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Comparative metabolic profile of maize genotypes reveals the tolerance mechanism associated in combined stresses

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Abstract
The two maize genotypes CML49 and CML100 were subjected to combined abiotic stresses concurrently (*drought and low-N / waterlogging and low-N*). The aim of study to reveal the differential response of metabolites of two maize genotypes in combined stress conditions and to understand the tolerance mechanism. Thus leaf and roots metabolites of two genotypes were detected and measured using GC-MS technique. The results of un-targeted metabolites analysis show, the accumulated metabolites of tolerant genotype (CML49) have a uniform pattern in response to combined abiotic stresses. Although most of the metabolites were related to defense, antioxidants, signaling and some metabolites indirectly involved in nitrogen restoration of the maize plant. Alternatively, few metabolites of sensitive genotype (CML100) were regulated in response to defense, while other metabolites were involved in membrane disruption and signaling antagonist also the pattern of metabolite regulation was random in sensitive genotype. Therefore, the present study provides insight into the molecular mechanisms of tolerance of maize plants in combined stresses.

Keywords: Combined stresses, GCMS, Metabolomics, drought, Low-N stress, waterlogging stress, and tolerant and sensitive

1. Introduction
Maize is the most important cereal, grown at the wide geographical ranges of latitude and longitude. In South Asia, particularly in tropical and subtropical environment maize is largely grown during summer-rainy season, in marginal areas which often faces extreme water availability such as waterlogging or water scarcity in a form of terminal drought. Correspondingly, co-occurrence of both the stresses often headed to the depletion in nitrogen content, thus causing low-N stress in the soil. Although on many occasions the stresses are for shorter duration and relatively mild in nature, but have a significant impact on plant growth and development. However, studies on combined stresses by Rizhsky *et al.* (2004) [19] revealed that the response of plant to combined stresses is unique and cannot be extrapolated from the response of plant to each individual stress. Though, plants belonging to the same genus showed different molecular response in combined stresses (Aprile *et al.* 2013) [1]. Besides, the composite responses of plants due to various concurrent stresses rely on signals, these varied and contrast signals may interact with each other to enhanced or obstruct one another (Vile *et al.* 2012; Suzuki *et al.* 2014) [26, 23]. Therefore, the nature of the interaction is an important aspect to fully identify the influences of combined abiotic stresses on crop plants. Among omics approach is metabolomics that has been neglected in crop improvement programs (Kumar *et al.* 2017). It has not be fully explored due to its complexity. Though, metabolites plays important role in plant metabolism that influences the plant biomass and architecture (Turner *et al.* 2016) [25]. Metabolomics study enable to identify wide variety of metabolites that gives the comprehensive view of the cellular process and physiological condition that display in a particular stress condition. Besides, the metabolites assign the functional gene and impact of the particular gene on the metabolic pathway, and that provides information of regulation as well interruptions of linked pathways (Wen *et al.* 2015) [27]. Further, metabolomics study enable to improve breeding resources through selecting the remarkable traits (Zivy *et al.* 2015) [29]. Therefore, the aim of metabolomics profiling is to compare the combined stress response in two maize inbred that differed in their stress adaptability and to unrevealed the complex molecular mechanism of tolerance at metabolomics levels in different stress conditions applied simultaneously.

2. Materials and Methods

2.1 Plant Material and Growth conditions

In the present work two maize inbred (CML49 & CML100) were selected that showed different adaptability to various combine stress treatments on this basis we have categorized them as 'tolerant and sensitive.' The selected two inbred (CML49 and CML100) plants with 60 pots each were grown in natural conditions in greenhouse up to 30 days. 30 DAS they were subjected to various stress treatment like, drought, waterlogging and plants were grown in low nitrogen, only 25% of the recommended dose of urea was applied to the treated pots. First 30 pots of each inbred were exposed to a drought and low-N stress for 10 days, while 30 control pots were supplied with full nutrient and water. After re-watering for two days normally, the same plants were exposed to waterlogging x low-N stress for up to 7 days, to maintain the water level 2-3cm above the soil surface of the pots plants were watered day and night. The roots samples from stressed and control replicates plants of each inbred (CML49 & CML100) were kept on the last day of combined stresses for extracting metabolites. Samples were quickly frozen in liquid nitrogen after removing from the plant and then kept at -80^0 C for further analysis.

