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PhD Thesis

EVALUATION OF PROLINE INCREASE IN PLANT PARTS STIMULATED BY CADMIUM AND DETECTED BY HONEY BEE AS ENVIRONMENTAL INDICATOR

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Ai miei genitori
non c'è parola che racchiuda
l'amore che provo per voi.
A mia sorella
pilastro della mia vita,
e fondamenta dei miei giorni.
Al Prof. Palumbo
Non c'è frase che renda
la stima e l'affetto che nutro nei suoi confronti
A Floriana compagna fedele
e alla mia dolce ***Titti.***
Questa tesi è per voi e a voi dedico la gioia, l'impegno e i pianti che si celano dietro questo traguardo, scusandomi se qualche volta vi ho deluso.
Al mio piccolo ***Daniel***, il mio amore, sapendo che sarò sempre
un passo indietro pronta ad aiutarti.
A Valerio R.
Alle tue mani, che spero, stringeranno sempre le mie.
Con gratitudine sconfinata.

INTRODUCTION

The accumulation in soils of most heavy metals such as Fe, Mn, Cu, Ni, Co, Cd, Zn, Pb is attributable to the long period through industrial waste and wastewater disposal. Although some of these metals are essential micronutrients responsible for many regular processes in plants, their excess, however, can have detrimental effects and can directly influence the plant growth, metabolism, physiology and senescence (Ghor *et al.,* 2019)**.** The cadmium (Cd) element, in particular, is usually found in pesticides sprayed to defend plants against pathogens in field; the critical limit of Cd is 5.33 mg/kg in light textured soils , 6.33 mg/kg in medium textured, and 9.29 mg/kg in heavy textured (Sukarjo *et al.,* 2019). Deficiency of nutrient elements in plants exposed to Cd stress showed disturbances in their macro- and micronutrients homeostasis, which indirectly affects the processes where these compounds are involved. Cd stress decreases the absorption of essential nutrient elements such as Ca, Mg, Zn, and Fe (Ros *et al.,* 1992, Ouariti *et al.,* 1997). As Cd is a non-essential element for plants; it can be transported via other metal transporters such as Ca, Mg, Zn, and Fe. Therefore, as Cd competes with these elements, in presence of a Cd excess the absorption of these nutrients is reduced, causing deficiency of essential elements. In addition, the inhibition of Fe (III) reductase induced by Cd results in Fe (II) deficiency (Chang *et al.,* 2003), seriously affecting the chlorophyll biosynthesis (Yasemin *et al.,* 2008) and Photosynthetic Electron Transport (Kalaji *et al.,* 2017)**.** In particular, the cytochrome b6f complex is a dimer with a low- and high-potential heme group, a 19 kDa Rieske iron-sulfur protein containing a [2Fe-2S] cluster. Iron is also a structural component of the metallo-enzymes that are involved in nitrate uptake, nitrite and nitrate reductase (Crichton, 2012). The activity of nitrate reductase is low in iron-deficient phytoplankton and experimental evidences shows a reduced activity of nitrate reductase also under Cd stress (Singh *et al.,* 2017). A direct or indirect conditioning of the stress from Cd on the processes of assimilation of carbon and nitrogen, profoundly alters the basic metabolism, compromising its vitality and places the plant in a different interactive context with the environment. The reproductive (gametophytic) phase in flowering plants is often highly sensitive to stress factors, probably to guarantee the progeny and conservation of the species in adverse environmental conditions.

In this context, the entomophilic pollination process can also be conditioned, on the basis of a biochemical communication between plant and insect, in a phase where metabolic indices of stress and products of secondary metabolism can play an important role in the transmission of the signal between plant and pollinator.

The most classic and functional pollinator of the Mediterranean scrub is the honeybee, *Apis mellifera* L. (Hymenoptera, Apidae). Insects need energy and protein that come from food; deep in a flower's center is a nectary which produces nectar, a sugary solution insects like that provides carbohydrates. Plant pollen is rich in protein, and free amino acids which can be a strong attraction for insects and in particular for honeybees. In addition to the floral system, also the leaves, through the emission of volatile organic compounds (VOC*s*), can play an important role in the process of attraction or repulsion of insects. The complexity of plant–insect chemical communication via VOC*s*, based on the sophisticated molecular perception mechanisms of insects, which can respond to one or more VOC*s* and thereby influence insect behavior in a manner that has yet to be fully elucidated. Development and survival of honeybee colonies are highly dependent on the quality of the environment and the availability of floral resources from which they obtain nutrients (especially pollen). The main chemical constituents of pollen are: proteins, carbohydrates, mineral content, callose, organic acids, amino acids, pigments, vitamins, hormones and steroids, whose percentages can vary greatly depending on plant species and environmental conditions (Herrera *et al.,* 2006) during pollen maturation and their subsequent release. Amino acids concentrations are present in the nectar of angiosperms lower than those of sugars; some authors showed as plants pollinated by insects contain a greater concentration of amino acids than those who use birds as pollinators (Gardener & Gillman, 2002). Unlike the other amino acids present in nectars, proline has a peculiar characteristic: it is able to stimulate the labellar salt receptor cells of some species of insects, which therefore seem to be able to recognize their taste (Wacht *et al.,* 2000). High amounts of proline are found in many types of nectar. In tobacco plants it can accumulate to levels of 45–60% of total amino acids (Carter *et al.,* 2006*)*. Hence, among the amino acids that are commonly found in the nectar, the proline has a unique feature: insects are able to recognize its taste (Carter *et al.,* 2006). Several studies suggest that a wide range of insects that feed on nectar preferentially use proline during the initial phases of flight (Micheu *et al.,* 2000).

An energetic substrate, readily available and suitable for intense flight phases can be an advantage for bees during long distance foraging. Proline is the most abundant amino acid in the hemolymph of many insects, including bees (Crailsheim & Leonhard, 1997), able to use proline as an energy substrate for flight muscles; even *Apis mellifera carnica* (Pollmann) is able to burn proline (Scaraffia & Well, 2003). The presence of proline in some nectars and the ability of some insects to perceive it suggest an evolutionary relationship that aims to increase the pollination of plants that produce nectar rich in proline by insects that prefer the taste of these nectars (Carter *et al.,* 2006).

Into the insects is called chemoreception the method of communication that refers to both the smell and the taste. Generally the olfactory stimuli are originated from volatile molecules, while the gustatory ones originate from water-soluble molecules that act directly by contact. In the insects there are two classes of polypeptides identified in the lymph of chemiosensilla: Obp (odorant binding protein) and Csp (chemosensory protein). These soluble proteins are able to reversely tie the smells and pheromones; in fact it is known that all chemical stimuli must interact with a large number of these proteins that can always modulate and modify the chemical message of origin (Pelosi *et al.,* 2006).

CHAPTER I - HEAVY METALS AND ACCUMULATION MECHANISMS

1.1 *Essential metals and contamination of heavy metals and metalloids in soil and plants*

To survive plants require some essential elements; many of them are necessary at low concentrations, some at higher concentrations, respectively defined as micronutrients and macronutrients. Elements such as C, H, O, N, P and K are considered macronutrient. These are the main elements as they constitute the biomass, in addition to becoming part of the protein and DNA structure and to carry out different metabolic functions. The micronutrients are instead B, Cl, Co, Cu, Fe, Mo, Mn, Na, Si, V, Zn and they perform important functions within the cellular metabolism being essential constituents of many enzymes. These are fundamental for the cell but they are toxic for the same if present at superior concentrations of the necessary (Barbieri & Bestetti, 2008; Clemens, 2000). Other metals, the potentially toxic trace elements, better known as heavy metals, are instead non-essential metals that cause serious damage to plants even to very small doses. Bertrand's graphic (Figure 1), shows the cell's letality if subjected to a high concentration of an essential element (green curve); the gray curve, on the other hand, shows that a cell submitted to minimum concentrations of a toxic element shows immediately inhibition of growth (Barbieri & Bestetti, 2008).

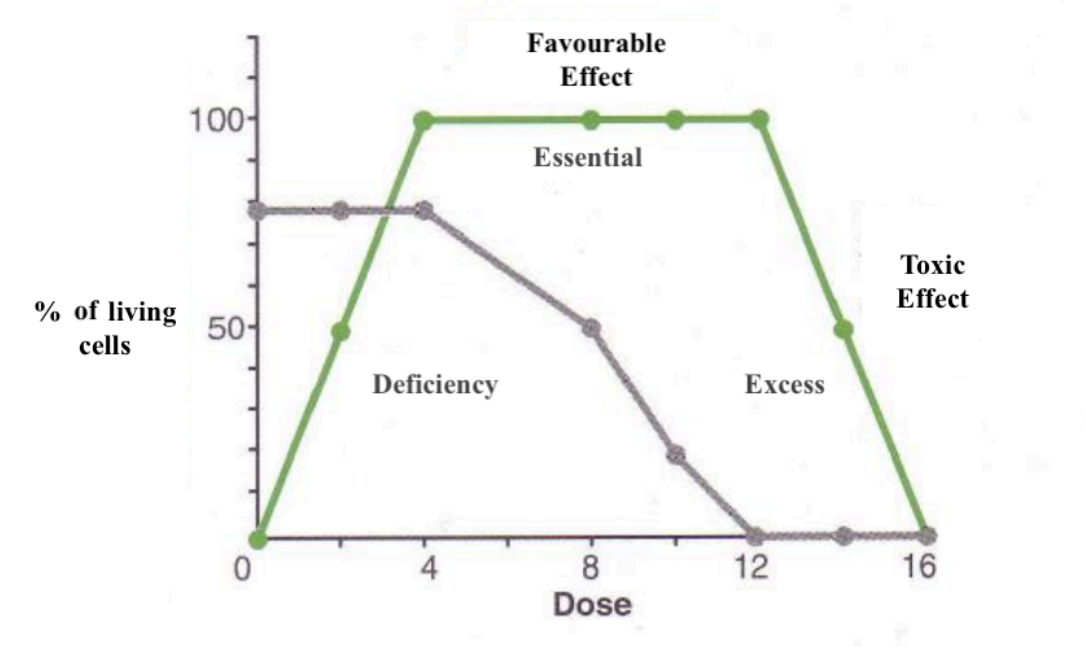


Fig. 1 - Physiological effects related to increasing concentrations of an essential (green line) or a toxic (gray line) element (by Barbers & Bestetti, 2008, modified).

Heavy metals are a group of elements with a density greater than 5.0 g/cm^3 with atomic weight greater than twenty, but in this group there are also elements with lower densities and non-metals (As, Mo, Se and B) jointed among them for some features:

- they generally behave like cations;
- their hydrate forms are poorly soluble, and have a good attitude to form complexes;
- they are very similar to sulphures with which they tend to react and change oxidation state in relation to pH and to Eh (Lorito & Vianello, 2010).

Some heavy metals are toxic to organisms over a certain threshold, others highlight toxicity already at very low concentrations (Bhargava *et al.,* 2012). A site is defined 'contaminated' when only one element exceeds the limit imposed by the law for the specific destination of that site (Barbers & Bestetti 2008). Contamination from trace elements is a serious problem due to the impacts that can have on human health and the ecosystem, since pollutants, potentially, could enter the biogeochemical cycles and then inside the trophic chain.

Once the pollutants deposited on the vegetation can be reached on the ground, they may suffer different processes such as adsorption, complexion and precipitation according to the chemical-physical characteristics of the soil (Aromolo *et al.,* 1999). The analysis of different vegetable species both terrestrial and aquatic has shown that they effectively bioaccumulate heavy metals, in pollution conditions. Many plants, but also bryophytes and algae, have evolved the ability to accumulate elements in trace at higher levels than those present in the soil or in water even compared to species that grow in the same area. The plants are divided into three groups for their ability to accumulate metals:

- accumulators, that accumulate metals mainly in their sprouts, by both low and high metal concentrations in the ground;
- indicators, where the metal concentration in plant tissues corresponds to the concentration in the environment;
- excluders, which maintain low metal concentrations in their sprouts despite the outer concentration of metals is high.

1.2 *Heavy metals storage mechanism in plants*

The accumulation mechanism of heavy metals in plants can be divided into three essential phases:

- radical absorption;
- transport of metals inside the plant;

- detoxing mechanisms (Salt *et al.,* 1995a).

Most metals in the soil are linked to its constituents, so that plants can accumulate them, but it is necessary that these elements are made soluble. The mobilization of soil-related metals can take place in different ways: many plants release, through the roots specific molecules (phytosiderophore) that have the ability to chelate and solubilize heavy metals (an example can be the avenic acid of the Graminaceae). Other plants are able to reduce and consequently mobilize metal ions, thanks to the intervention of specific metallic reducent linked to the plasma membrane of radical cells. Another mode of solubilization of heavy metals may be due to the acidification by the soil thanks to the release of protons from the roots: a low pH releases the metal ions in solution. These ions enter the roots, once solubilized, through or the apoplasty street (extra-mobile) or by simplasty (intracellular). Many of them enter plant cells thanks to an active transport mediated by carriers or specific channels. From the roots the metal ions can pass to the bud through xylem jars and, subsequently, be distributed to the rest of plant by means of the floema. To get to the xylem, these ions must cross the endodermis that thanks to the Caspary band allows the simplastic transport, given that the apoplasty pathway is blocked. Bioaccumulator plants must withstand toxic effects, and this is realized or limiting the absorption of metals at a cellular level, detoxifying the metal entered, or developing a biochemical mechanism that is able to make them resistant. Many plants have specific enzymes for heavy metal resistance and among these, for example, the acid phosphatases of the cell wall. Once these elements enter the cell, they must be detoxified; this process can take place by chelation, for precipitation or compartmentalization. In the case of Zn, it can be chelated from organic acids and accumulate in the vacuole, or be precipitated in the form of Zn-phitated. The Cd is also accumulated in the vacuole where it is associated with phytochelatine. The moment the plant is in contact with heavy metals inside vacuoles are accumulated glutathione-based peptides and phytochelatines, which bind the metal. The glutathione himself, binding at numerous potentially toxic compounds (through the residue of cysteine; enzyme glutathione s-transferred, GST), contributes to remove them from the cytoplasm, transporting them into vacuole (Alpi *et al.,*, 2000). The vacuole therefore can also perform a defense function.

1.3 *Heavy metals and cellular metabolism*

The plant organisms exposed to high concentrations of heavy metals tend to accumulate them in their tissues (Salt *et al.,* 1995). When their intracellular concentration exceeds a threshold value, there is a decrease in growth and reproduction of the exposed organism (Cobbett, 2000; Hall, 2002).

The effects of a certain metal vary from species to species, for example Cu acts at the level of the tilacoidal membranes, altering the structure and, moreover, can replace the chlorophyll magnesium atom with a consequent slowdown in the speed of photosynthesis (Stiborova *et al.,* 1987; Angel *et al.,* 1994; Gupta & Singhal, 1995, 1996; Singh *et al.,* 1997; Vinit-Dunand *et al.,* 2002). The Pb is also able to damage the tilacoidal membranes, with serious repercussions on the photosynthetic process. A short-term response in plants seems to be the increase in the synthesis of thermal shock proteins (HSP) (Tseng *et al.,* 1993; Hall, 2002). These proteins seem to have the task of protecting cell membranes from peroxidation damage caused by metal. The toxicity of heavy metals is therefore attributable to oxidative stress (de Pinto *et al.,* 2003), that induces the synthesis of the antioxidant defense system represented by enzymes, such as the superoxide dismutase (SOD), the catalasis (CAT), the peroxidase ascorbate (APX), the monodehydroascorbate reductase (MDHAR) and dehydrossiascorbed reductase (DHAR) (Rice-Evans *et al.,* 1996). These defenses can also involve water-soluble antioxidants such as ascorbate (ASC), glutathione (GSH), phenolic compounds and water-soluble molecules, such as carotenoids and tocopherols (Foyer *et al.,* 1994; Hodges & Forney, 2000; Pastorini *et al.,* 2000). The chloroplasts contain two dismutase superoxide isoforms (SOD): CuZnSOD, containing zinc and copper, and FeSOD, which contains iron; the superoxide dismutase catalyzes the dismutation of O_2- in H_2O_2, while the peroxidase (APX) ascorbate reduces hydrogen peroxide (H_2O_2), toxic, in H_2O (Fridovich, 1997). The reduction of hydrogen peroxide is associated with the oxidation of the ascorbate that produces dehydrossiascorbate normally reduced by the reductase dehydrossiascorbate (DHAR) with production of oxidized glutathione, which is reduced by a reductase with NADPH consumption. The coupling of the reactions catalyzed by the peroxidase-ascorbate enzymes (APX), glutathione reductase (GR) and dehydrossiascorbate reductase (DHAR), in plant organisms is called Haliwell-Asada cycle (Figure 2) and its function is to detoxify the cell from hydrogen peroxide (H_2O_2) through NADPH consumption (Ajay *et al.,* 2001).

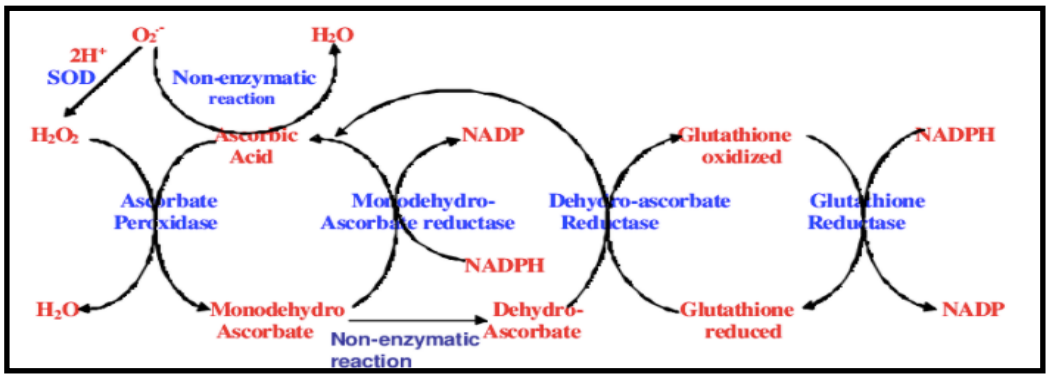


Fig. 2 - Haliwell-Asada cycle.

Therefore it seems likely to be the ascorbate and glutathione play a key role in dab the effects of oxidative stress in most eukaryotic systems (Noctor & Foyer, 1998; Smirnoff *et al.,* 2001). Oxidative stress damages the chloroplast, which is particularly sensitive to high concentrations of metal ions and reactive oxygen species (Foyer, 1996). The chloroplasts being made up of a system of membranes rich in polyunsaturated fatty acids, are potential peroxidation objectives (Halliwell & Gutteridge, 1999). Under normal conditions these molecules (ROS) manifest their toxic effect with extreme slowness, but this action is accelerated by the presence of xenobiotic molecules, such as heavy metals or from environmental factors such as light or exposure to UV rays. Another defense mechanism against the toxic effects of heavy metals is represented by the production of proline (PRO), a cyclical structure amino acid with osmoprotective functions (which will be discussed in the following chapters).

1.4 *The physiological systems of resistance and tolerance to metals in plants*

During the evolution, plants have developed mechanisms of tolerance and resistance to metals that are found in more and more elevated concentration inside the soils starting from the industrial revolution (Fusco *et al.,* 2005). Resistance and tolerance depend on the skill of the single plant to activate different molecular and physiological mechanisms against stress from heavy metals (Citterio *et al.,* 2003); in general we talk about homeostasis meaning the cell's ability to activate these defense systems at uptake level, accumulation and metal detoxification (Clemens, 2000).

The main mechanisms of homeostasis are: reduction of uptake by the roots through bonds with exudates (siderophores, organic acids, malate, citrate etc.) or with links on the outer part of the wall and cell membrane (peptides and carbohydrates), production of antioxidant enzymes in general and production of chelating compounds (metallothioneine and phytochelatine) for detoxification and compartmentalization of metals once entered the cell (Dalcorso *et al.,* 2010).

Figure 3A shows the chain of reactions caused by the presence of Cd inside the vegetable cell: the metal, once entered the cell induces the formation of reactive species of oxygen (ROS), calmodulin and calcium (which are 'messengers 'of external stimuli) and of some hormones, such as jasmonic acid (JA) and salicylic acid (AS). The presence of the ROS leads to the cascade phosphorylation of different kinases (MAPK) and these, with hormones and the Ca-calmodulin protection system converge into the core by adjusting the activity of some transcription factors (TF) (Dalcorso *et al.,* 2010).

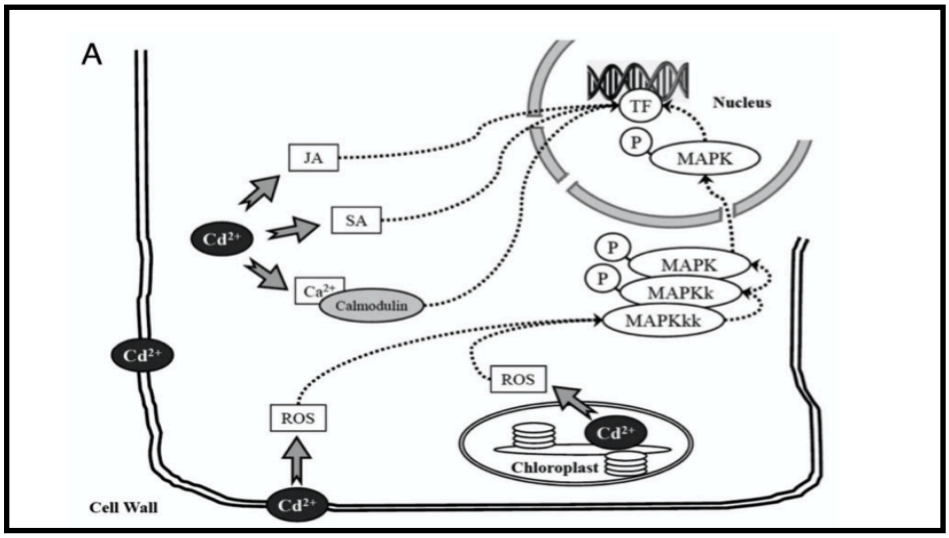


Fig. 3A - Reactions chain induced by the presence of cadmium in a vegetable cell (Dalcorso *et al.,* 2010).

Figure. 3B shows, therefore, the expression of genes responsible for the detoxification of Cd induced by the transcription factors through particular signal transduction routes. Specifically, the expression of the genes leads to the formation of many enzymes: membrane transport (mainly ABC protein), phytochelatine (PC) and metallothioneine (MT) (Fojta *et al.,* 2006; Dalcorso *et al.,* 2010). These molecules have the function of transporting the metal outside the cell or inside the vacuole, for its compartmentalization, and are the mechanisms most used by plants for the detoxification of Cd and arsenic (Clemens, 2006; Dalcorso *et al.,* 2008).

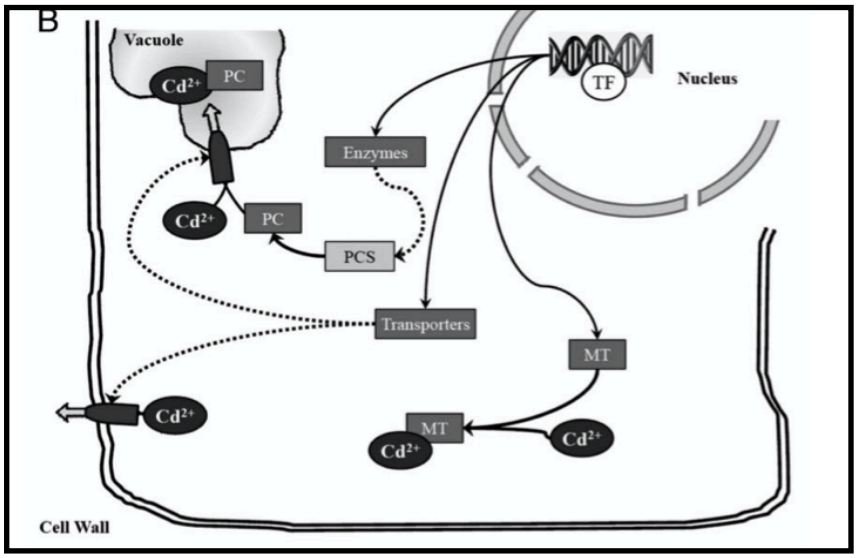


Fig. 3B - Reactions chain induced by the presence of cadmium in a vegetable cell (Dalcorso *et al.,* 2010).

The phytochelatins are peptides rich in cysteine, glutamic acid, glycine or alanine, and can include from 2 to 11 glutamilcysteinic units involved in the detoxification of heavy metals in plants, so they are synthesized in response to exposure to these substances (Trombetta *et al.,* 2008): glutathione (or another PM phytochelatin) is the starting block for the production of these molecules and is catalyzed by the enzyme phytochelatin-synthetase or PC-synthetase (PCS). The general formula of the phytochelatine structure (Figure. 4) is (ɣ-Glu-Cys)n -Gly, where Glu is glutamic acid, Cys is the cysteine, Gly is Glycine end n can vary from 2 to 11 (Fojta *et al.,* 2006). Thanks to the presence of cysteine's tiolic groups, the phytochelatins, such as metallothioneines that have the same function, chelate metals and form complexes of molecular weight between 2500 and 2600 Da.

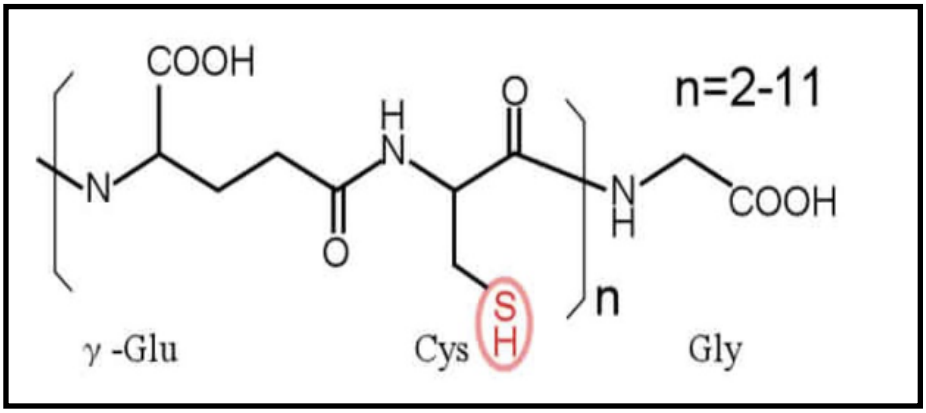


Fig. 4 - Structure of phytochelatine.

Even the metallothioneines are peptides rich in cysteine with the same function as phytochelatins, but, while the synthesis of the latter takes place by activation by the metal ions of the enzyme that promotes the polymerization process, the induction of metallothioneines takes place at genetic level, so they are synthesized starting from the m-RNA on ribosomes (Trombetta *et al.,* 2008). Many plants are not only able to tolerate high concentrations of heavy metals, but they are also able to hyperaccumulate them: about 400 species of different taxa were highlighted as heavy metal hyperaccumulator plants, i.e. plants capable of accumulating Ni, Co, Pb, in concentrations greater than 0.1% of their dry weight, Zn in greater concentration of 1% and Cd in concentration greater than 0.01% (Clemens, 2000). This plant capacity is exploited in phytoremediation technologies: with this term the use of plants for the decontamination of soils polluted by heavy metals is indicated, thanks to their ability to accumulate them and steal them inside the epigeal biomass (Mart, 2004).

