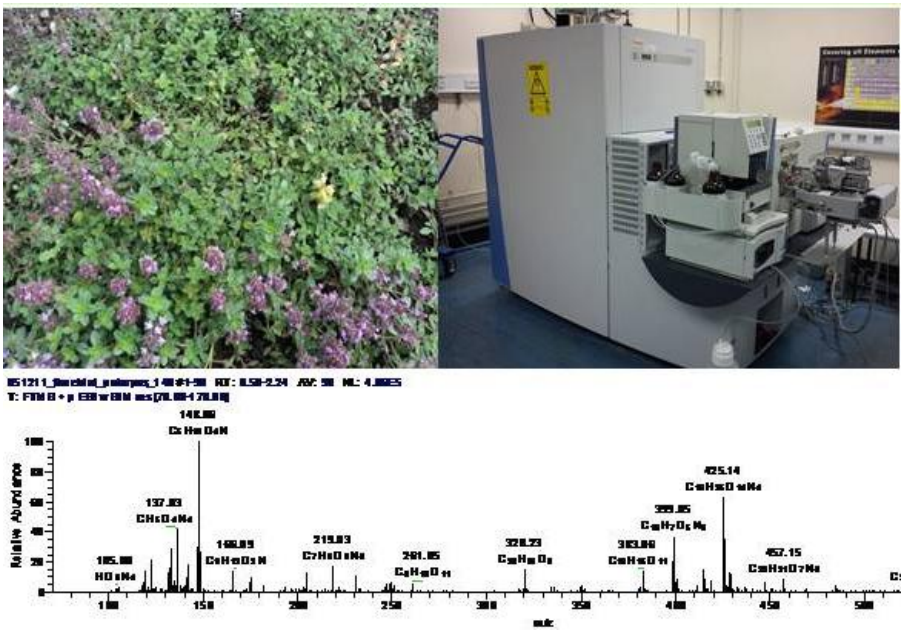
USE OF METABOLOMICS TO STUDY WATER DEFICIT STRESS ON THE MEDICINAL PLANT THYME



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ABSTRACT

Thyme is one of the best known genera within *Labiatae* (or *Lamiaceae)* family, because of it consists of more than 200 species and has diverse medicinal and culinary uses. Environmental factors such as drought can affect plant growth and production of secondary products. Understanding plant response to drought is of great importance and can facilitate the development of drought tolerant varieties. In the present thesis a range of genotypes of thyme was examined including *Thymus vulgaris*, *T. serpyllum, T. daenensis, T. kotchyanous, T. capitata and T. zygis* selected for differences in both drought tolerance and essential oil composition. Drought stress was imposed on 30 day old plants and traits such as leaf water potential, water content, root/shoot weight ratio and survivability were measured. Together these traits indicated that *T. serpyllum* was the most tolerant and *T. vulgaris* the most susceptible populations. A time-course of metabolite profiling using direct infusion FT-ICR mass spectrometry identified the most significantly changing metabolites in *T. vulgaris*. A comparison of metabolite finger print indentified differences in both polar and non-polar fractions. Metabolites including amino acids, carbohydrates, organic acids and lipids changed significantly during long-term water deficit. These results suggested that mechanisms adapting thyme to drought may include osmotic adjustment, ROS scavenging, cellular components protection, membrane lipid changes and hormone activity in which the key metabolites were proline, betaine, mannitol, sorbitol, ascorbate, JA, SA, ABA precursor, unsaturated fatty acids and tocopherol. Profiling of volatiles using GC/MS, showed an increasing -decreasing trend at major terpenes apart from thymol, alpha-cubebene and germacrene in sensitive plants. By contrast, tolerant populations had unchanged terpenes during the water stress period with an elevation on the last day. These results suggests that tolerant and susceptible populations of thyme employing different strategies in response to drought. In conclusion, the combination of metabolite profiling and physiological parameters contributed to a greater understanding of the mechanisms of thyme plant response at metabolomics level.

Keyword: Metabolomics, medicinal plant, thyme, water deficit, stress

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List of Abbreviations

Term	Definition
ABA	Abscisic acid
ANOVA	Analysis Of Variance
AsA	Ascorbate
CE	Capillary electrophoresis
CRD	Completely Randomized Design
CVOC	Constitutive Volatile Organic Compounds
Da	Dalton
DGDG	DiGalactosyl DiacylGlycerol
DI FT-ICR	Direct Infusion Fourier Transform Ion Cyclotron Resonance
DIMS	Direct infusion Mass Spectrometry
DMART	Duncan's New Multiple Range Test
EI	Electron Impact
ESI	ElectroSpray Ionisation
Eto	Evapotranspiration
FPR	False Positive Rate
FT-ICR	Fourier Transform Ion Cyclotron Resonance
Glog	Generalized Log Transformation
GL	Glycolipids
IAA	Indole-3-Acetic Acid

IAAId	Indol-3-AcetAldehyde
IVOC	Induced Volatile Organic Compounds
KEGG	Kyoto Encyclopedia of Genes and Genomes
KI	Kovat Index
KNN	k-nearest neighbour imputation method
LTQ	Linear Trap Quadrupole
MGDG	MonoGalactosyl DiacylGlycerol
MGT	Mean Germination Time
Mi-Pack	Metabolite Identification Package
M/Z	Mass to Charge
NMR	Nucleic Magnetic Resonance
PC	Principal Component
PC	Phosphatidyl Choline
PCA	Principal Component Analysis
PE	Phosphatidyl Ethanolamine
PEG	Poly Ethylene Glycol
PG	Phosphatidyl Glycerol
Phe	phenylalanine
PI	Phosphatidyl Inositol
PL	Phospholipids
PPM	Part Per Million

PQN	Probabilistic Quotient Normalization
QAC	Quaternary Ammonium Compounds
QC	Quality Control
QTL	Quantitative Trait Loci
RFO	Raffinose Family Oligosaccharides
ROS	Reactive Oxygen Species
RPM	Revolutions Per Minute
RT	Retention Time
Rubisco	Ribulose-1, 5-Bisphosphate Carboxylase/Oxygenase
SA	Salicylic Acid
SD	Susceptible Droughted
SEM	Standard Error of Mean
SIM	Selected Ion Monitoring
SNR	Signal to Noise Ratio
SW	Susceptible Watered
TCA	TriCarboxylic Acid
TD	Tolerant Droughted
TEV	Total Explained Variance
TGL	Triglycerides
TOF	Time Of Flight
Trp	Tryptophan

TW	Tolerant Watered
UV	Unit Variance
WC	Water Content
WP	Water Potential
WUE	Water Use Efficiency

CHAPTER I. GENERAL INTRODUCTION

1.1. *Thymus*: Botany, Essential oils and uses

1.1.1. Botany of thyme

The genus *Thymus* is one of the largest genera in the *Lamiaceae* family (original family name *Labiatae*) in terms of species numbers it has. According to present information, there are 214 species and 36 subspecies: more than 250 taxa. Thyme is the English word for the genus and sometimes for *Thymus vulgaris* too. Authors have different ideas about the origin of *Thymus*: some of them believe it comes from the Greek word *Thyo* (perfume) but others say it originated from the Greek word *Thymos* (courage, strength). Even though it has spread all over the old world, the Mediterranean area can be considered the centre of thyme.

This genus can be classified into two groups in regards to life-forms, which are the creeping and erect stem forms. Normally, the erect group comprises bushy and woody plants that are distributed in dry climates, while the creeping group live in humid climates. Stems are quadrangular like other *Lamiaceae*, covered by hair. *Thymus* has two types of essential oil glands: pedicellate glands with the upper cells full of essential oils, or big globe glands, with some basal cells, the latter one is typical of *Lamiaceae*. Generally the leaves are flat and sometimes wide. Flowers grow in clusters in nodes. The calyx is characterized by its five teeth, three upper short and two lower are longer. A common feature of thyme is gynodioecy, which means there are two types of flowers: female flowers (without stamens) and hermaphrodite (or perfect flowers). Depending on the ecology and species, more than fifty percent of each population produce female flowers (Stahl-Biskup and Sàez, 2002). Having female flowers gives advantages to thyme. They produce more viable seeds and the offspring are more vigorous than those of hermaphrodites (Assouad et al., 1978; Couvet et al., 1986). As female flowers cannot be self pollinated, so their offspring will not suffer from inbreeding depression.

Out of 250 species and subspecies, just five species are important economically namely *Thymus vulgaris* L. (common thyme), *Thymus serpyllum* L. (wild thyme or mother of the thyme), *Thymus zygis* L. (Spanish thyme), *Thymus capitatus* or recently *Thymbra capitata* L. Cav. (Spanish oregano or cone head thyme) and *Thymus mastichina* L. (Spanish marjoram or mastic thyme) (Stahl-Biskup and Sàez, 2002) .

Thyme as a commercial plant is traded in different ways (essential oils, fresh herb, dried herb and whole plant), but there are no specific statistics on these products, since governments put all the spices together in their reports. For instance, there are some statistics on essential oil production in Spain, as it is the main producer in the world. Between 1990-1998, Spain has produced 34-45 tons of thyme oil each year (Lawrence and Tucker, 2002). Regarding the dried herb, France is a main country of cultivating herbs, has imported 700-770 tons of dried herbs and Germany has imported 500 tons and produced 50 tons inside the country in 1990. In 1990 Netherlands and UK imported 90 and 220 tons respectively from Spain (Stahl-Biskup and Sàez, 2002).

1.1.2. Essential oils in *Thymus spp*

Essential oil study has increased in recent years because of their importance in medicine and for plant itself. It has been identified that they have roles in plant interactions with insects, other plants and environmental stresses. New chemical analytical techniques have opened up new topics for study. There is some evidence regarding their role in anti-leaf eating by insects and anti-microbial attack, and also their allelopathic effect in inhibition of germination of other plant seeds. Some suggested roles for essential oils are their interesting involvement in decreasing water loss on the leaf surface and the oils on flowers attracting the pollinators (Stahl-Biskup and Sàez, 2002). Glandular peltate trichomes are located on the both sides of leaves storing the essential oils in *Lamiaceae* family. *Thymus* genus essential oils secondary products have been investigated in chemistry for 160 taxa and the results show that this genus has two main categories: volatile compounds (which are 360 different compounds) and the polyphenols, especially the flavonoids (Stahl-Biskup and Sàez, 2002). Flavonoids act in hydrogen proxide scavenging (Brunetti et al., 2013; Yamasaki et al., 1997), pigmentation, protection against harmful UV light (Stapleton and Walbot, 1994), pollen fertility (Ylstra et al., 1994), regulation of auxin transport (Cheynier et al., 2013; Jacobs and Rubery, 1988).

Considerable progress in analytical techniques since the 1960s has identified 360 different volatile compounds in *Thymus* genus essential oils by GC/MS. Components of these volatiles are shown in Figure 1.1.

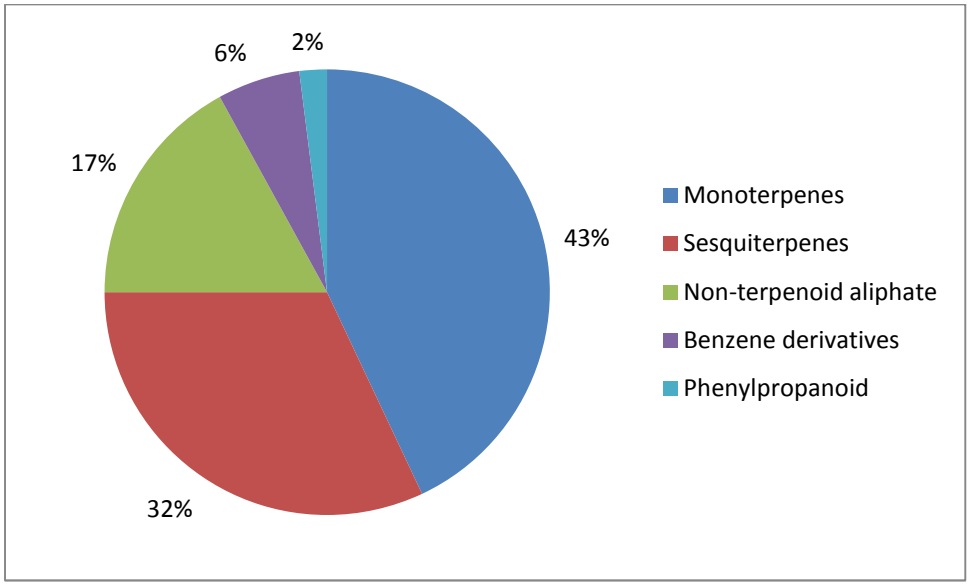


Figure 1.1. Components of volatile essential oils in *Thymus* genus

In 160 taxa studied of Thymus plants, their essential oil dominated by monoterpenes and sesquiterpenes. Next, non-terpenoid aliphate, benzene derivatives and phenylpropanoid compounds observed respectively (Stahl-Biskup and Sàez, 2002).

Main components of volatiles in 160 taxa studied in *Thymus* genus are including 43% monoterpenes, 32% sesquiterpenes, 17% non-terpenoid aliphate, 6% benzene derivatives and 2% phenylpropanoid.

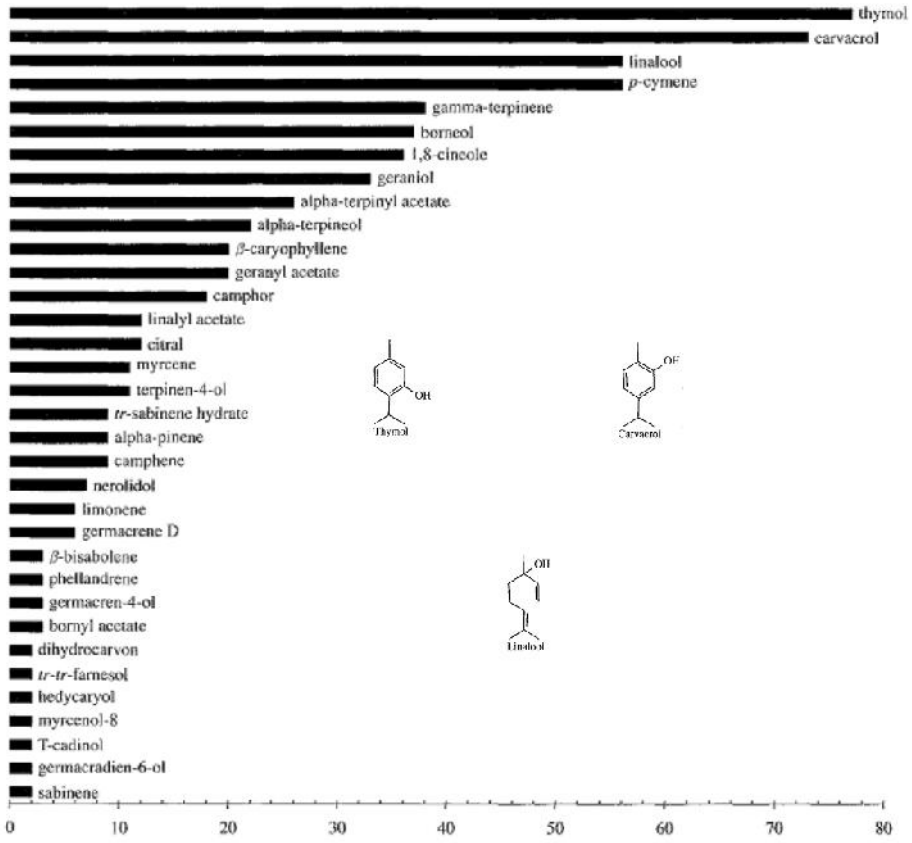


Figure 1.2. *Thymus* essential oils in order of their abundance. Adapted from Stahl-Biskup and Sàez, (2002).

X axis shows the number of *Thymus* taxa, which the compounds occurs. Out of the 160 taxa studied the essential oils, thymol has been observed in 77 taxa of *Thymus* with more than 10 percentages. Carvacrol has occurred in 73 taxa. linalool and ρ-cymene has occurred in 56 taxa of *Thymus* genus beyond 10 percent of the total volume (Stahl-Biskup and Sàez, 2002).

The most important compounds of *Thymus* essential oils namely thymol, carvacrol, ρ-cymene and linalool (all phenol) are monoterpenes. The numbers of taxa in which they occur are shown in Figure 1.2. According to occurrence, thymol has been found in 77 taxa of *Thymus* with more than 10%. Carvacrol has occurred in 73 taxa. Linalool and ρ-cymene are found in 56 taxa of *Thymus* genus (Stahl-Biskup and Sàez, 2002).

1.1.3. Uses

The history of thyme goes back to ancient Egypt with usage as perfume for unguents and embalming. A traditional usage, thyme essential oils have been used externally for treating injuries, infected ulcers and various types of dermatitis. As an internal usage, thyme has been used for treatment of illness such as influenza, cold, sinusitis, cough and asthma, because of its expectorant, antiseptic and spasmolytic properties. According to the EU regulations, Commissions E Monographs (2002) has evaluated two species, *Thymus vulgaris*

and *Thymus serpyllum* (WHO, 2002). In this monograph, both the species have been recommended for external uses as bath additive for supporting treatment of the upper respiratory tract. For internal use *T. vulgaris* can be used for bronchitis and whooping cough and catarrhs of the respiratory tract, but *T. serpyllum* is used just for the latter objective (Blumenthal, 1998).

The pharmacological effects of thyme are explained in detail by (Stahl-Biskup and Sàez, 2002) but can be summarized in the following categories.

1.1.3.1. Antimicrobial effects

Antibacterial effects

Numerous studies have shown this effect on a variety of bacteria particularly gram positive ones, and also some important food-borne pathogens *Salmonella enteritidis, Escherichia coli, Staphylococcus aureus, Listeria monocytogenes,* and *Campylobacter jejuni* (Blakeway, 1986; Farag et al., 1986; Deans and Ritchie, 1987b; Knobloch et al., 1988).

Antifungal effects

Different studies have indicated these effects against fungal diseases (Zambonelli et al., 1996; Blakeway, 1986; Deans and Ritchie, 1987b).

Antiviral effects

An investigation has shown this effect (Zeina et al., 1996).

1.1.3.2. Other effects

Antioxidant effects

Some of the compounds demonstrating the effects are phenol compounds of thyme oil, namely thymol, carvacrol and p-cymene (Deans and Ritchie, 1987a; Schwarz et al., 1996; Chung et al., 1997).

Spasmolytic effects

Several studies have focused on these effects on guinea pig ileum and rat duodenum contraction (Boccard et al., 2010; Cruz et al., 1989; Zarzuelo et al., 1989).

Antiparasitic (Schnitzler et al., 1995) and insecticidal effects

In insecticidal effects, thyme essential oil can be used as a component in pesticides as its compounds are toxic to adult insects by inhibiting their reproduction (Regnault-Roger et al., 1993; Karpouhtsis et al., 1998).

1.1.3.2. Non-medicinal uses of thyme

Food preservatives: The anti-microbial properties of thyme oil allow usage to prevent any alteration due to bacteria and fungi (Aureli et al., 1992). The antioxidant activity makes thyme oil good for preventing lipid oxidation in foods (Budinčević et al., 1995).

Cosmetics: thyme oil is used in cosmetic products like deodorants, perfumes, creams, milks, toothpaste and mouth washes (Marsh, 1992).

Culinary use: thyme because of its flavour and aroma is used as seasoning in fatty meats and bacon, pizzas, sausages, fish and soups (Stahl-Biskup and Sàez 2002).

1.2. Drought stress and its effects on plants

Twenty eight percent of earth's land is too dry for plants economically. Drought is one of the main abiotic stresses, because of reducing plant yield and growth. In spite of enormous numbers of studies of drought stress and massive improvements in modern agriculture, still there is eighty percent or more yield loss due to two main stresses namely drought and salinity (Jenks et al., 2007).

1.2.1. Definition of drought stress

There are different terms and expressions in contexts used for drought and related subjects.

"Drought" is a meteorological and agricultural term and it means a period of time without significant rainfall. In general, drought reduces the available water in the soil and allows loss of water by evaporation and transpiration continuously due to atmospheric conditions (Jaleel et al., 2007).

But "Water deficit" or "dehydration" is used when the water is insufficient for plant metabolism and therefore will affect its growth and development (Hirt and Shinozaki, 2003).

Another term - desiccation, which refers to losing free water entirely, namely less than ten percent water content, is left in the plant (equivalent to 0.1 g water per 1g dry matter (Alpert, 2005; Alpert, 2006).

1.2.2. Effects of drought on plants

Available water in most herbaceous plants is around 80-90 % of fresh weight (Kramer and Boyer, 1995), but drought conditions reduce water content which reduces plant water potential and turgor pressure. Water supply affects almost all plant processes directly or indirectly (Akıncı, 1997), hence water deficit stress due to reduction of available water will affect plants in various ways (Figure 1.3). The effect of drought on plants can be discussed relation to morphological, photosynthesis, proteins, lipids, mineral uptake and ROS factors (Lisar, 2011).

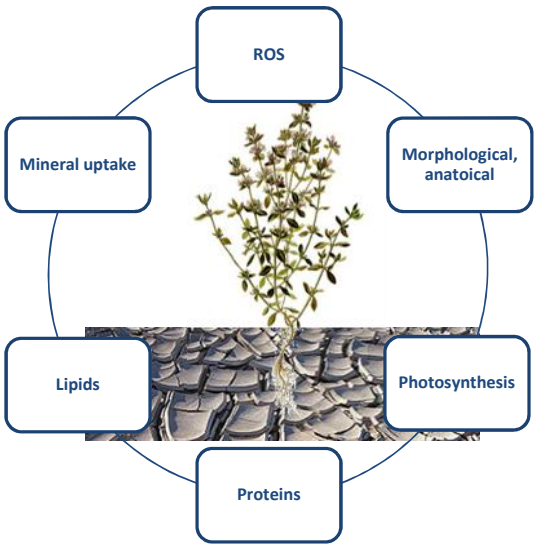


Figure 1.3. Effects of drought stress on plants.

Reducing available water in the plant environment, declines water content, water potential and turgor pressure. This condition affects lipids, proteins, photosynthesis, mineral uptake, morphology and anatomy and ROS.

In morphology, effects are on growth and establishment at early stages of plant growth. It is because of decrease in elongation and expansion growth (Kusaka et al., 2005; Hong-Bo et al., 2008; Specht et al., 2001). Numerous studies have reported a reduction in height and stem length of different plants in response to drought (Manivannan et al., 2007a; Sankar et

al., 2007; Sankar et al., 2008; Petropoulos et al., 2008). Several studies established a decline in leaf area growth due to drought in different plants such as poplar, soybean, maize and sunflower (Wullschleger et al., 2005; Farooq et al., 2009; Sacks et al., 1997; Manivannan et al., 2007a). Some studies showed drought stress significantly increased root growth (Jaleel et al., 2008) but in some studies drought decreased root growth (Wullschleger et al., 2005; Vandoorne et al., 2012). Arguably the most important effect is on plant fresh and dry matter under drought stress, which is economically important. It has been shown that fresh and dry weight of plants decreased due to drought in several studies (Farooq et al., 2009; Specht et al., 2001; Petropoulos et al., 2008).

Drought affects plant growth via some physiological and biochemical processes, such as declining photosynthesis, respiration, translocation, ion uptake, carbohydrates, nutrient metabolism and growth promoters (Farooq et al., 2009; Jaleel et al., 2008). Physiological effects start when the available water in soil decreases, and then the water potential is getting lower than that in the roots (Sunkar, 2010). In the early stages of dehydration, photosynthesis decreases due to CO_2 shortage, because of stomata closure (Chaves, 1991). Even though it is still arguable which CO_2 shortage is the main reason of photosynthesis declining. This maybe is due to difference drought treatment in different stages, species and leaf age (Chaves et al., 2003).

Gene expression, mRNA, transcription factors and protein synthesis change under water stress. These changes occur in LEA proteins (Late Embryogenesis Abundant), HSPs (Heat Shock Proteins), ABA-respondent proteins, dehydrins, proteases, cold regulation proteins, detoxification enzymes (SOD, CAT, APX, POD, GR), enzymes of various osmoprotectants (Lisar, 2011).

Drought stress through oxidative stress causes lipid peroxidation. Fatty acids composition changes under drought, for instance fatty acids with less than 16 carbons in chloroplast increase. In general, water stress alters enzyme activity, transport capacity and protein relations of membrane lipids (Lisar, 2011).

ROS such as O_2, H_2O_2 and OH^- are created by dissipation of excess light in photosynthesis apparatus under drought conditions. These ROS damage macromolecules, DNA, amino acids, proteins and photosynthetic pigment oxidation.

1.2.3. Strategies of plants to confront water deficit

Plants have different strategies to confront water limitation, which can be classified into three main categories (Figure 1.4). See (Bray, 2007) and (Verslues et al., 2006) for more details.

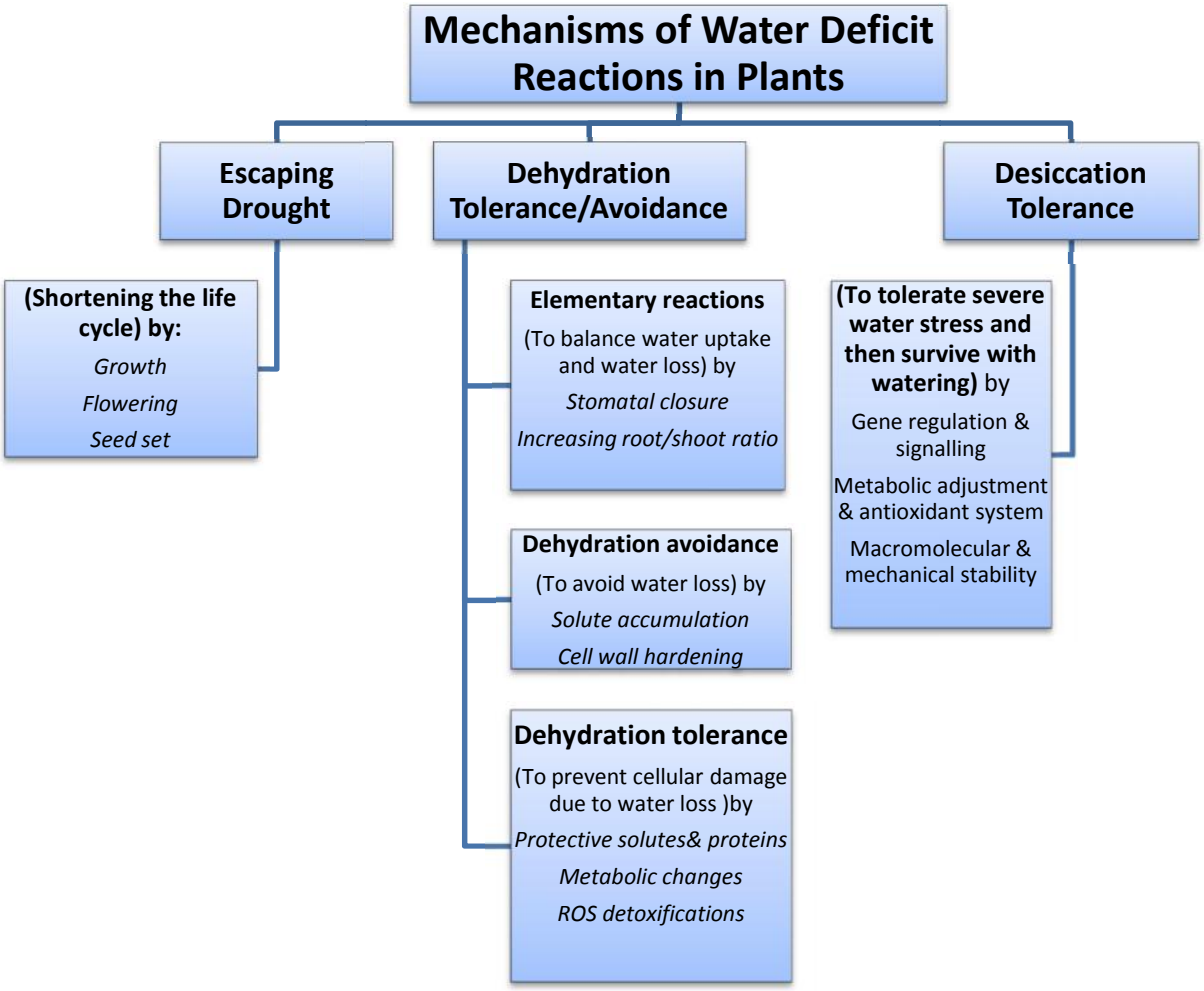


Figure 1.4. Main mechanisms of plant reactions to water deficit.

Plants and how they react to water deficit can be summarised in three ways i.e. escaping drought, dehydration tolerance/avoidance and desiccation tolerance. Escaping the drought is shortening the life cycle mainly in reproductive phase and desiccation tolerance can be performed in both reproductive and vegetative phases of some specific plants. Dehydration avoidance/tolerance mostly happens in crop plants in vegetative phase. Desiccation tolerance is observed in specific category of plant kingdom called resurrection plants. Possible processes of each mechanism have been indicated in the boxes.

1.2.3.1. Drought escape

The outstanding example is proceeding to the flowering phase when water is limited at late season and also germination at early season growth; basically the plant shortens the life cycle in this way. In this strategy the plant does not invest too much in upregulating the metabolic pathways against stress, instead, it maximizes the pathways for fast growth to pass the crisis (Verslues and Juenger, 2011).

1.2.3.2. Dehydration avoidance/tolerance

These mechanisms have been extensively reviewed by Verslues et al., (2006). Dehydration, because of reducing available water, can be defined as lower water potential (Ψw), so hence forth, this expression will be used. Classifying plant response to stress into tolerance and avoidance mechanisms was firstly suggested by Levitt (1972) and explained by Verslues et al (2006). Although plant responses most of the time cannot be categorized into this classification to design experiments for stress tolerance we have to understand these mechanisms (Verslues et al., 2006).

1.2.3.2.1. Dehydration avoidance

As the direction of water movement is from higher water potential to lower water potential, in dehydrated soil, roots will lose water. The first strategy of the plant is dehydration avoidance, by keeping a balance between water loss from the roots and water uptake. In other words, plants can avoid or postpone the water deficit stress for a short period of time using the following mechanisms (Ludlow et al., 1980): stomatal closure, increasing root/shoot ratio, morphological changes in roots and leaves, accumulation of solutes and cell wall hardening (Verslues et al., 2006).

Several morphological developments have been demonstrated previously to be correlated with avoidance mechanisms through minimizing water loss. These modifications include dense stomata (Larcher, 2003), longer roots with extensive branches (Arndt, 2000; Passioura, 1983), leaf rolling (Schwabe and Lionakis, 1996), dense leaf pubescence (Karabourniotis and Bornman, 1999; Liakoura et al., 1999; Bacelar et al., 2004), fewer intercellular spaces and smaller mesophyll cells (Bongi et al., 1987; Mediavilla et al., 2001),

epicuticular wax layer and thick cuticle (Leon and Bukovac, 1978) and lignified tissue (Richardson and Berlyn, 2002). Root structure is associated with dehydration avoidance mechanisms. For example, long roots with plentiful branches or low hydraulic conductance allow the plant to absorb more water sustainably and access a larger soil volume (Arndt, 2000; Passioura, 1983). Water transport from the roots to the leaves through the xylem in adaptive avoidance systems needs improved stomatal and root conductivity (Jones, 1992; Tyree and Ewers, 1991).

1.2.3.2.2. Dehydration tolerance

When low water potential persists, dehydration tolerance mechanisms begin to contribute to prevent cellular damage by water loss (Verslues et al., 2006). Moreover, dehydration tolerance is the final strategy that plants can employ to survive under drought stress conditions (Connor, 2005). There are four main mechanisms of dehydration tolerance, which are described herewith.

1.2.3.2.2.1. Osmotic adjustment

While available water is decreasing in the soil, accumulation of additional solutes and ions will occur (Taiz and Zeiger, 2002). This mechanism is referred to as osmotic adjustment, and these solutes are known as compatible solutes, because with their increase, plant metabolism is not disturbed (Yancey et al., 1982). When osmotic potential decreases due to osmolyte accumulation, water potential will decrease (Verslues et al., 2006; Zhang et al., 1999). This mechanism schematically is shown in Figure 1.5.

$\Psi=\Psi_p+\Psi_\pi$ / Water Potential= Turgor Pressure + Osmotic Potential (Nobel, 1999)

Cell wall hardening or cell wall deformability is quantified by the cell wall elastic modulus, ε. When ε is high, turgor pressure and then Ψ will decline, therefore this mechanism prevents water loss even without solute accumulation (Verslues et al., 2006).

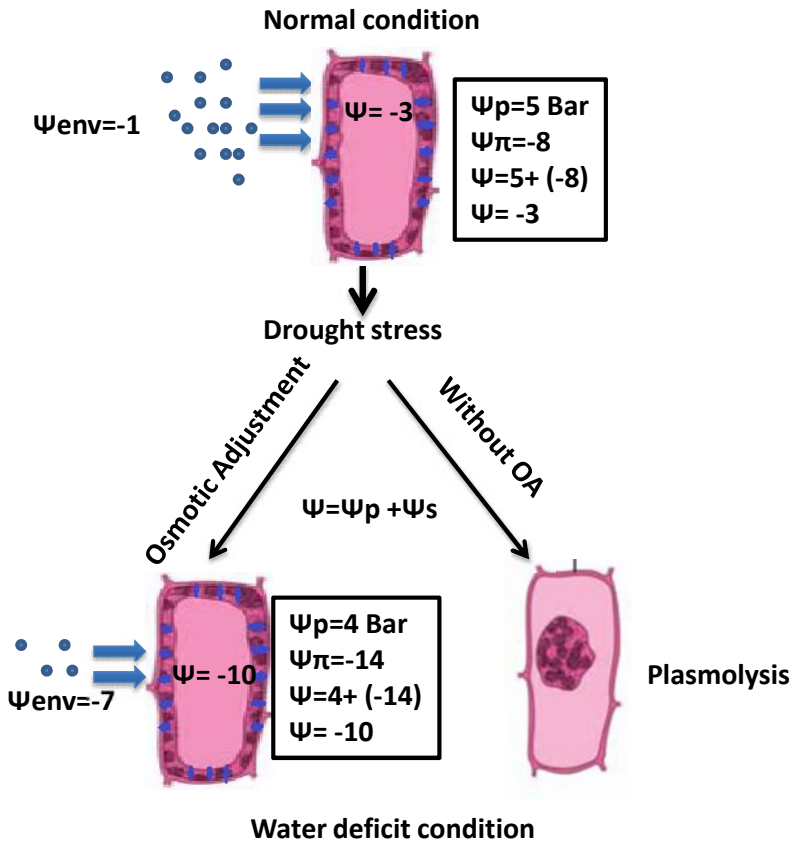


Figure 1.5. Osmotic adjustment as a key mechanism of plant response to water deficit stress.

Water always flows from higher water potential to lower water potential. Under normal conditions, water moves into the cell due to the difference in water potential of outside (-1) from inside (-3). Under droughted condition, two possible responses of cell are presented. Without osmotic adjustment, plasmolysis will occur. If the cell activates the osmotic adjustment mechanism, both turgor pressure and osmotic potential will decline. Therefore still water moves into the cell because of its lower water potential. $\Psi=\Psi_p+\Psi_\pi$: Water Potential= Turgor Pressure + Osmotic Potential

Compatible solutes, "osmolytes" or "osmo-protectants" known so far in plants are monosaccharides (fructose and glucose), sugar alcohols (mannitol, methylated inositol and pinitol), di- and oligo-saccharides (sucrose, trehalose, raffinose and fructan), amino acids (proline, glycine betaine, β-alanine betaine, proline betaine and citrulline), tertiary amines (ectoine; 1,4,5,6-tetrahydro-2-methyl-4-carboxylpyrimidine) and sulfonium compounds (choline o-sulfate, dimethyl sulfonium propironate) (Robinson and Jones, 1986; Pareek et al., 2010). These compounds contribute under drought stress by protection of cellular components (Chen and Murata, 2002), acting as osmoregulators with increasing osmotic pressure (Delauney and Verma, 1993). They prevent loss of water from cells by keeping

turgor pressure high and water content high and replacing water molecules in protein, nucleic acid structure because of their hydrophilic properties (Hoekstra et al., 2001). In general, these metabolites stabilize enzymes, protect membranes and produce osmotic adjustments to keep the turgor pressure (Chaves et al., 2003).

1.2.3.2.2.2. Activation of anti-oxidant systems against oxidative stress damage

Various environmental stresses such as drought, salinity, metal toxicity, cold and heat stress interrupt normal cell metabolism (Maheshwari and Dubey, 2009) and this unfavourable condition leads to enhanced production of Reactive Oxygen Species or ROS (Kele and Ünyayar, 2004). 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 chloroplasts, mitochondria and plasma membranes by exposure to high energy electron leak from electron transport activities (Foyer et al., 1994; Foyer, 1997; Luis et al., 2006; Blokhina and Fagerstedt, 2010; Heyno et al., 2011). These toxic molecules damage cells by oxidation of vital macromolecules such as proteins, membrane lipids, DNA, pigments and nucleic acids (Maheshwari and Dubey, 2009; Dat et al., 2000). Various studies have established the enhancement of ROS under osmotic stress conditions (Serrato et al., 2004; Borsani et al., 2005; Miao et al., 2006; Abbasi et al., 2007). In accordance, plants are equipped with complex defence mechanisms using enzymatic and non-enzymatic antioxidants to mitigate oxidative damages caused by ROS (Dat et al., 2000). Enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), enzymes of the ascorbate- glutathione (AsA-GSH) cycle such as ascorbate peroxidise (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) (Noctor and Foyer, 1998). Non-enzymatic antioxidants consist of ascorbate (AsA), glutathione (GSH), carotenoids, tocopherols, and phenolics (Sharma et al., 2012). Strong correlation has been reported between stress tolerance and higher concentrations of antioxidants (Zaefyzadeh et al., 2009; Chen et al., 2011).

1.2.3.2.2.3. ABA and its physiological role in drought stress response

In response to unfavourable environments such as drought, plants employ ABA (abscisic acid) to activate adaptive metabolic responses (Kusaka et al., 2005). In addition, ABA serves as a plant development optimizer (Cheng et al., 2002) and seed development regulator (Miransari and Smith, 2014). ABA chemically belongs to sesquiterpenes where 9′-cis-

epoxycarotenoid dioxygenase (NCED) produces C15 (xanthoxin) from cleavage of 9′-cis-neoxanthin and 9′-cis-violaxanthin. Then, ABA aldehyde converts xanthoxin to ABA (Finkelstein, 2013). Obviously water status is a stimulus of ABA formation (Petropoulos et al., 2008), but there is little knowledge on the signalling cascades leading to ABA induction. However, there is a different NCED gene family, which each gene activates the specific role for ABA (Sunkar, 2010). For instance, AtNCED3 in *Arabidopsis* induces ABA in response to stress and AtNCED6 and AtNCED9 play a role in seed dormancy and germination (Verslues et al., 2006). ABA activity results from synthesis, degradation and also translocation of ABA (Finkelstein, 2013). However, major roles of ABA in water deficit responses can be summarized as: causing stomata closure through guard cell regulation (Imber and Tal, 1970; Zhang et al., 1999) and also maintaining sustainable water uptake during water stress by continuous root growth through activation of various hormonal signalling pathways (Ludlow, 1989). In detail, stomatal closure is mediated by phospholipase activity and hyperpolarisation of membrane Ca^{2+} channels and tonoplast K^{+} channels of the guard cells (MacRobbie, 2000). Moreover, increase in root growth and decrease in shoot growth is due to low concentration of ABA in shoots and more ABA accumulation in roots; because ABA prevents the growth inhibition property of ethylene (Sharp and LeNoble, 2002; Sharp, 2002). In general, ABA enhances dehydration tolerance through induction of genes encoding tolerance proteins in most of the cells (Kusaka et al., 2005).

In *Arabidopsis* it has been shown that after osmotic stress that has followed water deficit, a transmembrane histidine kinase known as ‘osmometer’ called AtHK1 acts as receptor for water deficit (Osakabe et al., 2013). After the first stage of sensing, drought response directs to ABA-dependent and ABA-independent pathways. ABA-dependent pathways are activated following the accumulation of ABA, and leading to expression of two kinds of stress related genes, namely functional and regulatory. But ABA-independent pathways are not related to each other and seems there is some cross-talk between them (Chaves et al., 2003).

1.2.3.2.2.4. Other mechanisms of dehydration tolerance

Membrane proteins such as aquaporins can transport water (Chrispeels and Agre, 1994), solutes and gases through membranes or even throughout the plant (Bray et al., 2000). These membrane proteins form channels to pass water easily through the plasma

membrane (Kammerloher et al., 1994) and vacuolar membrane (Höfte et al., 1992). Therefore, under dehydration conditions, these membrane-associated transporters may serve as water movement facilitators (Bray et al., 2000).

1.2.3.3. Desiccation tolerance

This refers to organisms or tissues that can tolerate severe water deficit, and survive until the next rewatering (Bewley, 1979). This kind of tolerance has been seen in reproductive structures (seeds, spores and pollen) of bryophytes, lycophytes, pteridophytes, gymnosperms, and angiosperms (Oliver et al., 2000). Also desiccation tolerance has been reported in vegetative tissues of the bryophytes (Oliver et al., 2005; Proctor et al., 2007), pteridophytes, and angiosperms (Illing et al., 2005). An outstanding organism in this category is "common cushion moss" *Grimmia laevigata* (Brid.). This moss grows everywhere in the world, from wet areas to completely dry areas. This organism can be alive for a while in spite of an entirely dead appearance due to dry conditions, but after hydration, it will restart the life cycle. Various research has been done to find out the mechanism of desiccation tolerance by specific disciplines, namely physiological (Farrant et al., 2003; Vicre et al., 2004), metabolic (Whittaker et al., 2007), molecular genetics (Le et al., 2007; Bartels and Salamini, 2001), biochemical (Goyal et al., 2005; Moore et al., 2005) or ultra structural (Moore et al., 2006). In general desiccation tolerance can be discussed in three main topics: gene regulation and signalling mechanisms, metabolic adjustment and antioxidant systems and macromolecular and mechanical stability (Moore et al., 2009). As a main focus of this study is on dehydration, more details on desiccation tolerance can be referred to Proctor et al., 2007; Oliver et al., 2005; Oliver et al., 2000.

1.2.4.4. Key plant products involved in water deficit response

Many compounds contribute to adaptive response to drought stress (acclimation process). These compounds belong to three groups: the first group contains the major compounds with a key role in the adaptive response, such as osmolytes. The second group consists of by-products of stress responses generated through perturbation of normal metabolism. The third group are signalling molecules that regulate adaptive responses, such as salicylic acid (Mittler, 2002; Mittler et al., 2004).

1.2.4.4.1. Lipids

Lipids are the main compounds of membranes and water stress affects lipids and embedded proteins, therefore membrane permeability is important (Kuiper, 1985). Likewise, function and structure of membranes as well as enzyme activity and transport capacity are affected by the physical state and composition of lipids (Kuiper, 1985; Gronewald et al., 1982; Whitman and Travis, 1985).

The main lipids of membranes are phospholipids or PL (mostly in the mitochondrial and plasma membranes) and glycolipids or GL (mostly in the chloroplast membrane). Another form of lipids in plants is fats and oils, which belong to triglycerides (TGL) (Taiz and Zeiger, 2002). As an example of fatty acid composition in leaves, six classes have detected in bent grass leaves including palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3) (Liu and Huang, 2004). There are various studies on stress effects on plant lipids (Kameli and Lösel, 1995; Pham Thi et al., 1982; Anh et al., 1985; Navari-Izzo et al., 1989; Douglas and Paleg, 1981; Navari-Izzo et al., 1990; Pham Thi et al., 1987; Navari-Izzo et al., 1993; Liljenberg and Kates, 1985). Some investigations showed water stress reduced PL and GL in cotton (Wilson et al., 1987), wheat, barley (Chetal et al., 1981) and sunflower (Navari-Izzo et al., 1990), but some of the studies indicated PL increase in wheat (Kameli and Lösel, 1995), total lipid increase in alfalfa (Akıncı and Lösel, 2011) and free fatty acid increase in wheat (Quartacci and Navari-Izzo, 1992). In general, studies demonstrated that long water deficit causes decreasing PLs and GLs and linoleic acid contents but increasing triacylglycerols in leaf tissue. Likewise, previous investigations showed water deficit inhibits biosynthesis of polyunsaturated fatty acids which leads to reduced fatty acid unsaturation (Anh et al., 1985; Navari-Izzo et al., 1989; Martin et al., 1986). One of the tolerance mechanisms in response to drought stress is a modification in leaf cell membrane lipid composition (Lösch, 1993; Turner and Jones, 1980; Ferrari-Iliou et al., 1984; Anh et al., 1985; Quartacci and Navari-Izzo, 1992). These modifications include decreasing polar lipid contents and also polyunsaturated fatty acids, particularly the major leaf glycolipid (MGDG) (Chetal et al., 1981; Pham Thi et al., 1982). These alterations likely are due to increased lipolytic performance, lipid biosynthesis inhibition (Anh et al., 1985) and increased generating of free radical scavengers (Ferrari-Iliou et al., 1992). Previous studies established that the extent of these modifications in drought

tolerant plants are less than sensitive ones because of cell membrane more stability (Pham Thi et al., 1990).

1.2.4.4.2. Compatible solutes

As previously described in section 1.2.3.2.2., compatible solutes contribute to the well known tolerance mechanism called osmotic adjustment. These metabolites or "osmolytes" or "osmo-protectants" known so far in plants are monosaccharide (fructose and glucose), sugar alcohols (mannitol, methylated inositol and pinitol), di- and oligo-saccharides (sucrose, trehalose, raffinose and fructan), amino acids (proline, glycine betaine, β -alanine betaine, proline betaine and citrulline) tertiary amines (ectoine; 1,4,5,6-tetrahydro-2-methyl-4-carboxylpyrimidine) and sulfonium compounds (choline o-sulfate, dimethyl sulfonium propironate)(Robinson and Jones, 1986; Pareek et al., 2010).

1.2.4.4.4. Proteins

Drought stress induces a large number of genes encoding for the biosynthesis of low molecular weight proteins such as LEA proteins (Late Embryogenesis Abundant) and dehydrins (Ingram and Bartels, 1996). Dehydrins were observed in response to various abiotic stresses including drought, but their detailed function is not well understood (Cellier et al., 2000). LEAs have multiple tasks ranging from seed maturation to protection of membrane structure, stabilizing enzymes and promoting ion sequestration in vegetative organs (Close, 1997; Garay-Arroyo et al., 2000). Another class of proteins known as molecular chaperones (or originally known as HSP: Heat Shock proteins) has been reported under abiotic stress conditions such as drought (Alamillo et al., 1995; Alpert and Oliver, 2002). HSPs act in protein refolding and stabilizing proteins and membranes under stress conditions (Wang et al., 2004). The small HSP family (Hendrick and Hartl, 1995; Hong and Vierling, 2000), one of the five major families of molecular chaperones/HSPs, bind to partially folded or denatured substrate proteins and allows them to fold correctly (Sun et al., 2002).

1.2.4.4.5. Secondary metabolites

Plants in addition to primary metabolites (amino acids, carbohydrates and fatty acids) produce a large diversity of compounds known as secondary metabolites (Frey et al., 1999).

To date, 200,000 diverse compounds have been identified as secondary metabolites (Schwab, 2003) which are classified into three major groups, namely alkaloids, isoprenoids and phenylpropanoids (Frey et al., 1999). Apparently secondary metabolites are not involved directly in plant growth and development, but they have specific roles in adaptation and defence against biotic and abiotic stresses (Jaillais and Chory, 2010). According to their definition, secondary metabolites chemically are produced from primary metabolites and certain metabolites are synthesized in specific genera or species. Moreover, most accumulate in high quantities in specific structures such as trichomes, ducts, canals and lacticifers (Santner and Estelle, 2009).

Secondary metabolites can serve as protective agents or antioxidants under unfavourable environments such as drought. For example, in bean and tobacco under abiotic stress conditions, high concentrations of polyamines and phenyl amides have been observed (Jenks et al., 2007). In general, high accumulation of flavonoids and phenolic acids (Hirt and Shinozaki, 2003), polyamines consisting of putrescine, spermine and spermidine (Alpert, 2006) and anthocyanin (Alpert, 2004; Alpert, 2005) under abiotic stresses including drought has been reported.

Previous works established the key role of ABA, SA, JA and polyamines in biotic and abiotic responses (McCarty et al., 1989). Jasmonates, consisting of methyl jasmonate and jasmonic acid, are correlated with the induction of defence systems i.e. accumulation secondary metabolites such as alakaloids, terpenoids, coumarines and phenolic phytoalexins (van der Fits and Memelink, 2000). Recently, the contribution of various secondary metabolites in enhancing abiotic stress tolerance through functioning as antioxidants has been demonstrated. These compounds are saponin (Chan et al., 2010), melatonin (Tan et al., 2007) and serotonin (Anjum et al., 2008).

1.3. Metabolomics: definition, technologies and its applications in plant stress studies

There are several approaches to study plant stress which have been explained very well in reviews (Chaves et al., 2003; Wee and Dinneny, 2010). Within these approaches,

metabolomics has been engaged by researchers to investigate the metabolites involved in plant responses to stress.

1.3.1. Definition: Metabolomics or metabonomics is the high throughput measurement (identification and quantification) of small molecular weight (less than 1000 Dalton) compounds in a cell, tissue or system in a given condition (Viant, 2008; Ruan and Teixeira da Silva, 2010). In terms of 'Omics' technologies, metabolomics is placed at downstream of RNA analysis (transcriptomics) and proteins (proteomics), therefore can demonstrate an obvious understanding of complex biological interactions (Weckwerth, 2003; Bino et al., 2004) (Figure 1.6).

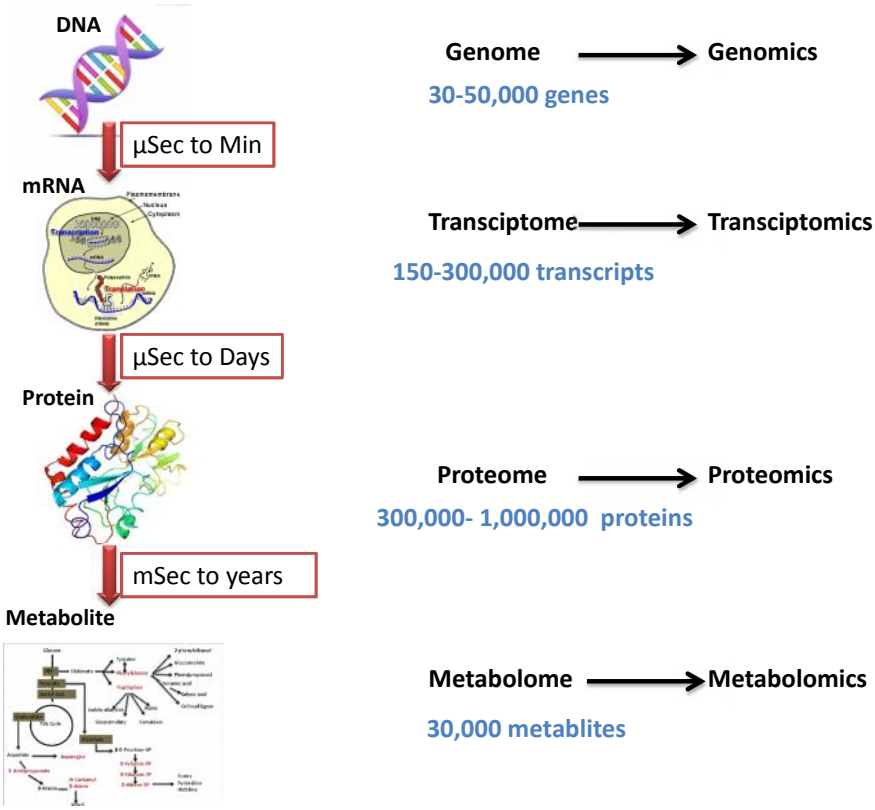


Figure 1.6. Metabolomics in the functional genomics heirarchy and their comparisons in dimensions (adapted from Lay Jr et al., 2006).