2.2 Sample Preparations and Metabolites extraction

The leaves and roots samples from three independent biological replicates each from control and treated maize plants (CML49 & CML100) was used for metabolite profiling. Harvested plant samples immediately frozen in liquid Nitrogen in a plastic bag (resistant to liquid N_2). Frozen samples were stored at -80^{0}C. The leaves and roots were homogenized separately in liquid nitrogen into a mortar and pestle to make fine powder. One gram powder of each sample was extracted three times with 5 ml of methanol at room temperature and sonicated for 15 min and centrifuged. The extract was concentrated to 1 ml by Speed Vac. The supernatant filter through whatman filter paper (No. 4), extracts collected and stored at 4 ^{0}C for further use. The prepared extracts of both leaves and roots of the control and treated plants were subjected to gas chromatography–mass spectroscopy (GC–MS) analysis. Samples were analyzed using GC-MS for the analysis of untargeted metabolites. For derivatization, methoxylamine in pyridine (10µl) and *N*-methyl-*N*-trimethylsilyltrifuoroacetamide MSTFA (90µl) were added.

2.3 Gas Chromatography and Mass spectrometry Analysis

The GC-MS analysis was performed with a GC-MS QP-2010 ULTRA equipped with an auto sampler (AOC-20i + s) from Shimadzu (Japan), using Equity-5 column, 30.0 m × 0.25 um × 0.25 mm for separation, and helium was used as a carrier gas at a constant flow rate of 1.0 mL/min. The 1µL sample was injected using 1/100 split-mode injections at a temperature of 260°C. The oven temperature program was initially set at 100°C and held for 2 min. The temperature was gradually increased to 250°C at a rate of 10°C/min, and 300°C at 15°C/min. Ions were generated by a 70ev electron beam at an ionization current of 2.0mA. Total ion chromatogram spectra were recorded in the mass range of 40–900 m/z at the rate of 2.5 spectra s^{-1}. For metabolite identification and annotation, peaks were matched against customized reference spectrum databases including the National Institute of Standards and Technology NIST and the Wiley Registry, internal libraries and further confirmed with ChEBI (http://www.ebi.ac.uk/chebi/init.do) and ChemSpider (http://www.chemspider.com/). Data obtained was then uploaded to the web-based tool Metabo Analyst for high throughput analysis.

3. Results and Discussion:

Metabolomics profiling was conducted using GC-MS of two different maize genotypes one of them was CML49 (tolerant) other one was CML100 (sensitive) after exposing them to combined stresses (drought, waterlogging and low-N stresses). In metabolomic profiling total 132 compounds were identified in two genotypes that includes, fatty acids, (long, medium and short chains), fatty acid derivatives, fatty aldehydes, fatty alcohols, carboxylic acids, terpenoids, glucosinolates, heterocyclic compounds, halohydrogens, alkaloids, phenolic acids, steroids and vitamins. The data was simplified by univariate analysis of two genotypes (CML49 and CML100) through fold change analysis, t-test and volcano plots. Fold change showed the comparison between the control group metabolites with that of treated group metabolites and important features selected by t-test with threshold (p< 0.05) level, the results was plotted in log 2 scale. Thus, same fold change have the same distance from zero base line**.** Sensitive genotype leaves shows higher accumulation of Tocopherol Isophytol, trans-squalene, nonacosane (Table 1) The non-volatile isoprenoid like Tocopherol acts as lipid soluble redox buffer and important scavenger of singlet oxygen species (Foyer *et al.* 2005) [4]. Further, Takshak and Agarwal (2015) [24] reported in UV-B treated leaf of *W somnifera* accumulates higher amount of phyto-constituents like Isophytol, trans-squalene, nonacosane and these plants were effective in scavenging free radicals. Sensitive genotypes leaves have Nonayl Iodide and Nonayl Bromide the signaling antagonists or disrupters of membrane potential. Leaves of sensitive genotypes also emits carveol, and citronellol these terpenes presented a rapid bactericidal effect against E. coli, S aureus, and S. Typhimurium (Guimarães A C *et al.* 2019) [6]. The below ground emission of volatiles the sesquiterpene (E)-β-caryophyllene from roots of sensitive genotype might have role to attract entomopathogenic nematodes to roots damaged by the ferocious maize pest Diabrotica virgifera. Maize varieties that lack this signal have been more vulnerable to maize pest (Rasmann *et al.* 2005; Rasmann and Turlings 2007) [17, 18]. However, metabolites such as Nitropropane, in plants, (3-NPA) is thought to serve as an anti-herbivore defense (Orth R, (1977; Chomcheon P, 2005; Strange R N, 2007) [15, 3, 22] and, in some legumes, to participate in nitrogen fixation (Hipkin C R, 2004) [7]. 2-Nonane have mild fruity, sweet odor may help to attract insects that help in pollination. Hence sensitive genotypes in response to multiple stress release various metabolites in random pattern.

