The effect of herbicide formulations and soybean genotype on the relationship between beneficial organisms and root pathogens

By

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ABSTRACT

There has been considerable speculation in the media that glyphosate has a negative impact on symbiotic micro-organisms, particularly in the case of genetically modified soybeans. This speculation coincides with the assumption that the presence of the RR® gene is detrimental to ability of rhizobacteria to infect genetically modified soybeans and stimulate nodule formation. Also postulated was that the presence of the gene weakens the resistance of the crop to soil borne pathogens. This thesis tested the hypothesis that glyphosate has an effect on soybean plants and its symbiotic rhizobacteria and that genetic modification of the plant is detrimental to successful rhizobium colonisation and disease resistance. A definite weakness in previous studies is that only one glyphosate formulation was used and that according to the literature no studies have used isolines of soybeans to compare interactions.

In trials utilising a strain of *Bradyrhizobium japonicum* recommended by the Agricultural Research Council of South Africa, (WB_{74}), and direct exposure of rhizobacteria to different glyphosate formulations showed no significant reduction of number of colonies. Neither did any of the treatments inhibit the ability of treated bacteria to infect both soybean isolines' plant roots and stimulate the formation of active nodules.

When exposing the RR® soybeans to different glyphosate formulations, the only negative effects on the different plant parts were found in cases where fertilisation with NH_4NO_3 was used instead of inoculation with the rhizobacteria. This result emphasised the importance of successful inoculation with the correct rhizobacterium. When the RR® soybeans were exposed to different glyphosate formulations in the presence of three soil pathogens, it was only plants treated with NH_4NO_3 that showed

detrimental effects. Since these trials were not taken to harvest, it is not possible to speculate on ultimate yield in these cases.

To investigate the presence of the RR® gene and its effect on soybean growth, both lines were treated in the exact same manner and cultivated under the same conditions. No significant differences were observed in any plant parameters, especially the mass of active nodules formed. When the growth parameters of the soybean lines were compared after exposure to soil pathogens, no significant differences in infection were observed. The presence of the RR® gene therefore does not appear to increase the susceptibility of soybean to soil borne pathogens.

PREFACE

Agrochemicals are a necessity in modern commercial agriculture, especially herbicides for the control of weeds. The development of crops with genetically modified resistance to certain herbicides has, therefore, resulted in glyphosate containing herbicides being widely used. It has been postulated that genetic modification of legumes, such as soybeans, can affect rhizodeposition, which in turn influences infection by rhizobacteria. There is also concern that the use of glyphosate could harm rhizobacteria directly, thereby affecting crop growth and yield.

The first chapter of this thesis is a literature review addressing the production of soybeans and its importance in food security, including the threat posed by climate change and factors affecting symbiosis with *Bradyrhizobium japonicum*. The review also provides an overview of the history of weed control, the development of chemical herbicides and the appearance of glyphosate containing herbicides. The general effect of herbicides on soil micro-organisms is discussed with emphasis on symbiotic microorganisms and soil-borne pathogens of soybeans. The insertion of the RR® gene is discussed and its effect on resistance to soil-borne pathogens after exposure to Roundup.

Chapter 2 addresses the question of whether glyphosate formulations when applied to soybean will affect symbiotic rhizobacteria. The chapter describes the effect of direct exposure of *B. japonicum* to recommended concentrations of glyphosate and also describes the effect of inoculating soybean seeds without the gene (A5409) and those that were genetically modified (A5409RG). There was no significant reduction in the numbers of treated rhizobia, nor, was their ability diminished to infect the roots and, cause nodulation.

Chapter 3 investigates the effect of various glyphosate containing herbicides on the growth of RR® soybeans in the presence or absence of symbiotic rhizobia in

order to elucidate whether the gene affects the performance of soybeans exposed to soil-borne pathogens. Soybean seeds were exposed to three soil-borne pathogens, *Fusarium oxysporum*, *Macrophomina phaseolina* and *Sclerotium rolfsii* that were previously isolated from diseased soybean plants. Seeds were either inoculated with *B. japonicum* or given NH_4NO_3 as nitrogen source prior to inoculation. The growth parameters of plants exposed to the pathogens showed a decrease in mass when NH_4NO_3 was used as a source of nitrogen. No significant differences were noted over any of the treatments in the dry mass of the aerial plant parts, roots or nodules when the plants were successfully inoculated with rhizobia.

Chapter 4 investigates the possible effect of the presence of the RR® gene by directly comparing the growth parameters of soybean isolines A5409RG and A5409, following prior inoculation with *B. japonicum* and exposure to *F. oxysporum*, *M. phaseolina* and *S. rolfsii*. The addition of arbuscular mycorrhizae inoculum to the rhizobia was also investigated. The isolines showed no significant differences in growth performance when grown under these conditions. When the isolines were exposed to the three pathogens, no significant differences were noted in the mass of the leaves. Both the root and nodule mass of A5409 were significantly increased when exposed to *S. rolfsii*. No other significances were noted between isolines over the other treatments.

Chapter 5 provides a general discussion of the foregoing chapters and attempts to place all outcomes into context with other similar studies while providing a future perspective for further research.

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Chapter 1

LITERATURE REVIEW

The effect of glyphosate formulations on symbiotic soil borne organisms and root pathogens with specific reference to soybean cultivation

1.1 Introduction

Modern food production is based on increasingly large areas of land being cultivated with a single cash crop. Since subsistence and smallholding farming cannot provide enough sustenance to ever-growing urban populations, multi-hectare mono-cropping is at present the only feasible method of providing food. This method of crop production necessitates the large scale use of chemicals to fertilise the soil and control plant pathogens, insects and weeds (Kremer, 2012).

Incorrect use of agricultural chemicals has a range of negative effects on the agroecosystem in general and more specifically, on soil health (Kalia & Gosal, 2011). Apart from these chemicals being directly toxic to beneficial micro-organisms (Kalia & Gosal, 2011), many additives in product formulations are also detrimental to other organisms (biodiversity) (Smith & Hallett, 2006). These products can result in changes to soil chemistry (Johansson *et al*., 2004). For example, inorganic nitrogen fertilisers are directly linked to a decrease in the numbers of rhizosphere microbes, loss of organic material and nitrogen from the soil (Jackson *et al*., 2012).

Researchers emphasise the importance of preserving and enhancing soil health by managing soil biodiversity, with special emphasis on the retention and cycling of nitrogen in agricultural bio-networks (Jackson *et al*., 2012). Many researchers have demonstrated high microbial diversity and biomass associated with perennial cover crops and intercropping (Balota & Auler, 2011; Jasa, 2011). Fields under commercial cash crop production with no cover-cropping showed a decrease in biodiversity as well as in soil nitrogen (Fields, 2004; Pisante & Stagnari, 2004).

There is, consequently an urgent need for development of biologically based fertilisers and pesticides that are efficient, easily to apply, cheap to produce and deliver, as well as having few negative effects on soil biology. These will include free living nitrogen fixing bacteria such as *Azotobacter* spp. and the Rhizobia symbionts,

phosphate solubilising arbuscular mycorrhizae, biological pesticides such as *Bacillus thuringiensis* and plant growth promoters such as certain *Trichoderma* spp. (Kalia & Gosal, 2011).

This literature review attempts to contextualise agricultural practices associated with the cultivation of soybean (*Glycine max* L.) with specific reference to those practices that can have a negative effect on soil health and crop yield. The use of herbicides, and in particular glyphosate, and its effect on soil biology is discussed with emphasis on endophytic root symbionts and soil borne pathogens of the crop. The review serves as a frame of reference for subsequent chapters of this thesis, which deal with specific issues relating to glyphosate and its effect on *Rhizobium* spp. associated with soybeans.

1.2. Production of soybean

Soybeans, family Leguminosae, have been produced in China for almost five thousand years and have been known in Europe from the seventeenth century, but mostly neglected till the end of the 19th century when many varieties were brought to America, becoming their second largest crop (Gibson & Benson, 2005). Today, soybean is the fourth largest cash crop in the world and was cultivated on 124 million hectares worldwide in the 2014/15 season, producing 312.97 metric tons equivalent to 2.5 tons per hectare (FAOSTAT beta, 2016). Soybeans are produced in the United States of America (ca 30%) as well as Brazil, Argentina, China and India (Martin *et al*., 2006). It is one of the world's fastest growing crops and can provide a cheaper source of sustainable protein, than animal protein. Since the 1920's intensive research has resulted in cultivars that are adapted to a wide range of environmental conditions and can deliver seed with a high oil content. Apart from producing high protein grain, soybeans play an important role in crop rotation, both with regard to nitrogen deposition in the soil and reduction in soil borne diseases.

Commercial soybean farming is based on monoculturing on a large area of land, which leads to the need for mechanical harvesting in order to bring the entire crop in at the same time. This term should not be confused with mono-cropping, in which the same crop is planted year after year on the same piece of land without the normal rotation between crops to control soil borne diseases (Jacques & Jacques, 2012). In monoculture production the use of mechanical harvesters relies on evenly distributed plants of similar height and size as well as the plants reaching maturity at

the same time. This is achieved by planting reputable seed of a single cultivar, which should deliver a crop that grows evenly and matures at the same time over the whole cultivated area. However, this outcome may be influenced by a variety of biotic and abiotic factors, including rainfall, cultivation methods, pathogens and weeds. In South Africa the production of soybeans reached 650 000 tons during 2012 with an average production of 1.38 tons ha^{-1} under dryland conditions over 472 000 ha (Dlamini *et al*., 2014), climbing to 784 500 tons in 2013 over 516 000 ha (ARC, 2014).

1.2.1 Agricultural Management Practices

In modern, large scale farming a series of choices have to be made before a crop is actually planted (Hua, 2005; Greig, 2009; Huh & Lall, 2013). In the case of soybeans, choices such as planting a short-, medium- or long-term cultivar and whether it grows determinately (stops elongating when flowering starts) or indeterminately, and whether the crop contains genes such as resistance to glyphosate, are crucial. The seed and inoculant have to be ordered and delivered well in time. The choice of cultivar is limited by soil type, soil depth, composition and soil pH (Hintz *et al*., 1992). If there are problems with soil in terms of the chosen crop, these will have to be remediated before planting (Hakeem *et al*., 2015). The next decision will be the time of planting, which is regulated by the general climate in the area and soil moisture. This decision relates to the availability of water, ie whether irrigation is available, or if the crop can mature with a good yield under dryland conditions. A soil test will reveal any deficiencies in terms of nutrients or pH and whether fertilisers are necessary and/or whether lime for pH amelioration needs to be ordered. Decisions have to be made as to the method of soil preparation, implements required for both planting and harvesting. Soil preparation is partly aimed at removing and/or destroying any weeds, however, pre- and post-emergence weed control will have to be continued using chemical herbicides (Pannell, 1994). All of the above decisions are determined by the environment in which the crop plants should flourish. If any of the above decisions are detrimental to inoculation with nitrogen fixing symbiotic rhizobia, extra nitrogen fertilisers will have to be applied (McConnell *et al*., 2002). Yields will probably be lower, beans may be deficient in certain nutrients and less nitrogen will be released into the soil for use by the follow-up crop.

1.2.1.1 Crop Rotation

Rotating crops on a specific piece of land over planting seasons reduces the possibility of disease transfer by removing the hosts of specific pathogens. This action can lessen disease incidence, but also disturbs root colonisation by arbuscular mycorrhizae (AM) (Johansson *et al*., 2004). Plants spend a large amount of energy on the production and secretion of root exudates (Haichar *et al*., 2008). Exudates attract beneficial microbes that symbiotically provide the plant with nutrients (Currier & Strobel, 1976) and also reduce populations of potential disease causing organisms (Goh *et al*., 2013). If a new crop species is planted every season, it takes time for specific root exudates to be produced and excreted, leading to a lag in the optimisation of beneficial organisms in the soil (Farrar *et al*., 2014). This may result in increased disease incidence, and consequently the use of more pesticides (Berendsen *et al*., 2012).

In the summer rainfall areas of South Africa, soybeans are often planted in rotation with maize in two-year-cycles or maize and sorghum in a three-year-cycle. This practice results in weed reduction, a lower incidence of disease and a general increase in the yields of all crops concerned (Nel, 2005). This trend is also seen in other countries, such as in Brazil where an increase of approximately a ton ha^{-1} in rice was recorded following soybean (Nascente *et al*., 2013).

1.2.1.2 Tillage

Tillage is the process whereby soil is prepared for planting. This may vary from just drawing a shallow line in the soil (Coolman & Hoyt, 1993) or digging the soil up to a depth of 30 cm to up-end and thereby killing any weeds and volunteer plants (Raper *et al*., 2000). Conventional tillage can cause physical damage to soil structure, leading to a disruption of fungal mycelia and the chemical composition of the soil (Johansson *et al*., 2004). Soil compaction leads to inadequate water and air movement (Kalia & Gosal, 2011), inhibited root penetration (Hoffmann & Jungk, 1995) and a reduction in microbial biomass and biodiversity (Johansson *et al*., 2004). Deep or conventional tillage disturbs soil aggregate structure and can lead to intensive soil compaction with concomitant loss of aggregation and air spaces (Nimmo & Perkins, 2002). These practices lead to a loss of carbon and nitrogen, further disturbing the balance between soil, microbes and plants.

In experiments covering seven growing seasons, van Groenigen *et al*. (2010) found that reduced tillage led to significantly higher levels of carbon in the soil. Although reduced tillage could lead to unwanted higher levels of soil moisture and lower soil temperatures, it ultimately leads to less soil erosion, better drainage and higher yields (Coolman & Hoyt, 1993).

1.2.1.3 Weed control

Weeds compete with crops for water and nutrients and they occupy physical space, rendering the plant unable to extend its root system or canopy. When weeds become established before the crop emerges, yield can be extensively compromised. If weeds are not eradicated before flowering and seed set, the weed seedbank in the soil can build up to levels where subsequent planting seasons may be lost due to weed numbers far exceeding that of the planted crop (Mirsky *et al*., 2010). Some weeds, including volunteer plants from the previous crop, exhibit allelopathy and may thereby inhibit the growth of subsequent crops (Fujii, 2001; Monaco *et al*., 2002). Weed seeds lend themselves to easy dispersal by wind, water, attachment to humans or animals, and sticking to tools and machinery not well cleaned (Sorensen, 1985). Many weeds can also reproduce vegetatively and pieces of the plant left in the soil after harvest or herbicide application, may germinate during the next season and thus interfere with the crop (Zimdahl, 2013).

Weeds directly affect plants by competing for nutrients, water and sunlight. In soybeans, this can lead to an uneven stand and loss of pods during mechanical harvesting (Harrison & Loux, 1995). Weeds may also impede harvesting by clogging mechanical harvesters (Harrison & Loux, 1995). Harvested products can be contaminated by toxic plant residues from weed seeds, leaves, or foreign particles, which can lead to grain being downgraded. Because it is not possible to weed large fields by hand, and mechanical weeding can damage a crop, chemical weed killers are used (Ware & Whitacre, 2004).

Herbicides are generally classified as pre-plant, pre-emergence or post-emergence, depending on the time of application. They are specific in either killing grasses or broadleaf weeds, and either systemic or contact, depending on their mode and site of action (Lin & Garry, 2002). Applying herbicide before planting or pre-emergence may not be sufficient, as the weed seedbank in the soil may be activated by irrigation or rainfall after the crop has been planted.

1.2.1.3.1. Chemical weed control

In 1896, a copper sulphate and lime mixture was sprayed on vineyards in the Bureaux region in France to deter school boys from picking the grapes and had the unexpected inadvertent effect of controlling mildew and other fungal diseases on grapes. The observation that this mixture also killed yellow charlock (*Brassica kaber* DC. L.C. Wheeler) stimulated an interest in the use of chemicals to control weeds as well (Swingle, 1894; Rao, 2000; Zimdahl, 2013). The first chemical herbicides in common use were either fertilisers such as $CaCN_2$ and a mixture of $MgSO_4$ and KCl or industrial chemicals such as metallic salts, $NaAsO_2$, H_2SO_4, $H_6N_2O_3S$ and $NaClO_3$ (Zimdahl, 2013).

Once the physiological function of the herbicide on these processes became better understood, research and development of herbicides were conducted to produce products that selectively killed target plants. One of the most common are herbicides that kill broadleaf weeds within cereal crops, such as Florasulam (triazolopyrimidine sulfonanilide) (De Boer *et al*., 2006) and herbicides that kill grasses amongst broadleaf crops and trees, such as Fluazifop (2-[4-(5-trifluoromethyl-2-pyridyloxy)-phenoxy] propionate) (Walker *et al*., 1988).

Although chemical control of weeds is an easy and relatively cheap way of controlling unwanted plants amongst crops, it does not always solve the problem. Certain weeds may become resistant to the specific herbicide used, prompting the producer to use more and often higher concentrations of herbicides, and crops may then be damaged due to spray drift or run-off (Nollet & Rathore, 2010). Such run-off can also enter the water table and surface bodies of water and may be harmful to aquatic creatures. Apart from reading herbicide labels carefully as to which weeds can be controlled and compatible crops, factors such as soil type and rainfall must also be taken into consideration (Moss, 2010). As in all agricultural endeavours, the use of more than one method for the control of weeds results in higher yields and has a less negative impact on soil health and biodiversity (Mueller-Schaerer, 2002; Duke & Powles, 2009). In addition to chemicals, mechanical removal of weeds should be practised as well as the use of biological control organisms and natural plant derived herbicides.

1.2.1.3.1.1 Characteristics of an ideal herbicide

An ideal herbicide should remain active for long enough to prevent weed competition with the crop and then quickly break down into harmless units in the soil. If a compound remains active in soil for a prolonged period, it may eventually harm the present crop or target the follow-up crop during the subsequent season, thus influencing the farmer's choice of rotation crops. For example, under optimum conditions the herbicide picloram may persist in the soil in damaging concentrations for more than a year (Keys & Friesen, 1968). Residual activity of a herbicide in soil is determined by biotic and abiotic degradation, and by transfer, which is affected by adsorption to soil particles, leaching, run-off on the surface, evaporation (volatility) and removal by other plants (Monaco *et al*., 2002). Microbes play an essential role in the breakdown of herbicides into harmless compounds (Colquhoun, 2006). If the herbicide, its active ingredient/s or additives, affect soil microbes, breakdown will take longer and increase harmful residual activity (Colquhoun, 2006).

In essence, any pesticide is a poison and even taking extreme care with production, transport, use and disposal, contamination and collateral damage is always a danger. Although labels are strictly regulated, the consumer may still take it into his/her own hands to apply a herbicide to a crop that is not registered, or to increase the dosages to get "better" results. Secondary suppliers of agricultural chemicals are not always well trained and may convince the consumer to disregard label information. Often the actual mixing and application of a particular herbicide is left to an illiterate or untrained worker (Olofsdotter, 1998). A common concern of researchers is that although the pesticide itself may not be toxic to non-target organisms, the additives that improve the efficacy of the product, such as surfactants, may be harmful to humans, animals and especially soil micro-organisms (Banks *et al*., 2013).

A systemic post-emergence herbicide, such as glyphosate, is designed to kill all plants it comes into contact with. A farmer has to make sure that the crop that is intended to be planted is genetically modified to be totally resistant to the herbicide. Spray drift should also be prevented, or surface run-off into lands with susceptible crops because the farmer may be held liable if a neighbour's crops are inadvertently damaged (Naylor, 2002)

A weed is either susceptible to a specific herbicide, naturally tolerant, or can become resistant after repeated treatments with a specific herbicide. Often within a target weed population a few plants may have inherent genetic immunity to a specific herbicide or group of herbicides. Thus, plants that survive will produce seed that in the next season will give rise to more resistant plants (Monaco *et al*., 2002).

Placing a herbicide on the soil surface after planting allows crop roots to grow away from the herbicide, which will therefore only affect shallow growing weeds. Fast leaching herbicides in shallow growing crops will target deep rooted weeds (Harrison & Loux, 1995). Shielded or directed spray physically protects the crop from damage. Such crops must have a slight tolerance to the herbicide (Kleemann & Gill, 2012). Differences between the leaf size, shape and orientation, as well as the presence of a waxy cuticle, may all affect retention of the herbicide, making it possible to target broadleaf weeds growing in grain crops. Grass roots tend to grow closer to the soil surface and are therefore more susceptible than the deep growing roots of dicotyledonous plants (Harrison & Loux, 1995).

The pinnacle of selectivity is the genetic manipulation of selected crop plants incorporating a gene that contributes to the detoxification of the herbicide in the crop plant. This allows a potential kill-all compound such as glyphosate to be applied over the crop plant, effectively killing all the weeds without damaging the crop (Dill *et al*., 2010).

1.2.1.3.1.2 Glyphosate

In 1974, a herbicide containing glyphosate as the active ingredient was registered as a weed killer (Henderson *et al*., 2010). This herbicide is structured to be sprayed on emerging weeds where, once absorbed, the chemical glyphosate (N-(phosphonomethyl) glycine interferes with the plant's ability to produce aromatic amino acids through the Shikimate pathway by acting as a competing inhibitor of 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase. EPSP can therefore not bind to its natural substrate and shuts down the pathway that leads to the production of proteins and, without these, the plant will ultimately die (Amrhein *et al*., 1980). This is a kill-all systemic herbicide that is successfully used on soybeans and other crops that have been genetically engineered to carry the Roundup Ready® gene (FAO, 2001). The engineered plant overproduces the enzyme, EPSP synthase, which negates the effect of glyphosate, and allows the plant to produce the necessary

amino acids to manufacture the proteins needed for growth. While it is still essential to treat weed in the 4-6 leaf stage, this can take place any time during the growing season without harming the crop. This herbicide is totally non-selective and applied post-emergence in cases where all vegetation has to be removed.

Human physiology does not utilise the Shikimate pathway, and therefore glyphosate should have no effect. However, some researchers have found that the glyphosate formulations may contain compounds toxic to higher life forms (Mesnage *et al*., 2012). Researchers found for example, that ethoxylated adjuvants used in glyphosate formulations may exhibit toxicity to human cells (Mesnage *et al*., 2012). Most countries have protective institutes that test new agricultural chemicals, not only for their activity within the function they were developed for, but also for side effects on humans, animals and microbes, as well as danger to the environment (WSDOT, 2006; European Commission, 2015). An extensive study carried out by West Midlands Poisons Unit, City Hospital, Birmingham, UK and the National Poisons Information Service (Birmingham Centre) concluded that most cases of poisoning by glyphosate formulations were due to deliberate intake of the concentrated product and that accidental exposure causes only mild symptoms, which can be treated symptomatically (Bradberry *et al*., 2004). Kubena *et al*. (1980) found growth reduction in broilers when fed different concentration of Roundup®, but stated that the amounts used are unlikely to ever be ingested by the animals other than being force-fed.

A large volume of popular information is available on the dangers of glyphosate (Mercola, 2013; Ho, 2012; Bodnar, 2013; KCMPR, 2015; Gammon, 2009). Many sources state that their information was supplied by scientists, without ever naming any individual. Furthermore, none of these publications are peer reviewed or accepted by the scientific community. These articles are mostly aimed at the farmers and the general public, and are difficult to counteract since most of the target market do not read peer reviewed scientific articles. Huber (2007) has spent a large amount of time researching the effect of glyphosate on non-target organisms. He has also published informative informal magazine articles in a fertiliser manufacturer's magazine on ways of counteracting some of the problems associated with the use of glyphosate containing herbicides (Huber, 2007; Johal & Huber, 2009). He also advocates judicial use of the product in later magazine editions (Huber, 2010).

N-phosphonomethyl glycine (2-(phosphonomethylamino) acetic acid) is a white powder that has no discernible odour and is stable at temperatures below 25°C. It has a molecular mass of 169.07 g mol^{-1}, with a solubility of 10.5 g L^{-1} (PubChem, 2015). It is never used in its powder form, but always formulated to form a salt (Smith *et al*., 1989), the best known being iso-propyoamine, ammonium, trimethylsulfonium, sodium, and the potassium salts. In order to ensure successful action of the herbicide and even enhancement of its performance, certain chemicals, known as adjuvants, are added directly to the herbicide itself (formulation adjuvants), or mixed into the tanks used for distribution (spray adjuvants). Formulation adjuvants can improve shelf life, improve compatibility of the herbicide with other chemicals, increase solubility, make it less volatile, while surfactants maximise leaf coverage and improve penetration into the leaf.

Iatrogenic effects of glyphosate are poorly documented and mostly lay emphasis on adverse effects, such as nerve damage to tadpoles and chicken embryos injected with glyphosate and glyphosate formulations (Paqanelli *et al*., 2010). Various claims have been made as to its damage to soil micro-organisms (Vinje, 2013; Mercola, 2013). However, Lane *et al.* (2012) found that glyphosate increases microbial respiration and has no effect on microbial biomass or biodiversity. In contrast Lancaster *et al*. (2010) found that four to five repeated applications of glyphosate caused an increase in biomass of soil microbes when compared to two and three applications, and also caused shifts in microbial diversity. These results are supported by work done by Nye *et al*. (2014) on the effect of glyphosate treated genetically modified soybean residues worked into the soil. Glyphosate adsorbs strongly to most soils and has a low desorbability, leaving very little of the chemical remaining in the soil (Wardle & Parkinson, 1989; Busse *et al*., 2000; Haney *et al*., 2000; Weaver *et al*., 2007; Partoazar *et al*., 2011; Duke *et al*., 2012). These authors also found that any changes were transient and had no long term effect on the biodiversity of the soil microbes.

Glyphosate is used almost exclusively to kill weeds before the crop is planted, before it emerges or after emergence of the crop (Allen, 2014). Fears have been expressed as to problems that might arise from certain weeds becoming resistant to glyphosate containing products. Such weeds will no longer have any competition from glyphosate sensitive weeds and will rapidly fill the empty niches resulting from the use of glyphosate (Rao, 2000; Powles, 2008; Kremer and Means,

2009). Some weeds may be genetically predisposed to resistance, and coupled with the constant use of glyphosate, have the ability to produce large amounts of viable seeds that facilitates increases in their numbers. Lack of crop rotation, limited tillage, constant usage of glyphosate only herbicides, and the use of lower than optimal concentrations of these herbicides will all contribute to an increase in weed resistance (Duke & Powles, 2008; Powles, 2008; Cedeira *et al*., 2011). It has thus become critical in some areas of the world to intersperse the use of glyphosate with other herbicides or to use non-chemical methods of weed control.

Due to long term use of glyphosate containing herbicides, accumulation of glyphosate in the soil has become a source of concern re possible effects on non-RR® follow-up crops (Meyer *et al*., 2016). Huber (2010) stated that glyphosate is not biodegradable, but Sviridov *et al*., (2015) published a review that analysis biodegradation via microbial transformation. The main product of spontaneous degradation is aminomethylphosphonic acid (AMPA). This is known to impair DNA reparation and mRNA synthesis in plants and animals, and low sub lethal concentration of both glyphosate and AMPA have been detected in cultured plants. AMPA translocates through the plant to root tips, shoots and nodule, acting as a sink for glyphosate and AMPA (Gomes *et al*., 2014). It was also found that the presence of glyphosate in the soil changed the biodiversity of the microbes (Wolmarans, 2013).

Certain bacteria can break down the C-P bond in glyphosate in order to utilise the phosphate (Fig 1.1), *Escherichia coli* being the best know organism to follow this pathway *Ochrobacterium anthropi* produces the enzyme glyphosate oxidoreductase (GOR) (Fig 1.2). This pathway leads to the production of AMPA which accumulates in the environment (Sviridov *et al*., 2015). Some bacteria that cannot break down the glyphosate molecule can utilise AMPA. Both *O. anthropi* and *Acromobacter* sp KG16 isolated from soils heavily contaminated by glyphosate and shown promise as bioremediants in other contaminated soils (Ermakova *et al*., 2010). *Agrobacterium radiobacter*, *Sinorhizobium melilioti*, *Bacillus pseudomallei* and *Nostoc* sp have been implicated in the biodegradation of glyphosate and the utilisation of phosphorous without assignment of a specific pathway (Hove-Jensen, *et al*., 2014). Sviridov *et al*., (2015) also mentioned that more pathways may exist.

