Early physiological mechanisms affecting soybean roots in response to neighbouring weeds

by

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ABSTRACT

EARLY PHYSIOLOGICAL MECHANISMS AFFECTING SOYBEAN ROOTS IN RESPONSE TO NEIGHBOURING WEEDS

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Plant competition studies rarely explore how plant to plant interaction can affect roots. In this study, we explored how the presence of aboveground neighbouring weeds could alter soybean seedling root structure and physiology. We hypothesized that in the presence of aboveground weeds, soybean root biomass and nodulation would be reduced, and that the reduction in nodulation would be caused by a loss in total flavonoid content. A non-limiting resource, growth chamber study was conducted to test this hypothesis. The results from this study supported our hypothesis. In the presence of aboveground weeds, soybean root biomass and nodulation were reduced. An accumulation of hydrogen peroxide and an increase in lipid peroxidation were also observed. In addition, total flavonoid content was reduced. This research begins to provide insight into the molecular pathway of how Far Red light affects crop plants. Understanding these mechanisms may aid in the development of soybean varieties that are more tolerant of the consequences of weed competition.
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List of Abbreviations

ANOVA: analysis of variance
APX: ascorbate peroxidase
CAT: catalase
DAB: 3, 3 diaminobenzidine
DAP: days after planting
DPPH: 1, 1-diphenyl-2-picrylhydrazyl
DW: dry weight
FR: far red
IFS: isoflavone synthase
LA: leaf area
MDA: Malondialdehyde
PPFD: photosynthetic photon flux density
R: Red
R:FR: red-to-far-red ratio
RLA: rate of leaf appearance
ROS: reactive oxygen species
SDS: sodium dodecyl sulphate
SOD: superoxide dismutase
TBA: thiobarbituric acid
Chapter 1

1.0 Literature Review

1.1 Soybean Roots

Roots play a variety of important roles in plant life, including: anchorage, water and mineral absorption, carbohydrate storage, synthesis of hormones and secondary compounds, and stem support (Graham et al., 2006). The health and vigour of the entire plant are conditioned by the distribution and function of its roots (Norman, 1963). The plant life cycle begins with the emergence of the embryonic root, known as the radicle, which demonstrates the significance of the root system to plant life (Graham et al., 2006). Not only does the root system nourish and support the plant, it also produces hormones, including cytokinins and gibberellins (Graham et al., 2006). These hormones are transported in the xylem to the shoot where they influence various growth and development processes (Graham et al., 2006).

The soybean (Glycine max (L.) Merr.) root system consists of a primary taproot, a large number of secondary roots, and root hairs (Scott & Aldrich, 1983; Murillo-Williams, 2007). The radicle, or primary taproot, is the first part of the embryo to penetrate the seed coat (Scott & Aldrich, 1983). Lateral roots, thought to comprise the majority of the root mass, begin to develop soon after the radicle begins to elongate, and root hairs (tubular extensions of single epidermal cells) develop quickly thereafter (Scott & Aldrich, 1983).

Root hairs are the primary sites of water and mineral uptake (Scott & Aldrich, 1983; Graham et al., 2006). These “fingerlike outgrowth[s]” are extensions of single cells that, in general, never reach a length greater than 1.3 cm, and a diameter greater than 10 µm (Graham et
The small nature of the root hair proves advantageous for the uptake of water and minerals from the soil pores, as they fit in many spaces too narrow for even the smallest roots to enter (Graham et al., 2006). These root hairs are also the key to the symbiotic relationship formed between legume species and nitrogen-fixing bacteria (Graham et al., 2006; Ohyama et al., 2009).

1.1.1 Nodulation – The Symbiotic Relationship

Nitrogen, an often limiting, very important plant nutrient, is obtained by most plants from soil nitrate, derived primarily from either chemical fertilizers or organic matter (Balestrasse et al., 2004). However, some plants, most notably legume species, are able to derive nitrogen from atmospheric N\textsubscript{2} through a symbiotic relationship with nitrogen-fixing bacteria (Balestrasse et al., 2004). Soybean (a legume) forms a symbiotic relationship with bacteria of the genera *Bradyrhizobium* (*B. japonicum*, *B. elkanii*, and *B. liaoningense*), *Sinorhizobium* (*S. fredii*, and *S. xinjiangense*), and/or *Mesorhizobium* (*M. tianshanense*) (El-Shemy, 2011). However, only *B. japonicum*, *B. elkanii*, and *S. fredii* have been utilized as commercial inoculants, with *B. japonicum* being most widely employed (El-Shemy, 2011).

These gram-negative, soil dwelling bacteria (*B. japonicum*) are able to convert gaseous nitrogen, derived from the air, into ammonium, which is then reduced to glutamine. This glutamine is utilized by the soybean plant (Graham et al., 2006; Singh, 2010; El-Shemy, 2011; Dolatabadian et al., 2013). In return, the host plant supplies the *B. japonicum* with carbon-rich energy compounds (Singh, 2010). The relationship between the bacterium and the host plant begins with a simple “chemical conversation” or “molecular dialog” (Graham et al., 2006; Ohyama et al., 2009; Singh, 2010; El-Shemy 2011; Abd-Alla et al., 2014). However, successful
symbiotic relationships are complex and require the coordination of multiple genes/gene families in both partners (Abd-Alla et al., 2014).

The first step in the initiation of the symbiotic relationship between the soybean plant and *B. japonicum* is the excretion of species-specific isoflavonoid compounds into the soil by the host plant (Graham et al., 2006; Ohyama et al., 2009). The two major isoflavonoid compounds released from soybean roots are daizein and genistein (Ohyama et al., 2009). These compounds serve as a signal to the *B. japonicum*, which respond by secreting small organic molecules into the soil (Graham et al., 2006). These organic molecules, called NOD factors (a modified lipochitine oligosaccharide), signal the host plant to begin to prepare for the process of nodule formation (Graham et al., 2006; Ohyama et al., 2009). Soybean perception of NOD factor requires a dimeric receptor protein and a complex downstream signalling cascade controlled by the plant (Dolatabadian et al., 2013).

Next, *B. japonicum* move toward the host plant’s roots, and proliferate near the root surface (Ohyama et al., 2009). The bacteria make the first contact with the host plant when they attach to a root hair (Graham et al., 2006; Ohyama et al., 2009). At this point, the host plant entraps the bacteria by curling the root hair around the bacterial cells (Graham et al., 2006; Ohyama et al., 2009). As the bacterial cells become enclosed by the curling root hair, an infection thread (a tunnel-like structure) is formed, and the bacteria can now enter into the host plant’s roots (Graham et al., 2006; Ohyama et al., 2009).

Upon entry, plant cell division and bacteria proliferation occur as the nodule structure begins to develop (Graham et al., 2006; Ohyama et al., 2009). As the nodule matures, nodular vascular bundles develop and eventually connect with root vascular bundles (Graham et al., 2006; Ohyama et al., 2009). At this point, the bacteroids (the symbiotic state of rhizobia) begin
to fix atmospheric N\textsubscript{2}, and material exchange between the nodules and the roots occurs through the vascular bundle connection via the xylem and phloem (Graham \textit{et al.}, 2006; Ohyama \textit{et al.}, 2009).

\textit{1.1.2 Flavonoids}

Produced by the phenylpropanoid pathway, flavonoids are a ubiquitous group of molecules that are diverse in form and function (Subramanian \textit{et al.}, 2006, 2007). They are widely distributed throughout the plant kingdom and are found in many fruits, flowers and leaves (Taylor & Grotewold, 2005). These molecules are characterized by the presence of two benzene rings that are connected by a 3-carbon bridge or by a pyrane or pyrone ring (Taylor & Grotewold, 2005). More than 4000 flavonoids have been discovered to date, based on the position of, and modifications to, the structure described above (Taylor & Grotewold, 2005). Flavonoids can be classified into several classes, including the flavonols, the flavones, the isoflavones, and the anthocyanin pigments (Taylor & Grotewold, 2005).

Flavonoids play a variety of roles in plants, including: protecting against UV damage and pathogenic microbes, acting as pigments or co-pigments in influencing flower colour, serving as signals for pollinators and other beneficial organisms, participating in plant hormone signaling, facilitating pollen-tube germination, modulating auxin distribution (including during nodulation), and acting as signal molecules to symbiotic microbes (Taylor & Grotewold, 2005; Subramanian \textit{et al.}, 2007 and references therein). Interestingly, flavonoid biosynthesis is controlled by various environmental variables, such as light, temperature, fungal elicitors, microbial pathogens, and wounding (Alokam \textit{et al.}, 2002).
In addition to the functions listed above, flavonoids play another crucial role during nodulation once the bacteria enter the plant, and exogenous flavonoids are no longer available (Subramanian et al., 2007). This includes stimulating the production of NOD factor inside the plant roots (Subramanian et al., 2007). In some legumes, nodule primordial cell division is preceded by an inhibition in auxin transport (Subramanian et al., 2007). It is believed that flavonoids are a crucial component of auxin transport regulation during nodulation (Subramanian et al., 2007).

A study was undertaken by Subramanian et al., (2006) investigating the possibility that isoflavones could be essential to symbiosis between B. japonicum and soybean, either because of their role as NOD gene inducers and/or because of their role in inhibiting polar auxin transport. RNA-interference (RNAi)-induced silencing of isoflavone biosynthesis (IFS) in soybean plants led to increased auxin transport in roots, thereby suggesting that isoflavones act as auxin transport inhibitors in soybean (Subramanian et al., 2006, 2007). This observation was solidified when the exogenous application of the isoflavone genistein inhibited auxin transport in wild-type soybean seedlings (Subramanian et al., 2006, 2007). However, this application of genistein was not sufficient to restore nodulation in the IFS RNAi roots (Subramanian et al., 2006, 2007). This result suggests that the accumulation of isoflavones in specific tissues is more important than the total root isoflavone levels (Subramanian et al., 2006, 2007).

The researchers went on to inoculate these same IFS RNAi soybean plants with a genistein-hypersensitive B. japonicum mutant (Subramanian et al., 2006, 2007). This B. japonicum mutant has the ability to synthesize the NOD factor signal in the presence of low levels of isoflavone NOD gene inducers (Subramanian et al., 2006, 2007). Inoculation of the IFS RNAi soybean roots with this B. japonicum mutant resulted in normal nodulation, indicating that the importance
of isoflavones in soybean nodulation is related to their role in NOD gene induction (Subramanian et al., 2006, 2007).

The study conducted by Subramanian et al., (2006) clearly determined that isoflavones modulate polar auxin transport in soybean roots. However, the IFS RNAi roots were able to nodulate normally when inoculated with the genistein-hypersensitive B. japonicum mutant. This response is either not essential to successful nodulation, or other flavonoids allow nodulation to occur even when isoflavone levels are indetectable (Subramanian et al., 2007).

1.1.3 Variables that Alter Nodulation

As with most living systems and processes, there are a variety of factors that can affect various parameters of the nodulation and nitrogen fixation processes in legumes. A reduction in fixation could be due to a simple reduction in host plant growth, but the symbiotic relationship is often more sensitive and first affected by changes to the surrounding environment (Singh, 2010). Some of the factors that have been observed to specifically inhibit soybean nitrogen fixation include: nitrogen, drought, soil acidity, autoregulation, light quantity, and light quality (Singh, 2010; El-Shemy, 2011). Each of these factors, and their effects on nodulation, will be discussed below.

1.1.3.1 Nitrogen

Soybean assimilates N from three primary sources: 1) atmospheric nitrogen by symbiotic N₂ fixation in root nodules, 2) soil mineralized nitrogen, and 3) fertilizer application (Ohyama et al., 2009). There are contrasting conclusions in the literature regarding a legume and the effect of nitrogen application on N₂ fixation. One can find different results depending on the species in
question, the amount of nitrogen applied, as well as the form of nitrogen applied (NO$_3^-$, NH$_4^+$, and urea). It has been clearly demonstrated that intermediate to high concentrations of nitrate and ammonium fertilizer have negative effects on N$_2$ fixation and legume-rhizobia symbiosis with regard to various parameters, including: nodule number, nodule weight, and nitrogenase activity (Bollman & Vessey, 2006; Ohyama et al., 2009). Repression of the induction of the regulatory nodD gene and nodABC genes in Bradyrhizobium japonicum (Wang & Stacey, 1990) and Rhizobium meliloti (Dusha et al., 1989) have been documented as an effect of NH$_4^+$ application. Application of NH$_4^+$ has also been shown to affect the exudation of flavonoid signalling compounds (Cho & Haper, 1991; Wojtaszek et al., 1993), and bacterial attachment to the root (Dazzo & Brill, 1978).

An experiment conducted by Gan and Liang (2010) on chickpea (Cicer arietinum L.) demonstrated that application of nitrogen fertilizer (84 kg ha$^{-1}$) negatively influenced nodule mass, showing reductions of 83% from the non-N control. In contrast, experiments conducted on pea (Pisum sativum L.) and white clover (trifolium repens L.) indicated that continuous low concentrations of ammonium (approximately ≤0.5 mmol L$^{-1}$) result in stimulation of both whole-plant nodulation (nodules plant$^{-1}$) and dry mass-specific nodulation (nodules g$^{-1}$ root DM) (Bollman & Vessey, 2006). The same concentration of ammonium that enhanced nodulation in pea and clover, however, had negative effects in soybean (Gulden & Vessey, 1998; Bollman & Vessey, 2006).

