Assessing provenances of *Moringa oleifera* L. for salt tolerance and low methane emission

A thesis presented in the partial fulfilment of the requirements for the degree of

Master of Science

In

Plant Breeding

at Massey University, Palmerston North,

New Zealand.

Adrian Mangar

2022

Abstract

Moringa oleifera has a high nutritional content and easy propagation. It is well known worldwide where it grows best in tropical and sub- tropical environments. *M. oleifera* originated from Northern India, but plants can be found growing in Guyana, South America, where most of the agricultural lands are on the coastal plains and sea water intrusion is common. The objective of this study was to carry out a pre-breeding characterization of six *M*. *oleifera* provenances, four from Guyana and two from India for salt tolerance and their effect on rumen methane emissions compared with high quality pasture species.

A salt tolerance trial using the six provenances was done in a greenhouse at the Massey Plant Growth Unit, Batchelar Road, Palmerston North. The experiment was set up in a randomised complete block design, containing five blocks and three levels of salinity (0 mM, 52 mM and 156 mM NaCl). For each treatment ten seeds were sown in two pots and the salt treatments were applied by immersing pots for 30 minutes in the respective salt solutions every seven days for 21 days. Plant growth and net photosynthesis were measured during the stress period, chlorophyll content measured before and after salt stress, and biomass measurements along with sodium concentration in leaves, were taken at the end of the experiment.

Methane and total gas production were measured using an *in vitro* batch culture system. Two experiments were done, a preliminary screening using oven dried *M*. *oleifera* planted in field and greenhouse, and a main experiment using the six provenances, a composite sample and *M*. *oleifera* leaves from greenhouse. Both experiments compared the different *M*. *oleifera* sources with high quality ryegrass and white clover. Real time gas production was recorded for 48 hours, total gas production, methane analysed at 12 and 24h. Short chain fatty acids concentration were also determined at the end of the fermentation.

The preliminary experiment showed that *M*. *oleifera* leaves grown in field and greenhouse have lower gas and methane production compared with ryegrass, but similar to white clover. The differences were driven by a high production of propionic and butyric acids. The six *M*. *oleifera* provenances also produced less methane than ryegrass but similar to white clover at 12 and 24 hours after the start of fermentation.

Salinity screening of the six *M*. *oleifera* provenances showed a significant ($P < 0.001$) reduction in plant growth at 156 mM NaCl. There was an increase in sodium in photosynthetic active leaves as salt stress increased. However, at 52 mM NaCl plants survived and showed no significant difference in root and above ground plant biomass when compared with the control. Net Photosynthesis was also not significantly affected by the application of 52 mM NaCl salt stress and there was also no significant reduction of plant percent dry weight at 52 mM NaCl when compared with control plants.

Future work should include screening of more *M*. *oleifera* provenances for salinity tolerance and determination of the genetic diversity among these provenances. This would help determine the origin of the seeds sourced from King Seeds (Katikati, New Zealand) and their relation to the seeds sourced from Guyana and India. Cold tolerance screening should also be carried out using provenances from Northern India, which will help widen the gene pool for the creation of a base population for breeding programmes.

Acknowledgements

Many persons have contributed towards the completion of this research. I would firstly like to thank my main supervisor Craig for giving me the opportunity to work with Moringa, his guidance and support throughout the entire research, especially thankful for him coming on Sundays to check up on the plants and hearing my concerns when I show up at his office unannounced, thank you.

I would also like to thank my other supervisors from Massey University, Svetla, Sarah and Vaughan for their advice on the project. Many thanks also to Stefan and German from AgResearch, for giving me the opportunity to work with them and learning new techniques and skills. Thanks to Adrian Molenaar for the suggestions throughout the experiments and Dr Axay for his suggestions on the literature review. Thanks to Anurag for his initial inputs and advice on planting Moringa.

To the staff of the Plant Growth Unit, thank you Mark, Leslie, Su and Sunil for their dedication towards the salinity trial and checking up on the plants when I could not. Especially Su, for doing the emergence count and watering the plants during lockdown. Thanks to Nick, and Saii for their advice on thesis writing, and Nick for guiding me towards my current supervisors. Thanks also to Jennifer Tate, for suggesting a special topic in my first year, it really helped a lot for the other courses that followed. Thanks to Ian for helping sort out the sodium and potassium analysis, and to Chris for his help in measuring photosynthesis. Thanks to Mesu for his help to measure plant biomass.

Thanks to Sato, Indira, Eon for gathering the Moringa seeds in Guyana and Indira for sending them to New Zealand and thanks to CCS Haryana Agricultural University for suppling the seeds from India.

Thanks to Jaime, Tina, Dandan and the team at International Students Affairs for always being there to help during my time at Massey. Thanks to my family for their support and understanding.

Finally, this work would not have been possible without the facilities provided by Massey University and AgResearch and the scholarship funded by the Ministry of Foreign Trade and Tourism.

Table of Contents

List of Tables

List of Figures

List of Photographs

Chapter 1 Introduction

The world population is expected to grow 19% by the year 2050 (United Nations Department of Economic and Social Affairs, 2015). This growing population along with climate change is affecting food security worldwide (Molotoks et al., 2021). Climate change, mostly caused by an increase in greenhouse gas in the atmosphere, results in increasing temperatures and consequently rise in sea level (Fawzy et al., 2020). These changes affect the environment where plants are cultivated and as such interfere with food production. Agriculture can provide a solution for global food security (Kopittke et al., 2019) by breeding crops for climate adaptation (Snowdon et al., 2021).

Guyana, a country located on the northern coast of South America is a developing country with a growing population. The government has embarked on an agricultural diversification programme to help satisfy local food demands and to make the country competitive in international markets (Ministry of Agriculture Guyana, 2021).

One of the foremost agricultural diversification programmes is to improve local cattle breeds. The local cattle breeds in Guyana are inferior and thrive on local pasture grasses that are low in nutritive value, but improved breeds will have a higher feed efficiency and need high quality feed to perform at their maximum potential (VandeHaar et al., 2016).

Feeding improved cattle breeds will bring challenges. Firstly, most of the prime agriculture lands are located on the low coastal plain. The low coastal plain is one of four natural regions located within Guyana. Lands on the low coastal plain are below sea level and prone to sea water intrusion which makes some areas saline. If existing high quality pasture species are to be introduced and cultivated to help meet the demands of improved cattle breeds, they may not grow well or not at all. Secondly, if low- nutrition inferior pasture grasses are continued as cattle feed for improved breeds, a supplemental fodder must be used to increase the nutritional value of diets.

Moringa oleifera also known as Saijan or Moringa in Guyana (Dyal, 2017) or drumstick tree in India where it originated (Dhakar et al., 2011), is a plant that grows well in tropical and subtropical zones (Palada, 1996). It has a high nutritional value (Stadtlander & Becker, 2017) and is used in some countries as a supplemental feed for animals (Su & Chen, 2020). This plant can be found growing in Guyana's coastal plain. It has been reported to tolerate growth in saline conditions, but little is known of its salinity tolerance. *Moringa oleifera* is a potential supplementary feed for cattle in Guyana.

A step to satisfy the feeding requirements of improved cattle breeds and help achieve their nutritional requirements is to screen local *Moringa oleifera* provenances for growth in saline conditions. This first step can be considered as a pre- breeding step and will help to identify potential salt tolerant genotypes that are essential to establish a base population. Subsequently, selection and/or improving existing varieties that will grow in a wide range of geographical environments can be undertaken through a breeding programme.

1.1 Aim of this research

The main aim of this study is to carry out a pre breeding assessment by exploring the potential for growing *M. oleifera* in saline soil for use as a fodder crop to feed cattle. The study has two broad objectives, the first objective is to address the question of the ability of *M. oleifera* grow on saline soils and the second objective will look at how well *M. oleifera* ferments in the rumen of cattle, with a special focus on methane production. Therefore, the objectives of this study are:

Objective 1: Determine the response of *M. oleifera* to salinity stress in a greenhouse experiment, using four provenances originating from Guyana and two from India.

Objective 2: Using young *M. oleifera* leaves of different provenances from Guyana, India and King Seeds, conduct an *in vitro* fermentation to establish and compare gas production among provenances compared with high quality ryegrass and white clover.

Chapter 2 Literature review

2.1 Agriculture and the need for diversification in Guyana

The agriculture sector of a developing country is very important because it helps with economic development and alleviating hunger (Pawlak & Kołodziejczak, 2020). Moreover, increasing population and the imminent effects of climate change, means that a country's agriculture sector must not only produce food, but do so in an efficient and sustainable manner (Jones & Ejeta, 2016). This implies that the sector must not only modernize but also needs to diversify. Agriculture modernization involves acquiring and implementing new technologies and techniques to help the sector achieve its goals. On the other hand, agriculture diversification is aimed to widen agriculture production, this may include the introduction of a wider range of crop varieties and/or animals species to increase the number of commodities and production to meet the demand of local and global consumers (De Roest et al., 2018).

Guyana, a country located in northern South America, has a 430 km Atlantic coastline. It has three main geographical zones, the coastal plains, the sandy belt, and the interior highlands. The daily temperature ranges between 16°C to 34°C with around 70 percent humidity annually. The agriculture sector consists mainly of rice and sugar production and livestock farming, and contributes to approximately 15 percent of the gross domestic product (Bubbico et al., 2020; FAO, 2015).

The present diversity of crops and livestock in the agriculture sector has been influenced by colonization. During colonial era, the agriculture sector was mainly focused on the cultivation of sugar cane *(Saccharum officinarum)* to produce sugar for United Kingdom (Canterbury, 2007). Presently, sugar is still being produced along with rice and poultry. These commodities occupy most of the agricultural productive lands on the coastal plains. Other commodities occupy a smaller land area, such as mangoes, coconuts, tomatoes, peppers and cattle. Fruits and vegetables are mostly cultivated for domestic consumption, and to a lesser extent export to other countries, whereas cattle rearing is done for local meat and milk production (Derlagen

et al., 2017). Although agriculture is relatively significant for Guyana's economy there is still not a wide range of crops and livestock which makes the country economically vulnerable.

Guyana is said to have economic vulnerability because it depends on a few export commodities, which can be subjected to unstable international prices and the local market is not large enough to compensate for any large changes in international market prices. To reduce this risk, the recommendation is to have a wider range of commodities for export and local markets. Increasing the number of commodities will help to reduce the risk that may arise if there are unstable market prices for one commodity. By distributing the risk across a wider range of commodities, any shocks arising from unstable prices from one commodity can be absorbed by other commodities and markets. Consequently, the bovine sector has been identified as a commodity sector with potential for diversification (Bubbico et al., 2020). Therefore, Guyana is starting to diversify its bovine sector by crossbreeding local breeds with exotic breeds of cattle, thereby increasing genetic potential to achieve higher meat and milk production (Ministry of Agriculture Guyana, 2021).

Crossbreeding will help to achieve increased milk and meat production through the introduction of new genetics into existing local breeds (Cundiff, 1970; Dezetter et al., 2019; Swalve, 2004). The new breeds will have different nutritional requirements as compared to the local inferior breeds. In addition, these genetically improved cattle breeds will be able to convert feed more efficiently than the local breeds (Kenny et al., 2018; VandeHaar et al., 2016). Also, the animals will need highly nutritive feed in order to perform at their potential and to produce quality milk and meat (Funston et al., 2012; Jelan & Sumarmono, 2019; T. Zhang et al., 2018).

Local pasture grasses cannot provide adequate nutrients in sufficient quantities and if used to feed cattle without supplementation will create a nutrient deficit in the diet of the animals. For example, *Echinochloa pyramidalis* commonly known as Antelope grass, is mostly used to feed cattle in Guyana. The grass contains approximately 11-13 % crude protein per dry matter (Smith et al., 1991). On average a heifer that weighs 100kg will need 17% crude protein daily for maintenance and growth (DairyNZ, 2021). Another problem is some areas located within the coastal plain have saline soils (Steele & Ramdin, 1971) and most of the grasses

that are recommended for improved pastures on the coastal plains of Guyana cannot withstand saline soils (Caribbean Agricultural Research and Development Institute, 1996). In addition, the geography of Guyana is very diverse. It consists of three main geographical zones, the coastal plains, the sandy belt and the interior highlands (FAO, 2015). Within Guyana, cattle rearing is done in four areas(Fernandes et al., 1975):

1. The coastal region, which stretches from west to east of northern Guyana and have a width of 65 kilometres and is 3 meters below sea level.
2. The intermediate savannahs, a region extending southwards 96 to 145km from the coastal region and 28 meters above sea level.
3. The northwest region of Guyana, which is mountainous.
4. The rupununi savannahs, the southernmost part of Guyana with vast stretches of open grassland with relatively poor soil characteristics.

As a result, there is a need for a forage crop to be used as supplemental feed that will help increase the nutritional value of the animal's diet and at the same time have tolerance to soil salinity, with adaptability to grow within diverse environments.

2.2 *Moringa oleifera*

Moringa oleifera (M. oleifera Lamarck) (Mabberley, 2017), a plant that is found growing on the coastland plain of Guyana can be used as a forage crop to supplement animal feed. Previous studies have supported its use as a supplemental feed (Su & Chen, 2020). In fact, it has been used as a feed supplement in Hubbard broiler chickens by adding *M. oleifera* leaf extract to the drinking water. By adding 90 ml of *M. oleifera* leaf extract in one litre of drinking water, feed intake was reduced by 12.83% and feed conversion ratio increased by 9.11% when compared with not adding extract (Alabi et al., 2017). Other studies have reported using *M. oleifera* for feeding fish. For example, freeze dried fresh *M. oleifera* leaves were used as an additive in fish diets, to supplement protein. The investigation showed *M. oleifera* can be added in the diet to supplement as much as 10% of the total dietary protein (Richter et al., 2003). In addition, *M. oleifera* was used to feed

Bangladesh Livestock Research Institute cattle breed-1 (BCB-1) dairy cows. The trial showed that cows fed dried *M. oleifera* leaf and twigs had significantly more milk production when compared with cows fed a diet containing no *M. oleifera* and primarily consisting of wheat bran, rice bran and soyabean meal (Bashar et al., 2020). Also, fresh *M. oleifera* biomass consisting of stems twigs and leaves were used as a supplement in the diets of Nubian goats. The *M. oleifera* was fed using a cut and carry system and the results showed does receiving 375g/kg dry matter of *M. oleifera* had a significantly higher milk production per day than does that did not receive any *M. oleifera (Kholif et al., 2018).*

Furthermore *M. oleifera* is known to have a high nutritional content and can grow in a wide range of environments (Trigo et al., 2021). Plants can be grown from seeds or from stem cuttings (Masih et al., 2019; Oluduro et al., 2016; Ramachandran et al., 1980), making the plant easy to propagate. This ease of propagation, along with its nutritional content and versatility of being able to grow in a wide range of climatic conditions, including dry areas, makes it a perfect candidate to fortify foods (Masih et al., 2019). As a result *M*. *oleifera* is also used as a supplement for humans suffering from malnutrition to help reduce hunger (Kunyanga et al., 2013). For example, the whole plant of *M. oleifera* is used in Guinea- Bissau, leaves are eaten fresh or cooked, seeds are eaten roasted as nuts or ground in food, flowers are cooked as a vegetable in food (Bancessi et al., 2020). In Nigeria the whole plant is also used for food, but most of the populace use the leaves and seeds for medicinal purposes ranging from treating malaria, diarrhea, diabetes, joint pain, ulcer and poor vision (Popoola & Obembe, 2013). The leaves are the most used part of the plant (Leone et al., 2015). Other countries that consume *M. oleifera* include Ghana, Malawi, Ethiopia and other East African countries (Agbogidi & Ilondu, 2012). A recent study conducted by Barichella et al. (2019) in Zambia showed that dried *M. oleifera* leaves consumed at up to 14g per day can be used as a supplement for children between the ages of 4 and 18 with mild or moderate malnutrition. In India it is also used for medicinal purposes (Sivasankari et al., 2014) and to combat malnutrition among children and babies.

2.2.1 Origin and Distribution of *Moringa oleifera* Lam.

M. oleifera originates within the region of northern India to south of the Himalayas, bordered by Bangladesh, Afghanistan and Pakistan (Dhakar et al., 2011). It was from here that it was distributed to other parts of the world, mostly by humans (Morton, 1991). For instance, it was introduced to eastern Africa in the early twentieth century (Foidl et al., 2001), possibly through the British taking it as an ornamental tree (Berger et al., 1984). Also, in 1915 the Office of Foreign Seed and Plant Introduction of the United States Department of Agriculture, introduced seeds from Cuba (Powell, 1927). Plants were introduced into Jamaica by the English in 1784 (Fawcett & Rendle, 1914). In a similar manner, it is suggested that it was brought to British Guiana, now Guyana, from India by the Indian indentured laborers, who brought their cultures and traditions (Ahyoung, 2017) when they came to settle and work after the emancipation of slaves. Presently, it is widely cultivated throughout tropical and subtropical areas (Su & Chen, 2020), particularly in the Americas, Caribbean, Africa, Asia and Pacific Islands (Fahey, 2005).

M. oleifera belongs to the Moringaceae family. Within this family there are twelve species: *Moringa drouhardii, Moringa hildedrandtii, Moringa ovalifolia, Moringa stenopetala, Moringa arborea, Moringa ruspoliana, Moringa concanensis, Moringa perigina, Moringa borziana, Moringa longituba, Moringa pygmae* and *Moringa rivae.* These species are remarkably diverse in their form and structure, ranging from large trees to slender trees, through to small shrubs with large underground tubers. Based on this diversity *Moringa* species are divided into four groups, namely, bottle trees, slender trees, tuberous shrubs and sarcorhizal trees. *M. oleifera* is grouped under slender trees and is known as Asian *Moringa* species along with *M. concanensis* and *M. peregrina* (Olson & Carlquist, 2001). This classification demonstrates there are different species within the genus *Moringa.* Therefore, there is a possibility of creating new cultivars from interspecific hybridization, effectively utilizing genes from different species within the Moringaceae family and ultimately increasing the potential future breeding programmes.

2.2.2 Description of *Moringa oleifera* plant.

(a)

(b)

(c)

(d)

(e) (f)

Photographs of *M*. *oleifera* provenance growing in Mon Repos, Guyana, South America. (a) mature plant, (b) inflorescence, (c) and (d) fruit, (e) leaf and (f) a single flower (Photographs taken by Adrian Mangar).

M. oleifera, has several common names based on its wide distribution. *M*. *oleifera* is commonly known as drumstick tree, ben tree, never die tree, horseradish tree (Ramachandran et al., 1980), kelor tree, sohanjna (Anwar & Bhanger, 2003), Shagara al Rauwaq in the Nile valley (von Maydell, 1986) and malunggay in the Philippines (Patricio & Palada, 2015). The tree is a medium sized, often fast growing perennial that can achieve a height of 7-12 meters (Foidl et al., 2001; Ramachandran et al., 1980). The leaves are arranged spirally on the branches that are 25-45 cm long. Leaves are an incomplete tripinnate. Leaflets are small, rounded, with lateral leaflets elliptic and terminal leaflets are obovate and slightly larger with the petiole 1-4 mm long. The tree has bisexual flowers that are scented and 0.7-1cm long. The calyx lobes are slightly unequal but somewhat angular, the sepals are unequal ranging in size from 0.7 to 1.4 by 0.25 to 0.5 cm. There are five petals that have a yellowish to white colour with a greenish base. Also, there are five stamens that can reach a length of 0.8-0.9cm. The ovary is densely packed with three furrows that are longitudinal,

bearing a double row of ovules. The fruits are pendulous capsules which are ribbed and linear, commonly referred to as pods, lobbed into three sections usually 20-45cm long but can reach as long as 120cm. The impressions of the seeds can be seen from the exterior of the fruit. The seeds are about 1cm in diameter, three winged with a brownish testa that is thick. The wings are at an angle of 120 degrees that run from the base to the apex of the seeds (Masih et al., 2019; Ramachandran et al., 1980).

2.2.3 Favoured traits of *Moringa oleifera.*

Moringa has been used for years by people for various reasons. The people of India have been using it in their diet for nearly 5000 years (Bhargave et al., 2015). Historical records indicated Moringa was used as early as 150 B.C. by royalty for skin and mental health. Additionally, Indian Maurian fighters used it for strength and vitality (Mahmood et al., 2010; Singh et al., 2020). While these references do not mention the species of *Moringa*, it is very possible they were referencing current *M. oleifera* as evidenced by its widespread use and cultivation. Early use of current *M. oleifera* is also an indication that it was known to have benefits to people, maybe because of its nutritional value.

2.2.4 Crude protein and Amino acids

The leaves of the *M. oleifera* are mostly used for consumption, because they contain a wide range of amino acids and vitamins as compared with other parts of the plant (Leone et al., 2015). The dried leaves of *M. oleifera* can contain as much as 30% crude protein (Busani et al., 2011; D. Wu et al., 2013). Olson (1976) has previously reported that the leaf protein content across eleven *Moringa* species (*M. peregrina, M. ovalifolia, M. borziana, M. rivae, M. ruspoliana, M. ruspoliana, M. concanensis, M. drouhardii, M. hilderbrandtii, M. longituba M. oleifera* and *M. stenopetala)* did not vary significantly when tested for total protein content. In contrast, crude protein was found to vary among species of *M. oleifera, M. drouhardii* and *M. hildebrandtii* which were sourced from Malawi, Madagascar and Gran Canary respectively (Stadtlander & Becker, 2017). This may indicate that the protein content of leaves varies among different species and /or with environment. However, which cannot be concluded because each of three species were sourced from

different environments. Other research comparing the nutritional content of *M. oleifera* with *M. stenopetala* showed a significant difference between the crude protein content of the leaves. *M. oleifera* leaves had a significantly higher crude protein (290g/kg dry matter) as compared with 268g/kg dry matter crude protein of *M. stenopetala,* growing within the same elevation and during the rainy season. Moreover, the elevation had significant effect on crude protein content of leaves within each species. Both species had high leaf crude protein content in the rainy season, but *M. oleifera* crude protein was higher in both rainy and dry seasons at low and mid elevations than *M. stenopetala* (Melesse et al., 2012). On the other hand, the crude protein content of fresh leaves was found to be similar among *M. oleifera* provenances growing at different locations, indicating the environment may not be an influencing factor on leaf protein content of *M. oleifera* provenances. For example, fresh leaves of *M. oleifera* plants from Nicaragua, Bangladesh, and India were found to have a crude protein content ranging from 26 % to 29 % (Foidl et al., 2001). While there was no statistical analysis to determine the significance difference, the range across provenance is very small (3%). Although the research did not compare amino acids among provenances of *M. oleifera*, a high crude protein content suggests that the leaves can be a good source of amino acids.

2.2.5 Amino acids

Amino acids whether essential or non-essential play diverse roles in the growth and development of animals. They are needed individually, or sometimes required along with other amino acids to carry out their functions (Li & Wu, 2018). Because of the role they play in the growth and development of animals, they are pivotal for the determination of production goals such as meat or milk quality (Wu, 2009).

Amino acids are required for protein synthesis. They are considered as non-essential when they can be produced by the animal in adequate amounts and for this reason, do not need to be provided in the diet. On the other hand, essential amino acids cannot be produced by the animal or are not made in enough quantities and must be provided in the animal's diet (Wu et al., 2013). Essential and non-essential amino acids found in *M. oleifera* leaves had similar values when compared with other species of Moringa (Stadtlander & Becker, 2017).

In ruminant animals, amino acids that are absorbed by the animal's digestive system comes from microbial protein synthesis and dietary source (Clark et al., 1992; Kung & Rode, 1996). Dietary source of amino acids are an important consideration when planning a ruminant's diet because some of the amino acids are used for microbial growth, making the nutrients supplied in the diet different from those that are available for absorption (Moro et al., 2020). Likewise, portion of amino acids are degraded in the rumen (Volden et al., 1998). Hence, diets that have a wider range and higher content of amino acids can help the animals be more efficient by making these amino acids more readily available to the animals.

Dried *M. oleifera* leaves contain nineteen amino acids, nine of which are essential, namely, threonine, tyrosine, methionine, valine, phenylalanine, isoleucine, leucine, histidine and lysine (Busani et al., 2011; Sánchez-Machado et al., 2010; Su & Chen, 2020). Leaves also contains arginine, which is considered an essential amino acid for young mammals and for foetal growth (Wu, 2009).

These essential amino acids have important roles in animals. Methionine, along with lysine and histidine, is required for muscular development and function and is considered a limiting amino acid for lactating cows (Kung & Rode, 1996; Volden et al., 1998). Histidine stimulates milk production and milk protein synthesis in ruminants (Moro et al., 2020). Valine, isoleucine and leucine are substrates for the production of alanine and glutamine which is found in milk (Wu & Knabe, 1994) and needed for the development of skeletal muscle, mammary brain and heart tissues (Lei et al., 2012; Li et al., 2009; Wu, 2009). Threonine is directly needed for making of the mucin protein which helps to maintain the structure and functioning of intestines, and glycine synthesis. Tryptophan plays an important role in the production of melatonin which helps in the inhibition of cytokines and superoxide. Phenylalanine is associated with the synthesis of tyrosine and is important for the animal's neurological system. Tyrosine is directly needed for protein phosphorylation and produces dopamine which also have neurological functions. Threonine, tryptophan and tyrosine are needed for immune function (Wu, 2009). *M. oleifera* has almost eight times the lysine content of cornmeal and two thirds the methionine content of soyabean meal (Su & Chen, 2020). The literature reviewed suggest that *M. oleifera* is a potentially good source of all these essential amino acids.

Common non-essential amino acids found in *M. oleifera* leaves are glycine and proline. Glycine is a simple amino acid and is part of the primary extracellular matrix protein (collagen), here it can account for one third of the protein. Proline is involved in making collagen and elastin, it is an important substrate for making arginine. Arginine helps to produce nitric oxide responsible for maintaining nutrient transport and haemodynamic in the animal's body (Wu & Meininger, 2009).

2.2.6 Vitamins and Minerals

Vitamins and minerals are important for animal health. Vitamin A is essential in biochemical pathways to help manage oxidative stress (Masters, 2018), vitamin C is also an antioxidant (Linster & Van Schaftingen, 2007), vitamin B1 or thiamine are cofactors for enzymatic reactions and is very important for cerebral metabolism (Fattal-Valevski, 2011). Again, vitamin B2 or riboflavin is an antioxidant against lipid peroxidation (Ashoori & Saedisomeolia, 2014), vitamin B6 is required for various chemical reactions such as the storage of carbohydrates in the form of glycogen and also releasing glucose from glycogen (Helmreich, 1992), and fatty acid metabolism (Horrobin, 1993). Likewise, vitamin K1 is necessary for blood coagulation because it is needed for the making of coagulation factors II, VII, IX and X (Buitenhuis et al., 1990). Dried *M. oleifera* leaves contain vitamins A, B1, B2, B6, C, E, K1, biotin, folic acid, niacin and pantothenic acid and minerals such as potassium, iron and calcium, phosphorus, magnesium, sodium, zinc, manganese, molybdenum, cobalt, selenium, copper, boron, aluminium and nickel (Stadtlander & Becker, 2017). These vitamins and minerals were found in leaves of *M. oleifera* originated from Malawai, India Nicaragua, Uganda. Also, dried leaves from *M. oleifera* plants grown in Brazil contained calcium, magnesium, zinc, potassium, iron and copper, respectively (Teixeira et al., 2014). These studies also show that there are mild variations among the vitamin and mineral content of *M*. *oleifera* leaves grown at different locations around the world (Stadtlander & Becker, 2017).

Dried *M. oleifera* leaves also contain vitamin E as α and γ -tocopherol, found at concentrations of 744µg/g and 5.7µg/g respectively, in the leaf material analysed (Sánchez-Machado et al., 2006). These are anti-oxidants and protect the animal's cells from stress, mainly oxidative damage (Jensen & Lauridsen, 2007).

Also, α -tocopherol can be found in cow plasma and milk (Meglia et al., 2006), this prevents oxidation of unsaturated fatty acids (Bendich, 1993). Other metabolically important vitamins include folic acid and biotin. Folic acid is responsible for gene expression and the neuro transmission (Ghoshal et al., 2006; Pogribny et al., 2008), it also has immune functions (Mikkelsen & Apostolopoulos, 2019). Whereas, biotin serve as a coenzyme and plays special roles in regulation of genes and chromatin structure (Zempleni, 2005).

Minerals are important for everyday growth and development of animals; calcium is needed for the formation of strong bones and teeth, cell defence, enzyme functioning and the regulation of metabolism (Moyo et al., 2011). Iron is a part of various proteins and is needed in various biochemical processes. Iron is bound to lactoferrin found in milk and is vital for the growth of calves. A deficiency can cause anaemia in calves in their early stages of life (Wysocka et al., 2020). Iron is also a vital part of haemoglobin. Haemoglobin is needed to for the delivery of oxygen to cells around the animals body (Giardina et al., 1995). A lack of iron can hinder physiological functions. Iron along with zinc is required for growth. Zinc helps metabolise protein, fats and sugars (Brisibe et al., 2009; Kozat, 2007). Magnesium is an essential element and has various functions in animals (Martens & Stumpff, 2019), it is a crucial element for the prevention of tetany in dairy cattle (McKinnon et al., 2003), needed for enzymatic reactions in the animal's body (Cowan, 2002) and functioning of nervous and skeletal muscular system (Lamb & Stephenson, 1994). Potassium is required for nerve and muscle functioning, the transport of other nutrients and is a major constituent of milk in dairy cattle. Copper aids in collagen formation gene regulation and immune function (National Research Council, 2001). Overall, the vitamins and minerals found in *M. oleifera* leaves are important for the normal function, growth and development of animals and can be supplied by *M. oleifera,* once it is included in the feeding system for animals.

2.2.7 *M. oleifera* anthelmintic properties

M. oleifera also have antiparasitic properties (Fatima et al., 2014). For example, a study reported that dry aqueous *M. oleifera* leaf extract had effective control against *Lchthyophthirius multifilis* – a protozoan parasite that affects fishes by damaging the gills and skin. In the study, the dry aqueous leaf extract of *M.*

oleifera used at a concentration at 4.5 g/L was found to effectively control the protozoan in its trophont stage (Chika et al., 2020). In another experiment, water soluble lectin obtained from Moringa seed extract at a concentration of 230 µg mL^{-1} had 80% mortality rate on *Aedes aegypti* fourth stage larvae (Coelho et al., 2009). Water soluble lectin obtained from *M. oleifera* seeds also showed an anthelmintic effect on goat gastrointestinal nematode eggs and larvae when used at 250 µg mL^{-1} (de Medeiros et al., 2018). These results further support the use of *M*. *oleifera* as a forage plant as it provides additional benefits for animal health, in addition to nutrition.

2.2.8 Limitations to the use of *M. oleifera* in different areas of the world

While *M. oleifera* is highly nutritious and is said to grow in tropical and subtropical areas, its growth is limited by some environmental factors.

2.2.8.1 Drought

M. oleifera can be found growing in sub-tropical areas, as far north as Spain, and as far south as Argentina and Chile (Godino et al., 2015). *M. oleifera* can be cultivated in arid and semi-arid areas (Boumenjel et al., 2020; Gopalakrishnan, 1978), on all types of soil (Masih et al., 2019; Ramachandran et al., 1980), but performs best on dry sandy soil (Duke, 1978; Palada, 1996), suggesting it may possess some tolerance to drought, an abiotic stress.

Morton (1991), stated *M*. *oleifera* plants can withstand stress caused by the limited availability of water. Furthermore, it has been found growing in countries where water is limited, such as Ethiopia, Kenya (Kumssa et al., 2017) and Egypt (Barakat & Ghazal, 2016), where climate patterns show a decrease in rainfall over the last century (Gado et al., 2019). Limited water has negative effects on stem and root length of *M. oleifera* plants, where plants exhibit a decrease in growth and cause a reduction in the overall growth rate *(Boumenjel et al., 2020).* To combat water stress, *M. oleifera* plants have been shown to have elevated isoprene content and reduced stomatal conductance (Brunetti et al., 2018), indicating it is an isohydric plant. An isohydric plant is a plant that has a water management system where the stomatal regulation maintains a minimum

leaf water potential from day to day (Klein, 2014). This implies that plants have a constant leaf water potential independent of changes in the environment and therefore can be advantageous for growing in a wide range of geographical locations including within Guyana.

It is also important to highlight that *M. oleifera* cells under stress caused by limited water were found to have an increased proline concentration (Chitiyo et al., 2021). Proline is a reactive oxygen species (ROS) scavenger (Liang et al., 2013) and a osmoprotectant (Matysik et al., 2002). When plants are stressed, such as having limited water availability, they tend to try and save water. This is achieved by the closure of the stomata, and because of this carbon dioxide supply is also negatively affected. This limits photosynthesis and increase the production of ROS, causing oxidative stress in the plant cells (Cruz de Carvalho, 2008). One way plants remove ROS is by producing secondary metabolites that can counteract these stress- causing compounds (Zhao et al., 2005). One such metabolite that counteract stress is proline. Having elevated metabolites to combat stress causing compounds demonstrates *M. oleifera* is an adaptive species which can survive semi-arid regions.

2.2.8.2 Temperature

Low temperature, another abiotic stress (Mahajan & Tuteja, 2005), is a climatic factor affecting the productivity and distribution of plants (Larcher, 2005). In low temperature zones there is a reduced phytodiversity of 1000-1500 species per 10^4 km^2 (Barthlott et al., 1996). This clearly suggests that low temperatures limit plant distribution. These temperatures can be chilling temperatures (0°C – 15°C) (Lukatkin et al., 2012; Sakai & Larcher, 1987) or freezing (<0°C) (Beck et al., 2007).

As the temperature decreases to chilling temperatures plants can show different symptoms such as wilting or yellowing of leaves (Beck et al., 2004), increase membrane viscosity, slowing of metabolism and development of oxidative stress (Beck et al., 2007). Chilling temperatures can also cause sterility if cold occurs during flowering (Wen et al., 2002).Furthermore, when temperatures are below 0°C, some plants die while others survive. Whether they suffer or die depends on their tolerance to ice formation within and

between plant cells (Mahajan & Tuteja, 2005). The formation of ice causes irreversible membrane damage (Gutierrez et al., 1992; Steponkus, 1984; Steponkus, 1993) which leads to a loss of the cell membranes functions and finally dehydration of the cell and ultimately plant death (Beck et al., 2007). The tolerance that all plants have to low temperatures varies with their origin and the damage that occurs depends on the temperature and the amount of time the plants are exposed to the low temperature (Lyons, 1973).

To avoid low temperatures, plants have adapted in different ways. Firstly, most plants that have adaptation to low temperatures have passed through a selection filter. For instance, freezing resistance is one such screen that most species pass through to survive or thrive in cold climates. In other words, a cold tolerant plant must be able to resist the low temperature extreme that exist in its environment, and therefore the varying temperature across the globe reflects different plant diversity, with diversity of plants varying as cold temperature extremes change. Secondly, some of plants adapt by fine tuning their phenology to maximize growth and development during higher temperatures and hence move to a period of dormancy during winter to protect plant tissues from being damaged during this period. Thirdly, is the adaptation of the plants physical structure. For example, some pants in cold climates have a short stature that reduces convective heat transfer. This is seen in alpine and artic species, most of which have short stature. This is a genetic adaptation to low temperatures, basically benefitting from the heat from the ground (Körner, 2016).

This means that for a plant to thrive within climates with relatively low temperatures it needs to develop under low growth constraining temperatures and hence can produce plant traits such as fast development, high metabolism and a below ground storage tissue. *M. oleifera* has been suggested to be able to withstand low temperatures, as much as -3°C (Godino et al., 2015). Meanwhile other sources stated that Moringa can withstand an absolute minimum temperature of -1°C and mean minimum temperatures of 4-10°C (Vélez-Gavilán, 2017). While these sources stated *M. oleifera* can withstand very low temperatures, in both instances for which the time the plants can withstand these temperatures was not stated.

As mentioned, *M*. *oleifera* originates from northern India. In fact, Ramachandran et al. (1980) indicated that genetic diversity may be available north of India specifically between Chenab River, then moving eastwards

to Sarda River and thence south of Sarda, to Tarai in Uttar Pradesh. In this region the average temperature during winter varies between 6.7°C and 26.2°C (Wani et al., 2017). This is evidence that there is possibility of identifying *M. oleifera* genotypes that possesses traits to enable them to tolerate lower temperatures as compared with previously stated temperature ranges of 19 °C to 29°C (Duke, 1978) and 25 °C to 35 °C (Saini et al., 2016).

2.2.8.3 Salinity

Soil salinization occurs because of the build-up of water-soluble salts in the soil around the plant roots. It reduces the water and soil quality, thereby affecting plant growth. Soil salinity can occur naturally or by human activities. Natural soil salinity occurs when the accumulation of soluble salts is accomplished by the weathering of rocks high in salts, such as sedimentary rocks that are normally high in carbonates and sulphates. Salinity caused by human activities is a build of soluble salts in the soil as a result of the actions of humans, such as, using irrigation water that contains high amount of salts under poor drainage conditions, modifying the landscape that will allow salt water intrusion or the use of chemicals or fertilizers that in time can increase the concentration of salts in the soil (Hopmans et al., 2021). Soil salinity contributes to high concentrations of soluble salts soils, and this creates an abiotic stress in plants that affects plants that affect their development and subsequently reduces productivity (Munns & Tester, 2008).

Soil salinity is usually measured by estimating the concentration of total soluble salts using electrical conductivity (EC). The EC of the soil is commonly expressed as deciSiemens per metre (dS/m), 1 dS/m = 1 millisiemen per centimetre (mS/cm)) and corresponds to the total dissolved solids in salt solution (Bresler et al., 2012). According to US Soil Salinity Laboratory Staff (1954), extracted saturated soil paste with an electrical conductivity (EC) value of less than 2 dS/m is considered non- saline, while EC values between 2 and 4, 4-8, 8 – 16 dS/m are considered slightly, moderately and extremely saline soils respectively. Other classifications of soil salinity can range from slightly saline (3g of salts), to highly saline (more than 12g of salts), in one litre of extracted water from saturated soil (Brouwer, 1985). Soils can also be classified as saline

when it contains a minimum of 40 mM Sodium chloride (NaCl). NaCl is a common cause of soil salinity (Munns & Tester, 2008).

Soil salinity can be caused by various salts, and the type of salt depends on the source of salinity. However, it is most times caused by sodium chloride (NaCl), because Na^+ and Cl^- ions are among the most common ions, and at high concentrations they negatively affect plant growth (Läuchli & Epstein, 1990; Maas & Hoffman, 1977). Sodium ions enter the plants through its roots either by a carrier or channel mediated entry. These entries are based on the fact that the transport of an ion is mostly determine by two driving forces, a chemical and electrical component that involves the concentration and electrical potential difference respectively between the cytoplasm and the extracytosolic compartment (Amtmann & Sanders, 1998). Other possible ways of entry are high affinity uptake of sodium when roots are starved of potassium, as was discovered in barley (Haro et al., 2005). However the main site of sodium entry in roots is uncertain (Munns & Tester, 2008).

