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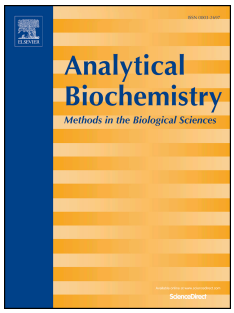

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Metabolomic approach reveals the biochemical mechanisms underlying drought stress tolerance in Thyme

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Abstract

Thyme as a perennial herb has been recognized globally for its antimicrobial, antiseptic and spasmolytic effects. In this investigation, we have used non-targeted metabolite and volatile profiling combined with the morpho-physiological parameters in order to understand the responses at the metabolite and physiological level in drought sensitive and tolerant thyme plant populations. The results at the metabolic level identified the significantly affected metabolites. Significant metabolites belonging to different chemical classes consisting amino acids, carbohydrates, organic acids and lipids have been compared in tolerant and sensitive plants. These compounds may take a role through mechanisms including osmotic adjustment, ROS scavenging, cellular components protection and membrane lipid changes, hormone inductions in which the key metabolites were proline, betain, mannitol, sorbitol, ascorbate, jasmonate, unsaturated fatty acids and tocopherol. Regarding with volatile profiling, sensitive plants showed an increased-then-decreased trend at major terpenes apart from alpha-cubebene and germacrene-D. In contrast, tolerant populations had unchanged terpenes during the water stress period with an elevation at last day. These results suggesting that the two populations are employing different strategies. The combination of metabolite profiling and physiological parameters assisted to understand precisely the mechanisms of plant response at volatile metabolome level.

Key words: Drought, FTICR, GC/MS, Metabolomics, *Thymus*, Tolerance

Abbreviations: DI-FTICR (Direct Infusion- Fourier Transform Ion Cyclotron Mass spectrometry), VOC (volatile compound), ROS (Reactive Oxygen Species), GC/MS-TOF (gas chromatography time of flight), KI (Kovat Index), QACs (Quaternary ammonium compounds), AsA (Ascorbaic Acid), PQN (Probabilistic Quotient Normalization), QC (quality control), KNN (K-Nearest Neighbour), SIM (Selected Ion Monitoring), Glog (Generalized Log Transformation), PCA (Principal Component Analysis), DT (Drought Tolerant population), TW (Tolerant population Watered), DS (Drought Sensitive population), SW (Susceptible population Watered), Mi-Pack (Metabolite Identification Package), KEGG (Kyoto Encyclopedia of Genes and Genomes),MGDG (MonoGalactosyl DiacylGlycerol), DGDG (DiGalactosyl DiacylGlycerol), PC (Phosphatidyl Choline), PI(Phosphatidyl Inositol), ABA (Abscisic Acid), CK (Cytokinin), IAA (Auxin), GA (Gibberelline), JA (Jasmonic acid), SA (Salicylic acid), NO (Nitric oxide), BR (Brassinosteroids), SL (Strigolactone).

1. Introduction

Thyme is a perennial herb belonging to the *Lamiaceae* (*Labiatae*) family consisting of more than 250 species and subspecies(Stahl-Biskup and Sàez, 2002). Thymus products and uses are widespread and include essential oils, oleoresins, fresh and dried herbs, and even landscape usage. As a medicinal plant, thyme extracts and essential oils can be used as antiseptic, antibacterial and spasmolytical agents (Sagdic *et al.*, 2002). Response to water deficit stress at the physiological level has been demonstrated in several species of the genus including *Thymus vulgaris* (Aziz *et al.*, 2008; Babaee *et al.*, 2010; Bahreininejad *et al.*, 2013; Letchamo *et al.*, 1994), *Thymus zygis* (Sotomayor *et al.*, 2004) and *T. hyemalis* (Jordan *et al.*, 2003), but no detailed study of the underlying metabolic changes under drought of this genus has been reported. In other plants such as soybean, wheat, eucalyptus, potato, *Arabidopsis*, grapevine and tomato, metabolite profiling has been used to study water deficit (Bowne *et al.*, 2012; Cramer *et al.*, 2007; Foito *et al.*, 2009; Levi *et al.*, 2011; Mane *et al.*, 2008; Rizhsky *et al.*, 2004; Sanchez *et al.*, 2012; Semel *et al.*, 2007; Silvente *et al.*, 2012; Vasquez-Robinet *et al.*, 2008). In the mentioned studies, they compared metabolites and transcriptional responses to drought in contrasting genotypes. They focused on metabolites contributed in primary metabolism and reported significantly altered metabolites between the genotypes along with their mechanism of action. The advantage of this approach is not only to demonstrate the common and adverse responses, but also at quantitaive level, the differences may account for difference in drought tolerance.

To cope with unfavourable conditions (the main factors limiting the plant productivity), plants have evolved mechanisms which allow them to maintain their productivity and/or survival (Rowley and Mockler, 2011). Drought stress is the main environmental factor that limits plant production worldwide (Boyer, 1976). Water supply affects almost all plant processes directly or indirectly (Akıncı and Lösel, 2012), hence water deficit stress due to reduction of available water will affect plants in various ways. The effect of drought on plants can be discussed relation to morphological, photosynthesis, proteins, lipids, mineral uptake and Reactive oxygen species (ROS) factors (Lisar *et al.*, 2012).

In spite of a detailed knowledge of plant responses to water deficit, there are many aspects that require further study, including strategies against dehydration and correspondingly biochemical

mechanisms. Comprehensive metabolite profiling through describing the molecular mechanisms underlying drought tolerance can inform future research to develop drought tolerant plants (Umezawa *et al.*, 2006; Valliyodan and Nguyen, 2006). Whilst considerable studies have been performed to understand plant responses to drought stress at the metabolic level (Bhargava and Sawant, 2013; Shao *et al.*, 2009), no comprehensive investigation has been carried out using metabolomics in thyme to date. In the current study, two thyme populations previously identified with differing drought tolerance (*T. vulgaris*, drought sensitive and *T. serpyllum*, drought tolerant, (Moradi *et al.*, 2014a) were subjected to water deficit stress in order to determine the major metabolites that might contribute to drought tolerance in thyme.

2. Results

2.1.Drought stress responses at physiological level

To monitor the morpho-physiological responses of thyme plants to water deficit, plants were grown under controlled conditions- as described in experimental section- subsequently water was withheld at 30th day after sowing from the pots containing plants that would be exposed to drought stress. Next, physiological parameters namely water content, water potential and shoot dry weight were recorded at 0, 4, 8, 12 and 15 days after water withholding in both controlled and treated plants. Soil moisture sharply decreased in both plants after 4 days but reached a plateau after 12 days of water limitation. The only difference was a slower rate of decline for DT plants. Water potential declined on 4th day and was around -4 bar until the end of stress period, except for *T. serpyllum*, where water potential dropped on day 15 to -10 bar. Tolerant plants had a water potential slightly higher than DSs on 8th and 12th days (Figure 1). Water content in sensitive plants (initially 94%) dropped to 88% on the 8th day and then 84% on 12th day. In contrast tolerant plants had 88% water content initially which remained constant until 12th day, then it dropped to 80% at 15th day. Sensitive plant shoot dry weight increased for 4 days but reached a plateau until the end of the stress period. The dry weight of tolerant plant shoots was initially lower than sensitive plants but the increase in weight continued for 11 days after withholding water.

2.2.Changes in primary metabolite level during water deficit stress

To assess the drought driven metabolome response, six biological replicates sampled from plants grown under well-watered and water-withheld pots for both tolerant and sensitive plants at the end of water deficit period. Subsequently, single-time point harvest materials were analysed by non-targetted metabolite profiling platform based on DI-FTICR mass spectrometry.

To visualize the differences between the metabolite profile of the plants grown under watered and droughted conditions and also to identify the major metabolites responsible for this difference, the dataset was subjected to Principal Component Analysis (PCA). This statistical approach is used to show similarities and differences between groups in addition to pattern recognition (Goodacre *et al.*, 2000). A score plot of all detected peaks over the first two PCs illustrated a good separation of four groups i.e. TD, TW, SD and SW (Figure 2). Figure 2.A showing polar positive ions, PC1 with 30.35% of the total variation clearly classified all samples into susceptible and tolerant groups, while PC2 explaining 13.33% of the total variation, just divided tolerant population into watered and stressed group. In figure 2.B, non-polar negative ions shows the first PC with 23.47% of the total variation categorized samples to tolerant and sensitive, while the second principal component described 16.04% of variation. The QCs (Quality Control consisting of an equal volume of random samples representative of all biological replicates) being centred supports the accuracy of this experiment.

