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Metabolic Response of Maize (Zea mays L.) Seedlings to both Drought and Heat Stress

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ACKNOWLEDGEMENTS

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ABSTRACT

The development of resistant maize (Zea mays) varieties against drought and heat is on the rise as these abiotic stress factors affect maize production. Plants including maize respond by altering their metabolism to produce LEA proteins (dehydrins), antioxidants, compatible solutes and some secondary metabolites as strategies to maintain plant metabolism. This study looks at identifying dehydrins and metabolites in maize leaves subjected to both drought and heat stress.

Two genotypes namely; Obaatampa a tropical variety and Sun Sweet, a temperate variety were subjected to drought, heat, combined stress (heat x drought) and a control condition over a period of 7 days in a 2x2 factorial design. From the same plant, samples were taken for metabolite profiling and protein extraction. GC-MS metabolite profiling detected 312 metabolites including amino acids and oligopeptides, carbohydrates, organic acids and terpenoids among others. Aconitic acid and asparagine were up regulated in all 3 conditions. Proline was also up regulated under heat and combined stress condition but drought observed a low regulation of proline. Drought generally observed a low regulation of metabolites; however, maleic acid was up-regulated under drought stress. Raffinose among other carbohydrates was up regulated under all conditions implying a possible osmotic function. Some terpenoids (B-amyrin) were also up regulated implying some defensive mechanisms are being employed against pathogens.

ANOVA-PCA and heatmap gave clear indications of the effect of heat on metabolite regulation. ANOVA-PCA and the heatmap showed that temperature stress regulated majority of these metabolites followed by combined stress then drought as this was evident from the fold-change values. PCA and Anova (Appendix 3) for metabolites show that there was significant ($P<0.05$) difference between specific metabolites for the two (2) genotypes (ANOVA- Appendix 3). However, the two genotypes reacted in a similar way though they observed some difference with specific metabolites. Furthermore, some dehydrins (DHNs) were also identified in the protein extraction. These identified proteins include 20, 23, 50 and 100 kDa weight dehydrin. These DHNs were identified in all stress conditions and in all genotypes. However, problems with proteins extraction prevented a complete picture of dehydrin responses to stress, though plants express unique proteins in all variety of stress conditions. In conclusion, heat stress has a high metabolite effect on plants than drought and combined stress. Furthermore, genotypes respond in

a similar way to different stress conditions and produce DHNs, as a tolerance mechanism against stress conditions.

1. INTRODUCTION

1.1 Stress and its Effects on Maize (*Zea mays* L.)

Maize or Corn (*Zea mays* L.) is the third most important cereal crop in the world and an essential staple food for both Sub-Saharan Africa and Latin America and thus plays a significant role in world food security (Bruce *et al*., 2002). Its production and consumption was about 989,608 and 953,133 thousand metric tons in 2013 and 2014, respectively (Cong *et al*., 2015). Maize production and distribution is highly rainfall dependent and yet current global climatic conditions reveal irregular rainfall patterns which result in stress conditions affecting its production (Bruce *et al*., 2002). Climate change which leads to stress conditions such us drought and heat stress causes a drastic reduction in the yield of maize, not only in Africa but also in other maize growing regions. This change can cause a major transformation in the agricultural system of maize cultivation (Jones & Thornton, 2003).

Stress as a general term has been defined in different forms. Nilsen & Orcutt (1996) define stress in plant physiological term "as the amount of environmental pressure for change that is placed on an organism's physiology". They also agree to the definition that "stress acts as a change in physiology that occurs when species are exposed to extraordinarily unfavourable conditions that need not represent a threat to life but will induce an alarm response". Lichtenthaler (1996) also defines stress as 'any unfavourable condition or substance that affects or blocks a plant's metabolism, growth or development'. Therefore, any change in the plants equilibrium condition induces stress in plants. Stress can be either abiotic (resulting from non-living factors such as drought, salinity, extreme temperature, metallic pollutants among others) or biotic (resulting from living organisms such as fungi and insects in the ecosystem). Whiles it is difficult to estimate the effects of stress, it is still evident that stress factors cause significant loss in plants and these include; decline in yields of food crops, leaf senescence, mechanical damage and excessive transpiration, imbalance in plants leading to deficiency or malfunction (Kranner, *et al*., 2010; Cramer, *et al*., 2011). Furthermore, stress induced water deficit in plants leads to growth limitation as a reduction in water uptake into the expanding cells of plants (Cramer, *et al*., 2011).

In this study, consideration will be given to abiotic factors but specifically drought and heat stress in plants. This is on the background that stress conditions that affect agriculture are largely

dominated by drought and temperature (cold or heat). Cramer *et al*., 2011, reveal that drought and temperature stress conditions affect 64% and 57% respectively of global land area and are thus relevant for consideration.

1.2 Drought Stress and its Effects on Plants

Water serves as a limiting factor to plant processes like germination, growth, seed development and storage of seeds. The insufficient amount of water in plants induces drought (Kalemba E. & Pukacka S., 2007). According to Farooq *et al.*, (2012), drought occurs as a result of reduced precipitation coupled with a high level of evapotranspiration. Drought undoubtedly is an important stress condition in maize production, resulting in a loss in maize yield (Bruce *et al*., 2002; Ashraf, 2010). Drought stress affects numerous growth and physiological processes in plants with a reduction in plant enlargement as a result of turgor loss (Farooq *et al*., 2012) as one of the main mechanisms. About 30 - 50% of maize during production, processing and postharvest processes globally (Aulakh, & Regmi, 2013) is lost due to drought and this threatens food security (Clare, 2012). Although many efforts have been carried out to improve or alleviate the problem of drought stress in maize, much more needs to be carried out.

Farooq *et al*. (2012) define growth as "an irreversible increase in volume, size or weight, which include the phases of cell elongation". But under conditions of drought, growth is limited in all forms. A decrease in plant water activates processes which leads to decreased vigour, viability loss and cell dehydration. Furthermore, under drought conditions an impaired enzyme activity leads to a number of events including changes in solute metabolism, chaperone synthesis and protein synthesis among other response mechanisms (Figure 1) (Hirt & Shinozaki, 2004).
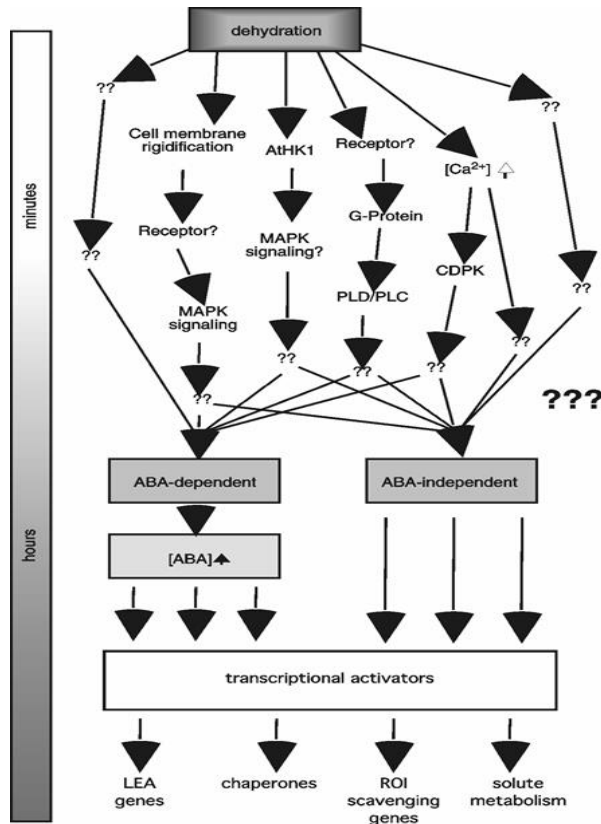
Figure 1.1. Dehydration stress induces a complex molecular response in plants. Upon dehydration plants induce a cascade of events which are complex but effective in combating against water stress. ABA among other pathways are stimulated and this activates transcription factors which leads to the synthesis of proteins, compatible solutes (carbohydrates, amino acids), chaperones, and ROI scavenging genes among other response mechanisms (Hirt & Shinozaki, 2004).

Loss of turgor in the plant causes a decrease in the energy supply of the plant. Major plant organs experience a reduction in dry matter accumulation. For example, drought reduces shoot, fresh and dry weight of marigold flowers. In Asian red sage, roots also undergo a reduction in dry weight. Drought also reduces the leaf area thus reducing photosynthesis and transpiration in plants. This can lead to a reduction in the number of leaves (Farooq *et al*., 2012, Nilsen & Orcutt, 1996).

1.3 Heat as a Stress Condition and its Effects on Plants

Heat is another stress condition that affects plant growth and development. Susceptibility to heat varies from one developmental stage/phase to another (vegetative and reproductive). It is also dependent on the species and genotype with a great deal of variation in critical temperatures and plant responses (Bita & Gerats, 2013). Heat in itself is a profitable and natural resource but becomes dangerous and poses harmful effects when it exceeds it limits for growth. Ambient conditions recommended for plant growth vary from one plant to another. Therefore, in defining heat stress or heat shock, this is simply defined as an increase in temperature above a threshold capable of triggering a negative response. However, the standard for plant growth should be about 25˚C. Therefore, heat stress is considered to be about 10-15˚C increase above the ambient condition (Wahid & Close, 2007; Wahid *et al*., 2007). This implies that heat stress in plants ranges between 35-45˚C (Wahid & Close, 2007; Wahid *et al*., 2007). This change in the plant causes an increase in respiration leading to exhaustion of more resources by the plant. Critical consideration of heat stress can be assessed as a function of intensity (temperature in degrees), duration and the rate of temperature increase (Wahid & Close, 2007; Wahid *et al*., 2007). The degree of heat in a climatic zone is dependent on probability and period of high temperature exposure (occurrence) during the hours of the day or the night. Heat tolerance is defined as the plant's ability to survive, performing its physiological processes under high temperatures which are a long existing problem that poses serious threats to crop production (Wahid *et al*., 2007). Physiologically high temperatures gradually or spontaneously lead to cellular collapse and cell injury. This effect is thus as a result of the degree and period of exposure of these plants to heat. The effect of high temperatures can be assessed based on their direct and indirect impacts on the plants. Direct impacts of high temperature include increase in fluidity of membrane lipids and protein denaturation and aggregation. Indirect impact includes enzyme inactivation, protein denaturation, inhibition of protein synthesis and loss of membrane integrity. Heat stress has the capacity to change the overall physiology or cellular arrangement (organization) as it causes splitting of microtubules and spindle elongation among other effects. Finally, these injuries eventually lead to starvation, growth inhibition and production of harmful compounds within the plants (Wahid *et al*., 2007).

Plants have different threshold temperatures for growth and development, but are dependent on the environmental conditions. Plants exposed to high temperatures above 35˚C significantly

affects their flowering and fruit set, seed germination, vegetative growth and crop yield. This occurs because plants channel resources to cope with heat stress, thus reducing photosynthates available for vegetative growth and development (Wahid *et al*., 2007). The tolerance of plants to high temperature depends on the relative humidity (RH) of the air, thus low relative humidity results in loss of water and causes dehydration in plants (Wahid *et al*., 2007). Furthermore, sensors positioned in various compartment of plants pick up signals in the change in ambient temperatures. Signaling between routes of increased Ca^{2+} influx and lipid based signaling cascades leads to the accumulation of osmolytes and antioxidants as a response mechanism against heat stress (Figure 1) (Bita & Gita, 2013).

1.4 Interaction between the two Stress Conditions

Drought is a complex phenomenon that depends on the occurrence of other factors. However, the concept of drought is built on the principle of water deficit, therefore factors that cause any form of water deficit directly aid in drought (McNab & Karl, 1988). Investigations on the two (2) prevalent causes of food loss reveal some level of dependency on one factor for occurrence. In my view, high temperature enhances the influence of drought in order to occur. This is because high temperatures increase the level of evaporation leading to water deficit as a function of drought. Research by AghaKouchak *et al*., (2014), supports the claim that heat or high temperatures contributes significantly to drought occurrence.

Research has focused on individual stresses but interaction stress has been given little attention. Generally combined stress adversely affects plant physiology and synergistically reduces crop yield and productivity. Studies show that metabolite in responses to a combination of two or more stress conditions produce unique responses in plants (Obata *et al.*, 2015). Furthermore, most studies focus on one stress factor, thus the combination of drought and heat stress could possibly produce unique responses in the plant.

Survival mechanisms can vary from plant to plant but on a general basis some plants like maize have a unique response mechanism among their species. Obaatampa, a drought tolerant species, from the tropics (DTMA, 2013) and sun sweet, a temperate crop were investigated in this experiment (Kaiser & Ernst, 2014). About 95% of maize grown in Ghana is Obaatampa, whereas

sun sweet corn is a popular variety grown in the temperature regions but the genotypes vary in their kernel colour and sugar content (DTMA, 2013; Kaiser & Ernst, 2014, 2014). Kaplan *et al*., (2004), reveals some similarity between heat and cold shock responses in Arabidopsis. Therefore, subjecting the 2 genotypes to the same growth condition might also reveal a similar response in both genotypes. It will therefore be interesting to verify how these 2 genotypes with different growth conditions respond biochemically to both drought and heat stress thus allowing plants adapt to thermal stress at the metabolite level.

1.5 Biochemical Response to Stress

All forms of stress whether abiotic or biotic stress stimulates different kinds of stress responses, which vary from one plant to another. Some response mechanisms adopted by plants under stress includes; protein synthesis, membrane lipid, low molecular weight metabolites, reactive oxygen species (ROS) and carbohydrate response mechanisms **(Hirt & Shinozaki, 2004).**

1.5.1 Reactive Oxygen Species (ROS) and Antioxidants

Reactive Oxygen Species (ROS) scavenging is the process where ROS accumulates in the cell as a result of different environmental stress factors and are detoxified by antioxidants or specialized enzymes (Taiza *et al*., 2015). Examples: H_2O_2 and superoxide peroxidase. Antioxidants are small peptides or organic compounds that receive electrons from ROS. Some antioxidants in plants include ascorbate, glutathione, lipid soluble α–tocopherol and β-carotene. Antioxidants and detoxifying enzymes of ROS forms a complex network, which is supported by an antioxidant recycling system that helps to maintain the reduced antioxidant levels. This function helps to maintain a minimal level of ROS which plays a significant role in plants such as signaling (Taiza *et al*., 2015).

Antioxidants function by preventing the accumulation of stress induced ROS such as superoxide which accumulates as a result of drought in wheat (Kellôs *et al*., 2008). Hydrogen peroxide (H_2O_2) and lipids peroxidase are also accumulated in tobacco as a result of drought (Kellôs *et al*., 2008). Rodriguez *et al*., (2002) states that ROS such as H_2O_2, play important physiological functions in plants, but excess of it must be detoxified. Therefore, the increased levels of ROS in

plants induce the activities of antioxidants (Kellôs *et al.*, 2008). The activity of these antioxidants contributes to an improved level of tolerance to stress (Kellôs *et al.*, 2008).

1.5.2 Lipid/Membrane Response

Cell membranes mediate all forms of interactions between the cell and its environment. The most fundamental functions of cellular membranes in biological organism is semi permeability. These membranes are structured by proteins inserted in between lipid layer components, allowing the in and out movement of molecules between cells. Among the numerous roles include storage of components, roles in membrane fission and fusion and elasticity which allows for deformation of cell structure. However, the function of the biological membrane largely depends on the combining properties of the various lipid molecules within membranes (Lee, 2005). Lipid membranes are largely constituted with phospholipids, sterols (cholesterol) and sphingolipids. Strong bonding between cholesterol and sphingolipids in a state can cause an association or dissociation within membranes (Thibault, *et al.*, 2012, Lee, 2005). Under stress conditions, membrane fluidity is affected thus affecting the normal physiological and biochemical functions of the plant. An increase in temperature, increases fatty acid saturation while low temperature increase unsaturation of fatty acid groups. Therefore, the removal of double bonds under high temperature prevents excess membrane fluidity whiles the insertion of double bonds increases fluidity of membranes at low temperature (Nilsen & Orcutt, 1996). Plants, microorganisms and insects all observe an increased level of unsaturated fatty acid when cultivated under low temperature. Marr and Ingraham (1962) therefore developed a concept that sets limit for temperatures of growth. That is, high temperature melts lipids and this indicates the maximum growth temperature, while low temperature solidifies lipids and therefore prevents cell growth indicating the lowest temperature of growth (Bita & Gerats, 2013). Taiz *et al.*, (2015), confirms this by stating that the decrease in membrane fluidity at low temperature causes a transition from a liquid-crystalline phase to a gel-phase in the cellular membranes, thus under high temperatures membrane fluidity reacts differently. This transition between lipid phases alters the metabolism of the plant to be either tolerant or susceptible (Taiz *et al.*, 2015). The removal or insertion of sterols may also contribute to membrane stability in plants as temperature changes (Nilsen & Orcutt, 1996). Polyunsaturated fatty acids in plants increases membrane fluidity because they

have a kink in their chain which prevents close packing unlike saturated fatty acids (Taiz *et al.*, 2015).

