PHYSIOLOGICAL AND MOLECULAR STUDIES ON CYANOGENIC POTENTIAL IN CASSAVA (*Manihot esculenta* Crantz) IN RESPONSE TO NITROGEN NUTRITION, WATER STRESS AND SHADE

By

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DECLARATION

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LIST OF ABBREVIATIONS

%	Percentage
A_{260}	Absorbance at 260 nm wavelength
A_{280}	Absorbance at 280 nm wavelength
bp	Base pair
cm	centimetre
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
et al.	et alia
EtBr	Ethidium bromide
F	Forward primer
g	gram
ICAR-CTCRI	ICAR- Central Tuber Crops Research Institute
kb	Kilo bases
kg	Kilogram
L	Litre
m	Meter
M	Molar
mg	milligram
$MgCl_2$	Magnesium Chloride
min	Minute
ml	Millilitre
mm	Millimeter
mM	Millimolar
NaCl	Sodium chloride

ng	Nanogram
nm	Nanometer
°C	Degree Celsius
OD	Optical density
PAGE	Poly Acrylamide Gel Electrophoresis
PCR	Polymerase chain reaction
R	Reverse primer
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	revolutions per minute
RT	Reverse transcriptase
RT-PCR	Reverse transcription-polymerase chain reaction
s	second
sp.	Species
spp.	Species (plural)
SDW	Sterile distilled water
Taq	*Thermus aquaticus*
TBE	Tris-borate EDTA buffer
TE	Tris-EDTA buffer
TM	Trademark
Tm	Melting temperature
Tris HCl	Tris (Hydroxy methyl) aminomethane hydrochloride
U	Enzyme units
UV	Ultra violet
V	Volt
v/v	volume/volume
W	Watt
w/v	weight/volume
µg	Microgram
µl	Microlitre
µM	Micromolar

1. INTRODUCTION

There are wide number of tropical roots and tuber crops, belonging to different genera and are used as food in different parts of the world. They are the main source of carbohydrate to a large population of the developing countries. The carbohydrates, primarily starch are mostly found in storage organs which can be enlarged rhizomes, corms or tubers. Most root and tuber crops are adapted to the specific environment and are confined to that area only, having a small role in world food production and are considered minor tuber crops. But some of them like cassava (*Manihot esculenta* Crantz) and sweet potato (*Ipomoea batatas* L.) are grown worldwide and play an important role in food security. Their importance in world hunger scenario lies in their ability to substitute cereals as the source of carbohydrate and in their yet to be defined processed products due to their under exploited nature (FAO, 1985). A number of compounds, namely, saponins, phenolic compounds, alkaloids, phytic acids, carotenoids, and ascorbic acid are known to be found in several tubers and root crops. Also they are said to have specific bioactivities, namely, antioxidant, immunomodulatory, antimicrobial, antidiabetic, antiobesity, and hypocholesterolemic activities, among others (Naczk and Shahidi, 2006).

Cassava is one of the foremost important staple food crops in tropical regions of the planet. Production of this crop plays an important role in the maintenance of food security in most of the developing world, as well as in Africa, the Asian Pacific and South America (Nassar and Ortiz, 2007; Montagnac *et al.*, 2009). Cassava is cultivated primarily within the tropic and sub-tropic regions of the globe, over a variety of environmental and soil conditions. It is highly tolerant to drought and heat stress and grows well on marginal soil, and provides food for quite 800 million individuals (FAO, 2007). A group of nitrile containing plant secondary metabolites which synthesizes cyanide on enzymatic breakdown called cyanogenic glycosides are found in plants. Although functions of it in most of the plants are yet to be determined some are said to perform role against herbivores and in transport of reduced nitrogen. (Belloti and Arias, 1993; Selmar, 1993; McMahon *et al.,* 1995). Every part of cassava plant except seeds

contain cyanogenic glycosides in the form of linamarin (90%) and lotaustralin (10%) (McMahon *et al.*, 1995). Due to the presence of this residual cyanide in food, various diseases can be caused especially in places where cassava forms part of their dietary staple. These disorders include hyperthyroidism, tropical ataxic neuropathy and konzo. HCN dose of 1-3 mg / kg of body weight can be harmful to humans as it inhibits cytochrome c oxidase, which is important in respiratory electron transport chain (Gleadow and Møller, 2014). Lower levels can also be harmful if taken continuously as it can lead to Konzo, a chronic neurological disease which leads to leg paralysis and is seen in some parts of Africa (Bhattacharya *et al.*, 2009).

Cyanogenic glycoside levels are highest in leaves (about 20 fold higher than in roots) as they are produced in leaves and are transported to different parts from there. Also cyanide levels differ according to different cultivars with root cyanide content ranging from 100 mg to 500 mg per kg of fresh weight of root tissue but no cultivar have been found cyanide free till now. Gleadow and Møller, in 2014 reported that cyanide content in roots are found to be affected by high nitrogen fertilizers and water stress with drought resulting in an increase in cyanogenic potential of cassava. Majority of detection methods of cyanogenic glycosides contain three steps like extraction of cyanogens in diluted acids (Bradbury *et al.*, 1994) then degradation of linamarin using endogenous linamarase (Rao and Hahn, 1984) to cyanohydrin and then to HCN and detection of cyanide using colorimetry (Cooke *et al.*, 1978; Essers *et al.*, 1993) or by titration with $AgNO_3$.

The present study is done with the aim of studying the response of cassava with respect to cyanogenic glycoside content in response to factors like nutrition, water stress and light intensity along with comparison of detection methods of the cyanides in plant. The main objectives are:

1. Physiological study of cyanogenic glycosides in the plant with response to nitrogen nutrition, water stress and shade
2. Molecular study of cyanogenic glycosides in the plant with response to nitrogen nutrition, water stress and shade
3. Comparison of methods like Near-Infra Red (NIR) spectroscopy and High Performance Thin Layer Chromatography (HPTLC) for cyanogenic glycoside estimation

2. REVIEW OF LITERATURE

2.1 CASSAVA

Cassava (*Manihot esculenta* Crantz) is a member of the genus *Manihot* which contains hundreds of trees, shrubs and herbs (Allem, 2002). It is distributed exclusively in tropical and sub-tropical areas of the globe. Although some studies assert that cassava has multiple centers of origin, others claim that origin of cultivated species was on the southern edge of the Brazilian Amazon (Olsen and Schaal, 1999). Botanically, it is a woody perennial shrub which grows upto 5 m in length. It is believed to have been cultivated, mainly for its tuber which is high in starch content, for 9000 years, making it one of the oldest crops to be cultivated (Hershey, 1987).

The crop has been in cultivation in India now for more than two centuries. Cassava was introduced into India by the Portuguese in the Malabar region, presently part of Kerala state during the17th century, from Brazil. Cassava was used as substitute to rice (staple) especially by the people of low income strata. All the major cassava growing countries in the Asia continent have the productivity more than the world average productivity. Indonesia, Thailand, Vietnam and India are the major countries growing cassava in Asia. India acquires significance in the global cassava scenario due to its highest productivity in the world (27.92 t/ha.) and cultivated in an area of 240,000 ha producing 6.7 million tonnes. In India, 60-70 per cent of the total cassava production is used commercially to produce sago, starch, dried chips, flour etc. Human consumption of cassava is common in Kerala and in northeastern states like Assam, Meghalaya etc. as raw/cooked tubers and as sago in Gujarat, Maharashtra, West Bengal states.

Cassava is an important source of Vitamin B complex and has high storage capacity and thus ensures food security even during adverse climatic conditions. The root is a physiological energy reserve with high carbohydrate content, which ranges from 32% to 35% on a fresh weight basis and from 80% to 90% on a dry

weight basis. Eighty percent of the carbohydrates stored in the roots is starch (Gil and Buitrago, 2002). The protein content is low at 1% to 3% on a dry weight basis (Buitrago, 1990) and between 0.4 and 1.5 g/100 g FW (Bradbury and Holloway, 1988). Cassava roots have calcium, iron, potassium, magnesium, copper, zinc, and manganese contents comparable to those of many legumes.

2.2 TAXONOMY

Kingdom	Plantae – plantes, Planta, Vegetal, plants
Subkingdom	Viridiplantae
Infrakingdom	Streptophyta – land plants
Superdivision	Embryophyta
Division	Tracheophyta – vascular plants, tracheophytes
Subdivision	Spermatophytina – seed plants, phanérogames
Class	Magnoliopsida spermatophytes
Superorder	Rosanae
Order	Malpighiales
Family	Euphorbiaceae – spurge, euphorbes
Genus	*Manihot* Mill. – cassava
Species	*Manihot esculenta* Crantz – manioc, cassava or tapioca

2.3 CYANOGENIC GLYCOSIDES

Cyanogenic glycosides are considered the most important defense-related secondary metabolites and natural plant toxin in plant foods. They are a group of nitrile- containing plant secondary compounds that on enzymatic breakdown yields cyanide and this process is called cyanogenesis. They are found in a large number of plant species of which a large portion is used as food source (Vetter, 2000). The toxicity of cyanogenic glycosides is depended on the ability of getting hydrolyzed naturally or by enzymatic action in which cyanide is the end product. Thus the level which causes toxicity is measured in terms of quantity of free cyanides generated which makes their estimation in diet difficult (FAO/WHO 2011; WHO, 2004). Almost every cyanogenic glycoside are derived from one of

six amino acids namely L-valine, L-isoleucine, L- leucine, L-phenylalanine, or L-tyrosine, and cyclopentenyl-glycine (a nonprotein amino acid). They are important in chemical defense organization and plant insect interactions (Ganjewala, 2010). The leaves and roots of cassava contain potentially toxic levels of cyanogenic glycosides linamarin (95%) and lotaustralin (5%) (Conn, 1979, 1994; Balagopalan *et al.*, 1988).

In cassava, cytochrome P_{450} enzymes CYP79D1 and CYP79D2 catalyzes first step of the pathway for biosynthesis of linamarin and lotaustralin (Figure 1).

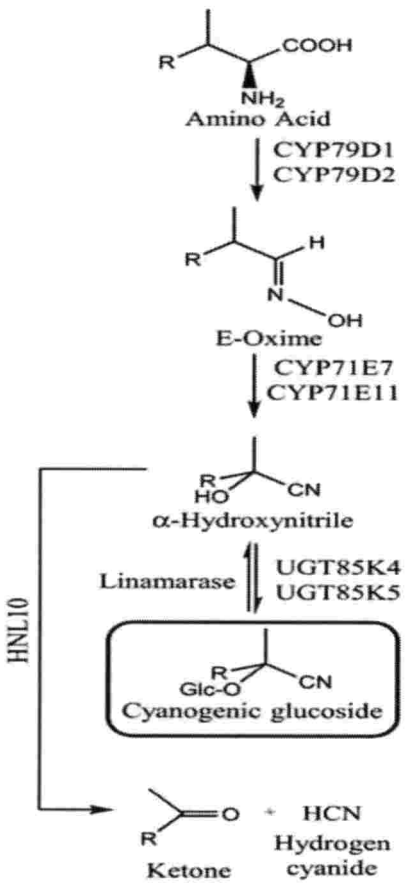


Figure 1- Pathway for biosynthesis of linamarin and lotaustralin

They convert valine and isoleucine to their corresponding oximes (Andersen *et al.*, 2000). CYP71E7 or CYP71E11 catalyzes the conversion of the two oximes into α-hydroxynitriles (Jørgensen *et al.*, 2010) then either UGT85K4 or

UGT85K5 glycosylates the hydroxynitriles to produce linamarin and lotaustralin (Kannangara *et al.*, 2011).

Doses between 0.5 and 3.5 mg HCN per kilogram body weight can lead to cyanide toxicity in both humans and animals and reported symptoms in humans are vomiting, stomach ache, diarrhea, convulsion, and in severe cases, it can be fatal. Their lesser body weight make children particularly more affected group (WHO, 1993). There is a wide variety of cyanogenic glycosides present in different plants like, taxiphyllin in bamboo shoots, linamarin and lotustraulin in cassava, dhurrin in sorghum and amygdalin in apple (Nhassico *et al.*, 2008). Age and other environmental factors affect cyanogenic glycoside production and accumulation in plants (Vetter, 2000).

2.3.1 Cyanogenesis in Cassava

Cyanogenesis in cassava starts when the plant tissue is damaged. Rupture of the vacuole releases linamarin, which is hydrolyzed by linamarase, a cell wall-associated b-glycosidase (McMahon *et al.*, 1995). Hydrolysis of linamarin yields an unstable hydroxy nitrile intermediate, acetone cyanohydrin and glucose. Acetone cyanohydrin spontaneously decomposes to acetone and HCN at pH > 5.0 or temperatures > 35°C and can be broken down enzymatically by hydroxyl nitrilase (HNL) (White and Sayre, 1995; Hasslacher *et al.*, 1996; Wajant and Pfizenmaier, 1994). Cyanogenic glycoside and its corresponding cyanogenic enzymes are localized in different cellular compartments or tissues in cynogenic plants. This strict compartmentalization prevents cyanogenesis until the tissue is disrupted. In some cyanogenic plants the separation of substrate and cyanogenic enzymes is at a subcellular level. In rubber tree, endosperm cells contain linamarin and HNL in the cytoplasm, whereas linamarase is located in the apoplast (Poulton, 1988; Selmar, 1993). In some cyanogenic plants the separation of substrate and cyanogenic enzyme(s) is at a tissue level. In sorghum (Sorghum bicolor) leaves, the cyanogenic glycoside dhurrin is located in vacuoles of leaf epidermal cells, whereas the β-glucosidase and HNL are localized in the cytoplasm and plastids of mesophyll cells (Wajant *et al.*, 1994). In cassava leaves

linamarin has been localized to the vacuoles, whereas linamarase is localized to cell walls and laticifers (Hughes *et al.*, 1994). It was also demonstrated that both linamarase and HNL were enriched (8- fold relative to whole leaves) in cassava leaf apoplast extracts (White and Sayre, 1995).

2.3.2 Health implications

Cassava contains high level of cyanogenic glycosides. In the leaves it ranged from 200 to 1,300 mg CN equivalents/kg dry weight and in roots 10 to 500 mg CN equivalents/kg dry weight. These levels are higher than the maximum levels (10 mg CN equivalents/kg dry weight) recommended for foods by the FAO. In Africa, a number of cyanide-associated health disorders have been attributed to eating poorly processed cassava, particularly by nutritionally compromised (low protein intake) individuals (Delange *et al.*, 1994). The severity of these disorders depends on the level and frequency of cyanogens exposure and the state of nutrition of the consumer. Chronic, low-level cyanide exposure resulting from eating poorly processed cassava has been associated with the development of goiter and tropical ataxic neuropathy (Oluwole *et al.*, 2000). Acute cyanogen poisoning can result from eating poorly processed high-cyanogen cassava varieties and is most commonly associated with famines.

The amount of HCN released determines the level of toxicity of cyanogenic glycosides in plants. Rapid respiration, decrease in blood pressure, headache, dizziness, vomiting, diarrhoea, blue discolouration of the skin due to lack of oxygen (cyanosis), twitching and convulsions are common clinical symptoms of acute cyanide poisoning in humans. Some of the effects are;

1. Konzo or spastic paraparesis is a motor neuron disease characterized by permanent weakening of the limbs. Severe condition will lead to loss of speech, loss of ability to walk etc. Commonly found in children and women of childbearing age in East Africa at times of famine when intake of cassava increases but that of protein decreases (Davis, 1991).

2. Tropical ataxic neuropathy (TAN) refers to several neurological diseases affecting mouth, eyesight, hearing or gait of mostly older males and females. TAN is mainly known to cause due to chronic consumption of foods derived from cassava.
3. Goiter and cretinism are diseases widely found in developing countries due to low intake of iodine. It is particularly rampant in Africa because of their overdependence on cassava as their staple food. Continuous exposure to dietary cyanide from cassava products can aggravate these diseases (Rosling, 1987) by the interferences of thiocyanate (the end products of cyanide detoxification in human system) with dietary iodine, thus leading to iodine deficiency.

2.4 CYANOGENIC GLYCOSIDES AND PLANT PHYSIOLOGICAL STRESSES

Complicated and elaborated mechanisms have been evolved in plants to cope with water stress by adjusting their metabolism and growth in response resulting in a variety of physiological, biochemical, and morphological changes at both molecular and whole-plant levels (McDowell *et al.*, 2008; Claeys and Inze, 2013). Thousands of years of cassava breeding by farmers and scientists have resulted in development of large number of cultivars of the crop which all contain the cyanogenic glycosides linamarin (approximately 93% of total glycosides; derived from valine) and lotaustralin (derived from isoleucine) (Nartey, 1968; Poulton, 1988). Highest concentrations are found in leaves and tubers (Nasser and Ortiz, 2007). The risk of cyanide poisoning is further increased by the fact that cyanide content can change according to environmental conditions such as drought and soil nutrient supply (Burns *et al.*, 2010).

2.4.1 Water stress

Biosynthesis of cyanogenic glycosides occurs in leaves and from there it is translocated to other tissues. In drought conditions the increase in cyanogens in cassava products is considered to be the result of high endogenous levels in plants

and in field studies it is generally found that there is an increase in the concentration of cyanogens in tubers in response to water stress but the degree depends on the cultivars used (Santisopasri *et al.*, 2001; El-Sharkawy, 1993; Bokanga *et al.*, 1994). Hular-Bograd *et al.* (2011) showed that cyanogenic glycosides content in the root was higher under the drought-stressed condition than under the well- irrigated condition as in a previous report. However, Vandegeer *et al.* (2013) reported an increase in the cyanogenic glycosides content in the root in response to soil moisture within 14 days of rewatering.

