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Going back to the soil: An integrated approach to farming.

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Going back to the soil: An integrated approach to farming.

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A

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Abstract

Agricultural productivity is often constrained by nutrient availability; as such, copious amounts of synthetic fertilizers are applied to maintain productivity. However, the intensive use of synthetic fertilizers has reduced the capacity of the soil to carry out crucial roles such as nutrient cycling because of shifts in the microbial community composition and structure. In addition, much of the applied synthetic fertilizers become lost to the environment through run-off, which contributes to soil degradation. With the increasing demand on agricultural systems to provide food and fibre and the adverse impacts of agricultural production on the soil resource, amendments that support soil productivity are required to supply plant nutrients. One way of sustainably improving nutrient acquisition and retention is by improving soil fertility; this can be achieved through integrative farming strategies that increase organic matter levels and stimulate the microbial community. In this greenhouse study, an integrative approach that relied on plant-soil-microbe interactions was used to evaluate the usage of an agromineral as a slow-release fertilizer. This assessment was done by comparing the effects of an agromineral, the Spanish River Carbonatite (SRC), and a synthetic fertilizer (Nitrogen-Phosphorus-Potassium (NPK), 20-20-20) on soil pH, microbial abundance and respiration, as well as on the legume-*Rhizobium* symbiosis. I hypothesized that the SRC, rich in calcium and other essential plant nutrients, would raise soil pH, stimulate microbial abundance and respiration, and enhance the legume-*Rhizobium* symbiosis in comparison to the synthetic fertilizer. In addition, the microbial abundance and respiration would differ depending on the plants grown. A mix of leguminous and non-leguminous cover crops was grown for 56 days in soils treated with three soil amendments: SRC, ammonium nitrate, and NPK synthetic fertilizer. The cover crops were grown in the following pairs: 1) alfalfa and chicory and 2) red clover and oilseed radish. At harvest, soil treated with SRC had higher pH

values (pH raised by 1.2 units) and exhibited a higher abundance of heterotrophic and symbiotic nitrogen-fixing bacteria than those soils lacking the SRC amendment. This effect of SRC was observed in the rhizosphere of both cover crop combinations. However, the effect of soil amendments on the phosphate-solubilizing bacteria varied between cover crop combinations. The relative abundance of phosphate-solubilizing bacteria was enhanced by the SRC amendment in the rhizosphere of red clover and oilseed radish plants; in contrast, in the rhizosphere of intercropped alfalfa and chicory, the NPK fertilizer was the amendment that stimulated the abundance of the phosphate-solubilizer bacteria. Additionally, microbial respiration was reduced in soils treated with SRC compared to that of ammonia nitrate- and NPK fertilizer-treated soils. The results indicate that soil amendments were the drivers of soil pH and abundance of symbiotic nitrogen-fixing and heterotrophic microorganisms, as well as the drivers of microbial respiration, while the plant combination had more pronounced effects on the abundance of phosphate-solubilizing bacteria. Furthermore, the SRC amendment appeared to have enhanced the legume-*Rhizobium* symbiosis compared to amendments lacking SRC. These preliminary findings suggest that SRC as a slow-release fertilizer may be useful as part of an integrative strategy to improve soil fertility by stimulating microbial activity.

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Chapter 1: General Introduction

1.1 Background

Many reports have indicated that agricultural production will need to increase by 70-100% to meet the demands of the growth in population (Coskun et al. 2017). However, arable lands required for production have become degraded and are no longer suitable for maximum productivity (Seufert et al. 2012; FAO 2016). To meet the food demand, agricultural intensification, i.e., increasing output over the same land area, and agricultural extensification, i.e., bringing more land into production (Gabriel et al. 2013), have been proposed. There are concerns with these suggestions; the former contributes to greenhouse gas emissions while the latter is a significant driver of biodiversity loss (Gabriel et al. 2013; FAO 2016). Furthermore, there have been reports that crop yields have plateaued despite using synthetic fertilizers (Cassman 1999; Brisson et al. 2010). The plateauing of crop yields are expected to worsen as land value increases (Pingali 2012) and as the non-renewable resources used in the manufacture of synthetic fertilizers decrease (Badgley et al. 2007; Manning 2010).

Although the Green Revolution of the 1950s and 1960s has remarkably transformed farming practices resulting in significant increases in crop productivity (Fitzgerald-Moore and Parai 1996; Pingali 2012), it has led to soil impoverishment (Tilman et al. 2002). Practices such as the misuse of agrochemicals, e.g., synthetic fertilizers and pesticides, and high-yielding monocropping have resulted in soil degradation as well as biodiversity loss (Tilman et al. 2002; Jacques and Jacques 2012; Pingali 2012). The continuous cultivation of crops has depleted the soil of its essential minerals (Stern et al. 2006; Drinkwater and Snapp 2007). To replenish the soil, farmers add synthetic fertilizers (Drinkwater and Snapp 2007), and this practice has modified

biogeochemical nutrient cycles such as those of N and P (Drinkwater and Snapp 2007; Parikh and James 2012). Soil degradation impacts the productive and functional capacities of the soils to meet human demands and perform several ecosystem services (Lehman et al. 2015a). Addressing the concerns of food insecurity and soil degradation is linked to improving the biological functioning of the soil resource (Lehman et al. 2015b). Restoring degraded soils can be achieved by integrating sustainable soil management strategies (Tilman et al. 2002; Pingali 2012) into current farming practices.

1.2 Research Gap and Goal

The adverse impact of agricultural intensification has been a cause for concern, and in recent times, the urgency to find ways to produce crops more sustainably has increased. The increased use of fertilizers has induced changes in soil properties, and over time these changes are thought to have had significant effects on the microbial community composition, structure and function (Zhong and Cai 2007; Treseder 2008; Tan et al. 2013; Geisseler and Scow 2014). Many reports have highlighted contrasting effects of synthetic fertilizer inputs on soil microbial properties such as the stimulation of particular microbial groups (Spehn et al. 2000; Borken et al. 2002; Zhong and Cai 2007; Zhao et al. 2016) and the reduction of others (Borken et al. 2002; Kennedy et al. 2004; Wu et al. 2005; Ramirez et al. 2012; Shen et al. 2016). The use of soil microorganisms and agrominerals represents a key strategy to improve crop productivity by potentially increasing nutrient uptake (von Wilpert and Lukes 2003; van Straaten 2006; Adesemoye et al. 2008; Adesemoye et al. 2009). However, before implementing an integrated soil management strategy, we need to evaluate the potential effects of these strategies on soil ecosystem processes such as nutrient cycling and decomposition. Studies have been done to investigate the

effects of agricultural intensification on the drivers of ecosystem processes, for example, on microbial diversity. The data indicate that plant species (Grayston et al. 1998; Paterson et al. 2007; Eisenhauer et al. 2010; Ladygina and Hedlund 2010), soil conditions such as pH (Kennedy et al. 2004; Rousk et al. 2010), and soil nutrient status (Zhong and Cai 2007; Ramirez et al. 2012; Shen et al. 2016) are all affecting soil microbial communities.

The purpose of this study, therefore, was to evaluate the effect of an agromineral and a commercial fertilizer on chemical and biological indices of soil health.

Chapter 2: Literature Review

2.1 The role of soil in ecosystem functioning

Agricultural production has been the cornerstone of many civilizations, both past and present, and optimal production remains vital for future generations. The soil is an essential driver of the functioning of natural and managed ecosystems, and it acts as a medium in which abiotic and biotic factors are at play (Grayston et al. 1998; Alkorta et al. 2003; Richardson et al. 2009a; Ladygina and Hedlund 2010). It represents a dynamic and complex ecosystem (Paterson et al. 2007; Zhong and Cai 2007) that supports not only crop growth but is also a habitat for a wide range of macro- and micro-fauna (Alkorta et al. 2003; Tian et al. 2015; Jacoby et al. 2017). The soil biota, e.g., nematodes, earthworms, bacteria, fungi, and protozoans, plays a key role in ecosystem functioning and services (Figure 2.1) (Richardson 2001; Alkorta et al. 2003; Chaparro et al. 2012; Lehman et al. 2015b; Holland et al. 2016).

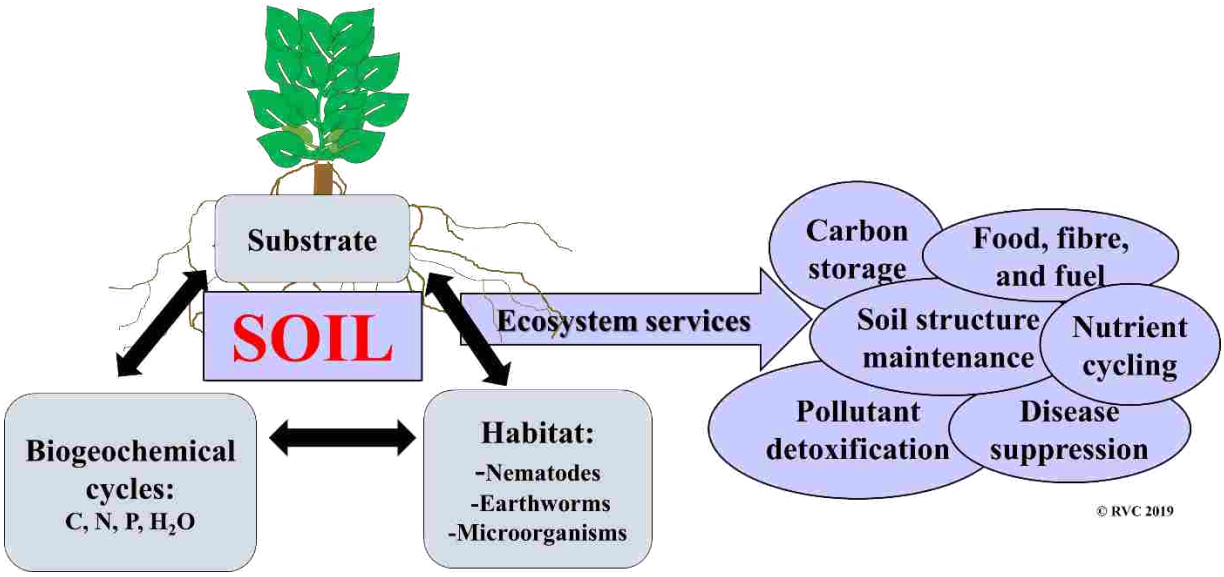

Figure 2.1 Soil functions and ecosystem services

The soil, as an ecosystem of macro-and microfauna, carries out many ecosystem functions and services that are key to agricultural productivity. For example, it allows nutrient cycling, helps in pest and pathogen suppression, and through stable aggregates formation, it reduces the risk of soil erosion and increases infiltration rates.

Soil health is often viewed as the continued capacity of the soil to serve as an essential living system within natural and managed ecosystem boundaries (Alkorta et al. 2003; Kibblewhite et al. 2008). Among all the ecosystems, the soil is thought to host the highest microbial diversity (Lehman et al. 2015b), and these microorganisms mediate many biochemical processes through enzyme-catalyzed reactions (Tabatabai 1982; Alkorta et al. 2003). Microbial communities are sensitive to disturbances in their environment; as such their activities and biomass are often used as indicators to assess soil health (Pankhurst et al. 1995; Alkorta et al. 2003; Nakhro and Dkhar 2010; Tian et al. 2015). Healthy soils can promote root growth, retain and release water, cycle nutrients, and promote optimal gas exchange and biological activity (Alkorta et al. 2003; Lehman et al. 2015b). Currently, most agricultural soils have lost their ability to carry out these roles, because of anthropogenic activities and poor management practices (Parikh and James 2012; Strecker et al. 2015; FAO 2016). Therefore, farmers must take an integrated approach to remediate degraded soils. Some of the current and suggested approaches are highlighted in Figure 2.2.

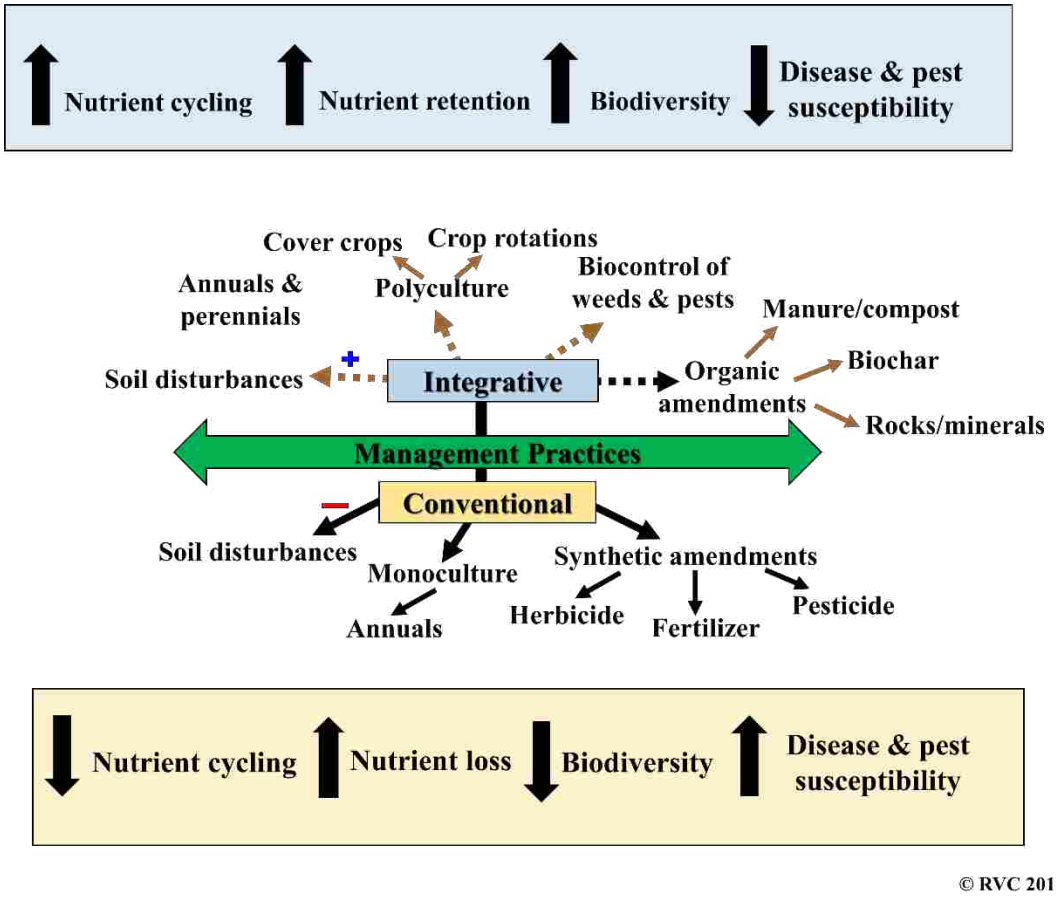

Figure 2.2 Management practices that influence soil health

The management practices referred to as integrative are the suggested practices that promote the biological activity of the soil, and thus soil health. The conventional practices are those that are primarily used in modern farming systems. The "+" sign represents methods that reduce soil disturbance while the "-" sign indicates an increase in mechanized tillage.

2.2 Nutrient requirement and crop growth

Plant growth is often limited by mineral nutrient availability, and under these circumstances, plants depend on the cycling of soil nutrients or external inputs (Paterson 2003). There are sixteen essential nutrients (Table 2.1)that have several biochemical and physiological functions in plants (Parikh and James 2012; Chen and Liao 2017). As such, a deficiency in any of these minerals, especially macronutrients, can severely limit crop growth and yield (White and Brown 2010; Sugiyama and Yazaki 2012). Mineral nutrients influence internal plant signals, many of which control plant developmental stages such as seedling establishment, which is a high nutrient demanding stage (Dakora and Phillips 2002). In many managed ecosystems, the nutrient requirements for crop growth are high, and N and P are often the most limiting nutrients (White and Brown 2010; Parikh and James 2012; López-Arredondo et al. 2014). Potassium is vital to increasing the plants' resistance to diseases such as those caused by the root-rotting fungi present in the soil (Wang et al. 2013). K^+ is not critically limiting in most soils, except in tropical regions where it has the highest depletion rate (Manning 2010). Going forward I will be focusing on N and P.

Table 2.1 Essential plant nutrients

Mineral nutrients	Chemical symbol
Macronutrients	
Nitrogen	**N**
Phosphorus	**P**
Potassium	**K**
Calcium	Ca
Magnesium	Mg
Sulphur	S
Silicon	Si
Micronutrients	
Boron	B
Chlorine	Cl
Copper	Cu
Manganese	Mn
Molybdenum	Mo
Nickel	Ni
Zinc	Zn
*Cobalt	Co
*Sodium	Na

The required nutrients are categorized as macro-and micronutrients based on their relative concentrations in the plant tissue. Macronutrients are further divided into primary (shaded rows, i.e., N, P and K) and secondary macronutrients, Ca, Mg, S and Si).

*Co and Na are essential to some plants, and although aluminum is not considered an essential mineral, the addition of small amounts to nutrient solutions stimulates plant growth.

2.2.1 Nitrogen

Nitrogen is one of the most crucial nutrients for plant growth and productivity (Beeckman et al. 2018), and although 78% of the atmosphere is made up of nitrogen, as N_2, this form is not directly available for plant use (Behie and Bidochka 2014; Hoffman et al. 2014; Oldroyd and Dixon 2014; Stein and Klotz 2016). A dynamic equilibrium exists between N_2 and the preferred useable forms nitrate (NO_3^-) and ammonium (NH_4^+) (O'Brien et al. 2016; Stein and Klotz 2016) but these forms are limited in agroecosystems (O'Brien et al. 2016). The assimilation of these available forms of N is known to have different effects on root growth (Beeckman et al. 2018). On the one hand, NH_4^+ is known to stimulate root branching (Lima et al. 2010; Xuan et al. 2017; Beeckman et al. 2018), increasing the surface area available for nutrient absorption. On the other hand, NO_3^- stimulates the elongation of lateral roots (Beeckman et al. 2018), enhancing the plant foraging capacity. Furthermore, NO_3^- has been reported to act as a signal molecule, modulating many processes such as root system architecture and seed dormancy (O'Brien et al. 2016; Xuan et al. 2017). Because of its negative charge, NO_3^- does not bind to the surfaces of soil particles and, hence, it is very mobile in the soil. As such, it is susceptible to leaching (Beeckman et al. 2018), rendering the application of NO_3^- less efficient than that of NH_4^+ under field conditions (Beeckman et al. 2018).

The majority of soil N exists in the form of organic matter (O'Brien et al. 2016), which cannot be directly assimilated by plants. However, through the activities of soil microorganisms, useable N is either assimilated in their biomass or made available for plant uptake (Lupwayi and Kennedy 2007) (Figure 2.3). Factors, such as temperature and soil moisture (Lupwayi and Kennedy 2007), C:N ratio and the quality of the residues (Watson et al. 2002), and root exudates (Faucon et al. 2017), influence the rates at which microbial immobilization or mineralization

occurs (Paterson 2003). For example, the release of root exudates or microbial biomass turnover may enhance the microbial mineralization of organic residue increasing the availability of inorganic N, and if microbes are N-limited, they will incorporate the available N into their biomass, reducing the N available for plant uptake (Faucon et al. 2017). As the exudates, the C:N ratio of the organic matter can stimulate microbial mineralization, e.g., if the residues have low C:N ratios and the microbes are C-limited (Crews and Peoples 2005); it can also stimulate microbial immobilization if the residue C:N ratios are high and the microbes are N-limited (Paterson 2003; Mommer et al. 2016). In the latter case, a temporary deficit is created, and useable N is only available for plant use if the microbes die and the N in their biomass is mineralized, or when other sources of useable N are made available (Crews and Peoples 2005).