Venn diagram shows total number of peaks increasing or decreasing in the populations (Figure 3). It shows that 53 peaks (in polar and non-polar fractions) increased significantly in tolerant plants, but in sensitive plants the increasing peaks were 342, which 17 peaks were common. In decreasing peaks, tolerant and sensitive plant had 480 and 295 peaks significantly changing respectively with 41 peaks in common.

The altered metabolites included amino acids, carbohydrates, organic acids, secondary metabolites and hormones. A summary of metabolite alteration with respect to their biological role was listed in Figure 4. For amino acids, sensitive plants had a decrease in all detected compounds except for tryptophan (m/z= 243.0529), while tolerant plants had elevation in all detected amino acids except for serine. Proline (m/z=221.0211) and citrulline (m/z=349.1709) had the largest increase. Moreover in sensitive plants, homomethionine (m/z=164.074) had the largest decrease. Regarding carbohydrates, all were elevated in tolerant population with the highest being xylulose (m/z=215.0143), while in sensitive plants, galactoglycerol

(m/z=255.1075) and erythrose (m/z=159.0054) were decreased and D-Xylulose-5-phosphate (m/z=253.0084) increased. Most of the organic acids had increased in both populations except for gibberellins (m/z=347.1864) in tolerant plants and homocitrate (m/z=247.0038) and aconitate (m/z=212.9796) in sensitive thyme. Various compounds were detected as significantly changing metabolites belonging to wide diverse metabolite categories mainly secondary metabolites. Membrane lipids had increased significantly in stressed tolerant plants except for lyso PC (m/z=523.3642), whereas most of the lipids in sensitive plants declined.

To understand the responses of DS population, the level of all detected peaks (3328 peaks) in control plants were compared to the same metabolite level in stressed plants. This comparison resulted in 605 peaks as significantly altered in susceptible population. Submission of the peak list (m/z along with intensities) to Metabolite Identification Package (Mi-Pack), putatively identified 92 metabolites which 57 increasing and 35 decreasing (for a complete list of significant metabolites see table 1 in (Moradi, submitted)).

These metabolites were broadly classified into amino acids, sugars, organic acids, phyto-hormones. Screening the complete list of identified metabolites, performed using literature review particularly through submitting in BioCYC and KEGG database. However only the metabolites with the available description of function were selected. Of the amino acids and sugars, the only compounds significantly increased were tryptophan and ribose respectively. The most pronounced elevated metabolites were compounds including guanine (m/z=152.0567), shikimate (m/z=213.016), isochorismate (m/z=247.0214), isojasmonic acid (m/z=249.0886), hydroxyferulate (m/z=249.0159) and dehydroquinate (m/z=243.0265). Within the significantly declined metabolites, outstanding compounds were amino acids including alanine (m/z=199.048), glutamate (m/z=148.0604), phenylalanine (m/z=204.0421), phospho-hydroxy-threonine (m/z=214.0112), aspartate (m/z=172.0007) and methionine (m/z=). This population had a decrease in organic acids including aconitate (m/z=212.9796), ascorbate (m/z=182.0578) and homocitrate (m/z=247.0038). Some sugars declined following water deficit included galactosylglycerol (m/z=255.1075) and erythrose (m/z=159.0054). The interesting compound detected within the decreased metabolites was linalool, since it is a commercially important volatile.

To profile lipids in DS plants, FT-ICR analysis was performed in negative ion mode of non-polar fraction in *T. vulgaris* extracts. Peak intensities of control plants were compared with those of droughted plants. Of 2527 metabolites detected, 695 peaks were statistically significantly different (compared by T-test), with 94 peaks putatively identified by Mi-Pack (Weber and Viant, 2010). For the complete list of non-polar metabolites affected by water stress in DS plant see table 2 in (Moradi, submitted). The most relevant metabolites among the non-polar metabolites were Methyl salicylate and 1-18:2 lyso PE for increased and decreased lipids across the diverse categories lipids including MGDG, DGDG, PC and PS as well as tocopeherol and gibberelline.

For polar metabolites of tolerant species, Statistical analysis revealed 144 peaks out of 3328 that were significantly altered between droughted and watered plants assessed by metabolite pool size. Those 144 peaks included known and unknown metabolites, enabling identification of 56 metabolites (see table 3 in (Moradi, submitted)). Of the carbohydrates significantly affected, all were elevated, including xylulose (m/z=215.0143), gluconic acid (m/z=417.1522), sorbitol and/or mannitol and/or iditol (m/z=169.0261). The amino acids betaine and/or valine (m/z=213.037), proline (m/z=221.0211), and citrolline (m/z=) increased in drought stressed plants in comparison to controls, except for serine. Organic acids mostly increased in tolerant compare to sensitive, including salicylate (m/z=138.0525), succinate (m/z=146.0924), oxoadipat (m/z=168.0421), shikimate (m/z=213.016), dehydroquinate (m/z=243.0265) and citrate (m/z=423.1053), while only gibberelline (m/z=331.1551) decreased.

Regarding non-polar metabolites, Metabolite profiling of DTs was undertaken following withholding water compared to control plants. Significant compounds changing were 591 in the non-polar fraction of which 61 metabolites were putatively identified and are listed in table 4 in (Moradi, submitted). The majority of lipids belonging to diverse classes increased in DTs (*T. serpyllum*) under drought. Notable lipids changing included classes of MGDG, DGDG, PD, PC, PI while lyso PC decreased. Moreover, elevating violaxanthin (m/z=599.4114) is very interesting. Since it is substrate of ABA and might increase the level of ABA concentration under stress condition (Frey *et al.*, 1999).

2.3.Changes in terpenes content during drought stress

In order to track VOCs in response to water deficit stress between drought tolerant and sensitive thyme, six independent biological replicates from plants sampled at 0, 4, 8 and 12th days, were analyzed using GC-MS volatile profiling platform. Our comparisons were including eleven major volatiles in total consisting three sesquiterpenes (alpha-cubebene, B-caryophyelene and germacrene) and eight monoterpenes (β-myrcene, O-cymene, β-pinene, alpha-thujene, ocimene, gamma-terpinene, thymol and alpha-phellandrene) (Figure 3). For volatiles with available external standards (p-cymene, B-myrcene, thymol and alpha-phellandrene) comparison was made on absolute quantities (pg/mg fresh weight), while for others relative abundances have been applied. Apart from alpha-cubebene, ten other metabolites exhibited significant differences between DT and DS plants. There was a high concentration of germacrene D in tolerant compared to DSs, while other compounds showed the same pattern which increased in intensity in DSs on 4th day and similar intensities throughout the stress period (Figure 6). In contrast, most of the terpenes of DTs were unaffected during the stress apart from the final day where there was a sharp elevation. When DSs are exposed to drought stress conditions, terpenes are elevated within 4 days, but return to the same intensity as prior to the stress. DTs did not change their terpenes except for germacrene and thymol which increased on 12th day. For *T. vulgaris* (susceptible) the 4th day was the turning point with increasing volatiles for all monoterpenes and sesquiterpenes at this point. The critical day for DTs was 12th day, since the increase in terpenes was been observed at this stage.

The altered metabolites are illustrated along with metabolic pathways perturbed to water deficit in both tolerant and sensitive populations (Figure 4).

3. Discussion

Water depletion in both sensitive and tolerant plants results in exhibition of various responses from metabolic to physiological and whole plant level. At the physiological level, soil moisture reduction lower leaf water content, which reduction rates in tolerant plants were slower than sensitive ones. Whilst lowering water potential maximize uptake of soil water particularly in tolerant plants (Chaves *et al.*, 2003). At metabolic level, sensitive plants exhibited more metabolites significantly altered rather than tolerant plants, but qualitavely tolerant plants showed increase in the accumulation of osmolytes aims to maintain water in cells and likewise,

antioxidants (including terpenes) help to protect plant cells from ROS (Reddy *et al.*, 2004; Seki *et al.*, 2007; Miller *et al.*, 2008).

