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Title:
Genetic manipulation of Rhizobium for tolerance to heat, acid and salt stress in soils impacted by climatic variability

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GENETIC MANIPULATION OF RHIZOBIUM FOR TOLERANCE TO HEAT, ACID AND SALT STRESS IN SOILS IMPACTED BY CLIMATIC VARIABILITY

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A dissertation submitted to the University of Bristol in accordance with the requirements for award of the degree of Doctor of Philosophy in the Faculty of Life Sciences

University of BRISTOL

17-Oct-20

School of Biological Sciences, United Kingdom
Abstract

Use of *Rhizobium* inoculants is a proven technology to increase legume crop yields, improve soil fertility through symbiotic biological nitrogen fixation (BNF). In Sub-Saharan Africa, BNF is challenged by increasing temperatures due to climatic variability. One possible solution is use of genetically modified heat tolerant strains since use of field selected tolerant strains has been unsuccessful. This research aims to understand genetic changes associated with heat stress in *Rhizobium* in order to enhance stress tolerance through genetic manipulation. Specific objectives are 1) to characterize selected soybean *Rhizobium* strains by 16S rRNA sequencing and identification of differentially expressed genes from heat tolerant strains; 2) to construct plasmids for introduction and expression of the identified genes into a susceptible *Rhizobium*. 3) to evaluate stress tolerance of transformed *Rhizobium* and impact of expressing these genes in *Rhizobium* on improvement of symbiotic growth parameters in soybeans.

Characterization identified USDA193 and HH103 as heat tolerant and susceptible strains respectively while 22 Malawi isolates as non-soybean *Rhizobium*. Proteomic study identified 3628 proteins, 8 differentially expressed at 20°C and 40°C by four-fold were selected, only hsp20, shsp, lbpA and 3 uncharacterised proteins (UC1, UC2 and UC3) were amplified from USDA193 for construction of pLMB51 plasmids for HH103 transformation. All transformants show phenotypic gene expression in normal liquid media. Under stress, the empty plasmid control, UC1, hsp20, UC2, and shsp transformants were acid and salt tolerant while hsp20, UC2 and shsp were heat tolerant. Non-stressed plants (20°C) showed no differences in nodule number (NN) and root dry weight (RDM), but nodule score (NS) except hsp20 and shoot dry mass (SDM) (except UC1 and UC2) were lower than the wild type. Heat stressed plants (32°C) show no differences in NN, NS, SDM and RDM but visual observations on plant vigor predict that hsp20, UC2, and shsp can maintain plant growth.
Dedication and Acknowledgements

I would like to acknowledge the great contribution from my family, especially my wife Jayne, my children Lonjezo Benedicto, Lemekezani Benjamin and Lingalathu Bernard because of moral support. Their company made my studies easier as it is always a challenge to study away from home, family and friends. Lastly, I would like to thank my brothers, sister and other family members for taking up family responsibilities in my absence, especially my brother George who has been a source of inspiration for me and finally my late parents Desiderio and Evarista for mentoring me with the hard working spirit which has allowed me to get this far.

I would like to express my most profound gratitude to my supervisors Prof Gary Foster and Dr. Andy Bailey for the wonderful job for their guidance throughout my study. I would like to thank the management of the University of Bristol for offering me a place as well as providing a conducive environment for study.

Special thanks go to my sponsor, the Department of Agricultural Research Services (DARS) through the Agricultural Productivity Program for Southern Africa (APPSA) a project that funded my PhD studies. Special mention goes to the director of DARS, Dr. Wilkson Makumba for his personal effort to ensure continued funding for my study after the expiry of the initial project funding period.

The Molecular Plant Pathology Laboratory (Lab 321) members at the University of Bristol require a special thanks for providing a safe and conducive environment for study. I would like to thank Drs. Amy James, Kate Heesom, and Mathew Avison for their technical support including all the lab members, Ian, Mona, Katherine, Suhad, Sarah, Farhana, Nisa, Katie, Luis, Anna, Bew, for walking with me through this journey. Finally, many thanks to members of Holy Cross Catholic Church in Bedminster Bristol especially Father Ripper for the spiritual support to me and my family whist in UK. Thank you all.

I dedicated this work to my late parents Desiderio and Evarista Liwimbi.

“Glory be to God.”
Author’s Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate’s own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

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### Acronyms and Abbreviations

- **ATP**: Adenosine Triphosphate
- **BLASTn**: Basic local alignment tool
- **BNF**: Biological nitrogen fixation
- **bp**: Base pair
- **DNA**: Deoxyribonucleic acid
- **FAO**: Food and Agriculture Organization
- **g**: Gram
- **hr**: Hour
- **ITS**: Internal transcribed spacer
- **Kg**: Kilograms
- **LBA**: Luria-Bertani agar
- **LBB**: Luria-Bertani broth
- **ml**: Milliliter
- **Mt**: Metric tons
- **NCBI**: National Centre for Biotechnology Information
- **NN**: Nodule number
- **NS**: Nodule score
- **OD**: Optical density
- **RDWt**: Root dry weight
- **RNA**: Ribonucleic acid
- **rpm**: Revolutions per minute
- **rRNA**: Ribosomal RNA
- **X g**: Gravitational force
1 Introduction

1.1 The biological nitrogen fixation symbiosis

1.1.1 Significance of biological nitrogen fixation

Nitrogen (N) is the main nutrient that limits crop production in most African soils as it is expensive and required in large quantities (Waddington, 2003). Nitrogen in soils is mostly derived from organic matter decomposition, chemical nitrogen fertilizers, nitrogen fixed by symbiotic biological nitrogen fixation (BNF) plus approximately 4% from reaction of electrical discharge from lightening with N₂ (Hoffman et al., 2014). Unfortunately, N supplied to soil as mineral fertilizers is easily lost through volatilization and soil leaching (Butterbach-Bahl et al., 2011). Approximately 50% of inorganic fertilizer applied to farm land is leached because it is applied in a few large doses and only 50% is used due to its low utilization efficiency in plants (Zahran, 1999). According to Dorward & Chirwa (2011), complete dependence of inorganic fertilizers for production of crop in Africa is unsustainable as it is costly and unaffordable for resource poor farmers. Furthermore, overuse of mineral fertilizer is often associated with environmental concerns on water pollution and has negative effects on soil fauna and soil health. Biological nitrogen fixation (BNF) is a cheap, sustainable and environmental friendly source of N, since nitrogen is naturally present in abundance as atmospheric N at approximately 78% (Van Cleemput et al., 2008). Some of the benefits of BNF include sustainable legume yield, soil fertility enhancement, savings on fertilizer costs and as a source of income from sale of farm produce (Waddington, 2003). Unlike other crops, leguminous crops, such as soybeans, associate with Rhizobium, a type of bacterium that lives in soil or root nodules of legumes and is able to fix nitrogen (Butterbach-Bahl et al., 2011). BNF is a natural process that converts atmospheric N (N₂) into usable forms of N such as ammonia and can reduce dependence on inorganic N-fertilizers and hence decrease water pollution (Oldroyd et al., 2011). The fixed form of N as ammonia may either be used directly by the same legume crop (Fig. 1.1) or other subsequent crops such as cereals (Butterbach-Bahl et al., 2011). Symbiotic BNF is estimated to be responsible for annual production of approximately 200 million metric tons (Mt) and approximately 90% of N₂ fixed on the global surface (Biswas and Gresshoff, 2014; Gage, 2004). Legumes of agricultural
importance annually fix between 40 to 60 million metric tons (Mt) of N\textsubscript{2} while other legumes in natural ecosystems fix an extra 3 to 5 million Mt (Graham, 2003). Under optimum soil environmental conditions, BNF can fix in the soil up to 300 kg N ha\textsuperscript{-1} (Gilbert, 2012).

Figure 1.1: Soybean field showing healthy green plants (inoculated with \textit{Rhizobia}) and N deficient yellow plants (non-inoculated), (Kananji et al., 2013)

1.1.2 The symbiotic legume- \textit{Rhizobia} relationship

According to Zahran (1999), BNF is the major source of N input in most agricultural soils in Africa. The BNF plays a vital role in sustainable agricultural systems due to reduced N fertilizer requirement resulting in sustainable production of vegetable protein. Gopalakrishnan (2015) reported that successful legume- \textit{Rhizobia} symbiotic relationships improve soil fertility, increase agricultural productivity, and reduce inorganic fertilizer requirements and water pollution. The symbiosis requires the development of root nodules as result of \textit{Rhizobium} infections for production of plant usable forms of N. According to Gage (2004), \textit{Rhizobium} receives isoflavone signals from plant root exudates which induces a \textit{nodD} gene to act as an activator in gene expression for several nodulation genes in the bacteria (eg. \textit{nodABC}). Gene expression is initiated when \textit{nodD} proteins bind to the conserved \textit{nod}-box with promoters of bacterial nodulation genes (Fig. 1.2). The nod factor
triggers a program that facilitates the construction of root nodules and entry of *Rhizobia* into the nodule via the infection thread. The N fixation system is coordinated by symbiotic fixation and nodulation genes present in the symbiont (Long, 2001). The *nif* and *fix* genes are responsible for N fixation while the *nod, nol* and *noe* genes are for nodulation (Shamseldin *et al.*, 2013) and these genes are carried either on plasmids or symbiotic islands (MacLean *et al.*, 2007).

![Figure 1.2: An illustration of nod factor production and regulation of nodulin genes by *Rhizobia* bacteria (Laranjo, *et al.*, 2014)](image)

Nodule formation is initiated when *Rhizobia* attached to the root hair tips are trapped between two root hair cell walls during curling of the root hair. This results in the formation of an infection thread that produce secondary growths through cell division which merge into visible nodules (Gage, 2004). The bacterium is then released as the cells expand and the infection threads penetrate the young cell walls. Finally, the bacteria transforms into bacteroid, an active state for nitrogen fixation where cells are transformed from rod-shaped...
to Y-shaped (differentiation) resulting in drastic increases in size, shape, and DNA content (Gage, 2004). Bacteroides in nodules are responsible for conversion of N\(_2\) to ammonia to supply the host plant in exchange for carbohydrates (Soto et al., 2006). An active N fixing nodule is characterised by a pink interior colour due to the presence of leghaemoglobin that regulates flow of oxygen in the nodule (Franche et al., 2009) (Fig. 1.3). Increased levels of oxygen are detrimental to the N fixation process as it affects the activity of the nitrogenase enzyme which requires anaerobic conditions.

There are also other important symbiotic nitrogen fixing systems that exist between N fixing bacteria and non-legume crops. According to Lesser et al., (2004), photosynthetic cyanobacteria can form symbiotic associations with *Azolla*, a water fern which helps maintain N levels in rice paddy field systems, while actinorhizal symbiosis occur between *Frankia* bacteria and non-legume trees such as *Myrica* which is important for soil erosion control and wood production (Benson & Dawson, 2007).

![Figure 1.3](http://orgprints.org): Roots of inoculated soybean plants with well-developed nodules with pink interior showing active nitrogen fixation (http://orgprints.org)
1.1.3 The symbiotic biological nitrogen fixation process.

BNF is a natural process facilitated by *Rhizobium* bacteria and other related organisms that possess a nitrogenase enzyme complex. The complex consists of an iron protein and a molybdenum-iron protein component (Zahran, 1999). During this process N₂ is transformed to plant usable forms such as ammonium (NH₃) and nitrate (NO₃⁻) (Butterbach-Bahl et al., 2011). Two molecules of NH₃ are produced from a reaction involving one molecule of N₂. 16 molecules of ATP are needed as an energy source and a supply of electrons and protons from hydrogen ions (Swain & Abhijita, 2013) (Fig. 1.4). BNF is very sensitive to changes in soil environmental conditions which affect both the microsymbiont and the host plant.

**Figure 1.4:** A simplified view of the mechanism of biological nitrogen fixation (Madigan et al., 2000)

Fischer & Newton (2002), in their review indicated that the BNF is initiated by reduced ferredoxin supplied from other metabolic processes. The ferredoxin donates electrons to the nitrogenase enzyme complex to which the N₂ is bound thereby reducing the Fe protein. The reduced Fe protein binds with ATP hydrolyzed into ADP and inorganic phosphate (Pi) that reduce the molybdenum-iron protein which in turn donates electrons to N₂ to produce HN=NH which is further reduced to H₂N-NH₂, and finally to 2NH₃ (Fig. 1.4). The nitrogenase enzyme complex is highly sensitive to oxygen as it reacts with the iron attachment of the
proteins. Supply of oxygen to the nodules is regulated by leghaemoglobin for maintenance of low oxygen levels within the nodules (Swain & Abhijita, 2013). Assimilation of ammonium by plants improves plant growth and productivity and the *Rhizobium* benefits from the exchange of carbohydrates produced by photosynthesis (Oldroyd et al., 2011).

1.1.4 Importance of the host legume soybean

Legumes unlike other non-legume plants, can naturally use nitrogen mostly derived from atmospheric N through symbiotic biological nitrogen fixation, so can grow on N-poor soils. BNF enables legumes to produce high protein plant tissue used as food for humans and animal fodder. Legumes are considered as an important building block for sustainable agriculture as they help to reduce plant nutrient deficiency and reduce environmental water pollution. As plants die, the N-rich debris increases the soil fertility allowing production of other crops (Stagnari, et al., 2017). According to Gepts *et al.*, (2005), legumes fall into three subfamilies: Mimosoideae, Caesalpinoideae and Papilionoideae. Papilionoideae is the largest subfamily which includes many food legumes crops like common bean (*Phaseolus vulgaris*), soybean (*Glycine max*) and chickpea (*Cicer arietinum*).

Soybean is a legume food crop of both nutritional and economic importance as it is a major source of vegetable protein. Soybean is rich in vitamins, contains about 40% protein and about 20% as a vegetable oil on dry weight basis (Yamada et al., 2012). Soybean plays an important role as a source of vegetable protein in both human diets and livestock feed (Graham, 2003). Soybean is grown in both tropical and temperate climates where average yields of 2.5 tons per ha may be achieved in good soils with enough moisture and better management (Mutegi et al., 2008). In most developing countries soybean is considered as a cash crop and sold on the world market as dry seed. Soybean, like most legume food crops, establish and participate in symbiotic BNF relationships as host to N\(_2\) fixing bacteria. However, there is high level of specificity between host legume and bacteria as most *Rhizobia* interact with specific types of legume to establish effective symbiosis (Wang *et al.*, 2012). Specificity may occur early during infection and nodule development or later during nitrogen fixation. Gage (2004) established that expression of lectins by other species of legumes help in host-microsymbiont specificity by simultaneously binding the compatible bacteria to the
plant cell wall. However, *Rhizobia* specificity may pose some challenges in areas like Sub-Saharan Africa where soils do not have a broad range of appropriate indigenous *Rhizobium* as soybean is an exotic crop domesticated from China. Fortunately, there are some *Rhizobium* species that nodulate a wide range of legume hosts. However, there are variable differences in their efficiency to fix N as this also depends on legume cultivar and environment, hence there is a need for optimization. On the other hand, there are also some legumes such as common bean (*Phaseolus vulgaris*) which may be nodulated by a wide range of *Rhizobium* species (Mora et al., 2014).

1.1.5 The characteristics of *Rhizobium* bacteria

*Rhizobia* are characterized as gram-negative beneficial bacteria that can symbiotically fix nitrogen in legume root nodules. *Rhizobia* are rod shaped, non-spore forming bacteria that live aerobically or as facultative anaerobes and have flagella for easy movement (Table 1.1). *Rhizobia* may also survive as free living bacteria after nodule senescence and during the dry season when plant hosts are few (López-Baena et al., 2016). However, other researchers classify *Rhizobium* as a plant growth promoting bacterium (PGPB) because they directly influence plant growth and development. This classification is also supported by Glick (2012), because N fixation is one of the mechanisms used by plant growth promoting bacteria.

According to Willems (2006), classification of *Rhizobium* is based on phenotypic characteristics (morphology, physiology and rate of growth) and DNA-based genotypic characterization (Janda & Abbott, 2007). *Rhizobia* bacteria belong to the class of *Proteobacteria* which has currently more than 98 species belonging to 14 genera α- and β-proteobacteria. Berrada & Fikri-Benbrahim (2014) reported that *Rhizobium*, *Mezorhizobium*, *Ensifer* (formerly *Sinorhizobium*), *Bradyrhizobium*, *Phyllobacterium*, *Microvirga*, *Azorhizobium*, *Ocrhobactrum*, *Methylobacterium*, *Devosia* and *Shinella* genera belong to the α-*Proteobacteria* class while *Burkholderia* and *Cupriavidus* (formerly *Ralstonia*) are in the β-*Proteobacteria* class characterized based on 16S rRNA (Kainth et al., 2005). Sequencing of the conserved internal transcribed spacer region (16S rRNA) has proved that it can accurately be used to differentiate *Rhizobium* strains within the same genus (Degefu
et al., 2013). However, complete genome sequencing indicates that the *Rhizobium* genome comprises of the chromosome and 2 major plasmid or symbiotic islands. The housekeeping genes are mainly required for proper functioning of cells such as metabolism and cell maintenance and are located on the chromosome (Villaseñor et al., 2011), while specific genes located on plasmids are responsible for symbiosis or adaptation to specific ecological zones (Zahran, 1999). The genes specifically required for nodulation are carried either on large plasmids or symbiosis islands (Gage, 2004). Phenotypic classification shows that members in this class are all gram-negative bacteria that grow mostly at very low levels of nutrients. *Rhizobia* in *Sinorhizobium* and *Bradyrhizobium* genera are classified as fast and slow growing bacteria respectively based on number of days to produce visible colonies on agar (Table 1.1).
<table>
<thead>
<tr>
<th>Character</th>
<th>Structure</th>
<th>Parameter</th>
<th>Sinorhizobium fredii</th>
<th>Bradyrhizobium japonicum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphology</td>
<td>colonial</td>
<td>Solid surface</td>
<td>Circular, convex, semi translucent, raised, 2-4 mm in diameter after 3-5 days</td>
<td>Circular, convex, opaque or white do not exceed 1 mm in diameter within 5-7 days</td>
</tr>
<tr>
<td>Cellular &amp; Liquid</td>
<td></td>
<td>Turbidity develops within 3-5 days in agitated broth cultures</td>
<td>Moderate turbidity develops after 3-5 days in agitated broth.</td>
<td></td>
</tr>
<tr>
<td>Morphology</td>
<td>Cellular</td>
<td>Staining</td>
<td>Gram-negative</td>
<td>Gram-negative</td>
</tr>
<tr>
<td>Morphology</td>
<td></td>
<td>Morphology</td>
<td>Rods (0.5-0.9 x 1.2-3.0 μm)</td>
<td>Rods (0.5-0.9 x 1.2-3.0 μm)</td>
</tr>
<tr>
<td>Motility</td>
<td></td>
<td>Motility</td>
<td>Motile by a single polar flagellum or 1 to ≥3 peritrichous flagella</td>
<td>Motile by one polar or subpolar flagellum</td>
</tr>
<tr>
<td>Growth parameters</td>
<td>Physiological</td>
<td>Tropism</td>
<td>Chemoorganotrophic, utilize a range of carbohydrates and salts of organic acids as carbon sources, without gas formation but pentoses are more preferred</td>
<td>Chemoorganotrophic, utilize a range of carbohydrates and salts of organic acids as carbon sources, without gas formation but pentoses are more preferred</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oxygen</td>
<td>Obligate aerobe</td>
<td>Aerobic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pH</td>
<td>Optimum pH 6-8 range pH 5.0-10.5</td>
<td>Optimum pH, 6-7, although lower optima may be exhibited by strains from acid soils</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lifestyle</td>
<td>Symbiotic nitrogen fixers</td>
<td>Intracellular symbiont</td>
</tr>
</tbody>
</table>

Source: [http://web2.uwindsor.ca/courses/biology/fackrell/Microbes/4610.htm](http://web2.uwindsor.ca/courses/biology/fackrell/Microbes/4610.htm) (2/10/19)
1.1.6 *Rhizobium* inoculation and soybean response

1.1.6.1 *Rhizobium* inoculation in Africa

The use of *Rhizobium* in Sub-Saharan Africa (SSA) was initiated with pasture legumes by colonial agricultural research to improve exotic cattle production. Commercial production of inoculants in South Africa started in 1952, however the quality was not good until it was improved in 1970s (Strijdom, 1998). While in Rwanda inoculant production started at Institut des Sciences Agronomiques du Rwanda (ISAR) in 1984 (Chianu et al., 2011). The United Nations Education Science and Cultural Organization (UNESCO) with support from United Nations Environment Programme (UNEP) and Food and Agricultural Organization (FAO) played a great role to promote biological nitrogen fixation (BNF) in Africa. According to Chianu et al. (2011), Microbial Resource Centre’s (MIRCENS) responsible for *Rhizobia* technology generation and dissemination were established in Dakar (Ghana), Cairo (Egypt) and Nairobi (Kenya). In 1990s, FAO supported initiatives at Sokoine University of Agriculture (SUA) in Tanzania to develop Nitrosua bio-fertilizer and University of Nairobi to develop Biofix. Around the same time, Uganda managed to initiate production of inoculants at Makerere University and Madhavani Ltd with support from United States Agency for International Development (USAID) while other initiatives to enhance BNF technology were established in Zimbabwe with support from International Atomic Energy Agency (IAEA) (Chianu et al. 2011; Bala, 2008). Most of the *Rhizobia* strains used for production of soybean inoculants were exotic because most of the soils in SSA had no indigenous rhizobium strains for soybean since the crop was adopted from China.

Sub-Saharan Africa is well-known for its diverse and unique environmental conditions such as arid and semi-arid regions as well as heterogenous soils and climates and is the origin of many legume crops. As a result, indigenous *Rhizobia* species present in SSA differ from those found in other continents. Most of the indigenous *Rhizobia* species present in SSA belong to the genus *Bradyrhizobium*, which often associate with pulses (cowpea, Bambara groundnuts and peanut), legumes of importance to smallholder farmers (Grönemeyer & Reinhold-Hurek, 2018). *Bradyrhizobium japonicum*, a species that nodulates soybeans, requires acclimatization to environmental soil conditions in SSA as it is mainly found in temperate regions (Vinuesa et al., 2008). Therefore, it is not surprising to note that *Bradyrhizobium*
*japonicum* is less competitive in soybean nodulation under heat stress (Suzuki et al., 2014). According to the study on soybean response to inoculation in Sub-Saharan Africa by van Heerwaarden et al. (2018), there was no response to inoculation over 3 years and across various locations. This suggests presence of some common environmental factors limiting response of soybeans to inoculation and establishment. Among them heat, acidity and salt stress are the main suspects since they are direct effects of climates change. Although, acidity may be also due to incorrect and over-use of inorganic fertilizers.

1.1.6.2 *Rhizobium* inoculation in Malawi

According to earlier studies by Chilimba et al., (2000), on soybean response to inoculation in Malawi, results show that inoculation significantly increased grain yield of soybean. However, no significant yield increases were achieved when promiscuous soybean varieties were used without inoculation. This result suggested the need to inoculate soybeans in Malawi since *Rhizobium* population in the soils was not adequate to effectively nodulate promiscuous cultivars. Njira et al. (2013) also reported significant increases in grain yield of soybeans. Yield increase of approximately 100% due to inoculation were observed over and above non-inoculated controls.

*Rhizobium* research and commercial inoculant production for legume crops such as soybean, ground nuts and cowpea in Malawi started at Chitedze Agricultural Research Station as early as 1970s (Chianu et al., 2011). Inoculants containing *Rhizobia* bacteria that nodulate soybean were required in Malawi soils since soybean is a non-native crop. The introduction of new *Rhizobium* strains and genetic material through use of commercial inoculants resulted in increased *Rhizobia* soil population and diversity (Loureiro et al., 2007). Until the late 1980s and early 1990s, the market was dominated by commercial seed-applied peat-based inoculants (Chilimba, 2008). Inoculant production increased from 450 to 1800 packets (50g) between 1976 and 1987 due to improved extension programs (Chianu et al., 2011). Since then, the production levels stagnated until 2010 when the production unit received a boost from FAO through a contract to supply 20,000 packets of soybean inoculant for the Productivity Improvement Project. After the project was phased out in 2013, production was cut back due to lack of resources until 2015 when production of inoculants was privatized in order to increase production capacity, distribution and access to inoculants.
Over recent years, the Ministry of Agriculture in Malawi through agricultural field days has been receiving feedback from farmers on inoculation failure. In order to verify this claim of soybean inoculation failure, recent studies have shown a sharp decline in soybean response to inoculation despite the presence of *Rhizobium* bacteria in soils after decades of inoculation. Van Heerwaarden et al. (2018), in their research conducted over a period of 3 years reported no significant differences in grain yield of inoculated soybeans. Furthermore, Phiri et al. (2016) also reported that inoculating soybean with *Rhizobia* as well as inoculation plus a starter dose of 30 kg N ha\(^{-1}\) did not significantly increase biomass yields. These results suggest the contribution of some other environmental factors as being responsible for non-response of soybean to inoculation, perhaps associated with high temperature, soil acidity and salts.

1.2 Climate change challenges in agriculture

The planet is surrounded by a blanket of gases that protects it from extreme temperatures and keeps it warm. Plants, animals and microbes would not survive on this planet without this gas as it would be 20–30°C colder. However, in recent years there has been a steady global temperature increase as the thickness of this blanket of gases is altering due to rapid accumulation of greenhouse gases (GHGs) in the atmosphere. The production of these GHGs which include carbon dioxide (CO\(_2\)), methane (CH\(_4\)) and nitrous oxide (N\(_2\)O) is due to human activity of which agriculture is the major contributor. These gases trap more heat and causes the planet to warm up, a phenomenon known as global warming. Global warming is associated with a shift in rainfall distribution locally, regionally or globally and is often linked to local temperature increases, and water scarcity which are major effects of climate change (Thomas et al. 2006).

Agricultural production heavily depends on predictable weather and climatic conditions as well as ecosystem services like genetic resources; water regulation; soil formation; pest regulation; nutrient cycling and primary production (Howard & Reading, 2011). Climate change causes unpredictable weather patterns and creates fundamental changes in temperature and precipitation. As a result, these changes negatively impact agricultural production through crop yield losses which often challenge food security. Furthermore, there is also increased prevalence of plant and animal diseases with more prevalent pests.
and fungal pathogens (Bank et al., 2008). The climate change effects which include drought, extreme temperatures, flooding, salinity and acidity affect natural processes which in turn constrain agricultural productivity (Zahran, 1999). High temperatures are detrimental to crop production particularly when the plants are flowering and often result in lower productivity, fertility and reduced immunity in animals hence more prone to certain diseases. Drought affects agricultural production as water stress alters soil chemical and physical properties as well as biological activities that are important for plant and soil health (Lehman et al., 2015). Flooding impacts on agriculture due to decrease in soil quality resulting from depletion of soil oxygen causing reduced soil conditions which affect soil-water chemistry and loss of soil aggregation (Bünemann et al., 2018). Acidity and salinity negatively affect agricultural production since very few plants and microbes can survive under these soil conditions. Elevated temperatures increase microbial activity and hence an increase in respiration, which produces more CO₂ as a waste product. Fortunately, there are natural chemical cycles mediated by both bacteria and archaea that control and balance these gases. For instance, in the nitrogen cycle; Rhizobium bacteria convert atmospheric nitrogen into plant usable forms of nitrogen. Research has shown that soil respiration and the release of CO₂ may double for every 5-10°C increase in temperature. An increase in the release of CO₂ causes more global warming which speeds up the activity of the soil microbes resulting in a vicious circle (El-Ramady et al., 2013.). Although this may result in more favorable conditions that could increase the yields of some crops, in some regions, these potential yield increases are likely to be restricted by extreme events, particularly extreme heat and drought, during crop flowering (European Environment Agency, 2015).

1.3 The effect of soil environment stress factors on symbiotic Rhizobia

Agricultural productivity globally is mostly constrained by soil environment stress factors which include among others high temperature, soil acidity and salinity (Zahran, 1999). According to Mhadhbi et al. (2015), these factors often affect agricultural land in arid and semi-arid climates which covers approximately 40% and 30% of earths arable land surface respectively. Natural environmental processes, such as BNF, play an important role in sustainable agricultural production. Unfortunately, under tropical environments, BNF is greatly compromised by these stress factors which negatively affect or completely hinder
legume root-nodule formation and function. However, there are clear variations in *Rhizobia* tolerance to most of these soil environment stress factors (Zahran, 1999).

