasonal changes in legume cover crop biomass and survival

Considering all species treatments across both sites, legume biomass was lower at TP 3 (March) than TP 1 (November), 2 (January), and 4 (May). However, interactions led to separate analyses by site and by species to isolate effects of seasonal change on legume biomass over the winter annual growing season (Table 3.7). Legume biomass at each site, separated by species, is reported in Figure 3.4.

Overall at GR red clover and hairy vetch followed a similar pattern of greater biomass both in the late fall and late spring, while Austrian winter pea and crimson clover had greater biomass in the late fall and early winter, but less in the spring. At Horst, crimson clover biomass was stable throughout the season, and some winter peas survived until termination (Figure 3.4).

Final cover crop biomass abundance

Across all species and at both sites, mixes produced statistically indistinguishable quantities of total biomass (Table 3.8), with also no differences between weed and legume biomass for any mix. When rye and legume biomass were compared for PM and VM, rye had greater biomass than the legume in both cases. In VM, recently winter-killed radish biomass was collected. Dead radish biomass was the same as rye biomass, but both were greater than legume and weed biomass in all VM plots (Table 3.8).

Seasonal changes in nodulation

Effects of TP and legume species on nodule number and nodule mass are reported in Table 3.9. Analysis of nodule number per plant for cover crop legumes over the season showed significant effects of TP and species. Nodule number decreased over time and then appeared to recover in spring. When analyzed over all sampling TPs, red clover had the greatest nodule number. When all legume species were combined, this large difference between red clover and the other legume nodule numbers masked potential interactions in statistical analysis (Table 3.7).

Similar to nodule number, analysis of nodule mass on a per plant basis revealed significant effects of TP and species (Table 3.7). All species had greater nodule mass later in the season (Table 3.9). Over all TPs, Austrian winter pea had the greatest nodule mass, and crimson clover had the least.

Pink nodule occurrence, or the proportion of plots with pink nodules, is reported in Table 3.10. Pink nodules occurred more frequently in the spring and fall than the winter, but were present at all sampling time-points for both sites.

Seasonal changes in symbiotic nitrogen fixation

Symbiotic nitrogen fixation was measured as percent nitrogen derived from the atmosphere (%Ndfa) using the Natural Abundance approach. Analysis of %Ndfa showed significant effects of sampling time, species, and site (Table 3.7). Because %Ndfa differed between sites, GR and Horst data were analyzed separately and in these analyses species did not differ in %Ndfa. Effects of sampling time on %Ndfa for each site are reported in Figure 3.5. At GR, there was high above-ground plant mortality for both crimson clover and Austrian winter pea at TP 3, and high mortality of Austrian winter pea at TP 4. Grand Rapids % Ndfa was compared among TP 1, 2 and 4, and only among crimson clover, red clover, and hairy vetch. At GR, SNF was the same at November and January sampling, 56.9 and 65.8 %Ndfa, respectively. By May and termination, SNF had dropped to 27.9 %Ndfa. At Horst, SNF at November sampling was greater than SNF in January and March. By May, SNF was indistinguishable from all earlier samplings, between the low of 9 %Ndfa in winter and high of 33 %Ndfa in November. Horst had lower SNF than GR at all TP (Figure 3.5).

Baby sites

Unreplicated baby site cover crop data are reported as observational data, and some seasonal differences are explored within site across the level of species (Table 3.11). Cover crops at the baby sites served important non-quantitative purposes as farmer-to-farmer learning and demonstration sites during on-farm workshops. Temperature data was replicated, and temperature difference between sites were analyzed.

Temperature differed by site

Temperature trends differed between the sites and between months of the year (Figure 3.6). Site comparisons were made across all three baby sites for the months of November through April, when each site had living plants. During this period, HAFA had the highest temperatures overall, followed by Cala and then PF. In remaining months, temperature was compared between HAFA and PF (no plants were present at Cala), where HAFA had greater mean daily temperatures than PF, except during August. In August, only a few days of the month were actually measured, possibly leading to more similar means.

Observed trends by site

Page and Flowers farm

Temperatures were lower at PF and there were fewer GDDs for the cover crops within this tunnel than the others (Table 3.1). However, PF was also the only baby site where Austrian winter pea survived until spring. Biomass does not tends to differ by sampling time, but trends suggest that nodule number and nodule mass were both greater in the late spring than in the fall and winter. Nitrogen derived from the atmosphere across legume species at termination was 64.5% (Table 3.11).

HAFA farm

Temperature and GDD trends at HAFA indicate that this tunnel was hotter than the others during the late winter and early spring (Table 3.6, Figure 3.6). Despite higher temperatures and amassed GDDs in the tunnel, none of the Austrian winter peas in the PM mix survived the winter. There were no observed differences between sampling times for the biomass, nodule number, or nodule mass. In nodule number especially, high variability is apparent within each sampling time point. Percent Nitrogen derived from the atmosphere across all legume species at termination was 80.9% (Table 3.11).

Cala farm

The cover crop trial at Cala farm was seeded in early November due to very late-season use of the tunnel by the farmer. Cala was cooler overall than HAFA, and warmer than PF (Figure 3.6). The seeds broadcast in this trial did not germinate until late winter/early spring, and cover crop mixes had very little biomass until late March/early April. Upon termination on May 8th, there was an average legume biomass per plant of 2.5 g, 15.3 nodules per root, 2.3 mg of nodule mass per root, and 8.8 %Ndfa (Table 3.11).