CHAPTER II - CADMIUM ACCUMULATION AND DEFENSE MECHANISMS

2.1. *Cadmium (Cd)*

Cadmium (Cd) was discovered almost simultaneously by Friedrich Stromeyer and Karl Hermann in 1817 in zinc oxide samples obtained by combustion of zinc carbonate from Salzgitter (Germany). Cd is a bivalent ion with a density of 8.6 gr cm^3 and in areas with high levels of anthropogenic pollution is among the most common metals. It is relatively rare and in nature it is not in pure state. It is quickly oxidized in Cd oxide if exposed to air, and reacts easily with carbon dioxide, water vapor, sulfur dioxide, sulfur trioxide, or hydrogen chloride producing carbonate, hydroxide, sulfide, or Cd chloride; Cd establishes weak bonds with carbon and other more electronegative atoms. Although in traces, the presence of the Cd in the various environmental sectors can cause a series of problems to all organisms and its bioaccumulation in the food chain can be highly harmful (Mishra *et al.,* 2006). In fact, the presence of this element in the food chain arouses enormous concern because of its known neurotoxic, mutagenic and carcinogenic effects.

2.2 *Cd absorption in plants*

In elementary form, heavy metals are not available for the plant, but if entered into aqueous solution they make themselves available for vegetables; it is therefore necessary that they react first with other elements to be solubilized. Cd is absorbed in plants mainly from the soil through the radical system (Salt *et al.,* 1995; Toppi *et al.,* 1999); in fact, on the surface of the root, they bind to the carboxyl groups of the uronic acids of the mucilage. The latter has the function of limiting the absorption of the metal by the root, establishing an important barrier to protect the radical apparatus; later, some of these ions linked by mucilage, will be released when the mucilage will be biodegraded (Yang *et al.,* 2013). To a lesser extent, CD ions enter the plants even through leaves, and the leaf ability to absorb these ions greatly depends on its morphology: for example the hairy leaves absorb the heavy metals from the atmosphere better (Godzik *et al.,* 1993). The soil pH is considered the most important parameter that influences the availability of metals, of the Cd in particular. Many studies show that there is a linear trend between the soil pH and the absorption of Cd: the decrease in soil pH leads to a growing concentration of Cd in plants (Kirkham *et al.,* 2006), therefore, the soil pH affects the availability of the Cd present in solution in the soil but a soil pH increase does not always reduce the amount of CD absorbed by plants (Singh *et al.,* 1995); in fact, it is demonstrated that in grain plants cultivated on the acid red earth in China (soil pH = 4.95), the content of

Cd in the grain is 0.36 mg / kg^{-1}, while, at pH of 6,54, the contents of the Cd in wheat is 0.43 mg / kg^{-1} (Li *et al.,* 2005). The absorption of Cd ions competes with that of elements such as Zn, P, Cl (Li *et al.,* 1994; Zhao *et al.,* 2002; Dheri *et al.,* 2007; Oporto *et al.,* 2009), Ca (Choi *et al.,* 2005), and Cu (Kudo *et al.,* 2011); furthermore, the addition of synthetic chelating agents, such as EGTA and EDTA, to the ground contaminated by CD, improves the absorption of heavy metal by the plant, obtaining a result of cleaning the contaminated soil (Salt *et al.,* 1995).

2.3 *Distribution of Cd in various plant organs*

As mentioned before, the main absorption pathway of the Cd is given by the roots, but it is located in all plant organs and tissues; in particular, the leaves represent the preferential site of accumulation of Cd, while in the roots the maximum absorption of this metal is manifested (Zheljazkov *et al.,* 1996). In the leaves, Cd is accumulated in different zones, which correspond to the metabolically less active parts, which will then meet necrosis. This suggests that there is an active metabolism whose function is to neutralize the toxic effects of this element, and, on the other hand, the absence of detoxication mechanisms induces the onset of necrotic events (Cosio *et al.,* 2005). Furthermore the age of the leaves affects a lot the ability to accumulate Cd; in fact, it is preferably accumulated in the young leaves of *Brassica juncea* L. and *Brassica caerulescens thlaspi* L. (Salt *et al.,* 1995), while in other species the Cd accumulates in the senescent leaves (Godzik *et al.,* 1993). The effects caused by Cd include morphological changes, reduction of photosynthesis rate, due to reduced perspiration, up to cellular apoptosis (Souza *et al.,* 2011). Cd carries out its toxic action on the transport chain of photosynthetic electrons by determining imbalances between the donor site and the acceptor site and reducing the levels of proteins involved (Sigfridsson *et al.,* 2004).

The content of heavy metals in the various organs of the plant decreases according to the following order: roots, leaves-stems, inflorescences and seeds. This succession can vary with different plant species. Seedling represents the first barrier against the absorption of Cd; it is shown, in fact, that the Cd is mainly present in the cell walls of the seed integuments and does not reach embryos, even with lethal metal concentrations (Salt *et al.,* 1995).

When the roots absorb the Cd, it accumulates above all in the rhizodermis and in the cortex of the roots (Vodnik *et al.,* 1999) and a considerable amount of Cd is found at the level of radical hair (Harad *et al.,* 2010); however, we do not know if these structures are more or less important for the absorption of the heavy metal (Seregin *et al.,* 1997); moreover, in the sites where the side roots penetrate the endoderm, the Cd penetrates the stele faster (Akhtera *et al.,* 2014). The protective struc-

ture of the bark seems to reduce the toxic effects of these ions on the other tissues, as most of these ions bind to components of cell walls (polygalacturonic acids) (Lux *et al.,* 2010), then the bark acts as a second barrier against the toxic effects of this metal. Therefore, most of the Cd is located in rhizodermis and in the cortex and does not cross the endodermic barrier to sublethal concentrations, while at lethal concentrations, this barrier is broken and the flow of Cd comes into the tissues of the stele (Akhtera *et al.,* 2014). Studies on the structure of the endodermic barrier help to understand plant resistance mechanisms, as well as identifying the different ways that heavy metals use to reach the sprout. Relatively high concentrations of Cd are found in the cell wall in many species (Seregin *et al.,* 1997; Lux *et al.,* 2010). The substantial difference between the monocotyledons and dicotyledons is the different content in pectin and hemicellulose, and this factor affects their binding capacity towards the cations (Lux, 2010). The presence of these heavy metals, therefore, promotes the deposition of callosium and suberin, which brake the absorption of heavy metals themselves (Samardakiewicz *et al.,* 1996; Schreiber *et al .,* 1999; Piršelová *et al.,* 2012) and cell walls can prevent Cd transport in the cytoplasm; a considerable part of absorbed heavy metals remains on the outer surface of the plasmalemma in the form of globular aggregates (Wierzbicka *et al.,* 1984); however, part of the Cd ions is absorbed up to the cytoplasm.

2.4 *Cd transport in the plant*

Cd ions enter cells by passive transport, but active absorption is apparently connected to the competition of Cd with other metals to membrane transporters and ion channels (Salt *et al.,* 1995); 96% of the absorbed metal is found inside vacuoles, which together with the cell wall manage to absorb these considerable element.

The deposition of Cd in vacuole takes place in the form of complexes and salts, therefore representing a detoxification mechanism (Mazen *et al.,* 1997). For understanding the concept of plant tolerance it is important to understand the transport mechanisms of heavy metals absorbed towards vacuol:

• first, Cd ions from the external solution can immediately enter the endoplasmic reticulum connected to the apoplast (Gamalei *et al.,* 1997);

• second, in vacuole, the accumulation of high affinity compounds towards heavy metals, such as organic acids and compounds that form low-solubility complexes with heavy metals, determines the predominant location of these elements in this organelle (Mazen *et al.,* 1997).

• third, the seizure of Cd may depend on the synthesis of phytochelatins (PC) in the cytoplasm (Carrier *et al.,* 2003).

In summary, the greater portion of Cd is linked to polygalacturonic acids of the cell wall and to the various ligands in the vacuol (Carrier *et al.,* 2003; Lux *et al.,* 2010). This bond lowers the metal concentration in the cytoplasm. The efficient exclusion of cytoplasmatic Cd through accumulation in vacuoles or translocation towards senescent tissues can be crucial for the tolerance of the plant to heavy metals.

2.5 *Effects of Cd on enzymatic activities*

Cd interacting with enzymes could inhibit their activities. In most cases, the inhibition by Cd is the result of the interaction between Cd ions and groups –SH, essential for the reaction center and for the stabilization of the enzyme tertiary structure. Over 100 known enzymes are inhibited when ions interact with groups -SH. Cd can also promote various enzymatic activities, but it is not clear whether this influences directly enzymatic activities, or if this stimulation is the result of changes in the synthesis of enzymes, or it is determined by the immobilization of inhibitors. It should be remembered that Cd stimulates the formation of reactive oxygen species (ROS) (Benavides *et al.,* 2005; Cho & SEO, 2005; Gratão *et al.,* 2005); in fact, in front of an oxidative stress the cell responds with an increase in antioxidant enzyme activities with the aim of neutralizing free radicals and peroxides (Uraguchi *et al.,* 2009). However, the resistance of the same enzyme towards heavy metals varies according to the plant species involved. The inhibition exercised by Cd is not specific, and it is also produced by other cations with bond affinity superimposable to that of Cd for the functional groups of proteins, but in some cases, Cd promotes enzymatic activities, such as direct stimulation of the catalase, peroxidase and dismutase superoxide (Uraguchi *et al.,* 2009).

2.6 *Effects of Cd on the photosynthesis*

On the photosynthetic parenchyma the accumulation of Cd can have repercussions both on the light phase processes and on the reactions of the dark phase; in fact, it has been shown that in many species, such as *Brassica napus* L. (Baryla *et al.,* 2001), *Helianthus annuus* L. (Di Cagno *et al.,* 2001), *Brassica caerulescens thlaspi* J&C (Kupper *et al.,* 2007), *Hordeum vulgare* L. (Popova *et al.,* 2008), *Vitis vinifera* L. (Wahid *et al.,* 2008), and *Triticum* spp. (Moussa & El- Gamal, 2010), photosynthesis is significantly inhibited after exposure to Cd both at long and short-term. Among the various multiprotein complexes immersed in the thylacoid membrane, photosystem II (PSII) is particularly sensitive to the action of Cd (Sigfridsson *et al.,* 2004) both at the level of the reducing site (acceptor) and at the level of the oxidant site (donor); the accumulation of Cd damages the

structure of the reaction centers and electrons transporters, compromising the functionality of the entire photosynthetic electronic transport. Other target of Cd contamination is the complex of the ferredoxin-NADPH $^{+}$ reductase that inactivating itself involves damage to photosystem I (PSI) (Siedlecka & Baszynki, 1993; Sárvári *et al.,* 1999). The deleterious effects of Cd on photosynthesis also highlight at ultrastructural level. In fact, leaves contaminated by Cd show a distorted ultrastructure of the chloroplasts, in which clear alterations appear in the synthesis of chlorophyll, plastochinones and carotenoids; furthermore, the development of plastids is directly influenced by Cd, and there are alterations of the membranes whose changes in lipid composition and fatty acids determine a reduction in functionality (Ouarites *et al.,* 1997; Sersen & Kr'ova, 2001; Popova *et al.,* 2009). The reduction of chlorophyll content, in particular of chlorophyll b, is a typical effect of Cd (Vodnik *et al.,* 1999), and it is attributable to the inhibition of enzymes responsible for the synthesis of chlorophyll and to the lack of Mg and Fe. On the leaf surface (stomatal guard cells), compared to mesophyll, the decrease in the content of pigments, induced by Cd, is higher.

Furthermore, the Cd can interfere directly at a structural level, interfering with the cell division both at the level of leaf tissues and at the functional level; this involves a reduction in stomatal conductance, which translates into a decompensation of gaseous exchanges. At parity of Cd concentration, the effect on chlorophyll varies in different plant species. The inhibition of the synthesis of chlorophyll by Cd ions is often associated with chlorosis phenomena but according to Baryla *et al.,* 2001, chlorosis observed in *B. napus* is not due to a direct interaction of Cd with the biosynthetic pathways of chlorophyll, and it is probably caused by the decrease in chloroplast density.

Cd performs its toxic action mainly on two key enzymes of the photosynthetic process:

- the ribulosium-1,5-bisphosphate carboxylase-oxygenase (Rubisco)
- the phosphoenolpyruvate carboxylase (PEPcase).

In particular, Cd ions reduce the activity of Rubisco, damaging the structure with the replacement of magnesium ions, important cofactors of the carboxylation reaction, and moving the activity of Rubisco towards oxygenation reactions (Siedlecka *et al.,* 1998).

2.7 *Effects of Cd on plant growth and morphogenesis*

All metabolic changes produced by Cd drastically modifies plant growth and development; a non-specific symptom of the effects exercised by different factors of stress to evaluate the phytotoxicity of these factors are morphogenetic distortions. During environmental tests, to assess the presence of heavy metals such as Cd, a parameter that is often used is the inhibition of the growth in the plants.

That inhibition is the result of metabolic diseases and direct effects on growth; for example, Cd interactions with cell wall polysaccharides cause a decrease in cell plasticity (Lux *et al.,* 2010). The germination of the seeds represents a phase of the development of plants more tolerant to heavy metals (Obroucheva *et al.,* 1998), while the growth of the roots is more sensitive to Cd with respect to the growth of the sprout (Seregin *et al.,* 1997; Obroucheva *et al.,* 1998). This evidence is correlated to the result that heavy metals accumulate predominantly in the radical apparatus; in particular, at low concentrations, Cd promotes the growth of the radical system, while at high concentrations inhibits the growth of the roots and these inhibitory effects are not observed within the first 3 days of exposure (Obroucheva *et al.,* 1998).

2.8 *Mechanism of response to Cd stress at cellular level*

The plants have evolved mechanisms of response to soil Cd contamination; furthermore, the data highlight that there is no universal tolerance mechanism to Cd; often, strictly related plants respond differently when exposed to Cd. Cd toxicity is often reduced through its seizure into specific cellular compartments, such as vacuoles, or within specialized cells, such as trichomes (Harad *et al.,* 2010); furthermore, the adaptation of plants to land contaminated by Cd improves through symbiotic collaboration with other organisms. In *Medicago sativa* L., the colonization of roots by arbuscular mycorrhizal fungi increases plant tolerance towards Cd.

Probably also fungi are likely to have a set of enzymatic activities and / or mechanisms capable of chemically modifying Cd, making it less toxic (Wang *et al.,* 2012). The protection mechanism based on the cell wall (as mentioned above) is only one of the processes used by plants to cope with the damage induced by Cd. Beyond the cellular wall, there are other protective mechanisms against Cd toxicity, participating to the biochemical responses and the intracellular seizure of Cd. Numerous studies show that when Cd ions entering the cell are bound to small peptides containing cysteine denominated phytochelatine (PC) through their groups -SH. These compounds form complexes containing Cd of about 3.6 kd. The base structure of these peptides is [γ-glutammil-cisteinyl] n-glycine, with n = 2-11 (Carrier *et al.,* 2003). The production of phytochelatine coincides with the activation of sulfur metabolism; the synthesis of the cysteine and reduced glutathione (GSH), an antioxidant precursor of phytochelatine, increases (Zhang & Shu *et al.,* 2006). The importance of sulphur in biosynthesis of sulphide proteins, suggests that higher sulphur content generates large amounts of protein capable of sequestring Cd in cytosol, thus reducing metal accumulation in the cell wall (Zhang *et al.,* 2014 c). Therefore, the availability of sulphur can control the synthesis

speed of these proteins (Loeffler *et al.,* 1989; Uegsegger *et al.,* 1990). The presence of phytochelatine in detoxification of plants subjected to Cd stress is considered an important mechanism and seems to be organ-dependent; another important class of cysteine rich proteins that bind heavy metals in plants and animals are metallothioneine (MTS). Their role in the stress by heavy metals in plants is known and their potential use in the field of biotechnology is supported by recent studies that analyzed the effects of the overexpression of the MTS in *Arabidopsis thaliana* L. and tobacco (Gu *et al.,* 2014) (Figure 5).

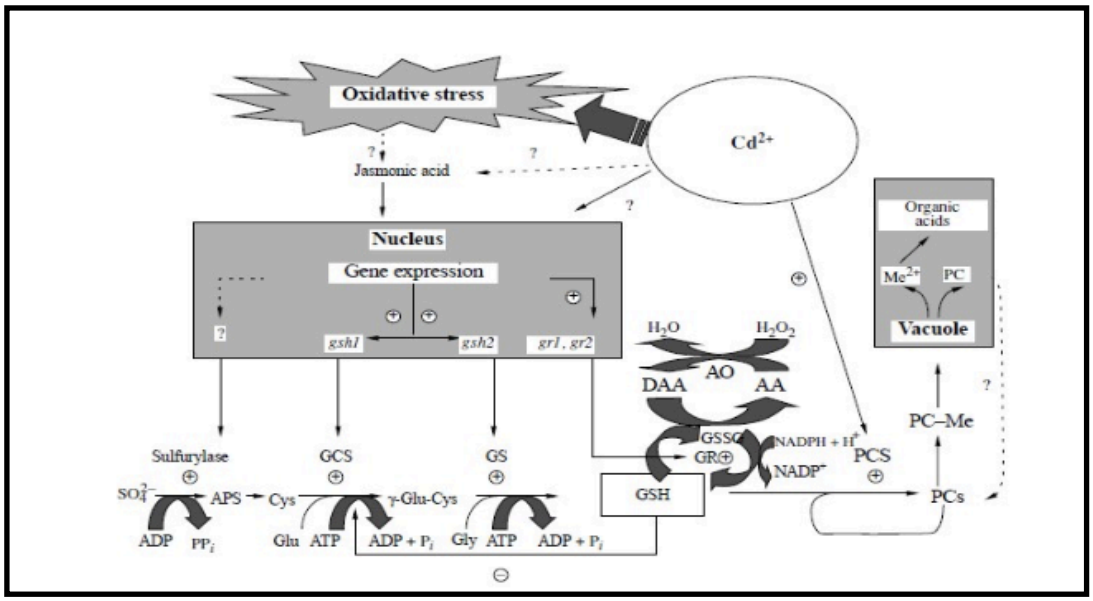


Fig. 5 - Oxidative stress induced by cadmium in plant.

This study showed that transformed plants exhibit an increased tolerance to Cd stress, as well as a lower accumulation of H_2O_2 in *A. thaliana* and greater ROS removal activity in tobacco. The based phytochelatines response appears little effective in Cd-sensitive plants, even if their total content increases following exposure to heavy metals (Uraguchi *et al.,* 2009).

2.9 *Cd and production of reactive oxygen species*

It is known that heavy metals cause oxidative damage to plants, both directly and indirectly, through the formation of reactive oxygen species (ROS). Some heavy metals, such as copper and iron, can be toxic through their participation in redox cycles such as Feston and / or Haber-Weiss reactions. On the contrary, Cd is a non-redox metal and does not produce ROS as superoxide anion (O_2^-), single oxygen (O_2), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH^-), but generates oxidative stress, interfering with the antioxidant defense system (Benavides *et al.,* 2005; Cho & Seo, 2005;

Gratão *et al.,* 2005). Cd inhibits photoactivation of photosystem II (PSII) inhibiting the transfer of electrons. The generation of ROS by Cd would be indirectly through the production of a disturbance in chloroplasts. Furthermore, other studies suggest that Cd can stimulate ROS production in the mitochondrial electrons transfer chain (Heyno *et al.,* 2008).

As suggested by Gill *et al.,* 2010, in *M. sativa* the exposure to Cd for 6-24h causes a rapid accumulation of peroxides and a reduction in glutathione (GSH) and homoglutathione (HGSH), carrying to a redox imbalance. DNA injury by Cd causes damages to the photosynthetic apparatus, but also the destruction of nucleic acids, lipids of cell membrane, proteins and a reduction in protein synthesis, consequently influencing the growth and development of the whole organism (Gill & Tutueja, 2010; Kranner & Colville, 2011). Many proteins with enzymatic activity have the function of reducing the presence of ROS, such as: superoxide dismutase (SOD), catalase (CAT), peroxidase ascorbate (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), peroxidase (POD) and glutathione reductase (GR) but also simpler molecules such as glutathione (GSH); SOD, GR, APX, POD and CAT, therefore, show variations in their activities depending on plant species and Cd concentrations used (Sandalio *et al.,* 2001; Yilmaz & Parik *et al.,* 2011).

2.10 *Cd and production of heat shock proteins*

Plant response to Cd requires enzyme activation capable of folding proteins, as one of the most dramatic intracellular effects caused by Cd is protein denaturation. These proteins are called thermal shock proteins (HSP) and their levels increase after treatment with Cd (Timperio *et al.,* 2008).

CHAPTER III: PROLINE INDUCTION AND ACCUMULATION MECHANISMS

3.1 *Introduction*

Proline (Figure 6), or (2S)-pyrrolidine-2-carboxylic acid presents the secondary amino group rather than primary and is classified as amino acid despite being a neutral pH imino acid.

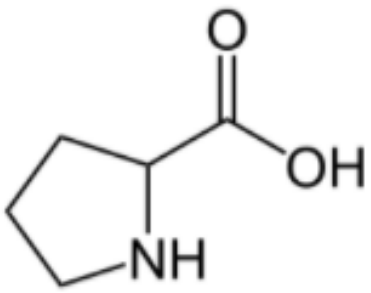


Fig. 6 - Proline structure.

It is a cyclic amino acid, it has not polar characteristics and does not possess α hydrogens, it can not form hydrogen bonds to stabilize the propeller or β leaflet and consequently does not participate in the formation of the secondary structure which, in fact, interrupts where there is a proline residue. It is often found at the end of the α-helix; unlike other amino acids that exist almost exclusively in the *trans* form in polypeptides, the proline can exist in the *cis* configuration. *Cis-trans* isomerization can play an important role in protein folding. It is a non-essential amino acid that is obtained by reduction from glutamic acid where the carboxylic group in γ is reduced to aldehyde giving glutamate semialdehyde. The aldehyde reacting with the α-amino group to eliminate a water molecule turns into a Schiff base that will be reduced by giving rise to proline (Figure 7).

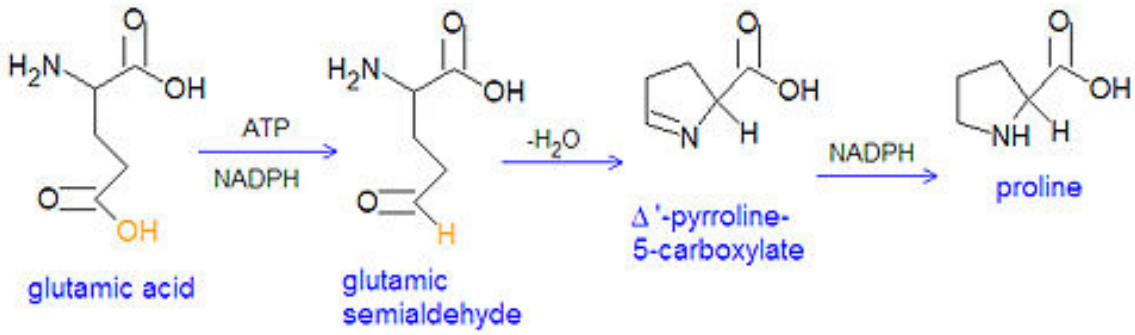


Fig. 7 - Proline reaction.

3.2 *Proline's metabolism*

The biosynthesis of proline (Figure 8) takes place with different modes in plants and bacteria (Csonka *et al.,* 1993; Delauney *et al.,* 1993). If the genes coding for the enzymes associated with the synthesis and catabolism of the proline were cloned and partially characterized, little is known about the regulatory aspects (Kavi Kishor *et al.,* 2005). The synthesis of this amino acid mainly happens starting from glutamate but seems to originate even through the pathway of arginine / ornithine. There are some differences between prokaryotes and eukaryotic relative to the biosynthetic ways involved as shown in figure 8 (Kavi Kishor *et al.,* 2005).

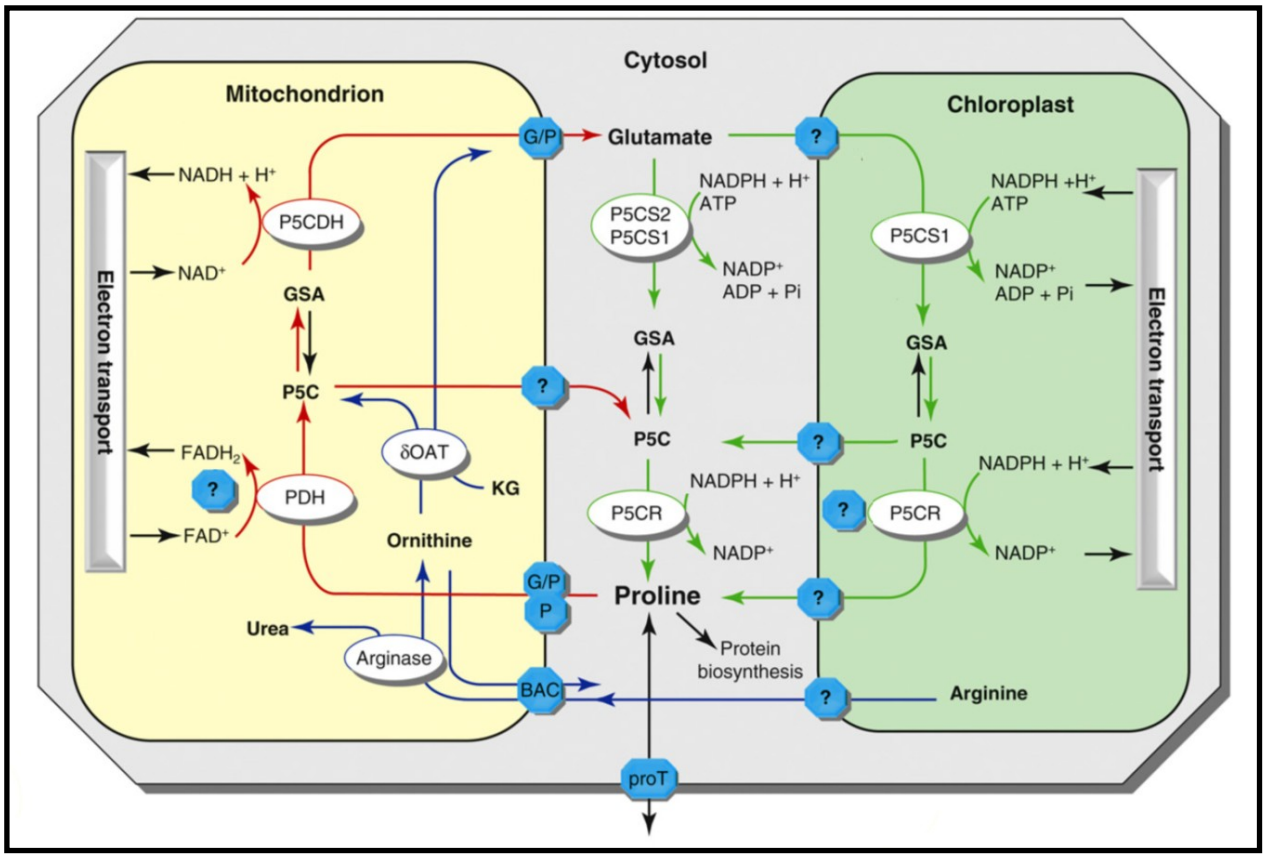


Fig. 8 - Proline's metabolism. The spatial separation of biosynthesis and catabolism is highlighted in the scheme. As can be seen from the question marks, still several aspects remain to be clarified. In particular the existence of a specific transporter for the P5C, as well as the location of the reductase P5C and the final acceptor of the electrons used by the ProDH remain critical points to be clarified (modified, by Szabados & Savouré, 2010).