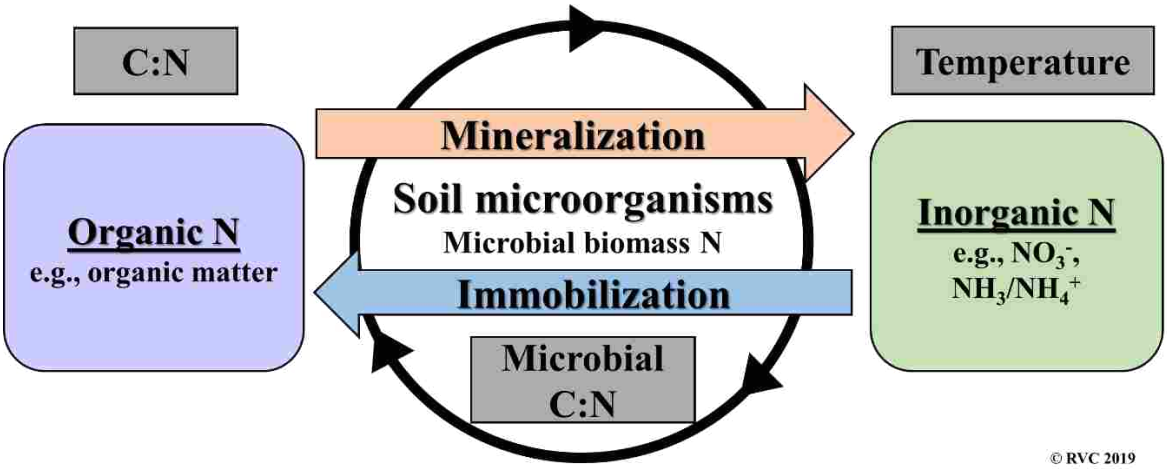

Figure 2.3 Transformation of nitrogen between soil pools

The soil contains substantial amounts of N, but most of it is "locked" up in organic forms. Soil microorganisms can either mineralize organic N forms, e.g., in plant residue, into plant useable NO_3^- and NH_4^+ or immobilize these inorganic mineral forms as they incorporate them into their biomass, rendering them unavailable for plant uptake. Factors that are known to influence microbial activity, e.g., moisture and elemental stoichiometry ratios among others, influence the rate and extent to which mineralization and immobilization occur.

2.2.2 Phosphorus

P is the second most limiting plant nutrient found in soils and is critical to plant development (Shepherd et al. 2016). P has roles in the division and organization of cells promoting root development and seedling establishment (Sugiyama and Yazaki 2012). Although P is essential to plants, the bioavailability of phosphate ion (PO_4^{3-}), the useable form of P, in soils is generally low (Roberts and Johnston 2015). Because PO_4^{3-} is bound to the mineral fractions of soils, it is highly insoluble, and its availability depends on soil chemistry (Mommer et al. 2016), pH in particular. In acidic soils, PO_4^{3-} is absorbed by the plants as dihydrogen phosphate, $H_2PO_4^-$, which is highly reactive with Al and Fe cations (Hou et al. 2018). In alkaline soils, it exists as hydrogen phosphate, HPO_4^{2-}, which complexes with Ca and Mg cations (Hinsinger 2001; Ros et al. 2017). Since these forms of PO_4^{3-} are quickly adsorbed onto clay surfaces and chelate with metal oxides, they become unavailable for plant uptake (López-Arredondo et al. 2014; Ros et al. 2017) (Figure 2.4). Up to 80% of the total P in the soil is organic P, and this form is not available to plants. As such, organic P must be mineralized into useable forms before being absorbed by plant roots (Hinsinger 2001; Chen and Liao 2017). The turnover of organic P is primarily carried out by plants and soil microorganisms (Hou et al. 2018) via the action of plant and microbial phosphatases (Tan et al. 2013), acidification (Dakora and Phillips 2002; Gyaneshwar et al. 2002; Burghelea et al. 2015), the release of exudates (Li et al. 2014), and the process of weathering (van Straaten 2006).

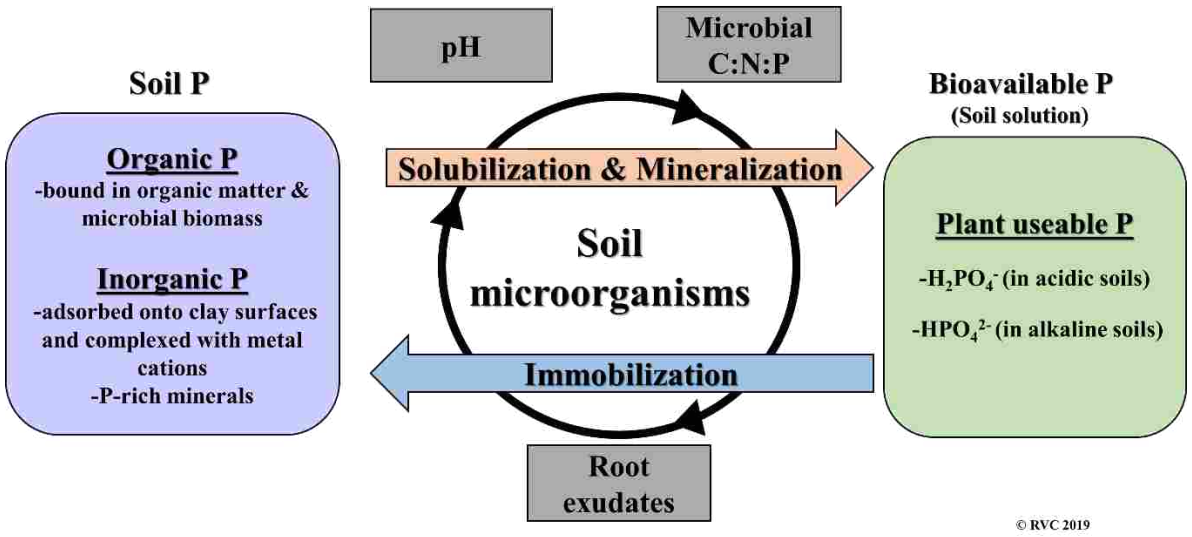

Figure 2.4 Transformation of phosphorus between soil pools

There are two forms of phosphate available for plant uptake, and the pH of the soil will influence the dominant form. The bioavailability of phosphate is low soil solution because it is often found in organic matter and microbial biomass as organic phosphorus or adsorbed onto clay surfaces, bound to metal cations or complexed in P-rich minerals as inorganic phosphorus. However, through natural weathering, exudation of H^+ and organic anions, as well as the activities of microorganisms, unavailable P is made available for microbial use and plant uptake. The microbial biomass, the C:N and C:P ratios, as well as the pH, influence the release or immobilization of bound P.

2.3 The impact of industrial agriculture on the ecosystem

The farming practices introduced during the Green Revolution have been the blueprint for many current small- and large-scale farms. These practices include, but are not limited to, the cultivation of a single high-yielding crop variety (Kiers et al. 2007) such as barley, wheat, corn, and rice (Rahman 2003; Jacques and Jacques 2012; Qiu and Li 2016). These crop varieties require copious amounts of synthetic fertilizers to maintain yield, and fertilizers are often composed of soluble salts of a single elemental nutrient, such as ammonia nitrate and triple superphosphate fertilizer, or a combination of N, P, and K (Nziguheba et al. 2000; Zhang et al. 2017a). Crops as monocultures have replaced many natural ecosystems that once flourished (Tilman 1999; Jacques and Jacques 2012), resulting in increases in the susceptibility of crop species to diseases because of biodiversity loss (Strecker et al. 2015). Synthetic herbicides and pesticides are, therefore, needed to control weeds and pests (Tilman 1999; Jacques and Jacques 2012; Pingali 2012). Even though these farming practices, particularly the use of synthetic N and P fertilizers, have significantly contributed to increased productivity, their use has reduced the N_2-fixing ability of microorganisms that provided a biologically-fixed source of useable N (Gan et al. 2004; Stein and Klotz 2016). This imbalance has led to the modification of many soil ecosystem processes, such as N and P cycling (Bennett and James 2011; Tkacz and Poole 2015), and has contributed to soil degradation (Diaz and Rosenberg 2008).

Furthermore, the manufacture of NPK fertilizers contributes to the depletion of non-renewable resources (Bohlool et al. 1992; Choudhary et al. 1994; van Straaten 2002a; Manning 2010; Baker et al. 2015; Rosemarin and Ekane 2016; Shepherd et al. 2016). Phosphorous rock (PR) refers to the diverse range of naturally-occurring minerals that contain relatively high levels of P, such as apatite (Zhang et al. 2017b). This non-renewable ore is the source from which

synthetic P fertilizers are manufactured (van Straaten 2002b; Shepherd et al. 2016; Talboys et al. 2016), and their manufacture consumes approximately 80% of the PR ore mined annually (Roberts and Johnston 2015). Therefore, long-term supplies of PR are critical to agricultural productivity if farmers wish to maintain the current rate of crop production, i.e., 160-170 million tonnes/year (Roberts and Johnston 2015), and keep pace with the projected demand for food.

Moreover, the industrial reduction of N_2 to NH_3 via the Haber Bosch process requires high temperatures (up to 1200 °C) and pressures (1000-3000 kPa) (Bohlool et al. 1992; Jensen and Hauggaard-Nielsen 2003; Manning 2010; White and Brown 2010). This process requires six times the amount of energy needed to produce P or K fertilizer (Santi et al. 2013), and this energy is often derived from non-renewable energy sources, for example, natural gas and coal (Bohlool et al. 1992; Jensen and Hauggaard-Nielsen 2003). As the price of oil derived from fossil fuel and the scarcity of PR increase, so will the price of N and P fertilizers (van Straaten 2006; Heppell et al. 2016). Indeed, the global fertilizer demand is steadily increasing and is estimated to reach 220 million tonnes by 2020 (FAO 2017; IFA 2018). Coupled with the economic and environmental costs of producing fertilizers, the transport and application of synthetic fertilizers to fields are energy-intensive and extremely costly (Jensen and Hauggaard-Nielsen 2003).

Farming practices are known to contribute to greenhouse gas emissions, accounting for up to 21% of the total annual global emissions (FAO 2016). The emission of N-based gases represents a loss of N from agroecosystems, which significantly contributes to N pollution (FAO 2016). The loss of N from agroecosystems often occurs in three ways: 1) global NO_3^- leaching and run-off (Ramos and Martínez-Casasnovas 2006; García-Díaz et al. 2017), 2) NH_3 volatilization (Coskun et al. 2017), and 3) microbial transformations such as nitrification and denitrification (Coskun et al. 2017). Microbial transformations of applied N sources result in the release of nitrous oxide, a

gas of great concern because it has 300 times the heat-trapping capacity of CO_2; thus, it has a significant potential for global warming (Ravishankara et al. 2009; Richardson et al. 2009b; Hu et al. 2015). These microbial processes are influenced by soil N availability, soil organic matter (SOM) content, temperature, and oxygen content (Steenwerth and Belina 2010). The loss of N from managed ecosystems and its subsequent contribution to environmental degradation are likely to increase as agricultural production increases unless strategies aimed at reducing the transformation of applied N fertilizers are incorporated. Such strategies include, but are not limited to, 1) reducing the use of NH_3/NH_4^+-based fertilizers (IPCC 2018), 2) using slow-released or time-released fertilizers as well as optimizing the timing and placement of N fertilizers (Reay et al. 2012), and 3) planting multiple crop species (Guo et al. 2009). These strategies represent sustainable farming practices that aim at increasing biodiversity, promoting nutrient cycling and, thus, reducing nutrient loss. In general, sustainable agriculture has been defined as the successful management of resources such as soil, water, environment, and non-renewable energy sources to satisfy the changing human needs, while maintaining or enhancing the quality of the environment, in addition to conserving these crucial resources (FAO 2016; Chen and Liao 2017).

2.4 Farming strategies that promote soil health and nutrient cycling

Healthy soils are central to sustainable agricultural productivity, and management practices that promote and maintain the optimal use of the soil resource overtime must be implemented (Singh et al. 2011). Soil organic matter is a central component of an integrated management strategy for improving soil health (Wood and Baudron 2018). The level of SOM often serves as a sensitive indicator of long-term modifications in the functioning of the soil ecosystems (Lehman et al. 2015a; Finney et al. 2016). SOM is a C-rich source of decomposing animal, microbial, and

plant residue that holds globally three times more C than the atmosphere and the vegetation (Schmidt et al. 2011). The addition of SOM has led to improved soil structure and tilth (Bacq-Labreuil et al. 2018), thus reducing the risk of soil erosion, and to increased infiltration rates and water-holding capacity of the soil (Williams and Hedlund 2013), leading to improved nutrient retention (Lupwayi and Kennedy 2007).

Moreover, SOM increases cation exchange capacity (Chang et al. 2007; Wood and Baudron 2018) which influences the soil's ability to retain essential nutrients and buffer against acidification (Chang et al. 2007; Wood and Baudron 2018). Since modern agricultural practices contribute to environmental degradation, practices that enhance SOM can potentially reduce soil degradation, decrease nutrient loss, and increase C storage (Lehman et al. 2015b). Organic inputs represent a viable option because they are obtained more sustainably than chemical inputs, often from agricultural, industrial, and municipal processes as nutrient-rich by-products (Jacoby et al. 2017). These industrial by-products contain organically-bound nutrients that are more stable in the soil, and when applied as organic fertilizer, e.g., as compost, they can increase SOM, reduce nutrient leaching, and NH_3 volatilization (Jacoby et al. 2017). There has been a resurgence in the use of several organic amendments as part of a sustainable management system to improve soil health (Lanza et al. 2016; Zhang et al. 2016; Jin et al. 2016). For example, biochar (Atkinson et al. 2010; Lehmann et al. 2011; Robertson et al. 2012), compost, and manure (Pinamonti 1998; Birkhofer et al. 2008; Coll et al. 2011; Sharma et al. 2013; Suja et al. 2017) can positively influence soil C content, biological activity, and nutrient retention.

Notwithstanding these positive effects, there have been contrasting reports, however, of the inability of organic amendments to support agronomic yield production when compared to their synthetic counterparts (Bengtsson et al. 2005; Badgley et al. 2007; Seufert et al. 2012;

Pradeepkumar et al. 2017; Suja et al. 2017). The application of these organic sources has been reported to have adverse effects on soil microbes because of contaminants associated with the source material (Mårtensson and Wetter 1990). However, these reported effects on soil microbes depend on the source of the materials used as amendments (Mårtensson and Wetter 1990). Because of potential contaminants such as antibiotics and heavy metals associated with these sources, other materials such as geological materials (van Straaten 2006) capable of supplying nutrients are being assessed.

2.5 The use of rocks in crop production

Most of the nutrients required for plant growth exist to varying degrees in naturally-occurring rocks and minerals (Harley and Gilkes 2000; Gillman et al. 2002; van Straaten 2006). The term agromineral has been used to describe geological complexes that can serve as low-cost, slow-release fertilizers (van Straaten 2006; Zhang et al. 2017b). The use of rocks and minerals or their by-products in farming as a soil amendment to remineralize the soil is not a new concept and has been in practice since the 19th century (van Straaten 2006; Ramos et al. 2015). For example, limestone and dolomite have been used as soil conditioners to raise the pH of acidic soils, perlite to enhance aeration in artificial substrates, and vermiculite and zeolite to maintain moisture in growth substrates (van Straaten 2002b; van Straaten 2006).

Although there are many promising reports on the direct use of different agrominerals as nutrient sources (Bakken et al. 1996; Bakken et al. 1997; Bakken et al. 2000; Hildebrand and Schack-Kirchner 2000; von Wilpert and Lukes 2003; Talboys et al. 2016), other studies found non-significant results (Bolland and Baker 2000; von Wilpert and Lukes 2003). In studies evaluating the use of K-based agrominerals, e.g., granite dust and a complex of dolomite and phonolite, as

slow-release fertilizers, the authors reported that although granite dust raised soil pH, it is inefficient in supplying adequate amounts of K and P to meet the demands of wheat plants growing in a K-deficient soil (Bolland and Baker 2000). The dolomite and phonolite mineral complex also ameliorated the pH of K-deficient forest soil, but the weathering of this K-based mineral resulted in the subsequent release of high doses of Na (von Wilpert and Lukes 2003). The simultaneous release of copious amounts of Na with the dissolution of these rock sources is of concern because large quantities of Na can lead to Na-loading of groundwater (von Wilpert and Lukes 2003); this would hinder the use of such complexes as supplements to synthetic fertilizers.

Carbonatites represent a highly reactive mineral complex that is primarily made up of apatite (P source), biotite (K source), and calcite ($CaCO_3$ source), as well as varying quantities of micronutrients and rare earth elements (Sage 1987). As carbonatites weather, they release adequate amounts of Ca, K, P and Mg over time (Heim et al. 2011; Myrvang et al. 2016). Bakken et al. (1997 and 2000) reported having recovered significant amounts of K from the biotite and nepheline fractions of a carbonatite absorbed in plants. Although carbonatites represent viable supplementary nutrient sources, their short-term use to support crop growth and yield in agricultural systems can be limited. One such limitation relates to the dissolution rates of these Ca-rich complexes compared to water-soluble synthetic fertilizers (Renforth et al. 2015). The slow release of nutrients may hinder seedling establishment and other early plant development processes that have high nutrient demands (Choudhary et al. 1994; Silwal 2013). Another limitation relates to their potential release and accumulation of toxic elements such as Ba and Sr (Renforth et al. 2015), which may pose an environmental risk or inhibit plant growth. Although most carbonatites are low in toxic and radioactive elements such as Cd, Pb, and U, there are some that are enriched with Ba and Sr (Heim et al. 2011; Myrvang et al. 2016). One such example is the Stjernøy carbonatite found in

Norway (Heim et al. 2011; Silwal 2013; Myrvang et al. 2016). The accumulation of Ba and Sr may result in undesired effects on plant growth upon their assimilation (Myrvang et al. 2016). For example, Ba has been shown to significantly inhibit germination, seedling establishment, photosynthetic activity, and growth of soybean (Iqbal and Ijaz 2002) and maize (Suwa et al. 2008) plants, while Sr was shown to decrease chlorophyll α in comparison to chlorophyll ß, inhibiting radicle elongation in corn (Moyen and Roblin 2010).

2.6 Spanish River Carbonatite

Spanish River Carbonatite (SRC) is a carbonatite primarily composed of apatite, biotite, and igneous calcite, a rare and highly reactive form of $CaCO_3$ (Boreal Agrominerals Inc.). SRC is also made up, to a lesser extent, of varying amounts of elemental nutrients and rare earth elements (three of the latter are highlighted in Table 2.2) (Sage 1997; Slack 2003; Boreal Agrominerals Inc.). SRC is an igneous, alkaline rock of magmatic origin that is located in the Sudbury region of Ontario, Canada (Sage 1987; Slack 2003). This agromineral is free of potentially harmful metal leachate, that is typically associated with mine waste facilities, according to the B.C. Waste Management Act-Special Waste Regulation Schedule 4, B.C. Reg. 63/88 (Boreal Agrominerals Inc.). This unique characteristic of SRC is attributed to its low fluorine content that inhibits the formation of pyrochlorate, and the subsequent accumulation of radioactive ions and heavy metals (Slack 2003). Because of its high $CaCO_3$ content and its wide range of minerals, SRC is currently sold to organic and conventional farmers as a soil fertilizer and conditioner (Boreal Agrominerals Inc.). The composition of SRC makes it an attractive slow-release plant useable nutrient source.

To my knowledge, Jones (2016) is the only researcher who has conducted studies on the agronomical value of SRC as a source of plant useable nutrients. This author reported positive and

promising effects of SRC as a soil amendment on the growth of pea plants and heterotrophic soil bacteria. Also, the author demonstrated that SRC could act as a suitable nutrient source provided that the plants are supplemented with N. Nitrogen supplementation is required because SRC and agrominerals in general are not a source of N (van Straaten 2006), and N is critical to plant growth (Chen and Liao 2017). The author reported comparable growth and yield of pea plants inoculated with N_2-fixing bacteria grown in soils amended with SRC and of non-inoculated plants supplemented with a soluble nutrient solution. In addition, the SRC amendment resulted in a two-fold increase in the number of colony-forming units of heterotrophic bacteria in the rhizosphere of pea plants and in a two-fold increase in the number of nodules formed on the inoculated pea roots. Because SRC acted in a manner comparable to the soluble nutrient solution on the growth of pea plants, Jones (2016) recommended that a combination of SRC amendment and rhizobia inoculation, in the case of legumes, supplement the use of synthetic fertilizer (Jones 2016).