Discussion

Legume biomass

Winter had a dramatic effect on legume productivity in high tunnels, with legume biomass overall greater in the fall and late spring than during the winter. At the northernmost site in Grand Rapids, most species had highest biomass in the fall, and then dropped to their lowest biomass in March, mimicking growth patterns of winter annual plants, with a winter dormant period when a portion or all aboveground tissue may senesce (Brandsaeter & Netland 1999). At Horst, in a warmer climatic zone and overall warmer tunnel, there was more biomass remaining in January and March for all species. Very low temperatures in high tunnels are known to inhibit growth of winter annual crops (Orde et al. 2018; Martin & Sideman 2012), therefore the colder minimum and mean temperatures in Grand Rapids during December, January, and February possibly inhibited biomass production more than at Horst. At the unreplicated baby sites, trends in legume biomass were similar to the mother sites, with low biomass at the March sampling, following the coldest winter period in the tunnels. Legume biomass also tended to be greater at the HAFA high tunnel in Rosemount, which had greater GDD accumulation and therefore overall warmer temperatures to support cover crop growth than PF in St. Paul or Cala in Turtle Lake.

While all evaluated species are known to be winter-hardy in various climatic conditions, this study found that Austrian winter pea and crimson clover were less productive and hardy in our high tunnels than red clover and hairy vetch. By termination in May, red clover and hairy vetch biomass regrew with warming temperatures, while Austrian winter pea had not. Pea may not have shown the winter-hardiness seen in other research (Markarian et al. 1968; Young-Mathews 2017) due to shallow seeding; other studies found better pea establishment in the open field when sown at depths of 25-75 mm (1-3 in) (Vann et al. 2018) to protect the growing points of the pea shoot and maintain moisture levels (Chen et al. 2006). Crimson clover had spring biomass equal to fall at Horst, but did not regrow to fall levels at Grand Rapids, suggesting that poor cold tolerance limits crimson clover as a high tunnel winter annual, but only in very cold zones (Young-Mathews 2013). Crimson clover is also slow to establish and possibly did not develop necessary root mass at Grand Rapids to drive spring re-growth following winter loss of aboveground biomass due to lower cold tolerance (Snapp et al. 2005; Curran et al. 2018). Overall, we observed biomass production in red clover and hairy vetch that suggest they are well suited to over-winter high tunnel growth, with greater cold tolerance and positive growth response to spring warming compared to other species, consistent with studies in temperate climates (Brandsaeter & Netland 1999; Teasdale et al. 2004; Perkus 2018).

We expected that cover crop survival and maintenance of plant biomass in high tunnels over the winter would be additionally challenging due to the large temperature fluctuations that are present compared to the open field. Our temperature data show wide fluctuations at Grand Rapids and Horst throughout the cover crop winter annual season. Warm temperatures in the midst of a cold period have been found to trigger plants to break dormancy and reverse cold-acclimation processes, re-purposing soluble tissue sugars that are used to protect cells from freezing (Johansson et al. 2015). Loss of acclimation and cryoprotectans due to frequent above-freezing day temperatures in December, January, and February in our high tunnels could leave plants vulnerable to the subsequent below-freezing night temperatures (Hekneby et al. 2006), which may have contributed to the mortality we saw in winter pea. Studies of perennial plants found some species and even cultivars may be more likely to break dormancy, even during short periods of warmth (Liu et al. 2015).

Spring biomass of plots at termination

When plot biomass was quantified at termination, our results suggested that winter annual cover crops in high tunnels produced more spring biomass earlier than the same crops in the field. Our high tunnel cover crop biomass at Grand Rapids in mid-May was comparable or greater than biomass measured in the field in early summer at the same site. We found over 2500 kg ha^{-1} of combined vetch and rye biomass in the VM mix and 123 kg ha^{-1} red clover, while only 1950 kg ha^{-1} vetch/rye mix and 80 kg ha^{-1} red clover under field conditions was quantified by Liebman et al. (2019). In high tunnels in warmer zones such as St. Paul and Rosemount, similar or greater biomass could be produced as early as April, but unreplicated results bar comparison.

At the mother sites, we observed that high tunnel conditions equally favored spring biomass production in legume monocultures and in mixes of legumes with non-legume species. There were no differences in spring biomass per area (kg ha^{-1}) at the time of termination in May between any treatments in our study. These results contrast with field studies (Liebman et al. 2019; Blesh 2018) that found lower biomass in legume monocultures than in mixes of legumes with cereals or brassicas. Though mixes of cover crop species can potentially contribute a greater diversity of ecosystem services to agricultural systems (Blesh 2018), mixes of cover crop legumes with other non-legume species can lead to lower legume biomass than legumes in monoculture due to greater competitive ability and fast growth of cereals and brassicas (Jannink et al. 1997; Baraibar et al. 2018). Mixes can therefore reduce N provision of legumes by reducing legume biomass, but could fulfill other functions such as increasing soil organic matter or alleviating compaction, which may be preferable in tunnels that need no extra N (Snapp et al. 2005; Lawson et al. 2015). Using cover crop mixes in our study may have influenced our biomass results for pea and vetch, reducing the individual biomass and biomass in kg ha^{-1} of these legumes through competition from other species. Indeed, winter rye in both the pea-rye mix and the vetch-rye-radish mix was the dominant species in those plots, consistent with other field studies (Silva 2014). We also observed cover crop biomass equal or greater than that of weeds, suggesting some weed suppression by all cover crop treatments did occur, another important function of winter annual cover crops (Alonso-Ayuso et al. 2018; Blesh 2018).

