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Metabolite profiling analysis by GC-MS of sugarcane culm (*Saccharum* ssp.) to reveal metabolites involved in the drought stress.

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ABSTRACT: water deficit is an abiotic stress that affects plant development and causes a reduction in the productivity of sugarcane. In this study, we analyzed the metabolite profile, by gas chromatography mass spectrometry (GC-MS), of sugarcane (*Saccharum* spp.) internodes (5 and 9) from two different cultivars with different ability to deal with stress: CTC15 (tolerant cultivar) and SP90-3414 (susceptible cultivar). Each cultivar was submitted to different field capacity (FC). Well-watered (100% FC), moderate stress (40 % FC) and stress severe (25% FC). By GC-MS analyzes 76 metabolites were identified, such as amino acids, sugars, polyamines, organic acids and other metabolites. There was a general increase in metabolite levels in the CTC15 cultivar and between treatments under drought stress, including changes in amino acids, sugars, organics acid and other metabolites. The CTC15 showed more amino acids in comparison to SP90-3414. In addition, amino acids, sugars, polyamine were significantly abundant in the severe stress condition. Therefore, an increase in amino acids, sugars and polyamine was observed under water stress conditions. In this work, in accordance with others studies, isoleucine and putrescine showed higher abundance when exposed to drought stress. Our results may encourage more detailed studies to understand the role of these amino acids in drought stress. Our data also contribute to further analysis aiming the identification of molecular markers to assist in plant breeding and suggests key metabolites to the development of transgenic sugarcane plants.

Key words: metabolite profiling, drought tolerance, sugarcane, GC-MS, culms.

1. INTRODUCTION

Sugarcane (*Saccharum* spp.) belongs to the Poaceae family, which is a C4 annual tropical plant specie. It needs appropriate temperature/humidity conditions to allow the

maximum growth during the vegetative phase and to accumulate sucrose in the culms (Inman-Bamber & Smith, 2010). Brazil is the largest producer of sugarcane in the world (the estimated harvested area is 8.84 million ha) and the country is expected to produce 647.6 million tonnes of sugarcane in the 2016/2017 crop (CONAB 2017) with an area of 9.110 ha in 2016. Brazil is also the largest sugar producer in the world, with 39.8 million tons produced in 2016, and is the second largest ethanol producer. In the 2016/2017 harvest, ethanol the volume produced was 27.8 million liters (Unica, 2017). Sugarcane is one of the crops with the higher productivity of biomass per planted area, and it has the higher amount of biofuel efficiency (Tew et al., 2008), due to its capacity to accumulate sucrose (a disaccharide), in the culms. The culms are formed by different physiological stage internodes. The immature culms are green, with more fibers, high concentration of hexoses and low concentration of sucrose (McCormick et al, 2006). However, the mature culms have higher levels of sucrose, the sucrose accumulation occurs during the maturation period, phase in which the plant has a decreased vegetative growth. This event depends on abiotic factors, such as water deficit and low temperature (Yao et al, 2002). However, sugarcane crop does not support long periods of water scarcity and this is one of the major abiotic stresses that damages the crop production, affecting several metabolic reactions, changing the rate of growth and sugarcane development. The water deficit is one of the main abiotic stresses that affect the productivities of the plants (Bolouri-Moghaddam et al, 2010). The water deficit in Brazil affect also the sugarcane productivity (Ramesh, 2000). Studies regarding the mechanism involved in sucrose accumulation in the culms are important to increase productivity and develop varieties more resistant to long periods of drought. Sucrose is the principal form of carbon storage in sugarcane. Synthesized in mature leaves and in stem parenchyma. Sugarcane has 12% - 17% total sugars, of which are composed by 90% of sucrose and 10% glucose/fructose (Wheals et al., 1999). In order to increase this sucrose concentration, genetic studies are being conducted. The Sugarcane EST Sequencing Project (SucEST) study the expression control genes in sugarcane and dataset are available at web. This data may be important to study the relationship between abiotic stresses and genic expression. In order, to provide a better understanding of metabolic routes and genes expression in response to environment change.

In the present work, we studied the metabolic profile of internodes 5 and 9 (I5 – I9) from two cultivars of sugarcane contrasting in tolerance to drought: CTC15 (drought tolerant) and SP90-3414 (susceptible drought), by GC-MS technique enabled the

identification of metabolites and enhanced our knowledge about metabolism pathways such as sugars, amino acids and organics acids.

2. RESULTS

The cultivars CTC15 and SP90-3414 were submitted to drought aiming to identify metabolic variation in the I5 and I9. In total, the metabolite profile, after manual inspection, resulted in the identification of 76 metabolites in sugarcane culms by GC/MS, including 24 carbohydrates, 16 organic acids, 15 amino acids, 3 fatty acyls, 3 amines, 2 alkanes, 2 fatty acid and others (for a complete list, see table 1). Therefore, with the aim to identify metabolites differentially abundant among the treatments, ANOVA was performed for each cultivar (I5 and I9). The metabolic profiling was separated into two groups and reported as a heat-map (Fig 1). The first group includes metabolite profiling of I5 and I9 from CTC15 (Fig. 1 A). The second group includes variation of metabolites in the I5 and I9 from SP90-3414 (Fig. 1 B).

2.1 Metabolites identified in CTC15 and SP90-3414

The number of total metabolites identified varied between cultivars and internodes (I5 and I9). In the cultivar CTC, I9 showed the highest number of metabolites in comparison with I5. In contrast, the I5 in cultivar SP90-3414 had a higher number of metabolites in comparison to I9 (Table 2).

2.1.1. Principal Component analysis (PCA)

Principal Component analysis (PCA) was performed with the aim to identify outliers and to analyze the trend of separation between treatments (WW, MS and SS) in the cultivars (CTC15 and SP90-3414).

2.1.2. PCA of the internode 5 from CTC15 with well-watered, moderate stress and severe stress treatments.

The results of PCA to internode 5 were 22.2 % to PC1 and 14.8 % PC2, the octadecenoic acid and aconitic acid were the principal metabolites that contribution in the PC1. In the PC2 were sorbose and maltitol (Fig. 2).

2.1.3. PCA of the internode 5 from CTC15 with well-watered and moderate stress.

The results of PCA to internode 5 were 33.5 % to PC1 and 20.7 % PC2, the octadecenoic acid and benzoic acid were the principal metabolites that contribution in the PC1. In the PC2 were aspartic acid and mannopyranose (Fig. 3).

2.1.4. PCA of the internode 5 from CTC15 with well-watered and severe stress.

The results of PCA to internode 5 were 27 % to PC1 and 23.9 % PC2, the sorbose and maltitol were the principal metabolites that contribution in the PC1. In the PC2 were ribitol and maleamic acid (Fig. 4).

2.1.5. PCA of the internode 9 from CTC15 with well-watered, moderate stress and severe stress.

The results of PCA to internode 9 were 21.2 % to PC1 and 12.7 % PC2, the octadecenoic acid and arginine were the principal metabolites that contribution in the PC1. In the PC2 were galactose and inositol-2-phosphate (Fig. 5).

2.1.6. PCA of the internode 9 from CTC15 with well-watered and moderate stress.

The results of PCA to internode 9 were 30.5 % to PC1 and 17.4 % PC2, the gluconic acid-1.5-lactone and KDG were the principal metabolites that contribution in the PC1. In the PC2 were mannopyranoside and lactobionic acid (Fig. 6).

2.1.7. PCA of the internode 9 from CTC15 with well-watered and severe stress.

The results of PCA to internode 9 were 25.5 % to PC1 and 16.6 % PC2, the xylitol and homoserine were the principal metabolites that contribution in the PC1. In the PC2 were inositol-2-phosphate and similar to fructose (Fig. 7).

2.1.8. PCA of the internode 5 from SP90-3414 with well-watered, moderate stress and severe stress.

The results of PCA to internode 5 were 22.1 % to PC1 and 14.8 % PC2, the galactopyranoside and gluconic acid-1.5-lactone were the principal metabolites that contribution in the PC1. In the PC2 were artemisinic and KDG (Fig. 8).

2.1.9. PCA of the internode 5 from SP90-3414 with well-watered and moderate stress.

The results of PCA to internode 5 were 29.4 % to PC1 and 16.8 % PC2, the putrescine and secologanin were the principal metabolites that contribution in the PC1. In the PC2 were fructose and hydroxymethylfurfural (Fig. 9).

2.1.10. PCA of the internode 5 from SP90-3414 with well-watered and severe stress.

The results of PCA to internode 5 were 28.7 % to PC1 and 22.5 % PC2, the oxamide and aspartic acid were the principal metabolites that contribution in the PC1. In the PC2 were xylitol and galactopyranoside (Fig. 10).

2.1.11. PCA of the internode 9 from SP90-3414 with well-watered, moderate stress and severe stress.

The results of PCA to internode 9 were 41.9 % to PC1 and 17.3 % PC2, the asparagine was the principal metabolite that contribution in the PC1. In the PC2 were galactitol and gulonic acid (Fig. 11).

2.1.12. PCA of the internode 9 from SP90-3414 with well-watered and moderate stress.

The results of PCA to internode 9 were 48.1 % to PC1 and 20.1 % PC2, the asparagine and pyroglutamic acid were the principal metabolite that contribution in the PC1. In the PC2 were aspartic acid and mannopyranose (Fig. 12).

2.1.13. PCA of the internode 9 from SP90-3414 with well-watered and severe stress.

The results of PCA to internode 9 were 59.8 % to PC1 and 15.2 % PC2, the asparagine was the principal metabolite that contribution in the PC1. In the PC2 were putrescine and xylitol (Fig. 13).

2.2. Metabolites common to I5 and I9 (CTC15 and SP90-3414)

In the comparison between metabolites common found from SP90-3414 and CTC15, in the internode 5 and 9 with different treatment only the metabolite pantothenic acid was common in the internode 5 in both cultivars (CTC15 and SP90-3414) and hydroquinone was common in the internodes 9 in both cultivars (see Fig. 14).

2.3 Internode 5: metabolic profile of drought tolerant sugarcane cultivar (CTC15) by GC/MS

Aiming to identify the exclusive and common metabolites for each treatment, a Venn diagram was performed (http://bioinfogp.cnb.csic.es/tools/venny/). In the I5 from CTC15 cultivars, 13 metabolites were exclusivelly associated to one of the treatments; five metabolites were exclusively found in WW treatment: galactitol and xylitol (carbohydrate), pantothenic acid and, KDG (organic acid), aspartic acid (amine). In MS plants we found four exclusive metabolites: proline and pyroglutamic acid (amino acid), similar to ribulose (carbohydrate) and butanoic acid (fatty acid). In addition, four metabolites were found only in SS treatment: gulonic acid and malic acid (organic acid), butylamine (amine) and secologanin (terpenoid) (Fig. 14 A).