### 1.3.1 High temperature

There are wide variations on the effects of high temperature on soil microbes as they depend on both soil type and environmental conditions. Some of the impacts of temperature on microbes include changes in population abundance, diversity, altered functions and gene selection. Nitrogen fixation is driven by complex microbial transformations which are often affected by changes in temperature (Schloter et al., 2018). The survival and persistence of *Rhizobium* bacteria in the soil is greatly influenced by temperatures. Temperature in the tropics often hit a maximum range of 40-50°C despite the fact that *Rhizobia* bacteria is only able to survive and fix biological nitrogen at a maximum temperature range of 32-47°C and 27-40°C, respectively (Hungria and Vargas, 2000). The reduction in number of *Rhizobia* in the soil, genetic alterations or deletion and poor physiological status of surviving *Rhizobia* may lead to loss in N fixation capacity (Hungria and Vargas, 2000). High temperature causes loss of N fixation due to a decrease in exchange of molecular signals and inhibition of root-hair formation so reducing the number of sites for nodulation. Elevated temperature prevents attachment of bacteria to the root hairs, root-hair penetration and formation of the infection-thread. Despite a successful infection, high temperature still affects nodule initiation by preventing the release of *Rhizobium* from the infection thread as well as development of the bacteroides. When nodule development is achieved, high temperature often results in sub-optimal nodule function due to decreased synthesis of leghaemoglobin and reduced nitrogenase activity. Characterization of PP201 a wild-type Pigeon Pea *Rhizobium* strain and heat-resistant mutants cultures at 30°C and 43°C by (Nandal et al., 2005) established cellular biochemical changes. At 43°C, mutants produced large quantities of exopolysaccharides (EPSs) and different kinds of lipopolysaccharides (LPSs) while wild type produced no LPSs. Furthermore, the protein pattern at 43°C indicated that wild-type formed very few proteins while mutants overproduced additional proteins such as a 63–74 kDa heat shock protein (*Hsp*). Bansal et al. (2014) observed prominent beneficial effects on symbiotic effectiveness on Mung bean (*Vigna radiata*) with thermal tolerant *Rhizobium* isolates at higher temperatures. At 45°C, *Rhizobia* isolate MR23 produced new polypeptide
bands of approximately 75 and 21 kDa while MS57 produced a new polypeptide band of approximately 20 kDa. Analysis of data highlighted above has shown that specific proteins produced under stress are responsible for the stress tolerance, hence the need for more research on genetic approaches. Hungria & Vargas (2000) also suggest that selection for effective *Rhizobium* strains adapted to local soil environments has not proved to be a good approach to ensure efficient N fixation under stress. However, there are a number of studies that demonstrate the impact of heat stress gene overexpression in *Rhizobium* to induce stress tolerance. Paço et al. (2016), show that addition of an extra copy of *clpB* in *Mesorhizobium mediterraneum* enhanced nodulation and nodulation competitiveness in chickpea under stress conditions. In other bacteria, some proteins which are responsible for temperature tolerance are to some extent also involved in acid tolerance as they are upregulated under acid stress. Lemos *et al.* (2001) demonstrated that *dnaK* and *groESL* genes in *Streptococcus* mutants are upregulated in response to acid shock and persistent acidification. While Mujacic & Baneyx (2007) indicated that Hsp31 a heat-inducible chaperone plays an important role in the acid resistance of starved *Escherichia coli*.

1.3.2 Salinity

The influence of climate change on rainfall patterns is responsible for alteration of soil salinity which has a huge impact on soil microbial populations. Unpredictable rainfall patterns and a reduced amount of total rainfall results in increased salinity, an accumulation of salts in the soil. Salinity is a global challenge as it affects almost 40% of land area in tropical and Mediterranean regions. Salinity threatens agricultural productivity as it causes a drastic decrease in productivity of most crops in arid and semi-arid regions where salt toxicity as well as osmotic stress heavily affect soil microbes (Zahran, 1999). According to Elsheikh (1998), the harmful effect of salts on the growth of *Rhizobium* is generally due to specific ionic effect of salts rather than their osmotic effect. Research by Fahmi *et al.* (2011), showed that increasing salinity of irrigating water affected inoculated faba beans though decreased nodulation, nodule N, nitrogenase activity and chlorophyll content. Interestingly, the effect of these salts is influenced by presence or absence of an energy source such as carbon since the type of growth media plays a significant role on tolerance of *Rhizobium* to salts (Zahran, 1999). Abdelmoumen *et al.* (1999) recommended NaCl as a good indicator for assessing
Rhizobium tolerance to salt as growth in susceptible strains does not stop completely even at 5%. According to Mashhady et al. (1998), exotic Rhizobia strains growing in liquid media are adversely affected by high salts compared to the wild type with a relatively slight salt tolerance. Salt tolerance may therefore be linked to genetics since fast-growing Rhizobium strains are more tolerant to salts than slow growers like Bradyrhizobium. In agreement with this, Cardoso et al. (2015) correlated gene presence on plasmids to salt tolerance after using multivariate approach to assess salt tolerance of Rhizobia and their N-fixing potential by relating salt tolerance to rainfall and then water availability to salt tolerance. In this research, the impact of salinity on the genetic profile and N fixation varied due to salt tolerance of strains. The results also showed that growth of Rhizobium decreases when electrical conductivity (EC) of growth media is raised by between 15% and 28%. Surprisingly, many salt-tolerant strains of Rhizobium may grow and survive at soil salt concentrations that are inhibitory to most agricultural legumes. Furthermore, Rhizobium bacteria exhibit wide diversity in salt tolerance due to increased microbial ecology diversity. This suggests availability of Rhizobium strains with inherent tolerance to salinity which may also be responsible for tolerance to other stress factors.

1.3.3 Acidity

Soil pH is a measure of how acidic or alkaline the soil is or the concentration of hydrogen ions that contribute to general soil conditions. Acidic soils have a pH of less than 5.5 as measured on the pH scale of 1(acidic) to 14 (alkaline). Soil acidity is currently one of the major challenges in tropical agriculture resulting from tropical conditions which often lead to highly weathered, leached and acidic soil containing high levels of aluminium (Al) and manganese (Mn) (Wood, 1993). The pH between 6.0 and 7.0 is considered as optimum for Rhizobium although a few strains can still grow well at pH of less than 5.0 (Hungria & Vargas, 2000). Low pH is responsible for low legume productivity as it affects plant growth and survival of Rhizobia in addition to a decrease in nodule development and hence low N fixation (Ferguson et al., 2013). Soil acidity affects solubility of soil nutrients and plant growth through Al and Mn toxicity or Ca, Mg, P and Mo deficiency since most mineral soil nutrients are readily available for plants near-neutral pH (Rahman et al., 2018). Simple mechanisms such as cytoplasmic buffering, proton exclusion and heat shock response are
used by microbes for survival when the soil pH is less than 5.5 (Ferguson et al., 2013). Acidic soil conditions are associated with alteration of genetic material of both plant and soil microbes (Morón et al., 2005). According to Hungria & Vargas (2000), acidity at pH 4.5 or less affects the root infection process and exchange of molecular signals as a result of a reduction in the release of nod-gene inducers by soybean and inactivation of nod genes like nodA. As a result, nodulation decreases and liming is therefore required to raise the pH above 5.2, where nutrients such as calcium and molybdenum become available to ensure good nodulation and N₂ fixation (Hungria and Vargas, 2000). Generally, it can be concluded that acid tolerant strains perform better than acid sensitive strains in acidic soils, however, Jida & Assefa (2014) found that nodulation in sand at pH (4.5-5.0) was completely inhibited in both acid tolerant and sensitive strains of B. leguminosarum bv. Viciae that had been isolated from strongly acidic soil pH (4.8-5.2) and neutral soil, respectively. According to O’Hara et al. (1989), Rhizobium tolerance to low pH depends on their ability to maintain the pH inside cells within 7.2-7.5 when the external pH is low. However, a number of findings suggest that genetics has a role in Rhizobium tolerance to acidic conditions. Dilworth et al. (2001), in their research on genes responsible for stress tolerance unveiled a sensor-regulator gene pair (actS-actR) essential for growth at acidic pH. Tiwari et al. (1996) reported that the actS protein acts both as a sensor and signal transmitter that influences gene transcription in Sinorhizobium meliloti. To confirm its role in acid tolerance, the workers were able to generate acid sensitive phenotypes by inactivation of the actS gene. Fenner et al. (2004) also proved that actS and actR are required for low pH since R. leguminosarum knockout mutations in actS and actR were acid sensitive and resulted in slower growth than the control at pH 7. Vinuesa et al. (2003) also identified lpiA in S. medicae as a gene upregulated by acid shock whose expression is crucial for enhancing viability of cells exposed to lethal acid (pH 4.5). However, its transcription requires the fused sensor-regulator product of the fsrR gene (Reeve et al., 2006). This evidence therefore highlights the need to explore more on genetic approaches to acid stress tolerance.
1.4 Importance and functions of molecular chaperones and small heat shock proteins

Environmental bacteria endure different types of stresses like heat, acid and salt stress that may cause damage to cell components. To overcome and survive the stressful conditions, bacteria use highly specialized structured proteins called chaperones to protect their intracellular materials. Chaperones are a large group of un-related protein families responsible for stabilization of unfolded proteins and assist in recovery from stress (Sugimoto et al., 2008). Verghese et al. (2012) in their review outlined some functions of molecular chaperones which include prevention of protein damage by heat from aggregation, unfolded aggregated proteins, and refolded damaged proteins for efficient degradation. Chaperones are important proteins that function to safeguard cell viability as they prevent the degradation of proteins under severe stress conditions and their expression is often upregulated as a feedback to cellular stress (Verghese et al., 2012). Baneyx & Mujacic (2004) indicated that the role of chaperones is to prevent unsuitable conformation of exposed non-polar amino acids by facilitating their correct and efficient folding, degradation or assembly before they are transported across membranes. Some chaperones are also called heat shock proteins as they act to protect proteins from heat stress. Furthermore, they provide the right environment in which correct protein folding can occur (Fig. 1.5). These proteins can recognise and correct mistakes in folding by binding to the non-polar surface. They also promote correct folding of their substrate proteins by unfolding incorrect polypeptide chain conformations and provide an environment in which correct protein folding can occur (Mayer & Bukau, 2005). However, some chaperones like Hsp 90 interact with only unfolded proteins while others like Hsp70 and Hdj-1 are non-specific as they interact with both unfolded and partially folded proteins (Baneyx & Mujacic, 2004).

Molecular chaperones have specific properties that assist them to achieve their functions. According to Sugimoto et al. (2008), molecular chaperones interact with unfolded newly synthesised protein subunits emerging from the ribosome or partially folded proteins being translocated across subcellular membranes. They stabilize non-native conformation and facilitate correct folding of protein subunits, but they do not interact with fully functional
native proteins which are in their folded form nor form part of the final folded structure (Baneyx & Mujacic, 2004).

Jolly and Morimoto (2000) reported that molecular chaperones present in bacterial, plant and animal cells are classified into six major families according to their molecular size. These are further divided into three functional subclasses considering their mechanism of action which include folding, holding and solubilization chaperones (Table 1.2). The foldase chaperones work to support the folding of proteins in an ATP-dependent manner. While holdase binds folding intermediates to prevent their aggregation (Baneyx & Mujacic, 2004).

![Figure 1.5: The role and functions of Heat Shock Proteins (HSPs) in improving heat stress tolerance in E. coli. Source; Reddy et al, (2015).](image-url)
### Table 1.2: Common molecular chaperones in bacteria, plant and animal cells and their role in stress tolerance

<table>
<thead>
<tr>
<th>Family</th>
<th>Organism</th>
<th>Chaperon</th>
<th>Location</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hsp100</td>
<td>E. coli</td>
<td>ClpA, B, C</td>
<td>Cytosol</td>
<td>Helps the re-solubilization of heat-inactivated proteins from insoluble aggregates</td>
</tr>
<tr>
<td></td>
<td>S. cerevisiae</td>
<td>HSP104</td>
<td>Cytosol</td>
<td></td>
</tr>
<tr>
<td>Hsp90</td>
<td>E. coli</td>
<td>HtpG</td>
<td>Cytosol</td>
<td>Refolding and maintenance of proteins <em>in vitro</em></td>
</tr>
<tr>
<td></td>
<td>S. cerevisiae</td>
<td>HSP83</td>
<td>Cytosol</td>
<td>Autoregulation of the heat shock response</td>
</tr>
<tr>
<td></td>
<td>Mammals</td>
<td>HSP90</td>
<td>Cytosol</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GRP94</td>
<td>Endoplasmic reticulum</td>
<td></td>
</tr>
<tr>
<td>Hsp70</td>
<td>E. coli</td>
<td>DnaK</td>
<td>Cytosol</td>
<td>Autoregulation of the heat shock response</td>
</tr>
<tr>
<td></td>
<td>S. cerevisiae</td>
<td>Ssa 1–4</td>
<td>Cytosol</td>
<td>Interaction with nascent chain polypeptides</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ssb 1,2</td>
<td>Cytosol</td>
<td>Functions in interorganellar transport</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kar2</td>
<td>Endoplasmic reticulum</td>
<td>Refolding and maintenance of denatured proteins <em>in vitro</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ssc1</td>
<td>Mitochondria</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mammals</td>
<td>HSC70</td>
<td>Cytosol/nucleus</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>HSP70</td>
<td>Cytosol/nucleus</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>BIP</td>
<td>Endoplasmic reticulum</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>mHSP70</td>
<td>Mitochondria</td>
<td></td>
</tr>
<tr>
<td>Hsp60</td>
<td>E. coli</td>
<td>groEL</td>
<td>Cytosol</td>
<td>Refolding and prevention of aggregation of denatured proteins <em>in vitro</em>;</td>
</tr>
<tr>
<td></td>
<td>S. cerevisiae</td>
<td>HSP60</td>
<td>Mitochondria</td>
<td>facilitates protein degradation by acting as a cofactor in proteolytic systems</td>
</tr>
<tr>
<td></td>
<td>Plants</td>
<td>cpn60</td>
<td>Chloroplasts</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mammals</td>
<td>HSP60</td>
<td>Mitochondria</td>
<td></td>
</tr>
</tbody>
</table>
Assembly of bacteriophages and Rubisco (an abundant protein in the chloroplast)

<table>
<thead>
<tr>
<th>Hsp40</th>
<th>E. coli</th>
<th>DnaJ</th>
<th>Cytosol</th>
<th>Essential cochaperone of Hsp70 proteins to enhance rate of adenosine triphosphatase activity and substrate release</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>Ydj1</td>
<td></td>
<td>Cytosol/nucleus</td>
<td></td>
</tr>
<tr>
<td>Mammals</td>
<td>Hdj1, 2</td>
<td></td>
<td>Cytosol/nucleus</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>small heat shock proteins</th>
<th>E. coli</th>
<th>lbp A and B</th>
<th>Cytosol</th>
<th>Suppresses aggregation and heat inactivation of proteins in vitro thermotolerance through stabilization of microfilaments</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>HSP27</td>
<td></td>
<td>Cytosol</td>
<td></td>
</tr>
<tr>
<td>Mammals</td>
<td>αA and αB crystallin</td>
<td>Cytosol</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HSP27</td>
<td></td>
<td>Cytosol</td>
<td></td>
</tr>
</tbody>
</table>

Source: Jolly & Morimoto (2000)
1.5 Molecular chaperones and shsps for tolerance to acid, heat and salt stress

*Rhizobium* bacteria naturally exhibit optimal growth at temperatures between 28-30°C. This range is below the extreme temperatures experienced in some parts of southern Africa as high as 42.4°C, which are above the critical temperature range of N₂ fixation in soybeans of 35 - 40°C (Masters (2010); Zahran (1999). Most locations in southern Africa frequently experience extreme temperatures. Fortunately, bacterial cells have the capability to tolerate variation in temperature, but temperatures out of the tolerable range often affect their cell structure and function (Verghese et al., 2012). Protein folding is impaired by heat and hence chaperones function to prevent or correct damage caused by misfolding. At temperatures above their tolerable range, cells survive by upregulation of heat shock proteins (HSPs) through heat shock response (HSR) as illustrated in Fig. 1.6. In order to perform this function, some of these molecular chaperones are involved in energy dependent reactions. These chaperones include an ATP-dependent disaggregate *clpB* which promotes the solubilization of protein aggregates and functions in collaboration with other proteins in the *DnaKJ* system to achieve efficiency (Paço et al., 2016). Chaperones like *dnaK* and *groEL*, through energy propelled conformational changes facilitate refolding or unfolding of affected proteins (Mayer & Bukau, 2005). According to Kilstrup et al. (1997), *DnaK* (fast-induced protein) is synthesized rapidly in the first 10 min following heat shock, while *groEL* and *groES* (slow induced proteins) are initially synthesized gradually but maintain a high synthesis rate after 15 min in *Lactococcus lactis*. Research by Mogk et al., (2003), show that partially affected proteins are initially supported on the surfaces of holding chaperones (*Hsp33, Hsp31* and *lbpB*) prior to folding while controlled inactivation of heat and prevention of aggregation is the function of *lbpA/B* chaperones.
Some of these protein chaperones have well-known characteristics and are also present in specific *Rhizobium* strains. The 10 kDa chaperone *groES2* is present in *Sinorhizobium fredii HH103* genome and is responsible for ATP binding as it binds to Cpn60 in the presence of Mg-ATP to suppress ATPase activity of Cpn60. The *groES* chaperonins assist substrate protein (SP) folding by cycling through several conformational states. In each cycle the SP is, in turn, captured, unfolded, briefly encapsulated (1/2 ~ 1 s), then released in its correct folding form by the chaperonin complex (Gomes et al., 2012).

The putative small heat shock proteins *hsp20* also known as *IbpB*, 16 kDa heat shock protein B or *shsps* belong to the *hsp20*-like_chaperone family and are present in *Sinorhizobium fredii HH103* genome. Other proteins are protected by these chaperones from heat-induced denaturation and aggregation by binding to protein kinase 1 (PDK1) and allow its nuclear

**Figure 1.6:** Network of Heat Shock Proteins (HSPs) during abiotic stress response. Source; Rowley & Mockler, (2011)
transportation. Shsps, an ATP-independent chaperone prevents irreversible protein aggregation and assists in subsequent protein renaturation by acting together with ATP-dependent chaperones (Basha et al., 2004).

The 60 kDa chaperon is also known as Protein Cpn60 or groEL gene is present in the genome of Sinorhizobium fredii USDA 205 and belongs to the Chaperonin_Cpn60 family. It is an ATP dependent protein and functions to prevent misfolding and promotes the refolding and proper assembly of unfolded proteins generated under stress conditions by binding to unfolded proteins and enabling them to fold in a protected environment where they do not interact with any other proteins. Research on the chickpea Mesorhizobium strain with low symbiotic effectiveness, strain ST-2 was modified by the addition of extra groEL copies from M. mediterraneum UPMCa36T, which were cloned in the expression vector pRK415 (Rodrigo da-Silva et al., 2017)

Bittner et al., (2007) observed growth differences in Sinorhizobium meliloti. Mutants in both groEL1 and groEL5 grew slightly slower than the wild type under heat stress conditions at 40°C. The small heat shock proteins, also known as immunoglobulin-binding protein A (IbpA) are present in the genome of Sinorhizobium fredii USDA 205 and they belong to the IbpA-B like family. Their function is adsorption of unfolded chains to prevent stress aggregation. Kitagawa et al., (2000) discovered that IbpA-, IbpB- and IbpAB-were overexpressing in E. coli strains grown at 40°C and these strains were found to be resistant not only to heat but also to superoxide stress.

1.6 Aims and Objectives

1.6.1 Aims

The main aim of this research project is to identify the genetic changes associated with acidity and heat stress in Rhizobium to enhance stress tolerance through genetic manipulation of Rhizobia. In addition, this research aims to understand the impact of genetic manipulation on symbiotic N fixation and tolerance of Rhizobium to harsh soil conditions emanating from high temperature, soil acidity and salinity.

The research will characterize selected soybean Rhizobium strains by sequencing the 16S rRNA, the Internal Transcribed Spacer (ITS) region of their DNA and identify heat stress
genes. The identified genes will be amplified from DNA of tolerant *Rhizobia* strain to include restriction enzyme site ends. These genes will then be used to make transformants in pLMB51 vector with *tauA* promoter by restriction digestion ligation with targeted genes from each stress tolerant strain. The targeted heat stress genes will be used to transform a heat sensitive *Rhizobia* strain to try to enhance its tolerance to heat stress and other stresses. The new transformants will be evaluated for heat, acid and salt stress tolerance as well as their effectiveness in N fixation under these stress conditions through inoculation experiments. Measurement on growth parameters will be done, and data analyzed using appropriate statistical packages. Further studies to assess the stability of the cloned plasmid DNA over time will be done to establish possible deletion of the insert from the new clone.

1.6.2 Specific objectives

1. To sequence the 16S rRNA gene of the Malawi isolates and selected *Rhizobium* strains sourced from Europe for identification and comparative study.

2. To assess relative stress tolerance of the selected *Rhizobium* strains to heat, acid and salt stress on survival and growth of *Rhizobium*.

3. To manipulate susceptible *Rhizobium* strain with heat stress genes from the heat tolerant *Rhizobium* strains expressing increased tolerance to heat stress.

4. To assess the effect of manipulating the *Rhizobium* strains on stress tolerance and nitrogen fixation potential in soybean.
2 Materials and Methods

Sigma, Thermo Scientific, VWR, Bioline and Melford supplied most of the analytical grade chemicals and consumables while ready mix growth media components were supplied by Oxoid, Melford and For Medium. Soybean seed was purchased from Jungle Seeds, UK. Media, solutions, glassware and plasticware were sterilized by autoclaving at 121°C for 15 minutes. Millipore water was used to prepare all culture media and buffer solutions.

2.1 Microbial techniques and protocols

2.1.1 Maintenance of bacterial isolates

*Rhizobia* strains *Bradyrhizobium* N2P5549 and *Sinorhizobium fredii* HH103, USDA 193 and USDA 205 were sourced from John Innes Centre, UK. The strains were sub-cultured on Tryptone Yeast-Extract Agar (2.5 g l\(^{-1}\) NaCl, 8 g l\(^{-1}\) tryptone, 5 g l\(^{-1}\) yeast extract, 16 g l\(^{-1}\) agar, pH 7, 0.025% w/vol Congo red) and grown at 28°C for two to three days. To obtain liquid cultures, a single colony was used to inoculate 10 ml of sterile Tryptone Yeast-Extract broth (2.5 g l\(^{-1}\) NaCl, 8 g l\(^{-1}\) tryptone, 5 g l\(^{-1}\) yeast extract, pH 7) and grown under similar conditions as above with shaking at 200 rpm. To confirm that the cultures contain *Rhizobium*, a loop full was streaked on to Peptone Glucose Agar (5 g l\(^{-1}\) glucose, 10 g l\(^{-1}\) peptone, 15 g l\(^{-1}\) agar pH 7, 10 ml l\(^{-1}\) bromocresol purple) and grown at 28°C for two to three days.

*Escherichia coli* DH5α (Life Technologies) was sub-cultured on Luria-Bertani agar (25g l\(^{-1}\) LB and 15g l\(^{-1}\) agar; pH 7) and incubated at 37°C. Liquid cultures from single colonies were grown in Luria-Bertani broth (25 g l\(^{-1}\) LB) and incubated at 37°C with shaking at 200 rpm. Selective media was prepared by adding either ampicillin (Melford) or tetracycline (ForMedium) at a working concentration of 50 μg ml\(^{-1}\) and 10 μg ml\(^{-1}\) respectively, but 15 μg ml\(^{-1}\) tetracycline for *Rhizobium* bacteria. Antibiotics were supplied as powder to prepare stock solutions of 50 mg ml\(^{-1}\) ampicillin in water (filter sterilized) and 5 mg ml\(^{-1}\) tetracycline in ethanol and stored at -20°C (Ausubel et al., 1994).

2.1.2 Storage condition of microbial isolates

For long term storage greater than 6 months of the microbial isolates, cultures were stored in 2 ml vials at -80°C in 15% glycerol. To prepare stocks, 1 ml of a mature culture grown in a
recommended growth media was mixed with an equal volume of 30% glycerol (filter sterilized) and frozen in liquid nitrogen before storage at -80°C.

2.1.3 Gram staining and cell microscopy

Cell microscopy was conducted to confirm that the subcultures were pure and authentic *Rhizobium*. At least 5 randomly selected single colonies were observed under a microscope using a 40x magnification objective (Leica, 020-519.010, Leica Microsystems). Gram staining was carried out on 3 single colonies to characterise the cultures according to their Gram reaction. A bacterial smear was fixed in a drop of water and stained for 1 min with crystal violet solution, rinsed in water before flooding for 1 min with Iodine solution. The iodine was drained, decolorized for 3 min with 95% alcohol and rinsed with water before counterstaining for 1 min with safranin. The slide was rinsed with water, blot dried and observed under the microscope using a 100x magnification objective with oil immersion (Leica, 020-519.010, Leica Microsystems).

2.2 Molecular methodologies

2.2.1 DNA extraction and purification

*Rhizobia* genomic DNA extractions were done according to the method of Chomczynski & Rymaszewski (2006) and modified by Chen et al. (2011). Cells were harvested from a 5 ml culture in Tryptone Soya broth by centrifugation for 5 min at 14,000 g and supernatant was discarded. The pellet was resuspended in 200 μl of solution 1 (2 mM EDTA and 0.01% Tween 20) by vortexing vigorously before centrifugation for 1 min at 14,000 g. 100 μl of alkaline lysis solution II (20 mM potassium hydroxide and 60% polyethylene glycol 200, pH 13.4) was added and vortexed before incubation for 15 min at 90°C on a heating block. The bacterial genomic DNA lysate was obtained after centrifugation for 1 min at 14,000g and directly used as a template for PCR amplification of the 16S rDNA or stored in a -20°C freezer.

A GeneJET Plasmid Miniprep Kit (Thermo Scientific) was used to extract and purify plasmid DNA from *E. coli*. Bacterial cell membranes were disrupted with SDS/alkaline lysis solution to release plasmid DNA and other cell components. Neutralization solution was added to precipitate chromosome, membranes and protein. Cellular debris was pelleted by centrifugation. The supernatant containing the DNA was bound on the silica membrane located in the spin column. Plasmid recovery using the GeneJET Plasmid Miniprep Kit was
highly efficient with a potential DNA yield of up to 20 μg from 5 ml of mature E. coli culture. Purified plasmid DNA was directly used in PCR amplification and restriction enzyme digestion reactions (Thermo Scientific).

### 2.2.2 Restriction digestion of DNA

To confirm that the plasmid was correctly constructed it was digested with restriction enzymes. Restriction digestion was also used to characterize the 16S rDNA and to prepare linearized vector for ligation reactions to insert specific genes. Restriction enzymes used to digest plasmid DNA (BglII), linearize vector (Ncol/BamHI and XbaI) and 16S rDNA characterization (EcoR1 and Sau3AI) were supplied by Fermentas and New England Biolabs. A typical restriction digestion was carried out in a 30 μl reaction mix with 3 μl 10X Buffer, 10 μl plasmid DNA, 1 μl restriction enzyme, and sterile distilled water to a final volume of 30 μl. The reaction time for Fast DigestTM enzymes (Fermentas) was 30 minutes and 2 hours for New England Biolabs enzymes at 37°C.

