Comparative Metabolic profiling of Drought-Tolerant and Drought-Sensitive Maize inbred lines under Drought stress

ABSTRACT

This study investigates the metabolic responses of plant species under mild drought stress, by comparing drought tolerant UASBM 13 and drought sensitive UASBM10 maize inbred lines. Study emphasized the synthesis and upregulation of crucial metabolites associated with antioxidant activities and cell wall lignification. Metabolites such as syringentin, naringenin chalcone, ferulic acid, sinapic acid, Sinapoyl Malate, and resveratrol were explored for their potential roles in mitigating drought-induced oxidative stress and fortifying cellular structures. Under mild stress, naringenin chalcone exhibited consistent upregulation, indicating its involvement in antioxidant activity and cell wall lignification. The study observed significant upregulation of sinapic acid, Sinapoyl Malate, and resveratrol in tolerant lines, suggesting their role in drought tolerance. Additionally, metabolites related to cell wall lignification were induced in response to drought stress, contributing to the overall drought tolerance in plants. The findings highlight the intricate metabolic mechanisms involved in plant adaptation to drought stress and the potential applications of these metabolites in enhancing drought resilience.

Keywords: drought tolerant, drought sensitive, lcms/ms, corn cync, metabololicprofiling

1. INTRODUCTION

Maize (*Zea mays*) holds a significant position as a crucial food and forage crop, contributing to various industries through the provision of raw materials. In India, it stands as the third most essential cereal crop after rice and wheat, showcasing the highest yield per hectare among all cereal crops. Despite its agricultural prominence, there has been a noticeable decline in the total area of maize production in India, primarily attributed to the adverse impact of drought [1]. Projections for the end of the 21st century indicate a substantial risk of yield loss in maize due to drought, ranging from 5.6 to 6.3% [2].

Drought stress significantly influences multiple stages of maize development, such as seedling growth, vegetative growth, flowering, fertilization, grain filling, and maturity. Particularly, drought stress during the vegetative period can lead to reduced growth rates and prolonged vegetative growth, impacting the overall productivity of maize [3]. Furthermore, drought stress during the early germination stage has been reported to have lasting effects on post-germination performance, emphasizing the critical nature of the initial stages of maize growth [4]. Neglecting the implications of drought stress during seedling establishment may result in decreased biomass accumulation, subsequently leading to a diminished yield [5].

Various strategies have been developed to mitigate the impact of drought stress on maize, including the use of drought-tolerant elite varieties, improved agronomic and water management practices, and the application of biological and chemical agents. While each strategy has its significance, the development of new maize varieties with desirable quantitative traits and the integration of associated biochemical, molecular, and physiological traits are of paramount importance. Achieving this requires an integrated approach driven by genomics, proteomics, and metabolomics to identify and understand metabolic pathways, regulations, cell signaling, and molecular interactions [6].

Among the diverse "omics" approaches, metabolomics and metabolic markers play a crucial role in functional genomics, offering insights into genotype-phenotype and phenotype-genotype interactions. Metabolomics, by studying changes in metabolites and their concentrations, provides a direct link to the phenotype, allowing a comprehensive understanding of the cellular and physiological behavior of crop plants under different environmental stimuli. In the context of the plant kingdom, which boasts an extensive diversity of metabolites—estimated to be around 200,000, with many yet to be explored—metabolomics emerges as a powerful tool to unravel the complexity of plant metabolites [7, 8].

This study aims to elucidate the metabolomic landscape associated with the influence of microbial biostimulants on growth promotion and enhanced drought tolerance in maize plants. By investigating the metabolic responses of biostimulant-treated plants, this research seeks to identify metabolic adaptability and distinctive metabolomic patterns characterizing the impact of microbial biostimulants on plant physiology. These insights serve as a foundational framework for predicting and comprehending the physiological shifts induced by microbial biostimulants in crop plants, particularly under drought conditions. Such an understanding represents a pivotal step in advancing the plant biostimulants industry and contributing to sustainable practices for global food security.

2. MATERIAL AND METHODS

2.1 Plant Material

The biosample for this study comprised homozygous inbred lines of maize exhibiting distinct characteristics in terms of drought tolerance at the reproductive stage. Seeds of reproductive stage drought-tolerant (UASBM13) and reproductive stage drought-sensitive (UASBM 10).Thebiosample for this study comprised homozygous inbred lines of maize exhibiting distinct characteristics in terms of drought tolerance at the reproductive stage. Seeds of reproductive stage drought-tolerant (UASBM13) and reproductive stage drought-sensitive (UASBM10) lines were sourced from the Department of Biotechnology. These seeds were individually packed and stored at 4°C in sealed containers until the initiation of the experiment

2.2 Soil Preparation:

A single lot of red loamy soil was collected from the Department of Biotechnology field at GKVK, Bangalore. The soil was manually cleaned of debris and clumps, followed by sieving using a mesh with a size of 3.0 mm. The soil was then mixed with fine vermicompost in a 4:1 ratio. Eight kilograms of uniformly mixed soil was filled into experimental pots measuring 30 x 25 cm [10].

