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Comparative profiling of drought induced root metabolic responses in sugarcane wild relative *Erianthus arundinaceus* (IND 04-1335) and a commercial variety Co 99004

R. Valarmathi*, H.K.M. Swamy, K. Preeti and C. Appunu

Division of Crop Improvement, ICAR-Sugarcane Breeding Institute, Coimbatore- 641 007, India

***Corresponding Author Email :** valarmathi.r@icar.gov.in

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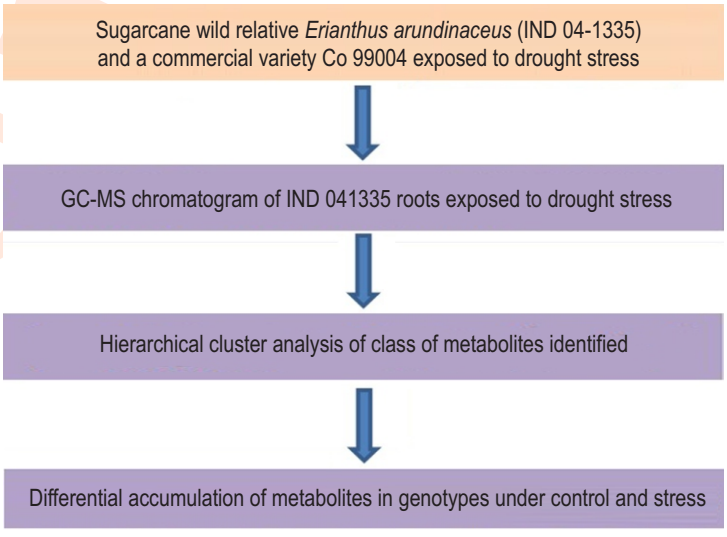
Abstract

Aim: To study the metabolic changes in roots of a drought tolerant wild relative of sugarcane *Erianthus arundinaceus* clone (IND 04-1335) and a commercial sugarcane cultivar Co 99004.

Methodology: Setts of *Erianthus arundinaceus* (IND 04-1335) and a commercial variety Co 99004 were planted in medium size pots in replication. After 45 days of planting, drought stress was imposed by withholding irrigation. The corresponding control pots were maintained under continuous irrigation. On 26th day of drought, stress morphological and physiological traits such as leaf drying, canopy temperature, leaf relative water content and chlorophyll fluorescence were recorded. Root samples were subjected to metabolomic analysis using GC-MS.



Results: After 26 days of drought exposure, IND 04-1335 were found to be tolerant without any drought induced morphological symptoms. The comparative metabolite profiling identified a total of 143 metabolites in the control and drought exposed roots. Hierarchical cluster analysis showed that roots of IND04-1335 control and Co 99004 stress had the most similar metabolite profiles, while the profile of IND04-1335 stressed root was distinctive.The metabolomic profile of IND04-1335 under drought stress showed an increased accumulation of sugars (melezitose, trehalose), sugar alcohols (mannitol), amino acid (proline) and carotenoids (rhodopin, carotene) as compared to Co 99004.

Interpretation: Differentially accumulated metabolites in IND04-1335 under drought stress may play an important role as osmoregulants, antioxidants and chelating agents thereby imparting better tolerance mechanism to this genotype.

Key words: Drought, *Erianthus arundinaceus*, Germplasm, Metabolomics, Sugarcane

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Introduction

Sugarcane (*Saccharum officinarum* L.) is being cultivated worldwide as a commercial crop for sugar. It is an economically highest tonnage yielding crop and accounts for more than 80% of world's total sugar production. Sugarcane as an efficient crop in converting solar energy into biomass is also cultivated marginally for ethanol and biomass production. Being an annual crop, sugarcane faces severe drought during summer. Drought is considered to be one of the most important abiotic stress limitations which effects crop production adversely. Sugarcane achieves 70-80% of yield during formative phase (60-150 days after planting; DAP), water deficit conditions during critical growth period badly affects cane yield (Hemaprabha *et al.,* 2013). Cultivars with significant levels of drought tolerance is reported to sustain growth as well as stabilize yield under drought (Chinnusamy *et al.,* 2004). Therefore, there is a continuous requirement of tolerant genotypes which can not only withstand extreme environmental stress like drought but also sustain yield.