3.1. Physiological basis of water saving and drought tolerance in *T. serpyllum* compared to *T. vulgaris*

Water content as a direct indicator of plant water status, clearly identified the tolerant population, since there was no significant difference of water content between watered and droughted plants in the tolerant population. Assessment of water potential and shoot dry weight identified *T. vulgaris* (sensitive) population as a water spender and *T. serpyllum* (tolerant) as a water saver. This strategy could result in less use of soil water and less shoot dry matter for tolerant. Therefore, the presented results indicated that *T. serpyllum* behaved as a water saver, while *T. vulgaris* exhibited water spender behaviour (Larcher, 2003). According to the definitions and concepts proposed by Levitt (1972), plants can employ one of two strategies: water spending or water saving (Levitt, 1972; Monson and Smith, 1982; Kalapos, 1994). Water savers close their stomata even in adequate soil moisture, hence reducing transpirational water loss (Reynolds *et al.*, 1997; Roark and Quisenberry, 1977). These plants in addition to having more rigid cell walls (due to higher modulus elasticity) and lower osmotic potential are less vulnerable to xylem cavitation (Gyenge *et al.*, 2005). Plant species classified as water spenders maintain open stomata and assimilate more CO_2, therefore have more yields (growth rate) than water savers (Dong and Zhang, 2001; Roark and Quisenberry, 1977). More growth rate is suitable trait for plant in general, but it seems for plants under stress condition or extracting certain products, this trait is not appropriate.

3.2. Metabolic mechanisms of drought tolerance in *T. serpyllum* compared to *T. vulgaris*

On the basis of the present results the tolerance mechanisms of thyme to water deficit stress could be divided into four categories as follows:

3.2.1. Osmotic adjustment as a key mechanism of drought response

Many plants employ this mechanism to cope with osmotic stress by large scale synthesis/accumulation of common solutes including amino acids such as proline, aspartic acid, and glutamic acid (Samuel *et al.*, 2000; Hamilton and Heckathorn, 2001; Bacelar *et al.*, 2009),

carbohydrates (Vijn and Smeekens, 1999), methylated quaternary ammonium compounds (Rathinasabapathi *et al.*, 2001) such as betaines, polyols (Smirnoff, 1998) and low molecular weight proteins (Ingram and Bartels, 1996).

In *T. serpyllum* (tolerant plants), proline, betaine, valine and alanine all increased. While in sensitive population the only increasing amino acid was tryptophan, all other amino acids decreasing. Amino acids are main product of inorganic nitrogen assimilation, and are components of proteins and nucleic acid (Greenway and Munns, 1980). Significant accumulation of free amino acids under drought stress has been observed in a number of plants (Shao *et al.*, 2009) such as wheat (Munns *et al.*, 1979), soybean (Fukutoku and Yamada, 1981), olive, rice and groundnut. Their accumulation enhances plant tolerance, probably by osmotic adjustment (Greenway and Munns, 1980). Increasing levels of proline have been detected in various drought tolerant plants (Hassine *et al.*, 2008; Parida *et al.*, 2008; Evers *et al.*, 2010). Large regulation of proline metabolism at the transcript level has demonstrated that proline accumulation is a stress-induced and adaptive response of plant (Verslues and Sharma, 2010). Considerable work has established some possible functions for proline accumulation under water deficit condition which include lowering of cytoplasmic osmotic potential (Voetberg and Sharp, 1991; Verslues and Sharp, 1999). Proline may also protect cellular structure by acting as a water substitute during dehydration (Yancey, 2005). Betaine (glycine betaine) is one of the four common zwitterionic QACs (Quaternary ammonium compounds) which can act as osmoprotectants under drought (Hanson *et al.*, 1994). The most common QACs (glycine betaine, proline betaine, β-alanine betaine, choline o-sulfate and 3-dimethylsulfoniopropionate) (Rhodes and Hanson, 1993; McNeil *et al.*, 1999) are amino acid derivatives with a fully methylated nitrogen atom (Chen and Murata, 2002).

All the carbohydrates including xylulose, gluconic acid, sorbitol and mannitol were increased in the tolerant population, while in sensitive plants erythrose elevated and D-Xylulose-5-phosphate decreased. Previous studies demonstrated that carbohydrates such as soluble sugars increases or at least being maintained fixed under stress condition (Pinheiro *et al.*, 2001). These sugars, in addition to their role as osmolytes (Hoekstra *et al.*, 2001; Jang and Sheen, 1994), might act as stress response signals (Jang and Sheen, 1994; Chaves *et al.*, 2003). Increases in xylose (a monosaccharide) and sugar acids such as gluconic acid in the tolerant population are consistent

with other studies such as eucalyptus (Warren *et al.*, 2012). These carbohydrates are major components of the cell wall (Keegstra *et al.*, 1973) and have been demonstrated to contribute to a drought stress response as protective function by changing cell wall composition (Joly and Zaerr, 1987; Zwiazek, 1991). Increases in acyclic polyols such as mannitol and sorbitol have been observed in response to water stress in many plants (Noiraud *et al.*, 2000). These compounds can act as osmoregulators as well as oxygen radical scavengers (Halliwell and Gutteridge, 1999).

3.2.2. ROS scavenging and cellular structure protection during water deficit

Ascorbate and tochoherol significantly increased in tolerant plants (Figure 4). These antioxidants have been observed to alter under various environmental stresses including drought (Sharma and Dubey, 2005; Maheshwari and Dubey, 2009; Mishra *et al.*, 2011; Srivastava and Dubey, 2011; Hernández *et al.*, 2001). ROS or free radicals ($O_2^{\cdot-}$, $^{\cdot}OH$, H_2O_2, $^1O_2$) are produced in cellular compartments as a by-product of various biochemical reactions or in the chloroplast, mitochondria and plasma membrane by exposure to high energy electron leakage from electron transport (Foyer, 1997; Foyer *et al.*, 1994; Luis *et al.*, 2006; Blokhina and Fagerstedt, 2010; Heyno *et al.*, 2011). Various studies have established an increase in ROS under osmotic stress (Serrato *et al.*, 2004; Borsani *et al.*, 2005; Miao *et al.*, 2006; Abbasi *et al.*, 2007). Plants have complex defence mechanisms using enzymatic and non-enzymatic antioxidants to mitigate oxidative damage caused by ROS (Dat *et al.*, 2000). Of the non-enzymatic compounds, low molecular weight ascorbate (AsA), is the most plentiful and powerful antioxidant in plants with a key role under oxidative stress by protecting macromolecules (Sharma *et al.*, 2012; Smirnoff, 2000).

3.2.3. Membrane lipid composition change in addition to fatty acid unsaturation

Different trends for a number of non-polar metabolites were observed when comparing stressed and control conditions for both sensitive and tolerant plants. Tolerant thyme plants that experienced drought stress showed an increase in membrane lipids in comparison with the watered except for lyso PC. However, leaf lipids decreased in the sensitive plants of all categories with the exception of 18:1 lyso PE and PA. The two populations with diverse tolerance to water stress had very different responses of lipid concentrations to stress. Declining leaf lipids, as in the sensitive plants, has been previously observed in various crop plants such as sunflower (Navari-Izzo *et al.*, 1993), lupin (Hubac *et al.*, 1989), oat (Liljenberg and Kates, 1985)

and cotton (Pham Thi *et al.*, 1982). The decrease in lipid contents is the consequence of deleterious effects of drought stress which include cell membrane degradation (Anh *et al.*, 1985; De Paula *et al.*, 1990), inhibition of lipid biosynthesis (Pham Thi *et al.*, 1987; Monteiro de Paula *et al.*, 1993) and lipolytic and peroxidant processes (Ferrari-Iliou *et al.*, 1994; Sahsah *et al.*, 1998; Matos *et al.*, 2001). Tolerant plants employ mechanisms to reduce the negative effects on lipid metabolism such as protoplasmic tolerance (Repellin *et al.*, 1997). Plants through this mechanism rearrange membrane lipids (Lösch, 1993; Turner and Jones, 1980) to maintain membrane structure and fluidity. Maintenance of appropriate membrane fluidity during stress allows continued functioning of membrane proteins such as the photosynthetic machinery (Upchurch, 2008). In contrast, previous experiments on drought-tolerant cultivars of tobacco and maize demonstrated that these plants are able to maintain or increase polyunsaturated level of fatty acids (Zhang *et al.*, 2005; Berberich *et al.*, 1998; Mikami and Murata, 2003). It has been observed under salinity stress that tolerance can be enhanced through increasing the level of polyunsaturated fatty acids (Rodríguez-Vargas *et al.*, 2007; Allakhverdiev *et al.*, 1999). In agreement with the previous results, increasing membrane lipid unsaturation occurs in response to various stresses including drought in tolerant plants.

