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Spanish River Carbonatite: Its benefits and potential use as a soil supplement in agriculture

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Its benefits and potential use as a soil supplement in agriculture

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A

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Abstract:

A critical problem facing agriculture today is being able to consistently and sustainably provide plants with adequate nutrients for growth. With this problem being exacerbated by the ever-increasing human population, new perspectives and techniques are required to ensure global food security. In order to fully realize potential solutions, however, plant growth and nutrition cannot be exclusively focused upon. The soil-microorganism-plant system is comprised of many interconnected and interdependent processes that together support plant growth: it is upon these processes that the focus must be placed. In this work, the agromineral Spanish River Carbonatite (SRC) is characterized using the framework of the soil-microorganism-plant system with the pea *Pisum sativum* L. as a model crop plant. The overall objective of confirming the potential usefulness of SRC as an agricultural amendment was divided into three sub-objectives. First, the optimal concentration of SRC that most benefits the soil, microorganisms, and plants, was determined. Second, the impact of SRC on the agriculturally-important symbiosis between pea and rhizobia was assessed. Third, the effect of storage conditions on SRC's usefulness as an agricultural supplement was preliminarily examined. Overall, it was hypothesized that the addition of SRC at an optimal concentration would result in increased plant growth, because SRC is a source of a wide variety of nutrients. Importantly, it was verified that SRC is capable of acting as such a nutrient source for plants, and that its addition into the soil enhanced the efficiency of the rhizobia-pea symbiosis resulting in benefits to plant growth. Preliminary results also indicate that beneficial microorganisms are present within SRC, but are negatively influenced by current storage methods. By using a perspective that took into account soil properties and microorganisms in addition to plant responses, it was possible to examine the effect of SRC on the underlying soil-microorganism-plant processes. The findings presented here provide evidence that agrominerals such as SRC are potentially powerful tools for agriculture and that in studying the complexities of plant nutrition the whole soil-microbe-plant system must be taken into consideration.

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Chapter 1: General introduction and literature review

By the year 2050, the total global population is projected to reach 9.1 billion persons and developing countries are expected to experience the most growth (Roy *et al.* 2006). Because of the population increase, there is a need for improvements in the efficiency of agricultural practices to meet the needs of the population without encroaching on land required for other uses such as environmental conservation (Sayer *et al.* 2013). The use of both chemical fertilizers and organic agricultural practices has allowed great advances in crop production. Despite these advances, however, a persistent problem is to provide plants with sufficient nutrients for their optimal growth and yield. This problem is exacerbated by the need to make agriculture less damaging to the environment. New approaches and tools are thus needed to ensure both agricultural sustainability and food security for future generations.

Section 1: The Challenge of Plant Nutrition

Two main strategies have been used to address plant nutrition in agricultural systems: the use of organic nutrient sources (e.g., compost) and that of synthetic nutrient sources (e.g., chemical fertilizers). The benefits and drawbacks of each of these approaches are covered below.

1.1: Organic nutrient sources

Commonly, animal wastes/manure (Brandt and Mølgaard 2001; Tilman *et al.* 2002; Ge *et al.* 2011), plant residues (Stanhill 1990), and crop rotations with nitrogen-fixing symbiotic legumes (Brandt and Mølgaard 2001; MacWilliam *et al.* 2014) are used singly or in combination as organic nutrient sources for plants. Several positive outcomes have resulted from the use of

these organic nutrient sources when compared to the use of synthetic nutrient sources. First, an increase in the diversity of predatory ground (Family Carabidae) and rove beetles (Family Staphylinidae) was seen when comparing organic and synthetic fertilization methods in a study by Pfiffner and Niggli (1996); these organisms feed on pest organisms and can act as biological pest control agents (Zehdner *et al.* 2007). Second, the use of manure as a nutrient source can mediate soil organic matter deficiency and enhance the diversity of the soil microbial community (Zhang *et al.* 2012). The more types of microorganisms that perform a given soil process, such as nitrogen fixation, the more the soil as a whole can maintain those processes in the event of disturbances (Allison and Martiny 2008). Finally, organic fertilization has also been linked with higher soil-aggregate stability (Mäder *et al.* 2002) and higher soil organic carbon levels (Fließbach and Mäder 2000; Marschner *et al.* 2003). More stable soil aggregates and increased soil organic matter can lead to an improved ability of the soil-plant system to utilize available nutrients (Mäder *et al*. 2002).

Although the use of organic fertilizers has been proposed as being vital for reducing the environmental impact of agriculture, it comes with drawbacks. First, the usefulness of organic nutrient sources depends on the specific environments and methods utilized (Flohre *et al.* 2011; Toumisto *et al.* 2012), and the yields obtained from organically-fertilized systems are typically at least 20% lower than those of chemically-fertilized systems (Trewavas 2001; Mäder et al. 2002). Furthermore, biodiversity may not actually be improved by organic nutrient sources systems over synthetically-fertilized systems. A critical meta-analysis of the impact of organic versus conventional fertilization on overall biodiversity indicated that the biodiversity gains depend largely on the type of organisms (e.g., predatory or non-predatory insects) in question and the landscape surrounding the study area (Bengtsson *et al.* 2005). Importantly, it was found that previous studies did not always properly account for landscape differences between compared plots and this likely skewed the obtained results. For example, a crop field next to a forest will

generally have higher overall biodiversity than a crop field next to more fields, regardless of agricultural practices. There are simply more organisms in forests that can migrate to these fields. Finally, it has also been suggested that because organic fertilizers rely on means other than chemical fertilizers to replace nutrients, they cause overall soil nutrient deficits that further impact yield (Trewavas 2001). Clearly, the use of organic nutrient sources does not appear sufficient for meeting the needs of the global population. But how do synthetic fertilizers compare?

1.2: Chemical/Synthetic nutrient sources

Conventional agricultural methods have taken advantage of industrialization, and these methods arose largely out of the need to use pre-existing farmlands more intensely (Matson *et al.* 1997). The prerequisite to agricultural intensification was the invention of chemical/synthetic fertilizers, which have since become a key tool for agriculture. These fertilizers are water-soluble, and used primarily to add nitrogen, phosphorous, and potassium to soil systems. Synthetic fertilizers are employed to ensure plants have sufficient nutrition for growth and also help to obviate nutrient depletion in the soil caused by removal of nutrients from plant harvesting (van Straaten 2007, p. 6). The premise behind fertilizer production is to turn large stores of nutrients that are not easily available to plants into forms that plants can easily access. Thus, atmospheric nitrogen gas is reacted through the Haber-Bosch process to create N fertilizers (White and Brown 2010); apatite minerals that are rich in P are treated with sulfuric acid to form superphosphate fertilizers (van Straaten 2007, p. 134-136); and potash is used for K fertilizers (Darst 1991). Compound fertilizers that contain N, P and K are the types most frequently employed, and these fertilizers are manufactured through combinations of the aforementioned processes (van Straaten 2007, p. 327).

The direct benefit of chemical fertilizers is that the nutrients required for plant growth are available for plant uptake as soon as the fertilizers are added to soils. This benefit is especially apparent when comparing the yield of crops given chemical fertilizers to that of crops that had access only to nutrients that were already present in the soil, as seen in the following examples. A two-fold increase in the kg ha^{-1} yield of rice and a four-fold increase in the kg ha^{-1} yield of wheat with NPK fertilizer use were seen in a study by Yadav *et al.* (2000). Significant increases from NPK fertilizer use on the kg ha^{-1} yields of cowpea, peanut, and maize were found by Yamato *et al.* (2006). Although chemical fertilizers have proven to be incredibly beneficial for increasing crop yield, their overuse has come with two major environmental costs both within and outside the area they are used in. First, when compared to that of organic methods, application of chemical fertilizers has been linked with decreases in biodiversity (Pfiffner and Niggli 1996; Oehl *et al.* 2004; Zhang *et al.* 2012) and detrimental soil changes (Mäder *et al.* 2002; Marschner *et al.* 2003) that decrease nutrient use efficiency (Mäder *et al*. 2002). Second, those nutrients not taken up by plants or not absorbed by the soil can be rapidly flushed out into nearby waterways during heavy rain (Royer *et al.* 2006; Savard *et al*. 2010). The influx of easily-accessible nutrients into neighbouring water bodies promotes their eutrophication (Schindler 1974) and the development of extensive algal blooms (Diaz and Rosenberg 2008) which can contaminate drinking water supplies (Savard *et al.* 2010). In particular, algal blooms can alter water quality and food-webs by creating hypoxic/anoxic water conditions over areas in excess of a thousand square kilometers (Diaz and Rosenberg 2008). Therefore, it is obvious that over-reliance on chemical fertilizers comes with drastic environmental consequences and as such, these fertilizers should not be relied upon exclusively as a nutrient source for crops.

1.3: An integrated approach

The benefits of organic fertilizer use are directly related to the drawbacks of chemical fertilizer use, and vice versa, the benefits of organic nutrient sources are related to the drawbacks of chemical nutrient sources. Each of these approaches is aimed at achieving the same end through different means, and an integrated method that takes advantage of techniques used by both fertilization practices could prove to be the most beneficial. Such an integrated approach has been emphasized by the Food and Agriculture Organization of the United Nations in a report on achieving food security (Roy *et al.* 2006). An example of the potential of this integrated approach is seen in the findings of Zhong *et al.* (2010) during a four-year fertilizer trial in China using maize as the model crop. Across all four years, a combination of organic manure and chemical fertilizer used together consistently resulted in a 1.7-fold increase in the kg ha^{-1} yields over those crops receiving only NPK fertilizer. However, in order for an integrated approach to achieve maximum potential, the plant-microbe-soil system must be understood as a set of interconnected and interdependent subsystems.

Section 2: The agropyramid - a visualization of the interconnected soil processes

The various interconnected soil processes that support plant growth can be viewed schematically as an 'agropyramid' (Figure 1.1) with four levels. The first level designated as such because this is where most humans focus their attention is plant growth. Plant growth is dependent upon proper plant

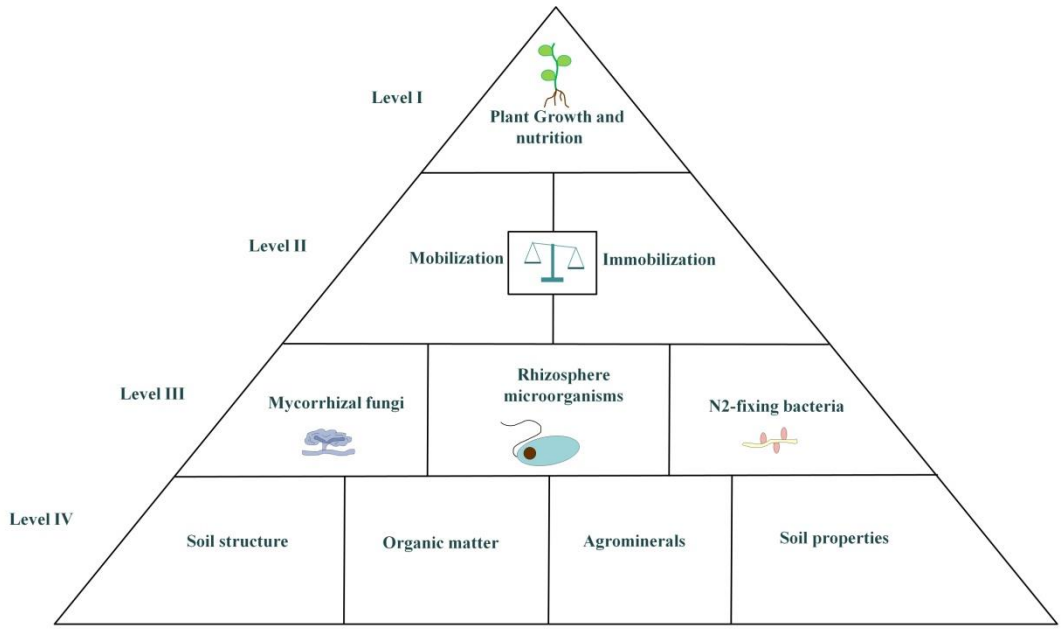

Figure 1.1: The ‘agropyramid’; a schematic representation of interacting soil system components that support plant growth and nutrition. Level I is the main focus of agriculture: plant growth and nutrition. Level II supports the first level, and reflects the balance between mobilization and immobilization of nutrients in the soil. Level III highlights some soil microorganisms that help mobilize nutrients for plant growth. The base of the pyramid, level IV, is made of the components and properties that make up the soil. Each level both depends on and interacts with those levels below it.

nutrition, which leads us to the second level: the balance between nutrient mobilization and immobilization. Although it is often necessary that nutrients be made available to plants through mobilization, excess mobilization must be avoided to minimize nutrient leaching. On the third level are soil microorganisms; these are crucial for cycling nutrients within soil ecosystems. However, microorganisms require organic matter and other soil substrates to maintain healthy populations. The soil influences on microorganism growth are found in level four: the physical structure of the soil, its organic and mineral components, and resulting chemical properties (such as pH). Although the layers underlying plant growth are often disregarded by the layperson, they are nonetheless essential. These four levels will be covered in further depth in the following sections.

2.1: Level I - Plant growth

2.1.1: Pea: a model crop plant

In this thesis, the focus will be on the pea plant (*Pisum sativum* L.). Pea is an important crop, with over 53,000 tonnes produced in Canada in 2013 (FAO 2015). In agriculture, pea is a food source for both humans and livestock, and it has use in crop rotations as a source of nitrogen (MacWilliam *et al.* 2014). In the lab, pea plants are relatively quick and easy to grow, and are smaller than many other crop plants. These attributes and others have led to the use of pea as a model organism in genetics (e.g., Weller *et al.* 1997), in plant physiology (e.g., Berry and Aitken 1979), and in examining the development of mutualisms with soil microorganisms (e.g., Voisin *et al.* 2010; Balzergue *et al*. 2011). Therefore, a large body of research for this species is available to draw upon, and the responses of both symbiotic and non-symbiotic plants to treatment can be assessed in a single system. The following sections will therefore be specific to peas where applicable.

2.1.2: Plant nutrients

Plants require at least fourteen essential nutrients in addition to carbon, oxygen and water, and if not present in adequate concentrations these can limit plant growth and yield (White and Brown 2010). Different plant species have different requirements for nutrients (Masclaux-Daubresse *et al.* 2010), and this must be taken into consideration during their cultivation as deficiencies or toxic excesses of nutrients can impact plant health. The nutrients most often limiting plant growth are nitrogen, phosphorus and potassium.

Nitrogen is an essential constituent of all nucleic acids and proteins and is therefore required in high amounts by plants. Nitrogen in the soil can take a number of different forms; of importance to plant nutrition are organic nitrogen compounds (such as amino acids), ammonium (NH_4^+), and nitrate (NO_3^-). These forms interchange constantly as a result of microbial action and environmental conditions (Richardson *et al.* 2009). Nitrogen enters the soil primarily through microbial fixation of atmospheric nitrogen or decomposition of biological material (Richardson *et al.* 2009). It can then be taken up by plant roots either as part of an organic compound or as ammonium or nitrate. This is accomplished through mass flow along with water into the root and through specialized transport proteins (Richardson *et al.* 2009).

Phosphorus is a principal component of plant cellular macromolecules, and it participates in both cell energetics and the regulation of many cytosolic and nuclear processes (Duff *et al.* 1994). It is most often taken up as the orthophosphate anion PO_4^-. Although it may be present in great concentrations within the soil system, only a small proportion of phosphorus is available for uptake by plants at a given time (Richardson *et al.* 2009). Natural phosphorus is made available through the slow physical and chemical weathering of minerals already present within the soil (Vitousek *et al.* 2010). Because the speed of weathering is often insufficient to support plant growth, the recycling of phosphorus in an ecosystem is typically necessary to ensure adequate

plant P nutrition (Richardson *et al.* 2009). This recycling is achieved through decomposition of organic matter which allows plants to access biologically active forms of phosphorus.

Also necessary for proper cellular functioning in plants is potassium, which is involved in balancing cell electrical neutrality, triggering chemical reactions and maintaining osmotic pressure (Maathuis and Sanders 1996). Potassium is directly available to plants in cationic form (K^+), and is taken up through mass action with water and through specialized protein channels (Maathuis and Sanders 1996).

2.2: Level II - Nutrient cycling

Although ensuring optimal concentrations of these nutrients for growth and yield is of prime importance, this cannot be achieved without the underlying processes that make these nutrients available. Therefore, directly supporting plant growth is the cycling of nutrients within a soil system, and the delicate balance between mobilization and immobilization of nutrients (Figure 1.1, level II). Mobilization encompasses the release of nutrients into forms that plants can take up, and these nutrients can be liberated from organic matter through decomposition or through the weathering of minerals (Dungait *et al.* 2012). Obviously, a certain level of mobilization is required for plants to achieve maximum growth and yield (Dungait *et al.* 2012), but excessive mobilization can lead to nutrient runoff (Savard *et al.* 2010). Mobilization of nitrogen is achieved through the breakdown of organic compounds such as amino acids by fungi and bacteria, or the interconversion of N_2 - NH_4^+ - NO_3^- through the action of both types of microorganisms (Powlson 1993; Richardson *et al.* 2009). Conversely, phosphorus and potassium are mobilized through weathering and solubilization of minerals (Richardson *et al.* 2009), or are released from organic matter. The process of mobilization is mimicked by the production of chemical fertilizers; both make nutrients available for plant uptake.

Acting counter to mobilization is immobilization or the sequestration of nutrients so that these become unavailable to plants. Nutrients can become immobilized when they are taken up by

organisms as part of their nutrition or become incorporated into the soil minerals (Dungait *et al.* 2012). Nitrogen, phosphorus, and potassium are typically immobilized when they are taken up by organisms and become part of the cellular composition, although adsorption of phosphorus (Vitousek *et al.* 2010) and potassium (Kayser and Isselstein 2005) onto soil particles can also immobilize these nutrients. If the soil system immobilizes nutrients too quickly, there will be insufficient amounts to sustain optimal plant growth (Dungait *et al.* 2012) and conversely, if not enough nutrients are immobilized, nutrient runoff can occur (Savard *et al.* 2010). In agricultural systems, the balance of mobilization and immobilization is shifted to favour mobilization, and nutrient leaching is often a consequence of this imbalance (Powlson 1993; Vitousek *et al.* 2010; Royer *et al.* 2006; Savard *et al.* 2010). The nutrient cycle thus has considerable ramifications on the sustainability of agriculture because of the potential environmental impacts an imbalanced cycle may have.

2.3: Level III - Soil Microorganisms

Nutrients are made available largely through the action of soil microorganisms, and a healthy soil microbial population can not only minimize leaching, but improve crop yield as well (Mäder *et al.* 2002; Lalfakzuala *et al.* 2008; Zhong *et al.* 2010; Ge *et al.* 2011; Lv *et al.* 2011). The beneficial soil microbial population can be broadly grouped into two main categories: specialized mutualists such as mycorrhizal fungi and rhizobia that engage in direct symbiotic interactions with plants, and generalists that make up most of the rhizosphere community and indirectly enhance plant growth (Figure 1.1, level III). Direct symbiotic associations are beneficial in that they allow the plants access to nutrient sources they are otherwise unable to take advantage of, whereas the presence of rhizosphere microorganisms typically enhances plant growth through more indirect means.