Plant response to salinity is very complex (Munns, 2002). But in summary salinity affects plants in two ways. Firstly, it causes an osmotic stress. Saline soils have an above normal concentration of salts and a low concentration of water. A plant with it roots exposed to saline soils will have the opposite- a lower concentration of salts (usually lower than the soil) and a high concentration of water. When this happens, an external osmotic pressure is created, making the intake of water through the roots difficult. Therefore, an abrupt increase in soil salinity will cause leaf cells to lose water but only for a short time until an osmotic adjustment occurs, however cell growth is reduced (Fricke & Peters, 2002; Passioura & Munns, 2000). As the salt stress continues, reduction in overall cell growth results in a slower leaf appearance. The cell dimensions also change, leading to a reduction in cell area, causing leaves to be smaller and thicker. Also, the decrease in water uptake also affects stomatal opening. Because as the plants detect the osmotic effect of the high salinity, the production of abscisic acid increases. Abscisic acid plays a main role in cell signalling in times of water related stress, and the increase in abscisic acid in the leaf leads to decreased transpiration by controlling stomatal conductance (Fricke et al., 2004). The hormone gibberellin also plays a role in plant

response during stress periods since it facilitates the destruction of DELLA proteins which negatively affect plant growth and link signals from hormones and abiotic stress (Achard et al., 2006)

In addition, photosynthesis is then affected by the reduced water uptake and stomatal conductance of the plant. However, in some salt stress plants the rate of photosynthesis per leaf area is not affected even though stomatal conductance is reduced (James et al., 2002) and this may be explained if photosynthesis is expressed as unit chlorophyll basis. In particular, the change in salt stressed leaf cause them to have a reduced area and become thick thereby increasing chloroplast density. However generally photosynthesis per plant is reduced because of reduced total leave area per plant (Munns & Tester, 2008).

Secondly, after a prolonged exposure to soil salinity, sodium ions start to accumulate in the leaf blade after being deposited in the transpiration stream (Munns, 2002). The sodium ions in most cases remain in the plant shoots because there is limited capacity of the phloem to recirculate the ions back to the root. As a result, the total accumulation of sodium ions is usually decided by the processes controlling sodium into the xylem of the roots (Munns & Tester, 2008). The ions in the leaves keep accumulating in the cells until they reach a point where the cell is no longer able to maintain an intra cellular ionic balance. In addition, sodium ions in the cell compete with potassium ions to bind to the active sites of enzymes causing the inactivation of some enzymes thereby affecting protein synthesis and ribosome functions. Other research has reported the replacement of potassium ions by sodium ions affected chloroplast functions (Slabu et al., 2009).

Salt tolerance varies for different and within crops. For instance, wheat plants belonging to different sub-species within the same species have varying responses when exposed to 150 mM (millimoles) of NaCl (Munns & James, 2003). Whereas in *Brassica napus*, salt tolerance can range between 50 to 173 mM NaCl among inbred lines (Wu et al., 2019). This implies that variations of tolerance to different levels of salinity can exist within the same species.

Previous research has shown two-week-old *M. oleifera* plantlets were able to tolerate 150 mM of NaCl for six weeks without dying. The salt-treated plants demonstrated a decrease in growth when compared with

plants not grown in conditions of salt stress. In this study, the NaCl was mixed in a nutrient solution and applied once. In the same study, it was also reported that plants treated with a 50 mM NaCl application showed similar leaf area, plant height and number of leaves as untreated plants (Al-Shoaibi & Boutraa, 2021) indicating that they could tolerate a level salt sufficient to define the soil as saline according to the classification by Munns and Tester (2008). Also, soil containing 150 mM NaCl equates to approximately 8.77grams of salt per litre of water extracted from saturated soil and is above the 3 grams of salt used to classify soils as saline as stated by Brouwer (1985).

Nouman et al. (2012b) also reported *M*. *oleifera* growth under saline conditions. In this research, young *M*. *oleifera* plants with only five-leaves, were found to withstand growth in saline conditions for thirty days. The salinity treatments of 4, 8 and 12 dS/ m were created by adding different amounts of NaCl to Hoagland's solution and applying this to the soil. Importantly, the results showed no significant difference between plants grown at 8 dS/ m and plants grown at 2 dS/ m for shoot length, shoot fresh weight and root fresh weight. This experiment suggests that *M*. *oleifera* plants can be grown in soils that are moderately saline according to the classification by US Soil Salinity Laboratory Staff (1954). Another study done by Fatima et al. (2018) showed *M*. *oleifera* plantlets survived growing in pots containing soil mixed with 150mM of NaCl from three weeks old to for five months.

2.2.8.3.1 Sodium and Potassium relationship in salt stress plants

Potassium is an important element for plant growth and development. It is required for protein synthesis, carbohydrate metabolism and enzyme activation. In addition, it is also required for the control of the stomata and photosynthesis. Potassium also helps decrease reactive oxygen species in plants by maintaining the electron transport during photosynthesis. Therefore, a deficiency of potassium in plant cells can reduce photosynthesis (Waraich et al., 2012). Another important role of potassium is maintaining osmotic balance and cytoplasmic homeostasis (Almeida et al., 2017). Under salinity stress sodium is accumulated within plant cells. Sodium then competes with potassium for potassium binding sites in biochemical pathways, disturbing the metabolism of the plant (Wang et al., 2013).

2.2.8.3.2 Biomass Indices

To measure the effect of salt stress on plant biomass, biomass indices can be used. These indices are derived from plant biomass measurements gathered from destructive sampling of plants. Salt stress Index, a common index used, is calculated as the percent of biomass produced over a specific time period and can compare salt stress plants with non-salt stress plants (Munns et al., 2002). Stress tolerance index is also used to assess plant biomass response to stress. Stress tolerance index uses both stress and non-stress populations, helping to make selection of genotypes that performs well in both conditions (Fernandez, 1992). Plant biomass indices help to identify plants that are more tolerant to salt stress than others.

2.3 Salinity in Guyana

Guyana is a native word which means "land of many waters". Guyana has an area of 216 000 Km^2. However, the coastal plain which contains most of the economic activity, population and agriculture has always been prone to soil salinity because of saltwater intrusion from the Atlantic Ocean. This happens because a major part of the coastal plain is below sea level and although it is protected by sea walls and mangrove forest system. At high tides sea water tends to enter inland (US Army Corps of Engineers, 1998).

Photo taken from https://www.climatehotmap.org/global-warming-locations/guyana.html showing overtopping of sea walls on Guyana's coastal plain.

Guyana's coastal plain consists of fertile low humic gleys (Braun, 1964). The plain represents approximately 4.2 percent of the total area of Guyana. Soils referred to as low humic gleys are also referred to as

hydromorphic soils that are rich in organic matter (Paula et al., 1987). However, hydromorphic soils are generally characterised by the reduction or localised segregation of iron because of the constant water logging. The ferrous iron (Fe^{2+}) in the soil gives it a bluish grey colour when it' accumulated because of the deprivation of oxygen by water logging. The soil can also have rusty looking patches if the iron moves towards the surface and oxidises (Duchaufour, 1982). Water logging is further supported by the land being below sea level in the low coastal plains. This makes draining the soil very difficult after heavy rain, as most of the drainage is by canals and the flow of the water depends on gravity and the tide. As a result, at high tides no water can drain off the land, simply because there is a negative gradient. So, land that is soaked with seawater from overtopping of the sea walls and saltwater intrusion must wait until the tide changes before the water can be drained.

Despite the land being below sea level, the country has most of its farmlands and about ninety percent of the population located on the coastal plain. Development in Guyana has been slow and as such there is limited access to other parts of the country because of the lack of roads (US Army Corps of Engineers, 1998), hence a densely populated coastal plain in relation to other areas within Guyana.

Apart from the threat of salinity from seawater over land, there are also other means by which soil salinity can occur. Guyana has three main rivers that flows into the Atlantic Ocean, exiting through the coastal plain, mixing fresh water with sea water, forming brackish water. Brackish water generally contains more salts than fresh water but lower than sea water and represents one percent of the overall water on earth. Moreover, brackish water is also formed by the intrusion of sea water into aquifers and can contain as much as 32.44 mM of NaCl as reported by Ortiz et al. (2005). This scenario is possible in Guyana where aquifers are the main source of water for crop irrigation (US Army Corps of Engineers, 1998), with the shallowest aquifer below the coastal plain having a salinity up to 12000mg of mixed salts per litre of water (FAO, 2015). In fact, in Guyana, salt water intrusion has been reported over the last ten years, especially during the dry season when there is less fresh water (Fraser, 2018). Currently there are patches of saline soil on the coastal plain of Guyana (Guyana Lands and Surveys Commission, 2013).

Sea water directly causes soil salinity because of two ions that are present within its composition, sodium ions (Na^+) and chloride ions (Cl^-) (Millero et al., 2008). Importantly, while high NaCl concentrations can cause soil salinity, soil salinity is not limited to NaCl ions alone, and can also refer to other salts or a combination of salts.

Soil salinity is a serious environmental issue (Shrivastava & Kumar, 2015) and as a result, the forages used to feed animals in Guyana must be able to grow in saline soil so that it can be cultivated widely along the coastal plain.

2.4 Forage and biogas production

The ability to grow in moderately saline soil and provide a high nutritional value are characteristics of an ideal forage crop for Guyana. However, there are other characteristics that a forage crop must have. Because a forage main purpose is for feed, in this case for cattle, its digestibility and fermentative behaviour in the rumen is also important.

Fermentative behaviour of forages or any feed as a whole, that is used in ruminant production, is important because different forages have different fermentation characteristics. Some forages produce more biogas than others. For example, a study evaluating the biogas production of sixty- two plant samples grouped into grasses, herbs, browse leaves, browse twigs, and legumes, grown in a temperate climate, showed that grasses can produce more biogas than herbs and legumes, while twigs produced an overall lower gas production in 72 hours of *in vitro* fermentation (Hummel et al., 2006). This study shows that there is difference in biogas production when different plant parts are used as feed. Another study showed a difference in biogas production among eight grasses grown in pastures in Iran (Kulivand & Kafilzadeh, 2015). In this research, all the substrates tested were grasses of different species and from different pastures located on different farms. This indicates that biogas production differences can also be found among different grasses, location and pasture mix. Similarly, Dal Pizzol et al. (2017) tested different species of grasses and legumes and a combination mixture of the two, sourced from tropical and temperate climates.

They found a difference in biogas production as a result of the different mixtures of forage and in addition, also showed that the climate where the forages were grown influenced the fermentation results. These experiments demonstrate the behaviour of forages in the rumen can vary as it relates to biogas production and that plant species, the climate in which they are grown, and plant parts affects rumen biogas production. In fact, Johnson and Johnson (1995) have suggested these reasons along with the level of feed intake, the type of carbohydrate in the feedstuff and ruminal microflora as deciding factors in ruminal biogas production.

Animals that are classed as ruminants such as cattle and goats can adapt to various dietary conditions especially if influenced by the environment. For example, ruminants can thrive on either fresh or dried grasses, or feeds high in fibre or starch. Unlike non-ruminant animals that have one stomach and a simple digestive system, ruminants have a stomach with four compartments: the reticulum, rumen, omasum and abomasum. The stomach in ruminants take up approximately 75% of the abdominal cavity. In ruminants, fermentation occurs in the reticulo- rumen which is before the abomasum and the small intestine. Fermentation is a process that occurs in the absence of oxygen. This absence of oxygen because of facultative bacteria and aerobic yeast using up oxygen that enters the rumen. This anaerobic condition is then maintained in the rumen and helps the ruminal microbes (bacteria, archaea, protozoa, fungi, yeast) thrive. These microbes ferment and break down plant cell walls into their carbohydrate fractions and produce volatile fatty acids (VFAs), such as acetate (used for fat synthesis), propionate (used for glucose synthesis), and butyrate. The animal later uses these VFAs for energy. It is within the rumen that substrates (animal feed) are broken down to monomers and these are converted relatively fast to carbon dioxide and methane. Some of the carbon dioxide produced will diffuse into the blood stream. However, majority of the methane is removed from the rumen by belching since this gas has a very low solubility in blood (Owens & Basalan, 2016).

2.5 Climate change and the greenhouse effect

The climate has changed over time because of the changes in the concentration and proportions of gasses in the atmosphere, particularly due to the actions of humans (Mikhaylov et al., 2020). This change is causing the earth's average temperature to increase, and this effect is termed the greenhouse effect. Any gas that absorbs infrared radiation emitted by the earth's surface and in turn emits radiation to the colder atmospheric temperatures is termed a greenhouse gas (Wuebbles & Hayhoe, 2002). The concentration of a gas (in the atmosphere) is a factor that decides how much effect the gas has in warming the atmosphere, along the wavelength at which it absorbs radiation and whether other gases absorb at the same wavelength. Some major greenhouse gasses include carbon dioxide, water vapor, nitrous oxide, chlorofluoromethanes, ozone and methane (Karakurt et al., 2012; Mitchell, 1989).

Although carbon dioxide is more abundant in the atmosphere than methane, methane is a major greenhouse gas because it has a larger warming potential than carbon dioxide (Myhre et al., 2013). Generally, methane has two major emission sources, natural sources and anthropogenic sources which contributes 40 and 60% of global emissions respectively (Karakurt et al., 2012). Natural sources of methane mostly include wetlands and other inland water systems. Anthropogenic sources of methane are sources deriving from human activities, such as the burning of fossil fuels, biomass burning, waste management and agriculture (Saunois et al., 2016). Because methane can be produced as a result of agricultural activities, it can be considered as a by-product of agriculture (Robertson et al., 2000).

In agriculture, the production of ruminant livestock, particularly cattle is a major source of methane (Johnson & Johnson, 1995). Other sources of methane include stored manure from these animals and from what is used in field to supply nutrients to crops. (Rotz, 2018). Methane emissions can be between 9 and 11 gram of methane per square meter (g CH_4/m^2) in pastures (Dumortier et al., 2017) and are mainly produced from the fermentation of the feed consumed by ruminants (Martin et al., 2010; Ramírez-Restrepo et al., 2016). Livestock contributes to approximately 44% of global methane emissions of which cattle is responsible for

28.6% out of the 44 % (Gerber et al., 2013). As such modifying the diets of ruminants can be part of a strategy to help lower methane production worldwide (Black et al., 2021; Dini et al., 2018; Johnson & Johnson, 1995) and help the fight against climate change.

Climate change is of great importance in the world and several initiatives were taken to address implications and future risks. The Intergovernmental Panel on Climate Change (IPCC) was created in 1988 by the World meteorological Organization (WMO) and the United Nations Environment Programme (UNEP) to provide governments with scientific information on climate, so that they can make and develop policies to address climate change (Intergovernmental Panel on Climate Change, 2021). Also, "taking urgent action against climate change" is one of the United Nations sustainable development goals (United Nations Department of Economic and Social Affairs, 2021). In support of these initiatives for the reduction of greenhouse gas emissions, searching for a low methane emission feed must be included as a pre- breeding prerequisite.

2.5.1 Approaches to lower methane production

Over the years there have been discussions on how the global emissions of greenhouse gasses can be reduced, particularly methane produced from cattle (Boadi et al., 2004). Methane emission is a function of the amount of cattle, how well they convert feed in to meat or milk and how the manure or waste from these animals are handled (Gerber et al., 2013). Consequently, majority of the existing strategies to help reduce methane emissions include increasing the productivity of the animal. Animal productivity involves improving the overall production system holistically through, nutrition management, improved genetics and reducing the unit of methane produced per unit of meat or milk. By increasing animal productivity, the production of meat or milk per animal will increase and thus reducing the number of animals needed to acquire a targeted total production, thereby reducing methane emissions. Nutritional and management strategies are also utilized. For instance, maximising the feeding frequency or adjusting the feed intake to get the best feed conversion and complimenting these with the appropriate grazing strategies (Boadi et al.,

2004). Another approach includes forage processing, such as the grinding or pelleting of feed to improve its consumption. In contrast, other strategies to modify and control rumen fermentation are more aggressive. For example, elimination of rumen protozoa by dietary or chemical agents (Van Nevel & Demeyer, 1996) or using chemicals to directly inhibit methane production (Mathison, 1997).

During the last decade consumer awareness has become higher with more people are becoming cautious of what is in the food they eat and what risks chemicals present can pose to human health (Ergönül, 2013; Hartmann & Klaschka, 2017; Sim et al., 2019). Coincidentally, leading up to the growing awareness of chemicals in food, in 2006 the European Union banned the use of antibiotics in livestock feeds because of the risk of passing antibiotics resistance from animals to humans (The European Parliment, 2003). These are some of the main reasons that helped pave the way for research into plant compounds to help reduce methane emissions and overall animal productivity. This can be illustrated by Cieslak et al. (2012) and (Makkar et al., 2009) by their work on searching for plant metabolites beneficial to animal production. In both studies by Makkar et al. (2009) and Cieslak et al. (2012) plants were evaluated for metabolites including tannins and their effect on livestock such as measuring palatability and effect on rumen microbial fermentation.

2.5.2 Measuring methane emission of feeds

Common methods to measure the methane and total gas produced from feeds involve the use of *in vitro* incubation systems. In *in vitro* incubation systems, feeds are mixed with rumen fluid and incubated to mimic the animal's digestive system. Early systems were designed to measure endpoint measurements of fermentation (Tilley & Terry, 1963). In the 1970s the Hohenheim gas test was developed. This system uses syringes made out of glass as incubation chambers, the feed and rumen fluid are then added and gas fermented is measured by the movement of the pistons inside the cylinders (Menke et al., 1979). In the 1990s automated systems were developed to provide real time measurements of gas from fermentation (France et al., 2005). Some of the first systems measured gas production for an entire 24 h releasing pressure build up using solenoid valve (Davies et al., 2000). Most recent a fully automated system was designed and

developed to measure methane, hydrogen and total gas production for screening feeds for low methane emission (Muetzel et al., 2014).

Previous studies had reported that *M. oleifera* is able to reduce methane (CH_4) emissions from enteric fermentation (Parra-Garcia et al., 2019; Pedraza-Hernández et al., 2019; Soliva et al., 2005; Soltan et al., 2019). But methods used to determine methane production can vary. For instance, Soliva et al. (2005) used a rumen simulation technique (RUSTEC) and a Hohenheim gas test with gas production measured using a gas chromatograph. Whereas Parra-Garcia et al. (2019) and Pedraza-Hernández et al. (2019) used pressure transducer to determine total biogas production and methane production was quantified with a tetra3 gas analyser. While, Soltan et al. (2018) estimated methane emission stoichiometrically based on the observed *in vivo* short chain fatty acids (SCFAs) from a prediction model. In general, methods that use bottles to measure gas production produce similar results, but automated systems that release gas constantly into a gas chromatograph can produce high density data that can be used to model gas data, and make comparisons between total gas and methane produced (Muetzel et al., 2014).

2.5.3 *M. oleifera* and greenhouse gas case reduction

Various *in vitro* fermentation experiments have shown *M. oleifera* can lower methane production. *M. oleifera* was added either as an extract or dried forage, while others used extracted root bark. For example, *M. oleifera* leaf extract was used in combination with soyabean hulls to feed Holstein steers. The *M. oleifera* leaf extract was made from leaves randomly collected from plants growing in Veracruz, Mexico. The leaves were crushed, and one gram mixed with eight millilitres of water. They were then left for seventy-two hours at 28°C then for one hour at 39°C. Three diets were used containing 0, 75 and 150 g/kg dry matter soya bean hull, and to each diet, *M. oleifera* was added at concentrations of 0.6, 1.2, 1.8 ml/g. Results showed that as the concentration of *M. oleifera* leaf extract increased in the diets, the asymptotic methane production decreased. This occurred in diets that contained 0 and 75 g/kg DM of soya bean hull, but was kept constant for the diet containing 150 g/kg DM. Nevertheless, in diets containing 150 g/kg DM of soya bean hull, the rate of methane production was reduced from 0.027 (mL/g DM)^2 to 0.012 (mL/g DM)^2 using 0 and 1.8 ml/g

leaf extract respectively, demonstrating a reduction in the rate by more than half (Parra-Garcia et al., 2019). Similarly, research on *M. oleifera* was found to lower methane production in goats. The leaf extract was prepared by mixing ten grams of grounded leaf with 90 ml of water and treatments included, 0, 0.6 and 1.8 ml /g DM. However, in this experiment live yeast cultures were also added at 0, 2 and 4mg/g DM. The basal diet to which these treatments were added contained oat straw, corn, soya bean paste, urea, molasses, sunflower oil, and vitamins and minerals. The results showed that *M. oleifera* leaf extract as a feed supplement in male goats (Nubia x Criollo) along with *Saccharomyces cerevisiae* (live yeast culture) at various doses, decreased methane production (Pedraza-Hernández et al., 2019).

Moreover, Soltan et al. (2019), contrary to the previous two experiments, used *M. oleifera* root bark extract. In this study, the root bark was collected from five to six months old *M. oleifera* plants grown in Alexandria, Egypt. The root bark extract was made by scraping the outer layer of the roots by hand, drying the material for 72h at 40°C and then making an ethanol extract using 100g of dried material in 1000ml of water and ethanol mix (700 ml/L). Results using data from the *in vitro* experiment showed that *M. oleifera* root bark extract added to the basal diet (clover hay, ground maize, soyabean meal, wheat bran and cotton seed meal) at 50 g/kg DM significantly reduced methane production at the end of 24h when compared to using *M. oleifera* extract at 25 g/kg DM and monensin (an ionophore antibiotic that modifies rumen microbes) (Soltan et al., 2019).

Another study showed that ethanol extract of *M. oleifera* leaf and stalk from mature two-year-old plants growing in Nicaragua lowers methane production. In this study the ethanol extraction was done by mixing 1 kg of fresh *M. oleifera* leaves with 3 litres of ethanol in a food blender. Afterwards, the mixture was filtered until the outflow was clear and the residue (ethanol extracted *M. oleifera*) was then extracted with acetone, dried and sieved. Four dietary treatments were used, soya bean meal, rapeseed meal, unextracted Moringa leaves and extracted Moringa leaves. These treatments were then added separately to diets consisting of hay, barley and straw meal. All the diets and their treatments were formulated to have the same amount of protein. The results showed diets containing *M. oleifera* had significantly lower methane production (ml per

day) compared with diets of soyabean and rapeseed meal (Soliva et al., 2005). These studies provide evidence that *M. oleifera* can be beneficial to help mitigate the methane emissions from cattle.

2.6 *M. oleifera* Genetics and Breeding

M. oleifera is a diploid, which means it has a paired chromosome, 2n, one from each parent. (Duke, 1978; Patel & Narayana, 1937; Ramachandran et al., 1980). Although *M. oleifera* plants can have twenty six percent selfing (Muluvi et al., 2004), the plants have a wide genotypic (Ravi et al., 2020) and phenotypic (Dao et al., 2017) variation if propagated sexually by seeds. *M. oleifera* is therefore adapted to both selfing (geitonogamy) and outcrossing (xenogamy), having a higher fruit and seed set when outcrossed (Jyothi et al., 1990). Research by Ramachandran et al. (1980) indicated variation in flowering time, where some varieties flower throughout the year and some during two distinct seasons. Other research has shown seeds from different plants possessing different values for the same traits. For example, a study conducted at Ouagadougou, Burkina Faso, demonstrated that seeds from twelve *M. oleifera* provenances from different ecological zones had highly significant differences for seed length, thickness and seed weight at a confidence level of $P < 0.001$ (Dao et al., 2017). In addition, Stevens et al. (2015), reported variations in anti- nutritional compounds such as oxalate, phytate and tannin throughout different provenances of *M. oleifera* from different parts of Nigeria. Also, significant variations in kernel oil were detected from seeds of different plants within a population of *M. oleifera* cultivated in the northwest region of Argentina (Ayerza, 2011). These differences signify that there is significant variation among *M. oleifera* provenances and cross pollination is dominant in *M. oleifera*. It obviously suggests an advantage of using stem cuttings (Palada, 1996) to obtain clones and reduce genetic variation once varieties with desirable characteristics have been developed from breeding programmes.

2.6.1 Varieties of *M. oleifera*

M. oleifera is grown for seeds or leaves. The leaves are mostly used as a food source for humans and animals and the seeds are used for their oil and water purification purposes. Accordingly, varieties have been developed to meet these needs, with some varieties better for seed production and others better for leaf production. For instance, two varieties have been developed in India, Periyakulam 1 (PKM-1) and Periyakulam 2 (PKM -2). PKM-1 is a variety that is grown for seeds, which are used mainly for oil (Lalas & Tsaknis, 2002). The variety was developed after inbreeding for six generations and released in 1989. The average yield is 220 fruits/tree/ year. PKM-2 was developed after PKM-1, also for seed production, from hybridization of two local varieties in India, the Eppothum vendran and the Arasaradi. Both PKM-1 and PKM-2 bear the same number of fruits per tree each year, but the fruits of PKM- 2 are larger and therefore can attain 280g/fruit as compared with PKM-1 which only has the potential to achieve 150g/fruit. Plants of both varieties are annuals and were developed within a span of 10 years (Ponnuswami, n.d).

M. oleifera plants are present in different countries and have grown there as naturally occurring populations without human intervention (selection), except for their introduction. This suggest that each population would have been subjected to natural selection. These ecotypes are as a result of the genotypical response of the species to the habitat (Gregor, 1944) and because of this, phenotypic differences among ecotypes have been encountered (Förster et al., 2015). In other words, *M. oleifera* plants that have grown in different locations within different countries and are exposed to different climates are sometimes referred as ecotypes. For instance, in India some ecotypes present are Jaffna, Chavakacheri Murungai, Chemmurungai, Palmurungai, Kodikalmurungai, Puna murungai and Kodikkal Murungai (Ramachandran et al., 1980) while in Mexico there are, Culiacan, Elota, Guerrero, Mojolo, Sinola and Sonora ecotypes (Pérez-Ángel et al., 2020). In South Africa, *M. oleifera* is known as the South African ecotype (Moyo et al., 2011), in Zambia it is known as Zambian *M. oleifera* and also referred as a wild plant from India (Barichella et al., 2019), while other studies have mentioned *M. oleifera* as provenances. Hence, the *M. oleifera* plants are named directly after the location where they thrived. For example, *M. oleifera* plants from -Tamale, Ghana is called provenance

from Tamale (Dao et al., 2017). Although *M. oleifera* plants are sometimes called ecotypes and provenances, both terms have a connection to the location where they were adapted. This signifies there is a vast diversity around the world which has adapted to different climatic conditions. As a result, there are various seed banks around the world that contains collections of *M. oleifera* seed material, mainly, the Educational Concerns for Haiti Organization (ECHO), Tamil Nadu Agricultural University, the United States National Plant Germplasm collection, the Svalbard Global Seed Vault, the World Vegetable Centre and International Moringa Seed Bank (Sustainable Bioresources LLC, 2021).

These seed banks provide long term storage of different Moringa accessions that were collected from locations around the world and help make the accessions more easily available, but material transfer agreements are still needed. In other words, the accessions stored in the seed banks can be useful to create base populations for breeding programmes, which can then be used to select desired traits to create an elite or improved cultivar. In the Guyana context an elite cultivar would be a cultivar that can be grown in a wide range of environments, has moderate salt tolerance and at the same time maintains its nutritional content. Ideally having one or two cultivars that can be grown in any location within the country.

2.6.2 Genetic Diversity

The first step in a breeding programme is the establishment of a base population to create genetic diversity. Except for clones, individuals occurring naturally of a given species are not genetically identical because their DNA differs. These differences are known as the polymorphism of a species and these polymorphisms are what make up the genetic diversity of the species (Lewontin & Hubby, 1966). It is this genetic diversity that enables a species to respond to environmental changes (Hake & Ross-Ibarra, 2015), and in most cases are important for the survival of the species (Booy et al., 2000).

Assessing genetic diversity is therefore important to determine the differences within and among species and will help to pinpoint the origin of the species where the centre of diversity exist. The place of origin of a species contains landraces and wild relatives of that species and is considered a gene bank in itself. At the

place of origin, the species have had to adapt in unfavourable conditions to survive. Hence, the species growing in its place of origin will contain numerous genes to tolerate various biotic and abiotic stresses (Bhandari et al., 2017). Knowing the genetic diversity will also help to explain how the species was domesticated and in the long term preserve the germplasm through the protection of the natural habitat (Badr & El-Shazly, 2012). Importantly, assessing the differentiation of *M. oleifera* provenances from Guyana, India and King seeds by detecting polymorphisms, will help to determine genetic diversity that exists. This will be the first step of knowing its potential to contain potential genes that can be used in crop improvement to create elite cultivars.

Chapter 3 Salinity screening of *M. oleifera* provenances

3.1 Introduction

Soil salinity is an abiotic stress that affects plant development and subsequently reduces productivity (Munns & Tester, 2008). Tolerance to soil salinity can vary for different crops (Munns & James, 2003; Wu et al., 2019). Previous studies have shown *M*. *oleifera* can grow in moderately saline soils (Al-Shoaibi & Boutraa, 2021; Fatima et al., 2018). What is similar among previous studies is that *M*. *oleifera* showed satisfactory growth in 50 mM of NaCl but at 150 mM NaCl a decrease in growth was observed. This suggests that *M*. *oleifera* at a young age cannot withstand a concentration of more than somewhere between 50 mM and 150mM of NaCl in its growth medium or soil. However, variation in salt tolerance across different plants of the same species indicates that more work is needed to screen genotypes before a conclusion is made on the salinity tolerance of a species (Negrão et al., 2017).

Therefore, in this study different *M*. *oleifera* provenances of known origin were screened for salt tolerance and survival by mimicking the soil and saline conditions in Guyana. It is hypothesised that different *M*. *oleifera* provenances can tolerate moderately saline soil at an age when it has true morphological leaves (9-10 weeks) and older after sowing.

3.1.1 Objectives

In this study, six provenances of *M*. *oleifera* were used to screen for salinity tolerance. Seeds of Queenstown, Mon Repos, Bush lot and Benab provenances were sourced from Guyana and PKM-t and PKM-g from India. The objectives were to: (1) determine the effect of salinity on plant height, visual morphological quality and biomass, (2) identify some causes of salinity responses by measuring the net photosynthesis of leaves of different provenances of *M*. *oleifera*; and quantify the residual salt in the potting mixture by measuring the electrical conductivity. (3) quantify the plant's uptake of Na^+, by measuring the concentration of Na^+ in the young mature leaves of *M*. *oleifera* plants.

3.2. Materials and methods

3.2.1 Location

The greenhouse experiment was conducted between August and October 2021 at the Plant Growth Unit (PGU), Massey University, Batchelar Road, Palmerston North (-40.37807, 175.61362), New Zealand.

3.2.2 Plant material

Seeds from six *M. oleifera* provenances were used in this experiment. Four of the provenances from Guyana (Queenstown, Mon Repos, Bush lot and Benab (see appendix 3)) and two provenances; PKM-t and PKM-g, originally sourced from Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India and villages of Deoria District, Uttar Pradesh India, respectively and had been stored at Massey University from 15 November 2019 in sealed containers at 5°C.

3.2.3 Greenhouse conditions

The greenhouse was maintained at an average temperature of 25.1 ^{o}C ±0.044, with a relative humidity of 60-70%. Natural light was not controlled. The temperature in the greenhouse was recorded every half an hour by a MicroLogger (Model T, Hortplus, New Zealand) that was placed at plant canopy height in the centre of the greenhouse and humidity was measured and monitored by a wireless thermometer and hydrometer (Model XC-0322).

3.2.4 Planting pots

The seeds were directly sown in pots and allowed to germinate. Each pot (24cm diameter, 9 litres) was filled with 8.5 litres Daltons Base mix + Osmocote Pro ® (see Annex 1 for details about potting mix). Five seeds were sown in the centre of each pot in a quincunx system at a spacing of 7.5 cm to simulate a high-density planting system that would be similar if the plants were used in a cut and carry method feed system. The plant spacing used also allowed the experiment to have adequate replication, because the available number of planting pots and greenhouse space were a limiting factor. One hundred and ninety seeds of each

provenance were sown in the first week of August 2021, 150 were sown directly in the planting pots and 40 in smaller containers for transplantation if needed. On September 13, 2021, seeds that did not germinated were replaced with plants grown from the extra seeds sown, ensuring there are at least 8 plants per treatment where possible.

3.2.5 Salt treatments

The application of salt treatment began on 27 September 2021 and ended on 20 October 2021. Three salt treatments were used, 0 mM NaCl (0g of NaCl in 1 litre of water (0 mS/cm)), 52 mM NaCl (3.04g NaCl in 1 litre of water (5.78 mS/cm)) and 156 mM NaCl (9.12g NaCl in 1 litre of water (16.79 mS/cm)). The electrical conductivity (EC) of the different concentrations used was measured using HI 8733 multi range EC meter by Hanna Instruments at 24 ^{o}C and adjusted to a conductivity using the formula EC at 25°C = EC of sample ÷ (1 + (0.02 × (temperature of sample °C – 25))). Salt solutions were made by either mixing 3.04g or 9.12g of NaCl in one litre of reverse osmosis (RO) water to obtain 52mM NaCl or 156 mM NaCl respectively.

The different salt treatments were applied once every seven days for a total of twenty-eight days by immersing plant pots in 20-litre buckets containing the respective salt solution. This allowed the salt solution to cover two thirds of the pots. For immersion, the buckets were filled with five litres of either 52 mM NaCl or 156 mM NaCl salt solution, and pots were immersed for thirty minutes. Pots were left for thirty minutes to simulate flash flooding that may be caused by sea water over topping and intrusion. The treatments that received 0 mM NaCl were irrigated with reverse osmosis water every two days during the experimental period. Treatments that received 52 mM NaCl and 156 mM NaCl were re-watered as soon as the surface of the potting mixture started to dry with 500 ml of RO water to maintain the potting mixture at field capacity. The volume of water used also helped to avoid the leaching of water through the bottom of the pots which may result in the loss of salts. Irrigation with reverse osmosis water was chosen over tap water because RO water has less salts than tap.

3.2.6 Experimental design

The experiment was set up in a randomised complete block design with five blocks. Each block contained eighteen treatments (6 provenances *3 salt concentrations) and for each treatment 10 seeds were sown in two pots (five seeds in each pot). The blocks were laid out parallel with each other along the length of the greenhouse. The location of the greenhouse vents may cause variations in temperature throughout the greenhouse and hence areas and hence plants closer to the vents may be cooler as compared to areas that are further away. The block orientation was chosen with the aim of having any variation due to position accounted for in the blocking. Variation in temperature within the greenhouse was possible because the greenhouse used was not fully sealed, and air flow was controlled by the opening of vents by hydraulic rams controlled by a temperature sensor. In addition, the greenhouse is situated parallel to another greenhouse that has extractor fans to cool it. As such the hot air from one greenhouse is sucked in and blown into the greenhouse where the trial was conducted when the vents were opened.

3.3 Measurements

3.3.1 Plant emergence

A count of plants that emerged was done 28 days after the sowing of seeds (3 September 2021). This was done in one count because of the restrictions by Covid-19 lockdown.

3.3.2 Plant height

Plant height measurements were done in millimetres using a graduated measuring tape. The height of each plant was measured once before and after the salt stress period. This approach was chosen to measure growth during the salt stress period alone and to exclude growth variations that occurred before the salt treatments. The height of each plant was measured from the surface of the potting mixture to the tip of the shoot. To ensure the accuracy and consistency of plant height measurements, each pot was filled with the

same amount of potting mixture and the surface levelled after filling. Each seed was also sown at a depth of 2 cm.

3.3.3 Chlorophyll

Soil plant analysis development (SPAD) measurements (a unitless measurement) were taken from each treatment to measure leaf chlorophyll content. Eight measurements were taken randomly using the SPAD CCM 200 plus by Opti sciences from the first fully open leaf of two plants within each treatment at the beginning and the end of the salt stress period. The leaves were inserted into the SPAD meter avoiding the leaf mid-rib and only taking measurements from the leaf blade where possible. SPAD measurements that include leaf mid-rib may be inaccurate because it does not represent the photosynthetic area of the leaf blade. However, in some cases the leaves were small, and the mid-rib was unavoidable, but the leaf area was still sufficient to obtain a SPAD value.

3.3.4 Visual morphological quality of plants

Each plant was subjected to a visual scoring method adapted from Niu et al. (2007) to score the leaves of plants at the end of the salt stress period (28 days after the first application of salt stress). The visual scoring contained a scale of 1-5, in which 1= severely stunted growth with over 50% foliage salt damage (leaf necrosis, browning) or dead; 2= somewhat stunted growth with moderate (25-50%) foliage salt damage; 3= average quality with slight (less than 25%) foliage salt damage; 4 = good quality with acceptable growth reduction and little foliage damage; and 5 = excellent with vigorous growth and no foliage damage. The scoring was done for all the plants by one person on the same day to help reduce any human error.

3.3.5 Plant Biomass

The fresh weight (FW) and dry weight (DW) of above ground plant biomass (referred in this study as plant shoots) and plant roots were measured after the completion of salt stress. Plants were harvested over five-

days (28th October to 1st November 2021). Each day, plants from one block were harvested. The plants were manually uprooted, and the shoots and roots were separated and weighed. Next, they were placed in individual paper bags in an oven (Contherm Scientific Precision Cabinet, New Zealand) at 60°C for five days. After five days, random samples of plant root and shoot were collected from different positions in the oven and weighed to track water loss. The same samples were then re-weighed two days after to ensure there was a constant weight. Once no change in weight was observed the shoots and roots were weighed again, and the dry weight recorded. The dry matter in percentage for the root and shoot of each plant was then calculated using the formula (DW/FW) / 100, where DW is the plant dry weight and FW is the fresh weight of the same plant.

3.3.5.1 Biomass indices

The relative decrease in plant biomass (RDPB), salt tolerance (ST) index and stress tolerance index (STI), root mass ratio (RMR) and the relative root mass ratio (RRMR) were calculated for all provenances (average for each provenance) according to the equations below (Negrão et al., 2017).

3.3.5.1.1 Relative decrease in plant biomass (RDPB)

The relative decrease in plant biomass (RDPB), was calculated using the fresh mass of plants (M_f) at the end of the experiment, grown under control and salt conditions for all provenances, according to the following equation:

$$\mathrm{RDPB} = \frac{\mathrm{M_{f,control}} - \mathrm{M_{f,salt}}}{\mathrm{M_{f,control}}}$$

Where $M_{f,\ control}$ is the plant's fresh mass under control conditions and $M_{f,\ salt}$ is the plant's fresh mass under salt conditions. The RDPB was calculated for each provenance at 52 mM NaCl and 156 mM NaCl using mean values for $M_{f,\ control}$ and $M_{f,\ salt}$ for each provenance obtained from data collected from each plant.

