How delayed soil warming affects nitrogen fixation, plant performance and growth in the nitrogen fixing shrub green alder (*Alnus alnobetula* subsp. *crispa* (Aiton) Raus)

by

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Abstract

The short growing season and cold climate of the Canadian boreal forest can result in restricted amounts of available soil nitrogen, limited plant growth and low ecosystem productivity. Nitrogen fixing plants, which form symbiotic relationships with specialised bacteria, should have an advantage in low nitrogen environments, yet, their abundance in the boreal forest is low. Cool temperatures limit nitrogen fixation activity. How nitrogen fixation is affected when temperature differences occur between the soil and air, especially in the spring when soil temperatures remain cool, have not been well documented. To investigate this, two studies were conducted on the nitrogen fixing shrub *Alnus alnobetula* subsp. *crispa* (Aiton) Raus. In 2017, nitrogen fixation was monitored in both open and forested sites in the southern boreal forest. Nitrogen fixation activity began in mid June, was highest in early August at a soil temperature of 20˚C, and ceased in early October. The difference between soil and air temperature was largest in the spring. Soil temperature did not have an effect on nitrogen fixation until after bud burst and leaf development. In the open site, soil temperature warmed at an earlier date compared to the forested site. However there was no difference in nitrogen fixation rate between the sites. Soil moisture had no effect on nitrogen fixation. To examine the effects of extended delays in soil warming, past leaf development, a lab study was performed. For a period of thirteen weeks, soil was cooled to 10˚C, 14˚C and 16˚C, independently of shoot temperature (20˚C). On average, soils at 14˚C and 10˚C inhibited nitrogen fixation (by 29% and 62%, respectively) and photosynthesis (by 43% and 39%). Reductions in photosynthetic rate were mainly attributed to a reduction in nitrogen supply and the formation of new chlorophyll pigment. Although this effect was not immediate, suggesting some utilization of stored nitrogen. Reduced amounts of fixed nitrogen and photosynthates resulted in lower growth and biomass production when exposed to low soil temperatures. The advantages of nitrogen fixation may be

constrained by soil temperature in cool environments, restricting the abundance of nitrogen fixing species in the boreal forest.

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1. Introduction

1.1 Nitrogen Availability, Nitrogen as a Limiting Resource

The net primary production (NPP) (carbon fixed – carbon lost) of an ecosystem can be affected by several environmental factors, both directly and indirectly. Temperature, precipitation and growing season (i.e., climate) can limit NPP directly by influencing plant physiological functions and overall growth (McGuire et al. 1992; Chu et al. 2016). Climate can also influence NPP through the quality of tissue biomass produced, overall stand age, (Chu et al. 2016), soil mineralization rates and nutrient availability (Reich et al. 1997). Reduced availability of phosphorus and nitrogen has been observed to limit NPP in many ecosystems (Alvarez-Clare et al. 2013; Elser et al. 2007; Niinemets and Kull 2005; Norby et al. 2010). Generally, the degree to which nitrogen limits NPP increases with latitude (McGuire et al. 1992), with little nitrogen limitation in many areas of the tropics (LeBauer and Treseder 2008). The rate of organic matter mineralization resulting in the production of inorganic nitrogen is the main driver of nitrogen limitation on productivity.

Nitrogen cycles between three major pools: atmospheric (N_2), organic (e.g., amino acids), and inorganic (NH_4^+, NO_X^-). Mostly, plants take up inorganic forms of nitrogen, with different species sometimes exhibiting preferential uptake of different inorganic forms (Gherardi et al. 2013; Nordin et al. 2001). Inorganic nitrogen uptake is also influenced by temperature, with studies observing declines in ammonium, and especially nitrate in certain species at low temperatures (Boczulak et al. 2014; MacDuff and Hopper 1986; Macduff et al. 1987) which may be due to several factors. At low temperatures membrane fluidity decreases (Simon 1974), which may decrease nitrogen membrane transport activity. Additionally, reduction in root growth can reduce overall soil nitrogen uptake (Tolley and Raper 1985; Osmond et al. 1982). For nitrate and

ammonium, the most drastic reduction in uptake has been observed at temperatures lower than approximately 15˚C and 11˚C, respectively (Clarkson and Warner, 1979; MacDuff and Hopper, 1986; Macduff et al. 1987). In addition to inorganic nitrogen, uptake of amino acids can occur via specialized root transporters, although generally only observed with simple amino acids (Nasholm et al. 2009; Williams and Miller 2001). Preferential uptake for organic nitrogen over inorganic nitrogen has been observed in forests with low productivity, potentially due to high soil availability of organic nitrogen over inorganic nitrogen in these areas (Nordin et al. 2001). However, preferential uptake for both inorganic nitrogen (Kielland, 1994) and organic nitrogen (Chapin et al. 1993) has been reported in different studies of arctic plants, suggesting preference may be partly species dependent in unproductive environments. After uptake, nitrate is converted to ammonium and incorporated into amino acids and plant tissues.

1.1.1 Climate and Mineralization Rate

Climate is the main driver of mineralization via both direct effects of temperature and precipitation, and indirectly through its impact on litter quality production. The effect of climate (temperature and precipitation) on the mineralization rates of organic matter has been well documented in many ecosystems (Aerts 1997; Mcguire et al. 1992; Meentemeyer 1978; Verburg et al. 1999). Increasing temperature results in increased organic matter decomposition and nitrogen release (Hobbie 1996). For example, optimal rates of mineralization in forest and grassland sites of Spain has been observed at 25˚C with 80% soil moisture (Guntinas et al. 2012). Climates which experience cool temperatures and low precipitation, such as the boreal, have lower reported nitrogen mineralization rates when compared to warmer and wetter climates, such as the tropics (**Table 1.1**). In addition, the litter quality (chemical composition) can also effect

mineralization, although to a lesser extent than climate (Aerts 1997; Moore et al. 1999). Certain components found in plant litter can be more difficult to breakdown by soil microbes than others. The nitrogen content, C:N ratio, lignin:N ratio and polyphenolic content have all be documented to effect mineralization (Gonzalez and Seastedt 2001; Mao et al. 2018; Melillo et al. 1982; Moore et al. 1999; Palm and Sanchez 1991; Scott and Binkley 1997). Generally, litter with a higher nitrogen content and less lignin and polyphenol content is broken down faster. Although not all species and vegetation types reported in these studies are affected in the same way by the compounds listed. For example, nitrogen content may not have a large influence on decomposition rates in conifer litter (Berg et al. 2000; Prescott 1995). Cool and dry climates can directly reduce mineralization and inorganic nitrogen release and indirectly effect litter quality, which further limits decomposition. This may result in a positive feedback between soil nitrogen availability and litter quality in areas which are sub-optimal for decomposition (Gosz 1981 in Gosz 1984).

In addition to mineralization, industrial nitrogen fixation and fuel combustion can increase soil nitrogen inputs (between 0.25-3 kg/ha/year) (Dentener et al. 2006), this deposition is usually lower than rates of nitrogen mineralization in most isolated forested environments of the Canadian boreal (**Table 1.1**). Leaching of inorganic soil nitrogen into lakes and rivers can also reduce the availability of nitrogen to plants. Inorganic nitrogen lost from leaching is usually below 1 kg/ha/year in forested boreal areas (Mustajarvi et al. 2008), but can increase after logging (Binkley et al. 1992; Jerabkova et al. 2011; Palviainen et al. 2014) and forest fires (Harden et al. 2003).

Table 1.1 Comparison between the nitrogen mineralization rates in temperate forest, boreal forest and tropical forest soils measured per soil mass or per land area.

Location (soil depth - type)	N Mineralization (μg/g/year)	Source
Temperate (10cm - surface)	116-192	Devito et al. 1999
Boreal (10cm - mineral)	13.0	Devito et al. 1999
Boreal (7cm - mineral)	15.2-28.5*	Jerabkova et al. 2006
Wet Tropics (5cm - surface)	863.37*	Neill et al. 1997
Wet Tropics (10cm - mineral)	395.12*	Neill et al. 1997
Dry Tropics (10cm - surface)	125-210*	Raghubanshi 1992
	N Mineralization (kg/ha/year)	
Boreal Not specified)	14.3	Gundale et al. 2011
Boreal (8cm - surface)	23-29	Pajuste and Frey 2003

(*) Data was converted from published values. The growing season was assumed to be 95 days for boreal environments and 365 days for tropical environments. Mineralization rate was assumed to be continuous.

1.1.2 Nitrogen Fixation – Infection, Anatomy and Oxygen Maintenance

Some prokaryote organisms (both free-living and in symbiosis) are specialized in the fixation of atmospheric nitrogen, allowing them and their hosts to survive in areas of low nitrogen availability. In terrestrial boreal systems on a per land area basis, rates of nitrogen fixation in free-living bacteria are low (≈1.2 kg/ha/year) (Reed et al. 2011). Fixation can operate at a much higher rate when a nitrogen fixing organism is in symbiosis with an autotrophic partner that can supply the bacteria with carbohydrates, in exchange for fixed nitrogen (**Table 1.2**). Additionally, in terrestrial systems nitrogen fixation can be measured in species of lichens and mosses (DeLuca et al. 2002; Hitch and Stewart 1973; Markham 2009) (**Table 1.2**) and in marine and terrestrial environments by cyanobacteria (Brauer et al. 2013; Stal 2015). Symbiotic partnerships between nitrogen fixing bacteria and specialized hosts occurs in several species. In plants, symbiotic fixation occurs in most members of the legumes (Fabaceae) via a symbiotic relationship with the bacterium *Rhizobia* (Bauer 1981). A second large group of nitrogen fixing

symbiotic plants are collectively known as actinorhizal. Members of the actinorhizal plant group come from 8 different plant families (24 genera) and nitrogen fixation is carried out by bacteria in the genus *Frankia* (generally with few identified species). In many actinorhizal plant species, close to all tissue nitrogen is derived from fixation, although this is not commonly observed in legumes (Andrews et al. 2011).

Table 1.2 Nitrogen fixation rates in mosses and plants.

Location	Stand Age (years)	Host Species	Symbiont	N Fixed (kg/ha/year)	Source
			Plants		
Temperate	–	*Myrica gale*	*Frankia*	28	Schwintzer and Tjepkema 1983
Boreal	38	*Alnus hirsuta*	*Frankia*	60	Lee and Son 2005
Boreal		*Alnus hirsuta*	*Frankia*	56.4	Tobita et al. 2013
Boreal	11	*Alnus sinuata*	*Frankia*	6.25	Mead and Preston, 1992
Pacific Costal	50	*Alnus rubra*	*Frankia*	75-85	Binkley et al. 1992
Boreal	7	*Alnus incana*	*Frankia*	115	Rytter et al. 1991
Crop Land	–	*Glycine max*	*Rhizobia*	40-224	Schipanski et al. 2010
Crop Land	–	*Glycine max*	*Rhizobia*	44-313	Peoples et al. 1995
			Mosses		
Boreal	–	*Hylocomium* sp.	N/A	1.6	Zackrisson et al. 2009
Boreal	–	*Pleurozium* sp.	N/A	2.0	DeLuca et al. 2002
Boreal	–	*Sphagnum capillifolium*	N/A	1.93	Markham 2009

Note: Mosses fix nitrogen via an association with cyanobacteria, not in symbiosis.

Symbiotic fixation in plants can occur via different pathways, depending on the host plant species. Bacterial infection of the root can begin at the root hairs (intracellular infection), or between root epidermal cells (intercellular infection) (Pawlowski and Demchenko 2012). Root cells differentiate around the bacteria, becoming specialized and begin forming the lobular structure, known as the nodule (**Figure 1.1**). Nodules are connected to the plants vascular system and are supplied with carbohydrates from the host plant for growth and maintenance (Huss-Danell 1990). Nodules of actinorhizal plants are perennial structures. New nodules are formed over the growing season as the activity of older nodules can decrease over time (Guofan and Tingxiu 1987). The nitrogen fixing enzyme (nitrogenase) is sensitive to oxygen and must be kept in an oxygen limited environment as exposure causes irreversible damage to the enzymes functionality via the oxidation of the metal carrier subunits (Goldberg et al. 1987). In *Rhizobia*, a cortical group of cells envelops a central zone of nitrogen fixation activity, with respiration and the vascular system lying outside this zone (Silvester al. 1990). In addition, specialised hemoglobin known as leghemoglobin, which has a high affinity for oxygen, are used to maintain the low oxygen state within the cortical zone (Ott et al. 2005). Most *Frankia* symbioses lack leghemoglobin and have a different way of maintaining a low oxygen environment. The nitrogenase enzyme is located within specialised structures, called vesicles (**Figure 1.1**). Vesicles are surrounded by a lipid layer, able to limit oxygen diffusion (Harris and Silvester 1992), similar to heterocysts found in cyanobacteria (Kleemann et al. 1994). Vesicles in nodules are arranged around the interior vascular system, allowing for respirational oxygen diffusion (Silvester et al. 1990). Whether the difference in anatomy and anaerobic maintenance between *Frankia* and *Rhizobia* changes the energetic costs related to nitrogen fixation is un-clear.

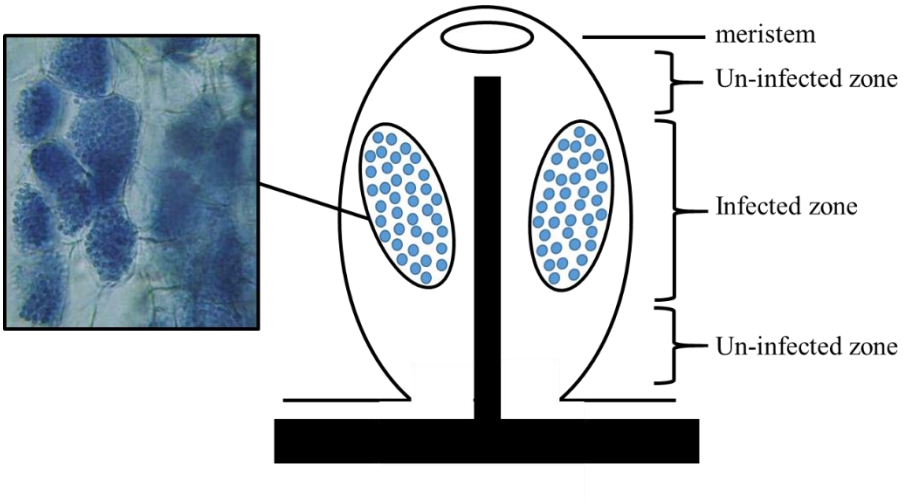

Figure 1.1 *Frankia* nodule longitudinal section (from *Alnus* sp.). Black bar represents the root vascular system. Vesicles stained blue using Fabils stain at 400X magnification. Photo courtesy of John Markham.

Comparing soil mineralization rates (**Table 1.1**) with nitrogen fixation rates (**Table 1.2**), it is obvious that nitrogen fixation adds ecologically important levels of nitrogen to an ecosystem. However, it should be noted that the mineralization rate and nitrogen fixation rate are not wholly comparable. While mineralization frees nitrogen in the soil for plant use, nitrogen fixation only directly benefits the host plant. Actinorhizal species are known to drop their leaves in the fall without reabsorbing much of their nitrogen, depositing litter that is high in nitrogen content (Killingbeck 1993). This can be potentially advantageous, as some studies have observed increased soil nitrogen content when nitrogen fixers are present (Tarrant and Miller 1963), but nitrogen availability is still ultimately dependent on the mineralization rate of the organic matter.

1.1.3 Nitrogen Fixation – Energetic Cost

While nodule anatomy can differ between plant species, the process of nitrogen fixation is the same. Atmospheric nitrogen is fixed via the enzyme nitrogenase. This enzyme contains a two protein complex of iron (Fe) and iron-molybdenum (Fe-Mo). Gaseous nitrogen (N_2) is reduced into ammonia (NH_3^+), along with a hydrogen by-product. The iron protein complex requires 2ATP/ electron (which have been supplied by ferredoxin) to break the N_2 bonds until $2NH_3^+$ are formed (Burns and Hardy 1975). In total, eight electrons and sixteen ATP are required for the reduction of nitrogen gas into two ammonia and hydrogen (Dixon and Wheeler 1983). However, this is only a theoretical baseline value for electron movement which neglects other physiological and chemical components of fixation and nitrogen consumption. Efficiency of electron transport, hydrogen formation rate, nodule maintenance and nitrogen assimilation add to the total energetic and respiratory costs (Schwintzer 1990; Dixon and Wheeler 1983). Thus, an accurate value of the energetic cost associated with nitrogen fixation can vary and is difficult to determine. The energetic requirement of fixation is usually reported as the carbon cost. At an optimal hydrogen evolution rate, the amount of ATP required is 28, which has a conversion to two grams of carbon for every gram of nitrogen fixed (assuming 36 moles of ATP/ glucose). Theoretically, the minimum carbon cost of nitrogen fixation, assimilation and transport is 2.34 gC/gN (Dixon and Wheeler 1983). Lab studies have reported various carbon costs for both legume and actinorhizal plants that range from 4.5-7.5 gC/gN (Lundquist 2005; Schwintzer and Tjepkema 1983). This is more energetically costly than nitrate uptake and reduction, and ammonium assimilation (Silsbury, 1977) (**Table 1.3**). Individual studies conducted on actinorhizal and leguminous fixers, have observed that around 50% of all root respiratory activity is localized to the nodules, further highlighting their high energetic requirement and oxygen

demand (Lundquist 2005; Tjepkema and Winship 1980). Rainbird et al. (1984) estimated that in soybean (*Glycine max* (L.) Merrill) (*Rhizobia*) 52% of total nodule energy was used in fixation, 22% in nodule maintenance and the reminder in hydrogen evolution and protein assimilation.

Table 1.3 Energetic cost of inorganic nitrogen uptake and assimilation. Carbon cost calculated from oxygen consumption during respiration, based on calculations from Robinson (2001).

Species	Nitrogen Source	gC/ gN	Source
Hordeum vulgare	NH_4^+ assimilation	0.28	Bloom et al. 1992
Hordeum vulgare	NO_3^- absorption	0.15	Bloom et al. 1992
Hordeum vulgare	NO_3^- reduction	1.37	Bloom et al. 1992
Not specified	NO_3^- reduction	1.62	Pate and Layzell 1990

1.1.4 Nitrogen Fixation – Inhibitors of Fixation (direct and indirect)

Energetic costs associated with fixation can also be increased due to shifts in abiotic stress, which may make fixation more costly as a nitrogen source for the plant host (Vitousek and Howarth 1991). Nitrogen fixation can be impacted by a number of environmental stresses. Direct effects on the functionality of the nodule include the temperature at which the nodule operates. Studies have shown that *Frankia* isolates fix nitrogen between 6˚C and 25˚C (Tjepkema and Murry 1989). The activation energy associated with the nitrogenase enzyme can change at certain temperature thresholds (Ceuterick et al. 1976). An optimal operating temperature of between 20˚C – 25˚C has been reported in both actinorhizal and legume species, in both lab and field studies (Hawkins and McDonald 1994; Lee and Son 2005; Waughman 1977). *Casuarina equisetifolia* (L.), a tropical actinorhizal plant, demonstrated a fixation optimum of 35˚C (Waughman 1977). In tropical legumes peak nitrogen fixation activity has been

observed as high as 40˚C and the abundance of leguminous trees diminishes around annual temperatures of 15˚C (Liao et al. 2017). In addition to reducing nitrogenase activity, cooler root temperatures (<20˚C) in legumes (*Rhizobia*) have been reported to reduce nodule infection and development rate (Gibson 1971). In soybean (*Glycine max*) and lupine (*Lupinus albus* (L.)), leguminous nitrogen fixing species, nitrogen fixation was found to be more temperature sensitive than inorganic nitrogen uptake (Legros and Smith 1994).