Table 2.2 Mineral composition of SRC

Mineral composition	% mean dry matter in deposit*
Calcite ($CaCO_3$)	**65**
Biotite & vermiculite mica (K source)	**15**
Apatite ($(Ca_3PO_4)_2$ source)	**12**
Magnetite & hematite (Fe source)	2
Magnesite (MgCO3)	1.5
Pyroxene (silicate source)	1.5
Orthoclase (K-feldspar source)	1
Thenardite (Na_2SO_4 source)	0.2
Admixture (ppm)	
Mn	1200
Zn	60
B	40
Mo	12
Cu	10
Rare Earth Elements (ppm)	
Lanthanium	161
Neodymium	130
Yttrium	33

*SRC can contain up to 7% moisture
Source: http://borealagrominerals.ca/products/composition.html

Although Jones (2016) indicated that SRC may serve as a supplement for synthetic fertilizer, the weathering of Ca-rich compounds may hinder the release of the P-bearing, apatite, component via the common ion effect (Choudhary et al. 1994; Zhang et al. 2017b). Indeed, as these Ca-rich complexes weather, they release Ca^{2+}, the common ion, into the soil solution that already contains Ca^{2+}. This release of Ca^{2+} results in an increase in soil pH, but P release from the apatite component is limited because, at alkaline pH, PO_4^{3-} becomes complexed with Ca^{2+} as calcium phosphates (Goulding 2016; Ritchey et al. 2016). Therefore, the simultaneous release of Ca with the weathering of P-bearing compounds under alkaline conditions may prevent the substantial use of mineral complexes rich in Ca in agricultural systems (Heim et al. 2011). However, their use on Ca-deficient and neutral soils or in combination with synthetic fertilizers may prove beneficial in ameliorating acidic, nutrient-depleted soils (Barea et al. 2002).

The weathering of agrominerals is a complex process that involves several physical, chemical, and biological factors (van Straaten 2006; Zhang et al. 2017b). These factors are often linked to the mineralogy, chemical reactivity, rate of application of the complex, soil pH, and microbial activity (Ba and Guissou 1996; Burghelea et al. 2015). The use of an integrated farming approach involving chemical and biological activities may enhance their long-term efficacy. It is well established that the interactions in the rhizosphere between roots and their associated microbiota, and those between microorganisms influence the weathering process of complex minerals, and the subsequent release and uptake of nutrients (Vanlauwe et al. 2000; Hinsinger 2001; Blum et al. 2002; Talboys et al. 2016). The rhizosphere is the volume of soil surrounding the roots, that is directly influenced by root activity and the microbial interactions (Haichar et al. 2014; Zwetsloot et al. 2018).

These rhizosphere interactions have been shown to enhance the weathering of agrominerals via 1) composition of compounds released from the roots (Vanlauwe et al. 2000; Calvaruso et al. 2006; Talboys et al. 2016), 2) the type of microorganisms present [e.g., phosphate-solubilizing bacteria (Toro et al. 1997; Omar 1998; Calvaruso et al. 2006), or ecto- and mycorrhizal fungi (Ba and Guissou 1996; Barea et al. 2002; Alves et al. 2010; Burghelea et al. 2015)]. Hinsinger and Jaillard (1993) reported that the exudation of H^+ and organic acids from rape roots acidifies the rhizosphere and leads to the weathering of K-bearing phlogopite mica releasing interlayer K. Additionally, forest trees with a low P status were shown to increase their foliar P content by using their mycorrhizae to exploit an apatite complex (Hagerberg et al. 2003), suggesting that apatite may be used to alleviate P-deficiency in forest soils. Mycorrhizae interactions in the rhizosphere modified soil pH, and soil pH is a key factor is determining the dissolution rates of agrominerals and their agronomic effectiveness (Hagerberg et al. 2003).

2.7 The role of root exudates in nutrient acquisition

Access to adequate amounts of soluble nutrients influences internal plant signals that control several plant developmental stages (Dakora and Phillips 2002). The availability of nutrients is a major constraint to agricultural productivity (Dakora and Phillips 2002). Plants release root exudates to enhance nutrient availability in their rhizospheres (Chen and Liao 2017). Root exudates are a complex mixture of low and high molecular-weight compounds selectively secreted by roots (Huang et al. 2014; Venturi and Keel 2016); they represent a significant C source for heterotrophic microbes (Chaparro et al. 2012; Haichar et al. 2014). The composition of the roots exudates varies from plant to plant and with soil conditions (Jacoby et al. 2017), and plants growing under nutrient-limiting conditions modify the composition of their root exudates (Hartman et al.

2009). To deal with deficiencies such as Fe, Mn, Zn, and P plants exude 1) carboxylic acids, H^+, phenols or siderophores to weather and acquire insoluble minerals making them available for uptake (Hartmann et al. 2009) or 2) sugars, amino acids, and organic acids to attract microorganisms involved in nutrient acquisition (Dakora and Phillips 2002; Jacoby et al. 2017).

Besides, some plants are more effective than others at mobilizing soil nutrients, thus improving the nutrient availability for themselves and their neighbouring less-effective mobilizers (Zhang et al. 2014; Faucon et al. 2017). White lupin and chickpea, for example, are effective mobilizers of P from unavailable soil sources (Kamh et al. 1999; Li et al. 2014) and are often referred to as P-mobilizing species (Zhang et al. 2014; Faucon et al. 2017). In response to P-limitation, P-mobilizing species release carboxylates, organic anions, and H^+ (Kamh et al. 1999; Sugiyama and Yazaki 2012; Li et al. 2014) (which are effective at solubilising P from Ca phosphates in alkaline soils) or release phosphatases and phytases to hydrolyse organic P or poorly available inorganic P sources (Li et al. 2014; Chen and Liao 2017). Therefore, growing a mix of crop species that are differentially capable at mobilizing nutrients may serve as a means to improve nutrient availability.

2.8 Plant root-microbe interactions exploited in nutrient acquisition

With the increasing costs and negative impacts of fertilizers, more attention has been placed on exploiting plant-microbe interactions to increase plant nutrient acquisition and improve soil health (Dakora and Phillips 2002; Sugiyama and Yazaki 2012). There is a group of microorganisms capable of influencing plant growth through direct and indirect interactions. These microorganisms are known as plant growth-promoting rhizomicrobes/bacteria (PGPB) (Haichar et al. 2014; Jacoby et al. 2017; Martínez-Hidalgo and Hirsch 2017; Mhlongo et al. 2018). PGPB

include species of several bacterial genera, for example, *Bacillus, Pseudomonas, Enterobacter, Arthrobacter, Burkholderia, and Paenibacillus* (Mhlongo et al. 2018). They are commonly found associated with many agriculturally important crops such as maize and rice (Berg and Smalla 2009; Bulgarelli et al. 2015) and lupin and rape (Zhang et al. 2017c). Because of their roles in plant protection and growth, PGPB are often used as biofertilizers and biocontrol agents (Pérez-Montaño et al. 2014; Mhlongo et al. 2018). However, before their use as biofertilizers in field environments, PGPB are screened for common plant growth-promoting characteristics such as phosphate-solubilization capacity (Gupta et al. 1994; Liu et al. 2015), organic acid production (Liu et al. 2015; Álvarez-López et al. 2016), and potassium-solubilization (Sheng and He 2006). Incorporating the use of PGPB, especially those involved in nutrient solubilization and mineralization, in farming may lead to reduced rates of fertilization. Adesemoye et al. (2009) reported yields of PGPB-inoculated tomato plants grown in soils supplemented with reduced rates of synthetic fertilizers statistically comparable to those of plants grown in soils supplemented with a full dose of fertilizer. Such findings are promising and provide insights into the use of soil microorganisms to increase nutrient acquisition and plant growth.

PGPB with the capacity to solubilize and mineralize inorganic and organic phosphate sources represent a solution for issues related to P-deficiency in agroecosystems. Bacterial strains, including *Pseudomonas, Bacillus, Rhizobium, Actinomycetes* (Guang-Can et al. 2008; Liu et al. 2015), and fungal strains of *Aspergillus, Penicillium* and *Rhizophagus,* formerly known as *Glomus,* are effective at increasing the availability of soil P (Martin et al. 2007; Arcand et al. 2010; Alori et al. 2017). These microorganisms are collectively referred to as phosphate-solubilizing microorganisms (PSM) (Richardson and Simpson 2011; Baas et al. 2016; Alori et al. 2017). A common mechanism used by PSM is acidification via the production and release of organic acids,

such as citric, oxalic, and keto-gluconic acids, into the surrounding environment (Richardson and Simpson 2011; Alori et al. 2017). Additional strategies to solubilize P include chelation reactions and the secretion of extracellular enzymes (Richardson et al. 2009a; Mohammadi 2012; Behera et al. 2017), as well as the use of hyphal networks to aid in P acquisition (Alori et al. 2017). The ability to solubilize insoluble P sources is being exploited in field studies to improve P acquisition and plant growth (Omar 1998; Rodriquez and Fraga 1999; Taktek et al. 2015; Baas et al. 2016).

2.9 The roles of cover crops in agroecosystems

Cover crops are herbaceous plants (Sullivan 2003; Steenwerth and Guerra 2012) often grown to provide soil coverage and reduce NO_3^- leaching during the periods when the land is left bare in annual cropping systems (De Baets et al. 2011; Finney et al. 2017; Shelton et al. 2018). They can also be harvested before flowering and incorporated into the soil as green manure to provide nutrients upon decomposition and increase SOM content (Abawi and Widmer 2000; Magdoff and van Es 2009). Incorporating plant residues into the soil is an effective method of building SOM levels (Li et al. 2015a), and the use of cover crops to do so is well documented (Fageria et al. 2005; McGourty and Reganold 2005; Baumgartner et al. 2008; Pérez-Bermúdez et al. 2016). Their use as a fertility-building tool has declined following the green revolution (McKenna et al. 2018). However, recently, there has been a renewed interest in using them because of their positive influence on soil properties such as increasing aggregate stability and reducing soil erosion (Dabney et al. 2001; Pérez-Bermúdez et al. 2016; Bacq-Labreuil et al. 2018). Different species of cover crops are selected depending on their functional traits and the ecosystem services (Table 2.3) they provide (Faucon et al. 2017; Sharma et al. 2018; Shelton et al. 2018).

Table 2.3 Ecosystem services provided by cover crops

Ecosystem services	Cover crop	Cropping system	Reference
Suppressing weeds & pests	Alfalfa/weed	Mix-crop	(Chung and Miller 1995b)
	Cowpea/Sudangrass	Rotation	(Ngouajio et al. 2003)
	Oilseed radish/nematode	Mix-crop	(Mennan et al. 2008)
	Oilseed radish/lettuce/weed	Mix-crop	(Lawley et al. 2012)
	Rapeseed/winter rye	Rotation	(Larkin et al. 2010)
Improving soil structure	Oats/rye	Monoculture/mix-crop	(Kabir and Koide 2002)
	Oilseed radish/cereal rye	Rotation	(Williams and Weil 2004)
	Ryegrass/oats/mustard	Mix-crop	(De Baets et al. 2011)
Reducing erosion & nutrient loss	Rye/hairy vetch/ crimson clover	Rotation	(Daniel et al. 1999)
	Brassica sp/rye	Mix-crop	(Dean and Weil 2009)
Scavenging nutrients	Red clover/soybean/maize	Mix-crop	(Gaudin et al. 2015)
	Brassica sp	Rotation	(Fageria et al. 2005)
	Red clover/bluegrass	Mix-crop	(Thilakarathna et al. 2012)
Supporting microbial populations	Forage radish/cereal rye	Mix-crop	(White and Weil 2010)
	Barley/clover/rapeseed	Rotation	(Larkin et al. 2010)

The families of Poaceae (cereal and grasses) and Fabaceae (legumes) are the most commonly-used cover crops and to a lesser extent the families of Brassicaceae (mustards) and Asteraceae (daisies) that contain broadleaved herbaceous plants (McGourty and Reganold 2005; Dabney et al. 2010; Steenwerth and Guerra 2012). Some desirable traits of cover crops include their ability to 1) establish quickly under less than ideal conditions (Sharma et al. 2018), 2) produce extensive aboveground biomass that effectively protects the fertile topsoil (Mennan et al. 2008), 3) act as a host for beneficial microorganisms (Watson et al. 2002; Fageria 2007), and 4) scavenge limited nutrients such as N and P (Fageria et al. 2005; Jacobs 2012). Selection of the types of cover crops to use as part of a management system requires a good understanding of the interactions that occur among plant species because growing cover crops together with the primary crops can influence these interactions and negatively affect productivity (Faucon et al. 2017; Finney et al. 2017). These interactions include temporal and spatial competition for light, water, and nutrients, as well as the release of compounds that inhibit the growth of other crops, i.e., allelopathic chemicals (Chung and Miller 1995a; Faucon et al. 2017). These specific chemicals have been shown to compromise the yield of the primary crop (Florentín et al. 2011; Lal 2015; Steenwerth et al. 2016).

To achieve a range of ecosystem services (Table 2.3), Shelton et al. (2018) recommend incorporating a mix of cover crops as part of an integrated management strategy. For example, grasses are ideal for building SOM because their residues have high C:N ratios and lignin content (McGourty and Reganold 2005; Olmstead 2006; Dabney et al. 2010). A high C:N ratio and high lignin content result in a slower decomposition rate, low net mineralization in the short-term, and a sustained release of nutrients for the primary crops (McGourty and Reganold 2005; Olmstead 2006; Wang et al. 2008a). Legumes are chosen for their role in symbiotic N_2-fixation (Dabney et

al. 2010; Alvarez et al. 2017). Compared to grasses, legumes have a lower C:N ratio, as such their decomposition rates are rapid once their residues have been incorporated into the soil (McGourty and Reganold 2005; Li et al. 2015a).

Several studies have reported that portions of the N assimilated by the non-legume are derived from the N_2-fixing legume grown in an intercropping system (Martin et al. 1991; Kurppa et al. 2010; Rusinamhodzi et al. 2012; Thilakarathna et al. 2016). However, the amount of N_2 fixed by the legume cover crop and available for uptake will depend on the cultivar of the plant, the effectiveness of the microbial inoculum, and the soil moisture and temperature (Steenwerth and Guerra 2012). The root system of many legumes allows them to absorb nutrients available in low concentrations deeper in the soil, increasing the nutrient levels in the topsoil (Fageria et al. 2005). Non-leguminous cover crops are often better at providing soil coverage and reducing soil erosion by improving soil structure (Dapaah and Vyn 1998; Williams and Weil 2004). These cover crops have a higher root scavenging capacity than leguminous crops, increasing their abilities to reduce NO_3^- leaching and soil erosion (Fageria et al. 2005). However, sometimes the growth of these scavenging cover crops is limited by N deficiency and growing them in a mixture with legumes can be the best strategy to obtain the maximum benefits from cover crops (Fageria et al. 2005). Several studies have highlighted the positive effects of leguminous crops on their companion crops, the soil properties, and on the subsequent crop in the rotation. These crop rotation studies include soybean-sugar cane (Li et al. 2013), soybean-maize (Rusinamhodzi et al. 2012; Alvarez et al. 2017), soybean-radish (Antoun et al. 1998), red clover-bluegrass (Thilakarathna et al. 2012; Thilakarathna et al. 2016), and wheat-corn-pea-hyacinth bean (Rochester et al. 2001).

2.10 Legume-*Rhizobium* symbiosis

Under N limitations, flavonoids are secreted by leguminous plants to signal to microorganisms capable of fixing N_2 (Antunes et al. 2006; Sugiyama and Yazaki 2012; Oldroyd 2013). These N_2-fixing microorganisms, collectively called rhizobia, engage in symbiosis with about ten angiosperm families (Abdel-Lateif et al. 2012). Successful colonization of the plants by these microbes leads to the formation of novel root organs, i.e. the nodules, that are the site of N_2-fixation (Oldroyd 2013). The plants supply the micro-symbionts with fixed C (Downie 2014; Sasse et al. 2018); in return, the micro-symbiont provides useable N. The ability to biologically reduce N_2 to NH_3 is limited to prokaryotes, i.e., bacteria and archaea (Gage 2004; Downie 2014; Martin et al. 2017), that encode nitrogenase, an O_2-sensitive N_2-fixing enzyme (Downie 2014; Stein and Klotz 2016; Martin et al. 2017). Rhizobia can be found growing endophytically in non-legume hosts while others are free-living in soils (Denison and Kiers 2011).

About 40-100 million metric tonnes of NH_3 entering the biosphere per annum does so through biological nitrogen fixation (Rubio and Ludden 2008; Downie 2014; Lira et al. 2015; Vimal et al. 2017). As such, plants associating with N_2-fixing bacteria have a selective advantage under conditions of N limitation (Downie 2014). It is estimated that about 80% of biologically-fixed nitrogen in agricultural systems is derived from symbiotic N_2-fixation (Yang et al. 2010; Vimal et al. 2017). As such, symbiotic N_2-fixation plays an essential role in maintaining soil fertility and crop productivity, and it has done so for centuries (Badarneh and Ghawi 1994). This biological process is sensitive to environmental factors such as soil pH, nitrogenous compounds, and phytohormones (Ferguson et al. 2013; van Noorden et al. 2016). These factors, especially soil pH and nitrogenous compounds, affect multiple aspects of nodulation ranging from flavonoid secretion (Dusha and Kondorosi 1993; van Noorden et al. 2016) to nodule initiation, development,

and distribution on the root system (Gan et al. 2004; Bollman et al. 2006). Additionally, various forms of mineral N adversely affect nodulation and N_2-fixation (Gulden and Vessey 1998; Gan et al. 2004; Oono and Denison 2010); for example, the presence of NO_3^- appears to have a more inhibitory effect on the legume-*Rhizobium* symbiosis than that of NH_4^+ (Gulden and Vessey 1997; Gulden and Vessey 1998; Bollman et al. 2006). Nevertheless, exploitation of the legume-*Rhizobium* symbiosis has been a critical strategy in reducing external N inputs, and its use has increased in recent times.

Chapter 3: The Use of an Integrative Strategy to Enhance Nutrient Acquisition and Restore Soil Health

Intensive agricultural production, particular the high rates of synthetic fertilizer application, has resulted in soil erosion, nutrient depletion, and biodiversity loss (FAO 2009; Strecker et al. 2015); as such, there is an urgent need to sustainably supply nutrients to plant and restore the fertility of degraded soils to avoid further degradation. An ideal farming approach enhances productivity while managing natural resources such as the soil (FAO 2009). Therefore, to sustainably farm, small- and large-scale farmers must reduce their use of synthetic fertilizers and other conventional agriculture practices through the implantation of an integrative management system (Figure 2.2). Farmers can protect the soil resource by maintaining diverse plant communities and undertaking practices that improve SOM and stimulate the activities of soil microorganisms, thus enhancing nutrient cycling. One such integrative strategy (Figure 3.1) uses cover crops with different traits, exploits plant-microbe interactions to enhance nutrient acquisition and plant growth, and utilizes SRC as a slow-release fertilizer to support both microbial and plant growth.