### 2.2.3 Polymerase Chain Reaction (PCR)

#### 2.2.3.1 Conventional PCR

Target gene fragments were amplified by conventional PCR from genomic DNA for identification purposes. DreamTaq Polymerase Master Mix (2X) (Thermo Scientific) was transferred to 1.5 ml Eppendorf tubes in 500 μl aliquots and stored at -20°C. A 20 μl PCR reaction was prepared with 10 μl of DreamTaq Master Mix (2X), 1 μl forward primer (10 μM), 1 μl reverse primer (10 μM), 1 μl DNA template (1-10 ng) and 7 μl sterile distilled water (Thermo Scientific). PCR was performed in an Eppendorf thermocycler with the following program: initial denaturation at 95°C for 3 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min; and a final extension at 72°C for 3 min. However, the annealing temperature (Ta) was optimised depending on primer stability and was calculated as 5°C lower than the melting temperature (Tm) of the primer with the lowest Tm. 10 μl of the PCR product was analyzed by gel electrophoresis alongside 5 μl of 1 kb hyperladder (Bioline).

#### 2.2.3.2 Colony PCR

Colony PCR was employed to identify positive E. coli transformants growing on a selective medium after transformation with plasmid DNA. Reactions were prepared as described
above in conventional PCR. The DNA template was prepared by suspending a single colony from a selection plate in 3 μl of sterile millipore water. 1 μl of suspension was used as a template in PCR reactions. Specific primers were used to amplify the region between the left and right borders of the plasmid. The PCR program used was the same as for conventional PCR except for the initial denaturation step which was extended to 10 min. The remaining 2 μl of colony suspension was used to set up an overnight culture in appropriate selective liquid medium to propagate more plasmid DNA from positive colonies that gave an expected size amplicon.

2.2.3.3 High-Fidelity PCR
Phusion (Thermo Scientific), a high-fidelity DNA Polymerase, was used to amplify PCR products for cloning into a vector for propagation or expression, to ensure error-free DNA fragments of the correct sequence. The reaction mix comprised of 10 μl Phusion GC buffer (5X), 1 μl dNTPs (10 mM), 1.5 μl forward primer (10 μM), 1.5 μl reverse primer (10 μM), 1.5 μl of DNA, 0.5 μl of Phusion DNA Polymerase and water to final volume of 50 μl. The PCR was performed using the following program: initial denaturation at 98°C for 30 sec; 35 cycles of denaturation at 98°C for 10 sec, annealing at 55°C for 30 sec, extension at 72°C for 45 sec; and a final extension at 72°C for 10 min. However, the annealing temperature (Ta) was optimized depending on the stability of the primers. Annealing temperature equal to the Tm of the primer with the lowest Tm was used when the primers sequences had ≤ 20 nucleotides or was calculated as 3°C less than the melting temperature (Tm) of the primer with the lowest Tm when the primers sequences had more than 20 nucleotides. 10 μl of PCR product was run on a 1% gel alongside 5 μl of 1 kb hyperladder. The expected sized band was cut and purified using the Gene Jet gel purification kit using manufacturer's protocol. The purified fragments were then used directly for cloning or ligation reactions.

2.2.4 Primer sequences
PCR primers were designed using primer 3 plus software (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) based on the protein coding sequences obtained from the UNIPROT database using protein code or vector sequence from Addgene. The protein codes used are A0A0T6ZGR3, G9AC37, G9AC38, G9AJ83, G9A3F1, A0A0T6ZUQ4, A0A0T6ZQ48 and A0A0T6ZGL3 for uncharacterized protein1, 10 kDa chaperonin groES2, 16 kDa heat shock
protein B, uncharacterized protein 2, Putative small heat shock protein, 60 kDa chaperonin \( \textit{groEL} \), Immunoglobulin-binding protein A and uncharacterized protein 3 respectively. These primers as listed in Table 2.1 were synthesized by Integrated DNA Technologies (IDT).

Table 2.1: Primer sequences used for amplification of heat stress genes, cloning and sequencing

<table>
<thead>
<tr>
<th>Name</th>
<th>DNA Sequence (5’-&gt;3’)</th>
<th>Tm (°C)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>fD1</td>
<td>AGA GTT TGA TCCTGG CTC AG</td>
<td>54.3</td>
<td>Amplification of ( \textit{Rhizobium} )</td>
</tr>
<tr>
<td>rD1</td>
<td>AAG GAG GTG ATC CAG CC</td>
<td>54.1</td>
<td>16SrDNA</td>
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<tr>
<td>UC1-F</td>
<td>ATGCACACGAAGATGTTTGACC</td>
<td>56.0</td>
<td>Amplification of uncharacterized protein 1</td>
</tr>
<tr>
<td>UC1-R</td>
<td>TTACGGGTGTACTCGACGC</td>
<td>59.1</td>
<td></td>
</tr>
<tr>
<td>groES2-F</td>
<td>ATGACGTCCGTCCCTTGCAT</td>
<td>59.2</td>
<td>Amplification of 10 kDa chaperonin ( \textit{groES2} )</td>
</tr>
<tr>
<td>groES2-R</td>
<td>CTAAGCTGCGTTTCTCGCTG</td>
<td>58.5</td>
<td></td>
</tr>
<tr>
<td>hsp20-F</td>
<td>ATGGCACACAACCTGGATTT</td>
<td>58.0</td>
<td>Amplification of 16 kDa heat shock protein B</td>
</tr>
<tr>
<td>hsp20-R</td>
<td>CGCCGCTTTATCGCCTTCAA</td>
<td>58.5</td>
<td></td>
</tr>
<tr>
<td>UC2-F</td>
<td>ATGGTTGCAAGGCAACCTC</td>
<td>58.5</td>
<td>Amplification of uncharacterized protein 2</td>
</tr>
<tr>
<td>UC2-R</td>
<td>CTATTATTGCAATAATTTTATTCGT</td>
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</tr>
<tr>
<td>Shsp-F</td>
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<td>Amplification of Putative small heat shock protein</td>
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<tr>
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<td>56.9</td>
<td>chaperonin ( \textit{groEL} )</td>
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<tr>
<td>IbpA-F</td>
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<td>56.1</td>
<td>Amplification of immunoglobulin-binding protein A (IbpA)</td>
</tr>
<tr>
<td>IbpA-R</td>
<td>TGCAGCTGCTGCTGCCCTC</td>
<td>58.5</td>
<td></td>
</tr>
<tr>
<td>UC3-F</td>
<td>ATGGAAACCGCCTGATGGA</td>
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<td>Amplification of uncharacterized protein 3</td>
</tr>
<tr>
<td>UC3-R</td>
<td>CCGATGCATCGCGGTTC</td>
<td>63.2</td>
<td></td>
</tr>
<tr>
<td>pJET1.2-F</td>
<td>CGACTCAGTATAGGGAGAGCGGC</td>
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<td>Sequencing pJET 1.2 cloning vector</td>
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<tr>
<td>pJET1.2-R</td>
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<td>TUC1-F</td>
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</tr>
<tr>
<td>Gene</td>
<td>Primer Location</td>
<td>Primer Sequence</td>
<td>GC Content (%)</td>
</tr>
<tr>
<td>----------</td>
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</tr>
<tr>
<td>TUC1-R</td>
<td>TCTAGGTCTAGATTACGGGTGTACTC</td>
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<td>Introducing Ncol and Xbal enzyme sites to uncharacterized protein1</td>
</tr>
<tr>
<td>TgroES2-F</td>
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<td>67.8</td>
<td>Introducing Ncol and Xbal enzyme sites to groES2 gene</td>
</tr>
<tr>
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<td>TCTAGGTCTAGACTAGGCTGCGTTTTC</td>
<td>60.3</td>
<td></td>
</tr>
<tr>
<td>Thsp20-F</td>
<td>CATGCCATGGGACCAACCTGAGATTTC</td>
<td>64.1</td>
<td>Introducing Ncol and Xbal enzyme sites to hsp20 gene</td>
</tr>
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<td>Thsp20-R</td>
<td>TCTAGGTCTAGACTACGGCAACCTGTCGC</td>
<td>58.8</td>
<td></td>
</tr>
<tr>
<td>TUC2-F</td>
<td>CATGCCATGGGTGTGCAAGCAACCTC</td>
<td>64.5</td>
<td>Introducing Ncol and Xbal enzyme sites to uncharacterized protein2</td>
</tr>
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<td>TUC2-R</td>
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<td>Introducing Ncol and Xbal enzyme sites to shsp gene</td>
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<tr>
<td>TgroEL-F</td>
<td>CATGCCATGGCTGCAAGAATGAGATTC</td>
<td>64.4</td>
<td>Introducing Ncol and Xbal enzyme sites to groEL gene</td>
</tr>
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</tr>
<tr>
<td>TibpA-F</td>
<td>CTAGCGGATCCATGCGTCACTTTGATTTT</td>
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<td>Introducing Ncol and Xbal enzyme sites to IbpA gene</td>
</tr>
<tr>
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<td>63.8</td>
<td></td>
</tr>
<tr>
<td>TUC3-F</td>
<td>CATGCCATGGAAAACCGGCTGATGGAAG</td>
<td>63.5</td>
<td>Introducing Ncol and Xbal enzyme sites to uncharacterized protein3</td>
</tr>
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<td>TUC3-R</td>
<td>TCTAGGTCTAGATCAGCCTGCGCATG</td>
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<td></td>
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<tr>
<td>PLMB51-F</td>
<td>GGTTAACGACATGATGGTACCAGT</td>
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<td>Amplification of the region between the left and right border of PLMB51</td>
</tr>
<tr>
<td>PLMB51-R</td>
<td>CAGGACGACCATGATTACCTAGT</td>
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<td></td>
</tr>
<tr>
<td>pLMB-51-F</td>
<td>CGATAGACGCCCTTCAGCAGA</td>
<td>56.8</td>
<td>Amplification of PLMB51 plasmid vector</td>
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<td>pLMB-51-R</td>
<td>TCTGCGCAAGGACGAGAATT</td>
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<tr>
<td>nifH1</td>
<td>AAGTGCCTGAGCTCCGCTG</td>
<td>62.4</td>
<td>Amplification of nifH gene</td>
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<tr>
<td>nifH2</td>
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<td>nolBT-F</td>
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<td>nolBT-R</td>
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<td>54.8</td>
<td></td>
</tr>
</tbody>
</table>
2.2.5 Gel electrophoresis and purification of PCR products

The quality of genomic DNA or plasmid DNA extracts and purified PCR products or restriction digest reactions was examined by gel electrophoresis. Molecular Grade Agarose (Bioline) was used to prepare a 1% agarose gel (w/v) in 1X TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) and 20 µl Midori Green. The cast gel was submerged in 1X TAE buffer while 5 µl Hyperladder I (Bioline) (Figure 2.1) was loaded alongside samples to help estimate band size (bp) and nucleic acids concentration (ng). The gel was run for 30 minutes at 120 volts using a PowerPac for Basic Power Supply (Bio-Rad) and visualised under UV light using a GelDoc Imager (Bio-Rad), comprising of a transilluminator and a camera to capture and save images. PCR products were purified to remove primers and primer dimers produced by the reaction. 20 µl of PCR product was loaded onto a gel, run using the same conditions as in gel electrophoresis above and visualised in the dark on a UV lightbox. A band of the expected size was excised and purified by GeneJET Gel Extraction Kit (Thermo Scientific) with a potential DNA yield of up to 25 µg. 3 µl of purified DNA was used to run a gel as above to help determine its concentration.

Figure 2.1: Image of 5 µl hyperladder 1 kb on 1% agarose (Bioline) gels, DNA size markers were used to determine DNA fragment sizes and estimate concentration. source bioline.com.
2.2.6 Cloning of PCR products

The cloning vector pJET1.2 (Figure 2.2) was used for cloning of PCR products and subsequent sequencing, propagation and maintenance of the internal transcribed spacer (ITS) sequence and heat stress gene fragments from Rhizobium. The PCR products were cloned into the multiple cloning site (MCS) of the pJET1.2 vector using the blunt-ended protocol of CloneJET PCR Cloning Kit (Thermo Scientific). The blunt-end PCR products were generated using Phusion ® High-Fidelity DNA Polymerase (Thermo Scientific), universal primers for ITS and specifically designed primers to introduce Ncol and Xbal enzyme sites to 5′ and 3′ ends of the PCR amplicons. The Phusion PCR reaction and program was as described in high fidelity PCR above. The PCR product of the ITS region or heat stress genes were cloned into the pJET vector. The ampicillin marker in the vector facilitated the selection of the transformed E. coli colonies as demonstrated by growth on ampicillin containing media. Cloning reactions were performed on ice in a 10 µl reaction mix containing 4 µl of PCR product, 5 µl of 2X buffer, 0.5 µl aliquot of blunt cloning vector (pJET1.2) and 0.5 µl of T4 DNA ligase. The reaction was incubated at room temperature for 30 min. The ligation reaction was transformed into E. coli competent cells for propagation, multiplication and maintenance.
Restriction enzyme ligation technique uses T4 DNA ligase to introduce genes in plasmid vectors using sticky ends assembly. The heat stress genes were amplified by Phusion a High-fidelity DNA polymerase (Thermo Scientific) using specifically designed primers to include enzyme sites. The PCR amplified gene fragments purified by GeneJET PCR purification kit and pLMB51 plasmid vector were digested with Ncol and Xbal and gel purified according to manufacturer’s protocol. A linearized pLMB51 (Addgene) vector backbone (Fig. 2.3) was re-circularized by inserting the genes by T4 DNA ligation. Restriction enzyme ligation was performed using 2 μl vector, 2 μl insert, 3 μl T4 DNA ligase buffer, 1 μl sterile water, 1 μl ATP and 1 μl T4 DNA ligase. Plasmid constructs were used to transform E. coli competent cells for further multiplication. Correct plasmid assemblies were confirmed by restriction digestion and PCR amplification of specific genes (Sigma-Aldrich).
Figure 2.3: pLMB51 plasmid vector map. The vector with $\text{tauA}$ promoter for $\text{Rhizobium}$ as shown in plasmid B (Tett et al., 2012)
2.2.7 Transformation of bacteria

2.2.7.1 Preparation of electrocompetent bacterial cells

Electrocompetent *E. coli* Top10 and S17 cells were prepared using a single colony to inoculate 5 ml of LB and grown overnight at 37°C at 200 rpm as described by Sharma & Schimke (1996). The next day, 100 ml flasks of LB were inoculated with 1 ml of culture and grown for 4 hrs at 37°C at 200 rpm until an OD$_{600}$ of 0.6 was reached. The bacterial cultures were subdivided into 50 ml falcon tubes, centrifuged at 4°C at 4000 x g for 10 min. The cells were washed twice in ice-cold sterile Millipore water, once in sterile ice-cold 10% glycerol then resuspended in a final volume of 1.5 ml ice-cold sterile 10% glycerol before it was divided in 50 µl aliquots and frozen in liquid nitrogen for storage at -80°C.

2.2.7.2 Transformation of DNA into *E. coli* by electroporation

For electroporation, 5 µl ligation product or 3 µl purified *E. coli* plasmid was mixed with 50 µl of ice thawed electro-competent *E. coli* cells and incubated on ice for 30 min. The mixture was transferred to a chilled sterile 0.2 cm electroporation cuvette and electrocuted using a GenePulser to deliver a 2.5kV pulse. 950 µl of SOC was added and transferred to a 1.5 ml tube before incubation for 1 hr at 37°C at 200rpm. 100 µl of the transformed *E. coli* cells were plated on LB agar plates containing 100 µg ml$^{-1}$ of ampicillin or 10 µg ml$^{-1}$ of tetracycline and incubated overnight at 37°C. 100 µl of transformed *Rhizobium* was plated on Tryptone Yeast-Extract Agar (TY) plates containing 10 µg ml$^{-1}$ of tetracycline and incubated at 28°C for 2-3 days. At least 10 colonies were screened by colony PCR for the presence of the insert using specific forward and reverse primers. PCR positive colonies having a band of the expected sizes were cultured in an appropriate media supplemented with an antibiotic for selection.

2.2.7.3 Introduction of plasmid constructs into *Rhizobium* by conjugation

In conjugation, 10 ml of TY broth was inoculated with *Rhizobium* HH103 strain and incubated for 3 days at 28°C at 200 rpm. On the second day, 10 ml of TY containing 10 µg ml$^{-1}$ of tetracycline was inoculated with each pLMB51 plasmid (donor) in S17 *E. coli* strain and incubated overnight at 37°C at 200 rpm. 1 ml of HH103 *Rhizobium* culture was aliquoted into six separate Eppendorf tube. Likewise, 1 ml of each plasmid culture was aliquoted into a separate Eppendorf tube. All the tubes were centrifuged at 4000 x g for 2 min and
supernatant was discarded. The cells were washed in 1 ml of 0.9% saline solution, centrifuged at 4000 x g for 2 min and supernatant was discarded. The cells were mixed in 100 µL of 0.9% saline solution, plated separately on LB plate and incubated for 2 days. Cell of each plasmid on the LB plate were washed with 1 ml of 0.9% saline solution in separate Eppendorf tubes and mixed vigorously. 100 µl of mixture was plated on Tryptone Yeast-Extract Agar (TY) plates containing 10 µg ml⁻¹ of tetracycline and incubated at 28°C for 2-3 days. At least 3-4 colonies were PCR screened using specific primers for presence of insert. Colonies showing expected sized band were cultured in Tryptone Yeast-Extract Agar (TY) broth containing 10 µg ml⁻¹ of tetracycline and incubated at 28°C for 2-3 days.

2.2.8 Sequencing

DNA sequences of gene fragments present in pJET1.2 plasmid transformants were confirmed by sequencing of plasmid DNA. The plasmid miniprep kit (Thermo Scientific) was used to extract plasmid DNA from an overnight culture according to the manufacturer's protocol. The plasmids with inserts of the expected sizes were prepared for sequencing using Mix2 seq kit (Eurofins) by combining 5 µl of plasmid DNA (100 ng µl⁻¹), 5 µl of pJET forward sequencing primer (5 µM) and 10 µl of deionized water. The sequence results were uploaded into Clone Manager Professional 7 and double digested with Ncol and Xbal restriction enzymes. The product sequence was translated by Sequencher 5.1 to determine the protein sequence. The amino acid sequence was used to BLASTn search the NCBI database for similar sequences for identification.

2.3 Rhizobia growth assay

2.3.1 Antibiotic sensitivity profiling

Antibiotic sensitivity profiling was conducted to evaluate Rhizobium sensitivity to different types and concentrations of antibiotics. Stock solutions of streptomycin, kanamycin, tetracycline, neomycin, ampicillin and chloramphenicol at different concentration ranging between 5-100 µg ml⁻¹ (Table 2.2). The water based antibiotic solutions were sterilized through 0.22µm filters. Tryptone Yeast Extract agar plates with variable concentrations of antibiotics ranging from 0.2-1000 µg ml⁻¹ were used with plates without antibiotics as the controls. The concentration of each culture was adjusted to an OD₆₀₀ of 0.5. 30 µl of each strain was spotted onto the plate. The spot was left to dry completely before incubation at
30°C for three and six days for fast and slow growing stains, respectively. Plates were then observed for bacterial growth in comparison to the control plate. The strain was categorized as highly resistant, resistant or susceptible for complete, partial and no growth respectively.

Table 2.2: Stock and working concentrations of antibiotics

<table>
<thead>
<tr>
<th>Stock</th>
<th>Working</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent</td>
<td>concentration</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>Water</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>Water</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>Ethanol</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>Ethanol</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>Water</td>
</tr>
<tr>
<td>Neomycin</td>
<td>Water</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>DMF</td>
</tr>
</tbody>
</table>

2.3.2 Abiotic stress tolerance

Optical density (OD) of culture is a reliable measure of cell density since a spectrophotometer can measure intensity of light very accurately. This may show tolerance of an organism to a specific stress when measured at optimum and extreme conditions. The OD was measured using Biochrom WPA Biowave II, USB x18 (50volts) UV-Vis Spectrophotometer. Measurement of OD of a culture using a spectrophotometer depends on the difference in intensity of the light that reaches the photoelectric cell between a cuvette filled with a clear sterile medium as the reference intensity, adjusted to zero and cuvette containing bacterial cells where a significant portion of light is scattered and no longer reaches the photoelectric cell. The weaker electric signal is converted to an OD value. Optical density (OD) is directly proportional to the biomass in the cell suspension in specific range of the exponential growth phase. Therefore, the relationship between OD and cell density at an OD ≤ 0.4 during the exponential growth phase is linear. However, samples with an OD above 0.4 were diluted by an appropriate dilution factor (Fig. 2.4). To evaluate the effect of high temperature, acidity and salt stress on *Rhizobium* transformed with various heat stress genes. 50 ml of TY broth
at pH 7, pH 4.5 or with 1% NaCl were prepared and inoculated in triplicates with 1 ml of overnight culture of each transformant whose absorbance was adjusted to ~0.1 at OD₆₀₀. For temperature stress, the transformed *Rhizobium* was grown at 28°C and 40°C for 48 hrs before measurement of cell density. For acid stress and salt stress, transformed *Rhizobium* was grown at 28°C for 48 hrs in TY broth adjusted to pH 4.5 and 1% NaCl respectively. Tetracycline and taurine were added to a final concentration of 10 μg ml⁻¹ and 1 mM, respectively, except for the controls.

![Figure 2.4: Relationship of Optical density and cell density of bacterial culture. Source: Widdel F. (2010)](image-url)
2.4 Proteomic methodologies

2.4.1 Protein extraction

Protein extraction was done by sonication of cells in a buffer solution in order to disrupt bacterial cells to release protein into solution. Two protein samples were prepared from USDA 193 grown at 20°C and 40°C. Cells were harvested from 100 ml of a mature culture for each treatment when the OD$_{600}$ reached 0.6, by centrifugation in a 50 ml falcon tube at 8000 g for 10 min at 4°C. The harvested cells were resuspended in 20 ml of 30 mM Tris-HCl, pH 8 and placed on ice to avoid heating up during cell lysis. Cells were lysed by sonication using a standardized cycle of 1 sec on, 1 sec off for 7 min at amplitude of 63% using a Sonics Vibracell VC-505TM (Sonics & Materials Inc., Newton, Connecticut, USA). After sonication, the samples were centrifuged at 8,000 rpm for 15 min at 4°C to pellet intact cells and large cell debris. The amount of protein extracted was estimated by UV spectrophotometry at 415 nm until the concentration of sample was ≥ 2mg/mL. The protein was concentrated using 3 kDa cutoff centicon filter to about 1 mg ml$^{-1}$ before sending for analysis at the proteomics facility, Cellular Molecular Medicine (CMM), University of Bristol.

2.5 Tandem Mass Tag Mass Spectroscopy

The protein analysis was done by Tandem Mass Tag (TMT) spectroscopy to identify proteins expressing highly at elevated temperatures. 100 µg of each sample of culture grown at 20°C and 40°C was digested with 2.5µg trypsin and incubated overnight at 37°C before being labelled with Tandem Mass Tag (TMT) six plex reagents according to the manufacturer’s protocol (Thermo Scientific). During labelling, moisture was removed through evaporation, the residue resuspended in 5% formic acid. The samples were filtered twice to remove the salts using a SepPak cartridge according to the manufacturer’s instructions (Waters) before evaporation and resuspension into 20 mM ammonium hydroxide (pH 10). High pH reversed-phase chromatography (Ultimate 3000 liquid chromatography system, Thermo Scientific) was used to obtain peptide fractionations. The peptide fractions were fractionated further using an Ultimate 3000 nano-LC system and the spectra was read by an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific).
2.6 Data analysis

In TMT spectroscopy, the spectra obtained were processed and quantified using Proteome Discoverer software v1.4 (Thermo Scientific) and the results searched using UniProt *Sinorhizobium fredii* database. A 10 ppm Peptide precursor mass tolerance and 0.6Da MS/MS tolerance was used as the cut-off point. Data analysis considered oxidation of methionine as a variable while carbamidomethylation of cysteine and TMT mass tag addition to peptide N-termini and lysine as fixed factors. Searches were performed with full tryptic digestion at 99% confidence limit and 95% confidence limit for the reverse database search.

2.7 Plant-based methodologies

2.7.1 Assessing root nodule formation

Root nodule formation was evaluated using soybean cultivar Black Jet or Edamame in a complete randomized block design (CRBD) experiment through growth observations and nodulation. To remove any surface microorganisms, soybean seeds were surface sterilized in ethanol (70%) for 1 min, washed in bleach (4%) for 1 min, then rinsed six times in sterile water before pre-germination in sterile vermiculite (Howieson & Dilworth, 2016). Each seedling was transferred to a 3.5” plastic pot filled with acid washed sterile sand as growth medium. The *Rhizobium* transformants were grown in 10 ml TY broth supplemented with 10 μg ml⁻¹ tetracycline for three days at 28°C at 200 rpm. Cells were harvested by centrifugation at 10,000 x g, washed, and resuspended in fresh TY broth. Seedlings in three replicate pots, one in each pot, were inoculated with 1 ml of a suitable *Rhizobium* culture two days after transplanting. Two replicated sets of pots were left without inoculation to serve as negative and positive controls. Deionized water was used to irrigate all the plants but once a week, 20 ml of growth medium containing 1mM taurine and all the plant nutrients excluding nitrogen was used to irrigate the plants except for the fertilizer treatment where 5 ml of 10 g/L KNO₃ was added. The nutrient growth medium was prepared by combining 50 ml of each stock solution (1–4), 0.5 ml of trace element solution 5 was added, made up to 1.6L with deionised water and autoclaved. 400 ml of sterile CaSO₄ solution (6) was added to make up to 2L (Table 2.3 ).

Soybean stressed plants were grown for six weeks at 32°C in a growth chamber, Labs Economic Lux Chamber (Microclimate-Series™) with a light cycle of 16/8 hour (night/day) from fluorescent tubes while non-stressed plants were grown under glasshouse conditions.
at 20°C or 28°C, 16/8 hour (night/day) with led lighting and a humidity of 60. Harvesting was done by uprooting the whole plant without disturbing the root system. The roots were carefully cleaned by washing with water and separated from stems before counting number of nodules (NN) and nodule scoring (NS) a chart with a range of 0-8 scores where 0=absent and 8= extremely abundant (Appendix1). Both roots and stems were immediately dried in an oven to a constant weight at 65°C and thereafter measurements taken on shoot dry mass (SDM) and root dry mass (RDM)(Paço et al., 2016).

**Table 2.3:** Preparation of stock solutions for plant growth nutrient solution

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity (g/L)</th>
</tr>
</thead>
<tbody>
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<td>12.3</td>
</tr>
<tr>
<td>2 KH₂PO₄</td>
<td>6.8</td>
</tr>
<tr>
<td>3 K₂SO₄</td>
<td>17.5</td>
</tr>
<tr>
<td>4 Fe-EDTA</td>
<td>2.5</td>
</tr>
<tr>
<td>5 trace element solution (store at 4°C)</td>
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</tr>
<tr>
<td>H₃BO₃</td>
<td>0.464</td>
</tr>
<tr>
<td>Na₂MoO₄.2H₂O</td>
<td>0.018</td>
</tr>
<tr>
<td>ZnSO₄.7H₂O</td>
<td>0.539</td>
</tr>
<tr>
<td>MnSO₄.4H₂O</td>
<td>0.042</td>
</tr>
<tr>
<td>CoSO₄.7H₂O</td>
<td>0.141</td>
</tr>
<tr>
<td>CuSO₄.5H₂O</td>
<td>0.125</td>
</tr>
<tr>
<td>6. CaSO₄ agitated solution</td>
<td>2.04</td>
</tr>
<tr>
<td>+N control</td>
<td>5 ml weekly of 10 g/L KNO₃</td>
</tr>
</tbody>
</table>

Source: Howieson & Dilworth (2016)
2.8 Statistical analysis

Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) software (IBM SPSS statistics version 24).

The data obtained from *Rhizobium* stress tolerance and from the soybean plant-growth assays were analyzed using one-way ANOVA and multivariate analysis (p < 0.05). The post hoc Tukey HSD test was used to compare the means and indicate which treatments have significant differences.
3 Characterization of *Rhizobium* and selection of differentially expressed proteins.

3.1 Introduction

*Rhizobium* is a gram-negative bacterium that produces structures known as nodules on the roots of legume plants. Its ability to infect the roots of its hosts and produce nodules led to the discovery of *Rhizobium*’s potential to utilize atmospheric nitrogen through biological nitrogen fixation (BNF). As an agent for symbiotic biological nitrogen fixation, *Rhizobium* can convert atmospheric nitrogen into plant usable forms such as ammonium. Since this discovery, *Rhizobium* has been used as a biological fertilizer to provide nitrogen to legume plants and fix nitrogen in agricultural soils. The root infection process starts when flavonoid signals released by the legume plant are recognized by a compatible *Rhizobium* leading to its attachment to the roots, causing root curling to trap the *Rhizobium* inside. The curled root develops into a complex structure (nodule) that house the *Rhizobia* and act as factories for nitrogen production (Abdel-Lateif et al., 2012).

