Using phytohormones and genotype selection to enhance biological nitrogen fixation in soybean

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This thesis is submitted to Lancaster University in partial fulfilment of the requirements for the degree of doctor of philosophy

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Declaration

I declare that this thesis is my own work, except where reference has been made to other sources, and that it has not been previously presented for a higher degree elsewhere.

Robert Kempster

Lancaster University, March 2021

Statement of authorship

Chapter 4 is largely based on an article which was published by Annals of Applied Biology, October 2020. An additional year of field trial data is also included in Chapter 4 and controlled environment hormone application data removed as this is reported in this thesis in Chapter 3. As such, there are amendments to the results and discussion herein, which are not included in the publication. The manuscript has multiple authors, and their contributions are indicated below.

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RK designed, analysed data and prepared the manuscript, and also assisted in the sowing, maintenance and sampling of 2018/2019 field trial from September to December 2018, and laboratory work January 2020. MB assisted in manintaining field trials, sampling and laboratory work. LB advised on controlled environment hormone application and field trial experiments. MR, LB and ICD gave advice on experimental design, contributed to the revision of the manuscript and provided laboratory space.

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Oral/ Poster presentations

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Kempster, R. Using phytohormone interaction to improve nitrogen fixation in legumes. CGE workshop. Lancaster University. 20th September 2017.

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Abstract

Soybean is the most important plant-based protein source and may be vital to sustainable agricultural intensification, required to support an ever-growing global population. Soybean, however, has a high nitrogen (N) demand which often limits yield. Biological nitrogen fixation (BNF), through symbiosis with rhizobia, is a natural process through which legumes can derive much of their N requirement, with many environmental benefits over chemical fertiliser application. Enhancement of BNF therefore offers an opportunity to sustainably increase yield. Nodules are root organs that form following legume perception of nod factors (NF) produced by compatible rhizobia species, leading to expression of NF response genes, including *ENOD40*. They are the sites of BNF, providing a highly controlled optimal environment for rhizobia to fix N. Primarily, work on symbiosis has focused on the signalling events that coordinate nodule initiation and development, with a key role of phytohormones tightly controlling this process. However, the effect of varying nodule morphologies on BNF capacity is little studied. This thesis uses two approaches, genotypic variation and phytohormone application, to enhance BNF and seeks to understand if differences in nodule traits (such as size, number or distribution) can explain differences in BNF. To better quantify nodule traits, a novel image analysis protocol was developed.

To determine whether genotypic variation in nodule traits could explain differences in BNF capacity, six soybean genotypes with contrasting BNF capacities (based on previous literature) were studied. Although BNF and nodule traits varied between genotypes by up to 60%, there was no simple relationship between nodule formation and BNF as hypothesised. Instead, both genotype and growth stage interacted to affect BNF capacity, with different nodule traits influencing BNF to different extents across the soybean genotypes when measured at different stages. Nodule traits (nodule area or diameter) derived from novel image analysis techniques were better correlated with BNF than traditional assessment methods (nodule number or weight).

To establish if phytohormone application could enhance BNF, three positive regulators (cytokinin, auxin and gibberellic acid) were applied at various concentrations via different application methods (seed coat, seed soak, root application and foliar spray). Of the phytohormones tested, only cytokinin (kinetin) gave promising results and was most effective when applied as a seed soak treatment (10^{-9} mol L^{-1}). This treatment increased total nodule area (32%), doubling BNF. Interestingly, cytokinin treatments led to the nodules being more tightly clustered near the root crown (81% decrease in

distance from root crown) suggesting that early nodule development was promoted. Indeed, cytokinin seed treatment increased the expression of *ENOD40* by 52% after 72 hours. This suggests that cytokinin seed soaking primes the seeds by promoting the symbiotic pathway.

As both genotype and cytokinin application changed nodule development and BNF in controlled environments, their agronomic potential was assessed in field trials in Argentina with early and conventional sowing dates. Low root zone temperature is a key limiting factor of nodule formation but is little studied despite growing interest in European grown soybean and early sowing dates elsewhere, where crops often experience cool growing conditions. Cytokinin treatment had limited positive effects on BNF but did not increase yield or total N uptake. Genotypic differences in yield were not explained by overall BNF across the growth period and were better associated with differences in soil N uptake, with 21% increase in higher yielding DM50I17. Interestingly, DM50I17 showed increased early nodule development (18% increase in nodule number) and correspondingly greater (52%) early BNF which might have improved yield through greater canopy N accumulation (9%), available for remobilisation. These field trials indicate BNF is an important N source during cold conditions, maintaining N supply leading to more consistent yield. In contrast to previous literature, soil N uptake was more sensitive to low root zone temperature than BNF.

Taken together, this thesis is the first detailed examination of whether nodule traits affect BNF in soybean. It explored the potential of genotype selection and phytohormone application to enhance BNF by altering the number or size of nodules. Differences in nodule morphologies, particularly nodules with 4 mm diameter, influence BNF with increased nodule development positively correlated with BNF. Cytokinin seed priming enhances root *ENOD40* expression and thus nodule formation and BNF. However, future work should further examine the effect of cytokinin priming on the symbiotic pathway to fully understand the mechanism(s) behind this treatment. Equally, greater understanding of changes in endogenous phytohormone concentration across genotypes and following cytokinin treatment may explain nodule variation seen in this study. Although genotype selection and phytohormone treatments did not always enhance BNF sufficiently to increase soybean yield, cytokinin application altered important nodule traits that may provide greater N (and yield) benefits in specific agronomic circumstances with limited N inputs.

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Chapter 1: General introduction

1.1 | Context

An ever-increasing world population with changing diets puts enormous pressure on agricultural systems to maintain food supply (Godfray *et al*., 2010). Food production must double by 2050 (Tilman *et al*., 2011), in a way that is environmentally and socially sustainable (von Braun, 2007), and also cope with the challenges of climate change.

In the past, expanding agricultural land area allowed increased production, keeping up with increasing demand. However, this greatly diminished biodiversity and carbon storage within global forests (Balmford *et al.,* 2005). Agricultural land is also shrinking due to urbanisation, and land degradation due to soil erosion and associated nutrient losses (Nellemann *et al.,* 2009). There is, therefore, a need to increase production on the current agricultural land area while limiting the environmental impacts, a process known as sustainable intensification (Royal Society of London, 2009).

Legume crops are globally important for food, feed and fuel, grown on 12-15% of the arable land with a production value of over $200 billion per year (Graham & Vance, 2003; Peoples *et al*., 2009; Jensen *et al*., 2012). However, global cereal cropping (724 million ha in 2019) far surpasses legume cropping (just 197 million ha 2019) (*FAOSTAT Statistical Database*, 2020). But European legume production has increased in recent years, following common agricultural policy reform in 2013 (Figure 1.2). Additionally, across Africa, legumes are becoming more important (Foyer *et al*., 2019).

1.2 | Benefits of legumes

Legumes have a number of important roles in sustainable agriculture (Figure 1.1) (Voisin *et al*., 2013; Stagnari *et al*., 2017) including: improving biodiversity in production systems (Nemecek *et al*., 2008; Jensen *et al*., 2010; Köpke & Nemecek, 2010), providing a sustainable N source that limits agricultural greenhouse gas emissions (Lemke *et al*., 2007) and an important plant-based protein source for humans and livestock (Tharanathan & Mahadevamma, 2003).

Legumes can improve several aspects of soil fertility, in addition to increasing soil N (detailed description below), which only accounts for around 20% of benefits following legumes in a rotation (Hesterman *et al*., 1986; Fyson & Oaks, 1990; Chalk, 1998). Soil organic carbon can be increased through continuous cropping, replacing the fallow period with a legume crop, so improving biological soil quality (Wu *et al*., 2003; Hernanz *et al*., 2009). Legumes can also mobilise phosphorus (P) through roots secreting organic acids including citrate and malate, allowing access to fixed soil P such as phytate (Hocking, 2001; Shen *et al*., 2011).

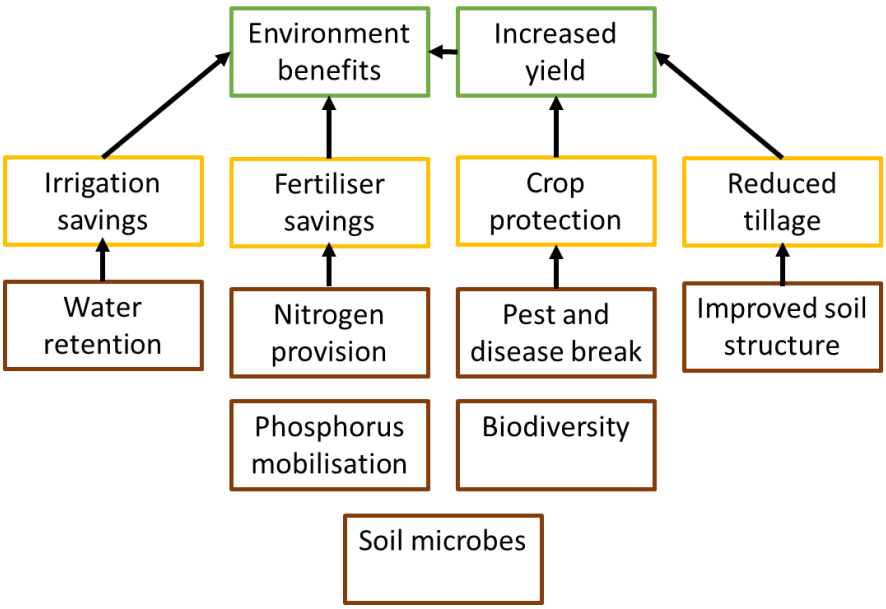


Figure 1.1 Benefits of legumes adapted from Preissel *et al*., 2015 showing multiple ecosystem services (brown) leading to agronomic benefits (yellow) which result in system benefits (green).

Soil water retention and availability can also increase as legume crops take up less water than cereals during growth (Miller *et al*., 2003) and post-harvest crop residue aids infiltration and retention of precipitation between crops (Kirkegaard & Ryan, 2014; Angus *et al*., 2015). As grain legumes generally have more shallow roots than cereals, soil water content can accumulate at lower soil depths (below 60 cm) (Miller *et al*., 2003; Liu *et al*., 2011; Wang *et al*., 2012).

Hydrogen gas, released as a by-product of BNF (Equation 1.1) (Hunt *et al*., 1988), can improve soil microbe community favouring the development of plant growth promoting bacteria.

Whether legumes are infected with rhizobia which have an active uptake hydrogenase enzyme (Hup) or inactive Hup determine if they emit hydrogen (Evans *et al*., 1987). Hydrogen released from nodules is rapidly oxidised by soil microbes (Dong & Layzell, 2001) and is a large energy input which substantially alters soil microbial activity and carbon balance (McLearn & Dong, 2002; Stein *et al*., 2005). Increased yield (48%) of barley following hydrogen emitting (Hup^-) soybean compared to non-emitting (Hup^+) or non-inoculated soybean (Dean *et al*., 2006) was either due to improved soil fertility or improved root growth caused by hydrogen emission (Dong *et al.,* 2003).

Legumes can also reduce the need for crop protection chemicals. As legumes and cereals are susceptible to different pests and diseases, they are a good break crop in wheat-based rotations (Zander *et al*., 2016). Additionally, legumes can help control weeds by stabilising weed composition due to differences in growth compared to cereals, disrupting different weed niches (Bàrberi, 2002; Seymour *et al*., 2012). Legume break crop benefits increase cereal yield by 24% compared to those grown continuously (Kirkegaard *et al*., 2008).

However, to benefit from legume crops, optimal crop management practices must be implemented including rates and timing of N fertiliser, soil management, weeding and irrigation. Low and inconsistent yield in legumes is a key reason for limited cultivation of these crops (Cernay *et al.*, 2015; Reckling *et al.*, 2016). There is therefore a need to improve legume yield and yield stability to increase adoption by farmers.

Soybean

Soybean represents 61% of global legume production (*FAOSTAT Statistical Database*, 2020; Herridge *et al*., 2008), as it is the best source of vegetable protein for both humans and livestock, with a seed made of up to 46% protein (Breene *et al.*, 1988). Due to these benefits, soybean is an important food for much of the world, enhancing the agricultural economies of many countries (Goldsmith, 2008). Unlike most other legume crops, supply chains and markets for soybean are well developed (Reckling *et al*., 2016). Global soybean production in 2019 was approximately 334 million metric tonnes (MMT; Figure 1.2) with over 121 million ha used, equating to 8.4% of all farmland (FAOSTAT *Statistical Database*, 2020). While cool growing conditions constrain European soybean production (Figure 1.2) (Zimmer *et al*., 2016), future crop improvement in soybean will have a global impact.

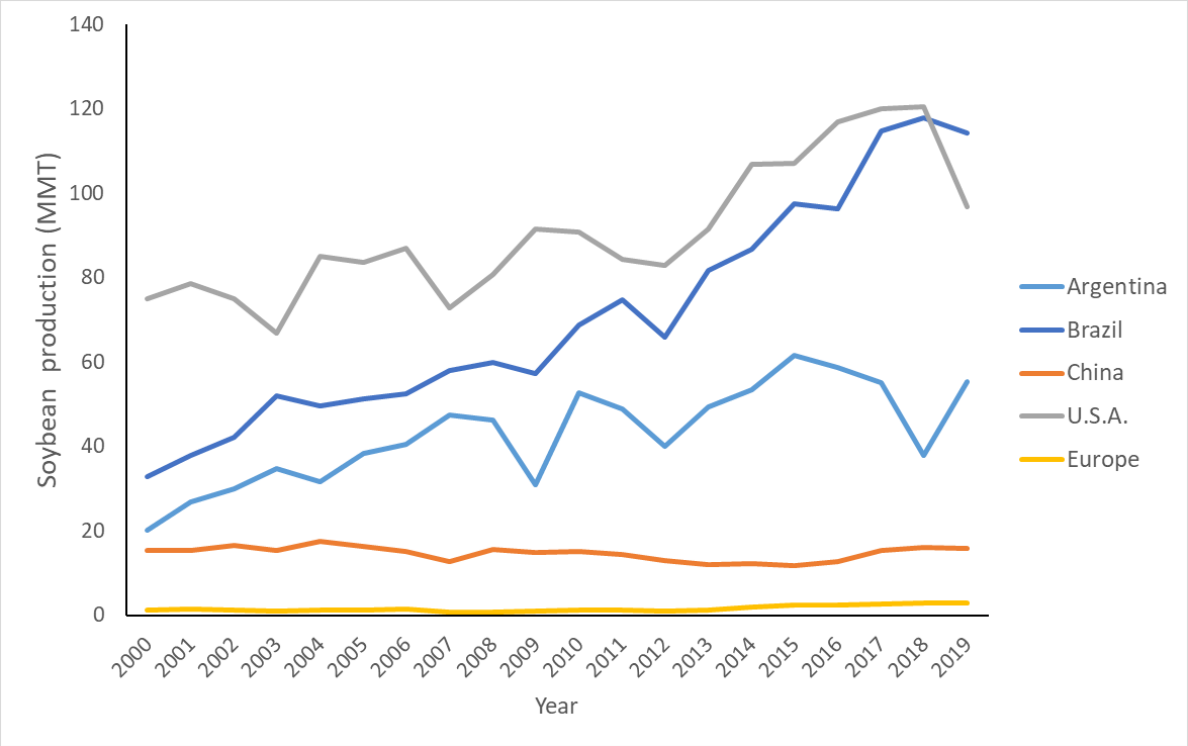


Figure 1.2 Soybean production in the top four producers (Argentina, Brazil, China and USA) compared to limited production in Europe (FAOSTAT *Statistical Database*, 2020).

A major problem associated with the expansion of soybean cultivation is deforestation (Costa *et al*., 2007) to increase cropping area. Also, despite its limited N fertiliser needs due to biological nitrogen fixation (BNF; see below), soybean production does have a significant carbon footprint from field operations, liming and N_2O emissions from residue decomposition (Adom *et al*., 2012). Therefore, increasing soybean yield per hectare has the potential to reduce its negative impact on biodiversity and relative carbon emissions on a yield to carbon basis (Appendix 1.1 CGE carbon report).

Benefit of soybean in cropping systems

Soybean can benefit subsequent crops in the rotation. Cereal crops grown after soybean can have enhanced yield of 0.49 tonnes ha^{-1} (Franke *et al*., 2018) compared to those grown continuously (Crookston *et al*., 1991; Agyare *et al*., 2006; Seifert *et al*., 2017). Soybean-maize rotations have more consistent yields, that do not decline like monoculture production of maize (Agyare *et al*., 2006; Uzoh *et al*., 2019).

Increasing yield depends on several factors, including more sustainable nutrient management. Remaining soybean residues can enhance soil N content, as soybean can fix up

to 450 kg N ha^{-1} (Peoples & Craswell, 1992; Osterholz *et al*., 2017), thereby reducing N fertiliser requirements by up to 150 kg ha^{-1} per year (Rembon & MacKenzie, 1997). Phosphorus availability is improved due to soybean root exudates (Nuruzzaman *et al*., 2005; Jemo *et al*., 2006; Pypers *et al*., 2007; Richardson *et al*., 2009; Franke *et al*., 2018). Soybean – maize rotations also disrupt disease cycles and prevent pest build up, so limiting biotic stress. Soybean is not a *Striga* host and root exudation of strigolactones by soybean reduces the prevalence of *Striga*, a major parasitic weed affecting maize, which can decrease yields by up to 80% (Kim *et al*., 2002; Gethi *et al*., 2005; Kanampiu *et al*., 2018). As native grasses act as Striga hosts, cultivating soybean is preferable to leaving fields fallow (Franke *et al*., 2018).

In areas with maize or soybean in consecutive years there may be negative consequences, such as soil degradation and water pollution, as additional agrochemical inputs are required to compensate for lack of ecosystem services provided through biodiversity (Davis *et al*., 2012; Sindelar *et al*., 2016; Hunt *et al*., 2017). Thus, the benefit of including soybean in rotation is far greater than N availability with N fertiliser input not sufficient to compensate and maintain yield (Karlen *et al*., 1994; Bowles *et al*., 2020). Greater accumulation of soil organic carbon occurs with soybean-maize systems due to greater microbial activity, stimulated by lower lignin content and a low C to N ratio of soybean residues (Bichel *et al*., 2016).

Soybean is therefore a valuable crop, because of both the high value grain it produces and multiple benefits it can have when used in crop rotations.

1.3 | Soybean nitrogen requirements

Lack of nitrogen commonly restricts soybean crop growth and yield more than any other factor, when water is available (Hirel *et al*., 2007). High N uptake is required to achieve high seed yield in all crops, but especially legumes due to their high seed protein content (Sinclair & de Wit, 1975; Giller & Cadisch, 1995). Soybean is particularly N demanding, more than all other major crops, requiring 29 mg of N per g of photosynthate whereas maize requires 11 mg (Sinclair & de Wit, 1975). Applying N fertiliser to soybean is not common due to the financial cost, with up to 69% of soybean N requirements typically supplied by nitrogen fixed from the air via symbiosis with soil bacteria (Salvagiotti *et al*., 2008). Agricultural production systems in Argentina and Brazil show that soybean cultivation can be achieved with minimal

N fertiliser inputs (54 kg N ha^{-1} annually) by relying on BNF (216 kg N ha^{-1} annually) (Herridge *et al*., 2008). The optimisation and effective use of BNF in agricultural systems will surely enhance the agricultural sustainability of this crop.

Soybean seed yield is determined by N uptake, from either mineral N absorption or BNF, N use efficiency and harvest index (Donald & Hamblin, 1976; Novoa & Loomis, 1981; Rotundo *et al*., 2014). Of these factors, N uptake and assimilation are highly correlated with, and have the greatest effect on, final soybean seed yield (Sinclair & Jamieson, 2006; Rotundo *et al*., 2014). To produce 5,000 kg of soybean grain per hectare, approximately 390 kg N ha^{-1} is required (Santachiara *et al*., 2017).

Variable N fixation rates with growth stage can restrict N supply at times with demand and supply of N mismatched. At early stages of legume growth there is a period of N starvation, usually between 15-20 days after emergence, as cotyledonary N supplies are depleted before N fixation begins (Hungria *et al*., 1991). As a result, applying 'starter N' (50 kg ha^{-1}) can increase soybean yield by approximately 3% (Gai *et al*., 2017), but this may be to the detriment of early nodule formation, thus is not advisable in low N input systems. The N demands of simultaneous leaf and nodule development is thought to cause this initial N starvation (Atkins *et al*., 1989). However, the greatest N requirement of soybean is at pod filling, when nodule senescence limits BNF (Van De Velde *et al*., 2006) also leading to a shortage of N. Achieving optimum yields requires improving the synchronicity of N uptake at different growth stages.

As little or no N fertiliser is applied to soybean, N must be supplied from native soil N and BNF; estimated to be worth $15 billion a year globally if required as N fertiliser (Hungria & Mendes, 2015). The supply of N from BNF is highly variable, providing from 0%, up to around 70% of soybean N uptake, with 68% average (Salvagiotti *et al*., 2008). Currently, the yield improvement rate in soybean is high (Figure 1.2), with global production around double that of 1990 (Ainsworth *et al*., 2012), but this mainly relies on applying nitrogen fertilisers (Specht *et al*., 1999; Salvagiotti *et al*., 2008) or increased land use (Masuda & Goldsmith, 2009); and is still not sufficient to meet future needs (Ray *et al*., 2013). It is likely that future yield increases in soybean will result from increased BNF, perhaps through altered timing of N supply from N fixation.

1.4 | Sources of nitrogen

Although dinitrogen gas (N_2) makes up most of the earth's atmosphere (78%), N is one of the most limiting nutrients for crop production. Atmospheric N_2 is unavailable to plants unless the highly stable triple covalent bond between two N atoms can be broken, producing ammonia (NH_3) or nitrate (NO_3^-). This can be achieved through natural BNF and industrial processes.

Haber-Bosch

The ability to industrially fix nitrogen, through the Haber-Bosch process, was one of the most important inventions of the twentieth century. It enabled the Green Revolution with its greatly increased crop yields, leading to increased global population (Smil, 1999; Peoples *et al*., 2009; Jensen *et al*., 2012). Nitrogen fertiliser produced in this way sustains around half the world population (Fryzuk, 2004; Erisman *et al*., 2008), overtaking BNF as the dominant N source in the 1950s. With an increasing population, industrially fixed N continues to be heavily relied upon as indicated by global N application rates increasing 10-fold from 1950 to 2008 (Robertson & Vitousek, 2009).

The environmental impact of such a high N application rate occurs at multiple stages, from the acquisition of fossil fuels needed to power the process of N fertiliser production to the release of N_2O gas from fields after fertiliser application, a greenhouse gas with 292 times more potent warming effect than carbon dioxide (Crutzen *et al*., 2008). Currently, 1% of world energy is used to provide 65% of global nitrogen fixation through this industrial process (Smith, 2002; Conley *et al*., 2009). The costs associated with this huge amount of N production equate to 50% of agricultural fossil fuel use and 5% of global natural gas consumption (Crutzen *et al*., 2008; Canfield *et al*., 2010). Additionally, fossil fuel prices will likely increase, increasing the cost of N fertilisers and putting them out of reach of many farmers in developing regions and reducing profit for all.

Additional negative impacts of N fertilisers are caused by over-use and inefficient uptake, with 30-50% lost due to leaching, resulting in additional environmental damage including eutrophication of waterways (Choudhury & Khanif, 2001; Vance, 2001; Halvorson *et al*., 2002). An estimated 48 Tg per year of anthropogenic N is lost from land to sea (Schlesinger, 2009), from atmospheric nitrous oxide, ammonia volatised from fertiliser and decomposition

and nitrate leaching to underground water. An agricultural system so reliant on the Haber-Bosch process cannot be considered sustainable for climate change or the nitrogen cycle, with the safe planetary boundaries for N already being exceeded (Rockström *et al*., 2009; Canfield *et al*., 2010; Charpentier & Oldroyd, 2010; Beatty & Good, 2011). Clearly, therefore, an alternative N source is required to satisfy future demand. Furthermore, improved technology to match supply with uptake is critical.

Biological nitrogen fixation

Biological nitrogen fixation is the natural process of turning atmospheric N_2 into plant available NH_3. The majority of BNF occurs in oceans with over 400 Tg N per annum (Codispoti, 2007). In agriculture, there are many symbiotic systems that are beneficial, not all of them occurring in legumes. BNF from non-legumes, in association with free-living bacteria, provides 23 Tg N fixation per year (Herridge *et al*., 2008), and can provide 75% of rice N requirements (Halvin *et al*., 2005).

Legume crops are, however, the main source of natural agricultural N fixation, forming endosymbiotic relationships with soil bacteria (rhizobia) in which BNF occurs to supply N to the host in exchange for reduced carbon. Increased N provision from this symbiosis benefits the host legume as well as subsequent crops (Peoples *et al*., 2009), which is particularly important where N fertiliser application is limited (Preissel *et al*., 2015). However, global industrially derived N inputs still outweigh that from symbiosis with 121 compared to 40 Tg N per year, respectively (Conley *et al*., 2009).

Biological nitrogen fixation is crucial for agriculture as industrial N production does not meet demand. The environmental damage of industrial N fixation will likely lead to increased reliance on legumes in future sustainable agricultural systems (Serraj *et al*., 1999). Nitrogen contribution from agricultural legumes is estimated to be between 33-46 Tg leading to N fertiliser reduction which equates to 277 kg ha^{-1} of CO_2 per year (1 kg N = 3.15 kg CO_2) across European cultivated cropland (Jensen *et al*., 2012). Legumes differ in their ability to fix N with the amount of plant N derived from nitrogen fixation (%Ndfa) ranging from an average of 40% in *Phaseolus vulgaris* (common bean) to 75% in *Vicia faba* (Faba bean) with soybean at 68% (Herridge *et al*., 2008). Higher rates of 69-94% Ndfa have been reported in Brazilian soybean, equating to 70-250 kg N ha^{-1} (Alves *et al*., 2003; Hungria *et al*., 2005). On average, soybean is

able to acquire 50-60% of its own N requirement through BNF (Salvagiotti *et al*., 2008). However, new soybean cultivars are less able to fix N than older ones as breeding and selection occur at sites of high N availability (van Kessel & Hartley, 2000; Nicolás *et al*., 2002) potentially making future soybean yield increases challenging without the use of N fertiliser input.

Enhancing symbiosis can therefore increase crop yields by improving nutrient supply, whilst limiting negative environmental impacts of N fertiliser use and thus improving agricultural sustainability (Vance, 2001; Peoples *et al*., 2009; Rockström *et al*., 2009; Canfield *et al*., 2010; Jensen *et al*., 2012).

1.5 | Nodules

Nodules are specialised root organs that evolved around 58 million years ago in certain legume species to host N_2 fixing rhizobia in a tightly controlled manner. In N-limiting conditions, a complex exchange of signals between symbionts leads to nodule formation. Nodulin (*Nod*) genes are plant genes that are specific to nodulation and nodulation (*nod*) genes are rhizobial genes used in nodule formation. During root nodule formation, two processes, infection and nodule organogenesis, occur simultaneously. During infection, rhizobia attached to the root hairs and cause them to curl. The infection thread, a tubular extension of the plasma membrane, transports rhizobia from the root hair tip to the root cortex. At the same time, cortical cells dedifferentiate and divide to form a distinct area in the cortex called a nodule primordia. When the infection thread enters the nodule primordia, rhizobia are released into membrane bound compartments, called symbiosomes (Udvardi & Day, 1997), and differentiate into nitrogen-fixing endosymbiotic organelles called bacteriods. Nodule biology is important to understand potential enhancements to BNF.

Nodule structure

There are two nodule types (Figure 1.3), determinate which form on soybean, common bean and *Lotus japonicus* roots and indeterminate forming on *Medicago truncatula*, *Medicago sativa* (alfalfa) and *Pisum sativum* (pea). Determinate nodule growth stops at maturity and BNF starts, whilst indeterminate nodules maintain a meristematic zone that allows continued growth alongside BNF. Determinate nodules begin with divisions in the outer and middle root

cortex cells and lose meristematic activity soon after initiation and instead grow through cell expansion leading to a globular form with a homogeneous inner core of infected cells. Indeterminate nodules are derived from pericycle and inner cortex cell division and maintain an active apical meristem leading to an elongated cylindrical morphology giving them a more complex structure divided into five zones (Figure 1.3). Products of BNF are also different, and are determined by the rhizobium species with which they are associated. Determinate nodules export ureides, while indeterminate nodules export amides. Nodule life cycle also differs between nodule types, core cells of determinate nodules develop simultaneously and senesce together whereas indeterminate nodules continue to grow with fixation zones becoming a senescence zone with time (Van De Velde *et al*., 2006).

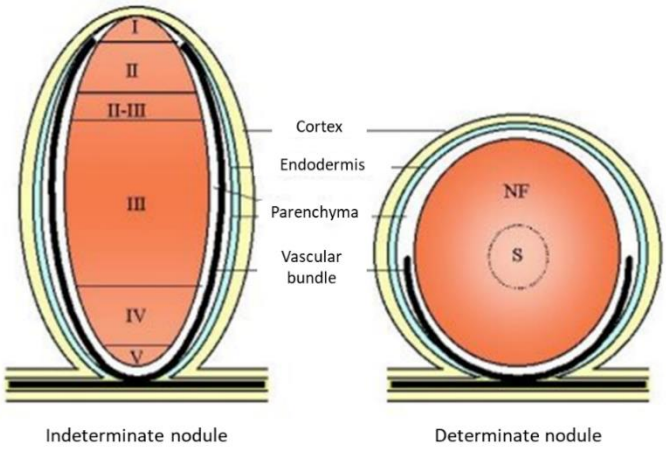


Figure 1.3 Structure of indeterminate and determinate nodules. I: meristematic region, II: infection zone, II-III: interzone II-III, III: nitrogen fixation zone, IV: senescent zone, V: saprophytic zone, S: senescent zone, NF: nitrogen fixation zone (adapted from Scholte, 2002).

Nitrogenase

The enzyme that catalyses BNF is nitrogenase (equation 1.1), using 52% of nodule respiratory energy (Rainbird *et al*., 1984). Two component proteins, the iron (Fe) protein and the iron-molybdenum cofactor (MoFe) protein, make up nitrogenase. The Fe protein is an ATP-binding site and MoFe the site of substrate binding and reduction. The nodulation genes *fix*ABCX encode proteins required for N_2 fixation (Hageman and Burris, 1978; Earl *et al.,* 1987), and structural genes *nif*H, *nif*D and *nif*K encode an iron protein subunit, MoFe protein α-subunit

and β-subunit, respectively (Sundaresan & Ausubel, 1981). Nitrogenase forms two products, ammonia (NH_3) and ammonium (NH_4^+) which diffuse first out of the bacteroid then diffuse or are transported across the symbiosome membrane (Bisseling *et al*., 1979). Assimilation by the host then takes place in the infected cell cytosol, with the enzymes glutamine synthetase and glutamate synthase producing glutamine from ammonium. These are then transported to the shoot as either amides (asparagine and glutamine) in species with indeterminate nodules, or as ureide (allantoin and allantoic acid) in soybean and other species with determinate nodules (Karr *et al*., 1990; Day *et al*., 2001).

Leghaemoglobin

As BNF is an energy intensive process it requires high rates of respiration to produce sufficient ATP to power it, so an adequate oxygen supply is essential. However, nitrogenase is very oxygen sensitive, being irreversibly inhibited through conformational changes or limited by decreased electron transfer (Robson & Postgate, 1980). Leghaemoglobin, giving active nodules a pink colour, maintains high oxygen flux in micro-aerobic conditions whilst also protecting nitrogenase by scavenging oxygen, so maintaining low oxygen concentrations (Hirsch, 1992).

1.6 | Plant regulation of BNF

As BNF is energy demanding, the host needs to balance N demands with the C cost. The energetic cost of symbiosis is 16 ATPs for N_2 reduction (equation 1.1) (Kahn *et al*., 1998).

$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi \qquad (1.1)$$

When comparing energy costs of nitrate uptake to BNF, other factors such as nodule development must be considered (Valentine *et al*., 2010). BNF is thought to require significantly more energy than nitrate uptake and reduction, for soybean 8.28 mg C per mg of N compared to 4.99 mg C per mg of N, respectively (Finke *et al*., 1982). The ability of legumes to grow in low N environments, however, clearly justifies these costs from an evolutionary perspective and now makes them important in low N fertiliser cropping systems.

Due to the cost of symbiosis, plants have a number of regulatory mechanisms to control nodulation. Key regulatory mechanisms are autoregulation of nodulation (AON), shoot

nitrogen, oxygen permeability, carbon supply and metabolism each of which are influenced by environmental stresses.

Autoregulation of Nodulation

Plants can regulate BNF by adjusting nodule number with autoregulation of nodulation (AON) controlling numbers (Reid *et al*., 2011), suppressing 90% of rhizobial infection events. This allows the host to balance the need for N with the energy cost of nodule establishment and maintenance of nodules. Both positive and negative regulators of nodulation are required for proper nodule formation (Ferguson *et al*., 2010), including hormonal control as outlined below. While the loss of any positive regulator usually inhibits nodulation, loss of a negative regulator enhances nodule numbers leading to a super-nodulation phenotype. Plants with non-functional AON are stunted and have low yields as excessive nodule development drains plant resources. Therefore, efforts to enhance BNF through nodules are unlikely to result simply from increased nodule number but rather in other traits such as nodule size.

Shoot nitrogen

Plant N status influences rates of BNF by altering nodule number and size, with maximum rates at times of high demand, such as pod filling (Fischinger *et al*., 2010) and lower rates due to N remobilisation (Fischinger *et al*., 2006). Shoot N may also regulate BNF in nodules directly, involving the nodule oxygen diffusion barrier, which responded to low-level ammonium application to soybean shoots (Neo & Layzell, 1997). Rates of BNF also vary with phenological stage (Cabeza *et al*., 2015). Certain amino acids, such as glutamate (Fischinger *et al*., 2006; Forde & Lea, 2007), glutamine (Neo & Layzell, 1997), aspartate (King & Purcell, 2005), asparagine (Sulieman & Schulze, 2010) and ureide (Ladrera *et al*., 2007) also have a role as long distance, phloem transported, signals of shoot N status (Sulieman & Tran, 2013). BNF is regulated by N demand, whereby if N demand is less than N supply from fixation, then these N signalling molecules enter the phloem and are carried to the nodule, causing a reduction in BNF.

Oxygen permeability

Adequate oxygen supply to the nodule interior is a key factor regulating nodule activity (Dong & Layzell, 2001). A high concentration of oxygen within the nodule can risk denaturing

nitrogenase, whilst a low concentration limits BNF, due to limited respiration and thus ATP production (Marchal *et al*., 2000). Despite being oxygen sensitive, nitrogenase activity increased as the root oxygen concentration was increased gradually, so indicating oxygen limitation in ambient conditions (Hunt *et al*., 1989). Oxygen concentration within a nodule is influenced by its morphology, as the size of intercellular spaces in the central nodule tissue alters internal oxygen gradients. Larger internal spaces allow greater oxygen gradients across nodule tissue, so limits oxygen diffusion out of the symbiosome (Bergersen, 1997; Vance *et al*., 1998). An oxygen diffusion barrier controls oxygen diffusion from the nodule cortical cells to the inner cells, creating sharp differences in oxygen concentration between these regions (Tjepkema & Yocum, 1974). The oxygen diffusion barrier responds to restricted plant growth, and limits BNF to an appropriate level for current N demand (Kleinert *et al*., 2017). Leghaemoglobin is required for BNF as it allows high levels of respiration whilst also buffering free O_2 concentration, maintaining very low O_2 concentrations in active nodules (Appleby, 1984; Ott *et al*., 2005).

Carbon supply and metabolism

Biological nitrogen fixation has a high energy demand consuming much photosynthate (Schulze, 2004). The amount of C is species and growth stage dependent ranging from 1.4-12 g C per g N (Schuize *et al*., 1999). Therefore, plants carefully regulate the need for N with carbohydrate reserves (Unkovich *et al*., 2010; Sulieman & Tran, 2013). Sucrose is the primary C source for nodules (Stacey, 2007), converted into malate and succinate by sucrose synthase, which are used as C source in bacteroids (Walsh, 1990). Nodule starch accumulation, indicating sufficient or excess supply (Redondo *et al*., 2009), is not interrupted by day-night cycles (Cabeza *et al*., 2015) showing that BNF is not synchronous with plant photosynthesis (Vance & Heichel, 1991; Schulze, 2004). Elevated CO_2 does, however, increase BNF rates (Rogers *et al*., 2009), associated with increased nodule number and mass as well as increased nitrogenase activity (Lam *et al*., 2012). Therefore, nodule metabolism is limited by phloem supply to nodules and not longer-term shoot carbohydrate status. Therefore, improved nodule vasculature, allowing increased phloem C supply, could enhance BNF.

Environmental constraints to BNF

Nitrogen fixation is very sensitive to abiotic stress. Whilst stresses like drought, salinity, acidity and phosphorus deficiency have all been well studied (Valentine *et al*., 2010), others like cold stress have not. Suboptimal root zone temperatures (RZT; <25°C) delay BNF onset and nodule initiation (Legros & Smith 1994; Mishra *et al*., 2009; Poustini *et al*., 2005; Zhang *et al*., 1995). Early stages of nodule establishment are restricted (Lindemann & Ham, 1979; Lynch & Smith, 1993; Matthews & Hayes, 1982), especially infection and early nodule development, due to limited *nod* gene expression and NF synthesis, see below (Shiro *et al*., 2016; Zhang & Smith 1994).

1.7 | Nodule development and phytohormones

Nodule formation first involves signalling between the host and rhizobia, followed by root hair curling, infection thread formation, cortical cell division and development of vasculature (Figure 1.4). Each stage of this process, explained below, is tightly controlled by phytohormones which are required for proper nodule development.

Signalling and perception

To initiate nodulation, both symbionts release diffusible molecules that must be mutually recognised to initiate further signalling (Dénarié *et al*., 1996). Flavonoids released by the host plant are recognised by compatible rhizobia, usually *Bradyrhizobium japonicum* for soybean (Oldroyd, 2013), activating rhizobial genes, including *nod* genes that encode Nod factors (NF) (Redmond *et al*., 1986; Caetano-Anollés & Gresshoff, 1993; Denarie & Debelle, 1996; Spaink, 2000). The NF initiates nodule development in compatible host legumes but only in a transient portion of the developing root known as the 'zone of nodulation' (Bhuvaneswari *et al*., 1980; Calvert *et al*., 1984). In this region, root hairs remain sufficiently plastic to allow deformation and invasion by rhizobia. Even before endosymbiosis, phytohormones secreted into the rhizosphere can influence rhizobia (Figure 1.4A). Strigolactone has been identified in *Medicago truncatula* root exudates (Liu *et al*., 2011) and the biosynthesis gene (*CCD8*) is induced in root hairs, suggesting strigolactones are secreted during infection (Figure 1.4A) (Breakspear *et al*., 2014). Additionally, gibberellic acid (GA_3) application to *B. japonicum in vitro* downregulates expression of nodulation genes *nif*A, nifH and *fix*A, with nitrogenase activity also decreased (Chen *et al*., 2015). Conversely, rhizobial gene expression (*nif* and *fix*

genes) is increased following auxin (IAA and 2,4-D) (Bianco & Defez, 2010) or jasmonate (jasmonic acid and methyl jasmonate) application (Rosas *et al*., 1998; Mabood & Smith, 2005; Mabood *et al*., 2006). This suggests that plants use phytohormones as additional signals to promote symbiosis.

Nod factors released by rhizobia are recognised by two LysM domain receptor-like kinases, in soybean NF response 1 and 2 (*GmNFR1* and *NFR5*) (Popp & Ott, 2011; Oldroyd, 2013; Zipfel & Oldroyd, 2017). NF perception by the host leads to cytokinin accumulation (Figure 1.4B) (Oldroyd, 2007; van Zeijl *et al*., 2015) and induces expression of cytokinin responsive genes (*NIN* and *EFD)* and cytokinin response regulators (*RR4* and *RR1*) (Gonzalez-Rizzo *et al*., 2006; Vernié *et al*., 2008; Plet *et al*., 2011), showing the effect of cytokinin signalling at several stages of the symbiotic pathway. Additionally, most early transcriptional changes after NF application (3 hours post inoculation) are dependent on the histidine kinase cytokinin receptor (CRE1) (van Zeijl *et al*., 2015) initiating the symbiotic signalling pathway.

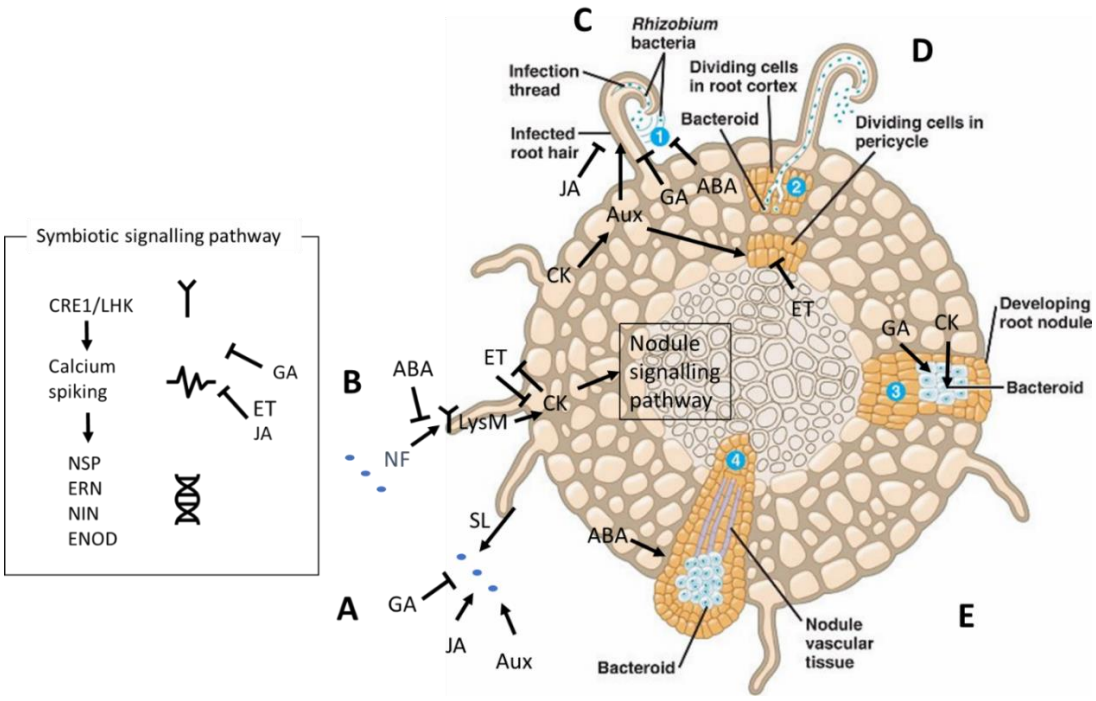


Figure 1.4 The role of phytohormone signalling through nodule development: host signalling (A), Nod factor (NF) perception leading to the symbiotic signalling pathway (B), root hair curling and infection thread formation (C), bacterial release and cortical cell division (D) and nodule differentiation and maturity (E). Hormone abbreviations are as follows: abscisic acid – ABA, auxin – Aux, cytokinin – CK, ethylene – ET, gibberellin – GA, jasmonic acid – JA and strigolactones – SL.

Nod factor application upregulates ethylene biosynthesis genes (van Zeijl *et al*., 2015) and salicylic acid accumulates following inoculation with incompatible rhizobia (Martinez-Abarca *et al*., 1998). Abscisic acid (ABA) regulates nodule formation with the balance between ABA and NF determining if nodulation will occur (Ding *et al*., 2008). Gibberellin biosynthesis genes (*GA20-oxidase a* and *GA 3-oxidase 1a*) are NF dependant (Lievens *et al*., 2005; Hayashi *et al*., 2012; Breakspear *et al*., 2014), with expression peaking 12 hours after inoculation before declining. This signalling involving a number of phytohormones is evident of the tight regulation involved during nodule initiation.

Symbiotic signalling pathway

Downstream signal transduction cascades, following NF perception, involve nuclear membrane potassium ion-channel proteins (Ané *et al*., 2004; Riely *et al*., 2007) and nucleoporins (Kanamori *et al*., 2006; Saito *et al*., 2007). These proteins cause calcium spiking, oscillations in cytosolic calcium, around 10 minutes post NF perception (Wais *et al*., 2000; Walker *et al*., 2000) through the rapid influx of calcium ions followed by membrane depolarisation efflux of chlorine and potassium in root hair cells (Felle *et al*., 1999). Calcium and calmodulin-dependent kinase (CCaMK) perceives calcium spiking leading to the activation of several transcription factors. These include nodulation signalling pathway 1 and 2 (*NSP1* and *NSP2*) (Kalo, 2005; Smit, 2005), Ets repressor factor (*ERF*) required for nodulation (ERN; Middleton *et al.*, 2007) and nodule inception (*NIN*) (Schauser *et al*., 1999; Borisov *et al*., 2003) which together act to promote early nodulation (*ENOD*) genes in the epidermis, regulating bacterial infection (Figure 1.4B). Cytokinin has an important role in initiating the symbiotic signalling pathway, discussed in Chapter 3.

Nodule initiation is regulated by ethylene and JA with plant responsiveness to NF reduced through a negative feedback loop. Upregulation of ethylene biosynthesis genes (ACC synthases) following NF-induced cytokinin accumulation negatively regulates further NF signalling and subsequent cytokinin accumulation (van Zeijl *et al*., 2015). GA inhibits early nodulation events at the epidermis, between *LHK1* and *NSP2* in the nodulation signalling pathway, with an opposite effect to cytokinin by downregulating *NSP1*, *NSP2*, *NIN* and downstream early nodulation (*ENOD*) genes (Maekawa *et al*., 2009; Fonouni-Farde *et al*., 2017).

Ethylene and jasmonic acid act together to inhibit nodulation by suppressing and altering calcium spiking (Oldroyd *et al*., 2001; Sun *et al*., 2006). Ethylene (shortens spiking period) and JA (lengthens spiking length) have antagonistic effects (Sun *et al*., 2006). This cross-talk between hormones tunes the nodulation process by inducing early nodulin gene expression (*EFR1*, *RIP1* and *ENOD11*) (Lorenzo *et al*., 2003; Sun *et al*., 2006). ABA application abolishes NF induced calcium spiking and gene expression (Ding *et al*., 2008). Activation of CCaMK leads to local cytokinin accumulation, stimulating cortical cell division of nodule primordia (Frugier *et al*., 2008).

Root curling and infection thread formation

Perception of NF causes swelling and growth of the root hair tip in the direction of NF, causing root hair curling (Figure 1.4C) (Heidstra *et al*., 1994; De Ruijter *et al*., 1998). As the root hair curls, 6-8 hours post inoculation, continuously dividing rhizobia are trapped (Callaham & Torrey, 1981; Turgeon & Bauer, 1985) and subsequently enter the root down the infection thread after 12 hours (Turgeon & Bauer, 1985; Gage, 2004). Root hair growth is induced by auxin (Pitts *et al*., 1998), which is known to increase cell wall extensibility allowing infection pocket formation and growth of infection threads (Oldroyd *et al*., 2001; Esseling *et al*., 2003). As cytokinins affect auxin transport (Dello Ioio *et al*., 2008; Plet *et al*., 2011; Marhavý *et al*., 2014), it is likely that auxin accumulation is induced through cytokinin activity (Suzaki *et al*., 2013). At the epidermis, ABA application decreased the number of bacterial colonies within root hair curls, and inhibited the step between root hair swelling and curling (Suzuki *et al*., 2004).

Auxin signalling is important for root hair infection, with auxin-response genes locally induced in infected cells (Breakspear *et al*., 2014). GA suppresses infection thread development in pea (McAdam *et al.,* 2018) and applying the GA biosynthesis inhibitor (UniP) increased nodulation and infection threads (Maekawa *et al*., 2009). JA application suppressed nodulation in the *har*1-4 super-nodulating mutant by inhibiting root hair curling, infection thread and nodule primordial development (Nakagawa & Kawaguchi, 2006).