The experiment conducted by Gulden and Vessey (1998) concluded that the growth of soybean plants supplied with nitrogen via N$_2$ fixation and NH$_4^+$ was “greatly superior” to that of soybean plants which received nitrogen from N$_2$ fixation only. However, negative effects of NH$_4^+$ supplementation on N$_2$ fixation were seen at concentrations ranging from 0.5 to 1.0 mM,
and on whole plant growth between 1.0 and 2.0 mM NH$_4^+$ (Gulden & Vessey, 1998). Contrary to what was observed in pea in the Gulden and Vessey experiment in 1997, a suppression of specific nodulation (nodules g$^{-1}$ root DW), and specific nitrogenase activity (nitrogenase activity g$^{-1}$ nodule DW) was recorded in soybean (Gulden & Vessey, 1998).

In both pea and soybean, a stimulation of whole plant nodulation (nodules plant$^{-1}$) and N$_2$ fixation was observed when NH$_4^+$ supplementation was utilized (Gulden & Vessey 1997, 1998). Gulden and Vessey (1998) argued these increases resulted from an increase in overall plant growth (i.e. fewer nodules g$^{-1}$ root DW, but much larger roots). Once NH$_4^+$ supplementation ceased, however, the nodules on these plants appeared to settle into a consistent level of nodule DW relative to root DW (Gulden & Vessey, 1998). These results lead to the conclusion that the effect of nitrogen on nodulation is specific to species, cultivar, nitrogen rate, and nitrogen type.

1.1.3.2 Drought

There are several environmental conditions that limit the efficient growth and activity of legume plants (Predeepa & Ravindran, 2010). Soil drying, or drought, is one such environmental condition (Ladrera et al., 2007). According to Ladrera et al., (2007), three major factors are thought to be involved in the effect of drought on N$_2$ fixation: oxygen limitation, carbon shortage, and regulation by nitrogen metabolism. Drought causes an increase in nodular oxygen diffusion resistance, and this phenomenon has been extensively examined by researchers (Durand et al., 1987; Diaz del Castillo & Layzell, 1995; Serraj & Sinclair, 1996; Minchin, 1997). Since N$_2$ fixation under drought conditions cannot be fully restored by increasing the O$_2$ concentration in the root zone, however, it is unlikely that oxygen limitation is the only cause of the decline in nitrogen fixation during dry conditions (Ladrera et al., 2007).
Another explanation for the down-regulation in nitrogen fixation under drought conditions is a shortage of carbon supplied to bacteroids (Arrese-Igor et al., 1999). Carbon is mainly transported from the shoots to the nodules as sucrose, and hydrolyzed by either sucrose synthase (SS) or alkaline invertase (AI) (Ladrera et al., 2007). As demonstrated by Gonzalez et al., (1995, 1998), SS is the first nodule enzyme in both soybean and peas to show a decrease in activity under drought conditions. This decrease in SS activity leads to an accumulation of sucrose and organic acids, particularly malate (Galvez et al., 2005). This buildup of compounds leads to a shortage of substrates for bacterial respiration (Ladrera et al., 2007).

Nitrogen metabolism is also thought to play a role in drought-related inhibition of nitrogen fixation by a nitrogen feedback mechanism involving shoot nitrogen status (Ladrera et al., 2007). Several molecules are thought to be involved (Ladrera et al., 2007). Sinclair and Serraj reported in 1996 that legumes which export ureides, such as soybean, are more sensitive to drought than legumes that export amides. Ureides, specifically allantoin and allantoic acid, are the major nitrogen transport compounds in N₂ fixing soybean (Patterson & LaRue, 1983; Thomas & Schrader, 1981). It has been discovered that nodulated soybean roots treated with \(^{15}\)N₂ incorporate the \(^{15}\)N₂ into ureides, and xylem transport the ureides from the roots to the shoot where they are metabolized (Thomas & Schrader, 1981; Patterson & LaRue, 1983). Various studies have concluded that there is an accumulation of ureides in both the shoots (Serraj & Sinclair, 1996b; Serraj et al., 1999) and nodules (Serraj et al., 1999; Vadez et al., 2000) under drought conditions. Ureide inhibition of nodule activity under drought conditions could be a result of either direct feedback within the nodule or indirect feedback from the shoots (Serraj et al., 2001).
In the study conducted by Ladrera et al., (2007), two soybean cultivars with different drought sensitivities were analyzed. ‘Jackson’ had “substantial” drought tolerance, and ‘Biloxi’ was sensitive to soil drying (Ladrera et al., 2007). The drought tolerance of ‘Jackson’ was confirmed under both controlled and field conditions, and was associated with low accumulation of ureides in the shoots (Ladrera et al., 2007). ‘Biloxi’ accumulated a higher concentration of ureides in its shoots under drought conditions when compared to ‘Jackson’ (Ladrera et al., 2007). Inhibition of nitrogen fixation caused by drought conditions occurred in both cultivars; however, the inhibition occurred earlier and more severely in ‘Biloxi’ (Ladrera et al., 2007). In this study, under mild drought stress conditions, no accumulation of ureides were found in the shoots of either cultivar, even though nitrogen fixation was inhibited (Ladrera et al., 2007). Therefore, Ladrera et al., (2007) concluded that leaf ureides are not involved in the early stage of nitrogen fixation inhibition under drought conditions; however, a role in the later stages of a more severe drought cannot be ruled out.

Complex amino acid cycling occurs between the plant and the bacteria in the nodules (Ladrera et al., 2007). It is still unknown at this time if this exchange of amino acids could be directly disrupted by drought-induced accumulation of nitrogen compounds (Ladrera et al., 2007). In this study, drought caused ureide (a nitrogen compound) accumulation in the nodules of both cultivars (Ladrera et al., 2007). Since this accumulation occurred earlier, and to a greater extent in ‘Biloxi’, it was thought to be correlated with the drought nitrogen fixation sensitivity of this cultivar (Ladrera et al., 2007). These results do not provide direct evidence that ureides are the actual compound which induce a decrease in nitrogen fixation under drought conditions; however, it is believed that an accumulation of fixation products is involved (Ladrera et al., 2007).
There is a high correlation between sucrose synthase inhibition and abiotic stress, such as salinity, defoliation, nitrate and oxidative stress in nodules (Arrese-Igor et al., 1999). In the study conducted by Ladrera et al., (2007), a reduction of sucrose synthase activity was noted in ‘Biloxi’ at the first day of reduced watering, before any effect was noted on nitrogen fixation. In the cultivar ‘Jackson,’ however, sucrose synthase activity rates were maintained at levels equal to those of the control plants until the third day of reduced watering (Ladrera et al., 2007). The decline in sucrose synthase activity was also concomitant with that of nitrogen fixation (Ladrera et al., 2007). In addition to drought, soil acidity is another abiotic stress that can affect legume nodulation.

1.1.3.3 Soil Acidity

Acidic soils (high concentrations of hydrogen ions [H\(^+\)] in the soil solution) are known to have low levels of both phosphorous (P) and calcium (Ca). This type of soil is also known to have high levels of exchangeable aluminum (Al), and manganese (Mn) (Wood et al., 1984; Taylor et al., 1991). A higher concentration of H\(^+\) ions in the soil solution increases the solubility of Al, Mn and iron (Fe) (El-Shemy, 2011). The growth and survival of many Rhizobia species are known to be significantly affected by soil stresses, such as acidity and the resulting elemental changes (i.e. low levels of P and Ca, and high levels of Al and Mn) (Wood et al., 1984; Buerket et al., 1990; Taylor et al., 1991; Cheng et al., 2002; El-Shemy, 2011).

Many studies have been conducted on soil acidity and its influence on legume and Rhizobia symbiosis (Wood et al., 1984; Buerket et al., 1990; Taylor et al., 1991; Cheng et al., 2002; El-Shemy, 2011). The results of these studies depend on a variety of factors, including: the host species, the growth medium (soil or culture solution), the strain of bacteria, and various
interactions among these factors (Wood et al., 1984; Buerket et al., 1990; Taylor et al., 1991; Cheng et al., 2002; El-Shemy, 2011). In a study conducted by Munns (1968) on the nodulation of alfalfa (*Medicago sativa* L.) in culture solution, it was concluded that root hair curling is an acid-sensitive step in the nodulation process. In contrast, a study conducted by Robson and Loneragan (1970) on barrel medic (*Medicago truncatula* Gaertn.) in soil determined that *Rhizobia* survival and colonization are acid-sensitive steps.

Cheng et al., (2002) studied the nodulation response of alfalfa and spiny medic (*Medicago murex* L.) to soil acidity. It was determined that nodulation of both host plant species was challenged by soil acidity (Cheng et al., 2002). At a pH of 4.3, spiny medic developed fewer nodules than plants grown at neutral pH; however, these nodules developed at a similar rate to those on plants grown at a pH of 7.0 (Cheng et al., 2002). When alfalfa was grown under the same acidic conditions, fewer nodules were produced, and nodules appeared later than those on plants grown in soil of pH 7.0 (Cheng et al., 2002). As a result, Cheng et al., (2002) concluded that growth medium acidity delays the initiation of infection, and hence, the appearance of nodules by decreasing the rhizobial population present.

The growth and survival of symbiotic bacterial species is an important component of the nodulation process at low pH, because the size of the bacterial population effects the concentration of the NOD factor produced (Cheng et al., 2002). In order for successful nodulation to occur, rhizobia need to accumulate and multiply in the host plant’s rhizosphere. This process ensures the critical concentration of NOD factor required for proper nodule formation (e.g. root hair deformation, cortical cell division, and the physical attachment of rhizobial cells to root hairs) is reached (Cheng et al., 2002). Cheng et al., (2002) suggested that spiny medic may be more efficient at increasing the pH of its rhizosphere, thus developing
conditions more favourable for rhizobial survival and growth, than alfalfa. This could potentially be the reason for the delayed nodulation in alfalfa (Cheng et al., 2002). Similar results were seen in soybean (Taylor et al., 1991), and white clover (Trifolium repens L.) (Wood et al., 1984). The overall conclusion that can be drawn from these studies is that the symbiosis between many host plants and bacterial species is challenged by soil acidity.

1.1.3.4 Autoregulation

Autoregulation (which can be defined as an internal, plant-mediated, feedback-regulated process) inhibits nodule formation on young root segments via pre-existing nodules on older root tissue (Pierce & Bauer, 1983; Delves et al., 1986; Francisco & Harper, 1995; Abd-Alla, 2001; Voisin et al., 2010). It is thought that once a critical number of subepidermal cell divisions are initiated in the root cortex, a precursor molecule is transported from the root to the shoot (Francisco & Harper, 1995; Abd-Alla, 2001). Once this molecule reaches the shoot, it is converted to a shoot-derived inhibitor, which is then sent back to the root system to suppress further subepidermal cell divisions from developing into nodules (Francisco & Harper, 1995; Abd-Alla, 2001). This is a process that continues throughout the host plant’s growth and development (Francisco & Harper, 1995).

The formation and maintenance of nodules is an energy expensive process involving consumption of carbon assimilates derived from photosynthesis (Voisin et al., 2010). Nodule growth and maintenance affects both the roots and shoots of the host plant, particularly during the early stages of plant growth as the nodules are competing with the small plant for carbon use (Voisin et al., 2010). This has been demonstrated by various researchers using hypernodulating mutant host plants (Voisin et al., 2010). These mutant plants do not employ autoregulation, and
the result is excessive nodulation, causing both a depression of shoot and/or root growth, and a reduction of final N uptake and yield (Voisin et al., 2010). Although the exact nature of the compounds involved in the autoregulation process has yet to be unambiguously elucidated, potential candidate compounds include phytohormones, like ethylene, auxin or brassinosteroids, jasmonic and abscisic acids (Voisin et al., 2010 and references therein).

Nodulation is delayed or inhibited throughout various stages of host plant growth and development (Voisin et al., 2010). This phenomenon is first recognized as the delayed onset of nodulation after host plant germination (Voisin et al., 2010). In a study conducted by Voisin et al., (2010), nodule initiation began after the appearance of the first leaf and before the exhaustion of seed reserves. Therefore, the authors concluded that during seed germination and early seedling growth, nodulation is suppressed not by autoregulation per se, but rather by a repression signal arising from the seed (Voisin et al., 2010). Voisin et al., (2010) thought that the typical “shoot-to-root signalling cross-talk” involved in autoregulation was inoperative due to the absence of a real shoot.

Once the host plant’s shoot developed, the early seed repression of nodulation was alleviated; however, in the study conducted by Voisin et al., (2010), nodule initiation only occurred if nitrogen was limiting. Nodule initiation, in other words, only commenced if mobilization of seed nitrogen reserves and nitrate uptake by the roots appeared to be insufficient to meet the plant’s needs (Voisin et al., 2010). Overall, Voisin et al., (2010) concluded that the relationship between the nodulation process and plant development are tight and complex.

It is thought that nodulation in soybean is primarily controlled by the shoot, but there is some indication that the shoot control can be modified by the root (Francisco & Harper, 1995; Aba-Alla, 2001). For example, when the shoot of ‘NOD1-3’ (a hypernodulating soybean
cultivar) was grafted to the root of ‘Crauford’ (a commercial cultivar with restricted nodulation), nodule formation on the ‘Crauford’ root was not enhanced; however, when the shoot of ‘NOD1-3’ was grafted onto the root of commercial cultivars ‘Clark’ and ‘Davis’, hypernodulation was induced (Abd-Alla, 2001). In contrast, when the shoots of these two commercial cultivars (‘Clark’ and ‘Davis’) were grafted onto the root of ‘NOD1-3’ in another trial, the number of nodules was significantly inhibited. These results imply that in the ‘Crauford’ cultivar, the autoregulation of nodulation is a root-controlled signal, while in the ‘Clark’ and ‘Davis’ cultivars, the signal is shoot-controlled (Abd-Alla, 2001). The conclusions of this study support the argument that both the shoot or/and root may be involved in the regulation of nodulation and their role is cultivar dependent (Abd-Alla, 2001).