2.3 Water Holding Capacity (WHC) Determination

The WHC of the soil-vermicompost mixture was determined using a standard method. Five hundred grams of the mixture were placed in a ceramic Buchner funnel with Whatman-2 filter paper. After adding 250 ml of water, the setup was left for 3 hours, and the collected water was measured. WHC was calculated by subtracting the drained water volume from the initially added amount, expressed as a percentage of water weight to soil-vermicompost weight (ml/100g) [10].

2.4 Study of Soil Dehydration Dynamics

The dehydration dynamics of the soil in experimental pots were studied under greenhouse conditions. Pots were saturated with water, and soil moisture content at different depths (1, 2, 3, 4, 5, and 6 inches) was measured every 3 days using the gravimetric method. Non-linear regression analysis was applied to understand the dehydration rate at different depths over time. The lost soil water, based on regression analysis, was replenished to maintain a desired moisture level during drought induction studies [10].

2.5 Drought Induction Experiment

Drought induction studies were conducted in pots under greenhouse conditions. Two groups of pots, labeled group 1 and group 2, each comprising 4 pots, were arranged randomly. Both groups were filled with 8 kg of premix soil and saturated with water according to the calculated water holding capacity. Seeds of drought-susceptible (UASBM10) and drought-tolerant (UASBM13) maize inbred lines were sown in group 1 and group 2, respectively. Pots were watered for the first 20 days to maintain soil moisture in the range of 30 to 32%. Afterward, water supply was withdrawn, reducing the mean soil moisture level to 15%. Moisture at this level was maintained for different durations to induce varying levels of stress. Sampling was conducted on the 10th, 20th, and 30th days under drought conditions, while control pots were maintained at 30 to 32% soil moisture [10].

2.6 Sample Extraction

Leaf samples (0.5 g) from UASBM10 and UASBM13 were collected after different drought induction periods and stored at -20°C. Samples were chopped into fine pieces and extracted using HPLC-grade methanol. Mechanical disruption with a mortar and pestle was employed, and each extract was made up to 25 ml using a standard volumetric flask. After centrifugation at 10,000 RPM, the supernatant was appropriately diluted and injected into the LCMS system for further analysis [11].

2.7 Liquid Chromatograph

Liquid chromatography was performed in reverse phase mode using a binary gradient solvent manager (Waters) with specific conditions for solvent A and B. The total flow rate was 1 ml/min, and a C18 column (250 x 2.1 mm, 3 microm particle size) was used. The detector wavelength range was set at 220 to 400 nm [11].

2.8 Mass Spectrometry

Tandem mass spectrometry was performed using a Waters Xevo TQD instrument with electrospray ionization in negative mode. Specific conditions included a capillary voltage of

3.5 kV, cone voltage of 30 V, desolvation temperature of 500°C, and a mass scan range of 150 to 1000 [11].

2.8 Partial Chemical Characterization

Chromatographic peaks responding to different drought levels were identified based on fragmentation patterns and tandem mass spectrum databases. Molecular ion peaks were selected, and putative identities of drought-responsive peaks were determined through database searches [11].

2.9 Pathway Mapping

Putative compounds were confirmed using the CornCys database and manually mapped into reference pathways of the KEGG database. Pathways were drawn using Dia [12].

3. RESULTS AND DISCUSSION

3.1 Pot soil moisture level optimization for induction of drought stress

The changes in soil moisture reduction at different levels of depth such as 2, 4, 5 and 6 inches from the top layer is shown in Fig 1. In the experimental pot, a reduction in the moisture of 2% was observed at 2 inches from top soil over a period of 15 days. The reduction rate was found to be following a logical mathematical trend in all 5 level of soil depth. In the 18 day study, the top soil moisture level reached its minimum level. At sub surface level of 3,4,5 and 6 inches depth, the moisture level was found to be 6, 8, 10 and 30 % respectively. The changes in moisture content with respect to time (0 to 18 days) varied soil moisture content from 30% to 7% at depth of 4 inch which is assume to be the middle of the test pot. As the desired drought stress level is 15% soil moisture and the same was calculated by integrating the curve in the Fig 1.