Similar to the mother sites, there were no discernable differences at termination in total kg ha^{-1} biomass among the mixes at the unreplicated baby sites, suggesting that the overall treatments performed equally well at these different high tunnels. Total cover crop biomass across mixes at termination tended to be greater at PF than at Cala and HAFA, different than the trends in individual legume biomass, which suggests that non-legume biomass may have been responding positively to factors like soil fertility rather than temperature and GDD in the PF tunnel. Similar to the mother sites, winter rye biomass tended to comprise the majority of the cover crop biomass at all sites, and weeds, though present, did not overwhelm most of the treatments in terms of biomass.

Nodulation

We observed that winter conditions in high tunnels dramatically affected nodulation. Winter measurements of field nodulation are rarely captured due to frozen soil and snow (Bergersen et al. 1963; Gurusamy et al. 2000), and high tunnel conditions offered a unique perspective of winter impacts on nitrogen fixation and nodulation. Nodule number was greatest in the fall and at spring termination, and lowest at the coldest sampling time-point (January). Our findings align with other studies that found nodule initiation and development are limited by cold temperatures, and that nodules also may die and slough off as plants age and new nodules are initiated (Zhang et al. 1995; Abd-alla & Issa 2014; Prévost et al. 2003). Similar to nodule number, nodule mass was greatest in spring and lowest in January, agreeing with literature supporting nodule sensitivity to cold temperatures (Lindemann & Ham 1979; Rice et al. 1995). For legumes, flowering and seed development require high levels of nitrogen, and so warm temperatures plus N demand may have also driven greater nodule numbers and nodule mass in spring (Marrou et al. 2017). Despite winter reduction of nodulation, non-frozen and active pink nodules were observed at all sampling time-points at both sites, including January. Nodules appear pink due to the leghemoglobin protein, which is present in effective, active nodules (Dakora 1995). Pink nodule occurrence suggests that in these high tunnels cover crop nodules remained active, even during the cold period of mid-winter, where mean daily temperatures were -5.5°C at Grand Rapids and 0°C at Horst. These observations contrast with older field and growth chamber studies that found nodule tissue even of cold-hardy legumes will senesce or become dormant during winters with average temperatures of 0°C or prolonged periods with night temperatures of -2°C

(Frankow-Lindberg 2001; Gurusamy et al. 2000; Bergersen et al. 1963). Our findings suggest that high tunnel conditions, with day temperatures rising above freezing even in December and January, may allow nodules to remain more active during winter months than their field-grown counterparts. Quantification of actual enzymatic activity in pink nodules in these species over the winter is required to validate our observations in this study.

At the baby sites, nodulation patterns were similar to the mother sites, with trends of greater nodule number and mass both in the fall and at spring termination, although greatest nodule mass occurred at different sampling times for each site. We also observed trends of greater overall nodule numbers and nodule mass at HAFA than at PF and Cala, which, similar to biomass trends, was probably due to the warmer overall tunnel temperatures at the southern Minnesota HAFA site.

Symbiotic nitrogen fixation

Symbiotic nitrogen fixation was found to decrease over the winter months, and did not fully recover by the time cover crops were terminated and incorporated in May. Overall, we observed lower SNF values at Horst than Grand Rapids, possibly due to greater concentration of initial soil N at Horst. High levels of plant-available soil nitrogen, especially nitrate, are known to reduce SNF (Voisin et al. 2002; Jones & Olson-rutz 2018; Ferguson et al. 2018), which is consistent with our observations of lower SNF values at Horst. SNF is very energy intensive and if plants, especially stressed plants, can satisfy their N demands with soil N, they will down-regulate nodulation and SNF in the nodules (Silsbury 1977; Carlsson & Huss-Danell 2003; Lim et al. 2014). Interestingly, Grand Rapids and Horst did not differ in measurements of legume nodulation success, nodule number and nodule mass were similar between sites at all samplings and for each species. Therefore, down-regulation of nodulation likely contributed less to the differences in SNF between Horst and Grand Rapids. Instead there may have been down-regulation of nodule activity following nodulation due to soil N availability (Voisin et al. 2002). Our results suggest that similar nodulation levels between high tunnel locations may not also be predictive of similar levels of N provision in these environments.

Though we observed reduced levels of SNF earlier in the season at Horst than GR, both sites had reduced SNF following the winter, not increasing by May termination. Daily mean temperatures in April and May were consistently above 10°C at both sites, but minimum temperatures were still falling below freezing until late April, which may have contributed to the limited spring recovery of SNF we observed. Our observations are consistent with other field studies that found lower levels of SNF in legumes at earlier termination dates versus later termination dates in cool spring climates (Lawson et al. 2015; McCauley et al. 2012). The extreme temperature fluctuations of the early spring we observed in the high tunnels may have also affected SNF levels. Although minimum temperatures in the high tunnel are usually only 2 to 4 degrees greater than those in the field (Ward & Bomford 2013; Jett 2000), we saw maximum temperatures in March and April at GR that were over 30°C greater than maximum field temperatures (NOAA 2019) and diurnal differences within the tunnel of 43-48°C. Levels of SNF were reduced at this site only after this period of intense diurnal temperature fluctuation, which suggests that these variable spring conditions may have compounded the negative effects of cold minimum temperatures to reduce SNF. Research has shown that very high temperatures (35-45°C) curtail SNF in legumes (Ofosu-Budu et al. 1992) and can damage the nitrogenase enzyme, which fixes atmospheric N in nodules (Bhandari et al. 2017), and that increasing temperature fluctuations can affect chlorophyll content and photosynthesis, which could limit plant resource allocation to nodules (Erwin & Heins 1995). Overall our results suggest that both cold night temperatures and large diurnal temperature fluctuations may limit SNF for high tunnel legumes in the spring, but that in late fall and early winter, warm maximum temperatures in the tunnel coupled with less extreme diurnal differences may in some cases extend plant growth and higher levels of SNF into the winter season.