In case of water stress it has been noted that building up of cyanogenic potential is depended on the growth stage of plant when the stress is affected like in active root bulking phase if there is no water stress low it results in low cyanide potential. de Bruijn's (1971) study showed that cyanoglucoside levels are highest in young cassava leaves and petioles and decline with age. He found no indications that the glycoside concentrations of the tuberous roots are directly related to plant age and considered that fluctuations in glucoside content during growth are mainly due to changes in ecological conditions. de Bruijn (1971) grew young plants in bags for 2 months, with water regimes which were two-thirds and one-third optimal and observed an increase in cyanide content per unit dry matter in both roots and leaves with increasing dryness. However, he states that in the field the glycoside content would be increased only after a very long dry period because plants can adapt to short droughts by abscission of some leaves. Compared with other field crops, cassava is more photosynthetically active under severe prolonged drought, an advantage that underlies its remarkable productivity and ability to endure harsh environments. Thus, it is beneficial to select for higher photosynthetic capacity, combined with other desirable plant traits such as longer leaf life (Lenis *et al.*, 2006).

It was found that cassava tolerates a relatively long period of drought once the crop is established. In these studies, using a limited number of varieties, a stress period of 2-3 months was imposed 3-4 months after planting. The crop was later allowed to recover from stress for the rest of the growing cycle with the aid

of rainfall and supplementary irrigation (Connor *et al.*, 1981; Porto, 1983; El-Sharkawy and Cock, 1987).

2.4.2 Nitrogen nutrition

Nitrogen fertilization increased the concentration of cyanogenic glycosides in leaves of sorghum, (Nelson, 1953) and cassava (Lancaster and Brooks, 1983) as much as eight-fold in some cases. Although these investigations were not designed to examine the effects of nutrient stress, since fertilization usually increased overall growth (though growth data were not reported in every study) the unfertilized controls must have been nitrogen-limited to some degree, and therefore were by definition (Greenwood, 1976) under nitrogen stress. A reduction in cyanogenic glycoside concentration under low nitrogen supply would not be surprising since cyanogenic glycoside molecules contain nitrogen. In *H.* arbutifolia, leaf cyanogenic glycoside accumulation is directly correlated with nitrogen availability, with the highest percentages of cyanogens being found during the warm, moist months of spring and summer when nitrogen is considered to be most available (Dement and Mooney, 1974). Leaves and stems being the centre of photosynthetic process, vigorous top growth would be indicative of a better development of storage roots and consequently of higher tuber yields.

In studies with other nutrients, the concentration of cyanogenic glycosides in Sorghum spp. increased under low phosphorus levels (Patel and Wright, 1958), but was not affected significantly by low potassium supplies (Clark *et al.*, 1979). Sinha (1969) reported similarly that the effect of N on cyanide content of cassava depended on the cultivar. There seems to be some irregularity in results obtained from experiments involving the relationships between some nutrients especially potassium (K) and the content of cyanogens in cassava roots. Many authors have reported significant reduction in the hydrocyanic acid (HCN) content of cassava tubers in response to potassium fertilization (John *et al.*, 2005; El-Sharkawy and Cadavid, 2000; Tandon and Sekhon, 1988). Attalla *et al.* (2001) described however results of a field experiment where high HCN level in tuber tissues of cassava was noticed with increasing rates of potassium fertilizer (K_2SO_4).

2.4.3 Light intensity

Shading to 35 and 70% daylight for 8 weeks increased the cyanoglucoside content of leaves and correspondingly decreased cyanoglucosides in roots (de Bruijn, 1971), perhaps by reduction of translocation to the roots either of cyanoglucosides or cyanoglucoside precursors. The role of N in agricultural production is intimately connected with photosynthesis (Lawlor, 2002). Numerous studies show decreases in both the rate of net CO_2 assimilation and the quantum yield of photosynthesis in plants grown with a limited N supply as compared with N-replete controls (Evans, 1989; Khamis *et al.*, 1990; Terashima and Evans, 1988). Some studies had demonstrated that N deficiency decreases the quantum yield of Photosystem II (PS II) electron transport and the maximum PS II photochemical efficiency (DaMatta *et al.*, 2002; Lu and Zhang, 2000), suggesting that N deficiency may affect the PS II photochemistry. On the contrary, other reports have indicated that N limitation has no effect on PS II photochemistry (Bungard *et al.*, 1997; Khamis *et al.*, 1990). Schmidt *et al.*, 2018 demonstrated that the levels of the biosynthetic enzymes CYP71E7/11 and UGT85K4/5 decreased upon the onset of morning light with minimum levels between 4 p.m. and 6 p.m. A similar decrease was observed for the transcripts of CYP79D1, CYP79D2, CYP71E7/11, and UGT85K5.

LC-MS analysis demonstrated that the linamarin content varied during the day and night cycle with a difference of 35% between the highest and the lowest levels. From 9 a.m. until noon, the linamarin content was found to decrease moderately. The content increased in the afternoon until 7 p.m., where it again decreased reaching a minimum at 10 p.m. The linamarin content increased slowly through the night and early morning. This demonstrated a diurnal variation in linamarin content. Some studies have suggested that cassava uses a C3–C4 intermediate form of photosynthesis on account of high leaf photosynthetic rates, low apparent rates of photorespiration, a chlorenchymatous bundle sheath and a high photosynthetic nitrogen (N) use efficiency (El-Sharkawy and Cock, 1987; El-Sharkawy, 2016).

Decreases in photosynthetic activity are often paralleled by a reduction in leaf chlorophyll content (Ekanayake *et al.,* 1998). Ekanayake *et al.* (1996) noted that low moisture condition, high air and soil temperatures at middays and very low temperatures at nights during the harmattan period negatively affect chlorophyll fluorescence activities, which contribute to reduction in growth rate of cassava during the dry season in the upland. Johnston and Onwueme (1998) showed that chlorophyll a:b ratio was less in plants grown under shade than in plants exposed to full sunlight, and that compared with chlorophyll a, chlorophyll b increased significantly under low light. This suggests that chlorophyll b concentration may increase under stressful conditions compared to chlorophyll a.

2.5 DETECTION METHODS

Different methods are available for the quantitative determination of cyanogenic compounds (linamarin, cyanohydrin and free cyanide). The majority require three steps. The first step, extraction of cyanogens, is normally carried out in dilute acid (Bradbury *et al.*, 1994) to stop the degradation of cyanogenic compounds. The second step involves degradation of linamarin to cyanohydrin and glucose and, subsequently, to HCN. This can be achieved either by autolysis, which relies on the endogeneous linamarase (Cooke, 1978), by enzymatic hydrolysis by adding exogenous linamarase (Rao and Hahn., 1984) or by alkalinic hydrolysis by addition of NaOH. For the third step, determination of HCN, various methods have been developed, such as titration with $AgNO_3$, reaction with alkaline picrate (Egan *et al.*, 1998), and, most widely used, the photometric method based on the König reaction (Cooke, 1978; Essers *et al.*, 1993).

Cyanogenic glycosides are generally estimated by measuring the amount of hydrogen cyanide liberated by hydrolysis. Efforts continue to be made to develop suitable methods for the determination of the intact individual glycosides. Use of as chromatography and high-performance liquid chromatography has also been reported (Bisset *et al.*. 1969).

2.5.1 High performance thin layer chromatography

High-performance thin layer chromatography (HPTLC) is an extension of TLC which is robust, simplest, rapid, and efficient tool in quantitative analysis of compounds. HPTLC is an analytical technique based on TLC, but with enhancements intended to increase the resolution of the compounds to be separated and to allow quantitative analysis of the compounds. Some of the enhancements such as the use of higher quality TLC plates with finer particle sizes in the stationary phase which allow better resolution (Reich and Schibli, 2005). The separation can be further improved by repeated development of the plate, using a multiple development device. As a consequence, HPTLC offers better resolution and lower Limit of Detection (LODs). HPTLC is a globally accepted practical solution to characterize small molecules in quality assessment throughout the developing world. HPTLC is used for purity control of chemicals, pesticides, steroids, and water analysis (Weber *et al.*, 2005). HPTLC is also widely used for analysis of vitamins, water-soluble food dyes, pesticides in fruits, vegetables, and other food stuffs (Verbitski *et al.*, 2006)

2.5.2 Near-infra red spectroscopy

Near Infrared Spectroscopy (NIR) is a type of vibrational spectroscopy that employs photon energy in the energy range of 2.65 x 10-19 to 7.96 x 10-20 J, which corresponds to the wavelength range of 750 to 2,500 nm (wavenumbers: 13,300 to 4,000 cm-1). This energy range is higher than necessary to promote molecules only to their lowest excited vibrational states (through a fundamental vibrational transition) and lower than typical values necessary for electron excitation in molecules (except for some rare earth compounds). Its overall objective is to probe a sample in order to acquire qualitative and/or quantitative information coming from the interaction of near- infrared electromagnetic waves with its constituents. NIR spectroscopy is not very sensitive. Most of the quantitative applications are targeted to determine major constituents in the sample. In general, the detection limit is about 0.1% (m/m), although, for some

specific applications and under favourable characteristics of the sample matrix and analyte, NIR can reach lower values.

2.6 ROLE OF UGT GENE IN BIOSYNTHESIS OF CYANOGENIC GLYCOSIDES

The biosynthetic pathway cyanogenic glycosides includes the key intermediates, oximes and a-hydroxynitriles, involves two multifunctional cytochrome P450 enzymes and a member of the family 1 glycosyltransferases. The final step of cyanohydrin glucosylation to produce cyanogenic glucosides has been characterized in sorghum and almond, and the genes involved belong to the UGT85 family (Jones *et al.*, 1999; Franks *et al.*, 2008). UDP-glucosyltransferases, UGT85K4 and UGT85K5, are responsible for the last step in the biosynthesis of linamarin and lotaustralin in cassava. UGT85K4 and UGT85K5 both catalyze the in vitro conversion of the aglycones, acetone cyanohydrin and 2-hydroxy- 2-methylbutyronitrile, into linamarin and lotaustralin.

3. MATERIALS AND METHODS

The study entitled "Physiological and molecular studies on cyanogenic potential in cassava (*Manihot esculenta* Crantz) in response to nitrogen nutrition, water stress and shade" was carried out at the Division of Crop Utilization, ICAR-Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during the year 2017-2018. Details regarding the experimental materials used and procedures followed in the study are elaborated in this chapter.

3.1 SOURCE OF GERMPLASM

Three released varieties namely Sree Vijaya, Sree Athulya and PDP CMR 1 were selected from ICAR-CTCRI for the present study. These were raised in pots using stem cuttings as planting material. Cuttings were planted and appropriate treatments were given for the study.

3.2 EXPRESSION PROFILING

3.2.1 Glass wares and other materials

1.5 ml and 2 ml Eppendorf tubes, PCR tubes, mortar and pestle, micropipette tips for 10 µl, 200 µl and 1000 µl were autoclaved and used. 1.5 ml tube stand, PCR tube holders, micropipettes, ice bags, measuring cylinder, bottles, spatula, polythene covers, labels, wipes, weighing pot and the other materials needed for molecular work.

3.2.2 Instruments

The equipments viz., water bath, weighing balance, microwave oven, ice machine, vortex mixer, spinner, cooling centrifuge, Nano Drop spectrophotometer, pH meter, deep freezer (-20 °C, -80 °C), refrigerator, electrophoresis apparatus, gel documentation system, PCR machine, hot air oven, autoclave and distilled water unit were used for the study.

3.2.3 RNA Extraction

RNA was extracted from fresh tender leaves of cassava planted in pots to which different mode of nitrogen nutrition treatment was given using GeNei plant RNA Isolation kit.

1g of plant tissue was pulverised in pre chilled mortar and pestle using liquid nitrogen and was transferred to a 30 ml sterile tube. Then pre-warmed solution A + phenol mixture (80 °C) is added to it in a ratio1:4 and is mixed for 5 minutes. Equal volume of chloroform is added to this mixture and incubated at room temperature for 30 minutes followed by centrifugation (Eppendorf) at 5000 rpm for 15 minutes. Upper phase is transferred to a new tube and solution B is added, mixed well and incubated for 4 hours at 4 °C. RNA is precipitated by centrifugation at 7000 rpm for 20 minutes at 4 °C. Pellet is dissolved in wash buffer and is suspended completely and centrifuged at 7000 rpm for 20 minutes at 4 °C. This step was repeated 5 times. After this pellet was washed in 70% ethanol twice and finally with 100% ethanol with centrifugation at 7000 rpm for 10 minutes at 4 °C. Pellet was air dried for 15 - 20 minutes and was dissolved in 150 µl of nuclease free water. This was kept at 55 °C for 2 minutes to dissolve in solution. All samples were checked for RNA in 1.2% agarose gel and confirmed.

3.2.4 Agarose gel electrophoresis

1.2% agarose gel was used to check the quality and integrity of the extracted DNA. 1.2% agarose solution was prepared by weighing out 1.2 g agarose in a conical flask and dissolving it using 100 ml 1X TBE buffer. Every reagents was prepared in DEPC treated water. Agarose was dissolved by heating and after that the flask was allowed to cool and when the temperature of the flask decreases, about 0.9 µl (10 mg/ml) of EtBr was added directly to the gel and gentle mixing was done. Casting tray was prepared with combs to which gel was poured and allowed to solidify. 4 µl of isolated DNA sample mixed with 2 µl of 1X loading dye was loaded into the wells of prepared gel. Horizontal gel electrophoresis unit was used to run the gel. The gel was run for about 30 minutes at 110V. The run

was stopped after the dye front reached 3/4th of the gel. Then it was visualized UV light using a gel documentation system.

3.2.5 Quantification of RNA

RNA was quantified using UV spectrophotometer (DenoVix DS 11+ NanDrop). Nuclease free water in which RNA was dissolved was used to calibrate the machine to blank i.e. zero absorbance. The advantage of NanoDrop is that it requires only 1 µl sample to measure its quantity and quality. The quantity of DNA was determined at OD_{260} and the purity was determined by OD_{260}/OD_{280} ratio.

3.2.6 cDNA synthesis

RNA is inherently susceptible to RNase degradation and it is a chemically unstable molecule. For qRT-PCR, mRNA is converted to cDNA by the reverse transcriptase enzyme as it is more stable. This enzyme catalyzes cDNA synthesis starting with the poly A tails on the individual mRNA molecules.

cDNA synthesis was done using Thermo Scientific Verso cDNA synthesis kit. Using this kit reaction mix was prepared for 20 µl reaction containing 4 µl 5x cDNA synthesis buffer, 2 µl dNTP mix, 1 µl RNA primer, 1µl RT enhancer, 1µl verso enzyme mix, 1-5 µl template RNA, and volume was made upto 20 µl nuclease free water. cDNA was synthesised by doing PCR in thermal profile of 1 cycle of 42 °C for 60 minutes and an inactivation step of 1 cycle of 95 °C for 5 minutes.

3.2.7 Primer Designing

Primer was designed for UGT85K4 gene using Primer 3+ software. A reference gene TBP (TATA binding protein) was also selected for qPCR. UGT85K4 was selected because UGT85K4 is responsible for the last step in the biosynthesis of linamarin in cassava.

CASSAVA UGT85K4

Forward Primer-5' TGCTTGGCTCCTTTCATTGA 3'

Reverse Primer-5'ACTTTCATCTTTGAAAGGGACGA 3'

TBP REFERENCE GENE

Forward Primer-5' ATGGCAGATCAAGGAGGCTTGGAA 3'

Reverse Primer-5' GCAGCGAAACGCTTAGGGTTGTAT 3'

3.2.8 RT- qPCR

Real-time quantitative polymerase chain reaction (Eppendorf realpex) is a tool used for gene expression studies. Cassava UGT85K4 primers and reference gene primers are used for relative quantification of expression of UGT85K4 gene. DyNAmo Flash SYBR Green qPCR kit was used for qPCR amplification (Table 1).

Table 1-qPCR mix for qPCR amplification

Components	**Volume**
Diluted cDNA	1.5 µl
Forward primer	1 µl
Reverse primer	1 µl
DyNAmo Flash SYBR Green qPCR master mix	5 µl
Double distilled water	1.5 µl

3.2.9 Thermal profile

Initial denaturation: 95 ºC 7 min

Denaturation : 95 ºC 10 s

Annealing : 55 ºC 30 s

Extension : 72 ºC 30 s

Melt curve analysis: 85 ºC

Number of cycles : 35 cycles, step 2-4

After completion threshold value (Ct) values were recorded.

3.3 PROTEIN PROFILE

3.3.1 Protein extraction

Protein was extracted using the protocol of Wang *et al.*, 2006. Leaves were crushed in mortar and pestle using liquid nitrogen and the powder was transferred into a 2 ml tube and filled with 10% acetone. This was mixed well and centrifuged at 16000 rcf for 3 min at 4 °C. To the pellet 80% methanol plus 0.1M ammonium acetate was added, mixed and centrifuged again. Supernatant was discarded and tube was filled with 80% acetone and suspended well. This was again centrifuged and the pellet was air dried for 10 minutes. To this, phenol and SDS sample buffer in 1:1 ratio was added, mixed thoroughly and incubated for 5 minutes this was centrifuged again and the upper phase was collected in a fresh tube and was filled with methanol and ammonium acetate and incubated at -20 °C for 10 minutes. This was centrifuged at 16000 rcf for 5 minutes at 4 °C. White pellet obtained was subjected to 100% methanol wash followed by 80% acetone wash. Protein was dissolved in SDS sample buffer and then run on 12% SDS – Poly acrylamide gel electrophoresis (SDS - PAGE).