2.3.1: Arbuscular-mycorrhizal fungi

Arbuscular mycorrhizal (AM) fungi form intimate associations with the roots of approximately 80% of known vascular plant species (Peterson *et al.* 2004). Though AM fungi may be of the *Arum* or *Paris* type depending on how the fungus progresses through the root, only the *Arum* type will be considered here as it relates to pea. In the association between AM fungi (Phylum Glomeromycota) and plants, the AM fungus is an obligate partner, while the plant is a facultative partner (Peterson *et al.* 2004). The overall symbiosis and its development are summarized in Figure 1.2. During this symbiosis, the fungus obtains phosphorus and nitrogen that would be otherwise inaccessible to the plant (Carbonell and Gutjahr 2014) and exchanges them for photosynthetic sugars and a protected environment in which to live. The AM fungus is based primarily within the cortical tissue of the plant root, though it maintains a broad hyphal network in the surrounding soil (Friese and Allen 1991). The hyphae external to the root, i.e., the extraradicular hyphae, allow the fungus access to phosphorus pools beyond the phosphorus-depletion zone surrounding the plant root, because in essence they extend the surface area of the root system (Li *et al.* 1991). The extraradicular hyphal network also contributes to improved soil particle stability through exudation of the protein glomalin (Rilling 2004; Singh *et al.* 2013), the low turnover rate of which helps sequester carbon within the soils (Singh *et al.* 2013). It is worth noting that this symbiosis does not increase the overall amount of nutrients present within a soil, it simply makes those already present more accessible to plants.

The mycorrhizal fungus is present as a dormant spore within the soil, and upon its perception of strigolactones exuded by nearby roots, the spore is induced to germinate and develop branched hyphae (Gutjahr and Parniske 2013). As the hyphae grow towards the plant root, they exude lipo-chito-oligosaccharide-based myc factors that are used by the plant to establish symbiotic compatibility and to prepare the root for colonization by the fungus (Maillet *et al.* 2011; Gutjahr and Parniske 2013). Once contact is established and compatibility is assessed by

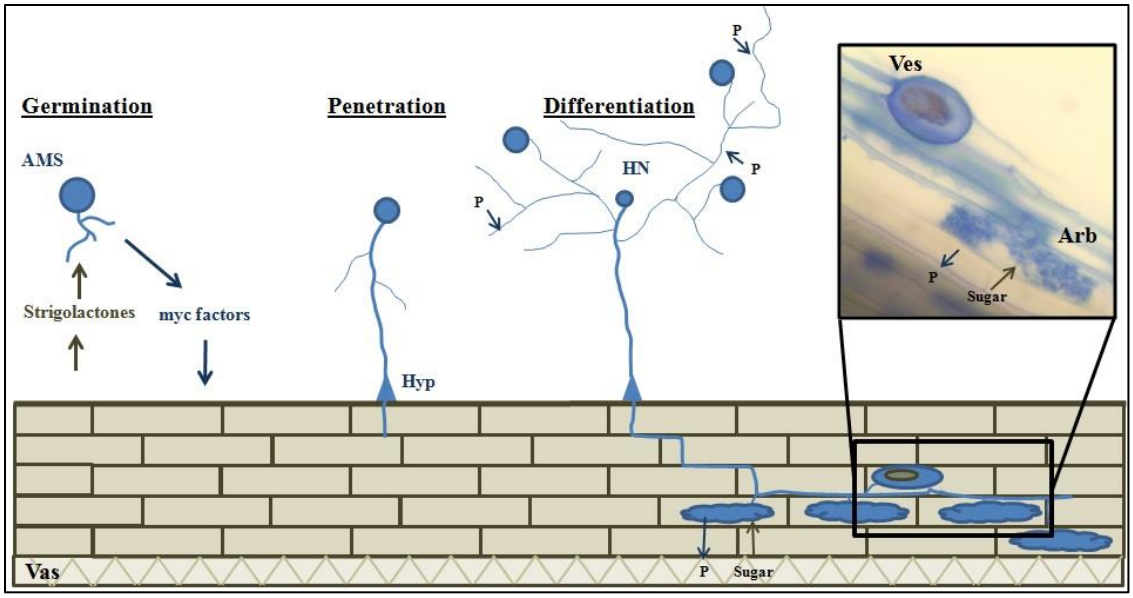

Figure 1.2: Overview of the arbuscular-mycorrhizal symbiosis. A longitudinal section of a plant root is shown at the bottom of the diagram. The mycorrhizal symbiosis is initiated by the germination of an AM fungal spore (AMS), and hyphal branching is promoted by strigolactones exuded by the plant. Fungal myc signalling molecules are also produced around this time. Following mutual recognition between the two symbionts, the symbiosis then proceeds with fungal penetration of the root via the formation of a hyphopodium (Hyp). The crux of the symbiosis is the differentiation of hyphae within the root into arbuscules (Arb) and storage vesicles (Ves). Phosphorus is taken up by the extraradical hyphal network (HN), transported through the fungus, and is exchanged primarily at the arbuscule for plant photosynthates. The phosphorus and sugars are transported to the appropriate sinks via the vascular tissue (Vas). Spores for the propagation of the fungi form on the extraradicular hyphal network when the symbiosis is well-developed.

fungal recognition of root cutin monomers (Wang *et al.* 2012), a fungal structure known as a hyphopodium is formed to assist in fungal penetration of the root epidermis. The fungus then grows within the cortical intercellular spaces of the root (Guinel and Geil 2002). Although it enters plant cells within the inner cortex, the fungus is never in direct contact with the plant cytoplasm as it is contained within a plant-based perifungal membrane whenever it breaches a cell (Bonfante and Perotto 1995). Once within the cell, the fungal hypha differentiates into a transient, tree-like structure known as an arbuscule; this structure helps facilitates the transfer of phosphorus and sugar between the two symbiotic partners (Carbonell and Gutjahr 2014). Arbuscules are short-lived structures, and persist for only 24-72h (Kobae and Hata 2010; Gutjahr and Parniske 2013).

Once the symbiosis is well-developed, additional fungal structures known as vesicles form; these have presumed roles in lipid storage (Peterson *et al.* 2004). The fungus is propagated through the production of spores formed on the extraradicular hyphae (Peterson *et al.* 2004). The development and maintenance of this association is dependent on the nutrient status of the plant. If phosphorus is plentiful in the soil environment, a hypothetical shoot-based signal inhibits fungal colonization of the root system (Carbonell and Gutjahr 2014). In addition, the amount of carbon allocated to the fungus is linked with the amount of phosphorus the plant receives from the fungus, and more productive fungal partners are allocated more carbon (Kiers *et al*. 2011).

2.3.2: Rhizobia

A more selective symbiosis occurs between nitrogen-fixing bacteria (Family Rhizobiaceae) and leguminous plants such as pea (Mylona *et al.* 1995). Unlike in the mycorrhizal mutualism between AM fungi and plants where the fungus is an obligate symbiotic partner, both partners in the rhizobia-legume mutualism are facultative symbionts. An overview of the rhizobia-legume symbiosis is illustrated in Figure 1.3, with the inset highlighting the flow of

nutrients between partners. The rhizobia, in exchange for dicarboxylic acids such as fumaric acid, provide the plant with nitrogen mainly as ammonium. In addition, glutamate is thought to be provided to the rhizobia in order to reduce their need for ammonium (Lodwig *et al.* 2003). The ammonium produced from nitrogen fixation that is not used by the bacteria can then be transferred to the plant to contribute to its glutamate pool (Lodwig *et al* 2003). The nutrient transfer is facilitated by the close association between plant cells and rhizobia in the nodules. The rhizobia-legume symbiosis requires the rhizobia to enter the root, and the underlying molecular mechanism by which rhizobia enter and colonize plant roots is thought to be a co-opted variation on the mycorrhizal infection process (Guinel and Geil 2002; Provorov *et al.* 2002; Oldroyd *et al.* 2011). An overview of nodule development is presented here and is briefly summarized in Figure 1.3. The focus will be on the indeterminate nodule type, as this is the type formed by *P. sativum* (Guinel 2009) and its specific partner *Rhizobium leguminosarum* bv. *viciae* (Skøt 1983).

The chemotactic attraction of rhizobia to roots is achieved through the secretion of phenolic flavonoids by the plant, which provides a level of specificity to the interaction (Ferguson *et al.* 2010). Upon sensing a suitable plant host, the rhizobia produce lipochitooligosaccharides (NOD factors) which in turn initiate developmental changes within the root (Tsyganov *et al.* 2002; Ferguson *et al.* 2010). When rhizobia contact the root hair, it curls over to form a pocket where the bacterial colony grows. Rhizobia enter the root hair via physical forces and chemical degradation of the cell wall. Rhizobia proceed through an infection thread, a structure composed of plant cell wall components and supported by microtubules (Oldroyd *et al.* 2011), towards the root's inner cortex (Ferguson *et al.* 2010). Within the inner cortex, the cortical cells will have already begun to divide in response to the NOD factors to form a nodule primordium (Tsyganov *et al.* 2002; Ferguson *et al.* 2010). As the cells making up the primordium continue to divide, they soon make contact with the infection thread and the rhizobia contained within. The nodule proper

is considered to have formed once the primordium begins developing its own vasculature and the rhizobia are released from the infection thread (Xiao *et al.* 2014).

The structure of a fully-formed indeterminate nodule has four to five distinct zones with one interzone (Guinel 2009), illustrated in Figure 1.3. The outermost zone I is the meristem, and is responsible for the continual growth that characterizes the indeterminate nodule type. Proximal to zone I, zone II is the location where the infection threads terminate. In this zone, the rhizobia are endocytosed by the plant cells (Łotocka *et* al. 2012) and are contained within a plant-derived peribacteroid membrane separating them from the plant cell cytoplasm (Whitehead and Day 1997). The encapsulated rhizobia are induced to terminally differentiate into bacteroids by plant nodule-specific cysteine-rich peptides which prevent further bacterial cell division; the resulting bacteroid is now part of an organelle-like structure known as a symbiosome (Kondorosi *et al.* 2013). Between zone II and zone III is the interzone II-III, which contains plant cells that are rich in starch and produce leghaemoglobin (Guinel 2009). Leghaemoglobin is an iron-heme protein that mediates oxygen levels in the nodule, thereby creating a microoxic environment that allows the bacteroid nitrogenase enzyme to fix nitrogen with minimal inhibition from oxygen binding (Downie 2005). However, zone III is the prime location where the bacteroids are fixing nitrogen. Zone IV is the most proximal zone where both bacterial and plant cells are senescing (Guinel 2009). An additional proximal zone has also been described, zone V, which contains rhizobia that have not differentiated and seem to feed on the senescent tissues (Timmers *et al.* 2000). This latter zone likely also contributes to the distribution of non-differentiated rhizobia into the soil environment after the nodule senesces entirely (Timmers *et al.* 2000).

Because the cost to the plant of forming and maintaining nodules is high, the number of nodules that form on the root is restricted primarily by a plant mechanism known as autoregulation of nodulation (AON; Voisin *et al.* 2010; Reid *et al* 2011). In pea, seedlings are susceptible to colonization by rhizobia only after the first leaves begin to develop (Voisin *et al.*

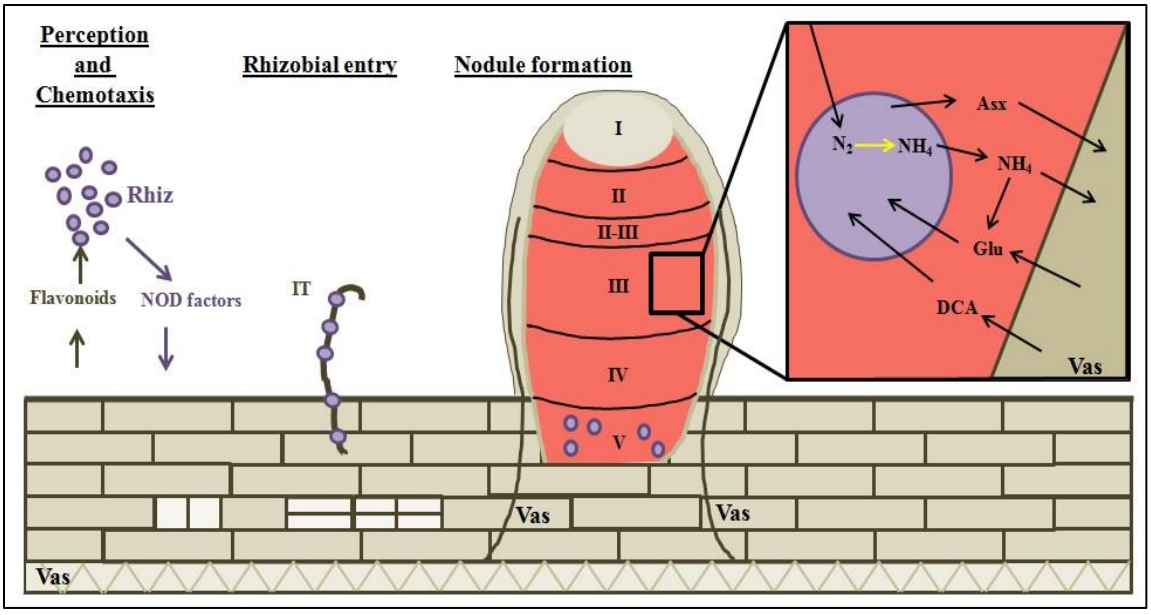

Figure 1.3: Overview of the rhizobia-legume symbiosis in pea. A longitudinal section of a plant root is shown at the bottom. The symbiosis is triggered by the sensing of the flavonoids by rhizobia (Rhiz) which then produce NOD factors and move chemotactically toward the root exuding the flavonoids. The NOD factors induce the dedifferentiation and cell division of some inner cortical cells (shown in lighter brown). Once in contact with a root hair, rhizobia alter the cell cytoskeleton so that the root hair curls. Through pressure and degradation of the cell wall, the rhizobia proceed down an infection thread (IT) that allows entry into cortical cells. The IT grows towards the nodule progenitor cells and brings rhizobia to the nodule primordia formed by the cortical cells, resulting in the formation of a nodule with its own vasculature. Indeterminate nodules can be divided into five (I-V) zones and one interzone (II-III). The inset from zone III, the fixation zone, illustrates the symbiosomes (purple) taking in atmospheric nitrogen (N_2) and fixing it (yellow arrow) into ammonia (NH_4). This, along with aspartate (Asx), is exchanged with glutamate (Glu) and dicarboxylic acids (DCA) received from the plant vasculature (Vas).

2010), and they form nodules only when the plant is in need of nitrogen. After the seedling stage, the colonization by rhizobia and the subsequent inhibition of nodulation via AON produce a characteristic zone of nodulation at the crown of the root system where nodules are first formed (Reid *et al* 2011). However, if the plant becomes limited by nitrogen, a second zone of nodulation may be formed lower on the root system (Voisin *et al*. 2010). As with the mycorrhizal symbiosis, the availability of the primary nutrient provided by the symbiont has a negative influence on the development of the rhizobial symbiosis, as seen when nitrate is made available to inoculated plants (Bollman and Vessey 2006). Curiously, in contrast to nitrate, when ammonium is provided to inoculated pea plants, nodulation is stimulated instead of inhibited (Bollman and Vessey 2006). This may be related to the aforementioned incorporation of ammonia into glutamate by the plant and subsequent promotion of nitrogen fixation by this amino acid (Figure 1.3). In absence of provided nitrogen, the rhizobial symbiosis is capable of providing sufficient nitrogen for plant growth (Voisin *et al.* 2002). Unlike the mycorrhizal symbiosis which only makes those nutrients already present more available, the rhizobial symbiosis increases the nitrogen levels in the soil after the nodules and/or roots decompose.

2.3.3: The rhizosphere community

Although root mutualists provide strong nutritional benefits to plants, the microorganisms in the rhizosphere community cannot be overlooked. These generalist species can promote plant growth either directly or indirectly. An excellent example are those bacteria, such as *Enterobacter cloacae* UW4 (Li *et al*. 2000) or various rhizobial species (Duan *et al.* 2009), that are able to metabolize 1-aminocyclopropane-1-carboxylic acid (ACC) using the ACC-deaminase enzyme. These bacteria are thought to lower plant production of ethylene by breaking down the ethylene-precursor ACC for use in their own metabolism (Glick *et al.* 1998). Ethylene is a plant

stress hormone that limits plant growth (Apelbaum and Burg 1972), and when ACC is degraded the levels of the product ethylene are lowered. Therefore, lower ACC levels mean that plant growth is not inhibited by ethylene (Glick *et al.* 2007) and symbioses with beneficial root microorganisms can continue to develop (Barnawal *et al.* 2014). Ethylene is a known inhibitor of both the mycorrhizal and rhizobial symbioses. It negatively affects processes involved in nodulation such as rhizobial entry into the root cortex and nodule primordia formation (Guinel and Geil 2002). Exogenous ethylene application has also been shown to reduce the progression of mycorrhizal fungi into pea roots and the subsequent development of intraradicular hyphae and arbuscules (Geil *et al.* 2001).

Rhizosphere microorganisms can also indirectly assist plants by secreting chitinase and cellulase enzymes in the rhizosphere to help protect plants against pathogens such as fungi (Kaplan *et al.* 2013). Finally, by promoting root growth, the rhizosphere community can enhance nutrient uptake, as demonstrated in tomato plants inoculated with *Bacillus amyloliquefaciens* IN937a and *B. pumilus* T4. With microorganism inoculation, these plants required 20-30% less of a N:P:K fertilizer (20:10:20) to maintain growth equal to that of plants given 100% of the recommended dose of fertilizer (Adesemoye *et al.* 2009).

The benefit of a healthy soil microorganism population is demonstrated in the cascade effect of improved plant growth; this can be seen clearly when organic matter is added to soils. Increases in microbial biomass, diversity and activity are observed with organic matter usage (Lalfakzuala *et al.* 2008; Zhong *et al.* 2010; Ge *et al.* 2011; Lv *et al.* 2011) and this increase contributes to higher crop yields (Mäder *et al.* 2002; Zhong *et al.* 2010) and soil fertility (Mäder *et al.* 2002; Ge *et al.* 2011). Overall, the presence of a healthy and diverse soil microbial population is paramount, and accordingly alternatives or alterations used to decrease the environmental impact of agriculture need to take into consideration the soil microorganisms. The compatibility between the soil microorganisms and agricultural practices is not always assured, and potential negative impacts should be examined before the practice is adopted. Even the

presence or absence of organic matter in the form of compost/manure can have profound effects on the composition and biomass of soil microorganisms, and the changed microorganism populations then impact the nutrition of plants.

2.4: Level IV - The soil matrix

Ultimately, it is the composition of the soil itself that is the foundation upon which microbial populations, nutrient cycling and plant growth are built (Figure 1.1, level IV). Although in most cases the soil structure cannot be easily altered, its physical properties such as pH can be manipulated for agricultural gain. For brevity, this section will be limited to soil pH as it plays a crucial role in the suitability of a soil system for growth of plants and microorganisms. It is well known that soil pH and the availability of nutrients are linked (e.g., Neumann and Römheld 2012) and that release of nutrients from minerals is enhanced under acidic (pH<5) conditions (Valsami-Jones *et al.* 1998; Guidry and Mackenzie 2003; Welch *et al.* 2010).

The diversity of soil bacterial populations is also linked with soil pH, with the highest diversity seen at neutral pH (Lauber *et al*. 2009). Agricultural practices often directly alter the soil pH as seen in the examples below. The consistent application of nitrogen fertilizers results in increased soil acidity (Conyers *et al.* 1996; Guo *et al.* 2010) that is exacerbated when cations are preferentially taken up by plant roots over anions (Bolan *et al.* 1991; Guo *et al.* 2010). Soil acidification is a major problem in agriculture; it affects phosphorus mobility and increases the availability of potentially-toxic aluminum (Guo *et al* 2010). The acidification of soils can be countered through addition of calcium carbonate in a process known as liming (Haynes and Naidu 1998), which often results in increased growth of crop plants (Mathur and Levesque 1983; Grewal and Williams 2003).