- Glutamate pathway

The biosynthesis of proline from glutamate, in bacteria begins by a γ-glutamyl-kinase (γ-gk) which catalyzes the phosphorylation of the γ-glutamyl-phosphate substrate, which is reduced to glutamic-γ-semialdehyde (GSA) from glutamic enzyme-γ-semialdehyde dehydrogenase (GSADH). For spontaneous cycling of the GSA in solution the δ-pyrroline-5-carboxylic acid (P5C) is formed. In the eukaryotes, on the other hand, the synthesis of GSA starting from glutamate is catalysed by a single bifunctional enzyme, the δ-pyrroline-5-carboxy-synthetase (P5CS), equipped with both γ-glutamil-chinasic and glutamyl-γ-semialdehyde dehydrogenasic activity (Hare *et al.,* 1999) showed the NADPH's P5CS enzyme as a cofactor. The kinase activity is limiting the synthesis speed of the proline and undergoes a negative feed-back adjustment from the latter, inhibition that is more marked in the bacteria than in eukaryotes (Hu *et al.,* 1992; Zhang *et al.,* 1995). There are different isoforms of P5CS whose adjustments, in terms of transcriptional feedback regulation, vary between species. For example, the gene of *A. thaliana* AtP5CS1 is expressed in many organs and differentiated tissues and its transcription is induced by dehydration, from the high salinity treatments and with abscisic acid (ABA), while its levels are low in cell cultures in active division, or in the absence of abiotic stress (Kavi Kishor *et al.,* 2005). In contrast, the AtP5CS2 gene is expressed in cell cultures in the division and its levels respond better to stress conditions (Strizhov *et al.,* 1997). The two P5CS genes in *Medicago truncatula* Gaertner show, however, a different adjustment in various organs and in response to osmotic stress. As for MtP5CS1, the transcript levels in various organs correlate well with those of free proline, but the transcript is not affected by osmotic stress. By contrast, levels MtP5CS2, very low in all organs, undergo a marked activation only in the roots of plants stressed with salt (Armengaud *et al.,* 2004). The last passage of the biosynthetic way that leads to the formation of proline for reducing P5C, is common to both prokaryotes and eukaryotes, and is catalysed from the enzyme pyrroline-5-carboxy-reductase (P5CR). The P5CR was discovered for functional complementation of a mutant of *E. coli* using a cDNA library obtained from potato nodules (Delauney & Verma, 1990).

In general terms, it seems that the activity of P5CR-1 and P5CR-2 is inhibited by a high concentration of cations rather than anion (Murahama *et al.,* 2001). Also for this enzyme the regulation shows differences between species. For example, in spinach the two isoforms are inhibited by an increase in the salinity of the vehicle, while in pea there is an increase, even if modest, of enzymatic activity (Kavi Kishor *et al.,* 2005).

The P5CR seems to have an important role in determining the OSMO-induced proline accumulation, since, as it was stressed, the limiting passage is the one controlled by the P5CS.

They generally use both NADPH and NADH as a reducing power donor, and it seems to have an important role in regulating cellular redox potential (Kavi Kishor *et al.,* 2005).

- Arginine/ornithine pathway

An alternative way to the production of proline is that of arginine / ornithine, which in turn can be synthesized starting from glutamate or arginine. The arginine is a very precious nitrogen reserve, and is converted from the enzyme arginase (ARG) into ornithine, which in turn is metabolized differently in bacteria and plants. In the former it is transformed from the enzyme ornithine-α-aminotransferase (α-OAT) to α-keto-δ-aminovalerate, which in turn cycles spontaneously in pyrroline-2-carboxylic acid (P2C). The latter is finally converted into proline by the P2C reductase (P2CR). In the plants these passages are not found, the ornithine can be converted directly into GSA (glutamic-γ-semialdehyde) from the enzyme ornithin δ-aminotransferase (δ-OAT). Recently, Sekhar *et al.* (2007) showed that the enzyme is inhibited by serine, isoleucine and valine, while it is insensitive to proline. The δ-OAT of *A. thaliana* (AT5G46180) has been identified for sequence homology with that of grapevine and has been seen that it is induced in the seedlings in response to saline stress (Roosens *et al.,* 1998). Transgenic tobacco and rice plants overespriments the δ-OAT have lower proline levels, higher and greater resistance at osmo-saline stress conditions, leading to the conviction that the conversion of ornithine can contribute to the accumulation of proline (Wu *et al.,* 2005). The reaction of the Δ-OAT determines the formation of GSA, which is in spontaneous equilibrium with its cyclic shape P5C. This could convince us that ornithine could represent an alternative starting point for the synthesis and accumulation of proline as suggested by Delauney *et al.* (1993). The studies of Hervieu *et al.* (1995) conducted on radish plants (*Raphanus sativus* L.) treated with gabaculin, an δ-OAT inhibitor, seem to confirm the contribution of the ornithine pathway to the proline synthesis. However, the glutamate pathway still seems to be the preferential way both in normal-osmotic conditions and lack of nitrogen, while when the availability of nitrogen is high the preferential way is that of the ornithine (Delauney *et al.,* 1993).

The presence of a transit peptide, predicted by eleven different programs, and the colocalization of δ-OAT-GFP of *A. thaliana* with mitochondria, are strong evidences in favor of its localization at the level of these organelles, which suggests that the P5C produced from the ornithine is placed in the catabolic pathway rather than in the biosynthetic one (Funck *et al.,* 2008).

A transport of P5C / GSA from mitochondria to cytosol does not seem plausible due to its chemical instability (Funck *et al.,* 2008); in fact, until today a specific transporter for this metabolite has not been cloned in any organism. This provides a further evidence at the expense of the idea that exists

a shortcut between arginine and proline synthesis that does not proceed through the formation of glutamate and the activity of cytosolic P5CS. In this perspective it seems more plausible than the P5C is metabolized to glutamate (Funck *et al.,* 2008).

3.3 *Proline's catabolism*

The re-oxidation of the imino acid proceeds to glutamate through only two steps. The first one is catalyzed by a proline dehydrogenase (ProDH) which produces P5C followed by that of P5C dehydrogenase (P5CDH), which converts the latter into glutamate. Into the plants, amino acid catabolism proceeds at mitochondrial level (Deuschle *et al.,* 2001). ProDH is localized in the internal mitochondrial membrane (Figure 9; Kiyosue *et al.,* 1996); this element seems to favor the electrons transfer directly to respiratory chain even if until today the physiological acceptor has not been identified.

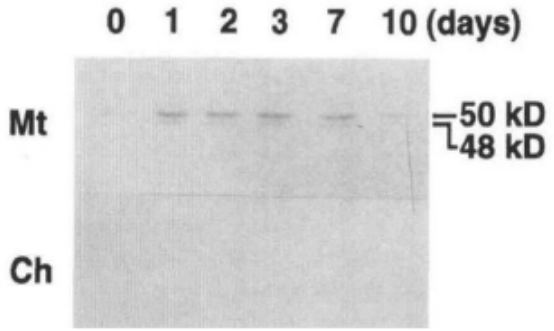


Fig. 9 - Localization of PDH.

The plastid fraction shows no hybridization while in the mitochondria are found two bands corresponding to the precursor and the mature form (Kiyosue *et al.,* 1996). According to some inhibition of the degradation of proline plays only a marginal role in determining its real accumulation (Hare *et al.,* 1999). The gene is expressed in the reproductive organs and in germinating seeds (Verbruggen *et al.,* 1996; Nakashima *et al.,* 1998). Ribarits *et al.* (2007) observed a different adjustment of the two isoforms present in *Nicotiana tabacum* L.

The two isoforms, NtPDH1 and NtPDH2, are expressed differentially in different organs both in normo-osmotic conditions and dehydration conditions, while in just germinated seedlings and subjected to dehydration isocore the two are co-regulated. The analysis of the sequence shows a high similarity with a *N. tabacum* gene (CIG1) induced by cytokinins (Kimura *et al.,* 2001). In fact both

isoforms are strongly induced in meristems and pollen, tissues that contain high levels of these hormones (Ribarits *et al.,* 2007). However it seems that in both species, one of the two isoforms have a constitutive role, while the other is more involved in the stress response. Ribarits *et al*. (2007) have also speculated that NtPDH1 is also involved in the growth and development of the plant, providing, therefore, energy and metabolites from the degradation of proline, while NtPDH2, strongly induced by water stress, would play a predominant role in this regard. This hypothesis is also reflected in the fact that the two isoforms are co-expressed at high levels in the pollen and seeds, highly dehydrated tissues but require a ready source of energy and nutrients. Genotypes in which one or both NtPDH genes have been silenced show defects in the formation of the seeds as well as disorders of the normal germination and development of seedlings (Ribarits *et al.,* 2007).

3.4 *Proline's transport*

The transport of amino acids is regulated not only by endogenous signals, but also by environmental signals such as biotic or abiotic stresses (water, salt, and heavy metal stresses, etc...). If the metabolism of proline in plants is well characterized, at least regarding the biosynthesis, little is known on its uptake and its transport within the plant. Several evidences suggest, however, that its transportation is crucial in determining the adjustment under certain conditions (Girousse *et al.,* 1996). In *A. thaliana* eight different transporters for amino acids have been cloned and characterized and three of those coding for the proline specific transporters (AtProT1, AtProT2; Rentsch *et al.,* 1996; AtProT3; Grallath *et al.,* 2005). The transport of proline is important not only for accumulation under stress but also during normal plant development. The fact that some mutations for the biosynthesis of this amino acid can be reverted by exogenous administration of the amino acid is a further indication of the existence of carriers (Nanjo *et al.,* 1999). In recent years, several coding genes for proline carriers have also been cloned in other species, including tomato (*Lycopersicon esculentum* L.; Leprot1-3; Schwacke *et al.,* 1999), rice (*Oryza sativa* L.; OsProT; Igarashi *et al.,* 2000), barley (*Hordeum vulgare* L.; HvProt; Ueda *et al.,* 2001) and mangrove (*Avicennia marina* Forssk; AMT1-3; Waditee *et al.,* 2002).

Contrary to those of *A. thaliana*, some of these can also carry glycine-betaine (Schwacke *et al.,* 1999; Waditee *et al.,* 2002) or γ-aminobutyric acid (GABA) (Breitkreuz *et al.,* 1999). The expression levels of these transporters often correlate with stress and / or conditions with high proline concentrations. Even the expression of the barley transporter (HvProT) increases in response to osmo-saline stress response (Ueda *et al.,* 2001), but in this species the carrier is extremely selective

and transports only proline. Instead in tomato the expression of LeProT1 depends on the levels of proline and is confined exclusively to pollen. The proline is synthesized and accumulated in cytosol and chloroplasts, while its degradation is located exclusively in the mitochondria. This implies the existence of localized transporters at the level of the organelles. The existence of two carriers responsible for proline mitochondrial transport was described in isolated mitochondria of wheat (Di Martino *et al.,* 2006). In this species transport seems to be mediated by a specific carrier for proline and a proline / glutamate antiport. The hypothesis schematized in Figure 10, provides that the proline enters the mitochondria initially thanks to the action of its specific transporter, once inside the mitochondria the proline is oxidized to glutamate, which can be exported to exchange of further proline thanks to an antiport (Di Martino *et al.,* 2006). The existence of these transporters makes the idea that the metabolism of the proline and its spatial separation of catabolism and biosynthesis constitutes a sort of shuttle system for the redox potential among cell compartments. The genes coding for these two transporters have not been identified, but the demonstration of their existence returns to transport phenomena an important role in regulating its accumulation even within the cell.

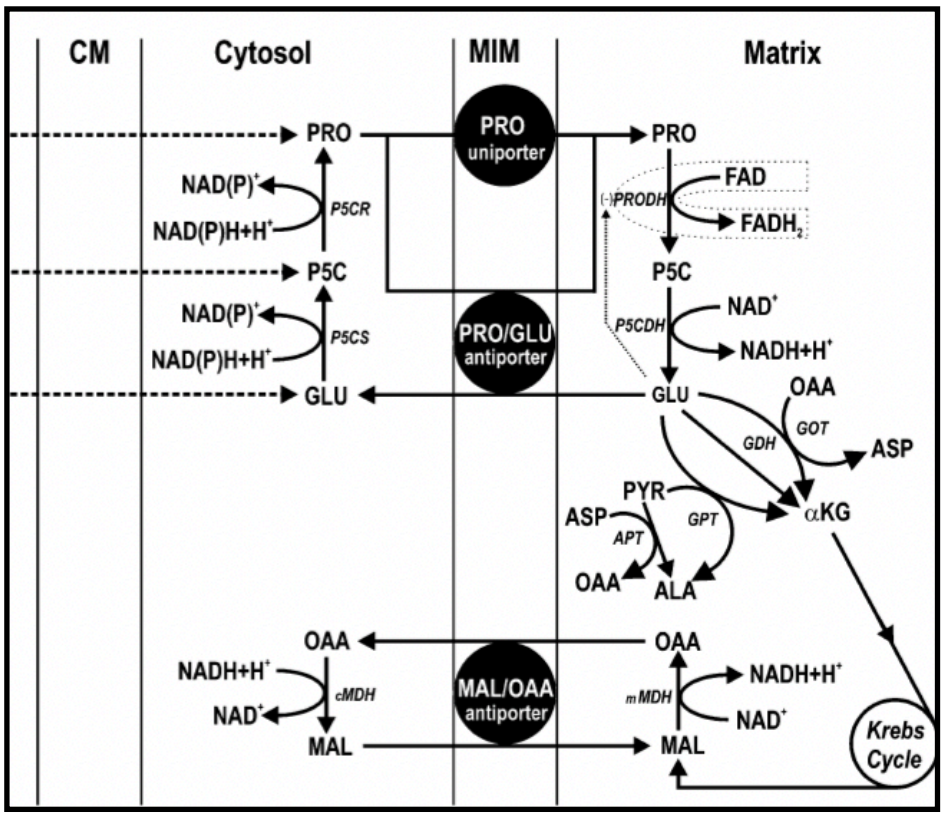


Fig 10 - Transport of proline in wheat mitochondria. A specific proline carrier transports from the cytosol to the mitochondria, where the proline is oxidized to glutamate. An antiport glutamate/ proline exports the product of oxidation and contributes to feed the catabolic pathway importing other proline (by Di Martino *et al.,* 2006).

3.5 *Regulation of proline's metabolism in* Arabidopsis thaliana *L.*

Most studies, in general, and in particular on proline have been conducted using *A. thaliana* as a model plant. This was the first plant species whose genome, small, has been entirely sequenced, making a vastness of biochemical and molecular data readily available. In addition, *A. thaliana* accumulates proline in response to osmotic stress (Verbruggen *et al.,* 1993; Yoshiba *et al.,* 1997). In *A. thaliana* there are two P5CS isoforms that cover specific roles in proline biosynthesis control (Fabro *et al.,* 2004; Székely *et al.,* 2008). Also in other plant species this enzyme is encoded by two nuclear localization genes that are considered not functionally redundant (Fujita *et al.,* 1998; Ginzberg *et al.,* 1998).

The P5CS represents the enzyme limiting the biosynthesis of proline and is controlled both at the transcriptional and allosteric level by inhibition at feed-back (Savoré *et al.,* 1995; Yoshiba *et al.,* 1995; Zhang *et al.,* 1995). This should be contrary to the accumulation of the imino acid in conditions of osmotic stress, when its cytosolic concentration exceed 130 mm, a level to which the P5CS is completely inhibited. To explain this apparent paradox, it was suggested that the conditions of osmotic stress can lead to the conformational changes of the P5CS such as to make the enzyme less sensitive to feedback inhibition by the final product. With regard to the two isoforms, it is believed P5CS1 to be necessary for the accumulation of proline induced by stress (Fabro *et al.,* 2004; Székely *et al.,* 2008). During osmotic and saline stresses, there are several signals that are responsible for the activation of the P5CS1. The role of the ABA in determining the accumulation of proline was studied by Verslues & Bray (2006). These scientists, using mutants in biosynthesis and the perception of the ABA, were able to see not only the dependence of the accumulation of proline from the levels of ABA, but also the sensitivity and / or the ability of plants to respond to this hormone. Subsequent results support the idea that the H_2O_2 is an integral part of the signal and responses regulated by the ABA (Verslues *et al.,* 2006). During osmotic stress the availability of CO_2 decreases due to the closure of the stomatas and this involves a reduction in the activity of the Calvin cycle with a consequent decrease in NADPH's consumption. The P5CS activity in the chloroplast can somehow booth this phenomenon, consuming NADPH and restoring NADP, and this allows a reduction in ROS production at Photosystem I level. In 1985, Phang proposed a model in which the interconversion of proline / P5C could modulate the cell redox state maintaining a correct NADP+ / NADPH ratio. To support this model there is the evidence that the path of phosphate is located in cytosol and chloroplasts, i.e. the two cellular compartments where proline synthesis can take place.

The catabolism of proline and especially the expression of ProDH, on the other hand, is positively regulated by the proline (Verbruggen *et al.,* 1996). In stress conditions, when proline's synthesis increases there will be an increase in the catabolic pathway, which would end up triggering a futile cycle, but this does not happen except to restore permissive conditions, suggesting a mechanism of repression of the pathway mediated by the conditions of stress.

In knockout or antisense mutants for ProDH there is no significant increase in the cytosolic levels of proline in permissive conditions, while in stress conditions significantly higher levels are detected than in wild type (Mani *et al.,* 2002).

The hypothesis formulated to explain this result is that the degradation and synthesis of the P5C / GSA intermediate acts as a signal limiting the biosynthesis of the proline. It must necessarily exist a specific transporter that allows its diffusion, or a signal must be generated that can be transduced and perceived from the inside of mitochondria to citosol (Deuschle *et al.,* 2004). It then becomes plausible to think of a communication mechanism between the biosynthetic pathway and the catabolic pathway that uses the P5C as a signal. Even if it is believed that proline levels depend mainly from the regulation of P5CS and ProDH genes, it seems that P5CDH regulation also plays an important role. Since there is an active transport of proline, it is believed that this amino acid also serves to transfer nitrogen, carbon and redox potential between various organs, such as from the flowers to seeds. In fact during the development of *A. thaliana* and in the absence of stress, the levels of free proline changes from organ to organ regardless of the pool of amino acids. The highest levels find themselves in the flowers, particularly in the pollen granules, and in the seeds, while in the roots the level of proline is decidedly lower.

3.6 *The proline in nectar and its importance for pollinators*

Although at concentrations that are much lower than those of sugars, amino acids are present in the nectar of angiosperms. The biological meaning of this presence was not completely clarified, and still today is a subject of debate in the scientific community. Some authors showed as plants pollinated by insects contain greater concentration of amino acids than those who use birds as pollinators (Gardener & Gillman, 2001). It is believed that the quantity and quality of the food source can strongly influence the longevity and fecundity of the insects. In particular, it is known that the adult female butterflies transfer the amino acids taken from the food source directly to the eggs and they prefer artificial pollens rich in amino acids compared to those that are devoid of them. This behavior cannot be found in males (Mevi-Schutz & Erhardt, 2005).

Among the amino acids that are commonly found in the nectar, the proline has a unique feature: insects are able to recognize its taste (Carter *et al.,* 2006). The levels of proline in pollen can reach 70% of total amino acid thanks to the expression of specific carriers for this amino acid (Schwacke *et al.,* 1999). Also in some nectars there are high levels of proline. In tobacco plants can be accumulated until it reaches concentrations around 2 mM and represent 45-60% of the total ones (Carter *et al.,* 2006). Both the bees and butterflies prefer sugar solutions enriched with proline than those in which the amino acid was omitted. Between proline doses it appears that those between 2 and 6 mMol, the closest ones to the physiological concentrations, are the most attractant (Carter *et al.,* 2006).

The proline is particularly important for insects, so as to be the most abundant amino acid in the hemolymph of many species, including bees. It has not yet been clarified if other amino acids can act as attractants, but the proline and hydroxyproline seem to be the only ones, among natural amino acids, capable of stimulating the chemoreceptors of the labellar chemosensilla of some insects (Hansen *et al.,* 1998; Wacht *et al.,* 2000). Several studies suggest that a wide range of insects is able to use proline as an energy substrate for flight muscles. Tse-tse fly (*Glossina morsitans* Westwood), *Drosophila* spp., Colorado potato beetle [*Leptinotarsa decemlineata* (Say)], japanese beetle (*Popillia japonica* Newman), *Pachnoda sinuata* (Fabricius) and *Hycleus* (*Decapotoma*) *lunata* (Pallas) are just some of the insects that can use this amino acid during the flight. Even the *A. m. carnica* is able to burn proline (Scaraffia & Wells, 2003). This amino acid is believed to be consumed during the intense flight phases or at the initials of muscle pre-heating (Micheu *et al.,* 2000; Gade & Auerswald, 2002). In individuals kept fasting for 14 days, when carbohydrates in the hemolymph and flight muscles are exhausted completions, proline can be used as the only energy source. This amino acid is also subject to circadian fluctuations with a maximum at morning hours. These fluctuations are under control of hormonal neuropeptide (AKH). In the insects there are different types of these peptides that can alternatively adjust a way rather than another. In fact, during the circadian fluctuations of proline in the hemolymph, there is no change in carbohydrate levels and despite this insects use amino acid as energy substrate (Gade & Auerswald, 2002). Proline seems to transport acetyl unity from fat bodies to the muscles during the insect's flight. In this way, acetyl-CoA, deriving from the metabolism of fatty acids, glucose or amino acids, is converted into α- ketoglutarate in the cycle of citric acid at the level of fatty bodies. When necessary, α-ketoglutarate is converted into proline to be transported to the muscles. Once in the muscles the proline is converted into α-ketoglutarate to enter the cycle of citric acid and produce ATP (Scaraffia & Wells, 2003).

However, the production of proline is metabolically more expensive than that of glucose. In fact the proline contains nitrogen, which is a limiting nutrient. The accumulation of both in the nectar provides two types of insect fuel: proline for short flights and glucose for longer ones. The presence of proline in some nectars and the ability of some insects to perceive it suggest an evolutionary relationship that aims to increase the pollination of plants that produce nectar rich in proline by insects that prefer the taste of these nectars (Carter *et al.,* 2006).

CHAPTER IV: HONEY BEE AS BIOINDICATOR

4.1 *Introduction*

The Apidae family is a large family that contains various genera including *Apis*; the species generally accepted and belonging to this genus are nine (*Apis mellifera* L., *A. florea* Fabricius, *A. dorsata* Fabricius, *A. cerana* Fabricius, *A. nigrocincta* Smith, *A. koschevnikovi* Enderlein, *A. adrenifromis* Smith, *A. nuluensis* Tingek, Koeniger, & Koeniger, *A. laboriosa* Smith) but only two are managed by man: *A. mellifera* and *A. cerana*. If two other species will be defenitively accepted, *Apis breviligula* Maa and *Apis indica* Fabricius, the genus *Apis* will have 11 species. These bees are classified as social insects showing differences, for example, in communication between workers. All bees, social or solitaries, are feeding nectar and play an important role in pollinating flowering plants.

4.2 *Apis mellifera* L.

The life cycle of *A. mellifera* begins in the late winter with the oviposition by the queen in the individual cells of the comb. From the egg can arise two types of caste: if it is fertilized it will give life to a worker bee, or if not fertilized it will originate a male bee, called drone.

The role of the queen, which morphologically is larger than all the other bees of the colony, is of fundamental importance to ensure cohesion in the nest and to oviposit the fertilized eggs to give rise to many workers able to rear the larvae; this is possible because the females (worker bees) of the hive secrete the royal jelly, rich in sugar and secretions, a very high quality food and produced in large quantities (Haydak, 1943). Most deposed eggs will be fertilized, while only a few will not be fertilized. For two days all the larvae are powered by royal jelly, after which the larvae of the drones and workers mainly receive honey and pollen, while the larvae of the queens continue to be fed with royal jelly. Each larva during the development undergoes 5 moults; the development time for each caste is standardized, the larvae intended to form future queens take place after 15-16 days of life; those that are destined to become workers will have metamorphosis at about 21 days, while males do not take up before they have reached 24 days of life. The members of the colony are divided, then in three castes: queen, workers and drones.

Each of these castes carries out a very precise tasks. Workers perform different roles based on age, feature defined as temporal polyethism; cleaners: clean the honeycombs and beehive; feeders: take care of the nutrition of the larvae; wax-making bees: produce wax; gravel bees: eliminate dead bees from the hive; collectors: receive nectar and pollen collected by the companions and then place it

inside the honeycombs; guard bees: defend the family from external attack; fanning bees: keep the temperature inside the hive stable; foragers: collect nectar and pollen.
The males have the only task of fertilizing the queen and coupling in flight die immediately after fertilization (Ribband, 1953). Recently drones were observed to feed larvae and ventilate the brood.

4.3 *Chemoreception in insects*

This term refers to the method of communication, both through smell and taste. Generally the olfactory stimuli are originated from volatile molecules, while the gustatory ones originate from water-soluble molecules that act directly by contact. As widely known in insects the communication is intra and interspecific and recognition depend largely on the olfaction (Kaissling, 1987), and between the different and multiple substances to which these organisms respond we often refer to chemical signals (semiochemicals), known as pheromones and allochemicals. The pheromones (from the greek *phérō* "carry" and *hormàō* "exciting") are chemical messengers active between individuals of the same species, produced to induce the communication of information related to sexual reception, to provide a response to a danger, to be able to orientate in the search for food, but also to be able to maintain the typical hierarchical division of social insects (Birch, 1974). Generally a pheromone consists of a set of chemical compounds produced in specific proportions (Starrat *et al.,* 1979; Linn and Roelofs, 1989; Tumlinson *et al.,* 1989,1994), more represented by small organic, hydrophobic and volatile molecules, pick up even over long distances (Wilson, 1971; Kennedy, 1983). However, there are also other semiochemicals that are not volatile and require direct contact (contact pheromones), like the cuticular hydrocarbons.
Allochemicals are chemical messengers active even among different species, including allomones, kairomones and synomones (Brown, 1970). Allomones are chemical messengers issued in order to bring an advantage to the same individual that emits them; they generally have defensive function as repellents or deterrents. The kariomones are favorable exclusively for the species that receives them, as they act as stimulants for oviposition and nourishment; they are produced by the plant which in this way directs on itself the phytophagous insects (Bernays & Chapman, 1994; Bernays, 1995). The synomones benefit both to those who emit them and to those who receive them; the most classic example is represented by insects that take nourishment and other resources from the plant, leaving them the benefit of being pollinated. In social insects the communication between individuals of the same colony mediated by chemical signals represents a sort of complex language.