3.3.5.1.2 Salt tolerance (ST) index and stress tolerance index (STI)

The salt tolerance (ST) index and stress tolerance index (STI) were calculated for all provenances at 52 and 156 mM NaCl using mean values according to the following formulas:

$$ST = \frac{Y_{salt\ at\ T_2}}{Y_{control\ at\ T_2}}$$

$$STI = \frac{Y_{control}}{Y_{av}} \times \frac{Y_{salt}}{Y_{av}}$$

Where, Y in these equations denotes dry weight as a trait, and Y_{av} refers to the average dry weight of all the genotypes under control conditions for trait Y. For the calculation of the salt tolerance for each provenance, $Y_{salt\ at\ T_2}$ is the mean dry weight of plant stressed at 52 mM or 156 mM NaCl and $Y_{control\ at\ T_2}$ is the mean dry weight of plants that were treated with 0 mM NaCl at the end of the stressed period.

For the calculation of stress tolerance index for each provenance, $Y_{control}$ is mean value of plants treated with 0 mM NaCl and Y_{salt} is mean values for either 52 or 156 mM NaCl at the end of the experiment.

3.3.5.1.3 Root mass ratio (RMR) and relative root mass ratio (RRMR)

The root mass ratio (RMR) and the relative root mass ratio (RRMR) were calculated for all provenances at 0, 52 and 156 mM NaCl using the following equations below:

$$RMR_{control} = \frac{M_{d,root,control}}{M_{d,control}}$$

$$RMR_{salt} = \frac{M_{d,root,salt}}{M_{d,salt}}$$

$$RRMR = \frac{RMR_{salt}}{RMR_{control}}$$

where $M_{d,root,control}$ and $M_{d,root,salt}$ are the root's dry mass under control and salt conditions, respectively, and $M_{d,control}$ and $M_{d,salt}$ are the total plant's dry mass under control and salt conditions, respectively. $M_{d,root,control}$, $M_{d,root,salt}$, $M_{d,control}$ and $M_{d,salt}$ were averaged for each provenance.

3.3.6 Net Photosynthesis

The net photosynthesis and stomatal conductance were measured using one plant selected randomly within each treatment using the LI- 6800 portable photosynthesis system (LI-COR Biosciences). The first fully opened, expanded leaf (counting from the top of the plant) of one random plant within each treatment was used. Measurements were taken once before the application of salt treatments and every seven days until 28 days (5 times). For each measurement, the leaf area was calculated and entered manually into the Li-COR 6800. *M. oleifera* leaves are tripinnate and each leaflet has the shape of an ellipse. Therefore, the leaf area = πab, where a = ½ width and b= ½ length of the leaflet. The process of calculating the individual leaf area is important because the Li-Cor system calculates the net photosynthesis in relation to the leaf area and therefore not adjusting the leaf area individually can produce inaccurate readings of photosynthesis and stomatal conductance.

These measurements were taken between 10 am and 2 pm to minimize variations in photosynthesis that may arise because of changing sunlight intensity throughout the day. A limiting factor was the time it takes for one measurement (5-10 minutes.) As a result, one measurement was taken from each treatment. Measurements were taken over a three-day period, with blocks 1 and 2 the first day, blocks 3 and 4 the second and block 5 on the third day.

3.3.7 Potting mixture EC measurements

The electrical conductivity of the potting mixture within each pot of each treatment was determined according to the US Soil Salinity Laboratory Staff (1954), with some modifications. After the extraction of the plants for biomass measurements, pots were left in the greenhouse with potting mixture to air dry for two weeks. Afterwards approximately 250 g of potting mixture were collected using a hand trowel from the centre of each pot to a dept of 10 cm. The potting mixture sample from each pot were placed in paper bags and left to air dry for a 24h. The samples were then taken for analysis in the laboratory. From each bag 50 g

of potting mixture was weighed using a digital balance, placed into a 1000 ml conical flask and 250 g reverse osmosis water added to make a 1:5 soil water extraction. The flask was then shaken at 200 rpm for 30 minutes using a Gyrotory orbital shaker (New Brunswick Scientific Company, Connecticut, USA). The extraction process was done in batches of 12, the maximum number of 1000 ml flask the shaker can accommodate. After 30 minutes of shaking, the contents of the flask were allowed to settle for 5 minutes. The potting mixture water solution was filtered using glass wool into a 100ml beaker and the electrical conductivity of the filtered liquid was measured at room temperature using a HI 8733 multi-range EC meter (Hanna instruments).

3.3.8 Determination of Sodium and Potassium concentration in plant leaves

3.3.8.1 Leaf selection and preparation

The first fully expanded mature leaf (counting from the shoot tip), from each plant was selected for the determination of sodium and potassium concentration. This was the same leaf used to measure photosynthesis in the last week of the experiment. The leaf of each plant was collected at the same time when the plants were uprooted for fresh weight measurements. They were then placed in separate paper bags and dried at 60 ^{o}C for 72h. When drying was complete, the leaves were stored at the PGU research Laboratory. After three months of storage, leaves were again placed in the oven set at 65°C for 24h. This was done to remove any moisture that had accumulated within the leaves during storage period, so they could be ground. The leaves were then ground using a mortar and pestle and then placed in resealable plastic bags (62mm x 75mm).

3.3.8.2 Acid digestion of leaves

Ground leaf samples were then weighed and placed in cation digest tubes. 0.1g of each leaf sample was weighed and place in separate cation digest tubes. These tubes were specially marked at 25 ml (for dilution

of plant digest). Leaf samples (each pertaining to one plant) were selected randomly within a treatment and placed in the digestion blocks in ascending order of the treatment number. Hence, starting with treatment one and ending with treatment eighteen and repeating this cycle until all digestion wells were filled. This was done in order to make sure that there are samples from each treatment within each digestion block.

There were three digestion blocks, each consisting of 32 wells. In each block, a blank sample was used (nitric acid), a standard (coconut fibre from Wageningen University) and two duplicates, which were randomly selected leaf samples that had enough dry material to be tested in duplicate. A total of five digestions were done, corresponding to the number of blocks in the greenhouse experiment. Each digestion consisted of 96 samples.

Digestion was done in a fume hood by adding 4 ml of concentrated nitric acid to each sample, placing a small funnel over each tube, and leaving it to digest with reflux overnight at 120°C on the heating block. After digestion, the contents of the tubes were evaporated to dryness by removing the funnels and the temperature increased 30°C every 30 minutes until 210°C and maintained at 210°C until all tubes were dried. The tubes were then removed and 5 ml of 2M HCL made up with deionised water was added to each tube. The tubes were left for 90 minutes to dissolve the residues. Afterwards deionised water was then added to each tube to make the volume up to 25ml. The solution was then placed in screw cap plastic containers for storage.

3.3.8.3 Analysis of digested plant material

The digested plant material was analysed using a Microwave plasma atomic emission spectrometer (Agilent MP-AES 4200). Samples were prepared for analysis by adding 0.5 ml of 20,000 parts per million (ppm) Caesium chloride solution to 9.5ml of digested plant material solution to a 10 ml tube to achieve 1000ppm.

Samples were analysed in batches of 96 for sodium and potassium using 568.263 nm and 769.897 nm wavelengths respectively. A first run was done using standard solutions containing 0,0.625, 1.25,2.5, 5,10,20 ppm of sodium and potassium with the first eighteen samples to determine potassium and sodium limits. This was then readjusted to 0, 5, 10, 20, 40, 80, 160, 320 and 640 ppm for potassium and 0, 0.625, 1.25, 2.5,

5,10, 20, 40 and 80 ppm for sodium. One mixture of each standard solution was made for both sodium and potassium. Each standard solution was made up to 20ml using 0.4M HCL (similar matrix of the digested plant material) and contained 1000ppm caesium as an anti-interference agent. For the plotting of standard curves, a correlation coefficient limit of 0.990 was used.

3.3.8.4 Conversion of results from parts per million to grams per kilogram

Results obtained from the MP- AES were converted from ppm to g/Kg. The results obtained were multiplied by a total dilution factor (df). The first dilution factor was calculated by dividing the total volume of the solution after digestion (in litres) by the weight of the respective sample. For example, 0.025 L/ 0.001 Kg. The second dilution factor was calculated by dividing the total sub- sample used for the analysis (10 ml, which contained 9.5 ml of digested solution and 0.5 ml caesium) by the sample of the digested solution used (9.5 ml) for example 10 ml/9.5 ml. The total dilution factor was then obtained by multiplication of both dilution factors. The total dilution factor was then multiplied by the result obtained from the MP-AES since 1 ppm = 1 mg/L and then further divided by 1000 to convert mg/Kg to g/Kg.

3.4 Statistical Analysis

All analyses of variance (ANOVA) were done using Genstat 21st edition (VSN International, 2021).

Data for seed emergence, net photosynthesis and SPAD were analysed by ANOVA, using all the data collected within each treatment. Initial plant height and plant growth (height) during the stress period were analysed separately using ANOVA but using an average per treatment. Summary statistics were done to present the mean, maximum and minimum value and standard error of the mean, least significant difference (LSD) and the significance value (P-Value). All multiple comparison tests for these parameters which showed significant differences were subsequently analysed using Tukey multiple comparison test.

Data obtained from evaluating plant visual score and biomass produced unbalanced results and hence analysed as individual plants. Unbalanced results were not obtained because of the design of the experiment. The experiment was set up as a balanced design with 18 treatments, 6 provenances and 5 blocks. The design became unbalanced because there was an uneven emergence which caused an uneven number of plants per treatment, and secondly, as the experiment progressed plants died as a result of salt stress. As such, the data cannot fit into a normal analysis of variance model because there was not the same number of plants per each treatment. The data was instead analysed using unbalanced ANOVA using Genstat 21st edition (VSN International, 2021). This fits the unbalanced data to completely general model. Where there was a significant difference, the Bonferroni multiple comparison test was used to compare means.

Charts to show plant visual score quality were done using SPSS version 27 (IBM Corpporation, 2020). For plant visual score, a provenance by salt concentration comparison was done. The standard error, least significant difference and significance were also calculated.

Biomass data were analysed as percent dry weight (%) and dry weight for shoots, roots and whole plant. Each biomass parameter was analysed individually to determine differences among provenances and among salt concentrations. The mean, least significant differences and significances were calculated. Multiple comparison test to compare means was done using Bonferroni multiple comparison test and the estimated standard error provided.

The relative decrease in plant biomass (RDPB), salt tolerance (ST) index and stress tolerance index (STI), root mass ratio (RMR) and the relative root mass ratio (RRMR) were calculated for all provenances from raw data using Microsoft Excel in Office 365.

Data for potting mixture electrical conductivity for the potting mixture of each pot after the twenty-eight days of stress was analysed using an analysis of variance. The means for provenances, salt concentrations, standard error of the mean, least significant difference, and significance (P-Value) were calculated and

presented. The Tukey multiple comparisons test was used to compare means among provenances and means among salt concentrations.

Plant leaf sodium and potassium concentrations were analysed using an analysis of variance using all data obtained from the MP-AES.

3.5 Results

3.5.1 Plant emergence

There were significant differences ($p < 0.001$, Table 3.1) for plant emergence among the different provenances. An analysis of variance showed that Mon Repos had the lowest emergence and Queenstown the highest. PKM-g, PKM-t and Queenstown had similar emergence values all above 8 plants per treatment and over 120 plants total for each.

Table 3.1 Mean number of plants for each provenance per treatment at 30 days after sowing. The minimum and maximum number of plants is also presented along with the standard error of the mean, least significant difference ($LSD_{0.05}$) and significance (P-Value).

Provenance	Mean number of plants per treatment	Minimum in any one treatment	Maximum in any one treatment	Maximum possible	Standard error of mean	Total plants emerged
Benab	4.3^{b}	2	7	10	0.360	65
Bush Lot	6.3^{c}	3	9	10	0.454	95
Mon Repos	1.9^{a}	0	3	10	0.305	27
PKM-T	8.5^{d}	7	10	10	0.191	128
PKM-G	8.2^{d}	6	10	10	0.315	124
Queenstown	8.4^{d}	7	10	10	0.236	127
$LSD_{0.05}$	0.85					**563**
P- Value	<0.001***					

Maximum number of plants in anyone treatment is 10.

Means with different letters are significant using Tukey's test. *** = $P < 0.001$

3.5.2 Number of plants after transplanting

There were no significant differences among mean number of plants per treatment for Queenstown, PKM-t and PKM-g. Mon Repos had the lowest mean number of plants per treatment and the lowest total number of plants (Table 3.2).

Table 3.2 Mean number of plants for each provenance per treatment after transplanting (at 45 days after sowing), representing the initial number of plants before the start of the salt stress. The minimum and maximum number of plants is also presented along with the standard error of the mean, least significant difference ($LSD_{0.05}$) and significance (P-Value).

Provenance	Mean number of plants per treatment	Minimum	Maximum	Standard error of mean	Total plants after transplanting	% Increase of plants after transplanting
Benab	4.6^{b}	2	8	0.335	69	5.8
Bush Lot	7.4^{c}	5	9	0.321	112	15.1
Mon Repos	2.4^{a}	0	4	0.289	36	33.3
PKM-T	9^{cd}	8	10	0.218	135	5.1
PKM-G	9.1^{d}	8	10	0.191	137	9.4
Queenstown	9.6^{d}	9	10	0.130	144	11.8
$LSD_{0.05}$	0.72				633	11.06
P- Value	<0.001***					

Means with different letters are significant using Tukey's test. *** =P < 0.001

3.5.3 Mean plant height of each provenance before the application of salt stress

PKM-g and PKM- t had the highest mean plant height (Table 3.3). Analysis of variance (ANOVA) of mean plant height before the start of the salt stress period showed no significant difference ($P > 0.05$) among provenances except for Mon Repos and PKM-t. Plant height varied greatly among and within each provenance (Figure 3.1)

Table 3.3 Summary statistics for plant height of the six provenances before the start of salt stress. Mean plant height in millimetres, minimum and maximum plant height, least significant difference ($LSD_{0.05}$), standard error of the means (SEM) and significance (P-Value) are presented.

Provenance	Benab	Bush Lot	Mon Repos	PKM-G	PKM-T	Queenstown	L.S.D (0.05)	P- Value
Mean plant height	156.5	183.3	145.1	187.4	192.4	150.3	39.8	0.067 NS
Median	147.5	170.5	115.0	175.0	171.7	148.5		
Minimum	92.5	110.7	95.0	133.8	129.0	106.4		
Maximum	240.0	299.8	297.5	274.7	311.6	188.1		
Range	147.5	189.1	202.5	140.9	182.6	81.7		
SEM	11.85	13.76	18.42	12.56	13.19	6.61		

NS, not significant = $P > 0.05$

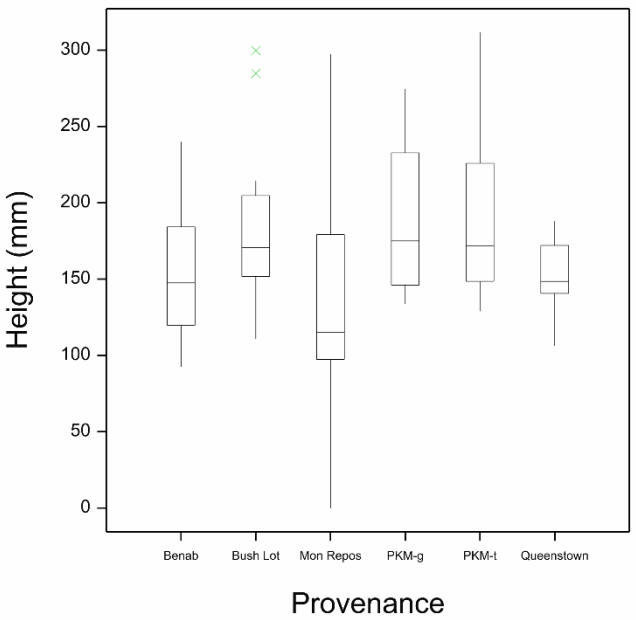


Figure 3.1 Plant height before the application of salt stress for each provenance.

3.5.4 Leaf chlorophyll content

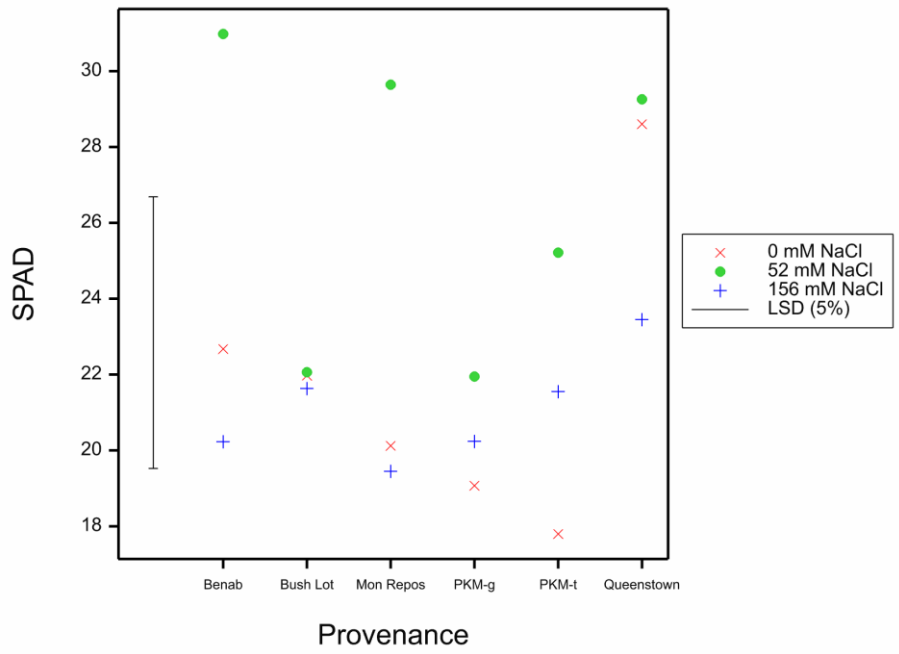
SPAD values varied for different salt concentrations before and after the exposure to stress (Table 3.4). SPAD measurements taken before the exposure of salt stress did not show any significant difference (Figure 3.2 (a)). Plants stressed at 156 mM (NaCl) had lower SPAD values than plants exposed to 0 or 52 mM (Figure 3.2 (b)). Analysis of variance also showed a significant difference ($P < 0.001$, Table 3.4) among SPAD values for the different provenances except for provenances at 156 mM NaCl before any stress was applied ($P < 0.05$). Also, SPAD values of plants stressed with 156 mM NaCl were lower when compared with plants stressed with 0 and 52 mM NaCl (Figure 3.3).

Table 3.4 Analysis of Variance (ANOVA) for the SPAD value of each provenance and salt treatment. The mean, least significant difference (LSD $_{0.05}$), standard error and significance (P-Value) are also presented.

		Before salt stress						After salt stress (28 days)					
Concentration	Provenance	Mean SPAD	Variance	Standard deviation	Standard error	LSD 0.05	P-Value	Mean SPAD	Variance	Standard deviation	Standard error	LSD 0.05	P-Value
0 mM NaCl	Benab	22.67	69.59	8.34	1.319	2.65	<0.001***	26.90	19.82	4.45	0.704	2.70	<0.001***
	Bush Lot	21.97	20.22	4.49	0.711			34.05	64.89	8.05	1.274		
	Mon Repos	21.21	28.60	5.34	1.017			24.96	26.19	5.11	0.809		
	PKM-g	19.07	20.82	4.56	0.721			25.90	27.67	5.26	0.832		
	PKM-t	17.80	21.0	4.58	0.725			28.13	60.12	7.75	1.226		
	Queenstown	28.60	58.97	7.68	1.214			33.15	61.74	7.85	1.242		
52 mM NaCl	Benab	30.98	185.62	13.62	2.154	3.85	<0.001***	24.59	29.43	5.42	0.857	2.41	<0.001***
	Bush Lot	22.06	43.18	6.57	1.039			32.10	43.76	6.61	1.046		
	Mon Repos	29.64	103.71	10.18	1.610			19.49	24.30	4.93	0.779		
	PKM-g	21.95	57.56	7.58	1.200			25.39	34.74	5.89	0.931		
	PKM-t	25.21	46.91	6.84	1.083			25.27	44.54	6.67	1.055		
	Queenstown	29.26	62.0	7.87	1.245			30.93	24.87	4.98	0.788		
156 mM NaCl	Benab	20.23	31.42	5.60	0.886	2.90	0.025*	16.60	80.99	8.99	1.423	3.05	<0.001***
	Bush Lot	21.63	27.0	5.19	0.822			17.67	35.23	5.93	1.049		
	Mon Repos	24.31	107.89	10.38	1.836			20.38	33.51	5.78	1.008		
	PKM-g	20.24	30.30	5.50	0.870			17.41	62.64	7.91	1.251		
	PKM-t	21.55	38.66	6.21	0.983			18.60	35.14	5.92	0.937		
	Queenstown	23.45	44.38	6.66	1.053			23.11	62.68	7.91	1.252		

***=P < 0.001, *=P < 0.05

(a)



(b)

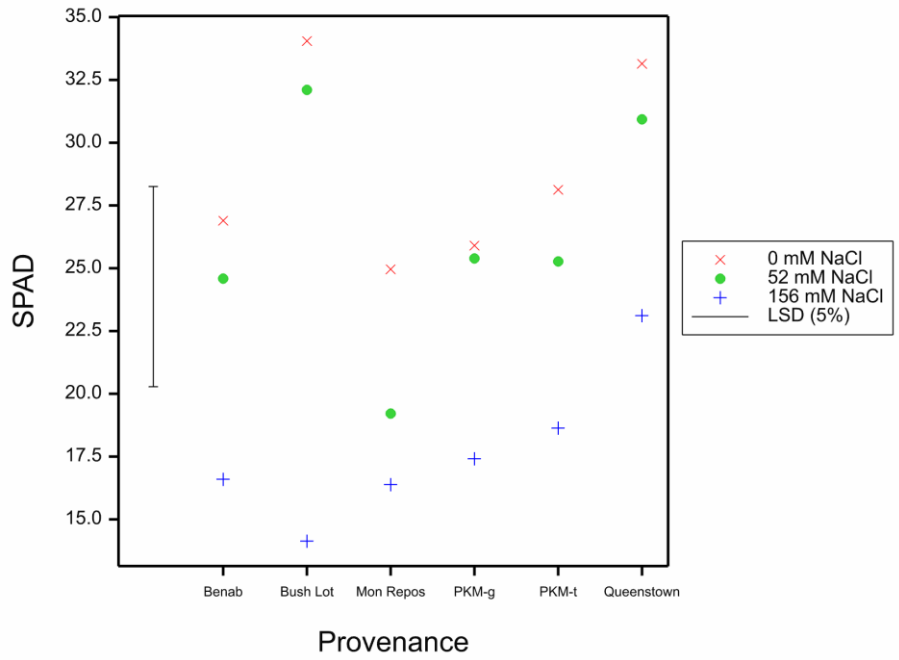


Figure 3.2 SPAD measurements for each provenance at different salt concentrations, (a) SPAD measurements before the application of salt stress, (b) SPAD measurements taken twenty-eight days after the start of the salt stress.

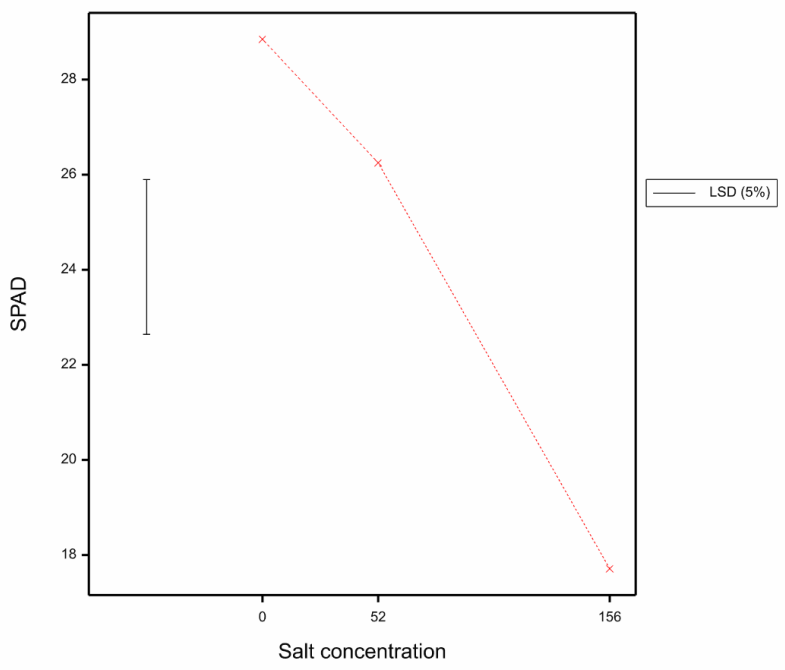


Figure 3.3 SPAD of plants treated with 0, 52 and 156 mM NaCl, values are mean for all provenances for each NaCl concentration.

3.5.5 Plant visual morphological characteristics

There was a salt stress by provenance interaction ($P < 0.001$) (Table 3.5). Plants not stressed (control plants) from the various provenances showed a significantly higher visual morphological quality ($P < 0.001$) than plants stressed with 156 mM NaCl (Figure 3.5). Results from the scoring also showed that there were plants from Mon Repos and PKM-t that had a score of 4 (good quality and acceptable growth) when stressed with 156 mM NaCl (Figure 3.4 (c)). At 52 mM NaCl all the provenances had plants with a visual score of 3 and above (Figure 3.4(b) and 3.5).

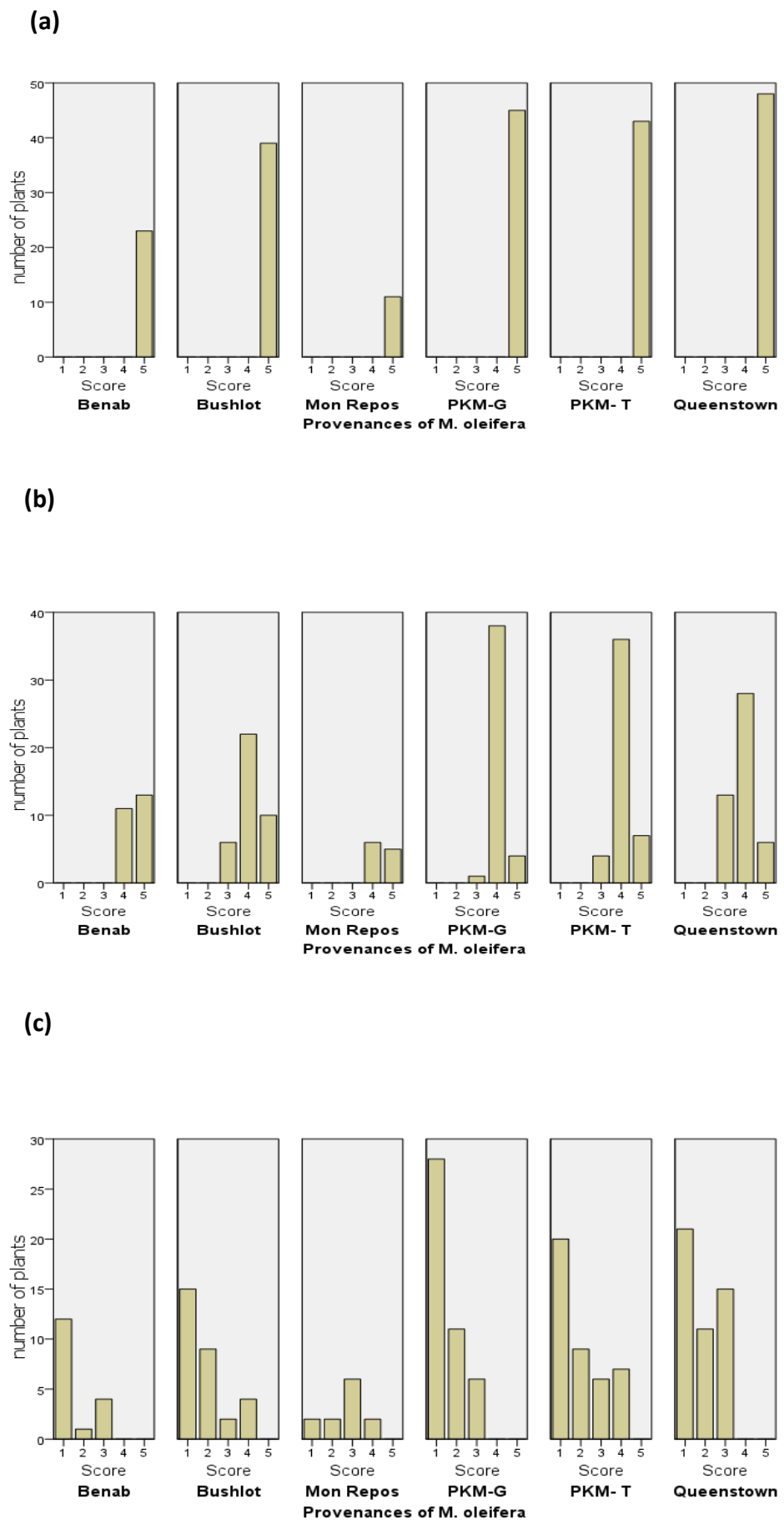


Figure 3.4 Plant visual score for each provenance (a) treated with 0 mM NaCl, (b) 52 mM NaCl and (c) 156 mM NaCl. Scores are 1 to 5, with 1 = severely stunted growth with over 50% foliage salt damage (leaf necrosis, browning) or dead; 2 = somewhat stunted growth with moderate (25%to 50%) foliage salt damage; 3 = average quality with slight (less than 25%) foliage salt damage; 4 = good quality with acceptable growth reduction and little foliage damage; and 5 = excellent with vigorous growth and no foliage damage.

Table 3.5 Analysis of Variance (ANOVA) for visual morphological quality score of the different provenances with their respective salt treatment. The provenance by salt concentration interactions were found to be significant and are shown. The mean score, standard error (SE), average least significant difference ($LSD_{0.05}$) and significance (P-Value) are presented.

Provenance	0 mM NaCl			52 mM NaCl			156mM NaCl				
	Mean plant score	Number of plants scored	SE	Mean plant score	Number of plants scored	SE	Mean plant score	Number of plants scored	SE	$LSD_{0.05}$ (average)	P-Value
Benab	4.98^{h}	23	0.1281	4.53^{gh}	24	0.1254	1.84^{ab}	17	0.1312		
Bush Lot	5.00^{h}	39	0.0984	4.09^{fg}	38	0.0997	1.87^{abcd}	30	0.1124		
Mon Repos	4.98^{h}	11	0.1855	4.45^{fgh}	11	0.1854	2.69^{e}	12	0.1775	0.32	<0.001***
PKM-G	5.00^{h}	45	0.0916	4.05^{fg}	43	0.0937	1.50^{a}	45	0.0916		
PKM-T	4.99^{h}	43	0.0937	4.05^{fg}	47	0.0896	2.39^{bde}	42	0.0948		
Queenstown	4.99^{h}	48	0.0887	3.85^{f}	47	0.0896	1.86^{abc}	47	0.0896		
Total		209			210			193			

Means with different letters are highly significant using Bonferroni multiple comparison test. ***= P < 0.001

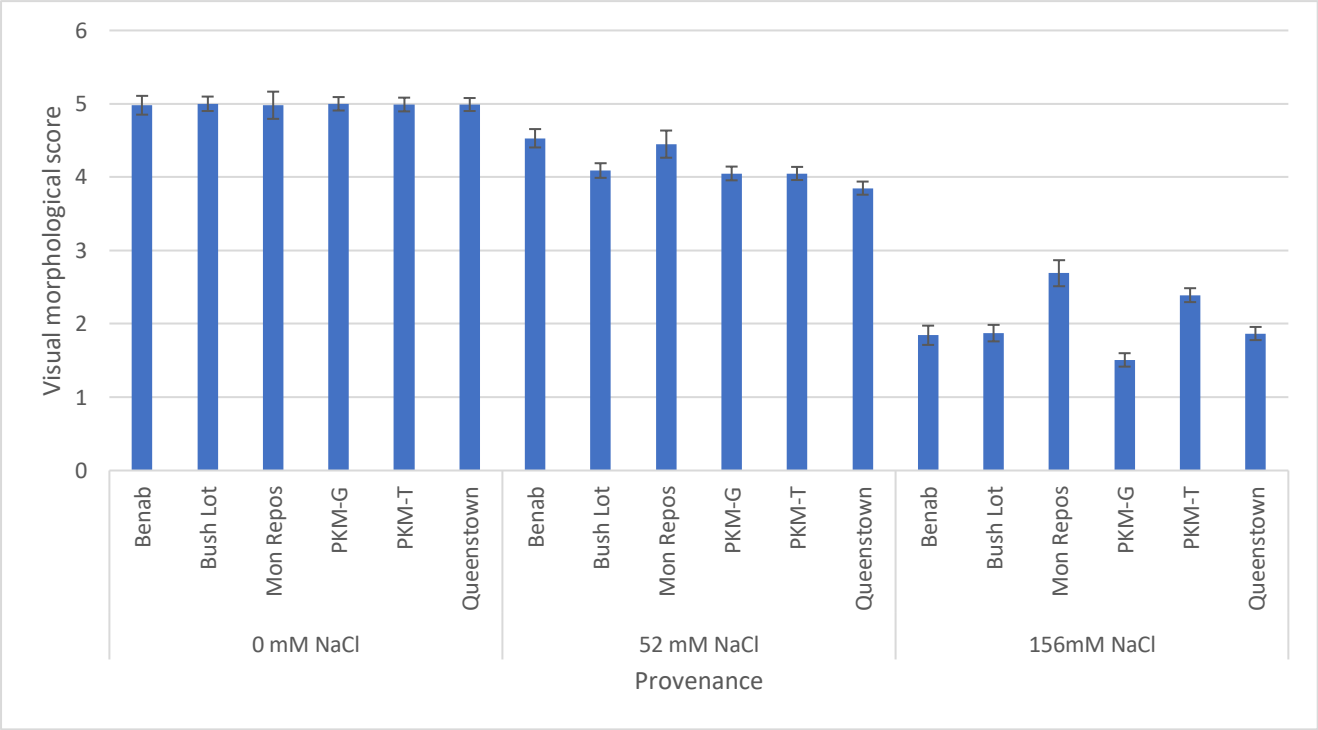


Figure 3.5 Mean plant score (visual morphological quality) for each provenance and salt concentration. Plant scoring was done 28 days after the start of the application of salt stress. Scores are 1 to 5, with 1 = severely stunted growth with over 50% foliage salt damage (leaf necrosis, browning) or dead; 2 = somewhat stunted growth with moderate (25%to 50%) foliage salt damage; 3 = average quality with slight (less than 25%) foliage salt damage; 4 = good quality with acceptable growth reduction and little foliage damage; and 5 = excellent with vigorous growth and no foliage damage.

3.5.6 Growth during salt stress period

There were significant differences ($P < 0.05$) for mean growth in height during the salt stress period across provenances. Queenstown showed the least growth and Mon Repos the highest (Table 3.6 and Figure 3.6). The height of plants treated with different salt stress differed significantly ($P < 0.001$) (Figure 3.7). Plants stressed with 156 mM NaCl had significantly lower growth than plants stressed with 0 and 52 mM NaCl. There was no interaction effect for provenance by salt stress

Table 3.6 Analysis of variance (ANOVA) of plant height after the experimental period of twenty-eight days. The mean height, least significant difference ($LSD_{0.05}$), standard error of the means (SEM) and significance are displayed.

	Mean plant growth in height (mm)						
Provenance	Salt concentrations			Mean	SEM	LSD (0.05)	P-Value
	0 mM NaCl	52 mM NaCl	156mM NaCl				
Benab	453.20	311.81	175.73	313.58^{ab}			
Bush lot	439.16	413.78	149.76	334.24^{ab}			
Mon Repos	552.20	439.07	154.27	381.84^{b}	26.66	75.28	0.030*
PKM-G	360.28	373.71	176.74	303.58^{ab}			
PKM-T	413.70	361.40	192.47	322.52^{ab}			
Queenstown	336.18	319.20	89.67	248.35^{a}			
Mean	425.79^{b}	369.83^{b}	156.44^{a}				
S.E.M	18.86						
$L.S.D_{0.05}$ =	53.23						
P-Value	<0.001***						

Means with different letters are significant using Tukey's test.

*= significant at $P < 0.05$, ***= significant at $P < 0.001$

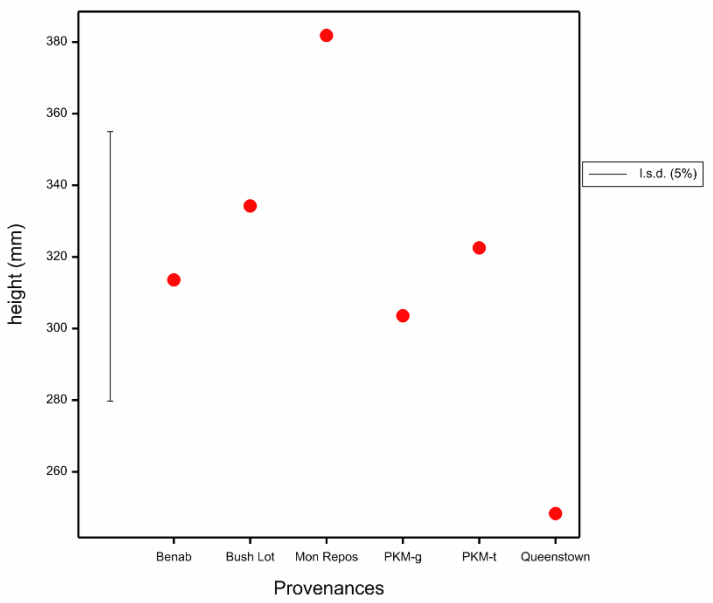


Figure 3.6 Growth in plant height (mm) after the salt stress period of 28 days for the six provenances. Values are averaged for all salt concentrations for each provenance.

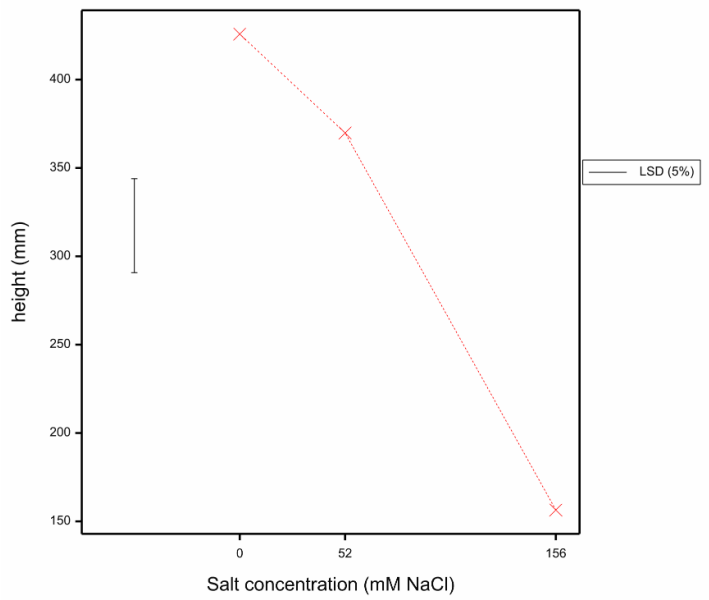


Figure 3.7 Growth in plant height (mm) after the salt stress period of 28 days for plants treated with 0, 52 and 156 mM NaCl. Values of all provenances for each salt concentration are averaged.