An indirect environmental influencer of nitrogen fixation is light (Rastetter et al. 2001; Vitousek et al. 2002; Vitousek and Howarth 1991). Under low light conditions, nitrogen fixing plants may not be able to meet the high energetic demands of fixation, leading to reductions in activity and supplied nitrogen. Because of this, shading can result in reduced nodule growth, nodule number and nitrogenase activity in both actinorhizal and leguminous fixing species (Gordon and Wheeler 1978; Sprent 1973; Ta and Faris 1988) and may result in the prevalence of nitrogen fixing species in recently disturbed areas (Vitousek and Field 1999).

High levels of soil inorganic nitrogen have been observed to cause reductions in nitrogenase activity, nodule formation and nodule growth in laboratory settings (Huss-Danell et al. 1982; Markham and Zekveld 2007; Waughman 1977), with values above 0.5mM N observed to have an inhibitory effect. Nitrate can limit the development, growth and nitrogenase activity of nodules in *Frankia* and *Rhizobia* symbionts (Arnone et al. 1994; Gentili et al. 2006; Xia et al. 2017). This may be due to a reduction in provided carbohydrates, as the application of nitrate has been observed to reduce the amount of photosynthates supplied to nodules in soybean (*Glycine max)* and pea (*Pisium sativum (L.)*) (Fujikake et al. 2003; Small and Leonard 1969). Soil phosphorus can also have a direct positive effect on nitrogen fixation in *Alnus* spp. (Gentili and

Huss-danell 2003; Reed et al. 2007). As increased phosphorus availability in *Alnus tenuifolia* (Nuttall) resulted in increased nodule biomass production (Uliassi et al. 2000; Uliassi and Ruess 2002).

Soil moisture can also affect nitrogen fixation. A reduction in soil moisture can cause a drought response by the host plant, which can involve the closure of the stomates (Agurla et al. 2018). This can reduce the photosynthetic rate, limiting photosynthates supplied to the nodules, and depressing overall nitrogen fixation activity (Wheeler 1971). Some studies in soybean (*Glycine max*) have also observed a buildup of root/nodule nitrogen content when exposed to drought stress, potentially due to a reduction in the movement on fixed nitrogen away from the nodules/ roots (King and Purcell 2005; Ladrera et al. 2007).

1.1.5 Nitrogen Fixation - Latitude Paradox

Given the fact that most terrestrial ecosystems are nitrogen limited, one would expect the abundance of nitrogen fixing plants to be high in almost all terrestrial ecosystems, especially areas of limited available soil nitrogen. In many tropical forests nitrogen fixing leguminous trees are common (Benson and Dawson 2007; Crews 1999; Tedersoo et al. 2018), but most tropical areas are not nitrogen limited (Jenny 1950; Martinelli et al. 1999). As you move further away from the tropics into cooler temperate and boreal forests the species composition shifts from leguminous trees to actinorhizal shrubs, and the overall abundance of nitrogen fixers decreases (Menge et al. 2014), with nitrogen fixing plants generally segregated to early successional environments (Huston and Smith 1987; Rastetter et al. 2001). The low abundance of nitrogen

fixing species at high latitudes, where they should have an advantage, is known as the latitude paradox of nitrogen fixation.

A number of models have been created to predict the conditions that limit and benefit nitrogen fixers in an attempt to explain the latitude paradox. From these models a number of hypotheses have been put forward. Menge et al. (2009) first proposed the idea of a facultative vs. obligate nitrogen fixation strategy, also known as the differential regulation hypothesis. Specifically, tropical nitrogen fixing species (often legumes) are facultative, able to stop fixation when no longer under nitrogen limited conditions. This would allow for the diversion of energy into plant growth to compete with non-fixing species until available soil nitrogen is consumed and fixation activity can be turned back on. Conversely Menge et al. (2009) also proposed that in high latitude temperate and boreal forests, nitrogen fixers (mainly actinorhizal species) are obligate, having a continual rate of nitrogen fixation regardless of soil nitrogen availability. Under this scenario non-fixing species may benefit from increased soil nitrogen deposition due to the presence of nitrogen fixing species. This could allow for increased plant growth in non-fixing species, without having to invest energy in fixation. Eventually out competing nitrogen fixing species, leading to the dominance of non-fixing species in later successional areas.

Nitrogen fixing species in low latitude forests may be environmentally disposed to this facultative type of fixation strategy. The facultative strategy requires the down regulation of nodule growth and in turn nitrogen fixation activity, as well as, the build-up of new nodules once fixation is no longer energetically unfavorable i.e., soil nitrogen is again limited. The short growing season and cool climate in high latitude forests may make the obligate strategy the only viable option in actinorhizal plants. The facultative strategy may simply be too costly in the long term for actinorhizal/ high latitude nitrogen fixing plants to benefit from in their environment.

Environmental constraints, such as the availability of additional nutrients, temperature and light level may increase the cost of fixation beyond limits that are beneficial to the host plant, resulting in a reduction in nitrogen fixer abundance at high latitudes (Vitousek and Howarth 1991). These environmental influences may lead to the favoring of an obligate strategy at high latitudes, thus limiting the prevalence of facultative leguminous fixers. Even though the obligate strategy may benefit non-fixing species in the long term, it may be the only solution, as the conditions that permit the growth of nitrogen fixing species may only be cost effective during early succession, limiting the abundance of actinorhizal plants in high latitude forests. Liao et al. (2017) used temperature and precipitation data to predict the change in fixer abundance based on computer models using collected data. Their model suggested that the abundance of nitrogen fixing leguminous trees peaked in environments with high mean annual precipitation (2500mm/year) and mean annual temperatures great than 15˚C. Their analysis of actinorhizal species showed abundance peaked at both low and high annual precipitation and at mean annual temperatures between 5-10˚C. Overall, climate (temperature and precipitation) explained 50% of the difference between the abundance of tropical legumes and boreal/ temperate actinorhizal species.

Results from field studies can both support and contradict the obligate fixation strategy. Mead and Preston (1992) observed that the amount of nitrogen derived from fixation in *Alnus sinuata* ((Regel) Rydberg) decreased after nitrogen fertilizer (100 kg/ha/year) was applied. A lab study by Zekveld and Markham (2011) observed reductions in nodule number in green alder (*Alnus crispa*) when 2mM nitrate was applied. These results are not consistent with an obligate fixation strategy. However, it should be noted that the levels of nitrogen used is higher than what is usually readily available in a natural boreal system. In *Alnus fruticosa* (Ruprecht) in interior

Alaska, overall plant growth rate was highest in early succession. However, the rate of nitrogen fixation (per nodule mass) did not change with successional age, even when the stand was dominated by white spruce (Mitchell and Ruess 2009a). In the Alaskan floodplains, nitrogen fixation activity (per nodule mass) in *Alnus tenuifolia* decreased in a poplar stand with succession, but not when activity was measured on a per plant basis (Uliassi et al. 2000). Additionally, *Alnus rubra* (Bongard) activity in the Alaskan floodplains did not decrease when inorganic nitrogen leaching in the soil was high (approximately 40 kg/ha/year) (Binkley et al. 1992). These results suggest an obligate strategy of fixation in alders at high latitudes. Because of the limited amount of nitrogen fixation data available in the boreal, it is necessary to further investigate the possible use of an obligate strategy by actinorhizal shrubs.

The use of a facultative strategy by tropical nitrogen fixing plants has been discussed further in a number of publications, whose authors agree with this strategy as an explanation of tropical nitrogen fixer abundance (Andrews et al. 2011; Hedin et al. 2009; Liao et al. 2017). Andrews et al. (2011) compared the amount of nitrogen derived from fixation (%Ndfa) between tropical legumes and temperate actinorhizal species and found that on average, actinorhizal plants derive 71% of their nitrogen from fixation, while this value was only 42% in tropical species, suggesting a facultative strategy in the tropics.

Additional predictive theories proposed by Menge et al. (2017) to explain the latitude paradox, include the nitrogen severity limitation hypothesis and the nitrogen fixation benefit cost hypothesis. The first hypothesis states that while soil nitrogen limitation is not common in the tropics, in the areas that it does occur this limitation is more severe than what is experienced in high latitude forests, leading to the persistence of nitrogen fixers in an environment that is not usually limited by nitrogen. The second hypothesis states that in lower latitude forests the cost of

fixation may be favorable under a wider range of soil nitrogen levels. Due to warmer overall temperatures that benefit nitrogenase activity, and the potential benefit of tropical fixers to synthesise more nitrogen based herbivory defensive compounds. This may allow for nitrogen fixation to be cost effective over a wider range of soil nitrogen levels. In high latitude forests the cool climate may increase the cost of fixation, making it only cost effective in extremely nitrogen limited environments, lowering species abundance. These theories are relatively new and have not benefited from any field tests or subsequent studies.

Houlton et al. (2008), proposed that nitrogen fixation in high latitude areas is limited based on the effects of low temperature on nitrogen fixation activity. In the tropics, they proposed that fixers were abundant due to soil phosphorus limitation. Tropical nitrogen fixers may be able to produce more phosphatase enzyme due to increased nitrogen availability from fixation, allowing for increased phosphorus availability around nitrogen fixers, giving them an advantage in phosphorus limited sites and allowing for their persistence in the tropics. More recent studies have found increased phosphatase activity in leguminous trees (Nasto et al. 2014), while other have failed to do so (Batterman et al. 2018). Thus, it has not been confirmed if this phenomenon is occurring in tropical fixers as a direct result of a plants nitrogen fixation status.

1.2 The Boreal Forest

The boreal forest is the dominant biome in northern latitudes, with the majority located within Russia (60%) and Canada (28%) (Brandt et al. 2013). In Canada, 75% all forested area is boreal (Natural Resources Canada, 2018) with average winter temperatures around -25˚C, and annual summer temperatures around 20˚C (Hogg 2003; Mellander et al. 2007). For the majority

of the Canadian boreal annual precipitation is low, but varies between the northern (478mm) and southern boreal (580mm), with 1/3 of precipitation accounting as snow (Hogg 2003; Smith et al. 1998). Annual precipitation can also differ between the eastern (1000mm) and western (400mm) boreal (Smith et al. 1998). The Canadian boreal forest contains deciduous, coniferous and mixedwood forests (Bergeron et al. 2014). In the northern most latitudes of the boreal the taiga is the transitional zone between the boreal and sub-arctic, with a conifer dominance. The southwestern regions contain aspen parkland, a transitional zone into southern temperate forests (Larsen 1980) with the southeast transitioning into deciduous forest (Rowe 1972). Plant species composition can differ between Eurasian and North American boreal forest communities, but on the genus level plants show similarities (Hare and Ritchie 1972). Common hardwood plants include *Betula* spp. (birch), *Populus* spp. (poplar), *Salix* spp. (willow), *Alnus* spp. (alder) and in the southern Canadian boreal, *Quercus* spp. (oak) (Brandt 2009). Common conifer species include *Picea* spp. (spruce), *Pinus* spp. (pine), *Abies* spp. (fir), and *Thuja* spp. (cedar) (Denneler et al. 2008; Hare and Ritchie 1972).

Low productivity values are usually observed in the boreal forest (Luyssaert et al. 2007). Boreal forests have low total net primary productivity (696 $gC/m^2/ha$) when compared to other ecosystems such as tropical forests (2,048 $gC/m^2/ha$) and grasslands (1,283 $gC/m^2/ha$) (Scurlock and Olson 2013). The low productivity of the boreal most likely stems from the effect that low temperature and precipitation has on the decomposition of dead organic matter combined with the short growing season. Residence times for nitrogen (how long nutrients remain inaccessible in the litter) are highest in the boreal when compared to temperate and tropical forests (138 years/kg, 5 years/kg, 2 years/kg respectively [Barnes et al. 1998]). Mineralization rates are usually highest in deciduous areas of the southern boreal, and lowest in coniferous sites (Moore

et al. 1999). Important nutrients become locked in the litter layer in forms which are not accessible to plants (Kimmins 1987). Consequently, the boreal region is low in the availability of important plant nutrients.

Succession in the boreal forest is an important process to allow for the colonization of disturbed/open areas. Many factors can influence the species trajectory and development in secondary succession. The type, severity and frequency of the disturbance (Heinselman 1981), the seed stock and soil characteristics of the land (Barnes et al. 1998), weather conditions during regrowth (Cayford 1963) and regional climate (Barnes et al. 1998) can all influence succession. The most common disturbance regimes in the boreal are fire and insect damage (Bogdanski 2008). Additional disturbance regimes can include wind storms and anthropogenic disturbances, such as logging and mining. A number of different paths may be taken in the regeneration cycle. Self-replacement occurs when adequate seed sock or belowground vegetative structures allow for the re-growth of a similar pre-fire stand. Cyclic succession occurs when a number of different species are dominant in the canopy at one point in time, usually starting with fast growing shade intolerant shrubs, which are eventually shaded out by larger and faster growing species (Bergeron et al. 2014; Bergeron and Dubuc 1989). Typical species composition during a cyclic successional event is as follows. Small herbs and grasses are the first to emerge, followed by fast growing small shrubs and nitrogen fixing actinorhizal species, such as Alder (*Alnus* spp.). In general, nitrogen fixing species are only dominant in the earliest stages of succession (Rastetter et al. 2001). Nitrogen fixation allows them to survive in areas of low soil nitrogen and increased access to light can allow for adequate photosynthesis to drive fixation (Huston and Smith 1987). Alder are generally considered to be shade intolerant (Menge et al. 2010) and are usually outcompeted by taller, non-fixing species over time (Clein and Schimel 1995). Early

successional shade intolerant tree species include *Pinus* spp. on dry cites and *Betula* spp. and *Populus* spp. on wet cites. Later on these are then replaced by shade tolerant tree species *Picea* spp., *Abies* spp. and/or *Thuja* spp. (Bergeron and Dubuc 1989). In addition, gap phase dynamics occurs when a small disturbance such as wind storms or the falling of dead trees forms an opening in the canopy, allowing for new species to grow. In these areas, increased light can allow for the growth of early successional species, such as actinorhizal shrubs, within older stands (McCarthy 2001; Mitchell and Ruess 2009a).

1.2.1 Nitrogen Fixation in the Boreal

The abundance of nitrogen fixing species in the boreal is low, especially compared to low latitude, tropical forests (Tedersoo et al. 2018). The majority of the nitrogen fixing species found in the boreal are actinorhizal, with the most common plant families being Betulaceae, Eleagnaceae and Myricaceae (Benson and Dawson 2007). Alder (*Alnus*) a member of the Betulaceae, are usually found in early successional sites and in forest canopy gaps of low soil nitrogen availability (Walker 1989). The genus alder contains 30 species, a few of which are native to the Canadian boreal [*Alnus incana* (L.) Moench ssp. *rugosa* (Du Roi) R.T. Clausen, *Alnus viridis* (Chaix) DC. ssp. *sinuata* (Regel) A. Löve and D. Löve and *Alnus alnobetula* subsp. *crispa* (Aiton) Raus)]. Additional actinorhizal plant species found in the boreal include *Shepherdia* spp. (buffalo berry), *Elaeagnus* spp. (silverberry), *Myrica gale* (L.) (sweet gale) and *Comptonia peregrina* ((L.) J.M. Coulter) (sweet fern).

During the fall, boreal deciduous species begin the process of dormancy and set buds to overwinter, beginning the breakdown of photosynthetic pigments in leaves to reabsorb nitrogen. However, alder, and other actinorhizal plants do not reabsorb a majority of their leaf nitrogen,

dropping green leaves (Cote and Dawson 1986; Cote et al. 1989). The reason for this is unknown. Tateno (2003), suggested that alders may keep their leaves green to increase photosynthetic gains in the fall, past what other species are capable of. The presence of alders has been observed to increase soil nitrogen availability through the sheading of nitrogen rich tissues. The dropping of nitrogen rich leaves allows the productivity of alder-conifer stands to have double the productivity of conifer only stands in non-boreal pacific coastal forests (Binkley et al. 1992). Soil under alder dominated stands has been observed to have higher rates of nitrogen mineralization in studies of pacific forests and in the open floodplains of Alaska (Binkley et al. 1992; Hobbie et al. 1998; Ruess et al. 2009; Tarrant and Miller 1963; Van Cleve et al. 1971). Although the ability of alder to enrich soil in Alaskan floodplains and pacific coastal forests has been well documented, there are few studies in boreal forest sites. The available data suggests some increase in nitrogen mineralization when alders are present (**Table 1.4**), although it should be noted that the European boreal is on average slightly warmer than the Canadian boreal, which may influence the mineralization rates shown. The extent to which alders increase mineralization rates and their effect on the nitrogen status of boreal forest soil as a whole is unclear. In the North American boreal, it has been observed that alders increase soil nitrogen availability (Mitchell and Ruess 2009a), while other studies have failed to documented any soil nitrogen enhancement in the presence of alders (Cortini and Comeau 2008; Essery 2010). The effect of alders on nitrogen availability and forest productivity may also be dependent on the area of the boreal studied. An experiment conducted on *Alnus crispa* in a pine (*Pinus banksiana* (Lambert)) stand, did not show large gains to site productivity in southern areas (18% increase with alder). However, in more northern study sites the presence of alder had a larger impact on site productivity (40% increase with alder) (Vogel and Gower 1998).

Table 1.4 Nitrogen mineralization rates in boreal forests soils, with and without the presence of a nitrogen fixing plant species.

Location	Soil Type (Depth)	Stand	N Fixer Present (Species)	N (kg/ha/year)	Source
Europe	Mineral (15cm)	Coniferous	Yes (*Alnus incana*)	2.85*	Myrold and Huss-Danell 2003
Europe	Mineral (15cm)	Coniferous	Yes (*Alnus incana*)	8.27*	Myrold and Huss-Danell 2003
Europe	Mineral (15cm)	Coniferous	Yes (*Lupinus nootkatensis*)	46.4*	Myrold and Huss-Danell 2003
Europe	Surface (10cm)	Old Field	Yes (*Alnus incana*)	84	Uri et al. 2003
Europe	Surface (10cm)	Coniferous	No	28.9	Persson and Wiren 1995
Europe	Mineral (10cm)	Coniferous	No	18.3	Persson and Wiren 1995
Europe	Mineral (20cm)	Coniferous	No	8.2	Persson and Wiren 1995
Canada	Surface (12cm)	Deciduous	No	37.1*	Ste-Marie and Houle 2006
Canada	Surface (7cm)	Coniferous	No	20.0*	Ste-Marie and Houle 2006
Canada	Surface (15cm)	Coniferous	No	3.80	Lamontagne 1998

(*) Data was converted from published values. The growing season was assumed to be 95 days and mineralization rate was assumed to be continuous.

1.2.2 Seasonal Changes and Temperature Effects

Previous field studies in boreal forests and northern temperate forests have observed nitrogen fixation ceases around late September or early October (Cote and Dawson 1989) with a complete absence of fixation in the winter (Pizelle 1984). Symbiotic fixation becomes detectable in May (Huss-Danell et al. 1992), with the highest rates of nitrogen fixation observed in July and August (Lee and Son 2005). At the start of the spring season air temperatures will increase at a faster rate than the ground temperature (**Figure 1.2**), leading to a potential miss match between the photosynthetic processes above ground, and nitrogen fixing process occurring in the soil. Delayed soil warming during the spring and its effect on nitrogen fixation may be further impacted in areas with a moss layer or other ground cover, as this may decrease the rate at which frozen soils thaw and begin to warm during the spring (Startsev et al. 2007), potentially delaying fixation. Nitrogen fixation in the spring is necessary to regain the nitrogen lost in the fall, but how nitrogen fixation acts during the spring, when cellular and enzymatic processes are negatively impacted, has not been well documented, especially in boreal actinorhizal species.