Initially, the research plan was to use *Rhizobium* isolates that nodulate Soybeans in the tropical soil environment of the Sub-Saharan Africa. The idea behind the use of these isolates was to directly infer the research finding to other interventions currently being practiced in tropical agriculture to increase crop productivity as well as mitigate the effects of climate change. A culture collection of 40 isolates presumed to be *Rhizobium* bacteria was sourced from Malawi in Southern Africa. However, there was need for characterization and confirming the identity of these isolates before they could be used in other research experiments.

Identification of *Rhizobium* strains can be achieved through phenotypic (morphology, physiology and rate of growth) and DNA-based genotypic characterization (Willems, 2006). The conserved internal transcribed spacer regions of the ribosomal RNA (ITS) and the 16S ribosomal RNA (rRNA), are the most studied and sequenced regions used in *Rhizobia* phylogenetic and taxonomic studies. This region is very important because it is present as an operon in most bacteria, it is conserved in its functions and large enough for bioinformatic studies (Větrovský & Baldrian, 2013). The 16S rRNA can accurately differentiate *Rhizobia* strains within the same genera while the ITS differentiates between genera which are closely
related as it is more variable. Other genes like \textit{nifH} and \textit{nolBT} can also be used since \textit{nifH} is specific for \textit{Rhizobium} while the \textit{nolXWBTUV} locus is conserved among \textit{Sinorhizobia fredii} strains (Pastorino et al., 2003). Four \textit{Rhizobium} strains sourced from John Innes Laboratory were also used as type strains. These strains were chosen to represent genetic and environmental diversity as they are either of different genera or isolated from different geographical locations. The strain N2P5549 is a \textit{Bradyrhizobium}, HH103 is a \textit{Sinorhizobium} originally from China while USDA193 and USDA205 are from warm and cold parts of the USA respectively. In this research, the 16S rRNA \textit{nifH} and \textit{nolBT} genes were PCR amplified from genomic DNA extract of each of the 4 \textit{Rhizobia} strains (Chomczynski & Rymaszewski, 2006).

Exposure of \textit{Rhizobium} bacteria to stressful growth condition allows for expression of stress tolerance proteins. In order to establish relative intrinsic \textit{Rhizobium} strain tolerance, the ability of \textit{Rhizobium} strains to grow under high temperature, acid and salt stress was evaluated. The expressed tolerance of \textit{Rhizobia} bacteria to various stress conditions allowed for selection of stress tolerant \textit{Rhizobium} strains. The identified stress tolerant strains were considered as possible candidates for identification of stress tolerance proteins.

According to Liang (2013), the proteomics is defined as the study of the types, quantities, roles and dynamics of all proteins present in a cell, tissue or an organism. Several proteomic studies have been conducted and mainly focus on legume- \textit{Rhizobium} symbiosis partners. Most of these studies have used LC-MS/MS-based approaches such as Tandem mass tag spectrophotometry as they are more sensitive. Sarma & Emerich (2005) used proteomics to establish the abundance of proteins related to nitrogen metabolism and transport in USDA110 bacteroids. While in 2006 they did a comparative study of proteins related to fatty acid and nucleic acid metabolism both in free-living USDA110 and bacteroids. Delmotte et al. (2010), identified a complete set of proteins related to nucleoside and nucleotide biosynthesis in USDA110 and in root and stem ORS27 bacteroids in 2014 using this approach. Tandem mass tag spectrophotometry allows for quantification and identification of proteins, peptides and nucleic acids from crude protein extracts using chemical labels, tandem mass tags (TMT or TMTs). In my research, stress genes were identified from total protein extracted from the cells of USDA 193, a heat tolerant \textit{Rhizobia} bacterium grown at 20\textdegree C and 40\textdegree C. Physical disruption of the cells suspended in an appropriate buffer with high intensity sound waves (sonication) facilitates the extraction of crude protein from the cells.
Direct absorbance measurement of the protein extract at 280 and 260 nm allowed quantification of protein concentration. The extract was initially digested, labelled with TMT and fractionated before running the composite sample through a Nano-LC Mass Spectrophotometer for efficient protein analysis. During labelling, the TMT chemical labels are attached to the macromolecules for subsequent detection in the spectrophotometer. The resulting spectra is quantified, and peptides identified by using Proteome Discoverer software v1.4 (Thermo Scientific) and results BLASTn searched against the UniProt Sinorhizobium fredii database. The highly expressed peptides (≥4x) were selected as targeted genes for generation of heat stress gene templates.

There are several stress genes found in E. coli species and other model bacteria that are responsible for various stress factors such as temperature, acid and salt stress. Among the heat shock proteins present in E. coli include groEL (Hsp60) and DnaK (Hsp70) and their respective co-chaperones groES and DnaJ-GrpE which are responsible for protein folding. However, in the absence of stress, these proteins assist in other metabolic process (Roncarati & Scarlato, 2017). The groEL (HSP60) chaperone assists in enzymatic degradation of heat damaged proteins in addition to protein folding and can interact with at least 250 proteins (Kerner et al., 2005). This chaperone is vital at all temperatures and prevents the degradation of polymerase, Pol V and Pol IV during UV mutagenesis (Layton et al., 2005). Some proteases which include ClpP and HslV and respective co-chaperones ClpA or ClpX and HslU are HSPs responsible for the removal of damaged polypeptides from stressed cells (Roncarati and Scarlato 2017). Some acid stress proteins (ASPs) genes present in E. coli include gadA, gadB and gadC genes which are part of the glutamate and arginine decarboxylase (GAD) system. ASPs are responsible for prevention or repair of acid caused damage on macromolecules such as glutamate, arginine, gamma-amino- isobutyrate and agmatine (Chung et al., 2006). The clpB, clpC, and clpE chaperones with ClpP as co-chaperone target heat and salt damaged proteins for degradation (Frees et al., 2004). The IbpA and IbpB chaperones which are 48% identical at the amino acid sequence level are required for efficient stabilization of thermally aggregated proteins in a disaggregation competent state (Matuszewska et al., 2005)
In the Rhizobium bacteria used in this research, I expect to find heat stress genes such as 
groEL in Mesorhizobium, shps groES, dnaK, dnaJ in B. japonicum and rpoH in S. meliloti 
(Rodrigues et al., 2006; Münchbach et al., 1999; Ono et al., 2001)

3.2 Aims

This chapter aims to confirm the identity of the Rhizobium strains by comparison of 
morphological characteristics and sequencing data of the 16S rDNA with data in databases. 
Although complete genome characterization by sequencing has not yet been done for some 
of the strains used in this study sequencing of closely related strains has already been done. 
In addition, morphological and partial genomic characterization of specific genes has been 
completed for all the strains used in this study. This study analyzes the phenotypic 
expression of these Rhizobium strains to heat, acid and salt stress to determine their 
tolerance to different stresses. Total protein analysis was conducted to identify the heat 
stress genes and to generate templates which could be used to construct plasmids for cloning 
into Rhizobium.

3.3 Results

3.3.1 Characterization of Rhizobium by 16S rRNA sequencing

3.3.1.1 Strain confirmation and maintenance

Rhizobia HH103, USDA193, USDA205 and N2P5549, strains obtained from the John Innes 
Center were evaluated and authenticated. When tested on TY-CR plates incubated in the 
dark, the colonies showed either weak or no absorption of the Congo Red dye (Fig. 3.1). In 
addition to their gram-negative reaction and having rod shaped cells, all the strains showed 
poor or no growth on PGA plates (Table 3.1). According to Sharma et al., (2010), this 
characteristic is indicative of Rhizobium bacteria. When grown on TY- BCP, three strains 
produced acid (fast growers) which turned the indicator yellow while one (N2P5549) 
produced alkaline metabolite (slow grower) with no change in color (Table 3.1). This result 
agrees with Hungria et al. (2001) who described acid producing Rhizobia as fast growers and 
alkaline producers as slow growers. The colonies were cream white with entire margins, 
smooth textured and convex with a dome shape (Table 3.1). The three fast growers had 
morphological characteristics matching with Sinorhizobium, while the slow grower resembles Bradyrhizobium.
Table 3.1: Morphological-cultural and biochemical characteristics of *Rhizobia* isolates

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>HH103</th>
<th>USDA193</th>
<th>USDA205</th>
<th>N2P5549</th>
</tr>
</thead>
<tbody>
<tr>
<td>Congo Red adsorption</td>
<td>Na</td>
<td>Na</td>
<td>Na</td>
<td>weakly absorb</td>
</tr>
<tr>
<td>Peptone Glucose growth</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Bromocresol Purple reaction</td>
<td>Yellow</td>
<td>Yellow</td>
<td>Yellow</td>
<td>Blue</td>
</tr>
<tr>
<td>Gram reaction</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Cell shape</td>
<td>Rod</td>
<td>Rod</td>
<td>Rod</td>
<td>Rod</td>
</tr>
<tr>
<td>elevation</td>
<td>Convex</td>
<td>Convex</td>
<td>Convex</td>
<td>Convex</td>
</tr>
<tr>
<td>Margin</td>
<td>Entire</td>
<td>Entire</td>
<td>Entire</td>
<td>Entire</td>
</tr>
<tr>
<td>Colony color</td>
<td>Cw</td>
<td>Cw</td>
<td>Cw</td>
<td>Cw</td>
</tr>
<tr>
<td>Colony texture</td>
<td>Smooth</td>
<td>Smooth</td>
<td>Smooth</td>
<td>Smooth</td>
</tr>
<tr>
<td>Presumed genus</td>
<td>Sinorhizobium</td>
<td>Sinorhizobium</td>
<td>Sinorhizobium</td>
<td>Bradyrhizobium</td>
</tr>
</tbody>
</table>

*Na* = Non-absorbing, *Cw* = Cream white
3.3.1.2 Genomic DNA extraction and PCR amplification of 16SrRNA and nolBT genes

Genomic DNA for PCR amplification of 16S rRNA, nifH and nolBT genes was extracted from *Rhizobia* cultures of HH103, USDA193, USDA205 and N2P5549 according to the method of Chomczynski & Rymaszewski (2006) and modified by Chen et al. (2011). According to Chen et al. (2011), such DNA extracted by the rapid gDNA extraction method can be directly used in PCR reactions. Gel electrophoresis was conducted to check for the presence of PCR bands of specific sizes.

The 16S rRNA gene, nifH and nolBT genes of these *Rhizobia* strains were amplified using universal primers adopted from Hassen et al. (2014) and Pastorino et al. (2003), respectively. The nolBT, nifH and 16S rRNA genes were used to identify and classify *Rhizobia* bacteria in order to confirm their taxonomy. The universal primers fD1 and rD1 were used for the 16S rRNA gene, nifH1 and nifH2 for the nifH gene while nolBT-F and nolBT-R primers were for the nolBT gene. For PCR amplification of 16S rRNA gene all the strains produce a strong band of 1500 base pairs for HH103, USDA193, and USDA205 but slightly faint for N2P5549 despite using similar quantities (Fig. 3.2). This result shows that N2P5549 is different from the rest of the strains. This finding is in agreement with Kwon et al. (2005) who amplified two bands of 16S rRNA genes from some *Sinorhizobium* *Rhizobia* strains. All the strains are presumed to be *Rhizobium* bacteria because according to Gröнемeyer et al. (2014), the 16S rRNA is one of the characteristics of *Rhizobia* bacteria. The DNA amplicon of 1500 bases was therefore excised from the gel, purified and cloned. PCR of *Rhizobia* DNA with nifH primers produced a band consistent with the predicted size of 601 base pairs in all the strains as it is a major characteristic gene for nitrogen fixation in *Rhizobium* bacteria (Fig. 3.3). For PCR amplification of nolBT gene, all the strains except N2P5549 produced a 730 base pair band (Fig. 3.4). These findings

![Figure 3.1: Biochemical characteristics of *Rhizobia* strains on TY with Congo Red. (A), HH103: (B) USDA193: (C) USDA205: (D), N2P5549](image-url)
agree with Pastorino et al. (2003) who identified that a 730 base pair band of nolBT is conserved and only present in Sinorhizobia fredii.

Figure 3.2: PCR amplified 16S rDNA of the Rhizobia strains electrophoresed in a 1% agarose gel at 120V for 30 min using 10 μl of sample from the total 20 μl of PCR product loaded to each well. Lane M, DNA ladder: Lane C, negative control (water): Lane 1, HH103: Lane 2, USDA193: Lane 3, USDA205: Lane 4, N2P5549.
Figure 3.3: PCR amplified \textit{nifH} gene of the \textit{Rhizobia} strains electrophoresed in a 1\% agarose gel at 120V for 30 min using \(10\ \mu l\) of sample from the total \(20\ \mu l\) of PCR product loaded to each well. Lane M, DNA ladder: Lane C, negative control (water): Lane 1, HH103: Lane 2, USDA193: Lane 3, USDA205: Lane 4, N2P5549.

Figure 3.4: PCR amplified \textit{nolBT} gene of the \textit{Rhizobia} strains electrophoresed in a 1\% agarose gel at 120V for 30 min using \(10\ \mu l\) of sample from the total \(20\ \mu l\) of PCR product loaded to each well. Lane M, DNA ladder: Lane C, negative control (water): Lane 1, HH103: Lane 2, USDA193: Lane 3, USDA205: Lane 4, N2P5549.
3.3.1.3 pJET Cloning of the 16S rRNA gene and *E. coli* transformation

In order to propagate more template for sequencing, the purified 1500 base pair, 16S rRNA amplicon was incorporated into a pJET cloning vector before transformation of *E. coli* electrocompetent cells. Direct sequencing was not used since the TM of some primers was not between 60°C and 70°C as recommended and the PCR reaction can sometimes amplify incorrect products. pJET cloning of the 16S rRNA gene was used as it provides a complete gene sequence from the start to stop codon. To select transformants that contain the 16S rRNA gene insert, colony PCR with pJET primers was conducted to screen *E. coli* colonies that grew on LB plates supplemented with the antibiotic, ampicillin (100 µg/ml). Less than 15 transformed *E. coli* cells were able to grow, and this small number of colonies shows that few of the *E. coli* cells successfully acquired antibiotic resistance from the pJET vector. pJET primers were used since universal 16S rRNA primers would perhaps amplify the 16S rRNA gene in *E. coli*. As expected, the pJET primers were able to amplify the 1500 base pair DNA amplicon from the plasmid of successfully transformed *E. coli* and this confirmed successful ligation of the insert into pJET (Fig. 3.5). The plasmid extracted from the positive transformants could be used for sequencing as it contains the 16S rRNA gene.
Figure 3.5: PCR of pooled plasmid DNA from *E. coli* transformed with 16S rRNA amplicons electrophoresed in a 1% agarose gel at 120V for 30 min using 10 μl of sample from the total 20 μl of PCR product loaded to each well. Lane M, 5 μl ladder; Lane C, negative control (water); Lane 1, HH103; Lane 2, USDA193; Lane 3, USDA205; Lane 4, N2P5549.

3.3.1.4 Sequencing of pJET plasmids

Sequencing was used to verify the correct 16S rRNA gene sequence cloned into pJET and used for comparison with reference sequences in the database. The extracted plasmid DNA was prepared and sent for sequencing at Eurofins Genomics with pJET forward and pJET reverse sequencing primers. Only one plasmid per strain was used for sequencing since a proof-reading polymerase was used for PCR. The results obtained from the two separate sequencing reactions were assembled using Sequencher 5.0. The data files were uploaded in sequencher, trimmed of vector ends, removed low confidence bases, merged and manually edited. The resulting sequences were aligned with the reference sequences obtained from NCBI database using Clone Manager Professional 7 (Sci. Ed software). The reference sequences used were AY260145.1 for HH103, NR036957.1 for USDA193, NR036957.1 for USDA205 and LC095721.1 from *Bradyrhizobium* Sp. VM-OKI for N2P5549. Since the genome of N2P5549 strain has not yet been sequenced, the 16S rRNA gene sequence of a closely related strain, *Bradyrhizobium* Sp. VM-OKI was used for the alignment. The N2P5549 plasmid BLASTn search in the NCBI database showed close
identity to *Bradyrhizobium* Sp. VM-OKI gene for 16S ribosomal RNA, partial sequence. Sequencing therefore confirmed the identity of all the *Rhizobium* strains since the sequences were like the reference sequences.

The alignment of HH103, USDA193 and USDA205 strains was generally good with a 100% similarity (Fig. 3.6, 3.7 and 3.8). However, alignment of N2P5549 indicated a lower value of 99% similarity since the 16S rRNA reference sequence used was of a closely related *Bradyrhizobium* strain and not N2P5549 (Fig. 3.9). The phylogenetic tree constructed by the neighbour-joining method using the 16S rRNA gene to show that HH103 and USDA193 are very closely related while USDA205 and N2P5549 are distantly related (Fig. 3.10).
Figure 3.6: Alignment of HH103 strain sequencing data with the HH103 strain reference sequences obtained from NCBI database using Clone Manager Professional 7. HH103R= reference sequence, HH103= sequencing data and the dots indicate matched bases.
Figure 3.7: Alignment of USDA193 strain sequencing data with the USDA193 strain reference sequences obtained from NCBI database using Clone Manager Professional 7. USDA193R= reference sequence, USDA193= sequencing data and the dots indicate matched bases.
Figure 3.8: Alignment of USDA205 strain sequencing data with the USDA205 strain reference sequences obtained from NCBI database using Clone Manager Professional 7. USDA205R= reference sequence, USDA205= sequencing data and the dots indicate matched bases.
Figure 3.9: Alignment of N2P5549 strain sequencing data with the VM-OKI strain reference sequences obtained from NCBI database using Clone Manager Professional 7. VM-OKI= reference sequence, N2P5549= sequencing data and the brown color indicates mismatched bases while dots indicate matched bases.
Figure 3.10: Phylogenetic tree constructed by the neighbour-joining method using the 16S rRNA gene to show relatedness for HH103, USDA193, USDA205 and N2P5549 strains. The evolutionary distances were computed using maximum composite Likelihood method and are in the units of the number of base substitutions per site. All ambiguous positions were removed for each sequence pair (MEGA X).

3.3.2 Characterization of Malawi collection of Rhizobium isolates

Having confirmed the identity of the four type strains of known origin, characterization of the 32 native Rhizobium isolates obtained from Malawi was therefore necessary in order to study phenotypic characteristics and identify stress tolerant strains. PCR for the 16S rRNA gene was performed on all the 32 isolates using fD1 and rD1 primers. PCR results for identification of these isolates using the 16S rRNA genomic tool indicated that 22 isolates were positively identified as true Rhizobium and belong to two distinct groups due to the presence of a 1500bp band on PCR gel and restriction digestion while 10 isolates were not Rhizobium (Table 3.2). In order to identify and select heat tolerant Rhizobium isolates, the 22 isolates were then evaluated using 40°C heat stress as the major factor to identify a heat tolerant strain that could be used to identify stress genes. The results showed that three isolates namely KU11C, NS17C and NS21B were heat stress tolerant (Fig. 3.11). As before, the PCR products of the three-heat stress tolerant isolates were cloned into pJET1.2 and sequenced. Unexpectedly, the five best hits of
BLASTn search of NCBI database show that NS21B was not a *Rhizobium* while KU11C and NS17C were *Rhizobium* but not related to *Rhizobium* that nodulates soybeans even though they were isolated from soybean root nodules (Fig. 3.12). BLASTn results documented in Appendix 2 confirmed that more isolates as non-*Rhizobium*. In order to overcome this challenge and meet the aims of this research, the research proceeded with phenotypic growth evaluation on the four *Rhizobium* type strains that nodulate soybean that were sourced from within Europe.

![Growth curve of *Rhizobium* isolates from Malawi collection grown in 50ml of TY broth inoculated with 1 ml of 2-day old culture of each isolate previously diluted to 0.1 OD₆₀₀. was grown in TY broth in an incubator under a heat stress of 40°C, 200 rpm. Optical Density measurements were taken at intervals over a period of 5 days. A dilution factor of 0.2 was used to measure samples except the three out of range samples where 0.05 and 0.1 dilutions were used.](image)

**Figure 3.11:** Growth curve of *Rhizobium* isolates from Malawi collection grown in 50ml of TY broth inoculated with 1 ml of 2-day old culture of each isolate previously diluted to 0.1 OD₆₀₀. was grown in TY broth in an incubator under a heat stress of 40°C, 200 rpm. Optical Density measurements were taken at intervals over a period of 5 days. A dilution factor of 0.2 was used to measure samples except the three out of range samples where 0.05 and 0.1 dilutions were used.
Table 3.2: Morphological characteristics of *Rhizobium* isolates from Malawi

<table>
<thead>
<tr>
<th>NAME</th>
<th>16S rRNA</th>
<th>Restriction Digest (bp)</th>
<th>Growth rate</th>
<th>Colony Size</th>
<th>Gum production</th>
</tr>
</thead>
<tbody>
<tr>
<td>KU11b</td>
<td>No</td>
<td>-</td>
<td>Fast</td>
<td>large</td>
<td>Yes</td>
</tr>
<tr>
<td>KU31B</td>
<td>Yes</td>
<td>800/600</td>
<td>Fast</td>
<td>large</td>
<td>Yes</td>
</tr>
<tr>
<td>KU47b</td>
<td>No</td>
<td>-</td>
<td>Fast</td>
<td>small</td>
<td>No</td>
</tr>
<tr>
<td>KU48B</td>
<td>No</td>
<td>-</td>
<td>Fast</td>
<td>small</td>
<td>No</td>
</tr>
<tr>
<td>KU11C</td>
<td>Yes</td>
<td>900</td>
<td>Fast</td>
<td>small</td>
<td>No</td>
</tr>
<tr>
<td>KU47C</td>
<td>No</td>
<td>-</td>
<td>Fast</td>
<td>large</td>
<td>Yes</td>
</tr>
<tr>
<td>KU20B</td>
<td>Yes</td>
<td>900</td>
<td>Fast</td>
<td>large</td>
<td>Yes</td>
</tr>
<tr>
<td>MC3B</td>
<td>No</td>
<td>-</td>
<td>Fast</td>
<td>small</td>
<td>No</td>
</tr>
<tr>
<td>MC5C</td>
<td>Yes</td>
<td>800/600</td>
<td>Fast</td>
<td>small</td>
<td>No</td>
</tr>
<tr>
<td>MC20C</td>
<td>Yes</td>
<td>900</td>
<td>Fast</td>
<td>small</td>
<td>No</td>
</tr>
<tr>
<td>MC22B</td>
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<td>800/600</td>
<td>Fast</td>
<td>small</td>
<td>No</td>
</tr>
<tr>
<td>NS20C</td>
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<td>-</td>
<td>Fast</td>
<td>small</td>
<td>No</td>
</tr>
<tr>
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<td>Fast</td>
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<td>No</td>
</tr>
<tr>
<td>NS2C</td>
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<td>Fast</td>
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</tr>
<tr>
<td>NS38C</td>
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<td>900</td>
<td>Fast</td>
<td>large</td>
<td>Yes</td>
</tr>
<tr>
<td>NS40B</td>
<td>Yes</td>
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<td>Fast</td>
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<td>Yes</td>
</tr>
<tr>
<td>NS35B</td>
<td>Yes</td>
<td>800/600</td>
<td>Fast</td>
<td>large</td>
<td>Yes</td>
</tr>
<tr>
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<td>Fast</td>
<td>large</td>
<td>Yes</td>
</tr>
<tr>
<td>NS21B</td>
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<td>800/600</td>
<td>Fast</td>
<td>large</td>
<td>Yes</td>
</tr>
<tr>
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<td>Fast</td>
<td>large</td>
<td>Yes</td>
</tr>
<tr>
<td>NS19C</td>
<td>Yes</td>
<td>900</td>
<td>Fast</td>
<td>large</td>
<td>Yes</td>
</tr>
<tr>
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<td>-</td>
<td>Fast</td>
<td>small</td>
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</tr>
<tr>
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<td>800/600</td>
<td>Fast</td>
<td>small</td>
<td>No</td>
</tr>
<tr>
<td>NS34C</td>
<td>Yes</td>
<td>900</td>
<td>Fast</td>
<td>small</td>
<td>No</td>
</tr>
<tr>
<td>NS22C</td>
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<td>-</td>
<td>Fast</td>
<td>small</td>
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<td>No</td>
</tr>
<tr>
<td>NS31C</td>
<td>Yes</td>
<td>800/600</td>
<td>Fast</td>
<td>large</td>
<td>Yes</td>
</tr>
<tr>
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<td>-</td>
<td>Fast</td>
<td>small</td>
<td>No</td>
</tr>
<tr>
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<td>900</td>
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<td>small</td>
<td>No</td>
</tr>
<tr>
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<td>900</td>
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</tr>
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<td>NS26B</td>
<td>Yes</td>
<td>900</td>
<td>Fast</td>
<td>large</td>
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</table>
**Figure 3.12:** The five best hit results of the BLASTn search of 16S rRNA sequences for KU11C, NS17C and NS21B heat tolerant *Rhizobium* isolates from Malawi collection.
3.3.3 Characterization of stress tolerance of *Rhizobium* strains

3.3.3.1 Response of *Rhizobium* strains under optimum growth conditions.

The growth conditions for the four strains of *Rhizobia* were optimized in order to establish the growth potential of *Rhizobia* used in this research. The four *Rhizobia* strains were cultured under optimum growth conditions where the pH of the growth medium was adjusted to pH 6.8, with a salt content to 0.05% NaCl and cultured for 4 days at 28°C, 200 rpm. The optimum growth conditions allow *Rhizobia* to fully express its growth potential as measured by absorbance at 600 nm. The growth curve of the strains at 28°C show that USDA205 grew vigorously after 40 hrs of growth (Fig 3.13). Significant differences in growth under optimum growth conditions was observed (Table 3.3). This difference in growth may be attributed to gum production, a characteristic exhibited mostly by USDA205. At 28oC, all strains exhibit increased growth with absorbances ranging from approximately 2.3 - 3.4 OD after 4 days incubation. Interestingly, among the four Rhizobia strains, USDA205 exhibited vigorous growth with absorbance significantly greater than HH103, USDA193 and N2P5549, which do not differ (Fig 3.14).

![Growth curve of HH103, USDA193, USDA205 and N2P5549 Rhizobium strains growing in TY broth under optimum growth conditions at 28°C for 4 days. A dilution factor of 0.2 was used to measure the Optical Density of the samples. The data correspond to the Optical Density (600 nm) vs. Hours]

**Figure 3.13:** Growth curve of HH103, USDA193, USDA205 and N2P5549 *Rhizobium* strains growing in TY broth under optimum growth conditions at 28°C for 4 days. A dilution factor of 0.2 was used to measure the Optical Density of the samples. The data correspond to the
mean while the bars are standard error values of three independent biological replicates (n=3).

Table 3.3: ANOVA table for means of Optical Density (600 nm) as a source of variation in growth for each strain.

