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Title

Improving heavy metal stress tolerance in tomato by grafting

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Presented By

PRADEEP KUMAR

Coordinator and Supervisor

Prof. Giuseppe Colla

Co-Supervisor

Dr. Menahem Edelstein

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Dedicated to my family

Preface

This thesis is submitted for the degree of Doctor of Philosophy in Horticulture at the University of Tuscia, Viterbo. The thesis has been written on the basis of experiments conducted at Department of Agriculture, Forestry, Nature and Energy (DAFNE), University of Tuscia from 2012-2015 under very supervision of Prof. Giuseppe Colla. Many inspiring people have been involved in the work leading to my PhD thesis. The first experiment was conducted under co-supervison of Dr. Menahem Edelstein, Researcher- Newe Ya'ar Research Center, Ramat Yishay, Israel during his deputation at University of Tuscia. Dr. Mariateresa Cardarelli, Researcher at CRA, Rome supervised the laboratory activities including biochemical analysis, mineral analysis and mycorrhizal studies of all experiments. The work related to metabolomics was performed under supervision of Dr. Luigi Lucini at Catholic University, Piacenza. Dr. Youssef Rouphael, Researcher at University of Naples has been guiding me throughout the PhD program and especially for constructive feedback related to preparation of manuscripts and thesis writing.

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(PRADEEP KUMAR)
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Abstract

Among the heavy metals, Cadmium (Cd) and nickel (Ni) are the most hazardous metals and their excess level in soil or irrigation water causes considerable damage to crop plants. Grafting has become an effective and sustainable tool in providing plant tolerance to various abiotic stresses, in particular of fruiting vegetables. In present research program, grafting potential was explored in mitigating adverse effects of elevated level of Cd or Ni in tomato (*Solanum lycopersicum* L.). The aim of this PhD dissertation was to improve tomato plants tolerance to heavy metals stress, in particular of Cd and Ni by grafting onto appropriate rootstock(s). In addition, the rootstock mediated plant tolerance mechanisms were studied at cellular and molecular level. The role of candidate genes (*LeNRAMP3 and LeFER*) function in Cd tolerance was also studied. Furthermore, in next step, the integrated response of grafting with arbuscular mycorrhizal (AM; *Rhizophagus irregularis*) inoculation in further enhancement of Cd tolerance was investigated.

Tomato plants whether non-grafted, self-grafted or grafted onto rootstocks of tomato (Maxifort and Unifort) or eggplant (Black Beauty) were adversely affected by excess concentration (i.e, 25 and 50 µM) of Cd or Ni in growing medium as evident by reduced plant growth and fruit yield. However, plants grafted onto tomato rootstocks, especially onto Maxifort shown better performance to mitigate adverse effects of heavy metals as shown by higher fruit yield and plant biomass, compared to others. We herein, demonstrate that the higher yield and biomass production in tomato rootstocks grafted plants, especially onto Maxifort was due to better ability of rootstock in particular of translocation of nutrient elements to shoot, higher leaf pigments contents, SPAD index and photochemical efficiency of PSII. In addition, their better ability to reduce oxidative stress, evident by lipid peroxidation and ion leakage, by induction of antioxidants- enzymes (CAT, APX or GPX) and -non-enzymes like carotenoid, proline and certain other metabolites. The over expression of *LeNRAMP3* gene in leaves support Maxifort grafted plants ability to Cd stress tolerance by providing higher availability of certain nutrient elements under Cd stress. Moreover, Maxifort rootstock could also efficiently limit the translocation of toxic metals, especially of Cd to the aerial plant parts (leaf + stem + fruit). Moreover, the expected positive response of AM fungi in providing plant tolerance to Cd stress especially under given moderate level of Cd stress (25µM), in later phase of study, could not be observed. Finally, from these investigations it can be concluded that tomato grafting onto appropriate rootstock such as Maxifort could alleviate Cd or Ni stress.

Abstract

Cadmio (Cd) e nickel (Ni) sono tra i metalli pesanti più pericolosi in natura ed elevati livelli di questi metalli nel terreno o nelle acqua irrigue possono causare notevoli danni alle colture e creare problemi di sicurezza alimentare a causa dell'accumulo dei metalli nella parte edule. L'innesto è diventato uno strumento efficace e sostenibile in *Solanaceae* e *Cucurbitaceae* per aumentare il grado di tolleranza delle piante a vari tipi di stress abiotici. L'attività di ricerca ha avuto come obiettivo quello di valutare il contributo potenziale dell'innesto nel mitigare gli effetti negativi di elevati livelli di Cd o Ni su pomodoro (*Solanum lycopersicum* L.). Lo scopo della tesi di dottorato è stato quello di verificare la possibilità di migliorare la tolleranza in pomodoro a metalli pesanti, in particolare di Cd e Ni, tramite l'innesto su appropriati portainnesti. Inoltre, i meccanismi che mediano la tolleranza delle piante a tali stress sono stati studiati a livello cellulare e molecolare. E' stato inoltre studiata l'espressione genica di alcuni geni (*LeNRAMP3* e *LeFER*) in funzione della tolleranza al Cd. Successivamente è stata valutata la possibilità di migliorare ulteriormente la tolleranza a stress da Cd attraverso l'integrazione dell'innesto su portinnesto tollerante con l'inoculazione di micorrizza arbuscolare (AM; *Rhizophagus irregularis*).

Piante di pomodoro non innestate, auto-innestate o innestate su portinnesti di pomodoro (Maxifort e Unifort) o di melanzana (Black Beauty) sono stati influenzati negativamente dalla eccessiva concentrazione di Cd o Ni (25 e 50 µM) nella soluzione, con evidente riduzione della crescita delle piante e della resa dei frutti. Tuttavia, piante innestate su portinnesti di pomodoro, in particolare su Maxifort hanno mostrato risultati migliori sulla mitigazione gli effetti negativi dei metalli pesanti avendo questi una maggiore resa in frutti e biomassa della pianta, rispetto alle piante innestate su altri portinnesti. La ricerca ha messo in evidenza che la più alta resa in frutti e biomassa in piante innestate su portinnesti di pomodoro, soprattutto sul Maxifort, è dovuta in particolare ad una migliore capacità dei portinnesti di traslocare gli elementi nutritivi verso gli apici vegetativi, un maggiore contenuto di pigmenti nelle foglie, un migliore indice SPAD ed una più alta efficienza fotochimica del PSII. E' stata inoltre evidenziata una loro migliore capacità di ridurre lo stress ossidativo tramite perossidazione lipidica e perdita di ioni per induzione di enzimi antiossidanti (CAT, APX o GPX), carotenoidi, prolina e alcuni altri metaboliti. La sovraespressione genica di *LeNRAMP3* nelle foglie supporta la tesi che piante innestate su Maxifort hanno maggiore tolleranza allo stress da Cd, garantendo alla pianta maggiore

disponibilità di alcuni elementi nutritivi. Inoltre, il portainnesto Maxifort limita in modo efficace la traslocazione di metalli tossici, in particolare dei Cd verso le parti aeree della pianta (come foglie, fusti e frutti). Nessun effetto positivo sulla tolleranza allo stress da Cd è stato riscontrato con funghi AM.

Dai risultati emersi quindi si può concludere che l'innesto di pomodoro su portainnesti appropriati come Maxifort può alleviare stress dovuti da metalli pesanti come Cd e Ni.

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Abbreviations and acronyms

µ	Micro
µM	Micro mole
µg	Microgram
ng	Nanogram
g	Gram
mg	Milligram
kg	Kilogram
%	Percent
Min	Minute
MT	Metric ton
mL	Milliliter
cm	Centimeter
m	Meter
m^2	Square meter
^oC	Degree Celsius
No.	Number
s	Second
α	Alpha
ß	Beta
<	Lesser than
>	Higher than
mha	Million hectare
±	Plus-minus
A	Alpha
B	Beta
P	Probability
cv.	Cultivar
DAT	Days after transplanting
N	Normality
DM	Dry matter
TSS	Total soluble solids
ICP	Inductively coupled plasma
MDA	Malondieldehyde
SI	Shape index
TA	Titratable acidity
FW	Fresh weight
DW	Dry weight
AM	Arbuscular mycorrhiza
W/V	Weight / volume

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Chapter 1

General Introduction:
Rationale, hypothesis and objectives

Chapter 1

General Introduction: Rationale, hypothesis and objectives

1. Heavy metals

The growing concerns over heavy metal pollution of soil and water especially, in the current era of industrialization have received increasing attention of scientists worldwide as its consequences can potentially endanger plants and humans health. In the advent of faster industrialization and certain human activities such as application of sewage-sludge, manures and phosphatic fertilizers, the metal industry and automobiles, their occurrence has increased in the soil environment (Yoon et al., 2006; Dal Corso, et al. 2013). Moreover, there are certain agricultural amendment which carry significant amount of heavy metals (Table 1) and thus polluting agriculture soils. Heavy metals persist in soils for a longer period after their introduction into environment as they could not be degraded naturally or by microbial activities (Liu et al., 2009). Vegetables grown in either soil contaminated with heavy metals or with water of low quality have higher concentrations of heavy metals compared to those grown in uncontaminated soil/water (Dowdy and Larson, 1975; Guttormsen et al., 1995; Mc Laughlin and Singh, 1999; Singh, et al. 2010). The long term intake of excess concentrations of heavy metals through food may lead to the chronic accumulation of heavy metals in the kidney and liver of humans causing disruption of numerous biochemical processes, leading to cardiovascular, nervous, kidney and bone diseases (WHO, 1992; Sharma, et al. 2009).

The exposure of plants to the toxic levels of heavy metals triggers a wide range of physiological and metabolic alterations (Dubey, 2011; Villiers et al., 2011). However, as different heavy metals have different sites of action within the plant, the overall visual toxic response differs between heavy metals. The most widespread visual sign of heavy metal toxicity is reduction in plant growth (Sharma and Dubey, 2007), including leaf chlorosis, necrosis, root activity, altered photosynthetic apparatus, reducing the biosynthesis of pigments and finally can lead to plant death (DalCarso et al., 2010; Carrier et al., 2003).

Among the heavy metals, Cadmium (Cd) and Nickel (Ni) are considered to be highly phytotoxic (Madhaiyan et al., 2007). According to the WHO, Cd and Ni are among those metals

that are of most immediate concern in the environment (Zafar et al., 2007). They are phytotoxic causing growth inhibition, disturbances in nutrient uptake and other metabolic and physiological processes of plants (Sanita di Toppi and Gabbrielli, 1999; Molas, 2002). Cd and Ni naturally occur at trace concentrations in most soils, except for serpentine soils where Ni is abundantly occur (Larcher, 2003). Unlike Cd, which is a non-essential element, Ni is known to play an important role, at low concentration, in nitrogen metabolism, and also found to stimulate seed germination and plant growth (Bollard, 1983) and therefore, Ni is considered to be an essential nutrient for plants (Marschner, 1995; Gerendás et al., 1999). Both, Cd and Ni elements are taken up through metal transporters with low specificity of those for nutrient elements and their excess accumulation can produce toxic effects (Clemens et al., 2002; Rogers et al, 2000).

Table 1. Heavy metal concentrations in agricultural amendments (modified from Nagajyoti et al., 2010)

Metals	Heavy metal concentrations (mg kg^{-1}) in agricultural amendments					
	Sewage sludge	Compost refuse	Farmyard manure	Phosphate fertilizers	Nitrate fertilizers	Lime
Cd	<1–3,410	0.01–100	0.1–0.8	0.1–190	0.05–8.5	0.04–0.1
Ni	6–5,300	0.9–279	2.1–30	7–38	7–34	10–20
Pb	2–7,000	1.3–2,240	0.4–27	4–1,000	2–120	20–1,250
Zn	91–49,000	82–5,894	15–556	50–1,450	1–42	10–450
Cu	50–8,000	13–3,580	2–172	1–300	-	2–125
Cr	8.40–600	1.8–410	1.1–55	66–245	3.2–19	10–15

1.1. Cadmium

The natural Cd concentration in the Earth's crust is 0.1–0.5 μg g^{-1}, although sedimentary rocks and marine phosphates may contain substantially higher levels (Cook and Morrow 1995; Kumar et al. 2000). Because of a strong demand for Cd worldwide, particularly in the nickel-Cd battery industry, approximately 30,000 t of Cd are released into the atmosphere each year, with an estimated amount of 4,000–13,000 t coming from industrial activities (ATSDR, 2005). The normal concentration of Cd in soil ranges from 0–1 μg g^{-1}, while 1–3 μg g^{-1} indicates slight contamination and Cd polluted soil may contain over 3–10 μg g^{-1} (Rodriguez-Flores and

Rodriguez-Castellon, 1982). Cd concentration above 3 µg g^{-1} in agriculture and horticulture soil (Mengel et al., 2001) and 10 µgL^{-1} in irrigation water (Pesod, 1992) are considered to be unsafe for crop production. Its toxicity is generally considered to be 2–20 times higher than that of other heavy metals (Vassilev et al., 1998; Shah and Dubey, 1995).

A number of reports of survey or production and market sites based study from different parts of world such as from China (Liu et al., 2006; Zhuang, et al., 2009), Turkey (Turkdogan et al. 2002), Japan (Arao et al., 2008) and India (Gupta et al., 2008; Sharma et al., 2009; Singh, et al., 2010), revealed that the content of heavy metal such as Cd in vegetables grown in contaminated soils or irrigated with water containing heavy metals were much higher than the International prescribed safe limits of 0.05 mgkg^{-1} (Yamaguchi et al., 2011). Moreover, Cd shown to have the highest capacity for transferring from soil into vegetables, e.g., the bio-concentration factors (BCF) value of Cd was 30-fold those of Hg and 50-fold those of Cr, Pb and As (Chang et al., 2014).

The FAO/WHO recommended maximum tolerable intake of Cd is 70 µg day^{-1} (John et al., 2009). Plant edible tissues constitute the main pathways for cadmium entry into the food chain (Ingwersen and Streck 2005). As assumed that at least 70% of the Cd intake by human is coming from plant foods (Wagner, 1993), and fruit and vegetable consumption is considered the primary source of human exposure to heavy metals (Chang et al. 2014). The long-term intake of Cd from food has raised a serious health concern (Yamaguchi et al., 2011). The major symptoms of chronic Cd toxicity are respiratory problems, renal dysfunction, disorders of calcium (Ca) metabolism, and bone disease such as osteoporosis and spontaneous bone fracture (WHO 1992). Cd causes both acute and chronic toxicity by adversely affecting to the kidneys, liver, heart, vascular and immune system. In short, Cd toxicity has turned into a potential agricultural and environmental issue worldwide (Obata and Umebayashi, 1997; Davis, 1984).

1.1.1. Cadmium uptake, translocation and distribution in plants

Cd can easily enter the roots through the cortical tissue and can reach the xylem via an apoplastic and/or a symplastic pathway (Salt et al., 1995). There are two different ways of Cd uptake have been reported: a) passive uptake, only driven by the concentration gradient across the membranes and b) inducible substrate specific and energy-dependent uptake (Nies, 1999;

Williams et al., 2000). In the soil solution, Cd mainly presents as Cd^{2+} but can also occurs as Cd-chelates (Tudoreanu and Phillips, 2004; Verbruggen et al., 2009). Cd uptake affected by several factors such as the bio-availability of Cd in soil, pH, temperature, redox potential (Bingham, 1979; Irfan et al., 2013), and the presence of chelating substances and other cations (Hardiman and Jacoby, 1984). Cd is highly available in acid soils and its solubility is increased by root exudates (Zhu et al., 1999; Gallego et al., 2012). Indeed at low pH, H^+ binds to soil particles more tightly than to other cations and hence other metals bound to soil particles get easily removed (Dube et al. 2001; McCauley 2009). The uptake and accumulation of Cd are also reported to be closely related to the amount of exchangeable Cd, rather than the total Cd (Dong et al., 2007).

There is no specific mechanism for the uptake or transport of Cd in plants known yet, and neither its involvement in plant metabolism as a moiety of metalloprotein (Irfan et al., 2013), except that in marine diatoms, where Cd has shown its involvement in the activity of carbonic anhydrase and photosynthesis (Thomine et al., 2000; Morant-Manceau et al., 2007). Cd is taken up by plants via cation transport systems normally engaged in the uptake of essential elements such as members of NRAMP and ZIP families' transporters or taken up via Ca uptake channel (Perfus-Barbeoch et al., 2002). Indeed, some of the metals that compete for same transmembrane carrier or uptake channel can inhibit Cd uptake from the rhizospheric solution and its accumulation in plant roots (Hart et al., 2002; Gallego et al., 2012). For instance, Ca levels affect Cd uptake because Cd competes with Ca for Ca uptake channels (Perfus-Barbeoch et al., 2002; Wojas et al., 2007).

After being taken up by roots, the allocation of Cd in different plant organs plays an important role in toxicity of Cd to plants. The larger fraction of the Cd entering in plants retained in the roots and only a small fraction is transferred to the aerial plant parts (Vitoria et al. 2001). A wide range of studies have demonstrated that the root is primary site of toxic metal accumulation in most plant species, including in tomato (Lopez-Millan et al., 2009; Djebali et al., 2010; Hediji et al., 2010). The higher accumulation of Cd in roots may occur due to the chelation with cellular ligands and their subsequent sequestration in the vacuoles, and the development of extracellular barriers, which restrict the translocation of toxic metal through the root symplasm (Lux et al., 2011). Out of the total Cd translocated to the aerial parts, mostly

abundant in older leaves, such a case that of in tomato (Hediji et al., 2010). Due to its mobility in phloem, Cd, in small amount, can also be accumulated in edible parts such as in fruits and seeds (Hart et al., 2002). Tomato plants raised either in contaminated soil or in nutrient medium shown to accumulate a little amount of Cd in their fruits (Gupta et al., 2008; Hediji et al., 2010). In contrast, Hartke et al., (2013), while evaluating 6 tomato cultivars under higher Cd rates in growth medium (90 $mgkg^{-1}$ added to substrate), found significant amount of Cd in the leaves (from 215 to 325 $mgkg^{-1}$ dry weight) and also a considerable amount in their fruits (from 9 to 16 mgkg-1 dry weight), this suggests concentration dependent change in Cd accumulation. In general, the content of Cd in plants decreases in the order roots>leaves>stems>fruits>seeds (Blum, 1997). However, the accumulation rate of Cd depends on the plant species and on the characteristics of soil or growth medium (John et al. 2009; He et al. 2013). Even within the same species, Cd can accumulate in different concentrations in the same organ depending on the cultivars (Alexander et al., 2006; McLaughlin et al., 1994). Different parts of the plants have different capacities to accumulate Cd because of the localization of specific ligands with affinity to bind to a particular metal (Prasad and Freitas, 2000).

