ZN STRESS IN *Tetradenia riparia*. ANALYSIS OF PHYSIOLOGICAL RESPONSES, INTERACTION WITH WATER STRESS AND VOCs EMISSION

“It is not the strongest of the species that survives,

but the most adaptable”

Charles Darwin

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GLOSSARY

Chl	chlorophyll
d.w.	dry weight
ETR	electron transport rate
f.w.	fresh weight
Fv/Fm	maximum quantum yield of photosystem II
m/z	mass-to-charge ratio
ncps	number of counts per second
ROS	reactive oxygen species
PS	photosystem
PTR-Ms-ToF	proton transfer reaction-time of flight-mass spectrometry
VOCs	volatile organic compounds

CHAPTER 1: GENERAL INTRODUCTION

1. 1 ABIOTIC STRESS IN PLANTs

Environmental factors affect an organism in many ways, at any time. Actually, the natural environment for plants is composed of a complex set of abiotic and biotic stresses. Plant responses to these stresses are equally complex.

Biotic environmental factors, resulting from interactions with other organisms, are, for example, infection or mechanical damage by herbivory or trampling, as well as effects of symbiosis or parasitism (Schulze et al., 2002); Instead, abiotic stress is defined as the negative impact of non-living factors on the living organisms in a specific environment. The non-living variable must influence the environment beyond its normal range of variation to adversely affect the population performance or individual physiology of the organism (Vinebrooke et al., 2004).

Abiotic stress is the most detrimental factor concerning the growth and productivity of crops worldwide (Gao, Ji-Ping, et al., 2007) and it is also known that abiotic stressors are most harmful when they occur together, in combinations (Mittler, 2006).

Abiotic stress comes in many forms. The most common of the stressors are the easiest for people to identify, but there are many other, less recognizable abiotic stress factors which affect environments constantly (Palta et al., 2006).

The most basic stressors include:

- Light stress
- Wind
- Rain
- Extreme temperatures
- Drought
- Flooding
- Other natural disasters, such as tornadoes and wildfires
- Chemicals and pollutants such as heavy metals or pesticides
- Nutrient deprivation in soil

Lesser-known stressors generally occur on a smaller scale. They include: poor edaphic conditions like rock content and inadequate pH levels, high radiation, compaction, contamination, and other, highly specific conditions like rapid rehydration during seed germination (Palta et al., 2006).
The effect of each abiotic factor depends on its quantity. Optimal intensities and concentrations of these may also differ not only for individual organisms, but also for particular organs of the same organism (Schulze et al., 2002).
The environmental factors that can cause a stressful condition in plants are more and more subject to fluctuations related also to the increase in human activities, including agriculture, mining, industrial, waste production.

In general, abiotic stress adversely affects the growth, development and productivity of plants, causing damage at the cellular, molecular, physiological and anatomical level.
Photosynthesis is severely affected in all its phases by environmental stress. Since the mechanism of photosynthesis involves various components, including photosynthetic pigments, photosystems, electron transport system, and CO_2 reduction pathways, any damage at any level caused by a stress may reduce the overall photosynthetic capacity of a green plant. (Ashraf et al., 2013).

Different stressful environments have been reported to reduce the contents of photosynthetic pigments. For example, salt stress can break down chlorophyll (Chl), the effect ascribed to increased level of the toxic cation, Na^+ (Ashraf et al., 2013). Reduction in photosynthetic pigments, such as Chl a and b has been reported in some earlier studies on different crops (Ashraf et al., 2013). Another class of pigments that play a key role for the plant under stress are carotenoids. They protect against oxidative stress through a scavenging mechanism and can receive energy from the chlorophylls in the triplet state, preventing this energy is transferred to oxygen (Finazzi et al., 2004). Thus, carotenoids are necessary for photoprotection of photosynthesis; Moreover, they play an important role as a precursor for signaling during the plant development under abiotic / biotic stress (Ashraf et al., 2013).
Previous studies have shown that these molecules are influenced by heavy metal stress such as cadmium. For example, in the green algae *Chlorella vulgaris* was found that the amount of carotenoids decreased with increasing cadmium over 18 days exposure. Another study highlighted that carotenoid concentration of *Sesuvium portulacastrum* leaves increased at 100, 200 and 300 mg kg-1 of Zn level in the soil,

but further increase in Zn level, significantly decreased the carotenoid concentration of the leaves of this plant (Kalaikandhan et al., 2014).

Many abiotic stresses can also affect, directly or indirectly, the synthesis, concentration, metabolism, transport and storage of sugars in plants. Soluble sugars do not only function as metabolic resources and structural constituents of cells, but they also act as signals regulating various processes associated with plant growth and development (Loreti et al., 2001).

Changes in environmental factors, such as light, water or temperature and attacks by herbivores or pathogens agents can lead to a significant decrease in photosynthetic efficiency in leaf tissues, reducing the supply of soluble sugars to sink tissues (Rosa et al., 2009). Soluble sugar fluctuations under abiotic stresses can involve changes in CO_2 assimilation and in activity of related enzymes as well as in the expression of specific genes. (Rosa et al., 2009). For example, it was observed that at least 10% of Arabidopsis genes are sugar-responsive (Kang et al., 2010). Therefore it is important to consider that sugar signaling pathways interact with stress pathways to modulate metabolic plant responses. (Tran et al., 2007).

1.1.1 PLANT RESPONSES

Plants must be able to sense the surrounding environment and give an appropriate response to each stimulus. The ability of plants to adapt and/or acclimate to different environments is directly or indirectly related with the plasticity and resilience of photosynthesis, in combination with other processes, determining plant growth and development (Turkan, 2011). A remarkable feature of plant adaptation to abiotic stresses is the activation of multiple responses involving complex gene interactions and crosstalk with many molecular pathways (Turkan, 2011).

Plants respond to stress through a variety of mechanisms. The mechanisms that allow the survival consent to the plant to avoid or tolerate stress.

Avoidance involves a reduction in metabolic activity during stress for not taking it.

Tolerance means maintain a high metabolic activity even during moderate stress, reducing it during the severe stress (Hasanuzzaman et al., 2013).

The stress response, and development with metabolic changes, starts from the moment in which the plant recognizes the existence of the stress at the cellular level.

Generally, plant stress responses depend on several factors, such as plant species, genotype, age of plant, stage of development and organ or tissue in question. For example, transcriptional responses to stress are tissue or cell specific in roots and are quite different depending on the stress involved (Dinneny et al., 2008). In addition, the severity and duration of stress (acute vs chronic), as well as number of exposures and the combinations of different stresses can have a significant effect on the complexity of the response (Kumar et al., 2014).

Plant abiotic stress responses include changes in both physiological and biochemical processes as well as in anatomical and developmental patterns. Despite the diversity in plant species and abiotic stresses, a generic “stress-induced” response at the plant anatomical level is reported which mainly comprises three components: inhibition of cell elongation, localized stimulation of cell division, and alterations in cell differentiation status. This results in changes in anatomical characteristics of basic plant organs, mainly roots, xylem, and leaves, which contribute in adaptation to unfavorable environmental conditions (Patakas, 2012).

One of the earliest metabolic responses to abiotic stresses and the inhibition of growth is the inhibition of protein synthesis (Good and Zaplachinski, 1994) and an increase in protein folding and processing (Liu JX et al., 2010). Energy metabolism is affected as the stress becomes more severe (e.g. sugars, lipids and photosynthesis, Pinheiro et al., 2011). Thus, complex changes, rapid or gradual, can occur in plant metabolism in response to stress.

1.2 HEAVY METAL STRESS IN PLANTs

The term "heavy metal" refers to those metallic elements that have density higher than 5 g/cm³ (Holleman and Wiberg, 1985). The 40 items that fall into this category usually behave as cations and are characterized by different oxidation states (transition metal elements), low solubility of their oxides, great ability to form complexes and great affinity for the sulphides (Riffaldi and Levi-Minzi, 1989).

These metals are natural components of the earth’s crust. High metal concentrations in soils can occur naturally, such as through ore outcropping, or anthropogenically, due to, for example, mining and smelting activities and the use of metal-containing fertilizers and pesticides. In the environment, heavy metals are generally more

persistent than organic contaminants, such as pesticides or petroleum products (Tchounwou et al., 2012).

The toxic metal pollution in water and soils is greatly increased as a result of anthropogenic activities. The processes taking place at high temperature introduce metals in the atmosphere in gaseous phase or in the form of particulate. The metals released into the atmosphere (mainly As, Cd, Cu, Hg, Pb, Sn and Zn), first to be deposited on the ground and at sea, are carried by winds as a function of their physical-chemical form. Most of the coarser particulate is deposited in a range of 10 km from the source emission, whereas for metals in the gas phase, the deposition can take place at much greater distances, up to 10,000 Km from emission sources. The trace metals, released in the environment, deposited on land, in the waters and sediments, are subject to global geochemical cycles which determine a continuous circulation among the various environmental media (Wuana and Okieimen, 2011). The heavy metals, into the ground, may undergo various processes such as adsorption, complexation and precipitation according to the chemical-physical characteristics of the soil (Aromolo et al., 1999).

Some heavy metals act as essential micro-nutrients for plants, just like for most other organisms. They include the metals from Mn to Zn in the fourth period of the periodic table, plus Mo in the fifth period. These elements take part in biological processes owing to their chemical properties, such as redox–activity under physiological conditions (Cu, Fe) or Lewis acid strength (Zn) (Frausto da Silva and Williams, 2001). However, the above-mentioned properties are also the reason why metals can be toxic when present in excess. For example, redox-active metals can participate in Haber-Weiss and Fenton reactions, triggering the formation of toxic hydroxyl radicals (Halliwell and Gutteridge, 1990); moreover, uncontrolled high-affinity binding to sulphur-, nitrogen- and oxygen-containing functional groups in biomolecules can cause their inactivation and damage.

The physiological range for essential metals between deficiency and toxicity is extremely narrow, therefore, a strictly controlled metal homeostasis network is necessary in all organisms, to adjust fluctuations in micronutrient availability (Nelson, 1999; Clemens, 2001).

1.2.1 THE ROLE OF ZINC IN PLANTs

Zinc is a crucial element for the metabolism of plant cells, being involved in a wide variety of physiological processes at the micromolar range (Marschner, 1995).
This metal plays an essential role in nucleic acid metabolism, in ribosome structure and functioning and, consequently, in protein synthesis and in the carbohydrate metabolism. Moreover, Zn-finger proteins, the largest class of Zn-binding proteins, are important factors regulating transcription (Broadley et al. 2007).
Zinc has protective effects against oxidative stress, peroxidative damages, loss of integrity and alteration of membrane permeability (Aravind and Prasad 2003). It is involved in the biosynthesis of chlorophyll carotenoids and in many metabolic reactions (Aravind and Prasad 2005). Most significantly, Zn acts as cofactor of number of enzymes such as dehydrogenases, oxidases, peroxidases (Vallee and Auld 1990) as well as anhydrases and performs significant role in the defense system. Indeed, it is required for the activity of more than 300 enzymes (McCall et al. 2000).
In several plant species, the activities of Zn-containing enzymes such as carbonic anhydrase, alcohol dehydrogenase and Zn/Cu superoxide dismutase are good indicators of the plant Zn nutrition (Talukdar and Aarts, 2008).

Zinc is present naturally in the environment, but the increase in human activities, related to extraction, industry and agriculture, has led to a concerning increase in the concentration of the metal in the environmental matrices (Duruibe et al., 2007).
Zinc availability in soils largely depends on the kind of clay minerals, pH and organic matter and its toxicity may occur in contaminated soils because of mining and smelting activities or due to long–term application of sewage sludge, manures, or Zn-rich municipal waste compost.
This metal can be toxic for plants at supra-optimal concentrations, and toxicity generally occurs when leaf concentrations reach 400–500 μg g–1 of dry mass (Marschner, 1995; Broadley et al., 2007). Common Zn toxicity symptoms can include a reduced plant water content, stunted plant growth, a decrease of stomatal conductance and photosynthesis, changes in root growth and morphology, severe nutrient imbalances and leaf chlorosis (Bazihizina et al., 2014).
Plants exhibiting Zn toxicity have smaller leaves than control plants and brown spots become apparent on the leaves of some species (Reichman, 2002). In severe cases, plants may exhibit necrotic lesions on leaves and eventually entire leaf death.

In roots, Zn toxicity is apparent as a reduction in the growth of the main root, fewer and shorter lateral roots and a yellowing of such organs (Reichman, 2002).

The toxic effects of Zn-excess have been reported in several plant species.
For example, in *Phaseolus vulgaris*, toxic concentrations of Zn resulted in inhibition of photosystems I and II and thus a decrease in photosynthesis (Reichman, 2002). It would appear that the mechanism of action involves the displacement of Mg by Zn at the water splitting site in photosystem II (Tsonev and Lidon, 2012).
In *Spinacia oleracea*, excessive Zn supply was found to greatly reduce ATP synthesis and activity in chloroplasts; In fact, after 50 minutes treatment in 1mM Zn the uncoupled electron transport rate was reduced by 26% and thylakoids treated with 1 to 2mM Zn for 30 minutes exhibited inhibition of the water splitting site of photosystem II (Reichman, 2002). Inhibition caused by Zn could be restored by benzidine which is an electron donor for photosystem II. However, after 60 minutes of exposure irreversible membrane damage occurred which could not be restored by benzidine suggested that the primary toxic action of Zn is the inhibition of ATP synthesis and therefore energy metabolism in plants (Reichman et al., 2002).
Interestingly, an excess of Zn has been shown to stimulate the production of a range of enzymes in *P. vulgaris*. Van Assche et al. (1988) suggested that this might be a compensation by the cell for the inhibition of physiological activity caused by high Zn, such as the inhibition of chloroplast NADPH production.
High external concentrations of Zn have been found to inhibit RuBP carboxylase activity (Van Assche and Clijsters, 1986). Several reports, in general, suggested that Zn partially substituted Mg in the RuBisCo-complex (Rout and Das, 2003).
Phosphorus and/or iron deficiency can be secondary stresses induced by excess Zn (White et al. 1979). Zn interferes both in the uptake and translocation process of Fe (Olsen 1972). At least in part, this can be attributed to the fact that both metal ions share common transport systems and binding molecules, especially organic acids and nicotianamine. In fact, maintenance of both Zn and Fe homeostasis is regulated at the uptake and transport level as well as by subcellular compartmentation and chelation (Lin and Aarts, 2012).
An excess of the metal also leads to increase in reactive oxygen species (ROS) that can induce oxidative stress thus damaging major biomolecules including lipid, protein, and nucleic acid (Peng at al., 2015).

Some species of plants have adapted to environments rich in Zn or other metals (Broadley et al., 2007). At a whole-plant scale, natural Zn hypertolerance is thought to be conferred by Zn exclusion or by compartmentalization, for example altering root-to-shoot translocation, binding the metal to cell wall and extracellular exudates or accumulating it in older leaves (Broadley et al., 2007). In particular, key subcellular processes enabling Zn hypertolerance are likely to be increased vacuolar compartmentalization (Broadley et al., 2007).

1.3 WATER DEFICIT STRESS IN PLANTs

Drought stress occurs when the transpiration rate exceeds the absorption of water and results in the occurrence of numerous changes in the plant at the physiological level, biochemical and molecular. Following the decrease of water potential of the leaf, the reduction of stomatal conductance can be observed, followed by a lower CO_2 assimilation and the consequent reduction of the photosynthetic rate (Cornic, 2000). As a result, a decrease in the production of carbohydrates occurs (McDowell et al., 2008).

If the period of drought is prolonged over time, it is generally observed the reduction of the total biomass, leaf area, height and diameter of the plants (Monclus et al., 2006).

Water deficit inhibits plant growth by reducing water uptake into the expanding cells, and alters enzymatically the rheological properties of the cell wall. The initial growth inhibition by water deficit occurs prior to any inhibition of photosynthesis or respiration (Muller et al., 2011).

An important role in the inhibition of growth is played by the newly divided cells that encase the xylem in the growing zone (Cramer et al., 2011). These cells act as a resistance to water flow to the expanding cells in the epidermis making it necessary for the plant to develop a larger water potential gradient.

In conclusion, growth is limited by the plant's ability to osmotically adjust or conduct water. The epidermal cells can increase the water potential gradient by osmotic adjustment and this is realized by solutes from the phloem, provided by photosynthesis (Cramer et al., 2011).

Nevertheless, photosynthesis declines due to stomatal limitations for CO_2 uptake and in parallel, increase photoinhibition from difficulties in dissipating excess light energy (Pinheiro et al., 2011).
At the cellular level, the water deficit causes a change in the concentration of solutes, in the volume of the cell and in the shape of the membrane, the loss of turgor, the destruction of membrane integrity and the denaturation of many proteins (Bray, 1997).
Furthermore, it is known that the water deficit leads to an increase in the formation of reactive oxygen species (ROS), which may directly damage the membrane lipids or inactivate enzymes following the reduction of the disulfide bridges (Bartoli et al., 1999).
This condition in the leaves is caused by the reduced availability of CO_2 and H_2O required for photosynthesis, to which the light energy absorbed by the antenna pigments cannot be used for the production of carbohydrates and gives rise to an increase in the production of oxidants in the chloroplasts (Arora et al., 2002).

The ability of plant to survive in water scarcity includes mechanisms put in place both at the level of whole plant at the cellular level. Plant responses change in particular depending on plant species and water deficit degree (Bray, 1997). Classically strategies of resistance are divided into escape, avoidance and tolerance (Chaves et al., 2003), although the differences between these three types appear to be not well-defined. Escaping means the plant's ability to prevent exposure to stress, for example by shortening its life cycle. Instead, the avoidance is referred to the occurrence of modifications both at the whole plant level and at the cellular level that prevent the occurrence of stress, such as the closure of the stomata or the osmotic adjustment (Xu et al., 2010). Finally, plants that are able to tolerate stress, actuate some defense mechanisms that allow the plant to cope with the stress alleviating the harmful effects, as the case of the increase in the activity of antioxidant enzymes to counteract oxidative stress. The resistance of a plant is still a complex phenomenon, which is often based on the presence of many combined strategies between them (Farooq et al., 2009).

The closure of the stomata is one of the first events that take place in the presence of poor water availability, since in this way the water potential is maintained the highest possible (Jarvis and McNaughton 1986), and sometimes, the rolling of the leaves or the change of the angle between the stem and the stem occur, allowing the plant to reduce absorption of light (Ehleringer and Cooper 1992).

The described modifications are likely induced following the recognition of a signal sent from the roots to the leaves. The molecule known signal may be the abscisic acid since there was a correlation between the content of abscisic acid in xylem and stomatal conductance (Xu et al., 2010).

Signal molecules also cause changes at the transcriptional level to increase the availability of protein products involved in various cellular processes of defense by water stress, for example in the synthesis of the osmotic solutes. These molecules can also have and antioxidant activity and concur to preserving the structure and the functionality of proteins and cell membranes (Bray 1997).

In the presence of prolonged water deficit over time plants can slow or stop the growth, using carbohydrates accumulated in the reserve tissues. A control of water losses can be achieved by reducing the leaf area and producing smaller leaves (Jarvis and McNaughton 1986), while a greater water supply can be guaranteed by the extension of the root system (Jackson et al., 2000).

1.4 VOLATILE ORGANIC COMPOUND EMISSION IN PLANTs

Volatile organic compounds (VOCs) are produced and emitted from various organisms (Rowan, 2011) and their total amount emitted globally into the atmosphere is estimated to exceed 1 Pg per year (Guenther et al., 1995).

Volatile organic compounds produced by vegetation are low-molecular-weight compounds (below 300 Da) and include a diverse set of chemical molecules belonging to four main groups: aliphatic and aromatic compounds, terpenes and phenols (Dudareva et al., 2006). They are synthesized via a few major biochemical pathways, though various forms of enzymatic modifications such as hydroxylations, acetylations, and methylations, add to the diversity of emitted volatiles by increasing their volatility at the final step of their formation (Dudareva et al., 2004).

Plant volatiles can be synthesized constitutively in plants (as the compounds that derive from aminoacids, present in scent of flowers and fruits (Dudareva, 2006), or *de novo* both in undamaged and damaged tissues.

In many cases, the variety of plant-emitted VOCs depends on the plant species, the distinctive parts of the plants, or the circumstances under which the plants are growing (Dudareva et al., 2006). Relatively to the latter feature, VOC profile emitted from plants

is reported to change in response to environmental factors (Laothawornkitkul et al., 2008); In fact, their production appear easily affected by various abiotic stresses, such as temperature, high light and drought (Penuelas and Staudt., 2010).

