# OXIDATIVE STRESS AND PROTEIN PROFILING IN CASSAVA (*Manihot esculenta* CRANTZ) UNDER ABIOTIC STRESSES

*by*

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## DECLARATION

I hereby declare that this thesis entitled **"OXIDATIVE STRESS AND PROTEIN PROFILING IN CASSAVA (*Manihot esculenta* CRANTZ) UNDER ABIOTIC STRESSES"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associate ship, fellowship or other similar title, of any other university or society.

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## CONTENT

## LIST OF TABLES

## LIST OF FIGURES

# LIST OF PLATES

# LIST OF APPENDICES

## EXPANSION OF ABBREVIATIONS AND SYMBOLS USED

| | |
|---|---|
| **$^{o}c$** | Degree Celsius |
| **$^{1}O_2$** | Singlet Oxygen |
| **µl** | Micro Li |
| **µg** | Micro Gram |
| **%** | Percentage |
| **AP or APX; (EC 1.11.1.11)** | Ascorbate Peroxidase |
| **AOS** | Active Oxygen Species |
| **AR** | Ascorbate Reductase |
| **AsA** | Acorbate |
| **CAT; (E.C 1.11.1.6)** | Catalase |
| **Cm** | Centimeter |
| **$CO_2$** | Carbon Dioxide |
| **CARs** | Carotenoids |
| **DAP** | Day After Planting |
| **DMSO** | Dimethyl Sulfoxide |
| **DCPIP** | Dichlorophenol Indophenols |
| **DTNB** | 5, 5'-Dithiobis Nitro Benzoic Acid |
| **EU** | Enzyme Unit |
| **GR; (EC 1.6.4.2)** | Glutathione Reductase |
| **GA** | Giberilic Acid |
| **$H_2O_2$** | Hydrogen Peroxide |
| **HSPs** | Heat Shock Proteins |
| **HTS** | High Temperature Stress |

| | |
|---|---|
| **IAA** | Indol Acetic Acid |
| **LEA** | Late Embryogenesis Abundant |
| **Mm** | Millimeter |
| **Ml** | Milliliter |
| **Mg** | Milligram |
| **Min** | Minute |
| **Nm** | Nanometer |
| **NADPH** | Nicotinamide Adenine Dinucleotide Phosphate |
| **NADH** | Nicotinamide Adenine Dinucleotide |
| **NR** | Nitrate Reductase |
| **$O^{2-}$** | Superoxide |
| **OH** | Hydroxyl Radicals |
| **PAGE** | Poly Acrylamide Gel Electrophoresis |
| **POX** | Peroxidase |
| **PSMs** | Plant Secondary Metabolites |
| **ROS** | Reactive Oxygen Species |
| **Rpm** | Red Hat Package Manager |
| **RWC** | Relative Water Content |
| **RGR** | Relative Growth Rate |
| **SOD; (EC 1.15.1.1)** | Superoxide Dismutase |
| **SDS-PAGE** | Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis |
| **TCA** | Trichloroacetic Acid |
| **150 V** | 150 Volt |
| **WDS** | Water Deficit Stress |
| **$WP_L$** | Water Potential |
| **UV** | Ultra Violet Radiation |
| **Sec** | Second |

# INTRODUCTION

# 1. INTRODUCTION

The cassava (*Manihot esculenta Crantz*) is cultivated mainly in the tropic and sub-tropic regions of the world, over a wide range of environmental and soil conditions. It is tolerant to drought and heat stress and grow well on marginal soils. It is an important dietary staple food in many countries within the tropical regions of the world (Perez and Villamayor, 1984), where it provides food for more than 800 million people (FAO, 2007). According to Alexandrite (1995), cassava plays an important role in alleviating food problems, because it thrives and produces stable yields under conditions in which other crops fail.

Cassava varieties are generally distinguished from each other by their morphological characteristics which include leaf, stem and tuber colour, leaf shape and number of storage roots per plant. Eze and Ugwuoke (2010) reported that tuber yield of cassava is influenced by both the quality of planting material used and the agronomic practices employed. All the major cassava growing countries in the Asian continent have the productivity more than the world average. Indonesia, Thailand, Vietnam and India are the major countries growing cassava in Asia. Although it is cultivated in India in 13 states, it is concentrated in the southern peninsular region and to a certain extent in northeast region of the country.

In India, cassava is largely grown under rain fed conditions and either the initial 6 months (as in Tamil Nadu) or the last 3-4 months (as in Andhra Pradesh and Kerala) of its growth period coincides with a seasonal drought (December-May) as well as contingent drought (failure of rain during normal monsoon) causing significant reduction in photosynthesis, dry matter production, tuber and starch yield. Ravi and James George (2003) reported that although cassava genotypes show tolerance to drought conditions, there is a wide variability in tuber yield among varieties. Unlike that of other crops, in cassava, the source and sink develops simultaneously and photosynthates are partitioned between these two. Under un-favourable, water deficit stress conditions, both vegetative growth as well as tuber bulking ceases and the plant becomes dormant. When conditions become favourable (adequate soil moisture) the plant resumes its growth and the tuber

continues to bulk. Under prolonged WDS, the plant sheds about 80% of leaves and becomes dormant. Once the soil regains moisture, the plant resurrects and develops canopy consuming considerable amount of carbohydrate from the tubers eventually reducing tuber starch content. Thus, if the sanesence of leaves due to water deficit can be prevented, then the remobilization of starch from tuber to shoot growth with availability of soil moisture can be prevented and thus reduction in starch content of tuber also can be prevented.

Besides drought, cassava crop is exposed to high temperatures (>30 °C) in Southern and central India which imposes an additional stress for cassava growth. In Kerala, the annual maximum (day) temperature has risen by 0.8°C and the minimum (night) temperature by 0.2 °C and the average by 0.5 °C over the past 43 years between 1961-2003 (The Hindu, October 6, 2005). A combination of drought and high temperature stress conditions were also found to lignify and reduce extractable starch content of cassava tubers. Foliar spray of calcium chloride ($CaCl_2$) has been reported to increase osmotic adjustment and antioxidative enzyme *viz.*, superoxide dismutase (SOD), catalase, ascorbate peroxidise (AP), and glutathione reductase (GR) activity and increased heat tolerance in grasses (Jiang and Huang 2001), *Zea mays* (Gong *et al.* 1997) and *Beta vulgaris* (Cooke *et al.* 1986).

Crop physiology of cassava has been well documented (Ravi and Mohankumar, 2000). The response of cassava to water deficit stress has been understood pretty well (El-Sharkawy *et al.*, 1990, El-Sharkawy, 2006. Ravi and Saravanan, 2001 reported that cassava was found to maintain nearly 50 % of its photosynthetic rate under drought conditions offering a better choice of crop in future warming climate conditions. Although cassava genotypes show tolerance (survival) under drought conditions, genotypes showed significant reduction in tuber yield and a wide variability was found in tuber yield among 62 genotypes under drought conditions (Ramanujam, 1990; Ravi and George, 2003).

The objective and aim of the present study was to assess oxidative stress caused by abiotic stresses such as water deficit stress and high temperature stress

and changes in the protein profile, growth parameter and yield parameter in cassava under abiotic stresses such as water deficit stress (WDS) and high temperature stress (HTS) and also to find the marker enzyme for developing abiotic stress tolerant varieties in different genotypes (short and long duration varieties) of cassava.

# REVIEW OF LITERATURE

## 2. REVIEW OF LITERATURE

*Manihot esculenta* Crantz is a monotypic species that is cultivated as a food crop. Cassava was domesticated during 2000- 4000 BC and originated in South America, and now distributed worldwide. Cassava was introduced to the Gulf of Guinea in Africa in the 16$^{th}$ century by the Portuguese. In the 18$^{th}$ century, they introduced it to the east coast of Africa, islands of Indian Ocean *viz.*, Madagascar, Zanzibar and Sri Lanka and India. Among the countries cultivating cassava, Africa is producing 108 million tonnes from an area of 12 million hectares. In India, cassava is mainly grown in southern states *viz.* Kerala, Tamil Nadu and Andhra Pradesh. In Kerala, it remains as a major food crop whereas in Tamil Nadu and Andhra Pradesh it is grown for industrial production of starch and sago. India ranks first and seventh in the productivity (35 t/ha) and production (7 million tonnes), respectively. Cassava is the fifth important starchy food crop in India after rice, wheat, potato and maize (FAO, 2005)

Abiotic stress factors such as drought, salinity and extremes of temperature have been known as major limitations to crop productivity (Boyer, 1982). One approach to improve stress tolerance in crops would be to transfer the genes for these adaptive traits from the tolerant organism to the crop. (Yeo and Flowers, 1989) reported that, this process has not been successful using conventional means, partly because the traits are poorly described and partly because of the transfer of unwanted genes during conventional crossing. Genetic transformation technology enables us to achieve gene transfer in a precise and, to some extent, predictable manner. (Yancey *et al.*, 1982) reported that certain plants, marine algae, bacteria and other organisms accumulate organic solutes such as sugar alcohols, the amino acid proline, quaternary ammonium and or tertiary sulphonium compounds in response to osmotic stress. These compounds are termed *"compatible solutes"*. (Johnson *et al.*, 1968) reported that even in high concentrations, they do not inhibit the activity of enzymes. They also protect enzymes and membranes against deleterious effects of destabilizing ions such as Na and Cl.

Accumulation of compatible solutes in response to stress is a metabolic adaptation found in a number of stress-tolerant, often unrelated taxa, suggesting convergent evolution for this trait (Wyn Jones and Storey, 1981; Yancey *et al.*, 1982; Rhodes and Hanson, 1993)

The *"osmoprotectant"* is synthesized in response to the stress and is localized in the cytoplasm; inorganic ions such as Na and Cl are preferentially sequestered into the vacuole Thus, this leads to turgor maintenance for the cell under osmotic stress (Flowers *et al.*, 1977, 1986; Bohnert *et al.*, 1995; Glenn *et al.*, 1999).

**2.1. Growth Parameter**

Cassava is grown under partially irrigated conditions or as a rainfed crop in areas where the annual rainfall varies from 700 to 3000 mm. Depending on the growing conditions and the variety, crop duration may vary between seven months (short duration) and 10 months (long duration). Cassava requires adequate soil moisture during initial one month for its establishment. In cassava, tuber (modified storage root) initiates within one month after planting and tuber bulking starts at two/three month after planting under adequate soil moisture conditions. Drought during this growth period may postpone tuber initiation as well as tuber bulking. In India where cassava is largely grown under rainfed conditions, either the initial six months (as in Tamil Nadu) or the last three-four months (as in Andhra Pradesh and Kerala) of its growth period coincides with a seasonal drought (December-May) as well as contingent drought (failure of rain during normal monsoon) causing significant reduction in photosynthesis, dry matter production, tuber and starch yield. Further, the crop is exposed to temperatures more than 35°C during the month of March- May along with water deficit stress. Under soil drought conditions, cassava plant sheds it's leaves (reduces it's leaf area) retaining few leaves at the apex, reduces its tuber bulking (growth) and remains dormant till the conditions become favourable. Once adequate soil moisture becomes available the tuber starts its growth and continues to bulk. Limited studies have been carried out in Central

Tuber Crops Research Institute (CTCRI) on the response of cassava to drought under field conditions. These studies revealed reduction in photosynthetic rate, total dry matter production and tuber yield. About 28 – 42 % of tuber yield was found to be reduced under drought conditions (Ramanujam, 1990). However, cassava was found to maintain nearly 50 percent of its photosynthetic rate under drought conditions. Although cassava genotypes showed tolerance under drought conditions, genotypes showed significant reduction in tuber yield and a wide variability was found in tuber yield among 62 genotypes under drought conditions (Ramanujam, 1990; Ravi and George, 2003).

**2.2. Free Radicals**

A free radical is any species capable of independent existence that contains one or more unpaired electrons (Halliwell and Gutteridge, 2006). An unpaired electron is one that occupies an atomic or molecular orbital by itself. The simplest free radical is atomic hydrogen. Since a hydrogen atom has only one electron, it must be unpaired. Many free radicals exist in living systems (some bad, some good and some both) although most molecules *in vivo* are non-radicals. Radicals can be formed by several mechanisms, such as adding a single electron to a non-radical. They can form when a covalent bond is broken if one electron from the bonding pair remains on each atom (hemolytic fission). Some bonds are hard to break, e.g. temperatures of 45°C to 60°C are often required to rupture C–C, C–H, or C–O bonds. Indeed, combustion of organic compounds proceeds by free radical mechanisms. Formation of OHC accounts for much of the damage done to living organisms by ionizing radiation (Von Sonntag, 1987).

**2.3. The Oxygen Radicals and Related Species**

There are many types of free radicals in living systems (Gilbert, 1981; Halliwell and Gutteridge, 2006), In fact, reactive species and antioxidants of the $O_2$ molecule is a free radical and has two unpaired electrons. The two electrons in $O_2$ have the same spin quantum number. This is the most stable state, or ground state, of $O_2$, and is the form that exists in the air around us. Oxygen is,

thermodynamically, a potent oxidizing agent. However, if $O_2$ tries to oxidize a non-radical by accepting a pair of electrons from it, both these electrons must have the same spin to fit into the vacant spaces in the p* orbitals. A pair of electrons in an atomic or molecular orbital cannot meet this criterion, since they have opposite spins (1½ and2½). This spin restriction makes $O_2$ prefer to accept its electrons one at a time, and helps explain why $O_2$ reacts sluggishly with most non radicals.

**2.4. Antioxidants**

Aerobic metabolisms generally depend on a stringent control of ROS by antioxidants (Finkel and Holbrook, 2000; Abele, 2002). However, as desiccation greatly enhances ROS formation, effective antioxidant machinery is bound to be an essential trait of desiccation tolerant organisms. Protection mechanisms that include sugars, glass formation and expression of LEA proteins (Alpert, 2005 and Crowe, 1998). The major water-soluble antioxidants found in biological systems are glutathione (g-glutamyl cysteinylglycine; GSH) and ascorbic acid (Asc) (Noctor and Foyer, 1998). According to Munne- Bosch and Alegre, (2002) Tocopherols and β-carotene are the main lipid-soluble antioxidants. An example of how antioxidants scavenge free radicals directly is given for GSH and XOH in equation 1. At physiological pH, GSH is present as the glutathiolate anion (GS2). Glutathione disulfide (GSSG), formed as shown in equation 2, is re-reduced by the enzyme glutathione reductase (equation 3). This way, XOH is scavenged and the antioxidant is recycled.

GS2 1 XOH 2OH 1 GSx (Eq. 1)

GSx 1 GSX GSSG (Eq. 2)

GSSG 1 NADPH 1 H1 2GSH 1 NADP (Eq. 3)

Ascorbate (Asc) also reacts rapidly with $Ox_2$, $XOH_2$ and $1O_2$ (Halliwell and Gutteridge, 1999), forming the rather unstable monodehydroascorbate (MDHA) and then dehydroascorbate (DHA). According to Foyer and Halliwell, (1976) to regeneration of Asc may occur via a Mehler-peroxidase reaction sequence or through the Asc-GSH cycle at the expense of NADPH. Asc also supports the

regeneration of membrane-bound antioxidants such as carotenoids and a-tocopherol. Plants also use pathways that dissipate light energy as heat. Frank *et al.*, (1999) and Demmig-Adams and Adams, (2000) found that in xanthophylls cycle solar radiation is dissipated as heat while violaxanthin undergoes de-epoxidation to antheraxanthin and then zeaxanthin. Thus formation of $O_2$ is prevented.

**2.5. ROS Scavenging Enzymes**

ROS scavenging enzymes include superoxide dismutase (SOD), ascorbate peroxidase (AP) and other peroxidases, mono- and dehydroascorbate reductase, glutathione reductase (GR) and Catalase. Elstner and Osswald, (1994) found that Superoxide dismutase catalyze the dismutation of to $H_2O_2$. Thus, $O_2$ is removed and further con- 2 2 version into OH is prevented (equation 4). Peroxidases catalase $H_2O_2$-dependent oxidation of substrates (S) (equation 5). Catalases break down high concentrations of $H_2O_2$ very rapidly (equation 6), but are much less effective than peroxidases at removing $H_2O_2$ present in low concentrations because of their lower affinity to $H_2O_2$.

OX2 1 OX2 1 2H1 H O 1 3O (Eq. 4) 2 2 2 2 2
SH 1 H O S 1 2H O (Eq. 5) 2 2 2 2
2H O 2H O 1 3O (Eq. 6) 2 2 2 2

Interplay between antioxidants and enzymes to scavenge ROS in plants were first suggested by Foye and Halliwell (1976). They postulated an ascorbate glutathione cycle for the scavenging of $H_2O_2$ produced from OX2 by SOD in which ascorbate and glutathione 2 play an important role as reductants. This cycle involves reactions of GSH, GR, AP and mono- and dehydroascorbate reductase. Moreover, it may also be linked to a-tocopherol (Finckh and Kunert, 1985).

**2.6. Intracellular origins of ROS and their multiple damaging effects**

Asada (1994) found that any circumstance in which cellular redox homeostasis is disrupted can lead to oxidative stress or the generation of ROS. Production of ROS during environmental stress is one of the main causes for

decreases in productivity, injury, and death that accompany these stresses in plants. (Halliwell and Gutteridge, 1989) reported that ROS are produced in both unstressed and stressed cells, and in various locations. They are generated endogenously during certain developmental transitions such as seed maturation and as a result of normal, unstressed, photosynthetic and respiratory metabolism. An initial oxyradical product, the superoxide radical ($O_2$-), upon further reaction within the cell, can form more ROS such as hydroxyl radicals and singlet oxygen. Superoxide is a charged molecule and cannot cross biological membranes. Subcellular compartmentation of defense mechanisms is, therefore, crucial for efficient removal of superoxide anions at their sites of generation throughout the cell. Hydrogen peroxide, on the other hand, which is formed as a result of SOD action, is capable of diffusing across membranes and is thought to fulfill a signaling function in defense responses (Mullineaux *et al.,* 2000). Found that ROS play an important role in endonuclease activation and consequent DNA damage. In the presence of metal ions such as Fe or Cu (II), hydroxyl radicals are formed very rapidly (Hagar *et al.,* 1996). Hydroxyl radicals can cause damage to all classes of biologically important macromolecules, especially nucleic acids. Hydroxyl radicals can also modify proteins so as to make them more susceptible to proteolytic attack. There is evidently considerable specificity associated with this degradative process since proteinshave widely differing susceptibilities to attack by ROS (Davies, 1987). Once damaged, proteins can be broken down further by specific end peptidases such as the thylakoid membrane (Casano *et al.,* 1994). A multicatalytic proteinase complex has been demonstrated in plant systems, with the capacity to selectively break down oxidatively damaged proteins (Van Nocker *et al.,* 1996).

### 2.7. Metabolic defense mechanisms: limiting ROS mediated Damage

Plant cells respond defensively to oxidative stress by removing the ROS and maintaining antioxidant defense compounds at levels that reflect ambient environmental conditions (Scandalios, 1997). Metabolic containment mechanisms for ROS involving antioxidant genes and associated processes are likely to have predated or coevolved with the appearance of aerobiosis and represent fundamental

adaptations of aerobic systems to an oxygen dependent metabolism. The mechanisms that act to adjust antioxidant levels to afford protection include changes in antioxidant gene expression (Cushman and Bohnert, 2000). ROS themselves play a role in intracellular redox sensing, activating antioxidant resistance mechanisms, among other adaptive processes (Toledano and Leonard, 1991; Karpinski *et al.*, 1997; May, *et al.*, 1998a). Number of redox sensitive transcription factors have been identified in animal, bacterial and plant cells (Pastori and Foyer, 2001). Functional roles of these responses include the protection of redox-sensitive enzymatic processes, the preservation of membrane integrity, and the protection of DNA and proteins (Scandalios, 1997). Redox-sensitive regulatory enzymes such as fructose-1, 6-bisphosphatase can be protected from oxidation/inactivation by the action of antioxidants such as glutathione. Under unstressed conditions, the formation and removal of $O_2$ are in balance. The defense system, when presented with increased ROS formation under stress conditions, can be overwhelmed when it is unable to remove the toxic molecular species with increased enzymatic or non-enzymatic antioxidant processes. Organelles such as the peroxisome and the chloroplast, where ROS are being produced at a relatively high rate, are especially at risk. In the case of the chloroplast, changes in light intensity and temperature or limitations in the substrates of photosynthesis occur frequently, resulting in increased production of ROS (Karpinska and Karpinski, 2000). ROS are produced at high levels in peroxisomes. Hydrogen peroxide is produced in the peroxisomal respiratory pathway by flavin oxidase. Fatty acid beta oxidation and glycolate oxidase action are other sources of hydrogen peroxide production in the peroxisome. Developmental transitions such as seed maturation, in which peroxisomes play an important role, also involve oxidative stress (Leprince *et al.*, 1990; Walters, 1998).

## 2.8. Drought stress

Drought stress is a complex syndrome involving not only water deprivation but also nutrient limitation, salinity, and oxidative stresses. Moreover, levels of light that is optimal for photosynthesis in well-watered plants become excessive in plants suffering water deprivation. Photosynthesis is particularly sensitive to water deficit because the stomata close to conserve water as available soil water declines. Stomatal closure deprives the leaves of carbon dioxide and photosynthetic carbon assimilation is decreased in favour of photo respiratory oxygen uptake. The process of stomatal closure and the enhancement of flux through the photo respiratory pathway increase the oxidative load on the tissues as both processes generate reactive oxygen species (ROS), particularly hydrogen peroxide ($H_2O_2$). Pei *et al.* (2000) found that hydrogen peroxide is also generated as a secondary messenger in abscisic acid (ABA)-mediated stomatal closure. In photorespiration, $H_2O_2$ is produced at very high rates by the glycollate oxidase reaction in the peroxisomes. Moreover, supeoxide production by the photosynthetic electron transport chain (via the Mehler reaction) is exacerbated by drought (Noctor *et al.*, 2002). Plants respond to diverse environmental signals in order to survive stresses such as drought (Pastori and Foyer, 2002). Strategies to minimize oxidative damage are a universal feature of plant defence responses. Hydrogen peroxides is eliminated by Catalase (CAT) and Ascorbate Peroxides (Chen and Asada, 1989; Scandalios *et al.*, 1997). These enzymes rapidly destroy the vast majority of $H_2O_2$ produced by metabolism, but they allow low steady state levels to persist presumably to maintain redox signaling pathways (Noctor and Foyer, 1998). Catalase is essential for the removal of $H_2O_2$ produced in the peroxisomes by photorespiration (Noctor *et al.*, 2000).