1.5.3 Protein Response

A common response to abiotic stress is the accumulation of proteins in plants including heat shock proteins, signal transduction proteins, enzymes involved in primary and secondary metabolic processes, chaperones and dehydrins (Lee Sang-Choon *et al.*, 2005; Sun *et al.*, 2016**b**). These processes involve the activities of proteins (inducible-stress genes) which confers direct and indirect protection to plants through the synthesis of osmo-protectants, detoxifying enzymes and to encode regulatory proteins such as protein kinases, transcription factors (TFs) and phosphatases (Figure 1) (Krasensky & Jonak, 2012). Heat Shock Proteins (HSPs) are a conserved evolutionary protein mechanism synthesized to confer tolerance against heat stress in plants (Wahid *et al.*, 2007). Under non-stress conditions, HSPs function by assisting in the synthesis, transport and proper folding of proteins. However, under stress conditions they function by promoting proper folding of denatured proteins and preventing aggregation of proteins as their conformation is important from the time of synthesis. The expressibility and broad distribution of HSPs in almost all compartments like cytosol, nucleus, plastids, endoplasmic reticulum and mitochondrion signifies their ability to protect all cellular compartments of the plants. HSPs just like dehydrins (DHN) have a conserved organizational structure. It contains an α-crystallin domain consisting of 90 amino acids with conserved N and C ends. This organization allows it to undergo dimerization. This domain allows it to function as a chaperone, preventing the aggregation of other proteins under a wide range of stress conditions by selection interaction with their hydrophobic surfaces (Kalemba & Pukacka, 2007). The regulation of HSPs could be achieved by a single master gene or a collective function of several Heat Shock Factors (HSFs), which is also dependent on the plant species. Another notable feature of HSPs is the subsequent defense mechanism initiated after mild heat exposure. This reveals the basal and acquired tolerance mechanisms adopted by plants (Hirt & Shinozaki, 2004). The acquired thermotolerance mechanism serves as basis to enhance basal thermotolerance and heat adaptability as it transitions to a performance stage of acclimatory homoestasis (Bokszczanin *et al.*, 2013; Kaplan *et al.*, 2004, Hirt & Shinozaki, 2004).

1.5.4 Dehydrins and their Regulation in Maize

Late embryogenesis abundant (LEA) proteins accumulate in high levels as a common mechanism against various abiotic stress conditions like heat, cold, drought, salinity, osmotic stress, leaf desiccation and seedling dehydration (Asghar *et al*., 1994; Brini *et al*., 2011; Hanin *et al*., 2011; Graether & Boddington, 2014). These proteins were originally discovered in cotton where they are expressed during the late stages of embryo (seed) development (Hanin *et al*., 2011). DHNs, a subgroup of the LEA proteins, accumulate in high concentrations during the late stages of seed development and also in vegetative tissues when subjected to drought and low temperature among other stress conditions. However, some DHNs are also constitutively expressed in the normal growth of plants. DHNs have been found in all major recognizable groups of land plants and thus seem to be almost universal (Close, 1997; Hara *et al*., 2005; Koag *et al*., 2009). The broad scope and spectrum for dehydrin identification in plants makes it significant for this study.

Multigene families under differential regulation encode DHNs in higher plants. DHNs are localized in various parts of the plants including cytosol, plasma membrane (Koag *et al*., 2003), mitochondria, protein bodies, nucleus, chloroplast and vacuoles (Hara *et al*., 2005; Koag *et al*., 2009). "The difference in expression and tissue location suggest that individual members of the DHN multigene family have somewhat distinct biological functions" (Koag *et al*., 2009). Thus the expression of a single protein has not been generally sufficient to confer resistance to stress (Koag *et al*., 2009). Dehydrins are unstructured proteins that constitute the biochemical group of LEA proteins "group 2" proteins (Asghar *et al*., 1994; Brini *et al*., 2011; Hanin *et al*., 2011) and are thought to play a significant role in cellular protection against dehydration (Hanin *et al*., 2011) leading to cell collapse. Dehydrins fill into spaces, accumulate and bind water which helps in maintaining cell volume during dehydration (Hanin *et al*., 2011). Although their specific functions are uncertain, it is believed that dehydrins contribute significantly to plant survival (Lee Sang-Choon *et al*., 2005). However, the exact functions of these proteins still remains unknown (Koag *et al*., 2009). Koag *et al*., (2009) states that, the specific biological function of the different DHN gene family depends on their expressibility and tissue location.
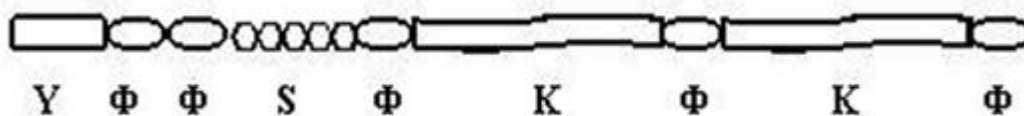
Figure 1.2. A linear representation of a YSK$_n$ dehydrin; Y – the Y-segment (T/VDEYGNP), S – a track of serine residues, K – the K-segment (EKKGIMDKIKEKLPG) and Ø - Ø-segment (Rorat, 2006)

Sub-classification of DHN is based on sequence motifs such as K-segment (Lys-rich sequence), Y-segment (N-terminal conserved), S-segment (a tract of ser residues) and Ø-segment (repeated glycine and polar amino acid). All dehydrins have one or more K-segments (EKKGIMDKIKEKLPG) (Hara *et al*., 2005; Mouillon *et al*., 2006; Koag *et al*., 2009; Koag *et al*., 2003). These Lys-rich motifs interact with membranes and macromolecules by forming an amphiphilic $\alpha-$helix, thus protecting membranes enabling them to survive stress (Hara *et al*., 2005). The domains are thought to confer tolerance and protect cellular membranes and organelles under dehydration stress (Wahid & Close, 2007). Dehydrins are hydrophilic with a high content of Gly thus giving the protein an unstructured form (Koag *et al*., 2003; Koag *et al*., 2009). The disordered or unstructured form of DHN gives it flexibility and malleability to withstand to changes in the protein environment such as pH, temperature and water potential (Koag *et al*., 2009). Additional work suggests that DHNs play significant roles in binding of lipid vesicles, metals, protection of lipid membranes against peroxidation, hydration or sequestration of ion molecules and aggregation of protein molecules (Rorat, 2006). Though many functions have been ascribed to dehydrins, I think one main function of a dehydrin is lipid membrane binding, as Koag *et al*., (2009) demonstrates the lipid vesicle membrane binding ability of dehydrins (K-segment).

1.5.5 Carbohydrate Response
Water limitation causes starvation in plants and this reduces photosynthesis, potentially resulting in a negative carbon balance. However, the levels of simple sugars will increase in stress plants

since starch will be broken down to produce sugars or vice versa (Nilsen & Orcutt, 1996). Carbohydrate may then be synthesized in different forms such as fructans, starch, oligosaccharides such as trehalose, raffinose and polyols (sugar alcohols) (Krasensky & Jonak, 2012). Osmolytes could be in the form of sugar alcohols (polyols) and sugars, which are accumulated as a pre-requisite tolerance mechanism for osmotic adjustments, increase in protein stability and stabilization of membrane layers. This maintains turgor pressure as it is common to most organisms under both drought and heat stress (Krasensky & Jonak, 2012; Bita & Gerats, 2013). Research carried out on a heat-tolerant tomato plant revealed that stabilization of the plant was enhanced by the increase in cell wall and vacuolar invertase activities and an increase in sucrose level (Bita & Gerats, 2013), thus important for plant tolerance. Furthermore, the rapid depletion of starch is necessary to produce sugar alcohols or sugars. They also function as osmolytes, accumulating in response to stress thus protecting membranes (Krasensky & Jonak, 2012).

1.5.6 Compatible Solutes in Stressed Plants

Compatible solutes are organic osmolytes that accumulate in cells in large quantity and concentrations without affecting the normal physiological processes of cells (Kuhlmann & Bremer, 2002). Plants like any other organism adapt to their changing environment through metabolic flux. Abiotic stress at the metabolic level can be defined as a change in the plants habitat that disrupts the plants metabolic homeostasis. Therefore, plants also regain homeostasis by adjusting metabolic pathways in responding to their growth conditions (Sun *et al*., 2016**a**).

In surviving abiotic conditions, stressed plants also induce the production of osmo-protectants. Osmo-protectants are important adaptive mechanisms that accumulate under extreme temperatures. These protectants include both primary and secondary metabolites. Primary metabolites are plant chemicals that are generally responsible for core housekeeping functions such as energy production, regulation of essential metabolites and molecules. Primary metabolites with osmoprotectant function include proline, glycine betaine, carbohydrates and soluble sugars which tend to protect cellular structures by regulating osmotic activity (Nilsen & Orcutt, 1996; Bita & Gerats, 2013). The secondary metabolites are plant chemicals that are produced in specific metabolic pathways and are necessary for growth or sometimes required

under specified conditions. A well understood function of secondary metabolite is plant defense, as they prevent pathogens from taking advantage of stressed plants to invade (Osbourn & Lanzotti, 2009). Secondary metabolites consist of phenolics, anthocyanins, flavonoids and plants steroids which plays a significant role in tolerance against heat stress. These metabolites regulate temperature increases by maintaining cell water balance, membrane stability and buffering cellular redox potentials (Bita & Gerats, 2013).

Glycine betaine (GB) is an amino acid derivative found in a wide range of crops and accumulates in some plants under various environmental stress conditions including drought (Zhang *et al*., 2012). Glycine betaine accumulates as a compatible solute in maize and sugarcane while others such as rice, mustard, Arabidopsis and tobacco do not naturally produce glycine betaine under stress conditions. The production of glycine betaine in the chloroplast maintains the activation of Rubisco and Rubisco activase which are inhibited even at a low heat levels (Bita & Gerats, 2013).

Understanding these mechanisms by which plants survive stress condition serves as pre-requisite in developing strategies to improve their tolerance to stress. Plants in surviving stress conditions activate various defense mechanisms of the plant. Plant response to stress varies with the level of stress condition it suffers, thus metabolites in plants also vary with the level of stress they are subjected to (Barchet *et al*., 2014). Therefore, maize in responding to stress conditions undergoes changes which are observed at various levels of plant growth (Barchet *et al*., 2014; Bowne *et al*., 2012).

1.6 Hypotheses

Comprehensive and complex mechanisms are therefore desired to properly correlate a metabolic effect with its abiotic stress, thus metabolomics and protein identification becomes relevant to this research. Metabolomics is a systematic approach where part or all of the metabolites in a sample are analyzed both quantitatively and qualitatively to elucidate any form of complex metabolite interaction network and their dynamic adjustments when tolerating stress, therefore this approach becomes relevant to this study (Sun *et al*., 2016**b**).

Previous research focused much on dehydrin in the seeds and tissue of maize plant with little emphasis on the leaf and which developmental stage of growth, since susceptibility to stress

varies from one developmental stage to another. These findings have the ability to form major standpoints for development of molecular markers as basis for improved crop tolerance (Obata *et al*., 2015). Given consideration to the extensive research carried out on maize but little or not enough findings could be attributed to the extreme complexity of drought resistance in maize (Bhanu *et al*., 2016). Therefore, the aim of the research is to identify dehydrins in maize at an early developmental stage under both drought and heat stress in two genotypes (Obaatampa and Sun sweet corn). Furthermore, to identify and establish the major pathways contributing to stress tolerance in maize. Also to understand and characterize metabolite effects and their levels in response to both heat and drought stress in maize leaves. Finally, to further understand the metabolite regulation under combined stress conditions of both drought and heat stress. Therefore, the hypotheses for this research are;

Hypotheses

- Drought and heat stress have different but overlapping effects on plant metabolites

  - Drought has a measurable effect on metabolites
  - Heat has a measurable effect on metabolites
  - Drought and heat have a measurable effect on metabolite

- Varieties bred for different conditions respond differently under drought and temperature stress

- Different and overlapping dehydrins are produced under both drought and heat stress

2. MATERIALS AND METHODS

2.1 Materials and Experimental Design

Experiments were carried out at the Norwegian Institute of Science and Technology (NTNU) - Faculty of Natural Sciences (Gløshaugen, Realfagbygget), Trondheim. Experimentally controlled conditions were used in order to get an answer to the outlined biological question and to reach the project goals.

Obaatampa (OB) maize is a common maize variety grown in Ghana. It is known to be drought tolerant and thrives under tropical conditions. Sun sweet (SS) corn is another popular variety grown in the temperate regions. These two genotypes; OB and SS were used in this experiment. The genotypes were nursed in a perlite growing medium. The seeds were watered until germination and transplanted into plastic pots (6cm x 6cm x 5cm). The planting medium was a mixture of soil and perlite in the ratio 3:1. The soil medium was filled into 120 plastic pots and arranged into 8 trays (15 pots per tray).

Half of the pots were sown with OB seedlings and the other half sown with SS. Genotypes were randomized to obtain 15 seedlings in each treatment. A growth chamber (Percival IntellusUltra Control System) was used for plant experiments, where trays were kept under the various conditions. In order to avoid positional differences within the growth chamber, genotypes and drought treatments in each tray were randomized. However, due to the limitations in the number of chambers, temperature treatments were not independently replicated but plants within chambers were treated as replicates for the purpose of analysis. Under the various treatments (Table 1) (control, drought, heat and drought x heat), seedlings were subjected to 400 $\mu mol/m^2/s$ of light, 12 h of light and 12 h of darkness with a relative humidity of 70%.

Table 2.1 Treatment conditions were carried out in a 2 x 2 factorial design

		Temperature	
		Low (L)	High (H)
Moisture	Watered (w)	Lw	Hw
	Drought (d)	Ld	Hd

2.1.1 Data Collection

From the start of the set-up, the low temperature, watered treatment (Lw) and high temperature, watered treatment (Hw) received 75 mL of water per week, while the Low temperature, drought (Ld) and high temperature, drought (Hd) received 35 mL of water per week. Furthermore, Lw and Ld were subjected to a temperature of 25˚C whiles Hw and Hd treatments were subjected to a temperature of 35˚C. Hoagland's solution was added in the watering process. After 1 week of growth in the chamber, time point 1 (T1) samples were taken; time point two (T2) samples were taken 2 weeks after start.

2.2 Protein Extraction

2.2.1 Sampling

Fresh leaves were taken from 7 plants as T1 samples after 1 week for both genotypes. However, these plants were not reused or resampled. T2 samples were taken from another 7 plants a week after T1 samples were taken. 100 mg of fresh leaf weight were sampled from each plant into 15 mL tubes and stored at -80˚C. By the end of the experiment many plants severely stressed and only T1 samples used in the analysis.

2.2.2 Protein Extraction and Western Blotting

Using the weight controlled extraction procedure, leaf samples were crushed into powder and a calculated average weight of sucrose extraction solution was added to the samples and kept on

ice (NB: Tube weight was recorded). The tubes were weighed to obtain the weight of tube, tissue and extraction solution. This allows the amount of tissue to be calculated, thus balancing the equivalent amount of phenol to be pipetted. 400 µL of phenol was added to the solution and vortexed. The mixture was centrifuged at full speed for 4 minutes. An equivalent amount of phenol from 10 mg of tissue was pipetted from the supernatant. 0.4 mL of ice cold 80% methanol with 0.1 M ammonium acetate was added to each tube and left overnight to precipitate. The mixture was centrifuged for 30 min at full speed and the supernatant discarded. Pellets were washed with a 0.4 mL of 100% methanol and centrifuged for 4 min at full speed. The supernatant was discarded and 0.4 mL of 80% acetone was added. The solution was centrifuged for 4 min at full speed and the supernatant discarded. The pellet was allowed to dry briefly to remove the remaining acetone. The pellets were dissolved in 25 µL of cracking buffer. In case solution was not enough to dissolve the pellet, it was increased to 50 µL. The dissolved samples were stored in a freezer at 21˚C.

Using the Laemmli discontinuous buffer system (LaemmLi UK., 1970), Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE) was used as a method of protein separation. Buffers were prepared and gel cassette assemblies were arranged. Electrophoresis was carried out according to the Mini-Protean®3 Cell Instruction Manual (BioRad). 25 µL of samples were loaded into each well alongside a 10 µL MagicMark™ XP marker (Invitrogen) and run at 200V for 35 min. SimplyBlue™ SafeStain was used to stain the gel for 1 h followed by gel washing with distilled water for another 1 h.

2.2.3 Western Blotting

The detected proteins were made accessible to the antibodies by the western blotting process. Western blotting allows proteins to be moved from the gel unto a nitrocellulose sheet or Polyvinylidene difluoride (PVDF) membrane. The other gel was equilibrated in a transfer buffer for 15 min. Using the Mini Protean 3 transfer system according to the manufacturer's instructions, transfer was carried out. However, we used a PVDF membrane in the transfer process. This allowed the charged proteins to move unto the PVDF membrane.

Bovine serum albumin (BSA) was used as a blocking solution to fill up all the places on the membrane where the target proteins were not attached. The nitrocellulose membrane was kept in the blocking buffer for about 30 – 45 min. 1% gelatin was melted into Tris Buffered Saline

(TBS). The nitrocellulose membrane was placed in a Rabbit Anti-Dehydrin (plant) Antibody (Agrisera) (primary antibody) solution of 1% gelatin TBS and incubated in a polythene bag for 2 h at room temperature (RT). The blot was rinsed in 1X TBS and 3 times in 1X TTBS for 7 min. Blot was placed in a polythene bag containing Goat Anti-Rabbit IgG (H+L)–AP Conjugate (secondary antibody) and incubated at RT for 45 min. The blot was again rinsed in ALPH buffer and further rinsed in distilled water. The blot was finally rinsed in a 10% acetic acid and then allowed to dry in the dark. This allows specific binding by the antibody, thus providing a clear result with minimal or no noise, but this was not true in our case.

2.2.4 Identification of Proteins
A comparative analysis of electrophoretic gels was carried out to identify the number of proteins detected on gels. Furthermore, quantification of protein abundance was done by observation. Electrophoretic gel and western blot images or pictures were taken using the G:BOX system.

2.3 Metabolite Profiling
For the analysis of leaf metabolites of maize plants, gas chromatography mass spectrometry (GC-MS) method was used. The GC-MS analysis was separated into 4 main parts namely; sampling, extraction, derivatization and GC-MS.

2.3.1 Sampling
Fresh leaf samples from each experimental condition were taken from healthy plants (n=7) as T1 samples after a week. T2 samples were taken from another 7 rep plants a week after T1 samples were taken. 150 mg weight of leaves were sampled from each plant into a 15 mL Eppendorf tubes and stored at -80˚C.