Cortical cell division

The infection thread elongates into the cortex cell below the infection site, which has already initiated cell division to form the nodule primordium (Figure 1.4D) (Calvert *et al*., 1984;

Mathews *et al*., 1989). Cell cycle responses occur and coincide with auxin responses and both strigolactone and GA biosynthesis (Breakspear *et al*., 2014). In determinate nodulation, cytokinin response genes are activated in the outer cortex cells (Lohar *et al*., 2004; Plet *et al*., 2011; Held *et al*., 2014). The positive effect of cytokinin application on cell division and expression of early nodulation gene is well documented (Jiménez-Zurdo *et al*., 2000; Mathesius *et al*., 2000; Tirichine *et al*., 2006; Murray, 2007) and this is likely through auxin, as cytokinin induces auxin transport inhibitors leading to auxin accumulation which drives cell division. As nodules form, three auxin transporter-like proteins are upregulated in developing primordia showing active auxin import (de Billy *et al*., 2001). Ectopic overexpression of the *microRNA160* gene in soybean, that negatively regulates auxin response factor genes, resulted in fewer nodules (Turner *et al*., 2013). In this case, nodule development and not initiation was inhibited, which could be because the transition from cell division and differentiation requires specific gradients of auxin distribution (Turner *et al*., 2013).

Applying very low concentrations of ethylene (0.07 ppm) greatly reduced cortical cell divisions, which leads to nodule primordium formation (Lee & La Rue, 1992). ABA also has a role in the regulation of cortical cell division by down-regulating cytokinin levels. This was proved using the *L. japonicus* gain-of-function LHK1 cytokinin receptor mutant, usually forming spontaneous nodules, was suppressed by 1 µM ABA treatment (Ding *et al*., 2008).

Mature nodule

When the infection thread reaches the dividing cortex, rhizobia are released into membrane bound compartments (symbiosome) (Udvardi & Day, 1997) where they differentiate into bacteroids, the nitrogen fixing form (Figure 1.4E). Cytokinins are required in certain expression windows to control both nodule numbers and differentiation (Mortier *et al*., 2014). The cytokinin response receptor (CRE1) controls the transition between meristematic and differentiation zones in mature nodules, suggesting a requirement for cytokinin at late as well as early stages of nodulation (Plet *et al*., 2011). Despite early negative regulation, GA appears necessary for proper nodule organogenesis, as GA deficient *na* pea mutants form limited numbers of small white nodules with reduced meristems (Ferguson *et al*., 2005; Ferguson *et al*., 2011). This demonstrates that nodule size and function is promoted by GA, as small nodules that formed on *na* mutants contained bacteria more like that of infection

threads than the large bacteriods of wild types and led to reduce BNF (McAdam *et al*., 2018). The dual role of GA can be explained spatially, whereby it limits infection thread formation in root hairs but promotes nodule organogenesis in the cortex (McAdam *et al*., 2018).

The nodule structure is formed of central nodule and vascular tissue (Newcomb *et al*., 1979; Calvert *et al*., 1984; Ferguson *et al*, 2005). Although ABA negatively regulates nodulation in early stages it is elevated in developing nodules (Ferguson & Mathesius, 2014), being required for proper nodule meristem formation with the ABA-insensitive *latd M. truncatula* mutant having small white nodules, suggesting a positive role in later nodule development (Liang *et al*., 2007). Ethylene is also a strong negative regulator of nodulation but does not alter the number of mature nodules (Nukui *et al*., 2004). Thus, hormones act at specific stages of nodulation. Nodules usually remain on the root for a few weeks then senesce and are replaced by new ones. Nodule senescence is triggered through nitric oxide (NO) accumulation in the active zone of nodules (Hichri *et al*., 2016).

1.8 | Summary

Sustainable intensification is required to enable future food supply with increasing population and unpredictable future climate. Soybean, as a protein source for humans and livestock, is a globally important crop. Its nitrogen fixation capacity may help mitigate some of the environmental damage associated with its production. Sufficient nitrogen is crucial for soybean yield therefore increasing BNF is key to sustainably reaching its yield potential. Nodules are the site of BNF and although symbiotic rhizobia and mechanisms behind plant signalling events have received much attention, nodules themselves and the impact of changes in their morphology on BNF are not well understood. Phytohormones are involved in each step of nodule initiation, development and function but the ability of phytohormone application to enhance BNF of soybean has not been explored.

The overall working hypothesis of this thesis is that increased nodule size or speed of establishment will alter BNF, and that genotype selection and phytohormone application may enhance BNF leading to increased yield in soybean.

Chapter 2 aimed to: (i) assess the performance of a novel image based nodule quantification method, (ii) examine genotypic differences in nodule development and BNF over time and (iii) determine the influence of different nodule traits on BNF. It was hypothesised that genotypes

would show genotypic differences in BNF, as seen previously, and that this could be explained by nodule traits. It was also hypothesised that the novel nodule quantification method would better detect such genotypic differences compared to conventional methods.

Chapter 3 aimed to: (i) determine an optimal hormone treatment that could enhance nodule traits, leading to increased BNF and (ii) determine the timing of treatment effects. It was hypothesised that the hormone treatments would enhance nodule traits and that this would, subsequently, increase BNF. As the hormone treatments were applied at early growth stage, it was hypothesised that they would alter early nodule development through a priming effect on the symbiotic pathway.

Lastly, Chapter 4 aimed to assess the agronomic potential of both genotype selection and phytohormone application. It was hypothesised that enhanced nodule development and BNF, seen in controlled environments (Chapter 2 and 3), would result in increased yield.

Chapter 2: Genotypic variation and importance of nodule traits in regulating BNF

2.1 | Introduction

BNF depends on successful nodulation and the efficiency of rhizobia to reduce atmospheric N_2. Nodulation is tightly regulated by the host plant, controlling the number, development and turnover of nodules (Ferguson *et al*., 2010; Sulieman & Schulze, 2010). There is a fine balance between plant N requirements and the C supply to nodules. Investment in and regulation of nodules is therefore important for N fixation, but whether the location of nodules, observed on legume root systems, affect fixation capacity is unclear. Soybean roots show a variety of nodule patterns that could comprise many small nodules or a few large nodules, perhaps due to variations in C allocation (Ikeda, 2003). Nodule size, spread across the root system (distribution) and timing may all influence the supply of N from BNF.

Defining 'successful' nodulation can be complex, as nodule morphology and physiology vary across legumes (Walsh, 1995), and even within species due to infection from different rhizobia strains (Ferreira *et al*., 2000). Various traits are used to quantify nodulation success, the most common being nodule number and total nodule weight. Of these, nodule number is most frequently reported (Ferreira *et al*., 2000; Albuquerque *et al*., 2012; Barros *et al*., 2013), but it is often not correlated with increased BNF (de Araujo *et al*., 2017). Nodule counting is also prone to human error. Instead, other factors such as nodule size have a greater influence on fixation (Hungria & Bohrer, 2000; Voisin *et al*., 2003; Tajima *et al*., 2007; de Araujo *et al*., 2017), although the biological reason for this is not fully understood. Total nodule weight is also frequently measured, but this does not reflect variation in individual nodule sizes comprising whole plant nodulation. Soybean appears to show preference to associate with certain strains, thereby increasing BNF capacity (Hungria & Bohrer, 2000) but the effect this has on nodule traits is also unknown. Irrespective of what causes variations in nodulation, this chapters seeks to better understand successful nodulation phenotypes for soybean.

High yielding soybean is dependent on nitrogen application to reach its yield potential (Specht *et al*., 1999; Salvagiotti *et al*., 2008). New genotypes have reduced capacity for BNF (Hungria & Bohrer, 2000; van Kessel & Hartley, 2000; Nicolás *et al*., 2002), reflecting the recent focus

of breeders on above-ground traits (Hungria *et al*., 2006). It is likely that genotypic variation in BNF capacity is determined by nodule phenotypes, as these organs host the symbiosis. Additionally, the timing of BNF affects yield and seed protein content with BNF in vegetative stages improving canopy development and in late reproductive stages allowing continued N accumulation, so reducing reliance on vegetative N remobilisation (Fabre & Planchon, 2000). Legume crop improvement efforts have recognised the importance of root traits (McPhee, 2005; Gonzalez-Rizzo *et al*., 2009; Schultz *et al*., 2010) and the efficiency of BNF (Shtark *et al*., 2010) but not nodulation. Understanding how variations in nodule phenotypes affect BNF could both guide plant breeding efforts, and inform the timing and success of biostimulant treatments, together these approaches will aid crop improvement efforts.

Nodule distribution reflects initial nodulation rate and efficacy (Bhuvaneswari *et al*., 1983; Pierce & Bauer, 1983; Crist *et al*., 1984; Heron & Pueppke, 1984; Malik *et al*., 1984) as only part of the root system, with newly formed root hairs, can be infected at any one time. In this way, it can be assumed that plants with less distributed nodules (close to the root crown) formed nodules sooner. It therefore follows that early nodule formation will lead to earlier BNF. It is unclear whether nodule distribution influences overall BNF, with conflicting results seen (Hardarson *et al*., 1989; McDermott & Graham, 1989). When nodule root position of soybean was manipulated by varying the inoculation technique (seed or soil), timing or depth, nodules that formed on lower root portions showed increased BNF compared to those at the crown (Hardarson *et al*., 1989). However, nodule occupancy with inoculant strains, compared to native rhizobia, led to increased BNF in upper root nodules (McDermott & Graham, 1989).

As well as the speed of nodule initiation, timing of BNF is important for plant growth as asynchronous N fixation and demand may limit soybean growth (Phillips & DeJong, 1984; Keyser & Li, 1992). Soybean genotypes have differing abilities to fix N at early and late stages (Burias *et al*., 1990; Herridge *et al*., 1990; Pazdernik *et al*., 1996; Fabre & Planchon, 2000; Hamawaki & Kantartzi, 2018). Changes in nodulation across these growth stages may help to explain this variation. Although genotypes able to form nodules in early growth may establish BNF sooner, this could incur a cost with earlier nodule senescence limiting N fixation in reproductive stages.

Individual nodule size appears important with greater fixation capacity in larger nodules shown through specific BNF, that is BNF per gram of nodule weight (Voisin *et al*., 2003; Tajima

et al., 2007). This could be due to increased import of C to nodules, due to increased nodule vasculature (Purcell *et al*., 1997; King & Purcell, 2001), as a linear relationship between C allocation and BNF occurs at all growth stages (Voisin *et al*., 2003). Photosynthate supply to individual nodules is determined by the rate of supply and nodule competition (Ikeda, 2003), such that increased nodule number will likely reduce specific BNF. However, since increased nodule size enhances BNF, there may be physiological changes enabling this.

When viewed in cross-sections, larger nodules have a greater proportion of infected tissue than small ones, with a linear relationship between nodule diameter and rhizobia infected area (Tajima *et al*., 2007). This equates to 25% of the inner nodule tissue being infected in 2 mm nodules, compared to 60% in 4 mm nodules (Weisz & Sinclair, 1988). Larger nodules, with more N fixing tissue, have a higher energy demands than small nodules, with increased sink capacity and thus photosynthate requirement (King & Purcell, 2001). As nitrogenase is highly oxygen-sensitive, oxygen permeability is tightly controlled by leghaemoglobin. Greater control of internal nodule oxygen may also lead to increased BNF. Larger nodules have a greater leghaemoglobin content and likely alter oxygen permeability due to their reduced surface area to volume ratio (Hunt & Layzell, 1993). The effect of nodule size on C supply and metabolism or O_2 permeability has, however, not been studied.

Classifying nodules into sizes makes it possible to predict their BNF capacity for different species, with 2-2.5 mm regarded as optimal in *Arachis hypogaea* (peanut) (Tajima *et al*., 2007) and 3-4 mm in common bean and soybean (Vikman & Vessey, 1993; King & Purcell, 2001). However, whether the frequency of these nodules alters whole root N fixation has not been shown. Detailed quantification of nodule distribution is possible (Remmler *et al*., 2014) but it is very time-consuming thus limiting its applications. Detailed quantification of nodule size is also lacking. Image-based systems have showed high accuracy when quantifying nodule number, especially when nodules are removed from roots (Lira & Smith, 2000), but again do not consider nodule size or apply this technique to better understand nodule physiology. Where nodules have been considered in terms of size (Vikman & Vessey, 1993; King & Purcell, 2001; Tajima *et al*., 2007) this has showed differences in nodules of varying sizes but not the number of nodules within size classes per plant. Determining size classifications of nodules could therefore be beneficial, but previous methods involve manual, time consuming methods using size guides. In this study, a novel high throughput image analysis protocol was

developed to calculate semi-automated whole root nodule number, size and spread across the root system (distribution), allowing timing to be inferred.

The USDA soybean germplasm is a valuable genetic resource, but its use has mainly been limited to plant pathology, morphology and seed composition (Posadas *et al*., 2014). This study aimed to establish if changes in nodulation could explain the variation in BNF across genotypes, as seen previously (Hamawaki & Kantartzi, 2018). It was hypothesised that certain nodule traits, such as size, may explain differences in BNF, and that there is an optimum nodule size for BNF. Genotypes with early nodulation are hypothesised to have greater BNF capacity during vegetative growth but limited fixation in reproductive stages. This chapter also aims to assess a newly developed image-based quantification method for its ability to help quantify nodule variation across genotypes; and the effect of this variability on BNF over time. It was hypothesised that image-based nodule quantification would be more sensitive and detect subtle differences in nodulation, not possible with weight based methods.

2.2 | Method

Developing high throughput nodule quantification

As nodule quantification is very labour intensive, involving counting large numbers of nodules, a method was developed here to increase the speed and accuracy of this process. Manually counting of nodules can be very error prone, leading to subtle fluctuations in nodule traits, crucial for detecting genotypic or treatment differences, being missed. While computational image analysis techniques can increase the speed and accuracy of this process, first an accurate method for removing nodules from roots and taking images had to be developed. This was because in whole root scans, nodules are often obscured, making it difficult to accurately estimate nodule number or area. Three imaging methods were used, two involving taking images on a mobile phone and the last using a scanner (Epson expression 11000XL Pro with transparent unit). These methods were correlated with the more labour-intensive conventional method of manually counting nodules. Extra care was taken to ensure that manual nodule counts were correct and could be used to compare the accuracy of other methods. Plants used for this comparison were taken from an experiment screening the effects of auxin and kinetin application as a root drench. Details of plant material and growth conditions are included in the methods section of Chapter 3. Plants were harvested at the

flowering stage and nodules removed and then imaged in one of three ways (Figure 2.1) as well as being manually counted.

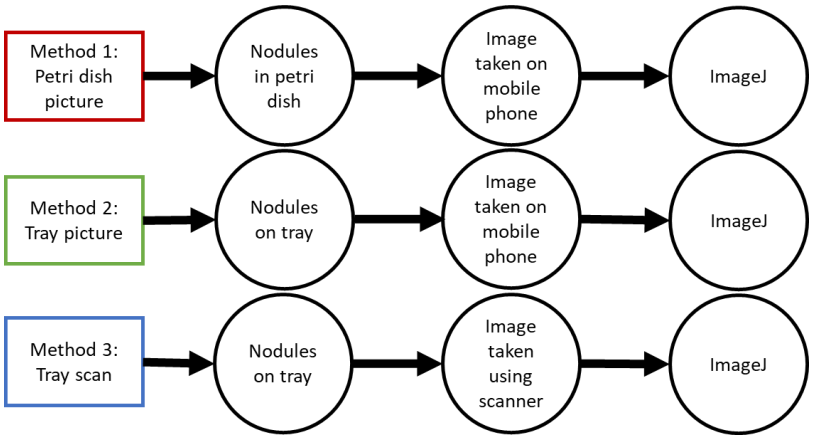


Figure 2.1 Imaging methods used to quantify nodule number and size.

The image processing software ImageJ (available at https://imagej.nih.gov/ij/) was used to analyse images taken previously (Figure 2.2). Images in JPEG format were imported and the scale set, via a plant label of known dimensions, as size reference in the picture (Figure 2.2A). Images were then converted into 8-bit format so that colour thresholds could be applied converting nodules and label markings to black and lighter coloured image noise to white. Plant label markings and additional noise, such as dust or growing substrate, were removed using the 'Flood Fill Tool' (Figure 2.2B). The 'Analyse particles' function can then be used to identify nodules with a size filter of 0.5 mm to 50 mm to remove any remaining contamination. Summary output gives the number of nodules, and total and average nodule area (mm^2); the area of each nodule is also given in a separate output (Figure 2.2C). Nodule distribution was estimated manually through digitally measuring the distance from the root crown to each nodule, this was then averaged to give per plant nodule distribution. A plant label was used as a size reference (Figure 2.2D).

Data was exported and further analysed to give specific nodule traits and number of nodules in size categories. Specific nodule traits are nodule number, weight or area divided by root weight per plant. To classify nodules into different sizes, the diameter was first estimated (assuming spherical shape) and then rounded to the nearest whole number and the number of each counted per plant. Nodules were categorised based on their diameter into size classes of 0.5 to 1.4 mm, 1.5 to 2.4 mm, 2.5 to 3.4 mm, 3.5 to 4.4 mm and 4.5 to 5.4 mm, and referred to as 1 mm, 2 mm, 3 mm, 4 mm and 5 mm nodules, respectively.

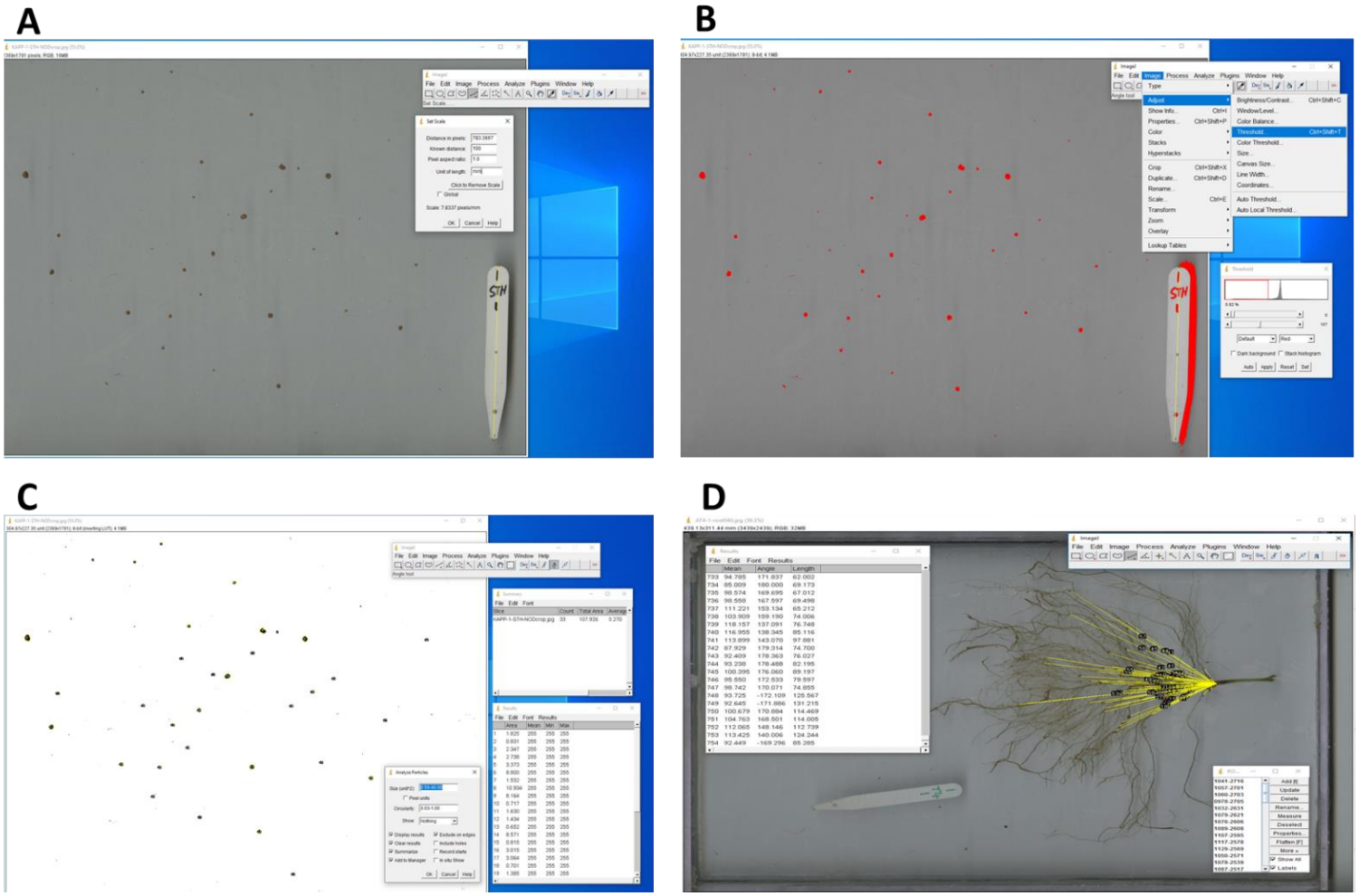


Figure 2.2 Semi-automated nodule quantification method. Image scale is set using plant label as a size reference (A), an image threshold is then set to identify dark regions so highlighting nodules (B), finally particles in the image are selected using size and roundness parameters and number, total and average size calculated (C). Nodule distribution determined by distance of each nodule from the root crown (D).

Plant material and growing conditions

Soybean (*Glycine max* L.) seeds of the genotypes (Table 2.1) Bossier, Davis, Enrei, Williams and 197 (provided by the USDA germplasm collection, GRIN) and the cultivar Viola (donated by Plant Impact) were sown in a randomised complete block design, with 10 pot replicates for each genotype and harvest stage. After autoclaving, fine grade vermiculite (Sinclair professional) was used to fill 2 litre pots. Before planting, seeds were surface sterilised with 1% sodium hypochlorite and then repeatedly washed. Seeds were inoculated with 10^8 CFU ml^{-1} of *B. japonicum*, isolated from commercial Biagro inoculant, that was previously cultured on YEM agar at 29°C for over 4 days. Plant were irrigated with N-limited Hoaglands solution (composition in Table 2.2) every two days with additional water irrigation as required. Greenhouse conditions were logged continuously. Average greenhouse temperature was 29.4°C (20-40°C) day and 21.2°C (16-32°C) night. Light was supplemented by high-pressure sodium lamps (600 W Greenpower, Osram, St Helens, UK) when photosynthetic photon flux density (PPFD) was less than 400 µmol m^{-2} s^{-1} for a 12 h photoperiod (7.00 hrs to 19.00 hrs). Plants were grown to yield with harvests at V2 (two trifoliate leaves), R1 (beginning bloom) and R6 (full seed) and maturity (Fehr & Caviness, 1977).

Table 2.1 Genotypes used with maturity group and BNF characteristics based on (Hamawaki & Kantartzi, 2018).

Genotype	Maturity group	BNF characteristics
Bossier	VIII	Late
Davis	VI	Early and Late
Enrei	IV	Early
Viola	OOO	-
Williams	III	Early
197	V	Poor early

Table 2.2 Nitrogen-limited Hoagland's solution used to irrigate plants.

Nutrient	Chemical name	Chemical formula	Conc. mM
Nitrogen	Calcium nitrate tetrahydrate	$Ca(NO_3)_2.4H_2O$	1
Magnesium	Magnesium sulphate heptahydrate	$MgSO_4.7H_2O$	0.5
Manganese	Manganese sulphate pentahydrate	$MnSO_4.5H_2O$	0.00157
Boron	Boric acid	H_3BO_3	0.01132
Copper	Copper sulphate pentahydrate	$CuSO_4.5H_2O$	0.0003
Molybdate	Ammonium molybdate tetrahydrate	$(NH_4)_6Mo_7O_{24}.4H_2O$	0.00003
Phosphorus	Mono potassium phosphate	KH_2PO_4	1.01
Potassium	Potassium chloride	KCl	1
Iron	Ferric sodium EDTA	NaFe EDTA	0.02547

Plant growth measurements

Plants were harvested when they reached V2 (approximately 20 days after sowing, DAS), R1 (42 DAS), R6 (77-105 DAS) and maturity, shoots were removed from the roots at the cotyledons and leaf area was measured using a leaf area meter (Model Li-3100C Li-Cor, NE, USA). Shoots were then dried at 60^{o}C for 72 hours to measure shoot dry weight.

Root samples were frozen at -20^{o}C until time of analysis. This was necessary as measuring each root system took 30 minutes with up to 70 plants harvested per stage. Roots were scanned (Epson expression 11000XL Pro with transparent unit), then nodules were removed from the roots and scanned again. Roots and nodules were then dried at 60^{o}C for 72 hours to measure nodule and root dry weight. ImageJ (1.51K) (Schneider *et al*., 2012) was used to analyse root and nodule scans as described above.

Biological nitrogen fixation

Oven dried stem samples were first finely cut with scissors and put in 2 ml round bottom Eppendorf tube for grinding using a ball mill at a vibrational frequency of 30 times per second for 2 minutes. Ureide products from fixation (allantoin and allantoic acid), nitrates and amino acids (asparagine and glutamine) were determined and the ratio of each was calculated. Ground stem samples (0.1 g) were used to extract ureide, nitrate and amino acid in a 0.1 mol

L^{-1} phosphate buffer and ethanol heated to 80°C. After cooling, extracts were filtered and centrifuged at 10,000 g then stored at -20°C until analysis. Three methods, Young-Conway's (Young & Conway 1942), Cataldo (Cataldo *et al*., 1975) and ninhydrin (Yemm & Cocking, 1955) were used to colorimetrically measure ureide, amino acid and nitrate N, respectively. Relative ureide was calculated as:

$$Relative\ ureide_N\%\ (RU) = (\frac{4U}{(4U+N+AA)}) \times 100$$

where U, AA and N are molar concentrations of ureide, amino acids and nitrate, respectively (Herridge & Peoples 1990). An estimate of BNF was calculated using the coefficients from Herridge & People (1990) as:

$$BNF = 1.56(RU - 15.9)$$

where RU is the percentage of relative ureide-N.

Data analysis

Two-way ANOVA was used to test for overall differences in the growth and nodulation of genotypes across harvest stages, with genotype and stage as main effects. Fishers least significant difference *post hoc* test was used to determine significant ($p < 0.05$) effects. A generalised linear model determined the effect of nodule traits on BNF and how each varies between genotypes at different stages. In addition, to explore which traits influence BNF in each genotype at different stages, Pearson's correlation between each trait and BNF were also calculated. The statistical package RStudio (RStudio Team, 2020) was used for all these analyses.

2.3 | Results

Quantifying nodulation

Nodule number, determined with all imaging methods, was strongly ($p < 0.001$) correlated with manual nodule counts. Scanned images (Method 3; Figure 2.1) gave the most accurate results (highest R squared values) so it was used in future experiments. These data give confidence that the imaging techniques detect all nodules and can be used in further studies to quantify number and size. Moreover, these imaging methods can determine individual nodule area and number of nodules within defined size categories.

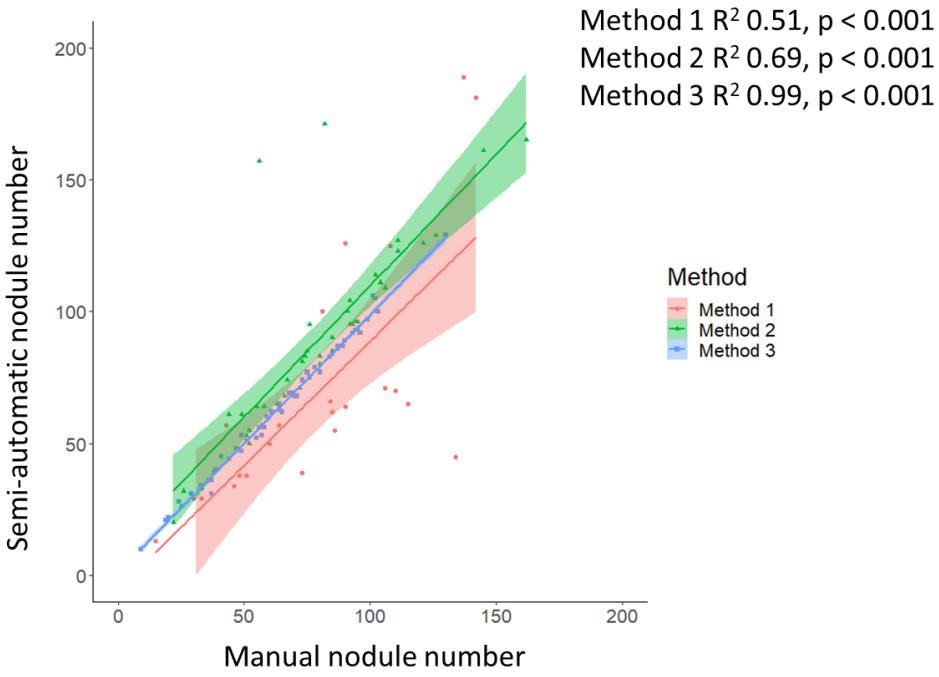


Figure 2.3 Accuracy of methods used to determine nodulation as determined by linear regression. Points are individual plants with line of best fit and shaded area indicating SE.

Genotypic variation in nodule phenotype and BNF

Across the three stages (V2, R1 and R6), BNF of Enrei, Viola and Williams were significantly (~62% more; $p < 0.05$, respectively) greater than that of Bossier. Nodule distribution at V2 did not vary across genotypes (Table 2.3). Genotypes only showed variation in total nodule area at R6, suggesting similar speed of nodule establishment despite differences in BNF (Figure 2.3). Nodule number was 56% ($p < 0.05$) higher in Williams than in the least nodulated genotype, Viola. Total nodule area of Williams was more than double that of both Bossier and Viola ($p < 0.05$). Specific nodule area was highest in Davis, Enrei and Williams, around 24% more than Viola and 197 ($p < 0.05$; Table 2.3). Total nodule weight of Williams was more than double that of Bossier, Viola and 197 ($p < 0.05$; Table 2.3). Specific nodule weight did not vary across genotypes thus the greater nodule weight of Williams was perhaps because of its more vigorous root system (Table 2.8). Genotype did not affect average nodule weight ($p = 0.099$), but average nodule area of Williams was 21% and 38% greater than Bossier and 197, respectively ($p < 0.05$; Table 2.3). Williams had almost five times the number of 4 mm nodules

than Bossier, Viola and 197 with the least ($p < 0.05$). High rates of BNF across all stages in Williams are consistent with high nodule numbers and size, and in Enrei by its specific nodule area. However, there are also inconsistencies such as limited nodule number, total weight or area of Viola despite high BNF and high specific nodule area of Davis despite low BNF. Taken together, the data shows that quantifying nodules across all stages is not representative and does not show the association between nodule traits and BNF capacity.

Overall, BNF was the same at V2 and R6 but was reduced at R1 (Table 2.3; ~38%; $p < 0.05$). Nodule number was higher (88%) in R6 than V2 ($p < 0.05$) and specific nodule number decreased by 49% from V2 to R1 then by 59% from R1 to R6. Again, differences between absolute and specific measures of nodules are explained by differences in root growth (Table 2.8). Total nodule area increased by 86% from V2 to R1 ($p < 0.05$) and by 30% from R1 to R6 ($p < 0.05$; Table 2.3). Specific nodule area decreased by 39% from V2 to R1 then by a further 45% from R1 to R6. Total nodule weight increased three-fold from V2 to R1 ($p < 0.05$) but not from R1 to R6, suggesting that nodules were fully developed by R1 with no further growth. Average nodule area increased by 27% ($p < 0.05$) from V2 to R1 but did not increase further from R1 to R6. BNF was similar at early and late stages despite increased absolute and decreased specific nodule number and weight, as such neither accounts for this. Equally, limited BNF at R1 cannot be explained through changes in nodules.

There was often a genotype x stage interaction, perhaps explaining why differences in nodules just considering one of these factors does not coincide with BNF. Genotypic effects on BNF were stage dependent ($p < 0.001$) with BNF of Davis increased by 91% from V2 to R6 while it decreased in Viola by 46% from V2 to R6 ($p < 0.05$; Figure 2.4A). Since genotypic effects on BNF are stage dependent, the speed of BNF onset also varies among genotypes (Figure 2.4A). Increase in total nodule area was marginally, but not significantly, more evident in certain genotypes, as shown by the genotype x stage interaction ($p = 0.053$; Figure 2.4B). Total nodule area in Williams and 197 increased from V2 to R6 by 4.2 and 3.7 times ($p < 0.05$) but other genotypes showed no significant changes. Again, there was a genotype x stage interaction ($p < 0.001$) with Davis, Enrei and Williams increasing the number of 4 mm nodules by 5.3, 3.6 and 13.1 times from V2 to R6, respectively ($p < 0.05$; Figure 2.4C).

Taken together, BNF and nodule traits varied between these genotypes but growth stage modulated the relationship between these variables. The greatest nodule development of

Williams was not reflected in increased BNF; with an interaction between genotype and stage affecting both nodules and BNF showing that nodule traits have different influence at different times. This suggests that growth stage affects the relationship between these traits.

Table 2.3 Changes in BNF and nodule traits in six genotypes at three stages (V2, R1 and R6). Values are averages (n = 10), with letters denoting significant difference at $p < 0.05$ as determined by least significant difference (LSD) test with p-values from a two-way ANOVA below.

Source of variation		BNF (%Ndfa)	Specific BNF (%Ndfa g^{-1})	Nodule number (#)	Specific nodule number (# g^{-1})	Total nodule weight (mg)	Specific nodule weight (mg g^{-1})	Average nodule weight (mg)	Total nodule area (mm^2)	Specific nodule area (mm^2 g^{-1})	Average area (mm^2)	4 mm nodule number (#)	Nodule distribution (mm)
Genotype	Bossier	20.5 b	3495	42.0 ab	82.7	68.8 b	102.2 ab	1.57	188 b	341 ab	4.97 bc	2.03 b	37.4
	Davis	27.4 ab	655	44.4 ab	78.4	90.2 ab	106.6 ab	1.83	239 b	349 a	5.23 ab	4.53 b	29.8
	Enrei	36.8 a	898	43.7 ab	78.2	139.0 ab	227.5 a	5.03	231 b	375 a	5.51 ab	5.07 b	44.8
	Viola	34.1 a	6212	27.4 b	61.7	45.8 b	82.9 ab	2.33	134 b	271 b	5.96 ab	1.80 b	45.7
	Williams	36.4 a	902	61.1 a	68.2	164.5 a	118.8 ab	2.46	382 a	353a	6.32 a	8.83 a	37.3
	197	27.2 ab	1445	50.2 ab	77.1	69.0 b	77.8 b	1.21	205 b	276 b	3.92 c	1.83 b	53.4
Stage	V2	35.6 b	4010 a	30.9 b	120.9 a	33.4 b	123.8	1.23	125 c	482 a	4.43 b	1.14 c	41.4
	R1	21.5 a	356 c	45.4 ab	62.9 b	103.3 a	140.6	2.80	232 b	296 b	5.63 a	3.44 b	-
	R6	33.9 b	2438 b	58.1 a	39.5 c	152.0 a	93.4	3.19	332 a	204 c	5.90 a	5.74 a	-
Genotype (G)		<0.001	0.281	0.021	0.521	<0.001	0.036	0.099	<0.001	0.024	0.001	<0.001	0.877
Stage (S)		<0.001	<0.001	<0.001	<0.001	<0.001	0.400	0.116	<0.001	<0.001	<0.001	<0.001	-
G x S		<0.001	0.016	0.340	0.479	0.003	0.844	0.801	0.053	0.105	0.397	<0.001	-

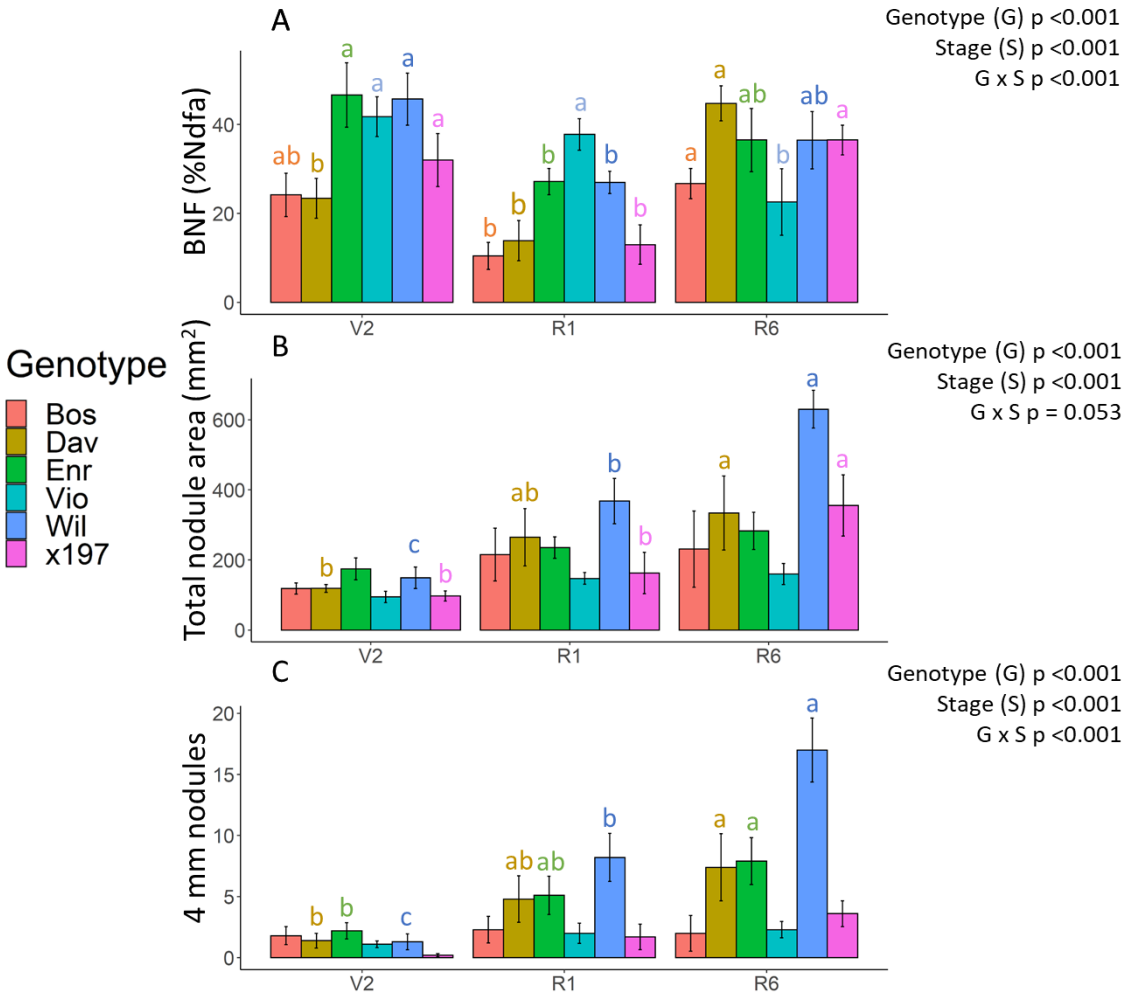


Figure 2.4 BNF (A), total nodule area (B) and number of 4 mm nodules (C) in 6 genotypes (Bossier, Davis, Enrei, Viola, Williams and 197) at three growth stages (V2, R1, R6). Letters denote significant difference for each genotype as indicated by letter colour, determined by LSD test. Bars are means ± SE of 10 replicates. ANOVA p-values from the genotype x stage date interaction are reported.

Importance of nodule traits to BNF for genotypes at different stages

Generalised linear model analysis showed that all nodule traits were positively associated with BNF, except for specific nodule area ($p < 0.05$; Table 2.4). The effect of nodule traits on BNF were all genotype and stage dependent, with triple interactions between these factors ($p < 0.001$; Table 2.4). When considering nodules in different size classes, only 3 and 4 mm nodules positively affected BNF ($p < 0.001$ and 0.007, respectively; Table 2.4). This suggests that nodule traits affect BNF, but this depends on both genotype and stage.

Table 2.4 Generalised linear model results assessing the effect of nodule traits, genotype and stage, as well as the interactions between these main effects on BNF.

Source of variation	Nodule number	Specific nodule number	Total nodule weight	Specific nodule weight	Average nodule weight	Total nodule area	Specific nodule area	Average nodule area
Nodule trait (NT)	**0.003**	**<0.001**	**<0.001**	**0.046**	**<0.001**	**<0.001**	0.544	**<0.001**
Genotype (G)	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Stage (S)	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
NT x G	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
NT x S	**0.043**	**0.001**	0.554	**<0.001**	**0.025**	**0.035**	**<0.001**	**0.044**
G x S	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
NT x G x S	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**

Source of variation	5 mm nodule number	4 mm nodule number	3 mm nodule number	2 mm nodule number	1 mm nodule number
Nodule trait (NT)	0.493	**<0.001**	**0.007**	0.993	0.432
Genotype (G)	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Stage (S)	**0.054**	**<0.001**	**0.003**	0.113	0.896
NT x G	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.022**
NT x S	**<0.001**	**<0.001**	0.346	0.906	**0.009**
G x S	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
NT x G x S	**0.002**	**<0.001**	**<0.001**	**<0.001**	**<0.001**

Across all genotypes, at V2, none of the nodule traits correlated with BNF. However, at R1 and R6, total nodule area, average nodule area and the number of 4 mm nodules positively correlated with BNF ($p < 0.05$; Figure 2.5). Additionally, at R6, total nodule weight also positively correlated with BNF ($p < 0.05$; Figure 2.5). This suggests that nodule traits have a greater effect on BNF at reproductive than vegetative stages. Interestingly, nodule traits were negatively correlated with specific BNF, suggesting that increased nodule development decreases efficiency.

When nodule traits for each genotype at V2 were correlated, only Bossier showed a positive correlation ($p < 0.05$) between BNF and average nodule area (Table 2.5). The number of 4 mm nodules was also positively correlated with BNF in Bossier ($p < 0.01$). This shows that early nodule development influences BNF more in certain genotypes, and that individual nodule size is most important for BNF, at least in Bossier. Additionally, in Davis at V2, the number of 1 mm nodules was positively correlated ($p < 0.01$) with BNF. In contrast, in Williams at V2, specific nodule area was negatively correlated ($p < 0.05$) with BNF. Thus, increased early nodule development benefits Bossier and Davis whilst negatively affecting BNF in Williams.

Figure 2.5 Correlation between nodule dry weight (nodw), average nodule weight (ave.nodw), nodule number (nn), specific nodule number (nn.rdw), total nodule area (tot.area), specific nodule area (tnod.rdw), average nodule area (ave.size), number of 5 to 1 mm nodules (x5-1mm) and BNF across all stages. Pearson's r values are shown with blue circle indicating a significance increase and red circles a significant decrease ($p < 0.05$, $n = 60$). Circle width shows the strength of correlation.

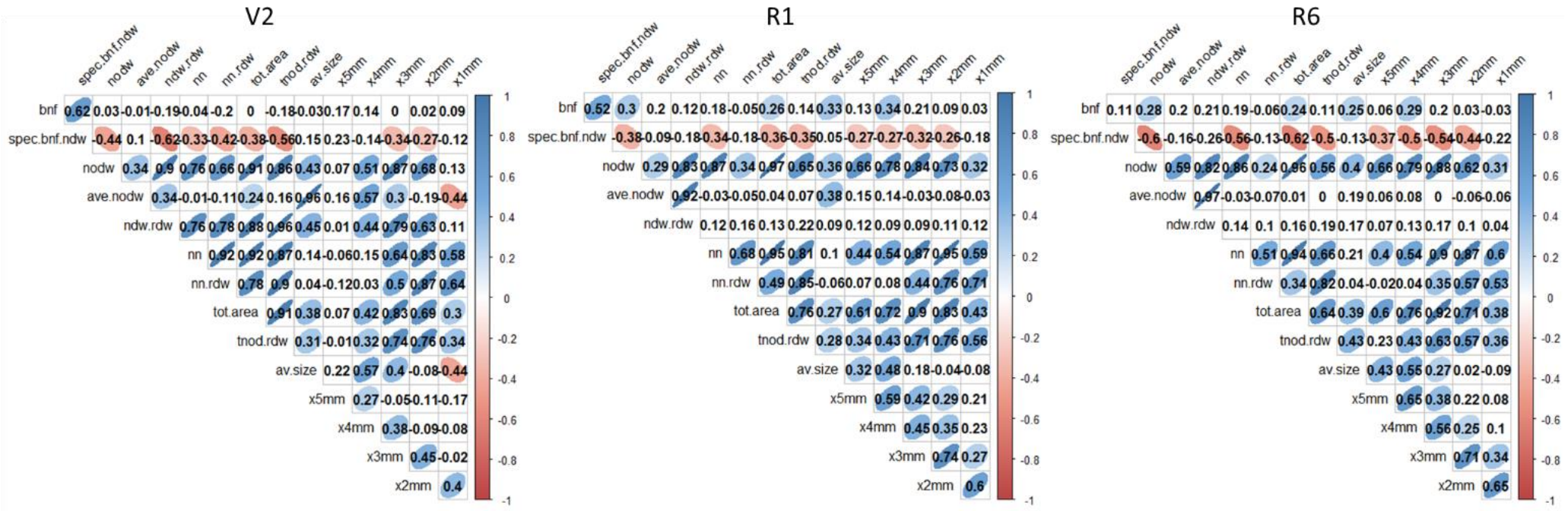


Table 2.5 Correlation between nodule trait and BNF at V2 in each genotype. Values are Pearson r values with significance indicated above (* = p < 0.05 and ** = p < 0.01; n=10).

Genotype	Nodule number	Specific nodule number	Nodule dry weight	Specific nodule weight	Average nodule weight	Total nodule area	Specific nodule area	Average nodule area	4mm nodule number	1mm nodule number
Bossier	-0.39	-0.44	0.28	<0.01	0.64	0.09	-0.09	**0.66***	**0.84****	-0.31
Davis	0.05	-0.25	0.54	0.31	0.54	0.44	0.17	0.59	0.38	**0.80****
Enrei	0.39	0.46	0.15	0.20	-0.48	0.30	0.40	-0.32	-0.09	0.46
Viola	-0.15	-0.32	-0.05	-0.23	-0.31	-0.09	-0.29	-0.36	-0.19	-0.35
Williams	-0.47	-0.59	-0.34	-0.62	0.24	-0.47	**-0.67***	0.20	0.10	-0.35
197	-0.24	-0.29	-0.33	-0.45	0.44	-0.36	-0.48	0.20	-0.11	0.40

At R1, increased nodule number and total nodule area were positively correlated with BNF in Enrei ($p < 0.05$ and $p < 0.05$, respectively; Table 2.6). BNF was positively correlated with a number of nodule traits in genotype 197, perhaps most interestingly with the number of 3 and 4 mm nodules (Table 2.6). Again, this suggests that increases in nodule traits are of particular importance to only certain genotypes at this stage.

Table 2.6 Correlation between nodule traits and BNF at R1 in each genotype. Values are Pearson r values with significance indicated above (* = p < 0.05, ** = p < 0.01 and *** = p < 0.001; n=10).

Genotype	Nodule number	Specific nodule number	Nodule weight	Specific nodule weight	Average nodule weight	Total nodule area	Specific nodule area	Average nodule area	4mm nodule number	3mm nodule number	1mm nodule number
Bossier	0.44	0.19	0.46	0.33	-0.25	0.47	0.35	-0.15	0.42	0.49	0.02
Davis	-0.06	-0.21	0.09	0.17	0.46	0.02	-0.04	0.36	0.25	-0.12	0.21
Enrei	**0.71***	0.45	0.17	0.08	0.04	**0.74***	0.47	-0.28	0.02	0.55	0.44
Viola	-0.02	0.18	-0.04	0.33	-0.13	0.02	0.28	0.08	0.56	-0.24	-0.21
Williams	0.14	0.20	0.15	0.25	-0.14	0.16	0.24	-0.18	0.52	-0.20	0.50
197	0.65*	-0.37	**0.81****	**0.84****	**0.78****	**0.76***	0.36	**0.66 ***	**0.89 *****	**0.72***	-0.49

At later stages (R6), nodule number ($p < 0.05$) and total nodule weight ($p < 0.05$), specific nodule weight ($p < 0.01$), total nodule area ($p = 0.05$), specific nodule area ($p = 0.05$) and

number of 3 mm nodules ($p < 0.01$) positively correlate with BNF in Bossier (Table 2.7). Whilst, in Davis, specific nodule weight, average nodule weight and area positively correlated with BNF ($p = < 0.05$, $p < 0.01$ and $p < 0.05$, respectively; Table 2.7). In Viola, only the number of 4 mm nodule positively correlated ($p < 0.05$) with BNF (Table 2.7). Interestingly, specific nodule weight negatively correlated ($p < 0.05$) with BNF in 197 at R6. Again, these data suggest that the relative importance of nodule traits on BNF capacity differ across genotypes.