The ecological roles of the plant-produced VOCs can include functions of defense and communication between plants and other organisms. Indeed, VOCs have various ecological and physiological roles in plants: antiherbivory and antimicrobial defense, pollinator attraction, allelopathic role (Owen and Penuelas, 2005), protection against high temperature (i.e. thermos-tolerance; Sharkey and Yeh, 2001), communication with other plants or organisms (i.e. plant-plant and plant-insect interactions; Penuelas and Llusia, 2003; Staudt and Lhoutellier, 2007). Moreover, many VOCs of the family of terpenes, such as isoprene and monoterpenes, having antioxidant properties, can protect cells from oxidative stress (Vickers et al., 2009).Volatile isoprenoids for example are able to protect plants from heat damage and allow them to maintain photosynthetic rates thus enhancing plant thermo-tolerance at elevated temperatures (Penuelas et al., 2005).

The influence of one of the most common types of abiotic stress, i.e. the presence of heavy metals in the environment, on plant VOC emission is still little known.

Generally, it seems that heavy metal stress can enhance secondary metabolites production, among which volatiles compounds (Mithöfer et al., 2004).

In particular, from previous studies, we know that in *Zea mays* Cu stress can prime for enhanced volatile production that can be triggered by caterpillar feeding (Winter et al., 2012). Moreover, we know that heavy metals can induce ethylene emission in plants (Spinelli et al., 2011). As it regards Zn, the few knowledge we have are related to its influence on *Martianthus leucocephalus* plant, in which it has been shown to increase VOC production (de Jesus et al., 2016). To the our best knowledge, nothing more is known about whether and how this metal can affect the biosynthesis and the emission of volatiles compound in other plant species.

1.5 PLANT ACCLIMATION

Abiotic stresses, such as temperature, flooding, drought, nutrient imbalance, and physical perturbations, require plants to continuously respond to the pressures of

environment. Several plant species are often able to alter their physiological response, thus managing unfavorable events (Crisp et al., 2016).

The duration of stress can be extremely variable. In fact, abiotic stress can persist for weeks or months or be part of a variable climate, promoting adaptation through resilience over subsequent generations (Crisp et al., 2016). Nevertheless, most abiotic stresses are transient.

As environmental perturbations may occur repeatedly, it is advantageous to plants to be able to remember past incidents and to use this stored knowledge to adapt to new challenges (Kinoshita and Seki, 2014).

One possible response of plants to exposure to stress is that they become more resistant to future exposure through the acquisition of memory, a response referred to as hardening, priming, conditioning, or acclimation.

Priming and acclimation have been defined as induction of a physiological state that allow a plant to deploy a more rapid and stronger defense responses compared with non-primed or not acclimated plants (Pandolfi et al., 2012).

Acclimation to external environmental changes can occur in plants thanks to internal adjustments within tissues and cells, enabling plant metabolism to proceed under these somewhat altered conditions (Pandolfi et al., 2016). In a contrast to adaptation that occurs in plant phylogeny, acclimation occurs during plant ontogeny and describes enhanced stress tolerance of a particular individual plant (Pandolfi et al., 2012).

Multiple examples of “memory” in higher plants have been shown across multiple species and have been discussed in detail (Crisp et al., 2016).

There is also evidence that priming can persist between generations, a process referred to as adaptive transgenerational plasticity (Crisp et al., 2016).

One of the best known of these “memory” systems is that termed ‘defense priming’, which controls the response to a pathogen or herbivore attack (Kinoshita and Seki, 2014). In defense priming, the plant displays a more rapid and robust response to the pathogen or herbivore in a second attack compared with the first one, thereby increasing its chances of survival. Priming can also be found for various abiotic stress responses such as drought stress (Bruce et al. 2007).

“Plant memory” is often characterized by heightened molecular responses upon exposure to a subsequent stress, which can be composed of an enhanced response, a more efficient response, or a more rapid response. Several molecular mechanisms

underpinning plant memory have been elucidated to date. One mechanism of memory formation may be sustained alterations in levels of key signaling metabolites or transcription factors, which provides an explanation for how plant metabolism is altered and maintained by exposure to various stresses (Crisp et al., 2016). Another possible avenue could be alterations to chromatin states, such as histone tail modifications, DNA methylation, or paused RNA polymerase II, which could play a further role in the coordinated changes in the patterns of gene expression that underpin memory responses (Crisp et al., 2016). Future multidisciplinary investigations will help to improve actual knowledge about involved mechanisms in “plant memory” development.

1.6 PLANT STUDIED: *Tetradenia riparia* (Hochst.) Codd

The plant object of the present study is the species *Tetradenia riparia* (Hochst.) Codd. It is a shrubby plant belonging to the Lamiaceae family and native of South-Africa. It is also known as *Iboza riparia* and *Moschosma riparium* (Gazim et al., 2014). In Brazil, *T. riparia* was introduced as an exotic ornamental plant and is cultivated in parks, gardens, homes, and botanical gardens, where it releases a very intense and pleasant aroma (Zelnik et al., 1978).

The Lamiaceae family has been studied to improve the production of essential oils and identify the compounds of these oils (Valmorbida et al., 2006). Gazim et al. (2010) report that the essential oil of *T. riparia* is a complex mixture of terpenoids: monoterpenes, sesquiterpenes, and diterpenes (hydrocarbons or oxygenated) and the most representative class of the oil composition is the oxygenated sesquiterpenes, especially 14-hydroxy-9-epi-caryophyllene.

The essential oil of *T. riparia* present biological activities reported in the literature as antispasmodic, larvicidal and insecticidal, anti-mycobacterial, antimalarial, repellent of *Anopheles gambiae*, antimicrobial, antinociceptive, and acaricidal against *Rhipicephalus (Boophilus) microplus* (Gazim et al., 2014). In fact this plant is used as a folk medicine in Africa for the treatment of inflammatory and infectious diseases.

1.7 OUTLINE OF THIS THESIS

This work has been carried out to elucidate some of the physiological mechanisms of responses in *Tetradenia riparia* plant subjected to abiotic stress.

In the second chapter the plant was cultivated in Zn excess condition to outline the effects that the metal has on some physiological parameters such as net photosynthetic rate, stomatal conductance, maximum quantum yield of photosystem II and electron transport rate.

Considering that in the natural environment plants interact with more than one environmental factor at the same time, the third chapter focused on how *T. riparia* reacts when exposed to water stress in combination with high concentrations of Zn.

A topic of increasing interest is the ability of a living organism to react more promptly to an external stimulus if previously exposed to that particular stimulus. So, in the fourth chapter, was investigated if the plant object of our studies, is able to maintain a "memory" of a past event, reacting differently to the recurrence of the same event. As experimented event by the plant, a prolonged period with Zn excess in the culture medium was chosen.

In all the three experimental parts, we paid particular attention on the relationship between abiotic stress and volatile organic compound emission in plant.

General discussion is presented in the fifth chapter.

CHAPTER 2: EXPERIMENTAL

"Tetradenia riparia under Zn excess: an analysis of the responses from growth impairment to VOC emission"

ABSTRACT

In this study we investigated the effect of Zinc (Zn) excess on *Tetradenia riparia*, considering plant growth, metal accumulation, photosynthetic parameters and volatile organic compound (VOC) emission.

Plants were hydroponically cultivated in presence of 100, 500 and 1000 µM $ZnSO_4$ for 4 weeks. Zinc application led to a significant negative effect on plant growth and decreased net photosynthetic rate and stomatal conductance. A Zn-induced decrease in the concentration of chlorophyll b and carotenoid was found, while the concentration of chlorophyll a and leaf total soluble sugars were not affected by metal treatment. Zinc accumulation in plant organs resulted proportional to the Zn concentration used in the growth medium. Furthermore, Zn concentration was lower in shoots than in roots, indicating a low metal translocation by the plant.

Thirteen VOCs including terpenes, monoterpenes, isoprene, acetates and aldehydes were identified and their intensity of emission resulted directly proportional to the Zn treatment. The only exception was the case of methanol, that presented a decreasing trend in presence of increasing Zn concentration.

This work represents one of the first evidences of Zn-induced volatiles and opens new possibilities for the studies of the role of VOCs in plant responses to heavy metal stress.

Keywords: Zn stress; volatile organic compounds; *Tetradenia riparia*

2.1 INTRODUCTION

Zinc is present naturally in the environment, but the increase in human activities, related to mining, industry and agriculture, has led to a concerning increase in the concentration of the metal in the environmental matrices (Friedland, 1990).

Zinc is also a crucial element for the metabolism of plant cells, being involved in a wide variety of physiological processes at the micromolar range (Marschner, 1995). However, it can be toxic for plants at supra-optimal concentrations, and toxicity generally occurs when leaf concentrations reach 400–500 μg g–1 of dry mass (Marschner, 1995; Broadley et al., 2007). Common Zn toxicity symptoms can include reduced plant water content and stunted plant growth (Sagardoy et al., 2009), changes in root growth and morphology, severe nutrient imbalances, leaf chlorosis (Marschner, 1995; Vaillant et al., 2005; Broadley et al., 2007; Sagardoy et al., 2009), and decrease of stomatal conductance and photosynthetic efficiency (Sagardoy et al., 2010). Photosynthesis impairment, appears to be mainly linked to the inhibition of photosystems caused by high concentrations of Zn. In fact, this metal seems to cause the displacement of Mg at the water splitting site in photosystem II (van Assche and Clijsters, 1986; Kupper et al., 1996). Furthermore, excessive Zn supply was found to greatly reduce ATP synthesis and activity in chloroplasts (Teige et al., 1990). At a more general level, an excess of this metal leads to increase in reactive oxygen species (ROS) that can induce oxidative stress (Schützendübel and Polle, 2002), thus damaging major biomolecules including lipids, proteins, and nucleic acids.

However, information about the toxic effect of Zn excess on plants and, especially, on the Zn-induced plant responses is still incomplete. In particular, one of the almost unexplored aspects of the relationship between Zn and plants is the effect of such metal, and of heavy metals in general, on the emission of volatile organic compounds (VOCs).

Volatile organic compounds are produced and emitted from various organisms (Rowan et al., 2011) and their total amount emitted globally to the atmosphere is estimated to exceed 1015 g for year (Guenther et al., 1995).

Volatile organic compounds produced by vegetation include a diverse set of chemical molecules belonging to four main groups: aliphatic and aromatic compounds, terpenes and phenols (Dudareva et al., 2013). The variety of plant-emitted VOCs depends on the plant species, the distinctive parts of the plants, or the circumstances under which

the plants are growing (Dudareva et al., 2013). Several researches, reported that VOC profile in plants is affected by environmental factors (Laothawornkitkul et al., 2008), being their production largely affected by various abiotic stresses, such as UV, ozone, drought, eutrophication and warming (Penuelas et al., 2010).

Volatile organic compounds have various ecological and physiological roles in plants, including plant defense against insects, pollinator attraction, plant-plant communication, plant-pathogen interactions, thermo-tolerance and environmental stress adaptation (Spinelli et al., 2011). Actually, many VOCs of the family of terpenes, such as isoprene and monoterpenes, are antioxidants and can protect cells from oxidative stress (Vickers et al., 2009). In any case, the phenomenon of VOC emission is quite complex and it is affected by interactions of plants with biotic and abiotic factors in constantly changing environments, at local and global level (Spinelli et al., 2011). Additionally, until now, the influence of one of the most common types of abiotic stress, i.e. the excessive presence of heavy metals in the environment, on plant VOC emission is not yet well known.

The species *Tetradenia riparia* (Hochst.) Codd belongs to the Lamiaceae family and is native of South-Africa, where it is widely used as a medicinal plant due to the antimicrobial, acaricidal and analgesic activities of the essential oil extracted from its leaves (Gazim et al., 2014). The essential oil of *T. riparia* has been shown to be a complex mixture of terpenoids, including monoterpenes, sesquiterpenes, and diterpenes (Gazim et al., 2014). Therefore, *T. riparia* can represent an easy-to-study model system to start investigating the relationship between heavy metal stress and volatile emission, since the natural richness of this plant in this kind of secondary metabolites has already been reported.

Furthermore, investigation of the effect of Zn on *T. riparia* will provide useful information also about the possibility of cultivating such a plant on marginal lands contaminated by this heavy metal. In fact, in South Africa, Zn pollution is relatively widespread (Mutune et al., 2014) and such lands, that are not suitable for the production of edible crops, could possibly be cultivated with plants used for medicinal purposes, thus obtaining an economic yield. However, an evaluation on whether in the final drug product the metal concentration exceeds or not the limits established by law will be strictly necessary.

For the proposed aim, after having characterized the effect of Zn excess on growth, photosynthesis and metal accumulation capacity, VOC emission was analyzed by PTR-MS (Proton Transfer Reaction - Mass Spectrometry), one of the most sensitive tools for real-time monitoring of volatile compounds.
One of the first evidences of Zn-induced volatiles in plants was reported, thus opening the research on the role of VOCs in plant responses to heavy metal stress.

2.2 MATERIALS AND METHODS

Plant material

Plant material was cultivated as in Bazihizina et al. (2016). *Tetradenia riparia* plants were grown in a naturally lit glasshouse in plastic pots with only demineralized water (25/25 °C day/night, 12 h day/12 h night, with an average photosynthetically active radiation at shoot height of 300 µmol m–2 s–1). After 2 weeks, seedlings were transferred to an aerated nutrient solution in pots of five liters capacity. Six plants were grown in each pot and all plants were supplied with half-strength Hoagland's nutrient solution (pH adjusted to 5.8 using KOH). The solutions were changed weekly.
Two weeks after transferring the plants to hydroponics, $ZnSO_4$ was added to the nutrient solutions to obtain the required final Zn concentrations (0µM, corresponding to the control treatment, 100µM, 500µM and 1000µM). Plants were grown for 4 weeks from the start of treatment.
Shoot length was measured every 5 days for the duration of the experiment to determine plant extension during the treatment period. At different time points (0 and 28 days), young fully expanded leaves (one for each plant in all treatments) were collected for subsequent analyses of total soluble sugars and leaf pigments. These leaf tissues were frozen using liquid N_2, stored at -80 °C, freeze-dried, and then stored at -20°C until the analyses were performed.

Measurement of leaf gas-exchange parameters

Leaf gas-exchange parameters were measured using the open gas-exchange system Li-6400 XT (Li-Cor, Lincoln, NE, USA) with an integrated fluorescence chamber head

(Li-6400–40; Li-Cor) as in Bazihizina et al. (2016). Leaf gas-exchange measurements were taken on all plants in each treatment, before the start of the treatment (time 0), after 24hours and 7, 14, 28 days. Measurements of net photosynthetic rate, stomatal conductance and electron transport rate were determined on the youngest fully expanded leaves at ambient relative humidity (40–50%), reference CO_2 of 400 µmol mol–1, flow rate of 400 µmol s–1, chamber temperature of 25 °C and photosynthetically active radiation of 300 µmol m–2 s–1. Using the integrated fluorescence chamber head (Li-6400–40) of the open gas-exchange system Li-6400 XT, chlorophyll fluorescence was measured on the same leaves used for gas-exchange measurements. The level of minimal fluorescence in the dark-adapted state (F0) was measured using a modulated pulse, and the level of maximal fluorescence in this state (Fm) was measured after applying a saturating actinic light pulse of 7000 µmol m–2 s–1. The values of the variable fluorescence Fv were calculated as Fm-F0 and the maximum quantum efficiency of photosystem II (PSII) was calculated as Fv/Fm (Bazihizina at al., 2016).

Plant harvest

Plants were sampled at 4 weeks after applying the treatments for the determination of shoot and root fresh and dry mass. The roots of plants were immersed in $Pb(NO_3)_2$ 10 mM for 10 min to desorb metals adhering to the root cell wall as in Barzanti et al. (2011), and then intact plants were carefully washed with de-ionized water. Then, each plant was separated into roots, leaves and stems and their fresh mass was recorded. Tissue samples were then oven-dried at 60°C for 1 week to determine their dry mass.

Elemental analysis

Roots, stems, and leaves were oven-dried at 60 °C for at least 24h. The dried plant material was then ground, using an electric grinder. For the acid digestion, aliquots (about 100 mg) of each sample were placed in a 25 ml beaker and mineralized with a mixture of HNO_3: $HClO_4$ (5:2 v/v) as in Pignattelli et al., (2013) on a heating plate (50-100° C). Zn concentration was determined in the digests by an atomic absorption spectrophotometer (AAnalyst 200, Perkin Elmer).

Leaf pigment analyses

Total chlorophyll and carotenoid concentrations were determined at two different times, at 14 and 28 days, in all treatments by reading the absorbance at 665, 652, and 470 nm of extracts obtained from randomly selected youngest fully expanded leaves. Samples were ground in liquid nitrogen, then 0,02 g were weighed and extracted with 1,25 ml methanol 100% for 30 min at 4°C in the dark. Chlorophyll and carotenoid concentrations were determined according to Wellburn and Lichtenthaler (1984) using a Tecan Infinite 200 Spectrophotometer (Männedorf, Switzerland).

Measurement of total soluble sugars

Freeze-dried young fully expanded leaves were extracted in boiling 80% ethanol, twice and the supernatant was collected and used to measure total sugars using anthrone (Yemm and Willis, 1954). Total sugars (as hexose equivalents) were determined by measuring the absorbance of the samples at 620 nm in a UV-visible spectrophotometer (UV-1601, UV-visible spectrophotometer, Shimadzu, Kyoto, Japan), using a standard curve for glucose. This method was judged reliable as it was determined the recovery of known amounts of glucose added to additional tissue samples immediately prior to extraction, as well as to ethanol alone, as in Bazihizina et al., (2012). The recovery of glucose from these samples was 100%, so data presented here have not been adjusted. Total soluble sugars were determined at two different time (14 and 28 days after the treatment).

PTR-TOF-MS analysis

The proton transfer reaction-time of flight- mass spectrometry (ptr-tof-ms) was used to identify volatile organic compounds emitted from the leaves of *Tetradenia riparia* for each treatment applied. Analyses were conducted on the twentieth day of treatment. Ptr-tof-ms instruments can generate entire mass spectra (snapshots) of complex trace gas mixtures in short response times with high sensitivity and high mass resolution (Lanza et al., 2015).

Ions are accelerated by an electric field of known strength. A heavy ion with the same charge of a light ion, will reach the detector later. The time that it takes for the ion to reach a detector at a known distance is measured. The ion velocity depends on the mass-to-charge ratio that is determined (Stephens, 1946).

This mass spectrometry technique uses gas phase hydronium ions as ion source reagents (Ellis and Mayhew, 2013). Ion source is directly connected to a drift tube and an analyzing system. The reaction takes place between $H3O^+$ ions and all the biogenic VOCs having a proton affinity higher than that of water (165.2 kcal mol−1) (Taiti et al., 2015).

Leaves were put in a glass jar (500 ml of capacity) provided with two tubes, the inlet and the outlet tube. The inlet tube was connected to a zero-air generator (Peak Scientific) and the outlet tube to the PTR-ToF-MS. Analyses were conducted in air conditioned room at 25 °C, making sure that the temperature and humidity did not change.

The sampling time for each channel of TOF acquisition was 0.1 ns, for a mass spectrum range between 20 and 170m/z. The VOCs in the headspace were measured by direct injection into the PTR-TOF drift tube inlet for 120 s.

Preliminary measurements on an empty jar were run before every sample measurement and used for background subtraction.

Raw data (count rate of the analytes recorded in number of counts per second, cps) were acquired with TofDaq software (Tofwerk AG, Switzerland), using a dead time of 20 ns for the Poisson correction. All data from each replicate and background signal were normalized, according to Jardine et al. (2010), by the primary ion signal (cps to ncps, normalized count per second).

For VOC identification, we used an average signal intensity recorded for 60 s, which allowed the acquisition of 60 average spectra.

The recognition of VOCs was based on the fragmentation patterns of pure standards available in the literature (Soukoulis et al., 2013) and compared with published VOCs emitted from different species of plants and other organisms.

Statistical analyses

Statistical analyses were conducted using GraphPad for Mac 6th Edition, and analysis of variance (ANOVA) was used to identify significant differences between treatments. Tukey post-hoc test was used for a posteriori comparison of individual means (with at least $P \leq 0.05$ as significant level).

2.3 RESULTS

Effects of Zn stress on plant growth

The elongation of shoot in *Tetradenia riparia* under different Zn treatments in hydroponics is presented in Table 1. Compared to control plants, the presence of increasing Zn concentration in the culture media caused a decrease in shoot growth. The decrease was more evident in plants grown with higher Zn concentrations.