Genomics has identified 30-50,000 genes which are transcribed to 150-300,000 mRNA, then are translated to 300,000-1,000,000 proteins and finally produce 30,000 metabolites. These dimensions are for humans.

There are some expressions used in context which are summarized in table 1.1 (Ruan and Teixeira da Silva, 2010). Metabolomics approaches have been classified by Fiehn (Fiehn,

2002) is presented in Table 1.1: metabolic profiling, metabolic fingerprinting and targeted metabolite analysis.

Table 1.1. Summary of expressions used in contexts of plant metabolomics.

Term	Definition
Metabolome	The total quantitative collection of small molecular weight compounds present in a cell, tissue or organism.
Metabolomics	The high throughput measurement (identification and quantification) of small molecular weight (less than 1000 Dalton) compounds in a cell, tissue or system in a given condition.
Metabolite fingerprinting	Rapid and high-throughput methods where global metabolite profiles are obtained from crude samples or simple cellular extracts. In general, metabolites are neither quantified nor identified.
Non-targeted metabolite profiling	The non-targeted quantification and identification of all metabolites within an organism or system under a given set of condition.
Targeted metabolite analysis	Quantitative analysis of a pre-selected list of metabolites. Either based on existing knowledge or following broad scope metabolomic analysis, in-depth biochemical profiling may be based on pre-defined groups of metabolites. Such an approach relies on optimised metabolites, extraction, separation and detection.
FT-ICR mass spectrometry	A method of obtaining accurate measurements of the mass to charge ratio of ions in a complex mixture sample, allowing the identification and measurement of the molecules.
Lipidomics	Specific metabolomic characterization of lipid species

Adapted from (Fiehn et al., 2001; Harrigan et al., 2007; Goodacre et al., 2003; Hall, 2006; Dunn and Ellis, 2005; Last et al., 2007; Fernie and Schauer, 2009).

1.3.2. Metabolomics workflow

Plant metabolomics experiments consist of a series of main steps, which are summarised in Figure 1.7 as five steps.

1.3.2.1. Plant growth: In plant metabolomics experiments, having identical plants is always a major challenge, even when they grow in a growth room with controlled conditions.

1.3.2.2. Sampling: To reduce the error, developmental stage, harvest time and the exact part of the organism should be the same for each sample, otherwise this will cause a large variance. More detail on harvest is presented in Fiehn et al. (2000).

1.3.2.3. Metabolite extraction*:* depends on the analytical equipment, but mostly a methanol: chloroform: water method is used to extract both hydrophilic and hydrophobic compounds (Fiehn, 2002). Moreover, for volatile compound extraction, SPME (Solid Phase Micro-Extraction) and using organic solvents are popular (Bewley, 1979; Moore et al., 2009).

1.3.2.4. Instrumentation and analysis (separation and detecting methods):

Separation methods: Electrophoresis or chromatography methods include Gas chromatography, High performance liquid chromatography (HPLC) and Capillary electrophoresis (CE).

Detecting methods: UV detection and electrochemical approaches include Mass spectrometry (MS), nuclear magnetic resonance (NMR) spectroscopy

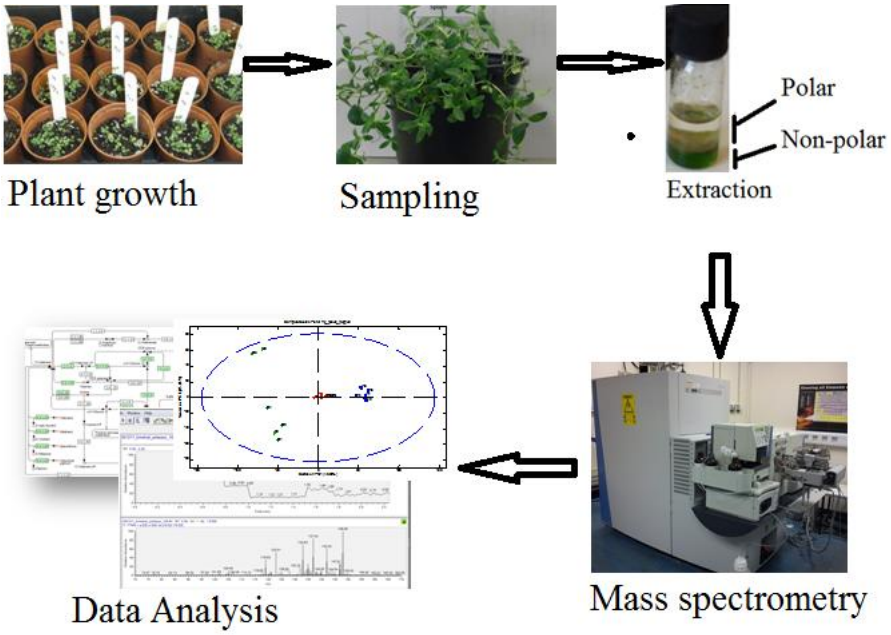


Figure 1.7. Five main steps in common plant metabolomics experiments.

Plants are grown under controlled conditions. Then, depending on the methodology, plant samples are harvested in a homogenous manner i.e. the same time, age, organ and genotype. Next, harvested samples are extracted using determined protocol. Extracts are analyzed by mass spectrometry or NMR based instruments. Finally, raw data are subjected to data analysis processes.

1.3.2.5. Data analysis (pre-processing and pre-treatment, data processing and statistical analysis):

Since metabolomics generates huge complex datasets, simple observational methods are not enough to analyze the data (Goodacre et al., 2007). Appropriate data analysis provides high quality identification and quantification of metabolites (van den Berg et al., 2006). In other words, separating biological variation from variations derived from unwanted and obscure sources is very important in metabolomics studies (Jonsson et al., 2004). Data handling in metabolomics can be further divided into 4 main steps: Pre-processing, Pre-treatment, Data analysis and statistical analysis.

1.3.2.5.1. Pre-processing and Pre-treatment

Pre-processing is referred to all low level process methods which transform raw instrumental data files to clean datasets for the next step (Goodacre et al., 2007), while the clean data are subjected to pre-treatment methods to go forward to the data processing step (Bro and Smilde, 2003). Various methodologies for each stage are briefly indicated here

(for more details see Goodacre et al, 2007). Common pre-processing methodologies include deconvolution (Jonsson et al., 2004; Kvalheim and Liang, 1992; Weljie et al., 2006), peak-picking (Katajamaa and Orešič, 2007; Windig et al., 1996), target analysis (Andreev et al., 2003), alignment (Skov et al., 2006; Forshed et al., 2005), phasing, base-line correction and bucketing (Holmes et al., 1992). Moreover, popular algorithms for pre-treatments are normalization, centring, mean-centring, scaling, auto scaling, range scaling, pareto scaling, transformations (log, root, square, box-cox), missing values, outliers (Goodacre et al., 2007).

1.3.2.5.2. Data processing and statistical analysis.

This step is actual data analysis by recruiting appropriate models, methods, parameter estimation, back-transformation and visualisation. The appropriate algorithm is selected based upon literature survey and local expertise (Goodacre et al., 2007). However, current statistical methods commonly used in metabolomics studies are categorized into univariate and multivariate analysis methods.

1.3.2.5.2.1. Univariate analysis

These methods are used to test significant changes of each metabolite between groups such as control and treated. If the dataset is distributed normally, one of the parametric tests such as t-test, z-test or ANOVA (Analysis of Variance) is employed. If the dataset is not following normal distribution, a non-parametric method such as the Kruscal-Wallis test is used (Steel and Torrie, 1960).

1.3.2.5.2.2. Multivariate analysis

Metabolomics generates large multivariate datasets with huge numbers of variables (Weckwerth and Morgenthal, 2005).There are many multivariate methods (Beebe et al., 1998; Chatfield and Collins, 1980) that can be recruited for data analysis in metabolomics. The appropriate multivariate method is chosen based on biological questions of the study and dataset structure (Boccard et al., 2010). However, these approaches such as dimensional reduction, correlation analysis or grouping samples are classified into unsupervised and supervised methods based on considering related response variable to dataset.

1.3.2.5.2.2.1. Unsupervised methods

Unsupervised methods don't need any additional information other than the data collected. Whereas these methods use only the x-data for classification of samples, the basis of this grouping is difficult to interpret. The most popular methods in this category include PCA (Principal Component Analysis) and Hierarchical cluster analysis (Goodacre et al., 2007).

1.3.2.5.2.2.1.1. PCA

Principal component analysis (Mártonfi et al., 1994) is likely the most popular statistical tool employed in metabolomics studies as the starting point for exploratory purposes before classification or supervised methods (Boccard et al., 2010). This multivariate method was initially proposed by Pearson (Pearson, 1956), and basically is used to interpret the underlying properties of data structure (Hotelling, 1933) by reducing dimensionality of dataset using projection into fewer dimensions, whilst maintaining minimum information loss (Kind and Fiehn, 2007; Bro and Smilde, 2003; Jackson, 2005). In practice, PCA concentrates on a few independent uncorrelated peaks that describe a large proportion of total variation and therefore makes a linear combination of original variables.

Finally, all the samples can be illustrated in a two or three dimensional (PC) space known as the score plot, in which the distance among the individual samples (in the Euclidean distance) indicates the variation of metabolites in samples. Likewise, a loading plot is used to demonstrate the contribution of each metabolite on the PCs (Boccard et al., 2010). Distribution of samples on score plots may help to group the samples, explore a trend or identify outliers.

1.3.2.5.2.2.1.2. Cluster analysis

Cluster analysis allocates individual samples (or metabolites) to a similar group based on a chosen distance parameter. This popular statistical method is used to reveal underlying patterns of data structure (Boccard et al., 2010). This multivariate analysis can be split into two forms - Hierarchical Cluster Analysis (HCA) and Non-Hierarchical Cluster Analysis (NHCA). Both forms group samples based on either similarity (e.g. Pearson correlation) or dissimilarity coefficients (e.g. Euclidean distance). In practice, in HCA each sample is considered as a single cluster, and then merges with another sample having the highest

similarity. These steps iterate until all of the samples are aligned. Finally results are shown as a dendrogram starting with all the individual samples, ending up with one cluster in addition to their distances (Eisen et al., 1998). In non-hierarchical algorithms such as K-means, samples are divided into a predefined number of clusters (K) without hierarchical organization (Boccard et al., 2010).

1.3.2.5.2.2.2. Supervised methods

Supervised methods require prior knowledge of the samples. In other words, there are subsets of samples with a known class or group and the rest of the samples are calibrated based on known samples scores. These methods are more powerful than unsupervised ones, since they are based on predefined groups (Goodacre et al., 2007).

1.3.2.5.3. Data analysis workflow used in this thesis

In this thesis, raw data was exported from Xcalibar (Version 2.0.7 Thermo Scientific) to MATLAB ver.7 (The Mathwork Inc., Natick, MA) and subjected to custom-written code including that sum of transient files and their process (Southam et al., 2007) (Figure. 1.8). These processes have been described already by Southam et al., 2007, but briefly they comprise averaging the triplicate transients, Hanning apodisation, zero filling once, and application of a fast Fourier transformation. Then, processed transient data files were submitted to custom written codes in MATLAB (SIM-stitch algorithm version 2.8). In this way, SIM windows were aligned together with existence peaks in the overlap region, then all the peaks with SNR (Signal to Noise Ratio) lower than 3.5 were removed. In addition, some known detected compounds (minimum three peaks for each mass spectrum) have been used to calibrate spectra (Southam et al., 2007). Three more MATLAB scripts were applied to the datasets, which referred to peak filtering (Payne et al., 2009). The first script, namely Replicate Filter, rejected all the peaks detected in just one replicate. In other words, peaks observed in at least two replicates were kept. The second script, called Flag Blank Peaks, removed all the peaks in the blank sample except for the peaks with double intensity of the same peak in biological samples. The third script, namely Sample Filter, retained peaks that presented in a minimum of 50% of all the samples (Payne et al., 2009, Taylor et al., 2009). At this stage, a peak list and peak matrix was 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. In mass spectrometry the mass to charge (m/z) formed by dividing the ion mass (m) in atomic mass unit and its ion charge number.

Next, the peak mass list, along with peak intensities, was submitted to the Mi-Pack software package (Weber and Viant, 2010) for identification. For each given accurate mass within the peak list, the correct numbers of empirical formulae were calculated by implication of seven 'golden rules' (Kind and Fiehn, 2007). Prior to statistical analysis, dataset normalization was performed based on the PQN (Probabilistic Quotient Normalization) method (Dieterle et al., 2006b) to diminish the effect of extreme peak intensities. Next, the data matrix was treated using the KNN imputation technique (k-nearest neighbour imputation method) (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 establishing the whole variance.

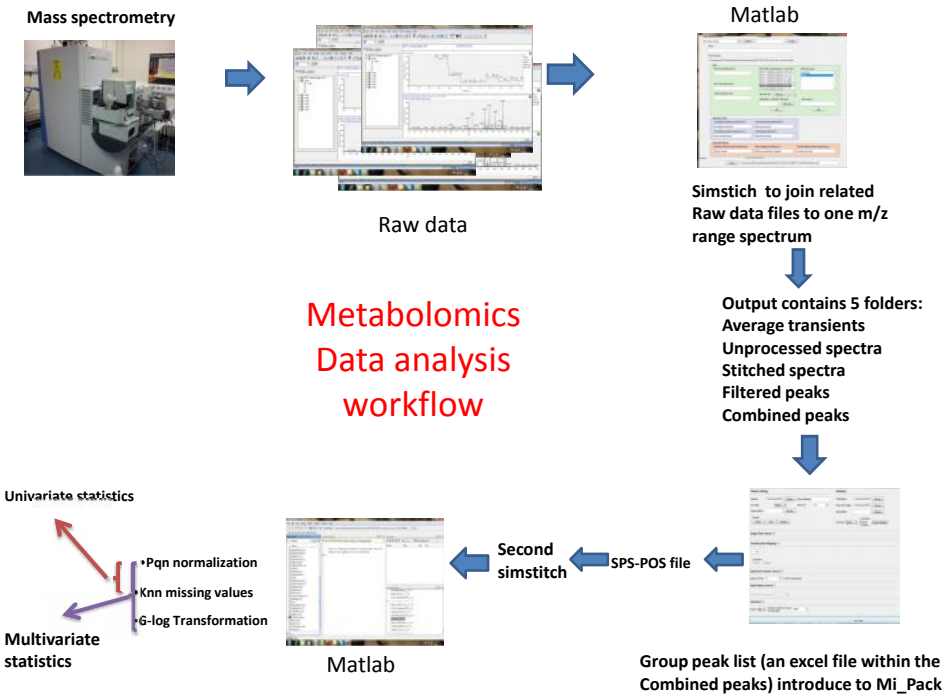


Figure 1.8. Data analysis workflow for DI FT-ICR metabolomics data performed on raw data of this study.

Raw data was loaded in MATLAB ver.7 and subjected to custom-written code to align the SIM windows along with the processes. This analysis results in five folders including average transients, unprocessed spectra, stitched spectra, filtered peaks and combined peaks. M/z along with their intensities were loaded in Mi-Pack software for metabolite identification, then based on the result, second sim-stitch was performed on dataset. Prior to univariate and multivariate statistical analysis, data matrix was subjected to normalization, missing values and transformation. For more details see Appendix 2.

1.3.3. Metabolomics Technologies

Due to variation of different technologies, considering speed, selectivity and sensitivity, suitable techniques are used (Sumner et al., 2003). Researchers mostly choose combinations of multiple techniques, as a single technology can not provide enough metabolite visualization (Sumner et al., 2003; Hall, 2006). For details of technologies see (Hagel and Facchini, 2007), but summarized herewith are some important techniques:

1.3.3.1. Nuclear magnetic resonance

This technique is usually used in biomedical metabolomics (metabonomics). NMR compared to MS-based techniques has less sensitivity (Kaddurah-Daouk et al., 2004). Especially for plant extracts, NMR can not detect some metabolites, as they can be below the NMR threshold. But this method is a uniform system and getting popular due to improvement. See review (Ratcliffe and Shachar-Hill, 2005) for more detail on this method.

1.3.3.2. GC/MS Mass spectrometry

Gas Chromatography/Mass Spectrometry is a popular method, because of robust separation, detection and software for metabolite identification. Even though it is principally for volatiles (eg monoterpenes, alcohols and esters), nowadays it is broadly used for non-volatile polar primary metabolites, such as sugars and amino acids, by converting them to volatiles prior to analysis by GC/MS (Hall, 2006). To confirm the metabolite identity, standard compounds are run and data compared with commercial databases.

1.3.3.3. LC/MS

Liquid Chromatography/Mass Spectrometry, in addition to primary metabolites can detect secondary metabolites too, which are usual in plant tissues. The only restriction of this technique is that metabolites should be ionized. This ionization can be done by combination of LC with mass spectrometry either by TOF (time of flight) or FT-ICR (Fourier transform ion cyclotron resonance). A disadvantage to GC/MS can be that only a few databases for LC/MS metabolite identification are available, which currently researchers are working on (Verhoeven et al., 2011; Hall, 2006).

1.3.3.4. CE/MS

Capillary Electrophoresis/ Mass Spectrometry is a powerful technique for microbial extracts and will have broad application for plants, because of good sensitivity and selectivity as a separation technique. This method can be combined with MS and is strong for analysis of primary and secondary metabolites (Sato et al., 2004; Hall, 2006).

1.3.3.5. FT-ICR mass spectrometry

Fourier Transform Ion Cyclotron Resonance (FT-ICR) mass spectrometry with ultra-high mass accuracy and resolution is employed popularly in biological science research (Allwood et al., 2012). The basic principle of FT-ICR mass spectrometry is illustrated in Figure 1.9. In fact, FT-ICR measures m/z (mass to charge) of ions using their cyclotron frequency in a strong magnetic field (Ohta et al., 2007). Figure 1.9 shows that subsequent to direct infusion of extracts to the instrument, metabolites are ionized using one of the common methods i.e. APCI, ESI and MALDI. Then, an ICR cell with a strong magnet traps ions, which depending on their m/z circulate in specific cyclotron frequency. Next, Fourier-transform converts the frequency spectrum to mass spectrum (Ohta et al., 2010).

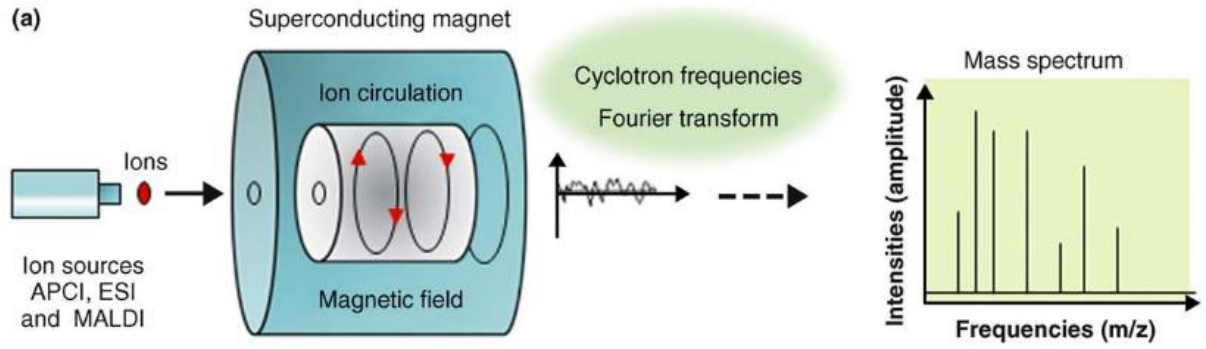


Figure 1.9. Schematic principle of the FT-ICR mass spectrometry system (Ohta et al., 2010).

FT-ICR measures m/z (mass to charge) of ions using their cyclotron frequency in a strong magnetic field. This diagram shows that subsequent to direct infusion of extracts to the instrument, metabolites are ionized using one of the common methods i.e. APCI, ESI and MALDI. Then, an ICR cell with strong magnet traps ions, which depending on their m/z circulate in specific cyclotron frequency. Next, Fourier-transform converts the frequency spectrum to mass spectrum (Ohta et al., 2010).

In principle, ionization source generates positively charged fragments by high-energy electron bombardements, and then ionized molecules based upon their masses are

separated in the mass analyser component. Next, ions are detected and quantified by detector unit. The most abundant ion is assigned as intensity of 100% in mass spectra (known as base peak) and all other ions aboundances are calculated relative to this value (Ryhage and Stenhag, 1960). The high mass accuracy offered by FT-ICR provides metabolite identification using unambiguous assignment of metabolite formula. For metabolites larger than 500 Da or ambiguously identified molecules, MS^n allows structural identification (Marshall, 2000). In addition to the above advantages, FT-ICR is becoming the most appropriate technology for complex mixtures of samples by detecting a diverse range of metabolites because of its high sensitivity, using developed ionization resources such as MALDI (Matrix-assisted laser desorption/ionization), nESI/APCI (Nanolectrospray Ionization/atmospheric pressure chemical ionization) and EI/CI (Electron Ionization/Chemical Ionization), separating mixtures into classes such as (polar/non-polar) or ionization methods (positive/negative) (Brown et al., 2004).

Most of the FT-ICR mass spectrometry based research is carried out using direct infusion rather than coupled to separation instruments (Han et al., 2008). Advantages of DI FT-ICR mass spectrometry can be summarized as follows: High mass accuracy (less than 1 ppm) and high mass resolution provides unambiguous discrimination between isobaric ions. This leads to detection of a larger number of metabolites compared with low-resolution instruments. Moreover, since there is only m/z column rather than m/z and retention time in LC/MS, pre-processing of datasets is becoming easier.

Fourier Transform Ion Cyclotron Resonance estimates metabolite mass more accurately, but has less chromatographic resolution, which for plant samples makes it difficult to distinguish the isomers (Murch et al., 2004; Brown et al., 2004). Orbitrap FT/MS is getting more popular rather than Cyclotron FT/MS, because it is faster, more sensitive and less expensive (Sumner et al., 2003; Dunn and Ellis, 2005) but still a problem with non-recognition the isomers exists.

1.3.4. Applications of metabolomics

This post-genomic technology has comprehensively and rapidly been used in natural sciences, ranging from food science to medicine, ecology and plant studies. Publications

show these applications vary from fundamental knowledge to applied objectives, but can be organized into five main categories (Hall, 2006) with some examples:

- Genotyping and phenotyping (metabotyping)
  Classification of populations (Tikunov et al., 2005).
- Population screening
  Testing for pathogenic infection (Allwood et al., 2006).
- Understanding physiological processes
  Studying metabolic changes during plant development (Tarpley et al., 2005).
  Characterizing the metabolic response to biotic (Desbrosses et al., 2005) or abiotic stresses (Bohnert et al., 2006).
- Biomarkers and bioactivity (Beekwilder et al., 2008)
  Identification of the genetic determinants of biochemical composition (Hall et al., 2008).
  Designing tools and markers for monitoring quality (Schauer and Fernie, 2006).
  Studying secondary metabolism (Goossens et al., 2003).
  Identifying plant natural products (Pauli et al., 2005).
- Quality and breeding (Overy et al., 2005).
  Effects of environmental conditions on quality (Carrari et al., 2006).
  Identifying metabolic quantitative trait loci (mQTLs) (Fernie and Schauer, 2009).
  Metabolic engineering (Facchini et al., 2000b; Kutchan, 2005).
- Substantial equivalence (Methodology to characterize the similarities and differences between samples, mostly transgenic and wild type) (Kristensen et al., 2005).

As this project is aimed at applying metabolomics to study water deficit stress, therefore after a brief explanation of abiotic stress applications will focus further on dehydration in detail.

1.3.4.1. Application of metabolomics in the study of plant abiotic stresses:

Metabolomics can characterise the interaction of plants with environment. This method can investigate the responses of organisms to abiotic pressure (Bundy et al., 2009).

Metabolomics was used to study temperature (Cook et al., 2004), water and salinity (Brosché et al., 2005), sulfur (Nikiforova et al., 2005a; Nikiforova et al., 2005b), phosphorus (Hernandez et al., 2007), oxidative (Baxter et al., 2007b) and heavy metal (Le Lay et al., 2006) abiotic stress with a combination of several stresses (Rizhsky et al., 2004) in plants. In this report, some of applications of metabolomics in important stresses are described.

In temperature stress, Kaplan et al. (2004) was used metabolite profiling to understand the dynamics of the *Arabidopsis* response. They used GC-MS profiling and identified a set of known metabolites and unknown mass spectral tags that particularly respond to heat or cold stress or to both (Kaplan et al., 2004). Morsy et al. (2007) used targeted profiling to study chilling tolerance in different populations of rice. The chilling-tolerant population could accumulate raffinose and galactose under stress, while the chilling-sensitive population had a lower level of these sugars (Morsy et al., 2007).

Both macronutrient-deficiencies such as nitrogen, potassium or phosphorus and micronutrient-deficiencies such as iron and zinc deficiency limit plant growth and development (Marschner and Marschner, 2011). Many studies have been conducted to understand the impact of nutrient deficiencies on plant metabolism. For instance, using HNMR it has been demonstrated that applying double the amount of nitrogen fertilizer leads to more amino acid and citric acid cycle intermediates (Lubbe et al., 2011). On sulphur-deficiency plants, Nikiforova et al. (2005) have performed untargeted GC-MS and LC-MS profiling to find out the response of known and unknown metabolites, then they combined this with transcriptomics to find metabolome-gene correlation (Nikiforova et al., 2005a; Nikiforova et al., 2005b).

To study heavy metal stress, some groups used NMR-based metabolic fingerprinting (Bailey et al., 2003) and metabolite profiling (Le Lay et al., 2006).

GC/MS of ^{13}C labelling along with microarray analysis was employed to investigate metabolic changes due to oxidative stress in *Arabidopsis*. Results indicated that the metabolite responses of plant cells are remarkably similar to that of microbial systems. Oxidative stress inhibited the metabolism of TCA (tricarboxilic acid cycle) and also reduced synthesis of amino acids such as glycine, serine and alanine (Baxter et al., 2007a; Budincevic et al., 1995).

Salt stress is one of the main factors affecting plant growth and development (Allakhverdiev et al., 2000; Koca et al., 2007). The negative effects subsequent to salt stress stem from lowering water availability, nutritional imbalance and ion toxicity (Ashraf, 1994). NMR technique demonstrated the clear increase of sugars and amino acids in shoots and roots of stressed plants of rice (Fumagalli et al., 2009). Metabolic fingerprinting was used to identify metabolic changes during salinity stress. In this study, FT-IR spectroscopy was used to fingerprint whole fruit flesh extracts (Johnson et al., 2003). Salt stress on maize using NMR resulted in elevation of alanine, glutamate, asparagines, glycine-betaine and sucrose and decline of malic acid, aconitic acid and glucose in shoots. In roots salt stress was associated with increased γ-amino-N-butyric acid (Parida et al.), malic acid, succinate and sucrose and decreased acetoacetate and glucose (Gavaghan et al., 2011). In tobacco, short-term low dose salt stress was correlated to pyrimidine and purine declining. While long-term salt stress caused accumulation of proline, myo-inositol, increased biosynthesis of aromatic amino acids and increased shikimate-mediated secondary metabolism. Salt stress altered various metabolism, including the TCA cycle, gluconeogenesis/glycolysis, glutamate-mediated proline biosynthesis, shikimate-mediated secondary metabolism and the metabolism of choline, pyrimidine and purine (Zhang et al., 2011).

1.3.4.2. Role of metabolomics in water deficit stress studies

Water deficit as a major abiotic stress, impacts plants at morphological, physiological and biochemical levels such as inhibited photosynthesis and growth, accelerated leaf senescence (Verslues et al., 2006). These negative effects are caused by induction of ABA synthesis in roots, its transportation to guard cells and closing of stomata (Bowne et al., 2012) leading to decreased photosynthesis, photo-inhibition and oxidative stress (Fraire-Velázquez and Balderas-Hernández, 2013). In return, the plant response depends on the genotype, developmental stage, type of cells and tissues, and duration and severity of stress (Barnabás et al., 2008). Therefore, to cope with unfavourable conditions, plants have evolved mechanisms which allow them to maintain their productivity and/or survival (Rowley and Mockler, 2011) such as osmotic adjustment (OA) which has been observed in plants such as cereals (González et al., 2008; Norouzi et al., 2008), canola (Norouzi et al., 2008) and maize (Hajlaoui et al., 2010).

Conventionally, to investigate the drought stress response, only a few metabolites were measured as key role players, while at present, metabolomics allows a less biased metabolite profiling of the response to identify contributed metabolites simultaneously (Fraire-Velázquez and Balderas-Hernández, 2013). Metabolomics has revealed ABA-dependent and ABA-independent pathways in response to dehydration stress (Yamaguchi-Shinozaki and Shinozaki, 2006). Metabolite profiling indicated that ABA concentration increases during dehydration and causes accumulation of sugars (like glucose and fructose) and amino acids (BCAAs branch-chain amino acids, saccaropine, proline, agmatine) (Urano et al., 2009). GC-TOF–MS, CE-MS in combination with transcriptomics revealed that during dehydration, ABA accumulated and regulated accumulation of sugars such as glucose and fructose and various amino acids (Urano et al., 2009). Metabolite profiling of *Eucalyptus* using GC/MS showed drought stress affected 30-40% of drought sensitive plant metabolites, and 10-20% of drought tolerant plant metabolites. In the *Eucalyptus* drought response, the accumulation of shikimic acid and two cyclohexanepentol stereoisomers was reported for the first time (Warren et al., 2012). Likewise, comparative analysis of soybean drought tolerant and sensitive cultivars using ^{1}HNMR showed different responses of genotypes to water stress. Not only did none of the cultivars accumulate the osmolytes, but also they differentially changed 2-oxoglutaric acid, pinitol, and allantoin suggesting their role as osmoprotectants (Silvente et al., 2012). Targeted metabolite profiling of three bread wheat cultivars using GC/MS demonstrated an increase of amino acids (proline, tryptophan, leucine, isoleucine and valine) as well as reduction in organic acids (Bowne et al., 2012).

1.4. Stress studies in the Thyme genus

There are limited studies on stress on the *Thymus* genus, and the results and achievements are summarised here. Letchamo et al. (1993) investigated three different soil water levels and two light regimes on two clonally selected *T. vulgaris*. Maximum plant yield and essential oil production was at 70 % soil water level for the two selections grown under supplemental lighting (Letchamo et al., 1994). In another study, Jordan et al. (2003) studied effects of 4 watering levels (20%, 40%, 60% and 80%) on *Thymus hyemalis* Lange. There were no significant differences in essential oil yield percentages between watering levels. Using GC-MS analysis, they showed that winter harvesting, with 40% watering gave the highest proportion of thymol (25%) but in spring harvesting the highest concentration was

for 80% watering level (the highest level) (Jordan et al., 2003). Another study on *Thymus zygis* subsp. *gracilis* was carried out on the basis of drought effect on phytomass production and essential oil quality. They had three different watering levels 63, 44, and 30% of the local potential evapotranspiration (Eto). Maximum plant dry matter production and essential oil yield was at 44% Eto, and the greatest thymol concentrations were obtained under the 30 and 44% Eto watering levels (Sotomayor et al., 2004). Babaee et al. studied the effect of 4 different levels of watering (100% field capacity), mild stress (85% field capacity), medium stress (70% field capacity) and severe stress (55% field capacity) on *Thymus vulgaris* growth, yield, one amino acid (proline) and oil composition of thyme (thymol). Water stress decreased plant height, the number of secondary branches, dry and fresh weight growth and root mass, dry and fresh weight of the root and length of the root. In addition, thymol percentage and proline content increased with severe stress (55% field capacity). Yield of the secondary metabolites proline and thymol were observed with water deficit changes (Babaee et al., 2010). Recently the impact of irrigation level on the morpho-physiology and the essential oil content of *Thymus daenensis* was investigated. With the increasing of water stress severity, they observed limited growth, herbage yield, chlorophyll and carotenoid levels, but elevated levels of proline, K^+, irrigation water use efficiency and essential oil content (Bahreininejad et al., 2013).

1.5. Aims

During a plant's life, there are many factors that affect growth, development and productivity (Levitt, 1972). These factors include: high or low temperature, high/low light, ozone, elevated CO_2, increased salt (Goyal et al. 2005) and lack of water (Valliyodan and Nguyen, 2006; Bohnert et al., 2006; Bohnert and Jensen, 1996). Water is arguably the most important environmental factor, as it is the main constituent molecule of most organisms. Lack of water will cause reduced growth rate due to lowering cell division and expansion (Skirycz et al., 2010; Skirycz and Inzé, 2010; Aguirrezabal et al., 2006; Pereyra-Irujo et al., 2008) and decrease carbon accumulation (Tardieu et al., 2011). Water stress is the main limiting environmental factor in most of the areas in the world (Valliyodan and Nguyen, 2006), and various investigations have been performed in several plant species.

In genetics and transcriptomics studies under water deficit stress conditions, a huge number of genes (QTLs) (Street et al., 2006; Kato et al., 2008; Mathews et al., 2008; Keppler and

Showalter, 2010; Ruan and Teixeira da Silva, 2010; Liu et al., 2011; Xue et al., 2011) and transcript changes (Ozturk et al., 2002; Gorantla et al., 2005; Talamè et al., 2007; Deyholos, 2010; Díaz et al., 2010; Yang et al., 2010) have been observed. Likewise, using proteomics, massive protein alterations (Hajheidari et al., 2007; Caruso et al., 2009; Peng et al., 2009; Kamal et al., 2010; Bazargani et al., 2011; Yang et al., 2011) have been reported. All these techniques can give important insights. However they have limitations. For example, mRNA up-regulation does not always lead to protein level increase (Gygi et al., 1999). Moreover, not all the proteins translated are functional (Sumner et al., 2003). However, study of changes in metabolites as the end-product of gene expression and protein translation can provide an alternative approach, and is important to understand the perturbations of metabolic pathways occurring during abiotic stresses such as drought. Even though some knowledge is available (Bhargava and Sawant, 2013), but current knowledge concerning the physiological and biochemical mechanisms of the adaptation of thyme to water deficit is not sufficient. Therefore, understanding the reaction of different populations of thyme in the metabolome to water deficit stress was the aim of this study. Most of the previous investigations on drought stress of thyme have focused on one or two species with few physiological and morphological traits along with a few commercial volatile compounds. Instead, the main objective of this project was basically metabolite profiling by FT-ICR Mass Spectrometry along with some basic physiological indices in *Thymus spp*. Currently, many questions are unresolved concerning response of thyme to drought stress at the metabolome level such as which metabolites are impaired by dehydration and how thyme responds to unfavourable conditions and finally what are the main mechanisms employed by drought stress tolerant thyme compared to sensitive population.

To achieve these objectives, this project has used a state-of-the-art approach, metabolomics, as this technique has been successfully utilized to study water deficit stress (Shulaev et al., 2008; Brosché et al., 2005; Cramer et al., 2007; Kim et al., 2007) particularly in comparative studies (Warren et al., 2012; Sanchez et al., 2012b; Silvente et al., 2012; Foito et al., 2009).

CHAPTER II. MATERIALS AND METHODS

2.1. Plant materials

The plant materials examined included eleven populations within 6 different species listed in the *Thymus* genus (Table 2.1).

Table 2.1. Plant materials collected from different countries during 2010.

No	Species	Origin	Provided by
1	*Thymus daenensis* (IR)	Iran	RIFR*
2	*T. kotchyanous (IR)*	Iran	RIFR
3	*T. vulgaris* (IR)	Iran	RIFR
4	*T. vulgaris* (GR)	Germany	Humber VHB®
5	*T. serpyllum*	Europe	Ball®
6	*T. serpyllum*	Spain	Semillas Silvestres®
7	*Thymbra capitata* (SP)	Spain	Semillas Silvestres®
8	*T. vulgaris* (SP)	Spain	Semillas Silvestres®
9	*T. zygis (SP)*	Spain	Semillas Silvestres®
10	*T. vulgaris* (Varico2)	Switzerland	Agroscope ACW
11	*T. vulgaris* (Varico3)	Switzerland	Agroscope ACW

* RIFR : Research Institute of Forest and Rangelands- Iran

2.2. Physiological experiments

2.2.1. Effect of drought on germination

To screen populations and determine their tolerance to drought during the germination phase, PEG6000 (Poly Ethylene Glycol 6000 MW) was used to make different osmotic potential solutions. The experiment was conducted in a Factorial design based on Completely Randomized Design (CRD) with populations as a main factor and PEG levels as a sub-factor (Steel and Torrie, 1980). The nine populations were *T. daenensis* (IR), *T.*

kotchyanous (IR), *T. vulgaris* (UK), *T. vulgaris* (Germany), *T. serpyllum* (Europe Union), *T. serpyllum* (Europe Union), *T. serpyllum* (Spain), *T. vulgaris* (Spain), *T. zygis* (SP). Four water stress treatments used were 0 bar, -2 bar PEG, -4 bar PEG and -6 bar PEG. Seeds were sown in petri dishes (9 populations, 4 PEG treatments with 3 replicates) on filter paper; each Petri dish containing 20 seeds of each population. Petri dishes were wetted (2 ml for each petri) with one of the four treatments. PEG solutions were made up according to a previous study (Michel and Kaufmann, 1973).

To calculate the PEG required making up the solution I used the formula:

$OP= (-1.18x10^{-2}) x C- (1.18x10^{-4}) x C + (2.67X10^{-4}) x C x T + (8.39xl0^{-7}) x C^2T$, where C=PEG concentration, T=Temperature (Michel and Kaufmann, 1973).

After sealing with lab film, dishes were placed in a growth room at 22 ^{o}C. After two days the number of germinated seeds was counted daily. After 10 days, the traits of fresh weight, dry weight radicle and plumule length were recorded. Dry weight was measured after drying at 70 degrees Celsius for 48 hours.

2.2.2. Effect of water stress at the early vegetative growth stage

Seeds were sown in 8cm diameter pots containing a soil mixture of 4 parts Humax Multipurpose peat based compost mixed in 1 part Perlite with Intercept 70wg insecticide added at 0.02g/l compost (about 120 g soil in each pot) and placed in the growth room with a 16:8 light/dark cycle at 22^{o}C and watered weekly with tap water. Drought stress was applied by withholding water from the 30th day. Growth, survivability, shoot water potential, water content and root to shoot ratio were recorded before, during and after drought treatment. To measure survivability, plant status was recorded daily after withholding water according to the following index: 5 (fresh plants), 4 (some leaves lost due to wilting), 3 (some leaves with dried tips), 2 (more than half the plant dried) and 1 (completely dried). Soil moisture levels were monitored daily using an HH2 Moisture meter, model SM300 by Delta-T Devices Ltd. The SM300 measures volumetric soil moisture content with 2.5% accuracy. Soil moisture was recorded at a sensor depth of 5 cm.

Shoot water potential was measured using a pressure chamber (Skye Company Model SKPM 1400) on shoots of about 10-30 cm length. To record the water potential, stems were cut

and sealed into the chamber. Chamber pressure was increased at a rate of bar (3-10 bar) per minute until sap was observed exuding from the cut point of the stem. This pressure was recorded as the equivalent of the xylem tension, and so the water potential, present before shoot excision. For a detailed description see Appendix 1.

2.3. Metabolite profiling experiments

2.3.1. Tissue harvesting and sample extraction for metabolite profiling

For metabolite profiling, plants were harvested (similar ages of leaves on one plant cut with scissors), flash frozen with liquid nitrogen, then weighed and returned back onto liquid nitrogen and stored in the -70^{o}C freezer. Six biological replicates per daily sample were freeze-dried for 48 hours. Freeze-dried samples were weighed and stored at -70^{o}C until extraction. The weight of dried samples ranged between 3 to 10 mg.

For extraction, a Methanol: Chloroform protocol was used, for very small and dried samples (<=10mg). To do this, first 32 µl MeOH and 12.8µl water per mg tissue were added to leaf sample and homogenised by a Precellys 24 homogeniser (Bertin Technologies Ltd, USA). Next, 32 µl $CHCl_3$ and 16µl Water were added and the mixture centrifuged. Each fraction of this 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 the steam of dried nitrogen gas. The dried extracts stored in the -70^{o}C freezer until mass spectrometry analysis took place.

2.3.2. FT-ICR Mass spectrometry

Prior to loading samples, dried samples were resuspended in dilution solution made up of 80:20 MeOH:H_2O (both HPLC grade) added with 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 Eppendorf 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.

2.3.3. Data analysis

Pre-processing. Raw data was exported from Xcalibar (Version 2.0.7 Thermo Scientific) to MATLAB ver.7 (The Mathwork Inc., Natick, MA) and subjected to custom-written code including sum of transient files and their process (Southam et al., 2007). These processes have been described already by Southam et al., 2007, but briefly comprise averaging the triplicate transients, Hanning apodisation, zero filling once, and application of a fast Fourier transformation. Then, processed transient data files were submitted to custom written codes in MATLAB (SIM-stitch algorithm version 2.8). In this way, SIM windows were aligned together with existence peaks in the overlap region, then all the peaks with SNR (Signal to Noise Ratio) lower than 3.5 were removed. In addition, some known detected compounds (minimum three peaks for each mass spectrum) have been used to calibrate spectra (Southam et al., 2007). Three more MATLAB scripts were applied to the dataset, which referred to peak filtering (Payne et al., 2009). The first script, namely Replicate Filter, rejected all the peaks detected in just one replicate. In other words, peaks observed in at least two replicates were kept. The second script, called Flag Blank Peaks, removed all the peaks in the blank sample except for the peaks with double intensity of the same peak in biological samples. The third script, namely Sample Filter, retained peaks that presented in a minimum of 50% of all the samples (Payne et al., 2009; Taylor et al., 2009). At this stage, a peak list and 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.

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). These rules briefly are: (1) Restriction of elements, (2) Lewis and Senior check (Taiz and Zeiger, 2002), Isotopic pattern filter, (4) H/C element ratio check, (5) Heteroatom ratio check, (6) Element probability check and (7) TMS check. As a summary, all the possible empirical formula ($C_C\ H_H\ N_N\ O_O\ P_P\ S_S$) considering elements ($^{12}C$ = 0–34, ^{1}H = 0–72, ^{14}N = 0–15, ^{16}O = 0–19, ^{31}P = 0–4 and ^{32}S = 0–3) were calculated. While detected peaks corresponded to adducts of neutral metabolites (charged molecular ions), elemental composition added by adduct mass of the seven most relevant positive ions i.e. $M\text{-}e^+$, $M+H^+$, $M+Na^+$, $M+^{39}K^+$, $M+^{41}K^+$, $M+2Na\text{-}H^+$, $M+2\ ^{39}K\text{-}H^+$. Finally all possible formulas were filtered to select the most accurate and correct elemental formula using rules mentioned above (Kind and Fiehn, 2007; Weber and Viant, 2010; Taylor et al., 2009; Allwood et al., 2012). It must be noted that, despite the high mass acuuracy, one mass may linked to different elemental formula, or even similar formula but different structures. Hence, in this thesis 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 can not distinguish between these isomers.

Statistical analysis. Prior to multivariate statistical analysis, datasets were processed by auto-scaling i.e. the mean of each spectrum was centred and divided by its standard deviation (Goodacre et al., 2007). Then PCA (Principal Component Analysis) was performed on the dataset using MATLAB software, PLS Toolbox.

2.4. Volatile profiling experiments

2.4.1. Experimental design

Seeds of *Thymus vulgaris* (drought sensitive) and *Thymus serpyllum* (drought tolerant) were grown in the condition described in section 2.2.2. 30-day old plants were treated with

drought stress at the vegetative stage by withholding water until soil moisture reached nearly zero (soil moisture measurement described in the next section). A set of plants with regular watering until the end of experiment were considered as control plants (watered plants). Morpho-physiological parameters were recorded at 4 day intervals from day 0 to the end (Figure 2.1).

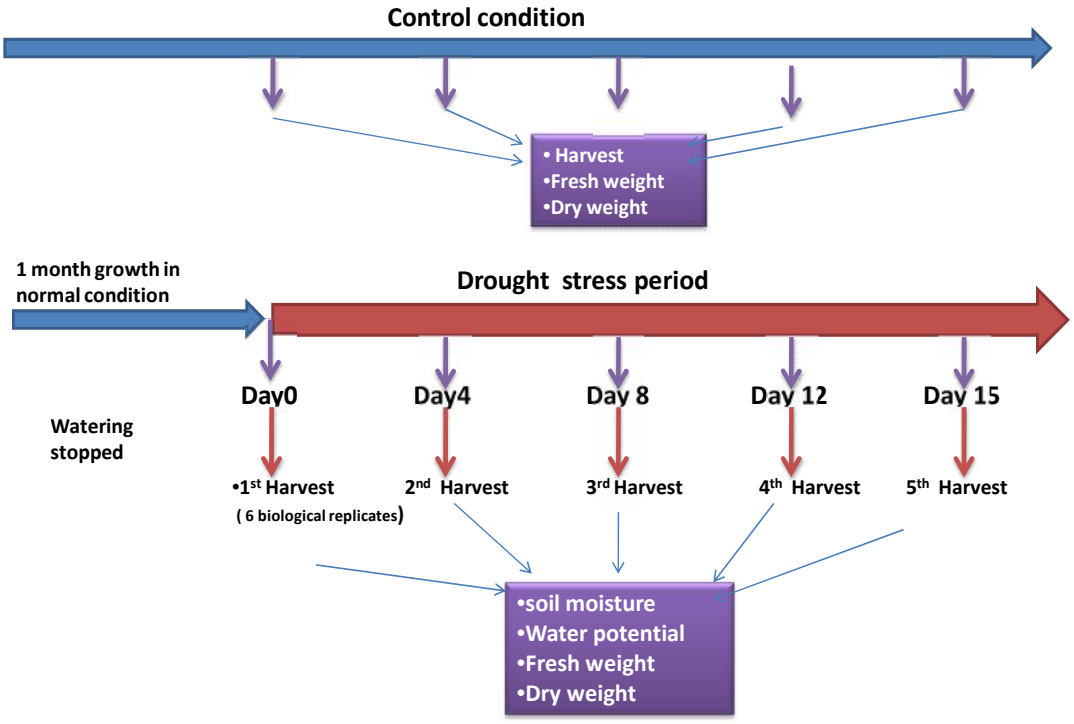


Figure 2.1. Experimental design to conduct the comparative study of volatiles along with physiological parameters in sensitive and tolerant thyme.

Thirty-day old plants (considered as droughted plants) were treated with drought stress at the vegetative stage by withholding water until soil moisture reached nearly zero. A set of plants with regular watering until the end of the experiment were considered as control plants (watered plants). Morpho-physiological parameters been recorded at 4 days intervals from day 0 to the end. Similar ages of leaves on individual plants harvested every four days for volatile profiling.

2.4.2. Sampling and extraction procedure for volatile profiling

From the first day of withholding water until day 15, plants were harvested every 4 days (similar ages of leaves on one plant cut with scissors), flash frozen with liquid nitrogen, then weighed and subjected to liquid nitrogen and stored in the -70^{o}C freezer. Six biological replicates per daily sample were at -70^{o}C until extraction. The weight of fresh samples

ranged between 30 to 100 mg. To do extraction, a modified liquid extraction method was used. Samples were taken from the freezer and put in liquid nitrogen immediately. The leaves were ground by pestle in microfuge tubes, and returned to liquid nitrogen tubes again. After weighing the samples 1 ml hexane including 10ng/µl internal standard (Benzyl Acetate) were added to each 1.5 ml eppendorf 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.

2.4.3. 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 with 20 m long, 0.180 mm id and 0.18 µm film thickness (Hewlett Packard, Palo Alto, CA) at 40 ^{o}C for 3 min and then raised at 30 ^{o}C min^{-1} to 250 ^{o}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 delay at 1597 eV at 20 scans per second. The mass range was 50-350 atomic mass units.

2.4.4. Volatile identification and quantification

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) and nine external standards including α-phellandrene, myrcene, α-terpinene, β-phellandrene, Cis,β-ocimene, γ-terpinene, terpinolene, linalool, α-humulene as

previously described (Kant et al., 2004). For each sample, five technical replicates were run by GC/MS.

CHAPTER III. PHYSIOLOGICAL RESPONSES OF DIFFERENT THYME POPULATIONS TO WATER DEFICIT DURING THE GERMINATION AND EARLY GROWTH STAGES

3.1. Introduction

There are limited studies on the effects of stress in *Thymus* species. Jordan et al. (2003) studied the effects of 4 watering levels on *Thymus hyemalis* Lange, showing no significant differences in essential oil yields between watering levels imposed by controlled irrigation (Jordan et al., 2003). Another study on *Thymus zygis* subsp. *gracilis* was carried out to study the effect of drought on biomass production and essential oil quality. Maximum plant dry matter production, essential oil yield and thymol concentrations were achieved with a moderate (30% and 44% evapotranspiration) watering level (Sotomayor et al., 2004). Babaee et al. (2010) studied the effects of 4 different watering levels. Water stress decreased plant height, number of secondary branches, dry and fresh weight, growth and root mass, dry and fresh weight and length of roots, and increased thymol percentage and proline content (Babaee et al., 2010). Letchamo et al. (1994) investigated three different soil water levels (50, 70 and 90% of field capacity) and two light regimes (natural light and natural light supplemented by a PPF of 200 µmol $m^{-2}s^{-1}$) on two clones of *T. vulgaris*. Maximum plant yield and essential oil yield was achieved at 70% soil water level for both clones when grown under supplemental lighting (Letchamo et al., 1994; Letchamo and Gosselin, 1995). Considering the effect of drought on germination, PEG has been used to screen different populations and varieties in plants such as wheat (Baalbaki et al., 1999), common bean (Hucl, 1993), barley (Al-Karaki, 1998) and pea (Okcu et al., 2005). For *Thymus* species, Bagheri et al. (2011) and Khoshsokhan et al. (2012) used PEG 6000 (molecular weight) to compare seed germination under water stress for *T. kotschyanus* and *T. daenensis* species (Khoshsokhan, 2012; Bagheri et al., 2011). They found a significant decline in the germination of both populations, but their results had some conflicts. However, previous studies demonstrated that a certain degree of drought stress is beneficial to essential oil production in thyme (Selmar, 2008). Moreover, there is a clear variation between the water stress tolerance of different species at least when considering germination.