2.3.1. Comparative metabolite profiling of well-watered versus moderate stress (I5)

Metabolite profiling of the CTC15 cultivar was performed to compare WW versus MS, considering the I5 and it was identified by Student´s t-test ($P \leq 0.05$). The analysis showed that the metabolites galactose, fructose, mannose1-phosphate, arabinose (carbohydrates), homoserine, methionine, asparagine (amino acids) were higher in WW plants. However, aspartic acid (amino acid) and maleic acid (organic acid) were more abundant in the MS treatment.

2.3.2. Comparative metabolite profiling of well-watered versus severe stress (I5)

GC/MS metabolite profiling showed a significant difference between WW and SS treatments. Mannose-1-phosphate (carbohydrate), asparagine, aspartic acid, homoserine (amino acids) and propanoato (alcohol) were found in higher abundance in WW plants. In contrast, the metabolites galactose, gluconic acid, sorbose (carbohydrate), cysteine (amino acid), benzoic acid (organic), glycerol (alcohol) were more abundant in SS treatment.

2.3.3. Comparative metabolite profiling of well-watered versus moderate stress versus severe stress (I5)

With the aim to identify the metabolites differentially abundant among the treatments, ANOVA was performed ($p< 0.05$), and we identified 15 metabolites. The metabolites maleic acid, ribose, homoserine, propane, maltitol and erythronic acid were differentially abundant in the WW cultivar. In contrast, the MS cultivar had only benzoic

acid and tartronic acid with high levels. In addition, in the SS cultivar the metabolites butanoic acid, octadecenoic acid, artemisinic acid, myristic acid amide, galactose and asparagine were high abundant in this treatment (see Fig. 1 A).

2.4. Internode 9: metabolic profile of drought tolerant sugarcane cultivar (CTC15) by GC/MS

The I9 from CTC15 cultivar showed in total 11 metabolites exclusively to one of the treatments, such as five metabolites exclusive to WW cultivar (lactose, gluconic acid, myristic acid, gulonic acid and estrone). The MS treatment showed three exclusive metabolites (aspartic acid, maleic acid and butylamine). In the SS cultivar we also found three exclusives metabolites (alpha-D-Glucopyranosyl, pantothenic acid and hydroquinone) (Fig. 14 B).

2.4.1. Comparative metabolite profiling of well-watered versus moderate stress (I9)

The metabolites differentially abundant in the WW cultivar were glucose, inositol-2-phosphate (carbohydrate), pyroglutamic acid, arginine (amino acids), benzoic acid, fumaric acid (organics). Whereas only botanic acid (fatty acid) was high abundant in the MS.

2.4.2. Comparative metabolite profiling of well-watered versus severe stress (I9)

In the WW, the metabolites more abundant were inositol-2-phosphate (carbohydrate), pyroglutamic acid, asparagine (amino acids), fumaric acid (organic). However, the metabolites ribitol, KDG, benzoic acid (organic acid), isoleucine (amino acid), glycerol (alcohol) showed high levels in the SS.

2.4.3. Comparative metabolite profiling of well-watered versus moderate stress versus severe stress (I9)

To identify metabolites differentially abundant among the I9 treatments from CTC cultivar, ANOVA was performed (p - value < 0.05), we identified 14 metabolites. The metabolites benzoic, hydroxymethylfurfural, pyroglutamic acid, arginine, fumaric acid, pentadecane were differentially abundant in the WW. In the MS, the metabolites abundant were butanoic acid, galactitol, 3-ketoadipate, lactobionic acid, octadecenoic acid. Included in the metabolites SS cultivar were phosphoric acid, propane and mannopyranoside (see Fig. 1 A).

2.5. Internode 5 metabolic profile of drought tolerant sugarcane cultivar (SP90-3414) by GC/MS

We used Venn diagram (http://bioinfogp.cnb.csic.es/tools/venny/) to identify the exclusive and common metabolites for each treatment. In the I5 from SP90-3414 cultivar showed a total of 10 metabolites exclusively. In the WW, the exclusive metabolites were glucose-6-phosphate and similar to fructose (carbohydrate), myristic-acid-amide (fatty acyl), pantothenic acid (organic acid) and estrone. Whilst, in the MS, the metabolites exclusives were galactose and alpha-D-Glucopyranosyl (carbohydrate), glutaric acid (organic acid) and hydroquinone (phenol). In addition, SS, only butanoic acid (fatty acid) were exclusive for this treatment (Fig. 14 C).

2.5.1. Comparative metabolite profiling of well-watered versus moderate stress (I5)

In relation to the WW, the metabolites fructose, inositol-2-phosphate (carbohydrate), asparagine (amino acid) were found in high levels. In contrast, the most abundant metabolites in MS were putrescine, homoserine (amino acids), butanoic acid (fatty acid), glycerol (alcohol) and maleic acid (organic).

2.5.2. Comparative metabolite profiling of well-watered versus severe stress (I5)

The metabolites fructose, inositol-2-phosphate (carbohydrate), asparagine (amino acid), octadecenoic acid (fatty acid) were more abundant in the WW. Whereas the metabolites xylitol (carbohydrate), aspartic acid, isoleucine (amino acids) were highly abundant in the SS.

2.5.3. Comparative metabolite profiling of well-watered versus moderate stress versus severe stress (I5)

In the SP90-3414 cultivar, with the aim to identify different metabolites abundances, ANOVA was performed ($p - value < 0.05$). The metabolites arginine and fructose were more abundant in the WW plant. Although that, the metabolites butanoic acid glycerol, putrescine, 5- hydroxymethylfurfural, maleamic acid, 3-ketoadipate, aconitic acid, homoserine lactone and artemisinic acid were high levels in the MS plant. In addition, the metabolites with high levels in the SS cultivar were oxamide, KDG, gluconic acid and ergocalciferol (Fig. 1 B).

2.6. Internode 9 metabolic profile of drought tolerant sugarcane cultivar (SP90-3414) by GC/MS

The I9 from SP90-3414 cultivar showed in total 11exclusive metabolites. Five of them were exclusively found in the WW, namely lactobionic acid (carbohydrate), octadecenoic acid (amino acid), secologanin (terpenoid), KDG (organic acid), and butylamine (amine). It was identified four exclusive metabolites in the MS: alpha-D-glucopyranosyl (carbohydrate), butanoic acid (fatty acid) tartronic acid (organic acid) and phosphoric acid (inorganic acid). The two remaining metabolites, severe stress myristic-acid-amide (fatty acyl) and hydroquinone (phenol) were found in the SS (Fig. 14 D).

2.6.1. Comparative metabolite profiling of well-watered versus moderate stress (I9)

The metabolites more abundant in the WW were ribose, ribitol, arabinose, fructose (carbohydrate), propanoato (alcohol), tropic acid (organic acid). In contrast, the metabolites more abundant in the MS were homoserine, isoleucine (amino acids), maleic acid (organic acid).

2.6.2. Comparative metabolite profiling of well-watered versus severe stress (I9)

In the WW cultivar, only tropic acid (organic acid) was high abundant. In contrast to SS cultivar that had isoleucine, aspartic acid, asparagine, putrescine, pyroglutamic (amino acids), glycerol (alcohol), butanoic acid (fatty acid), maleic acid (organic acid) as abundant.

2.6.3. Comparative metabolite profiling of well-watered versus moderate stress versus severe stress (I9)

ANOVA was performed to enable the identification of metabolites with different proportions in the I9 (SP90-3414). The metabolites propane, mannopyranose, arabinose, ribose, xylitol, galactitol and pantothenic acid were more abundant in the WW. In the MS, the metabolites maleamic acid, cadaverine and aspartic acid were the abundant ones. In contrast, the metabolites abundant in SS plant were oxamide, artemisinic acid, isoleucine, itaconic acid and gluconic acid (see Fig. 1 B).

3. DISCUSSION

Brazil is the largest producers of sucrose and ethanol in the world (Conab, 2017). These sucrose and ethanol are obtained from sugarcane culm. The sugarcane growth has

various stages and the initial accumulation of sucrose in sugarcane internodes begins when the vegetative growth decreases. Moreover, the irregularity of rainy season causes water deficit, the major abiotic stress in plantations that may interfere in the production of sugarcane (Lopes et al., 2011). Studies about metabolites are strategic to determine the genic function with integrate information in relation to the genetic alteration in response to environmental changes. According to Casu et al. (2007) there were changes in enzymatic activities and gene expression in the stem associated to the growth and accumulation of sucrose. In addition, metabolic routes can provide important features to the development of a gene marker (Rontein et al., 2002). However, a few studies aimed at understanding the metabolic profiling in culm of sugarcane (Glassop et al., 2007; Bosch et al. 2003), especially with a cultivar from Brazil.

Therefore, this work showed the levels of metabolites in response to drought stress in sugarcane culm. Revealing the relative metabolites abundance in different internodes of sugarcane aiming to understand the mechanisms involved in water stress conditions. We studied by GC-MS the metabolite profiling in internodes 5 and 9 of two different cultivars (CTC15 and SP90-3414). GC-MS is a technique that has been used to distinguish the metabolite profile in different plants or tissues. This can be useful in research dealing with metabolic process, transcription and regulatory signs as a way to provide clues to the identification of genetic markers related to water stress (Fiehn, 2002; Schauer et al. 2006).

3.1. Amino acids differentially abundant

In order to have a better visualization and reduce the general data extracted by GC-MS, it was generated a metabolic pathway (Fig. 15, 16) of the contrasting cultivars with significant abundance ($p \leq 0.05$) in the cultivars CTC15 and SP90-3414. Therefore, we showed the metabolites changes in internodes 5 and 9 when exposed to water stress. In total, the analysis yielded 76 metabolites. The CTC15 showed more amino acids in relation with the SP90-3414, demonstrating an increase in the synthesis of amino acids under water stress conditions. As Bowne et al. (2012), the levels of amino acids observed were statistically significantly higher in all cultivar of wheat exposed to water stress and the increase of these sugars, according to the author, may be an initial defensive response against water loss. Report by Hochberg et al. (2013) also observed increased amino acids levels in stress-induced plants in grapevine. In the report Kilian et al. (2007), the protein enzymes are important for the metabolism of amino acids, since they increase the

catabolic function in proportion to the abiotic stress at *Arabidopsis thaliana*. Pavli et al. (2013) stated that water stress alters plant metabolism and may result in reduction of protein synthesis in leafs and roots on sorghum. In addition, their work suggests that the high levels of amino acids may have been resulted by the reduction of protein synthesis or to the degradation of existing proteins.