In the following experiments, I exclusively utilized an artificial soil composed of 1:1 vermiculite:Turface™ in order to minimize externally-provided organic matter which could confound results. Vermiculite is a micaceous mineral composed primarily of magnesium and

potassium (Kalinowski and Schweda 2007) while Turface™ is a manufactured, heat-treated clay, the main components of which are silicon-dioxide, aluminum oxide, and iron (II) oxide (Turface™ composition report 2013; Table 1.1).

Section 3: Agrominerals for agriculture

Rock fertilizers, or agrominerals, are environmentally-friendly nutrient sources with great agricultural potential, especially in developing countries (van Straaten 2007, p. 149). One benefit of agromineral is that, unlike chemical fertilizers, they are not highly processed before use to solubilise nutrients (van Straaten 2007, p. 10) and are therefore cheaper than chemical fertilizers (Chien and Menon 1995; Labib *et al.* 2012). The problems associated with excessive chemical fertilizer use can be mitigated by their combined use with agrominerals. The benefit of an agromineral and chemical fertilizer combination is demonstrated in a study by Labib *et al*. (2012) where the growth of potatoes was highest when reduced levels of potassium-sulfate fertilizer application was combined with potassium-containing agrominerals.

There is, however, at least one drawback that can limit the use of agrominerals. Rocks are inherently complex combinations of minerals, and agrominerals can sometimes contain undesirable elements that would need to be first removed, two examples of which are radioactive uranium-238 (Sam *et al.* 1999) or toxic barium (Heim *et al.* 2012). If removal of these elements is not feasible, for example due to cost, it would mean that this particular mineral is unusable for agriculture. In this thesis, I will be focusing specifically on an agromineral that is relatively free of harmful elements and is already being sold as a soil amendment for agriculture: Spanish River Carbonatite (SRC). A brief overview of SRC is provided below. SRC is a complex alkaline rock that is primarily composed of calcite, apatite and biotite minerals (Sage 1987; Table 1.1), and is mined and sold by Boreal Agrominerals Inc. (Brampton, Ontario). The underlying carbonatite rock deposit that SRC is harvested from was formed through volcanic activity ~1880 Ma

(Rukhlov and Bell 2010) and is located in Northern Ontario, Canada. Because of the remoteness of the deposit, large amounts of SRC are removed only every few years; this SRC is then stored as an uncovered pile that is being packaged when needed. Anecdotal reports from commercial users of SRC have indicated that increases in plant growth, soil pH, and soil health accompany its use, but a scientific examination of the influence of SRC on plants, soils and microorganisms has not yet been conducted.

Table 1.1: Composition of two minerals used as part of the artificial soil mixture. Turface™ was used alongside vermiculite as the soil medium whereas Spanish River Carbonatite is an agromineral which was added to the soil in order to test for its usefulness as a nutrient source for plants.

Turface™	
% composition (average)	
SiO_2	74
Al_2O_3	11
Fe_2O_3	5
Miscellaneous (e.g., CaO)	10

Spanish River Carbonatite	
% of composition*	
Ca	19.50
Fe	2.82
Al	2.10
Mg	1.32
P	1.23
K	0.78
Na	0.51
N	0.30
S	0.05

*Retrieved from Boreal Agrominerals website, 2015. (http://www.borealagrominerals.com)

Section 4: Project Objectives

The main objective of this thesis was to confirm the potential usefulness of SRC as an agricultural amendment by examining how SRC addition affects the plant-soil-microbe system. Because of the broad scope of this objective, it was divided into the following sub-objectives:

1) To determine the optimal concentration of SRC based on plant growth, soil condition, and soil microorganism changes. To complement this objective, I hypothesize that plant growth, soil conditions and soil microorganisms will be positively impacted as the concentration of SRC is increased. Testing of this hypothesis is covered in chapter 2, which has been prepared as a stand-alone manuscript for later publication.

2) To assess how the rhizobia-legume symbiosis is altered, if at all, by optimal SRC addition. To complement this objective, I hypothesize that the introduction of SRC into the soil will improve nodulation efficiency; this would be due to the increased mineral nutrients provided by SRC and the subsequent proliferation of rhizobial bacteria. Testing of this hypothesis is also covered in chapter 2.

3) To examine how the storage conditions of SRC affect its usefulness as an agricultural supplement. To complement this objective, I hypothesize that, because SRC is a non-biological mineral, its usefulness will be unaffected by storage conditions. The testing of this hypothesis is covered in chapter 3, which although formatted as a stand-alone manuscript, contains preliminary findings that will need to be expanded on before publication.

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Chapter 2: Assessing the suitability of Spanish River Carbonatite for use in agricultural systems

Section 1: Introduction

Ensuring food security for the ever-growing global human population is contingent upon more efficient agricultural practices (Roy *et al.* 2006). A prevailing problem limiting the growth of crop plants is the inability to provide consistently sufficient nutrients to maintain plant growth and yield (Dungait *et al.* 2012). Two strategies are currently used to approach this problem, and can be broadly divided into the use of organic and the use of chemical nutrient sources. In the former, additives such as compost (Ge *et al.* 2011) and plant residues (Stanhill 1990) are used as nutrient sources. In the latter, raw materials such as phosphate rock (van Straaten 2007, p. 149) are processed to convert the nutrients into water-soluble forms that are easily taken up by plants. For example, treating phosphate rocks with sulfuric acid is the main means by which superphosphate fertilizer is produced (van Straaten 2007, p. 134-136). The use of organic or chemical nutrient sources comes with associated costs: organic fertilizers typically fail to reach the yields achieved by chemical fertilizers (Stanhill 1990; Trewavas 2001; Mäder et al. 2002), and the long-term use of chemical fertilizers in conventional agriculture leads to environmental damages due to nutrient runoff (Savard *et al.* 2010) and promotion of algal blooms/hypoxic conditions (Diaz and Rosenberg 2008).

The addition of rock fertilizers, also known as agrominerals, to soils is one means by which conventional and organic agricultural practices can be supplemented to increase plant growth and environmental friendliness (van Straaten 2007, p. 7; Labib *et al.* 2012). Agrominerals are locally-mined rock minerals that are generally not processed or modified prior to addition to soils, and they rely upon physical and biological weathering processes to release their nutrients for uptake by plants. Few studies have examined the use of agrominerals in agricultural systems,

however (Chien and Menon 1995; Sahu and Jana 2000; Liu *et* al. 2008; Labib *et al.* 2012), and the techniques required to maximize nutrient release have not been fully clarified. Agrominerals can also be 'contaminated' with undesirable elements such as uranium-238 (Sam *et al.* 1999) that require removal for agricultural use.

Spanish River Carbonatite (SRC) is an agromineral that is relatively free of harmful elements and is harvested from the Sudbury region in Northern Ontario. SRC is an excellent model to test the potential of agrominerals as it weathers quickly, its mineralogy has been characterized, and it has already been sold for a number of years as an agricultural soil supplement and liming agent (John Slack, personal communication 2014). It is a carbonatite mineral composed primarily of apatite, calcite, and biotite (Sage 1987). Here, a scientific examination of SRC is conducted to examine its effects on soil microorganisms, soil chemical properties, and plant growth. The overall objective of the study was to assess the suitability of SRC for agricultural use and this objective was divided into three sub-objectives: 1) To determine how SRC influences the growth of a model plant species throughout its life cycle, 2) to examine, during plant growth, how SRC affects the soil pH and soil microbial populations, and 3) to determine how SRC addition affects the rhizobial-legume symbiosis. The pea *Pisum sativum* (cv. Sparkle) was chosen for use here as a model plant for two reasons: it is an important agricultural crop in Canada, with over 53,000 tonnes produced in 2013 (FAO 2015), and because it forms agriculturally-relevant interactions with mycorrhizal fungi and nitrogen-fixing rhizobia. Thus, the use of pea allowed us to explore several agriculturally-relevant aspects of plant growth and how these may be altered upon SRC addition within a single easily-grown plant system.

In this study, it was found that SRC shows great promise both as a model agromineral and as a soil supplement for agricultural systems. SRC, added as a ratio of 1:10 SRC:soil, was capable of providing all required nutrients to plants except for nitrogen, with the agromineral acting as a mediator to a mutually beneficial positive feedback mechanism between soil

microorganisms and plants. Furthermore, when plants were given SRC and inoculated with rhizobia, nodulation was enhanced and the costs associated with nodulation were reduced when compared to inoculated plants not given SRC.

Section 2: Materials and Methods

2.1 Plant growth conditions

The following conditions were used for all plants regardless of the experiment unless otherwise noted. Surface-sterilization of *Pisum sativum* cultivar 'Sparkle' seeds was accomplished by swirling them in an 8% bleach solution for ten minutes. Seeds were then rinsed three times for one minute in sterile de-ionized water and left to imbibe for 16-18h in absence of light (Guinel and Sloetjes 2000). Following imbibition, seeds were individually planted 1cm deep with their radicles downwards in black Cone-tainers™ (656mL volume; Stuewe and Sons, Tangent, OR, USA) which had been filled with soil and fitted with a piece of fibreglass screen on the bottom to minimize soil loss. To avoid introduction of nutrient sources from organic matter that could confound results, a 1:1 (v:v) mixture of vermiculite:Turface™ (Plant Products Co. Ltd., Brampton, ON) was used as the soil medium. Soils were always autoclaved (sterilized for 75mins at 121°C) and left to cool to room temperature prior to use. Where indicated, the soil was supplemented with SRC prior to autoclaving. The SRC was obtained directly from Boreal Agrominerals Inc. (Brampton, ON) as 4.54kg retail packages. To ensure seedling establishment, the top of the soil was kept moist through either surface watering or the addition of a polyethylene plastic sheet held in place around each Cone-tainer™ with an elastic band until shoots emerged. Seeds that took longer than six days to establish (indicated by emergence of shoot from the soil; Knott 1987) were removed from the experiment and discounted. At planting, Cone-tainers™ were placed in sheet metal trays and watered by filling the tray with 1.5L of

deionized water every three to four days. The seed nutrient reserves are mostly depleted by 10 days after planting (DAP; Guardiola and Sutcliffe 1972), and so plants were given various nutrient solutions (as indicated under the specific experiment) every third watering after this time. All plants were grown in the growth-room facility at Wilfrid Laurier University under a 16h day (23°C) and 8h night (18°C) photoperiod cycle. Light was provided through high pressure sodium, metal halide and fluorescent bulbs giving 250$\mu molm^{-2}s^{-1}$ of photons (measured with LI250A LICOR Biosciences light meter, Lincoln, NE, USA).

2.2 Determination of optimal SRC concentration

To initiate the investigation into the usefulness of SRC as a soil additive, the optimal concentration of SRC was determined. A SRC:soil ratio of 1:10 (v:v) had been recommended by Boreal Agrominerals to its customers, however, this recommendation was based on the composition of the minerals and had not been verified as optimal for plant growth (John Slack, personal communication 2013). Four treatment groups were tested: soils with no SRC (control), soils with the recommended amount of SRC (1:10), soils with half of the recommended amount (1:20), and soils with twice the recommended amount (1:5). A quarter of the recommended amount (1:40) was also tested, but this treatment was discontinued as plants with this ratio were either equivalent to or worse than the control plants in terms of growth. Nutrients were provided to control plants in the form of a solution (as per Guinel and Sloetjes 2000, see Appendix A). The only non-SRC nutrient provided to SRC-treated plants was nitrogen in the form of 2.5mM NH_4NO_3 solution as SRC is a poor source of nitrogen (Sage 1987). Ammonium nitrate was chosen as the nitrogen form because it has been used previously in studies of pea growth (e.g., Bollman and Vessey 2006). The concentration of nitrogen in both the nutrient and nitrogen solutions was the same. Five plants were grown for each time period and treatment, and the

experiment was replicated four times. Three different life-stages of pea were examined: seedling (9 days-after planting; DAP), vegetative (21DAP), and reproductive (42DAP) (Knott 1987).At each stage, the soil pH and the number of nodes/plant dry weight were measured to indicate changes upon SRC addition in soil characteristics (Watson and Brown 1998) and plant growth (Voisin *et al.* 2002), respectively.

Plants were extracted from the soil by gently emptying each pot into separate, clean trays. Once the plants were freed from the pots, their root systems were washed clean of soil particles using deionized water and their cotyledons removed; the plants were then placed in paper towels to be dried for at least 72h at 60°C (Macdonald 2011). The soil left in the tray from each pot was homogenized by hand, and 20g collected for measuring the soil pH. To each individual soil sample, 20mL of deionized water were added; the solution was mixed by swirling for 5 seconds and then left to equilibrate for 10mins at room temperature before its pH was measured (Watson and Brown 1998).

Biomass allocation was examined at 21 and 42DAP by separating the root and shoot systems prior to drying. At 42DAP measurements of plant yield were undertaken to show agricultural potential. Three yield parameters were used: number of pods per plant, pod dry weight per plant, and number of seeds per plant. Pods were removed from the shoots prior to drying. Following drying, the number of non-aborted seeds (indicated by rounded and full appearance; Pigeaire *et al.* 1986) was counted.

Soil microorganisms were quantified using heterotrophic plate counts (HPC; Olsen and Bakken 1987) from soils of 42DAP plants. Soil sterility following autoclaving was also confirmed by this methodology. The soil samples were collected by emptying the pots into separate 70% ethanol-sterilized trays, homogenizing by hand, and placing 1g from each pot into autoclave-sterilized capped test tubes. Soil samples were stored overnight at 4°C and plated the

next day. Samples were combined with 9mL of sterile deionized water, vortexed, and then used to make a dilution series from 10^{-1} to 10^{-6}. For each dilution, 100µL was spread-plated onto 2.3% nutrient agar plates (Dicfo nutrient agar; BD, Mississauga, ON). All dilutions and plating were undertaken in a Labconco purifier class II biosafety cabinet. Plates were sealed with Parafilm® and incubated for 120h in darkness in the growth room (see §*2.1 plant growth conditions*). Resultant colonies were counted daily for 5 days, and the colony counts converted to colony-forming-units (CFU) per gram of soil by multiplying the observed colony number by the dilution factor (e.g., by 100 for 10^{-1} dilution) and then dividing by the volume plated (100µL). Although all dilutions and time points were examined, only the dilutions and time points which resulted in <300 adequately-spaced colonies were chosen for statistical analysis. Plates beyond these time points and dilutions frequently had colonies that merged together and this made colony counts unreliable (Figure 2.1).

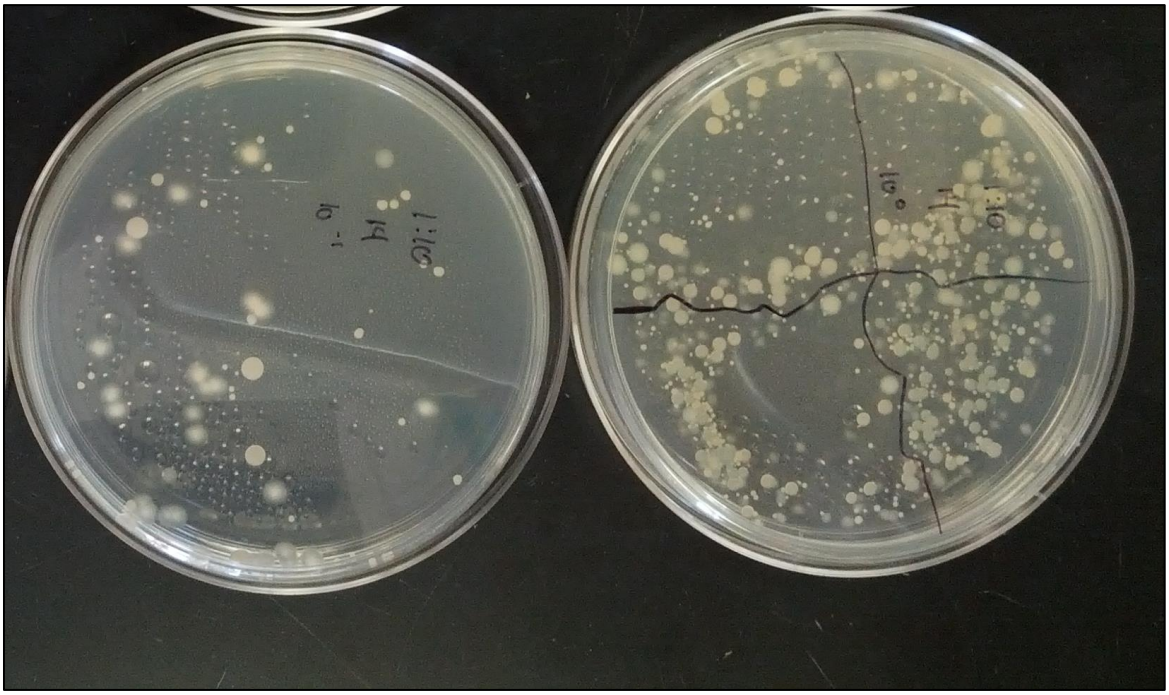

Figure 2.1: Comparison between nutrient agar plates with adequate (left) and excessive (right) bacterial growth. While both plates are from the 1:10 SRC:soil (v:v) treatment group from the optimal concentration experiments at 120h after plating, the left is with a 10^{-1} dilution, while the right is with a 10^{0} dilution. Plates left to grow longer than 48h or with concentrations above 10^{-1} had bacterial colonies that frequently coalesced as seen in the right plate.

2.3 SRC and the rhizobia-legume symbiosis

Two reasons motivated the examination of how SRC affects the rhizobia-legume symbiosis: first, this symbiosis is an agriculturally-important source of nitrogen, and second, the addition of rhizobia and SRC to the soil system should theoretically provide plants with all required nutrients. Three treatment groups were used for these experiments: control plants grown with no SRC and inoculated with rhizobia ($RSRC^-$), plants grown with 1:10 SRC and inoculated with rhizobia ($RSRC^+$), and plants grown with 1:10 SRC and given nitrogen solution ($NSRC^+$, as per §2.2). The latter group was included to distinguish changes induced by the symbiosis from those induced by the SRC treatment. Eight plants were included per treatment group, and the experiment set was replicated at least two times. The $RSRC^+$ plants were given only water, the $RSRC^-$ group received low nitrogen solution (nutrient solution with only 0.5mM $Ca(NO_3)_2$; Guinel and Sloetjes 2000) and the $NSRC^+$ group received 2.5mM NH_4NO_3 (all given as per §2.2).

At 4DAP, $RSRC^-$ and $RSRC^+$ plants were inoculated with *Rhizobium leguminosarum* bv. *viciae* (128C53K; kindly provided by Dr. Bernard Glick, University of Waterloo). Rhizobia were cultured from yeast-mannitol agar slant cultures by transferring two loops of bacteria into 20mL of yeast-mannitol broth (Appendix A) and then incubating the broth in a orbital-shaking water-bath (New Brunswick Scientific, Edison, NJ, USA) at 100rpm and 25°C for approximately 48h until bacteria reached stationary growth (indicated by a spectroscopic absorbance of 0.8-1.0 at 600nm; Macdonald 2011). A 5% rhizobia inoculant solution was then prepared from the broth and 5mL of this solution were given to each plant around the base of the epicotyl.