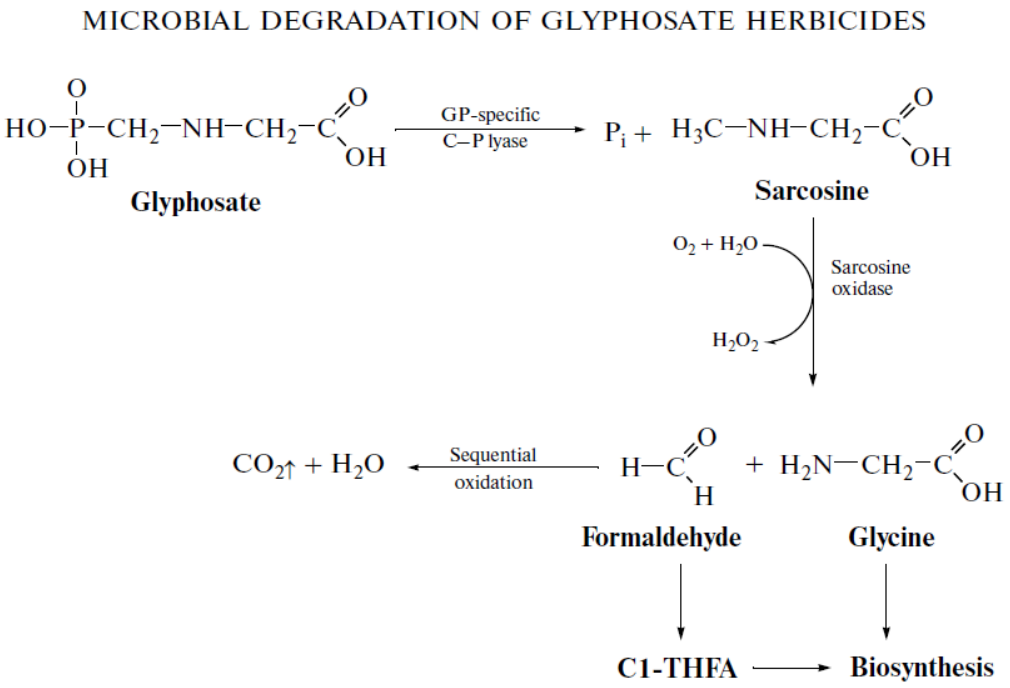

Figure 1.a C-P lyase-mediated glyphosate metabolism.
THF is tetrahydrofolic acid (From Sviridov et al., (2015))

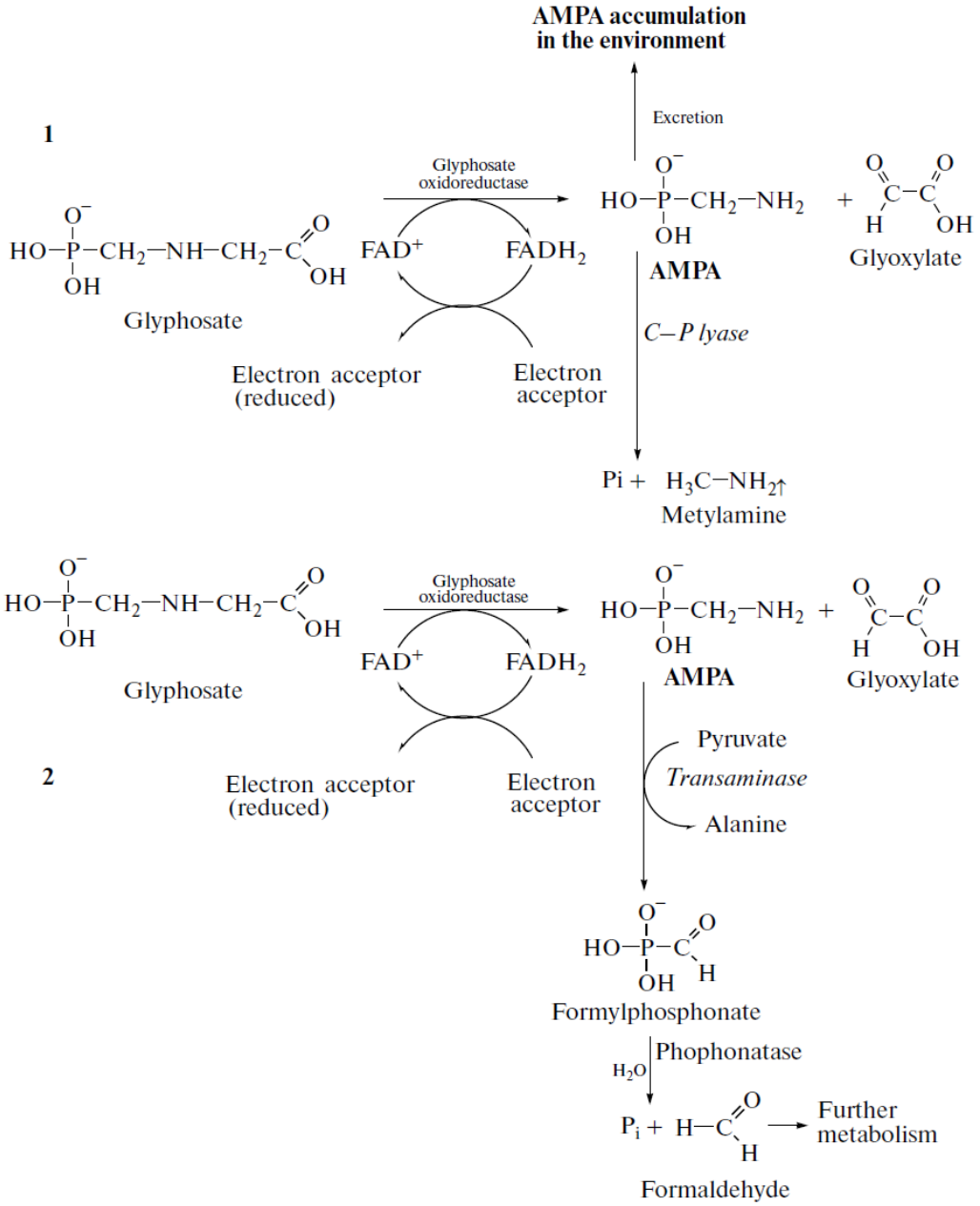

Fig 1.2 GOR pathway of glyphosate metabolism, best known in bacteria (From Sviridov et al., (2015))

1.3. Herbicides and Soil Health

In natural undisturbed soil, there is a delicate and sustained interdependence between beneficial soil microorganisms in the rhizosphere and their plant hosts. Mendes *et al*. (2013) view a plant as a super-organism that supplies soil organisms in its rhizosphere with photosynthates in exchange for growth limiting

elements and protection. These organisms are attracted by root exudates (Berendsen *et al*., 2012) and display a variety of benefits. They include symbiotic and free living nitrogen fixing bacteria as well as arbuscular mycorrhizae that supply nutrients to the plant, plant growth promoting bacteria (PGPRs) such as *Bacillus* spp. and *Pseudomonas* spp. which stimulate plant growth, and fungi that control or inhibit potential plant pathogens such as *Trichoderma* (Hayat *et al*., 2010). The total sum of the effects of the various beneficial rhizosphere microorganisms is the alleviation of biotic and abiotic stress on the plant, such as soil salinity, drought, flooding, low temperatures, low pH and toxic compounds. The biodiversity of these organisms is directly coupled to plant productivity and ultimately crop yield (Mendes *et al*., 2013). Kremer *et al.* (2005) reported that the use of glyphosate containing herbicides increased the biomass of various *Fusarium* spp., as well as that of a *Pseudomonas* strain, while *B. japonicum* numbers were adversely affected due to the exudation of glyphosate through the roots. Banks *et al.*, (2014) reported little effect after a single application, but warned that more studies need to be done when multiple applications are used.

Soil can be defined as the upper layer of the earth, consisting of mixture of organic degradation products, clay and rock particles. The amounts of these components can vary with very short distances of each other, and in South Africa 73 different types of soil are recognised (Frey, 2010). Glyphosate is strongly adsorbed by inorganic soil components often in competition with inorganic phosphates (Gimsing *et al*., 2003; da Cruz *et al*., 2007) and strongly correlated to pH. More glyphosate adsorbed at lower pH levels. Gimsing *et al*. (2003) also found organic carbon, clay content and mineralogy had no effect on glyphosate adsorption. Clay content and surface area plays a role in glyphosate adsorption (da Cruz *et al*., 2007). and Bergström *et al.* (2011) found that glyphosate degradation was slower, 110-151 days half-life, in clay soils, with low levels of leaching in both clay and sandy soils. Clay content varied from 1.3 in the topsoil to 60.6% at 60-90 cm.

Many agricultural chemicals have been shown to have a negative effect on soil arthropods. Förster *et al.* (2006) observed a decrease in numbers of two species of earthworm after field application of carbendazim and labda-cyhalothrin. These chemicals, however, had no effect on soil arthropods. The insecticides chlorpyrifos and dimethoate were found to be fatal to Collembola in field studies carried out by Endlweber *et al.* (2006), while El-Naggar and Zidan (2013) reported

varying effects of imidacloprid and thiamethoxam (insecticides) on arthropods, increasing populations of Collembola while decreasing those of the Arcaria. Although numbers of arthropods are initially decreased by the use of the organophosphate, dichlorov, they recover within five months after treatment (Iloba & Ekrakene, 2009). Lins *et al*. (2007) found similar effects after use of glyphosate on a zero-tillage production system. Glyphosate applied to soybeans twice, as recommended, had no effect on the biomass or diversity of soil arthropods (House *et al*., 1987). Nakamura *et al.* (2008) studied the use of glyphosate on weedy undergrowth during reforestation and found that even using levels higher than recommended had no negative effect on the soil arthropods. Earthworms placed in soil containing glyphosate in soil microcosms did not die, but showed a 50% decrease in body mass, although these results have not been duplicated under field conditions (Correla & Morelra, 2010). Genotoxic work showed that glyphosate does not cause gene mutations in earthworms (Muangphra *et al*., 2014).

1.3.1 Endophytic root symbionts

The Leguminosae have the ability to attract and form a stable symbiosis with an endophytic group of root bacteria (Beringer *et al*., 1979). These rhizobium bacteria infect the plant which then form a protective nodule on the root where the bacteria fix atmospheric nitrogen (N_2), and then convert it to ammonia (NH_4^+) and nitrogen dioxide (NO_2) via the nitrogenase enzyme. They supply excess ammonia to the plant that converts this into proteins. The plant also provides the bacteria with photosynthates, mostly in the form of carbohydrates. Legumes consequently do not need nitrogen fertiliser and usually have a heavier and longer root system that contributes nitrogen to the soil when the roots degrade after harvest (Fustec *et al*., 2010).

Beneficial endophytic symbionts need to successfully infect host plants, survive, thrive and reproduce. Some symbionts also need to survive in the soil or in alternative hosts, especially in seasonal crops. These organisms have to be fit enough to survive in the soil without triggering inhibitory reactions in the host (Denison & Kiers, 2011). Their success depends largely on abiotic conditions in the rhizosphere, such as temperature, pH, soil structure and absence of toxic substances.

Although undisturbed soils are normally rich in beneficial microbes, agricultural soil often becomes depleted through constant cultivation and the use of pesticides and fertilisers. Endophytic symbionts such as rhizobia, arbuscular mycorrhizae and *Trichoderma* spp. not only have a direct effect on the host crop, but increase soil microbe diversity and the general health of agricultural soil. It is, therefore, critical to ensure that agricultural practices do not have any negative effects on these beneficial micro-organisms (Mosttafiz *et al*., 2012).

For symbiosis to be successful, the plant needs to attract suitable microorganisms. This is achieved by exuding nutrients produced by photosynthates through its roots into the soil. Microorganisms are attracted along a nutrient gradient and move through the soil moisture towards the roots. This process, known as chemotaxis, can be controlled by the plant according to its needs, as well as in response to environmental pressure. A large variety of chemicals are released, including sugars, phenolics, amino acids and the flavonoids that rhizobia known to use to initiate infection (Chaparro *et al*., 2013). The type and concentration of the exudates varies throughout the life-time of the plant and it has been estimated that between 5% and 21% of carbon fixed by photosynthesis are moved to the rhizosphere by root exudates (Walker *et al*., 2003).

All amino acids and carbohydrates required for the growth, maintenance and reproduction of a plant is produced by photosynthesis, while water and the essential elements needed in these processes, including nitrogen, phosphate, sulphate, potassium and the microelements, are absorbed from the soil by the roots. Some of the photosynthate carbohydrates are produced in excess, and are exuded through the stems and leaves, as well as through the roots (Wollenweber & Jay, 1988). Root exudates migrate into the rhizosphere soil and become a source of nutrients for microorganisms (Walker *et al*., 2003). Strigolactones and flavonoids exudates have been shown to stimulate the germination of *Fusarium* spores (Steinkellner *et al*., 2009). Strigolactones also cause increased branching of arbuscular ectomycorrhizae, thereby increasing their ability to infect roots (Steinkellner *et al*., 2007).

1.3.1.1 *Rhizobium* spp.

The rhizobium associated with soybean, *Bradyrhizobium japonicum* Jordan, is commercially produced throughout the world and is constantly being tested for its

ability to aggressively infect and colonise soybean plants, stimulate the formation of nodules and fix atmospheric nitrogen. Supplied in either powder or liquid form, inoculation of soybean seed with the correct bacterial strain before planting each season ensures a high yielding, oil-rich seed harvest (Martin *et al*., 2006) and the release of bacteria and nitrogen from root residues into the soil at the end of the season.

Bradyrhizobium japonicum is attracted by flavonoid root exudates and exclusively infects soybean roots stimulating the plant to form a nodule in which a single infective bacterium multiplies to numbers exceeding 10^8 (Denison & Kiers, 2011). Inside the nodule, bacteria differentiate into distinct cell types called bacteriods which then actively fix atmospheric nitrogen as ammonia that the plant can use for the production of proteins (Oke & Long, 1999). The nodule also stores polyhydrozybuterate (PHB) and phosphates for the benefit of the bacteria. Once the nodule senesces or the plant dies, both the bacteria and bacteriods are released back into the soil. In soils where soybeans occur naturally, or are regularly planted, the numbers of bacteria are sufficient to successfully inoculate and supply the soybean plant with adequate nitrogen for the growth season. In areas where soybeans are planted for the first time, the seeds have to be inoculated with the correct strain of *B. japonicum* (Thelen & Schulz, 2011; Solomon *et al*., 2012).

It is advisable that soybeans be inoculated every time to negate any die-off of the rhizobia in the soil during the intervening season/s (Singleton & Tavares, 1986). This will increase the number of potential nitrogen-fixing nodules, especially where the native population of rhizobia may be numerous and infective, but not capable of efficient nitrogen fixing. Thies *et al*. (1991) carried out field experiments to assess the effect of the size of the indigenous population of rhizobia on inoculation and yield increase on some legumes. They found that high numbers of infecting, but inefficient native rhizobia, led to inoculation failing, and recommended further studies into effective inoculation of the correct rhizobium strain for the specific legume.

1.3.1.2 Arbuscular mycorrhizae

Arbuscular mycorrhizal fungi (AM) belong to the order Glomales (Glomeromycota), and occur worldwide in obligate symbiosis with most plants exhibiting true roots. Hyphae of the fungi penetrate the root cortex cell walls, forming haustoria-like structures inside the cells. These structures increase the surface area

of contact between the fungus and host cytoplasm. Active exchange of the nutrients P, N and C, between the plant and the fungus takes place here (Brundrett, 2002). Hyphal growth outside the plant root extends the potential reach of the roots into the soil, thus placing more micro- and macro-elements within reach of the plant. These hyphae play a role in the formation of soil aggregates, leading to a more stable soil structure and, thereby, increasing the water holding ability of the soil (Denison & Kiers, 2011; Johansson *et al*., 2004). Whiteside *et al*. (2012) determined that AM can access and remove recalcitrant and volatile N from organic debris, and make it available to its host in much larger concentrations that previously thought.

Arbuscular mycorrhizae are totally dependent on the plant host for photosynthetic carbon products, and have to be fit to successfully infect and colonise the host. In addition, they produce hyphae outside the root to access phosphates and nitrates, as well as micronutrients up to a 100 x more effectively than plant roots. Depending on the species of AM, storage vesicles containing lipids are formed either inside the roots or on the hyphae outside the roots (Smith *et al*., 2011). Multinucleate spores are formed on the external hyphae and can persist viably in the soil for up to ten years. These spores repeatedly extend and retract hyphae in search for a suitable host. This process entails the formation of primary hyphae, from which the cytoplasm is retracted back into the spores if no contact is made with a potential host root (Denison & Kiers, 2011).

Arbuscular mycorrhizae confer protection against pathogens to the host plant. The exact mechanism is still unknown, but it has been postulated that a high rate of colonisation can drastically reduce the amount of available infection points on the roots (Denison & Kiers, 2011). Lendzemo *et al*. (2007) reported lower rates of germination and attachment of *Striga hermonthica* (Del) in mycorrhizal sorghum. Various observations have shown that the presence of AM in the soil enhances nodulation of legumes. Increased P levels in the plant enhances N fixation, leading to increased root production and yield, while increased N fixation increases AM infection and growth (Johansson *et al*., 2004; Meghvansi *et al*., 2008; Wang *et al*., 2011; Gao *et al*., 2012;). Opinions differ as to the economic value of inoculating field crops with AM (Hayman *et al*., 1981; Lendzemo *et al*., 2005; White *et al*., 2008). It is, therefore, critical to maintain healthy soils that contain sufficient numbers of AM for natural inoculation to take place (Wang *et al*., 2011).

1.3.1.3 *Trichoderma* spp.

Trichoderma is an Ascomycete genus in the order Hyprocreales. Present in most soils, they are often the most prevalent group of fungi during sampling. Symbiosis occurs with many plant species., where the fungus forms an endophytic mutualistic relationship with a plant (Bae *et al*., 2011) Members of *Trichoderma* are also often isolated from bark and is also found growing on other macro fungi.

A variety of *Trichoderma* spp. have been extensively tested as biocontrol agents against plant disease. The most successful of these are *T. harzianum, T. viridae* and *T. hamatum* (Howell, 2003). In the early 1930's, it was postulated that *Trichoderma* reduces certain plant diseases by means of mycoparasitism. The fungal hyphae coil around the target fungus, penetrating the host hyphae and leading to dissolution of the host. As this was observed independent of external nutrients, *Trichoderma* used this as a method of growth and procreation (Howell, 2003; Cao *et al*., 2009; Badar & Qireshi, 2012). Effectiveness of a particular species may differ at different soil temperatures, in the presence of other rhizo-organisms, and under different soil and climatic conditions. The best solution is to isolate *Trichoderma* from an area with soil and climatic conditions similar to the site where it will be used (Howell, 2003). Various researchers have demonstrated the presence and effectiveness of kinases and proteases produced by *Trichoderma* in suppressing pathogenic fungi. *Trichoderma* has also been demonstrated to have the ability to metabolise fungal germination stimulants produced by cotton seed. In the absence of these compounds, pathogenic propagules did not germinate as well as when the compounds were present, and disease incidence dropped (Howell, 2003; Jantarach & Thanaboripat, 2010; Badar & Qureshi, 2012).

The ability of a rhizosphere organism to exploit a niche and flourish depends on its competence and ability to outgrow other organisms. When applied to the soil or a seedling, *Trichoderma* has shown the ability to outgrow other fungi and fully colonise plant roots. This is, however, difficult to prove exclusively, although selective fungicides active against only the *Trichoderma* could be used to prove suppression (Howell, 2003). Infection of roots by *Trichoderma* has been shown to lead to increased production of peroxides and chitinase, as well as callose enriched wall deposits in both the roots and leaves. Various terpenoids were also produced at higher levels after *Trichoderma* infection (Howell, 2003).

It has repeatedly been observed that the presence of *Trichoderma* spp*.* in soils improve the general growth, appearance, and well-being of plants. By stimulating root growth and the production of plant hormones, this endophytic fungus decreases plant stress and contributes to overall plant health (Badar & Qureshi, 2012; Sharma *et al*., 2012). A combination of *Bradyrhizobium japonicum* and *Trichoderma* inoculum stimulated root growth of soybeans more effectively than either of these in isolation (Howell, 2003; Deshmukh *et al*., 2016). Increase in root growth widens the area from which nutrients can be extracted, leading to an increase in the availability of micronutrients essential for healthy strong growth, leading to larger leaf area and increased photosynthetic activity.

1.3.2 Complexes between symbionts on soybeans

Bethlenfalvay *et al*. (1985) postulated that rhizobial infection of legume roots, followed by AM infection resulted in increased nodulation and phosphate uptake by the plant. AM do not seem to infect the nodules at all, maybe indicating an exclusion agent. This correlates with the procedure of inoculating seeds with rhizobia, while leaving the AM inoculation to fungi present in the soil (Powell *et al*., 2009). As the rhizobia are in the direct vicinity of the germinating seedling's roots, they would infect earlier than the AM that has to be attracted by the root exudates in order to find the root and start infection. The presence of rhizobia nodules does not seem to cause any inhibition to AM infection. Xie *et al*. (1995) found that nodule formation is not compulsory for AM infection. The presence of *B. japonicum* in the rhizosphere was enough to stimulate either an increased rate of infection or an increase rate of penetration growth of the hyphae. Powell *et al*. (2009) showed that the use of glyphosate at recommended rates had no ultimate effect on either microorganism or the plants.

Badar and Qureshi (2012) reported increases in the plant length and dry mass of beans infected with both the correct *Rhizobium* spp*.* and *Trichoderma hamatum*. Chlorophyll, carbohydrates and crude proteins were all at higher levels in the plant treated with both microbes, compared to the controls. In pot trails, Haque & Ghaffar (1992) applied rhizobia and *Trichoderma* spp*.* to Fenugreek and found that the combination of microbes reduced *Macrophomina phaseolina* (Tassi) Goid. infection in the plants by 50% compared to the controls and complete control was effected on *Rhizoctonia solani* Kühn.

Worldwide, legume crops such as *Vicia fabae, Cicer arietinum* and *Lupinese terms*, play a critical role in supplementing mainly starch rich staple diets with protein. Under mono-cropping, these legumes can be devastated by damping-off and root rot diseases caused, *inter alia,* by *Fusarium oxysporum, F. solani, M. phaseolina, R. solani* and *Sclerotinia rolfsii*. In glasshouse trials, Shaban and El-Bramawy (2011) showed that *Rhizobium leguminosarum* and *Trichoderma harzianum* both showed individual antagonism to all of these pathogens in varying degrees. However, when used in combination, greater degrees of antagonism were found for all the pathogens. The combined use of beneficial microbes led to improved survival rate of the legumes, an increase in plant height and number of branches, a higher number of pods, and an increase in the mass of seed harvested per plant. In addition to this, the use of *R. leguminosarum* eliminates the need for nitrogen fertilisers and increases the microbial biomass. *Trichoderma harzianum* increases phosphate flow in plants and its hyphae contribute to healthy soil aggregation (Powell *et al*., 2009).

1.3.3 Effect of herbicides on root symbionts of soybean

The inoculation of commercially planted legumes with rhizobia has become a standard operation in modern agriculture. However, the use of agricultural chemicals for fertilisation and pest control has become as essential as inoculation, and some of these practices may prove detrimental to rhizobia (Fox *et al*., 2007). Singh and Wright (2002) tested four herbicides containing terbuthylazine, simazine, prometryn as well as bentazone, and found that when used at recommended rates, had no effect on rhizobia in the laboratory. Sawicka and Selwet (1998) found that the pesticides, Imazethapyr (Pyvot 100) and linuron (Afalon), used in recommended dosage had an adverse effect on the activity of dinitrogen fixing in legume crops. They postulated that these pesticides may possibly have detrimental effects on the crop and/or the rhizobia. Fox *et al*. (2007) tested a range of agricultural chemicals in the field and found that methyl parathion (insecticide), dichlorodiphenyltrichloroethan (DDT insecticide), bisphenol A (environmental contaminant) and pentachlorophenol (insecticide), applied at recommended rates, all negatively affect the amount of nodules formed, as well as seedling biomass and total plant yield.

The development and use of a kill-all herbicide for use on genetically modified crops has evoked much debate in scientific as well as the lay communities, with many articles published concerning the possible negative effects on rhizobia during the use of glyphosate containing herbicides. Opinions range from reduction of numbers of rhizobia, to the inability of rhizobia to inoculate after exposure (Kremer and Means, 2009), to results showing no effect on either the bacteria or the plant (Drouin *et al*., 2010). Zobiole *et al*. (2010e) found that application of glyphosate significantly reduced nodule number, dry mass of above ground material and roots, and nodule mass in comparison to plants that had not been treated with glyphosate. They attributed these results to chelation of Ni in the soil by the herbicide, Ni being essential for nitrogen fixing by rhizobia. Duke *et al*. (2012) indicated that with correct usage, glyphosate binds so strongly to most soils that little is left to interact with metals. Kremer (2008) stated that the nodule numbers were always lower on RR soybeans when compared to non-RR soybeans, and attributed this to the presence of the gene. He also stated that the roots of soybeans that had been exposed to glyphosate always had a higher level of *Fusarium* infection than those not treated with the herbicide.

Field studies carried out by Means *et al.* (2007) showed no influence on soil microorganisms after the application of glyphosate to RR® soybeans. Powell *et al*. (2009) found that the use of glyphosate containing herbicides had no effect on the ability of rhizobia to infect the RR® soybean roots when applied at the recommended rates. Drouin *et al*. (2010) tested a variety of pesticides against 122 strains of rhizobia and found that glyphosate had no effect on these bacteria.

Abd-Alla *et al*. (2000) noted that a range of chemical pesticides inhibited root colonisation by AM, though these effects varied at different growth stages and with different plant species. Testing of biopesticides indicated that some of them can have serious deleterious effects on AM root colonisation and cause a shift in the AM community (Ipsilantis *et al*., 2012). Direct application of glyphosate to soil caused a significant decline of spore viability, and *Lolium multiflorum* (Lam.) planted in the treated soil showed a decrease in root colonisation (Druille *et al*., 2013). Sheng *et al.* (2012) reported similar findings, but neither of these authors looked at the ultimate effect on crop yield. Pasaribu *et al.* (2013) reported that glyphosate did not show significant effects on the AM when used at recommended rates, which is consistent with findings by Mujica *et al.* (1999).

Various *in vitro* studies have shown that certain *Trichoderma spp.* are sensitive to a range of pesticides including phosalone, amitraz, ethalfluralin, malathion, linuron, paraquat and glyphosate, but in each case glyphosate was the least toxic (Wilkinson & Lucas, 1969; Santoro *et al*., 2014; Mohammadi & Amini, 2015). Studies on the effect of glyphosate on soil fungi showed no effect on *Trichoderma* (Islam & Ali, 2013; Meriles *et al*., 2006), with Arfarita *et al*. (2013) demonstrating that *Trichoderma* can be used in the bioremediation of soils heavily contaminated with glyphosate.

1.4. Soilborne pathogens of soybean

Monoculture systems, where the same crops are planted on the same soils year after year, can lead to the build-up of pathogen populations resulting in catastrophic yield losses. Huang *et al*. (2002) reported that monoculture of kidney bean lead to reduced yield in addition to a significant increase in *Pythium* damping-off and *Fusarium* yellow, caused by *F. oxysporum*. Pérez-Brandán *et al*. (2014) reported that the incidence of both sudden death syndrome (SDS) (*F. crassistipitatum* Scandiani) and charcoal rot (*Macrophomina phaseolina* Tassi), were 7 to 35 x higher in soya monocultured for 24 years than in the soybean-maize rotation. Even when soybean mono-cropping is carried out in rotation with non-susceptible crops, certain root disease such as SDS can persist over a number of years and cause disease when soya are planted a year or two later (Babadoost, 2002). In South Africa, seedling death can vary from 50 - 65% with more than 60% of isolations from these root lesions identified as *Fusarium*, mostly *F. oxysporum.* Other fungi frequently isolated from soybean in South Africa include *Gliocladium* spp., *Phoma* spp., *Macrophomina* spp*., Phomopsis* spp., *Rhizoctonia* spp., *Alternaria* spp., *Trichoderma* spp., *Pythium* spp., *Sclerotium* spp. and *Sclerotinia* spp. A direct negative correlation was found between mono-cropping and the virulence of certain fungi. Most of the soil borne disease causing organisms found in South Africa are also known to cause disease worldwide, with a few being reported for the first time on soybeans (Tewoldemedhin, 2013).