Isoleucine is a component of branched chain of amino acids (BCAAs). In our work, isoleucine had significant abundance in the moderate and severe treatment in comparison with well-watered. In the internode 9 of cultivar CTC15, it appeared in high level in the severe stress treatment. In addition, from cultivar SP90-3414, internode 5, the isoleucine was significantly abundant in severe stress. Astonishingly, when we compared the treatments WW versus MS or WW versus SS in the internode 9 (SP90-3414), the isoleucine also had high levels in the moderate stress and severe stress in relation to well-watered treatment. Additionally, a comparison with the three treatments in the internode 9 (SP90-3414) showed that isoleucine had significant abundance in severe stress (see Fig.1 B). Isoleucine also had high levels in stress treatment (Urano et al., 2009; Less and Galili, 2008; Nunes-Nesi et al., 2010; Bowne et al.; 2012; Witt et al.; 2012). According to Taylor et al. (2004), the high levels of (BCAA) suggest an alternative route to energy in sugars in *Arabidopsis*.

3.2. Carbohydrates differentially abundant

In relation to carbohydrate metabolism, the CTC15 founded more sugars than SP 90-3414 cultivar. When comparing sugars within SP90-3414 plant, it showed in the I5 that fructose and inositol-2-phosphate were high levels in well-watered to both comparisons (WW versus MS; WW versus SS). Moreover, xylitol was abundant SS treatment compared with WW. Whilst, the I9, the metabolites significantly abundant in the WW were ribose, ribitol, arabinose, fructose compared with MS cultivar. The monosaccharide fructose was found as more abundant in the well-watered treatment from SP90-3414, between comparison I5 (WW-MS; WW-SS) and I9 (WW-MS). Although, CTC15 cultivar, fructose was high levels in the WW plant in comparison MS in internode 5. Although Pavli et al. (2013) observed increased sugars, organic acid in leaves and roots of stressed plants. Galactose and mannose showed increased in CTC15 with increase in the severe stress. Interestingly, these sugars were not abundant in the well-watered. According Bowne et al. (2012), to accumulated sugars may be an initial reaction against the water loss.

3.3. Polyamine differentially abundant

Additionally, an increase in putrescine in response to drought stress was highly abundant in moderate stress when compared with well-watered in the internode 5, and the same result was showed when compared the three treatments (WW versus MS versus SS). Thus, putrescine had high levels in moderate stress. In addition, in the I9 (SP90-3414), putrescine was highly abundant in SS in comparison with WW. A research conducted by Alcázar et al. (2014), which studied the effect of polyamine metabolic in response to drought stress in *Arabidopsis*, putrescine also showed high levels in drought stress conditions. According to Witt et al. (2012), polyamine, such as, putrescine, may become an interesting target in the future.

3.4. Drought stress and reactive oxygen species (ROS)

Metabolism which provides energy for plant growth affects the reactive oxygen species (ROS). When plants suffer from exposure to drought stress causes an accumulation of ROS with $^1O_2$, H_2O_2, O_2^-, OH as by-products. This may affect spatial configurations of proteins or enzymes and cause various metabolism perturbations (Fang and Xiong, 2014). This could be the cause of the increase in amino acids generally (Ford et al. 2011), such as, increased isoleucine. According DeBolt et al. (2007), some amino acids have been suggested to have a response under drought stress, including osmotic adjustment and reactive oxygen species.

4. CONCLUSION

Our results indicate that drought stress may interfere in metabolic synthesis in sugarcane culm. Amino acids and sugars increase in the drought stress treatment, demonstrating a possible mechanism of resistance to stress. This mechanism may be further investigated to obtain a molecular marker that allows an increase in sucrose accumulation and assist the development of new cultivars that are more resistant to drought.

5. METHODS

5.1. Plant materials

Drought stress treatments were carried out with two sugarcane cultivars: CTC15 (drought tolerant) and SP90-3414 (drought susceptible) to study changes metabolite

profiles in response it. These cultivars were previously reported as tolerant and susceptible (Thiebaut et al. 2014) to drought. Both cultivars were kindly provided by CTC (Centro de Tecnologia Canavieira, Piracicaba, Brazil). Five biological replicates from each treatment were sampled.

5.2. Stress treatment

A greenhouse experiment was conducted with temperature of 26˚C ± 2˚C and relative humidity of 60%. Single stalks were planted, germinated and growth in a 50 L pot with a mixture (2:1) of substrate (BasaPlant®, Brazil) and vermiculite. The plants were arranged in a completely randomized experimental design, under daily irrigation and the soil moisture was maintained near to 100% of field capacity (FC). After five months, plants were exposed to three levels of water availability in the soil:

- well-watered (WW – permanently irrigated and 100% FC);
- moderate stress (MS – 6 days without irrigation and 40% FC);
- severe stress (SS – 10 days without irrigation and 25% FC) (Fig. 17).

After this treatment, the internodes 5 and 9 of each cultivar were immediately frozen in liquid nitrogen and then stored at −80 °C until metabolite extraction. According to Ramesh et al. (2000), the growth phase of sugar cane at five months was identified as the critical period involving water demand. In addition, 70-80% of cane yield is produced during this phase (Singh and Rao, 1987).

5.3. Metabolites extraction and derivatization

The culm tissue was ground under liquid nitrogen using a ball mill with 50 mg of tissue for metabolomics analysis. In the next step, 500 µL of pre-chilled methanol (MeOH): chloroform ($CHCl_3$): water (H_20) (6:2:2 v/v/v) were added to the tissues. The extract was vortexed vigorously, sonicated at 40 Hz s^{-1} for 15 min and centrifuged at 14.000 x g for 10 min at 4 °C. The supernatant was filtered (0.2 µm) and 100 µL of each sample was transferred to vials and evaporate until dryness.

In the control treatment, blank samples were prepared and analyzed with each true groups. Blank control samples and a series of *n*-alkanes (C12–C40), which allowed retention indices to be calculated, were also used (Schauer et al. 2005). The derivation was according Hoffman et al (2010), with 30 µL of methoxyamine hydrochloride (15 mg mL^{-1}) in pyridine for 16 h at room temperature. The samples were trimethylsilylated by adding 30 µL of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) containing 1%

trimethylchlorosilane (TMCS), the resulting mixture stand at room temperature for 1h. After silylation, 30 µL of heptane was added. Stable isotope reference compounds [1 mg mL^{-1} each of ($^{13}C_3$)-myristic acid, ($^{13}C_4$)-palmitic acid and ($^{2}H_4$)-succinic acid] were added in samples prior to derivatization and used as external standard for quality control. In addition, the derivation analysis was in accordance with Gullberg et al. (2004).

5.4. Analysis and metabolites identification by GC-MS

To each sample with volumes with 1 µL was injected splitless into a gas chromatograph 7890A (Agilent Technologies, Santa Clara, USA) coupled with a Comb-xt Autosampler (Leap Technologies, Carrboro, USA). The injector temperature was 280 °C, the septum purge flow rate was 20 mL min-1 and the purge was turned on after 60 s. The gas flow rate through the column was 1 mL min-1, the column temperature was held at 80 °C for 2 min, then increased by 15 °C min to 305 °C, and held there for 10 min. The column effluent was introduced into the ion source of a GC×GC/TOFMS (Pegasus 4D, Leco Corp., St. Joseph, USA). The transfer line and the ion source temperatures were 280 and 250°C, respectively. Ions were generated by a 70 eV electron beam at an ionization current of 2.0 mA, and 10 spectra s-1 were recorded in the mass range 45–800 m/z.

The mass spectra were evaluated using the ChromaTOF software v. 4.51 (Leco Corp., St. Joseph, USA) was used to perform baseline correction and export all MS files in NetCDF format. Peak detection, retention time alignment and library matching were performed using the TargetSearch package (Cuadros-Inostroza et al., 2009). According Kopka et al. (2006) the metabolites were identified by comparing their retention indexes (± - 2 s) and spectra (similarity > 600) against the compounds stored in the Golm-Metabolome-Database (http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/gmd.html) and the metabolites had confirmed identity when with were present in three of five cultivars and were normalized by dry weight and total ion chromatogram (TIC).

5.5. Statistical analysis

The data from I5 and I9 samples were normalized with the division of values for each metabolite in each sample by the average value of all metabolites from each treatment. All experimental data were tested for Univariate (ANOVA and Student´s t-test) and multivariate (Principal Component Analysis - PCA) analyses were done in MetaboAnalyst 3.0 (Xia et al. 2015). To reduce systematic variance and to improve the performance for downstream statistical analysis data were normalized by the median, log-

transformed and Pareto scaled prior to data analysis. One-way ANOVA (FDR, adjusted p ≤ 0.05) was used to found significant differences among treatments, of each cultivar. After, Student's *t*-test (FDR ≤, adjusted p ≤ 0.05).