## 2.9. Antioxidant defense molecules have several roles

Ascorbic acid, glutathione, and α-tocopherol have each been shown to act as antioxidants in the detoxification of ROS. These compounds have central and interrelated roles, acting both non-enzymatically and as substrates in enzyme-catalyzed detoxification reactions (Foyer 1993; Hess 1993; Hausladen and Alscher, 1994; Winkler *et al.*, 1994; Chaudiere and Ferrari-Iliou, 1999). An anti-ROS

response includes the induction of genes that belong to ROS scavenging mechanisms.

Metabolic cycles located within the aqueous phase of the peroxisome, chloroplast, cytosol, and the mitochondrion successively oxidize and re-reduce glutathione and ascorbate, using NAD (P) H as the ultimate electron donor. Ascorbate, reduced glutathione (GSH), ascorbate peroxidase APX, glutathione reductase (GR), superoxide dismutase (SOD), and monodehydroascorbate reductase (MDHAR) are involved in several contexts in antioxidant regeneration throughout the plant cell. The enzymes involved are hydrophilic in nature, although in some instances they are known to be loosely associated with the membranes where the ROS are generated.

That is found in the chloroplast. Different isoforms of the antioxidant enzymes are located in different subcellular compartments. Evidence to date suggests a coordinated response to ROS among different members of the different SOD gene families.

### 2.10. Enzymatic antioxidants

The enzymatic antioxidants present work include in the study Superoxide dismutase (SOD), Catalase (CAT), Glutathione Reductase (GR), Ascorbate Peroxidase and Ascorbate Reductase.

### 2.10.1. Superoxide Dismutase Activity (SOD) (EC.1.5.1.1)

Within a cell, the superoxide dismutase (SODs) constitutes the first line of defense against ROS (Elstner, 1991). $O^{-}_{2}$ is produced at any location where an electron transport chain is present, and hence $O_2$ activation may occur in different compartments of the cell, including mitochondria, chloroplasts, microsomes, glyoxysomes, peroxisomes, apoplasts, and the cytosol. This being the case, it is not surprising to find that SODs are present in all these subcellular locations while all compartments of the cell are possible sites for $O^{-}_{2}$ formation, chloroplasts, mitochondria and peroxisomes are thought to be the most important generators of ROS Takahashi and Asada (1983) showed that phospholipids membranes are

impermeable to charged $O^{-}_2$ molecules. Therefore, it is crucial that SODs are present for the removal of $O^{-}_2$ in the compartments where $O_2$ – radicals are formed (Takahashi and Asada, 1983). Based on the metal co-factor used by the enzyme, SODs are classified into three groups: iron SOD (Fe SOD), manganese SOD (Mn SOD), and copper-zinc SOD (Cu-Zn SOD). Fe SODs are located in the chloroplast, Mn SODs in the mitochondrion and the peroxisome, and Cu-Zn SODs in the chloroplast, the cytosol, and possibly the extracellular space. Comparison of deduced amino acid sequences from these three different types of SODs suggest that Mn and Fe SODs are more ancient types of SODs, and these enzymes most probably have arisen from the same ancestral enzyme, whereas Cu-Zn SODs have no sequence similarity to Mn and Fe SODs and probably have evolved separately in eukaryotes (Kanematsu and Asada, 1990; Smith and Doolittle, 1992). The evolutionary reason for the separation of SODs with different metal requirements is probably related to the different availability of soluble transition metal compounds in the biosphere in relation to the $O_2$ content of the atmosphere in different geological eras (Bannister *et al.,* 1991).

**2.10.2. Peroxidase Activity (EC. 1.11.1.7)**

Peroxidase isoenzymes play an important role in eliminating $H_2O_2$ and are distributed in at least four distinct cell compartments, the stroma (PX) and thylakoid membrane (PX), the microbody (mPX), and the cytosol (c PX).

Scandalios (1993) reported that the plants under stress enhance the activities of enzymatic antioxidants especially Peroxidase. The increase Peroxidase activity reported in water stressed marigold (Karup *et al.*, 1994) and in sugarcane (Yang *et al.*, 1995). Zhang and Kirkham (1996) reported that decrease in activity of Peroxidase in sunflower and sorghum seedling under water stress.

Enhanced Peroxidase activity due to overexpression of CAPOAI gene in transgenic tobacco resulted in high tolerance to oxidative stress imposed by methyl viologen (Sarowar *et al.*, 2005)

### 2.10.3. Catalase Activity (EC. 1.11.1.6)

Catalase is a heme- containing enzyme that catalyses the dismutation of hydrogen peroxide into water and oxygen. The enzyme catalase found in all aerobic eukaryotes and is important in the removal of hydrogen peroxide generated in peroxisomes (microbodies) by oxidases involved in β-oxidation of fatty acids, the giyoxylate cycle (photorespiration) and purine catabolisim. Catalase is one of the first enzymes to be isolated in a highly purified state. Multiple forms of catalase have been described in many plants. Plants have three isoforms of catalase termed cat-1, cat-2 and cat-3 that are on separate chromosomes and are differentially expressed independently regulated. The cat-1 and cat-2 are localized in peroxisomes and the cytosol, whereas cat-3 is present in mitochondria.

Catalase activity decreased in pea plants under water stress (Moran *et al.*, 1994). An initial increase in CAT activity was found in pea plants under water stress. An initial increase in CAT activity was found in *Radix astrgali*, with the onset of ware stress, which decreased on subsequent days of stress treatment (Yong *et al.*, 2006). Lower CAT activity, was observed in low light intensities under WDS condition in *Picea asperata*. Catalase activity was not affected by mild drought in sorghum (Zhang and Kirkham, 1996). Over expression of catalase in transgenic rice enhanced tolerance for oxidative stress produced by chilling (Saryama *et al.*, 2005). Catalase activities decrease under conditions that suppress photorespiration, such as elevated $CO_2$ (Azevedo *et al.*, 1998). The importance of CAT in photosynthetic cells is demonstrated by observations in CAT-deficient mutants (Kendall *et al.*, 1983) and in transformed tobacco in which the major leaf CAT isoform is decreased by antisense technology (Takahashi *et al.*, 1997; Willekens *et al.*, 1997). When such plants are placed in conditions favoring high rates of photorespiration, photosynthesis is inhibited, the foliar antioxidant system is perturbed, and necrotic lesions appear on the leaves. Induction of defence-related proteins is observed, both locally in the necrotic regions and in leaves which do not suffer necrosis (Takahashi *et al.*, 1997; Willekens *et al.*, 1997). Shang and Feierabend (1999) found that the CAT protein is susceptible to photo inactivation upon exposure to high light

intensities. Leaf CAT activities have been shown to decline in certain stress conditions. There is now considerable evidence to show that CAT is one of the most rapidly turned over proteins in leaf cells particularly in stress conditions. Catalase gene expression and translation, as well as CAT protein turnover, are regulated in a complex manner that is far from resolved. Plant catalases are encoded by a small gene family usually composed of three isozyme genes which exhibit fairly complex spatial and temporal patterns of expression throughout the plant life cycle (Scandalios *et al.*, 1997; Willekens *et al.*, 1997). The wide diversity of plant CAT sequences has led to some discrepancies in the isozyme correlation in phylogenetic trees. This has been investigated and a model for the evolutionary divergence of monocot and dicot CAT genes has been proposed by (Iwamoto *et al.*, 1998). The presence of a G-box or ABRE (ABA responsive) element in the maize CAT1 promoter allows increased expression in response to exogenous ABA and osmotic stress (Guan and Scandalios, 2000). An antioxidant responsive element (ARE) is also present in the promoters of CAT1 and CAT3 underlying the important protective role of CAT in response to oxidative stress (Polidoros and Scandalios, 1999). Zhong and McClung (1996) and Polidoros and Scandalios (1998) found that the expression of some CAT genes is under circadian control.

### 2.10.4. Ascorbate peroxides Activity (APX) (EC 1.11.1.11)

Ascorbate peroxides (APX) belongs to the class I heme-peroxidases that is found in higher plants, chlorophytes red algae (Takeda *et al.*, 1998, 2000) (*Sano et al.*, 2001), and members of the protist kingdom (Shigeoka *et al.*, 1980; *Wilkinson et al.*, 2002). APX and other peroxidase sequences from all kingdoms of life are stored in the database Peroxibase (Oliva *et al.*, 2009), which also provides a series of bioinformatics tools useful for analyzing the peroxidases stored sequences.

Passardi *et al.* (2007) found that Genomic and cDNA APX sequences were obtained from a great variety of plant species, showing that APX are widely distributed in the vegetal kingdom. These enzymes are encoded by small gene families in these organisms. The different isoforms are classified according to their

subcellular localization. Soluble isoforms are found in cytosol (cAPX), mitochondria (mitAPX) and chloroplast stroma (sAPX), while membrane-bound isoforms are found in microbody (including peroxisome and glyoxisome) (mAPX) and chloroplast thylakoids (tAPX). The presence of organelle-specific targeting peptides and transmembrane domains found in the N- and C-terminal protein regions determine the final subcellular localization of the isoenzyme (Shigeoka *et al.*, 2002; Teixeira *et al.*, 2004, 2006).

Plant chloroplastic APX (chlAPX) isoenzyme encoding genes are divided into two groups. The first group comprises single genes encoding two isoenzyme through a post-transcriptional alternative splicing regulation. This group includes genes from spinach (*S. oleracea*), tobacco (*N. tabacum),* pumpkin (*Cucurbita sp*) and ice plant *(M. crystallium*). In the second group, individual genes codify different isoenzymes which are individually regulated. This group includes genes from *Arabidopsis,* rice, and tomato. The mechanism of alternative splicing in chlAPX has been studied in spinach (Ishikawa and Shigeoka, 2008) and the results showed that alternative splicing is fundamental for controlling the expression of stromal (sAPX) and thylakoid (tAPX) isoenzymes. This regulation occurs in a tissue-dependent manner.

Mittler *et al.* (2004) and Scandalous (2005) reported that extreme temperatures affect the growth, yield and quality of plant production. ROS levels tend to increase if plants are exposed to stressful conditions such as low or high temperatures. Potato tubers, the transient accumulation of cAPX mRNA after storage at low-temperature was greater than after high-temperature storage, showing that APX expression was induced in response to low temperature (Kawakami et al., 2002). Likewise, the two rice cAPX (OsAPX1 and OsAPX2) genes were induced after rice plants were exposed to low temperatures. Furthermore, OsAPX3, OsAPX4, OsAPX6 and OsAPX7 were also significantly induced, while OsAPX8 were repressed after 24 h under low temperature (unpublished data). Park *et al.* (2004) reported that the sweet potato cAPX gene was highly induced in leaves after exposure to high temperature. In cucumber plants

submitted to heat treatment, the activities of cAPX, sAPX and mAPX increased after an initial slight decline during the course of the experiment. The expression of sAPX followed a similar pattern (Song *et al.*, 2005). In response to cold, the expression of a peroxisomal APX gene increased slightly in Arabidopsis (*Zhang et al.*, 1997). In two cowpea (*Vigna unguiculata*) cultivars, one drought-tolerant and the other drought-sensitive, APX activity was 60 % higher in tolerant plants cultivated under control conditions. In response to drought stress, a higher increase in transcript levels of cytosolic and peroxisomal APX genes was observed in the sensitive cultivar (D'Arcy-Lameta *et al.*, 2006).

**2.10.5. Glutathione reductase Activity GR (EC 1.8.1.7),**

Glutathione reductase, also known as GSR or GR (EC 1.8.1.7), belongs to the family of NADPH-dependent oxidoreductase and occurs in both prokaryotic and eukaryotic organisms. Even though GR is located in chloroplasts, cytosol, and mitochondria, more than 80 % of its activity in photosynthetic tissues was reported to be of chloroplastic is form and GR and the content of GSH are intricately linked and are differentially modulated by various stressors. GR and GSH have been extensively reported to play crucial roles in determining stress tolerance in plants under various abiotic stresses. GR plays an essential central role in cell defense against reactive oxygen metabolites by efficiently maintaining the cellular reduced GSH pool through catalyzing the reduction of GSSG to GSH with the accompanying oxidation of NADPH. The over expression of GSH and/or GR is postulated to play an important role in plant protection against various forms of stress. GR catalyzes the NADPH-dependent formation of a disulphide bond in glutathione disulphide (GSSG) and is thus important for maintaining the reduced pool of GSH. Together, GSH and GR perform the scavenging of ROS and its reaction products; thereby provide tolerance to stress-exposed plants.

**2.10.6. Multiple defense roles for glutathione as antioxidant and redox sensor.**

Upon the imposition of oxidative stress, the existing pool of reduced glutathione (GSH) is converted to oxidized glutathione (GSSG) and glutathione biosynthesis is stimulated (May and Leaver 1993; Madamanchi *et al.,* 1994). The rate-limiting step for glutathione synthesis is thought to be gamma-glutamyl cysteinesynthetase, which is feedback regulated by GSH and is controlled primarily by the level of available L-cysteine (May *et al.,* 1998b). Increasing glutathione biosynthetic capacity has been shown to enhance resistance to oxidative stress (Arisi *et al.,* 1998; Zhu *et al.,* 1999). Glutathione reductase (GR) activities increase as the glutathione pool increases through a multi-level control mechanism, which includes coordinate activation of genes encoding glutathione biosynthetic enzymes and GR (Xiang and Oliver, 1998). Glutathione acts as a redox sensor of environmental cues and forms part of the multiple regulatory circuitry coordinating defense gene expression. There are two known genes encoding GR (Madamanchi *et al.*, 1992; Creissen *et al.,* 1995a; Creissen *et al.,* 1995b). The redox state of the GSH/GSSG couple may act as a direct link between environmental cues and crucial molecular adaptive responses of plant cells (Hausladen and Alscher, 1993; Broadbent *et al.*, 1995; Roxas *et al.,* 1997; May *et al.,* 1998a; May *et al.,* 1998b; Baginsky *et al.,* 1999). Glutathione has been reported to regulate rates of cell division (Sanchez- Fernandez *et al.,* 1997) and the induction of antioxidant defenses, as exemplified by the induction of Cu-Zn SOD (Herouart *et al.,* 1993). Glutathione has been suggested as an intermediary in a redox sensing signaling pathway in plants involving the ROS-mediated oxidation of membrane lipids to oxylipins as the initial step. It is also thought to act specifically as a regulator of the transcription of chloroplast genes (Baginsky *et al.,* 1999). The antioxidant genes known to date are all nuclear-encoded. However, previous biochemical data demonstrated the existence of multiple isoforms of GR, which have been assigned to various organelles, including the peroxisome, the chloroplast, the mitochondrion and the peroxisome (Creissen *et al.*, 1991). Only two Arabidopsis GR genes have been characterized, (Stevens *et al.,* 2000). A peroxisomal GR gene has not been

characterized to date, although a peroxisomal GR protein has been described (Corpas *et al.,* 2001). Consequently, it is very likely that other members of the GR gene family exist. Oxidative stress through impaired mitochondrial electrontransport in developing seeds. Seeds develop through a series of stages that may be tracked at morphological, physiological, and molecular levels. *Arabidopsis* seeds are orthodox (desiccation tolerant) and their ontogeny consists of some 20 distinct developmental stages. Embryogenesis in *Arabidopsis* is completed in approximately 9 days at 25 °C. During the final stage of embryogenesis, seeds must acquire desiccation tolerance to survive. As both the fruit and seeds dry, metabolism declines rapidly as water is lost. As respiratory activity slows during desiccation, electrons leak and react with oxygen to generate reduced oxygen species, so antioxidants are necessary to protect mitochondria from damage (Vertucci and Farrant, 1995). Desiccation tolerance is one of the unusual properties of seeds that, despite intensive investigation, remain rather poorly understood. Free radical scavengers may provide additional protection during desiccation because the development of desiccation tolerance, which involves a period of water stress, coincides with an increase in free radical scavengers in seeds (Vertucci and Farrant, 1995; Haslekas *et al.*, 1998). Water stress involves the production of ROS and their containment by antioxidants (Bohnert and Jensen, 1996; Bohnert and Shevelava, 1998). Thus, the availability of antioxidants such as GSH may be essential for seed maturation. Since GR is a major determinant in the maintenance of reduced glutathione levels, it is not surprising to find that seed aging has been linked to decreased GR activity (De Vos *et al.,* 1994; Bailly *et al.,* 1996; DePaula *et al.,* 1996). Lipid peroxidation and autoxidation are oxidative reactions associated with seed deterioration and death (Walters, 1998) that may be mitigated by radical scavenging systems.

### 2.10.7. Ascorbate Free Radical reductase Activity (AFR EC 1.6.5.4)

Bielski (1982) reported that AFR is relatively stable and reacts preferentially with itself thus prevnt the propagation of free radical reactions. Ascorbate plays a major role in the prevention of peroxidative damage by

scavenging hydroxyl, superoxide, and organic free radicals and, as a consequence produces its own free radical, AFR (also called monodehydroascorbate). Activated oxygen species in plant cells such as ($O_2$ and $H_2O_2$) is counteracted by catalase and by antioxidants including ascorbate, glutathione, a-tocopherol, carotenoids, and flavonoids (Elstner, 1982). Ascorbate peroxidase catalyzes the reduction of $H_2O_2$ to form water using the reducing power of ascorbate, producing AFR (Yamazaki and Piette, 1961; Hossain *et al.*, 1984). (Hossain and Asada, 1985) reported that AFR is directly reduced to ascorbic acid by the action of AFR reductase (monodehydroascorbate reductase, NADHAFR oxidoreductase, EC 1.6.5.4), using NADH or NADPH as electron donor Hossain and Asada (1985) because of these mechanisms, ascorbate is maintained in its reduced form as a protectant against cellular oxidative degradation. According to Hossain *et al.* (1984), high levels of AFR reductase activity, which regenerates partially oxidized ascorbate to its active reduced form, are found in chloroplasts but activity is also found in mitochondria, in the cytosol, and in microsome preparations and appears to be widely distributed among plant species and tissues (Arrigoni *et al.*, 1981). AFR reductase has also been cloned from cucumber fruit (Sano and Asada, 1994) and pea (Murthy and Zilinskas, 1994), but so far no information is available concerning the expression of this gene. Ascorbic acid is found in high amounts in chloroplasts, because of the production of superoxide anion radicals ($O_2$-) by photosynthesis and their dismutation by superoxide dismutase to molecular oxygen and $H_2O_2$, (Elstner,1982). (Tanaka *et al.*, 1985; Mehlhorn *et al.*, 1986; Luwe *et al.*, 1993) reported that ascorbic acid has also been implicated in plant detoxification of ozone and of respiratory-produced $H_2O_2$, during seed germination (Cakmak *et al.*, 1993), in the prevention of peroxide damage in nitrogen-fixing root nodules (Dalton *et al.*, 1993) in resistance to drought (Smirnoff and Colombe, 1988) and chilling (Schoner and Krause, 1990; Kuroda *et al.*, 1991) injury, and in plant resistance to nematode worm infection (Arrigoni *et al.*, 1979).

# MATERIALS AND METHODS

# 3. MATERIALS AND METHODS

The present study was carried out in the Division of Crop Production, Central Tuber Crops Research Institute (ICAR-CTCRI), Sreekariyam, Thiruvananthapuram during the period October 2014- June 2015. The details of experimental materials and methodology adopted for the study are presented in this chapter.

## 3.1. Plant materials

Four varieties/ genotypes of cassava (*Manihot esculenta* Crantz) *viz.*, H-165, Sree Athulya, Kunguma Rose and IPS -2-1 were planted in field with three replications, consisting 25 plants each (plate 1). The crop was planted on 18 January 2015 and grown under irrigated, field conditions in Block I of Central Tuber Crop Research Institute during 2014 (plate 2).Water Deficit Stress (WDS) was imposed during third, fourth and fifth month after planting by withholding irrigation for 10 days. High temperature stress was imposed by enclosing the plants in light transparent poly chambers. Plants were exposed to high temperature stress at 40°C ± 2 °C for six hours daily for a period of 10 days during $4^{th}$ and $5^{th}$ month. Activity of antioxidative enzymes like superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (AP), glutathione reductase (GR) and ascorbate reductase were assayed in the leaves of five verities of cassava using spectrophotometer EVOLUTION 201 UV-visible spectrophotometer (Thermo scientific) adopting standard procedures. Total protein was extracted from the leaves and tubers of Cassava plants and 1 D protein profiling was done using PAGE (Poly Acrylamide Gel Electrophoresis). Growth parameters *viz.*, leaf number, leaf retention percentage, plant height were recorded monthly whereas yield components such as tuber yield, tuber number and mean tuber weight were recorded at the time of harvest.