Arthropods are also often a serious problem, both in terms of herbivory causing injuries to a crop allowing entrance for pathogens, as well as being instrumental in the transfer of inoculum during feeding. Once the crop is harvested, many species. of arthropods use weeds as alternative hosts and as repositories for

their eggs and larvae which will emerge as soon as the next crop emerges (Norris & Kogan, 2005). Many microbial plant pathogens use weeds as hosts or intermediaries and if susceptible, the health and ultimate yield of the crop plants can be seriously compromised. These weeds also increase inoculum in the soil, leading to the possible compromising of follow-up crops, even if the current crop is not susceptible (Cobb & Reade, 2012). The most common soybean pathogens are shown in Table 1.

1.5. Genetically modified soybean

Roundup Ready® GMO soybeans express a bacterial variant of the cp4-epsps gene encoding for the formation of EPSPS, which is immune to the effect of glyphosate on the wild-type EPSPS activity, thereby allowing for use of this herbicide on genetically modified soybeans (Tu *et al*., 2001). EPSPS acts as a catalyst in an intermediary step in the Shikimate pathway that leads to the production of phenolic compounds (Hernandez *et al*., 1999). It is unknown how exactly the cp4-epsps gene can affect the concentration of phenolic compounds in the modified plant. These compounds are used as chemical messengers in the rhizosphere and a change in the levels of phenols may have unexpected consequences on soil micro-organisms and the crop (Powell *et al*., 2007). Motavalli *et al*. (2004) expressed concern over the possible effect of root exudates of genetically modified crops on the rhizosphere microbiome, as well as on the structure and nutritional value of soil. This may lead to fewer beneficial microbes being recruited, thereby and making infection sites available to pathogens (Berendsen *et al*., 2012).

Table 1. Common diseases of soybean

Symptom	Organism	Symptoms	Treatment	Reference
Bacterial blight	*Pseudomonas glycines*	Leaf spots, necrotic. Seed borne, leaf litter	Crop rotation, incorporate residue	**1**
Bacterial pustule	*Xanthomonas phaseoli*	Leaf spot, necrotic. Seed and leaf borne	Crop rotation, incorporate residue	1
Wildfire	*Pseudomonas tabaci*	Black/brown spot, yellow halo, with bacterial pustules	Resistance	2
Sclerotinia stemrot	*Sclerotinia sclerotiorum*	Blossoms, leaves grey-green. Plant residue/soil	None	1
Phytophthora root/stem rot	*Phytophthora sojae*	Seedling damping off, leaves yellow, wilt from stem. Secondary infections	Resistance	1
Brown stem rot	*Cephalosporium grgatum*	Decay inner stem	Rotation, 2-3 no soya	1
Stem canker	*Diaporthe phaseolorum var batatis*	Girdle stem	Resistance	1
Pod and stem blight	*Diaporthe phaseolorum var sojae*	Girdle stem, infect seed, stem and seed borne	Rotation, clean seed	1
Frog eye leaf spot	*Cercospora sojina*	Leaves, stem, pods, seeds lesion, residue	Clean seed, Resistance	1
Brown spot	*Septoria glycines*	Leaves, stem, pods, defoliation	Rotation, incorporate residue	1
Target spot	*Corynespora casiicola*	Reddish brown spots	Resistance	3
Sudden death syndrome	*Fusarium virguliforme*	Chlorotic mottling of leaves, loss of leaves	Seed treatment	1
Downy mildew	*Peronospora manchurica*	Infected seed, growth point mildewed	Rotation, incorporate residue, clean seed	1
Mosaic	Virus	Very common, leaves puckered, seed/pod reduced, aphids	None	1
Purple stain	*Cercospora* spp	Seed discolour	Clean seed	1
Sclerotinia blight	*Scleritinia rolfsii*	Rot base of stem	None	1
Charcoal rot	*Macrophomina phaseoli*	Rot base of stem, in soil	None	1
Soybean stunting	*Pythium debaryanum*	Dry necrotic lesions, stunting	Resistance	4
Soybean rot	*Rhizoctonia solani*	Pre and post emergence damping off	Crop rotation	1
Cotton root rot	*Phymatotrichum onmivoerium*	Plant dies suddenly in summer	Crop rotation	5
Anthracnose	*Glomerella glycines*	Stunting, yellow leafs	Resistance	1
Fusarium blight	*Fusarium oxysporum f. tracheiphilum*	Roots darken, decay, foliar stunting chlorosis	Resistance	1
Nematodes	**Organism**	**Symptoms**	**Treatment**	
Root knots		Stunted, rot	Rotation	1
Cyst nemtodes	*Heterodera glycines*	Stunted, leaves premature yellow	Rotation, prevent soil movement	1
Insects	**Organism**	**Symptoms**	**Treatment**	
Leaf feeding	Two-spotted spider mite Tetranychus urticae	White/yellow spots on leaves, webbing, leaf die	Monitor, single insecticide application	6
	Bean leaf beetle *Cerotoma trifurcata*	Defoliation, pod scarring, larvae roots, nodules, spread mottling virus	Knock-down insecticide, residual control	7
Pod flower feeding	Bollworm, stink bugs, grasshoppers,	Yield reduction	Foliar insecticide, natural enemies	8
Stem feeding	Three cornered alfalfa hopper, weed borer	Yield reduction	Seed treatment	9
Seed, seedling feeder	Seed corn maggot, seed corn beetle, wireworm	Cotyledon destruction	Seed treatment, soil insecticide	10

1. Wrather *et al*., 2010
2. Myung *et al*., 2009
3. Xavier *et al*., 2013
4. Strissel and Dunleavy, 1970
5. Gaxiola *et al*., 2010
6. Bueno *et al*., 2009
7. Hadi *et al*., 2012
8. Boethel *et al*., 2000
9. Reed and Delaney, 2010
10. DiFonzo and Jewett, 2006

1.5.1 Effect of gene on RR® soybean plants

Studies have shown that RR® soybeans treated with glyphosate, differ in various ways from RR® soybeans not treated with the herbicide. A decrease in polyunsaturated linoleic acid and linoleic acid was demonstrated, with associated increases in monosaturated fatty acids (Zobiole *et al*., 2010e). It was deduced that this was due to disturbance of photosynthesis, but the ultimate effect of these differences on the nutritional value on the grains remains unknown. Application of glyphosate early in the growth season seems to have less effect than when applied later during flowering (Zobiole *et al*., 2010b). This was also shown to be the case when the production of amino acids and the lignin content of the plant was measured after increased doses and more frequent applications of glyphosate (Zobiole *et al*., 2010a). Glasshouse studies indicated that early maturing soybean cultivars were more sensitive to glyphosate injury. This may be ascribed to the light sensitivity of these cultivars, as experiments were carried out under natural light (Zobiole *et al*., 2010d; Albrecht *et al*., 2012). Zobiole *et al*. (2010c; 2011) also reported that RR® soybeans would probably need additional nutrition such as exogenous amino acids to negate any deficiencies caused by use of the glyphosate, while Loeckner *et al*. (2009) found that the response of RR® soybeans to Mn application was due to cultivar differences and not influenced by the presence of the gene. Increasing usage of glyphosate and application at a later growth stage has the greatest effect on nutrients in the plant, nodulation, the size of the leaf area, and shoot mass (Zobiole *et al*., 2010d). Zobiole *et al*. (2012) advises use of minimum amounts of glyphosate applied less frequently and at earlier growth stages to prevent such deficiencies. It is, therefore, incorrect application of the herbicide, including too many applications, rather than the herbicide itself that contributes to the problems observed.

Both *B. japonicum* and AMs are highly dependent on root exudates to establish symbiosis with soybeans. A drastic change in the type or amount of phenolic compounds released by the plant may have a detrimental effect on the efficacy of the symbiosis of one or both of the microbes, leading to possible reduced yield and lower nitrogen concentration in the seeds produced (Powell *et al*., 2007). Pot trails by these authors showed no significant differences in nodulation or infection between genetically modified and conventional soybean lines.

A large number of researchers have demonstrated the effect of glyphosate on the efficacy of microbial infection and symbiosis, however, little consensus has been reached as summarised in the review by Wolman's and Swart (2014). Results from such studies found cases of both increase and decrease of diseases under certain conditions, due to the influence of glyphosate on the microbes in the soil. Few papers specify the formulation of glyphosate used, and even fewer explore the potential toxicity of the additives in the herbicide mix (Powell *et al*., 2009; Zablotowicz and Reddy, 2004). In many cases, the RR® soybean is not compared to its non-RR® line and any variations might be actually due to cultivar differences. Chelating properties of glyphosate can affect the availability of Ni to the rhizobia, but amelioration of the mineral will solve this problem (Zobiole *et al*., 2010e). Neither the presence of the gene nor use of glyphosate has any effect on mineral nutrition available to the plants, nor the yield of GM crops (Duke *et al*., 2012).

1.5.2 Effect of gene on pathogen resistance after exposure to glyphosate

Since glyphosate interferes with the shikimate acid pathway and the production of aromatic amino acids, it has been postulated that both the presence of the gene, and the use of glyphosate may impact negatively on the resistance of the plant to fungal and bacterial diseases (Keen *et al*., 1982). Sanogo *et al*. (2000) reported that both RR® soybeans and cultivars without the gene reacted similarly to infection by *F. solani* f. sp. *glycines* after treatment with glyphosate containing herbicides. This is consistent with Njiti *et al*. (2003) who found that the occurrence of *F. solani* was coupled to the soybean cultivar used and not to the herbicide itself. Experiments carried out in sterile soil with *Amaranthus rudis* suggested a possibility of higher infection by *Fusarium spp.* (Rosenbaum *et al*., 2014). However, the spread of RR® amaranth waterhemp may increase the amount of *Fusarium spp.* in crop production areas (Rosenbaum *et al*., 2014) and could, therefore, create disease problems. Harikrishnan and Yang (2002) also found that environmental factors such as rainfall had a much greater effect on disease caused by *R. solani* on RR® soybeans than use of the herbicide. *Macrophomina phaseolina*, causing charcoal rot on soybeans, especially on dry-land cultivations, showed neither a decrease nor increase (Mengistu *et al*., 2013). Duke *et al*. (2007) reported both increased resistance and increased susceptibility to certain diseases when synthetic herbicides were used on crop plants. Johal and Huber (2009) noted an increase in a variety of

diseases, but concluded that this was due to the misuse of glyphosate. The herbicide was often used on lands heavily infected with weeds, much older than the stages recommended for control on the herbicide label, making them difficult, if not impossible to control. This often leads to the use of higher concentrations of the herbicide. They also recommended the use of integrated pest management, such as tillage, to control weeds and lessen the dependence on glyphosate to control weeds. These weeds are often infected by pathogens or carriers of plant pathogenic organisms, which are released into the soil when they die due to the use of glyphosate (Wolmarans & Swart, 2014). Included in IPM is also rotation of crops, as continual monocropping is known to intensify the presence of plant pathogenic organisms (Duke *et al*., 2012).

1.6. Conclusions

There exists a body of apocryphal claims of glyphosate toxicity to symbiotic organisms. However, the scientific body of knowledge does not support these claims when glyphosate is applied according to the usage recommendations on the herbicide formulation label.

There is no indication of glyphosate resistant soybean plants having a heightened susceptibility to root pathogens after repeated exposure to glyphosate within a season when standard weed reduction techniques are used during crop production. Susceptibility can often be linked to differences between cultivars, rather than use of the herbicide. Nor is there any evidence of an increase in the number of root pathogens in the soil when glyphosate containing herbicides are used in combination with regulated crop rotation. The reported increase in root diseases is probably the result of continual cropping, irrespective of the crop or herbicide applied. The use of glyphosate may have transient effects on microorganisms, but these effects are mitigated by using integrated pest management, as well as healthy crop rotation systems. Agricultural seed and chemical companies recommend that glyphosate resistant cultivars should not be planted for more than three years consecutively before a standard cultivar is planted. This is done mainly to prevent the development of glyphosate resistant weeds, but will also mitigate against any possible negative effects of glyphosate on soil microbes. This discontinuity will thus allow populations of soil organisms to recover prior to the re-application of glyphosate. In the following chapters, a novel approach was thus adopted to

address the conflicting information appearing in the literature. In the first instance it is postulated that glyphosate is detrimental to the symbiotic microbes and may negate the inoculation process. In the second instance it is postulated that glyphosate can affect the resistance of genetically modified soybeans to soilborne diseases, and thirdly, that the presence of the gene leads to a higher susceptibility of genetically modified soybeans to soil pathogens when compared to its non-modified isoline.

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Chapter 2

Effect of glyphosate formulations on symbiotic rhizobia *in vitro* and in pot trails

2.1 Abstract

Rhizobial bacteria form an obligate endophytic relationship with legumes; the bacteria being given shelter within the nodules on the plant roots and the plant benefiting from excess nitrogen produced by the bacteria. This relationship negates or minimises the amount of nitrogen fertiliser needed during the lifetime of the plant. The use of any chemicals or production methods that can harm these bacteria may lead to the need for artificial nitrogen fertiliser with the concomitant costs in both production and labour, as well as an increase in pollution that occurs during the production of the fertiliser. The bacteria were directly exposed to the different glyphosate containing herbicides by infusing yeast extract mannitol agar with the recommended concentrations of the herbicides and plating different dilutions of known numbers of the bacteria thereon. After incubation, the numbers of colonies were determined. The treated bacteria were then collected and used to inoculate the soybean seed. *Bradyrhizobium japonicum* were in some cases affected by direct contact with glyphosate containing herbicides, but the ability to infect the soybean roots were not affected. The ability of the plants to produce nodules were also not affected and, although there were differences in some of the measured parameters, these were similar to results found by other researchers and are believed to not have any effect on ultimate yield.

2.2 Introduction

Glycine max (L.) Merr (soybean) belongs to the legume family, Leguminosae, a unique plant family, able to produce its own nitrogen with the help of symbiotic bacteria. It forms an endosymbiotic relationship with *Bradyrhizobium japonicum* Jordan by supplying shelter and photosynthates to the bacteria, and in return, receiving nitrogen in the form of NH^{+4} used for the production of protein rich vegetative material and grain (Denison, 2000; Hirsch *et al.*, 2001). The rhizobacteria supply nitrogen to the living plant and upon death and decomposition, the nitrogen released into the soil becomes available to the follow-up crop. The biodiversity of

the soil is also enriched by rhizobia released from the decaying nodules (Sylvester-Bradley & Kipe-Nolt, 1988). Soils where legumes have been repeatedly cultivated contain sufficient rhizobia inoculum to spontaneously infect follow-up seedlings. When legumes are planted commercially, it is important to inoculate seed with the correct rhizobia in order to ensure effective nodulation (Khonje, 1980). Damage to rhizobia during production, storage, transport or usage will lead to unsuccessful nodulation, which will necessitate the application of N fertilisers with its concomitant problems such as pollution and high financial input (Dogra & Dudeja, 1993).

Large scale planting of cash crops, such as soybeans necessitates chemical weed control (Zimdahl, 2013). Glyphosate (N-phosphonomethyl glycine) was developed in the 1970's and has since become indispensable in crop production (Battaglin *et al.*, 2014). The herbicide is exclusively used to combat weeds in fields of genetically modified crops including, soybeans, maize and cotton (Dill *et al.*, 2010). Legume farmers depend on rhizobia to supply nitrogen to their crops and inhibition of the rhizobia nodules by herbicides will thus have a negative effect on yield.

Duke *et al.* (2012) reported that glyphosate in the rhizosphere can originate from either root exudates or decaying plant material. Concentration in the rhizosphere is difficult to determine and its effect on the rhizosphere micro-biome is largely unknown. Kremer (2008) found that the number of root nodules were always lower in Roundup® Ready (RR®) soybeans compared to plants without the inserted gene. He observed that glyphosate treated soybeans displayed a higher degree of *Fusarium* infection than untreated plants (Kremer & Means, 2009). Glyphosate significantly reduced the number of nodules as well as the dry mass of plants compared to untreated plants (Zobiole *et al.*, 2010). This is attributed to the chelation of Ni and other nutrients in the soil, which is essential for nitrogen fixation. In contrast, Means *et al.* (2007) indicated no influence on soil microorganisms after the application of glyphosate to RR® soybeans. Powell *et al.* (2009) also found no effect on nodulation or ultimate yield of soybeans when the correct dosage of glyphosate was used. Duke *et al.* (2012), however, indicated that glyphosate binds so strongly to most soils that, with correct usage, very little is left to interact with soil microbes. This in contrast to the findings of Silva *et al*. (2018) that large amount of glyphosate and its breakdown product, aminomethylphosphonic acid on the soil surface can contribute to its dispersal to humans and animals leading to health problems.

Drouin *et al.* (2010) tested a variety of herbicides against 122 strains of rhizobia and found that glyphosate had no inhibitory effect.

The most common manner in which rhizobia come into contact with glyphosate is by spraying an inoculated plant. The first application is usually during pre-emergence, which will ensure that weeds are killed and any glyphosate that reaches the soil is de-activated (Duke *et al.*, 2012). The next application will normally occur six (6) weeks after emergence of the crop by which time most nodules would have been formed and the bacteria are inside the plant. Although commercial rhizobium inoculant should never come into contact with glyphosate, spillage and mishandling could lead to contamination of the bacteria before usage (De María *et al.*, 2006).

Another possible mode of contact is when rhizobia are added to the plant furrow, usually in liquid form. The general assumption is that, if the field had been sprayed with a glyphosate containing herbicide, it could harm the rhizobacteria (Kremer, 1999). Duke *et al.* (2012) observed that glyphosate is quickly inactivated by adhesion to clay and organic particles, and that the label explicitly states that planting should not take place directly after glyphosate application (Monsanto, 2008). Some glyphosate is usually translocated to the roots and then exuded, however, the resulting concentration in the soil has not previously been accurately measured. It is readily adsorbed to the mineral fraction of the soil and thus becomes harmless to the rhizobacteria (Duke *et al.*, 2012). Glyphosate levels in nodules are generally low, and although some inhibition of *B. japonicum* has been detected it had no effect on yield (King *et al.*, 2001).

The present study was conducted to determine the survival rate of rhizobia exposed to various glyphosate formulations *in vitro* and whether such exposure affected their ability to colonise soybeans and efficiently fix nitrogen *in vivo.*

2.3 Material and Methods

Soil for all the experiments were collected from the University of the Free State's experimental farm, Kenilworth near Bloemfontein, South Africa (-29.020397 S; 26.145308 E). The soil had been tilled annually, and was cultivated to conventional maize at time of the sampling. After removal of the growth, topsoil up to 15 cm was collected. The soil is classified as a Kenilworth-Bainsvlei ecotope. It is an example of a fine sandy loam soil in a semi-arid area (Soil Classification Working

Group 1991),. There is no history of glyphosate use. Analysis of the soil is given in Tabels 2.1 and 2.2.

2.3.1 *In vitro* herbicide evaluation of glyphosate

The glyphosate formulations, Slash Plus 540 SL, Slash Turbo 450 SL, Glygran 710 SG (Villa Crop Protection), Roundup® 360, Roundup® Ready Plus, Roundup® Turbo (Monsanto) and Kleen-Up (Environ-Crop) are currently registered for use on soybeans in South Africa and were used in the experiments at recommended concentrations. Herbicides with an acid equivalent (a.e) of 816 g a.e. (pre-emergence) and 5467.2 g a.e. (post-emergence) were placed in sterile 90 mm diameter plastic Petri dishes before approximately 20 mL cooled Congo Red yeast extract mannitol (CRYEM) agar was poured into the Petri dishes and swirled until the herbicide was evenly distributed. Sterile distilled water comprised control treatment.

Bradyrhizobium japonicum WB_{74} cultures, as approved by the Agricultural Research Council of South Africa (Act no 36 of 1947) were used as inoculant. Isolates of *B. japonicum* were maintained on yeast extract mannitol agar (YEMA) slants and inoculum was produced in YEM broth. Congo Red was added to YEMA in 90 mm Petri plates in order to differentiate between rhizobia and other bacteria for the herbicide tests. Rhizobia bacteria do not absorb the Congo Red resulting in cream coloured to white colonies (Vincent, 1970).

The number of bacterial cells in the broth was determined by viewing a 10 µL of a 7-day-old broth culture of *B. japonicum* under a microscope using a haemocytometer. The broth was diluted with sterile distilled water to give a final count of 1 x 10^8 colony forming units (cfu) per mL. This was further diluted in a logarithmic dilution series ranging from 10^{-3} to 10^{-6} in order to achieve the acceptable number of colonies when spread on a Petri dishes. From 30 - 300 cfu per dish is seen as the limit of the number of colonies on a plate that can be counted with ease and that prevents the occurrence of serious errors (Davis, 2014). The range of dilutions was used to compensate for any inhibitory effect by the herbicides.

Aliquots (100 µl) of the diluted broth were pipetted onto each Petri dish after the herbicide containing agar had set and was evenly distributed with a glass rod. The Petri dishes were incubated at 28°C ± 1°C for seven days the dark. The experimental design was a randomised block with six replications per treatment. Petri dishes were turned upside down onto the colony counter with a light and by

using a magnifying glass, colonies were marked by a felt tipped pen. Non-rhizobial colonies that absorbed the Congo red stain added to the agar were coloured red, only cream coloured and white colonies were counted. Colonies that were too small to be counted with ease were further incubated (up to 14 days), until no increase in colony numbers could be observed. Data were statistically analysed by conducting an ANOVA and Fisher's multiple comparison test (GenStat Release 17.1).

2.3.2 Inoculation of seed with treated rhizobia

Soybean seeds from two isolines (A5409 and A5409RG) were used for all experiments performed in a glasshouse maintained at a temperature regime of 25/18°C day and night and with natural sunlight.

Rhizobial colonies were harvested from the Petri dishes containing agar amended with herbicide, by flooding the surface with 1 ml sterile distilled water, scraping the growth off with surface sterilised glass rods, and collecting the liquid in sterile McCartney bottles (10 ml). The resultant broth was used to inoculate soybeans during planting. New black plastic pots (5 L) were filled with a mixture of Bainsvlei loam soil and compost (60:40) that had been steam sterilised and wet to field capacity. Five depressions, approximately 2-cm-deep were made in the soil in each pot and 500 µl of a 10^8 cfu broth of *B. japonicum* that had been exposed to glyphosate *in vitro* were pipetted into each depression. A single A5409 or A5407RG seed was dropped into each depression and covered with soil. The pots were laid out in a complete block design with three replicates per treatment in a glasshouse maintained at a day and night temperature regime of 25/18°C with natural sunlight. Pots were watered as needed and thinned out to three plants per plot once the second trifoliate leaf was fully developed. No observations of yellow flash or other symptoms were noted during the growing phase. The plants were harvested at eight weeks (R1) when nodulation ceases as the plant prepares for its reproductive period (Pedersen, 2009). The number of flowers per plant was noted when the plants were hand harvested, and the aerial fresh mass was determined. Soil was washed off the roots using a series of sieves to retain as much of the root mass as possible and any nodules that may have become detached during harvesting (Nissen et al., 2008). Dry mass of the aerial parts and roots was determined, the nodules were then

removed and weighed. Data were statistically analysed using ANOVA and Fisher's multiple comparison test (GenStat Release 17.1).

2.4 Results and Discussion.

2.4.1 *In vitro* evaluation of glyphosate on *B. japonicum*.

Glyphosate formulations and their recommended concentrations had no significant effect (P≤ 0.05) of reducing rhizobia colonies (Fig 2.3). The results from these experiments are consistent with those of dos Santos *et al.* 2005) and Schütte and Mertens (2010) and confirmed by Duke *et al.* (2012). Apart from accidental spillage of glyphosate on rhizobium inoculant during storage, direct contact is restricted to glyphosate leached from the roots of weeds treated with pre-emergence application of glyphosate. It has been postulated that this is where the rhizobia can come into direct contact with glyphosate (Boggaard & Gimsing, 2008). Barret *et al*. (2007) found that 85–95% of glyphosate was adsorbed to the soil, even when applied at higher than recommended rates. Desorption is very low, making very little of the chemical available to commercial rhizobium inoculant placed in the soil during planting of soybeans.

2.4.2 Inoculation of seed with treated rhizobia

Plants inoculated with rhizobia exposed glyphosate formulations showed no significant differences in aerial mass as compared to the control (Fig 2.4). None of the treatments had a significant effect on the mass of roots when compared with the control (Fig 2.5). Pre-emergence concentration of Kleen Up applied to rhizobia before inoculation had a significant (P≤0.05) effect on nodule mass, but none of the other treatments had a significant effect on nodule mass in comparison to the control (Fig 2.6). To the best of my knowledge no literature exists where rhizobia are pre-exposed to glyphosate before inoculation. However, the possibility of root exudates containing glyphosate accumulating in the soil over time and having an influence on rhizobia has often been discussed (Tu *et al.*, 2001; Huber, 2010; Duke *et al.*, 2012). Tu *et al.* (2001) and Duke *et al.* (2012) found that soil adsorption and microbial breakdown of glyphosate is rapid enough not to influence bacteria in commercial rhizobia inoculant. Suwa *et al.* (2011) reported that field rhizobia that had been exposed to glyphosate over long periods of time produced more nodules, whether these plants were treated with glyphosate or not.

A reduction in aerial mass was noted by Reddy *et al.* (2008) but has been shown to have no ultimate effect on yield. Duke *et al.* (2012) noted that reports of yield reduction were based on questionable research results and emphasises the importance of field work to verify results.

The present trials indicate that although, according to the above, some of herbicides tested had an effect on the growth of *B. japonicum*, the exposed bacteria are still able to infect soybean plants and successfully stimulate nodulation during the lifetime of the plant. Nitrogen fixing was not measured during this experiment, but sampled nodules showed the characteristic red centre, which is an indication of active nitrogen fixing (Downie, 2005). These findings are consistent with those of Duke et al. (2012) who reported that sensitivity to glyphosate containing herbicides of different *B. japonicum* lines varied from no sensitivity to a 50% decrease in bacterial numbers when exposed to the herbicides. Results also show that neither the active ingredient, ie. glyphosate, nor any of the additives in the formulations used, prevented successful nodulation. This is consistent with the early findings of Mallik and Tesfai (1985) and also with subsequent findings by Means *et al.* (2007), Powell *et al.* (2009), Drouin *et al.* (2010) and Duke *et al.* (2012). Zablotowicz and Reddy (2004) and Cassman et al. (1980) also demonstrated that inhibition of rhizobia bacteria had no effect on yield, which is in accordance with the fact that no correlation was found between aerial and nodule dry mass in the present study (Fig 2.5 and 2.6). This could either mean that glyphosate does not influence the total mass of nodules formed, or that there is a maximum limit in nodule mass that no longer influences the dry mass of the plant parts, which is correlated with yield (Skinner *et al.*, 1987; Zablotowicz & Reddy, 2004). There are varying opinions on the exact number or mass of nodules needed for successful nitrogen fixation, but the most general accepted number is 10 large or 15 small nodules per plant. This number is reached early in the vegetative growth of the plant, but nodules keep forming until the first reproductive stage, by which time the plant may contain hundreds of nodules (Staton, 2011; Kabahuma, 2013). Experiments were terminated at eight (8) weeks, thus no comment can be made as to any possible effect on the final yield.

2.5 Conclusions

The strain of *Bradyrhizobium japonicum* (WB_{74}) used in the present study exhibited no detrimental effect from direct exposure to herbicides, and neither was the dry mass of nodules affected. In order to preserve the symbiosis between legume and rhizobia, it is critical to test for bacterial strains that not only have the capability to infect, inoculate and successfully fix atmospheric nitrogen in the root nodules of the legumes, but which are also totally resistant to glyphosate formulations used either pre- or post-emergence. The ability of this herbicide in controlling competition between weeds and crop plants which leads to even germination and growth, as well as easing harvesting, should not interfere with the rhizobial symbiosis. Application of herbicides should, therefore, adhere strictly to the recommendations on the label with regard to time of application, size of the weeds to be treated, waiting time after planting sensitive crops, and the total amount of herbicide applied to tolerant crops during a particular season.