Table 2.7 Correlation between nodule trait and BNF at R6 in each genotype. Values are Pearson r values with significance indicated above (* = $p < 0.05$ and ** = $p < 0.01$; n=10).

Genotype	Nodule number	Specific nodule number	Nodule weight	Specific nodule weight	Average nodule weight	Total nodule area	Specific nodule area	Average nodule area	4mm nodule number	3mm nodule number	1mm nodule number
Bossier	**0.72***	0.37	**0.67***	**0.8****	0.12	**0.68***	**0.69***	-0.20	0.11	**0.78****	0.35
Davis	0.38	-0.24	0.52	**0.71***	**0.82****	0.42	0.15	**0.63***	0.47	0.41	0.13
Enrei	-0.28	-0.20	0.30	0.30	0.28	-0.14	0.01	0.28	0.09	-0.23	-0.61·
Viola	-0.28	-0.46	0.17	-0.06	0.45	0.06	-0.36	0.51	**0.73***	0.08	-0.56
Williams	-0.06	-0.18	-0.28	-0.42	0.07	-0.23	-0.49	-0.09	0.10	-0.19	-0.16
197	-0.32	-0.39	-0.39	**-0.64***	-0.46	-0.35	-0.59·	-0.45	-0.47	-0.33	-0.33

Yield and growth

Shoot dry weight of Williams was 63% higher than Bossier and Viola ($p < 0.05$; Table 2.8). Root dry weight of Williams was 60% and 70% higher than Bossier and Viola, respectively ($p < 0.05$; Table 2.8). Total seed weight and number were again highest in Williams with 197 and Davis next. Average seed weight of Williams and Enrei was 59% and 78% higher than Bossier, respectively ($p < 0.05$; Table 2.8).

Table 2.8 Changes in plant growth at three stages (V2, R1 and R6) and yield (R8) in six genotypes. Values are averages (n = 10) with letters indicating significant differences at p < 0.05 within each source of variation determined by LSD with p-values from a two-way ANOVA below.

Source of variation		Shoot dry weight (g)	Root weight (g)	Seed weight (g)	Seed number (#)	Average seed weight (g)
Genotype	Bossier	2.34 d	0.61 c	0.822 c	5.44 c	0.140 b
	Davis	3.90 bc	0.82 bc	3.788 b	22.44 b	0.162 b
	Enrei	3.92 bc	0.73 bc	2.033 c	8.30 c	0.249 a
	Viola	2.61 cd	0.56 c	1.729 c	11.20 c	0.158 b
	Williams	6.05 a	1.47 a	8.303 a	37.44 a	0.223 a
	197	4.42 b	0.89 b	4.894 b	25.88 b	0.164 b
Stage	V2	0.93 c	0.28 c	-	-	-
	R1	3.00 b	0.74 b	-	-	-
	R6	7.70 a	1.52 a	-	-	-
Genotype (G)		<0.001	<0.001	<0.001	<0.001	<0.001
Stage (S)		<0.001	<0.001	-	-	-
G x S		<0.001	<0.001	-	-	-

2.4 | Discussion

Nodulation and BNF

Overall, BNF was genotype-dependent with Enrei, Viola and Williams showing the greatest capacity. Corresponding differences in nodules in these genotypes were not consistently seen across all nodule traits (Table 2.3; Figure 2.4), suggesting a complex relationship between nodules and BNF. Specific nodule traits that account for differences in plant size (Unkovich *et al*., 2008) better allow comparisons of nodules in different genotypes, more closely corresponding with BNF capacity (Table 2.3). Surprisingly, specific nodule traits (nodule number, weight or area divided by root weight) are not often reported, perhaps due to difficulties in recovering and cleaning root systems accurately, with rare exceptions (Yashima *et al*., 2003), despite better representing the plants relative investment in nodules. However, comparisons across growth stages may be misleading as autoregulation of nodulation can suppress further nodule development whereas roots continue to grow.

BNF was not correlated with nodule number, but with nodule size, suggesting that increased size does improve nodule physiology in some way. This supports previous findings where nodule number does not correlate well with N accumulation (Neves *et al*., 1985; Hungria & Bohrer, 2000) but nodule weight does (Döbereiner, 1966; Hungria & Bohrer, 2000). However, the relationship is inverse, with nodule size altered by nodule number, as a finite photosynthate supply must be shared across all nodules. Thus, fewer nodules will have a greater C supply (Ikeda, 1999) allowing greater development (Mahon, 1977). Only a certain number of nodules can be supported, as super-nodulating mutants usually have a dwarf stature, again due to limitations of C supply to nodules. As seen previously (Hungria & Bohrer, 2000; Voisin *et al*., 2003; Tajima *et al*., 2007; de Araujo *et al*., 2017), greater BNF capacity can be achieved in larger nodules (Figure 2.5), suggesting they are in some way more efficient, likely due to altered physiology.

The greater C sink capacity of larger nodules may enhance photosynthate delivery (King & Purcell, 2001). Rhizobial symbiosis increases photosynthetic rates (28%), more than the cost of rhizobia, due to sink stimulation which improves photosynthetic nutrient use efficiency and harvest index (Kaschuk *et al*., 2009). Carbon is commonly not limiting to BNF (González *et al*., 2015), whereas accumulation of nitrogenous products do limit further fixation in a feedback loop (Bacanamwo & Harper, 1997; Sulieman & Schulze, 2010). This response has primarily been observed in drought experiments (King & Purcell, 2005; Sulieman & Tran, 2013) suggesting that the same could be true for nodules with different vasculature in non-stressed conditions, especially at high rates of BNF. Both C import and N export depend on nodule vasculature. Nodules have a complicated dual vasculature surrounding the nodule core (Livingston *et al*., 2019), which may allow larger infection areas that have sufficient import and export to maintain fixation. The majority but not all nodules have this dual vasculature, so it would be interesting to test whether differences in vasculature could be explained by nodule size. Alternatively, nodule oxygen concentration, which is highly regulated in the nodule inner cortex (5-60 μmol m^{-3}) (Millar *et al*., 1995) and adjustment of nodule permeability of the oxygen barrier by host plants may regulate BNF (Serraj *et al*., 1999; Sulieman & Tran, 2013). Differences in nodule size alter leghaemoglobin concentration (Hunt & Layzell, 1993) and may alter oxygen permeability. Future work should seek to understand how nodule traits influence nodule physiology, such as nodule oxygen concentration, carbon

supply or N export and the effect this has on BNF. Number of active nodules and rhizobial strain occupancy may also influence nodule size and subsequently BNF capacity. Nodule traits and BNF could be quantified in plants inoculated with either single or multi-strain inoculum to establish this link.

Nodulation and BNF over time

Different soybean genotypes vary in their ability to fix nitrogen at different stages, either early (around V1) (Pazdernik *et al.*, 1996), late (R5-R6) (Fabre & Planchon, 2000) or throughout (Hamawaki & Kantartzi, 2018) development. In this study, BNF varied across genotypes at both early and late stages but nodules differed only at later stages (Figure 2.4). This suggests that at early stages nodules of different genotypes have varying BNF capacities.

Only certain genotypes showed nodule development from early to late stages, with total nodule area increasing only in Davis, Williams and 197 from V2 to R6 (Figure 2.2), suggesting that nodules of the other genotypes were fully developed at V2. However, it may also reflect difference in plant N demand across stages. Increased nodule development did not necessarily lead to increased BNF. Only Davis showed increased BNF from V2 to R6, whilst the other genotypes did not change. Viola decreased BNF from V2 to R6 without changes in nodule traits. Therefore, the link between nodules and BNF in genotypes is more complex than a function of nodule number or size but instead is stage dependent. There is also evidence of varied nodule efficiency (Figure 2.4), which perhaps could be explained by quantifying aspects of nodule physiology or rhizobial nodule occupancy as mentioned above. Overall, this shows that for different genotypes "successful nodulation" can mean different things.

In general, BNF starts at around V2 and peaks at R1-R4 (Thibodeau & Jaworski, 1975; Keyser & Li, 1992) and it generally increases through the growth period but is genotype and environment dependent (Herridge *et al*., 1990). However, plant N demand and supply from BNF have different patterns (Phillips & DeJong, 1984; Keyser & Li, 1992) at early stages before nodule establishment and in late stages when nodules begin to senesce; altering the timing of nodule development may improve this balance. Previous characterisation of BNF in a controlled environment over time showed the same trend as seen herein, with high BNF in vegetative and late reproductive stages but declining at flowering (R1) (Pitumpe Arachchige

et al., 2020). Decreased BNF at R1 may result from floral initiation, with flower formation reducing the carbon available to meet nodule respiratory demand.

The speed of nodule establishment was the same in all genotypes with no differences in any of the nodule traits at V2. Nodule distribution also did not vary across genotypes, again suggesting that nodules form at the same time in all genotypes (Table 2.3). In this study, nodule traits were more influential at later stages in contrast to previous work where stronger correlations between nodule traits and BNF were seen at early vegetative stages (Voisin *et al*., 2003; Tajima *et al*., 2007). BNF in nodules depends on their developmental stage, therefore, changes in BNF must be due to subsequent development from V2 to R6 as nodule establishment (before V2) is consistent. Future work should determine if the speed of nodule initiation is indeed common to all genotypes by comparing the timing of nodulin gene expression. Due to the environmental sensitivity of nodule formation, this must be conducted in a controlled environment. Differences in endogenous hormones, such as cytokinin, through different stages may also explain differences in nodule development.

Correlations between nodule traits and BNF varied across genotypes and growth stages (Table 2.5 and 2.7). In early growth, increased nodule size correlated with increased BNF in Bossier, whereas in Williams, specific total nodule area and BNF were negatively correlated. Additionally, the number of small 1 mm nodules positively correlated with BNF in Davis at early growth stages. Equally, in later growth, increased nodule number and or size correlated with increased BNF in Bossier, Davis and Viola but negatively correlated with BNF in 197. Thus, increased nodule size was generally linked with increased fixation, as in other experiments (King & Purcell, 2001; Voisin *et al*., 2003; Tajima *et al*., 2007). This is especially noticeable at late reproductive stages when N demand is greatest. In this study, plants were N-limited so they were more reliant on N supply from BNF than that of field-grown plants, hence field trials that account for soil and atmospheric N should confirm these effects. Changes in BNF were not reflected in yield in Enrei and Viola (Table 2.8) but may affect yield quality. Again, field trials would be more appropriate to establish if changes in nodule traits can sufficiently increase BNF to increase yield.

Benefit and potential of novel nodule quantification

Nodule phenotyping techniques are important as future soybean breeding must focus on BNF to enable sustainable N supply to allow continued yield increases. High throughput, aboveground methods, based on the close relationship between N supply and photosynthetic rate, such as leaf traits (size or greenness) have been used as indicators to phenotype nodule traits (Gwata *et al*., 2004; Vollmann *et al*., 2011). However, these methods could only distinguish between nodulated or non-nodulated genotypes; thus more detailed quantification is required. Nodule quantification has advanced (Lira & Smith, 2000; King & Purcell, 2001; Tajima *et al*., 2007; Remmler *et al*., 2014) but to date does not include measures of individual nodule area that make up total nodule area. In this study, average nodule area detected subtle differences in nodules among genotypes (Table; 2.3; Table 2.5) that were not discriminated by average nodule weight. This appears biologically relevant as average nodule area but not average nodule weight correlated with BNF (Figure 2.4). Further, only nodules of a particular size class, not quantified previously, correlated with BNF. This data demonstrates that 4 mm nodules appear optimal at later growth stages (Figure 2.4), according to genotype and growth stage (Table 2.5-2.6).

2.5 | Conclusion

Genotype and growth stage influenced the relationship between BNF and nodule traits. Not all nodule traits are equally important for BNF, with size more influential than nodule number but again this varies across stages, between genotypes and at different stages within genotypes. More detailed quantification of nodule phenotypes will help determine the success of strategies to enhance BNF through altering nodules and establish the usefulness of this approach. The new nodule quantification method can detect differences between genotypes and additional nodule traits included herein (nodule area and size classification) are important for N fixation. This technique can now be used to detect changes in nodule traits caused by hormone application in controlled environments (Chapter 3) and in the field (Chapter 4).

Chapter 3: Phytohormone treatments enhance nodulation and biological nitrogen fixation.

3.1 | Introduction

The endosymbiotic relationship between legumes and rhizobia provides a valuable source of N, with nodules providing a suitable environment for BNF to take place (Ferguson *et al*., 2010; Oldroyd, 2013). In exchange for N, the host plant supplies rhizobia with carbohydrates (photoassimilates), the cost of which means tight regulation of nodule numbers is important. Root perception of NF alters plant hormone synthesis, transport, accumulation and how they are perceived, with hormone composition in the zone of nodulation leading to nodule initiation and development (Mathesius, 2008; Ding & Oldroyd, 2009; Desbrosses & Stougaard, 2011). Intracellular signalling cascades subsequently lead to the formation of nodules (see Chapter 1.7). Phytohormones are important regulators of nodule formation, having positive and negative effects depending on the stage of nodule formation, endogenous concentration and tissue (Ferguson & Mathesius, 2014). Ethylene, jasmonate, abscisic acid and salicylic acid all negatively affect nodulation (Penmetsa & Cook, 1997; Suzuki *et al*., 2004; Stacey *et al*., 2006), whilst gibberellin, auxin and cytokinin being important positive regulators (see Chapter 1.7).

Enhancing the efficiency of symbiosis by manipulating phytohormone signalling may increase or alter nodule traits, resulting in improved BNF with potential benefits to yield or yield quality. After the seedling exhausts its seed N reserves, a period of N starvation may occur 15 to 20 days post emergence before BNF begins (Hungria *et al.*, 1991; Atkins *et al*., 1989; Abendroth *et al.,* 2006). Thus, enhanced early nodulation and BNF may mitigate this.

Rhizobia are able to synthesise a number of phytohormones (Boiero *et al*., 2007). Rhizobia that secrete bioactive cytokinins (Phillips & Torrey, 1972; Sturtevant & Taller, 1989), cause cortical cell division (Cooper & Long, 1994) and enhance the speed of initiation and nodule number and size (Podlešáková *et al*., 2013). This evidence suggests that rhizobia have developed the ability to use hormones to enhance nodules and therefore their own fitness. This likely occurs through horizontal gene transfer similar to that shown experimentally with the transfer of the trans-zeatin secretion gene between *Rhizobium meliloti* that infects alfalfa

(Cooper & Long, 1994). This supports the idea that applying additional phytohormones could promote nodule formation; but the relative benefit to the plant must also be determined.

Gibberellin biosynthesis genes, such as *SrGA20ox1* in *Sesbania rostrata* (Lievens *et al*., 2005), are upregulated during infection thread and nodule development in a number of species (Dobert *et al*., 1992; Kouchi *et al*., 2004; Hayashi *et al*., 2014). Similarly, gibberellin biosynthesis pea mutants, particularly *na*, have low nodule formation; but nodulation can be restored to that of the wild type through GA_3 root application (10^{-6} M) (Ferguson *et al*., 2005). Further, applying gibberellin biosynthesis inhibitors (daminozide, CCC, or paclobutrazol) before rhizobial inoculation suppressed nodule organogenesis in *S. rostrata* (Lievens *et al*., 2005). However, gibberellin regulation of nodulation may be complex, as over production of gibberellin in pea mutants (*na-1*) also reduces nodule development (Ferguson *et al*., 2005). Gibberellin is therefore important for proper nodule development but only within certain concentration windows. Previous studies have linked gibberellin to early nodulation events, but few have explored the effect of application to enhance nodule traits (Bishnoi & Krishnamoorthy, 1991).

Auxin is an important regulator of plant development, with local accumulation leading to organ formation throughout the plant (Benková *et al*., 2003). Auxin and auxin influx transporter (AUX1) transcript accumulate in roots following rhizobial inoculation or NF application (Mathesius *et al*., 1998; de Billy *et al*., 2001; Pacios-Bras *et al*., 2003; Turner *et al*., 2013). This accumulation allows nodule formation also in the absence of rhizobia (Hirsch *et al*., 1989; Mathesius *et al*., 1998; van Noorden *et al*., 2006). Auxin transport is therefore required for early nodulation. Overproduction of auxin in S*inorhizobium meliloti* (expressing *rolAp-iaaMtms2*) and RD20 *Rhizobium leguminosarum bv. viciae* strain (expressing *p-iaaMtms2*) increased nodule number, size and BNF in *Medicago* (*M. truncatula* and *M. sativa*) and *Vicia hirsuta* (vetch), respectively (Pii *et al*., 2007; Camerini *et al*., 2008). However, auxin (IAA) overproducing *R. leguminosarum* had no effect on nodulation of common bean (Pii *et al*., 2007) and 5-methyltryptophan resistant *B. japonicum* mutants, with elevated IAA, restricted nodule mass and nitrogen fixation in soybean (Hunter, 1987), both with determinate nodules. Auxin application (10^{-6} to 10^{-10} M IAA) shortly after sowing (7 and 14 days) increased BNF by up to 76% in *Vigna radiata* (Mung bean) (Ali *et al*., 2008), suggesting that increased early auxin enhances nodulation leading to greater BNF capacity. As beneficial

nodule responses following auxin application vary among legume species, it is important to establish their effect on soybean.

Cytokinins stimulate cell proliferation and differentiation as well as other functions including organogenesis and delay of senescence. Increased cytokinin levels also contribute to nodule initiation. Nod factor application and activation of the calcium calmodulin receptor (CCaMK) led to cytokinin accumulation (Frugier *et al*., 2008; van Zeijl *et al*., 2015). Zeatin (10^{-6} M) and 6-Benzylaminopurine (BAP; 10^{-4} M) application lead to the expression of early nodulin genes *Enod2* and *Enod4*, respectively (Dehio & de Bruijn, 1992; Mathesius *et al*., 2000). Whole root application of low cytokinin concentrations (10^{-8} to 10^{-7}M BAP) stimulated local nodule formation through expression of early nodulin genes of *Lotus japonicus* (*nsp1*, *nsp2* and *nin*) and cortical cell division (Heckmann *et al*., 2011; Plet *et al*., 2011; Ariel *et al*., 2012; van Zeijl *et al*., 2015), leading to expression of early nodulin (*ENOD*) genes (Mathesius *et al*., 2000), indicating its mode of action in the symbiotic pathway. The addition of pTZs plasmid carrying a constitutive trans-zeatin secretion gene to normally non-nodulating *Rhizobium meliloti* also led to the formation of nodule like structures on alfalfa roots (Cooper and Long, 1994). Work with mutants showed the key role of cytokinin perception in nodulation with the gain-of-function in two cytokinin receptor genes (*snf2* and *lhk1-1)* giving spontaneous nodules, whilst loss-of-function showed very few nodules (Gonzalez-Rizzo *et al*., 2006; Tirichine *et al*., 2006; Murray, 2007). These studies show that cytokinin is necessary and sufficient for nodule formation. Applying kinetin (10^{-8} to 10^{-6} M) as a seed soak and foliar spray (at vegetative stage) to *Cicer arietinum* (chickpea) and mung bean increased nodule weight and N fixation at flowering and pod filling (Ali & Bano, 2008; Fatima *et al*., 2008), showing long lasting treatment effects. The effect of cytokinin application on nodule signalling, formation and BNF of soybean has not been shown experimentally.

The concentration of hormone treatments is an important factor determining effectiveness. Applying BAP at low concentration (10^{-6} M) increased nodule number in pea (cv. Sparkle) whereas higher concentrations (2.5 x 10^{-5} M) were inhibitory (Lorteau *et al*., 2001), since cytokinins increased ethylene production (Fuchs & Lieberman, 1968; Vogel *et al*., 1998) which inhibits nodulation. Similarly, GA application at high concentrations (10^{-3} M) decrease, whilst lower concentration increase nodule number (10^{-6} to 10^{-9} M) (Ferguson *et al*., 2005; Maekawa *et al*., 2009). Thus, each hormone may act as a positive or negative regulator depending on

its concentration. It is therefore important to apply a range of treatment concentrations to quantify hormonal effects.

The stage of the nodulation process influences the effect of hormones. Gene expression studies indicate that GA is a positive regulator during early infection and nodule development (Lievens *et al*., 2005; Hayashi *et al*., 2012) but not in mature nodules (Kouchi *et a*l., 2004). Cytokinin is required for nodule initiation and important for regulating nodule number and differentiation but is not involved in infection thread formation (Gonzalez-Rizzo *et al*., 2006; Murray, 2007; Plet *et al*., 2011). Auxin does not alter nodule number but is required for nodule development, particularly during transition from cell division to differentiation (Turner *et al*., 2013). Therefore, the timing of phytohormone application is likely to alter the effectiveness of the treatment but for gibberellin, auxin and cytokinin early application is likely most appropriate.

This study aimed to evaluate the effects of phytohormone application on soybean nodule traits. A limited number of studies have shown nodule and BNF enhancements are possible following hormone application (Table 3.1), but the optimal hormone and concentration has not been established in soybean. Since phytohormones regulate nodule initiation and development, it is hypothesised that hormone application could enhance nodule traits, in particular nodule size, leading to increased BNF, as shown in Chapter 2. More detailed characterisation of nodule development is needed to explain any changes in BNF (Chapter 2). Phytohormone application at early stages of nodulation was hypothesised to promote nodule development, leading to increased fixation. This study comprised three objectives: i) to test the effectiveness of candidate hormones (GA_3, IAA and kinetin) at hormone-specific concentrations (10^{-11} M to 10^{-7} M; Table 3.2), ii) assess different application methods (seed prime, foliar spray and root application) and their interactions with treatment concentration and iii) show the effect of hormone treatment over time, to better understand treatment effects on nodule signalling and development.

Table 3.1 Summary of hormone application studies showing effect on nodulation and BNF.

Phytohormone	Species	Application method	Concentration	Effect	Reference
Cytokinin (Kinetin)	Chickpea	Seed soak and foliar in pot trial	10^{-5} M seed and 10^{-6} M foliar 4 weeks after sowing	Increased infection zone area, BNF and yield. Delayed nodule senescence.	Ali & Bano, 2008
Cytokinin (BAP)	Pea	Root application	0.5x10^{-6}M to 2.5x10^{-5}M 4 and 6 DAS	Increased nodule number at 1x10^{-6}M. Reduced nodulation at high concentration	Lorteau *et al*., 2001
Cytokinin (kinetin) and Auxin (IAA)	Chickpea	Seed soak and foliar	10^{-5} M seed and 10^{-6} M foliar 4 weeks after sowing	Seed soaking with cytokinin and auxin increased nodule weight	Fatima *et al*., 2008
Auxin (IAA and 4-Cl-IAA)	Mung bean	Root application	10^{-10}, 10^{-8} or 10^{-6}M at 7 and 14 DAS	Increased nodule weight, BNF and yield at 10^{-8}M	Ali *et al.,* 2008
Gibberellin (GA_3)	Pea	Root application	10^{-9}, 10^{-6} or 10^{-3} M	Increased nodule number	Ferguson *et al*., 2005

3.2 | Methods

Plant material and growing conditions

Soybean (*Glycine max* L.) seeds cv. Viola (donated by Plant Impact) were used for each hormone application experiment (Table 3.2) with hormone types and concentrations based on the literature (Table 3.1). Seeds were sown into 1 L pots in a randomised complete block design with 12 biological replicates (one plant per pot) per treatment (Figure 3.1A). After autoclaving, fine grade (1-3 mm) vermiculite (Sinclair professional, Ellesmere Port, UK) was used as substrate. Before sowing, seeds were surface sterilised with 1% sodium hypochlorite and then repeatedly washed. Seeds were inoculated with 10^8 cells mL^{-1} of *B. japonicum* USDA110 that was previously cultured on YEM agar (Somasegaran and Hoben, 1994) at 29°C for four or more days. Two seeds were sown per pot, later thinned to one plant per pot just after emergence (VE). Pots were irrigated with modified N-limited Hoagland's nutrient solution that lacked NO_3^-, to prevent the inhibition of nodulation. Average greenhouse temperature was 29.8°C day and 21.3°C night. Light was supplemented by high-pressure

sodium lamps (600 W Greenpower, Osram, St Helens, UK) when photosynthetic photon flux density (PPFD) was less than 400 µmol m^{-2} s^{-1} for a 12 h photoperiod (7.00 hrs to 19.00 hrs).

For the gene expression experiments, Viola seeds were again used. Seeds were again surface sterilised, then soaked in kinetin solution (10^{-9} mol L^{-1}), water (hydroprimed) or allowed to dry (not primed) for four hours. Sterilised large plastic growth pouches (Mega International, West St. Paul, MN) 35 cm long and 16 cm wide were used to grow the seedlings (Figure 3.1). After treatment, four seeds per pouch were planted and 30 ml autoclaved water added to the pouch. To germinate, pouches were wrapped in foil and kept at 28ºC for 2 days. To keep roots dark, pouches were covered in aluminium foil and card was used for support. Pouches were put in growth racks at an angle to aid root contact with the pouch. Plants were grown in a Snijder growth cabinet set to 28°C day and 25°C night temperature with 16 h photoperiod at 80% humidity (Hayashi *et al*., 2012). The experiment was conducted in two runs, each with 4 racks (blocks) that contained a pouch with every treatment x harvest combination. Plants (2-4) from two pouches were pooled to give one biological replicate, giving 4 replicates in total across blocks and over the two runs.

Inoculation occurred 2-6 days after the seedlings were transferred to the pouches and location of root tip marked for harvest (Figure 3.1B-G). *B. japonicum*, isolated from commercial inoculant product Biagro, was grown on YEM agar at 29°C and diluted to OD600 = ~0.1, equivalent to 10^8 cells per mL. Inoculant (500 µl) was added to the portion of root below where the first mature root hairs were expected. Roots were inoculated at different times prior to harvest giving a time course with roots exposed to the inoculant for varying durations. Inoculation occurred at 72, 48, 24, 6 and 3 hours prior to harvest. This allowed plants to be harvested at the same developmental stage. To control for rhizobial contamination in pouches before time of inoculation and to show gene expression without rhizobia perception, additional uninoculated pouches were included.

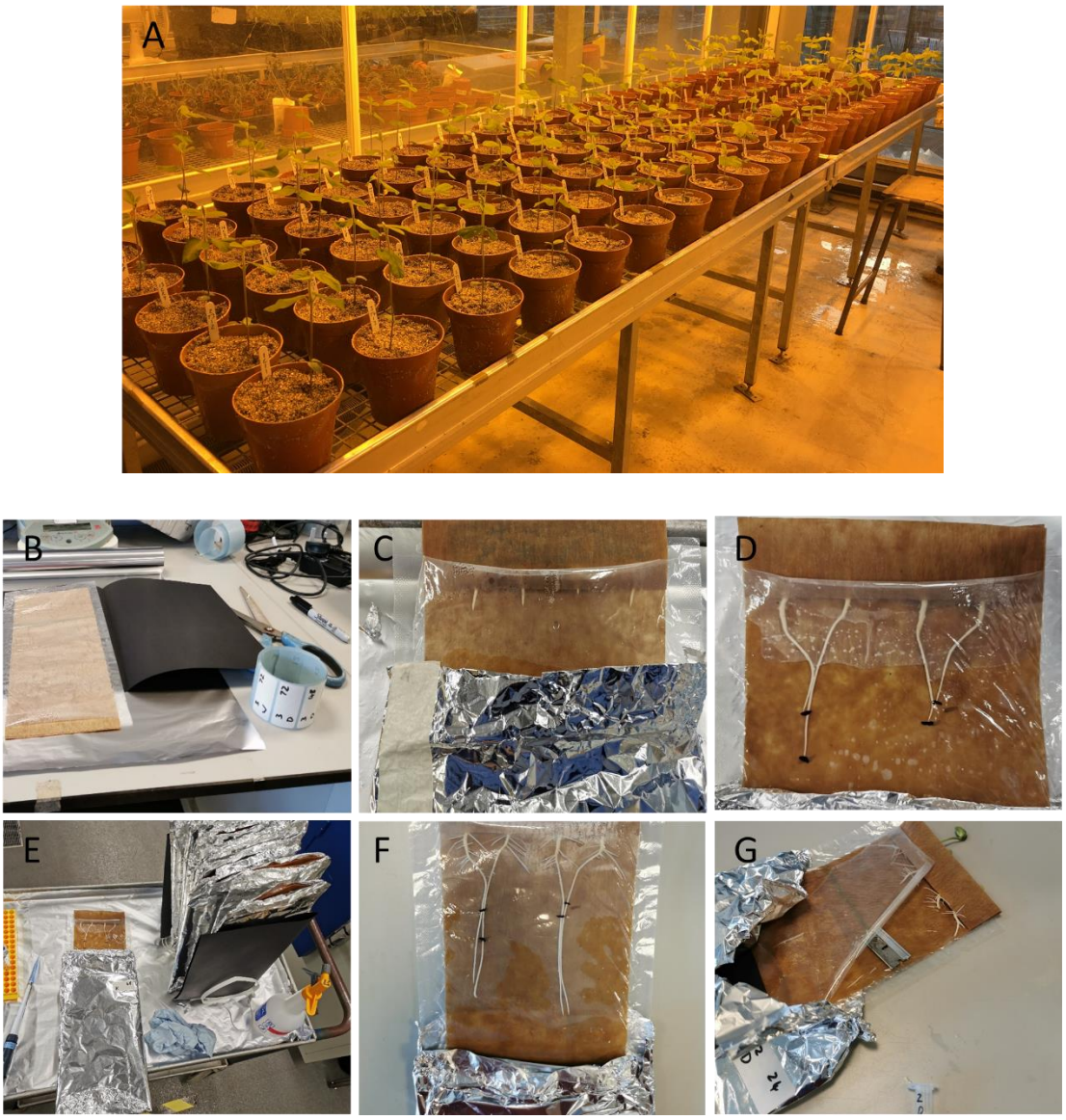


Figure 3.1 Greenhouse pot trial to screen hormone application effects (A) and plant growth method using growth pouches (B-G). Pouches, separated with blue roll, were autoclaved then wrapped in black card and foil (B). Seeds emerged through pouch after 2 days (C). At time of inoculation the root tip was marked on the pouch, indicating root section for harvest (D). Inoculation procedure with pouches of particular time pre-harvest taken from rack and inoculum added (E). Roots at time of harvest (F), harvest method (G).

Table 3.2 Phytohormone screening experiments with application method, experiment number, hormone tested, date, temperature and nodule traits measured in each.

Application method	Experiment number	Date	Average day/ night temperature (°C)	Hormone	Nodule measurements
Seed coat	1.C.1	08/11/17 – 04/12/17	23.6/ 19.7	Cytokinin	Nodule number and weight
	1.C.2	05/01/18 – 08/02/18	22.1/ 18.5	Cytokinin	
	1.A.1	09/08/17 – 01/09/17	32.7/ 24.8	Auxin	
	1.A.2	09/11/17 – 05/12/17	23.6/ 19.7	Auxin	
	1.G.1	04/08/17 – 31/08/17	32.8/ 25.2	Gibberellin	
	1.G.2	25/01/18 – 19/02/18	22.8/ 18.9	Gibberellin	
Root application	2.C	20/02/18 – 22/03/18	24.1/ 18.9	Cytokinin	Nodule number, weight, area, distribution and size
	2.A	02/03/18 – 03/04/18	24.3/ 19.0	Auxin	
	2.G	12/06/18 – 16/07/18	29.2/ 21.6	Gibberellin	
Seed prime, Foliar and Root application	3C	03/07/18 – 10/08/18	29.2/ 21.7	Cytokinin	BNF, nodule number, weight, area, distribution and size
Seed prime and foliar	4C	15/03/19 – 14/05/19	26.3/ 19.5	Cytokinin	BNF, nodule number, weight, area, distribution and size
Seed prime	5C	22/03/19 – 15/04/19	25.5/ 19.3	Cytokinin	Nodule number
Seed prime	6C	15/08/20 – 27/08.20	28/ 25	Cytokinin	Gene expression

Effects of cytokinin, auxin and gibberellin applied as seed coat and root application

In seed coat experiments, 75 µL of hormone treatment (Table 3.3) was added to a petri dish containing 25 g of seed and shaken; treatment is equivalent to commercial seed treatments at 3 g kg^{-1}. For root drench experiments, 20 mL of hormone treatment was applied directly to the growing substrate at growth stages VC and V1. Treatment concentrations varied with the hormone, as guided by literature (Table 3.1), with 4 concentrations (Table 3.3) and solvent and water control used for each except for kinetin which is water soluble thus did not have an additional solvent control.

Table 3.3 Cytokinin (kinetin), auxin (IAA) and gibberellin (GA_3) concentrations used in seed coat and root application experiments.

Hormone	Solvent	Treatment concentrations (mol L^{-1})			
Kinetin	-	10^{-7}	10^{-8}	10^{-9}	10^{-10}
IAA	Ethanol	10^{-8}	10^{-9}	10^{-10}	10^{-11}
GA_3	KOH	10^{-7}	10^{-8}	10^{-9}	10^{-10}

Effect of hormone application method on treatment success

As the most promising hormone treatment (Table 3.6), synthetic cytokinin kinetin (Sigma Aldrich) was applied via three application methods: seed priming, root (applied to substrate), and foliar spray. Seeds that were not primed in kinetin (root, foliar and control) were hydroprimed in water and plants not sprayed with kinetin (root, seed primed, and control) were sprayed with water (Table 3.4). For the seed priming treatment, 25 g of seed were submerged in 25 mL of 10^{-7} (high) and 10^{-9} mol L^{-1} (low) kinetin solution for 4 h. Seeds were air dried in the greenhouse before inoculation and sowing later that day. Foliar and root application took place at early growth stages, VC and V1, respectively. Foliar spray was applied with a handheld pump pressure sprayer and root application by pouring 20 mL of kinetin solution onto substrate. Again, concentrations of 10^{-7} (high) and 10^{-9} mol L^{-1} (low) kinetin solution were used for both foliar and root applications.

Table 3.4 Timing and method of kinetin application for each application method.

Treatment	Pre-planting	VC	V1
Kinetin seed prime	Kinetin 10^{-7} and 10^{-9} mol L^{-1}	Water	Water
Kinetin foliar	Water	Kinetin 10^{-7} and 10^{-9} mol L^{-1}	Kinetin 10^{-7} and 10^{-9} mol L^{-1}
Kinetin root	Water	Kinetin 10^{-7} and 10^{-9} mol L^{-1}	Kinetin 10^{-7} and 10^{-9} mol L^{-1}
Water control	Water	Water	Water

Plant growth measurements

At flowering stage (R1, approximately 30 DAS) plants were harvested, leaf chlorophyll (MC-100 chlorophyll concentration meter, Apogee Instruments, USA) leaf area, shoot and root dry weight and nodule quantification were all determined as described in Chapter 2. BNF was also determined as described in Chapter 2. In gene expression experiment 6C, 1.5-2 cm root sections containing the zone of emerging root hairs, previously labelled at inoculation, were harvested. One biological replicate comprised root sections from 4-6 plants (1 pouch from 2 blocks) pooled, wrapped in foil, snap frozen in liquid N, and stored at -80 °C.

RNA extraction and cDNA synthesis

Root sections (90 to 100 mg) were ground in liquid nitrogen in a pestle and mortar to give a fine powder and total RNA was extracted and purified using the RNeasy Plant Mini kit (Qiagen Ltd, UK) with optional DNase on column digestion (RNase free DNase set, Qiagen Ltd, UK) to eliminate DNA contamination. RNA integrity was checked using gel electrophoresis and RNA concentration on the SPECTROstar Nano Microplate Reader (BMG Labtech) at wavelength 260 nm.

Total RNA (1 µg) was then used to synthesise complementary DNA using the ProtoScript First strand cDNA Synthesis Kit (New England BioLabs, USA) using oligo(dT) primers in a 20 µL reaction. An initial incubation step at 25°C for 5 minutes followed by 1 hour at 42°C, then denaturation at 65°C for 20 minutes. The cDNA was diluted in a total volume of 100 µL.

Semi-quantitative reverse transcription polymerase chain reaction

Semi-quantitative reverse transcription PCR (RT-PCR) used actin as a control primer and *ENOD40a* was the target gene (Table 3.5) with the *Taq* DNA polymerase and inert red dye (BioMix Red reaction mix). A thermal cycler (PTC-100, MJ Research, Inc., USA) was used to perform reactions with 26 cycles of 1 minute at 94°C to denature primers, 1 minute at 55°C allowing primer to anneal to template and 30 seconds at 72°C to allow template to be copied.

Table 3.5 Soybean primer sequences *ENOD40a* target and actin control used in semi-quantitative RT-PCR reactions.

Gene name	Forward primer (5'→3')	Reverse
GmENOD40a	TCTCTCTTGAGTGGCAGAAGCA	TGGAGTCCATTGCCTTTTCG
GmActin	TCGTATGAGCAAGGAAATTGG	TAGAGCCACCAATCCAGACAC

Band intensity of target and control genes per sample were compared to account for potential differences in cDNA concentration due to pipetting error or RNA quantification inaccuracies. Three technical replicates were carried out per biological replicate also to reduce error.

Differences in levels of transcripts between target and control gene were detected by running PCR products using agarose gel electrophoresis (Appendix 3.9) to separated RT-PCR products using 10 µL of product on a 1.5% agarose gel, agarose dissolved in 1 x TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0), containing ethidium bromide (0.1 µg mL^{-1}) at 82 watts for 45 minutes. MassRuler DNA ladder, with a lower band of 80 bp, was used to estimate primer product sizes. A UV transilluminator with digital camera (Gel Doc 2000, Bio Rad, USA) was used to visualise RNA and DNA samples. Loading dye (6 x; MBI fermentas, USA) was added to RNA samples. PCR reactions with BioMix Red did not require additional loading dye. Differences in band intensity were quantified using ImageJ and relative expression calculated by dividing *ENOD40a* band intensity by that of actin to give relative expression.

Data analysis

One-way analysis of variance (ANOVA) was run with the data from hormone application experiments 1-3 (Table 3.2) with hormone treatment as the main effect. A two-way ANOVA was conducted for experiment 4 and 5 data with the model including hormone treatment and growth stage as main effects. Two-way analysis of variance (ANOVA) was run for experiment 6C, with harvest time and cytokinin treatment as the main effects. Protected Fishers least significant difference was calculated to detect significant ($p < 0.05$) effects. Models were validated by checking the normality of the residuals and by plotting residuals against fitted values. All data analysis was performed in R software (RStudio Team, 2020).

3.3 | Results

Seed coat application of cytokinin, auxin and gibberellin

Seed coat application of cytokinin, auxin or gibberellin did not significantly change any of the nodules or plant growth variables at any of the concentrations tested (Table 3.6; Appendix 3.1-3.3). Seed coat application therefore did not enhance any of the nodule traits assessed or BNF, as seen from lack of plant growth response (leaf area and shoot biomass) in these experiments.

Table 3.6 Effect of hormone seed coat application in nodule number, nodule dry weight, specific nodule number, average nodule and shoot dry weight, leaf area and chlorophyll content. Results of one-way ANOVA (p value reported) with residual SE showing variance of models and degrees of freedom for nodule and growth traits at R1.

		Nodule number	**Specific nodule number**	**Nodule weight**	**Specific nodule weight**	**Average nodule weight**	**Shoot weight**	**Leaf area**	**Chlorophyll content**
Cytokinin	Treatment	0.975	0.913	0.453	0.217	0.424	0.841	0.630	0.353
	Residual SE	31.4	156	13.7	58.2	0.70	0.140	21.19	40.92
	d.F	250	250	250	250	249	251	250	245
Auxin	Treatment	0.164	0.292	0.584	0.980	0.467	0.353	0.334	0.387
	Residual SE	12	39.8	10.56	22.31	1.04	116.9	14.15	44.77
	d.f	137	137	137	137	137	137	137	137
Gibberellin	Treatment	0.996	0.825	0.262	0.218	0.671	0.548	0.489	0.837
	Residual SE	25.8	108.9	12.98	39.96	0.372	109.2	19.66	35.33
	d.f	76	36	37	36	37	120	120	119

Root application of cytokinin, auxin and gibberellin

Root application of 10^{-10}, 10^{-9} and 10^{-7} mol L^{-1} cytokinin, in Experiment 2.C (Table 3.2), increased specific nodule number by 52%, 31% and 45%, respectively ($p < 0.05$; Table 3.7; Figure 3.2A). Total nodule area was increased by 66% and 92% ($p < 0.05$; Appendix 3.4) following kinetin application of 10^{-10} and 10^{-7} mol L^{-1}. Number of optimal 4 mm nodules (see Chapter 2) also increased more than four-fold after cytokinin application of 10^{-7} mol L^{-1} ($p < 0.05$; Figure 3.2B). Auxin application to roots (10^{-9}, 10^{-8}, 10^{-7} mol L^{-1}) reduced (approximately 70%; $p < 0.05$) the number of 4 mm nodules. Auxin application of 10^{-7} to 10^{-9} mol L^{-1} to roots

substantially decreased the number of 4 mm nodules ($p < 0.001$; Table 3.7; Appendix 3.5). Gibberellin did not significantly affect any nodule traits measured (Table 3.7; Appendix 3.6). Therefore, of the hormones tested here, nodule traits were most affected by cytokinin, with 10^{-7} mol L^{-1} cytokinin applied to roots having the greatest effect.

Table 3.7 Hormone root application. Results of one-way ANOVA (p value reported and bold where significant) with residual SE and degrees of freedom for nodule and growth traits at R1.

Source of variation		Specific nodule number	Specific nodule weight	Average nodule weight	Specific nodule area	Average nodule area	Nodule distribution	4 mm nodule number
Cytokinin	Treatment	**0.005**	0.118	0.223	0.065	0.552	0.209	**0.040**
	Residual SE	92.8	42.7	0.134	704.8	1.82	13.02	13.2
	d.f	54	52	49	42	42	15	52
Auxin	Treatment	0.161	0.453	0.793	0.201	0.835	0.543	**<0.001**
	Residual SE	130.3	80.2	0.507	56	1.45	22.68	1.42
	d.f	62	62	62	56	56	28	57
Gibberellin	Treatment	0.209	0.722	0.578	0.255	0.136	0.825	0.703
	Residual SE	56.1	0.033	0.853	105.9	0.399	21.81	1.54
	d.f	61	57	57	61	61	29	60

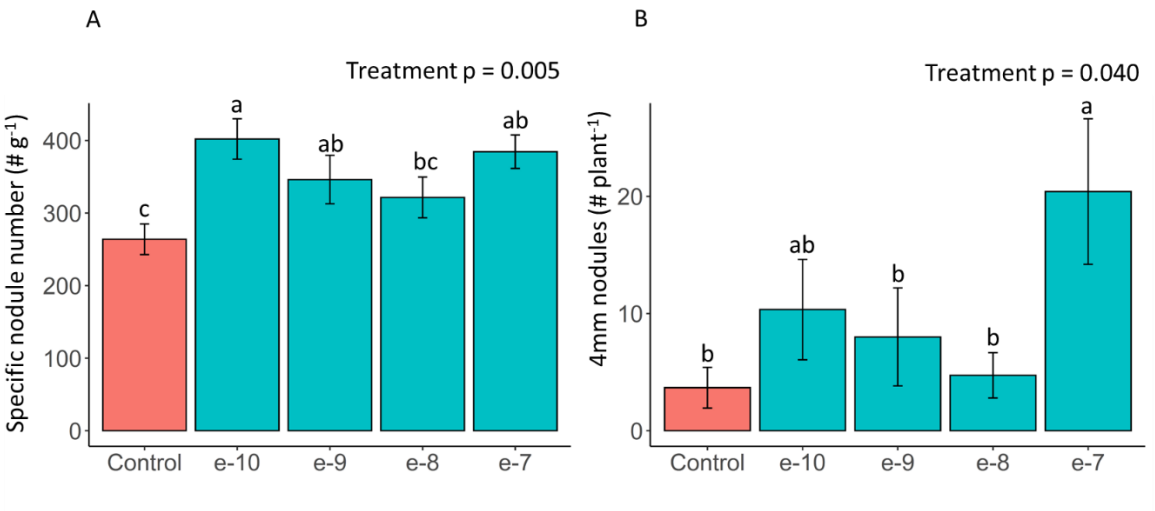


Figure 3.2 Specific nodule number and number of 4 mm nodules following cytokinin root application (teal) of 10^{-7} to 10^{-10} mol L^{-1} (e-7 to e-10) compared to untreated control (red). Letters denote significant difference as determined by LSD test. Bars are means ± SE of 12 replicates. ANOVA p-values from treatment are reported.

Hormone application method

Cytokinin seed priming treatment (10^{-9} mol L^{-1}) approximately doubled BNF (Table 3.8; $p = 0.05$) and increased total nodule area per root weight (32%; $p < 0.05$; Figure 3.3) compared to the control. Root cytokinin application (10^{-7} mol L^{-1}) also increased total nodule area (59%; $p < 0.05$; Table 3.8). Cytokinin treatments had no significant effect on total nodule weight. The mean distance of nodules from the root crown (mm) was roughly halved by cytokinin seed treatments ($p < 0.05$; Figure 3.3; Table 3.8), meaning nodules were less spread across the root system. Cytokinin treatments did not alter shoot weight ($p = 0.146$; Appendix 3.7), root weight ($p = 0.129$; Table 3.8) or leaf area per plant ($p = 0.126$; Appendix 3.7). Therefore, the cytokinin seed treatment (10^{-9} mol L^{-1}) was the most promising treatment, able to increase BNF and total nodule area.

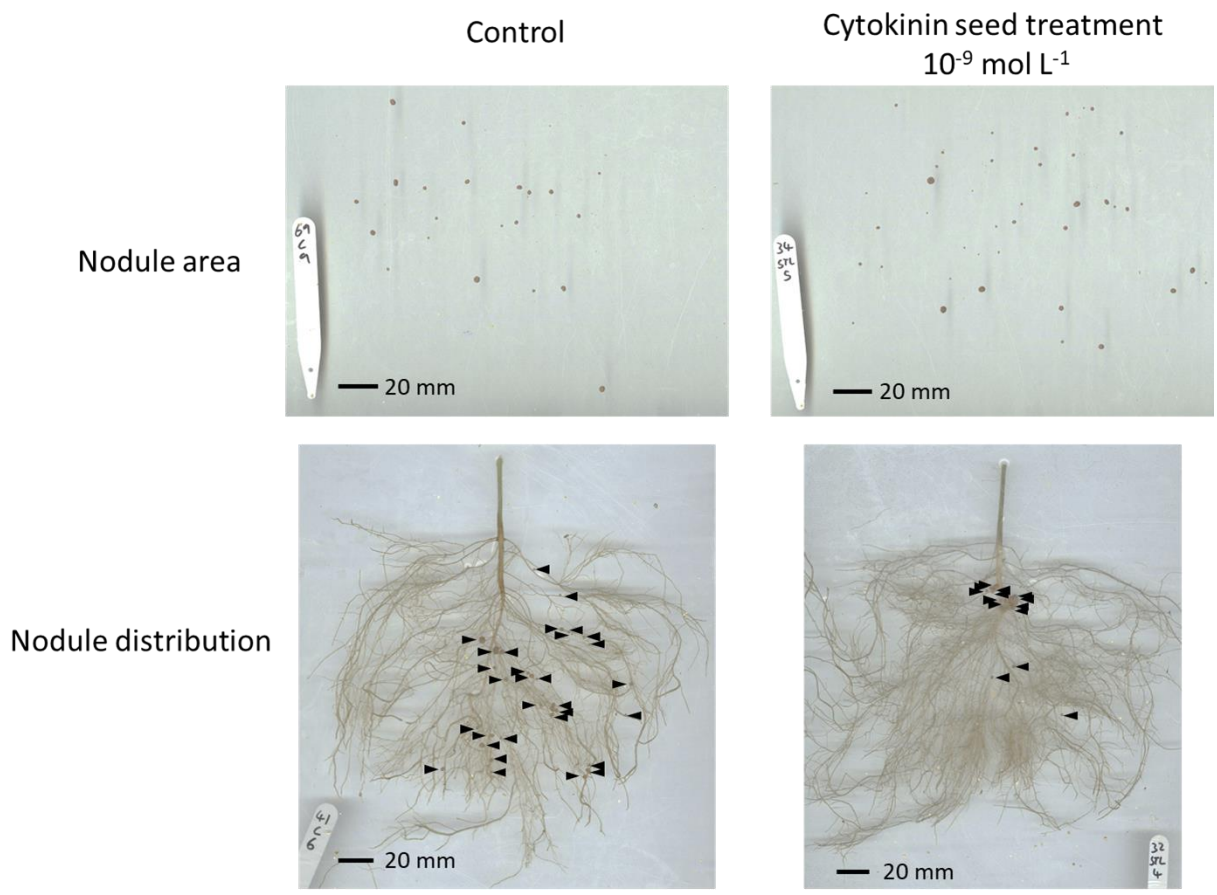


Figure 3.3 Nodule and root scans of plants with close to average nodule area and distribution for respective treatment. Size guide calculated from plant label with line representing 20 mm. Nodule locations indicated with arrows.