4.4 *The soluble chemoreception proteins*

The OBP*s*, the acronym of Odorant Binding Proteins, are small soluble olfactory proteins involved in the transport of odor molecules (=odorants) through the sensillum lymph to odorant receptors, which are housed on the dendritic membrane of olfactory sensory neurons of insects, also known as olfactory receptor neurons, as well as in the nasal mucosa of many vertebrates; even if the function is the same, they differ considerably from the structural point of view.

In vertebrates the OBPs are part of a lipocaline superfamily (Flower, 1996), an acidic nature folded in β-sheet domains to give rise to the typical barrel-β structure (Figure 11).

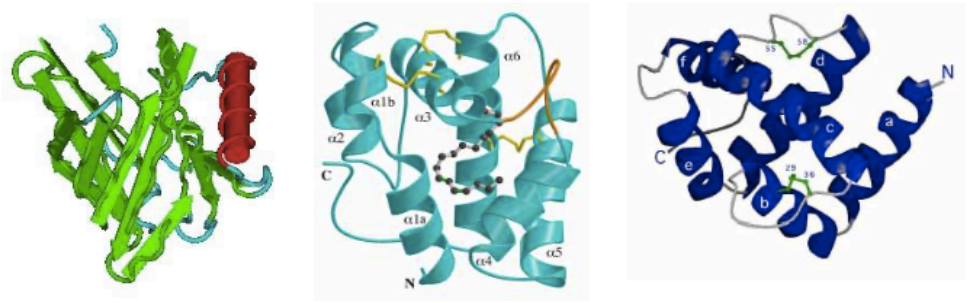


Fig. 11 - Three-dimensional structure of the vertebrate OBP (C), insect OBP (B) and CSP (C).

In insects there are two classes of polypeptides identified in the lymph of chemosensilla: OBP (odorant binding protein) and CSP (chemosensory protein).

These two families, such as vertebrate OBP*s*, are small and usually have a low isoelectric point but are greatly concentrated in perireceptory spaces. In any case, amino acid sequences and three-dimensional structure are different among these protein families; in the insects, both the OBP*s* and the CSP*s* have more α-propeller domains. The insect OBP*s* are formed by specific domains composed of six α-propeller connected by three disulfide bridges. This feature means that the structure is very compact; also the CSP*s* have a compact structure although they have only four α-propellers that form two small loops between adjacent residues.

These soluble proteins are able to reversely tie the smells and pheromones; in fact it is known that all chemical stimuli must interact with a large number of these proteins that can always modulate and modify the chemical message (Pelosi, 1994,1996, 1998, 2001; Pelosi & Maida, 1995; Steinbrecht, 1998; Tegoni *et al.,* 2000, Wanner *et al.,* 2004).

4.5 *OBP and CSP in insects, particularly in* Apis mellifera *L.*

The discovery of the OBP is relatively recent, in fact at the beginning of the 1980s the first OBP was observed in the giant *Antherea polyphemus* (Cramer) (Vogt & Riddiford, 1981) moth; this protein was called PBP, that is Pheromone Binding Protein. Initially the OBP study was limited only to moths and this allowed a moderately simple classification; only with the discovery of new OBP*s* in other insects we realized the divergence of these molecules.

The common feature, as observed by Scaloni *et al.,* 1999, resides in the fact that having a path of six α-propellers, in positions preserved in all the orders of insects such as to form three disulfuric bridges between the residues 1-3, 2- 5 and 4-6, makes the protein structure highly stable. Currently the availability of the complete sequences of the genomes of a large number of insects, and recently, also of *A. mellifera*, has allowed the identification of all the genes coding for the OBP*s* (Hummon, 2006; Pennisi *et al.,* 2007). It was possible to identify all coding genes for olfactory proteins. Compared to other insects the bees are equipped with a lower number of genes coding the OBP*s*; this highlights that the bees are equipped with other substances deputed to the discrimination of odors such as CSP*s* (chemosensory protein) that in Hymenoptera seem to be more specific to the smell with respect to the OBP (Ishida *et al.,* 2002; Calvello *et al.,* 2005). Furthermore, we must consider the large number of olfactory receptors present in the bee (Robertson *et al.,* 2006) compared with flies (Robertson *et al.,* 2003) and mosquitoes (Hill *et al.,* 2002); this could serve to compensate for reduced discrimination performed by carrier proteins. It should also be considered that the bee has extremely developed antennal lobes with about 160-170 glomeruli (Galizia *et al.,* 1999), which correspond to the 160-170 olfactory receptors (OR*s*).

4.6 *Bioindicators*

The instrumental and systematic use of biological events as indicators of anthropomorphic disturbances on the environment is relatively recent and, consequently, monitoring the conditions of the environment through bioindicators. As suggested by Celli & Porrini (1991) the definition of biological indicator, or bio-indicator, relates mainly to the biological structures able to indicate, through correlations of cause-effect an alteration of the environmental situation, attributable to a probable anthropic activity, especially of negative type.

An organic indicator is, by definition, therefore, a body that reacts in an observable, macroscopic or microscopic, visual or instrumentality, to the modifications of its ecological niche, or more generally of its biotope. More simply, with the term bioindicator we refer to any species particularly sensi-

tive to changes made by polluting factors in a given ecosystem; in theory, any living organism can be considered a potential biological indicator, as each organism responds to environmental modifications, but in reality a biological indicator must possess certain requirements such as adaptation, reperibility, economy of use, etc. (Celli & Porrini, 1991). One of the oldest suggestions regarding the possibility of resorting to organisms to obtain information on the state of health of a certain environment is the famous case of the *Biston betularia* (L.). The geometrid moth was the protagonist in England, at the end of the last century, of the phenomenon of industrial melanism.

Based on the type and entity of the reaction you can distinguish, in accordance with Ravera (1980), three categories of bio-indicators indicated below:

1. Indicator species: vegetable or animal organisms, whose presence or absence in an environment can be specifically associated with a certain type of pollution; they react with variations in the entity of populations to a specific type of pollution.
2. Real indicators: organisms that manifest morphological and / or structural modifications following the presence of a particular pollutant; the best ones, respond proportionally to the dose encountered.
3. Accumulators and / or collectors: accumulator organisms of pollutants. These are extremely useful in cases where pollutants are present in very low doses, since they concentrate them in their tissues (accumulators) or their products (collectors), without suffering lethal consequences, making them available for chemical analysis.

Plants, algae, insects, spiders, anellids etc... can be used as bioindicators. The use of plants is very frequent especially in the control of atmospheric and soil pollution. The techniques using vegetable biomonitors present a remarkable importance as they are relatively economical, they are applicable on quite vast and differentiated areas and allow to highlight the combined action of a pool of pollutants. The most frequently adopted option provides for the use of the so-called biaccumulator plants that respond with characteristic symptoms to a pollutant or / and accumulates it in its own tissues. One of the oldest examples is the use of the different cultivars of *N. tabacum* which result to be more or less resistant to pollution from ozone and, where present, show well-recognizable foliar symptoms, and the grapevine, that is particularly sensitive to the airborne compounds of the fluorine etc... As regards the anellids, for example, the importance of these terrestrial organisms is undoubted, if we consider the improvement they make to the structure of the soil and to the incorporation of organic matter. Their use as bioindicators lies in their sensitivity to the changes and imbalances of the edaphic environment; this makes them object to study as bioindicators for the soil ecosystem.

Excessive use, in agriculture, of copper products, has led to significant copper contributions in the soil with a consequent decrease in the populations of annellids, including the earthworms. Insects are also used effectively and efficiently as bio-indicators, and bees are extremely effective biological indicators for a number of reasons, which are illustrated in the following paragraph.

4.7 *Honey bee as bioindicator*

The honey bee is one of the insects on which many in-depth studies have been made and therefore the largest number of data is available. For these reasons the honey bee has been used for many years to test in laboratory the toxicity by ingestion or contact of products used in agriculture (Arzone *et al.,* 1980). In recent years the interest of the researchers has turned from the simple assessment of the risk connected to the introduction of new pesticides towards the honey bee until the assessment of agricultural pollution in its implications towards man. The use of bees in monitoring environmental pollution allows us to integrate and overcome some inherent limitations of chemical-physical methods, such as the expose of equipment and the need for specialized personnel. The honey bee is an effective bioindicator for the following reasons:

1) it indicates the chemical damage of the environment in which it lives, through two signals: high mortality in the case of pesticides and through the residues that can be found in their bodies, or in the products of the hive, in the case of pesticides and of other pollutants such as heavy metals and radionuclides, detected by laboratory analysis (Celli, 1994);

2) it is easy to breed;

3) it is an almost ubiquitous animal;

4) it does not have high food requirements;

5) it has the body relatively covered with hair that makes it particularly suitable for the interception of materials and substances with which it comes into contact and consequently these substances are carried inside the hive;

6) the high reproduction rate and the duration of the life cycle, relatively short, induces a fast and continuous regeneration in the hive;

7) it has a high mobility and a wide flight radius that allows to control a large area; in fact a hive perform numerous daily withdrawals, up to 10 million. Furthermore, withdrawals are homogeneous.

8) it scours all three environmental sectors (land, water, air, and also vegetation);

9) low management costs, especially in relation to the large number of samples made.

There are therefore real indicators as honey bees able to offer significant information on the status of a phenomenon to characterize and / or measure. Unlike other bioindicators, the bee can be defined as a travelling sensor and the covered area has a radius of 1.5 km (7 km^2).

The environment is constantly kept under control from the action of bees and if they come into contact with contaminated substances, they report them to the hive, thus making possible chemical analyses. It follows that the bees are able to quickly perceive all changes in environmental characteristics and also succeed in signalling it (Celli & Porrini, 1991).

It should also be reminded that the bee is not just a bio-indicator, but it is also:

- a biocollector: it stores extraneous substances by the environment (pollutants) in the products of excretion and / or secretion such as the royal jelly, wax, etc.
- a bioaccumulator: as pollutants are accumulated within their tissues in concentrations greater than those present in the surrounding environment.

The bees that are used as bioindicators are the foragers because they harvest pollen and nectar. Obviously there are some factors that limit the use of bee as an environmental bioindicator:

- flight activity depends on a temperature of at least 10°C, so, to our latitudes it cannot be used during the winter period;
- worker bees may not return to the origin hive for natural mortality, by drift (return to a different hive) or for mortality induced by pesticides;
- real-time detection of the entire family for stage and age is difficult to implement;
- it is not possible to control the choices of food sources by the entire family.

4.8 *Honey bee as heavy metal bioindicator*

The biomonitoring of heavy metals with bees is based on the principle that these contaminants can easily be "picked up" from the hair of the bees or be ingested through the pollen and nectar that will then be stored in the hive; in particular heavy metals can be picked up from the bees in the atmosphere through their hairy body and brought to the hive along with the pollen or the nectar of flowers, or water from puddle, ditches, fountains, streams or together with the honeydew of aphids. In this way, analyzing the recently deposited honey in the hive it is possible to determine the degree of contamination from heavy metals of a certain area. Bees and honey provide complementary information; in fact, while the amount of metals present in the second matrix derives from the nectar harvested by a wide area over several days, the information supplied by bees is relative to a few days preceding the capture. The fundamental characteristics that differentiate heavy metals from

other contaminants such as pesticides, is the type of placing in the territory and their environmental destiny. The pesticides are disseminated in a point-point manner and are degraded more or less by different environmental factors. Heavy metals are, on the other hand, continuously issued by the various sources, natural and anthropic and, not undergoing degradations, are continually remitted to "game" entering physical-biological cycles. The variables to consider during the use of the bees, or the alive products like the honey are several; among these we mention for example weather events (rain and wind are able to clean up the atmosphere or transfer heavy metals into other environmental sectors), seasonality (the nectariferous flow, usually greater in spring than in summer-autumn could dilute or not the contaminant) and the botanical origin of the honey (the honeydew of aphids, like nectar of flowers with open morphology, is much more exposed to contaminants than the nectar of flowers with closed morphology). However as suggested by Porrini *et al.,* 2000 it seems that honey is more reliable than the bees since the data are more repeatable.

4.9 *Factors conditioning worker honey bee activity*

The pollen collection follows a typical seasonal trend, rather constant from one year to another: in our regions it lasts from the end of february to late october, with a progressive increase during spring until it reaches the maximum peak that coincides with the months of May - June; and then follow a decrease during the late summer up to a recovery in September. The trend of the foraging activities is related to a series of internal and external factors to the hive intimately connected to each other, that can act as a stimulating or a limiting condition.

The internal factors that constitutes the fundamental stimulus for the harvest of pollen is the presence of miscellaneous brood: in fact, colonies with more consistent brood collect more pollen than others with less extensive hatching, while orphaned colonies that have exhausted the hatch completely cease the harvest. More simply, the pollen collection weight curve follows the progress of oviposition: it is no coincidence that the rhythm of activity of the queen is more intense in spring, slows down in the summer, up to suspend (summer diapause) and resumes within certain limits in autumn. Another internal factor that affects the collection is the geographical origin of the colony. Louveaux (1970) showed how, transferring families from different regions into the same location, some characteristics differences are obtained in the collection curves; in particular, families of Mediterranean origin started to harvest more early. The main external factors are represented by the biotic conditions (availability of flora) and abiotic (climate). As regards the weather conditions, as mentioned above, the temperature, which represents the main limiting factor at the end of winter, as

below 10° C the harvest does not take place, and even periods of bad weather can block momentarily the activity, which will resume as soon as adverse conditions end. The seasonal trend of blooms is another determining element for pollen harvest. In spring the abundance and variety of available species are highest, and this is in fact the period of the year in which there are the most huge crops. Starting from the end of June pollen sources become more limited and in the midst of summer only a small number of species (*Carduus* spp., *Verbascum* spp. etc.) manages to address the drought (period that coincides with the minimum point of the collection curve). It is obvious that the flora is also a limiting factor for the bees operative activity. Therefore there is a very close correlation between the activity of the hive and the external conditions: when the climate is more favorable and the flora is most abundant, the activity of the bees is maximum, and maximum is also the deposition by the queen and, of consequently, the development of the colony, which thus has the opportunity to fully exploit the most advantageous situation.

4.10 *Factors determining honey bee choice of plant species*

The worker bee performs a very precise selection towards the choice of a species at the expense of another, and the factors that orient the bee towards this choice are different. Mainly to be a target is the flora that is within a radius of 500 m from the hive, but in case of need the bee can still push itself towards more distant areas, an aspect that is conditioned, of course, also by climatic activity, as it is obvious that in case of bad weather the bee does not push much further than the position of the hive. The favorite species, which constitute the basis of the crops, are the most widespread and offering more abundant blooms. Different is the situation regarding secondary species: the latter are collected in a much greater number, but in a very varied way by the different beehives, and their presence in later years is much more discontinuous. The largest availability of a kind rather than another is not the only criterion on which the foragers base their choice. This is the case for example of anemogamous plants: although there are some of great beekeeping importance such as *Quercus* and / or *Lazea*, most of them are not visited or only in the absence of other resources, but always at very low levels. It is evident that other factors intervene in the choice; one of these resides in the biological value of the pollen, which is not the same for all pollens. Maurizio (1966, 1971, 1979) ranked three pollen groups: very active, poor and inactive; the latter in some cases can even be harmful, and include most anemophilic pollens, which are the least appetited by bees. As a pollen component it is responsible for its most or less high biological value it is not yet known completely, and certainly an essential role is determined by the protein constituents that are present-

ed to 15-25%. Another action that influences the choice of the species, and that sometimes appears to be decisive, is the action that man exercises on the possibility of foraging by the bees, and that one extrinsecates through changes of the environment in which the colony lives. If on the one hand the introduction to extensive territories of monoflora crops guarantees, although for a limited period of time, abundant nourishment, from the other the decisive reduction of the surface available to the spontaneous flora deprive these insects of a large number of resources. The most emblematic example is that of *Rhoeas papaver* L. which until a few years ago was the main component of the wheat crops while currently preserves this role only in a few places.

CHAPTER V: MATERIAL AND METHODS

5.1 *Plant material and experimental conditions*

The trials were conducted using alfalfa (Medicago sativa L.) because it is a very nectariferous and poliannual plant. Alfalfa seeds were surface sterilized with NaClO with 3% of active Cl. Three groups of seeds of alfalfa plants were sown in 10 plastic pots (1.2 dm^3) for group, filled with 1000 g Dry Weight (DW) of medium-textured soil [clay : peat : sand 40 : 40 : 20 (v/v/v/)] with a field water capacity of 35%. At 20 Days After Sowing (DAS), when five to six leaves were fully expanded, the first group of plants (Control: CNT) was grown under optimal water conditions by restoring evapotranspiration loss with water. The second group of plants (Cd mild treatment) was irrigated with a solution of cadmium sulphate sufficient to reach 10 ppm of Cd in the mass of the soil mixture (208/114 .10= 18,4 mg in 250 cc water). The third group of plants (Cd severe treatment) was irrigated with a solution of cadmium sulphate sufficient to reach 20 ppm of Cd in the mass of the soil mixture. (208/114 .20= 36,5 mg in 250 cc water as field capacity). Subsequently all 3 groups of plants were irrigated every 3 days with only spring water to compensate lost water for evapotranspiration until the end of the experiment. Evapotranspiration was estimated by the pot weight loss before each irrigation. The average daily evapotranspiration from the pots was approx. 15% of field water capacity. All plants were grown in a growth chamber under the same conditions of temperature, light and humidity (day/night temperatures: 27/20°C; light pe-riod: 16 h; day / night RH: 50 – 70%). When the plants were fully flowered (at 40 DAS), 3 leaves and 3 flowers ontogenetically similar of each group of 3 replicates were cutfrozen in liquid nitrogen, and used to determine the content of Cd, amino acids, proline and products of secondary metabolism. At the same time the Cd content in the mixture of soils of the three groups of pots was determined. The seedlings were harvested for: heavy metal quantification in leaves and flowers; proline quantification in plant materials; extraction by flowers and leaves and GC-MS analysis.

5.2 *Determination of Cd concentration in leaves, flowers and soil mixture*

Aliquots of the frozen powder of the 40 DAS samples of flowers and leaves were dried at 90°C for 24 h and were extracted by digestion in a 10 mL solution of HNO_3 (65%), $HClO_4$ (65%), and distilled water [1.0 : 5.0 : 2.5 (v/v/v)]. The solution was kept at 100°C for 4 h, diluted to 25 mL with 100 mM HCl, filtered through a Whatman No. 2 filter paper, and analyzed using ICP-OES spectrometry (ICP-OES Varian Liberty). The initial Cd contents, after added (24 DAS) and at 40 DAS of the soil mixture pulverized in an agata mortar were also determined.

The Cd soil element was

extracted according to Carter & Gregorich (2007) and analyzed. The samples of plant material were dried and grounded prior the mineralization. Mineralization was made with VELP DK6 using a solution of *aqua regia* (3:1 HCl:HNO_3) for 45' at 180°, weighing 0.7 mg of each sample.

5.3 *Proline contents*

Three plants randomly selected were used for amino acids and proline determination. The last leaves completely expanded and the last flowers completely blossomed were collected for investigations . Aliquots of finely powdered samples of leaves (100 mg fresh weight, fw) and roots (25 g fw) were suspended in 2 mL of ethanol/water [80 : 20 (v/v)]. After 30 min, the suspension was collected and centrifuged. The supernatant was used to determine amino acids. The primary amino acids were determined by autosampler-assisted pre-column derivatization by o-phthaldialdehyde (OPA), separation by reverse-phase HPLC, and fluorescence detection (excitation at 340 nm and emission at 450 nm) (Di Martino *et al.,* 2003). Proline was determined with HPLC as fluorescent 9-fluorenylmethoxycarbonyl derivative (P-FMOCcarbamate) on sample extracts that were previously derivatized by OPA reagent to remove the primary amino acids and was fluorimetrically detected (excitation at 266 nm and emission at 305 nm; Di Martino *et al.,* 2006). To test the effectiveness of the derivatization random samples known concentration of proline have been included; the retrieval represents the recovery % of increment; for retrieval has been used the following formula: Sample increment - Reference sample / Increment

• *Derivatizations*

The amino acid standards (Sigma Chemicals) and samples were derivatized in 6-ml glass vials with polythene press-caps with FMOC-CI by adding 0.1 ml of reagent and 0.1 ml of borate buffer solution to 0.4 ml of sample. The reaction was carried out at room temperature and was completed within a minute.

• *Instrumentation*

The chromatographic separations and determinations were carried out with a HPLC system. A 20 µL injection loop was used and separations were carried out with Zorbax Extended C-18 4.6 x 250 mm 5 micron Agilent. Detectors used were a DAD wavelength UV /VIS detector set at 263 nm for the samples with higher concentration. The column operated at 25°C with a flow rate of 1.0 mL/min using 50mM acetate buffer (pH 4.2) as eluent A and acetonitrile as eluent B. Amino acids were carried out in 47 minute; proline had a retention time of about 23 min.

5.4 *Plants VOCs and photosynthetic measurements*

VOC*s* emitted by plant samples were measured by using a PTR-ToF-MS 1000 ultra (Ionicon Analytik GmbH, Innsbruck, Austria) (Figure 12) connected on-line to a portable gas exchange system GFS-3000 (Waltz, Effeltrich, Germany). GFS-3000 system was equipped with an environmental-controlled cuvette (2 cm^2 area) and a fluorimeter for sample illumination and fluorescence measurements. Photosynthetic measurements were carried out with light intensity 1000 µmol m^{-2} s^{-1}, leaf temperature 30°C, chamber air humidity 50%, and CO_2 concentration 400 mmol mol^{-1}. The leaf was stabilized under the standard conditions until stomata opened and steady-state of CO_2 and water vapour exchange rates were reached. Steady-state values of net assimilation (A) and stomatal conductance to water vapor (gs) were detected. VOC*s* plant profiles were measured by direct injection of the volatile mixture that came out from the GFS-3000 cuvette into the PTR-qTOF-MS drift tube via a heated (80°C) peek inlet tube, with a flow rate of 100 sccm, in a mass range between 20-300 m/z for 360 s. The drift tube conditions were 3.8 mbar pressure, 80°C temperature and 1000 V resulting in a field density ratio E/N of 141 Td (Townsend: 1 Td = 10^{-17} V cm^{-2}). PTR-ToF-MS analyses were carried out in an air-conditioned room with a constant temperature of 25 ±1°C, the VOC*s* protonation was carried out by using H_3O^+ as proton donor in the transfer reaction and was effective for VOC*s* having a proton affinity higher than that of H_2O (691.7 kJ mol^{-1}). Three technical and three independent biological replicates were carried out for each condition.

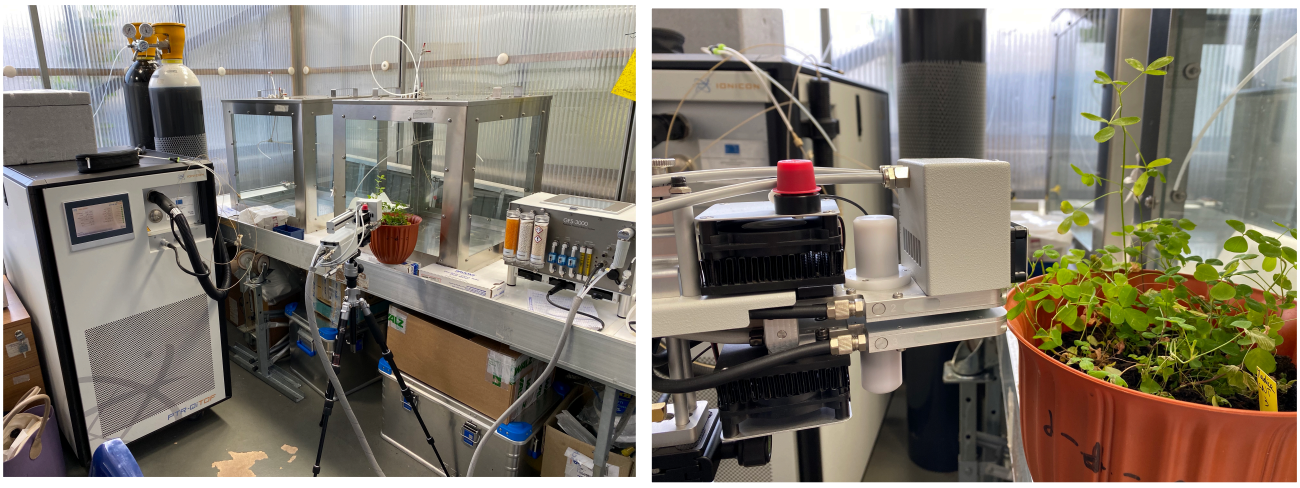


Fig. 12 - PTR-ToF-MS 1000 ultra (Ionicon Analytik GmbH, Innsbruck, Austria).

5.5 *Gas chromatography-mass spectrometry (GC-MS)*

The hexane extracts (100 µl) were concentrated to about 10µl under a gentle nitrogen stream, and 1µl volume was analyzed by a 7890B series gas chromatograph (Agilent Technologies) coupled with an Agilent 5977A Mass Selective Detector (MSD) (Figure 13) and equipped with a HP-5MS capillary column (30 m × 0.25 mm i.d., × 0.5 µm film thickness, J&W Scientific Inc., Folsom, CA, USA). The carrier gas was helium at a flow rate of 1.25 mL/min. The injection was made in the splitless mode, and the injector temperature was 250°C. The column oven temperature was initially programmed from 60°C to 250°C at 5°C/min, with a final holding time of 10 min. Spectra were recorded in the electron impact mode (ionization energy, 70 eV) in a range of 15–550 amu at 2.9 scans/s. A solvent delay time of 4 min was used. Each extract was analyzed in triplicate. Solvent controls were analyzed to check for interferences. The identification of VOC*s* was achieved by comparing mass spectra with those of the data system library (NIST08, p >90%), and, wherever possible, by comparing retention times (R.T.) and mass spectra with those of commercially available standards. Moreover, a mixture of a continuous series of straight-chain hydrocarbons, C5–C40 (Alkane Standard Solution C6–C40, Sigma Aldrich, Milan, Italy), was injected into an HP-5MS column under the same conditions previously described for the hexane extracts to obtain the linear retention indices (RI*s*) (Vandendool & Kratz, 1963). Component relative percentages were calculated based on GC peak areas. Each extract was analysed in triplicate. The relative abundance of each compounds was calculated using the integrated peak area data from the GC-MS trace.

Fig. 13 - GC-MS Agilent 5977A mass selective detector (MSD).