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DATA

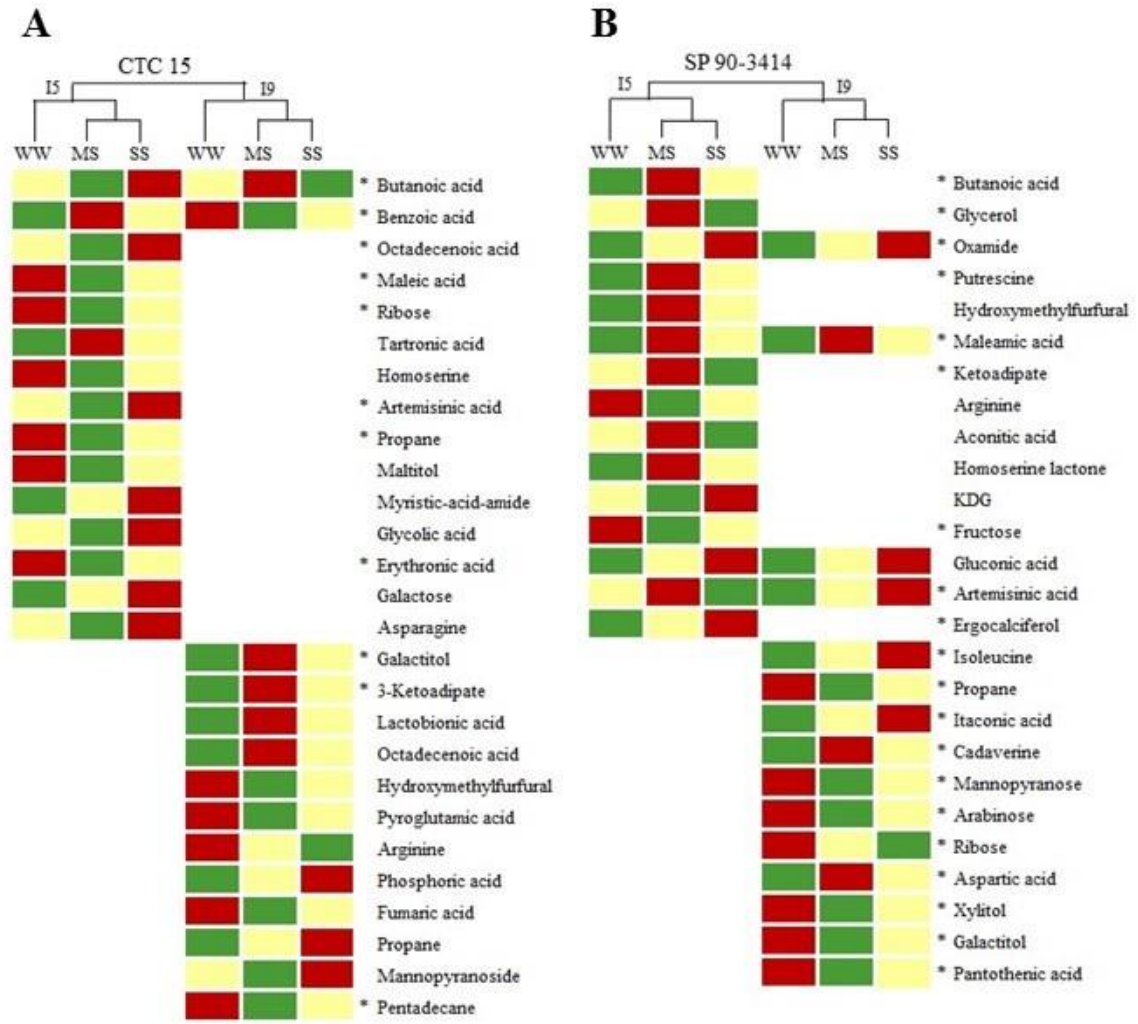

Figure 1: Heat map of metabolites differentially abundant among the treatments WW, MS, SS in the I5 and I9 from CTC 15 (**A**) and SP90-3414 (**B**). The red color represents the most abundant metabolites, green the less abundant metabolites and blank spaces means metabolites that were not detected. ANOVA results are showed with an asterisk (*) to indicate the metabolites significantly different ($P \leq 0.05$).

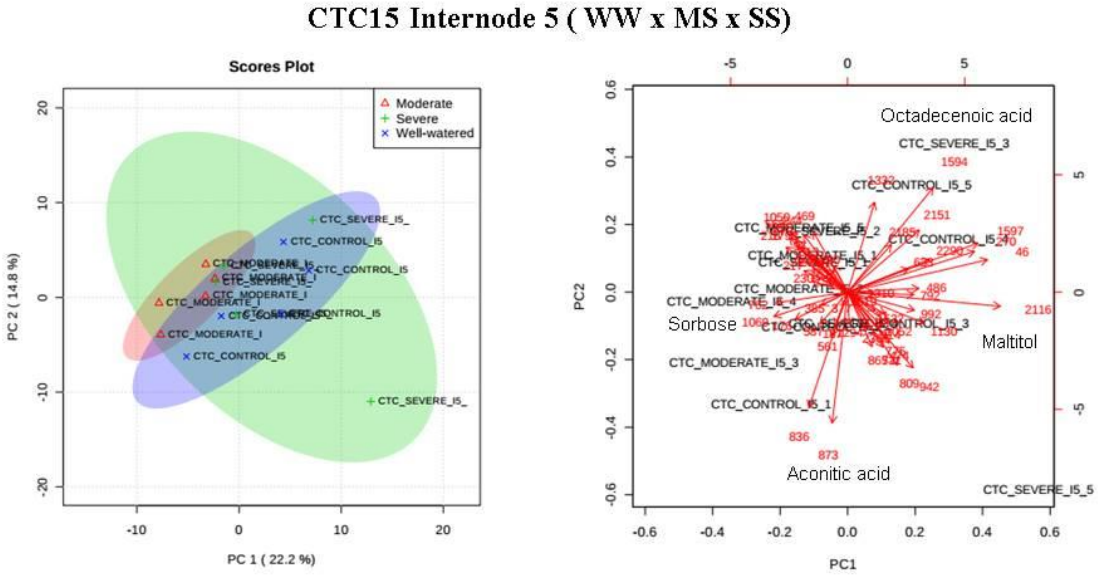

Figure 2: PCA of the CTC 15 from internode 5 with comparison well-watered versus moderate stress versus stress severe.

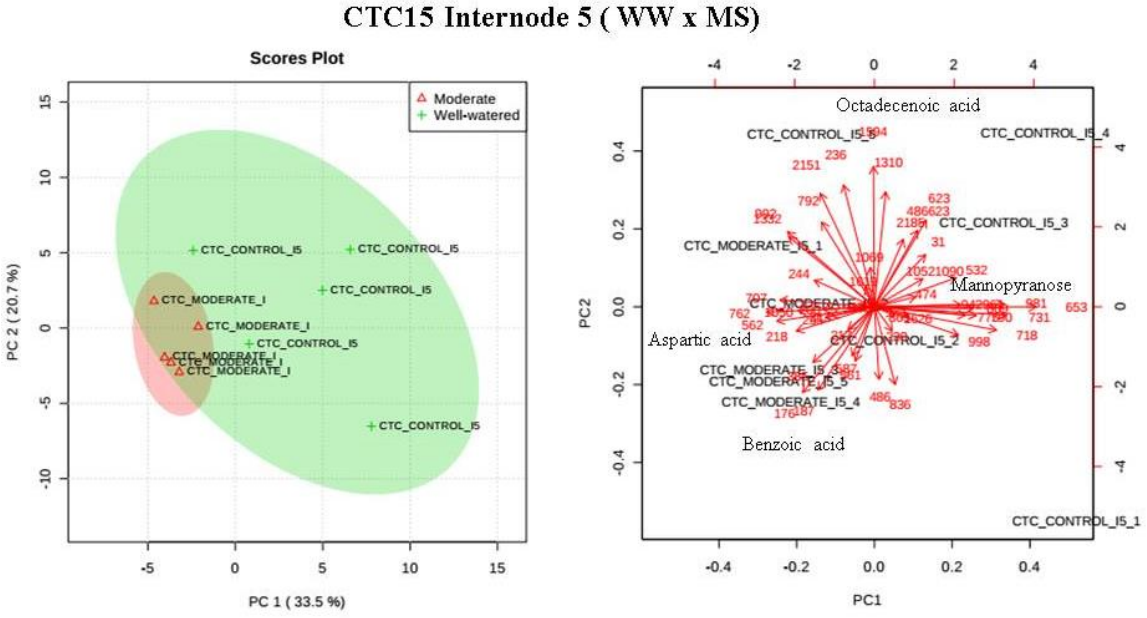

Figure 3: PCA of the CTC 15 from internode 5 with treatments (well-watered versus moderate stress).

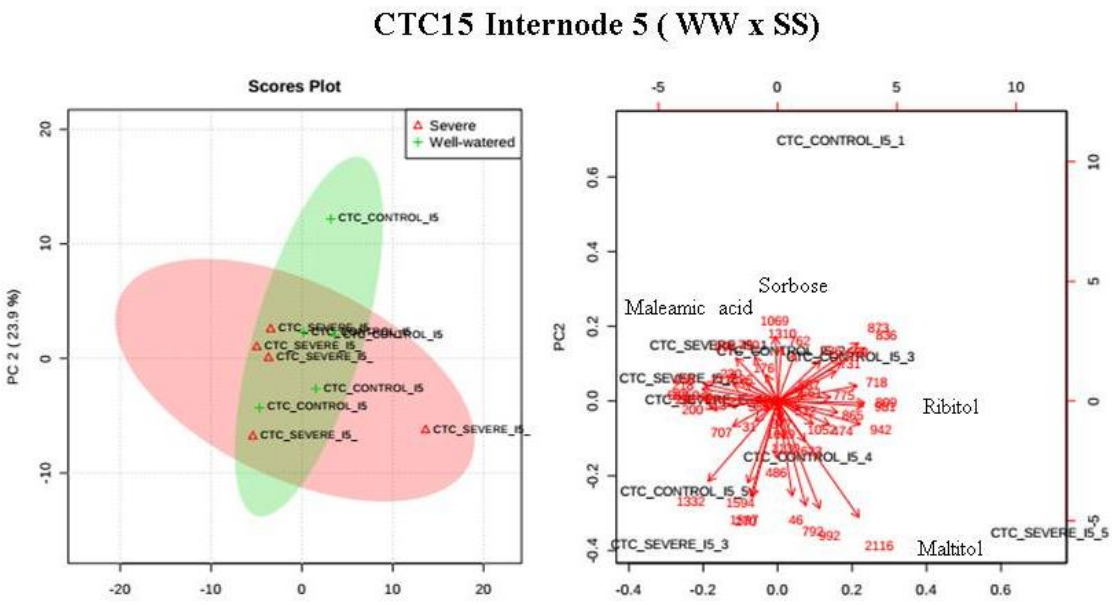

Figure 4: PCA of the CTC 15 from internode 5 with treatments (well-watered versus severe stress).