3.5.7 Plant Biomass

3.5.7.1 Percent dry weight

There were significant differences (P < 0.001) for shoot percent dry weight, and total percent dry weight across the different provenances. Root percent dry weight was significant at P < 0.05 (Table 3.7 (a)). PKM-g and Mon Repos contained the lowest percent dry weight when compared with the other provenances (Table 3.7 (a)).

Significant differences (P < 0.001) were also found for percent dry weight of plants treated with different levels of salt stress. At 156 mM NaCl, plants showed a significant reduction of shoot, root and total percent dry weight, but no significant difference was found between 0 and 52 mM NaCl. (Table 3.7 (b)).

Table 3.7 Analysis of percent dry weight of shoots, roots and whole plant using unbalanced ANOVA for (a) provenances (b) salt concentrations. The mean percent dry weight, average least significant difference ($LSD_{0.05}$) and P-Value are presented.

(a)

	Mean % Dry weight Provenances							
Parameters	Benab	Bush Lot	Mon Repos	PKM-g	PKM-t	Queenstown	$LSD_{0.05}$	P- Value
% dry weight shoot	17.68^{ab}	18.09^{b}	16.61^{ab}	16.51^{a}	17.25^{ab}	17.75^{b}	0.70	<0.001***
% dry weight root	15.91^{abc}	17.13^{abc}	13.82^{a}	14.88^{ab}	18.76^{ac}	17.28^{abc}	2.92	0.010*
% dry weight Whole plant	16.91^{ab}	17.66^{b}	15.86^{a}	15.94^{a}	17.37^{b}	17.61^{b}	0.78	<0.001***

Means with different letters are significant using Bonferroni multiple comparison test. ***= P < 0.001, *P < 0.05

(b)

Parameters	Mean % dry weight Salt concentrations 0 mM NaCl	52 mM NaCl	156 mM NaCl	L.S.D $_{0.05}$	P- Value
% dry weight shoot	18.38[b]	17.78[b]	15.24[a]	0.45	<0.001***
% dry weight root	17.42[b]	18.38[b]	13.33[a]	1.87	<0.001***
Total % Dry weight	18.06[b]	17.94[b]	14.18[a]	0.50	<0.001***

Means with different letters are highly significant using Bonferroni multiple comparison test.

***= P < 0.001

3.5.7.2 Dry weight

Plant dry weight varied significantly for shoot dry weight, total dry weight (P < 0.001) and for root dry weight (P < 0.05) across provenances (Table 3.8 (a)). Queenstown and Mon Repos had the lowest and highest whole plant dry weight respectively.

A significant difference was also found for whole plant dry weight at 0, 52, and 156 mM NaCl (P < 0.001, Table 3.8 (b)). Plants stressed with 156 mM NaCl weighed less than plants of the other two treatments (0 and 52 mM NaCl). Root dry weight did not differ significantly at 0 and 52 mM NaCl, but plants treated with the higher salt stress had lower root weight (P < 0.001, Table 3.8 (b)).

There were significant interactions between provenance and salt concentration for shoot dry weight but not for root dry weight. Shoot dry weight differs significantly (P < 0.05) for Benab and PKM-g plants stressed with 0 and 52 mM NaCl. Plants for all provenances stressed with 0 and 52 mM NaCl had a significantly higher shoot dry weight than plants stressed with 156 mM NaCl (Figure 3.8). Conversely, the root dry weight did not show a significant difference (P = 0.108) for any of the treatments (Figure 3.9).

Mean total dry weight for provenance by salt interactions showed a significant difference (P < 0.05) (Table 3.8 (c) and Figure 3.10). But mean total dry weight of PKM-g and Mon Repos plants stressed at 0 mM NaCl

and 52 mM NaCl showed no significant difference in total dry weight when compared using Bonferroni multiple comparison test and least significant difference (Table 3.8 (c)).

Table 3.8 Analysis of dry weight for shoots, roots and whole plant using unbalanced ANOVA, (a) Provenances, (b) salt concentrations, (c) salt by provenance interaction with standard error (SE). The mean dry weight, P-Value, and least significant difference ($LSD_{0.05}$) are presented.

(a)

Parameters	Mean dry weight Provenances Benab	Bush Lot	Mon Repos	PKM-g	PKM-t	Queenstown	$L.S.D_{0.05}$	P- Value
dry weight shoot	9.26^{c}	6.97^{bc}	13.18^{d}	6.47^{ab}	6.41^{ab}	4.92^{a}	1.64	<0.001***
dry weight root	6.01^{b}	5.47^{ab}	4.92^{ab}	4.32^{a}	4.90^{ab}	4.71^{ab}	1.14	0.036*
Total dry weight	15.27^{bc}	12.45^{ab}	18.11^{c}	10.80^{a}	11.92^{a}	9.63^{a}	2.59	<0.001***

Means with different letters are significant using Bonferroni multiple comparison test. ***= P < 0.001, *=P < 0.05. Shoot refers to above ground plant biomass.

(b)

Parameters	Mean dry weight Salt concentrations 0 mM NaCl	52 mM NaCl	156 mM NaCl	$L.S.D_{0.05}$	P- Value
dry weight shoot	9.39^{c}	7.03^{b}	2.49^{a}	1.05	<0.001***
dry weight root	5.86^{b}	5.42^{b}	2.86^{a}	0.73	<0.001***
Total dry weight	15.25^{c}	12.46^{b}	5.36^{a}	1.66	<0.001***

Means with different letters are significant using Bonferroni multiple comparison test, ***= P < 0.001.

(c)

	Mean dry weight						L.S.D $_{0.05}$	P-Value
Provenance	0 mM NaCl	SE	52 mM NaCl	SE	156 mM NaCl	SE		
Benab	22.11^{h}	1.672	13.61^{cdefg}	1.637	5.95^{abc}	2.007	4.50	0.047*
Bush Lot	15.22^{efgh}	1.285	14.07^{cefg}	1.302	6.01^{abc}	1.840		
Mon Repos	20.50^{egh}	2.422	21.13^{egh}	2.538	8.92^{abcde}	2.542		
PKM-g	13.06^{cdefg}	1.183	13.14^{cdefg}	1.196	4.06^{a}	1.546		
PKM-t	15.69^{efgh}	1.197	10.15^{abcdef}	1.170	6.35^{abcd}	1.446		
Queenstown	12.41cdefg	1.158	10.51^{acdef}	1.170	4.26^{ab}	1.253		

Means with different letters are significant using Bonferroni multiple comparison test, *= P < 0.05.

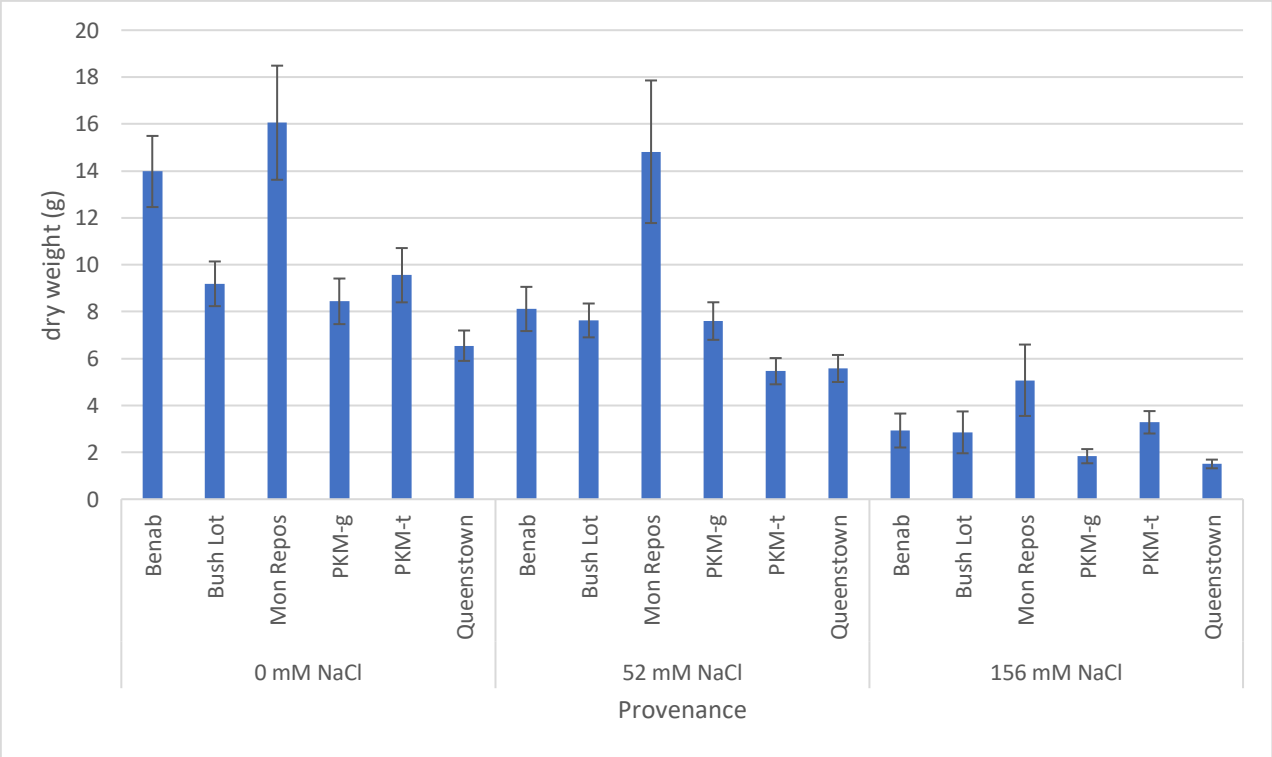


Figure 3.8 Mean shoot (above ground plant biomass) dry weight for each provenance for each salt treatment.

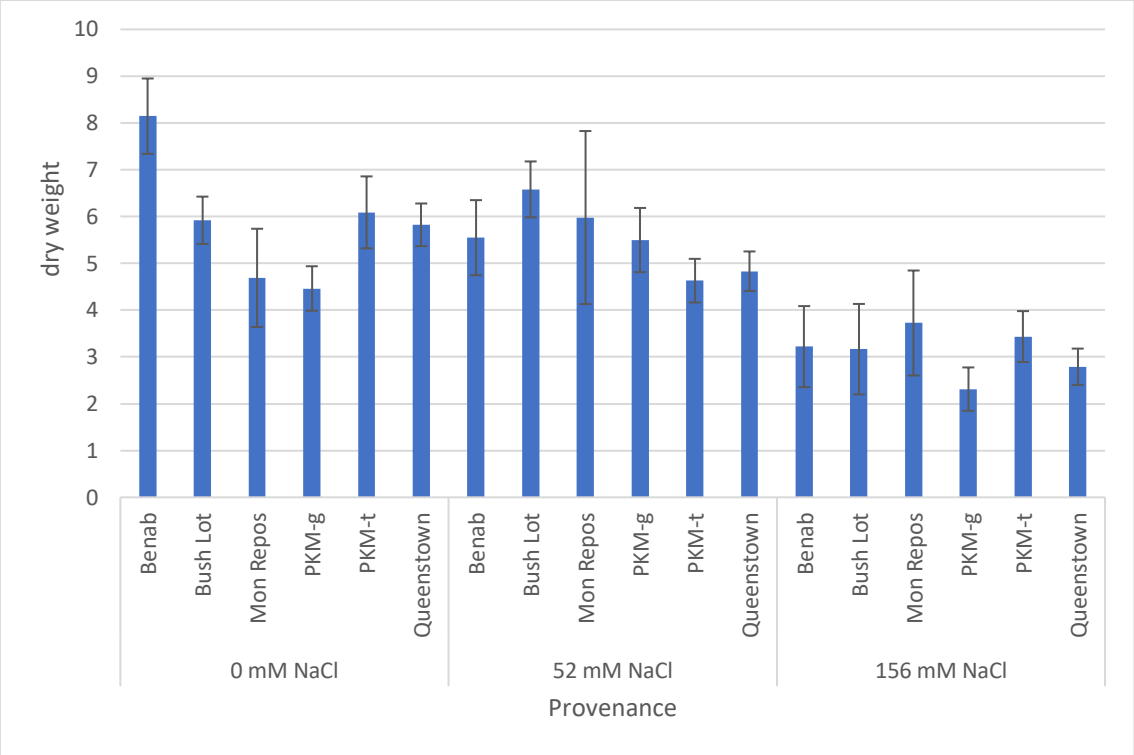


Figure 3.9 Mean root dry weight for each provenance for each salt treatment.

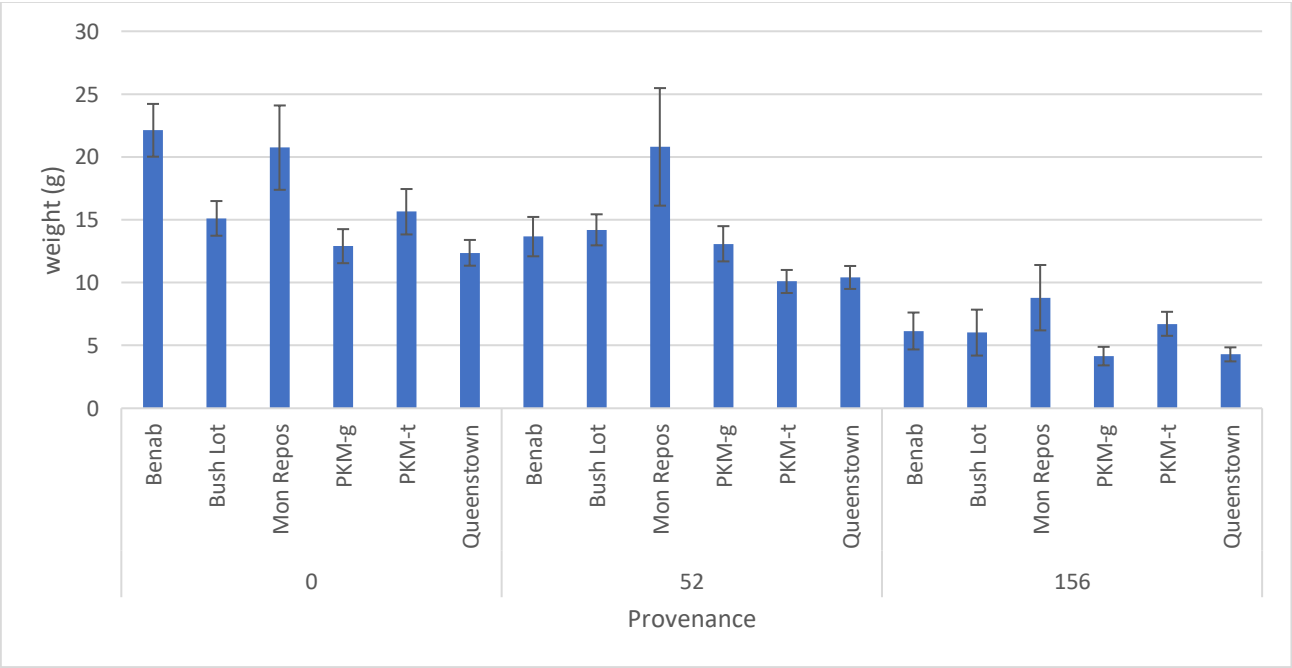


Figure 3.10 Mean whole plant dry weight for each provenance at different levels of salt concentrations.

3.5.8 Plant Biomass indices

3.5.8.1 Relative decrease in plant biomass (RDPB)

Plant biomass data were used to calculate stress indices, each provenance demonstrated a reduction in fresh weight when they were stressed at 52 and 156 mM NaCl. Bush Lot and PKM-g showed little or no decrease in biomass with values below 0.01 for a relative decrease in plant biomass (calculated based on fresh weight). In contrast, at 52 mM NaCl plants from Mon Repos showed an increase in the biomass (Table 3.9 (a)). At higher concentration (156 mM NaCl) the relative decrease in plant biomass were similar for all provenances, except for Benab and Queenstown, which had a higher relative decrease in plant biomass.

3.5.8.2 Salt and stress tolerance index

All provenances had a higher salt and stress tolerance index for dry weight at 52 mM NaCl than at 156 mM NaCl. Mon Repos and PKM-g both showed salt tolerance indices above 1 at 52 mM NaCl. However, Mon Repos and Benab had a higher stress tolerance index when compared with the other provenances at 52 mM NaCl (Table 3.9 (b)). Salt and stress tolerance indices for all provenances at 156 mM NaCl had values less than 1.

3.5.8.3 Root mass ratio (RMR) and relative root mass ratio (RRMR)

The root mass ratio increased for all provenance as the salt stress increased (Table 3.9 (c)). Queenstown had a higher root mass ratio than the other provenances at each salt stress. The relative root mass ratio (RRMR) was similar for all provenances at 52 mM NaCl and 156 mM NaCl, except for Queenstown which had the lowest value at both stress levels (Table 3.9 (a)).

Table 3.9 Indices derived from plant biomass measurements (a) relative decrease in plant biomass (RDPB), (b) salt stress index and stress tolerance index, and (c) root mass ratio and relative root mass ratio for each provenance.

(a)

Provenances	Relative decrease in plant Biomass	
	52 mM NaCl	156 mM NaCl
Benab	0.391	0.684
Bush Lot	0.079	0.557
Mon Repos	-0.028	0.572
PKM-g	0.014	0.598
PKM-t	0.365	0.519
Queenstown	0.143	0.608

(b)

Provenance	Salt tolerance index		Stress tolerance index	
	52 mM NaCl	156 mM NaCl	52 mM NaCl	156 mM NaCl
	DW	DW	DW	DW
Benab	0.618	0.278	1.312	0.591
Bush Lot	0.940	0.398	0.932	0.395
Mon Repos	1.002	0.424	1.873	0.793
PKM-g	1.015	0.321	0.733	0.232
PKM-t	0.645	0.429	0.685	0.456
Queenstown	0.841	0.347	0.559	0.231

(c)

Provenance	Root mass ratio			Relative root mass ratio	
	0 mM NaCl	52 mM NaCl	156 mM NaCl	52 mM NaCl	156 mM NaCl
Benab	0.368	0.406	0.524	1.103	2.996
Bush Lot	0.392	0.463	0.526	1.182	3.016
Mon Repos	0.226	0.288	0.423	1.272	5.625
PKM-g	0.346	0.420	0.558	1.213	3.507
PKM-t	0.389	0.459	0.512	1.179	3.028
Queenstown	0.471	0.464	0.650	0.986	2.095

3.5.9 Plant response mechanisms to salt stress

3.5.9.1 Net photosynthesis

There was no significant difference (P > 0.05, Table 3.10 (a)) for net photosynthesis of the different provenances before and twenty-eight days after the application of salt stress. However, significant difference (P < 0.001) was found among salt stressed plants (Table 3.10 (b), Figure 3.12). Results showed that the salt stress started to have a significant effect on the net photosynthesis 14 days after the first application (Table 3.10 (b)). While there was no difference detected in the salt stress by provenance interaction, the provenances Benab, PKM-g and Queenstown show a higher net photosynthesis under 52 mM NaCl salt stress as compared to 0 mM NaCl. Also, the mean net photosynthesis for each provenance at 156 mM NaCl was lower than at 0 and 52 mM NaCl (Figure (3.11)).

Table 3.10 Analysis of variance (ANOVA) for (a) net photosynthesis of the different provenances before and twenty-eight days after the application of salt stress, and (b), net photosynthesis of total plants for the respective salt stress used, before and at seven- days interval. Mean net photosynthesis in micromoles per second and square metre (µmol/ m² /s), least significant difference ($LSD_{0.05}$), standard error of the means (SEM) and significance (P-Value) are presented.

(a)

		Mean net photosynthesis (mol /m^2/s)						Average $LSD_{0.05}$	P-Value
Net photosynthesis	Salt stress (mM NaCl)	Benab	Bush Lot	Mon Repos	PKM-g	PKM-t	Queenstown		
Initial	0	16.84	17.41	14.16	17.41	13.54	16.89	4.92	0.408 NS
	52	17.64	16.15	11.26	17.80	16.94	16.86	4.97	0.107 NS
	156	15.54	18.70	12.73	17.28	19.05	16.29	5.64	0.241 NS
Final (28-days after)	0	14.64	13.49	13.11	11.68	12.28	11.27	3.41	0.363 NS
	52	15.07	12.74	12.35	9.60	12.62	12.10	4.16	0.224 NS
	156	10.34	4.37	9.10	8.02	8.50	11.94	5.96	0.207 NS

(b)

	Mean net photosynthesis (µmol /m/² s)			$LSD_{0.05}$	SEM	P-Value
Net photosynthesis	0 mM NaCl	52 mM NaCl	156 mM NaCl			
before salt stress	16.04	16.11	16.60	1.99	0.706	0.831 NS
7 days after salt stress	13.95	13.51	12.26	1.59	0.563	0.096 NS
14 days after salt stress	11.26^{b}	10.94^{b}	7.57^{a}	1.69	0.600	<.001***
21 days after salt stress	13.40^{b}	12.35^{ab}	9.34^{a}	1.13	0.614	<.001***
28 days after salt stress	12.74^{b}	12.41^{b}	8.17^{a}	1.78	0.632	<.001***

Means with different letters are highly significant using Tukey multiple comparison test. ***= P < 0.001.

NS = not significant

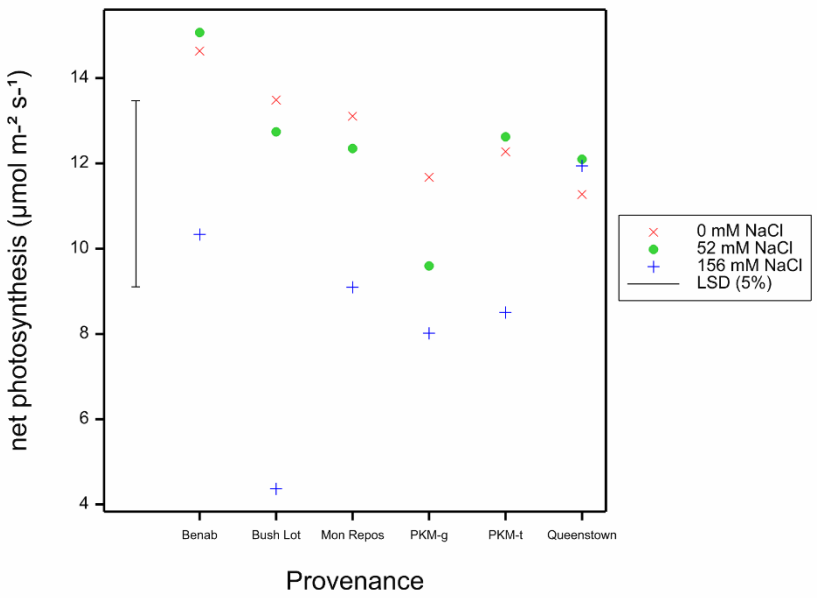


Figure 3.11 Net photosynthesis after 28 days of salt stress for each provenance treated with different salt concentrations. Values are mean, n = 5.

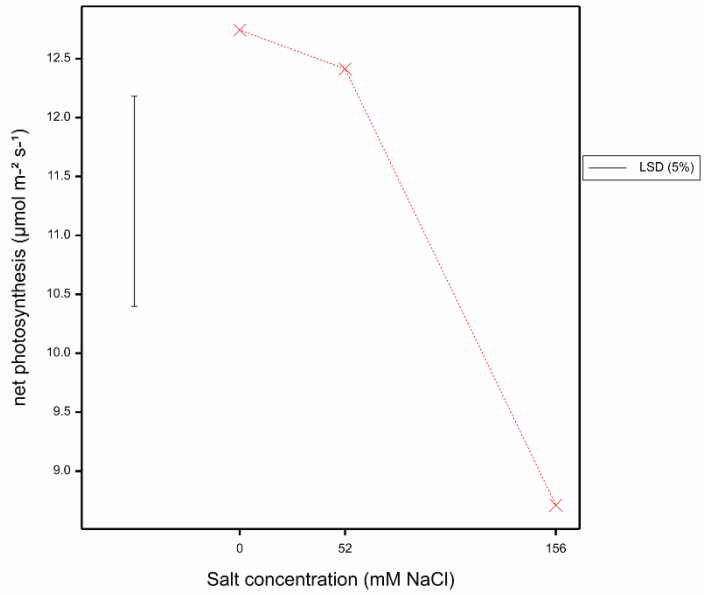


Figure 3.12 Net photosynthesis of plants that were stressed for a total of 28 days with 0, 52 and 156 mM NaCl. The net photosynthesis value corresponding to each salt concentration represents the average net photosynthesis of all six provenances.

Table 3.11 Mean transpiration of plants stressed with 0, 52 and 156 mM NaCl. The least significant difference (L.S.D $0._{05}$), standard error of the means and P-Value are presented.

	Mean transpiration mol /m^2/s			$LSD_{0.05}$	SEM	P-Value
	0 mM NaCl	52 mM NaCl	156 mM NaCl			
Transpiration mol /m^2/s	E	E	E			
14 days after salt stress	0.0028^b	0.0033^b	0.0017^a	0.0006	0.00053	<0.001***
21 days after salt stress	0.0046^b	0.0037^b	0.0025^a	0.0007	0.00027	<0.001***
28 days after salt stress	0.0036^b	0.0030^b	0.0020^a	0.0006	0.00022	<0.001***

Means with different letters are significant, using Tukey multiple comparison test. ***= P < 0.001

Table 3.12. Mean stomatal conductance of plants stressed with 0, 52 and 156 mM NaCl. The least significant difference (L.S.D $0._{05}$), standard error of the means and P-Value are presented.

	Mean stomatal conductance mol /m^2/s			$LSD_{0.05}$	SEM	P-Value
	0 mM NaCl	52 mM NaCl	156 mM NaCl			
Stomatal Conductance mol /m^2/s	gsw	gsw	gsw			
14 days after salt stress	0.1887^b	0.1952^b	0.0811^a	0.0437	0.0155	<0.001***
21 days after salt stress	0.3212^c	0.2412^b	0.1408^a	0.0639	0.0226	<0.001***
28 days after salt stress	0.2100^b	0.1666^b	0.1049^a	0.0464	0.0164	<0.001***

Means with different letters are significant, using Tukey multiple comparison test. *** = P < 0.001

3.5.9.2 Electrical conductivity (EC) of potting mixture across treatments

There were significant differences (P < 0.05) for the average EC of potting mixture for each provenance after the stressed period. There was also a significant difference (P < 0.001) for the EC of the potting mixture in pots that were exposed to different salt concentrations of 0, 52 and 156 mM NaCl. Potting mixture exposed to 156 mM NaCl stress had a higher EC (Table 3.13 and Figure 3.14). The results showed that at the end of the experiment, the potting mixture of plants from Mon Repos had a higher electrical conductivity when compared with the potting mixture of other provenances (Figure 3.13 and Table 3.13). The potting mixture of plants from Queenstown and PKM-g had the lowest mean EC (Table 3.13). There was no significant provenance by salt interaction for potting mixture.

Table 3.13 Analysis of variance (ANOVA) of mean electrical conductivity, measured for the potting mixture that was in the pots, determined after the trial finished. Values presented are mean electrical conductivity in milli Siemen per centimetre (mS/cm). The least significant difference ($LSD_{0.05}$), standard error of the means (SEM) and significance (P-Value) are presented.

Provenance	Mean electrical conductivity ($mScm^{-1}$) Salt concentrations						
	0 mM NaCl	52 mM NaCl	156mM NaCl	Mean (Provenance)	SEM	LSD (0.05)	P-Value
Benab	1.26	2.51	3.06	2.28ab			
Bush lot	1.02	2.08	3.34	2.15ab			
Mon Repos	1.41	2.58	2.99	2.32b	0.114	0.32	0.006*
PKM-G	1.04	2.53	3.03	2.22ab			
PKM-T	0.91	2.27	2.39	1.85a			
Queenstown	0.95	1.92	2.61	1.83a			
Mean (salt concentration)	1.10^{a}	2.32^{b}	2.90^{c}				
SEM	0.081						
$LSD_{0.05}$ =	0.22						
P-Value	<0.001***						

Means with different letters are significant using Tukey multiple comparison test. ***= P < 0.001, *= P < 0.05

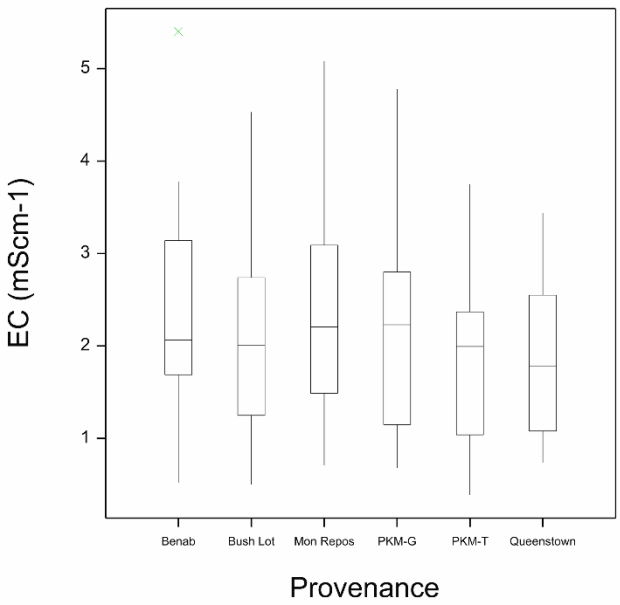


Figure 3.13 Electrical conductivity of the potting mixture after the salt stress period of twenty- eight days for each provenance.

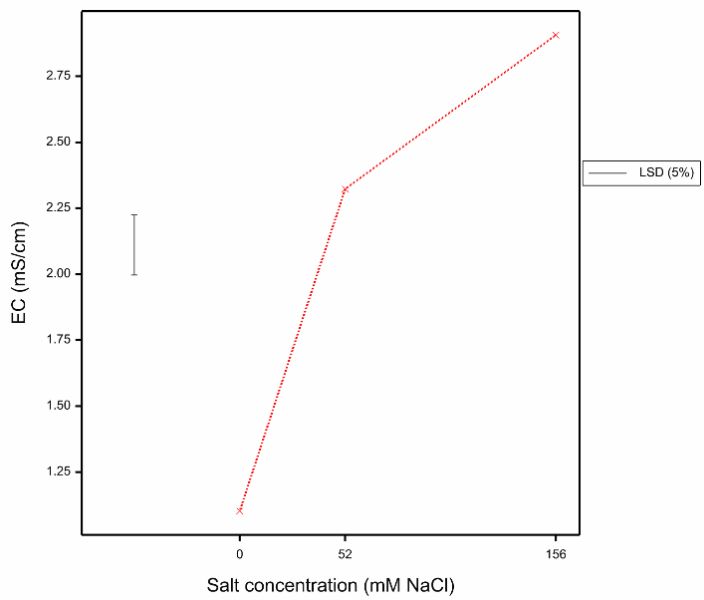


Figure 3.14 Mean electrical conductivity of the potting mixture that was used to apply salt stress using 0, 52, 156 mM NaCl, after 28 days. Each corresponding EC value for 0, 52 and 156 mM NaCl in the graph represents an average EC value of the potting mixture for the six provenances.

3.5.9.3 Leaf sodium and potassium concentration

Sodium concentration in leaves varied significantly ($P < 0.001$) for the provenances when plants were exposed to salt stress (Table 3.14). There was an increase in sodium concentration as salt stress increases (Figure 3.15). Potassium concentrations also varied across provenances with salt stress ($P < 0.05$, Table 3.14) but were in higher concentrations than sodium (Figure 3.15).

Table 3.14 Analysis of variance (ANOVA) comparing mean sodium and potassium concentration in *M. oleifera* leaves stressed at 0, 52 and 156 mM NaCl for the provenances. The mean concentration of sodium and potassium in grams per kilogram of leaves (g/Kg), least significant difference ($LSD_{0.05}$), and significance (P-Value) are presented.

		Mean concentration (g/Kg)						Average $LSD_{0.05}$	P-Value
	Salt stress (mM NaCl)	Benab	Bush Lot	Mon Repos	PKM-g	PKM-t	Queenstown		
Sodium	0	0.76	0.91	0.67	0.36	0.35	0.73	0.52	0.218NS
	52	4.45	3.89	3.73	3.32	2.55	4.70	0.61	<0.001***
	156	10.95	12.36	11.91	6.09	9.26	12.88	3.51	<0.001***
Potassium	0	31.05	32.07	33.23	30.03	30.04	36.99	15.11	0.899NS
	52	29.01	29.38	30.97	33.68	32.92	32.43	3.10	0.007*
	156	26.74	31.19	29.40	36.72	34.80	31.55	5.86	0.010*

NS= not significant, ***= significant at $P < 0.001$, * = significant at $P < 0.05$

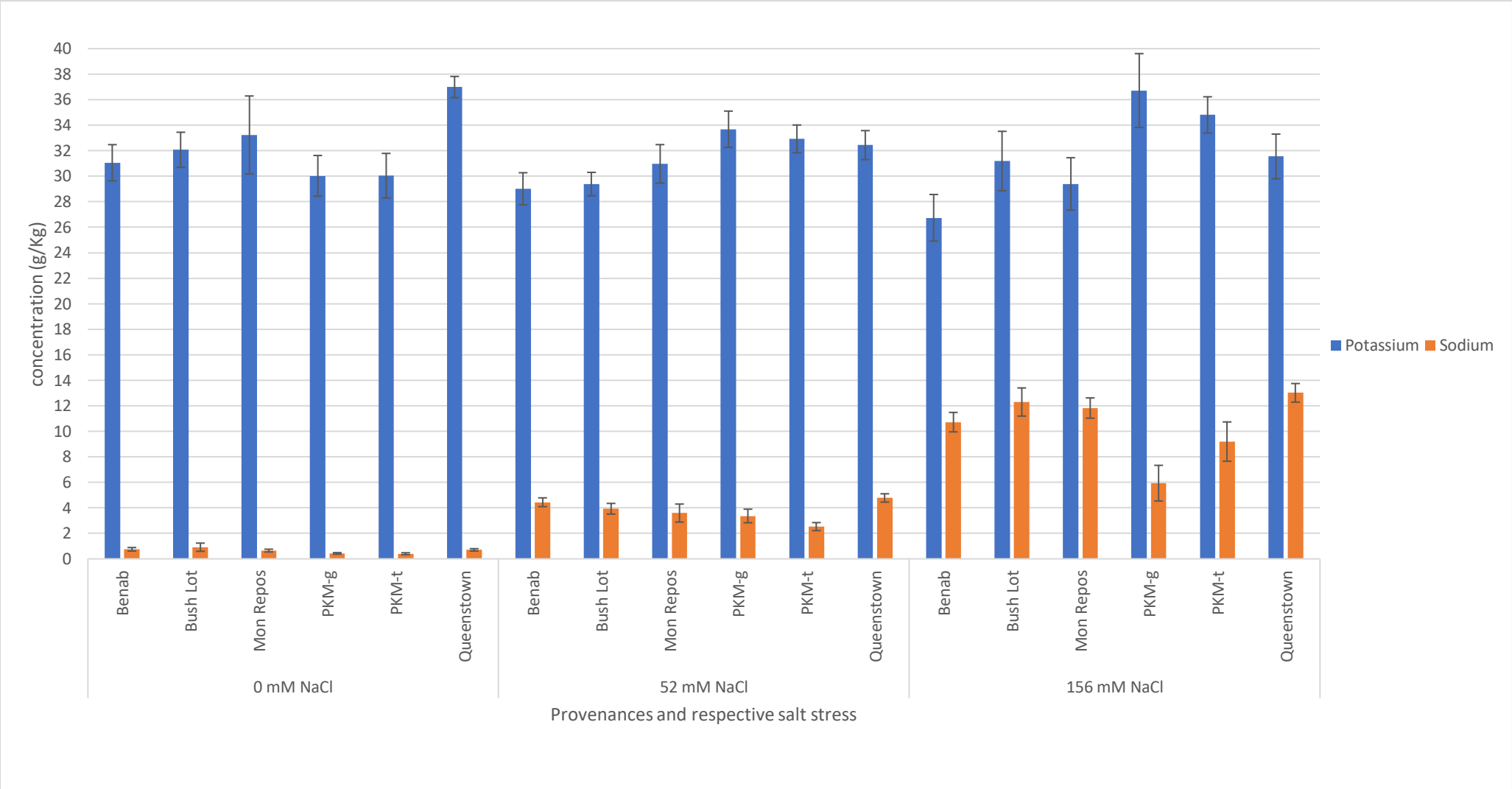


Figure 3.15 Mean sodium and potassium concentration in leaves of the different provenances stressed at 0, 52, 156 mM NaCl.

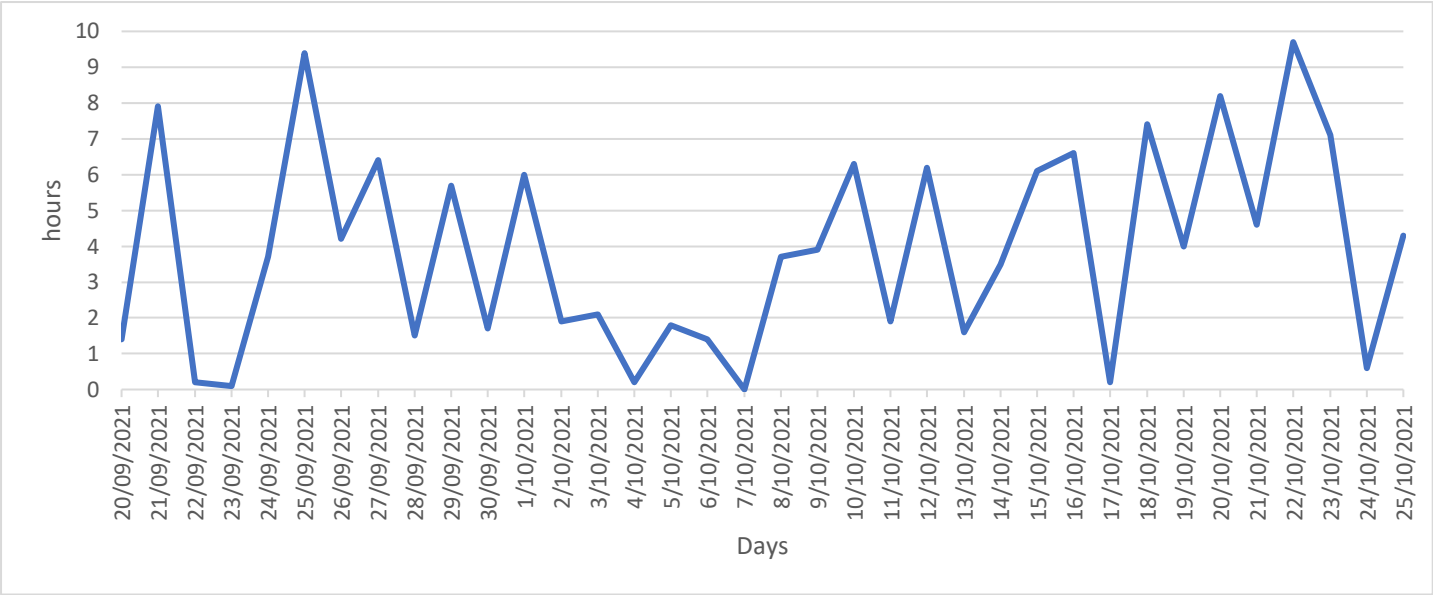


Figure 3.16 Sunshine in hours during the experimental period. The period above includes the first and last dates when net photosynthesis was measured are inclusive. Sunshine hours were recorded at the end of each day at 23:59 hours (National Institute of Water and Atmospheric Research, 2022).