Table 1: Metabolic profile of leaf and roots of tolerant and sensitive maize plants after exposure to combined abiotic stresses.

Tolerant Genotype (CML49)			Sensitive Genotype (CML100)		
Metabolites	**Class**	**Fold Change**	**Metabolites**	**Class**	**Fold Change**
Leaves Metabolites			**Leaves Metabolites**		
Octanoic acid	Saturated F A.	-2.003 ns	Nonyl Iodide	Liner allane	-2.0774**
Eiythrodiol	Triterpenoid	-1.8924 ns	Docosane	Linear Mane	-1.59**
9-Decen4-01	Fatty Alcohol	-1.9768 ns	Nonayl Bromide	Organic compound	-1.4269**
Oleic add	Saturated FA	-1.3913"	Isophytol	Terpenoid	-1.3326**
Linalool	Monoterpenoid	-1.1993 ns	Citionelly1 acetate	Fatty alco ester	-1.3162**
N-Pentadecane	Alkane hydrocarbon -1.0197***		Tocopherol acetate	Steroid	-1.2953**
Roots Metabolites			Canon	Terpenoid	-1.2777**
Furan carboxylic acid	CA	4.4828•*	Heptyl heni ether	Ether	-1.2772**
Acetic acid	carboxylic acid (CA) -1.4511**		Octadecanal	long chain aldehy3e	4.2726**
Pelargonic add	Saturated F A	-1.4303"	Napthalene acid	Organic compound	-1.2717**
Oxalic acid	CA	-1.4271**	Squalene	Steroid	-1.2623**
Caprylic acid	saturated F A	-1.4134"	Phthalate	Ester	1.0616 ns

The metabolomics profile of two different genotypes suggested that the response of maize plants to various stresses applied simultaneously was shared as well as also distinctive. However, tolerant genotype roots metabolite profile display large variance in metabolites accumulation, (mostly are halohydrocarbons, heterocyclic compounds, organic cations and saturated and unsaturated fatty acids, carboxylic acids, esters) compared to roots of sensitive genotype which have less diversity in metabolite accumulation. To reduce the multivariate data complexity, to highlight the similarities and difference patterns between samples, PCA was performed for metabolites concentration data of control and treated leaves and roots of tolerant and in sensitive plants. Also, to verify the difference between the metabolic profiles of the control and treated tissues statistically and to identify the main metabolites responsible for the differences. A score scatter plots of tolerant leaf showed the good separation of metabolites between treated and control plants. In PC1 the variance is greater (70.1%) and PC2 showed variance of (19.3%). Similar trend of metabolites separation was observed of sensitive genotype leaves. The treated plants exhibited higher variances in PC1 (91.2%) and control plants variance is less PC2 (5.2%) Fig 1a, b. However PCA of roots the trend was opposite for both genotypes, the score plot for PC1 (94.8%) and PC2 (4%) demonstrate clear separation between control (root metabolites) and treated (root metabolites) it may be due to the presence of many saturated and unsaturated fatty acids and halo-hydrocarbon in the samples. The presence of haloalkanes (2-Bromopentene, ethyl isocynates) heterocyclic compound (2-Nonyl-1,3-dioxalane), hetrocyclic aromatic compounds (N-Methyl-2-Iodopyrrol), Methyl ester (Thioacetic acid) and Methyl ketone (3-Nitropropane) all these compound processes strong, unpleasant and odor may involve in repelling herbivory insects, pest and bacteria, fungi, nematodes (Lamberth *et al.* 2012; Francis *et al.* 2013) [10, 5].