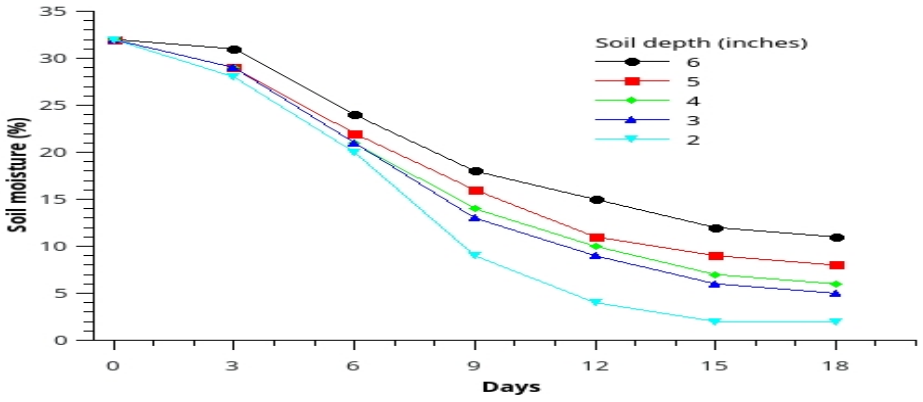

Fig 1: Changes in soil moisture content at different depth of a standard 1-foot-deep experimental pot under standard greenhouse condition.

The soil evaporation water loss happens from the top soil which is influences by the atmosphere temperature, humidity, wind current, etc. When the top layer evaporation happens, water from deep soil raises by capillary action and the evaporation continues. Therefore, to retain the soil moisture content just above the permanent wilting point of maize which is reported to be 15%, during the stress induction period, a calculate amount of water was added to the test pot.

3.2 Metabolic profiling of drought sensitive maize inbreed line with control

Metabolites extract from contrasting maize inbred lines subjected to drought stress were separated using liquid chromatography with reverse phase C-18 column coupled with a photo diode array detector. Every separated metabolite passed through the PDA detector recorded the respective absorption spectrum in the range of 200–450 nm. The max plot analysis showed that, majority of the metabolite had absorption in the range of 260 to 280 nm and hence, for all the subsequent analysis, chromatogram at 280 nm was followed. This wavelength selection ensured detection of most of metabolites having conjugation that include mainly semi polar compound like flavonoids, glycosylated steroid, alkaloids, phenolic acids, and glycosylated classes of molecule[15].

UASBM 10 (Sensitive) metabolic profile compare to control with 3 different time period for 10 days, 20 days and 30 days(different level). Here result of metabolic profile of drought stress for 30 days for conveniences. Fig 2 shows the drought treatment on UASBM10 test (A1) and the respective control (A2) at stress level after 30 days of drought stress. On analyzing the PDA profile of sensitive genotype under different levels of stress showed the presence of more than 100 metabolites with absorption at 280 nm. At drought stress level 1 comparison of PDA profiles of test and control showed similarity between the profiles except in certain segments. Among the four segments (B,C,D and E) of chromatogram, shown them D and E segment are not showing any change in chromatogram. segment B showed some variations between test and control at 6.38 retention time. However, on further examination, in the subsequent levels of drought stress, variations at this retention time was not significant and hence this peak was not taken into consideration. On the other hand, C segment showed variation in peak which is designated by the ID “peak 1” and highlighted with shading. Variations of areas of “peak 1” with respect to its control is given in Table 1. The drought sensitive UASBM10 showed only one peak which was responding proportional to the drought stress. The areas of “peak 1” at increasing stress level were; 10 days drought, 37; 20 days drought, 146 and 30 days drought, 317. Though more than 100 peaks were detected in the PDA profile at 280 nm, “Peak 1” showed a significant variation with respect to increased drought s