3.3.2 SDS – PAGE gel preparation

Stacking and Resolving gel composition for 12% SDS-Polyacrylamide gel preparation are described below in Table 2.

Table 2- 12% SDS-Polyacrylamide gel preparation

Resolving Gel (1 mm plate) 12%	
Water	1.76 ml
30% Acrylamide/Bisacrylamide solution	2.14 ml
1.5M Tris pH	1.4 ml
10% SDS	54 ml

10% APS (freshly prepared)	54 ml
TEMED	5.4 µl
Stacking gel (1mm) 12%	
Water	1.82 ml
30% Acrylamide/Bisacrylamide solution	454 µl
1M Tris pH	334 µl
10% SDS	27 µl
10% APS (Freshly prepared)	27 µl
TEMED	2.7 µl

3.3.3 Visualization of proteins in the gels

To visualize the fixed protein, the gel was placed in 40% distilled water, 10% acetic acid, and 50% methanol solution containing 0.25% Coomassie Brilliant Blue R- 250 and incubated over night at room temperature on a rocking shaker. Gel was transferred in to a mixture of 67.5% distilled water, 7.5% acetic acid, and 25% methanol and placed on a rocking shaker until the excess dye has been removed. The proteins in the gel appeared as deep blue bands.

3.4 PHYSIOLOGICAL AND BIOCHEMICAL STUDY

Potted plants were studied for the effect of light intensity and water stress on CNglc level by withholding water under open and shade and a different set of potted plants were studied for the effect of light intensity and N nutrition on CNglc level by providing N through selected form (NO_3) under open and shade.

3.4.1 Leaf CNglc (Linamarin) content

Fresh cassava leaf was cut into small pieces (2 g) and homogenized in 25 ml ortho phosphoric acid. The homogenates are centrifuged at 5000 rpm for 15 minutes and the supernatant was collected. The residue is re extracted with

another 25 ml ortho phosphoric acid and the supernatants combined and made up to 50 ml. From this 0.1 ml was added to test tubes followed by addition of 0.4 ml phosphate buffer (0.1 M, pH 7.0) and 0.5 ml phosphate buffer (pH 6.0) to make up it to 1 ml. Linamarase (0.5 ml) was added and the tubes are incubated at 30oc for 15 minutes. The reaction was stopped by adding 0.6 ml 0.2 NaOH and mixed well. After 1 minute 2.8 ml phosphate buffer (pH 6.0) was added and shaken. To this 0.1 ml chloramine T solution was added and mixed followed by 0.6 ml isonicotinic acid – barbiturate colouring reagent. Absorbance was measured in spectrophotometer (Perkin Elmer) at 605 nm after 10 minutes along with a reagent blank of colouring reagent and buffer.

A stock solution of KCN (24mg/100ml) is prepared and diluted 100 times (1µg CN/ml). A calibration curve is prepared in the concentration range 0.1 to 1µg CN.

3.4.2 Leaf nitrogen content

Leaf nitrogen content was measured using Kjeldahl method. Here 2g of leaf samples was weighed and grinded using mortar and pestle and sieved. 0.5g of sample was transferred into the digestion tube. 1g of catalytic mixture was added and was rinsed with a little distilled water. To this some carborandum beads were added followed by 5 ml of conc. Sulphuric acid and mixed well. The tubes were placed in digestion block and heated slowly until frothing stops. Then temperature was increased to 360 °C to digest the sample and was continued for 30 min. tubes were removed from the digestor and allowed to cool. 20 ml of distilled water was added and swirled. It was made up to 75 ml with distilled water. The digest was distilled in Kjelplus distillation unit and the distillate was collected in a 20 ml boric acid mixed indicator solution taken in a conical flask. Quantity of nitrogen liberated was determined by titration against standard acid.

3.4.3 Leaf protein content

The Bradford protein assay is used to measure the concentration of total protein in a sample. The principle of this assay is that the binding of protein molecules to Coomassie dye under acidic conditions results in a colour change

from brown to blue. This method actually measures the presence of the basic amino acid residues, arginine, lysine and histidine, which contributes to formation of the protein-dye complex (Bradford, 1976).

0.2 g of fresh leaf sample was weighed out and grated using a pre chilled mortar and pestle and made into a fine paste with help of 10 ml phosphate buffer (pH 7.6). The homogenate was then transferred into a 20 ml centrifuge tube. The sample was centrifuge for 5 min at 5000 rpm. Supernatant was collected and from this 0.8 ml was taken into a test tube, 0.2 ml of buffer added to it followed by 5 ml of Bradford's reagent. Absorbance was read at 595 nm. Protein content was measured using standard curve.

0.01, 0.02, 0.04, 0.06, 0.08, 0.1 ml of dilute Bovine serum albumin (1mg/ml) solution was taken and made upto 0.1 ml with phosphate buffer. 0.1 ml buffer alone served as blank. 5 ml of Bradford's reagent was added and mixed and absorbance was reads at 595 nm. The standard curve is prepared by plotting the concentration in x-axis and absorbance on the y-axis.

3.4.4 Leaf dry weight

Plant was uprooted and all the leaves present were weighed in a weighing balance and these were oven dried at 70 °C for 24 hours. It was then taken and weighed to obtain leaf dry weight.

3.4.5 Chlorophyll Fluorescence Measurements

Chlorophyll a fluorescence kinetics was measured on the ventral surface of intact leaves after a 20 min dark period with Li-cor-6400 portable photosynthesis system fitted with leaf chamber fluoremeter (Li-cor- Inc, USA) at ambient CO_2 concentration. Measuring beam intensity was 0.35μmol $m^{-2}s^{-1}$ actinic irradiance of 1500μmol $m^{-2}s^{-1}$. Seven hundred ms saturated flash of white light (5000 μmol $m^{-2}s^{-1}$) were applied at the beginning of measurement and after 300s. The nomenclature of Van kooten and Snel (1990) was used.

3.4.6 Photosynthesis measurements

Leaf photosynthesis parameter were measured in clear day between 10.30 to 2.30 hrs using portable, open circuit, infrared gas analysis system (Li-6400, Li-cor Inc, NE, USA. The mature fully expanded leaves from the exposed outer layer of the canopy were used for gas exchange measurements. Simultaneous measurements of CO_2 and water vapor flux, air (Tair) and leaf temperature allowed calculation of leaf carbon assimilation (A), stomata conductance (gs), transpiration (E), intercellular CO_2 partial pressure (Ci) and leaf to air vapour pressure difference calculated from the upper leaf (vpdL). Leaf chamber of 6 cm^2 was used. Artificial illumination was supplied to the leaf from a red-blue LED source attached to the sensor head. The irradiation was set according to the prevailing incident PAR.

3.4.7 Leaf Area

Leaf area was measured using leaf area meter (Li-3000 Li-cor Inc.) Plants were uprooted and leaves present at that time were used for leaf area measurement.

3.4.8 Nitrate reductase activity

Nitrate reductase activity assay was performed using fresh tissues (about 300 mg) according to Jaworski (1971). Small leaf discs were placed in vials containing incubation medium composed by 100 mM Potassium phosphate buffer (pH 7.5), 100 mM KNO_3 and 2% (v/v) n-propanol. Vials were vacuum infiltrated three times 5 minutes each time, then incubated under darkness at 30 °C for 20 or 40 min. Aliquots were withdrawn to quantify the NO_2-N released into the medium. Aliquot was added to a second medium containing 1% sulphanilamide in 1.5 mM HCl, 0.02% N-(1- naphthyl) - ethylenediamidedihydrochloride and 1.5 cm^3 distilled water. After 30 min, the resulting absorbance was read at 540 nm. Enzyme activity was expressed as NO_2-N produced. Amount was calculated on the basis of standard curve for KNO_2.

3.4.9 Nitrite reductase activity

Nitrite reductase activity assay was performed using fresh tissues (about 300 mg). Small leaf discs were placed in vials containing incubation medium composed by 100 mM Potassium phosphate buffer (pH 7.5), 100 mM KNO_3 and 2% (v/v) n-propanol. Vials were vaccum infiltrated three times 5 minutes each time, then incubated at 30 °C for 30 minutes. Aliquots were withdrawn for quantification of NO_2. Aliquot was added to a second medium containing 1% sulphanilamide in 1.5 mM HCl, 0.02% N-(1- naphthyl) - ethylenediamidedihydrochloride and 1.5 cm^3 distilled water. After 30 min, the resulting absorbance was read at 540 nm. Amount of nitrite was found out from standard curve.

3.4.10 High performance thin layer chromatography

A standard solution of linamarin (100 pg mL^{-1}) was prepared in 80% v/v methanol (100 mL). A sample solution was prepared by extracting from cassava leaf (1 g) with boiling 80% v/v methanol by homogenising in it; each extraction was performed for 10 min. The solution was filtered, evaporated, and dissolved in 80% v/v methanol (2.0 mL).

TLC was performed on 20 x 10 cm silica gel 60 F, HPTLC plates (Merck). A Camag HPTLC system comprising of Linomate V automatic sample applicator, Hamilton Syringe, Camag TLC Scanner-3, Camag Win CAT software, Camag Twin trough chamber and stationary phase precoated silica gel 60F 254 were used. The plates were first developed to a distance of 30 mm with ethyl acetate – acetone - water, 40 + 50 + 10 (v/v) as mobile phase, then to a distance of 85 mm with ethyl acetate - formic acid - water, 60 + 10 + 10 (v/v). Both developments were performed in an unsaturated 20 x 10 cm twin- trough TLC chamber. After each development the plates were dried in a stream of warm air. The spots on the plates were visualized by dipping into a 20 x 10 cm dipping chamber (Camag, Muttenz, Switzerland) containing aniline (2%) diphenylamine (2%), and orthophosphoric acid (15%) in acetone. The plates were air- dried and then heated at 105 °C for 60 minutes.

3.4.11 Fourier Transform Near infra-red spectroscopy

Fourier Transform Near infra-red spectroscopic (Perkin Elmer) techniques are routinely employed for quick and easy tools to evaluate the various internal quality attributes of fruits and vegetable products. NIR reflectance spectroscopy was used in the region of 10,000 - 4000 cm^{-1} to measure the cassava leaf tissue linamarin content. Linamarin standard was read in NIR and peaks obtained were noted. After that methanolic extract of linamarin from young leaves of cassava was read in the NIR. Peaks obtained were noted and compared for similarities.

4. RESULTS

The present study entitled the "Physiological and molecular studies on cyanogenic potential in cassava (*Manihot esculenta* Crantz) in response to nitrogen nutrition, water stress and shade" was conducted at the Division of Crop Utilization, Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram, Kerala. The results obtained from the study are summarized below.

4.1 EFFECT OF LIGHT INTENSITY AND WATER STRESS ON CNglc LEVEL IN CASSAVA

4.1.1 Chlorophyll content

The changes in biochemical constituents like chlorophyll a, b content (chl-a and chl-b) were determined in fresh leaves from plants grown in control and stressed conditions. Maximum amount of chlorophyll was recorded under plants grown in shaded and irrigated conditions followed by open and irrigated condition. Water deficit stressed conditions resulted in a large decrease in chlorophyll amount under both shade and open conditions. In case of chl-a also, maximum amount was observed under shaded irrigated conditions followed by open irrigated conditions. Sree Vijaya had the highest amount chlorophyll a content in both conditions with a maximum of 3.98 mg in shade irrigated condition. Lowest was observed in PDP CMR 1. But in case of chlorophyll b a notable change between treatments could not be observed. Maximum amount observed was in Sree Athulya in open irrigated condition while lowest was observed in PDP CMR 1. Sree Vijaya had the highest amount of total chlorophyll content with an average of 4.3 mg/g of fresh leaf tissue under open control condition and a 25.5% decrease was observed in open and water withheld condition. Also there was a 5.6% increase under shade irrigated condition while a 7% decrease under water stressed shade plants. PDP CMR 1 had the lowest amount of chl a content with an average of 2.6 mg/g fresh leaf tissue. However, there was an increase of 9% in open light - water stress condition (Figure 2).

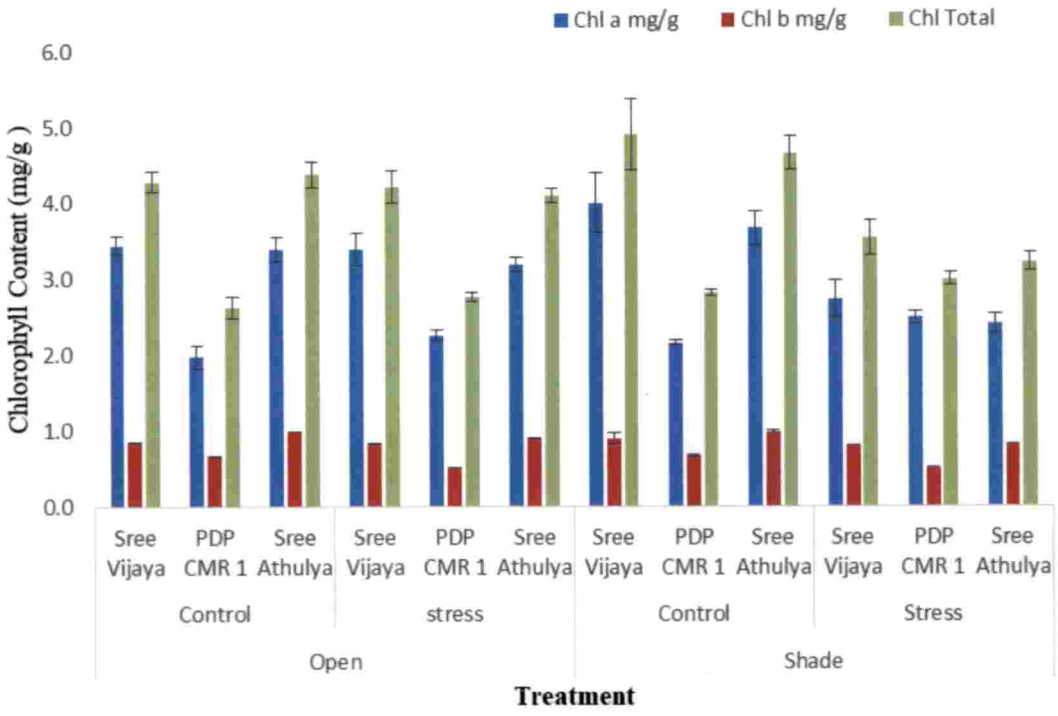


Figure 2 - Variation in chlorophyll content as affected by light intensity and water stress in selected varieties of cassava leaves

4.1.2 Changes in leaf protein content

Leaf protein content was measured using Bradford's method. Under open, well irrigated condition, plants did not showed any significant difference in protein content among the three varieties with all three of them having content of around 28 mg/g fresh leaf tissue. Maximum amount of protein was observed in water stressed shade plants with PDP CMR 1 showing greater increase of 30% reaching an amount of 41.15 mg/g fresh leaf tissue. Sree Vijaya showed an increase of 15% reaching an amount of 33.01 mg/g while Sree Athulya showing an increase of 11.9% reaches upto 31.54 mg/g. Shade treatment did significantly in cassava plants compared to open with changes in the range of 1%. Stressed plants under both open and shade conditions recorded an increase in protein content compared to no-stress treatment. Among the varieties, PDP CMR 1 showed highest increase protein content under both light treatments (Figure 3).

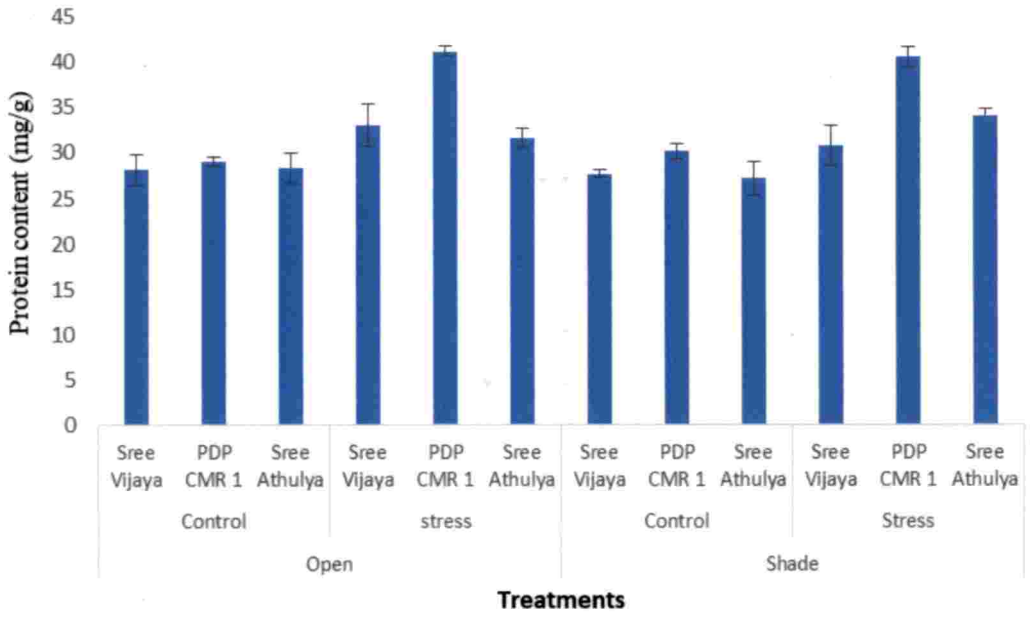


Figure 3 - Change in protein content as affected by light intensity and water stress in leaves of selected cassava varieties

4.1.3 Changes in leaf area

Total leaf area of cassava plants was determined at the end of the treatment period. Maximum leaf area was recorded in open irrigated conditions in Sree Vijaya with an average of 2627.7 cm^2 while the minimum leaf area was measured in PDP CMR 1 having an average of 1813.4 cm^2. Sree Athulya had a leaf area average of 2443.8 cm^2. Water stressed plants showed a sharp decrease in leaf area due to decrease in leaf number in plants. Sree Vijaya showed an 8.3 times decrease in leaf area compared to open irrigated condition. Sree Athulya's leaf area also decreased to 469.41 cm^2 while PDP CMR decreased to 285.2 cm^2 showing a decrease of 6.36 times. Shade irrigated plants also showed a decrease in leaf area compared to open irrigated plants with Sree Vijaya showing greatest decrease of 2.1 times that of open irrigated plants. PDP CMR 1 showed a decrease of 1.4 times and Sree Athulya of 1.1 times. A 13 times decrease was shown in

case of shade water withheld condition in Sree Vijaya reaching to 201.15cm^2. Similar trend was shown in other varieties also (Figure 4).

