Investigation of Guayule's Agronomic Performance and Agro-processing in South Africa

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A thesis submitted in partial fulfilment of the requirements for the degree of Doctor Philosophy in the Department of Biotechnology, University of the Western Cape.

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KEYWORDS

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ABSTRACT

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Guayule (*Parthenium argentatum*) is grown for its high quality hypoallergenic natural rubber latex production. The plant is native to the Chihuahua desert of North America and successful trials have been done in Europe, the United States of America (USA) and the Republic of South Africa (RSA). It is already undergoing industrial scale development in the USA producing good quality rubber products with impressive stretchiness and strength. The performance of guayule lines AZ1, AZ2, AZ3, AZ4, AZ5, AZ6, OSU1, and 11591 was investigated in the Eastern and Western Cape regions in RSA with an ultimate goal of leading to commercial production. To ensure continuous supply of plants, and avoiding documented seed dormancy issues, a micropropagation protocol was established using the OSU1 guayule line. In addition, laboratory scale latex extraction was accomplished using the Waring blender method with KOH pH 11 buffer and the amount of latex was quantified using the 1 ml latex quantification method. The extracted latex homogenate from the different guayule lines was pooled and purified using Sodium Carboxymethyl Cellulose. The efficiency of different molecular weights (90 000, 250 000 and 700 000) of Sodium Carboxymethyl Cellulose was determined in creaming guayule latex at room temperature and 4°C. The optimal creaming results were incorporated into creaming latex extracted from the different guayule lines during this study. Proteins from stem bark and leaf tissue were extracted using the phenol extraction method and separated using SDS

PAGE. The field data confirmed that the Eastern Cape and Western Cape regions of RSA can support the establishment of guayule lines AZ1-AZ6, OSU1 and 11591. The micropropagation process for OSU1 generated strong field plants which are still surviving in the field after two year. Purified latex was achieved by using Sodium Carboxymethyl Cellulose as a creaming agent. For proteome analysis, the AZ5 guayule line was chosen because of high latex production and its protein profiles of the leaf and stem bark revealed 75% and 45% identification respectively. Protein analysis of guayule will aid in the identification of rubber synthesis proteins which will be further characterized through cloning and molecular techniques.

DECLARATION

I declare that "Investigation of guayule's agronomic performance and agro-processing in South Africa" is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Rendani Daphney Mutepe August 2017

Signed  Date 18/0817

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DEDICATION

I dedicate this thesis to my beautiful daughter “Samantha Mutepe”, May God be with you in your studies and work hard to achieve.

“Life is not by chance, for every prize there is a price to pay and it involves hard work”

Love Mom

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ABBREVIATIONS

Am Bic	Ammonium bicarbonate
APPs	Allylic pyrophosphates
APX	Ascorbate peroxidase
ARC	Agricultural Research Council
BA	Benzyladenine
CPT	*cis*-prenyltransferase
CSIR	Council for Scientific and Industrial Research
DAFF	Department of Agriculture, Forestry and Fisheries
DCPA	Dimethyl 2, 3, 5, 6-tetrachloro–l, 4,-benzenedicarboxylate
DCPA	Dimethyl 2, 3,5, Gtetrachloro-l,4,-Benzenedicarboxylate
DHAP	Dihydroxyacetone phosphate
DMAPP	1, 1-dimethylallyl pyrophosphate
DRC	Dry rubber content
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
GA	Gibberellic acid
GA3P	D-glyceraldehyde-3-phosphate
GAP	Glyceraldehyde 3-phosphate
GO	Gene Ontology
GS	Glutamine synthetase
HCl	Hydrochloric acid
HIV/AIDS	Human Immunodeficiency Virus / Acquired Immune Deficiency Syndrome

HSP70	Heat shock protein 70
IBA	Indole-3-butyric acid
IDP	Incorporated isopentenyl diphosphate
IPG	Immobilized pH gradient
IPP	Isopentenyl pyrophosphate
KCl	Potassium chloride
KDa	Kilo Dalton
KOH	Potassium hydroxide
MALDI TOF	Matrix assisted laser desorption ionization-time of flight
MDH	Malate dehydrogenase
MPE	Methylerythritol 4-phosphate
MS	Murashige and Skoog
MVA	Mevalonate
MW	Molecular weight
Na-CMC	Sodium carboxymethyl cellulose
NCBI	National center for biotechnology information
NR	Natural rubber
NRL	Natural rubber latex
OEE1	Oxygen evolving enhancer 1
OSPs	Oxidative stress proteins
OSU1	Ohio State University 1
PAGE	Polyacrylamide gel electrophoresis
PCBER	Phenylcoumaran benzylic ether reductase
pI	Isoelectric point

REF	Rubber elongation factor
RSA	Republic of South Africa
RT	Room temperature
RuT	Rubber transferase
SAMS	S-adenosyl L. methionine synthase
SDS	Sodium dodecyl sulphate
SRPP	Small rubber particle protein
TIM	Triosephosphate isomerase
TPT	*trans*-prenyltransferase
TSC	Total solid content
UKZN	University of KwaZulu-Natal
USA	United States of America
USDA	United States Department of Agriculture
UWC	University of the Western Cape

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Chapter 1: Literature review

1.1 Introduction

Natural rubber (NR: *cis*-1, 4-polyisoprene) is a renewable bio-material used in large quantities by commercial, medical, transportation and defence industries. It displays many advantages over synthetic rubber, including higher elasticity, flexibility, resilience, abrasion resistance and heat dispersion. Because of these properties, the use of NR, especially in the manufacturing of heavy-duty truck and airplane tires, cannot be replaced (Ohya and Koyama, 2001). Most of the world's NR is produced from *Hevea brasiliensis*, the Para rubber tree, native to the Amazonian forest, but currently cultivated predominately in Southeast Asia (Beilen, 2006). There are more than 2500 other plants known to produce NR, but *Parthenium argentatum* (guayule) and *Taraxacum kok-saghyz* (Kazak or Russian dandelion, or Buckeye Gold) are known to produce rubber of a quality comparable to that of Hevea (Van Beilin and Poirier, 2007). *Helianthus annuus*, *Chrysanthemum nauseous*, *Cryptostegia_grandiflora*, *Solidago sp*., *Lactuca sp., Ficus sp.,* and *Euphorbia sp.* have also received some research attention (Venkatachalam *et al*., 2013; Cornish *et al*., 2007). The comparison of the important NR producing plant is shown in Table 1.1.

Table 1.1: Characteristics of important natural rubber producing plants

Plant	*Hevea bransiliensis Muell.Arg*	*Taraxacum Kok-Saghyz*	*Ficus carica*	*Helianthus annuus*	*Lactuca sativa*	*Parthenium argentatum*
Common name	Hevea	Russian dandelion	Fig tree	Sunflower	Lettuce	Guayule
Description	Tropical tree grown for industrial latex production	Perennial herb of the Aster family	Deciduous tree / large shrub	An annual plant of large proportions with few branches and a thick stem	A leafy annual / perennial herb	A drought tolerant, woody flowering plant
Family	Euphorbiaceae	Asteraceae	Moraceae	Asteraceae	Asteraceae	Asteraceae
Origin	South America	Uzbekistan and Kazakhstan	Southwest Asia and the Mediterranean	North America	Turkey and Middle East	Chihuahua desert (Mexico)
Height (m)	+/- 30	+/- 0.4	+/- 10	+/- 3.7	+/- 0.3	+/- 1.4
Source of latex	Stem	Roots	Fruit, leaves, stem	Leaves	Stem	Stem
Latex collection	Tapping	Mechanical extraction	Tapping	Mechanical extraction	Tapping	Mechanical extraction
Allergic response	Yes	No	Yes	No	Yes	No
Rubber/hectare	2000 Lg/ha	200 Kg/ha	N/A	896 Kg/ha	N/A	1500 Kg/ha
Picture						

Natural rubber production is increasing each year and to meet increasing demand (consumption) due to population growth increased production is needed. The production vs. consumption in the last six years is shown in Table 1.2.

Table 1.2: The world's production and consumption of natural rubber

Year	Production ('000 tonnes)	Consumption ('000 tonnes)
2009	9,723	9,289
2010	10,393	10,764
2011	11,216	10,997
2012	11,616	11,048
2013	12,217	11,386
2014	11,809	11,855

Source: International Rubber Study Group (RSG)

Natural rubber consumption exceeded production in the year 2010 and 2014, raising a question of where is the rubber going to come from to ensure enough supply in the future. Southeast Asia

is the leading NR producing region, accounting for 92% of the world's NR, followed by Africa (6%) and Latin America (2%) (Hayashi, 2009). The world's seven major NR producing countries are Thailand, Indonesia, Malaysia, India, Vietnam, China and Sri-Lanka. The Republic of South Africa (RSA) is one of many countries that are 100% dependent on NR imports. According to the Department of Agriculture's Strategic Plan (2010/11 – 2014/15), NR commands an annual import value of approximately R1.33 billion, making it the country's fourth largest agricultural import (Department of Agriculture, 2010). It is vital for RSA to have their own NR production in order to substitute rubber imports at least to some degree and with a goal to safeguard supplies for neighbouring African countries.

Challenges facing Hevea are diseases, such as the fatal pathogenic fungal disease caused by *Microcyclus ulei* (South American Leaf Blight), and for which there is currently no treatment. Other diseases are rapidly spreading, with *Rigidoporus microporus* (a white root rot), as a major concern (Beilen, 2006). In addition, although Hevea produces rubber of good quality and strength, life threatening latex allergies in many individuals have been reported (Potter *et al.*, 2001). Hevea rubber latex contains proteins which can induce Type I IgE-mediated allergic reactions that can lead to fatal anaphylaxis, with health workers at most risk. Studies have shown that South African hospital based staff and patients have developed allergies to NR (Potter, 2013). Amongst the populations which suffer from latex allergies, health workers are at major risk and approximately 8-20 % of allergic reactions have occurred in South African hospitals. Some data on the prevalence of NR allergy in RSA is indicated in Table 1.3.

Table 1.3: Natural rubber allergy prevalence in South African hospitals: Studies have shown that approximately 8-20 % allergic reactions have occurred in South African hospitals or laboratories.

Hospital / Laboratory	Reference	Prevalence (%)
NHLS	Ratshikhopha *et al*., 2015	20
Mankweng	Risenga *et al*., 2013	8.3
Groote Schuur	Potter *et al*., 2001	9
Red Cross	Brathwaite *et al*., 2001	5
Tygerberg	De Beer *et al*., 1999	20.8

*NHLS – National Health Laboratory Services

Table 1.3 confirms that natural rubber latex (NRL) allergy is a serious issue in South African hospitals. These allergy reactions may occur due to particulates derived from rub-off and wear of tear. The presence of NRL allergens increase the risk and may trigger asthma symptoms in patients sensitive to asthma. Based on these findings research has focused on finding alternative NR sources to cater for the increased need and those people who suffer from NRL allergy. Synthetic rubber is not an option as the chemical accelerators and other additives commonly used in the production of nitrile and non-latex gloves can also cause Type IV allergy. Patients often present with a chronic dermatitis on their wrists and the back of their hands (de Silva, 2014). Due to the increase in epidemic diseases such as HIV/AIDS and hepatitis, there is an increase in the global demand for NRL for the production of gloves and condoms. In addition, the movement away from NR agriculture to other prioritized agriculture (mainly food and energy) in

developing countries has increased the pressure on existing NR production and thus the development of an alternative source of NR is crucial. This review summarizes guayule's agronomy, factors influencing rubber synthesis, and latex extraction and processing methods, with the ultimate goal to argue for guayule rubber research, development and commercial production in the RSA.

1.2 Guayule plant

Guayule is a semi-desert shrub native to the Chihuahuan desert of the Trans-Pecos Regions of Southwestern Texas and Northern Mexico belonging to the Asteraceae family. Guayule plant height is highly dependent upon water quantity and can reach approximately 2 m and it may live as long as 40 years in its native habitat (George *et al*., 2005). This plant has been grown around the world including regions with rainfall as low as 230 mm per annum and in extreme temperatures ranging from as low as -18 to as high as 49.5°C (Bekaardt, 2002). Guayule produces high molecular weight rubber (Sundar *et al*., 2008) in commercially-viable yields. In addition, guayule is a low input perennial plant not used as a food source, and it can grow in diverse environmental and climatic conditions (Venkatachalam *et al*., 2013). These characteristics make guayule a very promising alternative source for the production of NR in arid and semi-arid areas and furthermore support the establishment and future expansion of guayule commercialization in RSA. It is usually grown for its hypoallergenic latex in the production of rubber products but it can also be useful in the production of resin, wood pellets and biofuel (Figure 1.1).

Figure 1.1: The products that can be obtained from guayule plant: Guayule can be grown for the production of hypoallergenic rubber products, resin, wood pellets and biofuel.

1.2.1 Discovery of guayule rubber

The presence of rubber in the guayule plant was first discovered by the North American Indians who used it for the production of bouncing balls. Their method of extraction involved chewing the stem/bark for several minutes and spitting out the coagulated rubber. Guayule rubber was scientifically noted in 1852 by Dr. J.M. Bigelouw of Harvard University, a member of the Mexican Boundary Survey party, and Professor Asa Gray, named the species *Parthenium argentatum* Gray. In 1876, guayule started to receive public attention as a rubber producing plant. The first commercial rubber extraction process on guayule was invented in the early 1900's by a New York belting and packaging company. This invention involved immersion of the ground/milled plant material in hot water and the rubber produced was determined to be of good quality (Salvucci *et al.*, 2010).

William Lawrence invented a mechanical method for rubber extraction in 1904 through projects sponsored by the United States Department of Agriculture (USDA). The Continental-Mexican Rubber Company, founded in 1906, used this mechanical extraction method, and ran several factories in Mexico and Texas. In 1909, Mexico exported approximately 9,500 tons of guayule rubber to the United States. Guayule rubber produced during this operation was mostly used in the tire industry. With time, the factories closed down due to limited wild guayule plant raw material and the Mexican Revolution. Research on improving rubber extraction from guayule continued, for example, by Dr McCullum at the USDA (Hammond and Polhamus, 1965).

The Emergency Rubber project of World War II led to a massive effort to produce rubber from guayule and other species. Areas suitable for guayule cultivation were identified in Texas, Arizona, and California where 32,000 acres were grown. The effort was discontinued after WWII when NR supplies from tropical Hevea trees were restored. Despite extensive cultivation and intensive research, guayule rubber was not commercially produced anywhere in the world after 1962 because it could not compete with Hevea (Hammond and Polhamus, 1965). Guayule research and development efforts resurged in response to the oil embargo which caused a massive increase in rubber prices, and in 1976 the National Science Foundation and Department of Defence provided funding to the USDA for the development of guayule's agronomy, breeding efforts and rubber evaluation (Steward and Lucas, 1986). However, once the embargo was over and prices reverted to normal levels, guayule rubber was still uncompetitive and this effort was abandoned.

During the 1980s, epidemic diseases such as HIV/AIDS and hepatitis emerged and an increased need for latex rubber arose, and billions of latex gloves had to be manufactured rapidly to supply the increased demand. New, inexperienced manufacturers did not include the in-line leaching process which had been standard in the industry, soluble proteins were entrained in the finished gloves at extremely high levels, and thousands of health workers and patients developed severe IgE-mediated allergic reactions towards Hevea NR products, including fatal anaphylactic reactions (Steward and Lucas, 1986). This led to a need for an alternative source of NR without allergy causing compounds. Synthetic gloves were introduced but did not match the performance of NRL gloves.

In 1991, Dr Katrina Cornish and colleagues at the USDA discovered that guayule latex does not contain allergy-inducing proteins and they initiated focused efforts to commercialize guayule allergy-safe latex for medical products. In 1996, a simple and fast aqueous latex extraction method was patented. In 1997, a license was awarded to Yulex Corporation for the extraction of guayule latex, and it was the first company to commercialize guayule as a hypoallergenic latex-producing crop in Arizona, USA (Steward and Lucas, 1986).

1.2.2 Guayule agronomy

Guayule can be grown successfully in arid and semi-arid areas and such environments are found in many continents including Africa, America, Europe, Asia and Australia (Beilen, 2006). Therefore guayule can be grown for hypoallergenic NRL production in many countries. The origin of guayule and successful trials around the world are shown in Figure 1.2.

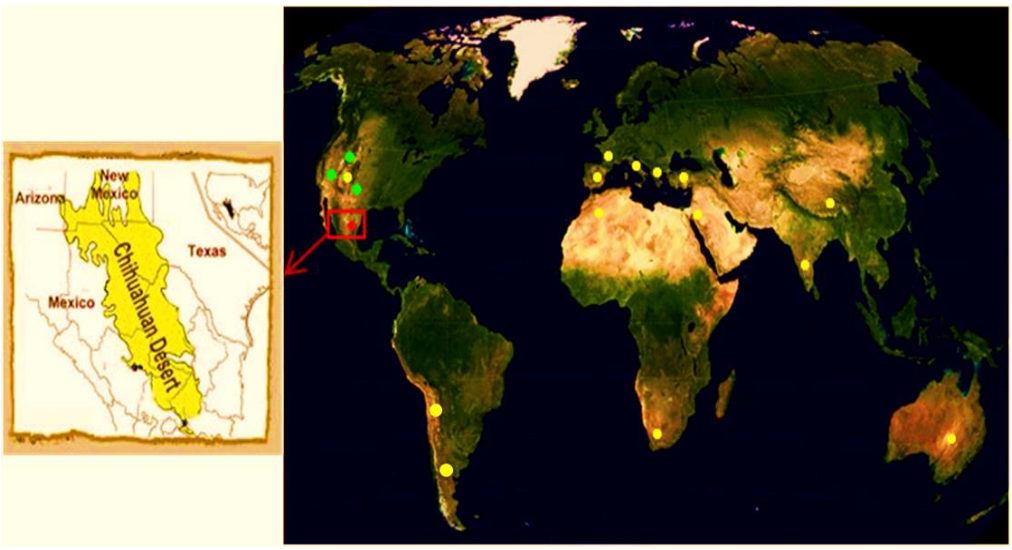


Figure 1.2: Guayule distribution map: Guayule plants are native in the Chihuahuan desert of Mexico and Texas (•). Successful field trials (•) have also been demonstrated in South Africa, Australia, Spain, Argentina, United States, Italy, France, Israel, Greece, Morocco, Chile, Turkey, China and India. Three companies including PanAridus, Yulex Corporation and Bridgestone are currently producing guayule natural rubber in the United States of America (•).

Guayule trials have been conducted in many countries including Argentina (Coates *et al*., 2001), Australia (Dissanayake *et al*., 2004), Israel (Mills *et al*., 1989), USA (Ray *et al*., 2005) and RSA (Bekaardt, 2002). Additional trials were conducted in Arizona using guayule lines AZ4, AZR2 and 11591, and these specifically investigated environmental, irrigation and fertilization parameters (Bekaardt *et al*., 2010). The RSA climate differs within each Province (Blignaut *et al*., 2009) thus trials were performed across the country in the Western Cape, Northern Cape, Eastern Cape and Limpopo with good success. The first guayule seeds entered RSA during World War II in 1939 and efforts began to establish guayule fields through research at the South African Council for Scientific and Industrial Research (CSIR) and Stellenbosch University (Milthorpe *et al.*, 1991). Other research was also conducted by Van Staden (Macrae *et al.*,1986) to determine the conditions for maximizing rubber producing potential in guayule. Guayule plants were grown in pots in an outside environment in this study, and it was shown that higher amounts of rubber were produced / synthesized during the winter season in the bark and root

tissue with little amounts in the leaves (0.5%). Guayule lines AZ101, AZ3, N565 and 11591 grown in different RSA regions including Elsenburg, Oudtshoorn, Graaff Reinet, Upington, Glen and showed a great potential for the production of latex (Bekaardt, 2002). The trial showed very promising results for guayule growth and establishment in RSA (Figure 1.3).

Figure 1.3: Mature guayule plants grown in RSA (Bekaardt *et al*., 2005): The image in the right was captured in 2012 at the Western Cape, after the plants were abandoned for over 5 years without artificial irrigation.

It is evident from Figure 1.3 that guayule plants grows and mature well within the RSA environment. From these trials plants survived for over 5 years without any artificial irrigation and continued to produce quality seeds. It therefore indicates the plant's ability to survive without artificial irrigation. Information on studies conducted in RSA will contributed to understanding on the agronomics of guayule within the RSA environment (Bekaardt *et al*., 2005; Milthorpe *et al*., 1991). Although guayule grows very well in a range of environmental and climatic conditions, root disease still remains the major limiting factor, especially in water logged fields (Macrae *et al*., 1986). Overall, sandy-loamy soil is most suitable for guayule cultivation. Rubber yields vary among guayule lines in different regions, soil types, planting

season and climatic conditions (George *et al*., 2005). Table 1.3 shows the latex yield obtained from trials conducted in the USA and Australia.

Table 1.4: The latex yield (Kg/ha/yr) obtained from different countries

Guayule line	United States of America						Australia		
	Arizona		Fort Stockton, Texas		Califonia		Gatton		Chinchila
	24 months	36 months	12 months	24 months	21 months	45 months	17 months	32 months	33 months
AZ101	-	-	-	-	-	-	-	-	-
AZ1	576.2	1310.8	-	-	-	-	611	789	816
AZ2	1235	1245.4	-	-	-	-	567	771	909
AZ3	611.1	1426	-	-	-	-	478	622	680
AZ4	788.9	1120.4	-	-	-	-	-	-	705
AZ5	675.8	1156.7	-	-	-	-	565	966	644
AZ6	808.2	1313.4	-	-	-	-	503	855	731
AZ-R1	-	-	293	436	-	-	-	-	-
AZ-R2	-	-	414	763	-	-	-	-	-
CAL-6	-	-	366	566	-	-	-	-	-
CAL-7	-	-	318	582	-	-	-	-	-
UC-101	-	-	409	550	-	-	-	-	-
UC-104	-	-	415	759	-	-	-	-	-
N565	492	845.2	-	-	400	348	379	675	480
11591	354.8	573.4	-	-	-	-	383	618	481
C250	-	-	-	-	908	828	-	-	-
C254	-	-	-	-	680	796	-	-	-
C215	-	-	-	-	682	701	-	-	-
593	-	-	-	-	325	362	-	-	-

Table 1.4 presents only a few guayule lines depending on the trials shown. From these trials, it is clear that yield depends on the guayule line, and on the environmental and climatic conditions. Suitable high rubber producing guayule lines have been identified to suit the respective climate

and soils in different countries. The improved AZ1-AZ6 lines produce higher rubber and latex yield than the older lines (N565 and 11591) in the USA (Ray *et al*., 1999) and Australian (George *et al*., 2005) trials. Rubber yield was observed to increase with time amongst all the lines. The data obtained for the South African trial is shown in comparison to Argentina (Table 1.5).

Table 1.5: The latex yields (%) obtained from different guayule lines in South Africa (12 months) and Argentina (24 months)

Guayule line	South Africa (12 months)			Argentina (24 months)
	Elsenberg	Oudtshoom	Graaff-Reinet	Catamarca
AZ2	1.4	0.9	0.8	-
AZ3	0.7	0.9	0.8	3.5
N565	0.8	0.8	0.8	4.4
11591	-	1.5	1.7	4.7

The differences observed can be attributed to the one year gap between the trials because guayule latex increases with time. The latex yield can be affected by so many factors including planting and harvesting season, climate and environmental conditions, watering, fertilisation, soil type and the guayule line. Different guayule lines performed differently in different regions of RSA and the AZ2 line produced more rubber in the Elsenberg trials, whilst 11591 was the best cultivar for the Oudtshoom and Graff-Reinet regions. Other South African regions also have the same soil and climate conditions as guayule's native habitat indicating potential for guayule field establishment (Milthorpe *et al*., 1991) across the country.

1.2.3 Guayule seeds

Good quality seed is very important for a successful plant establishment both in the field, green house or starting up in tissue culture. Guayule is a good seed producer; seeds are continually being produced throughout the growing season. The seed is an achene with attached bracts and a pair of sterile florets (Chandra and Bucks, 1986). Guayule seeds can be obtained from 2 year old plants, stored in the husks of the guayule flowers. Seeds are very small and approximately 1000-1500 can be obtained per gram. The average guayule seed has a length of 2.5 mm length and a width of 1.8 mm (Bucks *et al*., 1986). Threshing is involved in the process of separating the seeds from the husks. The most important factors to consider when handling guayule seeds are the quality, quantity and the cost of the seed. The seed colour may play a role in germination, as observed in some studies that show that some guayule varieties produce seeds with low embryo quality and thus contribute to low or no germination (Jorge *et al.*, 2006). Seeds obtained from guayule have different colours ranging from black to dark black and brown to yellowish colour (Figure 1.4).

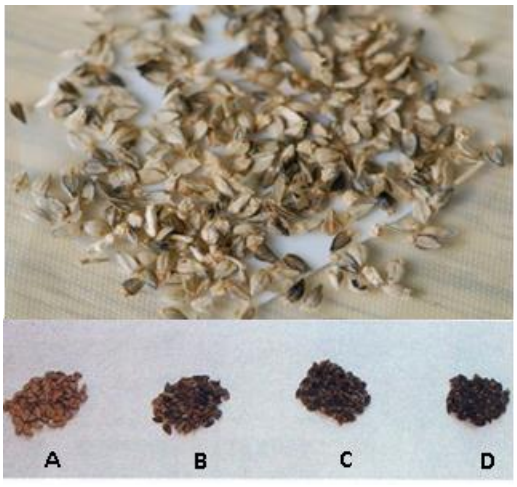
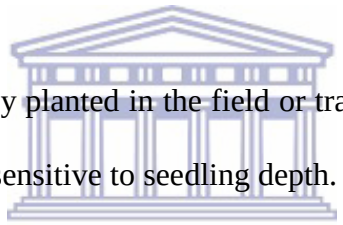


Figure 1.4: Guayule seeds (Gibson, 2013): Guayule seeds colour grades: **a**-yellow; **b**-gray; **c**-bright black; **d**-Dark black. As the colour intensifies from A to D, the percentage of empty seeds decreases and the rate of germination increases. The yellow seeds were found to be less viable as compared to gray and black seeds (Jorge, 2005).

Guayule seed is found enclosed in a hard pericarp, which is one of the major concerns when growing guayule since it slows germination rate. The delayed germination of guayule seeds may be due to natural or primary dormancy caused by embryo dormancy and inner seed coat dormancy. Freshly harvested seeds enter the natural dormancy stage of the embryo and seed coat which last up to two months (Jorge *et al*., 2007; Hammond and Polhamus, 1965). Germination rates also depend on the weight and the variety type (Jorge *et al*., 2007). The weight of the seeds depends on whether the seed is filled, partially filled with an embryo or empty. Guayule seeds can germinate beginning from 3–5 days after planting, followed by emergence within 10 days (Miyamoto and Bucks, 1985). Guayule seed dormancy and/or germination may also be influenced by climatic or environmental factors. Studies on seed quality effects based on environmental conditions, irrigation and fertilization of the guayule plant and the use of X-ray imaging, demonstrated that low germination rate of guayule is not only caused by the seed embryo and coat dormancy but may also be due to empty seeds (Bekaardt *et al*., 2010; Dissanayake *et al*., 2010; Jorge, 2005).

Pre-treatment methods for the seeds before planting are required to break dormancy with an advantage of fast and uniform germination. Different studies have been done to investigate the best pre-treatment method to overcome guayule seed dormancy. These methods include prior soaking the seeds in water for 12 hours to 7 days (McCallum, 1926; Jorge *et al*., 2006), and washing the seeds in sodium hypochlorite, calcium hypochlorite, abscisic acid, potassium nitrate and aqueous smoke solution (Bekaardt *et al*., 2004). Light and gibberellic acid (GA) has been observed to strongly interact and break dormancy in guayule (Chandra and Bucks, 1986; Dissanayake *et al*., 2010). A combination of yellow light with GA treatment yielded

approximately 82% of seed germination, overcoming both the guayule seed coat and the embryo dormancy. Different light colours ranging from yellow, red, green and blue have an effect on the germination rate of different seed cultivars (Smith, 2000). Guayule seed treatment with GA and smoke water solutions initiated faster and better germination compared to untreated seeds, which also depend on different lines (Bekaardt *et al*., 2004). The method that is currently applied and recommended to increase guayule seed germination include treatment with medium consisting of 25% polyethylene glycol (MW 8000), 0.1 mM GA, 0.05% potassium nitrate and 0.1% thiram (tetramethylthiuram disulfide) fungicide adjusted to pH 8.0 with calcium hydroxide. The seeds are treated under aerobic conditions in the light for 3-4 days (Foster and Coffelt, 2005).



Guayule seeds can either be directly planted in the field or transplanted using greenhouse grown seedlings. Guayule seeds are very sensitive to seedling depth. It is therefore crucial that seeds are not covered by more than 6 mm in soil because that reduces the number of seedlings produced (Hammond and Polhamus, 1965). Guayule stand establishment can be achieved by germinating seeds in the greenhouse and transplanting them in the field (Foster and Coffelt, 2005), however this method is costly. Direct seeding is more economical because the labour cost involved in raising the seedlings in the green house/nursery is omitted (Foster and Moore, 1992), however this method is much less reliable due to the small seed size and challenges faced with poor germination (Jorge *et al*., 2007).

Seed collection is one of the factors, which must be taken into account to obtain good plant establishment. Seed collection used to be done by means of using collecting pans put under the plant (Coates, 1986). A seed harvester was developed which showed promising results (Bedane

et al., 2008). During the harvest period, it was noted that only the mature seeds were harvested and they contained very little of the plant leaves reducing the energy required for seed cleaning. The seed harvester gives advantage and potential for guayule plant commercialization (Bedane *et al*., 2008). Harvested guayule seeds contain leaves, twigs, floral attachments and other plant parts. It is of importance to separate the viable seeds from these other plant parts and unfilled seeds (Coates, 1991). Guayule seed separation and processing is one of the most difficult and expensive operations. The belt threshing method for seed cleaning was observed to be effective in producing seeds with approximately 98% purity and 76% germination (Bedane *et al*., 2010).

1.2.4 Field / land preparation

Land preparation too is very important for guayule cultivation; it begins with the selection of the suitable sites, in terms of soil type and climatic or environmental conditions. The required soil type for guayule is sandy-loamy soil with a pH range of 6.0 to 8.0. Transplanted seedlings require well-drained soil, which will allow root penetration during initial establishment. Planting depth is a very important factor to consider when planting guayule because of its size. Planting depth influences the germination rate of guayule seeds and the rate at which seedlings emergence. Planting depth below 10 mm has been found to improve seed germination but with variation depending on the environmental or climatic conditions, soil moisture, water and seed quality (Dissanayake *et al*., 2008). These findings concur with those found by Hammond and Polhamus, 1965, showing that the depth of guayule seed in the soil can influence germination.

1.2.5 Soil pH / salinity and water quality

Soil salt content is an important factor for optimum growth, since guayule plants are slightly tolerant to soil salinity. Plants grow well in soil with pH from 6.0 to 8.5 with optimum growth occurring at pH 7.2 to 8.3. Soil salinity has been observed to increase rubber production but reduce the plant's total growth. However, very high soil salinity with concentrations above pH 8.5 is reported to decrease the rubber content as well as growth and biomass yield (Wadleigh *et al*., 1946). Salinity has been observed to hinder growth of young guayule seedlings; however transplants are much more tolerant of salinity than emerging seedlings (Miyamoto *et al*., 1985). Guayule plants can tolerate up to 0.3% salt in the soil, however a salt content higher than 0.3% greatly hinders growth or causes death (Siddiqui and Locktov, 1981).

Water supply is also a crucial factor in guayule establishment and production. Guayule has been reported to be salt sensitive during development and seedling stages (Miyamoto *et al*., 1990). Seedling mortality has been experienced due to rain showers despite the plant's tolerance to salt due to accumulation of soluble salts on the leaves. Increased salinity affects guayule production in many ways including reducing dry matter and plant growth, which contributes to low rubber and resin production (Foster and Coffelt, 2005).

1.2.6 Fertilization

Soil fertilization is not considered crucial in guayule plant management. Studies done in RSA indicated that soil fertilization does not improve seed viability (Bekaardt *et al*., 2010), but increase plant height and width (Cannell and Younger, 1983). Guayule plants respond more to N fertilizers than any other combination. For this reason when guayule is grown in soils with higher N levels, fertilizer input is not necessary (Foster and Coffelt, 2005). Nitrogen fertilization can be

applied (but is not limited to) as nitrate, urea and also ammonium sulphate (Dissanayake *et al*., 2007). Fertilizer application does not influence rubber accumulation and yield directly, however the significant effect is observed in plant height thus more rubber can be obtained because of the high plant biomass.

1.2.7 Weed control

Guayule competes very poorly against weed thus it is crucial for guayule establishers to find economic weed control. Weed limits guayule's maximum rubber and biomass yields (Foster *et al*., 1999). Weed competition is reduced for the fast guayule growing lines resulting in with high biomass yield rather than rubber yield (Coffelt *et al*., 2009). Manual weed control has been proven to be effective although labour intensive. There are some weed control chemicals, which can be applied to the field prior to planting guayule plants. Prodiamine (0.3-0.6 kg ha^{-1}) and dimethyl 2,3,5,Gtetrachloro-l,4,-Benzenedicarboxylate (DCPA 4.5-11 kg ha^{-1}) have been proven to be effective in guayule stands weed control (Foster *et al*., 1993) with Pendimethalin (0.6-22 kg ha^{-1}) being safe for weed control in transplanted plants (Foster and Coffelt, 2005).

1.2.8 Infections and diseases

Guayule plants produce significant quantities of resin useful as wood preservatives and protect the plant against infections or diseases. However, depending on the soil, environmental and climatic conditions, fungal diseases such as *Pythium dissortocum* root rot and *Sclerotinia sclerotiorum* might be observed, especially in warmer climates. *Rhizoctonia bataticola* and *Fusarium moniliform* may also affect the plant (Nakayama, 1992). Guayule plants were observed to contain natural products (argentine and partheniol), which have an anti-feeding effect against

termites. These natural products are useful in plant protection and preservation (Gutie´rrez *et al*., 1999). Since guayule is a desert plant, it requires less water for its development. Therefore, it is of importance to maintain the desirable soil moisture to avoid guayule plant infections or diseases, especially infections of the roots.

1.3 Natural rubber biosynthesis and proteins involved

Rubber biosynthesis in plants is an interesting biochemical system, which occurs in the lactiferous vessels (ducts) or parenchymal (single) cells of rubber-producing plants (Chotigeat *et al*., 2010). Rubber biosynthesis is catalysed by the rubber transferase enzyme, and requires two substrates and a divalent cation cofactor, to produces a high-molecular-weight isoprenoid polymer called natural rubber (Kang *et al*., 2000). The rubber biosynthesis process is influenced by high expression of genes such as those expressing important enzymes and proteins involved in the pathway(s) leading to rubber synthesis (Chow *et al*., 2012). Natural rubber biosynthesis is highly regulated by the induction of plant growth and its ability to regulate cellular metabolism and cell function. Through identification of proteins involved in rubber synthesis, their expression levels and factors that modulate them can also be identified. Genetic engineering can be employed to promote higher expression of genes encoding for these proteins, which will influence higher rubber production (Cornish and Siler, 1996). Despite a huge interest in finding alternative sources of rubber producing plants, the biochemistry and molecular mechanism of rubber biosynthesis, has not been fully studied (Gronover *et al*., 2011). Therefore understanding of the rubber biosynthesis mechanism will bring insight into how rubber yield can be increased.

Natural rubber is produced in the form of a *cis*-1, 4-polyisoprene polymer (Figure 1.5) from aggregation of isopentenyl pyrophosphate (IPP) units (Sundar and Reddy, 2008) to form an

elongated carbon-carbon double bonded structure with isoprene backbone (Saetung *et al*., 2011). It is therefore a polymer with a high molecular weight of incorporated isopentenyl diphosphate (IDP) units to form long carbon chains. The IDP units are switched from *trans*-configuration to highly stable *cis*-configuration by rubber transferase enzyme (Chow *et al*., 2007). There are about 320 to 35000 isoprene molecules making up natural rubber (Kang *et al*., 2000). The natural rubber composition is 94% *cis*-1, 4-polyisoprene, 6% protein and some fatty acids (Rahman *et al*., 2013).

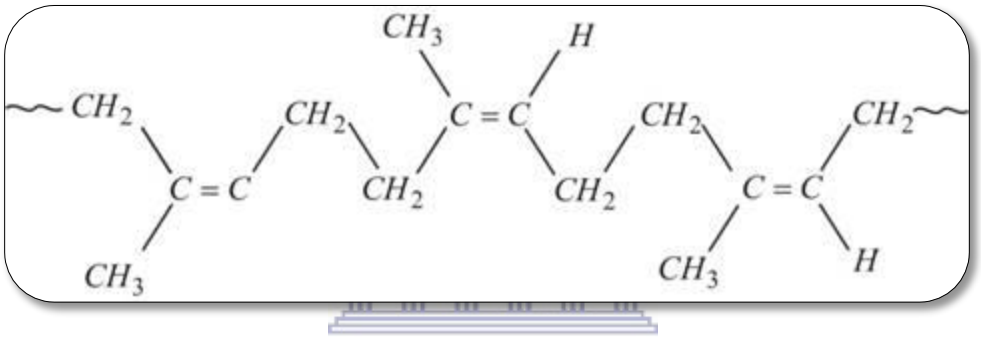


Figure 1.5: The *cis*-configuration chemical structure of natural rubber: The structure of the monomers is between methylene and is highly stable, displaying toughness and properties of a confined organization. The link between groups of one unit with those of nearby units and the bonds are not part of backbone structure (Gronover *et al*., 2011)

The rubber synthesis pathway has been shown to be similar in most rubber producing plants; however the yield of rubber is different across species (Cornish and Siler, 1996). The IPP is produced via two biosynthetic pathways, the cytosolic MVA pathway, which uses pyruvate as the substrate and the plastidic MPE pathway using pyruvate and D-glyceraldehyde-3-phosphate (GA3P) as substrates. IPP is converted into a rubber molecule and the reaction catalysed by rubber transferase enzyme (Chow *et al*., 2007).

During the biosynthesis, there are three steps involved, namely initiation, elongation and termination (Figure 1.6) (Cornish and Siler, 1996). During the initiation step the IPP is converted

into 1, 1-dimethylallyl pyrophosphate (DMAPP) by IPP-isomerase, to act as a substrate for the *trans*-prenyltransferase (TPT) to form Allylic Pyrophosphates (APPs). Studies done with *H. brasiliensis*, *F. elastica* and *P. argentatum*, indicated that initiation of rubber biosynthesis is most effective with farnesyl diphosphate (FPP) (Xie *et al*., 2008).