Zn affected also dry biomass of leaves and roots, detected at the end of treatment, as reported in Table 2. In particular, leaves and roots dry biomass decreased significantly with increasing Zn concentrations; Also stem dry biomass appeared reduced, even if in not significant manner.

Zn concentration	**Day 4** (cm)	**Day 8** (cm)	**Day 12** (cm)	**Day 16** (cm)	**Day 20** (cm)	**Day 24** (cm)	**Day 28** (cm)	**Day 32** (cm)
0 µM $ZnSO_4$	1,6 ± 0,2 **a**	3,03 ± 0,4 **a**	4,6 ± 0,3 **a**	7,2 ± 0,5 **a**	8,6 ± 0,6 **a**	10,6 ± 1,0 **a**	11,0 ± 1,0 **a**	13,2 ± 2,6 **a**
100 µM $ZnSO_4$	0,8 ± 0,1 **b**	2,04 ± 0,4 **ab**	2,4 ± 0,4 **ab**	4,4 ± 0,8 **b**	5,0 ± 0,7 **b**	6,2 ± 0,9 **b**	6,5 ± 0,9 **b**	7,9 ± 1,4 **ab**
500 µM $ZnSO_4$	0,7 ± 0,2 **b**	1,4 ± 0,4 **bc**	2,9 ± 0,5 **ab**	3,5 ± 0,6 **bc**	4,0 ± 0,5 **b**	4,1 ± 0,5 **bc**	4,1 ± 0,5 **bc**	4,1 ± 0,5 **bc**
1000 µM $ZnSO_4$	0,12 ± 0,07 **c**	0,4 ± 0,2 **c**	1,2 ±0,6 **b**	1,5 ± 0,5 **c**	1,8 ± 0,5 **c**	1,8 ± 0,5 **c**	1,8 ± 0,5 **c**	1,8 ± 0,5 **c**

Tab. 1: Shoot elongation in *Tetradenia riparia* in response to increasing $ZnSO_4$ concentration in the growth medium. Values are mean ± standard error (SE) and n=6. Letters indicate significant differences among treatments (at least $P < 0,05$).

	TREATMENTS			
	CONTROL	**100 µM $ZnSO_4$**	**500 µM $ZnSO_4$**	**1000 µM $ZnSO_4$**
LEAVES	9,5 ± 1,1 g **a**	5,9 ± 1,7 g **b**	1,2 ± 0,2 g **c**	0,7 ± 0,1 g **c**
STEMS	6,0 ± 2,1 g **a**	5,6 ± 1,9 g **a**	5,1 ± 0,8 g **a**	3,2 ± 1,0 g **a**
ROOTS	4,3 ± 0,3 g **a**	3,2 ± 0,3 g **a**	1,2 ± 0,2 g **b**	0,9 ± 0,2 g **b**

Tab. 2: Dry mass of leaves, stems and roots detected at the end of treatment in *Tetradenia riparia*. Values are mean ± SE (n=6). Different letters into the row indicate significant differences among treatments (at least P < 0,05).

Zinc accumulation in *Tetradenia riparia*

Figures 1a, 1b and 1c show respectively the concentration of Zn in roots, stems, and leaves of *Tetradenia riparia* grown for four weeks in hydroponics culture with Zn excess. Accumulation of Zn in all parts of the plant increased with increasing Zn concentration in the culture media. Roots showed the highest metal concentrations, whereas stems and leaves displayed similar and lower values.

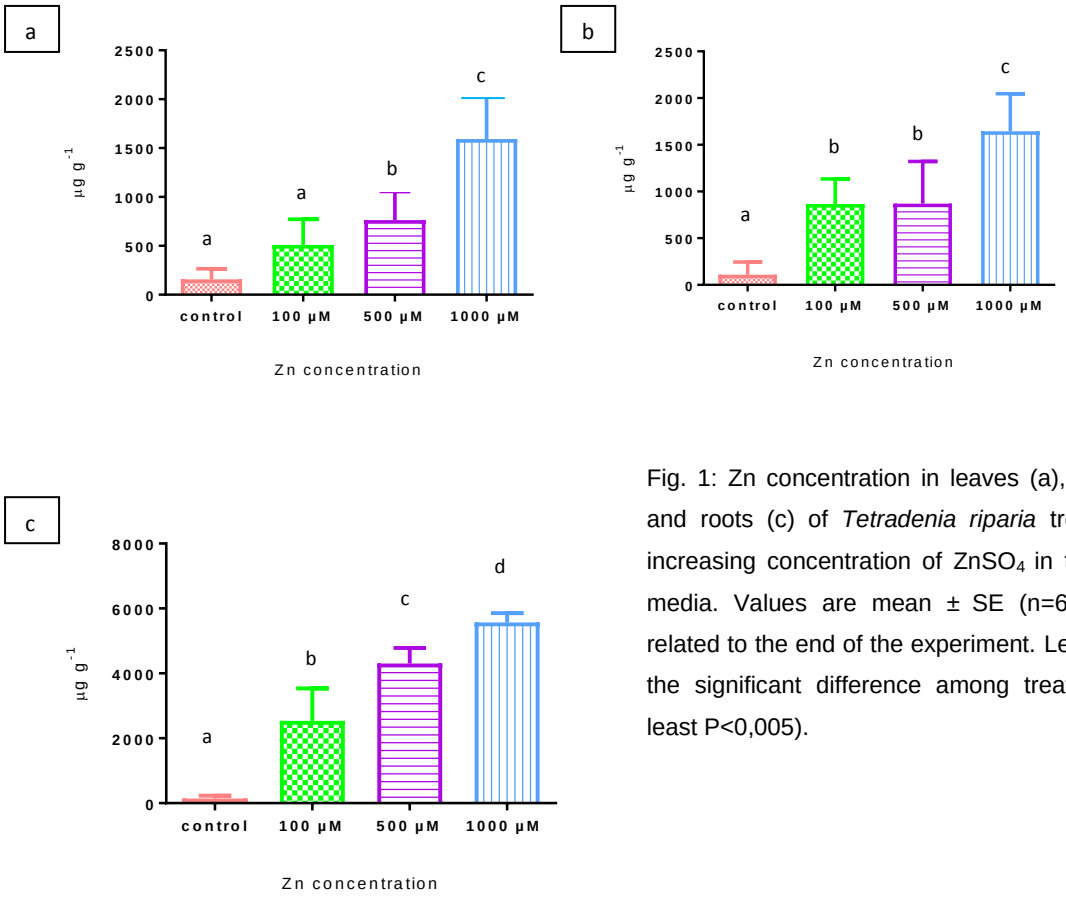


Fig. 1: Zn concentration in leaves (a), stems (b) and roots (c) of *Tetradenia riparia* treated with increasing concentration of $ZnSO_4$ in the culture media. Values are mean ± SE (n=6) and are related to the end of the experiment. Letters show the significant difference among treatments (at least P<0,005).

Translocation factor (TF) was calculated as the ratio between the concentration of the metal in leaves and roots. For each treatment, TF resulted <1, varying in the following increasing order: 500 µM $ZnSO_4$ (0,18), 100 µM $ZnSO_4$ (0,20), 1000 µM $ZnSO_4$ (0,29).

Leaf pigment concentration

Chlorophyll a (Figure 2a) and carotenoids (Figure 2b) concentrations declined with increasing Zn concentration in the root medium, with significant decline at 500 µM and 1000 µM $ZnSO_4$.

Conversely, the concentration of chlorophyll b did not change significantly among treatment. (Figure 2c).

Moreover, there was not any difference between first and second time sample, (after two weeks of treatment and at the end of treatment), regarding the amount of each pigment considered (Figure 3a, 3b, 3c).

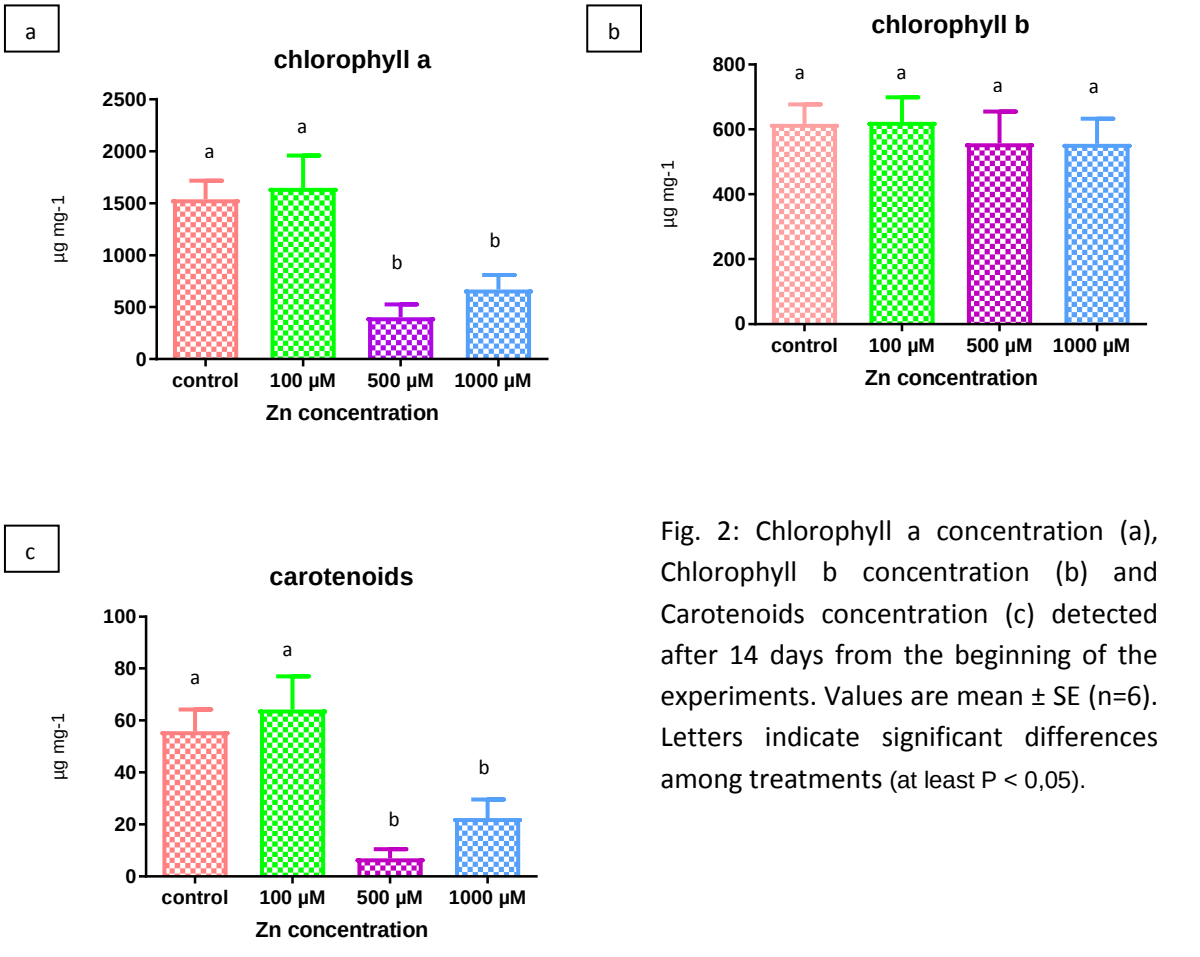
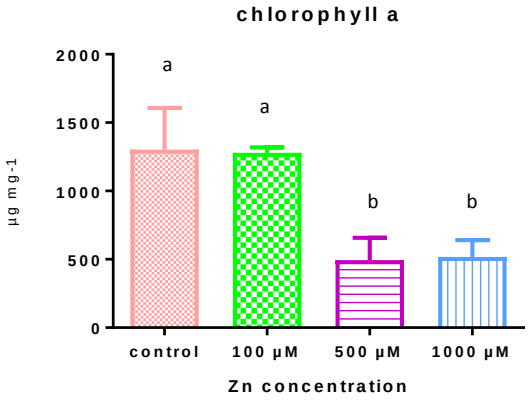
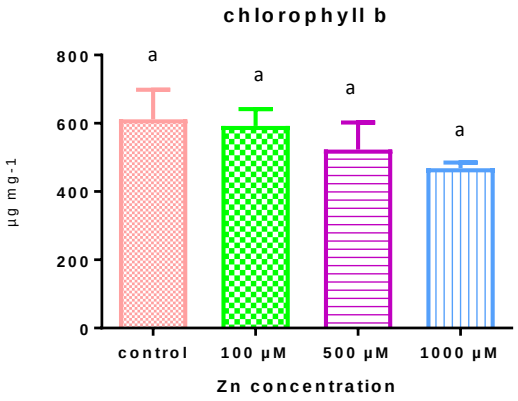


Fig. 2: Chlorophyll a concentration (a), Chlorophyll b concentration (b) and Carotenoids concentration (c) detected after 14 days from the beginning of the experiments. Values are mean ± SE (n=6). Letters indicate significant differences among treatments (at least $P < 0,05$).

a



b



c

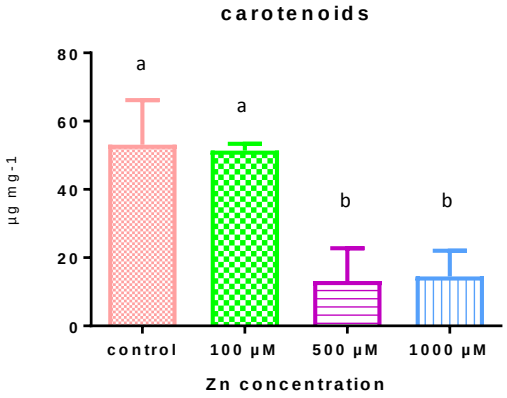


Fig. 3: Chlorophyll a concentration (a), Chlorophyll b concentration (b) and Carotenoids concentration (c) detected at the end of the experiment. Values are mean ± SE with n=6. Significant differences among treatments are indicated by letters (at least $P < 0,05$).

Photosynthetic efficiency

Increasing concentration of $ZnSO_4$ caused a decrease of net photosynthetic rate (Table 3a), stomatal conductance (Table 3b), maximum quantum yield of PSII (Table 3c) and electron transport rate (Table 3d). The efficiency of gas exchange was not immediately affected by the application of Zn to the culture medium. In fact, after 24 hours no significant differences were found between treated plants and control plants. In the period between 24 hours and 7 days after application of the treatment, the effect of Zn occurred most intensely in plants treated with 500 and 1000 µM, while no significant differences were observed in plants treated with 100 µM in comparison with control plants. However, after 14 days, even in plants treated with the lowest concentration of Zn, a significant decrease in all parameters measured was observed.

Treatments	**0h (µmol m-2 s -1)**	**24h (µmol m-2 s -1)**	**7 days (µmol m-2 s -1)**	**14 days (µmol m-2 s -1)**	**28 days (µmol m-2 s -1)**
Control	9,4 ± 1,4 a	9,6 ± 1,0 a	10,9 ± 1,3 a	9,8 ± 1,2 a	9,5 ± 1,3 a
100 µM $ZnSO_4$	8,2 ± 0,5 a	8,4 ± 0,5 a	11,2 ± 1,0 a	5,3 ± 0,5 b	4,2 ± 0,6 b
500 µM $ZnSO_4$	8,7 ± 0,7 a	8,9 ± 0,4 a	5,3 ± 0,6 b	1,6 ± 0,3 c	0,9 ± 0,1 c
1000 µM $ZnSO_4$	8,6 ± 0,6 a	8,5 ± 0,7 a	6,0 ± 0,5 b	0,4 ± 0,1 c	0,6 ± 0,2 c

Tab. 3a: Net photosynthetic rate detected in *Tetradenia riparia* before the treatment (0 h) and after 24 h, 7 days, 14 days and 28 days. Values are mean ± SE (n=6). Different letters into the row indicate significant differences between control and treatments (at least $P < 0,05$).

Treatments	**0h (µmol m-2 s -1)**	**24h (µmol m-2 s -1)**	**7 days (µmol m-2 s -1)**	**14 days (µmol m-2 s -1)**	**28 days (µmol m-2 s -1)**
Control	0,23 ± 0,05 a	0,20 ± 0,03 a	0,23 ± 0,03 a	0,16 ± 0,02 a	0,17 ± 0,03 a
100 µM $ZnSO_4$	0,19 ± 0,04 a	0,21 ± 0,05 a	0,22 ± 0,04 a	0,05 ± 0,01 b	0,06 ± 0,01 b
500 µM ZnSO4	0,18 ± 0,03 a	0,20 ± 0,05 a	0,09 ± 0,04 b	0,023 ± 0,003 b	0,024 ± 0,004 b
1000 µM ZnSO4	0,22 ± 0,05 a	0,18 ± 0,03 a	0,06 ± 0,01 b	0,011 ± 0,001 b	0,012 ± 0,001 b

Tab. 3b: Stomatal conductance in *Tetradenia riparia* at 0 h (before the treatment), and after 24 h, 7days, 14 days and 28 days. Values are mean ± SE (n=6). Different letters indicate significant differences among treatments (at least $P < 0,05$).

Treatments	0h (µmol m-2 s -1)	24h (µmol m-2 s -1)	7 days (µmol m-2 s -1)	14 days (µmol m-2 s -1)	28 days (µmol m-2 s -1)
Control	0,14 ± 0,01 a	0,13 ± 0,01 a	0,17 ± 0,01 a	0,16 ± 0,01 a	0,13 ± 0,01 a
100 µM $ZnSO_4$	0,15 ± 0,02 a	0,12 ± 0,02 a	0,17 ± 0,01 a	0,12 ± 0,01 b	0,08 ± 0,01 b
500 µM $ZnSO_4$	0,13 ± 0,01 a	0,128 ± 0,004 a	0,06 ± 0,01 b	0,05 ± 0,1 c	0,028 ± 0,003 c
1000 µM $ZnSO_4$	0,14 ± 0,01 a	0,134 ± 0,009 a	0,08 ± 0,01 b	0,034 ± 0,003 c	0,04 ± 0,01 c

Tab. 3c: Maximum quantum yield of PSII in *Tetradenia riparia* measured at 0 h (before the treatment) and after 24 h, 7days, 14 days and 28 days. Values are mean ± SE (n=6). Different letters into the row show significant differences among treatments (at least P < 0,05).

Treatments	0h (µmol m-2 s -1)	24h (µmol m-2 s -1)	7 days (µmol m-2 s -1)	14 days (µmol m-2 s -1)	28 days (µmol m-2 s -1)
Control	59,0 ± 5,3 a	58,3 ± 4,9 a	74,4 ± 6,6 a	70,2 ± 3,3 a	52,5 ± 0,3 a
100 µM $ZnSO_4$	56,5 ± 5,4 a	53,0 ± 8,0 a	74,4 ± 4,7 a	53,0 ± 3,0 b	35,8 ± 3,0 b
500 µM $ZnSO_4$	62,1 ± 4,8 a	56,1 ± 1,3 a	26 ± 3,1 b	23,7 ± 2,8 c	12,4 ± 1,3 c
1000 µM $ZnSO_4$	59,9 ± 5,4 a	58,9 ± 4,2 a	36,8 ± 2,8 b	15,0 ± 1,5 c	17,1 ± 4,4 c

Tab. 3d: Electron transport rate in *Tetradenia riparia* at 0h (before the start of the treatment), 24 h, 7days, 14 days and 28 days. Values are mean ± SE (n=6). Letters indicate significant differences among treatments (at least P < 0,05).

Total soluble sugar concentration in leaves

Total soluble sugar concentration in young fully expanded leaves of *T. riparia* did not change between plants treated with increasing Zn concentrations and control plants. No significant differences were found at the first and second sampling time, at day 14 (Figure 4a) and at day 28 (Figure 4b) after the application of Zn-excess.

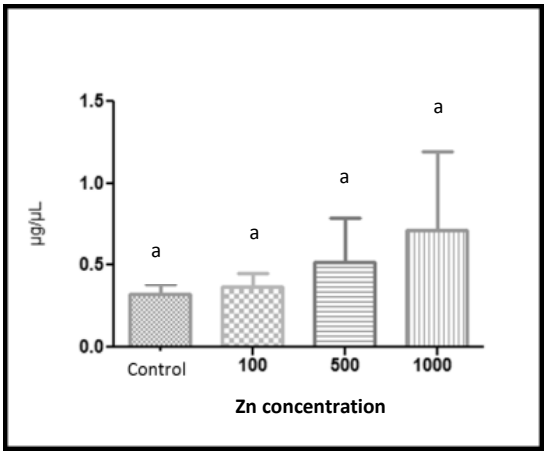


Fig.4a: Leaf total soluble sugars concentration in *T. riparia*, exposed to ZnSO4 increasing concentration, after 14 days of treatment. Values are mean ± SE (n=6). Letters show significant differences between control and treatment (at least P<0,05).