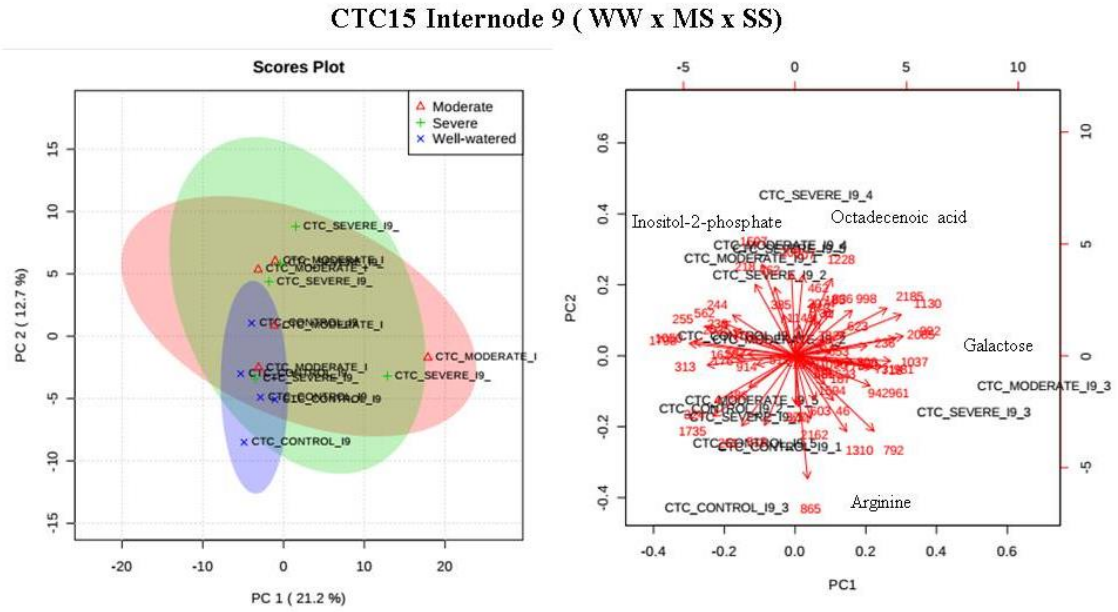

Figure 5: PCA of the CTC 15 from internode 9 with treatments (well-watered versus moderate stress versus severe stress).

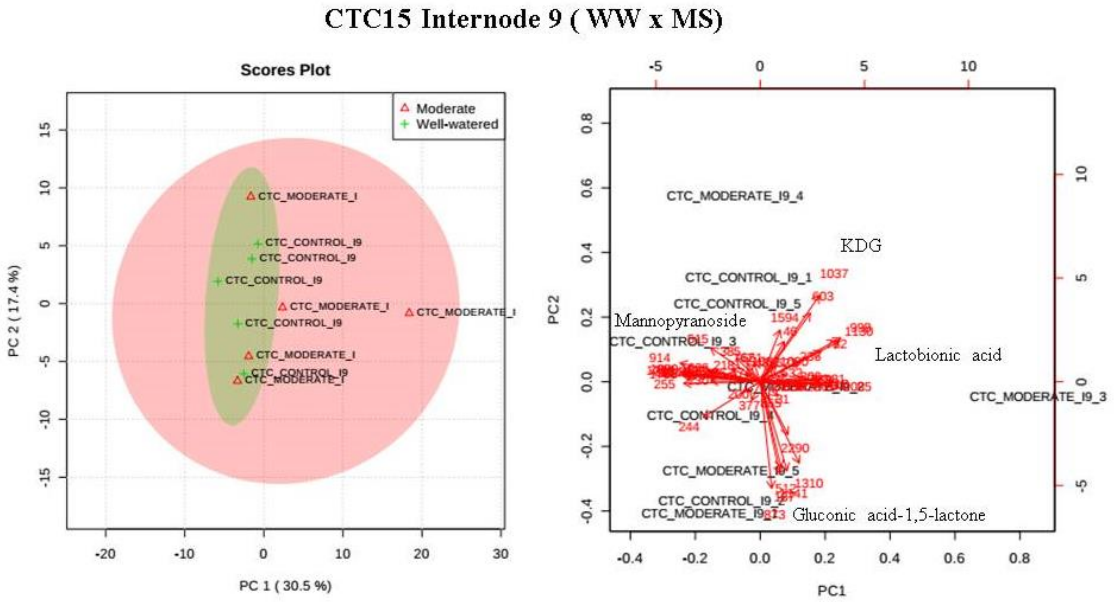

Figure 6: PCA of the CTC 15 from internode 9 with comparison (well-watered versus moderate stress).

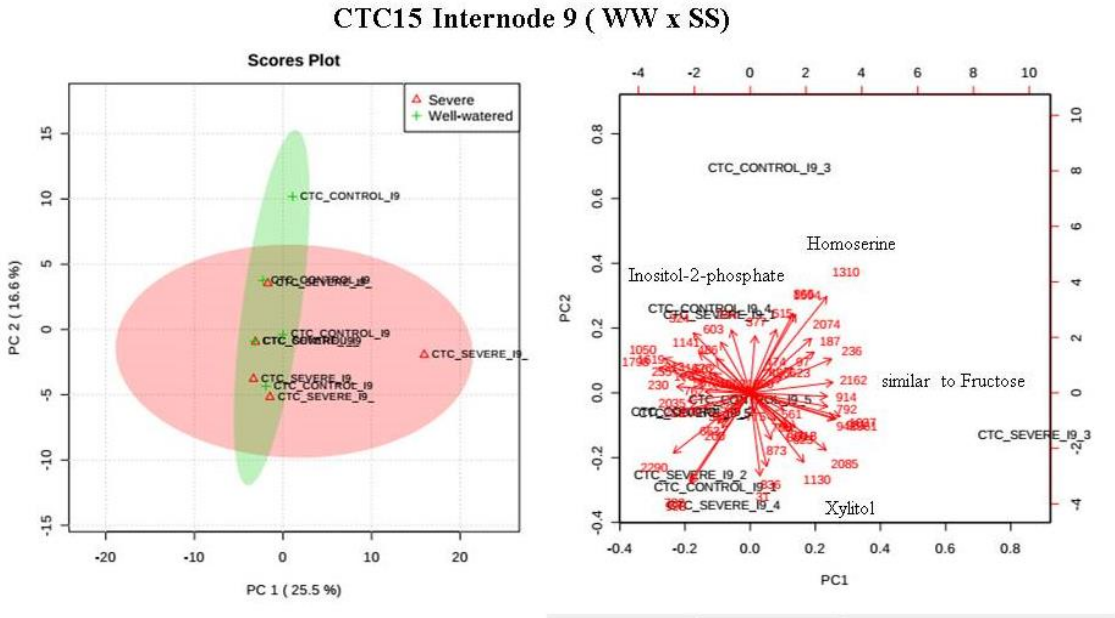

Figure 7: PCA of the CTC 15 from internode 9 with treatments (well-watered versus severe stress).

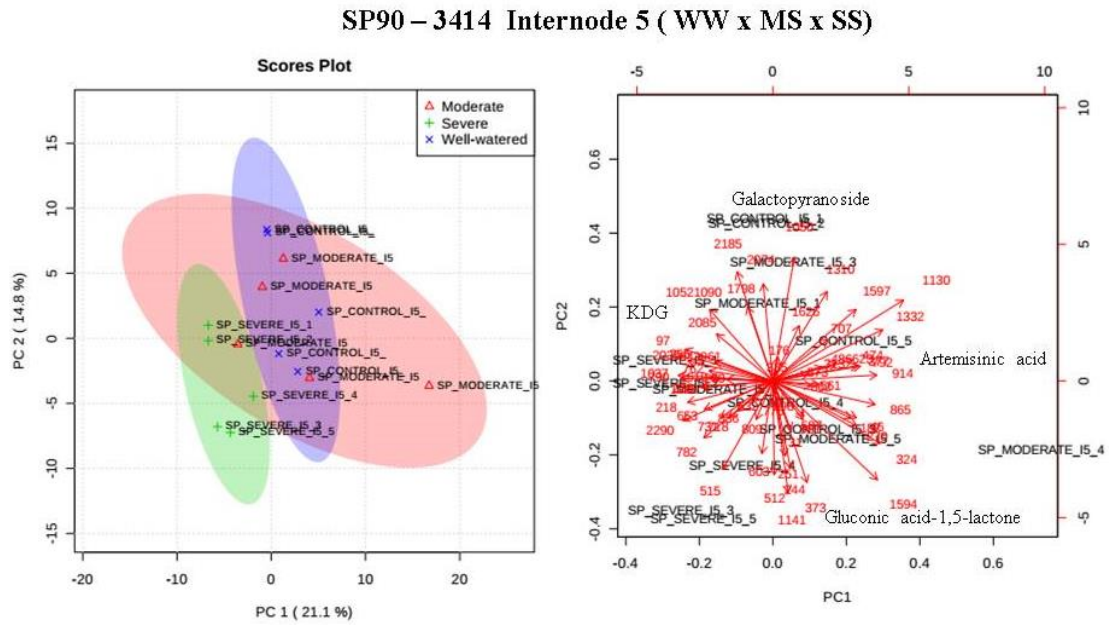

Figure 8: PCA of the SP90-3414 from internode 5 with comparison (well-watered versus moderate stress versus severe stress).

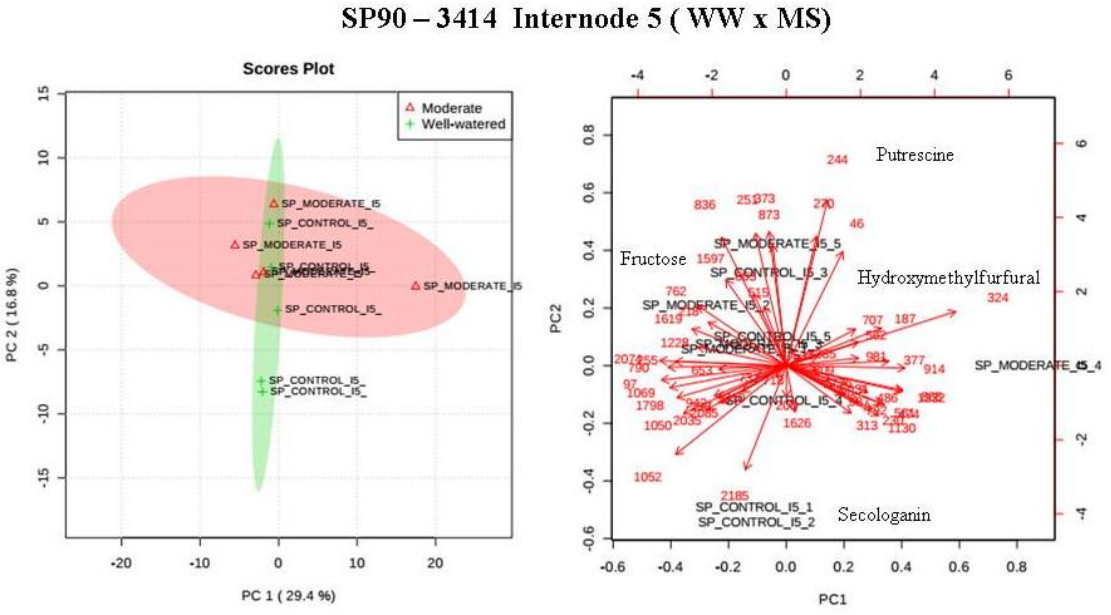

Figure 9: PCA of the SP90-3414 from internode 5 with well-watered versus moderate stress.