Based on the Cd accumulation pattern in plants of 28 vegetables across 7 families tested under Cd stressful condition, Yang et al. (2010) proposed the order from higher to lower accumulators of vegetable species: leafy vegetables > solanaceous vegetables > kale vegetables > root vegetables >alliums> melon vegetables > legumes. However, there was notable differences in Cd accumulation observed within the cultivars of same crop by Hartke et al., (2013) in tomato; Alexander et al. (2006) in carrot and pea; McLaughlin et al. (1994) in potato, Crews and Davies (1985) in lettuce.

1.1.2. Plant response to Cadmium stress

There are two types of causal relationships is reported to exist between higher toxic heavy metals concentrations in the soil/growing medium and the causal toxicity symptoms in plants: a) toxic metals compete with essential mineral nutrients for uptake thereby disturbing the mineral nutrition of plants and; b) after uptake, metal accumulates in plant tissue and cell compartments and hampers the general metabolism of the plant (Turner, 1997; Taylor, 1988). Therefore, Cd-stressed plants showed visible symptoms like leaf chlorosis and necrosis, root browning, reduction of shoot and root biomass, and finally the reduced crop yields (Wanger,

1993; Smeets et al., 2005); these toxicity symptoms could be due to a range of alterations at cellular/molecular levels caused by toxic metal (Hall et al., 2002).

Excess Cd accumulation in plants has shown adverse effect on plant growth by affecting the shoot and root biomass production (Hayat et al., 2012; López-Millán et al., 2009; Shekar, et al., 2011**)**. Roots are the primary victim of Cd damage as it enters first into the roots from growth medium (Sanita di Toppi and Gabbrielli, 1999). When roots exposed to excess Cd, the damage can occur in several ways including reduction in root elongation and root biomass, a decline in root metabolic activities, alterations in root architecture and development of a relatively compact and dense root system (Peralta et al. 2000 ; Shamsi et al. 2007). Liu et al., (2003) observed Cd inhibited growth of roots in garlic, which was ascribed to the direct or indirect effects of Cd on auxin metabolism or its carriers consequently affected cell division and cell expansion growth (Prasad, 1995). Djebali et al. (2010) have demonstrated that shoot growth was more sensitive than root growth, although Cd accumulation was higher in the roots. Sandalio et al., 2001, working with pea, observed a significant depression in plant growth as dry wright, especially in leaves, while the roots were affected only at higher Cd level (50 µM), the most affected part being the lateral roots. An increase in Cd concentration decreased the shoot biomass in pakchoi and vegetables-mustard (Chen et al., 2011) and tomato (Lopez-Millan et al., 2009; Djebali et al., 2010). Rehman et al (2011), found a linear-negative relationship with the dose of cadmium (from 10-50 µM) and leaf number and leaf area in tomato plants exposed to soil Cd. Cd-induced reduction in leaf area was observed in previous studies in tomato (Djebali et al., 2005, 2010; Hayat, et al., 2012). The reduced leaf area in tomato was associated with the reduced leaf expansion as a result of reduced cell size and small intercellular spaces (Djebali et al., 2005).

There has been substantial reduction in crop yield in many crop plants (Prasad, 1995; Yang et al., 1998; Irfan et al., 2013), including in tomato (Khan and Khan, 1983; Moral et al., 1994). In spite of observed reduced plant growth parameters, Shekar et al. 2011, in their study also found a significant reduction in flower numbers plant^{-1} and petals/sepals flower^{-1} and percent pollen viability; andfruit number plant^{-1} and mean fruit weight of tomato in response to increased Cd level in soil. This might explain the reduced fruit yield in other studies. Excess Cd and Ni inhibited growth and yield in cucumber through anatomical alterations and disturbed

physiological and metabolic processes caused by toxic metals (Feng et al., 2010; Matraszek et al., 2010; Savvas e al., 2013).

1.1.3. Mechanisms of Cadmium toxicity in plants

1.1.3.1. Leaf pigments and efficiency of PS II

The chlorophyll and carotenoid decreases by Cd application can be indicative of damage especially at the chloroplasts, where Cd is known to accumulate (Lagriffoul, et al. 1998). Several studies have demonstrated that the primary sites of action of Cd are photosynthetic pigments, especially the biosynthesis of chlorophyll and carotenoids (Baszynski et al., 1980; Prasad, 1995). The decline in chlorophyll content also related to Cd induced alteration in the supply of essential nutrient elements such as Mg, Fe, Zn and Mn (John et al, 2009). Furthermore, the reduction in chlorophyll is reported to a direct consequence of reduced carotenoids content, which is known to protect chlorophyll and other macromolecules by quenching excited triplet state of chlorophyll to avoid generation of free radicals (Bartey and Scolnik, 1995; Hediji et al., 2010). Djebali et al., (2005) have observed the decline in chlorophyll and carotenoids contents was in response to Cd concentration in nutrient solution (from 0 to 100 µM), which were accountable for the reduction in photosynthesis and growth produced by Cd (Stobart et al., 1985; Bazzaz et al., 1992).

The reduction of photochemical activity, as decreased maximal quantum efficiency of PSII (Fv/Fm) due to Cd stress, is one of the non-stomatal factors that affect photosynthesis (Maksymiec et al., 2007; Xu et al., 2012). Cd induced inhibition in photoactivation of PSII is reported to due to its competitive binding to the essential Ca^{2+} site (Faller et al., 2005). The inhibition of PSII was also quite evident under certain abiotic stresses (Calatayud and Barreno, 2004; He et al., 2009), including Cd stress in sunflower (DiCagno et al., 2001), pea (Chugh and Sawhney, 1999) and in model plant *Arabidopsis thaliana* (Maksymiec et al., 2007).

1.1.3.2. Oxidative damage

Once accumulated in excess in plant cells, Cd can adversely affect plant metabolisms and can induce oxidative damage (Gratao et al., 2008; Hediji et al., 2010). Cd-induced oxidative stress in plants may occur as a consequence of over production of reactive oxygen species (ROS) that disrupt cellular homeostasis and cause damage to proteins and pigments and lipid

peroxidation and ion leakage, which in turn leads to membrane damage and enzymes inactivation (Scandalios, 2005; Sharma and Dubey, 2005; Gratao et al., 2008). Given the fact, being non-redox active metal, Cd does not participate in Fenton type ROS-producing reactions; a great deal of research have demonstrated that excess Cd can indirectly induce ROS production in plants (Gallego et al., 2012). Moreover, Xu et al., (2012) have reported that the Cd related oxidative stress is more likely the consequences of inhibition of photosynthesis, as evidenced by the inhibition of photosynthetic electron transport and activity of photosystem (PS) II and decline in photosynthetic pigments. High amount of oxidative stress, as indicated by higher MDA content and ion leakage along with higher H_2O_2 accumulation, was observed in so called sensitive *Solanum* spp. (S. *torvum*) than the higher Cd tolerant spp (*S. nigrum*) (Xu et al., 2012). Increased amount of MDA under Cd stress also observed in rice leaves (Hsu and Kao, 2007), tomato seedlings (Mediouni et al., 2006), *Brassica juncea* (Mobin and Khan, 2007), probably as a result of increased H_2O_2 production (Gratao et al., 2008).

1.1.3.3. Interference with nutrient availability

Presence of Cd in root ambient can markedly alter the plant metabolism as a consequence of disturbance in the uptake and translocation of mineral nutrients (Nazar et al., 2012). Cd interferes mainly with the translocation of most of the micronutrients, especially those having same valences (Zn, Mn, Fe, Cu), to the leaves, though it has rather synergistic effects on their uptake (Sandalio et al., 2001). Some of these elements are important as constituents of many enzymes and proteins (Hall, 2002; Lopez-Millan et al., 2009) and their deficiency can result physiological disorders (Dong et al., 2006). The Cd-induced Fe deficiency in leaves are more conspicuous (Larbi et al., 2002; Lopez-Millan et al., 2009). The reduction of K, Ca and Mg in the tissue due to high concentrations of Cd has been reported in cucumber and tomato plants (Burzynski, 1988). Antagonistic effects between high Cd concentrations and Mn uptake and transport have been reported in many studies (Larbi et al., 2002; Dong et al., 2006; Wu et al., 2007; Lopez-Millan et al., 2009). It has been suggested that this interaction may be mediated either by competition for binding sites or transporters such as NRAMP (natural resistance-associated macrophage protein) and ZIP/IRT (zinc regulated transporter/iron-regulated transporter-related protein) families, which are able to transport Fe, Zn, Mn, Cu as well as Cd (Guerinot, 2000; Clemens et al., 2013). Cd competes with Ca for Ca channel for uptake (Perfus-

Barbeoch et al., 2002), easily replaced Ca because of their same ionic radius and valence (Hart et al. 2002). For example, Cd^{2+} can replace Ca^{2+} in calmodulin and result in the inhibition of calmodulin-dependent phosphodiesterase activity, where calmodulin acts as an important protein in cellular signaling (Rivetta et al., 1997).

1.1.4. Plant tolerance response to Cadmium stress

Plant tolerance mechanisms entail the coordination of complex physiological and biochemical processes, including changes in global gene expression (Urano et al., 2010; DalCorso et al., 2010). Plant tolerance response that can avoid or limit the harmful effects of heavy metals may include 'avoidance' (by excluding metals by roots) and/or 'tolerance' (through internal metal detoxification) strategy to enable plants to survive in presence of excess internal metal concentration. The avoidance involves restriction of metal entry to the cell by extracellular precipitation, biosorption to cell walls, reduced uptake, or increased efflux, whereas tolerance involves intracellular metal chelation through the synthesis of amino acids, organic acids, GSH, or metal binding ligands such as phytochelatins (PCs) and metalothiones (MTs), vacuolar compartmentation, and induction of the antioxidant defense system to counter the toxic effects caused by metal induced ROS (Hossain et al., 2012). However, plant tolerance response to heavy metals can vary for each heavy metal and plant species, and more than one mechanism can involve in mitigating the toxic effects (Hall, 2002).

1.1.4.1. Immobilization and cellular exclusion

A first barrier against heavy metal stress, operating mainly at root level, can be the immobilization of toxic metal by means of the cell wall (Nishizono et al., 1989) hence less metal transfer to the shoots. Indeed, because of their negative charge, cell walls have significant capacity for heavy metal binding and retention (Polle and Schutzendubel, 2003). Lozano-Rodriguez et al. (1997) found that the maize and pea had equal concentrations of Cd in their roots and shoots, but less pronounced toxicity symptoms appeared in maize, which was ascribed to the much of Cd bound to cell wall in maize than pea.

In a study of Ouariti, et al., (1997) with french bean, the only trace amount of Cd translocated to shoot (2%) is presumably due to much of the Cd in the root was in the apoplast or in the vacuoles (Akhter et al., 2014). Moreover, at equal external Cd levels, *Solanum melongena*,

had more symplastic Cd than the tolerant *S. torvum*, suggesting an exclusion mechanism (Yamaguchi et al., 2011).

1.1.4.2. Cellular detoxification- metal chelation and sequestration

Plant tolerance to Cd is thought to be related to plants ability in internal metal detoxification processes conferred by toxic metal chelation with cellular ligands and cellular and subcellular compartmentation of chelated complex (Lopez-Millan et al., 2009 and reference therein). Vacuolar compartmentalization plays an important role in Cd detoxification and tolerance by preventing the free circulation of Cd ions in the cytosol and restricting them to a limited area (Sanita di Toppi and Gabrielli, 1999). PCs are the best-characterized heavy metal chelators in plants, especially in the context of Cd tolerance; the general structure of PCs is (γ-Glu-Cys) nGly, where n = 2–11 (Cobbett 2000; Cobbett and Goldsbrough 2002). PCs are synthesized in the cytosol where thiol groups (-SH) of cysteine allow PCs to chelate metals and form complexes (PCs-Cd) and then transported to the vacuole through the activity of ABC transporters (Sanita di Toppi and Gabrielli, 1999; Cobbett, 2000). In the vacuole, because of the acidic pH, the high molecular weight complex dissociates and Cd can be complexed by vacuolar organic acids (citrate, oxalate, malate) (Krotz et al., 1989), and, possibly, by amino acids. Citrate, which is synthesized in plants, has a higher capacity for metal ions than malate and oxalate, and although its principal role is to chelate Fe^{2+} it also has a strong affinity also for Ni^{2+} and Cd^{2+} (Cataldo et al. 1988). Mishra et al. (2006) observed synthesis of PCs in bacopa plants in both roots and leaves at 10 μM and 50 μM Cd concentrations, respectively. An overview of absorption of Cd present in soil; its transportation, accumulation and detoxification has been shown in Figure 1.

1.1.4.3. Extracellular detoxification

As a first line of defense against heavy metals, plant roots secrete exudates into the soil matrix. Different amino acids and carboxylic acid are exuded from the roots to bind and detoxify heavy metals in soil, therefore, playing an important role in tolerance (Rauser, 1999; Clemens et al., 2001). For examples, oxalate secreted from the root apex helps to exclude Cd from entering tomato roots, thus contributing to Cd resistance in the tomato cv. Micro-Tom (Zhu et al., 2011).

Root exudation of malate and citrate in sorghum and maize, respectively have been reported in response to Cd stress (Pinto et al., 2008)

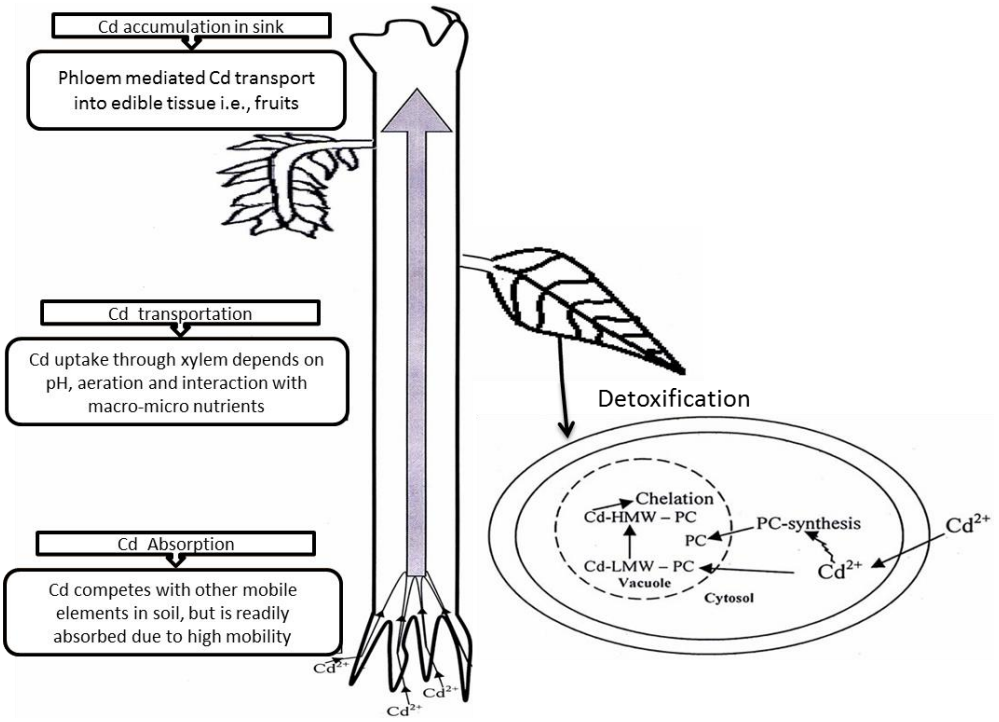

Fig. 1. An overview of Cd absorption, transportation, accumulation and detoxification in plants (adopted from Nazar et al., 2012).

1.1.4.4. Antioxidative defense system

To overcome the effect of ROS, plants have evolved integrative antioxidative defense systems comprised of enzymatic and non-enzymatic antioxidants (Gallego et al., 2012). Antioxidative enzymes play a crucial role in detoxification of ROS via a series of complex systems, which include the dismutation of $O_2^{\cdot -}$ to H_2O_2 by enzyme superoxide dismutase (SOD), and further detoxification of H_2O_2 by peroxidases such as catalases(CAT), ascorbate peroxidase (APX) and guaiacol peroxidase(GPX), (Asada 1992 ; Gratao et al., 2008, Gallego et al., 2012). CAT has been main player in the defense response to Cd in micro-tom (Gratao et al., 2008) and in mustard (Wang et al., 2008). The higher activity of CAT, together with APX, which is a key antioxidant enzyme in ascorbate-glutathione (AsA-GSH) cycle, play important role in H_2O_2 detoxification system (Asada, 1992). Cd-induced changes in the activities of these enzymes have also been reported in pea (Chaoui and El Ferjani 2005), bean (Chaoui et al. 1997), sunflower

(DiCagno et al. 1999), pepper (Leon et al., 2002) and also in tomato (Gratao et al., 2005; 2008; Djebali et al., 2010; Loez-Millan et al., 2009).

Certain metabolites as non-enzymatic compounds such as glutathione (GSH), ascorbic acid (AsA), tocopherol and carotenoids are produced in Cd stress (Gill and Tuteja, 2010; Gratao et al., 2l008; Gallego et al., 2012) and help in alleviating Cd-induced toxic effects in plants. For examples, the content of AsA and GSH increased following higher Cd dose in cucumber (Goncalves et al. 2007) and soybean (Srivastava et al. 2011) which could help in plants adoptive response to Cd stress. Moreover, GSH is an important constituent of the AsA–GSH cycle, a major pathway for the degradation of H_2O_2 produced under Cd stress (Smeets et al., 2008, 2009). Aside acting as osmolyte and its role in metal chelation, proline is known to play an important role as ROS scavenger, thereby reducing oxidative damage (Alia and Saradhi, 1991; Xu et al., 2009; 2012). The accumulation of Proline in stressed plants is reported to be associated with reduced damage to membranes and proteins (Alia et al., 1997; Shah and Dubey, 1998). Higher proline accumulation in tolerant *S. nigrum* plant was associated with higher antioxidants accumulation and lower oxidative damage than *S. melongena* (Sun et al., 2007) or *S. torvum* (Xu et al., 2012), in Cd stress condition. The enhanced accumulation of proline in the leaves might have represented a major biochemical adaptation under heavy metal stress conditions in wheat (Lalk and Dorfling, 1985), faba bean (Siddiqui et al., 2012), cowpea (Bhattacharjee and Mukherjee, 1994) and tomato (De and Mukherjee,1998). In addition to these, certain secondary metabolites such as polyphenol, glucosinolates, hormones, terpenes, and other compounds induced under Cd stress influence the metal toxicity responses in plants.