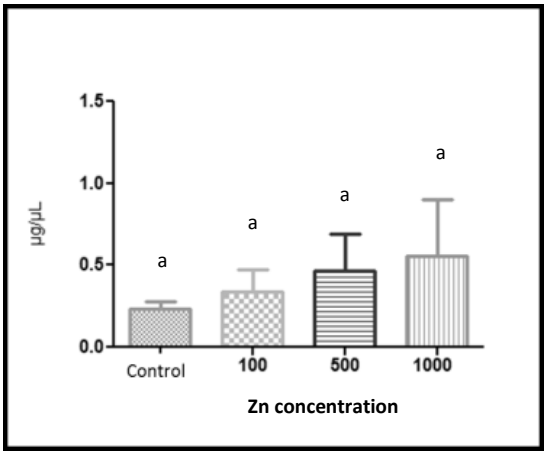


Fig.4b: Total soluble sugars concentration in leaves after 28 days of treatment in *T. riparia* grown with $ZnSO_4$ increasing concentration. Values are mean ± SE (n=6). Letters indicate significant difference between control and treatment (at least P<0,05).

Organic volatiles compounds emission

Analysis of volatiles from *T. riparia* has led to the identification of 13 compounds, detected by PTR-Tof-MS, using the data set comprising peaks from m/z= 20 to m/z= 170. Table 4 shows m/z ratio (both theoretical and measured), molecular formula, tentative of identification and bibliographic citations referred to volatile compounds identified in other studies by PTR-MS technologies showing the same molecular mass (Taiti et al., 2014; Taiti et al., 2015a,b; Infantino et al., 2015; Masi et al., 2016; Mancuso et al., 2015; Vita et al., 2015).

High concentration of Zn, induced a significant increase in the emission of the identified VOC (Table 5), except for m/z 33,03, tentatively identified as methanol, emitted with a greater intensity in control plants. The volatile with m/z 59,05, putatively propanal, was emitted in greater quantity in all Zn-treated plants.

Compound[a]	Measured m/z[b]	Protonated chemical formula[c]	Tentative of Identifications[d]	Theoretical m/z[e]	TOF References[f]
1	33,03	CH_5O+	Methanol	33,0335	Taiti et al., 2015
2	41,04	C_3H_5+	Alkyl fragment	41.0386	Taiti et al., 2015
3	43,05	C_3H_7+	Alkyl fragment (propene)	43.0542	Sánchez Del Pulgar et al., 2013
4	45,03	C_2H_5O+	Acetaldehyde	45,0334	Brilli et al., 2011
5	57,07	C_4H_9+	1-butene	57.0699	Galle et al., 2011
6	59,05	C_3H_7O+	Propanal/2-Propanone	59.0491	Infantino et al., 2014
7	61,03	$C_2H_5O_2$ +	Acetic acid	61.0284	Taiti et al., 2015
8	69,07	C_5H_9+	2-Methyl-1,3- butadiene (isoprene)	69,0698	Mancuso et al., 2014
9	81,07	C_6H_9+	Cis and trans-hexenal	81,07	Taiti et al., 2015
10	91,05	$C_4H_{11}O_2$+	2-3-Butanediol(or Isopropyl methyl sulfide)	91.0753	Vita et al., 2015
11	93,07	C_7H_9	Toluene	93.0699	Infantino et al., 2014
12	137,1	$C_{10}H_{17}$+	Monoterpenes	137.1325	Taiti et al., 2015
13	153,1	$C_{10}H_{17}O$+	Terpenoid-like compound	153.1273	Aprea et al., 2015

Tab. 4: Compounds identified through PTR-Analysis. [a]Number of identified compound. [b]Mass to charge ratio measured by the Mass Spectrometer. [c]Compound's chemical formula (H^+ added by protonation). [d]Putative identifications according to spectral properties. [e]Theoretical mass to charge ratio found in literature or PTR-TOF-MS manual. [f]PTR-TOF-MS articles where molecule was reported.

	Zn concentration			
VOLATILE COMPOUND	**0 µM $ZnSO_4$ (ncps)**	**100 µM $ZnSO_4$ (ncps)**	**500 µM $ZnSO_4$ (ncps)**	**1000 µM $ZnSO_4$ (ncps)**
33.03 **(Methanol)**	772,7 ± 41,5 **a**	581,8 ± 57,9 **b**	343,2 ± 19,5 **c**	179,5 ± 36,8 **c**
41.04 **(Alkyl fragment)**	145,5 ± 11,6 **a**	293,1 ± 32,7 **a**	499,6 ± 49,3 **ab**	781,3 ± 156,7 **b**
43.05 **(Propene)**	65,2 ± 18,1 **a**	134,5 ± 21,01 **ab**	221,5 ± 41,2 **b**	363,4 ± 7,3 **c**
45.03 **(Acetaldehyde)**	138,2 ± 20,5 **a**	328,0 ± 26,0 **ab**	559,2 ± 105,2 **bd**	664,8 ± 95,8 **cd**
57.07 **(1-butene)**	14,4 ± 2,15 **a**	45,7 ± 1,4 **ac**	153,9 ± 26,9 **b**	86,6 ± 4,9 **c**
59.05 **(Propanal/2-Propanone)**	501,8 ± 38,3 **a**	1149,0 ± 83,1 **ab**	2037,0 ± 373,8 **bc**	2921,0 ± 103,4 **c**
61.03 **(Acetic acid)**	60,8 ± 6,8 **a**	103,8 ± 9,4 **a**	328,0 ± 31,4 **b**	324,0 ± 32,9 **b**
69.07 **(Isoprene)**	18,9 ± 4,8 **a**	39,3 ± 7,9 **a**	57,2 ± 5,5 **a**	167,1 ± 30,6 **b**
81.07 **(Cis and trans-hexenal)**	316,7 ± 25,1 **a**	600,3 ± 60,5 **b**	818,4 ± 28,6 **c**	1064,0 ± 18,9 **d**
91.05 **(2-3-Butanediol)**	20,7 ± 3,5 **a**	49,5 ± 5,1 **a**	59,8 ± 6,2 **a**	140,5 ± 21,6 **b**
93.07 **(Toluene)**	33,7 ± 4,8 **a**	47,7 ± 9,8 **ab**	81,3 ± 10,8 **ab**	220,1 ± 74,8 **b**
137.1 **(Monoterpenes)**	34,6 ± 2,7 **a**	57,1 ± 1,14 **b**	96,7 ± 1,9 **c**	144,6 ± 8,1 **d**
153.1 **(Terpenoid-like compound)**	32,5 ± 2,2 **a**	47,5 ± 1,5 **a**	89,3 ± 8,9 **b**	143,7 ± 2,9 **c**

Tab. 5: Volatile organic compound emission in *Tetradenia riparia* under Zn treatment. Values are mean ± SE (n=3). Different letters into the row indicate significant differences among treatments (at least $P < 0,05$).

2.4 DISCUSSION

Zn significantly influenced the growth and development of *Tetradenia riparia* plants, already at the lowest concentration used and in a dose-dependent manner. Plants treated with 100 µM $ZnSO_4$ continued to grow for the entire duration of the experiment, even though at a lower extent than untreated plants, and showed a final reduction of shoots.

Compared to controls, in plants treated with 500 and 1000 µM $ZnSO_4$ shoot elongation was strongly inhibited. The presence of such Zn concentrations in the growth medium resulting in a significant reduction of the final dry biomass with a decrease of about 60% and 75% of the shoot and about 72% and 79% of the root weight, respectively.

The negative effect of Zn excess on growth has been already reported in many plant species, such as in tobacco, where concentrations of 250 and 500 µM $ZnSO_4$ led to a growth reduction of 50% compared to untreated plants (Bazihizina et al., 2014), or in other plants, such as *Sedum alfredii*, *Canavalia ensiformis* and *Brassica napus* L. (Yang et al. 2005; Andrade et al. 2009; Mourato, 2015). Among the different causes of Zn toxicity, its capacity of inhibiting the enzymatic activity and of interfering with the absorption of other nutrients, with the consequent inhibition of cell division and elongation, are frequently reported (Tsonev and Lidon, 2012).

Regarding metal accumulation, Zn concentration in plant organs increased with increasing the concentration of the element in the culturing solutions. In particular, the concentration of Zn in the roots was found to be greater than in the shoots, with concentrations up to around 5600 µg/g d.w. in plant grown with 1000 µM $ZnSO_4$, while in the leaves the highest concentration detected was around 1600 µg/g d.w. Therefore, the translocation factor resulted always lower than unity, highlighting a strong limitation in moving and accumulating Zn in the shoot in *Tetradenia riparia*. Actually, retention or immobilization of Zn in root tissues may be regarded as one important protection mechanism against excessive diffusion of heavy metals in plants (Aibibu et al. 2010), thus concurring to avoid the irreversible damage of the shoot tissues and to protect the photosynthetic parts.

Although Zn translocation to the shoot was low, even at the lowest Zn treatment, leaf Zn concentration (around 500 µg/g) exceeded by far the maximum permissible limit for this metal in medicinal plants (i.e. 50 µg/g according to the World Health Organization

as reported by Shah et al., 2013). Hence, our results discourage the cultivation of *Tetradenia riparia* for medicinal use on substrates with even moderate contamination by metals. Given that in soils of Southern Africa, where this plant typically grows, the contamination levels can be relevant, up to even more than 500 mg Zn/Kg (Yabe et al., 2010), the occurrence of shoot metal concentration above the permissible limit can be extremely realistic. In any case, the bioavailability of the metal in the soil may vary in relation to many factors (Greger, 1999) and can therefore be the most important factor in determining the final metal concentration of shoots. Actually, insoluble Zn comprises more than 90% of soil total Zn and is unavailable for plant uptake.

Regarding the effect of Zn on the photosynthetic machinery, our results confirm the negative effects of the metal excess shown in previous research on other plant species. Zn treatment induced a decrease in the net photosynthetic rate, affecting both stomatal conductance and photosynthetic parameters, even at the lowest concentration used. Probably, *T. riparia* plants readily closed the stomata to reduce transpiration and, thus, the absorption of the metal. Actually, it is already known that excessive Zn concentrations may limit the stomatal conductance and consequently the fixation of CO_2 (Tsonev end Lidon, 2012), impairing the photosynthesis (Medrano et al., 2002) and reducing the primary production of the plant.

In addition to such stomatal limitation of the photosynthesis, Zn excess negatively affected the efficiency of PSII and electron transport rate, as it was found also in isolated barley chloroplasts (Tripathy and Mohanty, 1980) and *Phaseolus vulgaris* (Van Assche et al., 1986). Actually, Rashid et al. (1991) proposed that elevated levels of Zn could perturb the conformation of the PSII core complex and might also affect the acceptor side of the photosystem. According to more recent studies, Zn seems to affect electron transport flow of PS II at the water splitting site, probably replacing Mg (Van Assche and Clijsters, 1986), although the mechanism of direct action of this metal is still in question (Rout and Das, 2003). The inhibition of the photosynthetic system is reported to be associated also to changes in chlorophyll structure and amounts (Tsonev and Lidon, 2012) and, actually, Zn excess led to a marked decrease in photosynthetic pigment concentrations also in *T. riparia* plants. Precisely, Chl a and carotenoid concentrations decreased significantly in plants treated with 500 and 1000 µM $ZnSO_4$ after two weeks of treatment. On the contrary, Chl b concentration appeared unchanged in treated plants, thus Chl a/Chl b ratio decreased. This corroborates the results shown in Ialelou et al., (2013), where in *Cucurbita pepo* the

reduction in Chl a was greater than Chl b. Regarding possible causes of the metal effect on pigment concentration, Zn is known to interfere with chlorophyll biosynthesis (Kalaikandhan et al., 2014) by inhibiting the chlorophyll synthesizing enzyme activity (Manios et al. 2003). Moreover, a possible reduction in the transport of Fe to the chloroplasts caused by Zn excess could determine the reduction of the Chl content in the leaves (Symeonidis and Karataglis, 1992).
On the other hand, Zn excess did not seem to significantly affect the amount of total soluble sugars in the leaves, whereas previous studies have shown that a decrease in photosynthetic efficiency could cause a reduction of the supply of soluble sugars to the tissues. For example, Zn excess significantly decreased the carbohydrate levels in *Triticum aestivum* leaves (Kumar et al., 2012) and in cluster bean (Manivasagaperumal et al., 2011). The lack of Zn effect on the concentration of such molecules in *T. riparia* might be due to a supposedly reduced ability to store simple sugars in the form of complex sugars under stress conditions, as demonstrated by Ialelou et al. (2013) in *Cucurbita pepo*. In such study, the concentration of soluble sugars increased while the starch content decreased under Zn stress.

Regarding the volatile emission, our data showed that *T. riparia* plants are able to emit at least thirteen different VOCs, including terpenes, monoterpenes, isoprene, acetates and aldehydes. These categories of molecules, in particular terpenes and monoterpenes, are actually known to characterize the essential oil composition of other officinal plants belonging to the Lamiaceae family, like *Rosmarinus officinalis* and *Thymus vulgaris* (Miladi et al., 2013).
Interestingly, Zn excess increased the intensity of the VOC emission in a dose-dependent manner, without affecting their qualitative profile. To the best of our knowledge, this is only the second report on a possible relationship between Zn excess and VOC emission in plants (de Jesus et al., 2016). The identified volatiles could play a role in protecting plants from oxidative stress under high heavy metal levels as suggested by de Jesus et al. (2016) for *Martianthus leucocephalus*, another plant from the Lamiaceae family (de Jesus et al., 2016). Actually, Lucini and Bernardo (2015) previously showed that Zn might increase the activity of enzymes involved in the biosynthesis of some compounds, for example dimethylallyl pyrophosphate isomerase and phenylalanine ammonia lyase, which intervene in the terpenes and phenylpropanoids biosynthesis (de Jesus et al., 2016). In any case, as the Zn-treated

plants can maintain volatile compound production even under adverse growth conditions and with limited photosynthetic capacities, our results support the important role of these molecules in plant responses to metal excess, as previously suggested by de Jesus et al. (2016).

In general, the emission of VOCs was significantly greater in plants treated with 500 and 1000 µM $ZnSO_4$ compared to control plants, but for compounds, such as cis- and trans-hexenal and monoterpenes, the emission was high also in 100 µM treated plants, thus showing variation in the Zn concentration-threshold able to induce VOCs. Actually, the defensive function of the above-mentioned molecules is well-known and linked, in particular, to the plant responses to biotic stress. Hexenal is released in large amount after wounding (Harren and Cristescu, 2013), while monoterpenes perform several function, having anti-microbial and antioxidant properties (Zengin and Baysal, 2014). Their emission would confirm that abiotic stress (in particular heavy metals) can induce the synthesis and accumulation of the same defense-related secondary metabolites, induced by biotic stress (Mithöfer et al., 2004), probably to counteract some possible similar detrimental effects of the two kind of stresses.

The VOC emitted at greater intensity under Zn stress was propanal, as compared to the other identified volatiles. This compound had been previously recognized as odorous substance in durum wheat, chili pepper (Infantino et al., 2014) and in *Phyllostackys nigra* (Crespo et al., 2013), and it was also identified as green leaf volatile in other plant species (Geron et al., 2006). This substance has been proposed to have a certain role in allelopathic interaction between plants (Roshchina, 1993), but results are still to be confirmed. In any case, our results suggested a promising role of such molecule in the defense toward Zn stress that deserves to be further investigated.

The only exception in the Zn-induced VOC production was represented by methanol, whose emission intensity decreased under Zn stress. This result could be explained by considering that the main source of methanol emission is cell wall pectin demethylesterification (Oikawa et al., 2011). Plants could have responded to metal treatment decreasing the methylesterification degree of pectins, in the effort of increasing the cell wall ability to bind Zn, consequently excluding the element from the symplast. Actually, pectins are known to be involved in plant defense strategy against metals (Krzesłowska, 2011), but their role in metal tolerance is still controversial (Colzi et al., 2011, 2012).

Overall, our results indicated a significant influence of Zn excess on the emission of VOCs in *Tetradenia riparia* and opened new research possibility to clarify the general assumption that heavy metal stress can enhance VOC emission rate and pattern (Loreto and Schnitzler, 2010).

CHAPTER 3: EXPERIMENTAL

"Interaction between zinc excess and water deficit in *Tetradenia riparia*. An analysis of growth, gas exchange and VOC emission"

ABSTRACT

Tetradenia riparia (Hochst) Codd. is a plant native of South Africa that typically grows in riparian areas subjected to variations in water availability consequently to climate and environmental changes. This plant is traditionally used as a medicinal herb to obtain essential oils with biological properties.

In the present work, the combined effect of two abiotic stresses, drought stress and Zn stress, was assessed in this plant species, evaluating its physiological responses with particular attention to the emission of volatile organic compounds.

Plants were grown in hydroponics in the presence of 500 µM $ZnSO_4$ and 35g/l of polyethyleneglycol (PEG) as simulator of water stress. These chemicals were applied separately or in combination.

The effect of these abiotic stresses was evaluated on plant growth, photosynthetic parameters and VOC emission.

Zinc application caused a significant negative effect on plant growth, net photosynthetic rate and stomatal conductance. In the presence of PEG, Zn had less inhibitory effect on these parameters. This could be due to a lower translocation of Zn to the shoots, avoiding compromising photosynthetic part.

Fifty different VOCs were identified, among which terpenes, monoterpenes, isoprene, acetates, aldehydes and alcohols. The intensity of emission was higher in plants treated only with Zn and lower in plants treated with PEG. In plants subjected to both treatments, the intensity of emission was similar to that of the control plants.

This work confirmed that the emission of VOCs is specifically influenced by different abiotic stresses, but at the same time revealed that the combination of different stresses may not result in changes in their emission.

Keywords: Zn stress; water deficit; volatile organic compounds; *Tetradenia riparia*

3.1 INTRODUCTION

Stress in plants could be defined as any change in growth conditions that disrupts metabolic homeostasis and requires an adjustment of metabolic pathways (Shulaev et al., 2008).

Intensity and duration of exposure are distinctive elements of stress and affect the response capacity of the plant.

The most basic abiotic stressors include light stress, wind, rain, extreme temperatures, drought, flooding, other natural disasters, chemicals and pollutants such as heavy metals or pesticides (Ramakrishna and Ravishankar, 2011).

Plant species that live in areas subject to low rainfall or with high temperatures are often subject to poor water supply conditions.

The productivity and growth are strongly influenced by drought stress. However, plants are able to develop responses and adaptations that allow to tolerate or withstand these conditions (Levitt, 1980).

For instance, there are some plants that perform vegetative functions only in favorable periods for the growth, avoiding the unfavorable periods. Other plants are able to maintain an adequate level of tissue hydration, through different mechanisms, for example by decreasing the water potential of the tissues, increasing the absorbing surface or the ability to carry the water to the shoots, or reducing the loss of water thanks to a lower transpiration surface and greater cutinization (Xu et al., 2010).

Drought, affects plants at various levels of their organization (Wentworth et al., 2006).

The physiological responses to drought at the whole plant level is highly complex and involves deleterious and/or adaptive changes. This complexity is due to several factors such as plant species and variety, the dynamics, duration and intensity of soil water depletion, changes in water demand from the atmosphere, environmental conditions, as well as plant growth and the phenological state in which water deficit is developed (Lisar et al., 2012).

Plant organs are influenced in different ways by water stress. For example, plants appear to allocate relatively more biomass to roots when their growth is limited by belowground factors, such as water, resulting in the leaf reduction and limitation of the growth due to a less available photosynthetic surface (Poorter et al., 2012).

At the shoot level, there can be a lowering of osmotic potential, due to the increase of the concentration of osmotically active solutes, such as organic acid, inorganic ions,

proline, soluble carbohydrates and the level of transpiration is regulated through the state of opening of the stomata (Boyer, 1985).
Plants reduce water loss at the expense of CO_2 absorption. The indirect consequence is the inhibition of cell division and cell expansion which results in a reduction of growth. Nevertheless, Ni and Pallardy (1991), observed that plants typical of arid habitat and regions show a delayed closing of stomata and increased carbon assimilation.