3.6 Discussion

In this study, plants were exposed to salt stress for four weeks (28 days). Salt was applied every seven days and final data collection was done seven days after the final application of salt. *M. oleifera* plants were evaluated before the stress period for shoot emergence and initial plant height. During the stress period, plant growth, chlorophyll content, visual quality, percent dry weight and dry weight were measured. Net photosynthesis was evaluated before, during and after the stress period along with transpiration and stomatal conductance of leaves. Sodium and potassium concentration in the leaves were also quantified and the electrical conductivity of the potting mixture in each pot was measured after the salt stress period.

3.6.1 Initial measurements before the application of salinity stress

3.6.1.1 Plant emergence

While it was not the objective of this study to investigate seed germination or seedling emergence, seedling emergence of the different provenances varied significantly and is an important factor that contributed to the outcome of this study. There were significant differences ($P < 0.001$) for the mean number of plants per treatment for each provenance (Table 3.1 and 3.2). This was because of poor seedling emergence which was most likely due to poor germination of seeds. Seeds from Benab, Bush Lot and Mon Repos had low seedling emergence. This affected the total number of plants in the experiment, causing unbalanced data to be obtained for plant scoring, plant height and plant biomass. Despite soaking seeds of all the provenances with distilled water for 24 hrs before sowing to help ensure uniformity in seedling emergence, Mon Repos, Benab and Bush Lot seeds had poor emergence. Soaking seeds before sowing was done to help rehydrate seeds (Finch-Savage & Leubner-Metzger, 2006) to ensure the seeds have sufficient water available to germinate.

For this experiment the four provenances from Guyana; Benab, Bush Lot, Mon Repos and Queenstown were relatively fresh seeds because they were harvested in April 2021 and sown in August the same year. In addition, the post-harvest handling of the seeds is unknown. Also, there was insufficient seeds to assess

germination prior to sowing. Furthermore, the harvest time of PKM- g and PKM-t is unknown, however these seeds were in cold storage at 4°C for almost a year before they were sown for this experiment.

Very little is known about germination in *M*. *oleifera* and reasons for poor germination are few. But previous studies have mentioned the germination of *M*. *oleifera* seeds to vary. For instance, Jahn et al. (1986) reported *M*. *oleifera* fresh seeds to have a germination ranging from 60% to 90 % and Nouman et al. (2012a) reported 54%. But in both studies, specific information of the seeds such as time harvested or time stored were not reported. However, Jahn et al. (1986) stated light affected germination in *M*. *oleifera*. Seeds of *M*. *oleifera* had a 40 % germination under full light as compared with 94 % when 50 % shade was provided. Ahmed et al. (2014) also found shade affected germination in *M*. *oleifera*. A 50 % light exposure is reported to give best germination. The light exposure was controlled using shade nets. However, the number of seeds that germinated under 50 % light was not significantly different ($P > 0.05$) from seeds germinated with no shade. Nevertheless, in this work light was not measured but the seeds were not exposed to direct sunlight and were sown directly in pots to a depth of 2 cm located in a greenhouse, where no artificial shade was provided, and natural lighting conditions prevailed. This suggest that while light may not penetrate the potting mixture at 2 cm it may be filtered and light reaching the seeds.

Other researchers reported a decrease in *M*. *oleifera* seed viability as seed storage time increased (Sharma & Raina, 1982). This was not the case in this experiment, because the seeds from India were in storage for 21 months and showed significantly higher emergence ($P < 0.001$, Table 3.1) than the seeds of the other provenances except for Queenstown, which showed similar emergence.

PKM-t and PKM- g are provenances of PKM-1. PKM-1 is an improved variety created by pure line selection and continuous selection for six generations. Selection in each generation involved only long pods and desirable characters, these were then advanced to create the next generation. The PKM-1 variety was developed for its fruit production (Ponnuswami, n.d). In fact, improved varieties are normally bred for higher performance above the average performance of traditional varieties or landraces or farmers varieties, as they are sometimes called (Fajardo Vizcayno et al., 2014). A possible reason for higher seedling emergence

by the provenances from India could be improved genetics. Also, these seeds may have been produced in a controlled production environment, where processes including post-harvest are designed to maximise and maintain seed quality. This may suggest a reason why the provenances sourced from India had better seed emergence than Benab, Bush Lot and Mon Repos. However, this does not explain why the Queenstown provenance had similar emergence as the improved varieties from India. Another possible reason could be that the seeds from Guyana were sourced from wild populations and selected by persons not trained in seed collection and therefore do not know how to select quality seeds. In addition, seeds from Mon Repos which showed poor emergence were sundried and this may be a possible cause for reduced viability because of the uncontrolled temperatures exposed to the seeds. The other provenances from Guyana were handpicked and were already dried on the plant. But some of the seeds from Benab and Bush Lot had small holes that may be a sign of weevil infestation and seed rot, which may be a reason for poor germination. Also, in Guyana, different persons with various educational background were involved in the sourcing of seeds. This may have affected the quality of seeds collected. But perhaps the main reason for improved seed emergence of PKM-t and PKM-g is that they were part of a commercial seed production system whereby *M. oleifera* plants were specifically grown for seed production. Whereas the seeds from Guyana were harvested randomly from plants growing on the Coastal plain.

3.6.1.2 Plant height before the application of salinity stress

The different provenances showed no significant difference ($P > 0.05$, Table 3.3) for mean plant height, though some amount of variation can be seen within populations of each provenance (Figure 3.1). Importantly, *M. oleifera* possess a 26 % chance of self-fertilisation (Muluvi et al., 2004), which can homogenise traits between parent plants and offspring. However, there is a 74 % chance that open pollination will occur in *M. oleifera*. This type of breeding system along with the fact that the provenances are from a wide geographical location, enables the plants to have a wide genotypic (Ravi et al., 2020) and phenotypic (Dao et al., 2017) variation when propagated sexually. Genetic variation may one of the causes for uneven plant height, because seeds of the provenances sourced from Guyana were taken from small

landrace populations across the coastline of Guyana with an average distance of 67 km between each population. Each provenance was given the name of the village from where it was sourced. In most cases these landrace populations were left to survive on their own favouring natural selection. In other words, the genes in each plant may vary, resulting in a different growth rate for each plant. While open pollination brings about a wide genetic variation in *M. oleifera*, which can cause variable plant height, plants with flowers that have been open pollinated have a higher fruit set than plants with self-pollinated flowers. (Manduwa et al., 2018).

Furthermore, in this study, to control and help reduce variations among individual plants, environmental conditions were maintained as constant as possible. The experiment was a greenhouse trial and the average air temperature inside the greenhouse during the experiment was 25.1 ^{o}C. The minimum and maximum temperature for the experimental period was 15.5 and 34.0 ^{o}C ±0.044 respectively. Before the start of salt stress application, the plants were irrigated daily to maintain field capacity of the potting mixture in each pot. Also, during the experimental period the green house floor was soaked once a day to help maintain relative humidity between 60 and 70 % except for the period between August 17 and September 13 where it was not possible because of covid-19 level 4 lockdown. The lockdown period coincided with the first month after the seeds were sown. This may have affected the seed germination and plant growth because of not being able to control the humidity within the greenhouse. Nevertheless, no significant difference among the mean height enabled the study plants to have a relatively equal starting point from which to accurately estimate the change in growth that salt stress can cause.

3.6.2 Effects of salinity stress

3.6.2.1 Leaf Chlorophyll content

Salt stress significantly reduced the chlorophyll content of the leaves and differences were detected across provenances ($P < 0.05$) and salt concentration ($P < 0.001$) (Table 3.4 and Figure 3.3). In this experiment leaf chlorophyll content was measured using a SPAD meter. These provide SPAD values by measuring the transmittance of red (650 nm) and infrared (940 nm) radiation through the leaf and calculate a SPAD value that should represent the chlorophyll content of the leaf (Minolta, 1989).

In this study, salt stress began when the plants were mature and already bearing true leaves (nine weeks after sowing of seeds). Increasing salt stress in *M*. *oleifera* plants lowered chlorophyll content in young fully expanded mature leaves. More specifically, *M*. *oleifera* plants treated with 156 mM NaCl stress showed a significantly lower chlorophyll content than plants treated with 0 mM and 52 mM NaCl (Table 3.4 and Figure 3.2 and 3.3). However, no significant differences were found between salt stress plants at 0 mM NaCl and 52 mM NaCl.

A reduction in leaf chlorophyll content in salt stressed plants may be caused by various factors. Firstly, the plants under stress may have a reduction in the synthesis of chlorophyll. Previous research done on salt stressed *Helianthus annus L.* plants, stressed with 100 mM NaCl, revealed a decrease in the of synthesis of 5-aminolaevulinic acid (ALA), a precursor of chlorophyll (Santos, 2004). Secondly, an increase in soil salinity by the addition of NaCl causes osmotic stress by increasing the concentration of ions in the soil and thereby making it difficult for the plant to take up water through its roots (Munns & Tester, 2008). Lack of water may cause yellowing in leaves and as a result give lower SPAD measurements. For this reason, to eliminate the possibility of lack of water field capacity of the potting mix was maintained as described in the methods. Suggesting that the cause for the reduced chlorophyll is salt stress and not lack of water.

The reduction in chlorophyll content in 156 mM NaCl -stressed plants was found in all provenances (Figure 3.3), demonstrating a common effect of high salt stress in *M*. *oleifera*. While previous work done by Nouman

et al. (2012b) did not use a SPAD meter to measure leaf chlorophyll content in *M. oleifera* leaves, their results also showed a significant decrease of leaf chlorophyll content when plants were exposed to 12 dS/m or approximately 150 mM NaCl salt stress, although the sampling number was low in the Nouman et al. (2012b) report. Fifteen plants were sampled as compared to seven hundred and twenty spad measurements in this current study. Also, in the work done by Nouman et al. (2012b), salt stress was initiated at an earlier five-leaf stage, while in this study salt stress started when the plants produced true morphological leaves at approximately nine weeks after sowing of seeds. But results obtained in this study and that of Nouman et al. (2012b) suggest that *M. oleifera* is affected by high salinity stress (more than 150 mM NaCl). Nevertheless, in this experiment, it was found that there was no significant difference between the mean chlorophyll content of plants treated with 0 mM and 52 mM NaCl (Table 3.4). This result also agreed with that of Nouman et al. (2012b) and is a good indication that *M. oleifera* can withstand moderate soil salinity as classified by US Soil Salinity Laboratory Staff (1954).

The mean chlorophyll content for the provenances also had a significant difference after the salt stress ($P < 0.001$). Queenstown showed a significantly higher chlorophyll content than Mon Repos at 52 mm NaCl (Table 3.4). However, plants of Queenstown were exposed to a significantly lower electrical conductivity which suggests a lower osmotic stress in theses than plants of Mon Repos measured by the final EC of the potting mixture (Table 3.13). However, lower EC of potting mixtures does not indicate that the salt stress was not properly applied, and it may be that the potting mixture (see appendix 1) used is inert and does not provide for the accumulation of salts. But a possible reason for the lower EC of the potting mix of Queenstown may be due to the number of plants. The number of plants of the provenance Queenstown was significantly higher than Mon Repos and this may cause more absorption of NaCl. This implies that more salt per plant is available to those of Mon Repos than Queenstown. Nevertheless, the EC of the potting mixture at 156 mM NaCl (Table 3.13) does not fall within the range of moderately saline soil but as slightly saline soil, according to US Soil Salinity Laboratory Staff (1954). This low potting mixture EC as compared to the high EC applied

through the salt solutions as stress, is an indication that the effects measured on the plants are mostly from the salt stress applied and not the gradual build-up of salts in the potting mixture.

Generally, the increase of salt stress reduces chlorophyll content in leaves, and this is demonstrated in other crops. For instance, 100 mM NaCl salt stress reduced total chlorophyll content in mature tomato plants (Al-aghabary et al., 2005). Also, salt stress at 200 mM NaCl to cow peas (*Vigna unguiculata* (L.) Walp.) at the first trifoliate leaf- stage significantly reduce chlorophyll content (Dong et al., 2019). This signifies that SPAD measurements can be used as a tool to help select salt tolerant Moringa genotypes for a breeding programme, because the susceptible genotypes will have a reduced chlorophyll content and therefore lower SPAD values than tolerant genotypes.

3.6.2.2 Plant morphological visual quality

In this experiment a total of 612 plants were scored at the end of the stress period (Table 3.5 and Figure 3.4). Plants of the different provenances stressed with 156 mM NaCl had a significantly ($P < 0.001$) lower visual quality than plants stressed with 0 and 52 mM NaCl (Table 3.5 and Figure 3.5). Most of the plants treated with 156 mM NaCl from the different provenances had a score ranging from 1 to 3, but there were some plants from Bush Lot, Mon Repos and PKM-t that had a score of 4 (Figure 3.4 (c)). This clearly shows that there is varying tolerance to salinity stress among and within the different provenances. While there was previous work done with salinity stress in *M. oleifera* (Al-Shoaibi & Boutraa, 2021; Elhag & Abdalla, 2012; Fatima et al., 2018; Hussein & Abou-Baker, 2013), none of them used a plant visual score to evaluate the effects of salinity on *M. oleifera* plants. The method of scoring used in this study was adopted from Niu et al. (2007). In this method, a 5-point scoring technique was used, with the best quality being 5. This was found to be more unbiased than other methods that employ a larger scale of 1-7 as described by Ravelombola et al. (2019). The scoring method by Niu et al. (2007) was also chosen because it takes into consideration the leaf damage along with plant growth as compared to other methods described by Niu et al. (2010) and Cai et al. (2014), which scores the plant based on leaf damage alone.

Scoring the plants also revealed a higher quality plant ($P < 0.001$) in Benab when compared with plants from Queenstown at 52 mM NaCl salt stress (Table 3.5). However, this may be because of a larger sample size of Queenstown than Benab (Table 3.5). The low number of plants of Benab may not accurately represent the population in situ. At 52 mM NaCl, Benab had a higher standard error than Queenstown, indicating that the data is more variable which may be a function of the smaller number of plants in the sample. Mon Repos also showed a high plant visual quality at 52 mM NaCl, but also had a high standard error, but again a low number of plants were assessed. As such, the scoring may not be as effective for those provenances that have a low sample size and care must be taken when interpreting the visual plant quality of these plants, also, variable data across provenances will make it more difficult to make comparisons. It also signifies that a low number of plants will not give accurate results in selection for salt tolerant genotypes when using plant morphological visual quality. Despite this, plants from all the provenances treated with a 52 mM NaCl stress had acceptable plant quality when compared to 156 mM NaCl (Figure 3.5), indicating that *M. oleifera* as a species will have relatively good growth under moderate salinity stress and visual morphological plant quality can be a fast way to identify salt tolerant genotypes.

3.6.2.3 Plant growth in height during the stress period

Plant height reduced significantly ($P < 0.001$) as the salt stress increased (Table 3.6 and Figure 3.7). One of the immediate responses of a plant to salinity stress is the rapid reduction of plant growth (Munns & Tester, 2008). In this experiment, plant height was measured at two points, the start of the stress period, and at the end. This was suggested by (Negrão et al., 2017), and by adopting this method to measure plant height, variations in height that would have occurred after germination but before the start of the salinity stress are eliminated. Metwally et al. (2021) and Al-Shoaibi and Boutraa (2021) both reported similar results that showed a reduction in plant height with increasing salinity stress. However, in their work, no mention is made of the subtraction of the initial plant height to avoid misrepresentation of the growth during stress. Also, in this study, six provenances were investigated, and the results showed significant differences ($P < 0.05$) for the plant height during the stress period. Overall, when taking into consideration all stress levels

(0, 52 and 156 mM NaCl) plants from Queenstown demonstrated lower plant growth in height (P = 0.03) when compared with plants from Mon Repos, but Mon Repos had a low number of plants. Other studies that have evaluated salinity stress in *M*. *oleifera* only compared plants within a population obtained from a single provenance (Metwally et al., 2021), while there was one study that compared *M*. *oleifera* with its sister species *Moringa perigina* (Al-Shoaibi & Boutraa, 2021). In the latter study, salt stress seems to affect both species, but plant growth in height was reduced more in *M. peregrina* than in *M*. *oleifera,* suggesting *M*. *oleifera* is more tolerant to salinity stress than *M*. *peregrina.*

Different plant species vary in tolerance to salinity stress and for this reason, they will have varying growth rates when stressed (Munns & Tester, 2008). Interestingly, there are reports of salinity tolerance variation within the same species. For instance, wheat plants belonging to different sub-species within the same species have shown varying responses when exposed to 150 mM of NaCl salt stress (Munns & James, 2003). In a similar manner, this study has reported different provenances (in this context *M*. *oleifera* seeds sourced from different geographic locations) having varying responses in growth to salinity stress (Figure 3.6).

Importantly, plants treated with 0 and 52 mM NaCl had similar heights (Table 3.6). This result suggest that if *M*. *oleifera* plants are stressed with moderate saline conditions as classified by US Soil Salinity Laboratory Staff (1954) there will be no significant difference in plant height when compared with non- stressed plants and *M*. *oleifera* plants can withstand a salt stress of 52 mM NaCl and possibly higher. The threshold level that the plants can tolerate before there is a drastic reduction in plant height is between 52 mM NaCl and 156 mM NaCl. In the present study an intermediate salt concentration was not used between 52 mM NaCl and 156 mM NaCl because the main aim of the study was to determine the possibility of growing *M*. *oleifera* for cattle in moderately saline soils and not determine the maximum salt concentration that *M*. *oleifera* can tolerate. At 156 mM NaCl, plant height was severely affected for all provenances (Table 3.6 and Figure 3.7), with the average height less than half that of the height of plants treated with 52 mM NaCl. In this study, the sodium concentration in leaves increased as the salt stress increased (Table 3.14 and Figure 3.15). At a high salt stress, there is no plant growth because the salt ions accumulate in the old leaves which are no longer

expanding and hence cannot help to dilute the ions as younger leaves do, ultimately resulting in the dropping of leaves. The results obtained in this study show that at high salinity stress the plants are not able to grow and develop at a faster rate than the occurrence of negative salinity effects, and if this stress level continues the plants will eventually die.

3.6.3 Biomass

Plant biomass percent dry weight (%) and dry weight (g) for shoots, roots and whole plant was assessed for each provenance and salt treatment. The following biomass indices were also calculated: relative decrease in plant biomass, root mass ratio, relative root mass ratio, salt tolerance index and stress tolerance index.

In general, there was a reduction in fresh plant biomass (Table 3.9 (a)) and a significant reduction in plant dry weight when plants were stressed with 156 mM NaCl (Tables 3.7 and 3.8). Salinity stress negatively affects plant biomass, because plant biomass is primarily made up of mostly plant cell walls (Somerville et al., 2010). In particular, the cell walls are primarily cellulose (McFarlane et al., 2014) and salinity affects the cellulose synthase complexes, the process by which cellulose is made (Wang et al., 2016). As a result of reduced cellulose synthesis, a disruption in the plant growth occurs and hence a reduction in plant biomass. At 52 mM NaCl, cellulose synthase complexes were not affected.

In this study, root dry weight and percent dry weight were significantly reduced ($P < 0.001$) when plants were stressed at 156 mM NaCl, but root dry weight was not reduced at 52 mM NaCl when compared with the control treatment (0 mM NaCl) (Tables 3.7 (b) and 3.8 (b)). This effect is a result of the plant's response to salt stress, which can be complex (Munns, 2002; Munns & Tester, 2008; Zhao et al., 2021). Geng et al. (2013) found that an immediate response by plants to increasing salt stress is a rapid decrease in the growth rate and a temporary pause in the growth of the main root. Results obtained in this experiment showed both plant shoot weight and root weight decreased significantly between stresses at 52 mM NaCl and 156 mM NaCl. Only the shoot weight was significantly reduced at 52 mM NaCl but not root weight, suggesting that the growth of roots is less affected by the application of 52 mM NaCl salt stress (Figure 3.9) and the above

ground plant biomass may be more sensitive to salt stress. *M. oleifera* root growth may become severely affected if the salt stress is above 52 mM NaCl.

While there was a significant reduction in shoot dry weight at 52 mM NaCl, the percent shoot dry weight did not follow this trend (Table 3.7 (b)). Both root and shoot percent dry weight were not significantly different at 0 and 52 mM NaCl (Table 3.7 (b)). In fact, the root mass ratio for all the provenances increased as the salt stress increased (Table 3.9 (c)). This is suggesting that 52 mM NaCl salt stress had no effect on root biomass and if there was a temporary pause in the growth of the roots as suggested by (Geng et al., 2013) from their work in Arabidopsis, it did not affect the root biomass production.

However, a significant reduction in shoot and root dry weight and percent dry weight at 156 mM NaCl means smaller *M. oleifera* plants when stressed with 156 mM NaCl. In this research, root weight data was taken from the majority of the roots extracted which includes the primary root and not the total roots of the plant, because there were a small number of tertiary roots that remained in pots. As such, the no significant difference in root biomass reported in this study reflects biomass for most of the plant roots extracted. The total reduction in root biomass is not known here, but Munns (2002) summarised from previous studies that there is a reduction in lateral roots after weeks of salinity stress and also pointed out leaf growth is more affected than root growth. The results of this study are in accordance with Munns (2002) and clearly show the effects of salinity on shoot growth are more than on root growth (Figure 3.8 and 3.9) which explains the higher root mass ratio with increasing salinity (Table 3.9 (c)).

Other studies found similar results that support the decrease of *M. oleifera* biomass as salinity stress increases, but these studies did not compare different provenances (Al-Shoaibi & Boutraa, 2021; Fatima et al., 2018). The six provenances used in this study showed significant differences in whole plant percent dry weight and dry weight (Table 3.7 (a) and 3.8 (a)). Bush Lot, PKM-t and Queenstown had similar whole plant percent dry weight, and these were significantly higher ($P < 0.001$) than whole the plant percent dry weight of Mon Repos and PKM-g (Table 3.8 (a)). However, Queenstown, Bush Lot and PKM-t showed a significantly lower whole plant dry weight than the other provenances. This shows that different provenances respond

differently to salinity and although the plants of Queenstown, Bush Lot and PKM-t were smaller, they had a higher biomass to water content ratio. However, plants from Mon Repos and Benab were larger but had a lower biomass to water content ratio. A higher biomass to water content is important because it will mean more production per unit area of land and means more nutrients for animals. It is evident that a direct cause of plant biomass reduction during salt stress is reduced growth, and this is as a result of reduced cellulose production as can be seen by the reduced plant weight and dry matter (Wang et al., 2016).

3.6.4 Net Photosynthesis

When there is salt stress, osmotic adjustment occurs, and this reduces the available resources for photosynthesis (Flexas et al., 2004). In this study net photosynthesis was lower across all provenances and salt treatments at the end of the salt stress period when compared to initial measurements before salt stress (Table 3.10 (a)). Also, there were no significant differences across provenances for each salt treatment. This indicates net photosynthesis may not be a good measure for screening *M*. *oleifera* for salt stress. However, measuring net photosynthesis in plants to determine plant response to different salinity levels is not new, and have been done in various crops, such as maize (Hessini et al., 2019), tomato (Singh et al., 2016), ornamental shrubs (Cirillo et al., 2016) and quinoa (Becker et al., 2017). But in *M*. *oleifera*, this is new, as of this date there is no research that evaluates the response of photosynthesis of different *M*. *oleifera* provenances at varying salinity stress. Most of the research done involving the screening of *M*. *oleifera* for salinity tolerance, evaluates plant growth parameters (see appendix 2). How a plant responds to salinity stress has been known to vary within plant species and is usually the first step in creating improved cultivars for salt tolerance. For instance, some researchers have reported net photosynthesis to vary significantly among the same species of Artichoke at different levels of salinity stress, but these were recognised as cultivars known to have contrasting responses to salinity (Xue & Liu, 2008). This signifies that salt tolerance must have existed in one genotype or a population of Artichoke, and then this tolerance was used to create an improved cultivar. Similarly, in this study, the provenances responded differently to salinity stress and

provenances that showed a higher net photosynthetic rate under salt stress could be used to breed a salt tolerant variety.

A variation in net photosynthesis may be caused by a number of factors, including the genetic profile of the provenances. Other variations among plants in the greenhouse may be as a result of the temperature variation that occurred during the experimental period. A study done by Alshoaibi (2021) evaluated the effect of temperature on photosynthesis. A comparison of *M*. *oleifera* with its sister species *M. peregrina* was made and the results showed no significant difference between species. However, the research found temperature affected the photosynthetic rate for both species. The research further demonstrated that lower temperature tends to reduce net photosynthesis in both species. This means that photosynthesis is affected by the temperature where *M*. *oleifera* plants are grown. In fact, although the experiment was done in a greenhouse, the temperature had a range of 18.5^{o}C, with a minimum and maximum temperature of 15.5 and 34.0 ^{o}C respectively. While temperature was not measured at different points within the greenhouse, but rather in the centre at plant canopy height, the blocking design was used to account for variations due to plant position within the greenhouse.

In this experiment, a sample size of one plant from each treatment, using the first fully expanded mature leaf was chosen to measure net photosynthesis. This sample size was chosen to ensure measurements were taken in the shortest possible time between the hours of 10 am and 2 pm to avoid variations in net photosynthesis measurements throughout the day caused by sunshine and temperature. While this is ideal for minimising variations, statistically it may cause a high standard error as a result of a low sample number and will cause a misrepresentation of the population. This low sample size may have been a reason for not detecting variations across provenances.

Notwithstanding the temperature variations during the experimental period, varying rates for net photosynthesis of *M*. *oleifera* provenances can be caused by other reasons. In fact, during net photosynthesis data collection, there was varying light intensity caused by cloud cover changing throughout the day, as evidenced by data collected by the National Institute of Water and Atmospheric Research (2022). The data

recorded showed that the amount of sunshine varied each day during the experimental period (Figure 3.16). Changes in the light intensity and amount of sunshine hours during the day can also affect photosynthesis in plants. When a leaf in the shade is suddenly exposed to high light intensity, photosynthesis slowly increases to a new steady state. This adjustment of photosynthesis is called photosynthetic induction and it takes between several minutes to an hour and depends, among others, on the species, ecological habitat and environmental factors such as CO_2 partial pressure, temperature and humidity (Kaiser et al., 2017; Kaiser et al., 2015; McAusland et al., 2016; Valladares et al., 1997). For example a study done by Y. Zhang et al. (2018) showed that as the period of sun and shade increased, photosynthesis was strongly inhibited and this effect was increased in salt stressed tomatoes leaves (*Solanum lycoperscicum*). In the same study, salt stressed leaves had a lower electron use efficiency and lower stomatal pore area per leaf on the adaxial side. This implies that the net photosynthesis of *M*. *oleifera* plant leaves was not only affected by salinity (Figure 3.11 and 3.12), but also by the fluctuations in sunshine duration each day and the effect of net photosynthesis maybe the summation of the effects of salinity and duration of sunshine.

This study showed salt stress significantly reduced ($P < 0.001$) net photosynthesis in *M*. *oleifera* plants (Table 3.10 (b)). Net photosynthesis was reduced significantly when plants were stressed with 156 mM NaCl fourteen days after the first application (Table 3.10 (b)). The first application of salt stress did not create any osmotic stress that affected photosynthesis and two applications were needed to induce a negative response. This also is evidenced by the reduction in transpiration and stomatal conductance (Tables 3.11 and 3.12). Photosynthesis comprises of two phases and is the process where plants use water and carbon dioxide to produce precursors of carbohydrates and oxygen. The first phase occurs during the day and requires light. The light is used to drive the electron transport system and in turn, the excited electron gets converted to ATP and NADPH, during this process water molecules are split to form half a molecule of oxygen, an electron and a proton. The electron is then re used to form more ATP and NADPH and the oxygen produced is released through the stomata (Shevela & Björn, 2017). The second phase does not require light energy and it takes place in the stroma of the chloroplast. In this phase, the carbon dioxide is converted to

precursors of carbohydrates and is partially driven by ATP and NADPH. The reaction is in a cycle where ribulose-1,5-bisphosphate goes through a series of oxidation and redox changes and at the end the ribulose-1,5-bisphosphate is regenerated for the process to continue (Biel & Fomina, 2015). Therefore, it may be that in the first two weeks of salt stress, the plants were taking up enough water and photosynthesis was not affected because there was not a build-up of salts in the potting mixture or the concentration of sodium in the plant was not yet at a level where it was affecting photosynthesis.

3.7 Conclusions

The reduction of net photosynthesis after two stress applications could be because of the build-up of salts in the potting mixture. However, in this experiment while the electrical conductivity of potting mixture used for the different salinity treatments was found to be significantly different (Figure 3.14), they were not classified as highly saline. In fact, potting mixture used to apply the high salt stress of 156 mM NaCl gave an average EC that is classified as slightly saline according to the US Soil Salinity Laboratory Staff (1954). This means that there was no significant and rapid build-up of salts in the potting mixture and if there was, it was very slow. Moreover, analysis of plant leaves revealed the provenances had significantly ($P < 0.001$) different amounts of sodium build-up (Table 3.14) when exposed to 52 mM NaCl and 156 mM NaCl. Also, potassium content in leaves was significantly different across provenances at 52 mM NaCl and 156 mM NaCl but was higher than the sodium content (Figure 3.15). This suggests that potassium in *M. oleifera* leaves is not being replaced by sodium in vital pathways and could be a reason for the satisfactory growth of *M. oleifera* when exposed to 52 mM NaCl.

The reduction of net photosynthesis at 156 mM NaCl also severely affected plant weight (Table 3.7 and 3.8). Conversely, at 52 mM NaCl salt stress, photosynthesis was not significantly reduced when compared with 0 mM NaCl, and as a result, plant percent dry weight at 0 mM NaCl and 52 mM NaCl did not differ significantly. However, at 52 mM NaCl there was a decrease in shoot weight but not in root weight when compared with 0 mM NaCl (Table 3.8 (b)). This decrease in shoot weight is also supported by the increasing root mass ratio

(Table 3.9 (c)), indicating a reduction in shoot growth, possibly by the diversion of energy towards the plant root.

The results obtained showed *M. oleifera* can tolerate moderately saline conditions and therefore can be grown in Guyana on lands that are cultivated and affected by saltwater intrusion. It also implies it is possible to use brackish water to irrigate *M*. *oleifera* when fresh water is not available.

Chapter 4 Moringa in the rumen, a greenhouse gas reduction?

4.1 Introduction

Globally, livestock emits 7.1 gigatonnes of $C0_2$ equivalent of greenhouse gas each year, with methane contributing to about 3.1 gigatonnes of $C0_2$ equivalent. This amount will increase as the demand for meat grows along with a growing global population (Food and Agriculture Organization of the United Nations, 2013). As such, reducing methane emission from ruminant livestock is important (Gerber et al., 2013). A key strategy to help reduce methane emissions is to increase ruminant efficiency by providing better quality diets, because forage quality has been associated methane emissions (Jonker et al., 2015; Lee et al., 2017).

The methane emissions from forage diets are most times 6-9% of the gross energy that the animal consumes (Johnson et al., 2000). This has prompted investigations into methane emission in major forages, as reducing methane emission by ruminants would help lower greenhouse gas emissions and may also make available more energy to animals. In New Zealand, the dairy industry is one of the largest contributor to agricultural greenhouse gas emissions (Ministry for the Environment, 2019). For this reason, the New Zealand dairy industry, which is based on pastoral grazing and consists of mainly of ryegrass mixed with white clover, chicory, plantain and sometimes red clover pastures, have been under investigation for their methane emissions (Jonker et al., 2018; Sun et al., 2011).

M. oleifera is already being used as feed for animals in some countries (Su & Chen, 2020). In Nicaragua it is used as a supplemental feed for cattle and research has proven it to be beneficial for both beef and dairy cattle when comprising of 50-60% of the total feed intake (Fuglie, 2000). It is also used in Zimbabwe by subsistence farmers to feed cattle (Maroyi, 2005), and has been proposed as a source of protein for cows in tropical areas (Mendieta-Araica et al., 2011). The use of *M. oleifera* is expanding in third world countries and

its use will increase as population grows. If *M. oleifera* is to be used as a forage crop to feed cattle, the quantification of methane emission during ruminal fermentation can be part of an important strategy to help New Zealand and other countries lower their greenhouse gas emissions to combat climate change and meet reduction targets. Therefore, the objective of this study was to:

4.1.1 Objective

Compare the gas and methane production of *M. oleifera* to ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.) using an *in vitro* incubation system.

4.2 Material and methods

4.2.1 Location

The analyses were undertaken at the AgResearch Grasslands Research Centre, Tennent Drive, 11 Dairy Farm Road, Palmerston North 4442, in May 2021 and in January 2022.

4.2.2 Ethics Statement

The collection of rumen fluid from fistulated Holstein Frisian heifers and the management of these animals was approved by the AgResearch Grasslands Animal Ethics Committee, Palmerston North, New Zealand (AE 15154) and is in accordance with the Animal and Welfare Act of 1999 and its amendments, New Zealand.

4.2.3 Source of dried *M. oleifera* leaves

Seeds of *M. oleifera* were sourced from King Seeds (Katikati, New Zealand), Guyana and India. There were seven provenances used in this experiment (King Seeds considered a provenance). Moringa seed from King Seeds (a local New Zealand seed company) is of unknown origin but was sourced from the United States (King Seeds, pers. Comm.), four provenances were sourced from Guyana and two from India. The four provenances sourced from Guyana were Benab, Bush Lot, Mon Repos and Queenstown, and the two from India were Periyakulam -g (PKM-g) and Periyakulam- t (PKM-t) as described in 2.2.

4.2.3.1 Preliminary Analysis

Dried leaves of *M. oleifera* were obtained from plants of King Seeds. Two hundred seeds from King Seeds were sown in potting bags on the 29 September 2020, at the Plant Growth Unit, Massey university, Batchelar Road, Palmerston North 4474. Seeds were germinated and plants left to grow for five months. Afterwards one hundred plants were transplanted in an open field located on Poultry Farm Road, Palmerston North. The plants were transplanted to the field using a spacing of 1m by 1m. The remaining plants were left in the greenhouse.

4.2.3.2 Main analysis

Dry leaf material to carry out the main analysis was obtained from plants used in a previous salinity trial, along with dried leaves of plants grown from seeds sourced from King Seed as described in 4.2.3.1. Seeds originated from Guyana and India were sown in the first week of August as part of a salinity screening trial and these seeds were sown directly in 9 litre- plant pots with a 24cm diameter.

The potting mixture used in all the bags and pots was Daltons Base mix + Osmocote Pro (see appendix 1 for detailed composition).

4.2.4 Age of plants and dried plant material

Dried *M. oleifera* plant material used in this experiment came from plants of different age. At the time of harvesting fresh plant material, plants of King seeds were seven months old (from the time of sowing the seeds). The fresh material obtained from the other provenances was taken from three months old plants.

The fresh leaves of plants grown from seed from King Seeds were harvested from plants that were in the field and in the greenhouse in April 2021 so that enough leaf material was obtained. The leaf material was dried separately. For the main analysis (provenances that originated from Guyana and India) leaf material from the untreated controls were harvested from the 0 mM NaCl (nil NaCl) treatments at the end of the salinity trial in October 2021.

All fresh plant material for both analyses were oven dried for 72 hours in a convection oven set to 60°C. Dried plant material was then ground finely using a coffee and spice grinder, labelled and stored in air- tight plastic containers.

4.2.5 Substrate collection

For the preliminary analyses there were a total of eight substrates used in addition to the control. *M. oleifera* transplanted in the field were in ten rows, each consisting of ten plants. Young fully expanded mature leaves were collected from the first three rows of plants, then another four rows and the last three rows and pooled

separately forming three composite samples. This procedure was also used for the plants in the greenhouse since the plants were in pots and consisted of ten rows. Dried grounded ryegrass and white clover were supplied by AgResearch Grasslands. These were harvested in spring 2010 from Aorangi Experimental Station, 315 Lockwood Road, Manawatu, Wanganui, Palmerston North.

For the main analyses, there were 34 different sources of *M. oleifera* used:

1. *M. oleifera* leaves from the preliminary experiment were re-tested with the exception that leaves originating from the field and greenhouse were pooled into one composite sample and mixed thoroughly by placing into a square container and rotating the container twelve times on its edges.
2. Young leaves were collected from all the plants in the greenhouse (plants grown from seeds sourced from King Seeds that remained in the greenhouse) and pooled into one composite sample.
3. The salinity trial consisted of 18 treatments and 5 blocks, set up in a randomised complete block design. Within each block there were 6 treatments of 0 mM NaCl that corresponded to the six provenances. From each 0 mM NaCl treatment, dried leaves from all the plants were combined, forming six samples within each block that represents the six provenances and thus making 30 samples in total (6 from each block * 5 blocks).
4. Ryegrass and white clover were the same as for the preliminary analysis.

4.2.6 Rumen Fluid

For this study, rumen fluid was collected from three fistulated seven-year-old dry Holstein Friesian cows. In all the experiment rumen fluid was collected in the morning before the animals were allowed to graze on pasture (around 8am) on the day of the incubation.

4.2.7 *In vitro* fermentation

The *in vitro* fermentation was done using a fully automated batch culture system according to the procedures outlined in Muetzel et al. (2014). For each experiment, each substrate (dried leaf material to be used in the

in vitro incubation) was duplicated and then replicated three biological times, with each biological replicate a mixture of rumen fluid from two different donor animals.

4.2.7.1 Preliminary Experiment: Comparing *M. oleifera* leaves from plants grown from King Seeds seed in the field and greenhouse with leaves from ryegrass and white clover.

The first experiment consisted of nine substrates; six substrates of *M*. *oleifera* (three from the field and three from greenhouse), white clover, rye grass and the internal control ryegrass sample that have been used repeatedly in incubations for over eight years. The preliminary experiment began on the 17 May 2021.