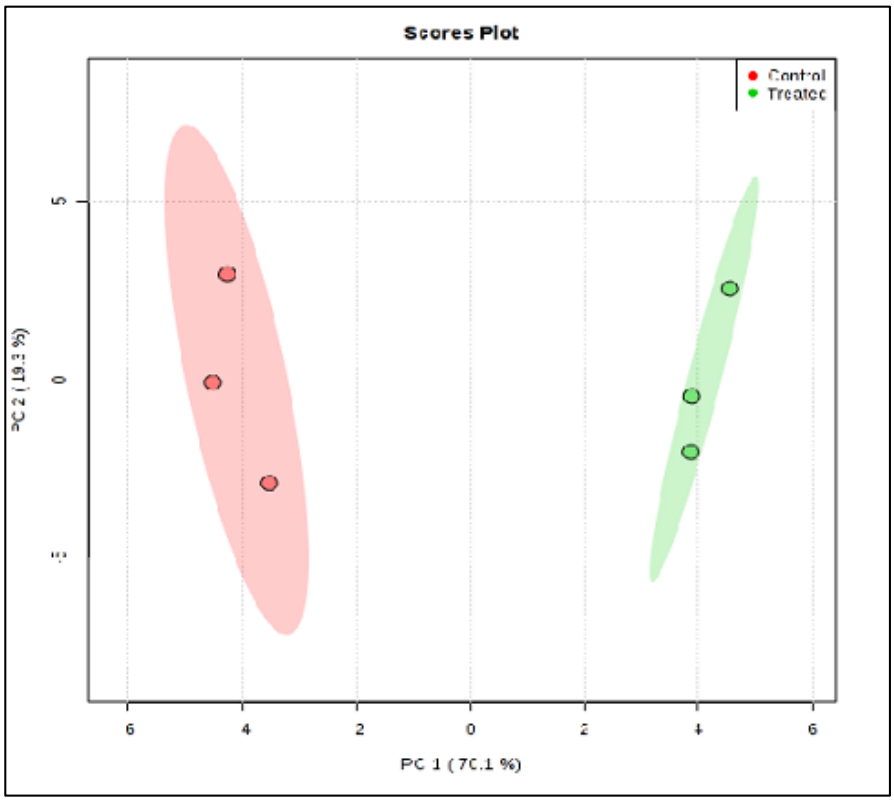

Fig 1a: Score plot of tolerant leaf shows higher variations in metabolites of PC1 than PC2

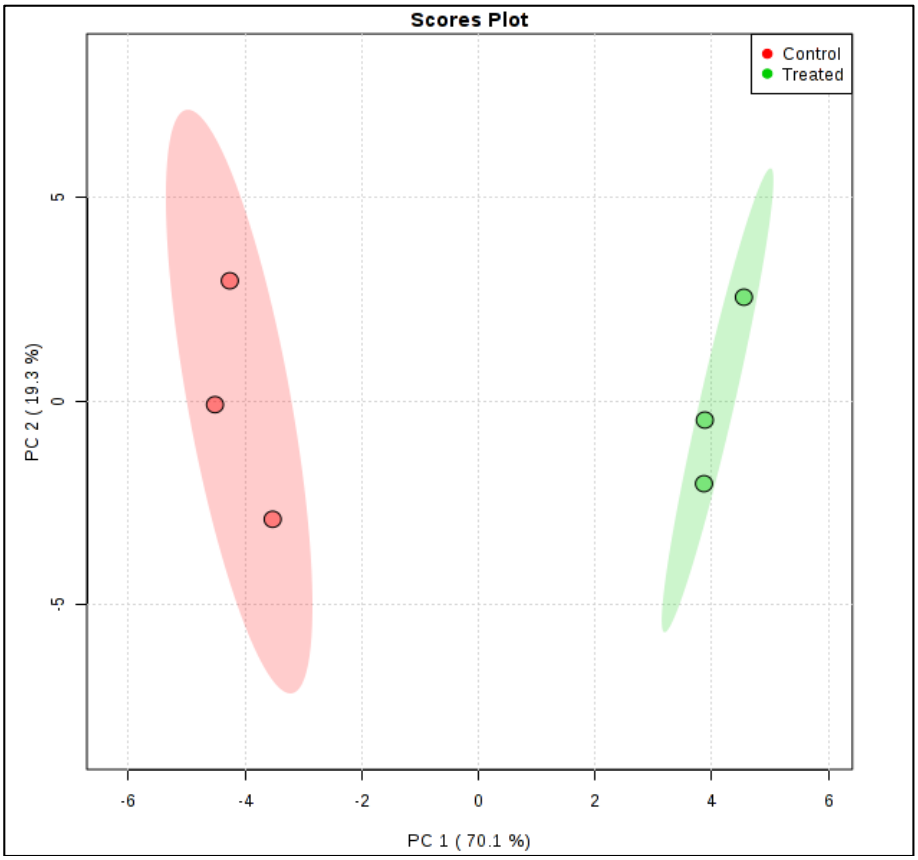

Fig 1b: Score plot of Sensitive leaf showing variations in metabolites of control and treated leaf. PC1 have higher variation than PC2

Correspondingly, sensitive (CML100) genotype PC1 (94%) and PC2 (5.3%) Fig 2a, b, control root metabolites were well separated and treated roots metabolites patterns shows less variance in separation because presence of similar metabolites like, 2-Furan carboxylic acid and Furan anhydride. Strikingly, in both genotypes an overlap of Furan's, and Tiglic acid was observed in roots. F-acids are mainly bound to phospholipids, they substitute for PUFAs and their strong capability (F-acids) to serve as radical scavengers suggests that plants use them to defend against oxidative stress (Spiteller 2005) [21].