The shoots and roots of plants respond to abiotic stress through different physiological, biochemical and molecular mechanisms (Zhu, 2016). Water-use efficiency, photosynthetic efficiency, leaf relative water content, transpiration efficiency, cell membrane stability and carbon isotope discrimination are some of the key traits which are reported to be affected at shoot level (Shulaev *et al.,* 2008). While at root level, traits such as root length, root biomass and root penetration ability are shown to be altered which can eventually reduce water uptake capacity thereby, causing stomatal closure, lower photosynthetic rate and finally effecting yield (Xiong *et al.,* 2002; Hirayama *et al.,* 2010).To respond and adapt to the extreme environmental conditions, expression of stress responsive transcripts, biosynthesis of compatible metabolites and its increased accumulation are some of the effective strategies evolved by plant system (Chen and Murata, 2008; Chmielewska *et al.,* 2016; Ullah *et al.,* 2017). With advancement in NGS technology to understand the molecular response and potential metabolic pathways, extensive transcriptomic studies have been carried out (Kang *et al.,* 2019; You *et al.,* 2019), while metabolomics has become a powerful tool to analyse stress induced metabolic changes which will supplement the transcriptome data and can also help in developing drought tolerant cultivars.

Metabolite profiling using GCMS technique has been widely used to understand the change in metabolites of several crops under extreme environmental conditions in recent years (Yang *et al.,* 2018). Studies clearly indicate that during stress conditions, metabolites play a major role as potential antioxidants, osmolytes, chelating agents, stabilize proteins and enzyme biosynthesis. They also help in maintaining cell membrane integrity, regulate cellular turgidity and regulate ion transport and thereby, improve resilience ability of crops under abiotic stresses, such as drought and salinity (Rai, 2002; Golldack *et al.,* 2011). The above functions are carried out through altered levels of sugars, soluble amino acids, organic acids, and low molecular weight compounds in both leaf and roots under drought stress (Tang *et al.,* 2017; Khan *et al.,* 2018). The concentration and allocation of metabolites like sugars, amino acids, and fatty acids among photosynthetic and root tissues are reported to be altered under drought conditions (Zhang *et al*., 2017). A significant reduction in the levels of several amino acids and intermediates of Krebs cycle and glycolysis, key metabolites involved in energy metabolism and photosynthesis are reported. These manifestation are predicted to be evolved by the plant system in order to save the energy budget of the plant and to develop distinct metabolic strategies to cope with the stress (Rai, 2002). However, amino acids which are building blocks for biosynthetic pathways, precursors of different secondary metabolites play an essential role in signalling under environmental stress and are significantly regulated during stress (Hildebrandt *et al.,* 2015). Amino acid such as proline is one among the extensively studied metabolites which plays a vital role in mitigating drought stress (Hayat *et al.,* 2012).

Higher accumulation of proline acts as osmolyte and is reported to guard cell membranes and other amino acids from ROS damage during drought stress. Several metabolites belonging to class of sugars and sugar alcohols accumulate in high concentration during drought stress. Significant accumulation of sugars such as sucrose, fructose, ribose and ketose are reported in roots under drought stress. Both sugar and sugar alcohols play an essential role in osmotic adjustment during drought (Kang *et al.,* 2019). Small molecules belonging to class of terpenoids and polyamines are reported to accumulate in high concentrations in roots of maize. Presence of root-associated terpenoids are reported in rice and plants belonging to Pinaceae and Asteraceae families indicate their potential physiological role under stress (Schmelz *et al.,* 2011; Zerbe *et al.,* 2013).

Though there is clear evidence that drought adaptive mechanism is regulated through the expression of a cascade of metabolic pathways, their regulation in sugarcane roots by and large is still unexplored. Comparative metabolomic studies between wild germplasm and cultivated varieties under drought will provide more significant information about crop tolerance mechanism. Such metabolomic data can be used to identify the underlying regulatory pathway and the corresponding functional genes (Tuberosa and Salvi, 2006). The initial large scale studies on identifying drought tolerant germplasm from *Erianthus arundinaceus* a wild related genus of sugarcane has led to the identification of drought tolerant clones. Hence, in view of the above, the present study was conducted to understand the metabolic changes in roots of tolerant *E. arundinaceus* clone (IND 04-1335) and a commercial sugarcane cultivar Co 99004 using GC-MS.

Materials and Methods

Genetic material and drought exposure: Setts of *E. arundinaceus* (IND 04-1335) and a commercial variety Co 99004 were planted in medium size pots and maintained at rainout

shelter. The genotypes were planted in five replications and 45 DAP drought stress was imposed by withholding irrigation. The corresponding control pots were maintained under continuous irrigation. During drought stress leaf drying was measured manually and scored based on the percentage of dried leaf. The leaf relative water content (LRWC) was measured during drought stress and compared with respective control plants. Fresh weight of third leaf from top was recorded and the leaves were saturated in distilled water for 5 hrs and turgid weight was recorded. Leaves were dried in oven at 65°C for 24 hrs and dry weight was recorded. LRWC was calculated from the equation of Schonfeld *et al.* (1988). Canopy temperature and chlorophyll fluorescence in third leaf from top was measured by chlorophyll fluorometer OS1P(OPTI-SCIENCES).