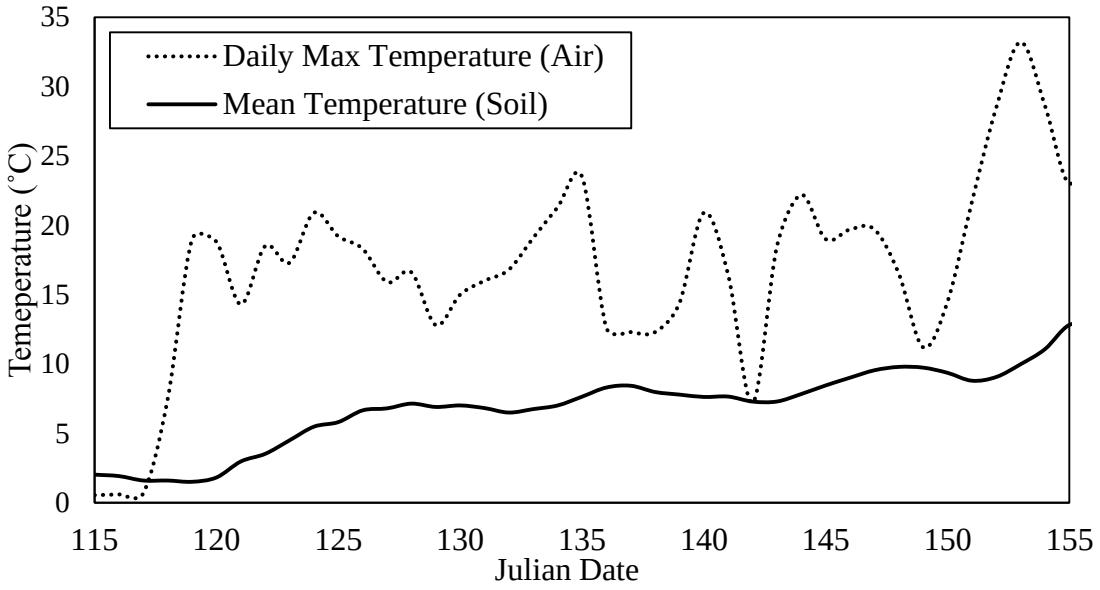

Figure 1.2 Soil and air temperature during the spring in the southern Canadian boreal. Air temperature shown is the midday average. Air temperature was measured using a HOBO U30 monitoring system (Onset, USA). Soil temperature was measured at 30cm of depth, using a four channel external temperature logger (Onset, USA). Measurements were recorded between April 25th (Julian date 115) and June 4th (Julian date 155).

1.3 Thesis Objective

The objective of this thesis was to investigate how soil temperature will affect nitrogen fixation, photosynthesis and overall growth of a boreal forest actinorhizal species. In particular, to determine how delays in soil warming will affect nitrogen fixation and how this may in turn effect whole plant function and growth in the actinorhizal shrub green alder (*Alnus alnobetula* subsp. *crispa*). A field study was conducted to monitor seasonal fluctuations in soil temperature and soil moisture in a boreal forest site, while also observing changes to nitrogen fixation rate in both an open and forest understory. A lab study also investigated nitrogen fixation activity, photosynthesis and other physiological parameters that may be effected in green alder when soil temperature is cooler than shoot temperature over a thirteen week experimental period.

2.0 Experiment #1 – Comparison of seasonal changes in nitrogen fixation activity in green alder (*Alnus alnobetula* subsp. *crispa* (Aiton) Raus) in a forest understory and adjacent burn site in the southern Canadian boreal.

The objectives of this study are to investigate nitrogen fixation activity in *Alnus alnobetula* subsp. *crispa* (*Alnus crispa*) in relation to seasonal temperature changes in a southern boreal forest environment. In particular I am interested in how soil temperature and nitrogen fixation may be effected by temperature delays between the air and soil, especially in the spring. In addition, to compare how nitrogen fixation may change with successional age, I am interested in how nitrogen fixation will differ between *Alnus alnobetula* subsp. *crispa* in an open site and a closed canopy understory site.

The temperature difference between the soil and air will be largest in the spring, as soil will warm at a slower rate compared to air. Low soil temperature will reduce nitrogen fixation activity. As soil temperatures increase during the growing season, a positive response in nitrogen fixation activity is expected. In addition, nitrogen fixation is expected to start earlier in the season in the open site compared to the closed site, due to a combination of increased light availability and earlier soil warming.

2.1 Materials and Methods

2.1.1 Experimental Site Description

Alnus alnobetula subsp. *crispa (*hereafter called *Alnus crispa)* shrubs were sampled at the Sandilands Provincial Forest, Manitoba, Canada (49°21'23.2"N 96°11'37.7"W). This area is in the boreal forest, close to the grassland transitional zone. Historical data (2001- 2007) shows an average growing season (May-August) precipitation of 362.7mm (weather station located 11.7 km south east of sampling area), daily temperature average of 22.1˚C, and nightly temperature

average of 9.5˚C (weather station located 53.6km south east of sampling area) (Government of Canada). Soils are low in inorganic nitrogen (7.2 mg/kg) (Markham, unpublished), have a shallow organic layer, with lower soil horizons consisting mainly of coarse sandy soil deposits of distal glaciofluvial sediment (Matile and Keller 2004), with a soil pH of 6.6-7.3 (Manitoba Agriculture 1999). Summer droughts are common in the region. Soil temperature was monitored at 30cm of depth in the forested (closed) area, using a HOBO four channel external temperature logger (Onset, USA).

Samples of nodules were taken from individual *Alnus crispa* shrubs in a recently fire disturbed area (open site) and a forested stand (closed site) consisting of a tree canopy of mainly of pine (*Pinus banksiana*). The two sites were originally part of a single forest stand. Trees in the open site were removed in a forest fire in 2008, most of the understory species re-sprouted after the fire (Markham and Essery 2015). The closed site was prevented from burning by a fire break between the sites. The two sites are similar in topography, soil texture and plant species composition. Measurements of the closed site in 2012 showed that on average pine trees were 97 years old, with a density of 400 trees/ha and 33% canopy openness (Markham et al. 2019). Canopy openness may have increased in subsequent years in certain areas of the forested site due to a recent outbreak of the *Ips* sp. beetle.

2.1.2 Measurements

Acetylene Reduction Assay

Nodules of *Alnus crispa* were dug up in the mineral soil layer, at approximately 10-20 cm depth. Nodules were then removed from the root and placed in 45mL glass jars. Using the acetylene reduction assay (ARA) technique to reduce acetylene to ethylene (Hardy et al. 1968)

the activity of the nitrogenase enzyme was calculated on a per nodule mass basis over time (specific nodule activity). The headspace in the jar was replaced with 10% Acetylene. The jars were then buried, with the organic layer and a bag of vermiculite placed on top to maintain the soil temperature around the jar. After 45 minutes, a 5mL sample of gas was taken and injected in a sealed glass vial, displacing water as to maintain atmospheric pressure in the vial. At the end of the day the samples were run through a Varian 3400 gas chromatograph (GC) (Varian, Canada) fitted with a 0.25 mL sample valve and a Haysep T column to quantify acetylene and ethylene (GC settings available in **Appendix 1.0**). Control samples of ethylene gas were also run through the GC. Nodules were freeze dried after the ARA measurement and weighed. New shrubs were measured each time, due to possible stress digging and removal of nodules had on plant health. Measurements were taken during the morning and afternoon, between 10am and 2pm, with open and closed sites switched each sampling day to avoid any temporal bias. On each sampling day between three and six shrubs were sampled per site (open and closed). Shrubs were selected at random. Measurements began in May of 2017 and continued until the end of October 2017. While ARA measurements were conducted, bud burst was monitored in the spring and leaf senescence was monitored in the fall. Bud burst was recorded when leaf emergence was first visible. Senescence was recorded if leaves dropped when the shrub was shaken lightly. The temperature of the soil and the soil moisture content around the shrubs was measured using a thermometer and a soil moisture probe (ML2x Theta Probe) approximately 20cm into the soil layer at the end of the ARA measurement.

Vesicle Infection

A sub sample of fresh nodules (approximately 20mg) were preserved in 80% alcohol. Samples were longitudinally sectioned to view vesicles at 100x magnification (Nikon, Japan). They were dyed with Fabils stain (0.5% aniline blue, 0.5% basic fuchsin, 24mM iodine and 36mM potassium iodide in lactophenol) (Noel 1964), and washed with 50% glycerol. The aniline blue stains the callose of the vesicle walls (**Figure 1.1**) Vesicle infection of the nodule was measured using a grid placed over the infection zone of the nodules. The percentage of grid boxes containing vesicles was then calculated.

2.1.3 Statistical Analysis

Soil temperature, measurement date, soil moisture content, site (open or closed) and amount of vesicle infection were included in least squares models to predict specific nodule activity. A number of models were calculated by combining the different measured parameters in both quadratic and linear fits. The AIC values of these models was used to select the best model, with non-significant variables kept in the model if they reduced the AIC value (AIC values for all models available in **Appendix 2.0**).

In addition to the least square models, the individual effects of soil temperature and soil moisture on nitrogen fixation was analysed using linear regression. All nitrogen fixation data was log+1 transformed. Some fixation data was measured at 0, necessitating the plus one transformation. Soil temperature, soil moisture and nitrogen fixation rate in the open vs. closed sites was analysed using two tailed t tests. All statistical analysis were conducted using JMP Pro 14 software (SAS Institute, USA)

2.2 Results

Bud burst occurred in most plants in both sites by late May (Julian date 144), with the expansion of new leaves occurring in late May and early June. Leaf senescence occurred by late October in most shrubs, although leaves would drop in some shrubs before this period if the shrubs were shaken, indicating abscission zone formation in some plants. Leaves remained mostly green when they fell. There was no observed difference in the timing of bud burst, leaf expansion or leaf senescence between the open and closed sites. In the 2017 growing season (May to August) precipitation was 191.2 mm (weather station located 11.7 km south east of the site). The daily temperature averaged 22.2˚C and the nightly temperature averaged 8.2˚C (weather station located 53.6 km south east of the site).

Soil temperature was significantly warmer in the open site in the spring when compared to the closed site until mid June. Around June 17th (Julian date 168,) a period of cool weather reduced the soil temperature of the open site. Soil temperature between the sites was not significantly different at any other period (**Figure 1.3a**). Soil temperature was lower than the air temperature throughout the season. This difference in temperature was largest in the spring around June 7th (Julian date 158) by approximately 14˚C. In addition, for a brief period in the fall, September 7th (Julian date 150), the soil and air temperature difference was also large. Throughout the summer, the temperature difference between the soil and air varied between 6˚C and 10˚C (**Figure 1.3b**).

The model that best predicted nitrogen fixation included all measured parameters (**Figure 1.4**). However, only soil temperature (as a linear variable) ($F_{1,114}$ = 9.89, p = 0.0402) and Julian date (as a quadratic variable) ($F_{6.144}$ = 4.31, p = 0.0022) were significant (**Table 1.5**). Date and temperature are confounding variables, as changes in date and air temperature are seasonally

linked. However, Julian date was kept in the model as it may also be a predictor of plant phenology. The model predicted peak nitrogen fixation activity (reported as specific nodule activity (SNA)) at Julian date 208 (July 27th), and an increase of 0.8μmol C_2H_4/g/h for every degree increase in soil temperature. The model formula along with additional models and their AIC values are available in **Appendix 2.0**.

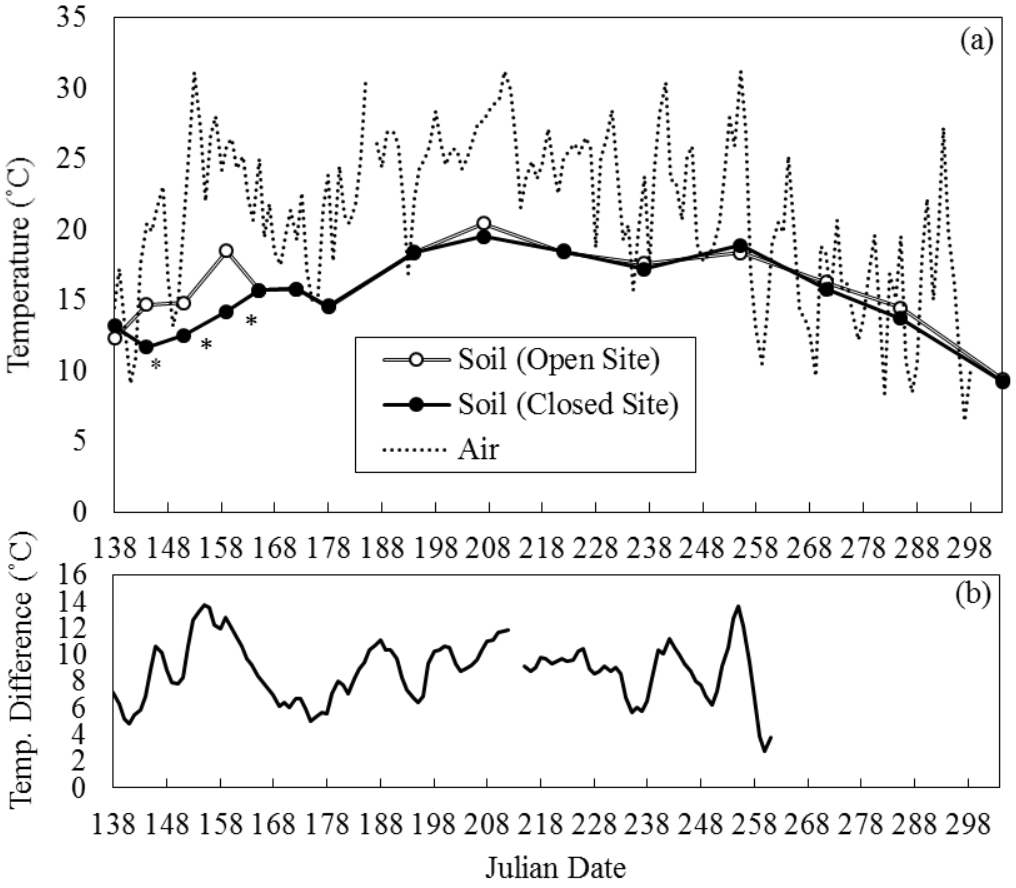

Figure 1.3 (a) Mean soil temperature from Sandilands Manitoba, measured at 10-20cm depth under *Alnus crispa* shrubs at the time of ARA measurements using a thermometer. Daily maximum air temperature data collected from the Sprague (Manitoba) weather station. (*) Significant temperature difference between sites, based on a t test. n = 3-6 per day per site. (b) Difference between the maximum air temperature (from Sprague weather station) and soil temperature (from Sandilands Manitoba) measured at 30cm depth using a HOBO four channel external temperature logger (Onset, USA).

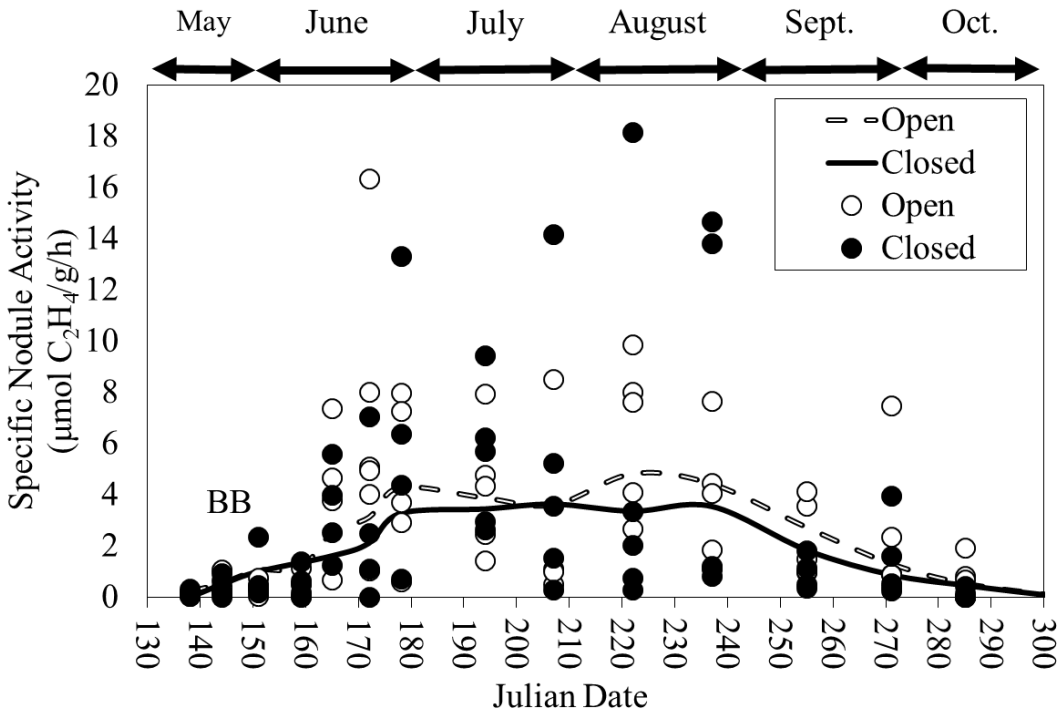

Figure 1.4 Specific nodule activity (SNA) of *Alnus crispa* nodules taken from field measurements in open (disturbed) and closed (forested) sites, measured over a growing season in 2017, using the ARA technique in Sandilands, Manitoba. Lines based on a least-squares predictive model for SNA ($r^2 = 0.49$), including date (as a quadratic variable), soil temperature, soil moisture, % vesicle infection and site (open or closed). Equation available in **Appendix 2.0**. (BB) = Bud burst. n = 3-6 on each sampling date per site.

Table 1.5 Significance of variables used in predictive model for nitrogen fixation in *Alnus crispa* shrubs from Sandilands Manitoba. AIC = 231.1 Additional models available in **Appendix 2.0**

Term	Estimate	Std Err	t Ratio	*p* Value	$F_{1,114}$ values
Intercept	1.955	0.678	2.88	0.0047	
Soil Temperature (˚C)	-0.084	0.04	-2.07	**0.0402**	9.89
Vesicle Infection (%)	$5.88x10^{-3}$	$4.24x10^{-3}$	1.39	0.1685	
Soil Moisture (%)	$-5.47x10^{-3}$	0.01	-0.53	0.5979	
Site (Open or Closed)	-0.101	$05.69x10^{-2}$	-1.78	0.0778	
Julian Date	$5.01x10^{-3}$	$1.59x10^{-3}$	3.14	**0.0022**	4.31
Julian Date^2	$-3.34x10^{-4}$	$4.9x10^{-5}$	-6.78	**<0.0001**	46.0

The specific nodule activity (SNA) was minimal in the spring months, before June 14th (Julian date: 165) at both sites. Specific nodule activity increasing steadily after this date, and remained similar throughout the rest of the summer months, with the highest SNA observed in early August (Julian date: 222). After this date, SNA showed a decline in early September, with no measurable activity by the end of October in either site (**Figure 1.4**).

Percentage of vesicle infection in the nodule lobes did not show any significance between sites (open: 50.2% ± 6.3; closed 48.1% ± 6.1, mean ± SE, $p = 0.3585$) nor was there any correlation between SNA and vesicle infection ($p = 0.1840$).

Soil temperature was significantly different between the open and closed sites in the spring, but not in the summer or fall (**Figure 1.3a**). No difference was observed between the open and closed sites for SNA at any period (**Table 1.6**). Specific nodule activity was relatively low (all rates <2μmol C_2H_4/g/h) below 14˚C. When all temperature data are included, a positive linear correlation between SNA and soil temperature is observed ($F_{1,128} = 35.73$, $p = <0.0001$; r = 0.47). When May and October values are removed, temperature no longer has a significant effect ($p = 0.5833$). When only dates after May 24th (Julian date 138) are included, temperature has a significant effect ($F_{1,111} = 19.25$, $p = 0.0001$, r = 0.47). The highest SNA was measured at 20˚C (**Figure 1.5**). The highest soil temperature recorded was 21˚C.

Soil moisture was significantly higher in the open site, compared to the closed site on only three days (July 13th, $p = 0.0008$; August 25th, $p = 0.0143$; September 12th, $p = 0.0361$). When days are grouped into seasons, soil moisture was significantly higher (by 20%) in the open site, only in the summer months (**Table 1.7**). The highest SNA values were recorded between 7% and 18% soil moisture, but the data does not show any clear trend or significant effect of soil moisture on SNA (**Figure 1.6**).