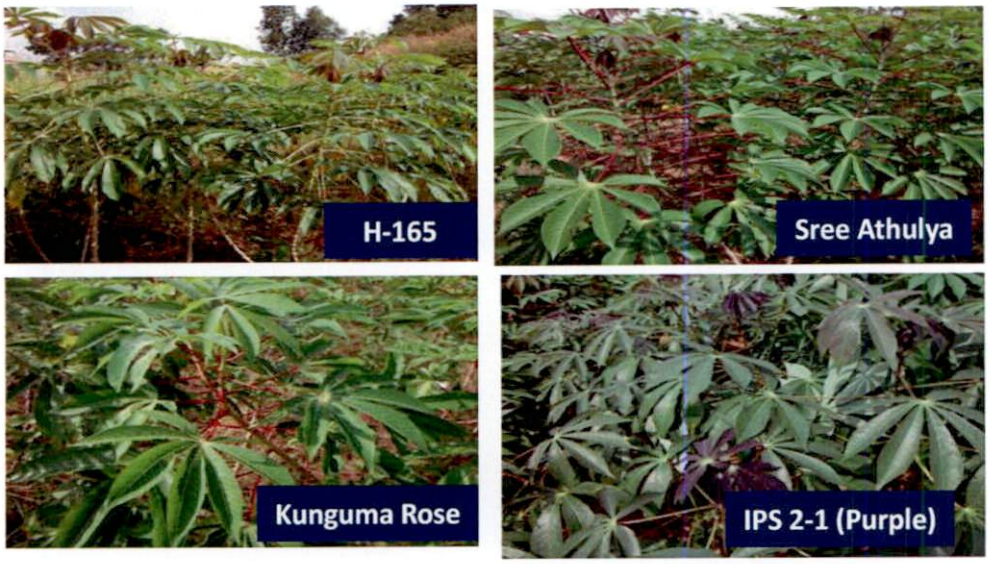


**Plate 1. Four varieties/ genotypes of cassava (*Manihot esculenta* Crantz) viz., H-165, Sree Athulya, Kunguma Rose and IPS -2-1**

**Plate 2. View of cassava field in Block I of Central Tuber Crop Research Institute.**

### 3.2. Growth parameter

#### 3.2.1. Leaf retention percentage

Leaf retention percentage were recorded in 3rd, 4th and 5th month according to the formula

$$\text{Leaf Retention Percentage} = \frac{Number\ of\ leaves}{Total\ umber\ leaves}\ X\ 100$$

#### 3.2.2. Plant Height

Plant height was recorded from the base of the plant to the apex of the plant using meter scale for 180 days with an interval of 60 days

#### 3.2.3. Number of leaves per plant

Leaf number was counted from the base of the plant to the apex of the plant recorded for 180 days with an interval of 60 days

### 3.3. Yield parameters were recorded after 3rd, 4th and 5th month after planting

#### 3.3.1. Number of tuber per plant

At the time of harvest, tubers were collected from the base of each plant and were counted.

#### 3.3.2. Mean tuber weight

The average weights of the tubers were recorded with the help of electronic weighing balance (Ultra-electronics).

#### 3.3.3. Tuber length

The length of each tuber was measured with the help of meter scale.

#### 3.3.4. Tuber width

The width of each tuber was measured using the help of meter scale

### 3.4. Quantitative estimation of protein

Protein content of leaf tuber and tuber forming roots samples was estimated by Bradford (1976) method. The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic

and ionic interactions stabilize the anionic form of the dye, causing a visible colour change. The assay is useful since the extinction coefficient of a dye-albumin complex solution is constant over a 10-fold concentration range.

**3.4.1. Total protein extraction for protein quantification and enzyme assay**

Cassava leaves and tuber were washed with distilled water and grated into a pre- chilled pestle and mortar containing liquid nitrogen and ground to a fine paste with the help of ice cold phosphate buffer (Appendix I) (pH 7.0, 2 ml per gram of fresh tissue). The homogenate was then transferred into a 20 ml centrifuge tube. The sample was spun for 2-3 minutes at 5000 rpm. The clear supernatant was transferred to a clean sterile tube and stored at 20°C till further analysed. This clear extract was used as enzyme source for the assay by suitable method and to determine amount of protein in the sample. 100 µl of protein extract was pipetted out into a test tube. To this 3 ml Bradford reagent (Appendix I) was added. The contents of the tube was mixed thoroughly and kept for 10 minutes in room temperature. The absorbance was read after 10 minutes at 595 nm. Three aliquots from each sample were used for the assay. The protein content was estimated using standard curve.

**3.4.2. Protein profiling using SDS PAGE**

The leaves of the control and stressed plants were collected and the protein fractions were prepared. The protein profile of these fractions was analysed by SDS-PAGE (Laemmli, 1970).

**3.4.2.1. Gel casting**

The electrophoresis glass plates, spacers and comb were cleaned with acetone to remove any grease. The spacers were placed between the two plates. The plates were fixed perfectly on to the gel casting apparatus. The resolving gel (Appendix II) was poured using a Pasteur pipette between the glass plates ensuring no leakage. The resolving gel was overlayed with ethanol to prevent oxygen from diffusing into the gel and inhibiting polymerization. After the polymerization was complete, the overlay was poured off and the top was washed several times with deionized water to remove any unpolymerized acrylamide. Care was taken to drain as much fluid as possible from the top of the gel and then the remaining water was

removed with the edge of paper towel. Over the polymerized resolving gel, the stacking gel (Appendix II) was applied. Immediately after pouring the stacking gel, the well template (Teflon comb) was placed on the gel to form well. After the polymerization completed, the comb was removed carefully and the wells were immediately washed with deionized water to remove the unpolymerized acrylamide.

**3.4.2.2. Preparation of the sample**

The protein extracted from the leaves and tubers, adopting the procedure stated above, and used for protein profiling. The protein concentration of the supernatant was adjusted such that an aliquot containing 200 µg per ml was taken in a microfuge tube and equal volume of the SDS sample buffer was added (Appendix II). The tubes were placed on a boiling water bath for 3 minutes and then cooled. Sample volumes containing 100 µg of the protein were used for the electrophoretic run. PageRuler ™ unstained protein ladder (Fermentas life sciences, Thermo scientific) was also loaded and run along the samples each time, for comparing the molecular weight of the protein bands separated after electrophoretic study.

**3.4.2.3. Electrophoretic run**

The protein samples and ladder were loaded on to the wells. The reservoir buffer (Appendix II) was filled in both the electrode chambers and the electrodes were connected to the power pack. The conditions were set initially at 150 V and 50 minute until the dye front reached the resolving gel and later at 100 V till the dye front reached the end. After running the gel was removed, washed in distilled water and stained with the coomassie blue solution (Appendix II).

**3.4.2.4. Staining with coomassie blue**

The gel was immersed in at least 100ml of staining solution and kept overnight at room temperature. The gel was then destained by soaking it in the destaining solution 3-4 times (Appendix II). After destaining, the gels were stored in 20 % glycerol in a sealed plastic bag. The molecular weight of the bands seen on the gel was indicated by the position of the molecular weight marker proteins.

### 3.5. Physiological Parameters

#### 3.5.1. Estimation of Total Chlorophyll and carotenoids

The total chlorophyll and carotenoids content of the leaves was estimated according to the procedure developed by Sadasivam and Manickam (1992). Fresh leaf sample (0.5 g) was taken and cut into small bits. These bits were put in test tubes and incubated overnight at room temperature with 10 ml DMSO: 80 % acetone mixture (1:1$V/V$). The coloured solution was decanted into a measuring cylinder and made up to 25 ml with the DMSO – acetone mixture. The absorbance was measured at 663, 645, 470 and 510 nm using a spectrophotometer. The chlorophyll content was measured by substituting the absorbance values in the given formula:

$$\text{mg chlorophyll a/g tissue} = 12.7\,(A663) - 2.69(A645) \times \frac{V}{1000 \times W}$$

$$\text{mg chlorophyll b/g tissue} = 22.9(A645) - 4.68(A663) \times \frac{V}{1000 \times W}$$

$$\text{mg total chlorophyll (per g tissue)} = 20.2\,(A645) + 8.02\,(A663) \times \frac{V}{1000XW}$$

$$\text{Total carotenoid} = \frac{(1000 \times A470) - (1.82 \times \text{chl a}) - (85.02 \times \text{chl b})}{198} X \frac{V}{1000 X W}$$

Where, A = Absorbance at specific wave lengths,

V = Final volume of chlorophyll extract in 80% acetone

And W = Fresh weight of the tissue extracted

#### 3.5.2. Relative water content (RWC)

RWC was calculated by measuring the fresh weight, dry weight and turgid weight of known number of leaf disc from the treatment plants. After measuring the fresh weight of the sample, it was submerged in the distilled water for 3 hours and then turgid weight was taken. The dry weight of the sample was measured after

keeping the sample in the oven at 80°C for three consecutive days. The RWC of the treatment was calculated using the following formula.

$$RWC = \frac{\text{Fresh weight} - \text{dry weight}}{\text{Turgid weight} - \text{dry weight}} \times 100$$

**3.6. Biochemical parameters**

**3.6.1. Estimation of Proline**

Proline content was estimated according to Bates et al., (1973).The midribs of a leaf were removed and 500 mg of the fresh leaf tissue was weighed. It was homogenised with 10 ml of 3% sulphosalicylic acid (Appendix III) in a mortar and pestle. The homogenate was filtered through a Whatmann No.2 filter paper. This procedure was repeated with the residue and the filtrates were pooled.

**3.6.1.1. Estimation**

2.0 ml of the filtrate solution was taken in a tube and 2.0 ml of acid ninhydrin (Appendix III) and 2.0 ml of glacial acetic acid were added. The tubes were incubated for 1 hour at 100°C on a water bath. The tubes were transferred to an ice bath to terminate the reaction. 4.0 ml of toluene was added and mixed vigorously for 15 to 20 seconds. The chromophore containing toluene was aspirated from the aqueous phase. It was allowed to reach room temperature and the absorbance measured at 575nm.A reagent blank was maintained. A standard curve was obtained using a known concentration of proline.

$$\mu\text{moles of proline/g tissue} = \frac{\mu\text{g proline/ml x ml toluene}}{115.5} \times \frac{5}{\text{g sample}}$$

Where, 115.5 is the molecular weight of proline.

**3.7. Antioxidant enzyme assays.**

**3.7.1. Glutathione reductase assay (GR, EC 1.6.4.2)**

Glutathione reductase activity was determined by following the method of Foyer and Halliwell (1976). The leaf tissue was homogenized in a pre-cooled mortar and pestle with the Glutathione reductase extraction mixture (Appendix IV),

homogenate was centrifuged at 15000 rpm for 20 minute and the supernatant was used for the enzyme assay.

### 3.7.1.1. Enzyme assay

2 ml of Glutathione reductase assay mixture (Appendix IV) was taken in a test tube. The reaction was initiated by the addition of NADPH at 25°C and the absorbance was recorded at 310 nm. For 2-3 min with an interval of 15seconds. One unit of GR is expressed as NADPH oxidized/ minute/gram. The GR activity was calculated by the formula.

$$U/ml = \frac{(\Delta A340nm/\min) \times 3 \times \text{wt of sample}}{6.22 \times \text{volume of enzyme}}$$

Where, $\Delta$ = change in absorbance

3 = volume of reaction mixture

6.22 = millimeter extinction co-efficient

### 3.7.2. Superoxide Dismutase assay (SOD, EC 1.15.1.1.)

The activity of superoxide dismutase was determined by the method of Beauchamp and Fridovich (1971).The leaf material was homogenized in a mortar and pestle with 5 volumes of the SOD extraction buffer (Appendix V) and the homogenate was centrifuged at 6000 rpm for 10 sec. The pellet was washed with extraction buffer and centrifuged again. The supernatants were pooled and used for the enzyme assay.

### 3.7.2.1. Enzyme assay

To the SOD assay mixture (2ml) (Appendix V), riboflavin was added as the last component and the reaction was initiated by placing the tubes under two 15-V fluorescent lamps. The reaction was terminated after 10 min by removing the reaction tubes from the light source. Non illuminated and illuminated reactions

without supernatant served as calibration standards and the absorbance was measured at 560 nm. SOD is calculated by the formula,

$$protein\ EU/mg = \frac{(\Delta A560nm/\min) \times 2.5\times \text{ wt of sample}}{0.0436 \times \text{ volume of enzyme}}$$

Where, 0.0436 mμ = Extinction co-efficient

2.5 = volume of reaction mixture

EU = Enzyme Unit

### 3.7.3. Catalase assay (CAT, EC 1.1 1.1.6)

The activity of catalase was assayed by a modified method of Luck, (1974).The leaf material was homogenized in a pre-cooled mortar and pestle with 5volumes Catalase extraction mixture (Appendix VI) and the homogenate was centrifuged at 8000 rpm for 10 min at 4°C. The pellet was stirred with cold phosphate buffer, allowed to stand with occasional stirring. The combined supernatants were used for the assay.

#### 3.7.3.1. Enzyme assay

$H_2O_2$-phosphate buffer (3.0ml) was taken in an experimental cuvette, followed by the rapid addition of 40μl of enzyme extract and mixed thoroughly. The time required for a decrease in absorbance by 0.05 units was recorded at 240nm in a spectrophotometer. The enzyme solution containing $H_2O_2$-free phosphate buffer served as control. One enzyme unit was calculated as the amount of enzyme required to decrease the absorbance at 240nm by 0.05 units. Catalase is calculated by the formula;

$$protein\ EU/mg = \frac{(\Delta A240/\text{min}) \times 3.04 \times \text{wt of sample}}{0.036 \times \text{ volume of enzyme}}$$

Where, 3.04 = volume of reaction mixture

0.036= extinction co-efficient

### 3.7.4. Ascorbate peroxidase assay (APX, EC 1.1 1.1.1 1)

The activity of ascorbate peroxidase was assayed by the method of Nakanoand Asada (1987). The leaf tissue was homogenized in a pre-chilled pestle and mortar with the ascorbate peroxidise extraction buffer (Appendix VII) the homogenate was squeezed through four layers of cheesecloth and then centrifuged at 20,000 rpm for30 min. The supernatants were used for the enzyme assay.

#### 3.7.4.1. Enzyme assay

In the Ascorbate peroxidise assay mixture (Appendix VII), the hydrogen peroxide-dependent oxidation of the ascorbate was followed by a decrease in the absorbance at 290 nm. The enzyme activity was calculated using the equation,

$$\text{protein EU/mg} = \frac{(\Delta A290/\text{min}) \times 2 \times \text{wt of sample}}{2.8 \times \text{volume of enzyme}}$$

Where, 2.8 = Extinction co-efficient

2 = volume of reaction mixture

### 3.7.5. Assay for Ascorbate Free Radical Reductase (EC 1.6.5.4)

The activity of ascorbate free radical reductase was assayed by the method of (Hossain et al., 1984). The leaf tissue of cassava of 1g was powdered in liquid N, using a pestle and mortar and then homogenized at 0°C for 4 min in ascorbate free radical reductase assay buffer 10 ml (Appendix VIII) using a Tekmar homogenizer. Extracts were centrifuged at full speed in a bench-top centrifuge for 30 min at O °C, the supernatants were filtered through Miracloth.

#### 3.7.5.1. Enzyme assay

15-pL aliquots were assayed in a final reaction volume of 1mL. AFR reductase activity was determined spectrophotometrically by following the decrease in A340 due to NADH oxidation.

### 3.8. Non enzymatic Anti-oxidant Assay

#### 3.8.1. Estimation of Ascorbic Acid

Ascorbic acid content was estimated volumetrically by the method of Sadasivam and Manickam (2008).

### 3.8.1.2. Extraction of Ascorbic acid

Fresh leaf was homogenized with 5 ml of 4% oxalic acid using pestle and mortar. The homogenate was centrifuged at 6000 rpm for 10 min. The Supernatant was saved and the pellet was re extracted twice with the same volume of the solvent. The supernatants were pooled and used for estimation of total content of ascorbic acid.

### 3.8.1.3. Estimation of Ascorbic acid

Working standard solution (5 ml) was pipetted out into a 100 ml conical flask. 4% oxalic acid was added to it and titrated against the dye ($V_1$ ml). End point was noted on appearance of pink colour which persisted for a few minutes. The sample (0.5 – 5g) was weighed and ground in a mortar with pestle using 15 ml of 4% oxalic acid. The homogenate was filtered through a double layered cheese cloth. The filtrate was made up to a known volume and centrifuged at 10,000 rpm for 10 min. The supernatant was collected and made up to 25 ml using oxalic acid. 5.0 ml aliquot was pipetted into a conical flask to which 10 ml of 4% oxalic acid was added. This was titrated against Dichlorophenol indophenols (DCPIP) solution, until the appearance of pink colour ($V_2$ ml.)

$$\text{Amount of ascorbic acid } \left(\frac{\text{mg}}{100\text{g}}\text{sample}\right) = \frac{0.5}{V1ml} \times \frac{V2}{5ml} \times \frac{100}{wt.of\ sample} \times 100$$

Whereas, $V_1$ = volume of standard

$V_2$ = volume of sample

### 3.3.6.4. Estimation of reduced glutathione

Reduced glutathione was determined by the method of Moron *et al.* (1979). Glutathione on reaction with DTNB (5, 5'-Dithiobis Nitro Benzoic acid) produces a yellow coloured product that absorbs at 412nm. The homogenate was prepared

with 0.5 g of the plant sample with 2.5 ml of 5% TCA. The precipitated protein was centrifuged at 1000 rpm for 10 minutes. The supernatant (0.1ml) was used for the estimation of GSH. The supernatant (0.1 ml) was made up to 1.0ml with 0.2M sodium phosphate buffer (pH 8.0). Standard GSH with concentrations ranging between 2 and 10 M were prepared. Two ml of freshly prepared DTNB solution was added and the intensity of the yellow colour developed was measured in a spectrophotometer at 412nm after 10 minutes. The values were expressed as moles GSH/g sample.

# RESULTS

# 4. RESULTS

The present study entitled the "Oxidative stress and protein profiling in Cassava (*Manihot esculenta*)" was conducted during the period 2014-2015 at the Division of Crop Production, Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram and the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram, Kerala. The results obtained from the study are summarized below.

## 4.1 GROWTH PARAMETER

### 4.1.1 Change in the plant height of four cassava varieties/genotypes as affected by WDS and high temperature stress conditions.

Under control condition the plant height of four varieties of cassava was measured for 180 days with the interval 60 days and the results are given in Fig.1. At 60 days after planting (DAP) the average plant height of four varieties/genotype recorded as: H-165-(57.33 ± 13.65 cm), Sree Athulya – (70.33 ± 7.91cm), Kunguma rose – (31.00 ± 12.17cm) and IPS 2-1 – (56.83 ± 28.27 cm). The maximum plant height was observed in the variety Sree Athulya (70.33± 7.91cm) and the minimum plant height was observed in the variety Kunguma rose (31.0± 12.15cm). After 120 DAP, the average plant height of four varieties of cassava increased: H-165 (114.33 ± 6.658 cm), Sree Athulya (121.67 ± 3.22cm), Kunguma rose (74 ± 8.19 cm) and IPS 2-1 (159.41 ± 15.94) cm. After 120 DAP, it was found that the variety IPS 2-1 showed maximum increase in height (102.57 cm) and minimum change in height was observed in the variety Kunguma rose (43 cm). At 180 DAP, the average plant height of varieties changed significantly H-165 (140.33 ± 24.54 cm), Sree Athulya (137.5 ± 23.70cm), Kunguma Rose (116.22± 32.01 cm) and IPS 2-1 (159.40± 12.54cm). From the study, it was observed that the variety IPS 2-1 had the maximum average height (159.40 cm) and Kunguma rose had the minimum average height (116.22 cm). Under 10 days of WDS for 3$^{rd}$, 4$^{th}$and 5$^{th}$ month and heat stress condition for 4$^{th}$and 5$^{th}$month the four varieties of cassava *viz.*, H-165, Sree Athulya, Kunguma Rose and IPS -2-1 did not show much variation in plant height.

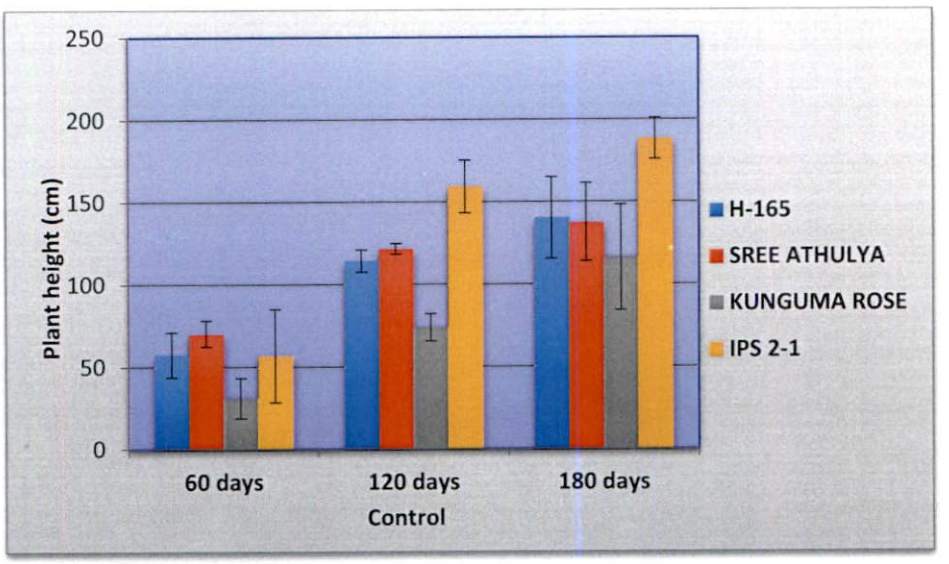


**Fig. 1.Changes in the plant height of four varieties/genotypes of cassava under irrigated condition.**

### 4.1.2 Change in the leaf production of four cassava varieties/genotype as affected by WDS and high temperature stress conditions

Under controlled condition plant leaf production at 60 DAP and 180 days was recorded and depicted in (Fig. 2). It was observed that at 60 DAP the average leaf production per plant of variety H-165 was (43 ± 10.82), Sree Athulya was (32± 2), Kunguma rose was (12.333 ± 3.51) and IPS 2-1 was (13 ± 1.732) the average leaf production per plant of the four cassava varieties varied between 43 and 12 respectively. At 60$^{th}$ (DAP) the maximum leaf production was observed in the variety H165- 43, and minimum leaf production was observed by the variety Kunguma rose-12. Similar pattern was maintained at 120$^{th}$ (DAP) and 180$^{th}$ (DAP) by H165 and Kunguma rose. From this observation it was found that the variety H-165 had the maximum leaf production and the variety Kunguma rose had minimum leaf production. Under 10 days of WDS applied for (3$^{rd}$, 4$^{th}$and 5$^{th}$month) it was found that there is a reduction in the leaf production in all the varieties H-165-(37 ± 0.03), Sree Athulya-(29 ± 0.14), Kunguma rose – (21 ± 0.04) and IPS 2-1 – (19 ± 0.26). Similar pattern was recorded in the case of high temperature stress condition for (4$^{th}$and 5$^{th}$ month) for 10 days and the leaf production was reduced to (H-165- ) (40± 0.02), (Sree Athulya) - (32 ± 0.10), (Kunguma Rose-) (25 ± 0.43) and IPS 2-1 – (21 ± 0.58). From the study it is concluded that the average leaf production was reduced under both stress conditions WDS (Fig.3) and high temperature Stress condition (Fig. 4) compared to irrigated conditions.