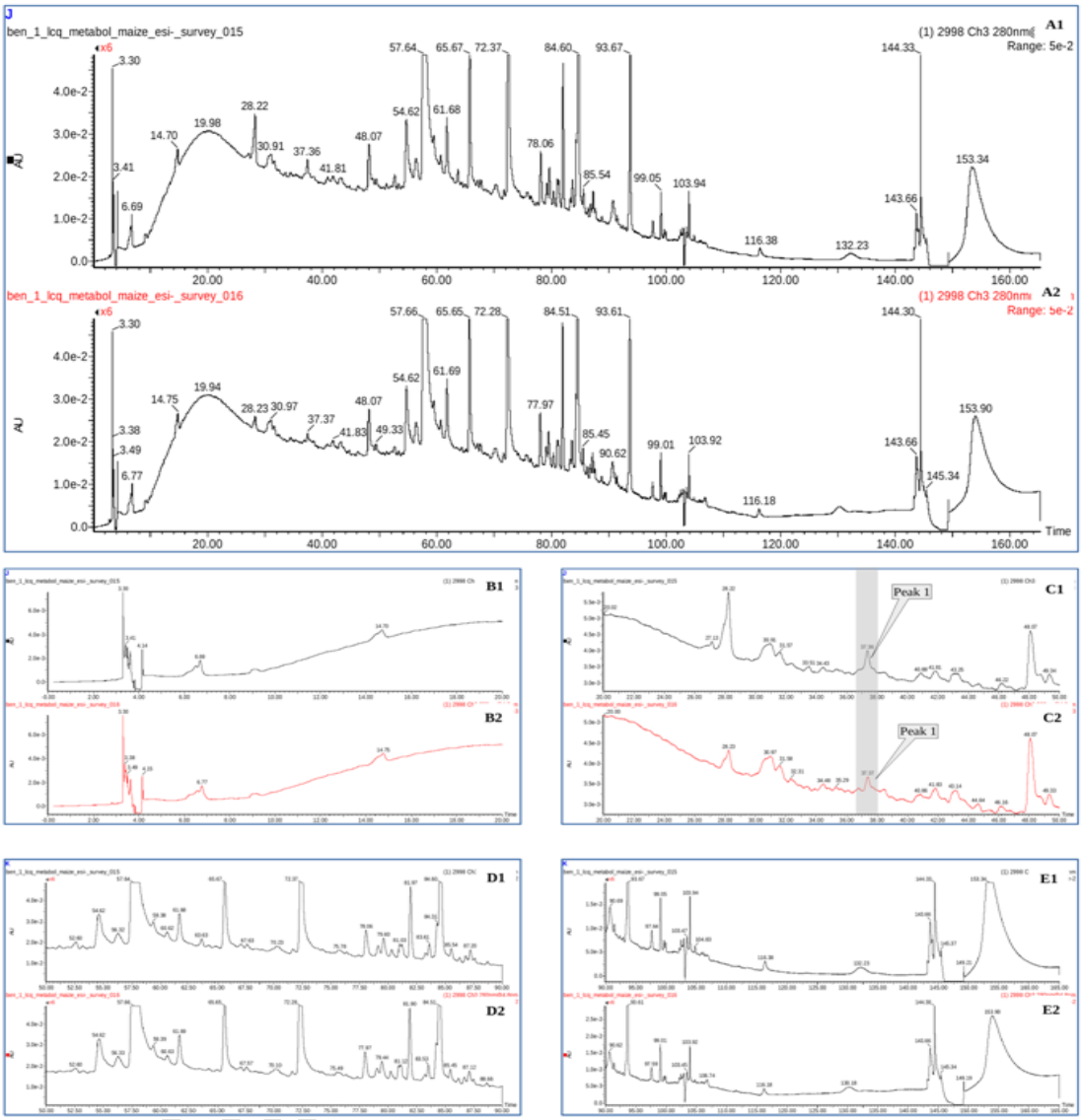

Fig 2 :- Photo diode array absorption profile (@ 280 nm) of leaf methanol extract of drought sensitive maize inbred lines UASBM10 at induces stress **for 30 days** (A1) and respective control (A2). B1 and B2, magnified profile retention time from 0.0 to 20 min of A1 and A2; C1 and C2, magnified profile retention time from 20 to 50 min of A1 and A2; D1 and D2, magnified profile retention time from 50 to 90 min of A1 and A2; E1 and E2, magnified profile retention time from 90 to 165 min of A1 and A2. Shaded region shows variations in peak profile.

3.3 Comparison of metabolite profiling of tolerant genotype at different levels of drought stress and their respective control.

Compared to the metabolite response of sensitive genotype UASBM10 wherein only one metabolite at 280 nm responded, the tolerant genotype UASBM13 showed a eight peaks at PDA 280 nm responsive. On comparison of control and drought exposed UASBM13, we found the existence of more than 100 metabolites as observed in the case of sensitive UASBM10. The magnified chromatogram in segment C showed significant