Understanding the response of plants to water deficit is of great importance, providing information to improve drought tolerance, particularly in screening germplasm for useful variation (Reddy et al., 2004). In this chapter I aimed to evaluate the drought tolerance of a range of thyme germplasm by examining morphological and physiological changes following

water stress during germination and the early vegetative growth phase. The germplasm examined was chosen because of its economic importance within the genus. I also aimed to determine the mechanism(s) of drought tolerance within thyme populations.

3.2. Results

3.2.1. Germination phase

Nine populations of thyme were tested for germination under 4 different levels of stress. Imposing drought using PEG had a significant effect on all traits studied (Figure 3.1). Radicle/plumule ratio increased in the -2 and -4 bar treatments compared to unstressed controls. Fresh and dry weight increased at -2 bar increased but decreased at -4 and -6 bar. Despite the apparently small increases, these differences were all significant statistically. According to analysis of variance (was carried out in Factorial design) as shown in Appendix 3, there were significant differences between the populations for all traits ($p<0.01$). Subsequently, the treatment means were compared with Duncan's New Multiple Range Test.

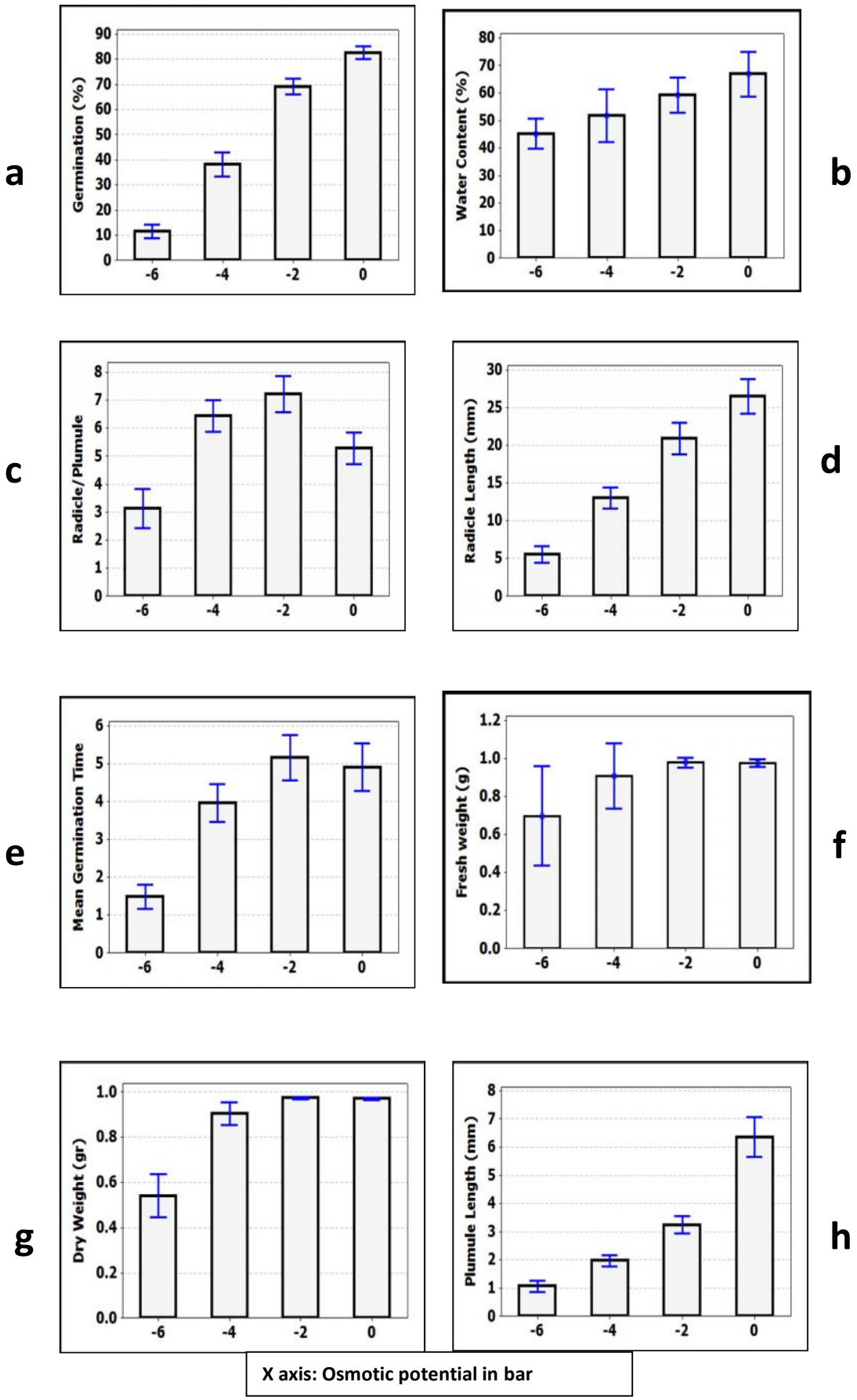
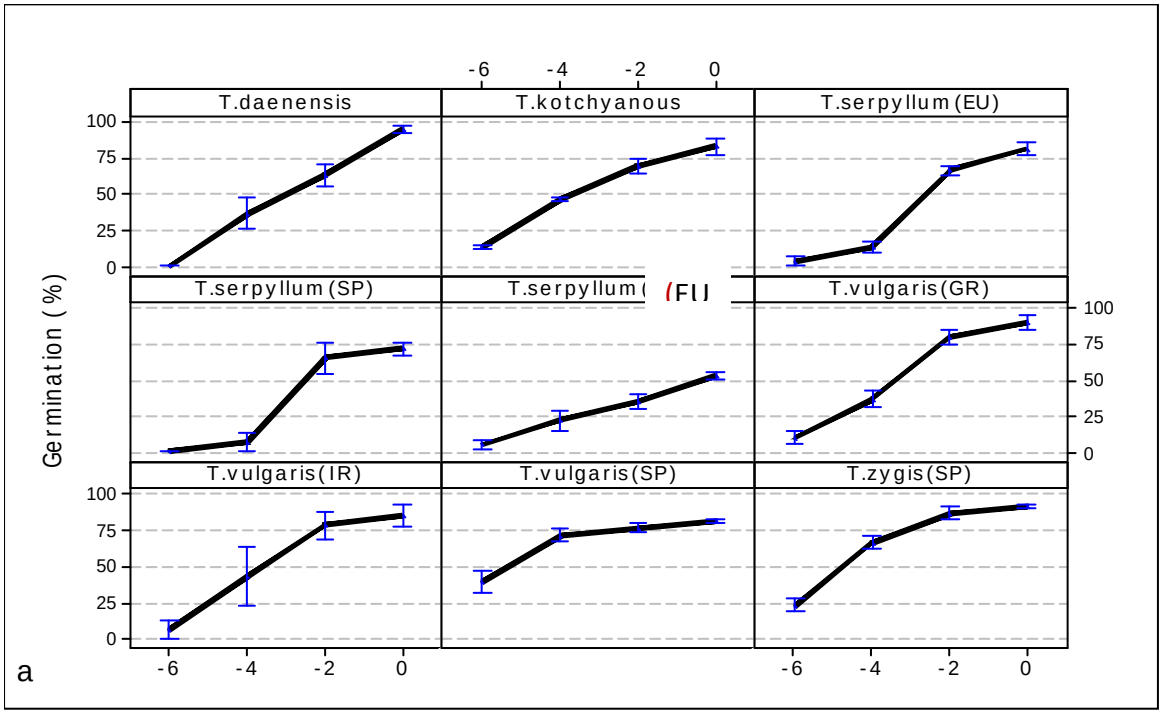
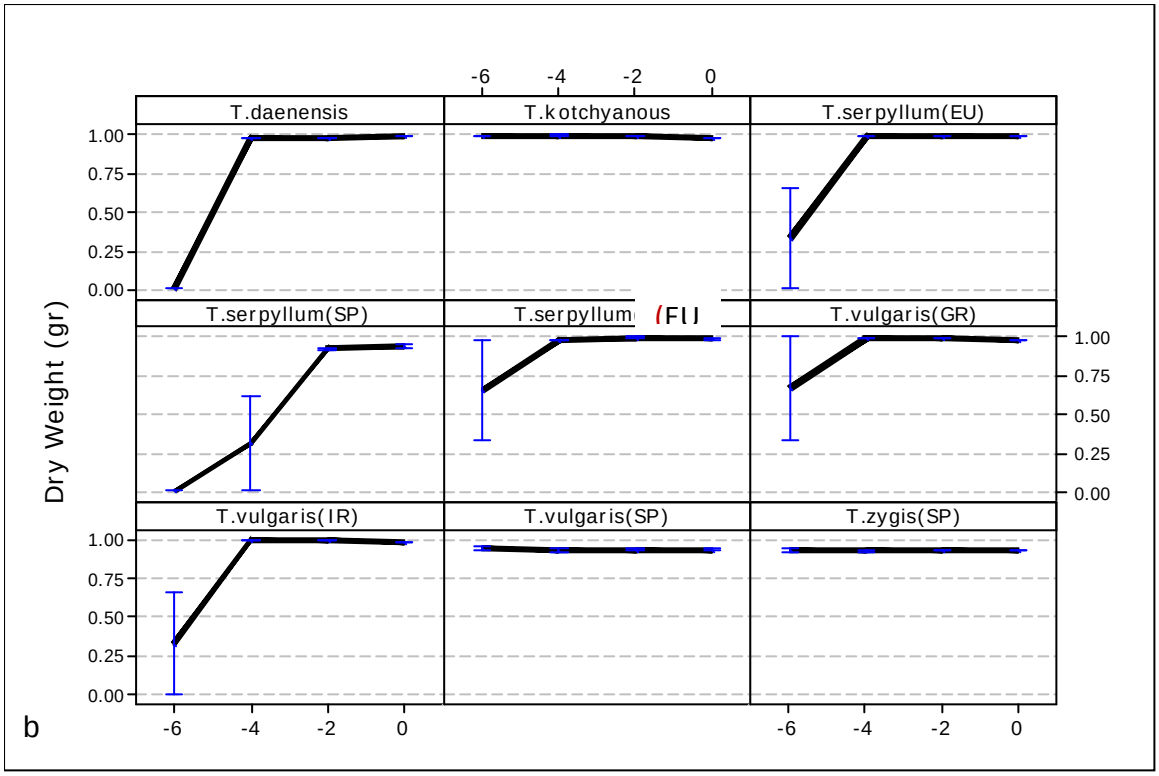


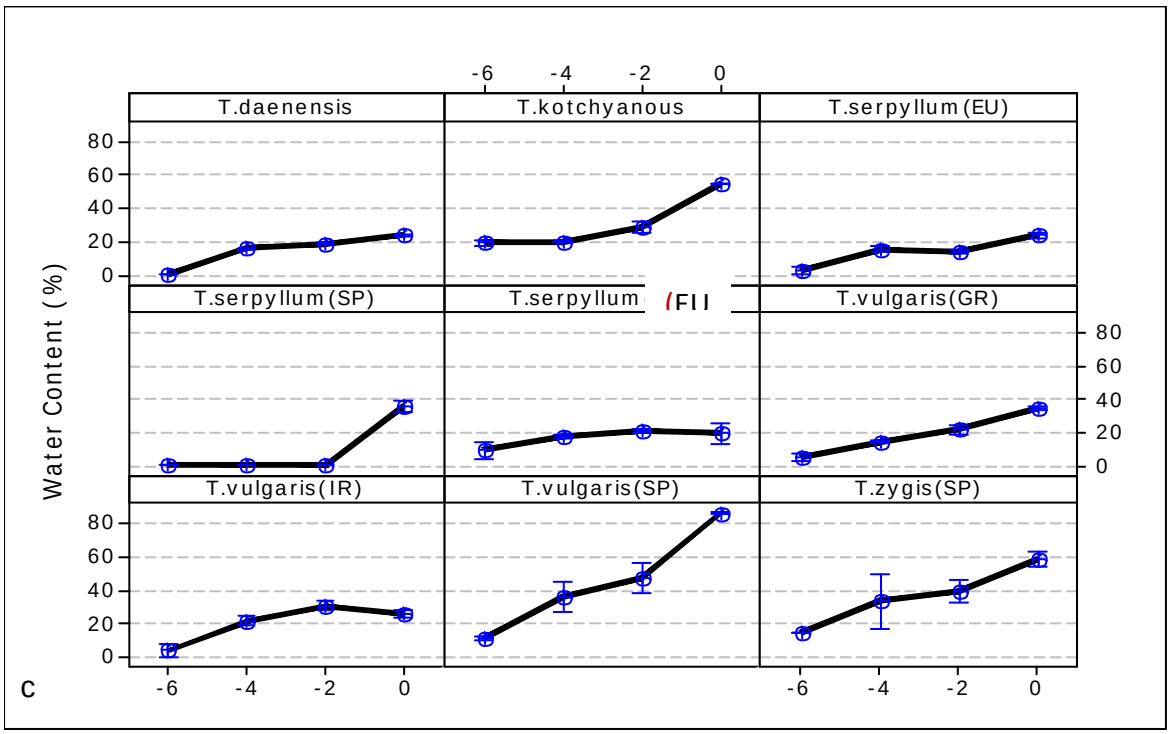
Figure 3.1. Four levels of PEG induce changes in all 8 recorded morpho-physiological traits across the nine thyme populations.

a) Germination percentage % b) Water content % c) Radicle/Plumule ratio d) Radicle length (mm) e) Mean Germination Time f) Fresh Weight (g) g) Dry Weight (g) h) Plumule Length (mm). Nine populations of thyme were exposed to 3 osmotic potential levels by different PEG concentrations, which made mild stress (-2 bar), moderate stress (-4 bar) and severe stress (-6 bar). These graphs show the means of traits affected by stress compared to the control condition. Fresh weight and dry weight did not alter at -2 bar under control conditions, but decreased in moderate and severe stress. Mean germination time (MGT) and Radicle/Plumule increased at -2 and -4 bar and decreased under severe osmotic stress. Other traits declined along with stress dose increase. X-axis shows stress levels in bar, where 0 is control and -6 bar is the highest level of osmotic stress. Error bar are means ± SEM.

Because there was a significant interaction (different behaviour of populations to treatment levels), statistical comparisons were not performed. However, for an overall view of the response of the different populations, the mean response of traits to different PEG levels is shown in Figure 3.2.




T.daenensis
T.kotchyanous
T.serpyllum(EU)
T.serpyllum(SP)
T.serpyllum
T.vulgaris(GR)
T.vulgaris(IR)
T.vulgaris(SP)
T.zygis(SP)
Dry Weight (gr)
1.00
0.75
0.50
0.25
0.00
-6
-4
-2
0
b


T.daenensis
T.kotchyanous
T.serpyllum(EU)
T.serpyllum(SP)
T.serpyllum
T.vulgaris(GR)
T.vulgaris(IR)
T.vulgaris(SP)
T.zygis(SP)
Water Content (%)
80
60
40
20
0
-6
-4
-2
0
c

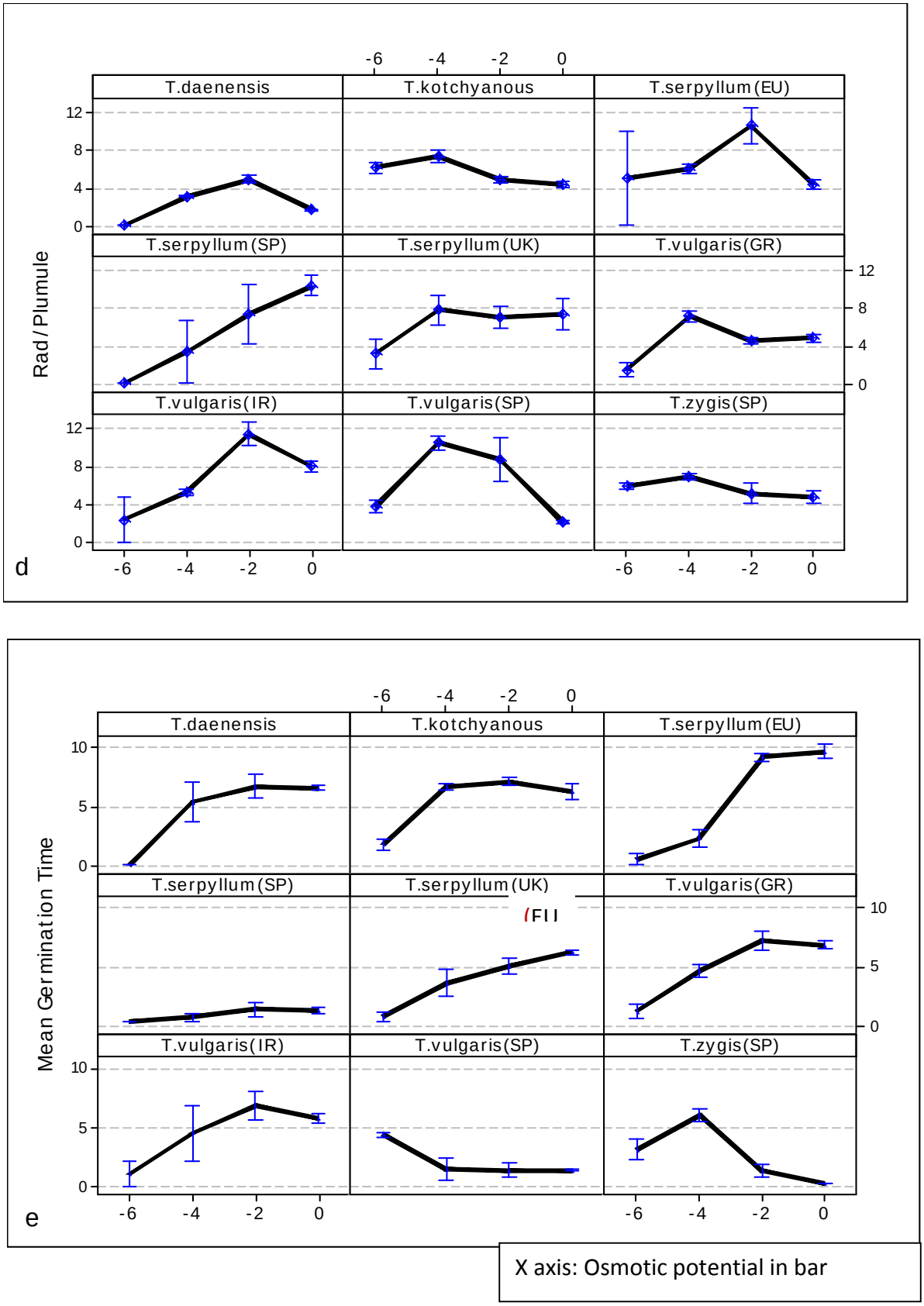


Figure 3.2. Effect of water deficit stress on germination and seedling traits in nine populations of thyme.

a) Germination percentage (%): Germination percentage decreased with increasing osmotic potential for all the species. However, *T. vulgaris* has almost the same germination as the control at -2 bar and *T. serpyllum* (SP) has the same reaction. b) Dry weight (g): There is no difference among stressed and control plants in *T. kotchyanous (IR)*, *T. zygis* and *T. vulgaris* (SP). Other species had similar dry weight at 0, -2 and -4 bar, then dropped down at -6 bar, except for *T. serpyllum* (SP), which had the same dry weight at 0 and -2 bar, which dropped in -4 bar and was unchanged for -6 bar. c) Water content (%): in all species the water content declined steadily, except for *T. serpyllum* (EU) and *T. vulgaris* (IR) which had a slightly increased at -2 bar then a decrease. d) Radicle/Plumule ratio: water stress increased this ratio in all species, but only in *T. serpyllum* (SP) it declined sharply. e) Mean Germination Time: there were no significant difference between the control and -2 bar, but with increasing stress to -4 bar, *T. zygis (SP)* increased the MGT, while some species such as *T. serpyllum* (EU) decreased MGT significantly, but the others had rather similar MGT util –4 bar. In the -6 bar treatment, *T. vulgaris* (SP) increased the MGT but the others decreased. The X-axis shows osmotic potential in bar and 0 is control condition without any PEG. Error bars are ± 1 SEM. Number of replicates varies from 3-15.

Percentage germination decreased with decreasing osmotic potential for all species. However some qualitative differences were noted. *T. vulgaris* and *T. zygis* (SP) species had almost the same germination as the control at -2 bar. No difference in dry weight was observed among stressed and control plants in *T. kotchyanous (IR)*, *T. zygis* and *T. vulgaris* (SP). The other species had similar dry weight at 0, -2 and -4 bar, but all decreased at a stress of -6 bar, except for *T. serpyllum* (SP). For water content, all species decreased their water content except for *T. serpyllum* (EU) and *T. vulgaris* (IR) both of which slightly increased at -2 bar then decreased as stress increased. Water stress increased radicle/plumule ratio in all species, apart from *T. serpyllum* (SP) in which it declined sharply. For Mean Germination Time, there were no significant differences between the control and -2 bar, but as stress increased to the -4 bar level, *T. zygis (SP)* had increased MGT. Some species such as *T. serpyllum* (EU) has significantly decreased MGT, but the others had similar MGT until a level of –4 bar. At -6 bar stress, *T. vulgaris* (SP) had increased MGT but all other species decreased.

3.2.1.1. Effect of Drought on Germination Percentage

There was considerable variation in germination between populations (Figure 3.3). Under severe water stress (-6 bar osmotic potential), *T. vulgaris* (SP) and *T. zygis* (SP) had the highest germination. In *T. daenensis* (IR) and *T. serpyllum* (SP) there was no germination at -6 bar stress. Other populations had intermediate germination. Treatment means comparison using DMRT (Duncan's New Multiple Range Test) in each population showed

that *T. vulgaris* (SP) was less affected by osmotic stress. In other words, it had the same germination at 0, -2 and -4 bar osmotic potential and lower germination at -6 bar level.

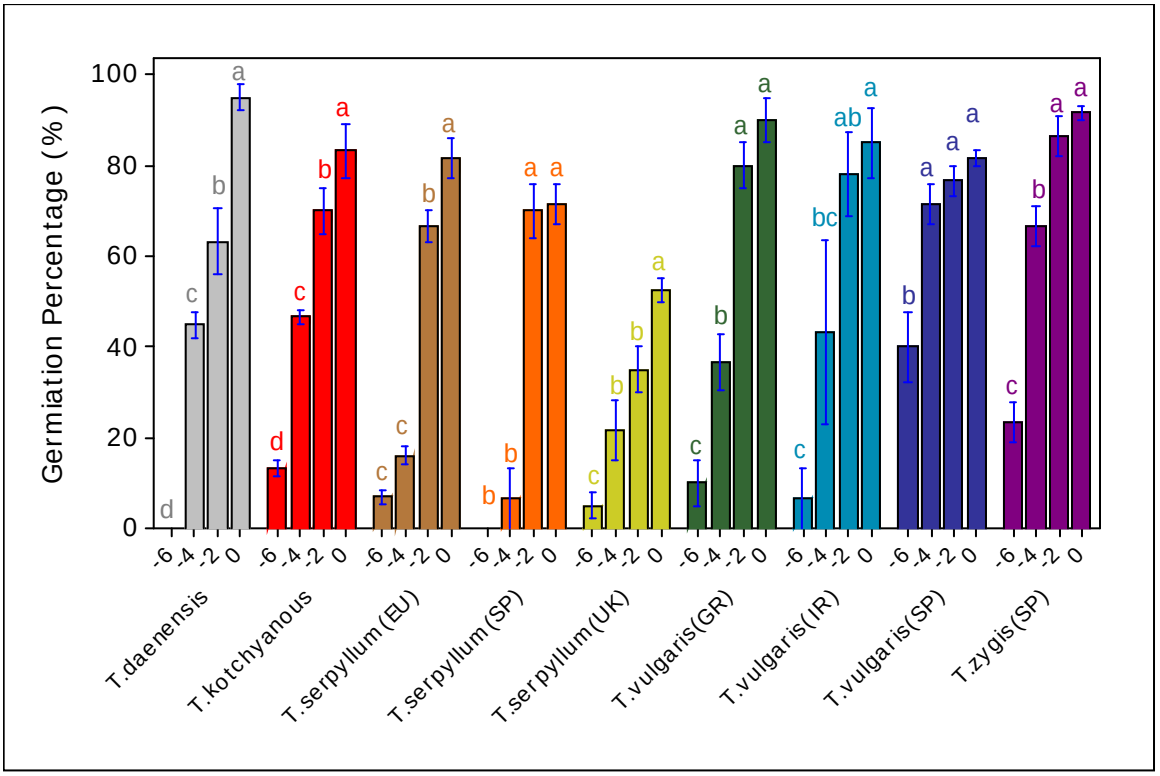


Figure 3.3. The nine thyme populations different responses to PEG levels at the germination phase.

Ten days after placing the seeds on filter paper, the percentage of germinated seeds (out of 20) was counted in 3 replicates. As indicated on the graph, *T. danensis* (IR) and *T. serpyllum* (SP) did not germinate at -6 bar osmotic potential. Importantly, *T. vulgaris (SP)* could germinate as well in -2 and -4 bar as in the control (0 bar/sterile water). There was no significant difference for the germination of this population at the applied levels of 0, -2 and -4 bar osmotic stress but a difference was seen at -6 bar. Error bars are Mean± SEM with 3 replicates and letters on the bar show the same group (i.e. means that are not significantly different from one another) within the same population shown with the same colors.

3.2.1.2. Monitoring a time course of germination.

Another important trait is how rapidly germination occurs and progresses, as if a population can germinate quickly and establishes roots, and then the seedling is more likely to avoid becoming droughted. Accordingly, percent germination was evaluated over time for seeds under control, mild, medium and severe water stress. In control conditions (without PEG) the trend of the populations indicated that most populations had the same behaviour (Figure 3.4). In other words, generally 60-90% of the seeds germinated during the first 3 days. However, *T. serpyllum* (EU) and *T. serpyllum* (UK) showed a different germination

profile. *T. serpyllum* (EU) and *T. serpyllum* (UK) seeds, gradually germinated (more slowly) over the nine days. This variation must be caused by species related traits, as all the environmental factors were the same. When osmotic potential decreased to -2 bar, seed germination displayed a different trend. At mild water stress of -2 bar, all populations were able to germinate, but the maximum germination obtained was less than 90%. Some populations, such as *T. vulgaris* (SP) had almost the same germination behaviour as the controls, so it can be defined as tolerant to this level of drought by this criterion. It seems that most of these plants could germinate at this level of PEG.

At moderate water stress, -4 bar, all populations showed some germination. However, the *T. serpyllum* (UK, SP, EU) populations were more affected than others, as they had the lowest germination at this level. Under more severe drought stress (-6 bar), *T. serpyllum* (SP) and *T. daenensis* (IR) showed no germination. In contrast, the populations of *T. vulgaris* (SP) and *T. zygis* (SP) had the highest germination among the populations studied at that level of stress.

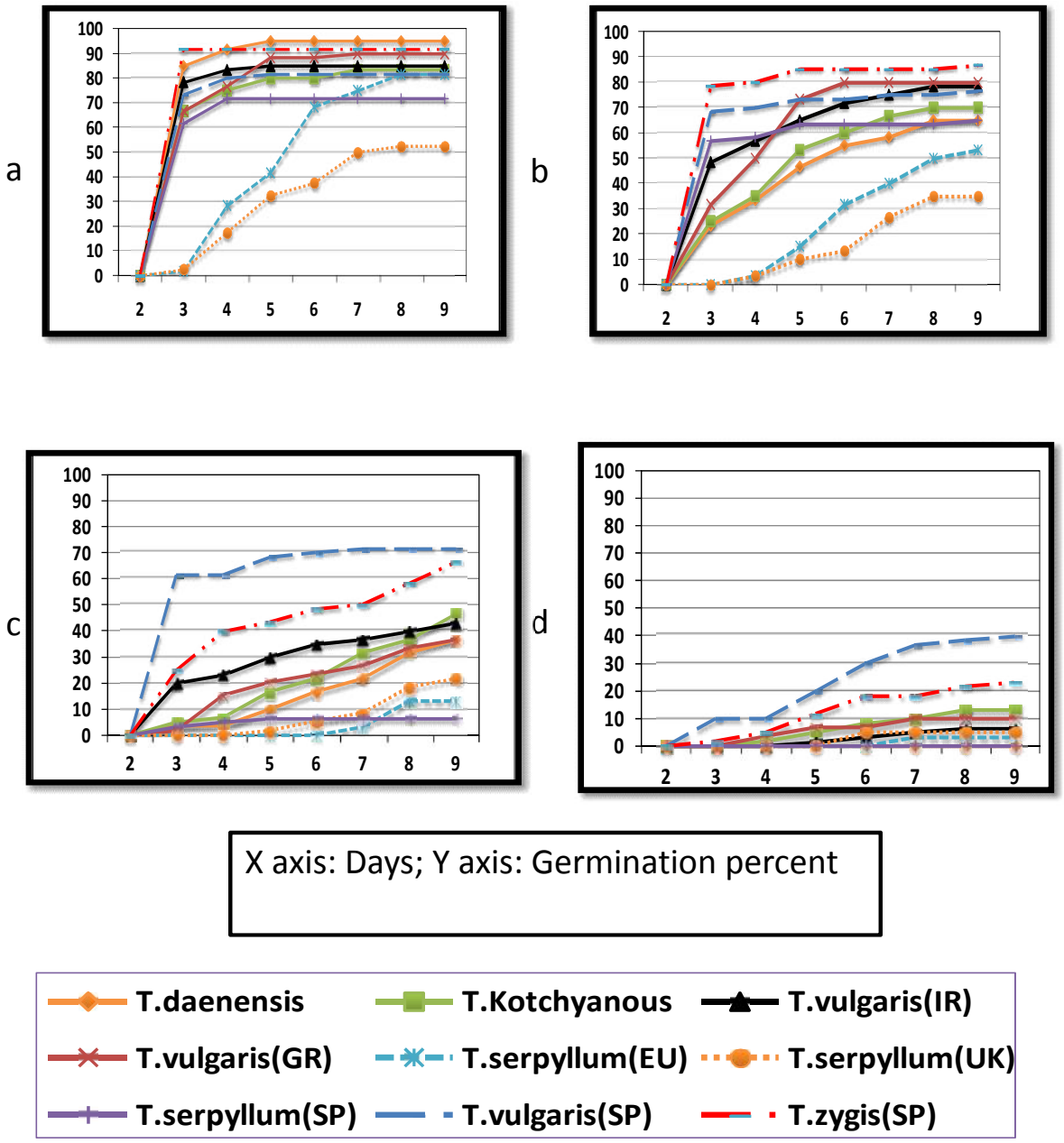


Figure 3.4. Monitoring germination percentage in 9 populations of thyme seeds at different levels of water stress.

a) Control condition (no PEG added). b) Osmotic potential -2 bar. c) Osmotic potential -4bar. d) Osmotic potential -6 bar. X axis shows the days after sowing the seeds on plate and Y axis indicates the counting number of germinated seeds on that day. In control conditions, most populations germinated about 40% during first 4 days, but they reached 50-95 % germination at the end. In -2 and -4 bar osmotic potential, the trend for germination was similar, with just *T. serpyllum* (EU, UK) still showing slower germination. In sever water stress (-6 bar) *T. vulgaris* (SP) and *T. zygis* (SP) had the highest germination, while *T daenensis* (IR) and *T. serpyllum* (SP) could not germinate at all.

3.2.1.3. Classification of populations based on early traits

To achieve a comprehensive analysis of each osmotic potential, cluster analysis was performed (Figure 3.5). This multivariate analysis was carried out using a Euclidean distance and Ward clustering method (Mohammadi and Prasanna, 2003; Ward JR, 1963). At an external water potential of -2 bar, 4 populations could be designated as tolerant: all (3 species) *T. serpyllum* and also *T. vulgaris* (IR). *T. zygis (SP)* and *T. vulgaris* (SP) were moderately tolerant while all the others could be classified as susceptible. A similar analysis was carried out at -4 bar stress but excluding traits Radicle/Plumule and Water content as they are product of linear combination of other traits. This analysis classified nine populations into 3 groups (Sensitive, Moderate and Drought tolerant). In this dendrogram, *T. serpyllum* (SP) was the most susceptible and *T. kotchyanous* (IR), *T. vulgaris* (SP), *T. zygis* (SP), *T. vulgaris* (GR) and *T. daenensis* (IR) were the most tolerant species. The others, namely *T. vulgaris* (IR), *T. serpyllum* (EU) and *T. serpyllum* (UK) could be classified as moderately tolerant. However, populations had a different response at osmotic stress levels. For instance, *T. daenensis* was sensitive at all levels, while *T. serpyllum* (SP) was tolerant at both -2 and -4 bar but appeared sensitive at -6 bar. Likewise, *T. vulgaris* (SP) was moderate at -2 and -4 bar but was placed in tolerant group at -6 bar. It seems every population had strategies against a specific level of osmotic stress. In other words, if it was tolerant at low levels, we can not predict it to be tolerant at higher levels and performing further experiments is necessary.

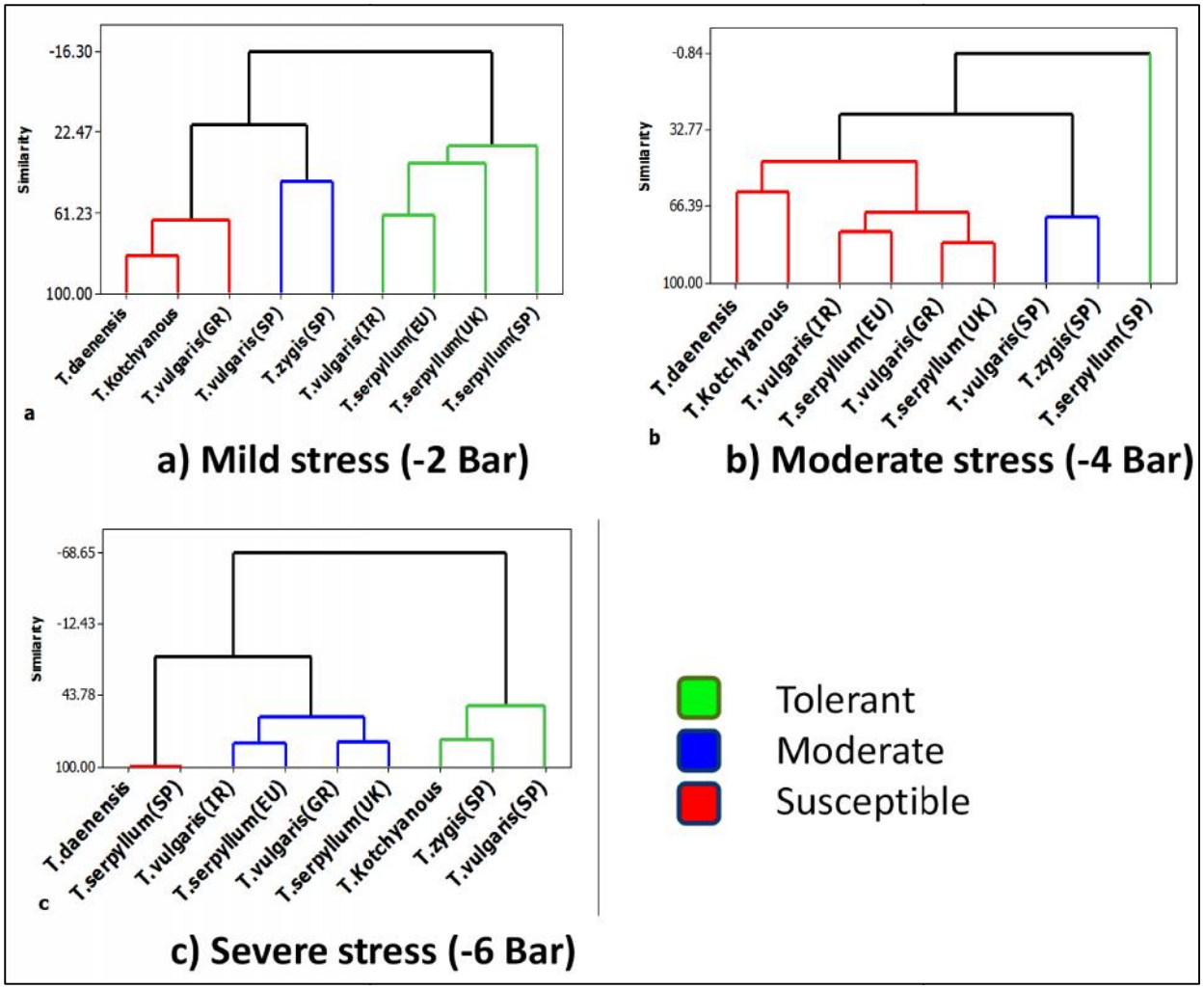


Figure 3.5. Classification of nine thyme populations using cluster analysis based on all traits recorded in 3 levels of osmotic potential.

X-axis shows the populations and Y-axis indicates the similarity between the individuals. To do this analysis, 6 traits were entered which were the following: fresh weight, germination percentage, radicle length, plumule length, radicle/plumule ratio and water content. Cluster analysis as a multivariate method was used to classify all the populations in mild stress conditions (a), moderate stress (b) and severe stress conditions (c). At a water stress of -2 bar, 4 populations were located in tolerant class, which were all *T. serpyllum* and *T. vulgaris* (IR). *T. zygis (SP)* and *T. vulgaris* (SP) was moderate and the others were susceptible. In moderate stress, *T. daenensis* (IR) and *T. serpyllum* (SP) were the most susceptible and *T. kotchyanous (IR)*, *T. vulgaris* (SP) and *T. zygis* (SP) were the most resistant species. In severe stress conditions, two populations namely *T. daenensis* (IR) and *T. serpyllum* (SP) did not germinate at all. Tolerant populations at this level were *T. kotchyanous (IR)* (IR), *T. zygis* (SP) and *T. vulgaris* (SP). The others were moderate.

Germination is multivariate and I recorded various traits, therefore to understand the underlying data structure and/or form, a smaller number of uncorrelated variables (for example, to avoid multicollinearity in regression), principal component analysis (Mártonfi et

al., 1994) was carried out on the -2, -4 and -6 bar data and results summarized as score plot of individuals for the first two principal components (Figure 3.6). The results of the PCA analysis agreed with the cluster analysis grouping.

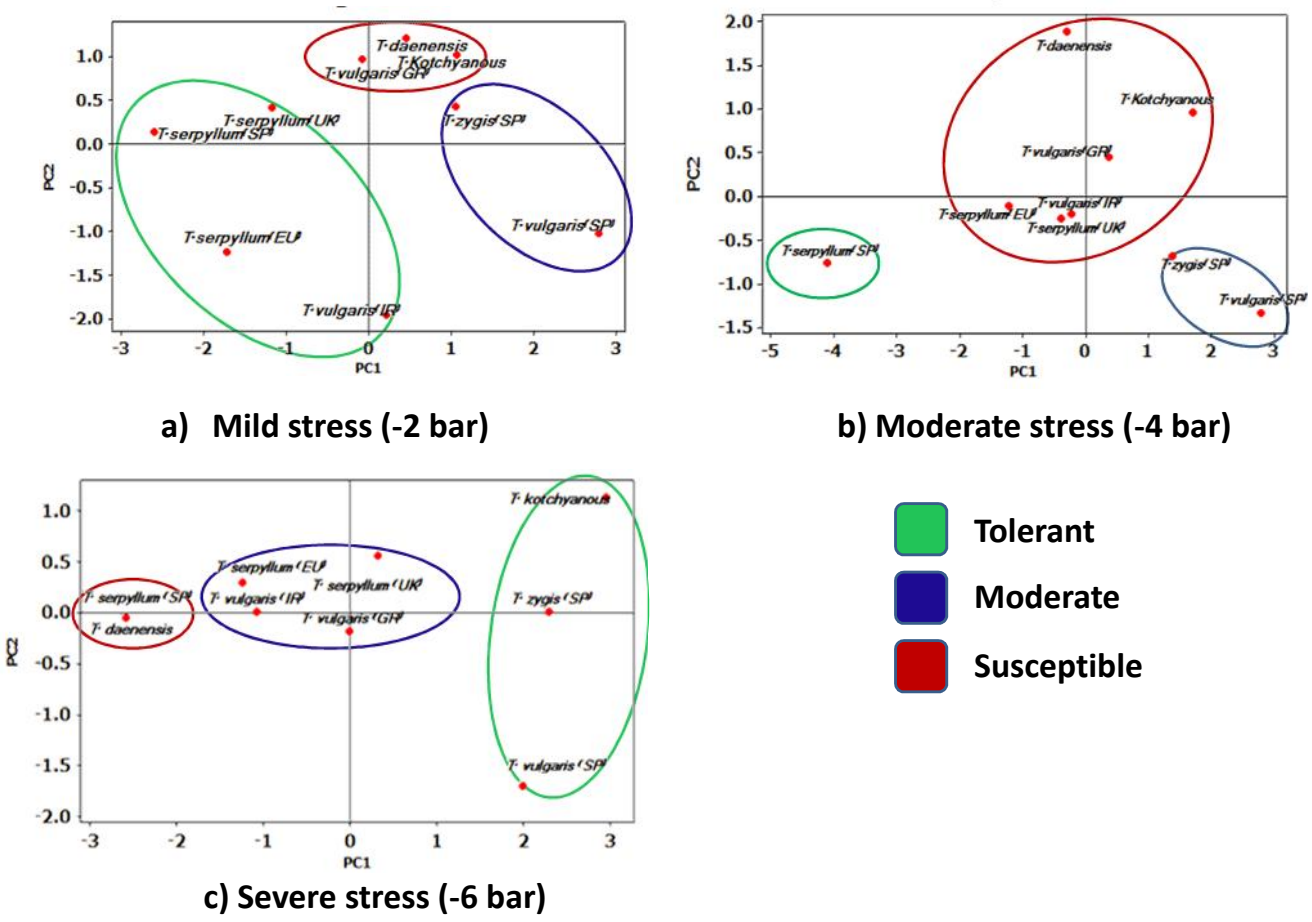


Figure 3.6. Principal component analysis of 9 populations of thyme based on 6 traits.

The traits for PCA analysis were: Water content, Germination percentage, Radicle length, Plumule length, Radicle/Plumule Ratio and Fresh weight. PCA as an unsupervised method breaks down the large data set to PC1 and PC2. PC1 described the largest variation in the data, which discriminates a tolerant group with large score of this component and susceptible group with small scores. PC2 has the second largest variation, orthogonal to PC1.

The traits involved in this analysis were water content, germination percentage, radicle length, plumule length, radicle/plumule ratio and fresh weight. PCA as an unsupervised method breaks down the large data set to PC1 and PC2. PC1 described the largest variation in the data, which discriminates a drought-tolerant group with a large score for this component and susceptible group with a small score. PC2 has the second largest variation, orthogonal to PC1. PC1, explaining 69% of total variation, clearly separated population

groups according to their tolerance. PC2, describing 16% of existing variation, could further separate populations within the tolerant group (Figure 3.6).

3.2.2. Physiological traits in the early vegetative growth stage

3.2.2.1. Root/Shoot ratio

Root/shoot length ratio was measured after the drought stress period (Figure 3.7) and compared to watered plants. Responses of the population were placed into three categories. In the first group (populations *T. vulgaris* (IR), *T. daenensis* (IR), *T. kotchyanous (IR)*, *T. vulgaris* (Varico2), *T. vulgaris* (Varico3) and *T. capitata* (SP) the root/shoot ratio was greater under drought than watered conditions. The second group consisted of populations that had lower root/shoot ratios in stressed conditions than in control conditions: this population was *T. serpyllum* (SP). The populations placed in the third group had the same root/shoot ratio in both well-watered and droughted condition (Figure 3.7). In general, comparison of the root/shoot under stressed condition using DMRT (Dauncan's New Multiple Range Test) classified all the populations to 4 categories. Group a had the greatest root/shoot ratio and group d was the lowest (Figure 3.7).

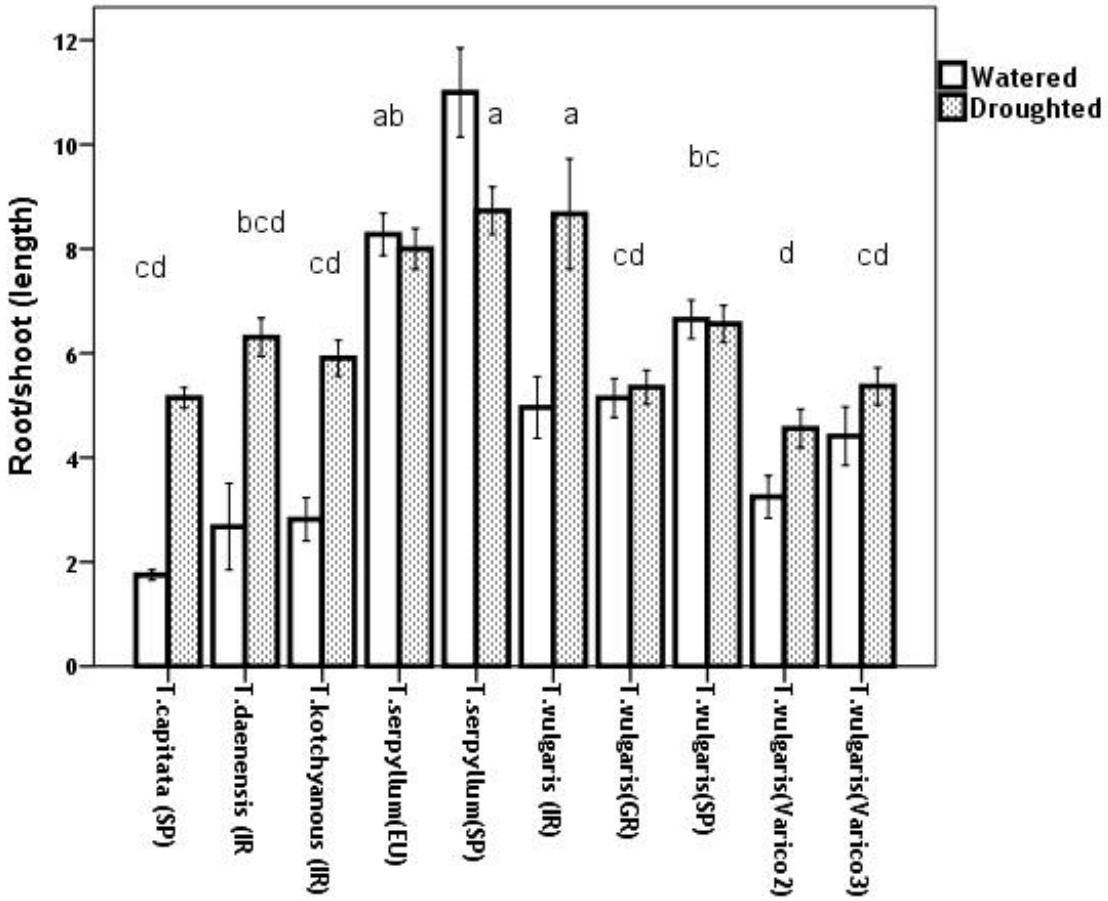


Figure 3.7. Three different root/shoot ratios (rd=rw, rd<rw and rd>rw) were observed in 11 thyme populations over a water deficit period in early stage growth.

Root and shoot length was recorded in 3 replicates. Studied population responses were placed in three categories. In the first group (populations *T. vulgaris* (IR), *T. daenensis* (IR), *T. kotchyanous (IR)*, *T. vulgaris* (Varico2), *T. vulgaris* (Varico3) and *T. capitata* (SP) ratios in droughted plants were greater than under watered conditions (rd>rw). The second group consists of populations which had root/shoot ratio in less than control condition under stress; this population was *T. serpyllum* (SP). The other populations were placed in the third group which had the same ratio in both conditions. Similar letters on bars show the same mean group based on ANOVA and Duncan mean comparison (p<0.05). Error bar give ±1 SEM. rd: ratio droughted rw: ratio watered.

3.2.2.2. Shoot water potential

All the populations studied in the present study had a significantly lower leaf water potential in drought compared to well watered plants ($p<0.01$); (Figure 3.8). Overall, the average water potential of leaves of well-watered plants was -3.4 bar which decreased to -10.5 bar following prolonged drought. The largest difference of around 10 bar occurred in *T. vulgaris* (GR) (-4 bar in watered decreasing to -14 bar in droughted material). The smallest decline

was observed in *T. capitata* (SP) where leaf water potential declined from -4 bar to -7 bar in water stressed material.

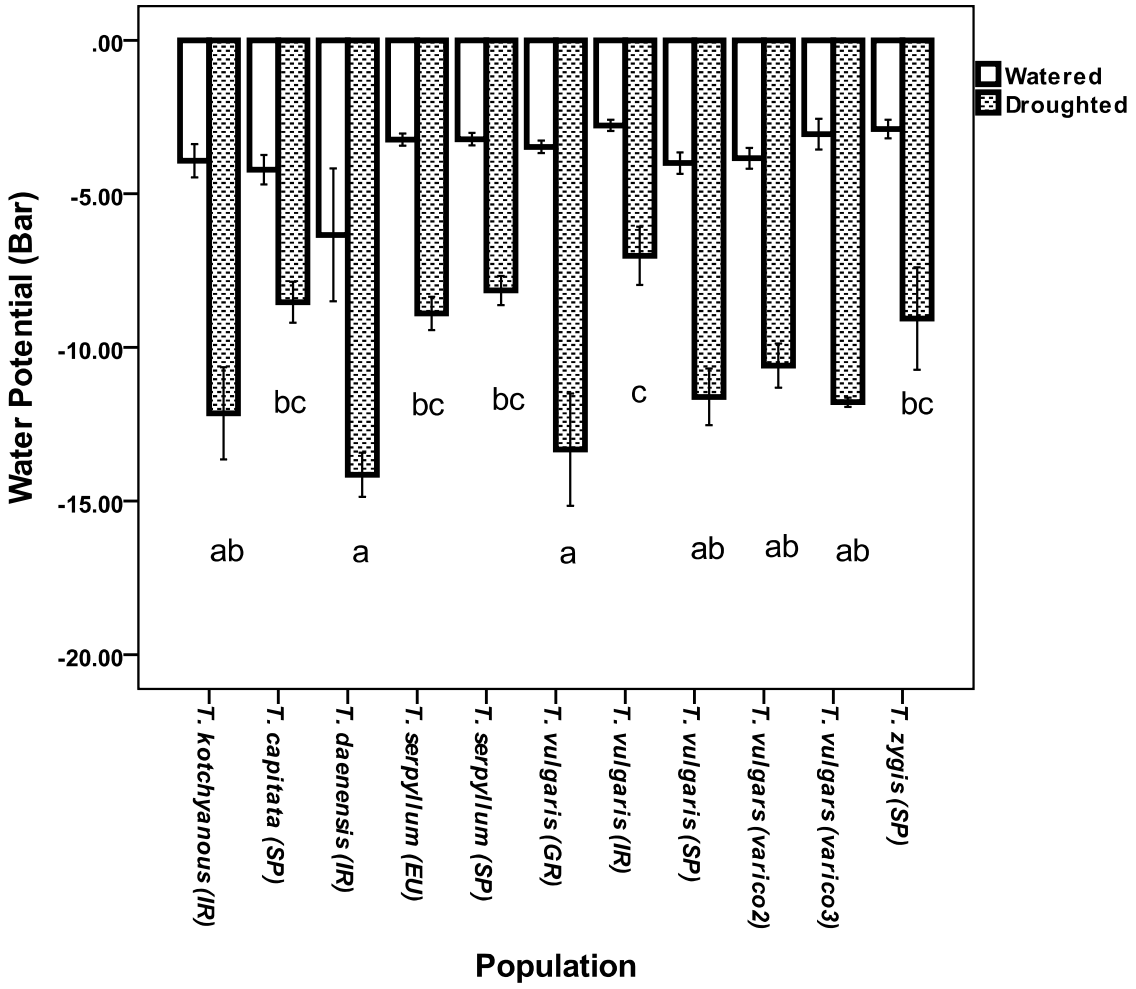


Figure 3.8. Water potential of shoots from well watered and drought stressed plants.