1.1.3.5 Light Quantity and Quality

The allocation of photoassimilates (products of photosynthesis) to roots is essential for nodule formation (Kasperbauer & Hunt, 1994). A number of researchers have demonstrated that the light environment the plant shoot is exposed to can influence biomass allocation among growing plant parts (Kasperbauer & Hunt, 1994; Kasperbauer, 2000; Rajcan & Swanton, 2001; Rajcan et al., 2004; Markham & Stoltenberg, 2009; Markham & Stoltenberg, 2010; Cressman et al., 2011; Page et al., 2011). In general, greater photoassimilate allocation to roots led to the formation of more nodules (Kasperbauer & Hunt, 1994). Several studies were conducted in the mid- to latter part of the twentieth century on the effects of altered light quality on legume nodulation (Lie, 1969; Sheehy et al., 1983; Kasperbauer et al., 1984; Balatti & Montaldi, 1986; Kasperbauer & Hunt, 1994). There have, however, been very few studies to-date on the effect of light quantity on legume nodulation.
A study on the effect of light quantity on legume nodulation was briefly mentioned by Sheehy et al., (1983). The investigation demonstrated that plants grown in irradiance levels as low as 7 W m\(^{-2}\) showed a reduction in total nitrogen fixation; however, the activity of the nodules were the same as those on plants grown at 28 W m\(^{-2}\) (Sheehy et al., 1983). The authors concluded these results indicated that growing plants over a wide range of irradiance levels would add little to current knowledge (Sheehy et al., 1983). Similar results were briefly mentioned in a study conducted on pea by Voisin et al. (2010). Experiments on light quality have been more numerous.

In an experiment on the southern pea (\textit{Vigna unguiculata} (L.) Walp.), Kasperbauer & Hunt (1994) determined that a higher ratio of far-red (FR) relative to red (R) light resulted in a higher shoot: root biomass ratio, longer stems, less massive roots and fewer nodules. The authors concluded that the adaptive response to a higher FR: R was to allocate more photosynthate to developing stems and leaves, and consequently less photosynthate for new root growth (Kasperbauer & Hunt, 1994). This lack of root growth also resulted in less nodulation (Kasperbauer & Hunt, 1994). Similar results were also found in soybean (Kasperbauer et al., 1984). In 1984, Kasperbauer et al., investigated nodule formation and photosynthate allocation in soybean plants that received red or far-red light at the end of the photosynthetic period. Results were consistent with those of Kasperbauer and Hunt (1994), in that the red light treated plants partitioned less dry matter to stems, and more to roots, than the far-red light treated plants. In other words, the root/shoot ratio of red light treated plants was higher than that of far-red light treated plants (Kasperbauer et al., 1984). Also, inoculated plants treated with red light developed more nodules than far-red light treated plants (Kasperbauer et al., 1984). The findings of
Kasperbaurer et al. (1984) thus supported the hypothesis that light quality influences the allocation of photosynthate to alter stem elongation, root development, and perhaps indirectly, nodulation.

Lie (1969) undertook a more in-depth experiment on the effects of red and far-red light on root-nodule formation in legumes. When leguminous plants were grown under constant PPFD, exposing the shoot to red light increased nodule production on the roots more than when they were exposed to blue light (Lie, 1969). Exposure to far-red light for a few minutes at the end of the photoperiod reduced the number of nodules formed (Lie, 1969). The inhibitory action of far-red light on nodule formation could be somewhat reduced by subsequently exposing the shoot of the same plant to red light (Lie, 1969). Lie (1969) therefore hypothesized that the nodulation process is controlled by the phytochrome system. This theory is supported by Furuya and Hillman (1964), who detected phytochrome in the roots of etiolated pea plants.

Lie (1969) conducted several different experiments on the effects of red and far-red light on nodulation. It was found that the nodulation of pea and broad bean plants were reduced when roots were exposed to far-red light for 5 to 15 minutes daily during five consecutive days following inoculation. Similar results were also obtained when roots were exposed to far-red light for 15 minutes on the 3rd or 4th day after inoculation (Lie, 1969). Interestingly, when roots were exposed to far-red light prior to inoculation, or during the first two days following inoculation, no alteration in nodulation was observed (Lie, 1969). The deleterious effects of root exposure to far-red light could be reduced with subsequent exposure to red light (Lie, 1969).

Within this experiment, not only did Lie (1969) expose the roots of two leguminous plant species to far-red light, the shoots were also exposed. Exposure of the roots to far-red light, however, reduced nodulation more markedly than exposure of the shoots (Lie, 1969).
Surprisingly, plants whose roots were irradiated with far-red light recovered from the treatment, and eventually developed a large number of nodules (Lie, 1969). In contrast, shoot exposure to far-red light had a more permanent effect, explained by the authors as a consequence of the excessive shoot elongation (Lie, 1969). Root exposure did not result in excessive shoot elongation (Lie, 1969). Exposure of the shoot to red light did not reduce the deleterious effect on nodulation of exposure of roots to far-red light (Lie, 1969).

Lie (1969) conjectured that the nodulation of leguminous plants is not simply affected by light via photosynthesis and carbohydrate partitioning, but rather that nodulation is controlled by the phytochrome system. The hypothesis that far-red light affects nodulation indirectly via differential distribution of carbohydrates to the roots and the shoots (i.e. altered root/shoot ratio) was rejected by the results of the experiments in which just the roots of the plants were irradiated (Lie, 1969). These findings help to solidify the objectives and hypothesis of the proposed study, as they clearly demonstrate that exposure of leguminous plants to far-red light reduces nodulation.

A variety of factors, including nitrogen, autoregulation, drought, soil acidity, light quantity and light quality have been observed to affect legume nodulation. However, despite some speculation into the mechanisms behind the cause for the alterations in nodulation due to these various factors, there has been very limited research.

1.2 Reactive Oxygen Species

Molecular oxygen was introduced into the environment about 2.7 billion years ago by the evolution of photosynthetic organisms (Gill & Tuteja, 2010). Reactive oxygen species (ROS), partially reduced forms of atmospheric oxygen (O₂), are typically the result of “the excitation of
O₂ to form singlet oxygen (¹O₂) or from the transfer of one, two or three electrons to O₂ to form, respectively, a superoxide radicle (O⁻₂), hydrogen peroxide (H₂O₂) or a hydroxyl radicle (HO⁻)” (Mittler, 2002). However, in contrast to O₂, ROS in plants are toxic, highly reactive, and cause damage to lipids, carbohydrates, proteins and DNA (Mittler, 2002; Halliwell, 2006; Gill & Tuteja, 2010).

ROS are generally thought to be produced from three different mechanisms: 1) unintended consequences of aerobic respiration, 2) pathways enhanced during abiotic stress, and 3) NADPH oxidases, amine oxidases, and cell-wall-bound peroxidases that participate in the production of ROS during processes such as programmed cell death (PCD) and pathogen defense (Mittler, 2002; Halliwell, 2006; Gill & Tuteja, 2010). Additionally, ROS influence the expression of a number of genes, thereby controlling many processes, such as: growth, cell cycle, systemic signalling and development. They are produced continually as by-products of various metabolic pathways in multiple cellular locations, including the chloroplast, mitochondria and peroxisomes (Gill & Tuteja, 2010; and references therein). Photosystem I and II are the major sites for the production of ¹O₂ and O⁻₂ in chloroplasts. Complex I, ubiquinone, and complex III of the electron transport chain are the major sites for the generation of O⁻₂ in the mitochondria (Gill & Tuteja, 2010).

In order to deal with the various sources of ROS production, plants possess “oxidative defense machinery” to protect against oxidative stress damage (Gill & Tuteja, 2010). However, because ROS are not only toxic, but also participate in some signalling events, plants have the ability to finely modulate low levels of ROS, as well as detoxify excess quantities generated during stress events (Mittler, 2002). Plants possess very efficient enzymatic (e.g. superoxide dismutase, SOD; catalase, CAT; ascorbate peroxidase, APX) and non-enzymatic (e.g. phenolic
compounds, ascorbic acid) antioxidant defense systems (Gill & Tuteja, 2010). The behaviour of ROS (i.e. whether they will act as damaging, protective or signalling factors) depends on the equilibrium between ROS production and scavenging at the proper time and location (Mittler, 2002; Gill & Tuteja, 2010).

1.3 Root Stress Physiology

Roots play a variety of important roles in plant life, including: anchorage, water and mineral absorption, carbohydrate storage, synthesis of hormones and secondary compounds, and stem support (Graham et al., 2006). Also, beyond the necessity of the root system to the plant itself, some plant roots, such as carrots, beets, parsnip, and rutabaga, provide a food source for humans (Graham et al., 2006). These fundamental roles of roots indicate the importance of healthy root structures and the potential negative ramifications of such plant stressors as drought, excess moisture, insect pressure, and weed competition on the root system.

The study of root stress physiology in various plant species, including such plants as soybean, wheat (Triticum aestivum L.) and corn (Zea mays L.) has been quite extensive (Sparkes et al., 2008; Sparkes & King, 2008; Alam et al., 2010; Alam et al., 2010b). These studies have been conducted at various levels of complexity and intensity, ranging from objective observation of specific characteristics to proteome analysis. The latter approach is the large scale study of proteins, particularly of their structure and functions (Sparkes et al., 2008; Sparkes & King, 2008; Alam et al., 2010; Alam et al., 2010b). The majority of the studies seem to reach similar conclusions, regardless of the stress (e.g. drought stress, water stress, etc.) studied (Sparkes et al., 2008; Sparkes & King, 2008; Alam et al., 2010; Alam et al., 2010b). The trends observed when
a plant is subjected to these various stresses appears to be morphological, physiological, and/or biochemical changes that tend to negatively impact that root system.

1.4 Light Quality, Phytochromes, and Shade Avoidance

Light energy, encompassing both light quantity and quality, is one of the most important ambient signals plants receive (Takano et al., 2008). Light quantity refers to the intensity, the number of photons incident on a surface, or the amount of light intercepted by the crop (Rajcan & Swanton, 2001). Light quantity is typically measured as the photosynthetic photon flux density (PPFD) within the spectral range of 400-700nm (Green-Tracewicz & Swanton, 2010). Light quality, in contrast, is a “driving variable of plant morphology,” and can be characterized as the ratio of red (600-700 nm) to far-red light (700-800 nm) (Rajcan & Swanton, 2001; Green-Tracewicz & Swanton, 2010). Light provides the energy source for photosynthesis, and because of this, plants need to be able to adapt to changes in light quantity and quality in order to optimize the efficiency of this essential process (Genoud et al., 1998).

During crop-weed interaction, a reduction in available PPFD can be caused by mutual shading of leaves (Rajcan & Swanton, 2001). This reduction in plant-available PPFD can lead to decreases in photosynthetic rate, leading to a potential reduction in leaf area (LA), and ultimately reducing dry matter accumulation in both the crop and the weed (Rajcan & Swanton, 2001). In an example cited by Rajcan and Swanton (2001), leaf area of maize at silking was reduced 15% by high pressure weed competition. The severity of this reduction depends on multiple factors, including the crop-weed mixture, population density of the weeds, and time of emergence of the weeds relative to the crop (Rajcan & Swanton, 2001).
Not only are plants grown underneath or within a canopy exposed to reduced light quantity (PPFD), they are also subjected to a different quality of light than plants grown in full sunlight (Rajcan & Swanton, 2001). Plants selectively absorb red light, and reflect and/or transmit far-red light (Rajcan & Swanton, 2001). Therefore, light in the lower part of a canopy is enriched with far-red light, causing the R:FR ratio of the light in this region to be lower than the R:FR ratio of the light above the canopy (Rajcan & Swanton, 2001). Plants growing in this FR light enriched lower canopy tend to have different architecture than plants growing in full sunlight (Rajcan & Swanton, 2001). The plant mechanism that senses this altered R:FR ratio is phytochrome (Rajcan & Swanton, 2001; Green-Tracewicz & Swanton, 2010).

Plants have evolved multiple natural photomorphogenic pigments, including the phytochromes, cryptochromes, and phototropins, that allow them to perceive light over a variety of wavelengths and intensities (Kasperbauer, 2000; Takano et al., 2008). Phytochromes, which are part of a family of photosensory proteins, have been determined to solely perceive red/ far-red light (Takano et al., 2008; Rockwell et al., 2009). They interconvert between two forms: a red light absorbing form, \( P_r \), and a far-red light absorbing form, \( P_{fr} \) (Rockwell et al., 2009). Cryptochromes and phototropins receive UV-A and blue light (Takano et al., 2008).

\( P_r \), the inactive form of phytochrome, absorbs light most efficiently around 665 nm, and \( P_{fr} \), the active form, absorbs optimally at 730 nm (Salisbury & Ross, 1991; Smith, 2000; Jiao et al., 2007). Phytochrome remains in the inactive \( P_r \) form until red photons are absorbed, allowing it to interconvert to the active \( P_{fr} \) form, which preferentially absorbs far-red photons (Rockwell, 2009; Green-Tracewicz & Swanton, 2010). The conformational change from \( P_r \) to \( P_{fr} \) enables translocation from the cytoplasm to the nucleus, allowing the phytochromes to interact with downstream transcriptional cascades (Quail, 2002; Jiao et al., 2007). The conversion of
phytochrome from its inactive to active forms occurs over various wavelengths of light; this conversion helps plants to sense the quality and quantity of light, and regulate various developmental stages (Smith, 2000).