1.2. Nickel

Ni concentration in environment is increasing in certain areas at alarming rates. It is released into the environment from various anthropogenic activities, including metal mining, smelting, fossil fuel burning, vehicle emissions, disposal of household, municipal and industrial wastes, fertilizer application and organic manures (Alloways, 1995; Chen et al., 2009). During the last two decades, soil and water contamination with Ni has become a serious environmental threat as its concentration reached up to 26 gkg^{-1} in soils and 0.2 mgL^{-1}in polluted water (Zwolsman and Van Bokhoven, 2007; Chen et al., 2009), which are much higher than the general range of Ni in natural soil (<0.1g kg^{-1}) and in surface water (<0.005 mgL^{-1}) (McIlveen

and Negusanti, 1994; McGrath, 1995). It has become widespread pollutants in soil and water and its occurrence has been reported from almost all the continents of the world (Chen et al., 2009).

Given the fact that Ni is readily taken up by plants and its high mobility, there is danger of its excessive accumulation in plant organs, and thus harmful for plants and also to the human being via food chain. In trace amounts, Ni is mostly a necessary element in animals; however, higher concentrations are toxic (Chen et al., 2009). The common damaging effects of Ni are damage of the vessels of the heart muscle, kidneys and central nervous system, and reduce the immune capacities of the animal organism. It may also cause eczemas, allergic inflammation and carcinomas of mucous membranes and lungs (Allenby and Basketter, 1994)**.**

Ni is considered as an essential element and plays a significant role in various metabolic processes, including urea and ureide metabolism, iron absorption, nitrogen fixation and seed development, however, the requirement of Ni in plants is very low (Rao, 2006) and it can be toxic at high concentrations. For most crop species the normal Ni concentration in plant tissues ranged from 0.05 to 10 µg g^{-1} dry weight and above this upper limit, toxicity symptoms are likely to occur (Yusuf et al., 2011). For instance, Ni toxicity is reported to occur at concentrations in the range of >10 µg g^{-1} in sensitive and >50 µg g^{-1} in moderately tolerant species (Marschner, 2002; Seregin and Kozhevnikova, 2006; Yusuf et al., 2011).

1.2.1. Nickel Uptake, translocation and distribution in plants

The uptake of Ni in plants is usually occur by root systems via passive diffusion and active transport (Seregin and Kozhevnikova, 2006), but its uptake ratio between active and passive transport can vary with species, form and concentration of Ni in growth medium (Dan et al., 2002; Vogel-Mikus et al., 2005). A number of reports suggested that Ni is more easily absorbed by the plants when present in ionic form than that of chelated form (Mishra and Kar, 1974). In contrary, DeKock and Mitchell (1957) demonstrated that the absorption rate of Ni was decreased when divalent cations including Ni^{2+} was supplied with EDTA in mustard and tomato. The bio-availability of Ni depends on form of Ni, pH, presence of organic matter and other cations presence in soil or nutrient solution (Mishra and Kar, 1974; Chen et al., 2009). Acidic medium (pH<5.6) favours the absorption of Ni.

Soluble Ni compounds can be absorbed via the cation transport system, including those involve in Cu^{2+} and Zn^{2+}; these two metals have inhibitory effects on Ni^{2+} uptake (Cataldo et al. 1978). Moreover, Mg^{2+} transport also known to involve in Ni^{2+} uptake because of the similar charge/size ratio of the two metal ions (Oller et al., 1997). The inhibitory effect of various metal ions on absorption and translocation of Ni^{2+} from roots to shoots varied as Fe^{3+}, Co^{2+}, Ca^{2+}, Mg^{2+}, NH^{4+}, K^{+}, Na^{+} (Yusuf et al., 2011).

Once taken up by the roots, Ni is transported from roots to the aerial parts in particular leaves through transpiration stream via the xylem (Krupa et al., 1993). Ni^{2+} ions are loaded into the xylem and transported to the shoots as complexes with various chelators. Organic acid such as citrate, although its principal role is to chelate Fe^{2+}, also it has a strong affinity for Ni^{2+} and Cd^{2+}in the xylem (Lee et al. 1977; Cataldo et al. 1988). Moreover, amino acids are potential metal ligands; Ni may also be chelated by histidine and translocated (Kramer et al. 1996). Cataldo et al. (1978) reported that more than 50% of the Ni absorbed by plants is accumulated in the roots, this might attributed to the sequestration of toxic metal in the cation exchange sites of the walls of xylem parenchyma cells and immobilization in root cells vacuoles (Seregin and Khozevnikova, 2006). There was higher Ni concentration in roots than the shoots of barely (Brune and Deitz, 1995) and maize (Baccouch et al., 2001). Ni has been suggested to be highly mobile in xylem and phloem by being its presence in high amount (> 80%) in vascular cylinder, while less than 20% is in the cortex of roots (Marschner, 1995; Page and Feller, 2005).

Several types of transporter proteins are involved in the root-to-shoot transport of metals. Metal ions are also translocated from source to sink tissue via phloem. High molecular weight compounds that chelate Ni has been found in the phloem of *Ricinus communis* plants (Wiersma and Van Goor 1979). Citrate, which is synthesized in plants by the enzyme citrate synthase, and although its principal role is to chelate Fe^{2+}, also it has a strong affinity for Ni^{2+} and Cd^{2+}(Cataldo et al. 1988).

1.2.2. Plant response to nickel stress

Once Ni is taken in excess by plants, diverse toxicity symptoms in plants may occur. The toxic symptoms generated by Ni include chlorosis, necrosis, inhibition of shoot and root growth and decrease in leaf area (Shaw et al. 2004). Moreover, excess concentration of Ni have been

reported to adversely affect plant growth (Molas 2002), plant water relation and photosynthesis (Chen et al. 2009), inhibition of enzymatic activities (Gajewska et al. 2009), interference with the uptake of other essential metal ions and induction of oxidative stress (Chen et al. 2009). All of these altered physiological processes ultimately results in reduced crop yield and quality (Gajewska et al. 2006). However, the responses to toxicity differ according to plant species, growth stage, cultivation conditions, Ni concentration and exposure time (Chen et al., 2009 and reference therein).

Plants response to elevated Ni concentrations in the soil or nutrient solution have been observed as inhibition of shoot and root growth, reduction in leaf area, and consequently a decrease in crop productivity (Shaw et al., 2004). Roots being the primary target of metal anions, their growth are usually more severely affected than that of the aerial parts as evident from two different studies, where excess Ni more severely affected the root growth of cabbage and maize than that of their aerial parts (Panday and Sharma, 2002; Seregin et al., 2003). The shoot and root dry matter production of tomato have reported to be adversely affected when plants were fed to5 mg L^{-1} Ni by supplying in root media (Palacios et al., 1998). Finding of Parida et al., (2003) showed that soil application of Ni at higher rate (>30-40 mg kg^{-1}) adversely affected fresh and dry weight yield of fenugreek. The crop performance in terms of growth and yield of tomato decreased in response to increasing Ni concentration from 0 to 20 mg L^{-1} in the nutrient solution (Balaguer et al. (1998). Others report show that the accumulation of Ni adversely affects the growth and yield of eggplant (Pandey and Sharma, 2002), wheat (Gajewska et al., 2006) and mustard (Gopal and Nautiyal, 2012).

1.2.3. Mechanism of nickel toxicity in plants

1.2.3.1. Leaf pigments and efficiency of PSII

Ni is readily taken up by root system and has shown to inhibit a large number of plant enzymes such as those of the Calvin cycle and chlorophyll biosynthesis and consequently decreases the photosynthetic activity (Ali et al., 2008; Pandey and Sharma, 2002). The primary functional mechanism of heavy metal toxicity is the displacement of essential ions from important molecules e.g. displacement of Mg^{2+} from Ni^{2+}, this leads to the alteration of the

structure and/or activity of chlorophyll molecule and that of ribulose-1,5-biphosphate carboxylatedoxygenase (RuBisCO) (Van Assche and Clijsters 1986; Kupper et al. 1996, 1998).

It is reported that Ni damages the photosynthetic apparatus at almost every level of its organization, including destroying cells of mesophyll and epidermal tissue and decreasing chlorophyll a, b, total chlorophyll and chlorophyll a/b ratio (Chen et al., 2009). Ni interferes with the photosynthetic electron transport chain and the light harvesting complex II (Singh et al., 1989; Chen et al., 2009).

1.2.3.2. Oxidative damage

Excessive Ni leads to significant increases in the concentration of hydroxyl radicals, superoxide anions, nitric oxide and hydrogen peroxide (Chen et al., 2009). Similar to Cd, also Ni is not a redox active metal that cannot directly generate these ROS. However it interferes indirectly with a number of antioxidant enzymes (Gajewska and Sklodowska, 2007; Hao et al., 2006; Chen et al., 2009), e.g. CAT, APX and GPX, which are involved in the scavenging of ROS to protect cells from oxidative damage (Ali et al., 2013). The changes in the membrane lipid composition as MDA content, which is an important indicator of oxidative stress, due to Ni stress have been also increased linearly as observed earlier in rice (Ros et al., 1992), wheat (Pandolfini et al., 1992), pigeonpea and cucumber (Madhava Rao et al. 2000; Khoshgoftarmanesh et al., 2014). Exposure to high level of Ni^{2+} enhanced MDA concentration in. However, the MDA content was higher in Ni^{2+} sensitive plants compared to a Ni^{2+} tolerant *Silene* (Gonnelli et al. 2001). High Ni^{2+} concentration adversely affected cell membrane functions and hence can affect the lipid composition and H-ATPase activity of the plasma membrane, as reported in rice shoots (Ros et al., 1992). Gajewska et al. (2013) observed that exposure to Ni resulted in enhancement of electrolyte leakage in shoot and root of wheat seedlings. In their earlier study, they have suggested that the destructive effect of Ni on membranes might be associated with increased lipid peroxidation as well as changes in fatty acid profile (Gajewska et al. 2012). Moreover, findings of Ouzounidou et al. (2006) indicated that membrane structure and functioning might also be affected due to displacement of Ca^{2+} from the membrane ligands by Ni^{2+} ions.

1.2.3.3. Interaction with nutrient elements

Ni, above a threshold level (5-10 µg g^{-1} DW), may inhibit the absorption, uptake and accumulation of essential macro and micro elements such as K, Ca, Mg, Fe, Cu, Zn, and Mn, leading to their deficiency in plants (Chen et al., 2009; Yusuf et al., 2011). Palacios et al. (1998) have reported that high concentration of Ni decreased significantly the uptake of several divalent cations (Mg^{2+}, Fe^{2+}, Mn^{2+}, Cu^{2+}, and Zn^{2+}). One of the possible mechanisms for the reduction in the uptake of these essential nutrients relies on the competition for the common binding sites due to similar ionic radii of Ni and the above mentioned cations (Seregin and Khozevnikova, 2006). The decrease in the uptake may also be attributed to Ni-induced metabolic disorders that affect root function (Seregin and Khozevnikova, 2006). For instance, in tomatoes Ni absorption predominately damaged the roots, causing a reduction in the subsequent absorption and translocation of all major nutrient elements (Knight and Crooke, 1956).

The displacement of one metal ion by any toxic heavy metal ion leads to the inhibition or loss of enzyme functions. For instance, displacement of Mg^{2+} from Ni^{2+}in RuBisCO that results in a loss of enzyme activity (Wildner and Henkel, 1979). Moreover, since SOD and CAT are metalloenzymes containing Fe, Cu or Zn (Chen et al., 2009), the decreasing in enzymatic activity could be expected as excess Ni has shown to decrease micronutrients (Fe, Cu and Zn) in the leaf tissues. Nalini-Panday and Sharma (2003) had pointed that the exposure to excess Ni concentration decreased uptake of Fe and its translocation to tomato leaves as well as chlorophyll content, associated with decrease in the activities of the Fe enzymes, catalase and peroxidase.

1.2.4. Mechanisms of plant tolerance to Ni stress

1.2.4.1. Cellular detoxification of Ni

In many hyperaccumulators, Ni is stored in vacuoles or transported through the cytoplasm by complexing with organic acids such as malate and citrate or amino acids such as histidine and glutamine (Bhatia et al. 2005; Krämer et al. 2000).Histidine is well recognized free amino acid in heavy metal metabolism and due to presence of carboxyl, amino, and imidazole groups form chelation to Ni and confer Ni tolerance (Callahan et al. 2006). Histidine levels also increase in the xylem of *Alyssum lesbiacum*, a Ni hyperaccumulator, when the plant is exposed to Ni (Kramer et al. 1996).

1.2.4.2. Extracellular detoxification

As a first line of defense against heavy metals, plant roots secrete exudates into the soil matrix. One of the major roles of root exudates is to chelate metals and to prevent their uptake inside the cells (Marschner 1995). For example, Ni-chelating histidine and citrate are present in root exudates and these reduce the uptake of Ni from soil (Salt et al. 2000).

1.2.4.3. Antioxidant system

Exposure of maize (Baccouch et al. 2001) and pigeonpea (Rao and Sresty 2000) to Ni provoked positive response to antioxidant systems. The increase in the leaf APX and GPX activities were observed in cucumber, rice, and *Luffa cylindrica* under Ni stress conditions (Gajewska and Sklodowska, 2008; Maheshwari and Dubey, 2009; Wang et al., 2010). The decrease in CAT activity recorded in cabbage and wheat, respectively in the findings of Pandey and Sharma (2002) and Gajewska and Sklodowska (2008). In contrast, Khoshgoftarmanesh et al. (2014) reported an increase in the CAT activity at toxic concentrations of Ni. An explanation for these contradictory results could be that the activity of antioxidant enzymes (CAT) may vary with the type and duration of stress treatment, growing conditions and also between plant species/genotypes (Chen et al. 2009 and references cited therein). Sharma and Bhardwaj (2008) also observed an increase in the activities of CAT and POX and SOD in mustard plants exposed to Ni. Short duration Ni exposure at around 0.05 mM has shown to increase the activities of certain antioxidant enzymes such as SOD, POD, GR, and GPX and led to the removal of ROS in different plant species (Freeman et al., 2004; Gajewska and Sklodowska 2005; Gomes-Juniora et al.,2006).

Another system of protection against Ni toxicity include the synthesis of osmolytes, such as proline (Seregin and Kozhevnikova, 2006). Proline enhancement was observed in response to heavy metal toxicity (Pandey and Sharma, 2002; Gajewska et al., 2006). The decrease in proline contents may be related with the oxidative stress resulting in hydrolysis of protein (Azmat and Khan, 2011).Accumulation of proline in plants exposed to Ni treatment has been observed in cabbage (Panday and Sharma 2002), pea (Gajewska and Sklodowska, 2005), wheat (Gajewska et al. 2006, 2009), and in rice plants (Maheshwari and Dubey 2007).

1.3. Vegetable grafting

1.3.1. Importance and background

Grafting has been practiced in woody plants such as fruit trees for thousands of years, but it is relatively a recent innovation in herbaceous vegetables. The first record of vegetable grafting comes from Japan, where a watermelon farmer reportedly developed watermelon [*Citrullus lanatus* (Thunb.) Matsum. and Nakai] for pest/disease and yield increase by grafting with squash rootstock (*Cucurbita moschata* Duch.) in the 1920s (Tateishi, 1927). For members of the *Solanaceae,* the first record was of eggplant (*Solanum melongena* L.) grafted on scarlet eggplant (*Solanum integrifolium* Poir.) in the 1950s (Oda, 1999). Grafting tomato (*Solanum lycopersicum* L.) was introduced commercially in the 1960s (Lee and Oda, 2003). In fact, vegetable production using grafted seedlings at commercial scale was started in Japan and Korea three decades ago (Lee et al., 2010). It was reintroduced in Europe in the early 1990s as a mean to control root diseases of soil or hydroponic systems (Lee et al., 2010). The adoption of vegetable grafting in the Western world (American, European and Middle East countries) has increased significantly since the banning of the fumigant methyl bromide in 2005 by the Montreal Protocol (Cohen et al., 2012). Because of this, grafting has been primarily used in fruiting vegetables to improve plant tolerance against soil borne pathogens (Lee, 1994; Crinò et al., 2007) but its application dramatically increased over the years in mitigating the negative effects of other biotic and abiotic stresses with expanding the reasons of grafting. In recent pasts, appreciable amount of success achieved and grafting has emerged as useful tool to increase plant vigor and yield, induce higher tolerance to abiotic stress conditions such as salinity, heavy metal, nutrient stress, thermal stress, water stress, organic pollutants, and alkalinity and may also to improve fruit quality (Colla et al., 2010a,b,c 2011, 2012, 2013; Savvas et al., 2010; Schwarz et al., 2010; Rouphael et al., 2008a,b, 2010).

1.3.2. Grafting in relevance to heavy metal stress

Given the fact, considering the difficulty in controlling heavy metal accumulation in environmental, the organisms have to cope with exposure to unwanted heavy metals (Gallego et al., 2012). In recent years, concerted efforts focused on reducing heavy metals uptake and accumulation in the aerial plant organs to mitigate the adverse effects of toxic metals.

Development of plant cultivars of genetic tendency to resist heavy metals stress is a constituent part of environmentally-friendly technologies which allow harvesting clean agricultural products on polluted soil (Alybayeva et al., 2014). However, it is too long process and takes several years to breed a variety of desired traits. Furthermore, it is still not clear whether plants heavy metals tolerance and hyperaccumulation abilities are genetically independent or related traits (Tsyganov et al. 2007). In fact, plants have evolved different defense mechanisms to mitigate the adverse effects of heavy metals on their metabolism through minimizing their absorption or detoxifying them when they enter into plant tissues (Savvas et al., 2013). Root genotypes play a crucial role in the defense mechanisms against heavy metals by way of controlling their uptake (Sarwar et al., 2010; Savvas et al., 2013). However, the efficiency of many elite commercial cultivars of fruit vegetables to exclude heavy metals and maintain high rates of nutrient uptake when they are exposed to heavy metal stress is rather low. One way to develop plants ability to tolerate the adverse effects of heavy metals would be by uniting the characteristics of both the elite cultivars and tolerant/hardy root genotypes by way of grafting. Grafting is regarded as an alternative and faster tool over relatively slow breeding methodology (Flores et al., 2010). Many rootstocks used in vegetable grafting were able of enhancing the uptake rates of some nutrients even under abiotic stress conditions (e.g. heavy metals) because they are characterized by more vigouros root systems. The ability of plant roots to control the uptake of nutrients and non-nutrient elements depends on both the root structure and the uptake mechanisms at biochemical level in the root cells (Savvas et al., 2010).