### 4.1.3 Change in leaf retention percentage of four cassava varieties/genotypes as affected by WDS and high temperature stress conditions

Under control condition the leaf retention of all four varieties of cassava *viz.*, H-165, Sree Athulya, Kunguma rose and IPS -2-1 was recorded for 180 days. Plants under stress free condition, at 60$^{th}$day, the leaf retention of the variety H-165 was 62.62% and it increased at 120 DAP to 78.5%. At 180 DAP, the leaf retention Percentage of H-165 decreased to 72.46%. In the variety Sree Athulya, at 60DAP, the leaf retention percentage was 82.59% whereas at 120 and 180 DAP the leaf retention percentage has reduced to 65.9% and 39.92% respectively. In both

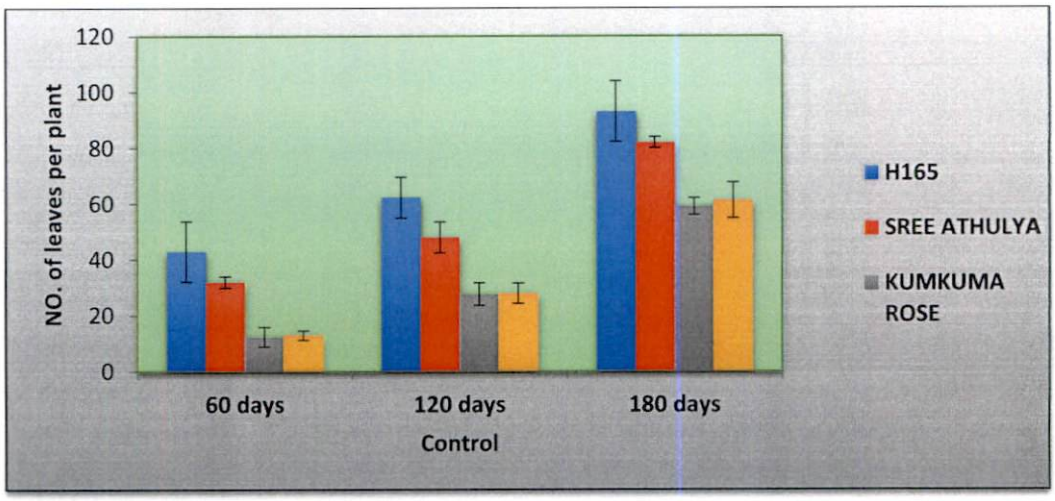


**Fig.2. Changes in the leaf production of four varieties/genotypes of cassava under control condition.**

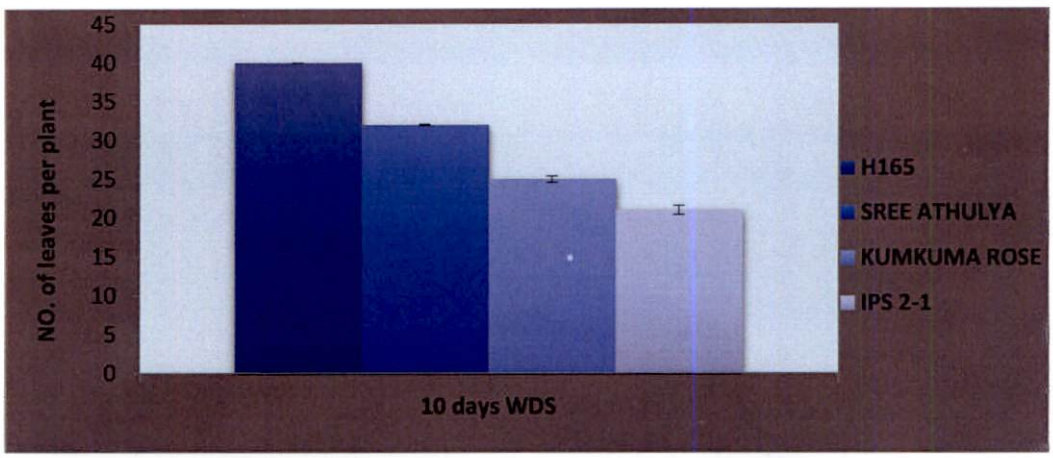


**Fig. 3: Changes in the leaf production of four varieties /genotypes of cassava under WDS condition. (Data average of 3rd, 4th and 5th month WDS).**

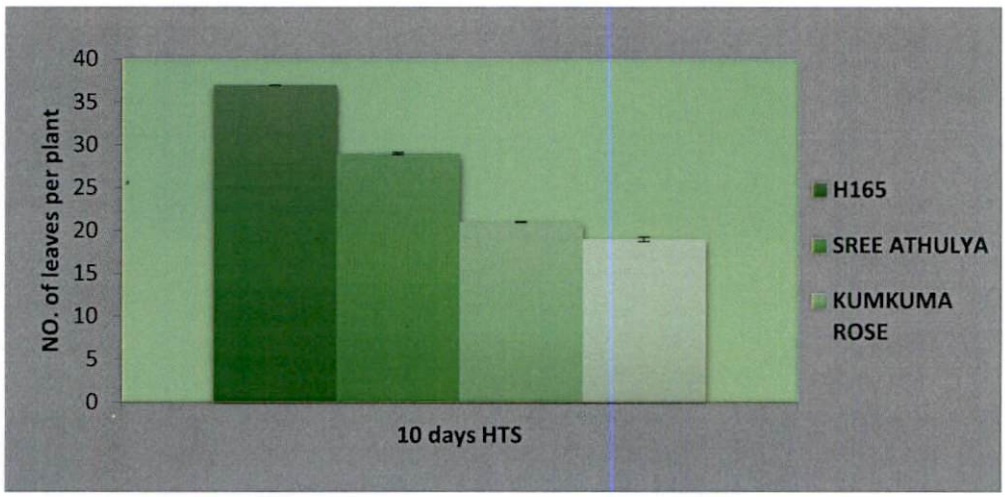


**Fig.4. Changes in the leaf production of four varieties/genotypes of cassava under high temperature stress condition. (Data average of 4th and 5th month HTS).**

varieties Kunguma rose and IPS 2-1, the reduction in the leaf retention percentage at 120DAP was 49.35 % and 43.24 % respectively compared to the 60$^{th}$ day reading 72.06 % and 82.42 %. At 180 DAP, there was a reduction in the leaf retention percentage in both the variety Kunguma rose and IPS 2-1 60.48 % and 63.40 % respectively (Fig.5). After 10 days of WDS condition applied for 3$^{rd}$, 4$^{th}$ and 5$^{th}$month, the leaf retention percentage of four varieties of cassava were H-165- (53.1%), Sree Athulya- (76.93%),Kunguma rose- (61.23%) and IPS 2-1-(68.08%) (Fig.6) similarly, in high temperature stress condition, leaf retention percentage of four varieties/genotypes of cassava was H-165- (49.32 %), Sree Athulya- (53.02 %), Kunguma rose-(47.09 %) and IPS 2-1- (54.21 %), respectively (Fig. 7).

## 4.2 YIELD PARAMETER

### 4.2.1 Tuber Number

The total number of tubers per plant was recorded under control condition, WDS and high temperature stress conditions (Table 1). Sree Athulya and H-165 had the maximum number of tubers per (7.33 ± 2.52) and (7 ± 1) plant and Kunguma rose had minimum number of tubers per plant (4.33 ± 1.53). In the plants under WDS conditions, after third, fourth and fifth month, it was observed that the Sree Athulya had the maximum tuber number (6.33 ± 1.14) and Kunguma rose had the minimum tuber number (3.67 ± 1.53). In the plants under high temperature stress treatment after 4$^{th}$ and 5$^{th}$ month, Sree Athulya had the maximum number of tubers (6.33 ± 1.53) and minimum numbers of tubers was observed in Kunguma rose (3.33 ± 2.31).

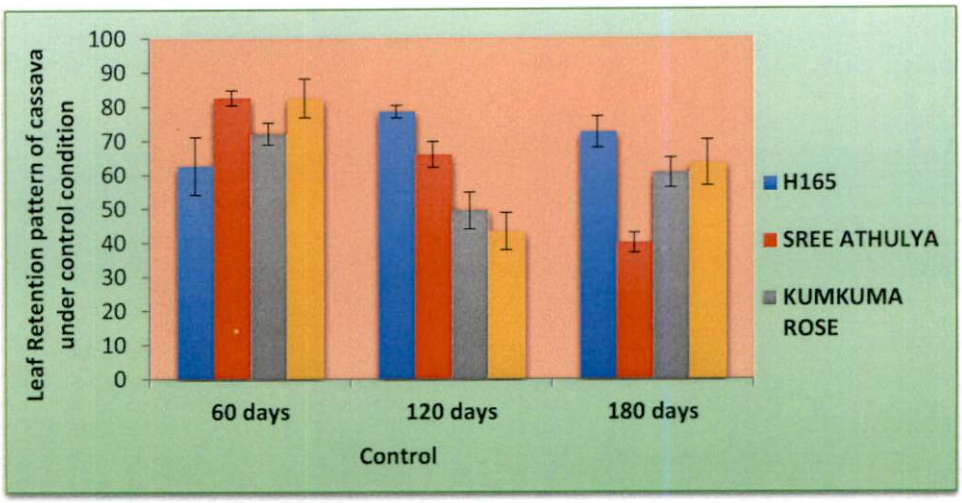


**Fig. 5. Changes in the leaf retention percentage in four varieties /genotypes of cassava under control condition.**

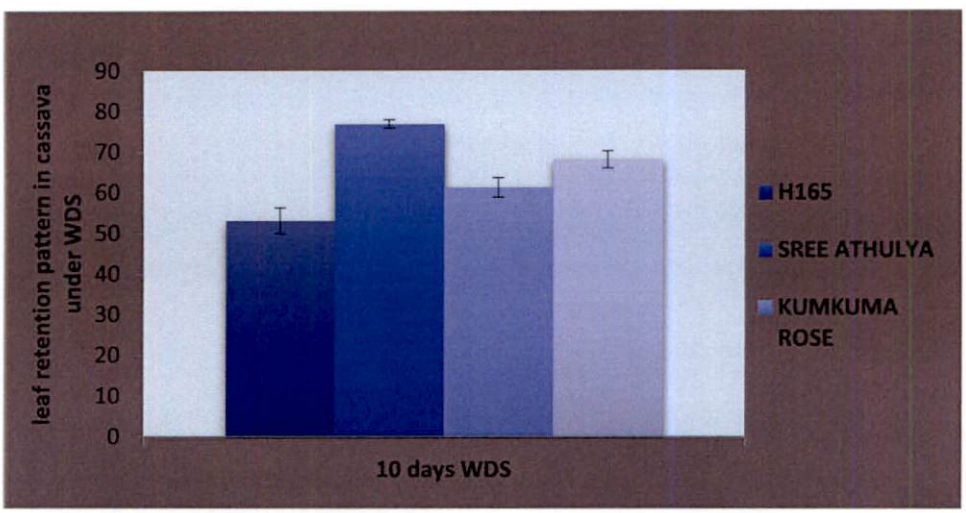


**Fig. 6. Changes in the leaf retention percentage in four varieties/genotypes of cassava under WDS condition. (Data average of WDS as 3rd, 4th and 5th month).**

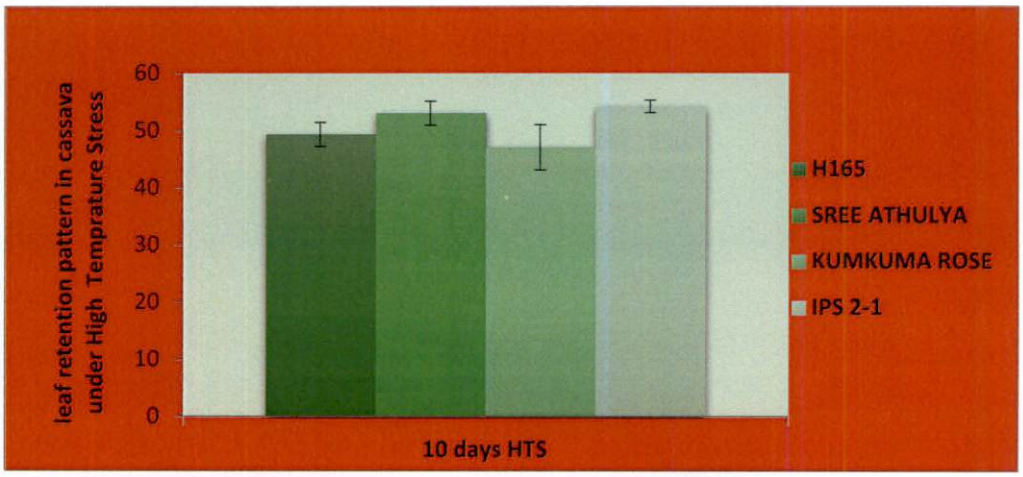


**Fig.7. Changes in the leaf retention percentage in four varieties of cassava under high temperature stress condition. Values are average of (data from 3rd and 4th month HTS treatments).**

**Table 1. Changes in number of tuber under different treatment condition**

| Variety/genotype | Number of tuber per Cassava Plant | | |
|---|---|---|---|
| | Control | High Temperature Stress ($40 \pm 2\ ^0C$ for 10 days) | WDS (10 days during $3^{rd}$,$4^{th}$ and $5^{th}$ month) |
| H-165 | 7 .00± 1.00 | 5.68 ± 2.08 | 6.00 ± 2.00 |
| Sree Athulya | 7.33 ± 2.52 | 6.33 ± 1.16 | 6.33 ± 1.53 |
| Kunguma rose | 4.33 ± 1.53 | 4.00 ± 1.00 | 3.33± 2.31 |
| IPS 2-1 | 5.00 ± 2.00 | 3.67 ± 1.53 | 4.33 ± 1.53 |

From the present study it was concluded that moisture stress affect tuber production in cassava

### 4.2.2 Tuber Length

Tuber length was recorded from the four varieties/genotypes of cassava under control condition, and after WDS and high temperature stress conditions (Table 2). Under control condition it was found that the variety Sree Athulya had the maximum tuber length (41.83 ± 7.90 cm) and minimum tuber length were observed in the variety H-165 (25.67 ± 3.06 cm). For the plants under WDS condition, after 3$^{rd}$, 4$^{th}$ and 5$^{th}$month, it was observed that the Sree Athulya had the maximum tuber length (41.25 ± 7.15 cm) and H-165 had the minimum tuber length (25.27 ± 2.53 cm). In the plants under high temperature stress treatment after 4$^{th}$ and 5$^{th}$ month, it was observed that the Sree Athulya had the maximum tuber length (41.17 ± 6.93 cm) and minimum tuber length was observed in H-165 (25.19 ± 2.31 cm). From this study it was found that stress has no influence in tuber length.

### 4.2.3. Tuber width

The tuber width per plant was recorded under stress free condition, WDS and high temperature stress condition (Table 3). Under stress free condition maximum tuber width was found in the variety H-165 (22.5 ± 1.80 cm) and minimum tuber width was found in the variety IPS 2-1(13.43 ± 0.21 cm). Under WDS the maximum tuber width was observed in the variety H-165 (20.73 ± 2.44 cm) followed by IPS 2-1 (11.83 ±1.76 cm). Similar results was obtained in high temperature stress condition the maximum tuber width were showed by the variety H-165 (21.53 ± 0.32, IPS 2-1 (9.93 ± 1.50 cm). From the study it was concluded that the variety H-165 had the maximum tuber width in stress free and treatment conditions and minimum tuber width were obtained in the variety IPS2-1.

**Table 2. Change in tuber length under different treatment condition**

| Variety/ genotypes | Tuber length (cm) | | |
|---|---|---|---|
| | Control | High temperature stress ($40 \pm 2^0$ C for 10 days) | WDS (10 days during $3^{rd}$,$4^{th}$ and $5^{th}$ month) |
| H-165 | 25.65 ± 3.06 | 25.19 ± 2.31 | 25.27 ± 2.53 |
| Sree Athulya | 41.83 ± 7.90 | 41.17 ± 6.93 | 41.25 ± 7.15 |
| Kunguma rose | 34.50 ± 4.92 | 34.00 ± 1.00 | 34.33± 2.31 |
| IPS 2-1 | 30.27 ± 0.64 | 30.21 ± 1.53 | 30.30 ± 1.53 |

**Table 3. Change in the tuber width under different treatment condition**

| Variety/ genotypes | Tuber width (cm) | | |
|---|---|---|---|
| | Control | High temperature stress ($40 \pm 2^0$C for 10 days) | WDS (10 days during $3^{rd}$,$4^{th}$ and $5^{th}$ month) |
| H-165 | 22.5 ± 1.80 | 20.73 ± 2.441 | 21.53 ± 0.32 |
| Sree Athulya | 15.16 ± 5.84 | 18.73 ± 4.833 | 16.33 ± 1.80 |
| Kunguma rose | 13.63 ± 0.96 | 15.56 ± 1.721 | 14.66 ± 0.28 |
| IPS 2-1 | 13.43 ± 0.20 | 11.83 ±1.755 | 9.93 ± 1.50 |

### 4.2.4. Mean Tuber yield per plant

The mean tuber yield per plant was recorded under control, WDS and high temperature stress conditions (Plate 3, Table 4). Maximum tuber weight was recorded for the variety Sree Athulya under control condition (3.33 ± 0.58 Kg) and minimum tuber weight was observed in the variety IPS 2-1 (0.85 ± 0.16 Kg). For the plants under WDS condition after 3rd, 4thand 5thmonth, it was observed that the Sree Athulya had the maximum tuber weight (2.93 ± 0.32 Kg) and IPS 2-1 had the minimum tuber weight (0.80 ± 0.08 Kg). Similar results were found in plants under high temperature stress for 4thand 5thmonth. Sree Athulya had the maximum mean tuber weight (3.09 ± 0.81 Kg) and minimum mean tuber weight was found in IPS 2-1 (0.82 ± 0.16 Kg). From this study it was found that there is almost no or negligible difference in the mean tuber weight in both treatment conditions (WDS and high temperature stress) and control conditions.

## 4.3 PROTEIN ESTIMATION

### 4.3.1 Total protein content in leaves of four cassava varieties/genotypes as affected by Water Deficit Stress (WDS) and high temperature stress condition.

Total protein was extracted from the leaves, tubers and tuber forming roots of four cassava genotypes/ varieties different treatment were analyzed (Fig. 8). In the control condition, it was observed that Kunguma rose had the maximum total leaf protein content (6.74 ± 0.51 mg/g leaf) compared to other three varieties H165 (4.62 ± 0.4 mg/g leaf) Sree Athulya (5.61±0.01 mg/g protein) and IPS 2-1 (2.10 ± 0.05 mg/g leaf).