The sensing of low R:FR light as an indicator of forthcoming competition by phytochromes induces morphological changes in many plant species which have been termed “shade avoidance response” (Kasperbauer, 2000; Rajcan & Swanton, 2001; Rajcan et al., 2004; Markham & Stoltenberg, 2009; Green-Tracewicz & Swanton, 2010; Markham & Stoltenberg, 2010; Cressman et al., 2011; Page et al., 2011). Some of these shade avoidance characteristics include: altered carbon allocation patterns (from roots to shoots); altered gene expression and metabolic pathways; internode elongation; and longer, thinner leaves (Kasperbauer, 2000; Rajcan & Swanton, 2001; Rajcan et al., 2004; Markham & Stoltenberg, 2009; Green-Tracewicz & Swanton, 2010; Markham & Stoltenberg, 2010; Cressman et al., 2011; Page et al., 2011). In many cases, the shade avoidance response occurs well before any direct competition for resources (Cressman et al., 2011).

1.5 Soybean Roots and Shade Avoidance

Much work has been done by Green-Tracewicz et al., (2011; 2012) on the expression of the shade avoidance response in soybean plants as a consequence of weed competition. In the first experiment, the characteristic shade avoidance responses were observed (Green-Tracewicz et al., 2011). These responses included: increases in plant height, internode length, and shoot: root ratio. These responses, however, were deemed temporary changes, as they did not persist throughout the entire life cycle of the plant (Green-Tracewicz et al., 2011). There were also some altered soybean plant traits that were thought to be permanent in nature (Green-Tracewicz et al.,
Some of these traits included: reduced rate of leaf appearance (RLA), root biomass, total plant biomass, leaf area (LA), and yield (Green-Tracewicz et al., 2011). Specifically pertinent to this study is the observation that “plants in the weedy treatment accumulated 36% less root biomass than plants in the weed-free treatment” (Green-Tracewicz et al., 2011).

Similar results were found in another study conducted by Green-Tracewicz et al., (2012). Soybean plants that had been exposed to weed competition were: 25% taller (up to the V6 stage); showed hypocotyl and epicotyl internode elongation (at V1 and V2 harvests); had larger shoot: root ratio; and showed reduced leaf area per plant, trifoliate number per plant, leaf biomass, root biomass, and total plant biomass, compared to the plants in the weed-free treatment (Green-Tracewicz et al., 2012). Green-Tracewicz et al., (2012) also found that leaf area and biomass (stem, leaf, and total biomass) of soybean plants were reduced progressively as the duration of time spent exposed to weed competition increased. Green-Tracewicz et al., (2011; 2012) established that weed competition influences a number of soybean growth parameters, including reducing total soybean root biomass; however, the exact root parameters that are altered by low R:FR light (e.g. root length, number of tips, root volume, and/or nodulation etc.) have yet to be identified.

Preliminary work was conducted during my undergraduate research project to determine the effects of low R:FR reflected from the neighbouring surfaces of perennial ryegrass (Lolium perenne L.) on soybean root relative growth rate and root structure. Experiments were conducted in controlled growth facilities under non-resource limiting conditions in pots designed to eliminate direct competition for resources. Soybean root surface area, volume, diameter, length and numbers of tips were analyzed using WinRhizo (Regent Instruments Inc., Sainte-Foy, QC, Canada). Under weedy conditions, a significant increase in plant height was observed at all
harvest intervals (VE, VC, V1, V2, and V3 stages); however, a significant reduction in the radicle root length was only observed at VC, V1, and V3 stages of development. Moreover, a significant reduction in root biomass was also recorded in the weedy treatment at the VE, V1 and V3 stages. At the V3 stage of development (22 days after planting) the reduction in R:FR ratio reflected from the neighbouring weeds caused a significant reduction in the total root volume, length, surface area, and the number of root hair tips. This research was, however, simply groundwork, and more extensive and intensive research is needed.
Chapter 2

Early physiological mechanisms affecting soybean roots in response to the presence of aboveground neighbouring weeds

2.1 Introduction

Crop-weed competition studies rarely explore how plant-to-plant interactions alter the structure and physiology of crop roots. Despite being immobile, plants have the ability to detect changes in their surrounding environment and rapidly integrate this information into alternative patterns of growth. Alternative growth patterns have been observed in response to aboveground signals of light quality, such as R:FR (Kasperbauer & Karlen, 1994; Skálová & Vosátka, 1998; Pecháčková, 1999; Liu et al., 2009; Green-Tracewicz et al., 2011, 2012; Yang et al., 2014). The ability to detect aboveground signals and transfer this information to roots is an essential survival strategy to ensure optimum fitness. Several studies have identified changes in FR, reflected from neighbouring aboveground vegetation, as a signal of pending competition.

Light quality signals, such as R:FR, have also been suggested to be a fundamental mechanism of crop-weed competition (Smith, 1982; Rajcan & Swanton, 2001, Rajcan et al., 2004, Page et al., 2009). Other variables, such as blue light or organic volatile compounds, such as ethylene, may also contribute to our understanding of the resource independent variables that play an important role in crop-weed competition (Pierik et al., 2004; Ballaré et al., 2009). Among all of the variables, however, changes in photosynthetic photon flux density (PPFD) or
changes in the R:FR have provided direct evidence which suggests a central role that light
signals play in modifying morphological strategies of plants and hence the outcome of plant
competition. Affirmation of this central role of low R:FR was recently reported in a study by
Afifi and Swanton (2012), in which changes in FR reflected from both biological (i.e.
neighbouring weeds) and non-biological (i.e. commercial red filter) sources triggered similar
physiological responses, notably the modulation of the phenylpropanoid pathway in maize
seedlings.

Low R:FR is known to reduce root biomass (Kasperbauer & Karlen, 1994; Skálová &
Vosátka, 1998; Pecháčková, 1999; Liu et al., 2009; Page et al., 2009). Wheat seedlings exposed
to FR light developed fewer roots and had a higher shoot: root than unshaded plants
(Kasperbauer & Karlen, 1994). Page et al., (2009) found that maize root biomass was reduced
progressively as the duration of exposure to neighbouring weeds increased. Afifi and Swanton
(2011) discovered that maize roots originating from seed or stem tissue differ in their response to
changes in the R:FR reflected from neighbouring weeds. Limited studies on root morphology
and associated physiological changes have been conducted on soybean.

In two different studies, Green-Tracewicz et al., (2011; 2012) confirmed that low R:FR
reflected from aboveground neighbouring weeds increased the shoot: root, and decreased total
soybean seedling root biomass by as much as 36%. In addition, a recent field intercropping study
conducted by Yang et al., (2014) examined the effects of light quantity and R:FR on the growth
of soybean seedlings. In agreement with previously reported results, these researchers found that
root length, total root biomass and root: shoot of soybean were significantly decreased by the
combined effects of altered light quality and reduced light quanta in a field environment (Yang et
Each of these studies, however, did not examine the effects of low R:FR on soybean nodulation.

A very limited number of studies have been published on the effects of FR on soybean root physiology and nodulation. It is well established that allocation of photoassimilates, products of photosynthesis, to roots is essential for nodule formation (Kasperbauer & Hunt, 1994). A study undertaken by Kasperbauer and Hunt (1994) determined that exposing southern pea to low R:FR resulted in higher shoot:root biomass ratio, less massive roots and fewer nodules. Similar results were also found in soybean (Kasperbauer et al., 1984). Lie (1969) conducted a more in-depth experiment and determined that the nodulation of pea and broad bean plants were reduced when roots were exposed to far-red light for 5-15 minutes daily during five consecutive days following inoculation. None of these studies, however, involved weed competition.

Understanding the physiological mechanisms that occur in soybean roots in response to the presence of neighbouring weeds is critical to our understanding of non-limiting resource competition. Studies to-date have not identified how root physiology, including nodulation, is affected by the presence of neighbouring weeds. Therefore, we hypothesized that the low R:FR signal reflected from the vegetative tissue of neighbouring weeds, and perceived by the phytochrome system, will influence negatively soybean root structure and biomass, and reduce nodule number.

It is well known that reactive oxygen species, such as H$_2$O$_2$, increase under conditions of biotic and abiotic stress. It has also been well established that FR irradiance decreases anthocyanin production in a variety of crop plants, including tomato, cranberry and, more recently, corn (Rabino et al. 1997, Zhou and Singh 2002, Afifi and Swanton 2012).
Anthocyanins belong to a class of phenolic compounds known as flavonoids and as such, we hypothesize that if a reduction in nodule number occurred, it would be the result of an accumulation of H$_2$O$_2$ and a subsequent reduction in total flavonoid content.

2.2 Materials and Methods

2.2.1 Effect of aboveground neighbouring weeds on soybean root morphology

University of Guelph soybean variety, OAC Wallace soybean seeds were planted 2 cm deep into Turface MVP, a clay baked medium (Profile Products LLC Buffalo Grove, IL, USA) in clean, 8 cm diameter, 10 cm tall, 355 mL plastic cups (one seed per cup) (Dart Container Corporation, Mason, MI) (see Green-Tracewicz, 2010). Each cup was positioned into a slightly larger, plastic cylinder, modified to be 18 cm deep with a diameter of 8 cm (Consolidated Bottle Co., Toronto, ON). Both containers, the larger cylinder containing the smaller cup, were centred within a clean 14 cm diameter, 19 cm tall, 2.5 litre, white plastic pot (Airlite Plastics Company, Omaha, NE, USA) (see Green-Tracewicz, 2010). The remaining area surrounding the larger cylinder in the 2.5 L container was occupied with either Turface MVP, or with established perennial ryegrass. Ryegrass was sown three to four weeks prior to the planting of the soybean, in order to ensure sufficient time for establishment and subsequently to reflect a consistent R:FR prior to soybean emergence.

Ryegrass was used as the model weed species. This potting arrangement isolated the roots of the soybean seedlings from those of the perennial ryegrass, thereby eliminating the effects of direct root competition for water, nutrients or any allelopathic effect (Green-Tracewicz
& Swanton, 2011). The soybean seedlings and the ryegrass were watered and fertilized separately to ensure these resources were supplied to each plant species in non-limiting quantities. The soybean seedlings were watered daily, and fertilized twice per week with the complete nutrient solution described by Tollenaar (1989). The ryegrass was watered every other day, and fertilized with the same nutrient solution, two times per week. In order to minimize shading potential, the ryegrass was manually clipped as required to remain below the soybean unifoliate leaves.

All pots were placed within the same growth cabinet (Conviron, model PGW36, Controlled Environments Ltd., Winnipeg, MB) set to a 23:15°C day: night temperature and a 16:8 h (day: night) photoperiod, at 60-65% humidity. Irradiance was supplied by a sliding bank of Sylvania F48T12/CW/VHO 115 W Hg tubes and 40 W tungsten bulbs delivering a total of 550 umol m⁻²s⁻¹ PPFD. A point quantum radiometer (LI-190SA, LI-COR Biosciences Lincoln, NE, USA) with a cosine-corrected sensor on a fiber-optic cable was used to measure incoming PPFD at the top of the soybean seedlings.

Light quality treatments included: (1) high R:FR (herein referred to as “weed-free”), measured as upward reflected light from Turface MVP and, (2) low R:FR (herein referred to as “weedy”), measured as upward reflected light from ryegrass. Light quality was measured frequently during the experimental period. The R:FR of the light reflected from the ryegrass canopy and the Turface MVP media were measured using a R:FR sensor (SKR 110, 660/730nm, Skye Instruments Ltd, Llandrindod Wells, Powys, UK). This measurement was recorded immediately after the soybean seeds were planted and at random intervals during the growth period. The R:FR of incoming irradiance did not differ between treatments (approximate values of 2.5-2.8). The R:FR reflected from the surface of the ryegrass and Turface MVP was
determined by positioning the sensor downward, 5 cm above either surface at four different points within each treatment. The R:FR in the weed-free treatment, containing only Turface MVP, was (R:FR ± S.E.) 1.33 ± 0.15 and this ratio was 0.67 ± 0.16 in the weedy treatment.

A total of 128 pots, representing 64 weedy and 64 weed-free were placed in the growth cabinet. Twenty-four of the pots were positioned as border rows around the outside of the growth chamber. Within the growth cabinet, weedy and weed-free treatments were separated by a white opaque plastic divider to minimize any potential contamination of R:FR between treatments. Eight plants from each treatment were selected randomly for harvest at emergence (VE), cotyledon (VC), unifoliate (V1), first trifoliate (V2), and second trifoliate (V3) stages of development. Shoot height, and root morphology (including root volume, surface area, diameter, length and number of root tips) were measured and recorded for both weedy and weed-free treatments. A total of five replicates (in time) were completed in the same growth cabinet for this experiment. The weedy and weed-free sides of the chamber were randomly assigned for each replicate.

At each harvest, soybean roots were washed with tap water and cut from the shoot. Shoot height was recorded in order to confirm a shade avoidance response (i.e. stem elongation). The soybean shoot from each seedling was then placed into a clean, labelled paper towel and dried at 80°C to a constant weight. Roots from each individual plant were tagged and placed immediately into a plastic container, covered with tap water and stored at 4°C. Roots from each individual seedling were placed into a thin layer of water (2-3mm in depth) on a transparent tray (20 x 30 cm) for analysis using WinRhizo software (Regent Instruments Inc., Sainte-Foy, QC, Canada). Each individual root system was spread as evenly as possible on the tray and imaged at a medium resolution (200-400 dpi) using a Epson Expression 10000XL scanner (Epson America
Inc., Long Beach, CA, USA) to determine root length, surface area, volume, average diameter, and number of root tips. Upon completion of the analysis, each root system was placed onto a clean, labelled paper towel and dried at 80°C to a constant weight. Once dried, the individual weight of each root and shoot from each seedling was recorded for further analyses.

2.2.2 Effect of aboveground neighbouring weeds on soybean root nodule number, \( H_2O_2 \) content, DAB staining, lipid peroxidation, flavonoid content, DPPH-radicle scavenging activity, and radicle scavenging enzyme gene expression.