At the baby sites SNF (%Ndfa) was only calculated at termination. We found SNF was lowest at Cala and greater at PF and HAFA. These SNF values at PF and HAFA were also much greater than those at termination at the mother sites; this is conceivable for Horst, which had very high levels of initial soil N that could have inhibited SNF throughout the season. However, GR had the lowest initial N levels of the sites, which suggests that colder mean and minimum temperatures at GR were the limiting factor for SNF compared to HAFA and PF. Low SNF at Cala may be partially attributed to lower nodulation of young plants at this site, but also to soil N fertility. Thought base-line soil N was not excessive at Cala, the farmer later applied 54 kg/ha of N to the tunnel, which is higher than the recommended rate (Sustane Natural Fertilizer Inc. 2016), and preferential legume uptake of applied N likely reduced SNF (Voisin et al. 2002; Silsbury 1977).

Late germination does not hinder spring cover crop growth

At Cala farm, where cover crops were seeded in November and did not germinate until March, we observed substantial biomass at termination, and surprisingly high levels of nodulation and SNF, though these tended to be less than at PF and HAFA. These results are comparable to another Minnesota study of high tunnel cover crops that observed vigorous spring growth following seeding in April (Perkus 2018), which may indicate that an early spring seeding of cover crops in a high tunnel could be beneficial for farmers who have missed the timing for fall-planting.

Conclusion

The results of this study suggest that winter annual legume cover crops can be a viable N-acquisition strategy for high tunnel growers in the Upper Midwest, and that some winter annual legumes are more suitable for overwintering in high tunnels in this region than others. In terms of biomass, winter survival/persistence, and nodulation at replicated Mother sites, red clover and hairy vetch best over-wintered in our high tunnels, and are most likely to do well for Upper Midwest farmers hoping to add legume cover crops into their winter annual rotation. We also found that legumes were crowded out by strong competitors such as rye in species mixes, suggesting that higher seeding rates of hairy vetch and Austrian winter pea may be necessary for farmers interested in these treatments for nitrogen provision.

With the replicated mother sites, high soil N at Horst, and cold temperatures plus large diurnal temperature fluctuation at GR reduced spring SNF from higher levels in the fall. Despite the reduction in SNF over the winter, our results did suggest that legumes fix nitrogen in high tunnels throughout the winter-annual season. Additionally, like cash crops in warmer regions, the active period of cover crop growth extends later into the fall and begins earlier in the spring than for plants in the field. However, temperature fluctuations in high tunnel environments appeared to delay recovery of SNF and, in some species, biomass. Slow spring recovery of SNF suggests farmers should delay termination of winter-annual cover crops as long as rotations allow to increase legume nitrogen contributions.

Lack of differences between sites for nodulation and per-area spring biomass (kg ha^{-1}) were surprising and warrant further study. However, these may be encouraging results for farmers in the coldest zones of the Upper Midwest; if soil tests indicate low soil N and they have a tightly-built high tunnel, they may see cover crop spring growth equal to that of growers in more southerly regions of the Midwest. Additionally, though one of our objectives was to see how overwintered legumes might contribute N to high tunnels through SNF and in many cases we observed low %Ndfa in these legumes, this may actually be an encouraging result. At Horst, residual soil N was high and the legumes planted did not initiate high levels of SNF. This response is beneficial for the high tunnel soil, which needs no additional N, but may need the organic matter, soil biological activity, and moisture retention potentially offered by a legume cover crop.

In summary, we found that legume cover crops may be successfully overwintered in high tunnels in the upper Midwest in mono-cultures or mixes, and that depending on high tunnel conditions, they can continue to accumulate N through SNF through most of the winter months, building capacity as a green manure for farmers in the spring.

Tables

Table 3.1. High tunnel location, climate, regional and spatial metrics

High tunnel location	**Orientation**	**Replications**	**Plot size (m^2)**	**Plant Hardiness zone**	**Average frost free days**	**Soil type**
Grand Rapids (GR)	N-S	3	5.9	3b	118	Shooker sandy loam/ Rosy sandy loam
Osceola (Horst)	N-S	4	15.1	4a	140	Hubbard sandy loam
Saint Paul (PF)	E-W	1	4.5	4b	160	Urban land-Waukegan complex
Rosemount (HAFA)	N-S	1	3.9	4b	144	Wadena loam
Turtle Lake (Cala)	N-S	1	7.4	4a	139	Crystal Lake silt loam

Table 3.2. Seed and rhizobia inoculant data

Seed variety	**Source**	**Seeding rate (kg ha^{-1})**	**Rhizobia species in Inoculant**
Crimson clover	Johnny's Selected Seeds, LOT: 54643	28.0	*Rhizobium leguminosarum* biovar *trifolii*
Medium red clover	Johnny's Selected Seeds, LOT: 16A-32IL	13.5	pre-inoculated (*Rhizobium leguminosarum* biovar *trifolii)*
Austrian winter pea	Albert Lea, LOT: 2017	42.0	*Rhizobium leguminosarum* biovar *viceae*
Winter rye	Johnny's Selected Seeds, LOT: 57277	42.0	N/A
Winter max Mix (Radish, Vetch, Rye)	Albert Lea Seed, LOT: 2017-OCC3	84.1	*Rhizobium leguminosarum* biovar *viceae* (for vetch)

Table 3.3. Results of baseline soil tests for all sites, Fall 2017.