Differences between the well-watered plants and the same populations under drought were significant (p<0.01, Student's t test). Y axis shows water potential in – bar. The largest difference recorded was for *T. vulgaris* (GR) at around 10 bar (-4 bar decreased to -14 bar) and the smallest for *T. capitata* (SP) at around 3 bar (-4 declined to -7). Similar letters on bars show the same mean group based on ANOVA and Duncan mean comparison (p<0.05). Numbers of replicates = 15, Error bars ±1 SEM.**

To determine significant water potential differences following drought, one-way ANOVA was used. Within the droughted plants, the group showing decreased water potential included *T. vulgaris* (Varico2), *T. vulgaris* (Varico3), *T. vulgaris* (SP), *T. vulgaris* (GR) *T. kotchyanous* (IR) and *T. daenensis* (IR), maintaining shoot water potential at a more negative value to uptake more water.

3.2.2.3. Shoot Water content as an alternative indicator of shoot water status

Populations exposed to different external water potentials varied in their water content (% WC) (Figure 3.9). Under unstressed conditions, *T. vulgaris* (GR), *T. vulgaris* (SP) and *T. zygis* (SP) had WC over 80%, which decreased by around 10% at the higher levels of drought. On average, water content for watered plants was 85% ± 3.7 and for droughted plants was 73.4% ± 15.5 after the droughted period and before wilting. The larger standard deviation for droughted plants (15.5%) is consistent with more diverse effects of water stress on different populations. ANOVA followed by Duncan mean separation identified *T. vulgaris* (SP) and *T. zygis* (SP) as a lower water content group and *T. serpyllum* (SP), *T. daenensis* (IR), *T. kotchyanous* (IR), *T. serpyllum* (EU), *T. vulgaris* (IR), *T. capitata* (SP) and *T. vulgaris* (GR) as forming a higher water content group.

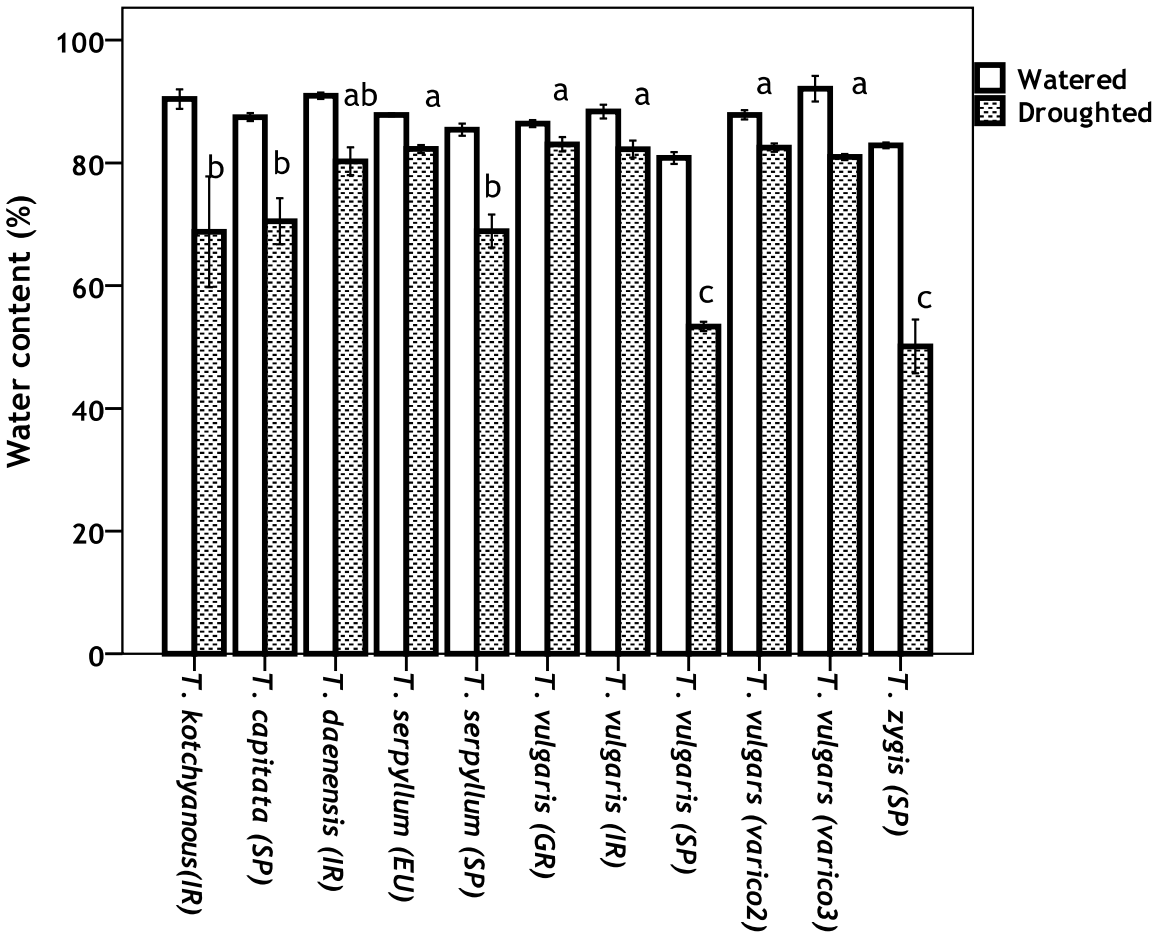


Figure 3.9. Effect of drought regime on the water content (WC) of plant shoots compared to well watered plants.

WC was calculated as the difference between fresh weight and dry weight divided by fresh weight. Y axis shows the soil moisture percentage. Similar letters on bar show the same mean group based on ANOVA and Duncan mean comparison ($p<0.05$). Error bars ±1 SEM. Replicate numbers = 4

3.2.2.4. Correlation of shoot water potential and water content

While water potential represents the real water status of the tissue, measuring water potential is technically more difficult, therefore, correlation between water content and water potential was calculated (Table 3.1).

Table 3.1. Association of water potential and water content (WC) in studied populations.

No.	population	Pearson Correlation (r)	R^2	Linear Regression model
1	*T.daenensis* (IR)	-0.48*	0.24	WC=96.19-WP
2	*T. kotchyanous (IR)*	-0.075n.s.	0.006	WC=81.9-0.33WP
3	*T. vulgaris* (IR)	-0.51*	0.26	WC=90.2-0.82WP
4	T. *vulgaris* (Germany)	0.61*	0.37	WC=87.8-0.37WP
5	*T. serpyllum* (EU)	-0.77**	0.59	WC=90-0.82WP
6	*T. serpyllum* (SP)	-0.91**	0.83	WC=96-3.3WP
7	*T. capitata* (SP)	-0.77**	0.59	WC=102.3-3.3WP
8	*T. vulgaris* (SP)	-0.81**	0.66	WC=95.5-3.18WP
9	*T. zygis* (SP)	-0.44*	0.17	WC=75.3-1.75WP
10	*Thymus* spp.	-0.27**	0.076	WC=86-0.8WP

P<0.05, ** P<0.01, n.s. non-significant

According to Pearson correlation there was a significant negative correlation between water potential and water content in all populations except for *T. kotchyanous (IR)*. The highest significant association between WP and WC was in *T. serpyllum* (SP) (-0.91**) and the lowest was for *T. zygis* (SP) which was still significant (-0.44**).

3.2.2.5. Survivability of thyme populations under drought stress condition

Survival was estimated as the number of days plants were alive following withholding of water. To follow the progress of soil drying, soil moisture was measured, and plant status was scored against a 5-point index. There was significant variation among populations with *T. serpyllum* (SP) surviving longer than all other populations (22 days) while soil moisture had declined to nearly zero percent (Figure 3.10). The most susceptible population was *T. vulgaris* (SP), which did not survive beyond 12 days, despite maintaining soil moisture of around 10%. There were both inter and intra species differences: within T. *vulgaris*, *T. vulgaris* (SP) was sensitive; but *T. vulgaris* (VARICO3) was more tolerant, but both *T. serpyllum* populations were tolerant.

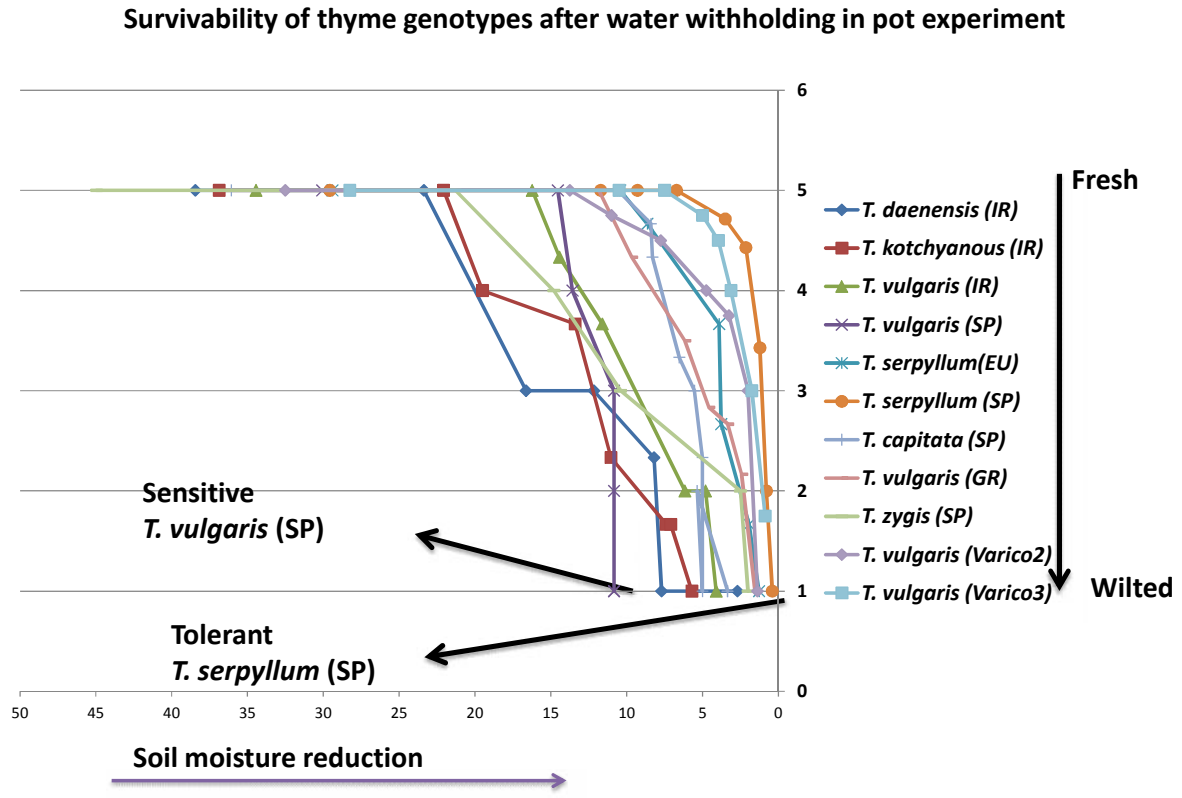


Figure 3.10. Survivability of 11 thyme populations after withholding water in one month old plants.

The y-axis shows the drought index from 5-fresh plant to 1-dead plant (as described in materials and methods). *T. serpyllum* (SP) survived longer than all other populations (22 days) with soil moisture declining to near zero percent. The most susceptible population was *T. vulgaris* (SP), which did not survive beyond 12 days, despite a soil moisture of around 10%.

3.3. Discussion

3.3.1. Germination

Germination and seedling establishment are the first, arguably the most important, but also the most vulnerable stage in the life cycle of plants (Roberto and Rodolfo, 2004). Water deficit stress like other environmental factors can compromise the seedlings establishment (Albuquerque and De Carvalho, 2003). There are several investigations involving the use of PEG to induce controlled drought stress in other plants. Murillo-Amador et al. (2002) indicated a significantly decreased germination percentage in cowpea (Murillo-Amador et al., 2002) with similar results demonstrated in pea (Okcu et al., 2005). One limitation of using PEG can be uptake by the plant, as shoots (with rate of 1 mg/g fresh weight per week) uptake PEG slower than the roots (Lawlor, 1970). But in this relatively short-term experiment of 10 days, the problem of uptake is likely to be less than in pot growth experiments. Likewise, we assess our plants at the germination phase, which initially depends on cell expansion in the radicle. PEG has advantages compared to other methods of dehydration, as it does not damage plant roots and it imposes low water potential stress more likely reflecting the type of stress caused by water loss from the soil (van der Weele et al., 2000; Verslues et al., 1998). It is also accurate and reproducible (Verslues et al., 2006).

Regarding seedling growth, previous studies have shown the increase of traits such as root growth under water stress conditions for some plants (Jaleel et al., 2008). The remaining the traits decline with decreasing in osmotic potential. Thus water stress imposed by PEG inhibits germination and other seedling growth parameters apart from at -2 bar. These results have been observed in plants such as pea (Okcu et al., 2005) and rice (Pirdashti et al., 2003). In thyme Bagheri et al. (2011) showed no change in germination under a -3 bar water stress, and MGT (Mean Germination Time) was unaffected at -3 bar, increased at -6 bar and subsequently decreased in -9 bar. Other traits such as root and shoot length declined at more negative osmotic potentials (Bagheri et al., 2011). This species variation may provide valuable germplasm for plant breeders, and also scientists investigating drought mechanisms in this genus (Chaves et al., 2002).

The results of the present study on germination percentage (Figure 3.3) showed general agreement with the only previous studies on *Thymus* species germination, in *T. daenensis*

(IR) and *T. kotchyanous (IR)* (Khoshsokhan, 2012; Bagheri et al., 2011), as those populations had around 90 percent germination under control (well watered) conditions and as *T. kotchyanous (IR)* was more tolerant than *T. daenensis* (IR) under severe water deficit condition (-6 bar osmotic potential).

To my knowledge there is currently no study screening different species of thyme under water stress for more than 2 species at germination level. An interesting point is the conflicting results of two published works. Bagheri et al. (2011) compared *T. denensis* and *T. kortchyanous* and concluded that, based on germination, *T. kotchyanous* was more tolerant than *T. daenensis* (IR), agreeing with the present study, (Bagheri et al., 2011), while Khoshsokhan et al. (2012) concluded the opposite (Khoshsokhan, 2012). The contrasting results might be explained by the use of different ecotypes of the species in the two studies, in addition to different treatment levels. The former used treatment levels from -3 to -18 bar while the latter used just -3 to -9 bar, even though both used PEG 6000 to impose the stress.

3.3.2. Physiological traits

When studying water stress the three most important traits have been argued to be plant growth, survival and plant water status (Verslues et al., 2006). Water stress is described by lower water potential, lower water content and reduced plant growth and finally plant death (Manivannan et al., 2007b; Ekanayake et al., 1985). In the present study plant shoot length, root length and root/shoot ratio were recorded as a plant growth index, water status was measured as both % water content and leaf water potential and to screen for overall drought tolerance, time of survival (survivability) was estimated.

Regarding root/shoot ratio (Figure 3.7), it seems first group members use one of the well known mechanisms of adaptation, which is development of the root system, which can enhance water uptake to support growth of above-ground organs (Passioura, 1981). But in second group, root/shoot decrease might follow the general rule of drought stress, which both elongation and expansion growth in shoot and root is affected by drought stress (Kusaka et al., 2005; Shao et al., 2008).

It must be noted that root and shoot length as a growth indicator might be affected by several factors including genetics, environment and the interactions between the two. However, recording these traits is crucial in evaluating plant yield and stress responses (Sestak et al., 1971; Manivannan et al., 2007b; Heath, 1972). Increasing the root to shoot ratio is one of the avoidance mechanisms that can maximize the water uptake while minimizing water loss when under water deficit circumstances (Chaves et al., 2003). Regulation of root to shoot ratio has explained by other researchers as one of the indirect functions of ABA. It has been demonstrated that ABA synthesis is increased in the roots after decrease in soil moisture (Cutler and Krochko, 1999; Thompson et al., 2007; Wilkinson and Davies, 2002). Synthesized ABA is transported to the shoots through the xylem (Davies, 2010; Davies and Zhang, 1991; Dodd et al., 2008; Wilkinson and Davies, 2002). Therefore under drought conditions, the ABA concentration in roots will be higher than in shoots. A higher concentration of ABA in roots limits the functioning of ethylene, which is a growth inhibitor. In contrast, a lower concentration of ABA in the shoots allows ethylene to inhibit shoot growth (Mahajan and Tuteja, 2005; Sharp, 2002). Therefore during water deficit root/shoot ratio will increase as a response to drought (Sharp and LeNoble, 2002; Chaves et al., 2003). There are, however, investigations in some other plants with conflicting results. For instance, Jaleel et al. (2008) has reported increasing root growth in sunflower and *Catharnthus roseus* (Jaleel et al., 2008), while the another group reported decreasing root dry weight in poplar (Wullschleger et al., 2005) and also Sacks et al. (1997) reported no significant difference in maize and wheat (Sacks et al., 1997).

The populations in the present study showed differences in response to drought regarding the root/shoot ratio. The highest root/shoot ratio in either control or stressed conditions was shown by *T. serpyllum* species (Figure 3.7) which shows these populations, with prolific root systems, can efficiently extract water from shallow soil layers and support aerial parts during vegetative growth. However, populations of *T. capitata (SP)*, *T. vulgaris* (IR) and *T. daenensis* (IR) responded to water stress with significant increases in root/shoot ratio compared to watered plants.

Using the pressure bomb to measure water potential offers high precision and represents measurement of a fundamental, not derived, parameter (Clarke and Simpson, 1977). The water potential range values in this thesis are similar to those of Sayar and others (2007) in

a study on wheat for watered and droughted plants (Sayar et al., 2007) although comparability is difficult due to differences in both species and treatment methods.

Water potential quantifies the water availability status (Kramer and Boyer, 1995) and as soil water potential decreases it becomes increasingly difficult for plants to take up water from the soil (Verslues et al., 2006). Significant correlation has been reported between high negative shoot water potential in durum wheat and both drought (Benlaribi et al., 1990) and salt stress (Haddad and Coudret, 1991) tolerance.

Different mechanisms can operate to maintain a lower water potential, (Chaves et al., 2003), allowing plants to take up water even under severe water deficit conditions. To take up more water one strategy is to accumulate more solutes: these solutes are referred to osmolytes or osmoprotectants (Pareek et al., 2010).

To complement shoot water potential measurements, water loss was measured as water content as a proportion of fresh weight, providing a less technically demanding method (Sunkar, 2010). Water content provides a rapid assessment of water in tissues. In two conditions water content is not an adequate measure of water status and water potential should be applied. Tissues with higher lipid contents have lower water content, and if sampling is done in non-equilibrated (heterogeneous) tissues, in both cases water potential will give a more accurate index of available water (Sunkar, 2010). In our study water potential is more accurate than water content because the leaves and stems used for water content estimation are heterogeneous tissues.

Overall analysis of all the data (Table 3.1.) found a significant negative association between water content and water potential. Since a pressure chamber instrument may not always be available, this correlation can be utilised to predict water potential based on water content using the regression formula in Table 3.1. This prediction is particularly reliable for *T. serpyllum* (SP), *T. vulgaris (SP), T. capitata (SP), T. serpyllum (UK).*

Several criteria have been suggested for screening plants for drought tolerance e.g. water potential (Sinclair and Ludlow, 1985), water use efficiency, seedling vigour (Nagarajan and Rane, 2000) and survivability (Singh et al., 1999). Survivability is rapid, simple and can be used for large samples of genotypes (Tomar and Kumar, 2004). The approach can accurately

be used to distinguish tolerant genotypes from susceptible (Singh et al., 1999). This tolerance indicator can be used effectively in plant improvement programs, since, as has been demonstrated in cowpea, it is free of environmental effects and is controlled by one gene, (Mai-Kodomi et al., 1999).

Survivability as a central trait in physiological screening stage, showed *T. serpyllum* (SP) is the most tolerant and *T. vulgaris* (SP) the most susceptible population of the plant material evaluated. In contrast, leaf water status trait indicated that the studied populations responded differently to water deficit (Figure 3.8 and Figure 3.9); these traits did not necessarily correlate with survival. This variation could be attributed to different underlying physiological mechanisms.

Given the importance of thyme for commercial secondary metabolite production (Stahl-Biskup and Sàez, 2002), it is important to consider the effects of drought stress on the populations studied here. The volatile content of studied species has been shown in 3.2 based on other investigations in the past. Some studies have reported a role for secondary metabolites in the adaptability of plants to particular environments (Stahl-Biskup and Sàez, 2002). These studies suggested that plants adapted to dry and hot conditions had enhanced phenolic chemotypes (carvacrol and thymol), but plants with non-phenolic chemotypes (geraniol, a-terpineol, tr-sabinene hydrate or thuyanol-4, linalool) were better adapted to wetter and cooler climates (Stahl-Biskup and Sàez, 2002; Echeverrigaray et al., 2001). According to the data acquired in this investigation, it appears there is no particular trend for association of phenolic compounds content and drought tolerance. The only possible reason for this non-association could be environmental effects on essential oils and these studies were reported from different part of the world with different climates. Hence, for accurate correlation, analysis of essential oils for the plants of this experiment is required.

Table 3.2. Volatile compound content of species: thymol and carvacrol.

species	Thymol %	Carvacrol %	Reference
T. vulgaris	49.7	48.8	(Morgan, 1989)
T. serpyllum	18.7	-	(Nickavar et al., 2005)
T. daenensis	73.9	3.6	(Nickavar et al., 2005)
T. kotchyanous	38.6	33.9	(Nickavar et al., 2005)
T. zygis	23.8	25	(Salgueiro et al., 2010)
T. capitata	39.3	12.7	(Ravid and Putievsky, 1986)

3.4. Conclusions

These experiments demonstrated that different external water potential revealed distinct genetic differences between populations with respect to their germination percentage. Different populations are genetically diverse; and also have different responses to drought. In conclusion, for mild stress (-2 bar) all *T. serpyllum* along with *T. vulgaris* (IR) were tolerant compared to the others, but in moderate stress (-4 bar) condition, *T. serpyllum* (SP) was tolerant; and finally in -6 bar, namely severe stress, *T. vulgaris* (SP), *T. zygis* (SP) and *T. kotchyanous (IR)* were also located in the tolerant class.

In the early vegetative phase, populations had different responses of their root/shoot ratio to drought. All droughted plants had lower leaf water potential. Survivability was a reliable tool to screen the germplasm for drought stress tolerance. In total, this series of physiological experiments identified *T. serpyllum* as being more tolerant than other species, and *T. vulgaris* (SP) as the most susceptible population.

Responses of populations to water stress at two different stages, namely germination and seedling, had agreement for mild and moderate stress. In other words, *T. serpyllum* (SP) as a tolerant plant at early vegetative growth stage had high germination at mild and moderate

stress at germination phase. Likewise, *T. vulgaris* (SP) as a sensitive plant in physiological experiments had medium germination at mild and moderate stress.

Further investigations including transcriptomics, proteomics and metabolomics are needed to understand the different mechanisms of drought stress tolerance in these plants. In addition, as these experiments were performed in a growth room, confirmation of these responses requires evaluation under field conditions.

3.5. Summary of chapter 3

Thymus, with more than 200 species, is familiar for its medicinal and culinary uses. There is an increasing demand for thyme and its products, requiring cultivation under different conditions, including drought. To understand the response of thyme to water deficit and its variation across the genus eleven populations from a range of species (*daenensis*, *kotchyanous*, *vulgaris*, *serpyllum*, *capitata* and *zygis*) were evaluated during germination and the early vegetative phase. In the germination phase, 3 levels of osmotic potentials (-2, -4 and -6 bar) were imposed by PEG 6000 along with control conditions in 3 replicates. 9 traits related to germination and seedling were recorded: fresh weight, dry weight, radicle and plumule length, water content, germination percentage and mean germination time. Drought stress for the seedling stage was imposed by water withholding after one month of planting; then root/shoot ratio, survivability, water content and water potential were recorded.

For mild stress (-2 bar) all *T. serpyllum* species along with *T. vulgaris* (IR) were rated tolerant compared to the others, but in moderate stress (-4 bar), *T. serpyllum* (SP) was tolerant; and finally in -6 bar, namely severe stress, *T. vulgaris* (SP), *T. zygis* (SP) and *T. kotchyanous* (IR) were designated in the tolerant class. In the vegetative growth phase, populations had different responses of their root/shoot ratio. All droughted plants had lower leaf water potential. Correlation of water potential with water content was discussed. Survivability was a reliable tool to screen the germplasm for drought stress tolerance. In total, this series of physiological experiments identified *T. serpyllum* as the most drought tolerant species, and *T. vulgaris* (SP) as the most susceptible.

In the next chapter, to develop the technique concerning time of harvest and understanding of general molecular mechanisms behind the drought responses of thyme, state of the art approach namely FT-ICR metabolomics was used. To use this technique, time course metabolite profiling combined with physiological parameters investigated on a moderately stress tolerant population, *Thymus vulgaris* (GR), based on my results from this chapter.

CHAPTER IV. INVESTIGATING THE METABOLIC RESPONSES OF *THYMUS VULGARIS* TO WATER DEFICIT STRESS USING FT-ICR MASS SPECTROMETRY-BASED METABOLOMICS

4.1. Introduction

Metabolomics, via a metabolic profiling approach, can be used to study plant stress. Specifically, time course profiling can give valuable information about metabolite changes during the development of a stress response (Shulaev et al., 2008). The various technologies of metabolomics have been reviewed in detail (Hagel and Facchini, 2007). FT-ICR (Fourier Transformed Ion Cyclotron Resonance) by DIMS (Direct-Infusion Mass Spectrometry), with less than 1 ppm mass accuracy and measuring compounds less than 1500 Daltons in weight, has been used in plant metabolomics (Aharoni et al., 2002). FT-ICR has two considerable advantages; namely high mass accuracy (the measured mass is close to true mass) and high mass resolution (it can distinguish two ions with similar masses) (Zulak et al., 2008). DI FT-ICR workflow along with SIM-stiching (SIM: Selected Ion Monitoring; described in materials and method section) has enabled an increase the dynamic range (ca. 3000 m/z) but still retained high mass accuracy (maximum absolute mass error of 0.29 ppm) (Southam et al., 2007; Weber et al., 2011). This technique has been used to study the effects of herbicide and light/dark treatments in *Arabidopsis* (Oikawa et al., 2006; Nakamura et al., 2007), the study of nitrogen metabolism in transgenic tobacco (Mungur et al., 2005), and metabolic response of opium poppy cell cultures to elicitor treatment (Zulak et al., 2008). However, to our knowledge, the current report is the first to utilize DI FT-ICR to investigate drought stress responses in plants.

This chapter aims to optimise the developed general scheme of DI FT-ICR metabolite profiling of plant extracts and data processing as well as monitoring the major metabolites changing over a drought stress period on a daily basis in *Thymus vulgaris*. The identified metabolites' role in drought stress through various metabolic pathways will be discussed.

4.2. Results

4.2.1. Physiological traits

In order to investigate the effects of water deficit on the water status of the soil and the plant, soil moisture, water potential, water content and fresh weight were recorded on a time course basis. Soil moisture was 80% on the first day, declined until the 6th day, and then was unchanged for a further 2 days (Figure 4.1). Again, after a further decrease, soil moisture remained around 5% for 4 days. Plants wilted after 14 days. Concomitant with the

decrease in soil moisture, the water content of leaves and leaf water potential significantly changed following the decrease in soil moisture. The water content of leaves was 87% on the first day and remained constant until the 7th day of withholding water (Figure 4.1.a). Thus, in spite of a 60% soil moisture decrease, plant water content was unchanged. However after 10-12 days of withholding water, the leaf water content decreased to 82%. At the end of the water stress period, leaf water content was 77%. The water potential of the leaves had the same pattern as water content during drought stress: a water potential of -4 Bar on the first day declined to -8 Bar over the experimental period (Figure 4.1.c). Likewise, fresh weight as a plant growth rate index was statistically unchanged during water deficit, particularly after the 4th day of water withholding (Figure 4.1.b).

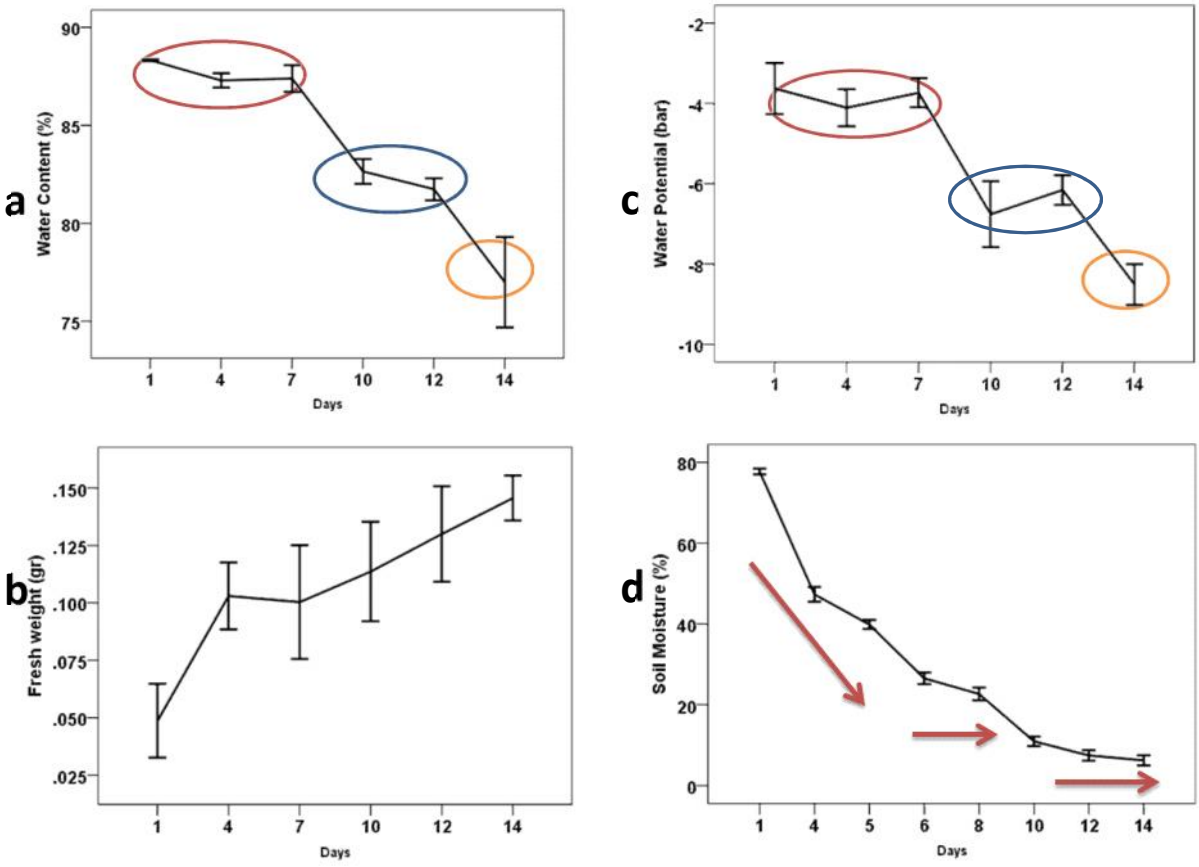


Figure 4.1. Physiological parameters recorded during drought stress of Thymus vulgaris.

Water withholding impact on soil moisture, water potential, water content and fresh weight were investigated over time. Soil moisture was 80% on fist day, had a sharp decline until the 6th day, and then was stationary unchanged for a further for 2 days (d). Again after another further decrease, soil moisture remained around 5% for 4 days. This population could not tolerate less than 5% soil moisture and plants wilted after 14 days. Concomitant with the decrease in soil moisture, water content of leaves and leaf water potential significantly changed following soil moisture

decreasing. Water content of leaves was 87% on first day and remained constant until the 7th day of withholding water (a). In spite of a 60% soil moisture decrease, plant water content was unchanged. However after 10-12 days of water withholding, water content decreased to 82% (second circle). At the end of the water stress period, recorded leaf water content was 77%. Water potential of leaves had the same pattern as water content during drought stress; a water potential of -4 bar on the first day gradually declined to -8 bar over the experimental period due to water deficit (c). Likewise, fresh weight as a plant growth rate index was statistically unchanged during water deficit, since there was no significant growth rate change after the 4th day of water withholding (b).

4.2.2. Metabolic profiling

My initial objective was to optimize the general scheme of DI FT-ICR. These modifications should achieve spectra with high reproducibility across the technical replicates within a biological replicate, as well as a high number of peaks. The optimum fresh matter (50-100 mg) was harvested and following 2 days of freeze-drying; the dry matter obtained was less than 10 mg. Next, FT-ICR analysis was performed for positive ion mode of polar fraction in 14 groups (days) with 6 biological replicates/day and 3 technical replicates. In order to process the generated mass spectra, 2 technical replicates out of 3 with an 80% sample filter were retained (peaks occurred at least 80% of samples within group independently). Examples of mass spectra for positive ion mode are shown in Figure 4.2.

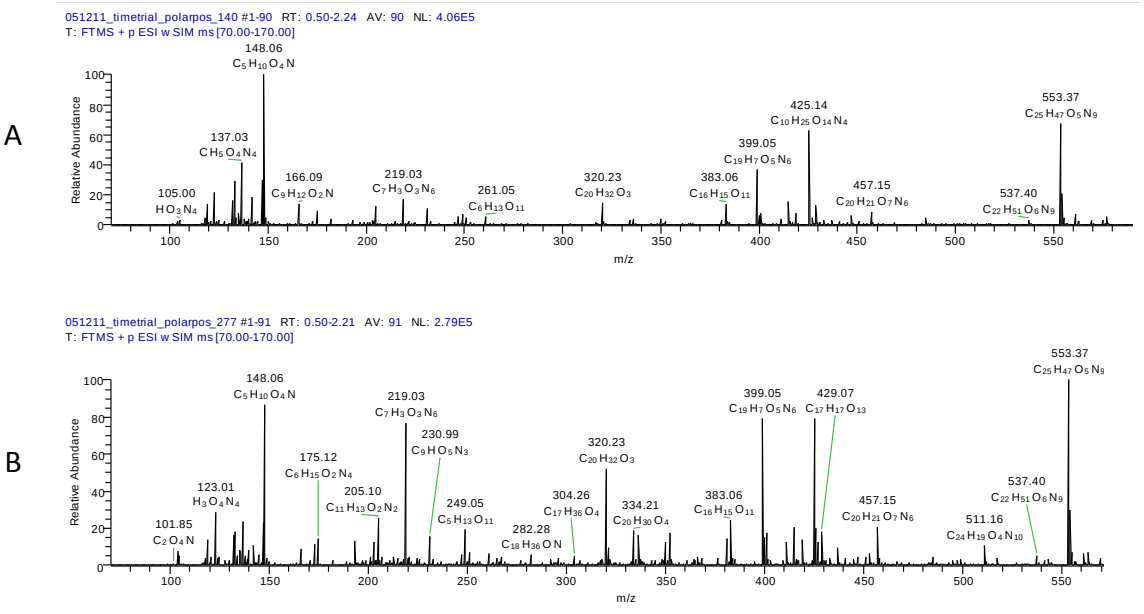


Figure 4.2. Examples of FT-ICR mass spectra for Thymus vulgaris (GR) leaf samples.

These mass spectra were generated by DI FT-ICR for the polar positive metabolites of *Thymus vulgaris*. (A) Second day after water withholding (B) 14th day of drought stress. FT-ICR analysis was performed in positive ion mode for the polar fraction in 14 groups (days) with 6 biological replicates/day and 3 technical replicates. In order to process the generated

mass spectra, 2 technical replicates out of 3 with 80 % sample filter were retained (peaks occurred at least 80% of samples within a group independently).

4.2.2.1. Whole metabolite overview following the water deficit stress

After removing missing values, a total of 4755 peaks with 51% CV (coefficient of variation which is normalized measure of dispersion) were incorporated into the data analysis. To visualize differences between the 14 time points at the metabolome level, the data was subjected to PCA (Principal Component Analysis). Prior to PCA, the data was pre-treated by auto-scaling i.e. the mean of each was spectrum centred and divided by its standard deviation (Goodacre et al., 2007). This scaling method, which is also referred to UV scaling, allocates equal weights to all variables. Therefore, since all peaks have equal unit variance (UV), they will equally contribute to the model (Boccard et al., 2010; Jackson, 2005; Eriksson, 2006).

PCA reduced 4755 metabolites to 2 main principal components (PC1 and PC2). Sample position according to score loadings is illustrated in figure 4.3. 37% of the total variation across the dataset was explained by these two components. The abscissa (PC1) accounted for 27% of the total variation, clearly separating time points into an early (first 10 days) and late phase (last 4 days) of the water deficit stress period. The ordinate (PC2) describes 10.4% of total variation, discriminating the early phase responses to 2 distinct groups, namely the first 4 days and days 5-10.

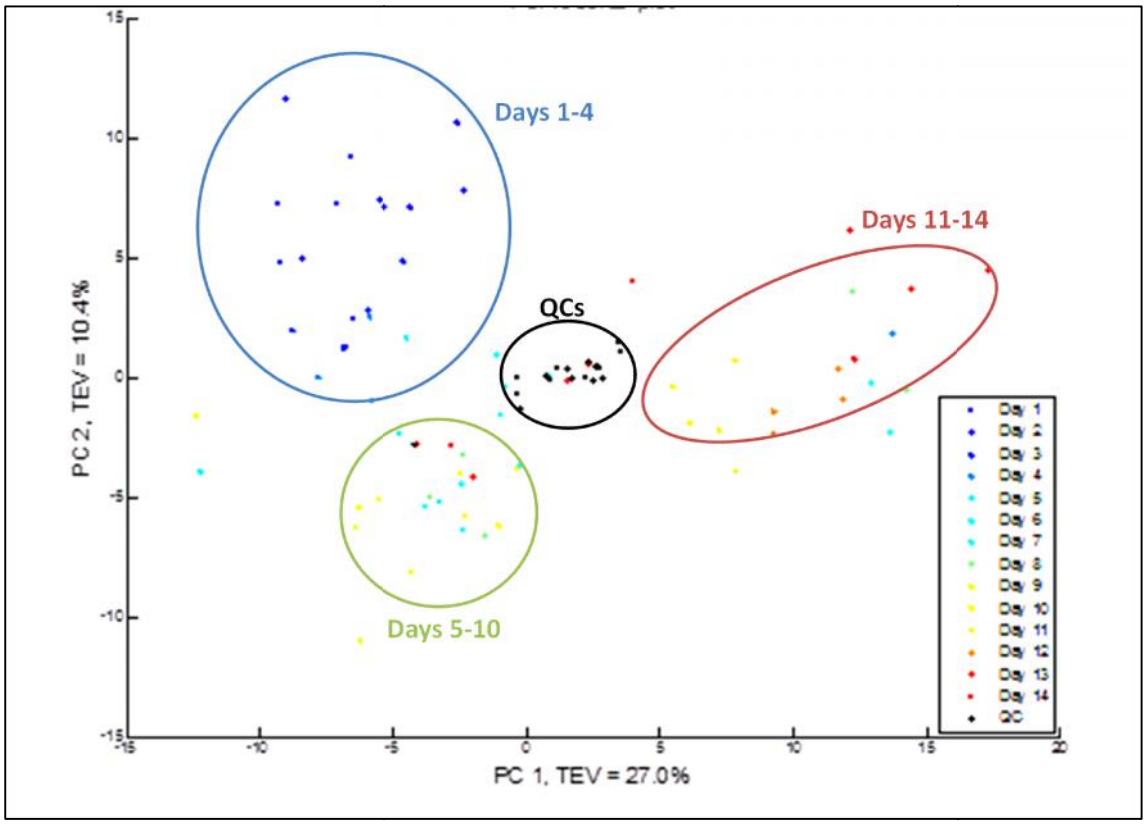


Figure 4.3. Score plot of PCA analysis on 14 day-harvested leaf samples exposed to drought including QCs and 4755 metabolites.

PCA as a way of data reduction reduced 4755 metabolites to 2 main principal components (PC1 and PC2). In this score plot, 37% of the total variation across the dataset was explained by these two components. The abscissa (PC1) accounts for 27% of the total variation, clearly separating time points into an early (first 10 days) and a late phase (last 4 days) of the water deficit stress period. The ordinate (PC2) explains 10.4% of the total variation, impling the separation of early phase responses into 2 distinct groups namely first 4 days and days 5-10. TEV: Total Explained Variance. QC : Quality Control samples consisting equal volume of randomly selected from all the analyzed samples.

4.2.2.2. The most significant peaks in drought stress responses

In order to determine highly important compounds affected by drought stress, 65 peaks were selected. These peaks had the most influence on the classification of samples, as they placed in the lowest and the highest values of PC1 and PC2 (Figure 4.4).

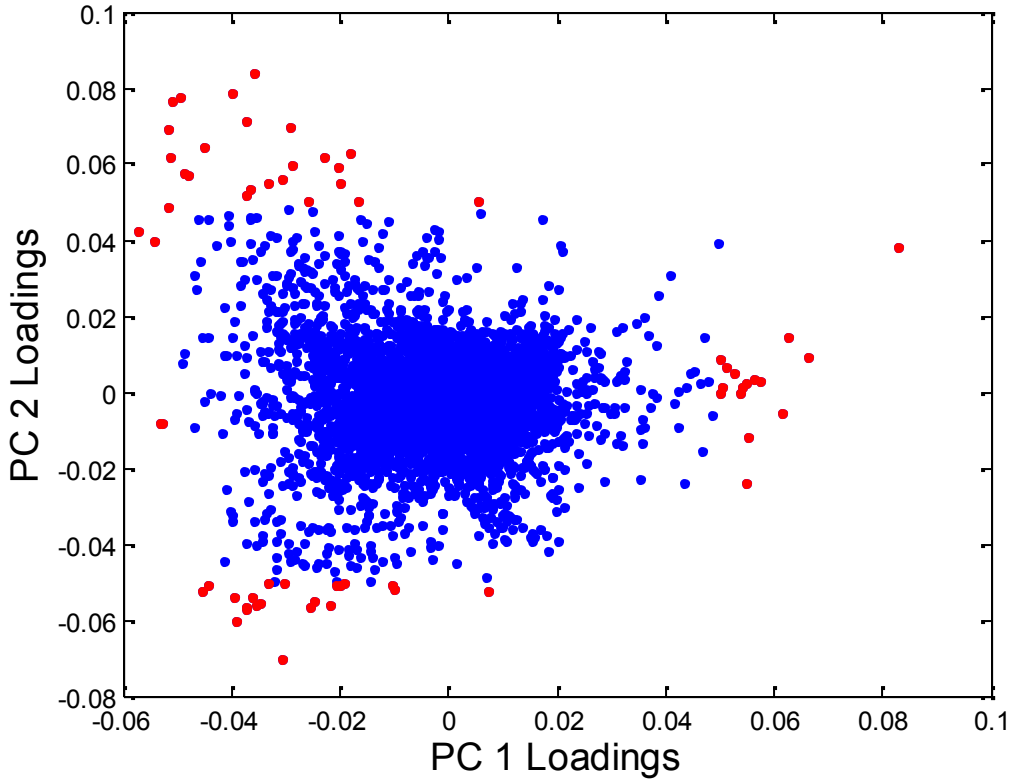


Figure 4.4. The most significant peaks based on high and low loading identified on the loading plot.

In order to determine highly important compounds, 65 peaks were selected (red dots). These peaks had the most influence on the classification of samples, as they were placed in the lowest and the highest values of PC1 and PC2. Cut off line has applied more than ±0.04 loading scores.

Box-plot analysis was performed to illustrate the changing pattern of the selected metabolites over the stress period (Figure 4.5). The most significant peaks (65 peaks) were classified into 3 main groups. Group A contained 26 peaks that were in their highest peak area (8) on the first day but decreased to 1 following drought stress from day 1 to day 6. Group B consisted of 22 peaks, which following the water stress period had increased by day 9 to peak area 7, but declined to 1 on day 12 and finally had a slight increase on the 13th and 14th days. Group C peaks were not affected by water deficit until the 10th day but suddenly increased more than two times over the remaining days.

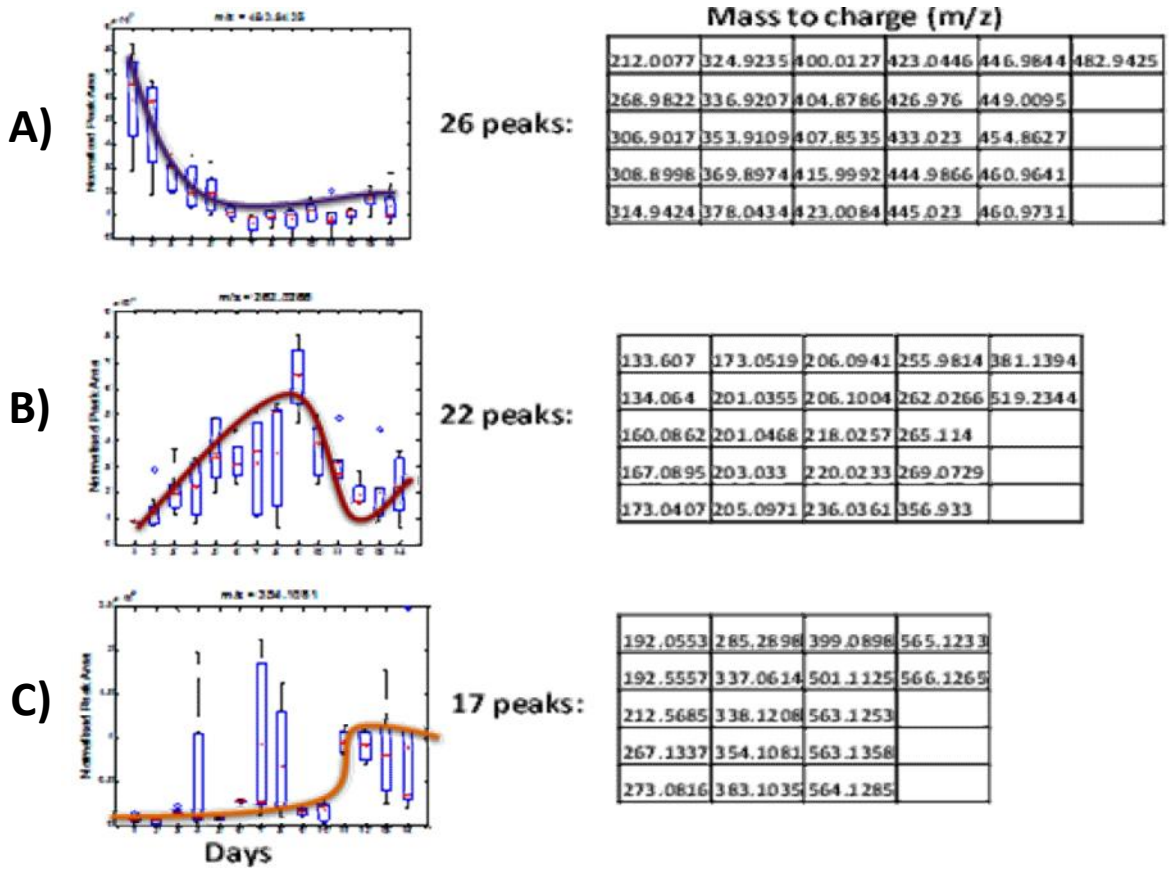


Figure 4.5. Three major peak change patterns following water deficit stress of Thyme for high loading score peaks.

The most significant peaks (65 peaks) could be classified into 3 main groups according to their pattern/profile over time. Group A contains 26 peaks that were in their highest peak area (8) on the first day but drastically decreased to 1 following drought stress imposed from the 1st to 6th day, while no further change occurred up to the end of the experiment (a). Group B, consisting of 22 peaks, following water stress period had a steady increase up to the 9th day to peak area 7, but again dramatically declined to 1 on 12th day and finally had a slightly increase on the 13th and 14th day (b). Group C peaks were not affected by water deficit until the 10th day but suddenly increased more than two times during the remaining days (c). Mass to charge of peaks within each group are shown in the tables.

4.2.2.3. Metabolite identification

In this investigation to identify the metabolites, semi-automated software known as Mi-Pack (Metabolite Identification Package) has been used (Weber and Viant, 2010). This package has been described in detail in the Materials and Methods chapter. Mi-Pack has improved the accuracy of metabolite identification using seven 'golden rules' (Kind and Fiehn, 2006), as well as prior knowledge of metabolite interconnectivity, to decrease incorrect assignment of empirical formulae (Weber and Viant, 2010).

Using Mi-Pack, 11 metabolites were putatively identified subsequent to submission of the most significant peak mass values (65 peaks). Likewise, 28 metabolic pathways have been identified as significant (Table 4.2). Characteristics of putatively identified metabolites are summarized in Table 4.1, consisting a mass to charge, intensity, empirical formula, mass value and putative name. However, 54 peaks remained unknown. Within the identified metabolites, regarding drought stress- related metabolic pathways and literature review, five compounds can be interpreted as having a biological role in response to water deficit stress; asparagine, phenylalanine, tryptophan, D-Xylose-5-phosphate, aspergillic acid (Tzin and Galili, 2010; Kutchan, 1995; Kusaka et al., 2005).

Table 4.1. Summary of putatively identified metabolites out of top weighted peaks for polar positive ions analysis of *Thymus vulgaris* exposed to water stress.