Plants were harvested 24 days after inoculation (DAI)/28DAP to compare nodulation and plant growth characteristics between treatment groups. Plants were examined as per the vegetative measurements in §2.2. The number of functional nodules (indicated by the presence of

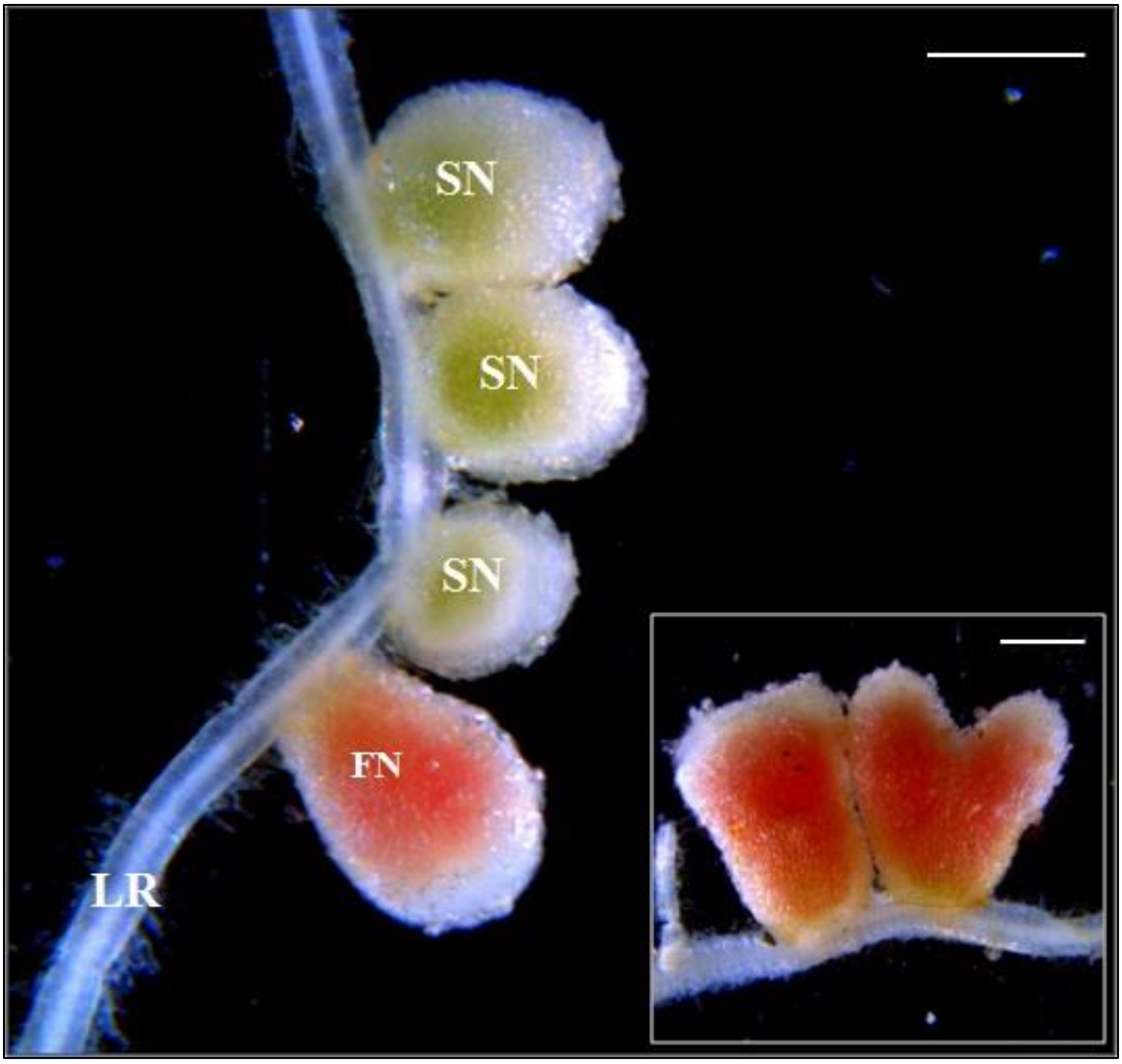

Figure 2.2: Photomicrograph showing a single lateral root segment (LR) with non-functional and functional nodules. The inset shows a single LR with both single (left) and multilobed (right) nodules. Senescent nodules (SN) are white with a green core, whereas functional nodules (FN) have a reddish-pink core due to the presence of leghaemoglobin. Both lateral roots are from 28 days-old plants that had been inoculated with *Rhizobium leguminosarum* bv. *viciae* 4 days after planting. The images were taken with a SMZ1800 stereomicroscope, and the scale bars are 1mm. Use of microscope courtesy of Dr. M. Costea, department of Biology, Wilfrid Laurier University.

leghaemoglobin; see Figure 2.2 and Bisseling *et al.* 1978), their location on the root system, and their dry weight were used to assess symbiotic status. Nodules were excised from the roots using a razor blade and placed in pre-weighed Eppendorf Tubes®, then dried as per other plant tissues (see §2.2). To assess the efficiency of the symbiotic association, plant return on nodule construction cost (host total dry weight/nodule dry weight; Oono and Denison 2010), specific nodulation (number of nodules/host root dry weight; Gulden and Vessey 1998) and specific nodule dry weight (nodule dry weight/host root dry weight; Gulden and Vessey 1998) were calculated. Plant return on nodule construction cost gives an estimate of how much plants were able to grow based on the amount of carbon they invested in nodules. Specific nodulation and specific nodule dry weight each indicates how numerous and how large nodules were, respectively, when the overall size of the root system is taken into account.

2.4 SRC, nodulation, and plant nutrition

Of further interest in the assessment of SRC's impact on the rhizobia-legume symbiosis is the nitrogen status of the plant. Symbiotic rhizobia are capable of fixing atmospheric nitrogen into ammonia (Lodwig *et al.* 2003) which is made available to plants for their nutrition. Determining the nitrogen status of a nodulating plant in absence of externally-provided nitrogen therefore provides an indirect indication of how much nitrogen is taken up by the plant as a result of the symbiosis.

Four treatment groups were used for these experiments and these were grown as per those in §2.3: $RSRC^-$ as a positive symbiotic control, $NSRC^+$ as a positive nitrogen control, N^-SRC^+ as a negative nitrogen control provided with 1:10 SRC but no nitrogen sources, and $RSRC^+$ as a symbiotic SRC treatment. Watering was done as per §2.3, with N^-SRC^+ treatment plants receiving only water. Each treatment group consisted of eight plants which were harvested at

24DAI/28DAP over one trial. Two additional 17DAI/21DAP trials were also conducted to examine chlorophyll differences over time, with the same groups and number of plants as above.

Plant nitrogen was estimated by levels of leaflet chlorophyll (Guinel and Sloetjes 2000) and nitrogen levels were quantified with dried shoot nutrient content determination (carried out by Actlabs, Agricultural division, Ancaster, ON). Following the removal of the plant from the soil (as per §2.2), the compound leaves at nodes four (fully developed) and six (recently developed) were separated from the parent plants. Leaflets from the same node and treatment group were pooled, and 0.5g of tissue (fresh weight) from each pool was homogenized in 80% acetone (Guinel and Sloetjes 2000) using a mortar and pestle. As pooling invariably resulted in more than 0.5g of fresh material available, consecutive samples were taken from each pool until material was depleted and these were considered as replicates. After homogenization, 3mL of the homogenate were diluted with 1mL of 80% acetone in a test tube, and 1.5mL of the dilution transferred to an Eppendorf Tube®. Samples were centrifuged for 10mins at 2500rpm (Porra *et al.* 1989), and then the supernatant was transferred to a quartz cuvette and its absorbance was read at 470, 647, 664 and 710nm using a Cary 50 UV-Vis spectrophotometer (Varian, Inc. Mississauga, ON). Absorbance readings were converted into mg chlorophyll per g fresh weight tissue using the Lichtenthaler equations (Lichtenthaler 1987). Dried shoot tissue (minus node four and six compound leaves) were weighed and the shoots were then divided equally into two samples per treatment group. These samples were sent to Actlab Laboratories for nutrient content analysis. The number of nodules present on the root systems was recorded prior to drying.

2.5 Statistical analyses

All statistical analyses were completed using the R software suite (version 3.2.1; http://www.r-project.org/) with the 'lme4', 'proto', 'multcomp' and 'lmerTest' library packages

as well as the Microsoft Excel software (2007). Measurements of treatment plants were compared to those of control plants using a one-way mixed-model ANOVA with replication as random effect to account for between-trial sources of variation. When treatments were shown to be a significant source of variation (95% confidence level), a Tukey Honest Significant Difference (HSD) post-hoc test was used to identify treatment-specific differences. Normality was tested using the Shapiro-Wilks test on residuals extracted from each ANOVA model. In instances of non-normality, the quantile-quantile plots and histograms for the data were examined to visualize the distribution of the data and identify any obvious outliers for removal. When necessary, the data were log-transformed to be normalized and the above analyses repeated. Statistical analyses were not undertaken on node counts because of the low variation between and within treatments.

Section 3: Results

3.1 Plant growth

Seedling establishment normally occurred three to four days after planting. All plants were typically flowering by 28DAP, and they completed their life cycles within approximately 75DAP. Under the growth conditions used, untreated, non-inoculated *P. sativum* plants usually had 3.5, 6.7 and 9.2 nodes at seedling, vegetative and reproductive stages (Table 2.1), respectively, and had produced seven to eight seeds in two or more pods per plant at 42DAP. Although initially beneficial in assisting seedling establishment, the continued use of polyethylene pot covers appeared to induce branching of the shoot across all treatments and controls with sometimes up to 60% of plants in a treatment group displaying a branching phenotype. Branches were observed developing most frequently from nodes two and three and though usually producing only a single extra compound leaf, in some instances two to four new nodes developed off of the new branch. As all plants were subjected to similar conditions, these

Table 2.1: Number of nodes, given as a mean ± standard error ($n \geq 10$), of plants harvested 9, 21 and 42 days after planting (DAP) with or without SRC administered at different concentrations. Because differences in node number between treatments were minimal at each time point, statistical analyses were not conducted.

	Nodes		
	9DAP	**21DAP**	**42DAP**
Control	3.5 ± 0.2	6.7 ± 0.3	9.2 ± 0.3
1:20	3.6 ± 0.2	7.1 ± 0.2	9.5 ± 0.2
1:10	3.8 ± 0.1	7.4 ± 0.3	9.5 ± 0.3
1:5	3.7 ± 0.1	7.2 ± 0.3	9.6 ± 0.2

plants were not excluded from the data set unless they displayed stunted growth. In instances of branching, the additional nodes developed on the extra stem were included in the node count for that particular plant as the node number was used as an indicator of plant growth, and the plants had developed these nodes, just in different areas. A model describing the development of the branching phenotype and the influence of polyethylene is given in Appendix B. Given the near-complete cessation of the branching phenotype when plastic covers were not used, the explanation for the branching phenotype was considered to be from the mechanisms in the above-mentioned model and not from any experimental treatment. Plastic covers were therefore not used for the fourth SRC/rhizobia trial, all of the SRC soil microbiota experiments, and the SRC/nitrogen experiment.

3.2 Optimal SRC concentration

Growth of *P. sativum* (cv. Sparkle) plants was supported with no visible nutrient-deficiency symptoms at all tested ratios of SRC:soil but only when plants were provided with supplemental nitrogen. The addition of SRC resulted in significant changes in plant growth across seedling (9DAP) and vegetative (21DAP) stages, but these changes were not observed in plants in the reproductive stage (42DAP). The number of nodes did not differ between plants at any time point (Table 2.1).

At 9DAP, only seedlings in the 1:5 treatment group produced significantly more total biomass (248 ± 20 mg) than seedlings in the control (203 ± 12) or other treatment groups (1:20 with 210 ± 11 mg and 1:10 with 201 ± 10mg). At 21DAP, plants in all treatments except for the 1:20 treatment group invested significantly more biomass in their root systems than those in the control group, although all treatment and control plants had similar shoot dry weights (Table

Table 2.2: Growth characteristics of plants at the vegetative stage (21DAP) which had been given various concentrations of SRC. The shoot dry weight (SDW), root dry weight (RDW), and shoot:root (S:R) ratio is given as mean ± standard error ($n \geq 10$). Superscripted letters indicate either no significant differences (same letter) or significant differences (different letters; mixed model ANOVA + Tukey HSD post hoc test at 95% confidence level) between treatment groups in that column.

	SDW (mg)	**RDW (mg)**	**S:R**
Control	236.7 ± 34.2^{a}	198.7 ± 52.0^{a}	1.41 ± 0.15^{a}
1:20	234.5 ± 24.5^{a}	178.1 ± 17.4^{b}	1.30 ± 0.06^{a}
1:10	311.1 ± 34.1^{a}	226.8 ± 20.1^{bc}	1.32 ± 0.07^{a}
1:5	285.6 ± 39.5^{a}	258.9 ± 28.1^{c}	1.07 ± 0.07^{a}

2.2). Calculated shoot:root ratios (Table 2.2), indicative of the allocation of carbon to nitrogen in a plant, were also similar at this time. During the vegetative stage, plants treated with SRC invested approximately 1.3x more biomass in shoots than in roots. At the reproductive stage (Table 2.3), investment in shoots appeared to increase with increasing SRC amounts; however, this trend was not significant. At this stage, no significant change in root or shoot dry weights was observed between treatment and control plants (Table 2.3). By this stage, plants across all treatments had nearly doubled their root dry weights and quadrupled their shoot dry weights compared to those plants at the vegetative stage. Interestingly, in the reproductive stage the S:R ratio of plants in the 1:5 SRC treatment group tended to be slightly lower than those in other treatments and control (Table 2.3), perhaps indicating impaired growth, although this was only significantly so when compared to plants in the 1:20 treatment group. Although root system sizes still tended to be increased with the addition of SRC, the large variation in the measured values means these differences were not significant. No significant changes in pod dry weight, number of pods, or number of seeds per plant were noted (Table 2.4).

The addition of even small amounts of SRC caused significant increases in soil pH, causing it to rise by 1.5-2.0 units over control soils at all measured time points (Figure 2.3). Additionally, changes in soil pH over time were observed for both treated and untreated soils (Figure 2.3). Soils without SRC showed a constant decrease in pH across the different time points, but the soils with SRC had more variable pH values over time. The pH of 1:20 soils increased from 9-42DAP, those of the 1:10 soils decreased, and those of the 1:5 soils first decreased from 9-21DAP then increased from 21-42DAP.

A distinct pattern in the 42DAP soil microorganism counts was observed, where a statistically-significant two-fold increase in the number of CFU per gram of soil was seen at the 1:10 concentration. The heterotrophic microorganism counts of the other tested concentrations were not significantly different from those of control (Figure 2.4). Because of the colony growth

Table 2.3: Growth characteristics of plants at the reproductive stage (42DAP) which had been given various concentrations of SRC. The shoot dry weight (SDW), root dry weight (RDW), and shoot:root (S:R) ratio are given as mean ± standard error ($n \geq 15$). Superscripted letters indicate either no significant differences (same letter) or significant differences (different letters; mixed model ANOVA + Tukey HSD post hoc test at 95% confidence level) between treatment groups in that column.

	SDW (mg)	**RDW (mg)**	**S:R**
Control	1071.9 ± 93.7^{a}	359.2 ± 18.1^{a}	3.02 ± 0.23^{ab}
1:20	1170.9 ± 120.9^{a}	337.1 ± 22.3^{a}	3.41 ± 0.21^{a}
1:10	1227.4 ± 93.4^{a}	405.0 ± 22.2^{a}	3.02 ± 0.14^{ab}
1:5	1209.9 ± 91.2^{a}	441.0 ± 30.4^{a}	2.78 ± 0.13^{b}

Table 2.4: Reproductive stage parameters used to estimate plant yield at 42DAP for optimal SRC concentration determination. Pod dry weight per plant (PDW), number of pods (Pods), and number of non-aborted seeds (Seeds) are given as means ± standard error ($n \geq 15$). Superscripted letters indicate no significant differences (same letter; mixed model ANOVA + Tukey HSD post hoc test at 95% confidence level) between treatment groups in that column.

	PDW (mg)	**Pods**	**Seeds**
Control	718.3 ± 52.1^{a}	2.6 ± 0.2^{a}	7.7 ± 0.6^{a}
1:20	734.5 ± 87.2^{a}	1.9 ± 0.2^{a}	6.1 ± 0.9^{a}
1:10	793.0 ± 72.7^{a}	2.2 ± 0.2^{a}	7.1 ± 0.6^{a}
1:5	925.3 ± 83.9^{a}	2.0 ± 0.1^{a}	8.3 ± 0.4^{a}

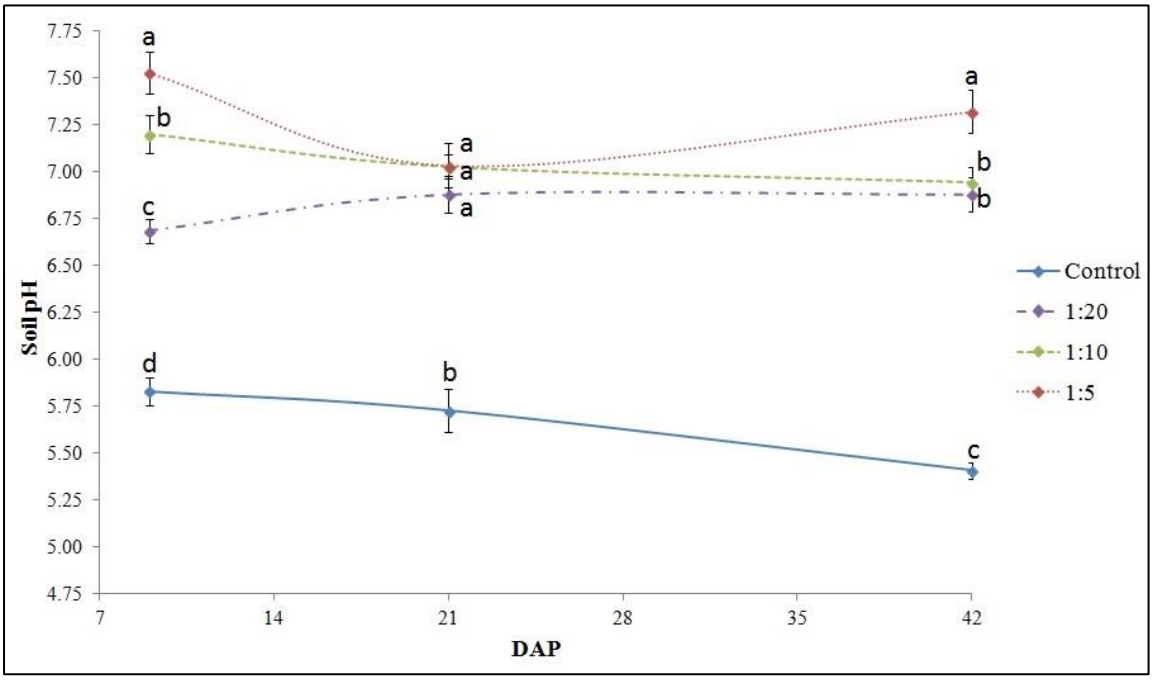

Figure 2.3: Variation in soil pH over time depending on SRC treatment. Soil pH was measured at 9, 21 and 42 DAP, and is shown as a mean ± standard error ($n \geq 15$). Significant differences between treatment groups at each time point are indicated by different letters (mixed model ANOVA + Tukey HSD post hoc test at 95% confidence level), whereas non-significant differences are indicated by identical letters.