Table 1.6 Specific nodule activity (SNA) of *Alnus crispa* from field measurements in Sandilands Manitoba, between open (disturbed) and closed (forested) sites. (a) Per date, (b) Per month. (c) Per season. (*) Data from June 19th added. Mean ± SE. P values from two tailed t-test. n = 3-6 for each date at each site.

(a)

Date	Julian Date	Open	Closed	*p* Value
		SNA (μmol C_2H_4 /g/h)	SNA (μmol C_2H_4/g/h)	
May 18th	138	0.184±0.02	0.126±0.07	0.3969
May 24th	144	0.518±0.20	0.395±0.17	0.6148
May 31st	151	0.394±0.15	0.758±0.41	0.4579
June 8th	159	0.546±0.33	0.573±0.24	0.9354
June 14th	165	4.13±1.4	3.36±0.93	0.8349
June 21st*	172	6.59±2.2	2.68±1.6	0.1787
June 27th	178	4.50±1.4	6.21±2.7	0.7466
July 13th	194	4.2±1.1	5.40±1.2	0.4639
July 26th	207	2.78±1.9	4.97±2.5	0.4915
Aug 10th	222	6.46±1.3	4.93±3.4	0.2487
Aug 25th	237	3.84±1.1	6.34±3.2	0.9174
Sept 12th	255	2.24±0.67	0.965±0.33	0.126
Sept 28th	271	2.51±1.3	1.36±0.70	0.4008
Oct 12th	285	0.716±0.34	0.109±0.11	0.1754

(b)

Month	Open	Closed	*p* Value
	SNA (μmol C_2H_4 /g/h)	SNA (μmol C_2H_4 /g/h)	
May	0.365±0.09	0.448±0.16	0.6587
June	4.46±0.95	3.05±0.85	0.2146
July	3.57±1.0	5.19±1.3	0.3605
August	5.15±0.94	5.63±2.2	0.4252
September	2.38±0.68	1.18±0.40	0.0979

(c)

Season	Open	Closed	*p* Value
	SNA (μmol C_2H_4 /g/h)	SNA (μmol C_2H_4 /g/h)	
Spring	2.82±0.68	1.87±0.52	0.2501
Summer	4.4±0.69	5.40±1.3	0.9645
Fall	1.44±0.43	0.740±.28	0.0683

Spring = May and June Summer = July and August Fall = September and October

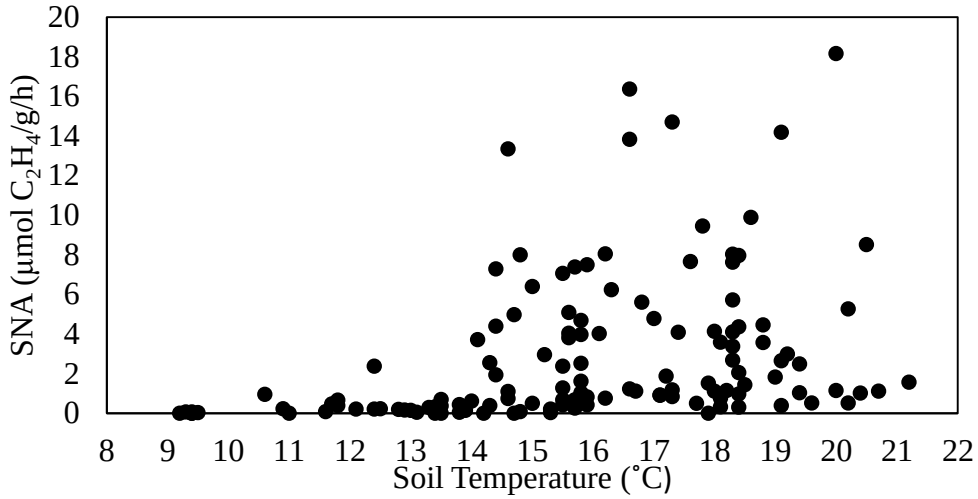

Figure 1.5 Specific nodule activity (SNA) of *Alnus crispa* nodules from Sandilands measured using the ARA technique against the soil temperature at the time of measurement (10-20cm depth). Data from open and closed (forested) sites were combined. n = 3-5 per day per site.

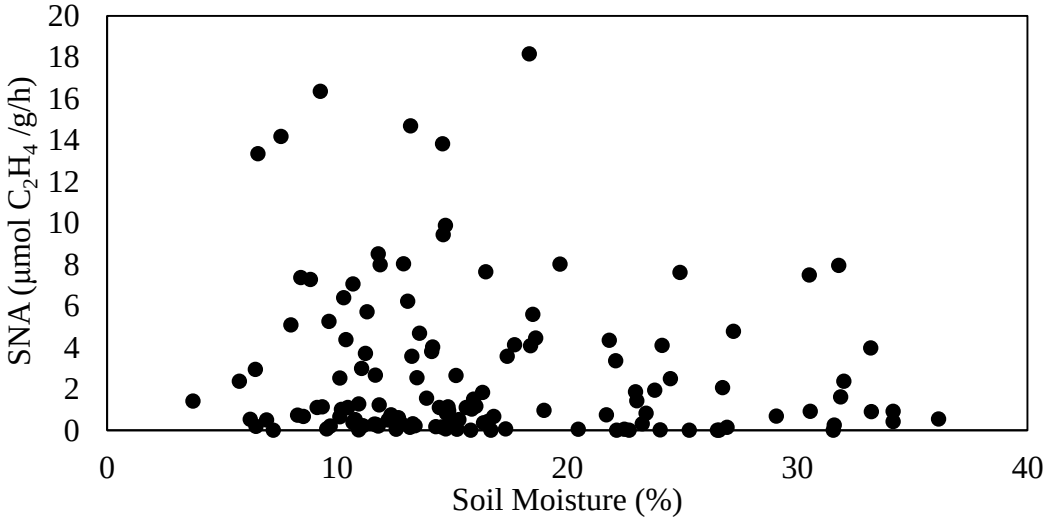

Figure 1.6 Specific nodule activity (SNA) of *Alnus crispa* nodules from Sandilands measured using the ARA technique against the soil moisture at the time of measurement (10-20cm depth). Data from open and closed (forested) sites were combined. n = 3-5 per day, per site.

Table 1.7 Soil moisture values measured at the time of SNA measurement at 10-20cm soil depth under *Alnus crispa* shrubs in Sandilands Manitoba between open and closed (forested). Mean ± SE. n = 3-6 for each day at each site.

Season	Open	Closed	*p* Value
	Soil Moisture (%)	Soil Moisture (%)	
Spring	11.9±0.56	11.1±0.63	0.3873
Summer	18.9±0.69	15.1±1.1	**0.0447**
Fall	24.5±1.4	24.5±2.1	0.9999

Spring = May and June Summer = July and August Fall = September and October

2.3 Discussion

The least squares model was able to predict 49% of the nitrogen fixation (SNA) response in *Alnus crispa* with soil temperature and date having a significant effect on nitrogen fixation (**Figure 1.4**). However, the most parsimonious model included all measured parameters regardless of their significance (**Table 1.5**). In the boreal forest, seasonal temperature changes are drastic, thus linking date and temperature together. This model is better able to predict nitrogen fixation than previous models developed by Mitchell and Ruess (2009b) on *Alnus fruticosa* in interior Alaska. Their model, containing date and soil temperature, was only able to predict 29% of a nitrogen fixation response.

As soil temperature increased it was expected to have a significant positive effect on nitrogen fixation activity in *Alnus crispa* shrubs measured over a growing season in the southern boreal forest. Generally, biological functions, including enzyme activity, decrease with temperature due to a reduction in kinetic rates under lower temperatures (Gillooly et al. 2001). Nitrogen fixation rate (SNA), when pooled between the open and closed sites, was highest when measured at soil temperatures above 14˚C. Below 14˚C little to no fixation activity (< 2 μmol/g/h) was found (**Figure 1.6**). This result alone suggests that nitrogen fixation is directly inhibited in *Alnus crispa* at soil temperatures lower than 14˚C. Lab studies on nitrogen fixation rates in soybean (*Glycine max*), and in the nitrogen fixing bacteria *Azotobacter* sp. have observed distinct breaks in the Arrhenius plots of nitrogenase at distinct temperatures, indicating increased activation energy requirements at low temperatures. These breaks were observed at temperatures of 15˚C and 22˚C, respectively (Ceuterick et al. 1978; Duke et al. 1979). The high energetic costs associated with fixation requires an adequate carbohydrate supply (Lundquist 2005). Increasing the activation energy associated with the nitrogenase enzyme would require additional

carbohydrate resources to be translocated to the nodules, effectively increasing the already high energy cost for the host species, thereby dampening any ecological advantage associated with nitrogen fixation. However, it is unlikely that this change in activation energy, if occurring, is the only mechanism affecting nitrogen fixation in my sampled plants, as this experiment was a field sampling study. Soil temperatures dropped below 14˚C only during the spring (May) and fall (October) (**Figure 1.3**). During the spring, photosynthesis does not function until buds break their dormant state. Even after bud-break, it would take time to develop mature leaves. At this time, regardless of soil temperature, fixation would be limited by the amount of photosynthates supplied to the nodules. When values below 14˚C (from May and some of October) are removed from analysis, soil temperature no longer has any significant effect on SNA. When only the dates before leaf expansion (before May 24th) are removed, the soil temperature effect on nitrogen fixation is again significant. This suggests that soil temperature does not influence nitrogen fixation activity until after some degree of leaf development occurs. The amount of stored carbohydrates in nodules is relatively low by the spring (Wheeler et al. 1983). Nodules mainly rely on newly captured carbon, which is utilized rapidly and can be supplied to the nodules within a few hours when leaves are actively photosynthesizing (Gordon and Wheeler 1978; Wheeler 1971; Lawrie and Wheeler 1975). Bud burst began in most plant samples by mid to late May, which would give plants enough time to develop leaves and begin photosynthesising around the same time as a rapid increase in nitrogen fixation activity that occurred in mid-June (Julian date 165) (**Figure 1.4**). A similar result was found by Johnsrud (1978) who reported no nitrogen fixation in *Alnus incana* until leaf development. Nitrogen fixation, while limited, is possible at soil temperatures lower than 14˚C. Results from experiment #2 of this thesis show nitrogen fixation in *Alnus crispa* can occur at 10˚C (**Figure 1.10**). In addition, lab studies on

free-living *Frankia* have also observed nitrogen fixation at temperatures as low as 10˚C (Burggraaf and Shipton 1982), and in symbiosis at temperatures as low as 8˚C (Winship and Tjepkema 1985), and 9˚C (Johnsrud 1978) in both lab and field studies, respectively. Thus, in the spring, soil temperature alone may not be the best direct indicator of nitrogen fixation response, due to a lack of plant development/carbohydrate resources available at this time. However, temperature could still indirectly effect nitrogen fixation in the spring by influencing the timing of plant development, as warmer spring weather would cause plants to break their dormancy earlier in the season.

When soil temperature data is analysed together (all months), a linear response of nitrogen fixation to temperature can be observed (**Figure 1.5**). When only summer months are analysed, temperature no longer significantly effects nitrogen fixation. This result suggests that in the summer, once soil temperatures become closer to the optimum operating temperature of the nitrogenase enzyme, temperature may not have as strong of an influence on fixation (this occurred around June 27th, Julian date 178) (**Figure 1.4**). Even though a temperature difference between the soil and air was still detected (approximately 6˚C), the soil temperature itself was around 16-20˚C during sampling days. Under these conditions nitrogen fixation would not be as severely impacted by temperature (Tjepkema and Murry 1989). The maximum soil temperature observed in the field did not exceed 21˚C **(Figure 1.5**), with the highest nitrogen fixation activity observed at 20˚C during early August (**Figure 1.5**). The overall optimum temperature of the nitrogenase enzyme, has been reported at 25˚C, estimated globally over a range of species (Houlton et al. 2008). However, this optimum may be biased, as temperature has been observed to be higher (30-35˚C) in tropical fixing species (Waughman 1977), and lower (~22˚C) in northern/arctic fixing species (Johnsrud 1978; Prevost et al. 1987). This most likely indicates a

difference in optimum operating temperatures in nitrogen fixing organisms at a regional scale. Studies in Norway on *Alnus incana* ((L.) Moench) and *Alnus glutinosa* ((L.) Gaertner) have observed optimum nitrogen fixation temperatures at 22˚C (Johnsrud 1978) and 20˚C (Akkermans 1971 in Johnsrud 1978), respectively. Because no values over 21˚C were recorded in this study, it is inconclusive what the temperature optimum for nitrogenase in *Alnus crispa* is. The optimum may be close to 20˚C, however no definite conclusion can be made from this study alone.

Soil temperature measurements were recorded between 10 and 20cm soil depth. The majority of nodules in *Alnus crispa,* in this area of the boreal, do not routinely have nodules in the upper layers of the soil horizon (personal observation). It is possible that some un-sampled nodules were operating at higher temperatures if they were present closer to the surface. It is also possible that other species of alder in the boreal may experience soil temperatures higher than the 21˚C recorded maximum from my study. For example, personal observation shows that in *Alnus incana* subsp. *rugosa* ((Du Roi) R.T. Clausen), a boreal forest alder prevalent in wet habitats, nodules are generally found at the soil surface. This may effect the response of *Alnus rugosa* to soil temperatures, as they may experience earlier soil warming in the spring and higher soil temperatures during the summer given the reduced soil depth at which nodules form relative to *Alnus crispa*. Additional studies of *Alnus rugosa* in a similar region of boreal forest, would be beneficial to understand the nitrogen fixation response of different species of alders to soil temperature in the Canadian boreal and how this response may compare to *Alnus crispa*.

During the fall, fixation gradually decreased and eventually ceased in late October (**Figure 1.4**). Unlike in the spring, no clear break in fixation activity occurred. The more gradual decline in nitrogen fixation activity suggests a stronger temperature effect on fixation as the plants and nodules move into dormancy. In the fall, most deciduous plants in northern climates

begin to lose their photosynthetic ability as chlorophyll breakdown begins, nitrogen is remobilized to the bark and roots, and leaves senesce (Chapin and Kedrowshi 1983). However, actinorhizal shrubs keep their chlorophyll for an extended period in the fall, usually dropping their leaves while still green (Cote and Dawson 1986; Cote et al. 1989). Tateno (2003) and Neave et al. (1989) both observed that photosynthetic activity was extended in *Alnus firma* (Siebold and Zucc) and *Alnus glutinosa* ((L.) Gaertner), respectively, almost a month past the non-fixing species *Morus bombycis* (Koidz.) and *Tilia heterophylla* (Ventenat). As the air and soil temperature drops, photosynthesis would be expected to decrease, resulting in reduced carbohydrates resources supplied to the nodules. Reduced carbohydrates, in combination with reductions in soil temperature would lead to a more gradual reduction in nitrogenase activity during the fall in *Alnus crispa*.

Alnus crispa shrubs in the open site were predicted to begin seasonal nitrogen fixing activity at an earlier date, compared to the closed site. Increased direct sunlight in the open was expected to increase the soil temperature, allowing for earlier soil warming and a consequential increase in nitrogen fixation activity in the spring. Even though a significant increase in soil temperature was observed in the open site in the spring (**Figure 1.3b**), fixation was not significantly different between the sites (**Table 1.6**). As discussed above, soil temperature most likely had a limited effect on fixation, as plants were emerging from their dormant state and would be limited by plant development rather than soil temperature. Results from experiment #2 of this thesis shows that when cool soil temperatures persist past bud burst and leaf development, temperature can have a significant effect on nitrogen fixation (**Figure 1.10**). More northern areas of the boreal, or sites that experience a more persistent state of cooler soil may observe a stronger soil temperature difference in the spring, leading to delayed soil warming and delayed nitrogen

fixation activity. For example, in the boreal of northern Alberta, Startsev et al. (2007) found that soil temperature at 5cm of depth did not reach 10˚C until June, and 15˚C until August, a lag of approximately 5˚C compared to my site in Southern Manitoba. The presence of an 8cm moss layer was also shown to further delay soil warming, with soils reaching 10˚C by August and not warming past 12˚C during the summer (Startsev et al. 2007).

While no significant moss layer was present in my study site to insulate the soil, a substantial amount of grass and sedge cover was present in the open site. This may have resulted in some insulating effect on the soil, which could have reduced soil warming rate in the spring. Additionally, the open site was measured nine years after a forest fire had removed all organic material (Markham and Essery 2015). In previous years, the lack of ground cover may have resulted in earlier spring soil warming in the open site, which may have increased nitrogen fixation activity during the very early stages of succession. Further studies on spring soil warming should be conducted on a different site which has been more recently disturbed. In addition, further study on my open site should be conducted, with the removal of ground cover, to further investigate this possibility.

Throughout the experimental period, no significant differences in nitrogen fixation activity was observed between the open and closed sites (**Table 1.6**). This result shows that nitrogen fixation in *Alnus crispa* was not effected by canopy shading in the closed site. The closed site had experienced ice storms in late 2012 that had decreased the canopy cover by approximately 13% (Markham et al. 2019). More recent disturbances caused by an *Ips* sp. beetle outbreak have most likely further increased the amount of light reaching the forest floor (Markham, personal communication). Thus, the closed site is not a completely closed canopy but does represent some shading which was expected to negatively impact nitrogen fixation. This

result suggests that the light level in both the open and closed sites was adequate for fixation, and not a limiting factor affecting nitrogen fixation over the growing season. This result is contrary to what was hypothesised, as increased light access was expected to benefit the nitrogen fixation potential of *Alnus crispa* in the open, as compared to the closed site. Under shaded conditions nitrogen fixation rates have decreased in *Alnus glutinosa* (Gordon and Wheeler 1978; Wheeler and Bowes 1974). In alfalfa (*Medicago sativa* (L.)) shading reduced the amount of nitrogen fixed being transferred to the host plant (Ta and Faris 1988) and in cover (*Trifolium* sp.) nodule growth was reduced under low light (Butler et al. 1959). Previous studies from the same site have found that *Alnus crispa* is able to compensate for lower available light in the forest understory by producing thinner leaves (Markham and Anderson, in preparation). Although no conclusions about leaf thickness can be made in regards to my study, it is likely that plants in the understory had a similar strategy to compensate for the reduction in light.

Low soil moisture was expected to have a significant negative effect on nitrogen fixation. Low moisture could induce a drought stress response in the host plant, reducing photosynthetic rate and leading to reduced carbohydrate allocation to nodules. It is possible that the period of low soil moisture observed in my study was not long enough to elicit any drought response that would affect nitrogen fixation.

While vesicle infection had no significant effect, previous studies on the same *Alnus crispa* site have measured the length of the vesicle infected zone in nodules, and found a significant increase during the most active summer months (Markham and Anderson, in preparation). In the summer, vesicle length may have also been increased within the nodules to allow for increased fixation during the most optimal (warmest) temperature conditions.

The nitrogen fixing activity found here is consistent with what has been reported previously from *Alnus* sp. in literature in northern temperate forests with nitrogen fixation rates peaking in mid-summer, reducing in rate until the fall, and stopping around October (Cote and Dawson 1989; Daly 1966; Lee and Son 2005; Stewart 1962). Because my model was only able to account for 49% of nitrogen fixation it is probable that additional factors impacted nitrogen fixation in this study. These factors may include, nodule age, plant reproductive status/age, herbivory, leaf area and amount of available light. *Frankia* nodules are perennial structures and age over time, where the activity of older nodules slows down compared to newer nodules (Guofan and Tingxiu 1987; Srivastava and Ambasht 1994). *Alnus crispa* that are producing reproductive structures may be shuttling more of their carbohydrate resources to those structures instead of the nodules for fixation, as observed in soybean (*Glycine max*) (Thibodeau and Jaworski 1975). Similarly, increased herbivory may affect the nitrogen fixing rate of plants, as nitrogen fixers may be targeted by herbivores as a nitrogen-rich tissue source (Vitousek and Field 1999; Vitousek and Howath 1991) a short term increase in nitrogen fixation rate can occur when exposed to herbivores (Zekveld and Markham 2011). Measuring these additional factors in future studies may help to create a better predictive model of nitrogen fixation activity in *Alnus crispa,* in this area of the southern Canadian boreal.