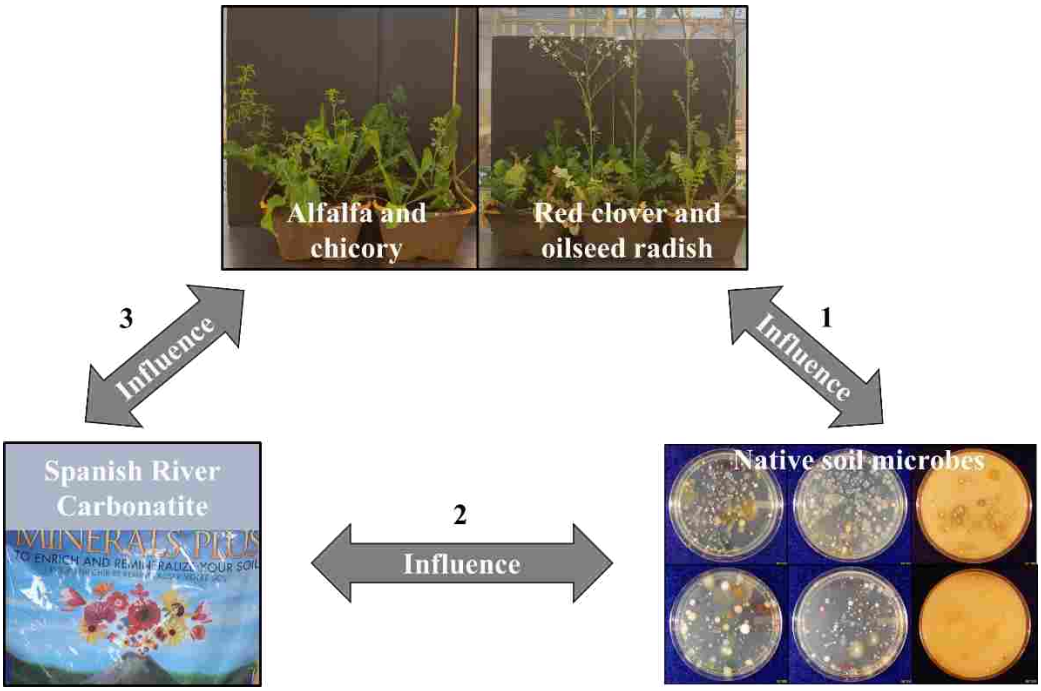

Figure 3.1 An integrated approach

This figure is a representation of the hypothesized interconnected interactions that enhance nutrient acquisition and plant growth. **Step 1** highlights the plant root-microbe interactions that indirectly increase nutrient uptake and promote plant growth. **Step 2** is the weathering process of the agromineral through the solubilization activities of rhizosphere microbes and S**tep 3** represents direct mechanisms that some plant species use to improve nutrient availability. Each step depends on the synergistic interaction with the others.

Soils in which leguminous and non-leguminous cover crops are grown are expected to harbour a diverse number of soil microorganisms such as symbiotic N_2-fixing, heterotrophic, and phosphate-solubilizing bacteria. The plant-microbe and microbe-microbe interactions in the rhizosphere are expected to enhance the weathering of the sparingly soluble SRC. I proposed that the release of nutrients from SRC will stimulate cover crop root growth by enhancing nutrient availability by gradually releasing nutrients over time in a manner that suits the cover crop demands (Harley and Gilkes 2000), whereas the soluble nutrients present in synthetic fertilizers would be immediately available for plant uptake as well as for immobilization and run-off (Gaiotti et al. 2017). The release of nutrients from SRC such as P, K, and the N_2 fixed by the leguminous cover crops and their respective *Rhizobium* species should support plant growth. In addition, soil pH is known to modify the microbial community composition (Rousk et al. 2009; Hou et al. 2018), and most of the cover crops previously mentioned grow best in near-neutral soils; the release of Ca as a result of SRC weathering should create an ideal environment for bacterial activities (Lauber et al. 2009; Rousk et al. 2010). The use of diverse cover crop species, nutrient acquiring microorganisms, and a slow-release nutrient source that is free from toxic elements represent a viable strategy to improve soil fertility and enhance nutrient acquisition.

This preliminary study was a collaborative effort with a team of researchers at Brock University. The researchers at Brock University focused on the aboveground interactions in a vineyard ecosystem, while I focused on those interactions that are occurring belowground. The cover crop combinations used in this study was determined by the collaborating team at Brock University. They grew the cover crops in different plant combinations, and identified the combination of plants that grew well together, i.e., alfalfa and chicory, red clover and oilseed (Heather VanVolkenburg, personal communication). The combination of cover crops I used for

this thesis was based on their findings. Informative details about the specific cover crops used can be found in Appendix A.

My overarching objective was to assess, under greenhouse conditions, the effects of three soil amendments on soil pH, on the relative microbial abundance, and on the microbial activity of three microbial functional groups in the rhizosphere of two cover crop combinations. I was particularly interested in understanding the influence of amendments on soil parameters, and how these parameters varied depending on the cover crop combination.

I used pot experiments to evaluate whether SRC represented an efficient slow-release nutrient source, and **the following hypotheses** were developed:

1. Because SRC is ~65% calcite (Slack 2003), I hypothesized that the pH in SRC-amended soils would be higher than those of ammonia nitrate- and NPK fertilizer-treated soils.
2. Because plant species are known to shape the microbial community structure (Eisenhauer et al. 2010), I hypothesized that microbial abundance and respiration vary depending on cover crop combination.
3. Because SRC has been proposed to enhance the legume-rhizobia symbiosis (Jones 2016), I hypothesized that the overall symbiotic efficiency of leguminous cover crops grown in SRC-treated soil is higher than cover crops grown in soils lacking the SRC amendment.

Chapter 4: Research Design and Methodology

A 3 x 2 factorial design was developed in which there were three levels of soil amendment: 1) Nitrogen as 2.5 mM ammonium nitrate (Sigma-Aldrich, Oakville, ON), 2) SRC in an SRC-to-soil ratio of 1:10, and 3) synthetic fertilizer as 20-20-20 N-P-K (Walmart Inc., St. Catharines, ON; Appendix B), with two combinations of cover crops: 1) alfalfa and chicory, and 2) red clover and oilseed radish, for a total of six treatments (Figure 4.1). All the seeds used in this study were obtained from Canadian Comfort Alpacas, Fenwick, ON. In pilot studies done to evaluate the growth of the cover crops, I noticed that the leguminous cover crops produced nodules, yet I had not inoculated the seeds with rhizobia. Although it is customary to sell inoculated legume seeds, this was not the case with the seeds bought. Therefore, I hypothesized that the purchased seeds were unintentionally inoculated with rhizobia possibly during the seed harvesting and packaging processes.

Eight replicates per treatment combination were used and were completed in four experimental runs. In each experimental run, 24 pots (FibreGrow planter pots (Volume: 6 L: Size: 30 cm x 30 cm)) were sterilized using the pre-vac 30 cycle of the STERIS AMSCO® Lab 250 Steam Sterilizer (Mississauga, ON), and were then disinfected with 1% Virkon® aqueous solution to minimize fungal contamination. Once pieces of debris (roots, twigs, and peat) larger than 1 cm were removed from SunGro Sunshine® Mix 1 potting soil (JVK®, St. Catharines, ON; Appendix C), the soil was saturated with non-sterile water. The pots were filled with water-saturated soil and left to settle in the greenhouse for 24-48 hours. For SRC treatments, SRC (90 mL) was applied to each corresponding treatment pot 24 h before sowing; the SRC was gently worked into the top 5 cm of the soil surface, and the soil was lightly watered and allowed to sit overnight.

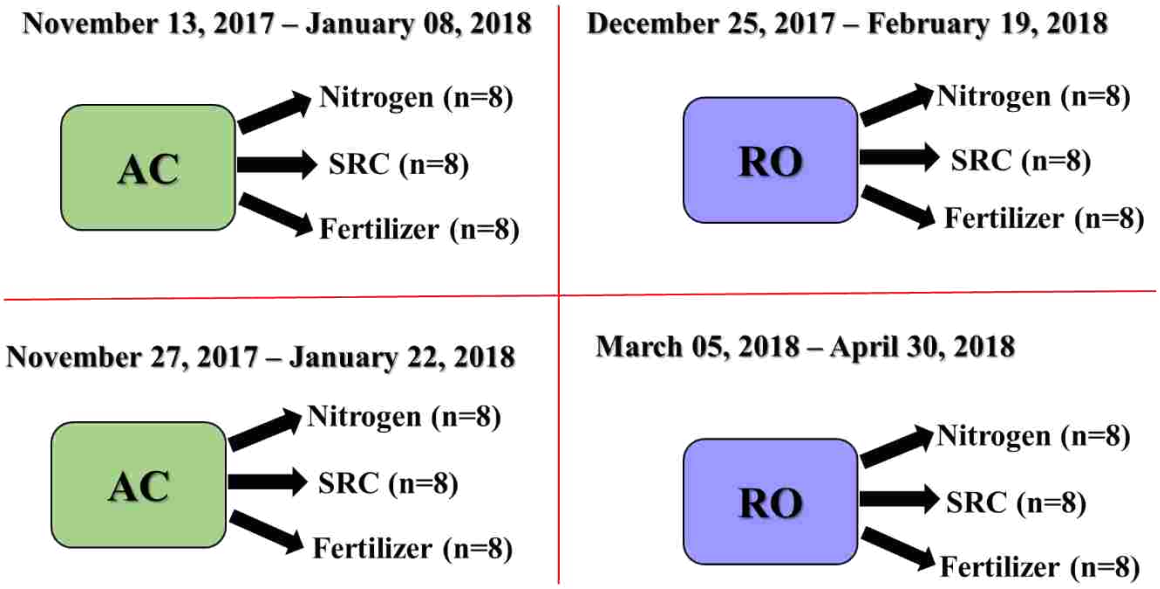

Figure 4.1 Experimental design

The cover crop combinations consisted of alfalfa and chicory (AC), and red clover and oilseed radish (RO). The soil amendments were nitrogen (as 2.5 mM ammonium nitrate), SRC (1:10 SRC:soil ratio), and fertilizer (as 20-20-20 NPK). The first set of intercropped alfalfa and chicory was grown from the week of November 13, 2017 to the week of January 08, 2018, while the second was grown from the week of November 27, 2017 to the week of January 22, 2018. Likewise, the first group of intercropped red clover and oilseed radish plants was grown from the week of December 25, 2017 to the week of February 19, 2018. The second group of plants was grown from the week of March 05, 2018 to that of April 30, 2018. For each cover crop combination and planting time, and for each soil amendment, there were eight pots with two plants of each species.

The alfalfa-chicory intercropped combination consisted of two alfalfa and two chicory plants per pot, whereas the red clover-oilseed radish combination consisted of two red clover and two oilseed radish plants per pot (Figure 4.2). Ten seeds were initially sown at about 5-8 cm from each corner (depth according to seed company directions) to ensure that at least one plant of each species was growing in each location. The seedlings were allowed to grow for fourteen days, at which time they were thinned to one per corner. Depending on treatments, pots received 500 mL of ammonium nitrate solution, water in the case of SRC, or NPK fertilizer biweekly (i.e., at days 14, 28, and 42). Also, all plants received 1 to 2 L of water every three to five days at the soil surface (the point where the plant emerges from the soil), and the amount was based on pot weight. Each pot was rotated 90° and randomly moved every three days to minimize greenhouse condition bias. The conditions in the greenhouse were control-regulated based on ambient conditions, with temperatures varying between 20.6 and 24.8 °C and the relative humidity between 52.1 and 21.7%. Each experimental run lasted for 56 days and was conducted in the greenhouse located on the roof of the Centre for Cold Regions and Water Science at Wilfrid Laurier University, Waterloo, ON.

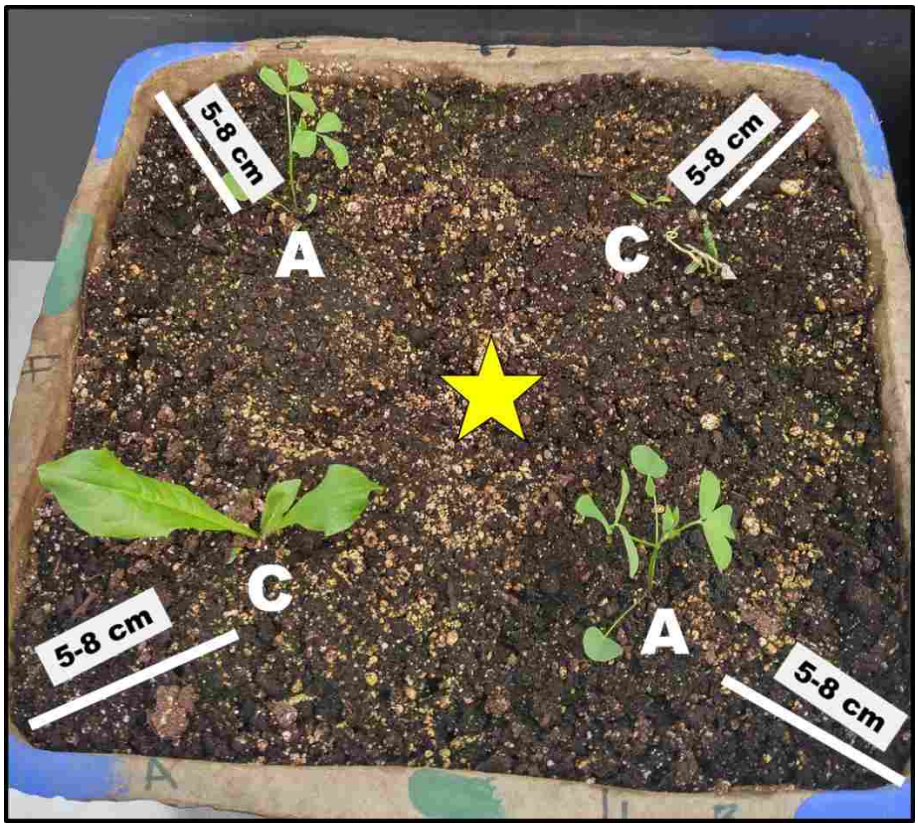

Figure 4.2 Sowing arrangement of cover crop seeds

Alfalfa (A) and chicory (C) seeds were sown in diametrically opposite positions. Here the plants are seen 18 days after planting. The star in the centre indicates where the soil samples were taken 56 DAP, while the solid red lines indicate the sowing distance from the corners of the pots.

4.1 Harvesting

Soil sampling and laboratory analyses were carried out in an aseptic manner by wearing latex gloves during sampling; the gloves and soil corer were sterilized with 70% ethanol before collecting the samples from each pot. Materials and utensils used during sampling and analyses (e.g., corer, sieves, incubation jars, flasks, and spatula) were washed with soap, rinsed in 10% sodium hypochlorite (bleach) solution, and then with deionized water before leaving them to air-dry. At day 56 after planting, before harvesting the plants, two soil cores (L: 15 cm; W: 1.9 cm) were taken from the centre of each pot (Figure 4.2), placed into 50 mL pre-labelled Falcon™ conical tubes, and stored at 4 °C until analysis. Because the pots were small and the roots of each plant were entangled, the soil in the entire pot was designated the rhizosphere. Sufficient soil was collected to measure soil pH, microbial abundance, and microbial respiration. After soil sampling, plants were gently removed from each pot and care was taken to preserve the roots and root nodules. Uprooted plants were placed into their respective emptied pots, and the shoots were separated from the roots at the point where the two joined. Nodules located on the roots of the legumes were detached, counted, and placed into pre-labeled envelopes. The roots were gently washed free of debris using water; in the case when more nodules became apparent, they were removed, counted, and added to the envelopes.

4.1.1 Evaluating the efficiency of the legume-*Rhizobium* symbiosis

To assess the use of SRC in combination with the N fixed in the legume-*Rhizobium* symbiosis as a strategy to support plant growth, the effect of soil amendments on the nodulation process was evaluated. The cost and gains of the symbiosis were assessed by calculating:

1. The plant return on nodule construction cost (i.e., host total dry weight (g) per g nodule dry weight).
2. Specific nodulation (i.e., the number of nodules per g root dry weight).
3. Specific nodule dry weight (i.e., nodule dry weight (g) per g root dry weight). This parameter (Gulden and Vessey 1998)

While the former parameter corresponds to the amount of C (from photosynthesis) invested into the symbiosis per nitrogen fixed, reflected in the host biomass (Oono and Denison 2010), the latter two parameters take into consideration the plant growth in response to the presence of the mineral nitrogen available (Gulden and Vessey 1998).

4.2 Measuring soil pH

Soil pH was measured according to Watson and Brown (1998). The measurements were done potentiometrically using an electric pH meter (FiveEasy™ FE20, Mettler-Toledo AG, Switzerland) in a soil/deionized water slurry. Twenty grams of soil collected from each pot per treatment were weighed out into sample cups. Twenty mL of deionized water was then pipetted into each cup. The soil/water slurry was mixed by swirling vigorously for 5 seconds, then left to equilibrate at room temperature for 10 minutes, before measuring the pH of the soil samples.

4.3 Quantifying relative microbial abundance

Assessment of the relative abundance of the three microbial groups was carried out according to standard methods (EPA 1978) via a series of 10-fold dilution. The extent of the dilution is a factor that determines the number of colonies formed on each of the nutrient media. The process was carried out under aseptic conditions to avoid the introduction of foreign microbes.

Three microbial media were used to allow the growth of the microbial groups that were of interest. A modified nitrogen-free Yeast Mannitol Agar or YMA (Vincent 1970) (Appendix D) was used to isolate *Rhizobium* species selectively (i.e., those bacteria that symbiotically fix N_2 with legumes) from the soil. Nutrient agar or NUTRA (Difco & BBL 2009) was employed for microorganisms that use organic carbon as a substrate and energy source, i.e., heterotrophic microbes. Finally, the National Botanical Research Institute Phosphate Media or NBRIP (Nautiyal 1999) (Appendix E) was used to select for bacteria that can solubilize a partially soluble phosphate source on solid media.

The isolation and enumeration of colony forming units (CFU) of symbiotic nitrogen-fixing, heterotrophic, and phosphate-solubilizing bacteria were conducted according to standard plate methods for aerobic bacteria (EPA 1978). To prepare the dilution series, culture tubes were filled with nine mL of water (diluent) and capped. These tubes, as well as micropipette tips, were autoclaved using the liquid 15 cycle (STERIS AMSCO® Lab 250 Steam Sterilizer) and allowed to cool. Since there were eight pots per treatment, a random number generator (https://www.random.org/) was used to produce a total of four samples per treatment. Each of the four generated samples was a composite from two random pots. From each of the ammonium nitrate-treated soil samples, a sub-sample of one gram was weighed out for use in the dilution series (Figure 4.3). A sterile working environment was maintained by working aseptically in a Labconco™ Purifier Class II Biosafety Cabinet. The one gram of soil was added to a culture tube containing nine mL of sterile water (10^{-1}), and the content was mixed via agitation for 30 seconds using a vortex (VWR, LAB DANCER S41).

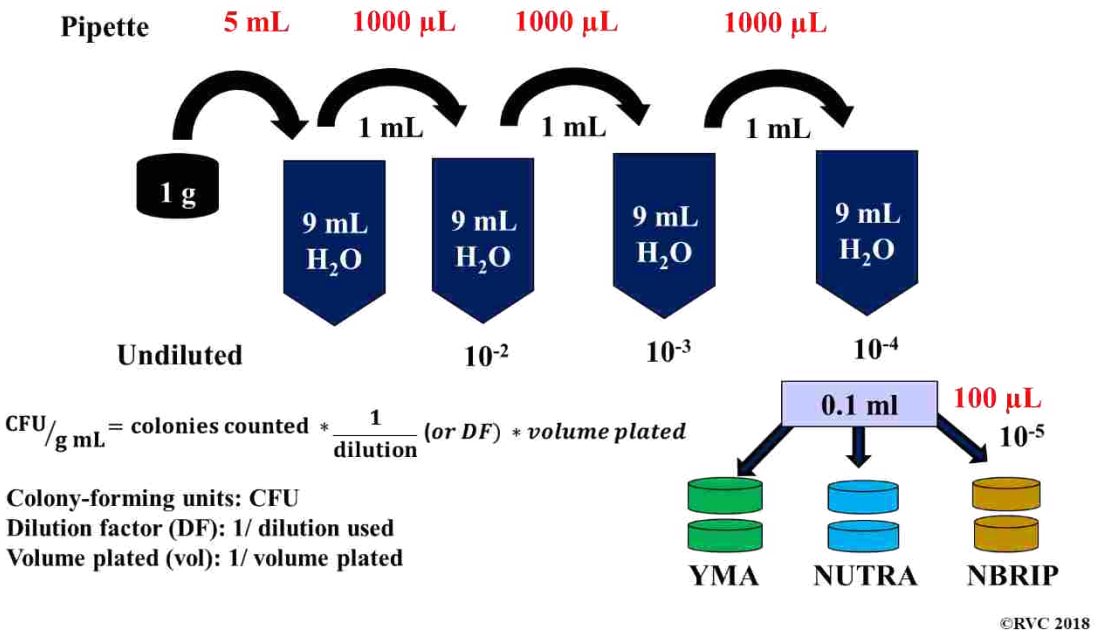

Figure 4.3 Serial dilution and plating

Flow diagram of the serial dilution and plating method used in quantifying the relative abundance of symbiotic nitrogen-fixers (YMA), heterotrophs (NUTRA), and phosphate-solubilizing (NBRIP) bacteria, where YMA is Yeast Mannitol Agar, NUTRA is Nutrient Agar, and NBRIP is the National Botanical Research Institute Phosphate media (n=8). There were three media types for each soil amendment and two replicates per media. Colony-forming units were counted five and ten days after incubation.