3.2.4. The role of phytohormones in response of thyme to water stress

In the present study plants cataorgised as tolerant , SA and neoxanthin (precursor of ABA) significantly increased and GA decreased under water deficit stress conditions. While sensitive plants had lowered neoxanthin and increased JA (Figure 4). Meanwhile indol-3-acetaldehyde (IAAId, (Woodward and Bartel, 2005)) as a precursor of IAA elevated in both populations under stress conditions. Increasing ABA in tolerant plants is consistent with a role for ABA in dehydration tolerance mechanisms which has previously established (Seo *et al.*, 2009; Ramírez *et al.*, 2009; Legnaioli *et al.*, 2009; Hong *et al.*, 2008; Li *et al.*, 2008; Wilson *et al.*, 2009; Mishra *et al.*, 2006). Moreover, accumulation of SA in tolerant plant under drought condition, correlates with the contribution of this hormone in enhancing drought tolerance (Munne-Bosch and Penuelas, 2003; Chini *et al.*, 2004), osmotic stress (Borsani *et al.*, 2001) and regulation of antioxidant enzyme activity (Durner and Klessig, 1995; Durner and Klessig, 1996). Currently known hormones are including ABA (abscisic acid), ethylene, CK (cytokinin), IAA (auxin), GA (gibberelline), JA (jasmonic acid), Sa (salicylic acid), NO (nitric oxide), BR (brassinosteroids) and SL (strigolactone) (Peleg and Blumwald, 2011). These hormones play a key role in the

adaptation to environmental stress in synergistic or antagonistic manner (Jaillais and Chory, 2010; Santner and Estelle, 2009). They play this role through regulating various adaptive responses (Messing *et al.*, 2010; Argueso *et al.*, 2009; Wang *et al.*, 2009). It is well established that IAA (Mahouachi *et al.*, 2007; Albacete *et al.*, 2008; Arbona and Gómez-Cadenas, 2008), ethylene (Pieterse *et al.*, 2009), JA (Wasternack, 2007) and SA (Raskin, 1992) are implicated in response to various biotic and abiotic stresses (De Diego *et al.*, 2012). Metabolic pathways can be altered due to the specific stress, the degree of alterations depends upon plant species and the type and length of stress (Krasensky and Jonak, 2012). Comparative analysis of metabolites in stress-sensitive plants along with the stress-tolerant species of the same plant is an appropriate way to demonstrate the role of metabolism in natural stress tolerance (Gong *et al.*, 2005; Hannah *et al.*, 2006; Zuther *et al.*, 2007; Janz *et al.*, 2010; Korn *et al.*, 2010; Lugan *et al.*, 2010).

3.3.Volatile compound alterations during water stress

Volatiles of thyme mainly consist of monoterpenes and sesquiterpenes, hence the major terpene intensities were compared throughout the stress period. The observed pattern for all of the eleven terpenes was similar apart from thymol, alpha-cubebene and germacrene. In sensitive plants all the terpenes were elevated at day four then decreased to previous levels. While tolerant plants maintained the same level of terpenes during the water stress period and elevated at 12^{th} day of stress period. These trends observed in sensitive plants can be explained by drought stress effects through declining photosynthesis and diversion of carbon allocation to defence molecule production systems. Since, following to decrease in shoot water content, photosynthesis starts to decline likely due to stomatal closure (CO_2 diffusion limitation) (Chaves, 1991; Cornic, 1994; Ort *et al.*, 1994) or metabolic perturbation (Boyer, 1976; Lawlor, 1995) such as declining Rubisco activity or concentration (Rennenberg *et al.*, 2006). Certain volatile compounds' carbon is provided mainly by photosynthesis (Schnitzler *et al.*, 2004) and drought stress affects photosynthesis (Bhagsari *et al.*, 1976; Flexas *et al.*, 2004). Therefore water stress influences volatile compounds indirectly (Šimpraga *et al.*, 2011). The increase at the end observed in tolerant plants can be attributed to oxidative stress and plant strategy against deleterious effects of ROS. In spite of the likely role of terpenes in the protection of leaves under drought, their exact mechanism in drought tolerance is unknown. However, results obtained in this investigation might suggest that sensitive plant photosynthesis was affected strongly by stress,

while tolerant plants having appropriate strategies for water use such as osmoregulation in addition to ROS scavenging, maintained the terpenes at similar levels even during severe stress.

The increasing and decreasing trend observed in our sensitive plants has been published previously in precise studies imposing water stress including monitoring water potential and water content on some of the Mediterranean plant species (Ormeno *et al.*, 2007). As previous researchers found, it seems changing the volatile composition might be due to either carbon diversion from photosynthesis to terpenes (Sharkey and Loreto, 1993; Peñuelas and Llusià, 2003) or serving terpenes as non-enzymatic antioxidants to scavenge ROS (Gershenzon *et al.*, 1978; Llusià and Peñuelas, 1998).

4. Experimental

4.1. Plant material and experimental design

Seeds of *Thymus vulgaris* and *Thymus serpyllum* (as representative of drought sensitive and drought tolerant plants respectively) were obtained from the company Semillas Silvestres®, Spain. Seeds were grown in a growth room with a 16:8 (light: dark) cycle and a temperature of 22°C and watered with tap water weekly. Drought stress was applied and measurement of soil moisture, water content, water potential and shoot dry weight were carried out as described previously (Moradi *et al.*, 2014b). Measurements were made at 4 day intervals from day 0. Similar aged leaves of individual plants were harvested every four days for volatile profiling. An additional harvest was carried out for FTICR profiling at the last sampling date. To illustrate the experimental design in a simple form, Figure 7 displays three platforms used to assess the response of morpho-physiologic (No.1 blue colour), non-targetted volatile profiling using DI-FTICR (No.2 in green colour) and volatile compounds profiling using GC/MS (No.3 in red colour).

4.2. Leaf sampling and extraction for DI FTICR

Leaf samples were harvested and flash frozen in liquid nitrogen. Frozen samples were freeze dried for 48 hours. For extracting metabolites, the freeze-dried samples were weighed and then extracted using the methanol: chloroform: water protocol. Briefly, 32 µl MeOH and 12.8µl water per mg tissue were added and tissue homogenised using a Precellys 24 homogeniser (Bertin Technologies Ltd, USA). Next, 32 µl CHCl3 and 16µl water were added and the mixture was

centrifuged. Each fraction of the biphasic solution was transferred to separate vials as polar (upper layer) and non-polar (lower layer) extracts. Polar extracts were dried with a vacuum concentrator (Thermo Savant, Holbrook, NY, USA) and non-polar extracts were dried under a stream of dried nitrogen gas. The dried extracts were stored at -70ºC until analysis.

4.3. Sampling and extraction procedure for volatile profiling

From the first day of withholding water until day 12, plants were harvested every 4 days. Similar aged leaves from one plant were removed with scissors, flash frozen in liquid nitrogen, weighed and stored at -70ºC. Six biological replicates were collected each sampling point. The weight of fresh samples ranged between 30 to 100 mg. Extraction was performed using a modified liquid extraction method: samples were taken from the freezer and immediately put in liquid nitrogen. Leaves were ground in a microfuge tube, and returned to the liquid nitrogen . After weighing, 1 ml hexane including 10ng/µl internal standard (Benzyl Acetate) was added to each 1.5 ml tube. Next, tubes were vortexed for 15s and centrifuged at 13000 rpm for 10 min. The supernatants were transferred into 1.5 ml brown glass vials for storage.