5.6 *Electroantennography (EAG)*

The antennal sensibility of honeybee foragers to increasing amounts (25, 50, 100 µL) of each stimulus was measured using the EAG technique (Figure 14) described in previous studies (De Cristofaro *et al.,* 2004; Germinara *et al.,* 2017). A glass micropipette (0.2 - 0.3 mm i.d.) filled with 0,1 M KCl solution was inserted into a head dissected from bee, and used as the indifferent electrode.

The recording electrode was put in contact with the last antennal segment from which the distal tip had been cut. The antenna has been exposed parallel to the airflow direction. Ag-Cl coated silver wires were used to maintain the electrical continuity between the antennal preparation and an AC/DC UN-6 amplifier in DC mode connected to a PC equipped with the EAG 2.0 program (Syntech Laboratories, Hilversum, The Netherlands). A stream of charcoal-filtered humidified air (500 ml / min) was directed constantly onto the antenna through a stainless steel delivery tube (1 cm i.d.) with the outlet positioned at approximately 1 cm from the antenna. Test stimuli were extracts in *n*-hexane of leaves and flowers of alfalfa plants (Figure 14). Twenty five microliters of each stimulus was absorbed onto a filter paper (Whatman No. 1) strip (1 cm x 2 cm) inserted in a Pasteur pipette (15 cm long) and used as an odour cartridge. Over 1 s, 2.5 cm^3 of vapour from an odour cartridge were blown by a disposable syringe into the air stream flowing over the antennal preparation. The control (100 µL *n*-hexane) and the standard stimulus [25 µL of (*Z*)-3-hexen-1-ol dissolved (10 µg/µl) in *n*-hexane] were applied at the beginning and at the end of the experiment, and in addition, the standard stimulus was applied after each group of test odours, to evaluate the gradual decrease in the antennal sensitivity over time. Intervals between stimuli were 1 min.

The extracts were stored at −20°C until use. Hexane extracts were presented in ascending doses. EAG responses were recorded from 12 right antennae and 12 from left ones of different honey bees. The absolute amplitude (mV) of the EAG response to each test stimulus was corrected to compensate for solvent and/or mechanosensory artefacts according to Raguso & Light (1998) lightly modified. The Student's *t*-test for independent samples was used to compare the mean EAG responses of the right antennae and those of the left ones. Corrected mean EAG responses, to the same dose of different extracts, were analysed by one-way ANOVA, followed by Tukey test ($P<0.05$) for separation of means.

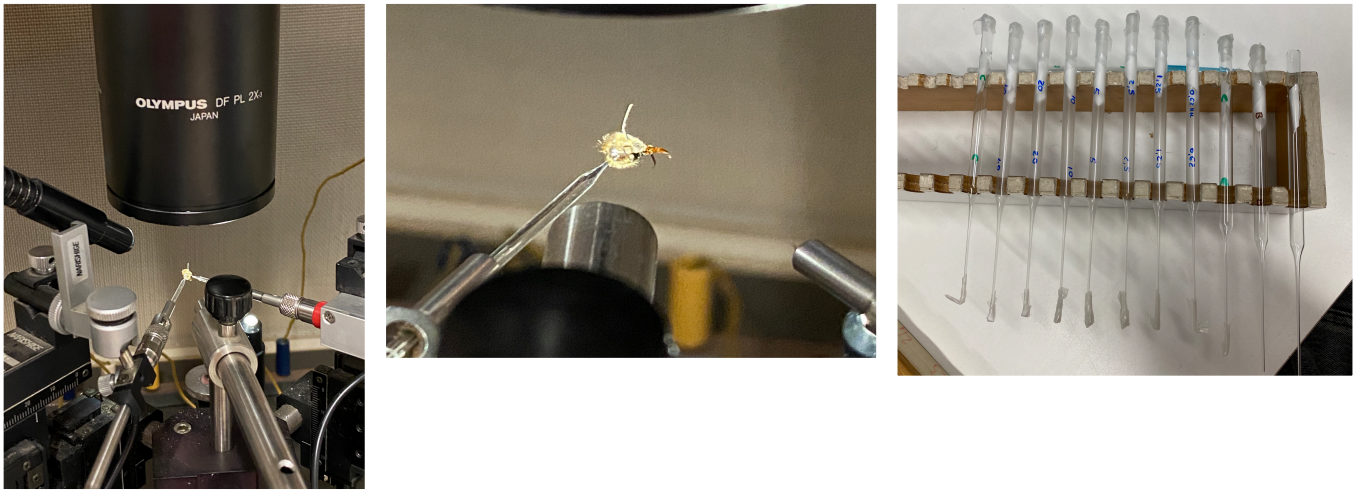


Fig. 14 - EAG apparatus and test stimuli.

Honey bees used in this study were from *A. m. ligustica*, maintained in the privately hives near the University of Molise (Campobasso, Italy).

5.7 *Honey bee feed test*

Honeybees used in this study were from *A. m. ligustica* (Figure 15), maintained in the experimental apiary near the IPSP-CNR. Newly emerged bees used in all of the trial were obtained daily from brood frames taken from the experimental hives and kept in controlled temperature room at 34°C and 60% RH.

Fig. 15 - Honey bees used for experiments.

This experimental part was conducted at Portici IPS-CNR. For each trial, 30 bees were placed in a plastic box (10 x 10 x 13 cm) (Figure 16) with five feeding tubes consisting of 2 ml Eppendorf tubes. Four 3 mm holes were drilled in the top side of each tube.

Fig. 16 - Plastic box (10 x 10 x 13 cm) used for feed test.

Two feeding tubes were filled with 1.0 M sucrose (342.3 g up to 1 liter with H_2O); two with 1.0 M sucrose plus a treatment solution [proline at 5 serial dilution from 0 (control) to 6,4 mMol] and one with water. Each one of the ten Essential Amino Acids (EAA: Arg, His, I-Leu, Leu, Lys, Met, Phe, Thr, Try, Val) was added to a 1.0 M sucrose to reach the ratio 1:50 (EAA:C solution).

The boxes were placed in a constant temperature room at 34°C and 60% RH. All experiments were continued for 14 days, while the number of dead bees in each box was counted daily. Evaporation control box containing no bees but the same solutions to monitor evaporation rates of each one were provided. These boxes are treated the same as those containing bees by replacing the diets on a daily basis. Feeding tubes have been weighed on day one after being filled, and on subsequent days before and after re-filling the tubes with solution. The difference equates to the amount consumed in a 24 hour period. All experiments were replicated three times. For diet preparation, to make a 1:10 ratio of amino acids to carbohydrate, we weigh out the following amounts of each amino acid and then make up to 100 ml with 1 M sucrose solution,

Amino acid	Weight (g)
Methionine	0.149
Tryptophan	0.204
Arginine	0.174
Lysine	0.146
Histidine	0.155
Phenylalanine	0.165
Iso-Leucine	0.131
Threonine	0.119
Leucine	0.131
Valine	0.117

and we dilute 1:10 amino acid stock solution further with 1M sucrose to make each of the appropriate ratio.

Ratio	V2	C1	C2	1:10 AA stock solution (ml)	1M ucrose stock (ml)
1:50	100	0.1	0.02	20	80

To work out any new ratios, the following formula can be used: $V_1C_1=V_2C_2$, and by algebric rearrangement: $V_1=(V_2 \times C_2) / C_1$ (V=volume, C=concentration, 1=stock solution, 2=new solution).

CHAPTER 6 – RESULTS, DISCUSSION AND CONCLUSION

6.1 *Cd accumulation in* Medicago sativa *L. flowers, leaves and roots*

At 24 DAS when Cd sulfate was added, the concentrations of Cd in the soil mixture reached 10 and 20 ppm in pots to generate conditions for mild and severe stress respectively. When flowering was completed, i.e. at 40 DAS (days after sowing), the Cd concentrations in the leaves were 5.3 and 10.2, whereas in the flowers were 4.3 and 9.3 ppm fw (fresh weight) in the two treatments respectively. Since the atomic weight of Cd is 112, with a simple stechiometric calculation it is possible to establish that the moles of Cd in the mesophyll and in the floral tissue reaches, under severe conditions stress, 35 and 80 nmol/g fw respectively which corresponds to 40μM and 95μM if it is assumed that the water content in plant tissues varies from 80 to 90% (Gonzalez 2001) Since Cd is not homogeneously distributed in the tissue, but confined in cellular compartments, it is presumed that the previously calculated concentrations can be significantly higher within cellular organelles such as chloroplasts and mitochondria. On the other hand the concentrations of Cd, in the roots at 40 DAS, reached 60 and 135 nmol/g fw in the two treatments respectively.

6.2 *Proline and amino acids pattern in leaves and flowers of Cd treated plants*

Since a state of stress always generates metabolic alterations, a biochemical index of this condition is given by the modifications of the amino acid pattern. For this reason amino acids determination and a comparative investigation was carried out on flowers and leaves of Cd treated plants and control plants.

At 47 DAS corresponding at 23 DAC (days after adding cadmium) all amino acids distribution and content, except for proline, were still similar in both mild stressed (Cd 10 mM) and control plants, in leaves as well as in flowers. The proline increase was 3 and 5 fold the control in leaves and flowers, respectively. In the amino acids pattern of leaves and flowers, the most plenty free amino acids were glutamate, aspartate, glutamine, alanine, glycine and serine. The pool of other amino acids (leucine, isoleucine, valine, lysine and tryptophan) was approximately 1% of the overall amount. Glutamate, glutamine and aspartate content also slightly increased, compared to controls, 0.9, 0.7 and 0.8 fold, respectively. These four amino acids accounted for 56% and 50% of the overall amino acid content of mild Cd stressed leaves and flowers, respectively (Figure 17).

At 23 DAC in severe Cd treatment (Cd 20 ppm), the proline increase was 7 and 9 fold the control in flowers and leaves, respectively. Glutamine, glutamate, and aspartate, decreased by 20% compared with 16 DAC conditions in both leaves and flowers. Among the most abundant amino acids, alanine

did not change. Leucine, isoleucine, valine and tryptophan also did not change significantly. Conversely, proline further increases in both leaves and flowers reaching 1.8 and 1.5 µmol/g fw and the 23% and 25% of the total amino acids in the leaves and flowers respectively. If we consider that the fresh weight in the leaves and flowers is made up of 90% water, 1.85 and 1.6 µmol/g fw correspond to (1.8/0.9) 2 mM and (1.6/0.9) 1.77 mM. Interestingly, the marked increase of the amino acids Gly and Ser in leaves, and aromatic amino acids Tyr, Trp and Phe in both leaves and flowers, was more 100% than control (Figure 17).

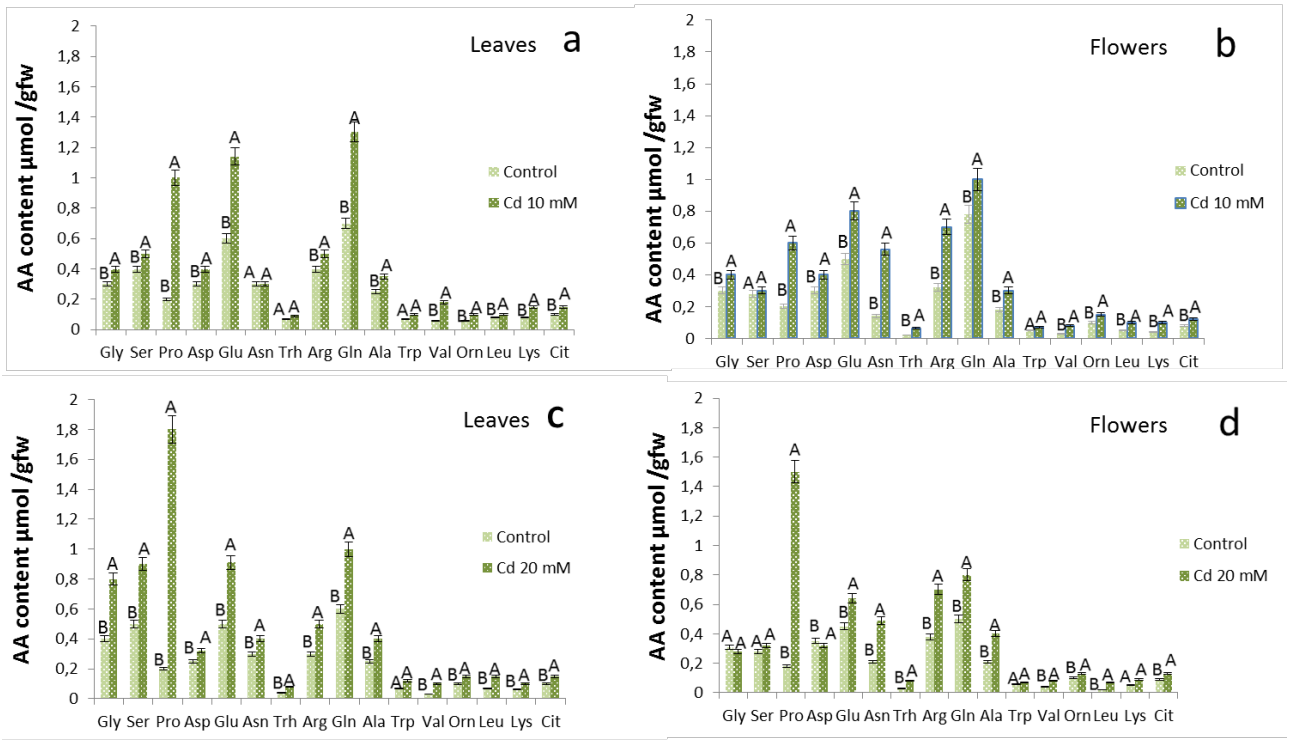


Fig. 17 – Amino acids contents in treated and control plants (leaves and flowers) of *M. sativa*.

6.3 *VOCs emission by Cd treated plants*

The analyses of the samples of *M. sativa* plants (CNT, 10 ppm Cd and 20 ppm Cd) were conducted at IPSP-CNR of Portici (Naples) (Table 1).

Samples	Features (positive)	Features (zero)
10ppmCd 1	30	14
10ppmCd 2	30	14
10ppmCd 3	30	14
20ppmCd 1	35	9
20ppmCd 2	35	9
20ppmCd 3	35	9
CNT 1	34	10
CNT 2	34	10
CNT 3	34	10

Table 1: Summary of data processing results.

The plant interactions with biotic and abiotic factors is often the cause of VOC*s* emissions which represent main molecular vectors for the communication between plants and the surrounding biological environment. VOCs analysis, using a VOCs analyzer on the third and fourth leaves completely expanded by the vegetative apex of plants of *M. sativa*, showed 34 different peaks associated with molecules or classes of molecules differently distributed between the leaves of control plants and plants treated with Cd. In the perspective and in the need to give significance to the data, as ANOVA only tells whether the overall comparison is significant or not, it is usually followed by post-hoc analyses in order to identify which two levels are different. For this purpose, Fisher's least significant difference test (Fisher's LSD) was applied. 34 peaks were significant ($p< 0.05$) (Figures 18 A, B, C). The Principal Component Analysis (PCA) is an unsupervised method aiming to find the directions that best explain the variance in a dataset (X) without referring to class labels (Y). The data are summarized into much fewer variables called scores which are a weighted average of the original variables. Before any analysis data were transformed and scaled (Data transformation: Log_{10} Normalization; Data scaling: Autoscaling). Univariate analysis methods are the most common methods used for exploratory data analysis.

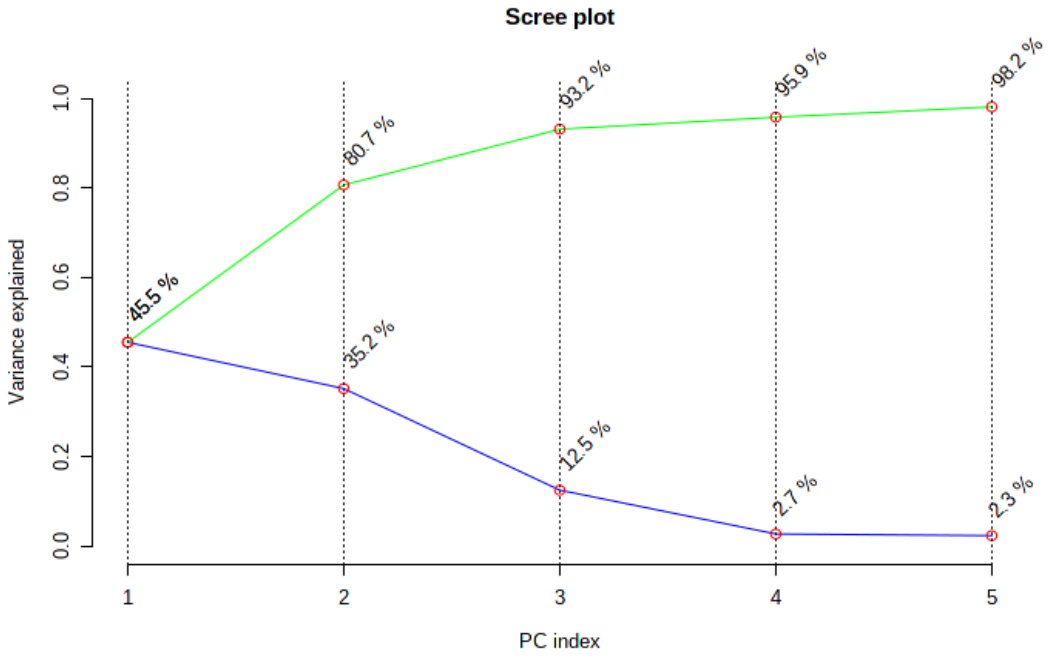


Fig. 18 A - The Scree plot shows the variance explained by PCs. The green line on top shows the accumulated variance explained; the blue line underneath shows the variance explained by individual PC.

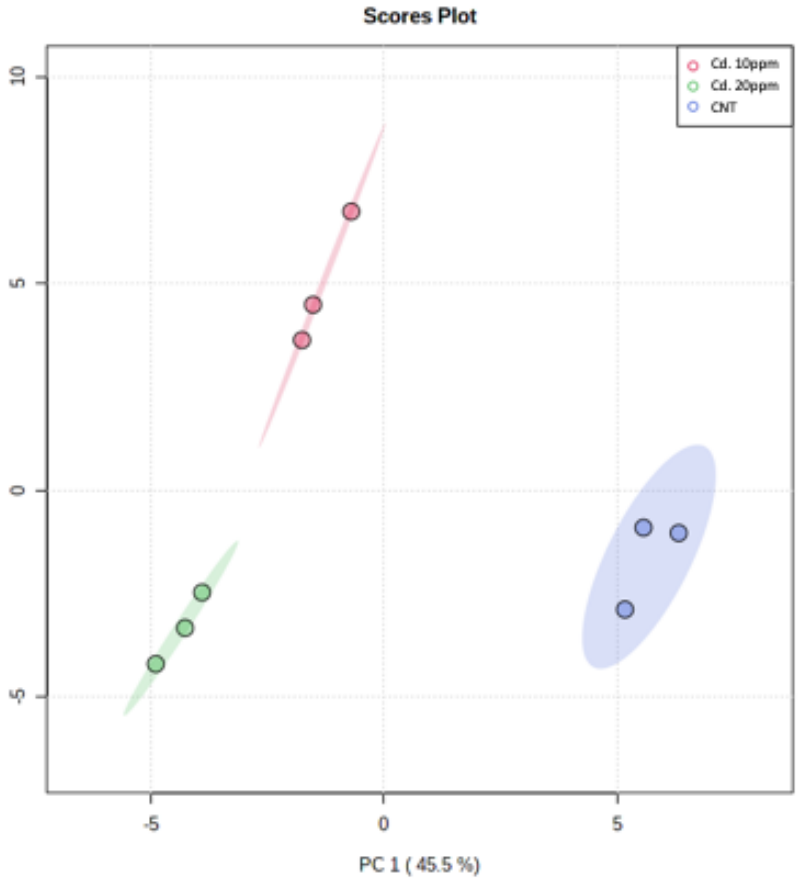


Fig. 18 B - Scores plot between the selected PCs. The explained variances are shown in brackets. 80,7 % of total variance is explained by the first two PCs. PCA analysis highlights that all samples belong to a different cluster depending on the treatment. Control is well separated by treatments on PC1 (Positive values), while the two different treatments with Cd are plotted separated each other on PC2.

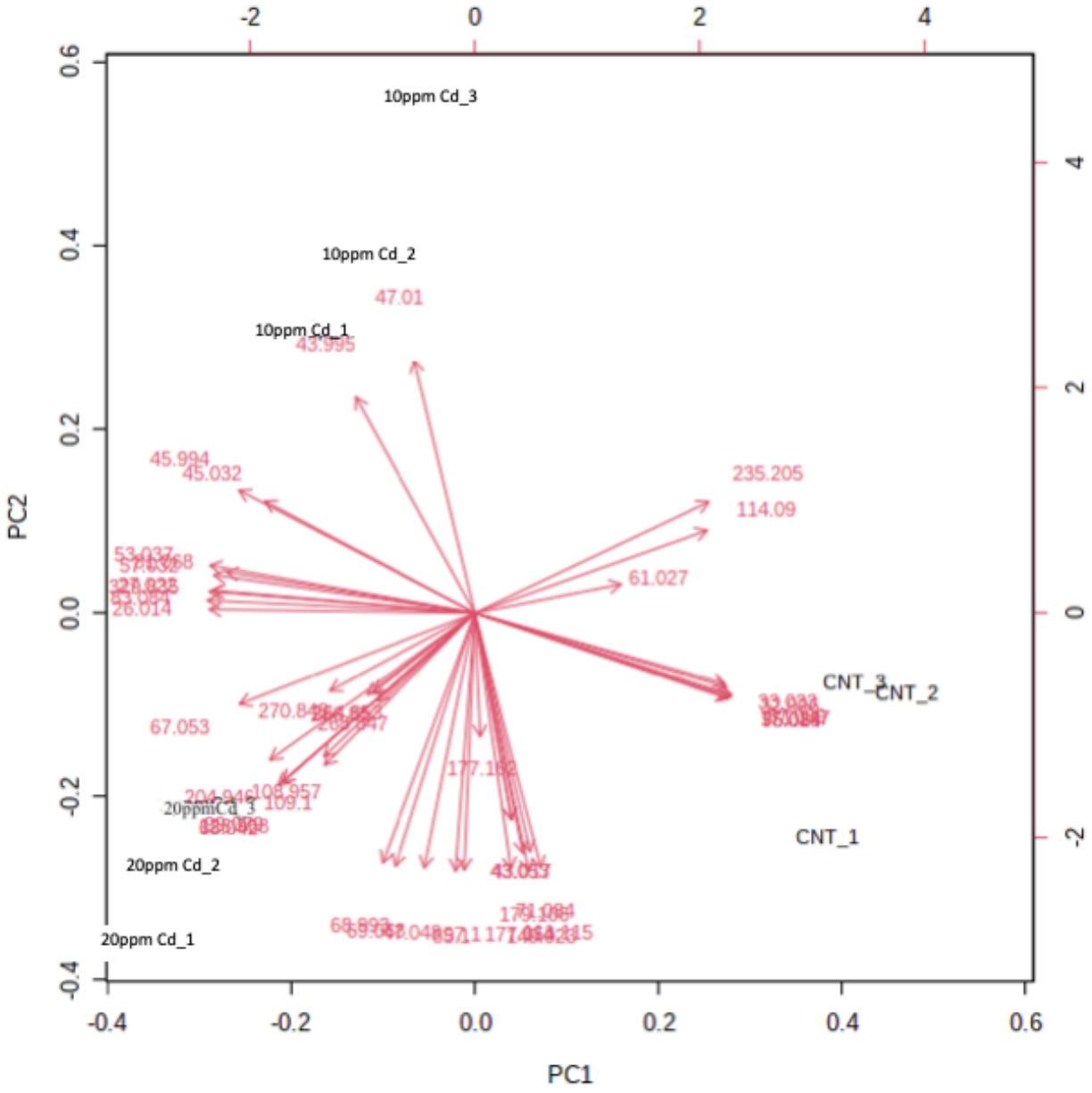


Fig. 18 C - PCA analysis. Positively correlated variables are grouped together. Negatively correlated variables are positioned on opposite sides of the plot origin (opposed quadrants). The distance between variables and the origin measures the quality of the variables on the factor map. Variables that are away from the origin are well represented on the factor map.

A better visualization of the results is achieved by hierarchical cluster analysis where each sample begins as a separate cluster and the algorithm proceeds to combine them until all samples belong to one cluster. Figure 19 shows the clustering result in the form of a heatmap.