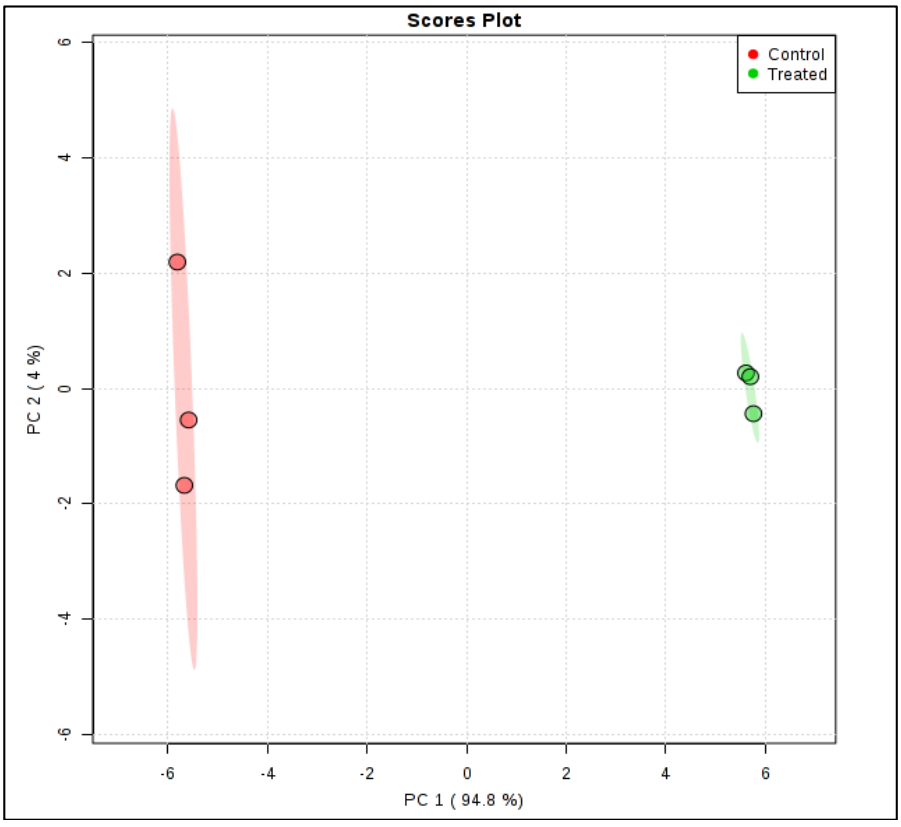

Fig 2a: Score plot of tolerant roots. Control roots shows larger variation in metabolites compared to treated roots

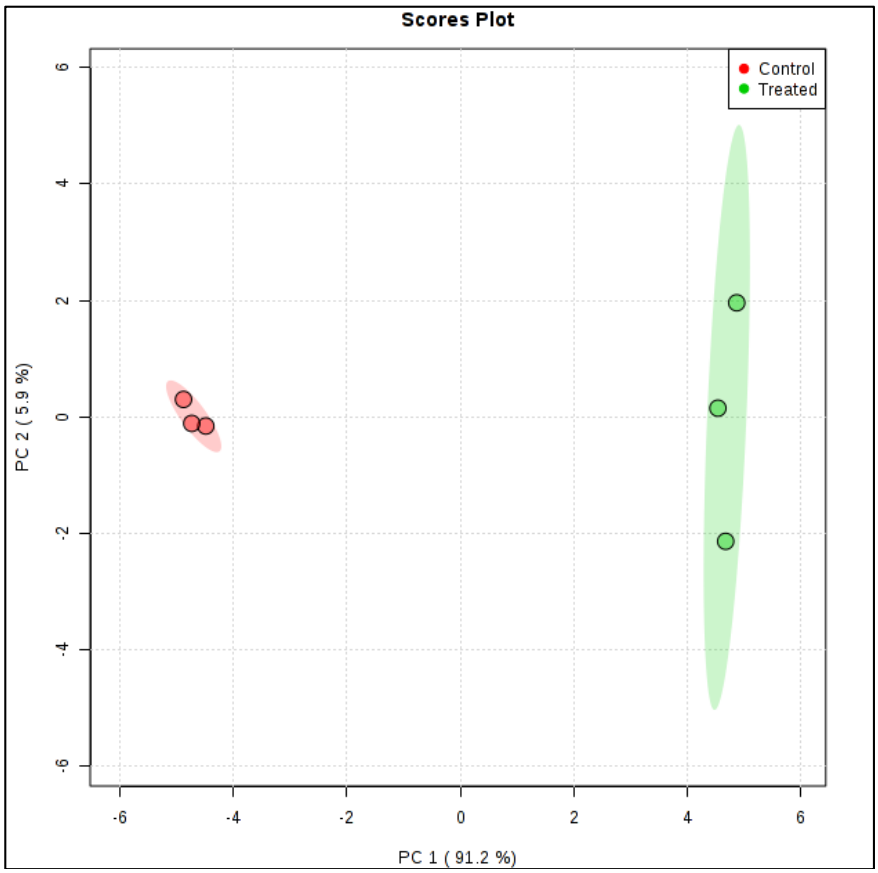

Fig 2b: Score plot of sensitive roots. Control roots shows less diversity in metabolites compare to treated roots.