In relevance to the heavy metals stress, recent studies have indicated that some rootstocks of *Solanaceae* and *Cucurbitaceae* species may efficiently restrict the uptake and/or translocation of heavy metals and toxic micronutrients in minimizing their accumulation in aerial plant biomass, including the fruits (Edelstein et al., 2007; Arao et al., 2008; Rouphael et al., 2008, Mori et al., 2009; Savvas et al., 2013). A few studies are there reporting the effectiveness of grafting in reducing fruit and/ or shoot Cd and Ni contents in vegetables. It has been reported that grafting can reduce the ions transport to the shoots due to mechanical incision (Kawaguchi et al., 2008; Martínez-Ballesta et al., 2010). While comparing the ability of four commercial inter-specific *Cucurbita* (*C. maxima* × *C. moschata*) rootstocks along with self-grafted and ungrafted cucumber plants under external concentration of Cd (10μM) and Ni (50μM), Savvas et al. (2013), showed that grafting *per se* significantly reduced Ni accumulation in shoots, with no

effect of root genotypes. Whereas, the lowest Cd concentration was in plants grafted with 'Power' rootstock, which was mainly due to Cd exclusion at the plasma membrane of root cells of 'Power' rootstock. Furthermore, a considerable reduction of Cd in fruit and shoot tissues of eggplant was noticed in plants grafted onto *S. torvum* (Arao et al., 2008), and this reduced Cd in *S. torvum* grafted plant was ascribed to restricted root-to-shoot metal translocation (Arao et al., 2008; Mori et al., 2009), and this limited root-to-shoot Cd transfer in *S. torvum* was due to suppressed symplastic uptake and xylem loading process (Yamaguchi et al., 2011). These studies were mainly focused on the reduction of Cd contents in fruits and shoots by use of grafting, it is still uncertain if the reduced Cd content in aerial parts of plant's is related to plant tolerance with plant growth and yield behaviours. However, grafting response in cucumber (Rouphael et al., 2008) and melon (Edlestein et al., 2005) to toxic level of Cu and B, respectively have shown that the plant tolerance of cucumber and melon, based on higher growth and fruit yield, achieved when plants were grafted onto commercial tolerant pumpkin rootstocks 'Shintoza' and 'TZ-148', respectively, compared to their respective ungrafted control. Beside the observed lower toxic concentration of Cu and B in leaves of grafted plants, the enhanced nutrient uptakes and availability concomitant with better physiological activities resulted by vigorous rootstocks might have led better plants performance under metals stress conditions. For examples, at higher external Cu, reductions in net assimilation, stomatal conductance, chlorophyll and carotenoid content and percentage of electrolyte leakage were more severe in ungrafted plants in comparison with those grafted on 'Shintoza' rootstock (Rouphael et al., 2008). A wide range of studies have demonstrated that the rootstock mediated positive effects in grafted plants under different abiotic stresses is possibly due to better nutrients uptake and translocation, biosynthesis and contents of photosynthetic pigments, photosynthetic activity, antioxidant activity, lower oxidative stress, etc. (Rouphael et al., 2008; He at al., 2009; Dietmar et al.,2010; Colla et al., 2013). In short, it can be due to better plant nutritional status, physiological and biochemical process, together with better internal toxic-ion detoxification ability provide by vigorous root system of specific rootstock. Studies have also shown that the rootstock significantly affects the gene expression in the scion, thereby indicating that some signals transported from the root to the shoot may also influence the heavy metals uptake and translocation (Si et al., 2010).

1.4. Arbuscular mycorrhizal fungi

1.4.1 Importance in heavy metal stress

Another promising and environmental friendly tool to overcome heavy metals toxicity in contaminated soils would be through the inoculation with beneficial microorganisms such as arbuscular mycorrhizal (AM) fungi. AM are beneficial associations between soil fungi and plant roots, related with 83% of higher plants (Ma et al., 2001). The association of AM fungi with plant roots can enhance plant growth and resistance to stressful edaphic conditions including toxicity produced by heavy metals (Ouziad et al., 2005; Shahabivand et al., 2012; Liu et al., 2015). It has been demonstrated that AM can constitute a biological barrier against transfer of toxic elements or heavy metals to shoots (Joner and Leyval, 1997). Improved nutritional status, reduced metal uptake, immobilization of metals in fungal biomass, dilution by increased shoot and root growth, and metal compartmentalization into plastids or other membrane rich organelles are among the most Cd-tolerance mechanisms induced by AM fungi (Turnau et al., 1993; Kaldorf et al., 1999; Gaur and Adholia, 2004; Andrade et al., 2008). The response of AM fungi in heavy metal uptake may differ with the level of heavy metal in soil (Rivera-Becerril et al., 2002). For examples, there was an enhancement in foliar Cd concentration observed in mycorrhized soybean and clover plants raised under low Cd condition. However, at higher Cd level, AM fungi either decreased or did not change foliar Cd concentration (Gildon and Tincker, 1983; Heggo et al., 1990; Tonin et al., 2001). Shoot and root development of three pea genotypes were decreased in the presence of Cd, but inoculation with the AM fungus (*Glomus intraradices*) attenuated this negative effect in all the genotypes (Rivera-Becerril et al. (2002).

There is no evidence in International literature, showing that the grafting has been used in response to alleviate plant tolerance in vegetables such as tomato to heavy metals, especially the two most important heavy metals of environmental concern i.e., Cd and Ni. Moreover, the response of mycorrhizal integration with grafting in response to heavy metals tolerance is lacking.

1.5. Tomato

Tomato (*Lycopersicon esculentum* L.) is the most important vegetable crop, next to potato. Present world production of tomato is about 164 million tons fresh fruit from 4.7 m ha

(FAOSTAT, 2013, Table 2). In addition to its economic importance, tomato is known for its nutraceutical importance being rich source of antioxidants, such as lycopene, β-carotene, phenolics and vitamin C in human diet (Toor et al., 2006). It has been associated with a lower risk of developing certain types of cancer, cardiovascular diseases and age-related macular degeneration (Dorais et al., 2008). Tomato is worldwide produced and consumed, grown in both the open-filed and greenhouse conditions, under soil or soil-less medium.

Due to ever increasing worldwide food demands and shrinking cultivated lands, it is likely that vegetables, in particular tomato production could face or occur adverse condition of polluted soil or water with heavy metals. Moreover, there are reports from different parts of the world where vegetables, including tomatoes exposed to heavy metals in natural soil and irrigation water particularly, in urban and sub-urban areas (Zhuang, et al., 2009; Singh et al., 2010).

Table 2. Tomato top producer in 2013 according to FAOSTAT data

Top Tomato Producer – 2013 Production (,000 MT)	
China	50,664
India	18,227
United States	12,575
Turkey	11,820
Egypt	8,533
World	163,964

1.6. Hypothesis

Considering the above, the research programme was developed with the hypothesis that grafting commercial tomato cultivar (cv. Ikram) onto selected rootstocks will increase plant tolerance to Cd and Ni stress either by limiting metal uptake by roots and/or its translocation to the aerial plant parts and/or facilitating internal toxic metal detoxification, while maintaining optimum uptake and mobilization of nutrient elements within the plants. Moreover, minimizing the intake of toxic metals by consumers due to lower fruit metal concentration could be achieved.

1.7. Objectives

To verify the proposed hypothesis, the research work planned to accomplish the following objectives:

1. to evaluate different grafting combinations under external heavy metals (Cd and Ni) concentrations;
2. to evaluate the performance of rootstock-grafted plants with or without mycorrhiza under Cd stress and
3. to study the mechanisms of rootstock-mediated plant tolerance at physiological, biochemical, metabolomic and molecular level.

To meet the above mentioned objectives, following research activities were organized under hydroponic system in greenhouse at University of Tuscia, Viterbo:

1) evaluation of grafting combinations under Cd stress (1st year, 2012);

2) evaluation of grafting combinations under Ni stress (2nd year, 2013);

3) evaluation of grafting with or without mycorrhiza under Cd stress (3rd year, 2014).

Chapter 2

Effects of Grafting on Growth, Yield, Elements Uptake and Distribution under Cd Stress in Tomato

Pradeep Kumar[1,2], Menahem Edelstein[3], Mariateresa Cardarelli[4], and Giuseppe Colla[2*]

[1] *ICAR-Central Arid Zone Research Institute, 342003 Jodhpur (Rajasthan), India*

[2] *Department of Agriculture, Forestry, Nature and Energy, University of Tuscia, 01100 Viterbo, Italy*

[3] *Newe Ya'ar Research Center, ARO, Ramat Yishay, 30095 Israel*

[4] *Consiglio per la Ricerca in Agricoltura e l'analisi dell'economia agraria, Centro di ricerca per lo studio delle Relazioni tra Pianta e Suolo, 00184 Roma, Italy*

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*Correspondence: Prof. G. Colla; e-mail: giucolla@unitus.it

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Chapter 2

Effects of Grafting on Growth, Yield, Elements Uptake and Distribution under Cd Stress in Tomato

Abstract

A greenhouse experiment was conducted to determine the influence of long-term Cd exposure (0, 25 or 50 µM) on crop productivity, fruit quality, leaf chlorophyll content, fluorescence and mineral composition of tomato plants cv. Ikram, either non-grafted, self-grafted or grafted onto rootstocks of tomato (Maxifort or Unifort) and eggplant (Black Beauty). Both moderate (25) and high (50 µM) concentration of Cd in root ambient considerably decreased the fruit yield and fruit number in response to Cd levels, whereas mean fruit weight decreased but was similar to both supply levels. Shoot and root biomass and leaf area were decreased with increased level of Cd in nutrient solution. The fruit yield, shoot and root biomass and leaf area were higher in plants grafted onto tomato rootstocks and especially onto Maxifort in comparison with non-grafted or self-grafted plants and especially grafted onto Black Beauty. The higher plant performance of tomato rootstocks grafted plants were related to higher SPAD index, chlorophyll fluorescence and photosynthetic pigments concentration in leaves associated with better nutrient translocation and availability (higher Ca, Mg, Fe, Mn and Cu) in leaves. Moreover, the content of Cd was also lower in leaves and fruits of Maxifort grafted plants. Concerning fruit quality, especially fruit color, fruit toxicity symptom and fruit Cd concentration, Black Beauty followed by Maxifort grafted plants were better. However, plants grafted onto Black Beauty rootstock were poorly performed for production related traits than rest other grafting combinations which was possibly due to lower nutrient elemental concentration along with toxic elements, indicating some incompatibility reaction with scion.

1. Introduction

The growing concerns over heavy metal pollution of soil and water especially, in the current era of industrialization have received increasing attention of researchers worldwide as its consequences can potentially endanger plant and human health. In particular, cadmium (Cd) is one of the most dangerous metals in agricultural soils (Dong et al., 2007). However, Cd

concentration above 3 µg g^{-1} in agriculture soil (Mengel et al., 2001) and 10 µgL^{-1} in irrigation water are considered to be unsafe for crop production (Pescod, 1992). It is suggested that Cd can cause damage even at very low concentrations, and healthy plants may contain Cd levels that are toxic for human and animals (Chen at al., 2007). Cd is non-essential element for plant growth but can easily taken up by plants and its accumulation in plants may result serious human health concern via food chain (Zhou and Qiu, 2005; Shah and Dubey, 1998)

In plants, Cd has been reported to severely affect morphological and physiological processes such as leaf chlorosis, and roots browning, (Lopez-Millan et al. 2009; Hasan et al., 2011), decline of plant growth (Lopez-Millan et al. 2009; Moral et al., 1994) nutritional deficiency, decrease in chlorophyll synthesis, photosynthetic efficiency, nitrogen assimilation, membrane integrity and enzymes activity (Chen et al., 2007; Hédiji et al., 2010; Xu et al., 2013). Among the possible underlying mechanisms of causing toxicity by Cd reported by Hasan et al. (2011) are the binding of Cd to sulfhydryl and/or carboxyl groups or replacement of essential co-factors e.g. Zn by Cd, and Cd induced oxidative stress either by producing oxygen free radical or by decreasing the level of enzymatic and non-enzymatic antioxidants. However, the intensity of Cd stress varies among plant species/ cultivars, plant tissues and its concentrations and duration of exposure (Grant et al., 1998; Alexander et al., 2006; Benavides et al., 2005; Groppa and Benavides, 2008).

In recent years, concerted efforts focused on reducing heavy metals uptake and accumulation in the aerial plant organs. Development of plant cultivars of genetic tendency to resist heavy metals stress is a constituent part of environmentally-friendly technologies which allow harvesting clean agricultural products on polluted soil (Alybayeva et al., 2014). However, it is too long process and takes years to breed a variety of desired traits. Moreover, it is still not clear whether plants Cd tolerance and hyperaccumulation abilities are genetically independent or related traits (Tsyganov et al. 2007).

One of the possible sustainable strategies for improving plant tolerance to Cd stress, particularly in fruit vegetables, would be by grafting them onto resistant/tolerant rootstocks. Grafting has been primarily used in fruiting vegetables to improve plant tolerance against soil borne pathogens (Lee, 1994; Crinò et al., 2007) but its application dramatically increased over the years in mitigating the negative effects of other biotic and abiotic stresses with expanding the

reasons of grafting. In recent pasts, appreciable amount of success achieved and grafting has emerged as useful tool to increase plant vigor and yield, induce higher tolerance to abiotic stress conditions such as salinity, heavy metal, nutrient stress, thermal stress, water stress, organic pollutants, and alkalinity and may also to improve fruit quality (Colla et al., 2010a,b,c 2011, 2012, 2013; Savvas et al., 2010; Schwarz et al., 2010; Rouphael et al., 2008a,b, 2010).

To our knowledge, few studies are there reporting the effectiveness of grafting in reducing fruit and/ or shoot Cd contents in vegetables (Arao et al., 2008; Mori et al., 2009; Savvas et al., 2013). A substantial reduction of fruit, and also in shoot Cd content in eggplant (*Solanum melongena* L.) was achieved by Arao et al. (2008) by grafting onto *S. torvum* rootstock. The reduced fruit and shoot Cd content was mainly due restricted root-to-shoot Cd transfer by *S. torum* rootstock, as also revealed by the study of Mori et al. (2009). Similarly, Savvas et al.(2013) were able to effectively reduce fruit Cd content in cucumber (*Cucumis sativus* L.) by grafting onto a commercial interspecific hybrid *Cucurbita* rootstock, 'Power' (*C. maxima x C. moschata*), where the reduced fruit Cd content was mainly due to limited Cd uptake by the roots apparatus. However, no information is available up to date regarding the impact of grafting and rootstock genotype on the uptake and accumulation of Cd by tomato, which is one of the world's most important vegetables, grown in both the open-filed and greenhouse conditions.

Taking above into consideration, in this study we exposed commercial tomato cv. 'Ikram', either non-grafted or grafted onto its own roots (self-grafted) or onto selected rootstocks of tomato and eggplant, to long term Cd stress conditions (0, 25 and 50 μM). The relative performance of different grafting combination to Cd stress was determined in terms of plant growth, fruit-yield and -quality, SPAD index and chlorophyll fluorescence, chlorophyll and carotenoids content, tissue metal distribution and nutrient element composition and assimilate partitioning in different plant organs.

2. Material and methods

2.1. Plant material and growing conditions

Tomato (*Solanum lycopersicum* L.) cv. Ikram (Syngenta, Milan, Italy), a leading cluster bearing cultivar in Italy and other Mediterranean countries, was selected as scion. Scion cv.

Ikram either self-grafted or grafted onto tomato or eggplant rootstocks, whereas ungrafted Ikram was used as a control plant. Plants were grown during autumn growing season over the period from August 4 to December 18, 2012 (136 days after transplanting) in a heated greenhouse located at the Experimental Farm of Tuscia University, Italy. The greenhouse was maintained at daily temperature between 18 and 33^{o}C, and day/night relative humidity of 55/85%.The selected tomato rootstocks were: Maxifort and Unifort (De Ruiter/Monsanto, Bergschenhoek, The Netherlands), where Maxifort provides higher plant vigour than the Unifort. However, the eggplant (*Solanum melongena*) Heirloom cv. Black Beauty was included based on its earlier shown capability as rootstock for tomato in alleviating adverse effects of certain abiotic stresses in grafted tomato plants (Black et al., 2003; Abdelmageed and Gruda, 2009).

Seedlings were produced by a commercial company (Centro SEIA, Sisily, Italy) using the slant cut (splice) graft technique. Tomato seedlings of good health at 3-4 true-leaf stage were transferred into pots (6 L) containing quartziferous sand. Pots were arranged in double rows in separate plastic covered channel leaving pot head open on a fixed platform providing sufficient slope to enable drainage collection into tanks fixed at the lateral end of each channel. The space between plants within a row was 0.45 m and the distance between the centers of double rows was 1.2 m. Pollination was facilitated by the bumble bees. Plants were grown as vertical cordons where all laterals branches were pruned and later the terminal bud was also removed after having 6th fruit cluster.

Plants were subjected to three Cd concentrations i.e. 0, 25 and 50 µM by adding appropriate amount of $CdCl_2$ to the basic nutrient solution. The Cd treatment was applied in form of $CdCl_2$ and initialized 27 days after the transplanting (Aug. 31). The basic (control) nutrient solution used in this experiment was a modified Hoagland and Arnon formulation, which was in the beginning applied at half concentration for 3 weeks and later shifted to full concentration. A composition of the basic nutrient solution, using salts of analytical grade, was: 14.0 mM N–NO_3, 1.6 mM S, 1.5 mM P, 6.0 mM K, 4.5 mM Ca, 1.5 mM Mg, 20 µM Fe, 9 µM Mn, 0.3 µM Cu, 1.6 µM Zn, 20 µM B, and 0.3 µM Mo. Deionized water was used for the preparation of all nutrient solutions. Nutrient solution, with or without Cd, was pumped from independent supply tanks of 478L through a drip irrigation system, with one emitter per plant of 2 l h^{-1} flow rate. Five to 15 fertigations were applied per day, each of 2-5 min duration. Timing of the irrigations was

increased to have at least 30% of the nutrient solution draining out from the pots (Rouphael et al., 2004; Rouphael and Colla, 2005). The nutrient solutions were changed at every 10-12 days intervals (in the beginning) to 5-6 days (full grown plants stage).The pH of the nutrient solutions was adjusted to 5.6–5.8 by adding proper amounts of 1N HNO_3 stock solution.

The three nutrient treatments solution (non-Cd control 25 and 50 µM Cd) were combined with the five grafting treatments (ungrafted Ikram; Ikram/ Ikram; Ikram/ Black Beauty; Ikram/ Maxifort or Ikram/ Unifort) in a factorial experimental design rendering 15 treatments. Each treatment was replicated three times with three plants in each.

2.2. Yield and plant biomass determination

Fully ripe fruit harvest was started on October 26 and continued until the termination of the experiment (December 18). The fruit yield, number of fruits and mean fruit weight were recorded for all individual plant. At the end of the experiment (136 DAT, Dec. 18), each plant was separated into different plant organs (stem, leaf, and root) and were dried in a forced-air oven at 80 ºC until constant weight for biomass determination. Shoot biomass was equal to the sum of aerial vegetative plant parts (leaves + stems). Leaf area (LA) was measured with an electronic area meter (Delta-T Devices Ltd., Cambridge, UK). At the termination of the experiment, stem girth of scion and rootstock were also measured above and below the graft union point, respectively.