No.	m/z (mass to charge)	intensity	Empirical formula (peak)	Ion form	Theoretical mass	Mass error	Putative metabolite identification
1	133.0607	342205.8	$C_4H_8N_2O_3$	M+H +	132.0535	-0.52	3-Ureidopropionate, Asparagine, Glycylglycine, Methylazoxymethanol acetate, N-Carbamoylsarcosine
2	166.0862	45310.2	$C_9H_{11}NO_2$	M+H +	165.079	-0.33	Phenylalanine
3	205.0971	2963827	$C_{11}H_{12}N_2O_2$	M+H +	204.0899	-0.27	Tryptophan
4	262.0266	26443.2	$C_{14}H_9NO_2$	M+K +	223.0633	0.43	2-7Aminoanthraquinone, 9-Nitroanthracene and Trisphaeridine
5	265.114	35499.9	$C_{12}H_{20}N_2O_2$	M+(41K) +	224.1525	0.93	Aspergillic acid
6	268.9822	69671.5	$C_5H_{11}O_8P$	M+K +	230.0192	-0.44	α-D-ribose-1-phosphate, D-xylulose-5-phosphate, α-D-Xylose 1-phosphate
9	381.1394	80442.4	$C_{20}H_{24}N_2O_3$	M+(41K) +	340.1787	-1.49	3-Hydroxyquinine
10	400.0127	35282.6	$C_{10}H_{19}NO_9S_2$	M+K +	361.0501	-1.47	Glucoputranjivin
11	563.1358	39343.8	$C_{25}H_{30}O_{12}$	M+(41K) +	522.1737	1.41	Melampodinin

Table 4.2. Metabolic pathways contain significant metabolites demonstrated by Mi-Pack derived from KEGG.

No	Mapname	Class of metabolism	Significant metabolite numbers *	Total number of Compounds**
1	Pentose phosphate pathway	Carbohydrate Metabolism	4	32
2	Ascorbate and aldarate metabolism	Carbohydrate Metabolism	3	47
3	Amino sugar and nucleotide sugar metabolism	Carbohydrate Metabolism	2	85
4	Pentose and glucuronate interconversions	Carbohydrate Metabolism	5	55
5	Phenylalanine metabolism	Amino Acid Metabolism	2	63
6	Beta-Alanine metabolism	Amino Acid Metabolism	1	31
7	Phenylalanine, tyrosine and tryptophan biosynthesis	Amino Acid Metabolism	2	27
8	Glycine, serine and threonine metabolism	Amino Acid Metabolism	1	49
9	Cyanoamino acid metabolism	Amino Acid Metabolism	1	43
10	Alanine, aspartate and glutamate metabolism	Amino Acid Metabolism	1	24
11	Arginine and proline metabolism	Amino Acid Metabolism	1	90
12	Tryptophan metabolism	Amino Acid Metabolism	1	81
13	Glucosinolate biosynthesis	Secondary Metabolites	3	75
14	Indole alkaloid biosynthesis	Secondary Metabolites	1	47
15	Biosynthesis of plant secondary metabolites	Secondary Metabolites	3	141
16	Biosynthesis of phenylpropanoids	Secondary Metabolites	2	97
17	Tropane, piperidine and pyridine alkaloid biosynthesis	Secondary Metabolites	1	68
18	Vitamin B6 metabolism	Antioxidant Metabolism	1	32
19	Riboflavin metabolism	Antioxidant Metabolism	1	23
20	Pantothenate and CoA biosynthesis	Antioxidant Metabolism	1	27
21	Carbon fixation in photosynthetic organisms	Energy Metabolism	3	23
22	Methane metabolism	Energy Metabolism	2	78
23	Nitrogen metabolism	Energy Metabolism	1	24
24	Purine metabolism	Nucleotide Metabolism	2	92
25	Pyrimidine metabolism	Nucleotide Metabolism	1	59
26	Biosynthesis of plant hormones	Hormones	2	68
27	ABC transporters	Membrane Transport	1	83
28	Aminoacyl-tRNA biosynthesis	Translation	3	53

* Number of metabolites which significantly changed in that pathway

** Total number of metabolites involved in that pathway

Mi-Pack using the prior information of metabolite interconnectivity from the KEGG databases has determined the pathways consisting of significantly changing metabolites.

4.2.2.4. Biological interpretation

Direct infusion FTICR mass spectrometry in the non-targeted metabolite profiling strategy provided an overall image of the metabolite changes occurring during response to water deficit. Transformation mapping algorithm of Mi-Pack by using KEGG databases (Kanehisa et al., 2008) extracted 29 metabolic pathways listed in Table 4.2. Water stress affected a total

of 29 metabolic pathways. These pathways classified into 4 Carbohydrate Metabolism, 8 Amino Acid Metabolism, 6 Secondary Metabolites, 3 Vitamins, 3 Energy Metabolism, 2 Nucleotide Metabolism and 1 pathway in Hormones, Membrane Transport and Translation (Figure 4.6).

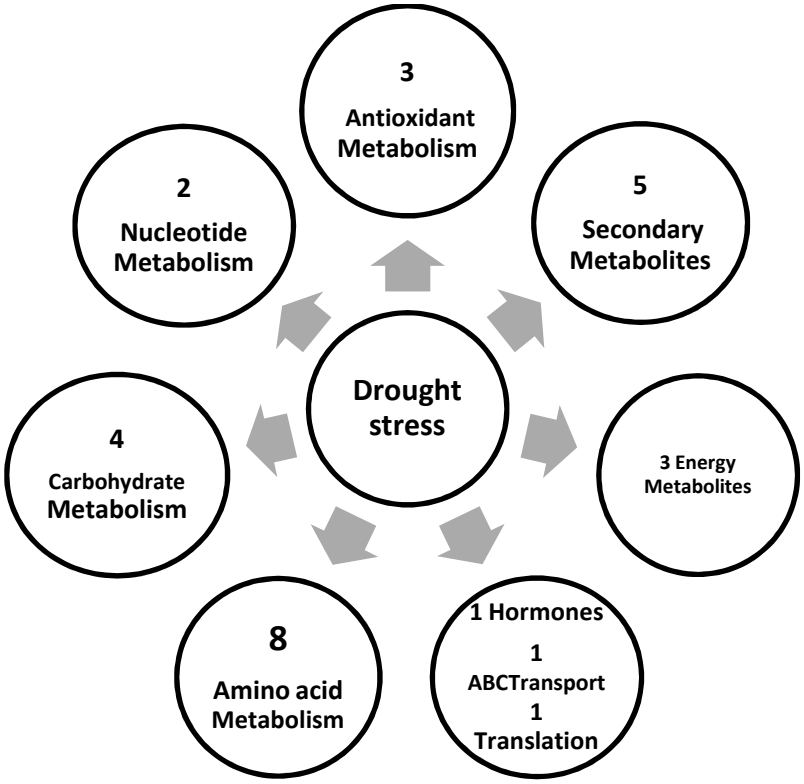


Figure 4.6. Metabolic pathways significantly affected by water deficit stress in *Thymus vulgaris*.

According to the list of metabolic pathways derived from KEGG databases, 28 observed pathways have been classified according to their major metabolism. These pathways classified into 4 Carbohydrate Metabolism, 8 Amino Acid Metabolism, 5 Secondary Metabolites, 3 Vitamins, 3 Energy Metabolism, 2 Nucleotide Metabolism and 1 pathway in Hormones, Membrane Transport and Translation.

The metabolic pathways affected by water deficit stress are illustrated in Figure 4.7. In these pathways, compounds that have been significantly affected are shown in red. Phenylalanine and Tryptophan are aromatic amino acids synthesized through the Shikimate pathway and have important roles in plant hormone metabolism and secondary metabolite biosynthesis (Korkina, 2007; Less and Galili, 2008). Asparagine, an amino acid amide, because of its chemical structure can act in nitrogen storage and transport (Lea et al., 2007). 3-Uridopropionate and N-Carbamyl-β-Alanine are intermediate compounds to synthesize

Uracil. D-Xululose-5P, D-Ribulose-5P and D-Ribulose-5P act through purine, pyrimidine and histidine metabolism (Figure 4.7).

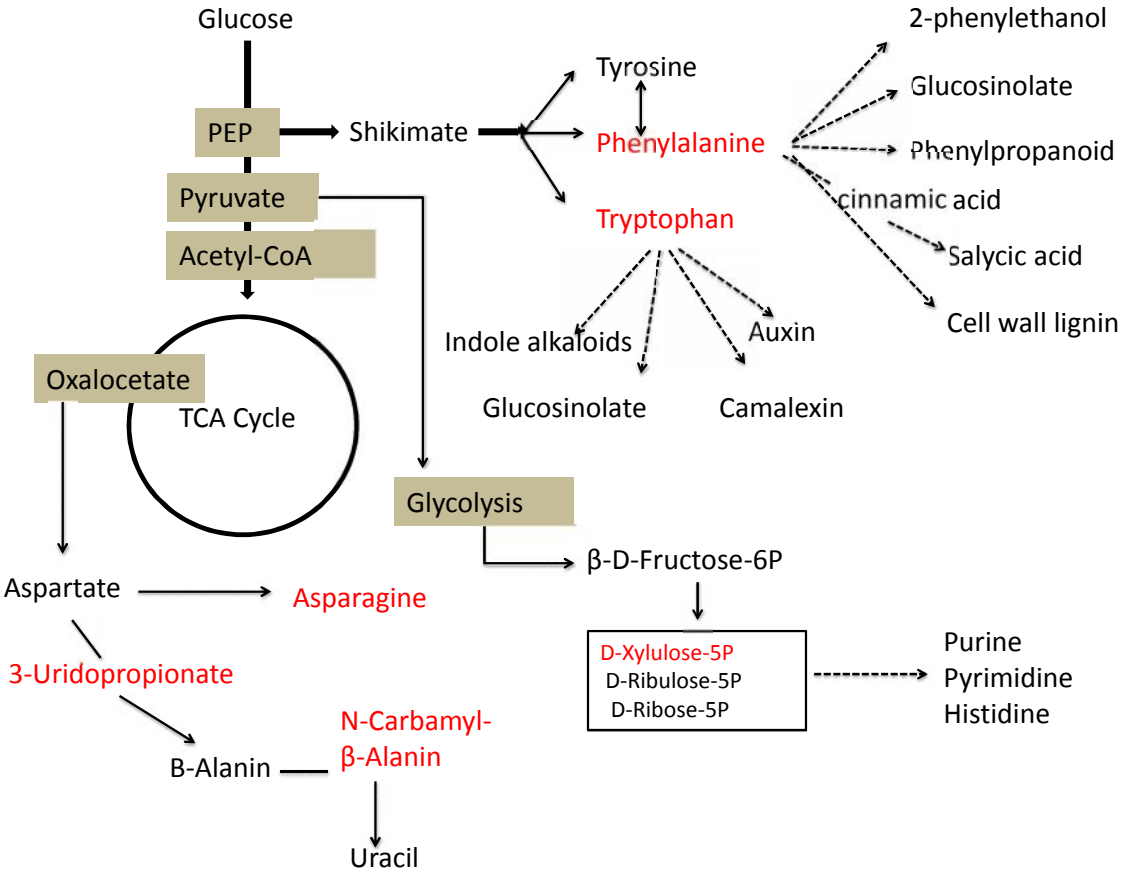


Figure 4.7. Major metabolic pathways affected by water deficit stress in *Thymus vulgaris*.

Impact of water deficit stress summarized as above pathways. In these pathways, identified compounds that have been significantly affected are shown in red. Phenylalanine and Tryptophan are aromatic amino acids which are synthesized through the shikimate pathway and have important roles in plant hormone- and secondary metabolite biosynthesis. Asparagine, as an amino acid amide, because of its chemical structure acts in nitrogen storage and transport in plant. 3-Uridopropionate and N-Carbamyl-β-Alanine are intermediate compounds to synthesize uracil. D-Xylulose-5P, D-Ribulose-5P and D-Ribulose-5P act through purine, pyrimidine and histidine metabolism. It must be noticed that Xylulose, Ribulose and Ribose are not distinguishable by FT-ICR mass spectrometry and therefore these compounds are placed in a box together.

4.3. Discussion

Three clear physiological phases, in this experiment can be recognized: Mild stress (1^{st}-7^{th} day), moderate (7^{th}-12^{th} day) and severe stress (12^{th} -14^{th} day). In a mild stress state, in spite of drastic soil moisture reduction (Figure 4.1), water potential and water content did not change. This status can be described by the stress avoidance terminology (Levitt, 1972;

Verslues et al., 2006), which describes the first response of plants to stress conditions: maintaining water status at unstressed levels. Under severe stress, the water status of the plants (i.e. water potential and water content) declined dramatically, providing potential triggers for the induction of stress tolerance mechanisms.

To conduct such pot experiments under growth room conditions it is important that a) sufficient numbers of replicates for physiological traits, particularly water potential are recorded, b) an even aged set of plant material is used, c) a similar density of plants is used and d) pots are rotated in the tray to ensure uniform illumination. This thesis main experiment performed once because of lack of time and resources which is important limitation. In other words, pot experiments are performed in controlled condition, but small variation during experiment might affect plant growth and cause serious bias in the experiment. Hence, ideally repeat of whole set of experiment to gain unbias results is suggested.

The results of the score plot (Figure 4.3) revealed two important points. First, all sampling time points clustered in three distinct groups illustrating the validity of the experimental setup including plant growth, drought imposition method, harvesting, extracting, FT-ICR metabolomics data acquisition and data processing strategy, since they were harvested at similar time points and supposed to have similar physiological status. Secondly, 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. Clustering of all QCs in the centre of the PCA diagram can indicate the metabolite responses not due to technical problems and errors. Interestingly, the current approach to separation of the time points in PCA was similar to a study of metabolite response to salt stress in *Arabidopsis thaliana* (Kim et al., 2007). In both of the investigations, PC1 classified the response to early and late phase, while PC2 described just the early phase.

Alongside the physiological changes occurring during water deficit stress (Figure 4.1), changes in metabolites during mild stress (short-term) can be interpreted as maintaining plant function at normal levels by using avoidance strategies (Kramer and Boyer, 1995) such as osmolyte accumulation and cell wall stiffening (Verslues et al., 2006). In contrast under severe water stress (long-term), plants may protect cellular compartments from

dehydration damage by mechanisms such as synthesis of dehydrins (protective proteins) and LEA (Late-Embryogenesis Abundant) proteins (Bravo et al., 2003; Hara et al., 2001) as well as osmoprotectants (Hincha and Hagemann, 2004). Another response of plants to drought is against damages caused by ROS (Reactive Oxygen Species) (Apel and Hirt, 2004; Hung et al., 2005; Laloi et al., 2004). Hence, it seems that metabolite groups A and B can be interpreted as drought avoidance mechanisms, as they have changed (up-regulated/ down-regulated) in the early days of stress (Figure 4.5). However, group c metabolites are up-regulated at the end of the drought stress period, where avoidance mechanisms were unable to maintain the balance between water uptake and water loss (Verslues et al., 2006).

Similar patterns of metabolite changes were found in forage legumes under drought stress (Sanchez et al., 2012a). 76 metabolites were observed increasing and 14 metabolites decreasing, but both changes were stress-dose dependent and followed different patterns of responses including fast and late responses, linear and non-linear trends. Interestingly, these stress-dose dependency patterns have been observed in salinity stress (Sanchez et al., 2008b). These authors proposed three fine tuning models of dose-dependent responses including linear, plateau and threshold. These models could interpret salt stress responses for various metabolites. Since plant response to time course style is more likely similar to the mentioned investigation with different level of stress dose.

FT-ICR MS is a leading technology in non-targeted metabolomics (Weber and Viant, 2010; Taylor, 2010; Iijima et al., 2008) and provides thousands of mass values with extremely high accuracy and resolution in biological samples comprising complex mixtures of metabolites (Brown et al., 2004; Han et al., 2008). However, one of the major obstacles in metabolomics is metabolite identification (Kind and Fiehn, 2006; Wagner et al., 2003). Putative metabolite identification can be implemented as an approach to overcome this challenge, i.e. accurate mass values are searched against databases in the peak-by-peak approach (also called Single Peak Search) (Kind and Fiehn, 2006; Smith et al., 2006). Metabolite identification, particularly Single Peak Search, potentially has a high false positive rate (FPR). False positive rate is generated by assigning one mass value to more than one empirical formula as well as correspondence of several chemical structures for each formula. For instance, formula $C_6H_{12}O_6$ at 180.06339 Da occurs in several carbohydrate compounds. Moreover, thousands of metabolites are found in any biological sample but there is a lack of species-specific

databases such as KNApSAcK and HMDB (Brown et al., 2004, Weber and Viant, 2010). It should be noted that putative metabolite names reported in this study cannot considered unambiguous, since they are assigned to empirical formulae calculated based upon just an accurate mass, which do not fulfil the criteria for Metabolomics Standards Initiative of metabolite identification (Sumner et al., 2007; Taylor et al., 2009). Despite the accurate mass measurement by FT-ICR, assigning unambiguous or high-confidence metabolite names to an observed ion is difficult. This is partly because for one exact mass with the same elemental formula, different structures are possible (Zhou, 2011).

Environmental stress can trigger a chain of responses at all levels of plant organization and can alter plant metabolism in several ways. For instance, examples include synthesis of osmoprotectants and redox metabolism (Krasensky and Jonak, 2012). Osmolyte production is used to adjust osmotic potential and also stabilize cellular compartments and proteins. Redox metabolism can adjust excess level of potentially damaging ROS (Valliyodan and Nguyen, 2006; Bartels and Sunkar, 2005; Janská et al., 2010).

Among the most effective metabolites, amino acids (i.e. phenylalanine, tryptophan and asparagine) changed according to pattern B in Figure 4.5. Amino acid changes have been observed in various plants exposed to water deficit stress (Barnett and Naylor, 1966; Draper, 1972; Handa et al., 1983; Rhodes et al., 1986; Fougère et al., 1991; Brosché et al., 2005; Zuther et al., 2007; Kempa et al., 2008; Sanchez et al., 2008a; Usadel et al., 2008; Lugan et al., 2010). These amino acid alterations can be interpreted as a “passive” or “active” effects of water shortage on plant metabolism. In passive effects, some enzymatic system has been impaired or even leads to protein breakdown under water stress conditions. Protein degradation along with lipid, chlorophyll, and nucleic acid breakdown occurs following ROS increase (Thompson et al., 1998). These degradation processes, which the plant exhibits in water deficit conditions, are similar to leaf senescence process, which result in cell death (Miller et al., 2008; John et al., 2001; Butt et al., 1998). However, if the response is adaptive, plants accumulate amino acids to cope with stress by acting in specific physiological roles such as osmoregulation, ion transport regulation, gene expression, redox balancing and stomata opening (Karamanos, 1995; Rai, 2002; Patterson et al., 2009). Application of exogenous amino acids resulted in relieving drought stress effects by regulating membrane permeability and ion uptake (Rai, 2002). In other words, amino acids

help to modulate inorganic solute (K^+ and Ca^{2+}) flow into the cells (Rana and Rai, 1996; Khanna and Rai, 1998) and therefore contribute stomatal opening and osmoregulation (Rai, 2002). Changes in individual amino acids and their possible role in plant responses to a developing drought stress are considered in the next section.

Phenylalanine (Phe), as well as being a component of proteins, is a precursor to synthesize various important metabolites such as flavonoids, phenylpropanoids, anthocyanins, cell wall lignin and other secondary metabolites (Tzin and Galili, 2010). Products of Phe metabolism have several functions, including protection against biotic and abiotic stresses, particularly via the antioxidant function of some products such as phenylpropanoid (Weisshaar and Jenkins, 1998; Vogt, 2010; Pichersky and Gang, 2000; D'Auria and Gershenzon, 2005; Casati and Walbot, 2005; Dixon, 2001). Among the secondary metabolites derived from Phe, about 1% are volatile and the major classes of secondary metabolites are Phenylpropanoids, Glucosinolates and 2-phenylethanol (Tzin and Galili, 2010). Phenylalanine can be converted into cinnamic acid and is involved in the biosynthesis of Salicylic Acid (SA). SA is well known in biotic stress as a stimulus of plant defence mechanisms, but recently various studies have confirmed that SA can act as a key molecule in signalling pathways of abiotic stress (Raskin, 1992; Klessig et al., 2000; Shah, 2003; Halim et al., 2006) including drought stress (Munne-Bosch and Penuelas, 2003; Peñuelas and Llusià, 2003; Chini et al., 2004). At low concentration SA improves plant stress tolerance, suggested to stem from the antioxidative properties of SA (Horváth et al., 2007).

The tryptophan (Trp) pathway ends with the phytohormone indole-3-acetic acid (IAA or auxin) and diverse secondary metabolites with various functions including participating in defence mechanisms against biotic and abiotic stresses (Tzin and Galili, 2010; Kutchan, 1995; Conn, 1995). The plant hormone IAA, in addition to its role in developmental process, has been observed in biotic and abiotic stress responses (Leyser, 2002; Woodward and Bartel, 2005; Lau et al., 2008). The latter has been confirmed during abiotic stress in rice including drought, salt and cold (Jain and Khurana, 2009). Diverse Trp-derived secondary metabolites are classified into 3 major classes, namely glucosinolates, indole alkaloids and camalexin (Tzin and Galili, 2010). Although the main pathway to synthesis of auxin is not known in detail, some auxins are synthesized from Trp (Gibson et al., 1972; Tsurusaki et al., 1997; Wright et al., 1991). Therefore some pathways have been suggested as possible IAA

pathways from Trp (Pollmann et al., 2002; Facchini et al., 2000a; Tao et al., 2008; Normanly et al., 1993). Glucosinolates have been observed in contribution to biotic stress defence mechanisms (Halkier and Gershenzon, 2006; Böttcher et al., 2009), while camalexin is associated with both biotic and abiotic stresses (Böttcher et al., 2009; Zhao and Last, 1996).

Another significant amino acid is asparagine (Asn). The chemical properties of Asn make it a major transportable source of nitrogen in xylem (Pate, 1980). Asn has been implicated in a few metabolic pathways, but was observed to accumulate under a number of abiotic stresses in different plants such as soybean (Fukutoku and Yamada, 1984; King and Purcell, 2005), alfalfa (Fougère et al., 1991), pearl millet (Kusaka et al., 2005) and wheat (Carillo et al., 2005). During drought stress, increased asparagine might be either a direct response of the plant via its playing role as an osmoprotectant in addition to a cytoprotectant (Kusaka et al., 2005) or an indirect response: i.e. it may be due to lack of protein synthesis under drought that leads to synthesis of another form of nitrogen (Lea et al., 2007).

Purine, pyrimidine and histidine metabolism has been perturbed by lowering either D-Xylulose-5P, D-Ribulose-5P or D-Ribulose-5P based on 'pattern A' Figure 3.5. This trend displays itself from the first day: these metabolites dramatically declined after the first day until the 7^{th} day and were fixed throughout the rest of the stress period (Figure 3.5). Decrease of the purine and pyrimidine pool affected by environmental stresses such as salt (Peterson et al., 1988; Peterson et al., 1987; Nieman et al., 1988) and drought has been reported in several plant species (Stasolla et al., 2003). It seems that impairment of the organic bases of nucleotides might be due to oxidative damage caused by ROS (Reactive Oxygen Species) (Sharma et al., 2012). ROS at low concentration serve as second messenger in various phytohormone responses such as biotic and abiotic stress (Torres et al., 2002; Miller et al., 2008), stomatal closure (Neill et al., 2002; Yan et al., 2007; Kwak et al., 2003), programmed cell death (Mittler, 2002; Bethke and Jones, 2001), root gravitropism (Joo et al., 2001), seed germination, hypersensitive responses and lignin biosynthesis (Sharma et al., 2012). High concentrations cause oxidative damage to biomolecules including lipids (Sharma and Dubey, 2005; Han et al., 2009; Tanou et al., 2009; Mishra et al., 2011), proteins (Sharma and Dubey, 2005; Tanou et al., 2009; Romero-Puertas et al., 2002; Maheshwari and Dubey, 2009) and DNA (Imlay and Linn, 1988). Oxidative damage to DNA ranges from

modification of nucleotides, deoxyribose oxidation, DNA strand breakage and nucleotide removal (Liu et al., 2000; Halliwell and Gutteridge, 1999).

Although we used FT-ICR as an ultra-high mass resolution technique and Mi-Pack identification software, we were still unable to putatively identify 50 out of 65 of the most effective peaks. To unambiguously identify the structure of the unknown metabolites or confirm the identity of the outstanding putatively identified metabolites, I suggest using tandem mass spectrometry (MS/MS) and/or NMR (Aharoni et al., 2002; Weber and Viant, 2010). Furthermore, using of SIM-stitching has improved DI FT-ICR platform yield with gaining higher dynamic range and lower mass error (Southam et al., 2007, Taylor et al., 2009). The traditional DI FT-ICR drawback was increased mass error due to trying to detect both low and high concentration metabolites (to get higher dynamic range)(Zhang et al., 2005a). Application of DI FT-ICR in this stress study strongly confirms the advantage of this approach over the current time-consuming analytical methods demonstrated by others (Aharoni et al., 2002, Taylor et al., 2009, Han et al., 2008). On the other hand, using a much smaller number of samples and also fast analysis makes this technique appropriate for a screening tool in stress studies particularly of large germplasm collections.

4.4. Conclusion

My drought stress investigation, conducted under growth room conditions with the stress imposed by withholding water, gives for the first time the possibility to investigate the combined physiological and metabolic responses of thyme. *Thymus vulgaris* drought tolerance was monitored under developing water deficit conditions. The traits measured included leaf water potential, shoot water content, fresh weight and soil moisture. Soil moisture decreased gradually, while water potential and water content were constant during the first week. Then both traits decreased significantly.

Daily harvest and extraction followed by DI FT-ICR allowed me to profile the metabolites of thyme. The clearly identified phases observed in the physiological responses could be correlated with the metabolic responses. It suggests that patterns in metabolite changes indicated by the results of DI FT-ICR may be causal to the physiological responses, since metabolic level precedes the underlying level of physiological status. Mass spectrometry allowed metabolite profiling in a small sample size and mixture of metabolites (Brown et al.,

2004). Within the all current mass spectrometry technologies, FTICR offers the highest mass accuracy and mass resolution, even with complex mixture samples. Therefore, without applying separation techniques prior to ionization (i.e. direct infusion) we could generate a list of massive peaks as m/z (Breitling et al., 2006; Breitling et al., 2008; Brown et al., 2004; Dunn, 2008; Southam et al., 2007; Takahashi et al., 2008).

In total, 28 metabolic pathways were affected by water deficit stress, which included carbohydrate, amino acid, energy, secondary metabolites, vitamins, and nucleotide metabolism. Within the identified metabolites, amino acids such as Phe, Trp and Asn were a key component in drought stress response act through phytohormones and secondary metabolites.

In conclusion, combined investigation at the physiological and metabolic level on a time course frame of *Thymus vulgaris* enabled us to identify significant metabolites and metabolic pathways in the sophisticated metabolic perturbations induced by water shortage. Moreover, the potential of this experimental design along with DI FTICR technology demonstrated that this approach can be a powerful tool for further studies on plant drought stress.

4.5. Summary of Chapter 4

During plant life, there are several factors affecting plant growth, development and finally their productivity. Water is one of the important environmental factors, as it is the main molecule in all living organisms. If soil water falls lower than a critical level, this will cause water deficit stress. This stress influences plant metabolism via both direct and indirect ways. Direct effects include effects on photosynthesis, growth and development, while indirect effects are those such as damage due to oxidative stress.

Thymus vulgaris or common thyme is well known worldwide since ancient times for its medicinal and culinary uses. Its extract has antiseptic, antibacterial and spasmolytic properties (Stahl-Biskup and Sàez, 2002, Sagdic et al., 2002).

To my knowledge, there are limited studies on the effects of stress of *Thymus* species. These studies mainly focused on morpho-physiological responses of thyme to water deficit stress. Moreover they have studied the effect of drought stress only on essential oil components.

However, there are no metabolomics studies on thyme subject to abiotic stresses. In this experiment we combined morpho-physiological parameters (including water potential, water content, shoot fresh weight and soil moisture) with metabolite changes during water deficit stress. All physiological parameters that significantly changed corresponded to the soil moisture decrease. Likewise, the patterns of metabolite changes indicated by the results of DI FT-ICR reflected the physiological responses. Non-targeted metabolite profiling was carried out by DI FTICR mass spectrometry. This approach could detect 4755 peaks, of which 65 were selected as the most effective peaks based on their PCA loading scores. The selected peaks followed 3 major patterns over time, which have been described in detail. To putatively identify the metabolites and metabolic pathways, those peaks were submitted to Mi-Pack analysis. Major compounds, namely phenylalanine, tryptophan, asparagine, N-carbamyl-β-Alanin and xylulose/ribulose/ribose were affected under water shortage conditions. We highlighted the important role of these compounds in drought stress tolerance via plant hormones, secondary metabolite biosynthesis and purine, pyrimidine and histidine metabolism. Here, the results confirm the application of high-throughput approach DI-FTICR to study drought stress responses of thyme populations.

In the next chapter, two extreme populations of thyme with respect to tolerance to drought stress have been selected following physiological experiments. Populations will be exposed to water deficit stress using a similar protocol of our previous experiments. Next, in addition to the physiological traits, leaf samples will be harvested to perform metabolite profiling. Based on observed responses in those groups, major metabolites and involved pathways will be discussed.

CHAPTER V. COMPARATIVE METABOLOMIC APPROACH TO UNDERSTAND THE MOLECULAR MECHANISMS UNDERLYING DROUGHT STRESS TOLERANCE IN THYME

5.1. Introduction

Response to water deficit stress at the physiological level in addition to essential oil content has been demonstrated in several species of the genus including *Thymus vulgaris* (Babaee et al., 2010; Letchamo et al., 1994; Aziz et al., 2008), *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 plant 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 (Cramer et al., 2007; Mane et al., 2008; Vasquez-Robinet et al., 2008; Rizhsky et al., 2004; Semel et al., 2007; Levi et al., 2011; Sanchez et al., 2012b; Bowne et al., 2012; Silvente et al., 2012).

In spite of a detailed knowledge of plant responses to water deficit, there are some aspects that require further study, such as strategies of the plant against dehydration. Comprehensive metabolite profiling through describing the molecular mechanisms underlying drought tolerance can facilitate future research to develop drought tolerant plants (Valliyodan and Nguyen, 2006; Umezawa et al., 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., 2009b), no comprehensive investigation has been carried out using metabolomics in thyme to date.

In the current study, two thyme populations I have previously identified as varying in drought tolerance (*T. vulgaris* as drought sensitive and *T. serpyllum* as an example of a drought tolerant population; Chapter 3 of this thesis), were subjected to water deficit stress in order to determine the major metabolite and metabolic pathways that might contribute to drought tolerance in thyme.

5.2. Results

5.2.1. Identification of the critical point for physiological traits comparison

To investigate the physiological responses of thyme to drought stress, both tolerant and susceptible populations were exposed to prolonged water deficit stress. To compare two populations with differing tolerance to drought, identifying the time at which they had a similar physiological status was necessary. To identify the proper time for recording traits

and also harvesting leaf samples for further metabolomics experiments, survivability index experiments (explained in chapter 3) was used. Both susceptible and tolerant populations were planted in pots and grown for 1 month, then watering was stopped and soil moisture and survivability indices were recorded daily until the plants wilted. Survivability indices were: 5 (fresh plants), 4 (some of the leaves fell due to wilting), 3 (some of the leaf tip dried), 2 (more than half the plant dried) and 1 (completely dried). Index 3 was chosen as a time point for recording physiological traits as well as for harvesting leaf samples for metabolomics (Figure 5.1). My data from chapter 4 showed that at earlier times, there is no much effects on plant metabolites, likewise later on plant will be wilted and it is not suitable for harvest. At the chosen time point, the tolerant population (*T. serpyllum)* had 1% soil moisture for and took 14 days to reach that physiological phase (a). The susceptible population (*T. vulgaris)* had 4.5% soil moisture and took 11 days to reach that physiological phase (b).

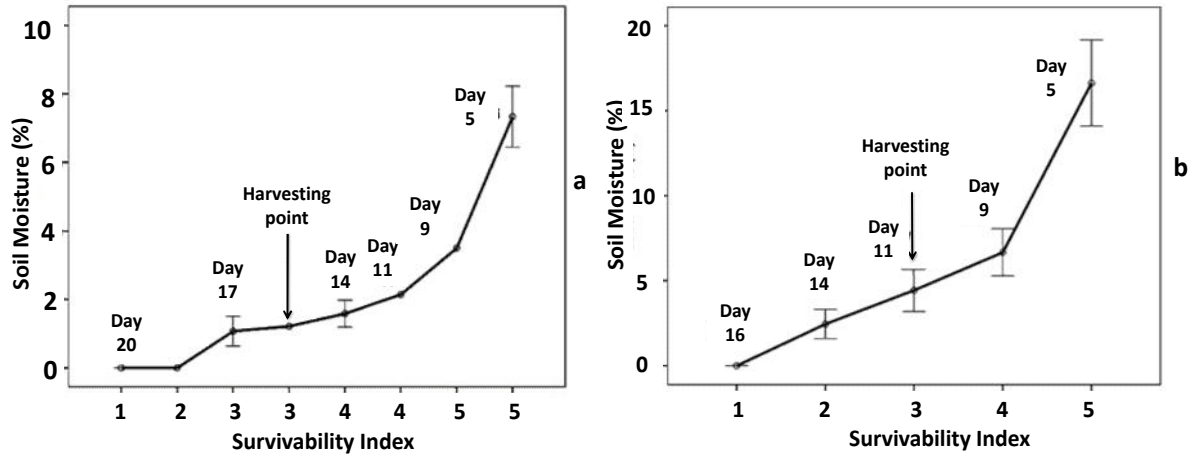


Figure 5.1. Identification of the critical point in water deficit stress based upon survivability index.

Critical point is the point at which both populations had the similar physiological status. To identify this point, both susceptible (b) and tolerant (a) populations were planted in pots and grown for 1 month, then watering was stopped and soil moisture and survivability indices were recorded daily until plants wilted. Survivability indices were from 5 (fresh plants) to 1 (completely dried) as described in material and methods section. Index 3 was chosen as a time point for recording physiological traits as well as for harvesting leaf samples for metabolomics. This point had 1% soil moisture for the tolerant population i.e. *T. serpyllum* and it took 14 days to reach that physiological phase (a). The susceptible population i.e. *T. vulgaris* had 4.5% soil moisture and it took 11 days to reach that physiological phase (b). Error bar= ± 1 SEM

5.2.2. Water relations and physiological responses to water deficit

Two main indicators of plant water status, along with soil moisture and shoot dry weight, were recorded at the end of stress period before wilting. Soil moisture was constant at the beginning of stress period, but started to differentiate during early days of stress period, (Figure 5.2). Soil moisture in pots of the tolerant population decreased more slowly than in the susceptible. Thus *T. serpyllum* utilised less water for growth and metabolism than *T. vulgaris*. Shoot dry weight was measured at the end of stress period in both watered and treated plants (Figure 5.2). Significant differences were observed between control and treated populations after water deficit stress period.

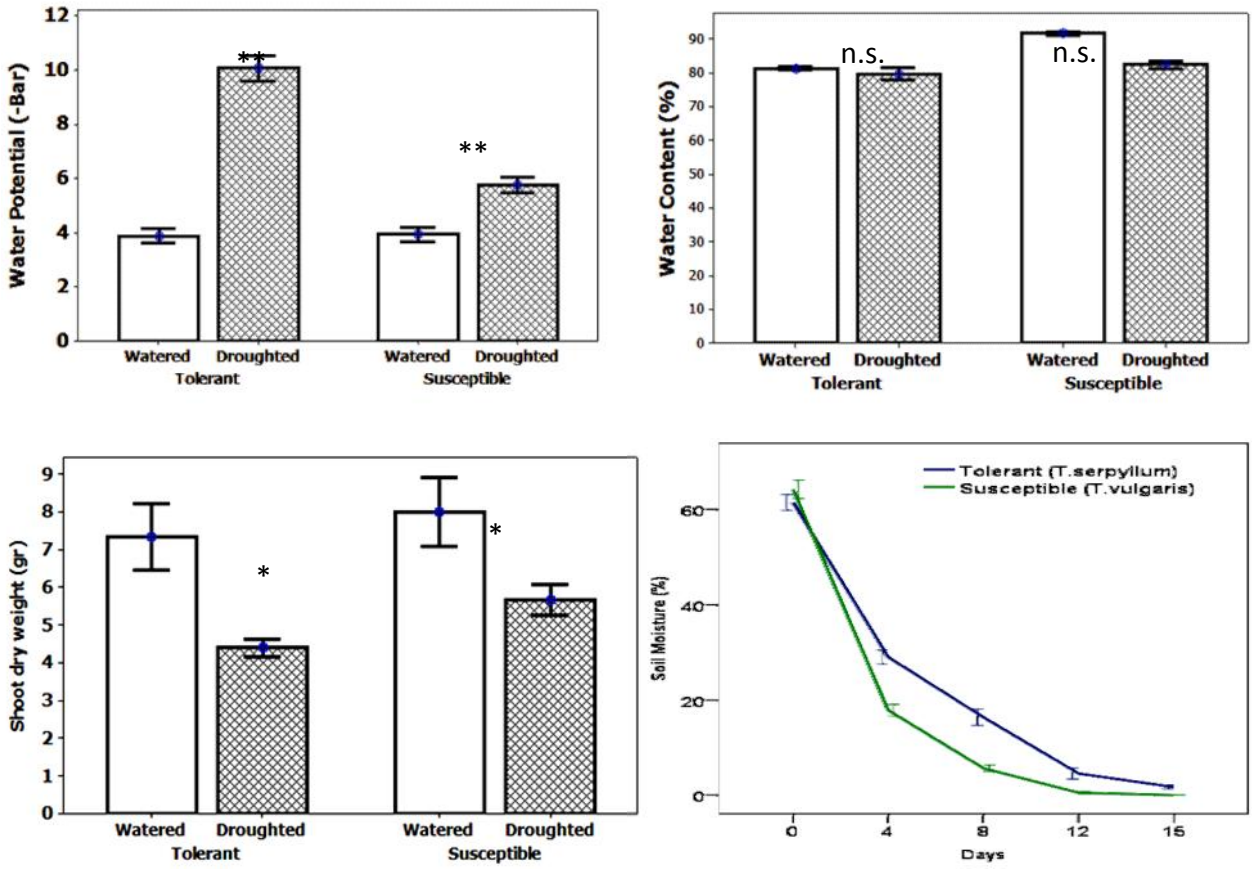


Figure 5.2. Growth and water status parameters affected by water stress in *T. vulgaris* (susceptible population) and *T. serpyllum* (tolerant population).

The growth parameter i.e. shoot dry weight decreased about 1.5 times. Water status parameters i.e. water content (WC), water potential (WP) and soil moisture (SM) were affected significantly after water withholding. WC declined from 90% to 80%, but water potential altered from -4 bar in watered to -6 bar in droughted plants for sensitive plants. Soil moisture was over 60% before stress period, but drastically decreased the following day util the 4th day to 20%. Soil

moisture gradually declined until the 12th day to nearly 0%. Treated traits between the two populations were compared by T-test and result indicated by ** (p<0.01), * (p<0.05) and n.s. (non-significant). Values are mean ± SEM of 5-10 replicates.

Even though water shortage significantly decreased the leaf water potential of stressed plants compared to watered (Figure 5.2), the tolerant and susceptible populations showed different responses. Both populations had similar leaf water potential in watered conditions; however under drought, *T. serpyllum* had lower leaf water potential than *T. vulgaris*.

5.2.3. Alteration in Metabolites following water tress in sensitive and tolerant populations

Non-targeted DI-FTICR mass spectrometry-based platform in positive ion mode for the polar fraction and in negative ion mode for the non-polar fraction was employed to measure metabolite changes over the drought stress period in 4 groups including 'Tolerant' population droughted (TD), tolerant population watered (TW), susceptible population droughted (SD) and susceptible population watered (SW) with 6 biological replicates/group and 3 technical replicates. In total 3328 peaks for polar and 2527 peaks for non-polar ions were detected by this approach.

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 5.3). The four groups (Tolerant Droughted, Tolerant Watered, Susceptible Droughted and Susceptible Watered) were well separated by the first two PCs. 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.

A) 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.

B) Non-polar negative ions: 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.

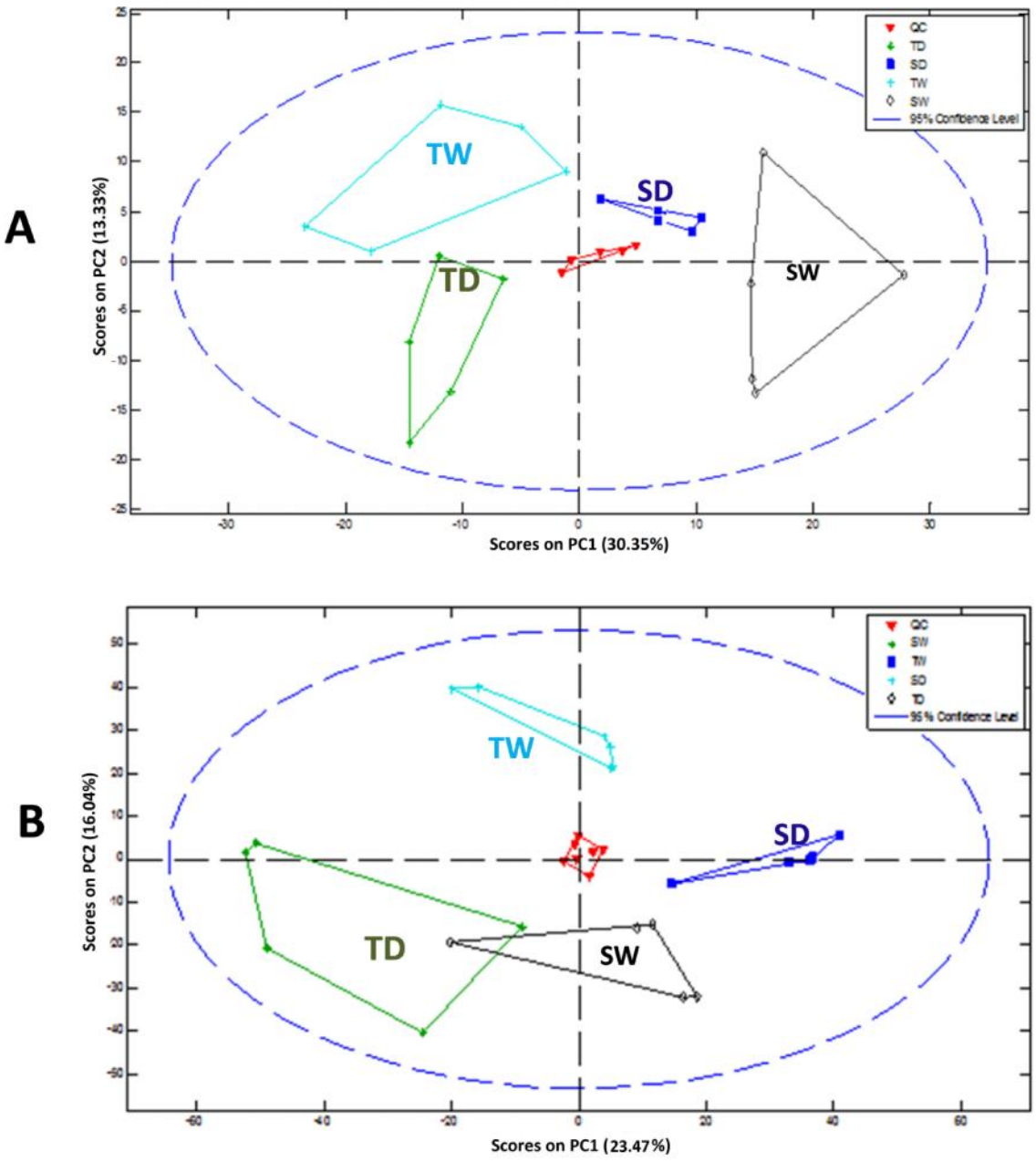


Figure 5.3. Score plot of PCA on polar and non-polar metabolite extracts for the tolerant and susceptible thyme plants grown in control and droughted conditions.

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: 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. B) non-polar negative ions: 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.

5.2.3.1. Metabolite profiling of *Thymus vulgaris* (SP) following water deficit stress

5.2.3.1.1. Polar metabolites

To understand the responses of sensitive 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 up-regulating and 35 down-regulating (for a complete list of significant metabolites see appendix 5). 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. Some selected metabolites are shown in Table 5.1. Of the amino acids and sugars, the only compounds significantly increased were tryptophan and ribose respectively. The most pronounced up-regulated metabolites were compounds including guanine, shikimate, isochorismate, jasmonic acid, ferulate and dehydroquinate. Within the significantly down-regulated metabolites, outstanding compounds were amino acids including alanine, glutamate, phenylalanine, threonine, asparate and methionine. This population had a decrease in organic acids including aconitate, ascorbate and homocitrate. Some sugars declined following water deficit included galactosylglycerol and erythrose. The interesting compound detected within the down-regulated metabolites was linalool, since it is a commercially important volatile (Table 5.1).

Table 5.1. Some selected significantly changed polar metabolites after water deficit stress in sensitive population (*T. vulgaris*).

No.	m/z	Metabolite Putative name	Fold change	Metabolism impaired
1	152.0567	Guanine	8.126021	Purine
2	383.053	3',5-Dihydroxy-3,4',7-trimethoxyflavone	6.82898	Secondary metabolites

3	297.0735	L-1-glycero-3-phosphocholine	3.835445	Glycerophospholipid
4	207.0054	Homogentisate	3.678512	Tyr and terpenoid
5	279.0628	Liquiritigenin	2.055584	Secondary metabolites
6	243.0529	Tryptophan	2.063225	Amino acid metabolism
7	163.039	Umbelliferone	1.950639	Secondary metabolites
8	249.0159	2-hydroxycaffeate, 5-Hydroxyferulate	1.926832	Phenylpropanoid biosynthesis
9	181.0495	Caffeic acid, frulate	1.895583	Phenylpropanoid biosynthesis
10	215.0401	indole-3-acetamide	1.731181	Tryptophan metabolism
11	213.016	Shikimate	1.576528	Hormones (IAA, SA)
12	223.0367	Choline phosphate	1.356969	Betaine biosynthesis
13	249.0886	(+)-7-Isojasmonic acid	1.318742	Hormones (JA)
14	247.0214	Isochorismate	1.279165	Phenylpropanoid, terpenoid and hormones
15	191.0141	α-D-xylose, α-L-arabinopyranose, L-ribulose, L-xylulose, Ribulose, pentose-ring	0.755605	Pentose interconversions
16	170.0214	5-Aminolevulinate, trans-4-hydroxy-L-proline, L-glutamate-gamma;-semialdehyde	0.734922	Amino acid metabolism
17	159.0054	Erythrose	0.717474	Carbohydrate metabolism
18	141.9901	Carbamoyl phosphate	0.687481	Amino acid metabolism
19	255.1075	Galactosylglycerol	0.677701	Galactose metabolism
20	212.9796	L-dehydro-ascorbate, cis-Aconitate	0.677697	TCA cycle, secondary met.
21	193.0989	Linalool, Menthone	0.66174	Volatile
22	259.0213	myo-Inositol 1-phosphate	0.654594	Inositol Phosphate met.
23	247.0038	homo-isocitrate, homocitrate	0.596293	Amino acid metabolism
24	128.0108	Alanine, Sarcosine, beta-Alanine	0.58143	Amino acid metabolism

25	148.0604	L-Glutamate, O-acetyl-L-serine	0.505933	Amino acid metabolism
26	220.0816	O-succinyl-L-homoserine	0.449596	Amino acid metabolism
27	204.0421	L-Phenylalanine	0.434908	Amino acid, hormones
28	130.0499	5-Oxoproline, Pyrroline hydroxycarboxylic acid	0.425213	Amino acid metabolism
29	172.0007	D-Aspartate	0.393028	Amino acid, hormones
30	214.0112	4-phospho-hydroxy-L-threonine, L-aspartyl-4-P	0.319532	Amino acid metabolism
31	164.074	Homomethionine	0.199405	Amino acid metabolism

In total, 60 metabolic pathways are implicated after imposing water deficit stress in *T. vulgaris* including 24 secondary metabolites, 18 amino acids, 8 carbohydrates, 3 nucleic acid, 2 lipid, 2 energy, 2 vitamin, 1 transport, 1 hormones and 1 translation (for full list of metabolites see appendix 6).

5.2.3.1.2. Non-polar metabolites changes by water deficit stress

To profile lipids in drought sensitive 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 sensitive plant see appendix 7), but some interesting compounds has been indicated in Table 5.2.

Table 5.2. Changes in non-polar metabolites of *T. vulgaris* leave after prolonged water deficit stress.

No.	m/z	Metabolite Putative name	Fold change	Metabolism impaired
1	471.3485	4α-formyl-5α-cholesta-8,24-dien-3β-ol, 2-hydroxyoleanolate	10.65649	terpenoid metabolism
2	453.338	5,7,22,24(28)-ergostatetraenol	3.255588	sphingolipid biosynthesis
3	607.292	all-trans-Hexaprenyl diphosphate	2.968147	secondary metabolites

4	229.0274	ferulic acid	2.850011	phenylpropanoid biosyn.
5	455.3527	5-dehydro episterol	2.836833	steroid biosynthesis
6	151.0402	Methyl salicylate	2.427382	phenylpropanoid biosyn.
7	423.4209	Octacosanoic acid	2.019534	saturated fatty acid
8	528.2689	1-18:2-lysoPE	1.716795	linoleate biosynthesis
9	437.4371	Nonacosanoic acid	1.662246	saturated fatty acid
10	451.4527	Melissic acid	1.619134	saturated fatty acid
11	479.484	Lacceroic acid	1.615193	saturated fatty acid
12	723.4256	18:3-16:3-PA	1.498831	unsaturated fatty acid
13	493.4997	Psyllic acid	1.430454	saturated fatty acid
14	379.2493	sphinganine 1-phosphate	1.360567	sphingolipid metabolism
15	293.2487	Sterculic acid	0.825311	lipid metabolism
16	373.2597	9,10-epoxystearate	0.75826	lipid metabolism
17	449.2551	1-16:1-lysoPE	0.711928	lipid metabolism
18	790.5221	18:2-18:3-MGDG, 18:3-18:2-MGDG	0.69887	lipid metabolism
19	357.2072	Delta;9-tetrahydrocannabinolic acid	0.657837	secondary metabolites
20	802.4649	18:2-18:3-PS	0.653111	lipid metabolism
21	281.2486	oleic acid, Stearate	0.64726	unsaturated fatty acid
22	225.186	Myristoleate, Myristate	0.641802	lipid metabolism
23	125.0357	Thymine	0.618435	pyrimidine metabolism
24	741.4721	18:3-t16:1-PG	0.616271	lipid metabolism
25	815.5279	18:1-18:3-MGDG, 18:2-18:2-MGDG	0.61589	lipid metabolism
26	742.476	1,2-dipalmitoyl-phosphatidylglycerol	0.612451	lipid metabolism
27	935.5757	18:3-18:3-DGDG,18:2-18:3-DGDG, 18:3-18:2-DGDG	0.585909	lipid metabolism
28	755.4754	16:0-18:3-PS	0.581752	lipid metabolism

29	356.2807	4,8-sphingadienine	0.581544	sphingolipid metabolism
30	817.5419	18:0-18:3-MGDG, 18:1-18:2-MGDG	0.57221	lipid metabolism
31	848.5681	18:0-18:1-PS	0.552131	lipid metabolism
32	745.5041	18:1-t16:1-PG, 18:2-16:0-PG	0.550731	lipid metabolism
33	820.5335	1-Hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phosphoserine	0.550426	lipid metabolism
34	973.6132	16:0-18:3-DGDG, 16:1-18:2-DGDG, 18:3-16:0-DGDG	0.537255	lipid metabolism
35	971.5966	18:2-16:2-DGDG, 18:3-16:1-DGDG	0.533996	lipid metabolism
36	441.2652	(3S,5R,6S)-5,6-epoxy-3-hydroxy-5,6-dihydro-12'-apo-β-caroten-12'-al	0.532922	trepenoid metabolism
37	429.374	α-Tocopherol	0.524467	antioxidant metabolism
38	819.5557	18:0-18:3-PC, 18:1-18:2-PC, 18:2-18:1-PC, 18:0-18:2-MGDG, 18:1-18:1-MGDG	0.509882	lipid metabolism
39	719.4881	16:0-16:1-PG, 1,2-dipalmitoyl-phosphatidylglycerol	0.508343	lipid metabolism
40	550.4848	N-(2-hydroxyhexadecanoyl)-4,8-sphingadienine	0.486931	sphingolipid metabolism
41	599.4114	9'-cis-Neoxanthin, Neoxanthin, Violaxanthin	0.476259	trepenoid metabolism
42	845.5516	20:2-18:3-PC	0.450911	lipid metabolism
43	742.5406	18:0-18:2-PE, 18:1-18:3-PE	0.433134	lipid metabolism
44	751.5381	18:0-16:3-MGDG, 18:1-16:2-MGDG, 18:2-16:1-MGDG, 18:3-16:0-MGDG	0.37511	lipid metabolism
45	331.1551	Gibberelline A20, Gibberelline A4, Gibberelline A51	0.361041	hormone metabolism
46	842.5207	18:2-18:2-PS, 18:2-18:3-PS	0.348773	lipid metabolism
47	414.2944	β-apo-8-carotenal	0.271468	terpenoid metabolism
48	995.5966	18:3-18:3-DGDG	0.256217	lipid metabolism

The most relevant metabolites among the non-polar metabolites were Methyl salicylate and 1-18:2 lyso PE for up-regulated and decreased lipids across the diverse categories lipids including MGDG, DGDG, PC and PS as well as vitamin E and gibberelline.