In the elongation step, the rubber tranferase requires divalent ions, Mg^{2+} or Mn^{2+} and catalyses the *cis*-1, 4 polymerization of isoprene units from IPP. During termination, rubber transferase enzyme is believed to release the rubber particle chain (Collins-Silva *et al*., 2012, Cornish and Siler, 1996). Studies have shown that rubber transferase is similar to *cis*-prenyltransferases, since it catalyses the synthesis of a *cis* molecule. Prenyltransferases are grouped into the *trans*-prenyltransferases and the *cis*-prenyltransferases, based on their amino acid sequence, protein fold and the reaction they catalyse. The formation of the *trans*-allylic pyrophosphate in the initiation step is catalysed by *trans*-prenyltransferases.

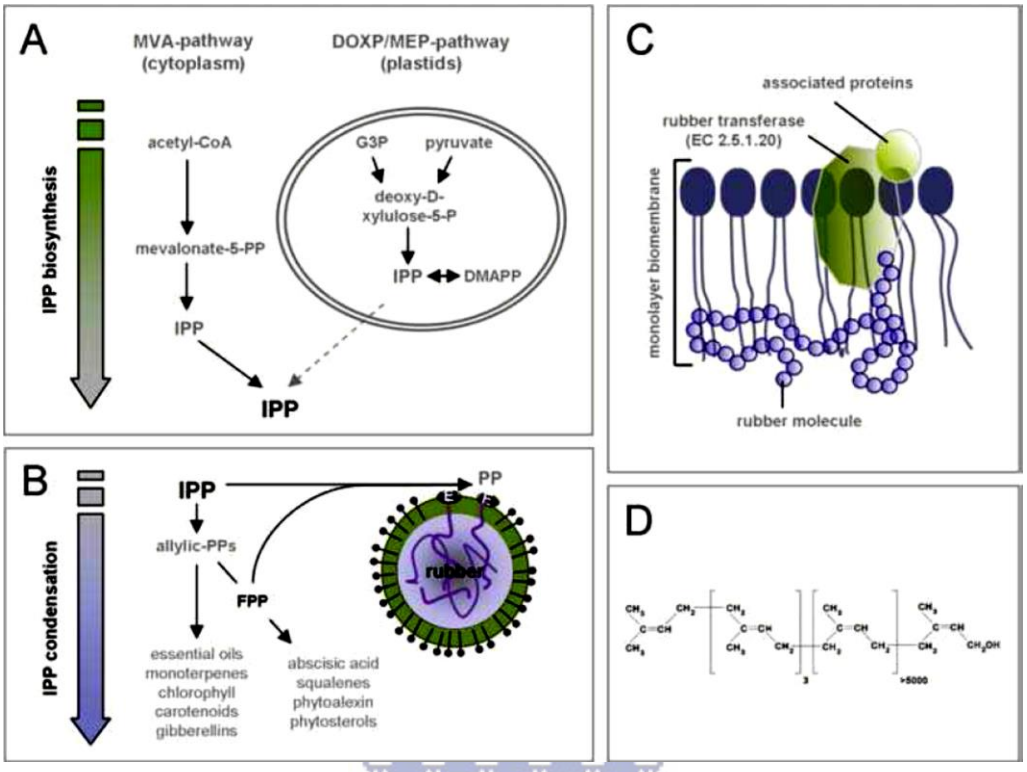


Figure 1.6: The natural rubber biosynthesis pathway: a The initiation step whereby the substrate subunit IPP is synthesised by the MVA and MEP pathway in the cytosol and plastid respectively. **b** Elongation step whereby IPP is used to synthesise isoprenoids including allylic diphosphate as side chains of natural rubber. **c** Synthesis of natural rubber is catalysed by rubber transferase enzyme on the membrane of the rubber particle. **d** The chemical structure of natural rubber showing IDP units condensing, thus forming a *cis*-configuration structure (Gronover *et al*., 2011).

In guayule, a group of proteins which include the Allen Oxide Synthase (AOS) (Pan *et al*., 1995; Backhaus *et al*., 1991), the *cis*-prenyltransferase (CPT) which is assumed to be a component of the rubber transferase (Cornish, 2001; Cornish and Backhaus *et al*., 2003) and the small rubber particle protein (SRPP, also known as guayule homolog: GHS) (Kim *et al*., 2004) have been identified so far and are believed to be associated with rubber biosynthesis.

The possible role of GHS in rubber biosynthesis was investigated using the recombinant protein *in vitro*. Based on properties such as sequence (although GHS is 37 amino acids longer than SRPP) and the catalytic nature of GHS as compared to that of the Hevea SRPP, it was suggested that GHS might have a role in rubber biosynthesis (Kim *et al.,* 2004). In Hevea, the SRPP and the Rubber elongation factor (REF) are key proteins involved in rubber biosynthesis (Gronover

et al., 2011); however the REF in guayule has not yet been identified. In guayule the roles of these three proposed guayule rubber biosynthesis proteins *in vivo* still remain elusive (Ponciano *et al*., 2012). Further studies on guayule's molecular properties will provide information and identities of the rubber biosynthesis proteins including the most abundant 50 KDa protein seen in the leaves and stem bark of guayule (Sundar and Reddy, 2008; Sundar and Reddy, 2001).

In guayule, rubber biosynthesis and deposition occurs in the cytoplasm and later accumulate in the vacuole where they are stored (Goss *et al*., 1984). The mechanism for the rubber particle transport into the vacuole is not well understood. The rubber particles first appear in the cytosol of a two and half month old guayule shrub and later accumulate in the central vacuole of three months old plants (Benedict *et al*., 2009). Most of the rubber particles are found in vacuoles of epithelial cells. Rubber particles are suggested to bud from the endoplasmic reticulum (ER) membrane along with cytochrome p450 proteins. After impounding in the vacuole, the membrane of the rubber particle is digested, leaving naked rubber particles accumulating into large masses (Benedict *et al*., 2009).

The large volume of the cytosol and the effective functioning of the plant's cellular organelles promote a high rate of rubber biosynthesis result in a large numbers of mitochondria to sustain these processes for rubber synthesis in guayule (Benedict *et al*., 2011). Rubber particles are also deposited in the pith, vascular rays, epithelial cells that are facing the resin ducts and the cortical parenchyma. Approximately 60-80 % of all rubber particles accumulate in the cortical parenchyma cells of guayule plants. Guayule harvested in RSA winter season was found to have high rubber content in epithelial cells covering the resin ducts (Benedict *et al*., 2013).

During guayule rubber biosynthesis, the structure and size of the initiator molecules can affect the condensation process and reaction rate of IPP during polymerization. Not only the excess amount of APP concentration, but also the carbon chain length of another initiator molecule FPP must be longer approximately ^{15}carbons (C_{15}) to promote rubber synthesis in guayule. This will result in a high rate of IPP incorporation into the guayule rubber transferase to enhance rubber production (Cornish and Scott, 2004).

1.4 Factors influencing rubber biosynthesis in guayule

Rubber formation in guayule occurs in cycles within the plant leaves, stems and roots. Rubber accumulation is influenced by various factors such as low temperatures, light intensity, moisture and water stress, the age of the tissue, nutrient availability, type of cultivars and the season. Rubber does not accumulate during the summer months but synthesis begins in the fall and winter as the plant experiences low night temperatures (Goss *et al*., 1984).

1.4.1 Temperature

Low temperature is a key factor influencing rubber biosynthesis in guayule (Angulo-Sánchez *et al*., 2002) and in regulating the expression of genes coding for enzymes involved in rubber biosynthesis (Ponciano *et al.,* 2012). The effects of low temperature treatment on guayule rubber biosynthesis showed that guayule plants treated with 60 cycles of very low temperatures (15/10°C) resulted in increased rubber transferase activity compared to untreated plants (Sundar and Reddy, 2008). Rubber particles with high transferase activity are known to contain abundant rubber particle protein (Cornish and Backhaus, 1990), the same protein with a molecular mass of 50 kDa was found accumulating at higher concentrations in the stem bark of cold treated guayule plants (Sundar and Reddy, 2008). It was therefore concluded that low night temperatures

positively influence rubber accumulation and increased rubber transferase activity (Benedict *et al*., 2010).

A study done on gene expression analysis of cold-acclimated guayule tissues, showed an increase in the levels of the rubber particle protein, AOS but not of the other proteins associated with rubber particles (CPT and SRPP). Based on this report it was concluded that gene expression does not control the activity of the transferase complex, instead post-translational modification might be a regulating factor (Ponciano *et al*., 2012). The fact still remains that proteins of the rubber biosynthesis complex in guayule need to be identified and characterized.

1.4.2 Light intensity

The effect of light intensity and low night temperatures on rubber production in guayule plants was investigated (Sundar & Reddy, 2001). The results showed that guayule plants grown under a radiation of 900 $\mu Em^{-2}s^{-1}$ and a night temperature of 15°C had a positive effect on guayule plants rubber synthesis implying high rubber transferase activity. A combination of an optimum low night temperature and light intensity throughout the year and not restricted to winter months enhanced rubber production in guayule plants. These conditions were observed to be critical for maximum rubber accumulation in guayule (Sundar and Reddy, 2001).

Mitchell *et al* (1944) investigated the effect of light intensity and nutrient supply on germination of the seeds, growth and hence rubber production by guayule. The observations were that light intensity effect also depends on the nutrients supply to the plants. Guayule plants with added nutrients and supply of direct light (100% light intensity) accumulated a higher percentage of rubber compared to those in low light intensity (≤ 75%) and without nutrient supply. When high level of nutrients was supplied, guayule plants were very sensitive to the reduction of light

intensity with respect to rubber accumulation. With reduced light intensity, the amount of rubber produced was reduced by 36%. A linear relationship was observed between the amount of rubber accumulated per guayule plant and the intensity of light to which the plants were exposed. An increase in light intensity resulted in an increase in rubber accumulation. Through this study, it was determined that light intensity together with nutrient supply is a limiting factor in rubber production for guayule plants (Mitchell *et al*., 1944).

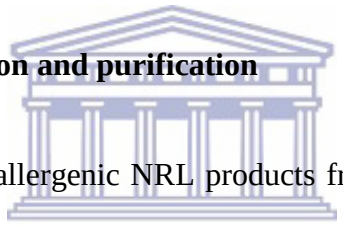
Macrae *et al* (1986) investigated guayule rubber production by determination its rubber producing potential. During this study, it was observed that acetate acts as a precursor for rubber biosynthesis in guayule. The acetate was incorporated into rubber however, the level of incorporation depended on the part of the plant (root, stem or leaves). Incorporation of acetate into rubber was highly influenced by light intensity and also low night temperatures. Based on the different studies mentioned above, it is clear that light intensity influence rubber accumulation in guayule plants.

1.4.3 Moisture and water stress

Moisture is also one of the important factors in guayule's growth and rubber accumulation. Guayule requires permeable well-drained, well-aerated soils that have a reasonably good supply of soil moisture most of the year. Rubber yield increases more rapidly during high moisture stress than during low moisture stress. The amount of rubber within a plant is further increased after each successive period of high stress compared to plants subjected to continuous low stress (Benedict *et al*., 1947). Based on studies conducted using 11591 guayule lines, it was indicated that the stem is the major tissue that accumulates rubber followed by the roots, with very small amounts found in the leaves. The influence of water stress on rubber accumulation was indicated by the high amount of rubber yield in water-stressed compared to low rubber yield in well-

watered guayule plants. In this report it was concluded that maintenance of low water regimes would be good for enhanced rubber synthesis. The season in which the guayule plants are cultivated and the tissue type has an effect on the amount of rubber produced. During summer, there was no significant difference in the rubber concentrations in the leaves between the water-stressed and well-watered plants but a very high concentration of rubber was observed in the stems of the water-stressed plants compared to the well-watered plants. It was observed that plants, which were grown/cultivated in winter, had low rubber accumulation compared to the ones grown/cultivated in summer. In addition, a combination of low water stress on plants cultivated in summer resulted in an enhanced rubber accumulation (Veatch-Blohm *et al*., 2006).

1.5 Latex extraction, quantification and purification



Commercial development of hypoallergenic NRL products from guayule for the manufacturing of medical devices requires knowledge and understanding of the extraction, quantification and purification methods and several methods have been used effectively.

1.5.1 Latex extraction

Extraction of latex from guayule parenchyma cells is a relatively simple and fast process. Fresh latex extracted from guayule shrubs can be concentrated up to 60% solids. Unlike Hevea rubber tree tapping, latex extraction from a guayule shrub requires mechanical tissue disruption to release the rubber particles from the parenchyma cells. The rubber particles are then separated from the homogenate, purified and concentrated (Cornish *et al*., 1999). At a laboratory scale, guayule latex is extracted from freshly harvested shrub with a blender. Larger-scale homogenizers or mills are needed for commercial-scale extraction. The latex content in

harvested guayule branches of different sizes is stable for at least 2 weeks when sealed in plastic and stored at 4^{o}C to prevent dehydration and spoilage. The quality of extracted latex also depends on the post-harvest storage. Processing methods depend on the branch and sample size and have been developed for branch sizes of <0.5 cm, 0.5-1 cm and >1 cm, whereas sample sizes of <1.6 kg and >1.6 kg (Cornish and Brichta, 2002). The blender extraction method uses ice-cold aqueous ammonium hydroxide (pH 10) or potassium hydroxide (pH 11) with sodium sulphite. The grinding of harvested guayule material for latex extraction must be done twice and the filtrates will be pooled together. Grinding time is one of the important factors to take into account because it affects the latex yield (Cornish *et al*., 2005). Guayule rubber (in solid not latex form) can also be extracted from dried guayule stems and roots using hexane, benzene, petroleum ether or cyclohexane (Salvucci *et al*., 2010). However, the most reliable, affordable and suitable method for extracting latex from guayule parenchyma cells is based on homogenization (Cornish and Brichta, 2002) and this method is most suitable for rural areas.

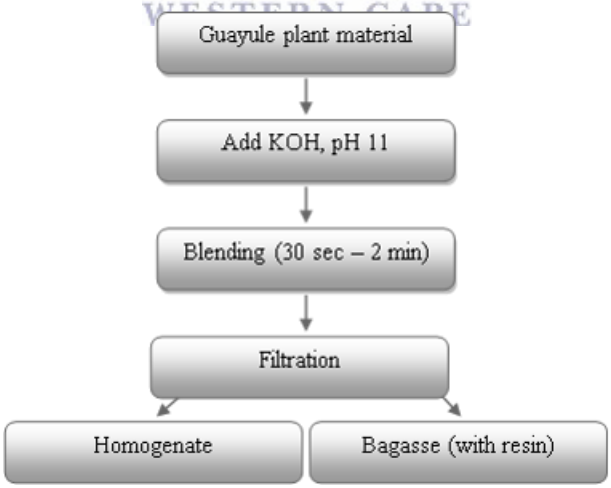


Figure 1.7: Guayule latex extraction using the blender method (Cornish *et al*., 1999): The Oster blender method uses pH11 ammonium / potassium hydroxide or sodium sulphite.

Guayule rubber can also be extracted from dried guayule tissue (Figure 1.8) using acetone, hexane, benzene, petroleum ether or cyclohexane whereby dissolved rubber is detected using evaporative light scattering (Salvucci *et al*., 2010). However, the use of dry plant material can cause a loss in the amount of extractable latex and the latex cannot be used for products manufacturing thus this method is normally used for yield quantification purposes.

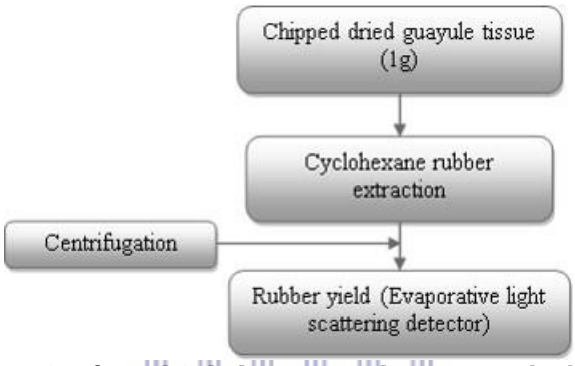


Figure 1.8: Guayule latex extraction from dried plant material: Latex can also be extracted from dried guayule tissue using acetone, hexane, benzene, petroleum ether or cyclohexane whereby dissolved rubber is detected using evaporative light scattering.

1.5.2 Latex quantification

Latex quantification measures the amount of latex in the living plant and is expressed on a dry weight basis. Latex quantification is best performed in triplicate and the yield is determined as an average. Quantification methods include 1 ml and 14 ml methods (Cornish *et al*., 2005) and latex coagulation is done using methanol or glacial acetic acid (Salvucci *et al*., 2009). The coagulated dry guayule rubber obtained is oven dried and weighed to identify the yield.

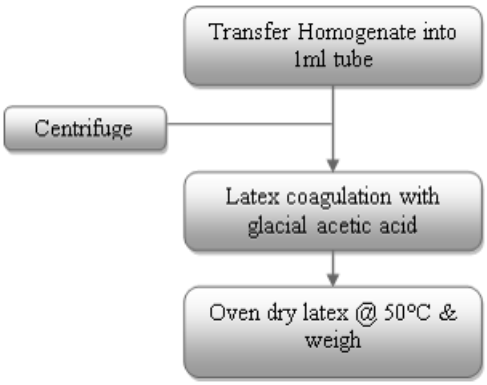


Figure 1.9: Guayule latex quantification: Latex quantification is best performed in triplicates and the yield is determined by the average. Either the 1 ml or 14 ml quantification method can be used.

Concerning the extraction process, any blender can be used but, for each variation, the time of blending at a specific blade rotation rate required to extract all the latex from the parenchyma cells must be determined. For example, the Oster blender requires 4x the residence time of the 1 litre Waring blender. Faster blade rotation requires shorter homogenization time than a slower rotation and over-blending can cause a decline in apparent latex yield (Cornish and Brichta, 2002). The addition of antifoam to prevent foaming during homogenization is usually an asset.

1.5.3 Latex concentration and purification

Latex concentration can occur slowly under natural gravity. In the USA guayule latex is separated from the homogenate and purified using a series of high speed centrifugations, which make the separations much faster. However, latex separation can also be achieved through the addition of creaming agents, allowing a much faster separation than natural gravity, but with a much lower cost process than centrifugation. Different creaming agents include casein, sodium carboxymethyl cellulose, polyvinyl alcohol, sodium alginate, methylcellulose, carboxymethyl cellulose, butadiene–styrene–methyl [methacrylate copolymer], ammonium alginate, ammonium oleate, aluminum cellulose glycolate, locust bean gum and tamarind seed powder.

Through the addition of a creaming agent of choice into the crude latex, two phases will be obtained; the upper creamy layer containing 50% rubber and the bottom layer which contains little or no rubber (Ochigbo *et al*., 2011). The concentrated latex can then be re-suspended in fresh creaming agent and re-concentrated to wash the latex and reduce the total protein content. The final concentrated latex is the raw material used for the manufacture of products such as gloves, condoms, foams, balloons, catheters, baby soothers, dental dams and latex thread.

The initial latex concentration and pH maintenance are important factors in the concentration of guayule latex homogenate (Cornish *et al*., 2005). The pH should be maintained at least 10 to achieve maximum latex concentration and the initial concentration obtained during quantification should be above 4 mg/ml, and under these conditions the latex is stable at room temperature of 16 weeks in stored homogenate. Initial latex concentrations of less than 4 mg/ml and unregulated pH cause rapid loss of latex in stored homogenates. Refrigeration has also been observed to maintain latex yield and maintenance of pH by the addition of ammonium hydroxide also inhibits microbial growth and enzyme activity.

Latex creaming is a cheaper concentration method than centrifugation and requires less energy input. The latex produced is environmentally friendly, has a higher viscosity and gives greater filterability than centrifuged latex. Moreover, creaming does not require heavy machinery. It is for these reasons that latex creaming will be the method of choice for latex purification in small-scale rubber manufacturing plants in rural RSA communities. Finally guayule latex extraction

and purification using a creaming agent is a very simple process that can be achieved at a relatively low cost.

1.6 Hypothesis

- Guayule field establishment can be achieved through direct seeding and micropropagation.
- Proteome profiling and identification is possible using proteomics tools.
- Latex purification is dependent on temperature, time and creaming agent concentration.

1.7 Objectives

The main aim of the study was to investigate the agronomic performance (survival, height, canopy and stem diameter) of guayule lines AZ1, AZ2, AZ3, AZ4, AZ5, AZ6, OSU1 and 11591 in the Eastern and Western Cape regions in RSA with an ultimate goal of leading to commercial production.

The objectives of the study:

- To evaluate plant survival, height, canopy and stem diameter for AZ1, AZ2, AZ3, AZ4, AZ5, AZ6 and OSU1 guayule lines in the Eastern and Western Cape regions of South Africa.
- To establish a micropropagation protocol for OSU1 guayule line.
- To extract, quantify and purify guayule latex.
- To optimise guayule latex creaming under South African conditions.
- To perform proteome analysis of guayule bark and leaves.

Chapter 2: Investigating the field performance of guayule lines in South Africa

Abstract

The very first guayule seeds entered RSA during World War II in 1939. The Republic of South Africa is one of many countries which depend 100% on NR imports. Several research institutions including the Council of Scientific & Industrial Research (CSIR), University of KwaZulu-Natal (UKZN) and the Agricultural Research Council (ARC) conducted research on guayule's potential as the RSA's first NR plant. Significant progress has been made on collecting data on field establishment and plant development; and the regions of RSA where the plant thrives well have been identified as well. Previous investigations for guayule establishment in RSA were done using only four lines (AZ101; AZ3; N565 and 11591). Over the years new germplasms with attractive traits in size (biomass) and latex yield have been developed including AZ1; AZ2; AZ4; AZ5; AZ6 and OSU1 lines. To investigate the agronomic performance of these lines, trials were done at the Western Cape (Stellenbosch) and the Eastern Cape (East London) ARC experimental farms. In addition to the new germplasms, 11591 and AZ3 were also grown in the glasshouse to serve as positive controls. After 6 months plants were transplanted into the experimental fields and drip irrigation was applied together with Kynitro-19 fertilizer on a weekly basis. Due to excessive rainfall during winter, weed pressure was a limiting factor but weeds were controlled by the application of preeglone herbicide. Plants were monitored for growth and development after 2 years in the field and data on the phenotypic characteristics, plant height, stem and canopy diameter was recorded. Different lines showed diversity in leaf

structure from narrow-long to broader-short leaves. When grown in the WC, AZ1–AZ3 lines were the tallest with an average of 0.90 m, followed by 11591 and OSU1 lines with an average 0.70 m, and the shortest plants were AZ5 and AZ6 lines at an average of 0.6 m. The canopy diameter showed an average of 1.0 m for AZ1–AZ3 lines, followed by 0.8 m for OSU1 and 11591 lines. The lines with the smallest canopy diameter were AZ5 and AZ6 with an average of 0.50 m. The stem diameter had an average 0.20 m for AZ1-AZ3 and 0.1 m for AZ5, AZ6, 11591, OSU1 lines. In the EC, AZ1 and AZ3 showed an average height of 1.6 m tall, whereas AZ4-AZ6 were the shortest with a height average of 1 m. The AZ1–AZ3 lines had the highest canopy diameter with an average between 1.5–2.0 m, followed by 1.0 m for AZ4-AZ6. The stem diameter averaged at 0.2 m for AZ1 and AZ3, followed by 0.18 m for AZ5 and AZ6 and lastly 0.10 m for AZ2. The OSU1 and OSU1 clonal plants all died in the EC however survived in the WC. These results show that these guayule lines consistently grew larger in the EC than in the WC area, which may be due to many factors including soil type, nutrients and environmental weather factors.

2.1 Introduction

Guayule (*Parthenium argentatum*) is grown for the production of high quality hypoallergenic natural rubber latex (NRL) (Siler and Cornish, 1994), or for natural rubber (George *et al*., 2005). The plant is native to the Chihuahua desert of North America, and many successful trials have been done in the United States of America (USA) (Ray *et al*., 1999; Dierig *et al*., 1989); Australia (George *et al*., 2005); Israel (Mills *et al*., 1989) and RSA (Beekardt *et al*., 2002). It has undergone commercial development in the USA producing good quality medical rubber products with impressive stretchiness and strength (Slutzky *et al*., 2013).

Field establishment can be achieved through direct seeding (Foster and Moore, 1992); transplantation of germinated seedlings (George *et al*., 2005; Coates *et al*., 2001) and micropropagated plants (Veatch *et al*., 2005). Research towards the establishment of guayule as the first commercial natural rubber (NR) source in RSA was initiated in 1979 (Gilliland and Van Staden, 1986) and continued through trial establishment and tissue culture experiments to study the mechanism of rubber synthesis (Beekardt *et al*., 2002; Trautmann *et al*.,1991; Macrae *et al*., 1986).

The RSA environmental conditions are favourable for the growth and development of guayule (Nix 1986). The RSA consists of 70% relatively level land suitable for crop production matched to the soil and climate variables (SAPAS, 2011-2025). It is classified as a "low agricultural potential" country because two thirds of the country receives less rainfall thus not many plants can thrive without irrigation and 66 million hectares are classified as semi- or true desert (ALSA,

2013). Since guayule is a desert plant, the mean annual rainfall of less than 500 mm per annum in RSA is sufficient to support a guayule crop.

The performance of the different guayule lines differ depending on the line selection, environment and climatic factors. After experiments done in Australia Queensland and Gatton, it was observed that in Queensland, AZ1 and AZ2 lines produced the highest yield in biomass whereas in Gatton AZ5 and AZ6 lines outperformed the other lines (George *et al*., 2005). Significant differences in plant development and yield were also observed in the trial done in the RSA in Elsenburg, Graaff-Reinet, Oudtshoorn, Bethulie, Glen Austin and Upington (Bekaardt *et al*., 2005).

Soil type or composition plays an important role in guayule development because the plant is sensitive to root rot disease and thus thrives in sandy-loamy or any well drained soil (Foster *et al*., 1991). In the Western Cape, weed can be a limiting factor in its development, especially during seedling emergence and in winter seasons because of high rainfall, it is thus important to identify herbicides not harmful to the plants. Suitable herbicides include dimethyl 2, 3, 5, 6-tetrachloro–l, 4,-benzenedicarboxylate (DCPA), Pendimethalin and prodiamine (Foster *et al*., 1993). In an effort to commercialize guayule, improved lines with high yielding and fast growing ability were developed (Ray *et al*., 1999). The objective of this study was to evaluate the performance of guayule lines AZ1, AZ2, AZ3, AZ4, AZ5, and AZ6, OSU1 and 11591 in the Western and Eastern Cape regions of RSA.

2.2 Materials and methods

2.2.1 Seed import

Guayule seeds were provided by the Ohio State University (OSU) in the USA. The import permit was obtained from the Department of Agriculture, Forestry and Fisheries (DAFF), RSA. The guayule lines used for this study were AZ1–AZ6, OSU1 and 11591; their characteristics are shown in Table 2.1 (George *et al*., 2005).

Table 2.1: The characteristics of guayule lines tested in RSA

Guayule Line	Characteristics
AZ1	This germplasm was selected from PI 478660 and tested as C16-1. It was developed from a single plant and showed increased rubber production after 2 years of growth compared to N565 and 11591. AZ1 is known for high resin content and rubber yield than 11591 after 2 and 3 years of growth.
AZ2 / AZ3	AZ2 and AZ3 lines have been develop from individual plant selections from PI 478640. They were selected for very vigorous growth within the first 2 years producing more plant dry weight after 2 and 3 years of growth and more resin and rubber after 2 years as compared to N565 or 11591.
AZ4 / AZ5	These lines were based on their higher rubber and resin content and yield after 2 and 3 years compared to N565 and 11591.
AZ6	This is a uniform line developed from one of the original selections and it incorporates rubber faster. It also produces high plant content that N565 and 11591 after 3 years.
OSU1	This is the first developed germplasm from Ohio. It was developed as a 3rd generation selection from the AZ great grandparents.
11591	An earlier released line which is also triploid compared to other lines.

2.2.2 Seed germination

Guayule seeds from the different lines (AZ1–AZ6, OSU1 and 11591) were germinated in the glasshouse in sterilized coconut coir-peat-vermiculite mixture (1:1 v/v) in trays of 120 wells. Approximately 5 seeds were placed in each well due to low germination rate. The glasshouse temperature was maintained between 25-30°C and watering was applied with a watering can every 2 days. Four weeks post germination; the seedlings were transplanted into bigger (diameter of 35 cm) pots (Agrimark, RSA). These were kept in the green house for 6 months, receiving irrigation and fertilization using the Kynitro-19 (Kynoch, RSA) fertiliser once per week.

2.2.3 Experimental sites

Guayule trials were conducted in the WC in Stellenbosch and the EC in East London (ARC experimental farms) from 2013 – 2015. In Stellenbosch, the climate is warm and humid with higher rainfall in winter than summer. The average annual rainfall is 802 mm while the temperature typically varies from 6°C to 34°C and is rarely below 4°C or above 35°C. East London receives approximately 593 mm of rain per year, with most rainfall occurring during summer. The highest rainfall is received at the beginning and towards the end of the year while the lowest rainfall is experienced in the winter seasons. It has an average relative humidity climate between 66-82% with warm summers and no dry season and the temperature varies from 10°C to 27°C, rarely below 8°C or above 29°C all year around (Figure 2.1). The environmental and climatic conditions of the selected locations are different and therefore it was expected that the guayule lines would develop differently.

Figure 2.1: Average temperatures in Stellenbosch (a, c) and East London (b, d) areas: The average annual rainfall is 802 mm while the temperature typically varies from 6°C to 34°C and is rarely below 4°C or above 35°C in Stellenbosch. East London receives approximately 593 mm of rain per year, with most rainfall occurring during summer. The highest rainfall is received at the beginning and towards the end of the year while the lowest rainfall is experienced in winter season. It has a mild humid temperate climate with warm summers and no dry season and the temperature varies from 10°C to 27°C, rarely below 8°C or above 29°C (http://www.worldweatheronline.com/East-London-weather-history/Eastern-Cape/ZA.aspx).

2.2.4 Site selection

The trial sites were selected in the WC in Stellenbosch and EC in East London; ARC experimental farm because of their differences in soil and climatic conditions. Prior research done by Bekaardt (2002) showed that both areas were suitable environments for guayule development. The current study was undertaken to investigate the performance of a larger variety

of lines, including a newly developed line OSU1. The trial sites are clearly shown on the map in Figure 2.2.

Figure 2.2: The trial location in the WC (a) and the EC (b) as shown on the South African map: The two experimental sites were selected based on prior research by Bekaardt (2002) showing that both areas were suitable environments for guayule development. This study investigated a wider variety of lines, including a newly developed line, in the environmentally different regions.

2.2.5 Soil analysis

Soil samples were collected in both fields using a soil plunger (man-made from the ARC: Potchefstroom), packaged in paper bags and immediately sent to Bemlab (Pty, Ltd, Strand, South Africa) for analysis. In the WC, soil was collected at a depth of 30, 60 and 90 cm whereas in the EC only 30 cm was collected due to clay the plunger was unable to pass through.

2.2.6 Management of greenhouse plants

Four weeks post germination; plants were transplanted into bigger pots of 35 cm total volume and kept in the greenhouse. Watering and fertilization with Kynitro-19 was applied once per week and the plants were left to grow in these conditions for 6 months while monitoring their developments.

2. 2.7 Transplantation to the field

After 6 months, plants transplanted in EC (East London, Mthiza) and WC (ARC, Nietvoorbij Heidenfontein) ARC experimental farms in RSA on 22 May 2013 and 12 June 2013 respectively. The spacing between the rows was 2 m and between the plants it was 1.5 m. The WC plants were simultaneously watered and fertilized with Kynitro-19 fertilizer once per week using drip irrigation while the EC plants were watered and fertilized manually. The number of plants transplanted per line in each trial depended on the germination rate and the plants available during the transplantation season. The number of plants transplanted in the different fields was according to Table 2. 2.

Table 2.2: The number of plants transplanted per location

Guayule Line	Number of Plants	
	WC	EC
AZ1	17	5
AZ2	16	5
AZ3	92	5
AZ4	4	5
AZ5	104	5
AZ6	104	5
11591	4	-
OSU1	7	-

2.2.8 Statistical analysis

Measurements for plant height, stem diameter and canopy diameter were performed three times, independently. Results are reported as the mean ± standard error (SE) of three independent determinants. For statistical analysis, a two-way analysis of variance (ANOVA) test was used to compare the plants performance for height, canopy and stem diameter in EC and WC (Appendix D).

2.3 Results

2.3.1 Soil analysis

Results of the soil samples from the respective trials are shown in Table 2.3, 2.4 and 2.5. Table 2.3 shows the analysis of the micro and macro soil nutrients. The availability of micro nutrients can determine soil fertility. In this study, soil analysis was measured for Fe, Mn, Zn, Cu and B. The Fe and Mn were found to be low in the WC and higher in the EC, while the content of Zn was recorded between 0.1 and 7.5 mg/kg in WC and EC respectively. Lower Copper content was recorded in the WC and but high in the EC and lower B concentrations were observed in both fields.

Table 2.4 shows the macronutrients which were analysed in this study including N, K, Ca, Mg and P. Overall the macronutrient content was high in both the WC and the EC except for N content. The soil N content was found to be very low (Table 2.1) to medium thus it was enhanced by fertilization with Kynitro-19 (19% N). Nitrogen is recognised as an element which contributes to guayule's developments and yields (Cannell *et al*., 1983). The K amount was found to be between 1.5–4% and Ca was recorded between 22–77% in both fields and Mg

content was 11–32%. The classification of soil in the WC trial was carried out in a bigger field therefore three points (represented as point WC part A, B and C) were selected across the field for soil analysis. The EC field was small because of the number of plants thus only two points were selected (represented as EC point A and B). The results in the WC showed that the soil at point A and B were classified as sandy-loamy with 27% and 40% clay content respectively. At Point C, the soil was classified sandy-loamy with a 29% clay concentration.

In the EC, both point A and B were found to have clay-loamy soil with an average of 36% clay soil. The high clay soil content contributed to a high water holding capacity thus slow water evaporation which can be beneficial during low rainfall seasons but detrimental in high rainfall seasons because guayule plants are susceptible to root rot diseases. The soil pH was between pH 3.7 – 5.4, the pH values decreased with the depth in soil collection and the pH of the topsoil was 5.4 in the WC and 5 in the EC (Table 2.5). The difference of soil pH throughout the field could have affected the growth and development of other lines because throughout the field growth variations were observed amongst the lines, especially in the WC.

The salt concentration was between 0.5– 1.3% and 1-2% in the WC and EC respectively however it was low in the upper soil but high in the subsoil (Table 2.3). Plants established for this trial showed tolerance to high salt concentration in both fields. The tolerance could be because they were transplanted while they were bigger and older as compared to small seedlings.

Table 2.3: Soil structure elements compositional analysis

Field Point	Depth (cm)	Mg/kg								Exchangeable cations (cmol(+)/kg)			
		P Bray II	K	Cu	Zn	Mn	B	Fe	NO_3-N	Na	K	Ca	Mg
WC Point A	30	10	63	0.91	1.1	14.8	0.15	65.3	8.52	0.03	0.16	2.91	0.47
WC Point A	60	9	35	0.47	0.5	4.5	0.1	41.03	3.84	0.04	0.09	1.79	0.6
WC Point A	90	5	25	0.21	0.1	1.2	0.15	24.83	4.00	0.03	0.07	0.81	0.86
WC Point B	30	20	76	1.2	2.2	11.5	0.23	62.66	14.49	0.03	0.2	4.24	0.65
WC Point B	60	12	41	0.59	0.7	4.4	0.14	24.99	6.83	0.05	0.1	3.5	1.04
WC Point B	90	4	34	0.19	0.1	0.8	0.14	17.88	4.97	0.07	0.09	1.25	1.45
WC Point C	30	24	66	1.54	6.5	9.5	0.16	49.06	11.42	0.04	0.17	3.14	0.72
WC Point C	60	21	29	1.54	4.6	7.7	0.19	41.45	5.43	0.05	0.07	2.73	0.92
WC Point C	90	9	46	0.27	0.3	1.1	0.18	12.66	3.04	0.07	0.12	1.5	1.86
EC Point A	30	7	282	7.85	5	491.6	0.72	249.88	8.86	0.24	0.72	13.46	5.18
EC Point B	30	8	192	6.99	7.5	406.7	0.35	224.32	10.49	0.32	0.49	8.46	3.54

Table 2.4: The soil classification compositional analysis

Field Point	Depth (cm)	Soil	pH (KCL)	Stone (%)	Clay (%)	Silt (%)	Fine Sand (%)	Medium Sand (%)	Coarse Sand (%)	Classification	Water holding capacity		
											10 kPa %	100 kPa %	mm/m
WC Point A	30	Sand	5.4	18.0	21.4	12.0	34.0	12.6	20.1	SaKlLm	24.19	15.22	89.7
WC Point A	60	Loam	4.8	13.0	29.4	12.0	28.1	10.5	20.0	Lm	27.02	18.12	89
WC Point A	90	Clay	4.2	9.0	33.4	12.0	26.4	10.6	17.6	KlLm	28.86	19.79	90.7
WC Point B	30	Loam	5.4	26.0	29.4	8.0	29.2	6.0	27.4	SaKlLm	23.15	15.32	78.3
WC Point B	60	Clay	5.0	22.0	43.4	10.0	18.3	7.3	21.0	Kl	26.12	19.08	70.4
WC Point B	90	Clay	3.7	10.0	49.4	18.0	9.2	6.2	17.2	Kl	33.21	25.57	76.4
WC Point C	30	Sand	5.3	22.0	23.4	10.0	30.8	13.0	22.8	SaKlLm	22.42	14.41	80.1
WC Point C	60	Clay	4.5	17.0	25.4	14.0	30.1	13.3	17.2	SaKlLm	25.4	16.67	87.3
WC Point C	90	Clay	3.7	6.0	36.8	18.0	16.8	11.4	17.0	SaKlLm	31.75	23.13	86.2
EC Point A	30	Loam	5.0	16.0	35.4	18.0	31.0	1.6	14.0	KlLm	31.37	21.34	100.4
EC Point B	30	Loam	4.6	21.0	37.4	32.0	24.7	0.7	5.2	KlLm	33.76	23.97	97.9

Table 2.5: The percentage of other elements in the soil

Field Point	Depth (cm)	Na (%)	K (%)	Ca (%)	Mg (%)	T-Value (cmol/kg)	C (%)
WC Point A	30	0.70	4.02	72.49	11.59	4.02	0.67
WC Point A	60	1.30	2.82	56.74	18.88	3.16	0.34
WC Point A	90	1.22	2.45	30.48	32.42	2.66	0.22
WC Point B	30	0.51	3.57	77.60	11.92	5.47	0.88
WC Point B	60	1.06	2.00	67.41	19.92	5.20	0.60
WC Point B	90	1.33	1.54	22.25	25.67	5.63	0.34
WC Point C	30	0.83	3.77	69.56	15.87	4.51	0.73
WC Point C	60	1.04	1.65	61.85	20.95	4.41	0.59
WC Point C	90	1.12	1.92	24.58	30.39	6.12	0.26
EC Point A	30	1.18	3.57	66.65	25.67	20.19	2.01
EC Point B	30	2.23	3.44	59.39	24.84	14.25	1.56

2.3.2 Germination and seedlings in trays

Seeds germination occurred between 7 – 14 days depending on the guayule line and the seed colour. Seeds of deeper black colour were found to germinate quicker with higher germination rates compared to brown-pale coloured seeds. Figure 2.3 shows the colour of seeds for the different lines.