3.0 Experiment #2 – The effects of delayed soil warming, past bud burst, on the nitrogen fixing woody species green alder (*Alnus alnobetula* subsp. *crispa* (Aiton) Raus)

The objective of this study was to investigate how long term delays in soil warming effects nitrogen fixation, overall plant physiology and growth in *Alnus alnobetula* subsp. *crispa* (green alder). I subjected dormant green alder to various degrees of delayed soil warming by allocating different temperatures to the soil while shoot temperatures remained under constant conditions. Low soil temperature will cause a reduction in the activity of nitrogen fixation, reducing available nitrogen supplied to the host plant. This will lead to reductions in above ground photosynthetic function and result in lower overall growth in green alder.

3.1 Materials and Methods

3.1.1. Experimental Set-up

Alnus alnobetula subsp. *crispa* (hereafter called *Alnus crispa*) seeds were collected near Beaver Creek Manitoba, Canada (51°09'49.4"N 95°53'47.5"W). Seeds were stratified by placing on wet turface (Profile Products, USA) in the dark at 6°C for one month. Seeds were then sown on peat moss for one month before being transferred to sandy soil in 13cm x 13cm x 15cm pots in September of 2017. Plants were grown under light levels of 100-120 μmol/s/m^2 with a 16 hour photoperiod at a temperature of 21˚C. Soil was obtained from the Sandilands provincial forest, in a jack pine (*Pinus banksiana*) stand, with natural *Alnus crispa* in the understory (49°22'58.2"N 96°12'56.6"W). Soils are low in inorganic nitrogen (7.2 mg/kg) (Markham, unpublished), consisting mainly of coarse sand, with a soil pH of 6.6-7.3 (Manitoba Agriculture 1999). Seedlings were fertilized with Rorisons nutrient solution with reduced nitrogen (1mM)

(**Appendix 1.0**) one month after transplanting (October 2017), and twice via a foliar spray two months after transplanting (November 2017). The seedlings were inoculated with nodules obtained from *Alnus rugosa* plants growing in the Sandilands Provincial Forest. Previously frozen nodules were crushed with a mortar and pestle and mixed with distilled water. Five mL of inoculant (1mg *Frankia*/plant) was injected below the soil surface close to the roots three times over a two month period. Rorisons nutrient solution containing zero nitrogen was applied two times over the inoculating period (**Appendix 1.0**). Plants were watered as needed with distilled water.

Starting at six months of age plants were forced into dormancy by placing them in A1000 growth chambers (Conviron, Canada) set at 10˚C with 10h of light set at approximately 100 μmol/m^2/s, with minimal watering. After a few weeks plants were moved to a cold room set at 5˚C, with no light. Seven months after transplanting all plants lad lost their leaves and bud scales were visible, indicating dormancy. The dormancy period lasted for 4-6 weeks. Plants were separated into four height groups and assigned randomly within their groups to experimental boxes (i.e. each box contained a subsample from each height group).

Growth boxes were built to thermally isolate the roots from the shoots. The pots (roots) were placed in plywood boxes and the shoots were enclosed in boxes made of Lexan (Sabic, Saudi Arabia), a clear polycarbonate plastic. An insulating buffer of foil lined bubble wrap (Reflectix Inc., USA) was placed between the root and shoot sections. Micro controllers (Arduino UNO [Arduino, Italy]) were used to independently manipulate the root and shoot temperatures, (code available in **Appendix 3.0**). Temperature probes were placed in the upper and lower sections. Motor actuated vents (also controlled by the microcontrollers) allowed for the intake of warm air when needed to keep the temperatures in the boxes at the desired values,

with heat supplied form a hairdryer. Plants were watered via tubing drilled through the wood base and connected to each pot (**Figure 1.7**). Plants were watered twice per week for all treatments with distilled water. Occasionally plants at soil temperatures of 16˚C were watered three times a week due to soil drying. Plants were always watered before nitrogen fixation and photosynthetic measurements were conducted. Plants were fertilized with Rorisons nutrient solution (0N) at the beginning, and half way through (week six) the experimental period.

Shoot temperature was maintained between 20˚C and 22˚C throughout the experimental period, in all treatments. The root temperature range of the constant treatments was 16˚C (this was the highest root temperature that was possible with the set-up), 14˚C and 10˚C. In a fourth treatment the soil temperature was gradually increased over time to mimicking a natural setting (from spring to summer), called the increasing temperature (IT) treatment. For this treatment the root temperature started at 10˚C and was increased by 2˚C every two weeks until a root temperature of 16˚C was achieved. This experiment lasted a total of thirteen -fourteen weeks. This range is due to the amount of time needed to harvest the plants and conduct the final physiological measurements. The boxes were laid out in a block design (**Figure 1.8**). A total of 20 boxes were used, with 5 replicates of the 4 treatments (each box contained 4 plants, i.e. subsamples). Light levels of the boxes ranged from 60 – 105 μmol/s/m^2 PAR measured using an Li-3000C light meter (Licor, USA) (**Figure 1.9**).

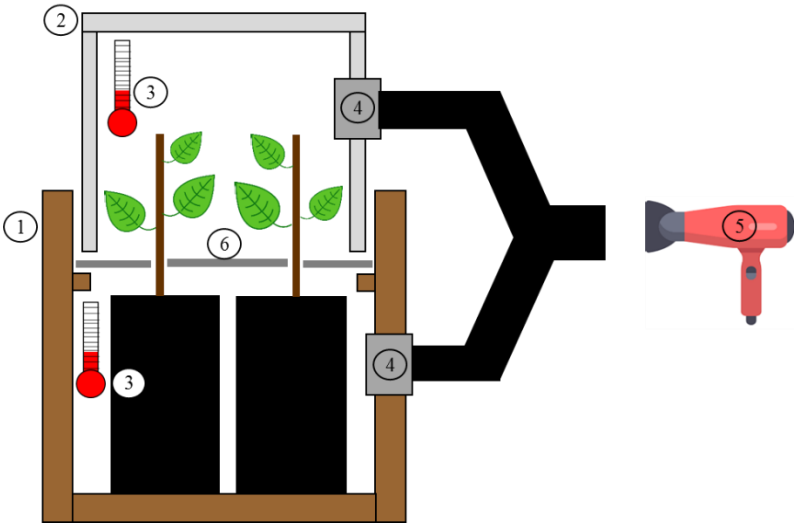

Figure 1.7 Set up of an individual growth box used during experiment #2. (1.) Wooden base. (2.) Lexan top. (3.) Temperature probe. (4.) Gates controlled by microcontroller (Arduino). (5.) Heat supply from hairdryer. (6.) Insulating material. Watering tubing not shown.

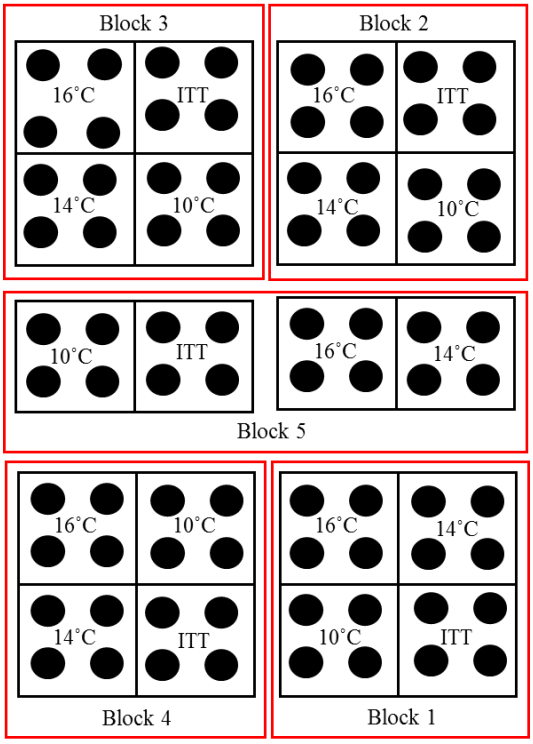

Figure 1.8 Layout of experiment #2. Circles represent individual pots. Black boxes represent an individual growth box the pots are placed in. Temperature values shown represent the root temperatures of the constant treatments. ITT = Increasing temperature treatment.

85	85	75	65
75	75	100	70
90	90	105	90
85	80	75	65
100	105	70	60

Figure 1.9 Light levels (μmol/s/m^2 PAR) for each growth box used in experiment #2.

3.1.2 Measurements

Acetylene Reduction Assay

Nitrogenase activity was measured using the acetylene reduction assay (ARA) technique (Hardy et al. 1968) to measure the nitrogen fixation rates of the nodules. Measurements were made between 10am and 4pm. Pots (containing roots) of whole plants were placed in glass boxes (1200-1600 mL) and sealed using modeling clay. Acetylene gas (200mL/box) was injected at 12-16% headspace volume. After a one hour period, a 5mL sample of gas was collected and analyzed in a Varian 3400 gas chromatograph (GC) (Varian, Canada) fitted with a 0.25 mL sample valve and a Haysep T column (GC setting details in **Appendix 2.0**). Nitrogenase activity was measured while the plants were in dormancy (week zero), and every second week up to and including week thirteen (harvest). At harvest, nodule weight was used to calculate nitrogenase activity on a nodule mass basis. Nodule mass was estimated for weeks prior to harvest by calculating the percentage of nodule mass allocation at harvest and using estimated plant weight (from height and diameter values) to estimate nodule mass at specific times (Hunt 1978). This method assumed that biomass allocation to nodules was constant over the course of the experiment.

Photosynthetic Rate

Photosynthetic rate was measured every two weeks (starting at week six) using a Li-6400 infrared gas analyser (IRGA) (Licor, USA). Measurements were conducted at a CO_2 flow rate of 400μmol/s and a light level of 700 μmol/m^2/s. Measurements were made on the second mature leaf from the top (or the first mature leaf if a second leaf was not developed) between 10am and 3pm. Any leaves too small for the IRGA chamber were traced onto clear plastic sheets and their leaf area was determined using a Li-3000C leaf area meter (Licor, USA).

Harvest

At harvest, plant tissues (leaf, stem, roots and nodules) were washed in distilled water then weighed. Total leaf area was measured using a Li-3000C leaf area meter (Licor, USA). Plant tissues were dried in a Labcono freeze drier (Labcono, USA) and their dry weight determined. Leaf tissues were then ground to homogenize samples in a Retsch 20.745.0001 mixer mill (Cole-Parmer, Canada).

Relative Growth Rate and Specific Leaf Area

Growth rate was calculated by plotting the least squares fit of the relationship between total dry tissue mass and the height of the plants multiplied by the diameter squared ($H*D^2$). The relationship was used to estimate plant mass during the experiment. Relative growth rate was then calculated as the difference in the log between the end of the experiment (week thirteen) and start of leaf development (week four) (Hunt 1978). The specific leaf area (SLA) of the plants was determined by dividing the total leaf area by the dry mass of the leaves.

Soluble Leaf Protein and Chlorophyll Content

Protein content in the leaves was determined using 100mg samples of ground dry leaf tissue. Leaf samples were added to cold phosphate buffer (chemical composition available in **Appendix 2.0**). Samples were homogenized in a tissue grinder for one minute and then placed on a shaker, on ice, for 30 minutes. Samples were then centrifuged (Avanti J-30I, Beckman Coulter, USA) at 15000g for 20 minutes (4˚C). The supernatant (200µL) was then transferred to a test tube containing 5mL of Coomassie G-250 Bradford protein dye (5 times diluted) (Bio-Rad, USA). Samples (in triplicate) were vortexed and left to react for 10 minutes. Samples were run in an Ultrospec 2100 pro spectrophotometer (Biochrom, USA) at 595 nm and compared to a bovine serum albumin (BSA) standard curve to determine soluble protein content.

Chlorophyll content of the leaves was measured by taking 20mg of ground dry leaf sample in 8mL of methanol and left overnight. The next day samples were centrifuged at 14,600rpm (Sorvall Legend 14, ThermoFisher Scientific, USA) and their absorbance read with an Ultrospec 2100 pro spectrophotometer (Biochrom, USA) at 650nm and 665nm. The following equations were used to calculate chlorophyll a and b content (MacKinney 1941):

$$\text{Chl a} = 16.5(A665) - 8.3(A650)$$

$$\text{Chl b} = 33.8(A650) - 12.5(A665)$$

^{15}N Isotope Analysis

Five mg of powdered leaf samples was sent to UC Davis Stable Isotope Facility and measured for ^{14}N and ^{15}N isotope content using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., UK). These values were then used to calculate the ratio of stable nitrogen isotopes ($\partial^{15}N$) and the nitrogen derived from fixation (%Ndfa) using the following formula:

$$\frac{\partial^{15}N(\text{Reference soil from field}) - \partial^{15}N(\text{Nitrogen fixing plant})}{\partial^{15}N(\text{Reference soil from field}) - B} * 100 = \%Ndfa$$

B = $\partial^{15}N$ of a nitrogen fixing plant grown on a medium free of nitrogen (*Alnus crispa* grown on turface in the lab).

Carbon Isotope Discrimination

The leaf ^{13}C and ^{12}C isotope content was measured using the same method as nitrogen isotope content, to determine the ratio of carbon isotopes ($\delta^{13}C$). This ratio was then used to calculate carbon discrimination (Δ) according to Farquhar et al. (1989) (shown below). Atmospheric carbon (δ_{air}) reference value was set at -0.008‰ according to the Pee Dee Belemnite carbon isotope standard.

$$\Delta = \frac{\delta_{air} - \delta_{plant}}{1 + \delta_{plant}} * 1000$$

3.1.3 Statistical Analysis

Three different types of statistical analyses were conducted. For all response variables at individual weeks (week two – week thirteen) I used least squares regression analysis with temperature as a continuous variable, and the box containing each of the four subsamples a nominal random variable (i.e. the true treatment replicate containing the four subsamples). The block effect (the location of each replicate in the growth chamber) was also added, but was removed if no significant block effect was observed. Results are shown without the block effect, except where noted. The effect of light (based on light map values, **Figure 1.9**) was added to the model (continuous variable), but was removed when not significant. Results are shown without the light effect, except where noted.

Second, photosynthesis, stomatal conductance and nitrogenase activity were analysed for overall treatment effect, time effect and treatment by time interactive effect, using a repeated measured analysis. Temperature was set as a continuous variable, time as a continuous variable and the boxes containing the subsamples as a nominal random variable. Because nodule mass was estimated from the beginning of the experiment until week ten, the nitrogen fixation values measured at harvest were not included in the repeated measures analysis. Nitrogen fixation at harvest (week thirteen) was analysed independently of other weeks, using actual nodule mass values.

Lastly, to compare the increasing temperature (IT) treatment to the constant temperature treatments, an ANOVA with each constant temperature and the increasing temperature treatment was run with the treatments as nominal variables (temperature was a continuous variable in earlier analyses when the IT treatment was not included). The boxes containing the subsamples

were set as a random nominal variable. Treatments where then compared using a Tukey post hoc test.

Nitrogenase activity values were analysed with a log (+1) transformation in all analyses. Dead and non-nodulated plants were removed from all analyses, this included two plants that died during the experiment and four additional plants that did not form visible nodules. All analyses were performed using JMP Pro 14 (SAS Institute, USA).

3.2 Results

3.2.1 The Constant Temperature Treatments

When compared within each sampling period, nitrogenase activity was significantly reduced in the 10˚C and 14˚C root temperature treatments by 80% and 37%, respectively, during week four ($F_{1,12}$ = 4.78, p = 0.0494, slope = 0.1373), by 76% and 17% during week six ($F_{1,12}$ = 5.17, p = 0.0439, slope = 0.1765), and by 70% and 40% during week eight, when compared to the 16˚C treatment (**Figure 1.10** and **Table 1.8**). No significant difference in nitrogenase activity was observed at week two, week ten, or at harvest (week thirteen). When all measuring periods are included in a complete analysis, nitrogenase activity was significantly reduced by root temperature treatments ($F_{1,12}$ = 9.78, p =0.0085, slope = 0.158). Additionally, nitrogenase activity significantly increased over time ($F_{1,222}$ = 105.1, p= <.0001, slope = 0.250). On average, between week two and week ten, the 10˚C treatment fixed 62% less ethylene than the 16˚C treatment, and 29% less ethylene was produced by plants in the 14˚C treatment.

At week four of the experiment, nitrogenase activity increased in all treatments, although this spike in activity was greater in the 16˚C (7.5 times) and 14˚C (7.0 times) root temperature treatments as compared to the 10˚C (5.5 times) treatment, on average.

By week six all plants had developed mature leaves. At weeks six and eight, no significant difference between the treatments was observed for photosynthesis. Photosynthetic activity (measured on a leaf area basis) was significantly reduced in weeks ten and thirteen (harvest) with decreasing root temperature (**Figure 1.11** and **Table 1.9**). At week ten, photosynthesis was on average reduced by 57% and 50% in the 10˚C and 14˚C treatments, respectively, compared to the 16˚C treatment. When all measurement periods (weeks) were

combined, root temperature had a significant negative effect on photosynthetic rate ($F_{1,12} = 6.63$, $p = 0.0233$, slope = 0.316). Photosynthetic rate was also significantly reduced over time ($F_{1,146} = 12.85$, $p = 0.0005$, slope = -0.190). On average, between week six and harvest (week thirteen), 39% less photosynthetic activity occurred in the 10˚C treatment and 43% less actively in the 14˚C treatment, when compared to the 16˚C treatment.

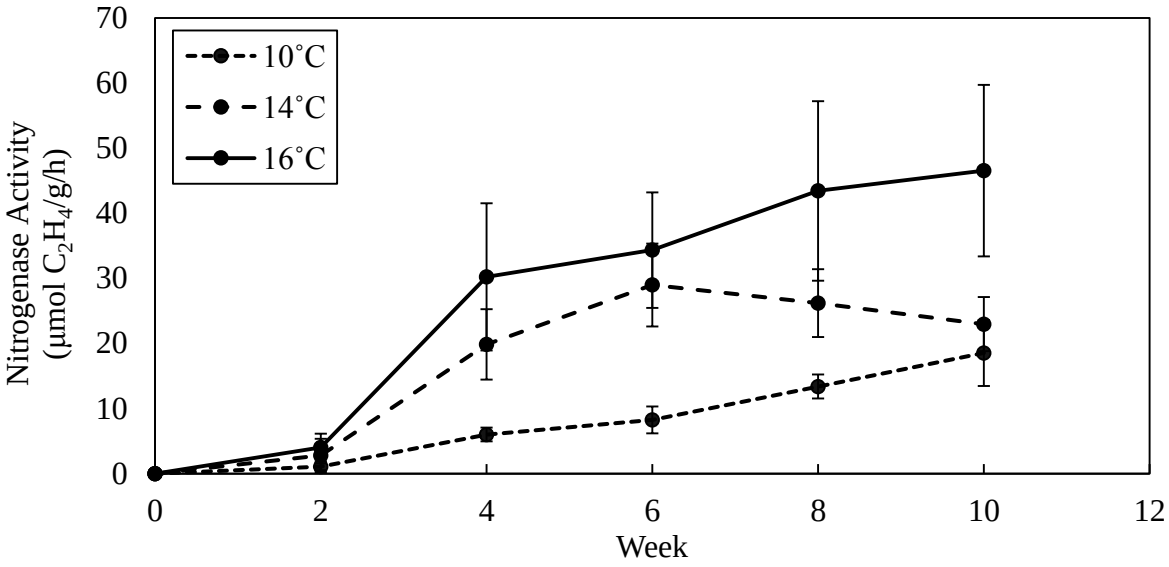

Figure 1.10 Nitrogenase activity of *Alnus crispa* nodules measured using acetylene reduction assay. Measurements began while plants were in dormancy (week zero). Plants were exposed to different root temperatures while the shoot temperature remained at 20°C. Nodule mass was estimated based on nodule mass allocation measured at harvest. Mean ± SE. n = 5.