Metabolite extraction and GC/MS analysis: Roots of *E. arundinaceus* (IND 04-1335) and commercial variety (Co 99004) were collected for metabolomics analyses from control and drought exposed plants on 26th day of drought stress. Root tissues in three replication were flash-frozen in liquid nitrogen immediately after collection; 40 mg of frozen root tissue was ground in liquid nitrogen; extracted with methanol and then with chloroform (Fiehn *et al.,* 2000). Ribitol was added as internal standards and filtered using 0.22 µm PVDF syringe filter (Millipore, Ireland). The extracts were concentrated using a Speed Vac and trimethylsilylation was performed at 37°C for 30 min with N-methyl-N-[trimethylsilyl] trifluoroacetoamide (80 µl; MSTFA). About 1µl of the samples were injected into GC injection port (AI3000 II, Thermo Fischer Scientific, USA) connected to a GC/MS (TRACE™ GC Ultra with DSQII Quadrupole mass spectrometer and capillary column of 30 cm in length, 0.25 mm diameter and 0.25 µm film thickness-Agilent, DB-5 ms Ultra Inert, 122-5532). The injection port temperature was maintained at 250˚C, the oven temperature was programmed at 70˚C (5 min hold) and increased @ 5°C min^{-1} to 300°C. Helium was used as a carrier gas at a flow rate of 1 ml sec^{-1}. The electron ionization source temperature was maintained at 250°C and the abundances of ions in the range of 50-600 m/z were scanned @ 1.1 scans sec^{-1}. The compounds were identified using NIST library and the data analysis was carried out using R software.

Hierarchical cluster analysis: Hierarchical cluster analysis was carried out using Permu Matrix Version 1.9.3 EN, dissimilarity was measured based on Euclidean distance and the cluster was generated using UPGMA method.

Results and Discussion

After 20 days of drought exposure, the first symptom of leaf rolling was observed in Co 99004. On 26th day of drought stress, Co 99004 showed more than 30% leaf drying while the plants of IND 04- 1335 showed only leaf rolling. At this stage the canopy leaf temperature was measured in the range of 23-24°C in control plants of IND 04-1335, Co 99004 as well as in IND 04-

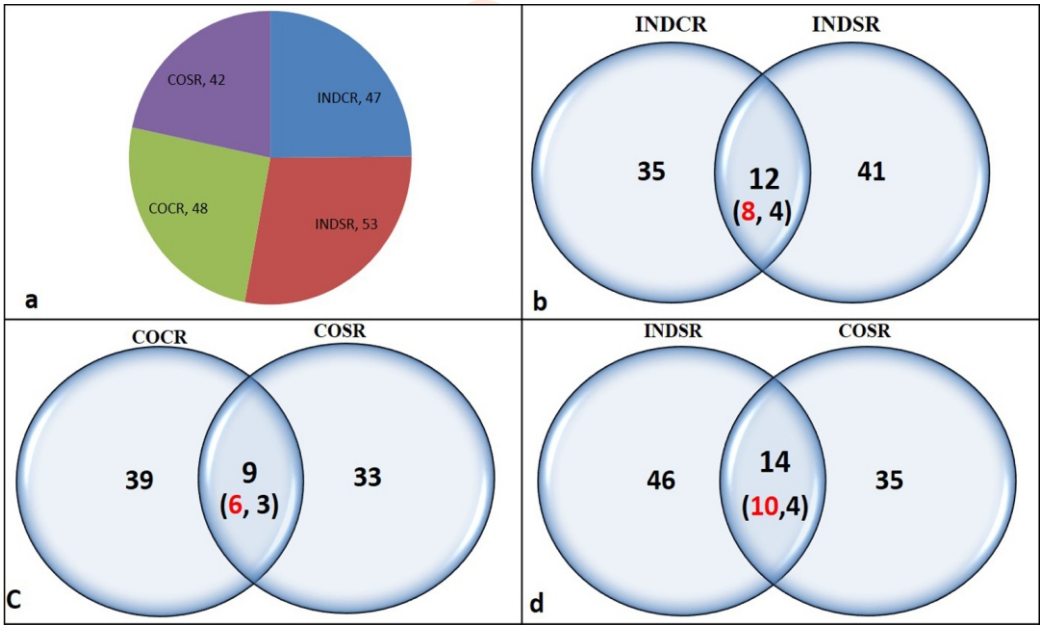

Fig.1: Total number of metabolites significantly accumulated in the roots IND04-1335 and Co 99004. Total number of metabolites in both the genotypes under control and 26 days after drought exposure. (a.) Number of metabolites identified in IND04-1335, (b.) Co 99004, (c.) Number of metabolites identified under drought stress in IND04-1335 and Co 99004, (d.) Numbers inside parenthesis indicate common metabolites and number of metabolites upregulated under stress are indicated in red. (INDCR – IND04-1335 control root, INDSR – IND04-1335 stress root, COCR - Co 99004 control root, COSR - Co 99004 stress root).