Figure 2.3: Guayule lines used in this project and the colour of the seeds: As the seeds were obtained, it was noticed that the seeds showed different colours from brownish to darker black depending on the line.

Guayule lines AZ4 and OSU1 seeds were pale brown in colour and the germination rate was very poor which can be noticed by the number of plants which were available for transplantation (Table 2.2). The brownish seed was observed to lack an embryo whereas the black seeds were filled with embryo thus significant germination difference between the two seeds. The watering system in the glasshouse was done manually using a hand spraying bottle as shown in Figure 2.4 and this resulted in high germination rate as compared to using the watering can. In large scale production, a very soft spraying is required since guayule seeds are very small and thus are planted 0.5 cm below the soil and guayule seedlings are very fragile and extra care should be taken when applying irrigation. Figure 2.4 shows the hand spraying and the emergence of seedlings.

Figure 2.4: Germination of guayule seeds in the glasshouse: a The process of using coconut peat and vermiculite together with hand spraying was efficient for faster and increased germination rate. **b** seedlings started to emerge after a week.

Guayule seeds showed high germination rate in the vermiculite mixture as compared to soil bark mixture which was used initially. Due to seed dormancy character, germination was observed to be none, partial or complete germination depending on the line. Therefore about 5 seeds were germinated into each cell at a time per guayule line.

2.3.3 Establishment of greenhouse plants

Four week old seedlings were kept in the greenhouse in pots for 6 months before transplanting into the field. Figure 2.5 shows guayule plants of 2 months old developing well in greenhouse conditions. Water and fertilizer applied once per week using a watering can was sufficient for their growth.

Figure 2.5: Two months old different guayule lines growing in the greenhouse: As the plants continued to receive irrigation and fertilization as described in section 2.3.2, they were developing very well without any plant infections.

2.3.4 Plant transplantation in the field

After 6 months in the greenhouse, the different lines were transplanted in the respective fields. The amount of transplanted plants per guayule line depended on the germination rate. Some lines did not germinate very well and thus very low number of plants tested in the trials on the germination rate of the lines. The field trials used a row and plant spacing of 1.5 x 1.0 m and

holes were dug 30 cm deep. The plants were transplanted into the individual holes in such a way that the roots and part of the stem were properly covered with soil (30 cm).

2.3.4.1 Field establishment

On the 12 June 2013, guayule lines listed in Table 2.2 were transplanted in the WC and the EC ARC farms. The transplantation process is illustrated in Figure 2.6 and 2.7 respectively for both fields. Immediately after transplantation the plants were watered using the drip irrigation system. The plants were found to perform significantly differently across the field amongst the lines. Although all the plants were six months old, there were some which were bigger in size however small plants of approximately <20 cm died at a higher rate than the larger plants.

Figure 2.6: Transplanting of plants in Stellenbosch farm: a Six month old plants were transplanted in the field, **b** After the transplantation process all the plant roots and the lower part of the stem were covered in soil and immediately irrigated, **c** The plants surviving in the field after a year.

2.3.4.2 Plants establishment in Eastern Cape

Figure 2.7: The transplanting process in East London farm: a Measurements were done 1m between the plants and 1.5 m between the rows and the holes dug 30 cm deep. **b** The plants were carefully removed from the pots, placed in the holes and covered with soil. **c** The plants maturity and development were followed for 2 years.

2.3.5 Flowering

Guayule lines flowered starting at 6 months and continued to flower throughout the year except in winter. Figure 2.8 shows the flowering cycle of guayule plants from bud initiation to seed development. During the first stage of flowering, green round balls are produced and they turn into beautiful yellow flowers during the second stage. Guayule seeds are produced within these flowers and the final stage shows dry flowers where seeds are embedded in husks. At this stage, seeds can be harvested, cleaned and prepared for germination.

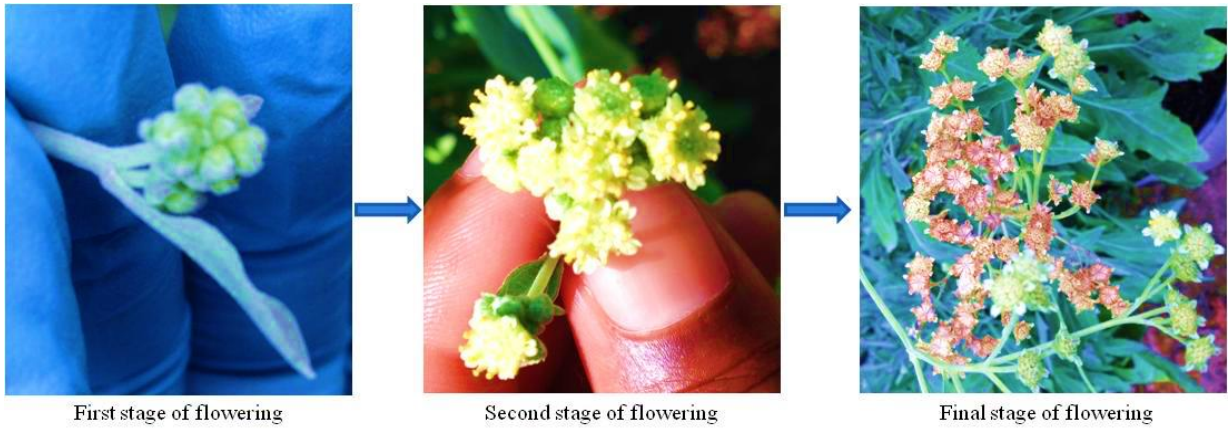


Figure 2.8: The flowering and seed development in guayule plants: Guayule plant flowers throughout the year producing seed except during the winter season. The development of seeds is shown in the different stages and as the flowers dry out the seeds mature and can be harvested and cleaned.

2.3.6 Weed control

Through the use of manual weed control, the field was kept clean for at least a month but during rainy seasons (winter) the weed growth was difficult to control. The use of Preeglone (200 g/L Paraquat) proved to be effective in the treatment of weed in the WC field however the EC field was always kept clean throughout the year manually. The process followed in weed management is shown in Figure 2.9.

Figure 2.9: Weed management in the guayule field: a Weed development in the field. **b** Hand hoes and spades were used as one form of removing weed. **c** Weed was also removed through spraying with Preeglone (active ingredient: Paraquat 200 g/L).

2.3.7 Plant survival

Many factors which contribute to guayule's development include environmental and climatic factors and also the line. Plant survival was 50% and 95% in the WC and the EC trial respectively. The AZ4 line did not survive in WC, while AZ5 and AZ6 had a low survival rate. The high biomass lines in the WC (AZ1, AZ2 and AZ3) thrived and appeared resistant to snails and adverse environmental factors. In the EC, all the lines showed better performance than in the WC. In some cases, branches dried up but new growth developed from the main stem (Figure 2.10). The causes of this response are not yet understood. It is possible that pests or pathogens affected the shoot but not the roots.

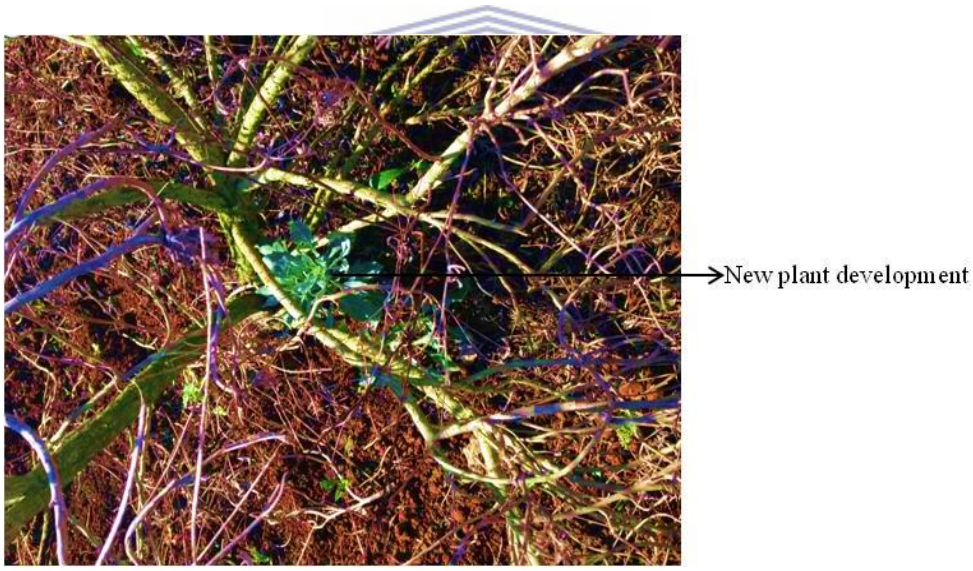


Figure 2.10: The development of a new plant from an older dehydrated shoot: In the EC field, it was observed that some plants started to dry out within 2 years, however a new plant started to develop from the main stem showing regeneration ability.

2.3.8 Leaf structure

The guayule lines showed a difference in the leaf structure, height and overall development throughout the field. The leaf structures for the different lines are shown in Figure 2.11. Lines

AZ1, AZ2 and AZ3 had a broad leaf structure with short petioles while the AZ4, AZ5, AZ6, OSU1 and 11591 leaf structures were narrow with long petioles.

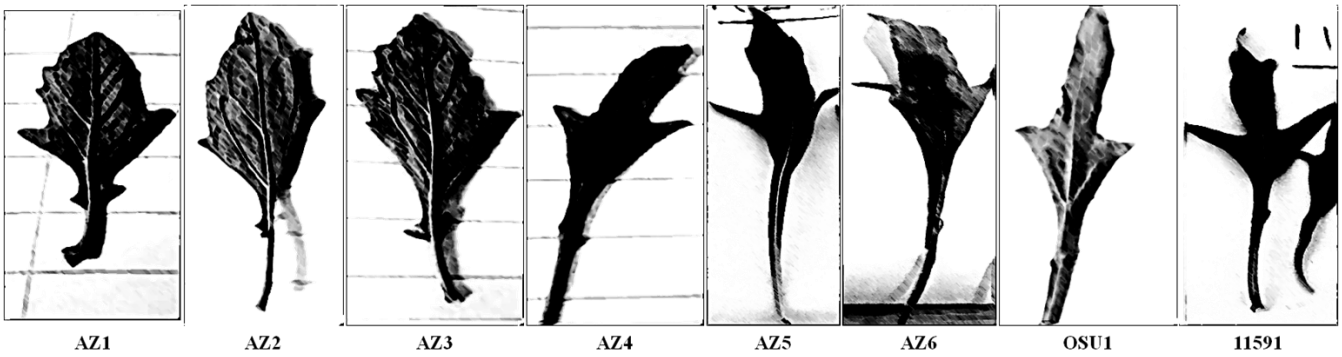


Figure 2.11: The structure of the leaves of guayule lines investigated: The AZ1, AZ2 and AZ3 had broad leaves whereas the AZ4 AZ6, OSU1 and 11591 had narrow leaves.

2.3.8.1 The plant height, canopy width and stem diameter measurements in the Eastern Cape

All the plant measurements in this study were taken using a measuring tape and reported in meters (m) for ease of comparison. Plant height significantly showed differences within the same line and amongst the other lines. The AZ1 and AZ3 lines showed increased average height as compared to the other lines with an average of 1.6 m. Variations were mostly observed within the AZ1 and the AZ2 cultivar where the plants did not grow consistently. The AZ4, AZ5 and AZ6 lines showed more uniformity as compared to AZ1 and AZ2. Uniformity is influenced by seed character and is one of the disadvantage in guayule field plants (Mills *et al*., 1989). Figure 2.12 shows measurements obtained from three randomly selected plants per line.

The plant canopy diameter was measured by circling the measuring tape around individual plants in the middle of the canopy. The AZ1, AZ2 and AZ3 lines had increased canopy diameter ranging between 1.5-2.0 m followed by AZ4 at 1.0-1.5 m. The AZ5 and AZ6 lines had decreased

canopy width average of 1.0 m. Figure 2.13 shows the individual canopy measurements from selected plants.

Stem diameter was measured as the diameter of the main stem just above the soil surface by circling with a measuring tape. The AZ1 and AZ3 lines showed a large stem diameter with an average of 0.2 m followed by the AZ5 and AZ6 lines showing similar stem width of 0.18 m. The small stem diameter average was observed for the AZ2 line with a diameter average of 0.10 m, these results are shown in Figure 2.14. A positive correlation was observed between plant height, canopy diameter and stem diameter, thus the taller plants showed a big stem diameter and a high canopy diameter as compared to the short plants.

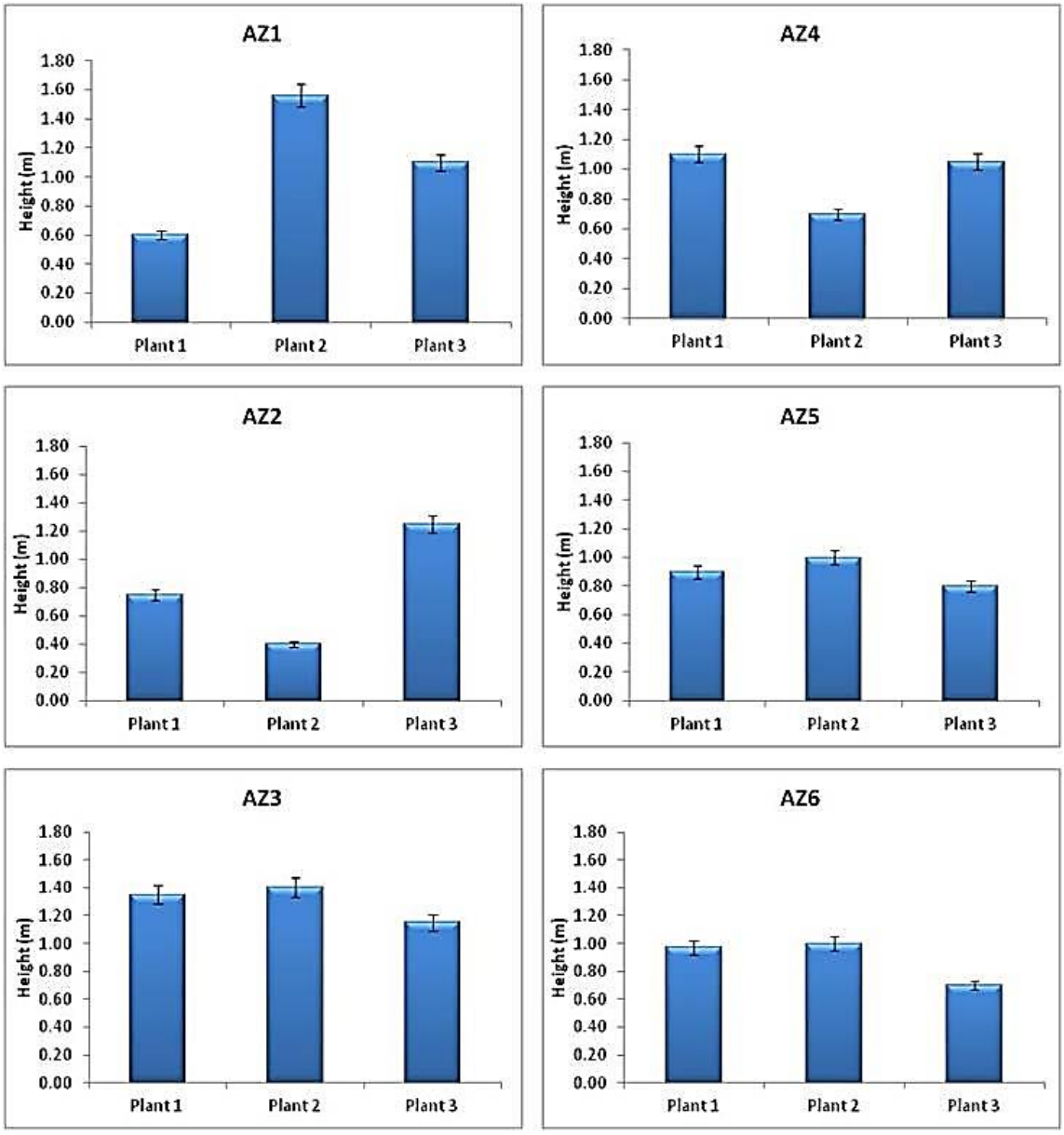


Figure 2.12: The plant height of 2 year old guayule lines at EC experimental farm: The plant height of lines was measured in triplicate and compared with the other lines. There were significant differences amongst the lines and within each line. Plants were less variable within each line than between lines.

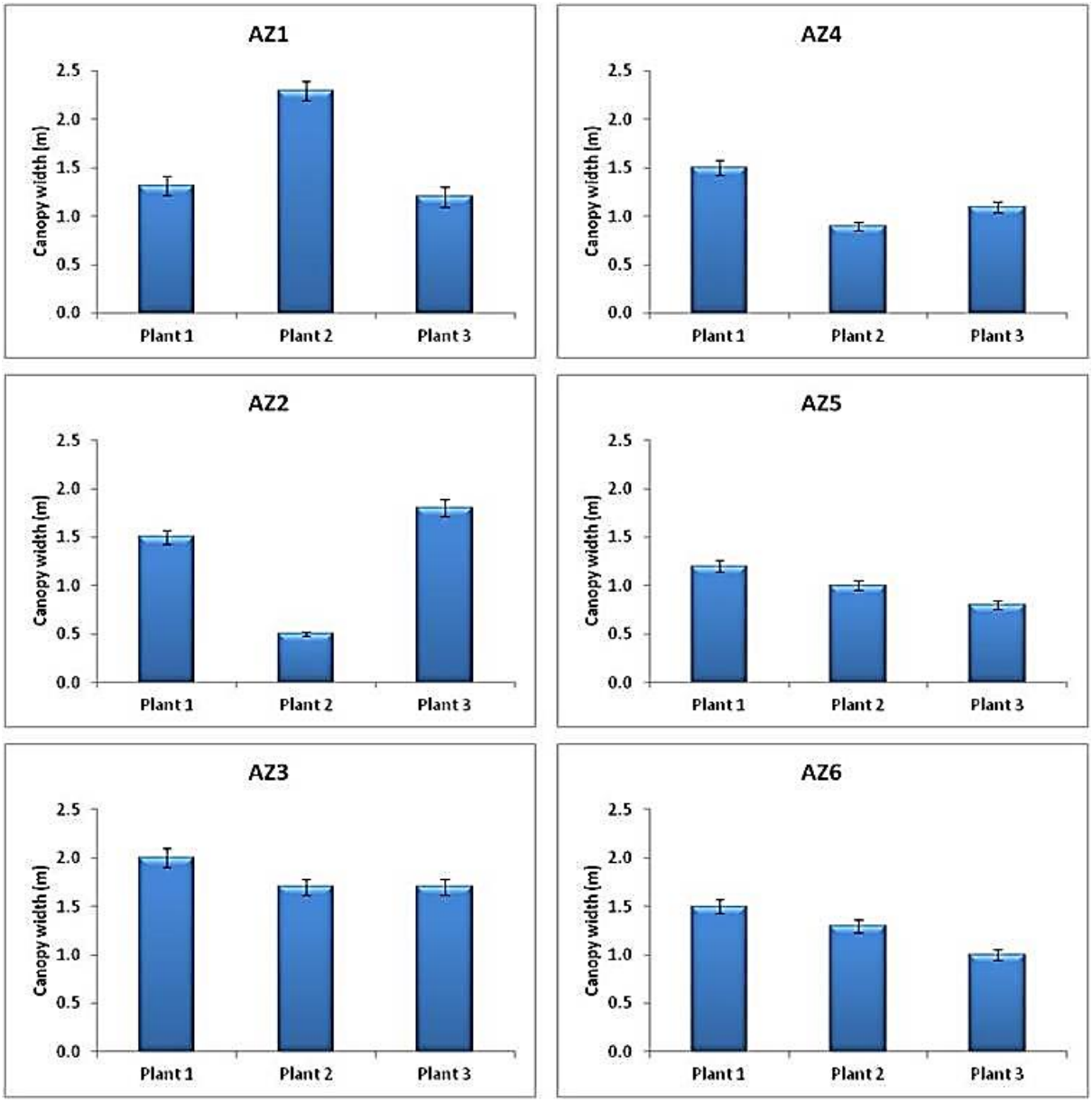


Figure 2.13: The width plant canopy measuremnts of 2 year old guayule lines at EC experimental farm: The plant height of lines was measured in triplicate and compared with the other lines. There were significant differences amongst the lines and within each line specially for the AZ1 and AZ2 line.

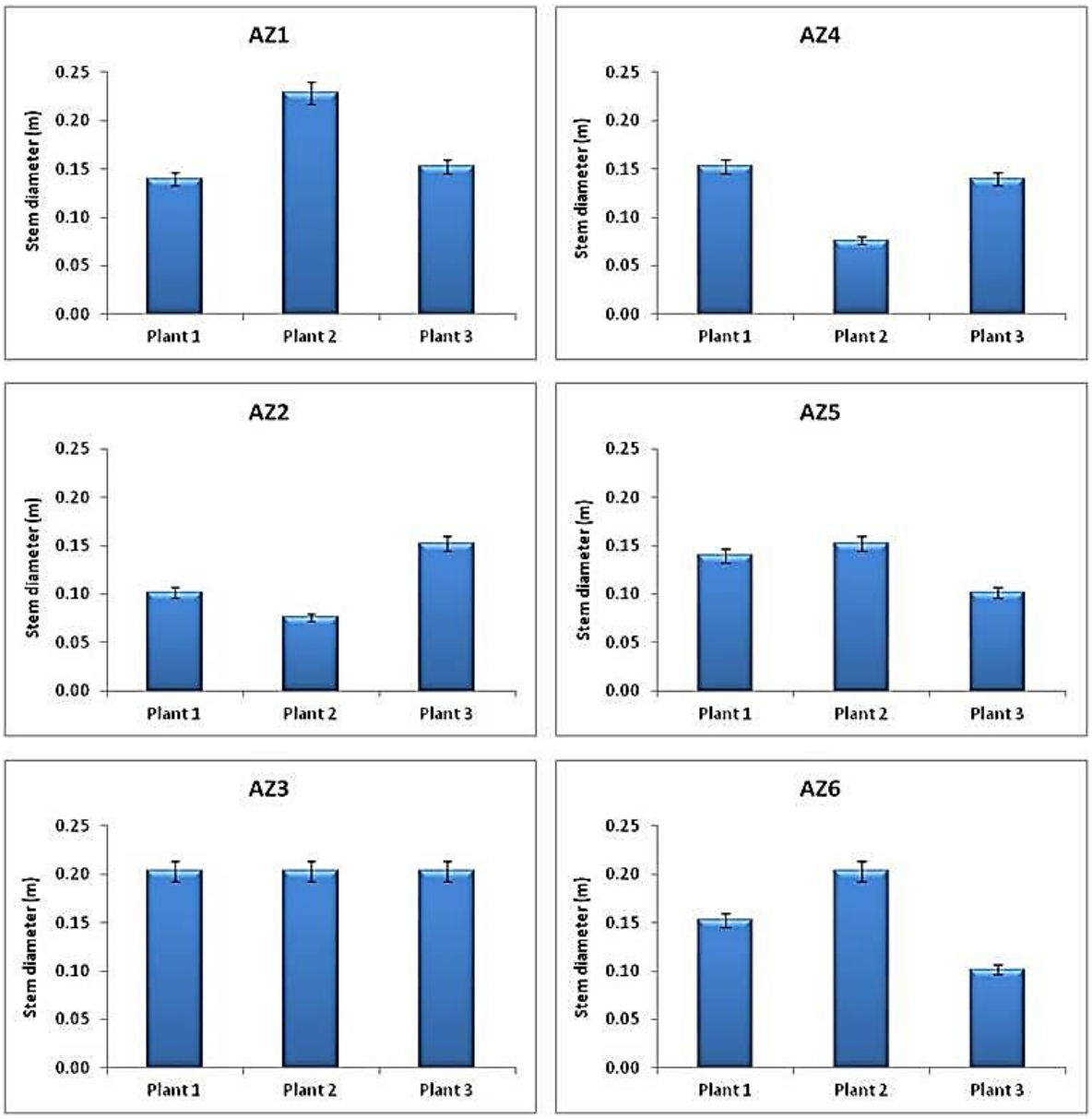


Figure 2.14: The main stem diameter measurements for 2 year old guayule lines: The main stem was measuered also in triplicate and compared amongst the other lines. The AZ3 showed a higher stem diameter but for the other lines were not significnatly different from each other, due to high variation within each line.

2.3.8.2 The plant height, canopy width and stem diameter measurements in the Western Cape

Guayule lines tested in this field were large in number as compared to the EC field. There are some lines which were tested only in the WC due to logistics issues to the EC. Measurements were taken as illustrated in Section 2.2.6.2 however the AZ4 line data is not available in the WC because there was a 100% mortality rate for this line. Observations in the WC were that the AZ1, AZ2 and AZ3 lines were taller with the height average of 0.9 m. The 11591 and OSU1 lines were recorded to have the average height of 0.7 m and the shortest plants were found to be the AZ5 and the AZ6 line. From these observations, it is clear that the EC plants performed better in plant height as compared to the WC plants. The stem diameter was high for those lines which are taller thus the AZ1, AZ2 and AZ3 stem diameter average was reported between 0.15-0.2 m. Other lines AZ5, AZ6, 11591, OSU1 had a stem diameter average of 0.1 m. Figure 2.15; 2.16 and 2.17 shows the individual plants height, stem and canopy diameter measurements in the WC region.

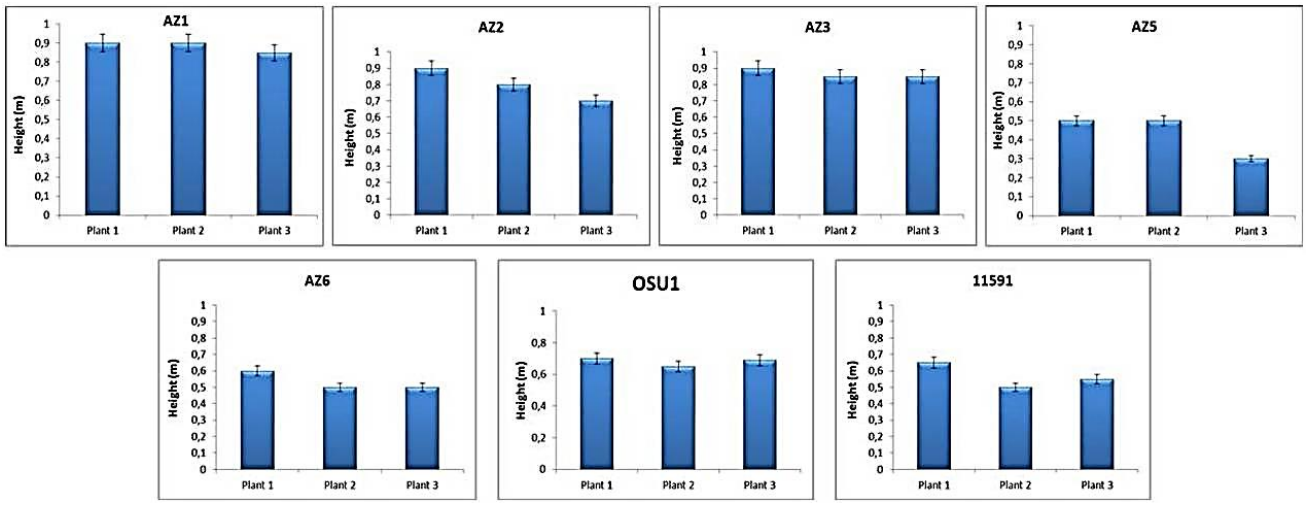


Figure 2.15: The plant height of 2 year old guayule lines in the WC experimental farm: The AZ1-AZ3 were taller, with an average of 0.9 m, than AZ5, AZ6, 11591 at an average of 0.6 m, and the OSU1 line with a plant height of 0.7 m. There were significant differences within each line and amongst the different lines.

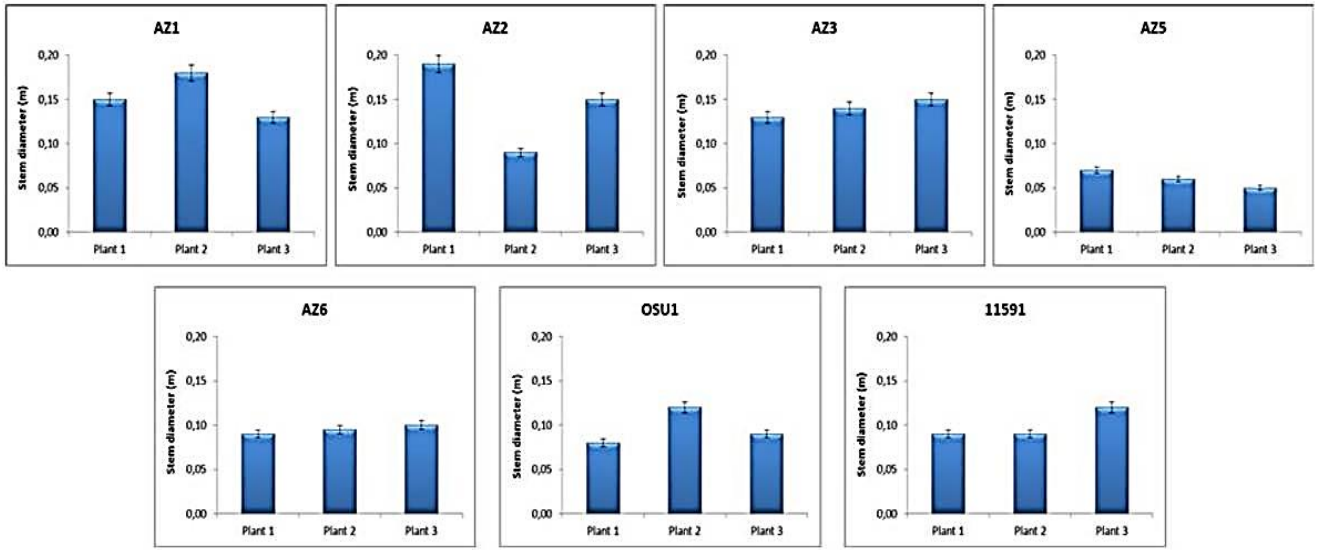


Figure 2.16: The canopy diameter measuremnts of 2 year old guayule lines in the WC experimental farm: The plant height of lines was measured in triplicate and further compared with the other lines. There were no significant differences amongst the lines however differences were observed amongst the lines. The AZ1, AZ2 and AZ3 were more bulkier followed by OSU1 while the AZ5, AZ5 and 11591 lines showed less perfomance in this area.

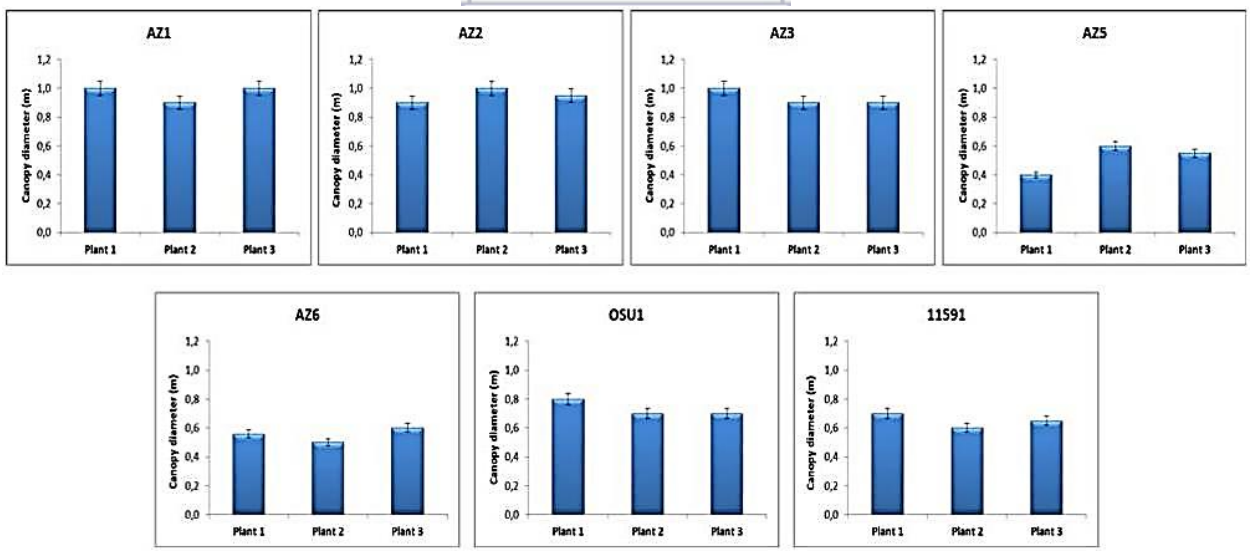


Figure 2.17 The main stem average diameter measurements for 2 year old guayule lines in the WC: The AZ1, AZ2 and AZ3 lines showed a larger stem diameter as compared to AZ5, AZ6, 11591 and OSU1. Increased variations within each line were observed with the AZ1 and AZ2 lines but other lines were constant.

2.3.8.3 The comparison of plant development in both fields

The plant height was compared between the field trials and the observations were that the EC plants showed great height as compared to the WC plants; however the AZ2 cultivar was the same height in both fields. The AZ3 line was the tallest with a 1.2 m height in the EC followed by the AZ1 line with a 1.1 m height whereas the AZ2, AZ5 and AZ6 lines were similar in plant height. The WC site the AZ1, AZ2 and AZ3 lines grew taller than the other lines but were similar to each other. The AZ5 and AZ6 lines were very short (0.6 m) and their developmental behaviour was similar in the WC. For the EC plants, guayule lines AZ1 and AZ3 exceeded the natural habitat height reported by Lloyd (1911) to rarely exceed 1 m. The comparison results are shown in Figure 2.18.

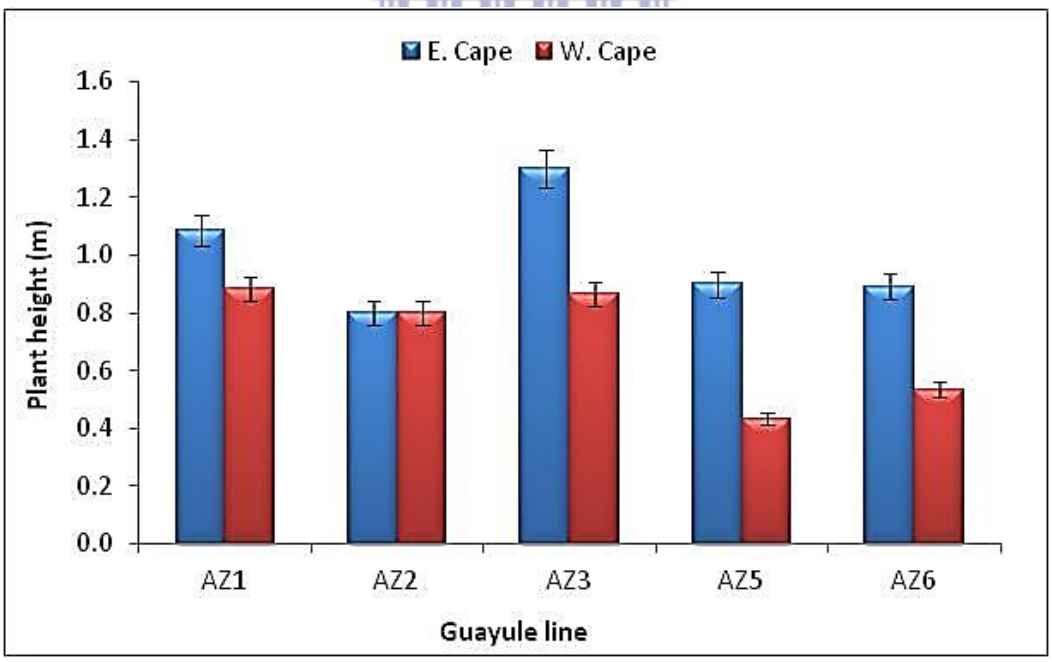


Figure 2.18: The performance of 2 year old guayule lines in plant height in the EC and WC: All the lines showed increased performance in the EC as compared to the WC plants with the AZ3 showing great height. The AZ1, AZ2 and AZ3 lines were observed to have increased height regardless of the location. The AZ2, AZ5 and AZ6 had the same performance in the EC however the AZ2 was higher in the WC as compared to AZ5 and AZ6. The AZ5 and AZ6 showed the least performance in the WC field.

The stem is the most important part of a guayule plant since 90% of the rubber is produced in the stem bark. Therefore, a plant with a big stem diameter will provide more stem mass. It would be also beneficial if the line showing big stem diameter is also a high rubber producing line as it will increase the rubber yield. The comparison of stem diameter showed significant differences between the lines and the study areas. The EC plants had a larger stem diameter than the WC plants except for the AZ2 line. There was also a correlation between plant height and stem diameter such that taller plants had higher tem diameter average. The AZ1, AZ3 and AZ6 lines had increased stem diameter average while the AZ2 and AZ5 showed reduced but similar, stem diameter measurements. Stem diameter average was similar for AZ1, AZ2, AZ3 and AZ6 lines grown in the WC however AZ5 had a reduced stem diameter average.