2.3. Fruit quality analysis

At peak harvesting period, nine full red-ripe fruits were selected per plot (3 from each plant) to determine the fruit quality. Fruit firmness (N cm^{-2}), by using a penetrometer (Bertuzzi FT 011; Brugherio, Milan, Italy), fitted with an 8 mm-diameter round-head probe and fruit shape index (SI), the ratio of width to length were determined for each fruit. Total soluble solids (TSS, ^{o}Brix) content of the fruit juice was determined by an Atago N1 refractometer (Atago Co. Ltd., Japan) and titratable acidity was determined by potentiometric titration with 0.1 M NaOH up to pH 8.1 and the results were expressed as percentage of citric acid in the juice. Fruit juice pH was also measured with a pH meter (HI-9023; Hanna Instruments, Padova, Italy). Fruits were dried in a forced air oven at 80 ºC until constant weight and weighed to determine the fruit dry matter (DM).

Fruit color measurements were performed on the surface of the tomatoes, around the equatorial region. The color was measured for 10 tomatoes per plot with 4 times for each tomato. A Minolta Chroma Meter CR-200 (Minolta Camera Co. Ltd., Osaka, Japan) tristimulus color analyzer, consisting of a head with an 8 mm diameter measuring area a diffuse illumination/0° viewing, was used. The chromameter was first calibrated with a white tile and checked for recalibration between measurements, although no adjustments were necessary. Readings are reported in the L*, a*, b* system.

2.4. SPAD index and chlorophyll fluorescence measurements

A portable chlorophyll meter (SPAD-502, Minolta corporation, Ltd., Osaka, Japan) was used to measure the leaf chlorophyll concentration, as a rational unit. Measurements were made at a central point on the leaflet between the midrib and the leaf margin of 3rdleaf from the top. Twelve random measurements per plot were taken and averaged to a single SPAD value for each treatment.

The Photosystem II (PSII) efficiency of leaves, estimated by the variable to maximum chlorophyll fluorescence ratio (Fv/Fm) was measured by chlorophyll fluorometer Handy PEA (Hansatech Instruments Ltd, UK) as described by Colla et al. (2013). The measurement done on the adaxial surface of six random leaves in the same leaflets per plot after at least 20 minutes dark adaptation. The maximum quantum yield of open PSII (Fv/Fm) was calculated as (Fm – F0)/Fm (Maxwell and Johnson, 2000). The SPAD and fluorescence measurements were done at 90 days after the initiation of Cd treatment.

2.5. Leaf pigment concentration determination

The leaf pigments (total chlorophyll and carotenoids) were extracted by homogenization of fresh leaf tissues (0.5g) in acetone (80%). The resulting extracts were centrifuged at 4,800 *g* for 20 min. The total chlorophyll and carotenoid contents were determined by taking absorbance of the supernatant at 470, 647, and 664 nm by a UV–Vis spectrophotometer (Perkin Elmer, Norwalk, CT, USA). The chlorophyll and carotenoids contents were calculated as were described by Lichtenhaler and Wellburn (1983) and the content was expressed in mgg^{-1}of fresh weight.

2.6. Analysis of Cd and mineral nutrient concentrations

The leaf, stem, fruit and root tissues were ground separately in a Wiley mill to pass through a 20-mesh screen. Then, 0.5 g of the dried plant tissues were analyzed for the following macro- and micro-nutrients and toxic element: N, P, K, Ca, Mg, Fe, Mn, Zn, Cu, B and Cd. Nitrogen concentration in the plant tissues was determined after mineralization with sulfuric acid by "Kjeldahl method" (Bremner, 1965), P, K, Ca, Mg, Fe, Mn, Zn, Cu, B and Cd concentrations were determined by dry ashing at 400 ºC for 24 h, dissolving the ash in 1:20 HNO3, and assaying the solution obtained using an inductively coupled plasma emission spectrophotometer (ICP Iris; Thermo Optek, Milano, Italy) (Karla, 1998).

2.7. Statistical analysis

All data were statistically analyzed by ANOVA using the SPSS software package (SPSS 10 for Windows, 2001). Duncan's multiple range test was performed at $P = 0.05$ on each of the significant variables measured.

3. Results

3.1. Visual toxicity symptoms

The Cd toxicity symptoms as leaf chlorosis was observed, especially at 50 µM Cd level only on older leaves with more conspicuously in Ikram/Black Beauty grafting combination or non-grafted tomato plants. The symptoms of Cd toxicity was also observed on fruits as stem-end yellowing, originating from fruit-stem attachment point, more pronounced under severe Cd stress condition (Photo.1). The symptoms were more pronounced in fruits from non-grafted plants followed by Ikram/Unifort and self-grafted plants. Fruits from Ikram/Maxifort showed appreciably lower, but it was scarcely observed in fruits from Ikram/Black Beauty combination. Indeed, the visible fruit toxicity symptoms were in response to the Cd contents detected in fruits of respective plants.

Photo.1. Fruit disorder symptoms under Cd stress

3.2. Fruit yield and biomass production

Tomato fruit yield of all grafted and non-grafted plants decreased in response to Cd concentration in nutrient solution; the decrease in yield was due to both the reduced fruit number and mean fruit weight caused by Cd treatments (Table 1). Compared to control, mean fruit yield reduction was 18 and 26%, at 25 and 50µM Cd level, respectively. In relevance of grafting, regardless of Cd level, the yield of the different grafting combinations was in the following order: Ikram/Maxifort > Ikram/Unifort > Ikram/Ikram >non-grafted Ikram> Ikram/Black Beauty combination (Table 1). The highest fruit yield of Maxifort grafted plants was mainly because of higher mean fruit weight. Shoot and root dry biomass, and LA were also decreased in response to Cd level (Table 1). The highest shoot and root dry biomass and LA were recorded in Ikram/Maxifort combination, while the lowest shoot dry biomass and LA was in Ikram/Black Beauty combination followed by non-grafted and self-grafted plants (Table 1). In contrast, the roots dry biomass, which was significantly higher in Ikram/Maxifort from non-grafted or self-grafted plants, but did not differ from Ikram/ Black Beauty or Ikram/Unifort combination (Table 1). The stem girth of rootstock and scion stock at below and above the graft union point, respectively also measured at the termination of experiment. Significant reduction in stem girth of both rootstock and scion observed, the reduction was in response to the level of Cd stress. Mean reduction of rootstock and scion stem girth varied from 13 to 17% and 6 to 20 %, at 25 and 50 µM Cd level, respectively in comparison with observed in control. However, grafting combination has no significant effects on stem girth on either side of graft union, which varied from 11.0 to 12.1mm and 11.0 to 12.20mm for rootstock and scion stem girth, respectively (Data

Table 1. Mean effects of Cd concentrationand grafting combination on yield, fruit number and mean weight, shoot and root dry weight and final leaf area of tomato plants.

Treatments	Fruit yield	Fruit number	Fruit mean weight	Shoot	Root	Leaf Area
	(kg plant^{-1})	(plant^{-1})	(g fruit^{-1})	dry weight (g plant^{-1})		(m^2 plant^{-1})
Cd level (Cd, µM)						
0	3.37 a[a]	38.7 a	93.7 a	210.4 a	19.2 a	2.36 a
25	2.77 b	32.6 b	87.8 b	154.7 b	15.5 b	1.78 b
50	2.51 c	29.8 c	85.6 b	136.3 c	13.6 c	1.62 c
Graft combination (G)						
Ikram	2.87 bc	33.96 ab	88.6 b	163.7 b	15.2 b	1.87 c
Ikram/Ikram	2.94 b	34.04 a	90.7 b	170.4 b	15.7 b	1.92 bc
Ikram/Black Beauty	2.19 c	32.48 b	70.3 c	147.7 c	16.3 ab	1.69 d
Ikram/Unifort	3.09 ab	34.08 a	93.1 b	173.0 ab	16.0 ab	2.00 ab
Ikram/Maxifort	3.32 a	33.89 ab	102.4 a	182.8 a	17.1 a	2.12 a
Significance[b]						
Cd	***	***	***	***	***	***
G	***	*	***	***	*	***
Cd x G	NS	NS	NS	NS	NS	NS

[a]Values are the means of three replicate samples.

[b]Means within columns separated using Duncan's multiple range test $P = 0.05$.

NS. *. ***Non significant or significant at $P < 0.05$ or 0.001, respectively.

Table 2. Mean effects of Cd concentrationand grafting combination on fruit shape index (SI), firmness, dry matter (DM),total soluble solids (TSS) content, juice pH and titratable acidity (TA) and Hunter colour values of tomato fruits.

Treatment	SI	Firmness (N cm^{-2})	DM (%)	TSS (°brix)	pH	TA (%)	L*	a*	b*
Cd level (Cd, µM)									
0	1.25 a[a]	2.28 c	6.00 b	5.46 b	4.31 a	0.24 b	36.4 b	30.7 a	28.3 b
25	1.20 b	2.56 b	6.11 ab	5.57 b	4.21 b	0.34 a	37.9 a	29.5 b	29.4 a
50	1.22 b	2.76 a	6.26 a	5.86 a	4.16 b	0.35 a	38.2 a	29.1 b	30.1 a
Graft combination (G)									
Ikram	1.21	2.56	6.08 ab	5.56 b	4.22	0.31	37.6 ab	29.5 b	29.1
Ikram/Ikram	1.23	2.55	6.11 ab	5.61 b	4.26	0.30	37.5 ab	29.1 b	28.6
Ikram/Black Beauty	1.20	2.50	6.29 a	5.84 a	4.20	0.33	37.0 b	31.4 a	29.9
Ikram/Unifort	1.23	2.51	6.13 ab	5.61 b	4.22	0.30	38.0 a	29.4 b	29.6
Ikram/Maxifort	1.22	2.56	6.00 b	5.54 b	4.22	0.32	37.5 ab	29.4 b	29.1
Significance[b]									
Cd	***	***	**	***	***	***	***	*	***
G	NS	NS	*	*	NS	NS	*	*	NS
Cd x G	NS	NS	NS	NS	NS	NS	NS	NS	NS

[a]Values are the means of three replicate samples.

[b]Means within columns separated using Duncan's multiple range test $P = 0.05$.

NS.[*], [***]Non significant or significant at $P<0.05$ or 0.001, respectively.

not shown). Fruit dry matter (DM), total soluble solids (TSS) content, color factor brightness (L*), redness (a*) and yellowness (b*) significantly influenced by both Cd level and grafting combination but not by their interaction, whereas fruit shape index (SI), fruit firmness, pH and titratable acidity (TA) were significantly affected by only Cd level (Table 2). Mean fruit SI and pH decreased but was similar between 25 and 50 µM Cd levels, while DM and TSS content increased only at 50 µM Cd. Fruit firmness increased in response to Cd level (Table 2). Averaged over Cd levels, measure of highest DM and TSS content was recorded in Black Beauty grafted plants, which were statistically similar to other grafting treatments, except Maxifort grafted plants, which was observed with lowest DM and TSS contents, Moreover, the fruit color indicators (L*, a* and b*) were significantly affected, but similarly for the two Cd levels (Table 2). The tomato fruit of Ikram/Black Beauty was characterized by higher redness (higher a*) and brightness (lower L*) as compared to the other grafting combinations (Table 2).

3.3. SPAD index and chlorophyll fluorescence and leaf pigments

The SPAD index, chlorophyll fluorescence and leaf pigments total chlorophyll and carotenoids contents significantly decreased in concentration dependent manner of Cd in nutrient solution (Table 3). Averaged over Cd levels, the SPAD index and Fv/Fm were distinctly higher in Ikram/Maxifort in comparison with other grafting combination; the lowest SPAD index and Fv/Fm was recorded in Ikram/Black Beauty combination (Table 3). Similarly, the chlorophyll contents were distinctly higher in Ikram/Maxifort combination while the lowest, while was recorded in Ikram/Black Beauty, regardless of Cd level (Table 3). Total carotenoids content was similar among all the grafting combinations, except Ikram/Black Beauty combination which showed significantly lower than others (Table 3).

3.4. Cadmium uptake and distribution

Cadmium content in all examined tomato plant parts (roots, leaves, stems, and fruits) increased in response to Cd concentration in nutrient solution (Table 4). The distribution of Cd within the plant was in order of Roots>Leaves>Stems>Fruits. Of the total Cd taken by plants (sum of contents in roots, stem, leaves and fruits), mean Cd retained by plant roots were 63.6, 64.8, 72.5, 74.1, and 77.7 % in plants grafted onto Black Beauty, Unifort, ungrafted, self-grafted plants and plants grafted onto Maxifort rootstock, respectively. The significantly lower root Cd content was recorded in

Ikram/Black Beauty or Ikram/Unifort combination, while higher but similar Cd content was in Ikram/Maxifort, non-grafted or self-grafted plants. However, the scenario of Cd accumulation in aerial plant parts was apparently different to that was observed in roots in response to grafting. There was marked effect of grafting incision *per se* on Cd transfer from root-to-shoot, especially to the leaf as indicated by reduced level of Cd (10.0 %) in leaves of self-grafted plants in comparison with ungrafted ones, which is main site for important physiological activities, but the difference in Cd content among these two was not clear in fruit (Table 4).

Table 3. Mean effects of solution Cd concentration and grafting combination on SPAD index, maximum quantum use efficiency of PSII (Fv/Fm), total chlorophyll and carotenoids of tomato leaves.

Treatment	SPAD Index	Fv/Fm	Chlorophyll	Carotenoids
			mg g^{-1} fw	
Cd level (Cd, µM))				
0	58.4 a[a]	0.829 a	0.91 a	0.19 a
25	49.4 b	0.809 b	0.76 b	0.17 b
50	46.6 c	0.791 c	0.66 c	0.15 c
Graft combination (G)				
Ikram	50.9 b	0.809 b	0.76 b	0.17 a
Ikram/Ikram	52.7 ab	0.813 b	0.77 b	0.17 a
Ikram/Black Beauty	47.7 c	0.794 c	0.75 b	0.16 b
Ikram/Unifort	52.1 ab	0.816 ab	0.79 ab	0.17 a
Ikram/Maxifort	54.1 a	0.824 a	0.83 a	0.18 a
Significance[b]				
Cd	***	***	***	***
G	**	*	*	*
Cd x G	NS	NS	NS	NS

[a]Values are the means of three replicate samples.

[b]Means within columns separated using Duncan's multiple range test $P = 0.05$.

NS. *, *** Non significant or significant at $P < 0.05$ or 0.001, respectively.

Higher Cd accumulation in aerial plant parts observed in ungrafted plants followed by Unifort grafted plants, indicating less control of Cd transfer from root-to-shoot. However, significantly lower Cd content in leaves, stems and fruits was detected in Ikram/ Black Beauty followed by Ikram/ Maxifort plants, in comparison with non-grafted or self-grafted plants and Ikram/Unifort combination, (Table 4). Conversely, compared to non-grafted plants, the mean

reduction in Cd contents in Ikram/Black Beauty and Ikram/Maxifort plants were 23%, 17 % and 39%, 23% in leaves and fruits, respectively (Table 4).

Table 4. Mean effects of Cd concentration and grafting combination on Cd distribution in leaf, stem, fruit and root of tomato plants.

Cd treatment	Leaf	Stem	Fruit	Root
	(mg kg^{-1} DW)			
Cd level (Cd, µM))				
0	0.20 c[a]	0.04 c	0.02 c	0.2 c
25	119.5 b	46.3 b	6.13 b	525.1 b
50	225.0 a	113.9 a	7.96 a	780.0 a
Graft combination (G)				
Ikram	131.3 a	60.5 a	5.51 a	519.7 a
Ikram/Ikram	118.3 b	55.7 ab	5.04 a	511.8 a
Ikram/Black Beauty	100.9 c	48.8 bc	3.35 c	267.4 b
Ikram/Unifort	115.1 b	57.4 a	5.36 a	327.3 b
Ikram/Maxifort	109.0 bc	44.7 c	4.25 b	549.4 a
Significance[b]				
Cd	***	***	***	***
G	*	*	***	***
Cd x G	NS	NS	***	***

[a]Values are the means of three replicate samples.
[b]Means within columns separated using Duncan's multiple range test $P = 0.05$.
NS, *, ***Non significant or significant at $P < 0.05$, or 0.001, respectively.

3.5. Mineral composition and partitioning

The content of N significantly increased in roots but decreased in leaves, while it remained unaffected in fruits. Non-grafted and self-grafted plants had higher N in their roots, especially under control (0µM Cd) than the grafted plants (data not shown). Leaf and fruit N did not differ among grafting combinations (Table 5). Roots P content decreased only at 25 µM Cd, contrarily, leaves P content increased similarly at both Cd levels, while in fruits it decreased only at higher Cd level (50 µM Cd). The content of P in roots and leaves was highest in non-grafted plants, though was similar to rest others (Table 5). Root K content was not affected by either Cd or grafting treatments. The contents of K in fruits increased at 25 µM Cd, while it decreased in leaves, but only at 50 µM Cd. Leaves K content was only influenced by grafting and was highest in Ikram/ Black Beauty followed

by Ikram/Unifort combination (Table5). The Ca content in Roots and leaves decreased at both Cd levels similarly, while in fruits it decreased at only higher Cd level (Table 5). Ikram/Maxifort or Ikram/Unifort plants had higher level of Ca in their leaves, fruits and roots, compared to other grafting treatments (Table 5). The Mg content in leaves was similarly decreased at both 25 and 50 µM Cd level, whereas in fruits Mg decreased at only higher Cd level. The Mg content in leaves was significantly higher in Ikram/Maxifort or Ikram/Unifort than rest other grafting combinations (Table 5).

As presented in table 6, in presence of Cd in root ambient, the contents of micro-nutrient elements like Fe, Zn, Cu and B content in roots significantly increased at variable Cd stress level. In contrast, root Mn content decreased with increase of Cd level. However, the opposite trend was observed in leaves for Fe, Zn, Mn and Cu contents. In fruits, the contents of Fe, Zn, Mn and Cu significantly decreased (Table 6). Concerning the grafting combination, the contents of Fe and Mn were significantly higher in leaves of Ikram/Maxifort or Ikram/Unifort as compared to other grafting combinations, whereas Zn content in roots was significantly higher in non-grafted or self-grafted tomato, while Cu content in roots was higher in Ikram/Black Beauty combination (Table 6). The Mn and Cu contents were significantly affected in only fruits by grafting, where distinctly higher Mn content was in Ikram/Maxifort and lower Cu content in Ikram/Black Beauty than rest other grafting combinations (Table 6). There was no significant effect of grafting combination on B content in any of the analyzed plant parts (Table 6).