1335 stress exposed plants, while drought stressed plants of Co 99004 showed a higher canopy temperature of 27-28°C. Canopy leaf temperature is considered as one of the important physiological trait to understand drought response of a genotype. The process of transpiration through open stomata maintains the canopy temperature at metabolically functional range, while closure of stomata due to high evaporative demands under drought stress leads to high canopy temperature (Siddique *et al.,* 2000; Anjum *et al.,* 2011). However, a tolerant genotype is reported to extract more available soil moisture thereby maintaining the transpirational cooling (Ludlow and Muchow, 1990; Reynolds *et al.,* 2009). Therefore, low transpirational cooling due to early stomatal closure could be responsible for a higher canopy temperature in the leaves of stress exposed Co 99004. The tolerant genotype IND 04-1335 was able to regulate photosynthetic rate as well as transpirational cooling under water stress conditions, which was also evidenced in the high leaf relative water content recorded in IND 04-1335 (Kapoor *et al*., 2020).

Leaf relative water content an important indicator of drought adaptability was measured during extreme drought condition. The LRWC ranged between 80-84% in well-watered plants of both IND 04-1335 and Co 99004, while 75-80% LRWC was measured in IND 04-1335 drought stressed plants. The plants of Co 99004 under drought stress showed an average LRWC content of 65%. A high chlorophyll fluorescence (Fv/Fm) was recorded in control plants of IND 04-1335, Co 99004 as well as in IND 04-1335 drought stressed plants (0.6-0.7), while Co 99004 drought stressed plants showed lesser Fv/Fm ratio (0.3). A 50% reduction in chlorophyll fluorescence in Co 99004 under drought stress compared to IND 04-1335 indicates down regulation of photosynthesis due to inactivation of PSII activity in Co 99004 (Wang *et al.,* 2003; Yao *et al.,* 2018), while at similar days of drought exposure higher Fv/Fm in IND 04-1335 indicates its significant photosynthetic efficiency and less photo inhibition. The morphological and physiological traits with intact green leaves, high chlorophyll fluorescence, lower canopy temperature and higher leaf relative water content was observed in IND 04-1335 under drought.

Stress adaptive mechanism is imparted through a cascade of metabolic pathways which are genetically regulated through the expression of genes and compatible metabolites (Harding *et al.,* 2003). Metabolite profiling is expected to provide information on the accumulation and biosynthesis pattern of compatible solutes which will complement as well as supplement the genomics data and lead to a better understanding on the stress responsive behaviour of plant. Specifically, a comparative study using wild germplasm resources and a commercial variety of crop subjected to extreme environmental conditions will provide more significant information about crop stress tolerance mechanism. The comparative metabolite profiling of a sugarcane germplasm IND 04-1335 and a commercial variety Co 99004 by GC-MS identified a total of 143 metabolites in the control and drought exposed roots. Among the genotypes IND 04-1335 stressed root (INDSR) showed maximum number of metabolite accumulation (53), followed by control roots of Co 99004 (COCR, 48), control roots of IND 04-1335 (INDCR, 47) and Co 99004 stressed root (COSR, 42) (Fig. 1a).

A total of 143 metabolites were identified among both the genotypes under control and stress conditions, the roots of IND 04-1335 under control and drought stress showed accumulation of 89 metabolites among those 35 metabolites were specific to INDCR and 41 were specific to INDSR, while only 12 metabolites were common to both control and stress (Fig. 1b). The commercial sugarcane variety Co 99004 showed a similar trend in which 39 were specifically accumulated under control and 32 were specific to drought conditions and only nine metabolites were common to both control and stress (Fig. 1c). Fourteen metabolites were found common among the drought stressed root samples of IND 04-1335 and Co 99004, while 46 were specific to INDSR and 35 were specific to COSR (Fig. 1d) respectively. In both the genotypes even though very less number of metabolites were common to control and drought conditions, the maximum number of commonly shared metabolites were found to be upregulated under drought condition. Earlier studies have reported altered levels of metabolites under drought conditions (Chmielewska *et al.,* 2016; Yang *et al.,* 2018; Kang *et al.,* 2019). In the present study, the roots of both genotypes rather than altered metabolite profile showed accumulation of different metabolites which was evident from the lesser number of metabolites commonly shared under stress condition. The total metabolites identified represented 40 different classes and maximum number of metabolites belonged to alcohols (19), followed by fatty acids (18) and ketones (16). Five metabolites in each compound classes such as sugars, esters, organic acid, carboxylic acid, amines, carotenoids and amino acid were identified and two to three compounds were identified from all other classes. Hierarchical cluster analysis showed that roots of INDCR and COSR had the most similar metabolite profiles, while the root profiles of COCR formed a second cluster along with INDCR and COSR (Fig. 2).