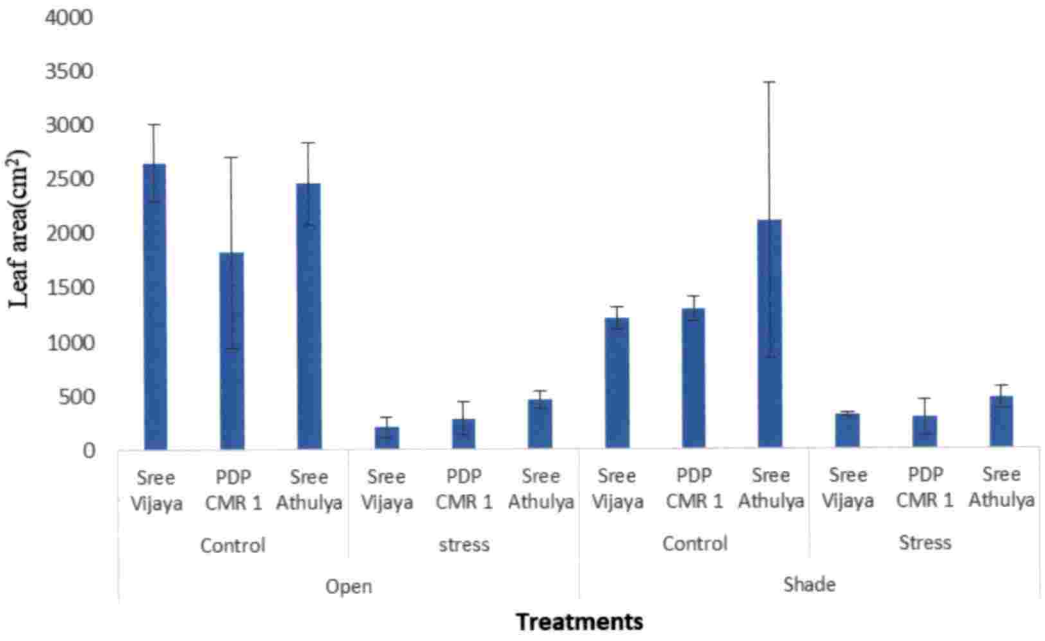


Figure 4 - Changes in leaf area as affected by light intensity and water stress in selected cassava varieties

4.1.4 Changes in leaf fresh and dry weight

Leaf fresh and dry weights were recorded in three cassava varieties at the end of the treatment period. Maximum leaf fresh weight was recorded in Sree Athulya under open irrigated conditions (34.14 g/plant) while minimum was observed in PDP CMR 1 at 20.95 g/ plant. Sree Vijaya had an average of 32.2 g per plant. In water stressed, open light grown plants leaf fresh weight decreased 6.59, 4.4 and 4.2 fold in Sree Vijaya, PDP CMR 1 and Sree Athulya, respectively. Control plants under shade treament had lower fresh weight compared to that of open conteol. The reduction in leaf fresh weight were 2.9, 1.7 and 1.3 fold in Sree Vijaya, PDP CMR 1 and Sree Athulya respectively (Figure 5).

Sree Athulya showed highest leaf dry weight of 9.88 g per plant followed by Sree Vijaya with 7.58 g per plant and PDP CMR 1 with 5.13 g per plant in open irrigated condition. There was a 5.22 fold decrease in dry weight in the stressed plants of Sree Athulya in open light condition and 6.17 fold decrease in case of Sree Vijaya, 4.58 fold in case of PDP CMR 1. In case of shade irrigated plants also there is a decrease in dry weight compared to open irrigated. In case of Sree Vijaya there is a 3 fold decrease thus reaching to 2.47g per plant. Similar trend is observed in all the plants.

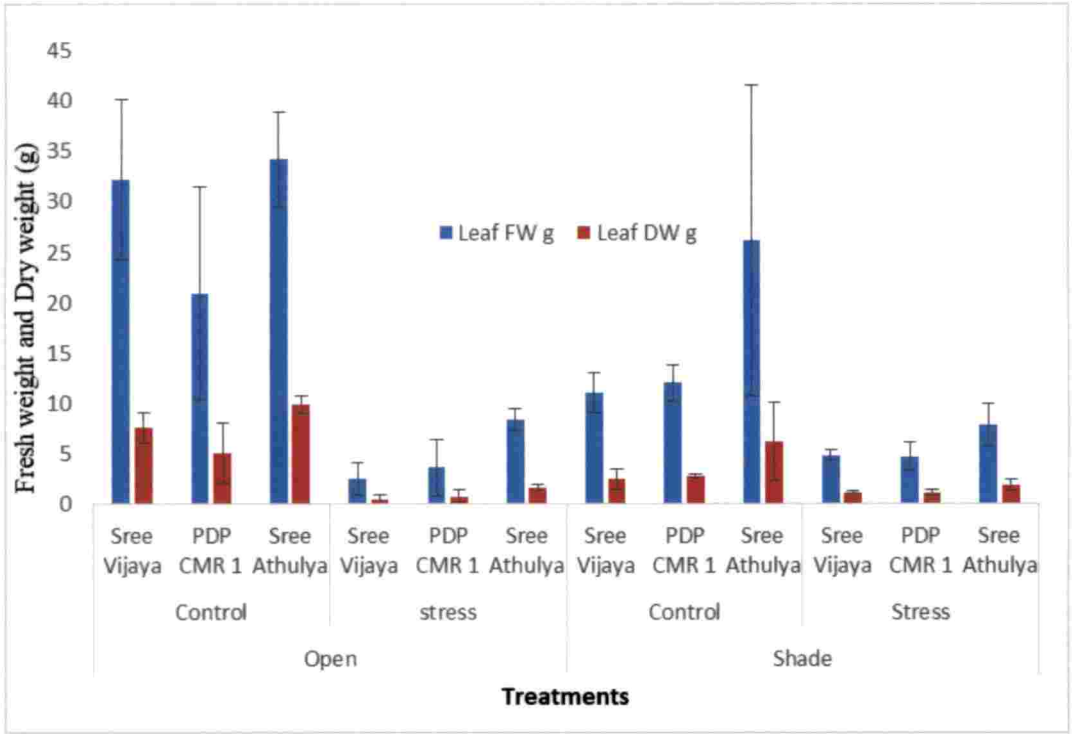


Figure 5 - Change in leaf fresh weight and dry weight as affected by light intensity and water stress in selected cassava varieties

4.1.5 Plant fresh weight and dry weight

Plant fresh weight was found maximum for Sree Athulya with 64.13g followed by Sree Vijaya with 60.74 g and minimum is for PDP CMR 1 with 35.65g. This is decreased under open water withheld condition by 20.962 for Sree Athulya, 21.584 for Sree Vijaya and 19.98 for PDP CMR 1. Plants grown under shade irrigated condition has also shown a decrease in fresh weight with 30.98g for Sree Vijaya, 24.94g for PDP CMR 1 and Sree Athulya has shown an increase

by 80.35g. Like that of open water withheld treatment, shade water withheld treatment also resulted in decrease in fresh weight (Figure 6).

In case of dry weight also same trend as of fresh weight wasobserved as Sree Athulya having the maximum value of 13.79 g, followed by Sree Vijaya with 12.35g and the minimum for PDP CMR 1 with 5.59g. An average 4 fold decrease is seen in each plant in case of open water stressed conditions. In shade irrigated plants a decrease is seen in dry weight compared to open irrigated plants in average of 2 folds. Same trend as that of fresh weight was observed here also in case of dry weigh with an average decrease of 2.5 fold than that of open irrigated plants.

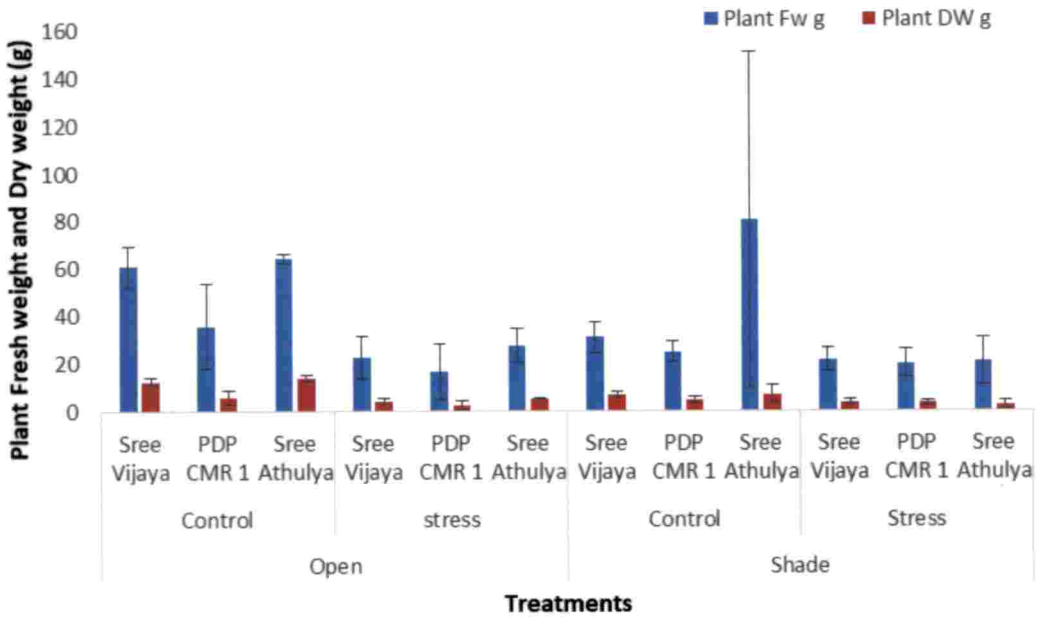


Figure 6 - Change in plant fresh weight and dry weight as affected by light intensity and water stress in selected cassava varieties

4.1.6 Changes in leaf gas exchange parameters

The gas exchange measurements were carried out in cassava leaves and the results are presented in Table 3. There were significant differences in gas exchange parameters among treatments. Net photosynthetic rate (Pn) was highest in Sree Vijaya (23.17 µmol m^{-2} s^{-1}) under control plants of open condition.. There

was a drastic decrease in Pn in stressed plants compared to control. There was a slight decrease in Pn under shade conditions compared to open light. Stressed plants showed similar reduction in Pn in both light conditions. Stomatal conductance (Gs) showed similar trend as Pn for both light conditions and stress treatment. Open control plants showed higher Gs compared to shaded plants of all the varieties. Under water stress, there was a decrease in Gs in all the varieties except PDP CMR1 which showed an increase. The transpiration rate of cassava leaves decreased in water stress in both open and shade condition. Compared to the Pn and Gs, the transpiration rate change was lesser in stressed plant.

Table 3 – Gas exchange measurements as affected by light intensity and water stress in selected cassava varieties

	Net photosynthetic rate (unit)				Conductance				Transpiration rate (unit)			
	Open		Shade		Open		Shade		Open		Shade	
	Control	Stress	Control	Stress	Control	Stress	Control	Stress	Control	Stress	Control	Stress
Sree Athulya	19.63	2.05	15.13	0.79	0.33	0.25	0.26	0.17	6.92	5.78	5.51	3.85
PDP CMR1	19.35	1.63	8.75	2.54	0.23	0.09	0.086	0.15	5.97	2.27	2.10	3.52
Sree Vijaya	23.17	2.09	18.96	1.63	0.35	0.13	0.28	0.09	7.33	3.13	5.76	2.27

4.1.7 Chlorophyll fluorescence

Chlorophyll fluorescence kinetics parameters were determined for the cassava plants under different nutrient regimes and light condtions. Chlorophyll fluorescence kinetics such as Fv/Fm, Fv'/Fm', PhiPS2, NPQ were presented in the table 4. The Fv/Fm, the maximum the maximum potential quantum efficiency of Photosystem II of cassava plants did not significantly vary among the treatments. It ranged from 0.75 to 0.78 in open conditions and it ranged from 0.76 to 0.82 in shaded plants of all the varieties. Shaded cassava plants showed higher Fv/Fm values compared to open light grown plants in all nutrition levels. The nutrition status of plants did not alter significantly the Fv/Fm values.

Table 4 - Chlorophyll fluorescence kinetics parameters as affected by light intensity and water stress in selected cassava varieties

	Fv/Fm				Fv'/Fm'				PhiPSII				NPQ			
	Open		Shade		Open		Shade		Open		Shade		Open		Shade	
	Control	Stress	Control	Stress	Control	Stress	Control	Stress	Control	Stress	Control	Stress	Control	Stress	Control	Stress
Sree Athulya	0.77	0.65	0.81	0.68	0.46	0.26	0.48	0.31	0.17	0.05	0.09	0.04	1.58	2.56	0.82	2.39
PDP CMR1	0.77	0.65	0.79	0.67	0.70	0.29	0.54	0.38	0.75	0.04	0.11	0.04	0.03	4.02	1.26	1.96
Sree Vijaya	0.82	0.66	0.79	0.64	0.48	0.30	0.47	0.42	0.17	0.04	0.10	0.06	1.93	3.30	2.09	0.71

4.1.8 Cyanogenic glycoside content

Maximum amount of CNglc was found in leaves of PDP CMR 1 with 0.5 mg/g fresh leaf tissue followed by Sree Athulya 0.32 mg/g and a minimum was found in Sree Vijaya with 0.18mg/g under open irrigated condition. In open water stress conditions 0.23 mg/g tissue was found in Sree Vijaya meaning an increase of 22%. In case of Sree Athulya a 9% increase amounts to 0.38 mg and 7% increase in PDP CMR 1 resulting in 0.58 mg. There is not much difference between plants grown in shade irrigated and open irrigated in level of CNglc content. Plants grown in shade water deficit stress have CNglc amount at par with plants grown in open water deficit stress with a maximum average of 0.54 mg for PDP CMR 1, 0.38 mg for Sree Athulya and 0.23 mg for Sree Vijaya.

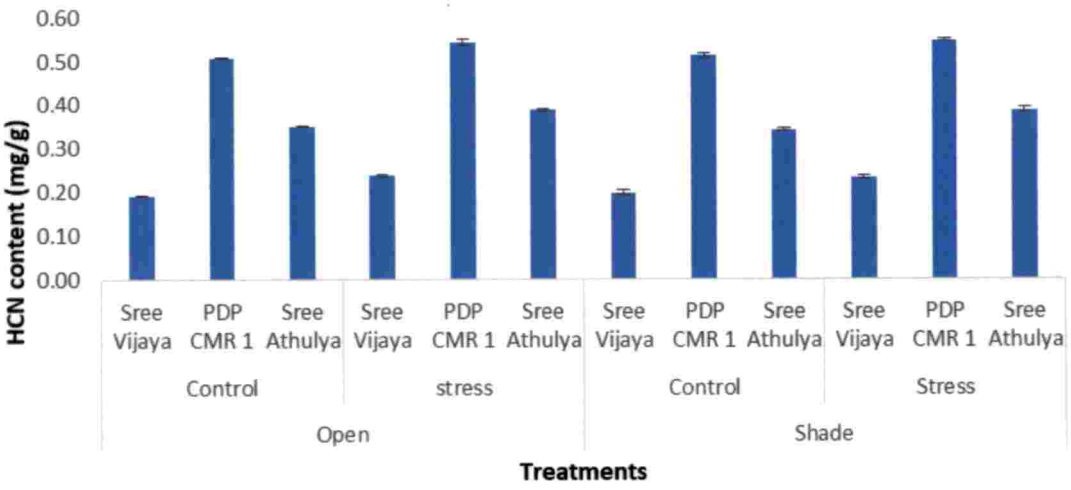


Figure 7 - Change in HCN content in fresh leaves as affected by light intensity and water stress in selected cassava varieties

4.1.9 Leaf nitrogen content

Maximum amount of nitrogen content was found in control water deficit stress treated plants with Sree Vijaya having a maximum amount of 4.4% nitrogen content. Sree Athulya had 4.37% nitrogen content whereas PDP CMR – 1 had the lowest with 4.29%. Minimum amount was observed in open irrigated plants with Sree Vijaya having an average of 4.37%, Sree Athulya with 4.28% and PDP CMR -1 with 4.3%. There is only a slight difference in nitrogen content between plants grown in shade water deficit stress and open water deficit stress conditions (Figure 8).