In nature, plants are simultaneously exposed to a combination of biotic and abiotic stresses. Nevertheless, in the study of environmental stresses, a poorly known aspect concerns the interaction between different types of stress, for example the simultaneous effect of drought and heavy metal stress.
High metal availability, arising from mining and industrial activities, disposal of sewage sludge or soil acidification, is an increasing problem in agriculture and forestry. Metal toxicity causes multiple direct and indirect effects in plants affecting practically all physiological functions (Barceló and Poschenrieder, 2008).
Among heavy metals, Zn is present naturally in the environment, but the increase in human activities has led to a concerning increase in the concentration of the metal in all the environmental matrices (Duruibe et al., 2007).
Zinc is an essential element for plants (Shier, 1994), being a constituent of metalloproteins or a cofactor for several enzymes, such as anhydrases, dehydrogenases, oxidases and peroxidases (Hewitt, 1983). This metal also plays an important role in regulating the nitrogen metabolism, cell multiplication, photosynthesis and the synthesis of nucleic acid, proteins and auxin in plants (Shier, 1994). Nevertheless, at high concentrations, Zn can be highly phytotoxic.
Growth inhibition is a general phenomenon associated with Zn toxicity (Collins, 1981); Moreover, an excess of this metal, can lead to nutrient imbalance, chlorosis of leaves, impairment of photosynthesis and increase of reactive oxygen species (Peng et al., 2015).
A poorly explored aspect is the relationship between abiotic stress and the emission of volatile organic compounds (VOCs) in plants. Stressful environmental conditions caused by high light, temperature, drought and , eutrophication (Penuelas and Staudt., 2010), can influence VOC production and realease (Laothawornkitkul et al., 2008). In fact, abiotic stress factors can cause changes in the primary and secondary

metabolism leading to changed emissions of VOCs (Loreto and Schnitzler 2010). As a generalization, stress appears to increase VOC emission (Holopainen and Gershenzon, 2010).

Currently, little is known about the effect of drought stress on emission of VOC by plants. Stomatal closure and reduced photosynthesis as a response to drought are expected to decrease VOC emission (Loreto and Schnitzler 2010). Nevertheless, Loreto and Schnitzler (2010) suggested that drought stress does not inhibit the emission of VOCs, *per se*, but leads to different VOC emission patterns, depending on the type of compound and stress intensity (Bourtsoukidis et al., 2014). Under moderate stress conditions, the emissions of VOCs remained rather unaffected, but under severe drought, emissions were reduced (Kesselmeier and Staudt 1999).

Concerning the influence of heavy metals on VOCs, it is known, from previous studies, that VOCs emission pattern can be altered by heavy metal stress. For example, trees naturally emit VOCs and such emission can be increased by metal pollution (Velikova et al., 2011).

The data available on the relationship between Zn and VOCs in plants are still few. From previous study we know that in *Martianthus leucocephalus* Zn excess cause an increase of leaves volatile compound concentration (de Jesus et al., 2016).

Chapter 2 of the present thesis, highlighted that *Tetradenia riparia* was able to emit several different VOCs under Zn stress conditions. Therefore, this study was performed to assess the combined effect of two abiotic stress, drought stress and Zn stress in *Tetradenia riparia*, in terms of physiological responses and especially VOC emission.

3.2 MATERIALS AND METHODS

Plant growth conditions

Cuttings of *Tetradenia riparia* plants were cultivated in aerated water for two weeks inside a greenhouse with natural light and an average temperature of about 30 °C during the day and about 20 °C during the night.

Rooted plants obtained from these cuttings were then relocated in a plastic pots of five liters capacity with aerated nutrient solution and cultivated for four weeks. In particular,

all plants were supplied with half-strength Hoagland's nutrient solution in demineralized water with the following compositions: potassium nitrate 3 mM, calcium nitrate 2 mM, ammonium dihydrogen phosphate 1 mM, magnesium sulphate 0,5 mM, EDTA ferric sodium-salt 40 µM, phosphorous acid 25 µM, Zn sulphate 2 µM, manganese (II) sulphate 2 µM, ammonium heptamolybdate 0,1 µM, copper (II) sulphate 0,1 µM and MES buffer 1 mM as reported in Bazihizina et al., 2016. When necessary pH was adjusted to 5.8 using KOH. Growth medium was changed once a week.

Afterwards, plants with similar shoot and root were selected and the treatments were added to the nutrient aerated solution. Three treatments were applied, the first constituted by $ZnSO_4$ 500 µM, the second constituted by PEG 6000 (35g/l), the third constituted by $ZnSO_4$ 500 µM + PEG (35g/l). Five replicates were considered for each treatment and were cultivated for 4 weeks after treatments application.

Plant length was measured every 7 days for all the duration of the experiment to determine its extension during the treatment period. At different time points (14 and 28 days), young fully expanded leaves (one for each plant in all treatments) were collected for subsequent analyses of total soluble sugars. Liquid N_2 was used to freeze this tissues, then they were stored at -80 °C, freeze-dried, and analyzed.

Plant harvest

Plants were sampled after 4 weeks from the beginning of the treatments. Each of them was carefully removed from the plastic pot and washed with deionized water. Plants roots were immersed in a solution containing deionized water and 20 mM Na_2 EDTA for about 20 min to remove Zn eventually adhered to root surface (Peng et al., 2015). Then, all plants were washed again with only deionized water. Afterwards, fresh weight of shoot and root was registered. Finally, plants were separated into leaves, stems and roots and placed in an oven at 60 °C for 1 week to dry. Dry weight of these organs was then registered.

Leaf gas-exchange parameters

During the 4 weeks of treatment, physiological parameters as net photosynthetic rate and stomatal conductance were measured. The measurement were taken before the start of the treatments and after their application at 24 hours, 48 hours and 10 days.
These measurement were made on all plants of each treatment, using the open gas-exchange system Li-6400 XT (Li-Cor, Lincoln, NE, USA), operating with a flow rate of 400 µmol s–1, reference CO2 of 400 µmol mol–1 and photosynthetically active radiation of 300 µmol m–2 s–1. Measurements were taken between 09.30 h and 11.30 h.

Zn analysis

Dried roots, stems and leaves of each plants, removed from the oven at 60 °C, were ground using an electric grinder. After being placed again in the oven at 60 °C for 24 hours, an aliquot of 100 mg was taken from each sample and placed in a 25 ml beaker. To each beaker a mixture of HNO_3: $HClO_4$ (5:2 v/v) was added for the acid digestion.
All the beakers were placed on hot plates at a temperature of 60-100 °C to accelerate the mineralization of the plant material. When the tissues were completely digested, the content of each beaker was added to 10 ml volumetric flasks and brought to volume with deionized water. Then, the solution in each flask was filtered with an appropriate filter paper and added to plastic tubes with a small funnel for subsequent determination of Zn using the atomic absorption spectrophotometer (AAnalyst 200 Perkin-Elmer).

Determination of carbohydrates by anthrone method

Total soluble sugars were determined at two different time (day 14 and 28 days after the treatment). It was used a quantitative colorimetric method, based on a coloring reaction of the reagent anthrone with carbohydrate in plant tissue that produces a green colored complex, whose intensity is directly proportional to amount of carbohydrates in solution. Spectrophotometric absorbance was detected at 620nm

utilizing a UV-visible spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan). The procedure derived from Yemm and Willis, 1954 (The Estimation of Carbohydrates in Plant Extracts by Anthrone).

Volatile organic compound determination

The volatile compounds emitted from the leaves of *T. riparia* were analyzed with a PTR-TOF 8000 (IONICON Analytik GmbH, Innsbruck, Austria) using H_3O^+ as reagent ion for the proton-transfer reaction. The reaction takes place between H_3O^+ ions and all VOCs having a proton affinity higher than that of water (165.2 kcal mol^{-1}). Plant leaves were inserted in a glass jar (500 mL at 25°C, with a dynamic headspace flushing flow rate of 200 ml/min) equipped with two Teflon inlet and outlet tubes on opposite side, which were, respectively, connected to a zero-air generator (Peak Scientific) and the PTR-TOFMS. Volatile organic compounds were then measured by direct injection of the head space mixture into the PTR-TOFMS drift tube via a heated (60°C) peek inlet tube with a flow rate of 100 sccm for 5 min. Measurements were carried out as previously described in Cappellin et al. 2011, using a PTR-TOFMS in its standard configuration. The sampling time for each channel of TOF acquisition was 0.1 ns, amounting to 350,000 channels for a mass spectrum ranging up to m/z=270. The conditions in the drift tube were: drift voltage 600 V, temperature 110°C, pressure 2.25 mbar, extraction voltage at the end of the tube (Udx) 32V. Raw data were acquired with the TofDaq software (Tofwerk AG, Switzerland) using a dead time of 20 ns for the Poisson correction, and peak extraction followed the methodology described in Cappellin et al. 2011, employing a modified Gaussian peak shape. For peak quantification, the resulting data were corrected according to the duty cycle and the signals were normalized to the primary ion signal (cps to ncps) (Cappellin et al. 2011). Volatiles organic compounds were determined 14 days after the beginning of the treatment. For VOC identification and data modeling, we used an average signal intensity recorded for 60 s, which allowed the acquisition of 60 average spectra with a high mass accuracy (Taiti et al., 2014). For such duty cycle, the limit of detection is expected to be in the single-digit pptv range, following the PTR-TOF 8000 technical specifications. The identification of VOCs was based on the fragmentation patterns of

pure standards available in the literature and compared with published VOCs emitted from different species of plants.

Statistical analyses

The raw data collected were analyzed using GraphPad software 7th edition for windows.

An analysis of variance (ANOVA) was performed to identify overall significant differences between treatments. All data shown are means of 5 replicates. Tukey post-hoc test was used for a posteriori comparison of individual means, with at least $P \leq 0.05$ as significant level).

3.3 RESULTS

Effect of Zn stress and water deficit on plant growth

Shoot elongation of *Tetradenia riparia* under Zn treatments and water deficit is presented in Table 6.

Plants growth continued throughout the duration of the experiment. However, compared to control plants, an application of 500 µM of Zn sulphate caused an evident slowdown in growth from the beginning of the treatment. In plants treated with PEG, an uninterrupted shoot elongation was recorded, but appeared decreased compared to untreated plants. Instead, plants grown with PEG and Zn, showed a similar growth trend to Zn treated plants, but less depressed.

Considering dry biomass, detected at the end of treatment, Zn caused a significant decrease of leaves, stem and roots dry weight compared to control plants, as reported in Table 7. The average value of dry weight of leaves, stems and roots in *T. riparia* control plants was of 8,0 g, 5,4 g and 1,8 g, while in *T. riparia* plants grown with Zn excess was respectively of 1,9 g, 2,1 g and 0,4 g. This means that leaf, stem and root biomass decreased respectively by more than 76%, 61% and 77%. Similar trend was observed when in combination with PEG, but the effect was less strong and the reduction percentages observed were of about 70% for leaves, 56% for stems and 39% for roots.

PEG applied alone in the culture medium induced a reduction of 14% in leaf dry biomass and of 11% in stem dry biomass compared to control plants, although not significantly, and caused a significant increase of root dry biomass, of about 68%, compared to untreated plants.

Treatment	**Day 7** (cm)	**Day 14** (cm)	**Day 21** (cm)	**Day 28** (cm)
Control	5,6 ± 0,8 **a**	16,7 ± 4,0 **a**	23,2 ± 4,9 **a**	25,7 ± 5,7 **a**
Peg	4,7 ± 0,8 **ab**	9,7 ± 2,8 **ab**	15,5 ± 3,3 **ab**	19 ± 4,7 **ab**
Zn	1,7 ± 1,1 **b**	3,9 ± 2,1 **b**	4,5 ± 2,7 **b**	5,6 ± 3,0 **b**
Peg+Zn	2,8 ± 0,3 **ab**	5,7 ± 0,8 **b**	8,6 ± 1,3 **b**	9,1 ± 1,2 **b**

Tab. 6: Elongation of shoot in *Tetradenia riparia* untreated plants (control) and in *Tetradenia riparia* treated plants (PEG, Zn and PEG+Zn). Values are mean ± SE (n=5). Letters indicate significant difference among treatments and control (at least P<0,05).

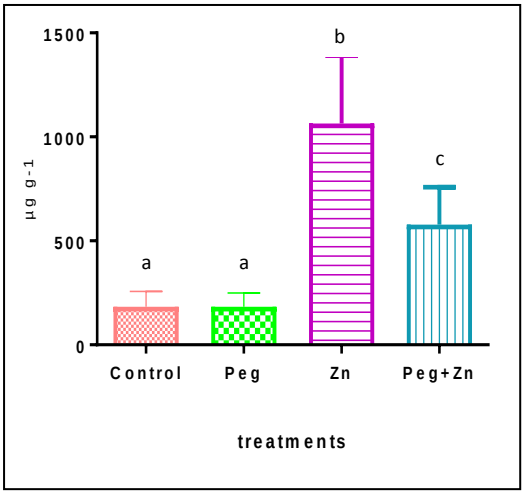
Treatment	**Leaves (g)**	**Stems (g)**	**Roots (g)**
Control	8,0 ± 0,3 **a**	5,4 ± 0,5 **a**	1,8 ± 0,1 **a**
PEG	6,9 ± 0,8 **a**	4,8 ± 0,7 **a**	3,03 ± 0,6 **b**
Zn	1,9 ± 0,03 **b**	2,1 ± 0,1 **b**	0,4 ± 0,03 **c**
PEG+Zn	2,4 ± 0,3 **b**	2,4 ± 0,2 **b**	1,1 ± 0,1 **ac**

Tab. 7: Dry mass in *Tetradenia riparia* detected at the end of the treatment. Values are mean ± SE (n=5). Different letters into the row indicate significant differences among treatments (at least P < 0,05).

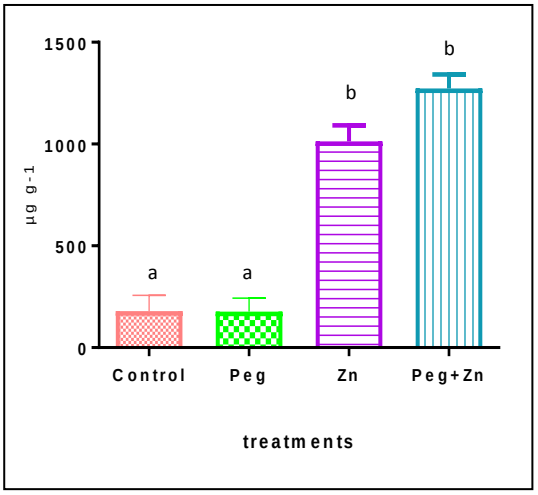
Zn concentration in plant organs

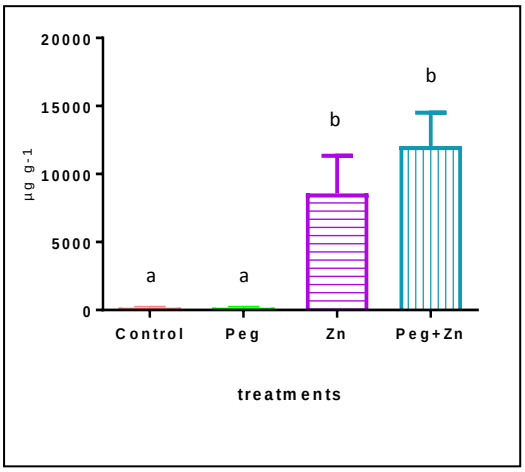
Figure 5 (a,b,c) shows respectively the concentration of Zn in roots, stems, and leaves of *Tetradenia riparia* grown for four weeks in hydroponic culture with different treatments applied. Zinc concentration was higher in roots than in shoot in plant grown under treatments with Zn. At the leaf level, Zn concentration in plants treated with Zn and PEG was lower than in plant treated with only Zn.

a



b





c

Fig.5. Zn concentration in leaves (a) stems (b) and roots (c) of *Tetradenia riparia* in control plants, in plants treated with PEG, in plant treated with Zn, and in plants treated with PEG+Zn. Values are mean ± SE (n=5) and are related to the end of the treatment. Letters show significant differences (at least P<0,05)

Photosynthetic efficiency

Net photosynthetic rate and the stomatal conductance trends of *T. riparia* plants are reported in Table 8 and Table 9 respectively. During the first 24h, the net photosynthetic rate was not affected by the treatments. Zn leads to a decrease of this parameter after 48h, both in plants treated with only the metal, and in those treated with PEG+Zn, while the negative effect of PEG was visible at the last detection.

Stomatal conductance resulted decreased early in plants grown with PEG and PEG+Zn, already after 24h of treatment, whereas the effect of Zn alone was visible after 48h. However, in PEG treated plants, a quick recovery occurred.

Treatment	**0h (µmol m-2 s -1)**	**24h (µmol m-2 s -1)**	**48h (µmol m-2 s -1)**	**10days (µmol m-2 s -1)**
Control	11,34 ± 0,42 **a**	12,19 ± 0,86 **a**	12,14 ± 0,36 **a**	11,77 ± 0,25 **a**
PEG	9,86 ± 0,49 **a**	11,95 ± 0,91 **a**	12,21 ± 0,34 **a**	10,51 ± 0,23 **b**
Zn	10,91 ± 0,30 **a**	9,65 ± 0,38 **a**	9,06 ± 0,31 **b**	7,13 ± 0,21 **c**
PEG + Zn	11,02 ± 0,44 **a**	11,05 ± 0,16 **a**	9,08 ± 0,32 **b**	7,74 ± 0,19 **c**

Tab. 8: Net photosynthetic rate detected in *Tetradenia riparia* at 0h (before the treatment), and after the beginning of the treatment, at 24 h, 48 h and 10 days. Values are mean ± SE (n=5). Different letters into the row indicate significant differences among treatments (at least P < 0,05).

Treatments	**0h (µmol m-2 s -1)**	**24h (µmol m-2 s -1)**	**48h (µmol m-2 s -1)**	**10days (µmol m-2 s -1)**
Control	0,27 ± 0,02 **a**	0,24 ± 0,01 **a**	0,24 ± 0,01 **a**	0,26 ± 0,02 **a**
PEG	0,31 ± 0,03 **a**	0,19 ± 0,01 **bc**	0,27 ± 0,01 **a**	0,25 ± 0,02 **a**
Zn	0,24 ± 0,01 **a**	0,22 ± 0,01 **ab**	0,12 ± 0,01 **b**	0,07 ± 0,01 **b**
PEG + Zn	0,29 ± 0,02 **a**	0,17 ± 0,02 **c**	0,10 ± 0,01 **b**	0,124 ± 0,003 **c**

Tab. 9: Stomatal conductance detected in *Tetradenia riparia* before the start of the treatment (0h) and after 24 h, 48 h and 10 days. Values are mean ± SE (n=5). Letters into the row indicate significant differences among treatments (at least P < 0,05).

No significant differences in total soluble sugar concentration detected in young fully expanded leaves were found among treatments. Besides, no differences were found between the first and the second sampling time, at day 14 (Figure 6a) and at day 28 (Figure 6b) after the application of treatments.

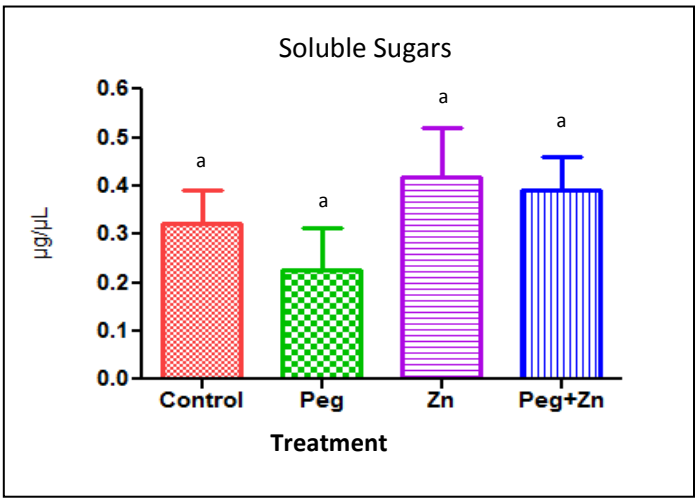


Fig.6a: Carbohydrates detected in *T. riparia* leaves by anthrone method, after 14 days of the treatment (Control, PEG, Zn and PEG+Zn). Values are mean ± SE (n=5). Letters indicate significant differences (at least P<0,05).