Site	**NO3 (mg kg^{-1})**	**% Organic Matter**	**Bray P (ppm)**	**NH4-K (ppm)**	**Soil water pH**
GR	11.8	3.0	120	311	6.7
Horst	178.7	4.1	85	344	6.9
Cala	21.3	2.6	80	160	6.6
HAFA	11.0	2.6	263	360	6.2
PF	43.1	8.9	105	207	7.0

Table 3.4. B values and source of values for legume species %Ndfa analysis.

Legume Species	**B value used**	**B value source**
Crimson clover	-1.55‰	Blesh 2018
Red clover	-1.65‰	Schipanski and Drinkwater 2011
Austrian winter pea	-0.3‰	Unkovich et al. 2008
Hairy vetch	-0.22‰	Perrone 2018

Table 3.5. Extractable N at Grand Rapids and Horst, Fall 2017.

Site	**Extractable N (mg N kg soil^{-1})**
GR	31.5 ± 2.3 b[1]
Horst	197.9 ± 42.4 a

[1] Means sharing a letter are not significantly different. Means were separated using Tukey's HSD, $\alpha = 0.05$.

Table 3.6. Cumulative GDD for all sites during each period between sampling. For comparison across different sampling dates, estimates were standardized to match the earliest sampling date from each time-point (TP). True cumulative GDD at each site at the time of termination are also reported.

Site	Page and Flowers	HAFA	Cala	Grand Rapids	Horst
TP					
		-------------------- Accumulated GDD -------------------			
1 (Sep1-Nov2)	892.1	994.9	NA[1]	937.3	982.5
2 (Nov2-Jan10)	34.2	223.6	39.0	55.4	167.4
3 (Jan11-Mar15)	68.4	337.7	72.5	179.2	177.8
4 (Mar16-Apr27)	304.9	549.8	404.9	609.2	352.8
Total GDD (Non-standardized)	1417.2	2153.9	720.0	2327.4	1830.0

[1] Not measured due to late cover crop seeding.

Table 3.7. Effect of treatments on response variables at Grand Rapids and Horst sites.

Fixed Effects	Legume Biomass	Nodule Number	Nodule mass	% Ndfa
	--P values	---		
Time-point (TP)	1.27e-09 ***	0.0043 **	0.0059 **	0.019 *
Species	0.0019 **	1.49e-14 ***	2.37e-06 ***	0.028 *
TP x Species	1.33e-10 ***	0.17 ns	0.39 ns	0.93 ns
Random effects				
Site	0.043 *	ns	ns	0.00034 ***
Block	ns	ns	ns	ns

*, **, and *** represents significance of p values at $\alpha = 0.05, 0.01, 0.001$ respectively.

Table 3.8. Estimated biomass kg ha^{-1} of mixes and individual cover crop species at termination (May 2018) for Grand Rapids and Horst sites.

Mix	**All Mixes, Total biomass minus weeds**	**All Mixes, Legumes v. Weeds**		**PM and VM, Leg. V. Rye**		**VM**	
	----------------------------------Biomass (kg ha^{-1}) ------------------------------------						
CC	122.2 ± 73.5 a[1]	Legume	122.2 ± 73.5 ab				
		Weed	509.0 ± 205 ab				
RC	440.4 ± 210.7 a	Legume	440.4 ± 210.7 a				
		Weed	978.1 ± 292 a				
PM	2337.9 ± 903.2 a	Legume	13.6 ± 13.6 b	Legume	13.6 ± 13.6 b		
		Weed	101.6 ± 73 ab	Rye	4662.1 ± 1317a		
VM	1572.9 ± 307.8 a	Legume	140.6 ± 85 ab	Legume	140.6 ± 85 b	Legume	140.6 ± 85 b
		Weed	128.9 ± 62 ab	Rye	2955.1 ± 468 a	Rye	2955.1 ± 468 a
						Radish	1622.3 ± 245 a
						Weed	128.9 ± 62 b

[1] Means sharing a letter within columns are not significantly different. Means separated by Tukey's HSD, α = 0.05.

Table 3.9. Nodule number and nodule mass at four sampling time-points and for four legume cover crop species at Grand Rapids and Horst sites.

Sampling Time-point	**Nodule Number**	**Nodule mass (mg)**
1 NOV	44.2 ± 6.3 a[1]	24.3 ± 8.8 ab
2 JAN	32.1 ± 8.1 b	7.4 ± 1.4 b
3 MAR	40.5 ± 10.6 ab	13.7 ± 3.7 a
4 MAY	107.8 ± 42.1 a	25.6 ± 6.3 a
Legume Species		
Crimson clover	23.5± 4.0 b[1]	4.2 ± 1.2 c
Red clover	164.9 ± 38.7 a	9.9 ± 2.1 b
Austrian winter pea	17.0 ± 4.3 b	26.8 ± 9.1 a
Hairy vetch	19.1 ± 2.6 b	18.9 ± 5.3 ab

[1] Means sharing a letter within columns for each variable are not significantly different. Means separated by Tukey's HSD, $\alpha = 0.05$.

Table 3.10. Percent of plots at both Grand Rapids and Horst with pink nodule occurrence at each sampling time-point. Both sites had at least one occurrence of pink nodules at every sampling.

TP	**---- Percent of plots with pink nodules ----**
1 (Nov)	79.2
2 (Jan)	35.4
3 (Mar)	44.8
4 (May)	75.0

Table 3.11. Mean values of response variables at each sampling time-point for Baby sites.