<table>
<thead>
<tr>
<th>Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
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<td></td>
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<tr>
<td>Total</td>
<td>1.412</td>
<td>11</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

df = degree of freedom, Sig. difference p < 0.05

Figure 3.14: Growth analysis of HH103, USDA193, USDA205 and N2P5549 Rhizobium strains in TY broth under optimum growth conditions (28°C and pH 6.8) after 4 days. The data correspond to the mean while the bars are standard error values of three independent biological replicates (n=3). The different letters (a and b) represent statistically significant difference at p< 0.05 using ANOVA, Tukey HSD.
3.3.3.2 Intrinsic response of *Rhizobium* to heat stress

In order to establish relative tolerance of *Rhizobium* strains to higher temperature and select the most tolerant strain, the four *Rhizobium* strains were grown for 4 days at 37°C, 40°C and 45°C, 200 rpm. At higher temperatures, the stressed *Rhizobia* normally respond by expressing heat shock protein to protect the cells from damage. Significant differences in growth due to temperature was observed (Table 3.4). A temperature shift from 28°C to 37°C could not distinguish between HH103 and USDA205 (Fig. 3.15). At 40°C, USDA 193 exhibit a pronounced stress tolerance as is demonstrated by a reasonable but steady growth (appx. 0.6 OD) measured at 600nm (Fig 3.16). The growth of USDA 193 was significantly greater than that of HH103, USDA205 and N2P5549. While, the growth of HH103 was significantly greater than that of USDA205 and N2P5549 (Fig 3.16). The results show that USDA193 is more tolerant to heat stress in comparison to the other 3 *Rhizobia* strains, hence it was chosen as a potential source of heat stress genes. This result was confirmed by exerting more heat stress at a temperature of 45°C again with USDA193 showing the most growth at this elevated temperature. (Fig 3.17). Significant differences in growth due to temperature was observed (Table 3.4). The results in Fig 3.18 show Rhizobia growth of the strains at a high temperature of 40°C. The increase in temperature from 28°C to 40°C resulted in a significant decline in growth (Fig 3.14 and Fig 3.18).
**Figure 3.15:** Growth curve of HH103, USDA193, USDA205 and N2P559 *Rhizobium* strains growing in TY broth under stressful conditions at 37°C for 4 days. A dilution factor of 0.2 was used to measure the Optical Density of the samples. The data correspond to the mean and while the bars are standard error values of three independent biological replicates (n=3).

**Figure 3.16:** Growth curve of HH103, USDA193, USDA205 and N2P559 *Rhizobium* strains growing in TY broth under stressful conditions at 40°C for 4 days. The data correspond to the mean and while the bars are standard error values of three independent biological replicates (n=3).
Figure 3.17: Growth curve of HH103, USDA193, USDA205 and N2P559 Rhizobium strains growing in TY broth under stressful conditions at 45\(^\circ\)C for 4 days. The data correspond to the mean and while the bars are standard error values of three independent biological replicates (n=3) per treatment.

Table 3.4: ANOVA table for means of Optical Density (600 nm) as a source of variation in response to temperature for each strain.

<table>
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<tr>
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<th>df</th>
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</tr>
<tr>
<td>Within Groups</td>
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<td>8</td>
<td>0.001</td>
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<td></td>
</tr>
<tr>
<td>Total</td>
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</tbody>
</table>

df = degree of freedom, Sig. difference p < 0.05
Figure 3.18: Growth of HH103, USDA193, USDA205 and N2P5549 Rhizobia strains in TY broth at high temperature, 40°C after 3 days. The data correspond to the mean and while the bars are standard error values of three independent biological replicates (n=3) per treatment. The different letters (a - c) represent statistically significant differences at p<0.05 using ANOVA, Tukey HSD.

3.3.3.3 Response of Rhizobium to acid stress

In order to establish relative Rhizobium tolerance to low pH, the four Rhizobium strains were cultured in TY for 4 days at pH 5.5, pH 5.0, pH 4.5, pH 4.0 and pH 3.5, 200 rpm. The initial absorbance of the culture was approximately 0.1 OD. Under acidic conditions, some acid stress tolerant Rhizobia strains respond by expressing the heat shock proteins to protect the cells from damage which results in visible growth of the culture (Münchbach et al., 1999). The results show growth of the Rhizobia strains in acidic conditions. The increase in acidity from pH 5 to pH 4.5 resulted in a significant decline in growth of all strains (Fig 3.19). Significant differences in growth due to acidic conditions was observed (Table 3.5). At pH 4.5, USDA 193 and N2P5549 exhibits a small but significantly greater acid stress tolerance.
than HH103 and USDA205. Notably, the absorbance of all the strains were very low between 0.1 and 0.15 OD measured at 600nm and this means that none of the strains can grow well at pH4.5 (Fig 3.20).

![Graph showing Mean Optical Density (600 nm) for HH103, USDA193, USDA205, and N2P5549 Rhizobium strains in TY broth adjusted to pH 5.5, pH 5.0, pH 4.5, and pH 3.5 at 28°C for 3 days. A dilution factor of 0.2 was used to measure the Optical Density of the out of range samples.]

**Figure 3.19:** Growth of HH103, USDA193, USDA 205 and N2P5549 *Rhizobium* strains in TY broth adjusted to pH 5.5, pH 5.0, pH 4.5 and pH 3.5 at 28°C for 3 days. A dilution factor of 0.2 was used to measure the Optical Density of the out of range samples.

**Table 3.5:** ANOVA table for means of Optical Density (600 nm) as a source of variation in response to acidic conditions for each strain.

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<th>df</th>
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<td>0.000</td>
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<td>Within Groups</td>
<td>0.000</td>
<td>8</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0.001</td>
<td>11</td>
<td></td>
<td></td>
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</tbody>
</table>

df = degree of freedom, Sig. difference p < 0.05
Figure 3.20: Growth of HH103, USDA193, USDA205 and N2P5549 *Rhizobia* strains in TY broth under acidic conditions, pH 4.5 after 3 days. The data correspond to the mean and while the bars are standard error values of three independent biological replicates (n=3) per treatment. The different letters (a and b) represent a statistically significant difference at p<0.05 using ANOVA, Tukey HSD.

3.3.3.4 Response of *Rhizobium* to salt stress

The relative tolerance of the *Rhizobia* to high salts was established by culturing the four *Rhizobium* strains in TY broth with 1% NaCl, 200 rpm. Significant differences in growth due to higher salt concentration was observed (Table 3.6). The increase in salt content from 0.05 to 1% resulted in a significant decline in growth in some strains (Fig. 3.21). At 1% NaCl, USDA205 exhibits tolerance as it showed good growth with an absorbance of approximately 2.5 OD_{600}. The absorbance of USDA205 was significantly greater than HH103, USDA 193 and N2P5549. The absorbance of N2P5549 at approximately 1.5 OD_{600} was significantly greater than HH103 and USDA 193 (Fig 3.13). The results show that USDA205 is the most tolerant strain to salt stress.
Table 3.6: ANOVA table for means of Optical Density (600 nm) as a source of variation in response to 1% NaCl for each strain.

<table>
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</tr>
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<td>Within Groups</td>
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<td>Total</td>
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<td></td>
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</tbody>
</table>

df = degree of freedom, Sig. difference p < 0.05

Figure 3.21: Growth of HH103, USDA193, USDA205 and N2P5549 Rhizobia strains in TY broth at a higher salt concentration, 1% NaCl after 3 days. A dilution factor of 0.2 was used to measure the Optical Density of the out of range samples. The data correspond to the mean and while the bars are standard error values of three independent biological replicates (n=3) per treatment. The different letters (a - c) represent statistically significant difference at p< 0.05 using ANOVA, Tukey HSD.
3.3.4  Nodulation of the identified Rhizobium strains

The research further evaluated the nodulation effectiveness of HH103, USDA193, USDA 205 and N2P5549 Rhizobium strains to nodulate the host soybean plant after establishing the relative tolerance of these strains to heat stress. Seedlings of the two soybean cultivars were separately inoculated with the 4 different strains and grown at 28°C for 4 weeks. After harvesting the soybean plants, the number of nodules formed on each plant was recorded. The results indicate that the heat susceptible strain HH103 is most effective in terms of nodulation in comparison to USDA193, USDA205 and N2P5549 as it produced the greatest number of nodules (Fig 3.22). Therefore, USDA193 the heat tolerant strain, was identified as a suitable donor of stress genes to a highly effective but heat susceptible HH103 Rhizobium strain.

Figure 3.22: Nodulation of Black Jet and Edamame soybean cultivars inoculated with HH103 and USDA193 Rhizobia strains and grown at 28°C for 4 weeks. The control= no inoculation and fertilizer= 5 ml of 10g/L KNO₃. The data correspond to the mean and while the bars are standard error values of three independent biological replicates (n=3) per treatment.
3.3.5 Proteins differentially expressed under high temperature gradient from heat tolerant strain USDA193

The aim of this experiment was to identify the different proteins that are expressing highly at high temperature in USDA193, a selected heat tolerant Rhizobium strain. The cultures were grown in TY media at 20°C for 24hrs and 48°C for 48hrs equating to mid-log phase for each condition, then harvested and proteins extracted. The different growth time points were used due to slow bacterial growth at 40°C. Proteomic analysis was done by the proteomics facility in Cellular and Molecular Medicine (CMM) at University of Bristol using Tandem mass Tag (TMT) spectrophotometry.

3.3.5.1 Protein extraction by sonication

Protein extraction procedure was conducted in order to obtain crude protein for total protein analysis. The proteins were extracted by sonication from the cells of the heat tolerant strain, USDA 193. The protein concentration of ≥2 mg/ml in the crude protein is the standard requirement for total protein analysis using Tandem Mass Tag. The concentration of the protein extract was estimated by using direct absorbance measurement at 280 and 260 nm. The results show that there was no significant difference in the amount of protein extracted at 20°C and 40°C because of the different growth periods (Table 3.7). However, the amount of protein extracted at both temperatures was lower than the minimum required concentration as it ranged from approximately 0.4 to 0.7 mg/ml with 3 replicates extractions (Fig. 3.23). In order to achieve the required total protein concentration of ≥2 mg/ml, the protein extract concentration was increased by reducing the 20 ml volume to a final volume of approximately 1 ml using centricon filters.

Table 3.7: ANOVA table for means of protein concentration (mg/ml) as a source of variation in response to elevated temperature.

<table>
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<td>0.105</td>
<td>7</td>
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</tr>
</tbody>
</table>

df = degree of freedom, Sig. difference p < 0.05
Tandem Mass Spectrometry (TMS) was used to identify the proteins expressed by the *Rhizobia* bacteria cells grown at 20°C and 40°C. The amount of each protein in each of the digested protein extracts from the two temperatures was measured by a Mass spectrophotometer. A search of the quantified spectra using molecular mass and charge in UniProt *Sinorhizobium fredii* database show that a wide range of proteins were being expressed (Fig. 3.24). Comparative analysis of the ratio of the proteins expressed at each of the two temperatures allowed calculation of a protein content ratio (40°C/20°C). The protein content ratio results show that several proteins were overexpressed at 40°C compared to 20°C (Fig. 3.24). Using the protein content ratio results, several stress response proteins

![Figure 3.23: Protein extraction from USDA 193 *Rhizobia* strain cells cultured at 20°C and 40°C. No statistical difference at p< 0.05 was observed. The data correspond to the mean and while the bars are standard error values of three independent biological replicates (n=3) per treatment. No statistical differences at p< 0.05 was observed using ANOVA, Turkey HSD.](image)
were identified as they were expressing highly at 40°C. The protein content ratio of 4.0 was used as the cut off point for selecting proteins which are differentially expressed. The eight most differentially expressed proteins were therefore selected as target genes for use in genetic manipulation of HH103, a heat susceptible *Rhizobia* strains to induce tolerance to heat stress. The selected proteins included Putative heat shock protein (*hsp20* family), 10kDa *groES2*, Immunoglobulin protein A (*lbpA*), putative small heat shock protein (*shsp*), 6kDa, *groEL* and three uncharacterised proteins (*UC1*, *UC2* and *UC3*). The results also show differentially expressed proteins such as histidine kinase, glyoxylate carboligase, cytochrome p450 and epoxyqueuosine reductase which function as kinetic, catalytic, oxidizing and reducing enzymes respectively (Fig. 3.24)
Figure 3.24: Comparison of protein concentration ratios of proteins being differentially upregulated by the USD193 strain cultured at 40°C compared to 20°C
3.4 Summary

Firstly, this research managed to evaluate the 32 presumed *Rhizobium* isolates collected from Malawi soils and 4 strains originating from United States of America (USA) and China. Characterization confirmed the identity of the four putative *Rhizobia* strains sourced from John Innes Centre using morphological, biochemical and genetic characteristics. Basically, the genetic tools were based on the 16S rRNA gene, *nolBT* gene, *nifH* gene and Sanger sequencing. Using these tools, the three *Rhizobia* strains (HH103, USDA193, and USDA205) were confirmed to belong to *Sinorhizobium* while N2P5549 was confirmed as a *Bradyrhizobium*. However, the Malawi isolates were identified either as non-*Rhizobium* or as fast-growing non-soybean nodulating *Rhizobium*. Using restriction digestion, the *Rhizobium* isolates were found to belong to two distinct groups.

This research established the intrinsic tolerance of the *Rhizobia* strains to heat, acid and salt stress. Among the *Rhizobia* strains under investigation, USDA193 was found to be the most heat tolerant strain while USDA205 was found to be the most salt tolerant strain. However, it was also found that none of the strains could thrive well under strong acid stress, although *Rhizobia* strain N2P5549 was slightly tolerant to acid.

Finally, using proteomics, this research identified several proteins in USDA 193 which are differentially up regulated under high temperature. Among the differentially expressed proteins, the top eight proteins with known or unknown functions were selected for the use of their sequences as stress protection genes.

3.5 Discussion

In this chapter, the identities of HH103, USDA193, USDA205 and N2P5549 *Rhizobium* strains were confirmed using morphological, biochemical and genomic characteristics where the 16S rRNA gene was amplified from the DNA extract and sequenced. Although most of the phenotypic characteristics agree with data from literature (Table 1.1, Chapter 2), N2P5549 was found to have slightly absorbed the Congo Red. However, some *Rhizobia* strains are known to weakly absorb Congo Red (Somasegaran & Hoben, 1994). However, the results for presumed *Rhizobium* isolates from Malawi collection contrasts with evidence of Parr et al., (2017) who found that *Rhizobium* present in Malawi soils are mostly slow growing *Bradyrhizobium*. This suggests that these isolates might be exotic strains of fast-growing *Rhizobium* such as USDA110 which have been used as soybean inoculants for decades.
Although there were few strains of *Rhizobia* used in this research, the tools used were able to identify the two main *Rhizobia* groups and define each *Rhizobia* strain to a specific group. As indicated by Kainth et al., (2005) on diversity of *Rhizobium*, this research was therefore able to establish that there is diversity in *Rhizobia* bacteria nodulating soybeans.

Several bioinformatic analysis were performed on the 16S rDNA gene sequence to confirm its accuracy. The NCBI BLASTn search of resulting sequence of each strain identified the 16S rRNA gene of that specific *Rhizobium* strain as the first hit while its alignment with the reference sequence from the NCBI database indicated clear homology with a high identity score. These two tests confirm the identities of the strains used in this research. The Malawi collection of *Rhizobium* isolates was of poor quality and could not be used since they mostly depend on morphology and biochemical method for identification of *Rhizobium*.

Although temperature affects *Rhizobium*, it was noted that there was no difference in the amount of protein being produced at 20°C and 40°C. This means that there was no change in the total amount of proteins being produced by the cells. This suggests the thinking that when some proteins are being highly expressed, other proteins may be down regulated to balance up the total cell protein (data not shown).

The fact that USDA193 was identified as the most heat tolerant strain was not surprising since USDA193 was isolated from the warm part of the USA. This means that this strain has evolved since it has the highest thermotolerance compared to the other strains which may be genetic or have acquired tolerance.

The identified source of heat stress genes, USDA193, has not yet been sequenced. I was able to identify other *Rhizobia* strains that possess the same sequences whose genome have already been sequenced. The sequences of all the proteins in *Rhizobia* bacteria can be obtained from UniProt database where most of the protein sequences are deposited indicating sources and other related characteristics (www.uniprot.org). This assisted in identification and amplification of all the targeted genes used in this research.
4 Construction of pLMB51 plasmids and generation of Rhizobium transformants for expression of stress genes

4.1 Introduction

In the previous chapter, several differentially expressed proteins were identified from a heat tolerant Rhizobium strain USDA193 grown at 20°C and 40°C. The aim of this chapter was to develop a way to manipulate expression of these genes in a heat sensitive Rhizobium strain HH103 with the hope that this might deliver more stress tolerance. Genes are effectively transferred from one bacterial cell to another cell, of either the same or a different species using a plasmid as a vehicle. Plasmid construction techniques allow incorporation of specific genes into plasmid vectors and their expression in the recipient organisms such as Rhizobium. Specifically designed primers are often used to introduce restriction enzyme sites by PCR to the terminal ends of the gene of interest in order to facilitate cloning (Hoseini & Sauer, 2015). Cloning was therefore used to introduce the stress gene into a pLMB51 vector for their expression in the recipient Rhizobium bacteria.

Antibiotic selection is an important tool for selection of transformants that harbor plasmids encoding a specific antibiotic resistance gene. A robust selection marker that is compatible with an appropriate vector is vital for genetic transformation (Jang & Magnuson, 2013). Antibiotic profiling of Rhizobia strains was therefore necessary in order to establish the appropriate antibiotic and concentration as a selectable marker for identification of transformed Rhizobia cells. The antibiotics such as ampicillin, neomycin, streptomycin, tetracycline, kanamycin and chloramphenicol are mostly used as markers for common plasmid vectors in genetic studies. In order to establish the most appropriate concentration for selection of resistant Rhizobium transformants, the four Rhizobium strains were screened against these antibiotics in this antibiotic profiling study. The antibiotic was therefore chosen as an appropriate marker since most Rhizobium strains are sensitive to this antibiotic hence efficient selection (Young & Chao, 1989).

In order to express the stress genes in Rhizobium bacteria, an expression vector, pLMB51 was chosen since it has a taurine dependent Rhizobium specific promoter, tauA (Tett et al.,
According to Tett et al. (2012), the pLMB51 plasmid vector contains an RP4 plasmid backbone. Self-transmissible plasmids such as RP4 plasmids are often shared through conjugation since they contain genes that encode proteins necessary for mobilization and transfer of plasmids from one bacterium to another (Woodall, 2003). In order to construct the plasmid, the gene fragments were cut from the cloning vector pJET1.2 and were cloned into the pLMB51 expression vector by restriction digest ligation according to Koelle (1998).

According to Griffiths et al., (2000), conjugation is the union of two bacterial cells, resulting in the transfer of chromosomal material from the donor to the recipient cell. Transformation of E. coli competent cells of a conjugative strain, S17 with the cloned pLMB51 expression vector allowed further multiplication of the plasmid DNA for effective conjugation. According to Woodall (2003), genes present on plasmids may be efficiently transferred from the donor bacterium to a recipient cell through bacterial mating. In this research, a conjugative E. coli strain S17 facilitated the transfer these genes into Rhizobia by conjugation where the cloned pLMB51 plasmid vector harboring the stress genes were used as a plasmid donor.

Colony PCR of transformed E. coli cells using pLMB51 vector primers allowed the identification of transformants having the stress gene insert. PCR of Rhizobium plasmid DNA, using gene and vector sequence primer pairs, assisted in the identification of positive Rhizobium transformants. PCR amplification of the nifH gene facilitates confirmation of the selected transformants as true Rhizobium (Pastorino et al., 2003).

PCR using specifically designed primers containing sequences of desired restriction enzymes introduced the enzyme recognition sequences into PCR products to allow for cloning into an expression vector. XbaI and Ncol/BamH1 restriction enzyme sites were introduced into targeted genes in order to facilitate the cloning of UC1, groES, hsp20, UC2, shsp, groEL, lbPA and UC3 heat stress genes into the expression vector, pLMB51. Specific primers were designed from sequences of targeted heat stress gene sequences using primer3 to introduce XbaI and Ncol/BamH1 sequences at the start and end of the gene. At least seven bases were included before and after the enzyme sequence in the forward and reverse primers to facilitate efficient cleavage at these sites for cloning purposes.
4.2 Aims

- To amplify the targeted genes that differentially expressed at high temperature.
- To identify a suitable selectable marker for screening *E. coli* and *Rhizobia* transformants that contain a pLMB51 plasmid vector.
- To sequence the targeted stress genes using pJET1.2 cloning vector.
- To construct plasmids containing specific targeted stress genes using the expression vectors pLMB51.
- To manipulate *Rhizobia* using pLMB51 plasmid constructs containing stress genes.

4.3 Results

4.3.1 Generation of heat stress gene (HSG) templates

4.3.1.1 Designing primers of targeted heat stress genes

The targeted genes were selected because of their predicted functions in *E. coli*, presumed but not tested in *Rhizobium* and their differential expression like the case of the uncharacterized proteins. *hsp20, IbpA* and small heat shock protein (*shsp*) are typically involved in response to stress resulting from conditions which among others include temperature, ionizing radiation and hypertonicity. While under stress conditions, *groES* and *groEL* are responsible for protein folding as they assist in assembly of single chain or unfolded polypeptides into the correct structure and hence reduces misfolding as they promote the refolding of unfolded polypeptides. The uncharacterized genes designated as *UC1, UC2* and *UC3* were selected due to high expression values despite having no known functions.

The heat tolerant *Rhizobium* strain USDA193 has not yet been sequenced, so it was necessary to isolate these genes based on the sequences of known *Rhizobium* strains like HH103 and USDA205. Specific primers were designed to amplify the complete gene sequence from start codon to the stop codon. The accession numbers, codes and sources of the heat stress genes sequences were used to design primers are shown in Table 4.1. The heat stress gene sequences were uploaded into Primer3Plus tool, to generate primers for specific genes. All the primers met the specific temperature requirement for primer design where the primer pairs should have Tm values that are within 5°C (Table 2.1).
Table 4.1: The identity and source of targeted stress proteins from the UniProt database that were used in designing primers for amplifying genes.

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<tr>
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<td>HH103</td>
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</tr>
<tr>
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<td>SFHH103_03407</td>
<td>HH103</td>
<td>G9A3F1</td>
</tr>
<tr>
<td>60 kDa, groEL</td>
<td>1629</td>
<td>N181_31305</td>
<td>USDA205</td>
<td>A0A0T6ZUQ4</td>
</tr>
<tr>
<td>Heat shock protein IbpA</td>
<td>462</td>
<td>N181_15470</td>
<td>USDA205</td>
<td>A0A0T6ZQ48</td>
</tr>
<tr>
<td>Uncharacterized protein 3</td>
<td>360</td>
<td>N181_22045</td>
<td>USDA205</td>
<td>A0A0T6ZGL3</td>
</tr>
</tbody>
</table>

4.3.1.2 PCR of heat stress genes from *Rhizobium* DNA

To generate the heat stress gene (HSG) template genes were initially amplified from the USDA 193, HH103 or USDA205 DNA with specifically designed primers (Appendix A) using Phusion polymerase. It proved problematic to amplify groES and groEL from USDA 193 despite optimization of PCR conditions even though proteomics results indicate their presence in this strain. Hence these two gene were amplified from HH103 and USDA205 respectively. PCR fragments amplified from USDA 193 DNA were of expected sizes ranging from 165 to 1649 bp (Table 4.1). The size of the PCR fragments was determined by start and stop codon to be full length for cloning as well as sequencing. Most of the fragments were between 300 bp and 500 bp except for UC2 and groEL which were approximately 200 bp and 1500 bp respectively. The open reading frames of all the eight-heat stress genes were successfully amplified from the USDA 193 DNA (Fig.4.1).
4.3.2 Synthesis of gene fragments and sequencing

4.3.2.1 Introduction of enzyme sites into stress gene

Restriction enzyme sites were introduced to both ends of the stress gene in order to facilitate cloning of the genes into the expression vector. Primers were specifically designed to include Ncol or BamH1 and Xbal enzyme sequences in the forward and reverse primers respectively. Using these primers, gene fragments with Ncol or BamH1 and Xbal enzyme sites at their ends were synthesized by PCR using Phusion High-fidelity DNA Polymerase (Thermo Scientific). The PCR products were run on an electrophoresis gel to confirm their sizes. The results indicate clear bands of corresponding to 321, 333, 480, 189, 528, 1647, 486 and 381 base pairs for UC1, groES, hsp20, UC2, shsp, groEL, IbpA and UC3, respectively (Fig. 4.2). The synthesized gene fragments were slightly larger than the original genes sizes indicated in Table 4.1 due to additional bases from restriction enzyme sequences and stop codon which were added at the end for hsp20 and UC3 since the sequences of these two genes had no stop codon. The bands were cut, and gel purified for cloning into pLMB51.
Figure 4.2: Gel documenting PCR products of stress genes adapted with Ncol or BamH1 and Xbal enzyme sites electrophoresed in a 1% agarose gel at 120V for 30 min using 10 µl of sample from the total 20 µl of PCR product loaded to each well. Lane M, 5 µl 2log DNA ladder: Lane C, negative control (water): Lane 1, UC1 (321 bp): Lane 2, groES (333 bp): Lane 3, hsp20 (480 bp): Lane 4, UC2 (189 bp): Lane 5, shsp (528 bp): Lane 6, groEL (1647 bp): Lane 6, IbpA (486 bp): Lane 8, UC3 (381 bp).

4.3.2.2 Cloning and Sequencing of stress genes

The purified PCR products of each gene were cloned into pJET 1.2 vector and transformed into Top10 E. coli competent cells for plasmid multiplication. The ampicillin marker in the pJET1.2 vector facilitates the selection of the transformed E. coli colonies on LB ampicillin (100 µg/ml) plates which demonstrates acquired resistance to ampicillin. As expected, the specific primers were able to amplify DNA amplicons of various sizes from the plasmid DNA of successfully transformed E. coli transformants and this confirms successful ligation of the insert into pJET1.2. The results show clear bands of expected sizes approximately 300, 300, 500, 200, 500,1500, 500 and 400 base pairs for UC1, groES, hsp20, UC2, shsp, groEL, IbpA and UC3 respectively (Fig. 4.3). The genes were PCR amplified from plasmid DNA obtained by miniprep from the positive E. coli transformants using pJET primers to ensure that these genes are not amplified from E. coli and confirm presence of the insert before sending for sequencing. The sequence data files resulting from the sequencing reactions were uploaded in Clone Manager Professional 7 (Sci. Ed software), trimmed to the Ncol or BamH1 and Xbal enzymes and used in a BLASTn search. The NCBI BLASTn search results using the sequences show that each sequence was positively identified, and each sequence related to its original gene sequence by 96-100% identity (Table 4.2). This result means that the pJET
transformants contained genes with the correct sequences. Furthermore, protein translation analysis of each sequence using Sequencher 5.0 show expected mutations on the 2nd amino acid of UC1, groES, hsp20 and shsp genes where there is a G instead of C in UC1 and hsp20 genes and instead of A in groES and shsp genes due to modifications to include the restriction site. However, the protein sequences indicate that the genes will be able to express the correct protein when incorporated into Rhizobia bacteria cells.