Because the Sunshine® mix contained sphagnum moss, which created a stopper in the tips of the micropipette used, a sterile five mL glass pipette was used to remove one mL from the 10^{-1} dilution tubes. The dilution series was continued by using a 1000 µL micropipette with a sterile tip to make three successive dilutions, the tubes were vortexed, and the micropipette tips changed between each dilution. A 100 µL micropipette was used to remove 0.1 mL of the sample from the 10^{-4} dilution and to plate it onto each of the three media plates. An L-shaped glass rod was ethanol- and flame-sterilized, then cooled for 10 seconds, before using it to spread the inoculum across the agar surface (Figure 4.3). Spread plating was carried out in duplicate according to standard methods (EPA 1978), and the plates were left to dry for 30 seconds before closing the lids and applying Parafilm®. The sealed plates were then inverted, placed in a dark box, and incubated at 23 °C for ten days in an ENCONAIR™ growth chamber. The above procedure was repeated for the SRC- and fertilizer-amended soil samples. The relative abundance of microbes (log CFU/g soil) in the undiluted sample was enumerated by counting individual colonies on each of the media.

Counting the CFU assumes that each colony is separate and is formed by a single microbial cell (EPA 1978). The counting range of CFU used in this study was 30-300 per standard plate count (Wollum II 1982). Therefore, colonies falling outside the lower and upper limits were excluded from the statistical analyses (Lee 2015). The total CFU on YMA, NUTRA, and NBRIP agar plates were counted five and ten days after incubation. The CFU obtained and the respective dilution factor was used to calculate the relative number of microorganisms in the original soil sample and was reported as log CFU per g soil (Lee 2015).

4.4 Measuring microbial biomass respiration

A simple laboratory approach that takes advantage of the reaction that occurs when CO_2 is converted to carbonate in the presence of excess hydroxonium ions (Figure 4.4) was used to assess microbial biomass respiration (Anderson 1982; Rowell 1995; ISO 2002; Haney et al. 2008). Soil samples taken at harvest were dried at 40 °C for three days and then ground in a 70% ethanol-sterilized mortar and pestle (Haney et al. 2008).

Twenty grams (± 0.01) of the soil was weighed out into pre-labelled, ethanol-sterilized air-tight Magenta™ jars, and for the controls, silica sand was used as the substrate. Fifty mL of deionized water were added to the twenty grams of soil to adjust the soil to a water content of ~ 20%, and the soil/water slurry was mixed well. A culture tube cap, serving as the CO_2 absorption vial, was filled with ten mL of 1 M NaOH solution, and once it was securely placed in the rewetted soil, the lid of the jar was quickly closed. The jars were positioned in a dark box at room temperature (~20 °C in the laboratory) for four days. Following the incubation of the jars, the amount of CO_2 released from microbial respiration was quantified via titration (Rowell 1995; Haney et al. 2008). An acid-base titration was done using 1 M HCl and the 1M NaOH in the vial and 1% phenolphthalein indicator (Sigma-Aldrich, Oakville, ON). Barium chloride ($BaCl_2$, 1 M) was added to precipitate the resulting sodium carbonate out of solution (Rowell 1995). The addition of $BaCl_2$ ensured that only the neutralization of NaOH by the HCl was occurring during titration. Complete neutralization was noted when a change of colour, from white to pink, occurred in the Erlenmeyer flasks. The standard rate of microbial respiration (RCO_2), i.e., CO_2 evolved per twenty grams of moist soil per second, was calculated and expressed as grams CO_2 per grams moist soil per second ($gCO_2\ g^{-1}$ moist soil s^{-1}) as per Rowell (1995).

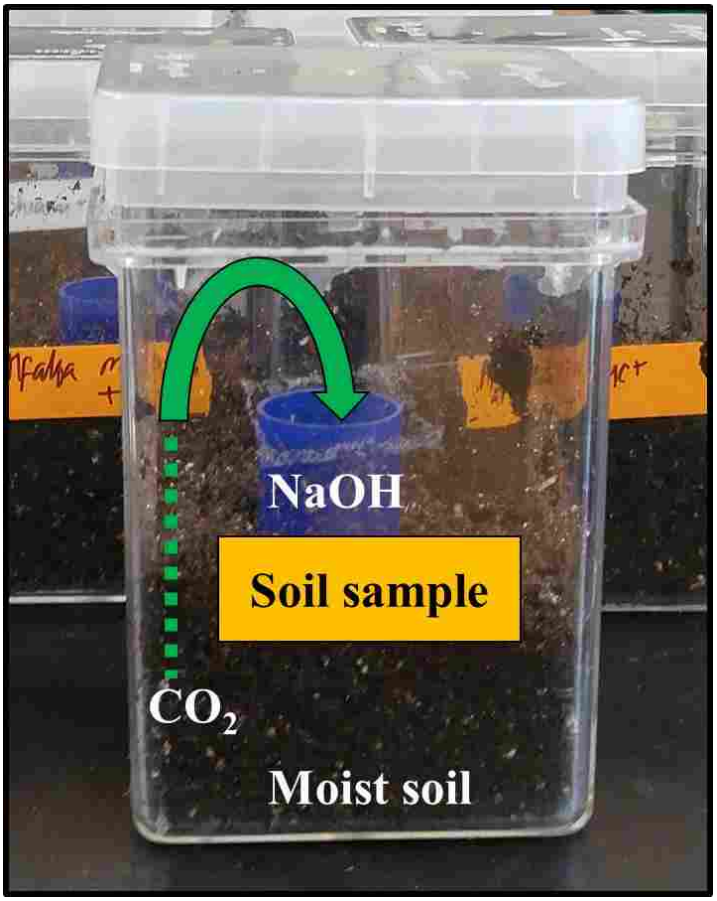

Figure 4.4 A simple laboratory respirometer

Each Magenta^{TM} jar (n=4) per soil amendment contained 20 ± 0.1 g moist soil while the control jars (n=3) contained silica sand instead of soil. A vial with ten mL 1M NaOH solution was securely embedded into the substrate to absorb the CO_2 released during the four days of incubation.

4.5 Statistical analyses

Since there were two plants of the same cover crop species per pot (alfalfa-chicory and red clover-oilseed radish), an average was first calculated to have a single value per pot. The effects of soil amendments on the legume-*Rhizobium* efficiency were assessed by using the non-parametric Kruskal-Wallis test. In the cases where the test indicated statistical significance results, a multiple comparisons ($\alpha = 0.05$) was done using the Wilcoxon rank sum test. A one-way analysis of variance (ANOVA) was used to assess the effects of the soil amendment on soil pH, microbial abundance and respiration. The assumptions of the ANOVA model were evaluated by using the Levene's test to assess the homogeneity of the variance and the Shapiro-Wilks test to assess normality. In cases where the assumptions of the ANOVA were violated, the non-parametric Kruskal-Wallis test was used. A post-hoc multiple range Tukey HSD test or the Wilcoxon rank sum test ($\alpha < 0.05$) was performed whenever a soil amendment was found to be significant. The analyses were done using R software suite (RStudio version 1.1.442-© 2009-2018 RStudio, Inc.; https://cran.rstudio.com/) with packages 'emmeans' (Type II Satterthwaite approximation), 'car', 'rcompanion', and 'multcomp'.

Chapter 5: Results

5.1 The effect of soil amendment on microorganisms

5.1.1 The rhizosphere of alfalfa and chicory

On average, the soil in which alfalfa and chicory plants was sown was slightly acidic (6.15 ± 0.04) before sowing and before the addition of the soil amendments. However, at harvest, the pH of the soils was raised (Appendix F). There was a significant effect of soil amendment on pH ($F = 87.618$, df =2, $p = 3.0E^{-15}$). The pH of SRC-treated soil was significantly higher than the pH of ammonium nitrate- and fertilizer-treated soils, and the pH of soils amended with fertilizer was significantly lower than those treated with ammonium nitrate (Figure 5.1).

There was no significant effect of soil amendments on the relative abundance (log CFU/g soil) of symbiotic nitrogen-fixing, heterotrophic, and phosphate-solubilizing bacteria in the rhizosphere of alfalfa and chicory plants (Table 5.1). Although soil amendment did not significantly affect microbial abundance, SRC-treated soils tended to have more symbiotic nitrogen-fixing and heterotrophic bacteria than soils treated with ammonium nitrate and fertilizer. However, fertilizer-treated soils tended to have more phosphate-solubilizing bacteria than SRC- and ammonium nitrate-treated soils (Figure 5.2). Soil amendment significantly affected the amount of CO_2 released during microbial respiration ($F = 6.6763$, df =2, $p = 0.003$). Microbial respiration was significantly higher in soils amended with fertilizer than in those amended with SRC while the respiration rate in ammonium nitrate-treated soil was statistically similar to that measured in fertilizer- and SRC-treated soils (Figure 5.3).

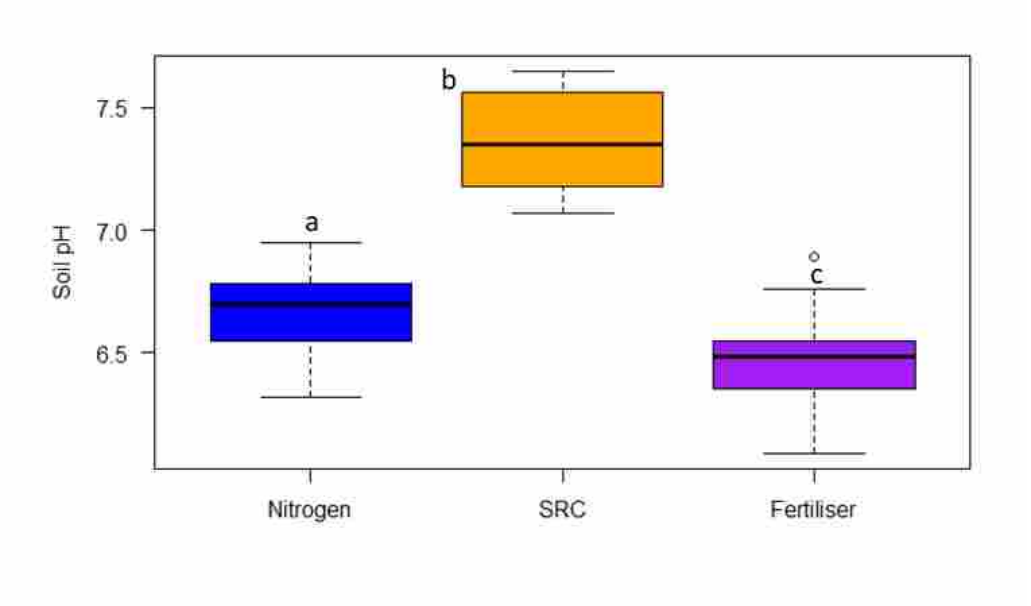

Figure 5.1 Soil pH in the rhizosphere of intercropped alfalfa and chicory plants

Boxplots of the effect of soil amendments: Nitrogen as 2.5 mM ammonium nitrate, SRC in a 1:10 SRC:soil ratio, and fertilizer as 20-20-20 NPK commercial fertilizer, on soil pH 56 days after planting (n =16). Different letters indicate statistical significance (1-way ANOVA + Tukey HSD post hoc test at $p \leq 0.05$). The small circle above the fertilizer boxplot whiskers represents an outlier, i.e., any point that is 1.5x greater or lesser than the interquartile range for that data group.

Table 5.1 Analysis of Variance table for the microbial abundance in the rhizosphere of intercropped alfalfa and chicory plants

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Symbiotic nitrogen-fixers			
Level	**df**	**F-value**	**p-value**
Treatment	2	0.4342	0.650
Heterotrophs			
Treatment	2	2.4059	0.110
Phosphate-solubilizers			
Treatment	2	2.4534	0.110

The relative abundance of heterotrophs, symbiotic nitrogen-fixers, and phosphate-solubilizers in the rhizosphere of intercropped alfalfa and chicory plants

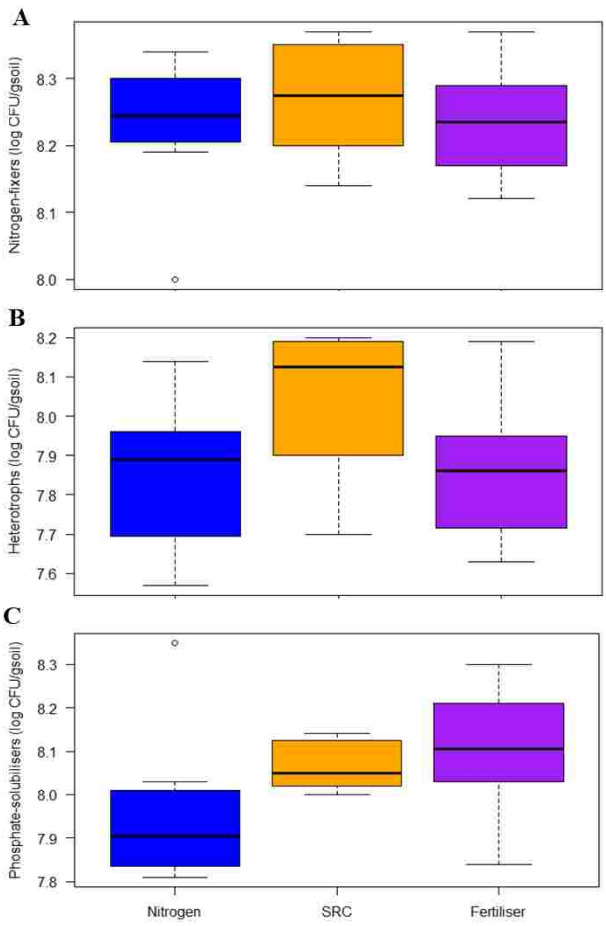

Figure 5.2 Microbial abundance in the rhizosphere of intercropped alfalfa and chicory plants

Boxplots of the effect of soil amendments: Nitrogen as 2.5 mM ammonium nitrate, SRC in a 1:10 SRC:soil ratio, and fertilizer as 20-20-20 NPK commercial fertilizer, on the relative abundance (log CFU/g soil) of **A:** symbiotic nitrogen-fixing, **B:** heterotrophic, and **C:** phosphate-solubilizing bacteria (n = 4) 56 days after planting. The 1-way ANOVA indicated no significant differences between soil amendments. The small circle above or below the boxplot whiskers represents an outlier, i.e., any point that is 1.5x greater or lesser than the interquartile range for that data group.

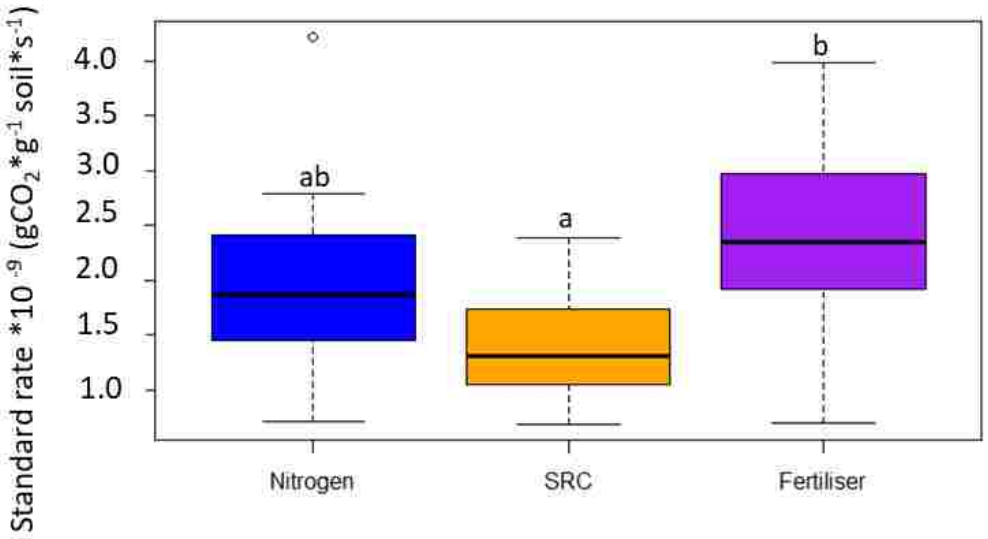

Figure 5.3 Microbial respiration in the rhizosphere of intercropped alfalfa and chicory plants

Boxplots of the effect of soil amendments: Nitrogen as 2.5 mM ammonium nitrate, SRC in a 1:10 SRC:soil ratio, and fertilizer as 20-20-20 NPK commercial fertilizer, on the standard rate of microbial respiration (gCO_2*g^{-1} $soil*^{s-1}$) 56 days after planting (n = 4). Different letters indicate statistical significance (1-way ANOVA + Tukey HSD post hoc test at $p \leq 0.05$). The small circle above the boxplot whiskers represents an outlier, i.e., any point that is 1.5x greater or lesser than the interquartile range for that data group.

5.1.2 The rhizosphere of red clover and oilseed radish

On average, the soil in which red clover and oilseed plants were sown was slightly acidic (6.08 ± 0.16) before sowing and the addition of the soil amendments. However, like for the rhizosphere of alfalfa and chicory, the pH of the soil was raised at harvest (Appendix F). At harvest, soil amendment significantly affected soil pH ($F = 77.648$, df =2, $p = 2.6E^{-15}$); a pattern similar to that of alfalfa and chicory soil was observed in red clover and oilseed radish soil. The pH of SRC-treated soils was significantly higher than that of ammonium nitrate- and fertilizer-treated soils, and the soil amended with ammonium nitrate had a significantly higher pH than that of the fertilizer-treated soil (Figure 5.4).

There was no significant effect of soil amendments on the relative abundance (log CFU/g soil) of symbiotic nitrogen-fixing, heterotrophic, and phosphate-solubilizing bacteria in the rhizosphere of red clover and oilseed radish plants (Table 5.2). Although soil amendments did not significantly affect the microbial abundance, SRC-treated soils tended to have more symbiotic nitrogen-fixing, heterotrophic, and phosphate-solubilizing bacteria than soils treated with ammonium nitrate and fertilizer (Figure 5.5). Likewise, soil amendment did not significantly affect the amount of CO_2 released during microbial respiration ($X^2 = 0.74662$, df =2, $p = 0.689$). Nevertheless, the amount of CO_2 released in soils treated with ammonium nitrate and fertilizer tended to be slightly higher than that of soil treated with SRC (Figure 5.6).