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Table 2.1 Chemical characteristics of Bainsvlei soil (Wolmarans, 2013)

Soil type	pH	Ca (mg/kg)	K (mg/kg)	Mg (mg/kg)	Na (mg/kg)	Zn (mg/kg)	Fe (mg/kg)	Cu (mg/kg)	P (mg/kg)	N (mg/kg)	C (mg/kg)
Bainsvlei	6.02	474.00	120.00	76.00	44.00	1.78	16.36	1.00	19.76	359.20	1390.00

Table 2.2 Textural characteristics of Bainsvlei soil (Wolmarans, 2013)

Soil type	Organic matter%	Silt %			Sand %		
		Coarse	Fine	Clay	Coarse	Fine	Clay
Bainsvlei	0.232	3.330	3.330	3.330	0.670	8.00	81.17

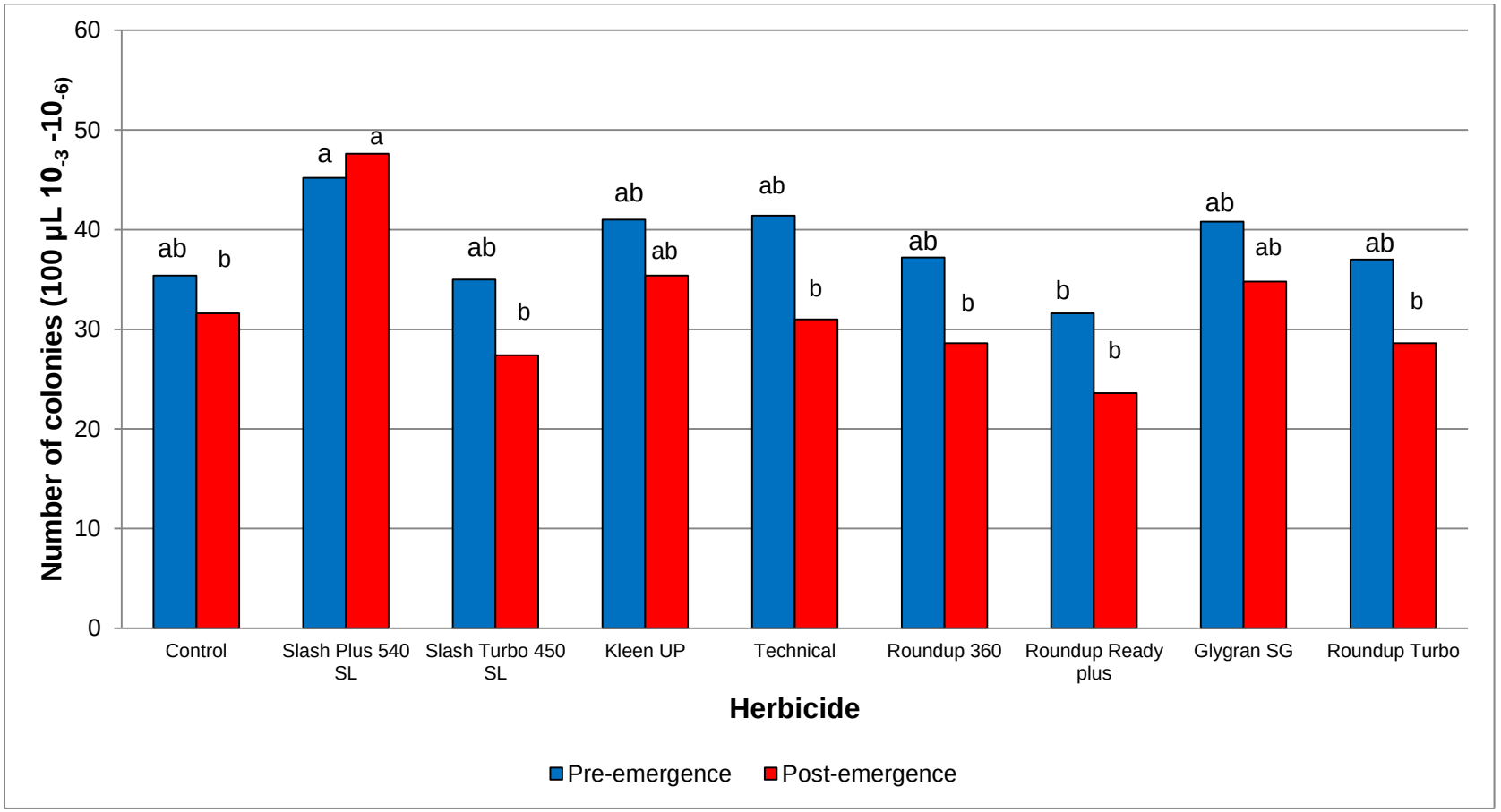

Figure 2.3: Effect of herbicide concentration on rhizobium *in vitro*. Bars with the same alphabetical letters do not differ from each other significantly using means separation of Fischer's LSD (P<0.05) for both pre- and post-emergence concentrations.

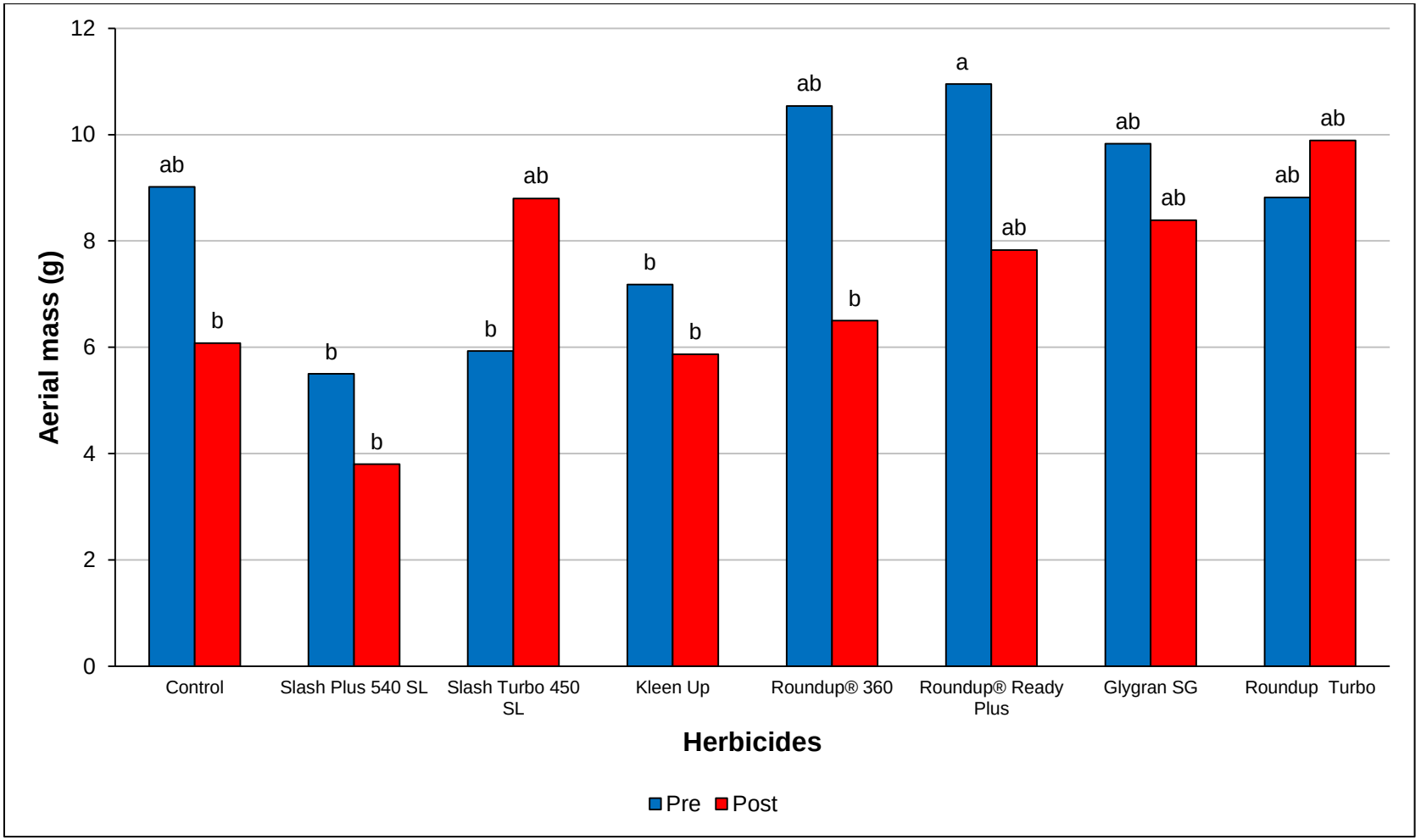

Figure 2.4: Aerial dry mass per plant at 8 weeks after inoculation with rhizobia exposed to two concentrations of glyphosate formulations. Bars with the same alphabetical letters do not differ from each other significantly using means separation of Fischer's LSD ($P<0.05$).

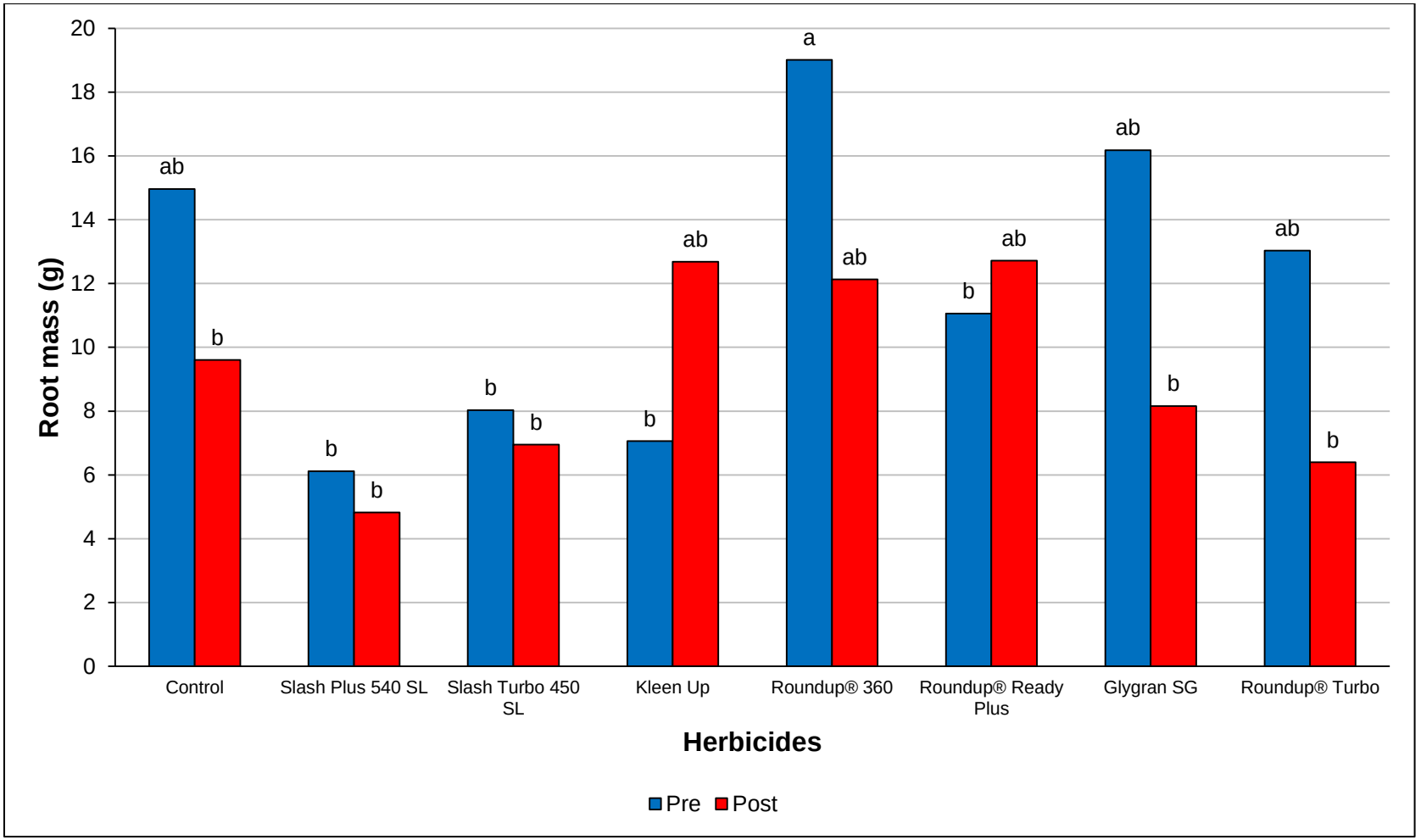

Figure 2.5: Dry mass of roots per plant at 8 weeks after inoculation with rhizobia exposed to two concentrations of glyphosate formulations. Bars with the same alphabetical letters do not differ from each other significantly using means separation of Fischer's LSD ($P<0.05$).

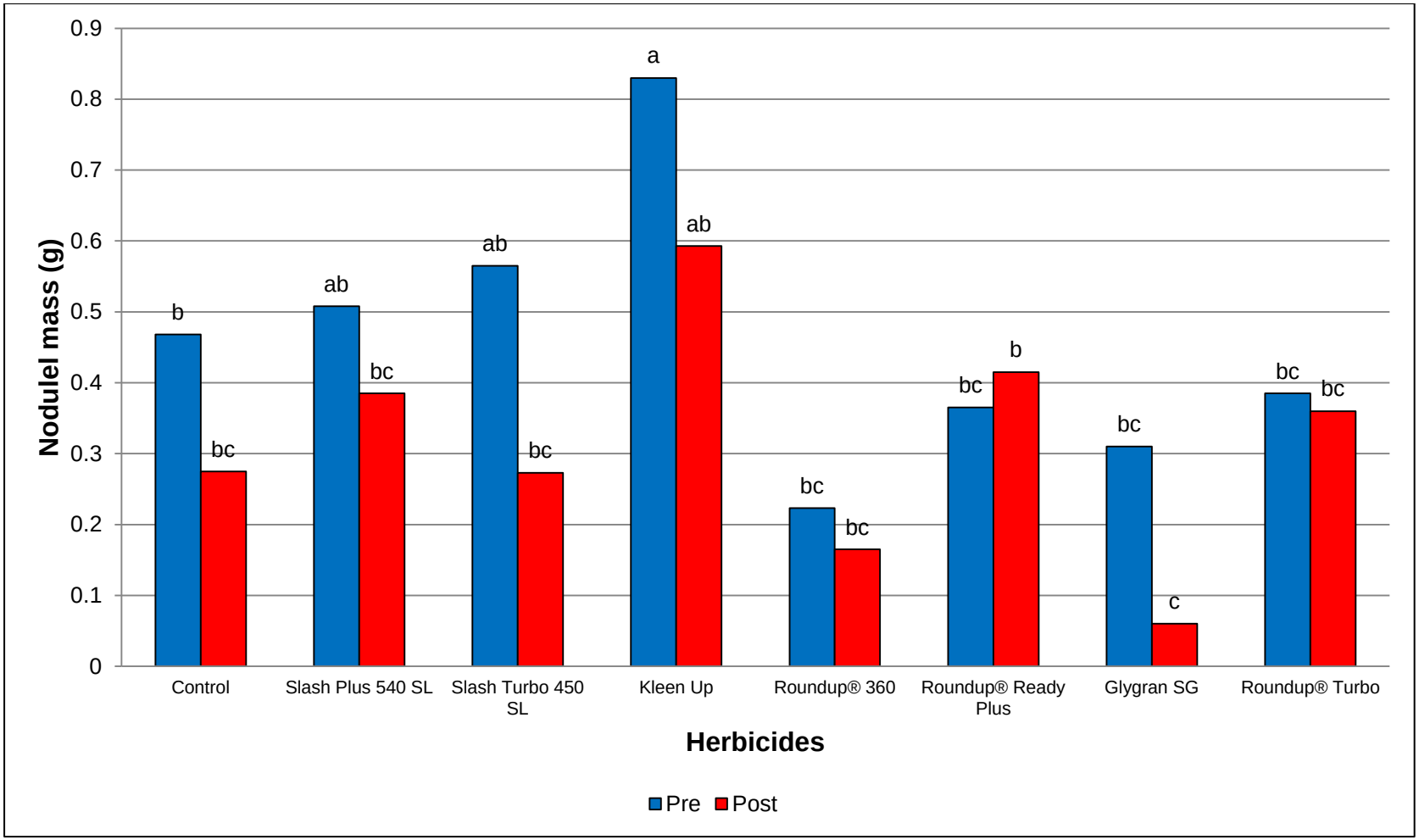

Figure 2.6: Nodule dry mass per plant after 8 weeks after inoculation with rhizobia exposed to two concentrations of glyphosate formulations. Bars with the same alphabetical letters do not differ from each other significantly using means separation of Fischer's LSD ($P<0.05$).

Chapter 3

Effect of glyphosate formulations on the growth of RR® soybean plants in the presence or absence of rhizobia when challenged by root pathogens.

3.1 Abstract

Soybean producers rely on rhizobial nodulation to supply the plants with sufficient nitrogen during the growing season. Most soybean cultivars now available in South Africa contain the Roundup® Ready gene that allows for the use of glyphosate containing herbicides to control weeds in commercial farming. Concern has been voiced as to the possibility that the use of glyphosate may influence both the nodulation efficacy of the plant, as well as its resistance to soil-borne pathogens. Various formulations of glyphosate were tested in the presence and absence of rhizobia, as well as the presence of three soil borne root pathogens; *Fusarium oxysporum*, *Macrophomina phaseolina* and *Sclerotium rolfsii.* Differences in dry mass of plant parts were only observed when the seeds were not treated with the correct rhizobium. The growth of soybeans exposed to soil borne pathogens was only affected when the nitrogen supply came from ammonia and not via the symbiosis with rhizobia.

3.2 Introduction

Soybean (*Glycine max* (L.) Merr) is mainly produced for oil and high protein meal (Liebenberg, 2012). In South Africa, commercial production takes place on a large scale with intensive monocropping being implemented over large areas of the country. In 2014/15 a total area of 687 000 ha was under soybean production, with 1.07 kt harvested. The province of Mpumalanga produced 0.389 kt (36.4%), Free State 0.366 kt (34.2%), KwaZulu Natal, 0.103 kt (9.6%), Limpopo 0.072 kt (6.7%), Gauteng 0.069 kt (6.4%), North West 0.032 kt (3.0%), Northern Cape 0.014 kt (1.3%), Eastern Cape 0.020 kt (0.2%), Western Cape 1 260 t (0.1%) (Dept of Agriculture, Forestry and Fisheries, 2016). The practice of monocropping can, however, quickly lead to a build-up of plant pathogens in the soil, causing yield reduction during follow-up planting of the same crop (Ware & Whitacre, 2004). It is, therefore, normal practice that soybean is rotated with other crops that are not susceptible to soil-borne pathogens infecting soybean. These crops are usually

cereals rather than broadleaf plants, and include maize and wheat, although sunflower is sometimes also planted in soybean rotation systems (Roth, 2014).

In an extensive monoculture system, manual control of weeds is impractical and has consequently led to intensive use of chemical herbicides. Herbicides are classified based on their activity in the plant, such as those that only target grasses growing among broadleaved plants and *vice versa*. Herbicides are usually tested on specific weeds amongst specific crops and should, therefore, be applied strictly according to specifications (Thompson *et al*., 2015). During the 1970's, glyphosate (*N*-phosphonomethyl glycine) was developed by Monsanto and has since become one of the main herbicides used in industrial farming (Battaglin *et al*., 2014). It kills all plants except those that have been genetically modified by insertion of the so called Roundup® Ready gene (Mesnage *et al*., 2012).

The symbiosis between legumes and their obligate endophytic rhizobia is a unique and delicate interaction. The host plant and its bacteria, however, do not exist in a vacuum and are sensitive to changes in their environment. These effects include abiotic factors such as rain, soil pH and air temperature, as well as biotic factors such as pathogens, arthropod activity and herbivory (Niste *et al*., 2013). The modern practice of large scale crop production also introduces the use of chemicals, either for fertilisation, or as protection against pests (Ahemad & Khan, 2012).

A specific legume species can only be successfully inoculated by a specific species of rhizobium, although some rhizobial species can effectively infect more than one species of legume (Wang *et al*., 2012). Soybeans form an endo-symbiotic relationship with the bacterium, *Bradyrhizobium japonicum* Jordan, whereby they shelter the bacteria in a nodule, formed on the roots. The bacteria in turn fix atmospheric nitrogen and supply excess nitrogen to the plant in the form of ammonia (Gage, 2004). Successful inoculation, therefore, negates the use of nitrogen fertilisers for the duration of a planting season. After mature soybean pods are harvested, decaying roots release the bacteria and together with aerial parts worked into the ground, supplement the soil with up to 150 kg of N for use by the follow-up crop (Corbeels *et al*., 2003). At the same time soil microbial biodiversity is increased (Zhang *et al*., 2011).

Successfully inoculated legume plants grow faster and are healthier than plants supplied with commercial nitrogen fertilisers (Namvar *et al*., 2011). A stronger plant will be more resistant to disease and because rhizobia occupy potential

infective sites on roots, it will be difficult for a pathogen to infect (Vance, 1983). Rhizobia infect plant roots in a process similar to pathogens, and stimulation of plant defences via systemic acquired resistance (SAR) may consequently increase disease resistance (Avis *et al*., 2008). It is standard practice to inoculate all cultivated legumes with commercially produced rhizobia before planting, since the presence in the soil of rhizobia specific to a particular legume species is not guaranteed (Liebenberg, 2012), especially where soybeans are not endemic. Inoculation with rhizobia may, however, fail for a number of reasons, with death of the bacterial inoculum being the most prevalent (Bennet, 2015).

One of the causes of yellowing of soybean leaf can be temporary nitrogen deficiency before the plant becomes well nodulated. This normally dissipates once the rhizobia actively starts to fix atmospheric nitrogen (Department of Agriculture, Forestry and Fisheries, 2010). If greening does not take place, it will be imperative to give the field nitrogen fertiliser in order to still produce a profitable yield (Semu & Hume, 1979). There are contradictory reports how nutrients added to the soil in the form of fertilisers affect soil pathogens and some responses are not well understood. However, modern use of chemical fertilisers have allowed for growing crop plant in area know for high levels of soil pathogens (Huber & Haneklaus, 2007). High levels of nitrogen will increase the severity of a disease caused by obligate pathogens, whereas high nitrogen levels will decrease the levels of obligate pathogens (Dordas, 2008). The three pathogens used throughout this study are all facultative pathogens, *Fusarium oxysporum* Schtdl, *Macrophomina phaseolina* (Tassi) Goid. and *Sclerotium rolfsii* (Curzi) Tu & Kimbrough. A crop plant that is deficient in any of the macro or micro elements needed for growth becomes more susceptible to soil borne diseases. An example of this is increased resistance to take-all disease (*Gaeumannomyces graminis*) in cereals when sufficient nitrogen is added (Huber & Haneklaus, 2007; Spann & Schumann, 2013). Microbial activity in soil may also influence the dynamics of available nutrients. Huber and Haneklaus (2007) warns that excess of nutrients can influence these dynamics, and change the response to the nutrient. Application of excess N may increase susceptibility of maize to stalk rot due to imbalance of other nutrients caused by the excess N or pathogen activity enhancement. Span & Schumann (2013) remarked that excess nitrogen leads to overproduction of amino acids and other nitrogen containing compounds, which also leads to a nutrient imbalance. This can cause a more favourable environment for

fungal pathogens. The chemical formulation of the nutrient can affect the way it is oxidised or reduced by soil microbes, affecting uptake by the plant. This can affect pH of the soil which could also have adverse effects on the plants ability to absorb the nutrient cations (Huber & Haneklause, 2007; Dordas, 2008).

Because glyphosate is taken up by a plant and systemically translocated in its tissue (Singh *et al*., 2011), it is understandable that possible toxicity of glyphosate to rhizobial bacteria may be a factor in rhizobial survival (Hetherington *et al*., 1999), and there are reports of damage to rhizobium bacteria (Duke *et al*., 2012; Kremer & Means, 2009). Concern has also been voiced as to the possible negative effect of the presence of the RR® gene on the ability of the rhizobia to infect the plant successfully, although scant evidence for this has been found (Zablotowicz & Reddy, 2004; Powell *et al*., 2007). The aim of the present study was to determine whether the use of different glyphosate formulations affects the efficacy of rhizobial inoculation in terms of nodule production as well as susceptibility of soybean plants to soil pathogens in the presence or absence of rhizobial inoculation.

3.3 Materials and Methods

Plant material was collected from an experimental field in Limpopo, South Africa (S24.454953 E28.161775) where soybeans had been planted and treated with a nematocide (Cureterr 5G [carbofuran]) during planting. Soybeans had successfully been inoculated with *B. japonicum* (WB_{74}), but showed stunting with many of the affected plants displaying mycelial growth on the root crown (no data collected).

Mature complete soya and dry bean plants from the adjacent plots displayed mycelial growth on the root crown. These were harvested in order to identify the pathogen/s. The samples were placed in brown paper bags and transported in insulated containers to the laboratory where they were stored at 8°C. Roots were washed under tap water to remove extraneous soil and cut into lengths of approximately 2 cm. Root sections were agitated for three minutes in 3.5% NaOCl, followed by three rinses in 65% ethanol for 30 seconds each, before being placed on a sterile paper towel to dry in a laminar flow unit. Five root pieces were placed on water agar (WA), containing streptomycin (0.3 ml L^{-1}), in each of twenty five 90-mm-diameter Petri dishes, and incubated at 25°C in the dark. Mycelium that emerged from root pieces was removed aseptically and sub-cultured on potato dextrose agar

(PDA), containing streptomycin (0.3 ml L^{-1}). A single spore or hyphal tip from emerging fungal colonies was transferred to a Petri dish containing PDA with streptomycin and incubated at 25°C ± 2°C. The resultant sub-cultures were divided into visually distinct groups once fungal colonies had grown sufficiently.

For the identification of the three pathogens, DNA was extracted from soybean roots using the Anatech Wizard Genomic DNA kit according to the manufacturers' instructions. Concentration was determined using the Thermo Scientific NanoDrop 2000 Spectrophotometer. Individual samples were diluted to 4 ng μL^{-1}. For the PCR reaction DNA was amplified using 10 pmol of each primer (*ITS* 1 and *ITS* 4 primer set), 10 ng template and 1 x concentration of the KAPA ready mix (Kapa Biosystems). The BIO-RAD CFX96™ Real-Time System, C1000™ Thermal Cycler PCR was set up for one minute at 94°C, followed by 35 cycles of: 15 s at 94°C, 20 s at 55°C, 1 min at 72°C and 5 min at 72°C. The resultant product was then amplified using ABI PRISM® Big Dye® Terminator V 3.1 Cycle Sequence Kit (Applied Biosystems) using 20 ng template, 3.2 pmol of the primer, 1 x sequence buffer and 1/8 of the sequence ready reaction mix. The mixture was denatured at 96°C for 1 minute followed by 25 cycles of: 96°C for 10 s, 56°C for ITS 1 and 58.3°C for ITS 4 for 5 s, and 60°C for 4 min. The product was cleaned up using FADF columns (FavorPrep™ GEL/PCR Purification Kit). The sequencing clean-up was accomplished by incubating a mixture of 10 µL of the cleaned up PCR product, 10 µL water, 5 µL EDTA and 60 µL of ice cold 100% ethanol at room temperature for 15 mins. The mixture was centrifuged at 12 000 *g* at 4°C for 15 mins. The supernatant was decanted into a fresh tube and stored in the dark for 48 h. Sequencing was achieved by aligning with MEGA v5.05 & CHROMAS LITE v2.01. The fungi were identified as: *Fusarium oxysporum* Schtdl, *Macrophomina phaseolina* (Tassi) Goid. and *Sclerotium rolfsii* (Curzi) Tu & Kimbrough.

Multiplication of inoculum of individual fungal pathogens was carried out according to the method developed by Miles and Wilcoxson (1984), and later modified by Letendre and Gibbons (1985). Approximately 200 g of sorghum seed was placed in each of 16 1 L Consul jars with a metal screw-top. Modified Fries medium (Kølmark, 1965) was poured over the seed until it was covered, and the lids were screwed on loosely. The jars were left to stand on the laboratory work surface overnight to allow absorption of the liquid. The jars were then sterilised in an autoclave for 20 minutes at 121°C and left to cool at room temperature. Sterilisation

was repeated the following day and the jars were cooled down before the lids were screwed down tightly to prevent contamination.