Figure 4.3: PCR of pJET plasmids containing stress genes electrophoresed in a 1% agarose gel at 120V for 30 min using 10 μl of sample from the total 20 μl of PCR product loaded to each well: Lane M, 5 μl 2log DNA ladder: Lane C, negative control (water): Lane 1, UC1 (321 bp): Lane 2, groES (333 bp): Lane 3, hsp20 (480 bp): Lane 4, UC2 (189 bp): Lane 5, shsp (528 bp): Lane 6, groEL (1647 bp): Lane 6, lbpA (486 bp): Lane 8, UC3 (381 bp).
Table 4.2: NCBI BLASTn search results showing source of best hit of similar sequence and % identity to the targeted stress genes.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Size (bp)</th>
<th>Similar Sequence</th>
<th>% DNA Identity</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncharacterized protein 1</td>
<td>303</td>
<td>Sinorhizobium fredii CCBAU 25509 plasmid pSF25509b, complete sequence</td>
<td>99</td>
<td>CP029452.1</td>
</tr>
<tr>
<td>groES2</td>
<td>315</td>
<td>Sinorhizobium fredii CCBAU 25509 plasmid pSF25509a, complete sequence</td>
<td>99</td>
<td>CP029453.1</td>
</tr>
<tr>
<td>Putative probable heat shock protein, hsp20</td>
<td>459</td>
<td>Sinorhizobium fredii CCBAU 25509 plasmid pSF25509a, complete sequence</td>
<td>96</td>
<td>CP029453.1</td>
</tr>
<tr>
<td>Uncharacterized protein 2</td>
<td>171</td>
<td>Sinorhizobium fredii HH103 plasmid pSFH103e complete sequence</td>
<td>100</td>
<td>HE616899.1</td>
</tr>
<tr>
<td>Putative small heat shock protein</td>
<td>510</td>
<td>Sinorhizobium fredii CCBAU 25509 plasmid pSF25509a, complete sequence</td>
<td>100</td>
<td>CP029453.1</td>
</tr>
<tr>
<td>groEL</td>
<td>1629</td>
<td>Sinorhizobium fredii CCBAU 83666 plasmid pSF83666b, complete sequence</td>
<td>99</td>
<td>CP023071.1</td>
</tr>
<tr>
<td>Heat shock protein IbpA</td>
<td>462</td>
<td>Sinorhizobium fredii CCBAU 25509 chromosome, complete genome</td>
<td>99</td>
<td>CP029451.1</td>
</tr>
<tr>
<td>Uncharacterized protein 3</td>
<td>360</td>
<td>Sinorhizobium fredii CCBAU 45436 plasmid pSF45436e, complete sequence</td>
<td>98</td>
<td>CP029235.1</td>
</tr>
</tbody>
</table>
4.3.3  Sensitivity of *Rhizobium* strains to antibiotics

4.3.3.1  Marker selection

*Rhizobium* strains were screened for resistance to antibiotics to choose the appropriate antibiotic resistance marker for selection of transformants. The results indicate that *Rhizobia* bacteria strains have varied capacity to tolerate different types of antibiotics and concentrations. All four strains were resistant to streptomycin at a concentration of 900 - ≥1000 µg/ml while HH103 and USDA205 strains were moderately resistant to ampicillin at 600 – 700 µg/ml which had an inhibitory effect on USDA193 and N2P5549 at 50 – 100 µg/ml. All the *Rhizobia* strains were highly sensitive to neomycin, tetracycline, kanamycin and chloramphenicol at concentration of ≤ 20 µg/ml (Fig. 4.4). These antibiotic concentrations are commonly used for cloning and expression vectors. Tetracycline was chosen as the best marker for selection of *Rhizobium* considering uniformity in its effect across different *Rhizobia* strains and availability of vectors encoding a tetracycline resistance marker and containing *Rhizobium* specific promoters. This facilitated the choice of pLMB51 expression vector having a *Rhizobium* promoter *tauA* for the expression of the targeted genes in *Rhizobia* bacteria and a tetracycline resistance marker (Tett et al., 2012).

![Figure 4.4: Minimum inhibitory concentration of ampicillin, neomycin, streptomycin, tetracycline, kanamycin and chloramphenicol antibiotics in TY media plates used to grow HH103, USDA193, USDA205 and N2P5549 Rhizobium strains.](image-url)
4.3.4  Restriction digestion analysis

4.3.4.1  Preparation of the expression vector and inserts

The expression vector, pLMB51 is a low copy plasmid capable of replication in *E. coli* and other gram negatives such as *Rhizobium*. Ncol or BamH1 and Xbal restriction enzymes were used in a double digestion to cut plasmid from pJET transformants and linearize the pLMB51 vector to produce compatible ends. The restriction digest was run on a 1% agarose gel to confirm fragment sizes and purity. The results show that a single band of above 10.0 kilobase corresponding to the digested pLMB51 plasmid vector was detected (Fig. 4.5).

![Image of gel illustrating circular and linearized pLMB51 plasmid vector digested with Ncol or BamH1 and Xbal enzyme electrophoresed in a 1% agarose gel at 120V for 30 min using 10 μl of sample from the total 30 μl of reaction mix loaded to each well. Lane M, 5 μl 2log DNA ladder: Lane 1, circular pLMB51 plasmid vector: Lane 2, linear pLMB51 plasmid vector.]

**Figure 4.5:** Gel illustrating circular and linearized pLMB51 plasmid vector digested with *N*col or *Bam*H1 and *X*bal enzyme electrophoresed in a 1% agarose gel at 120V for 30 min using 10 μl of sample from the total 30 μl of reaction mix loaded to each well. Lane M, 5 μl 2log DNA ladder: Lane 1, circular pLMB51 plasmid vector: Lane 2, linear pLMB51 plasmid vector.

4.3.5  Construction of expression vectors

4.3.5.1  T4 DNA ligation using compatible ends

In order to construct an expression plasmid vector, the purified digested gene fragments were cloned into pLMB51 vector by ligation. Ligation reactions require the use of a linearized vector and an insert with base overhangs which are complimentary to those produced by the digested vector. The linearized plasmid pLMB51 vector with a *Rhizobium* specific promoter tauA was re-circularized before cloning with a gene
fragment insert by T4 DNA ligation. The *Rhizobium* promoter, *tauA* in the pLMB51 vector is responsible for driving expression of the genes in the host cell and the tetracycline marker is responsible for the selection of transformed cells on antibiotic plates. The correct construction was confirmed by PCR using primers designed at the ends of the vector as double digestion did not work due methylation of the *Xba*I site. The six pLMB51 plasmids were successfully constructed using UC1, *hsp20*, UC2, *shsp*, *IbpA* and UC3 gene inserts as shown in the example below for putative heat stress protein (*hsp20*) (Fig. 4.6). Cloning for *groEL* and *groES* was stopped because they were only clonable from the heat sensitive strains.

**Figure 4.6:** An example of a pLMB51 plasmid construct (14.334kb) cloned with putative heat stress protein (*hsp20*), a stress gene cloned downstream of the *tauA* promoter.
4.3.5.2 Transformation of *E. coli*

The cloned pLMB51 plasmid vector construct was used to transform electrocompetent cells of *E. coli* strain S17. To select positive transformants that contains the plasmid construct, colony PCR using pLMB51 primers was conducted on three randomly selected transformants to screen *E. coli* colonies that grew on LB plates supplemented with tetracycline (10 µg/ml). PCR results indicate clear bands of approximately 300, 500, 200, 500, 500, 400 and 500 base pairs for UC1, *hsp20*, UC2, *shsp*, *IbpA*, UC3 and pLMB51, respectively (Fig 4.7). As expected, the pLMB51 primers were able to amplify the DNA insert from the plasmid of successfully transformed S17 *E. coli* transformants and this confirms successful ligation of the insert into pLMB51. The plasmid from the positive transformants could therefore be used for propagation of stress tolerant *Rhizobia* transformants as it contains the stress gene inserts.

![Figure 4.7: Colony PCR of DNA for E. coli strain S17 transformed with cloned pLMB51 vector electrophoresed in a 1% agarose gel at 120V for 30 min using 10 µl of sample from the total 20 µl of PCR product loaded to each well. Lane M, 5 µl 2log DNA ladder: Lane C, negative control (water): Lane 1, UC1: Lane 2, *hsp20*: Lane 3, UC2: Lane 4, *shsp*: Lane 5, *IbpA*: Lane 6, UC3: Lane 7 pLMB51](image)

4.3.6 Generation of stress tolerant *Rhizobia* transformants.

4.3.6.1 Development of stress tolerant *Rhizobium* transformants by conjugation

In order to generate stress tolerant *Rhizobia* transformants, the pLMB51 plasmid constructs were introduced into *Rhizobia* HH103 by conjugation using the donor strain *E. coli* S17. To select *Rhizobia* transformants that contains the plasmid vector construct,
PCR was conducted on DNA extracted from three randomly selected *Rhizobia* colonies that grew on TY agar plates supplemented with tetracycline (10 µg/ml) and rifampicin (25 µg/ml) using pLMB51- F and a reverse primer for each stress gene. The pLMB51- F primer was plasmid specific and included some bases of the regulator in the plasmid while the reverse primer was gene specific. The primer pair was selected to avoid amplification of the genomic version of the same gene. The PCR results on *Rhizobia* DNA, indicate the presence of the gene inserts and pLMB51 plasmid which appear as various sized bands of approximately 300, 500, 200, 500, 500, 400 and 500 base pairs for UC1, hsp20, UC2, shsp, IbpA, UC3 and pLMB51 genes and plasmid respectively (Fig. 4.8). Amplification of these inserts from the DNA of successfully transformed *Rhizobia* transformants confirmed successful transformation of HH103 *Rhizobium* strain. Furthermore, PCR results on *Rhizobium* DNA for the presence of the *nifH*, a nitrogen fixation gene using *nifH* primers show that all the positive transformants harbor this gene which appear as a 0.6kb band (Fig. 4.9). As expected, the *nifH* primers could not amplify the same gene in *E. coli* strain S17. The presence of this gene confirms that these colonies are true *Rhizobia* transformants since nitrogen fixation is the major identity of *Rhizobia* bacteria. The positive *Rhizobia* transformants could therefore be used for soybean inoculation experiments to assess nitrogen fixation and induced stress tolerance as it contains the stress gene inserts.

![Figure 4.8](image.png)

**Figure 4.8:** PCR of stress genes from DNA of *Rhizobium* HH103 with recombinant pLMB51 plasmid electrophoresed in a 1% agarose gel at 120V for 30 min using 10 µl of sample from the total 20 µl of PCR product loaded to each well. Lane M, 5 µl 2log DNA ladder: Lane C,

**Figure 4.9:** PCR of nifH gene from DNA of *Rhizobium* HH103 with recombinant pLMB51 plasmid electrophoresed in a 1% agarose gel at 120V for 30 min using 10 μl of sample from the total 20 μl of PCR product loaded to each well. Lane M, 5 μl 2log DNA ladder: Lane C, negative control (*E. coli* DNA): Lane 1, *UC1*: Lane 2, *hsp20*: Lane 3, *UC2*: Lane 4, *shsp*: Lane 5, *IbpA*: Lane 6, *UC3*: Lane 7, pLMB51.
4.4 Summary

The six stress tolerant genes which are *UC1, hsp20, UC2, shsp, lbpA* and *UC3* from a heat tolerant *Rhizobium* strain, USDA193, were successfully amplified for cloning into the expression vector pLMB51. Unfortunately, it proved difficult to amplify *groES* and *groEL* genes from this strain even though proteomics results predicted their presence in this strain. As a result, *groES* and *groEL* genes were amplified from HH103 and USDA205 respectively.

The targeted genes with enzyme sequences were successfully introduced into a cloning vector and sequences were confirmed using Sanger sequencing. The targeted stress genes which include *UC1, UC2, UC3, groES2, putative probable heat shock protein (hsp20), putative small heat shock protein, (groEL), heat shock protein, lbpA*, were positively confirmed as having similar sequences to reference proteins.

An appropriate selectable marker for screening *E. coli* and *Rhizobia* transformants having the pLMB51 plasmid vector was identified. This was done though antibiotic profiling of different *Rhizobia* strains against a variety of antibiotics over a range of concentrations. The results obtained indicate that kanamycin, neomycin and tetracycline were good markers for *Rhizobium* selection since all strains screened showed sensitivity to these antibiotics. However, tetracycline was chosen as a selectable marker for the identification and confirmation of positive transformants that contain the pLMB51 plasmid since the marker is also present in this plasmid.

I managed to construct 6 plasmids which were constructed with targeted stress genes in the pLMB51 expression vector. Stress gene inserts with cohesive sites were cloned into a linearized pLMB51 vector by T4 DNA ligation. The plasmid constructs were propagated and maintained in conjugative *E. coli* strain S17 to ensure efficient conjugation into *Rhizobium*.

The plasmid constructs were successfully transferred from *E. coli* strain S17 into the heat susceptible *Rhizobium* HH103. As a result, *Rhizobium* strain HH103 was hence genetically manipulated using cloned pLMB51 plasmid vector. Genetic manipulation facilitated probable expression of these stress genes in *Rhizobia* in order to induce stress tolerance.
*Rhizobium* HH103 transformants with potentially improved heat tolerance were therefore successfully generated by genetic manipulation. In conclusion, the results obtained indicate that it is possible to introduce stress genes into *Rhizobium* bacteria using expression vectors.

### 4.5 Discussion

Heat stress genes were selected and amplified from the DNA of heat tolerant *Rhizobium* strain USDA193 identified through phenotypic response of *Rhizobium* to heat stress. Since it was required to amplify the genes from the start codon to the stop codon, there was a limited choice on the primer sequences (Hoseini & Sauer, 2015). As a result, it was problematic to amplify some genes due to primers which required high temperature with possibility of dimer formation since they had complimentary sequences.

According to marker selection results, there were several antibiotics which could be used as selectable makers. The results obtained indicate that kanamycin, neomycin and tetracycline were good markers for *Rhizobium*. However, tetracycline was chosen since the pLMB51 vector having a *Rhizobium* promoter uses tetracycline as a marker. Tett et al. (2012), successfully used tetracycline as a selectable marker in the development of pLMB51 and pLMB509 expression vectors that uses an inducible taurine promoter (*tauAp*).

Plasmid extraction for pLMB51 often only gave low yield and this made purification of digestion vector difficult. This is not a surprising result since the expression vector, pLMB51 used in plasmid construction is a low copy plasmid with a yield of approximately 5-7 copies (Tett et al., 2012). Following the manufacturers protocol, 2-3 individual miniprep extractions were pooled in order to obtain enough plasmid for plasmid construction.

Although, colony PCR or PCR of plasmid DNA is often used to screen colonies that grow on antibiotic plates, I found it difficult to screen *Rhizobium* colonies that grew on TY tetracycline plates by colony or plasmid using pLMB51 plasmid primers designed from the vector ends flaking the gene. It is suspected that the introduced plasmid is integrated into the chromosome resulting in amplification of a similar sized band from the different types of transformants (Griffiths et al., 2000). PCR was therefore conducted on the DNA extracted from the cultured colonies using primers where one pair is designed from the gene sequence and the other from the vector. The sequences in the promoter region of
the vector and at the end of the gene facilitated confirmation of the insert since they are conserved and unique sequences within the plasmid construct.
5 Assessment of stress tolerance and nitrogen fixation potential of Rhizobium transformants

5.1 Introduction

The environmental bacterium, *Rhizobium* is often exposed to stress factors that may include elevated temperatures, acidity and salinity. In order to endure these stressful soil conditions and obtain physiological adaptation, *Rhizobium* responds to these changes through expression of certain genes. Physiological changes through gene expression are the major components of bacterial response to stress due to changes in the environment. Tolerance of *Rhizobia* to stress factors like pH, salinity and higher temperature present in the soil is also required for efficient and effective nitrogen fixation under challenging environmental soil conditions (Zahran, 1999). *Rhizobia* survival and growth is often compromised by temperatures above optimum, hence the need for a protective and survival mechanism against these hostile conditions. Osmotic stress induces the expression of NaCl-responsive loci, which slows down metabolism. This results in accumulation of amino acids and other carbon sources such as glycine betaine (GB) and glycogen that help to protect the cell from adverse effects of salts. Furthermore, osmotic stress upregulates expression of some genes like *IbpA* (Domínguez-Ferreras et al., 2006). Acid shock is regulated by a hydrogen defense mechanism located on the cell membrane. During acid shock, this mechanism blocks the entry of H\(^+\) into the cell to maintain an alkaline intracellular pH when the external pH declines from 7.2 to 5.6 (O’hara et al., 1989b). However, there are some genes such as *hsp31* which are directly associated with acid resistance (Mujacic & Baneyx, 2007). The changes due to stress may be monitored through absorbance measurements on bacterial growth. In order to achieve effective nodulation and efficient nitrogen fixation process, *Rhizobia* should be able to adapt and tolerate stressful environmental soil conditions such as elevated temperatures, acidity and increased salt concentration.

Bacterial exposure to sudden adverse environmental conditions often triggers a heat-shock response (Roncarati & Scarlato, 2017). Heat shock response is a repair mechanism employed by stressed cells to protect cell proteins resulting in rapid production of heat shock proteins (Tulin et al., 2003). Exogenous heat stress proteins can be recombinantly expressed in *Rhizobium* to improve stress tolerance. Expression of the introduced genes
encoding heat stress proteins may be evaluated using heat shock response thereby confirming their function as heat stress proteins. Heat shock response through production of heat shock proteins is key to tolerance of bacteria to stress (Roncarati & Scarlato, 2017). Tolerance to various stress factors can only be demonstrated when the bacterium is able to employ a short-term heat shock response. This response is controlled by a heat shock factor (Hsf) which forms a complex that initiates transcription when relocated to the site of the target gene (Tulin et al., 2003). Heat shock at 40 °C was used to attest the heat shock response of Rhizobia bacteria overexpressing putative heat stress proteins. The promoter tauA is specific for Rhizobium bacteria and is inducible by the addition of taurine which was added to switch on the promoter during heat shock. The improved heat shock response results in improved bacterial growth and confirms that stress proteins are being synthesized and are functional.

In order to assess the stress tolerance of transconjugated Rhizobia transformants, bacteria were grown under heat, acid and salt stress conditions. The optical density of the cultures of Rhizobia transformants grown in TY broth supplemented with tetracycline and rifampicin is indicative of their stress tolerance.

Nitrogen fixation potential is defined as the ability of Rhizobia to nodulate and provide the plant with usable forms of nitrogen mostly derived from atmospheric N. The BNF is required for increased plant growth and grain yield without use of inorganic fertilizer. In addition to measuring improved stress tolerance through improved bacterial growth, nitrogen fixation potential of Rhizobium transformants was evaluated both under stress and optimum growth conditions. The trials were performed to evaluate whether additional copies of heat stress genes would improve nitrogen fixation under stressful conditions. The N fixation assessment was based on plant growth and other parameters such as number of nodules, root dry weight, shoot dry mass, nodule dry mass and nodule score.

5.2 Aims

1. To assess whether heat stress genes introduced in Rhizobium transformants are expressing.
2. To evaluate the stress tolerance of Rhizobia transformants to heat, acid and salt stress.
3. To evaluate nitrogen fixation potential of stress tolerant Rhizobia transformants in soybean.
5.3 Results

5.3.1 Pilot study for selection of soybean cultivar and temperature for stress tolerance evaluation.

5.3.1.1 Selection of the soybean cultivar for inoculation experiments

Nodulation of the only two soybean cultivars available in Europe namely Edamame and Black Jet was conducted in order to assess their ability to be nodulated with *Rhizobium* strain HH103 at optimum temperature (28°C). The number of nodules per plant after harvesting soybean plants grown for 4 weeks was counted (Fig. 5.1). The results indicate that there were no significant differences between the two cultivars (Table 5.1). The non-significant result might be due to the small sample size since the total number of nodules in Black Jet was more than in Edamame. Therefore, Black Jet was selected as the cultivar to be used for the evaluation of *Rhizobium* transformants due to increased nodulation efficiency.

**Table 5.1:** ANOVA table for means of nodule number as a source of variation for each cultivar.

<table>
<thead>
<tr>
<th></th>
<th>Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Groups</td>
<td>748.167</td>
<td>1</td>
<td>748.167</td>
<td>2.564</td>
<td>.185</td>
</tr>
<tr>
<td>Within Groups</td>
<td>1167.333</td>
<td>4</td>
<td>291.833</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1915.500</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*df = degree of freedom, Sig. difference p < 0.05*
Figure 5.1: Quantification of nodulation of different soybeans cultivar’s grown at 28°C, inoculated with *Rhizobium* strain HH103. The data correspond to the mean and while the bars are standard error values of three independent biological replicates (n=3) per treatment. No statistical differences at p< 0.05 was observed using ANOVA, Turkey HSD.

5.3.1.2 Evaluation of soybean nodulation at elevated temperature

Nodulation of Black Jet, a soybean cultivar selected for conducting stress tolerance tests was evaluated at 28°C and 32°C in order to establish the symbiotic performance of this cultivar at elevated temperature when inoculated with a heat susceptible *Rhizobium* strain HH103. The number of nodules formed on the roots of each plant was quantified after harvesting the plants grown at 28°C and 32°C for 4 weeks in a growth chamber (Fig. 5.2). The results indicate that an average of 80 nodules formed at 28°C and this was significantly greater than the average 39 nodules that formed at 32°C (Table. 5.2). The results show that the elevated temperature of 32°C was high enough to challenge the *Rhizobium* and result in low nodule formation.
Table 5.2: ANOVA table for means of nodule number as a source of variation for each temperature

<table>
<thead>
<tr>
<th>Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
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</tr>
<tr>
<td>Total</td>
<td>3184.000</td>
<td>5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

df = degree of freedom, Sig. difference p < 0.05

Figure 5.2: Nodulation of Black Jet inoculated with *Rhizobium* strain HH103 growing at 28°C and 32°C. The data correspond to the mean and while the bars are standard error values of three independent biological replicates (n=3) per treatment. The different letters represent statistically significant differences at p < 0.05 using ANOVA, Turkey HSD.
5.3.2 Growth of pLMB51-containing *Rhizobium* transformants under optimum growth condition

Phenotypic gene expression was assessed in order to establish the impact of the genes introduced into the HH103 *Rhizobium* strain under optimum growth conditions. The optical density of *Rhizobium* cultures grown at 28 °C were measured after 72 hrs (Fig. 5.3). The results show that there were significant differences in optical density of the *Rhizobium* transformants (Table. 5.3). The results indicate that cell growth of the wild type (WT) and the empty vector (pLMB51) were significantly greater than UC1, hsp20, UC2, shsp, IbpA and UC3 *Rhizobia* transformants. However, the WT did not differ from the empty vector (pLMB51). Among the transformants, IbpA produced the greatest growth followed by hsp20 then UC3 and finally shsp, UC1 and UC2 which did not significantly differ (Fig. 5.3). The results show that the presence of the empty vector (pLMB51) plasmid alone does not affect the cell functions, but over-expression of the other genes did reduce growth under low stress conditions.

**Table 5.3:** ANOVA table for means of Optical Density (600 nm) as a source of variation for each transformant of the rhizobium strain.

<table>
<thead>
<tr>
<th></th>
<th>Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>F</th>
<th>Sig.</th>
</tr>
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</tr>
<tr>
<td>Within Groups</td>
<td>0.589</td>
<td>40</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>18.392</td>
<td>47</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

df = degree of freedom, Sig. difference p < 0.05
Figure 5.3: Growth of WT Rhizobium strain HH103, empty vector transconjugant (pLMB51) and Rhizobium transformants with the different recombinant plasmids under optimum growth conditions (28°C). The data correspond to the mean and while the bars are standard error values of six independent biological replicates (n=6) per treatment. The different letters represent statistically significant differences at p< 0.05 using ANOVA, Turkey HSD.
5.3.3 Evaluation of modified transformants for stress tolerance

5.3.3.1 Tolerance of pLMB51 Rhizobium transformants to heat stress

Optical density as an indicator of bacterial growth was measured following growth for 72 hrs at 40 °C to establish tolerance of HH103 Rhizobium transformants to heat stress (Fig. 5.4). The results show that cell growth at 40 °C was much lower than at 28 °C, hence there was a stress imposed (Table 5.4). Furthermore, unlike the significant reduction in IbpA, UC3 and UC1 at 28 °C, the WT did not differ from IbpA, UC3 and UC1. In addition, the results indicate that the cell growth of shsp, UC2 and hsp20 were significantly greater than both the WT and the empty vector (pLMB51). The transformant expressing shsp had the highest optical density followed by UC2 and then hsp20. The optical density of the empty vector (pLMB51) did not differ from IbpA, UC3 and UC1. The results show that hsp20, UC2 and shsp are heat stress tolerant transformants as they can survive and maintain relatively high cell numbers at 40°C (Fig. 5.4). Therefore, the elevated temperature of 40°C was detrimental to growth compared to growth at 28°C (Fig. 5.3). The experiment identified shsp, as the most tolerant transformant since it exhibits less reduction in cell growth at elevated temperatures.

Table 5.4: ANOVA table for means of Optical Density (600 nm) as a source of variation for each rhizobium transformant.

<table>
<thead>
<tr>
<th></th>
<th>Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Groups</td>
<td>5.154</td>
<td>7</td>
<td>0.736</td>
<td>836.714</td>
<td>0.000</td>
</tr>
<tr>
<td>Within Groups</td>
<td>0.035</td>
<td>40</td>
<td>0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>5.189</td>
<td>47</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

df = degree of freedom, Sig. difference p < 0.05
Figure 5.4: Growth of wild type *Rhizobium* strain HH103, empty vector transconjugant (pLMB51) and *Rhizobium* transformants with the different recombinant plasmid growing under heat stress at 40 °C. A dilution factor of 0.2 was used to measure the Optical Density of the out of range samples. The data correspond to the mean and while the bars are standard error values of six independent biological replicates (n=6) per treatment. The different letters represent statistically significant differences at p< 0.05 using ANOVA, Turkey HSD.

5.3.3.2 Tolerance of pLMB51 *Rhizobium* transformants to acid stress
Optical density was measured in response to acid stress to establish tolerance of the HH103 *Rhizobium* transformants to acid stress. The density of cultures grown at 28 °C in TY broth of pH 4.5 was measured after 72 hrs (Fig. 5.5) and show significant differences (Table 5.5). Unexpectedly the empty vector (pLMB51) transformants displayed the most tolerance under acidic conditions. As a result, the empty vector (pLMB51) had a greater cell density than *UC1, hsp20, UC2, shsp, IbpA* and *UC3* However, the expression of transgenes reduced this tolerance but *hsp20, shsp* and *UC1* were all better than the WT. Consequently, all of them produced more cells than *UC2, UC3* and *IbpA*. The results show that *UC1, hsp20* and *shsp* are acid stress tolerant transformants as they can survive and
maintain high cell numbers at pH 4.5 (Fig. 5.5). However, acidity affected growth of all the *Rhizobia* transformants except pLMB51 as the number of cells at this pH were much lower than at pH 6.8 (Fig. 5.3).

**Table 5.5:** ANOVA table for means of Optical Density (600 nm) as a source of variation for each rhizobium transformant.

<table>
<thead>
<tr>
<th></th>
<th>Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Groups</td>
<td>88.355</td>
<td>7</td>
<td>12.622</td>
<td>1224.308</td>
<td>0.000</td>
</tr>
<tr>
<td>Within Groups</td>
<td>0.412</td>
<td>40</td>
<td>0.010</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>88.767</td>
<td>47</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

df = degree of freedom, Sig. difference p < 0.05

**Figure 5.5:** Growth of wild type *Rhizobium* strain HH103, empty vector transconjugant (pLMB51) and *Rhizobium* transformants with the different recombinant plasmid growing under acid stress at pH 4.5. The data correspond to the mean and while the bars are standard error values of six independent biological replicates (n=6) per treatment. The different letters represent statistically significant differences at p< 0.05 using ANOVA, Turkey HSD.
5.3.3.3 Tolerance of pLMB51 *Rhizobium* transformants to salt stress

To establish the tolerance of the HH103 *Rhizobium* transformants to salt stress, the cell density of cultures grown in 1% NaCl TY broth at 28 °C was measured after 72 hrs (Fig. 5.6). As already observed, there were significant differences under acidic conditions (Table 5.6), the empty vector (pLMB51) displayed the best performance under salt stress. The results indicate that the cell growth of the empty vector (pLMB51) was significantly greater than *IbpA* and *UC3*. However, the expression of the transgenes reduced the tolerance compared to the empty vector but *UC1*, *hsp20*, *UC2* and *shsp* still performed better than WT. On the other hand, the optical density of *UC1* was significantly greater than *hsp20* and *shsp* but did not differ from *UC2*. The *UC2* transformant showed greater growth than *shsp* but did not differ from *hsp20* which did not differ from *shsp*. The result shows that *UC1*, *hsp20*, *UC2* and *shsp* are tolerant to salt stress as they can survive and maintain high optical density in 1% NaCl (Fig. 5.6). However, the salt generally affected growth of all the transformants as the optical density in 1% NaCl was much lower than at 0.05% NaCl (Fig. 5.3).

**Table 5.6**: ANOVA table for means of Optical Density (600 nm) as a source of variation for each rhizobium transformant.

<table>
<thead>
<tr>
<th>Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Groups</td>
<td>70.147</td>
<td>7</td>
<td>10.021</td>
<td>673.980</td>
</tr>
<tr>
<td>Within Groups</td>
<td>0.595</td>
<td>40</td>
<td>0.015</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>70.741</td>
<td>47</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*df = degree of freedom, Sig. difference p < 0.05*
Figure 5.6: Growth of wild type *Rhizobium* strain HH103, empty vector transconjugant (pLMB51) and *Rhizobium* transformants with the different recombinant plasmid growing under salt stress in 1%NaCl. The data correspond to the mean and while the bars are standard error values of six independent biological replicates (n=6) per treatment. The different letters represent statistically significant differences at p< 0.05 using ANOVA, Turkey HSD.