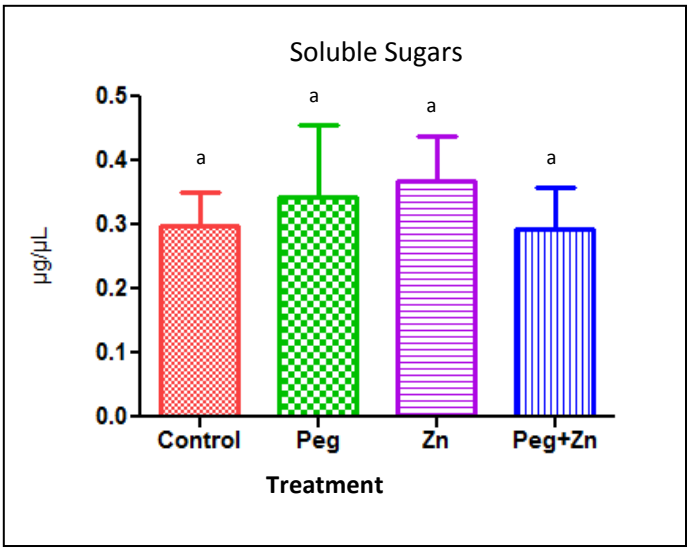


Fig.6b: Carbohydrates found in *T. riparia* leaves with anthrone method at the end of the treatment (Control, PEG, Zn and PEG+Zn). Values are mean ± SE (n=5). Letters indicate significant differences (at least P<0,05).

Organic volatiles compound emission

46 volatile organic compounds were detected with PTR-Tof-MS, using the data set comprising peaks from m/z= 30 to m/z= 210. The emission profile is shown in table 10, where are reported m/z ratio (both theoretical and measured), protonated chemical

formula and tentative of identification with the relative biographical reference (Taiti et al., 2015a,b; Masi et al., 2016). The emission intensity values (mean and standard error) are reported in Table 11. In the presence of Zn alone, the emission rate of the most identified VOCs increased compared to control plants. On the contrary, PEG led to a visible decrease of the detected compounds, despite not significant compared to untreated plants. In plant treated with PEG+Zn the emission rate followed a trend similar to that of the control plants.

Compound[a]	Measured m/z[b]	Protonated chemical formula[c]	Tentative of Identifications[d]	Theoretical m/z[e]	TOF references[f]
1	33,03	CH_5O+	Methanol	33,0335	Taiti et al., 2015
2	41,04	C_3H_5+	Alkyl fragment	41,0386	Taiti et al., 2015
3	43,02	C_2H_3O+	Alkyl fragment (ethenone)	43.0178	Sánchez Del Pulgar et al., 2013
4	43,05	C_3H_7+	Alkyl fragment (propene)	43,0542	Sánchez Del Pulgar et al., 2013
5	45,03	C_2H_5O+	Acetaldehyde	45,0334	Brilli et al., 2011
6	47,05	C_2H_7O+	Ethanol	47.0491	Hung et al., 2013
7	49,01	CH_5S+	Methanethiol	49.0106	Stotzky et al., 1976
8	53,04	C_4H_5+	Cyclobutadiene	53.0385	Vita et al., 2015
9	55,05	C_4H_7+	Alkyl fragment	55.0542	Sánchez Del Pulgar et al., 2013
10	57,07	C_4H_9+	Alcohol Fragment	57,0699	Galle et al., 2011
11	59,05	C_3H_7O+	Propanal/2-Propanone	59,0491	Infantino et al., 2014
12	61,03	$C_2H_5O_2+$	Acetic acid	61,0284	Taiti et al., 2015
13	63,03	C_2H_7S+	Dimethyl sulfide	63,026	Stotzky et al., 1976
14	67,05	C_5H_7+	3-Penten-1-yne	67.0542	Tirillini et al., 2000
15	69,03	C_4H_5O+	Furan	69.0336	Vita et al., 2015
16	69,07	C_5H_9+	2-Methyl-1,3- butadiene (isoprene)	69,0698	Mancuso et al., 2014
17	71,05	C_4H_7O+	3-Buten-2-one	71.0491	Pennazza et al., 2013
18	71,09	$C_5H_{11}+$	Alkyl fragment	71.0855	Özdestan et al., 2013
19	75,08	$C_4H_{11}O+$	2-Methyl-1-propanol (isobutanol)	75.0804	Tracey et al., 1989
20	80,05	C_5H_6N+	Pyridine	80.0494	Özdestan et al., 2013
21	83,09	C_6H_{11}	C6 compounds/ Hexenol fragment	83.0855	Brilli et al., 2011
22	87,04	$C_4H_7O_2+$	Butan-4-olide	87.0441	Schulz e Dickschat, 2007
23	87,08	$C_5H_{11}O+$	2-Methylbutanal	87.0804	Sánchez Del Pulgar et al., 2011
24	91,05	C_4H11O_2+	Isopropyl methyl sulfide	91.0753	Vita et al., 2015
25	93,07	C_7H_9	Toluene	93.0699	Infantino et al., 2014
26	95,09	C_7H_{11}	Toluene; Terpene fragment	95.0855	Romano et al., 2014
27	99,08	$C_5H_7O_2+$	4-Methyl-5 h-furan-2-one	99.080	Taiti et al., 2015

28	101,1	$C_6H_{13}O_b$	Hexanal	101.0961	Jardine et al., 2010
29	101,1	$C_5H_9O_2$ +	2,3-Pentanedione	101.0597	Pennazza et al., 2013
30	103	C_8H_7+	Ethynylbenzene	103.0543	March et al., 2006
31	103,1	$C_5H_{11}O_2$+	4-Hydroxy-3-methyl-2-butanone	103.0754	March et al., 2006
32	105,1	$C_4H_9O_3$+	4-Hydroxybutanoic acid	105.0546	Pennazza et al., 2013
33	107,1	C_8H_{11} +	1,3-Dimethylbenzene; Terpen fragment	107.0855	Galle et al., 2011
34	109,1	C_7H_9O+	Anisole	109.0647	Pfander and Rychener, 1982
35	109,1	$C_6H_9N_2$+	2,5-Dimethylpyrazine	109.0760	Pfander and Rychener, 1982
36	111	$C_6H_7O_2$+	2-Acetylfuran	111.0431	Groenhagen et al., 2013
37	121,1	C_9H_{13}+	1,2,4-Trimethylbenzene	121.0647	Taiti et al., 2015
38	123,1	$C_8H_{11}O$+	1-Methoxy-3-methylbenzene	123.0804	March et al., 2006
39	133,1	$C_{10}H_{13}$+	p-Cymenene	133.1011	Pennazza et al., 2013
40	135,1	$C_{10}H_{15}$+	p-Cymene	135.1168	Aprea et al., 2007
41	137,1	$C_{10}H_{17}$+	Monoterpenes	137.1325	Taiti et al., 2015
42	139,1	$C_9H_{15}O$+	2-Pentylfuran	139.1117	Hung et al., 2013
43	149,1	$C_{11}H_{17}$+	1-Ethylpropyl)benzene	149.1324	Spivallo et al., 2007
44	153,1	$C_{10}H_{17}O$+	Terpenoid-like compound	153.1273	Aprea et al., 2015
45	153,1	$C_{10}H_{17}O$+	Carveol	153.1273	Aprea et al., 2015
46	205,2	$C_{15}H_{25}$+	Sesquiterpenes	205.195	Ruuskanen et al., 2011

Tab. 10: Volatile organic compounds detected through PTR-Analysis. [a]Number of identified compound. [b]Mass to charge ratio measured by the Mass Spectrometer. [c]Compound's chemical formula (H^+ added by protonation). [d]Putative identifications according to spectral properties. [e]Theoretical mass to charge ratio found in literature or PTR-TOF-MS manual. [f]PTR-TOF-MS articles where molecule was reported

	TREATMENT			
VOLATILE COMPOUND	**Control (ncps)**	**PEG (ncps)**	**Zn (ncps)**	**PEG + Zn (ncps)**
33.03 **(Methanol)**	1263,0 ± 461,9 **a**	641,0 ± 74,3 **ab**	602,9 ± 89,8 **ab**	208,7 ± 27,0 **b**
41.04 **(Alkyl fragment)**	239,7 ± 20,9 **a**	117,3 ± 74,2 **a**	1891,0 ± 530,8 **b**	313,8 ± 58,6 **a**
43.02 **(Ethenone)**	863,0 ± 152,4 **a**	578,1 ± 52,5 **a**	3753,0 ± 305,6 **b**	1010,0 ± 140,5 **a**
43.05 **(Propene)**	331,5 ± 60,2 **a**	135,9 ± 30,4 **a**	1680,0 ± 464,3 **b**	323,9 ± 27,7 **a**
45.03 **(Acetaldehyde)**	256,1 ± 27,4 **a**	155,4 ± 27,9 **a**	1940,0 ± 150,5 **b**	366,7 ± 8,3 **a**
47.05 **(Ethanol)**	2,4 ± 0,3 **a**	2,7 ± 0,8 **a**	22,7 ± 2,9 **b**	5,5 ± 0,8 **a**
49.01 **(Methanethiol)**	-	-	4,1 ± 1,2 **a**	1,0 ± 0,4 **b**
53.04 **(Cyclobutadiene)**	73,9 ± 14,7 **a**	24,7 ± 5,2 **a**	337,0 ± 91,9 **b**	76,8 ± 9,0 **a**
55.05 **(Alkyl fragment)**	131,8 ± 25,6 **a**	61,4 ± 15,8 **a**	653,8 ± 130,3 **b**	129,4 ± 12,8 **a**
57.07 **(Alcool fragment or 1-butene)**	63,8 ± 9,6 **a**	26,7 ± 12,0 **a**	364,6 ± 74,1 **b**	65,0 ± 7,2 **a**
59.05 **(Propanal/2-Propanone)**	671,5 ± 112,6 **a**	463,3 ± 76,1 **a**	3430,0 ± 483,3 **b**	672,2 ± 33,5 **a**
61.03 **(Acetic acid)**	373,1 ± 59,7 **a**	322,2 ± 39,5 **a**	1139,0 ± 166,9 **b**	342,3 ± 41,3 **a**
63.03 **(Dimethylsulfide)**	2,0 ± 0,3 **a**	1,3 ± 0,3 **a**	5,9 ± 1,3 **b**	1,7 ± 0,4 **a**

67.05 **(3-Penten-1-yne)**	79,4 ± 16,5 **a**	27,7 ± 3,9 **a**	289,4 ± 73,5 **b**	74,6 ± 8,9 **a**
69.03 **(Furan)**	42,1 ± 10,6 **a**	15,1 ± 2,3 **a**	124,5 ± 20,1 **b**	41,1 ± 6,2 **a**
69.07 **(Isoprene)**	96,0 ± 22,4 **a**	38,0 ± 6,5 **a**	331,2 ± 63,0 **b**	90,7 ± 12,3 **a**
71.05 **(3-buten-2-one)**	17,4 ± 4,4 **a**	9,6 ± 1,3 **a**	66,2 ± 4,1 **b**	15,2 ± 2,2 **a**
71.09 **(Alkyl fragment)**	5,2 ± 1,5 **a**	1,7 ± 0,5 **a**	28,0 ± 3,0 **b**	6,0 ± 0,8 **a**
75.08 **(Isobutanol)**	-	-	2,9 ± 1,3 **a**	1,0 ± 0,3 **a**
80.05 **(Pyridine)**	9,5 ± 1,7 **a**	3,1 ± 0,9 **a**	40,5 ± 10,7 **b**	10,2 ± 1,1 **a**
83.09 **(C6 compounds/Hexenol fragment)**	14,8 ± 3,4 **a**	6,2 ± 1,2 **a**	60,6 ± 9,3 **b**	14,2 ± 1,6 **a**
87.04 **(Butan-4-olide)**	7,5 ± 1,3 **a**	6,5 ± 1,1 **a**	32,3 ± 2,4 **b**	7,8 ± 0,7 **a**
87.08 **(2-Methylbutanal)**	2,7 ± 0,3 **a**	3,9 ± 1,2 **a**	15,6 ± 1,6 **b**	2,1 ± 0,2 **a**
91.05 **(2-3-Butanediol or Isopropyl methyl sulfide)**	85,5 ± 13,9 **a**	34,8 ± 7,7 **a**	521,6 ± 151,2 **b**	96,8 ± 7,8 **a**
93.07 **(Toluene or p-cymene fragment)**	185,7 ± 31,2 **a**	73,2 ± 14,3 **a**	1023,0 ± 322,7 **b**	196,2 ± 15,8 **a**
95.09 **(Toluene or terpenes fragment)**	249,7 ± 53,3 **a**	86,9 ± 14,0 **a**	936,8 ± 268,5 **b**	236,2 ± 30,1 **a**
99.08 **(4-Methyl-5 h-furan-2-one)**	0,5 ± 0,3 **a**	-	5,3 ± 0,7 **b**	0,6 ± 0,3 **a**
101.1 **(Hexanal)**	0,5 ± 0,3 **a**	0,6 ± 0,3 **a**	4,4 ± 1,5 **b**	0,8 ± 0,4 **a**
103 **(Ethynylbenzene)**	249,7 ± 53,3 **a**	86,9 ± 14,0 **a**	938,8 ± 268,5 **b**	236,2 ± 30,1 **a**
103.1 **(4-Hydroxy-3-methyl-2-butanone)**	-	-	4,0 ± 0,3 **a**	-
105.1 **(4-Hydroxybutanoic acid)**	5,0 ± 1,0 **a**	1,7 ± 0,7 **a**	31,4 ± 5,5 **b**	6,0 ± 0,6 **a**
107.1 **(1,3-Dimethylbenzene or Terpen fragment)**	17,9 ± 4,3 **a**	6,0 ± 1,6 **a**	54,2 ± 4,4 **b**	16,7 ± 1,7 **a**
109.1 **(Anisole)**	22,2 ± 4,3 **a**	6,0 ± 0,3 **b**	51,4 ± 3,7 **c**	14,9 ± 2,0 **ab**
109.1 **(2,5-dimethylpyrazine)**	31,9 ± 6,9 **a**	8,9 ± 0,4 **b**	73,8 ± 7,2 **c**	22,0 ± 3,1 **ab**
111 **(2-Acetylfuran)**	2,7 ± 1,1 **a**	-	6,8 ± 0,3 b	2,6 ± 0,5 **a**
121.1 **(1,2,4-Trimethylbenzene)**	4,0 ± 0,9 **a**	1,4 ± 0,1 **a**	14,5 ± 3,1 **b**	3,5 ± 0,3 **a**
123.1 **(1-Methoxy-3-methylbenzene)**	5,5 ± 1,4 **ac**	0,8 ± 0,3 **b**	8,3 ± 0,8 **c**	2,6 ± 0,4 **ab**
133.1 **(p-Cymenene)**	0,7 ± 0,5 **a**	-	5,1 ± 1,5 **b**	1,4 ± 0,1 **a**
135.1 **(p-Cymene)**	17,4 ± 3,7 **a**	6,5 ± 0,4 **b**	38,5 ± 1,3 **c**	19,0 ± 3,8 **a**
137.1 **(Monoterpenes)**	55,1 ± 0,8 **a**	28,1 ± 6,3 **a**	304,3 ± 96,5 **b**	73,3 ± 6,7 **a**
149.1 **(1-(Ethylpropyl)benzene)**	5,8 ± 1,7 **a**	1,7 ± 0,2 **b**	12,1 ± 0,8 **c**	3,8 ± 0,5 **ab**
153.1 **(Carveol)**	177,0 ± 38,0 **a**	33,1 ± 3,8 **b**	88,7 ± 11,1 **b**	72,8 ± 15,1 **b**
205.2 **(Sesquiterpenes)**	6,9 ± 2,0 **a**	2,1 ± 0,2 **a**	14,9 ± 1,9 **b**	4,9 ± 0,9 **a**

Tab. 11: Emission intensity of volatile organic compounds detected in *Tetradenia riparia* under different treatments. Values are mean ± SE (n=6). Different letters into the row indicate significant differences among treatments (at least $P < 0,05$).

3.4 DISCUSSION

In this study, the simultaneous effect of Zn excess and water deficit was investigated in *Tetradenia riparia* with a special focus on plant response in terms of VOC emission.

The presence of 500 µM $ZnSO_4$ in the culture medium caused a marked inhibition of shoot elongation and a significant decrease of leaf, stem and root dry weight as compared to the control plants. Actually, the well-known adverse effect of Zn excess on plants height and weight has been previously reported in several species of plants, grown either in pots or in hydroponics (Tsonev and Lidon, 2012). For example, stunting of shoot associated with Zn toxicity (Rout and Das, 2003) was previously observed in *Zea mays* (Rosen et al., 1977), in pea plants (Stoyanova and Doncheva, 2002), in barley plants (Sridhar et al., 2007) and in *Phyllostachys pubescens* (Peng et al., 2015).
Regarding Zn concentration in the plant organs, *T. riparia* accumulated most of this metal in roots, as the majority of plants shows under heavy metal excess (Ali et al., 2012). For example, a largest Zn concentration in roots respect to shoot was detected in *Martianthus leucocephalus* (de Jesus et al., 2016), in *Trifolium alexandrinum* (Ali et al., 2012) and in alfalfa plants (Gbaruko and Friday, 2007). Zinc toxicity is generally associated with alteration of root physiology (Rout and Das, 2003) and, in agreement with this, in Zn-treated plants roots appeared less developed and necrotic. Actually, restricted Zn translocation, could help to protect shoot by limiting the diffusion of the metal itself (Aibibu et al., 2010). In any case, though lower than in roots, metal concentration detected in the shoots seemed to be enough to undermine the health of the plant, compromising plant photosynthetic capabilities.
Zinc excess affected net photosynthetic rate, causing a significant decrease of this parameter already after 48 hours of treatment. Photosynthetic disorders were often observed in plants exposed to high metal levels (Nagajyoti et al., 2010) and the consequent decrease in plant photosynthetic efficiency could be partly responsible for the overall decrease in plant growth and biomass production (Chandra and Kang, 2015). In parallel, an imbalance in stomatal conductance was detected. The early stomatal closure induced by Zn, may have contributed to limiting the translocation of the metal itself.
Zinc excess did not influence plants with regard to soluble sugar concentration in leaves, in contrast with other studies that showed that heavy metals, in general, lead to a decrease of it (Rosa et al., 2009), since a disturbed photosynthetic activity could

modify their metabolism (Subba et al., 2014). However, there are also some studies showing that certain Zn concentration cause an increase of soluble sugars in leaves. In these cases, sugars increasing has been related to the starch degradation as reported in Ialelou et al. (2013) for naked pumpkin.

In plants cultivated with PEG, a decrease in shoot elongation was recorded compared to control plants. Similar results were obtained in several plant species, such as potato plants (Büssis et al., 1998) and forage sorghum (O'Donnell et al., 2013), subjected to a reduced water potential of the nutrient solution.

PEG treatment did not affect negatively the dry biomass produced by the entire plant, but, taking into consideration the organs separately, it was evident that it caused an increase in the root biomass of approximately 68% and a decrease in shoot biomass of approximately 13%. Probably, an increased allocation of resources to the roots allowed the plants to increase the surface for water uptake, as proposed by Kozlowski and Pallardy (2002), thus adapting to the non-optimal growth conditions with limited availability of water. Actually, increased root growth due to water deficit has been previous reported in other plant species, such as, for example, sunflower and *Catharanthus roseus* (Jaleel et al., 2009).

PEG caused a negative effect on the net photosynthetic rate, significantly after 10 days of treatment. It is still unclear whether drought mainly limits photosynthesis through stomatal closure or through metabolic impairment (Tezara et al., 1999; Lawson et al., 2003), despite stomatal limitation was generally accepted to be the main determinant of reduced photosynthesis under drought stress (Reddy et al., 2004). In PEG-treated plants, stomatal closure occurs after 24h of treatment, but the net photosynthetic rate was unchanged, thus suggesting that, at the beginning of the treatment, photosynthetic efficiency was not affected by stomatal closure. To confirm this, while stomatal conductance increased after 10 days of treatment, photosynthetic activity declined.

Total soluble sugars concentration in leaves did not change in plants under drought stress, either after 14 days or after 28 days of treatment. Other studies found that drought, as well as salinity, low temperature and flooding, in general, increased soluble sugar concentrations (Rosa et al., 2009). Nevertheless, based on available data in literature, it is known that sugar changes do not follow a general model and vary with plant genotype and the stress factor (Rosa et al., 2009). Recent researches showed that the increase in soluble sugars concurred to rise plant tolerance to abiotic stresses such as drought (Rosa et al., 2009). Therefore, it might be convenient for the plant to

keep high certain soluble sugars level in the leaves, reducing the synthesis of complex sugars and adjusting the own water potential (Lemoine et al., 2013).