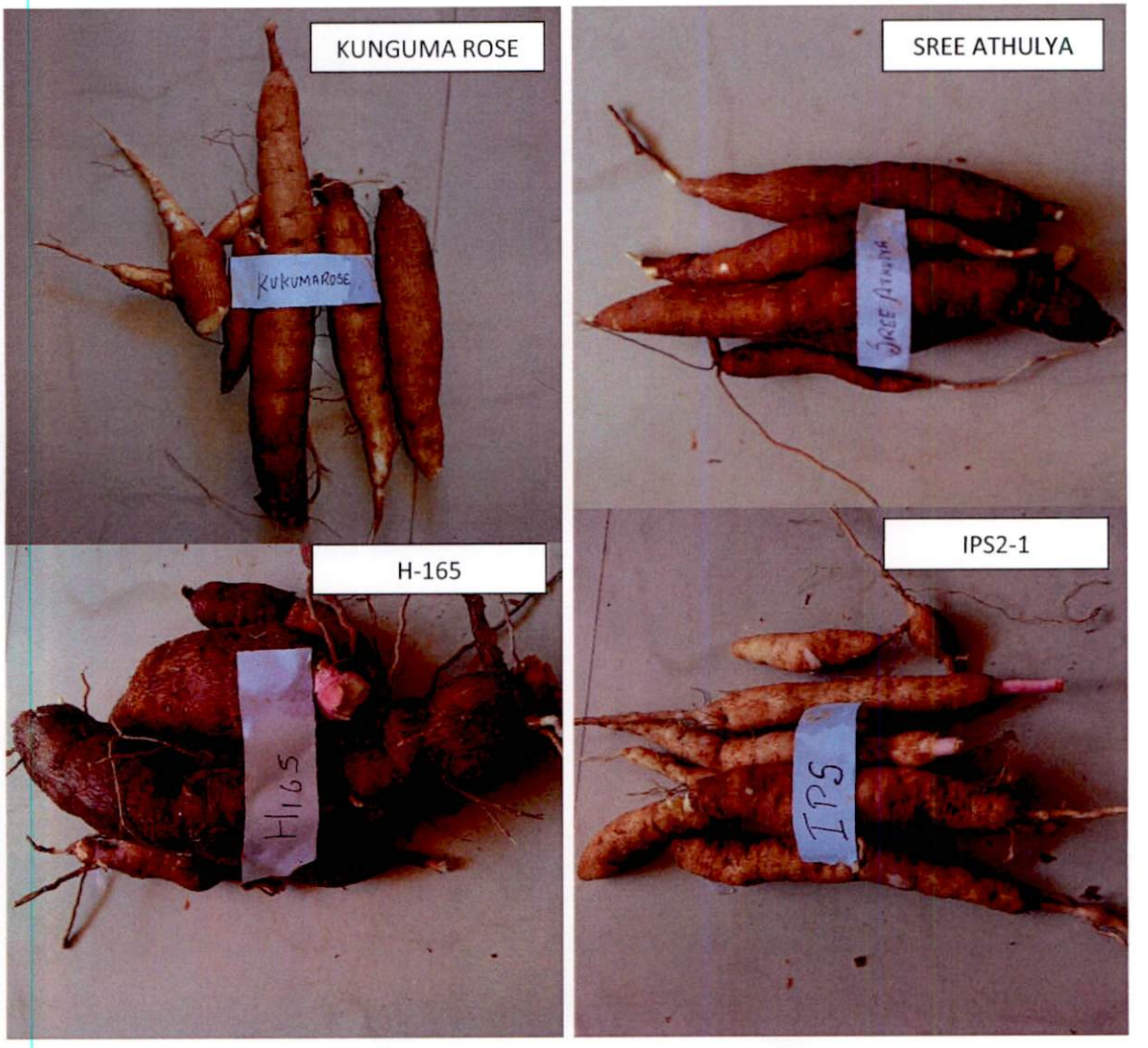


**Plate 3. Tubers of four varieties/genotypes of cassava Kunguma rose, Sree Athulya, H-165 and IPS 2-1**

**Table 4. Change in Mean tuber weight (Kg) under different treatment condition**

| Variety/ genotypes | Mean tuber weight (Kg) | | |
|---|---|---|---|
| | Control | High temperature stress (40 ± 2°C for 10 days) | WDS(10 days during 3rd,4th and 5th month) |
| H-165 | 1.86 ± 0.18 | 1.84 ± 0.17 | 1.73 ± 0.22 |
| Sree Athulya | 3.33 ± 0.58 | 3.09 ± 0.81 | 2.93 ± 0.32 |
| Kunguma rose | 2.46 ± 0.56 | 2.24 ± 0.66 | 2.16 ±0.71 |
| IPS 2-1 | 0.85 ± 0.16 | 0.82 ±0.16 | 0.80 ± 0.08 |

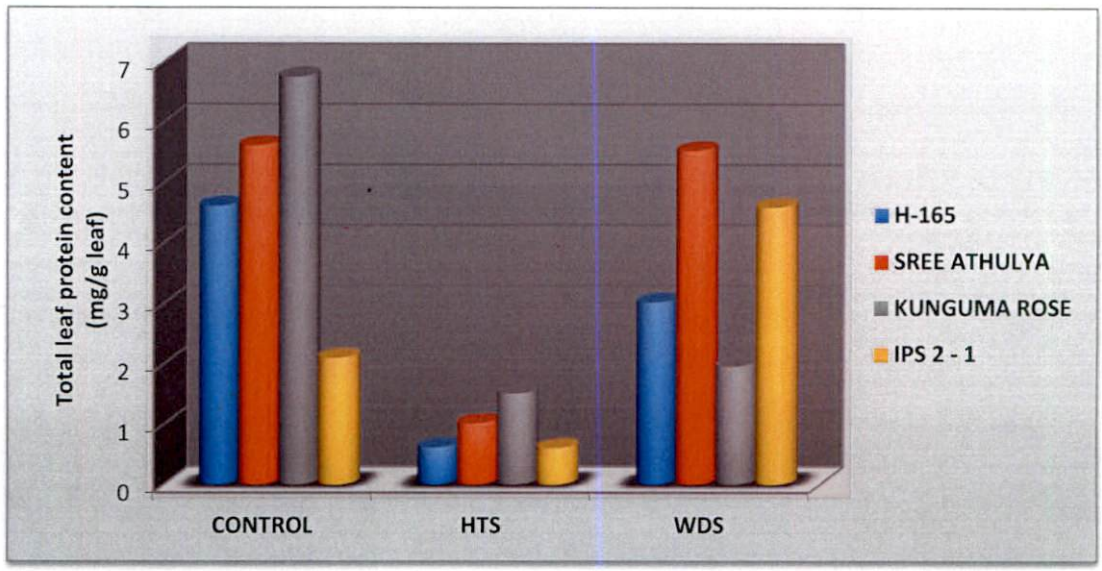


**Fig 8: Changes in leaf protein content of four cassava varieties/ genotypes as affected by WDS and high temperature stress condition**

Under WDS applied during 3rd, 4thand 5thmonth, it was observed that there is a reduction in the total leaf protein content H-165 (3.02 ± 0.11 mg/g leaf), Sree Athulya (5.53 ± 0.40 mg/g leaf), Kunguma Rose (1.96± 0.23 mg/g leaf). IPS 2-1 had high protein content (4.59 ± 0. 51 mg/g leaf) in WDS condition compared to control. Under high temperature stress applied during 4thand 5th month there was a drastic reduction in the total protein content in each varieties H-165(0.62±0.01mg/g leaf), Sree Athulya (1.02±0.08 mg/g leaf),Kunguma rose (1.51±0.14mg/g leaf and IPS -2-1(0.61 ±0.01 mg/g leaf) compared to control and WDS condition.When a comparison was made between the four varieties under high temperature stress the variation in total protein content was negligible between the two varieties *viz.*, H165 (0.62 ±0.01 mg/g leaf)and IPS 2-1(0.61 ±0.01 mg/g leaf) whereas there was a significant difference between the varieties Kunguma rose (1.51±0.14 mg/g leaf) and IPS 2-1(0.61±0.01 mg/g leaf). The result of total leaf protein content was statisfically analyzed using ANOVA and it was found that there was significant difference (P>0.001) between the treatments..

### 4.3.2. Total protein content in tubers of four cassava varieties as affected by water deficit stress (WDS) and high temperature stress condition

The total protein was extracted from the tubers of four cassava varieties/ genotypes *viz.*, H-165, Sree Athulya, Kunguma rose and IPS-2-1 grown under irrigated and water deficit stress (WDS)and high temperature stress condition (Fig. 9). In control it was observed that the variety Sree Athulya (14.92±0.08 mg/g tuber) had maximum tuber protein content compared to other three varieties H165 (12.37± 0.03 mg/g tuber), Kunguma rose (10.75 ± 0.08 mg/g tuber) and IPS 2-1 (15.88 ±0.04 mg/g tuber). In plants subjected to WDS condition it was observed that the variety H-165 had the maximum protein content (9.98 ± 0.13 mg/g tuber) compared to other three varieties and minimum protein content was observed in the variety IPS 2-1 (5.46 ± 0.27 mg/g tuber). Under WDS conditions all the four varieties H165 (9.98 ± 0.13 mg/g tuber), IPS 2-1 (5.46 ± 0.27 mg/g protein), Sree Athulya (7.65 ± 0.55 mg/g protein) and Kunguma rose (6.53 ± 0.12 mg/g tuber) had less amount of protein compared to control conditions. In plants subjected to high temperature

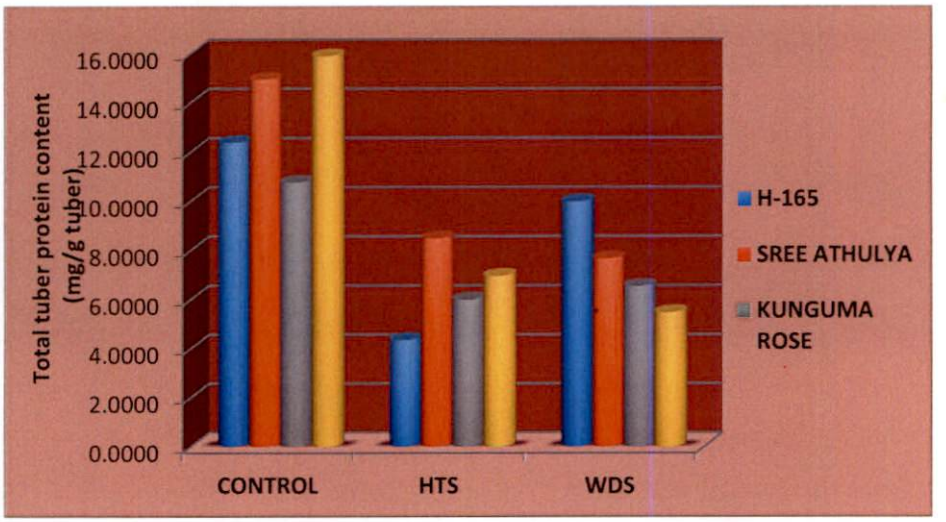


**Fig 9: Changes in tuber protein content of four cassava varieties/ genotypes as affected by WDS and high temperature stress conditions**

stress conditions it was observed that the variety Sree Athulya had the maximum protein content (8.46 ±0.31 mg/g tuber) and H-165 showed the minimum protein content (4.34 ±0.41 mg/g tuber). IPS 2-1 under high temperature stress condition recorded had tuber protein content (6.95 ± 0.94 mg/g tuber) compared to the plant in WDS condition (5.46 ± 0.27 mg/g tuber) .From the study, it is concluded that the total tuber protein content significantly decreased under stress condition (WDS and High Temperature Stress) compared to control. The total tuber protein content varied significantly (P>0.01) between the treatments.

### 4.3.3. Total protein content in tuber forming roots of four cassava varieties/ genotypes as affected by water deficit stress (WDS) and high temperature stress condition

Total protein were extracted from the tuber forming roots of four cassava varieties / genotypes *viz.*, H-165, Sree Athulya, Kunguma rose and IPS-2-1 grown under irrigated and water deficit stress (WDS) and high temperature stress condition (Fig. 10). It was observed that under control condition the variety H-165 tuber forming root had the maximum protein content (1.06 ± 0.18 mg/g) and minimum protein content (0.36 ± 0.21 mg/g) was observed in the variety Sree Athulya tuber forming root. In plants subjected to WDS condition for 3$^{rd}$, 4$^{th}$ and 5$^{th}$ month it was observed that the the variety H-165 (2.12 ± 0.26 mg/g tuber forming root) showed maximum protein content and minimum protein content were observed in the variety IPS 2-1(1.23 ± 0.27 mg/g tuber forming root). In plants under high temperature stress it was observed that the variety H-165 (1.03± 0.17 mg/g tuber forming root) had the maximum protein content and minimum protein was observed in Sree Athulya (0.38± 0.11 mg/g tuber forming root). From the study it was concluded that the varieties showed maximum protein content under high temperature stress condition compared to stress free condition and WDS and total protein content in control and WDS are almost similar. The result of protein content in tuber forming roots was analyzed using ANOVA and it was found that protein content significantly varied (P>0.01) between the treatments.

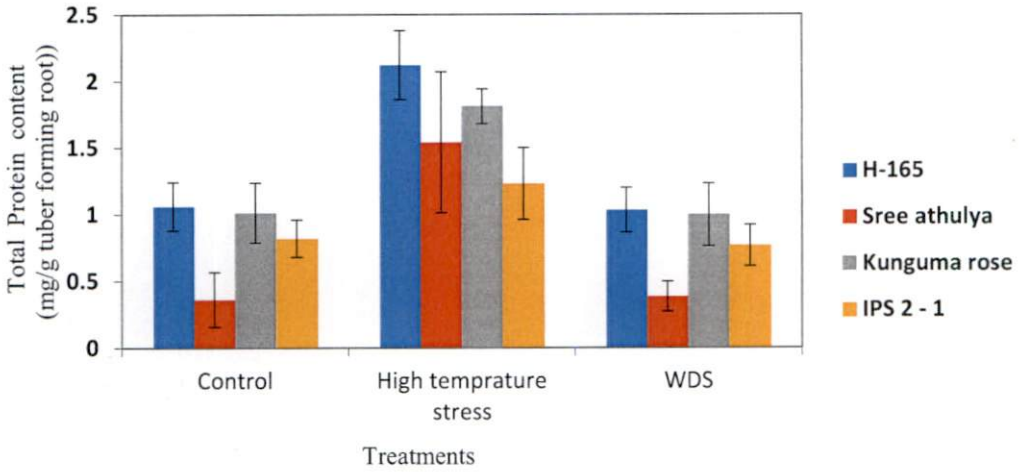


**Fig.10 Changes in tuber forming roots protein content of four cassava genotypes as affected by WDS and high temperature stress conditions**

## 4.4 PROTEIN PROFILING

### 4.4.1. Changes in the profile of leaf proteins as affected by WDS and high temperature stress conditions.

Leaf protein extracted from the leaves of four varieties under control and stress (WDS and high temperature stress) conditions. The protein profiling was done by PAGE. Extracted proteins were loaded into the wells of SDS gel along with the marker protein. After the electrophoretic run for 50 minutes at 150 V the gel was removed, stained in staining solution and kept overnight for destaining. After destaining, plants under irrigated condition were observed for protein and bands (Plate 4). After 10 days of WDS applied during 3$^{rd}$, 4$^{th}$and 5$^{th}$ months protein bands were observed for the varieties *viz.*, Sree Athulya and IPS 2-1 and no protein bands observed for H- 165 and Kunguma rose (Plate 5). Bands observed for plants exposed to high temperature stress condition because of low protein content in high temperature stress due to denaturation of protein (Plate 6). For the control plants thicker bands were observed near 50 KDa regions whereas plants under WDS condition showed clear bands at the region near to 70 KDa expect for plants exposed high temperature stress, no significant bands were observed in three varieties. In IPS 2-1 for which bands were observed below the region of 50 KDa.

### 4.4.2. Changes in the profile of tuber proteins as affected by WDS and high temperature stress conditions.

Total Protein were extracted from the tubers of four varieties/genotypes of cassava under control and stress (WDS and high temperature stress) conditions. The protein profiling was done by PAGE. Proteins extracted were loaded into the wells of SDS gel along with the marker protein. After the electrophoretic run for 50 minutes at 150V the gel was removed, stained in staining solution and kept overnight for destaining. After destaining, it was observed that plants under stress free condition had significant protein bands indicating high protein content in stress free condition (Plate 7). The variety Sree Athulya and Kunguma rose showed protein band in the region between 70 KDa and 50KDa whereas the variety IPS 2-1 showed band at the region near to 100 KDa and the variety H-165 had the band at the region near

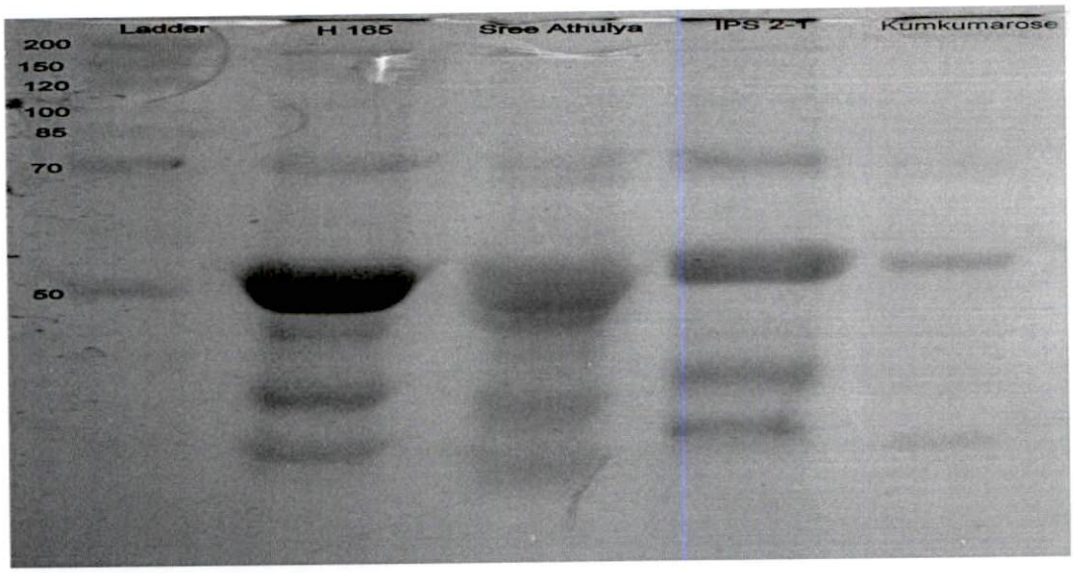


**Plate 4. Changes in the total leaf protein profiling pattern in four varieties/ genotypes of cassava under control condition.**

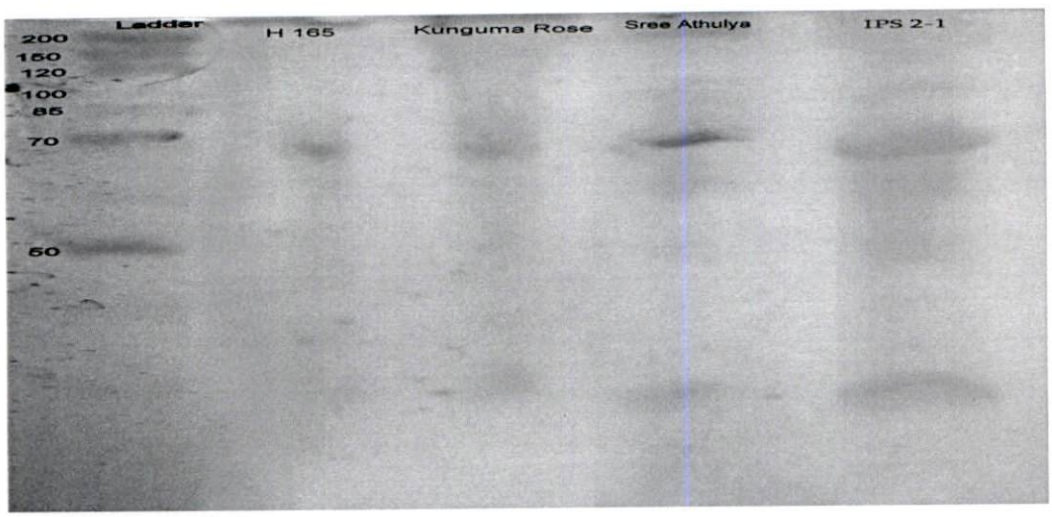


**Plate 5. Changes in the total leaf protein profiling pattern in four varieties/ genotypes of cassava under WDS condition.**

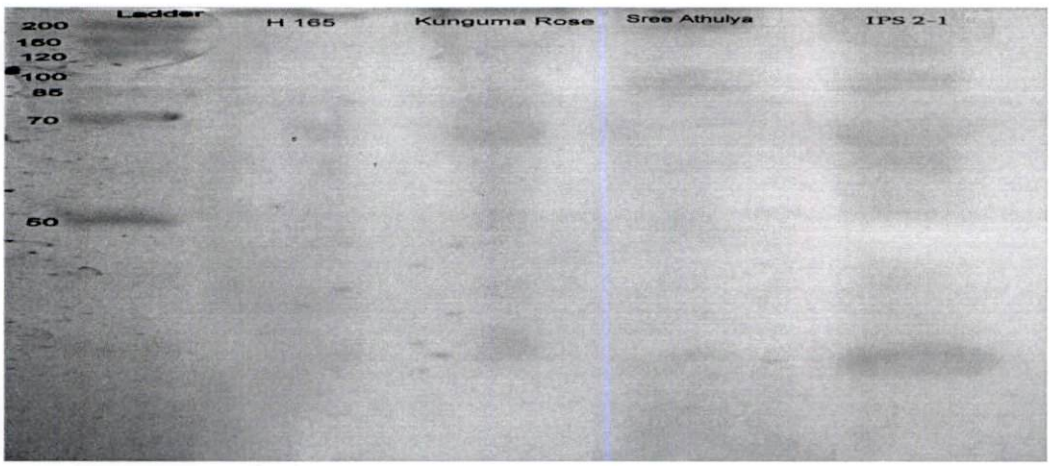


**Plate 6. Changes in the total leaf protein profiling pattern in four varieties/ genotypes of cassava under High temperature stress condition.**