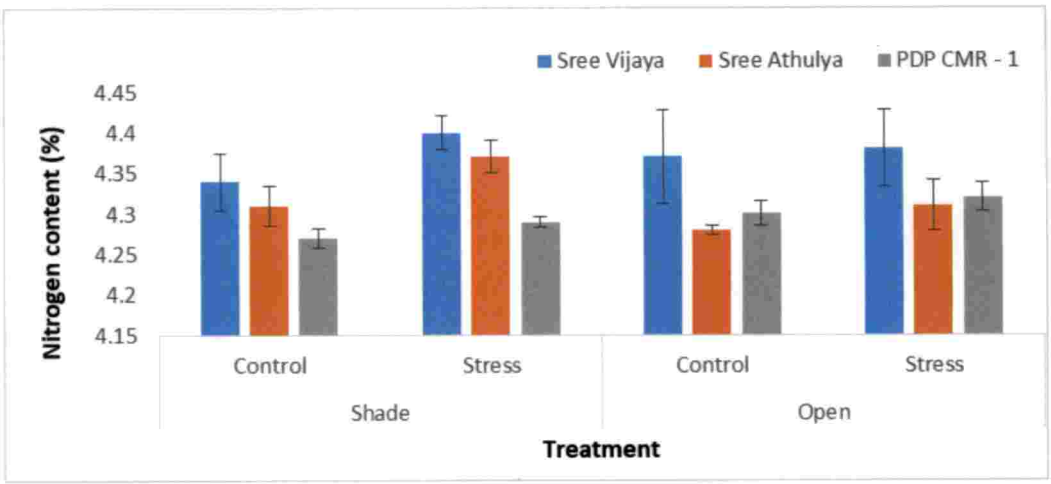
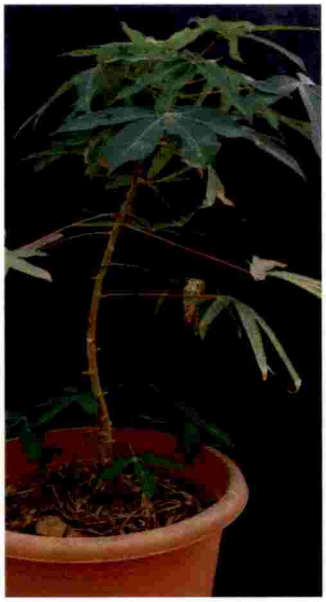


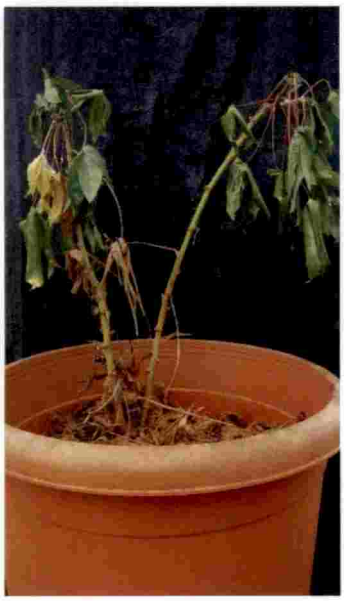
Figure 8 - Nitrogen content as affected by light intensity and water stress in cassava leaves in selected cassava varieties

Sree Athulya - shade - irrigated



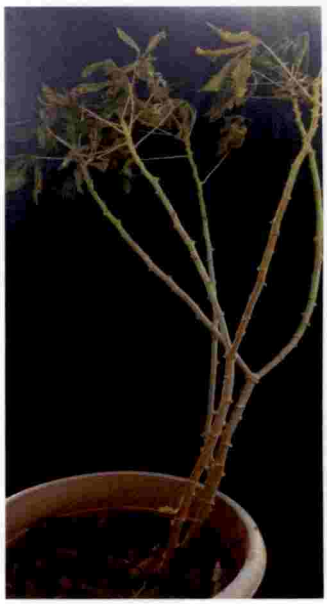
PDP CMR - 1 - shade - irrigated

Sree Vijaya - shade - irrigated



Sree Athulya – shade – drought stress

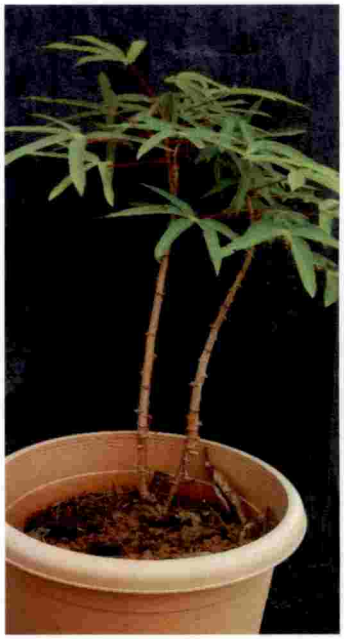
PDP CMR -1 – shade – drought stress



Sree Vijaya – shade-drought stress

Sree Athulya – open - irrigated

Sree Vijaya – open - irrigated



PDP CMR-1- open - irrigated

Sree Athulya – open – drought stress

Sree Vijaya – open – drought stress

PDP CMR – 1 – open – drought stress

4.2 EFFECT OF LIGHT INTENSITY AND N- NUTRITION ON CNglc LEVEL IN CASSAVA

4.2.1 Chlorophyll content

Chlorophyll content was measured in terms of chlorophyll a, chlorophyll b and total chlorophyll content. There was a slight increase in chlorophyll content according to level of nitrogen nutrition both under light and shaded condition. Sree Vijaya had the highest amount of chlorophyll a with a maximum of 3.61 mg/g in 2x strength Hoagland's solution and minimum amount was recorded in PDP CMR 1. Although there is an increase in amount of chlorophyll a for 2x strength Hoagland's solution from 0.5x strength not much difference was observed between 1x and 2x strength solution both showing around 3.6 mg/g fresh tissue. Sree Athulya also had chlorophyll a content of around 3.1mg/g similar to that of Sree Vijaya. PDP CMR 1 has the lowest amount of 2.5 mg/g and not much difference was observed in diiferent strength of Hoagland's solution. Highest amount of chlorophyll b was seen in Sree Athulya with 0.89 mg/g fresh tissue grown in shade and 2x strength Hoagland's solution followed by Sree Vijaya. Lowest amount was seen in PDP CMR 1 ranging to 0.65 mg/g with shade grown plants showing an increase in the amount (Figure 9).

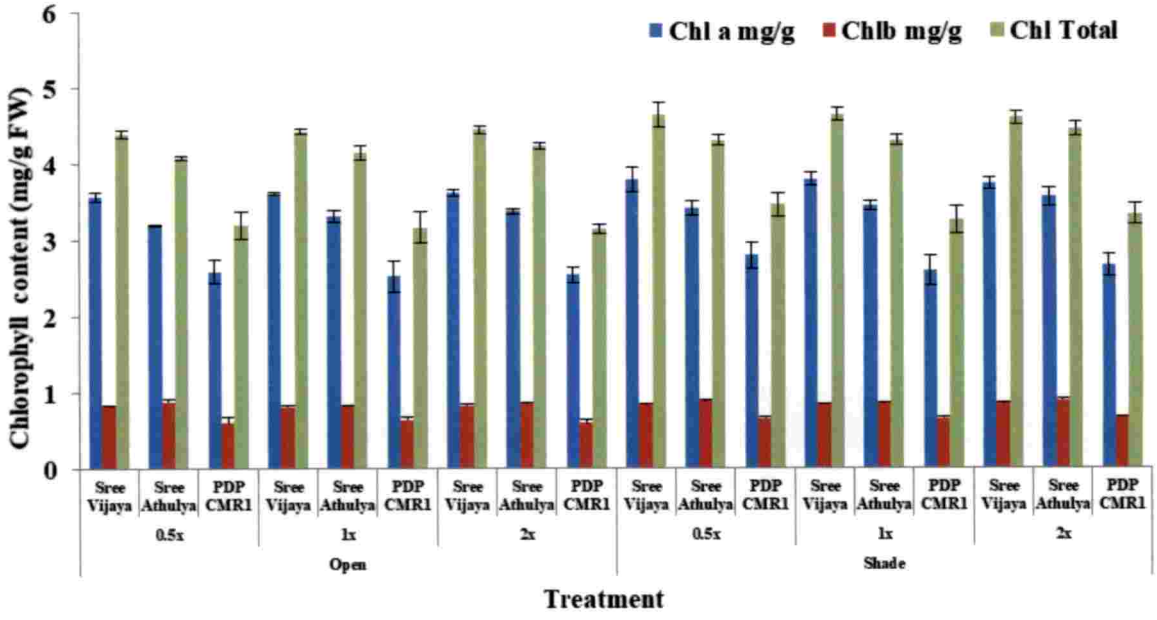


Figure 9 - Chlorophyll content as affected by light intensity and N- nutrition in selected cassava varieties

4.2.2 Changes in leaf protein content

Highest amount of protein content was recorded by PDP CMR 1 with an average of 30 mg/g in shaded condition. Sree Vijaya had 28.6 mg/g of protein in open condition with 0.5x strength Hoagland's solution. It was increased to 30.5 mg/g in 1x strength solution. Sree Athulya does not showed any increase in protein content according to nitrogen nutrition. Lowest amount of protein content was recorded in Sree Athulya with 28 mg/g in both shaded and open conditions. Nitrogen nutrition did not influence significantly the protein content in the leaves of cassava plants. Also there was no marked difference between PDP CMR 1 and Sree Vijaya in leaf protein content. Sree Vijaya closely follows PDP CMR 1 in protein content with an average of 29.5 mg/g fresh tissue. In most of the varieties higher protein content was seen in 1x strength Hoagland's solution (Figure 10).

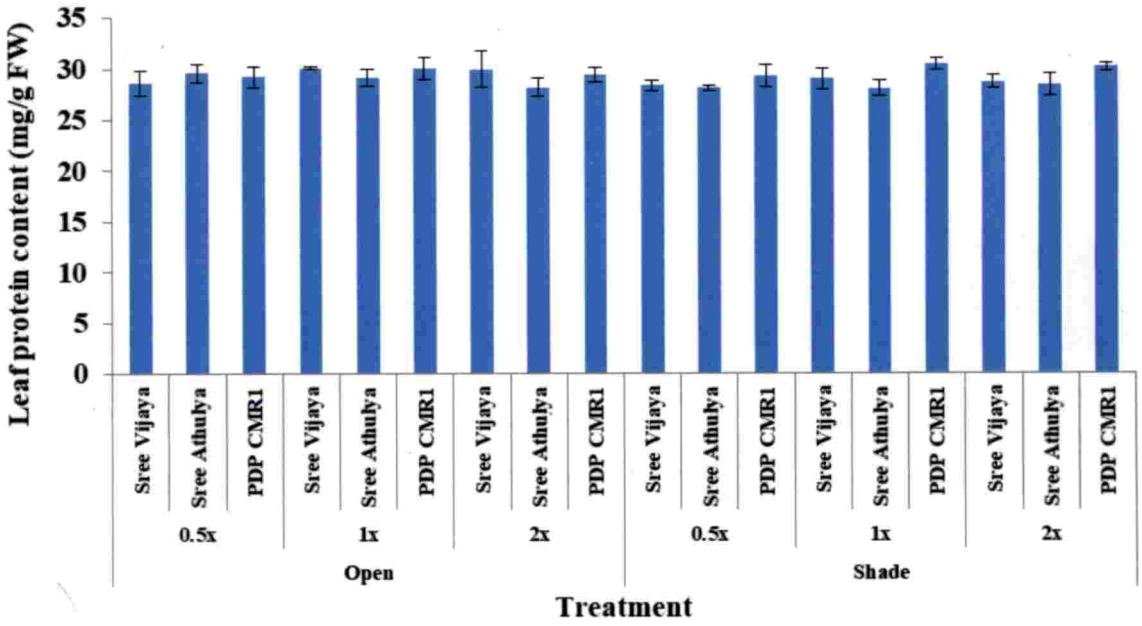


Figure 10 - Change in leaf protein content as affected by light intensity and N-nutrition in selected cassava varieties

4.2.3 Changes in leaf area

Sree Athulya had the largest leaf area in open conditions in all level of nitrogen nutrition with a maximum of 1415.4 cm^2. This is followed by Sree Vijaya with value ranging upto 1395.4 cm^2.

Lowest value was seen in PDP CMR 1 with a maximum of 1108.9 cm2. An increase in leaf area is seen in all varieties according to increase in strength in nitrogen nutrition. Plants kept in open have a slight increase in leaf area than kept under shade. Here also leaf area increased according to nitrogen nutrition with Sree Vijaya having the maximum leaf area under 2 x strength nutrient solution with 1391.5 cm^2. PDP CMR 1 had the minimum compared to the other two with 1122.7 cm^2 in 2x strength solution (Figure 11).

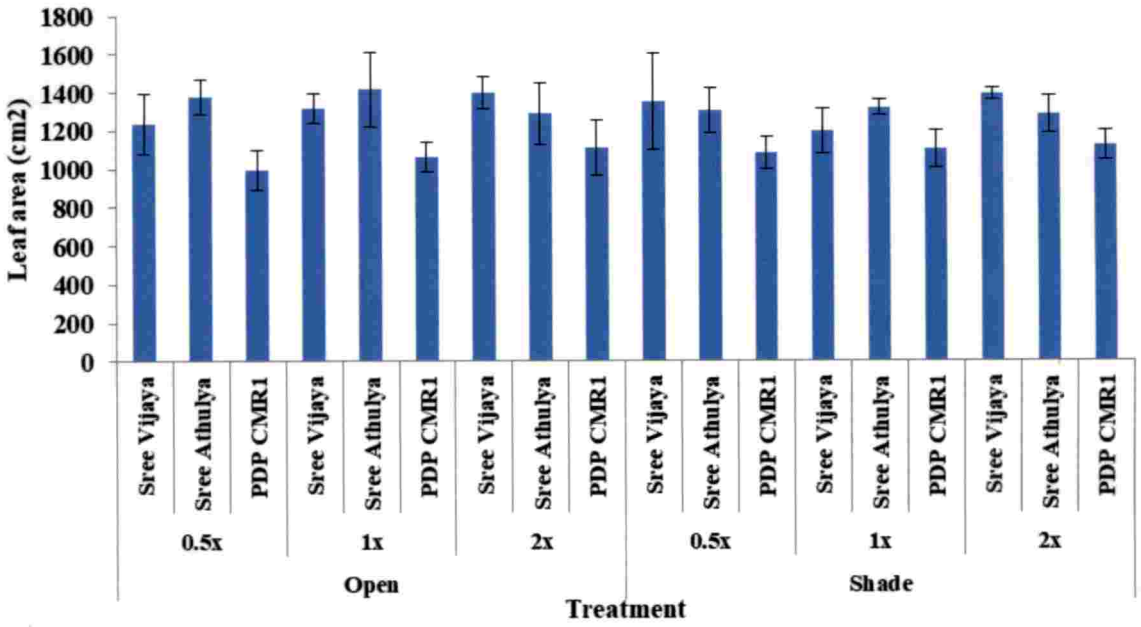


Figure 11 - Changes in leaf area as affected by light intensity and N-nutrition in selected cassava varieties

4.2.4 Leaf fresh and dry weight content

In open, control condition Sree Athulya had the highest leaf fresh weight with 30.813 g. Sree Vijaya also has a high leaf fresh weight of 30.31 g per plant. Lowest leaf fresh weight was seen in PDP CMR 1 ranging upto 24.89 g. There was an increase in leaf fresh weight according to an increase in strength in nitrogen nutrition with 2x strength solution having the maximum fresh weight. Plants grown in shade showed a

decrease in leaf fresh weight than those in open condition. Same trend as that of open is seen here also in the case of fresh weight and nitrogen nutrition relationship.

In leaf dry weight also plants grown in open condition had greater values than those under shade. There is an increase in fresh weight according to nitrogen nutrition level with 1x strength solution treated plants having highest values than 0.5x treated plants. Not much difference was observed between 1x strength solution and 2x strength solution treated plants though. Maximum dry weight was recorded in Sree Vijaya in 1x strength solution treated and in open condition with 6.9 g. Sree Athulya also had 6.8 g under similar condition. Minimum was recorded for PDP CMR 1 with 3.93 g in the same condition (Figure 18).

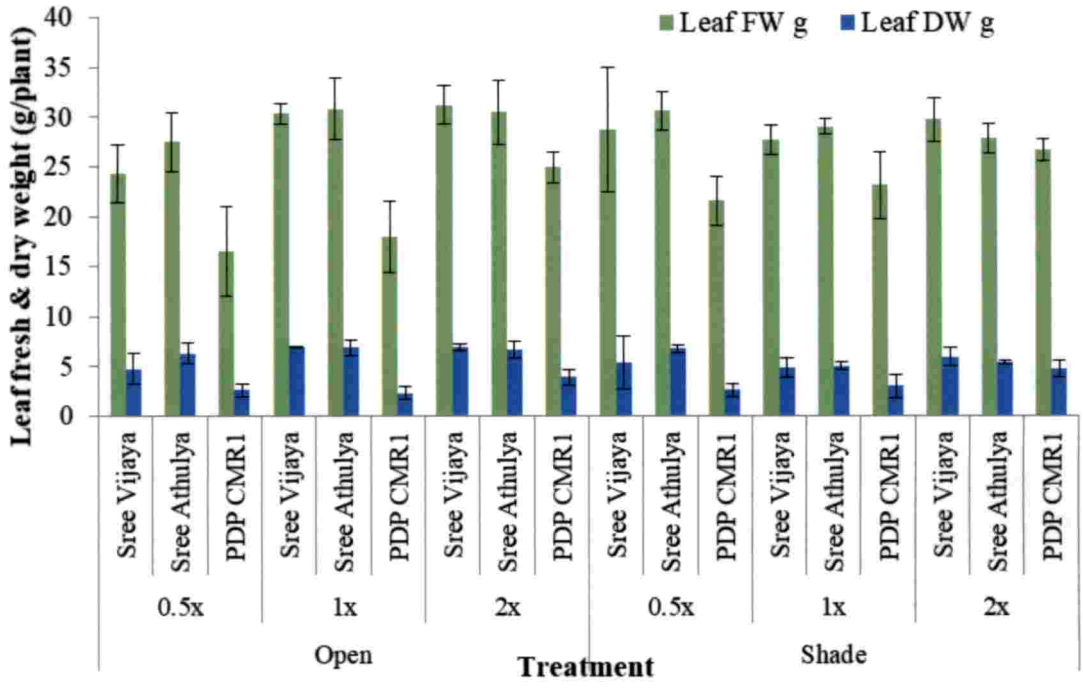


Figure 12 – Changes in leaf fresh and dry weight content as affected by light intensity and N- nutrition in selected cassava varieties

4.2.5 Plant fresh weight and dry weight

Maximum plant fresh weight was recorded in Sree Vijaya grown in 2x strength solution and open condition with 52.97 g followed by Sree Athulya in 51.36 g. Minimum was observed in PDP CMR 1 ranging upto 32.58 g. No considerable increase was seen according to nitrogen nutrition level in plant dry weight. Also plant fresh weight was found to be low under shaded condition than in open conditions.