OAC Wallace soybean seedlings were grown in the same growth cabinet conditions, exposed to the same light treatments, and maintained in the same manner as the soybean seedlings for the morphology experiment (see detailed explanation of conditions above). Also, light measurements were taken in the same manner, at the same time points, and with the same equipment as detailed in the morphology experiment. However, based upon experimental protocols outlined in the appendix, the soybean seedlings were grown in a 1:1 mixture of Turface MVP and grade 2a vermiculite (Therm-O-Rock East Inc., New Eagle, PA) and inoculated with HiStick N/T (Becker Underwood (BASF), Saskatoon, SK, Canada) commercial inoculant peat. All soybean seedlings were harvested at the unifoliate stage of development.

After the roots of the soybean seedlings were washed, the numbers of root nodules on each individual root system were carefully counted using a standard tally counter. Once the root and shoot of each plant was dried to a constant weight at 80°C, the two components were put into a labelled bag, and sent to the University of Guelph’s Laboratory Services and analyzed for total carbon and total nitrogen content using the combustion method.
2.2.2.1 Analysis of $H_2O_2$ concentration

Hydrogen peroxide in the ground root tissues was estimated according to the protocol reported by Patterson et al., (1984). One hundred mg of frozen ground tissue was homogenized in 200 µl cold acetone. After centrifuging for 5 min at 10,000g, the supernatant was mixed with 20 µl of Titanium reagent (2% TiCl$_2$ in conc. HCl). The Ti-$H_2O_2$ complex was precipitated by adding 40 µl of 15 M ammonia solution. This solution was centrifuged as described above, then the pellet was washed with cold acetone two times and then dissolved in 1mL of 4 N H$_2$SO$_4$. The absorbance of the solution was measured at 410 nm against blanks which had been prepared similarly but without plant tissue.

2.2.2.2 Detection of $H_2O_2$ using DAB staining method

Detection of $H_2O_2$ in the root tissue using the DAB staining method was completed using the protocol reported by Thordal-Christensen et al., (1997). Roots of soybean seedlings were cut carefully and placed in glass containers containing 1 mg ml$^{-1}$ of 3,3 diaminobenzidine (DAB)-HCl (Sigma, MO, USA). Samples were incubated within the DAB solution overnight. Root samples were washed with 70% ethanol and results were photographically documented. The presence of $H_2O_2$ was detected as a reddish-brown colour within the root tissue.

2.2.2.3 Analysis of lipid peroxidation

Malondialdehyde (MDA) is one of the final products of peroxidation of unsaturated fatty acids found in phospholipids and is responsible for cell membrane damage (Halliwell & Gutteridge, 1984). Lipid peroxidation was measured by determining the MDA content of the ground root tissues using a thiobarbituric acid (TBA) reaction as described by Hara et al., (2003).
One hundred mg of frozen, ground, unifoliate soybean seedling root tissues were homogenized in 1 ml of 5 mM potassium phosphate buffer (pH 7). After centrifuging at 4 ºC for 15 min at 12,000 g, an aliquot of the supernatant (900 μL) was mixed with 600 μL of TBA solution containing: 10% (w/v) sodium dodecyl sulphate (SDS), 20% (w/v) acetic acid, 0.8% (w/v) aqueous TBA, and deionized water. The control reaction was a mixture of 900 μL of 5 mM KP buffer and 600 μL of the TBA solution. These mixtures were incubated at 98 ºC for 60 min, and then cooled to room temperature. Mixtures were centrifuged at 12,000 g for 15 min at room temperature. The absorbance of the mixture was measured at 535 nm and 600 nm. The MDA content was calculated from the subtracted absorbance (A535-A600) using a molecular extinction coefficient of (1.56x10^5 M^-1 cm^-1).

2.2.2.4 Analysis of flavonoid content

Total flavonoid contents of root tissue were measured with the aluminum chloride colorimetric assay using the protocol reported by Patel et al., (2010). One mL of 100% ethanol was added to 0.1g of ground, frozen, unifoliate soybean seedling root tissue. The mixture was vortexed on high for one minute, three times. Samples were then put into the centrifuge at 12,000 rpm for 15 minutes. The supernatant was transferred to a fresh 1.5 mL eppendorf tube and stored in the dark, at 4ºC, for further analysis.

In a fresh 2 mL eppendorf tube, 800 μL of distilled water, 200 μL of the above ethanolic extract, and 60 μL of 5% NaNO₂ were added. The mixture was briefly vortexed, and incubated at room temperature for 5 minutes. After the addition of 60 μL of 10% AlCl₃, the mixture was incubated at room temperature for an additional 6 minutes. Before transferring 1 mL of the mixture to a fresh cuvette, 400 μL of 1M NaOH and 480 μL of distilled water were added, and
the sample was vortexed. The absorbance of the reaction mixture was measured at 510 nm. Total flavonoid content of the samples was expressed as a percentage of Quercetin equivalent per gram fresh weight.

2.2.2.5 Analysis of DPPH-radicle scavenging activity

The antioxidant capacity of the sample extracts was tested by the evaluation of the free radicle-scavenging effect on the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicle, according to the method of Abe et al., (1998). Briefly, 0.1 g of frozen ground tissue from each seedling tissue was extracted with 1 ml of 99.5% methanol. The extract was then centrifuged at 12,000 g for 15 min. An aliquot of (100 μL) of the methanolic extract was mixed with 400 μL of absolute ethanol, 250 μL of 0.5 mM DPPH and 500 μL of 100 mM acetate buffer (pH 5.5). The mixture was vortex-mixed and kept in the dark for 30 min. The absorbance of the solution was measured at 517 nm against blanks of DPPH solution which had been prepared similarly but without plant tissue. Results were expressed as the percentage of inhibition of the DPPH radicle, which was calculated according to the following equation:

\[ \text{% inhibition of DPPH} = \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100 \]

Where “\(A_{\text{control}}\)” is the absorbance reading of DPPH in the solution without extracts and “\(A_{\text{sample}}\)” is the absorbance reading of DPPH within the sample solution.

2.2.2.6 RNA Isolation

RNA isolation was completed using the protocol outlined on the TRIzol Reagent (Life Technologies Inc., Burlington, ON, Canada). One hundred mg of ground, frozen unifoliate
soybean seedling root tissue was homogenized in 1 mL of TRIzol Reagent. Samples were then centrifuged at 12,000 g for 10 minutes at 2°C. The cleared homogenate solution was transferred to a fresh tube and incubated at room temperature for 5 minutes. Two hundred µL of chloroform was added, and each tube was vortexed for 15 seconds. Samples were again centrifuged at 12,000 g for 15 minutes at 2°C. The upper aqueous phase was transferred to a fresh tube, and the RNA was precipitated from the aqueous phase by the addition of 0.5 mL of isopropyl alcohol. Samples were incubated at room temperature for 10 minutes and then centrifuged at 12,000 g for 10 minutes at 2°C. The supernatant was removed, and the remaining RNA pellet was washed with 1 mL of cooled, 75% ethanol. The sample was briefly vortexed and centrifuged again at 7,500 g for 5 minutes at 2°C. The supernatant was again removed, and the RNA pellet was dried under vacuum for 5 minutes. One hundred µL of RNase-free water was added to the pellet and the tube was incubated at 60°C for 10 minutes. The pellet was dissolved by passing the solution several times through a pipette tip.

2.2.2.7 Analysis of genes expression using the quantitative real time PCR (QRT-PCR)

Quantitative real time PCR was conducted to test the transcript response of GmMnSOD, GmGPX, GmAPX2, GmCAT4, GmCuZnSOD, GmFeSOD, GmN93, and GmIFS genes to the presence of neighbouring weeds. Total RNA from each treatment was isolated from the different seedling tissues using Tripure-Reagent (Sigma-Aldrich, MO, USA). To eliminate any residual genomic DNA, total RNA was treated with RQ1 RNase-free DNase (Promega, WI, USA). The first strand cDNA was synthesized from total RNA by using the Reverse Transcription System Kit (Quanta, MD, USA). Primer Express 2.0 software (Applied Biosystems, CA, USA) was used to design the primers for the target genes (see description of primer sequences in Table 4).
Results were standardized to the housekeeping gene *GmUbi* (Matthews *et al.*, 2014). As described in Livak and Schmittgen (2001), relative quantification (RQ) values for each target gene relative to the internal control tubulin was calculated by the $2^{\Delta\Delta C_T}$ method (Livak & Schmittgen, 2001).

2.3 *Statistical analysis*

2.3.1 *Morphology*

Experiments were designed as a randomized complete block. Statistical analyses were performed in SAS v9.3 (SAS Institute, Cary, NC, USA) with a Type I error rate set at the 5% significance level. Soybean seedlings harvested at VE, VC, V1, V2 and V3 developmental stages were analyzed using a repeated measures analysis of variance (ANOVA) carried out using PROC MIXED, generating means and standard errors for each treatment at each stage. No transformations were required for analysis. In this experiment, replications were defined as growth cabinet environments in time and were combined for analysis. Replications were partitioned as random effects. Fixed effects included treatment, sampling time, and the interaction between these effects. Residual analysis was performed to test for the assumptions of ANOVA. Residuals and predicted values were plotted to ensure the homogeneity of variance, and independence of errors. The mean residuals of physiological and morphological parameters (including height, root surface area, root volume, root diameter, and number of root tips, as well as the normality of the error distribution) were tested with PROC UNIVARIATE. The Shapiro-Wilk statistic was used to test the assumption of normality. The significance of the random and fixed effects was tested using an F-test.
2.3.2 Physiology

Experiments were designed as a randomized complete block. Statistical analyses were performed in SAS v9.3 (SAS Institute, Cary, NC, USA) with a Type I error rate set at the 5% significance level. Soybean seedlings harvested at the unifoliate stage of development were analyzed using a one-way analysis of variance (ANOVA) carried out using PROC MIXED, generating means and standard errors for each treatment. No transformations were required for analysis. Growth cabinets were partitioned as random effects. Treatment was a fixed effect. Residual analysis was performed to test for the assumptions of ANOVA. Residuals and predicted values were plotted to ensure the homogeneity of variance, and independence of errors. The mean residuals of nodule number, DPPH-radicle scavenging activity, H$_2$O$_2$ content, lipid peroxidation, and flavonoid content, as well as the normality of the error distribution, were tested with PROC UNIVARIATE. The Shapiro-Wilk statistic was used to test the assumption of normality. The significance of the random and fixed effects was tested using F-test.

2.4 Results

Soybean seedlings express shade avoidance characteristics in response to the presence of aboveground neighbouring weeds. This study was conducted to confirm that soybean seedlings grown under the described conditions would express shade avoidance characteristics in response to the proximity of neighbouring weeds. As expected, plant height of weedy seedlings increased. Seedlings were first examined at emergence, and although there was an increase in plant height at this stage, it was not significant; however, from the cotyledon to second trifoliate stages, differences were apparent (Table 1). For example, at the cotyledon stage, shoot height was
increased from 4.5 cm ± 0.42 in weed-free seedlings to 5.3 cm ± 0.42 in weedy seedlings. The longer seedlings were exposed to low R:FR as a consequence of neighbouring weeds, the greater the increase in shoot height. By the second trifoliate stage, shoot height in weedy plants was 19.5 cm ± 0.42, as compared to 14.5 cm ± 0.42 in weed-free plants, a difference of approximately 5 cm. Although plants exposed to neighbouring weeds displayed the classic shade avoidance response of increased shoot height, this was not reflected in measurements of shoot dry weight, regardless of developmental stage.

Shoot dry weight did not differ between treatments, however, differences were found with root dry weight and the root: shoot ratio. Despite a difference of approximately 5 cm in shoot height at the second trifoliate stage of development for plants growing in the weedy treatment compared to the weed-free treatment, this height differential was not indicative of an increase in shoot dry weight. Seedlings exposed to neighbouring weeds weighed 0.50 g ± 0.010, compared to 0.51 g ± 0.010 for the weed-free plants at the second trifoliate leaf stage (Table 1). Root dry weight, however, was reduced by the presence of weeds sampled at the second trifoliate leaf stage. At this stage, root dry weight per plant was 0.17 g ± 0.007, compared to 0.20 g ± 0.007 in the weed-free plants. Differences in root: shoot between treatments were dependent on stage of soybean development, and were detected only at the cotyledon and unifoliate stages of soybean development (Table 2). These changes in root dynamics were also reflected in additional root parameters.

Low R:FR reflected from aboveground neighbouring weeds reduced soybean seedling root length, surface area, and volume. Total root length was decreased by the presence of aboveground neighbouring weeds only at the second trifoliate stage of development, compared to soybean seedlings grown in a weed-free environment (Table 2). At the second trifoliate leaf
stage, total root length of seedlings exposed to aboveground neighbouring weeds was 975 mm ± 24.8, compared to those kept weed-free (1063 mm ± 24.8). Accompanying this reduction in total root length was a decrease in root surface area and volume, detected at the first and second trifoliate stages of soybean development. At the first trifoliate leaf stage, root surface area was reduced from 134.4 cm² ± 5.52 in weed-free plants to 122.0 cm² ± 5.48 in weedy plants (Table 2, see also Figure 1). Similar results were observed in root volume (Table 3). By the first trifoliate stage, the presence of aboveground neighbouring weeds reduced the root volume of soybean seedlings from 2.05 cm³ ± 0.095 in the weed-free treatment to 1.80 cm³ ± 0.094. By the second trifoliate, root volume was further decreased from 2.65 cm³ ± 0.090 in weed-free plants to 2.20 cm³ ± 0.090.