A distinct metabolite profile was observed in the root samples of IND 04-1335 exposed to drought stress. Metabolites belonging to class of alcohol, fatty acid, carboxylic acid, carotenoids, sugars, ketones and terpenoids were present in all the four samples. However, the number and concentration of compounds belonging to these classes were altered in one or other samples leading to a distinct profile in all the four samples. Hierarchical cluster analysis of individual metabolites also showed a distinct metabolite profile in the root samples of IND 04-1335 exposed to drought stress (Fig. 3). Among the different class of metabolites, sugars and carotenoids showed significantly higher accumulation under drought in both the genotypes, while the accumulation in IND 04-1335 under stress was four fold higher than Co 99004 (Fig. 4a, 4b).

The individual metabolites of sugars and carotenoids which were accumulated in high concentration in IND 04-1335

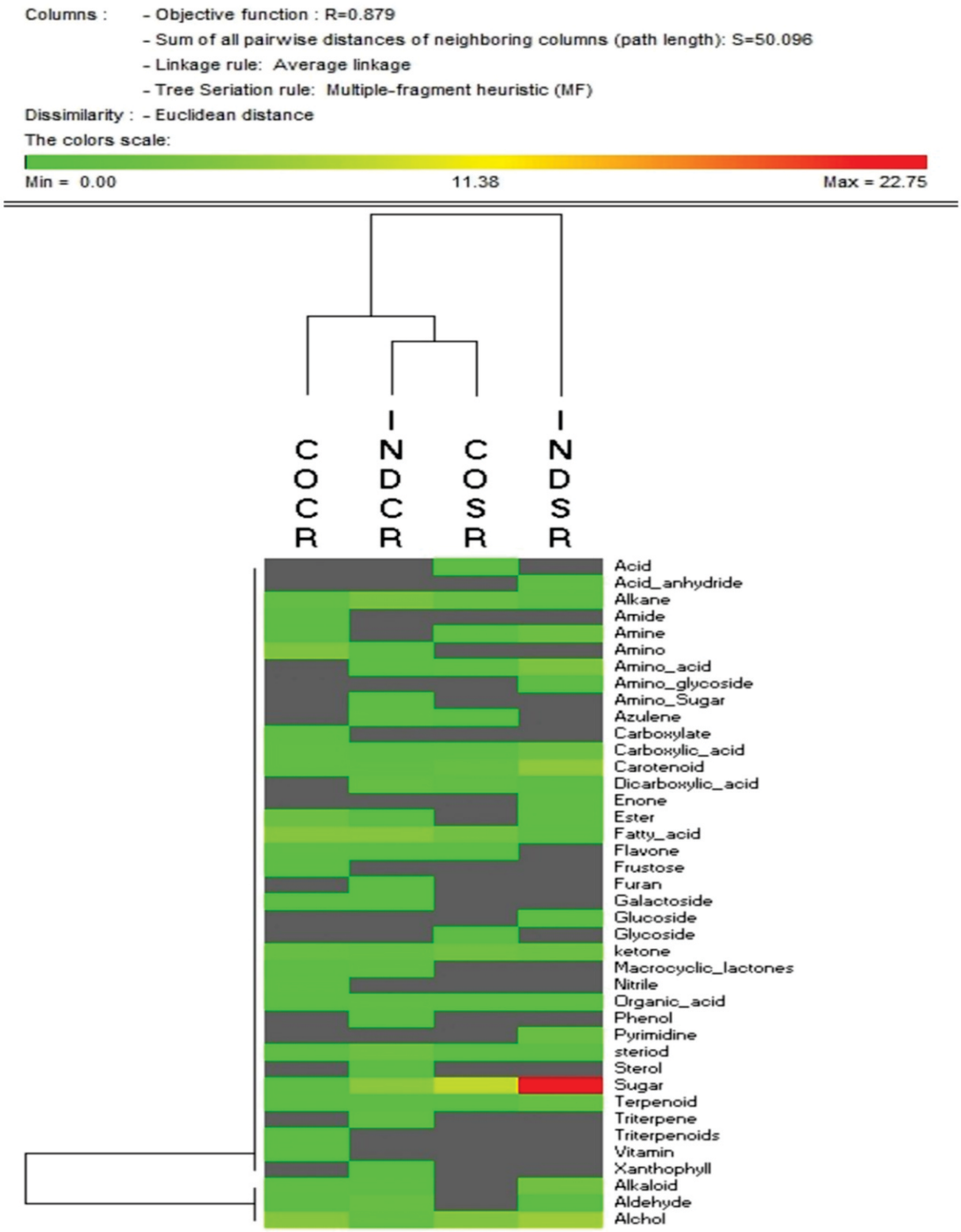

Fig. 2: Hierarchical cluster analysis of total content of different class of metabolites accumulated in the roots of *E. arundinaceus* (IND 04-1335) and Co 99004. INDCR - IND04 control root, INDSR – IND04-1335 stress root, COCR - Co 99004 control root, COSR - Co 99004 stress root. Colours in the heat map indicate the total content of the metabolite, red and green colour indicates higher and lower levels, respectively.

Fig. 3: Hierarchical cluster analysis of different metabolites accumulated in the roots *E. arundinaceus* (IND 04-1335) and Co 99004. Colours in the heat map indicate the total content of the metabolite, blue and yellow colour indicates higher and lower levels, respectively.