variations in four peaks. At this segment of Fig 3 significantly responding peaks were annotated as “peak 2”, :peak 3” and “peak 4”. Segment D of the chromatogram also showed measurable variations in four peak as a result of exposure to different levels of drought stress. Drought responsive peaks in segment D was annotated as “peak 6”, “peak 7”, “peak 8” and “peak 9”. Changes in areas of “peak 2” to “peak 9” in response to different drought stress is given in Table 1 for different drought stress. On the other hand, no peak in segment B as well as segment E showed significant variations in response to different levels of drought stress. The peaks annotated as “peak 2”, :peak 3” in the segment C were found to be decreasing the area in response to increasing drought stress . The areas of “peak 2” at stress level for 10, 20 and 30 were 390, 298 and 90 respectively. The same trend was observed in “peak 3” where the peak areas at stress of 10, 20 and 30 were found to be 403, 30 and 18 respectively. On the other hand other two peaks in this segment were found to be increasing with increasing stress for 10, 20 and 30 days. Peak 4; 45, 302 and 958; peak 5; 90 110 1021.It was quite interesting to note that, all four peaks in segment C were found in both control as well as in the stress treatment, but the segment D, all four peaks, annotated as Peak 6, 7,8 and 9 were not observed in control but observe only on exposure to drought stress to the tolerant genotype. From the result it is observed that, drought stress leads to variation in metabolic regulation that include up-regulation, down regulation as well as synthesis of new Molecules.