Table 3.8 Effect of different cytokinin application methods (foliar, root and seed) at two different concentrations (high 10^{-7} and low 10^{-9} mol L^{-1}) on plant nodule phenotype and root weight. Values are averages, ±SE with letters denoting significant difference at $p < 0.05$ as determined by least significant difference (LSD) test with p-values from a one-way ANOVA below with model residual standard error and degrees of freedom (df).

Treatment		BNF (%Ndfa)	Total nodule area (mm^2)	Specific nodule area (mm^2 g^{-1})	Specific nodule weight (g g^{-1})	Nodule distribution (mm from crown*)	Specific nodule number # g^{-1}	Average nodule size (mm^2)	Average nodule weight (mg)	Root weight (mg)
Control	Control	17.5 b ±3.1	93.7 b ±12.1	471 b ±61.8	10.54 ±1.77	84.9 a ±7.4	127 c ±36.1	4.25 ±0.40	1.41 ±0.18	203 ±15
Foliar	High	21.9 b ±3.2	121.2 ab ±12.6	468 b ±68.3	10.17 ±1.77	65.1 ab ±8.6	122 c ±39.9	4.47 ±0.42	1.47 ±0.20	0.254 ±16.7
	Low	24.8 ab ±3.6	124.8 ab ±13.6	599 ab ±68.3	6.12 ±1.87	74.5 a ±6.6	243 ab ±39.9	2.96 ±0.44	0.87 ±0.20	250 ±16.7
Root	High	22.3 b ±3.6	122.9 ab ±14.1	540 b ±72.5	8.01 ±1.98	77.2 ab ±6.4	172 bc ±42.3	3.70 ±0.47	0.92 ±0.21	237 ±17.7
	Low	22.0 b ±3.9	154.1 a ±12.6	748 a ±64.8	5.71 ±1.77	83.9 a ±6.1	295 a ±37.8	3.15 ±0.42	0.71 ±0.19	217 ±15.8
Seed	High	20.5 b ±3.6	135 a ±14.1	575 ab ±72.5	8.16 ±1.98	34.7 c ±7.4	174 bc ±42.3	3.69 ±0.47	1.03 ±0.21	237 ±17.7
	Low	34.0 a ±3.6	153.3 a ±13.3	625 ab ±68.3	7.16 ±1.87	46.7 bc ±6.6	189 abc ±39.9	3.70 ±0.44	0.98 ±0.20	260 ±16.7
Treatment		0.054	**0.014**	0.054	0.371	**<0.001**	**0.025**	0.147	0.065	0.129
Residual SE		10.21	39.85	205	5.61	14.83	119.7	1.31	0.60	50.1
df		53	58	57	58	24	57	58	57	57

*digitally calculated average distance of nodule from root crow

Cytokinin seed and foliar treatment over time

In Experiment 4C, cytokinin seed or foliar treatments did not significantly alter any of the plant or nodule traits measured (Table 3.9; Appendix 3.8). Cytokinin effects did not depend on growth stage (V2-R6) that plants were harvested (no significant growth stage x treatment interactions). Equally, the cytokinin seed treatment did not increase BNF across all stages ($p = 0.111$; Table 3.9). At V4, however, the cytokinin seed treatment more than doubled BNF ($p < 0.05$; Figure 3.4).

Table 3.9 Effect of different cytokinin application treatments (foliar 10^{-7} and seed 10^{-9} mol L^{-1}) on plant nodule phenotypes from V2 to R6. Values are averages, ±SE with letters denoting significant difference at $p < 0.05$ as determined by least significant difference (LSD) test with results of one-way ANOVA below with model residual standard error and degrees of freedom (df).

Source of variation		**BNF % Ndfa**	**Nodule number (#)**	**Specific nodule weight (mg g^{-1})**	**Average nodule weight (mg)**	**Specific nodule area (mm^2 g^{-1})**	**Average area (mm^2)**	**Nodule distribution (mm)**
Cytokinin	Control	15.5 ±3.03	134 ±6.67	1.15 ±0.039	9.2 ±0.603	608 ±22.3	4.61 ±0.171	68.7 ±3.68
	Foliar	20.2 ±2.99	130 ±6.37	1.19 ±0.039	10.04 ±0.603	621 ±22.3	4.81 ±0.171	67.3 ±3.68
	Seed	24.5 ±3.11	125 ±6.37	1.15 ±0.040	9.43 ±0.613	581 ±22.3	4.66 ±0.171	62.5 ±3.68
Stage	V2	-	59.2 c ±7.14	-	-	236 b ±25	1.06 c ±0.191	-
	V4	13.5 b ±3.16	132.6 b ±7.98	1.19 a ±0.039	7.19 c ±0.647	728 a ±28	4.48 b ±0.214	-
	R4	17.9 b ±2.89	159.8 a ±7.14	1.29 a ±0.039	11.79 a ±0.579	739 a ±25	6.51 a ±0.191	-
	R6	28.7 a ±3.09	167.4 a ±7.14	1.01 b ±0.040	9.69 b ±0.590	710 a ±25	6.73 a ±0.191	-
Cytokinin (C)		0.111	0.708	0.700	0.551	0.520	0.644	0.467
Stage (S)		**0.003**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	-
C x S		0.999	0.589	0.564	0.738	0.462	0.855	-
SE		15.14	39.11	0.216	3.171	137.1	1.047	11.65
d.f		66	102	80	74	102	102	27

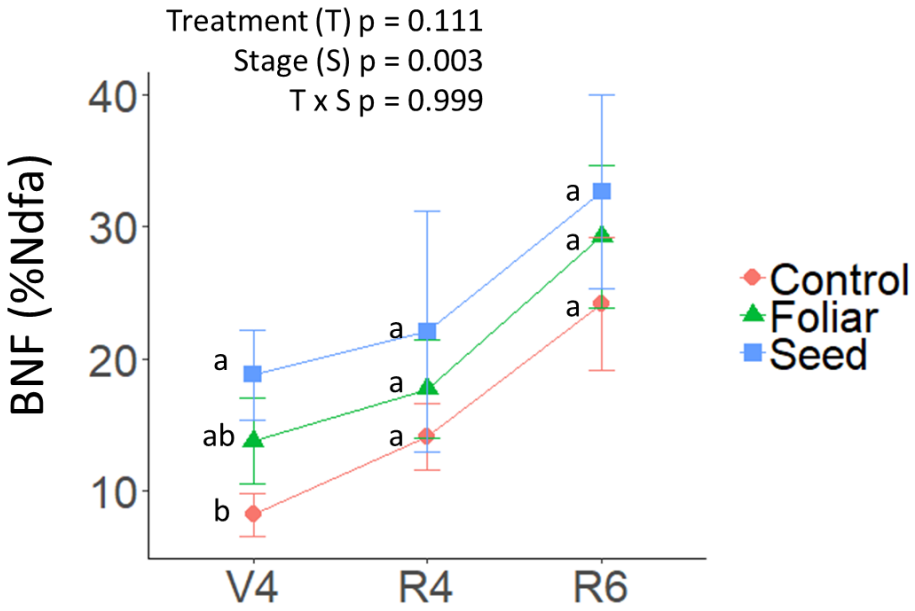


Figure 3.4 Effect of different cytokinin application treatment (control, foliar 10^{-7}or seed 10^{-9} mol L^{-1}) on BNF from V2 to R6. Values are averages, ±SE with letters denoting significant difference at $p < 0.05$ within stages as determined by least significant difference (LSD) test. ANOVA p-values from treatment, stage and their interaction are reported.

Biological nitrogen fixation was approximately 82% higher at R6 than at V4 or R4 ($p < 0.05$). Average nodule area showed corresponding increases from V2 to R6 ($p < 0.05$), however average nodule weight peaked at R4, 63% and 22% more than V4 and R6, respectively (Table 3.9). Thus, nodule mass and size change in different ways across growth stages, with average nodule size (area) corresponding to BNF. Plant growth, photosynthesis and yield were not altered by cytokinin application but did vary with growth stage (Appendix 3.8). Results highlight how different nodule traits vary over time as shown in Chapter 2. Cytokinin appears to increase early BNF but this is insufficient to affect plant growth or yield.

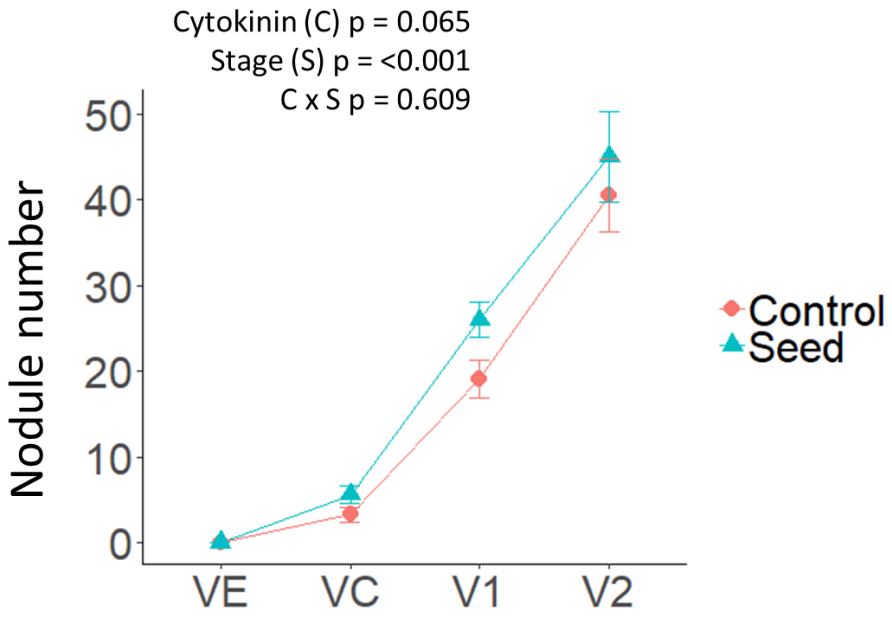


Figure 3.5 Effect of cytokinin seed priming (10^{-9} mol L^{-1}) on nodule number from VE to V2. Values are averages, ±SE with letters denoting significant difference at $p < 0.05$ within stages as determined by least significant difference (LSD) test. ANOVA p-values from treatment, stage and their interaction are reported.

In Experiment 5C, the cytokinin seed treatment did not increase the number of nodules at early (VE to V2) stages ($p = 0.065$; Figure 3.5). However, cytokinin seed priming, in experiment 6C, upregulated relative gene expression of *ENOD40a* by 32% and 52% compared to not primed and hydroprimed controls 72 hours post inoculation ($p < 0.05$; Figure 3.5). No changes in *Enod40a* gene expression were seen compared to uninoculated control before 72 hours. Taken together, these experiments show that the cytokinin seed treatment tend to increase BNF and early nodule signalling and formation.

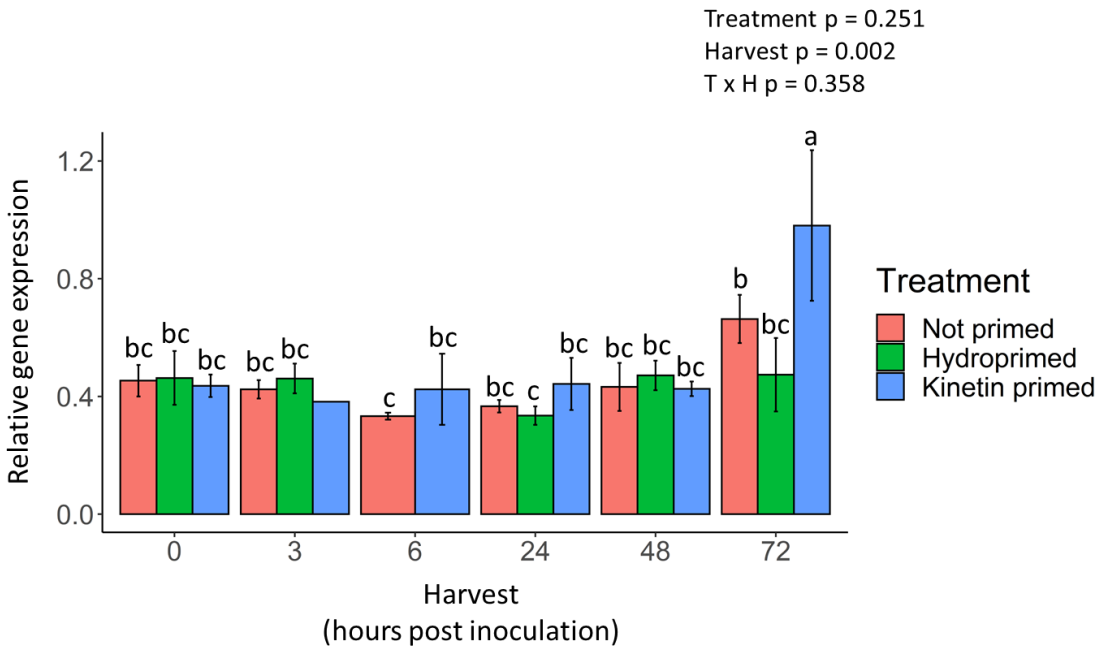


Figure 3.6 Effect of cytokinin seed priming (10^{-9} mol L^{-1}) on *Enod40a* gene expression relative to actin. Values are averages of 1-3 biological replicates each with 3 technical replicates, letters denote significant difference at $p < 0.05$ within stages as determined by least significant difference (LSD) test. ANOVA p-values from treatment, harvest and their interaction are reported.

3.4 | Discussion

In this study, three plant hormones (kinetin, IAA and GA_3) were tested as nodule and BNF biostimulants. Previous tests of phytohormone effects on nodules (Table 3.1) have included only a limited number of hormones and concentrations. Results herein comprehensively compared multiple hormone concentrations allowing an appropriate selection to be made for soybean. Cytokinin (kinetin) was the most promising hormone treatment, increasing nodule traits, including nodule number, specific nodule area and the number of optimal 4 mm nodules (see Chapter 2) (Figure 3.2) as well as BNF (Table 3.8), even though increases in nodule traits were not always consistently seen (Table 3.10). Thus, cytokinin application seems to enhance nodule development leading to increased BNF.

Table 3.10 Summary of hormone application effects on nodule traits and BNF. White cells indicate trait was not measured with different colours denoting either increase (green), negative (red) or no change (grey) following treatment. The shade of colour used indicates the strength of response.

Application method	Experiment number	Hormone	Nodule number	Total area/ weight	4 mm	BNF	Nodulin gene expression
Seed coat	1.C	Cytokinin					
	1.A	Auxin					
	1.G	Gibberellin					
Root application	2.C	Cytokinin					
	2.A	Auxin					
	2.G	Gibberellin					
Seed prime, Foliar, Root application	3C	Cytokinin					
Seed prime and foliar	4C	Cytokinin					
Seed prime	5C	Cytokinin					
Seed prime	6C	Cytokinin					

Although auxin has been reported to have central roles in nodule formation (Mathesius *et al*., 1998; de Billy *et al*., 2001; Pacios-Bras *et al*., 2003; Turner *et al*., 2013), in these experiments exogenous hormone application did not enhance soybean nodules (Table 3.6 and 3.7; Appendix 3.2 and 3.5). Auxin-overproducing rhizobia stimulated nodule formation and development in species forming indeterminate nodules such as *Medicago* and vetch (Pii *et al*., 2007; Camerini *et al*., 2008) but not in those forming determinate nodules such as common bean and soybean (Hunter & Hunter, 1987; Pii *et al*., 2007). Results of this study confirm these earlier observations, with auxin root application tending to reduce total nodule area and number of 4 mm nodules (Appendix 3.5). However, in other experiments exogenous auxin (using IAA and 4-Cl-IAA; 10^{-8} M) application increased nodule size and BNF of mung bean, which also forms determinate nodules (Ali *et al*., 2008), suggesting that nodule type does not always determine the effect of hormone treatments. Therefore, the effects of hormone treatments must be evaluated for each legume species. It could be that endogenous

auxin concentration is sufficient to not limit nodulation in soybean but this may not be the case in other species.

Gibberellin is also thought to be important for nodule formation, especially at early stages (Dobert *et al*., 1992; Kouchi *et al*., 2004; Lievens *et al*., 2005; Hayashi *et al*., 2012). GA_3 application (10^{-6} M) can restore nodule formation of GA biosynthesis mutants (Ferguson *et al*., 2005), but in the present experiments additional GA had no effect on nodule formation for the soybean cultivar Viola (Table 3.6 and 3.7 and Appendix 3.3 and 3.6). Therefore, results suggest that GA operates within a certain concentration range with additional application having no added benefit to nodulation. Thus, it cannot be assumed that additional application of hormones that positively regulate nodulation will enhance nodule traits and BNF.

Cytokinin application did, however, enhance some nodule traits, likely due to its effects on earlier nodule formation. Treatment effects over time indicate potential benefits at different plant stages. Active nodule number is a balance between formation and senescence, driven by the demand for N and the cost of photosynthates, thus harvesting at a single growth stage may not be representative of such changes in nodules over time. Cytokinin treatment may shift nodulation timings with potentially positive and negative consequences at different stages; earlier nodule establishment may result in early senescence. Although long-term changes in the timing of nodules were not detected (Experiment 4C; Table 3.9), early changes occurred. In Experiment 4C, even though the cytokinin treatment increased N_2 fixation at earlier stages (V4; Figure 3.4), there was no cytokinin x stage interaction, suggesting treatment effects on BNF were not stage-dependent (Table 3.9).

More concentrated nodule distribution following cytokinin application (Figure 3.3 and Table 3.8) is suggestive of earlier formation. At a certain distance up from the root tip, susceptibility to nodule initiation is greatest due to root hair formation (Bhuvaneswari *et al*., 1983; Calvert *et al*., 1984) thus less distributed nodules (closer to the root crown) resulted from earlier formation. Additionally, cytokinin increased nodule number in early stages (Figure 3.5). Following NF perception and calcium spiking, cytokinin is a second messenger (Tirichine *et al*., 2006; Murray, 2007; Oldroyd, 2007; Frugier *et al*., 2008) that induces inner cortical-cell divisions (Hirsch *et al*., 1997; Fang & Hirsch, 1998; Mathesius *et al*., 2000) and development of infection threads (Lorteau *et al*., 2001). Taken together, this shows the importance of cytokinin signalling in early nodulation. Cytokinin application (10^{-8} M BAP added to agar

growth media) enhanced nodulation in *L. japonicum* with upregulated expression of early nodulin genes (*NIN* and *ENOD40) (*Heckmann *et al*., 2011).

Results of these experiments confirm the same phenotypic response in soybean (Figure 3.5) and again show cytokinin application can induce NF response genes, in this study *ENOD40a* (Figure 3.6). This perhaps provides evidence that cytokinin seed soak has a priming effect on these genes, perhaps accounting for enhanced nodule development. Upregulation of *ENOD40a* by cytokinin occurred 72 hours post inoculation (Figure 3.6), slower than when NF were directly applied to *S. rostrata* or *Trifolium repens* (Dehio & de Bruijn, 1992; Mathesius *et al*., 2000). This could be due to host-rhizobia signalling events required before NF production by inoculants used in this study. However, *ENOD40a* upregulation 12 hours after inoculation with *B. japonicum* in the same pouch system has been reported (Hayashi *et al*., 2012). This may be explained by differences in the speed of nodule develoment seen across genotypes (Chapter 2) or differences in rhizobial strain used. Further work to establish if different responses are seen across genotypes or in a variety of inoculants would be beneficial. Additionally, as cytokinin application upregulated transcription factors (*nsp1*, *nsp2* and *nin*) upstream of *ENOD* genes in *Lotus japonicus* (Heckmann *et al.,* 2011*;* Plet *et al.,* 2011*;* Ariel *et al.,* 2012*;* van Zeijl *et al.,* 2015*),* expression of these genes should also be determined in soybean to further establish the effect to cytokinin on the symbiotic pathway.

Plant responses to various hormones depend on treatment concentration (Lorteau *et al*., 2001; Ali & Bano, 2008; Piotrowska-Niczyporuk & Bajguz, 2014). Additionally, variable application methods, legume species and cytokinin types across the literature make comparisons of treatment concentrations difficult. In this study, kinetin application to soybean was more effective at a low (10^{-9} mol L^{-1}) concentration than high (10^{-7} mol L^{-1}) for all application methods (in Experiment 3C; Table 3.8), with no concentration x application method interaction. This is in contrast to earlier results (Experiment 2C) where higher concentrations (10^{-7} mol L^{-1}) applied to roots also improved nodule traits (Figure 3.2; Appendix 3.4). Seed priming gave the most promising results of the application methods tested. Root application of 10^{-6} mol L^{-1} (BAP) increased nodule number in pea but higher concentrations had a negative effect due to ethylene production (Lorteau *et al*., 2001). However, kinetin seed soak of 10^{-5} M with chickpea improved nodule traits (Ali & Bano, 2008; Fatima *et al*., 2008). Therefore, treatment concentration should be species-specific with

application method of less importance. The type of cytokinin is also likely to affect plant responses and the concentration at which it should be applied and warrants further testing. For this study, kinetin was chosen based on previous promising results in legumes forming determinate nodules (Ali & Bano, 2008; Fatima *et al*., 2008). Further investigation of continued or later hormone application is recommended, similar to delayed or additional inoculation (from one week to flowering) studies which lead to increased nodule development and yield (Materon, 1994; Moretti *et al*., 2018).

Cytokinin application can result in non-functional spontaneous nodulation in the absence of rhizobia (Heckmann *et al*., 2011), perhaps to the detriment of plant growth. However, increased nodule area (Appendix 3.7) did not restrict plant growth and instead increased BNF (Table 3.8), suggesting that changes in nodule traits are beneficial. Nevertheless, plant growth did not increase, suggesting that the increase in BNF was insufficient to alter plant biomass but may have increased plant N content, altering C:N ratio and nitrogen use efficiency. Responses to cytokinin application are not seen in nitrate-sufficient growth media (Bauer *et al*., 1996), so the cytokinin treatment effects seen in these experiments could be triggered by the limited N availability. Testing cytokinin treatments in in the field is therefore required (Chapter 4).

Results of these experiments do not fully support previous findings (Chapter 2) that increased nodule size enhances BNF (Voisin *et al*., 2003; de Araujo *et al*., 2017) as here the highest BNF corresponds with the largest total nodule area (Table 3.8). This is likely due to differences in the timing of nodules and BNF seen across genotypes, with cultivar Viola showing correlations with 4 mm nodule number only at R6 (Chapter 2). Therefore, it is recommended that the effects of cytokinin treatments on BNF and nodule traits are explored in a wider range of genotypes.

Cytokinin application can increase transpiration, biomass accumulation, chlorophyll content and photosynthesis in non-legumes (Tassi *et al*., 2008; Cassina *et al*., 2011; Dobránszki & Mendler-Drienyovszki, 2014), suggesting that growth stimulation can occur irrespective of effects on BNF. In contrast, plant biomass, chlorophyll content and leaf gas exchange were not altered in these experiments (Appendix 3.7 and 3.8), indicating that increases in BNF are not likely to be due to increased N demand from larger plants or increased photosynthate supply due to increased photosynthesis.

Cytokinin interacts with many other phytohormones, including the negative nodulation regulator ethylene (Lorteau *et al*., 2001) and positive regulator, auxin (Mathesius *et al*., 2000). During nodulation, NF perception reduces the auxin to cytokinin ratio in roots (Cooper & Long, 1994; Bauer *et al*., 1996; Hirsch *et al*., 1997) leading to increased nodule formation (Caba *et al*., 2000). Both cytokinin and auxin act together to induce nodulin gene expression (Jiménez-Zurdo *et al*., 2000) with auxin accumulation dependent on cytokinin (Ng *et al*., 2015). Cytokinin treatments therefore likely work in association with auxin, but this was not measured in these experiments.

3.5 | Conclusion

Cytokinin application was the most promising hormone tested here, with other hormones having relatively little effect. Application method and concentration influenced treatment success with seed priming treatment at a concentration of 10^{-9} mol L^{-1} optimal here. Cytokinin treatment increased total nodule area leading to increases in BNF, supporting previous findings herein (Chapter 2). Promotion of early nodule signalling events also occurred following treatment, with *ENOD40* upregulation, suggesting that cytokinin enhances nodule establishment. However, plant growth was not increased, so, the agronomic value of these increases in BNF on N accumulation and yield were evaluated in Chapter 4.

Chapter 4: Genotype and cytokinin effects on soybean yield and biological nitrogen fixation across soil temperatures.

4.1 | Introduction

Soybean is one of the most important vegetable protein sources globally, contributing to the agricultural economies of many countries (Hungria & Mendes, 2015). Soybean has the highest nitrogen (N) requirement of all major crops (Sinclair & De Wit, 1975) with 80 kg canopy N required per metric tonne of seed and yield strongly correlated to N accumulation (Salvagiotti *et al*., 2008; Rotundo *et al*., 2014). As a legume, soybean uses two N sources, mineral soil N uptake and atmospheric BNF. Soybean can derive up to 70% of its N demand from BNF (Salvagiotti *et al*., 2008; Santachiara *et al*., 2017) but high soil N concentrations limit BNF (Santachiara *et al*., 2019).

Temperature also affects the contribution of the two N sources to plant N status, with BNF generally considered more cold sensitive than soil N uptake (Matthews & Hayes, 1982; Thomas & Sprent, 1984; Legros & Smith, 1994;;). In soybean, root zone temperatures (RZT) less than 25^{o}C delay the onset of BNF, with nodule initiation limited at 10^{o}C RZT and activity at 15^{o}C (Legros & Smith 1994; Zhang *et al*., 1995; Poustini *et al*., 2005; Mishra *et al*., 2009). However, low soil temperatures may also limit mineral N uptake by restricting root growth and/or nitrate uptake as seen in controlled environments (Rufty *et al*., 1981; Tolley & Raper 1985) but not in field trials.

Despite these limitations, which may affect early growth and subsequent yields, many regions recommend early sowing of soybean in cold soils (Purcell *et al*., 2014; Di Mauro *et al*., 2019; Rattalino Edreira *et al*., 2020) to take advantage of early rainfall, to avoid summer drought, reduce disease and insect damage and to extend the growing season. Local soybean production has the potential to improve protein self-sufficiency (De Visser *et al*., 2014), even though many European countries have suboptimal environments for soybean (Kurasch *et al*., 2017).

BNF depends on successful nodulation and rhizobial efficiency to fix atmospheric N_2 to ammonia. Previous work to mitigate the effects of low RZT on BNF have focused on identifying cold tolerant rhizobia (Zhang *et al*., 2002; Zimmer *et al*., 2016; Kühling *et al*., 2018; Yuan *et*

al., 2020). However, the success of rhizobial inoculants can depend on their persistence in the soil and competition with native rhizobia, with local strains better adapted to adverse conditions (Thilakarathna & Raizada, 2017). Early nodule establishment in low RZT may therefore improve the effectiveness of cold adapted inoculants. The photosynthetic cost of BNF (16 moles ATP per mole N) (Kahn *et al*., 1998), requires that plants balance this with their N requirements, but N is more limiting to growth than carbon (C) uptake under low (around 15^{o}C) temperatures (Thomas & Sprent, 1984; Walsh & Layzell, 1986). Thus, promoting nodule development in cold environments is likely to be beneficial.

In optimal soil temperatures, certain nodule traits are associated with increased BNF. Nodule size positively correlates with increased N fixation (Voisin *et al*., 2003; Tajima *et al*., 2007; de Araujo *et al*., 2017) and certain nodule sizes (4 mm diameter) are considered optimal (Chapter 2; Purcell *et al*., 1997; King & Purcell, 2001), with greater relative export of N products and import of C. Increased nodule weight following exposure to low RZT temperatures (15^{o}C), may compensate for lower nodule activity (Zhang & Smith, 1994), suggesting that increased nodule development is beneficial for the cold tolerance of BNF. The effects of early nodule establishment on soybean BNF have been studied previously (Chibeba *et al*. 2015; Cerezini *et al*. 2016) but not in early sown soybean experiencing low RZT.

Different soybean genotypes vary in their ability to fix N in low temperature (Lynch & Smith, 1993; Zhang & Smith, 1994). As new soybean cultivars show reduced BNF under normal conditions (van Kessel & Hartley, 2000; Nicolás *et al*., 2002), similar effects could occur under cold temperatures but with greater impacts on yield as N uptake is also limited. Maintaining N uptake during seed filling is important for high yield (Kumudini *et al*., 2002; Zimmer *et al*., 2016) especially in early sown soybean that experience low RZT. Although genotypes differed in BNF when grown in cool conditions, there was no effect on nodules (Zimmer *et al*., 2016) and nodule traits were not associated with genotypic differences in cold tolerance.

An alternative approach to enhance nodulation and to reduce the effects of cold is to manipulate endogenous hormone concentrations *in planta*, such as cytokinins (Lorteau *et al*., 2001; Ali *et al*., 2008; Fatima *et al*., 2008; Heckmann *et al*., 2011). Low RZT limit early nodulation signalling including perception of NF (see Chapter 1.6). Suboptimal RZT have been shown to decrease endogenous cytokinin in *Solanum lycopersicum* (tomato) and *Cucumis sativus* (cucumber) (Ali *et al*., 1996; Tachibana *et al*., 1997). Cytokinin application may

enhance nodulation by maintaining plant rhizobial communication in low RZT. Cytokinin induces early nodulin genes in plants (see Chapter 3) acting in a similar way to NF signalling, inducing cortical cell division genes (Dehio & Bruijn 1992; Bauer *et al*., 1996; Mathesius *et al*., 2000; Heckmann *et al*., 2011). Therefore, early cytokinin application during nodule formation may compensate for delayed bacterial signalling and stimulate higher rates of nodule development and BNF.

Exogenous cytokinin applications induced positive effects in a number of legumes depending on the application method, timing and concentration (Cho *et al*., 2002; Koprna *et al*., 2016; Liu *et al*, 2004) with high concentrations limiting nodule number (Lorteau *et al*., 2001; Mens *et al*. 2018). Cytokinin applications during early reproductive development (stages R1-R3) increased pod set (Ibrahim *et al*., 2007; Nonokawa *et al*., 2007; Yashima *et al*., 2005). Cytokinin seed priming or application to recently emerged seedlings also increased yield of other legumes (peanut, chickpea, *Lens culinaris* and pea) but effects are unknown in soybean (Schroeder, 1984; Naeem *et al*., 2004; Fatima *et al*., 2008; Dhruve & Vakharia 2013;;). Seed treatment with non-thermal plasmas increased soybean nodule nitrogenase activity, in part by increasing endogenous cytokinin concentrations (Pérez-Pizá *et al*., 2020). While cytokinin application can enhance BNF in chickpea (Fatima *et al*., 2008), no studies have considered cytokinin application to improve BNF of early sown soybean.

Since nitrogen supply is the most limiting factor to soybean yield (Rotundo *et al*., 2014) and suboptimal temperature (<25^{o}C) limits its uptake (Tolley & Raper, 1985; Rufty *et al*., 1981; Zhang *et al*., 1995), this study tested whether N uptake varied between different genotypes and with cytokinin application. A field experiment with early and conventional sowing dates aimed to: (i) examine low temperature responses of different commercial soybean genotypes and (ii) test whether cytokinin application could enhance BNF in cold temperature. Since nodule formation and BNF are sensitive to cold temperature, it was hypothesised that early sowing would limit BNF and any genotypic differences in cold tolerance will reflect differences in N uptake. Moreover, it was hypothesised that cytokinin treatment would enhance nodule traits, helping to maintain BNF during exposure to low soil temperature.

4.2 | Materials and methods

Site conditions, treatments and experimental design

Field trials were conducted to determine whether genotypes differed in, and whether cytokinin treatments enhanced, BNF. How genotype and cytokinin alter response to early sowing with low RZT under field conditions was also evaluated. Trials were sown during the 2018/2019 and 2019/2020 growing seasons, with three sowing dates of 25th September, 8th November (early November) and 25th November (late November) in the first trial and two sowings 19th December and 3rd January in the second. The 2019/2020 trial aimed to establish the effect of cytokinin treatment and did not consider temperature effects. Trials were carried out at Campo Experimental Villarino, located in Zavalla, Santa Fe, Argentina (33°1' S, 60°53 W; elevation 24.6 m). Soil and air temperature and potential evapotranspiration (Hargreaves and Samani, 1985) varied across sowing dates but precipitation did not (Figure 4.1; Table 4.1; Appendix 4.1-4.2). The USDA soil series was a silty clay loam Vertic Argiudoll, Roldan series. Soil (0 to 20 cm depth) had 2.86% and 3.05% organic matter, 13.9 and 18.9 mg kg^{-1} P and 5.8 pH in 2018/19 and 2019/20, respectively. N-NO_3^- was 12.5 mg kg^{-1} in September, 22.9 mg kg^{-1} in early November, and 7.1 mg kg^{-1} in late November in the first trial and 19.4 mg kg^{-1} in December (2019). This rainfed experiment was sown in a field having a double crop of *Triticum aestivum* (wheat) and soybean during the previous seasons.

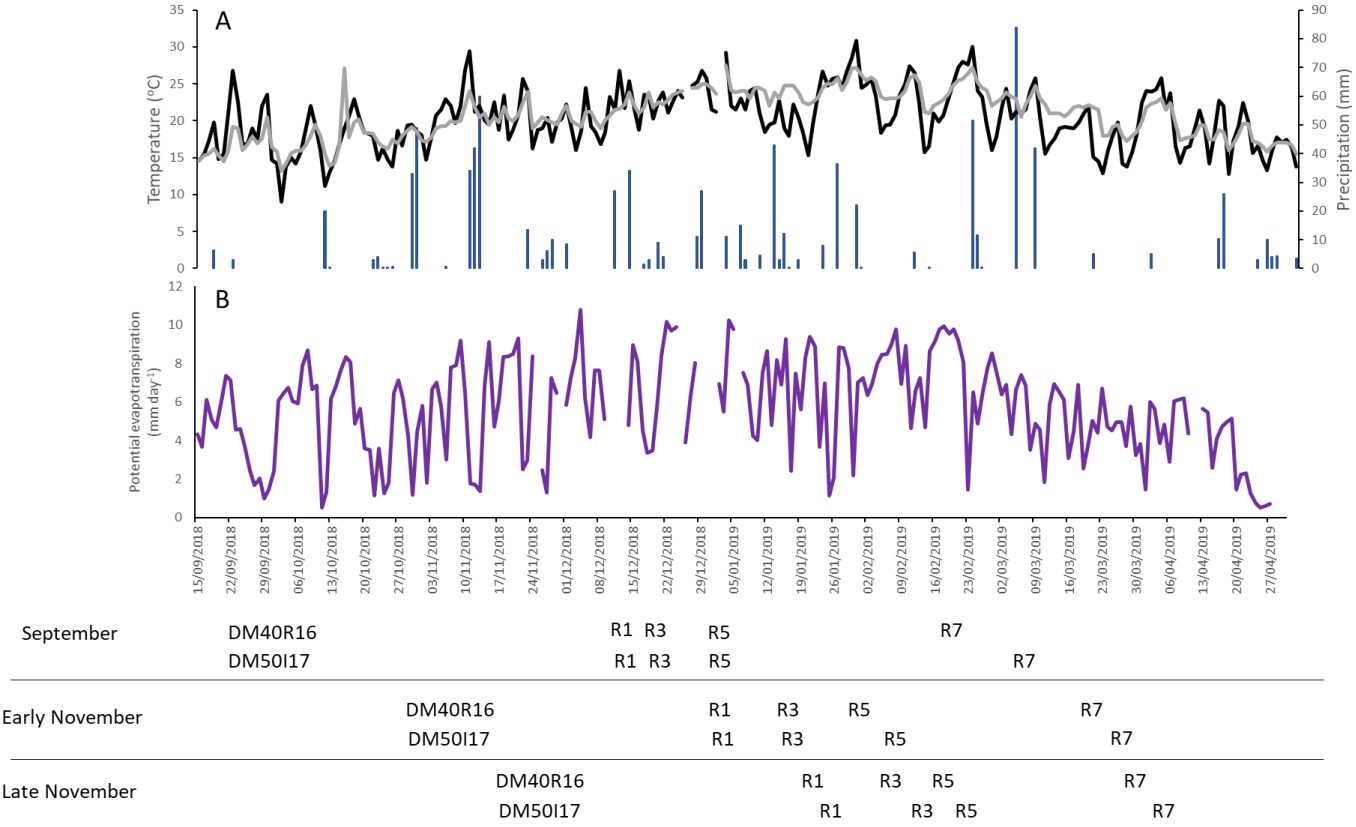


Figure 4.1 Daily air and soil (10 cm depth) temperature (black and grey line, respectively), precipitation (blue bars; A) and potential evapotranspiration (purple line; B). Phenological growth stages for each genotype (DM40R16 and DM50I17) in each sowing date, with genotype name denoting sowing date, are shown below.

Cytokinin treatments (kinetin; Sigma Aldrich) consisted of either seed priming (10^{-9} mol L^{-1}), foliar spray (10^{-7} mol L^{-1}) or water control. All seeds were submerged either in water (foliar and control) or cytokinin solution (seed) for four hours, air dried and stored at 4°C until sowing the following day. Cytokinin treatment did not significantly affect emergence, measured 22 days after sowing. Foliar cytokinin treatment was applied at VC and V1 (rate of 50 L ha^{-1}), with control and seed-treated plants sprayed with water. Two commercial soybean genotypes were used, DM40R16 and DM50I17 (Grupo Don Mario), with maturity groups IV and V, respectively. For the late November sowing date (2018/2019 trial), days from emergence to R7 (physiological maturity) for genotypes DM50I17 and DM40R16 differed by 12 days in the first trial. Figure 4.1 shows the phenology of genotypes from each sowing date for 2018/2019. After drying, seeds were coated with inoculant and osmoprotector at recommended rates with RizoLiq LLI ® (Rizobacter, Argentina) and seed insecticide and fungicide, Cruiser Advanced® (Syngenta, Argentina) at recommended rates. A randomised complete block

design was used with genotypes and cytokinin treatments randomised within blocks, resulting in three plot replicates for each cytokinin/genotype combination per sowing date. Plots were over-seeded and hand thinned to a target plant population of 20 plants per m^2. Manual sowing was necessary due to enlarged seed following seed priming, where seeds were evenly distributed into furrows approximately 3 cm deep. Each plot was 6 m long with 4 rows 0.52 m apart (plot size was 12.5 m^2), with all measurements comprising the two central rows. Weeds and pests were chemically controlled with commercially available products as needed.

Table 4.1 Field climate data for three sowing dates in the 2018/ 2019 field trial. Values are averages of each month, ±SE with letters denoting significant difference at $p < 0.05$ as determined by least significant difference (LSD) test with results of one-way ANOVA below with model residual standard error.

Sowing	**Air temperature (^{o}C)**	**Soil temperature (10 cm depth ^{o}C)**	**Precipitation (mm)**	**Potential evapotranspiration (mm day^{-1})**
September	17.0 b ±0.6	15.0 c ±0.4	0.32 ±1.89	3.93 c ±0.177
Early November	16.8 b ±0.6	17.4 b ±0.4	3.25 ±1.86	5.05 b ±0.172
Late November	20.8 a ±0.6	20.4 a ±0.4	5.60 ±1.89	5.41 a ±0.181
Sow	**<0.001**	**<0.001**	0.115	**0.043**
Residual SE	3.4	2.3	10.4	2.27

Biomass and nitrogen concentration

Above-ground biomass was sampled at the R1, R3, R5, and R7 phenological stages (Figure 4.1) (Fehr & Caviness, 1977) from a 0.5 m^2 area, leaving the first and last plant of the rows to prevent border effects. Due to poor germination in the second trial, plants were not harvested at R5 to ensure sufficient area for determining yield. From each harvest, leaf area was measured with a leaf area meter (Model Li-3100C Li-Cor), and plants were separated into leaves and stems and dried at 60^{o}C in an air forced oven. After drying, all plant parts were weighed to determine dry matter. Seed yield was determined at physiological maturity from the remainder of the plot (2.1 m^2) using an experimental static harvester. After weighing, all

plant biomass samples were milled to 1 mm. Nitrogen concentration in leaves and stems was determined using Kjeldahl procedure (McKenzie & Wallace 1954). Nitrogen use efficiency was calculated by dividing total above ground biomass by total N uptake (Xu *et al*., 2012). Nitrogen harvest index was calculated by dividing total seed N content by total canopy N uptake at R7.

Biological nitrogen fixation

Ground stem samples (0.4 g) were used to extract ureide and BNF was determined using the same method as described in Chapter 2. The amount of N fixed biologically (kg ha^{-1}), for each harvest, was calculated by multiplying relative ureide N (%) by aboveground total N (kg ha^{-1}) (Herridge & Peoples, 1990). By adding the amount of biologically fixed N at each harvest date plus the amount accumulated between each harvest date, total N coming from BNF at physiological maturity (kg ha^{-1}) was determined. The ratio between biologically fixed N (kg ha^{-1}) and total N uptake at maturity provided the final percentage of N derived from fixation (Ndfa%) for the growth period. The difference between aboveground total N (kg ha^{-1}) and biologically fixed N (kg ha^{-1}) was used to indicate soil mineral N absorption.

Nodulation

Nodules were quantified using the same method as described in Chapter 2. Roots were sampled when each plot reached at R1, R3 and R5. Three plant samples were taken and frozen at -20^{o}C until analysis. Root samples were thawed and washed before nodules were detached and photographed on a white surface with a size reference label. ImageJ (1.51K; Schneider *et al*., 2012) was used to count and measure nodule area (mm^2). Once imaged, nodules were dried at 60^{o}C and weighed.

Data analysis

Data from the first trial was used to see the effect of genotype and cytokinin on BNF across soil temperatures, the ANOVA included sowing date, genotype and cytokinin treatment as main effects, with Protected Fisher's least significant difference calculated for significant ($p < 0.05$) effects. To determine the effect of genotype and cytokinin (main effects) on nodule traits, BNF and yield data were combined across both field seasons and sowing date was included as a random effect in a linear mixed effect model. Models were validated by checking

the normality of the residuals and by plotting residuals against fitted values. All data analysis was performed in R software (RStudio Team, 2020).

4.3 | Results

Cytokinin and Genotype

Yield of DM50I17 was significantly ($p < 0.001$) higher than DM40R16 by 18% (Table 4.2). Cytokinin seed priming did not significantly alter yield, but foliar treatment reduced yield ($p < 0.05$) by 10% from control (Table 4.2). Grain quality, indicated by seed N content, was not significantly affected by genotype or cytokinin. Specific leaf area of DM50I17 was 15% higher than DM40R16 ($p = 0.007$; Table 4.2). Taken together, genotype DM50I17 performed better in this environment but cytokinin treatments did not benefit plant growth or yield.

Table 4.2 Seed yield at R8, specific leaf area at R1, grain nitrogen content, percent BNF, soil N uptake, N use efficiency (NUE; biomass/N uptake) and N harvest index (NHI; grain N/ N uptake) at R7. Yield data from five sowing dates (n= 30 for cytokinin and 45 for genotype), other variables from four sowing dates (n=24 for cytokinin and 36 for genotype), in two genotypes (DM40R16 and DM50I17) with cytokinin application (water control, seed soak or foliar spray). Values are averages with letters denoting significant difference at $p < 0.05$ as determined by least significant difference (LSD) test with mixed effect model results and degrees of freedom below.

Source of variance		Seed yield (kg ha^{-1})	Grain N (g)	Specific leaf area (cm^2 g^{-1})	BNF (% Ndfa)	Soil N uptake (kg ha^{-1})	Total canopy N (kg ha^{-1})	NUE (kg kg^{-1})	NHI (%)
Genotype	DM40R16	2903 b	4.13	255 b	40.5 a	170 b	292 b	29.0 b	78.0
	DM50I17	3420 a	4.30	292 a	33.5 b	205 a	321 a	30.2 a	79.3
Cytokinin	Control	3249 a	3.84	289	39.2	193	324	29.8	77.3
	Seed	3320 a	4.63	268	38.1	186	309	29.7	78.9
	Foliar	2916 b	4.17	264	33.7	183	288	29.3	79.8
Genotype (G)		**<0.001**	0.569	**0.007**	**0.019**	**0.001**	**0.027**	**0.034**	0.255
Cytokinin (C)		**0.023**	0.111	0.239	0.278	0.730	0.090	0.683	0.177
C x G		0.851	0.557	0.407	0.202	**0.017**	0.155	0.549	0.851
df		80	63	63	63	63	63	63	63

Overall, DM40R16 had higher BNF than DM50I17 (17%; p = 0.019) but lower soil N uptake (21%; p = 0.001) leading to lower total canopy N (10%; p = 0.027; Table 4.2). NUE was higher in DM50I17 (4%; p = 0.034) but NHI was not significantly different. Therefore, DM50I17 accumulated more N due to soil N uptake and greater N use efficiency.

Cytokinin treatment did not alter BNF in either genotype (Table 4.2). The effect of cytokinin treatment on soil N uptake was genotype-dependent (p = 0.017; Figure 4.2) with seed treatment reducing N uptake in DM40R16 by 23% (p < 0.05).

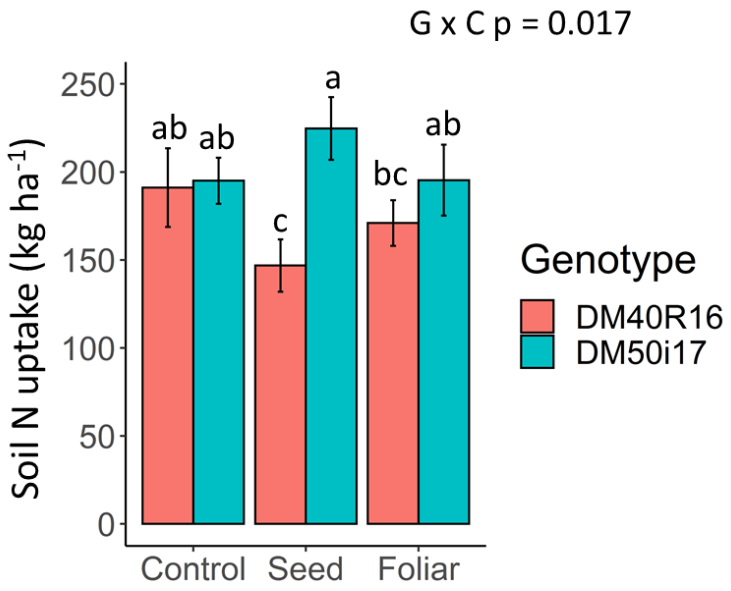


Figure 4.2 Soil N uptake in two genotypes, DM40R16 (red) and DM50I17 (teal), following cytokinin application. Data are means ± SE of 12 plots across 4 sowing dates. ANOVA p-values from the genotype x cytokinin interaction are reported.

At R1 and R5, DM50I17 derived 52% and 11% more N from fixation than DM40R16 (p < 0.001 and p = 0.026, respectively; Table 4.3). However, at R7, DM40R16 had higher BNF (26%; p = 0.008; Table 4.3). Thus, genotypes showed different BNF capacities at early and late stages.

Cytokinin foliar treatment reduced BNF by 26% (p < 0.05) across both genotypes, at R1. . At R5, there was a genotype x cytokinin interaction, with cytokinin seed treatment reducing BNF in DM40R16 but not DM50I17 (26%; p < 0.05). Thus, cytokinin treatments appear detrimental to BNF.

Table 4.3 Biological N fixation (BNF) at R1, R3, R5 and R7. Data from four sowing dates (September, early November and late November in 2018/19 and December 2019/20 minus R3), in two genotypes (DM40R16 and DM50I17) with two cytokinin applications (water control, seed soak or foliar spray). Values are averages (n=24 for cytokinin and 36 for genotype), with letters denoting significant difference at $p < 0.05$ as determined by least significant difference (LSD) test with ANOVA and degrees of freedom below.