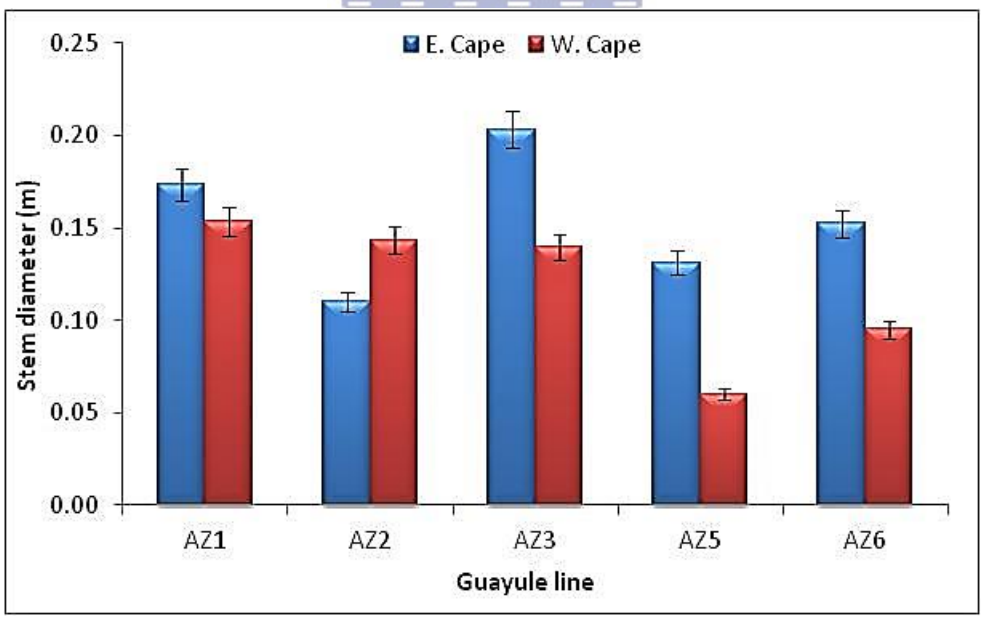


Figure 2.19: Stem diameter comparison between study sites and lines: The bigger stem diameter was observed for AZ3, AZ1 and AZ6 in the EC and for lines AZ1, AZ2 and AZ3 in the WC. The line with the least stem diameter was AZ2 in the EC and AZ5 in the WC.

The canopy diameter was observed to correlate with high biomass amongst the different lines (Bucks *et al*., 1985). The EC plants had larger canopy diameters than plants in the WC. The AZ3

and AZ1 lines had the highest canopy diameter followed by AZ2 and AZ6 with the same diameter, and AZ5 had the least. In the WC field, AZ1, AZ2 and AZ3 had similar canopy diameter, approximately 1.0 m, while AZ5 and AZ6 were much smaller at about 5.0 m.

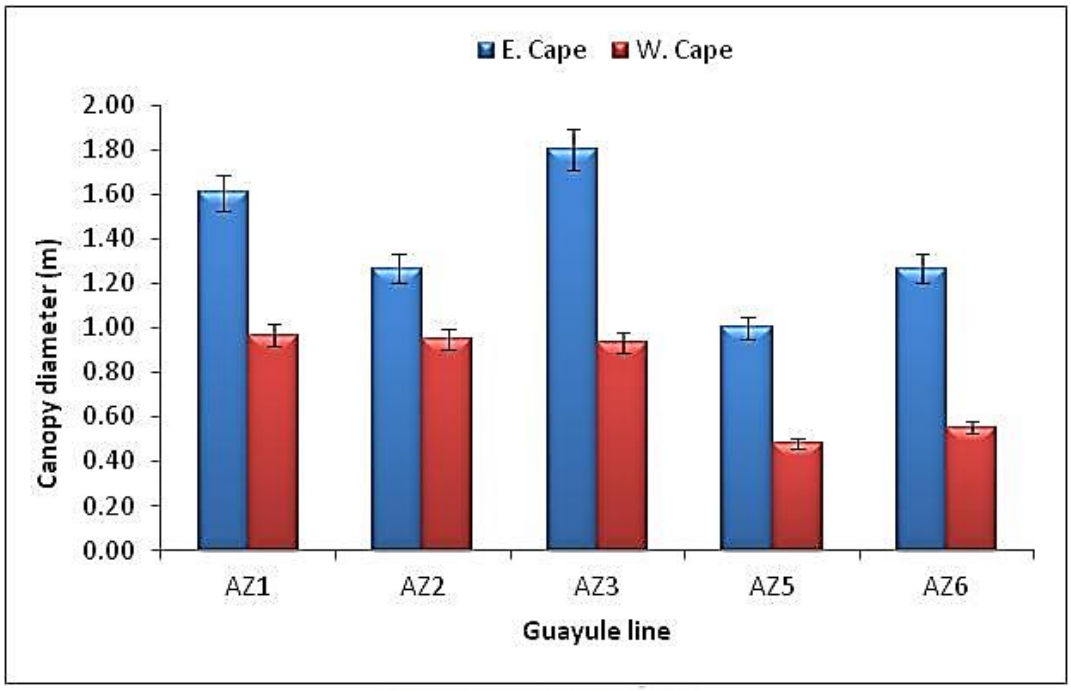


Figure 2.20: Canopy diameter averages for 2 year old guayule lines in the EC and WC: The AZ1, AZ2 and AZ3 were constant in the WC while AZ3 showed increased diameter followed by AZ1 and AZ2 with AZ6. The less canopy diameter measurements were observed for AZ5 and AZ6 in the WC field.

2.4 Discussion

Germination rate can be influenced by the seed colour as previously reported by Jorge (2005). The germination rate in the glasshouse was higher during summer than the winter season, consistent with reports that guayule germination is optimal between 20-30°C and greatly reduced below 15°C (Benedict and Robinson, 1946).

A large amount of biomass accumulation was obtained with the EC tested lines however a small biomass accumulation was observed with lines AZ1, AZ2 and AZ3 in the WC. One can be able

to determine a line's capabilities in terms of biomass production as the line with narrow-long pointing leaves showed low biomass whereas the lines with broader-rounder leaves produce high biomass (Mehta *et al*., 1979). The plant height measurements in this study were higher than those reported by Dissanayake *et al* (2004), a trial done in Australia where measurements were taken at 1 year 6 months. Guayule plants grow well in sandy loamy soil (Ray, 1993) and the soil should be well-drained with medium to fine textured sub-soils (George *et al*., 2005).

Research has shown that guayule has been found to react positively to N after the roots have reached layers of soil containing some clay thus the presence of some clay in the subsoil has been proven to be beneficial in guayule plant development (Muller, 1946). Different minerals and composition were obtained in the different sites and these results may have also influenced plant performance. Plants in the EC performed better in height, canopy and stems diameter as compared to those in the WC. The EC showed to have the best conditions for guayule plants to thrive. The soil composition in the EC was clay-loamy soil with an average of 36% clay soil and contained a pH of 4.6-5.0. Amongst the lines planted, AZ3 showed a greater height followed by AZ1. There was no difference in height between the AZ2, AZ5 and AZ6 lines. Reasons for lack of variations in the EC could be that only a small number of plants were tested on a small field as compared to the WC.

In this study, P content was observed to be low in the WC but high in the EC. A combination of high concentration of P and high N concentration were observed to increases the rubber yield in greenhouse plants (Bonner, 1944). The increase in growth / biomass / height in the EC could be the result of P and N content in high amounts although these were field experiments. The N content, applied as nitrate, has been observed to improve guayule growth (Foster and Coffelt, 2005). Other elements such as Ca, Fe, Mg, S, B, Cu, Mn and Zn were also measured however

Hammond and Polhamus (1965) reported that the presence of these elements does not affect guayule's growth and development however Bonner (1944) found contradicting observations indicating that B deficiency may reduce the guayule rubber content.

Salt is one of the limiting factors in guayule growth since it causes stress to the plants, it results in decreased growth / biomass but increased rubber yields per plant (Miyamoto *et al*., 1985). Salinity hinders growth of young guayule seedlings; however transplants are much more tolerant to salinity than emerging seedlings (Miyamoto *et al*., 1985). Guayule plants can tolerate low salt concentrations of 0.1 to 0.3% in the soil; however a salt content higher than 0.3% greatly inhibits growth or causes death (Siddiqui and Locktov, 1981). Despite the high salt concentration of between 0.5-2.2% in the WC and EC respectively, all lines showed good performance however they differed in height and canopy diameter based on the area.

The high mortality rate experienced in the WC could have been influenced by the weed competition and high rainfall which contributed to root rot. Small transplanted plants were eaten by snails and that also contributed in the number of dead plants, especially in the AZ5 and AZ6 lines. The high salt concentration together with the low soil pH of 3.7-5.4 could also negatively affect some lines since the preferred pH for guayule is between pH 6.0–8.0 (George *et al*., 2005; Dissanayake *et al*., 2008).

Flowering occurred throughout the year except in the winter dormancy season. During winter seasons plants became dormant however they recovered during summer seasons and continued to flower. Seeds were collected during these trials, are currently being cleaned, and will then be tested for germinability. It was observed that germination and transplantation must be done in summer and the plants need to acclimate to the environment 2 months before winter to ensure

survival. Guayule competes poorly against weed and its maximum growth and yield is affected (Foster *et al*., 1999; Coffelt *et al*., 2009). This observation together with the soil nutrient composition may have contributed to the poor performance of lines tested in the WC region.

The low or lack of germination of available seed impeded the determination of plant uniformity within and amongst the lines. Therefore, more data should be collected from a large scale trial in the EC. It can confidently be said that guayule plants can grow in RSA and stand establishment is possible on good soil / environment. Both EC and WC locations can support the establishment of guayule plants but specific lines should be chosen for each location for best performance and agronomic inputs (fertilizer usage, soil preparation before transplanting).

Chapter 3: Extraction, quantification and purification of guayule latex

Abstract

Approximately 90% of the latex is found in the parenchyma cells of the stem bark tissue, with the rest in the root bark parenchyma. Latex extraction is achieved by mechanical means using an alkaline buffer, usually potassium hydroxide (KOH) or ammonium hydroxide. Several extraction methods have been developed but the use of a Waring blender using KOH, pH 11 is a method of choice for rapid and efficient extraction on a laboratory scale. Guayule latex was extracted, quantified and purified from 2 year old plants harvested from both the EC and WC trials described in Chapter 2. The AZ4 and AZ6 lines contained most rubber in the EC while AZ5, AZ6, OSU1 and 11591 lines contained the most rubber in the WC. Latex purified by creaming with 0.5% sodium carboxymethyl cellulose (Na-CMC), 250 000 molecular weight (MW) at room temperature. Preliminary studies were conducted using guayule latex imported from Ohio State University, USA to determine the best creaming conditions, such as time, temperature and concentration of Na-CMC MW. Creaming efficiency was calculated based on latex volume (ml) and mass (g). Separation of latex from homogenate was observed in all the different Na-CMC MW (90 000, 250 000 and 700 000), and yield was affected by time and temperature. The creamed latex was preserved using 0.4% Ammonia and stored at 4°C.

3.1 Introduction

Commercial development of hypoallergenic NR products from guayule for the manufacturing of medical equipment requires knowledge and understanding of the extraction, quantification and purification methods. Several methods have been used effectively in the extraction, quantification and purification of guayule latex. Guayule latex is suspended within the cytoplasm and vacuoles of parenchyma cells in the stem and root bark and its extraction requires mechanical (homogenization and separation) techniques. Latex extraction from guayule parenchyma cells is a very simple and fast process and on a lab scale it can be done using the Waring or Oster blender method (Cornish *et al*., 1999; Cornish and Brichta, 2002). Latex production in guayule occurs in cycles within the plant's stems and roots. Rubber accumulation is influenced by various factors, such as low temperature, light intensity, moisture and water stress, the age of the tissue, nutrient availability, guayule line and the season. Low temperature is a key factor promoting rubber biosynthesis in guayule and in regulating the expression of genes coding for enzymes involved in rubber biosynthesis (Ponciano *et al*., 2012). Moisture is another important factor in guayule's growth and rubber accumulation. Guayule requires permeable well-drained, well-aerated soils, and high productivity depends on a reasonably good supply of soil moisture most of the year. Rubber concentration increases more rapidly during high moisture stress than during low moisture stress. The amount of rubber within a plant is further increased after each successive period of high stress compared to plants subjected to continuous low stress (Benedict *et al*., 1947). Latex quantification determines the amount of rubber which is in the form of latex in the living plant and is expressed on a dry weight basis. Latex concentration may occur slowly naturally, but it can be achieved rapidly through the addition of creaming agents. Different creaming agents include casein, sodium carboxymethyl cellulose, polyvinyl alcohol,

sodium alginate, methylcellulose, carboxymethyl cellulose, butadiene–styrene–methyl [methacrylate copolymer], ammonium alginate, ammonium oleate, aluminum cellulose glycolate, locust bean gum and tamarind seed powder (Ochigbo *et al*., 2011). Through the addition of a creaming agent of choice into the latex, two phases will be obtained; the upper creamy layer containing ~60% rubber and the bottom layer which contains little or no rubber (Cornish and Brichta, 2002). The latex concentration step acts as latex purification or washing step thus the total protein content in latex is reduced through the process. The final concentrated latex is above 60% dry rubber content, and is the raw material in the production of gloves, balloons, condoms, tires, foams, balloons, catheters, baby soothers, dental dams and latex thread. Refrigeration has also been observed to maintain latex yield and maintenance of pH by the addition of ammonium hydroxide prevents microbial growth and enzyme activity. The objectives of this chapter were to extract, quantify and purify (cream) guayule latex from different lines grown in EC and WC.

3.2 Materials and methods

3.2.1 Sample collection and transportation

Guayule stems, of the same appearance and size (10 mm), from 3 individual plants of each guayule line were harvested using a sharp cutter and all the leaves removed (Figure 3.1). The small stem size was selected to avoid breaking the Waring blender. The stems were stored in transparent plastic bags to avoid drying and immediately transported to the University of the Western Cape (UWC), department of biotechnology, proteomics unit. Upon arrival, samples were immediately processed for latex extraction and quantification.

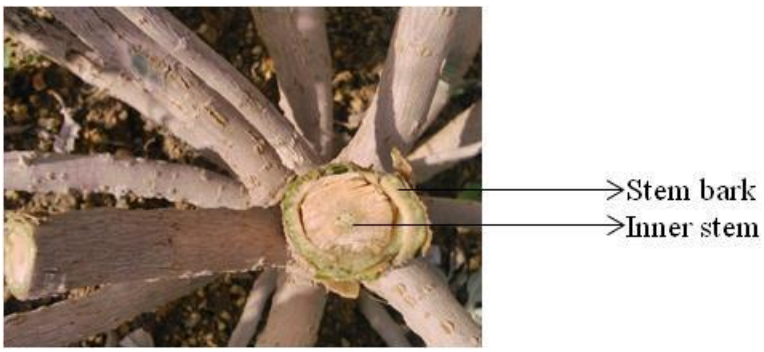


Figure 3.1: Harvesting guayule stems: A very sharp plant cutter was used to cut off the stems which were used in latex extraction process.

3.2.2 Latex extraction

Latex extraction was carried according to the method of Cornish *et al* (1999), with minor modification. About 100 g of stem was ground in 400 ml extraction buffer (KOH, pH 11) using a 1 L Waring blender (Waring Products Division, Torrington, CT, USA) for 1 min (Figure 3.2). The ground mixture was filtered through a double layer of Miracloth (Merck, Darmstadt, Germany) into a beaker to remove woody components (bagasse). The bagasse was dried at room temperature and the extraction process was done per each guayule line in triplicate.

Figure 3.2: Guayule latex extraction: Latex extractions were done in triplicate for all the lines using extraction buffer (KOH pH 11) in a Waring blender

3.2.3 Latex quantification

The latex homogenate was quantified using the 1 ml quantification method (Cornish and Brichta, 2002). These experiments were done in triplicate where 1 ml of the homogenate was transferred into 1.5 ml microfuge tubes and centrifuged at 4300 xg for 5 min at room temperature to float the latex (Figure 3.3). Approximately 200 µl 100% glacial acetic acid was added to coagulate the floated rubber and the tubes were centrifuged again. The coagulated latex plugs were removed from the tubes, air dried and weighed.

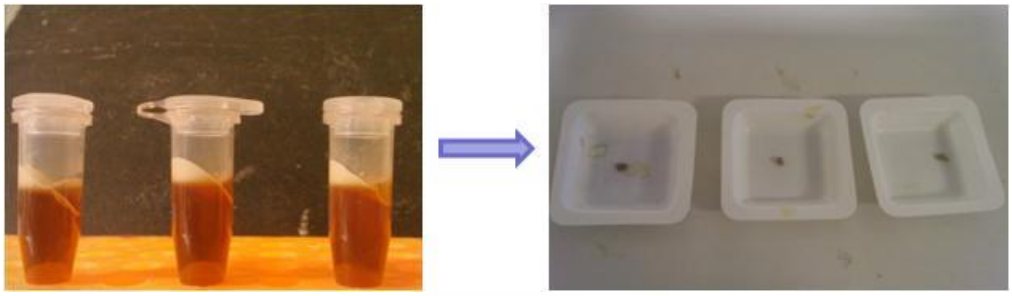


Figure 3.3:The quantification of latex homogenate: Using the 1 ml quantification method, the latex was quantified amongst the different lines.

3.2.4 Latex purification

3.2.4.1 Optimization of creaming agent conditions

Latex creaming using different MW of Na-CMC was done to determine the total solid content (TSC) and the dry rubber content (DRC) at 4°C and RT at different time intervals. Flotation of latex under natural gravity is a very slow process. This can be accelerated by using creaming agents. Creaming has been used in the production of latex concentrates because it is low cost, requires less energy input than centrifugal methods and it is environmentally friendly (Yumae *et al.*, 2010). Already extracted guayule latex (50% rubber content) preserved in ammonium hydroxide was provided by The Ohio State University, USA. Na-

CMCs of 90 000 MW; 250 000 MW and 700 000 MW (Sigma Aldrich, RSA) were tested and stock solutions of 2% (wt/v) were dissolved in KOH, pH 11 for each MW except for the 700 000 MW. Only 1% (wt/v) stock solution was prepared for Na-CMC 700 000 MW because of its low solubility. Creaming experiments were carried out at room temperature (RT) and 4°C inside 50 ml burettes. Different concentrations of the carboxymethyl cellulose (0.05%, 0.1%, 0.5%, 1% and 2%) were added respectively into a burette containing 2% latex and potassium hydroxide buffer to a total volume of 50 ml. Burettes were covered with foil and allowed to stand for 24 hours, while monitoring the latex separation, and yield readings were taken at 2, 4, 6 and 24 hours. Within 24 hours, the latex had separated into two layers, top layer containing concentrated rubber particles and the bottom layer containing little or no rubber particles (clear buffer). The concentration of latex in the separated rubber layer was quantified using the 1 ml quantification method described in Section 3.2.3.

3.2.4.2 Creaming of pooled latex homogenate

Latex extracted from individual guayule lines was pooled in a single beaker. Creaming is hindered by the presence of non-rubber solids (Cornish and Brichta, 2002) therefore the pooled homogenate was re-filtered using a layer of the Miracloth to reduce the solids. The homogenate was mixed with 0.01% (wt/v) SDS, 10% (v/v) glycerol, 0.4% (v/v) ammonia and 0.5% (wt/v) 250 000 MW Na-CMC [1 (homogenate): 4 (creaming agent)] ratio. The concentration of the creaming agent was selected from the optimized conditions determined in Section 3.2.4.1. The mixture was transferred into an airtight container to avoid air passing through, which could cause coagulation of the rubber in the latex, and monitored for separation at RT. It was of importance not to disturb the creaming container as this disrupts the separation. Scooped latex was

resuspended in fresh creaming agent, to wash the latex, and separated. Latex washing was repeated four times to obtain clear white latex.

3.2.5 Statistical analysis

Rubber extraction, quantification and purification was performed thrice independently. Results are reported as the mean ± SE of three independent determinants. For statistical analysis, ANOVA test was used to compare the extracted rubber amongst guayule lines AZ1- AZ6 plants performance for height, canopy and stem diameter in EC and WC (Appendix E).

3.3 Results

3.3.1 Latex extraction

Guayule lines (AZ1, AZ2, AZ3, AZ4, AZ5 and AZ6) were planted in both trials however the AZ4 line did not survive in the WC region and OSU1 / OSU1-clonal lines did not survive in the EC region. The 11591 was only tested in the WC trial because of low seed availability. An attempt was made to extract rubber from both leaf and stem but no rubber was obtained from the leaves. Grinding stem for 1 min in the Waring blender was sufficient to ground all the available stems into fine bagasse (Figure 3.4).

Figure 3.4: The latex homogenate from stem extractions: a The latex homogenate after filtration. **b** The bagasse with good hemicelluloses content was obtained as a by-product.

3.3.2 Latex quantification

The amount of latex rubber (mg/g) obtained for 3 individual plants sampled per line is shown in Figure 3.5 and Figure 3.6 for the WC and EC, respectively. In the EC (Figure 3.5) a higher rubber concentration was obtained from lines AZ4 (8 mg/g) followed by AZ5 and AZ6 (7 mg/g) while lines AZ1, AZ2 and AZ3 showed a lower rubber concentration of 3 mg/ml. Variation amongst plants of the same lines were observed especially for the AZ1, AZ2, AZ4 and AZ6 lines. Figure 3.6 represents the WC results and the line which produced more rubber was AZ6 (13 mg/g) followed by AZ5 and OSU1 (10 mg/g). The 11591, AZ2 and AZ3 lines produced an amount of 8 mg/ml with AZ1 producing the least rubber concentration of 6 mg/g. There were also significant differences in the amount of rubber produced in the same line for the AZ5 and AZ6 plants.

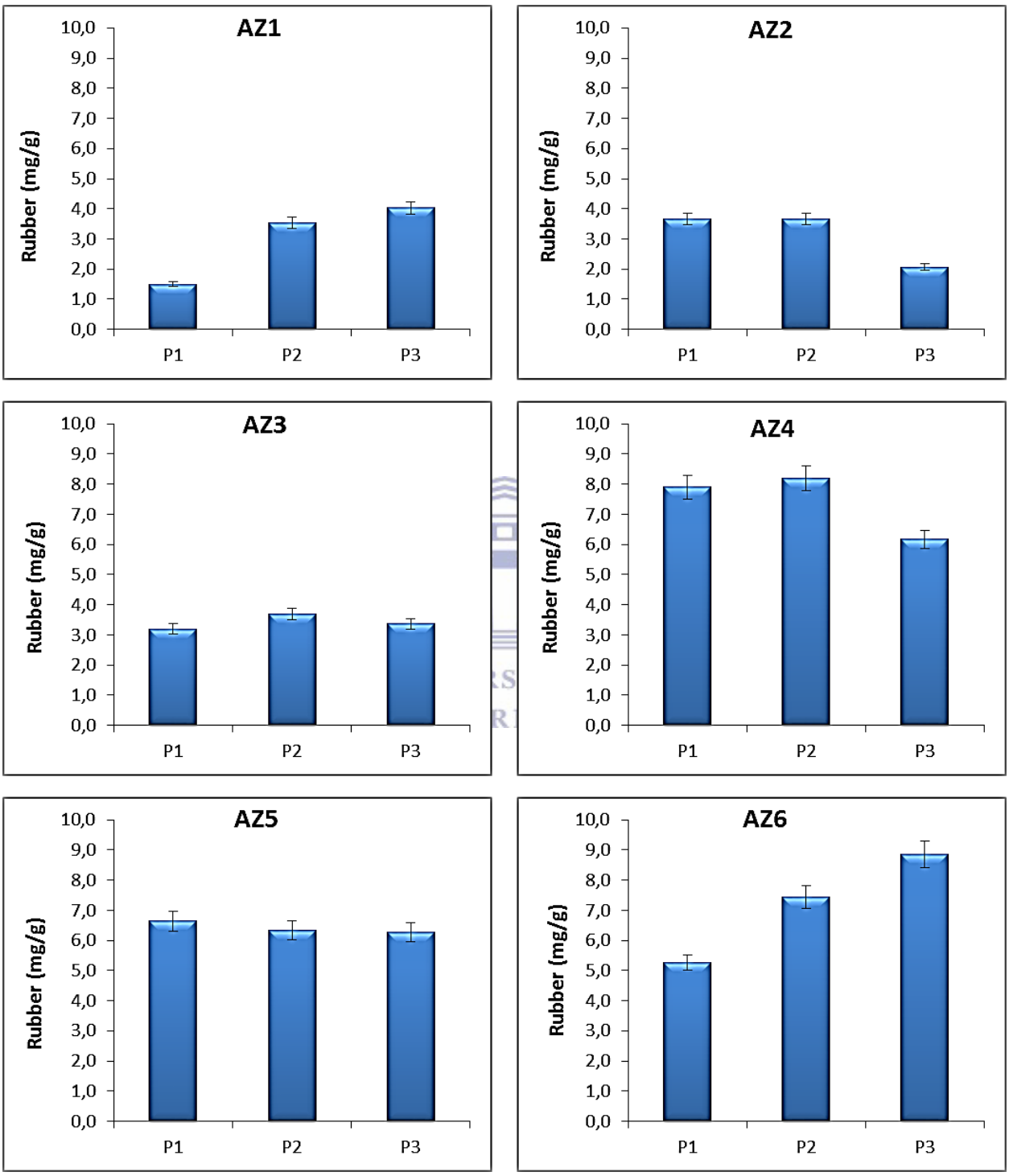


Figure 3.5: Rubber of 2 year old plants in the EC: The AZ4, AZ5 and AZ6 guayule lines showed maximum rubber concentration compared to other lines.

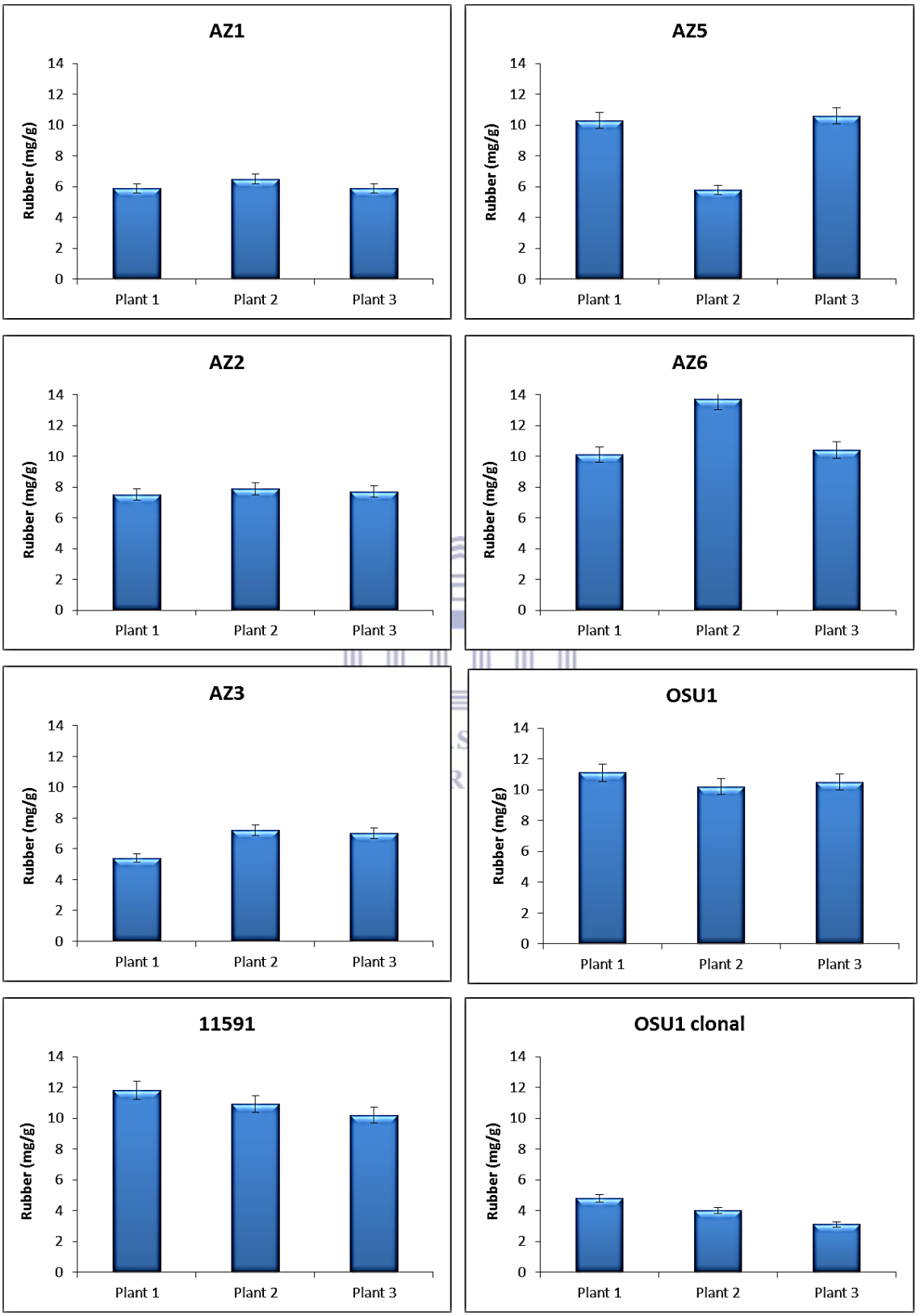


Figure 3.6: Rubber of 2 year old guayule lines in the WC: The AZ5, AZ6, OSU1 and 11591 lines produced more rubber compared to other lines. The OSU1 line rubber concentration measurements was at 1 year

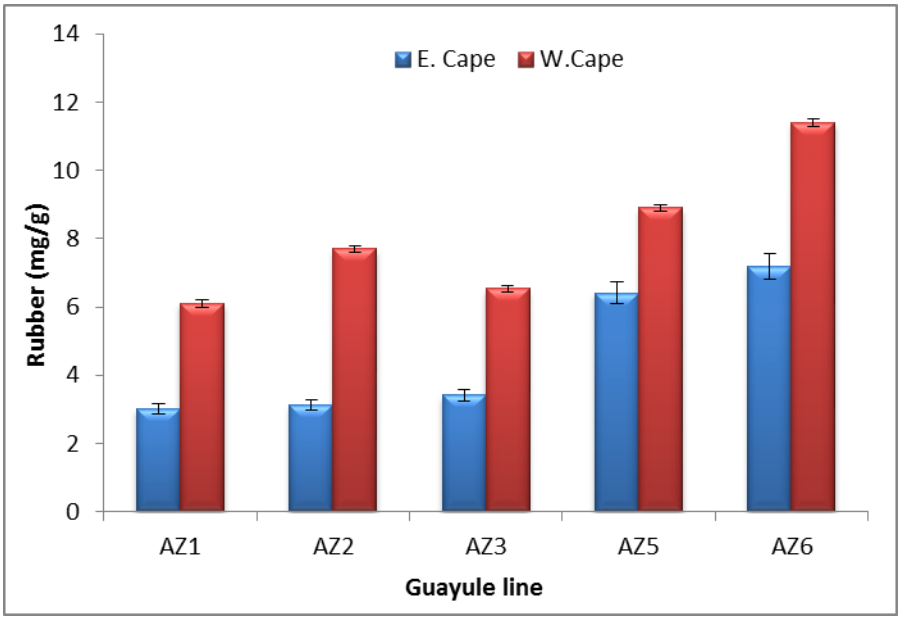


Figure 3.7: The rubber (mg/g) obtained from 2 year old plants: The yield of rubber obtained was compared between the provinces and the lines. The WC lines produced more rubber compared to the EC.

Because of 100% mortality observed with AZ4 line in the WC, the results presented in Figure 3.7 show the comparison of rubber concentration amongst lines AZ1, AZ2, AZ3, AZ5 and AZ6. The results in Figure 3.7 show that plants in the WC region/province produced a higher concentration of rubber as compared to those in the EC. However high rubber was produced by lines AZ6 followed by AZ5. The AZ1, AZ2 and AZ3 lines produced approximately the same concentration of rubber of 3 mg/ml in the EC and 7 mg/ml in the WC.

3.3.3 Latex purification

3.3.3.1 Optimization of creaming agent conditions

To determine the efficiency of Na-CMC as a creaming agent for guayule latex under different temperatures, different MW (90 000, 250 000 and 700 000) CMC were tested at room temperature and 4°C. The results showed that all the different MW of Na-CMC were able to

cream guayule latex although the concentration was affected by time and temperature as shown in Figure 3.8.

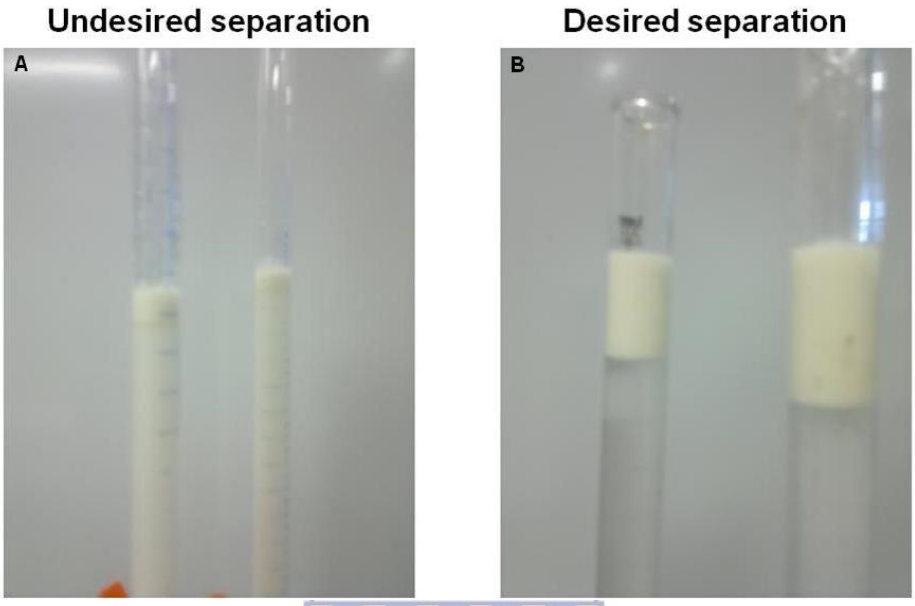


Figure 3.8: The separation observed during investigation of the different Na-CMC MW: a The undesired separation because a high concentration of rubber particles is still in the buffer. **b** The desired separation where the separated latex volume was clearly observed and recorded. All the rubber particles floated on top where carefully transferred into 1 ml Eppendorf tubes.

The volume of the separated latex was recorded and compared amongst the different concentrations of the different MW and the different temperatures. Figure 3.9 shows the creamed latex volume (ml) obtained at different time intervals.

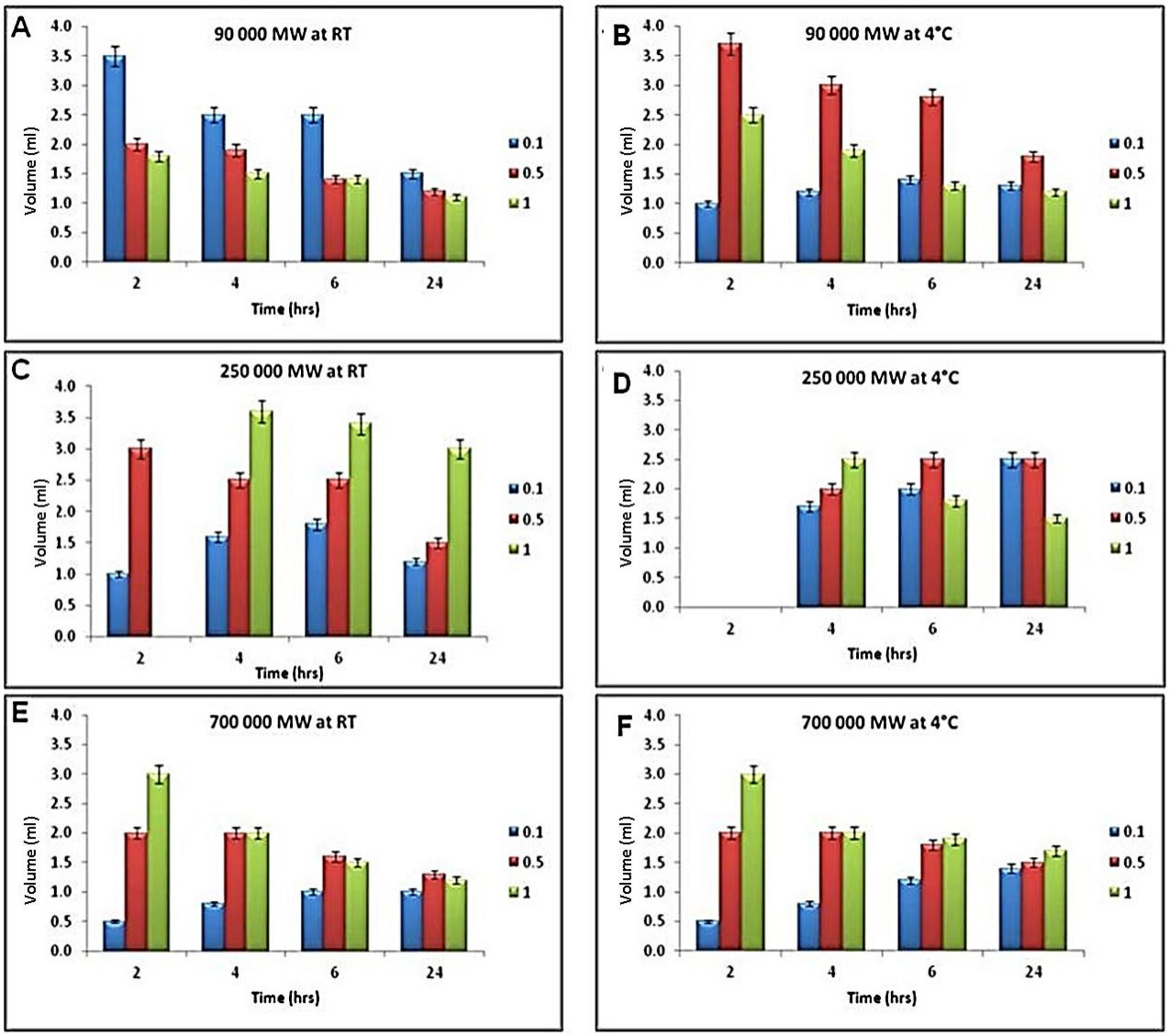


Figure 3.9: The TSC obtained using 90 000, 250 000 and 700 000 MW at room temperature and 4°C: a and **b** 90 000 MW at RT and 4°C, **b** and **c** represent 250 000 MW at RT and 4°C while **e** and **f** represent 700 000 MW at RT and 4°C respectively.