Potato dextrose agar was prepared and sterilised for 20 minutes at 121°C in an autoclave. When it had cooled down to approximately 50°C the agar was poured into sterile plastic Petri dishes and left to set inside a laminar flow unit. Three Petri dishes were seeded with the respective pathogens and placed inside an incubator at 25°C in the dark. These fungal cultures were left to grow until the surface of the agar was covered with mycelium. Four small blocks of agar (1 cm^3) were aseptically cut from each mature fungal culture and placed in a 1 L glass Consol jar containing sterilised sorghum seed, which was shaken by hand to mix the contents evenly. Treated jars were incubated at 25°C ± 2°C and agitated daily until mycelial growth had evenly colonised the seed. The colonised seed was then aseptically removed from the jars and evenly spread on a flat tray and allowed to dry in a laminar flow unit for three days, whereafter they were ground to a coarse powder and stored in airtight containers at room temperature (Mishra *et al*. 2013). This inoculum powder was used in all subsequent trials. Uninoculated jars were treated the same way and used as a control treatment in all subsequent experiments.

3.3.1 Effect of glyphosate formulations and rhizobia on the growth of RR® soybean.

Yeast extract mannitol (Merck) broth was prepared in 1 L flasks and aerated with a commercial pump. Flasks were inoculated with the *B. japonicum* isolate currently recommended in South Africa by the Agricultural Research Council (ARC), and previously sourced from Stimuplant CC. The bottles were aseptically aerated through a sterile filter using a small commercial pump for seven days, and a sample was aseptically drawn through the pipes. This sample was examined on a haemocytometer and the concentration of the bacteria was counted and determined to be 10^8 colony forming units (cfu) per mL of broth.

Black polythene 5 L pots were filled with a mixture of steam sterilised loam soil (Bainsvlei) (Tilahun *et al.,* 2004) and peat moss (60:40). The pots were placed in a glasshouse at 25/18°C day and night temperatures and natural sunlight, and watered to field capacity a day before planting. Five depressions of ca 2 cm deep were made in the soil in each pot with the base of a 15 mL Falcon tube. In total 500 µL of a broth of *B. japonicum* (10^8 cfu mL^{-1}) were pipetted into each depression. A

total of 24 pots were treated in this manner; a further 24 received 70.69 mg of ammonium nitrate (NH_4NO_3) (equivalent to 40 kg of nitrogen ha^{-1}) in place of the rhizobium inoculum. A single Roundup Ready® soybean seed (A5409RG) was placed in each soil depression and covered with soil. The pots were laid out in a randomised block design in the glasshouse and kept wet at field capacity. Emerging plants were thinned out to three per bag once the second trifoliate leaf had emerged, resulting in nine plants per treatment. Each treatment was replicated four times.

When hypocotyls broke through the soil surface (i.e. crack stage), each pot was sprayed with one of seven pre-emergence glyphosate containing herbicide formulations (Table 3.1) followed by a second post-emergence/pre-flowering spray at six weeks (V6) (Fig 3.1). Water was used as a control. During the first application, the herbicide was sprayed on the surface of the soil using a spray apparatus incorporating a travelling boom on which two Tee-Jet 8003-E nozzles were mounted. The boom travels at a constant speed of 2.88 km h^{-1}, delivering the spray mixture at 200 L ha^{-1}, as recommended with herbicide delivery, at a pressure of 2 bar. The pots were placed on the floor underneath the boom with the soil surface ca 30 cm below the nozzles. The procedure was repeated for the second spray, where herbicide was applied to the leaves and stems of the plants, totally wetting all surfaces (Allemann & Geronio, 2007). No note was taken of yellow flash or other symptoms during the growing phase.

At eight weeks, the plants were harvested and roots were carefully washed in a tub of tap water. The water was subsequently poured through a 1-mm sieve to capture any nodules that had become dislodged during the washing process (Nissen *et al*., 2008). Roots and aerial parts were weighed using a Sartorius Basic top scale and the nodules were weighed using a Shimadzo Libror AEG-220 chemical scale (LasecSA). Data were statistically analysed by conducting an ANOVA and Fisher's multiple comparison test (GenStat Release 17.1).

3.3.2 Effect of glyphosate formulations and rhizobial inoculation on root disease of RR® soybean.

Black polythene 5 L pots were filled with a mixture of steam sterilised loam soil (Bainsvlei) (Tilahun *et al.,* 2004) and peat moss (60:40). Inoculum of *F. oxysporum*, *M. phaseolina* and *S. rolfsii* as previously prepared was individually mixed into each pot to a final ratio of 3:100 (v/v). Powdered sterile sorghum seed

served as a control treatment and was also mixed to a final ratio of 3:100 (v/v). Sixteen pots were prepared for each pathogen as well as the control treatment. The seeds in eight of the pots were inoculated with *B. japonicum*, with the other eight receiving NH_4NO_3 as previously described. Within treatments, four of the pots were sprayed with herbicide and the remaining four with an equal volume of water. The same procedure as in the previous experiment was used, with Slash Plus 540 SL being applied at the crack stage at 2 L ha^{-1} and again at week six at 4.7 L ha^{-1} pre-flowering. The treatments were replicated four times. The pots were laid out in a randomised block design, watered as required and harvested at eight weeks. No observations of yellow flash or other symptoms were noted during the growing phase. Aerial plant parts were cut off at soil level and left to dry to equal mass. Roots were washed thoroughly in water and nodules were removed as previously described.

3.4 Results and Discussion

3.4.1 Effect of glyphosate formulations and rhizobia on the growth of RR® soybean.

There were no significant ($P \leq 0.05$) differences between the dry masses of any of the plant parts when exposed to the different glyphosate containing herbicides, whether the plants were inoculated with rhizobia or treated with NH_4NO_3 (Fig 3.2a and b, Fig 3.4a and b, Fig 3.4).

Roundup® 360 reduced the aerial (Fig 3.2a) and root dry mass (Fig3.3a) of plants by 40% in comparison to the control without any herbicide in plants treated with NH_4NO_3. This was not reflected in plants inoculated with rhizobia and is consistent with findings of Zablotowicz and Reddy (2004)., that plants successfully inoculated will be more resistant to any outside stresses. Glygran reduced dry root mass (Fig 3.3a) by 65% compared to the control with no herbicide when no rizobial inoculation took place, but this was not reflected in plants inoculated with rhizobia. Roundup® Ready Plus reduced aerial dry mass by 30% in both the inoculated and uninoculated plants (Fig 3.a and b). Although this seems high, it does not significantly differ from the mass of the control treatment without herbicide, and as these plants were harvested at eight weeks, it is not possible to extrapolate the ultimate effect of these decreases in mass of plant material on yield. In plants inoculated with rhizobia and treated with herbicides, all the plants, except those

treated with Roundup Turbo, showed an increase of nodule mass of 100-300% compared to that of the control (Fig 3.4). There is evidence that soil microbes can use glyphosate as a source of nutrients (Busse *et al*., 2000), and the results of this experiment show that none of the herbicides negatively affect the rhizobia bacteria.

Use of glyphosate containing herbicides on genetically modified soybeans negates the use of manual weeding or directionally aimed herbicide spraying, which is time consuming (Zimdahl, 2013). Manual weeding is impractical on very large monocropped fields. It is too labour intensive, takes too long, and always carries the risk of damage to the crop. Directed spraying is expensive and spray drift may again lead to crop damage. Being able to use a herbicide over the crop to target any post-emergence weeds is efficient and cost saving, which are important factors in modern large scale farming. Strong growth is coupled to higher yields, and effective nitrogen fixing by rhizobia ensures high protein yields in the grains (Zahran, 1999; Simon *et al*., 2014). Precise use of glyphosate containing herbicide formulations, coupled with adherence to dosage and time of use, will thus enable agriculture to rapidly and safely expand production in the face of climate change and population growth.

Although these results are reflected in various published articles as noted, these experiments were carried out in carefully controlled conditions in a glasshouse. The soil was steam sterilised to kill off any microorganisms, and temperature and lighting monitored. In the field, producers do not have control over either weather conditions or amount and types of microorganisms present in the soil (Wolmarans & Swart, 2014) and competition for resources in the soil may be deleterious to applied rhizobia (Wolmarans, 2013). The physical and chemical conditions of the soil such as acidity, clay and organic matter content, will have a definitive effect, not only on the native micro-organisms, but also on the added rhizobia (Helliwell *et al*., 2014), decreasing their numbers to below efficient inoculation levels. In the field, extreme temperature conditions, combined with high temperatures, may not only influence ultimate yield of soybeans (Thuzar *et al*., 2010), but also weaken the plant, thus making it more susceptible to pathogen infection (Vidić *et al*., 2013). Similar field experiments have been carried out (Borggaard & Gimsing, 2008)), though not with isolines and it may be advisable to repeat these, using isoline soybeans, under extreme field conditions.

3.4.2 Effect of glyphosate formulations and rhizobial inoculation on root disease of RR® soybean.

There is a perception that the presence of the RR® gene and the use of glyphosate herbicides can increase the susceptibility of soybean plants to infection by common root pathogens and may even cause infection by previously unreported pathogens (Huber, 2011). Analysis of data from these trials showed no significant differences in masses of aerial parts, roots or nodules in plants exposed to the three pathogens, whether treated with NH_4NO_3 or inoculated with rhizobia when treated with Slash 540 SL (Fig 3.5 a and b, Fig 3.6 a and b, Fig 3.7).

In the absence of rhizobia, *F. oxysporum* caused a 70% decrease in aerial mass of the soybean plants when compared to the control treatment without the pathogen, both with and without Slash 540 SL (Fig 3.5a). This decrease was not statistically significant ($P \leq 0.05$) and was not observed when the seed had been inoculated with rhizobium (Fig 3.5b). Both *F. oxysporum* and *S. rolfsii* decreased the dry root mass by more than 70% in comparison to the control when the plants were treated with NH_4NO_3 (Fig 3.3a) but not when inoculated with rhizobium bacteria (Fig 3.6b). The dry mass of the nodules of inoculated plants showed no significant differences ($P \leq 0.05$) when exposed to soil pathogens in the presence or absence of Slash 540 SL. This is consistent with findings by Deshwal *et al*. (2003) and Chao (1990) who showed that successful inoculation of legume roots leads to increased resistance of the plant against soil borne pathogens.

Glyphosate containing herbicides can now take care of weed problems, but cannot replace the main reason for well-planned crop rotation, which is the decrease of disease forming propagules in the soil by rotating susceptible plants with non-host crops. Increased attack by pathogens such as *Fusarium* spp. is, therefore, not due to the presence of the gene, changes in root exudates, or the use of glyphosate containing herbicides, but to suboptimal farming practices (Duke *et al*., 2012). In the present study, all growth conditions, including soil texture, water levels and light, were strictly controlled throughout the growth period. The seeds were individually inoculated with rhizobia, and the ground up pathogens were accurately measured and mixed into the growth medium to give an equal distribution of the propagules. In the field, seed is often mass inoculated, which could lead to some seed receiving less rhizobia, possibly leading to lower levels of inoculation of specific plants. This in turn can lead to lower yields and more susceptibility to soil borne disease. Larson

(2013) studied various conditions and types of inoculation and concluded that it is essential to use the correct methods of seed treatment and to use inoculant from reliable producers, especially in fields that had not previously been planted with soybeans. Most *in vitro* studies have shown that successful rhizobia inoculation increases the plant's resistance to soil pathogens (Sharif *et al*., 2003; Akhter, 2014). Field studies have shown that some rhizobia species actively kill or reduce the growth of pathogens (Dakora, 2004). However, Chen *et al*. (2012) found an increase in pathogenic fungal numbers in long term monocropping of peanuts. Although no direct literature references were found on the effect of clustered high concentrations of pathogens on the effectiveness of rhizobia, high pathogen load is known to increase disease in crop plants (Almquist, 2016). Rhizobia infection of a legume root stimulates a plant's resistant reaction which in turn inhibits the ability of pathogenic fungi to infect the plant (Dakora, 2003). Induced systemic resistance is a defence mechanism that is triggered in the plant through infection by potential pathogens. Rhizobacteria colonises the roots in the same way as potential pathogens and triggers this defence reaction. This induces resistance of uninfected sites on the roots through a variety of pathways, which leads to the roots resisting further infection by pathogens through the stimulation of production of various signalling molecules (Choudhary *et al*., 2007). Faessel *et al*. (2010) found that the application of acibenzolar S-methyl to the leaves of soybeans induced plant resistance to such a level that nodulation by rhizobia was significantly reduced. From this, it may be deduced that a high level of pathogenic fungi present in the soil may infect the roots of a soybean plant before the rhizobia could colonise the roots, thereby eliciting plant resistance to such a level that nodulation is diminished. This in turn may lead to higher levels of pathogenic infection and a lower yield, or even death of the plant.

3.5 Conclusions

The only negative effect of herbicides used on RR® soybeans occurred when no rhizobia were applied. Therefore it can be concluded that the use of glyphosate containing herbicides has no significant effect on plant growth and nodule formation when rhizobial infection is successful. When RR® soybean plants are exposed to soil borne fungal pathogens in conjunction with glyphosate containing herbicides, the only damage to the plants occur when they were not inoculated with rhizobia. Injury

to the plant is, therefore, not related to the presence of the RR® gene or the herbicides used, but solely to pressure of high concentrations of root pathogens in the soil due to monocropping with soybeans for more than 4 years continuously.

This result came from strictly controlled conditions in a glasshouse and only using two isolines of the same cultivar of soybean. The correct rhizobia were used in all experiments. The outcomes may have been very different using other cultivars and not using rhizobia at all. If no fertiliser were given in the place of the rhizobia, there is a very good chance that the plants would have been infected with the pathogens and may even have died due to a combination of disease and lack of nitrogen (Huber & Haneklause, 2007).

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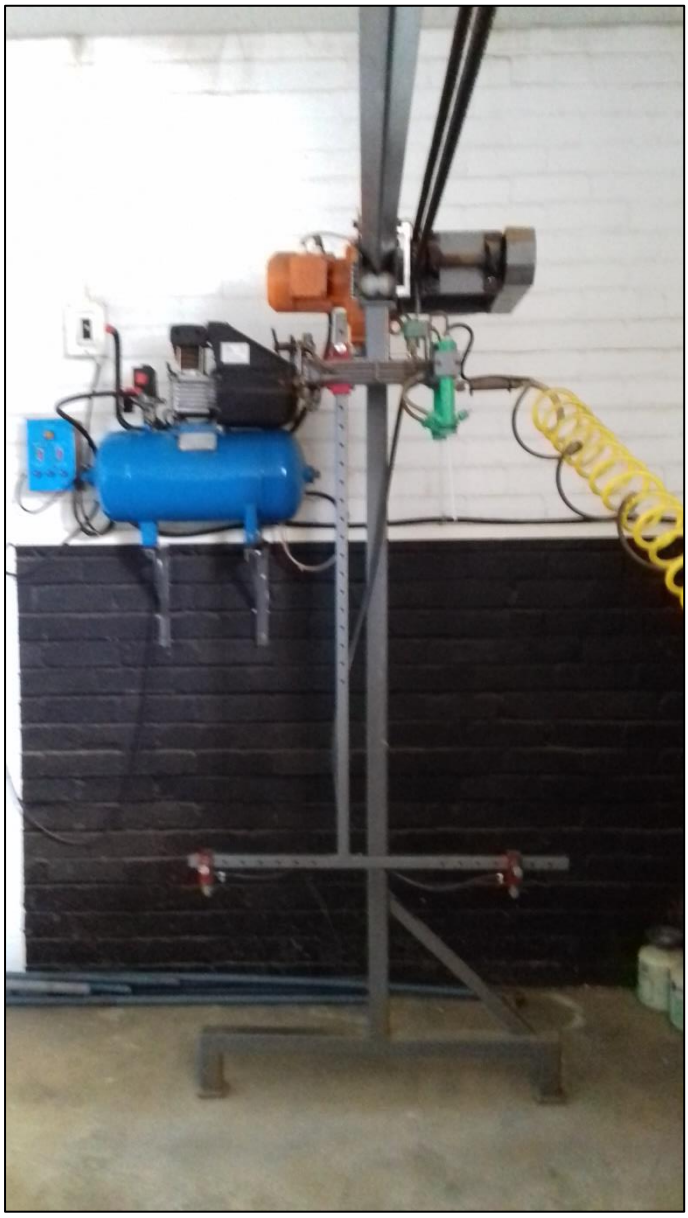

Figure 3.1: Herbicide sprayer, University of the Free State.
Photo: Dr J Allemann.

Table 3.1: Concentrations of herbicide applied to the soybean plants.

Herbicide	**Active ingredient***	**Pre emergence**	**Pre-flowering**
Liquid formulation		**L ha^{-1}**	**L ha^{-1}**
Slash Plus 540 SL	Glycine 540 g ae L^{-1}	2.0	4.7
Slash Turbo 450 SL	Glycine 450 g ae L^{-1}	0.8	3.2
Kleen Up	Glycine 360 g ae L^{-1}	2.0	7.0
Roundup® 360	Glycine 360 g ae L^{-1}	3.0	3.0
Roundup® Ready Plus	Glycine 540 g ae L^{-1}	2.0	4.7
Roundup® Turbo	Glycine 450 g ae L^{-1}	0.8	3.2
Granular formulation		**kg ha^{-1}**	**kg ha^{-1}**
Glygran	Glycine 710 g ae kg^{-1}	0.7	3.0

* ae = acid equivalent

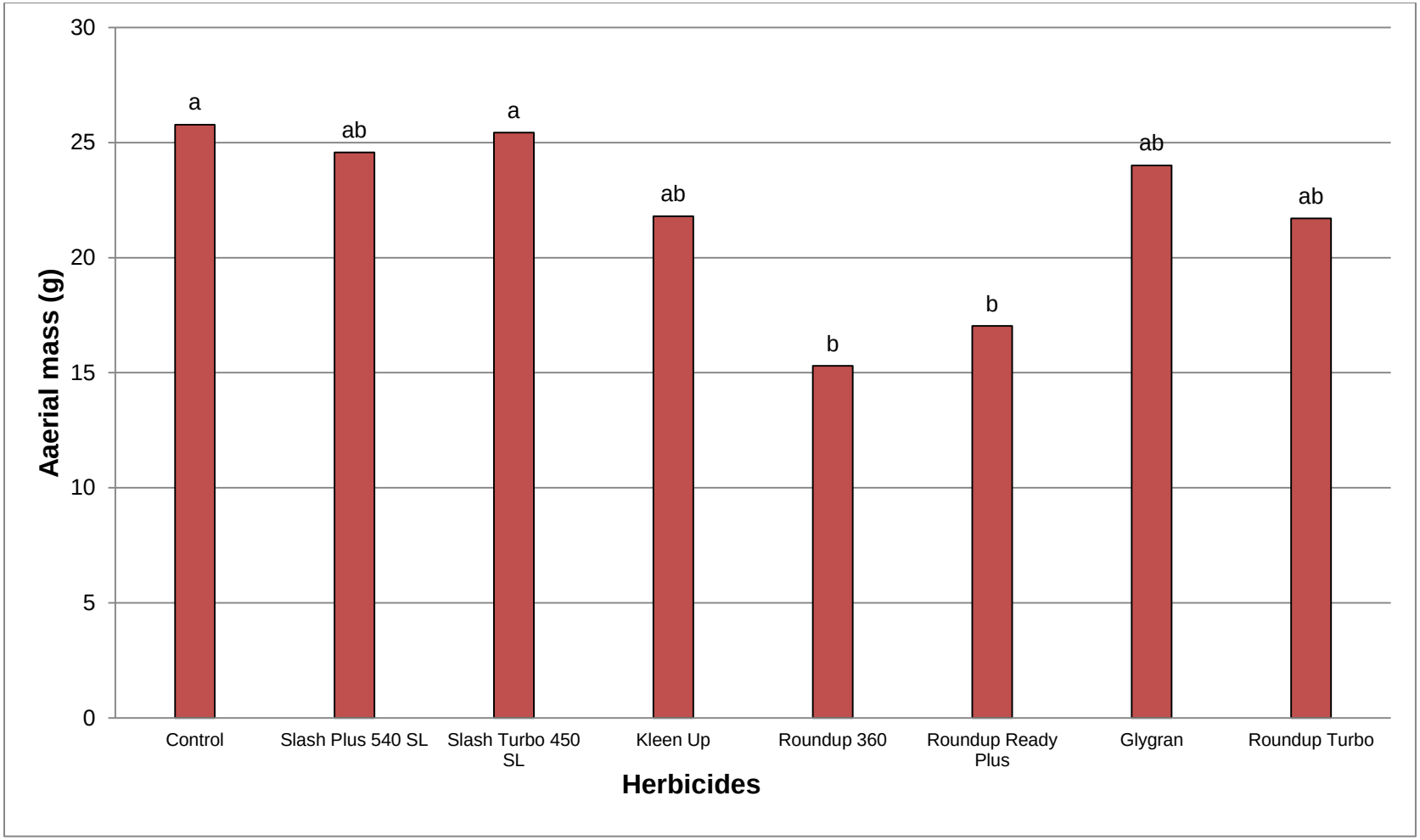

Figure 3.2a: Effect of herbicide formulations on the aerial dry mass per plant at V6 of plants treated with ammonium nitrate and not inoculated with rhizobium bacteria. Bars with the same alphabetical letters do not differ from each other significantly using means separation of Fischer's LSD ($P<0.05$).

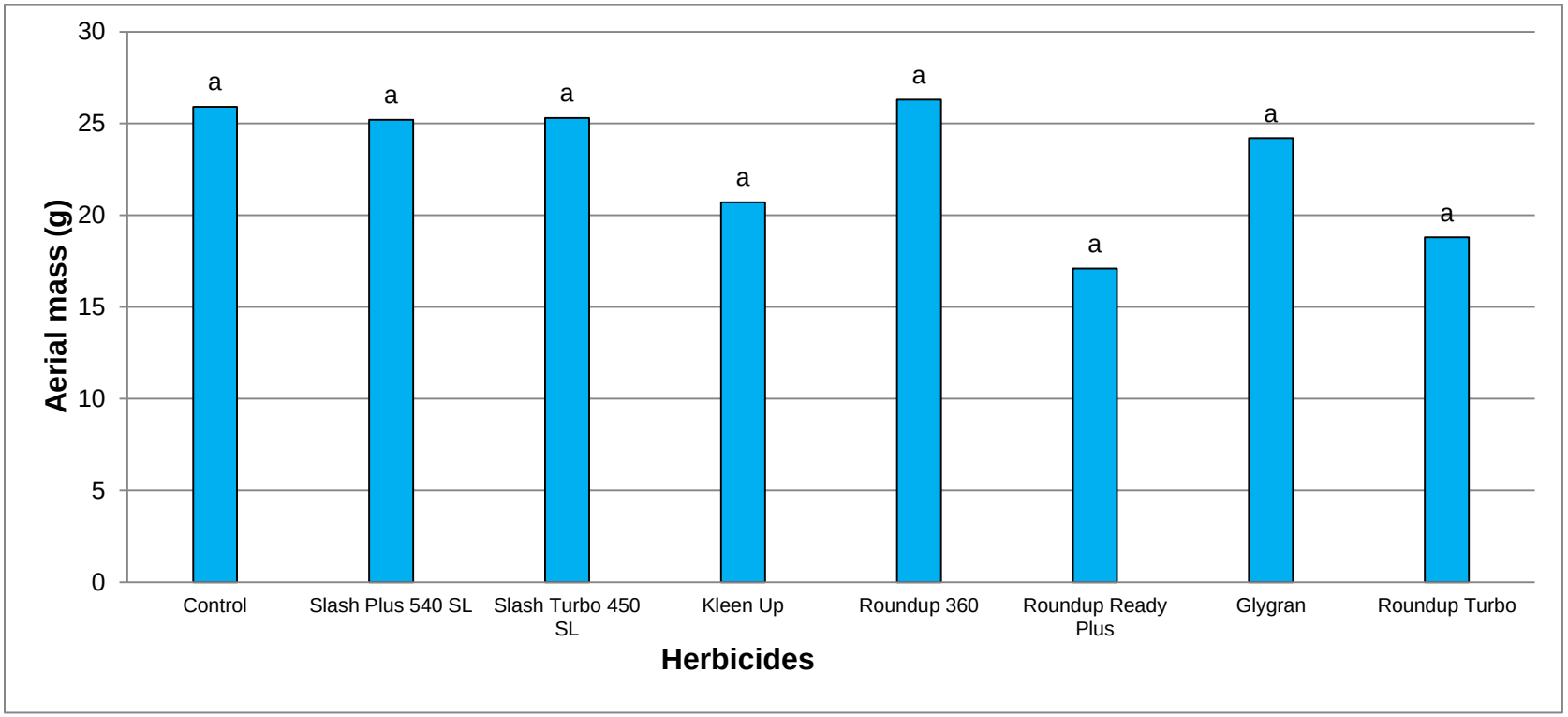

Figure 3.2b: Effect of herbicide formulations on the aerial dry mass per plant at V6 of plants inoculated with rhizobium bacteria. Bars with the same alphabetical letters do not differ from each other significantly using means separation of Fischer's LSD ($P<0.05$).

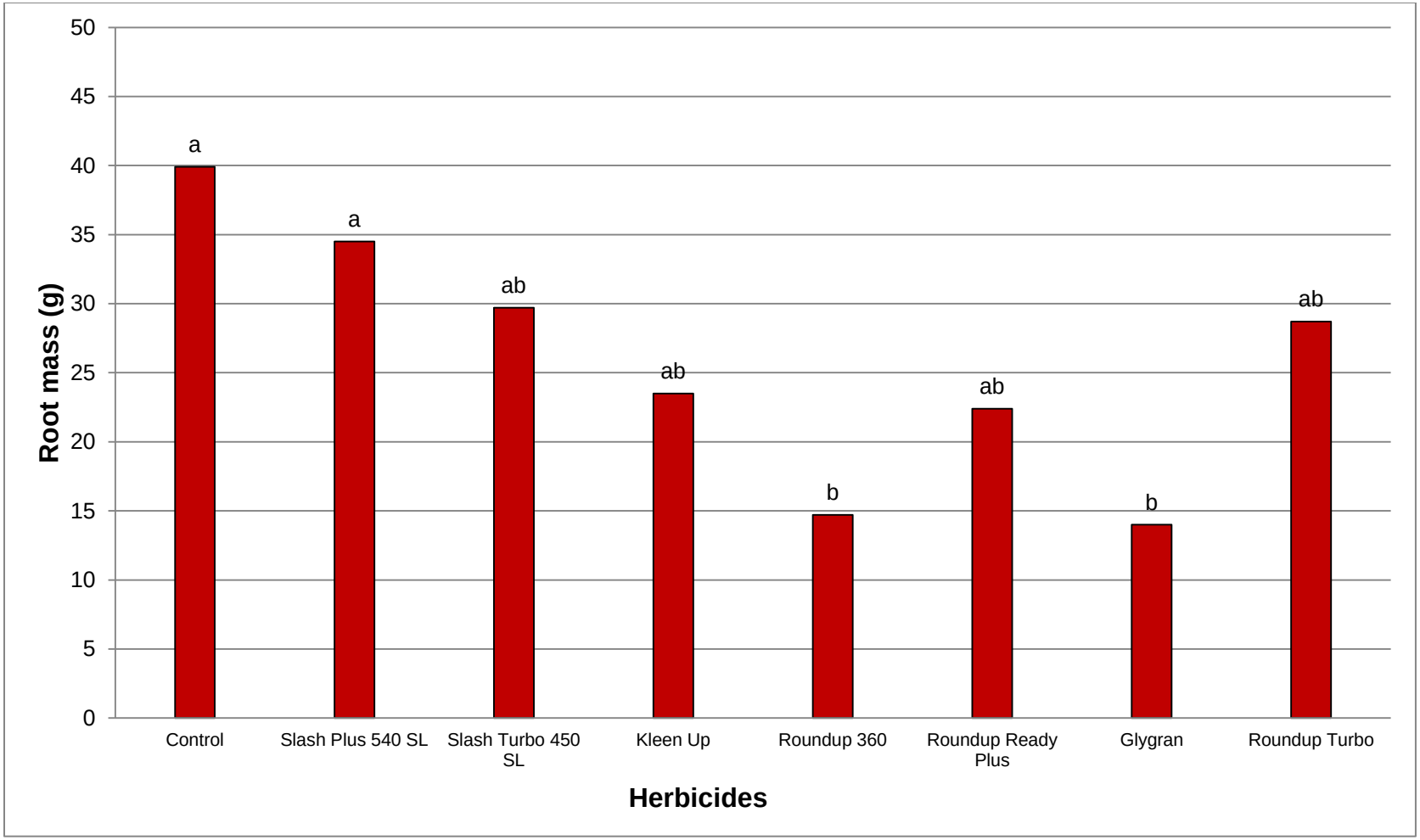

Figure 3.3a: Effect of herbicide formulations on the root dry mass per plant at V6 of plants treated with ammonium nitrate and not inoculated with rhizobium bacteria. Bars with the same alphabetical letters do not differ from each other significantly using means separation of Fischer's LSD ($P<0.05$).