Source of variance	**Sowing**	**BNF (%)**			
		R1	R3	R5	R7
Genotype	DM40R16	16.2 b	48.3	43.7 b	43.2 a
	DM50I17	24.6 a	50.3	48.5 a	31.9 b
Cytokinin	Control	21.7 a	47.2	46.3	40.5
	Seed	23.7 a	53.3	44.4	38.4
	Foliar	16.1 b	47.1	47.6	33.8
Genotype (G)		**<0.001**	0.430	**0.026**	**0.008**
Cytokinin (C)		**0.006**	0.058	0.488	0.405
C x G		0.239	0.900	**0.011**	0.200
df		63	46	63	63

Genotype DM50I17 had more nodules at R1 and R5 than DM40R16 (18% and 36%; $p = 0.041$ and $p = 0.002$, respectively; Table 4.4). Similarly, at R5, both total nodule area and number of 4 mm nodules were greater in DM50I17 (29% and 40%; $p = 0.009$ and $p = 0.011$, respectively).

Cytokinin seed treatment decreased average nodule size at R1 (7%; $p = 0.05$; Table 4.4). Cytokinin treatments therefore seemed to reduce nodule traits in field trials.

Table 4.4 Nodule number, average nodule size, total nodule area and number of 4 mm nodules at three growth stages (R1, R3 and R5). Data from five sowing dates across two years, in two genotypes (DM40R16 and DM50I17) with cytokinin application (water control, seed soak or foliar spray). Values are averages (n= 30 for cytokinin and 45 for genotype), with letters denoting significant difference at $p < 0.05$ as determined by least significant difference (LSD) test with ANOVA results below. Residual degrees of freedom are 80.

Source of variance	**Sowing date**	**Nodule number (#)**			**Average nodule size (mm^2)**			**Total nodule area (mm^2)**			**4 mm nodules (#)**		
		R1	R3	R5	R1	R3	R5	R1	R3	R5	R1	R3	R5
Genotype	DM40R16	27.6 b	48.5	38.6 b	7.47	7.30	8.11	229	353	309 b	5.90	12.5	6.40 b
	DM50I17	32.8 a	56.4	52.5 a	6.87	6.76	9.93	235	397	399 a	5.94	14.1	8.95 a
Cytokinin	Control	31.8	59.9	43.2	7.12 ab	6.90	8.01	251	423	339	6.36	15.1	8.18
	Seed	29.8	48.2	48.1	6.59 b	6.87	7.84	206	332	356	5.01	11.9	7.44
	Foliar	29.0	49.3	45.4	7.80 a	7.31	8.20	239	369	366	6.39	13.0	7.39
Genotype (G)		**0.041**	0.103	**0.002**	0.055	0.058	0.646	0.815	0.152	**0.009**	0.968	0.378	**0.011**
Cytokinin (C)		0.639	0.094	0.649	**0.008**	0.367	0.752	0.390	0.057	0.798	0.570	0.336	0.767
C x G		0.252	0.983	0.860	0.479	0.845	0.833	0.632	0.810	0.895	0.236	0.474	0.519

Sowing date

Sowing date did not significantly affect seed yield ($p = 0.252$). There was no significant genotype x sowing date interaction ($p = 0.513$), suggesting the genotypes were similarly cold tolerant (Figure 4.3). However, a treatment x sowing date interaction ($p = 0.03$) occurred, with cytokinin foliar treatment significantly ($p < 0.05$) decreasing yield only of early November sown crops. Thus, cytokinin treatments do not seem to benefit yield and may be detrimental in conventional sowing.

Grain quality, indicated by seed N content, was not significantly affected by sowing date. However, cytokinin seed treatment more than doubled grain N of DM40R16 in early November sowing compared to control ($p < 0.05$).

September sown crops had significantly ($p < 0.05$) lower specific leaf area, 30% and 45% less than the early November and late November crops, respectively (Table 4.5). Both genotypes significantly increased their specific leaf area from September to late November, DM40R16 by 69% and DM50I17 by 28% (Figure 4.3), without a significant genotype x sowing date interaction ($p = 0.128$) indicating no difference in cold tolerance.

Late November sowing accumulated more canopy N (12% and 21%) than the September and early November sowing (Table 4.5). However, September sown plants derived significantly more ($p = 0.001$; Table 4.5) of their N from BNF (Ndfa%) than later sown plants: 20% and 11% greater than in early November and late November sowing. Late November sown DM50I17 had lower (39%) BNF than September, while this effect was not seen in DM40R16 (Figure 4.4), as indicated by a genotype x sowing date interaction ($p < 0.001$). Percent BNF was also higher in DM50I17 than in DM40R16 in early November sowing date ($p < 0.05$; Figure 4.4). Therefore, early sowing increases plant reliance on BNF compared to those sown at more conventional times, with BNF of DM50I17 (but not DM40R16) significantly affected by sowing date.

Soil N uptake was 23% higher for the late November than the September sowing ($p < 0.05$; Table 4.5). Again, there was a genotype x sowing date interaction ($p < 0.001$), with increased soil N uptake (~32%) in the late November sowing of DM50I17 compared with other sowing dates of both genotypes (Figure 4.4). Therefore, soil N uptake is limited by early sowing date and only DM50I17 increased soil N uptake in response to later sowing.

Table 4.5 Seed yield at R8, specific leaf area at R1, grain nitrogen content, percent BNF, soil N uptake, N use efficiency (NUE; biomass/N uptake) and N harvest index (NHI; grain N/ N uptake) at R7. Data from three sowing dates (September, early November and late November), in two genotypes (DM40R16 and DM50I17) with cytokinin application (water control, seed soak or foliar spray). Values are averages (n=18 for sowing date and cytokinin and 27 for genotype)), with letters denoting significant difference at $p < 0.05$ as determined by least significant difference (LSD) test with ANOVA, residual SE and LSD results below. Residual degrees of freedom are 36.

Source of variance		Seed yield (kg ha^{-1})	Grain N (g)	Specific leaf area (cm^2 g^{-1})	BNF (% Ndfa)	Soil uptake (kg ha^{-1})	Total canopy N (kg ha^{-1})	NUE (kg kg^{-1})	NHI (%)
Sowing date	Sept	3922	5.18	226 b	44.6 a	191 b	348 b	31.1 a	79.6 b
	Early Nov	4210	4.27	294 a	35.8 c	197 b	310 b	32.3 a	86.8 a
	Late Nov	4254	4.70	327 a	39.5 b	235 a	397 a	24.2 b	74.1 c
Genotype	DM40R16	3895 b	4.78	264 b	40.4	195 b	337	28.1	81.3
	DM50I17	4362 a	4.66	301 a	39.4	219 a	366	30.3	79.0
Cytokinin	Control	4329 a	4.31	304	38.8	214	370	30.8	79.7
	Seed	4408 a	5.24	277	42.0	204	355	28.6	80.3
	Foliar	3649 b	4.61	266	39.0	199	329	28.1	80.5
Sow (S)		0.252	0.135	**<0.001**	**<0.001**	**0.003**	**<0.001**	**<0.001**	**<0.001**
Genotype (G)		**0.011**	0.737	**0.025**	0.401	**0.027**	0.095	0.127	0.086
Cytokinin (C)		**0.002**	0.114	0.154	0.102	0.216	0.141	0.266	0.979
G x S		0.513	0.636	0.128	**<0.001**	**<0.001**	0.638	0.067	0.465
C x S		**0.030**	0.099	0.069	**<0.001**	**0.042**	0.731	0.729	**0.010**
C x G		0.854	0.571	0.408	**0.002**	**0.009**	0.255	0.491	0.561
C x G x S		0.984	0.057	0.466	**<0.001**	0.923	0.109	0.162	0.067
Residual SE		638.9	1.3	59.2	4.9	38.8	61.9	5.2	3.4
LSD 5%		431.9	0.89	40.0	3.4	26.2	41.8	3.5	2.3

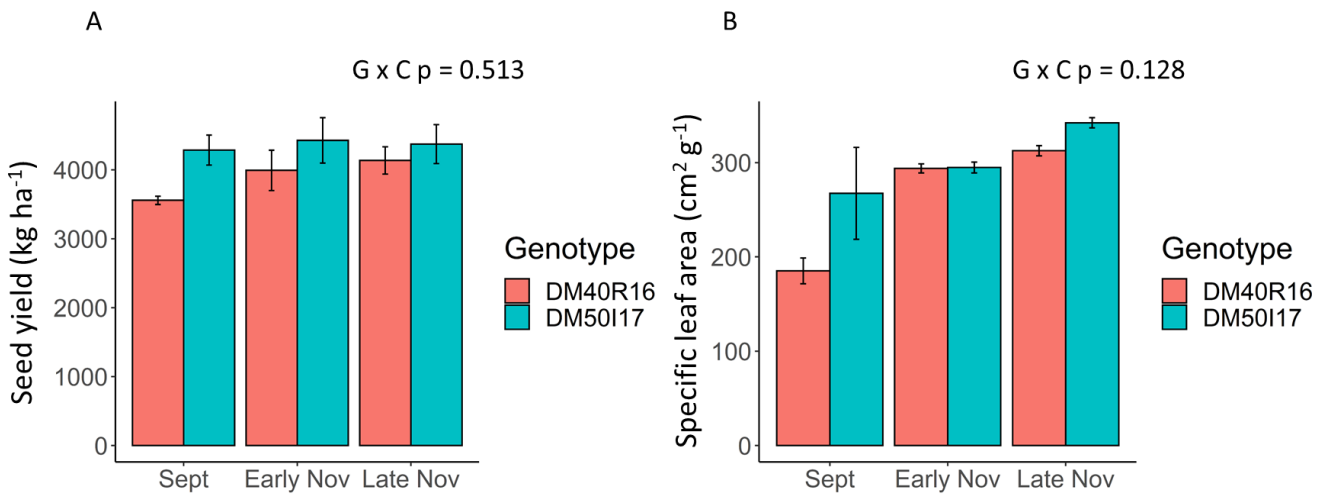


Figure 4.3 Seed yield (A) and specific leaf area at R1 (B) in two genotypes, DM40R16 (red) and DM50I17 (teal), across three sowing dates. Data are means ± SE of 9 plots, across 3 cytokinin treatments, with lack of letters denoting no significant difference at $p < 0.05$ as determined by least significant difference (LSD). ANOVA p-values from the genotype x cytokinin interaction are reported

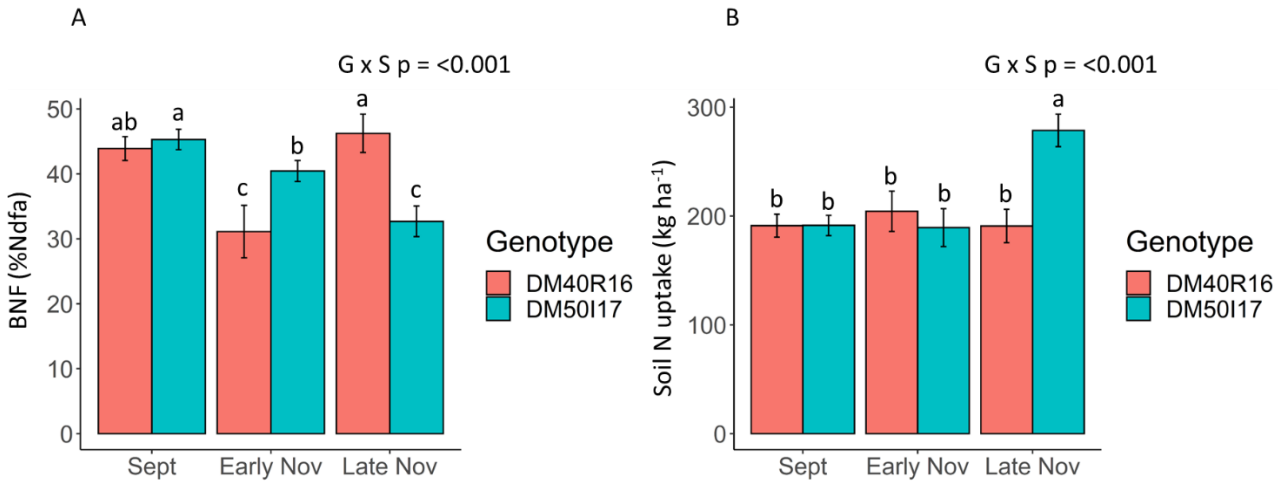


Figure 4.4 Percent BNF (A) and soil N uptake (B) in two genotypes, DM40R16 (red) and DM50I17 (teal), across three sowing dates. Data are means ± SE of 9 plots, with different letters above bars indicating significant differences according to LSD test. ANOVA p-values from the genotype x cytokinin interaction are reported

The effect of sowing date on BNF changed across the growth period (Table 4.6). At early reproductive stages (R1 and R3), BNF was higher in late November than September (74% and

40%, respectively; $p < 0.05$) sowing. However, at R7, BNF in September was 26% greater than late November ($p < 0.05$). Thus early sowing delayed BNF but led to increased BNF at maturity.

Table 4.6 Biological N fixation (BNF) at R1, R3, R5 and R7. Data from three sowing dates (September, Early November and late November 2018/19), in two genotypes (DM40R16 and DM50I17) with two cytokinin applications (water control, seed soak or foliar spray). Values are averages (n=18 plots for sowing date and cytokinin and 27 for genotype), with letters denoting significant difference at $p < 0.05$ as determined by least significant difference (LSD) test with ANOVA, residual SE and LSD results below. Residual degrees of freedom are 36.

Source of variance	Sowing	BNF (%)			
		R1	R3	R5	R7
Sowing date	Sept	8.21 c	43.4 b	38.2 b	47.4 a
	Early Nov	23.8 b	31.7 c	53.9 a	36.2 b
	Late Nov	31.7 a	72.5 a	54.9 a	35.1 b
Genotype	DM40R16	17.3 b	48.3	48.5	40.7
	DM50I17	25.2 a	50.2	49.5	38.5
Cytokinin	Control	23.5 a	47.2 b	49.8	38.0
	Seed	24.0 a	53.3 a	47.5	41.6
	Foliar	16.3 b	47.1 b	49.7	39.2
Sow (S)		**<0.001**	**<0.001**	**<0.001**	**<0.001**
Genotype (G)		**<0.001**	0.262	0.615	0.215
Cytokinin (C)		**<0.001**	**0.004**	0.523	0.233
G x S		**<0.001**	**0.009**	**0.006**	**<0.001**
C x S		**<0.001**	0.072	0.185	**<0.001**
C x G		**0.022**	0.806	**<0.001**	**0.002**
C x G x S		**0.034**	**<0.001**	**0.014**	**<0.001**
Residual SE		5.0	6.1	6.7	6.4
LSD 5%		3.4	4.1	4.5	4.3

Sowing date also significantly ($p < 0.001$) affected cytokinin response, with cytokinin seed treatment increasing BNF in September and early November sowing but not in late November ($p < 0.05$; Figure 4.5). Similarly, foliar cytokinin treatment increasing BNF in early November

but decreasing BNF in late November sowing ($p < 0.05$; Figure 4.5A). Thus the effect of cytokinin treatments was sowing-date dependent.

Foliar cytokinin treatment decreased soil N uptake by 47% ($p < 0.05$) compared with the control in the early November but not in other sowing dates, resulting in a significant cytokinin x sowing date interaction (Figure 4.5B; $p < 0.05$). Thus, cytokinin treatment reduces soil N uptake but this is sowing date dependent.

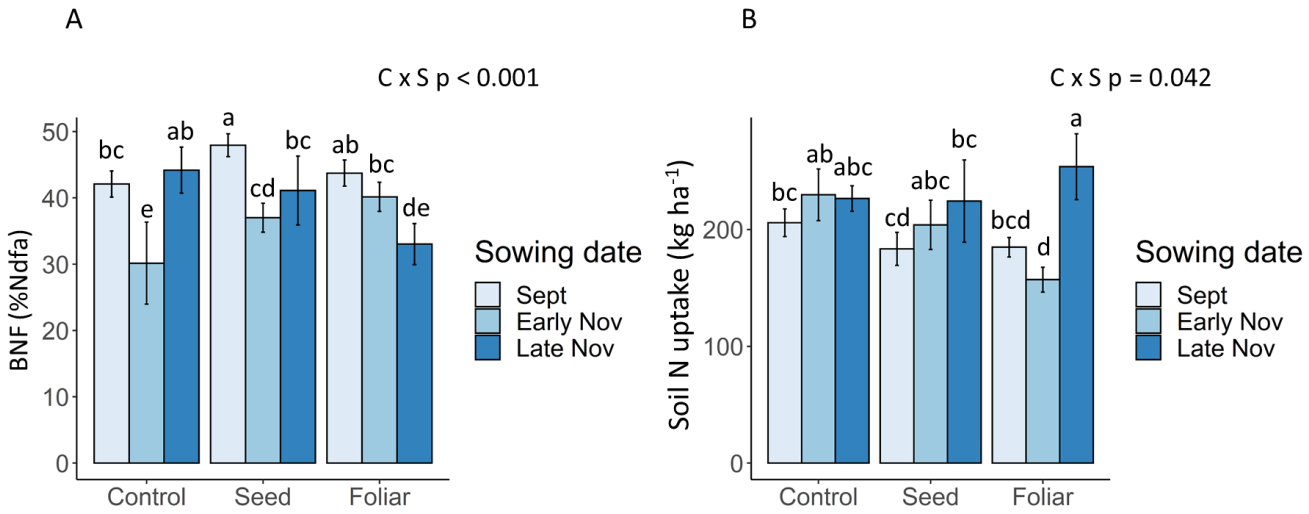


Figure 4.5 Effect of sowing date on the response of BNF (A) and soil N uptake (B) to cytokinin treatment in three sowing dates. Data are means ± SE of 6 plots, respectively. Different letters above bars indicate significant differences ($p < 0.05$) according to LSD test. ANOVA p-values from the cytokinin x sowing date interaction are reported.

Nitrogen use efficiency (NUE) was higher in September and early November than late November (approximately 24%; $p < 0.05$; Table 4.5). For the September sowing date, NUE was 25% greater in DM50I17 than DM40R16, but this effect was not significant (genotype x sowing date interaction $p = 0.067$; Figure 4.6). Nitrogen harvest index (NHI) was also higher in September and early November than late November (7% and 15%; $p < 0.05$; Table 4.5). Therefore, assimilation of N into canopy and grain tended to be more efficient in early sowing dates.

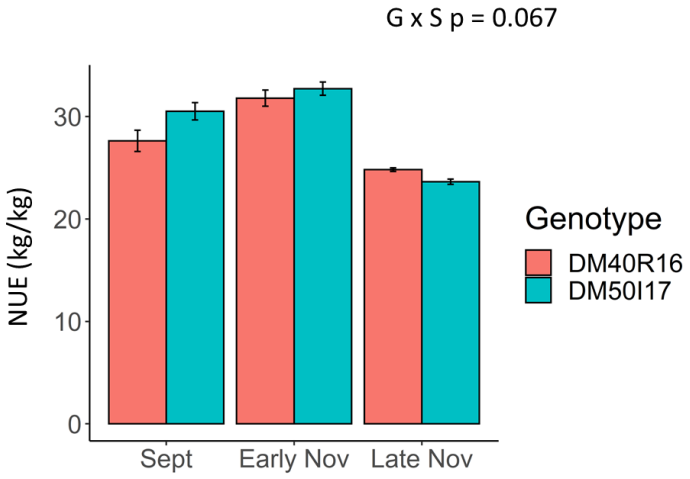


Figure 4.6 Nitrogen use efficiency (NUE; biomass/ N uptake) in two genotypes, DM40R16 (red) and DM50I17 (teal), across three sowing dates. Data are means ± SE of 9 plots with lack of letters denoting no significant difference at $p < 0.05$ as determined by least significant difference (LSD). ANOVA p-values from the genotype x sowing date interaction are reported.

At R1, the late November sowing had 63 and 46% more nodules than September and early November sowing, respectively ($p < 0.05$; Table 4.7). At R5 the opposite was evident, with nodule number increased in the September than late November sowing (by 38%; $p < 0.05$). Like BNF, early sowing date only affected nodules at R1 and R5 and not R3, decreasing nodule number at R1 but increasing it at R5.

Average nodule size followed a similar pattern with increased (37%; $p < 0.05$; Table 4.7) nodule size at R1 in late November than September sowing. At R3 and R5, nodules were larger in September than late November sowing (19 and 33%, respectively; $p < 0.05$). Thus, early sowing delayed both nodule development and senescence.

Similar trends occurred in other nodule traits (Table 4.7). At R1 and R3, the number of 4 mm nodules was greater in late November than September sowing, but at R5 the September sowing date had more than double the number of 4 mm nodules over the late November treatment. Equally, at R1, total nodule area in late November sowing was close to four times that of September while at R5 total nodule area in late November was more than 50% that of September. This gives further evidence that early sowing delayed nodulation.

Table 4.7 Nodule number and average nodule size at three growth stages (R1, R3 and R5). Data from three sowing dates (September, Early November and late November 2018/19), in two genotypes (DM40R16 and DM50I17) with cytokinin application (water control, seed soak or foliar spray). Values are averages (n=18 plots for sowing date and cytokinin and 27 for genotype), with letters denoting significant difference at $p < 0.05$ as determined by least significant difference (LSD) test with ANOVA, residual SE and LSD results below. Residual degrees of freedom are 36.

Source of variance	**Sowing date**	**Nodule number (#)**			**Average nodule size (mm^2)**			**Total nodule area (mm^2)**			**4 mm nodules (#)**		
		R1	R3	R5	R1	R3	R5	R1	R3	R5	R1	R3	R5
Sowing date	Sept	19.4 c	68.5	60.8 a	7.67 b	9.5 a	11.3 a	141 b	598	627.9 a	4.0 b	12.3 b	12.1 a
	Early Nov	28.2 b	60.6	40.4 b	9.53 a	8.65 ab	11.6 a	246 b	484	424 b	7.9 b	11.6 b	12.1 a
	Late Nov	51.9 a	61.7	37.5 b	10.47 a	7.7 b	7.54 b	544 a	461	270 c	15.8 a	33.2 a	5.5 b
Genotype	DM40R16	30.6	60.3	39.9	9.75 a	8.93	10.6	310	488	402	9.1	16.8	8.4
	DM50I17	35.8	66.9	52.5	8.70 b	8.32	9.74	310	541	479	9.4	21.3	11.3
Cytokinin	Control	36.3	73.9	45.9	9.03 ab	8.46	10.1	343	586	431	10.1	21.7	11
	Seed	31.8	59.1	47.5	8.49 b	8.39	9.8	320	500	430	7.9	17.9	9.1
	Foliar	31.4	57.7	45.2	10.2 a	9.02	10.6	268	457	461	9.7	17.5	9.5
Sow (S)		<0.001	0.618	**0.010**	**<0.001**	**0.005**	**<0.001**	**<0.001**	0.618	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Genotype (G)		0.123	0.364	0.057	**0.036**	0.144	0.591	0.994	0.364	0.166	0.889	0.112	0.060
Cytokinin (C)		0.421	0.132	0.959	**0.024**	0.400	0.192	0.349	0.132	0.872	0.646	0.394	0.546
G x S		0.605	0.449	0.256	0.269	0.373	0.741	0.373	0.449	0.349	0.809	0.532	0.537
C x S		**0.013**	0.641	0.574	0.863	0.071	0.527	0.058	0.641	0.614	0.683	0.781	0.542
C x G		0.450	0.886	0.602	0.349	0.969	0.900	0.478	0.886	0.606	0.353	0.318	0.562
C x G x S		0.582	**0.038**	0.448	0.659	0.415	0.410	0.602	0.038	0.701	0.278	0.139	0.097
Residual SE		12.1	26.0	23.5	1.78	1.52	2.25	157	26	202	7.58	9.98	5.33
LSD 5%		8.2	17.6	15.9	1.20	1.02	1.52	106	17.6	136	5.13	6.74	3.60

4.4 | Discussion

Effectiveness of cytokinin treatment and genotype selection

Higher yield was achieved in DM50I17 through greater canopy N accumulation due to higher soil N uptake. Although overall BNF was higher in DM40R16 than DM50I17, there was variation in the timing of BNF between genotypes (as seen in Chapter 2). Higher BNF capacity at R1 and R5 was seen in DM50I17 which also had correspondingly increased nodule number at R1 and R5, and 4 mm nodules at R5 (Table 4.3 and 4.3). Greater BNF capacity in these early reproductive stages may have aided canopy N accumulation with remobilisation of N during seed filling sustaining N supply and thus higher yields (Table 4.2). Although nodule number and total nodule area were increased in DM50I17, nodules tended to be on average smaller than those of DM40R16 (Table 4.4). This does not support previous findings that increased nodule size aids BNF but does support previous findings herein (Chapter 2) whereby optimal nodulation varies between genotypes at different stages.

Cytokinin treatments did not benefit crop yield, which appeared to be a consequence of limited N supply as canopy N tended to be limited by foliar cytokinin application (Table 4.2). This was perhaps because cytokinin application can limit root elongation and lateral root formation by increasing ethylene levels (Bertell & Eliasson 1992), thereby limiting N uptake. Although root growth was not measured in field trials, cytokinin application did not decrease root growth in pot experiments (Chapter 3) and cytokinin treatment had to be continuous to inhibit root growth previously (Bertell & Eliasson 1992).). Although cytokinin treatments show promise in enhancing BNF in a controlled environment (Chapter 3), the complexity of their response, seen in this study and previously (Koprna *et al*., 2016), makes determining their agronomic benefit challenging. Future trials should include a greater variety of genotypes, particularly of varying maturity groups (Salmeron *et al*., 2014), different treatment concentration (10^{-6} mol L^{-1}) or cytokinins (6-benzylaminopurine or N6-(Δ2-isopentenyl)-adenine) used previously (Mens *et al*., 2018). Cytokinin treatment longevity will likely vary depending on the type of cytokinin that is applied, with aromatic cytokinins, such as benzyladenine, more resistance to side chain cleavage by cytokinin oxidase than others, for example *cis* or *trans* zeatin.

Genotypic responses to early planting

Cold environments restrict plant N accumulation, with BNF thought to be more sensitive than soil N uptake. Although cold soil temperature (15-17.4°C; Table 4.1) limits total nitrogen accumulation (Table 4.5), surprisingly soil nitrogen uptake was more affected by low RZT than BNF, in contrast to previous findings in controlled environments (Legros & Smith 1994; Matthews & Hayes 1982; Thomas & Sprent 1984). In this study, BNF was 11% higher in September than the late November sowing, but soil N uptake was 23% lower (Table 4.5 and Figure 4.4). As early sowing reduces soybean root growth (Turman *et al*., 1995) thus limiting N uptake at low RZT (Alsajri *et al*., 2019; Ouertani *et al*., 2011; Rufty *et al*., 1981; Tolley & Raper 1985), this may explain why soil N uptake is more limited in the field compared to pot-grown plants in controlled environments. Differences in soil depth exploration affects the amount of N available to field-grown crops (Voisin, 2003), whereas root exploration in pots is unlikely to be limiting. Equally, reduced crop transpiration in cold conditions will limit water uptake, and thus nutrient uptake. Additionally, if soil nutrient concentration is low, high affinity active transporters will be required to pump ions across the cell membrane. This energy intensive process will also be limited by low root zone temperature. In cool growing conditions, increased BNF may compensate for limited soil N availability thus maintaining yield. BNF increases with evapotranspiration (Cleveland *et al.*, 1999), so increases in potential evapotranspiration across sowing dates (Figure 4.1) do not account for higher BNF from early planting. Differences in the timing and severity of cold stress might also explain the disparity between controlled and field environments, even though to my knowledge, the effects of early sowing on soybean N source have not been shown previously.

Despite different cycle lengths (Figure 4.1), early maturing DM40R16 (MG IV) was no more sensitive to cold than DM50I17 (MG V), both with similar yield and specific leaf area in response to early and conventional sowing dates (Figure 4.3). Previously, early maturing soybean genotypes appeared more sensitive to low temperatures, due to shorter vegetative growth (George *et al*., 1988; Heatherly, 2005; Salmeron *et al*., 2014) but this was not seen in this study. However, soil N uptake in DM50I17 was more cold-sensitive than DM40R16, requiring increased BNF from early sowing to maintain yield (Figure 4.4). As total canopy N at maturity was equal in genotypes in each sowing date, it was hypothesised that NUE could explain maintained yield in DM50I17. Despite a 25% increase in NUE in September sown

DM50I17, this effect was not significant (Figure 4.6), therefore cannot be confirmed. Therefore, increased BNF, enabling consistent N supply and enhanced NUE, overcame cold sensitivity. Maximising BNF may require decreased fertiliser N applications, as these inhibit nodulation (Santachiara *et al*., 2019), but this will depend on soil N levels at sowing as early canopy growth is critical for crop establishment. Available mineral N accumulates during the growing season as soil temperature increases, due to organic matter mineralization (Haynes *et al*.,1993).

Early sowing delayed BNF (Table 4.6), as previously reported (Zimmer *et al*., 2016), but also prolonged BNF in late reproductive stages resulting in higher rates of fixation. Increased BNF enhanced nitrogen harvest index (Santachiara *et al*., 2018) and early soybean sowing increased seed quality (Rahman *et al*., 2005) as in this study, and marginally increased grain N content in early sowing dates (Table 4.5). Biologically fixed N is more rapidly assimilated into pods and seed, whereas N from soil is first assimilated into vegetative tissue and then remobilised into reproductive parts (Ohyama, 1983). High N demand during grain filling promotes leaf senescence due to remobilisation of N from vegetative tissue, with high yielding varieties maintaining N supply during seed filling (Kumudini *et al*., 2002). Therefore, early sowing increased BNF at late reproductive stages, which likely helped maintain yield though low RZT had limited soil N supply.

Genotype and environment both affect nodule lifespan (Vessey, 1992), but the effect of early soybean sowing on nodule senescence has not been considered previously (Puppo *et al*., 2004). In this study, early sowing delayed nodule senescence (Table 4.7), perhaps due to more favourable RZT in later growth. Limited canopy N accumulation in early growth may limit later pod filling as less N is available for remobilisation, leading to increased N demand in reproductive stages. Carbon competition between pods and nodules was previously thought to occur, thus reproductive N supply from BNF would limit yield. However, male-sterile soybean show a similar decline in BNF in later growth, suggesting limited C competition between pods and nodules (Imsande & Ralston 1982; Riggle *et al*., 1984). Therefore delayed nodule senescence and prolonged BNF may benefit early soybean production.

Although nodule size has been suggested to influence BNF more than other nodule traits (de Araujo *et al*., 2017; Tajima *et al*., 2007; Voisin *et al*., 2003), in the present experiment, greater fixation was seen in DM50I17 with smaller nodules than DM40R16. Data herein confirm a

genotypic effect on the timing of BNF (Hamawaki & Kantartzi 2018) and additionally show that this occurs in nodules; however nodule traits and the timing of BNF were not correlated (Table 4.6 and 4.7). Low RZT is known to delay BNF and nodule formation (Zhang *et al*., 1995), which was confirmed in this study in both genotypes (Tables 4.5 and 4.6), and so it does not explain differences in N supply across sowing dates. To better understand N dynamics, nodules should be monitored at different stages as significant genotypic differences were detected only at R1 and R5 not R3 (Table 4.7). Differential N accumulation patterns seen in commercial genotypes (Rotundo *et al*., 2014), may in part be due to improved nodule traits, missed previously.

Cytokinin seed priming increased BNF, although this depended on genotype (in DM40R16), sowing date (Figure 4.5) and stage (Table 4.6). Cytokinin treatment was more effective in early sowing dates, with a cytokinin x sowing date interaction (Figure 4.5A). Thus, cytokinin effects in enhancing BNF are more beneficial in low temperature when plants depend more on N supply from BNF.

4.5 | Conclusions

Although the controlled environment trial suggested that cytokinin treatment can enhance BNF and early nodule establishment (Chapter 3), the field trials did not fully support its agronomic benefit, as cytokinin treatment did not increase either total N uptake or yield. Characterisation of soybean N uptake during cold stress showed that maintenance of N supply is important for maintaining yield in low temperature, with soil N uptake being more sensitive to cold than BNF, in contrast to much of the relevant literature. This may be due to limited root growth in early sowing. BNF was important in maintaining N supply in early sowing, leading to consistent yields across sowing dates. This is of great consequence to soybean N management as it emphasises the importance of strategies to enhance BNF in cool environments. Soil N supply was more sensitive in one genotype but was able to compensate with increased BNF to secure its N supply across soil temperatures, thus stabilising yields. This indicates the importance of appropriate selection for early sowing. Early sowing can delay nodule formation and BNF, but this may be beneficial by prolonging BNF and improving N harvest index at the end of the season.

Chapter 5: General discussion

5.1 | Context

Soybean is a globally important vegetable protein source and may prove to be a central part of sustainable food supply. Soybean has a high seed protein content giving it many uses but also a high N demand, and much of this can be provided through BNF. Early efforts to increase BNF did not make significant advances (Henzell, 1988; Shiferaw *et al*., 2004) and now the need to reduce reliance on N fertiliser is greater than ever. Efforts to increase BNF have focused on rhizobia (strain selection) but there is a need to shift attention to the host (Sinclair & Nogueira, 2018). Enhancing fixation through optimised rhizobia often fails (Dobbelaere *et al*., 2001) due to competition from native strains (Nazir *et al*., 2013), with inoculant rhizobia almost completely disappearing from soil after the first cropping cycle (Zilli *et al*., 2013). Equally, as rhizobial genomes are highly plastic, rapid adaptation of introduced strains can occur (Ferreira & Hungria, 2002; Mendes *et al*., 2004; Loureiro *et al*., 2007); selection favours survival in the soil and entry to hosts but not fixation. In addition, where soybean has previously been grown, bacterial inoculation does not tend to increase nodulation or crop yield (Schulz & Thelen, 2008; de Bruin *et al*., 2010; Mason *et al*., 2016) suggesting that subsequent infections are due to established native rhizobia. In Brazil and Argentina, annual inoculation of soybean increased yield by only 6-8%, whilst there was no change in the USA (Sinclair & Nogueira, 2018). Thus, in this study the focus was on plant regulation of nodules.

Nodules are the site at which BNF takes place, but the relative importance of nodule morphology is not fully understood. The aim of this thesis was to explore the importance of nodule traits for BNF and how this could be enhanced through genotype selection or hormone application to improve BNF and yield. As nodule initiation is delayed by cold stress, limiting soybean production in Europe and early sowing in South America, the mitigating effects of genotype selection and hormone application on delayed nodule development were also considered.

Different soybean genotypes have varying BNF capacities (approximately 20-90 %Ndfa at R7) (Hamawaki & Kantartzi, 2018), but this has not been linked to altered nodule traits, so this was the aim of Chapter 2. Hormones control the nodulation process, with cytokinin, auxin and gibberellin considered positive promoters and their ability to alter nodule traits and BNF

were explored in Chapter 3. In Chapter 4, field trials showed the effect of genotype selection and cytokinin application on nodules, BNF and yield and the extent that these factors could mitigate low RZT.

5.2 | Genotypic nodule variation and BNF

Different genotypes varied in their BNF (approximately 62% difference; Table 2.3) as seen previously (Keyser & Li, 1992; Peoples *et al*., 1995; Mapope & Dakora, 2016). Genotypes also had different BNF capacities at different stages, with some being characterised as either early or later fixers, also seen previously (Hamawaki & Kantartzi, 2018). Very few studies have characterised changes in both BNF and nodules across growth stages (Pitumpe Arachchige *et al*., 2020) and in this study nodule traits did not fully correspond with BNF. In the genotypes tested, nodules varied only at later stages (Figure 2.4B and C) despite variation in BNF at each stage. This suggests that the relationship between nodule traits and BNF varies across genotypes with the relative importance of nodule characteristics depending on both genotype and stage. Whether this reflects plant investment in nodules or difference in nodule efficiency across genotypes is unclear. It is often assumed that an increased investment in nodules will increase BNF, although super-nodulating genotypes have lower biomass and yield (Carroll *et al*., 1985; Song *et al*., 1995). Therefore, if increased nodule number is not necessarily beneficial, but genotypes vary in both nodules and BNF, it follows that certain nodule traits aid fixation.

One such nodule trait is nodule size, which increases BNF (Voisin *et al*., 2003; Tajima *et al*., 2007; de Araujo *et al*., 2017). Considering all genotypes in Chapter 2, total and average nodule area were positively correlated only at reproductive stages (Figure 2.5). Nodules of a certain size have also been considered optimal (Purcell *et al*., 1997; King & Purcell, 2001) and here only the number of 4 mm nodules per plant positively correlated with BNF but again only in reproductive stages (Figure 2.5). Additionally, positive correlations between nodule traits and BNF were seen only in certain genotypes at certain stages (Table 2.5 to 2.7). Nodule traits and BNF have been previously correlated during late stages of growth (8-14 weeks after sowing) (de Araujo *et al*., 2017; Pitumpe Arachchige *et al*., 2020) but here a wider range of stages and genotypes have been characterised. Taken together, this showed that BNF varied substantially across both genotype (62%) and growth stage (38%) and that the relationship

between nodules and BNF varied across genotypes. However, individual nodule size was important and in particular the 4 mm nodules, have a greater BNF capacity.

The way in which nodules are quantified also appears important, with specific nodule traits better corresponding to BNF (Table 2.3). This is perhaps unsurprising as differences in plant size are accounted for in these specific traits, but it is not frequently reported in the relevant literature. Correlations with BNF were seen only in nodule area traits and size classification, which are made possible through a novel imaging technique (Figure 2.1). Despite some efforts (Remmler *et al.*, 2014), quantifying nodules is not standardised across research in this field. Data herein suggests that image analysis is more accurate, able to detect subtle changes in nodule traits not possible by weighing or counting.

This study hypothesised that changes in nodule morphology would lead to increased BNF because of improved physiology (see Chapter 2) leading in turn to the variation in BNF seen in soybean genotypes. Alternatively, upregulation of BNF in nodules (Pradhan *et al*., 2018), through sensing of N, carbon supply to nodules or altered oxygen permeability (Schwember *et al*., 2019) may also explain differences in BNF capacity. Cytokinin has been linked to N sensing (Walch-Liu *et al*., 2005) so it may mediate this. BNF capacity will also be influenced by the genotype and by the rhizobia occupying nodules due to differences in N fixation efficiency across strains. Selection of rhizobia by legume hosts is not well understood and how nodule strain occupancy alters nodule traits has not been explored. It could be that larger nodules are due to occupancy with effective rhizobia or perhaps due to multi-occupancy, possibly by enhancing carbon sink capacity of nodules (Kaschuk *et al*., 2009). This would not be true for controlled-environment experiments but may explain variation seen in the field. Genotypic differences in nodulation competitiveness of *B. japonicum* strains depended on the region of adaptation of the soybean genotype (Payakapong *et al*., 2004), suggesting that soybean genotypes co-evolve with rhizobia and differences in BNF across genotype could be site dependent. This illustrates the importance of region-specific genotype selection and breeding efforts. It also highlights the benefit of strategies to enhance symbiosis with native rhizobia, instead of introduced inoculant strains.

5.3 | Effect of phytohormone application on nodulation and BNF

As nodule traits appear to affect BNF and phytohormones mediate nodule formation, positive regulators were identified (Ferguson & Mathesius, 2014) and applied using different methods (seed coat and soaking, foliar spray and root application) and concentrations (Chapter 3). Previous studies have applied various concentration of these hormones to a range of other legumes with various positive responses, but not to soybean (Table 3.1). Although previously auxin and gibberellin increased nodulation in other species (Ferguson *et al*., 2005; Ali *et al*., 2008; Fatima *et al*., 2008), this was not the case in this study. Nevertheless, cytokinin application enhanced nodule traits and BNF (Figure 3.2; Table 3.8).

Application method and cytokinin concentration influenced the success of treatments (see Chapter 3). Seed priming at 10^{-9} mol L^{-1} gave the best results, doubling BNF and increasing total nodule area by 32% (Table 3.8). Seed priming, inducing a physiological state with treatment before germination, has mainly been used to enhance germination and stress tolerance (Kumar *et al*., 2020). Cytokinin priming can improve germination and early seed vigour (Nikolić *et al*., 2006) but this was not seen in field trials, so it does not explain differences in BNF and nodules seen.

Cytokinin seed priming constrained nodule distribution (Table 3.8; Figure 3.3) which may increase BNF as the positioning of nodules influences BNF (McDermott & Graham, 1989; Wolyn *et al*., 1989; Hardarson & Danso, 1993). Soybean inoculants are often only competitive at the root crown, as seed inoculant application limits rhizobial distribution (McDermott & Graham, 1989; McLoughlin *et al*., 1990). Nodules on lateral roots are occupied by native strains, not optimised for BNF, as they are distributed across the rhizosphere (Weaver & Frederick, 1974; Wadisirisuk *et al*., 1989). Therefore, nodules forming earlier due to cytokinin treatment are more likely to be occupied by optimised inoculant strains so it would be interesting to quantify nodule occupancy following cytokinin application in the field.

Constrained nodule distribution following cytokinin application also led to the hypothesis that the treatment caused earlier nodule formation (see Chapter 3). Plants (*Aeschynomene afraspera* and *indica*) inoculated with cytokinin overproducing rhizobia formed nodules one day sooner than a mutant strain deficient in cytokinin synthesis (Podlešáková *et al*., 2013). Further testing of cytokinin seed soaking increased early (V4) BNF (Figure 3.4). To establish if

cytokinin did indeed promote early nodulation and to gain insight into a potential mechanism, gene expression over 72 hours of the NF response gene *ENOD40a* was determined after a kinetin seed soak. Cytokinin indeed led to increased relative gene expression of ENOD40a, suggesting a priming effect (Figure 3.6).

Attempts to enhance soybean-rhizobia symbiosis as an alternative to hormone treatment have been promising. Although not agronomically practical, hydrogen sulphide can act as a signalling molecule, regulating many physiological processes. Soybean seedlings treated with hydrogen sulphide showed increased nodule number (approximately 15%) and BNF (approximately 25%). This response was explained through upregulation of plant nodulin genes, early nodulin 40 (*ENOD40a*), ERF required for nodulation (*ERN*), nodulin signalling pathway 2b (*NSP2b*), and nodulation inception genes (*NIN*) and bacterial *nod* genes (Zou *et al*., 2019). Non-thermal plasma application also increased soybean nitrogenase activity leading to increased root cytokinin (Pérez-Pizá *et al*., 2020). Perhaps the same gene signalling will also be induced by the cytokinin treatment used herein and explain the mechanism behind early nodulation.

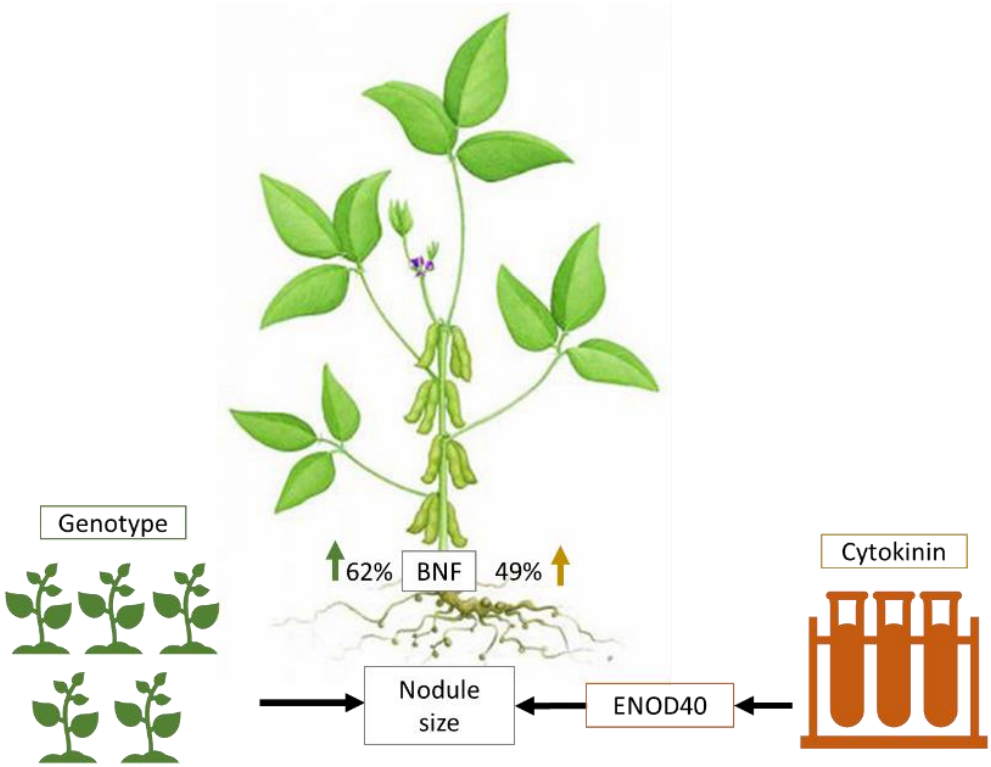


Figure 5.1 Effect of genotype selection and cytokinin application on BNF.

5.4 | Agronomic potential of genotype selection and phytohormone application

In Chapters 2 and 3, both genotype and cytokinin treatment altered nodules and BNF (Figure 5.1), thus in Chapter 4 the effects of both were assessed in field trials in order to better understand the agronomic potential of these strategies.

As in controlled environments, timing of BNF and nodules varied between genotypes in field trials (Table 4.3; Table 4.4) although the two did not correspond. Differences in the timing of BNF in genotypes or through cytokinin treatment may alter its effect on yield. Seed N demand is supplied by either N remobilisation, derived from N supplied before seed filling, or N accumulation during seed filling. Early BNF may give a larger N pool for remobilisation, important for seed N content as N accumulation during seed filling is less than N accumulation by seeds (Leffel *et al*., 1992; Vasilas *et al*., 1995; Egli & Bruening, 2007), with N remobilisation accounting for 59% of seed N. However, BNF is also an important source of N during seed development (Purcell *et al*., 2004), accounting for up to 95% of total N accumulation in low soil N, and 50% in fertile soil (Mastrodomenico & Purcell, 2012). Seed N accumulation can be double that taken from remobilisation, with most N (0.4 g N m^{-2} d^{-1}) from BNF. Continued BNF into late reproductive stages is therefore beneficial in achieving high seed yield. Taken together, it suggests that genotypes appear to use different N sources for grain filling, thus the timing of BNF will have different effects on yield depending on this.

Other aspects of soybean production may influence the effect of additional N from fixation. High phloem N concentration limits BNF (Neo & Layzell, 1997), so sufficient sequestration of N in the canopy is important for maintaining fixation. Ensuring high plant N demand may enable this, with decreased plant density leading to larger canopies which increased BNF four-fold (de Luca *et al*., 2014). Therefore, increased BNF without demand will not benefit plant growth.

Cold stress limits nodulation, but as nodule establishment is genotype-dependent (Chapter 2) and can be enhanced through cytokinin treatment (Chapter 3), the ability of each to mitigate this was tested in field trials (Chapter 4). Early sowing delayed the initiation of BNF and nodule development, but also prolonged these processes towards the end of the growing season (Table 4.6 and 4.7). Genotypes responded differently to early cold stress, in terms of N supply,

allowing DM50I17 to maintain consistent yield across all sowing dates whilst the other tended to be more sensitive (Figure 4.3). Surprisingly, however, low root zone temperature limited soil N uptake to a greater extent than BNF (Figure 4.4), perhaps by reducing root growth. This suggests that BNF is especially important in maintaining N supply in cold conditions. Increases in BNF caused by cytokinin were especially evident in early sowing dates, suggesting such treatment may mitigate cold stress (Figure 4.5).

5.5 | Directions for future study

Further work is required to understand the mechanism behind increased BNF of certain nodules shown here (Chapter 2). Variation in nodule BNF capacity is likely due to changes in physiology, as carbon sink capacity, oxygen permeability and N export all regulate BNF, thus future work should establish if there is a link between nodule morphology and physiology. Additionally, as the rhizobial strain occupying nodules determines BNF, it would be interesting to see if this also corresponds to differences in nodule traits. As the speed of BNF onset varies across genotypes (Chapter 2 and Chapter 4) and earlier nodule formation caused by cytokinin appears to be due to earlier nodulin gene expression (Chapter 3), future work could determine if the same is true across soybean genotypes.