The results presented in Figure 3.9 show the influence of temperature and time on the rubber particles separation efficiency. The 90 000 MW Na-CMC at RT showed a faster separation for the 0.1% concentration however with increase in time there was a decrease on the separated volume. This can be explained by the relationship of the rubber particles with the creaming agent. As time increases, the rubber phase becomes more concentrated as the rubber particles

bind closer together thus removing water / buffer and decreasing the separated volume. A high volume of 1.5 ml was observed for the 0.1% stock concentration after 24 hrs but there was no significant difference between the separated yield of 0.5% and 1% concentration. A faster separation was observed at 4°C for the 0.5% and 1% whereas there was a gradual increase for the 0.1% concentration. At 0.5%, high volume approximately 1.5 ml was obtained however no significant differences between 0.1% and 1% which showed just above 1 ml.

The 250 000 MW at RT shows that 0.1% stock gradually increased while a faster separation at 2 hrs was observed for 0.5% stock concentration. No clear separation was observed for the 1% stock concentration therefore it was recorded as "zero". After 4 hrs, there was a clear separation for the 1% and with time a slight decrease was observed. The 0.1% concentration showed an optimum yield after 6 hrs, however there was a slight decrease after 24 hrs. The performance of the 250 000 MW at 4°C shows no separation for all the concentrations at 2 hrs. After 4 hours, 0.1% showed gradual increase with time while the 1% concentration reached its optimum separation and started to decrease with time. After 24 hours, 0.1% and 0.5% produced the same volume (2.5 ml) while for 1% it was 1.5 ml.

The observations for the 700 000 MW at RT show a very slow separation for the 0.1% concentration. This MW was observed to behave in the same manner at both RT and 4°C and after 24 hrs there was no significant difference of the yield separated amongst the different concentrations. Approximately 1.2 ml and 1.5 ml was obtained at RT and 4°C respectively. After the yield was recorded, the 1 ml method was used for quantification of the separated latex, measuring the DRC. It is of importance to note that the yield which is lower than 1 ml was not

considered for the quantification experiments. Figure 3.10 shows the quantification of separated volumes at RT and 4°C for the different concentrations of different MW.

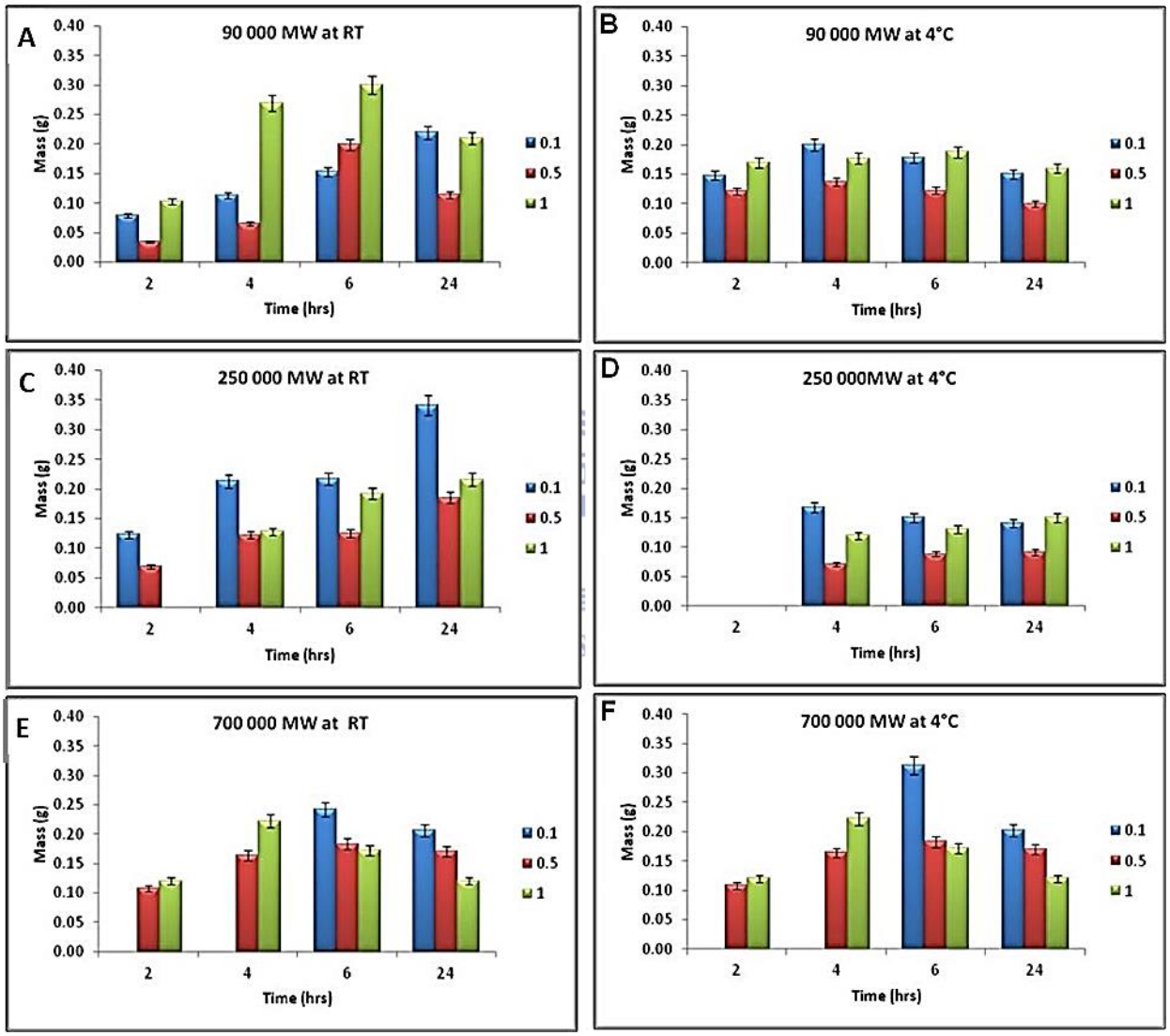


Figure 3.10: The quantification (DRC) of creamed latex at RT and 4°C using different concentrations of 90 000, 250 000 and 700 000 MW Na-CMC: a and **b** 90 000 MW at RT and 4°C, **b** and **c** represent 250 000 MW at RT and 4°C while **e** and **f** represent 700 000 MW at RT and 4°C respectively. The highest rubber mass was observed to be 0.35 g during the 250 000 MW (0.1%) at RT and also the 700 000 MW (0.1%) at 4°C Generally it was observed that the 0.1% concentration in all the MW produced a high mass rubber at RT and 4°C after 24 hrs.

A high mass of rubber was observed to be 0.35 g during creaming with 250 000 MW (0.1%) at RT and also for the 700 000 MW (0.1%) at 4°C. Generally it was observed that the 0.1% concentration produced a high mass rubber at both RT and 4°C after 24 hrs.

When 90 000 MW Na-CMC was used at RT, a higher rubber mass of 0.30 g was observed after 6 hrs for the 1% concentration followed by a slight decrease. The rubber mass showed an increase with time for the 0.5% and 1% and afterwards a decrease. The 0.1% concentration only showed increase with time and at the last sampling time (24 hrs) the mass was 0.20 g. For the 4°C experiments, the rubber mass was observed to remain constant throughout the time. There was no significant difference amongst the concentrations tested.

The 250 000 MW Na-CMC at RT showed no record for the 1% because of the < 1 ml yield obtained during Section 3.3.3.1 experiments. The 0.1% and 1% concentrations showed an increase in rubber mass even at 24 hrs. At 4°C, there was no good separation for all the concentrations and after 2 hrs, no significant difference was observed in rubber mass with increase in time. A higher mass of 0.35 g was observed at 0.1% concentration.

3.3.3.2 Creaming of pooled latex homogenate

After optimisation of the creaming experiments, the optimum concentration was selected due to results in Figure 3.11 for the purpose of creaming the pooled latex homogenate. The creaming agent concentration of 0.5% 250 000 MW was selected at room temperature and monitored for separation over time. It is important to note that the objective of this experiment was to determine the purification using the lower concentration to save on the costs. The high concentrations of creaming agents require high energy and time to dissolve. This step is also referred to as the "washing step" as the latex is being washed of all impurities including carbohydrates, resins and proteins. Immediately after all the chemicals were added and the mixture was allowed to stand, it was visible that the rubber particles started to move upward floating and with time a separated layer of latex was observed. After each separation the floated

latex was scooped into a fresh creaming solution mixture (1:4 ratio) and all the chemicals were kept constant during each wash. Figure 3.11 shows the separation of latex after each purification step and the improvement of latex colour.

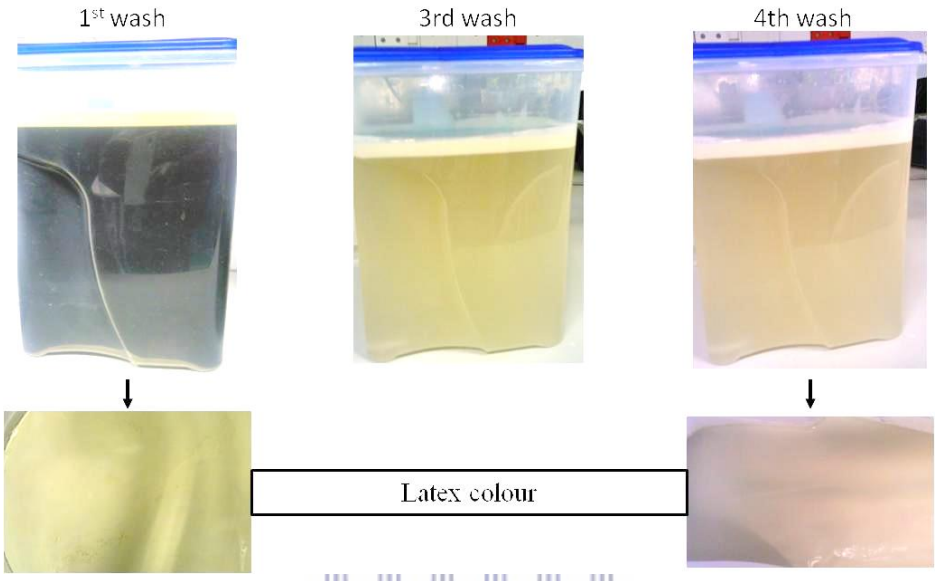


Figure 3.11: The purification of latex through creaming: The colour of the latex was improved after each washing and floating step.

During the first wash, it was observed that the homogenate was not 100% clarified, some solid particles still remained, however the creaming process was not hindered. The solid phase precipitated at the bottom while the rubber phase floated on top. The colour of the latex was recorded at the beginning where it was greenish and also the last wash where it was cream-white. About 800 ml of the washing buffer was added into 200 ml of the latex homogenate and this was observed to facilitate the washing of latex. The supernatant changed from dark-black to greenish to almost-clear at the fourth wash representing that impurities were being washed out of the latex. Figure 3.12 shows the quantification of creamed latex (A) preserved with 28% Ammonia and stored in 50 ml tubes at RT.

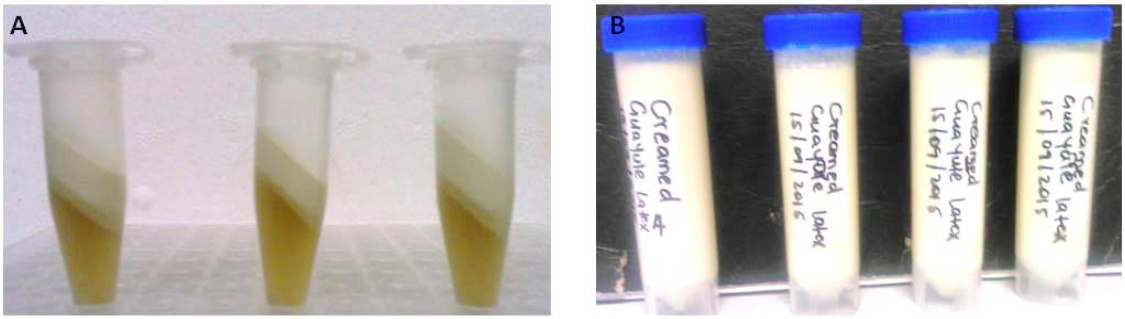


Figure 3.12: Quantification and preservation of creamed latex: a After a series of latex washing and floating, the creamed latex was quantified. **b** The creamed latex was preserved using ammonia and stored at 4°C.

The purified latex obtained after the fourth wash was referred to as the "creamed latex" was quantified as described in Section 3.2.3 packaged in 50 ml tubes. The results show that after the creaming process, the rubber was more concentrated and the supernatant in the Eppendorf tubes was clean. The rubber mass was approximately 12 mg/ml and after creaming it was ~ 150 mg/ml which represented a 12- fold increase. After 6 months of latex storage at RT, it is still in good condition, without any form of coagulation. Weekly monitoring showed that the latex continued to separate into two layers, the supernatant was at the bottom and the rubber phase floated on top.

3.4 Discussion

Guayule lines AZ4, AZ5 and AZ6 were found to produce high rubber yield as compared to AZ1, AZ2 and AZ3 lines, in agreement with an earlier report on AZ5 and AZ6 (George *et al*., 2005) and AZ1 (Ray *et al*., 1999). Performance is not always reproducible, however the AZ3 sometimes performs better than AZ5 in rubber yield (Coates *et al*., 2001). Overall, guayule rubber content depends on the interaction between guayule line and environmental factors and thus it will always differ amongst lines and regions.

The AZ1, AZ2 and AZ3 lines are characterized by broader leaf structures, bigger canopy sizes and higher biomass than AZ4, AZ5 and AZ6. Amongst the lines, higher rubber concentration occurred in lines with narrow leaves rather than broad leaves and amongst the trials higher rubber concentrations were achieved in the WC than the EC. Due to the need of plants for seeds in the future, individual plants were not cut and weighed, however it is of importance to note that a bigger plant with low rubber concentration may produce more rubber per plant. This might be because moderate stress conditions such as low temperature, salinity and drought can enhance the rubber content in guayule (Hoffmann *et al*., 1988).

The soil in the WC has a lower water holding capacity than the EC soil and is more likely to cause water stress conditions. According to the WC weather report, the province receives high rain in winter and experiences lower temperatures than in the EC. Water shortages occurred in some seasons in the WC, which could have influenced the rubber synthesis during drought stress. These plants were harvested in spring where the accumulation of rubber would have taken place during the winter seasons; therefore the sampling season is constant. Significant differences were observed amongst the plants in the same lines in rubber content.

Latex was successfully purified from homogenates using Na-CMC creaming agent (concentrated and washed) and after four months the creamed latex was still be in good condition, with no coagulation, when stored at RT or at 4°C. The advantage of the creaming method is that it does not require heavy machinery and using optimized laboratory conditions, the process can simply be done in the field in a drum with an airtight lid. This study has provided data on the lines with high rubber content in the EC and the WC. Guayule latex can rapidly be extracted using well

established methods and quantified using the 1 ml method. The latex can be easily purified using Na-CMC for the manufacturing of rubber products. Finally these processes and methods are easy and feasible, and can be easily trained and applied by both unskilled and semi-skilled individuals.

Chapter 4: *In vitro* micropropagation and field establishment of guayule line OSU1 from seedling explants

Abstract

Natural rubber (NR) is a renewable bio-material used in large quantities by commercial, medical, transportation and defence industries. Increased demand for NR products and latex allergy reactions has led to a search for an alternate NR source. Guayule is currently under commercialization for the production of circumallergenic medical rubber products at the United States of America (USA), but due to seed dormancy its cultivation is a challenge. The application of micropropagation techniques can be efficient for large scale plant production to ensure continuous supply for field establishment. The aim of this study was to develop a micropropagation protocol from tissue culture and establish the plants in selected local fields of RSA. This was achieved by generating guayule plants from seeds using the micropropagation procedure which includes germination on media, multiplication, rooting and acclimatization. Guayule line OSU1 seeds were disinfected with ethanol/sodium hypochlorite and germinated on Murashige and Skoog (MS) medium. Shoots were excised and transferred into multiplication medium supplemented with 1g/L indole-3- butyric acid (IBA) and 0.1g/L benzyladenine (BA) hormones for 4 weeks. The developed shoots (>2 cm) were transferred into rooting media supplemented with BA (0.4g/L) and gibberellic acid (GA_3) (0.1g/L) and roots grew within 1-2 weeks. The rooted plantlets were acclimatized by transferring them into a mixture of perlite and coconut peat (1:1 ratio), covered with transparent polybags and incubated in the glasshouse (20°C) for 4 weeks. Holes were gradually created on the polybags on a daily basis after 3 weeks to allow air to pass through the plantlets. On the fourth week of acclimatization, the plantlets

were transplanted into 25 cm pots containing sandy-loamy soil and kept in the greenhouse. Watering was done once per week or as necessary whereas fertilization using Kynitro-19 was done once per week. After 6 months, the plants were transplanted into the Western Cape (Stellenbosch) ARC experimental farm field and monitored for development. The plantlets produced during acclimatization were long and strong which contributed to a higher survival rate. The methodology followed during this study was successful and produced adequate results with >90% plant survival in the greenhouse and 50% in the field. The whole process from establishment using seeds to strong plants growing in the green house was achieved within 10 weeks.

4.1 Introduction

Guayule (*Parthenium argentatum*, Gray) is a hard wood shrub native to the Chihuahuan desert of Mexico (Stewart and Lucas 1986). Currently guayule is recognised as a "safe" alternative for the production of medical rubber products worldwide. It produces high quality latex from which circumallergenic rubber products can be made including gloves, tubing, condoms, and catheters with great strength, impressive stretchiness and softness (Cornish *et al*., 1999; Slutzky *et al*., 2013). Guayule's economic opportunities include utilisation of lignocellulose bagasse in biofuel production, development of termite resistant wood products and resin-based products, making it an attractive new crop in arid and semi-arid areas (Nakayama *et al*., 2003). However, it is a challenge to establish big stands of guayule to ensure a secure continuous supply of natural rubber in the future. Guayule is a good seed producer, producing seeds continually except in winter season. Mature guayule seeds can be obtained from 2 years old plants stored in the husks of the guayule flowers. Seeds are very small (2.5 mm length and 1.8 mm width) and approximately 1000-1500 seeds can be obtained in a gram (Bucks *et al*., 1986). Depending on the guayule line, environmental and climatic conditions, seeds can be half-filled or contain no embryo thus contributing to low or zero germination (Jorge *et al*., 2007). In addition guayule seed also exhibit poor germination due to natural dormancy of which pre-treatment methods are widely documented (Benedict and Robinson 1946; Bekaardt *et al*., 2004) but lack of a viable embryo, or empty seed, still remains a problem in field establishment. These challenges make it difficult to generate large number of plants for effective and efficient guayule establishment. In this case micropropagation method can be very useful in acquiring large number of plants from at least a few germinated shoots and it is also a useful method to generate clonal plants of specific high producers. Transplanting of green house or micropropagated plants in the field is

the most reliable method in successful guayule plant establishment (Foster and Coffelt 2005). Several investigations have been conducted on *in vitro* propagation of guayule plants (Castillon and Cornish 2000; Trautmann and Visser 1990; Dhar *et al*., 1989; Smith 1983) but the transfer of micropropagated plants from the greenhouse to the field where the environment is totally different from the well maintained conditions has not been reported in South Africa. Following the simplified and efficient method described by Castillón and Cornish (2000), this article presents an *in vitro* propagation protocol suitable for generating large numbers of guayule line OSU (Ohio State University) 1 transplants and demonstrates the successful transfer of the regenerated plants to the field.

4.2 Materials and methods

4.2.1 Seed germination and establishment of shoots

Guayule line OSU1 was used in the *in vitro* propagation and the field establishment process. OSU1 is the first developed germplasm from the Ohio State University (OSU), Arizona, with improved traits in biomass and latex yield. Seeds were obtained from the OSU Department of Horticulture and Crop Science. Approximately 10 seeds were surface sterilized with 70% ethanol followed by soaking for 20 minutes in 2.5% sodium hypochlorite, then rinsed three times with distilled water and dried at room temperature under a laminar flow. Plant tissue culture jars (Sigma-Alrich, Saint Louis, MO., USA) were sterilized at 121°C for 20 minutes, containing 50 ml of solidified half strength Murashige and Skoog (MS) basal media (Murashige and Skoog, 1969) consisting of 2.2 g/l MS; 1% (w/v) sucrose; 5 mM MES, 0.8% (w/v) agar, pH 5.8. After cooling, the sterilized seed were transferred to the jars and incubated in a growth room at 25 ± 2°C under a 16 hour light/8 hour dark regime with a light intensity of 47.29 µmol $m^{-2}s^{-1}$ provided

by cool-white fluorescent tubes (Bellco Electrical Co Pty Ltd, SA). The seeds were monitored for germination.

4.2.2 Multiplication

After 3 weeks post germination on media, the shoots had 3-4 leaves. Shoots were excised leaving all cuttings with at least one nodal point/one leaf and separately transferred on MS media supplemented with 0.1 g/L benzyladenine (BA) and 1 g/L indole-3-butyric acid (IBA) growth hormones, pH 5.7. Individual shoots multiplied into ~10 shoots which were maintained in the multiplication media and were sub-cultured every 2-3 weeks and maintained in the growth room conditions as described in section 4.2.1.

4.2.3 Rooting

Excised shoots from the multiplication media, above 2 cm in height, were transferred onto rooting media [MS media supplemented with 0.4 g/L BA and 0.1 g/L gibberellic acid (GA_3), pH 5.2]. These were incubated in the growth room for 1-2 weeks under the same conditions. Several roots appeared after 2 weeks. Prior to acclimatization, the rooted plantlets were rinsed with dH_2O to remove the agar and sterilized in a fungicide solution containing 0.5 g/L benomyl 500 WP (Villa protection, (Pty) Ltd) and 3.2 g/L proplant (Agriphar, RSA).

4.2.4 Acclimatization

The washed plantlets were transplanted into a mixture of autoclaved genulite and coconut peat (1:1) prepared in trays. Prior to mixing, the coconut peat bricks were soaked in water overnight (4 L of water per brick). A transparent polybag was used to cover the plants which were maintained in the growth chamber at 20°C for 3 weeks followed by transfer to glasshouse while still covered. After 4 days incubation in the glasshouse, holes were gradually made in the

polybag covering the plants and the polybag was completely removed within 1 week. After 2 weeks, seedlings were transplanted into 25 cm pots containing potting medium (20 L sand and 40 L potting bark mixture) followed by daily watering and weekly fertilized with Kynitro-19, K2588 (Kynoch, RSA) for the first three weeks while kept in the glasshouse.

4.2.5 Greenhouse plants establishment

After two month, acclimatized plants were transferred from the glasshouse and kept in the greenhouse. The plants were manually irrigated and fertilized once per week until they were 6 months old. To avoid root rot disease, irrigation was not supplied during raining days.

4.2.6 Field transplantation and plant measurements

For field trials, 103 fully acclimatized 6 months old OSU1 guayule plants were transplanted from the greenhouse to the field in January 2014, at the ARC-Nietvoorbij experimental farm, Helderfontein Block C (-33.918026, 18.869254), RSA. The field trials used a row and plant spacing of 1.5 x 1.0 m and holes were dug until 30 cm. The plants were transplanted into the individual holes and properly covered with soil. After a year in the field, clonal plants development was analysed and they were measured for their height, canopy and stem diameter.

4.3 Results

4.3.1 Seed germination and establishment of shoots

Pre-treatment by soaking the seeds in water or GA_3 while shaking at a lower speed on a vortex overnight was sufficient to induce and obtain adequate germination for the seeds used in this

study. Germination was observed within seven days but the shoots were left to grow for a further three weeks. Figure 4.1 shows guayule seeds in a petri dish and the germination on media.

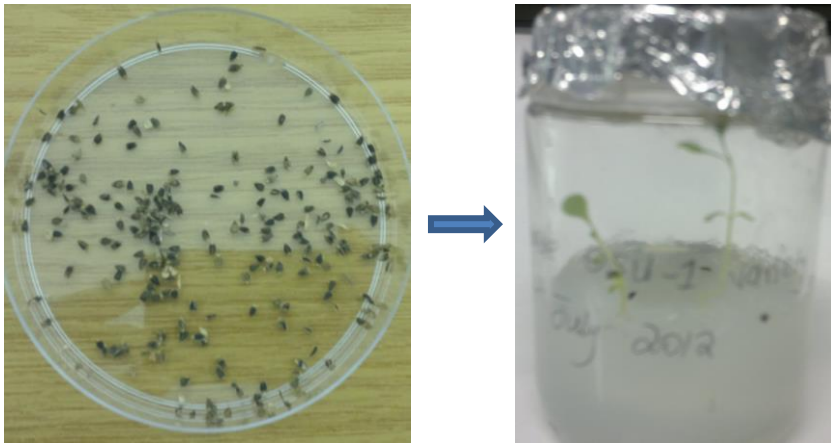


Figure 4.1: Germination of OSU1 guayule line on MS media: Guayule seeds were disinfected with standard methods using ethanol/sodium hypochlorite and germinated on MS media. Every week a new leaf developed and 4 leaves were observed after 4 weeks.

4.3.2 Multiplication

From the germinated shoots, multiplication and rooting processes were initiated through optimization of growth hormones. A combination of hormones for better development of micropropagated plants is an important factor. In this study maximum multiplication rate with longer shoots was obtained at a hormonal concentration and combination of 0.1 g/L BA and 1 g/L IBA which resulted in more than ten multiplied shoots from one initial shoot with length between 0.5-3 cm. It was also observed that the first multiplication process was achieved within four weeks however as the multiplied shoots were being multiplied further, adequate results were obtained within two weeks thus the multiplication rate increased with the number of cycles from the same shoot. Approximately ~2500 multiplied shoots were obtained within a period of nine months (Figure 4.2c).

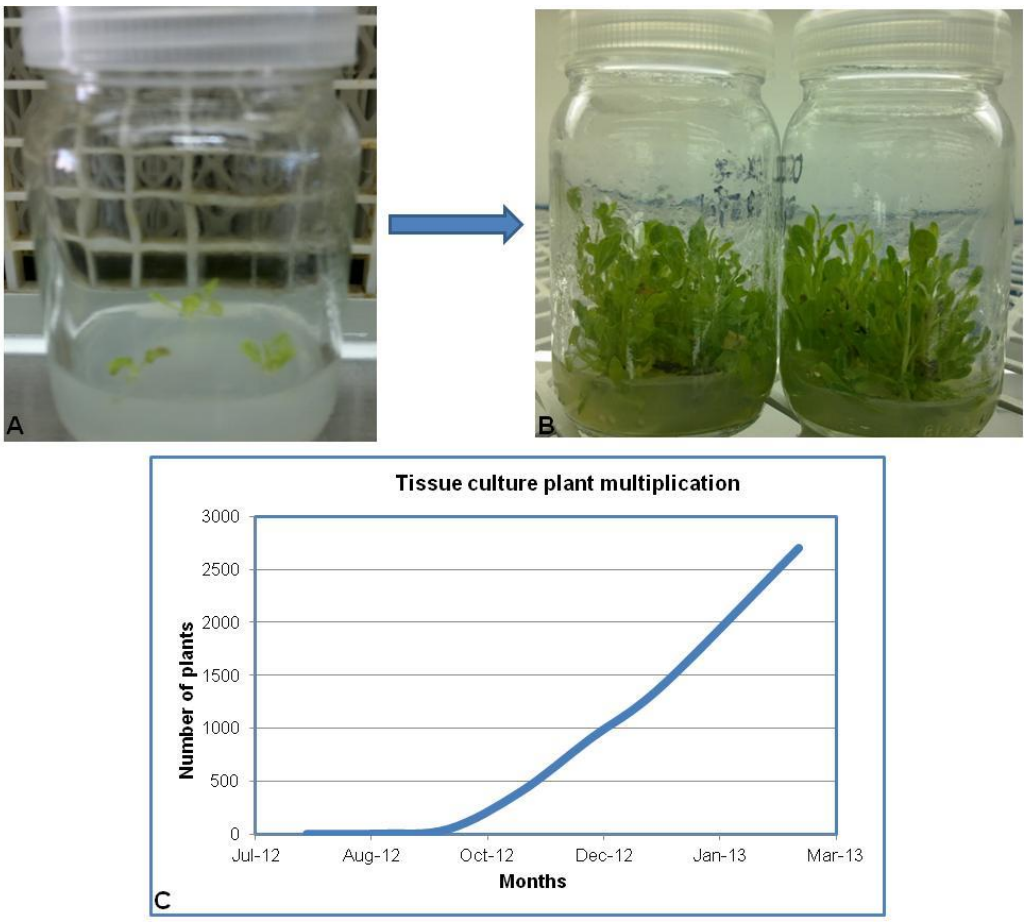


Figure 4.2: Multiplication of guayule shoots: a The excised shoots were transferred into multiplication media. **b** After 2-3 weeks, the shoots had multiplied represented by numerous shoots originating from 3 individual shoots. **c** The graph represents the number of plants obtained within 9 months from only 3 individual shoots, obtaining over 2500 shoots in seven months.

4.3.3 Rooting

Shoots of length above 2 cm were considered for the rooting step, since it was observed that shorter shoots had a lower survival rate during the acclimatization step and if survived they resulted in dwarf plants. In this study, a combination of 0.4 g/L BA and 0.1 g/L GA_3 successfully established about 90% of roots formation. It was also observed that washing off the agar from the rooted shoots with sterilized dH_2O only was not sufficient to maintain a sterile condition for the shoots during the acclimatization stage. Therefore, the use of fungicides, benomyl 500 WP and proplant was employed and successful in hindering fungal growth. This step was the most

fragile requiring careful washing to avoid root breakage and thus shoots death during acclimatization. Although Castillón and Cornish (2000) reported on the use of cellulose plugs to minimize the time of rooting and avoid breakage of newly roots, in this study we used agar in order to cut down on the cost, and care was taken as stated.

Figure 4.3: Induction of rooting: a Shoots from multiplication were transferred into rooting media **(**0.4 g/L BA and 0.1 g/L GA_3, pH 5.2 **b** Roots development after 2 weeks**. c** Two weeks after root induction the media was washed off with an fungicide solution mixture before transplanting into the genulite and coconut-peat mixture for acclimatization process.

4.3.4 Acclimatization and greenhouse plant establishment

Fully acclimatized guayule plants were obtained within 3 months, from the rooted plants to the transfer into perlite-peat mixture to greenhouse plants establishment in soil. Figure 4.4 shows the acclimatization process while Figure 4.5 shows the performance of greenhouse plants. A 90% plant survival rate was observed without diseases / infections episodes. Snails were observed to feed on the leaves of small plants but a snail repellent was effective in eliminating this problem.

Figure 4.4 Acclimatization process of rooted guayule seedlings: a Plantlets were transferred into a mixture of sterilized genulite and coconut-peat, covered with transparent polybag and incubated in the glasshouse (20°C). **b** Holes were gradually created on the bag on a daily basis after 3 weeks to allow air to pass through to harden the plantlets. **c** After 2 weeks the plants were transplanted into potting soil and left in the glasshouse for another 2 weeks before transferring them in the greenhouse.

Figure 4.5 Micropropagated plants in the greenhouse: The plants were kept in the greenhouse for 6 months before transplanting them in the field.

4.3.5 Field plants establishment

After 6 months in the greenhouse, acclimatized plants were transplanted in the field. The plant performance showed 50% survival rate. The plants are currently flowering and producing seeds which is being collected for germination purposes. The transplantation process and 1 year old plants is shown in Figure 4.6 below.

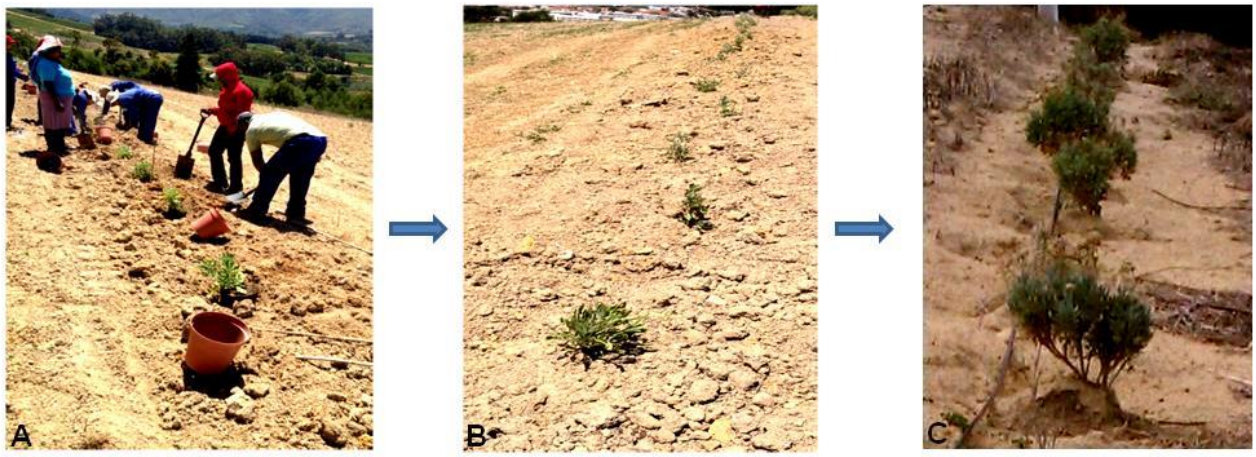


Figure 4.6: Field transplantation of OSU1 clonal plants: a six months old clonals were transplanted in Stellenbosch ARC experimental farm (Helderfontein, -33.918026, 18.869254). **b** Photos immediately after transplanting. **c** One year old plants in the field.

4.4 Discussion

Most previously published studies of *in vitro* propagation of guayule used BA concentration between (0.1 and 2.0 mg/L) for shoot multiplication (Castillon and Cornish 2000; Trautmann and Visser 1990; Smith 1983; Staba and Nygaard 1983). In this study maximum multiplication rate with longer shoots was obtained at a hormonal concentration and combination of 0.1 g/L BA and 1 g/L IBA which resulted in more than 10 multiplied shoots from one initial shoot with length between 0.5-3 cm. Other studies (Castillon and Cornish 2000; Trautmann and Visser 1990; Smith 1983) have shown the effectiveness of IBA in guayule roots induction. This study aimed to show that cytokinins (Kuroha and Satoh 2007; Soh *et al*., 1998) and gibberellins play an important role in root formation and development (Busov *et al*., 2006; Farquharson 2010) during *in vitro* rooting of guayule. Other studies have also emphasized on the importance of gibberellins in root development (Tanimoto 2005; Bidadi *et al*., 2010; Northcote and Haddon 1976). During the acclimatization stage, the covered plantlets were observed to grow in height, moreover after

holes were put through the polybag, the plantlets showed increased in colour intensity on the leaves. The acclimatization step showed to be a critical step in the micropropagation of guayule resulting in 70% survival rate of plantlets before transplanting into potting soil. The plantlets produced during acclimatization were long and strong which contributed to a higher survival rate. Guayule is an important plant known to produce circumallergenic natural rubber products. It is also important for the production of resins and its bagasse can be converted into high flammable wood pellets. However due to its natural dormancy character and mostly lack of embryo its establishment can be a challenge. The facultative apomixes of guayule and the higher E effects make guayule breeding challenging. If a high rubber producing line can be clonally propagated, it would be a big advantage and predominantly apomictic seed can also be obtained which would influence overall plant uniformity as shown in Chapter 2. This study was carried out to micropropagate a newly developed guayule line, OSU1 and further conducting some trials in the field. The results obtained show that this line can be micropropagated to produce plants which grow well in the greenhouse and the field. This study provides data for the establishment of OSU1 guayule line from seeds through *in vitro* propagation. Further research can focus on comparing the latex and resin yield of seed grown and micropropagated plants in the field and the data collected will provide insight of the best germination process for the desired product, either biomass, latex or resin.

Chapter 5: Proteome analysis of AZ5 guayule line leaf and stem bark tissue

Abstract

Proteomics techniques have been used as an important tool in plant research for advanced understanding of the regulation and function of proteins within a cell. Guayule is an important alternative in the natural rubber industry for the production of hypoallergenic rubber products especially in the medical industry. As a step towards molecular characterization, this study analysed the guayule leaf and stem bark proteome using gel based proteomics for the first time. Guayule proteins were extracted from leaf and stem bark using the phenol extraction method and quantified by Bradford assay. About 50 µg and 250 µg concentration of both leaf and stem bark total soluble proteins were separated respectively on one-dimensional and two-dimensional SDS PAGE. Approximately 32 leaf and 33 stem protein spots were manually selected and identified by matrix assisted laser/desorption ionization time in flight mass spectrometry. Protein sequences were analysed using Blast2Go PRO Version 3.3.5 through blasting, mapping and annotation. The theoretical pI/MW was computed using the ExPASy ProtParam tool. The Plant-mPLoc tool was used for the prediction of subcellular localization and the phylogenetic tree was generated using the PhyloT tool. Amongst the identified proteins, majority of them were stress responsive and anti-oxidative proteins which could have been influenced by the winter harvesting season. The identified proteins may lead to gene targets useful in understanding traits of interest in guayule. These protein profiles will provide reference data for comparison of leaf and stem bark protein changes under various South African conditions.

5.1 Introduction

Guayule is a desert plant native in the Chihuahuan desert of Mexico. In addition to its high quality hypoallergenic rubber, guayule is a perennial plant that is not used as a food source, and can grow in diverse environmental and climatic conditions including arid and semi-arid environments (Coates *et al*., 2001). These advantages make guayule a fascinating alternative source for the production of NR worldwide to date.

Plant proteomics is a useful tool to study the content and expression of proteins on a large scale (Wang *et al*., 2010). This tool will be very useful for analysing stem bark proteins where >90% of the rubber is synthesised in the cytoplasm of parenchyma cells. The protein expression profiles can provide information on rubber biosynthesis and other processes within the plant.