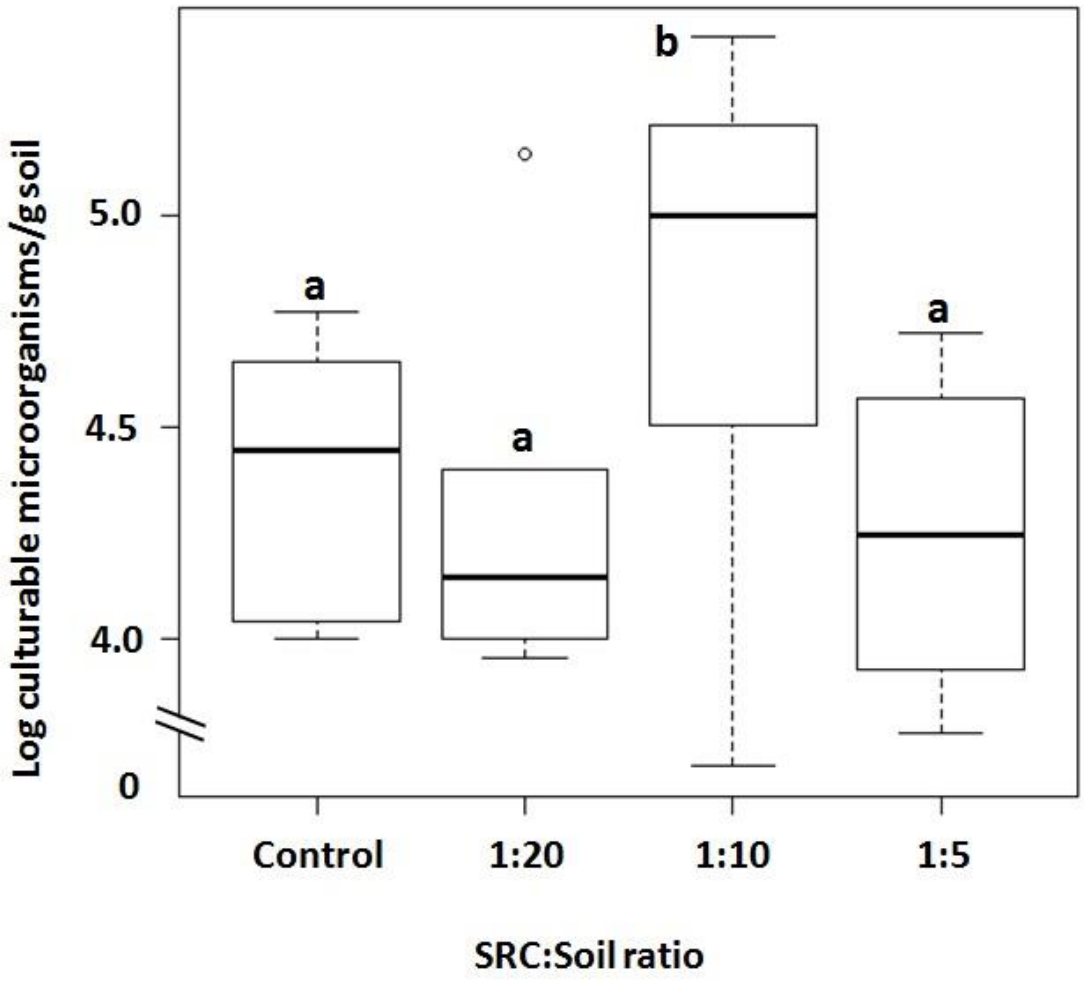

Figure 2.4: Colony-forming-units per gram of fresh soil collected at 42DAP. Counts chosen for the analysis were taken at 48h after samples were plated. Superscripted letters indicate either no significant differences (same letter) or significant differences (different letters; mixed model ANOVA + Tukey HSD post hoc test at 95% confidence level) between treatment groups. The circle indicates an outlier defined as being greater than 1.5x the interquartile distance for that treatment group.

patterns, only the 10^{-1} dilution at 48h after plating was analysed over the other concentrations and time periods. In the other dilutions and time periods, colony growth was either insufficient (<30 colonies per plate) or excessive (<300 colonies per plate) with the latter resulting in colonies coalescing together (Figure 2.1).

Because of the equivalent or greater growth/yield, the neutral soil pH and the dramatic increase in soil microorganism counts, the 1:10 ratio of SRC:soil was considered as the optimal concentration and used in all further experiments.

3.3 Enhancement of plant growth and nodulation with SRC treatment

The supplementation of soils with SRC had a dramatic effect on nodulation. An almost two-fold increase in the number of nodules was observed in 1:10 SRC-treated plants when compared to non-treated controls (Table 2.5).This translated into a significant increase in the total nodule biomass, although individual nodule dry weights were similar between SRC-treated and non-treated plants. Non SRC-treated plants only nodulated within the upper 10cm of the root system (Figure 2.5a), as is expected from the *P. sativum*/*R. leguminosarum* combination (Remmler *et al*. 2014). The increase in nodule number of SRC-treated plants is likely due to the unexpected development of a second nodulation zone that was observed in the lower 10cm of the roots of many SRC-treated plants (Table 2.5; Figure 2.5c).

The three measures of nodulation efficiency, plant return on construction cost, specific nodule dry weight, and specific nodulation indicated higher efficiencies in $RSRC^{+}$ plants than in $RSRC^{-}$ plants (Table 2.6), though only the first two parameters were significantly different. A lower plant return on construction cost indicates that SRC-treated plants were able to gain more biomass from their investment in the symbiosis than those plants without SRC, and a higher

Table 2.5: Nodulation parameters of inoculated, 28 day-old plants grown either without SRC ($RSRC^-$) or with SRC ($RSRC^+$). Values are means ± standard error ($n \geq 20$). Upper and lower nodules are defined as those formed in the upper or lower 10cm of the root system. Individual nodule dry weight (DW) per plant is calculated by dividing total nodule dry weight by total nodule number. Significant differences between treatments are indicated by superscripted differing letters (mixed model ANOVA + Tukey HSD post hoc test at 95% confidence level).

Treatment	Upper Nodules	Lower Nodules	Total Nodules	Total Nodule DW (mg)	Indv. Nodule DW (mg)
$RSRC^-$	74 ± 7.7^a	0 ± 0.0^a	74 ± 7.7^a	28.5 ± 2.4^a	0.469 ± 0.078^a
$RSRC^+$	87 ± 11.4^a	45 ± 14.4^b	132 ± 8.9^b	48.4 ± 4.3^b	0.393 ± 0.037^a

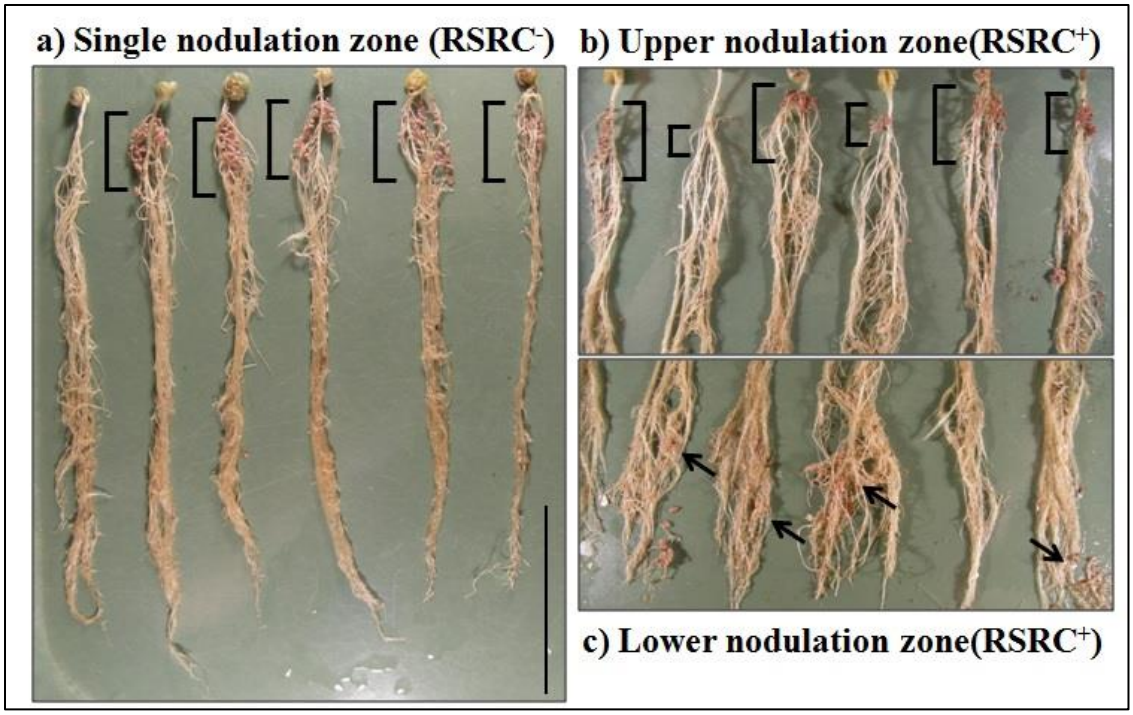

Figure 2.5: Comparison of nodulation zones in non SRC-treated ($RSRC^-$) and SRC-treated ($RSRC^+$) 28 day-old plants which were inoculated with *Rhizobium leguminosarum* bv. *viciae* 128C53K. Only a single nodulation zone (indicated by brackets) developed on the roots of inoculated plants without SRC (a). The plant on the far left of panel (a) did not develop any nodules. On $RSRC^+$ plant roots, an upper nodulation zone (b) was developed and was followed by a lower nodulation zone developed near the bottom of the root system (c). All plant root systems were approximately 30cm long, and the scale bar is 10cm.

Table 2.6: Calculated rhizobia/legume efficiency values for no-SRC ($RSRC^-$) and SRC-treated ($RSRC^+$) pea plants at 28DAP. Plant return on construction cost indicates how much the plant is able to grow given its investment in nodulation, whereas the specific nodule dry weight and specific nodulation are two ways of showing investment in nodulation based on the size of the root system. Higher plant return costs signify a less-efficient symbiosis, whereas higher specific dry weights and specific nodulation point toward a more-efficient symbiosis (Macdonald 2011). Values are a mean ± standard error ($n \geq 14$), and significant differences between treatments are indicated by superscripted differing letters (mixed model ANOVA + Tukey HSD post hoc test at 95% confidence level).

Treatment	Plant return on construction costs	Specific nodule dry weight	Specific nodulation
$RSRC^-$	17.73 ± 1.07^a	0.34 ± 0.02^a	1015.30 ± 160.09^a
$RSRC^+$	13.57 ± 0.62^b	0.46 ± 0.03^b	1270.32 ± 124.36^a

Table 2.7: Growth parameters of 28DAP plants that were either inoculated with rhizobia ($RSRC^-$) and not given SRC, inoculated and given SRC ($RSRC^+$), or not inoculated but given SRC and nitrogen solution ($NSRC^+$). Mean ± standard error values are given (n=7 for $NSRC^+$ and $n \geq 21$ for remainder). Sample size is smaller in $NSRC^+$ treatment due to rhizobial contamination of plants in that treatment group; the contaminated plants were not included. $NSRC^+$ is included as a non-rhizobial control to examine pattern of root/shoot growth with SRC as root dry weight is affected by SRC. Significant differences between treatments are indicated by superscripted differing letters (mixed model ANOVA + Tukey HSD post hoc test at 95% confidence level).

Treatment	Shoot Dry Weight (mg)	Root Dry Weight (mg)	Number of Nodes
$RSRC^-$	405.4 ± 28.3^a	83.3 ± 5.7^a	10 ± 0.3^a
$RSRC^+$	537.6 ± 32.8^b	106.5 ± 7.8^b	12 ± 0.5^a
$NSRC^+$	528.1 ± 54.5^b	171.4 ± 9.2^c	11 ± 0.8^a

specific nodule dry weight indicates that, when the size of the root system is accounted for, SRC-treated plants had more nodule biomass than the plants that were not given SRC. Although the three measures suggest increases in symbiotic efficiency, $RSRC^+$ plants did not grow larger than $NSRC^+$ plants. However, both $RSRC^+$ and $NSRC^+$ plants had higher root and shoot biomasses than $RSRC^-$ plants (Table 2.7).

3.4 SRC, nodulation, and plant nutrition

Three distinct trends were noticed during investigation of the effect of combined SRC/rhizobia on plant chlorophyll amounts, the most important of which regards chlorophyll b. First, regardless of treatment, age of plants, or node, the chlorophyll a (chl a) levels were constantly around 0.6mg/g of fresh tissue weight (Figure 2.6). Second, chlorophyll b (chl b) was the pigment most affected by the nitrogen treatments, though its concentration was typically around 0.7-0.8mg/g of fresh tissue weight. In all time points/nodes, the plants given no nitrogen sources had the lowest chl b levels, whereas the plants in the nitrogen and rhizobia treatments had similar chl b levels (Figure 2.6). One explanation for the effect of available nitrogen on chl b is that, under conditions of nitrogen deficiency, chl a levels are maintained at the expense of chl b levels. Chl b is a presumed accessory pigment that acts to transfer light energy to the primary photosynthetic pigment chl a (Taiz and Zaiger 2006). This is reflected in the biosynthetic and catabolic pathways of chl b: chl b can only be synthesized from chl a, and when it is broken down it is first transformed into chl a (Rüdiger 2002; Hörtensteiner and Kräutler 2011). When nitrogen is limiting, the plant must balance photosynthetic nitrogen need with metabolic nitrogen need. It is likely that, under N-limiting conditions, chl b is broken down to yield nitrogen and chl a is kept to maintain the photosynthetic capability of the plant. Third, the xanthophylls/carotenoids were generally not produced in high enough quantities to be detected except in the N^-SRC^+ treatment (Figure 2.6). An exception to this exists in node 4 at 28DAP, where xanthophyll/carotenoid levels were similar in N^-SRC^+ plants and $RSRC^-$ plants.

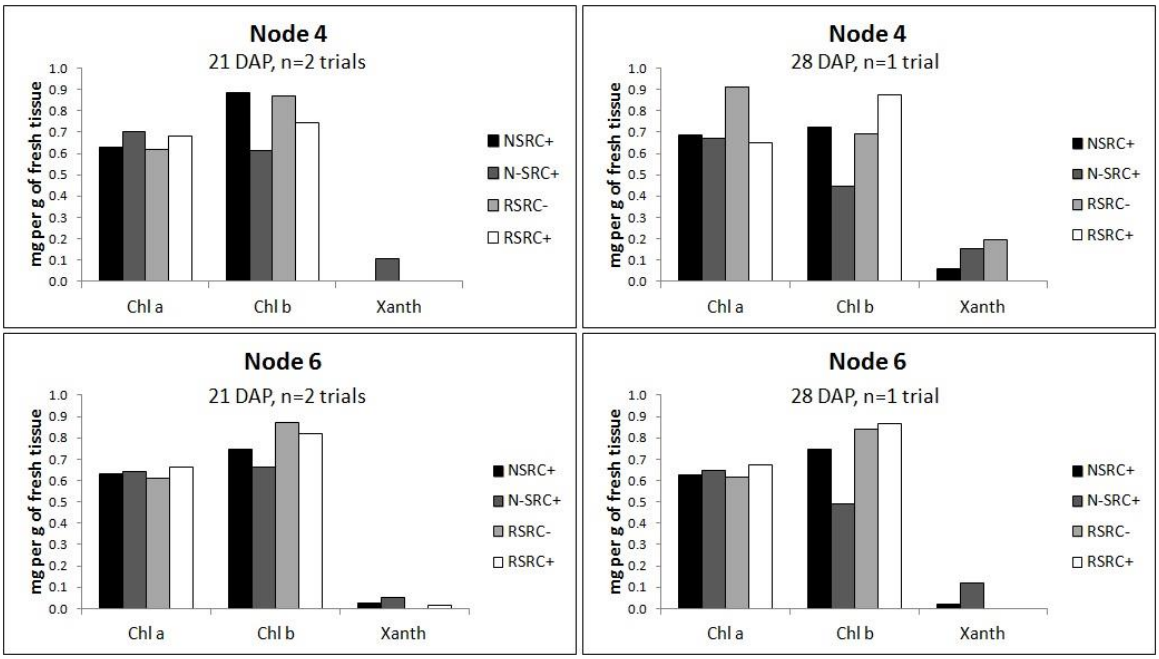

Figure 2.6: Mean levels of chlorophyll a (Chl a), chlorophyll b (Chl b), and xanthophyll/carotenoids (Xanth) from fully developed (node 4) and more-recently developed (node 6) leaflets. Four treatment groups were examined at both 21 and 28 days after planting (DAP): plants given a nitrogen solution and SRC ($NSRC^+$), plants given SRC but no nitrogen (N^-SRC^+), plants inoculated with rhizobia ($RSRC^-$) and without SRC, and plants inoculated with rhizobia and given SRC ($RSRC^+$). Because of small trial numbers, statistical analyses were not conducted. Measurements were taken from at least 8 plants per trial for each treatment, and were averages of 2-4 measurements per node per treatment.

The observed xanthophyll/carotenoid trends fit well with the role of these pigments as photo-protectants to dissipate excess light under low nitrogen conditions (Logan *et al*. 1999) and/or during leaf turnover to maximize nutrient reallocation from senescing leaves (Hoch *et al*. 2001).

The quantity of shoot nutrients was assessed in two different comparisons: nutrient levels between inoculated plants with and without SRC to show the effect of SRC addition, and between inoculated and non-inoculated plants given SRC to show the effect of nodulation. In the first comparison showing the effect of SRC addition, nodulated plants without SRC ($RSRC^-$) had significantly higher concentrations of N, P, S, Fe, B, and Zn, whereas only Ca levels were significantly higher in nodulated SRC-treated plants ($RSRC^+$; Figure 2.7). The K, Mg, Na, Al, and Mn amounts were not affected by SRC treatment. Therefore, except for Ca, plant macronutrient levels were higher when inoculated plants were provided with a low-N chemical fertilizer than when plants were given only SRC. In the second comparison showing the effect of nodulation on nutrient levels of SRC-treated plants, a curious difference is apparent. The levels of three nutrients, including nitrogen, were significantly different between $RSRC^+$ and $NSRC^+$ plants, indicating an effect of inoculation on plant nutrition (Figure 2.7). On one hand, the N and K shoot concentrations were significantly higher in shoots of $RSRC^+$ plants than in those of $NSRC^+$ plants. On the other hand, Zn was higher in $NSRC^+$ plants. The shoot levels of copper were also examined for all plants; however, concentrations were often below the detection limit (<5ppm) and so were not included in the analysis.