In the case of dry weight maximum was recorded for Sree Vijaya in open condition and 2x strength solution treated with 13.01 g. Sree Athulya had a maximum of 11.68 g in 0.5x strength solution treated in open condition and PDP CMR 1 had maximum of 7.28 g in 2x strength solution treatment. Same trend as that of leaf fresh weight was seen here with no considerable change according to nitrogen nutrition level and a decrease in plants grown in shade (Figure 13).

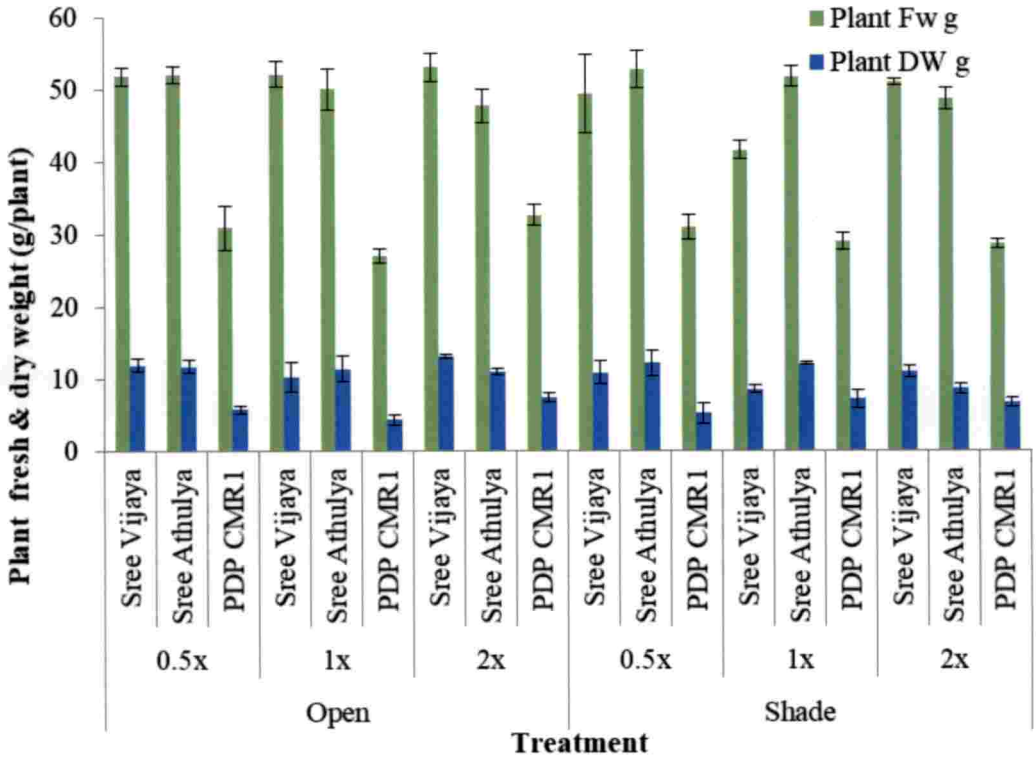


Figure 13 – Changes in plant fresh weight and dry weight according to light intensity and N- nutrition in selected cassava varieties

4.2.6 Photosynthesis measurements

The gas exchange measurements were carried out in cassava leave and the results are presented in Table 5. Photosynthesis rate seems to increase according to nitrogen nutrition level in open condition reaching 19.95 µmol m-2 s-1 for 2x strength treatment from 17.25 µmol m-2 s-1 for 0.5x strength treatment. In case of conductance a significant change was not seen. Same was observed in the case of transpiration rate also. Shade treated plants showed no change in photosynthetic rate according to increase in nitrogen nutrition level. Same was the observation for conductance and transpiration rate also.

Table 5 - Leaf gas exchange parameters of cassava varieties under different light conditions and different level of nitrogen nutrition treatments

Open	**Photosynthetic rate** ($\mu mol\ m^{-2}\ s^{-1}$)			**Conductance** ($mmol\ m^{-2}\ s^{-1}$)			**Transpiration rate** ($mmol\ m^{-2}\ s^{-1}$)		
	0.5x	1x	2x	.5x	1x	2x	0.5x	1x	2x
SreeVijaya	15.47	17.04	17.66	0.21	0.24	0.22	3.80	4.24	3.85
PDP CMR 1	15.13	15.84	16.98	0.19	0.18	0.23	3.48	3.58	4.28
Sree Athulya	17.25	19.81	19.95	0.29	0.25	0.25	5.38	4.92	4.54
shade	**photosynthetic rate**			**conductance**			**transpiration rate**		
	0.5x	1x	2x	0.5x	1x	2x	0.5x	1x	2x
SreeVijaya	3.42	3.39	2.13	0.06	0.05	0.04	0.79	0.71	0.13
PDP CMR 1	3.32	3.64	5.01	3.32	0.05	0.06	0.69	0.97	0.93
Sree Athulya	3.98	3.69	3.40	3.98	0.04	0.03	0.92	0.78	0.56

4.2.7 Chlorophyll fluorescence

Chlorophyll fluorescence kinetics parameters were determined for the cassava plants under different nutrient regimes and light condtions. Chlorophyll fluorescence kinetics such as Fv/Fm, Fv'/Fm', PhiPSII, NPQ were presented in the table 6. The Fv/Fm, the maximum potential quantum efficiency of Photosystem II of cassava plants did not significantly vary among the treatments. It ranged from 0.75 to 0.78 in open conditions and it ranged from 0.76 to 0.82 in shaded plants of all the varieties. Shaded cassava plants showed higher Fv/Fm values compared to open light grown plants in all nutrition levels. The nutrition status of plants did not alter significantly the Fv/Fm values. Similarly, Fv'/Fm' provides the maximum potential quantum efficiency of Photosystem II of light adopted leaves. Open and shaded plants showed marked difference in Fv'/Fm' values. It averaged 0.45 in open conditions, whereas the values were higher in shaded plants with an average of 0.53. Nutritional status did not influence significantly the Fv'/Fm' in cassava plants. PhiPSII, photochemical efficiency, was higher in open plants compared to shade. However, nutritional status did not significantly affect the parameters in both open as well as shade conditions. NPQ, the non-photochemical quenching values were not significantly affected by either light conditions or nutritional status of cassava plants.

Table 6 – Change in chlorophyll fluorescence as affected by light intensity and nitrogen nutrition level in selected cassava varieties

	Fv/F m			Fv'/F m'			PhiP S2			NP Q		
OPEN	0.5x	1x	2x	0.5x	1x	2x	0.5x	1x	2x	0.5x	1x	2x
Sree Athulya	0.77	0.76	0.76	0.45	0.46	0.46	0.23	0.21	0.2	1.73	1.78	1.65
PDP CMR 1	0.78	0.78	0.78	0.72	0.53	0.46	0.69	0.3	0.19	0.27	1.86	1.44
SreeVijaya	0.76	0.79	0.78	0.41	0.51	0.47	0.17	0.23	0.23	1.45	1.29	1.45

SHADE	Fv/F m			Fv'/F m'			PhiP S2			NP Q		
	0.5x	1x	2x	0.5x	1x	2x	0.5x	1x	2x	0.5x	1x	2x
Sree Athulya	0.82	0.81	0.82	0.61	0.56	0.56	0.14	0.15	0.16	1.29	1.6	1.3
PDP CMR 1	0.81	0.76	0.8	0.56	0.58	0.55	0.18	0.12	0.15	1.2	1.15	1.31
SreeVija ya	0.79	0.8	0.8	0.46	0.5	0.53	0.08	0.1	0.9	1.1	1.86	1.58

Maximum amount of cyanogenic glycoside was found in PDP CMR 1 with a maximum of 0.504 mg/g fresh tissue in 2x strength solution treatment and lowest was seen in Sree Vijaya in 0.1883 mg/g in 0.5x strength solution. Sree Athulya had a maximum CNglc content of 0.3607 mg/g. Amount of CNglc seems to be increased in plants grown in shade and the maximum amount is seen in PDP CMR 1 in 1x strength solution treatment with around 0.51 mg/g. CNglc content increase according to nitrogen nutrition and did not seem to follow a uniform pattern in the case of Sree Vijaya the CNglc content remains unchanged or a small decrease is seen. Also here although an increase is seen in CNglc content from 0.5x to 1x strength, such an increase was not seen in 2x strength solution (Figure 14).

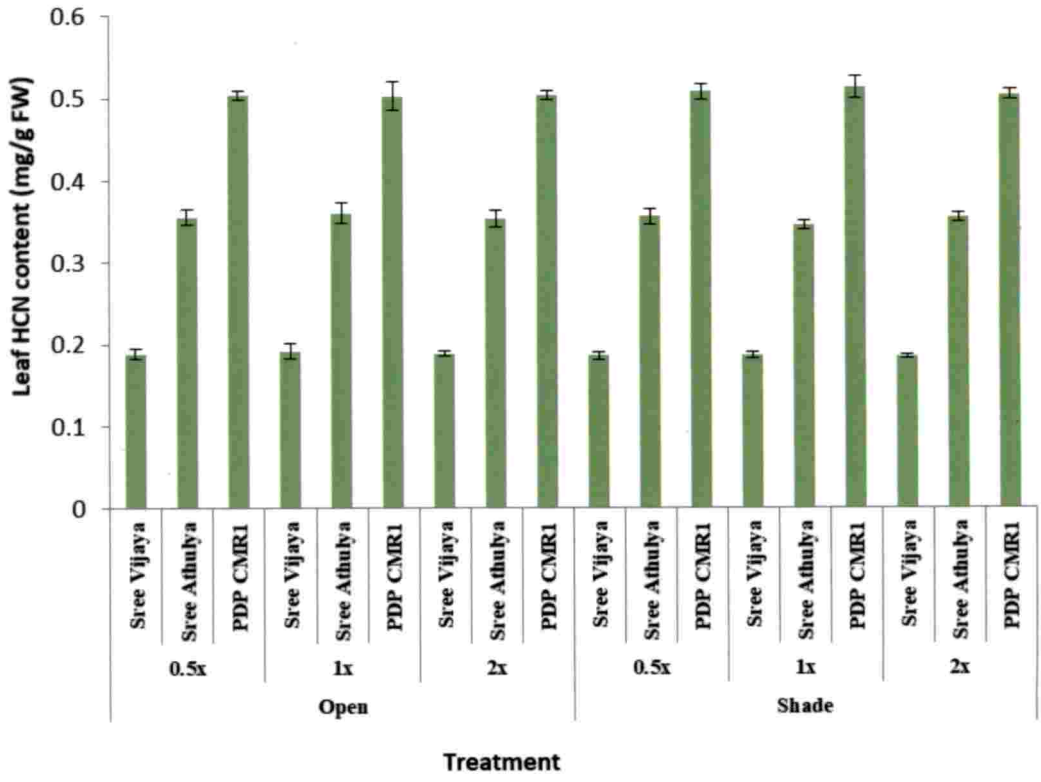


Figure 14 – Change in leaf HCN content as affected by light intensity and N-nutrition in selected cassava varieties

4.2.9 Leaf nitrogen content

Maximum amount of leaf nitrogen content was observed in plants grown in open 2x strength Hoagland's solution with an average of 4.41% for Sree Vijaya, 4.39% for Sree Athulya and 4.34% for PDP CMR -1. Lowest amount was observed in plants grown under shade 0.5x strength Hoagland's solution with an average of 4.3% for Sree Vijaya, 4.29% for Sree Athulya and 4.25% for PDP CMR -1. In both open and shaded cases not much difference was observed between 0.5x strength Hoagland's solution and 1x strength Hoagland's solution. But a significant difference was observed in 2x strength Hoagland's solution treated plants. Nitrogen content increases with level of nitrogen fertilizer application the effect is almost similar in plants grown in both shaded and open condition.

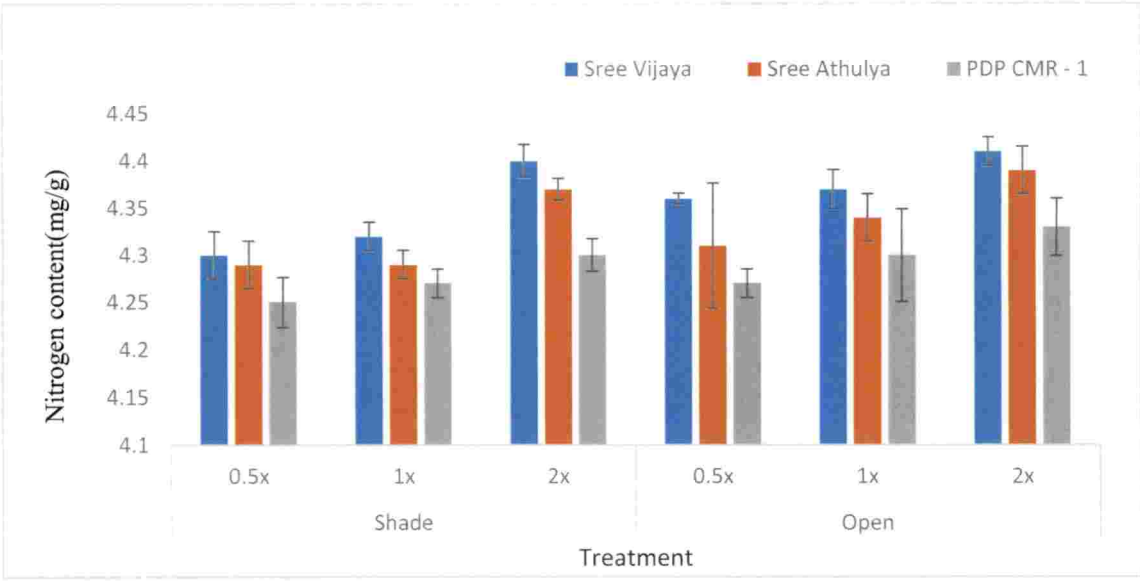


Figure 15 - Leaf nitrogen content as affected by light intensity and nitrogen nutrition in selected cassava varieties

4.3 STUDY THE PHYSIOLOGICAL AND MOLECULAR ASPECTS OF NITROGEN NUTRITION ON CNglc LEVEL IN CASSAVA

4.3.1 Cyanogenic glycoside content

Of the three modes of nitrogen nutrition, nitrate form was seen to cause a slight increase in CNglc content and the rest two does not show any significant change. PDP CMR 1 had the maximum CNglc content of 0.514 mg/g fresh weight. In ammoniacal form the CNglc content decreased to 0.508 mg/g and in amide form to 0.509 mg/g. Sree Vijaya had the lowest amount with 0.198 mg/g with nitrate form and 0.191 mg/g with ammoniacal and amide form. Sree Athulya had 0.365 mg/g with nitrate form and 0.363 mg/g with ammoniacal form and 0.359 mg/g with amide form.

4.3.2 Expression profile of Uridine diphosphate glycosyl transferase (UGT) gene

Here nitrate form of nutrition was taken as control and relative expression of the gene UGT85K4 was found out (Figure 16, 17 respectively).