4.4. Non-targeted metabolite profiling using FT-ICR Mass spectrometry

Prior to loading samples, freeze dried samples were resuspended in HPLC grade 80:20 MeOH:H_2O with addition of 0.25% formic acid for polar extracts and 20 mM ammonium acetate for non-polar extracts. Dilution ratios were 1.5:1 and 3:1 (dilution solvent: original extract volume) for polar and non-polar extracts respectively. The reconstituted samples were mixed by vortexing and then sonicated for 5 minutes. For quality control (QC), representative samples containing an equal volume of randomly selected samples were prepared. QCs in addition to other samples were centrifuged at 4˚C for 10 minutes at 14000 rpm. Three technical replicates containing 10 µl aliquots from each microfuge tube were loaded into auto-sampler plates. Samples were analyzed using a hybrid 7-T Fourier Transformed Ion Cyclotron Resonance Mass Spectrometer (LTQ FT, Thermo Scientific, Bremen, Germany) equipped with a chip-based direct infusion nanoelectrospray ionisation assembly (Triversa, Advion Biosciences, Ithaca, NY). ChipSoft software (version 8.1.0, Advion Biosciences) was controlling the Nanoelectrospray conditions which had 200 nL/min flow rate, 0.3 psi backing pressure, and +1.7 kV electrospray voltage for positive ion analysis and -1.7 kV for negative ions. A total range of 70- 590 m/z

range for polar and 70- 2000 m/z was scanned in 7 overlapping SIM scans which took 2 min, 15 sec in total.

4.5. Data analysis for FTICR

4.5.1. Pre-processing. In order to process the mass spectra generated, 2 technical replicates out of 2 with an 80% sample filter were retained (peaks occurred at least 80% of samples within group independently). Next, raw data was subjected to custom-written code including sum of transient files and their process (Southam *et al.,* 2007). Then, processed transient data files were submitted to custom written codes in MATLAB (SIM-stitch algorithm version 2.8). Three more MATLAB scripts were applied to datasets, which referred to peak filtering (Payne *et al.*, 2009). At this stage, a peak list and a peak matrix were generated. The peak list comprised two columns, namely m/z (mass to charge) and related intensities. The peak matrix consisted of a multivariate dataset that recorded all the peaks detected for each biological replicate.

4.5.2.Metabolite identification. The peak mass list, along with peak intensities, were submitted to the Mi-Pack software package (Weber and Viant, 2010) to identify. For each given accurate mass within the peak list, the correct number of empirical formulae were calculated by implication of seven 'golden rules' (Kind and Fiehn, 2007). It must be noted that, despite the high mass accuracy, one mass may linked to different elemental formula, or even similar formula but different structures. Hence, in this paper, for results tables, all the possible compounds have been inserted. For instance, for m/z=128.0108 all forms of alanine namely D-alanine, L-alanine and beta-alanine are considered and FTMS cannot distinguish between these isomers.

4.5.3. Statistical analysis. Prior to PCA, dataset normalization was performed based on the PQN (Probabilistic Quotient Normalization) method (Dieterle *et al.*, 2006) to diminish the effect of extreme peak intensities. Next, the data matrix was treated using the KNN imputation technique (K-Nearest Neighbour imputation method) (Dixon, 1979; Hrydziuszko and Viant, 2012) to estimate the missing values. Finally the samples were transformed using the GLog (Generalized Log Transformation) method (Parsons *et al.*, 2007) to remove the domination of highest intensity peaks through stabilising the whole variance. PCA (Principal Component Analysis) was then performed using MATLAB software, PLS Toolbox.

4.6. GC-MS analysis

One microliter of volatile extracts were injected into the GC/MS-TOF (gas chromatography time of flight) (Pegasus III, Leco, St. Joseph, MI) using the autosampler. Compounds were separated using a capillary column DB-5MS UI, 10 m long, 0.180 mm id and 0.18 µm film thickness (Hewlett Packard, Palo Alto, CA) at 40 °C for 3 min and then raised at 30 °C min^{-1} to 250 °C and maintained for 2 min. Helium was the carrier gas with a flow rate set to 3 mL min^{-1} for 2 min and 1.5 mL min^{-1} thereafter. The mass spectrometry was set to generate a mass spectrum at 70 eV with a 90s solvent delay at 1597 eV at 20 scans per second. The mass range was 50-350 atomic mass units. Volatile compounds were identified using either automatic identification based on spectral library of the instrument software (LECO Chroma TOF version 1.00 Pegasus driver 1.61) or literature survey. Peaks were identified by instrument software, confirmed by checking with volatile compound reference (Adams, 2007) and www.pherobase.com. For unknown peaks, the Kovat Index was calculated based on Retention Time and searched on references. Kovat Index (KI) for each compound was calculated using the formula KI (x) = 100 x ([log RT (x) – log RT(alkane on the left)] – log RT(alkane on the left)] x [number of carbon atoms of alkane on left]. Calculated KIs were then compared to those in reference (Adams, 2007) to confirm the identification. Identified peaks were quantified using correction of peak areas by an internal standard (benzyl acetate), sample weight and eleven external standards including α-phellandrene, myrcene, α-terpinene, β-phellandrene, Cis,β-ocimene, γ-terpinene, terpinolene, linalool, α-humulene, thymol and carvacrol as previously described (Kant *et al.*, 2004). For each sample, five technical replicates were run by GC/MS.

5. Conclusion

The present study demonstrated that tolerant and sensitive populations had different responses to water stress at both physiological and metabolic levels. Water content as a direct indicator of plant water status, clearly identified the tolerant population, since there was no significant difference of water content between watered and droughted plants in the tolerant population. Assessment of water potential and shoot dry weight identified *T. vulgaris* (sensitive) population as a water spender and *T. serpyllum* (tolerant) as a water saver. This strategy could result in less use of soil water and less shoot dry matter for tolerant.

The general picture of metabolites shows that the major classes of metabolites consisting amino acids, carbohydrates, lipids and organic acids were all differentially affected in the thyme populations at early vegetative growth stages in response to water stress. However, increase in the major metabolites pool size in tolerant populations (*T. serpyllum*) was associated with increased tolerance. This is likely to occur through several mechanisms which are including osmotic adjustment, ROS scavenging and cellular structure protection and membrane lipid composition change. Osmotic adjustment might include metabolites such as proline, betaine, mannitol and sorbitol. Likewise, ROS scavenging is probably carried out by enhanced ascorbate and tocopherol levels and also cellular protection by metabolites such as proline and mannitol. Membrane lipid changes might be resulted by increasing poly unsaturated fatty acids.

The highlighted differences between the tolerant and sensitive group of samples are demonstrated by the first component of PCA. Further investigations on the selected metabolites may provide more information on the biochemical pathways under water stress conditions. Eventually, with genetic engineering of the involved genes or by exogenous application of key metabolites it may be possible to enhance plant stress tolerance in sensitive thyme plants which is the end target, as the metabolites synthesized under drought by tolerant plants were not produced by sensitive plants. Some of these metabolites are including osmolytes, antioxidants and phytohormones.

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Figure legends

Figure 1. Physiological parameters influenced by long-term water stress in tolerant and sensitive thyme. One month old plants of tolerant and sensitive populations (*T. serpyllum* and *T. vulgaris* respectively) were exposed to long-term water limitation by water withholding. Next, physiological parameters were recorded at 4 day intervals. Soil moisture

and water content drastically declined in DS plants, while those parameters in tolerant plants were gently decreased. Moreover, shoot dry weight of DSs was greater at similar time points.

Figure 2. Score plot of PCA on polar and non-polar metabolite extracts for the tolerant and susceptible thyme plants grown in control and droughted conditions and harvested at the end of stress period. DI FT-ICR spectral data of control and droughted leaves derived from two thyme populations with varied tolerance to drought subjected to PCA. Four groups (TD: Tolerant Droughted, TW: Tolerant Watered, SD: Susceptible Droughted and SW: Susceptible Watered) well separated by the first two PCs. A) polar positive ions. B) non-polar negative ions. The QCs (Quality Control consisting).