5.2.3.2. Metabolite changes in *T. serpyllum* exposed to long-term water limitation

5.2.3.2.1. Polar metabolites which significantly changed in *T. serpyllum*

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 appendix 8 for full list). Selected metabolites are listed in table 5.3 (selection procedure described in section 5.2.3.1.1.

Table 5.3. Select polar metabolites altered due to water deficit stress in tolerant population (*T. serpyllum*).

No.	m/z	Metabolite Putative name	Fold change	Metabolism involved
1	221.0211	D-Proline	3.675039	amino acid
2	146.0924	Succinate, erythronic acid lactone	3.206736	amino acid, hormones and secondary metabolites
3	409.0646	4-Guanidinobutanoate	2.616624	arginine and proline metabolism
4	213.037	Betaine, L-Norvaline, L-Valine, N,N-dimethyl-β-alanin	2.501454	amino acid
5	213.016	phenylacetonitrile oxide	2.181992	glucosinolate
6	215.0143	α-D-xylose, α-L-arabinopyranose, L-ribulose, L-xylulose, Ribulose, pentose-ring	1.925916	pentose phosphate pathway
7	221.0421	(S)-3-Methyl-2-oxopentanoic acid, 4-Methyl-2-oxopentanoate	1.884176	amino acid, secondary metabolite
8	219.0475	Coumarin	1.751052	phenylpropanoid
9	138.0525	4-hydroxybenzoate, salicylate	1.730372	hormone

10	156.0421	Indole-3-acetaldehyde	1.660766	triptophan metabolism
11	229.0108	3-dehydroquinate	1.627809	Phe, Tyr and Trp biosyn.
12	215.0401	3-dehydro-shikimate	1.600713	leucine biosynthesis
13	183.0418	1-deoxy-D-xylulose 5-phosphate	1.545268	amino sugar, nucleotide sugar and Secondary metabolites
14	211.0003	Shikimate	1.538656	hormone
15	213.0636	N-alpha-acetylornithine	1.474433	proline biosynthesis
16	182.0578	Indol-3-acetamide, Indole-3-acetaldehyde oxime, L-ascorbate, Coniferyl aldehyde	1.459458	glucosinolate biosynthesis, hormone biosynthesis
17	168.0421	2-Oxo-5-methylthiopentanoic acid, 2-Oxoadipate	1.443312	glucosinolate biosynthesis
18	423.1053	Citrate, Isocitrate	1.400747	TCA cycle
19	417.1522	D-Gluconic acid	1.385645	pentose phosphate pathway
20	383.1106	Choline phosphate	1.261673	betaine biosynthesis
21	169.0261	D-Iditol, D-Sorbitol, Mannitol	1.260879	fructose and mannose metabolism
22	243.0265	D-arabinose 5-phosphate, D-ribulose-1-phosphate	1.175745	carbohydrate metabolism
23	138.0316	shikimate-3-phosphate	1.149642	amino acid, terpenoid biosyn
24	255.0264	o-succinylbenzoate	1.142614	terpenoid-quinone biosyn
25	275.0163	gibberelline A28	0.561308	hormone

Of the carbohydrates significantly affected, all were up-regulated, including sorbitol, mannitol, xylulose, gluconic acid and iditol. The amino acids betaine, proline, valine and citrolline increased in drought stressed plants in comparison to controls, except for serine. Organic acids mostly increased in tolerant compare to sensitive, including salicylate, succinate, oxoadipate, shikimate, dehydroquinate and citrate, while only gibberelline decreased.

5.2.3.2.2. Alterations in non-polar metabolites following drought in the tolerant plant (*T. serpyllum*)

Metabolite profiling of tolerant plants 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 appendix 9. Selected compounds are indicated in Table 5.4.

Table 5.4. Effect of water deficit stress on the non-polar metabolite changes of tolerant population *T. serpyllum.*

No.	m/z	Metabolite Putative name	Fold change	Metabolism involved
1	347.1864	Gibberelline A14, Gibberelline A53	2.391962	hormone
2	845.5516	20:2-18:3-PC	2.315085	lipid metabolism
3	429.374	alpha-Tocopherol	2.180552	antioxidant metabolism
4	840.5053	18:2-18:3-PS	2.138315	lipid metabolism
5	331.0824	Pinobanksin, licodione, naringenin, naringenin chalcone	1.953487	secondary metabolites
6	347.0773	dihydrokaempferol, eriodictyol	1.891305	secondary metabolites
7	747.6096	plastoquinone-9	1.789968	terpenoid biosynthesis
8	817.5419	18:0-18:3-MGDG, 18:1-18:2-MGDG	1.754854	lipid metabolism
9	445.3246	5-Dehydroavenasterol, 4,4-Diapophytoene	1.598501	terpenoid biosynthesis
10	844.5362	18:0-18:3-PS, 18:1-18:2-PS	1.569699	lipid metabolism
11	842.5207	18:2-18:2-PS	1.539988	lipid metabolism
12	819.5557	18:0-18:3-PC, 18:1-18:2-PC, 18:2-18:1-PC, 18:0-18:2-MGDG, 18:1-18:1-MGDG	1.530815	lipid metabolism
13	815.5279	18:1-18:3-MGDG, 18:2-18:2-MGDG	1.498786	lipid metabolism
14	417.3222	1-monostearin	1.493887	lipid metabolism
15	751.5381	18:0-16:3-MGDG, 18:1-16:2-MGDG, 18:2-16:1-MGDG, 18:3-16:0-MGDG	1.453924	lipid metabolism
16	816.5037	16:0-18:3-PS	1.439862	lipid metabolism
17	419.3294	Pentacosanoic acid, brassicasterol, campest-5-	1.432926	lipid metabolism

		en-3-one, crinosterol, episterol		
18	697.4829	18:0-18:3-PA, 18:1-18:2-PA	1.431091	lipid metabolism
19	791.5746	2-nonaprenyl-6-methoxy-1,4-benzoquinol	1.418327	secondary metabolites
20	792.5774	1,2-dipalmitoyl-phosphatidylcholine	1.388556	lipid metabolism
21	973.6132	16:0-18:3-DGDG, 16:1-18:2-DGDG, 18:3-16:0-DGDG	1.381646	lipid metabolism
22	599.4114	9-cis-violaxanthin, 9-cis-Neoxanthin, Neoxanthin, Violaxanthin	1.333432	terpenoid metabolism
23	936.5793	18:2-18:3-DGDG, 18:3-18:2-DGDG	1.310341	lipid metabolism
24	971.5966	18:2-16:2-DGDG, 18:3-16:1-DGDG	1.280008	lipid metabolism
25	832.5106	16:0-18:2-PI, 20:1-18:3-PS, 20:2-18:2-PS	1.159914	lipid metabolism
26	523.3642	1-18:0-lysoPC, Castasterone	0.878067	lipid metabolism
27	509.3851	6-deoxocastasterone	0.78317	Hormone metabolism
28	662.6107	N-(2-hydroxytetracosanoyl)-4,8-sphingadienine	0.715574	lipid metabolism
29	636.5944	4-hydroxysphing-8(E)-enine-22:0, ceramide, 4-hydroxysphing-8(Z)-enine-22:0, ceramide	0.66973	lipid metabolism
30	692.6577	4-hydroxysphing-8(E)-enine-26:0, ceramide, 4-hydroxysphing-8(Z)-enine-26:0, ceramide	0.668364	lipid metabolism
31	521.3488	1-Oleoylglycerophosphocholine	0.634523	lipid metabolism
32	505.3536	3-dehydroteasterone	0.587623	secondary metabolites
33	519.3331	1-Linoleoylglycerophosphocholine	0.52619	lipid metabolism
34	581.3709	1-Oleoylglycerophosphocholine	0.508969	lipid metabolism
35	623.2868	Presqualene diphosphate, all-trans-Hexaprenyl diphosphate	0.446375	lipid metabolism
36	295.2279	16-oxo-palmitate, 18-hydroxyoctadeca-9Z,12Z-dienoate, vernoleate	0.42548	lipid metabolism
37	327.2905	Octadecanal	0.170724	lipid metabolism

The majority of lipids belonging to diverse classes increased in tolerant plants (*T. serpyllum*) under drought. Notable lipids changing included classes of MGDG, DGDG, PD, PC, PI while lyso PC decreased. Moreover, elevating violaxanthin is very interesting. Since it is substrate of ABA and might increase the level of ABA concentration under stress condition (Frey et al., 1999).

5.2.4. Differences between susceptible and tolerant populations at the metabolite level

Using DI FT-ICR as an ultra-high mass resolution technique, non-targeted metabolites profiling detected 3328 polar metabolites. Uni-variate statistical test showed significantly changed metabolites following water deficit. Drought stress affected more metabolites in sensitive plants than tolerant ones. In sensitive plants, 605 polar and 695 for non-polar metabolites were significantly changed while for tolerant plants the corresponding figures were 144 and 591 for polar and non-polar metabolites respectively. Mi-Pack software (Weber and Viant, 2010) allowed identification of the significant metabolites. As indicated in Figure 5.4, out of 3328 peaks in polar fraction, 56 and 92 metabolites were identified in tolerant and sensitive populations. Likewise, the non-polar metabolites had 61 and 94 metabolites putatively identified for tolerant and sensitive respectively (Figure 5.4).

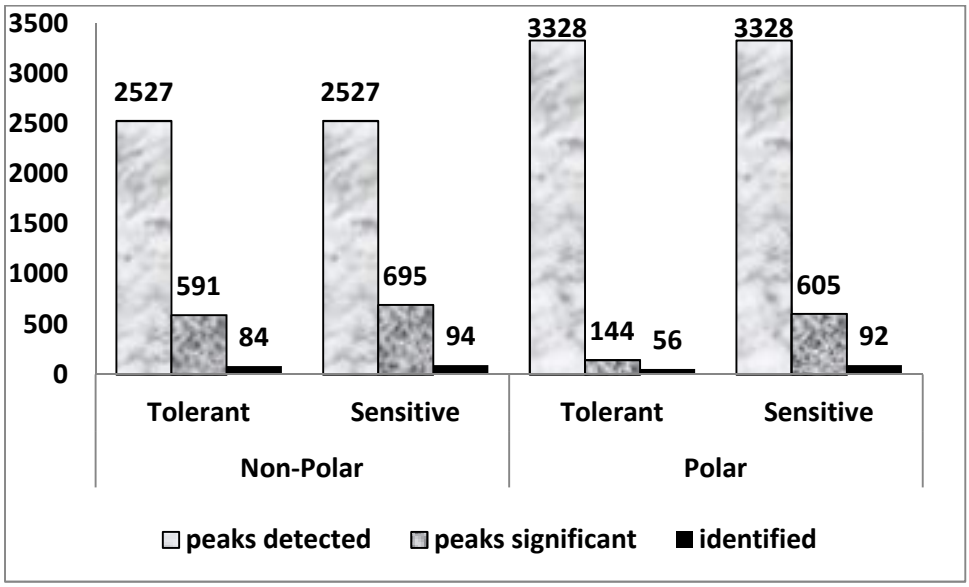


Figure 5.4. Summary of metabolite numbers detected, identified and affected by water deficit stress in tolerant and sensitive thyme plants.

This graph shows the scale of metabolite profiling considering polarity and significance. FT-ICR mass spectrometry based method detected 3328 and 2527 peaks in polar and non-polar fractions respectively. Sensitive plant had more significant metabolites than tolerant ones.

Venn diagram shows total number of peaks increasing or decreasing in the populations (Figure 5.5). 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.

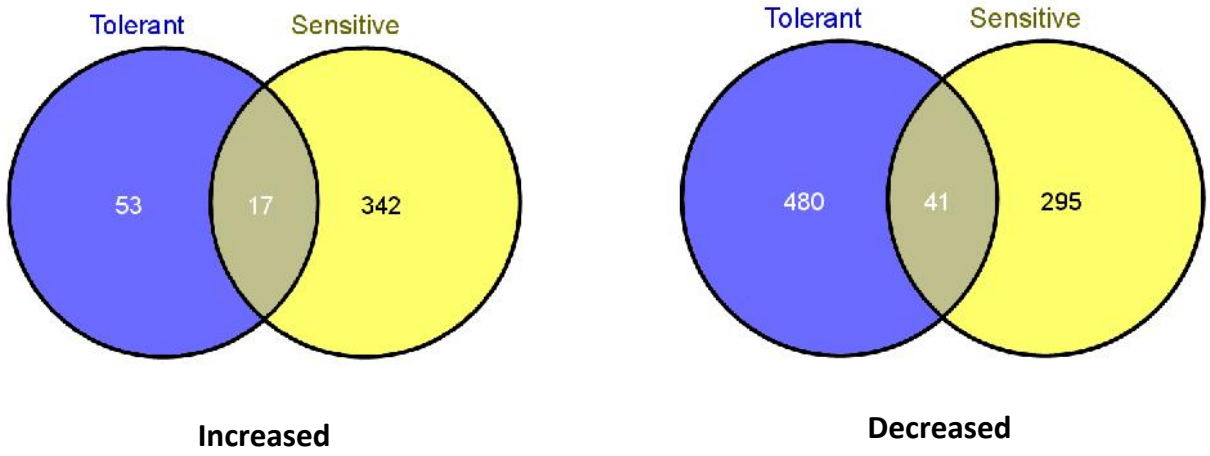


Figure 5.5. Total number of peaks significantly increased/decreased in droughted plants compared to watered.

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.

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 5.6 For amino acids, sensitive plants had a decrease in all detected compounds except for tryptophan, while tolerant plants had elevation in all detected amino acids except for serine. Proline and citrulline had the largest increase. Moreover in sensitive plants, homomethionine had the largest decrease. Regarding carbohydrates, all were up-regulated in tolerant population with the highest being xylulose, while in sensitive plants, galactoglycerol and erythrose were down-regulated and D-Xylulose-5-phosphate up-regulated. Most of the organic acids had increased in both

populations except for gibberellins in tolerant plants and homocitrate and aconitate 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, whereas most of the lipids in sensitive plants declined.

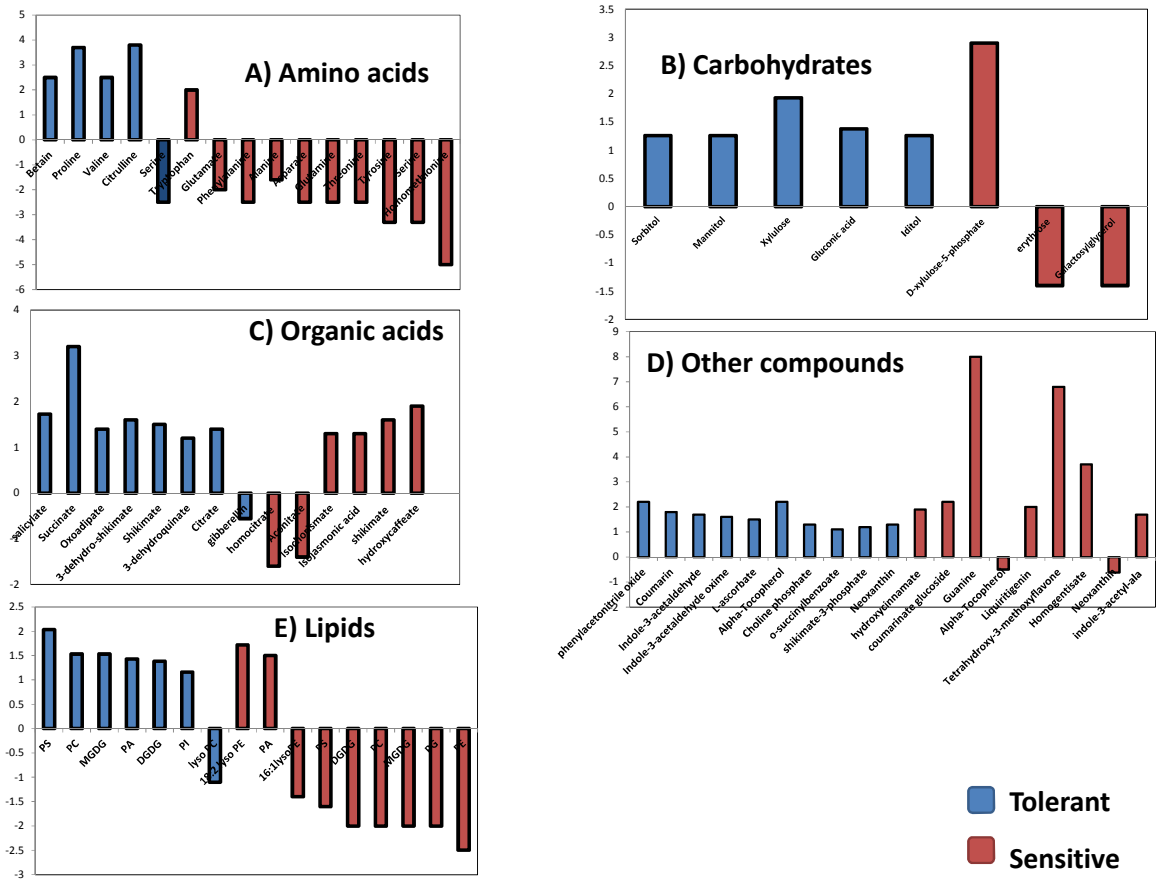


Figure 5.6. Metabolite changes regarding with their major classes of compounds.

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 up-regulated in tolerant population with the maximum of xylulose, while in sensitive plants galactoglycerol and erythrose down-regulated and D-Xylulose-5-phosphate up-regulated. 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

5.2.5. Metabolic pathways are altered in the plants subjected to water limitation

The altered metabolites are illustrated along with metabolic pathways perturbed to water deficit in both tolerant and sensitive populations (Figure 5.7).

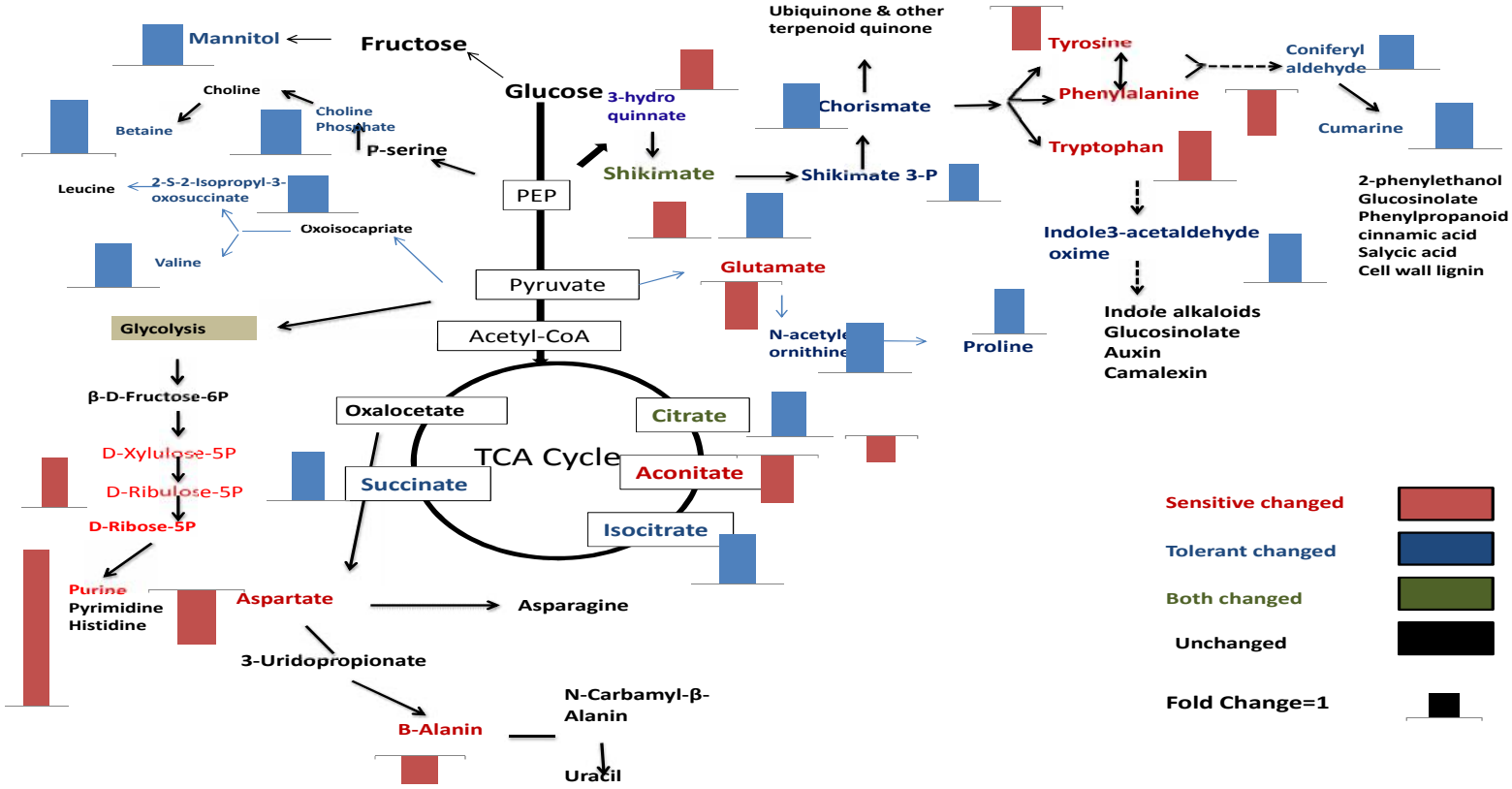


Figure 5.7. Presentation of the selected metabolites and metabolic pathways affected by drought stress in tolerant and sensitive thyme plants.

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.

5.3. Discussion

These results indicate the efficiency of physiological indicators in drought tolerance experiments. Soil water content as a quantified and controlled water deficit stress enables the performing of accurate drought stress experiments (Granier et al., 2006). Morpho-physiological platform has been proven as a robust tool of water deficit stress evaluation across the different populations (Aguirrezabal et al., 2006; Reymond et al., 2003). Likewise, reduction of shoot dry weight as an adverse effect of water stress has been reported in many plants (Duan and Zhao, 1996; Pan et al., 2003). It has been suggested that reduced plant growth, photosynthesis and leaf senescence (Bhatt and Srinivasa Rao, 2005) as well as altered biomass distribution due to changed resources cause dry matter reduction (Weiner, 1985; Wu and Wang, 1999; Pan et al., 2002; Shao et al., 2009a). In conclusion, *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.

Based on these results, main tolerance mechanisms can be categorized into four classes as follows:

5.3.1. Osmotic adjustment as a key mechanism of drought response

One of the mechanisms of drought tolerance is known as osmotic adjustment. This mechanism, by lowering the internal cellular osmotic potential (Turner and Jones, 1980) enhances plant tolerance to water deficit through allowing continued water uptake from the environment (Kishor et al., 2005), stomatal and photosynthetic adjustment (Ludlow et

al., 1980) as well as maintaining cell expansion (Bhatnagar-Mathur et al., 2008; Miller et al., 2008; Mittler, 2006) hence resulting in more plant growth and yield (Morgan, 1983). 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). These compatible (non-toxic) compounds are also referred as osmoprotectants or osmolytes and generally are small electrically neutral molecules (Alonso et al., 2001) and accumulate in cytoplasmic compartments of the cells (Heuer, 1999). Recent studies have established a key role of ABA in the regulation of metabolic adjustment (Krasensky and Jonak, 2012) and ABA induces accumulation of many osmolytes such as proline (Kempa et al., 2008).

5.3.1.1. The role of amino acids as osmoregulators

In 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).

5.3.1.2. The role of carbohydrates as osmoregulators

All the carbohydrates including xylulose, gluconic acid, sorbitol and mannitol were up-regulated in the tolerant population, while in sensitive plants galactoglycerol and erythrose up-regulated and D-Xylulose-5-phosphate down-regulated. 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).

5.3.2. ROS scavenging and cellular structure protection during water deficit

Ascorbate and tochoherol increased in tolerant plants (Figure 5.6). 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 et al., 1994; Foyer, 1997; 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).

Various studies have demonstrated the role of glycine betaine and other compatible osmolytes in protection of membrane and stabilizing the quaternary structure of proteins and enzymes (Papageorgiou and Murata, 1995).

5.3.3. 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 plants such as tobacco and maize demonstrated that these plants are able to maintain or increase polyunsaturated level of fatty acids (Zhang et al., 2005b; 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.

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

In tolerant plants, SA and neoxanthin (precursor of ABA; Figure. 5.8) significantly increased and GA decreased under water deficit stress conditions. While sensitive plants showed lowering neoxanthin and increasing JA (Tables 5.1-5.4). Meanwhile indol-3-acetaldehyde (IAAId; Figure 5.8) as a precursor of IAA elevated in both populations under stress conditions. Increasing ABA in tolerant plants reconfirms ABA role 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, confirms 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 (gibberellin), 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). ABA is a well-known hormone which rapidly causing stomatal closure subsequent to water stress (Wilkinson and Davies, 2010). Nevertheless, recent evidence suggests that other hormones such as BR, JA, SA and NO involved in closing stomata also (Ribeiro 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).

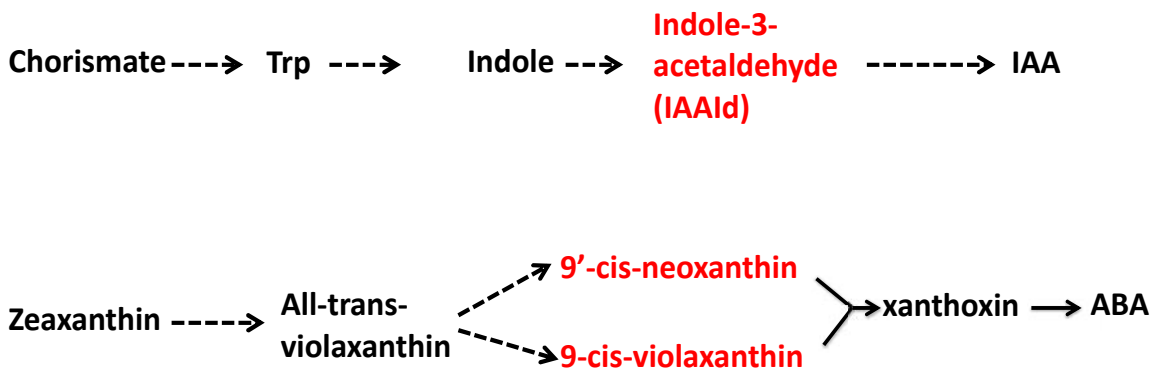


Figure 5.8. Potential pathway of ABA and auxin biosynthesis in plants.

To synthesize ABA, Zeaxanthin is converted to either 9'-cis-neoxanthin or 9-cis-vioxanthin. Next, these C40 carotenoids are cleaved into xanthoxin then to ABA (Christmann et al., 2006). Auxin (IAA) synthesis initiates from chorismate, then Trp (tryptophan). Finally indole-3-acetaldehyde (IAAId) is converted to IAA (Woodward and Bartel, 2005).

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). In general, tolerant plants alter metabolism under unfavourable conditions by accumulating or maintaining the high levels of particular metabolites (Krasensky and Jonak, 2012). FT-ICR mass spectrometry-based approach allowed comprehensive metabolic profiling, which resulted in insight into the complex manner in which metabolic pathways respond to stress.

5.4. Conclusion

To study the stress-induced metabolite modifications in thyme, a comparative metabolomics study combined with physiological indices was conducted. One month old plants of *Thymus serpyllum* as a drought-tolerant and *Thymus vulgaris* as a sensitive population were subjected to prolonged drought by water withholding.

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.

Non-targeted metabolite profiling with further multivariate analysis allowed comparison the metabolite changes in early stage between control and droughted of thyme plants. Water deficit stress affected about 600 and 150 polar metabolites in sensitive and tolerant population while 700 and 440 non-polar metabolites were altered following water stress in sensitive and tolerant plants respectively. 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. These observations

resulted from one set of experiment which might be affected by uncontrolled fluctuations of environmental factors such as temperature and light. Therefore it is suggested that to get valid and unbias results, whole set of experiment perform in more replications.

5.5. Summary of chapter 5

Thyme as a perennial herb belonging to *Lamiaceae* family has been recognized globally for its antimicrobial, antiseptic and spasmolytic effects. Rapidly increasing demand for various kinds of thyme products indicates importance of research on this plant. A wide diversity of environmental stresses affects plants in the field. The impact of water stress can be observed at several levels from cells to whole plant function. Therefore, parameters measuring changes in water relations, biochemical and physiological processes, membrane structure and ultra-structure of subcellular components can be employed to characterize the dehydration tolerance processes. In this investigation, we have used non-targeted metabolite profiling utilizing Mass spectrometry FT-ICR combined with the morpho-physiological parameters to assess the effects of prolonged water shortage on metabolite changes as well as plant water status and growth in drought sensitive thyme plant (*Thymus vulgaris*) and tolerant population (*Thymus serpyllum*) in order to understand the metabolite adjustment in relation to the responses at physiological level. Morpho-physiological parameters including water content, shoot water potential, soil moisture and shoot dry weight indicated the clear differences between these populations at physiological level. Considering the pattern of soil moisture changes and shoot dry weight decline, it seems *T. serpyllum* behaved as water saver, while *T. vulgaris* exhibited water spender behaviour. The results at the metabolic level identified the major metabolites in addition to main metabolic pathways that are significantly affected by long-term water deficit in thyme plants. Significant metabolites belonging to different chemical classes consisting amino acids, carbohydrates, organic acids and lipids have been compared in tolerant and sensitive plants. These mechanisms may include 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.

In the next chapter, we will assess the effect of water stress on the volatiles in both tolerant and sensitive thyme plants using time course experimental design. Since, FT-ICR mass

spectrometry was not able to detect these metabolites; we have to use GC-MS volatile profiling methodology. To understand the whole plant response at physiological level, morpho-physiological parameters will be recorded at each time point.

Chapter VI. IMPACT OF LONG-TERM WATER DEFICIT STRESS ON THE VOLATILE COMPOSITION OF THYME (*THYMUS* SPP.) USING GC/MS

6.1. Introduction

According to present information, 1700 volatile compounds are emitted from organs of plants (Knudsen et al., 2006; Knudsen et al., 1993) including roots (Steeghs et al., 2004). These volatile compounds can be categorized into constitutive (CVOCs) and induced (IVOCs) volatile compounds (Loreto and Schnitzler, 2010). IVOCs are synthesized only under stress conditions (*de novo* biosynthesis) and may take a role in stress response or adaptation (Dicke and Loreto, 2010), while CVOCs are produced and stored in specific organs and emitted from healthy plants (Niinemets et al., 2004). Another classification is based upon chemical structure; in this scheme, plant volatiles are grouped in four groups (i) C_5 (hemiterpenes) including isoprenes and methylbutenol (ii) C_{10} (monoterpenes) (iii) C_{15} (sesquiterpenes) (iv) C_{20} (diterpenes) (Vickers et al., 2009; Wu et al., 2006).

The largest and most diverse group of plant metabolites are isoprenoids (also known as terpenoids or terpenes) of which 20,000 compounds have been identified so far (Sacchettini and Poulter, 1997). These secondary metabolites mostly function as aroma, plant defence against pathogens and insects, communication to other plants, scavenging ROS and adaptation to environmental stresses (Spinelli et al., 2011). These functions are not essential for plant growth and survival. Nevertheless, there are some terpene-based compounds which are essential for plant growth such as the antioxidant (vitamin E) (DellaPenna, 2005), protein labels, electron carriers, hormones (cytokinin, abscisic acid, gibberellins, brassinosteroids) (Sakakibara, 2006; Hedden and Proebsting, 1999; Lange and Ghassemian, 2003), pigments (chlorophyll, carotenoids) (Lange and Ghassemian, 2003). Terpenes are synthesized in two separate pathways namely MVA (mevalonic acid) and MEP (2-C-methyl-D-erythritol 4-phosphate). MVA occurs in the cytosol while MEP occurs in plastids (Rohmer, 1999; Lange et al., 2000) (Figure 6.1).

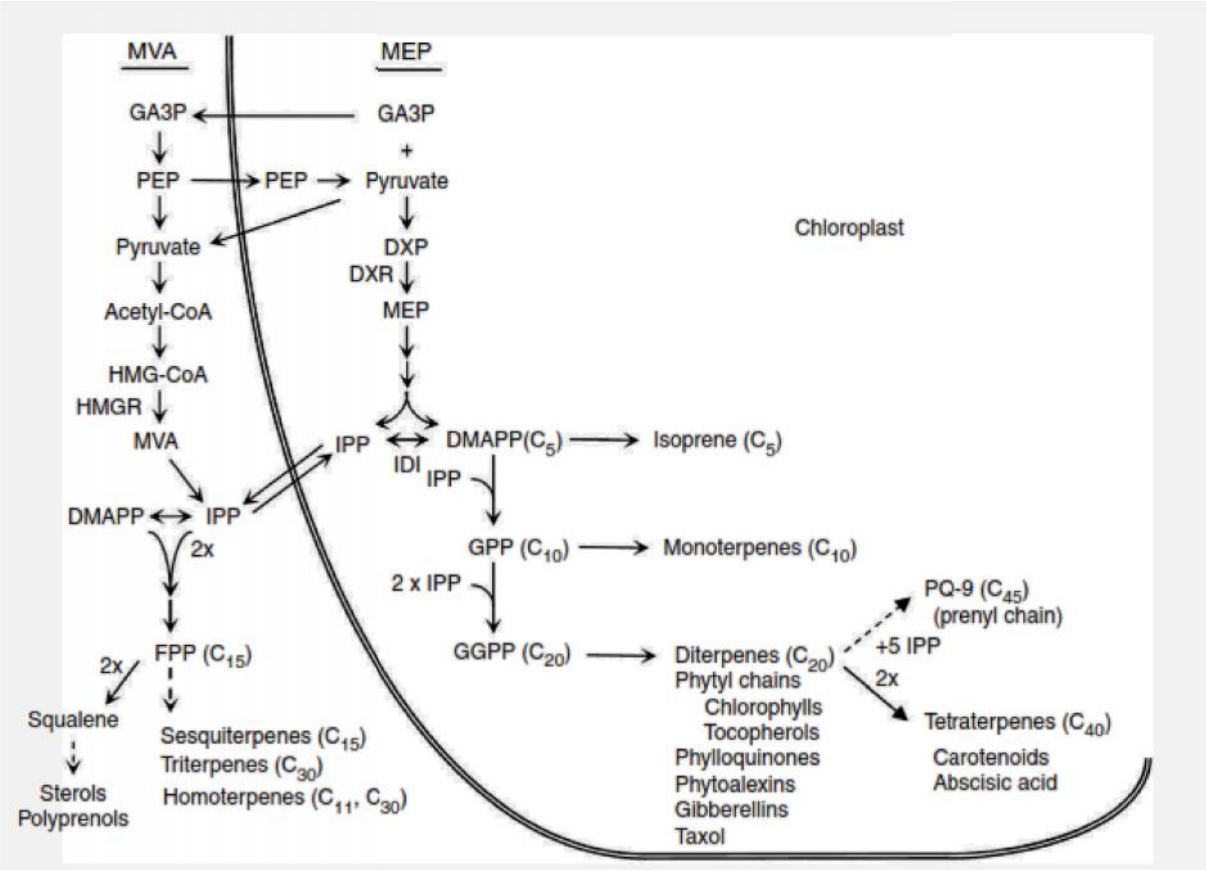


Figure 6.1. Two separate pathways for biosynthesis of terpenes in plastids and the cytosol. Adapted from (Vickers et al., 2009).

MVA (mevalonic acid) is found in the cytosol but MEP (2-C-methyl-D-erythritol 4-phosphate) occurs in plastids. MVA pathway synthesizes sesquiterpenes (C_{15}) and triterpenes (C_{30}) from GA3P converted to PEP then pyruvate and acetyl-CoA. But in MEP isoprene (C_5), monoterpene (C_{10}), diterpene (C_{20}) and tetraterpene (C_{40}) are synthesized from GA3P + pyruvate then converted to DXP and finally MEP. GA3P (glycerol 3-phosphate), DXP (1-deoxy-D-xylulose 5-phosphate), IPP (isopentenyl pyrophosphate), IDI (isopentyl diphosphate isomerase), DMAPP (dimethylallyl pyrophosphate), HMG-CoA (3-hydroxy-3 methylglutaryl-CoA), HMGR (3-hydroxy-3 methylglutaryl reductase), PEP (phophoenolpyruvate).

About 360 different volatile compounds have been identified across the 162 taxa of *Thymus* genus belonging to *Lamiaceae* family. Of these compounds, monoterpenes at 43% and sesquiterpenes at 32% are the dominant volatiles. To date, 270 terpenes have been detected in *Thymus* oils which thymol and carvacrol having the most economical importance (Stahl-Biskup and Sàez, 2002).

Plant volatiles are significantly altered by biotic and abiotic stresses (Holopainen and Gershenzon, 2010). Previous studies established the impact of abiotic stresses including temperature (Tingey et al., 1980), light (Schuh et al., 1997), water stress (Ebel et al., 1995;

Vallat et al., 2005), salt (Loreto and Delfine, 2000) and oxidative stress (Heiden et al., 1999) on VOCs. These stresses generally increase the emission of a wide range of terpenes including isoprene (Sharkey and Yeh, 2001), monoterpenes (Loreto et al., 1996) and sesquiterpenes (Duhl et al., 2008). Nevertheless, there are some studies that indicated no influence of stress on release of VOCs, such as salt stress on poplar (Teuber et al., 2008) and moisture stress on isoprene (Sharkey et al., 2008). Previous investigations on thyme indicated that volatile composition is affected by environmental factors (Loziene and Venskutonis, 2005) including drought (Jordan et al., 2003; Sotomayor et al., 2004).

The emission of VOCs may act as part of a plant response to mitigate deleterious effects of stress (Wenda-Piesik, 2011). Despite the obvious changes of thyme essential oil components in response to drought stress, the investigation on the underlying mechanisms at biochemical and physiological level is lacking (Selmar, 2008). To our knowledge, there has been no published time course of water deficit effects on volatile composition in thyme combined with morpho-physiological parameters. Therefore, this study aimed to monitor and compare the monoterpenes and sesquiterpenes of thyme in drought tolerant (*Thymus serpyllum*) and drought sensitive populations (*T. vulgaris*) during long-term water stress.

6.2. Results

6.2.1. Physiological parameters affected by long-term drought stress

Physiological parameters in this investigation namely water content, water potential and shoot dry weight were affected in both populations (Figure 6.2). 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 tolerant plants. Water potential declined on 4^{th} 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 sensitive plants on 8^{th} and 12^{th} days (Figure 6.2).

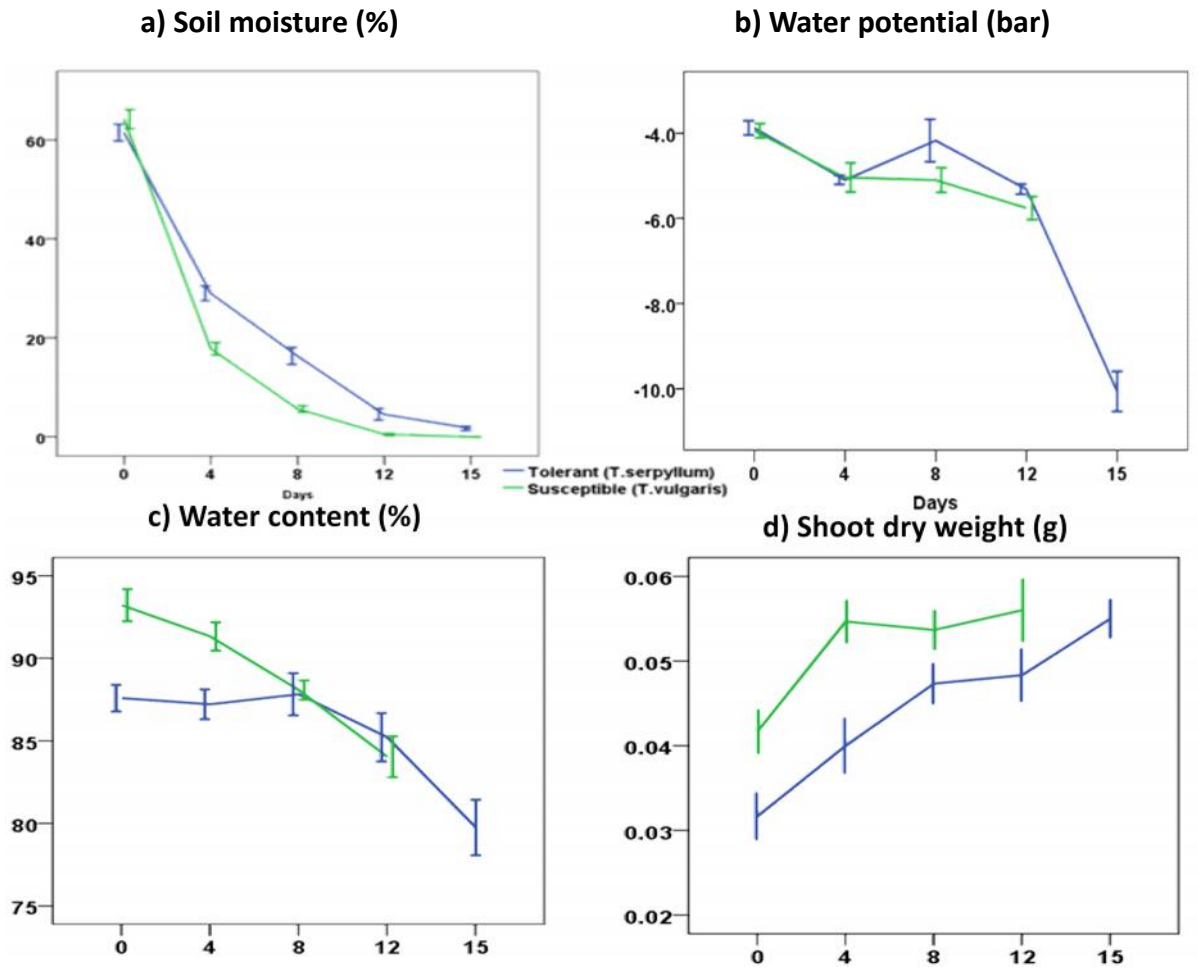


Figure 6.2. Physiological parameters influenced by long-term water stress in tolerant and sensitive thyme.

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 sensitive plants, while those parameters in tolerant plants were gently decreased. Moreover, shoot dry weight of sensitive plants was greater at similar time points.

There was a significant difference between populations, as 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, when it dropped to 85%. 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 for sensitive plants but the increase in weight continued for 8 days after withholding water.

6.2.2. Non-targeted volatile profiling of thyme extracts using GC/MS

The main objective was to determine the response of the volatiles in thyme during water stress. We employed a non-targeted GC/MS-based approach to simultaneously measure a wide range of terpenes. This approach detected various compounds in the range of 200s-400s of scanning, but the major components are listed in table 6.1. A complete list of volatile components including the structural formulae and MS profiling can be found in Appendix 10.

Table 6.1. Major volatile components detected in *T. vulgaris* and *T. serpyllum* by modified liquid extraction method from fresh leaves.

No	Compound	Formula	Major Ion	RT**	KI***
1	Beta-Pinene	C10H16	93	193	980
2	Beta-Myrcene	C10H16	41,93, 69	217	991
3	Alpha- Phellandrene	C10H16	93	225	1005
4	Alpha-Thujene	C10H16	93	233	931
5	P-Cymene	C10H14	119	240	1026
6	Alpha-Cubebene	C15H24	161	410	1351
7	Ocimene	C10H16	93	242	1050
8	Gamma-Terpinene	C10H16	93	260	1062
9	Benzyl acetate*	C9H10O2	108	320	1163
10	Thymol	C10H14O	135	355	1290
11	B-Caryophylene	C15H24	93,133	383	1418
12	Germacrene D	C15H24	161,105	407	1480

*** Benzyl acetate was used as internal Standard; ** Retention times are based on the GC-MS under the conditions mentioned in materials and methods; *** Reference for KI: Adams,R.P. (2007) on DB-5 column.**

In order to understand the influence of water deficit stress on the composition of monoterpenes and sesquiterpenes, the quantity of major compounds in tolerant plants was compared to sensitive under stressed conditions. We sampled six independent biological replicates for each time point and analyzed 5 technical replicates by GC-MS for each biological replicate. 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 6.3).

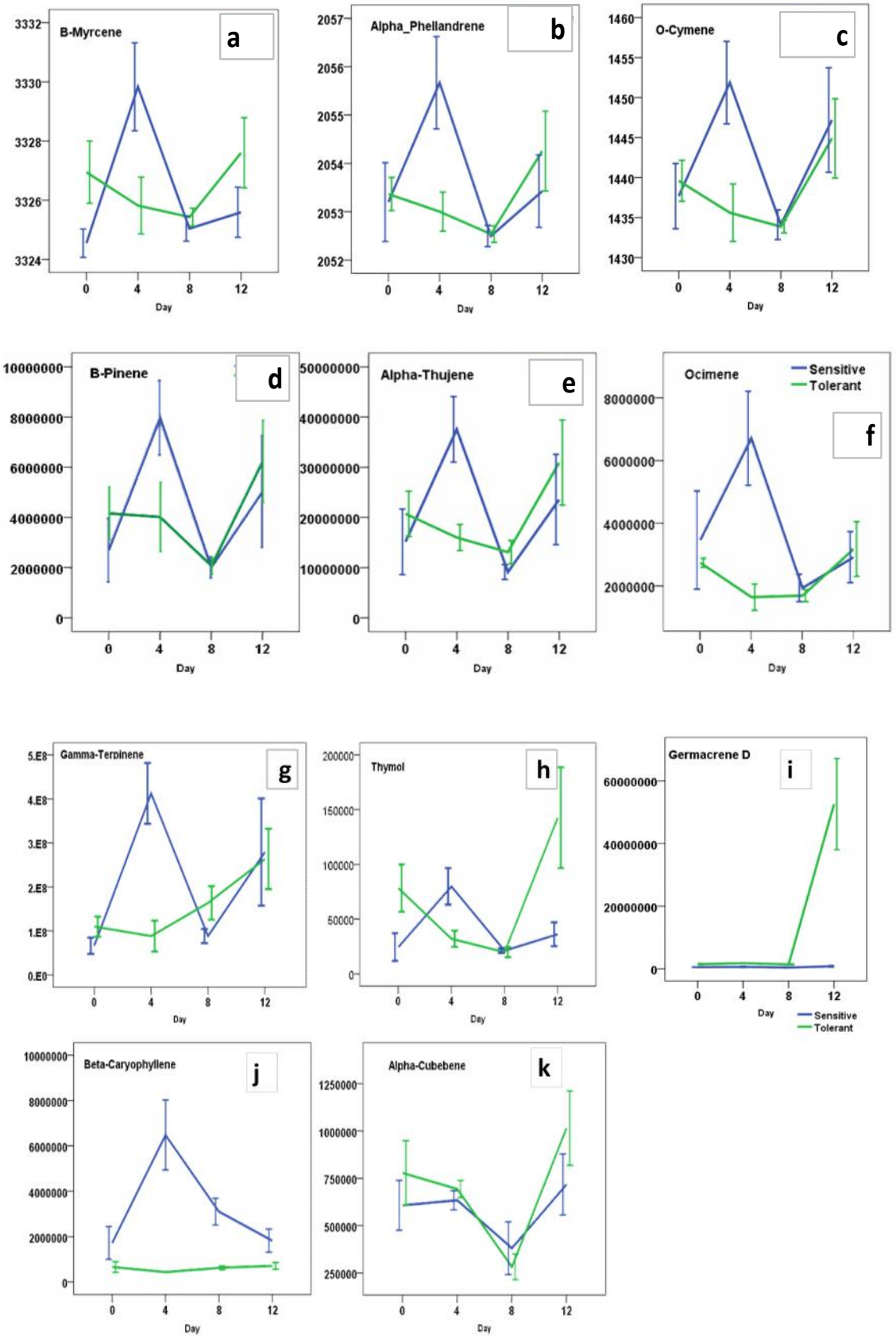


Figure 6.3. Volatile compounds affected in 4 week old tolerant and sensitive thyme plants under water deficit stress.

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) β-myrcene; b) alpha-phellandrene; c) O-cymene; d) β-pinene; e) alpha-thujene; f) ocimene; g) Gamma-terpinene; h) thymol; i) germacren; j) B-caryophylene; k) alpha-cubebene. Blue lines represent sensitive plants and green tolerant ones. Error bar= ±SEM, Rep=5.

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 thymol and alpha-cubebene, nine other metabolites exhibited significant differences between tolerant and sensitive plants. There was a high concentration of germacrene D in tolerant compared to sensitive plants, while other compounds showed the same pattern which increased in intensity in sensitive plants on 4^{th} day and similar intensities throughout the stress period (Figure 6.3). In contrast, most of the terpenes of tolerant plants were unaffected during the stress apart from the final day where there was a sharp elevation.