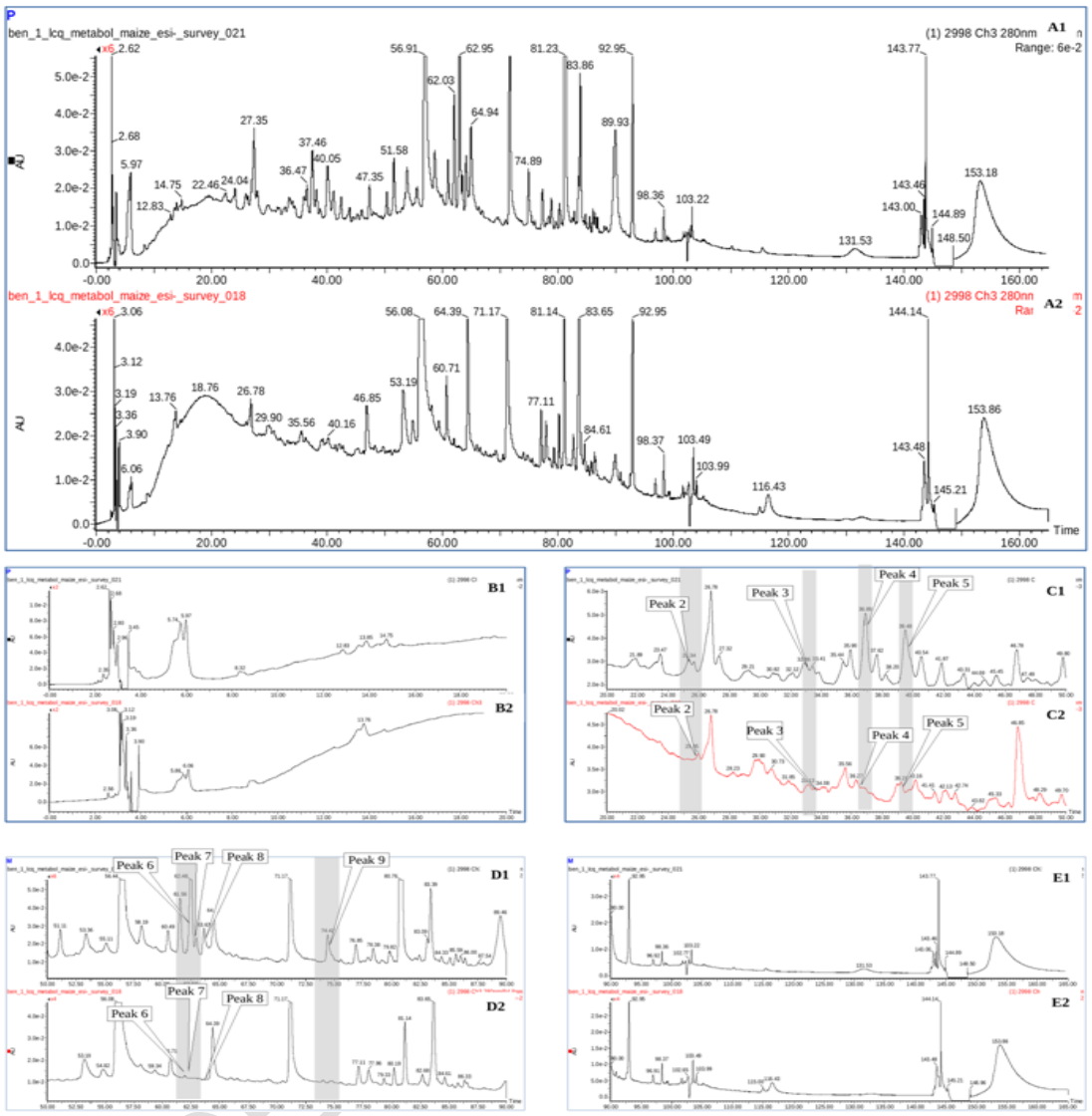

Fig 3 :- Photo diode array absorption profile (@ 280 nm) of leaf methanol extract of drought tolerant maize inbred lines UASBM 13 at induces stress for 30 days (A1) and respective control (A2). B1 and B2, magnified profile retention time from 0.0 to 20 min of A1 and A2; C1 and C2, magnified profile retention time from 20 to 50 min of A1 and A2; D1 and D2, magnified profile retention time from 50 to 90 min of A1 and A2; E1 and E2, magnified profile retention time from 90 to 165 min of A1 and A2. Shaded region shows variations in peak profile.

3.4 Comparison of Metabolite profiling between sensitive genotype and tolerant genotype at three different levels of drought stress

UASBM10 and UASBM13 grown under well watered condition (no stress), no measurable change was observed in the metabolite profile recorded at 280 nm of PDA detector. However, when these genotype were exposed to different level of drought stress, certain variations in peak profile was observed.

The peaks annotated as “peak 6”, “peak 7” and “peak 8” of Fig 4 ,observed in drought tolerant line .At the same time it was observed that, these peaks (peaks 6, 7 and 8) were not detected in sensitive lines exposed different days of drought stress. Hence, based on the above observation, it is understood that the peaks 6, 7 and 8 were normally not expressed in both tolerant and sensitive. However, they are expressed only in tolerant line under drought stress (Table 2).

In the above comparison certain peaks that were found only in the UASBM13 (tolerant) under induced drought stress was discussed. Besides the peak 6, 7 and 8, certain other peaks that were found in both sensitive as well as tolerant lines have shown measurable changes in response to drought stress. Such peaks were annotated as peak 9,11,12,13 ,14 and 15. Among these six peaks, peak 9, 11, 13 and 15 were found to be relatively in higher concentration in drought tolerant lines compare to sensitive lines under drought condition (Table 2). However, it was also noted that, all the above peaks in tolerant line showed a proportional decrease in the area in response to drought condition. On the other hand, peak 12 and 14 were found to be higher in sensitive (UASBM10) compared to tolerant line (UASBM13), but did not show any proportional change with respect to the drought stress.

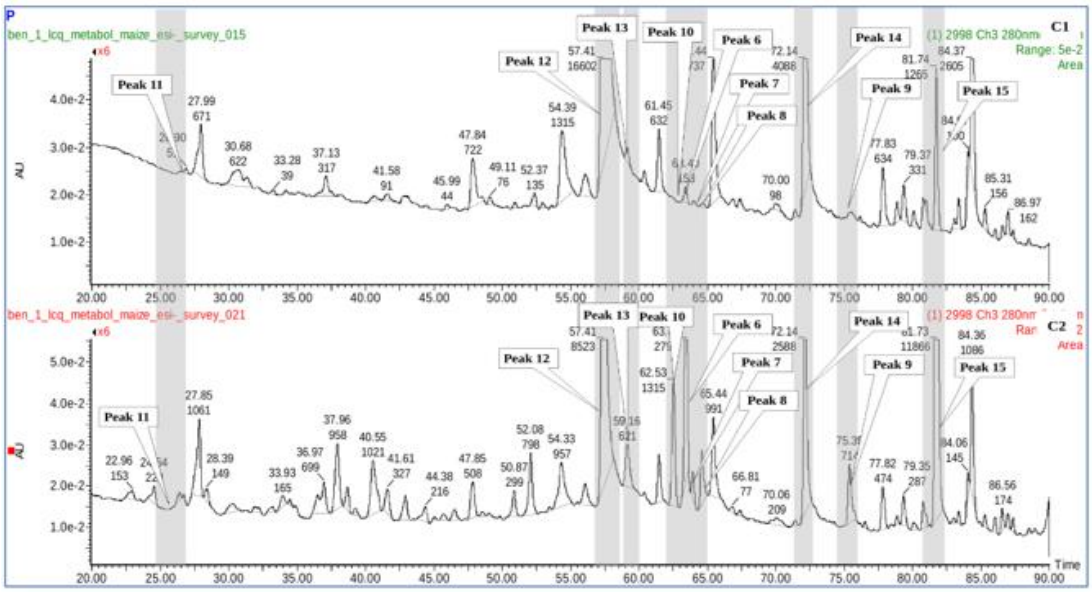

Fig 4:- Metabolic profile comparison between sensitive and tolerant after 30 days. A1 represent the sensitive and A2 represent the tolerant genotype chromatogram.