As the effect of cytokinin application showed promise but was not consistently beneficial (Chapter 3 and Chapter 4), further investigation of cytokinin-based treatments is required. This may include further investigation into their timing. This study focused on the effect of cytokinin on early nodule development, but cytokinin application may also delay nodule senescence, potentially providing an important N source during seed filling. Stay-green, characterised by delayed chlorophyll catabolism, may also delay soybean nodule senesce by extending photosynthate supply. However, this would be at the expense of N-remobilisation during seed filling. Thus, the relative importance of these N-sources on yield should be established. Since the effect of nodule traits on BNF, and nodulation itself, varies across genotypes (Chapter 2), determining the effect of cytokinin on a wider range of genotypes is recommended. Additionally, this study only applied kinetin, but it may be beneficial to establish whether other cytokinins show beneficial responses (Mens *et al*., 2018).

Cytokinin appears to upregulate early nodulation genes (Chapter 3) but the mechanism leading to this was not determined here, so future work should aim to better understand the

effect of cytokinin on this pathway. Other mechanisms, such as the interaction between cytokinin and other hormones, in particular ethylene and auxin, should also be determined.

To meet food demands in 2050, crop yields must increase by around 50% on current agricultural land to avoid massive conversion of natural ecosystems (Cassman & Grassini, 2020; Ray *et al* 2013). For soybean, yield increases will be most limited by inadequate N supply (Cafaro La Menza *et al*., 2017), that in high yielding areas currently producing 4.5 Mg ha^{-1} the 50% increase to 6.8 Mg ha^{-1} would require 540 kg N ha^{-1}. Soil N could provide up to 150 kg N ha^{-1} (Cafaro La Menza *et al*., 2019) but this leaves 400 kg N ha^{-1} which must come from BNF, something not commonly possible based on current reviews (Salvagiotti *et al*., 2008; Ciampitti & Salvagiotti, 2018). Soil N and BNF provide insufficient N for soybean especially in highly productive environments, with an upper limit of 5.5 Mg ha^{-1} in Nebraska (Cafaro La Menza *et al*., 2020), likely to continue as soybean N requirements and average yields increase (Grassini *et al*., 2015; Specht *et al*., 2015). Thus, BNF must be increased sustainably to supply sufficient N for these high yielding soybeans, helping to meet increasing food demand without further destruction of natural ecosystems (Cassman & Grassini, 2020).

References

Abendroth, L. J., Elmore, R. W., & Ferguson, R. B. (2006). Soybean Inoculation: Understanding the Soil and Plant Mechanisms Involved Historical Materials 2078. Univ. of Nebraska, Lincoln. http://digitalcommons.unl.edu/extensionhist/2078 (accessed 15 Sept. 2019).

Adom, F., Maes, A., Workman, C., Clayton-Nierderman, Z., Thoma, G., & Shonnard, D. (2012). Regional carbon footprint analysis of dairy feeds for milk production in the USA. *International Journal of Life Cycle Assessment*, 17(5), 520–534.

Agyare, W. A., Clottey, V. A., Mercer-Quarshie, H., & Kombiok, J. M. (2006). Maize Yield Response in a Long-term Rotation and Intercropping Systems in the Guinea Savannah Zone of Northern Ghana. *Journal of Agronomy*, 5(2), 232–238

Ainsworth, E. A., Yendrek, C. R., Skoneczka, J. A., & Long, S. P. (2012). Accelerating yield potential in soybean: Potential targets for biotechnological improvement. *Plant, Cell and Environment*, 35(1), 38–52.

Albuquerque, H. C. De, Pegoraro, R. F., Vieira, N. M. B., Amorim, I. de J. F., & Kondo, M. K. (2012). Capacidade nodulatória e características agronômicas de feijoeiros comuns submetidos à adubação molíbdica parcelada e nitrogenada. *Revista Ciência Agronômica*, 43(2), 214–221.

Ali, S., & Bano, A. (2008). Leaf and Nodule Senescence in Chickpea (*Cicer Arietinum* L .) and the Role of Plant Growth Regulators. *Pakistan Journal of Botany*, 40(6), 2481–2492.

Ali, B., Hayat, S., Hasan, S. A., & Ahmad, A. (2008). IAA and 4-Cl-IAA Increases the Growth and Nitrogen Fixation in Mung Bean. *Communications in Soil Science and Plant Analysis*, 39(17–18), 2695–2705.

Ali, I. A., Kafkafi, U., Yamaguchi, I., Sugimoto, Y., & Inanaga, S. (1996). Effects of low root temperature on sap flow rate, soluble carbohydrates, nitrate contents and on cytokinin and gibberellin levels in root xylem exudate of sand-grown tomato. *Journal of Plant Nutrition*, 19(3–4), 619–634.

Alsajri, F. A., Singh, B., Wijewardana, C., Irby, J. T., Gao, W., & Reddy, K. R. (2019). Evaluating Soybean Cultivars for Low- and High-Temperature Tolerance During the Seedling Growth Stage. *Agronomy*, 9(1), 13.

Alves, B. J. R., Boddey, R. M., & Urquiaga, S. (2003). The success of BNF in soybean in Brazil. *In Plant and Soil,* 252, 1–9.

Ané, J. M., Kiss, G. B., Riely, B. K., Penmetsa, R. V., Oldroyd, G. E. D., Ayax, C., Lévy, J., Debellé, F., Baek, J. M., Kalo, P., Rosenberg, C., Roe, B. A., Long, S. R., Dénarié, J. & Cook, D. R. (2004). *Medicago truncatula DMI1* Required for Bacterial and Fungal Symbioses in Legumes. *Science*, 303(5662), 1364–1367.

Angus, J. F., Kirkegaard, J. A., Hunt, J. R., Ryan, M. H., Ohlander, L., & Peoples, M. B. (2015). Break crops and rotations for wheat. *Crop and Pasture Science*, 66(6), 523–552.

Appleby, C. A. (1984). Leghemoglobin and *Rhizobium* Respiration. *Annual Review of Plant Physiology*, 35(1), 443–478.

Ariel, F., Brault-Hernandez, M., Laffont, C., Huault, E., Brault, M., Plet, J., Moison, M., Blanchet, S., Ichante, J. L., Chabaud, M., Carrere, S., Crespi, M., Chan, R. L. & Frugier, F. (2012). Two Direct Targets of Cytokinin Signaling Regulate Symbiotic Nodulation in *Medicago truncatula*. *The Plant Cell*, 24(9), 3838–3852.

Atkins, C. A., Pate, J. S., Sanford, P. J., Dakora, F. D., & Matthews, I. (1989). Nitrogen Nutrition of Nodules in Relation to `N-Hunger' in Cowpea (*Vigna unguiculata* L. Walp). *Plant Physiology*, 90(4), 1644–1649.

Bacanamwo, M., & Harper, J. E. (1997). The feedback mechanism of nitrate inhibition of nitrogenase activity in soybean may involve asparagine and/or products of its metabolism. *Physiologia Plantarum*, 100(2), 371–377.

Balmford, A., Green, R. E., & Scharlemann, J. P. W. (2005). Sparing land for nature: exploring the potential impact of changes in agricultural yield on the area needed for crop production. *Global Change Biology*, 11(10), 1594–1605.

Bàrberi, P. (2002). Weed management in organic agriculture: Are we addressing the right issues? *Weed Research*, 42(3), 177–193.

Barros, R. L. N., De Oliveira, L. B., De Magalhães, W. B., Médici, L. O., & Pimentel, C. (2013). Interação entre inoculação com rizóbio e adubação nitrogenada de plantio na produtividade do feijoeiro nas épocas da seca e das águas. *Semina: Ciencias Agrarias*, 34(4), 1443–1450.

Bauer, P., Ratet, P., Crespi, M. D., Schultze, M., & Kondorosi, A. (1996). Nod factors and cytokinins induce similar cortical cell division, amyloplast deposition and *MsEnod12A* expression patterns in alfalfa roots. *The Plant Journal*, 10(1), 91–105.

Beatty, P. H., & Good, A. G. (2011). Future Prospects for Cereals That Fix Nitrogen. *Science*, 333(6041), 416–417.

Benková, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertová, D., Jürgens, G., & Friml, J. (2003). Local, Efflux-Dependent Auxin Gradients as a Common Module for Plant Organ Formation. *Cell*, 115(5), 591–602.

Bergersen, F. J. (1997). Regulation of nitrogen fixation in infected cells of leguminous root nodules in relation to O_2 supply. *Plant and Soil*, 191(2), 189–203.

Bertell, G., & Eliasson, L. (1992). Cytokinin effects on root growth and possible interactions with ethylene and indole-3-acetic acid. *Physiologia Plantarum*, 84(2), 255–261.

Bhuvaneswari, T. V., Turgeon, B. G., & Bauer, W. D. (1980). Early Events in the Infection of Soybean (*Glycine max* L. Merr) by Rhizobium japonicum. *Plant Physiology*, 66(6), 1027–1031.

Bhuvaneswari, T. V, Mills, K. K., Crist, D. K., Evans, W. R., & Bauer, W. D. (1983). Effects of culture age on symbiotic infectivity of *Rhizobium japonicum*. *Journal of Bacteriology*, 153(1), 443–451.

Bianco, C., & Defez, R. (2010). Auxins upregulate *nif* and *fix* genes. *Plant Signaling & Behavior*, 5(10), 1290–1294.

Bichel, A., Oelbermann, M., Voroney, P., & Echarte, L. (2016). Sequestration of native soil organic carbon and residue carbon in complex agroecosystems. *Carbon Management*, 7(5–6), 261–270.

Bishnoi, N. R., & Krishnamoorthy, H. N. (1991). Effect of waterlogging and gibberellic acid on growth and yield of chickpea (*cicer arietinum* L.). *Indian J. Plant Physiology*, 34(2), 186–191.

Bisseling, T., Van Den Bos, R. C., Weststrate, M. W., Hakkaart, M. J. J., & Van Kammen, A. (1979). Development of the nitrogen-fixing and protein-synthesizing apparatus of bacteroids in pea root nodules. *Biochimica et Biophysica Acta (BBA) - Nucleic Acids and Protein Synthesis*, 562(3), 515–526.

Boiero, L., Perrig, D., Masciarelli, O., Penna, C., Cassán, F., & Luna, V. (2007). Phytohormone production by three strains of *Bradyrhizobium japonicum* and possible physiological and technological implications. *Applied Microbiology and Biotechnology*, 74(4), 874–880.

Borisov, A. Y., Madsen, L. H., Tsyganov, V. E., Umehara, Y., Voroshilova, V. A., Batagov, A. O., Sandal, N., Mortensen, A., Schauser, L., Ellis, N., Tikhonovich, I. A. & Stougaard, J. (2003). The *Sym35* gene required for root nodule development in pea is an ortholog of *Nin* from *Lotus japonicus*. *Plant Physiology*, 131(3), 1009–1017.

Bowles, T. M., Mooshammer, M., Socolar, Y., Calderón, F., Cavigelli, M. A., Culman, S. W., Deen, W., Drury, C. F., Garcia, A., Gaudin, A. C. M., Harkcom, W. S., Lehman, R. M., Osborne, S. L.,

Robertson, G. F., Salerno, J., Schmer, M. R., Strock, J. & Grandy, A. S. (2020). Long-Term Evidence Shows that Crop-Rotation Diversification Increases Agricultural Resilience to Adverse Growing Conditions in North America. *One Earth*, 2(3), 284–293.

Breakspear, A., Liu, C., Roy, S., Stacey, N., Rogers, C., Trick, M., Morieri, G., Mysore, K. S., Wen, J., Oldroyd, G., Downie, J. A. & Murray, J. D. (2014). The Root Hair "Infectome" of *Medicago truncatula* Uncovers Changes in Cell Cycle Genes and Reveals a Requirement for Auxin Signaling in Rhizobial Infection. *The Plant Cell Online*, 26(12), 4680–4701.

Breene, W. M., Lin, S., Hardman, L., & Orf, J. (1988). Protein and oil content of soybeans from different geographic locations. *Journal of the American Oil Chemists' Society*, 65(12), 1927–1931.

Burias, N., Planchon, C., & Paul, M. H. (1990). Phenotypic and genotypic distribution of nodules on soybean root system inoculated with *Bradyrhizobium japonicum* strain G49. *Agronomie*, 10, 57–62.

Caba, J. M., Centeno, M. L., Fernández, B., Gresshoff, P. M., & Ligero, F. (2000). Inoculation and nitrate alter phytohormone levels in soybean roots: differences between a super-nodulating mutant and the wild type. *Planta*, 211(1), 98–104.

Cabeza, R. A., Liese, R., Fischinger, S. A., Sulieman, S., Avenhaus, U., Lingner, A., Hein, H., Koester, B., Baumgarten, V., Dittert, K. & Schulze, J. (2015). Long-term non-invasive and continuous measurements of legume nodule activity. *Plant Journal*, 81(4) 637-648.

Caetano-Anollés, G., & Gresshoff, P. M. (1993). Nodule distribution on the roots of soybean and a super-nodulating mutant in sand-vermiculite. *Plant and Soil*, 148(2), 265–270.

Cafaro La Menza, N., Monzon, J. P., Lindquist, J. L., Arkebauer, T. J., Knops, J. M. H., Unkovich, M., Specht, J. E. & Grassini, P. (2020). Insufficient nitrogen supply from symbiotic fixation reduces seasonal crop growth and nitrogen mobilization to seed in highly productive soybean crops. *Plant Cell and Environment*, 43(8), 1958–1972.

Cafaro La Menza, N., Monzon, J. P., Specht, J. E., & Grassini, P. (2017). Is soybean yield limited by nitrogen supply? *Field Crops Research*, *213*(August), 204–212.

Cafaro La Menza, N., Monzon, J. P., Specht, J. E., Lindquist, J. L., Arkebauer, T. J., Graef, G., & Grassini, P. (2019). Nitrogen limitation in high-yield soybean: Seed yield, N accumulation, and N-use efficiency. *Field Crops Research*, *237*(April), 74–81.

Callaham, D. A., & Torrey, J. G. (1981). The structural basis for infection of root hairs of *Trifolium repens* by *Rhizobium* . *Canadian Journal of Botany*, *59*(9), 1647–1664.

Calvert, H. E., Pence, M. K., Pierce, M., Malik, N. S. A., & Bauer, W. D. (1984). Anatomical analysis of the development and distribution of *Rhizobium* infections in soybean roots. *Canadian Journal of Botany*, *62*(11), 2375–2384.

Camerini, S., Senatore, B., Lonardo, E., Imperlini, E., Bianco, C., Moschetti, G., Rotino, G. L., Campion, B. & Defez, R. (2008). Introduction of a novel pathway for IAA biosynthesis to rhizobia alters vetch root nodule development. *Archives of Microbiology*, *190*(1), 67–77.

Canfield, D. E., Glazer, A. N., & Falkowski, P. G. (2010). The Evolution and Future of Earth's Nitrogen Cycle. *Science*, *330*(6001), 192–196.

Carroll, B. J., McNeil, D. L., & Gresshoff, P. M. (1985). A Supernodulation and Nitrate-Tolerant Symbiotic (*nts*) Soybean Mutant. *Plant Physiology*, *78*(1), 34–40.

Cassina, L., Tassi, E., Morelli, E., Giorgetti, L., Remorini, D., Chaney, R. L., & Barbafieri, M. (2011). Exogenous cytokinin treatments of an NI hyper-accumulator, alyssum Murale, grown in a serpentine soil: Implications for phytoextraction. *International Journal of Phytoremediation*, *13*(SUPPL.1), 90–101.

Cassman, K. G., & Grassini, P. (2020). A global perspective on sustainable intensification research. *Nature Sustainability*, *3*(4), 262–268.

Cataldo, D. A., Maroon, M., Schrader, L. E., & Youngs, V. L. (1975). Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. *Communications in Soil Science and Plant Analysis*, *6*(1), 71–80.

Cerezini, P., Kuwano, B. H., dos Santos, M. B., Terassi, F., Hungria, M., & Nogueira, M. A. (2016). Strategies to promote early nodulation in soybean under drought. *Field Crops Research*, *196*, 160–167.

Cernay, C., Ben-Ari, T., Pelzer, E., Meynard, J. M., & Makowski, D. (2015). Estimating variability in grain legume yields across Europe and the Americas. *Scientific Reports*, *5*, 1–11.

Chalk, P. M. (1998). Dynamics of biologically fixed N in legume-cereal rotations: a review. *Australian Journal of Agricultural Research*, 49(3), 303.

Charpentier, M., & Oldroyd, G. (2010). How close are we to nitrogen-fixing cereals? *Current Opinion in Plant Biology*, *13*(5), 556–564.

Chen, W., Zheng, D., Feng, N., Liu, T., Liu, Y., Gong, S., Cui, H. & Xiang, H. (2015). The effects of gibberellins and mepiquat chloride on nitrogenase activity in *Bradyrhizobium japonicum*. *Acta Physiologiae Plantarum*, 37(1), 1723.

Chibeba, A., de Fátima Guimarães, M., Brito, O. R., Nogueira, M. A., Silva Araujo, R., & Hungria, M. (2015). Co-Inoculation of Soybean with *Bradyrhizobium* and *Azospirillum* Promotes Early Nodulation. *American Journal of Plant Sciences*, 06(10), 1641–1649.

Cho, Y., Suh, S. K., Park, H. K., & Wood, A. (2002). Impact of 2,4-DP and BAP upon pod set and seed yield in soybean treated at reproductive stages. *Plant Growth Regulation*, *36*(3), 215–221.

Choudhury, T. M. A., & Khanif, Y. M. (2001). Evaluation of effects of nitrogen and magnesium fertilization on rice yield and fertilizer nitrogen efficiency using 15N tracer technique. *Journal of Plant Nutrition*, *24*(6), 855–871.

Ciampitti, I. A., & Salvagiotti, F. (2018). New insights into soybean biological nitrogen fixation. *Agronomy Journal*, *110*(4), 1185–1196.

Cleveland, C. C., Townsend, A. R., Schimel, D. S., Fisher, H., Howarth, R. W., Hedin, L. O., Perakis, S. S., Latty, E. F., Von Fischer, J. C., Elseroad, A. & Wasson, M. F. (1999). Global patterns of terrestrial biological nitrogen (N_2) fixation in natural ecosystems. *Global Biogeochemical Cycles*, *13*(2), 623–645.

Codispoti, L. A. (2007). An oceanic fixed nitrogen sink exceeding 400 Tg N a^{-1} vs the concept of homeostasis in the fixed-nitrogen inventory. *Biogeosciences*, *4*(2), 233–253.

Conley, D. J., Paerl, H. W., Howarth, R. W., Boesch, D. F., Seitzinger, S. P., Havens, K. E., Lancelot, C. & Likens, G. E. (2009). Controlling Eutrophication: Nitrogen and Phosphorus. *Science*, *323*(5917), 1014–1015.

Cooper, J. B., & Long, S. R. (1994). Morphogenetic Rescue of *Rhizobium meliloti* Nodulation Mutants by trans-Zeatin Secretion. *The Plant Cell Online*, *6*(2), 215–225.

Costa, M. H., Yanagi, S. N. M., Souza, P. J. O. P., Ribeiro, A., & Rocha, E. J. P. (2007). Climate change in Amazonia caused by soybean cropland expansion, as compared to caused by pastureland expansion. *Geophysical Research Letters*, *34*(7), 2–5.

Crist, D. K., Wyza, R. E., Mills, K. K., Bauer, W. D., & Evans, W. R. (1984). Preservation of *Rhizobium* viability and symbiotic infectivity by suspension in water. *Applied and Environmental Microbiology*, *47*(5), 895–900.

Crookston, R. K., Kurle, J. E., Copeland, P. J., Ford, J. H., & Lueschen, W. E. (1991). Rotational Cropping Sequence Affects Yield of Corn and Soybean. *Agronomy Journal*, *83*(1), 108–113.

Crutzen, P. J., Mosier, A. R., Smith, K. A., & Winiwarter, W. (2008). N_2O release from agro-biofuel production negates global warming reduction by replacing fossil fuels. *Atmospheric Chemistry and Physics*, *8*(2), 389–395.

Davis, A. S., Hill, J. D., Chase, C. A., Johanns, A. M., & Liebman, M. (2012). Increasing Cropping System Diversity Balances Productivity, Profitability and Environmental Health. *PLoS ONE*, *7*(10), 1–8.

Day, D. A., Kaiser, B. N., Thomson, R., Udvardi, M. K., Moreau, S., & Puppo, A. (2001). Nutrient transport across symbiotic membranes from legume nodules. *Australian Journal of Plant Physiology*, *28*(7), 667–674.

de Araujo, A. S. F., de Almeida Lopes, A. C., Teran, J. C. B. M. y, Palkovic, A., & Gepts, P. (2017). Nodulation ability in different genotypes of *Phaseolus lunatus* by rhizobia from California agricultural soils. *Symbiosis*, *73*(1), 7–14.

de Billy, F., Grosjean, C., May, S., Bennett, M., & Cullimore, J. V. (2001). Expression Studies on *AUX1* -like Genes in *Medicago truncatula* Suggest That Auxin Is Required at Two Steps in Early Nodule Development. *Molecular Plant-Microbe Interactions*, *14*(3), 267–277.

de Bruin, J. L., Pedersen, P., Conley, S. P., Gaska, J. M., Naeve, S. L., Kurle, J. E., Elmore, R. W., Giesler, L. J. & Abendroth, L. J. (2010). Probability of yield response to inoculants in fields with a history of soybean. *Crop Science*, *50*(1), 265–272.

de Luca, M. J., Nogueira, M. A., & Hungria, M. (2014). Feasibility of lowering soybean planting density without compromising nitrogen fixation and yield. *Agronomy Journal*, *106*(6), 2118–2124.

de Ruijter, N. C. A., Rook, M. B., Bisseling, T., & Emons, A. M. C. (1998). Lipochito-oligosaccharides re-initiate root hair tip growth in *Vicia sativa* with high calcium and spectrin-like antigen at the tip. *Plant Journal*, *13*(3), 341–350.

de Visser, C. L. M., Schreuder, R., & Stoddard, F. (2014). The EU's dependency on soya bean import for the animal feed industry and potential for EU produced alternatives. *OCL Oilseeds and Fats, Crops and Lipids*, *21*(4), D407.

Dean, C. A., Sun, W., Dong, Z., & Caldwell, C. D. (2006). Soybean nodule hydrogen metabolism affects soil hydrogen uptake and growth of rotation crops. *Canadian Journal of Plant Science*, *86*(5), 1355–1359.

Dehio, C., & de Bruijn, F. (1992). The early nodulin gene *SrEnod2* from *Sesbania rostrata* is inducible by cytokinin. *The Plant Journal*, *2*(1), 117–128.

Dello Ioio, R., Nakamura, K., Moubayidin, L., Perilli, S., Taniguchi, M., Morita, M. T., Aoyama, T., Costantino, P. & Sabatini, S. (2008). A genetic framework for the control of cell division and differentiation in the root meristem. *Science*, *322*(5906), 1380–1384.

Dénarié, J., Debellé, F., & Promé, J. C. (1996). Rhizobium Lipo-Chitooligosaccharide Nodulation Factors: Signaling Molecules Mediating Recognition and Morphogenesis. *Annual Review of Biochemistry*, *65*(1), 503–535.

Desbrosses, G. J., & Stougaard, J. (2011). Root Nodulation: A Paradigm for How Plant-Microbe Symbiosis Influences Host Developmental Pathways. *Cell Host & Microbe*, *10*(4), 348–358.

Dhruve, J. J., & Vakharia, D. N. (2013). Influence of water stress and benzyl adenine imposed at various growth stages on yield of groundnut. *International Journal of Plant and Animal Sciences*, *1*(1), 5–10.

Di Mauro, G., Borrás, L., Rugeroni, P., & Rotundo, J. L. (2019). Exploring soybean management options for environments with contrasting water availability. *Journal of Agronomy and Crop Science*, 205(3), 274–282.

Ding, Y., Kalo, P., Yendrek, C., Sun, J., Liang, Y., Marsh, J. F., Harris, J. M. & Oldroyd, G. E. D. (2008). Abscisic acid coordinates nod factor and cytokinin signaling during the regulation of nodulation in *Medicago truncatula*. *The Plant Cell*, *20*(10), 2681–2695.

Ding, Y., & Oldroyd, G. E. D. (2009). Positioning the nodule, the hormone dictum. *Plant Signaling & Behavior*, *4*(2), 89–93.

Dobbelaere, S., Croonenborghs, A., Thys, A., Ptacek, D., Vanderleyden, J., Dutto, P., Labandera-Gonzalez, C., Caballero-Mellado, J., Aguirre, J. F., Kapulnik, Y., Brener, S., Burdman, S., Kadouri, D., Sarig, S. & Okon, Y. (2001). Responses of agronomically important crops to inoculation with *Azospirillum*. *Functional Plant Biology*, *28*(9), 871.

Döbereiner, J. (1966). Evaluation of Nitrogen Fixation in Legumes by the Regression of Total Plant Nitrogen with Nodule Weight. *Nature*, *210*, 850–852.

Dobert, R. C., Rood, S. B., & Blevins, D. G. (1992). Gibberellins and the Legume- Rhizobium Symbiosis. *Plant Physiology*, *98*(1), 221–224.

Dobránszki, J., & Mendler-Drienyovszki, N. (2014). Cytokinin-induced changes in the chlorophyll content and fluorescence of in vitro apple leaves. *Journal of Plant Physiology*, *171*(16), 1472–1478.

Donald, C. M., & Hamblin, J. (1976). The Biological Yield and Harvest Index of Cereals as Agronomic and Plant Breeding Criteria. *Crop Science*, *59*(3), 361–405.

Dong, Z., & Layzell, D. B. (2001). H_2 oxidation, O_2 uptake and CO_2 fixation in hydrogen treated soils. *Plant and Soil*, *229*(1), 1–12.

Dong, Z., Wu, L., Kettlewell, B., Caldwell, C. D., & Layzell, D. B. (2003). Hydrogen fertilization of soils - Is this a benefit of legumes in rotation? *Plant, Cell and Environment*, 26(11), 1875–1879.

Earl, C. D., Ronson, C. W., & Ausubel, F. M. (1987). Genetic and structural analysis of the *Rhizobium meliloti fixA*, *fixB*, *fixC*, and *fixX* genes. *Journal of Bacteriology*, 169(3), 1127–1136.

Egli, D. B., & Bruening, W. P. (2007). Nitrogen accumulation and redistribution in soybean genotypes with variation in seed protein concentration. *Plant and Soil*, *301*(1–2), 165–172.

Erisman, J. W., Sutton, M. A., Galloway, J., Klimont, Z., & Winiwarter, W. (2008). How a century of ammonia synthesis changed the world. *Nature Geoscience*, *1*(10), 636–639.

Esseling, J. J., Lhuissier, F. G. P., & Emons, A. M. C. (2003). Nod factor-induced root hair curling: Continuous polar growth towards the point of nod factor application. *Plant Physiology*, *132*(4), 1982–1988.

Evans, J., O'Connor, G. E., Turner, G. L., & Bergersen, F. J. (1987). Influence of mineral nitrogen on nitrogen fixation by lupin (*Lupinus angustifolius*) as assessed by 15N isotope dilution methods. *Field Crops Research*, *17*(2), 109–120.

Fabre, F., & Planchon, C. (2000). Nitrogen nutrition, yield and protein content in soybean. *Plant Science*, *152*(1), 51–58.

Fang, Y., & Hirsch, A. M. (1998). Studying early nodulin gene *ENOD40* expression and induction by nodulation factor and cytokinin in transgenic alfalfa. *Plant Physiology*, *116*(1), 53–68.

FAOSTAT Statistical Database. (2020). Retrieved November 2, 2020, from http://www.fao.org/faostat/en/#data/QC

Fatima, Z., Bano, A., & Aslam, M. (2008). Response of chickpea to plant growth regulators on nitrogen fixation and yield. *Pakistan Journal of Botany*, *40*(5), 2005–2013.

Fehr, W. R., & Caviness, C. E. (1977). Stages of soybean development. *Special Report*, *87*, 1–12.

Felle, H. H., Kondorosi, É., Kondorosi, Á., & Schultze, M. (1999). Elevation of the cytosolic free [Ca_2^+] is indispensable for the transduction of the Nod factor signal in alfalfa. *Plant Physiology*, *121*(1), 273–279.

Ferguson, B. J., Foo, E., Ross, J. J., & Reid, J. B. (2011). Relationship between gibberellin, ethylene and nodulation in *Pisum sativum*. *New Phytologist*, *189*(3), 829–842.

Ferguson, B. J., Indrasumunar, A., Hayashi, S., Lin, M. H., Lin, Y. H., Reid, D. E., & Gresshoff, P. M. (2010). Molecular Analysis of Legume Nodule Development and Autoregulation. *Journal of Integrative Plant Biology*, *52*(1), 61–76.

Ferguson, B. J., & Mathesius, U. (2014). Phytohormone Regulation of Legume-Rhizobia Interactions. *Journal of Chemical Ecology*, *40*(7), 770–790.

Ferguson, B. J., Ross, J. J., & Reid, J. B. (2005). Nodulation phenotypes of gibberellin and brassinosteroid mutants of pea. *Plant Physiology*, *138*(4), 2396–2405.

Ferreira, Magda C., De S. Andrade, D., Ligia, L. M., Takemura, S. M., & Hungria, M. (2000). Tillage method and crop rotation effects on the population sizes and diversity of *bradyrhizobia* nodulating soybean. *Soil Biology and Biochemistry*, *32*(5), 627–637.

Ferreira, Magda Cristiani, & Hungria, M. (2002). Recovery of soybean inoculant strains from uncropped soils in Brazil. *Field Crops Research*, *79*(2–3), 139–152.

Finke, R. L., Harper, J. E., & Hageman, R. H. (1982). Efficiency of Nitrogen Assimilation by N_2 -Fixing and Nitrate-Grown Soybean Plants (*Glycine max* [L.] Merr.) . *Plant Physiology*, *70*(4), 1178–1184.

Fischinger, S. A., Drevon, J. J., Claassen, N., & Schulze, J. (2006). Nitrogen from senescing lower leaves of common bean is re-translocated to nodules and might be involved in a N-feedback regulation of nitrogen fixation. *Journal of Plant Physiology*, *163*(10), 987–995.

Fischinger, S. A., Hristozkova, M., Mainassara, Z.-A., & Schulze, J. (2010). Elevated CO_2 concentration around alfalfa nodules increases N_2 fixation. *Journal of Experimental Botany*, *61*(1), 121–130.

Fonouni-Farde, C., Kisiala, A., Brault, M., Emery, R. J. N., Diet, A., & Frugier, F. (2017). *DELLA1*-mediated gibberellin signaling regulates cytokinin-dependent symbiotic nodulation. *Plant Physiology*, *175*(4), 1795–1806.

Forde, B. G., & Lea, P. J. (2007). Glutamate in plants: Metabolism, regulation, and signalling. *Journal of Experimental Botany*, *58*(9), 2339–2358.

Foyer, C. H., Siddique, K. H. M., Tai, A. P. K., Anders, S., Fodor, N., Wong, F. L., Ludidi, N., Chapman, M. A., Ferguson, B. J., Considine, M. J., Zabel, F., Prasad, P. V. V., Varshney, R. K., Nguyen, H. T. & Lam, H. M. (2019). Modelling predicts that soybean is poised to dominate crop production across Africa. *Plant Cell and Environment*, *42*(1), 373–385.

Franke, A. C., van den Brand, G. J., Vanlauwe, B., & Giller, K. E. (2018). Sustainable intensification through rotations with grain legumes in Sub-Saharan Africa: A review. *Agriculture, Ecosystems and Environment*, 261(September 2017), 172–185.

Frugier, F., Kosuta, S., Murray, J. D., Crespi, M., & Szczyglowski, K. (2008). Cytokinin: secret agent of symbiosis. *Trends in Plant Science*, *13*(3), 115–120.

Fryzuk, M. (2004). Ammonia Transformed. *Nature*, *427*(February), 498–499.

Fuchs, Y., & Lieberman, M. (1968). Effects of Kinetin, IAA, and Gibberellin on Ethylene Production, and Their Interactions in Growth of Seedlings. *Plant Physiol*, *43*, 2029–2036.

Fyson, A., & Oaks, A. (1990). Growth promotion of maize by legume soils. *Plant and Soil*, *122*(2), 259–266.

Gage, D. J. (2004). Infection and Invasion of Roots by Symbiotic, Nitrogen-Fixing Rhizobia during Nodulation of Temperate Legumes. *Microbiology and Molecular Biology Reviews*, *68*(2), 280–300.

Gai, Z., Zhang, J., & Li, C. (2017). Effects of starter nitrogen fertilizer on soybean root activity, leaf photosynthesis and grain yield. *PLoS ONE*, *12*(4), 1–15.

George, T., Singleton, P. W., & Bohlool, B. Ben. (1988). Yield, Soil Nitrogen Uptake, and Nitrogen Fixation by Soybean from Four Maturity Groups Grown at Three Elevations. *Agronomy Journal*, *80*(4), 563–567.

Gethi, J. G., Smith, M. E., Mitchell, S. E., & Kresovich, S. (2005). Genetic diversity of *Striga hermonthica* and *Striga asiatica* populations in Kenya. *Weed Research*, *45*(1), 64–73.

Giller, K. E., & Cadisch, G. (1995). Future benefits from biological nitrogen fixation: An ecological approach to agriculture. *Plant and Soil*, *174*(1–2), 255–277.

Godfray, H. C. J., Beddington, J. R., Crute, I. R., Haddad, L., Lawrence, D., Muir, J. F., Pretty, J., Robinson, S., Thomas, S. M., & Toulmin, C. (2010). Food Security: The Challenge of Feeding 9 Billion People. *Science*, *327*(5967), 812–818.

Goldsmith, P. D. (2008). Economics of Soybean Production, Marketing, and Utilization. In *Soybeans: Chemistry, Production, Processing, and Utilization* (pp. 117–150). Elsevier Inc.

Gonzalez-Rizzo, S., Crespi, M., & Frugier, F. (2006). The *Medicago truncatula CRE1* Cytokinin Receptor Regulates Lateral Root Development and Early Symbiotic Interaction with *Sinorhizobium meliloti*. *The Plant Cell Online*, *18*(10), 2680–2693.

Gonzalez-Rizzo, Silvina, Laporte, P., Crespi, M., & Frugier, F. (2009). Legume Root Architecture: A Peculiar Root System. In *Root Development* (Vol. 37, pp. 239–287). Oxford, UK: Wiley-Blackwell.

González, E. M., Larrainzar, E., Marino, D., Wienkoop, S., Gil-Quintana, E., & Arrese-Igor, C. (2015). Physiological responses of N_2-fixing legumes to water limitation. In *Legume Nitrogen Fixation in a Changing Environment: Achievements and Challenges* (pp. 5–33). Springer International Publishing.

Graham, P. H., & Vance, C. P. (2003). Legumes: Importance and Constraints to Greater Use. *Plant Physiology*, *131*(3), 872–877.

Grassini, P., Specht, J. E., Tollenaar, M., Ciampitti, I., & Cassman, K. G. (2015). High-yield maize-soybean cropping systems in the US Corn Belt. *Crop Physiology: Applications for Genetic Improvement and Agronomy: Second Edition*, (April 2018), 17–41.

Gwata, E. T., Wofford, D. S., Pfahler, P. L., & Boote, K. J. (2004). Genetics of Promiscuous Nodulation in Soybean: Nodule Dry Weight and Leaf Color Score. *Journal of Heredity*, *95*(2), 154–157.

Hageman, R. V., & Burris, R. H. (1978). Nitrogenase and nitrogenase reductase associate and dissociate with each catalytic cycle. *Proceedings of the National Academy of Sciences of the United States of America*, *75*(6), 2699–2702.

Halvin, J. L., Tisdale, S. L., Nelson, W. L., & Beaton, J. D. (2005). *Soil Fertility and Fertilizers: An Introduction to Nutrient Management* (7th editio). Upper Saddle River, NJ: Pearson Prentice Hall.

Halvorson, A. D., Follett, R. F., Bartolo, M. E., & Schweissing, F. C. (2002). Nitrogen fertilizer use efficiency of furrow-irrigated onion and corn. *Agronomy Journal*, *94*(3), 442–449.

Hamawaki, R. L., & Kantartzi, S. K. (2018). Di-nitrogen fixation at the early and late growth stages of soybean. *Acta Scientiarum. Agronomy*, *40*(1), 36372.

Hardarson, G., & Danso, S. K. A. (1993). Methods for measuring biological nitrogen fixation in grain legumes. *Plant and Soil*, *152*(1), 19–23.

Hardarson, G., Golbs, M., & Danso, S. K. A. (1989). Nitrogen fixation in soybean (*Glycine max L. merrill*) as affected by nodulation patterns. *Soil Biology and Biochemistry*, *21*(6), 783–787.

Hargreaves, G. H., & Samani, Z. A. (1985). Reference Crop Evapotranspiration from Temperature. *Applied Engineering in Agriculture*, *1*(2), 96–99.

Hayashi, S., Gresshoff, P. M., & Ferguson, B. J. (2014). Mechanistic action of gibberellins in legume nodulation. *Journal of Integrative Plant Biology*, *56*(10), 971–978.

Hayashi, S., Reid, D. E., Lorenc, M. T., Stiller, J., Edwards, D., Gresshoff, P. M., & Ferguson, B. J. (2012). Transient Nod factor-dependent gene expression in the nodulation-competent zone of soybean (*Glycine max* [L.] Merr.) roots. *Plant Biotechnology Journal*, *10*(8), 995–1010.

Haynes, R. J., Martin, R. J., & Goh, K. M. (1993). Nitrogen fixation, accumulation of soil nitrogen and nitrogen balance for some field-grown legume crops. *Field Crops Research*, *35*(2), 85–92.

Heatherly, L. G. (2005). Midsouthern USA Soybean Yield Affected by Maturity Group and Planting Date. *Crop Management*, *4*(1), 1–8.

Heckmann, A. B., Sandal, N., Bek, A. S., Madsen, L. H., Jurkiewicz, A., Nielsen, M. W., Tirichine, L. & Stougaard, J. (2011). Cytokinin Induction of Root Nodule Primordia in *Lotus japonicus* Is Regulated by a Mechanism Operating in the Root Cortex. *Molecular Plant-Microbe Interactions*, *24*(11), 1385–1395.

Heidstra, R., Geurts, R., Franssen, H., Spaink, H. P., van Kammen, A., & Bisseling, T. (1994). Root Hair Deformation Activity of Nodulation Factors and Their Fate on *Vicia sativa*. *Plant Physiology*, *105*(3), 787–797.

Held, M., Hou, H., Miri, M., Huynh, C., Ross, L., Hossain, M. S., Sato, S., Tabata, S., Perry, J., Wang, T. L., Szczyglowski, K. (2014). *Lotus japonicus* Cytokinin Receptors Work Partially Redundantly to Mediate Nodule Formation. *The Plant Cell*, *26*(2), 678–694.

Henzell, E. F. (1988). The role of biological nitrogen fixation research in solving problems in tropical agriculture. *Plant and Soil*, *108*(1), 15–21.

Hernanz, J. L., Sánchez-Girón, V., & Navarrete, L. (2009). Soil carbon sequestration and stratification in a cereal/leguminous crop rotation with three tillage systems in semiarid conditions. *Agriculture, Ecosystems and Environment*, *133*(1–2), 114–122.

Heron, D. S., & Pueppke, S. G. (1984). Mode of infection, modulation specificity, and indigenous plasmids of 11 fast-growing *Rhizobium japonicum* strains. *Journal of Bacteriology*, *160*(3), 1061–1066.

Herridge, D. F., & Peoples, M. B. (1990). Ureide Assay for Measuring Nitrogen Fixation by Nodulated Soybean Calibrated by 15N Methods. *Plant Physiology*, *93*(2), 495–503.

Herridge, David F., Peoples, M. B., & Boddey, R. M. (2008). Global inputs of biological nitrogen fixation in agricultural systems. *Plant and Soil*, *311*(1–2), 1–18.

Herridge, David F, Bergersen, F. J., & Peoples, M. B. (1990). Measurement of Nitrogen Fixation by Soybean in the Field Using the Ureide and Natural 15N Abundance Methods. *Plant Physiology*, *93*(2), 708–716.

Hesterman, O. B., Sheaffer, C. C., Barnes, D. K., Lueschen, W. E., & Ford, J. H. (1986). Alfalfa Dry Matter and Nitrogen Production, and Fertilizer Nitrogen Response in Legume-Corn Rotations. *Agronomy Journal*, *78*(1), 19–23.

Hichri, I., Meilhoc, E., Boscari, A., Bruand, C., Frendo, P., & Brouquisse, R. (2016). Nitric Oxide. Jack-of-All-Trades of the Nitrogen-Fixing Symbiosis? *Advances in Botanical Research*, *77*(February), 193–218.

Hirel, B., Le Gouis, J., Ney, B., & Gallais, A. (2007). The challenge of improving nitrogen use efficiency in crop plants: towards a more central role for genetic variability and quantitative genetics within integrated approaches. *Journal of Experimental Botany*, *58*(9), 2369–2387.

Hirsch, A. M. (1992). Developmental biology of legume nodulation. *New Phytologist*, *122*(2), 211–237.

Hirsch, A. M., Bhuvaneswari, T. V., Torrey, J. G., & Bisseling, T. (1989). Early nodulin genes are induced in alfalfa root outgrowths elicited by auxin transport inhibitors. *Proceedings of the National Academy of Sciences*, *86*(4), 1244–1248.

Hirsch, A. M., Fang, Y., Asad, S., & Kapulnik, Y. (1997). The role of phytohormones in plant-microbe symbioses Plant Soil. *Plant Ans Soil*, *194*, 171–184.

Hocking, P. J. (2001). Organic acids exuded from roots in phosphorus uptake and aluminum tolerance of plants in acid soils. *Advances in Agronomy*, *74*, 63–97.

Hungria, M., Barradas, C. A. A., & Wallsgrove, R. M. (1991). Nitrogen fixation, assimilation and transport during the initial growth stage of *Phaseolus vulgaris* l. *Journal of Experimental Botany*, *42*(7), 839–844.

Hungria, M., & Bohrer, T. R. J. (2000). Variability of nodulation and dinitrogen fixation capacity among soybean cultivars. *Biology and Fertility of Soils*, *31*(1), 45–52.

Hungria, Mariangela, Franchini, J. C., Campo, R. J., & Graham, P. H. (2006). The Importance of Nitrogen Fixation to Soybean Cropping in South America. In D. Werner & W. E. Newton (Eds.), *Nitrogen Fixation in Agriculture, Forestry, Ecology, and the Environment* (pp. 25–42). Werner D, Newton WE: Dordrecht: Springer.

Hungria, Mariangela, Franchini, J., & Soja, E. (2005). Nitrogen Fixation in Agriculture, Forestry, Ecology, and the Environment, (February 2016).

Hungria, Mariangela, & Mendes, I. C. (2015). Nitrogen Fixation with Soybean: The Perfect Symbiosis? In *Biological Nitrogen Fixation* (Vol. 2–2, pp. 1009–1024). Hoboken, NJ, USA: John Wiley & Sons, Inc.

Hunt, N. D., Hill, J. D., & Liebman, M. (2017). Reducing Freshwater Toxicity while Maintaining Weed Control, Profits, and Productivity: Effects of Increased Crop Rotation Diversity and Reduced Herbicide Usage. *Environmental Science and Technology*, *51*(3), 1707–1717.

Hunt, S., Gaito, S. T., & Layzell, D. B. (1988). Model of gas exchange and diffusion in legume nodules. *Planta*, *173*(1), 128–141.

Hunt, S., King, B. J., & Layzell, D. B. (1989). Effects of Gradual Increases in O_2 Concentration on Nodule Activity in Soybean. *Plant Physiology*, *91*(1), 315–321.

Hunt, S., & Layzell, D. B. (1993). Gas exchange of legume nodules and the regulation of nitrogenase activity. *Annual Review of Plant Physiology and Plant Molecular Biology*, *44*(1), 483–511.

Hunter, W. J. (1987). Influence of 5-methyltryptophan-resistant *Bradyrhizobium japonicum* on soybean root nodule indole-3-acetic acid content. *Applied and Environmental Microbiology*, *53*(5), 1051–1055.

Hunter, William J, & Hunter, W. J. (1987). *Bradyrhizobium japonicum* on Soybean Root Nodule Indole-3-Acetic Acid Content Influence of 5-Methyltryptophan-Resistant *Bradyrhizobium japonicum* on Soybean Root Nodule Indole-3-Acetic Acid Contentt, *53*(5), 1051–1055.

Ibrahim, M., Bekheta, M. A., El-Moursi, A., & Gaafar, N. A. (2007). Improvement of Growth and Seed Yield Quality of *Vicia faba* L. Plants as Affected by Application of Some Bioregulators. *Australian Journal of Basic and Applied Sciences*, *1*(4), 657–666.

Ikeda, J. (1999). Differences in numbers of nodules and lateral roots between soybean (*Glycine max* L. Merr.) cultivars, Kitamusume and Toyosuzu. *Soil Science and Plant Nutrition*, *45*(3), 591–598.

Ikeda, J. (2003). Mathematical description for nodule size distribution, and number, weight, and diameter of nodules deduced from a theoretical model of photosynthate competition among nodules. *Soil Science and Plant Nutrition*, *49*(6), 805–815.

Imsande, J., & Ralston, E. J. (1982). Dinitrogen Fixation in Male-Sterile Soybeans. *Plant Physiology*, *69*(3), 745–746.

Jemo, M., Abaidoo, R. C., Nolte, C., Tchienkoua, M., Sanginga, N., & Horst, W. J. (2006). Phosphorus benefits from grain-legume crops to subsequent maize grown on acid soils of southern Cameroon. *Plant and Soil*, *284*(1–2), 385–397.

Jensen, Erik S., Peoples, M. B., & Hauggaard-Nielsen, H. (2010). Faba bean in cropping systems. *Field Crops Research*, *115*(3), 203–216.

Jensen, Erik Steen, Peoples, M. B., Boddey, R. M., Gresshoff, P. M., Hauggaard-Nielsen, H., J.R. Alves, B., & Morrison, M. J. (2012). Legumes for mitigation of climate change and the provision of feedstock for biofuels and biorefineries. A review. *Agronomy for Sustainable Development*, *32*(2), 329–364.

Jiménez-Zurdo, J. I., Frugier, F., Crespi, M. D., & Kondorosi, A. (2000). Expression profiles of 22 novel molecular markers for organogenetic pathways acting in alfalfa nodule development. *Molecular Plant-Microbe Interactions*, *13*(1), 96–106.

Kahn, M. L., McDermott, T. R., & Udvardi, M. K. (1998). Carbon and Nitrogen Metabolism in Rhizobia. In *The Rhizobiaceae* (pp. 461–485). Dordrecht: Springer Netherlands.

Kaló, P., Gleason, C., Edwards, A., Marsh, J., Mitra, R. M., Hirsch, S., Jakab, J., Sims, S., Long, S. R., Rogers, J., Kiss, G. B., Downie, J. A. & Oldroyd, G. E. D. (2005). Nodulation signaling in legumes requires *NSP2*, a member of the *GRAS* family of transcriptional regulators. *Science (New York, N.Y.)*, *308*(5729), 1786–1789.

Kanamori, N., Madsen, L. H., Radutoiu, S., Frantescu, M., Quistgaard, E. M. H., Miwa, H., Downie, J. A., James, E. K., Felle, H. H., Haaning, L. L., Jensen, T. H., Sato, S., Nakamura, Y., Tabata, S., Sandal, N. & Stougaard, J. (2006). A nucleoporin is required for induction of Ca^2+ spiking in legume nodule development and essential for rhizobial and fungal symbiosis. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(2), 359–364.

Kanampiu, F., Makumbi, D., Mageto, E., Omanya, G., Waruingi, S., Musyoka, P., & Ransom, J. (2018). Assessment of management options on *striga* infestation and maize grain yield in Kenya. *Weed Science*, *66*(4), 516–524.

Karlen, D. L., Varvel, G. E., Bullock, D. G., & Cruse, R. M. (1994). Crop rotations for the 21st century. *Advances in Agronomy*, *53*(C), 1–45.

Karr, D. B., Suzuki, F., Waters, J. K., & Emerich, D. W. (1990). Further Evidence for the Uniformity of the Microsymbiont Population from Soybean Nodules. *Journal of Plant Physiology*, *136*(6), 659–663.

Kaschuk, G., Kuyper, T. W., Leffelaar, P. A., Hungria, M., & Giller, K. E. (2009). Are the rates of photosynthesis stimulated by the carbon sink strength of rhizobial and arbuscular mycorrhizal symbioses? *Soil Biology and Biochemistry*, *41*(6), 1233–1244.