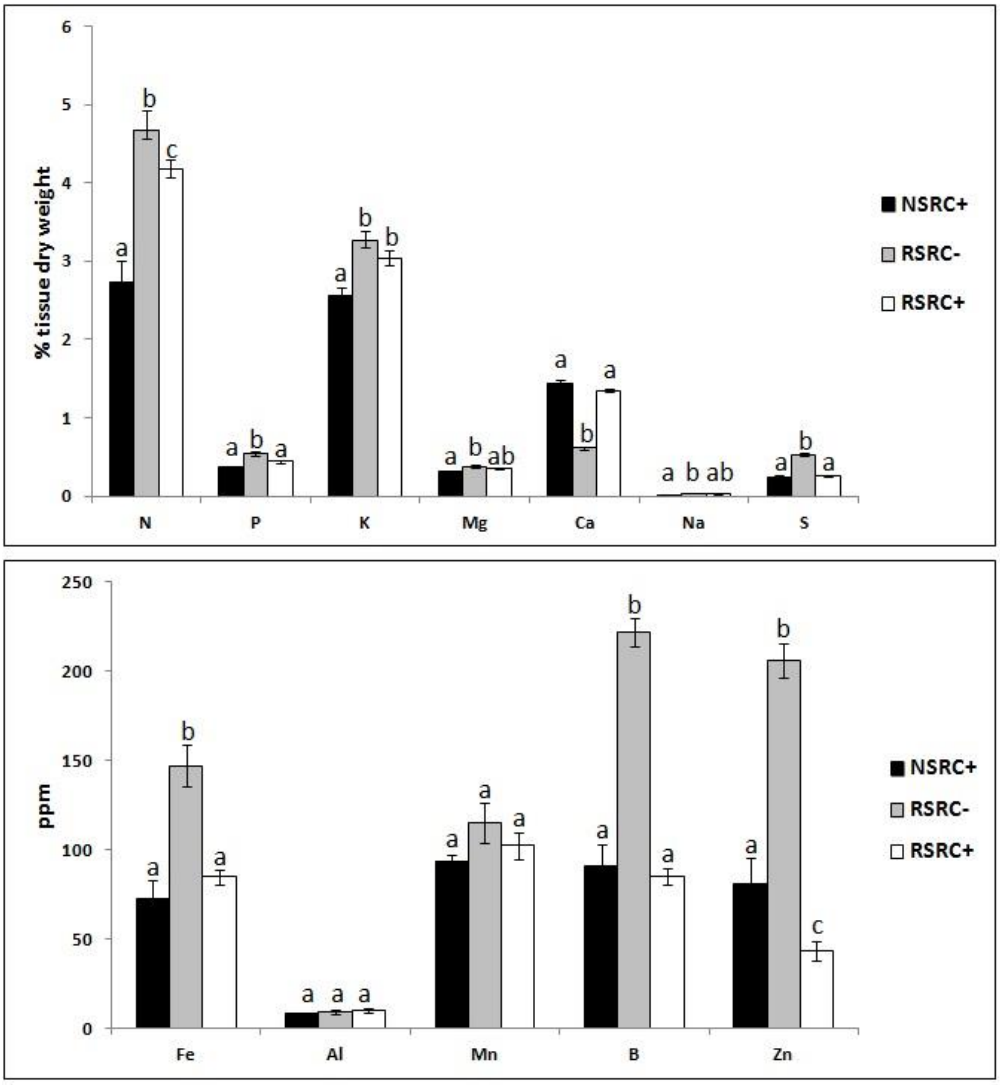

Figure 2.7: Dried shoot nutrient-content levels from 28 day-old plants (averages with lines indicating standard error; n=4 for $NSRC^+$ due to rhizobial contamination, and n=6 for $RSRC^-$ and $RSRC^+$, over two trials). Three treatment groups were examined: plants given a nitrogen solution and SRC ($NSRC^+$), plants inoculated with rhizobia ($RSRC^-$), and plants inoculated with rhizobia and given SRC ($RSRC^+$). Although copper was also examined, levels were typically below the detection limit (<5ppm) and these results were therefore not included. Significant differences between treatment groups for each element are given by different letters (mixed model ANOVA + Tukey HSD post hoc test at 95% confidence level), whereas non-significant differences are given by identical letters.

Section 4: Discussion

4.1 SRC supports plant growth

Here it is reported that SRC mixed with an artificial soil is capable of acting as a nutrient source for the growth of pea plants under laboratory conditions. With the addition of supplemental nitrogen or rhizobial inoculant, plants given SRC were able to complete their life-cycle with no visible nutrient deficiency symptoms, such as leaf yellowing/necrotic spots indicative of magnesium deficiency or leaf-edge scorching symptomatic of potassium deficiency. Plants with SRC had growth and yield similar to those plants given a full chemical nutrient solution. After testing several concentrations of SRC, it was found that the ratio of 1:10 SRC:soil (i.e., 5.42 kg/ ha) was the most promising for use in agricultural systems. Interestingly, this ratio is the one recommended by Boreal Agrominerals based on the stoichiometry of the mineral (John Slack, personal communication 2014).With this ratio, plant growth was slightly higher than that of control plants, the acidity of the soil was neutralized, and a significant increase in the number of culturable soil microorganisms was found. To demonstrate that SRC could be used as a nutrient source, externally-provided nutrient sources were minimized in these experiments by limiting SRC-treated plants to deionized water or nitrogen solution (prepared with deionized water). However, the Turface™ and vermiculite soil mixture could have weathered during the experiments, and thus the soil components cannot be excluded as potential sources of nutrients. Given their composition, Turface™ could have provided plants with aluminum and iron (Turface™ composition report 2013), and vermiculite may have released magnesium and potassium (Kalinowski and Schweda 2007).

Unlike typical chemical fertilizers which are processed to increase solubility of nutrients, SRC is not processed or chemically-activated (John Slack, personal communication 2014). Therefore, in our study, it is assumed that physical or biological weathering of SRC must

have occurred for nutrients to become available for plant growth. Because temperature and pressure were not altered in these experiments, the primary abiotic means by which mineral dissolution would have been promoted is soil solution acidity (Guidry and Mackenzie 2003; Kalinowski and Schweda 2007; Welch *et al*. 2010), though consistent watering may have contributed (Berner 1978; Palandri and Kharaka 2004). The dissolution of minerals is enhanced the most at low bulk solution pH (<5), well below the levels observed in treatment soils (Figure 2.3), and thus biological weathering mechanisms are suggested to have been the major contributors. Plant roots release compounds such as organic acids which can affect nutrient availability in the rhizosphere (Dakora and Phillips 2002; Bertin *et al.* 2003). Soil microorganisms can also increase solubility of nutrients by creating acidic microsites on the surface of minerals (Banfield *et al.* 1999). Cumulatively, these microsites help in providing nutrients to the soil solution. Some examples of increased nutrient availability from biological action include the release of K^+ and Mg^{+2} from biotite by bacteria and fungi (Hopf *et al*. 2009), the liberation of PO_3^- from apatite by mixed bacterial and fungal cultures (Welch *et al.* 2010), and the release of Ca^{+2} from calcite by *Burkholderia fungorum* (Jacobson and Wu 2009). However, microorganisms may also negatively affect the breakdown of minerals in some instances, as seen with *Shewanella oneidensis* biofilms which inhibit the dissolution of calcite (Lüttge and Conrad 2004). The minerals mentioned above (biotite, apatite, and calcite) are all major components of SRC.

4.2 SRC mediates a feedback loop between soil microorganisms and plant roots

In order for microbial breakdown of minerals to occur, soil conditions must be conducive to the growth of microorganisms. Growth of soil bacteria is often limited by carbon (Aldén *et al.* 2001; Demoling *et al.* 2007; Hobbie and Hobbie 2013) or co-limited by carbon and nitrogen (Demoling *et al.* 2007). Phosphorus can also be limiting under certain conditions such

as in fertilized or calcareous soils (Aldén *et al.* 2001; Griffiths *et al.* 2012). Soil microorganisms are capable of utilizing a wide variety of carbon sources, from carbohydrates and amino acids to aliphatic and aromatic compounds (Garland and Mills 1991; Campbell *et al.* 1997), many of which are exuded by plant roots. Root systems are major contributors to the soil carbon pool (Rasse *et al.* 2005) and it appears as though microorganism population structures are dictated by the exudate patterns of different plant species (Campbell *et al*. 1997; Grayston *et al.* 1998; Morgan *et al.* 2005).

The following is presented to explain the increased root growth observed upon addition of SRC to soils. In contrast to the nutrients of the chemical fertilizer solution, the nutrients within SRC were less accessible and had to be actively sought out by the plants through expansion of root systems. Root systems are developmentally 'plastic' and plants can selectively increase root growth to exploit high nutrient patches (Hodge 2004). All nutrients, with the exception of nitrogen that was added through irrigation, were homogenously distributed throughout the soil mixture, and a larger root system would have allowed plants to more effectively gather nutrients through increased contact with SRC.

I propose the following model to describe the mutually-beneficial interaction of microbes and plants; it is an expansion of a model presented by Banfield *et al.* (1999) describing the fungal-algal symbiosis within lichens and its influence on mineral weathering. The soil microorganism populations preferentially colonize the nutrient-rich SRC particles, which also contain the nutrients sought out by plant roots. When the root encounters the inhabited SRC particle, the carbon it exudes allows the local microorganism population to increase. The larger microorganism populations, provided they are not limited by available mineral surface area or other nutrients, are then able to break down SRC more rapidly, releasing nutrients that can be taken up by both microbes and plants. With improved nutrient uptake from the root, plant growth is promoted, and further expansion of the root system occurs. The

mineral-rich SRC particles thus act as a mediator between microorganisms and plants, and help to establish a positive-feedback mechanism between the two organisms. The effect of this positive feedback can be seen in the 1:10 SRC:soil treatments, where a larger root system likely provided sufficient carbon to remove the carbon limitation on the microorganism populations within the entire pot-bound soil system.

To test this model, a solution-based examination of microbial SRC dissolution could be performed. Microorganisms would be cultured in a media solution where the majority of nutrients are provided only as un-weathered SRC, then microorganism growth could be assessed both over time and with the addition of carbon compounds that mimic the natural exudates of roots. The demonstration that, when compared to abiotic weathering, the addition of microorganisms can enhance the dissolution of SRC would provide strong evidence to support the proposed model. This could be further expanded by measuring whether or not the microorganism-based dissolution is increased with the addition of carbon compounds. One evidence against this model is the decrease in both shoot biomass and culturable microorganisms despite root system increases in the 1:5 SRC:soil ratio. However, in that instance, the amount of SRC is double that of the optimal concentration, i.e., twice the amount of calcium is present in the soil system. This is reflected in the significantly increased soil pH at two of the three examined time points. The pH levels measured at this concentration are consistent with levels associated with a loss in soil microorganism biodiversity observed by Lauber *et al.* (2009), though without closer examination of the microbe-soil-plant system used here it is impossible to determine the mechanism by which SRC may inhibit microorganism and plant at high concentrations. Increases in soil moisture level can increase the number of culturable soil microorganisms (Lund and Goksøyr 1980), and this may also have played a role here. Because there was no positive trend linking soil microorganism counts with increasing amounts of SRC, I tentatively conclude that the promotion of soil microorganisms is the result

of the optimal SRC concentration, although I acknowledge that the combination of several other factors (e.g., high pH, water activity, etc…) may have been influential to some degree.

4.3 SRC enhances the efficiency of the rhizobia-legume symbiosis

Evidence that SRC can enhance mutually-supportive interactions between plants and microorganisms was also seen when plants were inoculated with rhizobia. Plants with SRC and rhizobia displayed significant increases in both root and shoot systems when compared to inoculated plants that were only given low-nitrogen solution (Table 2.7). The inclusion of SRC also resulted in a nearly two-fold increase in the number of nodules compared to control plants (Table 2.5). Part of the reason for this increase in nodulation is that a second nodulation zone was seen in SRC-treated plants, a situation that can occur in pea when plant growth requires more than the available reserves of nitrogen (Voisin *et al.* 2010).

Nodules are strong carbon sinks (Hacin *et al.* 1997), and supernodulating mutants where the autoregulation of nodulation is impaired have lower growth rates than wild type plants (Sagan and Duc 1993). This decrease in plant growth is also seen in wild-type plants inoculated with different strains of rhizobia because some rhizobia are more costly than others for the plant to partner with (Skøt 1983). Although a control treatment where plants were not given SRC or rhizobia was not used here, an approximation can be made to estimate the cost of nodulation on pea growth. Because no significant differences were seen between the shoots of non-inoculated plants grown with and without SRC, the SRC-treated, non-inoculated control can be used as a baseline to compare roughly the cost of nodulation on plant growth. With this approximation in mind, a decrease in the shoot dry weight is apparent between those plants given nitrogen and SRC and those inoculated with rhizobia and given no SRC (Table 2.7). What is especially curious is that there are no significant differences between inoculated ($RSRC^{+}$) and

non-inoculated plants given SRC ($NSRC^+$), i.e., there does not appear to be a cost of nodulation for the plant when SRC is part of the soil mixture.

Two non-mutually exclusive explanations are possible to describe the lack of nodulation cost: first, the efficiency of the symbiotic interaction is increased (more nitrogen gained for carbon invested) by the addition of SRC, and second, the plant is more photosynthetically active because of SRC addition and is thus able to better cope with the carbon loss associated with nodulation. Three calculations of symbiotic efficiency were performed to help distinguish between these two possibilities: plant return on construction cost, specific nodulation, and specific nodule dry weight. All three calculations indicated increases in the efficiency of the rhizobia-legume symbiosis in the presence of SRC, though only plant return on construction cost and specific nodule dry weight were significantly different from those of plants not given SRC (Table 2.6). These results suggest that plants grown with SRC gain more nitrogen per amount of carbon invested in nodulation (plant return on construction cost), and, when root system size is accounted for, produce larger nodules (specific nodule dry weight) than those growth without SRC.

It seems that the efficiency of nitrogen fixation may have been enhanced by the more nutrient-rich soil environment provided by SRC. Metal ions such as iron, copper, manganese, zinc, and nickel play important roles in nitrogen fixation (González-Guerrero *et al*. 2014) and so improved metal uptake could have affected the efficiency of the nodules on SRC-treated plants. Iron is an integral part of both the nitrogenase enzyme complex responsible for fixing atmospheric nitrogen (Scott *et al*. 1983) and the protein leghaemoglobin that minimizes the detrimental effect of oxygen on nitrogenase (Harutyunyan *et al.* 1995). Copper is a cofactor of rhizobial cytochromes involved in energy metabolism (Seliga 1993) and, along with manganese and zinc (Rubio *et al.* 2007), is part of the superoxide dismutase complexes that alleviate the amount of free radicals produced during nitrogen fixation. However, the aforementioned metals

were not found to be in higher concentrations in the shoots of SRC-treated inoculated plants than in those of non-SRC treated plants making it difficult to establish a direct connection; however, the levels of metals in the root were not examined. The shoot concentrations of zinc were significantly lower in inoculated, SRC-treated plants than in non-inoculated SRC plants, and this metal may have been preferentially allocated to the root systems to benefit nodulation. Aside from plant return on construction cost, the other measure of nodulation efficiency that was significantly different in SRC-treated plants was that of specific nodule dry weight which indicated that in those plants more photosynthetic carbon was available to be invested in larger nodules than in non SRC-treated plants. Although nitrogen levels were higher in the shoots of plants that were not given SRC, overall chlorophyll levels between SRC-treated and non SRC-treated plants were similar, implying equivalent photosynthetic capabilities between plants in both treatments. It is known that carbon investment in nodulation is positively linked with the plant's need for nitrogen and available carbon (Voisin *et al.* 2010). When this is taken together with the presence of a second wave of nodulation in SRC-treated plants, it suggests that SRC-treated plants were nitrogen-limited in their growth and yet had adequate carbon to allocate to symbiotic nitrogen production. Shoot nitrogen levels of inoculated plants in both treatments were higher than those of plants given only chemical nitrogen, which indicates that nodulation was capable of providing more than sufficient nitrogen to maintain plant growth. The shoot nitrogen levels may, however, simply indicate that plants provided with chemical nitrogen were nitrogen-limited, but not nitrogen-deficient.

Therefore, improved nitrogen efficiency and improved photosynthesis both likely explain the increase in growth. When nitrogen fixation efficiency is increased, more nitrogen is available to invest in photosynthesis and more photosynthetic carbon is produced. When growth is limited by nitrogen, this carbon can be allocated back to the rhizobia in the nodule, increasing the levels of nitrogen fixation.

4.4 Conclusions

Based upon the results obtained from these experiments, it is concluded that SRC can be used as a nutrient source for plants, at least under the conditions examined. Because pea growth is supported by SRC supplementation and enhanced with the combination of rhizobia/SRC in an artificial soil mixture, it is suggested that SRC could be a strong candidate for use in sustainable agricultural practices. However, caution must be employed in recommending the use of SRC due to its high levels of calcium and the effect this calcium has on soil pH. If the plants being grown are calcifuges, or the soils in question are alkaline, then SRC should not be applied. To properly take advantage of SRC in promising agricultural settings, further research that more closely examines the role of soil microorganisms, the effect of SRC on other rhizobia-legume pairings, and the nitrogen-fixation rates associated with those pairings are recommended. Additional areas for future experiments are the mechanism(s) by which SRC enhances nodulation, and the use of combined SRC plus organic/chemical nutrient sources, especially in a field setting. With knowledge of the various means by which SRC affects the microbe-soil-plant system, other agrominerals with similar stimulatory properties can be discovered and utilized.