In guayule, a group of proteins, which include the allene oxide synthase (AOS) (Pan *et al*., 1995; Backhaus *et al*., 1991), the rubber transferase (RuT) (Cornish, 2001; Cornish and Backhaus *et al*., 2003), and the small rubber particle protein (SRPP, also known as guayule homolog GHS) (Kim *et al*., 2004) have been identified and may be associated with rubber biosynthesis.

In Hevea, the SRPP and the rubber elongation factor (REF) proteins may be involved in rubber biosynthesis (Gronover *et al.,* 2011), however REF has not yet been identified in guayule. In guayule the roles of these three putative rubber biosynthesis proteins still remain elusive (Ponciano *et al*., 2012). Further studies on guayule's protein profiles will provide information and identities of the rubber biosynthesis proteins (Sundar and Reddy, 2008; Sundar *et al*., 2003).

In guayule, rubber biosynthesis and deposition of rubber particles occurs in the cytoplasm and the particles later accumulate in the vacuole where they are stored (Goss *et al.*, 1984). The mechanism of rubber particle transport into vacuoles is not well understood. Rubber particles

first appear in the bark parenchyma cytosol of two and half month old guayule shrubs and begin to accumulate in the central vacuole in three month old plants (Benedict *et al*., 2009) and those containing Cytochrome p450 proteins bud from the endoplasmic reticulum (ER). After impounding in the vacuole, the membrane of the rubber particle is digested, releasing the rubber particles which accumulate into large masses (Benedict *et al*., 2009). Rubber accumulation in guayule is influenced by factors such as low temperature, light intensity, water stress and moisture. Together with the knowledge on these factors, which influence rubber accumulation, a clearer understanding of guayule's proteome will aid in the manipulation of guayule genetics to produce high rubber yields.

To further exploit guayule as an alternative source of NR, one must acquire working knowledge of guayule's overall proteome and/or genome. So far, no research has been published on the 2D SDS PAGE of guayule plants, a technique that is able to separate complex protein mixtures. Through this technique, known and novel proteins will be identified to generate a basic reference proteome. This study will extract leaf and stem bark proteins using the phenol extraction method which extracts high amounts of protein with few impurities through washing with methanol and acetone. Protein identifications will be generated using 2D SDS PAGE, MALDI TOF mass spectrometry and Blast2GO software.

5.2 Materials and methods

5.2.1 Sample collection and storage

From the Stellenbosch trial as discussed in Chapter 2, guayule line AZ5 was selected for proteome analysis because of its high latex yield content. Two year old plants were randomly

selected during winter and leaf and stem bark tissues were cut using a plant cutter (Tabor tools, Israel) and immediately transferred into 50 ml tubes. The tubes were transferred into an ice box containing liquid nitrogen. These samples were transported to the University of the Western Cape where they were stored at -80°C until further use.

5.2.2. Extraction of total soluble proteins from leaf and stem bark tissue

The stored tissue materials were removed from -80°C and ground into fine powder in liquid nitrogen using a pestle and mortar. The ground tissue (1 g) was transferred into a 15 ml falcon tube and 3 ml of the extraction buffer (500 mM Tris-HCl, 50 mM EDTA, 700 mM Sucrose, 100 mM KCl and adjust pH to 8.0 with HCl) was added. The sample was vortexed and incubated on a roller at 4°C for continuous mixing. Thereafter, 3 ml of Tris-buffered phenol was added, and the sample was incubated on a shaker for 10 min at room temperature. The sample mixture was centrifuged at 4300 xg for 10 min at 4°C and three separated layers were observed after centrifugation. The upper layer was carefully recovered and transferred into a fresh 15 ml falcon tube. About 3 ml of the extraction buffer was added to the recovered sample and thoroughly mixed for 3 min. This mixture was centrifuged at 4300 xg for 10 min at 4°C. The upper layer was recovered and transferred into a fresh tube and 5 volumes of precipitation solution (0.1 M ammonium acetate in cold methanol) were added, briefly vortexed and incubated at -20°C overnight. The samples were removed from the -20°C and centrifuged at 4300 xg for 10 min at 4°C. Cold acetone (5 ml) was added into the obtained pellet and vortexed until thoroughly mixed and centrifuged at 4300 xg for 10 min at 4°C. The acetone washing step was repeated twice and the pellet was dried at room temperature for 5 min. The 2X sample buffer was added to the pellet and vortexed until completely dissolved and kept at -20°C until further use.

5.2.3 Protein quantification

Protein concentration was determined using a modified Bradford assay method (Bradford, 1976). From the stock solution (2 mg/ml), BSA standards ranging from the concentration of 0 – 0.05 mg/ml and the total soluble protein extracts were prepared in 2 ml plastic cuvettes in triplicate as show in Table 5.1. About 900 µl of the 1X Bradford reagent dye (Biorad, USA) was added to all the standards and protein samples. These mixtures were incubated at room temperature for 5 min and the absorbance was measured at 595 nm using the Milton Roy Spectronic GENESYS 5 Spectrophotometer (Spectronic Analytical Instruments). The cuvette without BSA was used as a blank and the average of the obtained absorbance values were used to construct a standard curve which was used to determine the concentration of the total soluble protein samples.

Table 5.1: The preparation of BSA standards

BSA Conc. (mg/ml)	BSA (µl)	Urea buffer (µl)	0.1 M HCL (µl)	Distilled H_20 (µl)
0	0	10	10	80
0.005	1	9	10	80
0.01	2	8	10	80
0.02	4	6	10	80
0.04	8	2	10	80
0.05	10	0	10	80

5.2.4 Separation of proteins by 1D SDS PAGE

5.2.4.1 Gel preparation

A 1D gel was prepared using the 1 mm Mini PROTEAN® 3 System (Bio-Rad Laboratories) for gel casting containing a running tank with lid, casting frame and stand, spacer plate, short glass plate and comb. The 12% separating with 5% stacking gels were prepared as shown in Table 5.2. A volume of 4 ml resolving buffer was added into the glass plate and layered with 1 ml isopropanol until solidified. The isopropanol was blotted using a paper towel, stacking buffer was added and the comb carefully inserted avoiding bubbles. This setup was left at room temperature until completely solidified. The glass plate containing the gel with the comb was wrapped using a paper towel, immersed in 1X running buffer (100 ml 10X running buffer in 900 ml H_20), covered with foil and stored at 4°C overnight or until further use.

Table 5.2: The components of gel preparation

	12% Resolving gel (ml)	5% Stacking gel (ml)
Distilled Water	6.4	3.6
40% Acrylamide/Bis stock solution 37:5:1	4.5	0.6
1.5 M Tris-HCl, pH 8.8	3.8	-
0.5 M Tris-HCl, pH 6.8	-	0.6
10% SDS	0.15	0.05
10% APS	0.15	0.05
TEMED	0.006	0.005

5.2.4.2 Sample preparation and gel loading of protein sample

Protein samples were mixed separately in 1.5 ml Eppendorf tubes with 2X loading buffer (60 mM Tris pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 200 mM DTT, 0.025% (w/v) bromophenol blue) in 1:1 ratio. The mixture was incubated at 95°C for 3 min and pulse spinned for 10 sec before loading. The gel cassette was taken from 4°C, the comb removed and the wells gently rinsed under tap water before being transferred into the electrode chamber. The electrode chamber with the gel setup were placed inside the tank and 1X SDS running buffer was poured into the tank taking into account that the electrode chamber was filled without any leaking. The total concentration of 50 µg protein from leaf and stem bark from the biological replicates was loaded onto the gel in triplicate. The lid was placed on top on the tank and connected to the power source (PowerPac™ Universal Power supply, Biorad, USA). The gel was initially ran at 50 V for the first 30 min and then the voltage increased to 120 V until the loading dye reached the end of the gel cassette.

5.2.4.3 Gel staining

The gel was carefully recovered by separating the spacer plate from the short glass plate and stained with Coomassie brilliant blue stains prepared according to Table 5.3. The gel was immersed in Coomassie I stain in a container and microwaved for 40 sec then placed on an orbital shaker for at least 1 hr or overnight. The Coomassie I stain was carefully poured out and replaced with Coomassie II stain, microwaved again for 40 sec and placed on the orbital shaker. This process was also repeated using Coomassie III stain and thereafter the destaining solution was poured over the gel, microwaved for 40 sec and placed on the orbital shaker. The destaining process was repeated until the stain was removed sufficiently and a paper towel was placed by the sides of the container for fast removal of access stain. The destained gel was transferred into

a container with distilled water. After gel staining, individual gels were washed in distilled water, imaged using PharosFX Plus System (BIO-RAD).

Table 5.3: Preparation of Coomassie stain and destaining solution

	Coomassie I	Coomassie II	Coomassie III	Destainer
Acetic acid (ml)	250	250	250	250
1.25% (w/v) Coomassie stock (ml)	50	6.25	6.25	-
Isopropanol (ml)	625	250		-
1% Glycerol (ml)	-	-	-	25
dH_20 (ml)	1575	1993.75	1743.75	2225

5.2.5 Separation of proteins by 2D SDS PAGE

5.2.5.1 Sample preparation and re-swelling

Protein samples stored in urea buffer at -20°C were used for the 2D dimensional protein separations. About 250 µg of protein was prepared for all the individual samples in triplicate for 7 cm IPG strip pH range 4-7 (Biorad, USA). Protein samples were mixed with 2 µl BioLyte (3/10) ampholytes (Biorad, USA), 2 µl of 50% DTT dissolved in urea buffer with a pinch of bromophenol blue. Urea buffer was added to bring the samples to a total volume of 125 µl per strip. Samples were briefly vortexed and pulse spun in the centrifuge followed by placement in the individual channels in the Immobiline™ Dry Strip Reswelling Tray (GE Healthcare, Amersham, UK). Strips were placed gel side down in direct contact with the sample using forceps and avoiding bubbles followed by addition of enough mineral oil (Biorad, USA) to cover

the strips and retain the moisture. These were left for 12 hrs at room temperature for passive rehydration to reswell to the original thickness of 0.5 mm.

5.2.5.2 Iso-Electric focusing

After 12 hrs of reswelling, strips were removed using forceps, rinsed with distilled water and the remaining unabsorbed protein and mineral oil blotted out on a filter paper. Moistened wicks were placed at the extreme ends of the negative and the positive electrodes for the removal of impurities during the focusing process. Strips were individually placed gel side down in the focusing tray of the Biorad Protean® IEF Cell. Mineral oil was again added on top of the strips to avoid evaporation and the focusing process was done at 20°C using conditions in Table 5.4.

Table 5.4: Isoelectric focusing conditions

Step	Volts (V)	Duration
1	250	0.15 hr
2	4 000	1 hr
3	4 000	12 000 Vhrs

5.2.5.3 Strips equilibration

After IEF, IPG strips were equilibrated in SDS-containing buffers in order to solubilize focused proteins and allow SDS binding prior to second dimension SDS-PAGE. Focused IPG strips were incubated gel side up in reswelling tray channels containing 2.5 ml equilibration buffer I (6 M urea, 2% (w/v) SDS, 50 mM Tris/HCl, pH 8.8, 20% (v/v) glycerol and 1% (w/v) DTT) for 20

min followed by equilibration buffer II (6 M urea, 2% (w/v) SDS, 50 mM Tris/HCl, pH 8.8, 20% (v/v) glycerol and 2.5% (w/v) iodoacetamide) for another 20 min with gentle agitation at room temperature. Equilibrated gel strips were rinsed in 1X SDS PAGE running buffer and gently blotted on a filter paper. Isoelectric focused proteins were ready for separation on second dimension SDS-PAGE on mini gels as prepared in Section 5.2.5.1 however without the stacking gel.

5.2.5.4 2D SDS PAGE gel strips loading and staining

Leaf and stem bark proteins (250 µg) were separated on 12% resolving gels using the Mini-PROTEAN® 3 Electrophoresis Cell (Biorad, USA) (Laemmli, 1970) and a PowerPac™ Universal Power supply (Biorad, USA). Protein standards of known MW (PageRuler™ Unstained Protein Ladder Thermo Scientific, UK) were loaded together with the protein samples and electrophoresed under the same conditions. The voltage was first applied at 120 V and the protein sample was left to run until the bottom of the gel, gels were stained as illustrated in Section 5.2.5.3 and scanned using PharosFX Plus System (BIO-RAD).

5.2.6 Sample preparation for mass spectrometry analysis

Approximately 29 and 33 spots were randomly selected for the leaf and stem bark proteins, as shown in Figure 5.1, for analysis by mass spectrometry. The selection was based on the intensity of the protein spots which were excised and transferred into 500 µl Eppendorf tubes. Spots were destained using 25 mM ammonium bicarbonate (Am Bic) in 50% acetonitrile at room temperature with gentle vortexing. This process was repeated to ensure thorough cleaning of the spots so that the Coomassie stain will not interfere with mass spectrometry analysis. After all the

stain was removed, the washing solution was carefully taken out of the tubes making sure not to remove the gel piece. Afterwards, 100% acetonitrile was added into the gel pieces and left at room temperature for approximately 10 minutes until the gel pieces turned white. Acetonitrile was removed and the gel pieces left at room temperature to dry for at least 3 hrs. Approximately 10 µl of trypsin solution (10 µg/µl) was added into the gel pieces and incubated on ice for 45 min. The trypsin solution was gently removed and enough solution of 25 mM Am Bic was added making sure the gel pieces were completely covered and incubated at 37°C for 18 hrs. After the incubation period, an equal volume of TA30 (30% acetonitrile in 0.1% trifluoroacetic acid) was added into the tubes and incubated for 45 min and room temperature while vortexing. The sample was now ready for analysis and 0.5 µl of the sample was spotted on the PAC II HCCA pre-spotted anchor chip MALDI target plate for peptide mass fingerprinting. Peptides were ionized with a 337 nm laser and spectra were acquired in reflector positive mode at a 28 kV using 500 laser shots per spectrum with a scan range of m/z = 700 – 4000 Da. Spectra were internally calibrated using PAC II peptide calibration standard (Bruker Daltonics). This calibration method provided a mass accuracy of 50 ppm across the mass range 700 to 4000 Da. Peptide spectra of accumulated 3,000 shots were automatically processed using Biotools 3.2 software (Bruker Daltonics). Database search was performed with the MASCOT algorithm using the SwissProt and NCBInr database on a Bio-tools 3.2 workstation. The search parameters were: taxonomy - other green plants; enzyme - trypsin; missed cleavages -1; fixed modification - carbamidomethyl (C); variable modification - oxidation (M); precursor tolerance - 50 ppm; fragment tolerance - 0.7 Da. Candidate protein matches with molecular weight search score greater than 50 were considered as positive identifications.

5.2.7 Protein sequences data analysis

Protein lists were subjected to Uniprot knowledge base (http://www.uniprot.org/) to obtain the sequence. Obtained sequences were loaded into Blast2Go PRO Version 3.3.5 (Conesa and Gotz, 2008) (www.blast2go.org) for analysis through blasting, mapping and annotation. The theoretical pI/MW was computed using the ExPASy ProtParam tool (http://web.expasy.org/protparam/). The Plant-mPLoc tool was used for the prediction of subcellular localization of plant proteins including those with multiple sites. A phylogenetic tree was generated using the PhyloT tool (http://phylot.biobyte.de/) based on NCBI taxonomy in order to observe the relationship between guayule and the top hits species.

5.3 Results

5.3.1 Protein analysis

5.3.1.1 1D SDS PAGE

The separation pattern of the protein bands from leaf and stem bark tissue was uniform in all biological and technical triplicate showing reproducibility amongst the extractions. In the leaf protein separation, there was an intense band, which was observed between 35 and 45 KDa. A number of bands were observed <25 KDa and others in 45 and 66 KDa.

In the stem bark proteins, an intense band around 45 KDa was observed and a few faint bands at this concentration were visible around 35 KDa, above 45 KDa and at 66 KDa. Figure 5.2 shows the stem protein separation on a 1D gel.

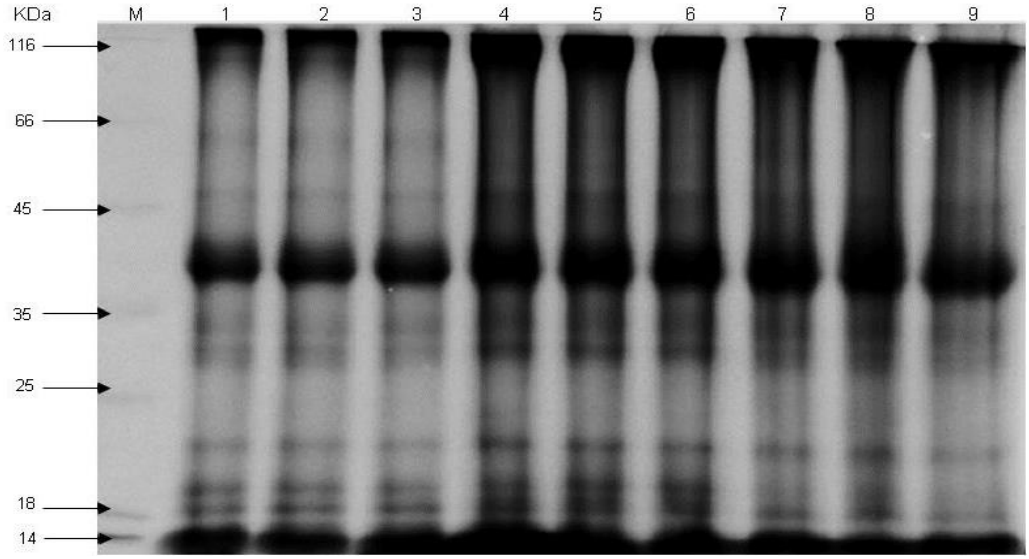


Figure 5.1: Leaf protein separation on 1D SDS PAGE: M-unstained marker, Lane 1-3 plant 1, 4-6 plant 2 and 7-9 plant 3.

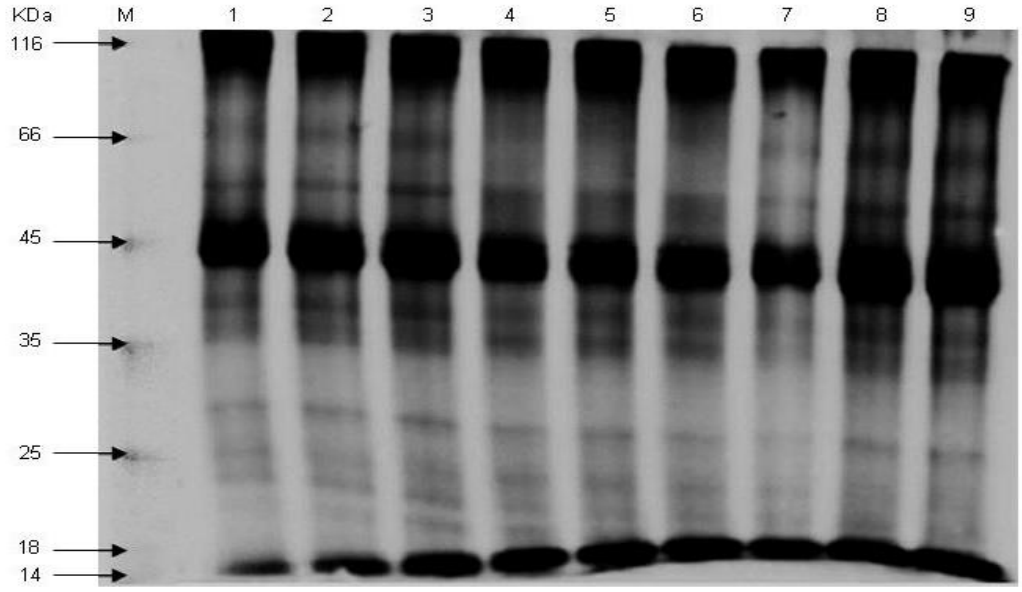


Figure 5.2: Stem bark protein separation on 1D SDS PAGE: M-unstained marker, Lane 1-3 plant 1, 4-6 plant 2 and 7-9 plant 3

5.3.2 2-DE SDS PAGE

This study reports the very first profile of guayule leaf proteins on 2D SDS PAGE. Figure 5.3 and 5.4 shows the separation of 250 µg leaf and stem bark proteins respectively on 2D Coomassie stained gel. Isoelectric points of the proteins were between pH 4-7 for both leaf and stem bark.

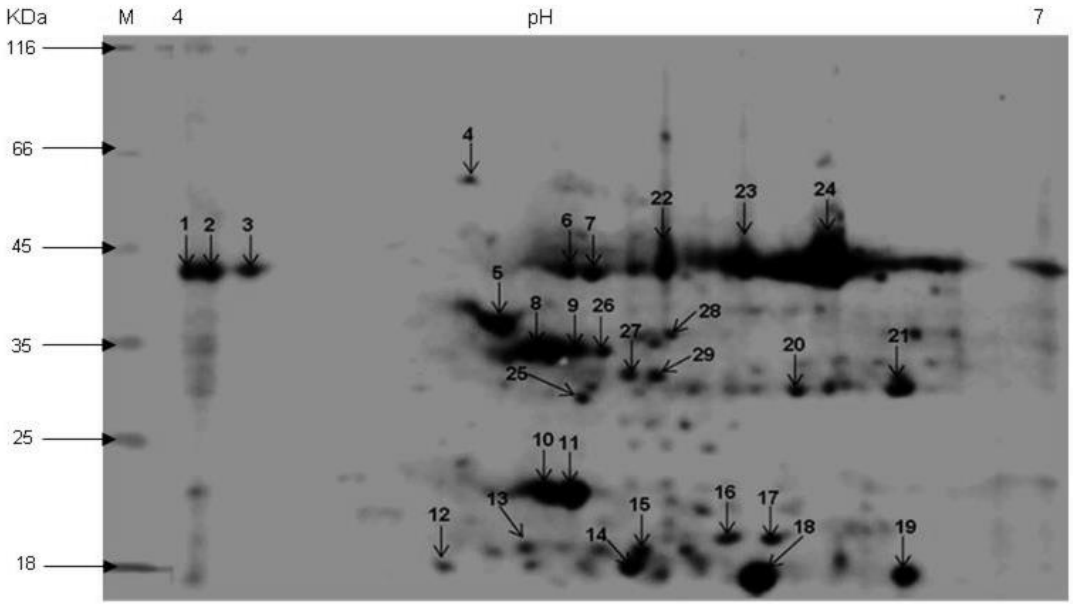


Figure 5.3: The 2D gel representing 250 µg of guayule leaf tissue: A representative gel image of leaf protein spots. About 28 spots were picked for the identification of proteins by mass spectrometry.

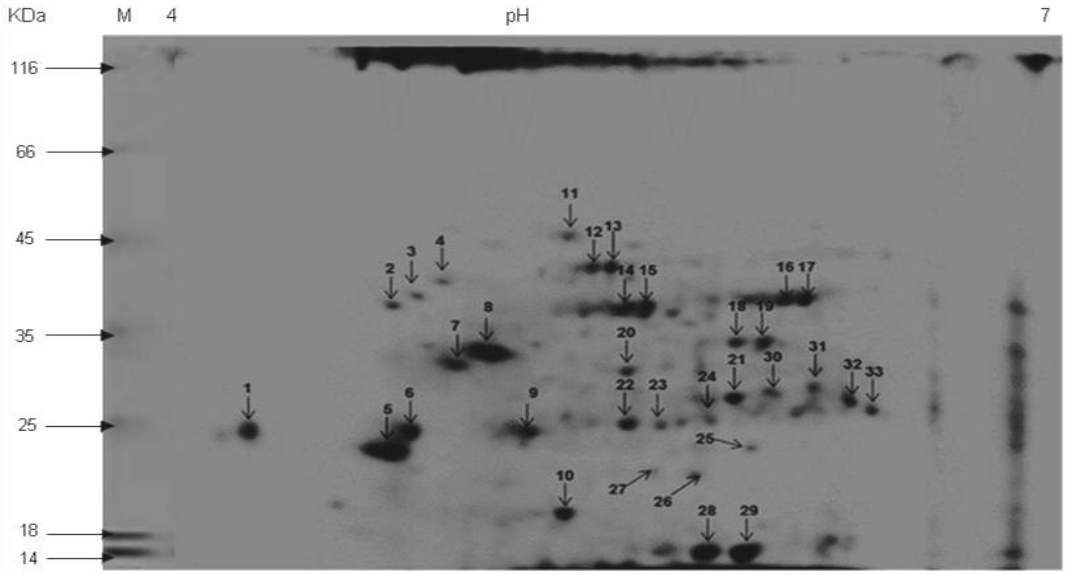


Figure 5.4: The 2D gel representing 250 µg of guayule stem tissue: A representative of stem bark protein spots. About 33 spots were picked for the identification of proteins by mass spectrometry.

5.3.2.3 Mass spectrometry analysis

After the spots of interest were selected, these samples were prepared according to the method in Section 5.2.7. Table 5.5 shows the proteins positively identified with the score, pI, MW, accession number and the reference species. About 22 and 15 of the selected spots were positively identified using mass spectrometry from leaf and stem bark tissue, respectively. Table 5.5 and 5.6 shows the proteins identified from leaf and stem bark respectively.

Table 5.5: Guayule leaf protein identification using mass spectrometry

Spot No.	Protein	Score	Pi		MW		Species	Location	Accession No.
			Theo.	Exp.	Theo.	Exp.			
1	RuBisCO	347.74	6.00	5.99	53.8	53.8	*Bartlettina sordida*	Chl	gi|62287181
2	RuBisCO	333.80	6.00	5.99	53.8	53.8	*Bartlettina sordida*	Chl	gi|62287181
3	RuBisCO	232.67	6.00	5.99	53.8	53.8	*Bartlettina sordida*	Chl	gi|62287181
4	Stromal HSP70	289.8	5.22	5.08	75.5	75.5	*Pisum sativum*	Chl	gi|399942
7	ATP synthase subunit beta	652.33	5.12	4.98	53.5	53.5	*Helianthus annuus*	Chl	gi|118573542
8	RuBisCO	287.6	6.09	6.06	48.6	48.6	*Flaveria bidentis*	Chl	gi|158726716
9	Glutamine synthetase	80.53	5.75	5.71	19	19	*Helianthus annuus*	Chl	gi|2197158
10	Oxygen-evolving enhancer protein 1	331.74	5.02	5.25	34.2	34.2	*Helianthus annuus*	Chl	gi|302595735
11	Oxygen-evolving enhancer protein 1	458.36	5.4	5.25	34.2	34.2	*Helianthus annuus*	Chl	gi|302595735
14	Triosephosphate isomerase	355.02	5.28	5.15	20.5	20.5	*lactuca sativa*	Chl	gi|1351274
16	Ascorbate peroxidase 2-like protein	128.21	4.61	4.46	13.2	13.2	*Tragopogon porrifolius*	Per	gi|290796650
17	Ascorbate peroxidase 2-like protein	194.81	4.59	4.44	13.2	13.2	*Tragopogon dubius*	Per	gi|290796648
21	Malate dehydrogenase	99.5	5.57	5.45	27.3	27.3	*Other green plants*	Mit	gi|3193222
22	Enolase	55.19	5.41	5.3	47.6	47.6	*Alnus glutinosa*	Cyto / Nuc	gi|3023685
23	RuBisCO	518.97	6.01	5.99	53.8	53.8	*Guizotia abyssinica*	Chl	gi|218546879
24	RuBisCO	728.58	6.00	5.94	53.8	53.8	*Helianthus annuus*	Chl	gi|116242748
28	RuBisCO	411.07	6.09	6.06	48.6	48.6	*Flaveria bidentis*	Chl	gi|158726716
29	Glutamine synthetase	70.95	5.63	5.55	47.7	47.7	*Daucus carota*	Chl / Mit	gi|8928128
30	Glutamine synthetase	142.18	5.75	5.71	19	19	*Helianthus annuus*	Chl / Mit	gi|2197158
31	Phosphoribulokinase	115.60	4.27	4.11	9.8	9.8	*Populus trichocarpa*	Chl	gi|145566786
32	Elongation factor TuB	381.26	5.69	5.62	46.7	46.7	*Nicotiana sylvestris*	Chl	gi|218312
33	Malate dehydrogenase	160.57	5.57	5.45	27.3	27.3	*Other green plants*	Mit	gi|3193222

Abbreviations: Chl – Chloroplast; Cyt – Cytoplasm; Mit – Mitochondrion; Nuc - Nucleus

Table 5.6: Guayule stem bark protein identification using mass spectrometry

Spot No.	Protein	Score	Pi		MW		Species	Location	Accession No.
			Theo.	Exp.	Theo.	Exp.			
9	Fructokinase-1	42.08	4.87	4.72	34.7	34.7	*Zea mays*	Chl	gi|162460362
10	Oxygen-evolving enhancer protein 1	252.47	5.40	5.25	34.2	34.2	*Helianthus annuus*	Chl	gi|302595735
11	HSP70	120.14	5.22	5.07	70.57	70.50	*Zea mays*	ER / Mit	gi|229464991
12	Glutamine synthetase	97.89	5.69	5.63	38.3	38.3	*Daucus carota*	Cyt	gi|8928127
14	Enolase	116.84	5.56	5.47	47.9	47.9	*Ricinus communis*	Cyt	gi|1169534
15	Enolase 1	106.43	5.57	5.48	47.8	47.8	*Hevea brasiliensis*	Cyt	gi|14423688
17	RuBisCO	391.04	6.00	6.13	53.8	52.6	*Guizotia abyssinica*	Chl	gi|62287181
18	S-adenosyl-L-methionine synthase	277.42	6.19	6.21	42.7	42.7	*Nicotiana suaveolens*	Chl	gi|223635283
19	S-adenosylmethionine synthase 4	299.93	5.96	5.73	42.5	42.5	*Populus trichocarpa*	Chl	gi|115361537
20	Actin-51	249.10	5.28	5.16	37.1	41.6	*Solanum lycopersicum*	Cyt	gi|3219772
25	Phenylcoumaran benzylic ether reductase	79.75	5.78	5.73	33.7	33.8	*Eucalyptus pilularis*	Cyt	gi|383081911
28	Ascorbate peroxidase 2-like protein	177.15	4.61	4.45	13.2	13.2	*Tragopogon porrifolius*	Per	gi|290796650
30	Glutamine synthetase leaf isozyme	63.59	6.77	6.93	47.2	39.7	*Phaseolus vulgaris*	Chl / Mit	gi|121353
31	Glutamine synthetase	103.51	5.71	5.66	38.9	38.9	*Stevia rebaudiana*	Cyt	gi|93280042
32	Malate dehydrogenase	104.07	5.89	5.86	35.4	35.4	*Beta vulgaris*	Chl	gi|11133601

Abbreviations: Chl – Chloroplast; ER – Endoplasmic Reticulum; Cyt – Cytoplasm; Per- Peroxisome; Mit – Mitochondrion

Using Blast2GO, enzyme class prediction was done for both the leaf and stem bark proteins. Approximately 4 and 5 classes of enzymes were identified from the 2D spots profiles in leaf and stem bark tissue, respectively, and the results are represented in Figure 5.5 and 5.6 below.

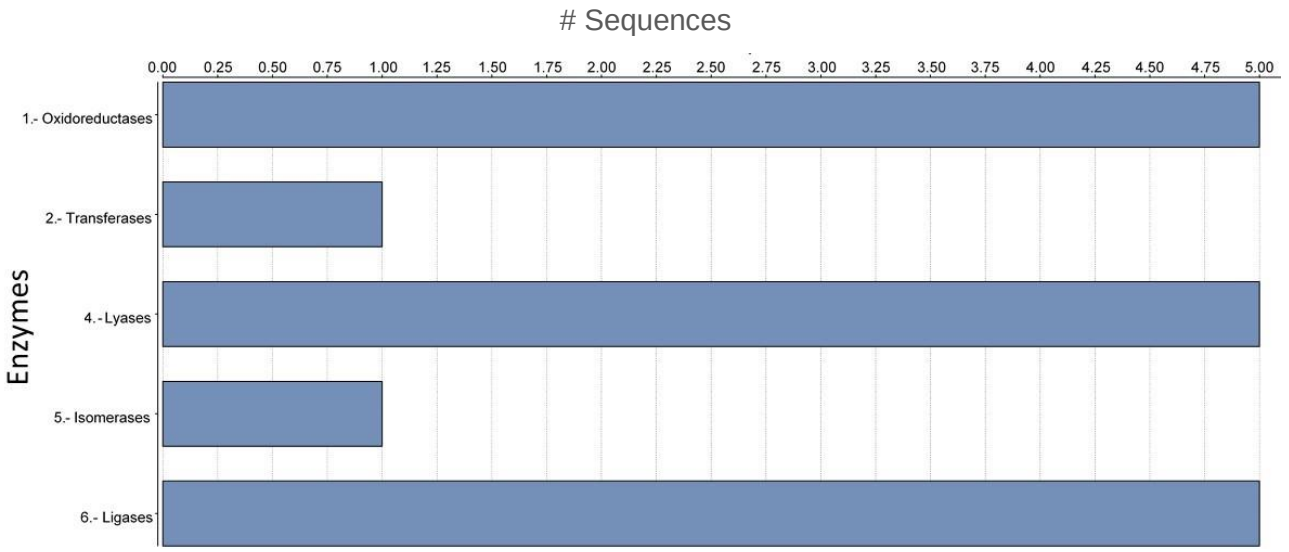


Figure 5.5: Prediction of protein classes observed in leaf tissue: Five classes of proteins were identified in leaf tissue. Oxidoreductases, lyases and ligases accounted for 50% and transferases and isomerases accounted for 10%.

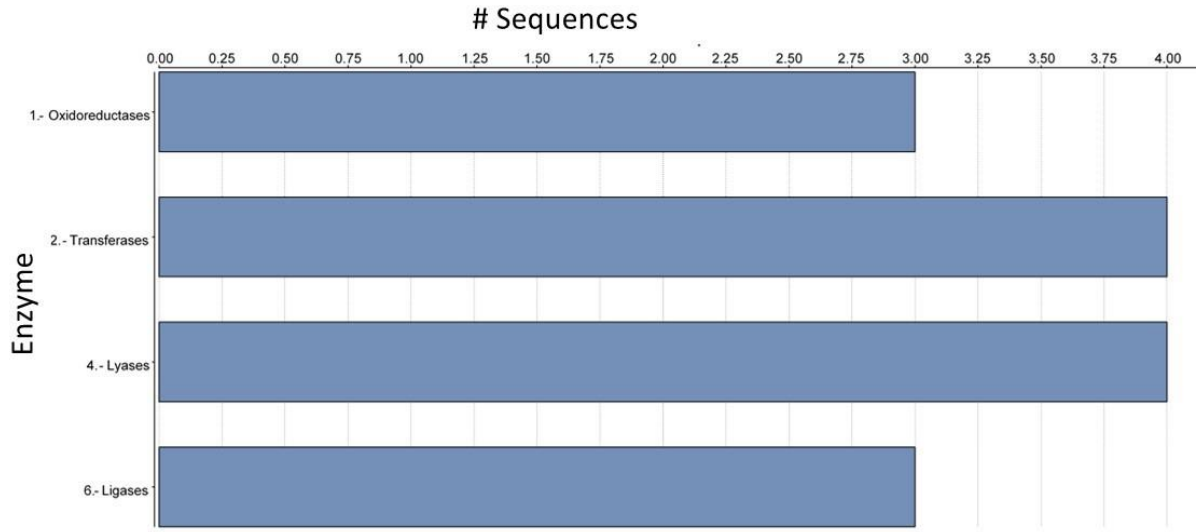


Figure 5.6: The prediction of enzyme classes observed in stem bark tissue: Majority of identified proteins belong in transferases and lyases class of proteins accounting for 40% followed by oxidoreductases and ligases at 30%.

5.4 Subcellular localization

Subcellular localization was done using the Plant-mPLoc tool which is able to identify even proteins with multiple sites as shown for protein spots 22, 29 and 30 in leaf proteins (Table 5.5) and also spot 11 and 30 in stem bark proteins (Table 5.6). Figure 5.7 and 5.8 shows the subcellular location of the leaf and stem bark proteins respectively.

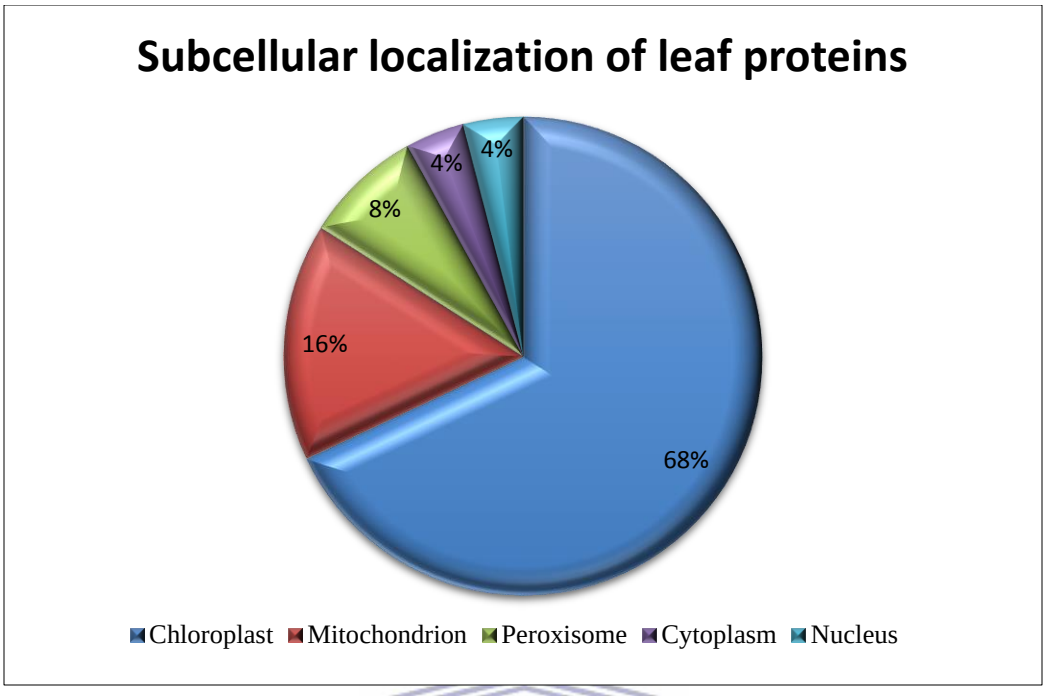


Figure 5.7: Subcellular localization of guayule leaf proteins using Plant-mPLoc tool: About 68% of identified proteins are located within the chloroplast; 16% within the mitochondrion; 8% within the peroxisome and 4% in both the cytoplasm and nucleus.