Figure 3. Total number of peaks significantly increased/decreased in droughted plants compared to watered. Venn diagram shows that 53 peaks (in polar and non-polar fractions) increased significantly in tolerant plants, but in sensitive plants the increasing peaks were 342, which 17 peaks were common. In decreasing peaks, tolerant and sensitive plant had 480 and 295 peaks significantly changing respectively with 41 peaks in common.

Figure 4. Metabolite changes regarding with their major classes of compounds. Vertical axis represents the fold change between control and treated plants. There are striking quantitative and qualitative differences between populations with the profile of amino acids, carbohydrates, organic acids and other compounds. In amino acid class, sensitive plants have decreased all the detected compounds except for tryptophan, while tolerant plants have increased all detected amino acids except for serine. Proline and citrolline had the largest increase. Moreover in sensitive plants, homomethionine had the largest decrease. Regarding with carbohydrates, all the carbohydrates increased in tolerant population with the maximum of xylulose, while in sensitive plants galactoglycerol and erythrose decreased and D-Xylulose-5-phosphate elevated. Most of the organic acids have increased in both populations except for Gibberellins in tolerant plants and homocitrate and aconitate in sensitive thyme. Various compounds were detected as significant metabolites belonging to wide diverse metabolite categories mainly secondary metabolites. Membrane lipids have increased significantly in stressed tolerant plants except for lyso PC,

whereas most of the lipids in sensitive plants have declined. Tolerant plant: Thymus serpyllum and Sensitive plant: Thymus vulgaris. Y axis: Fold change

Figure 5. Volatile compounds affected in 4 week old DT and DS thyme plants under water deficit stress. After withholding water, we harvested the leaves at 4 day intervals. For p-cymene, B-myrcene, thymol and alpha-phellandrene the graphs show absolute quantities (pg/mg fresh weight), while for others show relative abundance. a) Gamma-terpinene, B-Myrcene, Alpha-Phellandrene, O-Cymene, B-Pinene, Alpha-Thujene, Ocimene, b) Thymol, c) Germacrene, d) B-caryophylene, e) Alpha-cubebene. Blue lines represent DSs and green tolerant ones. Error bar= ±SEM, Rep=5.

Figure 6. Presentation of the selected metabolites and metabolic pathways affected by drought stress in tolerant and sensitive thyme plants. Diagram representing the response of tolerant and sensitive thyme plants to water stress at metabolite level. Bar charts illustrate the nearby metabolite fold change in droughted plants compare to watered plants. Blue coloured metabolites represent for alteration in tolerant, red-coloured for sensitive population and green-coloured metabolites referred to metabolites changed in both populations. Image made using powerpoint and excel.

Figure 7. Experimental design illustrated for both sensitive and tolerant thyme plants. Morpho-physiologic (No.1 blue colour), DI-FTICR (No.2 in green colour) and GC/MS (No.3 in red colour).