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Chapter 3: Preliminary findings on how storage affects microorganisms in SRC

Section 1: Rationale

After SRC is harvested from the deposit, it is stored uncovered for a number of years, packaged, and then transferred to an unheated warehouse (Figure 3.1). Of note, SRC is never sterilized or pasteurized prior to sale (John Slack, personal communication 2014). Most soils have a diverse microbiome including arbuscular-mycorrhizal (AM) fungi (van der Heijden *et al.* 1998; Kernaghan 2005), nitrogen-fixing symbiotic bacteria (Guimaraes *et al.* 2012), and plant growth-promoting bacteria (PGPB; Kaplan *et al.* 2013). Because of this knowledge, we had to alter the initial hypothesis, that storage conditions do not affect SRC usefulness. Our new hypothesis is that SRC contains beneficial microorganisms, and that these microorganisms are negatively affected by the storage conditions of SRC. Given the importance of soil microorganisms to the potential usefulness of SRC in agriculture (chapter two), we thought it imperative to assess whether microorganisms are present in packages of SRC and to determine, if present, whether the microorganisms affect plant growth. Furthermore, all of the experiments conducted in chapter two of this thesis were completed using sterilized SRC and soils. Although this was necessary to minimize contamination and to maintain conditions as equal as possible between trials and treatments, the soil systems used do not accurately reflect agricultural conditions. Because of the above, I therefore conducted some preliminary experiments to examine the microorganisms within SRC and how these might be affected by storage conditions. These experiments were based around the following objectives, whi

1) To assess whether packaged SRC contains viable root symbionts and PGPB using pea as a trap plant

2) To determine whether the behaviour of root symbionts is altered in the steps between the harvesting and the sale of SRC

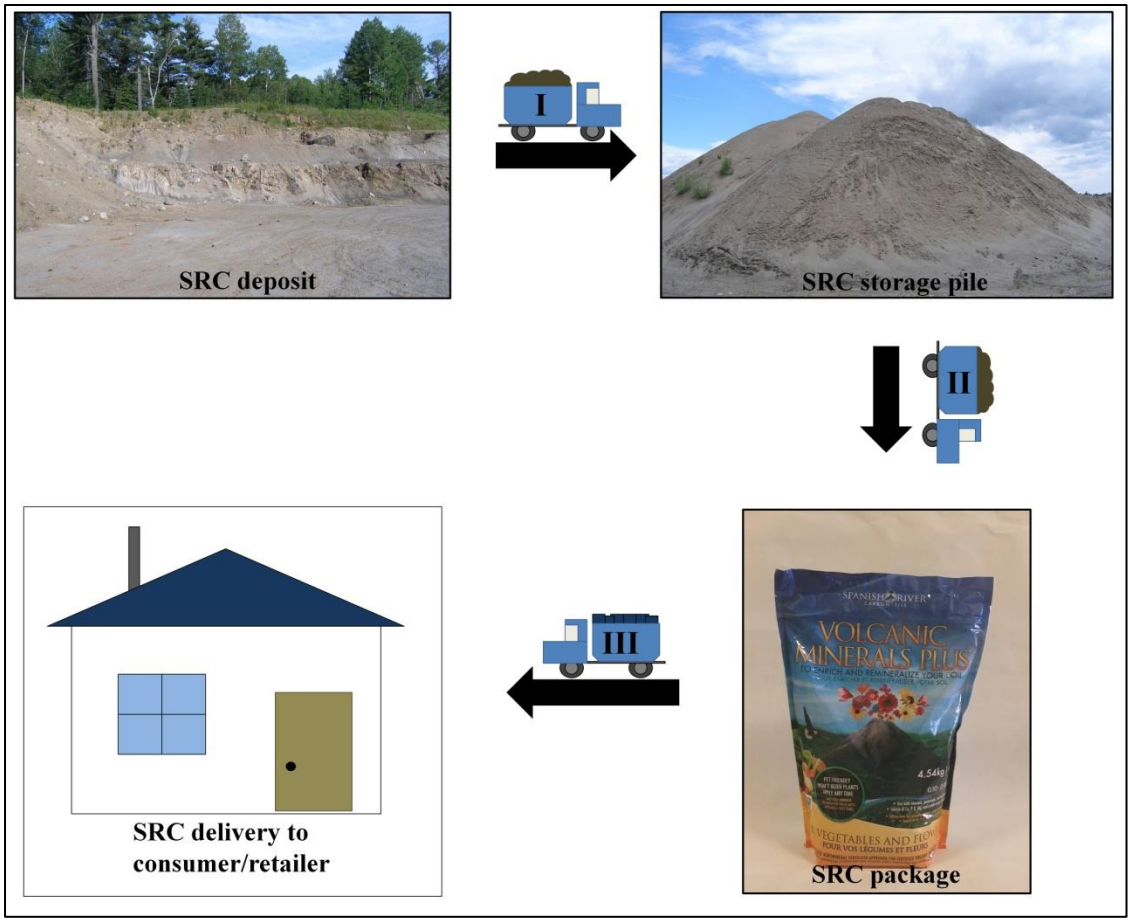

Figure 3.1: Overview of the SRC distribution procedure. Extraction of SRC from the deposit takes place only every few years as the remote location of the deposit limits mining. Therefore, large amounts of SRC are collected during each extraction and are kept as an uncovered, outdoor storage pile (I). SRC is then removed as-needed from the storage pile and packaged (II). Once in bags, it is kept in an unheated building until delivery to the distributor or consumer (III).

Section 2 Materials and methods

2.1 Trap pea plants grown with non-sterile SRC

2.1.1 Preliminary trial

A small preliminary trial was conducted using plants grown for 21 days after planting (DAP) in soils supplemented with 1:10 SRC. Two treatments, one with non-sterile SRC (P-NOST) and one with sterilized SRC (P-STER), were used. For each treatment, six plants were grown three to a pot in two pots (2624mL volume). Plants were grown in a 1:1 mix of vermiculite:Turface™ (as per §2.2). To fulfill plant nitrogen requirements, plants in both treatments were given 2.5mM NH_4NO_3 nitrogen solution as part of their watering. Only root/shoot dry weight, and nodule number were measured. Note that in this preliminary trial, I used a different bag of SRC than in the other trials in this chapter. Although the subsequent trials had slightly different conditions, the preliminary trial is included here to demonstrate how different bags of non-sterile SRC can lead to different results.

2.1.2 Main trials with non-sterile SRC

Pea plants were grown and the soils were prepared as per §2.2. Three experimental groups were used: one where both soil and SRC were autoclaved (STER), one where only the soil was autoclaved (NOST; SRC added after soil cooled) and one where sterile soil was used but plants were given chemical nutrients (CF) instead of SRC. All SRC was given in the recommended 1:10 SRC:soil ratio, and was from the same retail SRC package. This package was different from the one used in the preliminary trial. The STER group plants received water and N-solution, the NOST group plants received only water, and the CF group plants received water, nutrient solution, and chemical fertilizer (N:P:K, 17:5:19). Ten plants were harvested at 21DAP

to determine their shoot/root dry weights, number of nodes (chapter 2, §2.2), nodule numbers (chapter 2, §2.3), and number of colony-forming units (CFU) per gram of soil (chapter 2, §2.2). Six to seven (depending on survival) additional plants were left to seed (approx. 70 days). Seeds were collected once pods had dehisced, and were left to dry at room temperature for at least two weeks prior to weighing. Only fully formed seeds were collected, as in chapter 2, §2.2.

2.1.3 Assessing the colonization of trap plant roots by rhizobia and mycorrhizal fungi

The colonization of trap plant roots by rhizobia was assessed through the counting of nodules, the recording of the nodule locations, and the observation of basic nodule morphology. Only functional nodules, indicated by a red or pink colouration (Bisseling *et al.* 1978), were counted. Individual root systems were divided for nodule location record along the primary root according to distance from cotelydons: the 10cm distal from the cotelydons was defined as the "upper" area, the 10cm distal to that was defined as the "middle" area, and the last 10cm or more of the root was defined as the "lower" area. The upper area corresponds to where the single nodulation zone typical of rhizobia-pea associations is expected (Bollman and Vessey 2006; Macdonald 2011). Following nodule characterization, lateral roots were removed from the root systems and cut into 3cm segments. Of these, seven segments were randomly chosen to be examined for mycorrhizal colonization. The remaining lateral roots and the primary root were then dried for measuring the root biomass. The presence of mycorrhizal fungi in the root system was determined by microscopy on segments cleared by KOH and then stained using the ink-vinegar staining method (Vierheilig *et al.* 1998).

2.2 Assessing the rhizobial and mycorrhizal colonization of plants collected from the SRC deposit

Native plants that were growing on the SRC deposit were also assessed for root colonization by rhizobia and mycorrhizal fungi. This was done to estimate whether potential mutualistic microorganisms were actually present in the SRC deposit, and these plants were treated as a positive control to the trap plants grown above. During two visits to the SRC mining site, plant specimens from a variety of families were randomly collected. All collected plants were growing directly on exposed SRC. In addition, one legume was collected from the storage pile (see Figure 3.1).

2.3 Statistical analysis

The two main trials consisted of 20 vegetative growth plants plus ~12 plants that were left to seed per treatment, whereas the preliminary trial consisted of only 6 plants per treatment. Because of the small sample sizes of the preliminary treatment, statistics were not conducted on these data. Where applicable, treatments were compared using a one-way mixed-model ANOVA with replication as random effect as per chapter 2. No statistical analysis was conducted for data from the plant species collected from the mine.

Section 3: Results

3.1 Plant growth/yield and soil microorganism counts

No differences in shoot or root biomass allocations were found between STER and NOST plants, though roots in both of these treatments were significantly more extensive than those of CF plants (Table 3.1). In the preliminary trial treatments, plants grown with sterile SRC tended to be larger than those grown with non-sterile SRC as illustrated by their shoot and root

dry weights (Table 3.1). Curiously, the biomass of plants during the two main trials was different from that of plants assessed in the determination of optimal SRC (chapter 2). The root systems of plants in this set of experiments were nearly two times smaller than those of plants grown in the earlier set (chapter 2). However, despite the differences seen in roots, plant shoots were approximately equal between the two sets of experiments. The addition of SRC, regardless of sterilization, resulted in plants producing fewer seeds than plants given chemical fertilizer (Table 3.2). These fewer seeds were, however, significantly larger than those produced by plants given chemical fertilizer (Table 3.2). Plants with non-sterile SRC may have produced slightly more seeds than plants with sterile SRC; however, this needs to be confirmed with additional trials. At 21DAP, STER soils harboured nearly twice as many culturable microorganisms than NOST soils (Table 3.1).

Table 3.1: Growth characteristics of 21 day-old pea plants from all trials either given chemical fertilizer (CF), given sterilized-SRC and nitrogen solution (STER) or given only non-sterile SRC (NOST). For comparison, results from the preliminary trial treatment groups are included and prefaced with 'P-' (n = 6). The shoot dry weight (SDW), the root dry weight (RDW), and the number of nodes are given as a mean ± standard error ($n \geq 20$). The number of colony-forming units per gram of fresh soil is also included to indicate microbial abundance in soils supplemented with sterilized or non-sterilized SRC (n=10 plates). Superscripted letters indicate either no significant differences (same letter) or significant differences (different letters; mixed model ANOVA + Tukey HSD post hoc test at 95% confidence level) between treatment groups in that column. ND = not determined.

	SDW (mg)	**RDW (mg)**	**Nodes**	**CFU/g soil**
CF	255.6 ± 19.5^{a}	85.0 ± 6.9^{a}	8.5 ± 0.2	ND
STER	291.7 ± 23.4^{a}	117.3 ± 9.3^{b}	8.6 ± 0.2	4540 ± 254^{a}
NOST	290.9 ± 12.7^{a}	114.3 ± 6.1^{b}	8.2 ± 0.3	2250 ± 378^{b}
P-STER	326.3 ± 161.3	161.2 ± 125.2	ND	ND
P-NOST	315.7 ± 26.0	119.0 ± 9.2	ND	ND

Table 3.2: Yield characteristics from plants given nutrient solution and chemical fertilizer (CF), sterilized SRC and nitrogen solution (STER), or non-sterile SRC only (NOST). Individual seed dry weight is an average (± standard error) of between 51-103 seeds produced by 12-13 plants across two trials. Seeds per plant were calculated dividing the total number of seeds by the number of plants in that treatment and as such, no statistical analysis was conducted for this parameter. Superscripted letters indicate either no significant differences (same letter) or significant differences (different letters; mixed model ANOVA + Tukey HSD post hoc test at 95% confidence level) between treatment groups.

	Seed DW (mg)	**Seeds/plant**
CF	153.3 ± 4.8^{a}	6.4
STER	225.3 ± 7.6^{b}	3.9
NOST	227.4 ± 11.5^{b}	4.5

3.2 Symbiotic characteristics of non-sterile SRC trap plants and comparison with plants collected from the mine.

The 21DAP pea plants grown in non-sterile SRC formed nodules that were typically in the upper and middle portions of the root systems (5 ± 3 and 10 ± 3, respectively). No nodules were developed in the lower 10^{+}cm of the roots. The total number of nodules formed (15.0 ± 4.5) was low compared to that of the plants grown for the preliminary trial (111 ± 19) and to that of plants of the same age that had been grown in sterile SRC and had been inoculated with *Rhizobium leguminosarum* (140 ± 15 nodules at 17DAI; data not shown). The nodules formed on NOST plants in the two main trials displayed three different morphologies. In addition to "single" and "multilobed" nodules similar in their morphology to those seen earlier (Figure 2.2, chapter 2), a 'complex multilobed' type was observed (Figure 3.2a). These complex nodules were flatter and more fan-shaped than the other nodules. Additionally, there were sometimes nodules which were pale or green in colour, and without discernible meristems (Figure 3.2b).

A total of seven plants were collected from the SRC mine area: six from the SRC deposit and one from the SRC storage pile. Of these seven, most were mycorrhizal (Table 3.3); some examples are *Comptonia peregrina* (Figure 3.3a) and the Poaceae family grasses (Figure 3.3b). Neither the trap pea plants grown with non-sterile SRC nor the *M. alba* were colonized by mycorrhizal fungi, though both species are known to form mycorrhizal associations (Geil *et al*. 2001 and Lum *et al.* 2002, respectively). Because *C. peregrina* (family Myricaceae) is known to form nodules with actinorhizal bacteria of the genus *Frankia* (Family Frankiaceae; Benson and Silvester 1993), the roots of these plants were inspected for the presence of these nodules. Thus, two of the collected plant species, *M. alba* and *C. peregrina*, had numerous nodules that visually appeared to be functioning normally (see Figure 3.3c and d, respectively). Although the trap pea plants were poorly nodulated overall, the majority of these (15/20) had nodules on their roots. All six plants in the preliminary trial were nodulated, and the nodules were typical in appearance.

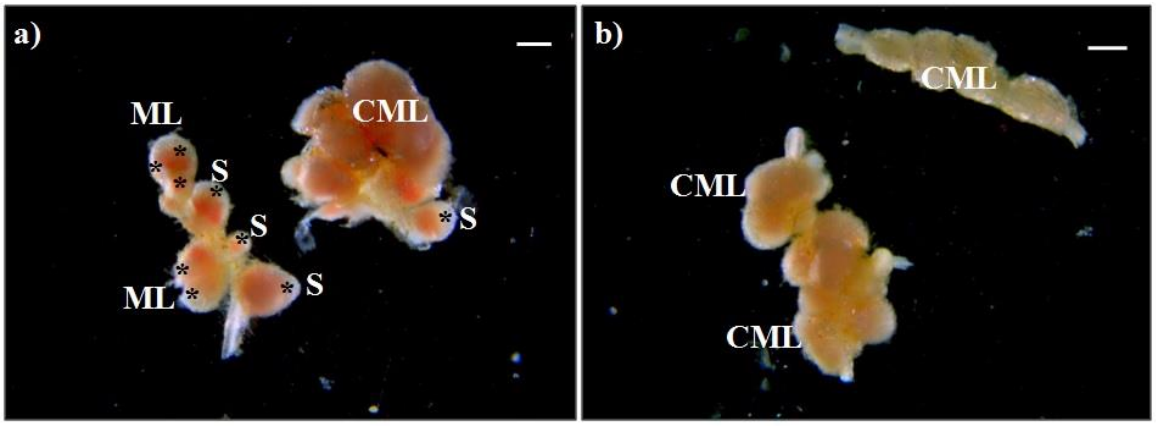

Figure 3.2: Photographs illustrating the nodule morphologies observed on pea roots grown with non-sterile SRC. Typically, pea forms single (a; S) or multilobed (a; ML) nodules. Single nodules have a cylindrical, elongated shape with a visibly-red leghaemoglobin core and lightly-coloured, terminally-positioned meristem (*). Multilobed nodules are characterized by having two or more meristems. Complex multilobed nodules (a and b; CML) were less elongate and more fan-shaped than the other nodule types, and in some cases appeared to be senescent. The complex nodules were often much lighter in colour than the other nodule types. All images were taken with a SMZ1800 stereomicroscope and the white scale bar represents 1mm. Use of microscope courtesy of Dr. M. Costea, Department of Biology, Wilfrid Laurier University.

Table 3.3: Symbiotic status of native plants randomly collected from the SRC sites and of trap plants that had been grown using non-sterile SRC (preliminary trial: P-NOST; main trials: NOST) in the growth room at Wilfrid Laurier University. For both mycorrhizal (Myc) and nitrogen-fixing symbioses (Nod), the symbiotic status of plants is given as the number of plants colonized by the appropriate micro-symbiont over the total number of plants examined.

	Growth/Collection details		**Symbiotic colonization**	
Plant type	**Location**	**Date collected**	**Myc**	**Nod**
Poaceae ssp.	SRC deposit	Aug 12/2014	1/1	NA
Poaceae ssp.	SRC deposit	June 25/2015	1/1	NA
Comptonia peregrina	SRC deposit	Aug 12/2014	1/1	1/1
Comptonia peregrina	SRC deposit	June 25/2015	1/1	1/1
Picea abies	SRC deposit	Aug 12/2014	1/1	NA
Melilotus alba	SRC deposit	Aug 12/2014	0/1	1/1
Melilotus alba	SRC storage pile	June 25/2015	0/1	1/1
P-NOST treatment group	Growth room	NA	0/6	6/6
NOST treatment group	Growth room	NA	0/20	15/20

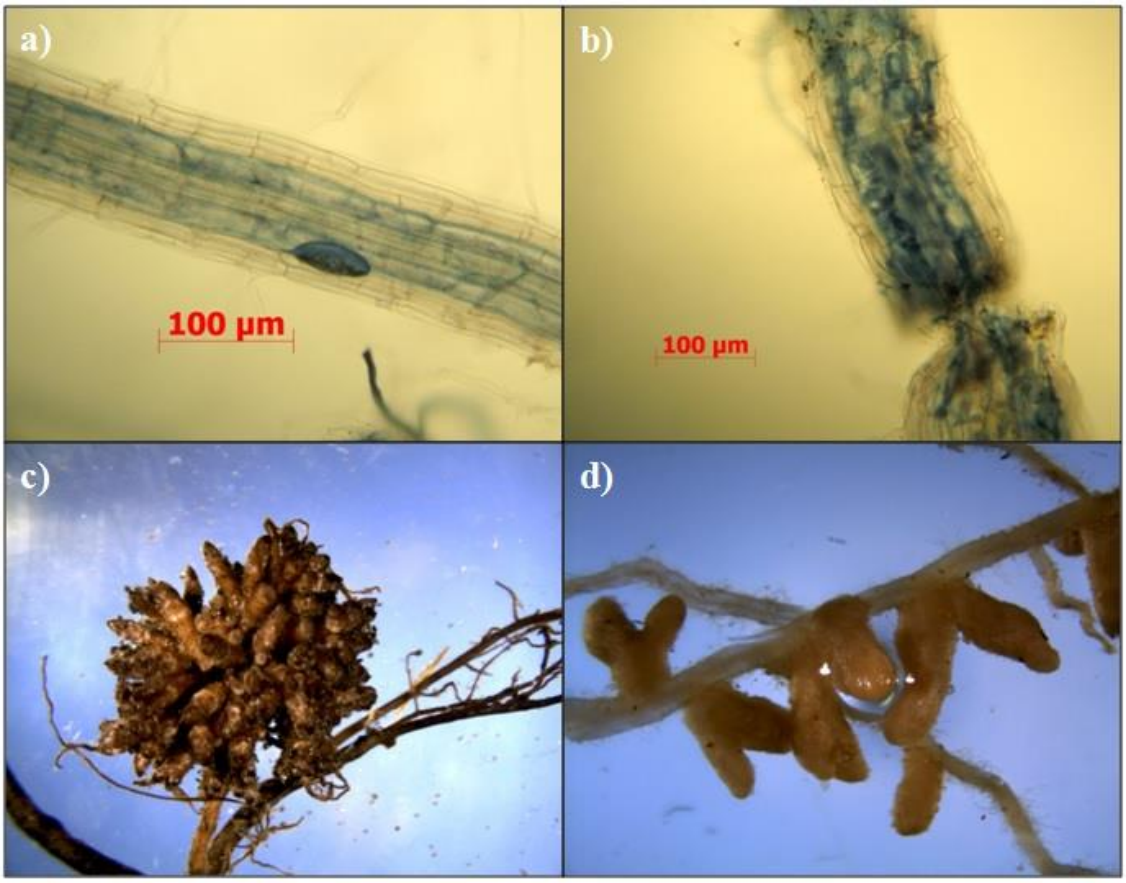

Figure 3.3: Photographs depicting root symbioses on roots of plants collected from the SRC mine. Mycorrhizal fungi (here stained blue) were found to be colonizing most of the collected plants, such as *Comptonia peregrina* (a) and Poaceae grass (b). Two kinds of nodules were also seen in collected plants: Actinorhizal nodules were observed on roots of *C. peregrina* (c), and rhizobial nodules on the roots of *Melilotus alba* (d). Photographs in c) and d) courtesy of Rajaa Alshikhy.