Despite observed changes in root volume, the average root diameter and the number of root tips were not affected by presence of aboveground neighbouring weeds (Table 3). At emergence, for example, the average root diameter was 1.05 mm ± 0.022, compared to 1.08 mm ± 0.021 in weed-free plants. No differences were detected by the second trifoliate stage of soybean development. The average number of root tips followed a similar trend, as no differences were observed between treatments at any stage of soybean growth.

**Low R:FR decreased the number of nodules per plant.** Low R:FR reflected from neighbouring weeds decreased the number of root nodules per plant when sampled at the unifoliate stage of soybean development. Nodule numbers per plant were reduced from an average of 28 ± 2.7 in the weed-free treatment, compared to 20 ± 2.8 nodules per plant when grown in the presence of weeds, a difference of approximately 29%. At this stage of seedling development, however, this reduction in nodule number per plant did not result in any difference
in root nitrogen concentration (5.36 % dry ± 0.234 – weedy; 5.16 % dry ± 0.237 – weed-free). It did, however, result in a decrease in nodule number per gram of root dry weight (RDW) (151 ± 24.8 nodules g⁻¹ RDW – weedy; 260 ± 25.2 nodules g⁻¹ RDW – weed-free). This reduction or possible delay in nodule development may have occurred as a result of physiological changes within the root system in response to exposure to low R:FR from aboveground neighbouring weeds.

**Low R:FR increased H₂O₂ content and lipid peroxidation in soybean seedling roots.** Low R:FR reflected from aboveground neighbouring weeds resulted in an increase in H₂O₂ and MDA content in root tissue of unifoliate soybean seedlings. The H₂O₂ content increased from 0.54 nM g⁻¹ FW ± 0.023 in the weed-free treatment, compared to 0.72 nM g⁻¹ FW ± 0.023 in the weedy treatment (Figure 2). This increase in the root tissue H₂O₂ content was visualized and confirmed by DAB staining at both the unifoliate and 1st trifoliate stages of soybean development (Figure 3). A similar response was observed for MDA content (Figure 4). In the weed-free treatment, MDA content of the root tissue was 32.8 nM g⁻¹ FW ± 1.45 vs. 41.4 nM g⁻¹ FW ± 1.45 in the weedy treatment.

**Low R:FR decreased flavonoid content and DPPH-radicle scavenging activity in soybean seedling roots.** Root flavonoid content and DPPH-radicle scavenging activity was lower in unifoliate soybean seedlings exposed to above-ground neighbouring weeds, compared to soybean seedlings grown in a weed-free environment (Figure 5). Total flavonoid content in the weed-free treatment was 3.18 mg g⁻¹ FW ± 0.122, compared to 2.38 mg g⁻¹ FW ± 0.122 in the weedy treatment. In addition, this decline in flavonoid content was accompanied by a similar
decrease in DPPH-radicle scavenging activity. The DPPH-radicle scavenging activity in the root tissue was reduced from 33.07 % of control ± 0.743 in the weed-free treatment to 21.15 % of control ± 0.743 in the weedy treatment (Figure 6).

In order to explore the molecular mechanisms contributing to this finding, a quantitative real time PCR was conducted to investigate the transcription level of *GmIFS*, a key gene involved in flavonoid biosynthesis (Yoo *et al.*, 2013), and *Gm*N93, a key gene involved in nodule formation (Reddy *et al.*, 1998) (see Table 4). The presence of neighbouring weeds caused a significant reduction in the expression of the *GmIFS* gene, and of the *Gm*N93 gene. For example, *GmIFS* gene expression was reduced to more than half-fold (0.43-fold) in soybean seedlings growing under weedy conditions, compared to those under weed free conditions (Figure 7). This inhibition of *GmIFS* gene expression would contribute to the observed reduction of total flavonoid content under weedy conditions. In addition, the *Gm*N93 transcription level was reduced to (0.49-fold) under the weedy treatment vs. one fold under weed free treatment. This gene inhibition may account for the lower number of nodules formed on soybean roots growing in the presence of neighbouring weeds and exposed to low R:FR signal.

**Low R:FR regulated the transcription of the scavenging enzymes genes in soybean roots.**

Qualitative real time PCR was conducted to test the effect of neighbouring weeds on the transcript levels of *GmAPX3, GmCAT, GmCuZnSOD, GmGPX, GmFeSOD, GmMnSOD* (see Table 4). These six common genes are known to encode for the production of scavenging enzymes. Interestingly, the transcript level of all six genes increased in the root tissue of soybean seedlings exposed to low R:FR, compared with the weed-free control (Figure 7). The transcript levels for the six genes, *GmAPX3, GmCAT, GmCuZnSOD, GmGPX, GmFeSOD,* and
GmMnSOD were 1.86-, 1.61- 1.40-, 1.58-, 1.91-, and 1.54-fold higher, respectively, in seedlings exposed to low R:FR relative to seedlings kept weed-free.

2.5 Discussion

The expression of shade avoidance characteristics in response to a low R:FR caused by the presence of neighbouring weeds has been well documented in the literature (Kasperbauer & Karlen, 1994; Skálová & Vosátka, 1998; Pecháčková, 1999; Sparkes & King, 2008; Liu et al., 2008; Page et al., 2009; Afifi & Swanton, 2011). Much work has also been done to confirm this phenomenon using a far red filter (Afifi & Swanton, 2011). In 2011, Afifi & Swanton confirmed that the shade avoidance response caused by a far red filter was “comparable to the weedy treatment.” Therefore, despite the fact that a far red filter was not included on the treatment list in this experiment, we believe it is reasonable to assume that the shade avoidance characteristics and resulting biochemical changes documented in this experiment are attributable to a reduction in the R:FR.

Under conditions of non-limiting resources, the presence of aboveground neighbouring weeds reduced soybean seedling root biomass, length, surface area and volume. Reductions in these root parameters were first evident in root surface area and volume at the first trifoliate leaf stage. Reductions in root length were detected by the second trifoliate leaf stage. A reduction in root biomass attributed to low R:FR has been reported in several studies (Kasperbauer & Karlen, 1994; Skálová & Vosátka, 1998; Pecháčková, 1999; Sparkes & King, 2008; Liu et al., 2008; Page et al., 2009; Afifi & Swanton, 2011). Sparkes and King (2008) observed a reduction in the root volume of wheat under low R:FR, even though the light quantity (PPFD) was held constant.
Afifi and Swanton (2011) found a reduction in total root volume, surface area, and biomass of maize seedlings exposed to low R:FR reflected from aboveground neighbouring weeds.

Prior to this study, limited work had been done to understand how root morphology in soybean changes in response to low R:FR reflected from neighbouring weeds. For example, Green-Tracewicz et al., (2011; 2012) conducted two studies on the expression of the shade avoidance response in soybean plants as a consequence of weed competition. This previous research established clearly that FR light had a significant negative influence on soybean root biomass. These researchers did not study, however, the influence of FR light on specific root morphological parameters, such as length, surface area, and volume. The reduction found in root morphological parameters in the present study would explain the loss in total root biomass reported in this and previous studies (Green-Tracewicz et al., 2011; 2012). Results from the present study indicate that changes in root growth and morphology can be influenced negatively very early in soybean seedling development. The soybean root system is responsible for providing anchorage and uptake of water and nutrients; therefore, any alteration in length, surface area, volume, and/or biomass will reduce the seedling’s ability to explore for and capture soil nutrients. In addition, these early changes may limit the ability of soybean seedlings to respond appropriately to additional biotic and abiotic stresses. Changes in root growth and morphology observed in response to FR reflected from aboveground neighbouring weeds may contribute to the rapid yield loss observed under field conditions in response to weeds that emerge with the soybean crop.

In this study, soybean seedlings continuously exposed to aboveground neighbouring weeds, a biological source of FR, exhibited a reduction in nodule number per plant. The inhibition of the GmN93 gene would, in part, account for this reduction. A few studies were
conducted to examine the effects of FR light on legume nodulation (Sheehy et al., 1983; Kasperbauer et al., 1984; Balatti & Montaldi, 1986; Kasperbauer & Hunt, 1994; Lie, 1969). These researchers, however, examined the effects of pulses of supplemental FR on various crop plants, including pea and soybean, and concluded similarly that a reduction in R:FR reduced legume root nodule numbers.

In addition, the roots of soybean seedlings exposed to FR light as a consequence of aboveground weed competition exhibited a reduction in total flavonoid content, relative to those seedlings kept weed-free. Flavonoids serve two essential roles in soybean seedlings: 1) acting as signal molecules to symbiotic microbes, and 2) non-enzymatic ROS scavenging (Taylor & Grotewold, 2005; Subramanian et al., 2007 and references therein). It has been well established that several flavonoids exuded from plant roots act as signal molecules inducing the transcription of bacterial genes, initiating the infection process (Treutter, 2006; and references therein). This is a significant role that flavonoids play in improving plant growth and fitness (Treutter, 2006). The reduction in total root flavonoid content found in soybean seedlings exposed to aboveground weed competition may also help to explain the reduction in nodule number per plant, as the seedling’s ability to communicate with the Rhizobia bacteria in the soil and to establish a symbiotic relationship is affected negatively. Reducing the nodule number per plant of soybean seedlings could lead to a nutrient deficiency, which could decrease crop growth rate, and ultimately reduce yield. Molecular mechanisms behind the reduction of soybean seedling nodulation as a consequence of exposure to aboveground neighbouring weeds were also explored.

Exposure to low R:FR conditions triggered the accumulation of H$_2$O$_2$ and subsequent lipid peroxidation of cell membranes in root tissue of soybean seedlings at the unifoliate stage of
development. Hydrogen peroxide is a well-known reactive oxygen species (ROS), which has been observed to increase under conditions of biotic and abiotic stress (Gill & Tuteja, 2010). Interestingly, under growth conditions in which all resources (light, water and nutrients) were supplied in sufficient quantities in order to eliminate direct competition, the production of H$_2$O$_2$ was triggered by the FR signal. As with the rapid changes in root growth and morphology, changes in H$_2$O$_2$ content were detected very early in soybean development. Similarly, Afifi and Swanton (2012), and Afifi et al., (2014) reported accumulation of H$_2$O$_2$ in the first leaf and crown root tissue of maize seedlings exposed to the presence of aboveground neighbouring weeds. The accumulation of H$_2$O$_2$ in the roots of soybean seedlings can result in major cellular damage, such as DNA alterations, oxidation of proteins and lipid peroxidation (Gill & Tuteja, 2010).

Levels of lipid peroxidation have been used widely as an indicator of ROS mediated damage to cell membranes under stress conditions (Tanou et al., 2009, Mishra et al., 2011). Recently, Afifi et al., (2014) found an increase MDA content in the first leaf and crown root tissue of maize seedlings exposed to low R:FR as a consequence of the presence of aboveground neighbouring weeds. The buildup of H$_2$O$_2$ and the subsequent cellular damage would reduce soybean seedling vigour and invariably reduce the ability of soybean seedlings to respond to further biotic and abiotic stress.

In this study, the transcript level of GmAPX3, GmCAT, GmCuZnSOD, GmGPX, GmFeSOD, and GmMnSOD in the roots of unifoliate soybean seedlings were found to increase under low R:FR conditions. Similar results were found in maize by Afifi and Swanton (2012) and Afifi et al., (2014). Enhanced production of ROS during stress can damage cells; however, it is also thought to act as a signal for the activation of stress-response and defense pathways of
scavenging enzymes (Gill & Tuteja, 2010). Therefore, the accumulation of H$_2$O$_2$ in the root tissue of unifoliate soybean seedlings exposed to the above-ground low R:F ratio is consistent with an increase in the transcript levels of ($Gm$APX3, $Gm$CAT, $Gm$CuZnSOD, $Gm$GPX, $Gm$FeSOD, $Gm$MnSOD).

Plants have several mechanisms that can reduce H$_2$O$_2$ production during a stressful period. These mechanisms include anatomical adaptations, and physiological and molecular changes (Mittler, 2002). Plants with the ability to scavenge and/or control the level of cellular H$_2$O$_2$ will be better adapted to survive (Gill & Tuteja, 2010). Efficient scavenging of ROS requires the action of both enzymatic and non-enzymatic scavenging mechanisms (Sharma et al., 2012). The enzymatic mechanism includes enzymes such as: ascorbate peroxidase (APX); catalase (CAT); various forms of superoxide dismutase, (SOD); and glutathione peroxidise (GPX). Non-enzymatic compounds, such as ascorbic acid, phenols, flavonoids, and anthocyanins, are known to be involved in antioxidant defence systems (Treutter, 2006).

Polyphenols, such as flavonoids, can chelate transition metal ions, directly scavenge ROS, delay diffusion of free radicles, limit peroxidative reactions and inhibit lipid peroxidation (Perveen et al., 2013). For example, transgenic potato plants with an increased concentration of flavonoids showed improved antioxidant capacity (Sharma et al., 2012).

Unifoliate soybean seedlings that were exposed to low R:F ratio reflected from neighbouring weeds had a reduction in both flavonoid content and DPPH-radicle scavenging activity in root tissue. This reduction of flavonoid content may be attributed to the observed down regulation of $Gm$IFS. Similar results were reported by Afifi et al., (2014), who investigated the effects of aboveground neighbouring weeds on the total phenolic content, of which flavonoids are a component, and DPPH-radicle scavenging activity of maize seedlings. It was found that the
presence of neighbouring weeds reduced total phenolic content in the first leaf, stem and crown roots of maize seedlings (Afifi et al., 2014). An identical response was observed in the DPPH-radicle scavenging activity in the same tissues (Afifi et al., 2014). Thus, the reduction found in soybean root flavonoid content and DPPH-radicle scavenging activity of seedlings exposed to weedy conditions is indicative of a decline in the plant’s ability to non-enzymatically scavenge for ROS. This decline increases the potential for these molecules to cause damage within the seedling, potentially decreasing the ability of the seedling to deal with subsequent stresses, both biotic and abiotic.