The combine data of tolerant genotype of leaf and roots shows higher fold change in octanoic acid, that are involved in fatty acid biosynthesis, 9-Decen-1-ol related to alcohol dehydrogenase and Erythrodiol a pentacyclic triterpenes, leaf surface contains triterpenoids as constituents of waxes being involved in different roles such as maintenance of leaves structure, provide water, permeability, and plant insect interactions (Bauer *et al.* 2004; Mintz-Oron *et al.* 2008; Wilson *et al.* 2008) [2, 12, 28].

In roots, fold change was high of carboxylic acids (Pelargonic acid, Valeric acid, oxalic acid, sebacic acid, acetic acid) most of them have unpleasant odor might plays defensive role against various biotic stresses. Low molecular weight organic acids (Acetic acid, Veleric acid, Butyric acid) may restricted the damage caused by root-knot nematode (R G McBride *et al.* 2000) [11]. Survival of *Fusarium oxysporum* f. sp. *lycopersici* or *Ralstonia solanacearum* was suppressed by acetic acid and/or butyric acid (Noriaki Momma *et al.* 2006) [14]. Therefore it seems that metabolites of tolerant genotype are more uniform, shows coherent pattern.

Comparative metabolomics analyses of two combined stresses response have highlighted a number of metabolites involved in diverse metabolic pathways. The Pathway analysis of the tolerant leaf and roots showed biosynthesis of unsaturated fatty acids, Glyoxalate and dicarboxylate metabolism, linoleic metabolism, fatty acid metabolism, Nicotinate and nicotinamide metabolism, Sulfur metabolism, pyruvate metabolism, and Glycolysis and gluconeogenesis metabolism. However, the pathway impact is highest of Nicotinate and Nicotinamide metabolism, sulfur metabolism and Pyruvate metabolism.

Whereas, Sensitive genotype leaf and roots the number of pathways are more compared to tolerant genotype. Mainly the impact was high of the following pathways, Steroid biosynthesis, Sulfur metabolism, Panthothenate and Co-enzyme biosynthesis, Sesquiterpine and terpenoids biosynthesis, Arginine and Proline metabolism, Valine, leucine and isoleucine degradation, While, Aminoacyl t-RNA synthesis, Glucosinolate biosynthesis and Valine, Lucin and Isoleucine synthesis had no impact **(**Supplemetary Table S1),.

4. Heat map and correlation studies of metabolites

The heat map leaf and roots metabolites of tolerant genotype shows unique pattern. Although, the leaf metabolites of the tolerant genotypes differed with root metabolites. Almost all metabolites tolerant genotype leaf have higher concentration (=1), and some metabolites of treated leaf have concentrations above one (>1) like, Octanoic acid, 9-Decen1-ol, Erythediol. The concentration >1 of the following metabolites 1,16 dichloro hexadecane, 1-Pentanol, Eicosternoic acid, Lenolenic acid and ethyl Nonane.

On the contrary, treated roots metabolites have very high concentrations the same metabolites in control roots shows v low concentration. Therefore the roots and leaf metabolites have no correlation with each other Fig 3.