4.2.7.2 Main Experiment - Comparing different provenances of *M*. *oleifera* used in the salinity trial, a composite sample of King Seeds from the field and greenhouse, leaves of King Seeds from the greenhouse and comparing them with ryegrass and white clover. Three separate incubations were done, each time using a mixture of different donor animals to yield the three biological replicates. The first incubation was done on the 8 December 2021, the second on the 15 December 2021 and the third on the 17 January 2022 and making 5 litres of BC Buffer for each incubation.

4.2.7.3 Incubation

Before the incubation, 0.5 grams of substrate was placed in 100-ml serum bottles. The bottles were randomized, then placed on a reciprocal shaker in a fan- driven incubator and pre warmed to 39°C. For incubation, batch culture (BC) buffer listed in Table 4.2 (Mould et al., 2005) was used. The composition of the final buffer is given in Table 4.1. Each BC buffer contained a mixture of two different rumen fluid from different donor animals. The buffers were gassed with CO_2 for at least 30 minutes before the reducing agent (NaOH 2.5 mM and cysteine-HCl 2.5 mM) was added just prior to collection of rumen fluid. Before rumen fluid collection, calibration gases were injected to the gas chromatograph.

The rumen fluid was collected in pre-warmed thermos flask. The flasks were filled completely with rumen fluid, leaving no head space. This was to exclude oxygen and maintain an active microbial community. The rumen fluid was then filtered through one layer of cheese cloth and added immediately to the buffer. CO_2

was then bubbled through the medium for 15 minutes to equilibrate the rumen fluid before adding to serum bottles and to also maintain an anaerobic environment for the microbes. A sample of the medium without any substrate was collected for the analysis of short chain fatty acids (0 h sample).

The serum bottles were flushed with CO_2 and 50 ml of medium was then added to each respective pre warmed bottle and sealed with a butyl rubber stoper. The bottles were filled one at a time and returned to the incubator where each bottle was connected to the automated system by inserting a 23-gauge needle through the rubber stoper. When all the bottles were filled, and needles inserted, the oscillating shaker was set to 120 rpm and turned on.

Gas was measured every minute by the increase in pressure in the bottles. When a threshold pressure representing a gas volume to flush the system was reached, a solenoid valve was opened, and the gases were injected in a gas chromatograph. The incubation was then left to run for 48h.

Data was analysed for methane and total gas production at 12 hours and 24 hours to eliminate the time variability. Time point at 48h was not used because at this time fermentation has already plateaued and finished. Although the batch culture experiment gives gas production in real time and is run for a 48- hour period, each serum bottle had a different recorded time when gas is measured. In the system designed by Muetzel et al. (2014), a computer records gas production from each serum bottle every minute. However, if the pressure inside a bottle exceeds the pressure that is equivalent to 6ml of accumulated gas, that bottle is prioritize for venting, as such there may have a queue of bottles waiting to vent. In addition, the starting point of fermentation for each bottle differs because the rumen fluid was added to each bottle one at a time.

4.2.7.4 Short chain fatty acids

After Incubation, a sample for short chain fatty acids analysis was taken from each bottle. Each serum bottle was shaken by hand before pipetting 1.8ml of sample from each into 2ml Eppendorf tubes. The samples were centrifuged (21,000 × g for, 10 minutes at, 4°C) and 900 µl of the supernatant was transferred into new

1.5µl tubes and 100 µl of internal standard solution (19 mM ethylbutyrate in 20%(v/v) phosphoric acid) added.

The samples were kept in a −20°C freezer until the following day, thawed and centrifuged as above. An aliquot of 750 µl of the supernatant was transferred into a 2 ml crimp cap gas chromatography vial and crimped immediately. Short chain fatty acids were analysed by gas chromatography as described by Attwood et al. (1998).

Table 4.1. The quantity of each compound used in one litre of medium

BC Buffer	Volume
10x Buffer 1	80 ml
10x Buffer 2	80 ml
dH_2O	640 ml
Cystein HCL	0.316g
10 N NaOH	0.2 ml
Rumen Fluid	200 ml

Table 4.2 The quantity of each component used in one litre (a) Buffer 1 and (b) buffer 2

(a)

10x Buffer 1 (Macrominerals)			Volume (1 L)
Buffer component	MW	Conc. (mM)	amount (g)
Na_2HPO_4	142.0	6	8.51
KH_2PO_4	136.1	9.6	13.02
$MgCl_2$ $6xH_2O$	203.3	0.5	1.05

(b)

10x Buffer 2			volume (1 L)
Buffer component	MW	Conc. (mM)	amount (g)
$NaHCO_3$	84.0	64.5	54.18
NH_4CHO_3	79.0	17.8	14.07

4.2.8 Experimental design

In both experiments each substrate (plant material) was duplicated then replicated three biological times. The substrates were completely randomised in the incubator. For the preliminary experiment *M. oleifera*

samples (3 from the field and 3 from greenhouse) was compared with high quality perennial ryegrass and white clover. (8 substrates * 2 replicates * 3 mixtures of rumen fluid (three biological replicates)).

In the main experiment, each provenance from Guyana and India sourced from the salinity experiment (0 mM NaCl) from within each block was considered as a separate substrate, hence were 6 substrates from each block (6 *5 blocks). Those along with young *M. oleifera* leaves from plants in the greenhouse, a composite *M. oleifera* sample from the first experiment was compared again with perennial ryegrass and white clover. The incubation also included a run control, making it 35 substrates * 2 replicates * three biological replicates.

4.2.9 Statistical analysis

The real time gas production data collected and used to calculate total gas and methane production in millilitre per gram (ml/g) of substrate at 12 and 24 hours. The biological replicates were used as blocks. Because of the unbalanced data produced from both incubations, Data were analysed using analysis of variance for unbalanced designs using GenStat regression in GenStat 21st edition (VSN International, 2021). The total gas, methane, proportion of methane, average standard error of the differences of means and P-value were also calculated.

4.3 Results

4.3.1 Initial comparison of *M. oleifera* with perennial ryegrass and white clover

Real time gas production showed a lower gas production by *M*. *oleifera* and white clover than in ryegrass (Figure 4.1 and 4.2). Raw data showed gas production was similar for all substrates for the first 12 hours. Raw data for methane production showed ryegrass with a higher production throughout the entire incubation period followed by white clover and *M*. *oleifera*.

Further analysis of data revealed *M*. *oleifera* had a lower ($P < 0.001$) methane and total gas production in millilitre of gas per gram of substrate (ml/g) (Table 4.3) at 12 and 24 hours. Methane production (ml/g) at 12 and 24 hours were significantly lower ($P < 0.001$) in all *M*. *oleifera* substrates than ryegrass (Table 4.3), but similar to white clover. In addition, analysis of short chain fatty acids at the end of incubation showed *M*. *oleifera* had a higher percentage butyrate and propionate but lower Acetate than ryegrass and white clover (Table 4.4). Importantly, *M*. *oleifera* took a shorter time to reach half the maximum gas production (GP t 1/2 max) (Table 4.3).

The results also showed that *M*. *oleifera* leaves from the greenhouse had over all lower gas production at 12 and 24 hours (Table 4.3). This was significantly different from ryegrass but not when compared with *M. oleifera* grown in the field.

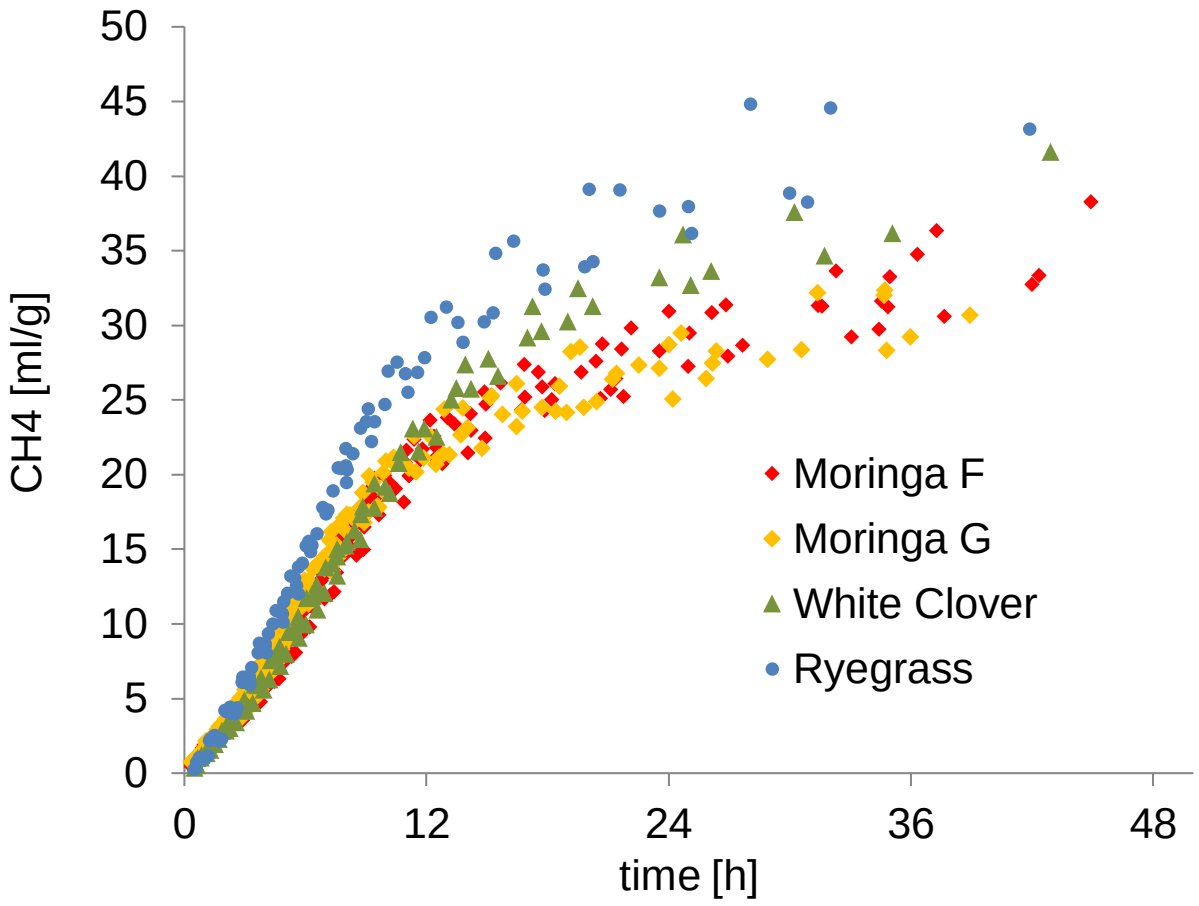


Figure 4.1 Real time production of methane (ml/g) for *M. oleifera* in field (Moringa F) and in greenhouse (Moringa G), ryegrass and white clover

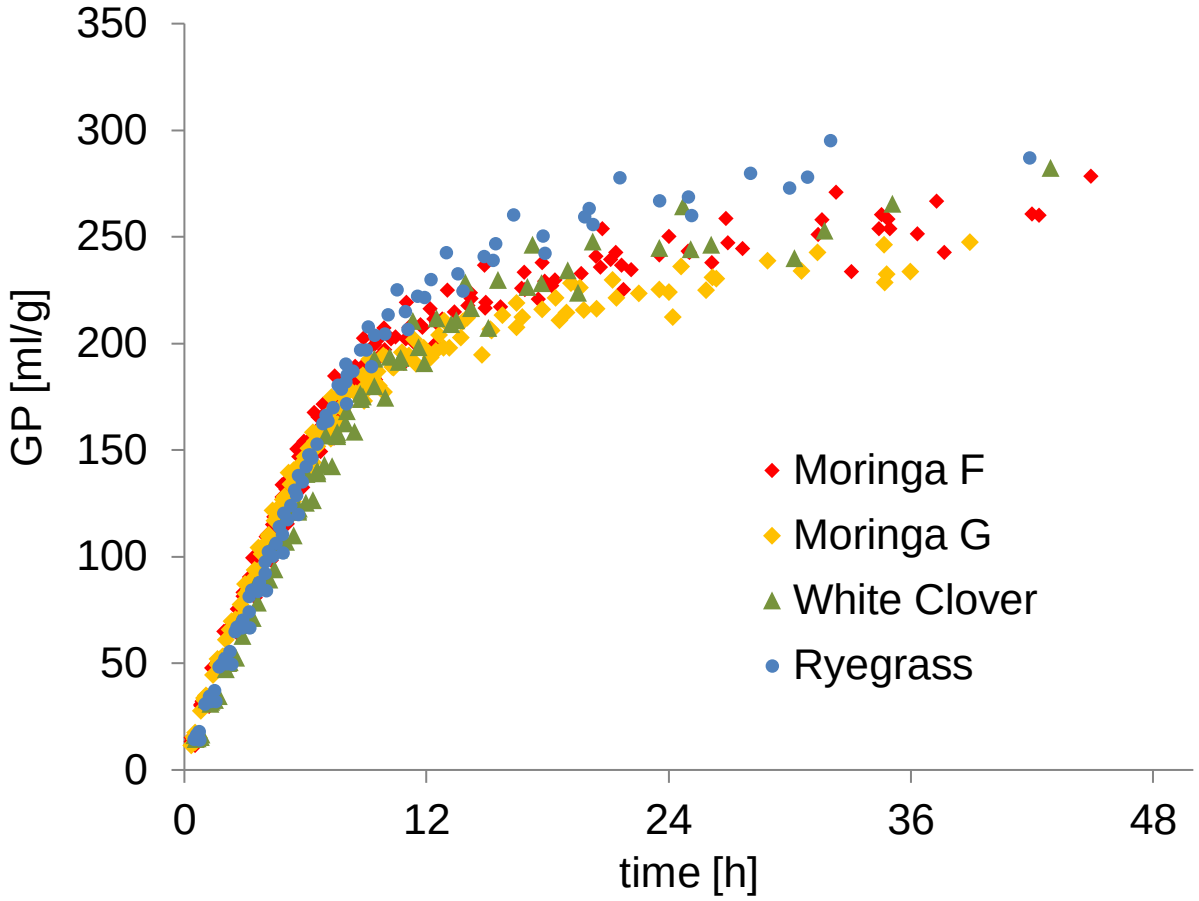


Figure 4.2 Real time production of gas production (ml/g) for *M. oleifera* in field (Moringa F) and in greenhouse (Moringa G), ryegrass and white clover.

Table 4.3 Total gas production, methane and proportion of methane at 12 and 24 hours for perennial Ryegrass (*L. perenne*), White clover (*T. repens*) and *M. oleifera.* The average standard error of the differences of means (SED) and P-Value are presented. GP t 1/2 max(h) and CH4 t 1/2 max(h) (half for *M. oleifera* in open field and greenhouse are the average for F1, F2, F3 and G1, G2, G3 respectively. t 1/2 max signifies half the maximum time.

	Time	12h			24h				
	Substrate	GP ml/g	CH4 ml/g	CH4(% GP)	GP ml/g	CH4 ml/g	CH4(% GP)	GP t1/2 max(h)	CH4 t1/2 max(h)
	Ryegrass	223.7	28.17	12.58	267.13	37.60	14.05	5.81	8.11
	White clover	202.3	22.78	11.25	244.83	33.13	13.54	5.91	9.56
M.oleifera Open field	F1	205.6	21.88	10.63	237.11	28.66	12.08		
	F2	210.1	21.41	10.16	243.70	28.46	11.66	4.97	8.51
	F3	210.6	20.89	9.90	243.82	27.57	11.28		
M.oleifera Greenhouse	G1	196.2	21.53	11.03	224.62	27.79	12.35		
	G2	198.6	21.42	10.76	227.52	27.52	11.90	4.48	7.13
	G3	195.3	21.30	10.88	223.59	26.82	11.99		
	SED	3.903	0.323	0.138	4.947	0.506	0.181	0.084	0.296
	P-Value	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***

*** significant difference P<0.001

Table 4.4 Short chain fatty acids at the end of fermentation. Moringa F and G are the mean for *M. oleifera* samples, F1, F2, F3 and G1, G2 and G3 respectively.

Acetate, propionate and butyrate are presented along with total short chain fatty acids. The mean concentration in millimoles (mM), average standard error of the differences (SED) and P-value are presented.

	Acetate	Propionate	Butyrate	Total SCFA			
Substrate	[mM]	[mM]	[mM]	[mM]	Acetate [%]	Propionate [%]	Butyrate [%]
Ryegrass	53.42	15.05	8.19	81.26	65.75	18.52	10.07
White Clover	49.44	12.89	7.16	72.98	67.75	17.66	9.81
Moringa F	49.03	15.56	8.48	77.48	63.27	20.08	10.94
Moringa G	49.21	14.55	8.14	76.19	64.59	19.10	10.69
SED	0.881	0.273	0.150	1.365			
P-value	<0.001***	<0.001***	<0.001***	<0.001***			

*** significant difference P<0.001

4.3.2 Comparison of *M. oleifera* provenances with perennial ryegrass and white clover

4.3.2.1 Gas production

Data analysis for methane and gas production at 12 and 24h showed significant ($P < 0.001$, Table 4.5) differences. Methane production of the composite sample (King Seeds) of *M. oleifera* at 12h did not differ significantly from white clover, but both was significantly lower than ryegrass and all other *M. oleifera* provenances. However, at 24h the methane production was similar for Bush Lot, Mon Repos, Queenstown and the composite sample. But the composite sample was lower ($P < 0.001$) than white clover and both were significantly lower than ryegrass (Table 4.5). The mean methane production at 24h was 30.5 ml/g, all samples tested were below this mean, except for white clover, PKM-t, ryegrass, benab, PKM-g and young leaves (King Seeds) from greenhouse. Bush Lot had the lowest gas production at 24 h which was not significantly different from Mon Repos, Benab and Queenstown, but significantly lower than ryegrass which showed a higher ($P < 0.001$) gas production than all other substrates.

4.3.2.2 Short chain fatty acids

PKM-g and leaves from plants from seeds sourced from King Seeds had the highest concentration of short chain fatty acids which was significantly higher than white clover but not for ryegrass (Table 4.6). PKM- g and ryegrass had similar acetate, propionate and butyrate concentration which were not significantly different.

Table 4.5 Total gas production, methane and proportion of methane at 12 and 24 hours for, *M. oleifera* composite sample, young leaves of *M. oleifera*, Queenstown, Benab, PKM-t, PKM, g Mon Repos, Bush lot, Ryegrass (*L. perenne*) and white clover (*T. repens*). Gas and methane production rate is shown (GP t1/2 max and CH4 t ½ max). The average standard error of the differences of means (SED) and P-Value are presented.

Time	12h			24h				
Substrate	GP ml/g	CH4 ml/g	CH4%	GP ml/g	CH4 ml/g	CH4 %	GP t1/2 max(h)	CH4 t1/2 max(h)
Ryegrass	215.5	25.69	11.91	260	32.84	13.13	6.30	8.30
White clover	199.2	21.63	10.79	241.1	31.60	13.08	5.90	9.82
Composite	206.31	20.19	9.74	241.3	28.27	11.11	5.07	8.18
F+G leaves from greenhouse	200.5	22.74	10.98	241.7	31.17	12.78	4.84	8.47
Queenstown	197.1	22.79	11.52	229.3	29.50	12.81	4.63	7.11
Mon Repos	195.7	22.10	11.23	229.9	29.99	12.74	4.75	7.73
Bush Lot	194.6	22.93	11.73	225.5	29.82	13.20	4.63	7.66
Benab	202.3	24.06	11.86	235	30.79	13.22	4.64	7.60
PKM-t	205.6	23.71	11.50	240.5	30.87	12.64	4.67	7.46
PKM-g	208.6	24.76	11.58	243	31.76	12.82	4.72	7.44
SED	5.094	0.770	0.3293	5.675	1.082	0.3961	0.121	0.556
P-Value	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***

*** significant difference P<0.001

Table 4.6. Short chain fatty acids at the end of fermentation for different *M. oleifera* sources. Acetate, propionate and butyrate are presented along with total short chain fatty acids. The mean concentration in mM, average standard error of the differences (SED) and P-value are presented.

	Acetate	Propionate	Butyrate				
substrate	[mM]	[mM]	[mM]	SCFA [mM]	Acetate [%]	Butyrate [%]	Propionate [%]
Ryegrass	56.40	15.02	8.02	83.39	67.64	9.62	18.01
White clover	52.95	12.98	7.35	75.09	70.52	9.79	17.29
leaves from greenhouse	55.27	14.98	9.31	85.45	64.68	10.90	17.53
F+G Composite	49.55	14.47	8.66	76.27	64.97	11.35	18.98
Queenstown	55.10	13.56	8.46	82.49	66.79	10.25	16.43
Benab	54.49	14.09	8.62	83.18	65.51	10.36	16.94
PKM-t	52.75	13.27	8.49	77.87	67.74	10.90	17.04
PKM-g	54.89	14.34	9.21	84.48	64.98	10.91	16.97
Mon Repos	52.60	13.63	8.22	78.30	67.17	10.49	17.41
Bush Lot	53.55	13.64	8.14	80.51	66.52	10.11	16.94
SED	1.814	0.483	0.332	2.635			
P-value	0.015*	<0.001***	<0.001***	0.005*			

*** significant difference P<0.001, * significant difference P<0.05

4.4 Discussion

In vitro fermentation results showed variation in gas production for provenances of *M. oleifera* (Table 4.5). Variations in gas production during fermentation can be caused by the nutritional composition of the plant. Plant nutrition is affected by genetics (Fritsche et al., 2017) and soil fertility. The different provenances may have genetic variations, and these may cause variations in the plant nutritional content (see 2.2). Because fermentation is a function of the cellulose content (Ahring et al., 2018), a change in cellulose content can affect methane production. In fact, a study done by Lima et al. (2018) reported that high cellulose content observed in *Megathyrsus* species led to an increase in methane production and species with low cellulose content showed lower methane production.

In this study the cellulose content of the leaves of the different provenances of *M*. *oleifera* was not measured but Dhakad et al. (2019) had reported the fibre content to differ in different geographic locations, ranging as low as 7g per 100 g of leaf material in Nigeria to 37 g per 100 g in Haiti. This implies that the geographical locations of the *M*. *oleifera* used in this study may have influenced the gas production and provide a reason for the variations among provenances from Guyana and India.

In this study ryegrass had a significantly higher gas production ($P < 0.001$) when compared with *M*. *oleifera* and white clover. However, in this study, ryegrass is reported to have a high gas production and this is explained by its high digestibility (70-85%) (DairyNZ, 2022).

Ulyatt et al. (2002) had reported grass nutritional content changes with climate, which affects the amount of methane produced during fermentation. This may be the reason for different methane emission from plants in field compared with plants in greenhouse i.e different contrasting environmental conditions. Plants in open field are exposed to the weather while plants in greenhouse are in a more controlled environment. Conversely, raw data and analysis after 12- and 24 hours fermentation from this study showed *M*. *oleifera* from the field and the greenhouse preformed similarly for methane and total gas production (Table 4.3 and

Figures 4.1 and 4.2). In the preliminary analyses, the composition of the plants was not measured because of the lack of sufficient plant material, but previous literature has shown nutritional composition of *M*. *oleifera* to vary (see 2.2). The results obtained may suggest that methane emission from ruminants fed *M*. *oleifera* grown in greenhouse may differ from ruminants fed *M*. *oleifera* grown in open field.

In this study *M. oleifera* samples from the field and greenhouse had a pooled average methane and total gas production of 27.73 ml/g and 233.39 ml/g (millilitres of methane per gram of substrate) respectively after 24h hours. A work done by Meale et al. (2012) reported total gas production of 187 ml/g DM (dry matter) incubated and a methane production of 6.4 mg/g digested DM after 24 hrs. The fresh material used was sixty-days old regrowth from mature trees grown in Ghana (no mention was made whether the regrowth included leaves alone or leaves and stem). The dry material was obtained by oven drying for 72 h at 55°C. However, the technique used by (Meale et al., 2012) was not automated and gas production was not measured in real time, which may explain the extremely low methane production reported. This indicates that different techniques used to measure gas production during ruminal fermentation can cause variations in gas production. Hall and Mertens (2008) showed that different *in vitro* fermentation methods affected results of gas production and fibre digestibility estimates. However, in this study, all *M*. *oleifera* dried leaves were measured using the same *in vitro* batch culture system by Muetzel et al. (2014) and therefore the technique used does not provide an explanation for variations in gas production among provenances.

All gas production results in this study are based on the fermentation of dried *M. oleifera* leaves only and excludes effects that may be caused by other feed additives. Other studies measured methane production from *M*. *oleifera* by using its leaf extract and incorporating it in diets. Adding *M*. *oleifera* leaf extract at a concentration of 1.8 ml/DM and 0.6 ml/g DM to balanced diets containing 75% forage and 25 % concentrate, resulted in a total gas production of 108.2 ml/0.5 g dry matter and 85 ml/0.5g dry matter respectively at 24 hours. But this was not significantly different than providing feed with no additives Pedraza-Hernández et al. (2019). This suggests that the effect of lower gas and methane production from *M*. *oleifera* leaves is caused by the leaves and variations is a result of their composition.

M. *oleifera* fermented faster than both ryegrass and white clover (Table 4.3 and 4.5). Preliminary fermentation revealed that *M. oleifera* from the field and greenhouse had a higher percentage propionate and butyrate in relation to total short chain fatty acids when compared with ryegrass and white clover, but lower acetate (Table 4.3). Short chain fatty acids or volatile fatty acids are the product of anaerobic microbial fermentation of complex carbohydrates. The major short chain fatty acids in abundance are acetate, propionate and butyrate. These are absorbed as nutrients which constitutes up to 80% of the ruminant maintenance energy requirement (Bergman, 1990). This implies that they are important measure of the quality of feed for ruminants and should be included in *in vitro* fermentation studies. More propionate at the end of fermentation for *M*. *oleifera* could mean that the hydrogen produced is not diverted to methane production, but for the formation of propionate (Pereira et al., 2022). And could imply there are some compounds in *M. oleifera* that drives alternative pathways to rid hydrogen and thereby produce less methane. *M. oleifera* has been known to contain condensed tannins (Gu et al., 2020; Tshabalala et al., 2020) a compound in white clover flowers and seeds, reported to reduce ammonia and methane *in vitro* (Roldan et al., 2021).

In the main experiment some *M. oleifera* provenances also had lower acetate concentration after fermentation than high quality ryegrass and white clover but the values were not significant. Similarly, a lower acetate content was found when comparing *M. oleifera* with other pasture species such as *Andropodon gayanus*, *Brachiaria ruziziensis*, *Pennisetum purpureum*, *Cajanus cajan*, *Cratylia argentea*, *Gliricidia sepium*, *Leucaena leucocephala* and *Stylosanthes guianensis*, *Annona senegalensis*, *Securinega virosa* and *Vitellaria paradoxa* (Meale et al., 2012). Ryegrass belongs to Poaceae plant family (Tamura & Yamada, 2007) along with *Andropodon gayanus*, *Brachiaria ruziziensis* and *Pennisetum purpureum and are all called grasses*. While white belongs to the Fabaceae plant family (Coombe, 1961; Zoric et al., 2012) together with *Stylosanthes guianensis* and *Cratylia argentea*. This relationship may be the reason why ryegrass and white clover had a higher similar acetate concentration as

the species reported. Similar results were found by Soliva et al. (2005), while some authors did not quantify short chain fatty acid content during their *in vitro* study (Pedraza-Hernández et al., 2019).

A high acetate production may be caused by the high cellulose content. While *M. oleifera*, ryegrass and white clover are all plants and contain cellulose, the high gas production obtained from the fermentation of ryegrass implies that it is more digestible than *M*. *oleifera* and white clover and coincides with the acetate concentration obtained after fermentation. However, it has been reported that, plant-based diets are primarily constituted of cellulose and a satisfactory amount of sugars. As a result ruminants on plant based diets will have a microbial population that will consist mostly of cellulolytic and saccharolytic bacteria causing more cellulose digestion and sugar formation that will result in a higher acetate production (Owens & Goetsch, 1988). This implies that generally, fermentation of plants high in digestible cellulose will yield a high acetate content.

4.5 Conclusions

This study has shown that dried *M. oleifera* leaves fermented faster than high quality ryegrass and had less methane and total gas produced. The fermentation of *M. oleifera* was similar to that of white clover but there were also some variations in gas production among the different provenances evaluated, suggesting there is potential to select for low methane genotypes. *M. oleifera* leaves also produced a higher concentration of short chain fatty acids than ryegrass or white clover at the end of fermentation but this was not significant. In addition, growing location (greenhouse or field) did significantly affect methane production among *M. oleifera* plants.

Chapter 5 Conclusions

The main aim of this study was to carry out a pre-breeding assessment of *M*. *oleifera* for its potential to grow in saline soils and use as a low-methane emission fodder crop to feed cattle. There has been reports in the literature of *M*. *oleifera* to grow in saline soils (Al-Shoaibi & Boutraa, 2021; Fatima et al., 2018; Metwally et al., 2021) but these were inconclusive. In addition, work on *M*. *oleifera* and its potential to be used as a low-methane emission fodder crop is lacking.

This study has determined that *M*. *oleifera* at an age of nine weeks after sowing, can grow in slightly saline soils without significant loss of biomass. The plants stressed at 52 mM NaCl had similar growth to non-stressed plants, but at 156 mM NaCl there was a significant reduction in plant growth. Net photosynthesis was not significantly affected until after 14 days of 156 mM NaCl salt stress and there was no significant difference between control plants and plants stressed with 52 mM NaCl. Salt stress did not significantly ($P < 0.001$) lower the potassium concentration in photosynthetic active leaves, but sodium concentration increased significantly ($P < 0.001$) as the salt stress increased.

This study concluded that there are various ways to screen and assess Moringa genotypes for salt tolerance. Plant breeders can use plant visual morphological quality along with SPAD measurements as a rapid tool to screen for salt tolerant genotypes. But other measurements such as plant biomass and net photosynthesis are more accurate, and while takes a longer time, can be used to validate results from rapid assessments. Nevertheless, the method and data obtained in this study can be used to select salt tolerant genotypes which can help plant breeders create a base population for selection in future breeding programmes.

This study also showed that *M*. *oleifera* whether grown in open field or greenhouse has lower methane and total gas production than high quality ryegrass but similar gas kinetics to white clover. The proportion of methane produced was less ($P < 0.001$) in *M*. *oleifera* leaves grown from seeds sourced from King Seeds than

ryegrass after 24 hours of fermentation. *M. oleifera* also fermented faster than ryegrass and white clover and produced similar total fatty acid content to both ryegrass and white clover at the end of fermentation.

Data obtained from this study provides evidence that *M. oleifera* can grow in moderately saline conditions and has the potential to produce less methane than high quality pasture species, this makes it worthy of further investigation.

Chapter 6 Future work

This study has demonstrated that *M. oleifera* is tolerant to slightly saline soils and has low methane emission. However, to broaden our knowledge some additional work can be done:

1. In this study there was a high variation in seed emergence. Future work should include investigating optimal harvesting and post-harvest methods for ensuring that seed germination in *M. oleifera* is not lost. This would help to better understand the behaviour of *M. oleifera* seeds in response to harvest and post-harvest processes and more importantly help to establish and preserve *M. oleifera* germplasm.
2. Further screening of accessions sourced from cold regions to identify cold tolerant genotypes. This would help build a base population for breeding with a broad variation of genes and make possible the creation of improved varieties that can be grown in a wider range of environments than now, for example temperate environments.
3. This study brings new insights into the methane production of various *M. oleifera* provenances using a fully automated batch culture system and has shown the fermentation of *M. oleifera* results a lowered methane emission compared with temperate pasture species. But it is important to study the interactions of feeds (Castro-Montoya et al., 2012), in a mixed diet including *M. oleifera*, and measuring the resulting gas production. This is because stock will not be fed a diet containing one

species but a mixture of species. Therefore, future work should include the evaluation of different mixtures of feed that include *M*. *oleifera* for total gas and methane emission.

4. In addition, to better understand the gas production variations of the different provenances of *M*. *oleifera.* The genetic diversity among provenances should be studied. With a wide range of genotypes across the world, genetic studies can be done to evaluate the diversity of *M. oleifera* using molecular markers. Genetic diversity studies will also help to determine the origin of the seeds sourced from King Seeds New Zealand that were bought from the United States of America and how related they are to the provenances obtained from Guyana and India.
5. Further screening of larger *M. oleifera* populations in Guyana located close to the sea Should be undertaken. This will help in the identification of more genotypes that are salt tolerant at 52 mM NaCl and help search for genotypes that has a higher salt tolerance for use in crop improvement.
6. The nutritional composition of different provenances sourced from Guyana and India should be measured to provide a better understanding of the variations observed in gas production in this study.

Chapter 7 References

Achard, P., Cheng, H., De Grauwe, L., Decat, J., Schoutteten, H., Moritz, T., Van Der Straeten, D., Peng, J., & Harberd, N. P. (2006). Integration of plant responses to environmentally activated phytohormonal signals. *Science, 311*(5757), 91-94.

Agbogidi, O. M., & Ilondu, E. M. (2012). *Moringa oleifera* Lam: Its potentials as a food security and rural medicinal item. *J. Bio. Innov, 1*(6), 156-167.

Ahmed, L. T., Warrag, E. I., & Abdelgadir, A. Y. (2014). Effect of shade on seed germination and early seedling growth of *Moringa oleifera* Lam. *Journal of Forest Products & Industries, 3*(1), 20-26.

Ahring, B. K., Murali, N., & Srinivas, K. (2018). Fermentation of cellulose with a mixed microbial rumen culture with and without methanogenesis. *Fermentation Technology, 7*(1), 1-7.

Ahyoung, O. (2017). Guyana. In *South America, Mexico, Central America, and the Caribbean* (pp. 441-451). Routledge.

Al-aghabary, K., Zhu, Z., & Shi, Q. (2005). Influence of silicon supply on chlorophyll content, chlorophyll fluorescence, and antioxidative enzyme activities in tomato plants under salt stress. *Journal of plant nutrition, 27*(12), 2101-2115.

Al-Shoaibi, A. K., & Boutraa, T. (2021). Comparative study on germination, growth and gas exchanges of the tropics' tree *Moringa oleifera* and its desert relative *Moringa peregrina* under saline conditions. *South African Journal of Botany, 139*, 374-385.

Alabi, O. J., Malik, A. D., Ng'Ambi, J. W., Obaje, P., & Ojo, B. K. (2017). Effect of aqueous *Moringa oleifera* (Lam) leaf extracts on growth performance and carcass characteristics of hubbard broiler chicken. *Brazilian Journal of Poultry Science, 19*(2), 273-280.

Almeida, D. M., Oliveira, M. M., & Saibo, N. J. M. (2017). Regulation of Na+ and K+ homeostasis in plants: Towards improved salt stress tolerance in crop plants. *Genetics and molecular biology, 40*(1), 326-345.

Alshoaibi, A. (2021). Seed germination, seedling growth and photosynthetic responses to temperature in the tropical tree *Moringa oleifera* and Its relative desert, *Moringa peregrina*. *Egyptian Journal of Botany, 61*(2), 541-551.

Amtmann, A., & Sanders, D. (1998). Mechanisms of Na+ uptake by plant cells. *Advances in botanical research, 29*, 75-112.

Anwar, F., & Bhanger, M. I. (2003, Oct 22). Analytical characterization of *Moringa oleifera* seed oil grown in temperate regions of Pakistan. *Journal of Agriculture and Food Chemistry, 51*(22), 6558-6563. https://doi.org/10.1021/jf0209894

Ashoori, M., & Saedisomeolia, A. (2014). Riboflavin (vitamin B2) and oxidative stress: A review. *British journal of nutrition, 111*(11), 1985-1991.

Attwood, G. T., Klieve, A. V., Ouwerkerk, D., & Patel, B. K. C. (1998). Ammonia-hyperproducing bacteria from New Zealand ruminants. *Applied and Environmental Microbiology, 64*(5), 1796-1804.

Ayerza, R. (2011). Seed yield components, oil content, and fatty acid composition of two cultivars of moringa (*Moringa oleifera* Lam.) growing in the Arid Chaco of Argentina. *Industrial Crops and Products, 33*(2), 389-394.

Badr, A., & El-Shazly, H. (2012). Molecular approaches to origin, ancestry and domestication history of crop plants: Barley and clover as examples. *Journal of Genetic Engineering and Biotechnology, 10*(1), 1-12.

Bancessi, A., Bancessi, Q., Baldé, A., & Catarino, L. (2020). Present and potential uses of *Moringa oleifera* as a multipurpose plant in Guinea-Bissau. *South African Journal of Botany, 129*, 206-208.

Barakat, H., & Ghazal, G. A. (2016). Physicochemical properties of *Moringa oleifera* seeds and their edible oil cultivated at different regions in Egypt. *Food and Nutrition Sciences, 7*(06), 472.

Barichella, M., Pezzoli, G., Faierman, S. A., Raspini, B., Rimoldi, M., Cassani, E., Bertoli, S., Battezzati, A., Leone, A., & Iorio, L. (2019). Nutritional characterisation of Zambian *Moringa oleifera*: acceptability and safety of short-term daily supplementation in a group of malnourished girls. *International Journal of Food Sciences and Nutrition, 70*(1), 107-115.

Barthlott, W., Lauer, W., & Placke, A. (1996). Global distribution of species diversity in vascular plants: Towards a world map of phytodiversity (globale verteilung der artenvielfalt höherer pflanzen: Vorarbeiten zu einer weltkarte der phytodiversität). *Erdkunde*, 317-327.

Bashar, M. K., Huque, K. S., Sarker, N. R., & Sultana, N. (2020, Sep). Quality assessment and feeding impact of Moringa feed on intake, digestibility, enteric CH4 emission, rumen fermentation, and milk yield. *Journal of Advanced Veterinary and Animal Research, 7*(3), 521-529. https://doi.org/10.5455/javar.2020.g449

Beck, E. H., Fettig, S., Knake, C., Hartig, K., & Bhattarai, T. (2007). Specific and unspecific responses of plants to cold and drought stress. *Journal of Biosciences, 32*(3), 501-510.

Beck, E. H., Heim, R., & Hansen, J. (2004). Plant resistance to cold stress: mechanisms and environmental signals triggering frost hardening and dehardening. *Journal of Biosciences, 29*(4), 449-459.

Becker, V. I., Goessling, J. W., Duarte, B., Caçador, I., Liu, F., Rosenqvist, E., & Jacobsen, S.-E. (2017). Combined effects of soil salinity and high temperature on photosynthesis and growth of quinoa plants (*Chenopodium quinoa*). *Functional Plant Biology, 44*(7), 665-678.

Bendich, A. (1993). Physiological role of antioxidants in the immune system. *Journal of dairy science, 76*(9), 2789-2794.