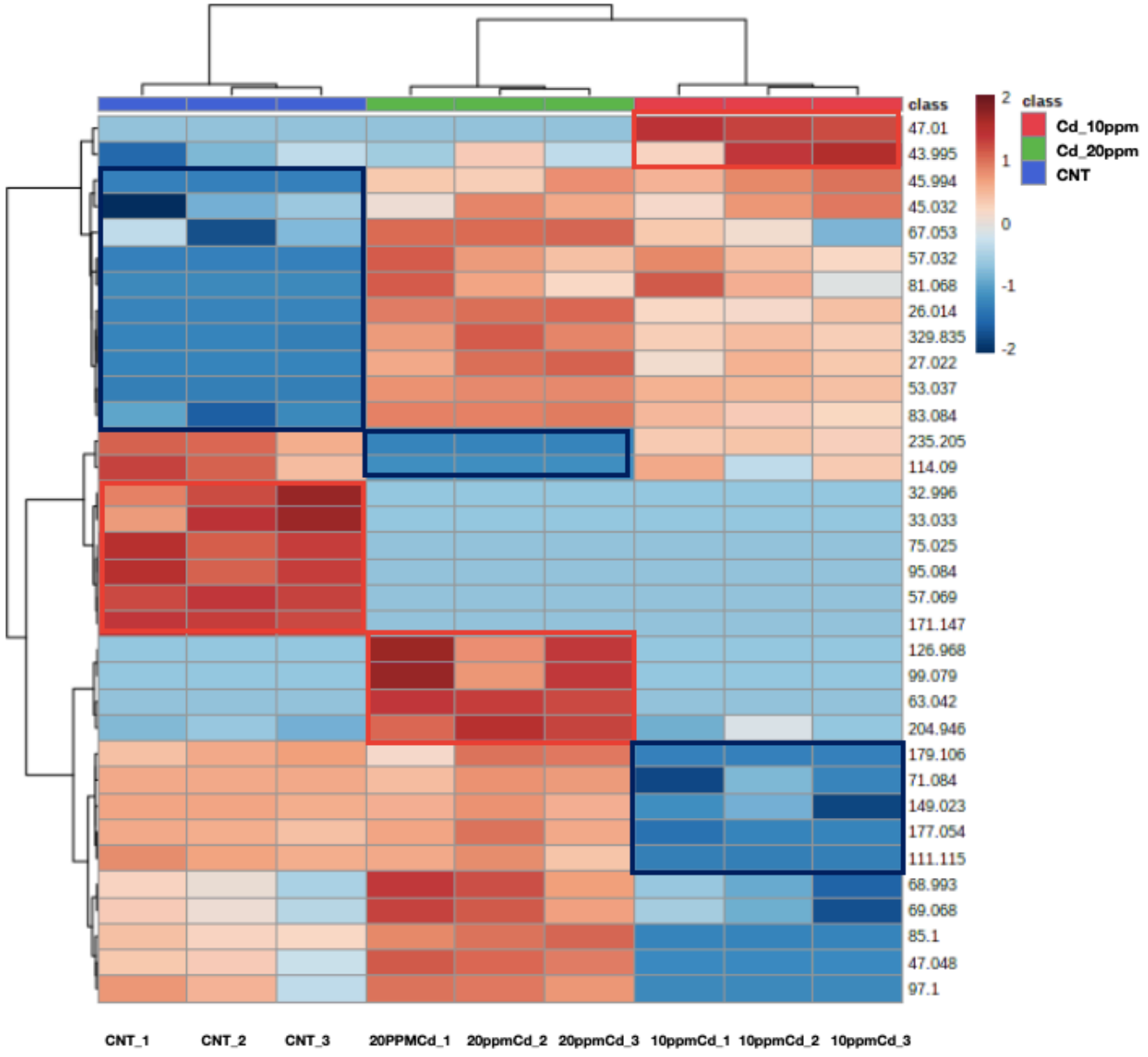
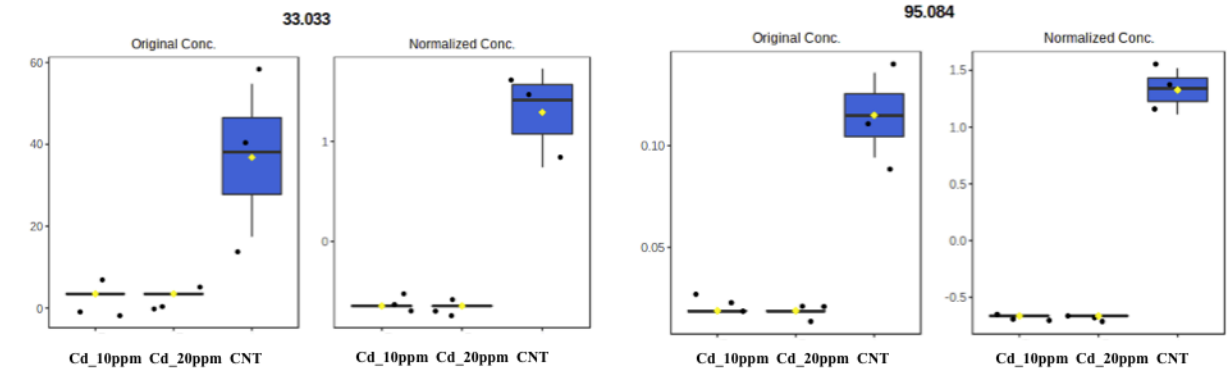
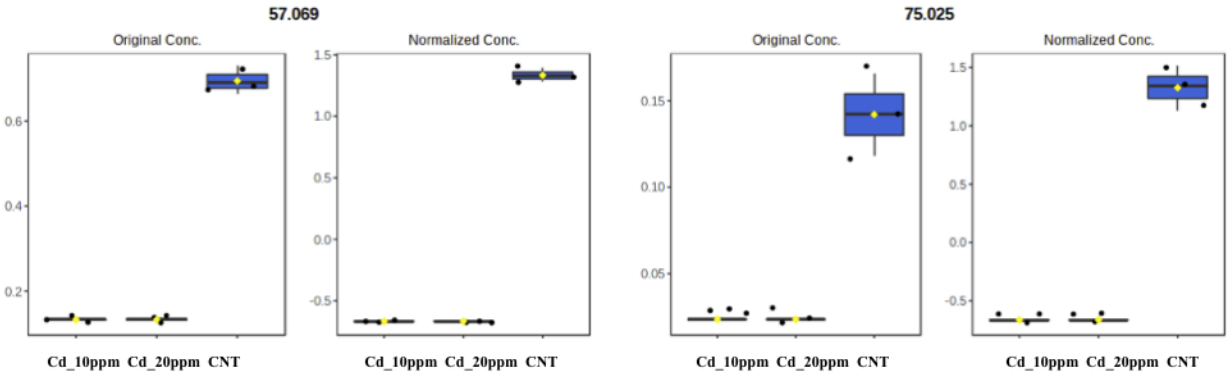
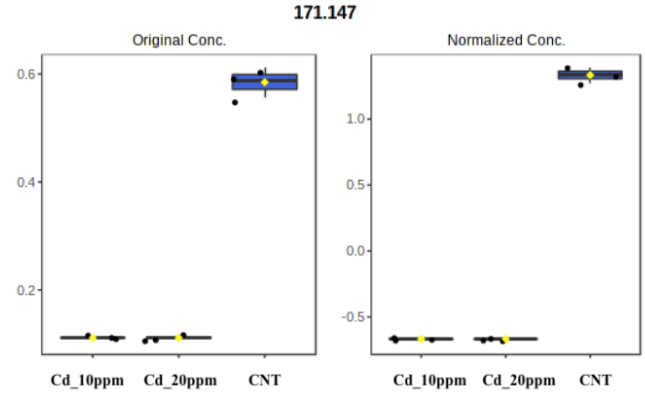


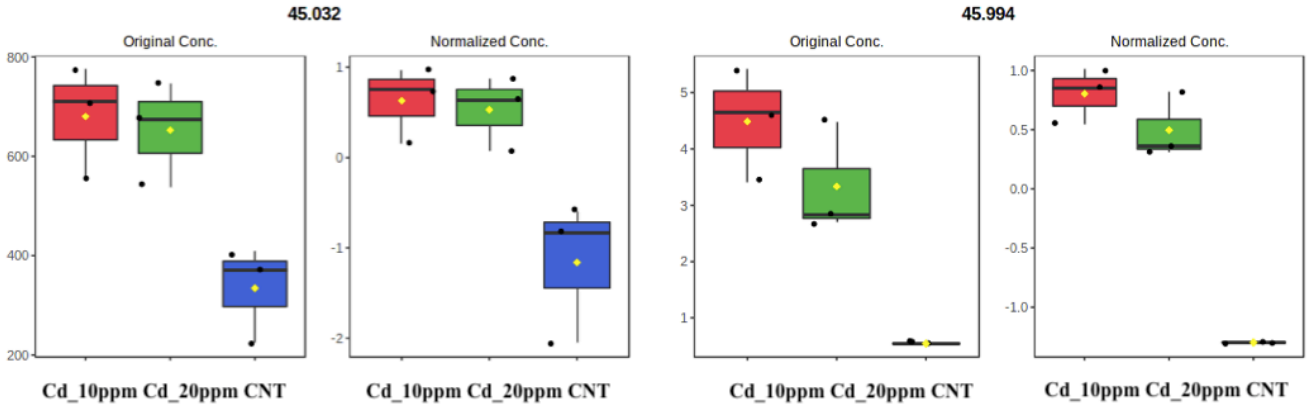
Fig. 19 - Clustering result in the form of a heatmap with samples grouped in a dendrogram (distance measure using Pearson, and clustering algorithm using Ward. D). Only ANOVA significant peaks are represented.There are clusters of peaks over emitted in only one kind of samples, as those in red squares, or that are under emitted in some cases (blue squares).

Molecules with different behavior can be identified, both by using PCA analysis and heat maps and, just as an example, they are shown in the following figures, were molecular weigh are indicated. In fact, a group of volatiles are strongly emitted by CNT, and inhibited in Cd treated plants, or vicev-ersa.

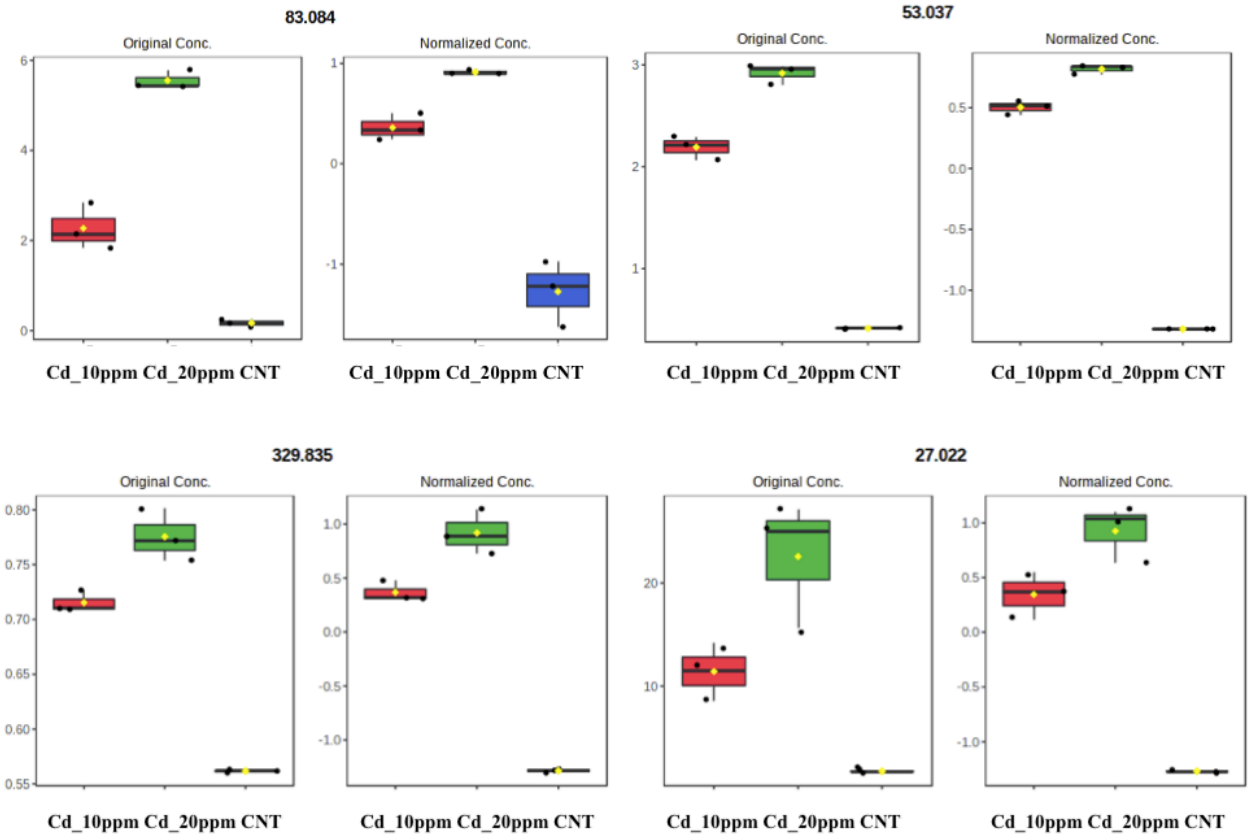




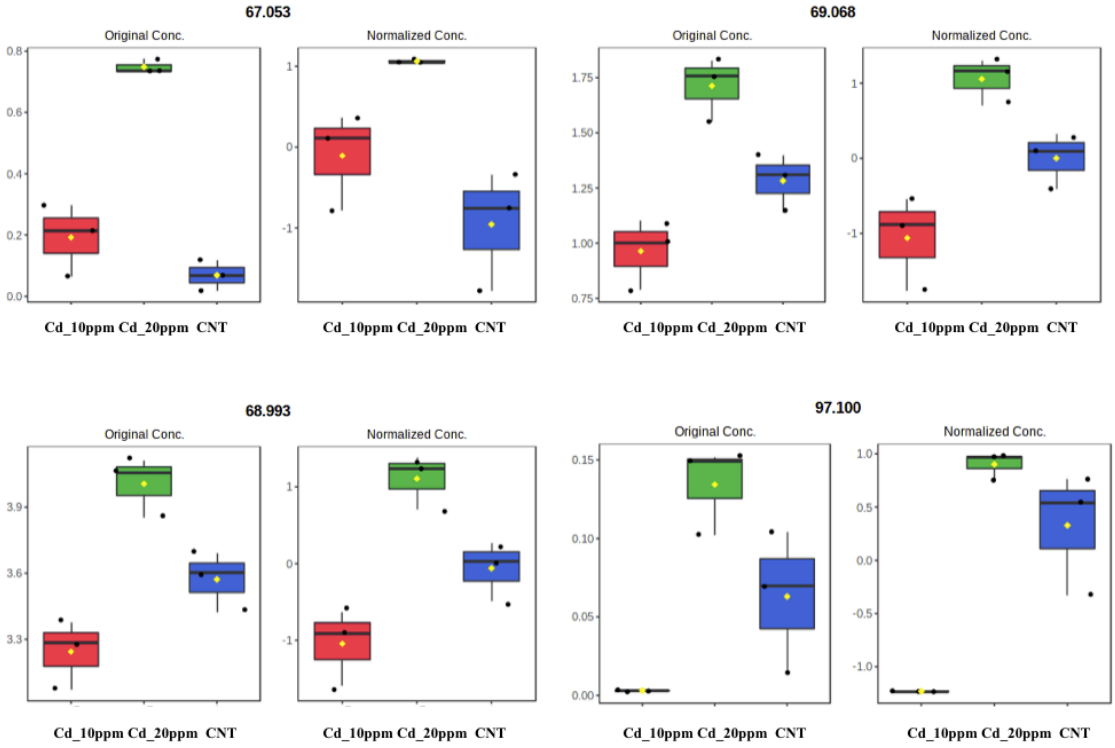




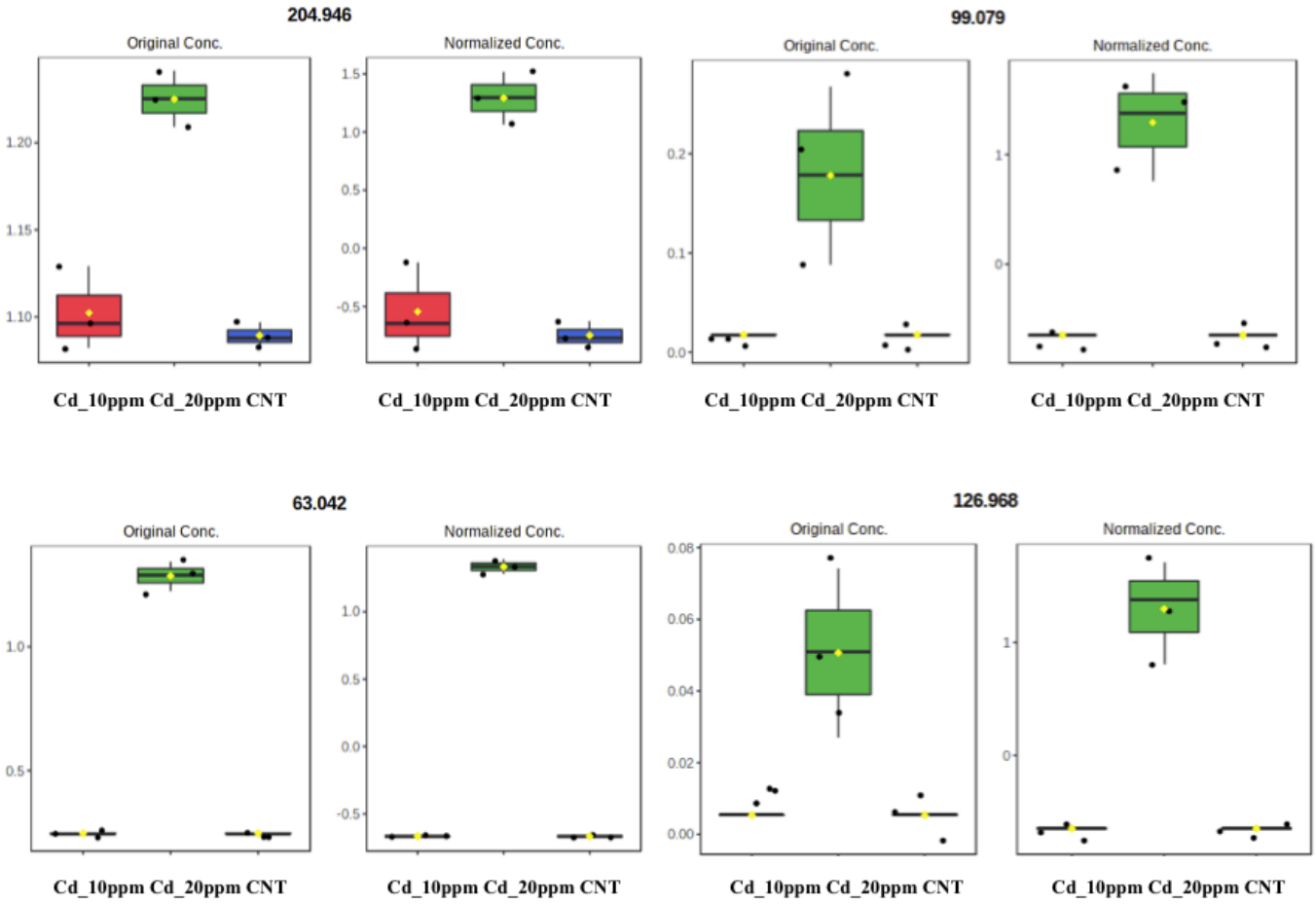
There was a group of volatiles with higher emission in Cd treated plants and the emission was dose dependent.



A group whose emission is inhibited by 10 ppm Cd treatment but stimulated by a higher dose of Cd, was compared to the control.



A group of volatile compounds whose emission seems to be enhanced only by 20 ppm Cd is also showed.



From mass analysis, 25% belongs to volatile substances that are not produced from leaves of plants contaminated with Cd, such as: methanol, alkyl fragment and propanoic acid. 50% of emerging peaks belongs to a group of volatiles with higher emission in Cd treated plants, and dose dependent such as acetaldheyde, hexanal fragment, terpene fragment, 2-methyl-1,3-butadiene (isoprene), and 25% belongs to a group of volatiles with higher emission only by 20 ppm Cd treated plants such as hexenal isomers. Quantitative analysis of VOC*s* leaves emissions shows an increase of 70% in treated plants compared to control (Figure 20).

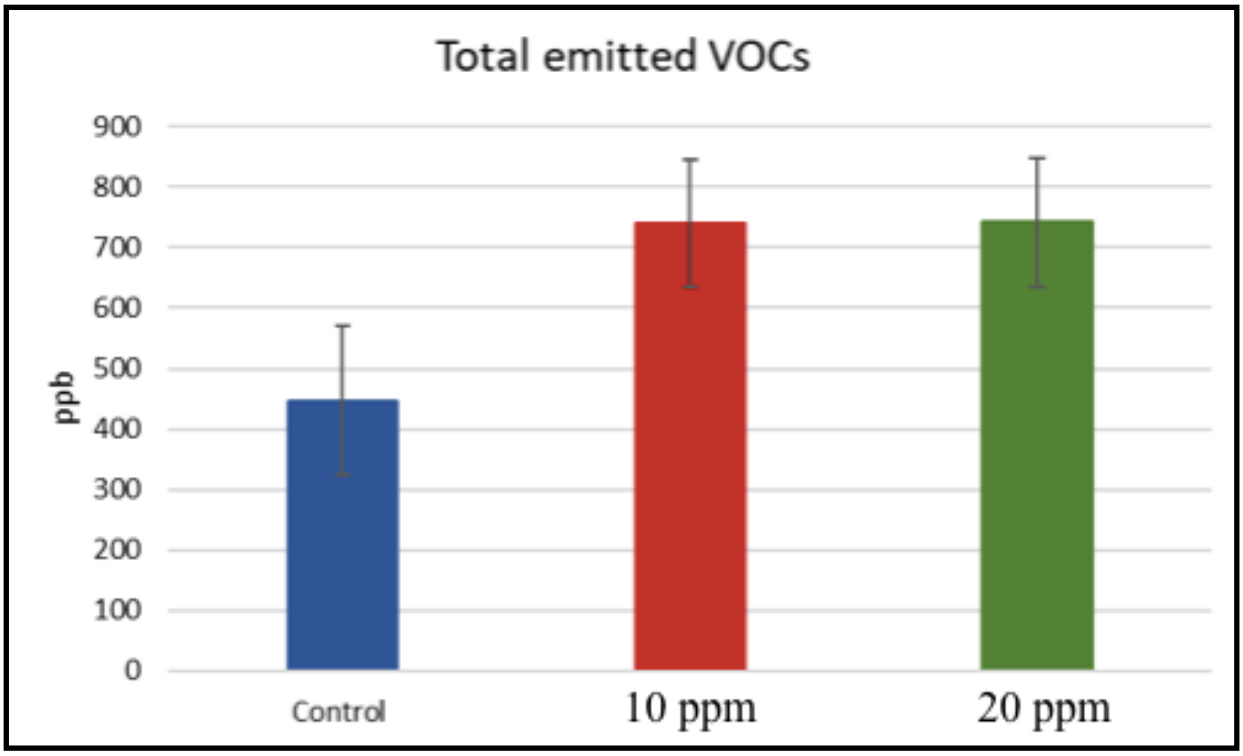


Fig. 20 - Total amount of emitted VOC*s* by CNT and treated plant (10-20 ppm of Cd) was significantly different (ANOVA, $p < 0.05$). Tukey's Multiple Comparison Test was carried out to evaluate mean difference between pairs of groups.

6.4 *GC-MS*

GC-MS analysis, used to detect the main components in the VOC*s* profile of the different hexane extracts, was conducted at the Department of the Sciences of Agriculture, Food and Environment, University of Foggia, Italy. Figures 21 and 22 show the chromatograms related to the different extracts of leaves and flowers.

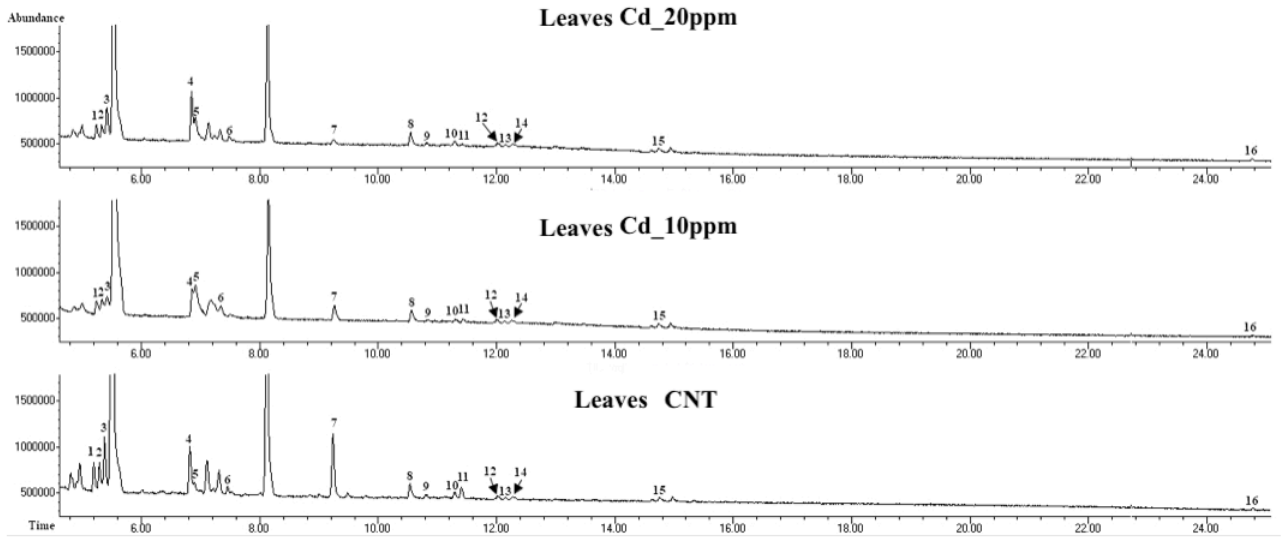


Fig. 21 - Chromatogram of leaves extract of CNT, 10 and 20 ppm Cd.

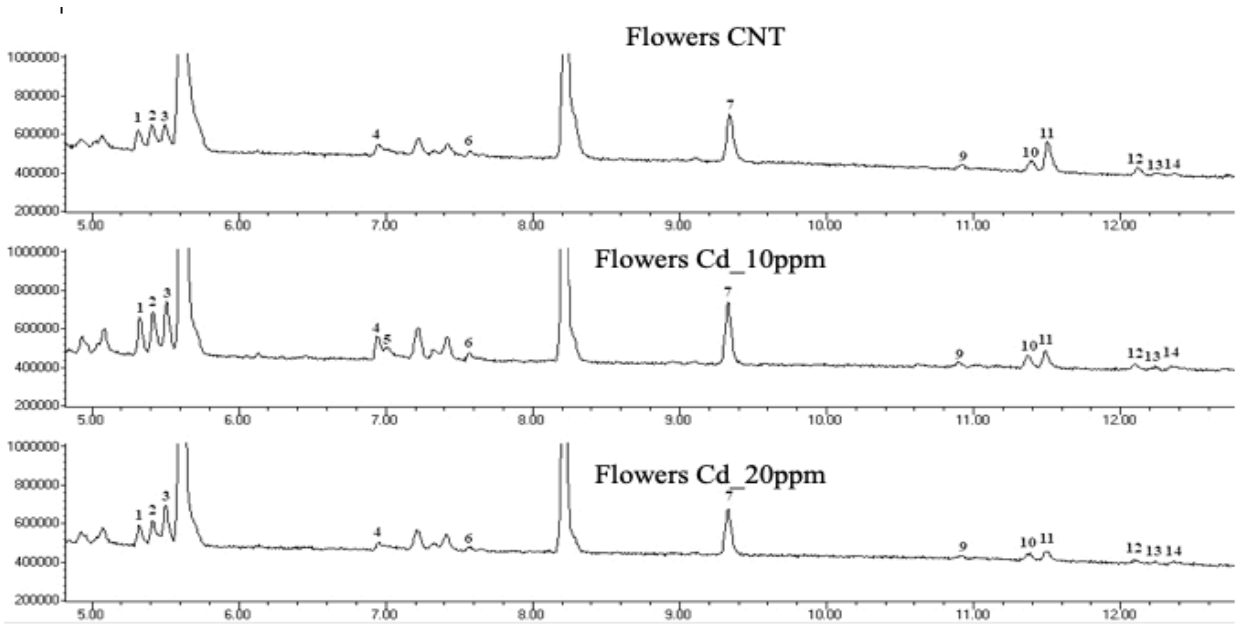


Fig. 22 - Chromatogram of leaves extract of CNT, 10 and 20 ppm

The percentages of specific compounds, expressed as relative abundance, are reported in Table 2. Across all six extracts, a total of 16 volatile compounds in the chemical classes of alcohols, aldehydes, ketones, esters and terpenes were detected. A total of 16 volatile compounds were identified in the extracts of CNT, 10 ppm and 20 ppm samples, obtained from different extracts of flowers, whereas 11, 13 and 12, were identified from the CNT, 10 ppm, and 20 ppm extracts of leaves samples. In flowers (Table 2), terpenes were the most represented chemical class, both in terms of the number of compounds (7) and relative abundance (38.39 - 45.89%), followed by ketones, esters, alcohols, and aldehydes.