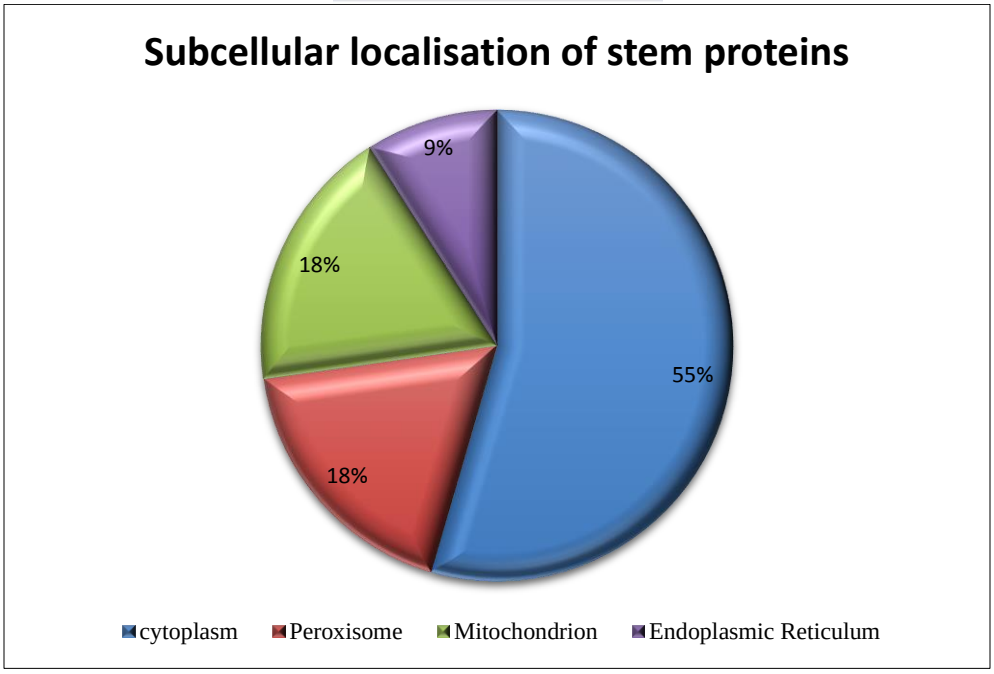


Figure 5.8: Subcellular localization of guayule stem bark proteins using Plant-mPLoc tool: About 55% of identified proteins are located within the cytoplasm; 18% within both the peroxisome and mitochondrion and 9% within the endoplasmic reticulum.

5.5 Protein mapping

Mapping of identified proteins was done using Blast2GO PRO tool where blast hits of functional data were retrieved from the Gene Ontology (GO) database. The GO database contains several million functionally annotated gene products for hundreds of different species and Blast2GO PRO used the NCBI database for sequence analysis. Functional categories from leaf and stem bark were grouped according to molecular function, biological process and cellular components as shown in Figure 5.9 and 5.10.

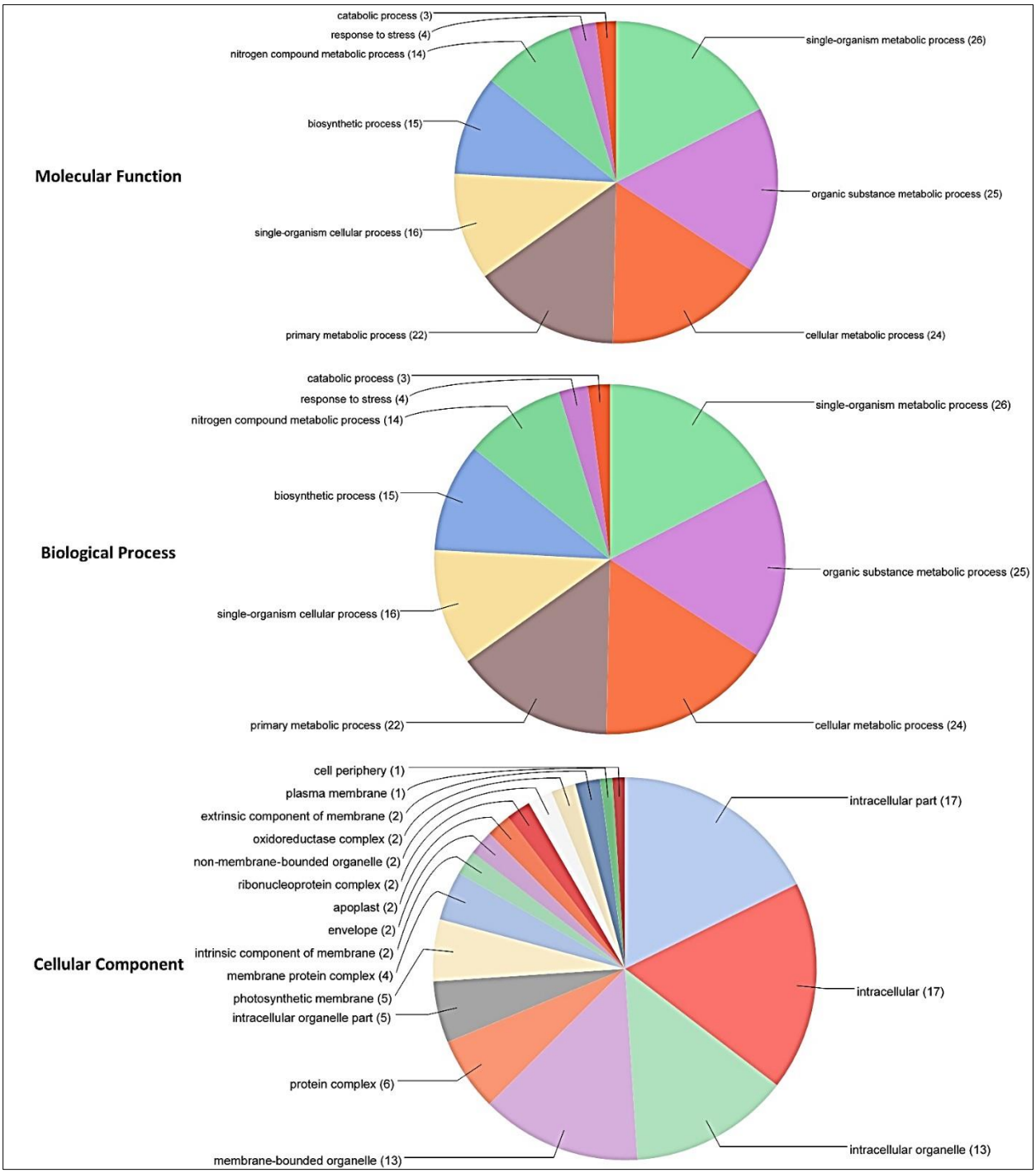


Figure 5.9: GO terms associated with leaf proteins: Level 3 Pie chart for molecular function, biological process and cellular component.

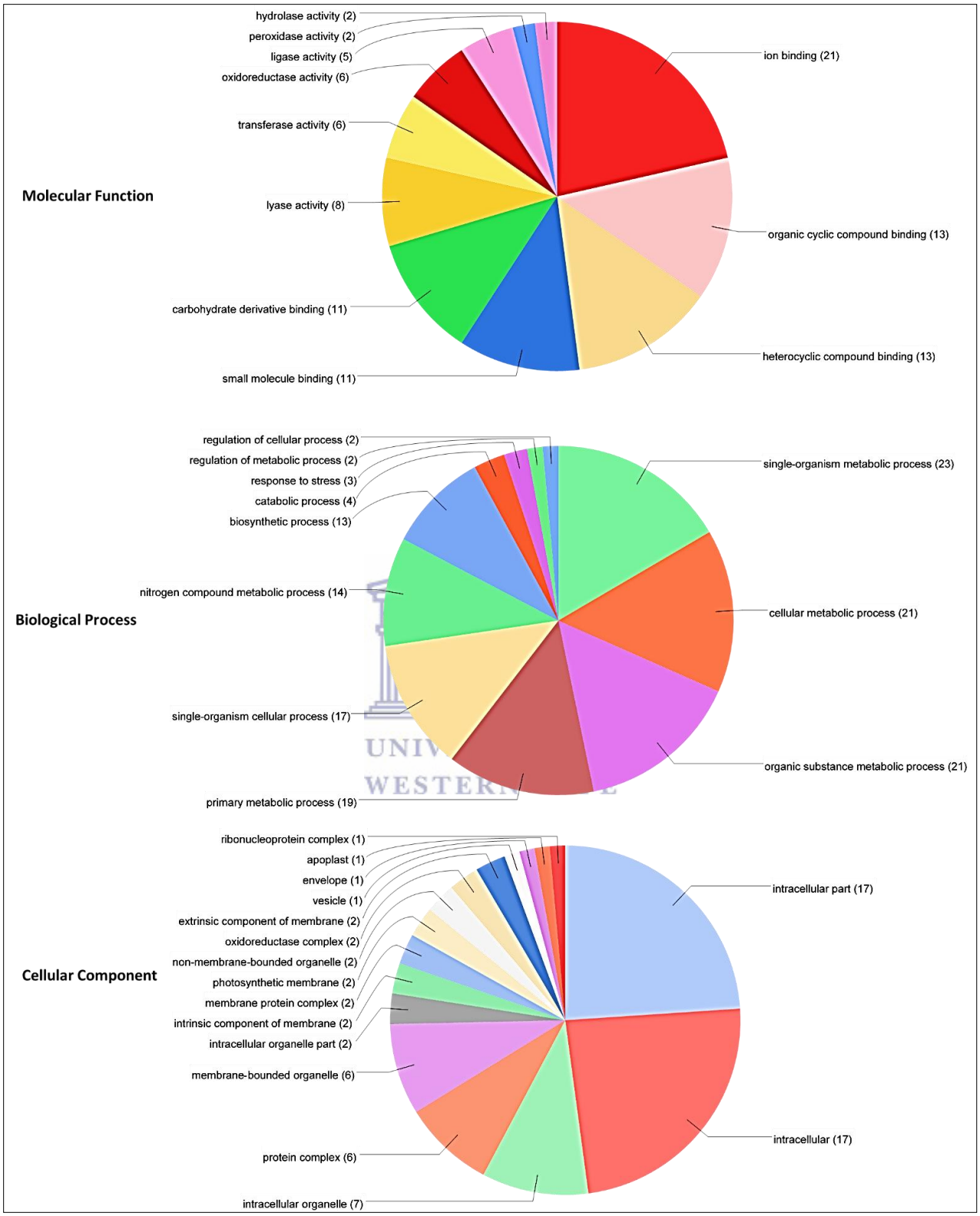


Figure 5.10: GO terms associated with stem bark proteins: Level 3 Pie chart for molecular function, biological process and cellular component.

The majority of assigned GO terms within leaf proteins with molecular function were largely involved in single organism metabolic processes, organic substance metabolic processes, cellular metabolic processes, single organism cellular processes and biosynthetic processes. Within the biological processes, their major functions include single organism metabolic processes, cellular metabolic processes, organism substance metabolic processes, single organism cellular processes and N compound metabolic processes. The major cellular component was intracellular, protein complexes and membrane bound organelles (Figure 5.9). For stem proteins GO terms association, the molecular functions included ion binding, lyase activity, organic cyclic compound binding, heterocyclic compound binding and carbohydrate derivative binding. The biological process and cellular component terms were identical to those of the leaf tissue (Figure 5.10). Using Blast2GO, the top hits species for the protein identified during this study was analysed. Figures 5.11 and 5.12 show the top hits species with leaf and stem bark proteins, respectively.

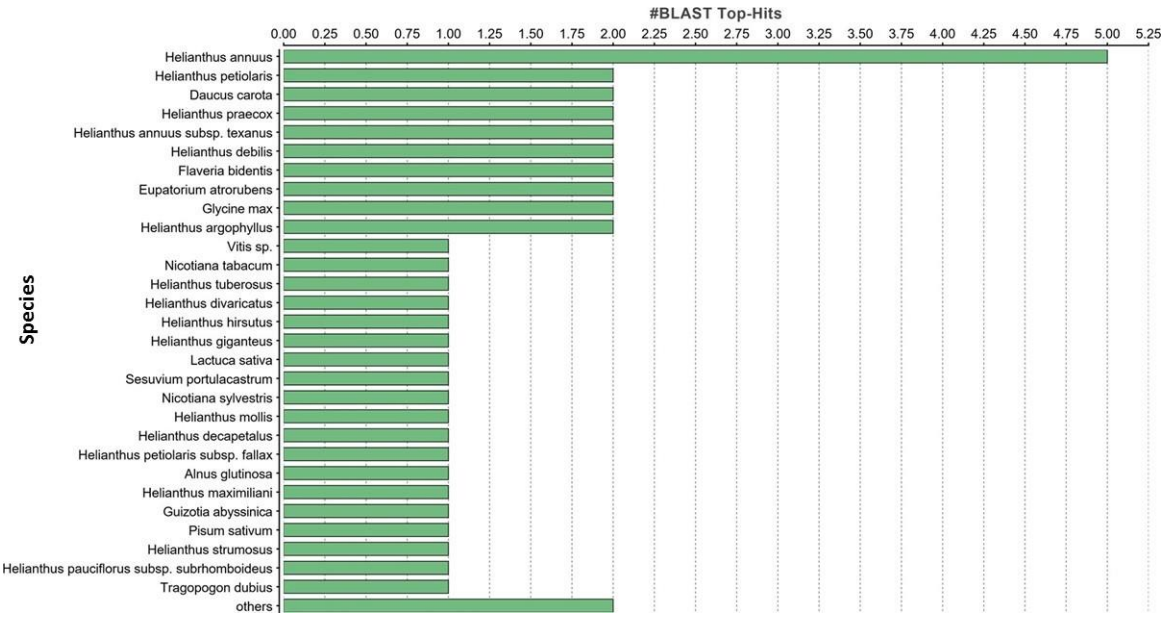


Figure 5.11: Top species classification in leaf protein sequences: The majority of the sequences were similar to those from Helianthus species, *Daucus carota* and *Flaveria bidentis*.

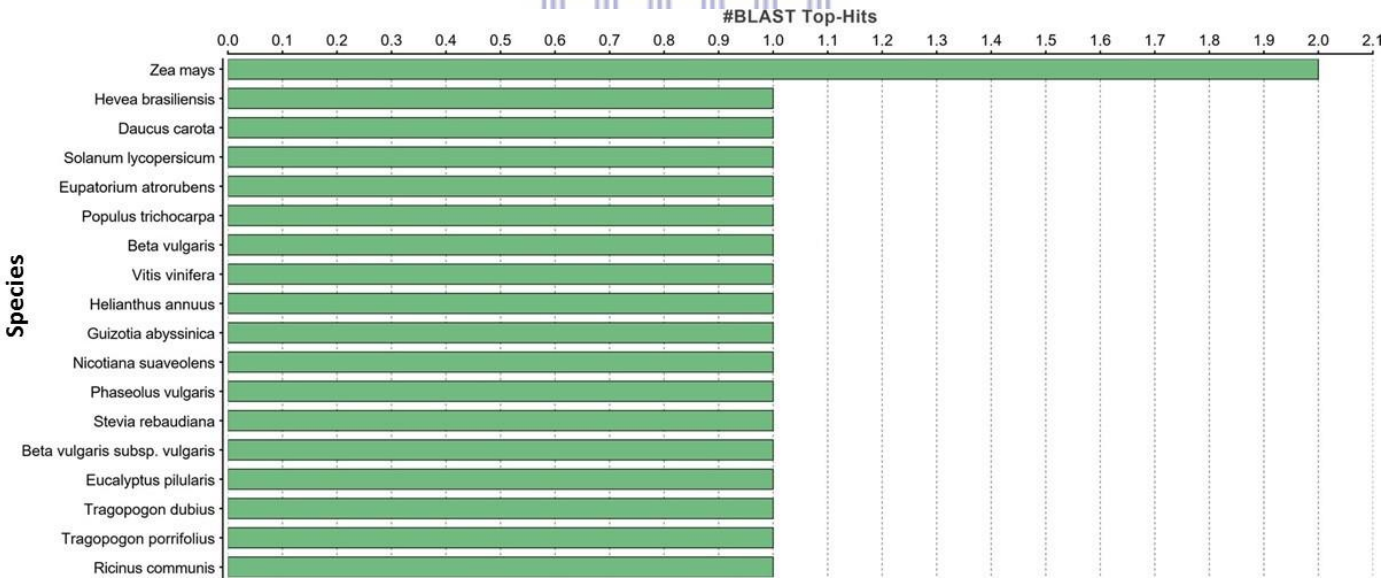


Figure 5.12: Top species classification in stem bark protein sequences: Most proteins sequences were similar to those of maize and *Hevea*

To establish the relationship of guayule with the top hits of plant species with high protein sequence identity, the PhyloT tool was used. Figures 5.13 and 5.14 show the species relationship in leaf and stem respectively.

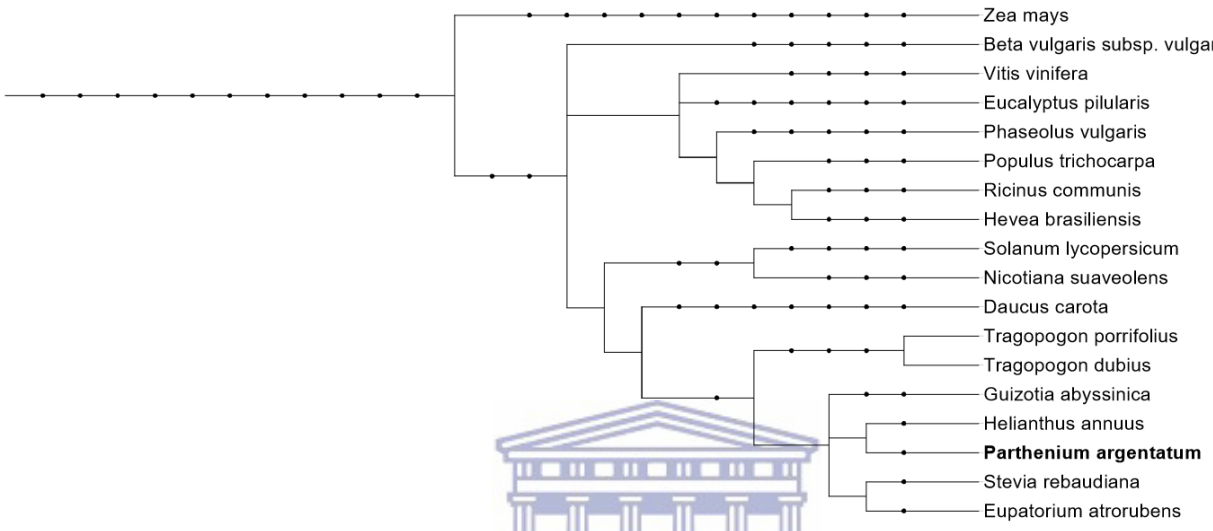


Figure 5.13: Phylogenetic tree of plant species associated with guayule leaf proteins: *Parthenium argentatum* was found to be closely related to Helianthus species, *Guizotia abyssinica*, *Eupatorium atrorubens* and *Flaveria bidentis.*

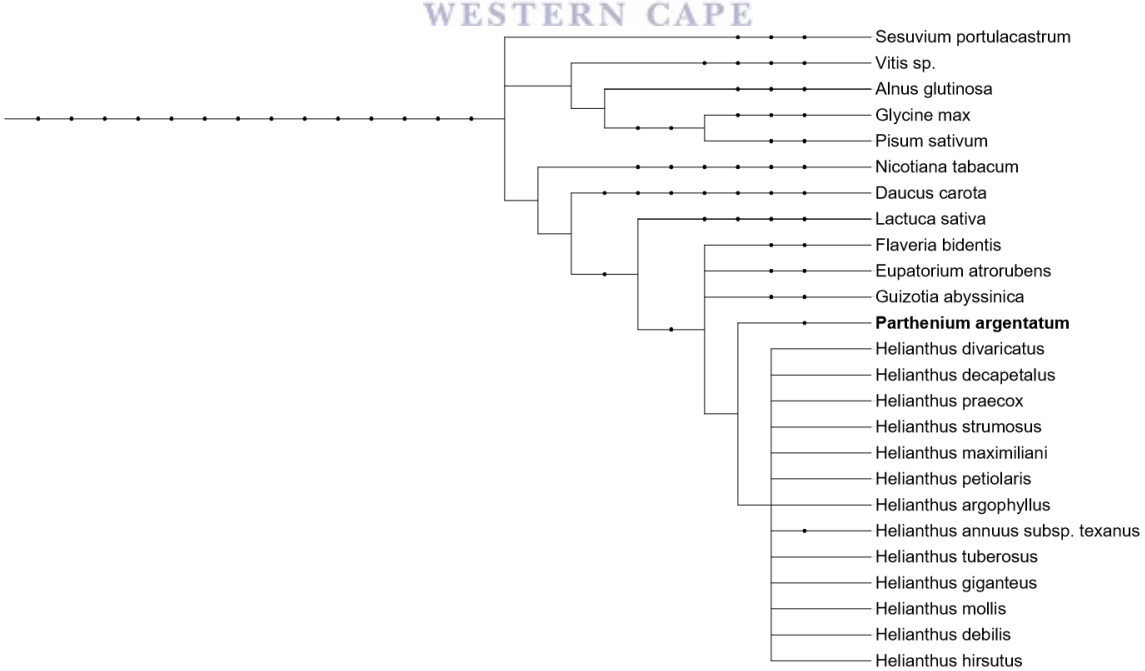


Figure 5.14: Phylogenetic tree of plant species associated with guayule in stem bark proteins: *Parthenium argentatum* proteins extracted from stem bark tissue were found to be closely related to those found in *Helianthus annuus*, *Stevia rebaudiana* and *Eupatorium atrorubens*.

5.6 Discussion

Extraction of both leaf and stem bark was established using the phenol extraction method and the protein concentrations were 1.6 and 1.2 µg/µl respectively. Guayule plants contain resins and high lipids which can interfere with 2D SDS PAGE spot separation, however a clean sample was obtained with this extraction method which resulted in good protein resolution.

Protein spots separated during 2D SDS PAGE were identified using the MALDI TOF MS together with NCBInr and Swissprot database searches. Guayule genome information is not yet available therefore the identification of its proteins was obtained based on the sequence information of other green plants. From the 29 and 33 protein spots which were selected from leaf and stem bark tissues, 22 and 15 were positively identified respectively (Table 5.5 and 5.6) which resulted in 75% and 45% protein identification. The higher protein identification in leaf can be explained by the photosynthetic functions which is the same for other green plants. A lower protein identification rate was observed in stem tissue which may be due to the lack of the guayule genome sequence data.

Protein identification using MALDI TOF MS revealed that about 90% of the identified proteins were stress related which might be due to the fact that samples were harvested during winter at low temperatures. Research done on the guayule protein profiles under stress conditions revealed protein expression changes (Sundar *et al*., 2003). Most of these proteins belonged to the classes of transferases, isomerases, lyases, ligases / synthetase and oxidoreductases. The identification of transferases can be associated with the rubber synthesis within the plant especially in the stem bark. Majority of the identified proteins in leaf tissue showed subcellular localization within the chloroplast, cytoplasm, peroxisome and mitochondrion and nucleus (Figure 5.7) whereas in the

stem tissue there was no chloroplast localization. High number of proteins showed localization within the cytoplasm, peroxisome, mitochondrion and endoplasmic reticulum (Figure 5.8).

Majority of the protein identified was ribulose bisphosphate carboxylase (RuBisCO) large subunit in the leaf tissue which belongs to the lyase class of enzymes together with ATP synthase, enolase and actin. RuBisCo is the most abundant expressed protein in plants especially in the leaves (Jensen, 2000). It has been found to play an important role in limiting carbon dioxide assimilation depending on environmental conditions (Parry *et al*., 2012). Actins are highly conserved proteins that are involved in various types of cell motility and are ubiquitously expressed in all eukaryotic cells. They play an important role in cytoplasmic streaming, cell shape determination, cell division, organelle movement and extension growth (Uniprot.org).

The oxidoreductase identified in this study include malate dehydrogenase (MDH) and phenylcoumaran benzylic ether reductase (PCBER). The MDH catalyses the NAD/NADH-dependent interconversion of the substrates malate and oxaloacetate. This reaction plays a major role in the malate/aspartate shuttle across the mitochondrial membrane, and in the tricarboxylic acid cycle within the mitochondrial matrix (Minárik *et al*., 2002). MDH enzyme have also been identified in guayule leaf extract as a marker for cultivar identification (Estilai *et al*., 1990). Research has found that PCBER helps in the reduction of phenylpropanoid dimers in plants to produce antioxidants that shield the plant against oxidative stress (Nicolas *et al*., 2014). The expression of PCBER may be influenced by the presence of oxidative stress proteins (OSPs) identified in both leaf and stem tissues.

A high number of OSPs was also observed and these proteins are involved in the defence mechanism of plants by any form of stress (Wassim *et al*., 2013). These proteins include

ascorbate peroxidase (APX), oxygen evolving enhancer 1 (OEE1) and ATP synthase. Expression of APX genes can be activated by specific factors such as pathogen attack (Rao *et al*., 1996), water deficiency (Mittler and Zilinskas, 1994) and salt stress (Mittova *et al*., 2004). The OSPs response are directly involved in the protection of plant cells against adverse environmental conditions (Caversan *et al*., 2012). The presence of OSPs is not surprising because plant tissues were harvested during winter season. This can be explained by the effect of low temperature stress on plant tissues. Similarly, APX was previously identified in guayule leaves which were exposed to low temperature (Sundar *et al*., 2004).

The presence of isomerases has been identified in other latex producing plants including *Eucommia ulmoides* (Chen *et al*., 2012) and *Hevea brasiliensis* (Han *et al*., 2000). Triosephosphate isomerase (TIM) is an enzyme which is involved in many metabolic pathways including glycolysis and catalyses the conversion of dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GAP) (Cui and Karplas, 2001). This enzyme have also been identified in guayule leaf extract as a marker for cultivar identification (Estilai *et al*., 1990).

Ligases, also known as synthetases, are a class of about 50 enzymes that catalyse reactions involving the conservation of chemical energy and provide a couple between energy-demanding synthetic processes and energy-yielding breakdown reactions. They catalyse the joining of two molecules, deriving the needed energy from the cleavage of an energy-rich phosphate bond. The glutamine synthetase (GS) protein is involved in N metabolism and its regulation (up or down) within the plant can have an effect on plant yield (Seabra *et al*., 2013). This can be one of the proteins of interest in this study to better understand guayule plants yields.

The heat shock protein 70 (HSP70) was also identified in both the stem and leaf tissues. The HSP70 is a housekeeping gene, which is involved in the maintenance and repair of proteins in response to stress and plays a major role in the environmental stress tolerance and adaptation (Mayer and Bukau, 2005). The presence of this protein may be influenced by the season of harvest which was in winter. Low temperature has been observed to activate the release of HSP70 (Sung *et al*., 2001; Mayer and Bukar, 2005; Qiao *et al*., 2015).

Transferases, including S-adenosyl L. methionine synthase (SAMS), phosphoribulo kinase and fructokinase-1 were identified. Transferases play a very important role during the phosphorylation process. The SAMS which is also called methionine S-adenosyltransferase is involved in the synthesis of ethylene from methionine during the ethylene biosynthesis pathway. This gene has been identified in Hevea to have weak ethylene-synthesizing ability in latex (Tang *et al*., 2016). Ponciano *et al* (2012) identified SAMS in guayule as a stress response protein as a result of cold. It has been observed that SAMS plays a role in polyamine synthesis which are secondary metabolites with roles in regulation of cold, drought and salt stress tolerance (Roje, 2006). Zeng *et al* (2000) observed that SAMS play a role in both the ethylene and caffeine biosynthesis in Hevea which has an influence in latex production. The presence of SAMS in guayule may be influenced by winter conditions as a source of stress which in turn contributes to high latex production. Fructokinases are involved in sucrose and fructose metabolism and are important for growth and development. Fructokinase can be expressed as Fructokinase 1 or 2 and in this study the Fructokinase 1 protein was identified. Depending on the plant, these fructokinases may have the same or different roles. It has been identified in *Solanum lycopersicum* (Odanaka *et al*., 2002), *Pisum sativum* (Turner *et al*., 1977) and *A. thaliana* (Gilkerson *et al*., 2012). Phosphoribulokinase is vital to the reductive pentose phosphate pathway

of CO_2 assimilation. It has been identified in maize, wheat and rice in relation to stress response in response to low temperatures (Nguyen *et al*., 2009) and drought (Zhao *et al*., 2011). Most of the proteins from the stem tissue were cytoplasmic, which is the same location where rubber biosynthesis occurs. The GO terms associated with leaf and stem sequences were observed using Blast2GO and these provided the information on the most important functions of the identified proteins in molecular function, biological process and cellular component.

In conclusion, leaf and stem bark proteins were successfully extracted using the phenol method. This resulted in approximately ≥ 50 protein spots separated in 2D SDS PAGE of which 22 (75%) and 15 (45%) were positively identified respectively. Subcellular localization provided us with data on where most of the leaf and stem proteins are localized. Enzyme classification showed the main functions within the plant. This study presents the first attempt at profiling guayule leaf and stem bark proteins using 2-DE SDS PAGE spots. These profiles allows us to embark in future research targeting one protein at a time in characterization towards genome sequencing. Furthermore this study will lead to research the guayule protein profile in summer for comparison between summer and winter protein expressions.

Chapter 6: Overall conclusion

Natural rubber is an important commodity used for the production of more than 40,000 products, including medical products and its use cannot be replaced by synthetic rubber (Venkatachalam *et al.*, 2013). Currently the global supply of NR comes from Hevea however worldwide allergic reactions have been reported (Sell and Visentainer, 2012). Guayule has been identified as an alternative for hypoallergenic rubber with comparable qualities to Hevea (Steward and Lucas, 1986). The study was influenced by how other countries embarked on finding different plants suited for their environmental / climatic condition for the purpose of reducing NR imports. The US also relied on NR import until they discovered guayule and the Russian dandelion NR producing plants. Natural rubber import in RSA is one of the crucial issues which must be tackled firstly through the establishment of its own NR plant.

The study evaluated the germination and development of guayule lines AZ1-AZ6 and OSU1 in the EC and WC regions of RSA. The results showed that these guayule lines grow well in these regions however seed quality and season of germination is crucial for increased germination. Darker-black seeds germinated in summer season showed higher germination rate as compared to those germinated in winter season. Furthermore darker blue seeds germinated better that pale-brown seeds. All the lines tested in this study showed increased height and canopy diameter in the EC as compared to the WC region. It can confidently be said that guayule plants can grow in the South African EC and WC regions and stand establishment is possible. Both EC and WC locations can support the establishment of guayule plants but specific lines should be chosen for each location for best performance. The findings from this study supports previous studies by Beekardt *et al* (2005) which showed that guayule cultivation is possible in RSA.

Natural rubber has been shown to start accumulating in guayule parenchyma cells within six months and mature plants can be observed within a year (Ponciano *et al*., 2012). The study investigated the extraction and quantification of guayule latex from 2 year old lines AZ1-AZ6, OSU1 and 11591 grown in the EC and WC. This chapter provided data on the lines with high rubber content in the EC and WC. Guayule latex can rapidly be extracted using well-established methods and quantified using the 1ml method (Cornish and Brichta, 2002).

Latex purification or creaming is an essential step for the production of concentrated latex, a raw material used for the manufacture of rubber products (Ochigbo *et al*., 2011). This study investigated the effect of temperature, time and Na-CMC concentration on latex creaming. Of the three molecular weights used in this study the optimal concentration for 90 000MW was observed to be 0.1% and 0.5% at RT and 4°C respectively. The optimal concentrations for 250 000MW were 1% and 0.5% at RT and 4°C respectively whereas creaming with 700 000MW, the results showed that high separation was observed using 1% at both RT and 4°C. Creaming can occur at both temperatures used however separation will depend on the concentration of the creaming agent. This purification process is ideal to use in the rural or developing areas as no heavy machinery is required thus its achievement is at a lower cost.

Although this study demonstrated that guayule can grow well in RSA, seed dormancy continues to be a challenge for future establishment of large fields (Jorge *et al*., 2006). This study established an *in vitro* propagation protocol suitable for generating large numbers of guayule line OSU1 transplants and demonstrated the successful transfer of the regenerated plants to the field. The results showed the importance of using micropropagattion techniques to surplus the seed grown plants for stands establishment.

Rubber biosynthesis is influenced by high expression of genes expressing important enzymes and proteins involved in the pathways leading to rubber synthesis (Chow *et al*., 2012). Despite identifying guayule as an alternative source of hypoallergenic rubber producing plant, the biochemistry and molecular mechanism of rubber biosynthesis, has not been fully studied and its genome is not yet sequenced (Gronover *et al*., 2011). Profiling its proteome will pave a way in characterizing important proteins involved in latex or biomass yields. The study investigated protein profiles of leaf and stem bark tissues during the winter season, a high rubber producing season for guayule. In conclusion, leaf and stem bark proteins were successfully extracted using the phenol method. This resulted in 75% and 45% of positively identified protein spots from the leaf and stem bark respectively. Subcellular localization provided us with data on where most of the leaf and stem bark proteins are localized. Enzyme classification showed the main functions within the plant. This study presents the first attempt at profiling guayule leaf and stem bark proteins using 2-DE SDS PAGE spots. These profiles allows us to embark in future research targeting one protein at a time in characterization towards genome sequencing. Furthermore this study will lead to research guayule's protein profile in summer for comparison between summer and winter protein expressions. Future studies will also use these novel 2D protein profiles as a reference map to further characterize guayule proteins.

The study has shown that guayule field can be established in RSA using direct seeding and micropropagation, however it is of importance to obtain an apomictic viable seed for establishment of uniform plants throughout. This will provide consistency in plant yields with less significant differences. As shown in Chapter 4 agro-processing can easily be done to produce guayule latex as a raw material for rubber products. Proteome profiling and identification can also be achieved using proteomics tools.