Figures

Figure1

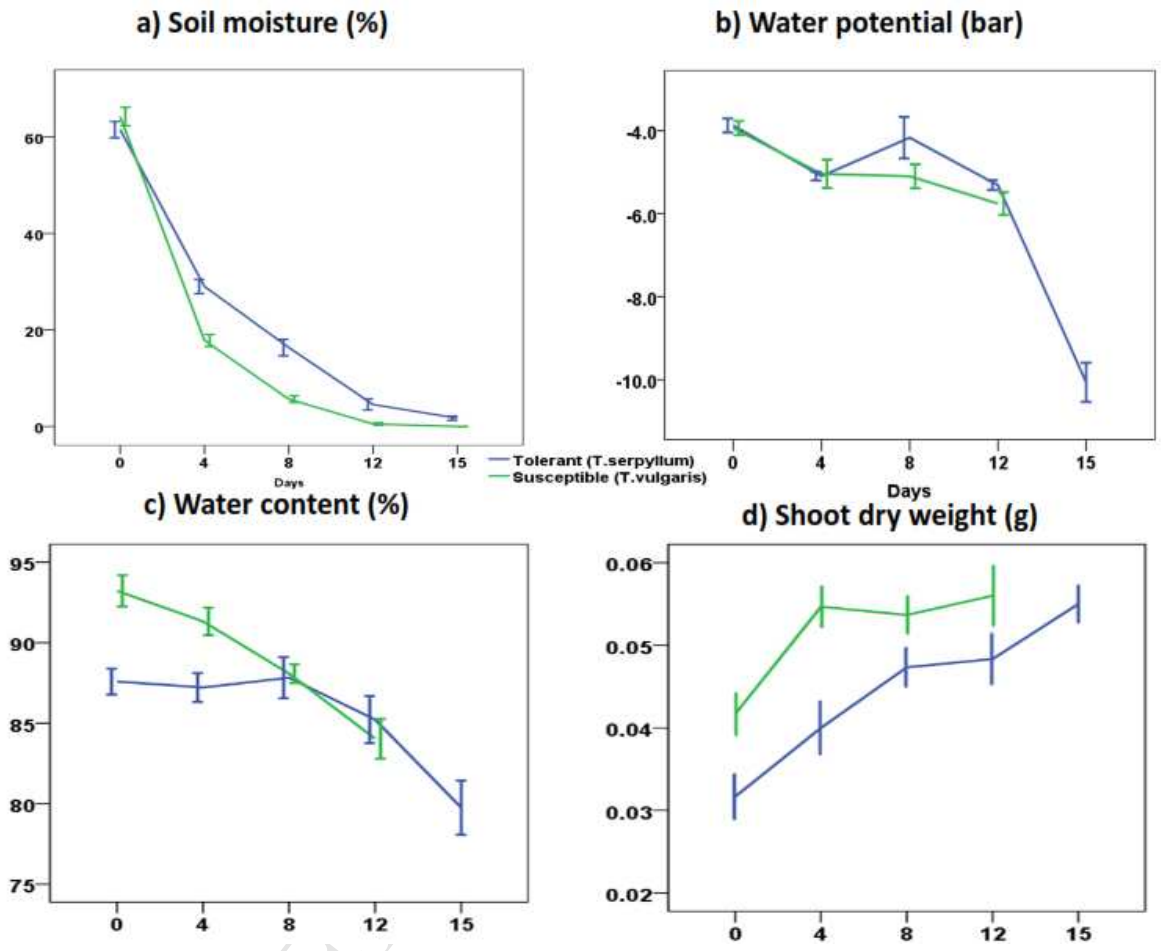

Figure2

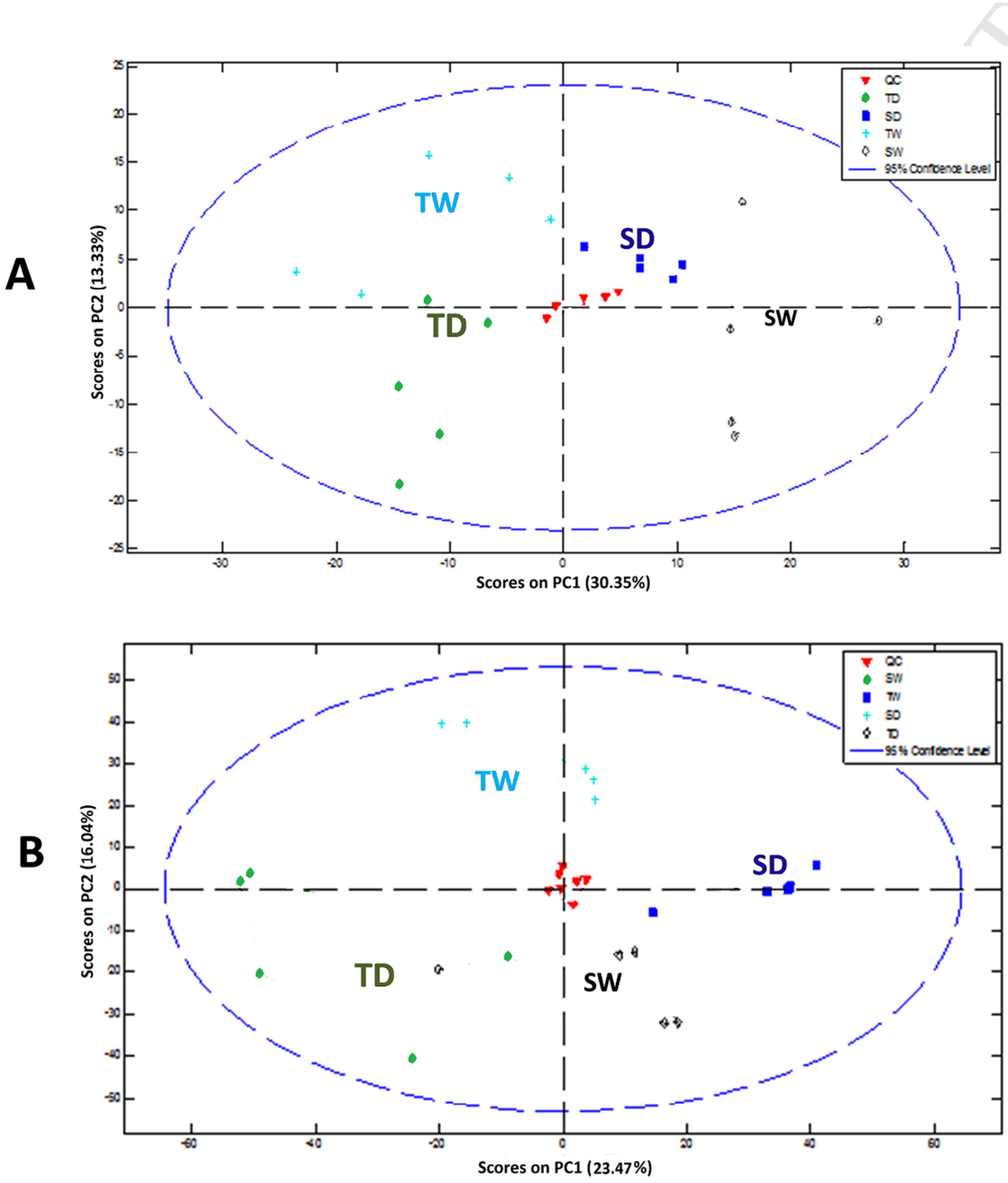
A
QC
TD
SD
TW
SW
95% Confidence Level
TW
SD
TD
SW
Scores on PC2 (13.33%)
Scores on PC1 (30.35%)
B
QC
SW
TW
SD
TD
95 % Confidence Level
TW
SD
TD
SW
Scores on PC2 (16.04%)
Scores on PC1 (23.47%)

Figure 3.

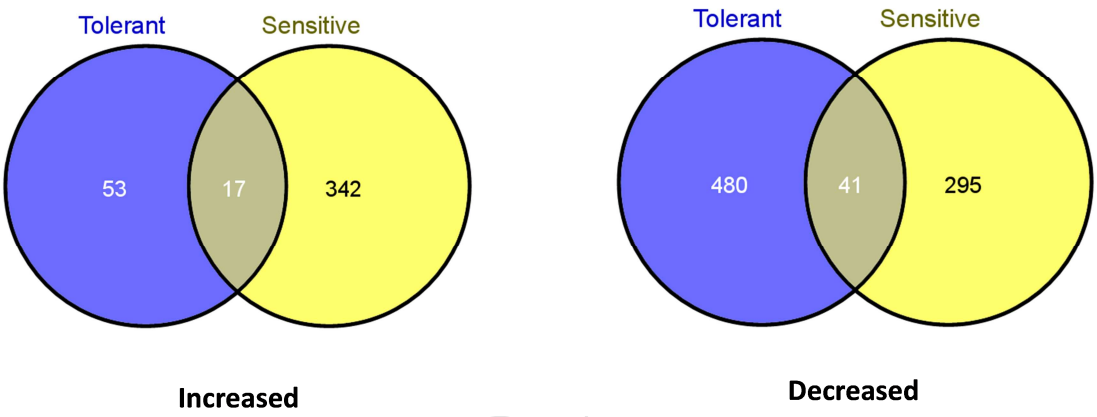

Figure 4.