2.3.2. Extraction
2000 µL of H_2O:MeOH:$CHCl_3$ in the ratio 1:2.5:1 plus internal standard (IS; ribitol) was added to the pre-cooled solvent and vortexed for 10 s. The solvent mixture contained internal standard concentration: 50 µg ribitol/mL. The tubes were treated in a 50˚C ultrasonic bath for 60 min. After 5 min, solvent pressure was released and tubes closed tightly again until ultrasonic treatment was completed. The samples were finally cooled down to room temperature. Samples were centrifuged at 3,100 rpm at 4°C for 10 min. 850 µL aliquots from the clear supernatant (the polar phase) were transferred into a 1.5 mL round-bottomed Eppendorf tubes. Extra lids (with 5

holes each) were prepared in advance to close the tubes. Samples were finally closed with the prepared lids whiles the attached lids were kept on the sample tubes. Samples were dried at room temperature in a SpeedVac for 16 - 24 h. Samples were stored at -80˚C before further processing prior to derivatization and GC-MS analysis.

2.3.3 Derivitization of Extracts

Derivatization is the process where compounds are changed into forms amenable to a particular analytical method (Kristi Sellers, 2010). The dried residue was redissolved in 80 µL of 20 mg/mL methoxyamine hydrochloride in pyridine and derivatized at 30˚C for 90 min in incubator. The temperature in incubator was adjusted to 37˚C, when removing samples. Finally, samples were treated with 100 µL of MSTFA (N-Methyl-N-(trimethylsilyl) trifluoroacetamide), short vortex and incubated at 37˚C for 30 min. Samples were transferred to 1.5 mL autosampler vials with glass inserts and stored at 20˚C prior to GC-MS.

2.3.4. Gas Chromatography- Mass Spectrometry (GC-MS)

Separations were performed on an Agilent 6890/5975 GC/MS (Agilent Technologies, Palo Alto, CA) equipped with an HP-5MS capillary column (30 m × 0.25 mm i.d., film thickness 0.25 µm) (Agilent Technologies, Palo Alto, CA). Sample volumes of 1 µL were injected with a split ratio of 15:1. Injection and interface temperature were set to 230˚C and 250˚C, respectively. The GC temperature program was held isothermically at 70˚C for 5 min, ramped from 70 to 310˚C at 5˚C/min, and finally held at 310˚C for 7 min (run time: 60 min). The MS source was adjusted to 230˚C and a mass range of m/z 70–700 was recorded (EI mode).

2.3.5 Data Analysis

Data alignment and processing was carried out using the MetAlign_041012 software (Rikilt, Wageningen, Netherland). Compound identification was achieved using MS libraries, such as NIST/EPA/NIH MassSpectralLibrary NIST05 (National Institute of Standards and Technology, Gaithersburgh, MD), the Golm Metabolome Database containing MS spectra of derivatized

metabolites (Hummel *et al*., 2010), in combination with an in-house retention index library of trimethylsilylated (TMS) metabolites. The Automated Mass Spectral Deconvolution and Identification System (AMDIS; National Institute of Standards and Technology, Boulder, CO) software was used to interpret GC-MS data.

Metabolite content (µg/g) expressed on wet weight basis was used for statistical analysis. Statistical analysis and graphical representation of data (multivariate data analysis, t-test, Analysis of Variance-Principal Component Analysis (ANOVA-PCA) was carried out using various software packages. ANOVA-PCA was performed using a two-factor csv script in the BioStatFlow online tool (http://biostatflow.org/). A three-factor analysis of variance (ANOVA) ($p<0.05$) was performed using Minitab 17 statistical software. MultiExperiment Viewer (version MeV_4_9_0) was used for hierarchical clustering (Pearson correlation) to generate a heatmap of metabolite levels (312 identified, structurally annotated compounds and non-identified MS tags) in the maize leaves. This analysis was performed using log2(n) ratio amended concentration levels to the median for each single metabolite. This analysis was performed using log2(n) transformed data. Mean values and calculated fold-change (FC) were done based on estimated concentrations (µg/g) of metabolites. Diagrams for mean representation of drought and temperature stress against the various genotypes were generated using R-Software.

3. RESULTS

Two maize genotypes were subjected to heat, drought and combined stress (heat x drought). Presented in this section is the sample data on metabolic and protein response to the varied stress regimes. I present first data on metabolite response followed by protein response to these stress conditions. However, the complexity of this representation is likened to the story of the elephant as observed/viewed by individuals from different points with their own perspectives and understanding of what an elephant looks like. I am therefore using multivariate analysis to get the best picture of what the whole elephant looks like. The Biostatflow software analyzes only 2 factors at a time, so I combined some factors to compensate for a 3-way ANOVA-PCA. A single analysis gives 2 PCA plots; one for factor 1 and the other for factor 2. But for easy understanding for the reader I present only some selected diagrams with the remaining diagrams at the Appendix 1 section.

3.1 Multivariate-statistical analysis of GC-MS profiling data

This section presents a number of diagrams indicating the varied combinations of stress factors as generated and analyzed by the Biostatflow software. Using the multivariate analysis (Analysis of Variance-Principal Component Analysis (ANOVA-PCA)), the Biostatflow analyzed 312 metabolites detected in 56 samples to generate this separation along the various PCs. Each point on a diagram represents a specific plant under a particular treatment.
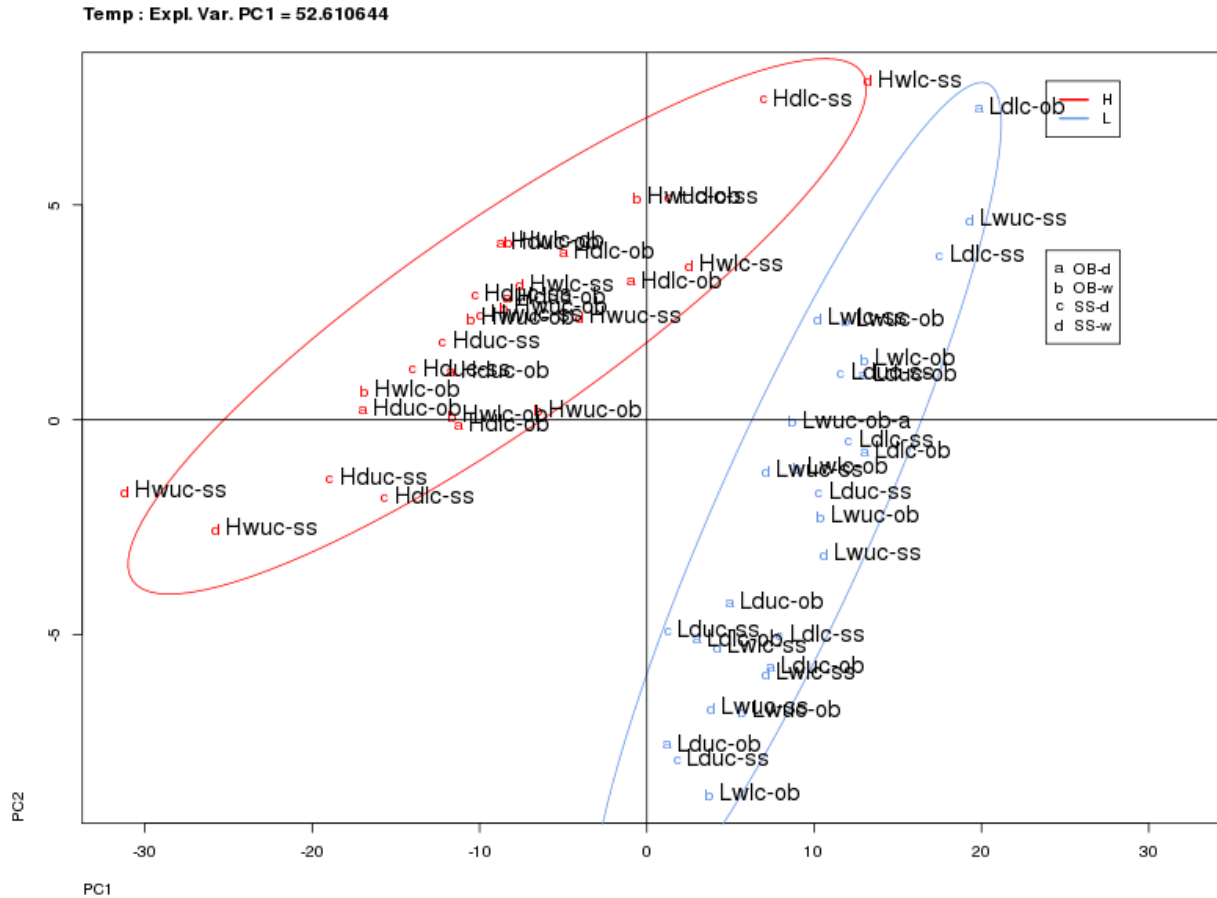
Figure 3.1. Factor 1 results of two-way ANOVA-PCA for temperature against combined genotype + water (Factor 2). Hand L represent high temperature and low temperature treatments respectively. OB+d, OB+w, SS+d and **SS+w** represent OB drought plants, OB watered plants, **SS** drought plants and **SS** watered plants.

Based on ANOVA-PCA analysis presented in Figure 3.1, a distinct separation between high and low temperature-treated plants could be shown. Both temperature clusters contain equal proportions of both OB and SS from water or drought treatments. However, the various genotypes treated under different moisture conditions are not distinctly separated within the temperature treatments groups. Temperature differentiates mainly along PC1, which explains 52.61% of variation between the sample groups.
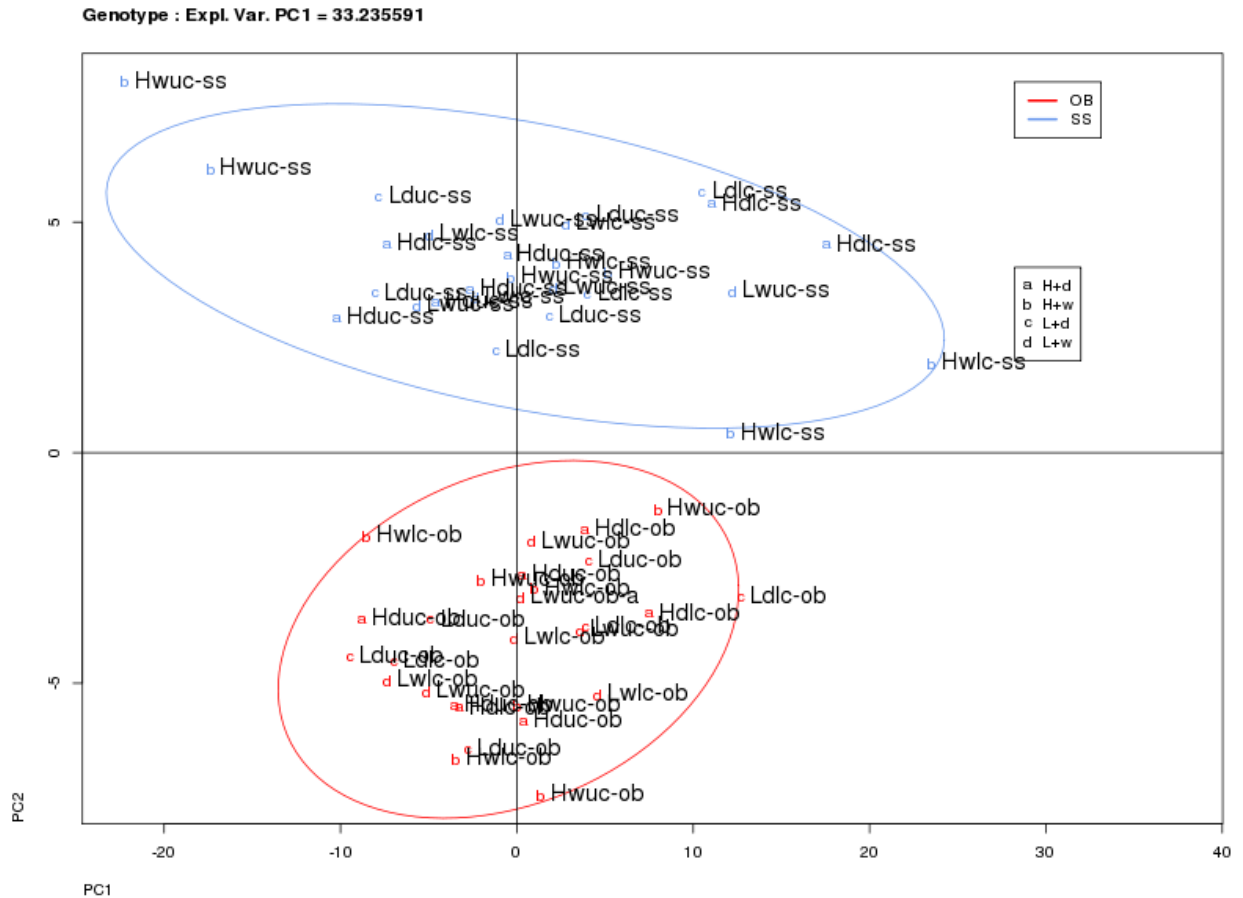
Figure 3.2. Two-way ANOVA-PCA for genotype (Factor 1) against combined (temperature + water) (Factor 2). H+d, H+w, L+d and L+w represent high temperature + drought plants, high temperature + watered plants, low temperature + drought plants and low temperature + watered plants respectively. Where OB and SS represent obaatampa and sun sweet respectively.

Observation from the diagram shows a distinct separation between the two genotypes with dispersed treatments. OB seems more clustered while SS observes a wide spread of treatments. Variety differentiates mainly along PC2, which explains 33.24% of variation in Figure 3.2.

Figure 3.3. Two-way ANOVA-PCA for moisture against combined (genotype + temperature) (Factor 1). Abbreviations: **d** and **w** represent drought and water treatments respectively. OB+H, OB+L, SS+H and SS+L represent OB-high temperature, OB-low temperature, **SS**-high temperature and **SS**-low temperature, respectively.

Figure 3.3 confirmed a possible overlap between drought and moisture treated plants, but showed a dispersed distribution of the two genotypes with their respective temperature treatments. Moisture doesn't really differentiate, but explains 35.57% of variation in Figure 3.3.

Figure 3.4. Two-way ANOVA-PCA for combined (genotype + temperature) against moisture (Factor 2). OB+H, OB+L, SS+H and SS+L represent OB-high temperature, OB-low temperature, **SS**-high temperature and **SS**-low temperature, respectively. Abbreviation: d and w represent drought and water treatments respectively.

Figure 3.4 showed that drought and watered plants under low temperature for both genotypes are separated unto the positive quadrant of PC 1with an overlap between the 2 genotypes. Furthermore, OB-high temperature plants are clustered to the top left of the diagram while SS-high plants are separated in the lower part of the PCA. Consistently, OB-high temperature treated samples have shown similar grouping in figure A1-1 & 2 (Appendix 1), and makes it easier to believe that OB-high temperature to have a strong effect on figure 3.1. Combined (Genotype and temperature) differentiate mainly along PC1, which explains 48.33% of variation in the between sample groups.

ANOVA-PCA analysis showed distinct levels of separation between the two genotypes thereby confirming variatal between OB and SS. An extended comparison between the two genotypes (Figures 2 and 4) revealed differences in the way each genotype responded to environmental factors. But observations from Figure 3.6 show that both genotypes (OB and SS) may regulate some metabolites in a similar manner. However, in situations of differences, this difference was not great as the majority of the metabolites between these two genotypes were not significantly different ($p < 0.05$) (ANOVA table). In Figure 3.1, the effect of temperature is seen as it shows a high level of separation between high temperature and low temperature treated plants with a small level of overlap (Figure 2 and 4) between some treatments. Surprisingly, drought was not distinctly separated as it overlaps with the well-watered plants (Figure 3.3). This may imply that the effect of drought was not strong to cause significant effects.

Metabolite regulation in maize

Figure 3.5. Hierarchical clustering heatmap depicting metabolite levels in maize leaves from different temperature and watering treatments. The colour spectrum, red to deep blue/black, signifies a high to low metabolite levels (log2 (n) ratio amended concentration levels to the median) based on 312 detected compounds. The thick yellow lines indicate a separation between the various treatments. A clear separation is shown as red colour dominares the right half of the heatmap which is associated with Hd and Hw plants (both genotypes). LW= Control condition, LD=Low temperature + watered treatment, HW= High temperature + water treatment and HD= High temperature + drought treament

Generally, a clear difference of plant metabolite levels across the various treatments could be observed. GC-MS-based metabolite profiling revealed a total of 312 metabolites (identified, structurally-annotated and non-identified MS tags), which are largely constituted by primary metabolites and to some extent, secondary metabolites. The primary metabolites included amino acids, organic acids, amines and carbohydrates which contribute to plant growth and function. These are largely synthesized in specific pathways of the plant like the glycolytic pathway, the Krebs cycle and the Calvins cycle. Furthermore, concentrations of several secondary metabolites belonging to the groups of phenolics (aromatic) compounds, sterols and terpenes were clearly affected by genotypic variation and environmental effects.

Hierarchical clustering indicated the significance of the effect of heat stress on metabolite concentrations in maize plants (Figure 3.5). Observations from the heatmap revealed a distinct separation between high temperature treated plants (35°C) and low temperature treatments at 25°C. Considering temperature, heated plants showed higher levels of metabolites as compared to normal temperature treatments (figure 3.5 and 3.6). Some red patches were also shown for some samples under low temperature condition in the heatmap (left half). These patches represent the LD plants showing the effect of drought on metabolite levels in maize plants. However, there is not much difference between LW and LD plants since they both showed similar patches of red in some metabolites (Figure 3.5). Under high temperature treatments, 264 out of 312 metabolites were significantly different ($P<0.05$) (Appendix 3) giving a strong indication that heat significantly affects the majority of the metabolites depicted in the heatmap. Out of these 264 metabolites, a number of metabolites overlap between heat and drought treatments. A total of 43 and 75 metabolites were significantly different under drought and

combined stress (temperature x drought) respectively (Appendix 3). This implies that drought affects the least number of metabolites whereas temperature has a significant effect on a large number of metabolites followed by combined stress (temperature x moisture).