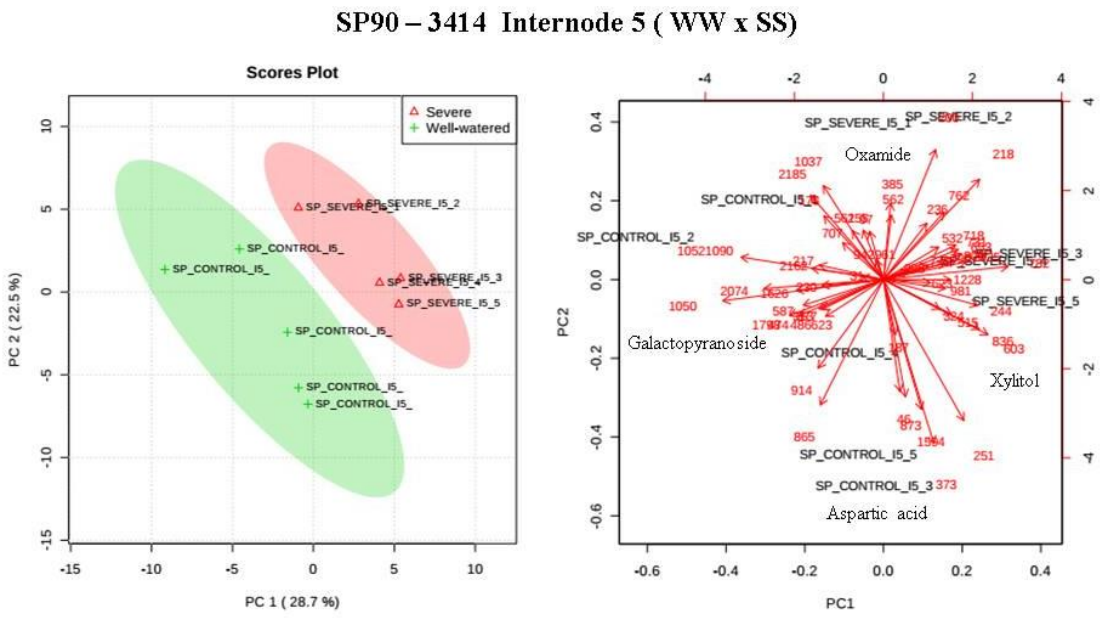

Figure 10: PCA of the SP90-3414 from internode 5 with well-watered versus severe stress.

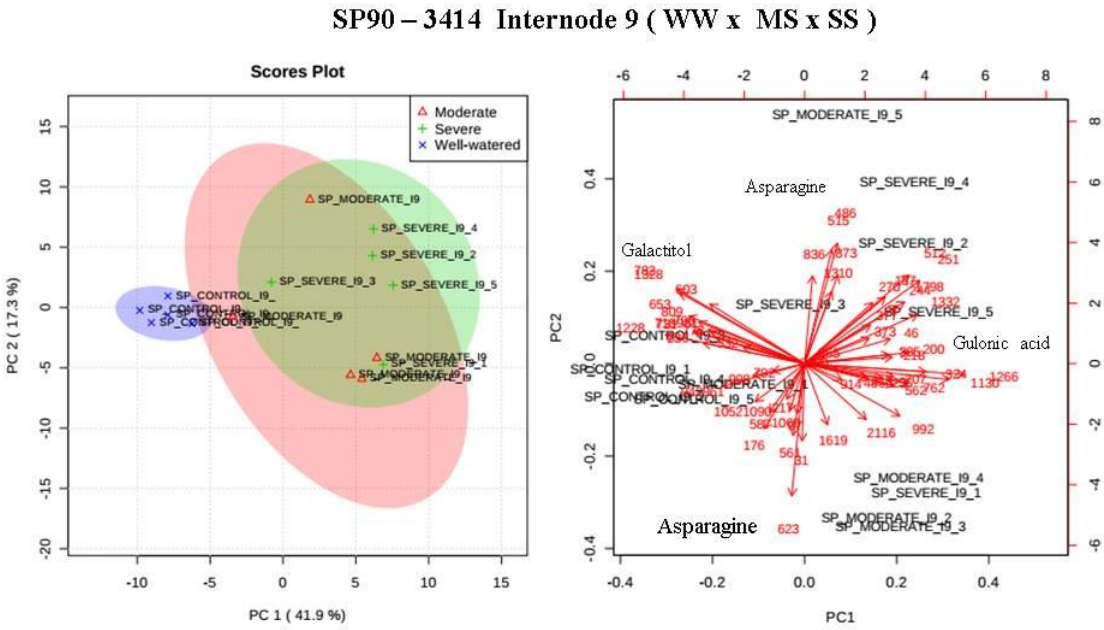

Figure 11: PCA of the SP90-3414 from internode 9 with well-watered versus moderate stress versus severe stress.

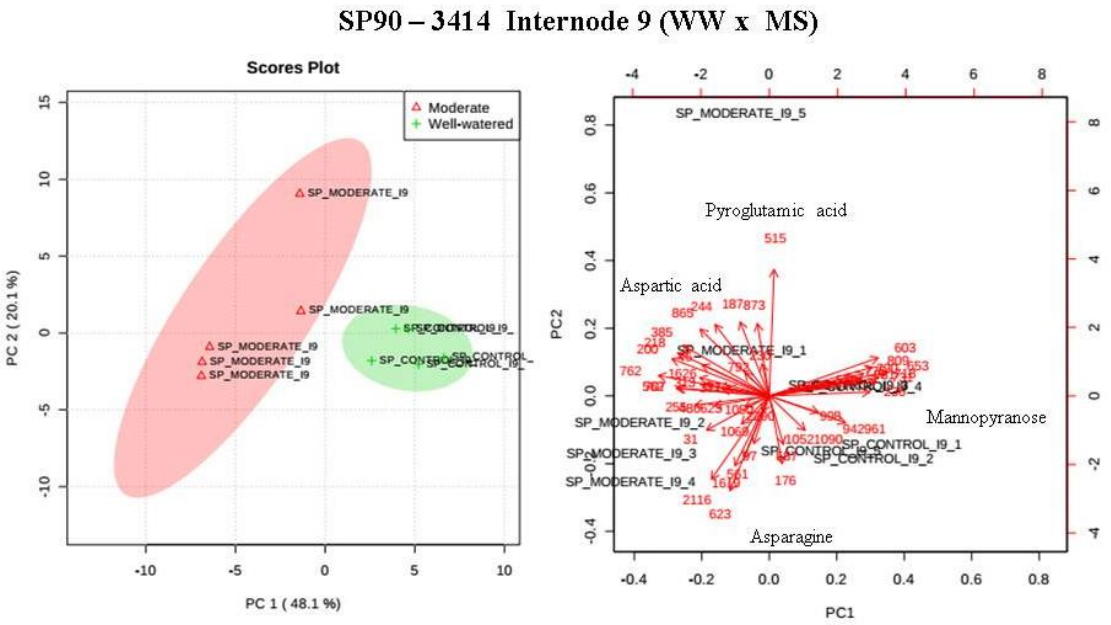

Figure 12: PCA of the SP90-3414 from internode 9 with well-watered versus moderate stress.

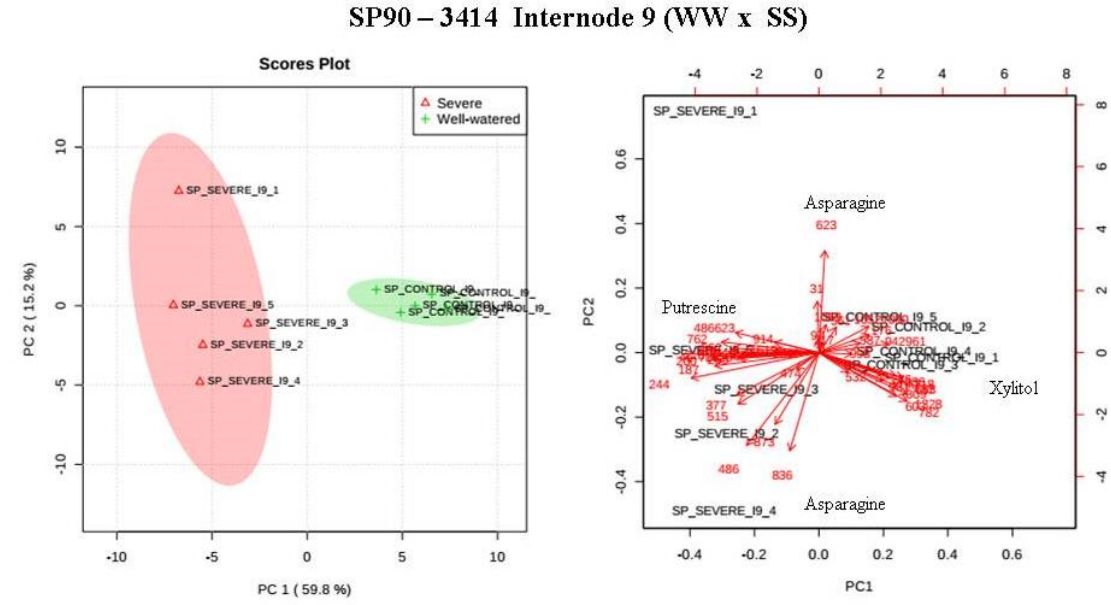

Figure 13: PCA of the SP90-3414 from internode 9 with well-watered versus severe stress.