Keyser, H. H., & Li, F. (1992). Potential for increasing biological nitrogen fixation in soybean. *Plant and Soil*, *141*(1–2), 119–135.

Kim, S. K., Adetimirin, V. O., Thé, C., & Dossou, R. (2002). Yield losses in maize due to *Striga hermonthica* in West and Central Africa. *International Journal of Pest Management*, *48*(3), 211–217.

King, C. A., & Purcell, L. C. (2001). Soybean Nodule Size and Relationship to Nitrogen Fixation Response to Water Deficit. *Crop Science*, *41*(4), 1099–1107.

King, C. A., & Purcell, L. C. (2005). Inhibition of N_2 fixation in soybean is associated with elevated ureides and amino acids. *Plant Physiology*, *137*(4), 1389–1396.

Kirkegaard, J. A., & Ryan, M. H. (2014). Magnitude and mechanisms of persistent crop sequence effects on wheat. *Field Crops Research*, *164*(1), 154–165.

Kirkegaard, J., Christen, O., Krupinsky, J., & Layzell, D. (2008). Break crop benefits in temperate wheat production. *Field Crops Research*, *107*(3), 185–195.

Kleinert, A., Le Roux, M., Kang, Y., & Valentine, A. J. (2017). Oxygen and the regulation of N_2 fixation in legume nodules under P scarcity. In *Legume Nitrogen Fixation in Soils with Low Phosphorus Availability: Adaptation and Regulatory Implication* (pp. 97–109). Springer International Publishing.

Köpke, U., & Nemecek, T. (2010). Ecological services of faba bean. *Field Crops Research*, *115*(3), 217–233.

Koprna, R., De Diego, N., Dundálková, L., & Spíchal, L. (2016). Use of cytokinins as agrochemicals. *Bioorganic & Medicinal Chemistry*, *24*(3), 484–492.

Kouchi, H., Shimomura, K., Hata, S., Hirota, A., Wu, G. J., Kumagai, H., Tajima, S., Suganuma, N., Suzuki, A., Aoki, T., Hayashi, M., Yokoyama, T., Ohyama, T., Asamizu, E., Kuwata, C., Shibata, D. & Tabata, S. (2004). Large-scale analysis of gene expression profiles during early stages of root nodule formation in a model legume, *Lotus japonicus*. *DNA Research*, *11*(4), 263–274.

Kühling, I., Hüsing, B., Bome, N., & Trautz, D. (2018). Soybeans in high latitudes: effects of *Bradyrhizobium* inoculation in Northwest Germany and southern West Siberia. *Organic Agriculture*, *8*(2), 159–171.

Kumar, A., Droby, S., White, J. F., Singh, V. K., Singh, S. K., Zhimo, V. Y., & Biasi, A. (2020). Endophytes and seed priming: agricultural applications and future prospects. *Microbial Endophytes*. January 107-124.

Kumudini, S., Hume, D. J., & Chu, G. (2002). Genetic Improvement in Short-Season Soybeans: II. Nitrogen Accumulation, Remobilization, and Partitioning. *Crop Science*, *42*(1), 141–145.

Kurasch, A. K., Hahn, V., Leiser, W. L., Vollmann, J., Schori, A., Bétrix, C., Mayr, B., Winkler, J., Mechtler, K., Aper, J., Sudaric, A., Pejic, I., Sarcevic, H., Jeanson, P., Balko, C., Signor, M., Miceli, F., Strijk, P., Rietman, H., Muresanu, E., Djordjevic, V., Pospišil, A., Barion, G., Weigold, P., Streng, S., Krön, M. & Würschum, T. (2017). Identification of mega-environments in Europe and effect of allelic variation at maturity E loci on adaptation of European soybean. *Plant, Cell & Environment*, *40*(5), 765–778.

Ladrera, R., Marino, D., Larrainzar, E., Gonzalez, E. M., & Arrese-Igor, C. (2007). Reduced carbon availability to bacteroids and elevated ureides in nodules, but not in shoots, are involved in the nitrogen fixation response to early drought in soybean. *Plant Physiology*, *145*(October), 539–546.

Lam, S. K., Chen, D., Norton, R., Armstrong, R., & Mosier, A. R. (2012). Nitrogen dynamics in grain crop and legume pasture systems under elevated atmospheric carbon dioxide concentration: A meta-analysis. *Global Change Biology*, *18*(9), 2853–2859.

Lee, K. H., & La Rue, T. A. (1992). Exogenous ethylene inhibits nodulation of *Pisum sativum* L. cv sparkle. *Plant Physiology*, *100*(4), 1759–1763.

Leffel, R. C., Cregan, P. B., Bolgiano, A. P., & Thibeau, D. J. (1992). Nitrogen Metabolism of Normal and High-Seed-Protein Soybean. *Crop Science*, *32*(3), 747–750.

Legros, T., & Smith, D. L. (1994). Root zone temperature sensitivity of nitrogen fixing and nitrate-supplied soybean (*Glycine max* (L.) Merr. cv maple arrow) and lupin (*Lupinus albus L.* cv ultra) plants. *Environmental and Experimental Botany*, *34*(2), 117–127.

Lemke, R. L., Zhong, Z., Campbell, C. A., & Zentner, R. (2007). Can pulse crops play a role in mitigating greenhouse gases from North American agriculture? *Agronomy Journal*, *99*(6), 1719–1725.

Liang, Y., Mitchell, D. M., & Harris, J. M. (2007). Abscisic acid rescues the root meristem defects of the *Medicago truncatula latd* mutant. *Developmental Biology*, *304*(1), 297–307.

Lievens, S., Goormachtig, S., Den Herder, J., Capoen, W., Mathis, R., Hedden, P., & Holsters, M. (2005). Gibberellins Are Involved in Nodulation of *Sesbania rostrata*. *Plant Physiology*, *139*(3), 1366–1379.

Lindemann, W. C., & Ham, G. E. (1979). Soybean Plant Growth, Nodulation, and Nitrogen Fixation as Affected by Root Temperature. *Soil Science Society of America Journal*, *43*(6), 1134–1137.

Lira, M. D. A., & Smith, D. L. (2000). Use of a standard TWAIN scanner and software for nodule number determination on different legume species. *Soil Biology and Biochemistry*, *32*(10), 1463–1467.

Liu, F., Jensen, C., & M., A. (2004). Pod Set Related to Photosynthetic Rate and Endogenous ABA in Soybeans Subjected to Different Water Regimes and Exogenous ABA and BA at Early Reproductive Stages. *Annals of Botany*, *94*(3), 405–411.

Liu, W., Kohlen, W., Lillo, A., den Camp, R. O., Ivanov, S., Hartog, M., Limpens, E., Jamil, M., Smaczniak, C., Kaufmann, K., Yang, W. C., Hooiveld, G. J. E. J., Charnikhova, T., Bouwmeester, H. J., Bisseling, T. & Geurts, R. (2011). Strigolactone biosynthesis in *Medicago truncatula* and rice requires the symbiotic *GRAS*-type transcription factors *NSP1* and *NSP2*. *Plant Cell*, *23*(10), 3853–3865.

Livingston, D., Tuong, T., Nogueira, M., & Sinclair, T. (2019). Three-dimensional reconstruction of soybean nodules provides an update on vascular structure. *American Journal of Botany*, *106*(3), 507–513.

Lohar, D. P., Schaff, J. E., Laskey, J. G., Kieber, J. J., Bilyeu, K. D., & Bird, D. M. K. (2004). Cytokinins play opposite roles in lateral root formation, and nematode and Rhizobial symbioses. *Plant Journal*, *38*(2), 203–214.

Lorenzo, O., Piqueras, R., Sánchez-Serrano, J. J., & Solano, R. (2003). *ETHYLENE RESPONSE FACTOR1* integrates signals from ethylene and jasmonate pathways in plant defense. *Plant Cell*, *15*(1), 165–178.

Lorteau, M.-A., Ferguson, B. J., & Guinel, F. C. (2001). Effects of cytokinin on ethylene production and nodulation in pea (*Pisum sativum*) cv. Sparkle. *Physiologia Plantarum*, *112*(3), 421–428.

Loureiro, M. D. F., Kaschuk, G., Alberton, O., & Hungria, M. (2007). Soybean *[Glycine max (L.)* Merrill] rhizobial diversity in Brazilian oxisols under various soil, cropping, and inoculation managements. *Biology and Fertility of Soils*, *43*(6), 665–674.

Lynch, D. H., & Smith, D. L. (1993). Early seedling and seasonal N_2-fixing symbiotic activity of two soybean (*Glycine max* (L.) Merr.) cultivars inoculated with *Bradyrhizobium* strains of diverse origin. *Plant and Soil*, *157*(2), 289–303.

Mabood, F., & Smith, D. L. (2005). Pre-incubation of *Bradyrhizobium japonicum* with jasmonates accelerates nodulation and nitrogen fixation in soybean (*Glycine max*) at optimal and suboptimal root zone temperatures. *Physiologia Plantarum*, *125*(3), 311–323.

Mabood, F., Zhou, X., Lee, K.-D., & Smith, D. L. (2006). Methyl jasmonate, alone or in combination with genistein, and *Bradyrhizobium japonicum* increases soybean (*Glycine max* L.) plant dry matter production and grain yield under short season conditions. *Field Crops Research*, *95*(2–3), 412–419.

Maekawa, T., Maekawa-Yoshikawa, M., Takeda, N., Imaizumi-Anraku, H., Murooka, Y., & Hayashi, M. (2009). Gibberellin controls the nodulation signaling pathway in *Lotus japonicus*. *Plant Journal*, *58*(2), 183–194.

Mahon, J. D. (1977). Root and Nodule Respiration in Relation to Acetylene Reduction in Intact Nodulated Peas. *Plant Physiology*, *60*(6), 812–816.

Malik, N. S. A., Pence, M. K., Calvert, H. E., & Bauer, W. D. (1984). *Rhizobium* Infection and Nodule Development in Soybean Are Affected by Exposure of the Cotyledons to Light . *Plant Physiology*, *75*(1), 90–94.

Mapope, N., & Dakora, F. D. (2016). N_2 fixation, carbon accumulation, and plant water relations in soybean (*Glycine max* L. Merrill) varieties sampled from farmers' fields in South Africa, measured using 15N and 13C natural abundance. *Agriculture, Ecosystems & Environment*, *221*, 174–186.

Marchal, K., Vanderleyden, J., & Janssens, F. A. (2000). The "oxygen paradox" of dinitrogen-fixing bacteria. *Biology and Fertility of Soils*, *30*(5–6), 363–373.

Marhavý, P., Duclercq, J., Weller, B., Feraru, E., Bielach, A., Offringa, R., Friml, J., Schwechheimer, C., Murphy, A. & Benková, E. (2014). Cytokinin controls polarity of *PIN1*-dependent Auxin transport during lateral root organogenesis. *Current Biology*, *24*(9), 1031–1037.

Martinez-Abarca, F., Herrera-Cervera, J. a, Bueno, P., Sanjuan, J., Bisseling, T., & Olivares, J. (1998). Involvement of salicylic acid in the establishment of the *Rhizobium meliloti* - Alfalfa symbiosis. *Molecular Plant-Microbe Interactions*, *11*(2), 153–155.

Mason, S., Galusha, T., & Kmail, Z. (2016). Soybean yield and nodulation response to crop history and inoculation. *Agronomy Journal*, *108*(1), 309–312.

Mastrodomenico, A. T., & Purcell, L. C. (2012). Soybean Nitrogen Fixation and Nitrogen Remobilization during Reproductive Development. *Crop Science*, *52*(3), 1281–1289.

Masuda, T., & Goldsmith, P. D. (2009). World soybean production: Area harvested, yield, and long-term projections. *International Food and Agribusiness Management Review*, 12(4), 143–162.

Materon, L. A. (1994). Delayed inoculation and competition of *Rhizobium meliloti* in annual *Medicago* species. *Applied Soil Ecology*, *1*(4), 255–260.

Mathesius, U. (2008). Auxin: at the root of nodule development? *Functional Plant Biology*, *35*(8), 651.

Mathesius, U., Charon, C., Rolfe, B. G., Kondorosi, A., & Crespi, M. (2000). Temporal and Spatial Order of Events During the Induction of Cortical Cell Divisions in White Clover by *Rhizobium leguminosarum* bv. *trifolii* Inoculation or Localized Cytokinin Addition. *Molecular Plant-Microbe Interactions*, *13*(6), 617–628.

Mathesius, U., Schlaman, H. R. M., Spaink, H. P., Sautter, C., Rolfe, B. G., & Djordjevic, M. A. (1998). Auxin transport inhibition precedes root nodule formation in white clover roots and is regulated by flavonoids and derivatives of chitin oligosaccharides. *Plant Journal*, *14*(1), 23–34.

Mathews, A., Carroll, B. J., & Gresshoff, P. M. (1989). Development of *Bradyrhizobium* infections in supernodulating and non-nodulating mutants of soybean (*Glycine max* L. Merrill). *Protoplasma*, *150*(1), 40–47.

Matthews, D. J., & Hayes, P. (1982). Effect of root zone temperature on early growth, nodulation and nitrogen fixation in soya beans. *The Journal of Agricultural Science*, *98*(2), 371–376.

McAdam, E. L., Reid, J. B., & Foo, E. (2018). Gibberellins promote nodule organogenesis but inhibit the infection stages of nodulation. *Journal of Experimental Botany*, *69*(8), 2117–2130.

McDermott, T. R., & Graham, P. H. (1989). *Bradyrhizobium japonicum* Inoculant Mobility, Nodule Occupancy, and Acetylene Reduction in the Soybean Root System. *Applied and Environmental Microbiology*, *55*(10), 2493–2498.

McKenzie, H., & Wallace, H. (1954). The Kjeldahl determination of Nitrogen: A critical study of digestion conditions-Temperature, Catalyst, and Oxidizing agent. *Australian Journal of Chemistry*, *7*(1), 55.

McLearn, N., & Dong, Z. (2002). Microbial nature of the hydrogen-oxidizing agent in hydrogen-treated soil. *Biology and Fertility of Soils*, *35*(6), 465–469.

McLoughlin, T. J., Hearn, S., & Alt, S. G. (1990). Competition for nodule occupancy of introduced *Bradyrhizobium japonicum* strains in a Wisconsin soil with a low indigenous *bradyrhizobia* population. *Canadian Journal of Microbiology*, *36*(12), 839–845.

McPhee, K. (2005). Variation for seedling root architecture in the core collection of pea germplasm. *Crop Science*, *45*(5), 1758–1763.

Mendes, I. C., Hungria, M., & Vargas, M. A. T. (2004). Establishment of *Bradyrhizobium japonicum* and *B. elkanii* strains in a Brazilian Cerrado oxisol. *Biology and Fertility of Soils*, *40*(1), 28–35.

Mens, C., Li, D., Haaima, L. E., Gresshoff, P. M., & Ferguson, B. J. (2018). Local and Systemic Effect of Cytokinins on Soybean Nodulation and Regulation of Their Isopentenyl Transferase (*IPT*) Biosynthesis Genes Following Rhizobia Inoculation. *Frontiers in Plant Science*, *9*, 1150.

Middleton, P. H., Jakab, J., Penmetsa, R. V., Starker, C. G., Doll, J., Kalo, P., Prabhu, R., Marsh, J. F., Mitra, R. M., Kereszt, A., Dudas, B., Vanden Bosch, K., Long, S. R., Cook, D. R., Kiss, G. B. & Oldroyd, G. E. D. (2007). An *ERF* Transcription Factor in *Medicago truncatula* That Is Essential for Nod Factor Signal Transduction. *The Plant Cell Online*, *19*(4), 1221–1234.

Millar, A. H., Day, D. A., & Bergersen, F. J. (1995). Microaerobic respiration and oxidative phosphorylation by soybean nodule mitochondria: implications for nitrogen fixation. *Plant, Cell and Environment*, *18*(7), 715–726.

Miller, P. R., Gan, Y., McConkey, B. G., & McDonald, C. L. (2003). Pulse crops for the northern Great Plains: I. Grain productivity and residual effects on soil water and nitrogen. *Agronomy Journal*, *95*(4), 972–979.

Mishra, P. K., Mishra, S., Selvakumar, G., Kundu, S., & Shankar Gupta, H. (2009). Enhanced soybean (*Glycine max* L.) plant growth and nodulation by *Bradyrhizobium japonicum*- SB1 in presence of *Bacillus thuringiensis*- KR1. *Acta Agriculturae Scandinavica, Section B - Plant Soil Science*, *59*(2), 189–196.

Moretti, L. G., Lazarini, E., Bossolani, J. W., Parente, T. L., Caioni, S., Araujo, R. S., & Hungria, M. (2018). Can additional inoculations increase soybean nodulation and grain yield? *Agronomy Journal*, *110*(2), 715–721.

Mortier, V., Wasson, A., Jaworek, P., De Keyser, A., Decroos, M., Holsters, M., Tarkowski, P., Mathesius, U. & Goormachtig, S. (2014). Role of *LONELY GUY* genes in indeterminate nodulation on *Medicago truncatula*. *New Phytologist*, *202*(2), 582–593.

Murray, J. D., Karas, B. J., Sato, S., Tabata, S., Amyot, L., & Szczyglowski, K. (2007). A Cytokinin Perception Mutant Colonized by *Rhizobium* in the Absence of Nodule Organogenesis. *Science*, 315(5808), 101–104.

Naeem, M., Bhatti, I., Ahmad, R. H., & Ashraf, M. Y. (2004). Effect of some growth hormones (GA3, IAA and kinetin) on the morphology and early or delayed initiation of bud of lentil (*Lens culinaris medik*). *Pakistan Journal of Botany*, *36*(4), 801–809.

Nakagawa, T., & Kawaguchi, M. (2006). Shoot-applied MeJA suppresses root nodulation in *Lotus japonicus*. *Plant and Cell Physiology*, *47*(1), 176–180.

Nazir, R., Semenov, A. V, Sarigul, N., & Elsas, J. D. van. (2013). Bacterial community establishment in native and non-native soils and the effect of fungal colonization. *Microbiology Discovery*, *1*(1), 8.

Nellemann, C., MacDevette, M., Manders, T., Eickhout, B., Svihus, B., Prins, A. G., Kaltenborn, B. P. (2009). The Environmental Food Crisis: the environment's role in averting future food crises. GRID Arendal. Retrieved from www.grida.no (accessed 20 Aug. 2019).

Nemecek, T., von Richthofen, J. S., Dubois, G., Casta, P., Charles, R., & Pahl, H. (2008). Environmental impacts of introducing grain legumes into European crop rotations. *European Journal of Agronomy*, *28*(3), 380–393.

Neo, H. H., & Layzell, D. B. (1997). Phloem Glutamine and the Regulation of O_2 Diffusion in Legume Nodules. *Plant Physiology*, *113*(1), 259–267.

Neves, M. C. P., Didonet, A. D., Duque, F. F., & Doöbereiner, J. (1985). *Rhizobium* strain effects on nitrogen transport and distribution in soybeans. *Journal of Experimental Botany*, *36*(8), 1179–1192.

Newcomb, W., Sippell, D., & Peterson, R. L. (1979). The early morphogenesis of *Glycine max* and *Pisum sativum* root nodules . *Canadian Journal of Botany*, *57*(23), 2603–2616.

Ng, J. L. P., Hassan, S., Truong, T. T., Hocart, C. H., Laffont, C., Frugier, F., & Mathesiusa, U. (2015). Flavonoids and auxin transport inhibitors rescue symbiotic nodulation in the *Medicago truncatula* cytokinin perception mutant *cre1*. *Plant Cell*, *27*(8), 2210–2226.

Nicolás, M., Arrabal Arias, C., & Hungria, M. (2002). Genetics of nodulation and nitrogen fixation in Brazilian soybean cultivars. *Biology and Fertility of Soils*, *36*(2), 109–117.

Nikolić, R., Mitić, N., Miletić, R., & Nešković, M. (2006). Effects of Cytokinins on In Vitro Seed Germination and Early Seedling Morphogenesis in *Lotus corniculatus L*. *Journal of Plant Growth Regulation*, *25*(3), 187–194.

Nonokawa, K., Kokubun, M., Nakajima, T., Nakamura, T., & Yoshida, R. (2007). Roles of Auxin and Cytokinin in Soybean Pod Setting. *Plant Production Science*, *10*(2), 199–206.

Novoa, R., & Loomis, R. S. (1981). Nitrogen and plant production. *Plant and Soil*, *58*(1–3), 177–204.

Nukui, N., Ezura, H., & Minamisawa, K. (2004). Transgenic *Lotus japonicus* with an ethylene receptor gene *Cm-ERS1/H70A* enhances formation of infection threads and nodule primordia. *Plant and Cell Physiology*, *45*(4), 427–435.

Nuruzzaman, M., Lambers, H., Bolland, M. D. A., & Veneklaas, E. J. (2005). Phosphorus benefits of different legume crops to subsequent wheat grown in different soils of Western Australia. *Plant and Soil*, *271*(1–2), 175–187.

Ohyama, T. (1983). Comparative studies on the distribution of nitrogen in soybean plants supplied with N_2 and NO_3 - at the pod filling stage. *Soil Science and Plant Nutrition*, *29*(2), 133–145.

Oldroyd, G. E. D. (2007). Nodules and Hormones. *Science*, *315*(5808).

Oldroyd, G. E. D. (2013). Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants. *Nature Reviews Microbiology*, 11(4), 252–263.

Oldroyd, G. E. D., Mitra, R. M., Wais, R. J., & Long, S. R. (2001). Evidence for structurally specific negative feedback in the Nod factor signal transduction pathway. *The Plant Journal*, *28*(2), 191–199.

Oldroyd, G. E., Engstrom, E. M., & Long, S. R. (2001). Ethylene inhibits the Nod factor signal transduction pathway of *Medicago truncatula*. *The Plant Cell*, *13*(8), 1835–1849.

Osterholz, W. R., Rinot, O., Liebman, M., & Castellano, M. J. (2017). Can mineralization of soil organic nitrogen meet maize nitrogen demand? *Plant and Soil*, *415*(1–2), 73–84.

Ott, T., Van Dongen, J. T., Günther, C., Krusell, L., Desbrosses, G., Vigeolas, H., Bock, V., Czechowski, T., Geigenberger, P. & Udvardi, M. K. (2005). Symbiotic leghemoglobins are crucial for nitrogen fixation in legume root nodules but not for general plant growth and development. *Current Biology*, *15*(6), 531–535.

Ouertani, K., Washington, E., Lage, P., Kantartzi, S. K., Lightfoot, D. A., & Kassem, M. A. (2011). Comparison of Early and Conventional Soybean Production Systems for Yield and other Agronomic Traits. *Atlas Journal of Plant Biology*, *1*(1), 1–5.

Pacios-Bras, C., Schlaman, H. R. M., Boot, K., Admiraal, P., Langerak, J. M., Stougaard, J., & Spaink, H. P. (2003). Auxin distribution in Lotus japonicus during root nodule development. *Plant Molecular Biology*, *52*(6), 1169–1180.

Payakapong, W., Tittabutr, P., Teaumroong, N., & Boonkerd, N. (2004). Soybean cultivars affect nodulation competition of *Bradyrhizobium japonicum* strains. *World Journal of Microbiology and Biotechnology*, *20*(3), 311–315.

Pazdernik, D. L., Graham, P. H., Vance, C. P., & Orf, J. H. (1996). Host Genetic Variation in the Early Nodulation and Dinitrogen Fixation of Soybean. *Crop Science*, *36*(5), 1102.

Penmetsa, R. V., & Cook, D. R. (1997). A legume ethylene-insensitive mutant hyperinfected by its rhizobial symbiont. *Science*, *275*(5299), 527–530.

Peoples, M. B., Brockwell, J., Herridge, D. F., Rochester, I. J., Alves, B. J. R., Urquiaga, S., Boddey, R. M., Dakora, F. D., Bhattarai, S., Maskey, S. L., Sampet, C., Rerkasem, B., Khan, D. F., Hauggaard-Nielsen, H. & Jensen, E. S. (2009). The contributions of nitrogen-fixing crop legumes to the productivity of agricultural systems. *Symbiosis*, *48*(1–3), 1–17.

Peoples, M. B., Ladha, J. K., & Herridge, D. F. (1995). Enhancing legume N_2 fixation through plant and soil management. *Plant and Soil*, *174*(1–2), 83–101.

Peoples, Mark B., & Craswell, E. T. (1992). Biological nitrogen fixation: Investments, expectations and actual contributions to agriculture. *Plant and Soil*, *141*(1–2), 13–39.

Pérez-Pizá, M. C., Cejas, E., Zilli, C., Prevosto, L., Mancinelli, B., Santa-Cruz, D., Yannarelli, G. & Balestrasse, K. (2020). Enhancement of soybean nodulation by seed treatment with non–thermal plasmas. *Scientific Reports*, *10*(1), 4917.

Phillips, D. A., & DeJong, T. M. (1984). Dinitrogen Fixation in Leguminous Crop Plants (pp. 119–132). John Wiley & Sons, Ltd.

Phillips, D. A., & Torrey, J. G. (1972). Studies on Cytokinin Production by *Rhizobium*. *Plant Physiology*, *49*(1), 11–15.

Pierce, M., & Bauer, W. D. (1983). A rapid regulatory response governing nodulation in soybean. *Plant Physiology*, *73*(2), 286–290.

Pii, Y., Crimi, M., Cremonese, G., Spena, A., & Pandolfini, T. (2007). Auxin and nitric oxide control indeterminate nodule formation. *BMC Plant Biology*, *7*(1), 21.

Piotrowska-Niczyporuk, A., & Bajguz, A. (2014). The effect of natural and synthetic auxins on the growth, metabolite content and antioxidant response of green alga *Chlorella vulgaris* (*Trebouxiophyceae*). *Plant Growth Regulation*, *73*(1), 57–66.

Pitumpe Arachchige, P. S., Rosso, L. H. M., Hansel, F. D., Ramundo, B., Torres, A. R., Asebedo, R., Ciampitti, I. A. & Jagadish, S. V. K. (2020). Temporal biological nitrogen fixation pattern in soybean inoculated with *Bradyrhizobium*. *Agrosystems, Geosciences & Environment*, *3*(1), 2–11.

Plet, J., Wasson, A., Ariel, F., Le Signor, C., Baker, D., Mathesius, U., Crespi, M. & Frugier, F. (2011). *MtCRE1*-dependent cytokinin signaling integrates bacterial and plant cues to coordinate symbiotic nodule organogenesis in *Medicago truncatula*. *Plant Journal*, *65*(4), 622–633.

Podlešáková, K., Fardoux, J., Patrel, D., Bonaldi, K., Novák, O., Strnad, M., Giraud, E., Spíchal, L. & Nouwen, N. (2013). Rhizobial Synthesized Cytokinins Contribute to But Are Not Essential for the Symbiotic Interaction Between Photosynthetic Bradyrhizobia and Aeschynomene Legumes. *Molecular Plant-Microbe Interactions*, *26*(10), 1232–1238.

Popp, C., & Ott, T. (2011). Regulation of signal transduction and bacterial infection during root nodule symbiosis. *Current Opinion in Plant Biology*, *14*(4), 458–467.

Posadas, L. G., Eskridge, K. M., & Graef, G. L. (2014). Elite performance for grain yield from unadapted exotic soybean germplasm in three cycles of a recurrent selection experiment. *Crop Science*, *54*(6), 2536–2546.

Poustini, K., Mabood, F., & Smith, D. L. (2005). Low root zone temperature effects on bean (*Phaseolus vulgaris* L.) plants inoculated with *Rhizobium leguminosarum* bv. *phaseoli* pre-incubated with methyl jasmonate and/or genistein. *Acta Agriculturae Scandinavica, Section B - Soil & Plant Science*, *55*(4), 293–298.

Pradhan, D., Sinclair, T. R., & Alijani, K. (2018). Nitrogen Fixation Establishment during Initial Growth of Grain Legume Species. *Journal of Crop Improvement*, *32*(1), 50–58.

Preissel, S., Reckling, M., Schläfke, N., & Zander, P. (2015). Magnitude and farm-economic value of grain legume pre-crop benefits in Europe: A review. *Field Crops Research*, *175*, 64–79.

Puppo, A., Groten, K., Bastian, F., Carzaniga, R., Soussi, M., Lucas, M. M., De Felipe, M. R., Harrison, J., Vanacker, H. & Foyer, C. H. (2004). Legume nodule senescence: roles for redox and hormone signalling in the orchestration of the natural aging process. *New Phytologist*, *165*(3), 683–701.

Purcell, L. C., De Silva, M., King, C. A., & Kim, W. H. (1997). Biomass accumulation and allocation in soybean associated with genotypic differences in tolerance of nitrogen fixation to water deficits. *Plant and Soil*, *196*(1), 101–113.

Purcell, L. C., Salmeron, M., & Ashlock, L. (2014). Soybean Growth and Development - Chapter 2 Arkansas Soybean Production Handbook - MP197. In *Soybean Growth and Development* (pp. 2–9).

Pypers, P., Huybrighs, M., Diels, J., Abaidoo, R., Smolders, E., & Merckx, R. (2007). Does the enhanced P acquisition by maize following legumes in a rotation result from improved soil P availability? *Soil Biology and Biochemistry*, *39*(10), 2555–2566.

Rahman, M. M., Hampton, J. G., & Hill, M. J. (2005). The effect of time of sowing on soybean seed quality. *Seed Science and Technology*, *33*(3), 687–697.

Rainbird, R. M., Hitz, W. D., & Hardy, R. W. F. (1984). Experimental Determination of the Respiration Associated with Soybean/ *Rhizobium* Nitrogenase Function, Nodule Maintenance, and Total Nodule Nitrogen Fixation . *Plant Physiology*, *75*(1), 49–53.

Rattalino Edreira, J. I., Mourtzinis, S., Azzari, G., Andrade, J. F., Conley, S. P., Lobell, D., Specht, E. J. & Grassini, P. (2020). From sunlight to seed: Assessing limits to solar radiation capture and conversion in agro-ecosystems. *Agricultural and Forest Meteorology*, *280*(October 2019), 107775.

Ray, D. K., Mueller, N. D., West, P. C., & Foley, J. A. (2013). Yield Trends Are Insufficient to Double Global Crop Production by 2050. *PLoS ONE*, 8(6).

Reckling, M., Hecker, J. M., Bergkvist, G., Watson, C. A., Zander, P., Schläfke, N., Stoddard, F. L., Eory, V., Topp, C. F. E., Maire, J. & Bachinger, J. (2016). A cropping system assessment framework—Evaluating effects of introducing legumes into crop rotations. *European Journal of Agronomy*, *76*, 186–197.

Redmond, J. W., Batley, M., Innes, R. W., Kuempel, P. L., Djordjevic, M. A., & Rolfe, B. G. (1986). Flavones Induce Expression of the Nodulation Genes in *Rhizobium*. In *Recognition in Microbe-Plant Symbiotic and Pathogenic Interactions* (pp. 115–121). Berlin, Heidelberg: Springer Berlin Heidelberg.

Redondo, F. J., De La Peña, T. C., Morcillo, C. N., Lucas, M. M., & Pueyo, J. J. (2009). Overexpression of flavodoxin in bacteroids induces changes in antioxidant metabolism leading to delayed senescence and starch accumulation in alfalfa root nodules. *Plant Physiology*, *149*(2), 1166–1178.

Reid, D. E., Ferguson, B. J., Hayashi, S., Lin, Y. H., & Gresshoff, P. M. (2011). Molecular mechanisms controlling legume autoregulation of nodulation. *Annals of Botany*, *108*(5), 789–795.

Rembon, F. S., & MacKenzie, A. F. (1997). Soybean nitrogen contribution to corn and residual nitrate under conventional tillage and no-till. *Canadian Journal of Soil Science*, 77(4), 543–551.

Remmler, L., Clairmont, L., Rolland-Lagan, A. G., & Guinel, F. C. (2014). Standardized mapping of nodulation patterns in legume roots. *New Phytologist*, *202*(3), 1083–1094.

Richardson, A. E., Hocking, P. J., Simpson, R. J., & George, T. S. (2009). Plant mechanisms to optimise access to soil phosphorus. *Crop and Pasture Science*, *60*(2), 124–143.

Riely, B. K., Lougnon, G., Ané, J. M., & Cook, D. R. (2007). The symbiotic ion channel homolog *DMI1* is localized in the nuclear membrane of *Medicago truncatula* roots. *Plant Journal*, *49*(2), 208–216.

Riggle, B. D., Wiebold, W. J., & Kenworthy, W. J. (1984). Effect of Photosynthate Source-Sink Manipulation on Dinitrogen Fixation of Male-Fertile and Male-Sterile Soybean Isolines 1. *Crop Science*, *24*(1), 5–8.

Robertson, G. P., & Vitousek, P. M. (2009). Nitrogen in Agriculture: Balancing the Cost of an Essential Resource. *Annual Review of Environment and Resources*, *34*(1), 97–125.

Robson, R. L., & Postgate, J. R. (1980). Oxygen and Hydrogen in Biological Nitrogen Fixation. *Annual Review of Microbiology*, *34*(1), 183–207.

Rockström, J., Steffen, W., Noone, K., Persson, Å., Chapin, F. S., Lambin, E. F., Lenton, T. M., Scheffer, M., Folke, C., Schellnhuber, H. J., Nykvist, B., de Wit, C. A., Hughes, T., van der Leeuw, S., Rodhe, H., Sörlin, S., Snyder, P. K., Costanza, R., Svedin, U., Falkenmark, M., Karlberg, L., Corell, R. W., Fabry, V. J., Hansen, J., Walker, B., Liverman, D., Richardson, K., Crutzen, P. & Foley, J. A. (2009). A safe operating space for humanity. *Nature*, *461*(7263), 472–475.

Rogers, A., Ainsworth, E. A., & Leakey, A. D. B. (2009). Will elevated carbon dioxide concentration amplify the benefits of nitrogen fixation in legumes? *Plant Physiology*, *151*(3), 1009–1016.

Rosas, S., Soria, R., Correa, N., & Abdala, G. (1998). Jasmonic acid stimulates the expression of nod genes in *Rhizobium*. *Plant Molecular Biology*, *38*(6), 1161–1168.

Rotundo, J. L., Borrás, L., De Bruin, J., & Pedersen, P. (2014). Soybean Nitrogen Uptake and Utilization in Argentina and United States Cultivars. *Crop Science*, *54*(3), 1153–1165.

Royal Society of London. (2009). *Reaping the benefits: Science and the sustainable intensification of global agriculture*. *Chemical Engineer*. London. Retrieved from

https://royalsociety.org/~/media/royal_society_content/policy/publications/2009/4294967719.pdf

RStudioTeam. (2020). RStudio: Integrated Development for R. RStudio. Boston, MA: PBC. Retrieved from http://www.rstudio.com/

Rufty, T. W., Raper, C. D., & Jackson, W. A. (1981). Nitrogen assimilation, root growth and whole plant responses of soybean to root temperature, and to carbon dioxide and light in the aerial environment. *New Phytologist*, *88*(4), 607–619.

Saito, K., Yoshikawa, M., Yano, K., Miwa, H., Uchida, H., Asamizu, E., Sato, S., Tabata, S., Imaizumi-Anraku, H., Umehara, Y., Kouchi, H., Murooka, Y., Szczyglowski, K., Downie, J. A., Parniske, M., Hayashi, M. & Kawaguchi, M. (2007). *Nucleoporin85* is required for calcium spiking, fungal and bacterial symbioses, and seed production in *Lotus japonicus*. *Plant Cell*, *19*(2), 610–624.

Salmeron, M., Gbur, E. E., Bourland, F. M., Buehring, N. W., Earnest, L., Fritschi, F. B., Golden, B. R., Hathcoat, D., Lofton, J., Miller, T. D., Neely, C., Grover, S., Udeigwe, T. K., Verbree, D. A., Vories, E. D., Wiebold, W. J. & Purcell, L. C. (2014). Soybean maturity group choices for early and late plantings in the midsouth. *Agronomy Journal*, *106*(5), 1893–1901.

Salvagiotti, F., Cassman, K. G., Specht, J. E., Walters, D. T., Weiss, A., & Dobermann, A. (2008). Nitrogen uptake, fixation and response to fertilizer N in soybeans: A review. *Field Crops Research*, *108*(1), 1–13.

Santachiara, G., Borrás, L., & Rotundo, J. L. (2017). Physiological Processes Leading to Similar Yield in Contrasting Soybean Maturity Groups. *Agronomy Journal*, *109*(1), 158–167.

Santachiara, G., Salvagiotti, F., Gerde, J. A., & Rotundo, J. L. (2018). Does biological nitrogen fixation modify soybean nitrogen dilution curves? *Field Crops Research*, *223*(April), 171–178.

Santachiara, G., Salvagiotti, F., & Rotundo, J. L. (2019). Nutritional and environmental effects on biological nitrogen fixation in soybean: A meta-analysis. *Field Crops Research*, *240*(September), 106–115.

Schauser, L., Roussis, A., Stiller, J., & Stougaard, J. (1999). A plant regulator controlling development of symbiotic root nodules. *Nature*, *402*(6758), 191–195.

Schlesinger, W. H. (2009). On the fate of anthropogenic nitrogen. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(1), 203–208.

Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature Methods*, *9*(7), 671–675.

Scholte, M. (2002). *T-DNA tagging in Medicago truncatula.* Wageningen University.

Schroeder, H. (1984). Effects of Applied Growth Regulators on Pod Growth and Seed Protein Composition in *Pisum sativum* L. *Journal of Experimental Botany*, *35*(6), 813–821.

Schuize, J., Adgo, E., & Merbach, W. (1999). Carbon Costs Associated with N_2 Fixation in *Vicia faba* L. and *Pisum sativum* L. over a 14-Day Period. *Plant Biology*, *1*(6), 625–631.

Schultz, C. J., Kochian, L. V., & Harrison, M. J. (2010). Genetic variation for root architecture, nutrient uptake and mycorrhizal colonisation in *Medicago truncatula* accessions. *Plant and Soil*, *336*(1), 113–128.

Schulz, T. J., & Thelen, K. D. (2008). Soybean Seed Inoculant and Fungicidal Seed Treatment Effects on Soybean. *Crop Science*, *48*(5), 1975–1983.

Schulze, J. (2004). How are nitrogen fixation rates regulated in legumes? *Journal of Plant Nutrition and Soil Science*, *167*(2), 125–137.

Schwember, A. R., Schulze, J., del Pozo, A., & Cabeza, R. A. (2019). Regulation of Symbiotic Nitrogen Fixation in Legume Root Nodules. *Plants*, *8*(9), 333.

Seifert, C. A., Roberts, M. J., & Lobell, D. B. (2017). Continuous corn and soybean yield penalties across hundreds of thousands of fields. *Agronomy Journal*, *109*(2), 541–548.

Serraj, R., Vadez, V., Denison, R. F., & Sinclair, T. R. (1999). Involvement of Ureides in Nitrogen Fixation Inhibition in Soybean. *Plant Physiology*, *119*(January), 289–296.

Seymour, M., Kirkegaard, J. A., Peoples, M. B., White, P. F., & French, R. J. (2012). Break-crop benefits to wheat in Western Australia – insights from over three decades of research. *Crop and Pasture Science*, *63*(1), 1.

Shen, J., Yuan, L., Zhang, J., Li, H., Bai, Z., Chen, X., Zhang, W. & Zhang, F. (2011). Phosphorus dynamics: From soil to plant. *Plant Physiology*, *156*(3), 997–1005.

Shiferaw, B., Bantilan, M. C. S., & Serraj, R. (2004). Harnessing the potentials of BNF for poor farmers: Technological, policy and institutional constraints and research needs. In: (Ed.) Symbiotic Nitrogen Fixation: Prospects for Enhanced Application in Tropical Agriculture. (pp.3–27). Oxford & IBH Publishing, New Delhi.

Shiro, S., Kuranaga, C., Yamamoto, A., Sameshima-Saito, R., & Saeki, Y. (2016). Temperature-Dependent Expression of *NodC* and Community Structure of Soybean-Nodulating *Bradyrhizobia*. *Microbes and Environments*, *31*(1), 27–32.

Shtark, O. Y., Borisov, A. Y., Zhukov, V. A., Provorov, N. A., & Tikhonovich, I. A. (2010). Intimate associations of beneficial soil microbes with host plants. In *Soil Microbiology and Sustainable Crop Production* (pp. 119–196). Springer Netherlands.

Sinclair, T. R., & de Wit, C. T. (1975). Photosynthate and Nitrogen Requirements for Seed Production by Various Crops. *Science*, *189*(4202), 565–567.

Sinclair, T.R., & Jamieson, P. D. (2006). Grain number, wheat yield, and bottling beer: An analysis. *Field Crops Research*, *98*(1), 60–67.

Sinclair, Thomas R., & Nogueira, M. A. (2018). Selection of host-plant genotype: The next step to increase grain legume N_2 fixation activity. *Journal of Experimental Botany*, *69*(15), 3523–3530.

Sindelar, A. J., Schmer, M. R., Jin, V. L., Wienhold, B. J., & Varvel, G. E. (2016). Crop rotation affects corn, grain sorghum, and Soybean yields and nitrogen recovery. *Agronomy Journal*, *108*(4), 1592–1602.

Smil, V. (1999). Detonator of the population explosion. *Nature*, *400*(6743), 415.

Smit, P. (2005). *NSP1* of the *GRAS* Protein Family Is Essential for Rhizobial Nod Factor-Induced Transcription. *Science*, *308*(5729), 1789–1791.

Smith, B. E. (2002). Nitrogenase Reveals Its Inner Secrets. *Science*, *297*(5587), 1654–1655.

Somasegaran, P., & Hoben, H. J. (1994). Handbook for Rhizobia. Handbook for Rhizobia. New York, NY: Springer New York.

Song, L., Carroll, B. J., Gresshoff, P. M., & Herridge, D. F. (1995). Field assessment of supernodulating genotypes of soybean for yield, N_2 fixation and benefit to subsequent crops. *Soil Biology and Biochemistry*, *27*(4–5), 563–569.

Spaink, H. P. (2000). Root Nodulation and Infection Factors Produced by Rhizobial Bacteria. *Annual Review of Microbiology*, *54*(1), 257–288.

Specht, J. E., Hume, D. J., & Kumudini, S. V. (1999). Soybean Yield Potential - A Genetic and Physiological Perspective. *Crop Science*, *39*(6), 1560–1570.

Specht, James E., Diers, B. W., Nelson, R. L., de Toledo, J. F. F., Torrion, J. A., & Grassini, P. (2015). Soybean (pp. 311–355). John Wiley & Sons, Ltd.

Stacey, G. (2007). The *Rhizobium*-Legume Nitrogen-Fixing Symbiosis. In *Biology of the Nitrogen Cycle* (pp. 147–163). Elsevier.

Stacey, G., McAlvin, C. B., Kim, S.Y., Olivares, J., & Soto, M. J. (2006). Effects of Endogenous Salicylic Acid on Nodulation in the Model Legumes *Lotus japonicus* and *Medicago truncatula*. *Plant Physiology*, *141*(4), 1473–1481.

Stagnari, F., Maggio, A., Galieni, A., & Pisante, M. (2017). Multiple benefits of legumes for agriculture sustainability: an overview. *Chemical and Biological Technologies in Agriculture*, *4*(1), 2.

Stein, S., Selesi, D., Schilling, R., Pattis, I., Schmid, M., & Hartmann, A. (2005). Microbial activity and bacterial composition of H_2-treated soils with net CO_2 fixation. *Soil Biology and Biochemistry*, *37*(10), 1938–1945.

Sturtevant, D. B., & Taller, B. J. (1989). Cytokinin Production by *Bradyrhizobium japonicum*. *Plant Physiology*, *89*(4), 1247–1252.

Sulieman, S., & Schulze, J. (2010). The efficiency of nitrogen fixation of the model legume *Medicago truncatula* (Jemalong A17) is low compared to *Medicago sativa*. *Journal of Plant Physiology*, *167*(9), 683–692.

Sulieman, S., & Tran, L. S. P. (2013). Asparagine: an amide of particular distinction in the regulation of symbiotic nitrogen fixation of legumes. *Critical Reviews in Biotechnology*, *33*(3), 309–327.

Sun, J., Cardoza, V., Mitchell, D. M., Bright, L., Oldroyd, G., & Harris, J. M. (2006). Crosstalk between jasmonic acid, ethylene and Nod factor signaling allows integration of diverse inputs for regulation of nodulation. *Plant Journal*, *46*(6), 961–970.

Sundaresan, V., & Ausubel, M. (1981). Nucleotide Sequence of the Gene Coding for the Nitrogenase Iron Protein from *Klebsiella pneumoniae*. *The Journal of Biological Chemistry*, 256(6), 2808–2812.

Suzaki, T., Ito, M., & Kawaguchi, M. (2013). Genetic basis of cytokinin and auxin functions during root nodule development. *Frontiers in Plant Science*, *4*(March), 1–6.

Suzuki, A., Akune, M., Kogiso, M., Imagama, Y., Osuki, K. I., Uchiumi, T., Higashi, S., Han, S. Y., Yoshida, S., Asami, T. & Abe, M. (2004). Control of nodule number by the phytohormone abscisic Acid in the roots of two leguminous species. *Plant & Cell Physiology*, *45*(7), 914–922.

Tachibana, S., Du, Y. C., Wang, Y. H., & Kitamura, F. (1997). Implication of Endogenous Cytokinins in the Growth Inhibition of Cucumber Plants by Supraoptimal Root-zone Temperature. *Journal of the Japanese Society for Horticultural Science*, 66(3–4), 549–555

Tajima, R., Lee, O. N., Abe, J., Lux, A., & Morita, S. (2007). Nitrogen-Fixing Activity of Root Nodules in Relation to Their Size in Peanut (*Arachis hypogaea* L.). *Plant Production Science*, *10*(4), 423–429.

Tassi, E., Pouget, J., Petruzzelli, G., & Barbafieri, M. (2008). The effects of exogenous plant growth regulators in the phytoextraction of heavy metals. *Chemosphere*, *71*(1), 66–73.

Tharanathan, R. N., & Mahadevamma, S. (2003). Grain legumes - A boon to human nutrition. *Trends in Food Science and Technology*, 14(12), 507–518.

Thibodeau, P. S., & Jaworski, E. G. (1975). Patterns of nitrogen utilization in the soybean. *Planta*, *127*(2), 133–147.

Thilakarathna, M. S., & Raizada, M. N. (2017). A meta-analysis of the effectiveness of diverse rhizobia inoculants on soybean traits under field conditions. *Soil Biology and Biochemistry*, *105*, 177–196.

Thomas, R. J., & Sprent, J. I. (1984). The Effects of Temperature on Vegetative and Early Reproductive Growth of a Cold-Tolerant and a Cold-Sensitive Line of *Phaseolus vulgaris* L. Nodular Uricase, Allantoinase, Xylem transport of N and Assimilation in Shoot Tissues. *Annals of Botany*, *53*(4), 589–597.

Tilman, D., Balzer, C., Hill, J., & Befort, B. L. (2011). Global food demand and the sustainable intensification of agriculture. *Proceedings of the National Academy of Sciences*, *108*(50), 20260–20264.

Tirichine, L., Sandal, N., Madsen, L. H., Radutoiu, S., Albrektsen, A. S., Sato, S., Asamizu, E., Tabata, S. & Stougaard, J. (2006). A Gain-of-Function Mutation in a Cytokinin Receptor Triggers Spontaneous Root Nodule Organogenesis. *Plant Cell Mol. Plant Microbe Interact. D. P. Lohar et Al. Plant J*, *18*(38), 2680–203.

Tjepkema, J. D., & Yocum, C. S. (1974). Measurement of oxygen partial pressure within soybean nodules by oxygen microelectrodes. *Planta*, *119*(4), 351–360.

Tolley, L. C., & Raper, C. D. (1985). Cyclic Variations in Nitrogen Uptake Rate in Soybean Plants. *Plant Physiology*, *78*(2), 320–322.

Turgeon, B. G., & Bauer, W. D. (1985). Ultrastructure of infection-thread development during the infection of soybean by *Rhizobium japonicum*. *Planta*, *163*(3), 328–349.

Turman, P. C., Wiebold, W. J., Wrather, J. A., & Tracy, P. W. (1995). Cultivar and planting date effects on soybean root growth. *Plant and Soil*, *176*(2), 235–241.