When sensitive plants are exposed to drought stress conditions, terpenes are elevated within 4 days, but return to the same intensity as prior to the stress. Tolerant plants did not change their terpenes except for germacrene which increased on 12^{th} day. For *T. vulgaris* (susceptible) the 4^{th} day was the turning point with increasing volatiles for all monoterpenes and sesquiterpenes at this point. The critical day for tolerant plants was 12^{th} day, since the increase in terpenes was been observed at this stage.

6.3. Discussion

All the changes in morpho-physiological parameters defined two different types of water use strategy by plants. *T. serpyllum* behaved as a water saver, while *T. vulgaris* exhibited water spender behaviour (Larcher, 2003).More details on water use strategies described in previous chapters.

Although there have been previously studies on the effect of environmental factors on the chemical composition of thyme, only a few of them have focussed on specific stress such as drought with quantified water status. In previous works they analyzed the essential oils of

the samples collected from different climatic characteristics. Observed differences in essential oil composition were attributed to differences in these climatic features (Loziene and Venskutonis, 2005; Adzet et al., 1977; Salgueiro et al., 1997; Salgueiro et al., 1995; Mártonfi et al., 1994; Yavari et al., 2010; Letchamo and Gosselin, 1995).

Among the few detailed studies which imposed drought precisely, there are some conflicting results. Jordan et al. (2003) investigated the effect of drought stress on essential oil composition of *Thymus hyemalis*. They imposed water stress by four different watering levels and observed no significant differences for terpenes apart from thymol which showed a significant difference at the lowest watering from other levels. They proposed that the variability in the chemical composition of plants stems from watering level effects. In contrast, Sotomayor et al. (2004) observed considerable differences between the three different watering levels on *Thymus zygis* essential oil composition (Sotomayor et al., 2004). They observed that volatiles such as alpha-thujene, beta-pinene and p-cymene decreased with increasing drought. Myrcene, alpha-phellandrene and gamma-terpinene were significantly elevated with lowering watering level (Sotomayor et al., 2004). These findings were partly confirmed by Aziz et al. (2008), who they reported a decline in p-cymene, germacrene and caryophyllene in *Thymus vulgaris* affected by 4 different irrigation levels (Aziz et al., 2008). These conflicting results may be explained by the different species of thyme and different methods of drought imposition. Studies on other plants on emission of volatiles after imposing drought stress are more confusing, since some reported decreases (Brilli et al., 2007; Lavoir et al., 2009), increases (Delfine et al., 2005; Blanch et al., 2009) and also decrease following increase (Sharkey and Loreto, 1993; Bertin and Staudt, 1996). 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 (Ormeno et al., 2007).

6.3.1. Possible explanations for changing volatile composition during drought stress

Comparison of volatile fluctuation patterns (Figure 6.3) for tolerant and sensitive thyme plants confirmed their varied physiological responses. As described above, tolerant populations (*T. serpyllum*) behaved as water savers and sensitive populations (*T. vulgaris*) as

spenders. Therefore, possible mechanisms for their response at the level of volatiles can be explained as follows.

6.3.1.1. Carbon diversion from photosynthesis to terpenes

Plants naturally allocate up to 2% of assimilated carbon to the biosynthesis of volatiles (Sharkey, 1995; Firn and Jones, 2006; Tani and Kawawata, 2008; Šimpraga et al., 2011). But under stressed conditions particularly multiple stresses they allocate up to 10 % (Peñuelas and Llusià, 2003) or in some cases up to 67% (Sharkey and Loreto, 1993). This allocation of carbon to monoterpenes might be occurring in two phases during the stress period (Figure 6.4).

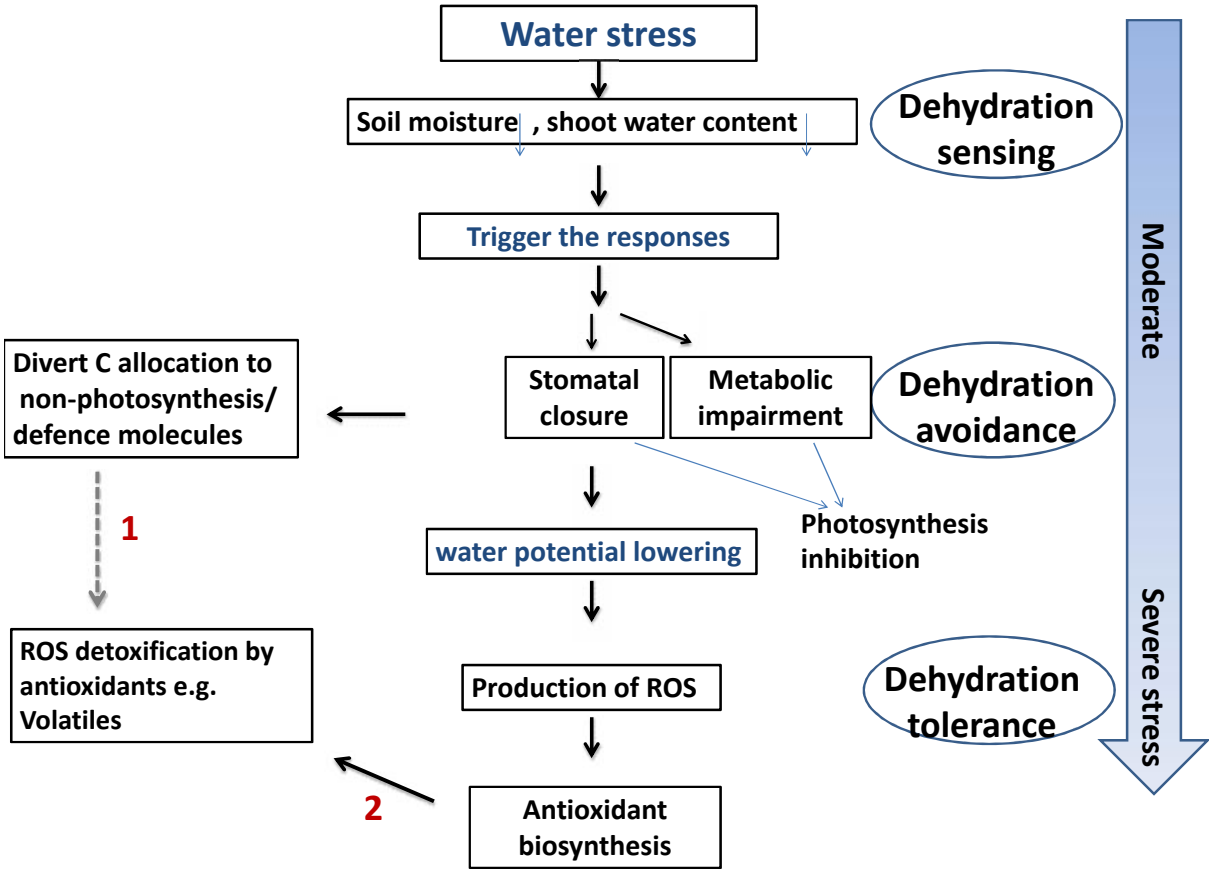


Figure 6.4. Water stress responses from dehydration sensing to water stress tolerance.

Drought stress may enhance terpenes through two different mechanisms. The first (number 1) is through diverting allocation of carbon from photosynthesis to defence molecule production. In this step, the plant takes the avoidance mechanism against moderate stress by closing stomata. Stomatal closure in addition to metabolic impairment such as declining Rubisco inhibits photosynthesis. Mechanism 2 is activated following severe stress. Severe stress leading to

oxidative stress produce ROS, therefore the plant activates a defence system including non-enzymatic antioxidants such as terpenes.

Subsequent to imposing water stress, soil moisture and later shoot water content decrease. This decline triggers the plant responses such as stomatal closure and various metabolic impairments. 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). The first possible mechanism for terpene production occurs in this circumstance (dotted arrow no.1 Figure 6.4). 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., 2004a). Therefore water stress influences volatile compounds indirectly (Šimpraga et al., 2011). Carbon allocation diverts to non-photosynthetic organs and/or biosynthesis of defence molecules (Chaves et al., 2003; Pinheiro and Chaves, 2011; Llusià et al., 2006). In this way, terpenes because of their antioxidant properties (Gershenzon et al., 1978; Llusià and Peñuelas, 1998) will increase. It seems in this circumstance, plants trade-off between growth and defence (Ormeno et al., 2007; Turtola et al., 2003). This is in agreement with the hypothesis of carbon/nutrition balance (Turtola et al., 2003) and growth differentiation balance (Lorio, 1986). The subsequently decline in terpenes might be attributed to lowering photosynthetic substrates due to continued stress conditions (Ormeno et al., 2007). Response of sensitive plants in terms of the terpenes followed this pattern.

6.3.1.2. ROS scavenging by non-enzymatic antioxidants

If lowered water potential persists, dehydration tolerance mechanisms can be employed by plants (Verslues et al., 2006). Decline in photosystem II activity will be lead to the imbalance of production and utilization of electrons, generating ROS (Peltzer et al., 2002). These free radicals will cause oxidative damage to the plant including lipid peroxidation, DNA, amino acid and protein oxidation (Johnson et al., 2003; Asada, 1999). In response to ROS deleterious effects, plants activate enzymatic and non-enzymatic antioxidant defence systems (Conklin, 2001). During severe water stress, antioxidants are will be elevated (Eskling et al., 1997; Depka et al., 1998). Terpenes can serve as antioxidants (Gershenzon et

al., 1978; Llusià and Peñuelas, 1998). This mechanism is shown in Figure 6.4. The result of this study shows that tolerant plant behaviour follows this potential mechanism.

6.4. Conclusion

To investigate the response of thyme plants at volatile metabolome level, one month old plants of tolerant and sensitive populations (*T. serpyllum* and *T. vulgaris* respectively) were exposed to long-term water limitation by withholding water. Physiological parameters were recorded in addition to leaf sampling for volatile profiling at 4 day intervals. Physiological assessments identified sensitive plants as water spenders and tolerant plants as savers. Shoot dry weight of sensitive plants was greater at similar time points.

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. 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 thesis 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.

6.5. Summary of chapter 6

Thyme (*Thymus spp*.) volatiles predominantly consisting monoterpenes and sesquiterpenes, serve as antimicrobial, antiseptic and antioxidant. Plant volatiles like other chemicals are affected by genetic make-up and environmental factors. The effect of prolonged water deficit stress on volatile composition was studied on tolerant and sensitive thyme plants (*T. serpyllum* and *T. vulgaris* respectively). Volatile sampling along with morpho-physiological parameters such as soil moisture, shoot water potential, shoot dry weight and water content performed on one month old plants subsequent to water withholding at 4 days intervals until the plants wilted. Tolerant and sensitive plants had clearly different response at physiological level. Sensitive plants showed an increased-decreased trend at major terpenes apart from Thymol, Alpha-cubebene and Germacrene. 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. Increasing terpenes for sensitive plants can be attributed to divergence of carbon allocation from photosynthesis to produce defence molecules and further decrease is likely related to photosynthesis substrate limitation due to water stress effects. Likewise, maintaining volatiles at the fixed levels with a later increase for tolerant plants is consistent with plant response to oxidative stress by producing antioxidant agent. The combination of volatile profiling and physiological parameters assisted to understand precisely the mechanisms of plant response at volatile metabolome level.

CHAPTER VII. GENERAL CONLCLUSIONS AND FUTURE WORK

7.1. General Conclusions

In this thesis, several approaches were utilized to comprehensively understand the responses of thyme to long-term water deficit stress at physiological and metabolome level. First, for screening germplasm consisting eleven population of *Thymus* spp., simple, non-expensive but efficient approach i.e. morpho-physiological parameters employed at germination and early vegetative growth phase (Chapter2). These simple indicators help to interpret the mechanisms observed. In germination phase, PEG allowed imposition of different osmotic potential levels and revealed distinct differences between populations with respect to percentage germination. *T. serpyllum* (SP) was the most susceptible, and *T. vulgaris* (IR), *T. serpyllum* (UK) and *T. serpyllum* (EU) had moderate susceptibility. The remainder of the populations were tolerant to water deficit stress during the germination period.

In the seedling stage, the morpho-physiological parameters of root/shoot ratio, survivability, water content and water potential were used to assess effects of drought. *T. serpyllum* (SP) was the most tolerant and *T. vulgaris* (SP) the most susceptible of the evaluated material. Different responses of populations to drought regarding water status were noted. Generally, *serpyllum* sp. was more tolerant than *vulgaris* species and within the *vulgaris* species, *T. vulgaris* (SP) was susceptible and *T. vulgaris* (GR) the most tolerant. *T. serpyllum (SP)* was susceptible in germination stage, was tolerant in seedling stage. In contrast *T. vulgaris (SP)* which was tolerant when assessed by seed germination was susceptible population at seedling stage. Between the tolerant populations in seed germination, only *T. zygis* had a tolerance to water deficit in the older plant phase. Hence, there was no consistent pattern of drought tolerance between the different phases of plant development.

A metabolomics approach was utilized to investigate the response of thyme at the metabolomics level. The time course metabolite profiling in moderately tolerant species (*T. vulgaris*) highlighted 66 peaks categorized into three groups based on their pattern of change during the stress period (chapter 3). Asparagine, phenylalanine, tryptophan, D-Xylose-5-phosphate and aspergillic acid were the most significant metabolites among the identified peaks.

A comparative analysis of water stress between tolerant and sensitive thyme plants (*T. serpyllum* and *T. vulgaris* respectively) revealed large number of significant altered metabolites including carbohydrates, amino acids, organic acids, secondary metabolites and lipids which changed in tolerant plants but not in sensitive population. These metabolites may be involved in enhancing tolerance through various mechanisms including osmotic adjustment, hormones activity, membrane lipid composition change, ROS scavenging and cellular structure protection. Osmotic adjustment in tolerant plant might include accumulation of metabolites such as proline, betaine, mannitol and sorbitol. Likewise, ROS scavenging is probably carried out by the observed elevation in ascorbate and tocopherol and also cellular protection by metabolites such as proline and mannitol. Accumulation of these non-enzymatic antioxidants may help tolerant plants to mitigate the deleterious effects of oxidative stress which occurs in severe drought stress. Tolerant plants also exhibited increasing lipid contents particularly unsaturated fatty acids. The roles of phytohormones were implicated in the alteration of SA, JA and precursors of ABA and IAA in both populations. SA and precursors of ABA were elevated in tolerant plant supporting a key role in enhancing tolerance to drought.

Thyme as an officinal plant belonging to *Labiatae* family with more than 360 species and subspecies produces large amount of secondary metabolites dominated by terpenes (monoterpenes and sesquiterpenes). In phytomedicine terpenes serve as antimicrobial, antifungal and antioxidants. Also, they play a key role in plants as secondary metabolite via their potential role against herbivores, attracting pollinators and abiotic stress tolerance.

There have previously been studies examining the effect of environmental factors on the chemical composition of thyme volatiles, but only a few have focussed on a specific stress such as drought. These studies attributed environmental factors to essential oil composition variation. Therefore, monoterpenes and sesquiterpenes of thyme monitored on time course manner in drought tolerant (*Thymus serpyllum*) and drought sensitive populations (*T. vulgaris*) during long-term water stress complemented by physiological parameters (chapter 5). Comparisons of major terpenes indicated that thymol and alpha-cubebene were not affected by significantly water stress, but other compounds followed two main patterns. In sensitive plants all terpenes were elevated at day four then decreased to the initial levels. In contrast, tolerant plants maintained the same abundance of terpenes until; elevation at 12th

day of stress period. These increases and decreases in sensitive plants can be explained by effects of drought stress on declining photosynthesis and diversion of carbon allocation to defence molecule production systems. The increase observed in tolerant plants may be attributed to plant strategies against deleterious effects of ROS. Thymol, the most economically important compound of thyme, is not affected under drought stress, but other volatiles such as germacrene were elevated in tolerant plants. Therefore, tolerant plants at the end of long term water stress period produce the largest quantity of germacrene. Likewise, ocimene (economically important in perfumery) and beta-myrcene and alpha-phellandrene (both well known in fragrance industry) can be extracted in the highest quantity from sensitive plants on the 4^{th} day.

In conclusion, survivability was the most effective and sustainable indicator of drought tolerance among the various morpho-physiological markers used to screen the thyme germplasm. For the first time we report large number of metabolites contributed in the response of thyme to water deficit stress. Among those compounds, asparagine, phenylalanine, tryptophan, D-Xylose-5-phosphate, aspergillic acid, proline, betaine, mannitol, sorbitol, ascorbate, tocopherol, SA, JA and precursors of ABA and IAA were noticeable which were acting through highly sophisticated mechanisms. Methodology of time course non-targeted volatile profiling was used to monitor the major volatiles during the water deficit period. The results indicated significant changes terpenes apart from thyme and alpha-cubebene. However, metabolomics-based approaches through providing overall image of metabolites could be of great interest of a better understanding of plant responses to drought stress particularly for non-model plants without any genome information. For this purpose, the present thesis indicates DI FT-ICR mass spectrometry combined with morph-physiological parameters may be an effective approach to demonstrate metabolite changes subsequent to water scarcity.

7.2. Future Work

Combined omics: The most comprehensive study of plant response to stress can be achieved using a combination of all three omics; namely metabolomics, transcriptomics and proteomics. Such a combined study would provide information on gene-to-gene, gene-to-metabolite and gene-to-protein networks. A major challenge in such omics-combined studies is an integrated sample collection, preparation and data analysis. These issues lead

to poor correlation of RNA-Protein-Metabolites. However, newly developed sampling techniques are required to minimize this error (Maltese and Verpoorte, 2009). Likewise, data should be analyzed using mathematical modelling of biological systems known as system biology. Therefore, comparative analysis of two diverse populations of thyme with metabolomics, transcriptomics and proteomics may allow elucidation of metabolite, genes and proteins contributed to stress tolerance in thyme. In addition, the data provided by this thesis could facilitate the transcriptomics study to identify the genes responsible for drought-related responses in thyme such as osmolytes we mentioned.

Targeted analysis on major metabolites: Non-targeted metabolite profiling can be performed to obtain general view of the metabolism or identify new metabolite/pathways involved in dehydration response of thyme. The main disadvantage of non-targeted approach is measuring relative concentration of metabolites. It means to validate those results, performing targeted analysis is necessary. Targeted analysis could be used to measure the precise quantity of some key metabolites determined in chapters 3, 4 and 5 namely asparagine, phenylalanine, tryptophan, D-Xylose-5-phosphate, aspergillic acid, proline, betaine, mannitol, sorbitol, ascorbate, tocopherol, SA, JA, ABA, IAA and terpenes. For this, combination of NMR and GC/MS with standards can be recruited to measure precisely the abundance of mentioned compounds in treated and control plants. However, precisely measuring the key metabolites will allow testing the hypothesis generated by non-targeted analysis.

Extension of current results to other *Thymus* species, medicinal plants and economical crop plants: Even though certain plants have evolved specific adaptive mechanisms to alleviate adverse effects of water deficit stress, there are some common metabolic adaptation are observed in diverse economical plants. Therefore, it seems these results regarding key metabolites and identified mechanisms could be used in plant breeding programs for other economical plants including medicinal plants to improve stress tolerance. Since metabolomics-assisted approach can be used to shortening the plant breeding procedures to release new improved varieties, measuring mentioned metabolites as biomarker using targeted analysis can help plant breeders to select the individuals in segregation generations which is the most time consuming part of the plant breeding procedures. Meanwhile, within the germplasm evaluated, Varico3 as a drought tolerant and

T. zygis and *T. capitata* as other economically important species are good candidates for further drought stress studies and breeding purposes.

Application of exogenous metabolites to confirm the role in stress: Exogenous application of compounds involved in adaptive responses to drought stress might alleviate adverse effects of stress. Whereas previous works support the enhancement of water deficit stress tolerance by foliar application of major compounds such as glycine betaine, kinetin, nitric oxide and salicylic acid (Rao et al., 2012), therefore it is suggesting further experiments to examine the influence of key metabolites (or generally compounds) in the alleviation of drought stress negative effects. Therefore, foliar applying (spraying or fumigating) of the selected metabolites with their available commercial products forms might help sensitive plants enhance their drought stress tolerance.

Transfer the responsible genes to sensitive plants to enhance the tolerance: As recent works demonstrated, transferring genes responsible for dehydration tolerance is a reliable approach to achieve sustainable tolerance. Metabolomics-based approaches combined with other omics identified the molecular mechanisms of adaptive response to water deficit stress in addition to stress-related genes. These genes are contributing in osmoprotectants and antioxidant metabolism. Osmoprotectants are responsible for protection of cellular compartments and structures and antioxidants are scavengers of free radicals and toxic compounds generated during water stress. However, the list of key compounds and pathways underlying in the response to drought stress in thyme resulted from the highly accurate FT-ICR mass spectrometry with further experimentation may lead to identify the candidate responsible genes for transformation. Then transferring identified genes might enhance the tolerance level to water deficit stress.

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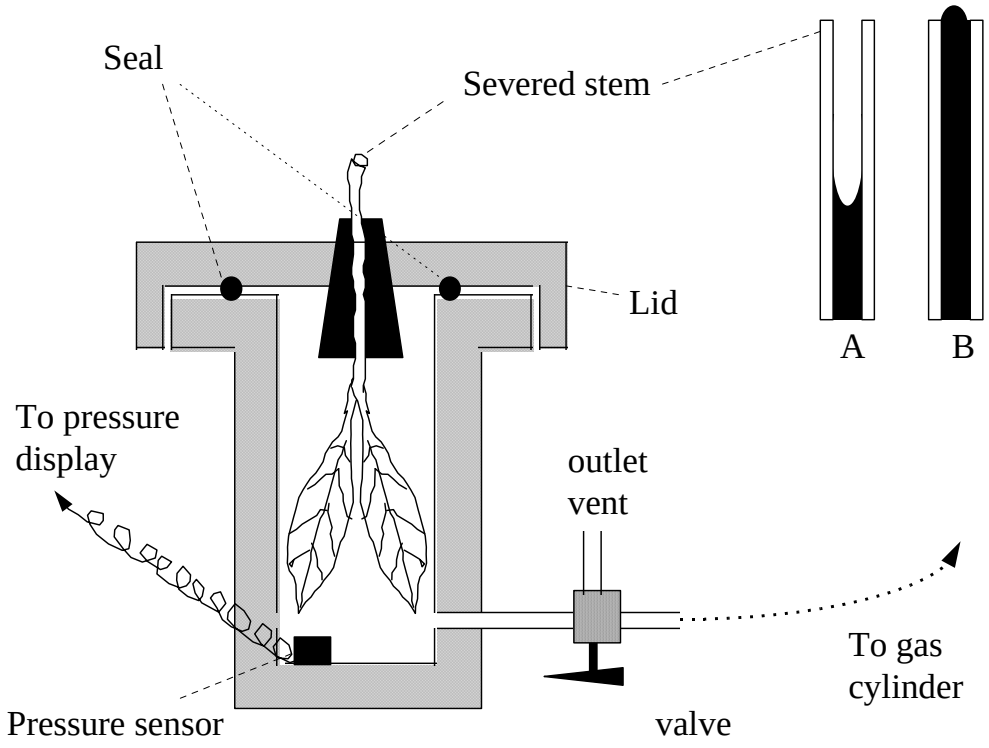
Appendix 1. Protocol for measurement of shoot water potential with the pressure bomb

Reference: BIO237 Plant Sciences: From Cells to the Environment. Practical Handbook 2010-11. Dr. Jeremy Pritchard

The pressure chamber operates using air at very high pressure and it is possible to cause an accident by incorrect use. Danger comes from unexpected and uncontrolled air release due to opening the valves in the wrong sequence or failure to secure the lid or the bung holding the plant specimen in the hole in the lid. Never put your head or hand over any part of the chamber while in use and always wear the face masks provided when operating the equipment. Wait to have the equipment explained by a demonstrator. Do nothing until you are sure of operation details.



Switch on the unit and put switch to 0-20 bar range (1 bar = 0.1 MPa).

Fix a plant specimen into a split bung and fix into the lid. The lid of the bomb screws off: make sure the rubber seal is always present when you put the lid on. Press the red 'free' button and use the 'display' knob to adjust the chamber pressure display to zero. Turn the 'flow rate' knob fully clockwise - do not over tighten - then release one quarter of a turn. Switch to 'fill chamber' The pressure in the bomb will rise you can control the rate of rise - listen for leaks and replace the leaf if necessary. **Wearing eye protection** watch the cut plant stem closely. When fluid is seen to emerge from the cut stem press the green 'hold' button, record the value shown in the window, this is the xylem pressure. When this pressure has been determined to your satisfaction (the point can be found several times by lowering the pressure and raising it again), leave the apparatus at zero pressure with the cylinder turned off. Press free 'display button' Slowly turn master switch to vent chamber. Check that bomb pressure is zero.

Appendix 2. Data analysis for metabolomics data

Data analysis in this thesis was comprised:

1) Sim-Stitch
2) Metabolite identification
3) Data set normalizationa and transformation

Softwares needed: Xcalibar, MATLAB, excel

1) Sim-Stitch

First, dataset subjected to Matlab scripts by following order:

SumTransients_0_9(8)

ProcessTransients_0_4(8)

Stitch_1_16(8)

ReplicateFilter_0_11(8)

FlagBlankPeaks_0_3(8)

SampleFilter_1_4(8)

Notice: For Nonpolar the following changes in scripts are necessary:

Stitch: line 194: Region.Bound = 70 2000 ***** for polar: 70 590

Replicate.Filter: 2 out of 2

FlagBlank Peaks: number of replicates=2

Sample filter: number of replicates=2

GetNoiseLevel_03m: line 35: minimum level change from 400 to 200

2) **Metabolite identification by Mipack**

In MI_Pack folder, run the GUI.bat

Browse and find your peak list (combined peaks folder/group peak list)-make sure filename doesn't have any space and inside the file, at the end there is no space). Ppm error for non-internal calibrated data is 1.25 and internal calibrated is 1.00. Browse compound and find the KeggDB files folder and select compound. Do the same for reaction pairs but select reaction_map formula. Browse organisms and select the Organisms folder in MiPack (it can be left). Browse subset and go and fine the organism's subset (MiPack/subset) that we built the other day (it can be left). Atoms, Ions and Isotopes have set up for positive polars.

Tick Single Peak Search and Empirical Formula search. Then click on run tasks. The results will save in a SPS-POS file within the folder that my group list there is.

3) Normalization, missing values and Glog transformation:

Step 0: load the peak matrix(in the combined peaks folder) – I'll call the matrix my_peaks.

Step 1: my_peaks_norm, coeffs = normalise_pqn_100(my_peaks);

Step 2: my_peaks_norm_knn, time = impute_knn(my_peaks_norm, 5, 1, 90);

Step 3: create a new matrix (my_QCs) that only includes the QCs from my_peaks_norm_knn.

Step 4: my_QCs_glog, lambda, scale, power = glog_total(my_QCs);

Step 5: my_peaks_glogged = glog(my_peaks, lambda, scale, power);

the lambda, scale and power values should all be produced by glog_total.m

To make my_QCs: open my_peaks_norm_knn file in workspace (by typing Browse in command line) and add labels and untick all rows except for QC, save it as my_QCs.

Appendix 3. Analysis of variance 9 populations treated by 4 level of PEG6000

Source	DF	Mean of squares						
		fresh weight	dry weight	radicle	plumule	rad/pl	Germ. percent	Water content
Species	8	0.22**	0.22**	481.38**	28.64**	23.48**	2092**	2169**
Treat	3	1.14**	1.13**	2115.23**	132.03**	79.86**	25555.7**	3371.1*
species*treat	24	0.12**	0.12**	146.52**	7.25*	17.67**	347.1**	334.6**
Error	71	0.04	0.05	18.37	0.77	6.41	144.9	124.9

Appendix 4. Dilution test and running Mass spectrometry

Dilution test for polar positive ions

Prior to Mass spectrometry analysis, dilution test was carried out to find out the proper dilution ratio. To do this, for Positive ions of polar metabolites, solution made up was including 4ml methanol+1ml water + 12.5 ul Formic acid (80% Methanol+ 20%Water+ 0.25% Formic acid). Then serially diluted solutions were made according to original volume: 1in2 1in4 1in8 1in16 1in32 1in64 and another series 1in 1.5 1in3 1in6. Samples were put in sonicator for 5 minutes to dissolve. After putting the eppendorfs in centrifuge for 14000RPM/10min/5C, 10 ul of samples were added to PCR multiwell plate with three first wells blank, then samples randomly to 3 wells each 10ul(3 technical replications).

Table 3. Summary of CV and number of peaks for dilution ratio carried out

Dilution ratio	CV	No. of Peaks
1.5	13.5	3232
2	18.8	2233
3	14.6	2709
4	20.6	2173
6	11.5	2705
all	27.1	1356

Based on less CV and high number of peaks, 1.5 dilution ratio has been selected.

Dilution test_Polar Negative ions

It needs to make them up in 80:20 MeOH: (Water including 100mM ammonium acetate) – It means needs to make the amm acetate up before beginning the final solution. Easiest way is to make up a 100mM solution in HPLC grade water and then you can use that as your 20% water fraction. As dilution ratio was 1.5 and we had totally 12 samples with originally 300ul, so we needed max 20 ml dilution solution. To make 4 ml (with concentration100mM), add 30.832 mg ammonium acetate to 4 ml HPLC grad water, then add to 16ml Methanol.

Running FTI-CR Mass spectrometry

This protocol is for polar positive ions, for non-polar negative ions is identical except for solution make up. This analysis was on with 1.5 dilution. Dilution solution (100ml) made with mixture of 80% Methanol(HPLC grade)+ 20% water (HPLC grade) then add %0.25 Formic acid. Then dried polar extracts were diluted in 1:1.5 (original volume: dilution solution). After vortex, all the samples placed in sonicator for 5 minutes. For quality control, 14 samples out of 84 samples (total) randomly selected and 100ul taken from each selected samples and pour in 1 eppendorf and mixed it. 800 ul from the top of the eppendorf taken and pour in new eppendorf, this is the QC. All samples including QC and Blank, centrifuged in 5C, 14000 RPM for 10 minutes. All samples loaded with 10ul aliquots with 3 replicates in 384-hole plate with the order of 3 Blank and QC at first and the end, then 3 QC in each 5 triplicate actual samples. Plate covered with a foil and sealed by Thermo-sealer (Agbene, Epsom, UK). Samples were analyzed using a hybrid 7-T Fourier transform 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). Nanoelectrospray conditions comprised of a 200 nL/min flow rate, 0.3 psi backing pressure, and +1.7 kV electrospray voltage (for positive ion analysis), controlled by ChipSoft software (version 8.1.0, Advion Biosciences). Raw data first checked by Xcalibar, if there was failed spray, those samples repeated afterwards. Finally raw data transferred to hard drive for data analysis.

Appendix 5. Complete list of metabolites with their intensities affected by water deficit stress in *T. vulgaris* for polar positive ions

m/z	watered	Droughted	Fold change	p_value	Metabolite name
152.0567	4804.58	39042.12	8.126021	2.03E-05	Guanine
383.053	39401.52	269072.2	6.82898	0.005439	3 ,5-Dihydroxy-3,4 ,7-trimethoxyflavone
404.1342	16925.02	68421.46	4.042621	0.006841	isopentenyladenine-7-N-glucoside
297.0735	40467.24	155209.9	3.835445	0.017317	(-)-Epiafzelechin, afzelechin
297.0735	40467.24	155209.9	3.835445	0.017317	L-1-glycero-3-phosphocholine
207.0054	23900.72	87919.08	3.678512	0.004191	Homogentisate
221.0211	6830.11	21126.6	3.093157	0.001329	benzaldehyde, 4-hydroxy-3,5-dimethoxy-
253.0084	33947.72	99540.4	2.932167	0.015001	α-D-ribose-1-phosphate , D-Xylose-5-phosphate , D-xylulose-5-phosphate, alpha-D-Xylose 1-phosphate, beta-L-Arabinose 1-phosphate
231.0265	1094217	3137004	2.866895	0.001404	α-D-ribose-1-phosphate, D-Xylose-5-phosphate , D-xylulose-5-phosphate, alpha-D-Xylose 1-phosphate, beta-L-Arabinose 1-phosphate
239.014	15123.42	41023.4	2.712574	0.013875	R(+)-3,4-dihydroxyphenyllactate, Syringic acid
383.0739	738691	1998454	2.705399	0.013744	Chrysosplenol C
237.0159	218735.7	539198.6	2.465069	0.032146	R(+)-3,4-dihydroxyphenyllactate, Syringic acid
209.0813	6578.208	15943.55	2.423693	0.007183	Sinapoyl aldehyde
263.0429	150747.2	360727.8	2.392933	0.014362	3-Hydroxykynurenine
261.0454	1150522	2639618	2.294279	0.015213	5-Hydroxytryptophan
399.0562	14201.62	31640.04	2.227918	0.012005	7-methylguanosine-5-phosphate
366.0538	47417.94	105047.2	2.215346	0.020769	cis-coumarinic acid-β-D-glucoside
444.0866	19277.42	42296.26	2.194083	0.0399	isopentyenyladenine riboside monophoshate
279.0628	26168.58	53791.72	2.055584	0.04834	Isoliquiritigenin, Liquiritigenin, Pinocembrin, Pinocembrin chalcone

244.0927	26750.54	54607.98	2.041379	0.026926	cytidine
243.0529	29157.88	60159.28	2.063225	0.044	Tryptophan
546.1013	35182.62	70114.64	1.992877	0.001586	TDP-rhamnose
413.0635	68470.38	136183.2	1.988936	0.020925	Chrysosplenetin
337.0557	229626.1	448350	1.952522	0.023791	nicotinate mononucleotide
163.039	22932.36	44732.76	1.950639	0.036206	Umbelliferone
384.0646	490728.8	954680.2	1.945433	0.011699	2-hydroxylamino-4,6-dinitrotoluene-C-glucoside, 4-hydroxylamino-2,6-dinitrotoluene C-glucoside
249.0159	9169.104	17667.32	1.926832	0.002184	2-hydroxycaffeate, 5-Hydroxyferulate
181.0495	9205.324	17449.46	1.895583	0.002973	2,4-dihydroxycinnamate, 3-(4-Hydroxyphenyl)pyruvate, Caffeate, Caffeic acid
481.1108	45624.36	85327.32	1.870214	0.025634	Reduced FMN
367.079	18128.24	32346.14	1.784296	0.007181	3 ,5-Dihydroxy-3,4 ,7-trimethoxyflavone
249.0636	29439.66	52297.66	1.776436	0.048694	Pyridoxamine phosphate
215.0401	156954.8	271717.2	1.731181	0.002962	(Indol-3-yl)acetamide, Indole-3-acetaldehyde oxime
227.0873	13711.49	22295.04	1.626011	0.031978	methyl 9-oxononanoate
327.2296	22137.8	35117.62	1.586319	0.007612	(5Z,8Z,11Z,14Z)-Icosatetraenoic acid
213.016	41200.6	64953.92	1.576528	0.014857	1-deoxy-D-xylulose 5-phosphate, shikimate
228.082	11412.4	17959.28	1.573664	0.026785	8-amino-7-oxononanoate, 8-methylthiooctanaldoxime
275.0163	66010.26	102433.4	1.551779	0.010849	1-Phospho-alpha-D-galacturonate
262.0507	6547.464	10126.51	1.546631	0.041933	N-Acetyl-D-mannosamine
235.0366	13395.76	20453.7	1.526879	0.036475	5-Hydroxyconiferyl alcohol, phlorisobutyrophenone
245.0421	34398.16	52369.68	1.522456	0.01178	β-L-fucose 1-phosphate
226.0839	159613.8	232042	1.453772	0.043164	8-amino-7-oxononanoate

399.0584	23011	32096.42	1.394829	0.046608	Pantetheine 4-phosphate
233.021	9491.358	13056.88	1.37566	0.022388	5-Hydroxyconiferaldehyde, ferulic acid
223.0367	11114.24	15081.68	1.356969	0.00606	Choline phosphate
249.0886	12089.48	15942.9	1.318742	0.008596	(+)-7-Isojasmonic acid, phenylethylbenzoate
343.079	16177.12	21158	1.307897	0.03088	4-Coumaroylshikimate
219.0418	5896.98	7702.57	1.306189	0.031285	Coniferyl alcohol
247.0214	478716.8	612357.6	1.279165	0.03135	isochorismate
199.0367	55078.2	66446.68	1.206406	0.037723	4-Methylumbelliferone, Herniarin
221.0575	44131.44	48364.98	1.09593	0.025143	Dihydroconiferyl alcohol
191.0141	17378.66	13131.4	0.755605	0.037511	α-D-xylose, α-L-arabinopyranose, L-ribulose, L-xylulose, Ribulose, pentose-ring
170.0214	4942.074	3632.04	0.734922	0.037896	(S)-4-Amino-5-oxopentanoate, 5-Aminolevulinate , *trans*-4-hydroxy-L-proline, L-4-hydroxy-proline, L-glutamate γ-semialdehyde
159.0054	11054.47	7931.298	0.717474	0.035106	erythrose
198.0761	7409.7	5255.304	0.709247	0.034427	3,4-Dihydroxy-L-phenylalanine, N-Hydroxy-L-tyrosine
201.0676	4943.748	3465.65	0.701017	0.018352	Phenanthrene
199.048	5523.458	3819.918	0.691581	0.001704	D-Alanyl-D-alanine, homoglutamine
474.1731	16460.16	11330.03	0.68833	0.02546	5-formyl-tetrahydrofolate
141.9901	3356.502	2307.532	0.687481	9.6E-05	Carbamoyl phosphate
255.1075	36357.94	24639.82	0.677701	0.032063	Galactosylglycerol
212.9796	12511.33	8478.886	0.677697	0.004899	L-dehydro-ascorbate, cis-Aconitate
193.0989	10802.65	7148.544	0.66174	0.027311	(+)-Isomenthone, Linalool, (-)-Menthone, alpha-Terpineol, 1,8-Cineole, Geraniol
248.1127	12085.19	7932.87	0.656413	0.046214	Linamarin
259.0213	73629.2	48197.2	0.654594	0.037558	β-D-glucose 1-phosphate, 1D-*myo*-inositol (1)-monophosphate, 1D-*myo*-inositol (2)

					monophosphate, 6-phospho-D-glucono-1,5-lactone , glucose-1-phosphate
137.0363	5293.034	3368.906	0.636479	0.011384	3-Hexenal, Leaf aldehyde
207.9983	9594.778	5894.282	0.614322	0.008395	DL-O-Phosphoserine, *cis*-2-methylaconitate
247.0038	32269.36	19242	0.596293	0.024667	homocitrate, 2-(2-Methylthio)ethylmalic acid
128.0108	6986.248	4062.012	0.58143	0.03141	D-alanine, L-Alanine, Sarcosine, beta-Alanine
298.9929	17019.58	8969.17	0.526991	0.001006	2-carboxy-D-arabinitol 1-phosphate, D-Glucose 1-phosphate, D-Hexose 6-phosphate, D-Mannose 1-phosphate, D-galactose 6-phosphate, D-mannose 6-phosphate, D-sorbitol-6-phosphate, Fructose 1-phosphate, L-galactose-1-phosphate, alpha-D-Galactose 1-phosphate, alpha-D-Glucose 6-phosphate, beta-D-Fructose 6-phosphate, mannitol-1-phosphate
123.0553	4976.548	2601.198	0.522691	0.027715	*E*-pyridine-3-aldoxime, Nicotinamide
148.0604	1162012	587900.6	0.505933	0.015987	L-Glutamate, O-acetyl-L-serine
298.0968	42409.32	21390.52	0.504383	0.014682	5-Methylthioadenosine
417.0281	34133.32	16140.68	0.472872	0.015079	7-methylguanosine-5-phosphate
332.046	31382.54	14730.58	0.469388	0.039444	S-(indolylmethylthiohydroximoyl)-L-cysteine
131.0532	22535.22	10166.79	0.451151	0.022286	N-dimethylethanolamine
220.0816	8749.24	3933.622	0.449596	0.000192	O-succinyl-L-homoserine
204.0421	15.35994	14.58274	0.434908	0.049416	L-Phenylalanine
130.0499	276286	117480.4	0.425213	0.016818	5-Oxoproline, Pyrroline hydroxycarboxylic acid
172.0007	42420.52	16672.46	0.393028	0.009082	D-Aspartate
214.0112	15076.01	4817.272	0.319532	0.007221	4-phospho-hydroxy-L-threonine, L-aspartyl-4-P
164.074	144433	28800.63	0.199405	0.002296	Homomethionine

Appendix 6. Complete list of metabolic pathways perturbed by water deficit in sensitive population (*T. vulgaris*).

ko00010	energy	Glycolysis / Gluconeogenesis	http://www.genome.jp/kegg-bin/show_pathway?ko00010+C00103+C00668+C05345
ko02010	transport	ABC transporters	http://www.genome.jp/kegg-bin/show_pathway?ko02010+C00025+C00041+C00079+C00188
ko00970	translation	Aminoacyl-tRNA biosynthesis	http://www.genome.jp/kegg-bin/show_pathway?ko00970+C00025+C00041+C00078+C00079+C00188
ko00020	sugar	Citrate cycle (TCA cycle)	http://www.genome.jp/kegg-bin/show_pathway?ko00020+C00417
ko00030	sugar	Pentose phosphate pathway	http://www.genome.jp/kegg-bin/show_pathway?ko00030+C00668+C05345
ko00040	sugar	Pentose and glucuronate interconversions	http://www.genome.jp/kegg-bin/show_pathway?ko00040+C00103
ko00051	sugar	Fructose and mannose metabolism	http://www.genome.jp/kegg-bin/show_pathway?ko00051+C00636+C05345
ko00052	sugar	Galactose metabolism	http://www.genome.jp/kegg-bin/show_pathway?ko00052+C00103+C00446+C00668
ko00500	sugar	Starch and sucrose metabolism	http://www.genome.jp/kegg-bin/show_pathway?ko00500+C00103+C00668+C05345
ko00520	sugar	Amino sugar and nucleotide sugar metabolism	http://www.genome.jp/kegg-bin/show_pathway?ko00520+C00103+C00446+C00636+C00668+C03737+C03906+C04037+C05345
ko00660	sugar	C5-Branched dibasic acid metabolism	http://www.genome.jp/kegg-bin/show_pathway?ko00660+C00025+C00417
ko00130	secondary metabolites	Ubiquinone and other terpenoid-quinone	http://www.genome.jp/kegg-bin/show_pathway?ko00130+C00544+C01179

		biosynthesis	
ko00281	secondary metabolites	Geraniol degradation	http://www.genome.jp/kegg-bin/show_pathway?ko00281+C01500
ko00591	secondary metabolites	Linoleic acid metabolism	http://www.genome.jp/kegg-bin/show_pathway?ko00591+C00219
ko00592	secondary metabolites	alpha-Linolenic acid metabolism	http://www.genome.jp/kegg-bin/show_pathway?ko00592+C16310+C16317
ko00901	secondary metabolites	Indole alkaloid biosynthesis	http://www.genome.jp/kegg-bin/show_pathway?ko00901+C00078
ko00902	secondary metabolites	Monoterpenoid biosynthesis	http://www.genome.jp/kegg-bin/show_pathway?ko00902+C00843+C01500+C09844+C11388+C11393+C11952
ko00908	secondary metabolites	Zeatin biosynthesis	http://www.genome.jp/kegg-bin/show_pathway?ko00908+C00170
ko00940	secondary metabolites	Phenylpropanoid biosynthesis	http://www.genome.jp/kegg-bin/show_pathway?ko00940+C00079+C00590+C01197+C02947+C05619+C12204+C12205
ko00941	secondary metabolites	Flavonoid biosynthesis	http://www.genome.jp/kegg-bin/show_pathway?ko00941+C02947+C08650+C09762+C09827+C12128+C16404
ko00943	secondary metabolites	Isoflavonoid biosynthesis	http://www.genome.jp/kegg-bin/show_pathway?ko00943+C09762
ko00944	secondary metabolites	Flavone and flavonol biosynthesis	http://www.genome.jp/kegg-bin/show_pathway?ko00944+C04443+C04444
ko00945	secondary metabolites	Stilbenoid, diarylheptanoid and gingerol biosynthesis	http://www.genome.jp/kegg-bin/show_pathway?ko00945+C02947
ko00950	secondary metabolites	Isoquinoline alkaloid biosynthesis	http://www.genome.jp/kegg-bin/show_pathway?ko00950+C00355+C01179
ko00960	secondary	Tropane, piperidine and pyridine	http://www.genome.jp/kegg-bin/show_pathway?ko00960+C00079

	metabolites	alkaloid biosynthesis	
ko00965	secondary metabolites	Betalain biosynthesis	http://www.genome.jp/kegg-bin/show_pathway?ko00965+C00355
ko00966	secondary metabolites	Glucosinolate biosynthesis	http://www.genome.jp/kegg-bin/show_pathway?ko00966+C00078+C00079+C17210+C17213
ko01110	secondary metabolites	Biosynthesis of secondary metabolites	http://www.genome.jp/kegg-bin/show_pathway?ko01110+C00025+C00078+C00079+C00099+C00103+C00188+C00355+C00417+C00590+C00636+C00668+C00843+C01179+C01197+C01500+C02947+C03004+C05345+C05619+C08650+C09315+C09762+C11388+C11393+C12128+C12204+C12205+C17210+C17213
map01060	secondary metabolites	Biosynthesis of plant secondary metabolites	http://www.genome.jp/kegg-bin/show_pathway?map01060+C00025+C00041+C00078+C00079+C00103+C00170+C00188+C00355+C00417+C00668+C01179+C01500+C05345
map01061	secondary metabolites	Biosynthesis of phenylpropanoids	http://www.genome.jp/kegg-bin/show_pathway?map01061+C00078+C00079+C00417+C00590+C00668+C05345+C05619+C08650+C09315+C09762+C12204
map01062	secondary metabolites	Biosynthesis of terpenoids and steroids	http://www.genome.jp/kegg-bin/show_pathway?map01062+C00417+C01500+C05345+C11388+C11389+C11393
map01063	secondary metabolites	Biosynthesis of alkaloids derived from shikimate pathway	http://www.genome.jp/kegg-bin/show_pathway?map01063+C00078+C00079+C00355+C00417+C00668+C01179+C05345
map01064	secondary metabolites	Biosynthesis of alkaloids derived from ornithine, lysine and nicotinic acid	http://www.genome.jp/kegg-bin/show_pathway?map01064+C00025+C00079+C00417+C00668+C05345
map01065	secondary metabolites	Biosynthesis of alkaloids derived from histidine and purine	http://www.genome.jp/kegg-bin/show_pathway?map01065+C00417+C00668+C05345

map01066	secondary metabolites	Biosynthesis of alkaloids derived from terpenoid and polyketide	http://www.genome.jp/kegg-bin/show_pathway?map01066+C00417+C00668+C01500+C05345
ko00230	nucleic acid	Purine metabolism	http://www.genome.jp/kegg-bin/show_pathway?ko00230+C00169+C00242
ko00240	nucleic acid	Pyrimidine metabolism	http://www.genome.jp/kegg-bin/show_pathway?ko00240+C00099+C00169
ko00340	nucleic acid	Histidine metabolism	http://www.genome.jp/kegg-bin/show_pathway?ko00340+C00025
ko00564	lipids	Glycerophospholipid metabolism	http://www.genome.jp/kegg-bin/show_pathway?ko00564+C00588
ko01040	lipids	Biosynthesis of unsaturated fatty acids	http://www.genome.jp/kegg-bin/show_pathway?ko01040+C00219
map01070	hormones	Biosynthesis of plant hormones	http://www.genome.jp/kegg-bin/show_pathway?map01070+C00078+C00079+C00417+C00668+C05345
ko00710	energy	Carbon fixation in photosynthetic organisms	http://www.genome.jp/kegg-bin/show_pathway?ko00710+C00041
ko00250	amino acid	Alanine, aspartate and glutamate metabolism	http://www.genome.jp/kegg-bin/show_pathway?ko00250+C00025+C00041+C00169+C00402
ko00260	amino acid	Glycine, serine and threonine metabolism	http://www.genome.jp/kegg-bin/show_pathway?ko00260+C00078+C00188+C00213+C03232+C05519
ko00270	amino acid	Cysteine and methionine metabolism	http://www.genome.jp/kegg-bin/show_pathway?ko00270+C00041+C00170
ko00290	amino acid	Valine, leucine and isoleucine biosynthesis	http://www.genome.jp/kegg-bin/show_pathway?ko00290+C00188
ko00330	amino acid	Arginine and	http://www.genome.jp/kegg-

		proline metabolism	bin/show_pathway?ko00330+C00025+C00169+C00213
ko00350	amino acid	Tyrosine metabolism	http://www.genome.jp/kegg-bin/show_pathway?ko00350+C00355+C00544+C01179
ko00360	amino acid	Phenylalanine metabolism	http://www.genome.jp/kegg-bin/show_pathway?ko00360+C00079
ko00380	amino acid	Tryptophan metabolism	http://www.genome.jp/kegg-bin/show_pathway?ko00380+C00078+C00632
ko00400	amino acid	Phenylalanine, tyrosine and tryptophan biosynthesis	http://www.genome.jp/kegg-bin/show_pathway?ko00400+C00078+C00079+C01179
ko00410	amino acid	beta-Alanine metabolism	http://www.genome.jp/kegg-bin/show_pathway?ko00410+C00099
ko00450	amino acid	Selenocompound metabolism	http://www.genome.jp/kegg-bin/show_pathway?ko00450+C00041
ko00460	amino acid	Cyanoamino acid metabolism	http://www.genome.jp/kegg-bin/show_pathway?ko00460+C03004
ko00471	amino acid	D-Glutamine and D-glutamate metabolism	http://www.genome.jp/kegg-bin/show_pathway?ko00471+C00025
ko00473	amino acid	D-Alanine metabolism	http://www.genome.jp/kegg-bin/show_pathway?ko00473+C00041
ko00480	amino acid	Glutathione metabolism	http://www.genome.jp/kegg-bin/show_pathway?ko00480+C00025+C01879
ko00630	amino acid	Glyoxylate and dicarboxylate metabolism	http://www.genome.jp/kegg-bin/show_pathway?ko00630+C00417