Sree Vijaya – 0.5x strength Hoagland's solution - Open

Sree Vijaya – 1x strength Hoagland's solution - Open

Sree Vijaya – 2x strength Hoagland's solution- Open

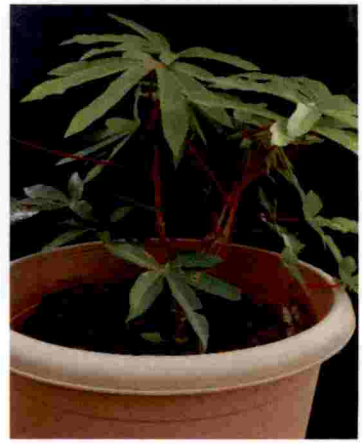
Sree Athulya – 0.5x strength Hoagland's solution- Open

Sree Athulya – 1x strength Hoagland's solution- Open

Sree Athulya – 2x strength Hoagland's solution- Open

PDP CMR – 1- 0.5x strength Hoagland's solution- Open

PDP CMR – 1 - 1x strength Hoagland's solution- Open

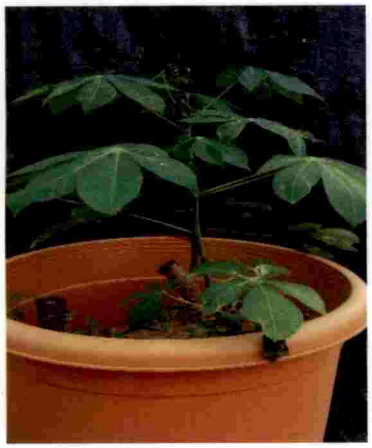


PDP CMR – 1 - 2x strength Hoagland's solution- Open

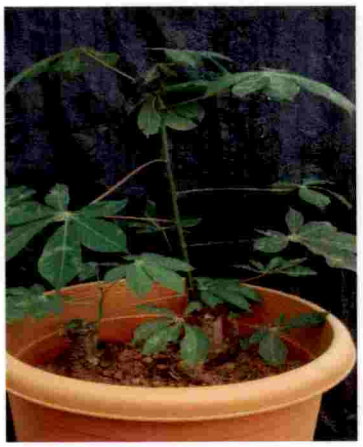
Sree Vijaya – 0.5x strength Hoagland's solution - Shade

Sree Vijaya – 1x strength Hoagland's solution - Shade

Sree Vijaya – 2x strength Hoagland's solution- Shade



Sree Athulya – 0.5x strength Hoagland's solution- Shade



Sree Athulya – 1x strength Hoagland's solution- Shade

Sree Athulya – 2x strength Hoagland's solution- Shade

PDP CMR – 1- 0.5x strength Hoagland's solution- Shade

PDP CMR – 1 - 1x strength Hoagland's solution- Shade

PDP CMR – 1 - 2x strength Hoagland's solution- Shade

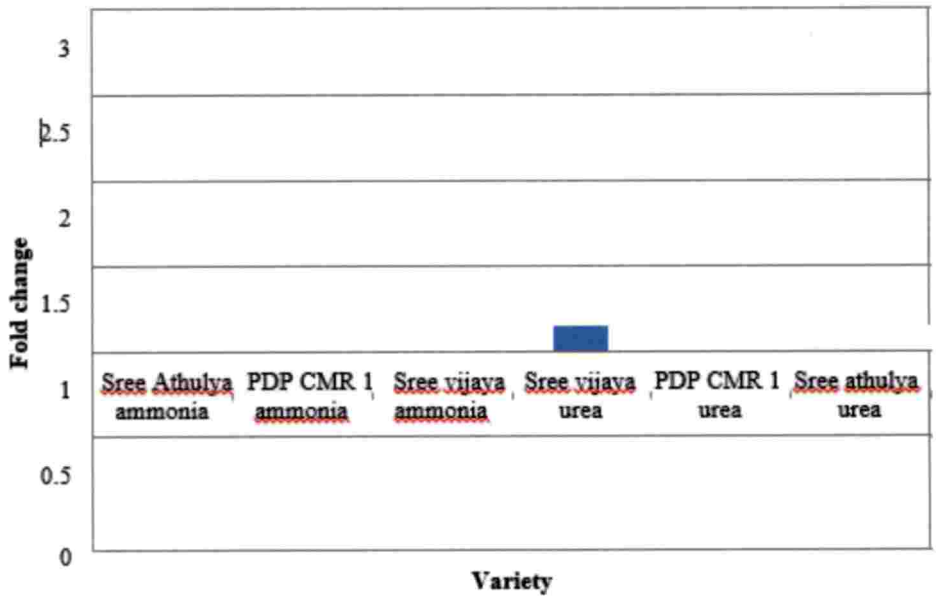


Figure 16 - Fold change graph of UGTK54 gene according to different form of nitrogen nutrition

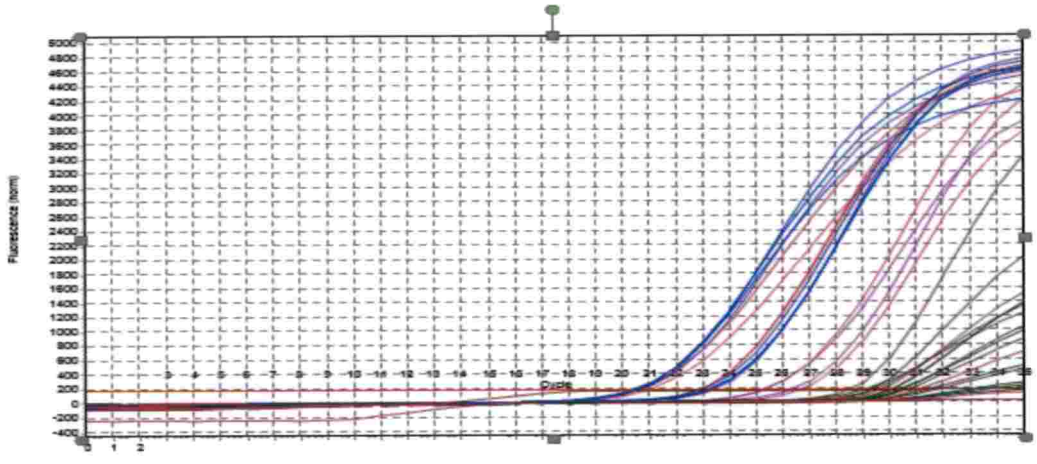
The expression levels of gene UGT85K4 for ammoniacal form of nitrogen nutrition and amide form of nitrogen nutrition was studied with respect to nitrate form. With different form of nitrogen nutrition no considerable change in gene expression is observed. All the varieties under treatments did not show any significant fold change in the expression levels.

Table 7- Table of Ct values

Pos	Name	Ct SYBR	Ct Mean SYBR	Ct Dev. SYBR
D1	V-1	34.78	33.06	2.44
D2	V-1	31.34	33.06	2.44
D3	V-2	29.34	28.91	0.62
D4	V-2	28.47	28.91	0.62
D5	V-3	20.31	20.59	0.40
D6	V-3	20.87	20.59	0.40
D7	V-4	25.26	25.25	0.01
D8	V-4	25.25	25.25	0.01
D9	V-5	20.55	20.54	0.01
D10	V-5	20.54	20.54	0.01
D11	V-6	20.39	20.40	0.01
D12	V-6	20.41	20.40	0.01
E1	V-7	30.42	29.86	0.78
E2	V-7	29.31	29.86	0.78
E3	V-8	28.95	29.30	0.49
E4	V-8	29.64	29.30	0.49
E5	V-9	31.00	30.47	0.75

E6	V-9	29.93	30.47	0.75
E7	V-1-C	32.89	33.12	0.32
E8	V-1-C	33.35	33.12	0.32
E9	V-2-C	30.66	31.18	0.74
E10	V-2-C	31.71	31.18	0.74
E11	V-3-C	22.79	22.85	0.08
E12	V-3-C	22.91	22.85	0.08
F1	V-4-C	26.61	26.44	0.24
F2	V-4-C	26.27	26.44	0.24
F3	V-5-C	23.35	23.10	0.35
F4	V-5-C	22.86	23.10	0.35
F5	V-6-C	23.20	23.19	0.02
F6	V-6-C	23.18	23.19	0.02
F7	V-7-C	30.05	30.25	0.29
F8	V-7-C	30.46	30.25	0.29
F9	V-8-C	26.24	26.27	0.02
F10	V-8-C	26.60	26.27	0.02
F11	V-9-C	30.21	30.36	0.21
F12	V-9-C	30.43	30.36	0.21
Analysis Parameters				
Type of Application	Quantification			
Dye(s)	SYBR			
Inverted Data	OFF			
Threshold setting	Noiseband			
Threshold level	166			
Baseline setting	Automatic			
Baseline range (from cycle no. ... to)	n/a			
Drift Correction	OFF			

V-1	Sree Vijaya - Ammoniacal form
V-2	Sree Vijaya - Nitrate form
V-3	Sree Vijaya- Amide form
V-4	Sree Athulya - Ammoniacal form
V-5	Sree Athulya - Nitrate form
V-6	Sree Athulya - Amide form
V-7	PDP CMR 1 - Ammoniacal form
V-8	PDP CMR1 - Nitrate form
V-9	PDP CMR 1 - Amide form
C	Control



Threshold: 166 (Noiseband)
Baseline settings: automatic, Drift correction OFF

Figure 17 - Ct graph of UGTK54

4.3.3 Protein profile

Leaf protein profile was carried out for the three cassava varieties with different nitrogen nutrition. The PAGE profile of cassava was presented in Figure 18. The PAGE showed similar protein profile for leaf proteins. However, one band at approximately 100 kDa differed considerably for Sree Vijaya variety under amide form of nitrogen nutrition. There were very prominent bands corresponding to large and small subunits of RUBISCO proteins in 55 kDa and 13 kDa respectively. Several other bands were visible in addition to these protein bands.

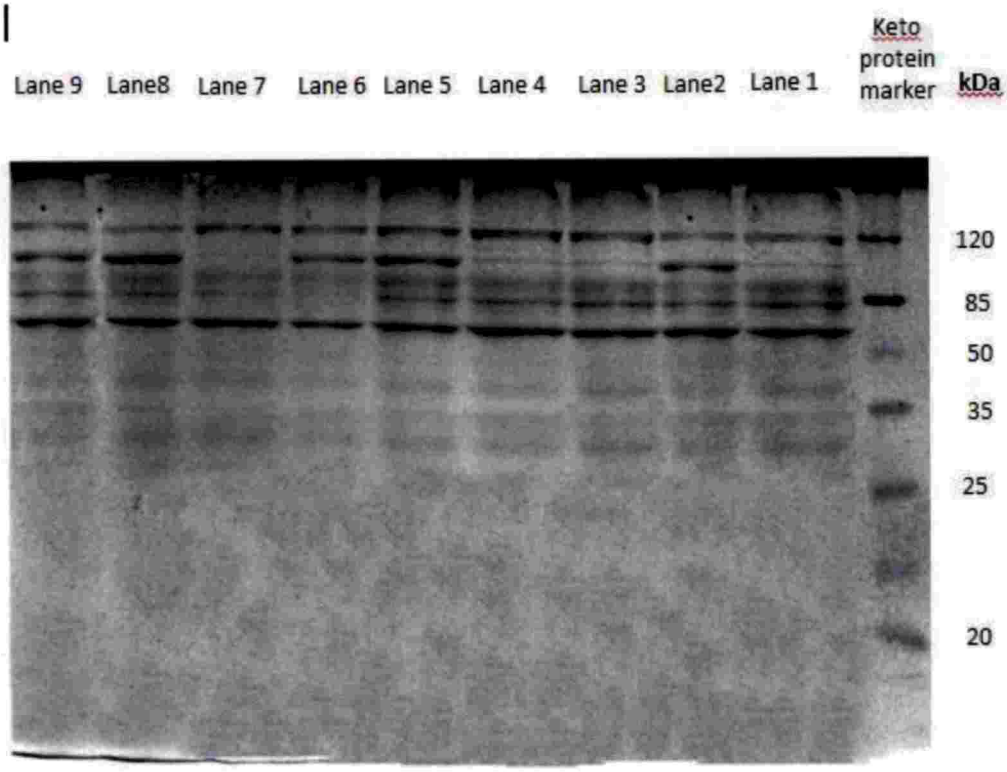


Figure 18 - PAGE profile of cassava leaves according to different forms of nitrogen nutrition

4.3.4 Nitrate reductase assay and Nitrite reductase assay

Highest nitrate reductase activity was seen in PDP CMR – 1 with 0.44 µg NO_2- g^{-1} fresh leaf h^{-1} in plants treated with nitrite form of nutrition. In ammoniacal form and nitrate form an activity of 0.39 µg NO_2- g^{-1} fresh leaf h^{-1} and 0.41 µg NO_2- g^{-1} fresh leaf h^{-1} respectively was observed. Sree Athulya showed 0.4 µg NO_2- g^{-1} fresh leaf h^{-1}, highest in nitrate form and 0.37 µg NO_2- g^{-1} fresh leaf h^{-1} in nitrite form and 0.38 µg

NO_2- g^{-1} fresh leaf h^{-1} in ammoniacal form. Sree Vijaya had a highest of 0.35 µg NO_2- g^{-1} fresh leaf h^{-1} in ammoniacal form and 0.32 µg NO_2- g^{-1} fresh leaf h^{-1} and 0.34 µg NO_2- g^{-1} fresh leaf h^{-1} in nitrite and nitrate form respectively.

In case of nitrite reductase activity also PDP CMR – 1 showed highest activity with 18.59 µg NO_2- g^{-1} fresh leaf h^{-1} in nitrate form of nutrition, 18.39 µg NO_2- g^{-1} fresh leaf h^{-1} in nitrite form and 18.2 µg NO_2- g^{-1} fresh leaf h^{-1} in ammoniacal form. Sree Athulya had a top value of 17.06 µg NO_2- g^{-1} fresh leaf h^{-1} in nitrite form. It had 16.98 µg NO_2- g^{-1} fresh leaf h^{-1} and 16.01 µg NO_2- g^{-1} fresh leaf h^{-1} in ammoniacal form. Sree Vijaya had a top value of 17.06 µg NO_2- g^{-1} fresh leaf h^{-1} in nitrite form and 16.98 µg NO_2- g^{-1} fresh leaf h^{-1} in nitrate form and 16.01 µg NO_2- g^{-1} fresh leaf h^{-1} in ammoniacal form.

Table 8 - Nitrate reductase assay as affected by different form of nitrogen nutrition in selected cassava varieties

Variety	Nitrate reductase activity (µg NO_2- g^{-1} fresh leaf h^{-1})		
	Nitrate form	**Nitrite form**	**Ammoniacal form**
Sree Vijaya	0.34	0.32	0.35
Sree Athulya	0.4	0.37	0.38
PDP CMR - 1	0.41	0.44	0.39

Table 9 - Nitrite reductase assay as affected by different form of nitrogen nutrition in selected cassava varieties

Variety	Nitrite reductase activity (µg NO_2- g^{-1} fresh leaf h^{-1})		
	Nitrate form	**Nitrite form**	**Ammoniacal form**
Sree Vijaya	16.98	17.06	16.01
Sree Athulya	18.09	18.31	17.48
PDP CMR - 1	18.59	18.39	18.2

4.4 THE DETECTION TECHNIQUES FOR CNglc USING HPTLC, NIR

4.4.1 High Performance Thin Layer Chromatography (HPTLC) of linamarin

Detection of linamarin the major cyanogenic glycoside of cassava was carried out using HPTLC. This technique is very sensitive can be used for very small quantities of CNglc detection. The silica gel plates were used for separating the CNglc. The mobile phase was ethyl acetate- acetone- water (6:4:1). The separation was good with the mobile phase. The spots on the plate were visualized using a developing reagent of containing aniline (2%) diphenylamine (2%), and orthophosphoric acid (15%) in acetone. The absorption intensity of linamarin was maximum at A = 366 nm. It showed good degree of detection with separation. Different standard volume from 2µl, 4 µl,6 µl,8 µl,10 µl and 12 µl were plotted in silica gel plates (lane 1 to 6) and three replicates of samples were analyzed each in 2 µl, 4 µl and 6 µl volume (lane 7 to 9). Linamarin was detected in all samples. Peak of detection in samples and of standard was also obtained. The HPTLC rnethod can be accurate and specific for the direct determination of linamarin in extracts of cassava plant and can be used for the assay of linamarin from plant samples. The chromatographic details are provided in Figure (19 and 20).

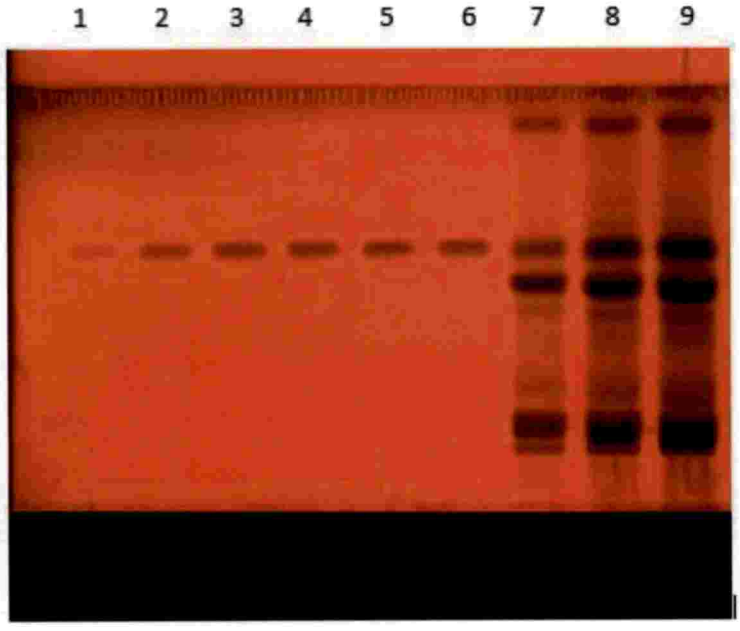


Figure 19 - HPTLC image of linamarin 366 nm after derivatization

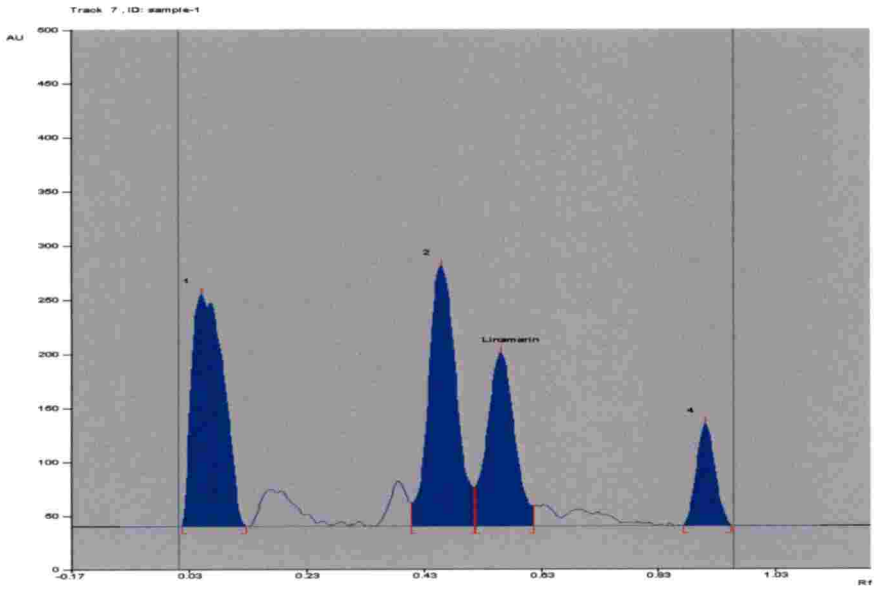


Figure 20 - Peak diagram of linamarin standard and sample

4.4.2 Near Infra-red (NIR) Spectrophotometric Analysis

NIR techniques are routinely used for analysis various biomolecules. NIR spectroscopic methods normally use the near-infrared region of the electromagnetic spectrum (from 780 nm to 2500 nm). Here, the analysis was done in the range of 1100nm to 4500nm. Linamarin standard was directly placed on the NIRA attachment of the FT-NIR system. The samples were measured in the range of 4000 wave number cm-1 to 10000 wave number cm^{-1}. Multiple scans were made to average out the spectra obtained. There were several unique peaks such as wave number 4835, 4745, 4633, 5110, in pure linamarin standard used for the detection method (Figure 21). The methanol extract of young cassava leaves showed similar characteristic peaks (Figure 22) which are unique to linamarin and these peaks can be used for detecting the linamarin from plant samples directly without much sample preparation techniques.