3.2. Impact of Abiotic Factors on Selected Metabolites

In the following, consideration will be given to certain groups of metabolites, including amino acid, organic acids, carbohydrate, phosphorylated structures, aromatic compounds and terpenes based on selection of strongly affected metabolites from hierarchical clustering analysis and fold-change calculations. i.e. amines, B-amyrin among other metabolites. This was done by identifying highly regulated metabolites from the heatmap and fold-change.

Figure 3.6 shows how genotypes OB and SS differently responded upon the various treatments. A consistent increase under the combined stress condition was observed for OB, while SS observes a constant decrease under the combined stress conditions. This may possibly explain the reductive response of metabolites under the combined condition. The effect of temperature is seen in some metabolites (Proline, putrescine, raffinose, asparagine among others) as that of drought is slightly observed under some metabolites (aconitic acid).
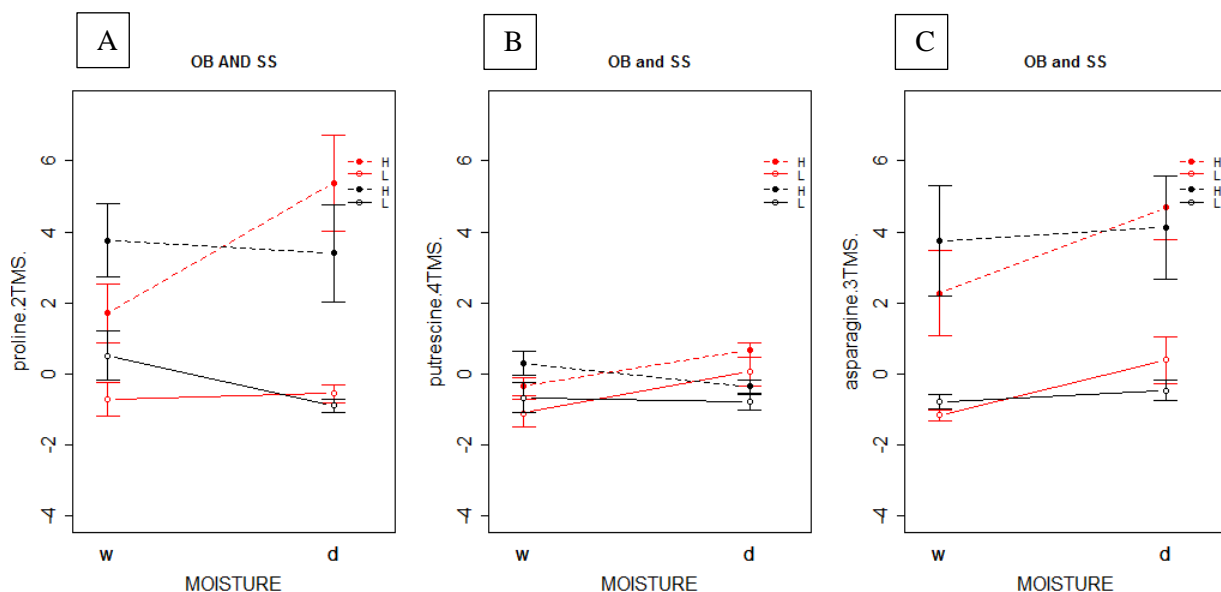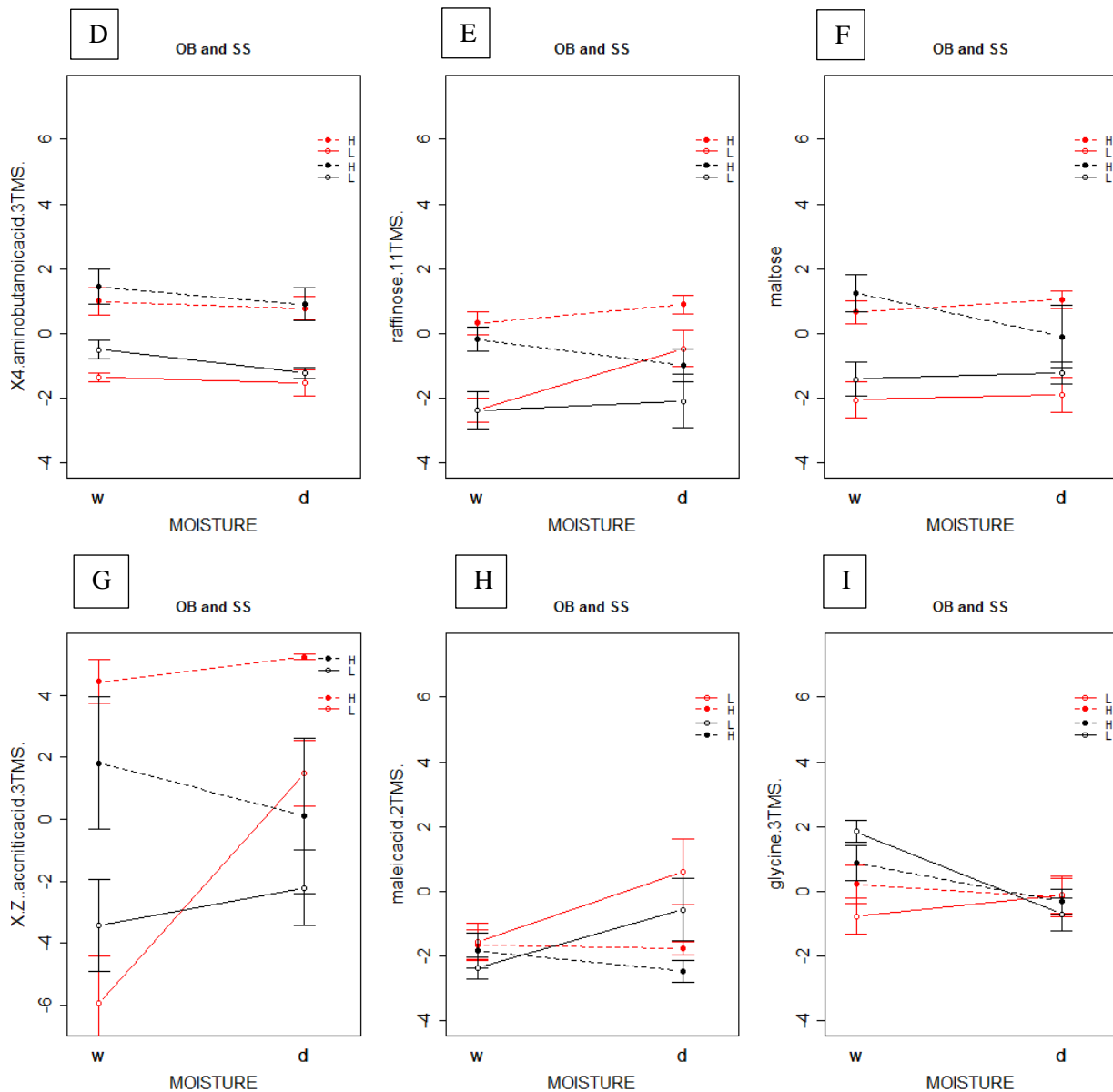
Figure 3.6. Mean representation of drought and temperature stress against the various genotypes. Red and black colour represents maize genotypes Obaatampa (OB) and Sun Sweet (SS), respectively. H and L represent high temperature and low temperature with w and d representing moisture and drought respectively. Dotted and non-dotted lines represent high and low temperature respectively. A, B, C, D, E, F, G and H represent the metabolites proline, putrescine, asparagine, GABA, raffinose, maltose, aconitic acid, maleic acid and glycine betaine. The Y-scale represents concentration levels for metabolites.

Table 3.1. Mean values and calculated fold-changes (FC) based on estimated concentrations for some metabolites; Abbreviations: HW=high temperature + water treatment, LD=low temperature + drought treatment and HD= combined stress (high temperature + drought). LW= control (low temperature + water treatment)

Metabolite	Class	Temperature [HW/LW]	Drought LD/LW	Combined Stress [HD/LW]	Additive or Reductive Response on Combined stress ([HD/LW]-[HW/LW + LD/LW])
asparagine	aa	142.10*	3.17	100.70	-44.56
(Z)-aconitic acid	ac	122.12	19.46	105.29	-36.29
tryptophan	aa	47.37	3.37	24.01	-26.73
β-amyrin	ter	21.93	3.85	17.97	-7.81
phenylalanine	aa	19.28	1.24	11.81	-8.72
histidine	aa	18.27	1.34	18.81	-0.80
coumaroyl glucoside	aro	16.71	3.72	19.94	-0.49
serine	aa	16.62	0.73	9.26	-8.09
fructose-6-phosphate	pho	15.01	2.23	10.06	-7.18
ribulose	pen	14.03	1.82	5.81	-10.05
proline	aa	12.69	0.37	79.22	66.16
α-amyrin	ter	12.11	2.18	9.81	-4.48
caffeic acid deriv.	aro	11.47	4.14	12.47	-3.14
2-aminobutanoic acid	aa	11.28	0.85	10.50	-1.62
isoleucine	aa	10.35	0.48	6.21	-4.63
luteolin	aro	10.09	1.06	8.63	-2.51
glutamine	aa	10.00	0.81	8.15	-2.65
2-methyl-2,4-bis-hydroxy-glutaric acid	ac	9.62	2.85	7.72	-4.75
α-tocopherol	aro	9.47	1.26	7.39	-3.34
threonine	aa	9.18	0.59	5.83	-3.94
γ-tocopherol	aro	9.18	1.15	3.26	-7.08
β-lactic acid	ac	9.02	1.29	9.28	-1.03
tyrosine	aa	8.87	1.22	6.88	-3.21
galactonic acid	ac	7.01	2.07	2.22	-6.86
maltose	disac	6.83	1.03	4.88	-2.99
aspartic acid	aa	6.49	0.53	6.70	-0.32
pyrrole-2-carboxylic acid	ac	6.31	1.27	3.80	-3.78

cysteine	aa	6.19	1.18	3.73	-3.64
glycerol-2-phosphate	pho	6.18	1.69	13.27	5.40
itaconic acid	ac	5.94	2.26	7.82	-0.38
(E)-aconitic acid	ac	5.84	1.94	4.59	-3.19
valine	aa	5.78	0.47	4.15	-2.09
leucine	aa	5.66	0.47	2.94	-3.18
lysine	aa	5.44	0.99	3.72	-2.71
4-aminobutanoic acid	aa	5.34	0.74	3.79	-2.29
glycerophosphoglycerol	pho	5.21	1.79	3.96	-3.03
glutaric acid	ac	5.04	1.31	2.61	-3.74
raffinose	trisac	5.02	3.03	5.29	-2.76
galactinol	alc	4.73	1.24	3.24	-2.73
5,6-dihydrothymine	pym	4.73	1.43	6.54	0.38
glycerol-3-phosphate	pho	4.58	1.38	2.62	-3.35
myo-inositol-2-phosphate	pho	4.55	1.20	3.57	-2.18
myo-inositol	alc	4.49	1.25	3.47	-2.27

* (red) values indicate highly affected metabolites with their fold-changes (FC) above 10, under temperature with their corresponding FC under moisture and the combined stress.

Table 3.1 shows the ranked order of fold-changes (FC) of metabolites according to temperature effects, with their corresponding FCs under both moisture and combined stress (temperature x moisture) conditions. Generally, very high FC levels (only metabolite level increase) were observed for both temperature and combined stress, while levels of certain metabolites were distinctly decreased under drought (FC<0). (Z)-aconitic acid, an intermediate compound in the Krebs-cycle, showed strongly increased levels under all treatments, making it a unique metabolite to be investigated. The amino acids asparagine, tryptophan, histidine and phenylalanine also showed high fold-changes under heat and combined stress, but were less affected under drought stress. Raffinose (Table 3.1) which increases in stress plants, showed fairly high FC increase under both heat and the combined stress condition, but showed a slightly lower level under drought condition. However, general reductive response was observed under combined stress (Table 3.1: last column). Surprisingly, proline, dihydrothymine, acetopyruvic acid, glycerol-2-phosphate and (Z)-4-hydroxycinnamic acid were the only metabolites that showed a high additive response implying that it played some significant role in plant survival.

Drought stress led to accumulated levels of certain amino acids (tryptophan, asparagine, adenine), organic acids ((Z)-aconitic acid, maleic acid, 2-methyl-2,4-bis-hydroxy-glutaric acid, ,

itaconic acid, galactonic acid, carbonic acid, arabinonic acid), aromatic compounds (coumaroyl glucoside, caffeic acid derivative, (E)- 4-caffeoylquinic acid), carbohydrates and phosphorylates (raffinose, glycerolaldopyranosid, fructose-6-phosphate), secondary metabolites (B-amyrin, a-amyrin) and other structures (9,12,15-(Z,Z,Z)-octadecatrienoic acid) and. High regulation of some acids under drought implies some connection with the citric cycle. Furthermore, some major amino acids are also regulated under this condition.

High temperature affects a large number of metabolites including; amino acids (asparagine, tryptophan, phenylalanine, histidine, serine, proline, 2-aminobutanoic acid, isoleucine, glutamine, threonine), organinc acids ((Z)-aconitic acid, 2-methyl-2,4-bis-hydroxy-glutaric acid), aromatic compounds (caffeic acid derive, coumaroyl glucoside, α-tocopherol, γ-tocopherol luteolin), carbohydrates and phosphorylates (fructose-6-phosphate, ribulose,) and secondary compounds (B-amyrin, a-amyrin).

Under combined stress, the following metabolites were affected; amino acids (asparagine, proline, tryptophan, phenylalanine, 2-aminobutanoic acid, glutamine, serine, histidine, tyrosine, aspartic acid), organic acids ((Z)-aconitic acid, 2-methyl-2,4-bis-hydroxy-glutaric acid, itaconic acid, B-lactic acid), aromatic acids (coumaroyl glucoside, caffeic acid, luteolin, α-tocopherol), carbohydrates and phosphorylates (glycerol-2-phosphate, fructose-6-phosphate) and secondary compounds (B-amyrin, a-amyrin).

3.3 Identification of Different Dehydrins under the Various Treatment

This section of the results experienced a number of problems with proteins extraction and western blotting. The stained gel with bands indicated the presence of dehydrin proteins in both genotypes (Figure 3.7). However, 3 lanes (Lw-OB, Lw-SS and Ld-SS) within the blot membrane (Figure 3.7B) showed faint indications of proteins. Observation from the blot membrane (Figure 3.7B) showed that the first 4 lanes (1-4) were the low temperature-treated plants while the last 4 lanes (5-8) are the high temperature plants. Though the first 4 lanes showed low traces, lane 3 (Ld-OB) detected some dehydrins whilst small amounts were found in lanes 1, 2 and 4. Observation from the gels indicated the potential presence of some dehydrins with varying bands (molecular weights) in both genotypes. All the lanes produced similar bands, thus the same dehydrins were produced in these plants. There is indication of some prospective dehydrins with specific weights. The band weights with specific molecular weights are 100, 50 (Mohammadkhani, & Heidari, 2008) 23 (Wahid & Close, 2007) and 20 kDa (Ceccardi, *et al.*, 1994).
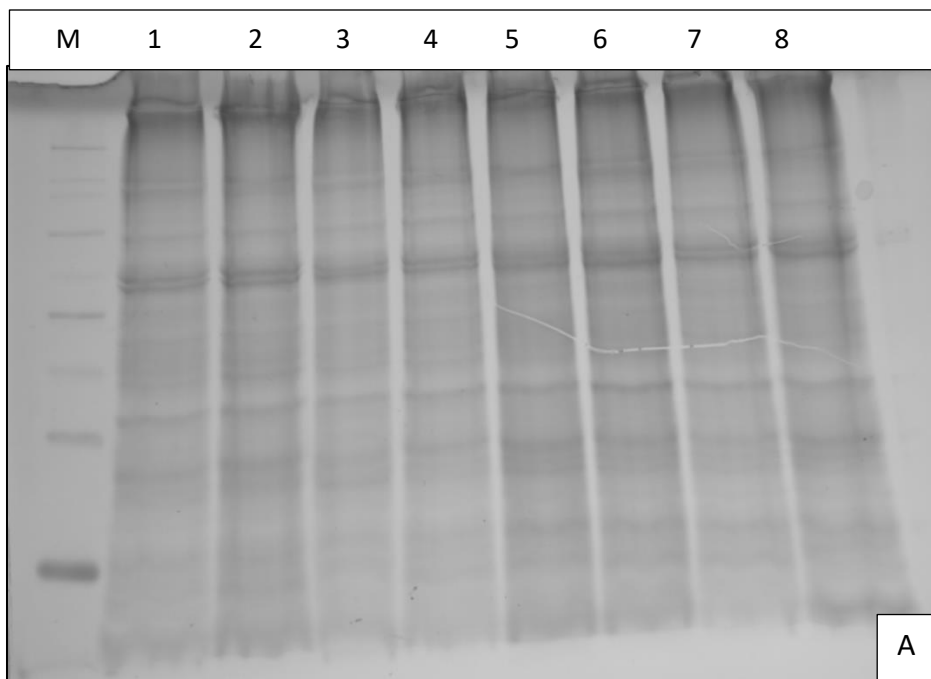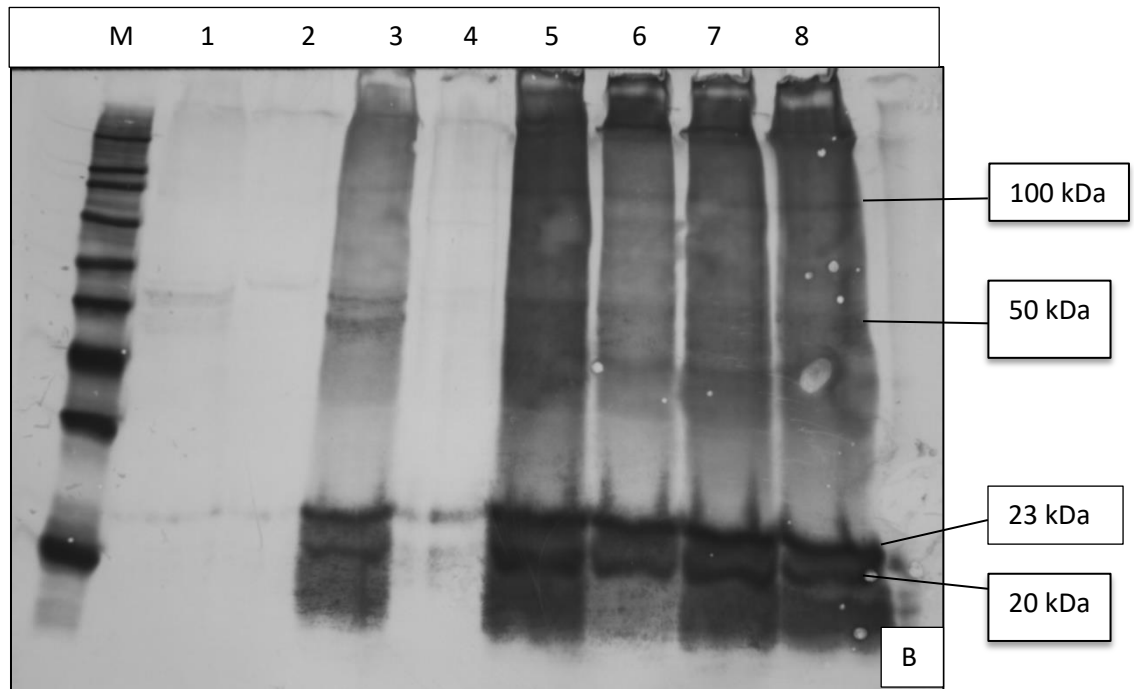
Figure 3.7. Stained Gel (A) and Western blot membrane (B) for the two genotypes under the various treatments. M represents Marker lane while 1, 2, 3, 4, 5, 6, 7 and 8 represent Lw-OB, Lw-SS, Ld-OB, Ld-SS, Hw-OB, Hw-SS, Hd-OB and Hd-SS respectively. Pictures were taken with G-BOX.