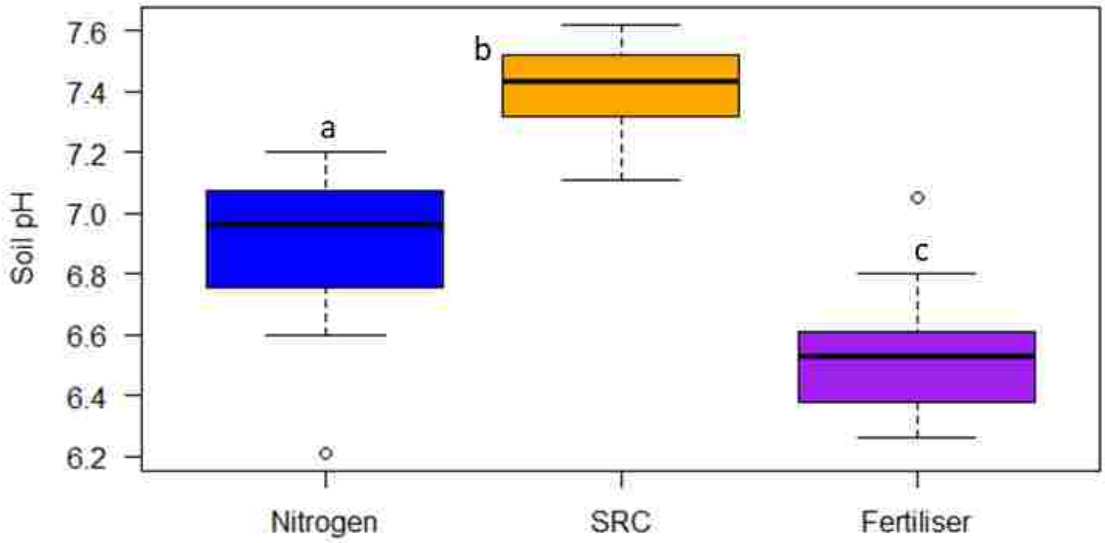

Figure 5.4 Soil pH in the rhizosphere of intercropped red clover and oilseed radish plants

Boxplots of the effect of soil amendments: Nitrogen as 2.5 mM ammonium nitrate, SRC in a 1:10 SRC:soil ratio, and fertilizer as 20-20-20 NPK commercial fertilizer, on soil pH 56 days after planting (n =16). Different letters indicate statistical significance (1-way ANOVA + Tukey HSD post hoc test at $p \leq 0.05$). The small circle above or below the boxplot whiskers represents an outlier, i.e., any point that is 1.5x greater or lesser than the interquartile range for that data group.

Table 5.2 Analysis of Variance table for the microbial abundance in the rhizosphere of intercropped red clover and oilseed radish plants

Symbiotic nitrogen-fixers			
Levels	**df**	**F-value**	**p-value**
Treatment	2	3.6585	0.043
Heterotrophs			
Treatment	2	2.6312	0.090
Phosphate-solubilizers			
Treatment	2	0.2508	0.781

The relative abundance of heterotrophs, symbiotic nitrogen-fixers, and phosphate-solubilizers in the rhizosphere of intercropped red clover and oilseed radish plants.

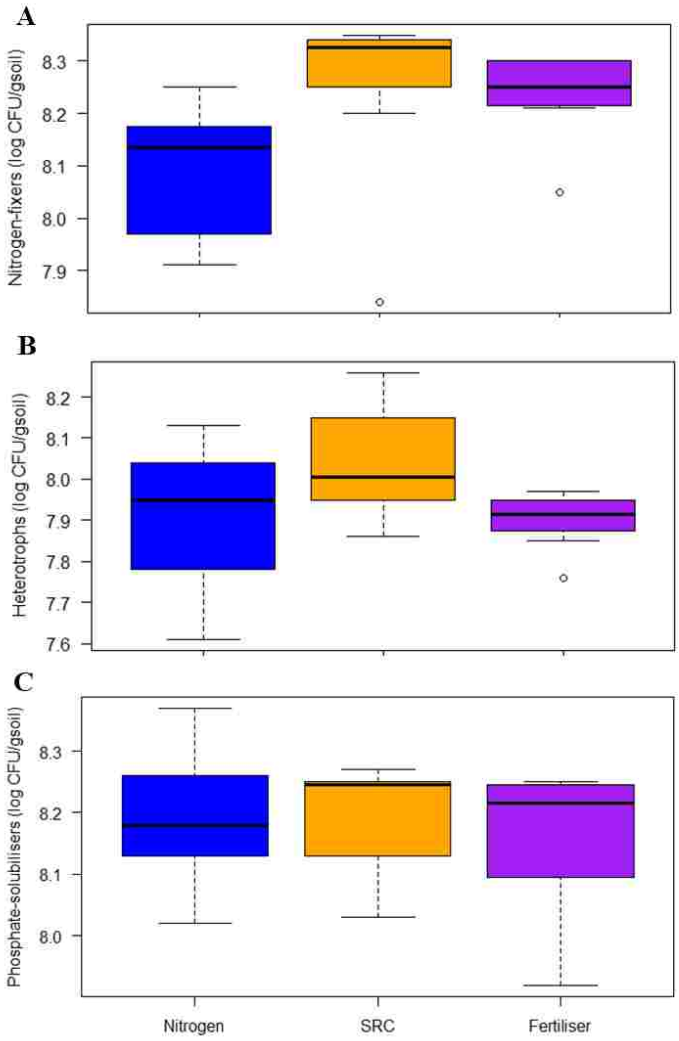

Figure 5.5 Microbial abundance in the rhizosphere of intercropped red clover and oilseed radish plants

Boxplots of the effect of soil amendments: Nitrogen as 2.5 mM ammonium nitrate, SRC in a 1:10 SRC:soil ratio, and fertilizer as 20-20-20 NPK commercial fertilizer, on the relative abundance (log CFU/g soil) of **A:** symbiotic nitrogen-fixing, **B:** heterotrophic, and **C:** phosphate-solubilizing bacteria (n = 4). The 1-way ANOVA indicated no significant differences between soil amendments. The small circle above or below the boxplot whiskers represents an outlier, i.e., any point that is 1.5x greater or lesser than the interquartile range for that data group.

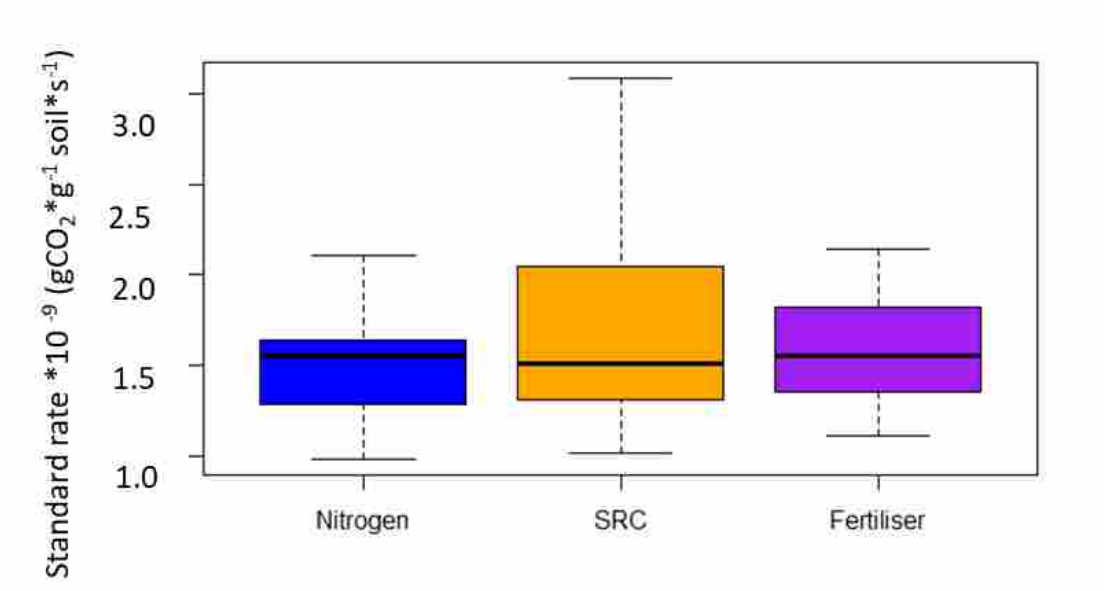

Figure 5.6 Microbial respiration in the rhizosphere of intercropped red clover and oilseed radish plants

Boxplots of the effect of soil amendments: Nitrogen as 2.5 mM ammonium nitrate, SRC in a 1:10 SRC:soil ratio, and fertilizer as 20-20-20 NPK commercial fertilizer, on the standard rate of microbial respiration (gCO_2*g^{-1} soil$*^{s-1}$) 56 days after planting (n = 4). The 1-way ANOVA indicated no significant differences. Indicated no significant differences between soil amendments.

5.2 Plant growth

Alfalfa and chicory plants grown under fertilization exhibited little to no signs of nutrient deficiency, such as chlorosis of the leaves and stunted growth. However, some ammonium nitrate- and SRC-grown plants were stunted, and their leaves were pale-green; these symptoms are often indicative of deficiencies in P and N, respectively (Figure 5.7A). Regardless of soil amendments, the growth of red clover plants appeared to be inhibited; their stunted growth may be attributed to the shading caused by their companion crop, the oilseed radish (Figure 5.7B). Also, red clover plants grown in ammonium nitrate-amended soil were characterized by leaves that were pale and with scorched margins, indicative of N and P deficiencies, respectively. As for red clover plants grown under fertilizer, they had pale-green leaves, but those plants grown in SRC-amended soils did not display any signs of nutrient deficiency. In fact, the leaves of SRC-grown red clover plants were much greener than those of plants grown in the other amendments. The aboveground biomass of the oilseed radish plants was so large, especially under NPK fertilization, that the red clover plants were obscured from view (Figure 5.7B). Regardless of soil amendment, oilseed radish plants displayed signs of N deficiency; however, the yellowing of the lower leaves was more pronounced in SRC-grown plants. Also, SRC-grown plants lost more of their leaves than plants grown in treatments lacking SRC (Figure 5.7B). The biomass of the cover crops used in this study is found in Appendix G.

Figure 5.7 Cover crops at harvest

These photographs illustrate the growth of intercropped plants. **A**: alfalfa and chicory and **B**: red clover and oilseed radish. "A" represents alfalfa, "C" chicory, "R" red clover, and "O" oilseed radish plants. The plants were grown in soils amended with 2.5 mM ammonium nitrate (referred to as Nitrogen), 1:10 SRC:soil ratio (referred to as SRC), or 20-20-20 NPK fertilizer (referred to as Fertiliser) for 56 days (n = 16). The circle below the letter is to help with identifying the cover crops.

5.3 The effects of soil amendments on the efficiency of the legume-*Rhizobium* symbiosis

At harvest, I observed that the nodules formed on SRC-grown alfalfa and red clover plants were pink compared to those on ammonium nitrate- and fertilizer-grown plants that were white. Additionally, SRC-grown alfalfa and red clover plants produced six- and eight-times more nodules than plants grown without SRC amendment, respectively. The means ± standard deviations of nodules borne by SRC-grown alfalfa and red clover plants were significantly higher with values of 19 ± 11 and 26 ± 13, respectively, than those of plants grown with ammonium nitrate (alfalfa: 1 ± 4 and red clover: 1 ± 2), and fertilizer (alfalfa: 4 ± 6 and red clover: 3 ± 0.4).

Plant return on nodule construction cost, specific nodulation, and specific nodule dry weight were used to assess the effects of soil amendments on the symbiotic efficiency between alfalfa plants and the colonizing *Rhizobium*. For the alfalfa plants, the Kruskal-Wallis test showed that soil amendments significantly affected the plant return on nodule construction cost (X^2 = 14.044, df = 2, p = 8.9E^{-04}), specific nodulation (X^2 = 21.815, df = 2, p = 1.8E^{-05}), and the specific nodule dry weight (X^2 = 20.597, df = 2, p =3.4E^{-05}). The plant return on nodule construction cost was significantly less for ammonium nitrate-grown alfalfa plants than for SRC-grown plants. The specific nodulation and specific nodule dry weight values of SRC-grown alfalfa plants were significantly higher than those of plants grown with the other two amendments (Table 5.3).

For the red clover plants, the Kruskal-Wallis test showed that there was no significant effect of soil amendment on the plant return on nodule construction cost (X^2 = 5.0844, df = 2, p = 0.08); however, soil amendments significantly affected the specific nodulation (X^2 = 32.703, df = 2, p = 7.9E^{-08}) and the specific nodule dry weight (X^2 = 32.705, df = 2, p =7.9E^{-08}) of red clover plants.

SRC amendment resulted in significantly higher values for specific nodulation and specific nodule dry weight than those of ammonium nitrate and fertilizer amendments (Table 5.3). Overall, the SRC amendment enhanced nodulation efficiency for both legumes; however, the effectiveness of SRC was better for the red clover than for the alfalfa plants.

Table 5.3 Nodulation parameters reflecting the legume-*Rhizobium* efficiency

	n = 16	Nitrogen	SRC	Fertilizer
Alfalfa	**Plant return on nodule construction cost**	0.01 ± 0.03^{a}	0.05 ± 0.05^{b}	0.06 ± 0.11^{a}
	Specific nodulation	30 ± 113^{a}	213 ± 167^{b}	34 ± 58^{a}
	Specific nodule dry weight	0.00 ± 0.01^{a}	0.05 ± 0.04^{b}	0.02 ± 0.05^{a}
Red clover	**Plant return on nodule construction cost**	0.12 ± 0.13^{a}	0.20 ± 0.20^{a}	0.14 ± 0.16^{a}
	Specific nodulation	24 ± 39^{a}	458 ± 209^{b}	56 ± 83^{a}
	Specific nodule dry weight	0.02 ± 0.04^{a}	0.32 ± 0.15^{b}	0.04 ± 0.06^{a}

Values are mean ± standard deviation, and different letters indicate statistical significance (Kruskal-Wallis and the Wilcoxon Rank sum post hoc test, $\alpha = 0.05$).
NB: Lower values for plant return on nodule construction cost (providing that plant growth is not reduced), and higher values for specific nodulation and specific nodule dry indicate an efficient symbiosis.

Chapter 6: Discussion

The general findings of this greenhouse study (Table 6.1) confirm the hypotheses that were tested (Chapter 3). Regardless of the cover crop combination grown, the SRC amendment significantly increased soil pH, and tended to increase the relative abundance of symbiotic nitrogen-fixing and heterotrophic bacteria; as well, it enhanced the efficiency of the legume-*Rhizobium* symbiosis. In contrast, the effect of the SRC amendment on the abundance of phosphate-solubilizing bacteria depended on the rhizosphere from which the microbes were cultured. The SRC amendment tended to increase their abundance in the rhizosphere of red clover and oilseed radish; however, in the rhizosphere of alfalfa and chicory plants, NPK fertilizer stimulated their abundance. Furthermore, NPK fertilizer stimulated microbial activity (measured as microbial biomass respiration) in the rhizosphere of both cover crop combinations and supported the growth of alfalfa, chicory, and oilseed radish plants. In contrast, the SRC amendment supported the growth of red clover plants.

There were four primary findings of this greenhouse study, and I will discuss them in the order found below:

- SRC amendment alkalinized soil pH
- Soil amendment is a stronger driver of heterotrophs, symbiotic nitrogen-fixers, and microbial respiration than cover crop combination
- The influence of soil amendment on phosphate-solubilizing bacteria depends on the cover crop combination
- Nodulation appeared to be promoted by SRC in comparison with the other two amendments

Table 6.1 Summary of findings

Parameters	AC	RO
pH	SRC increases pH	SRC increases pH
Heterotrophic bacteria	SRC supports greater abundance	SRC supports greater abundance
Symbiotic nitrogen-fixing bacteria	SRC supports greater abundance	SRC supports greater abundance
Phosphate-solubilizing bacteria	Fertilizer supports greater abundance	SRC supports greater abundance
Microbial respiration	SRC results in the lowest rate	SRC results in the highest rate
Plant return on nodule construction cost	**Alfalfa:** SRC enhances investment	**Red clover:** SRC enhances investment
Specific nodulation and specific nodule dry weight	**Alfalfa:** SRC enhances symbiotic gains	**Red clover:** SRC enhances symbiotic gains
Cover crop growth	Fertilizer supports growth	**Red clover:** SRC supports growth **Oilseed radish:** Fertilizer supports growth

NB: AC represents intercropped alfalfa and chicory, while RO represents intercropped red clover and oilseed radish.
Cover crop growth: sum of shoot and root dry weights (g) and all parameters were subjected to a 1-way ANOVA or the non-parametric Kruskal-Wallis test.

SRC had a liming effect on soil pH

The pH of the potting soil in which the intercropped cover crops were grown was slightly acidic before planting and treatment, and at harvest, all soils had a higher pH; thus the three soil amendments raised the soil pH (Appendix F). In the case of the alfalfa and chicory soil, the addition of ammonium nitrate, SRC, and fertilizer raised the pH by 0.56, 1.16, and 0.31 units, respectively. The pH of soil in which red clover and oilseed radish plants were grown was increased similarly by 0.91, 1.15, and 0.53 units, respectively. The alkalinizing effect of SRC on soil pH was expected because of the $CaCO_3$ content of the agromineral. SRC is 65% calcite which acts as a liming agent, and $CaCO_3$-bearing compounds are widely used to ameliorate acidic agricultural soils (van Straaten 2006; Goulding 2016). The effect of SRC on soil pH confirms my hypothesis as well as the findings of Jones (2016) who reported that even the additions of small amounts of SRC (1:20 SRC:soil ratio) resulted in significant increases in soil pH. NH_4^+-based fertilizers, such as the 2.5 mM ammonium nitrate and 20-20-20 NPK fertilizer used in this study, are known to have acidifying effects on soil (Bolan et al. 1991). However, the acidifying effect of NH_4^+-based fertilizers was not observed in this study since supplementing the soils with ammonium nitrate and NPK fertilizer raised the soil pH by 0.74 and 0.42, respectively. Soil acidification often results from the bacteria-mediated process of nitrification when NH_4^+-based fertilizers are oxidized to nitrate via nitrite releasing H^+ (Geisseler and Scow 2014). Since soil acidification was not observed at harvest, the slight increase in the pH of ammonium nitrate- and NPK fertilizer-treated soils may be attributed to the hardness of the water in the greenhouse, weathering of dolomite particles in the Sunshine® mix, and/or the absence of nitrifying bacteria in the potting soil.

SRC amendment stimulates the microbial abundance of heterotrophic and symbiotic nitrogen-fixing bacteria

Soil pH is a key environmental factor that influences soil microorganisms (Lauber et al. 2009; Rousk et al. 2010) via its influence on plant community composition, and nutrient solubility and availability (Fernández-Calviño and Bååth 2010). Higher bacterial and fungal diversities are often observed at near-neutral and acidic soil pHs, respectively (Lauber et al. 2009; Rousk et al. 2010; Geisseler and Scow 2014). In this study, although the direct effects of pH on microbial community composition were not evaluated, it was found that supplementing soils with SRC resulted in two things; firstly, it raised soil pH to near neutral levels and secondly, SRC-amended soil had higher relative abundances of heterotrophic and symbiotic N_2-fixing bacteria. This finding may suggest that the higher abundance of these two bacterial groups in SRC-treated soil is pH-related. This inference is plausible since bacterial species are known to have a narrow pH range for optimal growth, and any deviations outside this range can immediately result in reductions in bacterial growth (Fernández-Calviño and Bååth 2010; Rousk et al. 2010). Lauber et al. (2009) reported a proportional increase in the abundance of members of the *Alphaproteobacteria* class with increases in soil pH. The genus *Rhizobium* belongs to this bacterial class, and since rhizobia grow optimally in a pH range of 6.5 – 7.5 (Lei et al. 2011), it is possible that in my study the positive effects of SRC on soil pH favoured the growth of these neutrophilic bacteria. Kennedy et al. (2004) found that the addition of lime to the soil not only raises the pH but also increases microbial activity and biomass. Through raising the pH of the soil, lime may act as a selective agent on the soil bacterial community, leading to an increase in biomass and dominance of the neutrophilic bacterial species (Kennedy et al. 2004).