4. Discussion

4.1. Plant growth, fruit yield and quality

A general response of Cd toxicity in plants has been the inhibition and reduction of shoot and root biomass production (López-Millán et al., 2009; Hayat et al., 2012**)** and yield reduction in many crop plants (Prasad, 1995; Yang et al., 1998; Irfan et al., 2013), including tomato (Khan and Khan, 1983; Moral et al., 1994). In the present study, a significant depression in yield, shoot and root biomass and leaf area was observed, however, the effects varied in response to Cd concentration in the nutrient solution (Table 1). The decrease in fruit yield was directly related to the decrease in fruit number plant^{-1} and mean fruit weight (Table 1). This result is supported by the findings of Shekar et al. (2011), who observed the reduction in flower and fruit number plant^{-1} and mean fruit weight of tomato in response to increased Cd level in soil. Moreover, Cd toxicity

Table 5. Mean effects of solution Cd concentration and grafting combination on macronutrient concentration in leaves, fruits, and roots of tomato plants

Treatment	Macronutrients (g kg^{-1} DW)														
	N			P			K			Ca			Mg		
	Leaves	Fruits	Roots	Leaves	Fruits	Roots	Leaves	Fruits	Roots	Leaves	Fruits	Roots	Leaves	Fruits	Roots
Cd level (Cd, µM))															
0	32.4 a[a]	26.1	27.0 b	5.9 b	6.1 a	4.5 a	35.3 a	20.8 b	16.0	38.4 a	1.2 a	10.0 a	7.1 a	1.5 ab	1.8
25	30.3 b	24.9	30.0 a	7.0 a	5.5 ab	3.7 b	34.3 a	30.4 a	14.4	34.1 b	1.2 a	8.7 b	5.0 b	2.0 a	1.7
50	30.1 b	24.9	31.2 a	7.2 a	4.5 b	4.5 a	29.8 b	20.6b	16.6	34.6 b	0.8 b	8.3 b	4.9 b	1.3 b	1.8
Graft combination (G)															
Ikram	30.35	25.03	31.5 a	7.74 a	5.27	4.8 a	30.04 c	24.10	16.9	31.83 b	1.03 ab	9.6 ab	5.66 b	1.53	1.8
Ikram/Ikram	30.01	24.83	31.5 a	7.21 ab	5.77	4.7 a	31.02 bc	23.63	15.8	32.59 b	1.07 ab	8.4 bc	5.89 b	1.47	1.7
Ikram/Black Beauty	30.59	24.60	28.5 ab	5.01 c	4.70	3.6 b	37.44 a	23.10	14.3	37.60 ab	0.87 b	7.7 c	4.42 c	1.57	1.6
Ikram/Unifort	32.27	26.63	27.5 b	6.76 b	5.57	4.0 b	34.40 ab	24.50	16.2	36.34 ab	1.17 a	10.1 a	6.03 ab	1.57	1.8
Ikram/Maxifort	31.30	24.90	27.8 b	6.95 ab	5.53	4.1 ab	32.79 bc	24.67	15.1	38.13 a	1.27 a	9.3 ab	6.29 a	1.67	1.8
Significance[b]															
Cd	*	NS	**	**	***	**	*	***	NS	*	***	**	***	***	NS
G	NS	NS	*	***	NS	**	***	NS	NS	*	*	*	*	NS	NS
Cd x G	NS	NS	*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

[a]Values are the means of three replicate samples.

[b]Means within columns separated using Duncan's multiple range test $P = 0.05$.

NS. * **. ***Non significant or significant at $P< 0.05$, 0.01 or 0.001, respectively.

Table 6. Mean effects of solution Cd concentration and grafting combination on micro nutrient composition of leaves, fruits, and roots of tomato plants.

Treatment	Micronutrients (mg kg^{-1} DW)														
	Fe			Zn			Mn			Cu			B		
	Leaves	Fruits	Roots	Leaves	Fruits	Roots	Leaves	Fruits	Roots	Leaves	Fruits	Roots	Leaves	Fruits	Roots
Cd level (Cd, µM))															
0	42.1 a[a]	24.7 a	219.7 b	32.2 a	13.3 a	25.2 b	192.8 a	11.1 b	85.4 a	2.93 a	4.23 a	15.6 c	57.1	8.09 b	10.5 b
25	35.2 b	23.2 a	479.3 a	29.7 a	14.1 a	57.3 a	184.2 b	19.1 a	64.1 b	2.21 b	4.17 a	25.5 b	60.4	9.11 a	11.3 a
50	30.4 c	12.4 b	466.3 a	24.3 b	10.2 b	56.3 a	168.2 c	10.4 b	43.1 c	1.24 c	2.39 b	35.2 a	65.0	8.32 b	11.4 a
Graft combination (G)															
Ikram	35.2 b	19.0	401.9 b	29.2	12.0	56.1 a	178.7 bc	12.7 b	68.3 b	1.91 a	4.14 a	24.9 bc	59.0	8.21	12.1
Ikram/Ikram	33.1 b	19.3	391.9 b	28.4	12.1	52.2 ab	163.2 c	13.1 b	59.4 bc	2.02 a	3.44 a	21.0 c	61.1	9.32	11.2
Ikram/Black Beauty	31.3 b	21.8	250.5 c	27.3	13.2	32.2 c	174.3 bc	12.6 b	55.5 c	1.25 b	2.33 b	31.4 a	58.3	7.38	11.4
Ikram/Unifort	38.8 ab	20.2	373.9 b	30.2	11.8	44.3 b	190.1 a	13.4 b	57.8 c	2.11 a	4.21 a	23.3 bc	64.4	9.20	10.9
Ikram/Maxifort	40.1 a	22.4	524.1 a	28.8	12.4	46.4 b	202.9 a	15.7 a	80.0 a	2.35 a	3.38 a	26.7 ab	60.2	9.22	11.3
Significance[b]															
Cd	***	***	***	**	***	***	*	***	***	***	***	***	NS	**	*
G	**	NS	***	NS	NS	***	**	*	***	*	**	**	NS	NS	NS
Cd x G	NS	NS	*	NS	NS	*	NS	NS	*	NS	NS	***	NS	NS	NS

[a]Values are the means of three replicate samples.

[b]Means within columns separated using Duncan's multiple range test $P = 0.05$.

NS. * **. ***Non significant or significant at $P< 0.05$, 0.01 or 0.001, respectively.

reduced leaf area in tomato plants (Djebali et al., 2005, 2010; Hayat, et al., 2012), this was reportedly due to reduction in leaf expansion as a result of reduced cell size and small intercellular spaces (Djebali et al., 2005). There was also a reduction in stem girth in rootstock as well as scion stock, measured below and above graft union observed in grafted and non-grafted plants under Cd stress (data not shown).

Plants exposed to higher Cd level (50 µM Cd) exhibited leaf chlorosis symptoms, especially on older leaves as also reported in earlier studies on tomato in response to Cd stress (López-Millán et al., 2009; Hasan et al., 2011). More Cd accumulation in lower leaves than in upper leaves in previous studies (Cho and Park, 1999; Hediji et al., 2010) explains the appeared higher toxicity symptoms on older leaves. In addition to leaf chlorosis, we observed a typical fruit disorder in this study as stem-end-fruit yellowing (Photo 1); the severity was in response to Cd concentration in nutrient solution and to those detected in the fruits among grafting combinations. Despite the fact that the reduction in vegetable crop productivity under abiotic stresses, the increase in some quality attributes reported to occur (Rouphael et al., 2010; 2012b). Presence of Cd in nutrient solution increased DM, TSS, TA and firmness, while others shown adverse effects like fruit SI and pH, and fruit color factors described by decreased fruit redness (a*) and increased lightness (L*) and yellowness (b*) (Table 2). Nevertheless, excess level of Cd in the growth medium can lead to an increase of Cd content in fruits which represent a serious risk for human health.

Recent studies have shown that rootstock grafted plants had lower concentration of Cd in shoots and fruits of eggplants (Arao et al.,2008; Mori et al., 2009), melon (Edelstein and Ben-Hur, 2012) and cucumber (Savvas et al., 2013) as compared to non-grafted plants, Moreover, working with melon and cucumber under toxic concentration of B and Cu, Edelstein et al.(2007) and Rouphael et al. (2008), respectively found that the better plant growth and fruit yield in these crops were when plants grafted onto vigorous rootstock of ‘pumpkin’ as compared to non-grafted plants. The lower level of toxic metals elements (Edelstein et al., 2007) along with better plant physiological functioning associated with better availability of nutrient (Rouphael et al., 2008) in rootstock-grafted plants ascribed to the overall better performance of grafted plants. Similarly, in current study, fruit yield and plant biomass production were higher in grafted plants, especially onto tomato rootstocks Maxifort or Unifort as compared to other grafting combination. The

response of grafting combination in relation to yield and biomass production was partly related to the toxic metal concentration in leaves with exception of Ikram/Black Beauty combination, which was worse affected even with having lowest level of Cd in all analyzed plant parts. In spite of lower level of Cd in Ikram/ Black Beauty plants, the contents of certain essential elements (P, Mg, Mn, Cu and Fe) in leaves were also lower and showing severe leaf chlorosis symptoms. The nutritional deficiency in Ikram/Black Beauty plants might have led to the nutrient imbalance and physiological disorders, thereby poor plants performance (Dong et al., 2006). These could possibly due to some incompatibility reaction between scion cv. Ikram with Black Beauty rootstock. There is report that rootstock/scion incompatibility induces under- or over-growth of scion that can result to decreased water and nutrient flow through the graft union and eventually lead sometimes to wilting of the plant (Davis et al., 2008). However, unlike plant growth and yield, fruits from Ikram/Black Beauty were exceptionally better than other grafting combinations as evident by higher DM and TSS content and better fruit color (lower fruit lightness, L* and higher redness, a*), associated with least Cd toxicity symptoms (stem-end yellowing); these could be directly related to lowest detected Cd contents in the fruits. Next to the Ikram/Black Beauty, fruits from Ikram/Maxifort detected with less Cd content (Table 5), thus lower appearance of toxicity symptoms on fruits. However, further study is needed to elucidate the relation of Cd ion localization and damage to specific part or tissues of fruit.

4.2. SPAD index, chlorophyll fluorescence and leaf pigments

The higher yield and biomass production in plants grafted onto Maxifort or Unifort could be in part due to maintained higher SPAD index and-maximum quantum yield of PSII (Fv/Fm), compared to other grafting combinations. This higher Fv/Fm suggests that grafted plants onto appropriate rootstocks can delay photoinhibition under heavy metal stress, as has been also reported under salt stress condition (Colla et al., 2013; He et al., 2009). Moreover, the Maxifort or Unifort grafted plants could have maintained the higher chlorophyll content in than rest other grafting combinations (Table 3). Similar response of grafting was observed for Cu toxicity (Rouphael et al., 2008) and salinity toxicity (Colla et al., 2013), where chlorophyll content was significantly higher in grafted plant than those of ungrafted ones. The higher activity of PSII together with higher chlorophyll contents in tomato rootstock grafted plants, especially onto Maxifort ascribed to higher leaf area and finally the overall biomass production than other grafting combinations.

4.3. Cadmium uptakes and distribution

Cd accumulation in grafted and ungrafted plants largely occurred in roots (75 and 69%) and partly transported to the aerial parts, particularly to the leaves (17 and 20%), at the two Cd supply level (25 and 50 µM Cd). The high amount of Cd accumulation in roots ascribed to the chelation and sequestration process and the development of extracellular barriers (Lux et al., 2011). The Cd ions can also be transported to the growing fruits by mediated transport through the phloem (Hart et al., 1998) and the fruit Cd level can exceed to the proposed safe level, thus causing a serious health concern (Yamaguchi et al., 2011). Several reports where vegetables such as tomato shown to be accumulated significant level of Cd in their edible parts (Gratão et al., 2008; and references therein; Hartke et al., 2013).

In present study, the root-to-shoot Cd translocation was restricted by grafting *per se* as indicated by the observed lower Cd concentration, especially in leaves of self-grafted plants than non-grafted plants, though having similar concentration in their roots (Table 4). Similar was observed in the finding of Savvas et al. (2013), where Ni contents in the shoot of self-grafted cucumber plants was much lower than that of ungrafted plants, however, they did not observe clear difference in Cd contents between ungrafted and self-grafted plants. The Black Beauty and Unifort rootstocks displayed better ability to restrict Cd entry into roots by being observed much lower Cd concentration in their roots as compared to other grafting combinations. Further, the lower Cd in leaves and fruits of Black Beauty grafted plants showed limited root-to-shoot Cd transfer, but it was not the case for Unifort grafted plants as having higher Cd content in their leaves. Although, Cd concentration in roots of Maxifort grafted plants was higher, though similar to those of self-grafted plants or non-grafted plants, it was much lower in the leaves and fruits that indicate the better ability of Maxifort rootstock in limiting root-to-shoot transfer of toxic metal. Plant can deploy different mechanisms to reduce the level of toxic metals in the shoots as suggested by the results of earlier studies. For examples, the lower Cd contents in aerial parts of cucumber plants grafted onto pumpkin rootstock (‘Power’) was due to restricted Cd uptake (Savvas et al., 2013), whereas, it was due to limited root-to-shoot transfer, but not by restricted uptake in eggplant grafted onto *Solanum torvum* rootstock (Arao et al., 2008; Mori et al., 2009), compared to non-grafted or self-grafted plants.

4.4. Nutrient uptake, translocation and accumulation

In presence of Cd in nutrient solution, substantial inhibition in translocation of N, K, Ca, Mg, Fe, Zn, Mn and Cu to the leaf (contents in leaves), though their uptake by roots (content in roots) rather found increased including of B, with exception of Mn and Ca which decreased, or K and Mg of which uptake remained unaffected under Cd stress (Table 5, 6). In general, most of the nutrient contents were decreased in fruit tissues, especially at 50µM Cd application. Similar, differential response in mineral elements uptake by roots and their accumulation in leaves have been also observed in previous studies under variable Cd stress (Sandalio et al., 2001; Dong et al., 2006; Lopez-Millan et al., 2009). The changes in nutrient uptake possibly due to affected permeability of plasma membranes by Cd toxicity (Dong et al., 2006). It has been reported that Cd interferes mainly with the translocation of most of the micronutrients, especially those having same valences, to the leaves, despite having rather synergistic effects on their uptake (Sandalio et al., 2001). Higher plant biomass and yield in tomato-rootstocks grafted plants (Maxifort or Unifort) under Cd stress could partly be related to better nutritional status, especially of Ca, Mg, Fe, Mn and Cu (Table 5, 6); some of these are important constituents of many enzymes and proteins and thus helping in better physiological functioning (Hall, 2002; Lopez-Millan et al., 2009). In contrast, the severe reduction in growth and yield and the appeared phytotoxicity symptoms of Cd stress in plants grafted onto Black Beauty mainly ascribed to the inhibition of certain nutrient elemental availability in the leaf tissue (P, Mg, Fe, Mn, Cu, Table 5, 6)

5. Conclusions

In conclusion, the yield and biomass production decreased in grafted and non-grafted plants in response to an increase of Cd concentration in the nutrient solution. However, grafting onto appropriate rootstocks could minimize the toxic effects of Cd. Plants grafted onto tomato rootstocks, especially onto Maxifort were able to reduce toxic metal contents in aerial parts while maintaining better nutritional status (i.e. higher Ca, Mg, Fe, Mn and Cu) in leaves and higher chlorophyll content and F_v/F_m ratio in comparison with non-grafted or self-grafted and especially Ikram/Black Beauty plants. Their plant performance was also partly due to lower leaf Cd contents. In particular Maxifort, which efficiently limited translocation of Cd to leaves and even fruits. The lower level of Cd in all plant parts in Black Beauty grafted plants could not result in better plant performance, except fruit quality, may be because of lower leaf nutritional status which possibly occurred due to some incompatibility reaction.

Chapter 3

Effect of nickel and grafting combination on yield, fruit quality, antioxidative enzyme activities, lipid peroxidation and mineral composition of tomato

Pradeep Kumar[1,2], Youssef Rouphael[3], Mariateresa Cardarelli[4], and **Giuseppe Colla[1*]**

[1]*Department of Agriculture, Forestry, Nature and Energy, University of Tuscia, 01100 Viterbo, Italy*

[2] *ICAR-Central Arid Zone Research Institute, 342003 Jodhpur (Rajasthan), India*

[3]*Department of Agricultural Sciences, University of Naples Federico II, 80055 Portici, Italy*

[4]*Agricultural Research Council - Research Centre for the Soil-Plant System, 00184 Roma, Italy*

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*Correspondence: Prof. G. Colla; e-mail: giucolla@unitus.it

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Chapter 3

Effect of nickel and grafting combination on yield, fruit quality, antioxidative enzyme activities, lipid peroxidation and mineral composition of tomato

Abstract

Soil contamination by heavy metals negatively affects crop productivity, besides representing serious threat to human health. Grafting tomato, onto appropriate rootstocks may raise Ni tolerance through limiting heavy metal uptake by roots and/or its translocation to the shoot, and by detoxification. A greenhouse experiment was conducted to determine the influence of long-term Ni exposure (0, 25 or 50 µM) on crop productivity, fruit quality, leaf chlorophyll content, fluorescence, electrolyte leakage, catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX) activities in leaf, proline content, membrane lipid peroxidation, and mineral composition of tomato plants cv. Ikram, either self-grafted or grafted onto three rootstocks: Black Beauty, Unifort, and Maxifort. Significant reduction in yield was observed in response to increase in Ni concentration with more detrimental effects at 50 µM Ni. The fruit dry matter and total soluble solids content increased under severe Ni stress. The depression of crop performance under Ni toxicity was attributed to a decrease in leaf pigments (SPAD index), efficiency of PSII, macro and microelements, and increase in lipid peroxidation and membrane damage. Plants grafted onto tomato rootstocks Maxifort and Unifort exhibited higher chlorophyll content, photochemical activity of PSII, antioxidant activity of APX and GPX, lower accumulation of MDA, and a better nutritional status (higher Ca and Fe, and lower Ni) in the leaf tissues in comparison with self-grafted plants and those grafted onto 'Black Beauty'. Plants grafted onto tomato rootstocks Unifort and especially Maxifort could minimize the nickel toxicity by improving nutritional status and detoxification processes.

1. Introduction

Unlike several heavy metals such as Ag, Cd, Cr, Hg and Pb that have no biological function in plant (Seregin and Kozhevnikova, 2006), Ni is considered an essential microelements for higher plants since it is involved in nitrogen metabolism as the metal component of the

enzyme urease (Eskew et al., 1985; Brown et al., 1987; Marschner, 2002). For most crop species the normal Ni concentration in plant tissues ranges from 0.05 to 10 µg g^{-1} dry weight and above this upper limit, toxicity symptoms are likely to occur (Yusuf et al., 2011). During the last two decades, Ni has become a serious concern as its concentration has reached up to 0.2 mg L^{-1} in polluted waters (Zwolsman and VanBokhoven, 2007) and 26,000 mg kg^{-1} in polluted soils (Alloway, 1995). Nickel is readily taken up by plants and being highly mobile is easily translocated to different plant parts (Poulik, 1999). Consequently, the heavy metal is able to enter the food chain by plant edible tissues, causing serious damage on human health (Liu et al., 2013).