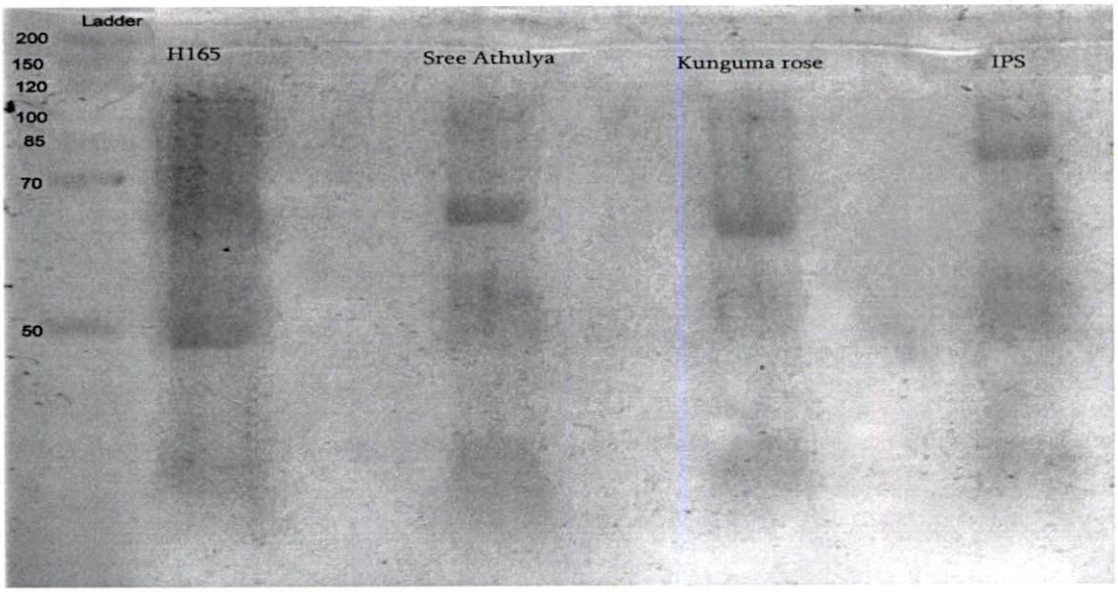


**Plate 7. Changes in the total tuber protein profiling pattern in four varieties/ genotypes of cassava under stress free condition**

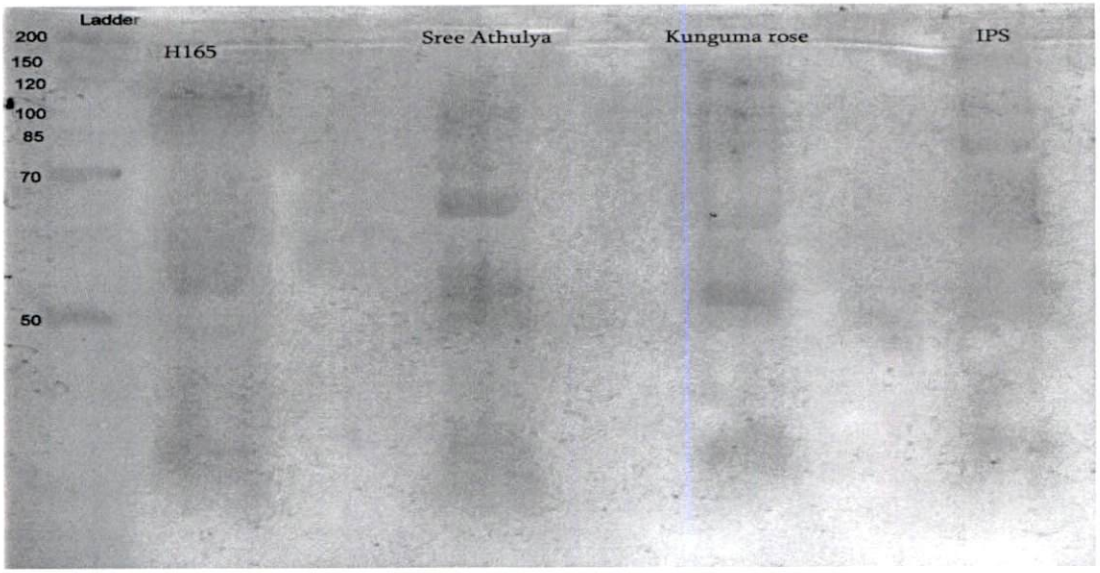


**Plate 8. Changes in the total tuber protein profiling pattern in four varieties/ genotypes of cassava under WDS condition.**

to 50KDa. After 10 days of WDS applied during 3rd, 4thand 5th months clear and significant amount of protein bands were observed in the varieties Sree Athulya and Kunguma rose, for which protein band were obtained at the region between70 KDa and 50KDa (Plate 8). Bands obtained are not much clear compared to the plants under stress free condition. No significant bands obtained in the varieties H-165 and IPS 2-1. Under high temperature stress condition for 4thand 5th it was observed that there were no significant bands were obtained for all the four varieties indicating low protein content due to denaturation of protein (Plate 9).

### 4.4.3. Changes in the profile of tuber forming roots proteins as affected by WDS and high temperature stress conditions.

Total protein were extracted from the tuber forming roots of four varieties of cassava under control and stress (WDS and high temperature stress) conditions. The protein profiling was done by PAGE. Proteins extracted were loaded into the wells of SDS gel along with the marker protein. After the electrophoretic run for 50 minutes at 150V the gel was removed, stained in staining solution and kept overnight for destaining. After destaining, it was observed that plants under stress free condition showed no significant protein bands because of low protein content in tuber forming root. Similar banding pattern were observed in plants under WDS stress condition. In plants under high temperature stress significant protein bands was observed on the gel plate 10. Brighter bands obtained in H-165 and Sree Athulya due to high protein content. The variety H-165 and Sree Athulya showed protein band in the region below 50 KDa whereas the variety IPS 2-1 showed protein band in the region between 70 KDa and 50 KDa and the variety H-165 had the band below 50 KDa.

## 4.5. PHYSIOLOGICAL PARAMETER

### 4.5.1. Change in the relative water content of four varieties/ genotypes of cassava as affected by WDS and high temperature stress conditions.

Under control condition relative water content in the leaves of four varieties/genotypes of cassava was estimated during third, fourth and fifth month.

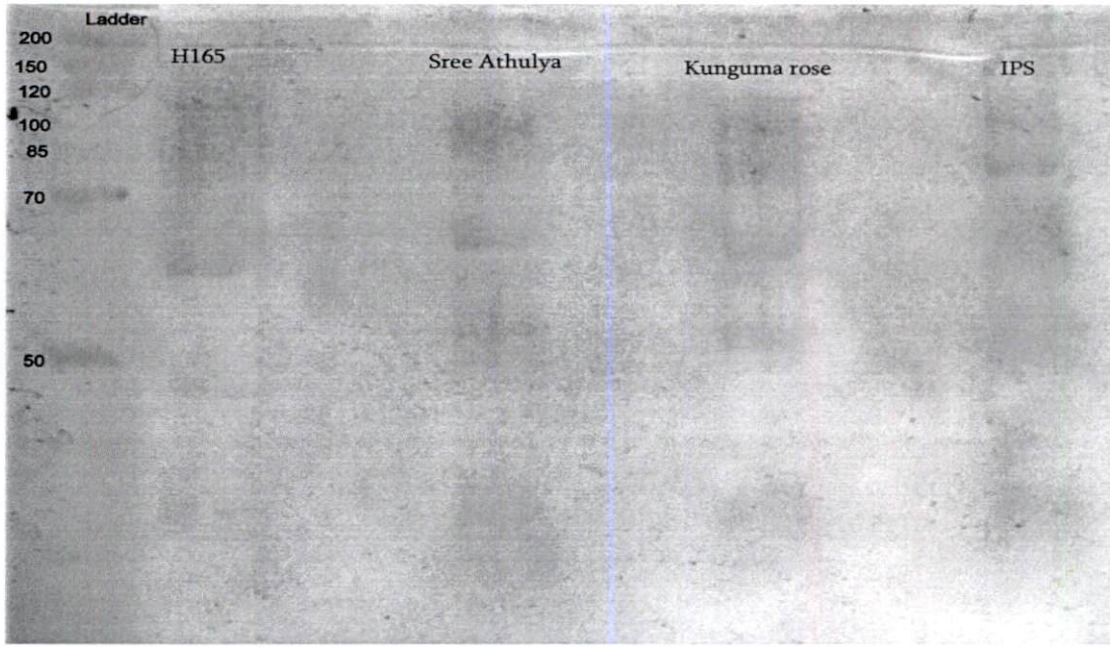


**Plate 9. Changes in the total tuber protein profiling pattern in four varieties/ genotypes of cassava under high temperature stress condition**

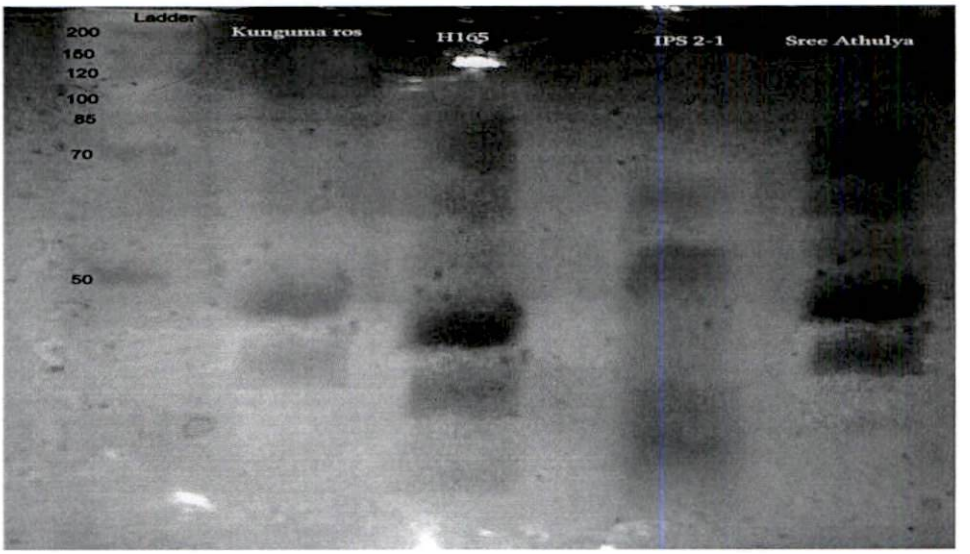


**Plate 10. Change in the total tuber protein profiling pattern in four varieties of cassava under high temperature stress condition.**

It was observed that the varieties Kunguma rose and IPS 2-1 had the higher RWC 84.09% and 84.5% and it was found during third month and minimum RWC showed by the varieties Sree Athulya (70.5%) and IPS2-1 (72.94%) respectively (Fig. 11). At WDS condition applied for 3rd, 4thand 5thmonth it was observed that RWC reduced drastically. The variety Sree Athulya had the maximum RWC (69.31%) and the variety H-165 had the minimum RWC (49.11%) (Fig. 12). Under high temperature stress applied for 4thand 5thmonth it was observed that the RWC is high compared to WDS treatment. The maximum RWC showed by the variety Sree Athulya (76.02%) and minimum RWC observed in the variety H-165 (54.21%) (Fig. 13). From the study it was concluded that RWC reduced at WDS and high temperature stress compared to control condition.

### 4.5.2. Changes in total chlorophyll and carotenoid content in the leaves of four varieties/ genotypes of cassava as affected by WDS and high temperature stress.

The total chlorophyll (Chl) and carotenoid content of the leaves of four varieties of cassava was estimated in control and under stress conditions (Fig. 14). In control plants, the total Chl content varied between (0.83 ± 0.08 mg/g) leaf and (0.53 ± 0.04 mg/g) leaf. Maximum Chl content was observed in the variety IPS2-1 (3.03 ± 1.42 mg/g leaf) and the minimum Chl content was observed the variety Sree Athulya (0.53 ± 0.04 mg/g leaf Under water deficit stress (WDS) (3rd, 4thand 5thmonth) the variety Kunguma Rose had the maximum Chl content (0.15 ± 0.02 mg/g leaf) and IPS 2-1 had the minimum Chl content (0.08 ± 0.01 mg/g leaf). Compared to control the Chl content in the variety IPS2-1was reduced drastically under WDS conditions. When plants were subjected to high temperature stress conditions (4thand 5thmonth) the maximum Chl content was observed in the variety (0.05 ± 0.01 mg/g leaf) when they were subjected to high temperature stress condition during fourth and fifth month and minimum Chl was in the variety Sree Athulya (0.03 ± 0.02 mg/g leaf. The total chlorophyll content of leaves from cassava plant significantly decreased under WDS and high temperature stress conditions compared to plant under control conditions. Maximum carotenoid

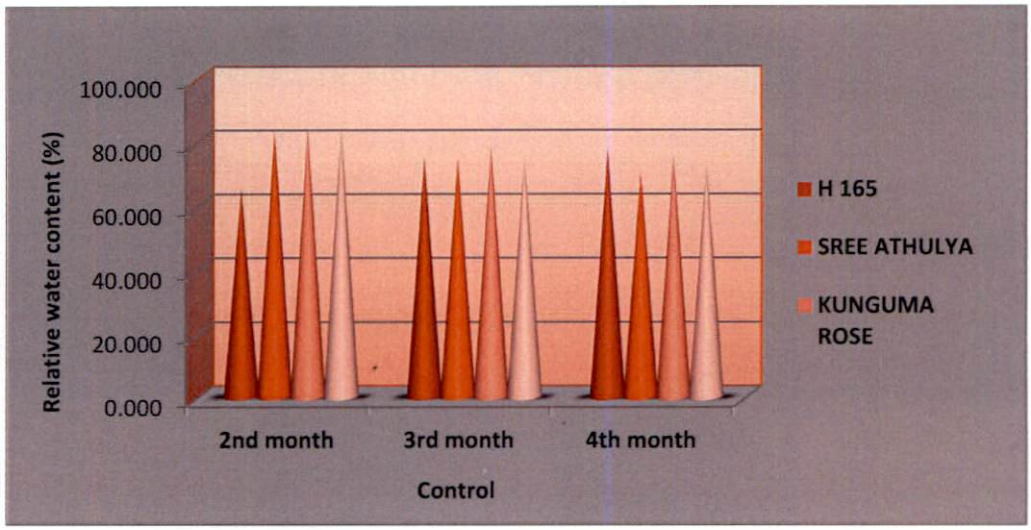


**Fig.11. Changes in the relative water content of four varieties/ genotypes of cassava under control condition**

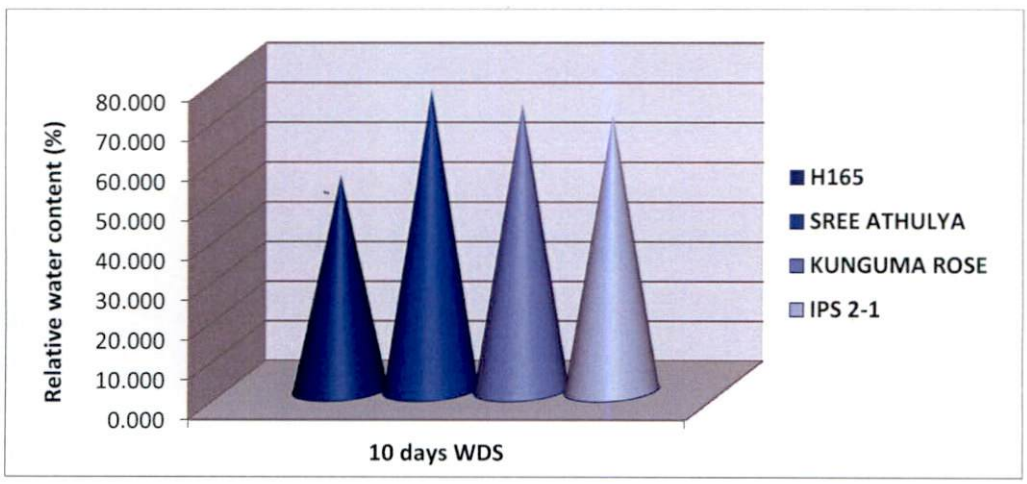


**Fig.12. Changes in the relative water content of four varieties/ genotypes of cassava under WDS condition.**

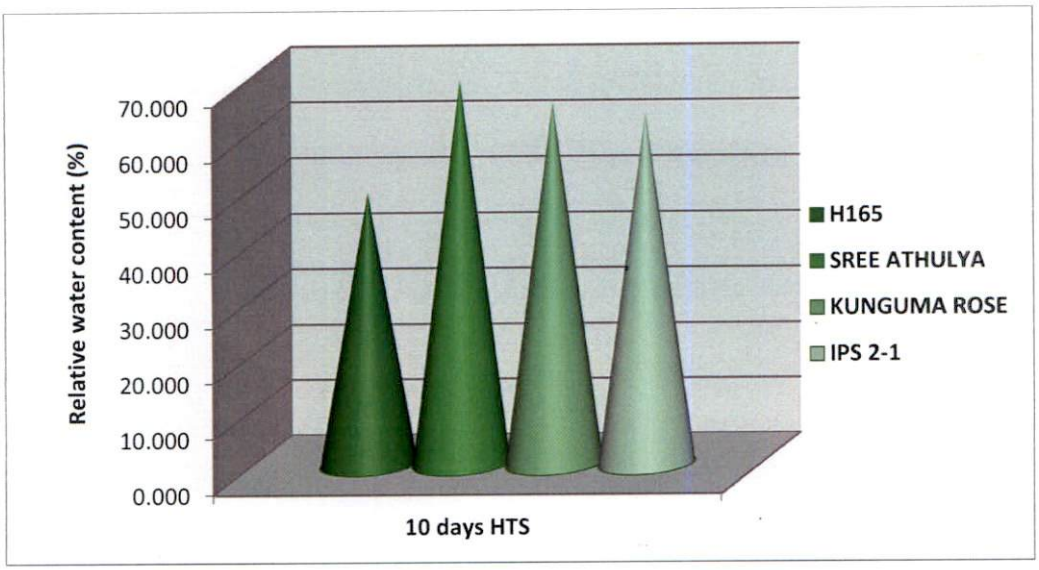


**Fig.13. Changes in the relative water content of four varieties/genotypes of cassava under high temperature stress condition.**

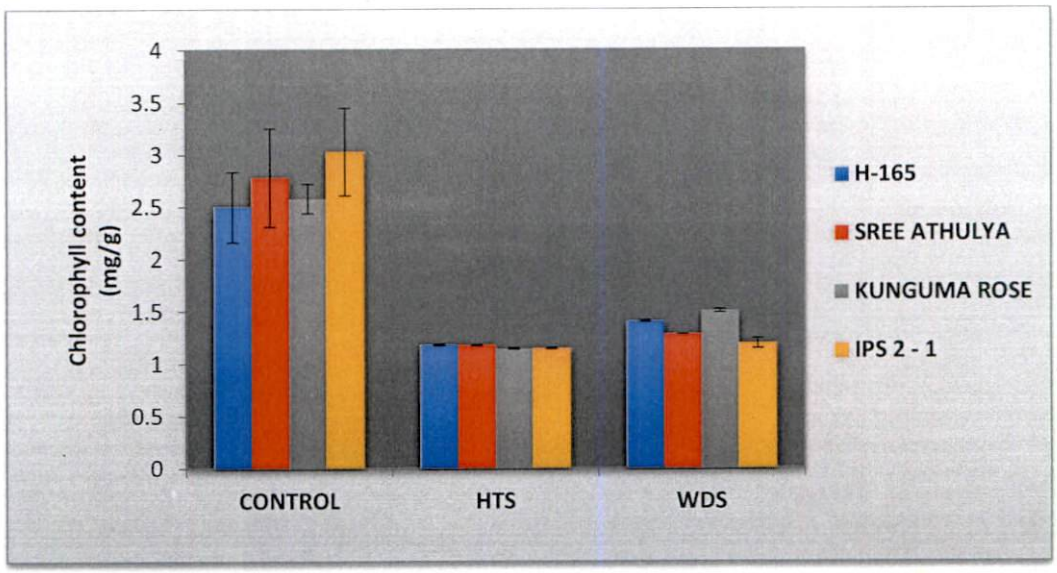


**Fig.14. Changes in total chlorophyll content in the leaves of four varieties/ genotypes of cassava as affected by WDS and high temperature stress conditions**

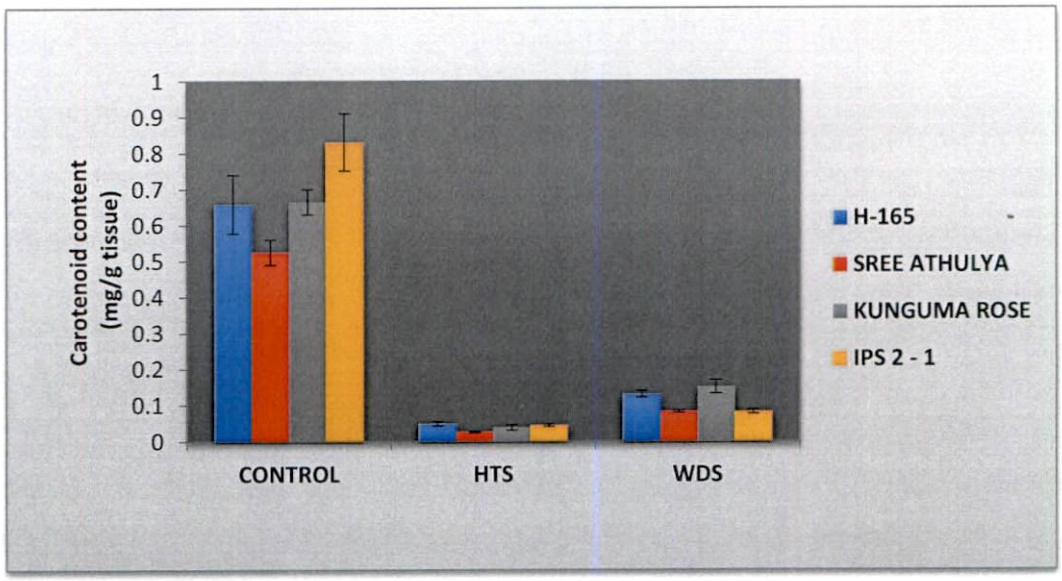


**Fig.15. Changes in carotenoid content in the leaves of four varieties/genotypes of cassava as affected by WDS and high temperature stress conditions.**

content was observed in the variety IPS 2-1 (0.83 ± 0.08 mg/g leaf) under control condition. Again heat stress lead to more degradation of carotenoid than water deficit stress in all varieties (Fig.15). The result of Chl and carotenoid indicated that there was significant difference (P>0.0001) in Chl and carotenoid content between the treatments. Mean Chl and carotenoid were analysed using Tukey contrast indicates decreased amount Chl and carotenoid under stress which was significantly different from that of control condition (3rd, 4thand 5th month) and high temperature stress (4thand 5thmonth) conditions (Fig.16). Proline is an indicator of amino acid and its level increases during the stress conditions. Under control condition the variety H-165 had the maximum proline content (19.01 ± .87 μ moles/g leaf) compared to other three varieties *viz.*, Sree Athulya (13.67 ± .21 μ moles/g leaf), Kunguma rose (12.01 ±.0 μ moles/g leaf) and IPS 2-1 (6.03 ± 0.26 μ moles/g leaf). In plants subjected to WDS conditions, the Variety Sree Athulya had the maximum proline content (24.54 ± .42 μ moles/g leaf), whereas Kunguma rose had the minimum proline content (14.53 ± 0.79 μ moles/g leaf). In plants under high temperature stress H- 165 had lower proline content (21.12 ± .49 μ moles/g leaf) and IPS 2-1 showed minimum proline content (11.25 ± .95 μ moles/g leaf). From the results it was clear that stress condition increased proline content in all the varieties selected for the study compared to control. Result of proline content analysis was cross checked with ANOVA. It was found that there was significant difference (P>0.001) in proline content between the treatment, Proline content varied significantly between the varieties (P>0.001) used for the study.