Furthermore, Fernández-Calviño and Bååth (2010) proposed that a change in soil pH (e.g., due to liming) outside the optimal range of bacterial growth can decrease the growth of the native bacterial community, making way for an increase in the growth of bacterial species that are better adapted to the new soil pH. This account may explain the positive effect of SRC on the relative abundance of the symbiotic nitrogen-fixing and heterotrophic bacteria. However, this explanation for pH-related microbial community shifts could not be confirmed in this study because of the methods used to culture the microbial groups. Several studies evaluating the influence on pH on microbial community composition (Lauber et al. 2009; Rousk et al. 2009; Rousk et al. 2010; Hartman and Richardson 2013) used methods that offer higher taxonomic resolutions, such as metagenomics, pyrosequencing, ribotyping, and phospholipid-derived fatty acid analyses, than the culture-based methods used in this greenhouse study.

Cover crops are drivers of the abundance of phosphate-solubilizing bacteria

Notwithstanding the role of soil pH in shaping microbial community, other factors such as C inputs (Grayston et al. 1998; Paterson et al. 2007; Hartman and Richardson 2013; Strecker et al. 2015) and soil nutrient status (Suzuki et al. 2009; Fierer et al. 2012; Ramirez et al. 2012; Li et al. 2014) have been shown to influence the composition of microbial communities. Plants are known to shape the soil microorganism community structure via their influence on soil chemistry and organic matter inputs (Berg and Smalla 2009; Bakker et al. 2014). However, there have been contrasting reports of the impact of plant community on shaping the soil microbial community. Some have reported that plant species effects are not significant drivers of soil microbial community structure (Kennedy et al. 2004; Ladygina and Hedlund 2010; Bakker et al. 2014), while others have reported strong plant effects on bacterial communities (Grayston et al. 1998; Zak et al.

2003; Steinauer et al. 2016). Paterson et al. (2007) argued that the addition of a root exudate solution to an organic soil increases the soil microbial biomass and supports the dominance of bacterial populations in the rhizosphere of ryegrass. This observed increase in soil microbial biomass corresponds with the greater utilization of rhizodeposition of the perennial ryegrass. These different plant species effects on microbial community composition and structure may indicate that plant species have a collective, non-specific influence on microbial communities in the soils tested (Paterson et al. 2007). These authors suggested that the contrasting findings can be explained by differences in the type of C released into the soil, i.e., labile sources of C common to plants, for example, organic acids and sugars, versus more recalcitrant organic matter (Paterson et al. 2007). Other authors proposed that edaphic factors such as soil organic matter, nitrogen, carbon, and potassium content can indirectly influence the structure of the microbial community, such as that of *Streptomyces* (Bakker et al. 2014).

Phosphate-solubilization is a key process of increasing the bioavailability of useable P (Richardson et al. 2009; Spohn 2016). This process occurs via acidification, complexation and chelation of bound inorganic P sources (Richardson et al. 2009a; Spohn 2016). In this study, fertilizer-treated soil in which alfalfa and chicory plants were grown had higher abundance of phosphate-solubilizing bacteria than SRC-treated soil. The elemental stoichiometry of soil microorganisms is constrained, and the microbial biomass maintains this homeostasis by modifying their microbial rates to acquire the limiting element (Cleveland and Liptzin 2007; Griffiths et al. 2012; Spohn 2016). It is possible that the phosphate-solubilizing bacteria in the rhizosphere of alfalfa and chicory plants were P-limited; as such, in response to a readily available P source, their activities were increased. It is also possible that these microorganisms shifted their

abundance and became acclimatized to high nutrient conditions under NPK fertilization (Kaminsky et al. 2018).

Conversely, SRC-treated red clover and oilseed radish soil had a higher abundance of phosphate-solubilizing bacteria. In some plants, phosphate deficiency is known to stimulate the production and release of phenolic compounds such as flavonoids and caffeic acid to solubilize inorganic sources of soil phosphate (Chishaki and Horiguchi 1997; Hartmann et al. 2009). For example, red clover plants in response to P-limitation are known to release strigolactones (Yoneyama et al. 2007), flavonoids, and carboxylates (Cesco et al. 2010) to aid in P acquisition. As such, the higher abundance of phosphate-solubilizing bacteria in the soils of SRC-grown red clover and oilseed radish plants could be explained by the following:

i. Since these plants did not receive any additional P than that in the potting soil, and because SRC contains a relatively insoluble P source. It is likely that the red clover and oilseed radish plants, as well as the microbes present, might have been P-limited. In response to this P limitation, the cover crops may have recruited microorganisms such as phosphate-solubilizing bacteria that can enhance the weathering of SRC via the release of exudates.

ii. Alternatively, the microbes themselves might have produced organic acids or phosphatases to acidify the soil or cleave P bound to organic matter to supplement their P nutrition.

Indeed, Yoneyama et al. (2007) reported that red clover plants release more orobanchol as a signal for arbuscular mycorrhizal fungi when grown under low P availability. It is possible that a similar strategy, such as the exudation of organic acids, phenolic compounds, or sugars, is utilized: red

clover exudates could act as signals for specific microorganisms involved in phosphate-solubilization.

Furthermore, the amount of CO_2 released by the microbes was the lowest in SRC-grown alfalfa and chicory and red clover and oilseed radish plants. Microbial respiration is one measure of the metabolic activity of the microbial community (Haney et al. 2008) and provides insight into the C and N mineralizing potentials of soil microorganisms (Borken et al. 2002). The C and N mineralizing activities of the soil microbial community allow the predictions of changes in the reserves of SOM (Moinet et al. 2016), thus having implications for terrestrial C cycling (Spohn 2015). Also, it has been shown that microbial respiration (Lanza et al. 2016; Moinet et al. 2016; Zwetsloot et al. 2018), as well as phosphate solubilization (Spohn 2016), are closely related to microbial access to C.

Plant productivity in agricultural systems is often limited by low availability of useable N and P, and nutrient additions via synthetic fertilizers usually result in increases in net primary plant productivity; this nutrient supplementation, however, may have negative consequences on the microbial community (Wardle 1992; Treseder 2008; Strecker et al. 2015). The positive effects of fertilization on plant growth influence the amount of C compounds released into the soil (Griffiths et al. 2012; Strecker et al. 2015) and thus microbial activity (Wardle 1992). Although soil microorganisms, especially bacteria, are often C- and N-limited, their activities are not always restricted by the same nutrients that those limiting plant growth (Cleveland and Liptzin 2007; Griffiths et al. 2012). Because soil microbes may be limited by C, N, and P, they may respond to nutrient inputs differently from plants. In this preliminary study, growth of alfalfa and chicory plants were higher under NPK fertilization than that of plants under ammonium nitrate- and SRC-amendments (Appendix G). Furthermore, NPK fertilizer stimulated the abundance of phosphate-

solubilizers as well as microbial respiration in the rhizosphere of alfalfa and chicory plants. Geisseler and Scow (2014) in a recent meta-analysis found that under long-term fertilization, the microbial biomass C not only increases, but results in shifts in the microbial community composition, and this is most likely related to changes in the C and N availability as a result of fertilization.

Ramirez et al. (2012) and Fierer et al. (2012) using pyrosequencing techniques reported that fertilization modified the microbial community composition by selecting for specific taxonomic groups. For example, supplementing soils with soluble nutrients resulted in a shift from microbes that often thrive in low-nutrient environments, such as the slow-growing oligotrophs, to those often found in high-nutrient conditions, such as the fast-growing copiotrophs. Is this shift from oligotrophs to copiotrophs present in my study, hence explaining the increase in the abundance of phosphate-solubilizers in the rhizosphere of alfalfa and chicory plants under NPK fertilization? Other studies have reported that fertilization results in shifts in the community composition (Kennedy et al. 2004; Nakhro and Dkhar 2010; Li et al. 2015b; Shen et al. 2016). For example, Paterson et al. (2007) and Li et al. (2015b) reported reductions in the abundance of gram-negative bacteria in soils under fertilization. Others reported that there are no significant effects of fertilization on microbial biomass and that the observed inhibitory effects were more pronounced in long-term studies (Treseder 2008).

Although culture-based techniques like those used in this study are fast and cost-effective, and provide useful information about microbial physiology, habitat, and potential functions (Hill et al. 2000; He et al. 2008), they are limited in their ability to describe a specific microbial ecosystem (Hill et al. 2000). Culture-based techniques tend to be limited in unravelling functional roles of microbial groups because less than 0.1% of soil microorganisms are estimated to be

cultured with these methods (Hill et al. 2000). Nevertheless, they are ideal for preliminary studies, and a combination of culture-based and molecular techniques should be used in follow-up studies to provide information about microbial community shifts in response to management practices. Furthermore, microorganisms do not exist in isolation, as such their roles in N mineralization and P solubilization, are often carried out by several microbial groups rather than being confined to a single phylum (Hill et al. 2000; Bakker et al. 2014).

SRC enhanced legume-*Rhizobium* efficiency

The use of fast-release fertilizers is a key management strategy in many farming systems to maintain crop productivity (He et al. 2008; Hartman and Richardson 2013). The growth of alfalfa, chicory, and oilseed radish was supported by NPK fertilizer, while the growth of red clover plants was enhanced by SRC amendment (Appendix G). The positive effects of SRC amendment on red clover growth may be attributed to the near-neutral pH of these soils and the absence of external N sources.

Low soil pH has been shown to reduce the survival of rhizobial strains and the efficiency of the resulting symbiosis (Morón et al. 2005); the severity of the inhibition depends on the legume species, cultivar, as well as the rhizobial strain (Tang and Thomson 1996; Ferguson et al. 2013). Furthermore, several studies have demonstrated the inhibitory effects of low pH on the growth and persistence of several rhizobial species, such as the microsymbionts of alfalfa (Rice et al. 1977; Draghi et al. 2016) and clover (Rice et al. 1977; Zahran 1999). In my study, soil amendment had a similar effect on pH, specific nodulation, and specific nodule dry weight, i.e., SRC amendment raised soil pH, specific nodulation, and specific nodule dry weight, while ammonium nitrate and NPK fertilizer lowered these three parameters. The direct effects of low pH on rhizobia were not

assessed in this study. However, the legume-*Rhizobium* symbiosis was enhanced in soils amended with SRC, the same amendment that raised soil pH to near-neutral levels. At harvest, the number of nodules formed on alfalfa and red clover roots grown in SRC-treated soils was significantly higher than that of nodules formed on plants grown in soils lacking SRC, confirming the preliminary findings of Jones (2016).

These results may suggest a pH-related effect on rhizobia, as stated by Draghi et al. (2016) who observed a reduction in the growth of *Sinorhizobium meliloti* at pHs between 6.0 and 6.1 and enhanced growth at pH 7.0. Soil pH of 6.0 and 6.1 is close to the threshold pH at which *S. meliloti* and N_2-fixation are impaired in alfalfa (Rice et al. 1977; Kaminsky et al. 2018). However, the positive effect of SRC amendment on specific nodulation and specific nodule dry weight could be a result of the presence of high Ca levels and the absence of nitrogenous compounds. The experimental design used in this study did not control for pH- or N- or their combined effects on nodulation and nitrogen fixation. Many reports have indicated that the addition of lime or CaCO3 pellets not only raise soil pH but also enhances nodulation and N_2-fixation (Buerkert et al. 1990; Brauer et al. 2002; Grewal Singh and Williams 2003; Ferguson et al. 2013). Therefore, further studies comparing the effects of agricultural lime and SRC on nodulation should be done to distinguish the effects of pH to that of the SRC amendment. Experiments should also be performed to evaluate the combined effect of lime, SRC, and external nitrogen inputs on nodulation.

The presence of mineral N, e.g., NH_4^+, NO_3^-, and urea, have been shown to have complex, and diverse, often adverse, effects on nodule formation, growth, and function (Nelson and Edie 1991; Gan et al. 2004; Mortimer et al. 2012; Xia et al. 2017). However, variations exist in the sensitivity of the legume-*Rhizobium* symbiosis to N additions (Nelson and Edie 1991) and several factors are known to impact nodulation. Such factors include legume genotype (Gan et al. 2004),

rhizobial strain (Nelson 1987; Mortimer et al. 2012), pH and temperature in the rhizosphere (Ferguson et al. 2013), and the concentration and form of N added (Nelson 1987; Gan et al. 2004; Bollman and Vessey 2006). The findings of this study may indicate that the SRC amendment promoted nodulation because of 1) the high Ca levels, 2) the absence of an external N. Furthermore, P is required for nodulation (Kaminsky et al. 2018); therefore, SRC represented a source of P that was not inhibitory to the nodulation process.

Since legumes are agriculturally-important crop plants that rely on N from symbiotic N_2-fixation for growth and development (Mortimer et al. 2012; Ferguson et al. 2013), the legume-*Rhizobium* symbiosis represents a sustainable approach to supply useable plant N in agricultural systems. The interaction is usually beneficial to both partners, but the legume places restrictions on the partnership (Oldroyd et al. 2011) for several reasons. One such reason may be related to the C cost of the N_2-fixation process, i.e., up to 25% of the legume's total C fixed may be required by the rhizobial strain for N_2-fixation (Oono and Denison 2010). The legume host may also be C-limited leading to the diversion of photosynthetically-derived C from the nodules (Nelson and Edie 1991; Oono and Denison 2010). As such, it is better to measure both the gains and the cost of the association to get an accurate understanding of the symbiotic efficiency (Oono and Denison 2010) and inform our use of this approach in the field.

The findings of this study indicate that in general SRC amendment enhanced the gains garnered from the symbiosis by alfalfa and red clover plants. In addition to promoting whole plant nodulation, SRC amendment promoted specific nodulation and specific nodule dry weight, indicating an efficient symbiosis. Specific nodulation provides a better understanding of factors regulating the nodulation process, especially in its early stages, for instance, nodule initiation and development (Gulden and Vessey 1998). The same authors used specific nodule dry weight as an

indicator of symbiotic efficiency, they hypothesized that since legumes rely on the legume-*Rhizobium* symbiosis as their sole N source, the plant will modify the extent of root growth and nodule formation to achieve a balanced ratio between the mass of the root system and the mass of the nodules. As such, a reduction in specific nodulation and specific nodule dry weight in the presence of external mineral N is indicative of a suppressive effect of mineral N on nodule initiation (Gulden and Vessey 1998; Gan et al. 2004).

Although SRC amendment enhanced the respective legume-*Rhizobium* efficiency in terms of gains, the plant return on nodule construction cost varied between alfalfa and *S. meliloti* and red clover and *R. trifolii*. The plant return on nodule construction assumes that an increased investment into the symbiosis (measured by nodule dry weight) should correspond to a proportional increase in nitrogen fixation (reflected in the total host dry weight) (Oono and Denison 2010). In this study, the plant return on nodule construction cost was lower in SRC-grown alfalfa than in fertilizer-grown plants, while the cost was higher for SRC-grown red clover plants than for fertilizer-grown plants. Although fertilizer-grown alfalfa plants had a higher plant return on nodule construction value than SRC-grown plants, this investment of fixed C in nodule construction was not at the expense of plant growth because these plants had significantly higher growth than SRC-grown alfalfa plants (Appendix G). The suppressive effect of mineral N on nodulation would make an investment in the symbiosis redundant for ammonium nitrate- and fertilizer-grown plants. The higher cost to produce nodules in SRC-grown plants did not significantly reduce plant growth compared to ammonium nitrate-grown plants. However, red clover plants grown in soils lacking the SRC amendment had lower plant return on nodule construction cost than plants grown in SRC-amended soils. A higher plant return on nodule construction cost is indicative of a less efficient symbiosis if the growth of the legume is reduced (Oono and Denison 2010). However, this was

not the case for SRC-grown red clover plants compared to ammonium nitrate- and fertilizer- grown plants. The overall growth of these plants was significantly higher than those of plants grown in soil lacking SRC (Appendix G).

Additionally, more nodules may not translate into greater efficiency since a legume colonized by less-effective rhizobia strains will produce more nodules than with a more effective strain, and thus have a higher plant return on nodulation cost (Oono and Denison 2010). Moreover, I did not inoculate the legumes, and there was no information about the expiration date or the effectiveness and quality of the rhizobial strains on the seeds. This information is vital because non-viable rhizobia and less effective strains will impact the nodulation process. The cost to produce nodules is often increased when a legume interacts with ineffective rhizobial strains (Oono and Denison 2010). Non-nitrogen-fixing strains of bacteria may also be present in nodules (Martínez-Hidalgo and Hirsch 2017); the different bacteria residing in the nodule microbiome could compete for fixed C or synergistically enhance nodulation and plant growth (Martínez-Hidalgo and Hirsch 2017). Therefore, if ineffective strains are colonizing the nodule and there is competition for the limited C from the plant, a higher plant return on nodule construction is likely. Since external N sources are known to suppress nodulation and nitrogen-fixation (Gan et al. 2004; van Noorden et al. 2016), and the parameters that provide insights into these processes were reduced under ammonium nitrate and NPK fertilizer, it is expected that plants grown under these conditions would have lower plant return on nodule construction costs.

Even though the cost to produce nodules in SRC-grown plants was higher than plants grown in soil lacking SRC, it appeared that the N_2-fixed by SRC-grown red clover and *R. trifolii* in combination with the nutrients obtained from SRC supplementation were adequate to support red clover growth. SRC-grown red clover plants had significantly higher above- and below-ground

biomass than ammonium nitrate- and NPK fertilizer-grown plants. The enhanced growth of red clover plants with little to no signs of nutrient deficiency under SRC amendment suggest that in addition to effectively fixing N_2 these plants were able to acquire essential nutrients such as P and K from SRC. These findings although unsupported by shoot and root tissue nutrient content and root exudate evaluation, or knowledge about the viability and effectiveness of the rhizobial strains used, could suggest that red clover plants utilized mechanisms to increase nutrient acquisition. They may do so by exuding H^+ and organic acids to acidify the rhizosphere and/or to recruit nutrient-acquiring microbes (Richardson and Simpson 2011; Álvarez-López et al. 2016; Alori et al. 2017), thus increasing the weathering of the SRC releasing P and K among several micronutrients. In fact, red clover plants are known to release polyphenolic compounds, flavonoids, orobanchol, and carboxylates under P-limiting conditions (Yoneyama et al. 2007; Cesco et al. 2010).

Research limitations and Future directions

This study was rather complex, and in retrospect the experimental designed should have been less ambitious. For example, instead of having eight pots for each of the three soil amendments and this for each of the cover crop combinations, I could have reduced the number of pots per amendment so that the two plant combinations could have been cultivated at the same time. Growing the two cover crop combinations at the same time would provide more breadth to the study and a better strength in the statistical analysis as the combinations would have been subjected to the same external factors. This may have allowed me to get insight into which of the two cover crop combinations used as part of the integrated strategy provided the most benefits in stimulating microbial activity.

Furthermore, nutrient analyses of the shoot and root tissues should be conducted to see if those plants grown in soil amended with SRC were able to acquire nutrients for growth. Performing studies similar to mine comparing the effects of SRC to those of agricultural lime on the indicators of soil health would also be useful to determine if the microbial response I saw was caused by the liming property of the SRC or by something else. Further work should not only look at "what are the factors shaping the microbial community composition?" but should also ask "how do these factors influence the functions/services these microbial groups provide?" These questions can be answered by using broad scale and more targeted methods to evaluate specific microbial taxa and their related functions. The use of culture-dependent methods, similar to those used in this study and enzyme activity study, and culture-independent methods, e.g., molecular-based methods, would be required to have a more complete picture of the microbial taxa that are

responding to the changes in soil amendments. Collaboration with farmers, microbial ecologists, soil scientists, and plant physiologists should continue to ensure that the current stresses on the soil ecosystem is alleviated, thus enabling a bottom-up approach to supply nutrients required by plants.