Biotic and abiotic stresses, including insect and disease infestation, have been reported to influence the flavonoid content in various crops (Treutter, 2006). Both frost hardiness and drought tolerance have been attributed to “flavonoids or other phenolic compounds with respect to functions in the cell wall and membranes” (Tattini et al., 2004; Treutter, 2006; and references therein). It has also been reported that flavonoids may play a role in toxic metal, such as aluminum, tolerance (Barceló & Poschenrieder, 2002). In 2002, Ryan et al., found that flavonoids play a predominant role in photo-protection in Petunia leaves. The present study, however, is the first to report that the low R:FR signal reflected from aboveground neighbouring weeds can affect root flavonoid content. Exposure to aboveground neighbouring weeds reduced flavonoid content in the roots of unifoliate soybean seedlings.

2.6 Conclusion

Early physiological mechanisms of weed competition were explored in this study. These mechanisms occurred in soybean seedlings grown under conditions of non-limiting resources in
response to the detection of the R:FR signal reflected from aboveground neighbouring weeds. The detection of low R:FR triggered a series of physiological changes that occurred within the soybean seedlings very early in development in response to pending weed competition. Low R:FR altered soybean root morphology, caused a reduction in nodule number per plant, an accumulation of H₂O₂, an increase in MDA content, a reduction in flavonoid content, and a decrease in DPPH-radicle scavenging activity. The reduction in flavonoid content was accompanied by a decrease in the transcription of GmIFS and GmN93. It was also found that the transcript levels of GmAPX3, GmCAT, GmCuZnSOD, GmGPX, GmFeSOD, GmMnSOD were increased (relative to weed-free plants) in the roots of soybean seedlings exposed to aboveground neighbouring weeds. It is hypothesized that these changes resulted in a physiological cost to the crop plant, which will contribute to the yield loss observed in weed competition studies conducted under field conditions. Studying interactions between aboveground signals and below ground plant responses is expected to improve our integrative understanding of the mechanisms of plant competition.
### 2.7 Tables

**Table 1.** The effect of above ground neighbouring weeds on shoot height (cm), shoot dry weight (g), and root dry weight (g) measured from soybean emergence until the 2nd trifoliate stage of soybean development. Data are means (± SE). Type I error rate set at 5% significance level.

<table>
<thead>
<tr>
<th>DAP</th>
<th>Soybean Stage</th>
<th>Shoot Height (cm)</th>
<th>Shoot Dry Weight (g)</th>
<th>Root Dry Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Treatment</td>
<td>Treatment</td>
<td>Treatment</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Weedy</td>
<td>Weed-Free</td>
<td>P-value</td>
</tr>
<tr>
<td>6</td>
<td>VE</td>
<td>2.7 (0.44)</td>
<td>2.4 (0.44)</td>
<td>0.59</td>
</tr>
<tr>
<td>8</td>
<td>Cotyledon</td>
<td>5.3 (0.42)</td>
<td>4.5 (0.42)</td>
<td>0.03</td>
</tr>
<tr>
<td>12</td>
<td>VC</td>
<td>9.4 (0.42)</td>
<td>7.4 (0.41)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>17</td>
<td>V1</td>
<td>15.1 (0.44)</td>
<td>10.8 (0.44)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>21</td>
<td>V2</td>
<td>19.5 (0.42)</td>
<td>14.5 (0.42)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

DAP – Days after Planting
Table 2. The effect of above ground neighbouring weeds on root: shoot, total root length (mm), and root surface area (cm$^2$) measured from soybean emergence until the 2$^{nd}$ trifoliate stage of soybean development. Data are means ($\pm$ SE). Type I error rate set at 5% significance level.

<table>
<thead>
<tr>
<th>DAP</th>
<th>Soybean Stage</th>
<th>Root: Shoot Treatment</th>
<th>Root: Shoot P-value</th>
<th>Total Root Length (mm) Treatment</th>
<th>Total Root Length P-value</th>
<th>Root Surface Area (cm$^2$) Treatment</th>
<th>Root Surface Area P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>VE</td>
<td>Weedy 0.11 (0.053)</td>
<td>0.5</td>
<td>Weedy 7 (26.8)</td>
<td>0.93</td>
<td>Weedy 2.9 (5.61)</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Weed-Free 0.16 (0.050)</td>
<td></td>
<td>Weed-Free 5 (26.2)</td>
<td></td>
<td>Weed-Free 2.7 (5.48)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Cotyledon</td>
<td>Weedy 0.18 (0.045)</td>
<td>0.05</td>
<td>Weedy 74 (24.8)</td>
<td>0.83</td>
<td>Weedy 18.2 (5.23)</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Weed-Free 0.30 (0.045)</td>
<td></td>
<td>Weed-Free 80 (24.7)</td>
<td></td>
<td>Weed-Free 19.3 (5.20)</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>VC</td>
<td>Weedy 0.31 (0.046)</td>
<td>0.04</td>
<td>Weedy 224 (25.0)</td>
<td>0.30</td>
<td>Weedy 49.6 (5.26)</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Weed-Free 0.44 (0.045)</td>
<td></td>
<td>Weed-Free 248 (24.7)</td>
<td></td>
<td>Weed-Free 53.8 (5.20)</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>V1</td>
<td>Weedy 0.37 (0.050)</td>
<td>0.39</td>
<td>Weedy 662 (26.1)</td>
<td>0.13</td>
<td>Weedy 122.0 (5.48)</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Weed-Free 0.43 (0.051)</td>
<td></td>
<td>Weed-Free 702 (26.4)</td>
<td></td>
<td>Weed-Free 134.4 (5.52)</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>V2</td>
<td>Weedy 0.35 (0.045)</td>
<td>0.54</td>
<td>Weedy 975 (24.8)</td>
<td>0.0002</td>
<td>Weedy 163.4 (5.23)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Weed-Free 0.39 (0.045)</td>
<td></td>
<td>Weed-Free 1063 (24.8)</td>
<td></td>
<td>Weed-Free 187.5 (5.23)</td>
<td></td>
</tr>
</tbody>
</table>

DAP – Days after Planting
**Table 3.** The effect of above ground neighbouring weeds on root volume (cm$^3$), average root diameter (mm) and number of root tips measured from soybean emergence until the 2$^{nd}$ trifoliate stage of soybean development. Data are means (± SE). Type I error rate set at 5% significance level.

<table>
<thead>
<tr>
<th>DAP</th>
<th>Soybean Stage</th>
<th>Root Volume (cm$^3$)</th>
<th>Average Root Diameter (mm)</th>
<th>Number of Root Tips</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Weedy</td>
<td>Weed-Free</td>
<td>P-value</td>
</tr>
<tr>
<td>6</td>
<td>VE</td>
<td>0.10 (0.096)</td>
<td>0.10 (0.094)</td>
<td>0.97</td>
</tr>
<tr>
<td>8</td>
<td>Cotyledon</td>
<td>0.36 (0.090)</td>
<td>0.38 (0.090)</td>
<td>0.80</td>
</tr>
<tr>
<td>12</td>
<td>VC</td>
<td>0.88 (0.090)</td>
<td>0.93 (0.089)</td>
<td>0.53</td>
</tr>
<tr>
<td>17</td>
<td>V1</td>
<td>1.80 (0.094)</td>
<td>2.05 (0.095)</td>
<td>0.006</td>
</tr>
<tr>
<td>21</td>
<td>V2</td>
<td>2.20 (0.090)</td>
<td>2.65 (0.090)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

DAP – Days after Planting
Table 4. Primers sequences used in performing quantitative real-time polymerase chain reaction.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Forward Primer Sequence</th>
<th>Reverse Primer Sequence</th>
<th>Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>GmMnSOD</td>
<td>5’ GGTCTGGACAAAGAGTTGAAGA 3’</td>
<td>5’ GCATGCTCCCCAACATCAATAC 3’</td>
<td>EF587264</td>
</tr>
<tr>
<td>GmGPX</td>
<td>5’ GACAAAGCTGCTCCACTGTA 3’</td>
<td>5’ GATCAAACCACATTCCCTTTTATC 3’</td>
<td>Glyma05g37900.3</td>
</tr>
<tr>
<td>GmAPX3</td>
<td>5’ CCCTGGACCTCTAATCCTCTTA 3’</td>
<td>5’ CTTGTCAGAAGGTAGCTGAAAG 3’</td>
<td>U56634.1</td>
</tr>
<tr>
<td>GmCAT14</td>
<td>5’ ATTGGAGGAGAGGGGATTAAAG 3’</td>
<td>5’ CTAACAGTTTCCACTCAGGATAG 3’</td>
<td>NM_001250642.1</td>
</tr>
<tr>
<td>GmCuZnSOD</td>
<td>5’ CTGGACCAACTCCATCATAGG 3’</td>
<td>5’ TACTTCGCCACCAGCATTTTCC 3’</td>
<td>NM_001248369</td>
</tr>
<tr>
<td>GmFeSOD</td>
<td>5’ GCTTGATGGAGATCAGTAG 3’</td>
<td>5’ CATGCACCTCCCAGAAGATG 3’</td>
<td>M64267</td>
</tr>
<tr>
<td>GmN93</td>
<td>5’ GCAGTTGTTGGACGATGTGG 3’</td>
<td>5’ GAGAGCTTGGAGCTGTGTGATT 3’</td>
<td>D13506</td>
</tr>
<tr>
<td>GmIFS</td>
<td>5’ GGAGAGAAGAGAAGAAGACAA 3’</td>
<td>5’ TGTCACCTCCACTTCTTAG 3’</td>
<td>FJ770473.1</td>
</tr>
</tbody>
</table>
2.8 Figures

Figure 1. First trifoliate soybean seedling roots (weedy – left; weed-free – right) as imaged at a medium resolution (200-400 dpi) using an Epson Expression 10000XL scanner for analysis by WinRhizo software.
Figure 2. Hydrogen peroxide (H$_2$O$_2$) content in the root tissue of unifoliate soybean seedlings as influenced by the above-ground neighbouring weeds. WF and W refer to soybean seedlings grown under weed-free and weedy conditions, respectively.
Figure 3. $\text{H}_2\text{O}_2$ detection in unifoliate (A) and first trifoliate (B) root tissue of soybean seedling using DAB staining method.
**Figure 4.** Malondialdehyde (MDA) content in root tissue of soybean seedlings as influenced by the above-ground neighbouring weeds at the unifoliate stage of soybean development. WF and W refer to soybean seedlings grown under weed-free and weedy conditions, respectively.
Figure 5. Flavonoid content in root tissue of soybean seedlings as influenced by the above-ground neighbouring weeds at the unifoliate stage of soybean development. WF and W refer to soybean seedlings grown under weed-free and weedy conditions, respectively.
Figure 6. DPPH-radicle scavenging activity in the root tissue of soybean seedlings as influenced by the above-ground neighbouring weeds at the unifoliate stage of soybean development. WF and W refer to soybean grown under weed-free and weedy conditions, respectively.
Figure 7. QRT-PCR analysis of the transcripts level of isoflavone synthase (GmIFS), an early nodulin gene (GmN93), ascorbate peroxidase (GmAPX3), catalase (GmCAT), copper-zinc superoxide dismutase (GmCuZnSOD), glutathione peroxidase (GmGPX), iron superoxide dismutase (GmFeSOD), and manganese superoxide dismutase (GmMnSOD) as influenced by the presence of above-ground neighbouring weeds at the unifoliate stage of soybean development. Data presented relative to weed-free treatment.
Chapter 3

3.0 General Discussion

3.1 Summary and Research Contributions

A plant’s ability to detect aboveground signals and transfer this information to roots is an essential survival strategy to ensure optimum fitness. Studies to-date have not identified how root physiology, including nodulation, is affected by the presence of aboveground weeds. This was the first experiment to examine early physiological mechanisms affecting soybean roots and nodules in response to the presence of aboveground neighbouring weeds. This research discovered that aboveground plant communication can alter root structure and physiology. The root characteristics studied included detailed morphology, nodule number per plant, $\text{H}_2\text{O}_2$ content, lipid peroxidation, total flavonoid content, DPPH-radicle scavenging activity, and the transcription of the major scavenging enzyme genes. This research found that soybean seedlings express shade avoidance characteristics, and a reduction in nodule number in response to aboveground neighbouring weeds. In addition, it was also determined that low R:F:R increased $\text{H}_2\text{O}_2$ content and lipid peroxidation, decreased flavonoid content and DPPH-radicle scavenging activity, and FR light regulated the transcription of the scavenging enzymes genes in soybean seedling roots.

Arguably, a major objective of the research process is to answer questions and acquire innovative knowledge to provide explanations and increase the understanding of the world, its components and how it got to be the way it is now. Prior to this study, there was very limited knowledge on how plant competition affected root morphology, physiology and nodulation. This
research begins to provide insight into the molecular pathway of how FR affects crop plants. Understanding these mechanisms may aid in the development of soybean varieties that are more tolerant of the consequences of weed competition, and an increase in the yield potential of the soybean crop plant.

3.2 Research Limitations

This study was limited by the measurements and observations that were taken. The experimental design did not allow for the incorporation of a non-biological (i.e. commercial red filter) source of FR light. This study was also unable to include a field study component. As this was the first study to begin examining the molecular changes at the root level in soybean seedlings exposed to aboveground weed competition, particularly detailed exploration into each molecular avenue studied was not possible (e.g. the specific flavonoid(s) that was reduced by the low R:FR).