	Legume Biomass(g)			Nodule Number			Nodule mass(mg)			% Ndfa		
TP Site	PF	HAFA	Cala	PF	HAFA	Cala	PF	HAFA	Cala	PF	HAFA	Cala
1 (Nov)	2.4±0.7	14.5±5.6	NA	25.3 ± 0.5	133.9 ± 37.5	NA	5.3 ± 2.4	63.8 ± 38.3	ND	NA	NA	NA
2 (Jan)	2.2±1.4	8.0±2.4	NA	3.5 ± 0.9	18.8 ± 10.8	NA	0.38 ± 0.26	37.2 ± 0.6	ND	NA	NA	NA
3 (Mar)	1.3±0.3	2.0±1.7	NA	33.3 ± 21.2	253 ± 209.2	NA	18.7 ± 11.7	18.5 ± 10.6	ND	NA	NA	NA
4 (Apr/May)	2.3±0.8	9.1±6.3	2.5± 0.8	110.3 ± 90.1	399.3 ± 358.3	15.3± 8.4	12.6± 5.1	42.3 ± 28.8	2.3±1.5	64.5 ± 11.4	80.9 ± 14.6	8.8 ± 37.8

[1] Means are across species, within Site and Time-point, plus or minus 1 SE.

Figures

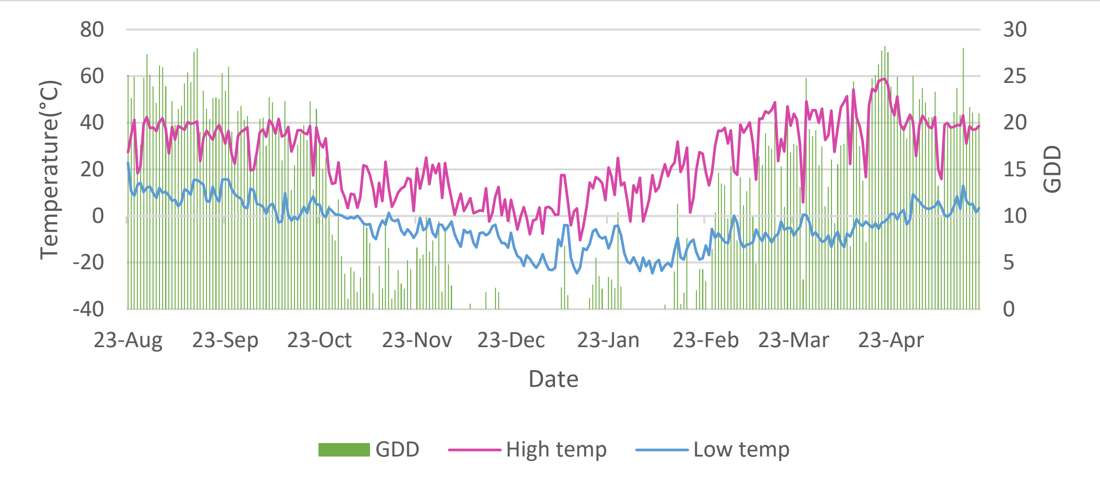

Figure 3.1. Daily high and low air temperatures (lines) and respective GDDs (vertical bars) throughout the cover crop growing season at the North Central Research and Outreach Station in Grand Rapids, MN. Temperatures are drawn from readings taken every 30 minutes, GDDs are calculated using a base temperature of 4°C.

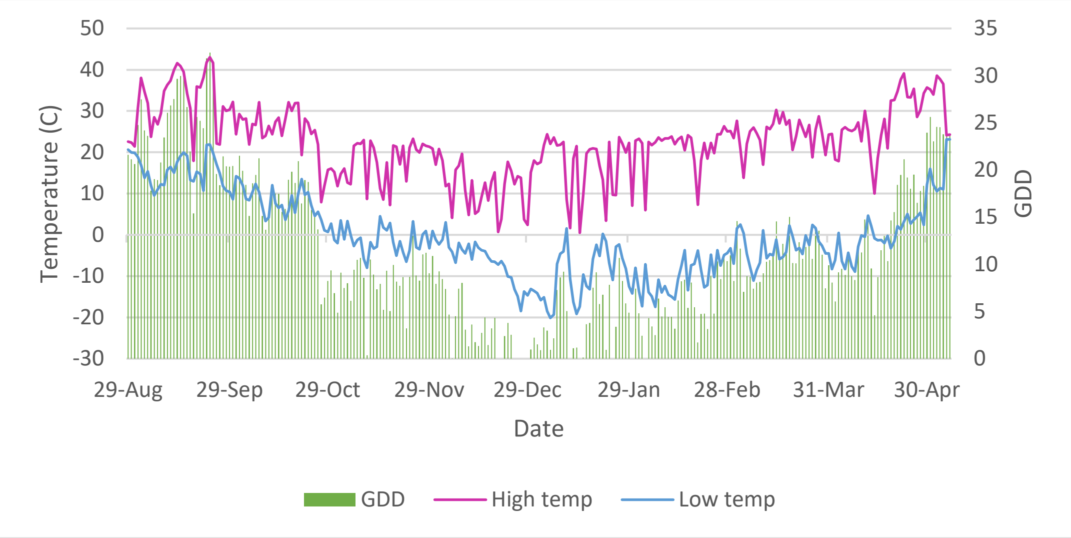

Figure 3.2. Daily high and low air temperatures (lines) and respective GDDs (vertical bars) throughout the cover crop growing season at the Horst Farm in Osceola, WI. Temperatures are drawn from readings taken every 30 minutes, GDDs are calculated using a base temperature of 4°C.