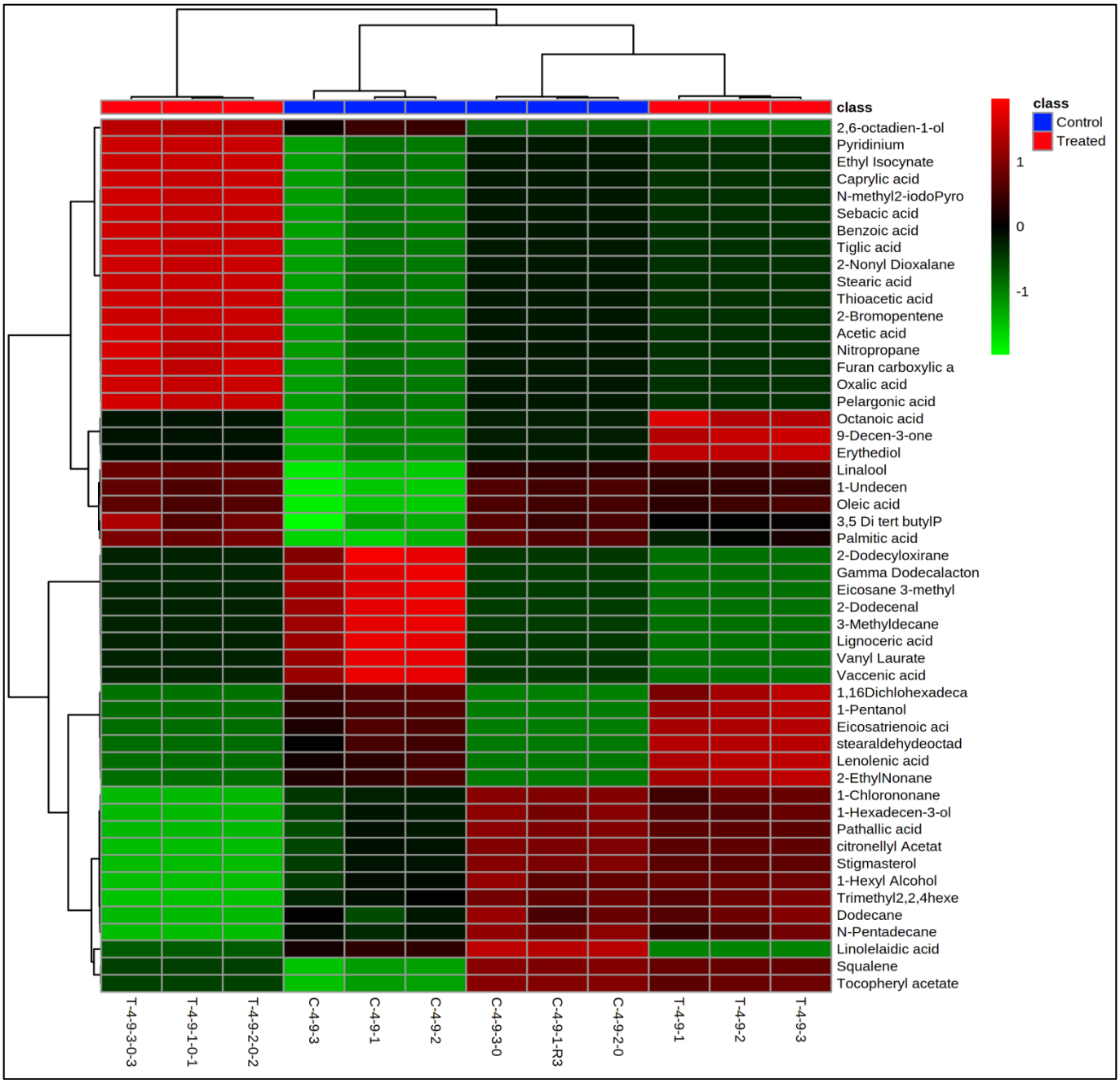

Fig 3: Heat map and Hierarchical clustering showing correlation between leaf and roots metabolites of tolerant genotype in response to combined abiotic stresses. The Red colors scale represents the high and Green -low concentrations of metabolites in control and treated plants.

Whereas, leaf and roots metabolites of sensitive genotype categorized into low and high concentrations Fig 4. Some of the metabolites of control roots are correlated with control and treated leaf (Dodecane, N-Pentadecane and Pthalate) shows higher concentration. Besides, Pentadectrine, 1-Undecen, oleic acid, palmatic acid, Dimethoxy propane, caprylic acid and 1-Hexene are common metabolites in control and treated roots with high concentrations. Therefore, in sensitive genotypes the leaf, roots metabolites are co-related or common. Thus there was less variations of metabolites in sensitive genotypes.