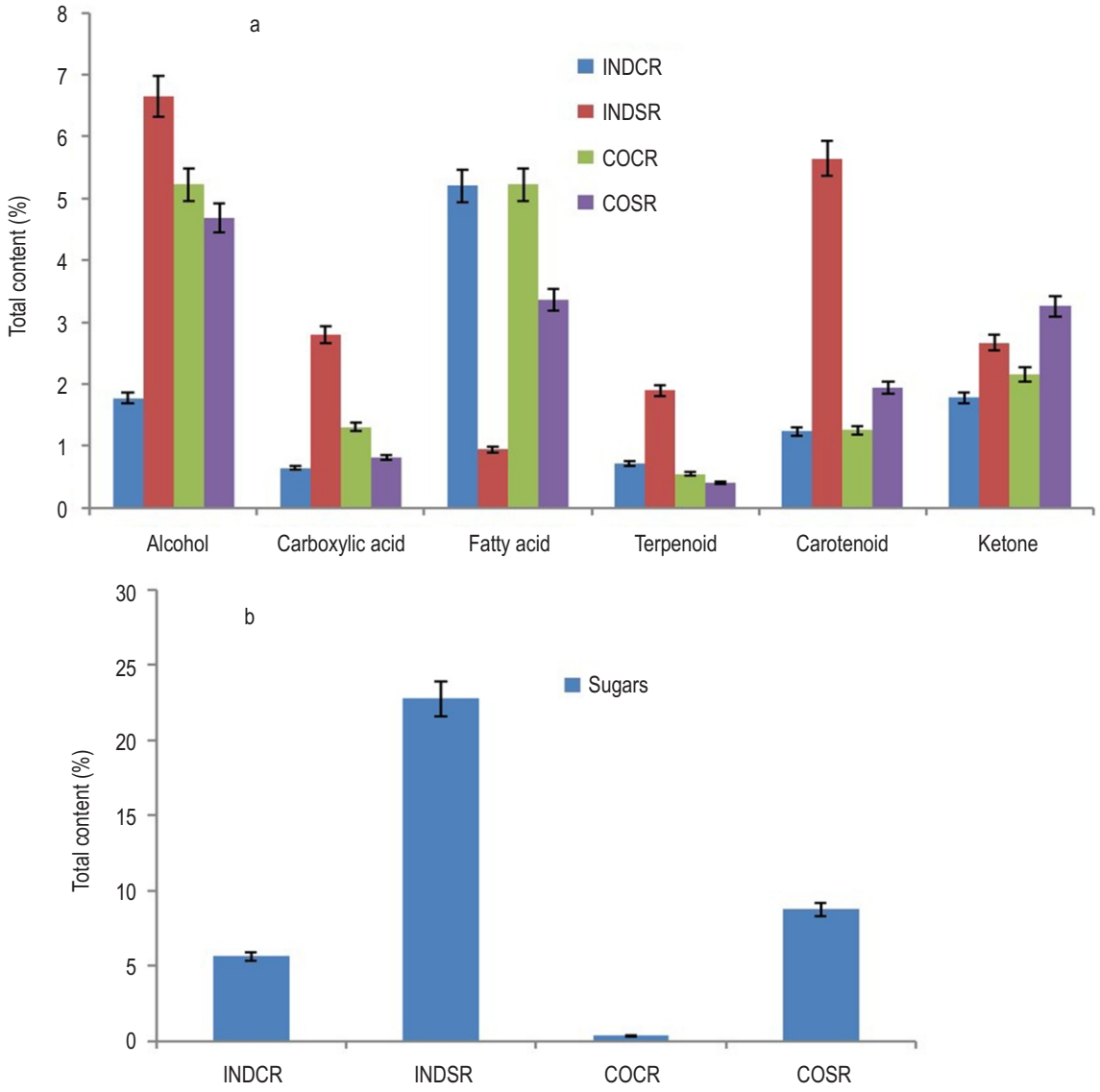

Fig. 4: Accumulation of different class of metabolites in the roots of IND 04-1335 and Co 99004 under control and stress. (a.) Various classes of metabolites and (b.) sugars. INDCR – IND04-1335 control root, INDSR – IND04-1335 stress root, COCR - Co 99004 control root, COSR - Co 99004 stress root.

stressed root are discussed separately. Alcohols, carboxylic acid, ketones and terpenoids significantly increased in IND 04-1335 under drought, while these class of metabolites were significantly reduced under drought in the commercial variety Co 99004 (Fig. 4a). Small molecules belonging to class of terpenoids were reported to be accumulated in high concentration in roots of maize. These metabolites function as potential antioxidants and thereby improve resilience to abiotic stresses, such as drought and high salinity (Obata *et al.,* 2015). Several fatty acids were found to be considerably reduced under drought stress. Drought is expected to damage the cell membranes which serves as the first receptors of stress. Due to the damage in the cell membrane all the membrane-bound enzyme activities and the level of membrane fatty acids are affected and reduced under drought (Elena Petcu *et al.,* 2001; Van Meer *et al.,* 2008; Sánchez-Martín *et al.,* 2018). Higher accumulation of fatty acids was observed only in the control roots of both the genotypes, while the inverse was witnessed in drought exposed plants of both the genotypes. Several differentially accumulated metabolites identified in both the genotypes are reported to function as osmolytes, antioxidants, free radical scavengers, potential protein stabilizers etc., under drought stress (Yobi *et al.,* 2012; Yang *et al.,* 2015). Sugars are reported to function as potential osmoregulants and prevent membrane fusion in response to drought stress (Hoekstra *et al.,* 2001; Sakurai *et al.,* 2008). Melezitose belonging to class of sugars accumulated higher quantity in both the