In the leaves samples (Table 3), alcohols were the most abundant (21.69 - 43.34%) among different chemical classes, followed by aldehydes, terpenes, ketones, esters and aromatics. In the leaves extracts, the percentage of the most abundant compounds varies according to the content of heavy metals present in the soils. In particular, dominant compounds in the extract from CNT included (*E*)-2-hexanal (17.27 %), 3-hexanol (13.62 %) and α-pinene (10.10 %) while, the extracts 10 ppm contains (*Z*)-3-hexen-1-ol (30.14 %), (*E*)-2-hexanal (17.80 %) and α-pinene (11.73 %) and 20 ppm (*E*)-2-hexanal (31.16 %), 1-octen-3-ol (15.10 %) and 3-hexanol (13.47 %). The flowers extracts contained α-pinene (30.19 %), 3-hexanol (10.56 %) and 2-hexanone (8.95 %), 10 ppm α-pinene (26.04 %), 3-hexanol (13.19 %) and 20 ppm α-pinene (30.19 %), 3-hexanol (17.36 %) and 2-hexanone (12.35 %).

					%Value		
Peaks n°	Compound	RT	RI_{cal}	RI_{ref}	**Flowers CNT**	**Flowers Cd_10ppm**	**Flowers Cd_20ppm**
	Ketones						
1	3-Hexanone	5.24	788	787	10.56 ± 0.36	11.99 ± 0.41	10.77 ± 0.29
2	2-Hexanone	5.34	792	791	8.95 ± 0.18	12.71 ± 0.25	12.35 ± 0.28
	Total Ketones				19.51 ± 0.31	24.7 ± 0.23	23.12 ± 0.27
	Alcohols						
3	3-Hexanol	5.42	801	801	6.79 ± 0.19	13.19 ± 0.22	17.36 ± 0.21
5	(*Z*)-3-hexen-1-ol	6.91	857	856	-	4.64 ± 0.18	-
8	1-octen-3-ol	10.56	975	971	-	-	-
	Total alcohols				6.79 ± 0.19	17.83 ± 0.19	17.36 ± 0.21
	Esters						
6	Isoamyl acetate	7.50	876	875	-	2.82 ± 0.09	2.74 ± 0.08
11	(Z)-3-Hexenol Ac	11.44	1005	1007	18.45 ± 0.39	6.84 ± 0.24	6.13 ± 0.22
	Total esters				18.45 ± 0.21	9.66 ± 0.31	8.87 ± 0.31
	Terpenes						
7	α-Pinene	9.26	928	927	30.19 ± 0.47	26.04 ± 0.41	32.15 ± 0.47
9	Sulcatone	10.86	980	982	2.87 ± 0.05	2.02 ± 0.07	2.82 ± 0.07
10	2-Carene	11.31	1001	1003	5.94 ± 0.22	5.33 ± 0.25	5.01 ± 0.24
12	m-Cymene	12.03	1022	1021	4.77 ± 0.22	2.09 ± 0.13	2.34 ± 0.08
13	Limonene	12.18	1027	1024	1.20 ± 0.05	1.10 ± 0.03	1.26 ± 0.05
14	Eucaliptol	12.04	1032	1033	0.92 ± 0.01	1.81 ± 0.08	1.63 ± 0.12
16	trans-β-ionone	24.76	1490	1486	-	-	-
	Total terpenes				45.89 ± 0.67	38.39 ± 0.61	45.2 ± 0.76
	Aromatics						
15	Phenethyl alcohol	14.65	1121	1122	-	-	-
	Aldehydes						
4	(*E*)-2-Hexenal	6.86	854	854	6.19 ± 0.21	8.61 ± 0.23	4.79 ± 0.18

Table 2 - VOC*s* levels detected in the hexane extracts of flowers.

					%Value		
Peaks n°	Compound	RT	RI_{cal}	RI_{ref}	**Leaves CNT**	**Leaves Cd_10ppm**	**Leaves Cd_20ppm**
	Ketones						
1	3-Hexanone	5.24	788	787	9.89 ± 0.28	9.11 ± 0.31	7.57 ± 0.23
2	2-Hexanone	5.34	792	791	8.69 ± 0.25	6.164 ± 0.21	5.43 ± 0.24
	Total Ketones				16.58 ± 0.18	15.27 ± 0.19	13.00 ± 0.22
	Alcohols						
3	3-Hexanol	5.42	801	801	13.62 ± 0.35	5.30 ± 0.21	13.47 ± 0.41
5	(*Z*)-3-Hexen-1-ol	6.91	857	856	5.91 ± 0.12	30.14 ± 0.43	13.03 ± 0.20
8	1-octen-3-ol	10.56	975	971	6.16 ± 0.24	7.90 ± 0.15	15.10 ± 0.28
	Total alcohols				21.69 ± 0.28	**43.34 ± 0.65**	**41,60 ± 0.61**
	Esters						
6	Isoamyl acetate	7.50	876	875	2.46 ± 0.08	1.37 ± 0.07	2.44 ± 0.14
11	(Z)-3-Hexenol acetate	11.44	1005	1007	4.03 ± 0.22	1.89 ± 0.02	0.90 ± 0.01
	Total esters				6.49 ±0.24	3.26 ± 0.05	3.34 ± 0.04
	Terpenes						
7	α-Pinene	9.26	928	927	10.10 ± 0.31	11.73 ± 0.23	3.47 ± 0.17
9	Sulcatone	10.86	980	982	1.65 ± 0.04	1.47 ± 0.05	1.97 ± 0.07
10	2-Carene	11.31	1001	1003	2.64 ± 0.09	1.90 ± 0.04	2.19 ± 0.04
12	m-Cymene	12.03	1022	1021	1.04 ± 0.05	0.57 ± 0.01	1.40 ± 0.04
13	Limonene	12.18	1027	1024	0.62 ± 0.10	0.77 ± 0.05	1.11 ± 0.06
14	Eucaliptol	12.04	1032	1033	1.30 ± 0.04	0.59 ± 0.01	1.15 ± 0.02
16	trans-β-ionone	24.76	1490	1486	0.72 ± 0.02	0.93 ± 0.09	1.30 ± 0.05
	Total terpenes				18.07 ± 0.28	17.96 ± 0.25	12.59 ± 0.21
	Aromatics						
15	Phenethyl alcohol	14.65	1121	1122	1.48 ± 0.11	0.39 ± 0.01	1.30 ± 0.02
	Aldehydes						
4	(*E*)-2-Hexenal	6.86	854	854	17.27 ± 0.38	17.80 ± 0.20	**31.16 ± 0.54**

Table. 3 - VOC*s* levels detected in the hexane extracts of leaves.

6.5 *EAG*

EAG responses were evaluated by measuring the maximum amplitude of negative polarity deflection (-mV) elicited by a stimulus (Light *et al.,* 1992). The resulting EAG amplitude was corrected according to the reduction of the EAG response to the standard. The extracts were concentrated to 25-50-100 µl. The responses of the right and left antennas of *A. m. ligustica* forager bees to the extracts were obtained by electroantennographic technique (EAG).

Student's *t*-test showed that the mean EAG amplitudes, evoked by each dose of the different extracts in the right antennae, were not significantly different ($p > 0.05$) from those evoked in the left ones, respectively (CNT_Flowers antennae: t = -1.16 – 0.23; d.f. 22; P > 0.05; Flowers_2g_10 ppm antennae: t = 0.16 – 0.86; d.f. 22; P > 0.05; Flowers_2g_20 ppm antennae: t = -1.55 – -0.26; d.f. 22; P > 0.05; CNT_Leaves antennae: t = 0.17–1.29; d.f. 22; P > 0.05; Leaves_5g_10 ppm antennae: t = -0.81 – 1.33; d.f. 22; P > 0.05; Leaves_5g_20 ppm antennae: t = 0.13–0.31; d.f. 22; P > 0.05). Therefore, the EAG responses of right and left antennae to the same stimulus were merged. The dose-response values elicited by the extracts tested towards *A. m. ligustica* antennae, are reported in Figure 23 A and B.

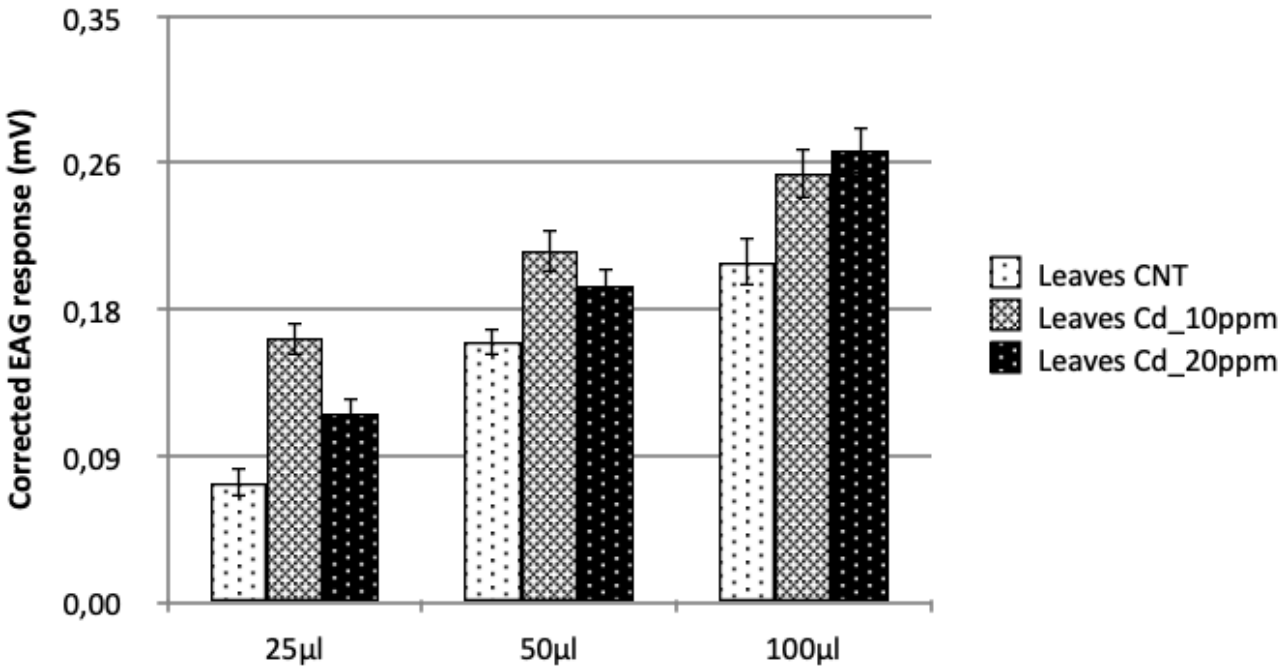


Fig. 23 A - Dose-response values elicited by all extracts tested towards forager honey bee antennae.

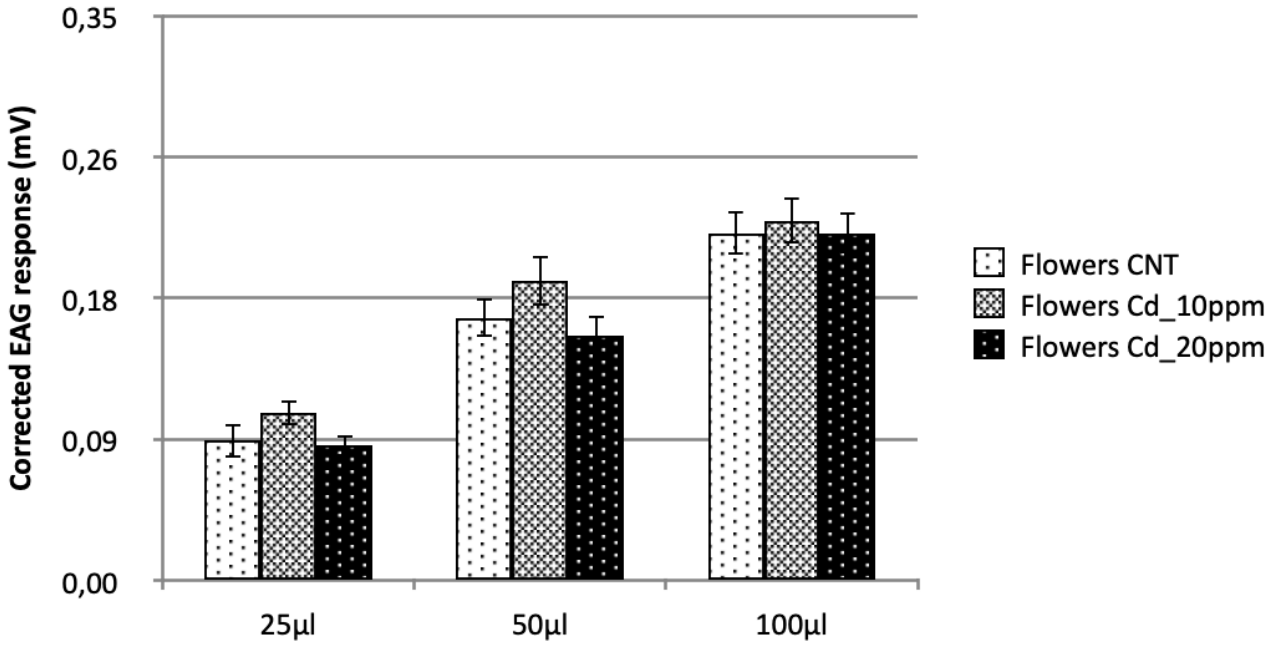


Fig. 23 B - Dose-response values elicited by all extracts tested towards forager honey bee antennae.

The one-way ANOVA, followed by Tukey test revealed significant differences in the EAG responses to the same concentrations of different extracts only for leave extract (CNT_Leaves, Leaves_5g_10ppm, Leaves_5g_20ppm C 25 µl: $F_{(2,63)}$ = 19.27; P < 0.01; CNT_Leaves, Leaves_5g_10ppm, Leaves_5g_20ppm C 50 µl: $F_{(2,63)}$ = 6.58; P < 0.01; CNT_Leaves, Leaves_5g_10ppm, Leaves_5g_20ppm C 100 µl: $F_{(2,63)}$ = 5.902; P < 0.01) (Figure 24).

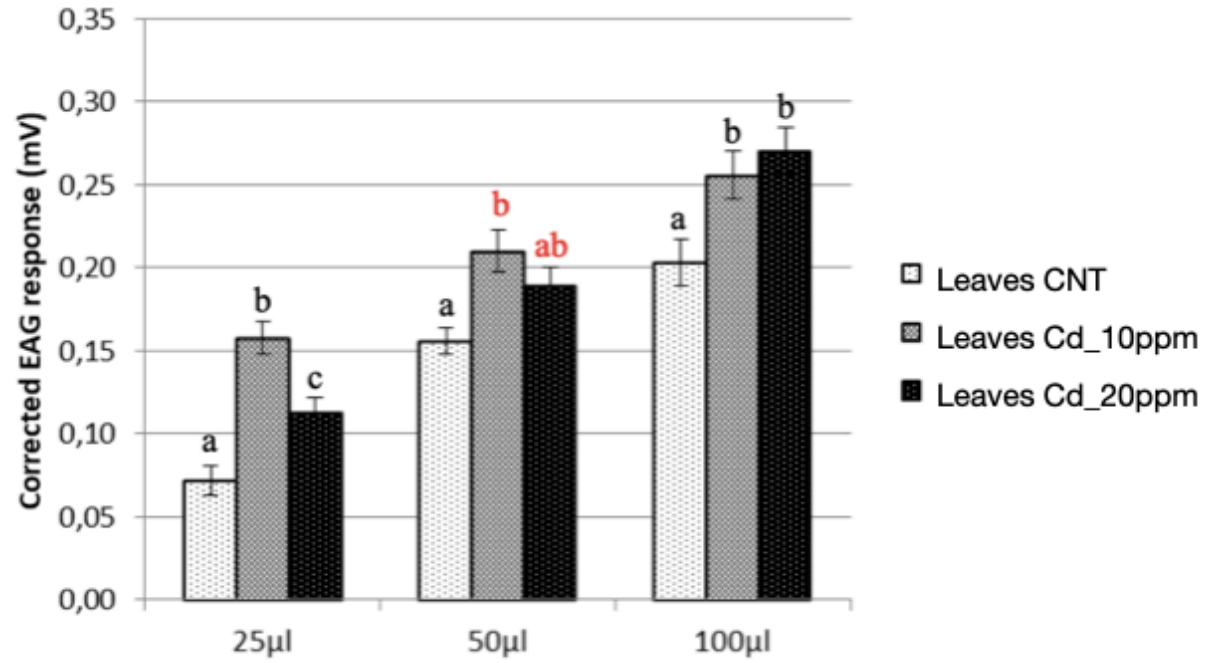


Fig. 24 - EAG responses of forager honey bee antennae to the same concentrations of the two different extracts

Regarding flower extracts (CNT_Flowers, Flowers_2g_10ppm, Flowers_2g_20ppm 25µl: $F_{(2,69)}$ = 1.78; P >0.05; CNT_Flowers, Flowers_2g_10ppm, Flowers_2g_20ppm 50µl: $F_{(2,69)}$ = 1.69; P >0.05; CNT_Flowers, Flowers_2g_10ppm, Flowers_2g_20ppm 100µl: $F_{(2,69)}$ = 0.12; P >0.05;), the mean EAG responses were not significantly different (Figure 25).

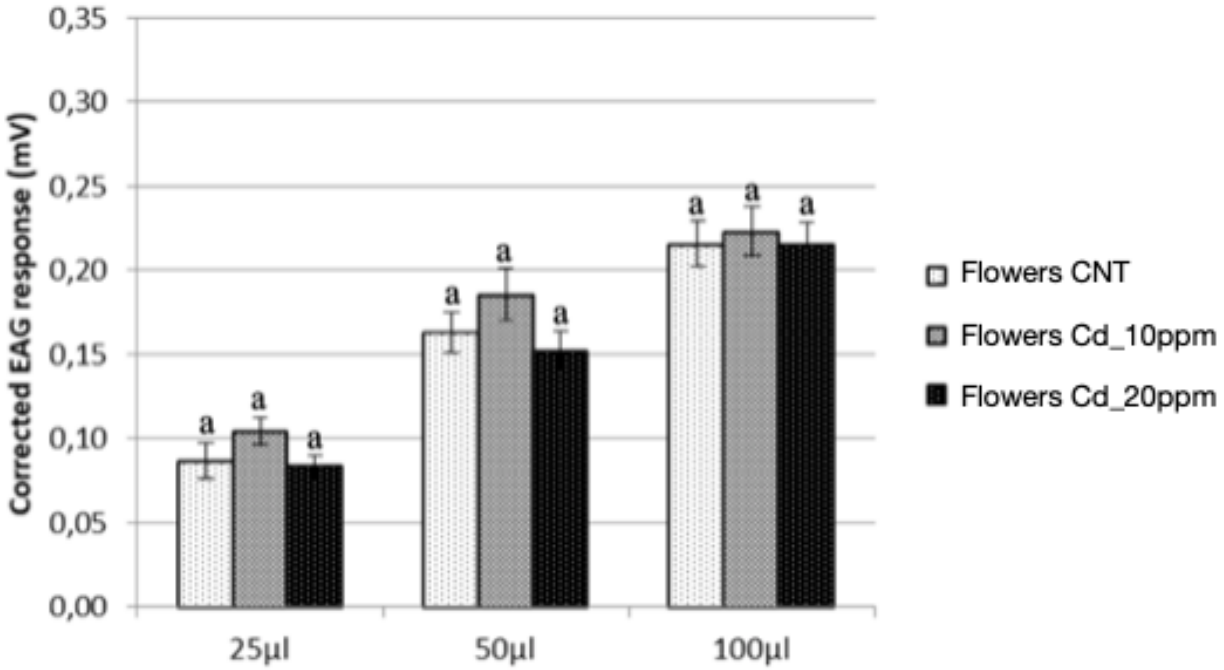


Fig- 25 - EAG responses of forager honey bee antennae to the same concentrations of the two different extracts.

6.6 *Feed test*

For these experiments proline was tested at different concentrations from 0 (control) to 6,4 mMol and Figure 26 shows the bees consumption of proline. The higher consumption (30.49 and 28.94 mg of solution), particularly on the third day, were recorded at proline concentrations of 1.6 and 3.2 mMol; on the contrary, at the other concentrations (0.2, 0.4, 0.8 and 6.4 mMol) lower amounts of solutions were consumed. However from the dose-response curves it is clear that the concentration of 0,2 mMol is the least favorite by bees in proline terms. Over time a dose/response relationship until 3.2 mMol was observed, followed by a consumption decrease. Despite the bees had also an amino acid solution, they still prefers solutions enriched in proline when compared to the other solutions; in fact Figure 27 shows an almost constant trend to feed on various concentrations of this imino acid, with a maximum consumption of 20.63 mg of amino acids solution, corresponding at 0.4 mM.

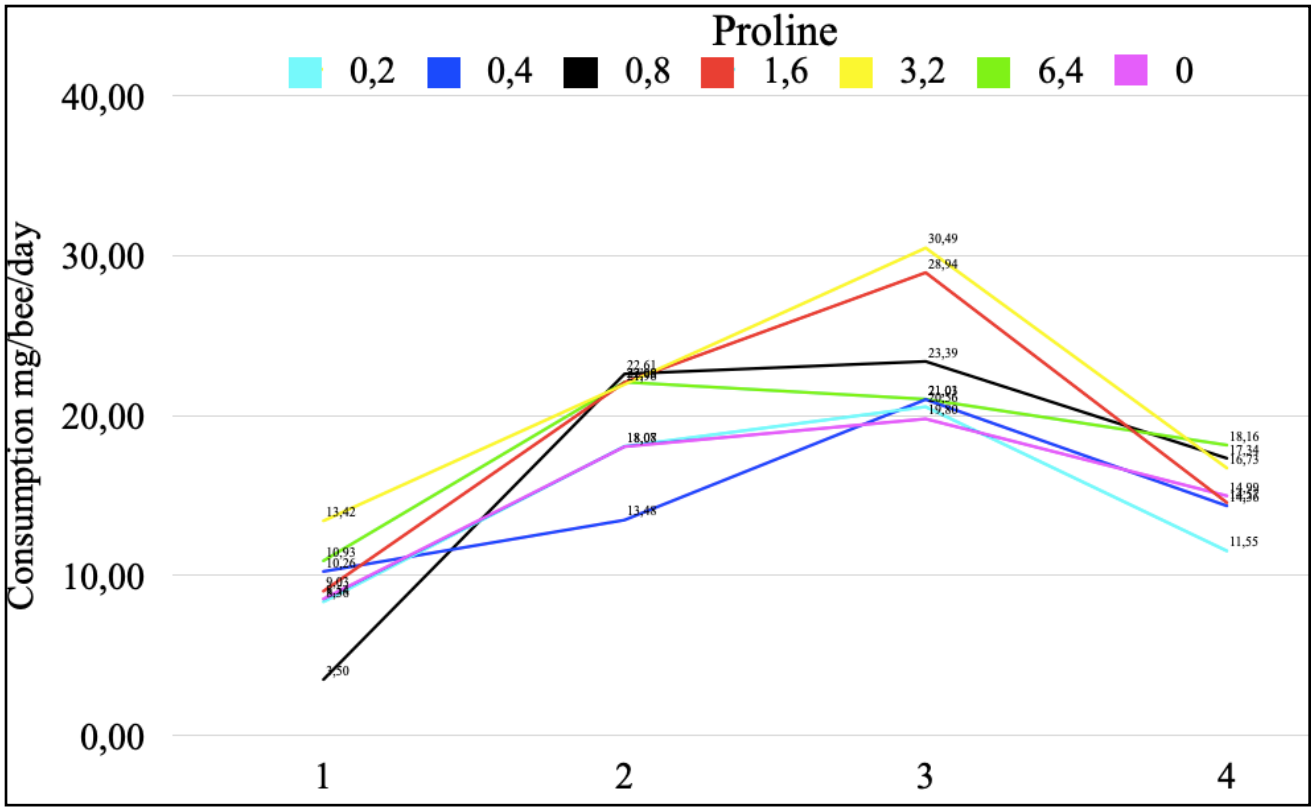


Fig. 26 - Average consumption of proline at different days.

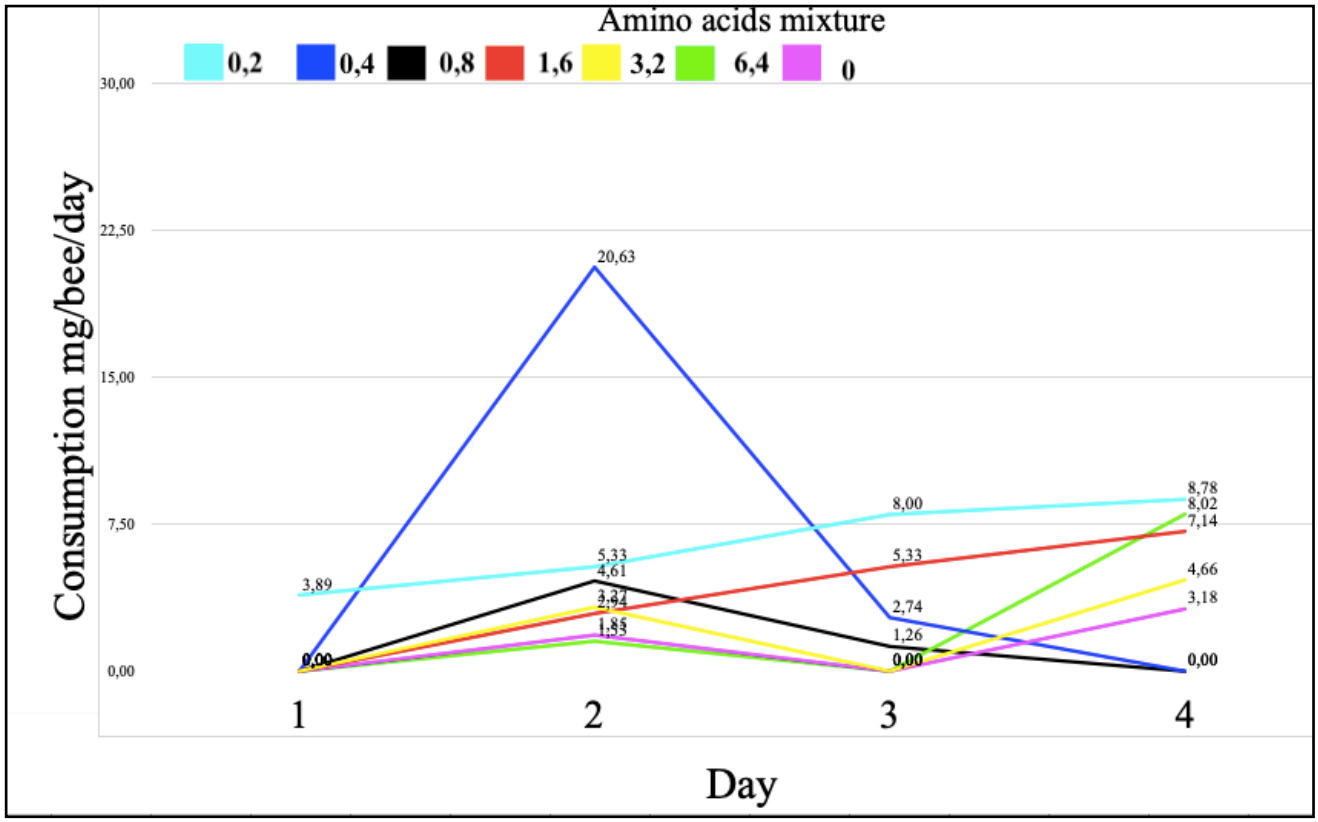


Fig. 27 - Average consumption of amino acids at different days.