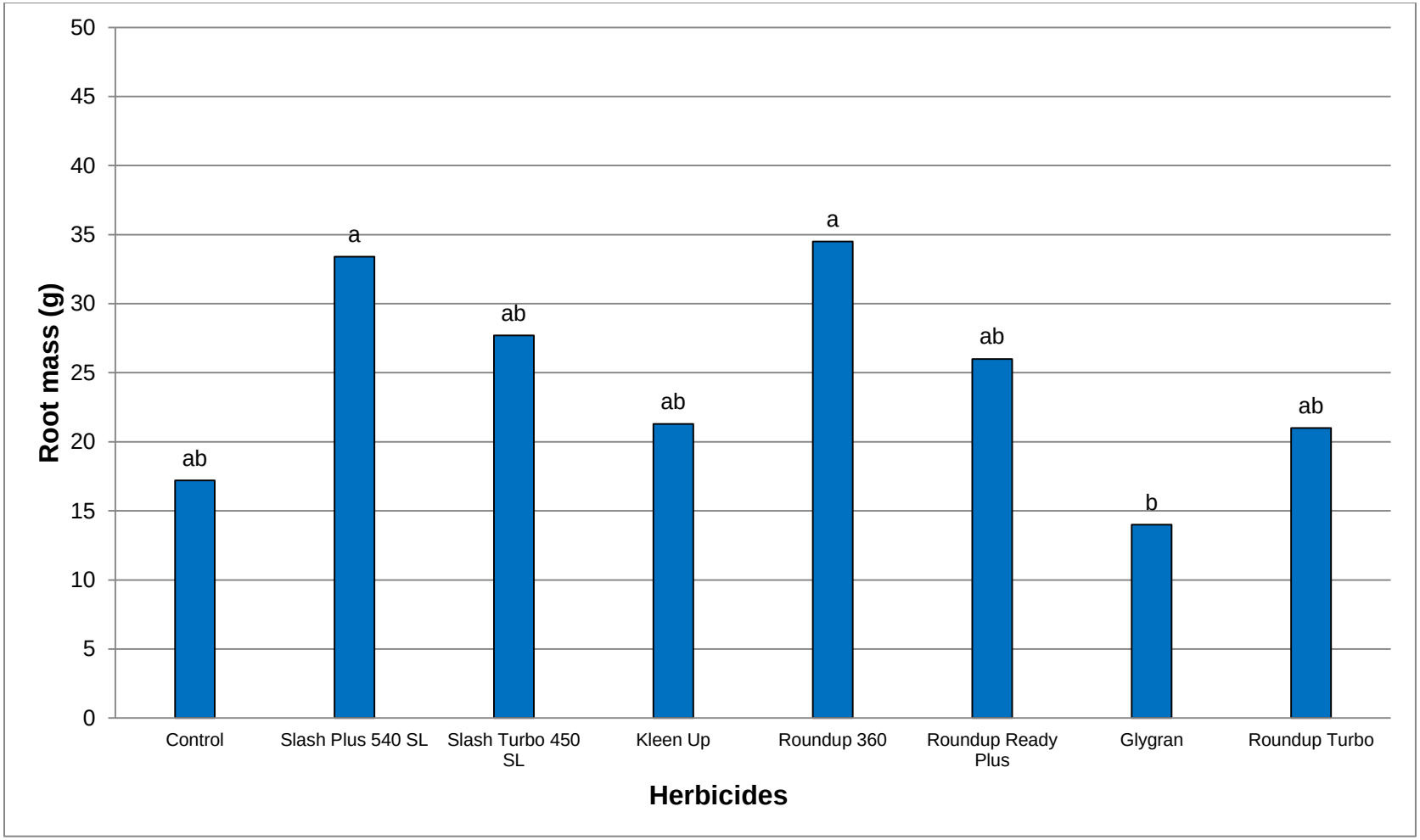

Figure 3.3b: Effect of herbicide formulations on the root dry mass per plant at V6 of plants inoculated with rhizobium bacteria. Bars with the same alphabetical letters do not differ from each other significantly using means separation of Fischer's LSD ($P<0.05$).

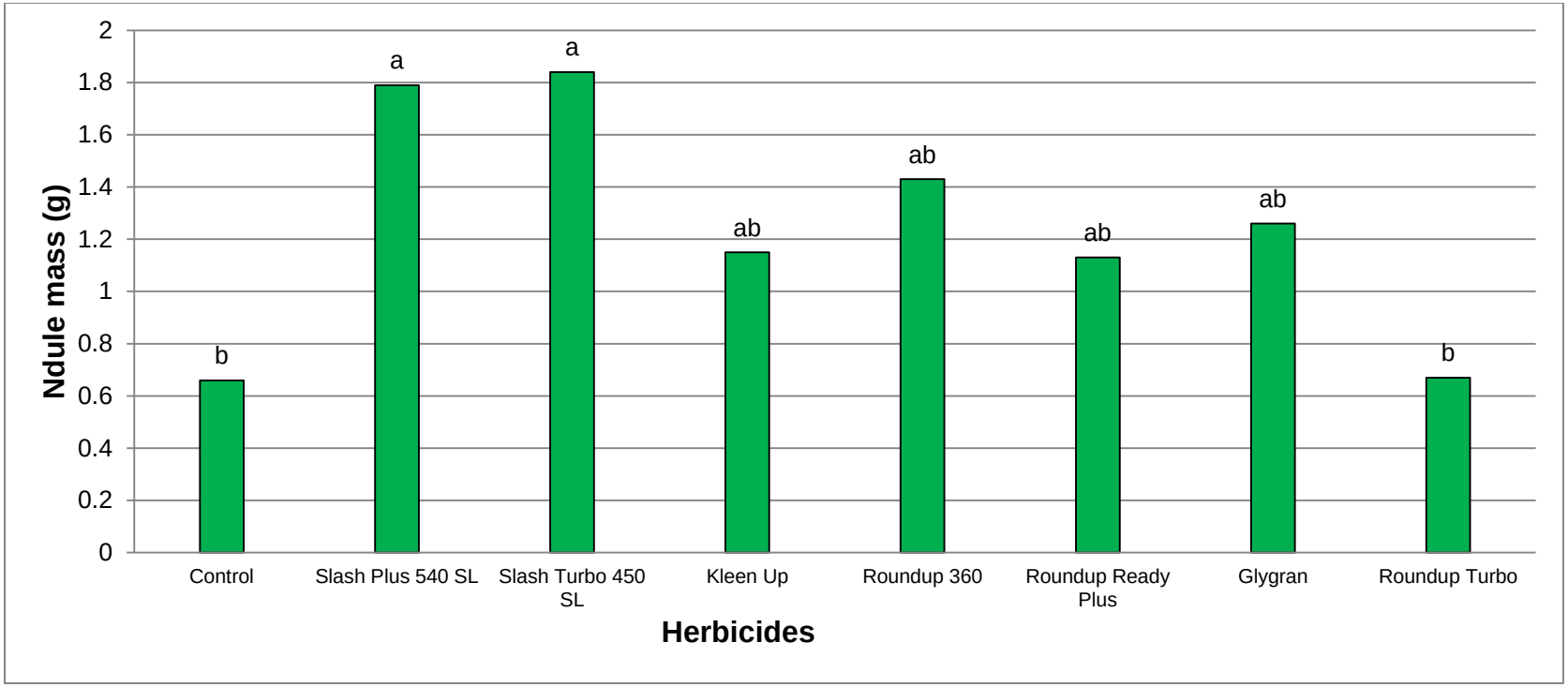

Figure 3.4: Effect of herbicide formulations on the nodule dry mass per plant at V6 of plants inoculated with rhizobium bacteria. Bars with the same alphabetical letters do not differ from each other significantly using means separation of Fischer's LSD ($P<0.05$).

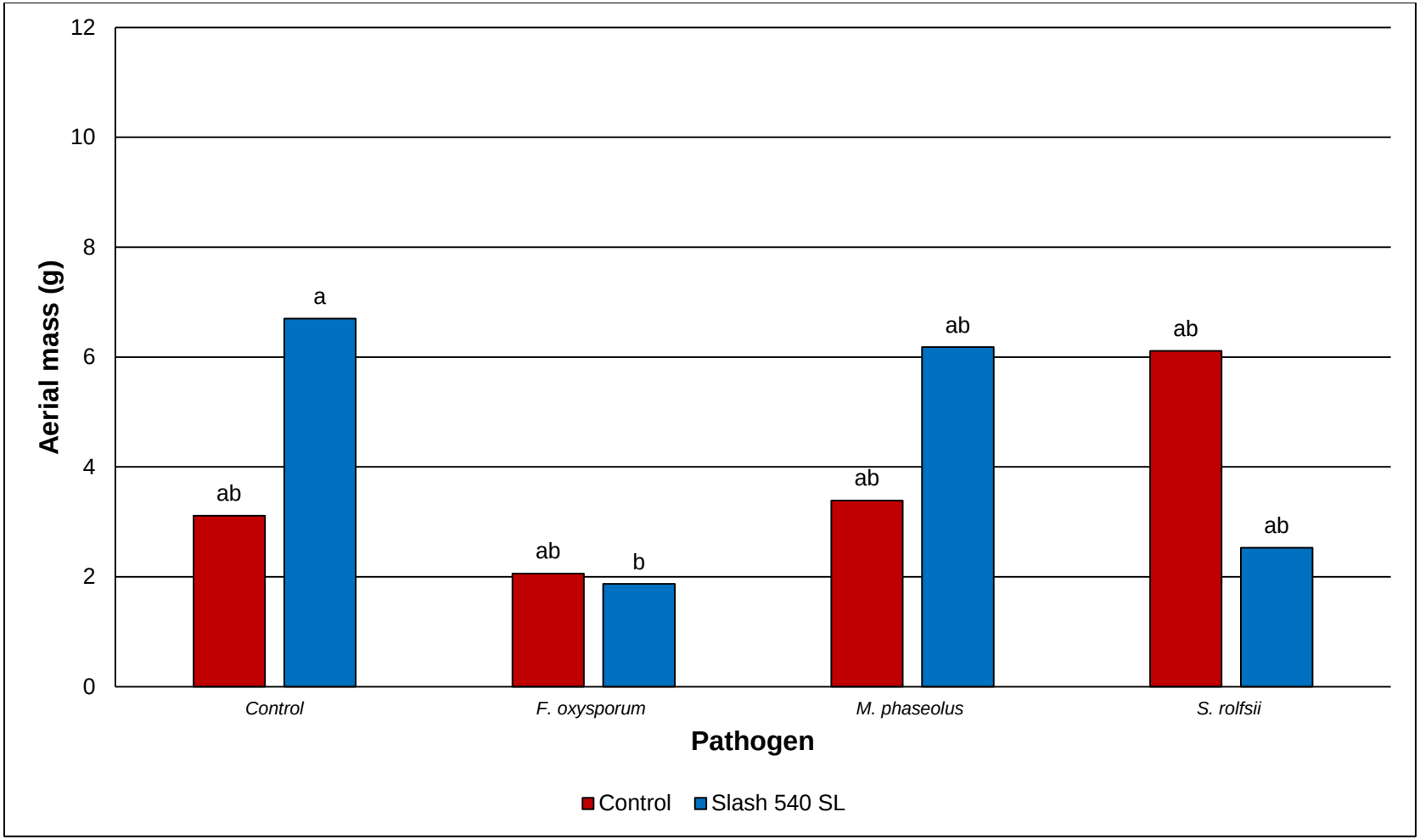

Figure 3.5a: Effect of pathogens on aerial mass per plant at V6 in grams when treated with herbicide in the absence of rhizobia. Bars with the same alphabetical letters do not differ from each other significantly using means separation of Fischer's LSD ($P<0.05$).

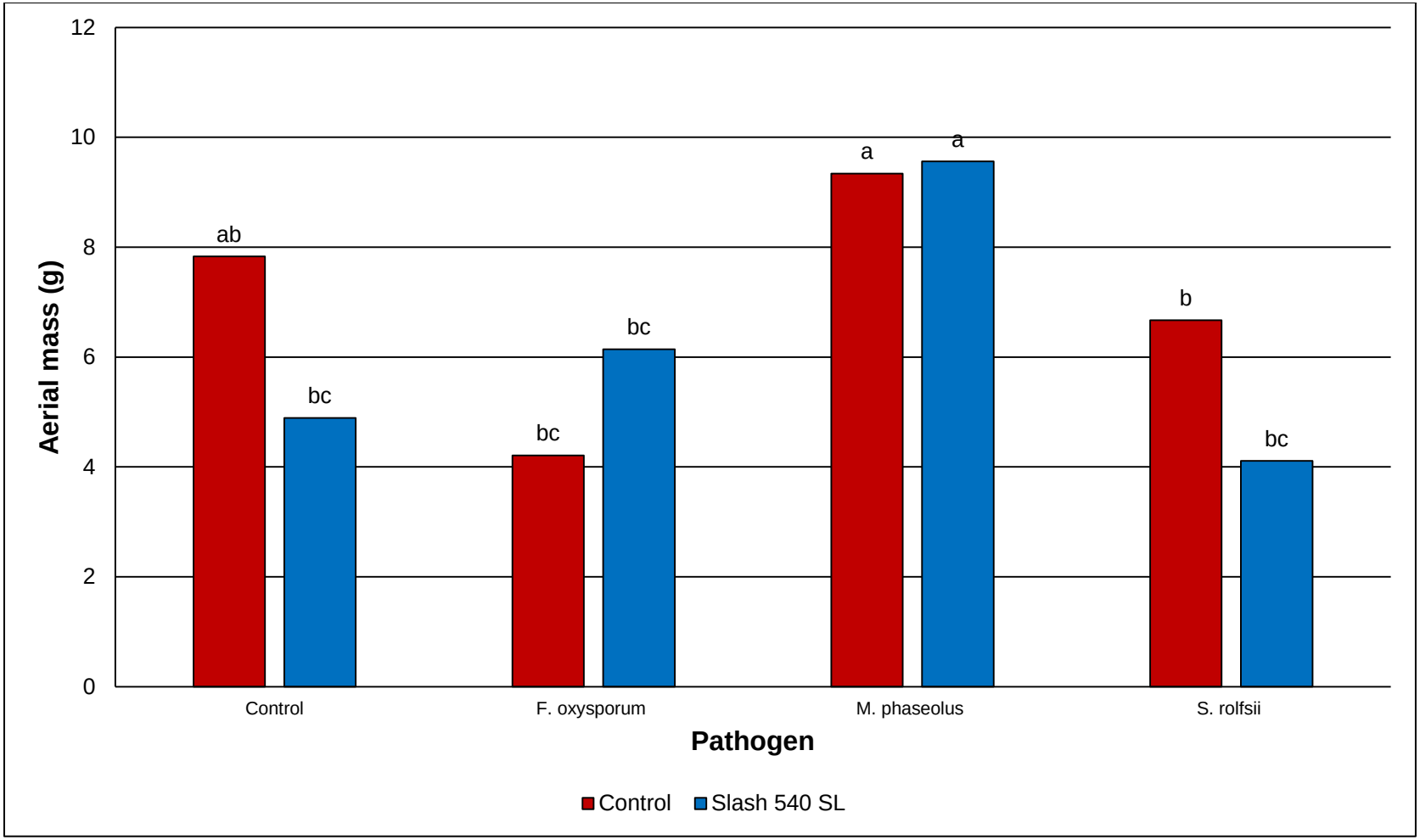

Figure 3.5b: Effect of pathogens on aerial mass per plant at V6 in grams when treated with herbicide in the presence of rhizobia. Bars with the same alphabetical letters do not differ from each other significantly using means separation of Fischer's LSD ($P<0.05$).

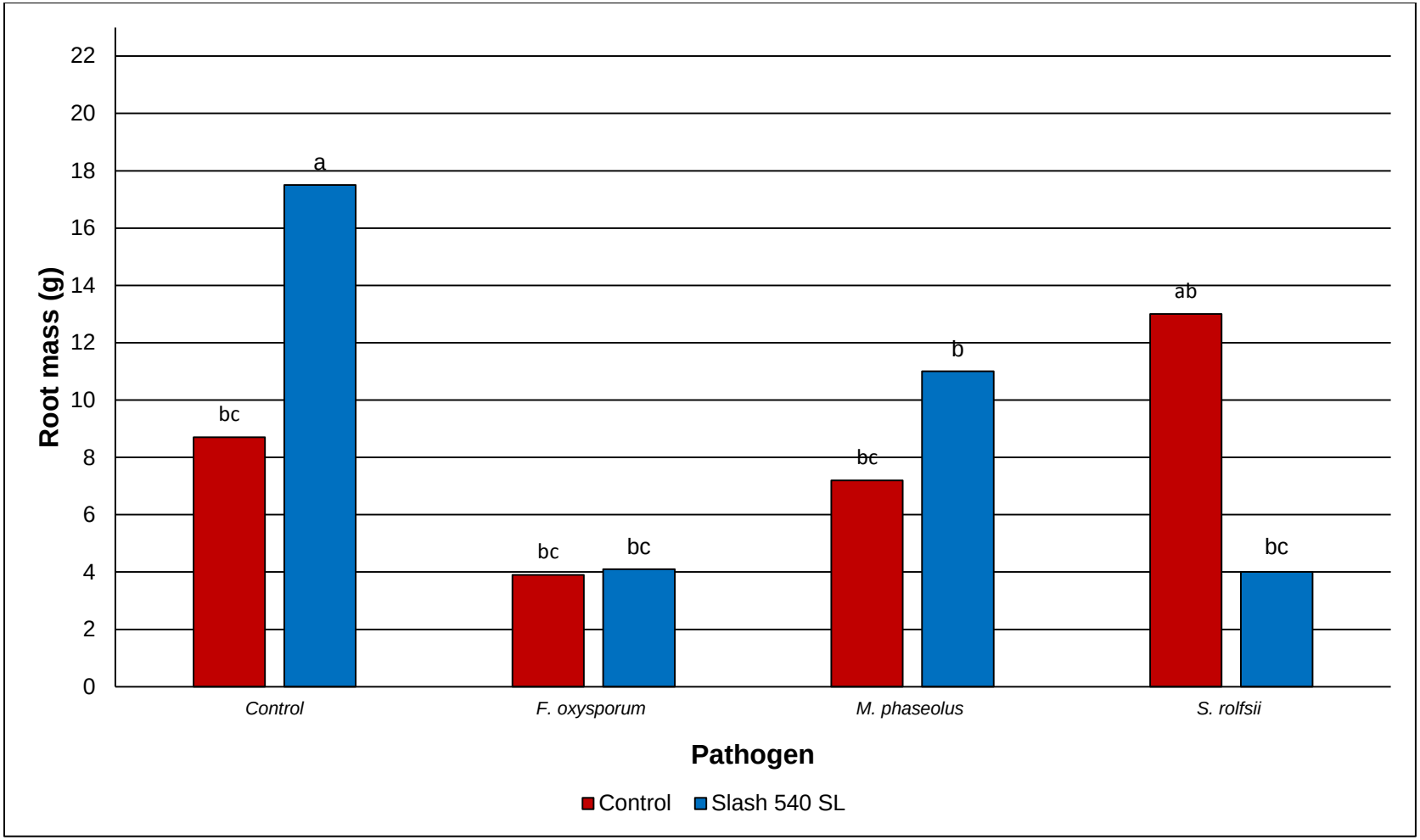

Figure 3.6a: Effect of pathogens on root mass per plant at V6 in grams when treated with herbicide in the absence of rhizobia. Bars with the same alphabetical letters do not differ from each other significantly using means separation of Fischer's LSD (P<0.05).

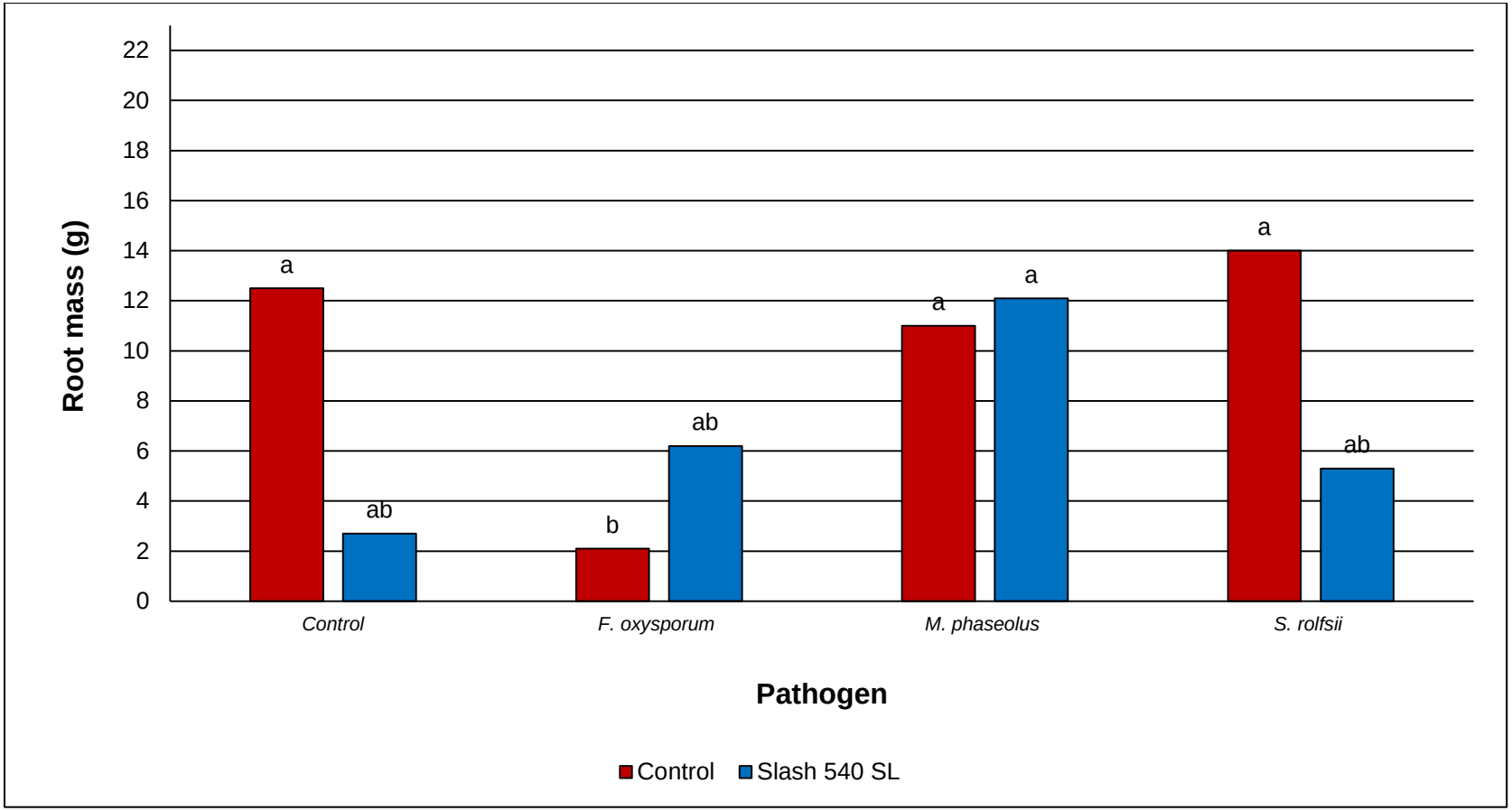

Figure 3.6b: Effect of pathogens on root mass in grams per plant at V6 when treated with herbicide in the presence of rhizobia. Bars with the same alphabetical letters do not differ from each other significantly using means separation of Fischer's LSD ($P<0.05$).

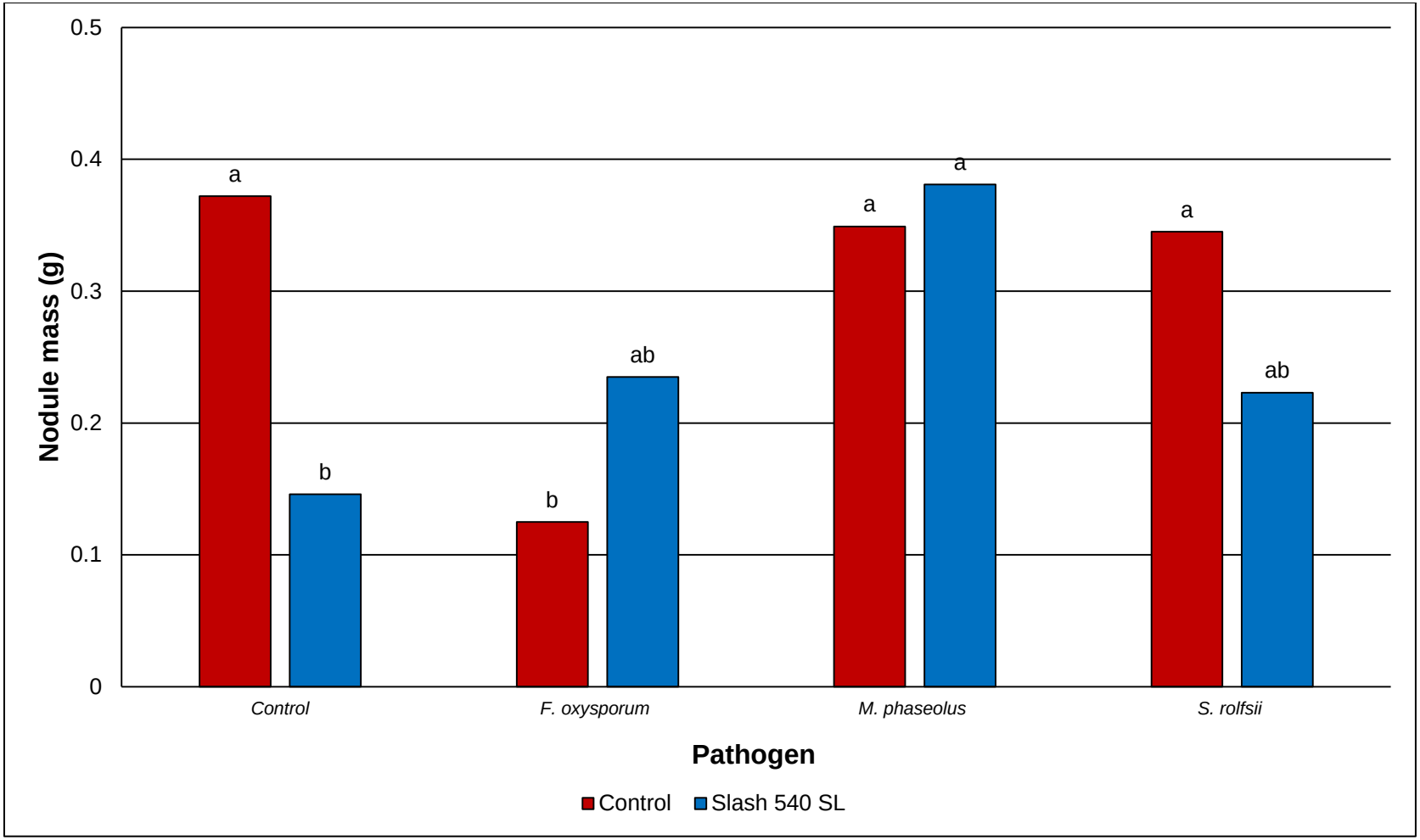

Figure 3.7: Effect of pathogens on nodule mass per plant at V6 when treated with herbicide in the presence of rhizobia. Bars with the same alphabetical letters do not differ from each other significantly using means separation of Fischer's LSD ($P<0.05$).

Chapter 4

Response of soybean isolines to infection by three soilborne pathogens in the presence of arbuscular mycorrhizae and *Bradyrhizobium japonicum*.