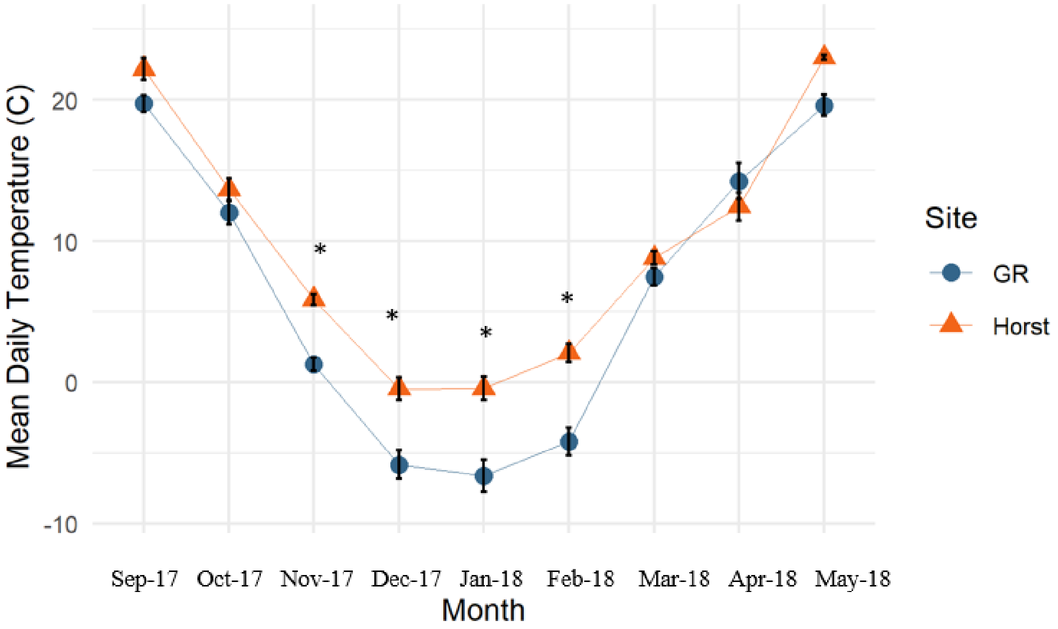

Figure 3.3. Mean daily temperatures per month for two sites. Error bars are 1 SE. Stars indicate significant temperature differences at $p<0.05$ (Tukey's HSD).

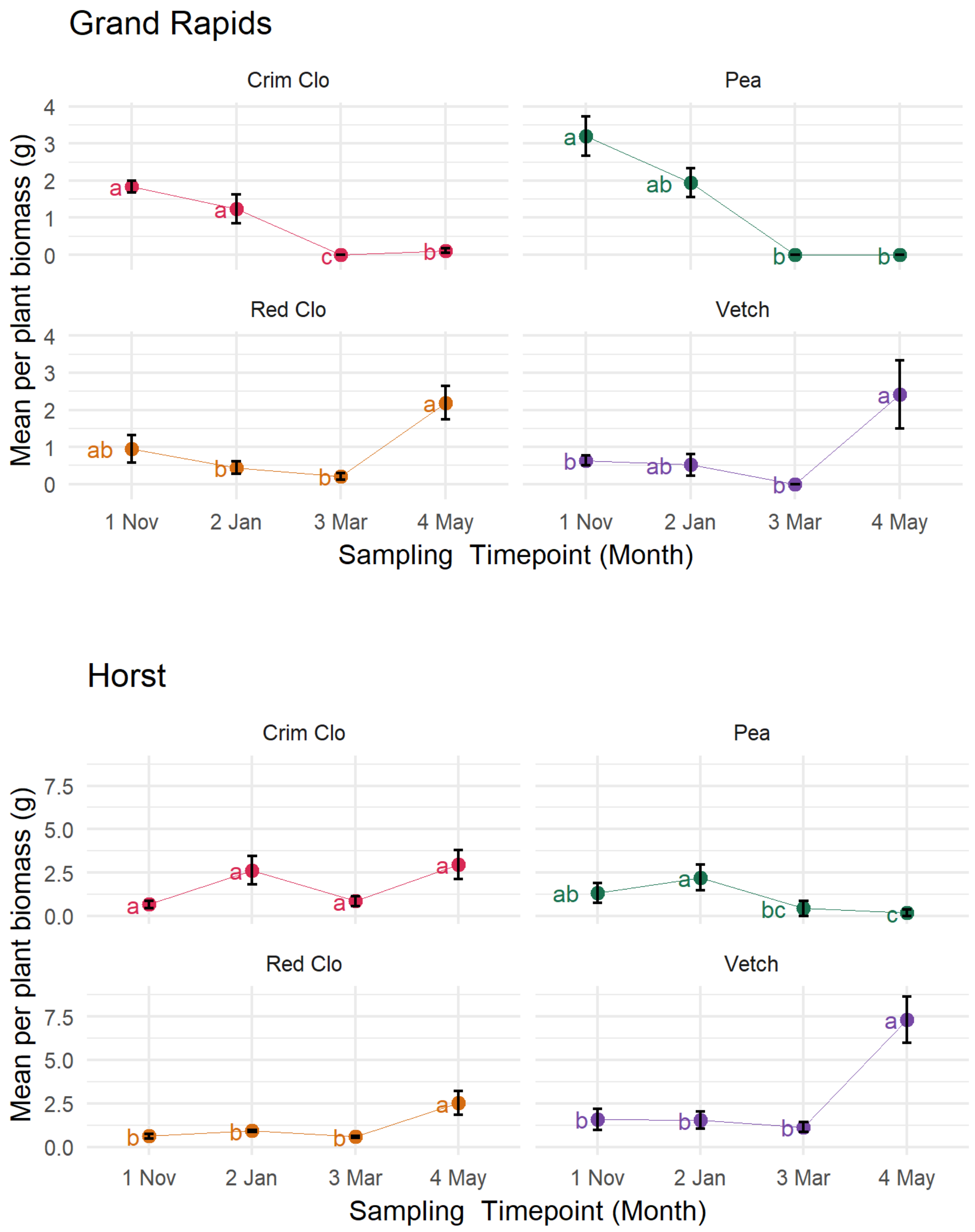

Figure 3.4. Aboveground legume biomass at four sampling time-points over the winter season. Sites and species were analyzed separately due to interactions. Error bars are 1 SE of the mean. Means sharing a letter within site and species are not significantly different, means were separated by Tukey's HSD, α = 0.05.