Section 4: Discussion

The two objectives of this study were to establish if packaged SRC contains viable root symbionts, and to determine if these microorganisms are lost between the harvesting and the sale of SRC. To accomplish this, pea plants were used as a trap for mycorrhizal fungi and rhizobia from non-sterile SRC. The colonization of these trap plants was assessed and characterized, and was compared to the colonization of plants that were collected from the SRC mine. The results obtained here indicate that packaged SRC does contain viable beneficial microorganisms which may be negatively affected by storage conditions, thus confirming our hypothesis regarding SRC storage. Compared to the various well-colonized plant species that were growing directly on the exposed SRC deposit, pea plants grown in the lab with non-sterile packaged SRC were inconsistently nodulated, were not colonized by mycorrhizal fungi, and had lower CFU/g soil than plants growing in soils with sterilized SRC.

4.1 Inconsistent nodulation of trap plants

The size of the nodulation zone in plants grown with non-sterile SRC was similar to that reported for the *P. sativum/R. leguminosarum* combination (Macdonald 2011; Bollman and Vessey 2006), with the majority of nodules forming less than 20cm from the cotelydons (<12.5cm - Macdonald 2011; 14cm - Bollman and Vessey 2006), though the nodulation zone here was only roughly resolved in 10cm increments. The nodules on the root system of trap peas were few, exhibited an unusual morphology, and had a generally lighter colouration than those on plants inoculated with *Rhizobium leguminosarum* bv. *viciae*. Furthermore, they also displayed signs of early senescence (green/pale colour, no visible meristems). In contrast, the nodules on the preliminary trial plants were an order of magnitude more numerous, displayed the expected morphologies, and were much darker in colouration. It should be noted here that when the SRC

was delivered, several bags were frozen solid, though unfortunately no note was made of which bags these were.

Despite the slightly different conditions used, the simplest explanation for the divergence in nodulation characteristics seen between preliminary trap plants and the full trial plants is that viable rhizobia were originally present in SRC, but were lost during storage before delivery. Both high (30°C or greater, Evans *et al.* 1993) and low (4°C or lower, Meade *et al.* 1985) extremes of temperature can impact the viability of *Rhizobium* cells, and unfavourable storage conditions (e.g., being frozen solid) are a prime suspect in the decreased viability seen here. Two alternative explanations must be proposed, however, but I think they can be discounted. First, the plants given non-sterile SRC in the preliminary trial could have been contaminated by *R. leguminosarum* from other experiments running at the same time in the growth room. This is doubtful though as no plants given sterile SRC ever nodulated in any trial. Second, the ammonium nitrate used in the preliminary trial could have affected the nodule numbers, as nodulation in pea has been shown to be stimulated by addition of ammonia (Bollman and Vessey 2006). However, in the 2006 study when ammonia was provided as ammonium nitrate, the number of nodules was not changed from the number seen on plants with no added nitrogen. Therefore, this possibility is also discounted. It is worth mentioning that atypical nodule morphologies have been reported when plants are associated with ineffective symbiotic partners, as seen with the *Medicago truncatula* /*Sinorhizobium meliloti* 1021 combination (Terpolilli *et al.* 2008); the nodules there were smaller and paler than expected. The altered nodule types seen in my experiments here may therefore represent the attempts of less-efficient or incompatible rhizobia to partner with the trap plants. It has been well documented that in nature competition between different rhizobial strains for colonization of roots occurs (Dowling and Broughton 1986; Triplett and Sadowsky 1992; Laguerre *et al.* 2003). Freezing of the SRC may have damaged the rhizobia most compatible with the trap plants, and left the less-compatible but more

cold-tolerant rhizobia behind to colonize the roots. Normally, rhizobia can be stored at both -20°C and -80°C in our lab without problems, but under more controlled conditions. Cultures that are stored at -20°C for the purposes of creating inoculant require only a few cells to survive, as subsamples of the frozen cultures are first grown up at 25°C before their use to inoculate plants. When stored at -80°C, glycerol is used as a cryoprotectant to prevent rupture of bacterial cells from the formation of ice crystals.

4.2 Mycorrhizal colonization

Although present in nearly all collected plants, mycorrhizal fungi were never found in the roots of the trap pea plants grown with non-sterile SRC. Given the observed nodulation phenotype, it is tempting to conclude that excess cold during storage conditions is also responsible for the loss of viable mycorrhizal fungi. However, two points make adoption of this explanation unwise. First, mycorrhizal spores are known to be cold-tolerant, and have been shown to resist temperatures of at least -80°C (Varga *et al.* 2015). It is highly improbable that storage conditions ever exceeded or even matched -80°C, and therefore freezing of SRC seems unlikely to have affected mycorrhizal spore viability. However, the rate at which freezing occurred, the level of moisture in the soil, and the number of freeze/thaw cycles are potential factors that could make the above explanation more reasonable. Second, mycorrhizal fungi are obligate symbionts (Peterson *et al.* 2004), and long-term storage without the protection of a symbiotic partner may have left them vulnerable to other factors besides cold temperatures. Predation or parasitism by other soil microorganisms such as amoebae or chytrids (Fitter and Garbaye 1994) is one possibility. The SRC I obtained was still moist when it was used despite several weeks or months of my storing it at room temperature, and thus the conditions within the bag would be favourable for the growth of some microorganisms. Another possibility is that the

combined moist and warm conditions were detrimental to the survival of mycorrhizal spores; a negative effect of continual warm/moist temperatures on mycorrhizal spore viability has been reported by Lekberg and Koide (2008). I therefore propose that, in contrast to the cold temperatures that negatively affected rhizobia survival, it was the warm and moist conditions within bags of SRC that negatively affected mycorrhizal spore survival. I am doubtful of the possibility of predation/parasitism by other microorganisms within SRC as no indications of these were ever observed in root segment slides. Interestingly, the notion that warm/moist temperatures negatively affect mycorrhizal viability is consistent with the observation from experiments in our lab that mycorrhizal fungi fare poorly when vermiculite is used as a soil medium for plants: plants are grown at 23°C and vermiculite is good at retaining moisture.

4.3 Conclusions

Although the results of these initial exploratory experiments indicate that storage conditions may affect the viability of potentially beneficial microorganisms, the impact these microorganisms may have on plant growth with non-sterile SRC is still unclear. Despite this, it is recommended that the conditions under which SRC is stored are changed. Refrigeration of SRC packages at continual low (minimum 4°C) temperatures in a controlled environment would likely contribute to the greater survival of beneficial microorganisms. Both mycorrhizal fungi and rhizobia are beneficial to plants and therefore attempts should be made to preserve their presence in retail packages of SRC. A project aimed at characterizing the microorganisms within the soil at the SRC mine is already underway, and will build upon the results presented here to help clarify how microorganisms and SRC may together improve plant growth.

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Chapter 4 - General conclusions

The overarching objective of this project was to assess the suitability of the agromineral Spanish River Carbonatite for use in agricultural systems by examining how it influences the growth and yield of pea plants, alters the soil conditions, and affects the microorganisms in the soil. This large objective was divided into three sub-objectives aimed at determining the optimal concentration of SRC for use, assessing the impact of SRC on the rhizobia-legume symbiosis, and investigating how the conditions under which SRC is stored affect its usefulness. These objectives were all achieved during the course of this project.

Regarding the first sub-objective, it was found that a mix of 1:10 SRC:soil was optimal for promoting root system growth, mediating potentially-acidic soils, and increasing the number of soil microorganisms. This ratio had been recommended by Boreal Agrominerals based on the stoichiometry of the mineral and here, it has been verified for best growth of plants. Because only one concentration (1:10) proved optimal, the hypothesis that SRC positively impacts soils, microorganisms, and plants in a concentration-dependant manner is rejected. Regarding the second sub-objective, an increase in the efficiency of the rhizobial-legume symbiosis was seen with the 1:10 SRC:soil mixture as compared to the normal nutrient regime. Both nitrogen fixation efficiency and the photosynthesis/growth of plants appeared to benefit from SRC addition. The hypothesis that SRC addition causes marked increases in the growth of nodulating plants is thus confirmed. Finally, regarding the third sub-objective, evidence for a loss of viability of beneficial soil microorganisms from storage of SRC was found. The hypothesis that SRC contains beneficial microorganisms that are negatively impacted by storage conditions is tentatively confirmed, although further studies on this topic are necessary. Therefore, based on the above findings, I conclude that SRC holds strong potential for direct use in agricultural systems. Furthermore, this study provides more evidence for the power of agrominerals as viable nutrient

sources, especially in combination with mutualistic soil microorganisms such as mycorrhizal fungi (Liu *et al.* 2008) or the rhizobia used here.

Agrominerals show great promise for use in the agriculture of developing countries (van Straaten 2007, p. 6-7). The costs of chemical fertilizers can be limiting in these countries, and more cost-efficient agromineral resources have the demonstrated ability to maintain or improve crop plant growth in conjunction with other techniques (e.g., Chien and Menon 1995). However, the soil processes that may be enhanced by agrominerals must always be kept in mind. Understandably, the end goal is the maintenance or improvement of crop plant growth, but as emphasized in the introduction, several interconnected processes work together to support plant growth. In order to take full advantage of SRC, agrominerals, and other agricultural techniques, a multidisciplinary and multi-perspective approach must be taken. In this project the addition of a single ingredient, SRC, to the artificial soil produced effects on several processes throughout the agropyramid mentioned in the introduction (Figure 1.1).

- The additional calcium provided by SRC helped buffer the soils against acidic conditions that could impact nutrient availability, as evidenced by the soil pH.

- The increase in root growth caused by SRC indirectly made carbon available for soil microorganisms to flourish, as seen in the 1:10 treatment CFU counts.

- The soil conditions, made more optimal than those of the control soils by the addition of SRC, allowed for increased nodulation and plant growth as demonstrated when plants were given both rhizobial inoculant and SRC.

Only by examining the microorganisms, the soils, and the plants together were these interconnected benefits made clear. To fully realize the potential of agrominerals and other

techniques that can improve agriculture, this integrated approach must be mirrored on a larger scale. Those scientists with a strong knowledge of soil systems must work together with those who understand microorganism ecology and those who are familiar with plant growth. Furthermore, the industrialists who discover and mine agrominerals must work together with both the aforementioned scientists and the farmers who use these agrominerals. Only through a synchronized effort can a new perspective be seen. Perhaps what should be done is an inversion of our perspective of the agropyramid (Figure 4.1) to focus on soils as a foundation. By using a bottom-up approach that takes advantage of techniques and ingredients that optimize the soil processes instead of using a top-down approach that focuses on plant growth, this new perspective will reveal innovations that allow for truly sustainable agriculture to become a reality.

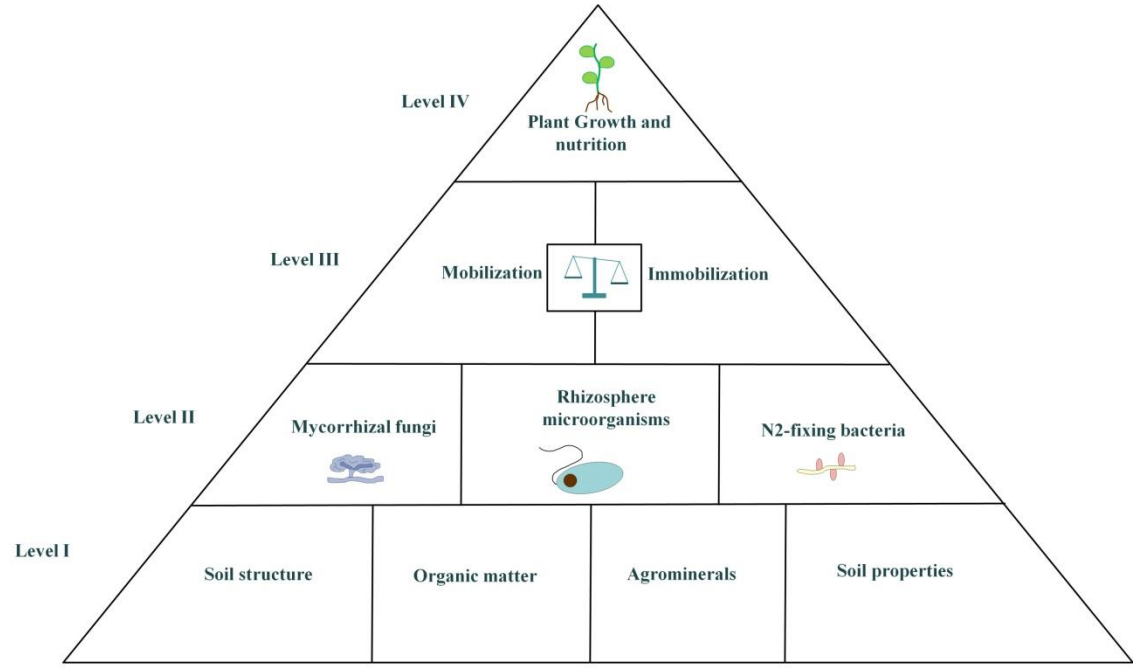

Figure 4.1: A new perspective: the levels (I-IV) on which we focused have been reversed from those in Figure 1.1 (IV-I). Instead of focusing on plant growth directly, emphasis should first be placed on the interconnected processes that work to support plant growth. Improvements to these processes would not only make agriculture more sustainable, but would initiate a cascade effect that would enhance plant growth and yield overall.

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Appendices

Appendix A: Nutrient solution and yeast mannitol broth compositions.

Table A1: Chemical composition of nutrient solutions and yeast mannitol broth. Low nitrogen solution is chemically identical to nutrient solution with the exception of only 0.5mM added $Na(CO_3)_2$ instead of 2.5mM. Yeast-mannitol broth was made to pH 6.8, and autoclaved prior to use.

Nutrient Solution	
Chemical	**Concentration (mM)**
KH_2PO_4	2
$Ca(NO_3)_2*4H_2O$	2.5 (0.5 in low N)
K_2SO4	2
$MgSO_4*7H_2O$	1
Fe II EDTA	0.2
KCl	0.05
H_3BO_3	0.025
$ZnSO_4*7H2O$	0.002
$MnSO_4*H2O$	0.002
$CuSO_4*5H2O$	0.0005
$NaMoO_4*2H2O$	0.0005

Yeast Mannitol Broth	
Chemical	**Amount (g/100mL)**
D-Mannitol	1
K_2HPO_4	0.05
$MgSO_4*7H2O$	0.02
NaCl	0.01
Yeast Extract	0.04

pH 6.8

Appendix B: Model for branching in pea induced by the photocatalytic degradation of polyethylene

Polyethylene is catalytically degraded by light (Zhao *et al.* 2007), and the release of gaseous degradation products such as carbon dioxide or ethylene likely affected seedling growth.. Carbon dioxide has a higher molar mass than air (44.01g m^{-1} vs. 28.97g m^{-1}), so liberated molecules form a pool at the soil surface (Figure B1a). Ethylene has approximately the same molar mass as air, and so would be homogenously spread via diffusion. The emerging apical meristem encounters both gases (Figure B1b), but while ethylene is known to have an inhibitory effect on cell division (Apelbaum and Burg 1972) and bud growth (Burg and Burg 1968), carbon dioxide promotes plant growth (Pritchard *et al.* 1999) and lateral bud development (Andersen 1976; Paez *et al.* 1980). Because in this stage, the carbon dioxide levels encountered by the shoot apical meristem are expected to be much higher than ethylene, any inhibitory effect of ethylene on growth is likely mitigated through the promotion of growth by carbon dioxide. However, once the apical meristem passes the carbon dioxide-rich region (Figure B1c), ethylene may act to inhibit cell division in the shoot apical meristem. At the same time, the auxiliary meristems would then be forming in the carbon dioxide-rich region and be subjected to growth promotion. Apical dominance is thus reduced because of carbon dioxide-induced promotion of auxiliary bud growth and ethylene-based inhibition of apical meristem growth; this results in a branched phenotype (Figure B1d).

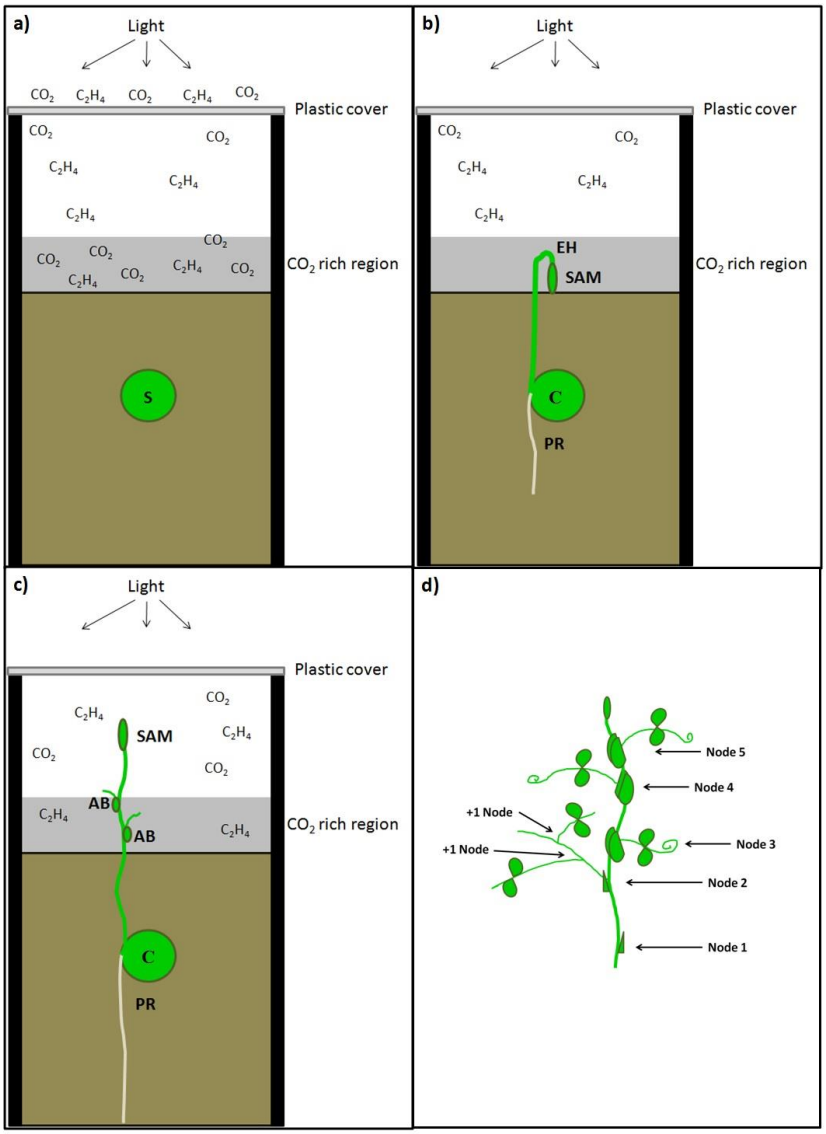

Figure B1: Model for the effect of CO_2 and C_2H_4 gasses released by photocatalytic degradation of polyethylene on seedling pea plant growth. a) Degradation of polyethylene releases C_2H_4 and CO_2. b) As the seed (S) puts out a primary root (PR) and develops into a seedling, its shoot apical meristem (SAM) is brought into a CO_2-rich region by the growth of the epicotyl hook (EH). c) With the further growth of the seedling, the SAM is brought out of the CO2-rich region while auxiliary buds (AB) are developed within it. d) The temporal and spatial effects of the degradation products of polyethylene induce a branching phenotype, where two additional nodes develop off of node 2.

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