Higher concentration of Ni has been reported to disturb physiological and biochemical processes in plant, these include reduction in growth (Molas, 2002), photosynthesis and water relations (Chen et al., 2009), alteration of enzymatic activities and proline content (Gajewska et al., 2006; 2009) , interference with the uptake and translocation of macro and micronutrients (Ca, Mg, Mn, Fe, Cu, and Zn; Chen et al., 2009) and induction of oxidative damages, e.g. increase in lipid peroxidation products, membrane permeability, and chlorophyll degradation (Chen et al., 2009; Gajewska and Sklodowska, 2007; 2008; Gajewska et al., 2012). All these factors alter biochemical and physiological processes, leading to a reduction in fruit yield and quality (Yusuf et al., 2011). However, under abiotic stress conditions including excessive Ni concentrations, plants have different scavenging mechanisms to cope with the formation of reactive oxygen species (ROS) that cause oxidative stress. The scavenging system controlling ROS includes both non enzymatic antioxidants (e.g. ascorbic acid, phenolic compounds) and antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and guaiacol peroxidase (GPX) (Noctor and Foyer, 1998).

The efficiency of defense mechanisms based on minimizing heavy metal uptake and translocation or by facilitating metal detoxification process depends mainly on root genotype (Sarwar et al., 2010). However, the efficiency of many commercial vegetable cultivars to limit root uptake of heavy metals and to improve nutritional status under metal stress conditions is rather low (Savvas et al., 2013). One of the possible sustainable strategies for reducing Ni accumulation in fruiting vegetable plants (Cucurbits and Solanaceous crops), would be by grafting them onto rootstocks able to reduce uptake and translocation of Ni to the shoots. Grafting has been successfully used as a tool to induce tolerance to many abiotic stresses in particular salinity, drought, alkalinity, acidity, nutrient and thermal stress (Edelstein et al., 2007;

Colla et al., 2008; Colla et al., 2010a, b, c; Colla et al., 2011; Schwarz et al., 2010; Savvas et al., 2010; Rouphael et al., 2008a, b, 2012a; Borgognone et al., 2013). Few reports on the uptake of heavy metals by grafted vegetable plants have been published (Savvas et al., 2010). Arao et al. (2008), reported an appreciable decrease in root-to-shoot Cd translocation in eggplants grafted onto its wild relative *Solanum torvum* rootstock. The Cd concentrations in the shoots and xylem sap of *S. torvum* were lower than those of eggplant; this was attributed to either lower xylem loading or reduced Cd flux in *S. torvum* (Arao et al., 2008; Mori et al., 2009; Yamaguchi et al., 2011). Nevertheless, few published data are available concerning Ni stress tolerance in vegetable crops. Savvas et al. (2013) showed that the Ni uptake and translocation to the aerial parts of cucumber was restricted by grafting; however, the response of grafted cucumber was only investigated at early development stage, whereas information on the effects of long-term Ni exposure is lacking. Moreover, rootstock mediated physiological and biochemical changes to excessive external concentration of Ni have not been investigated.

Our hypothesis is that grafting tomato, one of the world's most important vegetables, onto appropriate rootstocks may raise Ni tolerance through limiting heavy metal uptake by roots and/or its translocation to the shoot, and by detoxification processes. To verify our hypothesis, self-grafted and grafted tomato plants onto selected rootstocks were grown under greenhouse conditions with three levels of Ni concentration. Grafted and self-grafted plants were compared in terms of yield, growth, fruit quality, leaf pigment content, SPAD index, leaf fluorescence, leaf electrolyte leakage, CAT, APX, GPX in leaf, proline content, membrane lipid peroxidation, and mineral composition and assimilate partitioning.

2. Material and methods

2.1. Plant material, growth conditions and experimental design

Tomato (Solanum lycopersicum L. cv. Ikram, Syngenta, Milan, Italy) plants were either self-grafted, or grafted onto the following commercial rootstocks: Maxifort and Unifort (S. lycopersicum × S. habrochaites, De Ruiter/Monsanto, Bergschenhoek, The Netherlands) and eggplant (Solanum melongena L.) Heirloom cv. Black Beauty. Maxifort and Unifort were selected as being the most representative commercial rootstocks used in Italy. Eggplant cv. Black Beauty was included as rootstock due to its tolerance against abiotic stresses (Abdelmageed and Gruda, 2009).

The experiment was carried out in a greenhouse situated on the experimental farm of Tuscia University, Central Italy (42°25′ N; 12°08′ E) from April 23 to August 8, 2013. The air temperature and the day/night relative humidity inside the greenhouse were maintained between 18/33 °C and 55/85%, respectively. Seedlings were produced by a commercial company (Centro SEIA, Sicily, Italy) using the slant cut (splice) graft technique. At three-four true leaf stage tomato seedlings were transferred into pots (6 L) containing quartziferous sand. Pots were arranged in double rows in separate plastic covered channel leaving pot head open on a fixed platform providing sufficient slope to enable drainage collection into tanks fixed at the lateral end of each channel. The space between plants within a row was 0.45 m and the distance between the centers of double rows was 1.2 m, giving a plant density of 3.7 plants m-2. Pollination was facilitated by bumble bees. Plants were grown as vertical cordons where all laterals branches were pruned and later the terminal bud was also removed after the 5th fruit cluster.

The experiment was designed as a factorial combination of three Ni concentrations [0 (control), 25 or 50 μM] and four grafting combinations (self-grafted Ikram/Ikram, Ikram/Black Beauty, Ikram/Unifort, or Ikram/Maxifort). The treatments were arranged in a randomized complete-block design with three replicates per treatment. The Ni treatments were initialized on May 20 (28 days after transplanting, DAT). The basic (control) nutrient solution used in this experiment was a modified Hoagland and Arnon formulation. All chemicals used were of analytical grade, and composition of the basic nutrient solution was: 14.0 mM $N–NO_3$, 1.6 mM S, 1.5 mM P, 6.0 mM K, 4.5 mM Ca, 1.5 mM Mg, 20 μM Fe, 9 μM Mn, 0.3 μM Cu, 1.6 μM Zn, 20 μM B, and 0.3 μM Mo. The enriched-Ni treatments had the same nutrient composition plus 25, or 50 μM Ni supplied as $NiCl_2$. The pH of the nutrient solution for all treatments was 5.8 ± 0.3. Deionized water was used for the preparation of all nutrient solutions. Nutrient solution was pumped from independent supply tanks of 478 L through a drip irrigation system, with one emitter per plant of 2 L h^{-1} flow rate. Irrigation scheduling was performed using electronic low-tension tensiometers (LT-Irrometer, Riverside, CA, USA) that controlled irrigation based on substrate matric potential (Norrie et al., 1994). The duration of each irrigation event was tuned to provide at least 35% of the nutrient solution draining from the pots (Rouphael et al., 2004; Rouphael and Colla, 2005). The drainage solutions were collected in tanks placed at the lateral end of each channel. The drainage solutions were not reused in the system.

2.2. Yield and biomass measurements

Fully ripe fruits were harvested from June 26 and continued until the termination of the experiment (August 8). The fruit yield, number of fruits and mean fruit weight were recorded on all plants. At the end of the experiment (108 days after transplanting, DAT), each plant was separated into different plant organs (leaf, stem, and root) and their tissues were dried in a forced-air oven at 80 ºC until constant weight for biomass determination. Shoot biomass was equal to the sum of aerial vegetative plant parts (leaves + stems). Leaf area (LA) was measured with an electronic area meter (Delta-T Devices Ltd., Cambridge, UK).

2.3. Fruit quality analysis

At peak harvesting period, nine full red-ripe fruits were selected per plot (3 from each plant) to determine the fruit quality. Fruit shape index (SI) defined as the ratio of width to length was measured. Fruit firmness (N cm-2), was determined by using a penetrometer (Bertuzzi FT 011; Brugherio, Milan, Italy), fitted with an 8 mm-diameter round-head probe. Hunter colour values parameters: L* (brightness), a* (redness) and b* (yellowness) were measured on the surface of the external pulp with a Minolta colourimeter (Model CR-300; Minolta, Osaka, Japan). Total soluble solids (TSS, °Brix) content of the fruit juice was measured by an Atago N1 refractometer (Atago Co. Ltd., Japan) and titratable acidity was determined by potentiometric titration with 0.1 M NaOH up to pH 8.1 and the results were expressed as percentage of citric acid in the juice. Fruit dry matter (DM) was also determined.

2.4. Leaf pigment content

At the termination of the experiment (August 6, 106 DAT), the leaf pigments (total chlorophyll and carotenoids) were extracted by homogenization of fresh leaf tissues (0.5 g) in acetone (80%). The resulting extracts were centrifuged at 4,800 × *g* for 20 min. The total chlorophyll and carotenoid contents were determined by taking absorbance of the supernatant at 470, 647, and 664 nm by a UV–Vis spectrophotometer (Perkin Elmer, Norwalk, CT, USA). Formula and extinction coefficients used for the determination of leaf pigments were described by Lichtenhaler and Wellburn (1983) and the content of chlorophyllous pigments was expressed in mg g^{-1} of fresh weight.

2.5. SPAD index and fluorescence measurements

Both the SPAD index and the fluorescence measurements were recorded 106 DAT. A portable chlorophyll meter (SPAD-502, Minolta corporation, Ltd., Osaka, Japan) was used to measure the relative leaf chlorophyll concentration, as a rational unit. Measurements were made at a central point on the leaflet between the midrib and the leaf margin of the 3rd leaf from the top. Twelve random measurements per plot were taken and averaged to a single SPAD value for each treatment.

The chlorophyll fluorescence was recorded on 15 min dark-adapted leaves by means of a chlorophyll fluorometer Handy PEA (Hansatech Instruments Ltd,King's Lynn, UK) with an excitation source intensity higher than 3000 μmol $m^{-2}s^{-1}$ at the sample surface. The minimal fluorescence intensity (F_0) in a dark-adapted state was measured in the presence of a background weak light signal (about 2-3 μmol photons·m^{-2}·s^{-1}). The maximal fluorescence level in the dark-adapted state (Fm) was induced by 0.8 s saturating light pulse (3000 μmol photons $m^{-2}s^{-1}$). The maximum quantum yield of open photosystem II (PSII) (Fv/F_m) was calculated as $(F_m - F_0)/F_m$, as described by Maxwell and Johnson (2000). Six measurements per experimental unit were performed in the 3rd leaf from the top as were used for SPAD.

2.6. Assay of antioxidant enzyme activity

At the same date of the SPAD and fluorescence measurements, fresh leaf samples from the 3rd leaves from top were harvested from each plant and immediately frozen in liquid nitrogen and stored at -80°C for later antioxidant enzyme activity, proline and lipid peroxidation analysis. Enzyme extractions were performed using a pre-chilled mortar and pestle with two volumes of an ice-cold extraction buffer (0.05 M potassium phosphate buffer, pH 7.0) containing 0.1% (w/v) ascorbic acid, 1% (w/v) polyvinilpolypirrolidone, 1 mM Na_2-EDTA and 0.1% (v/v) Triton X-100. After centrifugation (15,000 × *g*, 30 min, 4°C) the supernatant was set aside for the determination of the enzyme activity and protein content by a spectrophotometer (Perkin Elmer, Norwalk, CT, USA). Catalase (CAT, EC 1.11.1.6) activity was measured according to Havir and McHale (1989). Assay mixture (1 ml) contained 0.1 ml of 125 mM H_2O_2 and 20 μl of the crude extract in 0.05 M potassium phosphate buffer (pH 7.0). Enzyme activity was evaluated by following the decomposition of H_2O_2 at 240 nm for 1 min and calculated using the extinction coefficient (0.036 mM^{-1} cm^{-1}). Activity of ascorbate peroxidase (APX, EC 1.11.1.11) was measured following the decrease of absorbance at 290 nm for 1 min (Nakano and Asada, 1981)

corresponding to the oxidation of ascorbic acid. APX activity was calculated using its extinction coefficient (2.8 mM^{-1} cm^{-1}). Activity of guaiacol peroxidase (GPX, EC 1.11.1.7) was measured according to with Chance and Maehly (1955). Assay mixture (1 ml) contained 0.1 ml of 90 mM guaiacol, 0.1 ml of 125 mM H_2O_2 and 50 µl of the crude extract in 0.05 M potassium phosphate buffer (pH 7.0). Enzyme activity was evaluated following the increase of absorbance at 470 nm for 40 s due to guaiacol oxidation and calculated using the extinction coefficient (26.6 mM^{-1} cm^{-1}). The specific enzyme activity for all enzymes was expressed as mmol mg^{-1} protein min^{-1}.

2.7. Proline determination

Free proline content was determined according to the method of Bates et al. (1973) Around 0.5 g of leaf material was homogenized in 10 mL of 30 g L^{-1}sulfosalicylic acid (Sigma Aldrich) and the homogenate was filtered through Whatman No. 2 filter paper. Then 2 mL of filtrate was reacted with 2 mL of acid-ninhydrin (1.25 g of ninhydrin in 30 mL of glacial acetic acid and 20 mL of 6 mol L^{-1} phosphoric acid) and 2 mL of glacial acetic acid in a test tube at 100 °C for 1 h. The reaction was terminated in an ice bath and then 4 mL of toluene was added and the product of the reaction was extracted by vortex mixing. The absorption of the upper phase was read at 520 nm using toluene as a blank. Proline concentration was calculated on a fresh weight (FW) basis using L-proline for the standard curve.

2.8. Determination of lipid peroxidation

The Ni-induced oxidative damage (membrane lipid peroxidation) in fresh leaf tissue was estimated by measuring the malondialdehyde (MDA) concentrations. Fresh leaf tissues were homogenized in 0.1% (w⁄v) trichloroacetic acid (TCA) solution in 1:3 ratio. After centrifugation (15 min, 12,000 g at 4°C), an aliquot of the supernatant was added to 0.5% thiobarbituric acid (TBA) made in 20% TCA and heated at 95°C for 30 min. After rapid cooling on ice, the mixture was centrifuged at 10,000 g for 10 min. The absorbance was recorded at 532 and 600 nm using UV-Vis spectrophotometer. The MDA concentration was calculated from the difference between the absorbance values at 532 and 600 nm (Ben Amor et al., 2005).

2.9. Mineral analysis

Dried plant tissues (leaf, fruit and root) were ground separately in a Wiley mill to pass through a 20-mesh screen, then 0.5 g of the dried plant tissues were analyzed for the following elements: N, P, K, Ca, Mg, Fe, Mn, Zn, Cu and Ni. Nitrogen concentration in the plant tissues

was determined after mineralization with sulfuric acid by "Kjeldahl method" (Bremner, 1965). P, K, Ca, Mg, Fe, Mn, Zn, Cu and Ni concentrations were determined by dry ashing at 400 ºC for 24 h, dissolving the ash in 1:20 HNO_3, and assaying the solution obtained using an inductively coupled plasma emission spectrophotometer (ICP Iris; ThermoOptek, Milano, Italy; Karla, 1998).

2.10. Statistical analysis

All data were statistically analyzed by ANOVA using the SPSS software package (SPSS 10 for Windows, 2001). To separate treatment means within each measured parameter, Duncan's multiple range test was performed at P = 0.05.

3. Results

3.1. Fruit yield components, biomass production and partitioning

In grafted and self-grafted plants, fruit yield decreased in response to an increase of Ni concentration in the nutrient solution (Table 1). The lowest yield observed under Ni enriched solutions (25 and 50 µM Ni) in comparison to the control was due to a reduction in both the number of fruits per plant and the fruit mean weight (Table 1). Moreover, irrespective of Ni level, the yield of the different grafting combinations was in the following order: Ikram/Maxifort>Ikram/Unifort>Ikram/Ikram>Ikram/Black Beauty (Table 1). Similarly, the final LA significantly decreased with increasing the Ni concentration in the nutrient solution, with no significant difference between 25 and 50 µM Ni treatments (Table 1).

Shoot and root dry biomass were highly affected by the interaction Ni × grafting combination (data not shown). At 0 µM Ni, shoot dry weight was highest in Ikram/Maxifort plants (291.4 g $plant^{-1}$) while at 25 µM Ni the highest shoot dry weight was recorded in Ikram/Maxifort and Ikram/Unifort grafting combinations (avg. 248.8 g $plant^{-1}$). The Ikram/Unifort combination gave the highest shoot dry weight biomass at 50 µM Ni (241.8 g $plant^{-1}$). At 0 µM Ni, the root dry weight was highest in Ikram/Maxifort and Ikram/Unifort grafting combinations (avg. 17.3 g $plant^{-1}$) while at 25 µM Ni no significant differences were recorded among grafting combinations (avg. 12.7 g $plant^{-1}$). Finally, Ikram/Black Beauty plants gave the highest root dry weight at 50 µM Ni (13.0 g $plant^{-1}$).

Table 1: Effects of Ni concentration in the nutrient solution and grafting combination on yield, fruit number and mean weight, shoot and root biomass dry weight and final leaf area (LA) of tomato plants.

Treatment	Fruit			Dry biomass (g plant^{-1})		LA (m^2 plant^{-1})
	Yield (kg plant^{-1})	Number Plant^{-1}	Mean weight (g fruit^{-1})	Shoot	Root	
Ni concentration (µM)						
0	3.20 a[a]	35.0 a	91.1 a	261.8 a	16.5 a	2.11 a
25	3.07 b	34.2 b	89.6 b	228.1 b	12.7 b	1.81 b
50	2.93 c	33.7 b	86.8 c	223.5 b	11.8 c	1.74 b
Graft combination (G)						
Ikram/Ikram	2.99 c	34.5 b	86.9 b	227.5 b	13.1	1.79 c
Ikram/Black Beauty	2.52 d	32.5 c	77.6 d	214.0 c	13.9	1.79 c
Ikram/Unifort	3.22 b	34.7 b	93.1 b	252.9 a	13.8	1.92 b
Ikram/Maxifort	3.53 a	35.5 a	99.2 a	256.9 a	13.9	2.06 a
Significance[b]						
Ni	***	***	***	***	***	***
G	***	***	***	***	NS	***
Ni × G	NS	NS	NS	**	*	NS

[a]Means within columns separated using Duncan's multiple range test, P = 0.05.
bNS, *, **, ***Non-significant or significant at P <0.05, 0.01, and 0.001, respectively.