Berger, M. R., Habs, M., Jahn, S. A., & Schmahl, D. (1984, Sep). Toxicological assessment of seeds from *Moringa oleifera* and *Moringa stenopetala*, two highly efficient primary coagulants for domestic water treatment of tropical raw waters. *East African Medical Journal, 61*(9), 712-716. https://www.ncbi.nlm.nih.gov/pubmed/6535725

Bergman, E. N. (1990). Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. *Physiological reviews, 70*(2), 567-590.

Bhandari, H. R., Bhanu, A. N., Srivastava, K., Singh, M. N., & Shreya, H. A. (2017). Assessment of genetic diversity in crop plants-An overview. *Advances in Plant and Agriculture Research, 7*(3), 279-286.

Bhargave, A., Pandey, I., Nama, K. S., & Pandey, M. (2015). *Moringa oleifera* Lam.—Sanjana (horseradish tree)—A miracle food plant with multipurpose uses in Rajasthan-India—an overview. *Int J Pure App Biosci, 3*(6), 237-248.

Biel, K., & Fomina, I. (2015). Benson-Bassham-Calvin cycle contribution to the organic life on our planet. *Photosynthetica, 53*(2), 161-167.

Black, J. L., Davison, T. M., & Box, I. (2021). Methane Emissions from Ruminants in Australia: Mitigation Potential and Applicability of Mitigation Strategies. *Animals, 11*(4), 1-20.

Boadi, D., Benchaar, C., Chiquette, J., & Massé, D. (2004). Mitigation strategies to reduce enteric methane emissions from dairy cows: Update review. *Canadian Journal of Animal Science, 84*(3), 319-335.

Booy, G., Hendriks, R. J. J., Smulders, M. J. M., Van Groenendael, J. M., & Vosman, B. (2000). Genetic diversity and the survival of populations. *Plant biology, 2*(04), 379-395.

Boumenjel, A., Papadopoulos, A., & Ammari, Y. (2020). Growth response of *Moringa oleifera* (Lam) to water stress and to arid bioclimatic conditions. *Agroforestry Systems, 95*(5), 823-833.

Bresler, E., McNeal, B. L., & Carter, D. L. (2012). *Saline and sodic soils: principles-dynamics-modeling* (Vol. 10). Springer Science & Business Media.

Brisibe, E. A., Umoren, U. E., Brisibe, F., Magalhäes, P. M., Ferreira, J. F. S., Luthria, D., Wu, X., & Prior, R. L. (2009). Nutritional characterisation and antioxidant capacity of different tissues of Artemisia annua L. *Food chemistry, 115*(4), 1240-1246.

Brouwer, C. G., A. Heibloem, M. (1985). Salty Soils. In *Introduction to Irrigation*. FAO. http://www.fao.org/3/r4082e/r4082e08.htm#chapter%207%20%20%20salty%20soils

Brunetti, C., Loreto, F., Ferrini, F., Gori, A., Guidi, L., Remorini, D., Centritto, M., Fini, A., & Tattini, M. (2018). Metabolic plasticity in the hygrophyte *Moringa oleifera* exposed to water stress. *Tree physiology, 38*(11), 1640-1654.

Bubbico, A., Keller, M., & Morales Opazo, C. (2020). *Perspectives on diversification prospects for the agrifood industry in Guyana* (FAO Agricultural Development Economics Technical Studies, Issue 6). FAO. https://www.fao.org/documents/card/en/c/ca9754en

Buitenhuis, H. C., Soute, B. A. M., & Vermeer, C. (1990). Comparison of the vitamins K1, K2 and K3 as cofactors for the hepatic vitamin K-dependent carboxylase. *Biochimica Et Biophysica Acta (BBA)-General Subjects, 1034*(2), 170-175.

Busani, M., Patrick, J., Arnold, H., Voster, M., Moyo, B., Masika, P., Hugo, A., & Muchenje, V. (2011). Nutritional characterization of Moringa (*Moringa oleifera* Lam.) leaves. *African journal of biotechnology, 10*(60), 12925-12933.

Cai, X., Sun, Y., Starman, T., Hall, C., & Niu, G. (2014). Response of 18 Earth-Kind® rose cultivars to salt stress. *HortScience, 49*(5), 544-549.

Canterbury, D. (2007). Caribbean agriculture under three regimes: Colonialism, nationalism and neoliberalism in Guyana. *The journal of peasant studies, 34*(1), 1-28.

Caribbean Agricultural Research and Development Institute. (1996). Grasses for improved pasture on coastal clay soils of Guyana. http://www.cardi.org/wp-content/uploads/downloads/2015/05/Grasses-for-improved-pasture-on-coastal-clay-soils-of-Guyana.pdf

Castro-Montoya, J., De Campeneere, S., Van Ranst, G., & Fievez, V. (2012). Interactions between methane mitigation additives and basal substrates on *in vitro* methane and VFA production. *Animal Feed Science and Technology, 176*(1-4), 47-60.

Chika, I. B., Onyekachi, O. J., Chioma, I. F., Emmanuel, A., Rose, O. N., & Bernard, M. O. (2020). Antiparasiticidal potential of aqueous leaves extract of *Moringa oleifera* against ichthyophthirius multifiliis infestation on clarias gariepinus. *Journal of Animal Health and Production, 8*(3), 113-121.

Chitiyo, S. T., Ncube, B., Ndhlala, A. R., & Tsvuura, Z. (2021). Biochemical responses of *Moringa oleifera* Lam. plants to graded moisture deficit. *South African Journal of Botany, 138*, 41-49.

Cieslak, A., Zmora, P., Pers-Kamczyc, E., & Szumacher-Strabel, M. (2012). Effects of tannins source (*Vaccinium vitis idaea* L.) on rumen microbial fermentation in vivo. *Animal Feed Science and Technology, 176*(1-4), 102-106.

Cirillo, C., Rouphael, Y., Caputo, R., Raimondi, G., Sifola, M. I., & De Pascale, S. (2016). Effects of high salinity and the exogenous application of an osmolyte on growth, photosynthesis, and mineral composition in two ornamental shrubs. *The Journal of Horticultural Science and Biotechnology, 91*(1), 14-22.

Clark, J. H., Klusmeyer, T. H., & Cameron, M. R. (1992). Microbial protein synthesis and flows of nitrogen fractions to the duodenum of dairy cows. *Journal of dairy science, 75*(8), 2304-2323.

Coelho, J. S., Santos, N. D., Napoleão, T. H., Gomes, F. S., Ferreira, R. S., Zingali, R. B., Coelho, L. C., Leite, S. P., Navarro, D. M., & Paiva, P. M. (2009). Effect of *Moringa oleifera* lectin on development and mortality of *Aedes aegypti* larvae. *Chemosphere, 77*(7), 934-938.

Coombe, D. E. (1961). *Trifolium occidentale*, a new species related to *Trifolium repens* L. *Watsonia, 5*, 68-87. https://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.669.8241&rep=rep1&type=pdf

Cowan, J. A. (2002). Structural and catalytic chemistry of magnesium-dependent enzymes. *Biometals, 15*(3), 225-235.

Cruz de Carvalho, M. H. (2008). Drought stress and reactive oxygen species. *Plant Signaling and Behaviour, 3*(3), 156-165.

Cundiff, L. V. (1970). Experimental results on crossbreeding cattle for beef production. *Journal of animal science, 30*(5), 694-705.

DairyNZ. (2021). *Heifer feeding and nutrition*. DairyNZ. Retrieved 09-04-2021 from https://www.dairynz.co.nz/animal/heifers/heifer-feeding-and-nutrition/

DairyNZ. (2022). *Ryegrass as a feed*. DairyNZ. Retrieved 18-03-2022 from https://www.dairynz.co.nz/feed/nutrition/ryegrass-as-a-feed/

Dal Pizzol, J. G., Ribeiro-Filho, H. M. N., Quereuil, A., Le Morvan, A., & Niderkorn, V. (2017). Complementarities between grasses and forage legumes from temperate and subtropical areas on *in vitro* rumen fermentation characteristics. *Animal Feed Science and Technology, 228*, 178-185.

Dao, M. C. E., Taita, P., & Walsh, D. (2017). Germination and seed traits variations among West African provenances of *Moringa oleifera* Lam.(Burkina Faso). *African Journal of Agricultural Research, 12*(9), 730-739.

Davies, Z. S., Mason, D., Brooks, A. E., Griffith, G. W., Merry, R. J., & Theodorou, M. K. (2000). An automated system for measuring gas production from forages inoculated with rumen fluid and its

use in determining the effect of enzymes on grass silage. *Animal Feed Science and Technology, 83*(3-4), 205-221.

de Medeiros, M. L. S., de Moura, M. C., Napoleão, T. H., Paiva, P. M. G., Coelho, L. C. B. B., Bezerra, A. C. D. S., & da Silva, M. D. C. (2018). Nematicidal activity of a water soluble lectin from seeds of *Moringa oleifera*. *International journal of biological macromolecules, 108*, 782-789.

De Roest, K., Ferrari, P., & Knickel, K. (2018). Specialisation and economies of scale or diversification and economies of scope? Assessing different agricultural development pathways. *Journal of Rural Studies, 59*, 222-231.

Derlagen, C., Tas, J., Boyce, R., Shik, O., & De Salvo, C. P. (2017). *Analysis of agricultural policies in Guyana* (Agricultural Policy Reports, Issue 1). IDB. https://publications.iadb.org/publications/english/document/Analysis-of-Agricultural-Policies-in-Guyana.pdf

Dezetter, C., Boichard, D., Bareille, N., Grimard, B., Mezec, P. l., & Ducrocq, V. (2019). Dairy crossbreeding: pros and cons for Holsteindairy systems. *INRA Productions Animales, 32*(3), 359-378.

Dhakad, A. K., Ikram, M., Sharma, S., Khan, S., Pandey, V. V., & Singh, A. (2019, Nov). Biological, nutritional, and therapeutic significance of *Moringa oleifera* Lam. *Phytotherapy Research, 33*(11), 2870-2903. https://doi.org/10.1002/ptr.6475

Dhakar, R. C., Maurya, S. D., Pooniya, B. K., Bairwa, N., & Gupta, M. (2011). Moringa: The herbal gold to combat malnutrition. *Chronicles of Young Scientists, 2*(3), 119-125.

Dini, Y., Gere, J., Cajarville, C., & Ciganda, V. S. (2018). Using highly nutritious pastures to mitigate enteric methane emissions from cattle grazing systems in South America. *Animal Production Science, 58*(12), 2329-2334.

Dong, L., Ravelombola, W., Weng, Y., Qin, J., Zhou, W., Bhattarai, G., Zia, B., Yang, W., Shi, L., & Mou, B. (2019). Change in Chlorophyll Content Over Time Well-differentiated Salt-tolerant, Moderately Salt-tolerant, and Salt-susceptible Cowpea Genotypes. *HortScience, 54*(9), 1477-1484.

Duchaufour, P. (1982). Hydromorphic soils. In *Pedology* (pp. 335-372). Springer.

Duke, J. A. (1978). The quest for tolerant germplasm. *Crop tolerance to suboptimal land conditions, 32*, 1-61.

Dumortier, P., Aubinet, M., Beckers, Y., Chopin, H., Debacq, A., de la Motte, L. G., Jérôme, E., Wilmus, F., & Heinesch, B. (2017). Methane balance of an intensively grazed pasture and estimation of the enteric methane emissions from cattle. *Agricultural and Forest Meteorology, 232*, 527-535.

Dyal, P. (2017). Saijan- the miracle tree. *Guyana Chronicle*. https://guyanachronicle.com/2017/05/21/saijan-the-miracle-tree/

Elhag, A. Z., & Abdalla, M. H. (2012). Effect of sodium chloride on germination and emergence of moringa (*Moringa oleifera* L.) seeds. *Journal of Science and Technology, 13*(2), 62-68.

Ergönül, B. (2013). Consumer awareness and perception to food safety: A consumer analysis. *Food control, 32*(2), 461-471.

Fahey, J. W. (2005). *Moringa oleifera*: A review of the medical evidence for its nutritional, therapeutic, and prophylactic properties. Part 1. *Trees for life Journal, 1*(5), 1-15.

Fajardo Vizcayno, J., Hugo, W., & Sanz Alvarez, J. (2014). *Appropriate seed varieties for small-scale farmers: Key practices for DRR implementers* (A Field Guide for Disaster Risk Reduction in Southern Africa: Key Practices for DRR Implementers, Issue 1). https://www.fao.org/3/i3768e/i3768e.pdf

FAO. (2015). *AQUASTAT Country Profile – Guyana.* (AQUASTAT - FAO's Global Information System on Water and Agriculture, Issue. FAO. http://www.fao.org/3/ca0428en/CA0428EN.pdf

Fatima, N., Akram, M., Shahid, M., Abbas, G., Hussain, M., Nafees, M., Wasaya, A., Tahir, M., & Amjad, M. (2018). Germination, growth and ions uptake of Moringa (*Moringa oleifera* L.) grown under saline condition. *Journal of plant nutrition, 41*(12), 1555-1565.

Fatima, T., Sajid, M. S., Jawad-ul-Hassan, M., Siddique, R. M., & Iqbal, Z. (2014). Phytomedicinal value of *Moringa oleifera* with special reference to antiparasitics. *Pakistan Journal of Agricultural Sciences, 51*(1), 251-262.

Fattal-Valevski, A. (2011). Thiamine (vitamin B1). *Journal of Evidence-Based Complementary & Alternative Medicine, 16*(1), 12-20.

Fawcett, W., & Rendle, A. B. (1914). *Flora of Jamaica: containing descriptions of the flowering plants known from the island* (Vol. 3). Order of the Trustees of the British Museum.

Fawzy, S., Osman, A. I., Doran, J., & Rooney, D. W. (2020). Strategies for mitigation of climate change: A review. *Environmental Chemistry Letters, 18*(6), 2069-2094.

Fernandes, P., Laohman, W., & Edwards, C. (1975). *The Problems of Pasture Managements in the Development of the Livestock Industry in Guyana*.

Fernandez, G. C. J. (1992, August 13-18). Effective selection criteria for assessing plant stress tolerance. International Symposium on Adaptation of Food Crops to Temperature and Water Stress, Taiwan.

Finch-Savage, W. E., & Leubner-Metzger, G. (2006). Seed dormancy and the control of germination. *New Phytologist, 171*(3), 501-523.

Flexas, J., Bota, J., Loreto, F., Cornic, G., & Sharkey, T. D. (2004). Diffusive and metabolic limitations to photosynthesis under drought and salinity in C3 plants. *Plant biology, 6*(03), 269-279.

Foidl, N., Makkar, H., & Becker, K. (2001). *The potential of Moringa oleifera for agricultural and industrial uses*. Retrieved 2021-09-21 from https://www.moringatrees.org/moringa-doc/the_potential_of_moringa_oleifera_for_agricultural_and_industrial_uses.pdf

Food and Agriculture Organization of the United Nations. (2013). *Major cuts of greenhouse gas emissions from livestock within reach*. FAO. Retrieved 2022-01-22 from https://www.fao.org/news/story/en/item/197608/icode

Förster, N., Ulrichs, C., Schreiner, M., Arndt, N., Schmidt, R., & Mewis, I. (2015). Ecotype variability in growth and secondary metabolite profile in Moringa oleifera: Impact of sulfur and water availability. *Journal of agricultural and food chemistry, 63*(11), 2852-2861.

France, J., Lopez, S., Kebreab, E., Bannink, A., Dhanoa, M. S., & Dijkstra, J. (2005). A general compartmental model for interpreting gas production profiles. *Animal Feed Science and Technology, 123-124*, 473-485.

Fraser, J. (2018). *When Salt Water Intrusion is Not Just a Threat But a Reality for Guyanese Farmers*. Inter Press Service News Agency. Retrieved 09-07-2021 from http://www.ipsnews.net/2018/08/salt-water-intrusion-not-just-threat-reality-guyanese-farmers/

Fricke, W., Akhiyarova, G., Veselov, D., & Kudoyarova, G. (2004). Rapid and tissue-specific changes in ABA and in growth rate in response to salinity in barley leaves. *Journal of Experimental Botany, 55*(399), 1115-1123.

Fricke, W., & Peters, W. S. (2002). The biophysics of leaf growth in salt-stressed barley. A study at the cell level. *Plant physiology, 129*(1), 374-388.

Fritsche, S., Wang, X., & Jung, C. (2017). Recent advances in our understanding of tocopherol biosynthesis in plants: An overview of key genes, functions, and breeding of vitamin E improved crops. *Antioxidants, 6*(4), 99.

Fuglie, L. J. (2000). New uses of Moringa studied in Nicaragua. *ECHO Development Notes, 68*, 1-25.

Funston, R. N., Summers, A. F., & Roberts, A. J. (2012). Alpharma Beef Cattle Nutrition Symposium: Implications of nutritional management for beef cow-calf systems. *Journal of animal science, 90*(7), 2301-2307.

Gado, T. A., El-Hagrsy, R. M., & Rashwan, I. (2019). Spatial and temporal rainfall changes in Egypt. *Environmental Science and Pollution Research, 26*(27), 28228-28242.

Geng, Y., Wu, R., Wee, C. W., Xie, F., Wei, X., Chan, P. M. Y., Tham, C., Duan, L., & Dinneny, J. R. (2013). A spatio-temporal understanding of growth regulation during the salt stress response in Arabidopsis. *The Plant Cell, 25*(6), 2132-2154.

Gerber, P. J., Steinfeld, H., Henderson, B., Mottet, A., Opio, C., Dijkman, J., Falcucci, A., & Tempio, G. (2013). *Tackling climate change through livestock: A global assessment of emissions and mitigation opportunities*. Food and Agriculture Organization of the United Nations (FAO).

Ghoshal, K., Li, X., Datta, J., Bai, S., Pogribny, I., Pogribny, M., Huang, Y., Young, D., & Jacob, S. T. (2006). A folate-and methyl-deficient diet alters the expression of DNA methyltransferases and methyl CpG binding proteins involved in epigenetic gene silencing in livers of F344 rats. *The Journal of nutrition, 136*(6), 1522-1527.

Giardina, B., Messana, I., Scatena, R., & Castagnola, M. (1995). The multiple functions of hemoglobin. *Critical reviews in biochemistry and molecular biology, 30*(3), 165-196.

Godino, M., Arias, C., & Izquierdo, M. I. (2015, 15 November). *Moringa oleifera*: potential areas of cultivation on the Iberian Peninsula. I International Symposium on Moringa, Acta Horticulturae

Gopalakrishnan, P. K. (1978). Drumstick. *Kalpadhenu, Kerala Agricultural University Trichur*, p184.

Gregor, J. W. (1944). The ecotype. *Biological Reviews, 19*(1), 20-30.

Gu, X., Yang, Y., & Wang, Z. (2020). Nutritional, phytochemical, antioxidant, α-glucosidase and α-amylase inhibitory properties of *Moringa oleifera* seeds. *South African Journal of Botany, 133*, 151-160. https://doi.org/10.1016/j.sajb.2020.07.021

Gutierrez, M., Mar Sola, M. d., Pascual, L., Rodriguez-Garcia, M. I., & Vargas, A. M. (1992). Ultrastructural changes in cherimoya fruit injured by chilling. *Food structure, 11*(4), 4.

Guyana Lands and Surveys Commission. (2013). *Guyana national land use plan*. GLSC. https://observatorioplanificacion.cepal.org/sites/default/files/plan/files/2013%20NLUP%20Guyana.pdf

Hake, S., & Ross-Ibarra, J. (2015). The natural history of model organisms: genetic, evolutionary and plant breeding insights from the domestication of maize. *elife, 4*, 1-8.

Hall, M. B., & Mertens, D. R. (2008). *In vitro* fermentation vessel type and method alter fiber digestibility estimates. *Journal of dairy science, 91*(1), 301-307.

Haro, R., Bañuelos, M. A., Senn, M. E., Barrero-Gil, J., & Rodríguez-Navarro, A. (2005). HKT1 mediates sodium uniport in roots. Pitfalls in the expression of HKT1 in yeast. *Plant physiology, 139*(3), 1495-1506.

Hartmann, S., & Klaschka, U. (2017). Interested consumers' awareness of harmful chemicals in everyday products. *Environmental Sciences Europe, 29*(1), 1-19.

Helmreich, E. J. (1992). How pyridoxal 5'-phosphate could function in glycogen phosphorylase catalysis. *BioFactors (Oxford, England), 3*(3), 159-172.

Hessini, K., Issaoui, K., Ferchichi, S., Saif, T., Abdelly, C., Siddique, K. H. M., & Cruz, C. (2019). Interactive effects of salinity and nitrogen forms on plant growth, photosynthesis and osmotic adjustment in maize. *Plant Physiology and Biochemistry, 139*, 171-178.

Hopmans, J. W., Qureshi, A. S., Kisekka, I., Munns, R., Grattan, S. R., Rengasamy, P., Ben-Gal, A., Assouline, S., Javaux, M., & Minhas, P. S. (2021). Critical knowledge gaps and research priorities in global soil salinity. *Advances in Agronomy, 191*, 1-191. https://doi.org/10.1016/bs.agron.2021.03.001

Horrobin, D. F. (1993). Fatty acid metabolism in health and disease: the role of Δ-6-desaturase. *The American journal of clinical nutrition, 57*(5), 732-737.

Hummel, J., Südekum, K. H., Streich, W. J., & Clauss, M. (2006). Forage fermentation patterns and their implications for herbivore ingesta retention times. *Functional ecology, 20*(6), 989-1002.

Hussein, M. M., & Abou-Baker, N. H. (2013). Growth and mineral status of moringa plants as affected by silicate and salicylic acid under salt stress. *International Journal of Plant & Soil Science*, 163-177.

IBM Corpporation. (2020). *IBM SPSS Statistics for windows, Version 27.0*. Armonk, NY: IBM Corp. https://www.ibm.com/support/pages/how-cite-ibm-spss-statistics-or-earlier-versions-spss

Intergovernmental Panel on Climate Change. (2021). *About the IPPC*. Retrieved 2022-01-13 from https://www.ipcc.ch/about/

Jahn, S. A., Musnad, H. A., & Burgstaller, H. (1986). The tree that purifies water: Cultivating multipurpose Moringaceae in the Sudan. *Unasylva, 38*(152), 23-28.

James, R. A., Rivelli, A. R., Munns, R., & von Caemmerer, S. (2002). Factors affecting CO2 assimilation, leaf injury and growth in salt-stressed durum wheat. *Functional Plant Biology, 29*(12), 1393-1403.

Jelan, Z. A., & Sumarmono, J. (2019, 6-8 August). Nutritional Manipulations and Its Effects on Yield and Quality of Beef. The 1st Animal Science and Food Technology Conference, Indonesia.

Jensen, S. K., & Lauridsen, C. (2007). α-Tocopherol stereoisomers. *Vitamins & Hormones, 76*, 281-308.

Johnson, D. E., Johnson, K. A., Ward, G. M., & Branine, M. E. (2000). Ruminants and other animals. In *Atmospheric Methane* (pp. 112-133). Springer.

Johnson, K. A., & Johnson, D. E. (1995). Methane emissions from cattle. *Journal of animal science, 73*(8), 2483-2492.

Jones, A. D., & Ejeta, G. (2016). A new global agenda for nutrition and health: the importance of agriculture and food systems. *Bulletin of the World Health Organization, 94*(3), 228.

Jonker, A., Farrell, L., Scobie, D., Dynes, R., Edwards, G., Hague, H., McAuliffe, R., Taylor, A., Knight, T., & Waghorn, G. (2018). Methane and carbon dioxide emissions from lactating dairy cows grazing mature ryegrass/white clover or a diverse pasture comprising ryegrass, legumes and herbs. *Animal Production Science, 59*(6), 1063-1069.

Jonker, A., Muetzel, S., Molano, G., & Pacheco, D. (2015). Effect of fresh pasture forage quality, feeding level and supplementation on methane emissions from growing beef cattle. *Animal Production Science, 56*(10), 1714-1721.

Jyothi, P. V., Atluri, J. B., & Reddi, C. S. (1990). Pollination ecology of *Moringa oleifera* (Moringaceae). *Plant Sciences, 100*(1), 33-42.

Kaiser, E., Kromdijk, J., Harbinson, J., Heuvelink, E., & Marcelis, L. F. M. (2017). Photosynthetic induction and its diffusional, carboxylation and electron transport processes as affected by CO2 partial pressure, temperature, air humidity and blue irradiance. *Annals of botany, 119*(1), 191-205.

Kaiser, E., Morales, A., Harbinson, J., Kromdijk, J., Heuvelink, E., & Marcelis, L. F. M. (2015). Dynamic photosynthesis in different environmental conditions. *Journal of Experimental Botany, 66*(9), 2415-2426.

Karakurt, I., Aydin, G., & Aydiner, K. (2012). Sources and mitigation of methane emissions by sectors: A critical review. *Renewable energy, 39*(1), 40-48.

Kenny, D. A., Fitzsimons, C., Waters, S. M., & McGee, M. (2018). Invited review: Improving feed efficiency of beef cattle–the current state of the art and future challenges. *Animal, 12*(9), 1815-1826.

Kholif, A. E., Gouda, G. A., Olafadehan, O. A., & Abdo, M. M. (2018). Effects of replacement of Moringa oleifera for berseem clover in the diets of Nubian goats on feed utilisation, and milk yield, composition and fatty acid profile. *Animal, 12*(5), 964-972.

Klein, T. (2014). The variability of stomatal sensitivity to leaf water potential across tree species indicates a continuum between isohydric and anisohydric behaviours. *Functional ecology, 28*(6), 1313-1320.

Kopittke, P. M., Menzies, N. W., Wang, P., McKenna, B. A., & Lombi, E. (2019). Soil and the intensification of agriculture for global food security. *Environment international, 132*, 105078.

Körner, C. (2016). Plant adaptation to cold climates. *F1000Research, 5*, 1-5. https://doi.org/10.12688/f1000research.9107.1

Kozat, S. (2007). Serum T3 and T4 concentrations in lambs with nutritional myodegeneration. *Journal of veterinary internal medicine, 21*(5), 1135-1137.

Kulivand, M., & Kafilzadeh, F. (2015). Correlation between chemical composition, kinetics of fermentation and methane production of eight pasture grasses. *Acta Scientiarum. Animal Sciences, 37*, 9-14.

Kumssa, D. B., Joy, E. J. M., Young, S. D., Odee, D. W., Ander, E. L., Magare, C., Gitu, J., & Broadley, M. R. (2017). Challenges and opportunities for Moringa growers in southern Ethiopia and Kenya. *PloS one, 12*(11), 1-15.

Kung, L., & Rode, L. (1996, 06/01). Amino acid metabolism in ruminants. *Animal Feed Science and Technology, 59*, 167-172. https://doi.org/10.1016/0377-8401(95)00897-7

Kunyanga, C. N., Imungi, J. K., & Vellingiri, V. (2013). Nutritional evaluation of indigenous foods with potential food-based solution to alleviate hunger and malnutrition in Kenya. *Journal of Applied Biosciences, 67*, 5277-5288. https://doi.org/10.4314/jab.v67i0.95049

Lalas, S., & Tsaknis, J. (2002). Characterization of *Moringa oleifera* seed oil variety "Periyakulam 1". *Journal of food composition and analysis, 15*(1), 65-77.

Lamb, G. D., & Stephenson, D. G. (1994). Effects of intracellular pH and [Mg2+] on excitation-contraction coupling in skeletal muscle fibres of the rat. *The Journal of physiology, 478*(2), 331-339.

Larcher, W. (2005). Climatic constraints drive the evolution of low temperature resistance in woody plants. *Journal of Agricultural Meteorology, 61*(4), 189-202.

Läuchli, A., & Epstein, E. (1990). Plant responses to saline and sodic conditions. In *Agricultural salinity assessment and management* (Vol. 71, pp. 113-137). American Society of Civil Engineers New York.

Lee, M. A., Davis, A. P., Chagunda, M. G. G., & Manning, P. (2017). Forage quality declines with rising temperatures, with implications for livestock production and methane emissions. *Biogeosciences, 14*(6), 1403-1417.

Lei, J., Feng, D., Zhang, Y., Dahanayaka, S., Li, X., Yao, K., Wang, J., Wu, Z., Dai, Z., & Wu, G. (2012). Regulation of leucine catabolism by metabolic fuels in mammary epithelial cells. *Amino acids, 43*(5), 2179-2189.

Leone, A., Spada, A., Battezzati, A., Schiraldi, A., Aristil, J., & Bertoli, S. (2015). Cultivation, genetic, ethnopharmacology, phytochemistry and pharmacology of *Moringa oleifera* leaves: An overview. *International Journal of Molecular Sciences, 16*(6), 12791-12835.

Lewontin, R. C., & Hubby, J. L. (1966). A molecular approach to the study of genic heterozygosity in natural populations. II. Amount of variation and degree of heterozygosity in natural populations of *Drosophila pseudoobscura*. *Genetics, 54*(2), 595.

Li, P., Knabe, D. A., Kim, S. W., Lynch, C. J., Hutson, S. M., & Wu, G. (2009). Lactating porcine mammary tissue catabolizes branched-chain amino acids for glutamine and aspartate synthesis. *The Journal of nutrition, 139*(8), 1502-1509.

Li, P., & Wu, G. (2018). Roles of dietary glycine, proline, and hydroxyproline in collagen synthesis and animal growth. *Amino acids, 50*(1), 29-38.

Liang, X., Zhang, L., Natarajan, S. K., & Becker, D. F. (2013). Proline mechanisms of stress survival. *Antioxidants & redox signaling, 19*(9), 998-1011.

Lima, D. M., Abdalla, A. L., Lima, P. d. M. T., Sakita, G. Z., McManus, C., & Louvandini, H. (2018). Morphological characteristics, nutritive quality, and methane production of tropical grasses in Brazil. *Pesquisa Agropecuária Brasileira, 53*, 323-331.

Linster, C. L., & Van Schaftingen, E. (2007). Vitamin c. *The FEBS journal, 274*(1), 1-22.

Lukatkin, A. S., Brazaityte, A., Bobinas, C., & Duchovskis, P. (2012). Chilling injury in chilling-sensitive plants: A review. *Agriculture, 99*(2), 111-124.

Lyons, J. M. (1973). Chilling injury in plants. *Annual Review of Plant Physiology, 24*(1), 445-466.

Maas, E. V., & Hoffman, G. J. (1977). Crop salt tolerance—current assessment. *Journal of the irrigation and drainage division, 103*(2), 115-134.

Mabberley, D. J. (2017). *Mabberley's plant-book: A portable dictionary of plants, their classification and uses*. Cambridge University Press.

Mahajan, S., & Tuteja, N. (2005). Cold, salinity and drought stresses: An overview. *Archives of biochemistry and biophysics, 444*(2), 139-158.

Mahmood, K. T., Mugal, T., & Haq, I. U. (2010). *Moringa oleifera* : A natural gift-A review. *Journal of Pharmaceutical Sciences and Research, 2*(11), 775.

Makkar, H. P. S., Norvsambuu, T., Lkhagvatseren, S., & Becker, K. (2009). Plant secondary metabolites in some medicinal plants of Mongolia used for enhancing animal health and production. *Tropicultura, 27*(3), 159-167.

Manduwa, D. M., Du Toit, E. S., & Robbertse, P. J. (2018, 2016). Study on the effect of temperature and flower age on pollen performance, stigma receptivity and fruit-set of *Moringa oleifera* Lam. VII International Symposium on Seed, Transplant and Stand Establishment of Horticultural Crops, Pretoria, South Africa.

Maroyi, A. (2005). The utilization of *Moringa oleifera* in Zimbabwe. *Journal of Sustainable Development in Africa, 8*(2), 160-168.

Martens, H., & Stumpff, F. (2019). Assessment of magnesium intake according to requirement in dairy cows. *Journal of animal physiology and animal nutrition, 103*(4), 1023-1029.

Martin, C., Morgavi, D. P., & Doreau, M. (2010). Methane mitigation in ruminants: From microbe to the farm scale. *Animal, 4*(3), 351-365.

Masih, L. P., Singh, S., Elamathi, S., Anandhi, P., & Abraham, T. (2019). Moringa: A multipurpose potential crop–A review. *Indian National Science Academy, 85*(3), 589-601. https://doi.org/10.16943/ptinsa/2019/49579

Masters, D. G. (2018). Practical implications of mineral and vitamin imbalance in grazing sheep. *Animal Production Science, 58*(8), 1438-1450.

Mathison, G. (1997). Effect of canola oil on methane production in steers. *Canadian Journal of Animal Science, 77*, 545.

Matysik, J., Alia, Bhalu, B., & Mohanty, P. (2002). Molecular mechanisms of quenching of reactive oxygen species by proline under stress in plants. *Current Science, 82*(5), 525-532.

McAusland, L., Vialet-Chabrand, S., Davey, P., Baker, N. R., Brendel, O., & Lawson, T. (2016). Effects of kinetics of light-induced stomatal responses on photosynthesis and water-use efficiency. *New Phytologist, 211*(4), 1209-1220.

McFarlane, H. E., Döring, A., & Persson, S. (2014). The cell biology of cellulose synthesis. *Annual review of plant biology, 65*, 69-94. https://doi.org/10.1146/annurev-arplant-050213-040240

McKinnon, A. E., Bell, S. T., & Sykes, A. R. (2003). Using a model of magnesium dynamics in cows to predict the risk of tetany in dairy herds. In (pp. 1-5). Modelling and Simulation Society of Australia and New Zealand.

Meale, S. J., Chaves, A. V., Baah, J., & McAllister, T. A. (2012). Methane production of different forages in in vitro ruminal fermentation. *Asian-Australasian Journal of Animal Sciences, 25*(1), 86.

Meglia, G. E., Jensen, S. K., Lauridsen, C., & Waller, K. P. (2006). [alpha]-Tocopherol concentration and stereoisomer composition in plasma and milk from dairy cows fed natural or synthetic vitamin E around calving. *The Journal of dairy research, 73*(2), 227.

Melesse, A., Steingass, H., Boguhn, J., Schollenberger, M., & Rodehutscord, M. (2012). Effects of elevation and season on nutrient composition of leaves and green pods of *Moringa stenopetala* and *Moringa oleifera*. *Agroforestry Systems, 86*(3), 505-518.

Mendieta-Araica, B., Spörndly, R., Reyes-Sánchez, N., & Spörndly, E. (2011). Moringa (*Moringa oleifera*) leaf meal as a source of protein in locally produced concentrates for dairy cows fed low protein diets in tropical areas. *Livestock Science, 137*(1-3), 10-17.

Menke, K. H., Raab, L., Salewski, A., Steingass, H., Fritz, D., & Schneider, W. (1979). The estimation of the digestibility and metabolizable energy content of ruminant feedingstuffs from the gas production when they are incubated with rumen liquor in vitro. *The Journal of Agricultural Science, 93*(1), 217-222.

Metwally, S. A., Ezzo, M. I., Abou Leila, B. H., & Abdalla, A. M. (2021). Effect of Diluted Red Sea Water on Growth Behavior and Chemical Component of Moringa Plants. *Annual Research & Review in Biology, 36*(4), 72-82.

Mikhaylov, A., Moiseev, N., Aleshin, K., & Burkhardt, T. (2020). Global climate change and greenhouse effect. *Entrepreneurship and Sustainability Issues, 7*(4), 2897-2931.

Mikkelsen, K., & Apostolopoulos, V. (2019). Vitamin B12, folic acid, and the immune system. In *Nutrition and immunity* (pp. 103-114). Springer.

Millero, F. J., Feistel, R., Wright, D. G., & McDougall, T. J. (2008). The composition of Standard Seawater and the definition of the Reference-Composition Salinity Scale. *Deep Sea Research Part I: Oceanographic Research Papers, 55*(1), 50-72.

Ministry for the Environment. (2019). *New Zealand's Greenhouse Gas Inventory 1990-2019* (ME 1559). https://environment.govt.nz/publications/new-zealands-greenhouse-gas-inventory-1990-2019/

Ministry of Agriculture Guyana. (2021). *Ministry of Agriculture*. Retrieved 25-08-2021 from https://agriculture.gov.gy/glda/

Minolta, K. (1989). Chlorophyll meter SPAD-502 instruction manual. *Minolta Co., Ltd., Radiometric Instruments Operations Osaka, Japan*.

Mitchell, J. F. B. (1989). The “greenhouse” effect and climate change. *Reviews of Geophysics, 27*(1), 115-139.

Molotoks, A., Smith, P., & Dawson, T. P. (2021). Impacts of land use, population, and climate change on global food security. *Food and Energy Security, 10*(1), 1-20.

Moro, J., Tomé, D., Schmidely, P., Demersay, T.-C., & Azzout-Marniche, D. (2020). Histidine: A systematic review on metabolism and physiological effects in human and different animal species. *Nutrients, 12*(5), 1-20.

Morton, J. F. (1991). The horseradish tree, *Moringa pterygosperma* (Moringaceae)—a boon to arid lands? *Economic botany, 45*(3), 318-333.

Mould, F. L., Morgan, R., Kliem, K. E., & Krystallidou, E. (2005). A review and simplification of the *in vitro* incubation medium. *Animal Feed Science and Technology, 123*, 155-172.

Moyo, B., Masika, P. J., Hugo, A., & Muchenje, V. (2011). Nutritional characterization of Moringa (*Moringa oleifera* Lam.) leaves. *African journal of biotechnology, 10*(60), 12925-12933.

Muetzel, S., Hunt, C., & Tavendale, M. H. (2014). A fully automated incubation system for the measurement of gas production and gas composition. *Animal Feed Science and Technology, 196*, 1-11. https://doi.org/10.1016/j.anifeedsci.2014.05.016

Muluvi, G. M., Sprent, J. I., Odee, D., & Powell, W. (2004). Estimates of outcrossing rates in *Moringa oleifera* using Amplified fragment length polymorphism (AFLP). *African journal of biotechnology, 3*(2), 145-151.

Munns, R. (2002). Comparative physiology of salt and water stress. *Plant, cell & environment, 25*(2), 239-250.

Munns, R., Husain, S., Rivelli, A. R., James, R. A., Condon, A. G., Lindsay, M. P., Lagudah, E. S., Schachtman, D. P., & Hare, R. A. (2002, 2002). Avenues for increasing salt tolerance of crops, and the role of physiologically based selection traits. *Plant and soil, 247*, 93-105.

Munns, R., & James, R. A. (2003). Screening methods for salinity tolerance: a case study with tetraploid wheat. *Plant and soil, 253*(1), 201-218.

Munns, R., & Tester, M. (2008). Mechanisms of salinity tolerance. *Annual review of plant biology, 59*, 651-681. https://doi.org/10.1146/annurev.arplant.59.032607.092911

Myhre, G., Shindell, D., Bréon, F. M., Collins, W., Fuglestvedt, J., Huang, J., Koch, D., Lamarque, J. F., Lee, D., & Mendoza, B. (2013). *Anthropogenic and natural radiative forcing, climate change 2013: the Physical science basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change, 659–740*.