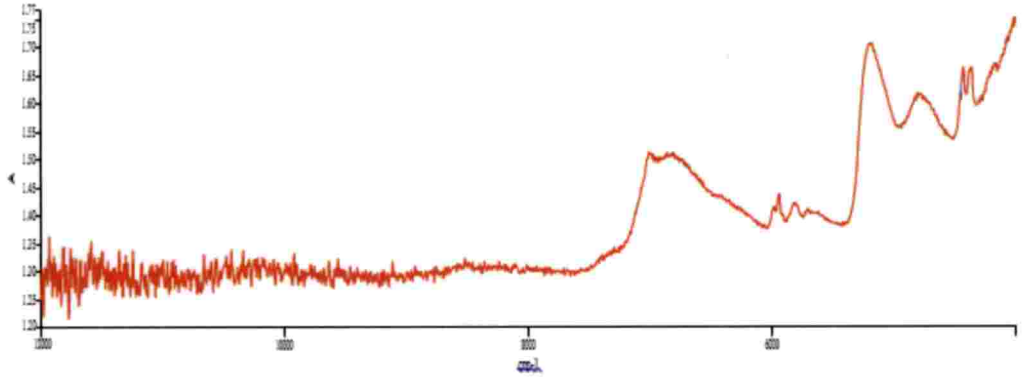


Figure 21 - NIR reading of pure linamarin standard

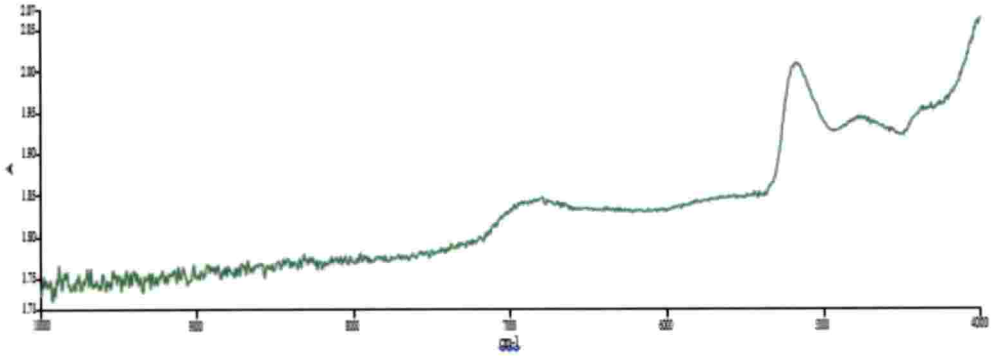


Figure 22 - NIR reading of methanolic extract of young cassava leaves

5. DISCUSSION

Cassava is one of the foremost important staple food crops in tropical areas of the planet. It is cultivated primarily within the tropic and sub-tropic regions of the globe, over a variety of environmental and soil conditions. It is highly tolerant to drought and heat stress and grows well on marginal soil, and provides food for quite 800 million individuals (FAO, 2007). A group of nitrile containing plant secondary metabolites which synthesizes cyanide on enzymatic breakdown called cyanogenic glycosides are found in cassava plants. Cyanogenic glycoside levels are highest in leaves (about 20 fold higher than in roots) as they are produced in leaves and are transported to different parts from there.

In this investigation, the CNglc production in cassava leaves are examined along with various physiological and biochemical constituents. There were changes in many of the biochemicals such as chlorophyll, protein in the leaves under open as well as shaded plants which were either well watered or stressed. Chlorophyll is the main photosynthetic pigment and the content in leaf is influenced by many factors such as light intensity and water availability. Leaf area and photosynthetic parameters were severely affected in cassava varieties under stress conditions. Din *et al* (2011) studied the response of cassava under drought stress and found similar reduction in photosynthetic rate as well as other gas exchange parameters.

In the present study, there was an increase of 22% in CNglc in Sree Vijaya in stressed plants grown under open light condition. Gleadow and Møller (2014) reported that cyanide content in cassava roots are found to be affected by high nitrogen fertilizers and water stress with drought resulting in an increase in cyanogenic potential of cassava. In all cyanogenic plants surveyed to date it is apparent that the cyanogenic glycoside and its corresponding cyanogenic enzymes are localized in different cellular compartments or tissues. This compartmentalization prevents cyanogenesis until the tissue is disrupted. In cassava leaves linamarin has been localized to the vacuoles, whereas linamarase is localized to cell walls and laticifers (Mkpong *et al.*, 1990; Pancoro and Hughes, 1992; Hughes *et al.*, 1994; McMahon *et al.*, 1995). According to Burns *et al.*, (2013) leaf cyanogenic glycosides content varied with plant water status which was estimated using carbon isotope discrimination (δ13C).

In the present study, three accessions were used to understand the response of cassava with respect to cyanogenic glycoside content in response to factors like nutrition, water deficit stress and light intensity along with comparison of detection methods of the cyanides in plant.

It is known that plant species and position of leaves affect the amount of chlorophyll in leaves (Gond *et al.*, 1999). Kurtar (2012) states that leaves getting sun light and leaves not getting sun light (shadow) have different internal and external structures, tissues providing strength of plants growing in extensive light conditions thrives and number of chloroplasts is low but they are big and the amounts of chlorophyll are too much. Increase in chlorophyll a/ b ratio may provide greater intensity on colour of the leaves, and it has been considered as adaptive characteristic for enabling reduction in the number of peripheral complexes responsible for intercepting solar radiation (Liu *et al.*, 2010). In this study maximum amount of chlorophyll was observed in plants grown under shaded and irrigated conditions followed by open and irrigated conditions. Water deficit stressed condition resulted in a large decrease in chlorophyll amount under both shade and open condition. In case of chlorophyll a also, maximum amount was observed in shaded irrigated condition followed by open irrigated conditions. But in the case of chlorophyll b a notable change between treatments could not be observed.

Paez *et al.*, (2000) studied the effects of light on growth of Aloe vera, a traditional medicinal plant. Plants were vegetatively propagated and grown under full sun, partial sun (30% full sun) or deep shade (10% Full sun) for 12 to 18 months. After one year of growth, 5 plants from each treatment were harvested to determine total above and below ground dry mass. Plants grown under full sun produced more numerous and larger axillary shoots, resulting in twice the total dry mass than those grown under partial shade. In this study, water stress condition showed a sharp decrease in leaf area due to decrease in leaf number in plants. Sree Vijaya showed an 8.3 fold decrease in leaf area compared to open irrigated conditions. Shade irrigated plants also showed a decrease in leaf area compared to open irrigated plants with Sree Vijaya showing greatest decrease of 2.1 fold than that of open irrigated. PDP CMR 1 showed a decrease of 1.4 fold and Sree Athulya of 1.1 fold. Leaf formation and growth in cassava are known to be highly sensitive to even small decreases in soil moisture (Connor and Cock, 1981; Connor *et*

al., 1981; Baker *et al.*, 1989; De Tafur *et al.*, 1997; Okogbenin *et al.*, 2003; Alves and Setter, 2004; Vandegeer *et al.*, 2013)

Generally, water deficit stress decreases protein synthesis and increases the concentration of free amino acids. In this study maximum amount of protein was observed in plants grown in shade water withheld condition with PDP CMR showing greater increase of 30% reaching an amount of 41.15 mg/g fresh leaf tissue. Shade irrigated condition have little effect on protein content showing only around 1% change. Open water withheld conditions also resulted in an increase in protein content.

de Bruijn (1973) reported that when leaves were shaded, the content of cyanogenic glucosides increased in leaves and the amount of cyanogenic glucosides transported to the tubers decreased. Here not much difference was observed between plants grown in shade irrigated and open irrigated in level of CNglc content. Plants grown in shade water stress have CNglc amount at par with plants grown in open water stress with a maximum average of 0.54 mg for PDP CMR 1, 0.38 for Sree Athulya and 0.23 mg for Sree Vijaya.

Increased nitrogen supply stimulates plant growth and productivity (Lawlor, 1995), as well as photosynthetic capacity of leaves (Evans and Terashima, 1988; Fredeen *et al.*, 1991; Makino *et al.*, 1992), through increased amounts of stromal and thylakoid proteins in leaves (Evans, 1989; Bungard *et al.*, 1997). Here an increase in leaf area is seen in all varieties according to increase in strength in nitrogen nutrition. Plants kept in open have a slight increase in leaf area than those kept under shade. Also there is an increase in leaf fresh weight according to an increase in strength in nitrogen nutrition with 2x strength solution having the highest fresh weight. Plants grown in shade showed a decrease in leaf fresh weight than those in open. Plants grown in open condition have greater leaf dry weight than those in shade. Highest plant fresh weight was seen in Sree Vijaya grown in 2x strength solution and open condition with 52.97g followed by Sree Athulya with 51.36g. No considerable increase was seen according to nitrogen nutrition level in plant dry weight. Also plant fresh weight was found to be low in shaded condition than in open condition. The effect of increasing nitrogen supply on the increase in total biomass was an expected result since nitrogen is fundamental to the process of photosynthesis and biomass accumulation (Evans, 1983; Hilbert, 1989). The interaction of nitrogen fertilization rate with the shade levels resulted in a maximal

average leaf mass at 20% followed by 40% shade at the highest nitrogen fertilization rate.

The application of nitrogenous fertilizers as a foliar spray reduced cyanogenic potential levels in the tuberous roots to some extent (Sinha, 1969). However, de Bruijn (1971) has shown that N fertilizer increases the cyanogenic potential of tuberous roots. Here CNglc content increase according to nitrogen nutrition doesn't seems to follow a uniform pattern as in case of Sree Vijaya CNglc content remains unchanged or a small decrease is seen. Also here although an increase is seen in CNglc content from 0.5x to 1x strength, increase is not seen in 2x strength solution. Nitrate reductase activity was found to increase in response to added N and paralleled with an increase in reduced N in vegetative parts in wheat (Eilrich and Hageman, 1973) and cassava (Cruz *et al.*, 2004). There was no uniform pattern detected in nitrate reductase activity but the highest was seen in PDP CMR – 1.

Detection of CNglc in plant samples are important for not only quality control but for many other aspects of biochemical research. Various techniques are used for estimating CNglc in cassava samples and the enzymatic and spectro-photometric techniques are routinely used. However, these techniques are time consuming and costly. NIR and HPTLC techniques are robust and easy to perform and more sophisticated. Hence, NIR data obtained from this investigation can be used for developing methods for quantifying the CNglc content directly in plant samples in the future without cumbersome sample preparation techniques.

6. SUMMARY

The study entitled "Physiological and molecular studies on cyanogenic potential in cassava (*Manihot esculenta* Crantz) in response to nitrogen nutrition, water stress and shade" was carried out at division of crop utilization, ICAR - Central Tuber Crops Research Institute, Sreekariyam. Thiruvananthapuram during the period 2017-18. The synthesis and regulation of bioactive natural products are influenced by changes in the nutritional availability of both micro and macro. The objective of the study was to understand the physiology and molecular aspects of biosynthesis of cyanogenic glycosides in cassava in response to plant nitrogen status, light intensity and water stress and to compare detection methods such as Near-infra red (NIR) spectroscopy and High Performance Thin Layer Chromatography (HPTLC) for cyanogenic glycoside estimation.

In the present study three accessions were used to understand the response of cassava with respect to cyanogenic glycoside content in response to factors like nutrition, water deficit stress and light intensity along with comparison of detection methods of the cyanides in plant. Parameters like photosynthetic rate, chlorophyll fluorescence were measured. Biochemical parameters like protein content, cyanogenic glycoside content etc. was done through spectrophotometric method. The same parameters were examined for study with different levels of nitrogen nutrition. In this study not much difference was observed between plants grown in shade irrigated and open irrigated in level of CNglc content. Plants grown in shade water stress have cnglc amount at par with plants grown in open water stress. But an overall increase in CNglc content was seen in water stress affected plants. In some a 22% increase was observed due to water stress.

In this study effect of different forms of nitrogen nutrition on CNglc content was studied by analyzing CNglc content and by analyzing gene expression data of UGT8K54 gene. No significant change in CNglc level could be detected by spectrophotometric method. Also significant change in expression level was not observed for different forms of nitrogen nutrition. In protein profile analysis of leaf crude protein also no significant change could be observed for different form of nitrogen nutrition for three varieties of cassava.

Detection of CNglc in plant samples are important for not only quality control but for many other aspects of biochemical research. Various techniques are used for estimating CNglc in cassava samples and the enzymatic and spectrophotometric techniques are routinely used. However, these techniques are time consuming and costly. NIR and HPTLC techniques are robust and easy to perform and more sophisticated. HPTLC is a proven technique for linamarin detection worldwide. NIR data obtained from this investigation can be used for developing methods for quantifying the CNglc content directly in plant samples in the future without cumbersome sample preparation techniques.

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APPENDIX 1

TBE Buffer	(10 X)
Tris base	107 g
Boric acid	55 g
0.5 M EDTA (pH 8.0)	40 ml

Final volume made up to 1000 ml with distilled water and autoclave before use

APPENDIX II

70% ethanol	
100% ethanol	70 ml
Distilled water	30 ml

APPENDIX III

Phosphate buffer	(pH-7.5, 0.1 M)
K_2HPO_4 (1 M)	80.2 ml
KH_2PO_4 (1 M)	19.8 ml

Dilute the combined stock to 1 litre with distilled water

APPENDIX IV

SDS sample buffer	(2X)
Tris (1 M, pH 6.8)	2 ml
Glycerol (50%)	4.6 ml
SDS (10%)	1.6 ml
Bromophenol blue (0.5%)	0.4 ml

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β-mercaptoethanol	0.4 ml

APPENDIX V

Hoagland solution	(1X)
KNO_3 (1 M)	6 ml
$Ca(N0_3)2.4H_20$ (1 M)	4 ml
$(NH_4)2\ HPO_4$ (1 M)	1 ml
$MgSO_4.7H_20$ (1M)	1 ml

Combine the above and make up to 1litre with distilled water

PHYSIOLOGICAL AND MOLECULAR STUDIES ON CYANOGENIC POTENTIAL IN CASSAVA (*Manihot esculenta* Crantz) IN RESPONSE TO NITROGEN NUTRITION, WATER STRESS AND SHADE

By

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Abstract of Thesis

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ABSTRACT

The study entitled "Physiological and molecular studies on cyanogenic potential in cassava (*Manihot esculenta* Crantz) in response to nitrogen nutrition, water stress and shade" was carried out at the Division of Crop utilization, ICAR-Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during the year 2017-2018. To study the physiology and molecular aspects of biosynthesis of cyanogenic glycosides in cassava in response to plant nitrogen status, light intensity and water stress and to compare detection methods such as Near-infra red (NIR) spectroscopy and High Performance Thin Layer Chromatography (HPTLC) for cyanogenic glycoside estimation.

Cassava (*Manihot esculenta* Crantz) contains cyanogenic glycosides like linamarin, acetone cyanohydrin, and hydrocyanic acid. The concentration of cyanogens in roots and leaves differ in the same plant and is known to be more abundant in the leaves than the stem and roots. The synthesis and regulation of bioactive natural products are influenced by changes in the nutritional availability of both micro and macro. It is important to be aware of and understand the consequences of such changes so that appropriate measures can be taken to either reduce risks or capitalize on benefits.

Here in this study not much difference was observed between plants grown in shade irrigated and open irrigated in level of CNglc content. Plants grown in shade water stress have CNglc amount at par with plants grown in open water stress. Increased nitrogen supply stimulates plant growth and productivity as well as photosynthetic capacity of leaves through increased amounts of stromal and thylakoid proteins in leaves. Here CNglc content increase according to nitrogen nutrition doesn't seems to follow a uniform pattern as in case of Sree Vijaya CNglc content remains unchanged or a small decrease is seen. Also here although an increase is seen in CNglc content from 0.5x to 1x strength, increase is not seen in 2x strength solution.

Detection of CNglc in plant samples are important for not only quality control but for many other aspects of biochemical research. Various techniques

are used for estimating CNglc in cassava samples and the enzymatic and spectrophotometric techniques are routinely used. From this study NIR data and HPTLC data obtained from this investigation can be used for developing methods for quantifying the CNglc content directly in plant samples in the future without cumbersome sample preparation techniques.