3.3 Future Research Directions

There are several future studies that could be proposed out of this work. One study may examine the physiological responses of soybean seedling roots exposed to a non-biological source of FR light. Another experiment might be utilized to determine the physiological response of field-grown soybean seedlings to low R:FR. The potential role of blue light in the R:FR response could also be explored as the blue light and red/far-red light receptors are known to work in conjunction (Lin, 2002). A further study could examine the response of specific flavonoids to the low R:FR signal, and perhaps the associated gene expression. While there are many physiological responses that could be investigated in more detail, that of specific flavonoid
production is particularly interesting as certain flavonoids are required for successful nodulation, as well as aid in pathogen defense, among other roles. It would be beneficial to soybean production and yield to determine exactly what impact the presence of aboveground weeds has on legume roots on a genetic, molecular and physiological level.
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Appendix

Materials & Methods

Optimizing growth conditions for soybean nodulation

Growth Medium

An experiment was conducted in order to select the most appropriate growth medium to study the effects of weed competition on soybean nodulation. Four different growth media/combinations of growth media were tested: (1) Turface MVP, (2) grade 2a vermiculite, (3) 1:1 mixture of Turface MVP: grade 2a vermiculite, and (4) 1:1 mixture of Turface MVP:QuickDry (Profile Products LLC Buffalo Grove, IL, USA). Twelve white plastic pots, 14 cm in diameter, 19 cm tall, and 2.5 L in volume (Airlite Plastics Company, Omaha, NE, USA), were filled with each medium, for a total of 48 pots. The pots were randomly arranged in a growth cabinet. Three replications of this experiment were performed. Each pot, containing the appropriate medium, was watered before planting one soybean seed (OAC Wallace) 2 cm deep. Before covering the seed, 5 mL of inoculant slurry was added using a syringe. The slurry consisted of 63 mL of HiStick N/T commercial inoculant peat mixed with 125 mL of water. Seeds were then covered, and watered again.

Plants were watered and fertilized daily using a Dosmatic Advantage A20 water-driven liquid injector (Dosmatic U.S.A., Carrollton, TX, U.S.A.) set to inject 40:1 (water:1% solution of 20-20-20) to ensure these resources were supplied in non-limiting quantities throughout the duration of the experiment. All plants were harvested when the first trifoliate leaves were fully
expanded. Each root system was cut from the stem and washed with tap water prior to analysis. The length of the root, and number of root nodules, was documented for each plant. As was done for the morphology experiment, the roots were analyzed using the WinRhizo system. Upon completion of the analysis, each root was placed into a clean, labelled paper towel and put in the oven to be dried at 80°C to a constant weight. Once the root and shoot of each plant was dried, the weight of each component was taken and recorded for analysis.

_Inoculation Method_

An experiment was conducted in order to select the most appropriate method of inoculation to study of the effects of weed competition on soybean nodulation. Two different inoculation methods were tested on two different growth mediums, Turface MVP, and a 1:1 mixture of Turface MVP:Vermiculite. The first inoculation method was the application of 5 mL of inoculate slurry, as described above, to each seed (hereafter referred to as “seed inoculated”). The second inoculation method involved the application of HiStick N/T commercial inoculant peat directly to the growth medium (hereafter referred to as “whole pot inoculated”). Twelve pots (30 L total volume), 14 cm in diameter, 19 cm tall, and 2.5 L in volume (Airlite Plastics Company, Omaha, NE, USA), of Turface MVP were mixed with 230 g of HiStick N/T inoculant peat to create the whole pot inoculated Turface MVP treatment, while six pots of Turface MVP (15 L) and six pots (15 L) of Vermiculite were mixed with 230g of HiStick N/T inoculant peat to create the whole pot inoculated Turface MVP:Vermiculite treatment.

Twelve white plastic pots (30 L total), 14 cm in diameter, 19 cm tall, and 2.5 L in volume (Airlite Plastics Company, Omaha, NE, USA), were filled with each growth medium/ inoculation method combination, for a total of 48 pots. The pots were randomly arranged in a growth cabinet
and watered. Three replications of this experiment were performed. One OAC Wallace soybean seed was planted 2 cm deep in each pot. Depending on the inoculation method, seeds were either immediately covered (for the whole pot inoculated plants), or had 5 mL of inoculant slurry applied before covering the seed (seed inoculated plants). Each pot, regardless of treatment, was watered again immediately after planting. Plants were watered and fertilized daily using a Dosmatic Advantage A20 water-driven liquid injector (Dosmatic U.S.A., Carrollton, TX, U.S.A.) set to inject 40:1 (water:1% solution of 20-20-20) to ensure these resources were supplied in non-limiting quantities throughout the duration of the experiment.

All plants were harvested when the first trifoliate leaves were fully expanded. Each root system was cut from the stem and washed with tap water prior to analysis. The length of the root and the number of root nodules was documented for each plant. The roots were analyzed using the WinRhizo system. Upon completion of the analysis, each root was placed into a clean, labelled paper towel and put in the oven to be dried at 80°C to a constant weight. Once the root and shoot of each plant was dried, the weight of each component was taken and recorded for analysis.

**Fertilizer Blend**

An experiment was conducted in order to select the most appropriate type of fertilizer to study the effects of weed competition on soybean nodulation. Three different fertilizer blends were tested: (1) 1% solution of 20-20-20, (2) solution described in Tollenaar (1989), and (3) half strength Hoagland’s solution. The solution described in Tollenaar (1989) was prepared by adding 160 g of 28-14-14, 160 g of 15-15-30, 80 g of NH₄NO₃, 160 g of MgSO₄·H₂O, and 12 g of Plant-Prod Chelated Micronutrient Mix (Plant Products, Ancaster, Ontario, Canada) to a 20 L pail
filled with water, and mixing thoroughly. Thirty-six white plastic pots, 14cm in diameter, 19cm tall, and 2.5 litres in volume (Airlite Plastics Company, Omaha, NE, USA), were filled with a 1:1 mixture of Turface MVP: Vermiculite. The pots were randomly arranged in a growth cabinet. Three replications of this experiment were performed. Each pot was watered before planting one soybean seed (OAC Wallace) 2 cm deep. Before covering the seed, 5 mL of inoculant slurry was added using a syringe. Seeds were then covered, and watered again.

Plants were simultaneously watered and fertilized to capacity with the appropriate fertilizer to ensure these resources were supplied in non-limiting quantities throughout the duration of the experiment. All plants were harvested when the first trifoliate leaves were fully expanded. Each root system was cut from the stem and washed with tap water prior to analysis. The length of the root, and number of root nodules was documented for each plant. The roots were analyzed using the WinRhizo system. Upon completion of the analysis, each root was placed into a clean, labelled paper towel and put in the oven to be dried at 80°C to a constant weight. Once the root and shoot of each plant was dried, the weight of each component was taken and recorded for analysis.

*Nodulation Timing*

An experiment was conducted to determine when nodules began to form on soybean seedlings grown in a controlled environment in a 1:1 mixture of Turface MVP: Vermiculite, inoculated with 5mL of inoculant slurry, and fertilized with the solution described in Tollenaar (1989). Twenty-four OAC Wallace soybean seeds were planted and inoculated as described above. Four plants were harvested when the seedlings reached the cotyledon stage, and each following day for the next five days. Each time a harvest was performed, the roots were washed
in tap water and the presence/absence of nodules was documented. This experiment was replicated three times.

_Growth Medium and Shade Avoidance_

A study was conducted in order to confirm that the soybean plants grown in the 1:1 Turface MVP: Vermiculite mixture were still receiving the low R:FR signal. The plants were grown in the same controlled environment conditions described above. Sixty-four cups were filled with 1:1 Turface MVP: Vermiculite and 64 cups were filled with only Turface MVP. Thirty-two cups of each growth medium were placed in the weedy half of the growth chamber, and the other 32 cups of each were placed on the weed-free side. OAC Wallace soybean seeds were planted 2 cm deep in each cup. All plants were harvested when the 1\textsuperscript{st} trifoliate leaves were fully expanded. Shoot height was recorded in order to confirm a shade avoidance response. The soybean shoot from each plant was then placed into a clean, labelled paper towel and dried at 80°C to a constant weight. Roots from each individual plant were tagged and placed immediately into a plastic container and covered with tap water and stored at 4°C until WinRhizo analysis could be performed. WinRhizo analysis was performed on all root systems.

**Results**

_Growth medium considerably affected soybean seedling root surface area and the number of root nodules._ Changing the type of medium soybean seedlings were grown in reduced the root surface area by as much as 62% by the first trifoliate stage of development. For example, when seeds were sown into a 1:1 mixture of Turface MVP and vermiculite, root surface area was
203.8 cm$^2 \pm 35.01$, as compared to 77.1 cm$^2 \pm 35.30$ when grown in a 1:1 mixture of Turface MVP and QuickDry. Similarly, the number of root nodules was also affected by choice of growth medium. Changing from a mixture of Turface MVP and QuickDry to Turface MVP and vermiculite saw a 72% increase in root nodule number. Growing seedlings in only Turface MVP or vermiculite resulted in a moderate reduction in both root surface area and number of root nodules, as compared to a mixture of Turface MVP and vermiculite. Based upon the results described, we decided to proceed with the mixture of Turface MVP and vermiculite.

**Inoculation procedure can drastically reduce the number of root nodules formed on soybean seedlings.** Soybean seeds planted into a mixture of Turface MVP and vermiculite, with only the seed being inoculated, resulted in an average of 60.0 ± 4.19 nodules by the first trifoliate stage of development. In contrast, when seeds were planted into Turface MVP and the growth medium was inoculated, rather than only the seed, 20.8 ± 4.22 nodules were formed, a difference of over 65%. However, inoculating the mixture of Turface MVP and vermiculite resulted in the formation of more nodules (46.4 ± 4.22) than inoculating only the seed when plants were grown in Turface MVP (38.0 ± 4.24). Therefore, based on root nodule number and root surface area, we decided to proceed with the mixture of Turface MVP and vermiculite and inoculate only the seed. We chose to include the Turface MVP only treatment in this experiment in addition to the mixture of Turface MVP and vermiculite, as all previous work controlled-environment work done in our lab has utilized Turface MVP only.

**Type of fertilizer does not affect the number of root nodules present on OAC Wallace soybean seedlings at the first trifoliate stage of development.** This study was undertaken
specifically to examine the effect of fertilizer type on the number of root nodules present on OAC Wallace soybean seedlings. No differences in nodule number were discovered based on type of fertilizer alone. Therefore, we chose to proceed with the complete solution described in Tollenaar (1989), as it was the fertilizer solution utilized for Experiment I.

OAC Wallace soybean seedlings produce visible nodules at the unifoliate stage of development under controlled conditions. When OAC Wallace soybean seeds were planted 2cm deep into a 1:1 mixture of Turface MVP and vermiculite, inoculated with 5mL of inoculant slurry, and grown under controlled conditions detailed above, visible root nodules were produced at the unifoliate stage of soybean development. Nodule number continued to increase as plants developed. As the unifoliate stage of development is the first stage at which nodules are present and seedlings respond to the low R:FR signal with an increase in shoot height, we decided to examine the effects of low R:FR on soybean root nodulation at this stage.

Soybean seedlings grown in a 1:1 mixture of Turface MVP and vermiculite respond to the low R:FR signal from aboveground neighbouring weeds with the classic shade avoidance response. This study was conducted in order to ensure that OAC Wallace soybean seeds planted into a mixture of Turface MVP and vermiculite were receiving and responding to the low R:FR signal reflected from aboveground neighbouring weeds, as was discovered when soybean were grown in only Turface MVP. Seedlings grown in Turface MVP and vermiculite and exposed to low R:FR showed an increase in shoot height, a classic shade avoidance characteristic, greater than those seedlings grown in only Turface MVP. In addition, as was consistent with the results of Experiment I, weedy seedlings grown in Turface MVP and vermiculite also showed a
reduction in total root length, as compared to weed-free seedlings in the same growth medium. Overall, seedlings grown in a mixture of Turface MVP and vermiculite performed better (i.e. had increased root length, volume and surface area) than seedlings grown in only Turface MVP; however, seedlings grown in the mixture of growth mediums did still respond to the low R:FR signal with classic shade avoidance characteristics.
Title "Morphological Study";
Data First;
Input Rep Harvest ID$ Trt$ ShootHeight RootLength ShootDW RootDW RS TotalM Length SurfArea AvgDiam RootVolume Tips Forks;

Cards;
1 1 8.2 W . 3.5 0.1238 0.0054 0.0436
    0.1292 3.7869 1.843 1.5491 0.0714 0 0
1 1 6.5 W . 1 0.1927 0.0054 0.0280
    0.1981 1.1791 0.6749 1.822 0.0312 0
;
Title "Shoot Height";
Proc Mixed Covtest Maxiter=2000;
   Class Rep Trt Harvest;
   Model ShootHeight= Trt Harvest Trt*Harvest/ influence outp=second residual;
   Random Rep;
   Repeated Harvest;
   LSMeans Trt*Harvest/ pdiff Slice=Harvest;
   ODS output diffs=ppp lsmeans=mmm;
   ODS listing exclude diffs lsmeans;
Run;
   %include "E:\Data\pdmix800.sas";
   %pdmix800 (ppp,mmm,alpha=0.05,sort=no,slice=Harvest);
Run;

Proc Plot Data=Second;
   Plot resid*pred resid*rep rep*ShootHeight resid*trt resid*ShootHeight/vref=0;
Run;

Proc Univariate Normal plot data=second;
   Var Resid;
   Histogram/normal (color=black w=3) barwidth=5 cfill=gray height=4
   font='arial';
Run;

Proc sort data=second; by studentresid;
proc print data=second;
Run;

Proc glm data=first;
   class trt;
   model surfarea = trt;
   means trt / hovtest = bf;
Run;