National Institute of Water and Atmospheric Research. (2022). *The National Climate Database* https://cliflo.niwa.co.nz/pls/niwp/wgenf.genform1

National Research Council. (2001). *Nutrient requirements of dairy cattle*. National Academy Press Washington, DC.

Negrão, S., Schmöckel, S. M., & Tester, M. (2017). Evaluating physiological responses of plants to salinity stress. *Annals of botany, 119*(1), 1-11.

Niu, G., Rodriguez, D. S., Aguiniga, L., & Mackay, W. (2007). Salinity tolerance of *Lupinus havardii* and *Lupinus texensis*. *HortScience, 42*(3), 526-528.

Niu, G., Rodriguez, D. S., Crosby, K., Leskovar, D., & Jifon, J. (2010). Rapid screening for relative salt tolerance among chile pepper genotypes. *HortScience, 45*(8), 1192-1195.

Nouman, Siddiqui, M., Basra, S., Afzal, I., & Rehman, H. (2012a). Enhancement of emergence potential and stand establishment of *Moringa oleifera* Lam. by seed priming. *Turkish Journal of Agriculture and Forestry, 36*(2), 227-235.

Nouman, Siddiqui, M. T., Basra, S. M. A., Khan, R. A., Gull, T., Olson, M. E., & Hassan, M. (2012b). Response of *Moringa oleifera* to saline conditions. *International Journal of Agriculture and Biology, 14*(5).

Olson, M. E. (1976, Dec). Worldwide spread of infections with Yersinia enterocolitica. *WHO Chron, 30*(12), 494-496. https://doi.org/10.1371/journal.pone.0159782

Olson, M. E., & Carlquist, S. (2001). Stem and root anatomical correlations with life form diversity, ecology, and systematics in Moringa (Moringaceae). *Botanical Journal of the Linnean Society, 135*(4), 315-348.

Oluduro, A. O., Ambrose, D. C., Abiodun, A. O., & Daunty, A. L. (2016). Moringa. In *Leafy Medicinal Herbs: Botany, Chemistry, Postharvest Technology and Uses* (pp. 163-169).

Ortiz, J. M., Sotoca, J. A., Expósito, E., Gallud, F., Garcia-Garcia, V., Montiel, V., & Aldaz, A. (2005). Brackish water desalination by electrodialysis: batch recirculation operation modeling. *Journal of Membrane Science, 252*(1-2), 65-75.

Owens, F., & Goetsch, A. (1988). Ruminal fermentation. In D. Church (Ed.), *The Ruminant Animal Digestive Physiology and Nutrition* (pp. 145-171). Englewood Cliffs.

Owens, F. N., & Basalan, M. (2016). Ruminal fermentation. In *Rumenology* (pp. 63-102). Springer.

Palada, M. C. (1996). Moringa (*Moringa oleifera* Lam.): A versatile tree crop with horticultural potential in the subtropical United States. *HortScience, 31*(5), 794-797.

Parra-Garcia, A., Elghandour, M., Greiner, R., Barbabosa-Pliego, A., Camacho-Diaz, L. M., & Salem, A. Z. M. (2019, May). Effects of Moringa oleifera leaf extract on ruminal methane and carbon dioxide production and fermentation kinetics in a steer model. *Environ Sci Pollut Res Int, 26*(15), 15333-15344. https://doi.org/10.1007/s11356-019-04963-z

Passioura, J. B., & Munns, R. (2000). Rapid environmental changes that affect leaf water status induce transient surges or pauses in leaf expansion rate. *Functional Plant Biology, 27*(10), 941-948.

Patel, J. S., & Narayana, G. V. (1937). Chromosome numbers in some economic flowering plants. *Current Science, 5*(9), 479-479.

Patricio, H., & Palada, M. (2015). Adaptability and horticultural characterization of different moringa accessions in Central Philippines. I International Symposium on Moringa, Manila, Philippines.

Paula, M. B., Nogueira, F. D., Andrade, H., & Pittis, J. E. (1987). Effect of liming on dry matter yield of wheat in pots of low humic gley soil. *Plant and soil, 97*(1), 85-91.

Pawlak, K., & Kołodziejczak, M. (2020). The role of agriculture in ensuring food security in developing countries: Considerations in the context of the problem of sustainable food production. *Sustainability, 12*(13), 5488.

Pedraza-Hernández, J., Elghandour, M. M. M. Y., Khusro, A., Camacho-Diaz, L. M., Vallejo, L. H., Barbabosa-Pliego, A., & Salem, A. Z. M. (2019). Mitigation of ruminal biogases production from goats using *Moringa oleifera* extract and live yeast culture for a cleaner agriculture environment. *Journal of Cleaner Production, 234*, 779-786. https://doi.org/10.1016/j.jclepro.2019.06.126

Pereira, A. M., de Lurdes Nunes Enes Dapkevicius, M., & Borba, A. E. S. (2022). Alternative pathways for hydrogen sink originated from the ruminal fermentation of carbohydrates: Which microorganisms are involved in lowering methane emission? *Animal Microbiome, 4*(1), 1-12.

Pérez-Ángel, R., Pérez-Tamayo, N. M., Castro-Martínez, C., & Contreras-Andrade, I. (2020). Yields and chemical composition of biomass, seed and oil of ecotypes of *Moringa oleifera* Lamarck introduced in Sinaloa, Mexico. *AGROProductividad, 13*(7), 21-29.

Pogribny, I. P., Karpf, A. R., James, S. R., Melnyk, S., Han, T., & Tryndyak, V. P. (2008). Epigenetic alterations in the brains of Fisher 344 rats induced by long-term administration of folate/methyl-deficient diet. *Brain research, 1237*, 25-34. https://doi.org/10.1016/j.brainres.2008.07.077

Ponnuswami, V. (n.d). Genetic improvement and varietal status of moringa. In *Advances in Production of Moringa* (pp. 7). Tamil Nadu Agricultural University. https://agritech.tnau.ac.in/horticulture/pdf/Moringa%20English%20book.pdf

Popoola, J. O., & Obembe, O. O. (2013). Local knowledge, use pattern and geographical distribution of *Moringa oleifera* Lam.(Moringaceae) in Nigeria. *Journal of Ethnopharmacology, 150*(2), 682-691.

Powell, F. W. (1927). *The Bureau of Plant Industry: Its history, activities and organization* (Vol. 47). Johns Hopkins Press.

Ramachandran, C., Peter, K. V., & Gopalakrishnan, P. K. (1980). Drumstick (*Moringa oleifera*): A multipurpose Indian vegetable. *Economic botany*, 276-283.

Ramírez-Restrepo, C. A., Clark, H., & Muetzel, S. (2016). Methane emissions from young and mature dairy cattle. *Animal Production Science, 56*(11), 1897-1905.

Ravelombola, W., Qin, J., Weng, Y., Mou, B., & Shi, A. (2019). A simple and cost-effective approach for salt tolerance evaluation in cowpea (*Vigna unguiculata*) seedlings. *HortScience, 54*(8), 1280-1287.

Ravi, R. S. D., Siril, E. A., & Nair, B. R. (2020, Apr). The efficiency of Cytochrome P450 gene-based markers in accessing genetic variability of drumstick (*Moringa oleifera* Lam.) accessions. *Mol Biol Rep, 47*(4), 2929-2939. https://doi.org/10.1007/s11033-020-05391-w

Richter, N., Siddhuraju, P., & Becker, K. (2003). Evaluation of nutritional quality of moringa (*Moringa oleifera* Lam.) leaves as an alternative protein source for Nile tilapia (*Oreochromis niloticus* L.). *Aquaculture, 217*(1-4), 599-611.

Robertson, G. P., Paul, E. A., & Harwood, R. R. (2000). Greenhouse gases in intensive agriculture: contributions of individual gases to the radiative forcing of the atmosphere. *Science, 289*(5486), 1922-1925.

Roldan, M. B., Cousins, G., Muetzel, S., Zeller, W. E., Fraser, K., Salminen, J.-P., Blanc, A., Kaur, R., Richardson, K., & Maher, D. (2021). Condensed tannins in white clover (*Trifolium repens*) foliar tissues expressing the transcription factor TaMYB14-1 bind to forage protein and reduce methane emissions *in vitro*. *Frontiers in Plant Science, 12*(777354), 1-19.

Rotz, C. A. (2018). Modeling greenhouse gas emissions from dairy farms. *Journal of dairy science, 101*(7), 6675-6690.

Saini, R. K., Sivanesan, I., & Keum, Y.-S. (2016). Phytochemicals of *Moringa oleifera*: A review of their nutritional, therapeutic and industrial significance. *3 Biotech, 6*(2), 1-14.

Sakai, A., & Larcher, W. (1987). Low temperature and frost as environmental factors. In *Frost Survival of Plants* (pp. 1-20). Springer.

Sánchez-Machado, D., Lopez-Cervantes, J., & Vázquez, N. R. (2006). High-performance liquid chromatography method to measure α-and γ-tocopherol in leaves, flowers and fresh beans from *Moringa oleifera*. *Journal of chromatography A, 1105*(1-2), 111-114.

Sánchez-Machado, D. I., Núñez-Gastélum, J. A., Reyes-Moreno, C., Ramírez-Wong, B., & López-Cervantes, J. (2010). Nutritional quality of edible parts of *Moringa oleifera*. *Food analytical methods, 3*(3), 175-180.

Santos, C. V. (2004). Regulation of chlorophyll biosynthesis and degradation by salt stress in sunflower leaves. *Scientia horticulturae, 103*(1), 93-99.

Saunois, M., Bousquet, P., Poulter, B., Peregon, A., Ciais, P., Canadell, J. G., Dlugokencky, E. J., Etiope, G., Bastviken, D., & Houweling, S. (2016). The global methane budget 2000–2012. *Earth System Science Data, 8*(2), 697-751.

Sharma, G. K., & Raina, V. (1982, 1982). Propagation techniques of *Moringa oleifera* Lam. In Improvement of forest biomass: symposium proceedings, India.

Shevela, D., & Björn, L. O. (2017). Evolution of the Z-scheme of photosynthesis: a perspective. *Photosynthesis research, 133*(1), 5-15.

Shrivastava, P., & Kumar, R. (2015). Soil salinity: A serious environmental issue and plant growth promoting bacteria as one of the tools for its alleviation. *Saudi journal of biological sciences, 22*(2), 123-131.

Sim, S., Lee, J., Uhm, Y., Kim, S., Han, E. J., Choi, K., Choi, J., Ban, Q. m., Cho, T., & Kim, A. Y. (2019). Korean consumers' awareness of the risks of chemicals in daily consumer products. *Environmental Sciences Europe, 31*(1), 1-12.

Singh, A. K., Rana, H. K., Tshabalala, T., Kumar, R., Gupta, A., Ndhlala, A. R., & Pandey, A. K. (2020). Phytochemical, nutraceutical and pharmacological attributes of a functional crop Moringa oleifera Lam: An overview. *South African Journal of Botany, 129*, 209-220.

Singh, M., Singh, V. P., & Prasad, S. M. (2016). Responses of photosynthesis, nitrogen and proline metabolism to salinity stress in Solanum lycopersicum under different levels of nitrogen supplementation. *Plant Physiology and Biochemistry, 109*, 72-83.

Sivasankari, B., Anandharaj, M., & Gunasekaran, P. (2014). An ethnobotanical study of indigenous knowledge on medicinal plants used by the village peoples of Thoppampatti, Dindigul district, Tamilnadu, India. *Journal of Ethnopharmacology, 153*(2), 408-423.

Slabu, C., Zörb, C., Steffens, D., & Schubert, S. (2009). Is salt stress of faba bean (*Vicia faba*) caused by Na+ or Cl–toxicity? *Journal of Plant Nutrition and Soil Science, 172*(5), 644-651.

Smith, J., Seaton, J., Osuji, P., D'Aguiar, P., Chesney, P., McBean, M., Haynes, A., Harding, C., & Cumberbatch, N. (1991). Dry matter and crude protein yields of Echinochloa pyramidalis on coastal clay soil of Guyana. *Turrialba, 41*(1), 69-75.

Snowdon, R. J., Wittkop, B., Chen, T.-W., & Stahl, A. (2021). Crop adaptation to climate change as a consequence of long-term breeding. *Theoretical and Applied Genetics, 134*(6), 1613-1623.

Soliva, C., Kreuzer, M., Foidl, N., Foidl, G., Machmüller, A., & Hess, H. (2005). Feeding value of whole and extracted *Moringa oleifera* leaves for ruminants and their effects on ruminal fermentation *in vitro*. *Animal Feed Science and Technology, 118*(1-2), 47-62.

Soltan, Y. A., Hashem, N. M., Morsy, A. S., El-Azrak, K. M., El-Din, A. N., & Sallam, S. M. (2018). Comparative effects of *Moringa oleifera* root bark and monensin supplementations on ruminal fermentation, nutrient digestibility and growth performance of growing lambs. *Animal Feed Science and Technology, 235*, 189-201.

Soltan, Y. A., Morsy, A., Hashem, N. M., & Sallam, S. (2019). Impact of supplementary *Moringa oleifera* leaf extract on ruminal nutrient degradation and mitigating methane formation *in vitro*. *Egyptian Journal of Nutrition and Feeds, 22*(1), 55-62.

Somerville, C., Youngs, H., Taylor, C., Davis, S. C., & Long, S. P. (2010). Feedstocks for lignocellulosic biofuels. *Science, 329*(5993), 790-792.

Stadtlander, T., & Becker, K. (2017). Proximate composition, amino and fatty acid profiles and element compositions of four different Moringa species. *Journal of Agricultural Science, 9*(7), 46-57.

Steele, J. G., & Ramdin, H. N. (1971). *Soil resources of Guyana*. https://ideas.repec.org/p/ags/cfcs71/263363.html

Steponkus, P. L. (1984). Role of the plasma membrane in freezing injury and cold acclimation. *Annual Review of Plant Physiology, 35*, 543-584.

Steponkus, P. L. (1993). A contrast of the cryostability of the plasma membrane of winter rye and spring oat-two species that widely differ in their freezing tolerance and plasma membrane lipid composition. *Advances in low temperature biology, 3*, 211-312.

Stevens, C., Ugese, F., Otitoju, G., & Baiyeri, K. (2015). Proximate and anti-nutritional composition of leaves and seeds of *Moringa oleifera* in Nigeria: a comparative study. *Agro-Science, 14*(2), 9-17.

Su, B., & Chen, X. (2020). Current status and potential of *Moringa oleifera* leaf as an alternative protein source for animal feeds. *Frontiers in veterinary science, 7*(53), 1-13. https://doi.org/10.3389/fvets.2020.00053

Sun, X. Z., Hoskin, S. O., Muetzel, S., Molano, G., & Clark, H. (2011). Effects of forage chicory (*Cichorium intybus*) and perennial ryegrass (*Lolium perenne*) on methane emissions *in vitro* and from sheep. *Animal Feed Science and Technology, 166*, 391-397. https://doi.org/10.1016/j.anifeedsci.2011.04.027

Sustainable Bioresources LLC. (2021). *Building an International Moringa seed Bank*. Retrieved 11/06/2021 from https://sustainablebioresources.com/moringa-projects/moringa-seed-bank/

Swalve, H. H. (2004). Crossbreeding as an alternative approach in dairy cattle breeding. *ZUCHTUNGSKUNDE, 76*(6), 412-420.

Tamura, K., & Yamada, T. (2007). A perennial ryegrass CBF gene cluster is located in a region predicted by conserved synteny between Poaceae species. *Theoretical and Applied Genetics, 114*(2), 273-283.

Teixeira, E. M. B., Carvalho, M. R. B., Neves, V. A., Silva, M. A., & Arantes-Pereira, L. (2014). Chemical characteristics and fractionation of proteins from *Moringa oleifera* Lam. leaves. *Food chemistry, 147*, 51-54.

Regulation (EC) No 1831/2003 of the European Parliment and of the Council 15 (2003). https://ec.europa.eu/jrc/sites/default/files/EC-1831-2003.pdf

Tilley, J. M. A., & Terry, d. R. A. (1963). A two-stage technique for the *in vitro* digestion of forage crops. *Grass and forage science, 18*(2), 104-111.

Trigo, C., Castelló, M. L., Ortolá, M. D., García-Mares, F. J., & Desamparados Soriano, M. (2021). *Moringa oleifera* : An Unknown Crop in Developed Countries with Great Potential for Industry and Adapted to Climate Change. *Foods, 10*(1), 1-14.

Tshabalala, T., Ndhlala, A. R., Ncube, B., Abdelgadir, H. A., & Van Staden, J. (2020). Potential substitution of the root with the leaf in the use of *Moringa oleifera* for antimicrobial, antidiabetic and antioxidant properties. *South African Journal of Botany, 129*, 106-112.

Ulyatt, M. J., Lassey, K. R., Shelton, I. D., & Walker, C. F. (2002). Seasonal variation in methane emission from dairy cows and breeding ewes grazing ryegrass/white clover pasture in New Zealand. *New Zealand Journal of Agricultural Research, 45*(4), 217-226.

United Nations Department of Economic and Social Affairs. (2015). *The World Population Prospects: 2015 Revision*. United Nations. Retrieved 2022-01-23 from https://www.un.org/en/development/desa/publications/world-population-prospects-2015-revision.html#:~:text=The%20current%20world%20population%20of,2015%20Revision%E2%80%9D%2C%20launched%20today.

United Nations Department of Economic and Social Affairs. (2021). *The 17 Goals*. United Nations. Retrieved 2022-01-13 from https://sdgs.un.org/

US Army Corps of Engineers. (1998). *Water Resource Assessment of Guyana*. https://www.sam.usace.army.mil/Portals/46/docs/military/engineering/docs/WRA/Guyana/Guyana%20WRA.pdf

US Soil Salinity Laboratory Staff. (1954). *Diagnosis and improvement of saline and alkali soils*. US Department of Agriculture.

Valladares, F., Allen, M. T., & Pearcy, R. W. (1997). Photosynthetic responses to dynamic light under field conditions in six tropical rainforest shrubs occuring along a light gradient. *Oecologia, 111*(4), 505-514.

Van Nevel, C. J., & Demeyer, D. I. (1996). Control of rumen methanogenesis. *Environmental Monitoring and assessment, 42*(1), 73-97.

VandeHaar, M. J., Armentano, L. E., Weigel, K., Spurlock, D. M., Tempelman, R. J., & Veerkamp, R. (2016). Harnessing the genetics of the modern dairy cow to continue improvements in feed efficiency. *Journal of dairy science, 99*(6), 4941-4954.

Vélez-Gavilán, J. (2017). *Moringa oleifera* (horse radish tree). In *Invasive Species Compendium*. CABI.

Volden, H., Velle, W., Harstad, O. M., Aulie, A., & Sjaastad, Ø. V. (1998). Apparent ruminal degradation and rumen escape of lysine, methionine, and threonine administered intraruminally in mixtures to high-yielding cows. *Journal of animal science, 76*(4), 1232-1240.

von Maydell, H.-J. (1986). *Trees and shrubs of the Sahel, their characteristics and uses*. CABI.

VSN International. (2021). *Genstat for Windows 21st Edition*. VSN International, Hemel Hemstead,UK. www.genstat.co.uk

Wang, Zheng, Q., Shen, Q., & Guo, S. (2013). The critical role of potassium in plant stress response. *International Journal of Molecular Sciences, 14*(4), 7370-7390.

Wang, T., McFarlane, H. E., & Persson, S. (2016). The impact of abiotic factors on cellulose synthesis. *Journal of Experimental Botany, 67*(2), 543-552.

Wani, J. M., Sarda, V. K., & Jain, S. K. (2017). Assessment of trends and variability of rainfall and temperature for the district of Mandi in Himachal Pradesh, India. *Slovak Journal of Civil Engineering, 25*(3), 15.

Waraich, E. A., Ahmad, R., Halim, A., & Aziz, T. (2012). Alleviation of temperature stress by nutrient management in crop plants : A review. *Journal of soil science and plant nutrition, 12*(2), 221-244.

Wen, J.-Q., Oono, K., & Imai, R. (2002). Two novel mitogen-activated protein signaling components, OsMEK1 and OsMAP1, are involved in a moderate low-temperature signaling pathway in rice. *Plant physiology, 129*(4), 1880-1891.

Wu, Wu, Z., Dai, Z., Yang, Y., Wang, W., Liu, C., Wang, B., Wang, J., & Yin, Y. (2013, 2013/04/01). Dietary requirements of "nutritionally non-essential amino acids" by animals and humans. *Amino acids, 44*(4), 1107-1113. https://doi.org/10.1007/s00726-012-1444-2

Wu, D., Cai, Z., Wei, Y., Zhang, C., Liang, G., & Guo, Q. (2013). Research advances in moringa as a new plant protein feed. *Chinese Journal of Animal Nutrition, 25*(3), 503-511.

Wu, G. (2009). Amino acids: metabolism, functions, and nutrition. *Amino acids, 37*(1), 1-17.

Wu, G., & Knabe, D. A. (1994). Free and protein-bound amino acids in sow's colostrum and milk. *The Journal of nutrition, 124*(3), 415-424.

Wu, G., & Meininger, C. J. (2009). Nitric oxide and vascular insulin resistance. *Biofactors, 35*(1), 21-27.

Wu, H., Guo, J., Wang, C., Li, K., Zhang, X., Yang, Z., Li, M., & Wang, B. (2019). An effective screening method and a reliable screening trait for salt tolerance of *Brassica napus* at the germination stage. *Frontiers in Plant Science, 10*(530), 1-12.

Wuebbles, D. J., & Hayhoe, K. (2002). Atmospheric methane and global change. *Earth-Science Reviews, 57*(3-4), 177-210.

Wysocka, D., Snarska, A., & Sobiech, P. (2020). Iron in cattle health. *Journal of Elementology, 25*(3), 1175-1185.

Xue, Y. F., & Liu, Z. P. (2008). Antioxidant enzymes and physiological characteristics in two Jerusalem artichoke cultivars under salt stress. *Russian Journal of Plant Physiology, 55*(6), 776-781.

Zempleni, J. (2005). Uptake, localization, and noncarboxylase roles of biotin. *Annual Review of Nutrition, 25*, 175-196.

Zhang, T., Si, B., Deng, K., Tu, Y., Zhou, C., & Diao, Q. (2018). Effects of feeding a Moringa oleifera rachis and twig preparation to dairy cows on their milk production and fatty acid composition, and plasma antioxidants. *Journal of the Science of Food and Agriculture, 98*(2), 661-666.

Zhang, Y., Kaiser, E., Zhang, Y., Yang, Q., & Li, T. (2018). Short-term salt stress strongly affects dynamic photosynthesis, but not steady-state photosynthesis, in tomato (*Solanum lycopersicum*). *Environmental and Experimental Botany, 149*, 109-119.

Zhao, J., Davis, L. C., & Verpoorte, R. (2005). Elicitor signal transduction leading to production of plant secondary metabolites. *Biotechnology advances, 23*(4), 283-333.

Zhao, S., Zhang, Q., Liu, M., Zhou, H., Ma, C., & Wang, P. (2021). Regulation of Plant Responses to Salt Stress. *International Journal of Molecular Sciences, 22*(9), 1-16.

Zoric, L., Merkulov, L., Lukovic, J., & Boza, P. (2012). Comparative analysis of qualitative anatomical characters of *Trifolium* L. (Fabaceae) and their taxonomic implications: preliminary results. *Plant Systematics and Evolution, 298*(1), 205-219.

Chapter 8 Appendices

Appendix 1 Composition of potting soil

The potting soil is made up of Daltons Base Mix and Osmocote© Pro

Daltons Base Mix	
Ingredients	Quantity
Calcium Ammonium Nitrate (C.A.N)	Fines A Grade 50.00%
Coconut fibre	30.00%
Pacific Pumice 7mm	20.00%
Description of Ingredients	
Calcium. Ammonium Nitrate, a common additive to raw bark as a nitrogen source for the microbial activity during composting. A grade C.A.N. fines is *Pinus radiata* bark size 0-12mm with added C.A.N. and moisture pH adjusted and composted usually 15-18 weeks.	

ICL

Printsheet /AU-EN

Osmocote Pro

3-4M NEW

19-3.9-8.3+1.2Mg+TE

3-4 2nd

Nitrogen Total (N)	**19%**
Nitrate nitrogen (N03-N)	6.3%
Ammoniacal nitrogen (NH4-N)	8.2%
Urea nitrogen (Ur-N)	4.5%
Phosphorus (P)	**3.9%**
Water soluble	3.0%
Potassium (K)	**8.3%**
Water soluble	8.3%
Magnesium (Mg)	**1.2%**
Water soluble	0.8%
Iron (Fe)	**0.30%**
Chelated by EDTA	0.06%
Manganese (Mn)	**0.04%**
Boron (B)	**0.01%**
Water soluble	0.01%
Copper (Cu)	**0.037%**
Water soluble	0.023%
Molybdenum (Mo)	**0.015%**
Water soluble	0.010%
Zinc (Zn)	**0.011%**

A second-generation coated fertilizer with controlled release. Fully coated NPK and trace elements. Osmocote Pro features orange / white color coded granules for easy recognition.

Advantages

- High nitrogen content, greener plants!;
- Fully coated NPK + full package of trace elements;
- Proven ICL coating technology;
- Efficient release;
- Orange / white color coded granules for easy recognition;
- Can be applied at full rates or base rates with additional water soluble fertilizers;
- Cost efficient 2nd generation.

Characteristics

Longevity

3-4 Months

Appendix 2. Previous work done on *M. oleifera* and salinity

Year	Authors	Title and brief description of methods	Ecotype/ origin of seed	Key Results	Journal
2021	Sami A. Metwally, M. I. Ezzo, Bedour H. Abou Leila, Aboelfetoh M. Abdalla	Title*:* Effect of Diluted Red Sea Water on Growth Behaviour and Chemical Component of Moringa Plants *Method* Greenhouse experiment The experiment included salt treatment in the form of different concentrations of red sea water and application of proline in the form of a spray. i.e four levels of salinity (0, 4000, 8000, 16000) ppm in combination with three levels of proline 200,400 and 600 ppm. Thus, the treatments included 13 treatments with 7 replicates for each. For cultivation, pots of 50 cm diameter and 50 cm in depth were filled with a mixture of loamy and sandy soil 2:1 by volume (30 kg). The treatments were applied to 6-week-old plants. Diluted sea water was irrigated for two weeks, followed by two weeks fresh water, alternating for a period of 11 months. Growth parameters were taken at 6 months and then at 12 months after sowing. Link: https://www.journalarrb.com/index.php/ARRB/article/view/30364	The seeds were obtained from *M. oleifera* Plants Production Association in National Research Centre, Egypt	Parameters measured: plant height (cm), leaves no., stem diameter (mm), leaves fresh weight (g), leaves Dry weight (g), stem fresh weight (g), stem dry weight, The major finding: Salt water had negative effects on plant parts even at 4000ppm.	Annual Research & Review in Biology

2021	Abdul Khaliq Al-Shoaibi, Tahar Boutraa,	Title: Comparative study on germination, growth and gas exchanges of the tropics' tree *Moringa oleifera* and its desert relative *Moringa peregrina* under saline conditions Method Germination: Seeds were subjected to 5 NaCl treatments: 0, 50, 100, 150 and 200 mM. Four replicates for each treatment were used, and the Petri dishes were kept inside an environmental controlled incubator, under 25 °C in darkness for two weeks. Germination was checked on daily basis for the whole period of the experiment, until no further seeds germinate, and seeds are considered germinated, when the radicle emerged. Plant Growth: Plants were grown under a temperature of 25 °C, a photoperiod (light/dark) of 14/10 h, a relative humidity of 60 % and illumination by fluorescent and halogen lamps of 400 µmol m^{-2} s^{-1}. After two weeks from germination, plants were subjected to NaCl concentrations 0, 50, 100 and 150 mM, in full-strength Hoagland nutrient solution for 6 weeks. Four replicates for each Moringa species of each salinity treatment were applied, leading to a total of 32 pots. One plant in a pot.	*M. oleifera* seeds were obtained from Al-Hilali Agricultural Establishment, Almadinah Almunawarah, Saudi Arabia and the seeds *M. peregrina* were donated by Jeeda charity community, Al-Ula district, Saudi Arabia where the seeds were collected from their natural areas.	Parameters measured: photosynthesis, chlorophyll fluorescence and chlorophyll content were measured. The growth parameters measured at the end of the experiment were plant height, number of leaves, number of branches, leaf area, root length, and root volume. Fresh weight of root and shoot were measured, and dry weight was measured by oven dried at 80 °C for 48 h. Major findings: Both species had decreasing germination rates as the salt concentration increased. Seeds did not germinate at 200 mM. The plant height and number of leaves of *M. oleifera* treated with 50 mM were significantly	South African Journal of Botany

		https://www-sciencedirect-com.ezproxy.massey.ac.nz/science/article/pii/S0254629921000843		greater than control plants. Dry mass of both species decreased with increasing salt concentrations	
2018	Noreen Fatima, Muhammad Akram, Muhammad Shahid, Ghulam Abbas, Mubshar Hussain, Muhammad Nafees, Allah Wasaya, Muhammad Tahir, Mauhammad Amjad,	Title: Germination, growth and ions uptake of moringa (*Moringa oleifera* L.) grown under saline condition *Method* Germination: 8 seeds in petri dishes, 5 treatments of 0, 50, 100, 150 and 200 mM NaCl. Five replications Pot experiment Three weeks old plants were transplanted in pots. The treatments were 5, 10, 15 and 20 dSm^{-1} using refine sodium chloride. Four replications. Treatments lasted for 5 months. https://www-tandfonline-com.ezproxy.massey.ac.nz/doi/pdf/10.1080/01904167.2018.1459690	University of Agriculture Faisalabad, Pakistan	Parameters measured: Germination percentage, time taken for 50 % germination, mean germination time, germination index, seed vigour index. Root and shoot lengths (cm), root and shoot dry weights (g) and Na^+, K^+, Ca^{2+} Germination occurred at 5 and 10 dSm^{-1}. The seeds took longer to germinate under salt treatments and the germination rates were lower in salt treatments. Plant growth parameters decreased with increasing salt treatment along with Na^+ uptake in roots and shoots.	Journal of plant Nutrition

2012	Ayoub Zeyada Elhag, Maha Hussien Abdalla	Title: Effect of sodium chloride on germination and emergence of moringa (*Moringa oleifera* L.) seeds Method Two experiments were done: 1. Germination of seeds was done at 40, 80 120 and 160 mM NaCl. 2. Seeds were sown in plastic bags containing soil with 0.2, 0.4 and 0.8 % w/w NaCl, to measure seedling emergence. http://repository.sustech.edu/bitstream/handle/123456789/16858/Effect%20of%20Sodium%20Chloride....pdf?sequence=1	Forestry Research Station, Soba, Agricultural Research Cooperation, Federal Ministry of Agriculture, Sudan.	Parameters measured: Germination percent, germination rate, emergence percent and emergence rate, seedling shoot length, root length, seedling fresh and dry weight. Major findings: Salinity affected seed germination and plant growth.	Journal of Science and Technology
2012	Wasif Nouman, Muhammad Tahir Siddiqui, Shahzad Maqsood, Rashid Ahmed Khan, Tehseen Gull, Mark Earl Olson, Hassan Munir	Title*:* Response of *Moringa oleifera* to saline conditions Method Plant Growth was measured in greenhouse, plants were sown in pots. The experiment consisted of 5 replicates, 3 plants in each and 3 random plants were measured. Treatments were: 2, 4, 8, 12 dS m^{-1} salinity and applied at 5 leaf stage for 30 days Salt was added by means of Hoagland's solution. https://www.researchgate.net/publication/256298230_Response_of_Moringa_oleifera_to_Saline_Conditions	University of Agriculture Faisalabad, Pakistan	Parameters measured: shoot length, root length, number of leaves, branches per plant, root and shoot fresh and dry weight. Chlorophyll a and b and beta carotene. Nitrogen and crude protein Major findings: Decreasing growth parameters as salt concentration increase. At 12 dS m^{-1} most plant growth parameters were significantly different from the control.	International Journal of Agriculture and Biology

Appendix 3. Basic information for provenances sourced from Guyana

General Information of climate and a plant

Climate of Low Coastal plain (Guyana)	
Annual rainfall: 1600 mm to 3000 mm	Min/Max annual temp: 22°C/31°C
Annual temperature: 27.5 °C.	

Plant description	
Family: Moringaceae	Common Name: Moringa, Saijan, Saigan
Genus: *Moringa*	
Species: *oleifera*	Plant Height (m): 3-4
Sub-species: *Var. Subsp. forma*. Other	Sub-species Name: Nil
Plant Form: Tree	Or Other: *(specify):*

Provenance specific data

Queenstown	
Date Collected: 28-03-2021	Date received at Massey: 8-05-2021

Name of collector	Affiliation (organisation)
Usha Ramlakhan (U.R)	National Agricultural research and Extension Institute NAREI)- Guyana
Position: Assistant Crop Extension Officer	

Geographic

Country: Guyana		
Province: Region 2, Pomeroon -Supenaam		Local Area Name: Queenstown
Collection site: residential area with houses. Close to the Sea (approx. 2 km)		
Site Notes: Housing scheme, flat land, with perennial fruit trees growing in the yards of homeowners.		
Latitude DDMMSS:	Longitude DDMMSS:	
GPS used (NO)	or Grid reference:	Altitude (m): 2 m below sea level

Habitat

Habitat & Associated Species: *Mangifera indica*	
Habitat modification: Habitat Loss, Water level, Fire, Pollution, Invasive spp., Harvesting, Livestock: or Other: (specify): habitat prone to flooding	
Land Use:	land is used for housing purposes
Landform:	flat land
Soil Colour & texture: dark brown and fine texture	
Aspect of Site: site located south of the Atlantic Ocean	Slope: none
Site Notes: the land is currently used for the building houses and the seeds collected were found growing isolated.	

Population & Sampling

Material collected: Fruit (Pods)	
Material identified in the field by: U.R	
No. of Plants Collected from: 1	% of plants sampled: 33
% Population producing seed: 30	Local abundance of plants: (very few, few, abundant): few
Estimated No. of plants in sampled population: 3	Area collected from (m^2):

	1200
Is there evidence of recruitment of seedlings into the adult population? NO	

Mon Repos	
Date Collected: 3-03-2021	Date received at Massey: 8-05-2021

Name of collector	Affiliation (organisation)
Indira Persaud (I.P)	National Agricultural research and Extension Institute NAREI)- Guyana
Position: Research Assistant	

Geographic

Country: Guyana		
Province: Region 3, West Demerara Islands		Local Area Name: Mon Repos
Collection site: the site is flat and situated next to a drainage canal.		
Site Notes: the plants are growing 3 m next to a canal that leads to the Atlantic Ocean.		
Latitude DDMMSS:	Longitude DDMMSS:	
GPS used (NO)	or Grid reference:	Altitude (m): 2 m below sea level

Habitat

Habitat & Associated Species: *Mangifera indica, Cocos nucifera*	
Habitat modification: Habitat Loss, Water level, Fire, Pollution, Invasive spp., Harvesting, Livestock: or Other: (specify): habitat prone to flooding	
Land Use:	Government compound for office buildings
Landform:	flat land
Soil Colour & texture: black to dark brown and medium texture	

Aspect of Site: south of the Atlantic Ocean (3 km)	Slope: none
Site Notes: Trees are 4 years old and and are growing in a single row. The land is well drained.	

Population & Sampling

Material collected: Seed or Inflorescences (complete flower head) etc: Fruit (Pods)		
Material identified in the field by: I.P		
No. of Plants Collected from: 6		% of plants sampled: 60
% Population producing seed: 100	Local abundance of plants: (very few, few, abundant): few	
Estimated No. of plants in sampled population: 10		Area collected from (m^2): 600
Is there evidence of recruitment of seedlings into the adult population? NO		

Bush Lot	
Date Collected: 15-03-2021	Date received at Massey: 8-05-2021

Name of collector	Affiliation (organisation)
Yashma Subhai (Y.S)	National Agricultural research and Extension Institute NAREI)- Guyana
Position: Crop Extension Officer	

Geographic

Country: Guyana	
Province: Region 5, Mahaica Berbice	Local Area Name: Bush Lot
Collection site: the site is flat and situated next to a major road.	

Site Notes:		
Latitude DDMMSS:	Longitude DDMMSS:	
GPS used (NO)	or Grid reference:	Altitude (m): 2 m below sea level

Habitat

Habitat & Associated Species: trees are among vegetable crops (*Brassica rapa, Allium spp. Phaseolus vulgaris*)	
Habitat modification: Habitat Loss, Water level, Fire, Pollution, Invasive spp., Harvesting, Livestock: or Other: (specify): *habitat prone to some sea water intrusion*	
Land Use:	Household farming (each household has a vegetable garden)
Landform:	flat land
Soil Colour & texture: black to dark brown and fine texture	
Aspect of Site: south of the Atlantic Ocean (4 km)	Slope: none
Site Notes: the area is generally a rural area with each household having a vegetable garden. Some fruit trees are present.	

Population & Sampling

Material collected: Seed or Inflorescences (complete flower head) etc: Fruit (Pods)	
Material identified in the field by: Y.S	
No. of Plants Collected from: 5	% of plants sampled: 100
% Population producing seed:100	Local abundance of plants: (very few, few, abundant): abundant
Estimated No. of plants in sampled population: 5	Area collected from (m^2): 1800
Is there evidence of recruitment of seedlings into the adult population? NO	

Benab	
Date Collected: 20-03-2021	Date received at Massey: 8-05-2021

Name of collector	Affiliation (organisation)
Shanelli Chand (S.C)	National Agricultural research and Extension Institute NAREI)- Guyana
Position: Plant Nursery attendant	

Geographic

Country: Guyana		
Province: Region 6, East Berbice Corentyne		Local Area Name: Benab
Collection site: the site is flat and situated next to beach		
Site Notes: the site is used as a plant nursery		
Latitude DDMMSS:	Longitude DDMMSS:	
GPS used (NO)	or Grid reference:	Altitude (m): 1 m below sea level

Habitat

Habitat & Associated Species: the trees are planted with a plant nursery where various crops are propagated and sold.	
Habitat modification: Habitat Loss, Water level, Fire, Pollution, Invasive spp., Harvesting, Livestock: or Other: (specify): habitat is a plant nursery	
Land Use:	Plant nursery located next to a beach.
Landform:	flat land
Soil Colour & texture: dark brown and fine texture	
Aspect of Site: south of the Atlantic Ocean (0.5 km)	Slope: none

Site Notes: the site is located at the entrance of a beach

Population & Sampling

Material collected: Seed or Inflorescences (complete flower head) etc: Fruit (Pods)		
Material identified in the field by: S.C		
No. of Plants Collected from: 3		% of plants sampled: 60
% Population producing seed:60	Local abundance of plants: (very few, few, abundant): few	
Estimated No. of plants in sampled population: 5		Area collected from (m^2): 1000
Is there evidence of recruitment of seedlings into the adult population? NO		