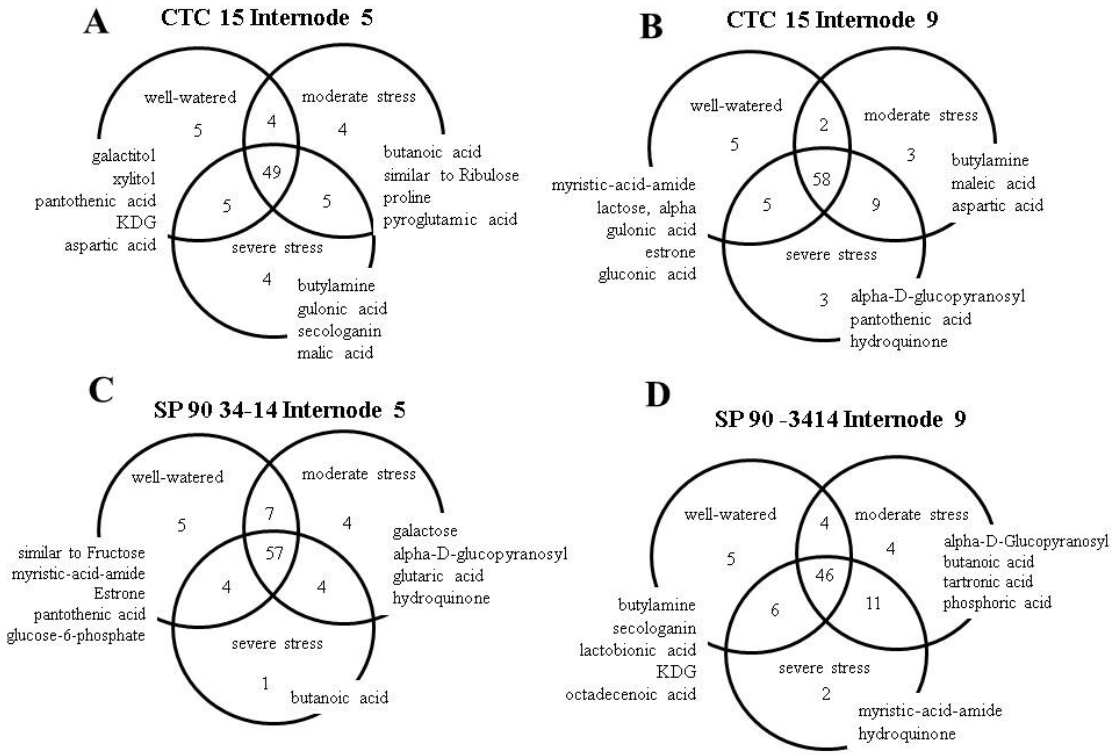

Figure 14: Venn diagram to show the numbers of exclusive metabolites identified in the different treatments of sugarcane. In the CTC 15, internode 5 (**A**) and internode 9 **(B)**. In addition, SP 90-3414, internode 5 (**C**) and internode 9 (**D**).

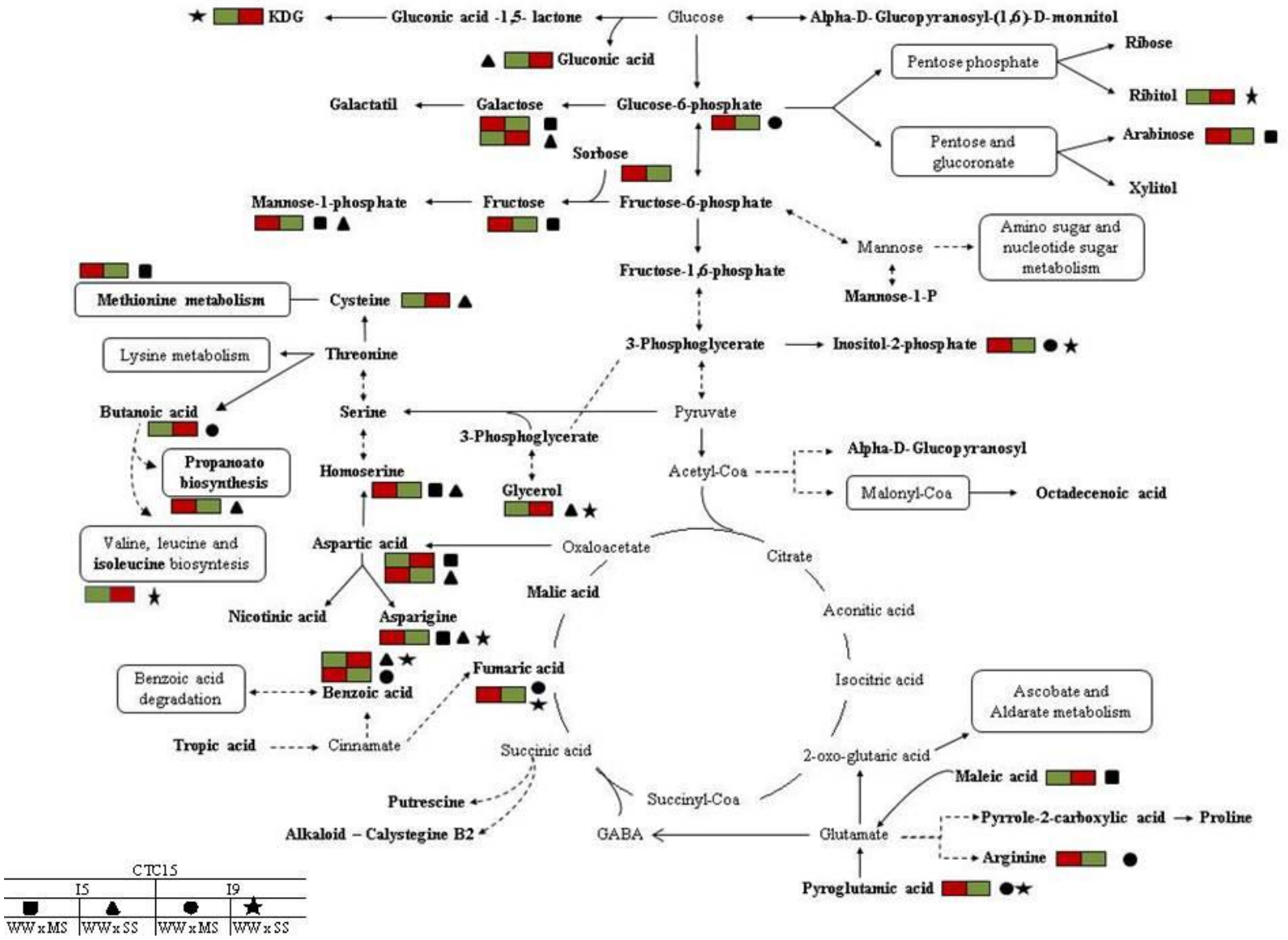

Figure 15: Metabolite profile of sugarcane internodes 5 and 9 from CTC15 cultivar submitted to different levels of water deficit**.**

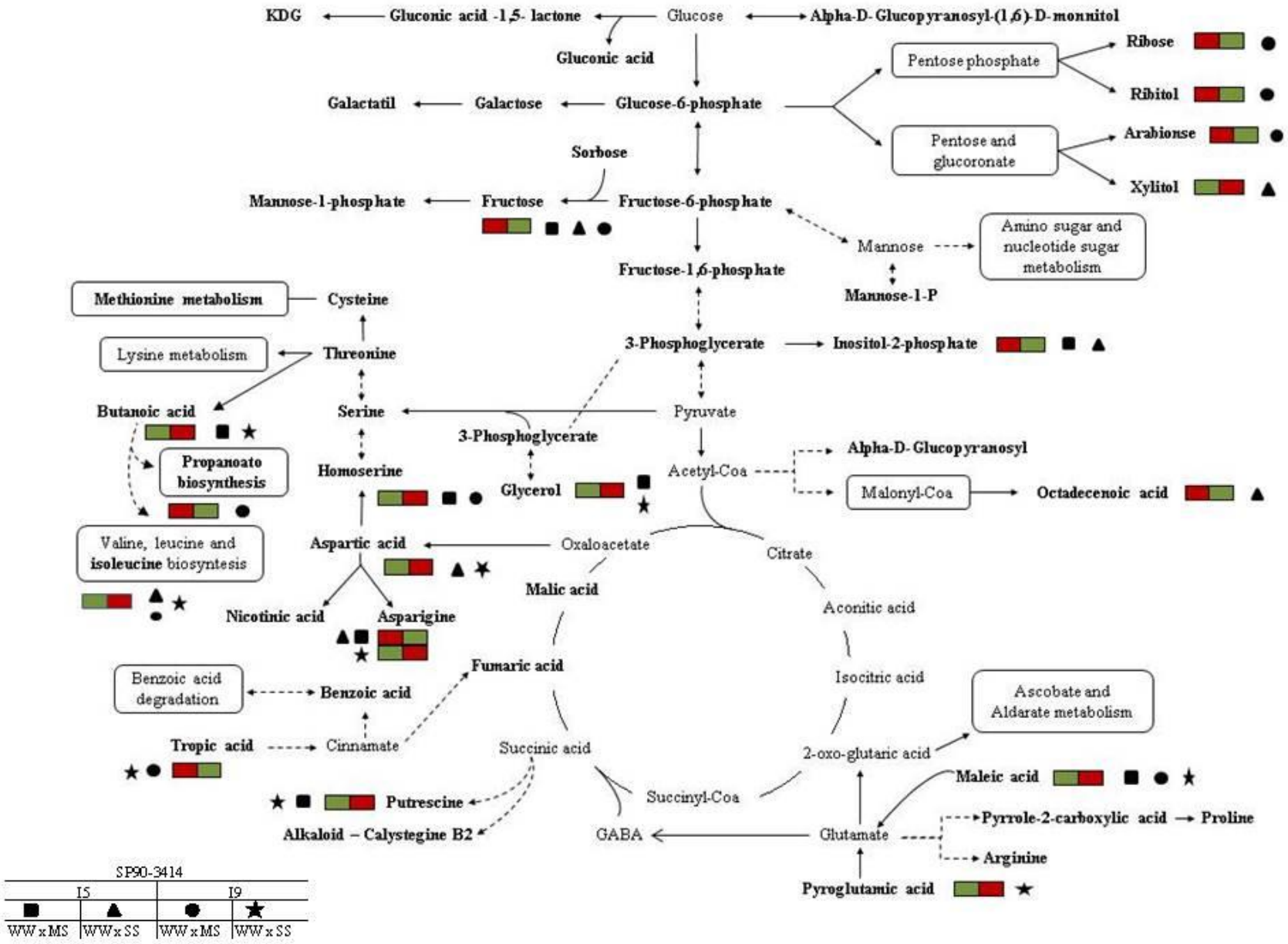

Figure 16: Metabolite profile of sugarcane internodes 5 and 9 from SP90-3414 cultivar submitted to different levels of water deficit.