Table 1.8 Nitrogenase activity of *Alnus crispa* plants under different root temperature treatments at individual weeks. Measurements began while plants were in dormancy (week zero). Nodule mass was estimated based on nodule mass allocation measured at harvest. At week thirteen (harvest) nitrogenase activity values were calculated using actual nodule dry mass values. Mean ± SE. n=5.

Nitrogenase Activity (µmol C_2H_4/g/h)	Soil Temperature (˚C)			*p* Value	Slope
	16	14	10		
Week 0	0	0	0	N/A	
Week 2	4.03±2.11	2.80±2.57	1.06±0.52	0.2128	
Week 4	30.23±11.31	19.86±5.39	6.02±1.07	**0.0494**	0.1373
Week 6	34.33±8.89	28.98±6.36	8.28±2.06	**0.0439**	0.1765
Week 8	43.43±13.80	26.19±5.20	13.39±1.84	**0.0037**	0.191
Week 10	46.54±13.18	22.98±4.13	18.54±5.05	0.1044	
Week 13	32.86±11.74	18.08±3.23	13.52±2.92	0.1106	

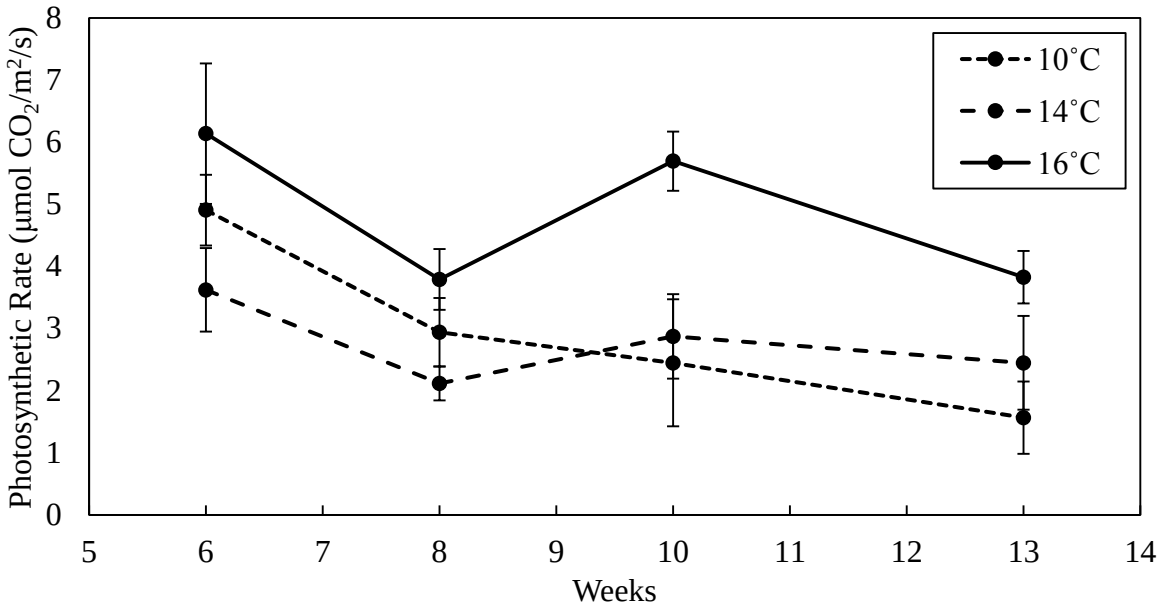

Figure 1.11 Photosynthetic rate of *Alnus crispa* over time. Plants were exposed to different root temperatures while the shoot temperature remained at 20°C. Samples were measured using an IRGA on the second mature leaf at 700 µmol photons /m^2/s. Mean ± SE. n = 5, n = 3-5 for week six.

Table 1.9 Photosynthetic rate of *Alnus crispa* plants at individual weeks. Plants were exposed to different root temperatures while the shoot temperature remained at 20°C. Measured using an IRGA on the second mature leaf. Mean ± SE. n=5, n = 3-5 for week 6 only.

Photosynthetic Rate (µmol $CO_2/m^2/s$)	Soil Temperature (˚C)			*p* Value	Slope
	16	14	10		
Week 6	6.13±0.57	3.62±0.67	4.91±1.13	0.4727	
Week 8	3.79±0.55	2.12±0.27	2.94±0.49	0.3677	
Week 10	5.69±1.02	2.87±0.68	2.45±0.48	**0.0259**	0.4805
Week 13	3.82±0.58	2.45±0.76	1.57±0.42	**0.0221**	0.3568

Leaf stomatal conductance was only significantly reduced at week ten by root temperature ($F_{1,12}$ = 10.5, p=0.0066, slope = 0.1679) (**Table 1.10**). When analysed over the complete experimental period, stomatal conductance was significantly reduced with low temperature ($F_{1,13}$ = 9.03, p = 0.0101, slope = 0.089). Transpiration followed the same trend as stomatal conductance (data not shown).

At harvest, the dry mass of the leaves, stem and roots all decreased with cooler root temperature treatments (**Table 1.11**). Compared to the 16˚C treatment, leaf mass was reduced by 40% and 50% in the 14˚C and 10˚C treatments ($F_{1,12}$ = 6.83, p = 0.0216, slope = 0.0455). Stem mass was reduced by 28% and 42% ($F_{1,12}$ = 6.44, p = 0.0248, slope = 0.0230), and by 17% and 32% in roots ($F_{1,12}$ = 7.83, p = 0.0155, slope = 0.0335). No change in nodule mass was observed. No change in the root: shoot ratio was observed. Relative growth rate (RGR) decreased by 37% and 60%, compared to the 16˚C treatment ($F_{1,12}$ = 8.73, p = 0.0115, slope = 2.080) (**Table 1.11**).

Table 1.10 Stomatal conductance of *Alnus crispa* plants under different root temperature treatments at individual weeks. Measured using an IRGA on the second mature leaf. Mean ± SE. n=5, n = 3-5 for week 6 only.

Stomatal Conductance ($mmol\ CO_2/m^2/s$)	Soil Temperature (˚C)			*p* Value	Slope
	16	14	10		
Week 6	1.34±0.14	0.68±0.14	0.98±0.27	0.3619	
Week 8	0.89±0.22	0.42±0.06	0.65±0.16	0.4834	
Week 10	1.66±0.30	0.78±0.14	0.55±0.11	**0.0066**	0.1679
Week 13	1.10±0.08	0.98±0.14	0.67±0.21	0.0592	

Table 1.11 Dry biomass and growth rate of *Alnus crispa* plants under different root temperature treatments. Dry mass and root:shoot ratio measured at harvest. Relative growth rate (RGR) measured between week 4 and harvest. Mean ± SE. n=5.

	Soil Temperature (˚C)			*p* Value	Slope
	16	14	10		
Leaf DW (g)	0.61±0.12	0.36±0.04	0.31±0.04	**0.0216**	0.0455
Stem DW (g)	0.36±0.05	0.26±0.03	0.21±0.02	**0.0248**	0.0230
Root DW (g)	0.65±0.06	0.54±0.06	0.44±0.04	**0.0155**	0.0335
Nodule DW (g)	0.034±0.009	0.020±0.003	0.020±0.005	0.2306	
RGR (mg/g/week)	22.0±3.26	13.9±2.88	8.86±2.90	**0.0115**	2.081
Root:Shoot	0.79±0.06	0.94±0.03	0.95±0.03	0.0760	

Total leaf area was significantly lower under cooler root temperatures by 56% and 67% ($F_{1,12}$ = 5.98, p = 0.0295, slope = 16.75) (**Table 1.12**). The specific leaf area (SLA) was significantly decreased (by 23% and 34%) with decreased root temperature ($F_{1,12}$ = 14.36, p = 0.0043, slope = 12.05) (**Table 1.12**), a block effect was also significant ($F_{4,9}$ = 3.57, p = 0.0496).

The chlorophyll content was significantly reduced (by 13% and 28%) with lower root temperatures when analysed for chlorophyll a ($F_{1,11}$ = 19.19, p = 0.0011, slope = 0.2184). Variation in light level in the room also had a significant effect ($F_{1,11}$ = 7.22, p = 0.0201, slope = -0.0259) on chlorophyll a (**Table 1.12**). Chlorophyll b was also significantly reduced under low root temperature treatments (by 10% and 23%), ($F_{1,10}$ = 5.33, p = 0.0420, slope = 0.0723), with a significant light level effect ($F_{1,11}$ = 4.90, p = 0.0487, slope = -0.0130) (**Table 1.12**). The ratio of chlorophyll a/b was not significant different between treatments (data not shown). Leaf protein did not change between root temperature treatments (**Table 1.12**).

The amount of nitrogen derived from fixation in the leaves decreased with lower root temperatures, by 6% and 12.5% ($F_{1,12}$ = 11.37, p = 0.0051, slope = 1.862) (**Table 1.13**) . The carbon to nitrogen ratio of the leaves increased with lower root temperatures by 9% and 19% ($F_{1,12}$ = 28.14, p = 0.0002, slope = -1.047) (**Table 1.13**). Carbon isotope discrimination was reduced with low root temperature by 2% and 4% ($F_{1,12}$ = 7.41, p = 0.0177, slope = 0.1844) (**Table 1.13**).

Table 1.12 Leaf area, chlorophyll and soluble protein content of *Alnus crispa* plants under different root temperature treatments. Values measured at harvest. Mean ± SE. n=5. (*) Block effect (*p* =**0.0496**). (**) Light effect (*p* = **0.0201**[Chl a]) (*p* = **0.0487**[Chl b]).

	Soil Temperature (˚C)			*p* Value	Slope
	16	14	10		
Leaf Area (cm^2)	164±44.5	71.8±12.6	54.3±9.1	**0.0295**	16.75
SLA (cm^2/g)	239±26.2	185±12.7	160±10.5	**0.0043***	0.0043
Chl a (mg/g)	4.63±0.25	4.01±0.39	3.34±0.12	**0.0011****	0.2184
Chl b (mg/g)	1.68±0.11	1.52±0.19	1.29±0.18	**0.0420****	0.0723
Protein (mg/g)	41.9±2.88	33.4±3.64	37.78±3.05	0.6594	

Table 1.13 Carbon and nitrogen measurements from leaves of *Alnus crispa* plants under different temperature treatments. Values measured at harvest. Ndfa = nitrogen derived from fixation. CΔ = carbon isotope discrimination. Mean ± SE. n = 5

	Soil Temperature (˚C)			*p* Value	Slope
	16	14	10		
Ndfa (%)	88.8±2.17	83.4±2.55	77.7±2.48	**0.0051**	1.862
C:N	27.7±0.56	30.4±1.22	34.1±0.69	**0.0002**	-1.047
C Δ (‰)	25.18±0.26	24.73±0.36	24.06±0.25	**0.0177**	0.1844

3.2.2 The Increasing Temperature (IT) Treatment

At weeks four, six and eight no difference for nitrogenase activity was detected between the increasing temperature (IT) treatment and the other treatment means based on a Tukey post hoc test (**Table 1.14**). In the IT treatment, 53% less ethylene on average was fixed compared to the 16˚C treatment. Using a Tukey test, no significant difference was observed between the IT treatment and other root temperature treatments for photosynthesis (**Table 1.15**). Stomatal conductance in the IT treatment was only significantly different from the 16˚C treatment during week ten (**Table 1.10**). No significant difference between the IT treatment and constant temperature treatments was observed for tissue dry mass, RGR, root:shoot ratio (**Table 1.17**), leaf area or SLA (**Table 1.18**). The C:N ratio was significantly lower in the IT treatment compared to the 10˚C treatment by 18% (**Table 1.19**). Nitrogen derived from fixation and carbon discrimination were not significantly different in the IT treatment compared to the regular treatments. Chlorophyll a was significantly higher (28%) in the IT treatment from the 10˚C treatment at harvest (**Table 1.18**). Chlorophyll b and leaf soluble protein showed no significant difference.

Table 1.14 Nitrogenase activity of *Alnus crispa* plants under an increasing temperature (IT) treatment at individual weeks. Plants began in dormancy (week 0). Nodule mass was estimated based on nodule mass at harvest. At week thirteen nitrogenase activity values were calculated using actual nodule dry mass values. Letters represent significant difference based on a Tukey post hoc test. Mean ± SE. n=5.

Nitrogenase Activity (μmol C_2H_4/g/h)	Soil Temperature (˚C)			ITT	ITT soil temperature at measurement (˚C)
	16	14	10		
Week 0	0	0	0	0	N/A
Week 2	4.03±2.11a	2.80±2.57a	1.06±0.52a	1.23±0.77a	10
Week 4	30.23±11.31a	19.86±5.39a	6.02±1.07a	16.07±4.83a	12
Week 6	34.33±8.89a	28.98±6.36a	8.28±2.06a	20.16±7.29a	14
Week 8	43.43±13.80a	26.19±5.20ab	13.39±1.84b	30.54±8.15ab	16
Week 10	46.54±13.18a	22.98±4.13a	18.54±5.05a	16.74±4.34a	16
Week 13	32.86±11.74a	18.08±3.23a	13.52±2.92a	24.11±5.13a	16

Table 1.15 Photosynthetic rate of *Alnus crispa* plants under an increasing temperature treatment (ITT) at individual weeks. Measured using an IRGA on the second mature leaf. Letters represent significant difference based on a Tukey post hoc test. Mean ± SE. n=5, n = 3-5 for week 6 only.

Photosynthetic Rate (μmol $CO_2/m^2/s$)	Soil Temperature (˚C)			ITT	ITT soil temperature at measurement (˚C)
	16	14	10		
Week 6	6.13±0.57a	3.62±0.67a	4.91±1.13a	3.92±0.78a	14
Week 8	3.79±0.55a	2.12±0.27a	2.94±0.49a	2.67±0.55a	16
Week 10	5.69±1.02a	2.87±0.68ab	2.45±0.48b	3.15±0.36ab	16
Week 13	3.82±0.58a	2.45±0.76a	1.57±0.42a	2.53±0.68a	16

Table 1.16 Stomatal conductance in *Alnus crispa* plants under an increasing temperature treatment (ITT) at individual weeks. Measured using an IRGA on the second mature leaf. Mean ± SE. n=5, n = 3-5 for week 6 only.

Stomatal Conductance (mmol $CO_2/m^2/s$)	Soil Temperature (˚C)			ITT	ITT soil temperature at measurement (˚C)
	16	14	10		
Week 6	1.34±0.14a	0.68±0.14a	0.98±0.27a	0.92±0.25a	14
Week 8	0.89±0.22a	0.42±0.06a	0.65±0.16a	0.49±0.14a	16
Week 10	1.66±0.30**a**	0.78±0.14**b**	0.55±0.11**b**	0.66±0.08**b**	16
Week 13	1.10±0.08a	0.98±0.14a	0.67±0.21a	0.81±0.12a	16

Table 1.17 Dry biomass and growth rate of *Alnus crispa* plants under an increasing temperature treatment (ITT). Dry mass and root:shoot ratio measured at harvest. Relative growth rate (RGR) measured between week 4 and harvest. Letters represent significant difference based on a Tukey post hoc test. Mean ± SE. n=5.

	Soil Temperature (˚C)			ITT
	16	14	10	
Leaf DW (g)	0.61±0.12a	0.36±0.04ab	0.31±0.04b	0.45±0.07ab
Stem DW (g)	0.36±0.05a	0.26±0.03ab	0.21±0.02b	0.26±0.03ab
Root DW (g)	0.65±0.06a	0.54±0.06ab	0.44±0.04b	0.53±0.05ab
Nodule DW (g)	0.034±0.009a	0.020±0.003a	0.020±0.005a	0.022±0.005a
RGR (mg/g/week)	22.0±3.26a	13.9±2.88ab	8.86±2.90b	14.7±2.49ab
Root:Shoot	0.79±0.06a	0.94±0.03ab	0.95±0.03b	0.79±0.03ab

Table 1.18 Leaf area, chlorophyll and soluble protein content of *Alnus crispa* plants under an increasing temperature treatment (ITT). Values measured at harvest. Letters denote significant difference from a Tukey Post Hoc test. Mean ± SE. n=5.

	Soil Temperature (˚C)			ITT
	16	14	10	
Leaf Area (cm^2)	164±44.5a	71.8±12.6ab	54.3±9.1b	99.1±24.2ab
SLA (cm^2/g)	239±26.2a	185±12.7ab	160±10.5b	198±16.8ab
Chl a (mg/g)	4.63±0.25**a**	4.01±0.39**ab**	3.34±0.12**b**	4.62±0.28**a**
Chl b (mg/g)	1.68±0.11a	1.52±0.19a	1.29±0.18a	1.93±0.30a
Protein (mg/g)	41.9±2.88a	33.4±3.64a	37.78±3.05a	40.46±2.30a

Table 1.19 Carbon and nitrogen measurements from leaves of *Alnus crispa* plants under an increasing temperature treatment (ITT). Values measured at harvest. CΔ = carbon isotope discrimination. Letters denote significance from a Tukey Post Hoc test. Mean ± SE. n = 5

	Soil Temperature (˚C)			ITT
	16	14	10	
Ndfa (%)	88.8±2.17a	83.4±2.55ab	77.7±2.48b	83.1±1.80ab
C:N	27.7±0.56**b**	30.4±1.22**ab**	34.1±0.69**a**	27.8±1.40**b**
C Δ (‰)	25.18±0.26a	24.73±0.36a	24.06±0.25a	24.8±0.21a

3.3 Discussion

3.3.1 The Constant Temperature Treatments

The relative growth rate, total leaf area and overall biomass of *Alnus crispa* was reduced with decreasing soil temperature (**Table 1.11** and **Table 1.12**). These results were expected, as low soil temperatures effected both nitrogenase activity and photosynthetic activity. Inadequate availability to fix nitrogen at low soil temperatures would reduce nitrogen availability, reducing chlorophyll formation and subsequent photosynthetic activity. Overall this reduction in nitrogen and carbohydrates would limit new tissue development resulting in a low growth rate.

Nitrogen fixation rate (as measured by nitrogenase activity) was expected to decrease when exposed to root temperatures lower than 16˚C. When analysed on a per week basis, nitrogen fixation activity remained minimal by week two, with no significant temperature effect (**Table 1.8**). As was discussed in experiment #1, lack of leaf development at this time would most likely limit nitrogen fixation activity, regardless of soil temperature. By this stage of the experiment most plant samples had not yet burst bud to allow for the assimilation of necessary carbohydrates to promote nodule function. By week four, the nitrogen fixation rate had increased in all treatments. A significant temperature effect was observed at weeks four, six and eight (**Table 1.8**), and when analysed over the entire experimental period. The reason for reduced nitrogen fixation activity at low temperatures most likely stems from a number of influencing factors. In general, enzyme kinetics slow at low temperatures, resulting in reduced activity (Gillooly et al. 2001). In regards to the nitrogenase enzyme, a number of studies have also attributed reductions in fixation activity to additional inhibitory influences at specific low temperatures. Duke et al. (1979) observed an increase in the activation energy associated with the nitrogenase enzyme at soil temperatures below 15˚C in soybean (*Glycine max*). In *Alnus*

rubra Winship and Tjepkema (1985) observed a rapid increase in nodule respiratory cost at soil temperatures below 16˚C. Increasing the already high cost associated with nitrogen fixation can make nitrogen fixation unfavorable to the host plant. In studies involving free living nitrogen fixing bacteria, Thorneley et al. (1975), attributed changes in fixation activity below 17˚C in *Klebsiella pneurnoniae*, to reductions in the stability of the nitrogenase protein sub-complex. Additionally, in *Azotobacter vinelandii*, Ceuterick et al. (1978) concluded that changes in lipids associated with the nitrogenase enzyme played an important role in effecting functionality at temperatures below 22˚C. The exact mechanism by which the nitrogenase enzyme was effected at low temperatures was not examined in this study, and therefore I was unable to evaluate if activation energy, protein stability or lipid composition of the nitrogenase enzyme had any effect on fixation activity at the soil temperatures studied.