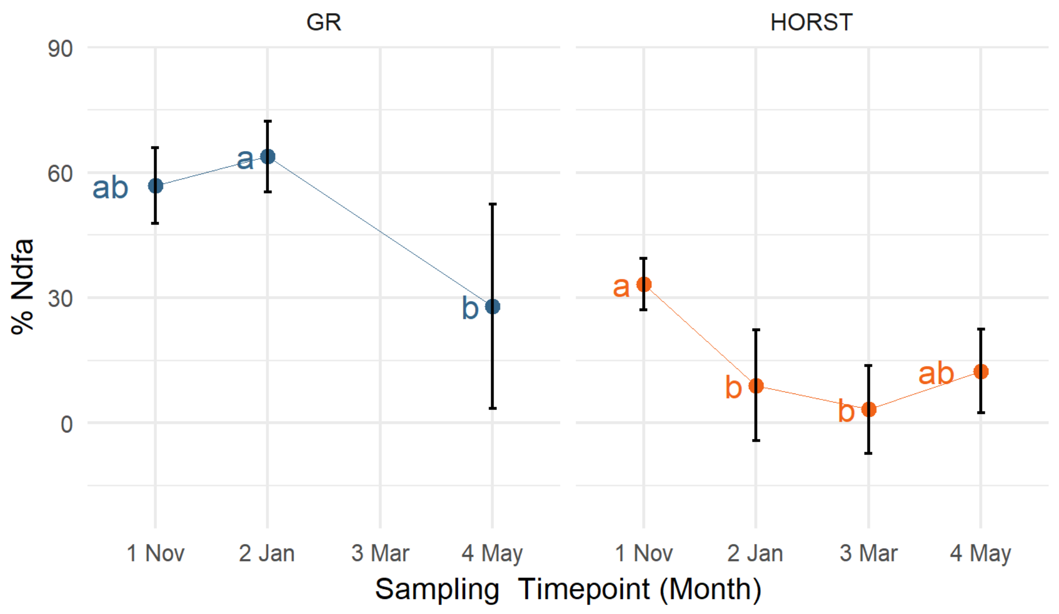

Figure 3.5. SNF measured by % Ndfa at each sampling time-point for two sites. Sites were analyzed separately. Sampling TP 3 was omitted from the Grand Rapids analysis due to high plant mortality. Error bars are 1 SE. Means sharing a letter within site and between time-points are not significantly different, means were separated by Tukey's HSD, $\alpha = 0.05$.

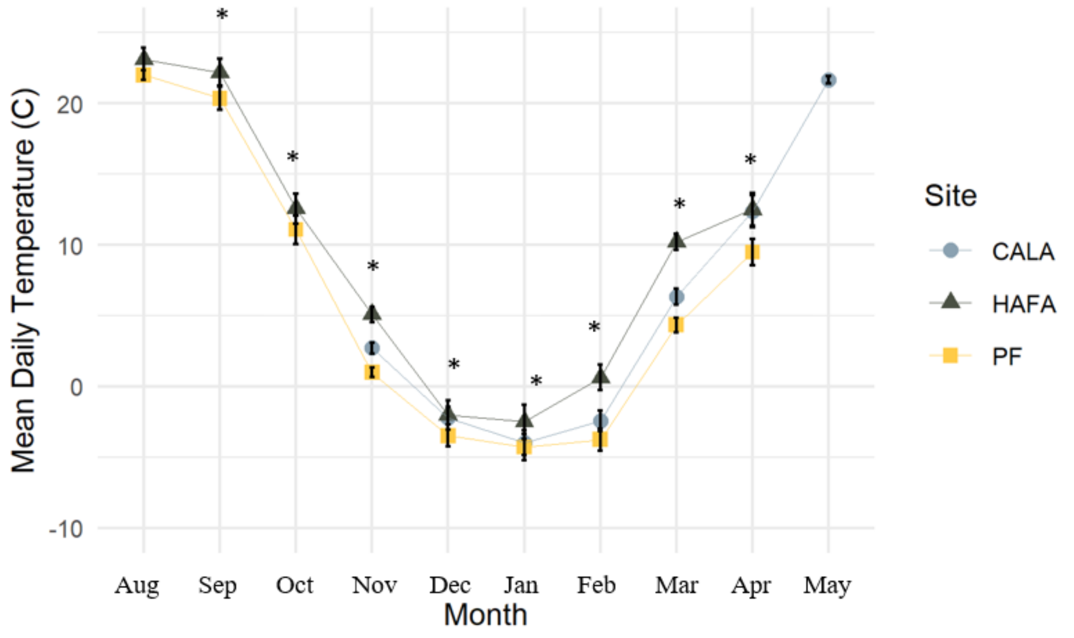

Figure 3.6. Mean daily temperatures at each month during the winter annual season for three baby sites. Stars indicate differences between sites within month (Tukey's HSD, $p<0.05$).

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