## 4.7. ENZYMATIC ANTIOXIDANTS

### 4.7.1. Changes in glutathione reductase (GR), (EC 1.6.4.2) activity as affected by WDS and high temperature stress conditions.

Glutathione reductase enzyme was extracted from the leaves of four cassava varieties/genotypes *viz.*, H-165, Sree Athulya, Kunguma rose and IPS -2-1 under control, WDS and high temperature stressed condition. GR activity varied between 1.25 ± 0.3 Eu/mg protein (IPS2-1) and 0.39 ± 0.01 Eu/mg protein (Kunguma rose))

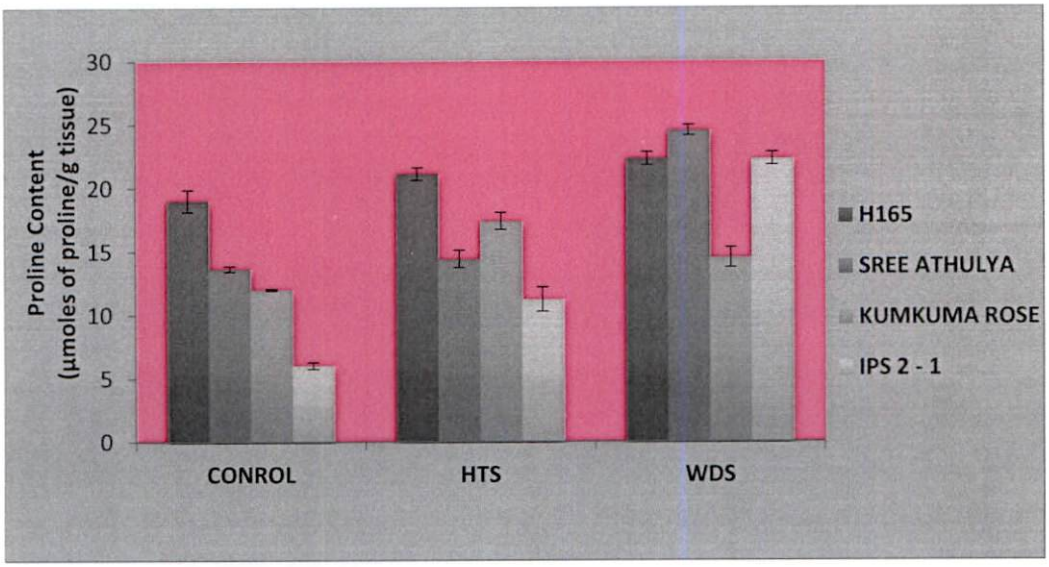


**Fig.16. Changes in proline content of leaves of four cassava varieties/genotypes as affected by WDS and high temperature stress conditions.**

in the leaves of control plants. H-165 (1.09 ± 0.33 Eu/mg protein), Sree Athulya (1.22 ± 0.02 Eu/mg protein) had negligible variation compared to IPS -2-1.GR activity varied between (4.31 ± 0.35 Eu/mg protein) and (1.961 ± 0.051 Eu/mg protein) in the leaves of cassava plant under WDS conditions applied for 3rd, 4thand 5thmonth. Kunguma rose (4.31 ± 0.35 Eu/mg protein) had maximum GR activity whereas Sree Athulya had the minimum GR activity (1.96 ± 0.05 Eu/mg proteins). GR activity varied between (1.25±0.03 Eu/mg protein) and (0.39 ± 0.01 Eu/mg protein) in the leaves of cassava plants under high temperature stress condition applied for 3rd, 4thand 5th month. The variety Sree Athulya had the maximum GR activity (2.92 ±0.03 Eu/mg protein) whereas minimum GR activity was observed in Kunguma rose (1.25 ± 0.05 Eu/mg proteins). It was found that during stress conditions, GR activity increased marginally than the control conditions. On comparing the GR activity between stress conditions it was observed that Kunguma rose had maximum GR activity in WDS condition (4.30 ± 0.35 Eu/mg protein) than in high temperature stress. Similar observation was made in H-165 and IPS 2-1, whereas the variety Sree Athulya had maximum GR activity (2.92 ± 0.03 Eu/mg protein) in WDS condition compared to high temperature stress (Fig17). The result indicated that GR activity varied significantly (P>0.01) between the treatments. The plants also showed significant varietal variation (P>0.05) in GR activity.

### 4.7.2. Changes in superoxide dismutase (SOD), (EC 1.15.1.1) activity in the leaves of four cassava varieties/genotypes as affected by WDS and HTS conditions.

The superoxide dismutase (SOD) activity was assayed from the leaves of four cassava varieties/genotypes grown under control, WDS (3rd, 4th and 5th month) and high temperature stress (4th and 5th month) and the result are presented in Fig.18. Under control conditions variety Kungumarose had the maximum SOD activity (6.48 ± .30 Eu/mg protein) whereas minimum SOD activity was observed in the variety H-165 (3.89 ± .03 Eu/mg protein).The SOD activity significantly varied among the four genotypes of cassava under control condition. Under WDS

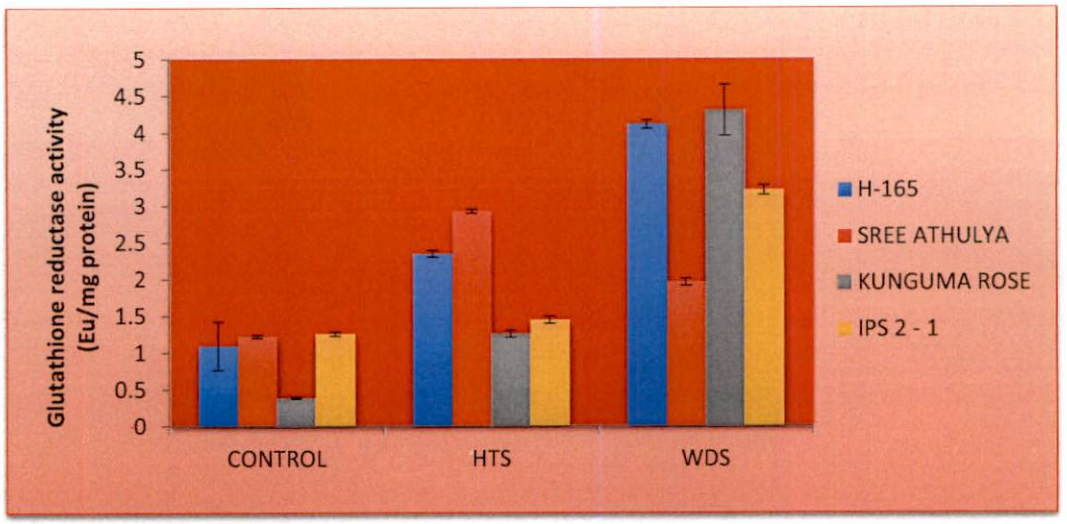


**Fig. 17: Changes in glutathione reductase activity in leaves of four cassava varieties/ genotypes as affected by WDS and high temperature stress conditions.**

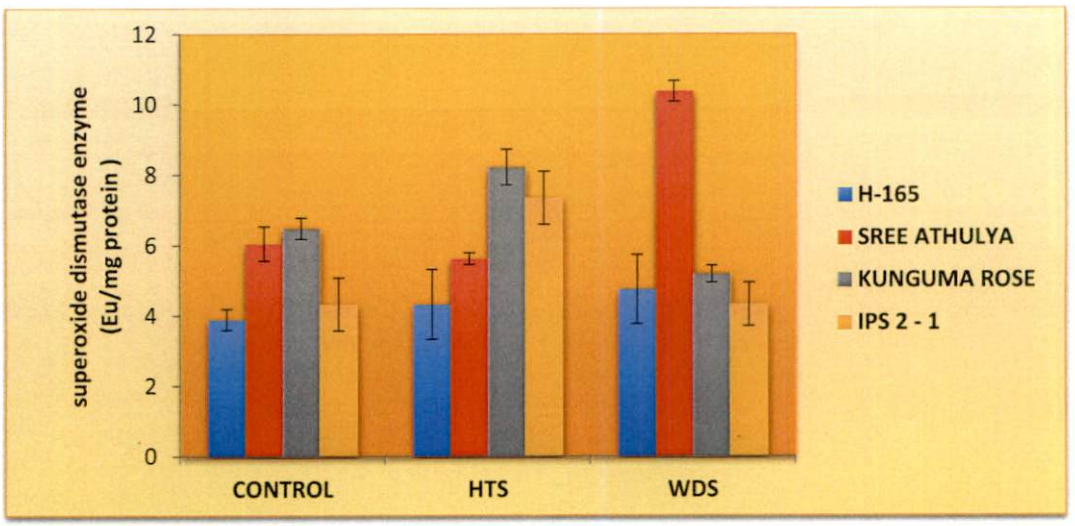


**Fig.18 Changes in Superoxide Dismutase Activity in the leaves of four varieties of cassava as affected by WDS and high temperature stress conditions.**

conditions the varieties Sree Athulya had maximum enzyme activity (10.37 ± .30 Eu/mg protein), whereas IPS 2-1 had the minimum enzyme activity (4.32± .62 Eu/mg protein). Under WDS conditions SOD activity significantly varied among the four varieties /genotypes of cassava H-165(4.75 ± .98 Eu/mg protein), 5.18 ±0.24 Eu/mg protein), Sree Athulya (10.37 ± 0.30 Eu/mg protein) and IPS 2-1 (4.32 ± 0.62 Eu/mg protein). (In plants under high temperature stress, the variety Kunguma rose had highest SOD activity (8.21± 0.50 Eu/mg protein) and the variety H165 had lowest SOD activity (4.32 ± 0.99 Eu/mg protein). In IPS 2-1 and Kunguma rose high SOD activity was shown in heat stressed plants whereas in H-165 and Sree Athulya high SOD activity was recorded under WDS. The result of SOD activity was analyzed using ANOVA and it was found that SOD activity showed significant difference (P>0.0001) between treatments and also SOD activity varied significantly (P>0.05) among four varieties cassava used for the study

**4.7.3. Changes in catalase (CAT), EC 1.1 1.1.6) activity in leaves of four cassava varieties/ genotypes as affected by WDS and high temperature stress conditions.**

The results of CAT activities in the leaves of four varieties of cassava are given in Fig.19. Under control conditions the CAT activity varied between 1.28 ±0.59 Eu/mg protein and 0.47 ±0.10 Eu/mg protein. The maximum catalase activity was observed in the variety Sree Athulya (1.28±0.91Eu/mg protein) and minimum activity was observed in the variety H165 (0.47 ±0.10Eu/mg protein). The CAT activity increased when plants were subjected to WDS (3$^{rd}$, 4$^{th}$and 5$^{th}$month) and high temperature stress conditions (4$^{th}$and 5$^{th}$month). Under WDS conditions CAT activity varied between 1.35 ± 0.05 Eu/mg protein (Sree Athulya)and0.937 ± 0.05Eu/mg protein (H-165).The CAT activity of Sree Athulya (1.35 ± 0.05Eu/mg protein), Kunguma rose (1.33 ± 0.75Eu/mg protein) and IPS 2-1(1.32 ± 0.53 Eu/mg protein) did not differ significantly. Under high temperature stress plants had significantly different CAT activity and it varied between 3.17 ± 0.67 Eu/mg protein (IPS 2-1) and 1.22 ± 0.21 Eu/mg protein (Kunguma rose). The variety IPS 2-1 had maximum enzyme activity in high temperature stress compared to WDS

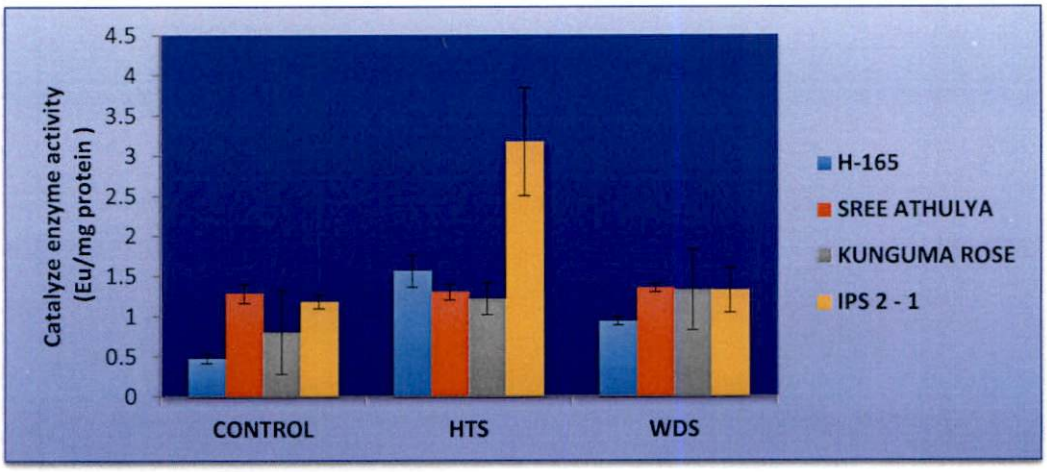


**Fig.19: Changes in Catalase activity in the leaves of four varieties of cassava as affected by WDS and high temperature stress condition.**

(1.32 ±0.53 Eu/mg protein) and control condition (1.18 ± 0.59 Eu/mg protein). From the analysed using ANOVA and it was found that there was no significant difference in CAT activity of plants between treatments.

### 4.7.4. Changes in Ascorbate peroxidase (APX), EC 1.1 1.1.1 1) activity in the leaves of four cassava varieties as affected by WDS and high temperature stress conditions.

The activity of Ascorbate Peroxidase (APX) was assayed from the leaves of cassava plants under control, WDS and high temperature stress conditions (Fig. 20). Among the four varieties of cassava *viz.*, H-165, Sree Athulya, Kunguma rose IPS 2-1 (4.35±0.45Eu/mg protein) and the variety H-165 had the minimum enzyme activity (3.29 ± 0.19 Eu/mg protein).Under WDS condition the variety IPS 2-1(4.70 ± 0.53EU/mg protein) had the maximum APX activity and the variety Sree Athulya had the minimum enzyme activity (3.98 ± 0.46EU/mg protein).Compared to control conditions, all the selected varieties had the enzyme activity more under WDS condition applied on 3$^{rd}$, 4$^{th}$and 5$^{th}$ month. Under high temperature stress conditions for 4$^{th}$and 5$^{th}$ month Sree Athulya had maximum APX activity (6.02 ± 0.56 EU/mg protein) and the variety H-165 had the minimum enzyme activity (5.30 ± 0.35 EU/mg protein). The variety Sree Athulya had maximum enzyme activity compared to the plant under WDS conditions. From the present study, it was concluded that APX enzyme activity is more under high temperature stress conditions compared to WDS and control conditions Results indicated that APX activity was showed significant difference (P>0.001) between treatments. The means of different treatment were compared using Tukey contrast and it was clear that the APX activity of plants under high temperature stress differ significantly (P>0.001) from that of plants under control and WDS condition.

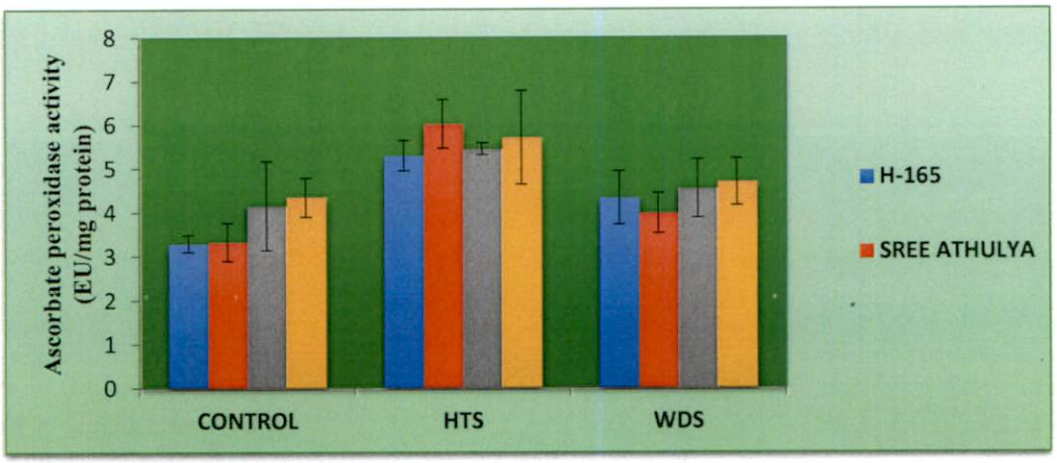


**Fig. 20: Changes in Ascorbate Peroxidase activity in the leaves of four varieties of cassava as affected by WDS and high temperature stress condition.**

**4.7.5. Measument of Ascorbate reductase (AFR), EC 1.6. 5. 4) activity in the leaves of four cassava varieties as affected by WDS and HTS conditions.**

The activity of ascorbate free radical reductase was assayed by the method of (Hossain *et al.*, 1984). The leaf tissue of cassava of 1 g was powdered in liquid N, using a pestle and mortar and then homogenized at 0 °C for 4 min in ascorbate free radical reductase assay buffer 10 ml. Extracts were centrifuged at full speed in a bench-top centrifuge for 30 min at 0oC, the supernatants were filtered through Miracloth, 15-pLaliquots were assayed in a final reaction volume of 1mL. To determine the AFR reductase activity spectrophotometrically the decrease in A340 due to NADH oxidation was monitored. However, there was no change in absorbance and therefore, the enzyme activity could not be measured.

## 4.8. NON ENZYMATIC ANTIOXIDANTS

**4.8.1. Ascorbic acid content in leaves as affected by WDS and HTS conditions.**

Ascorbic acid (AA) content was estimated by volumetric analysis. In the presence of AA the dye (Dichlorophenol indophenol) will develop a pink colour. From the study it was observed that extract of all the four genotypes/varieties of cassava *viz.*, H-165, Sree Athulya, Kunguma Rose and IPS -2-1 leaves developed faint pink colour in control and both stress condition. From the observations it was clear that the AA content in cassava leaves was negligible or below detectable amount under both control and stress conditions.

**4.8.2. Glutathione content in leaves of cassava plant as affected by WDS and HTS conditions.**

To determine reduced glutathione the method of Moron et al. (1979) was followed. Reduced glutathione on reaction with DTNB (5, 5'-dithiobis nitro benzoic acid) produces a yellow coloured product that absorbs at 412nm. The homogenate was prepared with 0.5 g of the sample with 2.5ml of 5% TCA. The precipitated protein was centrifuged at 1000rpm for 10 minutes. The supernatant (0.1ml) was used for the estimation of GSH. The supernatant (0.1ml) was made up

to 1.0 ml with 0.2 M sodium phosphate buffer (pH 8.0). Standard GSH corresponding to concentrations ranging between 2 and 10 M were also prepared. Two ml of freshly prepared DTNB solution was added and the intensity of the yellow colour developed was measured in a spectrophotometer at 412 nm after 10 minutes. There was no change in absorbance the glutathione content could 'not be determined. Other method tried also did not give results.

# DISCUSSION

## 5. DISCUSSION

Basic abiotic stresses such as drought, salinity, temperature, and chemical pollutants are simultaneously acting on the plants causing cell injury and producing secondary stresses such as osmotic and oxidative ones (Wang *et al.*, 2003). Plants could not change their sites to avoid such stresses, but have different ways and morphological adaptations to tolerate these stresses. Defense at the molecular level are very important for the survival and growth of plants. Plants show a series of molecular responses to these stresses. This effect of heat stress reflects the temperature dependence of Michaelis–Menton constant (Km) of every enzyme participating in the process (Mitra and Bhatia, 2008). Plants must cope with heat stress for survival, so they developed different mechanisms including the maintenance of cell membrane stability, capturing the reactive oxygen species (ROS), synthesis of antioxidants, accumulation and osmoregulation of osmoticum, induction of some kinases that respond to stress, Ca-dependent kinase proteins, and enhancing the transcription and signal transfer of chaperones (Wahid *et al.*, 2007).