Table 1:- Changes in peak profile of drought tolerant and sensitive maize lines in response to drought stress respective to control.

Peak ID	Maize Lines	Drought Stress Level	Chromatogram Segment	Retention Time	Peak Area @ 280 nm (Control)	Peak Area @ 280 nm respective sensitive and tolerant	Observed Molecular Ion Mass	Compound Name
1	Sensitive	10 days	C	36.8 to 37.4	20	37		
		20 days			27	146		Not Identified
		30 days			32	317		
2	Tolerant	10 days	C	24.5 to 25.5	40	390		
		20 days			60	298		Not Identified
		30 days			35	90		
3	Tolerant	10 days	C	32.5 to 33.5	20	403	223.2	
		20 days			26	30		Sinapic acid
		30 days			22	18		
4	Tolerant	10 days	C	35 to 36	37	45	338.27	
		20 days			90	302		Snapoyl Malate
		30 days			30	958		
5	Tolerant	10 days	C	38.5 to 39.5	85	90		
		20 days			70	110		Not Identified
		30 days			91	1021		
6	Tolerant	10 days	D	62.5 to 63.2	0	622	227.24	
		20 days			0	582		Resveratrol
		30 days			0	1315		
7	Tolerant	10 days	D	63.3 to 63.8	0	181	271.25	
		20 days			0	75		Naringenin Chalcon
		30 days			0	2795		
8	Tolerant	10 days	D	63.9 to 64.6	0	576	344.28	
		20 days			0	392		Syringetin
		30 days			0	222		
9	Tolerant	10 days	D	74.7 to 75.4	0	594	208.17	
		20 days			0	0		Hydroxy ferulate
		30 days			0	714		

Table 2 :- Changes in peak area of comparatively between drought tolerant and drought sensitive.

Peak ID	Drought Stress Level	Chromatogram Segment	Retention Time	Sensitive	Tolerant	Observed Molecular Ion Mass	Compound Name
6	10 days	D	62.6 to 63.2	0	622	227.24	Resveratrol
	20 days			0	392		
	30 days			0	3210		
7	10 days	D	63.3 to 63.7	0	181	271.25	Naringenin Chalcon
	20 days			0	47		
	30 days			0	2295		
8	10 days	D	63.9 to 64.6	0	576	344.28	Syringetin
	20 days			0	392		
	30 days			0	222		
9	10 days	D	74.5 to 75.1	35	595		Not Identified
	20 days			0	27		
	30 days			31	71		
10	10 days	D	61.82 to 62.5	0	123	207.2	Sinapaldehyde
	20 days			0	75		
	30 days			0	1315		
11	10 days	C	26.0 to 27.2	37	395		Not Identified
	20 days			102	298		
	30 days			51	0		
12	10 days	D	56.4 to 58.70	18426	9469		Not Identified
	20 days			18525	17875		
	30 days			18205	8523		
13	10 days	D	58.9 to 59.5	102	765		Not Identified
	20 days			133	99		
	30 days			122	621		
14	10 days	D	71.9 to 72.5	5112	3120	335.29	Caffeyl Shikimate
	20 days			5153	4405		
	30 days			4535	2791		
15	10 days	D	81.8 to 82.3	1055	12131	319.29	Coumaryl Shikimate
	20 days			1123	1088		
	30 days			1265	11866		

4. Discussion

Drought, a pervasive abiotic stressor, significantly impacts plant productivity by disrupting key physiological, biochemical, and cellular processes. This study delves into the intricate metabolic responses of plant species under mild drought stress, focusing on the synthesis and upregulation of crucial metabolites associated with antioxidant activities and cell wall lignification. The exploration of these metabolites, such as syringentin, naringenin chalcone, ferulic acid, sinapic acid, Sinapoyl Malate, and resveratrol, sheds light on their potential roles in mitigating drought-induced oxidative stress and fortifying cellular structures.

Drought stress has been reported to increase the superoxide production by absisic acid mediated stomata closer and incomplete electron transport from NADPH to O2. This ROS production is known to be scavenged by endogenous enzymatic defenses system of plants**[16]**. Syringentin is probably one of the components of endogenous non enzymatic antioxidant deference system which could be found in larger quantity in plants with inherently better antioxidant capacity. However , in certain plants species, this is expected to by synthesized by mild drought induced oxidative stress.