4.1 Abstract

The presence of rhizobacteria (*Bradyrhizobium japonicum* Jordan.) specific to soybean (*Glycine max)* and arbuscular mycorrhizae (AM) is important for protecting the plant against fungal pathogens. It has, therefore, become standard practice to inoculate soybean seed with both these organisms before planting. Field and glasshouse trials were conducted to compare the performance of two soybean isolines and 25 Roundup® Ready cultivars in the presence of AM, *B. japonicum* and three soybean root pathogens viz., *Fusarium oxysporum* Schtdl (Tassi) Goid, *Macrophomina phaseolina* and *Sclerotium rolfsii* (Curzi) Tu & Kimbrough. The nodulation capability of Roundup® Ready isoline A5409RG and non-Roundup® Ready isoline A5409 was tested by treating them with rhizobium prior to planting, and then harvesting plants eight weeks later to determine the dry mass of aerial parts, roots and nodules. No significant differences (P≤0.05) between the two isolines were observed. Only isoline A5409, prior inoculated with rhizobia, had a significantly higher (P≤0.05) root mass and nodule mass in the presence of *S. rolfsii*. The reaction of the two isolines when inoculated with both rhizobium bacteria and arbuscular mycorrhizae, and grown in the presence of the three pathogens, also showed no significant differences with regard to dry mass of aerial parts, roots or nodules.

4.2 Introduction

Numerous soil inhabiting microbes play a role in promoting the growth of crops to the detriment of plant pathogens (Ahmad *et al*., 2008). Rhizobia form an endophytic symbiosis with plants of the family Leguminosae (Martin *et al*., 2006). Each plant species is associated with a specific rhizobium, and *Glycine max* (L.) Merrill A5409 is exclusively infected and colonised by *B. japonicum* Jordan. The bacterium is attracted

by flavonoid-containing root exudates resulting in infection of the root and stimulation of the plant to produce a nodule (Gage, 2004). The differentiated bacteroids within nodules actively fix nitrogen from the atmosphere in the form of ammonia, which the plant uses in protein production (Oke & Long, 1999).

Fungi from the family, Glomeraceae, form an endophytic symbiosis with most terrestrial plants, by infecting the plant roots and forming arbuscules inside the plant cells (Sikes, 2010). This extensive plant/fungal interface allows for interchange of minerals, especially phosphate, from the soil into the plant and photosynthates into the hyphae of the fungus. The fungal hyphae extend outwards into the soil, expanding the reach of the plant roots and allowing for uptake of minerals beyond the root zone. Apart from occupying infection sites on the root and excluding these from potential pathogens, arbuscular mycorrhizae (AM) also absorb root exudates that might attract and maintain the growth of potential plant pathogens (Berruti *et al*., 2014). AM are regularly found in soybean roots (Kojima *et al*., 2014) and are now commercially available as a seed treatment in many countries.

Zaller *et al*., (2014) found that application of glyphosate containing herbicides to legumes inoculated with AM lead to significant decrease in root mycorrhisation and lowering of soil AM spore, vesicle and propagules. Helander *et al.,* (2018) reported a decrease in mycorrhizal colonisation of a non-target crop plant, *Festuca pratensis*, during a study of tillage, glyphosate use and cultivation history highlighting the importance of a healthy soil environment for crop production. Druille et al. (2013) reported a drop in spore viability when glyphosate was applied to the soil, and root colonisation decreases when glyphosate was applied to the foliage.

The presence of the Roundup® Ready gene in a specific crop purportedly changes the crop's physiology, which can in turn affect the ability of rhizobia and AM to successfully infect the roots (Huber, 2010; Zobiole *et al.,* 2012). Hungria *et al*. (2014) investigated nearly isogenic soybean cultivars, with one containing the RR® gene and the other not. The RR® soybeans were treated with glyphosate or conventional herbicide application and the non-RR® soybeans with conventional herbicide. Although there were effects on some rhizobia, there were no significant differences in ultimate yield over a three-year period. Johal and Huber (2009) reported that it is not the

presence of the gene itself that affects resistance to root disease, but over-use of glyphosate that leads to chelation of micro-nutrients in the soil. Addition of these micro-nutrients and the judicious use of herbicides will presumably ameliorate these effects (Johal & Huber, 2009), and will contribute to higher disease resistance (Duke *et al*., 2012). This will also have less harmful effects on the soil environment. Duke *et al*. (2012) emphasised that chemical weed control should be part of an integrated pest management system.

The presence of the Roundup® Ready gene in soybean does not seem to have a negative effect on the ability of a plant to attract rhizobia and successfully form nodules (Duke *et al*., 2012). Soil conditions, such as acidity or high levels of salt, can however affect this ability, and may under certain circumstances lead to yield failure (Zaharan, 1999). Rhizobia are stimulated by legume root exudates to form an infection tread which can enter the roots via root hairs in a similar fashion as a potential root pathogen, and will, therefore, activate the plant's immune system (Zamioudis & Pieterse, 2012). The same holds true for mycorrhizae, which not only protect the root from infection by pathogens, but also compete with potential pathogens for plant root exudates. It is, therefore, postulated that failure of nodulation increases a plant's susceptibility to pathogen infection (Muthomi *et al.,* 2007). Although the addition of nitrogen to replace that loss via ineffective fixation, may allow for successful growth, an increase in susceptibility to pathogens will nevertheless occur (Muthomi *et al.,* 2007).

The present study compared the growth and performance, in terms of nodulation as well as aerial and root mass, of a genetically modified RR® soybean cultivar to its isoline under ideal conditions and when challenged by three root pathogens.

4.3 Materials and Methods

4.3.1 Nodulation ability of RR® and non-RR® soybean plants.

Nodule production potential was investigated between two soybean isolines, Pannar A5409RG (Roundup® Ready) and Pannar A5409 (minus the gene). Plants were cultivated in a glasshouse with natural sunlight at a temperature regime of 25/18°C day and night. An isolate of *B. japonicum* (WB_{74}), as approved by the Agricultural

Research Council of South Africa*,* was maintained on yeast extract mannitol agar (YEMA) slants and inoculum was produced in yeast extract mannitol (YEM) broth. Mycoroot supplied SuperGro arbuscular mycorrhizae (AM) which was used according to instructions on the label. A loam soil Bainsvlei (Tilahum *et al.*, 2004) was mixed with peat moss (60:40) and steam sterilised (CM Bender, personal communication). Black plastic growth pots (5 L) were filled with the soil mixture and watered to field capacity one day before planting the soybean seeds.

Five small depressions of ~2 cm were made on the surface of the soil into which 500 µL of *B. japonicum* (2 x 10^8 cfu) broth was placed. A single seed was placed in each depression and covered with soil. Sterile water served as the control treatment for the rhizobia. The plants were watered every second day and thinned out to three per pot after the second trifoliate leaf had appeared. The trial was laid out as a complete randomised block design with four replications and maintained in the glasshouse at 25/18°C with natural daylight. No symptoms of yellow flash was recorded during the growing season. After eight weeks, when nodulation usually start decreasing (Bergersen, 1958), the plants were harvested by hand and the foliage was cut off just above the root crown with a pair of shears. The roots were washed through successive sieves to retain as much of the small roots and nodules that had become detached during harvest. The aerial parts and roots, plus recovered nodules of each pot, were placed in separate brown paper bags that were left open and left to dry in a glasshouse maintained at 25°C for 4 days. Nodules, still attached to the roots, were carefully removed and respective plant parts were weighed to determine mass per plant. An analysis of variance (ANOVA) was performed using GenStat Release 17.1 (Copyright 2014, VSN International Ltd) and means were subjected to Tukey's test.

4.3.2 Effect of rhizobia on resistance to root pathogens

The effect of rhizobial infection on the resistance of the two soybean isolines to three soilborne pathogens was evaluated. Isolates of *Fusarium oxysporum* Schltdl.*, Macrophomina phaseolina* (Maubl.) S.F. Ashby, and *Sclerotium rolfsii* Sacc.*,* previously isolated and identified from soybean fields in Limpopo, South Africa (S24.454953 E28.161775), were grown on sterile sorghum seed, dried on a lab bench, and

pulverised to be used as inoculum (Blum & Rodríguez-Kábana, 2006). Inoculum of each of the three pathogens was mixed with steam sterilised soil (3:100) in 5 L growth pots with pulverised sterile sorghum seed as a control treatment. Isolines, A5409RG and A5409, were planted and half the number of seeds were inoculated with *B. japonicum* as described previously. Uninoculated pots received the equivalent of 40 kg of N ha^{-1} as ammonium nitrate, six weeks after emergence applied directly to the soil and leached into the soil with 1 L of water. The treatments were replicated four times and the experiment was repeated over two seasons (laid out as a complete randomised block design). Pots were watered every two days and plants were harvested at eight weeks as previously described. No symptoms of yellow flash was recorded during the growing season. An analysis of variance (ANOVA) was performed using GenStat Release 17.1 (Copyright 2014, VSN International Ltd) and means were subjected to Tukey's test.

4.3.3 Effect of rhizobia and AM on resistance to root pathogens

The effect of rhizobial infection on the resistance of the two soybean isolines to three soilborne pathogens was evaluated as above but with the addition of AM at 1 g of inoculum powder per seed at planting. Seeds were, therefore, treated with either *B. japonicum* or AM, a combination of the two or, with neither as control treatment. At harvest, a 6 g sample of each of the root samples was removed and stained for detection of AM arbuscules or hyphae as described by INVAM (2013. This method is based on microscopic examination of the stained roots for the presence of hyphae and arbuscules. Another 6 g tissue sample was taken from the fresh roots and dried simultaneously with the other material. The dry mass of these samples was determined and the values added to the final dry mass of the roots. Yellow flash was not recorded during eight weeks of growing. Data were analysed using GenStat Release 17.1 (PC/Windows 8) Copyright 2014, VSN International Ltd. Analysis of variance was conducted together with Tukey's test of least significant differences.

4.4 Results and Discussion

4.4.1 Nodulation ability of RR® and non-RR® soybean plants

Comparison of the dry aerial parts, roots and nodules of A5409RG and A5409 after statistical analysis showed no significant differences (P≤0.05) (Fig 4.1). The two isolines are genetically identical except for the insertion of the glyphosate resistance gene and, therefore, no differences in growth would be expected (Raymer & Bernard, 1988). The presence of the gene also showed no detrimental effect on the plant's ability to attract *B. japonicum*, nor any hindrance in terms of the ability of the bacteria to infect the roots. Both lines also proved capable of forming the nodules that protect the bacteroids during nitrogen fixing. The aim of the single gene insertion was to confer resistance to glyphosate and not aimed at enhancing any growth parameters or disease resistance. This also includes any detriment to growth and inoculation as reported by Huber (2010), Ho (2012) and KCMPR (2015). These findings are consistent with those of Loeckner *et al*. (2009), Zobiole *et al*. (2010) and Duke *et al*. (2012) who found that any growth differences observed were attributable to cultivar differences and not the presence of the gene. Most of the latter research was performed on near identical cultivars and not isolines, which could also lead to disparity in results.

4.4.2 Effect of rhizobia on resistance to root pathogens

The dry mass of aerial parts per pot were converted to mass per plant and subjected to analysis of variance, where the effect of the three soil pathogens on the aerial mass was determined on both cultivars when inoculated with *B. japonicum.* Both the roots and nodules were treated in the same way. The roots were not examined for discolouration or the presence of the pathogens. The analysis indicated no significant differences (P≤0.05) between the masses of the aerial parts of the two cultivars in the presence of any three soil pathogens present at an infection rate of 3% of the total soil volume (Fig 4.2). These results were consistent for both the plants inoculated with *B. japonicum* and those treated with ammonium nitrate to replace nitrogen that the bacteria could fix.

When treated with *B. japonicum* and grown in the presence of *S. rolfsii*, line A5409 had a significantly (P≤0.05) higher root mass than all the other treatments over

both cultivars (Fig 4.3). All other treatments over both cultivars, all three the soil pathogens and with ammonium nitrate as replacement, showed no significant differences (P≤0.05) in mass.

The nodule mass of A5409 exposed to *S. rolfsii* was significantly higher (P≤0.05) than that of A5409R also exposed to *S. rolfsii* and A5409 exposed to *F. oxysporum*. There were no other significant differences (P≤0.05) between the nodule masses of the other treatments over both cultivars. This might relate to the higher root mass of A5409 as previously described, which is consistent with conclusions of Vasileva and Pachev (2015).

The lack of significant differences in the performance of the isolines in the present study are consistent with Horak *et al*. (2015). The study compared two soybean cultivars from a similar genetic background, Roundup Ready 2 Yield® soybean (MON 89788) and A3244, a conventional variety, in an ecological risk assessment. The present study found no differences in dry aerial, root or nodule mass. *Fusarium solani* and *M. phaseolina* were amongst the pathogens tested and also showed no differences in susceptibility between the two cultivars tested. The findings of the present study are consistent with Johal and Huber (2009), Zablotowicz and Reddy (2004) and Duke *et al*. (2012). Although the latter authors included the use of glyphosate containing herbicides. Kremer and Means (2009) and Johal & Huber (2009) found that RR® soybeans are more susceptible to certain soilborne fungal diseases than the traditional varieties, but they also included glyphosate, non-glyphosate and no herbicides in their experiments. Research into disease resistance is also often aimed at resistance comparison between various RR® cultivars to establish suitability of a specific cultivar and no comparison is done with non-modified cultivars (Kelly, 2013; Sciumbato, 2014). Since the RR® gene was developed solely to confer resistance to glyphosate containing herbicides, its insertion into the plant should not affect the resistance of plants to soilborne pathogens (Johal & Huber, 2009).

4.4.3 Effect of rhizobia and AM on resistance to root pathogens

The dry mass of aerial parts of A5409 grown in the presence of *S. rolfsii* and not inoculated by *B. japonicum* and AM, was not significantly different (P≤0.05) to

A5409RG. It however, displayed a significantly (P≤0.05) higher mass than the control treatment and treatments including *F. oxysporum* and *M. phaseolina* in the absence of *B. japonicum* and AM. The aerial mass of A5409 inoculated with *B. japonicum* and grown in the presence of *S. rolfsii* was significantly (P≤0.05) higher than that of A5409RG inoculated with *B. japonicum* in the control treatment. None of the other plants inoculated with *B. japonicum* and exposed to the different pathogens, indicated significant (P≤0.05) difference in terms of aerial dry mass.

The aerial dry mass of A5409 inoculated with AM and grown in the presence of *S. rolfsii* shows no significant differences (P≤0.05) to that of its identically treated isoline, the A5409 and the A5409RG in the control treatment inoculated with AM, or the A5409, inoculated with AM and grown in the presence of *F. oxysporum*. The aerial dry mass is, however, significantly (P≤0.05) less than that of A5409RG inoculated with AM and infected with *F. oxysporum*, and both cultivars inoculated with AM and grown in the presence of *M. phaseolina*. The aerial dry mass of A5409 inoculated with both *B. japonicum* and AM in the control treatment is significantly (P≤0.05) lower than that of all the other treatments (Fig 4.5). In none of the treatments did application of the symbionts, singularly or combined, significantly increase the aerial dry mass. In A5409 grown in the presence of *S. rolfsii* there was a significant (P≤0.05) reduction in aerial mass when rhizobia was the only symbiont added.

In the presence of *S. rolfsii* A5409RG, and the absence of the two symbionts, a significantly (P≤0.05) higher dry root mass was observed compared to plants that were not inoculated with the symbionts but exposed to *F. oxysporum* and *M. phaseolina*, or not (Fig 4.6). None of the treatments with either or both of the symbionts present resulted in a significantly higher (P≤0.05) dry root mass than the control treatment. Although not significant at the P≤0.05 level, inoculation with *B. japonicum* showed a tendency to result in a higher root mass than the control treatments when applied to A5409 in the presence of *F. oxysporum*. When grown in soil inoculated with *M. phaseolina*, A5409 displayed a tendency to have increased root mass when treated with *B. japonicum*, as well as when treated with both of the symbionts. Another tendency at the P≤0.05 level is the lower values of A5409 grown in the presence of *F. oxysporum*. None of the stained roots showed any signs of AM infection. All experiments were

harvested at 8 weeks (V6) and according to Dr Dames (personal communication 2014), this might have been too early to observe visual signs of AM infection.

The dry mass of nodules from A5409 inoculated with both symbionts, as well as that of A5409RG inoculated with only *B. japonicum* both grown in the presence of *M. phaseolina*, showed a significantly higher dry mass ($P \leq 0.05$) than A5409 inoculated with both symbionts in the control treatment and A5409RG inoculated with both symbionts in the presence of *M. phaseolina*. No other significant differences ($P \leq 0.05$) were evident for any of the treatments.

The RR® plants used in this study did not display more sensitivity to the pathogens in any of the treatments. This observation is consistent with Johal and Huber (2009) and Duke *et al*. (2012) who observed that the presence of the RR gene had no effect on the sensitivity of gmo plants to soilborne pathogens. It is, however, in contrast to Kremer and Means (2009) who reported that the presence of the RR® gene causes increased sensitivity to pathogens. Johal and Huber (2009) reported increased sensitivity of various RR® crops, including soybeans, to pathogens, but stated that these only occurred when glyphosate dosages were higher than usually recommended.

The mass of nodules formed by A5409RG was significantly ($P \leq 0.05$) lower than that of its isoline when treated with both symbionts and grown in the presence of *M. phaseolina*. This is in contrast to the findings of Duke *et al*. (2012) and Johal and Huber (2009), but may be explained by the findings of Tu (1978), who stated that soil heavily infested with pathogens might decrease the ability of the symbionts to find infection points on the root. The stimulated resistance reaction of the plant by pathogen infection may also render the symbionts incapable of infecting the roots.

Although samples of fresh roots were taken at harvest, stained and microscopically studied, no signs of AM infection were noted. As these plants were harvested at eight months to coincide with the other experiments, this may have been too short a time for development of visible hyphae and arbuscules (personal communication, Dr J Dames, 2014). The role played by the AM can therefore just be induced from dried mass comparison.

4.5 Conclusions

No significant differences in growth parameters were observed between two soybean isolines, one of which had the RR® gene. Nodulations was unaffected, as was the mass of aerial parts and roots. Isolines reacted similarly in the presence of root pathogens, whether they were inoculated with *B. japonicum* or given ammonium nitrate as a source of nitrogen, indicating that the presence of the gene does not affect disease susceptibility. The two isolines reacted in a similar fashion to pathogens when inoculated with AM, suggesting that the RR® gene does not influence disease resistance. Plant growth promoting rhizobacteria (PGPR), including legume symbiotic rhizobia, are known to positively influence the general health and resistance to pathogens in plants. The chapter confirms that inoculation with both rhizobia and AM is critical in the cultivation of legumes.

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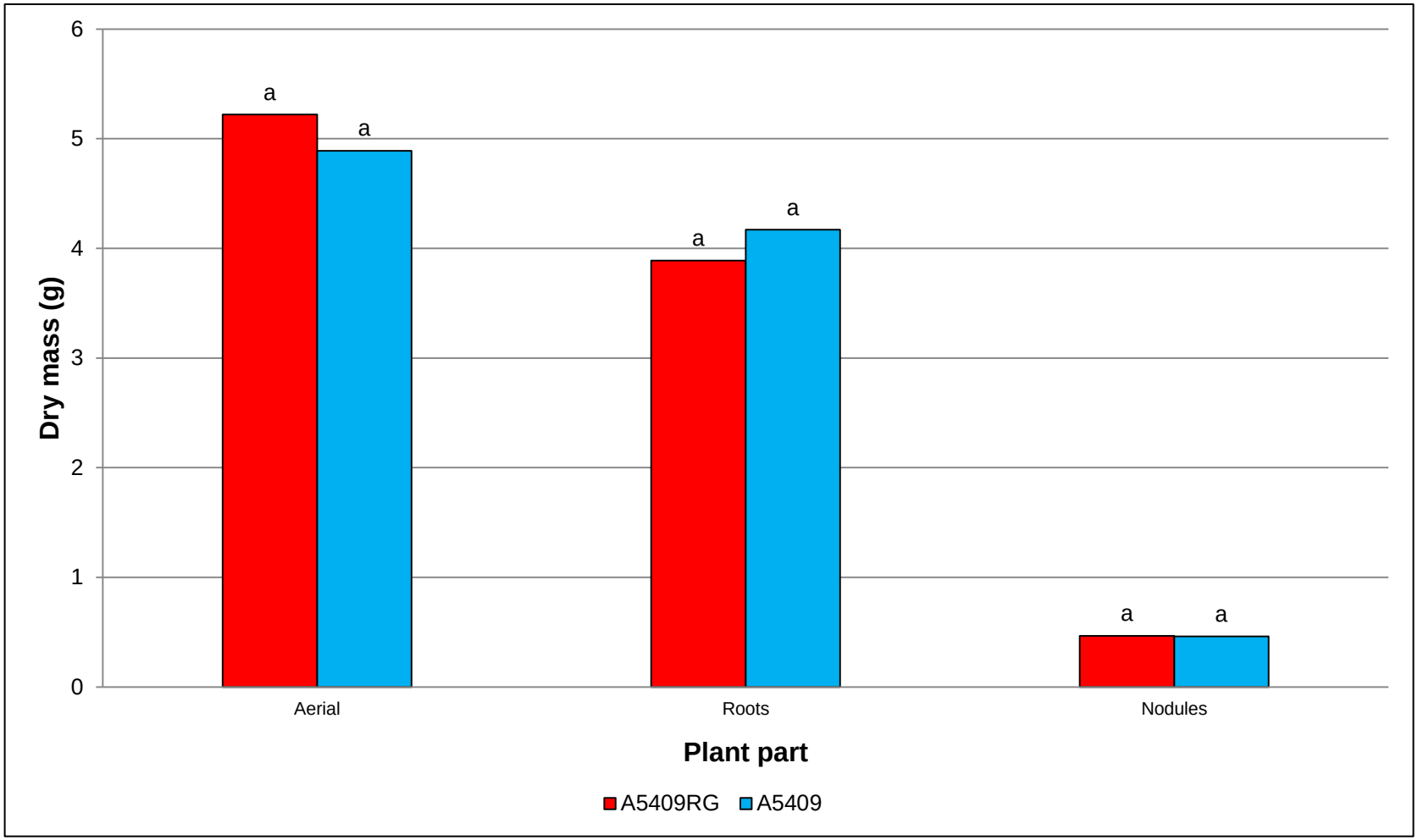

Figure 4.1: Comparison between A5409RG and A5409 in terms of dry mass of organs per plant at V6. Bars with the same alphabetical letters do not differ from each other significantly using means separation of Fischer's LSD ($P<0.05$).

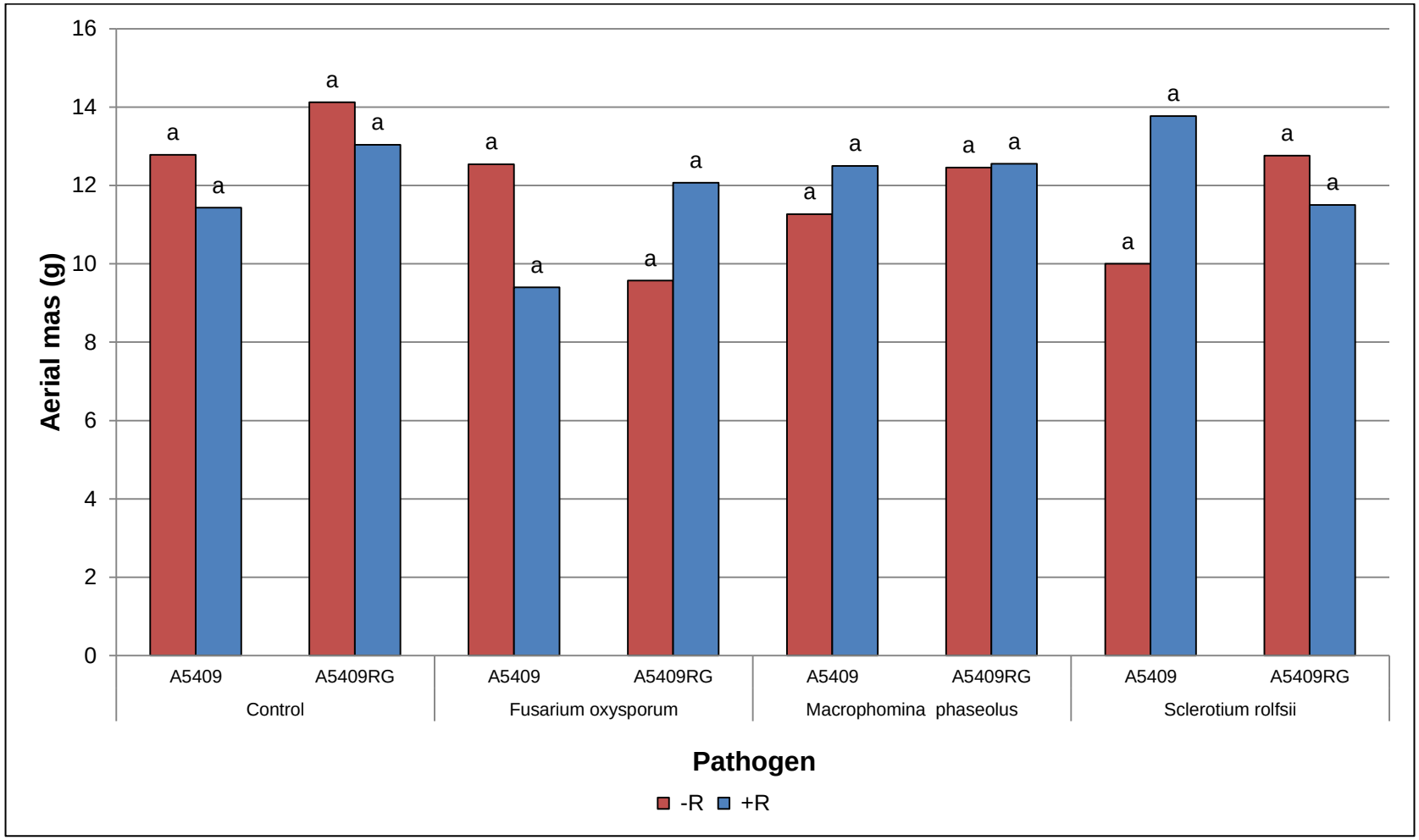

Figure 4.2: Mass of stems, leaves and pods per plant at V6 of the two isolines inoculated with the three pathogens and either inoculated with rhizobia (+R) or not (-R). Bars with the same alphabetical letters do not differ from each other significantly using means separation of Fischer's LSD ($P<0.05$).

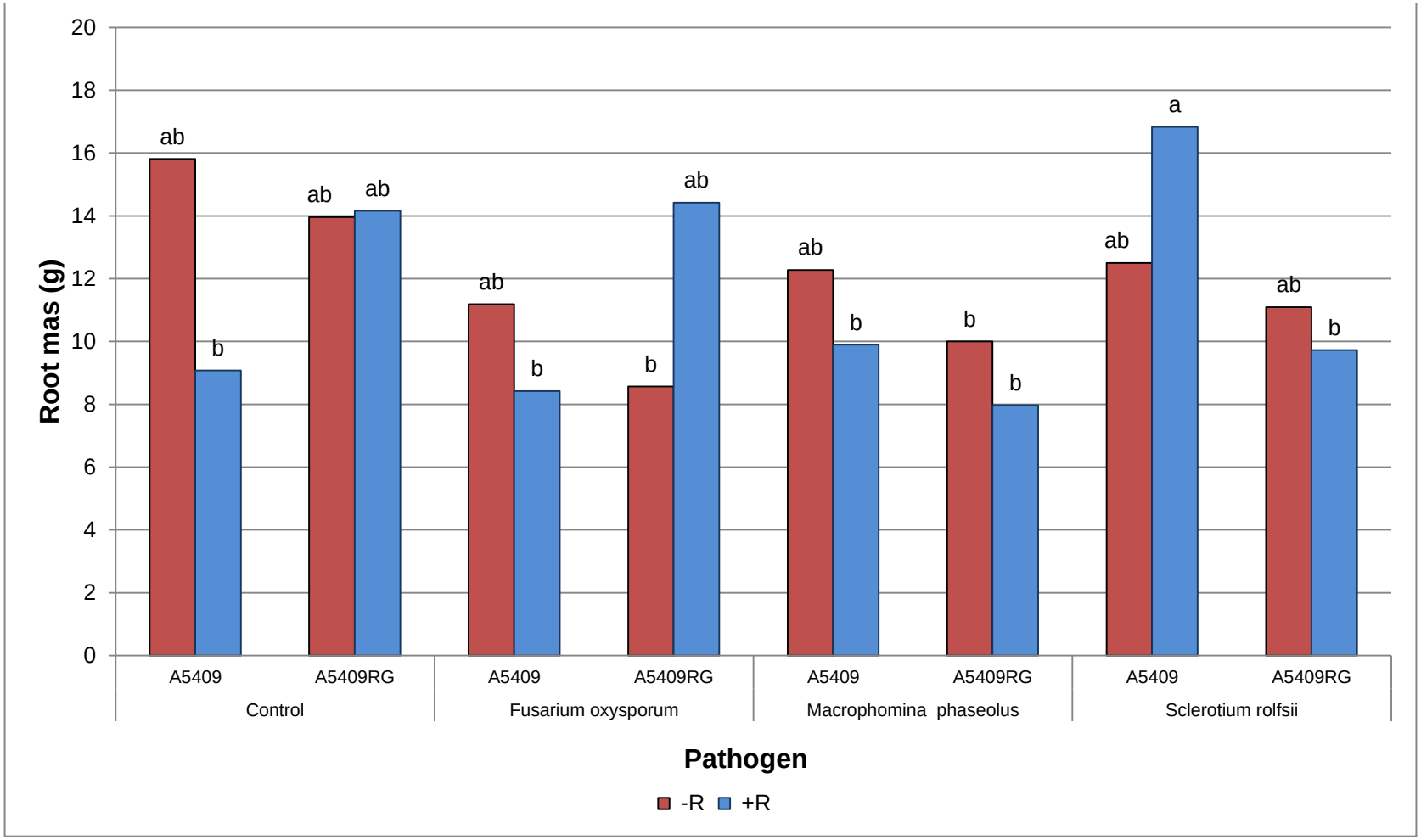

Figure 4.3 Root mass per plant at V6 of the two isolines planted with the three pathogens and either inoculated with rhizobia (+R) or not (-R). Bars with the same alphabetical letters do not differ from each other significantly using means separation of Fischer's LSD ($P<0.05$).