5.3.4 Analysis of Nitrogen fixation potential of pLMB51 *Rhizobium* transformants.

5.3.4.1 Nitrogen fixation potential of pLMB51 *Rhizobium* transformants at low temperature

The impact of stress genes cloned into the HH103 *Rhizobium* strain on biological nitrogen fixation was evaluated through nodulation to promote plant growth at low temperature conditions. Black Jet soybean cultivar inoculated with WT, empty vector (pLMB51) transconjugant and the different recombinant plasmids were grown in a greenhouse under unstressed conditions at 20°C. The WT strain (HH103) and empty vector
(pLMB51) transformants were used as negative and positive controls. The results indicate that there are significant differences between the pLMB51 transformants and the controls on nodule number (NN), nodule score (NS), shoot dry mass (SDM) and root dry mass (RDM) (Table 5.7 and 5.8). The NS for WT was significantly greater than the empty vector (pLMB51) and the different recombinant plasmid except hsp20. However, NS for the different recombinant plasmid do not differ. This result agrees with the visual assessment on plant vigor which shows that soybean plants inoculated with the WT were much taller and more vigorous than the empty vector (pLMB51) transconjugant and the different recombinant plasmids (Fig 5.7 and 5.8). There was a pronounced reduction in NN of the transformants compared to WT despite statistical non-significance in NN. However, there were no significant differences in SDM except UC1 and UC2 and RDM between WT and the transformants despite the observed significance on NS. This result means that plant growth may be limited as the Rhizobium are expressing these genes since it requires energy to maintain them. In this case, the soybean plants with minimal supply of nutrients is the only source of nutrient as they were grown in sand.

Table 5.7: ANOVA table for means of nodule Score, Nodule number, Shoot Dry Mass and Root Dry Mass as a source of variation for each strain.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nodule Score</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between Groups</td>
<td>17.917</td>
<td>7</td>
<td>2.560</td>
<td>5.119</td>
<td>0.000</td>
</tr>
<tr>
<td>Within Groups</td>
<td>20.000</td>
<td>40</td>
<td>0.500</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>37.917</td>
<td>47</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nodule Number</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between Groups</td>
<td>11618.479</td>
<td>7</td>
<td>1659.783</td>
<td>2.280</td>
<td>0.047</td>
</tr>
<tr>
<td>Within Groups</td>
<td>29121.500</td>
<td>40</td>
<td>728.037</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>40739.979</td>
<td>47</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shoot Dry Mass</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between Groups</td>
<td>0.617</td>
<td>7</td>
<td>0.088</td>
<td>4.017</td>
<td>0.002</td>
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<tr>
<td>Within Groups</td>
<td>0.878</td>
<td>40</td>
<td>0.022</td>
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<tr>
<td>Total</td>
<td>1.495</td>
<td>47</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Root Dry Mass</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between Groups</td>
<td>0.033</td>
<td>7</td>
<td>0.005</td>
<td>0.505</td>
<td>0.825</td>
</tr>
<tr>
<td>Within Groups</td>
<td>0.368</td>
<td>40</td>
<td>0.009</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0.401</td>
<td>47</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

df = degree of freedom, Sig. difference p < 0.05
Table 5.8: Growth parameters of soybean inoculated with HH103 transformants and grown at 20°C for 4 weeks

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nodule score</th>
<th>Nodule number</th>
<th>Shoot Dry Mass (g/plant)</th>
<th>Root Dry Mass (g/plant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>5.500&lt;sup&gt;a&lt;/sup&gt;</td>
<td>92.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.168&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.427&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>pLMB51</td>
<td>3.667&lt;sup&gt;b&lt;/sup&gt;</td>
<td>46.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.907&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.423&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>UC1</td>
<td>3.833&lt;sup&gt;b&lt;/sup&gt;</td>
<td>46.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.823&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.433&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>hsp20</td>
<td>4.000&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>45.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.987&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.420&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>UC2</td>
<td>3.833&lt;sup&gt;b&lt;/sup&gt;</td>
<td>46.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.780&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.413&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>shsp</td>
<td>3.833&lt;sup&gt;b&lt;/sup&gt;</td>
<td>54.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.932&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.497&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>IbpA</td>
<td>3.667&lt;sup&gt;b&lt;/sup&gt;</td>
<td>42.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.002&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.448&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>UC3</td>
<td>3.333&lt;sup&gt;b&lt;/sup&gt;</td>
<td>61.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.872&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.412&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mean</td>
<td>3.979</td>
<td>54.5</td>
<td>0.942</td>
<td>0.434</td>
</tr>
<tr>
<td>p&lt;0.05</td>
<td>0.000</td>
<td>0.047</td>
<td>0.002</td>
<td>0.825</td>
</tr>
<tr>
<td>SE</td>
<td>0.295</td>
<td>11.00</td>
<td>0.063</td>
<td>0.039</td>
</tr>
</tbody>
</table>

Means with the same letter (s) for each parameter, in each column are not significantly different at p<0.05 using ANOVA, Turkey HSD and n-6 plants.
Figure 5.7: Growth characteristics of soybean plants inoculated with different transformants of *Rhizobium* and grown at 20°C. (a) inoculated with wild type, pLMB51 and *UC1*, (b) inoculated with wild type, pLMB51 and *hsp20* and (c) inoculated with wild type, pLMB51 and *UC2* transformants.
Figure 5.8: Growth characteristics of soybean plants inoculated with different transformants of *Rhizobium* and grown at 20°C. (a) inoculated with wild type, pLMB51 and *shsp*, (b) inoculated with wild type, pLMB51 and *lbpA* and (c) inoculated with wild type, pLMB51 and *UC3* transformants.
5.3.4.2 Nitrogen fixation potential of pLMB51 *Rhizobium* transformants under heat stress

In order to evaluate the effect of the stress gene introduced into HH103 *Rhizobium* strain on symbiotic Nitrogen fixation under heat stress, a plant assay was performed to compare growth and nodulation in plants inoculated with the transformants and the wild-type strain. Soybean cultivar Black Jet was inoculated with the different transformants of HH103 and grown in a growth chamber at 32°C. The negative and positive controls for this experiment included WT strain and the empty vector (pLMB51). A comparison of plant growth parameters of soybeans inoculated with WT grown at 32°C to that at 20°C indicate that there was a reduction in all parameters under stress. The nodule score (NS) and root dry mass (RDM) at 32°C indicated that there were no significant differences between different recombinant plasmids and the controls (Table 5.9 and 5.10). However, results on nodule number (NN) and Shoot dry mass (SDM) show an increase compared to the WT though not significant, but *IbpA* had less nodules than the rest but do not differ from WT. However, visual observations on plant vigor and leaf color indicated that soybean plants inoculated with *UC1, hsp20, UC2* and *shsp* recombinant plasmids were more vigorous and had much greener leaves in comparison to the controls (Fig 5.9 and 5.10). The non-significant differences observed on the evaluated symbiotic parameters may be due to the small sample size, hence need more replication.

Comparing results of symbiotic parameters measured at 32°C and 20°C confirm that the recombinant plasmids can improve the capacity to withstand heat stress (Table 5.12). At 32°C, the NN, NS, SDM and RDM in WT reduced by 67%, 40%, 42% and 2% respectively. This result demonstrates that elevated temperature has a big impact on nodulation and presumably on nitrogen fixation. In contrast to the WT, the results for recombinant plasmids under stress indicate smaller reductions and in some cases an increase of some parameters. The maximum reduction was 46.18% for NN, 16.67% for NS and 19.26% for SDM. However, SDM of *UC1* and *UC2* increased by 5.1 and 12.8% respectively and there was an increase in RDM in all the transformants except *shsp* which reduced by only 6%.

The results for WT show that nitrogen fixation was presumably affected by the temperature increase since the NN and SDM of the WT were significantly greater at 20°C than at 32°C. Furthermore, the SDM and NN for *IbpA* and *UC3* respectively were significantly greater at 20°C than at 32°C. Other than the *hsp20* transformant which show an increase in RDM at 32°C than at 20°C, there was no significant difference on NN, NS,
SDM and RDM of all the recombinant plasmids for the two temperatures (Table 5.12). This suggests that increased temperature had neutral or positive effect on all the recombinant plasmids except IbpA and UC3. These results confirm the stress tolerance to heat stress conferred by expression of heat stress genes.

Table 5.9: ANOVA table for means of nodule Score, Nodule number, Shoot Dry Mass and Root Dry Mass as a source of variation for each strain.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nodule Score</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between Groups</td>
<td>0.544</td>
<td>7</td>
<td>0.078</td>
<td>0.443</td>
<td>0.869</td>
</tr>
<tr>
<td>Within Groups</td>
<td>7.009</td>
<td>40</td>
<td>0.175</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>7.553</td>
<td>47</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nodule Number</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between Groups</td>
<td>3388.444</td>
<td>7</td>
<td>484.063</td>
<td>10.949</td>
<td>0.000</td>
</tr>
<tr>
<td>Within Groups</td>
<td>1768.431</td>
<td>40</td>
<td>44.211</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>5156.874</td>
<td>47</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shoot Dry Mass</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between Groups</td>
<td>0.264</td>
<td>7</td>
<td>0.038</td>
<td>2.171</td>
<td>0.058</td>
</tr>
<tr>
<td>Within Groups</td>
<td>0.696</td>
<td>40</td>
<td>0.017</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0.961</td>
<td>47</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Root Dry Mass</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between Groups</td>
<td>0.160</td>
<td>7</td>
<td>0.023</td>
<td>2.823</td>
<td>0.017</td>
</tr>
<tr>
<td>Within Groups</td>
<td>0.324</td>
<td>40</td>
<td>0.008</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0.484</td>
<td>47</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

df = degree of freedom, Sig. difference p < 0.05
Table 5.10: Growth and nodule parameters of soybean inoculated with HH103 transformants grown at 32°C for 4 weeks

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nodule score</th>
<th>Nodule number</th>
<th>Shoot Dry Mass (g/plant)</th>
<th>Root Dry Mass (g/plant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>3.306&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30.528&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.671&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.417&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>pLMB51</td>
<td>3.333&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40.167&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.829&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.439&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>UC1</td>
<td>3.333&lt;sup&gt;a&lt;/sup&gt;</td>
<td>44.278&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.837&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.477&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>hsp20</td>
<td>3.333&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32.389&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.883&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.619&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>UC2</td>
<td>3.333&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39.528&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.880&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.476&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>shsp</td>
<td>3.583&lt;sup&gt;a&lt;/sup&gt;</td>
<td>42.694&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.859&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.467&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>IbpA</td>
<td>3.167&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.528&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.813&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.468&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>UC3</td>
<td>3.333&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32.917&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.704&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.520&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mean</td>
<td>3.340</td>
<td>34.878</td>
<td>0.807</td>
<td>0.485</td>
</tr>
<tr>
<td>p&lt;0.05</td>
<td>0.869</td>
<td>0.000</td>
<td>0.058</td>
<td>0.017</td>
</tr>
<tr>
<td>SE</td>
<td>0.171</td>
<td>2.714</td>
<td>0.054</td>
<td>0.037</td>
</tr>
</tbody>
</table>

Means with the same letter(s) for each parameter, in each column are not significantly different at p<0.05 using ANOVA, Turkey HSD and n-6 plants.
Table 5.11: Table for testing the effect of temperature using means of nodule Score, Nodule number, Shoot Dry Mass and Root Dry Mass as a source of variation for each *Rhizobium* transformant.

<table>
<thead>
<tr>
<th>Source</th>
<th>Dependent Variable</th>
<th>Type III Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corrected Model</td>
<td>Nod Score</td>
<td>27.628(^a)</td>
<td>15</td>
<td>1.842</td>
<td>5.456</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>Nodule Number</td>
<td>24227.416(^b)</td>
<td>15</td>
<td>1.615.161</td>
<td>4.183</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>Shoot Dry Mass</td>
<td>1.252(^c)</td>
<td>15</td>
<td>0.083</td>
<td>4.242</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>Root Dry Mass</td>
<td>0.255(^d)</td>
<td>15</td>
<td>0.017</td>
<td>1.969</td>
<td>0.028</td>
</tr>
<tr>
<td>Intercept</td>
<td>Nod Score</td>
<td>1278.473</td>
<td>1</td>
<td>1278.473</td>
<td>3786.771</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>Nodule Number</td>
<td>191634.903</td>
<td>1</td>
<td>191634.903</td>
<td>496.304</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>Shoot Dry Mass</td>
<td>72.938</td>
<td>1</td>
<td>72.938</td>
<td>3707.170</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>Root Dry Mass</td>
<td>20.295</td>
<td>1</td>
<td>20.295</td>
<td>2345.984</td>
<td>0.000</td>
</tr>
<tr>
<td>Strain</td>
<td>Nod Score</td>
<td>9.078</td>
<td>7</td>
<td>1.297</td>
<td>3.841</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Nodule Number</td>
<td>6958.284</td>
<td>7</td>
<td>994.041</td>
<td>2.574</td>
<td>0.019</td>
</tr>
<tr>
<td></td>
<td>Shoot Dry Mass</td>
<td>0.224</td>
<td>7</td>
<td>0.032</td>
<td>1.623</td>
<td>0.141</td>
</tr>
<tr>
<td></td>
<td>Root Dry Mass</td>
<td>0.079</td>
<td>7</td>
<td>0.011</td>
<td>1.304</td>
<td>0.259</td>
</tr>
<tr>
<td>Temperature</td>
<td>Nod Score</td>
<td>9.168</td>
<td>1</td>
<td>9.168</td>
<td>27.155</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>Nodule Number</td>
<td>9220.493</td>
<td>1</td>
<td>9220.493</td>
<td>23.880</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>Shoot Dry Mass</td>
<td>0.370</td>
<td>1</td>
<td>0.370</td>
<td>18.817</td>
<td>0.000</td>
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<tr>
<td></td>
<td>Root Dry Mass</td>
<td>0.063</td>
<td>1</td>
<td>0.063</td>
<td>7.287</td>
<td>0.008</td>
</tr>
<tr>
<td>Strain * Temp</td>
<td>Nod Score</td>
<td>9.383</td>
<td>7</td>
<td>1.340</td>
<td>3.970</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Nodule Number</td>
<td>8048.639</td>
<td>7</td>
<td>1149.806</td>
<td>2.978</td>
<td>0.008</td>
</tr>
<tr>
<td></td>
<td>Shoot Dry Mass</td>
<td>0.658</td>
<td>7</td>
<td>0.094</td>
<td>4.778</td>
<td>0.000</td>
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<tr>
<td></td>
<td>Root Dry Mass</td>
<td>0.113</td>
<td>7</td>
<td>0.016</td>
<td>1.873</td>
<td>0.085</td>
</tr>
<tr>
<td>Error</td>
<td>Nod Score</td>
<td>27.009</td>
<td>80</td>
<td>0.338</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nodule Number</td>
<td>30889.931</td>
<td>80</td>
<td>386.124</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Shoot Dry Mass</td>
<td>1.574</td>
<td>80</td>
<td>0.020</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Root Dry Mass</td>
<td>0.692</td>
<td>80</td>
<td>0.009</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>Nod Score</td>
<td>1333.111</td>
<td>96</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nodule Number</td>
<td>246752.250</td>
<td>96</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Shoot Dry Mass</td>
<td>75.764</td>
<td>96</td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Root Dry Mass</td>
<td>21.243</td>
<td>96</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected Total</td>
<td>Nod Score</td>
<td>54.638</td>
<td>95</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nodule Number</td>
<td>55117.347</td>
<td>95</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Shoot Dry Mass</td>
<td>2.826</td>
<td>95</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Root Dry Mass</td>
<td>0.948</td>
<td>95</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(df = \text{degree of freedom}, \text{Sig. difference } p < 0.05\)

a. R Squared = 0.506 (Adjusted R Squared =0.413), b. R Squared = 0.440 (Adjusted R Squared = 0.334)
c. R Squared = 0.443 (Adjusted R Squared = 0.339), d. R Squared = 0.270 (Adjusted R Squared = 0.133),
Table 5.12: The effect of heat stress on symbiotic nitrogen fixation parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Temperature (°C)</th>
<th>Wild Type</th>
<th>pLMB51</th>
<th>UC1</th>
<th>hsp20</th>
<th>UC2</th>
<th>shsp</th>
<th>lbpA</th>
<th>UC3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nodule number</td>
<td>20</td>
<td>92.833a</td>
<td>46.500a</td>
<td>46.833a</td>
<td>45.000a</td>
<td>46.167a</td>
<td>54.667a</td>
<td>42.667a</td>
<td>61.167a</td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>30.528b</td>
<td>40.167a</td>
<td>42.444a</td>
<td>32.389a</td>
<td>39.528a</td>
<td>42.694a</td>
<td>16.528a</td>
<td>32.917b</td>
</tr>
<tr>
<td>% difference</td>
<td>67.11</td>
<td>13.61</td>
<td>9.37</td>
<td>28.02</td>
<td>14.38</td>
<td>21.90</td>
<td>61.26</td>
<td>46.18</td>
<td></td>
</tr>
<tr>
<td>p&lt;0.05</td>
<td>0.000</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Nodule score</td>
<td>20</td>
<td>5.500a</td>
<td>3.667a</td>
<td>3.833a</td>
<td>4.000a</td>
<td>3.833a</td>
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<td>3.333a</td>
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<tr>
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<td>32</td>
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<td>3.333a</td>
<td>3.333a</td>
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<td>3.333a</td>
<td>3.583a</td>
<td>3.167a</td>
<td>3.333a</td>
</tr>
<tr>
<td>% difference</td>
<td>39.89</td>
<td>9.10</td>
<td>13.04</td>
<td>16.67</td>
<td>13.04</td>
<td>6.52</td>
<td>13.63</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>p&lt;0.05</td>
<td>0.000</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Shoot Dry Mass</td>
<td>20</td>
<td>1.168a</td>
<td>0.907a</td>
<td>0.823a</td>
<td>0.987a</td>
<td>0.780a</td>
<td>0.932a</td>
<td>1.002a</td>
<td>0.872a</td>
</tr>
<tr>
<td>(g/plant)</td>
<td>32</td>
<td>0.671b</td>
<td>0.829a</td>
<td>0.865a</td>
<td>0.883a</td>
<td>0.880a</td>
<td>0.859a</td>
<td>0.813b</td>
<td>0.704a</td>
</tr>
<tr>
<td>% difference</td>
<td>42.55</td>
<td>8.59</td>
<td>5.10</td>
<td>10.53</td>
<td>12.82</td>
<td>7.83</td>
<td>18.86</td>
<td>19.26</td>
<td></td>
</tr>
<tr>
<td>p&lt;0.05</td>
<td>0.000</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.038</td>
<td></td>
</tr>
<tr>
<td>Root Dry Mass</td>
<td>20</td>
<td>0.427a</td>
<td>0.423a</td>
<td>0.433a</td>
<td>0.420b</td>
<td>0.413a</td>
<td>0.497a</td>
<td>0.448a</td>
<td>0.412a</td>
</tr>
<tr>
<td>(g/plant)</td>
<td>32</td>
<td>0.418a</td>
<td>0.439a</td>
<td>0.455a</td>
<td>0.619a</td>
<td>0.476a</td>
<td>0.467a</td>
<td>0.468a</td>
<td>0.520a</td>
</tr>
<tr>
<td>% difference</td>
<td>2.10</td>
<td>3.78</td>
<td>5.08</td>
<td>47.38</td>
<td>15.25</td>
<td>6.03</td>
<td>4.46</td>
<td>26.21</td>
<td></td>
</tr>
<tr>
<td>p&lt;0.05</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.000</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Means with the same letter (s) for each parameter, in each column are not significantly different at p<0.05.
Figure 5.9: Growth characteristics of soybean plants inoculated with different transformants of *Rhizobium* and grown under stress at 32°C. (a) inoculated with wild type, pLMB51 and *UC1*, (b) inoculated with wild type, pLMB51 and *hsp20* and (c) inoculated with wild type, pLMB51 and *UC2* transformants
Figure 5.10: Growth characteristics of soybean plants inoculated with different transformants of *Rhizobium* and grown under stress at 32°C. (a) inoculated with wild type, pLMB51 and *shsp*, (b) inoculated with wild type, pLMB51 and *IbpA* and (c) inoculated with wild type, pLMB51 and *UC3* transformants.
5.4 Summary

Firstly, this chapter successfully demonstrated the impact of the introduced genes on *Rhizobium* growth. Based on the phenotypic change in the results shown, it can be assumed that the introduced genes were being expressed under optimum growth conditions. The results show that all the genes impose a penalty under non-stressed conditions, since the *Rhizobium* transformants having recombinant plasmid grew slower than the WT and positive control (pLMB51). Based on the NN/NS/SDM result, an assumption can be made indirectly that the effect of these genes on *Rhizobia* growth under optimum conditions may also have an impact on BNF and finally result in reduced plant growth.

Secondly, this chapter determined the tolerance of the transformants to stress conditions such as elevated temperatures (40°C), acidity (pH4.5) and salt (1% NaCl) by measuring the growth of HH103 transformants under stressful growth conditions. Among the targeted genes, *hsp20*, *UC2* and *shsp* were identified as heat stress tolerant genes while expression of *UC1*, *hsp20*, *UC2*, and *shsp* improved acid and salt stress tolerance.

Thirdly, this research evaluated the ability of the *Rhizobium* transformants to nodulate their specific legume host and promote plant growth at low temperature. All the transformants were able to infect and produce nodules on the roots of soybean plants. Nodule measurements, NN and NS, indicated that nodulation was much better at low than at high temperature. At low temperature, most of the genes negatively affected plant growth as shown by reduced plant growth. However, assessment of plant symbiotic parameters identified *hsp20* and *UC3* as transformants that can maintain plant growth in comparison to the WT strain.

Fourthly, the effect of introduced stress genes on symbiotic Nitrogen fixation was determined under heat stress conditions. At high temperature of 32°C, all the transformants were able to effectively nodulate soybeans just like the WT strain and pLMB51. However, visual assessment on plant vigor showed that some transformants were able to effectively increase plant growth at elevated temperatures despite non-significant differences on SDW and RDW. Therefore, *hsp20*, *UC2* and *shsp* transformants were identified as transformants that can improve nitrogen fixation under stressful conditions as demonstrated by vigorous plant growth and effective nodulation.
5.5 Discussion

In this research, I have assessed stress tolerance of Rhizobia transformants generated using pLMB51 plasmids constructs to introduce stress genes. The reduction in growth of the Rhizobium with recombinant plasmids under non-stressed conditions indicate that all the genes were expressed. This means that the plasmid used, pLMB51 can easily be integrated into the Rhizobium cell without affecting the cell function. Furthermore, the introduced genes were readily expressed by the cells since the genes were sourced from another Rhizobium strain. The variability in amount of fitness penalty paid due to gene expression from the different recombinant plasmids may be due to different functions of the specific genes in Rhizobium. The same gene may have different functions in different organisms due to difference in promoters (Alexandre et al., 2014). The fitness penalty from gene expression on growth of Rhizobium with recombinant plasmids displayed by these transformants at optimum growth temperature may also mean that the genes may function better either at low or high temperature.

The experiment on heat stress unveiled some genes that can induce tolerance of Rhizobium to elevated temperatures. The high temperature of 40°C used to evaluate these transformants effectively confirmed that hsp20, UC2 and shsp transformants were heat stress tolerant. This result is not surprising as hsp20 and shsp are responsible for heat stress response in E. coli. Khaskheli et al. (2015) used hsp20 a small heat shock protein to induce heat and salt tolerance in Bifidobacterium. Li et al. (2012) used hsp20 gene from the archaeon S. solfataricus P2 to generate E. coli transformants which could withstand thermal stress of 50°C and cold stress of 4°C. However, there is no information regarding the function of uncharacterized protein (UC2) in Rhizobium or any other model organism. Hence there is a need for more research in order to understand the characteristics and functions of this protein. The low bacterial cell growth at a high temperature of 40°C indicate that there was a stress imposed. Research has shown that there are very few strains that are able to survive and grow at temperatures above 40°C. Unlike the significant reduction of IbpA, UC3 and UC1 at 28°C, the non-significance to WT may mean that these genes are not responsible for heat stress in Rhizobium. Domínguez-Ferreras et al., (2006) established that IbpA in E. coli is upregulated under osmotic stress.
Rhizobium bacteria grow well in growth media at pH 6.8 to pH 7 and is unable to grow at pH 4.5. The acidity level of pH 4.5 effectively identified Rhizobium with recombinant plasmids that can tolerate acid stress. The increased tolerance displayed by the empty vector may be due to functional genes present in the pLMB51 plasmid such as the tauA promoter or tetracycline resistant gene. We suspect the promoter gene tauA to be responsible for the acid tolerance since tetracycline resistance gene is known to reduce tolerance to acid in E. coli (Hung et al., 2006). However, Javaux, et al., (2007) suggested that tauA, a binding protein, could serve as taurine catcher in biological fluids since it plays a major role in ABC transport system. Research has shown that small heat shock proteins hsp20 and shsp are also responsible for other stress factors such as such acid and salt stress in E. coli and other model bacterial species. Hassan et al., (2003) found that Streptococcus thermophilus cured from the shsp plasmids were less resistant to heat and acids. Furthermore, the difference in cell growth between the empty vector (pLMB51) and the acid tolerant Rhizobium with recombinant plasmids defines the penalty that the Rhizobium pays to maintain the genes. Although these genes are not directly associated with acid stress, hsp31 a member of the same family is known for its role in acid resistance of starved E. coli (Mujacic & Baneyx, 2007). However, currently there is no research related to acid stress tolerance induced by the UC2, uncharacterized protein.

Rhizobium bacteria are not able to grow under saline conditions however, they grow well in 0.05% salt growth media. The salt concentration of 1% effectively identified Rhizobium with recombinant plasmids that can grow under salt stress. The increased tolerance to salt displayed by the empty vector may be due to functional genes present in the pLMB51 plasmid such as the tauA promoter or tetracycline resistant gene. The tetracycline resistance gene is the main suspect conferring tolerance to salts in the Rhizobium transformant with the empty plasmid (pLMB51). According to Hassan et al., (2003) proteins of the TetR family are responsible for controlling transcription of many drug efflux pumps and response to osmotic stress and toxic chemicals. The small heat shock proteins hsp20 and shsp have been associated with other stress factors such acid and salt stress in E. coli as well as in other model species of bacterial. Genome analysis of Bifidobacterium breve UCC2003 demonstrated that hsp20 is involved in management of severe heat shock and by osmotic...
shock (Ventura et al., 2007). However, the uncharacterized proteins UC1 and UC2 have not linked with salts stress in any research, since they have not been characterized.

The major characteristic of *Rhizobium* bacteria is its ability to infect and nodulate host legume plants and produce effective nodules (Gage 2004). However, genetic manipulation of *Rhizobium* genes may result in loss of this ability to nodulate (Wang et al., 2018). However, the results generated in this research show that all transformants were able to nodulate their host, soybean.

Visual assessment on plants grown at 20°C showed that all the transformants except *hsp20* produced less vigorous plants than the WT. This may be due to the fact that *hsp20* is also up-regulated by cold temperatures and has greater thermotolerance in response to temperature shock (Li et al., 2012). Furthermore, plant growth may be limited as the *Rhizobium* are expressing the genes. These reductions are in line with the reduced bacterial growth observed in Fig. 5.1 under the non-stressed condition. Although there is no data on SDM and RDM for non-inoculated plants, we expect significantly lower values in comparison to the inoculated plants. Currently there are no research data showing that UC1 and UC2 uncharacterized proteins are associated with any known stress factors, hence the need for more research on their functions. Generally, nodulation and nitrogen fixation are affected by both low and high temperature (Montañez et al., 1995). According to Schweitzer & Harper (1980) nodule activity decreases with a decrease in temperature. The results on SDM at 20°C show that the nodules formed by the transformant were not active despite being classified as adequate for N fixation as indicated by NS (Howieson & Dilworth, 2016). This means that temperature has an impact on nitrogen fixation as displayed by the difference in NS and plant vigor at low temperature. According to Zhang et al., (1995), a temperature shift from 25 to 15°C may delay or even shut down nitrogen fixation. However, this impact was observed on the transformants rather than the WT and this suggests the presence and expression of the introduced gene.