4. DISCUSSION

Metabolites play significant roles in plant growth and development. These in addition to primary metabolism may vary from signaling to defense or plant tolerance. This research gives us new insight into the survival or tolerance mechanisms between tropical and temperate varieties of maize with their dehydrins and metabolite regulation under different stress conditions.

4.1. Effects of Heat, Drought and Combined Stress on Metabolite Regulation in OB and SS

4.1.1. Effect of Heat

In our attempt to find out which stress factor regulates the majority of metabolites, we revealed that heat stress has a strong influence on plant metabolism. The major observation was the big differences in metabolite levels in heat stressed versus unstressed plants being consistent in both moisture treatments. Heat caused a major change in the metabolite pool in the leaf tissue leading to enhanced or decreased levels of certain metabolites (Figure 3.5, 3.6 and table 3.1). These findings did not agree to studies in Arabidopsis thaliana (Rizhsky *et al*., 2004) and maize (Obata *et al*., 2015), as they found temperature to be a weak regulator of metabolic responses. Moreover, the findings from several studies conducted point drought to be a stronger factor in the perturbation of metabolite pools in plants (Obata *et al*., 2015; Kaplan *et al*., 2004; Rizhsky *et al*., 2004). This difference in our findings could be attributed to the variations in temperatures observed in maize experiments of Obata *et al*., (2015), where plants experienced a heat treatment range (22 to 33˚C on average) in field trials. However, results from metabolite analysis agreed largely with the other section of this study conducted by Torgbor (2017), who described the effects of high temperature (35˚C) on photosynthesis and growth using the same treatments and plants as in this study. In his master thesis, he also found that high temperature had greater effects than drought. To another large extent this study agrees with the findings of Obata *et al*., (2015). Amino acids were a major part of the response mechanisms adopted by maize plants under heat stress as revealed in our study (Obata *et al*., 2015; Table 3.1 and Appendix 2A-1). Particularly, consideration will be given to the amino acid asparagine and the Krebs-cycle intermediate (Z) aconitic acid. These two compounds were highly affected under all three conditions. However, the strong positive response of aconitic acid does not agree with results of

Obata *et al*., (2015) and Kaplan, *et al*., (2004). Clark (1968) shows that aconitic acid increases in the leaves of young corn with high mineral elements, thus providing a possible explanation for the high levels of organic acid in the Krebs cycle. Asparagine on the other hand is synthesized by glutamine and both metabolites function by mediating nitrogen storage and transport. High accumulation of asparagine has been correlated with stress treatment in young wheat leaves (Lea, *et al*., 2007). It is also evident that asparagine highly accumulates in plants as proline also increases. The regulation of asparagine in these plants possibly suggests a biological mechanism in maintaining osmotic functions in the plant (Lea, *et al*., 2007). However, there is little information available about the osmotic mechanisms of both aconitc acid and asparagine in plants.

4.1.2 Effect of Drought

The effect of drought (moisture) in this study did not yield the same results as described in literature. However, drought stress caused variations in the levels of certain metabolites known to function as osmoprotectants in plants under abiotic stress. This finding therefore did not agree to those of (Obata *et al*., 2015; Rizhsky *et al*., 2004) as they found drought to be a major regulator of metabolites in plants. This difference could be attributed to some factors such as, difference in the level of drought stress treatment applied. Organic acid among some other amino acids were a major part of the response adopted by drought stressed plants (Table and metabolite regulation (Appendix 2)). Among the up regulated metabolites under drought stress includes aconitic acid and maleic acid. The high regulation of these two metabolites agrees with the findings of Kaplan, *et al*., (2004), as it was observed that aconitic and maleic acid were up regulated under cold stress Arabidopsis. Aconitic acid is shown to accumulate differently in plants, thus it is highly accumulated in the leaves of young plants (Clark, 1968; Brauer & Teel., 1982; Lea, *et al*., 2007). Maleic acid on the other hand is an organic acid that acts as precursor for the synthesis of fumaric acid and malate. However, there was low regulation of maleic acid under both heat and drought stress (Metabolite regulation (Appendix 2)). Though lots of functions have been assigned to organic acids, there is little report on the contribution of these 2 metabolites to aid plants survive stress. The change observed in aconitic acid across all treatments may suggest a genetic manipulation of an enzyme thus a possible strategy in future breeding of tolerant maize. Furthermore, it was observed that raffinose, asparagine, tryptophan and B-amyrin were up regulated under drought stress. Raffinose by evidence from many studies

has been implicated in stress tolerance in plants (Koster, 1991). Therefore, the up regulation of raffinose signifies that it plays an osmotic function in aiding plants against stress (Minorsky, 2003; Obata *et al.*, 2015).

4.1.3 Effect of Combined Stress (Heat x Drought)

The synergistic effect of temperature and drought has been focused on for some time as it threatens food security due to its economic effect on crop production. In this study, we obtained high fold-changes, i.e. increase in metabolite levels, under combined stress as compared to the single-stress treatments for the individual metabolites (Table 3.1 and Figure 3.6). Obata *et al.*, (2015) points out that metabolite change under combined stress is considered as the total sum of the individual stresses, thus the combined stress should observe higher (additive) regulation of metabolites (Appendix 2: last column). It turned out that drought negatively affected heat response under combined stress. This was not entirely observed in our study, though fold-change values for the combined stress were high. This could be explained by a consistent decrease in metabolite concentrations in SS plants under combined stress conditions (Figure 3.6) and thus suggests catabolic processes (breakdown in metabolic processes). Proline, glycerol-2-phosphate, 5,6-dihydrothymine, acetopyruvic acid, phosphoric acid and (Z)-4-hydroxycinnamic acid were the only metabolite that received an additive response under combined stress (Table 3.1 and Appendix 2). However, their up regulation supposes a functional role in plant tolerance and survival to stress conditions. Up regulation of proline has been correlated with stress tolerance and survival (Witt *et al.*, 2012; Krasenkey & Jonak, 2012; Hayat *et al.*, 2012). However, there is a contrast between this finding and that of Kaplan *et al.*, (2004) who recorded low levels of proline in heat shocked Arabidopsis plants: though combined stress led to a significant increase in proline levels. They emphasize that low temperature (cold-shock), drought and salinity rather enhance proline content, but not heat stress. Our finding suggests that combined stress and heat stress alone in SS positively affects proline levels. The same was stated by Krasensky & Jonak, (2012) concerning the findings of Kaplan *et al.*, (2004), that proline is not accumulated under high temperature, which was also confirmed in maize studies by Obata *et al.*, (2015). Furthermore, the high levels of proline under heat and combined stress confirms with Witt *et al.*, (2012) who revealed a strong correlation of proline with heat but a negative correlation with

drought. This possibly explains the low levels of proline accumulated under drought stress (Table 3.1). Glutamine levels were also relatively high under single heat or combined stress, thus converted to produce proline and 4-aminobutanoic acid (GABA). GABA is further channeled back into the TCA cycle to synthesize succinate. This possibly explains the majority of organic acids and amino acid regulation in the mitochondrion. This finding largely agrees with the results of Kaplan *et al*., (2004). However, it is possible the variation in these results can be attributed to the difference in plant type (Obata *et al*., 2015) since species from different plant families might react differently in their metabolic response to stress.

Plants have distinct ways of managing their stress conditions and this was evident in our study. The accumulation of some amines, proteins and non-proteins were not out of place. Putrescine, a-tocopherol, β-alanine and GABA are specific metabolites implicated in the protection of membranes under stress conditions. Bita & Gerats (2013) state that, high levels of stress (heat) affect the activities of thylakoids leading to leakage of ions and a possible breakdown of the metabolism pathway. The presence of reactive oxygen species (ROS) signals the activities of these scavenging metabolites which reduces the risk posed to plants. These osmolytes and antioxidants regulate ROS levels thus protecting and alleviating oxidative stress (Kransensky & Jonak, 2012; Kellôs *et al*., 2008). Furthermore, the high levels of these metabolites indicate the presence of ROS in plants.

Furthermore, every stressed plant inevitably becomes a target for opportunistic pathogens and other organisms (Osbourn & Lanzotti, 2009). Therefore, one of the first responses upon abiotic stress is to defend plants against pathogens. Plants perceiving their susceptible nature induce high levels of secondary metabolites to confer resistance against pathogens that might take advantage of the stress nature of plants to invade. B-amyrin and a-amyrin are terpenoids which were regulated under all conditions, implying the need to protect plants from pathogens (Kaplan *et al*., 2004; Osbourn & Lanzotti, 2009).

Carbohydrates such as glucose, raffinose, maltose, sucrose and some polyols with their precursors (myo-inositol and galactinol) were increased under stress conditions. The high levels of carbohydrates regulated implicate them to play an osmolytic function in protecting plants against stress (Koster, 1991; Kaplan *et al*., 2004; Obata *et al*., 2015). Increased levels of carbohydrate precursors; myo-inositol and galactinol positively affected the accumulation of

raffinose, glucose, glucose-6-phosphate, fructose-6-phosphate among other carbohydrates. Raffinose, reported in many studies plays significant roles in plant tolerance to stress, as they function by preserving the functional integrity of plants (Koster, 1991¸Minorsky, 2003). Many other carbohydrates also play osmotic functions in aiding plants against stress (Kranskey & Jonak, 2012). Combined stress affected metabolites in a similar way to that of heat stress, as they positively affected the levels of amino acids and organic acids. This is because heat being the dominant factor even under combined stress, affects plant metabolism leading to a breakdown and release of metabolites while drought regulates mostly carbohydrates and organic acids.

4.1.4 Difference Between Varieties

PCA and Anova analyses (Appendix 3A-1) for metabolites showed that there was some level of difference between specific metabolites for the two genotypes (Appendix 3A-1). OB produced higher concentrations of metabolites than SS. However, there was significant difference among specific metabolites but generally this difference was not significant implying that the 2 plants may actually behave in a similar way. The findings of Torgbor (2017) confirmed that biomass from the two plants showed no significant differences under both heat and drought stress, however, OB performed slightly better than SS under the moist condition at high temperature. Furthermore, the photosynthetic data generally showed that, both varieties have different mechanism of dealing with the various stress factors in order to survive (Torgbor, 2017). The findings of Kaplan *et al*., (2004) further confirmed that both tropical and temperate plants regulate similar metabolites though these metabolites may vary in their concentrations (Figure 3.6). Furthermore, it was observed that SS generally showed a decline under combined conditions thus explaining the negative response of metabolites under combined stress (Table 3.1: last column).

4.2. Dehydrins are produced under Stress Conditions

Furthermore, the survival of these plants is also attributed to the presence and activities of LEA proteins specifically dehydrins in stressed plants. Our study clearly identified some dehydrins with molecular weights of 20, 23, 50 and 100 kDa ((Figure 3.7B). These dehydrins showed a thick band size and were visible in all genotypes and under all treatments though the bands were not sharp and some appeared in small traces. Figure 3.7B clearly showed plant response to

temperature as 4 lanes showed high indication of proteins. Lane 3 (Ld-OB) and 4 (Ld-SS) were drought plants; however, OB indicated higher levels of dehydrins compared to SS thus, OB might show better tolerance under stress conditions. This finding confirms the work of Wahid & Close (2007), who also identified DHNs of 21, 23 and 27 kDa under heat stress in sugarcane. It is therefore significant that plants express unique proteins (DHNs) in all variety of stress conditions including heat and drought stress. The same could be said for Borovskii *et al.*, (2005) who also identified DHNs with molecular weights of 52 and 63 kDa in wheat plants. Furthermore, the findings of Ceccardi *et al*., (1994) who identified a dehydrin with the same molecular weight from a maize kernel inbred line G50 also confirms this study. Inferring from the blot, we can suggest that the response of dehydrins to stress vary and these may be dependent on the level of stress imposed on plants. However, the unsuccessful nature of the blot makes it difficult to clearly identify bands thus making reference to DHNs with specific treatments. This problem was a big challenge to this study. However, this difficulty may be attributed to a number of factors which includes; failure in the transfer process, though some lanes showed successful protein transfer. It is also possible for a mix-up in the extraction process, since the samples were extracted in 2 different batches. Furthermore, small traces of protein bands may also indicate the non-stress condition of the plants thus not producing proteins. Though some attempts have been made to link some visible bands, I think a better blot will help to properly discuss this DHN section. Therefore, the identification of DHNs indicates that stressed plants in their tolerance mechanisms against stress, produce LEA proteins (dehydrins) which aid in their survival.

5. CONCLUSION

Climate is a complex phenomenal with the potency of affecting global food security. It is therefore important this study be carried to a higher level as it brings a level of security into the foods and agricultural industry. Since global trends indicate irregular climatic conditions, it is therefore necessary for the development of resistant maize varieties. Developing a maize variety that can thrive under all conditions should be our next line of research after we understand this complex phenomenon of single and combined stress conditions.

In conclusion, heat stress had a strong effect on metabolite regulation than drought and combined stress. Drought in this study showed low effects on metabolites regulation, however, there was up regulation in two metabolites; aconitic and maleic acid. Drought regulated a number of organic acids whiles heat stress regulated mostly amino acids. Combined stress on the other hand responded differently by showing an up regulation in aconitic acid and proline.

The two genotypes were expected to differ in their metabolite regulation since they were bred under different conditions. However, it turned out that generally both genotypes regulated the same metabolites except for variation in the concentrations of individual metabolites. Therefore since the genotypes reacted in a similar way to stress, it may suggest that maize plants have a mechanism in responding to drought and heat stress.

Dehydrins were identified in almost all the conditions except for our challenges as stated above. However, the same band sizes of DHNs were identified in all treatments. The identified weight bands were 20, 23, 50 and 100 kDa DHN sizes. Notwithstanding the challenges faced, it can be stated that the same DHNs were identified under both drought and heat stress.

6. CHALLENGES

The treatments were not independently replicated because only two growth chambers were available for the study.

It was obvious there was a challenge with the protein aspect of the study, as this aspect affected the quality of dehydrin detection in this study.

7. RECOMMENDATION

I suggest that this study be replicated and thus there should be a change in the watering regime to ascertain these findings, though some positive confirmations have been made in reference to other studies.

Furthermore, multiple varieties from the same or similar locations can be considered in future experiments to properly ascertain if response were due to adaptability or varietal differences.