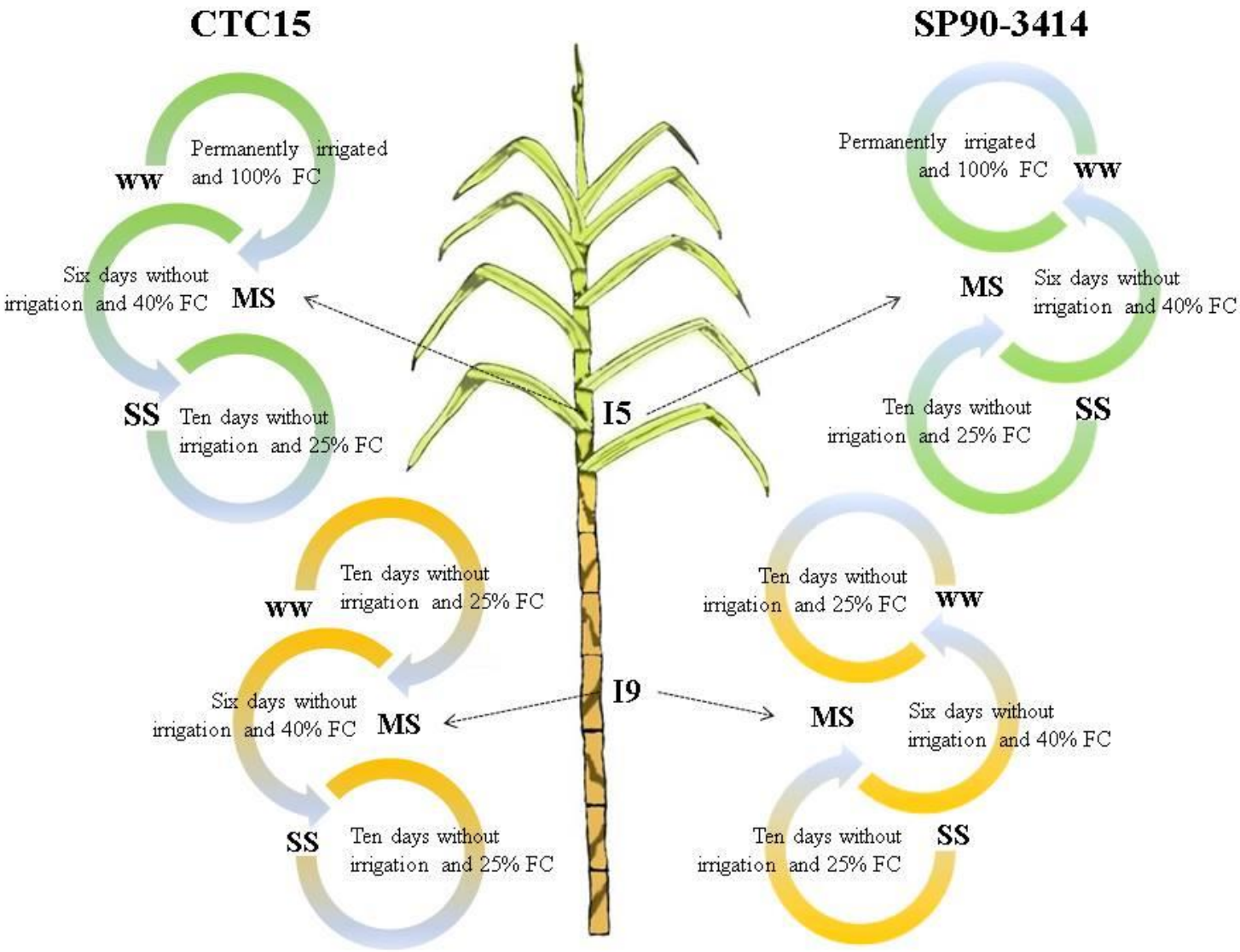

Figure 17: Scheme of the study material and the type of treatment applied in the internodes of the cultivars (CTC15 and SP90-3414).

Table 1: Metabolites identified in sugarcane (CTC 15 and SP90-3414) internodes 5 and 9 by GC/MS.

Class	Metabolites	Lib_ID	Kegg	Lib_RI	RI	Score
Alcohol	Propane-1,3-diol, 2-hydroxymethyl-	236	-	1334.1	1329.9	889
	Glycerol	187	C00116	1262.3	1260.8	817
Aldehydes	5-Hydroxymethylfurfural	324	C11101	1404.6	1401.8	829
Alkaloid	Calystegine B2	998	C10851	1811.1	1809	872
Alkane	Heptadecane	773	C01816	1698.5	1697.9	735
	Pentadecane	474	C08388	1500	1501.3	917
	Putrescine	244	C00134	1344.2	1344	790
Amine	Butylamine	44	-	1095.1	1095.1	620
	Cadaverine	385	-	1434.9	1437.7	671
	Pyroglutamic acid	515	C02238	1521.7	1523.5	878
	Threonine	217	C00820	1290.5	1286.6	929
	Proline	512	C01015	1524.1	1526.5	762
	Arginine	865	C00062	1742.5	1746.1	764
	Octadecenoic acid	1594	C08363	2216.6	2217.3	827
	Isoleucine	218	C00407	1286.7	1282.9	837
	Aspartic acid	373	-	1429.8	1431.1	887
Amino acids	Cysteine	792	C06809	1716.5	1713.7	649
	Methionine	775	C02712	1704	1704.1	894
	Homoserine, O-succinyl-	1310	-	1744.7	1747.5	730
	Aspartic acid	373	C00049	1422.1	1422.4	843
	Similar to Aspartic acid	377	-	1429.8	1431.1	887
	Homoserine lactone	836	-	1744.7	1747.5	730
	Asparagine	486	C00152	1501.3	1501.8	640
	Nicotinic acid	230	C00253	1300.8	1303.7	942
	Gluconic acid, 2-amino-2-deoxy-	1141	C03752	1980.1	1979.2	752
	Alpha-D-Glucopyranosyl-(1,6)-D-mannitol	2151	C00252	2894.4	2894.7	667
	Glucose-6-phosphate	1735	C00092	2371.3	2369.7	875
	Galactose	992	-	1815.5	1819.2	629
	Inositol-2-phosphate	1798	C01177	2466	2465.4	958
	Fructose	1090	C00095	1830.1	1834.3	619
	Similar to Fructose Derivate	961	C00247	1797	1799.7	601
	Arabinose	718	C00181	1660.4	1658.9	852
	Ribose	731	C00652	1666	1669.6	625
	Mannose-1-phosphate	1619	C00103	2238.3	2237.7	761
	Lactose	1970	C00185	2686.5	2689.7	633
Carbohydrate	Mannopyranoside	914	C03619	1768.4	1768.6	807
	Sorbose	1069	C00247	1852.5	1853.4	786
	Leucrose	2035	-	2753	2756.5	797
	Galactitol	1228	C00031	1924.1	1923.8	650
	Ribitol	809	C00121	1712.7	1712.8	737
	Xylitol	782	C00121	1694.7	1698.2	691
	Arabitol	790	C00121	1707.6	1705.6	737
	Alpha-D-Galactopyranosyl-(1,4)-D-galactopyranoside	981	C00252	2894.4	2894.7	667
	Lactobionic acid	2085	-	2798.1	2795.8	666
	Galactopyranoside	1050	C01083	2782.3	2777.4	900
	Similar Ribulose	723	C00309	1671.5	1674.9	755
	6-deoxy-Mannopyranose	653	-	1626.4	1628.1	647
Fatty acid	Octadecanoic acid	1626	C01530	2243.5	2245.1	918
	Butanoic acid	358/46	-	1404	1407.4	680

(Continued)

Table 1:
Continued

Class	Metabolites	Lib_ID	Kegg	Lib_RI	RI	Score
Fatty acyl glycosides	Maltitol	2116	C00185	2811.3	2810.9	719
Fatty Acyls	Myristic-acid-amide	1294	-	1967.8	1971.4	619
	3-Ketoadipate	707	-	1654.9	1650.7	730
	Succinic semi aldehyde	97	C00232	1177.1	1177.3	849
Inorganic acid	Phosphoric acid	185	C00009	1262.4	1264.3	784
Lactone	Gluconic acid-1,5-lactone	1141	C00198	1871.9	1875.3	731
Organic acids	KDG	1037	-	1837.1	1833	790
	Glutaric acid	808	-	1729	1728	712
	Tropic acid	603	C01456	1590.9	1594.1	701
	Tartronic acid	469	C02287	1520.6	1515.7	753
	Glycolic acid	31	C00160	1062.9	1059.2	700
	Benzoic acid	176	C00180	1251.3	1255.9	858
	Itaconic acid	251	C00490	1339.1	1342.8	807
	Maleic acid	270	C01732	1345.1	1344.4	739
	Fumaric acid	313	C01732	1397.1	1401.6	856
	Gulonic acid	1266	C00191	1952.8	1953.4	649
	Malic acid	462	C00149	1479	1480.5	775
	Erythronic acid	532	C01620	1528.6	1526.7	877
	Pantothenic acid	1328	C00864	1987.7	1986.8	788
	Acetic acid	561	C05852	1569.6	1571.5	750
	Aconitic acid	873	C00417	1747.8	1747.4	655
	Maleamic acid	562	C01596	1562.6	1561.7	912
Phenol	Hydroquinone	315	C00530	1401.3	1401.8	918
Prenol lipids	Ergocalciferol	2290	C05441	3066.1	3070.1	737
Pyrroles	Pyrrole-2-carboxylic acid	255	C05942	1350.7	1349.3	891
Sesquiterpene	Artemisinic acid	1130	-	1880.3	1880.9	716
Terpenoid	Secologanin	2185	C01852	2909.5	2911	663
Estrone	Others	2004	C00468	2734.1	2733.4	652

Table 2: Number of metabolites identified in internode 5 (I5) and internode 9 (I9) among the treatments in SP90-3414 and CTC15 cultivars.

	CTC15		SP90-3414	
	I5	**I9**	**I5**	**I9**
Well-watered	55	61	62	53
Stress moderate	55	62	64	59
Stress severe	55	64	56	57