Nodule growth was also anticipated to reduce under low root temperature treatments. However, no change in the dry mass of the nodules was observed at the end of the experimental period (**Table 1.11**). In soybean (*Glycine max*), nodule infection and growth was delayed below 17˚C (Zhang and Smith 1994). However, the perennial nature of actinorhizal nodules makes it difficult to compare growth rates to soybean. Winship and Tjepkema (1985) observed no growth in isolated *Frankia* at 10˚C. However, because I used symbiotically associated *Frankia*, the growth response to temperature may be different compared to free-living bacteria. The only conclusion that can be made from the available data is that at soil temperatures below 16˚C negatively impact the rate of nitrogen fixation in *Alnus crispa*, resulting in reduced fixation of atmospheric nitrogen, but do not effect nodule growth.

Under low root temperature treatments, the photosynthetic rate was anticipated to follow a similar trend to nitrogenase activity. Low soil temperature was anticipated to reduce the

availability of fixed nitrogen, resulting in a decrease in the formation of nitrogenous compounds such as chlorophyll, leading to reduced photosynthetic activity. When analysed over the experimental period, root temperature had a significant effect on photosynthetic rate (**Figure 1.10**). However this was not immediately observed when photosynthetic rate was analysed on a per week basis (**Table 1.9**). In the herbaceous annual plant soybean (*Glycine max*), low root temperature (13˚C) negatively impacted both nitrogen fixation and photosynthesis continuously throughout a fifteen week experimental period (Duke et al. 1979), suggesting an immediate negative effect of reduced nitrogen fixation on photosynthetic rate. Even though nitrogen fixation was reduced in my study, at weeks six and eight (**Figure 1.9**), a resulting decrease in photosynthetic rate was not detected until week ten (**Figure 1.10**). Photosynthetic rate was expected to decrease early on in the experiment, as less nitrogen fixation would mean less nitrogen was available for chlorophyll production. This result suggests that initially as *Alnus crispa* were coming out of dormancy and forming leaves, they were utilizing a supply of nitrogen not immediately derived from fixation. Non-actinorhizal woody species often utilize stored nitrogen in the spring for new leaf development. Millard (1994) found that in maple (*Acer pseudoplatanus* (L.)), 1/3 of nitrogen from newly developed leaves in the spring, was derived from overwintering stored nitrogen. Actinorhizal species however, do not generally reabsorb large amounts of leaf nitrogen, compared to non-actinorhizal species (Dawson and Funk 1981; Kurdali 2000). Over wintering storage of nitrogen in tissues has not been well documented in alder or other actinorhizal species. However, Kurdali (2000) did not observe a large change in the nitrogen content of the bark leading up to winter dormancy in oriental alder (*Alnus orientalis* (Decne.)). Thus, it is difficult to conclude that *Alnus crispa* was utilizing stored nitrogen for chlorophyll development during weeks six and eight. By week ten, photosynthetic rate was

significantly reduced by low soil temperature, with the lowest photosynthetic rate observed at 10˚C (**Table 1.9**). At week thirteen, a significant reduction in chlorophyll concentration was also observed (**Table 1.12**). These results suggest that plants under warmer root treatments were able to produce more chlorophyll due to an increased supply of nitrogen derived from fixation (**Table 1.13**), resulting in a higher photosynthetic rate. Even though the shoots of all the treatments were at 20˚C, soil temperatures below 16˚C had a significant effect on the ability of *Alnus crispa* to photosynthesise.

The photosynthetic rate of *Alnus crispa* was reduced under low soil temperatures, most likely via a reduction in chlorophyll production. However, additional stresses may have also impacted photosynthetic rate. Under cooler root temperatures the absorption of water may decline due to an increase in viscosity (Wan 2000; Wan et al. 1999) and a reduction in new root growth (Andersen et al. 1986). This can result in a drought stress response at low soil temperatures. A common drought response in plants is the closure of their stomates to reduce water loss, which consequently decreases photosynthetic activity (Agurla et al. 2018). Stomatal conductance and transpiration were significantly reduced by low soil temperature when analysed over the experimental period and at week ten when analysed on a per week basis (**Table 1.10**). In cotton (*Gossypium hirsutum* (L.)) and clover (*Triflorium repens* (L.)) root temperatures of 10˚C induced rapid reductions in stomatal conductance, transpiration and photosynthesis (Cox and Boersma 1967; Troughton 1969). In birch (*Betula papyrifera* (Marshall)), stomatal conductance and photosynthesis was reduced at root temperatures below 15˚C (Landhausser et al. 1996). However, this response to low soil temperatures has not always been observed. In willow (*Salix scouleriana* (Barratt ex Hooker)) at root temperatures of 3˚C, (Anderson and McNaughton 1973) and in poplar (*Populus balsamifera* (L.)) at a root temperature of 10˚C (Landhausser et al. 1996),

no change in stomatal conductance was observed. In addition to reductions in stomatal conductance, decreased carbon discrimination values at cooler root temperature treatments were also observed (**Table 1.13**). The rubisco enzyme discriminates against the ^{13}C isotope during photosynthesis (Farquhar et al. 1982). This discrimination is thought to decrease when under conditions that necessitate the closure of the stomates (Farquhar et al. 1989). Thus, carbon discrimination can potentially indicate some degree of stomatal closure and water stress. In addition to low temperature induced stomatal closure, low nitrogen availability may also influenced stomatal closure to some extent. In hydroponically grown lettuce (*Lactuca sativa* (L.)) and tomato (*Lycopersicon esculentum* (Miller)), decreases in stomatal conductance were observed when nitrogen was removed from the hydroponic solution. The authors attributed this this to increases in leaf abscisic acid, (Broadley et al. 2001; Chapin et al. 1988), although this phenomenon has not been studied in any nitrogen fixing species. The reduction in nitrogen fixation activity (**Table 1.8**) and increase in leaf C:N ratio at harvest (**Table 1.13**) suggests some degree of nitrogen stress in *Alnus crispa* under low root temperature treatments, which may have had a minor effect on stomatal conductance. In non-inoculated *Alnus crispa* grown under root temperature of 5˚C, no change in photosynthetic activity was observed when supplied with a full strength nitrate fertilizer (15 mM) (Lawrence and Oechel 1983). This suggests that photosynthesis in *Alnus crispa* at lowered root temperatures was most likely primarily inhibited by a lack of nitrogen derived photosynthetic pigments. However, photosynthesis may have also been effected via stomatal closure, which may have resulted from either a water stress response, a nitrogen stress response, or a combination of both. In addition, a reduction in the uptake and translocation of other nutrients from the soil may have also occurred at low root temperatures. In banana (*Musa* sp. (L.)) 10°C soil reduced the uptake of boron, iron, potassium, calcium and

sodium (Turner and Lahav 1985). However, because no analysis of the nutrient content in *Alnus crispa* (except for nitrogen) was conducted, no conclusions can be made to the uptake of other nutrients at low soil temperature in this study, and their possible effect on photosynthetic rate.

Water stress has also been observed to directly affect nitrogen fixation in some leguminous species. Limited transpiration may result in the buildup of nitrogenous compounds in the nodules and roots. Nitrogen, when at a high enough concentration in the tissues, can limit both the activity of the nitrogenase enzyme, and the formation of new nodules (Arnone et al. 1994; Waughman 1977). Studies on soybean (*Glycine max*), observed increase in the ureide content of the roots and nodules as well as decreases in nitrogen fixation activity when under drought conditions (King and Purcell 2005; Ladrera et al. 2007). However, separate studies under drought in common bean (*Phaseolus vulgaris* (L.)), and cool root temperature conditions in lupine (*Lupinus albus*), showed a reduction in fixation activity, but no corresponding accumulation of nitrogenous compounds in the nodules or roots (Coleto et al. 2014; Legros and Smith 1994). No studies could be found in regards to the accumulation or lack thereof of nitrogenous compounds in *Alnus* spp. or other actinorhizal species when exposed to either low temperature or drought conditions. Thus, it cannot be concluded if any nitrogen buildup is occurring in *Alnus crispa* at low soil temperatures that would impact nitrogen fixation rate. Nitrogen analysis of the nodules and root tissues in future studies of *Alnus crispa* under low root temperatures would be beneficial to investigate this possibility.

The protein and nitrogen content in the leaves was expected to reduce under cool root treatment due to a lack of nitrogen fixation. The increase in C:N ratio in the leaves of plants at 10˚C and 14˚C suggests less nitrogen was available. However, no change in soluble leaf protein was observed (**Table 1.12**), which was not expected. Approximately 20%-30% of soluble protein

in the leaves is found in the photosynthetic enzyme rubisco (Evans 1989). The reduction in chlorophyll concentration, but not soluble protein concentration in the low temperature treatments suggests that non-photosynthetic leaf proteins were being produced at a higher quantity under low root temperatures. In leaves of cucumber (*Cucumis sativus* (L.)) and lettuce (*Lactuca sativa* (L.)), cool root temperatures of 12˚C and 10˚C, respectively, resulted in observed increases in the concentration of the reactive oxygen species (ROS) hydrogen peroxide and the antioxidant enzyme superoxide dismutase (Qiu-Yan et al. 2013; Sakamoto and Suzuki 2015), potentially due to disruptions in cellular respiration in the roots at low temperatures. In *Alnus crispa*, plants exposed to cooler root temperatures may have increased their antioxidant production to combat the formation of ROS. However, further studies investigating this would have to be conducted to investigate this possibility.

Nitrogen fixing plants can supplement nitrogen fixation by obtaining additional nitrogen from the soil. When compared between treatments, plants at low soil temperatures obtained less of their overall leaf nitrogen from atmospheric fixation (**Table 1.13**). The cost of nitrogen uptake is much lower than nitrogen fixation (**Table 1.3**). Additionally, nitrogen fixation costs are intensified at low temperatures (Winship and Tjepkema 1985). As nitrogen fixation becomes more energetically costly at low temperatures, a strategy of preferentially increasing root growth was expected to occur in *Alnus crispa* to enable increased inorganic nitrogen uptake at low temperature treatments. Under low nutrient conditions it is generally thought that plants will selectively favour increasing root biomass, over shoot biomass, to facilitate nutrient capture (Bloom et al. 1985). Root dry mass was significantly reduced with low root temperature (**Table 1.11**), as low temperature and reduced availability of nitrogen and carbohydrates limited the formation of new roots. However, no significant difference between the root:shoot ratio was

observed at the end of the experimental period (**Table 1.11**). Differences in root biomass and growth between individual plants before the experiment began may have impacted the results of the root:shoot ratio measurement. The root mass was not known when the experiment started, as plants were grown without any temperature stresses for a six month period before being placed into dormancy. Thus, it is difficult to conclude if *Alnus crispa* were, or were not allocating more biomass to the roots when exposed to low root temperature treatments, as a way to supplement nitrogen fixation.

The specific leaf area (SLA) was significantly reduced with low root temperature treatments (**Table 1.12**). Studies have found that plants with lower specific leaf areas can be indicators of increased leaf toughness and defence against herbivores, as thicker leaves are harder to ingest (Caldwell et al. 2016). Fast growing plants are often more targeted by herbivores due to increased palatability and nutrient content (Coley 1988). The lower specific leaf area, low relative growth and high C:N ratio in the 10˚C treatment indicate some resilience to herbivores. Any nitrogen lost to herbivores would be difficult to get back at low root temperatures, due to a reduction in nitrogen fixation. Increases in leaf toughness may have resulted as an inadvertent defensive strategy under suboptimal growth conditions, due to a lack of overall leaf growth and available nitrogen. Future studies would benefit from the inclusion of herbivory choice trials to understand the effect low root temperatures may have on herbivore defence in *Alnus crispa*.

3.3.2 Increasing Temperature (IT) Treatment

The increasing temperature (IT) treatment was conducted to examine *Alnus crispa* response to a soil temperature regime that more closely follows seasonal soil temperature changes in a natural boreal forest environment. Under this treatment, the root temperature began at 10˚C and was slowly increased to 16˚C. Roots were set at 16˚C for only the second half of the experimental period (seven weeks in total). It was expected that once a soil temperature of 16˚C was reached nitrogen fixation rate would increase and by the end of the experimental period and become significantly higher than the nitrogen fixation rates of the constant treatments set at 14˚C and 10˚C. Increased fixation would result in increased nitrogen supplied to the host plant and in turn, increased chlorophyll production. This would then lead to an increase in photosynthetic rate in the IT treatment by the end of the experimental period. The nitrogen fixation rate was not significantly different between the IT treatment and any of the constant treatments, based on a Tukey test. The acetylene reduction assay method used to determine nitrogenase activity is a short term measurement, and can produce results with a high variability. Leaf C:N, because it is calculated using isotopes, can be a more reliable indicator of long term nitrogen fixation activity. Even though the fixation activity did not significantly differ between the treatments, the significant difference in the leaf C:N ratio between the IT treatment and the constant 10˚C treatment at harvest, suggests that in the IT treatment more nitrogen was supplied to the host plant (**Table 1.19**). A significant increase in chlorophyll formation in the leaves under the IT treatment by the end of the experimental period was also observed when compared to the 10˚C treatment, most likely resulting from increased nitrogen availability (**Table 1.18**). However, this increase in chlorophyll did not result in a corresponding increase in photosynthetic rate, as was expected (**Table 1.15**). It is difficult to determine why photosynthetic rate would not become

significantly higher in the IT treatment compared to the 10˚C treatment when more chlorophyll was present. A significant difference in stomatal conductance between the IT treatment and the 16˚C treatment suggest some stomatal closure at week ten. However, the carbon discrimination in the IT treatment was not different from the other treatments, which suggests no long term stomatal closure (**Table 1.19**). Additionally, no difference in the root mass at harvest or the root:shoot ratio suggest no reduction in root growth which may have occurred during the initial weeks of cool soil temperatures that could have led to lower water uptake and subsequent drought stress (**Table 1.17**). Thus, drought induced stomatal closure is not a likely explanation for the lack of increased photosynthetic rate.

Based on data from experiment #1, the growing season of *Alnus crispa* in the southern boreal is approximately twenty weeks (**Figure 1.3**). By week sixteen the air temperature starts to decrease, which may impact photosynthetic rate and nitrogen fixation. Approximately three - four weeks of additional photosynthetic activity can potentially be utilized by *Alnus crispa* in the field. If the experimental period was lengthened, past thirteen weeks, a significant difference in photosynthetic rate in the IT treatment may have been observed.

Overall, delayed soil warming that more closely reflected natural variations in soil temperature resulted in nitrogen fixation, photosynthesis and relative growth rate that were not statistically different from the constant root treatments at 16˚C, 14˚C or 10˚C.

4.0 Conclusions

Results from experiment #1 show that in the southern Canadian boreal, soil temperature was constantly below air temperature during the spring and summer months, with the greatest difference in temperature occurring during the spring. Nitrogen fixation did not begin until leaf development had occurred. The soil temperature in the open site warmed faster than the closed site in the spring, however this increase in soil temperature had no effect on nitrogen fixation rate, most likely due to the lack of leaf development. In the summer soil temperatures were closer to the optimum temperature of the nitrogenase enzyme, which would result in increased fixation activity. At this time soil temperature may not be as significant in effecting nitrogen fixation compared to the spring and fall.

Future studies that measure additional factors, including reproductive status, light level and herbivory may be beneficial in producing a better predictive model of nitrogen fixation activity response in *Alnus crispa* at this site. It is also recommended that future studies be conducted on alternate sites that are at an earlier stage of succession (<10 years after disturbance), as reductions in ground cover during this stage of development may increase soil temperature and nitrogen fixation response.

Results from experiment #2 show that extended periods of delayed soil warming, past leaf development, result in reductions in the ability of *Alnus crispa* to fix nitrogen. Additionally, the photosynthetic rate, even though it was kept at a constant temperature, was also effected by delayed soil warming, via a reduction in chlorophyll production and to a lesser extent by some degree of stomatal closure. After dormancy, for a short period, *Alnus crispa* may have been relying on stored nitrogen for the formation of photosynthetic pigments, however, additional studies will need to be conducted to confirm this. Future studies on extended delays in soil

warming would also benefit from measuring the nitrogen content in the nodules to further understand how low soil temperature impacted nitrogen fixation in *Alnus crispa*.

Overall, delays in soil warming and low soil temperatures reduce nitrogen fixation. When these delays are extended past bud burst and leaf development they can also cause reductions in the photosynthetic rate. Together these reductions in nitrogen fixation and photosynthesis can lead to reduced growth in *Alnus crispa*. This may limit the success and abundance of *Alnus crispa* and other alder species in the boreal forest compared to non-fixing species that do not rely on fixed nitrogen.

In the future, soil warming in the spring is anticipated to occur at an earlier date in the boreal forest due to climate change (Serreze et al. 2000). Earlier and faster soil warming may limit the negative effect that low soil temperature has on the nitrogen fixation response in *Alnus crispa* in the spring, allowing for potentially extended period of nitrogen fixation to occur. However, climate change may also cause soil temperatures in the summer to reach values which may limit nitrogen fixation activity (great than the 25°C optimum), which may also result in the continued limitation of nitrogen fixing species in the boreal forest.

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Appendix 1.0

Modified Rorison Nutrient Solution (Final concentration in 1L of H_2O)

1mL of 1M $MgSO_4 7H_2O$ (1mM Mg)
1mL of 1M K_2HPO_4 (2mM K, 1mM P)
3mL of 6.53g/L FeEDTA (0.05mM Fe)
1mL trace:
- 2.028 g/L $MnSO_4 H_2O$ (9µM Mn)
- 2.863 g/L H_3BO_3 (4.5µM B)
- 0.025 g/L $Na_2MoO_2 2H_2O$ (1µM Mo)
- 0.440 g/L $ZnSO_4 7H_2O$ (1.5µM Zn)
- 0.393 g/L $CuSO_4 5H_2O$ (1.5µM Cu)

For half strength N solution:
- 0.5mL of 2M $Ca(NO_3)_2 4H_2O$ (1mM Ca, 1mM N)
- 0.5mL of 2M $Ca_2Cl_2 2H_2O$ (1mM Ca)

For 0N solution:
- 1mL of 2M $Ca_2Cl_2 2H_2O$ (2mM Ca)

Soluble Protein Assay Solution

Buffer (1L)

- 21.1mL of 1M KH_2PO_4
- 28.9 mL 1M K_2HPO_4

Extraction Solution (250mL of buffer)

- 0.073g Ethylenediaminetetraacetic acid (EDTA)
- 0.044g Ascorbic Acid
- 2.0g Polyvinylpyrrolidone (PVP)

Gas Chromatograph Settings

Column oven: 80˚C for 2.5 minutes
Small valve oven: 80˚C
Pneumatics: 22psi for 2.5 minutes
Detectors: 250˚C He mL/min, H_2 mL/min, Air 300mL/min

Appendix 2.0 Formula for experiment #1 models. AIC values for various models from experiment 1. In order from best to worst AIC value.