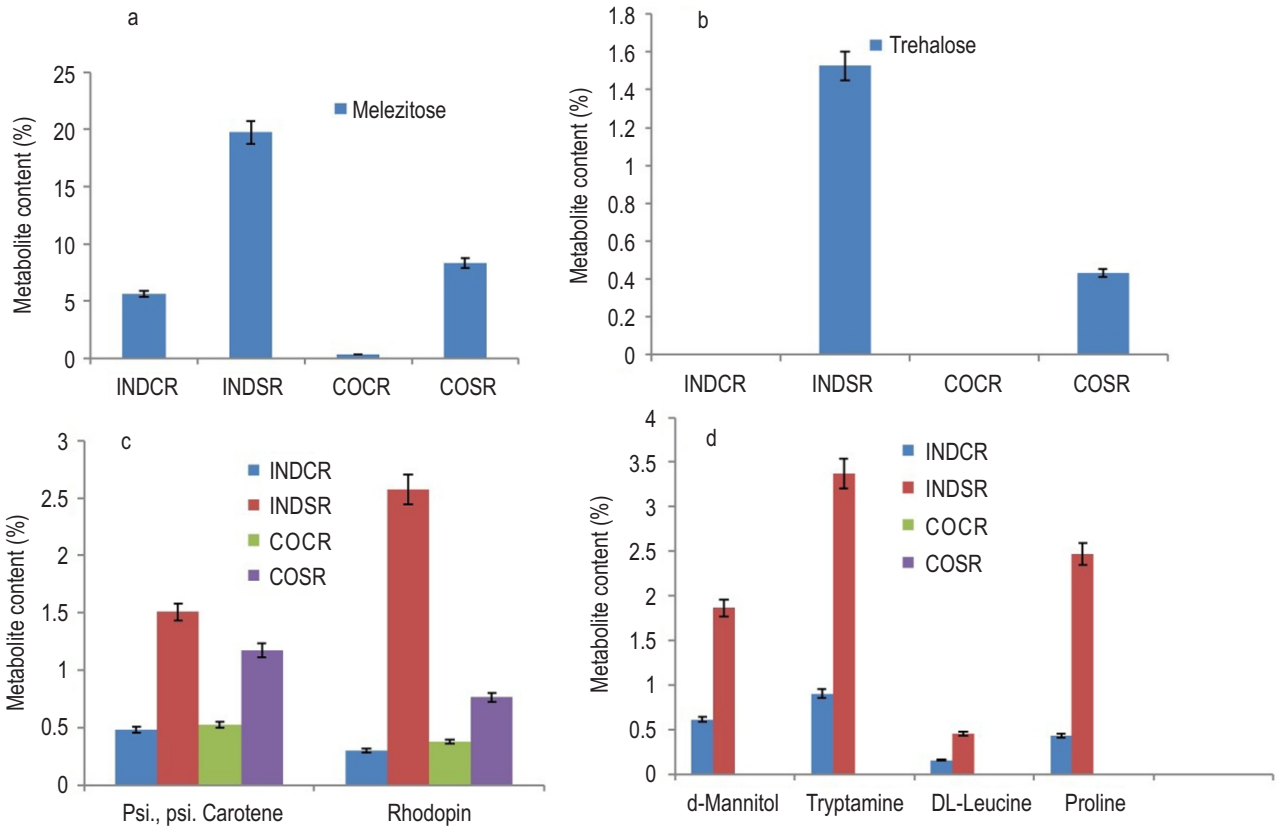

Fig. 5: Accumulation of different metabolites in the roots of IND 04-1335 and Co 99004 under control and stress. (a.) Melezitose, (b.) Trehalose, ©.and Carotenoids , (d.) Mannitol and amino acids. INDCR –IND04-1335 control root, INDSR – IND04-1335 stress root, COCR - Co 99004 control root, COSR - Co 99004 stress root.

genotypes under drought, while the concentration of melezitose in IND 04-1335 was two times higher than Co 99004 (Fig. 5a).

Higher accumulation of melezitose was observed in several crops and found to be significantly associated with desiccation tolerance (Harb *et al.,* 2015). Trehalose is a non-reducing disaccharide that acts as a protective compound to stabilize membranes and protein under abiotic stress conditions, such as dehydration, high salinity, hypoxia and nutrient starvation (Luo *et al.,* 2008; Paul *et al.,* 2018). In this study, significant accumulation of trehalose was observed only under drought condition in both the genotypes, while trehalose content in IND 04-1335 stressed root was three times higher in comparison with Co 99004 stressed roots (Fig. 5b). Carotenoids function as strong antioxidants in plant system and genes involved in regulation of carotenoid biosynthesis are significantly upregulated in leaves under drought stress. In this study, significantly increased accumulation of two carotenoids (rhodopin and psi, psi carotene) were observed in IND 04-1335 and Co 99004 under stress, while the concentration of these metabolites were found to be significantly higher in IND 04-1335 (Fig. 5c). This is the first report on the significant drought responsive accumulation of carotenoids in roots of sugarcane. Mannitol a potential sugar molecule which prevents cellular damage during drought stress through osmoregulation or through ROS scavenging was observed only