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APPENDIX 1

Multivariate-statistical analysis of GC-MS profiling data
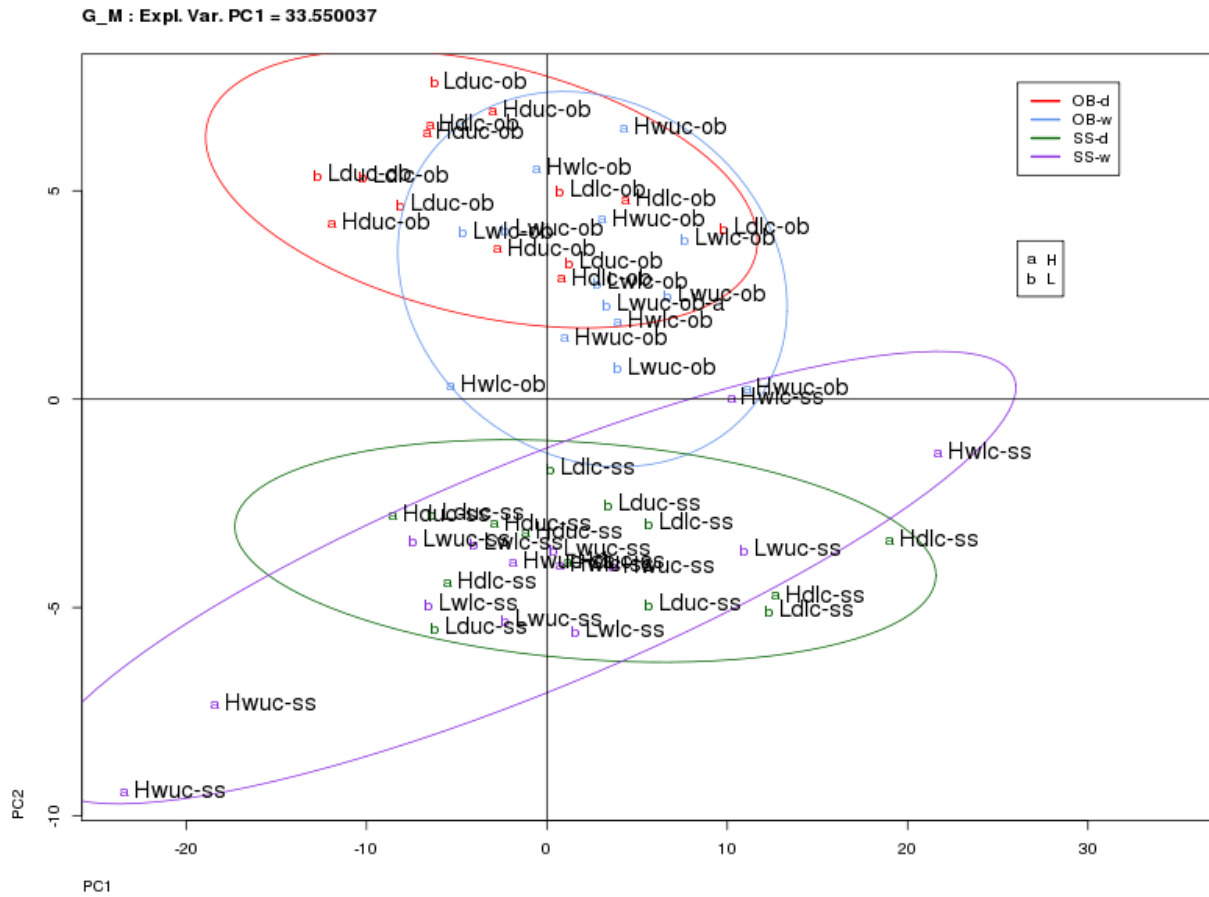
Figure A1-1. Two-way ANOVA-PCA for combine (genotype + water) against temperature (Factor 2). OB+d, OB+w, SS+d and SS+w represent OB drought plants, OB watered plants, SS drought plants and SS watered plants. H and L represent high temperature and low temperature treatments, respectively.
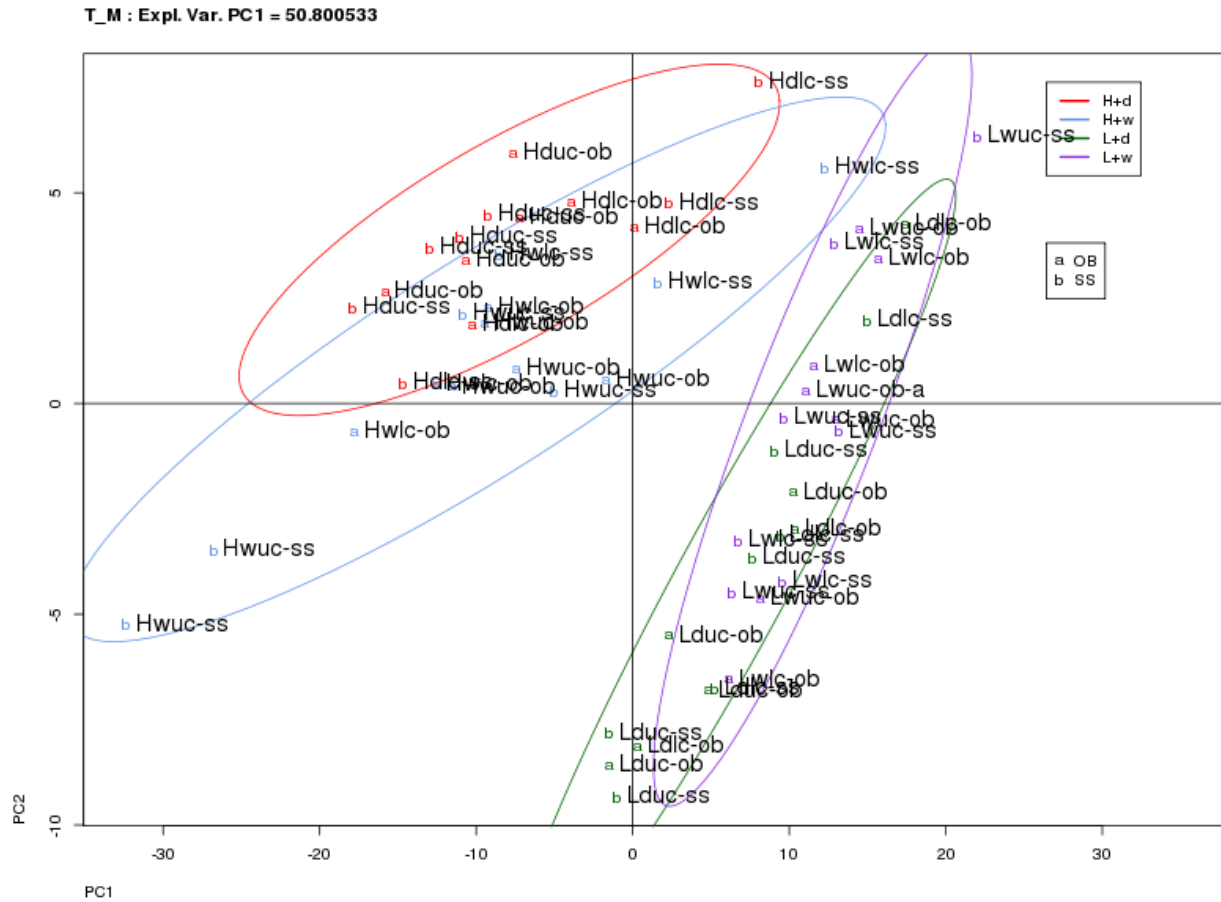
Figure A1-2. Two-way ANOVA-PCA for genotype against combine (temperature + water) (Factor 2). H+d, H+w, L+d and L+w represent high temperature + drought plants, high temperature + watered plants, low temperature + drought plants and low temperature + watered plants respectively.

APPENDIX 2

Table A2-1. Mean values and calculated fold-changes (FC) based on estimated concentrations for all metabolites under drought, heat and combined stress.

Metabolite	Class	Drought Effect LD/LW	Temperature Effect HW/LW	Combined Effect HD/LW	Additive or Reductive Response on Combined stress ([HD/LW]-[HW/LW + LD/LW])
asparagine	aa	3.17	142.10	100.70	-44.56
(Z)-aconitic acid	ac	19.46	122.12	105.29	-36.29
tryptophan	aa	3.37	47.37	24.01	-26.73
proline [+CO2]	aa	1.17	35.92	32.98	-4.11
NA_58	na	4.40	26.41	22.59	-8.23
disaccharide_10	disac	2.75	25.74	28.99	0.50
disaccharide_32	disac	2.40	25.21	16.86	-10.75
disaccharide_4	disac	2.99	24.35	20.28	-7.06
phosphate_3	pho	8.46	24.00	25.15	-7.32
NA_18	na	1.09	23.70	28.17	3.38
β-amyrin	ter	3.85	21.93	17.97	-7.81
disaccharide_5	disac	4.09	19.54	19.80	-3.83
phenylalanine	aa	1.24	19.28	11.81	-8.72
NA_55	na	3.58	19.07	23.56	0.92
histidine	aa	1.34	18.27	18.81	-0.80
coumaroyl glucoside	aro	3.72	16.71	19.94	-0.49
serine	aa	0.73	16.62	9.26	-8.09
disaccharide_3	disac	3.53	16.03	12.79	-6.77
fructose-6-phosphate	pho	2.23	15.01	10.06	-7.18
disaccharide_17	disac	1.89	14.76	7.82	-8.83
Isoleucine	aa	0.74	14.32	9.06	-6.01
NA_57	na	4.06	14.13	16.15	-2.04
ribulose (4TMS)	pen	1.82	14.03	5.81	-10.05
NA_42	na	5.61	14.02	3.00	-16.63
valine	aa	1.09	13.69	10.14	-4.64
NA_36	na	13.68	12.94	2.03	-24.59
NA_5	na	1.27	12.82	12.27	-1.82
proline	aa	0.37	12.69	79.22	66.16
hexose_4	hex	3.95	12.38	16.74	0.42
α-amyrin	ter	2.18	12.11	9.81	-4.48
farnesyl deriv.	ter	1.46	11.91	6.22	-7.15
caffeic acid deriv.	aro	4.14	11.47	12.47	-3.14

disaccharide_14	disac	3.38	11.39	13.01	-1.76
2-aminobutanoic acid	aa	0.85	11.28	10.50	-1.62
NA_26	na	1.17	10.95	6.82	-5.30
NA_33	na	3.33	10.86	3.24	-10.94
NA_43	na	2.07	10.80	5.14	-7.73
tryptophan	aa	1.99	10.70	8.67	-4.02
isoleucine	aa	0.48	10.35	6.21	-4.63
luteolin	aro	1.06	10.09	8.63	-2.51
serine	aa	1.34	10.07	3.09	-8.31
glutamine	aa	0.81	10.00	8.15	-2.65
Serine	aa	1.57	9.84	8.38	-3.02
NA_2	na	4.21	9.74	2.62	-11.33
2-methyl-2,4-bis-hydroxy-glutaric acid	ac	2.85	9.62	7.72	-4.75
NA_1	na	2.97	9.51	2.56	-9.92
NA_44	na	2.96	9.51	8.85	-3.62
α-tocopherol	aro	1.26	9.47	7.39	-3.34
threonine	aa	0.59	9.18	5.83	-3.94
γ-tocopherol	aro	1.15	9.18	3.26	-7.08
disaccharide_16	disac	3.67	9.05	13.55	0.83
β-lactic acid	ac	1.29	9.02	9.28	-1.03
tyrosine	aa	1.22	8.87	6.88	-3.21
kaempferol deriv.	aro	1.40	8.86	5.88	-4.39
disaccharide_26	disac	2.44	8.52	7.50	-3.46
disaccharide_7	disac	2.93	8.08	10.23	-0.78
NA_52	na	5.08	7.96	6.45	-6.58
NA_14	na	1.48	7.86	16.96	7.61
NA_11	na	1.27	7.80	2.52	-6.55
leucine	aa	0.73	7.53	4.28	-3.98
trisaccharide_2	trisac	1.07	7.02	5.03	-3.06
galactonic acid	ac	2.07	7.01	2.22	-6.86
disaccharide_12	disac	1.50	6.91	6.20	-2.22
Maltose	disac	1.03	6.83	4.88	-2.99
disaccharide_24	disac	1.50	6.56	2.86	-5.20
aspartic acid	aa	0.53	6.49	6.70	-0.32
guaiacyl deriv.	aro	2.39	6.48	8.58	-0.29
pyrrole-2-carboxylic acid	ac	1.27	6.31	3.80	-3.78
aromatic/sugar	aro	2.20	6.22	10.94	2.52
cysteine	aa	1.18	6.19	3.73	-3.64
NA_32	na	1.61	6.19	4.23	-3.57
glycerol-2-phosphate	pho	1.69	6.18	13.27	5.40
disaccharide_21	disac	2.96	6.11	4.81	-4.27
glutamine [-H2O]	aa	1.27	6.10	2.71	-4.67

NA_27	na	3.07	6.03	1.79	-7.31
itaconic acid	ac	2.26	5.94	7.82	-0.38
disaccharide_25	disac	1.20	5.89	4.16	-2.92
(E)-aconitic acid	ac	1.94	5.84	4.59	-3.19
valine	aa	0.47	5.78	4.15	-2.09
leucine	aa	0.47	5.66	2.94	-3.18
NA_41	na	1.71	5.51	4.85	-2.38
lysine	aa	0.99	5.44	3.72	-2.71
NA_9	na	1.11	5.40	3.24	-3.27
feruloyl deriv.	aro	1.53	5.35	7.72	0.84
4-aminobutanoic acid	aa	0.74	5.34	3.79	-2.29
disaccharide_28	disac	5.43	5.32	11.66	0.91
glycerophosphoglycerol	pho	1.79	5.21	3.96	-3.03
trisaccharide_5	trisac	1.14	5.21	6.77	0.42
hexose_3	hex	1.43	5.20	5.62	-1.01
glutaric acid	ac	1.31	5.04	2.61	-3.74
raffinose	trisac	3.03	5.02	5.29	-2.76
NA_39	na	1.51	5.01	7.55	1.02
NA_31	na	1.86	5.01	4.14	-2.74
phenylalanine	aa	1.16	4.99	4.07	-2.07
glutamic acid	aa	0.91	4.96	3.84	-2.03
glycerol-3-phosphate	pho	1.25	4.91	4.19	-1.96
acid_1	ac	1.30	4.84	3.29	-2.86
galactinol	alc	1.24	4.73	3.24	-2.73
5,6-dihydrothymine	pym	1.43	4.73	6.54	0.38
NA_16	na	1.59	4.71	4.81	-1.49
NA_37	na	1.26	4.61	3.56	-2.30
disaccharide_29	disac	1.95	4.60	4.97	-1.59
glycerol-3-phosphate	pho	1.38	4.58	2.62	-3.35
NA_40	na	2.09	4.57	4.82	-1.84
amine_3	am	1.14	4.56	3.32	-2.38
myo-inositol-2-phosphate	pho	1.20	4.55	3.57	-2.18
NA_22	na	1.35	4.53	3.56	-2.32
myo-inositol	alc	1.25	4.49	3.47	-2.27
disaccharide_1	disac	1.29	4.48	3.13	-2.64
acid_3	ac	1.54	4.48	2.17	-3.84
asparagine [-H2O]	aa	1.27	4.42	2.39	-3.30
NA_45	na	1.55	4.37	3.13	-2.79
disaccharide_13	disac	1.90	4.35	3.81	-2.43
acid_4	ac	1.85	4.29	5.06	-1.08
threonic acid-1,4-lactone	ac	1.73	4.25	1.98	-4.01
NA_50	na	2.82	4.25	10.35	3.29
β-alanine	aa	0.82	4.24	4.00	-1.06

threonine	aa	1.01	4.22	4.27	-0.96
saccharic acid	ac	1.02	4.20	3.47	-1.75
disaccharide_2	disac	1.85	4.20	3.63	-2.42
4-hydroxybutanoic acid	ac	1.24	4.19	1.99	-3.45
hexacosanoic acid	fa	1.29	4.18	2.44	-3.03
disaccharide_9	disac	2.01	4.07	3.07	-3.00
amine_1	am	0.95	4.05	2.16	-2.85
ethanolamine-O-phosphate	pho	0.99	4.04	3.36	-1.68
glucose-6-phosphate	pho	2.36	3.98	2.62	-3.72
NA_17	na	1.17	3.92	2.00	-3.09
Dodecanol	alc	1.18	3.88	2.77	-2.29
2-aminoadipic acid	aa	1.31	3.87	2.38	-2.79
disaccharide_30	disac	1.24	3.85	3.02	-2.06
threonic acid	ac	1.38	3.83	2.17	-3.04
acetopyruvic acid	ac	1.02	3.77	5.29	0.50
amine_2	am	0.90	3.76	2.70	-1.96
3-deoxy-2,4,5-trihydroxypentonic acid	ac	1.46	3.64	3.51	-1.59
aromatic_2	aro	1.24	3.58	2.36	-2.46
disaccharide_6	disac	1.35	3.57	3.04	-1.88
6-deoxy-mannopyranose	hex	1.40	3.52	2.77	-2.15
aspartic acid	aa	0.69	3.52	5.05	0.84
sugar acid_1	ac	1.36	3.44	2.98	-1.82
(E)- 4-caffeoylquinic acid	aro	1.96	3.44	4.49	-0.92
disaccharide_18	disac	1.11	3.43	2.21	-2.33
2-methylfumaric acid	ac	1.88	3.41	3.11	-2.18
ethanolamine	am	1.30	3.35	3.04	-1.61
NA_47	na	1.32	3.34	3.22	-1.44
feruloyl deriv.	aro	1.37	3.34	1.60	-3.10
NA_20	na	1.56	3.29	2.27	-2.58
disaccharide_11	disac	1.61	3.27	4.65	-0.23
phytol	ter	0.66	3.26	1.57	-2.35
NA_49	na	1.22	3.24	2.65	-1.81
trisaccharide_6	trisac	1.29	3.16	3.01	-1.44
disaccharide_33	na	1.63	3.15	4.10	-0.67
carbonic acid (2TMS)	ac	2.09	3.13	1.62	-3.59
glycerolaldopyranosid	glsug	2.51	3.12	2.44	-3.19
pyruvic acid	ac	1.91	3.11	2.59	-2.42
adenine	pur	2.35	3.09	2.35	-3.09
NA_12	na	1.34	3.07	2.51	-1.90
phosphoric acid monomethyl ester	pho	1.13	3.06	5.03	0.84
gluconic acid	ac	1.11	3.05	2.72	-1.45
disaccharide_36	disac	1.92	3.03	2.21	-2.73