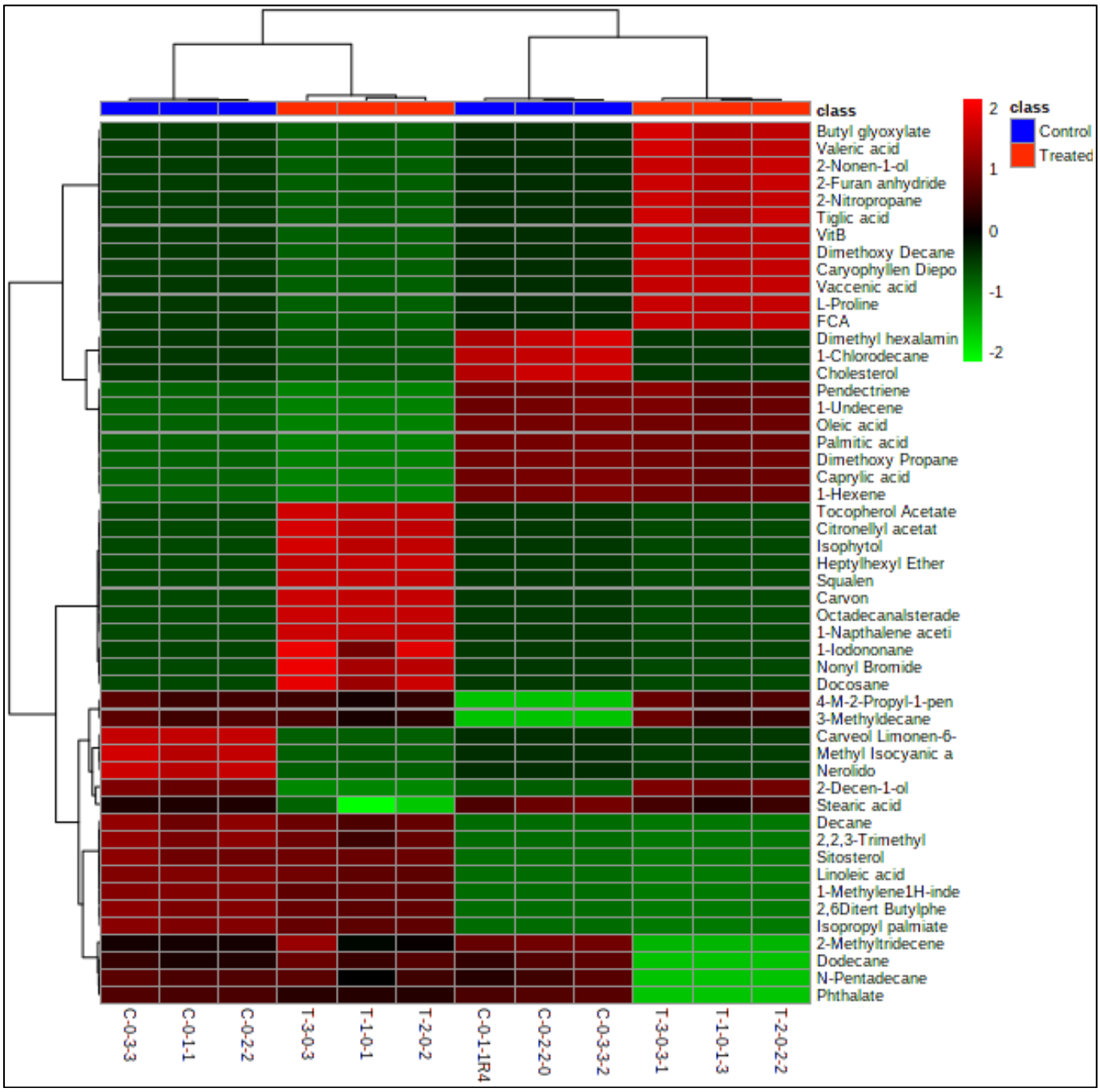

Fig 4: Heat map and Hierarchical clustering displays correlation between roots and leaf of sensitive genotype in response to combined abiotic stresses in control and treated. Red represent the –high concentration of metabolites and green represent the low concentration of metabolites.

5. Conclusion

The metabolomics study of leaf and roots in response to combined abiotic stresses of two maize genotypes tolerant and sensitive showed the varied metabolic profile. Metabolomic profile of tolerant genotype have uniform pattern of metabolites mostly involved in the biosynthesis of fatty acid metabolism, saturating and unsaturated fatty acids, sulfur metabolism, thereby, regulated signaling, re-fix soil nitrate metabolism and defense against the wide variety of pathogens. Therefore, enhancing the tolerant mechanism of the genotype. In contrast, sensitive plant metabolites mostly involved in leaking or disrupting the membranes that disrupt signaling between various organelles and also for various stresses, defense related metabolites were accumulated in random pattern. either defense against the particular pathogens or pollination attracters. In present metabolomics study we can say, the metabolites somehow help to survive the sensitive genotype in multiple stress conditions but most of them are the causes of susceptibility. On the basis of the above observation, we can conclude that tolerant genotype plants by remodeling of the cell wall, maintain metabolic homeostasis, and proper signaling thus able to tolerate multiple stress conditions. Therefore, the study provides a comprehensive analysis of tolerance mechanism.

6. Abbreviations

GC-MS- Gas chromatography mass spectrometry
Low-N- Low nitrogen stress
DAS- Days after sowing
PCA-Principal component analysis
"The authors have declared no conflict of interest".

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