Concluding remarks

Meeting the food demands of the growing population requires scientific innovations, and since reports have indicated that agricultural intensification, particularly the use of synthetic fertilizers, results in ecosystem degradation and biodiversity loss overtime (Strecker et al. 2015), strategies capable of supplementing soil nutrients without adverse effects are required. Enhancing the productivity of soil by increasing nutrient availability and nutrient use efficiency in managed ecosystems could offset the adverse effects of agricultural intensification (Lehman et al. 2015a; Lehman et al. 2015b). An understanding of the microbial community contributions to plant nutrient acquisition is key to developing sustainable farming practices. An integrated strategy involving the use of cover crops with different plant traits and an agromineral was used here to assess the response of microbial groups to the agromineral. The findings of this preliminary study indicate that SRC amendment could be incorporated by farmers into best management practices that aim at enhancing plant growth by stimulating the ecosystem services provided by the soil. Also, when particular species of leguminous cover crops are grown in the presence of the SRC amendment, nodulation efficiency is enhanced and hence it is expected that nitrogen fixation would increase.

The strategy used in this study drew on a multidisciplinary team because it relied on the partnership and expertise of industry personnel, the vigneron, and a collaboration between members of the Biology Departments of Brock University and Wilfrid Laurier University. The study was made possible through the contributions of the Ontario-China Innovative Research Fund and operators of the Spanish River Carbonatite quarry, Boreal Agrominerals Ltd. As for the study site, Hughes vineyard, I visited it to gain a better understanding of the holistic management practices the vigneron has implemented to improve its health. The vineyard represents an ideal ecosystem, in contrast to the greenhouse, since it contains a diverse plant community (different

vine stalks and cover crops), many invertebrates (insects and nematodes), and plant growth-promoting and pathogenic microbial communities that are interacting both above- and below-ground through nutrient cycling, plant growth, and energy transfers. Therefore, knowledge of ecological principles, plant biology, microbiology, soil science and chemistry and to a lesser extent an understanding of anthropology was required to complete this study successfully. Partnering with collaborators on such an integrated project although rewarding was not without its challenges; for example, there were different schedules and differing ideas that required flexibility from the individual parties to achieve the goal set. Integration of ideas fosters a learning environment whereby each person, an expert in his/her field, learns from the other. As for me, I entered the Master's degree programme with a background in biotechnology and zoology; as I have approached the end of this thesis, I have learnt a great deal which will make me into a better scientist. It is this shared knowledge and understanding that are required to restore soil health successfully.

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Appendix A

Supplementary information about the four cover crops used in this greenhouse study

The proposed integrated approach involves the use of commonly grown leguminous, namely *Medicago sativa* L. (alfalfa), *Trifolium pratense* L. (red clover), and non-leguminous, i.e., *Cichorium intybus* L. (chicory) and *Raphanus sativus* L. (oilseed radish) cover crops (Table A, Figure A). Alfalfa and red clover are important forage crops in the family Fabaceae that form N_2-fixing nodules in associations with *Sinorhizobium meliloti* (Maxwell et al. 1989; Catford et al. 2003; Nxumalo et al. 2010) and *Rhizobium leguminosarum* bv. *trifolii* (Russell and Jones 1975; Mårtensson 1990; Jin et al. 2006), respectively. Alfalfa and red clover can fix up to 500 kg N per ha per year (Watson et al. 2002) and between 389-460 kg N per ha per year (Watson et al. 2002; Lewis 2015), respectively. However, the amount of N_2 fixed depends on the cultivars, farming practices, and soil properties, e.g., pH and nutrient availability (Nxumalo et al. 2010; Wyngaarden 2015). These perennial cover crops proliferate in favourable conditions with adequate nutrients, such as N, P, K, Mg and Ca (Undersander et al. 2011), and near-neutral pHs (Lacefield et al. 1997; Lacefield et al. 2006). Under favourable conditions, these legumes (Figure A and B) produce high biomass, and their root systems (Figure E and F) increase the water infiltration rates in compacted, no-till soils (Williams and Weil 2004). In particular, the fibrous root system of red clover plants (Figure F) is ideal for improving soil structure and stability (Wyngaarden et al. 2015) as it enmeshes the soil particles, which reduces soil erosion (Watson et al. 2002). This root phenotype also renders red clover more efficient at absorbing soil or applied nutrients, especially P and K (Undersander et al. 1990), than other cover crops such as alfalfa that have fewer lateral roots (Figure E).

Chicory is an erect biennial forage herb (Figure C) in the family Asteraceae (Li 1997; Kunelius and McRae 1999; Bais and Ravishankar 2001). These plants are hardy, tolerating extreme temperatures during their vegetative and reproductive growth stages (OMAFRA 2003). They grow best on well-drained medium to fertile soils with pH ranging from 4.5 to 8.3 (Li 1997; Hall and Jung 2008) and have a high demand for K and P (Hall and Jung 2008). Also, chicory plants are very responsive to N additions; there is a proportional relationship between N fertilization and stem growth (Hall and Jung 2008). The root system of chicory plants (Figure G) makes them effective scavengers of N from the subsoil levels (Fageria et al. 2005). Because of their deep taproot system, chicory plants can be implemented as part of a management system to reduce nitrate leaching (Mårtensson et al. 1998; Druart et al. 2000) and increase soil drainage and aeration (Li and Kemp 2005). The taproot contains a large percentage of the plant's phytochemical compounds including many glucosides, acids, and phenolic compounds (Bais and Ravishankar 2001). Chicory stores up to 80% of its root biomass as these phytochemicals; therefore, the taproot represents an abundant source of carbohydrates supporting shoot emergence and plant development (Li and Kemp 2005; Street et al. 2013).

Oilseed radish is a fall or winter annual cover crop in the mustard family, the Brassicaceae (Mutch et al. 2004). Forage radish is often used as a cover crop to maintain soil fertility and crop productivity (Magdoff and van Es 2009; Jacobs 2012), improve soil aggregate stability (Dapaah and Vyn 1998), and reduce soil erosion (Williams and Weil 2004; De Baets et al. 2011; Jacobs 2012). The plant is intolerant to shade (Jacobs 2012; Verhallen et al. 2012), standing water, and severely N-deficient soil (Jacobs 2012). However, limitation by N levels depends on soil texture and history of nutrient addition (Jacobs 2012). Like chicory, oilseed radish is highly responsive to N fertilization (Jacobs 2012). Also, it grows best in cool, moist conditions and is adapted to soils

with pHs between 6.0 to 7.5 (Jacobs 2012). This cover crop establishes quickly, producing large aboveground biomass (Figure D) in a short period (Mutch et al. 2004; Florentín et al. 2011; Jabnoun-Khiareddine et al. 2016) (Figure 3.2 D). Because of this trait, oilseed radish plants provide good soil coverage during periods when the soil is in fallow or left bare (Mutch et al. 2004; Mennan et al. 2008; Jacobs 2012). This cover crop is often used as a biological solution to suppressing weeds (Charles et al. 2006; Wang et al. 2008b) because it establishes quickly (Charles et al. 2006), thus outcompeting the weed seedlings for resources (Snapp et al. 2003; Charles et al. 2006). Furthermore, these plants secrete glucosinolate compounds, that break down to produce volatile molecules like the active chemical metham sodium in the commercial fumigant Vampam® (Mutch et al. 2004). These compounds discourage the infestation of soil-borne diseases by fungi as well as suppress the growth of nematodes (Mennan et al. 2008; Jacobs 2012). Oilseed radish plants are characterized by their large taproots (Figure H) that can penetrate compacted soils, and upon their decomposition they leave behind large holes reducing the need for tillage (Jacobs 2012), improving aeration, water infiltration (Jacobs 2012), and potentially stimulating microbial activity (Mutch et al. 2004). Like chicory, oilseed radish is an effective scavenger of residual N, assimilating 100 to 150 lb/acre of N (Jacobs 2012). Oilseed radish plants can, therefore, be planted to reduce NO_3^- leaching, and as a source of soil N upon their decomposition in the spring when used in crop rotation (Isse et al. 1999; Wang et al. 2008b; Weil and Lawley 2009). Although there are many benefits to using oilseed radish as a cover crop, it can become an invasive or weedy species in some regions, because its seeds often remain viable in the soil for many seasons (Jacobs 2012).

Table A Characteristics and growth conditions of the cover crops

Characteristics	Alfalfa	Chicory	Red clover	Oilseed radish
Family	Fabaceae	Asteraceae	Fabaceae	Brassicaceae
Binomial	*Medicago sativa*	*Cichorium intybus*	*Trifolium pratense*	*Raphanus sativus var. oleiformis*
Growth cycle	Perennial	Biennial	Perennial	Annual
Rhizobia	*Sinorhizobium meliloti*	No	*Rhizobium leguminosarum bv. trifolii*	No
C:N ratio	13:1 to 25:1*	48	13.6:16.7	19-20
Exudates	Caffeic acid, medicarpin	Succinic acid, glucosides	Orobanchol, genistein	Glucosinolates
Optimal pH	6.5 - 7.0	4.5-8.0	6.0 - 7.6°	6.0-7.5

* C:N (Carbon-to-nitrogen ratio) of alfalfa varies from young hay (13:1) to mature hay (25:1).

°Optimal pH: Although the optimal pH for red clover growth is between 6.0 and 7.6, red clover plants can survive in soils with pHs between 5.0-8.5.

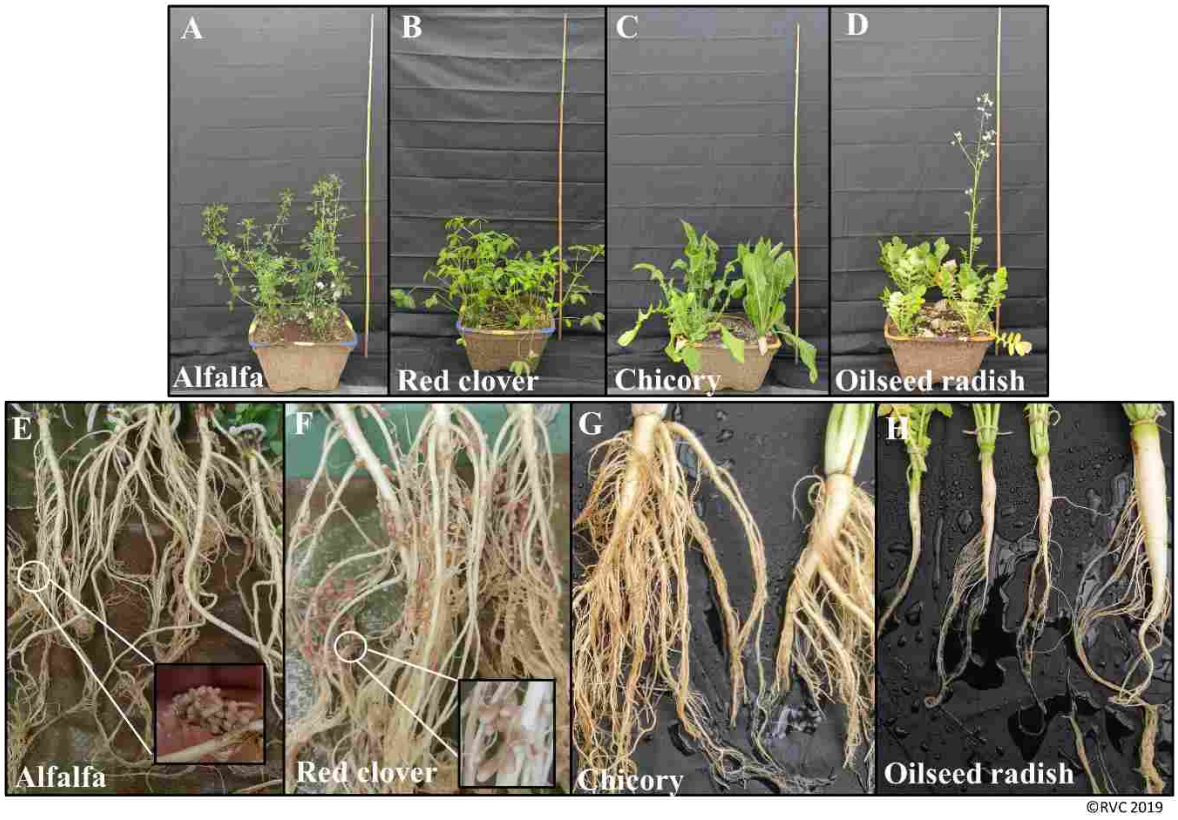


Figure Above-and below-ground cover crop characteristics

A-D depict the aboveground phenotype, while **E-H** phenotype depict the belowground phenotype of the alfalfa, red clover, chicory, and oilseed radish cover crops, respectively.
Alfalfa plants are characterized by small trifoliate leaves that alternate on the stem (**A**), and the root system (**E**) is characterized by a primary root with several lateral roots, which bear multi-lobed nodules (magnified) in the upper 5-10 cm the roots. Red clover plants are characterized by large trifoliate compound leaves that alternate on the stem (**B**). The taproot system of red clover plants is characterized by a primary root with several lateral roots, bearing single-and bi-lobed nodules (magnified) along the length of the roots (**F**). Chicory plants are characterized by a rosette of many broad, oblong, or oblanceolate leaves (**C**). The taproot system of chicory is characterized by a primary root that bears several lateral roots (**G**). Oilseed radish plants are characterized by a rosette of large deeply dissected leaves and a flowering stalk that emerges from the centre of the rosette (**D**). These plants have a large, thick taproot system with few lateral roots (**H**).

Appendix B

Guaranteed minimum analysis of 20-20-20 Farm Prod® commercial fertilizer

Mineral composition	% minimum analysis
Total nitrogen (N)	**20**
Available phosphoric acid (P_2O_5)	**20**
Soluble potash (K_2O)	**20**
Actual boron (B)	**0.02**
Actual copper (Cu)*	**0.05**
Actual iron (Fe)*	**0.1**
Actual manganese (Mn)*	**0.05**
Actual molybdenum (Mo)	**0.0005**
Actual Zinc (Zn)*	**0.05**
Ethylene diamine tetra-acetate (EDTA)°	**1**

**NB: ***Chelated with EDTA
°Chelating agent

Appendix C

Typical characteristics of SunGro Sunshine® Mix 1

Properties	Range
pH	5.0 - 5.8
EC	0.75 - 1.75
***Extractable nutrient (ppm)**	
Nitrate-N	4 - 67
Ammonium-N	1.- 31
P	9 - 42
K	36 - 129
Ca	37 - 158
Mg	17 - 77
S	70 - 225
Mn	0 - 1.5
Fe	0 - 0.5
Cu	0 - 0.05
B	0 - 0.36
Zn	0 - 0.16
Mo	0 - 0.07
Ingredients	
Coarse perlite Dolomite Gypsum Coarse Canadian sphagnum peat moss	

NB: The potting soil contains proprietary starter nutrient with major and minor nutrients as well as proprietary wetting agent. *Saturated extract procedure two weeks after production (https://www.tlhort.com/p-21606-sunshine-mix-1-lc-mix.aspx)

Appendix D

Modified Yeast Mannitol Agar medium (nitrogen-free) for *Rhizobium*

Reagents	Quantity (g/L)
D-mannitol	10
K_2HPO_4	0.5
$MgSO_4*7H_2O$	0.2
NaCl	0.1
Yeast extract	0.4
Agar	15

NB: Reagents were dissolved in deionized water, pH to 6.8 before adding agar then autoclaved at 121 °C for 15 minutes.
Ref: Vincent (1970)

Appendix E

National Botanical Research Institute Phosphate Medium for phosphate-solubilizing bacteria)

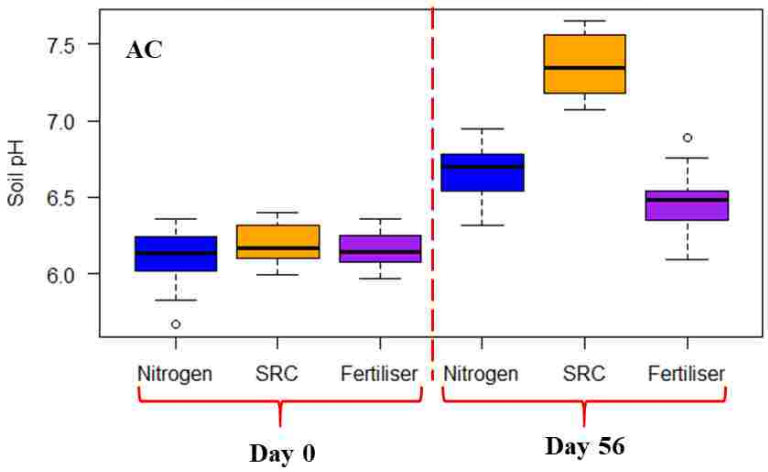
Reagents	Quantity (g/L)
Glucose	10
$MgCl_2.6H_2O$	5
$MgSO_4.7H_2O$	0.25
KCl	0.2
$(NH_4)_2SO_4$	0.1
$Ca_3(PO_4)_2$	5
Agar	15

NB: Reagents were dissolved in Milli-Q water, pH to 7.0 before adding agar then autoclaved at 121 °C for 15 minutes.
Ref: Nautiyal 1999

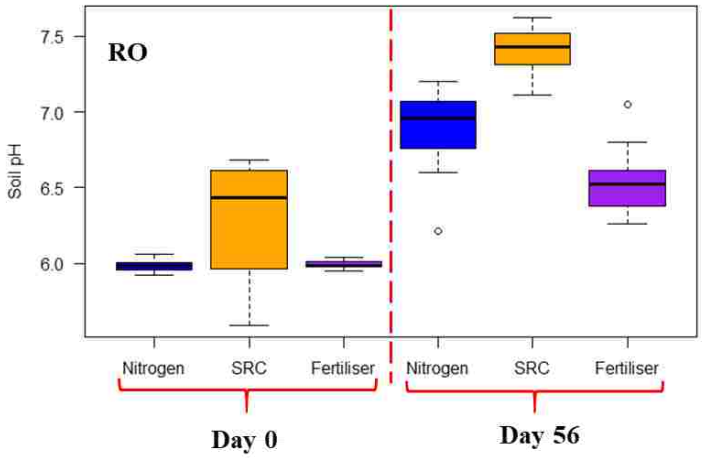
Appendix F

Soil pH before planting cover crops and applying soil amendments

AC pH	DF	F-value	p-value
Treatment	2	86.27	<0.0001
Trial	1	235.47	<0.0001
Treatment x trial	2	68.47	<0.0001



RO pH	DF	F-value	p-value
Treatment	2	68.9	<0.0001
Trial	1	293.72	<0.0001
Treatment x trial	2	18.2	<0.0001



NB: Results are of a repeated measures ANOVA taken at day 0 (before sowing and treatment i.e., each of the assigned pots were sampled) and day 56 (harvest i.e., each of the assigned pots were sampled). Soil pH at day 56 was consistently higher than that of day 0. AC: represents alfalfa and chicory plants, while RO represents red clover and oilseed radish plants.

Appendix G

Cover crop biomass

Kruskal-Wallis	X^2-value	DF	p-value
Alfalfa	6.111	2	0.047
Chicory	10.933	2	0.004
Alfalfa + chicory	8.985	2	0.01
One-way ANOVA	**DF**	**F-value**	**p-value**
Red clover	2	8.091	0.001
Oilseed radish	2	100.828	$<2E^{-16}$
Red clover and oilseed radish	2	92.68	3.90E-16

n = 16	Nitrogen	SRC	Fertiliser
Chicory	2.2 ± 1.4^{a}	2.1 ± 1.8^{a}	4.4 ± 1.8^{b}
Alfalfa + Chicory	2.7 ± 1.6^{a}	2.8 ± 2.1^{a}	5.3 ± 2.1^{b}
Red clover	0.2 ± 0.1^{a}	0.4 ± 1.6^{b}	0.2 ± 0.1^{a}
Oilseed radish	12.4 ± 4.7^{a}	9.1 ± 3.9^{b}	16.8 ± 3.5^{a}
Red clover + Oilseed radish	12.6 ± 4.7^{a}	9.5 ± 4.0^{b}	17.0 ± 3.6^{a}

NB: The total plant biomass (g ± SD) was subjected to a 1-way ANOVA or the non-parametric Kruskal-Wallis test.