shikimic acid	ac	1.42	2.95	3.00	-1.36
acid_2	ac	1.39	2.94	1.58	-2.74
disaccharide_22	disac	1.06	2.94	3.34	-0.66
disaccharide_34	disac	1.34	2.93	3.38	-0.89
NA_38	na	1.40	2.92	3.23	-1.09
sugar acid_3	ac	1.32	2.86	2.69	-1.49
1,2-propanediol	alc	1.68	2.83	2.08	-2.42
campesterol	ster	0.99	2.80	2.37	-1.42
(E)-ferulic acid	aro	1.07	2.72	3.08	-0.71
oxalic acid	ac	1.13	2.72	1.34	-2.51
p-coumaric acid	aro	0.90	2.72	2.98	-0.64
acid_5	ac	2.44	2.71	3.60	-1.55
NA_46	na	1.19	2.71	2.04	-1.85
hydrogen sulfide	sul	1.03	2.66	2.36	-1.33
2-hydroxy pyridine	pyr	1.46	2.64	1.28	-2.82
caffeoylquinic acid deriv._2	aro	1.43	2.64	3.09	-0.98
3-aminobutanoic acid	aa	1.44	2.60	1.68	-2.37
diglycine	amd	0.47	2.59	2.56	-0.50
phosphate_1	pho	1.97	2.48	1.57	-2.88
6-kestose	trisac	0.93	2.47	2.34	-1.06
stigmasterol	ster	0.97	2.43	1.73	-1.67
tetracosanoic acid	fa	1.09	2.42	2.03	-1.48
hexose_1	hex	1.25	2.41	2.45	-1.21
NA_56	na	1.41	2.36	2.45	-1.32
lyxonic acid	ac	1.17	2.36	2.45	-1.08
β-sitosterol	ster	0.90	2.35	1.60	-1.65
9,12,15-(Z,Z,Z)-octadecatrienoic acid	fa	1.96	2.33	1.77	-2.53
NA_51	na	1.27	2.33	1.77	-1.84
NA_54	na	1.41	2.33	4.24	0.50
hexose_8	hex	2.13	2.32	6.49	2.04
glycerol	alc	1.13	2.32	2.00	-1.44
caffeoylquinic acid deriv._3	aro	1.19	2.29	1.22	-2.25
NA_35	na	1.19	2.28	3.35	-0.12
docosanoic acid	fa	1.54	2.24	1.68	-2.09
disaccharide_35	disac	1.03	2.23	2.91	-0.35
glucopyranose [-H2O]	hex	1.24	2.20	1.82	-1.62
3-hydroxybutanoic acid	ac	1.01	2.20	2.15	-1.06
NA_34	na	1.44	2.20	1.48	-2.16
trisaccharide_4	trisac	1.08	2.19	1.72	-1.55
NA_19	na	1.32	2.18	2.14	-1.36
NA_21	na	1.31	2.18	1.96	-1.52
trisaccharide_1	trisac	1.31	2.16	1.88	-1.60

disaccharide_8	disac	1.08	2.14	1.98	-1.24
homoserine	aa	1.19	2.12	1.39	-1.92
feruloylquinic acid deriv.	aro	1.41	2.08	1.75	-1.74
carbodiimide	amd	0.88	2.08	1.58	-1.38
NA_28	na	1.36	2.06	1.72	-1.70
(Z)-4-hydroxycinnamic acid	aro	1.93	2.05	4.27	0.30
heneicosan-1-ol	alc	1.05	2.04	1.49	-1.61
glycine derivative	aa	1.27	2.04	10.86	7.55
caffeoylquinic acid deriv._1	aro	0.75	2.03	3.35	0.57
Allantoin	pur	1.27	2.02	2.74	-0.55
carboxyglycine	aa	1.35	2.00	1.18	-2.17
Diglyceride	digl	1.27	2.00	4.39	1.11
adipo-2,6-lactam	ac	1.11	1.97	1.17	-1.91
2-methylcitric acid	ac	1.27	1.97	1.67	-1.57
NA_3	na	1.19	1.96	3.31	0.16
ditertbutylphenol	aro	1.00	1.92	1.46	-1.46
succinic acid	ac	1.57	1.91	1.27	-2.21
(E)-caffeic acid	aro	0.94	1.89	2.22	-0.61
NA_13	na	1.13	1.86	1.35	-1.64
NA_7	na	1.19	1.86	1.41	-1.63
NA_6	na	1.38	1.85	2.54	-0.69
mannitol	alc	0.83	1.85	1.93	-0.75
NA_24	na	1.09	1.84	1.32	-1.61
disaccharide_31	disac	1.26	1.83	4.13	1.04
o-methylbenzoic acid	aro	1.08	1.81	1.43	-1.46
hexadecanoic acid	fa	0.96	1.80	1.37	-1.40
glutamic acid	aa	0.64	1.80	1.67	-0.77
octadecanoic acid	fa	0.94	1.80	1.33	-1.41
isocitric acid	ac	1.33	1.80	1.13	-1.99
acid_6	ac	1.42	1.78	2.38	-0.82
sugar acid_2	ac	0.93	1.78	1.41	-1.30
benzoic acid	aro	0.96	1.77	1.22	-1.51
tetradecanoic acid	fa	0.89	1.75	1.34	-1.31
1,2-ethanediol	alc	0.96	1.75	1.60	-1.11
putrescine	am	1.42	1.74	1.89	-1.26
NA_15	na	0.48	1.73	1.23	-0.99
sucrose	disac	1.40	1.73	1.56	-1.57
fructose-6-phosphate	pho	1.09	1.71	1.38	-1.42
9-12-(Z,Z)-octadecadienoic acid	fa	0.99	1.71	1.46	-1.25
octadecan-1-ol	alc	0.91	1.71	1.52	-1.10
NA_8	na	1.69	1.71	1.59	-1.81
citric acid	ac	1.32	1.71	1.16	-1.86
hexose_7	hex	1.27	1.67	1.56	-1.38

trisaccharide_8	trisac	1.27	1.67	1.42	-1.52
aromatic_1	aro	0.97	1.67	1.20	-1.44
caffeoylquinic acid deriv._4	aro	1.22	1.64	1.33	-1.53
NA_60	na	1.05	1.63	1.81	-0.87
disaccharide_19	disac	1.06	1.63	2.16	-0.53
malonic acid deriv.	ac	1.05	1.62	1.09	-1.58
tyrosine	aa	0.50	1.62	2.09	-0.03
2,5-dimethyl-2,5-bis-hydroxyhexane	alc	1.19	1.61	1.40	-1.40
fumaric acid	ac	0.88	1.61	1.01	-1.48
glucose-6-phosphate	pho	1.08	1.59	1.41	-1.26
sugar acid_4	ac	1.20	1.59	1.74	-1.05
trimethylenediamine	am	0.77	1.55	1.19	-1.14
NA_10	na	1.07	1.54	1.17	-1.44
hexanol	alc	0.95	1.53	1.14	-1.34
glucose-6-phosphate	pho	1.00	1.53	1.30	-1.23
1-monopalmitin	mgl	0.80	1.51	1.62	-0.70
2-hydroxybutanoic acid	ac	1.16	1.51	1.28	-1.39
NA_53	na	1.05	1.48	2.44	-0.10
NA_48	na	1.63	1.48	0.86	-2.26
dehydroascorbic acid dimer	ac	1.14	1.47	1.32	-1.29
malonic acid	ac	0.65	1.47	1.13	-0.99
NA_4	na	1.57	1.46	1.50	-1.53
hentriacontanoic acid	ac	0.89	1.45	0.72	-1.62
hexose_5	hex	1.55	1.45	1.47	-1.52
disaccharide_27	disac	0.84	1.38	13.15	10.93
phosphoric acid	pho	0.74	1.36	1.51	-0.59
NA_30	na	1.00	1.35	0.88	-1.47
feruloyl deriv.	aro	1.19	1.35	1.20	-1.33
hexose_6	hex	10.63	1.32	5.67	-6.29
disaccharide_20	disac	0.42	1.29	0.96	-0.75
maleic acid	ac	10.28	1.27	0.70	-10.84
trisaccharide_7	trisac	0.81	1.25	1.44	-0.62
NA_25	na	1.02	1.24	0.81	-1.45
ribose	pen	1.28	1.22	0.96	-1.55
disaccharide_15	disac	1.33	1.17	0.79	-1.71
lactic acid	ac	0.69	1.13	1.17	-0.66
glucose	hex	1.89	1.10	0.85	-2.13
quinic acid	ac	1.27	1.09	1.46	-0.91
glyceric acid	ac	1.30	1.08	0.80	-1.58
9-(Z)-octadecenoic acid	fa	0.82	1.08	0.93	-0.97
NA_59	na	0.52	1.06	1.35	-0.24
glucose	hex	1.92	1.01	0.76	-2.17

NA_23	na	0.73	0.95	1.26	-0.42
Glycine	aa	0.45	0.87	0.48	-0.84
malic acid	ac	1.13	0.87	0.55	-1.45
NA_29	na	1.60	0.73	0.76	-1.57
arabinonic acid	ac	2.92	0.66	0.50	-3.08
(E)-5-caffeoylquinic acid	aro	0.90	0.64	0.98	-0.57
Pentose	pen	1.64	0.64	1.76	-0.52
dodecanoic acid	fa	1.04	0.64	0.61	-1.07
lyxose	pen	1.13	0.59	0.43	-1.29
disaccharide_23	disac	1.64	0.57	0.68	-1.53
fructose	hex	0.80	0.54	0.42	-0.91
phosphate_2	pho	0.64	0.52	0.30	-0.86
2,4-dihydroxy-butanoic acid	ac	1.52	0.50	0.31	-1.71
2-hydroxyglutaric acid	ac	1.06	0.50	0.37	-1.19
glycolic acid	ac	0.43	0.44	0.32	-0.55
L-alanine	aa	0.35	0.43	0.24	-0.54
trisaccharide_3	trisac	3.42	0.39	0.33	-3.47
glucose 2	hex	1.17	0.38	0.22	-1.33
galactose	hex	1.00	0.37	0.30	-1.06
hexose_2	hex	1.06	0.36	0.17	-1.25
sorbitol	alc	1.10	0.34	0.20	-1.25
fructose	hex	1.11	0.33	0.15	-1.29
fructose _3	hex	1.10	0.33	0.15	-1.27
Glycine	aa	0.30	0.23	0.06	-0.46
pyruvic acid	ac	0.69	0.03	0.02	-0.70
allantoin	pur	0.88	0.02	0.03	-0.87

aa- amino acid, ac- organic acid, am- amine, amd- amide, ter- terpenoid, hex-hexose, alc-alcohol, pur-purine, pyr- pyrimidine, pho-phosphate, disac-disaccharide, fa-fatty acid, ster- sterol, trisac-trisaccharide, aro-aromatic compound, pen-pentose, hex-hexose, na-not identified,

APPENDIX 3

Table A3-1. Combined 3-way Factorial ANOVA.

A summary table of all P-values from the analysis of the various parameters (ANOVA Table)

				P-Values			
METABOLITES	MOISTURE	GENOTYPE	TEMP	MOISTURE*GENOTYPE	MOISTURE*TEMP	GENOTYPE*TEMP	MOISTURE*GENOTYPE*TEMP
NA 1	0.614	0.876	0.014	0.222	0	0.059	0.105
hydrogen sulfide	0.748	0.204	0	0.063	0.961	0.664	0.297
carbodiimide	0.058	0.25	0	0.043	0.957	0.537	0.159
NA_2	0.924	0.063	0.002	0.577	0	0.074	0.595
1,2-ethanediol	0.97	0.436	0.012	0.294	0.743	0.738	0.989
hexanol	0.045	0.473	0	0.164	0.129	0.442	0.048
1,2-propanediol	0.016	0.574	0	0.977	0.007	0.498	0.872
2-hydroxy pyridine	0.269	0.05	0.006	0.367	0	0.575	0.651
pyruvic acid	0.209	0.258	0	0.567	0.31	0.898	0.941
lactic acid	0.349	0.044	0.011	0.22	0.036	0.614	0.494
NA_3	0.187	0.005	0.001	0.005	0.874	0.083	0.047
glycolic acid	0.416	0.56	0.221	0.008	0.827	0.067	0.096
pyruvic acid	0.734	0.075	0.024	0.859	0.22	0.39	0.352
valine	0.676	0.55	0	0.137	0.803	0.532	0.348
NA_4	0.107	0.006	0.297	0.983	0.286	0.559	0.477
L-alanine	0.089	0.741	0.249	0.836	0.843	0.591	0.393
glycine	0.005	0.027	0.003	0.004	0.578	0.237	0.001
oxalic acid	0.153	0	0.019	0.689	0.191	0.224	0.753
carbonic acid	0.494	0.235	0.015	0.592	0	0.571	0.783
2-hydroxybutanoic acid	0.248	0.489	0.011	0.353	0.14	0.201	0.425
β-lactic acid	0.443	0.095	0	0.509	0.591	0.913	0.123
amine_1	0.812	0	0	0.031	0.127	0.051	0.72
leucine	0.53	0.944	0	0.074	0.822	0.94	0.102

3-hydroxybutanoic acid	0.506	0.397	0.017	0.002	0.673	0.448	0.456
2-aminobutanoic acid	0.678	0.775	0	0.431	0.745	0.108	0.345
phosphoric acid monomethyl este	0.021	0.79	0	0.012	0.345	0.974	0.848
isoleucine	0.689	0.922	0	0.028	0.902	0.785	0.115
glycine derivative	0.004	0.006	0	0.003	0.197	0.001	0.056
malonic acid	0.311	0.074	0.007	0.087	0.931	0.62	0.857
acid_1	0.682	0.661	0	0.356	0.078	0.818	0.973
NA_5	0.274	0.375	0	0.689	0.951	0.215	0.693
valine	0.122	0.568	0	0.054	0.257	0.422	0.244
3-aminobutanoic acid	0.615	0.537	0	0.98	0	0.569	0.432
NA_6	0.103	0.338	0	0.008	0.295	0.199	0.435
4-hydroxybutanoic acid	0.449	0.001	0	0.036	0.024	0.269	0.309
benzoic acid	0.22	0.676	0.001	0.068	0.585	0.23	0.278
NA_7	0.992	0.336	0	0.132	0.014	0.348	0.711
serine	0.384	0.657	0	0.044	0.153	0.268	0.732
ethanolamine	0.747	0.916	0	0.094	0.4	0.716	0.131
phosphoric acid	0.817	0.039	0.001	0.974	0.141	0.667	0.267
leucine	0.166	0.612	0	0.064	0.483	0.569	0.123
glycerol	0.691	0.74	0	0.025	0.926	0.417	0.974
2,5-dimethyl-2,5-bis-hydroxyhex	0.18	0.546	0.005	0.195	0.154	0.302	0.325
isoleucine	0.224	0.937	0	0.056	0.535	0.398	0.378
threonine	0.603	0.789	0	0.158	0.707	0.749	0.66
proline	0.367	0.549	0	0.015	0.073	0.597	0.42
maleic acid	0.172	0.04	0.012	0.975	0.03	0.281	0.566
glycine	0.083	0.025	0.48	0.002	0.761	0.105	0.03
succinic acid	0.606	0.581	0.071	0.721	0	0.228	0.041
glyceric acid	0.504	0.241	0.024	0.975	0.074	0.788	0.296
itaconic acid	0	0.143	0	0.447	0.056	0.51	0.154
pyrrole-2-carboxylic acid	0.873	0.724	0	0.857	0.188	0.415	0.233
fumaric acid	0.009	0.003	0.121	0.051	0.797	0.749	0.025
serine	0.544	0.714	0	0.171	0.71	0.278	0.874

o-methylbenzoic acid	0.462	0.48	0	0.471	0.06	0.869	0.418
threonic acid-1,4-lactone	0.911	0.021	0	0.977	0	0.971	0.735
acid_2	0.569	0.12	0.008	0.086	0.031	0.35	0.796
NA_8	0.221	0.017	0.71	0.764	0.364	0.158	0.923
threonine	0.554	0.389	0	0.465	0.818	0.893	0.922
malonic acid deriv.	0.05	0.286	0	0.304	0.001	0.339	0.421
2-methylfumaric acid	0.039	0.469	0	0.941	0.035	0.835	0.193
glutaric acid	0.853	0.589	0.002	0.566	0.21	0.823	0.581
NA_9	0.858	0.237	0	0.11	0.528	0.338	0.967
2,4-dihydroxy-butanoic acid	0.921	0.059	0	0.313	0.041	0.339	0.58
NA_10	0.626	0.603	0.029	0.273	0.224	0.575	0.119
aspartic acid	0.729	0.344	0	0.041	0.002	0.753	0.002
β-alanine	0.834	0.012	0	0.218	0.397	0.642	0.706
NA_11	0.382	0.579	0	0.594	0.02	0.306	0.613
5,6-dihydrothymine	0.009	0.147	0	0.059	0.394	0.093	0.868
carboxyglycine	0.829	0.946	0.315	0.163	0.046	0.45	0.33
acid_3	0.528	0.187	0.008	0.971	0.15	0.34	0.225
NA_12	0.433	0.865	0	0.049	0.282	0.824	0.23
malic acid	0.333	0.167	0	0.856	0.176	0.546	0.996
acetopyruvic acid	0.147	0.001	0	0.19	0.346	0.043	0.836
asparagine [-H2O]	0.558	0.478	0	0.332	0.211	0.885	0.788
NA_13	0.724	0.187	0	0.726	0.022	0.857	0.946
adipo-2,6-lactam	0.066	0.054	0.036	0.749	0.016	0.326	0.989
aspartic acid	0.324	0.702	0	0.688	0.031	0.228	0.018
4-aminobutanoic acid	0.09	0.056	0	0.273	0.835	0.415	0.662
glutamic acid	0.781	0.088	0	0.976	0.851	0.132	0.106
ditertbutylphenol	0.072	0.057	0	0.919	0.34	0.858	0.445
Phenylalanine	0.885	0.488	0	0.283	0.356	0.987	0.823
diglycine	0.433	0.964	0	0.061	0.649	0.066	0.312
cysteine	0.722	0.301	0	0.993	0.134	0.091	0.019
threonic acid	0.873	0.748	0	0.038	0.057	0.639	0.336