in IND 04-1335 and drought enhanced its accumulation (Chan *et al.,* 2011; Yang *et al.,* 2015) (Fig. 5d). Elevated levels of certain amino acids such as proline are supposed to enhance cellular tolerance and protein stabilization during osmotic stress (Zhu *et al.,* 1998; Yobi *et al.,* 2012; Guo *et al.*, 2020).

Two amino acids leucine and proline showed higher accumulation in IND 04-1335 roots under drought conditions, while these amino acids were not detected in Co 99004 roots (Fig. 5d). Amino acid such as leucine and isoleucin have been demonstrated to function as alternate donors of electron in the respiratory chain or become alternate source of respiratory substrate under stress condition (Harding *et al.,* 2003; You *et al.,* 2019). Similarly, alkaloid tryptamine was detected only in IND 04-1335 and showed ncreased accumulation under drought condition. Specific role of this alkaloid under drought is still not very clear. Increased accumulation or biosynthesis of

compatible metabolites is one among the efficient strategy evolved by plant system to respond and adapt to extreme environmental conditions. The present study provides valuable information on the metabolomic responses of sugarcane roots under drought stress which can be further utilized in genetic improvement of drought tolerance in sugarcane.

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Add-on Information

Authors' contribution: R.Valarmathi: Conceptualization, methodology execution, data analysis and supervision; **H.K.M. Swamy:** Data compilation and Analysis; **K. Preeti:** GC MS analysis and manuscript preparation; **C. Appunu:** Supervision and manuscript editing.

Research content: The research content of manuscript is original and has not been published elsewhere.

Ethical approval: Not Applicable

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