Appendix 7. Complete list of metabolites with their intensities affected by water deficit stress in *T. vulgaris* for non-polar positive ions

m/z	watered	Droughted	Fold change	p_value	Metabolite name
471.3485	203394.6	2167474	10.65649	0.01461	4α-formyl-5α-cholesta-8,24-dien-3β-ol, 2-hydroxyoleanolate
487.3436	52654	451330	8.571618	0.001077	4α-carboxy-5α-cholesta-8,24-dien-3β-ol
144.0456	2363.78	10566.84	4.470315	0.007611	8-Hydroxyquinoline, Indole-3-carboxaldehyde
453.338	11366.92	37006.02	3.255588	0.000149	5,7,22,24(28)-ergostatetraenol
607.292	20135.29	59764.52	2.968147	0.048568	Presqualene diphosphate, all-trans-Hexaprenyl diphosphate
229.0274	19934.78	56814.33	2.850011	0.02786	5-Hydroxyconiferaldehyde , ferulic acid
455.3527	7445538	21121750	2.836833	0.002145	3-keto-4-methylzymosterol, 5,7,24(28)-ergostatrienol, 5-dehydro episterol
151.0402	16947.57	41138.22	2.427382	0.019944	4-Hydroxy-3-methoxy-benzaldehyde, Methyl salicylate, vanillin
331.0824	30076.72	72740.83	2.41851	0.020842	Pinobanksin, licodione, naringenin, naringenin chalcone
473.3642	17615.18	42271.83	2.399739	2.38E-05	22α-hydroxy-campest-4-en-3-one, 4Alpha-hydroxymethyl-5alpha-cholesta-8,24-dien-3beta-ol , 4alpha-formyl-5alpha-cholesta-8-en-3beta-ol
455.3172	11675.13	26522.23	2.271686	0.001153	2-methyl-6-geranylgeranyl-1,4-benzoquinol
327.2905	20234.74	44815.95	2.214802	0.000164	2-hydroxy-eicosanoate, 20-hydroxyeicosanoate
519.3331	6711.288	13783.15	2.053726	6.64E-05	1-Linoleoylglycerophosphocholine
423.4209	51972.3	104959.8	2.019534	0.000647	Octacosanoic acid
311.0562	11847.41	23687.45	1.999378	0.010442	2-carboxyanthraquinone
555.2241	6372.042	12406.72	1.947055	0.005108	all-trans-Pentaprenyl diphosphate
387.1298	5096.452	9064.102	1.778512	0.03454	Secologanin

528.2689	4398.192	7550.793	1.716795	0.004067	1-18:2-lysoPE
437.4371	16754.66	27850.37	1.662246	7.86E-05	Nonacosanoic acid, heptacosanoate, 25-methyl-methyl ester, 4-heptacosene, 6-heptacosene
465.4683	26499.64	43721.08	1.649875	0.004847	4-nonacosene, 6-nonacosene
227.0925	59410.12	97535.83	1.641738	0.006305	1,3,5-Trimethoxybenzene (JAN)
451.4527	111281	180178.8	1.619134	0.000991	Melissic acid
309.2072	191441.6	309367.2	1.615987	0.021926	13(S)-hydroperoxylinolenate, 13S-HpOTrE, 2-R-hydroperoxy-linolenate, 6,9-octadecadienedioate
479.484	155592.2	251311.5	1.615193	0.025574	Lacceroic acid
531.3696	23063.6	37240.65	1.614694	0.022813	2-hydroxyoleanolate
137.0245	175879.6	279732.2	1.590475	0.011472	3,4-Dihydroxybenzaldehyde, 3-Hydroxybenzoate
383.3532	40514.32	64362.5	1.588636	0.013338	24-hydroxytetracosanoate, DL-Cerebronic acid
195.03	25590.75	40201.57	1.570941	0.048558	3,4,6-trihydroxy-cis-cinnamate, 3-(2-propenoic acid)-4,6-hydroxy cyclohexa-2,5-dienone, 3-(3,4-Dihydroxyphenyl)pyruvate
521.3488	9868.386	15372.68	1.557771	0.001486	1-Oleoylglycerophosphocholine
475.4163	16054.54	24767.2	1.542691	0.016467	sitostanol
723.4256	29781.92	44638.07	1.498831	0.020108	18:3-16:3-PA
505.3536	6890.696	9959.795	1.445398	0.01092	3-dehydroteasterone
493.4997	57140.14	81736.37	1.430454	0.047129	4-hentriacontene, 6-hentriacontene, Psyllic acid
449.2915	12274.57	17203.25	1.401536	0.031002	1,2-benzenedicarboxylate acid, diisooctyl ester
537.3799	14605.23	20234.3	1.385415	0.010343	(22R,23R)-28-homocastasterone
395.075	6368.138	8809.118	1.383311	0.003692	Chrysosplenetin
553.3751	7229.77	9987.982	1.381508	0.000397	28-homobrassinolide

379.2493	13613.8	18522.48	1.360567	0.008125	sphinganine 1-phosphate
313.0616	5380.02	7155.098	1.329939	0.038502	Geranyl diphosphate
465.3228	13367.36	17164.7	1.284076	0.044735	28-norbrassinolide
293.2487	19099.48	15763.02	0.825311	0.015758	Sterculic acid
373.2597	12143.86	9208.202	0.75826	0.009809	9,10-epoxystearate
449.2551	22361.24	15919.6	0.711928	0.025997	1-16:1-lysoPE
790.5221	1251113	874365.2	0.69887	0.045534	18:2-18:3-MGDG, 18:3-18:2-MGDG
357.2072	9952.18	6546.91	0.657837	0.042633	Δ-9-tetrahydrocannabinolic acid
802.4649	86220.08	56311.3	0.653111	0.038749	18:2-18:3-PS
281.2486	2808600	1817895	0.64726	0.038825	(6Z)-Octadecenoic acid , (9Z)-Octadecenoic acid , cis-2-octadecenoate, octadecadiene-1,18-diol, stearate
225.186	31607.32	20285.65	0.641802	0.011224	Myristoleate, myristate
407.2595	58763.64	37071.92	0.630865	0.044034	3,5-dihydroxy-6,7-didehydro-12-apo-β-caroten-12-al, 5,6-epoxy-3-hydroxy-12-apo-β-caroten-12-al
467.2805	60564.08	37767.33	0.623593	0.030855	3,5-dihydroxy-6,7-didehydro-12-apo-β-caroten-12-al, 5,,6-epoxy-3-hydroxy-12-apo-β-caroten-12-al
659.4329	1186391	738191.5	0.622216	0.033141	9-cis-violaxanthin, 9-cis-Neoxanthin, Neoxanthin, Violaxanthin
125.0357		1931.222	0.618435	0.0478	Thymine
741.4721	2142180	1320164	0.616271	0.046998	18:3-t16:1-PG
815.5279	21781.74	13415.16	0.61589	0.035435	18:1-18:3-MGDG, 18:2-18:2-MGDG
742.476	912712.8	558991.7	0.612451	0.045539	1,2-dipalmitoyl-phosphatidylglycerol
935.5757	6622716	3880308	0.585909	0.01558	18:3-18:3-DGDG
936.5793	3221806	1882470	0.58429	0.017801	18:2-18:3-DGDG, 18:3-18:2-DGDG
563.39	45515.12	26478.6	0.581754	0.040648	3-hydroxy-4-ketotorulene, Canthaxanthin

755.4754	88418.5	51437.68	0.581752	0.01707	16:0-18:3-PS
356.2807	12341.92	7177.372	0.581544	0.01485	4,8-sphingadienine
817.5419	9019.24	5160.898	0.57221	0.002539	18:0-18:3-MGDG, 18:1-18:2-MGDG
848.5681	23007.46	12703.14	0.552131	0.017143	18:0-18:1-PS
745.5041	923812	508771.5	0.550731	0.041668	18:1-t16:1-PG, 18:2-16:0-PG
820.5335	1481526	815470.8	0.550426	0.009297	1-Hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-8-phosphoserine
973.6132	3814426	2049320	0.537255	0.003821	16:0-18:3-DGDG, 16:1-18:2-DGDG, 18:3-16:0-DGDG
971.5966	146426	78190.83	0.533996	3.98E-05	18:2-16:2-DGDG, 18:3-16:1-DGDG
441.2652	17671.96	9417.78	0.532922	0.000393	3S,5R,6S-5,6-epoxy-3-hydroxy-5,6-dihydro-12-apo-β-caroten-12-al
429.374	19947	10461.54	0.524467	0.001441	22α-hydroxy-sitosterol, 4alpha-hydroxymethyl-4beta-methyl-5alpha-cholesta-8-en-3beta-ol, 4beta-(hydroxymethyl)-4alpha-methyl-5alpha-cholest-7-en-3β-ol, alpha-Tocopherol
319.2224	10857.35	5646.377	0.520051	0.002864	(6Z)-Octadecenoic acid, (9Z)-Octadecenoic acid , cis-2-octadecenoate, octadecadiene-1,18-diol
819.5557	16730.4	8530.533	0.509882	0.002335	18:0-18:3-PC, 18:1-18:2-PC, 18:2-18:1-PC, 18:0-18:2-MGDG, 18:1-18:1-MGDG
719.4881	772442	392665.3	0.508343	0.007946	16:0-t16:1-PG, 1,2-dipalmitoyl-phosphatidylglycerol
550.4848	8592.794	4184.098	0.486931	0.014303	N-(2-hydroxyhexadecanoyl)-4,8-sphingadienine
599.4114	175734	83694.87	0.476259	0.006062	9-cis-violaxanthin, 9-cis-Neoxanthin, Neoxanthin, Violaxanthin
845.5516	60542.18	27299.15	0.450911	0.02456	20:2-18:3-PC
742.5406	33131.66	14350.44	0.433134	0.036355	18:0-18:2-PE
738.5097	141906.7	57158.47	0.402789	0.002177	18:1-18:3-PE
581.4009	91686.26	35552.62	0.387764	0.001258	4-ketolutein

751.5381	183447	68812.72	0.37511	5.88E-05	18:0-16:3-MGDG, 18:1-16:2-MGDG, 18:2-16:1-MGDG, 18:3-16:0-MGDG
331.1551	162101	58525.08	0.361041	0.022945	Gibberellin A20, Gibberellin A4, Gibberellin A51
842.5207	330417	115240.5	0.348773	0.008365	18:2-18:2-PS
414.2944	173079.8	46985.6	0.271468	0.001308	β-apo-8-carotenal
840.5053	314830.8	84746.6	0.269181	0.010424	18:2-18:3-PS
995.5966	2666850	683293.3	0.256217	0.000371	18:3-18:3-DGDG

Appendix 8. Complete list of metabolite intensities affected by water deficit stress in *T. serpyllum* for polar positive ions

m/z	watered	Droughted	Foldchange	p_value	Metabolite name
221.0211	8263.49	30368.65	3.675039	0.047204	D-Proline
146.0924	10026.64	32152.8	3.206736	0.010101	Succinate, erythronic acid lactone
409.0646	15509	40581.22	2.616624	0.011523	4-Guanidinobutanoate
213.037	4556.264	11397.28	2.501454	0.034691	Betaine, L-Norvaline, L-Valine, N,N-dimethyl-β-alanine, *cis*-2-hydroxy-6-oxohepta-2,4-dienoate, phenylacetonitrile oxide
407.0671	143485.5	311246.8	2.169187	0.013496	phenylglyoxal
215.0162	228634.6	473619.4	2.071512	0.074215	vanillate
215.0143	4254.032	8192.91	1.925916	0.042995	α-D-xylose, α-L-arabinopyranose, L-ribulose, L-xylulose, Ribulose, pentose-ring
221.0421	57409.94	108170.4	1.884176	0.0046	8-Hydroxyquinoline, Indole-3-carboxaldehyde, (R)-Pantolactone, (S)-3-Methyl-2-oxopentanoic acid, 4-Methyl-2-oxopentanoate
219.0475	9475.778	16592.58	1.751052	0.041627	Coumarin
138.0525	11032.8	19090.85	1.730372	0.020158	4-hydroxybenzoate, salicylate
156.0421	8266.11	13728.08	1.660766	0.028835	Indole-3-acetaldehyde, 4-(Trimethylammonio)but-2-enoate, 4-hydroxyphenyllactate, alpha-Ketopantoate
229.0108	60578.34	98609.98	1.627809	0.092693	3-dehydroquinate, 1-aci-nitro-2-indolylethane
215.0401	90898.66	145502.7	1.600713	0.012844	S2-isopropyl-3-oxosuccinate, 3-dehydro-shikimate
183.0418	6391.966	9877.3	1.545268	0.068014	1-deoxy-D-xylulose 5-phosphate
211.0003	5910.17	9093.716	1.538656	0.074853	shikimate
213.0636	5267.458	7766.516	1.474433	0.046136	N-α-acetylornithine, shikimate
182.0578	4887.514	7133.122	1.459458	0.041538	(Indol-3-yl)acetamide , Indole-3-acetaldehyde oxime, L-ascorbate, Coniferyl aldehyde
168.0421	4075.734	5882.556	1.443312	0.015164	1,2-Dihydroxy-5-(methylthio)pent-1-en-3-one, 2-Oxo-5-methylthiopentanoic acid, 2-Oxoadipate

423.1053	56017.08	78465.74	1.400747	0.049579	Citrate, Isocitrate
417.1522	250170.4	346647.4	1.385645	0.009964	D-Gluconic acid
223.0367	17256.56	23446.9	1.358724	0.039435	1,10-Phenanthroline
216.9934	5172.294	7023.028	1.357817	0.010544	benzaldehyde, 4-hydroxy-3,5-dimethoxy-
383.1106	23140.96	29196.32	1.261673	0.0488	Choline phosphate
169.0261	19690.74	24827.64	1.260879	0.046556	D-Iditol, D-Sorbitol, Mannitol, R(+)-3,4-dihydroxyphenyllactate, Syringic acid
243.0265	29509.86	34696.06	1.175745	0.013691	D-arabinose 5-phosphate, D-ribulose-1-phosphate, L-ribulose-5-phosphate, 3-dehydroquinate
239.1043	44138.88	51299.5	1.162229	0.052528	4,9-dimethyldodeca-2,4,6,8,10-pentaene-1,12-dial
138.0316	15208.18	17483.96	1.149642	0.030251	shikimate-3-phosphate
255.0264	15920.46	18190.94	1.142614	0.04547	o-succinylbenzoate
409.1107	62745.84	71612.38	1.141309	0.028658	1-Phospho-alpha-D-galacturonate
172.9999	7910.294	8995.928	1.137243	0.017699	D-myo-Inositol 1,2-cyclic phosphate
124.9999	13489.24	14981	1.110589	0.014328	2-Ethylhexyl phthalate, 4-prenylphlorisovalerophenone
317.1149	802484.8	834983.2	1.040497	0.059182	dihydroconiferyl alcohol glucoside
141.0158	25156.88	25565.18	1.01623	0.08998	2-hydroxy-2-methylbutyronitrile
140.0682	7276.2	6718.392	0.923338	0.061149	N-Methylethanolamine phosphate
219.0246	83730.02	69933.68	0.835228	0.038227	diacetyl
219.032	5448.026	3235.298	0.593848	0.013307	6-O-β-D-glucosyl-6-hydroxyflavone, 7-O-β-D-glucosyl-7-hydroxyflavone
200.9984	7793.326	4616.022	0.592304	0.04985	indolylmethyl-desulfoglucosinolate, 1-O-Sinapoyl-beta-D-glucose
275.0163	130107.7	73030.42	0.561308	0.046299	GA_{43} , gibberellin A_{28}
249.0454	3558132	1965156	0.5523	0.035953	indolylmethyl-desulfoglucosinolate

Appendix 9. Complete list of metabolites with their intensities affected by water deficit stress in *T. serpyllum* for non-polar positive ions

m/z	watered	Droughted	Fold change	p_value	Metabolite name
347.1864	9066.04	21685.62	2.391962	0.006483	GA_{110}, Gibberellin A_{14}, Gibberellin A_{15} open lactone, Gibberellin A_{53}
845.5516	48197.96	111582.4	2.315085	0.002148	20:2-18:3-PC
429.374	10292.61	22443.58	2.180552	2.70E-05	22α-hydroxy-sitosterol, 4alpha-hydroxymethyl-4beta-methyl-5alpha-cholesta-8-en-3beta-ol, 4beta-(hydroxymethyl)-4alpha-methyl-5alpha-cholest-7-en-3beta-ol, α-Tocopherol
840.5053	257555.2	550734.2	2.138315	0.000372	18:2-18:3-PS
227.0925	45123.5	92371.6	2.047084	0.01218	1,3,5-Trimethoxybenzene (JAN)
802.4649	76863.28	156135.7	2.031343	0.02901	18:2-18:3-PS
331.0824	16443	32121.18	1.953487	0.014556	Pinobanksin, licodione, naringenin, naringenin chalcone
347.0773	47995.92	90774.9	1.891305	0.018068	dihydrokaempferol, eriodictyol
331.1551	49478.8	89202.2	1.802837	0.026517	Gibberellin A_{20}, Gibberellin A_4, Gibberellin A_{51}
747.6096	52743.8	94409.74	1.789968	0.028254	plastoquinone-9
817.5419	7503.368	13167.31	1.754854	0.00745	18:0-18:3-MGDG , 18:1-18:2-MGDG
445.3246	9454.32	15112.74	1.598501	0.00112	4α-methyl-5α-ergosta-8,14,24(28)-trien-3β-ol, 4,4-dimethyl-5-α-cholesta-8,14,24-trien-3-β-ol , -Dehydroavenasterol, 4,4-Diapophytoene
844.5362	279976.4	439478.8	1.569699	0.001309	18:0-18:3-PS, 18:1-18:2-PS
820.5335	861404.4	1328790	1.542586	0.001153	1-Hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phosphoserine

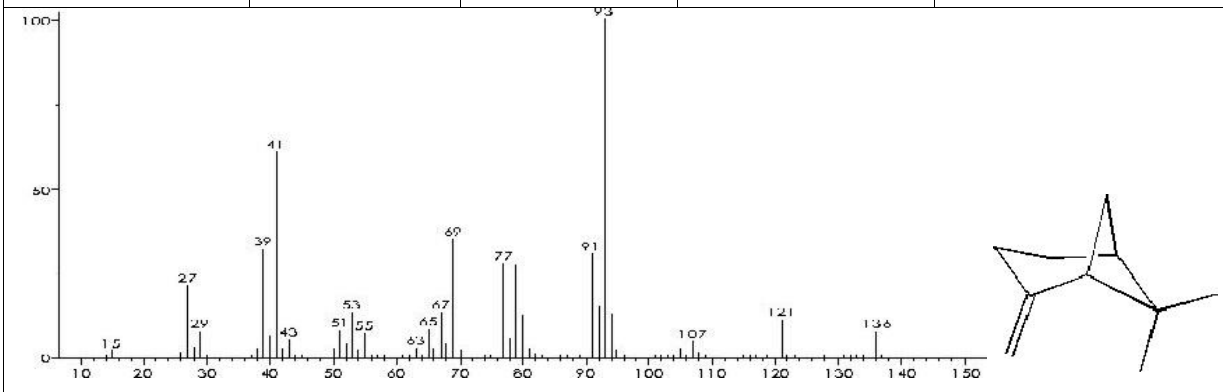
842.5207	345632.4	532269.8	1.539988	0.027269	18:2-18:2-PS
819.5557	13019.98	19931.18	1.530815	0.001727	18:0-18:3-PC, 18:1-18:2-PC, 18:2-18:1-PC, 18:0-18:2-MGDG, 18:1-18:1-MGDG, 18:1-18:3-MGDG, 18:2-18:2-MGDG
417.3222	1650804	2466114	1.493887	0.017791	1-monostearin
751.5381	72862.88	105937.1	1.453924	0.004963	18:0-16:3-MGDG, 18:1-16:2-MGDG , 18:2-16:1-MGDG, 18:3-16:0-MGDG
816.5037	2942034	4236122	1.439862	0.006599	16:0-18:3-PS
419.3294	35622.18	51043.94	1.432926	0.021547	24-methyldesmosterol, Campest-4-en-3-one, brassicasterol, crinosterol, episterol, Pentacosanoic acid, 24-hydroxytetracosanoate, DL-Cerebronic acid
697.4829	548727.2	785278.4	1.431091	0.048417	18:0-18:3-PA, 18:1-18:2-PA
791.5746	64026.96	90811.16	1.418327	0.008214	2-nonaprenyl-6-methoxy-1,4-benzoquinol
581.4009	26686.38	37276.54	1.396838	0.016164	4-ketolutein
792.5774	17411.36	24176.64	1.388556	0.021756	1,2-dipalmitoyl-phosphatidylcholine
973.6132	2413822	3335048	1.381646	0.003221	16:0-18:3-DGDG, 16:1-18:2-DGDG, 18:3-16:0-DGDG
599.4114	58569.04	78097.82	1.333432	0.027119	9-*cis*-violaxanthin , 9-*cis*-Neoxanthin , Neoxanthin, Violaxanthin
936.5793	1980274	2594834	1.310341	0.041392	18:2-18:3-DGDG, 18:3-18:2-DGDG
971.5966	116958.2	149707.4	1.280008	0.030809	18:2-16:2-DGDG, 18:3-16:1-DGDG
832.5106	570955.8	662259.8	1.159914	0.048879	16:0-18:2-PI, 20:1-18:3-PS, 20:2-18:2-PS
523.3642	16943.44	14877.48	0.878067	0.016729	1-18:0-lysoPC, castasterone
509.3851	16916.44	13248.44	0.78317	0.006516	6-deoxocastasterone
297.1861	148886.6	116494.4	0.782437	0.035449	retinoate

662.6107	7441.712	5325.092	0.715574	0.00795	N-(2-hydroxytetracosanoyl)-4,8-sphingadienine
271.1705	8594.666	6102.842	0.710073	0.047346	estradiol
636.5944	19099.28	12791.36	0.66973	0.002439	4-hydroxysphing-8(E)-enine-22:0, ceramide, 4-hydroxysphing-8(Z)-enine-22:0, ceramide
692.6577	14371.58	9605.452	0.668364	0.000426	4-hydroxysphing-8(E)-enine-26:0, ceramide, 4-hydroxysphing-8(Z)-enine-26:0, ceramide
521.3488	15213.86	9653.548	0.634523	0.001961	1-Oleoylglycerophosphocholine
489.3591	11692.45	7328.398	0.626763	0.005765	4α-carboxy-5-α-cholesta-8-en-3β-ol
309.2072	436312.2	269937.2	0.618679	0.029825	13(S)-hydroperoxylinolenate, 13S-HpOTrE, 2-R-hydroperoxy-linolenate, 6,9-octadecadienedioate
505.3536	12441.32	7310.812	0.587623	0.000142	3-dehydroteasterone
752.679	16650.02	9465.278	0.568484	0.000863	4-hydroxysphing-8(E)-enine-26:0, ceramide, 4-hydroxysphing-8(Z)-enine-26:0, ceramide
519.3331	18177.02	9564.566	0.52619	0.007652	1-Linoleoylglycerophosphocholine
552.5003	16387.96	8409.272	0.513137	0.004599	4-hydroxysphing-8(E)-enine-16:0, ceramide, 4-hydroxysphing-8(Z)-enine-16:0, ceramide
581.3709	14564.52	7412.89	0.508969	1.39E-05	1-Oleoylglycerophosphocholine
291.1967	285580.6	142493	0.498959	0.037567	(9Z)-(13S)-12,13-epoxyoctadeca-9,11-dienoate, 12,13(S)-epoxylinolenate, 12-oxo-cis-10,15-phytodienoate
623.2868	17793.46	7942.556	0.446375	0.012151	Presqualene diphosphate, all-trans-Hexaprenyl diphosphate
696.6162	46949.42	20093.02	0.427972	0.005757	4-hydroxysphing-8(E)-enine-22:0, ceramide, 4-hydroxysphing-8(Z)-enine-22:0, ceramide
295.2279	111585.4	47477.4	0.42548	0.004673	16-oxo-palmitate, 18-

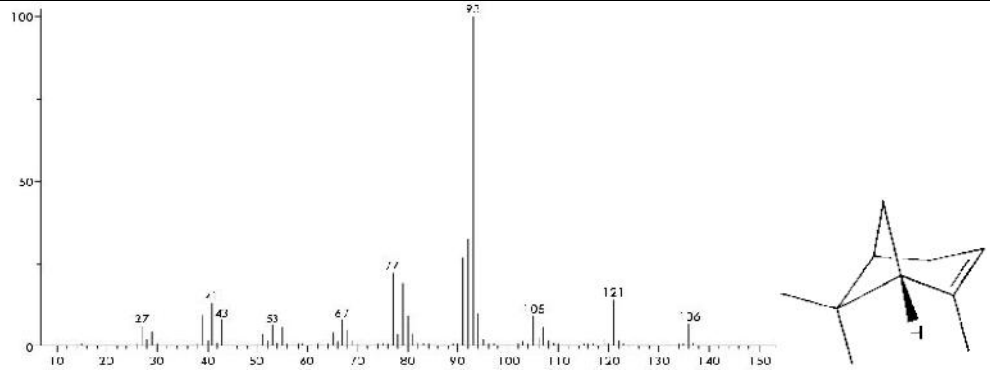
					hydroxyoctadeca-9Z,12Z-dienoate, vernoleate
311.2229	129411.1	52809.2	0.408073	0.007721	13(S)-hydroperoxyoctadeca-9,11-dienoate, hexadecanedioate
559.2356	52789.83	11030.52	0.208952	0.012233	protoporphyrin IX
327.2905	127945.3	21843.36	0.170724	0.009202	2-hydroxy-eicosanoate, 20-hydroxyeicosanoate, Octadecanal

Appendix 10. Complete list of volatiles with their formulas and profiling detected by GC-MS in *T. serpyllum* and *T. vulgaris*.

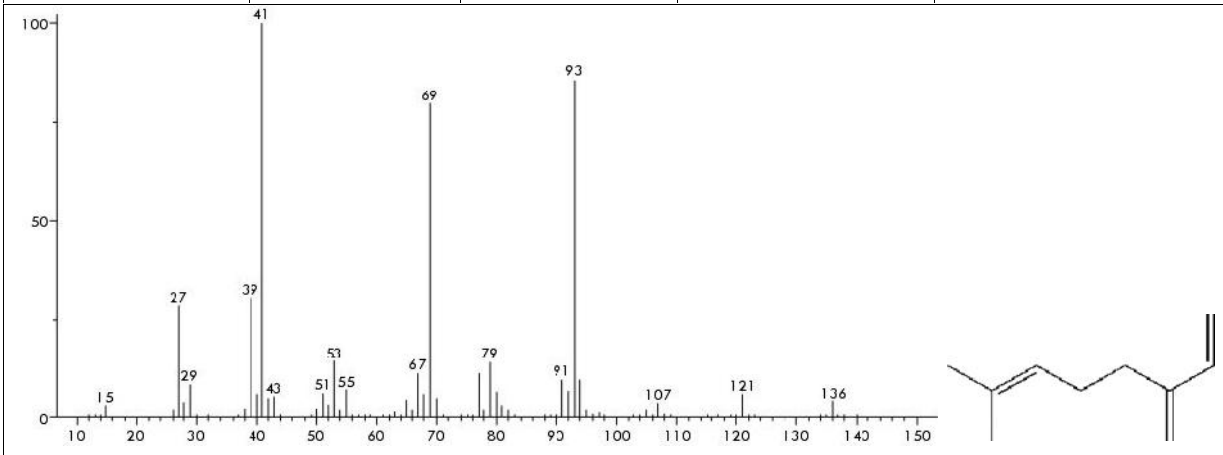
name	RT	KI	Formula	Ion
Beta-Pinene		980	C10H16	93



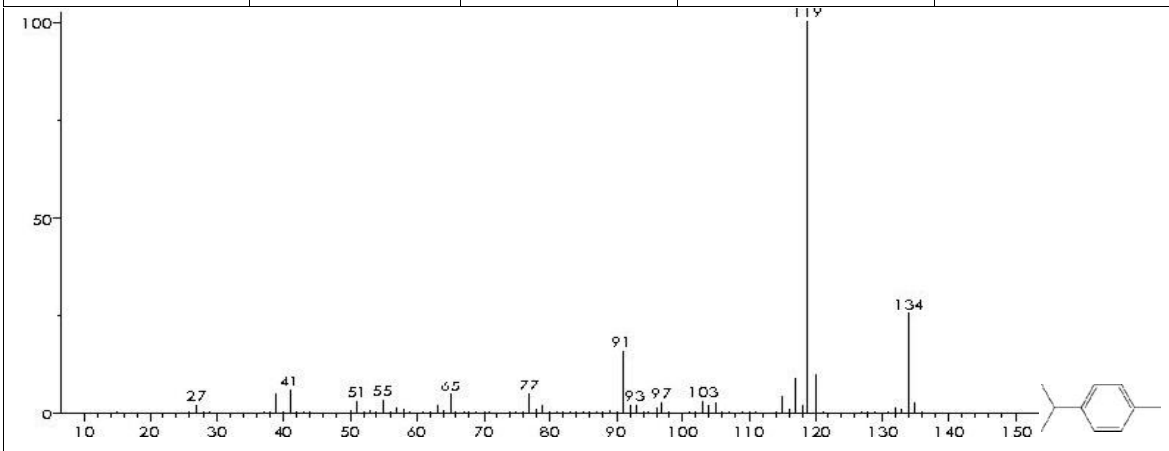
name	RT	KI	Formula	Ion
Alpha-Pinene	195		C10H16	93



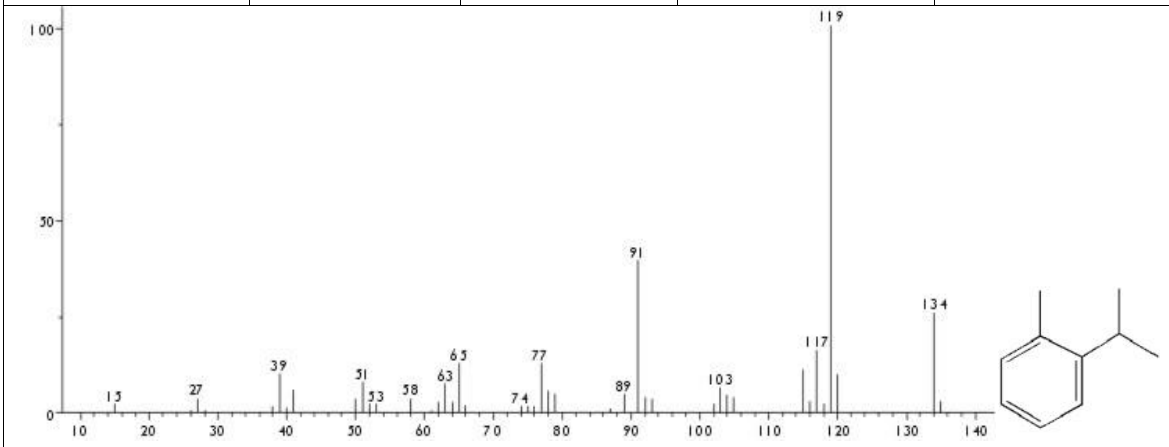
name	RT	KI	Formula	Ion
Beta- Myrcene	220	991	C10H16	41,93, 69



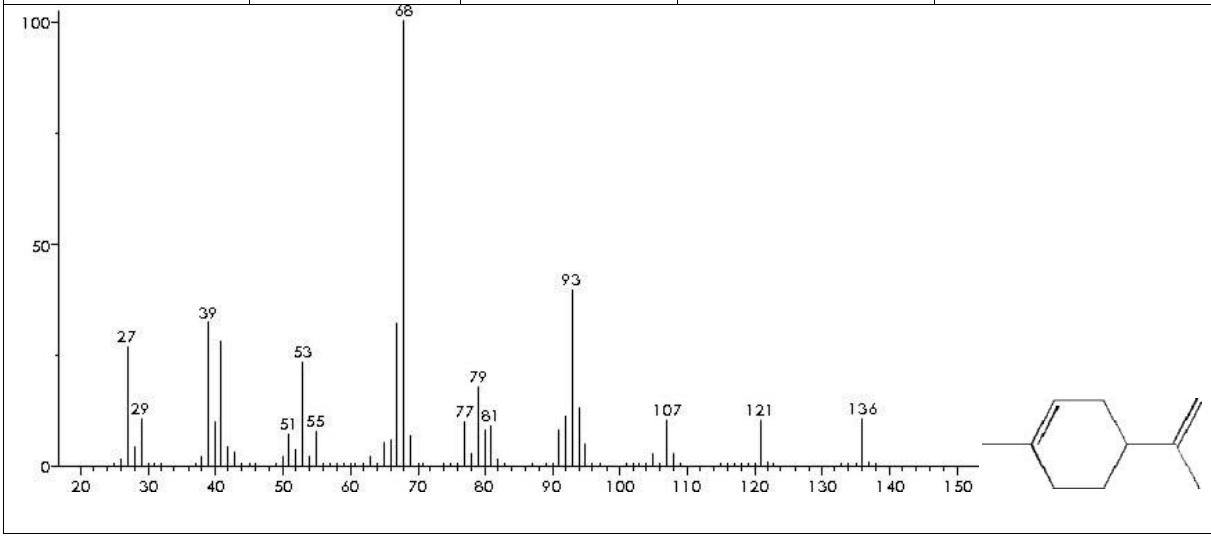
name	RT	KI	Formula	Ion
P- Cymene		1026	C10H14	119



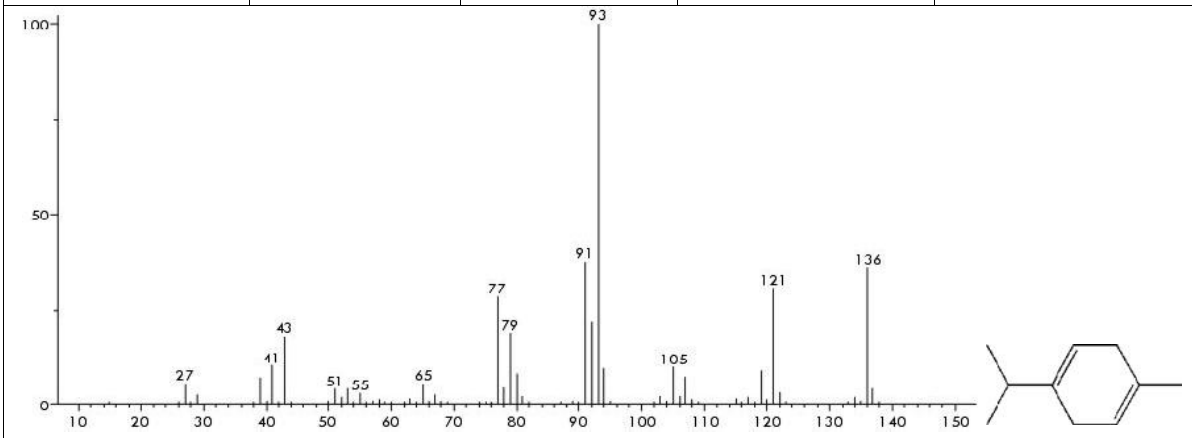
name	RT	KI	Formula	Ion
O- Cymene			C10H14	119



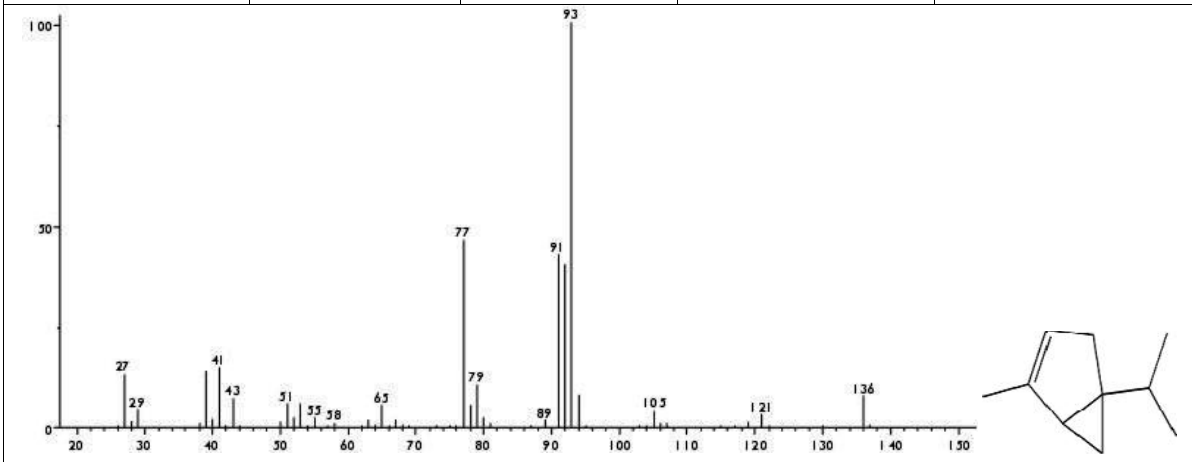
name	RT	KI	Formula	Ion
Limonene			C10H16	68



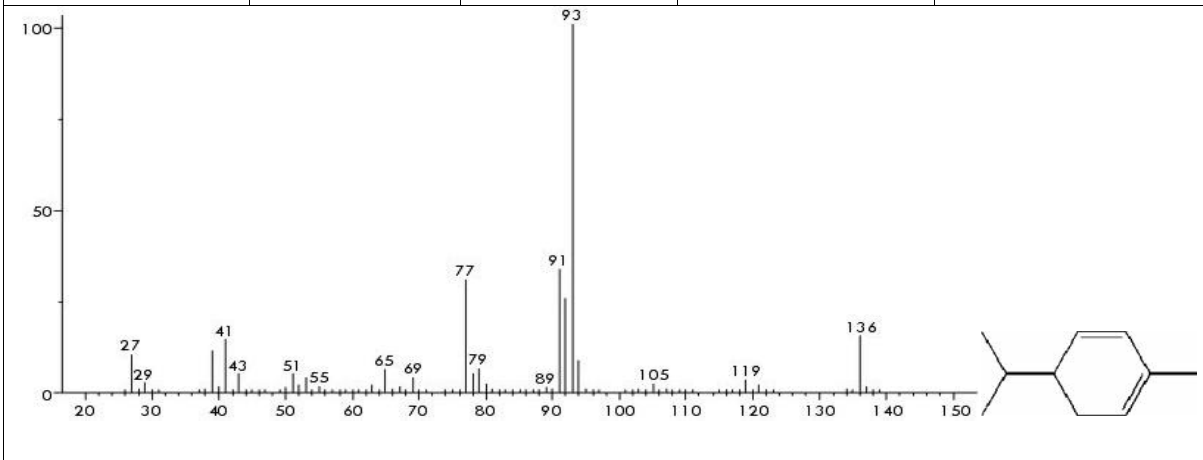
name	RT	KI	Formula	Ion
Gamma-Terpinen			C10H16	93



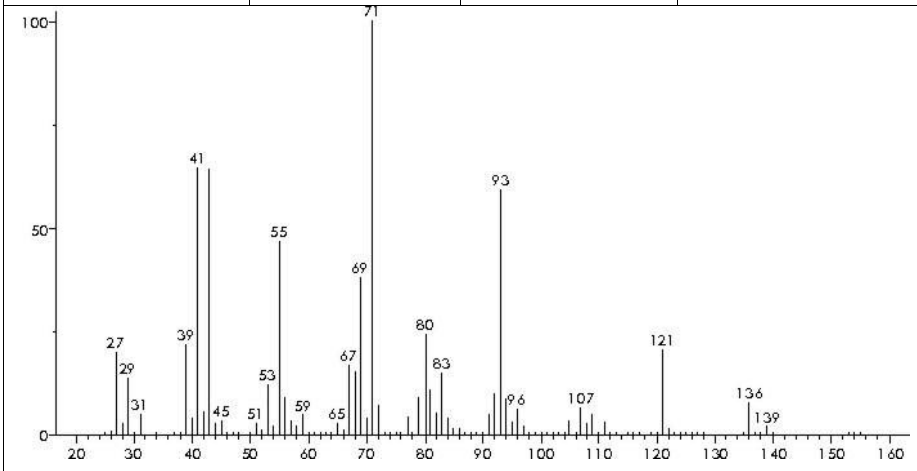
name	RT	KI	Formula	Ion
Alpha-Thujene			C10H16	93



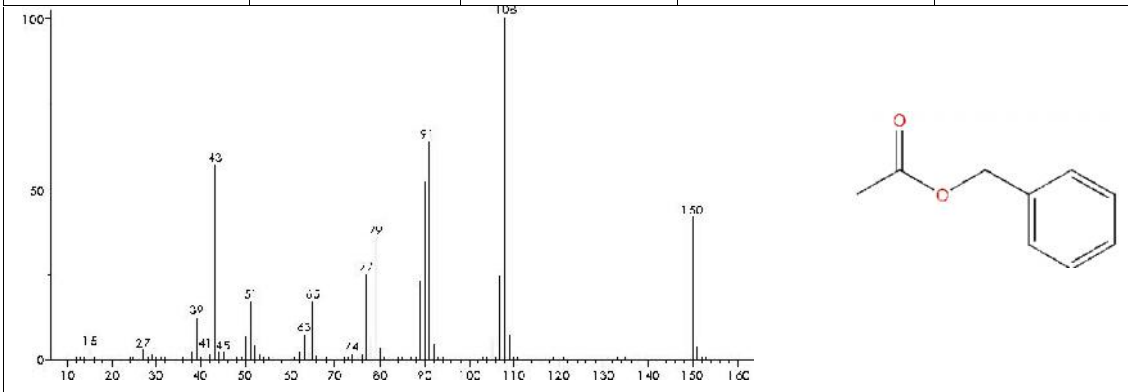
name	RT	KI	Formula	Ion
Alpha-Phellandrene		1005	C10H16	93



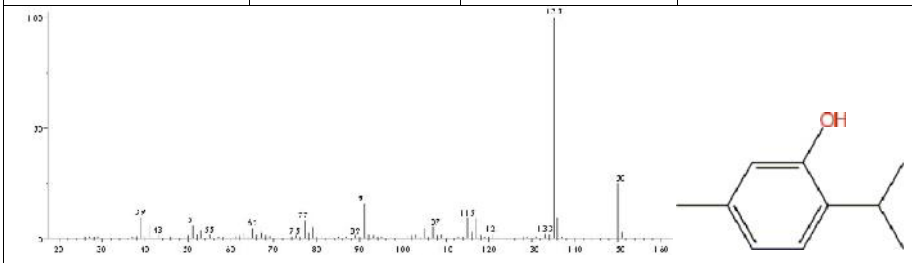
name	RT	KI	Formula	Ion
Linalool		1098	C10H18O	71



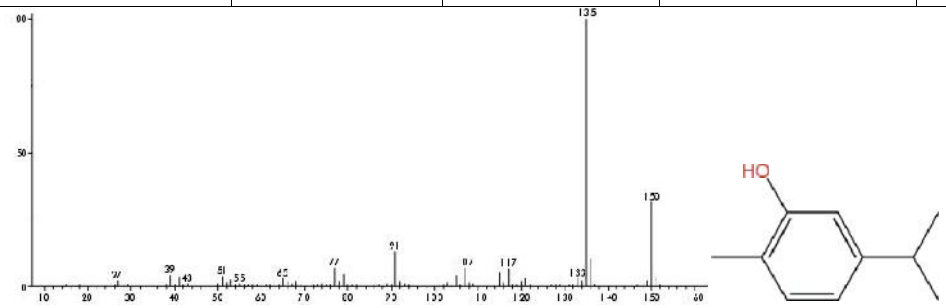
name	RT	KI	Formula	Ion
Benzyl Acetate		1098	C9H10O2	108



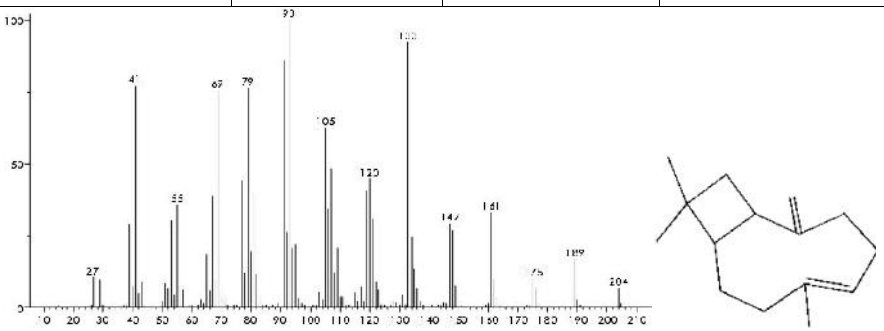
name	**RT**	**KI**	**Formula**	**Ion**
Thymol		1290	C10H14O	135



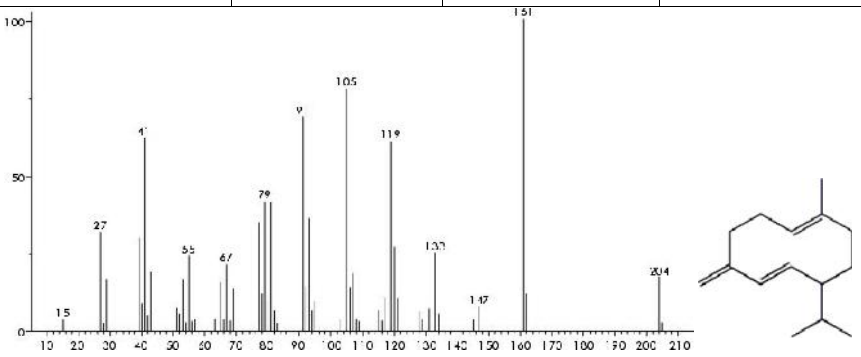
name	RT	KI	Formula	Ion
Carvacrol		1290	C10H14O	135



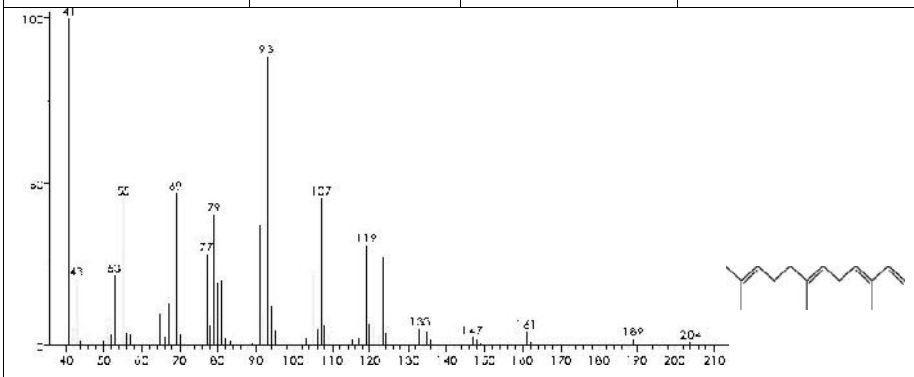
name	RT	KI	Formula	Ion
Beta-Caryophyllene		1418	C15H24	93,133



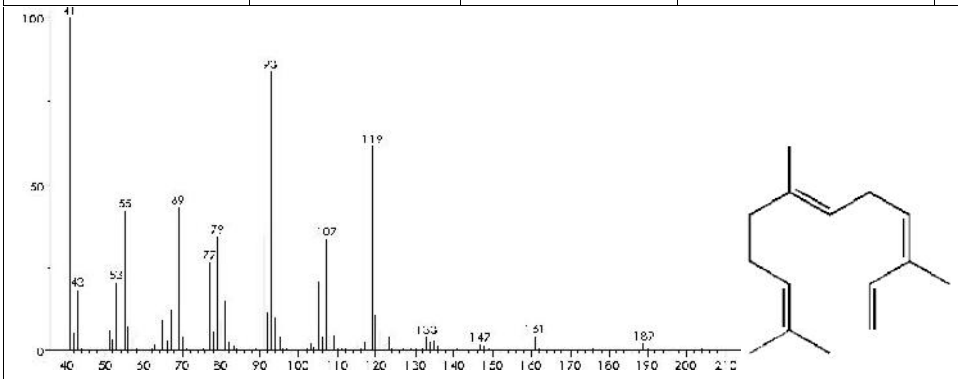
name	RT	KI	Formula	Ion
Germacrene D		1480	C15H24	161,105



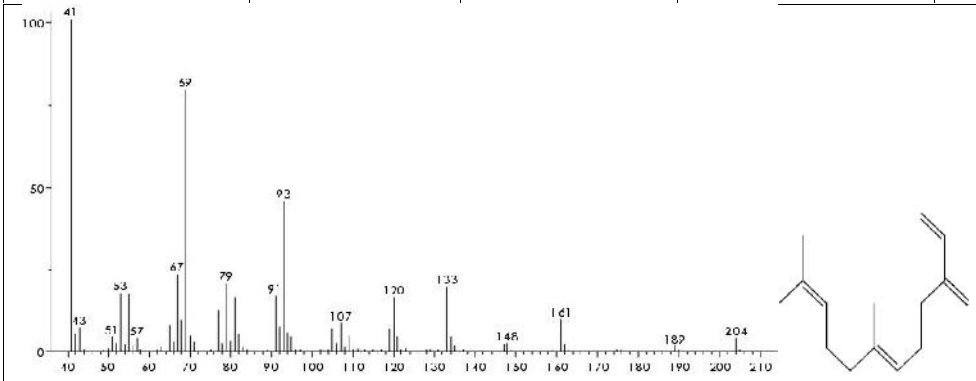
name	RT	KI	Formula	Ion
E,E Alpha-Farnesene		1508	C15H24	41,93



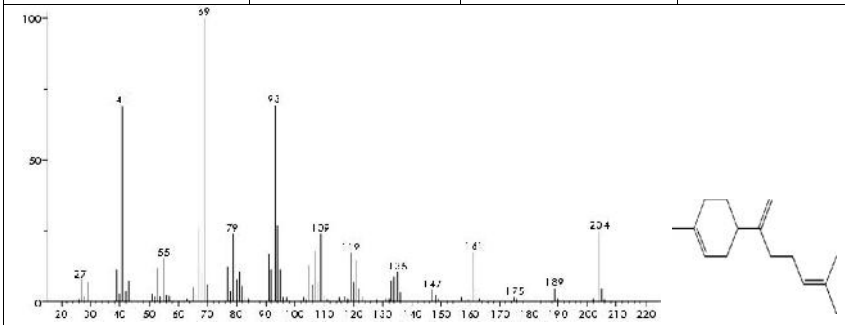
name	RT	KI	Formula	Ion
Z,E Alpha-Farnesene			C15H24	41,93



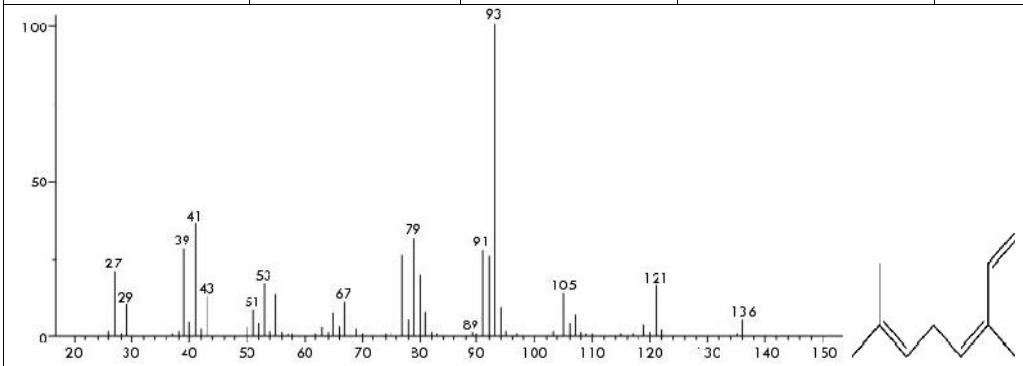
name	RT	KI	Formula	Ion
E Beta-Farnesene		1458	C15H24	41,69



name	RT	KI	Formula	Ion
Beta-Bisabolene		1509	C15H24	69,93



name	RT	KI	Formula	Ion
Ocimene, cis beta		1040	C10H16	93



name	RT	KI	Formula	Ion
Alpha-Caryolphyllene/ alpha-Humulene	392	1454	C15H24	93