NA_14	0.013	0.018	0	0.603	0.18	0.009	0.911
dodecanol	0.817	0.894	0	0.378	0.379	0.462	0.059
serine	0.186	0.345	0	0.249	0.017	0.448	0.187
2-hydroxyglutaric acid	0.837	0.935	0	0.667	0.18	0.666	0.697
proline [+CO2]	0.843	0.811	0	0.434	0.808	0.088	0.921
glutamine [-H2O]	0.598	0.484	0	0.813	0.038	0.829	0.396
NA_15	0.623	0.943	0.184	0.124	0.709	0.729	0.359
phosphate_1	0.682	0.94	0.937	0.669	0.054	0.797	0.812
NA_16	0.358	0.007	0	0.004	0.114	0.129	0.074
NA_17	0.377	0.431	0	0.009	0.023	0.428	0.848
acid_4	0.032	0.787	0.047	0.624	0.449	0.193	0.981
glutamic acid	0.685	0.295	0	0.85	0.512	0.066	0.247
phenylalanine	0.742	0.634	0	0.066	0.475	0.29	0.126
NA_18	0.119	0.946	0	0.797	0.473	0.033	0.414
acid_5	0.002	0	0	0.266	0.114	0.795	0.46
trimethylenediamine	0.027	0	0	0.007	0.68	0.801	0.045
3-deoxy-2,4,5-trihydroxypentoni	0.232	0.505	0.001	0.983	0.718	0.502	0.855
NA_19	0.041	0.011	0.002	0.01	0.52	0.721	0.787
dodecanoic acid	0.922	0.171	0.012	0.311	0.909	0.812	0.855
lyxose	0.501	0.548	0	0.128	0.203	0.046	0.842
NA_20	0.936	0.001	0	0.204	0.032	0.229	0.942
homoserine	0.176	0.007	0	0.894	0	0.783	0.929
asparagine	0.068	0.856	0	0.208	0.774	0.607	0.784
NA_21	0.342	0.656	0	0.158	0.065	0.897	0.585
ribose	0.409	0.377	0.54	0.017	0.046	0.031	0.655
pentose	0.138	0.086	0.467	0.02	0.968	0.132	0.396
NA_22	0.499	0.166	0	0.045	0.077	0.263	0.465
6-deoxy-mannopyranose	0.555	0.183	0.005	0.416	0.202	0.594	0.576
NA_23	0.927	0.491	0.169	0.391	0.5	0.566	0.714
glycerol-2-phosphate	0.005	0.019	0	0.011	0.571	0.042	0.939
ribulose	0.478	0	0.002	0.732	0.002	0	0.575

acid_6	0.944	0	0.18	0.048	0.373	0.062	0.416
NA_24	0.076	0.075	0	0.914	0.003	0.858	0.313
2-aminoadipic acid	0.75	0.059	0	0.752	0.231	0.296	0.915
2-methyl-2,4-bis-hydroxy-glutar	0.386	0.029	0	0.273	0.275	0.502	0.6
glycerol-3-phosphate	0.471	0.772	0	0.487	0.069	0.073	0.503
NA_25	0.567	0	0.031	0.555	0.817	0	0.08
putrescine	0.154	0.187	0.004	0.004	0.547	0.651	0.394
NA_26	0.923	0.285	0	0.234	0.55	0.428	0.498
NA_27	0.343	0.889	0.088	0.032	0.002	0.1	0.878
(Z)-aconitic acid	0.099	0.019	0	0.092	0.041	0.229	0.588
(E)-aconitic acid	0.647	0.255	0.203	0.635	0.546	0.093	0.923
NA_28	0.279	0.033	0.088	0.553	0.211	0.084	0.707
glycerol-3-phosphate	0.579	0.33	0	0.338	0.256	0.034	0.368
glucopyranose [-H2O]	0.83	0.005	0	0.993	0.152	0.004	0.022
lyxonic acid	0.273	0.897	0	0.001	0.319	0.704	0.18
glutamine	0.149	0.911	0	0.181	0.184	0.473	0.87
arabinonic acid	0.333	0.627	0.248	0.003	0.076	0.34	0.383
ethanolamine-O-phosphate	0.694	0.832	0	0.031	0.441	0.378	0.855
NA_29	0.137	0.086	0.675	0.032	0.398	0.34	0.311
NA_30	0.034	0	0.469	0.925	0.367	0.074	0.132
(Z)-4-hydroxycinnamic acid	0.019	0.827	0.002	0.176	0.968	0.684	0.519
NA_31	0.13	0.96	0	0.644	0.169	0.905	0.734
NA_32	0.548	0.962	0	0.866	0.204	0.353	0.542
fructose	0.512	0.07	0.331	0.131	0.775	0.294	0.231
sugar acid_1	0.157	0.727	0	0.189	0.104	0.107	0.694
NA_33	0.285	0.24	0.002	0.471	0.001	0.021	0.315
shikimic acid	0.331	0.085	0	0.001	0.075	0.003	0.856
NA_34	0.947	0.001	0.702	0.905	0.23	0.486	0.915
citric acid	0.953	0	0.115	0.332	0.069	0.578	0.096
isocitric acid	0.631	0	0.104	0.451	0.024	0.408	0.058
NA_35	0.696	0.019	0.042	0.003	0.963	0.353	0.87

2-methylcitric acid	0.377	0.41	0	0.058	0.054	0.094	0.99
dehydroascorbic acid dimer	0.615	0.001	0.164	0.593	0.205	0.23	0.584
tetradecanoic acid	0.054	0.527	0	0.644	0.579	0.75	0.107
quinic acid	0.949	0.002	0.304	0.002	0.186	0	0.106
adenine	0.437	0.183	0	0.276	0.002	0.026	0.02
fructose _2	0.796	0.394	0.039	0.001	0.003	0.016	0.351
fructose _3	0.8	0.441	0.045	0.001	0.003	0.017	0.358
allantoin	0.629	0.181	0.504	0.013	0.824	0.758	0.027
Galactose	0.613	0.3	0.352	0.01	0.573	0.148	0.005
tyrosine	0.092	0.026	0	0.187	0.036	0.454	0.147
glucose _1	0.187	0.286	0.649	0.007	0.008	0.007	0.284
glucose _2	0.862	0.787	0	0.001	0.004	0.003	0.674
allantoin	0.027	0.102	0	0.689	0.79	0.38	0.198
sorbitol	0.854	0.667	0	0	0.005	0.005	0.597
histidine	0.437	0.959	0	0.282	0.794	0.848	0.913
lysine	0.638	0.591	0	0.03	0.787	0.738	0.141
NA_36	0.979	0.063	0.187	0.324	0	0.716	0.205
NA_37	0.106	0	0	0.381	0.157	0.647	0.467
mannitol	0.821	0.668	0	0.37	0.24	0.855	0.146
tyrosine	0.678	0.675	0	0.034	0.372	0.342	0.397
p-coumaric acid	0.869	0.535	0	0.07	0.714	0.407	0.176
sugar acid_2	0.691	0.008	0	0.446	0.132	0.36	0.609
NA_38	0.011	0.002	0	0.039	0.722	0.27	0.6
NA_39	0.092	0	0	0.494	0.385	0.635	0.163
NA_40	0.226	0.057	0	0.382	0.069	0.083	0.431
Glucose	0.066	0.371	0.634	0.006	0.005	0.005	0.15
NA_41	0.064	0.039	0	0.125	0.044	0.094	0.876
hexose_1	0.253	0.959	0	0.021	0.236	0.322	0.632
galactonic acid	0.803	0.55	0.005	0.672	0.005	0.791	0.442
gluconic acid	0.815	0.518	0	0.974	0.708	0.442	0.7
NA_42	0.144	0.716	0.049	0.706	0.001	0.022	0.223

NA_43	0.82	0.693	0	0.876	0.033	0.389	0.642
hexose_2	0.704	0.314	0	0.001	0.002	0.002	0.277
saccharic acid	0.903	0	0	0.34	0.471	0.242	0.526
hexadecanoic acid	0.014	0.164	0	0.467	0.171	0.88	0.087
NA_44	0.086	0.171	0	0.032	0.11	0.498	0.885
hexose_3	0.2	0.007	0	0.511	0.927	0.743	0.479
amine_2	0.974	0	0	0.741	0.906	0.007	0.279
myo-inositol	0.934	0.76	0	0.086	0.177	0.769	0.559
(E)-ferulic acid	0.052	0.372	0	0.022	0.608	0.551	0.099
NA_45	0.894	0.323	0	0.298	0.453	0.318	0.979
phosphate_2	0.037	0.197	0.004	0.029	0.245	0.689	0.01
NA_46	0.642	0.585	0	0.541	0.376	0.503	0.652
NA_47	0.646	0.111	0	0.93	0.277	0.045	0.976
NA_48	0.66	0.528	0.523	0.884	0.094	0.668	0.95
(E)-caffeic acid	0.175	0.89	0	0.036	0.17	0.977	0.011
octadecan-1-ol	0.312	0.741	0.005	0.893	0.793	0.3	0.226
hexose_4	0.004	0.363	0	0.352	0.537	0.578	0.807
phytol	0.134	0.839	0	0.119	0.532	0.993	0.783
hexose_5	0.651	0	0.008	0.985	0.663	0.004	0.259
hexose_6	0.074	0.356	0.656	0.229	0.193	0.241	0.389
glycerophosphoglycerol	0.886	0	0	0.87	0.395	0.076	0.733
tryptophan	0.324	0.05	0	0.053	0.214	0.156	0.383
phosphate_3	0.214	0.133	0	0.809	0.35	0.415	0.316
9-12-(Z,Z)-octadecadienoic acid	0.39	0.765	0	0.033	0.664	0.391	0.077
tryptophan	0.306	0.35	0	0.153	0.182	0.332	0.108
9-(Z)-octadecenoic acid	0.544	0.113	0.63	0.8	0.817	0.333	0.187
9,12,15-(Z,Z,Z)-octadecatrienoi	0.115	0.066	0.001	0.714	0.001	0.805	0.053
octadecanoic acid	0.007	0.072	0	0.407	0.154	0.884	0.204
NA_49	0.747	0.05	0	0.006	0.038	0.699	0.1
hexose_7	0.285	0.219	0.001	0.007	0.033	0.029	0.559
NA_50	0.002	0.001	0	0.011	0.745	0.024	0.235

fructose-6-phosphate	0.656	0.536	0	0.305	0.113	0.028	0.266
Glycerolaldopyranosid	0.034	0.004	0	0.872	0.002	0.591	0.175
glucose-6-phosphate	0.581	0.575	0.004	0.843	0.304	0.133	0.032
hexose_8	0.014	0.144	0.003	0.198	0.862	0.072	0.484
glucose-6-phosphate	0.758	0.473	0.004	0.903	0.335	0.158	0.037
fructose-6-phosphate	0.843	0.599	0	0.028	0.02	0.001	0.52
NA_51	0.733	0.341	0	0.035	0.03	0.084	0.671
disaccharide_1	0.782	0.136	0	0.361	0.048	0.868	0.929
glucose-6-phosphate	0.541	0.083	0.002	0.427	0.006	0.92	0.992
disaccharide_2	0.107	0.129	0	0.242	0.528	0.149	0.524
disaccharide_3	0.132	0.212	0	0.297	0.26	0.537	0.687
myo-inositol-2-phosphate	0.829	0.988	0	0.168	0.165	0.347	0.572
disaccharide_4	0.222	0.802	0	0.44	0.594	0.938	0.768
disaccharide_5	0.253	0.439	0	0.067	0.34	0.616	0.981
heneicosan-1-ol	0.051	0.177	0	0.644	0.039	0.346	0.45
disaccharide_6	0.234	0.551	0	0.202	0.225	0.902	0.729
disaccharide_7	0.16	0.069	0	0.463	0.717	0.424	0.71
disaccharide_8	0.4	0.118	0	0.409	0.658	0.651	0.1
disaccharide_9	0.038	0.823	0	0.959	0.002	0.849	0.426
disaccharide_10	0.028	0.713	0	0.606	0.884	0.921	0.78
disaccharide_11	0.361	0.22	0.003	0.184	0.379	0.3	0.995
disaccharide_12	0.636	0.004	0	0.494	0.334	0.828	0.228
farnesyl deriv.	0.981	0.798	0	0.705	0.092	0.309	0.87
1-monopalmitin	0.293	0.018	0	0.067	0.058	0.655	0.083
sucrose (8TMS)	0.325	0.82	0.168	0.296	0.208	0.247	0.755
docosanoic acid	0.795	0.947	0.003	0.363	0.022	0.525	0.987
disaccharide_13	0.217	0.126	0	0.996	0.157	0.865	0.339
disaccharide_14	0.071	0.038	0	0.934	0.945	0.301	0.91
disaccharide_15	0.751	0.761	0.119	0.023	0.091	0.033	0.884
disaccharide_16	0.029	0.002	0	0.584	0.986	0.886	0.96
disaccharide_17	0.958	0.013	0	0.707	0.163	0.069	0.731

disaccharide_18	0.744	0.327	0.048	0.218	0.173	0.98	0.854
maltose	0.842	0.321	0	0.121	0.293	0.095	0.491
disaccharide_19	0.444	0.002	0.029	0.364	0.786	0.834	0.822
disaccharide_20	0.159	0.747	0.149	0.382	0.37	0.289	0.598
disaccharide_21	0.001	0.953	0	0.891	0	0.914	0.098
disaccharide_22	0.959	0.586	0.001	0.243	0.221	0.141	0.77
disaccharide_23	0.247	0.331	0.294	0	0.074	0.258	0.096
disaccharide_24	0.497	0.909	0.002	0.584	0.026	0.402	0.428
disaccharide_25	0.925	0.178	0	0.116	0.387	0.765	0.436
disaccharide_26	0.17	0.605	0	0.418	0.498	0.83	0.959
disaccharide_27	0.135	0.003	0.004	0.017	0.053	0.084	0.06
tetracosanoic acid	0.559	0.287	0	0.043	0.59	0.231	0.946
disaccharide_28	0.062	0.004	0.001	0.225	0.582	0.297	0.679
coumaroyl glucoside	0.1	0.004	0	0.162	0.264	0.545	0.32
disaccharide_29	0.138	0.277	0.001	0.733	0.422	0.599	0.668
disaccharide_30	0.925	0.33	0.002	0.133	0.375	0.28	0.69
aromatic/sugar	0.019	0.092	0	0.483	0.795	0.272	0.895
sugar acid_3	0.15	0.61	0	0.003	0.263	0.324	0.214
guaiacyl deriv.	0.032	0.006	0	0.104	0.37	0.466	0.863
disaccharide_31	0.002	0.311	0	0.017	0.216	0.156	0.232
disaccharide_32	0.192	0	0	0.813	0.413	0	0.774
amine_3	0.595	0	0	0.389	0.226	0	0.776
galactinol	0.715	0.524	0	0.029	0.175	0.137	0.152
aromatic_1	0.003	0.101	0	0.59	0.038	0.546	0.205
NA_52	0.007	0	0	0.416	0.014	0.77	0.684
γ-tocopherol	0.272	0	0	0.328	0.128	0.201	0.366
caffeic acid deriv.	0.029	0.899	0	0.188	0.352	0.523	0.428
NA_53	0.353	0	0.008	0.431	0.356	0.196	0.433
disaccharide_33	0.044	0.849	0	0.284	0.521	0.894	0.807
hexacosanoic acid	0.925	0.722	0	0.037	0.043	0.511	0.412
disaccharide_34	0.132	0.831	0	0.122	0.554	0.927	0.965

disaccharide_35	0.764	0	0.001	0.211	0.613	0.308	0.489
caffeoylquinic acid deriv._1	0.662	0	0.177	0.452	0.994	0.267	0.619
luteolin	0.751	0.509	0	0.995	0.682	0.725	0.004
caffeoylquinic acid deriv._2	0.449	0	0.083	0.055	0.113	0	0.942
NA_54	0.222	0	0	0.151	0.393	0.006	0.875
feruloylquinic acid deriv.	0.418	0.02	0	0.028	0.015	0.007	0.936
feruloyl deriv.	0.66	0	0.001	0.258	0.017	0.062	0.324
disaccharide_36	0.329	0.181	0	0.654	0.011	0.879	0.233
feruloyl deriv._1	0.057	0.015	0.468	0.323	0.363	0.097	0.19
caffeoylquinic acid deriv._3	0.769	0	0.016	0.371	0.064	0.081	0.517
caffeoylquinic acid deriv._4	0.643	0.123	0.002	0.031	0.016	0.058	0.795
α-tocopherol	0.719	0.246	0	0.271	0.285	0.654	0.915
feruloyl deriv._2	0.212	0.001	0	0.181	0.354	0	0.769
(E)- 4-caffeoylquinic acid	0.246	0	0.002	0.105	0.213	0.137	0.116
kaempferol deriv.	0.922	0.229	0	0.498	0.033	0.796	0.259
NA_55	0.406	0	0	0.048	0.132	0.001	0.587
(E)-5-caffeoylquinic acid	0.125	0	0	0.028	0.222	0	0.012
sugar acid_4	0.777	0	0.6	0.008	0.287	0	0.649
NA_56	0.103	0.478	0	0.209	0.195	0.06	0.935
campesterol	0.462	0.029	0	0.242	0.917	0.427	0.801
NA_57	0.367	0.906	0	0.109	0.385	0.843	0.797
NA_58	0.058	0.607	0	0.27	0.378	0.261	0.216
aromatic_2	0.82	0.552	0	0.095	0.038	0.281	0.665
stigmasterol	0.214	0.677	0	0.674	0.624	0.556	0.862
trisaccharide_1	0.472	0.054	0	0.013	0.043	0.079	0.479
trisaccharide_2	0.459	0.01	0	0.118	0.427	0.076	0.394
β-sitosterol	0.031	0.121	0	0.023	0.459	0.75	0.022
raffinose	0.154	0.002	0	0.062	0.077	0.778	0.921
trisaccharide_3	0.167	0.316	0.066	0.012	0.061	0.998	0.112
β-amyrin	0.001	0.93	0	0.121	0.001	0.537	0.593
trisaccharide_4	0.342	0.162	0.121	0.126	0.617	0.947	0.008

NA_59	0.235	0.301	0.263	0.057	0.413	0.604	0.358
α-amyrin	0.006	0.264	0	0.083	0.011	0.719	0.624
6-kestose	0.6	0.976	0.06	0.089	0.833	0.848	0.603
trisaccharide_5	0.811	0.003	0	0.01	0.93	0.052	0.642
trisaccharide_6	0.303	0.387	0	0.071	0.115	0.131	0.805
trisaccharide_7	0.389	0.108	0.397	0.004	0.994	0.619	0.334
hentriacontanoic acid	0.021	0.837	0.74	0.288	0.034	0.859	0.005
NA_60	0.963	0.076	0.09	0.04	0.393	0.059	0.868
diglyceride	0.004	0.125	0	0.074	0.352	0.032	0.685
trisaccharide_8	0.372	0.28	0.001	0.008	0.013	0.036	0.684

Red represents significant values (P<0.05)