Turner, M., Nizampatnam, N. R., Baron, M., Coppin, S., Damodaran, S., Adhikari, S., Arunachalam, S. P. Yu, O. & Subramanian, S. (2013). Ectopic expression of *miR160* results in auxin hypersensitivity, cytokinin hyposensitivity, and inhibition of symbiotic nodule development in soybean. *Plant Physiology*, *162*(4), 2042–2055.

Udvardi, M. K., & Day, D. A. (1997). Metabolite transport across symbiotic membranes of legume nodules. *Annual Review of Plant Biology*, *48*(July), 493–523.

Unkovich, M., Herridge, D., Peoples, M., Cadisch, G., Boddey, B., Giller, K., Alves, B & Chalk, P. (2008). Measuring plant-associated nitrogen fixation in agricultural systems. Australian Centre for International Agricultural Research.

Unkovich, M. J., Baldock, J., & Peoples, M. B. (2010). Prospects and problems of simple linear models for estimating symbiotic N_2 fixation by crop and pasture legumes. *Plant and Soil*, *329*(1), 75–89.

Uzoh, I. M., Igwe, C. A., Okebalama, C. B., & Babalola, O. O. (2019). Legume-maize rotation effect on maize productivity and soil fertility parameters under selected agronomic practices in a sandy loam soil. *Scientific Reports*, *9*(1), 1–9.

Valentine, A. J., Benedito, V. A., & Kang, Y. (2010). Legume Nitrogen Fixation and Soil Abiotic Stress: From Physiology to Genomics and Beyond. In *Annual Plant Reviews Volume 42* (Vol. 42, pp. 207–248). Oxford, UK: Wiley-Blackwell.

Van De Velde, W., Guerra, J. C. P., De Keyser, A., De Rycke, R., Rombauts, S., Maunoury, N., Mergaert, P., Kondorosi, E., Holsters, M. & Goormachtig, S. (2006). Aging in legume symbiosis. A molecular view on nodule senescence in *Medicago truncatula*. *Plant Physiology*, *141*(2), 711–720.

van Kessel, C., & Hartley, C. (2000). Agricultural management of grain legumes: Has it led to an increase in nitrogen fixation? *Field Crops Research*, *65*(2–3), 165–181.

van Noorden, G. E., Ross, J. J., Reid, J. B., Rolfe, B. G., & Mathesius, U. (2006). Defective long-distance auxin transport regulation in the *Medicago truncatula* super numeric nodules mutant. *Plant Physiology*, *140*(4), 1494–1506.

van Zeijl, A., Op den Camp, R. H. M., Deinum, E. E., Charnikhova, T., Franssen, H., Op den Camp, H. J. M., Bouwmeester, H., Kohlen, W., Bisseling, T., & Geurts, R. (2015). *Rhizobium* Lipo-chitooligosaccharide Signaling Triggers Accumulation of Cytokinins in *Medicago truncatula* Roots. *Molecular Plant*, *8*(8), 1213–1226.

Vance, C. P., & Heichel, G. H. (1991). Carbon in N_2 fixation: Limitation or exquisite adaptation. *Annual Review of Plant Physiology and Plant Molecular Biology*, *42*(1), 373–392.

Vance, C. P., Miller, S. S., Driscoll, B. T., Robinson, D. L., Trepp, G., Gantt, J. S., & Samas, D. A. (1998). Nodule Carbon Metabolism: Organic Acids for N_2 Fixation (pp. 443–448). Springer, Dordrecht.

Vance, C. P. (2001). Symbiotic Nitrogen Fixation and Phosphorus Acquisition. Plant Nutrition in a World of Declining Renewable Resources. *Plant Physiology*, *127*(2), 390–397.

Vasilas, B. L., Nelson, R. L., Fuhrmann, J. J., & Evans, T. A. (1995). Relationship of Nitrogen Utilization Patterns with Soybean Yield and Seed-Fill Period. *Crop Science*, *35*(3), 809–813.

Vernié, T., Moreau, S., De Billy, F., Plet, J., Combier, J. P., Rogers, C., Oldroyd, G., Frugier, F., Niebel, A. & Gamas, P. (2008). *EFD* is an *ERF* transcription factor involved in the control of nodule number and differentiation in *Medicago truncatula*. *Plant Cell*, *20*(10), 2696–2713.

Vessey, J. K. (1992). Cultivar differences in assimilate partitioning and capacity to maintain N_2 fixation rate in pea during pod-filling. *Plant and Soil*, *139*(2), 185–194.

Vikman, P., & Vessey, J. K. (1993). Ontogenetic Changes in Root Nodule Subpopulations of Common Bean (*Phaseolus vulgaris* L.). *Journal of Experimental Botany*, *44*(3), 579–586. https://doi.org/10.1093/jxb/44.3.579

Vogel, J. P., Woeste, K. E., Theologis, a, & Kieber, J. J. (1998). Recessive and dominant mutations in the ethylene biosynthetic gene *ACS5* of *Arabidopsis* confer cytokinin insensitivity and ethylene overproduction, respectively. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(April), 4766–4771.

Voisin, A.-S., Guéguen, J., Huyghe, C., Jeuffroy, M. H., Magrini, M. B., Meynard, J. M., Mougel, C., Pellerin, S. & Pelzer, E. (2013). Legumes for feed, food, biomaterials and bioenergy in Europe: a review. *Agronomy for Sustainable Development*, *34*(2), 361–380.

Voisin, A. S., Salon, C., Jeudy, C., & Ware. (2003). Root and Nodule Growth in *Pisum sativum* L. in Relation to Photosynthesis: Analysis Using 13C-labelling. *Annals of Botany*, *92*(4), 557–563.

Voisin, A. S., Salon, C., Jeudy, C., & Warembourg, F. R. (2003). Symbiotic N_2 fixation activity in relation to C economy of *Pisum sativum* L. as a function of plant phenology. *Journal of Experimental Botany*, *54*(393), 2733–2744.

Vollmann, J., Walter, H., Sato, T., & Schweiger, P. (2011). Digital image analysis and chlorophyll metering for phenotyping the effects of nodulation in soybean. *Computers and Electronics in Agriculture*, *75*(1), 190–195.

von Braun, J. (2007). The World Food Situation New Driving Forces and Required Actions. International Food Policy Research Institute (IFPRI). Available: https://www.ifpri.org/publication/world-food-situation-new-driving-forces-and-required-actions. (accessed 10 Oct 2020).

Wadisirisuk, P., Danso, S. K. A., Hardarson, G., & Bowen, G. D. (1989). Influence of *Bradyrhizobium japonicum* Location and Movement on Nodulation and Nitrogen Fixation in Soybeans. *Applied and Environmental Microbiology*, *55*(7), 1711–1716.

Wais, R. J., Galera, C., Oldroyd, G., Catoira, R., Penmetsa, R. V, Cook, D., Gough, C., Denarié, J. & Long, S. R. (2000). Genetic analysis of calcium spiking responses in nodulation mutants of *Medicago truncatula*. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(24), 13407–13412.

Walch-Liu, P., Filleur, S., Gan, Y., & Forde, B. G. (2005). Signaling mechanisms integrating root and shoot responses to changes in the nitrogen supply. *Photosynthesis Research*, *83*(2), 239–250.

Walker, S. A., Viprey, V., & Downie, J. A. (2000). Dissection of nodulation signaling using pea mutants defective for calcium spiking induced by Nod factors and chitin oligomers. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(24), 13413–13418.

Walsh, K. B. (1990). Vascular transport and soybean nodule function. III: Implications of a continual phloem supply of carbon and water. *Plant, Cell and Environment*, *13*(9), 893–901.

Walsh, K. B. (1995). Physiology of the legume nodule and its response to stress. *Soil Biology and Biochemistry*, *27*(4–5), 637–655.

Walsh, Kerry B., & Layzell, D. B. (1986). Carbon and Nitrogen Assimilation and Partitioning in Soybeans Exposed to Low Root Temperatures. *Plant Physiology*, *80*(1), 249–255.

Wang, Y., Marschner, P., & Zhang, F. (2012). Phosphorus pools and other soil properties in the rhizosphere of wheat and legumes growing in three soils in monoculture or as a mixture of wheat and legume. *Plant and Soil*, *354*(1–2), 283–298.

Weaver, R. W., & Frederick, L. R. (1974). Effect of Inoculum Rate on Competitive Nodulation of *Glycine max* L. Merrill. II. Field Studies. *Agronomy Journal*, *66*(2), 233–236.

Weisz, P. R., & Sinclair, T. R. (1988). A rapid non-destructive assay to quantify soybean nodule gas permeability. *Plant and Soil*, *105*(1), 69–78.

Wolyn, D. J., Attewell, J., Ludden, P. W., & Bliss, F. A. (1989). Indirect measures of N_2 fixation in common bean (*Phaseolus vulgaris* L.) under field conditions: The role of lateral root nodules. *Plant and Soil*, *113*(2), 181–187.

Wu, T., Schoenau, J. J., Li, F., Qian, P., Malhi, S. S., & Shi, Y. (2003). Effect of tillage and rotation on organic carbon forms of chernozemic soils in Saskatchewan. *Journal of Plant Nutrition and Soil Science*, *166*(3), 328–335.

Xu, G., Fan, X., & Miller, A. J. (2012). Plant Nitrogen Assimilation and Use Efficiency. *Annual Review of Plant Biology*, *63*(1), 153–182.

Yashima, H., Fujikake, H., Sato, T., Ohtake, N., Sueyoshi, K. & Ohyama, T. (2003). Systemic and local effects of long-term application of nitrate on nodule growth and N_2 fixation in soybean (*Glycine max* [L.] Merr.). *Soil Science and Plant Nutrition*, *49*(6), 825–834.

Yashima, Y., Kaihatsu, A., Nakajima, T., & Kokubun, M. (2005). Effects of source/sink ratio and cytokinin application on pod set in soybean. *Plant Production Science*, *8*(2), 139–144.

Yemm, E. W., Cocking, E. C., & Ricketts, R. E. (1955). The determination of amino-acids with ninhydrin. *The Analyst*, *80*(948), 209–214.

Young, G. E., & Conway, C. F. (1942). Estimation of Allantoin. *Journal of Biological Chemistry*, *142*, 839–843.

Yuan, K., Reckling, M., Ramirez, M. D. A., Djedidi, S., Fukuhara, I., Ohyama, T., Yokoyama, T., Bellingrath-Kimura, S. D., Halwani, M., Egamberdieva, D. & Ohkama-Ohtsu, N. (2020). Characterization of Rhizobia for the Improvement of Soybean Cultivation at Cold Conditions in Central Europe. *Microbes and Environments*, *35*(1), 1-13.

Zander, P., Amjath-Babu, T. S., Preissel, S., Reckling, M., Bues, A., Schläfke, N., Kuhlman, T., Bachinger, T., Uthes, S., Stoddard, F., Murphy-Bokern, D. & Watson, C. (2016). Grain legume decline and potential recovery in European agriculture: a review. *Agronomy for Sustainable Development*, *36*(2), 1-20.

Zhang, F., Lynch, D. H., & Smith, D. L. (1995). Impact of low root temperatures in soybean (*Glycine max*. (L.) Merr.) on nodulation and nitrogen fixation. *Environmental and Experimental Botany*, *35*(3), 279–285.

Zhang, F., & Smith, D. L. (1994). Effects of low root zone temperatures on the early stages of symbiosis establishment between soybean [*Glycine max* (L.) merr.] and *bradyrhizobium japonicum*. *Journal of Experimental Botany*, *45*(10), 1467–1473.

Zhang, H., Charles, T. C., Driscoll, B. T., Prithiviraj, B., & Smith, D. L. (2002). Low temperature-tolerant *Bradyrhizobium japonicum* strains allowing improved soybean yield in short-season areas. *Agronomy Journal*, *94*(4), 870–875.

Zilli, J. É., Pereira, G. M. D., França Júnior, I., Silva, K. da, Hungria, M., & Rouws, J. R. C. (2013). Dinâmica de rizóbios em solo do cerrado de Roraima durante o período de estiagem. *Acta Amazonica*, *43*(2), 153–160.

Zimmer, S., Messmer, M., Haase, T., Piepho, H. P., Mindermann, A., Schulz, H., Habekuß, A., Ordon, F., Wilbois, K. F. & Heß, J. (2016). Effects of soybean variety and *Bradyrhizobium* strains on yield, protein content and biological nitrogen fixation under cool growing conditions in Germany. *European Journal of Agronomy*, *72*, 38–46.

Zipfel, C., & Oldroyd, G. E. D. (2017). Plant signalling in symbiosis and immunity. *Nature*, *543*(7645), 328–336.

Zou, H., Zhang, N. N., Pan, Q., Zhang, J. H., Chen, J., & Wei, G. H. (2019). Hydrogen sulfide promotes nodulation and nitrogen fixation in soybean–rhizobia symbiotic system. *Molecular Plant-Microbe Interactions*, *32*(8), 972–985.

Appendices

Appendix 1.1. Centre for global Eco-innovation carbon report.

CENTRE FOR GLOBAL ECO-INNOVATION CO_2e Calculator

Name: Robert Kempster
Project: Using phytohormone interaction to improve nitrogen fixation in legumes
Industry Partner: Plant Impact
Academic Supervisors: Ian Dodd and Mariana Rufino
Description of project: This project aimed to develop a product that will lessen the environmental impact of agriculture by reducing reliance on industrially fixed nitrogen fertilisers by increasing biological nitrogen fixation (BNF). Soybean, the focus of this project, has high nitrogen (N) demand with N supply highly correlated with yield. Plant hormones are known to regulate the formation of nodules, in which BNF occurs. Signalling events leading to nodule establishment involving plant hormones have been studied, but not the effect of hormone application in enhancing BNF. As N supply is so crucial to soybean and with the environmental consequences of N fertilisers, BNF is key to allowing high but sustainable future yield.
Summary of GHG emission reduction: Agricultural production produces 12,000 mega tonnes of CO_2e per year accounting for 86% of food related anthropogenic greenhouse-gas emissions (Vermeulen, Campbell, & Ingram, 2012). These emissions are derived from a number of sources including fertiliser and crop protection manufacture and application and field operations. Relative carbon emissions can be reduced, on a per yield basis, through increasing yield for the same inputs.

Baseline carbon emissions are based on results from the AHDB Carbon Footprint Decision Tool, using data from a questionnaire completed by a Brazilian farmer (since Brazil is a key target market for Plant Impact). This was combined with results of an Argentinean field trial which tested the effectiveness of the biostimulant product developed in this project.

The potential carbon savings from a BNF biostimulant product developed in this project are the result of increased legume yield for the same carbon inputs, giving a relative reduction in carbon emissions on a per yield basis. For a Brazilian soybean farm (650 ha) case study, carbon savings are associated with relative carbon emissions (from fertilisers, agrochemicals and farm machinery) on a per yield basis. From this, the CO_2e emissions of the Plant Impact (PI) Brazilian soybean market were calculated using data on PI's share of the total yield.

GHG emissions before support 821,604 tCO_2e per annum	**Current GHG emissions** 806,883 tCO_2e per annum	**Total GHG reduction** 14,721 tCO_2e per annum	**Percentage of reduction** 1.79%
Section one – Baseline of CO_2e emissions relating to original process, service or product			
Scope one – Direct emissions from company owned and controlled operations			
Scope two – Indirect emissions purchased by company			
Scope three – Other indirect emissions from the supply chain owned and/or purchased by suppliers and consumers			

Downstream e.g. consumers (sold products)

CO_2e embodied **use of sold products**

A case study of a 650 ha farm was used to calculate the CO_2e emissions per ha, from which to calculate the CO_2e emissions of the Plant Impact (PI) Brazilian soybean market using data on PI's share of the total yield.

Emissions from fertiliser production

Although nitrogen is not applied to fields other fertilisers, superphosphate and potassium sulphate, are applied at a rate of 150kg/ha each. Combined, these give carbon emissions of 20.4 kg/ha (AHDB Carbon Tool), multiplied by 650ha case study farm gives **13.26 tCO_2e per annum**.

Emissions from agrochemical production

Application of crop protection products, i.e. pesticides (6kg active ingredient per ha) indicated by Brazilian farm case study lead to 246 kg/ha (AHDB Carbon Tool), multiplied by 650 ha gives **159.9 tCO_2e per annum**.

Emissions from agricultural machinery

Carbon emissions from field operations equate to 127.4 kg CO_2e/ha of fuel use (AHDB Carbon Tool). This includes emissions from 1 direct drilling of fertiliser (24.9 litres/ha = 66.8 kg CO_2e/ha), 9 spray applications of agrochemicals (7.2 litres/ha = 19.3 kg CO_2e/ha) and 1 combine at harvest (15.4 litres/ha = 41.3 kg CO_2e/ha) as indicated by Brazilian farm case study. The total of 127.4 kg CO_2e/ha multiplied by 650 ha gives total farm emissions of **82.81 tCO_2e per annum**.

Per farm scale:

Emissions from: Fertiliser production + Agrochemical production + Agricultural machinery

13.26 + 159.9 + 82.81 = **255.97 tCO_2e per annum**

Per yield scale:

Per ha emissions: Total farm CO_2e / number of Ha

255.97 / 650 = **0.3938 tCO_2e per ha per annum**

Per yield emissions: tCO_2e per ha / yield tonnes per ha

Yield data are from control plots in an Argentinian field trial, carried out as part of this project.

0.3938 / 4.329 = **0.09097 tCO_2e per tonne yield**

Plant Impact have indicated that they predict a 20% market share of the Latin American soybean biostimulant market which accounts for 30% of farmers.

Predicted yield of Plant Impact Brazilian soybean market (FOASTAT 2018 data):
(yield tonnes per ha x harvested area ha) x Biostimulant market x Plant impact market share

(4.329 x 34,771,690 = 150,526,646) x 0.3 x 0.2 = 9,031,599 tonnes total yield of predicted market.

Total baseline emissions:
Calculated tCO_2e per tonne yield x Predicted yield of Plant Impact Brazilian soybean market

0.09097 x 9,031,599 = **821,604 tCO_2e per annum**

Biogenic emissions – Other emissions related to flora, fauna, land and water
Total baseline emissions figure
821,604 tCO_2e per annum
Section two – Reduction of CO_2e emissions relating to new process, service or product
Scope one – Direct emissions from company owned and controlled operations
Scope two – Indirect emissions purchased by company
Scope three – Other indirect emissions from the supply chain owned and/or purchased by suppliers and consumers
Downstream e.g. consumers (sold products)
CO_2e embodied **use of sold products**

The product developed in this project decreases carbon emissions indirectly, as it is not common to apply nitrogen to Brazilian fields. Additionally, biological nitrogen fixation is known to be reduced by application of industrial fertiliser. Thus, the same increases in biological fixation from product developed here are not likely with N application. Another potential carbon saving is a reduction of nitrogen fertiliser applied to subsequent crops. However, the relative impact on subsequent crops was not tested experimentally and so will also not be included as saving associated with this project.

Carbon savings that can be attributed to this project are based on yield increases achieved through application of the product in Argentinean field trials. In this trial, yield increased with no (or negligible 76g/ha kinetin) increase in carbon input, thus relative carbon per tonne of soybean grain produced can be considered to be reduced.

Whereas control plots yielded 4.329 tonnes per ha, plots treated with the new product yielded the equivalent of 4.408 tonnes per ha.
Thus carbon emissions from the treated plots:

0.3938 tCO_2e per ha/ 4.408 tonne yield per ha = **0.08934 tCO_2e per tonne yield**

New emissions figure:
Calculated CO_2e emissions per tonne yield after treatment x Predicted yield of Plant Impact Brazilian soybean market:

0.08934 x 9,031,599 = **806,883 tCO_2e per annum**

Biogenic emissions – Other emissions related to flora, fauna, land and water

Total reduction emissions figure (savings)

Carbon baseline for Plant Impact's predicted market share based on yield of control plots is 821,604 tCO_2e per annum.

New emissions figure for Plant Impact's predicted market share based on yield of treated plots is 806,883 tCO_2e per annum.

The GHG emissions savings from this project are:

Baseline emissions - New emissions

821,604 - 806,883 = **14,721 tCO_2e per annum**

References

1. AHDB Carbon Footprint Decision Tool (available: https://ahdb.org.uk/carbon-footprint-decision-tool). Data based on results of 'Carbon baseline questionnaire' from a Brazilian soybean grower
2. Canfield, D. E., Glazer, A. N., & Falkowski, P. G. (2010). The evolution and future of earth's nitrogen cycle. *Science*, *330*(6001), 192–196. https://doi.org/10.1126/science.1186120
3. Jensen, E. S., Peoples, M. B., Boddey, R. M., Gresshoff, P. M., Henrik, H. N., Alves, B. J. R., & Morrison, M. J. (2012). *Legumes for mitigation of climate change and the provision of feedstock for biofuels and biorefineries. A review*. *Agronomy for Sustainable Development* (Vol. 32). https://doi.org/10.1007/s13593-011-0056-7
4. FOASTAT (available: http://www.fao.org/faostat/en/#home)
5. Vermeulen, S. J., Campbell, B. M., & Ingram, J. S. I. (2012). Climate Change and Food Systems. *Annual Review of Environment and Resources*, *37*(1), 195–222. https://doi.org/10.1146/annurev-environ-020411-130608

Appendix 3.1 Effect of cytokinin (kinetin) seed coat at five different concentrations (high 10^{-7} to low 10^{-11} mol L^{-1}) on plant nodule traits and plant growth with a water control. Values are averages, ±SE with results of one-way ANOVA below with model residual standard error and degrees of freedom (df).

Source of variation		Nodule number	Specific nodule number (# g^{-1})	Nodule weight (mg)	Specific nodule weight (mg)	Average nodule weight (mg)	Shoot weight (mg)	Leaf area (cm^2)	Chlorophyll content (µMol m^2)
Cytokinin	Control	56.7 ±4.58	239 ±22.8	40.1 ±2.00	168 ±8.49	0.911 ±0.103	455 ±20.4	68.8 ±3.16	223 ±6.03
	10^{-11}	58.1 ±4.63	236 ±23.0	38.2 ±2.00	158 ±8.49	0.964 ±0.103	447 ±20.4	66.1 ±3.09	217 ±5.97
	10^{-10}	53.8 ±4.58	216 ±22.8	38.9 ±2.00	159 ±8.49	0.898 ±0.103	457 ±20.4	66.2 ±3.09	224 ±5.97
	10^{-9}	57.4 ±4.58	254 ±22.8	41.8 ±2.00	174 ±8.49	1.013 ±0.103	475 ±20.6	70.2 ±3.12	235 ±6.31
	10^{-8}	55.2 ±4.68	240 ±23.3	39.1 ±2.07	175 ±8.78	1.00 ±0.107	450 ±20.6	64.8 ±3.16	221 ±6.10
	10^{-7}	59.6 ±6.41	248 ±31.9	34.6 ±2.08	144 ±11.89	0.654 ±0.144	428 ±28.5	72.7 ±4.33	232 ±8.35
Treatment		0.975	0.913	0.453	0.217	0.424	0.841	0.630	0.353
SE		31.4	156	13.7	58.2	0.7	0.140	21.19	40.92
d.f		250	250	250	250	249	251	250	245

Appendix 3.2 Effect of auxin (IAA) seed coat at four different concentrations (high 10^{-8} to low 10^{-11} mol L^{-1}) on plant nodule traits and plant growth with water and solvent controls. Values are averages, ±SE with results of one-way ANOVA below with model residual standard error and degrees of freedom (df).

Source of variation		Nodule number	Specific nodule number (# g^{-1})	Nodule weight (mg)	Specific nodule weight (mg)	Average nodule weight (mg)	Shoot weight (mg)	Leaf area (cm^2)	Chlorophyll content (µMol m^2)
Auxin	Control	25.7 ±2.45	85.0 ±8.12	26.8 ±2.16	88.5 ±4.55	1.16 ±0.213	401 ±23.9	59.7 ±2.89	398 ±9.14
	Solvent	27.3 ±2.45	99.3 ±8.12	24.8 ±2.16	88.9 ±4.55	1.15 ±0.213	399 ±23.9	60.8 ±2.89	420 ±9.14
	10^{-11}	28.2 ±2.45	88.5 ±8.12	28.6 ±2.16	86.8 ±4.55	1.15 ±0.213	440 ±23.9	65.0 ±2.89	411 ±9.14
	10^{-10}	22.8 ±2.45	77.2 ±8.12	26..2 ±2.16	85.3 ±4.55	1.53 ±0.213	438 ±23.9	65.2 ±2.89	404 ±9.14
	10^{-9}	22.6 ±2.50	77.4 ±8.30	27.8 ±2.20	90.3 ±4.65	1.61 ±0.217	421 ±24.4	60.8 ±2.95	413 ±9.33
	10^{-8}	30.5 ±2.45	95.3 ±8.12	30.2 ±2.16	87.1 ±4.55	1.26 ±0.213	464 ±23.9	67.3 ±2.89	423 ±9.14
Treatment		0.164	0.292	0.584	0.98	0.467	0.353	0.334	0.387
SE		12	39.8	10.56	22.31	1.04	116.9	14.15	44.77
d.f		137	137	137	137	137	137	137	137

Appendix 3.3 Effect of gibberellic acid (GA_3) seed coat at four different concentrations (high 10^{-7} to low 10^{-10} mol L^{-1}) on plant nodule traits and plant growth with water and solvent controls. Values are averages, ±SE with results of one-way ANOVA below and model residual standard error and degrees of freedom (df).

Source of variation		Nodule number	Specific nodule number (# g^{-1})	Nodule weight (mg)	Specific nodule weight (mg)	Average nodule weight (mg)	Shoot weight (mg)	Leaf area (cm^2)	Chlorophyll content (µMol m^2)
Gibberellic acid	Control	52.6 ±5.91	227 ±44.5	38.6 ±3.47	171 ±10.7	0.889 ±0.010	443 ±24.4	77.0 ±4.10	236 ±7.71
	Solvent	50.5 ±7.43	251 ±44.5	38.4 ±5.3	175 ±16.3	0.955 ±0.152	419 ±24.4	78.2 ±4.19	228 ±7.71
	10^{-10}	51.1 ±8.14	225 ±54.4	42.6 ±6.49	155 ±20.0	0.764 ±0.186	382 ±22.3	74.5 ±4.40	226 ±7.71
	10^{-9}	50.3 ±7.14	223 ±44.5	34.5 ±5.30	148 ±16.3	0.662 ±0.152	406 ±22.8	76.2 ±4.40	228 ±8.10
	10^{-8}	50.4 ±7.14	187 ±54.4	25.4 ±6.49	129 ±20.0	0.772 ±0.186	417 ±26.5	71.5 ±4.29	221 ±8.10
	10^{-7}	55.1 ±6.65	274 ±38.5	44.2 ±4.33	186 ±14.1	0.714 ±0.124	429 ±23.3	83.8 ±4.40	229 ±7.21
Treatment		0.996	0.825	0.262	0.218	0.671	0.548	0.489	0.837
SE		25.8	108.9	12.98	39.96	0.372	109.2	19.66	35.33
d.f		76	36	37	36	37	120	120	119

Appendix 3.4 Effect of cytokinin (kinetin) root application at four different concentrations (high 10^{-7} to low 10^{-10} mol L^{-1}) on plant nodule phenotype with water control. Values are averages, ±SE with letters denoting significant difference at $p < 0.05$ as determined by least significant difference (LSD) test with results of one-way ANOVA below with model residual standard error and degrees of freedom (df).

Source of variation		Nodule number	Specific nodule number (# g^{-1})	Nodule weight (mg)	Specific nodule weight (mg g^{-1})	Average nodule weight (mg)	Total nodule area (mm^2)	Specific nodule area (mm^2 g^{-1})	Average area (mm^2)	Nodule distribution (mm)	4 mm
Cytokinin	Control	69.1 b ±7.41	264 c ±26.8	43.9 ±3.98	168 ±12.3	0.525 ±0.039	239 c ±55.9	883 c ±235	3.38 ±0.606	63.6 ±6.51	3.67 b ±3.8
	10^{-10}	99.3 a ±7.41	402 a ±26.8	50.5 ±3.98	206 ±12.3	0.525 ±0.039	397 ab ±53.1	1583 ab ±223	4.00 ±0.575	74.7 ±6.51	10.33 ab ±3.8
	10^{-9}	77.2 b ±7.41	346 ab ±26.8	44.4 ±3.98	200 ±12.3	0.599 ±0.042	273 bc ±53.1	1182 abc ±223	3.63 ±0.575	74.2 ±6.51	8.00 b ±3.8
	10^{-8}	89.4 ab ±7.74	322 bc ±28.0	50.4 ±4.36	181 ±13.5	0.566 ±0.042	258 bc ±55.9	997 bc ±235	2.98 ±0.606	55.8 ±6.51	4.73 b ±3.97
	10^{-7}	99.8 a ±7.41	384 ab ±26.8	50.3 ±3.98	208 ±12.9	0.576 ±0.042	458 a ±55.9	1703 a ±235	4.34 ±0.606	73.8 ±6.51	20.4 a ±4.16
Treatment		**0.016**	**0.005**	0.568	0.118	0.223	**0.027**	0.065	0.552	0.209	**0.040**
SE		25.7	92.8	13.8	42.7	0.134	167.8	704.8	1.82	13.02	13.2
d.f		54	54	53	52	49	42	42	42	15	52

Appendix 3.5 Effect of auxin (IAA) root application at four different concentrations (high 10^{-7} to low 10^{-10} mol L^{-1}) on plant nodule phenotype with water and solvent control. Values are averages, ±SE with letters denoting significant difference at $p < 0.05$ as determined by least significant difference (LSD) test with results of one-way ANOVA below with model residual standard error and degrees of freedom (df).

Source of variation		Nodule number	Specific nodule number (# g^{-1})	Nodule weight (mg)	Specific nodule weight (mg g^{-1})	Average nodule weight (mg)	Total nodule area (mm^2)	Specific nodule area (mm^2 g^{-1})	Average area (mm^2)	Nodule distribution (mm)	4 mm
Auxin	Control	51.5 ±7.14	251 ±37.6	30.9 ±5.92	154 ±23.1	0.712 ±0.153	139 ±13.6	682 ±78.3	2.42 ±0.47	71.2 ±10.14	3.6 a ±0.450
	Solvent	45.2 ±7.46	223 ±39.3	35.5 ±6.18	164 ±24.2	0.898 ±0.153	81.7 ±13.0	437 ±74.7	2.21 ±0.448	62.2 ±9.26	1.364 bc ±0.429
	10^{-10}	67.1 ±7.46	352 ±39.3	38.0 ±6.18	191 ±24.2	0.651 ±0.153	126.4 ±15.2	700 ±87.6	2.11 ±0.526	50.7 ±10.14	2.250 ab ±0.503
	10^{-9}	65.7 ±7.14	318 ±37.6	40.3 ±5.92	182 ±23.1	0.730 ±0.146	113.3 ±12.4	542 ±71.5	2.39 ±0.429	49.5 ±9.26	1.00 bc ±0.411
	10^{-8}	62.5 ±7.46	313 ±39.3	44.2 ±6.18	219 ±24.2	0.846 ±0.153	112.4 ±13.6	559 ±78.3	1.76 ±0.470	59.7 ±9.26	1.091 bc ±0.429
	10^{-7}	65.3 ±7.46	331 ±39.3	38.5 ±6.18	196 ±24.2	0.636 ±0.153	110.1 ±12.4	569 ±74.7	1.76 ±0.448	49.1 ±9.26	0.727 c ±0.429
Treatment		0.204	0.161	0.733	0.453	0.793	0.085	0.201	0.835	0.543	**<0.001**
SE		24.7	130.3	21	80.2	0.507	43.0	56	1.45	22.68	1.42
d.f		62	62	62	62	62	57	56	56	28	57

Appendix 3.6 Effect of gibberellic acid (GA_3) root application at four different concentrations (high 10^{-7} to low 10^{-10} mol L^{-1}) on plant nodule phenotype with water and solvent control. Values are averages, ±SE with results of one-way ANOVA below with model residual standard error and degrees of freedom (df).

Source of variation		Nodule number	Specific nodule number (# g^{-1})	Nodule weight (mg)	Specific nodule weight (mg g^{-1})	Average nodule weight (mg)	Total nodule area (mm^2)	Specific nodule area (mm^2 g^{-1})	Average area (mm^2)	Nodule distribution (mm)	4 mm
Gibberellic acid	Control	30.5 ±4.83	104.4 ±16.9	37.2 ±5.28	106 ±10.45	1.75 ±0.270	71.4 ±9.17	241 ±31.9	2.57 ±0.12	64.1 ±8.90	0.909 ±0.463
	Solvent	39.6 ±4.83	131.7 ±17.7	41.9 ±5.91	124 ±11.69	1.18 ±0.301	81.3 ±9.62	265 ±33.5	2.12 ±0.126	63.9 ±8.90	1.00 ±0.486
	10^{-10}	28.3 ±4.41	79.2 ±16.2	43.2 ±5.04	119 ±9.97	1.76 ±0.257	66.6 ±8.78	186 ±30.6	2.45 ±0.115	57.3 ±8.9	1.667 ±0.444
	10^{-9}	30.2 ±4.41	91.4 ±16.2	38.6 ±4.82	109 ±9.54	1.39 ±0.246	66.9 ±8.78	200 ±30.6	2.26 ±0.115	48.1 ±9.75	1.417 ±0.444
	10^{-8}	24.2 ±4.83	78.6 ±17.7	34.1 ±5.28	103 ±10.45	1.52 ±0.270	53.8 ±9.62	172 ±33.5	2.24 ±0.126	59.6 ±8.90	1.111 ±0.512
	10^{-7}	26.3 ±4.41	78.6 ±17.7	38.4 ±4.82	110 ±9.54	1.75 ±0.246	58.9 ±9.62	175 ±30.6	2.36 ±0.115	63.5 ±8.90	1.750 ±0.444
Treatment		0.300	0.209	0.855	0.722	0.578	0.410	0.255	0.136	0.825	**0.**703
SE		15.3	56.1	0.017	0.033	0.853	30.42	105.9	0.399	21.81	1.54
d.f		61	61	57	57	57	61	61	61	29	60

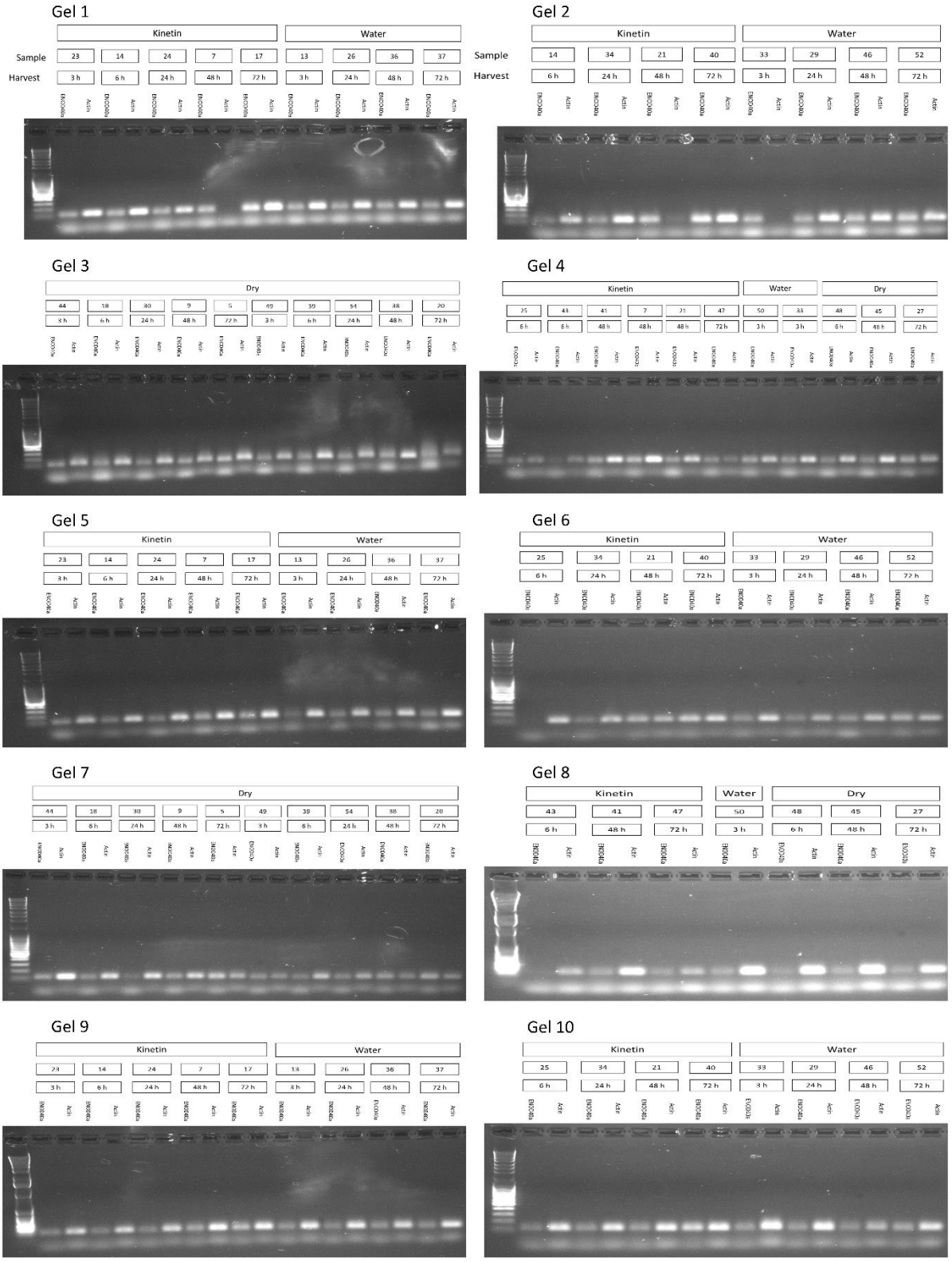
Appendix 3.7 Effect of different cytokinin application methods (foliar, root and seed) at two different concentrations (high 10^{-7} and low 10^{-9} mol L^{-1}) on plant growth and nodulation. Values are averages, ±SE with letters denoting significant difference at $p < 0.05$ as determined by least significant difference (LSD) test with results of one-way ANOVA below with model residual standard error and degrees of freedom (df).

Treatment		Shoot weight (g)	Leaf chlorophyll (µMol m²)	Leaf area (cm²)	Nodule weight (mg)	Nodule number
Control	Control	0.530 ±0.045	238 ab ±5.17	119 ±7.92	28.4 ±3.1	24.6 b ±7.2
Foliar	High	0.651 ±0.047	244 ab ±5.43	141 ±8.31	34.7 ±3.3	33 b ±7.6
	Low	0.601 ±0.050	227 b ±5.72	131 ±8.75	38.2 ±3.3	55.8 a ±8.0
Root	High	0.590 ±0.053	231 ab ±6.07	128 ±9.29	28.8 ±3.6	38.1 ab ±8.53
	Low	0.579 ±0.047	244 ab ±5.43	129 ±8.31	30.8 ±3.2	59.5 a ±7.6
Seed	High	0.638 ±0.053	256 a ±6.07	138 ±9.29	32.6 ±3.6	41.5 ab ±8.5
	Low	0.723 ±0.050	244 ab ±5.72	154 ±8.75	34.5 ±3.4	44.4 ab ±8.0
Treatment		0.146	**0.021**	0.126	0.349	**0.024**
SE		0.149	17.16	26.26	10.08	23.89
df		58	58	58	57	58

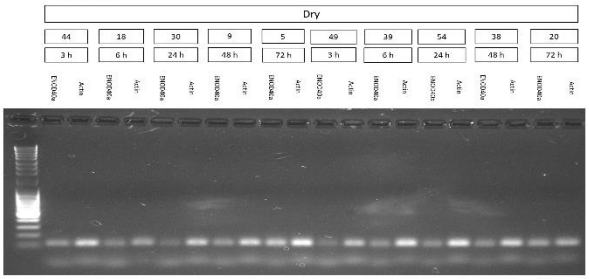
Appendix 3.8 Effect of different cytokinin application treatments (foliar 10^{-7} and seed 10^{-9} mol L^{-1}) on plant growth and yield from V2 to R6. Values are averages, ±SE with letters denoting significant difference at $p < 0.05$ as determined by least significant difference (LSD) test with results of one-way ANOVA below with model residual standard error and degrees of freedom (df).

Source of variation		**Shoot weight (g)**	**Root weight (g)**	**Leaf area (cm_2)**	**Leaf chlorophyll (µMol m^2)**	**Photosynthesis (µMol m^2 s^{-1})**	**Seed number**	**Seed weight (g)**
Cytokinin	Control	5.14 ±0.148	1.031 ±0.034	466 ±11.8	322 ±7.49	18.1 ±0.941	46.2 ±3.14	7.26 ±0.347
	Foliar	4.93 ±0.148	0.977 ±0.034	446 ±11.8	326 ±7.49	18.5 ±0.941	43.7 ±3.14	6.66 ±0.347
	Seed	4.94 ±0.148	0.993 ±0.034	446 ±11.8	317 ±7.59	17.7 ±0.941	45.4 ±3.14	6.89 ±0.347
Stage	V2	0.529 d ±0.171	0.282 d ±0.040	114 c ±13.6	246 d ±8.80	-	-	-
	V4	1.861 c ±0.171	0.782 c ±0.040	348 b ±13.6	283 c ±8.64	19.3 a ±0.76	-	-
	R4	4.994 b ±0.171	1.356 b ±0.040	655 a ±13.6	357 b ±8.64	20.7 a ±1.02	-	-
	R6	12.636 a ±0.171	1.582 a ±0.040	692 a ±13.6	401 a ±8.64	14.4 b ±1.02	-	-
Cytokinin (C)		0.549	0.514	0.389	0.784	0.700	0.849	0.477
Stage (S)		<0.001	<0.001	<0.001	<0.001	<0.001	-	-
C x S		0.786	0.980	0.848	0.845	0.567	-	-
SE		0.939	0.217	74.49	47.34	3.95	9.93	1.10
d.f		108	108	108	107	48	27	27

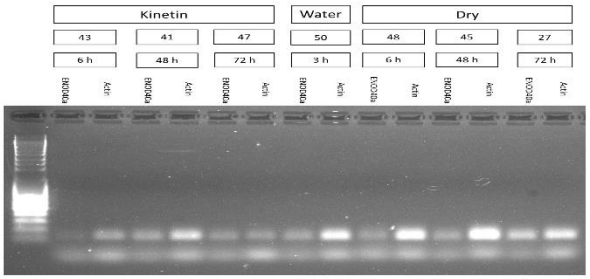
Appendix 3.9 Agarose gel electrophoresis from semi-quantitative RT-PCR analysis of *ENOD40a* transcript levels in root samples of seedling grown in growth pouches after either kinetin or hydro-priming or non-primed (dry) control. Lower band of DNA ladder represents 80 bp with *ENOD40a* product 100 and Actin 191 bp.



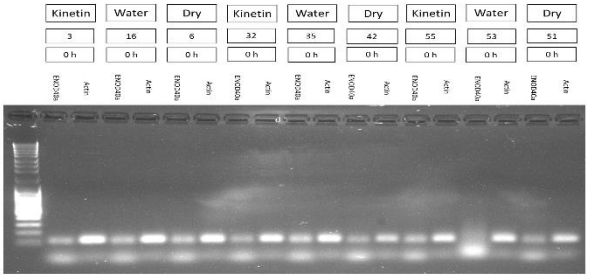
Gel 11



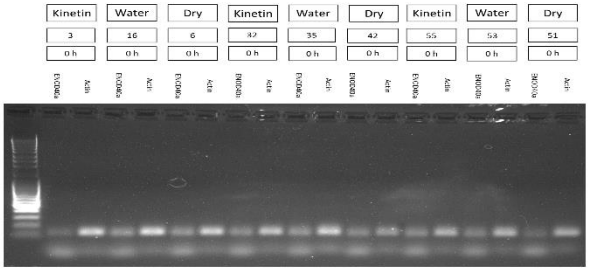
Gel 12



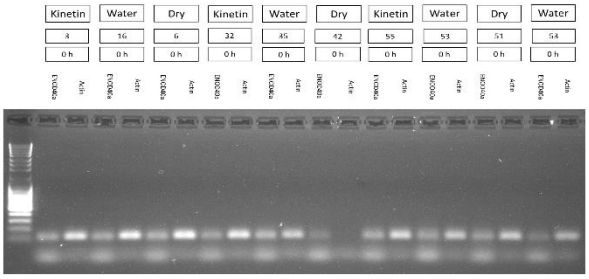
Gel 13



Gel 14

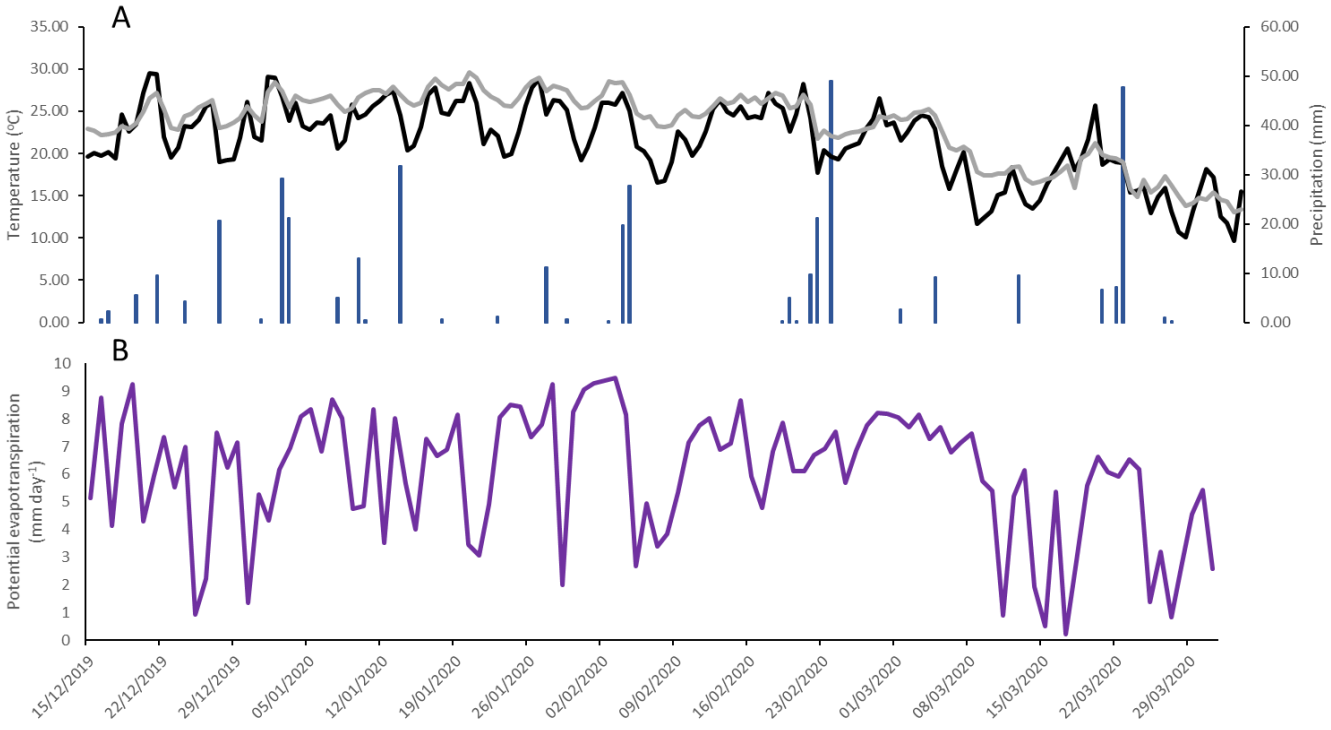


Gel 15



Appendix 4.1 Field climate data for three sowing dates in the 2019/ 2020 field trial. Values are averages, ±SE with letters denoting significant difference at $p < 0.05$ as determined by least significant difference (LSD) test with results of one-way ANOVA below with model residual standard error.

Sowing	Air temperature (°C)	Soil temperature (10 cm depth °C)	Precipitation (mm)	Potential evapotranspiration (mm day^{-1})
Dec	23.5 ±0.76	24.7 b ±0.38	2.73 ±1.98	5.97 ±0.54
Jan	24.0 ±0.76	27.0 a ±0.38	2.92 ±1.98	6.67 ±0.54
ANOVA (p value)	0.607	**<0.001**	0.947	0.365
Residual SE	3.05	1.53	7.93	2.16



Appendix 4.2 Environmental data for the 2019-2020 field trial. Daily air and soil (10 cm depth) temperature (black and grey line, respectively), precipitation (blue bars; A) and potential evapotranspiration (purple line; B).