The results on NN and NS were generally much lower at high temperature than at low temperature, but non-significant difference observed on SDM at 32°C mean that the genes are mitigating against the effect of high temperature. Furthermore, the fact that SDM had higher values at 32°C and lower at 20°C shows that the genes can increase heat stress tolerance. Further evidence on the impact of the stress genes is observed on NN of
transformants at 20°C which was approximately half of the WT, but it was more than the WT at 32°C. The huge reductions in NN, NS, SDM at 32°C confirmed the big impact elevated temperature impose on nodulation and presumably on nitrogen fixation.
6 General Discussion

6.1 Introduction

Soybean is a crop of great economic importance as it is a main source of protein, a source of vegetable oil and can be used for production of animal feed (Dunstan, 2016). Soybeans like any other legume play an important role in integrated nutrient management, a strategy being promoted in agricultural production in developing countries where fertilizer costs often unaffordable (Jonas et al., 2008). Traditionally, inorganic N fertilizers are used to increase productivity of this crop even though fertilizer use is costly and often associated with environmental concerns such as water pollution. Unfortunately nitrogen as nitrate or ammonium is the most expensive nutrient element in the soil as it requires a lot of energy to produce and can easily be lost through leaching with only 20 to 50% of the applied N absorbed by crops (Graham & Vance, 2000). Alternatively, Rhizobia bacteria are used to provide the necessary soil N through symbiotic biological nitrogen fixation (BNF) in order to reduce the cost of production as well as environmental impacts. BNF is the best possible solution since the N produced is utilized in situ and is not susceptible to leaching and the crop residue of the legumes (roots etc.) contribute N to the following crop. Unfortunately, using Rhizobium inoculants to increase soybean production is greatly impacted by climatic variability (Zahran, 1999). Predictions on climate change show that by the year 2050, Sub-Saharan Africa (SSA) where Malawi is located, will experience constantly higher temperatures and fluctuations in rainfall, resulting in reduced crop yields (Ringler et al., 2010). High temperature is reported to impact on BNF and reduce yield by approximately 30% (Onat et al., 2017). Although Rhizobium inoculants have previously been successfully used to increase legume production in Malawi, recently their performance has been negatively affected by challenging climatic environmental conditions. In order to mitigate against this phenomenon, Rhizobium bacteria must be able to survive, adapt and persist under such environments if they are to establish an effective symbiotic nitrogen fixation system. Most Rhizobia research programs that focused on selection of heat tolerant strains were hampered by very low adaptability of the selected Rhizobium strains to new
environments. The use of heat stress tolerant *Rhizobium* strains obtained by random field selection have been unsuccessful in mitigating the impact of high temperature likely because of competition from native *Rhizobium* populations (Rodrigo et al., 2017). The alternative strategy is use of biotechnology for genetic manipulation of heat susceptible *Rhizobium* with heat stress genes sourced from stress tolerant *Rhizobium* strains.

The use of biotechnology to improve agricultural production has increased tremendously over recent years due to increased food demand resulting from rapid population growth. This thesis has demonstrated that the performance of *Rhizobia* bacteria for inoculation of legume crops under challenging environmental conditions can be improved using genetic manipulation. Genetic manipulation allows use of genetic material from indigenous *Rhizobium* strains which are adapted to the specific environment. Furthermore, this technique allows the generation of genetically stable tolerant transformants because the stress genes are carried in plasmids which are maintained as bacteria cells undergo replication.

Specific stress response genes known to mitigate the impacts of various stresses such as increased temperatures, acidity and salinity may be introduced into *Rhizobium*. As a result, the generated *Rhizobia* transformants will be tolerant to specific types of stress upon overexpression of these genes.

Few stress response genes have been used to date in cloning experiments to improve *Rhizobium* performance under various growth conditions. For instance, *groEL* (Cpn60), an ATP dependent chaperone was used in the modification of *Sinorhizobium meliloti* for heat tolerance and nitrogen fixation. Studies have shown that mutants of *Sinorhizobium meliloti* in *groEL1* and *groEL5* grew slower than the wild-type under 40°C heat stress (Bittner et al., 2007). While expressed *groEL1* into *Sinorhizobium meliloti* is reported to improve symbiotic performance of *Rhizobia* (Ogawa & Long, 1995). Furthermore, Rodrigo et al., (2017) established that modification of chickpea *Mesorhizobium* strain ST-2 with additional copies of *groEL* improved symbiotic effectiveness. Paço et al., (2016) used *ClpB* to improve both acid stress tolerance and symbiotic performance of a chickpea microsymbiont, *Mesorhizobium mediterraneum* UPM-Ca36T strain. These results show that effectiveness of symbiotic nitrogen fixation at pH 5 increased by ~60% in comparison to the wild-type strain. Minder et al. (1997) successfully expressed *dnak* in *Bradyrhizobium japonicum* cloned with
However, the researchers observed a reduction in growth rate for the *dnaJ* transformants although the final cell density did not differ from the wild type. Moussaid et al., (2015) found that overexpression of otsA/B in *Mesorhizobium ciceri* (Rch125) respectively improved cell growth and nitrogenase activity in chickpea plants grown in saline media. Boscari et al., (2006) successfully improved nitrogen fixation activity of *Medicago sativa* growing under saline conditions by overexpressing betS in *Sinorhizobium meliloti*.

The focus of this thesis was therefore understanding the phenotypic changes associated with heat stress in order to explore better options to enhance stress tolerance through genetic manipulation of *Rhizobia*, but now that some stress tolerances are broad so might also help with acid and salt stress. In addition, to understand the impact of induced genetic manipulation on symbiotic N fixation and tolerance of *Rhizobium* to harsh soil conditions. This thesis therefore presents a study that addresses four major objectives. The first objective was to sequence the 16S rRNA gene of the *Rhizobium* strains selected according to source of origin for identification and comparative study. The 16S rRNA gene sequence has been used in most studies to identify and differentiate phylogenetically closely related strains of *Rhizobium* bacteria into specific groups (Rajendhran & Gunasekaran, 2011). The second objective was to assess relative stress tolerance of the selected *Rhizobium* strains to heat, acid and salt stress on survival and growth of *Rhizobium*. According to Alexandre & Oliveira (2013), evaluation of *Rhizobium* tolerance to temperature stress in culture medium has been used as a rapid way to select the most tolerant strains. The third objective was to identify candidate genes and manipulate susceptible *Rhizobium* strain with presumed heat stress genes from the heat tolerant *Rhizobium* strains expressing increased tolerance to heat stress. It has been established that that stress response genes can be used to improve *Rhizobium* response to stress and symbiotic performance in nitrogen fixation (Rodrigo da-Silva et al., 2017). The fourth objective was to assess the effect of manipulating the *Rhizobium* strains on stress tolerance and nitrogen fixation potential in soybean. The use of stress tolerant species of *Rhizobium* as inoculants, can greatly improve biological nitrogen fixation (Atieno & Lesueur, 2019).
To my knowledge, this thesis is therefore the first to report on genetic manipulation of HH103 *Rhizobium* strain with *hsp20, shsp, IbpA* stress genes and uncharacterized (*UC1, UC2* and *UC3*) genes in order to improve stress tolerance.

### 6.1.1 Characterization of stress tolerant strains and identification stress genes

The native soil *Rhizobia* might be highly inefficient in promoting plant growth under non-stress conditions but they are usually well adapted to more adverse conditions in the soil (Rodrigo da-Silva et al., 2017). Most soils in Malawi contain *Bradyrhizobium* as a native *Rhizobium* strain that nodulate Soybean (Parr et al., 2017). The adaptation and survival of these strains under such conditions suggests that the resistance might be due to altered expression which assist them to survive harsh soil condition. The initial strategy for this research was to use native strains for selection of stress genes for genetic manipulation of *Rhizobium* strains currently used as inoculants. However, the Malawi collection of isolates proved not good for this purpose, hence type strains were used to identify a suitable gene donor. *Rhizobium* strain USDA 193 was therefore identified through stress evaluations as the most heat tolerant strain that could be used to identify differentially expressed genes.

Proteomics was therefore used for identification of proteins differently expressed in USDA 193 at low and high temperature. Proteomics studies allows for identification of differentially regulated genes in response to a particular stimulus (Rodrigo da-Silva et al., 2017). Research has shown that higher transcription of the major chaperone induced under stress results in higher tolerance on a chickpea bacterial strain to that particular stress (Alexandre & Oliveira, 2011). It was therefore necessary to find possible proteins responsible for heat tolerance in a heat tolerant *Rhizobium* strain USDA 193. Analysis of protein extract from bacteria cultured at low and high temperatures identified groES, *hsp20, shsp, IbpA* and three uncharacterized proteins that differentially expressed highly at high temperature. The presence of some uncharacterized genes agrees with Rodrigo da-Silva et al., (2017) who predicted that there are many more genes of unknown function which are expressed in response to changes in the environment and might play an important role on survival of bacteria under stress. Most of the proteins expressed in *Rhizobium* were like those previously identified in *E. coli* and have specific known functions. Whilst comparative proteomics was successful in this study, there are other methods to identify differentially
expressed genes in bacteria which include RNA sequencing (RNA-seq), genome wide transcription analysis, Tandem Mass Tag and two-dimensional gel electrophoresis. Guerrero-Castro et al., (2018) used RNA-seq to identify genes differentially expressed in acid sensitive and acid tolerant strains of *Rhizobium* strain CIAT 899 grown at pH 4.5. Genome wide transcription analysis is an indirect method of gene identification because it identifies specific regulators that control genes related to particular stress (Vercruysse et al., 2011). Gomes et al., (2012) discovered differentially expressed stress proteins which included dnaK and groEL in *Rhizobium* strain CIAT 899 grown at 28 and 35°C using two-dimensional gel electrophoresis (2DE) and matrix-assisted laser desorption/ionization (MALDI-TOF) to identify the proteins. However, Tandem Mass tag spectrophotometry was used to identify differentially expressed proteins due to its sensitivity and robustness. Most of the predicted heat stress proteins expressed in *E. coli* such as dnaJ, dnaK and clpB were also detected in USDA 193 but were not differentially expressed. Further research is therefore required to establish the main functions of the three uncharacterized proteins which were differentially expressed in *Rhizobium* bacteria.

Heat shock proteins do not need to be over expressed but must always be ready to switch on by a heat stress inducible promoter in order to achieve heat tolerance. Once the promoter is on, the gene will be active to protect the protein depending on the intensity of the stimuli. The results in this research suggests that some proteins such as hsp20 and shsp are broad spectrum as they are expressed in response to three stresses while UC2 and UC1 are narrow spectrum since they respond to only two stresses. However, their response expression may sometimes be indirect while addressing the most pressing need since they respond to several different other stresses.

We anticipate that these genes are already upregulated under stress in sensitive strains like HH103 since *Rhizobium* poses σ^{32} transcription regulators such *rpoH* that respond to temperature upshifts. However, the level of expression may not be high enough to protect the cells from the imposed stress. The increased stress tolerance in the tolerant strain USDA193 may be due to the difference in protein sequences present in the two strains or the promoters driving the expression of these genes. The ideal expression profile may be achieved by using a heat inducible promoter which will not impose a fitness penalty unlike the *tauA* promoter as it is induced by taurine. According to Alexandre, & Oliveira (2011), a
higher level of induced transcription results in higher stress tolerance that ensure that bacterial cells are protected from high level of stress as well as ensure that energy is conserved by downregulation of genes responsible for other function other than stress response.

6.1.2 Generation of stress tolerant *Rhizobium* transformants

In this study, stress tolerant *Rhizobium* transformants were generated using pLMB51 (Tett et al., 2012) plasmid constructs for further evaluation in liquid culture and on soybean under stress and non-stressful conditions. Plasmid constructs generated through restriction digest ligation facilitate the expression of the stress genes in bacteria such as *Rhizobium* (Sigma-Aldrich). Restriction digestion ligation has previously been used in the development of *Rhizobium* transformants using other stress genes such as *clpB* (Rodrigo da-Silva et al., 2017). There are several methods which include electroporation, transformation, transduction and conjugation that can be used to manipulate *Rhizobium* (Patel & Sinha, 2011). However, conjugation was considered as a chosen method for transferring the genes since the plasmid vector used had the RP4 vector backbone. However, RP4, incP plasmids are difficult to work with in comparison to the smaller cloning vectors (Long, 1989). The *E. coli* strain S17 was therefore used to transfer the pLMB51 plasmid into *Rhizobium* by conjugation since it has a chromosomally integrated copy of the RP4 vector that can provide the transfer functions in *trans*, hence it is more efficient (Davison, 2002). The advantage of the incP plasmid, RP4 is that it is a broad host range expression vectors are stable when large inserts are cloned. In order to ensure gene expression in *Rhizobium*, the respective genes were cloned after the *tauA* promoter. There are several other promoters such LacZ, Ptac, trp and T7 which can be used to drive gene expression in gram negative bacteria. So a different promoter might avoid the fitness penalty under good conditions. However, the *tauA* promoter was chosen because it is able to control gene expression in several alphaproteobacteria and can be switched on by a relatively cheap inducer, taurine (Mostafavi et al., 2014). The generated HH103 *Rhizobium* transformants facilitated the production of the stress proteins in *Rhizobium* to study further and understand the induced stress tolerance in HH103 *Rhizobium*. The *Rhizobium* transformants were evaluated for their phenotypic growth under heat, acid and salt stress conditions. Unlike the wild type, in liquid media,
hsp20, UC2 and shsp induced heat tolerance, UC, hsp20, shsp induced acid tolerance while UC1, hsp20, UC2 and shsp induced salt tolerance in the transformants. The gene expression results indicate that each of the 4 genes namely UC1, hsp20, UC2 and shsp individually, have an added benefit in terms of stress tolerance of the transformed Rhizobium. This suggests that co-expression two or more genes in the same Rhizobium could be of great benefit due to synergy or additive effect. Alternatively, regulation of a single transgenic gene using a heat inducible promoter might also induce transcription of the other stress genes already present in the Rhizobium, hence increased stress tolerance.

The σ^{32} transcription regulators, rpoH1 and rpoH2 are responsible for expression of over 300 gene S. melilot under heat shock and stationary phase (Barnett et al., 2012). This regulator/transcription factor could be used to activate all the genes used in this research.

There is correlation between heat tolerance and other stress because the effects of heat stress at the cellular level which include protein denaturation and aggregation are common to the other stress factors (Alexandre et al., 2014). Coincidentally, Li, et al., (2012) established that hsp20 and shsp are also responsible for heat and acid stress in Sulfolobus solfataricus.

Attempts to construct plasmids with groES and groEL proved futile as these genes were difficult to amplify from USDA193 despite proteomics confirming their presence. This might be due to differences in sequences as the template sequences used to design primers were from HH103 and USDA205 Rhizobia strains respectively since USDA193 genome is not yet sequenced. Cloning of these genes extracted from HH103 and USDA205 Rhizobium strains into an expression vector was unsuccessful since PCR of the genes produced a small sized band when cloned. The possible reason for this was thought to be due to the presence of similar or repeat sequences in the plasmid.

6.1.3 Gene expression in Rhizobia cells and in plants

Several different approaches are used to analyze gene expression in bacterial cells, and these include DNA microarrays and RNA sequencing (RNA-seq). The major strength of DNA microarrays is that it can analyze hundreds to thousands of genes even the whole genome of an organism (Adn & Recientes, 2017). Guerrero-Castro et al., (2018) highlighted the advantage of RNA-seq as highly sensitive, specific and does not require special probes since
it can directly sequence the whole complementary DNA (cDNA) but prone to errors in the presence of ribosomal RNA (rRNA). Pérez-Montaño et al., (2016) used RNA-Seq to detect transcription of genes induced by salt in *Rhizobium* strain, CIAT 899. On the other hand, DNA microarrays was used to identify differentially expressed genes between *Bacteroides* and free-living cells of *Mesorhizobium huakuii*, 7653R (Peng et al., 2014). Other than using these methods, cell density as a measure of phenotypic response to various stresses may also be used to establish gene expression despite its low efficiency.

In plants, several methods which include seed coating, and direct inoculation with liquid culture of *Rhizobium* are used to infect soybean (Chamber, 1983), but each method has its advantages and disadvantages. Direct inoculation with liquid culture was used to inoculate soybean seedlings with HH103 transconjugant transformants. Plant root nodulation is a sign of successful plant infection to initiate the symbiotic biological nitrogen fixation process. Observations on plant growth, measurements and analysis of symbiotic plant growth parameters are used to assess nitrogen fixation. This was confirmed by analysis of symbiotic parameters such as nodule score (NS), nodule number (NN), shoot dry mass (SDM) and root dry mass (RDM) upon harvesting the plants at 4 weeks post inoculation. However, gene expression in a bacteria-plant symbiosis may vary under different growth conditions due to cell dynamics. In this research, plant growth was retarded under normal conditions probably due to plasmid penalty since energy is required to maintain the plasmid, while under stress, growth was either improved or maintained probably due to gene expression. Rodrigo da-Silva et al., (2017) established that wild type chickpea *Rhizobium* performed better than their transformants under optimum conditions. However, in this research, transformants expressing *hsp20* and *shsp* produced more SDM than the wild type and positive control under temperature stress. This result suggests gene expression in the *Rhizobium* transformants as observed by Rodrigo da-Silva et al., (2017) in chickpea *Rhizobium* bacteria. The result in table 5.3 indicates a decrease of 42.6% on SDM for WT strain against an average loss of 13.0% on the *Rhizobium* transformants at 32°C. In the dry/wet season between September and March, the daytime temperatures in Malawi normally range between 25-37°C (www.metmalawi.com). However, due to climate change it is likely that there will be an increase in daytime temperature as well as number of days of experiencing high temperatures. According to Onat et al. (2017), a maximum temperature above 35°C cause
heat stress that negatively affects soybean production. Considering that some transformants like UC1 and UC2 can increase SDM by 5.1% and 12.8% respectively at 32°C, this will be of great benefit to the farmer as the WT continues to lose SDM at higher temperature.

The same stress genes namely UC1, hsp20, UC2 and shsp with the greatest differential expression in proteomic also produce the most vigorous soybean plants. However, the order of ranking was different where UC1 and shsp ranked 1 and 4 respectively in proteomics and interchange positions in planta. In overexpression results, the order was the reverse of the proteomic expression except for UC1 which did not show expression. Surprisingly, UC1 had increased heat tolerance in planta despite not being over expressed in liquid at 40°C. This might be due to high expression of tolerance to salt since increased temperature results in accumulation of salts. Similarly, Rahmani et al. (2009) found a similar correlation between tolerance to elevated temperatures in liquid medium to symbiotic performance under heat stress.

6.2 Biosafety and economic implications

Biosafety is one of the key aspects to be considered when dealing with the release of genetically modified organisms into the environment. Biosafety is defined as efforts that aim at reducing and eliminate potential risks from biotechnology products. The management and use of biotechnology products in Malawi are guided by the Biosafety Act (2002). According to this Act, biotechnology is defined as any technique that uses living organisms to develop products or modify microorganisms for specific purpose. The use of genetically modified (GM) materials is heavily opposed by both organic farming advocates and farmers due to safety concerns upon exposure or consumption even though World Health Organization (WHO) and other organization certified them as safe (http://science-union.org/articlelist/2017/4/5/staple-crops). However, the main concern on the release of GM Rhizobium is their suspected impact on native population and the fate of the modified genes (Morrissey et al., 2002). To this effect, Morrissey et al. (2002) observed horizontal transfer of GM plasmid among S. meliloti strains as well as persistence of GM strains as long as ~6 years. Hirsch (2005) indicated that the potential of horizontal gene transfer should be considered for biological safety as they may easily be transferred to native microorganisms. This may increase the costs in management of plant, animal and human diseases due to
increase in cases. However, Hirsch (2005) in his research on chickpea found no evidence of gene transfer to the native *Rhizobium* after screening 4000 chickpea nodules for indigenous strain that acquired the Tn5-marked symbiotic plasmid. To the contrary, Amarger (2002) reported that in soybeans inoculated with GM *Sinorhizobium*, genes were transferred from the native strains to the introduced bacteria. However, the transformants were only localized to the phytosphere (Amarger, 2002). As a solution to avoid introduction of antibiotic resistance genes (ARGs) into the environmental bacteria, there is need to completely remove the selectable maker gene. Site specific recombination systems, zinc finger nuclease (ZFN) and transcription activator-like nuclease (TALEN) are some useful strategies for removal of selectable marker genes (SMG) as the most probable solution to this challenge posed by ARGs in plasmids incorporated into bacteria meant for release into the environment (Yau & Stewart, 2013). Furthermore, the use of non-drug selectable markers such as resistance to ionic heavy metals such as mercury or enzymes like URA3 and thymidine kinase, since acquired any resistance to these will have no impact on the environmental bacteria. However, the GM technology is a convenient tool in the current drive for sustainable agriculture in Africa which aims at reducing energy requirement and enhance use of non-toxic inputs for crop production (Amarger, 2002). The use of these stress tolerant *Rhizobium* transformants for soybean production in agriculture may result in greater economic gains. This may be due to expansion of soybean production to areas previously unsuitable for its production because of temperature, acid and salt stress conditions. Their use may lead to increased resilience and production of soybean crop under challenging environmental conditions.

6.3 Applicability of genetic modification of *Rhizobium* in Agriculture

Currently, the use of genetically modified (GM) *Rhizobium* bacteria in inoculant production and its field application in Malawi is prohibited by the Biosafety Act, 2002. However, section 17 and 18 of this Act allows use of genetic modification (GM) organisms for scientific research and/or experimental purposes (FAO, 2007). This may allow generation of data to prove their effectiveness and safety for clearance and approval with the Agricultural technology clearing committee (ATCC) in Malawi. Currently, Malawi is conducting confined field trial of three GM crops namely cotton (registration trial stage), cowpea (2nd yr.) and
banana (1st yr.) (https://allianceforscience.cornell.edu/blog/2017/04/malawi-progresses-in-gm-crop-trials/). This shows that the Malawi government is now ready to embrace GM technology if safety and economic benefits in using the interventions are guaranteed.

Genetic modification (GM) technology has been used on beneficial bacteria in many aspects such as improving plant nutrition, improving plant health and modulating plant growth in order to increase agricultural productivity (Hirsch, 2005). According to Bosworth et al. (1994), GM Rhizobium strains are used to improve plant nutrition through increased nitrogen fixation hence improving plant nutrition efficiency. The genetically modified (GM) stress tolerant transformants developed in this research may be used for production of high quality and effective Rhizobium inoculants when approved by ATCC in Malawi. This inoculant formulation will be able to meet minimum required standard of \(1 \times 10^9\) Rhizobium cells g\(^{-1}\) of freshly prepared inoculant (Lupwayi et al., 2000). Use of these transformants may increase the shelf life of the Rhizobium inoculants due to their heat stress tolerance. Furthermore, this will reduce loss of inoculant quality often encounter during transportation and storage since current formulations of Rhizobium inoculants require cold storage after production (Berninger et al., 2018).

According to Rodrigo da-Silva et al. (2017) successful performance of inoculants in the field is greatly compromised if inoculant formulation cannot survive abiotic stresses. As a rule of thumb, a good inoculant must adapt to environmental field conditions, effectively fix nitrogen, persist and compete well with native populations (Rodrigo da-Silva et al., 2017). At field level the use of these transformants will increase inoculant effectiveness since more Rhizobium cells will be able to survive stressful soil conditions. Furthermore, the cells that survive will be able to establish effective symbiosis through the development of root nodules despite stressful soil conditions. As a result, the net benefit will likely be increased crop yield as a result of increased plant growth due to increase in nitrogen fixation. In a bad year when effects of climate change are more pronounced these Rhizobium transformants will act as a buffer and maintain the crop yields however, they do not perform well under no stress.
6.4 Summary

This research has shown that there is diversity in the response of *Rhizobium* bacteria to heat, acid and salt stress. The research undertaken managed to characterize *Rhizobium* isolates and strains sourced from Malawi and within Europe respectively. Using morphological, biochemical and genetic characteristics, the research established the identities of these isolates and strains. Furthermore, the tolerance of these isolates and strains to variable stressful conditions namely high temperature, acidity and salt was established. Using the selected heat tolerant strain, USDA193, stress response genes that differentially expressed were identified.

Secondly, this research managed to PCR amplify all the targeted gene except *groES* and *groEL* from the host strain and used them in the construction of cloning and expression vectors. The six stress response genes that differentially expressed highly in the construction of plasmids were successfully cloned into cloning vector by restriction ligation cloning. Furthermore, this research also achieved in the selection of tetracycline an appropriate selectable marker for screening the recombinant transformants having the constructs.

Thirdly, this research successfully introduced each of the six plasmid constructs into a heat susceptible *Rhizobium* strain HH103 using a conjugative *E. coli* strain. This resulted in the development of HH103 *Rhizobium* transformants having an additional copy of the respective stress genes which were proved to be expressing in *Rhizobium*. This was ably demonstrated in this research as some of the transformants developed were able to display tolerance to variable stresses. This research established that the transformants harboring *hsp20*, *UC2* and *shsp* expressed heat tolerance, *UC1*, *hsp20* and *shsp* expressed acid tolerance and *UC1*, *hsp20*, *UC2* and *shsp* expressed salt tolerance.

Finally, this research has successfully demonstrated the impact of these genes in a *Rhizobium* - plant symbiotic interaction at elevated temperature. The results in this research has shown that expression of these genes *Rhizobium* has a positive impact on inoculated soybean plants. Analysis of symbiotic growth parameters on the plants inoculated with the stress tolerant transformants indicate that temperature stress has less negative effect in comparison to the WT strain. The stress tolerant strains for *Rhizobium* either maintained or improved the symbiotic performance of the soybean plants.
6.5 Conclusion

The following conclusions can be drawn from the findings and observation in this study:

- The findings contribute to the general understanding of the stress response and tolerance of *Rhizobium* to variable stress factors.
- In addition to the presence of well-known heat stress genes such as *groES, hsp20 shsp* and *IbpA* in *Rhizobium* it was discovered that *Rhizobium* harbors three other heat stress genes which have not yet been characterized.
- The uncharacterized differentially expressed genes will eventually be added to the current pool of knowledge on stress genes when their functions are fully studied and characterized.

6.6 Recommendations for future research work

In order to understand the functions of the uncharacterized genes and the impact of these stress genes on symbiotic biological nitrogen fixation under stress, there is need for further manipulation and studies of *Rhizobium* transformants as follows; 1) Generate *Rhizobium* transformants with 2 or more stress genes to investigate potential synergistic interaction from the stress genes. 2) Introduce the stress genes into a *Rhizobium* strain which does not harbor a similar gene in order to generate stress tolerant transformants. 3) Using a regulated promoter to drive the expression of the stress genes in order to benefit more from the effect of the stress genes and 4) Use of promiscuous soybean varieties to evaluate the impact of these genes on BNF since nodulation is most efficient with this type of cultivars.
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8 Appendices

8.1 Appendix 1: Nodule scoring chart used for scoring soybean plants grown in glasshouse and growth chamber (source: Howieson & Dilworth, 2016).
## 8.2 Appendix 2: NCBI BLASTn search results showing description of the best hit and % identity to the 16S rRNA of the Malawi collection of *Rhizobium* isolates.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Description</th>
<th>% Identity</th>
<th>Accession number</th>
</tr>
</thead>
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<td>Select seq KF863842.1 <em>Bacillus</em> sp. hb43 16S ribosomal RNA gene, partial sequence</td>
<td>98.63</td>
<td>gi</td>
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<tr>
<td>KU20B</td>
<td><em>Bacillus</em> sp. (in: Bacteria) strain SASC1 16S ribosomal RNA gene, partial sequence</td>
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<td>gi</td>
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<tr>
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<td>Bacterium strain BS1214 16S ribosomal RNA gene, partial sequence</td>
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<tr>
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<td>gi</td>
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<tr>
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<td><em>Bacillus cereus</em> strain MCM1Y2 16S ribosomal RNA gene, partial sequence</td>
<td>97.85</td>
<td>gi</td>
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<tr>
<td>NS39C</td>
<td><em>Bacillus cereus</em> strain LB073 16S ribosomal RNA gene, partial sequence</td>
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<td>gi</td>
</tr>
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