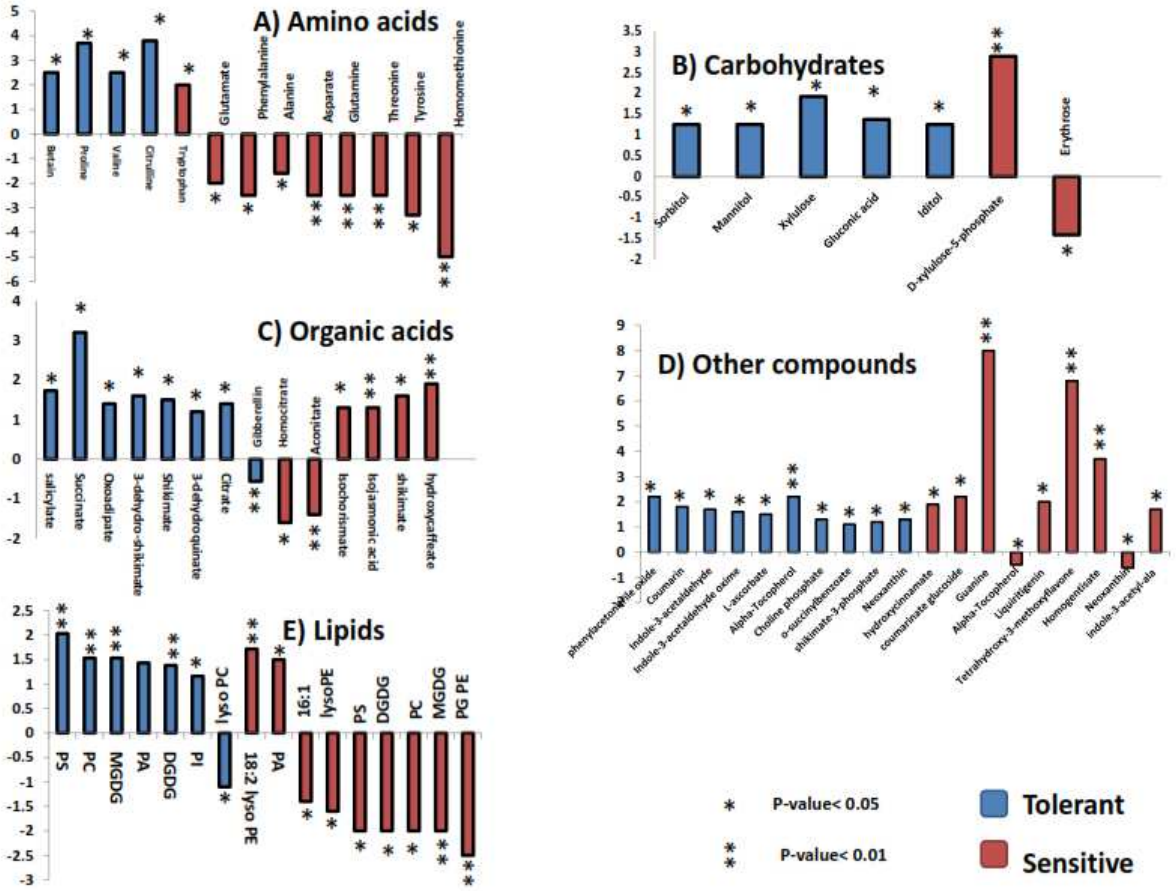

Figure5

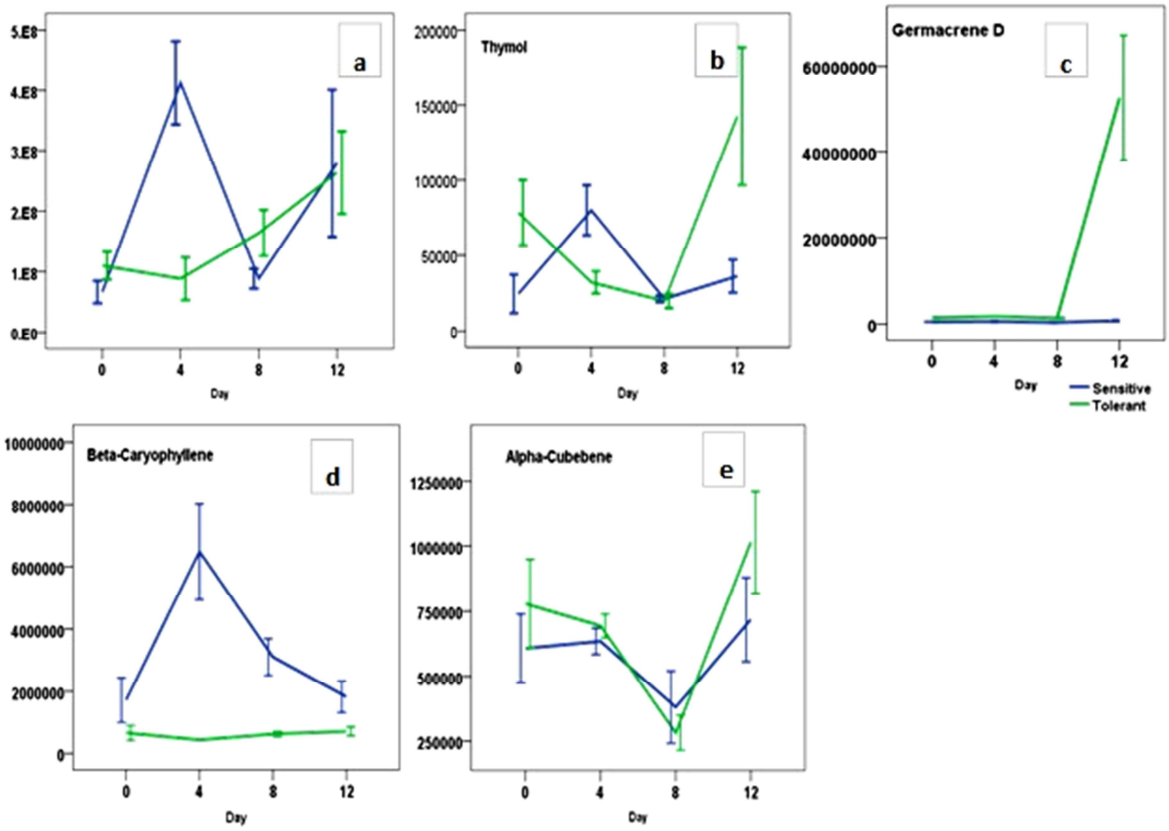

Figure6

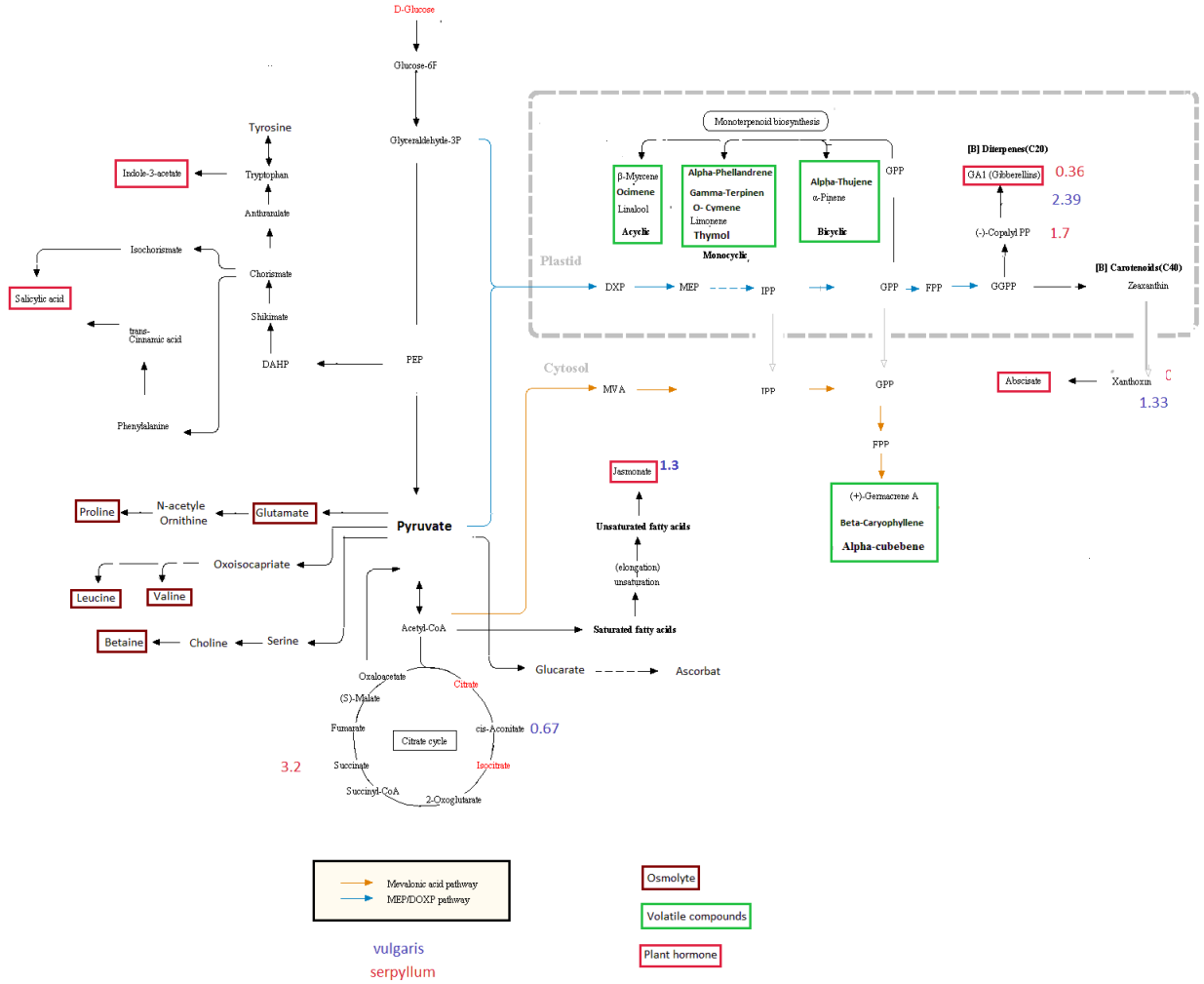
D-Glucose
Glucose-6F
Glyceraldehyde-3P
Tyrosine
Indole-3-acetate
Tryptophan
Anthranilate
Isochorismate
Chorismate
Salicylic acid
Shikimate
trans-Cinnamic acid
DAHP
PEP
Phenylalanine
Monoterpenoid biosynthesis
β-Myrcene
Ocimene
Linalool
Acyclic
Alpha-Phellandrene
Gamma-Terpinen
O- Cymene
Limonene
Thymol
Monocyclic
Alpha-Thujene
α-Pinene
Bicyclic
GPP
[B] Diterpenes(C20)
GA1 (Gibberelline)
0.36
2.39
(-)-Copalyl PP
1.7
Plastid
DXP
MEP
IPP
GPP
FPP
GGPP
[B] Carotenoids(C40)
Zeaxanthin
Cytosol
MVA
IPP
GPP
Abscisate
Xanthoxin
1.33
FPP
Jasmonate
1.3
(+)-Germacrene A
Beta-Caryophyllene
Alpha-cubebene
Unsaturated fatty acids
(elongation)
unsaturation
Saturated fatty acids
Proline
N-acetyle Ornithine
Glutamate
Pyruvate
Oxoisocapriate
Leucine
Valine
Betaine
Choline
Serine
Acetyl-CoA
Glucarate
Ascorbat
Oxaloacetate
Citrate
(S)-Malate
Fumarate
Citrate cycle
cis-Aconitate
0.67
3.2
Succinate
Isocitrate
Succinyl-CoA
2-Oxoglutarate
Mevalonic acid pathway
MEP/DOXP pathway
Osmolyte
Volatile compounds
Plant hormone
vulgaris
serpyllum

Figure 7