6.7 *Discussion*

Heavy metals contamination from natural sources, industrial effluents and man-made activity on agricultural land represents one of the most alarming ecological threats. Uptake and accumulation by crops represent the main entry pathway for potentially health-threatening toxic metals into human and animal food (Ali *et al.,* 2020). Among the heavy metals in soil, Cd accumulation is an irreversible process, since it remains in the soil for 15-1.100 years (Pendias, 1992). Many plants adopt cytological defense mechanisms by sequestering metal ions in cell compartments avoiding their exposure to sensitive cellular components. A second line of defense of metabolic type involves chelating molecules that reduce the toxicity and favor the sequestration in cellular vacuoles. Like all plant abiotic alterations, heavy metals toxicity also causes oxidative stress, production of stress-related proteins, antioxidants, ROS scavengers, activation of secondary metabolism, VOC*s* production and cellular defense mechanisms.

Cd accumulation in M. sativa *flowers, leaves and roots*

Although the apoplastic pathway for ion diffusion is relevant in the cortical parenchyma, the physi ological action of the endodermis, initiates three main stages of hyperaccumulation involving symplastic transport: active transport of metal through plasma endoderm membranes, metal entering the symplast of the leaf mesophyll, metal chelation and seizure in specific cell compartments within the leaves.

The concentration of Cd at 10 ppm and 20 ppm in the soil mixture results in 0.5 mM and 1.0 mM in the circulating solution occupying 20% of the total volume at ground water saturation. These concentrations are high enough to generate a gradient of apoplastic diffusion in the root parenchyma and of symplastic translocation through the plasma membrane. At 47 DAS the concentrations of Cd in the roots reach 60 and 195 μmol/g fw at 10 and 20 ppm Cd in the soil, respectively. The xylematic translocation, pushed also by a favorable osmotic potential, transfers Cd ions to the leaves and therefore to the flowers.

At 23 DAC the concentrations of Cd in mild and severe stress in the leaves reach 35 and 83 nmol/g fw, and in the flowers 26 and 73 nmol/g fw respectively. At 23 DAC the concentrations of a total pool of amino acids in mild and severe stress causes both in leaves and flowers a marked increase; in particular, in the leaves increased of 65% and 110% and in flowers of 70 % and 100% more than the control. An evident increase in the pool of free amino acids in plants treated with Cd can be interpreted as a metabolic response to the stress condition. Glutamate in the leaves of treated plants reached concentrations greater than 100 compared to control, and along with higher levels of glutamine claims that GOGAT enzymes have been highly operational in the synthesis of N compounds from carbon and energy skeletons, provided by photosynthesis (Noctor & Foyer, 2000). It should be noted that photosynthetic activity, although not impaired, is significantly slowed down in Cd-contaminated plants. In fact, Cd greatly reduces gas exchange parameters including stomatal conductance (Sc), transpiration rate (Tr) and photosynthetic rate (Pr). A mechanism of defense and adaptation to strong Cd stresses of plants is a partial closure of the stomata through which the translocation of Cd to the upper parts of plants slows down. Under severe Cd stress, plants adapt by closing stomata and reducing the uptake of Cd to the upper parts of the plants. The strong interference of Cd with K^+, Ca^{2+} and abscisic acid in the guard cells indirectly affects stomatal movements (Barcelo *et al.,* 1990). Stomatal closure is followed by a subsequent decrease in Tr and Pr. These physiological changes caused by Cd stress, are largely supported by the amino acid response, which indicates photosynthetic activity still sufficient to support the assimilation of nitrogen into organic

compounds. Instead, the apparent increase in Gly and Ser in leaves testifies an increase in photorespiratory metabolism. It is also interesting to note a significant increase in aromatic amino acids such

as Tyr, Trp and Phe, which indirectly indicate an active synthesis of shikimic acid, precursor of Tyr and Phe, indole and indole derivates, aromatic amino acid Trp, many flavonoid alkaloids etc. Shikimic acid is the product of biosynthesis between phosphoenolpyruvic acid and erythroso-4-phosphate, (metabolic intermediates of glycolysis and the Calvin cycle) and a transit stage between the basic metabolism and the secondary metabolism. In the amino acid pattern, the amino acid that shows the most sensitive variation in Cd stress condition is the proline that increases by 900% and 800% in the leaves and flowers, respectively. The vital hold of plants is based on sophisticated mechanisms, which allow optimal responses to environmental conditions. Metabolic tolerance via the accumulation of proline is often regarded as a basic strategy for the protection and survival of plants under abiotic stress (Hossain *et al.,* 2014). In recent years, proteomic and metabolomic gene evidence show that proline, produced under stressful conditions, can act as a scavenger of free radicals, a metal chelator, an activator of the ROS detoxification pathways, a cellular redox buffer and a source of energy, nitrogen and carbon.

Proline and amino acids pattern in leaves and flowers of Cd treated plants

As mentioned above, heavy metal toxicity significantly inhibits the process of nitrogen assimilation. The level of inhibition are highly dependent on heavy metal levels and enzyme sensitivity. That way, the exposure to metals at higher concentrations could result in severe damage to nitrogen assimilation and various metabolic activities. In Cd middle stress condition, the significant increase of glutamate, glutamine, proline, aspartic and alanine, could account for an active process of nitrate reduction and assimilation in amino acids such as Glu and Gln via GS-GOGAT. In severe stress conditions, however, when the inhibition of nitrogen assimilation becomes a limiting factor for the synthesis of amino acids, a further conversion of the metabolic precursors Glu and Gln into proline, justifies their decrease in both leaves and roots. On the other hand, the conversion of glutamate into proline in the plastidic pathway, when the deficiency of the oxidized form $NADP^+$ is a cause of oxidative stress, confirms its beneficial role, as an electron sink, to maintain redox balance in adverse situations. Finally, a reduction of the Glu also reduces the biosynthesis of the aspartic by the glutamic oxaloacetic transaminase, while the increase of Ala can account for an active glycolytic metabolism and oxidation of glucidic resources in conditions of Cd stress.

*VOC*s *emission by Cd treated plants and GC-MS analyses*

Most of the natural products accumulated by plants are involved in their interactions with the environment. They play an active physiological role in functioning as molecular messengers in plant communications especially with pollinating insects and even other plants. These interactions can be mediated by VOCs which are allelochemical which include volatile terpenes (VT) (Spinelli *et al.,* 2011). Volatile compounds produced by *M. sativa* plants can be classified by functional groups. These groups include alcohols (e.g., methanol, hexanol, 3-hexen-1-ol 1-octen-3-ol), aldehydes (e.g., benzaldehyde, acetaldehyde, hexanal and hexenal isomers), terpenes fragment (e.g. limonene, eucaliptol), isoprene (2-methyl-1,3-butadiene), aromatics (phenethyl alcohol), alkyl fragment and propanoic acid, esters (isoamyl acetate, 3-hexenol acetate).

The secondary metabolism takes origin from key molecules of the basic metabolism such as the pyruvate from glycolysis that is the substrate for the plastidic methylerythritol phosphate (MEP) pathway (Beyraghdar Kashkooli *et al.,* 2018) through which monoterpenoids and diterpenoids are obtained. Other volatile organic compounds such as benzenoids, benzaldehyde etc. are the result of the shikimate pathway which draws on its substrates from glycolysis and the pentose phosphate pathway (Widhalm *et al.,* 2015; Santos-Sanchez *et al.,* 2019).

On the other hand, it is also conceivable that an alteration of the photosystems as a consequence of Cd stress, increases phosphorylation at the substrate level and the production of reduction equivalents through the glycolytic process.

These compounds were distributed with different percentages and as total content of VOC*s* between treated plants and control. In particular, the quantitative analysis showed an increase of 30% and 70% of the total VOC*s* in the plants treated with 10 ppm and 20 ppm Cd, respectively, compared to control. In angiosperms, the main site of secondary metabolism is represented by the flower system that synthesizes volatile aromatic substances as an attractive signal for pollinating insects. A qualitative investigation carried out through the gas mass chromatography, has allowed to discriminate in the flowering phase the nature and the entity of the VOC*s* produced between leaf and flower. The emission of floral terpenes plays a key role in pollination in many plant species. The content of total terpenes in the flowers is about 3 times greater than the leaves. Among principal terpenes, α-pinene and 2-carene reach in the flower increases from 2 to 10 times greater than the leaves in conditions of severe Cd stress. On the contrary, hexanal was the most abundant VOC in the leaves and was

about 6 times greater than the flowers. These survey of VOC*s* both in quantitative and qualitative results lead to 2 rational conclusions: Cd contamination affects secondary metabolism by increasing the production of VOC*s*, which is a symptom of stress; the increase of VOC*s* following the stress condition, when of biological significance, could amplify the messenger signals between plant and pollinator.

EAG

In this study, electroantennographic responses of *A. m. ligustica* forager bee to extracts, obtained from flowers and leaves of plants treated with different amount of heavy metal (10 ppm and 20 ppm of Cd), including the control, were also investigated. The dose-response values obtained proved that the complex of volatile compounds of all extracts tested elicited antennal responses, showing the capability of forager bees peripheral olfactory system to perceive them. Moreover, data showed that the responses were dependent on the extracts and their concentrations. However, only the electro-physiological responses to the same concentrations of leaves extracts are significantly different from each other.

Feed test

Despite bees having at their disposal H_2O and amino acids mixture bees have expressed a dinamic proline intake dose-dependent until 3,2 mMol. Ours experiments show that the maximum trend to assume the nutrient solution at 6,4 mMol drops down of 30% with respect to 3,2mMol. From a bio-chemical point of view probably bees have been satisfied in energetic and nutritional terms, because giving proline means giving potentially a wide range of proteic amino acids because proline can be interpreted as reduced form of glutamate, and then by glutamate commutated in all other free amino acids by transamination.

6.8 *Conclusion*

The possibility that a heavy metal comes into biogeochemical cycles is the reason of the importance to deepen the research on integration system environment-plant-pollinator-human. The aim of this work was to link the proline increase, induced by Cd contamination, and how the volatile compounds pattern changes the ability of bees to perceive these substances at sensorial level. Analyses conducted on *M. sativa* plants grown on polluted substrate with Cd (10 and 20 ppm) showed an increase on proline concentration especially at leaves tissue, explainable by the fact that the anabolism and catabolism of this amino acids originates principally at leaf parenchyma. From the VOC*s* analyses it appears that the major changes, in terms of secondary metabolism, has been always at leaf tissue, with an increase in VOC*s*, respect to the flowers, probably because the Cd, generating through the photosynthetic system an oxidative stress, stimulates the proline production, that has a scavenger function, to overcome at such stress. Moreover it should be remembered that these metabolic changes induced by Cd drastically modifies both the plant growth and the development, influencing also the flowering. The proline presence has changed, therefore, the pattern of volatile compounds more in leaf then in flower, probably because in the first one there has been an increase in terms of concentration of that amino acid. These analyses have brought to light a significant increase in aromatic amino acids such as Tyr, Trp and Phe, which indirectly indicate an active synthesis of shikimic acid, a precursor of Tyr and Phe, indole and indole derivates, aromatic amino acid Trp, many flavonoid alkaloids etc. Shikimic acid, is the product of biosynthesis between phosphoenolpyruvic acid and erythroso-4-phosphate (metabolic intermediates of glycolysis and the Calvin cycle) and a transit stage between the basic metabolism and the secondary metabolism; this can, probably, partially explain the change of the VOC*s* emission, more abundant in leaf then in flower. The EAG tests highlighted that only the electrophysiological responses to the same concentrations of leaves extracts are significantly different from each other, allowing to speculate an attraction of this insect towards polluted plants. However the feed test, the purpose of which was to simulate the flower's nectar, shows that at 3.2 mM, in a range from 0 to 6.4, is the concentration to which it corresponds the major quantity of foraged nectar after 3 day. Thus, in our experiment, the tendency to take nutrient solutions at concentrations of 6.4 mM is already reduced by 30% compared to the solution containing 3.2 mM proline, thus generating a gaussian profile between the quantity of nutrient solution taken and the proline concentration. This response lends itself to two interpretations: 1) the proline at high concentration in the nectar generates a repellent (or antifeedant) effect threshold and leads the bees to stop feeding; 2) the proline at high concentration in the nectar generates

a metabolic saturation both in bioenergetics terms and azotorganic substances. Further studies will be needed to clarify this crucial aspect since only in the first case bees will quite completely avoid feeding on polluted plants and, consequently, a) they will not produce polluted honey and other beehive products, b) such behavior does not limit the adoption of phytodepuration techniques of soils polluted with Cd.

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Influence of Tomato Plant Mycorrhization on Nitrogen Metabolism, Growth and Fructification on P-Limited Soil

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Abstract
The application of mycorrhizal fungi in agricultural soils as bio-fertilizers is going to be established as an agronomic practice for enhancing crop nutrients acquisition and production. In this work, the effects of tomato root colonization by arbuscular mycorrhizal fungus *Glomus mosseae*, on nitrogen metabolism, fructification and environmental sustainability without P soil fertilization have been studied. At the harvesting fruit stage, the mycorrhizal (M) plants present a significantly higher concentration of mineral nutrients and organic nitrogen compounds. In particular, GLU, GLN, ASP and ASN have risen about 35% more than non-mycorrhizal (NM) plants. Tomato root mycorrhization improved nitrogen metabolism in plants, too by increasing the nitrate reductase and the glutamine synthetase enzymatic activity. Moreover, mycorrhization affects many aspects of vegetative and reproductive growth. In particular, the fruit production turns from inoculated (M) plants into non-inoculated (NM) plants, rising up to 50%.

Keywords Nitrogen-metabolism · Mycorrhizal plant · Tomato · P-limited soil

Introduction

In the Mediterranean area, as well as in many parts of the world, tomatoes (*Lycopersicon esculentum* L.) represent the major vegetable crop, growing in protected or in field conditions (Al-Karaki 2006). Italy is the leading producer of tomato industry varieties in the Mediterranean area. For its commercial importance, tomato has been selected as a model in this study. Growth and reproduction of tomato plants are severely affected by phosphorus deficiencies, a typical condition of many agricultural soils (López-Bucio et al. 2002; Güsewell 2004). Under these conditions, infection of plant roots by arbuscular mycorrhizal (AM) fungi is particularly advantageous (Cavagnaro et al. 2005). Mycorrhizal fungi establish mutualistic relationships with about 85% of terrestrial angiosperm species (Wang and Qiu 2006), and they enhance P, N, and other mineral nutrient uptake from the soil, primarily by absorptive surface increasing (Marschner and Dell 1994; Teste et al. 2014). Therefore, generally mycorrhizal plants have a higher concentration of these elements compared to non-mycorrhizal plants (Koide et al. 1998; Nouri et al. 2014). Mycorrhiza development is strongly influenced by nutrient availability in the soil, in particular phosphorous (Grant et al. 2005; Aissa et al. 2016). So, wider mycorrhizal colonization occurs when soil P concentration is limiting for plant growth, but it is often highly restricted when soil P is largely available and it is often detected under high P availability (Smith et al. 2011; Balzergue et al. 2013). It is well known that the exchange of nutrients (sugar and phosphate) between the fungus and the host plant is at the base of the symbiotic relationship. Mycorrhizal symbioses are closely connected to a genetic predisposition and to the interaction of genes between the fungus and the host plant. In particular, it has been demonstrated that in the arbuscular mycorrhizal fungus *Glomus* sp. monosaccharide transporter2 (*MST2*) expression is closely correlated with that of the mycorrhiza-specific phosphate transporter4 (*PT4*) (Helber et al. 2011). Moreover, high phosphate acts systemically by affecting essential symbiotic genes, in particular genes coding enzymes of carotenoid and

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plants

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Article

Joint Selenium–Iodine Supply and Arbuscular Mycorrhizal Fungi Inoculation Affect Yield and Quality of Chickpea Seeds and Residual Biomass

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Abstract: The essentiality of selenium (Se) and iodine (I) for the human organism and the relationship between these two trace elements in mammal metabolism highlight the importance of the joint Se–I biofortification to vegetable crops in the frame of sustainable farming management. A research study was carried out in southern Italy to determine the effects of the combined inoculation with arbuscular mycorrhizal fungi (AMF) and biofortification with Se and I on plant growth, seed yield, quality, and antioxidant and elemental status, as well as residual biomass chemical composition of chickpea grown in two different planting times (14 January and 28 February). The AMF application improved the intensity of I and Se accumulation both in single and joint supply of these elements, resulting in higher seed yield and number as well as dry weight, and was also beneficial for increasing the content of antioxidants, protein, and macro- and microelements. Earlier planting time resulted in higher values of seed yield, as well as Se, I, N, P, Ca, protein, and antioxidant levels. Se and I showed a synergistic effect, stimulating the accumulation of each other in chickpea seeds. The AMF inoculation elicited a higher protein and cellulose synthesis, as well as glucose production in the residual biomass, compared to the single iodine application and the untreated control. From the present research, it can be inferred that the plant biostimulation through the soil inoculation with AMF and the biofortification with Se and I, applied singly or jointly, proved to be effective sustainable farming tools for improving the chickpea seed yield and/or quality, as well as the residual biomass chemical composition for energy production or beneficial metabolite extraction.

Keywords: *Cicer arietinum* L.; AMF; biofortification; proteins; antioxidants; mineral elements; waste chemical composition

1. Introduction

I and Se are essential microelements for mammals. Se is known to be a part of the tri-iodothyronine deiodinases that participate in thyroid hormones synthesis [1]. These two trace elements are significant

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Environmental sustainability fruit quality and production in mycorrhizal tomato plants without P fertilizing

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Abstract. The influence of root colonization by arbuscular mycorrhizal (AM) fungus *Funelliformis mosseae*, on fruit quality, production and environmental sustainability were evaluated in field-tomato plants grown exposed to P-limited soil 5 µg g^{-1} soil (basal-soil) with nitrate fertilization (50 µg g^{-1} soil), after greenhouse germination and fungus colonization. After 60 days sowing (DAS), when the percentage of mycorrhizal root length (% RLC) raised at about 50%, the plants were transplanted in open field.
During the experiment, the mycorrhization has affected a lot of physiological aspects like vegetative and reproductive growth, improving them and ended the fruiting with a major fruit production that was 50% higher than not mycorrhizal (NM) plants. The ripening process of the fruits was also followed by testing sugars content and ß-Amylase activity in fruits of NM and mycorrhizal (M) plants fruits. At 140 DAS, in the harvesting fruits stage, fruits of M plants showed significantly higher mineral nutrient sugars and organic nitrogen compounds as amino acids and protein, compared to fruits from NM plants. In particular, GLU-GLN-ASP and ASN raised about 35% more than fruits from NM plants, improving nutritional aspect and flavor of the product. THR-ILEU-LEU-VAL and LYS, essential amino acids in man nutrition, increased around 25% more than fruits from NM plants, too. In this contest, lycopene, total carotenoids, ascorbic acid and glutathione (GS) and reduced form (GSH) were also tested in ripe fruits. The overall results suggest that tomato roots colonization by mycorrhizal fungus *Funelliformis mosseae* affects host plant nutritional status, modifying reproductive behavior, fruits production and nutritional quality.

Key words: *Funelliformis mosseae,* mineral nutrients, amino acids, mycorrhizal plants, *Solanum lycopersicum*.

INTRODUCTION

The tomato *(Solanum lycopersicum* Mill.) fruit is one of the most popular, as well as one of the most important food, of the Mediterranean gastronomic culture based

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Article

Application of Novel Microorganism-Based Formulations as Alternative to the Use of Iron Chelates in Strawberry Cultivation

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Abstract: The strawberry is a low-growing, herbaceous perennial plant, sensitive to iron deficiency. The iron deficiency represents a nutritional disorder, leading to a decreased content of photosynthetic pigments, which determines the yellow color characteristic of chlorotic leaves. Therefore, in calcareous soils, the use of synthetic iron chelate is often mandatory in strawberry cultivation. The employment of novel microorganism-based formulations as alternatives to the use of iron chelates, was evaluated during strawberry cultivation by monitoring the morpho-biometric parameters, chlorophylls, the iron content in leaves and roots, and the Fe chelate reductase activity involved in absorption of iron during the chlorosis event in plants using the strategy I. The experimental design envisaged growing strawberry seedlings on an inert substrate (pumice), irrigated with Hoagland solution iron-free, with a 12 h photoperiod. After 42 days, at the first appearance of chlorosis symptoms, plants were transplanted into a calcareous soil, and after seven days, they were treated, by a single application, with a microorganism-based formulations (MBF), an inoculum (In) of *Trichoderma* spp. and *Streptomyces* spp., or Sequestrene (Sq). Strawberry plants were sampled and analyzed at 5, 10, 15, and 20 days from the treatments. The results showed that microorganism-based formulations positively affected the strawberry seedlings, by reducing the chlorosis symptoms, producing comparable effects to the Sequestrene treatment.

Keywords: EDDHA; FC-R; Fe-deficiency; *Fragaria* × *ananassa*; *Glomus*; *Trichoderma*; *Streptomyces*

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1. Introduction

Iron deficiency is an important nutritional disorder in plants, resulting from the altered acquisition and use of Fe, rather than from a low level of Fe in soils, determining as the primary effect a decreased content of photosynthetic pigments, which leads to the characteristic yellow color of chlorotic leaves [1]. The ferric chlorosis is determined by iron necessity for the correct functionality of proteins involved in the synthesis of chlorophylls. Indeed, the synthesis of δ-aminolevulinic acid, precursor of chlorophylls, is regulated by the presence of iron [2]. Iron is also necessary for the synthesis of the protochlorophyllide from Mg-protoporphyrin. Moreover, in the thylakoid membrane, 20 atoms of iron are needed for photosynthetic electron transport chain of the PSII and PSI photosystems [3–5]. Such metabolic disorders induced by Fe deficiency cause chloroplast disorganization. This effect is shown by decreasing of photosynthetic units, granules, and stromal lamellae of the chloroplast, and by the decrease of thylakoids [6].

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Article

Mycorrhized Wheat Plants and Nitrogen Assimilation in Coexistence and Antagonism with Spontaneous Colonization of Pathogenic and Saprophytic Fungi in a Soil of Low Fertility

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Abstract: The aim of the work was to study the biological interference of the spontaneous colonization of pathogenic and saprophytic endophytes on the nitrogen assimilation of mycorrhized wheat plants cultivated in soils deficient in N and P. The nitrogen assimilation efficiency of mycorrhized plants was determined by measuring the activities of nitrate reductase assimilatory and glutamine synthetase enzymes and free amino acid patterns. Mycorrhizal plants at two different sites showed an assimilative activity of nitrate and ammonium approximately 30% greater than control plants. This activity was associated with significant increases in the amino acids Arg, Glu Gln and Orn in the roots where those amino acids are part of the inorganic nitrogen assimilation of mycorrhizal fungi. The nutrient supply of mycorrhizal fungi at the root guaranteed the increased growth of the plant that was about 40% greater in fresh weight and 25% greater in productive yield than the controls. To better understand the biological interaction between plant and fungus, microbiological screening was carried out to identify colonies of radicular endophytic fungi. Fourteen fungal strains belonging to nine different species were classified. Among pathogenic fungi, the genus *Fusarium* was present in all the examined roots with different frequencies, depending on the site and the fungal population present in the roots, providing useful clues regarding the principle of spatial conflict and fungal spread within the root system.

Keywords: amino acids; nitrate reductase; glutamine synthetase; plants mycorrhized; dark septate

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1. Introduction

About 80% of terrestrial plant roots are closely associated with mycorrhizal fungi [1], and many aspects of the physio-ecological roles played by these mycorrhizal fungi, such as plant nutrition, soil biology and soil chemistry, are well described [2]. The cytological interaction between fungus and root occurs at the interface between the plasma membranes of the arbuscular and cortical cells. Through these contact surfaces, nutritional exchanges take place between the plant that supply the fungus with carbonaceous skeletons and the fungus that transfers the mineral nutrients to the plant [3,4]. It has been reported that phosphate deficiency in soil stimulates mycorrhizal colonization [5]. The suppression of mycorrhizae arises from the high concentration of P and from other mineral nutrients present in the soil. In addition, a limitation of nitrogen in the soil also stimulates root colonization by mycorrhizal fungi [6]. Moreover, mycorrhizal fungi are implicated in absorbing inorganic nitrogen from the soil and moving it to the roots to be partly assimilated

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Article

Honeybees as Bioindicator of Heavy Metal Pollution in Urban and Rural Areas in the South of Italy

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Abstract: Honeybees (*Apis mellifera L.*) has been used in several papers for monitoring the environmental health status in terms of pollution, due to their wide-ranging foraging flights. Based on this consideration, this study aims to analysed the heavy metals' pollution in Molise region (Italy), investigating five sites characterized by different levels of contamination. Furthermore, the authors carried out a sampling activity for a long period, in order to obtain a complete dataset. In this way, detailed information about the status of the environments can be obtained. The main purpose of this work is to assess the health status of Molise region and to confirm the goodness of honeybees as environmental bioindicators of heavy metals' pollution, analysing their variability over time and space. Furthermore, the paper focuses on a comparison of the health status contamination in terms of heavy metals with two different areas of Italy, by using the Hierarchical Cluster Analysis and Principal Component Analysis, for evaluating the correlation existing among the three different areas of Italy.

Keywords: heavy metals; biomonitoring; pollution; *Apis mellifera*; honeybees.

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1. Introduction

Heavy metals are from years contaminants of interest of scientific communities. They represent, in fact, a significant risk for the environment, considered the potential drastic effects deriving from their deposition and moving through soil, water and air [1]. A work by Briffa et al. reported that the most common heavy metals in humans' everyday life are vanadium, chromium, cobalt, nickel, copper, molybdenum, titanium, manganese, iron and arsenic [2]. These inorganic substances are naturally present into the environment but, due to the astounding increase of the use of heavy metals, heavy metals presence in terrestrial (but also aquatic) ecosystem is significantly increased. As a matter of fact, industrial activities are responsible of the heavy metals' emission; products used in agricultural activities such as herbicide, fertilizers and insecticides led to an increase of environmental pollution. For this reason, the monitoring of environment health status is required [3]. From available scientific literature, it is known that most of traditional methodologies for the environmental monitoring could cover just a small area of interest. Furthermore, the costs for maintaining and installing the instruments onsite are high [4]. On the basis of this consideration, living organisms were taken into account such plant and insects [5]. The living organisms used for the biomonitoring can be classified depending on the type of interaction with the pollutant [6]. Among the biomarkers, in fact, it is possible to distinguish the bioindicators and the bioaccumulators. The first group consists of living

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