Under mild stress, naringenin chalcone was up regulated and found the same trends even at highest level of stress. As this intermediate is involved in the synthesis of different groups of flavanoids with antioxidant activity as well as component of cell wall lignification[17], it is understood that due to drought stress, up-regulation of naringenin chalcone and other downstream metabolites may be facilitating in the tolerance of drought stress by scavenging drought induced free radicals as well as reinforcement cell wall integrity by the synthesis of cell wall lignification metabolites.

drought stress induced significant amount of this meatabolite and probably these metabolites attributes drought stress by involving in cell wall lignification and hence reducing cell wall water permeability**[18]**. however it was interesting to note that sinapic acid and Sinapoyl Malate found to be negligibly low in control plants compared to tolerant plants. All these metabolites got up regulated proportional to the stress though sinapicacid was found to be declining at very high stress. Therefore, it is understood that the tolerant lines expected to have the ability to synthesis the cell wall lignification metabolites to tolerate the drought stress. Another important metabolite found upregulated in UASBM13 is resveratrol in response to drought stress, in a dose dependent manner which is not detected in tolerant control (withought drought stress) as well as sensitive genotype (UASBM10). This shows that resveratrol synthesized in tolerant genotype as result of drought stress and with the same amount of drought stress this metabolite was not

detected in sensitive and hence, probably the synthesis of reseveratrol is under the regulation of drought stress only in the tolerant line (UASBM13). Resveratrol has been reported to have a broad spectrum of antioxidant activities in many in vitro as well as in vivo modules. Hence, the resvertrol in UASBM13 could also act as an antioxidant that might provide activities, one of the recent study reported s that reseveratrol is a monolignoid and involved in cell wall lignification and play an important role in drought tolerance**[19]**.

The metabolite such as sinapaldehyde, Coumaroyl shikimate and Caffeoyl shikimate have been found to be have different responses to the drought stress in both UASBM10 and UASBM13. Sinapaldehyde which is involved in synthesis of sinapyl alcohol and the terminal metabolite syringenin**[20]** (Liu *et a*l.,2020)lignin was not detected in sensitive genotype but found upregulated in tolerant line as a result of drought stress in a dose dependent manner.

Coumaroyl shikimate which is an intermediate in the synthesis of ferulic acid and other terminal metabolites for the synthesis of cell wall lignification **[21]** process was found to be induced in sensitive genotype as well as in tolerant genotype. However, in the sensitive genotype the level of induction was found to be very low compared to the high level of induction in the tolerant genotype.

Similarly, 4- Coumaroyl shikimate which is an intermediate in the synthesis of ferulic acid and other terminal metabolite in lignin biosynthesis**[21]** was also induced in both sensitive and tolerant genotype. However when the level of caffeoyl shikimate was analyzed, we found a high level of caffeoyl shikimate in sensitive line than that of tolerant line. The observation of less amount of caffeoyl shikimate in tolerant line could be due to the high turnover of the same for the synthesis of subsequent metabolites that are in the down stream of ferulic acid.

Drought tolerance in crop plants are contributed by many different traits that include physiological features (root, shoot, leaves)**[22]**, biochemical features (osmolytes, endogenous antioxidants, etc.)**[23]**and cellular features (cell wall lignification, pectin synthesis, membrane permeability, etc)**[22]**. Certain metabolites that got up regulated in the tolerant line under the drought stress in the current study, have been reported to get integrated during cell wall lignification. Lignin is known to contain major metabolites synthesized from p-coumaryl, coniferyl, and sinapyl alcohols **[24]**. As the current study

also showed synthesis of the above said metabolites in response to drought stress, the tolerant line UASBM13 is synthesizing the metabolites required for cell wall lignification. Cell wall lignification is a reinforcement process in plants that provide many beneficial effects to the plant growth, development and tolerance to different biotic and abiotic stress. There are many reports stating the high level of lignification in plant and increased drought tolerance **[25]**.

CONCLUSION

The inbred lines UASBM10 (drought sensitive in reproductive stage) and UASBM13 (drought tolerant in reproductive stage) are genetically distinct and reported to have differences in 18 quantitative traits The variance in drought response showed that, UASBM13 is tolerant to drought stress during reproductive stage. As there are many qualitative traits contributing for drought tolerance such as; number of stomata, leaf thickness, size and number of root cortical cell, high osmolyte concentration, intrinsic enzymatic antioxidants, intrinsic non-enzymatic antioxidant, cell wall lignification, membrane integrity, water holding capacity of calcium pectate in middle lamella, etc., selection of genotype for breeding based crop improvement should consider pyramiding all such traits using the metabolite markers along with the core quantitative traits that are selected based on DNA markers.

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