Ethylene (μmol/g/h) (Log (+1) transformed) = 1.955+(Open or Closed)+(0.00501*(Julian Date))+(0.00588*(Vesicle Infection%))+(-0.00547*(Soil Moisture%))+(-0.08396*(Soil Temperature))+(Julian Date – 208.537)*((Julian Date-208.537)*(-0.000334))

Closed = -0.10127
Open = 0.10127

Term	Estimate	Std Error	t Ratio	Prob>|t|	AIC	R^2
Intercept	1.95512	0.67808	2.88	0.0047	231.1	0.49
Soil Temperature (˚C)	-0.084	0.04046	-2.07	0.0402		
Vesicle Infection (%)	0.00588	0.00424	1.39	0.1685		
Soil Moisture (%)	-0.0055	0.01034	-0.53	0.5979		
Site (Open or Closed)	-0.1013	0.0569	-1.78	0.0778		
Julian Date	0.00501	0.0016	3.14	0.0022		
Julian Date^2	-0.0003	4.93E-05	-6.78	<.0001		

Term	Estimate	Std Error	t Ratio	Prob>|t|	AIC	R^2
Intercept	1.69115	0.66787	2.53	0.0127	232	0.48
Julian Date	0.00471	0.0016	2.94	0.004		
Soil Temperature (˚C)	-0.0707	0.04014	-1.76	0.081		
Soil Moisture (%)	-0.0033	0.01037	-0.32	0.7489		
Vesicle Infection (%)	0.00674	0.00426	1.58	0.1158		
Julian Date^2	-0.0003	4.9E-05	-6.51	<.0001		

Term	Estimate	Std Error	t Ratio	Prob>|t|	AIC	R^2
Intercept	1.91724	0.63626	3.01	0.0032	233.2	0.48
Vesicle Infection (%)	0.00586	0.00412	1.42	0.1579		
Site (Open or Closed)	-0.0999	0.05487	-1.82	0.0712		
Soil Temperature (˚C)	-0.0885	0.03946	-2.24	0.0267		
Julian Date	0.0052	0.00139	3.73	0.0003		
Julian Date^2	-0.0003	4.76E-05	-7.17	<.0001		

Term	Estimate	Std Error	t Ratio	Prob>|t|	AIC	R^2
Intercept	1.95354	0.68132	2.87	0.0049	233.5	0.49
Vesicle Infection (%)	0.00588	0.00426	1.38	0.1703		
Soil Moisture (%)	-0.0052	0.01086	-0.48	0.6323		
Site (Open or Closed)	-0.1013	0.05715	-1.77	0.0789		
Soil Temperature (˚C)	-0.0837	0.04075	-2.05	0.0423		
Julian Date	0.00497	0.00168	2.96	0.0037		
Julian Date^2	-0.0003	5.07E-05	-6.61	<.0001		
Soil Temperature^2	0.00057	0.00701	0.08	0.9352		

Term	Estimate	Std Error	t Ratio	Prob>|t|	AIC	R^2
Intercept	2.07973	0.55666	3.74	0.0003	245.6	0.45
Soil Temperature (˚C)	-0.0906	0.03757	-2.41	0.0174		
Julian Date	0.00582	0.00147	3.97	0.0001		
Julian Date^2	-0.0003	4.7E-05	-7.12	<.0001		

Term	Estimate	Std Error	t Ratio	Prob>|t|	AIC	R^2
Intercept	0.64066	0.22792	2.81	0.0057	256.4	0.42
Julian Date	0.00461	0.00118	3.9	0.0002		
Julian Date^2	-0.0002	2.47E-05	-9.71	<.0001		

Term	Estimate	Std Error	t Ratio	Prob>|t|	AIC	R^2
Intercept	-1.2416	0.52981	-2.34	0.0208	267	0.29
Soil Temperature (˚C)	0.14331	0.02625	5.46	<.0001		
Vesicle Infection (%)	0.00497	0.00492	1.01	0.3147		
Soil Moisture (%)	-0.0131	0.00886	-1.48	0.1418		
Soil Temperature^2	-0.007	0.00732	-0.96	0.3401		

Term	Estimate	Std Error	t Ratio	Prob>|t|	AIC	R^2
Intercept	-1.4084	0.49141	-2.87	0.0049	267.68	0.28
Vesicle Infection (%)	0.00474	0.00497	0.95	0.3418		
Soil Moisture (%)	-0.014	0.009	-1.56	0.1214		
Site (Open or Closed)	-0.0334	0.06578	-0.51	0.6126		
Soil Temperature (˚C)	0.15239	0.02425	6.28	<.0001		

Term	Estimate	Std Error	t Ratio	Prob>|t|	AIC	R^2
Intercept	-1.3731	0.49182	-2.79	0.0061	268.6	0.29
Soil Temperature (˚C)	0.15199	0.02423	6.27	<.0001		
Site (Open or Closed)	-0.0416	0.0661	-0.63	0.5303		
Soil Moisture (%)	-0.022	0.01144	-1.93	0.0565		
% Vesicle (%)	0.00548	0.00501	1.09	0.2758		
Soil Moisture^2	0.00131	0.00116	1.13	0.2607		

Term	Estimate	Std Error	t Ratio	Prob>|t|	AIC	R^2
Intercept	-1.3354	0.54675	-2.44	0.0161	268.7	0.29
Soil Temperature (˚C)	0.13841	0.02718	5.09	<.0001		
Julian Date	0.00133	0.00186	0.72	0.4741		
Vesicle Infection (%)	0.00523	0.00494	1.06	0.2918		
Soil Moisture (%)	-0.0192	0.01228	-1.56	0.1208		
Soil Temperature^2	-0.0093	0.00799	-1.16	0.2476		

Term	Estimate	Std Error	t Ratio	Prob>|t|	AIC	R^2
Intercept	-1.46962	0.533805	-2.75	0.0069	269.8	0.28
Soil Temperature (˚C)	0.15172	0.024452	6.2	<.0001		
Vesicles Infection (%)	0.004852	0.005002	0.97	0.3341		
Soil Moisture (%)	-0.01643	0.012051	-1.36	0.1753		
Site (Open or Closed)	-0.03421	0.066092	-0.52	0.6058		
Julian Date	0.000515	0.001713	0.3	0.7645		

Term	Estimate	Std Error	t Ratio	Prob>|t|	AIC	R^2
Intercept	-1.2933	0.55372	-2.34	0.0213	270.7	0.29
Soil Temperature (˚C)	0.1373	0.02733	5.02	<.0001		
Vesicle Infection (%)	0.0049	0.00499	0.98	0.3288		
Soil Moisture (%)	-0.0202	0.01246	-1.62	0.1073		
Site (Open or Closed)	-0.0363	0.06601	-0.55	0.5834		
Julian Date	0.00138	0.00186	0.74	0.4599		
Soil Temperature^2	-0.0094	0.00802	-1.17	0.2432		

Term	Estimate	Std Error	t Ratio	Prob>|t|	AIC	R^2
Intercept	-0.6035	0.43688	-1.38	0.1696	278.7	0.26
Soil Temperature (˚C)	0.12079	0.02401	5.03	<.0001		
Soil Moisture (%)	-0.0135	0.00864	-1.56	0.1208		
Soil Temperature^2	-0.0124	0.00665	-1.87	0.0643		

Term	Estimate	Std Error	t Ratio	Prob>|t|	AIC	R^2
Intercept	-0.8535	0.41076	-2.08	0.0398	283.2	0.24
Soil Temperature (˚C)	0.13479	0.02292	5.88	<.0001		
Site (Open or Closed)	-0.0374	0.06449	-0.58	0.5631		
Soil Moisture (%)	-0.0211	0.0116	-1.82	0.0716		
Soil Moisture^2	0.00111	0.00115	0.96	0.3369		

Term	Estimate	Std Error	t Ratio	Prob>|t|	AIC	R^2
Intercept	-0.8169	0.40355	-2.02	0.0451	284.8	0.24
Soil Temperature (˚C)	0.12074	0.02395	5.04	<.0001		
Soil Temperature^2	-0.013	0.00662	-1.97	0.0516		

Term	Estimate	Std Error	t Ratio	Prob>|t|	AIC	R^2
Intercept	-1.1643	0.3668	-3.17	0.0019	286.5	0.22
Soil Temperature (˚C)	0.13645	0.02283	5.98	<.0001		

Term	Estimate	Std Error	t Ratio	Prob>|t|	AIC	R^2
Intercept	-0.9061	0.43183	-2.1	0.0379	287.4	0.23
Julian Date	-0.0014	0.00125	-1.13	0.2607		
Soil Temperature (˚C)	0.13853	0.02288	6.06	<.0001		

Term	Estimate	Std Error	t Ratio	Prob>|t|	AIC	R^2
Intercept	0.69743	0.45113	1.55	0.1248	306	0.04
Vesicle Infection (%)	0.00689	0.0057	1.21	0.229		
Soil Moisture (%)	-0.021	0.01357	-1.55	0.1241		
Site (Open or Closed)	-0.068	0.07511	-0.91	0.3673		
Julian Date	0.00148	0.00195	0.76	0.4506		

Appendix 3.0 Code (C++) uploaded to Arduino UNO's for experiment #2.

```
//CODE F with microSD//
#include <Servo.h>
#include <SPI.h>
#include <SD.h>
#define RELAY_ON 1
#define RELAY_OFF 0
#define Relay_1 9
int SensorValue = digitalRead(9);

Sd2Card card;
SdVolume volume;
SdFile root;
const int chipSelect = 8;

Servo myservo1; //BOX2TOP(22C)
Servo myservo2; //BOX2BOT(10C)
Servo myservo3; //BOX4TOP(22C)

int Servo_Close_TOP_2 = 90;
int Servo_Open_TOP_2 = 57;
int Servo_Close_BOT_2 = 110;
int Servo_Open_BOT_2 = 65;

int Servo_Close_TOP_4 = 54;
int Servo_Open_TOP_4 = 14;

//BOX#2 10
int ThermistorTOP2 = A0;
float C1a = 229.34;
float C2a = 10.526;
int Ya;
float BOX2TOP;

int ThermistorBOT2 = A1;
float C1b = 234.17;
float C2b = 10.401;
int Yb;
float BOX2BOT;

//BOX#4 10
int ThermistorTOP4 = A2;
float C1c = 233.42;
float C2c = 10.439;
int Yc;
float BOX4TOP;

int ThermistorBOT4 = A3;
float C1d = 235.74;
float C2d = 10.351;
```

```
int Yd;
float BOX4BOT;

void setup() {
  Serial.begin(9600);
  digitalWrite(Relay_1, RELAY_OFF);
  pinMode(Relay_1, OUTPUT);

    while (!Serial) {
    ; // wait for serial port to connect. Needed for native USB port only
  }


  Serial.print("Initializing SD card...");
  pinMode(10, OUTPUT);
  pinMode(chipSelect, OUTPUT);

if (!SD.begin(chipSelect)) {
    Serial.println("Card failed, or not present");
    // don't do anything more:
    return;
  }
  Serial.println("card initialized.");
}

void loop() {

  static int count = 0;

//BOX#2
  Ya= analogRead(ThermistorTOP2);
  BOX2TOP = (((Ya)-(C1a))/(C2a));
  Serial.print("BOX2(22)  ");
  Serial.println(BOX2TOP);

  Yb= analogRead(ThermistorBOT2);
  BOX2BOT = (((Yb)-(C1b))/(C2b));
  Serial.print("BOX2(10)  ");
  Serial.println(BOX2BOT);

  myservo1.attach(2);
  myservo2.attach(3);

  if((BOX2TOP > 22)){
    myservo1.write(Servo_Close_TOP_2);
  }
  else if((BOX2TOP < 20)){
    myservo1.write(Servo_Open_TOP_2);
  }
  if((BOX2BOT > 18)){
    myservo2.write(Servo_Close_BOT_2);
```

```
  }
  else if((BOX2BOT < 16)){
    myservo2.write(Servo_Open_BOT_2);
  }
 delay(2000);

//BOX#4
  Yc= analogRead(ThermistorTOP4);
  BOX4TOP = (((Yc)-(C1c))/(C2c));
  Serial.print("BOX4(22)  ");
  Serial.println(BOX4TOP);

  Yd= analogRead(ThermistorBOT4);
  BOX4BOT = (((Yd)-(C1d))/(C2d));
  Serial.print("BOX4(10)  ");
  Serial.println(BOX4BOT);

  myservo3.attach(4);

  if((BOX4TOP > 22)){
    myservo3.write(Servo_Close_TOP_4);
  }
  else if((BOX4TOP < 20)){
    myservo3.write(Servo_Open_TOP_4);
  }
  delay(2000);

  if((BOX2TOP < 20) || (BOX2BOT < 16) || (BOX4TOP < 20)){
    digitalWrite(Relay_1, RELAY_ON);
  }
  if((BOX2TOP > 21) && (BOX2BOT > 17) && (BOX4TOP > 21)){
    digitalWrite(Relay_1, RELAY_OFF);
  }

  int SensorValue1 = digitalRead(9);
  Serial.print("RELAY STATUS:");
  Serial.println(SensorValue1);

  File dataFile = SD.open("temp.txt", FILE_WRITE);
  if (dataFile) {
    dataFile.print("BOX2TOP:");
    dataFile.println(BOX2TOP);
    dataFile.print("BOX2BOT:");
    dataFile.println(BOX2BOT);
    dataFile.print("BOX4TOP:");
    dataFile.println(BOX4TOP);
    dataFile.print("BOX4BOT:");
    dataFile.println(BOX4BOT);
    dataFile.print("RELAY STATUS:");
    dataFile.println(SensorValue1);
    dataFile.print("LOOP NUMBER:");
```

```
    dataFile.println(count++);
    dataFile.close();
    Serial.println("Done Upload");
  }
  else {
    Serial.println("error opening temp.txt");
  }

  delay(116000);
}

//CODE A-E with SD card//
#include <Servo.h>
#include "DHT.h"
DHT dht1(18, DHT11);
DHT dht2(8, DHT11);
#include <SPI.h>
#include <SD.h>
File myFile;
#define RELAY_ON 1
#define RELAY_OFF 0
#define Relay_1 9

Servo myservo1; // BOX1ALL(22C)
Servo myservo2; //BOX2TOP(22C)
Servo myservo3; //BOX2BOT(10C)
Servo myservo4; //BOX3TOP(22C)
Servo myservo5; //BOX3BOT(15C)
Servo myservo6; //BOX4TOP(22C)

int Servo_Close_ALL_1 = 180;
int Servo_Open_ALL_1 = 125;

int Servo_Close_TOP_2 =40;
int Servo_Open_TOP_2 = 5;
int Servo_Close_BOT_2 = 108;
int Servo_Open_BOT_2 = 70;

int Servo_Close_TOP_3 = 53;
int Servo_Open_TOP_3 = 11;
int Servo_Close_BOT_3 = 70;
int Servo_Open_BOT_3 = 30;

int Servo_Close_TOP_4 = 96;
int Servo_Open_TOP_4 = 52;

//BOX#1 22
int ThermistorALL1 = A0;
float C1a = 237.63;
float C2a = 10.917;
int Ya;
```

```
float BOX1ALL;

//BOX#2 10UP
int ThermistorTOP2 = A1;
float C1b = 237.87;
float C2b = 10.742;
int Yb;
float BOX2TOP;

int ThermistorBOT2 = A2;
float C1c = 234.46;
float C2c = 10.995;
int Yc;
float BOX2BOT;

//BOX#3 15
int ThermistorTOP3 = A3;
float C1d = 237.51;
float C2d = 10.782;
int Yd;
float BOX3TOP;

//BOX#4 10
int ThermistorTOP4 = A5;
float C1f = 237;
float C2f = 10.8;
int Yf;
float BOX4TOP;

void setup() {
  Serial.begin(9600);
  dht1.begin();
  dht2.begin();
  digitalWrite(Relay_1, RELAY_OFF);
  pinMode(Relay_1, OUTPUT);

    while (!Serial) {
    ; // wait for serial port to connect. Needed for native USB port only
  }


  Serial.print("Initializing SD card...");

  if (!SD.begin(10)) {
    Serial.println("initialization failed!");
    while (1);
  }
  Serial.println("initialization done.");
}

void loop() {
```

```
static int count = 0;

//BOX#1
  Ya= analogRead(ThermistorALL1);
  BOX1ALL = (((Ya)-(C1a))/(C2a));
  Serial.print("BOX1(22)  ");
  Serial.println(BOX1ALL);

  myservo1.attach(2);

  if((BOX1ALL > 22)){
    myservo1.write(Servo_Close_ALL_1);
  }
  else if((BOX1ALL < 20)){
    myservo1.write(Servo_Open_ALL_1);
  }
 delay(2000);

//BOX#2
  Yb= analogRead(ThermistorTOP2);
  BOX2TOP = (((Yb)-(C1b))/(C2b));
  Serial.print("BOX2(22)  ");
  Serial.println(BOX2TOP);

  Yc= analogRead(ThermistorBOT2);
  BOX2BOT = (((Yc)-(C1c))/(C2c));
  Serial.print("BOX2(10)  ");
  Serial.println(BOX2BOT);

  myservo2.attach(3);
  myservo3.attach(4);

  if((BOX2TOP > 22)){
    myservo2.write(Servo_Close_TOP_2);
  }
  else if((BOX2TOP < 20)){
    myservo2.write(Servo_Open_TOP_2);
  }
  if((BOX2BOT > 18)){
    myservo3.write(Servo_Close_BOT_2);
  }
  else if((BOX2BOT < 16)){
    myservo3.write(Servo_Open_BOT_2);
  }
  delay(2000);

//BOX#3
  Yd= analogRead(ThermistorTOP3);
  BOX3TOP = (((Yd)-(C1d))/(C2d));
  Serial.print("BOX3(22)  ");
```

```
  Serial.println(BOX3TOP);

  delay(2000);

  float BOX3BOT = dht1.readTemperature();
  Serial.print("BOX3(15)  ");
  Serial.println(BOX3BOT);

  myservo4.attach(5);
  myservo5.attach(6);

  if((BOX3TOP > 22)){
    myservo4.write(Servo_Close_TOP_3);
  }
  else if((BOX3TOP < 20)){
    myservo4.write(Servo_Open_TOP_3);
  }

  if((BOX3BOT > 15)){
    myservo5.write(Servo_Close_BOT_3);
  }
  else if((BOX3BOT < 14)){
    myservo5.write(Servo_Open_BOT_3);
  }
delay(2000);

//BOX#4
  Yf= analogRead(ThermistorTOP4);
  BOX4TOP = (((Yf)-(C1f))/(C2f));
  Serial.print("BOX4(22)  ");
  Serial.println(BOX4TOP);

  delay(2000);

  float BOX4BOT = dht2.readTemperature();
  Serial.print("BOX4(10)  ");
  Serial.println(BOX4BOT);

  myservo6.attach(7);

  if((BOX4TOP > 22)){
    myservo6.write(Servo_Close_TOP_4);
  }
  else if((BOX4TOP < 20)){
    myservo6.write(Servo_Open_TOP_4);
  }
  delay(2000);

  if((BOX1ALL < 20) || (BOX2TOP < 20) || (BOX2BOT < 16) || (BOX3TOP < 20) || (BOX3BOT < 13) ||
(BOX4TOP < 20)) {
    digitalWrite(Relay_1, RELAY_ON);
```

```
  }
  if((BOX1ALL > 21) && (BOX2TOP > 21) && (BOX2BOT > 17) && (BOX3TOP > 21) &&
(BOX3BOT > 15) && (BOX4TOP > 21)){
    digitalWrite(Relay_1, RELAY_OFF);
  }
  delay(2000);

  int SensorValue1 = digitalRead(9);
  Serial.print("RELAY STATUS:");
  Serial.println(SensorValue1);

  myFile = SD.open("temp.txt", FILE_WRITE);

  // if the file opened okay, write to it:
  if (myFile) {
    myFile.print("BOX1ALL:");
    myFile.println(BOX1ALL);
    myFile.print("BOX2TOP:");
    myFile.println(BOX2TOP);
    myFile.print("BOX2BOT:");
    myFile.println(BOX2BOT);
    myFile.print("BOX3TOP:");
    myFile.println(BOX3TOP);
    myFile.print("BOX3BOT:");
    myFile.println(BOX3BOT);
    myFile.print("BOX4TOP:");
    myFile.println(BOX4TOP);
    myFile.print("BOX4BOT:");
    myFile.println(BOX4BOT);
    myFile.print("RELAY STATUS:");
    myFile.println(SensorValue1);
    myFile.print("LOOP NUMBER:");
    myFile.println(count++);
    myFile.close();
    Serial.println("Done Upload");
  } else {
    // if the file didn't open, print an error:
    Serial.println("error opening");
  }
  delay(106000);
}
```