Zn and PEG combination in the culture medium led to a mitigation of the toxic effects of Zn when administered alone, with a slight improvement of shoot growth and an increase of leaves, stem and roots dry biomass, though statistically not significant.
On the other hand, plant treated with PEG+Zn showed a slowdown in shoot elongation compared to plant grown with PEG only, probably caused by the toxic action of the metal itself.

Zinc accumulation in plant tissues varied when PEG was present in the culture medium. Plants grown with PEG+Zn accumulated more metal in the roots than in shoot, as when Zn was administered alone, but their Zn concentration in shoots was lower than in plants treated with only Zn. Probably, the greater root accumulation of Zn in PEG+Zn treated plants and their lower Zn concentration in shoots could have allowed the reported ameliorated growth of the above ground parts, even in the presence of the same metal excess in the culture medium.
Regarding the net photosynthetic rate, in PEG+Zn-treated plants a significant decrease was detected after 48h of the treatment as compared to control plants, but after 10 days such reduction was slightly less evident than in Zn-treated plants. Again, the presence of PEG in the culture medium seemed to dampen the effect of Zn. Nevertheless, in comparison with plant treated with PEG only, photosynthetic efficiency of plants grown with PEG+Zn was reduced, even if without an additive effect of the two stresses.
After 24h of treatment, stomatal conductance of plants grown with PEG+Zn diminished, as compared to control plants, with a similar pattern to that of plants grown with PEG only and differently from Zn-treated plants, which closed stomata later. The presence of PEG could have induced an early stomatal closure with the consequent effect of a lower Zn accumulation, at least at the beginning of treatment.
Zinc and PEG in combination did not seem to affect the amount of total soluble sugars in leaves, compared to control plants. The amount of detected soluble sugars in leaves could be the result of the balancing between decreased CO_2 assimilation and reduction and starch degradation caused by applied stresses (Rosa et al., 2009).

Regarding VOC emission, in this experiment 46 volatile organic compounds were revealed, from m/z 30 to m/z 210, using the PTR-TOF spectrometer. Among the detected compounds, there were aromatic hydrocarbons, alcohols, aldehydes, terpenes and monoterpenes. Many of these molecules were previously identified in essential oils of several aromatic plant species and they seem to have a major role in preventing insect feeding, in giving protection from fungal and bacterial attack and in the scavenging of reactive oxygen species (Siddiqui et al., 2017).

Generally, the emission rate varied in relation to the treatments applied, even if almost all the compounds were detected in both control plants and treated plants. This can indicate that the applied stresses act mainly on the rate of constitutive emission, influencing storage emission from specialized compartments (storage emission) or *de novo* emission (Copolovici and Niinemets, 2016).

In Zn-treated plants, a significant increase in intensity was observed, with respect to control plants and the other treatments. Exceptions were represented only by methanol (m/z 33.03) and carveol (m/z 153.1), emitted with greater intensity in untreated plants. Methanol emission has been found also in *Tuber magnatum* (Taiti et al., 2015) and in food matrices (Galle et al., 2001; Sánchez Del Pulgar et al., 2013). This compound derives from the demethylation of pectin cell wall. When the carboxylic groups of the homogalacturonans are not methylated, they can form ionic bonds with positively charged ions, such as Zn, thus increasing the ability of the cell wall to chelate metals in the effort of reducing their uptake (Oikawa et al., 2011).

According to Mithöfer et al. (2004), several plants species synthesize and accumulate secondary metabolites after treatment with metals. However, their influence can vary depending on the type of metal and the plant species. In fact, it was showed that in *M. leucocefalus* plant, exposed to increasing Cd, Cu and Zn concentration, only this latter metal was able to induce an increase in the production of volatile compounds (de Jesus et al., 2016), thus supporting our results about a possible functional relationship between VOC emission and Zn stress.

A few VOCs, such as methanethiol, isobutanol and 4-hydroxy-3-methyl-2-butanone, were detected only in plants grown with Zn. This might suggest that the metal is able to induce *de novo* the production of these volatiles, even if it cannot be excluded a direct release after a substrate damage caused by the metal itself.

In plants grown with only PEG, a lower VOC emission intensity was found in comparison to untreated plants, although not statistically significant for most

molecules. Previous studies have suggested that, in general, a mild water stress, leading to a moderate reduction in stomatal conductance and thus to a light loss of CO_2 in chloroplasts, can lead to increase in volatile emission (Copolovici and Niinemets, 2013). However, the results are sometimes controversial. For example, Ebel et al. (1995) reported the opposite, showing that VOC emitted from apple trees increased under severe drought stress, but not under mild stress. Most recently, Bourtsoukidis et al. (2014) found that in *Quercus robur* and *Prunus serotina* drought stress reduced emission rate of most VOCs. A similar result was reported also by Staudt et al. (2002), showing that monoterpenes emission decreased in *Quercus ilex* under water deficit. All these results seem to suggest that the effect of drought stress on VOC emission changes depending on the stress severity and timing, plant species and considered compounds. Some authors suggested that determined conditions, such as an increase in oxidative stress due to stomata closure, can lead to a major build-up of volatiles inside the leaves (Niinemets et al., 2009). Nevertheless, as the available CO_2 quantity decreases, it is necessary an equilibrium between increasing VOCs with protective function and energy saving. In our results, even if the amount of CO_2 assimilated might be enough, as suggested by the recovering in stomatal conductance, to support VOC production, the plant seemed to invest resources in primary metabolism, rather than in the secondary, thus resulting in a lower emission of volatile compounds.

Scarce information is present about the co-occurrence of multiple stress factors on plant volatile emission. Their effect are sometimes additive, while in other case one stress has priority. In our case, the responses of plants analyzed seem to indicate an intermediate effect of the two applied stresses on the VOC emission rate, thus confirming the difficulty in extrapolating the response of plants to multiple stress combinations from responses to individual stress factors, given their unpredictability (Holopainen and Gershenzon, 2010).

Most of the identified compounds appear to have a protective role for plant cells and tissue and there are many evidences that they might play a role in relieving stress, minimizing damage caused by a disrupted growth environment (Turtola et al., 2003).

As Zn and PEG in combination did not cause additive adverse effects in treated plants, this might explain the detected emission rate similar to that of untreated plants.

Only further investigation about multiple stress occurrence will allow us to understand the real ability of plants to respond to the surrounding environmental conditions.

CHAPTER 4: EXPERIMENTAL

"Acclimatation to Zn stress in Tetradenia riparia following prolonged exposure"

ABSTRACT

The environment is a dynamic reality and living organisms are often exposed to variable environmental parameters. Plants, being sessile organisms, must be able to respond effectively to growing conditions unfavorable that may occur several times.

The aim of this study was to evaluate if in plants obtained by cuttings from plants that were previously treated with Zn, there was a greater capacity to respond to the recurrence of the same conditions, i.e. Zn excess.

Plants were grown hydroponically for ten month with a concentration of 500 µM $ZnSO_4$ applied in the growth medium. Subsequently they were placed in a Zn excess-free media for two weeks to observe the ability of recovery once stress receded.

Then, cuttings were obtained from these plants and cultivated for 4 weeks with nutrient solution. These seedlings were then treated again with the same Zn concentration used previously.

Physiological parameters, such as net photosynthetic rate, stomatal conductance and electron transport rate were monitored and plants growth, in terms of the length of shoot, was recorded.

Independently from priming, Zn caused the same negative effect on the growth development and the physiological parameters analyzed of the treated plants, both primed and not primed. Nevertheless, Zn can induce root primordia formation in *T. riparia* plants, thus allowing a considerable resumption of shoot elongation when plants are placed in culture media with normal Zn concentration.

Keywords: Zn stress; acclimation; *Tetradenia riparia*

4.1 INTRODUCTION

Living organisms, in the course of their life cycle, come into contact with external factors that may affect, in a positive and negative way, growth, development and reproductive success. Environmental factors may vary, in a periodic or occasional manner, and the deviation from the optimal growth conditions may be more or less prolonged in time.

An organism must be able to implement effective response mechanisms, which may relate to a behavior or their physiology (Krasensky and Jonak, 2012). Consequently, metabolic, physiological and behavioral processes can mutate, so allowing living organisms to adapt to environmental conditions in which they live. In this way it was possible to occupy a large variety of environments. Even plants have adapted to live in areas with unfavorable characteristics, through the development of metabolic pathways, morphologies and/or specialized life cycles. In particular, plants may respond to the stressful conditions by employing escape, tolerance or survival strategy. (Bazihizina et al., 2016).

A period of acclimatization, a process of adaptation to environmental change that is realized by changing the physiological balance, prior to the recurrence of stress, can be able to confer resistance to an organism, otherwise susceptible. In fact, it is known that prior exposure to stress can make a plant more resistant to future exposure (Conrath et al., 2006).

Primed plants show a better response to stress, through a faster or stronger activation of the defense systems involved, induced by the subsequent recurrence of stress (Conrath et al., 2006). In addition, the memory of previous exposures to factors such as drought, heat, cold, pathogens, allows plants to distinguish a transient event from one prolonged, such as a frost at night from the prolonged winter cold.

In fact, if a stress is demonstrated occasional, it may not be not convenient for the plant to keep a physiological state associated with the memory of that particular event, with the answers involved constantly active, considering the energy costs (Walter et al., 2013). On the other hand, some types of stress can occur transiently, but repeatedly; Consequently, it becomes useful for an organism to remember the past unfavorable event, so that it can respond more effectively.

It is also known that the memory of plants can be transmitted through subsequent generations, a process referred to as adaptive transgenerational plasticity (Herman et al., 2011).

A previous study showed that a plant callus, when exposed to cold, grew into a new plant that behaves as if it had been a hard winter, without ever really having experienced it. This suggested that priming could acts on the phenotype of individuals, leaving unchanged the genetic information (Hilker et al., 2016).

Not all kind of stress, however, seem to be involved and priming stress does not always occur (Crisp et al., 2016). The ability to form a memory of stress seems to be influenced by the type of stress, by the repetition of stress in question and by how much time elapses between an adverse event and the other.

Several studies are based on a previous mild exposure of short duration. For example, it was observed that tobacco plants, exposed to non-toxic concentrations of Zn for one week and subsequently treated with high concentrations of the metal, showed an increase of leaf area and dry mass compared to plants not previously acclimated (Bazihina et al., 2014). However, in nature, plants do not always initially experience adverse events of mild intensity, but it happens that stress occurs with a certain force.

In our study, it was chosen to apply a stress of high intensity (Zn excess) for a prolonged period to observe if, cuttings originated from plants grown under these conditions, were able to respond more effectively to the recurrence of the same stimulus. A concentration of 500 µM $ZnSO_4$ was chosen on the basis of a previous evaluation carried out on *Tetradenia riparia*.

4.2 MATERIALS AND METHODS

Plants cultivation

During the month of April, *T. riparia* plants were grown in a naturally lit glasshouse in aerated plastic basins with deionized water and half strength Hoagland's nutrient solution. Every day, pH was checked and, if necessary, adjusted to 5.8 with KOH or HCl. Solutions were changed once a week. After four weeks, $ZnSO_4$ was added to the nutrient solutions to half of the cultivated plants, to obtain the final Zn concentrations of

500µM. Treated and not treated plants were grown under these conditions in a semi-controlled climatic chamber and monitored for ten month.

Subsequently, plants were transferred in a culture medium without Zn excess for two weeks to observe the ability of recovery. Then, from these plants, 48 cuttings were obtained (24 from plants previously treated and 24 from plants previously untreated), and transferred in a naturally lit glasshouse. Cuttings were grown in deionized water with half strength Hoagland's nutrient solution for four weeks. After this period, $ZnSO_4$ was added to the nutrient solution of 24 plants, (12 derived from treated plants and 12 derived from untreated plants), to obtained again the final Zn concentrations of 500 µM. Thus, finally, were obtained: 12 cuttings treated (PznZn) and 12 cuttings untreated (PznC), both derived from previous treated mother plants; 12 cuttings treated (PcZn) and 12 cuttings non treated (PcC), both derived from previous untreated mother plants. New seedlings, treated and untreated, were grown for other four weeks in order to observe any differences in terms of development and physiological responses.

Shoot length was measured every 7 days to determine its extension during the treatment period.

Detection of leaf gas-exchange parameters

Net photosynthetic rate, stomatal conductance and electron transport rate were determined using the open gas-exchange system Li-6400 XT (Li-Cor, Lincoln, NE, USA) with an integrated fluorescence chamber head (Li-6400–40; Li-Cor) as in Bazihizina et al (2015). Leaf gas-exchange measurements were taken for all plants in each treatment on the youngest fully expanded leaves and they were conducted before the application of treatment (T0) and after 24h, 48h, 7days, 14days, 21days and 28days, with ambient relative humidity (40–50%), reference CO2 of 400 µmol mol–1, flow rate of 400 µmol s–1, chamber temperature of 25 °C and photosynthetically active radiation of 300 µmol m–2 s–1. Chlorophyll fluorescence was measured on the same leaves used for gas-exchange measurements.

Plant harvest

Cuttings were sampled at 4 weeks after applying the treatments, for the determination of shoot and root fresh and dry mass. The roots of plants were immersed in $Pb(NO_3)_2$ 10 mM for 10 min to desorb metals adhering to the root cell wall as in Barzanti et al (2011), and then intact plants were carefully washed with de-ionized water. Then, each plant was separated into roots, leaves and stems and their fresh mass was recorded. Tissue samples were then oven-dried at 60°C for 1 week to determine their dry mass.

Statistical analyses

The data related to the growth and the leaf gas exchange measurements were analyzed with statistical software GraphPad (for Windows 7th). One-way ANOVA was used to identify any significant differences between treatments. The means were separated using Tukey post-hoc, with $P \leq 0.05$.

4.3 RESULTS

Stress recovery and root primordia formation

Once placed in a culture medium containing only aerated nutrient solution, plants previously grown with Zn excess, showed a recovery of growth, in term of shoot elongation (Table 12).

	5 days	10 days	14 days
Shoot elongation rate (cm)	3,17 ± 0,28 **a**	6,63 ± 0,48 **b**	13,73 ± 0,76 **c**

Tab. 12: Shoot elongation rate in *T. riparia* plants previously grown with 500 µM $ZnSO_4$, then place in a Zn excess-free media. Values are mean ± SE (n=24). Letters into the row indicate differences among sampling times (at least P<0,05).

Zn excess elicited root primordia formation on *T. riparia* green stem, which, once free from Zn excess, developed into adventitious roots (Figure 7).

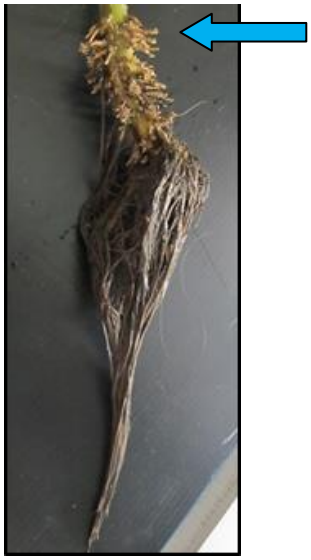


Fig. 7: Zn-induced root primordia developed into adventitious roots in *T. riparia,* once stress receded

Plant growth

Shoot elongation in *Tetradenia riparia* plants primed and not primed is presented in Table 13. $ZnSO_4$ 500 µM caused a halt to growth in a short time and primed and not-primed plants grew similarly with Zn treatments in hydroponics; In fact, PznZn and PcZn groups did not show significant differences from the beginning to the end of the treatment.

In the two groups of control plants, shoot elongation increased over time without any difference. The same trend was observed for dry biomass detected at the end of treatment in all groups (Table 14).

Treatments	Day 7 (cm)	Day 14 (cm)	Day 21 (cm)	Day 28 (cm)
PcC	10,4 ± 0,6 **a**	19,2 ± 1,1 **a**	25,6 ± 1,1 **a**	44,7 ± 1,7 **a**
PcZn	4,0 ± 0,5 **bc**	4,9 ± 0,6 **b**	5,2 ± 0,6 **b**	6,1 ± 0,8 **b**
PznC	5,1 ± 1,1 **b**	15,0 ± 2,1 **a**	23,7 ± 2,2 **a**	43,1 ± 4,31 **a**
PznZn	1,6 ± 0,6 **c**	2,3 ± 0,7 **b**	2,9 ± 0,9 **b**	3,9 ± 1,0 **b**

Tab. 13: Shoot elongation in *Tetradenia riparia* plants. Measurements were conducted at 7, 14, 21 and 28 days after treatment. Letters indicate difference among plant groups (at least P<0,05). Values are mean ± SE (n= 12).

Treatments	Leaves (g)	Stems (g)	Roots (g)
PcC	9,7 ± 0,9 **a**	8,3 ± 0,7 **a**	3,4 ± 0,3 **a**
PcZn	1,03 ± 0,1 **b**	1,5 ± 0,1 **b**	0,9 ± 0,1 **b**
PznC	8,8 ± 1,6 **a**	7,9 ± 1,6 **a**	3,2 ± 0,7 **a**
PznZn	0,6 ± 0,1 **b**	0,9 ± 0,1 **b**	0,1 ± 0,08 **b**

Tab. 14: Dry mass in *Tetradenia riparia* (control plants: PcC; PznC and treated plants: PcZn; PznZn) measured at the end of the experiment. Values are mean ± SE (n= 12). Different letters show significant differences among plant groups (ate least P<0,05).

Photosynthetic efficiency and gas exchange

A concentration of 500 µM $ZnSO_4$ caused an early decrease in net photosynthetic rate, stomatal conductance and electron transport rate both in primed and in not primed plants (Table 15, 16, 17).

Treatments	0h (µmol m-2 s -1)	24h (µmol m-2 s -1)	48h (µmol m-2 s -1)	7days (µmol m-2 s -1)	14days (µmol m-2 s -1)	21days (µmol m-2 s -1)	28days (µmol m-2 s -1)
PcC	9,4 ± 0,3 **a**	9,9 ± 0,4 **a**	10,2 ± 0,3 **a**	11,9 ± 0,3 **a**	12,1 ± 0,4 **a**	11,0 ± 0,4 **a**	10,3 ± 0,4 **a**
PznC	8,6 ± 0,4 **a**	9,9 ± 0,3 **a**	9,8 ± 0,5 **a**	11,8 ± 0,3 **a**	11,8 ± 0,4 **a**	10,1 ± 0,6 **a**	9,4 ± 0,6 **a**
PcZn	8,3 ± 0,6 **a**	6,9 ± 0,4 **b**	6,6 ± 0,4 **b**	4,9 ± 1,5 **b**	3,7 ± 1,4 **b**	3,3 ± 0,9 **b**	2,2 ± 0,8 **b**
PznZn	8,4 ± 0,4 **a**	7,6 ± 0,5 **b**	4,1 ± 0,6 **c**	1,5 ± 0,5 **c**	1,4 ± 0,5 **b**	1,6 ± 0,4 **b**	1,1 ± 0,2 **b**

Tab. 15: Net photosynthetic rate in control and treated *T. riparia* plants detected during the experiment at time 0 (before treatment), and at 24h, 48h, 7 days, 14 days, 21 days, and 28 days after the beginning of the treatments. Values are mean ± SE (n=12). Different letters into the row indicate significant differences between control plants and treated plants (at least P < 0,05).

Treatments	0h (µmol m-2 s -1)	24h (µmol m-2 s -1)	48h (µmol m-2 s -1)	7days (µmol m-2 s -1)	14days (µmol m-2 s -1)	21days (µmol m-2 s -1)	28days (µmol m-2 s -1)
PcC	0,18 ± 0,01 **a**	0,19 ± 0,01 **a**	0,18 ± 0,02 **a**	0,20 ± 0,01 **a**	0,23 ± 0,01 **a**	0,28 ± 0,01 **a**	0,28 ± 0,02 **a**
PznC	0,19 ± 0,01 **a**	0,20 ± 0,01 **a**	0,19 ± 0,01 **a**	0,23 ± 0,01 **b**	0,25 ± 0,01 **a**	0,28 ± 0,02 **a**	0,28 ± 0,03 **a**
PcZn	0,17 ± 0,01 **a**	0,09 ± 0,01 **b**	0,09 ± 0,01 **b**	0,019 ± 0,002 **c**	0,012 ± 0,002 **b**	0,014 ± 0,002 **b**	0,005 ± 0,001 **b**
PznZn	0,18 ± 0,01 **a**	0,11 ± 0,02 **b**	0,054 ± 0,008 **b**	0,02 ± 0,01 **c**	0,019 ± 0,005 **b**	0,018 ± 0,003 **b**	0,03 ± 0,01 **b**

Tab. 16: Stomatal conductance detected in control and treated plants of *T. riparia*. Values are mean ± SE (n=12). Different letters into the row indicate significant differences between control, and treated plants (at least P < 0,05).