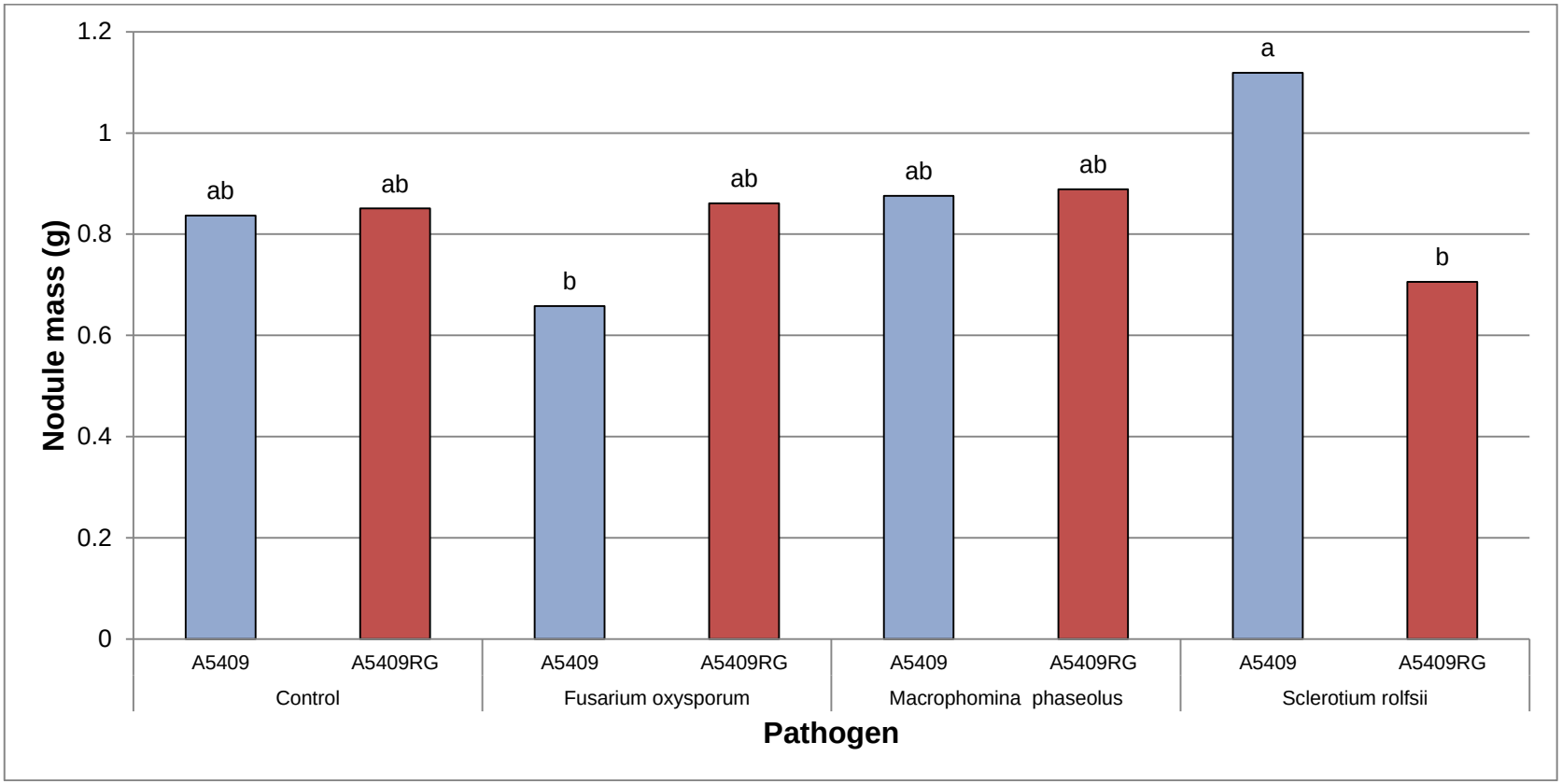

Figure 4.4 Nodule mass per plant at V6 of the two isolines planted with the three pathogens. Bars with the same alphabetical letters do not differ from each other significantly using means separation of Fischer's LSD ($P<0.05$).

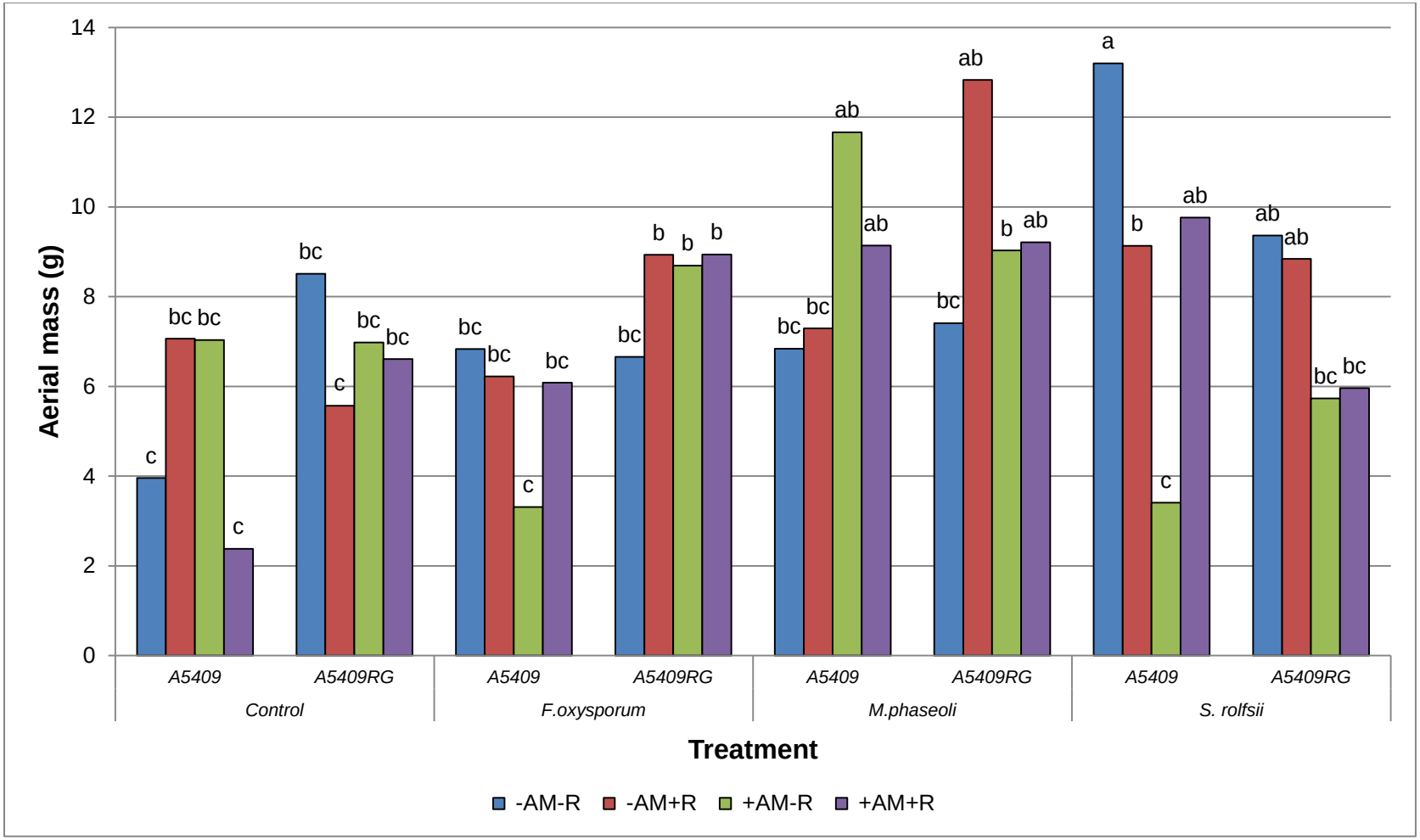

Figure 4.5: Effect of three root pathogens on the mass per plant at V6 of stems, leaves and pods when inoculated with arbuscular mycorrhizae (+M) and *B. japonicum* (+R). -M and -R indicates no inoculation. Bars with the same alphabetical letters do not differ from each other significantly using means separation of Fischer's LSD (P<0.05).

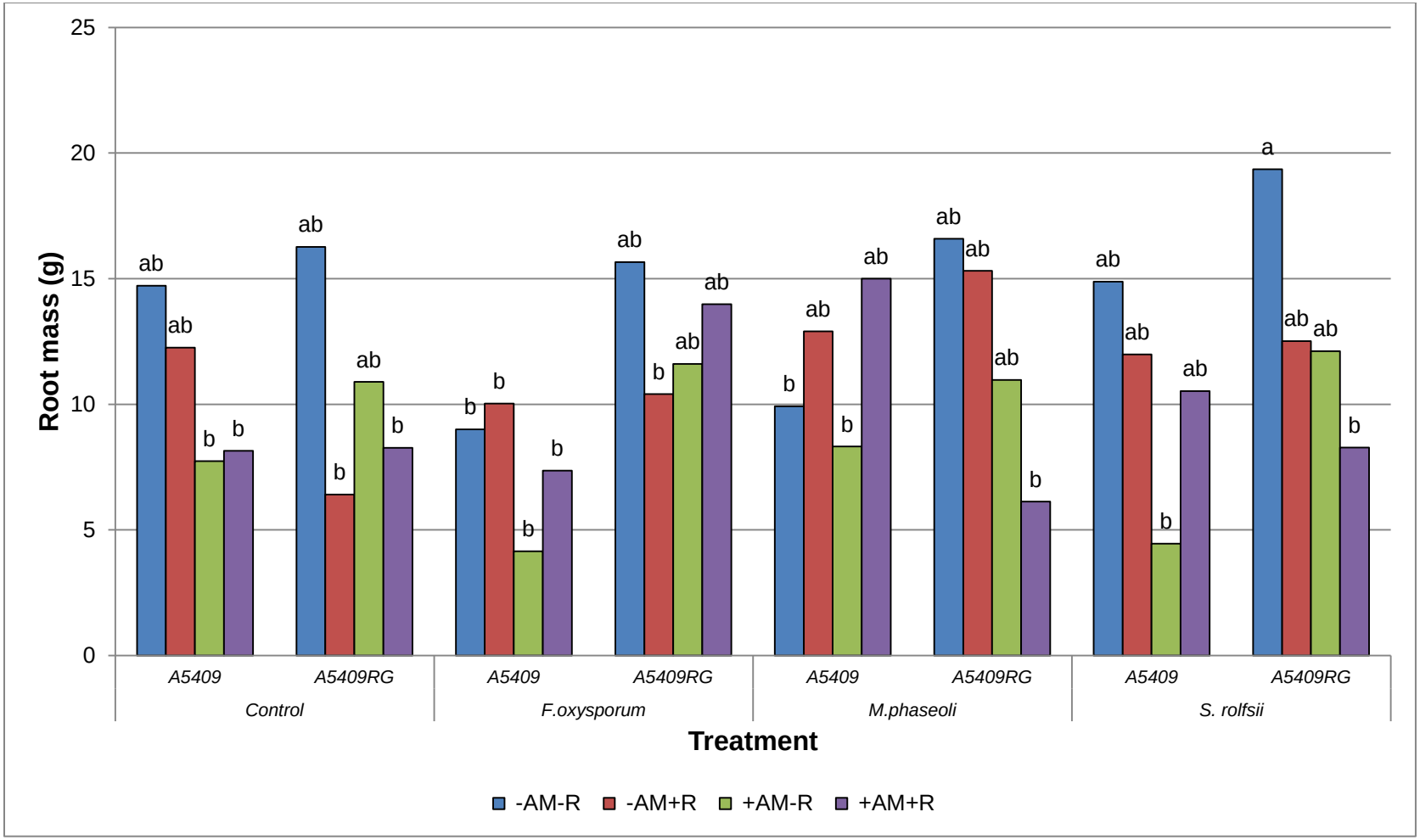

Figure 4.6: Effect of three root pathogens on roots mass per plant at V6 when inoculated with arbuscular mycorrhizae (+M) and *B. japonicum* (+R). -M and -R indicates no inoculation. Bars with the same alphabetical letters do not differ from each other significantly using means separation of Fischer's LSD (P<0.05).

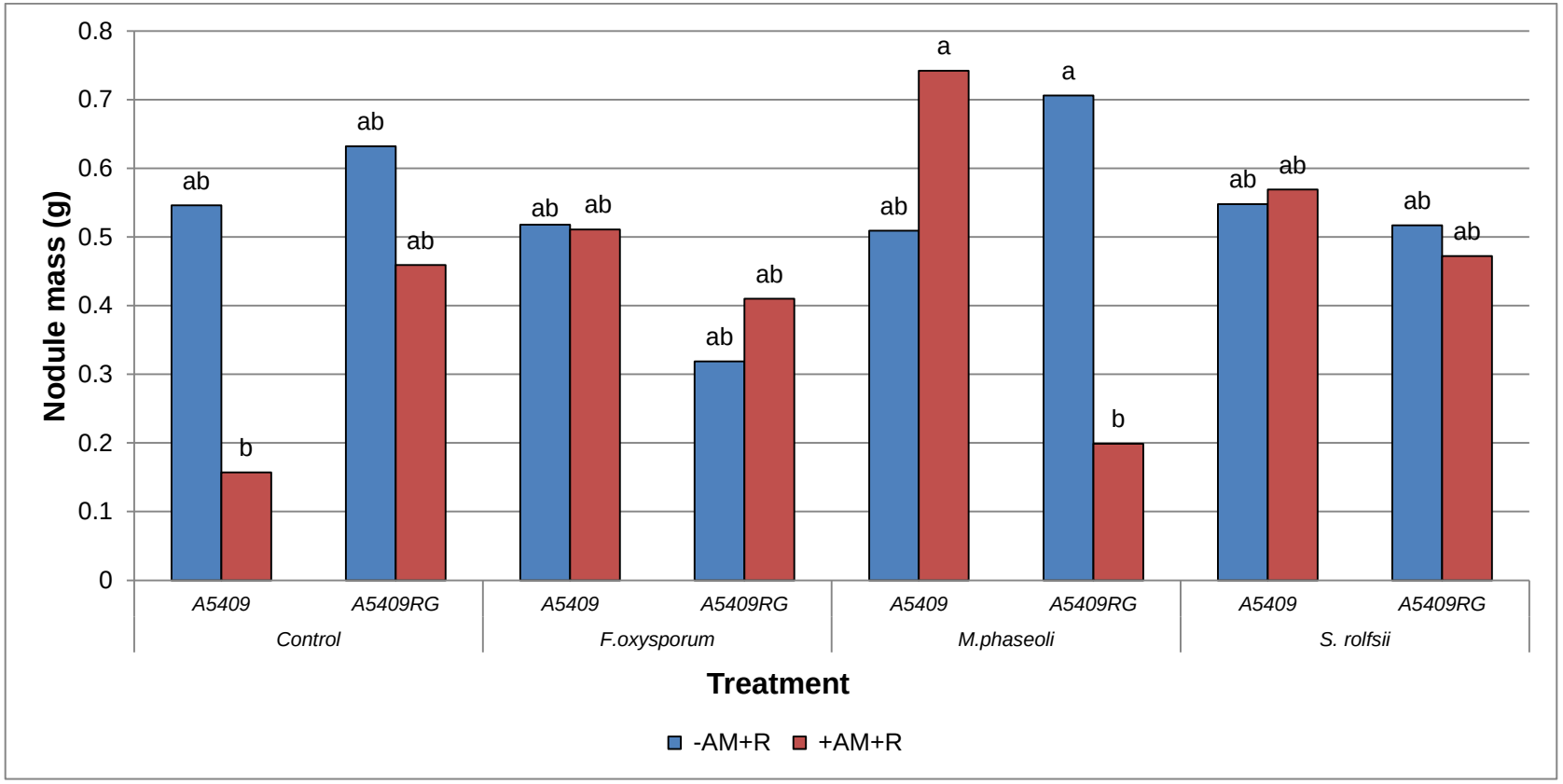

Figure 4.7: Effect of three root pathogens on nodule mass per plant at V6 when inoculated with arbuscular mycorrhizae (+M) and *B. japonicum* (+R). -M and -R indicates no inoculation. Bars with the same alphabetical letters do not differ from each other significantly using means separation of Fischer's LSD (P<0.05).

Chapter 5. General Discussion

5.1 Introduction

Glyphosate containing herbicides act as a kill all herbicide on all plants except those that had been genetically modified. Fears have been expressed that the use of genetically modified soybeans and the glyphosate containing herbicides may have a detrimental effect on the endophytic symbiosis between soybeans and *Bradyrhizobium japonicum* Jordan. There have also been concerns as to effect of the gene on the resistance of soybean to soilborne fungal pathogens. The objectives of the present study were to explore the effect of gene insertion and the use of the herbicide on two isoline soybean cultivars on rhizobia and resistance to three soilborne pathogens.

5.2 Effect of glyphosate and rhizobia on the growth of RR® soybean

5.2.1 *In vitro* evaluation of glyphosate on *B. japonicum*

None of the seven glyphosate formulations had a significant ($P \leq 0.05$) effect on the numbers of colony forming bacteria when applied at the recommended rates (Fig 2.3). The possibility is small that bacteria in their carrier medium would be exposed to glyphosate before inoculation of the seeds during planting. Root exudates from weeds treated during pre-emergence herbicide treatment could be a source of contamination, although glyphosate is strongly adsorbed by soil particles. Ayanalem and Assefa (2017) tested the effect of glyphosate on rhizobia isolated from *Vicia faba* L, and reported an 80% survival rate of the bacteria which is consistent with the results from this experiment.

5.2.2 Inoculation of seeds with treated rhizobia

Treated bacteria were then harvested and used as inoculum on both soybean species. There were no significant differences ($P \leq 0.05$) in the dry masses of any of the plant parts when compared to the controls when the above treated bacteria were used to inoculate both cultivars (Fig 2.4, 2.5, 2.6).

Ayanalem and Assefa (2017) found that *Vicia faba* L. inoculated with *Rhizobium leguminosarum bv. viceae* previously exposed to glyphosate, showed 90-100% inoculation and nitrogen fixing when compared to control plants. Various

authors have found that glyphosate containing herbicides had no effect on rhizobia, although is reportedly some increase in certain other soil microbes and shifts in biodiversity (Wolmarans & Swart, 2014) after use of glyphosate containing herbicides. As the bacterial numbers in commercial inoculum is routinely 10% higher than theoretically necessary for successful colonisation, any die-off by residual glyphosate or other toxins will be negated.

5.3 Effect of glyphosate on the growth of RR® soybeans in the presence or absence of rhizobia when challenged by root pathogens

5.3.1 Effect of glyphosate formulation and rhizobia on the growth of RR® soybeans

Although not significant ($P \leq 0.05$), there was a decrease in plant part masses when the herbicides were applied to plants treated with NH_4NO_3 in place of rhizobium inoculation (Fig 3.2a, 3.3a). No other significant differences were noted in the plant part masses (Fig 3.2b, 3.3b, 3.4).

The decrease in plant mass following NH_4NO_3 addition instead of rhizobial inoculant, is consistent with work done by Namvar *et al*. (2011) where plants that were successfully inoculated thrived when compared to those given fertilisers. In contrast, Duke *et al*. (2012) and Kremer and Means (2009) reported damage to rhizobia. This experiment was carried out in a glasshouse under carefully controlled conditions using only one cultivar of soybean and it isoline containing the RR® gene. Only one soil was used so as to negate the effect variation in soil characteristics, and it may be necessary to repeat this trial in the field.

5.3.2 Effect of glyphosate formulations and rhizobial inoculation on root disease of RR® soybeans

As in the previous experiment, the only decreases in the dry masses of aerial parts, roots and nodules were noted when NH_4NO_3 replaced bacterial inoculation (Fig 3.5a, 3.6a). These differences were not significant at the $P \leq 0.05$ level. No significant differences were found in any of the other treatments (Fig 3.5b, 3.6b, 3.7).

These results are supported by results by Deshwal *et al*. (2003) and Chao (1990), who found that successfully inoculated plants were more resistant to plant

diseases. However, findings by Chen *et al*. (2012) showed increase in disease in pathogenic fungal number when peanuts were monocropped without rotation.

Both these experiments were carried out in a glasshouse under strictly controlled conditions with only the isoline cultivars, using only one type of soil. It may be more meaningful to repeat these under field conditions on different soils and using more isoline cultivars. These two experiments emphasis the importance of successful rhizobial inoculation and colonisation. Once the plants have gone through the six week yellow flash period and do not recover, it is essential to expose the roots of a few plant in each field to assertain successful nodules. If no nodules are found, or the nodules do not have the characteristic red centres, it will be neccesary to add nitrogen fertilisation to the affected fields.

5.4 Response of soybean isolines to infection by soilborne pathogens in the presence of arbuscular mycorrhizae and *Bradyrhizobium japonicum*.

5.4.1 Nodulation ability of RR® and non-RR® soybean plants

The two cultivars did not differ significantly ($P \leq 0.05$) in dry mass of the aerial parts, roots or nodules (Fig 4.1). As successful nodulation is linked to healthy plant development (Raymer & Bernard, 1988), this lack of difference in nodule as well as aerial and root mass, is an indication that the presence of the gene has no effect on the nodulation ability of the plant. These results are consistent with those of Loeckner *et al*. (2009), Zobiole *et al*. (2010) and Duke *et al*. (2012) who found that differences in plant growth was only attributable to cultivar differences.

Although only a single cultivar and it isoline was used in all the experiments, these results emphasise that the insersion of the gene only has an effect on the plant resistance to glyphosate, and does not affect growth and development. As these plants were cultivated under strictly controlled conditions, and harvested at eight week, it may be advisable to repeat this experiment under field conditions.

5.4.2 Effect of rhizobia on resistance to root pathogens

Apart from a significant ($P \leq 0.05$) higher dry root mass of A5409 treated with *B. japonicum* and in the presence of *Sclerotium rolfsii*, no significant differences were noted between the dry masses of the respective plant parts (Fig 4.2, 4.3, 4.4).

New crop cultivars are being developed continiously to enhance and strengthen characteristics such as growth time, amount of protein and oil, number of pods, seeds per pod and many more, the RR® gene was inserted only to provide resistance of the plant to glyphosate (Monsanto, 2008). Few studies comparing the conventional cultivar and its RR® isoline have been carried out without the use of glyphosate containing herbicides. Horak *et al*. (2015) compared two near genetically identical cultivars, and also did not find any significant differences in plant growth.

This experiment was again carried out in a glasshouse under controlled condititons, and even spreading of the pathogen inoculant throughout the soil. It may be advisable to repeat this trail in the field where there could be concentrated areas of pathogen concentrations, which may adversley affect nodulation and disease development.

5.4.3 Effect of rhizobia and AM on resistance to root pathogens

Although no physical presence of AM hyphae or arbuscules were noted in the microscopic evaluation of the roots, there is lack of significant ($P \leq 0.05$) differences in the masses of the plant parts over all four treatments (Fig 4.5, 4.6, 4.7). The lack of significant ($P \leq 0.05$) differences between growth parameters of the two cultivars again emphasises the essential role of the insertion of the RR® gene. The only function of the gene is resistance against glyphosate containing herbicides, and the presence of the gene has no effect on the general growth parameters of the plant, nor its resistance or susceptibility to soilborne fungal diseases. These results are consistent with work done by Johal and Huber (2009) but are in contrast to findings by Kremer (1999), although all authors included glyphosate in their experiments.

The plants were harvested at eight weeks as in all the other experiments to keep the timeline constant. Eight weeks may have been too short a time for AM infection of the roots to have been visible in most of the roots. It may therefore be useful to repeat the glasshouse experiment, allowing the plants to reach maturity, R8, before harvesting, and using more root samples for staining. These experiments were also carried out soley in a glasshouse under controlled conditions. For a more practical and complete evaluation, it is recommended that these experiments be carried out under field conditions, using more than one isoline and in different soil types.

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Summary

The soybean plant has a unique symbiotic relationship with *Bradyrhizobium japonicum* Jordan, an endophytic bacterium that produces ammonia from atmospheric nitrogen and supply the excess to the plant for the production of proteins. These bacteria and other beneficial soil microbes such as the fungal group called arbuscular mycorrhizae and the fungus *Trichoderma*, are attracted to the plant through root exudates in the process of chemotaxis. Fears have been expressed that genetically modified soybeans may lead to a change in types and amounts of exudates produced, affecting the attraction for the soil microbes. Reservation has also been expressed that the presence of the gene may alter the plant's ability to form nodules, depriving the bacteria from shelter and food sources. Another concern was possible toxicity of the glyphosate containing herbicides towards the bacteria, either killing them or inhibiting their ability to inoculate the plant and fixing the nitrogen. In this study, two identical soybean lines, one with the RR® gene and the other without, were used. A variety of experiments were carried out to address the above concerns. When testing the growth parameters, both soybean isolines were used and the same was done when testing the soybeans' resistance to the three soil pathogens. During experiments using application of glyphosate containing herbicides, only the RR® soybean cultivar was used, as the unprotected cultivar would have been killed outright. *Bradyrhizobium japonicum* numbers were not affected by exposure to different glyphosate herbicides, and neither was the ability of these bacteria to inoculate the plants and stimulate nodulation affected. There were no differences in the appearance or in growth parameters between the two soybean isolines when grown under the same conditions. In order to simulate the failure of successful inoculation by *B. japonicum*, ammonium nitrate was used as a source of nitrogen to negate the effect of insufficient N for plant growth. The growth of RR® soybeans was only affected by application of glyphosate herbicides when no inoculation with rhizobia bacteria took place, and ammonium nitrate was used as a source of nitrogen. Infection of soybeans by *F. oxysporum*, *M. phaseolina* and *S. rolfsii* when the plants were exposed to Slash 540 SL (540 g acid equivalent L^{-}) only affected the dry mass of the aerial parts, roots and nodules when no inoculation with rhizobia bacteria took place ie, when ammonium nitrate was used as a source of nitrogen. It can be concluded that neither the presence of the RR® gene, nor the use

of glyphosate containing herbicides have an affect on the growth of the isolines of A5409 soybean plants when rhizobium inoculation was successful. All the experiments were carried out in glasshouses under controlled condition in one type of soil. All of these results were obtained by using the correct amount of herbicide as prescribed on the label and applied at the prescribed times. Correct farming methods, including crop rotation and use of herbicides as recommended in terms of concentration, time of application and weather conditions are of high importance for the herbicides to perform as claimed.

Keywords: Herbicide, glyphosate, legumes, rhizobia, arbuscular mycorrhiza, Roundup® Ready gene, soybean, pathogens