References

Angulo-Sánchez JL, de Rodríguez DJ, Rodriguez-García R (2002) Relationship between guayule biomass production, rubber synthesis and climatic conditions. Reprinted from: Trends in new crops and new uses. Janick, J., and Whipkey, A. (eds.). ASHS Press, Alexandria, VA

Backhaus RA, Cornish K, Chen SF, Huang DS, Bess VH (1991) Purification and characterization of an abundant rubber particle protein from guayule. Phytochemistry 30:2493-2497

Bedane GM, Gupta ML, George DL (2008) Development and evaluation of a guayule seed harvester. Industrial Crops and Products 28 (2): 177-183

Bedane GM, Gupta ML, George DL (2010) Development and evaluation of a guayule seed processing system. Industrial crops and products 31 (2): 378-384

Beilen V (2006) EPOBIO: Realising the economic potential of sustainable resources-bioproducts from non-food crops. Available from: http://cordis.europa.eu/publication/rcn/11572_en.html, accessesd 09 August 2016

Bekaardt CR (2002) Establishment of guayule (*Parthenium argentatum Gray*). M.Sc Agric thesis, University of Stellenbosch, South Africa

Bekaardt CR, Coffelt T, Fenwick JR, Wiesner LE (2010) Environmental, irrigation and fertilization impacts on the seed quality of guayule (*Parthenium argentatum Gray*). Industrial Crops and Products 31(3):427–36

Bekaardt CR, Pieterse PJ, Coetzee JH, Agenbag GA (2004) The breaking of seed dormancy of guayule (*Parthenium argentatum Gray*) by treatment with gibberellic acid and smoke water solution. African Journal of Plant and Soil 21(2):101–3

Bekaardt CR, Pieterse PJ, Reinten EY, Agenbag GA (2005) Rooting responses of guayule (*Parthenium argentatum Gray*) cuttings to hormone solutions. African Journal of Plant and Soil 22(4):266–8

Benedict CR, Goss R, Foster MA, Greer PJ (2009) The formation of rubber particles in developing cortical parenchyma of *Parthenium argentatum* plants exposed to the low temperatures of fall and winter of the Chihuahuan Desert. Industrial Crops and Products 30(3):403-406

Benedict CR, Goss R, Greer PJ, Foster MA (2011) The ultrastructure of low temperature stimulated rubber-producing cortical parenchyma in guayule. Industrial Crops and Products 33 (1): 89-93

Benedict CR, Goss R, Greer PJ, Foster MA (2013). A biochemical and ultrastructural analysis of rubber biosynthesis in *Parthenium argentatum Gray* in the Chihuahuan Desert. Industrial Crops and Products 45: 447-454

Benedict CR, Goss R, Paul J, Foster MA (2010) The formation of rubber producing cortical parenchyma cells in guayule (*Parthenium argentatum Gray*) by low temperature. Industrial Crops and Products 31:516–520

Benedict HM, McRary WL, Slattery MC (1947) Response of guayule to alternating periods of low and high moisture stresses. Botanical Gazette 108 (4): 535-549

Bidadi H, Yamaguchi S, Asahina M, Satoh S (2010) Effects of shoot-applied gibberellin/gibberellin-biosynthesis inhibitors on root growth and expression of gibberellin biosynthesis genes in *Arabidopsis thaliana*. Plant Root 4: 4-11

Blignaut JN, Ueckerman L, Aronson J (2009). Agriculture production's sensitivity to changes in climate in South Africa. South African Journal of Science 105: 61-68

Bonner J (1944) Effect of varying nutritional treatments on growth and rubber accumulation in guayule. Botanical Gazette 105:352-364

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72:248-54

Brathwaite N, Motala C, Toerien A, Schinkel M, Potter PC (2001) Latex allergy - The Red cross Children Hospital experience. South African Medical Journal 9 (9):750-751

Bucks DA, Roth RL, Powers DE, Chandra GR (1986) Direct seeding for economical guayule field establishment, In: Fangmeier, D.D., and Alcorn, S.M. Edition. Proc. Fourth Int. Guayule Res. Dev. Conf., Tucson, AZ, 16-19 Oct. 1985. Guayule Rubber Soc., Inc., College Station, TX pg. 77-87

Bucks DA, Nakayama FS, French OF, Rasnick BA, Alexander WL (1985) Irrigated guayule-Plant growth and production. Agricultural Water Management 10 (1): 81 - 93

Busov V, Meilan R, Pearce D, Rood S, Ma C, Tschaplinski T, Strauss S (2006) Transgenic modification of gai or rgl1 causes dwarfing and alters gibberellins, root growth, and metabolite profiles in Populus. Planta 224:288-299

Cannell GH, Youngner VB (1983) Irrigation, nitrogen and water use in guayule. Guayule rubber society annual meeting 20 - 23 June. Riverside, California; 1983. pg. 48

Castillón J, Cornish K (2000) A simplified protocol for micropropagation of guayule (*Parthenium argentatum Gray*). *In Vitro* Cellular & Developmental Biology-Plant 36(3):215-219

Caverzan A, Passaia G, Rosa SB, Ribeiro CW, Lazzarotto F, Margis-Pinheiro M (2012) Plant responses to stresses: role of ascorbate peroxidase in the antioxidant protection. Genetic Molecular Biology 35:1011-1019

Chandra GR, Bucks DA (1986) Improved quality of chemically treated guayule (*Parthenium argentatum Gray*) seeds, In: Fangmeier, D.D., and Alcorn, S.M. Edition. Proc. Fourth Int. Guayule Res. Dev. Conf., Tucson, AZ, 16-19 Oct. 1985. Guayule Rubber Soc., Inc., College Station, TX pg. 59-68

Chen R, Harada Y, Bamba T, Nakazawa Y, Gyokusen K (2012) Overexpression of an isopentenyl diphosphate isomerase gene to enhance trans-polyisoprene production in *Eucommia ulmoides Oliver*. BMC Biotechnology 12:78

Chotigeat W, Duangchu S, Phongdara A (2010) cDNA library from the latex of *Hevea brasiliensis*. Journal of Science and Technology 32 (6):555-559

Chow KS, Mat-Isa MN, Barahi A, Ghazali AK, Alias H, Zainuddin ZM, Hoh CC, Wan KL (2012) Metabolic routes affecting rubber biosynthesis in *Hevea brasiliensis* latex. Journal of Experimental Botany 63(5):1863-1871

Chow KS, Wan KL, Mat Isa MN, Bahari A, Tan SH, Harikrishna K, Yeang HY (2007) Insights into rubber biosynthesis from transcriptome analysis of *Hevea brasiliensis* latex. Journal of Experimental Botany 58(10):2429-2440

Coates W, Ayerza R, Ravetta D (2001) Guayule rubber and latex content - seasonal variations over time in Argentina. Industrial Crops and Products 14(2):85-91

Coates WE (1986) A guayule seed harvesting device. ASAE Applied Engineering in Agriculture 2(2): 70-72

Coates WE (1991) Chapter 10: Guayule harvesting equipment, guayule natural rubber - A technical publication with emphasis on recent findings. Guayule administrative Management Committee and USDA-CSRS pp. 241-260

Coffelt TA, Nakayama FS, Ray DT, Cornish K, McMahan CM, Williams CF (2009) Plant population, planting date, and germplasm effects on guayule latex, rubber, and resin yields. Industrial Crops and Products 29: 255–260

Collins-Silva J, Nural AT, Skaggs A, Scott D, Hathwalk U, Woolsey R (2012) Altered levels of the *Taraxacum kok-saghyz* (Russian dandelion) small rubber particle protein, TkSRPP3, result in qualitative and quantitative changes in rubber metabolism. Phytochemistry 79: 46-56

Conesa A, Götz S (2008) Blast2go: a comprehensive suite for functional analysis in plant genomics. International Journal of Plant Genomics 1: 13

Cornish K (2001) Similarities and differences in rubber biochemistry among plant species. Phytochemistry 57(7):1123-1134

Cornish K, Backhaus RA (1990) Rubber transferase activity in rubber particles of guayule. Phytochemistry 29:3809-3813

Cornish K, Backhaus RA (2003) Induction of rubber transferase activity in guayule (*Parthenium argentatum Gray*) by low temperatures. Industrial Crops and Products 17:83-92

Cornish K, Brichta JL (2002) Purification of hypoallergenic latex from guayule. Rubber crops pg 226- 233

Cornish K, Chapman MH, Nakayama FS, Vinyard SH, Whitehand LC (1999) Latex quantification in guayule shrub and homogenate. Industrial Crops and Products 10:121–136

Cornish K, Pearson CH, Rath DJ, Dong N, McMahan CM, Whalen M (2007) The potential for sunflower as a rubber-producing crop for the United States. Helia 30(46):157–66

Cornish K, Scott DJ (2005) Biochemical regulation of rubber biosynthesis in guayule (*Parthenium argentatum Gray*). Industrial Crops and Products 22:49-58

Cornish K, Siler DJ (1996) Hypoallergenic guayule latex: research to commercialization. American Chemical Society 10:141-156

Cornish K, Whitehand LC, Van Fleet JE, Brichta JL, Chapman MH, Knuckles BE (2005) Latex yield and quality during storage of guayule (*Parthenium argentatum Gray*) homogenates. Industrial Crops and Products 22:75-85

Cui Q, Karplus M (2001) Triosephosphate Isomerase: A Theoretical Comparison of Alternative Pathways. Journal of American Chemistry Society 123:2284-2290

De Beer C, Cilliers J, Truter EJ, Potter PC (1999) Latex gloves: More harm than good. Technology South Africa 13(1):282-288

de Silva RC (2014) Rubber additive contact dermatitis. Current Allergy and Clinical Immunology 27 (3):200-206

Dhar AC, Kavi Kishor P B, Rao AM (1989) *In vitro* propagation of guayule (*Parthenium argentatum*) - a rubber yielding shrub. Plant Cell Reproduction 8:489-492

Dierig DA, Ray DT, Thompson AE (1989) Variation of agronomic characters among and between guayule lines. Euphytica 44:265-271

Dissanayake P, George DL, Gupta ML (2004) Performance of improved guayule lines in Australia. Industrial Crops and Products 20(3):331-8

Dissanayake P, George DL, Gupta ML (2007) Improved guayule lines outperform old lines in southeast Queensland. Industrial Crops Products 25:178-189

Dissanayake P, George DL, Gupta ML (2008) Direct seeding as an alternative to transplanting for guayule in Southeast Queensland. Industrial Crops and Products 27:393-399

Dissanayake P, George DL, Gupta ML (2010) Effect of light, gibberellic acid and abscisic acid on germination of guayule (*Parthenium argentatum Gray*) seed. Industrial Crops and Products 32:111-7

Estilai A, Hashemi A, Waines JG (1990) Isozymes in diploid, triploid and tetraploid guayule. Horticulture Science 25: 346

Farquharson KL (2010) Gibberellin-auxin crosstalk modulates lateral root formation. The Plant Cell 22(3):540

Foster MA, Coffelt T (2005) Guayule agronomics: establishment, irrigated production, and weed control. Industrial Crops and Products 22(1):27-40

Foster MA, Fowler JL, Kleine LG, Puppala N (1999) Establishing new guayule lines by direct seeding. Industrial Crops and Products 9:93–100

Foster MA, Gabel SE, Ward TS, Kleine LG, McCann PK, Moore J (1991) Morphological characters as indicators of rubber content in guayule (*Parthenium argentatum*-Compositae). SIDA 14:339-367

Foster MA, Kleine LG, Moore J (1993) Response of direct-seeded guayule to pre-emergence herbicides. Journal of the American Oil Chemists Society 70:12

Foster MA, Moore J (1992) Direct-seeding techniques for guayule stand establishment in West Texas. Journal of Production Agriculture 5:163-167

George D, Gupta M, Dissanayake P (2005) Evaluating new guayule varieties for low-allergenic rubber production: a report for the Rural Industries Research and Development Corporation, Rural Industries Research and Development Corporation, Barton, A.C.T. Available from: http://trove.nla.gov.au/work/20318716?q&versionId=46559814+210627072,Accessed 08 August 2016

Gibson R (2013) Seed of the week - guayule. Growing with science blog. Available from: http://blog.growingwithscience.com/2013/01/seed-of-the-week-guayule. Accessed 19 December 2016.

Gilkerson J, Perez-Ruiz JM, Chory J, Callis J (2012) The plastid-localized pfkB-type carbohydrate kinases fructokinase-like 1 and 2 are essential for growth and development of *Arabidopsis* thaliana. BMC Plant Biology 12:102

Gilliland MG, Van Staden J (1986) Cyclic patterns of growth and rubber deposition in guayule *Parthenium argentatum*. Suggestions for a management programme. South African Journal of Plant and Soil 3(1):21-26

Goss RA, Benedict CR, Keithly JH, Nessler CL, Stipanovic RD (1984) cis-Polyisoprene synthesis in guayule plants (*Parthenium argentatum Gray*) exposed to low, nonfreezing temperatures. Plant Physiology 74:534-537

Gronover CS, Wahler D and Prüfer D (2011) Natural rubber biosynthesis and physic-chemical studies on plant derived latex. Biotechnology of biopolymers, Prof. Magdy Elnashar (Ed.), InTech, Available from: https://www.intechopen.com/books/biotechnology-of-biopolymers/natural-rubber-biosynthesis-and-physic-chemical-studies-on-plant-derived-latex

Gutie´rrez C, Gonzalez-Coloma A, Hoffmann JJ (1999) Anti-feedant properties of natural products from *Parthenium argentatum*. Industrial Crops and Products 10: 35–40

Haddon L, Northcote DH (1976) The influence of gibberellic acid and abscisic acid on cell and tissue differentiation of bean callus. Journal of Cell Science 20(1):47-55

Hammond B, Polhamus L (1965) Research on guayule (*Parthenium argentatum*): 1942 - 1959. Technical. Washington D.C.: Agricultural Research Service, United States Department of Agriculture

Han KH, Shin DH, Yang J, Kim IJ, Oh SK and Chow KS (2000) Genes expressed in the latex of *Hevea brasiliensis*. Tree Physiology 20:503-510

Hayashi Y (2009) Production of natural rubber from Para rubber tree. Plant Biotechnology 26(1):67-70

Hoffman GJ, Shannon MC, Maas EV, Grass L (1988) Rubber production of salt-stressed guayule at various populations. Irrigation Science 9:213-226.

Jensen RG (2000) Activation of Rubisco regulates photosynthesis at high temperature and carbon dioxide. Proceedings of the National Academy of Sciences 97(24):12937-12938

Jorge MH (2005) Germination and characterization of guayule (*Parthenium argentatum Gray*) seed. Doctor of Philosophy thesis, University of Arizona, Department of Plant Science

Jorge MHA (2005) Germination characterization of guayule seed by morphology, mass and, x-ray analysis. Industrial Crops and Products 22:59-63

Jorge MHA, Veatch-Blohm ME, Ray DT (2007) Quality of guayule seed separated by physical attributes. Industrial Crops and Products 25:55-62

Jorge MHA, Veatch-Blohm ME, Ray DT, Foster MA (2006) Guayule seed germination under different conditioning treatments. Industrial Crops Production 24:60-65

Kang H, Kang MY, Han KH (2000) Identification of natural rubber and characterization of rubber biosynthetic activity in fig tree. Plant Physiology 123:1133-1142

Kim IJ, Ryu SB, Kwak YS, Kang H (2004) A novel cDNA from *Parthenium argentatum Gray* enhances the rubber biosynthetic activity *in vitro*. Journal of Experimental Botany 55:377-385

Kim IJ, Ryu SB, Kwak YS, Kang H (2004) A novel cDNA from *Parthenium argentatum Gray* enhances the rubber biosynthetic activity *in vitro*. Journal of Experimental Botany 55:377-385

Kuroha T, Satoh S (2007) Involvement of cytokinins in adventitious and lateral root formation. Plant Root 1:27-33

Macrae S, Gilliland MG, Van Staden J (1986) Rubber production in guayule: Determination of rubber producing potential. Plant Physiology 81:1027-1032

Mayer MP, Bukau B (2005) Hsp70 chaperones: cellular functions and molecular mechanism. Cellular and Molecular Life Science 62(6):670-684

McCallum WB (1926) The botany and cultural problems of guayule. Industrial Engineering Chemistry 18:1121-1124

Mehta IJ, Dhillon SP, Hanson GP (1979) Trichome morphology as an indicator of high rubber-bearing guayule *Parthenium argentatum* plants in native populations. American Journal of Botany 66:796-804

Mills D, Benzioni A, Forti M (1989) Performance of USDA guayule lines in the Northern Negev of Israel 1. Economic Botany 43:378-85

Milthorpe P, Paterson-Jones JC, Whitworth JW, Abel G, Miller WP (1991) Case histories of guayule production in Australia, South Africa, and the United States. In: Whitworth, J.W.,Whitehead, E.E. (Eds.), guayule natural rubber. Office of Arid Lands Studies, University of Arizona, Tucson, AZ, pp. 367–385

Minárik P, Tomásková N, Kollárová M, Antalík M (2002) Malate dehydrogenases-structure and function. General Physiology and Biophysics 21(3):257-65

Mitchell JW, Geraldine Whiting A, Benedict HM (1944) Effect of light intensity and nutrient supply on growth and production of rubber and seeds by guayule. Botanical Gazette, The University of Chicago Press, pg 83 -95

Mittler R, Zilinskas BA (1994) Regulation of pea cytosolic ascorbate peroxidase and other antioxidant enzymes during the progression of drought stress and following recovery from drought. Plant Journal 5:397-405

Mittova V, Guy M, Tal M, Volokita M (2004) Salinity upregulates the antioxidative system in root mitochondria and peroxisomes of the wild salt-tolerant tomato species *Lycopersicon pennellii*. Journal of Experimental Botany 55:1105-1113

Miyamoto S, Bucks D (1985) Water quantity and quality requiments of guayule:current assessment. Agricultural Water Management 10:205-19

Miyamoto S, Davis J, Madrid L (1990) Salt tolerance of guayule. Texas Agricultural Experiment Station, Texas A&M University, Bull. 1651, College Station, TX, pg 18

Muller CH (1946) Root development and ecological relations of guayule. Technical Bulletin No. 923. December 1946.United States Department of Agriculture, Washington, D.C.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiology of Plant 15:73-93

Nakayama FS (1992) Chapter 25: Guayule as an alternative source of rubber. Developments in Crop Science 23:568-596

Nguyen HT, Leipner J, Stamp P, Guerra-Peraza O (2009) Low temperature stress in maize (*Zea mays L.*) induces genes involved in photosynthesis and signal transduction as studied by suppression subtractive hybridization. Plant Physiology Biochemistry 47(2):116-22

Nicolás C, Hermosa R, Rubio B, Mukherjee PK, Monte E (2014) Trichoderma genes in plants for stress tolerance- status and prospects. Plant Science 228:71-78

Nix HA (1986) Land evaluation for potential guayule rubber production in Australia. In: Stewart, G. A., and Lucas, S. M. (Eds.) potential production of natural rubber from guayule (*Parthenium*

argentatum) in Australia. Commomwealth Scientific and Industrial Research Organization. Melborne, Australia, pg 27-58

Ochigbo SS, Lafia-Araga RA, Suleiman MAT (2011) Comparison of two creaming methods for preparation of natural rubber latex concentrates from field latex. African Journal of Agricultural Research 6(12):2916-2619

Odanaka S, Bennett AB, Kanayama Y (2002) Distinct physiological roles of fructokinase isozymes revealed by gene-specific suppression of Frk1 and Frk2 expression in tomato. Plant Physiology 129:1119-1126

Ohya N, Koyama PT (2001) Biosynthesis of natural rubber and other natural polyisoprenoids. Biopolymers 73: 81

Pan Z, Durst F, Werck-Reichhart D, Gardner HW, Camara B, Cornish K, Backhaus RA (1995) The major protein of guayule rubber particles is a cytochrome P450. Characterization based on cDNA cloning and spectroscopic analysis of the solubilized enzyme and its reaction products. Journal of Biological Chemistry 270:8487-8494

Parry MAJ, Andralojc PJ, Scales JC, Salvucci ME, Carmo-Silva AE, Alonso H, Whitney SM (2012) Rubisco activity and regulation as targets for crop improvement. Journal of Experimental Botany 64(3):717-730

Ponciano G, McMahan CM, Xie W, Lazo GR, Coffelt TA, Collins-Silva J (2012) Transcriptome and gene expression analysis in cold-acclimated guayule (*Parthenium argentatum*) rubber-producing tissue. Phytochemistry 79:57-66

Potter PC (2013) Latex - Increasing allergy. Allergy Society of South Africa. Available from: http://www.allergysa.org/C_OL_Latex_001.asp

Potter PC, Crombie I, Marian A, Kosheva OBM, Schinkel M (2001) Latex allergy at Groote Schuur hospital - prevalence, clinical features and outcome. South African Medical Journal 91(9):760-5

Qiao L, Wu JX, Qin DZ, Liu XC, Lu ZC, Lv LZ, Li GW (2015) Gene expression profiles of heat shock proteins 70 and 90 from *Empoasca onukii* (Hemiptera: Cicadellidae) in response to temperature stress. Journal of Insect Science 15(1):49

Rahman AYA, Usharraj AO, Misra BB, Thottathil GP, Jayasekaran K, Feng Y, Hou S, Ong SY, Ng FL, Lee LS, Tan HS, Sakaff MKLM, Teh BS, Khoo BF, Badai SS, Aziz NA, Yuryev A, Knudsen B, Dionne-Laporte A, Mchunu NP, Yu Q, Langston BJ, Freitas TAK, Young AG, Chen R, Wang L, Najimudin N, Saito JA, Alam M (2013) Draft genome sequence of the rubber tree *Hevea brasiliensis*. Biomedical Central Genomics 14:75

Rao MV, Paliyath G, Ormrod DP (1996) Ultraviolet-B- and ozone- induced biochemical changes in antioxidant enzymes of *Arabidopsis thaliana*. Plant Physiology 110:125-136

Ratshikhopha ME, Singh TS, Jones D, Jeebhay MF, Lopata AL (2015) High concentrations of natural rubber latex allergens in gloves used by laboratory health personnel in South Africa. South African Medical Journal 105(1):43-46

Ray DT (1993) Guayule: A source of natural rubber. p. 338-343. In: J. Janick and J.E. Simon (eds.), New crops. Wiley, New York

Ray DT, Coffelt T, Dierig DA (2005) Breeding guayule for commercial production. Industrial Crops and Products 22(1):15-25

Ray DT, Dierig DA, Thompson AE, Coffelt TA (1999) Registration of six guayule germplasms with high yielding ability. Crop Science 39:300

Risenga SM, Shivambu G, Rakgole M, Makwela M, Ntuli S, Malatji T (2013) Latex allergy and its clinical features among healthcare workers at Mankweng Hospital, Limpopo Province, South Africa. South African Medical Journal 103(6):390-394

Roje S (2006) S-Adenosyl-L-methionine: beyond the universal methyl group donor. Phytochemistry 67:1686-1698

Saetung N, Campistron I, Pascual S, Soutif JC, Pilard JF, Fontaine L (2011) Synthesis of natural rubber-based telechelic cis-1,4-polyisoprenes and their use to prepare block copolymers via RAFT polymerization. European Polymer Journal 47:1151-1159

Salvucci ME, Barta C, Byers JA, Canarini A (2010) Photosynthesis and assimilate partitioning between carbohydrates and isoprenoid products in vegetatively active and dormant guayule: physiological and environmental constraints on rubber accumulation in a semiarid shrub. Physiology Plant 140:368-379

Seabra AR, Silva LS, Carvalho HG (2013) Novel aspects of glutamine synthetase (GS) regulation revealed by a detailed expression analysis of the entire GS gene family of *Medicago truncatula* under different physiological conditions. BMC Plant Biology 13:137

Sell AM and Visentainer JEL (2012) Natural rubber latex allergy, allergic diseases - Highlights in the Clinic, mechanisms and treatment, Prof. Celso Pereira (Ed.), ISBN: 978-953-51-0227-4, InTech, Available from: http://www.intechopen.com/books/allergic-diseases-highlights-in-the-clinicmechanisms-and-treatment/natural-rubber-latex-allergy

Siddiqui IA, Locktov P (1981) A feasibility study on the commercialization of guayule in NSW, Australia. Division of Plant Industry, California Department of Food and Agriculture, Sacramento, California

Siler DJ, Cornish K (1994) Hypoallergenicity of guayule rubber particle proteins compared to Hevea latex proteins. Industrial Crops and Products 2:307-313

Smith H (2000) Photochromes and light signal perception by plants an emerging synthesis. Nature 497: 585-591

Smith MK (1983) *In vitro* propagation of guayule (*Parthenium argentatum* Gray). Plant Science Letters 31:275-282

Soh WY, Choi PS, Cho DY (1998) Effects of cytokinin on adventitious root formation in callus cultures of *Vigna unguiculata* (L.) walp. In Vitro Cellular & Developmental Biology - Plant 34 (3):189-195

Spanò D, Pintus F, Mascia C, Scorciapino MA, Casu M, Floris G, Medda R (2012) Extraction and characterization of a natural rubber from *Euphorbia characias* latex. Biopolymers 97(8):589-94

Staba EJ, Nygaard BG (1983) *In vitro* culture of guayule. Z. Pflanzenphysiol. Bd. 109:371–378

Steward GA, Henderson K (2013) Propagation .In Stewart GA and Lucas S (eds) Potential production of natural rubber from guayule (*Parthenium argentatum*) in Australia. CSIRO, Australia 59-76

Sundar D, Chaitanya KV, Reddy AR (2003) Stress-induced proteins in *Parthenium argentatum* leaves. Biologia Plantarum 46 (2):313-316

Sundar D, Reddy AR (2001). Interactive influence of temperature and growth light intensity on rubber accumulation and rubber transferase activity in guayule (*Parthenium argentatum Gray*). Journal of Plant Physiology 158:1291-1297

Sundar D, Reddy AR (2008) Detection of a rapidly accumulating 50 kDa polypeptide and increased rubber accumulation in guayule under low temperatures. Scientific Research and Essays 3(8): 338-342

Sundar D, Chaitanya K, Jutur P, Reddy AR (2004) Low temperature-induced changes in antioxidative metabolism in rubber-producing shrub, guayule (*Parthenium argentatum* Gray). Plant Growth Regulation 44: 175

Sung DY, Kaplan F, Guy CL (2001) Plant Hsp70 molecular chaperones: protein structure, gene family, expression and function. Physiology Plant 113:443-451

Tang C, Yang M, Huang H (2016) The rubber tree genome reveals new insight into rubber production and species adaptation. Nature plants 2(6):16073

Tanimoto E (2005) Regulation of root growth by plant hormones: Roles for auxin and gibberellin. Critical Reviews in Plant Science 24: 249-265

The South African Department of Agriculture Forestry and Fisheries (2 March 2010) Strategic plan for the fiscal years 2010/11-2014/15. South Afica, Western Cape

Trautmann IA, Visser JH (1990) An *in vitro* study of organogenesis in guayule (*Parthenium argentatum* Gray). Plant Science 72:275-281

Trautmann IA, Visser JH, Spies HSC (1991) Detection of rubber in cultured material of *Parthenium argentatum* Gray (guayule). Plant Science 73: 97-102

Turner JF, Harrison DD, Copeland L (1977) Fructokinase (fraction IV) of pea seeds. Plant Physiology 60:666-669

Van Beilin JB, Poirier Y (2007) Guayule and Russian dandelion as alternative sources of natural rubber. Critical Reviews in Biotechnology 27(4):217-231

Veatch ME, Ray DT, Mau CJD, Cornish K (2005) Growth, rubber, and resin evaluation of two – year - old transgenic guayule. Industrial Crops and Products 22:65-74

Veatch-Blohm ME, Ray DT, McCloskey WB (2006) Water-stress-induced changes in resin and rubber concentration and distribution in greenhouse-grown guayule. Agronomy Journal 98 (3):766-773

Venkatachalam P, Geetha N, Sangeetha P, Thulaseedharan A (2013) Natural rubber producing plants: An overview. African Journal of Biotechnology 12 (12):1297–310

Wadleigh CH, Gauch HG, Magistad OC (1946) Growth and rubber accumulation in guayule as conditioned by soil salinity and irrigation regime. US Department of Agriculture Tech Bull 925

Wassim A, Ichrak BR, Saïda A (2013) Putative role of proteins involved in detoxification of reactive oxygen species in the early response to gravitropic stimulation of poplar stems. Plant Signaling and Behavior 8:1

Xie W, McMahan CM, Degraw AJ, Distefano MD, Cornish K, Whalen MC, Shintani DK (2008) Initiation of rubber biosynthesis: *In vitro* comparisons of benzophenone-modified diphosphate analogues in three rubber-producing species. Phytochemistry 69:2539-2545

Zeng RZ, Li Y, Bai XQ (2000) Synthetase and the ACC content of latex in rubber tree. Chinese Journal of Tropical Crops 21:64-68

Zhao B, Liang R, Ge L, Li W, Xiao H, Lin H, Ruan K, Jin Y (2007) Identification of drought-induced microRNAs in rice. Biochemical and Biophysical Research Communications 354:585-590

Appendices: Chemicals, solutions, buffers and protein annotations

Appendix A: Chemicals used during this study

Chemical	Supplier
Acetone	Merck
Ammonium acetate	Merck
40% acrylamide/Bis Solution, 37.5:1(2.6% C)	BIO-RAD
Ammonium bicarbonate	Merck
Acetonitrile	Merck
Ammonium Persulfate	BIO-RAD
Bio-Lyte 3/10 Ampholyte	BIO-RAD
1X Bradford reagent dye	BIO-RAD
Bovine Serum Albumin (BSA)	Roche
Bromophenol blue	Sigma
3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)	Sigma
Coomassie® brilliant blue (CBB) R-250	Bio-RAD
Dithiothreitol (DTT)	Fermentas
Glacial acetic acid	Merck
Ethanol	Kimix
Ethylenediaminetetracetic acid (EDTA)	Merck
Glycerol	Merck

Isopropanol	Merck
Hydrochloric acid	Merck
Iodoacetamide	BIO-RAD
2-(N-morpholino) ethanesulfonic acid (MES)	Sigma
Methanol	Merck
Mineral Oil	BIORAD
MS Medium	Sigma
Indole-3-butyric acid (IBA)	Sigma
PageRulerTM unstained protein ladder	Fermentas
Benzyladenine (BA)	Sigma
Gibberellic acid (GA_3)	Sigma
N,N,N',N'-Tetramethylethylenediamine (TEMED)	Sigma
Potassium chloride	Sigma
Potassium hydroxide pellets	Merck
Sodium carboxymethyl cellulose 90000 MW	Sigma
Sodium carboxymethyl cellulose 250000 MW	Sigma
Sodium carboxymenthyl cellulose 700000 MW	Sigma
Sodium hypochlorite	Kimix
Sodium dodecyl sulfate (SDS)	BIO-RAD
Ammonia 28%	Lab Chem
Sucrose	Merck
benomyl 500 WP	Villa protection, (PTY) Ltd
Thiourea	Sigma

Trifluoroacetic acid (TFA)	Merck
Tris (hydroxymethyl)-HCL	BIO-RAD
Trypsin	Promega
Urea	Sigma
Proplant	Agriphar, RSA
TRis-buffered phenol	Thermo Fischer Scientific

Appendix B: Preparation of stock solutions and buffers

0.5 M Tris-HCl, pH 6.8: 0.5 M Tris in distilled water adjusted to pH 6.8 with concentrated HCl

1 mg/ml BSA stock solution: 1 mg/ml BSA in urea buffer

1% sodium carboxymethyl cellulose 700 000 MW: 1g in 10 ml of KOH pH 11

1.25% (w/v) CBB stock solution: 1.25% (w/v) CBB R-250 in distilled water

1.5 M Tris- HCl, pH 8.8: 1.5 M Tris in distilled water adjusted to pH 8.8 with concentrated HCl

10% APS: 10% (w/v) APS in distilled water.

10% SDS: 10% (w/v) SDS in distilled water

1X SDS-PAGE running buffer: 25 mM Tris, 192 mM glycine containing 0.1% (w/v) SDS.

2% sodium carboxymethyl cellulose 250 000 MW: 2g in 10 ml of KOH pH 11

2% sodium carboxymethyl cellulose 90 000 MW: 2g in 10 ml of KOH pH 11

2X SDS sample loading buffer: 60 mM Tris pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 200 mM DTT, 0.025% (w/v) bromophenol blue, stored in 1 ml aliquots at -20°C

50% DTT: 50% (w/v) DTT in urea buffer

80% Acetone: 80 (v/v) in distilled water

Bradford reagent: 1 part BIO-RAD Protein assay dye reagent concentrate diluted with 4 parts distilled water

Equilibration base buffer I: 6M urea, 2% SDS, 0.375M Tris-HCL (pH 8.8); 20% glycerol and 2% DTT

Equilibration base buffer II: 6M urea, 2% SDS, 0.375 M Tris-HCL (pH 8.8), 20% glycerol and 2.5% iodoacetamide

Murashige and Skoog (MS) basal media: 2.2 g/l MS; 1% (w/v) sucrose; 5 mM MES, 0.8% (w/v) agar, pH 5.8

Urea buffer: 9 M urea, 2 M thiourea and 4% 3 (Cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS)

Appendix C: Protein annotation in Blast2Go

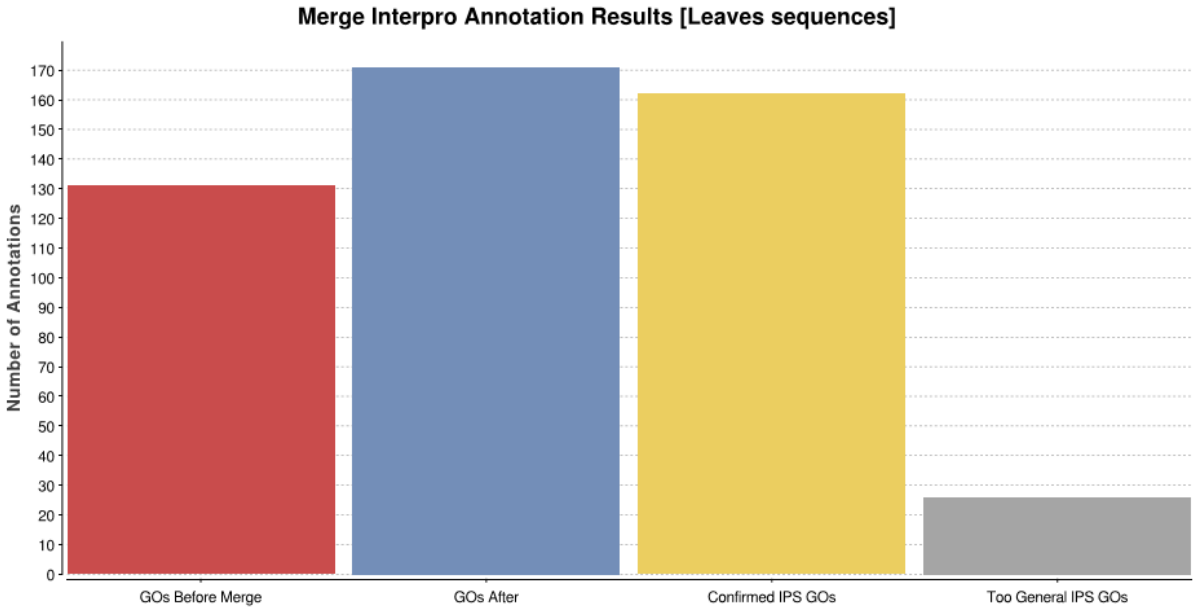


Figure A: Annotation of leaf proteins in Blast2go

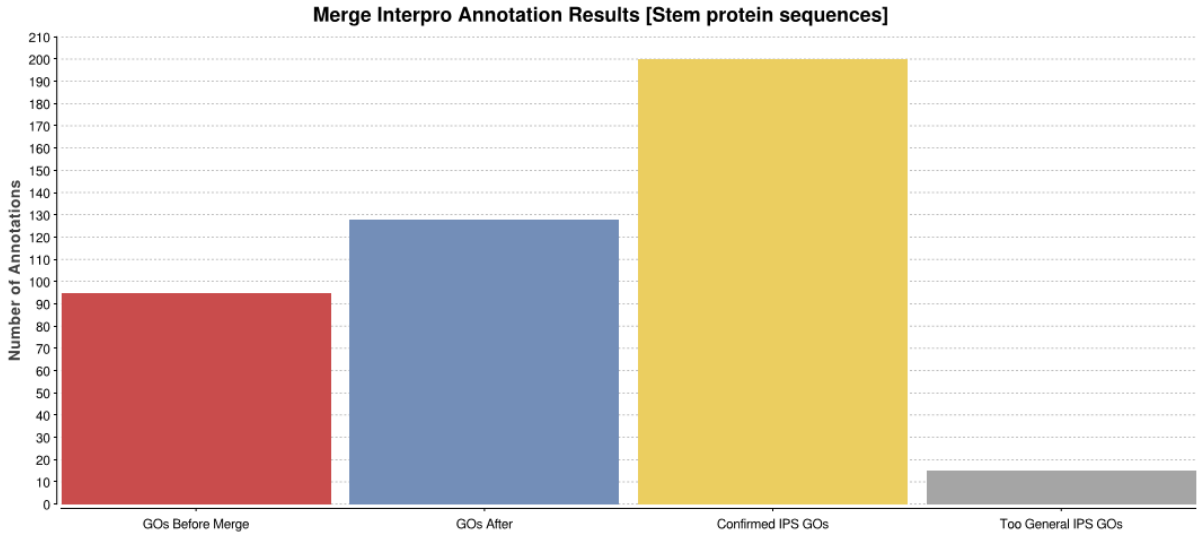


Figure B: Annotation of stem protein in Blast2go

Appendix D: Chapter 2 - statistical analysis on plants performance

Statistical analysis for Eastern Cape plants

Table D1: Plant height

Plant Height						
	AZ1	**AZ2**	**AZ3**	**AZ4**	**AZ5**	**AZ6**
Plant 1	0.60	0.75	1.35	1.10	0.90	0.97
Plant 2	1.56	0.40	1.40	0.70	1.00	1.00
Plant 3	1.10	1.25	1.15	1.05	0.80	0.70
Average	1.09	0.80	1.30	0.95	0.90	0.89
Std Dev	1.567	1.227	1.432	1.168	1.000	1.055
Error %	0.90	0.71	0.83	0.67	0.58	0.61

Table D2: Plant diameter

Plant diameter						
	AZ1	**AZ2**	**AZ3**	**AZ4**	**AZ5**	**AZ6**
Plant 1	1.32	1.50	2.00	1.50	1.20	1.50
Plant 2	2.30	0.50	1.70	0.90	1.00	1.30
Plant 3	1.20	1.80	1.70	1.10	0.80	1.00
Average	1.61	1.27	1.80	1.17	1.00	1.27
Std Dev	2.21	1.95	1.97	1.47	1.20	1.52
Error %	1.28	1.12	1.14	0.85	0.69	0.88

Table D3: Stem diameter

Stem diameter						
	AZ1	**AZ2**	**AZ3**	**AZ4**	**AZ5**	**AZ6**
Plant 1	0.1397	0.1016	0.2032	0.1524	0.1397	0.1524
Plant 2	0.2286	0.0762	0.2032	0.0762	0.1524	0.2032
Plant 3	0.1524	0.1524	0.2032	0.1397	0.1016	0.1016
Average	0.17	0.11	0.20	0.12	0.13	0.15
Std Dev	0.22	0.15	0.20	0.16	0.16	0.20
Error %	0.13	0.09	0.12	0.09	0.09	0.12

Statistical analysis for Western Cape plants

Table D4: Plant height

Plant height							
	AZ1	AZ2	AZ3	AZ5	AZ6	11591	OSU1
Plant 1	0.9	0.9	0.9	0.5	0.6	0.65	0.7
Plant 2	0.9	0.8	0.85	0.5	0.5	0.5	0.65
Plant 3	0.85	0.7	0.85	0.3	0.5	0.55	0.64
Average	0.88	0.80	0.87	0.43	0.53	0.57	0.66
Std Dev	0.91	0.90	0.90	0.55	0.59	0.64	0.70
Error %	0.53	0.52	0.52	0.32	0.34	0.37	0.40

Table D5: Canopy diameter

Canopy diameter							
	AZ1	AZ2	AZ3	AZ5	AZ6	11591	OSU1
Plant 1	1	0.9	1	0.4	0.56	0.7	0.8
Plant 2	1	1	0.9	0.6	0.5	0.6	0.7
Plant 3	0.9	0.95	0.9	0.55	0.6	0.65	0.7
Average	0.97	0.95	0.93	0.52	0.55	0.65	0.73
Std Dev	1.02	1.00	0.99	0.62	0.60	0.70	0.79
Error %	0.59	0.58	0.57	0.36	0.35	0.40	0.46

Table D6: Stem diameter

Stem diameter							
	AZ1	AZ2	AZ3	AZ5	AZ6	11591	OSU1
Plant 1	0.15	0.19	0.13	0.07	0.09	0.09	0.08
Plant 2	0.18	0.09	0.14	0.06	0.095	0.09	0.12
Plant 3	0.13	0.15	0.15	0.05	0.1	0.12	0.09
Average	0.15	0.14	0.14	0.06	0.10	0.10	0.10
Std Dev	0.18	0.19	0.15	0.07	0.10	0.12	0.12
Error %	0.10	0.11	0.09	0.04	0.06	0.07	0.07

Appendix E: Chapter 3 statistical analysis

Table E1: Latex extraction quantification for plants in Eastern Cape

	AZ1	AZ2	AZ3	AZ4	AZ5	AZ6
Plant 1	1.50	3.67	3.20	7.90	6.63	5.27
Plant 2	3.53	3.67	3.70	8.20	6.33	7.43
Plant 3	4.03	2.07	3.37	6.17	6.27	8.87
Average	3.02	3.13	3.42	7.42	6.41	7.19
Std Dev	1.34	0.92	0.25	1.10	0.20	1.81
Error %	0.77	0.53	0.15	0.63	0.11	1.05

Table E2: Latex extraction quantification for plants in Western Cape

	AZ1	AZ2	AZ3	AZ5	AZ6	11591	OSU1	OSU1 Clonal
Plant 1	5.9	7.5	5.4	10.3	10.1	11.8	11.1	4.8
Plant 2	6.5	7.9	7.2	5.8	13.7	10.9	10.2	4
Plant 3	5.9	7.7	7	10.6	10.4	10.2	9.9	3.1
Average	6.1	7.7	6.5	8.9	11.4	11.0	10.4	4.0
Std Dev	0.35	0.20	0.99	2.69	2.00	0.80	0.62	0.85
Error %	0.20	0.12	0.57	1.55	1.15	0.46	0.36	0.49