Treatments	0h (µmol m-2 s -1)	24h (µmol m-2 s -1)	48h (µmol m-2 s -1)	7days (µmol m-2 s -1)	14days (µmol m-2 s -1)	21days (µmol m-2 s -1)	28days (µmol m-2 s -1)
PcC	82,8 ± 3,4 **a**	78,3 ± 2,4 **a**	72,9 ± 2,6 **a**	83,9 ± 2,8 **a**	85,9 ± 2,2 **a**	74,0 ± 2,9 **a**	82,1 ± 3,4 **a**
PznC	81,6± 3,4 **a**	77,6 ± 2,9 **a**	74,2 ± 1,9 **a**	88,6 ± 2,5 **a**	92,5 ± 2,9 **a**	76,5 ± 4,4 **a**	82,4 ± 3,7 **a**
PcZn	77,8 ± 2,4 **a**	59,8 ± 3,2 **b**	54,8 ± 2,8 **b**	33,7 ± 4,9 **b**	20,4 ± 3,0 **b**	28,6 ± 1,8 **b**	23,4 ± 2,9 **b**
PznZn	85,3 ± 5,6 **a**	75,2 ± 3,1 **a**	50,0 ± 4,8 **b**	26,1 ± 4,7 **b**	28,0 ± 6,0 **b**	29,7 ± 4,6 **b**	22,9 ± 2,2 **b**

Tab. 17: Electron transport rate in control and treated plants of *T. riparia*. Values are mean ± SE (n=12). Different letters into the row indicate significant differences between control and treated plants (at least P < 0,05).

4.4 DISCUSSION

The present study investigated some physiological responses to Zn stress in *Tetradenia riparia* plants primed and non-primed, through previous metal exposure, under the hypothesis that acclimation could decrease the symptoms generally associated with Zn toxicity, increasing shoot and root growth and photosynthetic efficiency in primed individuals compared to non-primed ones.

T. riparia displayed a remarkable survival capacity after stress caused by Zn excess. In fact, plants grown with 500 µM $ZnSO_4$ for ten months, after the stress removal, showed a visible recovery of growth, evaluated through shoot elongation. It is conceivable that, during the period of prolonged stress, plant was still able to keep the key cellular functions intact, which allowed a rapid recovery following stress removal (Lawlor, 2013). This form of stress resistance was previously indicated as “quiescence”, through it plants seem to be able to improve survival rate and generation of new

tissues after the metal stress has receded (Bazihizina et al., 2016). Several examples have been reported in particular for plants exposed for a long time to drought, flooding and salinity (Granot et al., 2009; Bailey-Serres et al., 2012; Julkowska et al., 2015). This survival strategy may be linked to the Zn-induced root primordia formation on stem tissues, developed into adventitious root under Zn normal conditions. Actually, the formation of adventitious roots can help the plant to take up nutrients and water ensuring plant survival (Sauter, 2013).

In advance, a similar result was found by Bazihina et al. (2016), in which study *T. riparia* plants treated with Cd were able to recover once the stress receded, thanks mainly to the Cd-induced root primordia formation on green stems, then developed into adventitious roots in a Cd-free media. These outcomes appeared to confirm previous findings. In fact, it is known that stressful environment conditions can induce root adventitious formation from stem tissue, which support the main root system when damaged by the stress (Bazihizina et al., 2016). In particular, it was suggested that heavy metal excess can affect ethylene biosynthesis and production, which is involved in the regulation of adventitious root growth (Steffens and Rasmussen, 2016). Moreover, the influence of the metals on the adventitious root system seems to be related to the exposure time and the age of the plant (Keunen et al., 2016).

Zn treated plants, primed and non-primed, showed similar response capacity towards the applied stress. Shoot elongation both kind of plants was prematurely inhibited by Zn excess according with previous studies (Peng et al., 2015), and no improvement was detected in primed plants compared with non- primed plants. Plant productivity was remarkably limited by an excessive application of Zn to the root zone, with a similar reduction of leaf, stem and root dry weight at the end of the experiment.

Zinc toxicity caused photosynthetic imbalance and gas-exchange impairment. Both in primed and non-primed plants, a significant decrease in net photosynthetic rate was observed compared to control plants from the first 24h of treatment. This trend persisted throughout all the duration of the experiment, in disagreement with our working hypothesis. In addition, this parameter appeared to be more negatively influenced in primed plants. In fact, during the first week of the treatment, net photosynthetic rate showed a greater decrease in primed plants compared to non-primed plants.

In primed plants, deriving from plants previously grown with Zn excess, Zn concentration in shoot might be greater than that of not primed plants and this is could

be the main cause of the detected damage, since Zn phytotoxicity occurs in a dose-dependent manner (Reichman, 2002).
Stomatal conductance appeared decreased in all treated plants. An early stomata closure commonly occurs in plants under metal stress, as previously observed in *Beta vulgaris* (Sagardoy et al., 2010) and tea (Li et al., 2011) regarding Zinc. Such metal, similarly influenced electron transport rate both in primed plants and non-primed plants. The inhibition of ETR by high Zn concentration was previously observed in several plant species and seem to be linked to the production of toxic oxygen species induced by heavy metals in general (Tripathy and Mohanty, 1980, Kappus, 1985).

For the considered physiological parameters and for the experimental conditions used, Zn priming was not able to ameliorate plant responses to a following Zn stress, suggesting that a previous exposure was not able to reinforce the plant defenses and/or reduce the time required for their activation. However, we must consider that this result cannot be generalized, because it may depend on an inappropriate duration of the priming or on a need of multiple priming events. Indeed, for the memory formation, can be necessary more similar events, or stress has to be repeated within a certain time to trigger a memory. This could serve to plants to really understand the nature of stress (Hilker et al., 2016).
In fact, in some cases, for plants it may be advantageous to learn to forget (Crisp et al., 2016). Thus, plants appeared be able to discriminate between the different stress conditions and to choose which strategy to adopt.
Moreover, it is know that memory can be maintained for a specific time. For instance, Baldwin and Schmelz (1996) observed that *Nicotiana sylvestris* plants, previously primed with methyl jasmonate, were able to store the information for at least 6 days, whereas Gagliano et al. (2014) showed that in *Mimosa pudica* the ability to remember the previous stimuli was kept up to 40 days. They concluded that the time between one exposure and another might affect the ability of the plant to remember a past event.
In addition, some exposure factors can also increase the sensitivity of plants, the opposite of the increased resistance associated with priming (Bruce et al., 2007).
It would be of great interest to be able to define the ways in which plants can store information about stress exposure, as both abiotic and biotic stress can limit plants productivity. By recreating such mechanisms, it would be possible to improve the

response capacity of plants towards such stresses and we could cultivate areas with sub-optimal conditions of growth.

CHAPTER 5: GENERAL DISCUSSION

Plants are continuously exposed to variable environmental conditions and not always favorable; these conditions can affect their growth and development. Therefore, plants must be able to perceive the chemical and physical stimuli of the surrounding environment and react effectively to the environmental stress (Levitt, 1980).

The main objective of this thesis was investigating the effect of abiotic stresses, such as Zn excess and water deficit, in *Tetradenia riparia* with a special focus on the emission of volatile organic compounds under stressful conditions. In addition, the effect of Zn stress was analyzed also on *T. riparia* cuttings originated from plants previously acclimated with Zn excess.

T. riparia was chosen as the study model because of its well-known ability to produce volatile compounds that are the major constituents of the essential oil extracted from its leaves (Gazim et al., 2010). This oil has important antimicrobial and acaricidal properties, thus explaining the medicinal uses of *T. riparia* plants.
Moreover, as *T. riparia* plants typically grow in African riparian zones, with variable availability of water, they are probably exposed to periodic water deficit conditions. Since the soils of these areas can be frequently contaminated by heavy metals, such as Zn (Yabe et al., 2010), it represent also an interesting model to study the interaction between Zn and water stress.

Zn effect on *Tetradenia riparia*

The effect of increasing Zn concentrations on *Tetradenia riparia* plants was investigated in Chapter 2.

Zn affected significantly the physiology of *T. riparia*, limiting growth and productivity proportionally to the metal concentration applied to the culture medium and for the entire duration of the treatment, with intensity always proportional to the applied metal concentration.

Data from literature suggest that exposure to certain Zn concentration leads to different effects in relation to the tested species. Sagardoy et al., (2009), and more recently de Jesus et al., (2016), demonstrated that an application of 100 and 150 µM $ZnSO_4$ led to a negative effect on the development of *Beta vulgaris* and *Marthiantus leucocephalus* respectively. Instead, in *Pisum sativum* (Doncheva et al., 2001), growth became inhibited after 1000 µM $ZnSO_4$, as also observed for *Populus nigra* (Di Baccio et al., 2003). As previously suggested by Tsonev and Lidon (2012), threshold toxicity appear to vary dependently on plant species and exposure time and *T. riparia* proved to be in the average range of Zn sensitivity, at least for the experimental conditions used.

The reported Zn-induced reduction in length in *T. riparia* might be due to the reduction in meristematic cell number and enzyme activities and to interference with the absorption of some other nutritional elements, as suggested by previous researches, but the action mechanisms are still unclear (Glińska et al., 2016). Moreover, in Zn-treated plants, the decrease in growth seemed to be correlated with the inhibition of the net photosynthetic rate, PS II efficiency, electron transport rate and stomatal conductance.

The drastic reduction in the net photosynthesis rate and the almost identical reduction in the stomatal conductance suggested that the main factor limiting photosynthesis in Zn-treated plants implicates stomatal limitations, as reported in Vassilev et al. (2011) for *Phaseolus vulgaris.*

Zinc was accumulated in the plant organs in amounts proportional to the metal concentration present in the growth medium. The largest metal concentration was accumulated in the roots, probably as a strategy to limit the Zn amount in photosynthetic tissues not to completely damage them. Nevertheless, Zn moved to the shoot was enough to cause a significant decrease of chlorophyll a and carotenoids content in leaves, probably explaining part of the photosynthetic dysfunctions.

In the recent years, the use of herbal drugs is increased, along with the need for the safety and quality of the plants used (Ekor, 2013). Thus, it was interesting to evaluate if *T. riparia*, as medicinal plant, could be suitable for the cultivation in contaminated areas, by comparing the amount of metal accumulated in the leaves with the known limits for the maximum permissible concentration of the metal in plants used in pharmaceutical field.

Unfortunately, Zn concentration detected in leaves exceeded, in all treated plants, the recommended limit of 50 mg/Kg (Shah et al., 2013). Therefore, *Tetradenia riparia* is not suitable for pharmaceutical uses when cultivated in areas contaminated by Zn, given that contamination levels can frequently reach elevated values, around 500 mg/kg in many African soils (Yabe et al., 2010).

Previously, Bazihizina et al. (2016), investigated about the potential and safe use of *T. riparia* in heavy metal contaminated soils cultivating the plants with different cadmium concentration in the nutrient solution. Similar results were obtained, concluding that this species is not cultivable in substrates contaminated by the metal. Nevertheless, other studies on such topic have led to different and encouraging results, depending on the plant species in question. An example was reported by Hajar et al., (2014) for *Stevia rebaudiana*, used as food product or therapeutic agent in traditional medicine. Therefore, further studies are needed to integrate these findings, as the bioavailability of the metals can change in the different soils, as well as the plant responses.

Combined effect of Zn excess and drought stress in *Tetradenia riparia*

In chapter 3 we aimed to investigate the combined effect of Zn stress and water deficit on the growth and some physiological parameters in *T. riparia*.

During development, plants encounter a large variety of environmental stress (Ji et al., 2014) that often occur concurrently, but this aspect is rarely addressed and the effects of the different stresses are usually investigated separately.

In order to simulate a possible water deficit situation and heavy metal stress, PEG 6000 35% and 500 µM $ZnSO_4$, alone or in combination, were added to the hydroponic medium. Then, we investigated whether the combined action of the two stresses could lead to an additive damaging effect, creating even more disadvantageous conditions than those of plants exposed to a single stress.

Zn excess inhibited plant development, limiting photosynthetic efficiency and causing gas exchange imbalance. In fact, net photosynthetic rate and stomatal conductance decreased for all the duration of the monitoring. As a result, growth appeared soon inhibited, with a produced biomass significantly lower than untreated plants at the end of the experiment. In several species of higher plants treated with equal Zn concentrations similar symptoms were found, including stunted plant growth (Sagardoy et al., 2009), decreased stomatal conductance and photosynthesis (Sagardoy et al., 2010) and changes in root growth and morphology (Marschner, 1995), although the toxicity range varies according to the species.

Similarly, plants subjected to water stress, showed a reduced shoot growth, even if at a lower extent in respect to Zn-treated plants, at least for the experimental conditions used. At the end of the treatment, an increase in the root biomass was recorded, probably indicating an adaptive mechanism that, through allocating more resources to root, could allow an increased water uptake (Kozlowski and Pallardy 2002). This phenomenon has been previously observed in plants exposed to moderate water deficit conditions (Xu et al., 2010), but there are also some studies that reported the opposite. For example, in some *Populus* species, root dry weight decreased under mild and severe water stress (Jaleel et al., 2009). The reactions of plants to water stress probably differ depending upon intensity and duration of stress, as well as upon plant species and stage of growth (Chaves et al., 2002).

During the first hours of treatment, in PEG-treated plants, net photosynthetic rate remained unaltered and comparable with the control plants, while the most sensitive

parameter was stomatal conductance, significantly declined after 24 hours of treatment. Actually, in response to water deficit, leaves quickly close their stomata to limit the loss of water through transpiration (Daszkowska-Golec and Szarejko, 2013). Nonetheless, stomatal conductance returned to levels comparable to those of control plants already after 48h, suggesting that plants were adapting to the unfavorable conditions.

In plants exposed to both stresses, a slight improvement of shoot elongation was detected in comparison to plants treated with only Zn. An improvement of dry weight was recorded at the end of the experiment as well, though statistically not significant. Moreover, stomatal conductance increased significantly compared to Zn-treated plants, after 10 days. Unexpectedly, the contemporary presence of Zn and PEG did not cause more severe damage compared with plants treated with Zn alone, conversely Zn stress seemed to be alleviated.

In plants grown with PEG+Zn, Zn concentration in leaves, at the end of experiment, was lower than in plants treated with Zn alone, probably explaining the contained injures to the photosynthetic apparatus.

Previous studies investigating the combined effect of drought and salinity showed a detrimental synergistic effect observed in potato (Levy et al. 2013), wheat (Yousfi et al. 2012) and barley (Yousfi et al. 2010). Similar results were showed in de Silva et al., (2012) where heavy metals and drought stress reduced red maple growth in additive manner. Nevertheless, Rivero et al., (2014) reported that *S. lycopersicum*, exposed to combined salinity and heat stress, performs better than when subjected to these stresses separately.

As observed for single stress, the response of plants to a combination of two or more different abiotic stresses can depend on plant species, stress intensity and exposure period. This work provides further evidence on how the combination of two stresses can paradoxically not be deleterious, confirming that the responses of plants to different stresses occurring simultaneously cannot always be deduced from the response of plants to each individual stress.

Volatile organic compounds emitted under stressful conditions

Plants produce and emit a large spectrum of volatile organic compounds (Loreto and Schnitzler, 2010), that have different properties and functions (Dudareva et al., 2006). Their production and the emission rate and patterns can be influenced by different abiotic and biotic factors (Loreto and Schnitzler, 2010).

In our studies, VOC emission profiles in *Tetradenia riparia* was investigated in relation to Zn and water stress, separately and in combination.

Compounds belonging to different categories of molecules were found, including alcohols, aldehydes, acetates, ketones, terpenes, monoterpenes and isoprene.

Each treatment affected the emission intensity of the detected compounds and some of these were exclusively emitted from plants exposed to certain treatments. Particularly, Zn enhanced VOC emission rate in *T. riparia* proportionally to the concentration of metal added to the nutrient solution. Interestingly, how *T. riparia* leaves maintained VOC biosynthesis and emission under Zn stress conditions, despite impaired photosynthetic CO_2 fixation (Teuber et al., 2008), would deserve to be further investigated. Comparable results were obtained in other plants species, such as Grey poplar (Teuber et al., 2008), and *Eucalyptus globulus* (Loreto and Delfine 2000), in which isoprene emission did not change, although salt stress significantly affected carbon assimilation of these plants. It was deduced that maybe, the processes leading to the emission of isoprene were much more resistant to salinity than photosynthesis and a secondary isoprene carbon source, probably independent of photosynthesis, was stimulated by salt stress.

Selmar and Kleinwächter, (2013) suggested a further explanation, according to which, following the decrease of consumption of reduction equivalents for CO_2 fixation through the Calvin cycle, metabolic processes are shifted towards biosynthetic activities that consume them. Thus the synthesis of reduced compounds, such as isoprenoids, phenols or alkaloids, is enhanced (Selmar and Kleinwächter, 2013).

Volatile organic compounds emission pattern appear to be positively influenced by Zn-excess, thus suggesting that they might have a role in stress defense.

A different result was obtained in plant exposed to water deficit. In fact, drought stress, simulated by PEG 6000, led to a decrease in the intensity of VOC emission in *T. riparia*, although not significant compared to untreated plants.

In previous studies, concordant and discordant data were reported. For example, in *Betula verrucosa* most of the compounds found remained at a very low level (Pag et al., 2013), while *Zea mays* plants released more volatiles when standing in dry soil than in wet soil (Gouinguenè and Turlings, 2007). These and other findings suggest that abiotic stresses have a different influence on VOC emission in plants depending on the species considered.

Regarding the effect of stress combination, in *T.riparia* PEG+Zn treatment led to a very similar VOC emission to that of control plants, suggesting a possible balancing between stomata closure and increased production of the secondary chemical compounds.

In general, our results confirm that abiotic stress factors can affect the secondary metabolic pathways (Selmar and Kleinwächter, 2013). Moreover, as some factors, such as Zn excess, seem to be able to stimulate an increase in volatile compounds emission, there can be the possibility to exploit changes in plant growth conditions for the optimization of the production of metabolites used as pharmacological components.

The collected data can set the stage to further experimentations to find the water regime able to reduce the metal concentration in leaves, below the legal threshold, since our results suggested the combination of the two stresses not to modify the composition of the volatile compounds. To compensate for any reductions in biomass produced, the density of cultivated plants could be increased.

Acclimation process

Plants are steadily exposed to many stress factors, because of their sessile nature, and they response to environmental conditions through modifications of their own development and physiology.

The experience of environmental stimuli can prepare (prime) plants to future stress, improving their ability to respond and making them more resistant to future exposure (Bruce et al., 2007). In addition, plants can altering the phenotypes expressed by their offspring (Herman and Sultan, 2011).

In chapter 4, the process of priming was investigate in *T. riparia*, testing the hypothesis that Zn-treated cuttings, originated from plants previously exposed to the same stress, would be more tolerant than Zn-treated cuttings originated from untreated plants, by retaining memory of stress.

Unlike what was expected, primed plants showed the same degree of tolerance of non-primed plants against the metal. Zn excess caused an alteration in metabolic pathways, such as net photosynthetic rate, stomatal conductance, electron transport rate and growth in both plant groups.

It can be possible that the hardening process has not been triggered with the conditions applied. In fact, a single past event of exposure might not be enough, so that phenotypic modifications associated to the memory of plants, occurred.

In addition, heritable modifications, such as histone modifications or chemical changes at the DNA (Walter et al., 2013), may not have occurred.

The question arises of which conditions make it advantageous to keep a memory and to be always prepared, as the maintenance of the primed state has a cost. This depends on internal state and age of organism, life strategy, specificity of the stimuli applied with respect to the triggering stress and predictability of stress (Hilker et al., 2016).

After Zn stress, a period of recovery followed. During it, plants must strike a balance between investing resources in continued priming versus resetting. Probably, in this case, resetting was the strategy used by plants to maximize growth under restored favorable conditions.

It is worth pursuing further research, considering repeated periods of stress exposure, since primed plants could better tolerate adverse environmental conditions and could be used in agriculture or for pharmaceutical crops.

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