This study was carried out to find out the antioxiative enzyme activity and protein profiling of different varieties of cassava (H165, Sree Athulya, Kumkuma Rose and IPS) under different stress treatments (heat and drought) and identify physiological parameter as a marker for identifying / developing genotypes / varieties for abiotic stress tolerance such as drought and high temperature. Plant response studies to oxidative stress and protein profiling provide information on the physiological basis of mechanism by which they adapt to the ever changing environment. Heat stress, water stress and salinity are the most important environmental factors that regulate plant growth and development and contribute to an average yield loss of more than 50 % for most major crop plants (Bray *et al.*, 2000). Plant can respond and adapt to these stress to certain limit by altering their cellular metabolism and evoking various defense mechanisms. Therefore, understanding the mechanism underlying plant abiotic stress response and generation of stress tolerance plants has received much attention in recent years. Cassava, being a tropical crop which is highly prone to heat stress, water stress and

other oxidative stress assessing these oxidative stress and changes in the protein profile in cassava under abiotic stress in contrasting genotypes (short and long duration varieties) of cassava gain importance. Identification of marker for physiological parameter can help to identifying / develop varieties for tolerance to abiotic stress condition.

## 5.1 EFFECT OF ABIOTIC STRESS FACTOR ON GROWTH PARAMETER

Abiotic stress *viz.*, WDS and HTS were imposed on four different varieties of cassava plant at 3rd, 4th, 5th month and 4th, 5th month after planting, respectively for a period of 10 days. Observations on plant height (cm), leaf number and leaf retention (%) were recorded for 180 days with the interval 60 days. The influence of WDS and HTS on these parameter is discussed in following section.

Plant height of four different cassava varieties were not significantly affected by heat and water stress. However, an increasing trend in plant height were observed under both stress and control conditions.

The leaf production of four different cassava varieties were affected significantly by both heat and water stress, and the same is maximum in the case of water stress. In the case of leaf production, among four varieties H-165 showed higher tolerance to both the stress condition. In a similar study Ravi and Indira, (1995), reported that sweet potato leaves of permanently wilt when $WP_L$ decreases to -1.3 MPa and at $WP_L$ between -1.6 to -2.0 MPa the leaves senesence. The leaf retention of four varieties/ genotypes were differentially affected by both heat and water stress and leaf retention is maximum under WDS. A decreasing trend in leaf retention was observed in stress condition.

## 5.2 EFFECT OF ABIOTIC STRESS FACTOR ON YIELD PARAMETER

Observations on tuber number, tuber length (cm), tuber width (cm) and mean tuber weight (Kg/plant) were recorded in experimental material.The influence of WDS and HTS on these parameters are discussed in the following section. There is only negligible difference in yield parameters between stress and control condition. However, significant difference in yield parameters were observed among the varieties. Among the different cassava varieties, Sree Athulya showed

higher yield parameters except tuber width under stress condition. The slight difference in yield parameter between the control and stress conditions may be due to the increased oxidative stress which might have reduced the assimilatory activities in plant resulting in reduced yield characteristics. Finding of present study was in agreement with Ramanujam (1990); Ravi and George, (2003). It was also reported that the induction and synthesis of heat-shock proteins due to high temperature are common phenomena in all living organisms (Parsell and Lindquist, 1993; Vierling, 1991; Gupta *et al.*, 2010). The synthesis of this proteins is costly energy wise that is reflected on the yield of the organism Mohamed (2010).

## 5.3 EFFECT OF ABIOTIC STRESS FACTOR ON TOTAL PROTEIN CONTENT AND PROTEIN PROFILING

Total soluble protein of leaf and tuber was found to be lower in plants exposed to stress conditions. This is in favour of earlier reports by Parthasarathi (2004) and Agami (2013). The decrease in protein and amino acid content was due to impaired protein metabolism and synthesis. The protein fraction were converted into compatible cytoplasmic osmotic agents (Walter, 1995). Total soluble protein in the tuber forming roots showed an increasing trend in HTS condition. This is due to the low water content experienced by the plants. Similar finding was reported by Kosakivska *et al.*, 2008. Under the HTS conditions of short-term heat stress condition, observed a 2-fold increase of protein content in seedlings of *Rumex patienta L. x R. tianshanicus* was observed.

Heat stress as well as other stresses can trigger some mechanisms of defense such as gene expression that was not expressed under normal conditions (Morimoto, 1993). The sudden changes in genotypic expression resulting in an increase in the synthesis of protein groups. These groups are called "heat-shock proteins' (Hsps), "Stress-induced proteins" or "Stress proteins" (Lindquist and Crig, 1988; Gupta *et al.*, 2010). Almost all kinds of stresses induce gene expression and synthesis of heat-shock proteins in cells that are subjected to stress (Feder, 2006). Single dimensional protein profile was done in the present study for leaves and tubers. It was found that brighter bands were appeared at for protein from

control condition and under WDS and HTS condition failed to show such a banding pattern. In the case of tuber forming root brighter bands appeared in the HTS condition compared to control and WDS condition. So one dimensional protein profiling could not reveal the HSP in cassava.

## 5.4 EFFECT OF ABIOTIC STRESS FACTOR ON PHYSIOLOGICAL PARAMETER

The effect of abiotic stress factors on various physiological parameter like relative water content, chlorophyll and carotenoid content was studied. Among the given treatments with increase in WDS and HTS the chlorophyll and carotenoid content were found to be reduced in all the four varieties/ genotypes. When compared to HTS and WDS chlorophyll and carotenoid content was found to be lower in plants exposed to HTS. The results were in conformity with the finding of Gargi (2014) and Parthasarathi (2004). According to Walter (1995), the reduction of the chlorophyll might be due to the destruction of thylakoids and the chloroplast swelling and breaking down under stress condition. The generation of ROS induced by WDS also have resulted in the damage of membrane and cause photooxidation this can be the reason for reduced chlorophyll under stress conditions (Lambers *et al.*, 1998). It was also reported that a significant reduction in chlorophyll b was observed due to WDS in *Grevellia robusta* (Nautiyal *et al.*, 1993) and a similar result was reported in tea (Rajasekar *et al.*, 1991). The total chlorophyll content of leaves decreases in sweet potato plants subjected to WDS (Indira and Kabeerathumma, 1988; 1990; Chowdhury and Ravi, 1987; 1988).

Relative water content of plant content varied under both WDS and HTS condition. A Higher RWC was observed in plants exposed to HTS condition compared to WDS condition. Rahimi *et al.* (2010). Reported that RWC is more sensitive to drought stress than heat stress which is similar to present finding. Under WDS conditions, water potential ($WP_L$) or relative water content (RWC) of sweet potato leaves decreases (Indira and Kabeerathumma, 1988; Ravi and Indira, 1995; Chowdhury and Naskar, 1993). Cassava being a C3 plant needs appropriate strategies for efficient water management under stress condition. The plant may

able to tolerate drought with available better moisture with the deeper root system and increased dry matter production.

## 5.5 EFFECT OF ABIOTIC STRESS FACTOR ON BIOCHEMICAL PARAMETER

Proline status of plants under stress condition has followed an increasing trend suggesting that proline can be considered as an indicator of stress for cassava. This was in arrangement reported by with the earlier findings Agami, (2013) and Slabbert and Kruger (2014). Severe abiotic stress can also be the reason for major increasing trend of proline content in the plant.

## 5.6 EFFECT OF ABIOTIC STRESS FACTORS ON ENZYMATIC ANTIOXIDANTS

The effect abiotic stress factors on various enzymatic antioxidants such as glutathione reductase (GR), Superoxide dismutase (SOD), Catalase (CAT) and ascorbate peroxidase (APX) were studied.

Among the four different cassava varieties/genotypes, s Kunuguma rose showed maximum GR activity. Among the treatments GR activity showed an increasing trend in WDS compared to HTS and stress free conditions. The plant try to maintain the equilibrium of ROS generation to defend the oxidative damage during stress condition. Similar results were reported by (May and Leaver, 1993; Madamanchi *et al.*, 1994) that while the imposing the oxidative stress, the existing pool of reduced glutathione (GSH) is converted to oxidized glutathione (GSSG) and glutathione biosynthesis is stimulated.

Catalase activity of four varieties of cassava plant varied among different stress condition (WDS and HTS) compared to control. The catalase activity was lower under control condition. Enhanced production of ROS might not have been occurred in the plant to induce an intensified CAT activity. The CAT activity increased under WDS. This is in agreement with the earlier finding of Hertwig *et al.* (1992). In general, an increase in catalase activity was observed with an

increased stress level with the purpose of hunting the free radical produced during the stress.

Abiotic stress, particularly HTS and WDS induces oxidative injury and alter the activity of the SOD, CAT and GR in many plant species (Foyer et al.1994; Gong *et al.*, 1997). In the present study SOD activity showed an increasing trend in stress conditions. This finding was in agreement with Tsang *et al.* (1991). There was an increase in SOD in tobacco leaves during heat shock due to the accumulation of CU/Zn SOD mRNA.

The Ascorbate Peroxidase (APX) activities were found to have significant variation with the given treatment (WDS and HTS). Plants under stress treatment might have experienced considerable level of oxidative stress. This might be the reason why the ascorbate peroxidase activity increased under stress conditions. Findins of the present study was in agreement with the finding of Park *et al.*, (2004). Sweet potato cAPX gene was highly induced in leaves after exposure to high temperature. In cucumber plants subjected to heat treatment, the activities of cAPX, sAPX and mAPX increased after an initial slight decline. The expression of sAPX followed a similar pattern (Song *et al.*, 2005).

## 5.7 CONCLUCSION

Under HTS condition GR activity increased in H-165 and Sree Athulya all the four varieties showed increase GR activity under WDS. Kunguma rose showed maximum SOD activity in HTS whereas Sree Athulya had highest SOD activity in WDS. APX activity was high in all the varieties under HTS. I concluded that GR can be used as a biochemical marker for developing WDS tolerant varieties as it has showed consistent changes under both WDS and HTS. APX activity can be used as a marker for identifying HTS tolerant varieties. Detailed molecular biochemical and physiological information is necessary for number of varieties for using approximate marker enzyme to the suitable abiotic stress tolerant varieties.

# SUMMARY

## 6. SUMMARY

A study on "Oxidative stress and protein profiling in cassava (*Manihot esculenta* CRANTZ) under abiotic stress" (drought stress and heat stress) was conducted at CTCRI (Central Tuber Crops Research Institute) Sreekariyam, Thiruvananthapuram and Department of Biotechnology Collage of Agriculture, Vellayani, during 2014- 2015.

The present study was focused on four varieties/genotypes of cassava *viz.*, H165, Kunguma rose, Sree Athulya and IPS 2-1 in a field trail with three replications, each replication with eight plants. There were three treatments *viz.*, control, (water deficit stress (WDS) condition, and High temperature stress conditions. In WDS free conditions, plants were well irrigated and WDS for 10 days was imposed during $3^{rd}/4^{th}/5^{th}$ month after planting by withholding irrigation. For high temperature stress conditions, plants were incubated under the light transparent poly chambers for 10 days during $4^{th}/5^{th}$ month. Plants were exposed to high temperature stress at 40 °C ± 2 during day time for six hours between 9.0 AM to 4.0 PM daily for a period of 10 days.

Growth parameters such as leaf production, leaf retention, plant height were recorded for 180 days with an interval of 60 day and yield components such as tuber number, mean tuber weight, tuber length and tuber width were recorded.

Total protein extracted from the leaves, tubers and tuber forming of four varieties of cassava plants were estimated by Bradford method and protein profiling was done by PAGE. Chlorophyll was estimated from four varieties/genotypes by DMSO method. Proline content was estimated by spetrophtometrically using standard procedure. Activity of enzymatic antioxidants like superoxide dismutase, catalase, Ascorbate peroxidise, glutathione reductase and ascorbate reductase were assayed from the leaves of four varieties of cassava by standard procedures. Non enzymatic antioxidant like ascorbate, glutathione was also evaluated.

Growth parameters such as plant height, leaf number and leaf retention showed gradual increase in each interval of time. From the study it was concluded that there is no change in the plant height in stress condition compared to control. However reduction in leaf number and leaf retention were observed in stress

treatment condition compared to stress free condition. Yield parameter such as tuber number showed negligible variation in treatmented plants. The variety Sree Athulya had the maximum tuber number in both stress condition and control and minimum tuber number was observed in the variety Kunguma rose. The maximum tuber length was observed in the variety Sree Athulya (WDS and high temperature stress) and minimum tuber length was recorded in the variety H-165. The maximum mean tuber weight was observed in Sree Athulya under control and treatment condition and minimum mean tuber weight was observed in IPS 2-1. The maximum tuber width was found in the variety tuber H-165 under control and treatment condition and minimum tuber width was observed in the variety IPS 2-1.

From the study it was observed that all the four varieties/genotype of cassava *viz.*, H165, Sree Athulya, Kunguma Rose and IPS 2-1 showed variation in their total leaf protein content when plants were subjected to stress conditions. Three varieties namely H165, Sree Athulya, Kunguma Rose had the maximum leaf protein content in the control condition whereas IPS 2-1 had its maximum leaf protein content in WDS condition. In the case of total tuber protein content all those varieties showed maximum protein content in the control condition compared to WDS and high temperature stress condition. Total tuber forming root content increased at high temperature stress compared to control and WDS condition. Protein profiling had revealed in the case of leaf and tuber, clearer band were observed in control condition indicating high protein content in the control condition. Using WDS and HTS no clear cut protein bands were observed, for tuber forming root clear bands were obtained in high temperature stress condition. Physiological parameters like relative water content (RWC), chlorophyll and carotenoid content were maximum at stress free condition and gradual reduction were observed in treatmental conditions.

Enzymatic antioxidants (GR, SOD, CAT, APX and AR) showed maximum activity in treatment condition. The present study revealed that the AA content in of cassava leaves was negligible or below detectable amount under both stress and control conditions in all the verities.

Under HTS condition, GR activity has increased in H-165 and Sree Athulya and all the four varieties showed increase GR activity under WDS. Kunguma rose showed maximum SOD activity in HTS whereas Sree Athulya had highest SOD activity in WDS. APX activity was high in all the varieties under HTS. It is inferred that GR can be used as a biochemical marker for developing WDS tolerant varieties as it showed consistent changes under both WDS and HTS. APX activity can be used as a marker for identifying HTS tolerant varieties. Detailed molecular biochemical and physiological information will be necessary for more varieties for getting appropriate activities as the marker screen abiotic stress tolerant varieties.

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# APPENDICES

## APPENDIX - I

### Reagents for total protein extraction and quantification

**a. Bradford's reagent**

Dissolve 100 mg Coomassie Brilliant Blue G-250 in 50 ml 95 % ethanol

100 ml 85% (w/v) phosphoric acid

Whatman filter paper

**b. Phosphate buffer**

PH - 7.0

Volume of 1M $K_2HPO_4$ - 61.5 ml

Volume of 1M $KH_2PO_4$ – 38.5 ml

Make up to 100 ml

## Appendix-II

## Reagents for protein profiling using SDS PAGE

**a. Resolving gel (12%)**

Distilled water - 6.7 ml

1.5 M Tris-HCL - 5.0ml (PH 8.8)

10% SDS - 0.2ml

Acrylamide Stock - 8.0 ml

Freshly prepared ammonium persulphate - 0.1 ml

TEMED - 0.01ml

**b. Stacking Gel (4%)**

Distilled water - 6.1ml

1.5M Tris-HCL 2.5 ml (PH 8.8)

10% SDS - 0.1 ml

Acrylamide Stock - 1.3 ml

Freshly prepared ammonium persulphate - 0.05 ml

TEMED - 0.01ml

**c. Acrylamide stock solution (30%)**

Acrylamide - 30g

Bisacrylamide - 0.8g

100 ml of distilled water

**d. Protein Extraction buffer**

Tris - 50mM

Sodium chloride - 50mM

Ethylene diamine tetra acetic acid - 2mm

P-mercaptoethanol - 5mM

Phenylmethylsulphonylfluoride - 1mM

Polyvinylpyrrolidone - 0.5% (pH 8.0)

**e. SDS solution (10%)**

SDS - 10 g

100 ml of distilled water

**f. Electrophoresis buffer (pH.8.3)**

Tris (0.025M) - 3.02 g

Glycine (97M) - 14.428g

SDS (0.1%) - 1.0 g

Distilled water - 1.0 litre

**g. Coomassie blue staining solution**

CBB R-250 - 0.25 g

Methanol: water (1: l) - 90 ml

Glacial acetic acid - 10 ml

**h. Destaining solution**

Methanol: water (1:1) - 90 ml

Glacial acetic acid - 10 ml

## Appendix – III

## Reagents for estimation of proline

**a. Aqueous sulphosalicylic acid (3%)**

Sulphosalicylic acid - 3 g

Distilled water - 100 ml

**b. Acid Ninhydrin**

Ninhydrin - 1.25 g dissolved in a warm mixture of 30 ml of glacial acetic acid. Phosphoric acid - 20 ml (6 M)

Stored 24 hours at 4°C

## Appendix – IV

## Reagents for glutathione reductase assay

**a. Glutathione Reductase Extraction buffer**

Phosphate buffer - 50 mM (pH7.0)

EDTA - 1mM

TritonX-100 - 0 05%

PVP-40 - 2%

Ascorbic acid - 1mM

**b. Glutathione reductase assay mixture**

Tris-buffer - 0.1 M (pH 7.8)

EDTA – 2 mM

NADPH – 50 mM

GSSG - 0.5 mM

Enzyme extract – 20 µl

## Appendix – V

## Reagents for Superoxide dismutase assay

**a. SOD extraction buffer**

Tris-HC1 - 20 mM (pH 7.5)

$MgCl_2$ - 5mM

NaC1 - 10mM

**b. SOD Assay Mixture**

Phosphate buffer - 50mM (pH 7.8)

EDTA - 0.1mM

Methionine - 13mM

Nitroblue tetrazolium (NTB) - 75µM

Riboflavin - 2 µM

Supernatant - 100 µl

## Appendix – VI

## Reagent for catalase assay

**a. Catalase Extraction Mixture**

Phosphate buffers - 50 mM (pH 7.0)

$H_2O_2$ – 3ml (2mM)

## Appendix – VII

## Reagents for ascorbate peroxidase assay

a. **Ascorbate Peroxidase Extraction Buffer**

Phosphate buffer - 1mM (pH 7.0)

Sorbitol - 70%

EDTA - 1mM

PMSF - 0.1%

b. **Ascorbate peroxidase assay mixture**

Ascorbate - 0.25 M

$H_2O_2$ - 1 mM

Phosphate buffer – 50 mM (pH 7.0)

Enzyme extract - 37.5 µl

## Appendix VIII

## Reagents for Ascorbate free radical reductase assay

**a. Ascorbate free radical reductase assay buffer**

0.2 M potassium phosphate

PH 7.8, 1 mM EDTA

5 mM MgCl, 0.1% (w/v) BSA

10 mM 2-mercaptoethanol

0.005% (v/v) Triton X-100

# ABSTRACT

# OXIDATIVE STRESS AND PROTEIN PROFILING IN CASSAVA (*Manihot esculenta* CRANTZ) UNDER ABIOTIC STRESSES

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## Abstract of the thesis

**Submitted in partial fulfilment of the requirement for the degree of**

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## ABSTRACT

The present study an "Oxidative stress and protein profiling in cassava (*Manihot esculenta* CRANTZ) under abiotic stresses" was conducted during the period 2014- 2015 in the Division of Crop Production, CTCRI (Central Tuber Crop Research Institute) Thiruvananthapuram. The objective of the study was to elict information on the antioxidative enzyme activity, protein profiling, growth parameter and yield parameter of four varieties / genotypes of cassava under irrigated, high temperatures stress (40 °C ±2 °C) and water deficit stress (WDS) conditions and identify marker physiological parameter which can be used for identifying / developing cassava varieties for tolerance to abiotic stress conditions. The study indicated that the plant height of four different cassava varieties were not significantly affected by heat and WDS. The leaf production of four different cassava varieties were significantly affected by both heat and water stress, and the same was maximum in the case of water stress. The leaf retention of four varieties / genotypes were differentially affected by both heat and water stress. There was negligible difference in yield parameters between stress and control condition. However, there was significant difference was yield observed for parameters among the four varieties. The effect of abiotic stress on various physiological parameters like relative water content, chlorophyll and carotenoid content showed significant difference in control plants. Among the given WDS and HTS treatments, the chlorophyll and carotenoid content decreased in all the four varieties. Chlorophyll and carotenoid content were significantly reduced in plants exposed to HTS.

Relative water content of leaves varied under both WDS and HTS conditions. A higher RWC was observed in plants exposed to HTS condition compared to WDS condition. Total soluble protein content of leaf and tuber was found to be lower in plants exposed to stress conditions. Total soluble protein in tuber forming roots had increased under HTS conditions. Proline status of plants under stress conditions followed an increasing trend compared to stress conditions. The effect of abiotic stress on various enzymatic antioxidants such as Glutathione Reductase (GR), Superoxide Dusmutase (SOD), Catalase (CAT) and Ascorbate

Peroxidase (APX) were studied. Among the four different cassava varieties. Kunguma rose showed maximum GR activity showed an increasing trend in WDS compared to HTS and stress free conditions. Catalase activity of four varieties of cassava plant varied among different stress condition (WDS and HTS) compared to control. The catalase activity was significantly reduced due to stress conditions and. SOD activity increased under stress conditions. The ascorbate peroxidase (APX) activities had significant variation in WDS and HTS compared to control. The AA content in cassava leaves was negligible or below detectable amount under both control and stress conditions.

Under HTS condition GR activity increased in H-165 and Sree Athulya alone whereas all the four varieties showed increase GR activity under WDS. Kunguma rose showed maximum SOD activity in HTS whereas Sree Athulya had highest SOD activity in WDS. APX activity was high in all the varieties under HTS. From the present study it is inferred that GR can be used as a biochemical marker for developing WDS tolerant varieties as it showed consistent changes under both WDS and HTS. APX activity can be used as a marker for identifying HTS tolerant varieties. Detailed molecular biochemical and physiological study will be necessary using different varieties for getting appropriate activities as the marker to screen abiotic stress tolerant varieties.