3.2. Fruit quality

When averaged over grafting combinations, increasing the concentration of Ni from 0 to 50 µM in the nutrient solution significantly enhanced the fruit firmness, fruit dry matter (DM) and total soluble solids (TSS) contents with the highest values recorded under severe Ni conditions, whereas an opposite trend was observed for titratable acidity TA. Moreover, the brighter color (i.e. higher yellowness, b*) with higher lightness (L*) was observed under severe Ni stress (Table 2). Irrespective of Ni level, the lowest fruit shape index (SI) was recorded in Ikram/Black Beauty, whereas the higher fruit DM and TSS contents were observed in Ikram/Unifort and Ikram/Balck Beauty, respectively. Finally, the tomato fruit of Ikram/Black Beauty was characterized by lower redness (a*) and yellowness when compared to the other three grafting combinations (Table 2).

Table 2: Effects of Ni concentration in the nutrient solution and grafting combination on fruit shape index (SI), firmness, dry matter (DM), total soluble solids (TSS) content, titratable acidity (TA) and Hunter colour values (L*, a*, b*) of tomato fruits.

Treatment	SI	Firmness ($N\ cm^{-2}$)	DM (%)	TSS (°brix)	TA (%)	L*	a*	b*
Ni concentration (µM)								
0	1.25	2.39 b[a]	6.47 b	5.30 b	0.47 a	39.5 b	32.0	31.0 b
25	1.27	2.53 a	6.52 b	5.31 b	0.45 b	39.8 b	31.7	31.7 b
50	1.27	2.49 a	6.76 a	5.47 a	0.45 b	40.7 a	31.4	32.7 a
Graft combination (G)								
Ikram/Ikram	1.26 a	2.49	6.63 ab	5.33 b	0.44 b	40.3	32.1 a	32.3 a
Ikram/Black Beauty	1.23 b	2.47	6.56 ab	5.47 a	0.45 b	39.7	30.4 b	31.0 b
Ikram/Unifort	1.28 a	2.47	6.70 a	5.34 b	0.44 b	40.1	32.0 a	32.0 ab
Ikram/Maxifort	1.29 a	2.45	6.44 b	5.30 b	0.48 a	40.0	32.2 a	31.9 ab
Significance[b]								
Ni	NS	*	**	**	*	***	NS	***
G	**	NS	*	*	**	NS	***	*
Ni × G	NS	NS	NS	NS	NS	NS	NS	NS

[a]Means within columns separated using Duncan's multiple range test, P = 0.05.
[b]NS, *, **, ***Non-significant or significant at P <0.05, 0.01, and 0.001, respectively.

3.3. Leaf pigments, SPAD index, chlorophyll fluorescence and electrolyte leakage

Increasing the Ni from 0 to 50 µM in the nutrient solution decreased the leaf pigments in both self-grafted and grafted plants. Similarly, the SPAD index and the maximum quantum use efficiency of PSII measured as F_v/F_m ratio decreased with increasing Ni level with the lowest values recorded under severe Ni stress conditions, whereas an opposite trend was observed for the electrolyte leakage (EL, Table 3). Irrespective of Ni concentration, the highest SPAD index and F_v/F_m ratio values were recorded in both tomato rootstocks (Unifort and Maxifort), whereas an opposite trend was observed for the Chl a/b ratio. The highest EL was observed in both self-grafted and Ikram/Black Beauty combinations (Table 3).

3.4. Activity of antioxidative enzymes, proline and MDA content

The changes in the antioxidative enzyme activities were mainly due to the Ni stress treatment and to a lesser degree to the effect of grafting combination (Table 4). CAT activity in

leaf crude extract declined gradually with the increase in Ni concentration from 0 to 50 µM. By contrast, the APX and GPX activities in the leaves of severe Ni-exposed tomato plants were significantly higher by 14.9% and 26.8%, respectively than in the control (Table 4).

Table 3: Effects of Ni concentration in the nutrient solution and grafting combination on total chlorophyll, carotenoids, chlorophyll (Chl) a/b ratio, SPAD index, maximum quantum use efficiency of PSII (F_v/F_m), and the percentage of electrolyte leakage (EL) of tomato leaves.

Treatment	Leaf pigments (mg/g fw)		Chl a/b	SPAD Index	F_v/F_m	EL (%)
	Chlorophyll	Carotenoids				
Ni concentration µM)						
0	0.64 a[a]	0.16 a	2.31	62.6 a	0.80 a	80.7 c
25	0.59 ab	0.15 ab	2.34	60.7 b	0.78 b	86.1 b
50	0.56 b	0.15 b	2.31	56.4 c	0.75 c	89.9 a
Graft combination (G)						
Ikram/Ikram	0.56	0.15	2.34 ab	59.4 b	0.77 b	87.1 a
Ikram/Black Beauty	0.61	0.16	2.40 a	58.9 b	0.77 b	88.5 a
Ikram/Unifort	0.64	0.16	2.24 c	61.0 a	0.79 a	84.5 b
Ikram/Maxifort	0.58	0.15	2.30 bc	60.2 a	0.79 a	82.4 b
Significance[b]						
Ni	*	*	NS	***	***	***
G	NS	NS	**	***	**	*
Ni × G	NS	NS	NS	NS	NS	NS

[a]Means within columns separated using Duncan's multiple range test, P = 0.05.

[b]NS, *, **, ***Non-significant or significant at P <0.05, 0.01, and 0.001, respectively.

Among the grafting combinations, the lowest APX and GPX activities were recorded in Ikram/Black Beauty combination. Analysis of proline in leaves revealed significant differences between the Ni treatments with the highest values recorded in the control (Table 4). The proline content in leaves of Ikram/Black Beauty was significantly higher by 30.8% in comparison to the Ikram/Maxifort combination (Table 4). The severe Ni treatment produced a consistent increase in the level of lipid peroxidation in leaves determined by measuring the amount of MDA. The MDA content recorded on plants receiving 50 µM Ni was significantly higher by 20% and 6.7%, in comparison to tomato plants treated with 0 and 25 µM Ni, respectively. When averaged over Ni level, the MDA production reached maximum value with Ikram/Black Beauty, followed by

Ikram/Ikram and Ikram/Unifort, whereas the lowest MDA content was recorded when Ikram was grafted onto the Maxifort rootstock (Table 4).

3.5. Mineral composition and partitioning

The concentration of N in leaves and fruits was significant affected by Ni level where the lowest value of leaf concentration was at 50 µM Ni. Moreover, the concentration of N in leaves was higher in Ikram/Black Beauty than in the other grafting combinations, whereas an opposite trend was recorded for the concentration of N in root (Table 5). The concentration of P in leaves decreased under Ni-exposed treatments. Concerning the effect of grafting combination, the lowest concentration of P in leaves, fruits and roots were recorded when the cultivar Ikram was grafted onto the eggplant rootstock Black Beauty (Table 5).

Table 4: Effects of Ni concentration in the nutrient solution and grafting combination on catalase (CAT), ascorbate peroxidase (APX) and guaiacol peroxidase (GPX) activities, proline content, and malondialdehyde content (MDA) in tomato leaves.

Treatment	Antioxidant enzymes (mmol mg^{-1} protein min^{-1})			Proline (mg g^{-1} FW)	MDA (nmolg^{-1} FW)
	CAT	APX	GPX		
Ni concentration (µM)					
0	279.2 a[a]	10.7 b	2.39 b	6.82 a	4.80 c
25	227.2 b	10.4 b	2.76 a	6.00 b	5.40 b
50	189.3 c	12.3 a	3.03 a	5.14 c	5.76 a
Graft combination (G)					
Ikram/Ikram	230.7	11.46 ab	2.77 a	5.91 b	5.40 ab
Ikram/Black Beauty	242.6	10.33 b	2.27 b	6.70 a	5.48 a
Ikram/Unifort	209.9	10.51 ab	2.92 a	6.22 ab	5.32 ab
Ikram/Maxifort	244.3	12.14 a	2.95 a	5.12 c	5.07 b
Significance[b]					
Ni	***	*	**	***	***
G	NS	*	**	**	*
Ni × G	NS	NS	NS	NS	NS

[a]Means within columns separated using Duncan's multiple range test, P = 0.05.
[b]NS, *, **, ***Non-significant or significant at P <0.05, 0.01, and 0.001, respectively.

In leaves, K concentration decreased as solution Ni concentration increased from 0 to 50 µM. The concentration of K in fruits and roots tissues was significantly influenced by grafting combination, with the lowest values recorded on grafting combination Ikram/Black Beauty. At 50 µM Ni level, the leaves and roots had the lowest Ca concentration in comparison to those recorded in the control treatment. The highest Ca concentration in leaves, fruits and roots were observed in Ikram/Unifort and Ikram/Maxifort combinations. Similar to N and K, increasing Ni concentration in the nutrient solution decreased significantly the Mg concentration in leaves especially under severe Ni stress conditions. Moreover, the concentration of Mg in leaves and roots were lower in Ikram/Black Beauty in comparison to those recorded in plants self-grafted and grafted onto both tomato rootstocks (Table 5).

The effect of Ni-supply level on tissue micronutrient concentrations Fe, Zn, Mn and Cu was highly significant. The leaf and fruit tissue concentrations of Fe, and Cu decreased as the external Ni concentration increased from 0 to 50 µM, whereas an opposite trend was observed for the root tissue (Table 6). The fruit tissue of Zn decreased with increasing Ni level, whereas an opposite trend was recorded for Mn in fruits (Table 6). Overall, the accumulation of micronutrient was significantly lower in Ikram/Black Beauty in comparison to the Ikram/Ikram, Ikram/Unifort and Ikram/Maxifort combinations.

Irrespective of treatments, most of the Ni absorbed by tomato plants was recorded in root tissue. Ni concentration in leaves, fruits and roots was significantly raised by increased Ni levels from 0 to 50 µM (Table 6). Finally, the highest accumulation of Ni in leaf tissue was observed in both Ikram/Ikram and Ikram/Black Beauty combinations (Table 6), whereas in root tissue the highest values were recorded on self-grafted tomato plants treated with 50 µM Ni (data not shown).

4. Discussion

4.1. Crop productivity and growth responses

The requirement of Ni in higher plants is quite low, whereas toxicity occurs at concentrations in the range of 10 µg g-1 in sensitive and 50 µg g-1 in moderately tolerant species (Marschner, 2002; Seregin and Kozhevnikova, 2006; Yusuf et al., 2011). In the present study, significant depression in fruit yield, leaf area, shoot and root biomass production was recorded (Table 1), and that effect varied as a function of Ni concentration in the nutrient solution. Root growth was inhibited more severely (reduction of 23-28%) than the growth of the shoot (reduction of 14%).

Table 5: Effects of Ni concentration in the nutrient solution and grafting combination on macronutrient composition of leaves, fruits, and roots in tomato plants.

Treatment	Macronutrients (g kg^{-1} DW)														
	N			P			K			Ca			Mg		
	Leaf	Fruit	Root	Leaf	Fruit	Root	Leaf	Fruit	Root	Leaf	Fruit	Root	Leaf	Fruit	Root
Ni concentration (µM)															
0	34.2 a[a]	22.4 a	32.0	9.95 a	4.65	5.50 a	28.7 a	32.0	32.1 c	26.9 a	1.07	11.2a	5.28 a	1.29	2.5 ab
25	32.1 b	20.7 b	33.4	8.49 b	4.52	5.25 ab	25.9 b	30.3	35.7 b	25.8 ab	1.10	9.6 b	4.76 b	1.27	2.7 a
50	30.3 c	20.6 b	30.6	8.02 b	4.35	4.69 b	23.0 c	30.0	40.4 a	23.5 b	1.13	9.4 b	4.24 c	1.25	2.3 b
Graft combination (G)															
Ikram/Ikram	31.1 b	20.3 b	33.0 a	9.37 a	4.66 a	5.37 a	25.1	30.7 ab	36.7 a	25.7 ab	1.10 ab	9.7 b	4.86 a	1.28	2.5 a
Ikram/Black Beauty	33.1 a	22.1 a	27.5 b	7.54 b	4.18 b	4.52 b	25.6	29.5 b	29.5 b	22.2 b	1.01 b	9.8 b	3.86 b	1.23	2.0 b
Ikram/Unifort	32.2 ab	21.5 a	32.9 a	9.23 a	4.65 a	5.17 ab	26.4	32.2 a	38.9 a	26.4 a	1.16 a	10.0 ab	5.12a	1.29	2.7 a
Ikram/Maxifort	32.3 ab	21.1 a	34.6 a	9.14 a	4.52 a	5.53 a	26.3	30.8 ab	39.1 a	27.2 a	1.13 ab	10.8 a	5.20 a	1.28	2.7 a
Significance[b]															
Ni	***	***	NS	**	NS	*	***	NS	***	*	NS	***	**	NS	*
G	*	**	**	*	*	*	NS	*	***	*	*	*	***	NS	**
Ni × G	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

[a]Means within columns separated using Duncan's multiple range test, P = 0.05.

[b]NS, *, **, ***Non-significant or significant at P <0.05, 0.01, and 0.001, respectively.

Table 6: Effects of Ni concentration in the nutrient solution and grafting combination on micronutrient and nickel (Ni) composition of leaves, fruits, and roots in tomato plants.

Treatment	Micronutrients (mg kg^{-1} DW)														
	Fe			Zn			Mn			Cu			Ni		
	Leaf	Fruit	Root	Leaf	Fruit	Root	Leaf	Fruit	Root	Leaf	Fruit	Root	Leaf	Fruit	Root
Ni concentration (µM)															
0	42.9 a	30.1 a	139.2 b	24.4	17.8 a	24.2 b	159.4 a	11.7 b	62.9 b	3.98 a	4.33 a	12.6 b	0.08 c	0.04 c	0.68 c
25	39.0 b	26.7 b	185.7 b	24.2	15.7 b	27.4 b	166.4 a	13.0 a	62.0 b	3.02 b	3.89 a	14.9 b	7.05 b	3.22 b	84.4 b
50	35.3 c	21.3 c	287.2 a	25.6	13.3 c	47.7 a	147.5 b	13.2 a	66.6 a	2.40 c	3.25 b	24.9 a	25.7 a	12.33 a	174.7 a
Graft combination (G)															
Ikram/Ikram	38.2 bc	24.7 b	210.5	26.5 a	15.9	37.0 a	171.8 a	13.7 a	61.6 b	2.98 b	4.03 a	18.2 b	11.1 a	4.83	111.7 a
Ikram/Balck Beauty	36.1 c	25.0 b	161.3	21.4 b	14.8	25.9 b	125.2 c	10.9 b	54.4 c	1.76 c	3.02 b	13.7 c	11.1 a	4.94	52.1 c
Ikram/Unifor	40.2 ab	27.1 a	205.5	24.7 a	15.7	37.4 a	155.8 b	12.8 a	69.1 a	3.56 a	4.18 a	17.4 b	10.9 ab	5.62	99.1 b
Ikram/Maxifort	41.8 a	27.2 a	238.7	26.3 a	16.1	32.0 a	178.2 a	13.2 a	70.2 a	4.23 a	4.07 a	20.6 a	10.6 b	5.39	83.4 b
Significance[b]															
Ni	***	***	***	NS	***	***	**	*	*	***	**	***	***	***	***
G	**	NS	NS	***	NS	**	***	***	***	***	**	***	*	NS	***
Ni × G	**	NS	NS	*	NS	NS	NS	NS	***	*	NS	**	NS	NS	**

[a]Means within columns separated by different letters using Duncan's multiple range test, P = 0.05.

[b]NS, *, **, ***Non-significant or significant at P <0.05, 0.01, and 0.001, respectively.

These results are consistent with the findings of Panday and Sharma (2002) and Seregin et al. (2003), who reported that under Ni stress conditions, the root growth of cabbage and maize was more severely affected than that of the aerial parts. Overall, the decrease in crop performance due to Ni agrees with the findings of Balaguer et al. (1998) who observed that increasing the Ni concentration from 0 to 20 mg L-1 in the nutrient solution, affected negatively tomato growth and yield. Others report show that the accumulation of Ni adversely affects the growth and yield of eggplant (Panday and Sharma, 2002), wheat (Gajewska et al., 2006), cowpea (Kopittke et al., 2007) and mustard (Gopal and Nautiyal, 2012). In general abiotic stress (i.e. drought, salinity, heavy metals) reduces vegetable crop productivity, but can improve some quality attributes of the product as observed in plants grown in soilless and soil conditions (Rouphael et al., 2010; 2012b). In the current experiment, plants receiving 50 µM Ni enhanced some fruit quality aspects of tomatoes which are particularly important for marketing and consumer satisfaction in particular visual appearance (firmness and brightness) and taste (DM and TSS contents) (Table 2). This result is in agreement with findings of Gad et al. (2007) who observed that increasing Ni up to 30 mg kg-1 soil improved the fruit DM, vitamin C and TSS concentration in tomato. However, excessive applications of Ni in the rootzone lead to an increase of Ni content in fruits which represent a serious risk for human health.

Recent studies have indicated that some rootstocks of Solanaceae and Cucurbitaceae species may restrict the uptake of heavy metals and toxic micronutrients (Edelstein et al., 2007; Rouphael et al., 2008a; Mori et al., 2009). In fact, our study showed that grafting Ikram onto tomato rootstocks in particular Maxifort, improved the yield and growth parameters in comparison to self-grafted plants or Ikram/Black Beauty combination (Table 1). Ni-tolerance of plants grafted onto tomato rootstocks, in particular Maxifort may be attributed to a reduction of Ni concentration in leaf tissue and the better uptake and translocation of mineral elements in particular iron to the shoots. Edelstein and Ben-Hur (2006) and Rouphael et al. (2008a) demonstrated that grafting fruit vegetables particularly, melon and cucumber onto appropriate rootstocks (i.e. pumpkin) may limit the heavy metals (Cr and Ni) and trace elements (Cu) concentration in the aerial parts, thus mitigating their adverse effects on crop performance and human health (Savvas et al., 2010).

4.2. Physiological and biochemical responses

Considering that the response to Ni relies on the plant ability to counteract the negative effects caused by Ni toxicity, the different changes induced by the eggplant (Black Beauty) and tomato (Unifort and Maxifort) rootstocks on crop performance (i.e. yield and growth) should be also reflected at physiological and biochemical levels. In fact, the higher yield and biomass production in grafted plants in particular those grafted onto Unifort and Maxifort rootstocks could be attributed to the capacity of maintaining higher SPAD index and maximum quantum efficiency of PSII (F_v/F_m) (Table 3). This result, suggests that grafted plants onto appropriate rootstocks can delay photoinhibition under heavy metal stress especially, that at biochemical level, Ni interferes with the photosynthetic electron transport chain and the light harvesting complex II (Singh et al., 1989; Chen et al., 2009). Moreover, the lower Ch a/b ratio recorded in both tomato rootstocks demonstrated less damage to chlorophyll b, indicating better protection of PSII under metal stress conditions (Ünyayar et al., 2005).

The electrolyte leakage method for assessing cell membrane stability has been widely adopted as a benchmark to differentiate stress susceptible and tolerant species/genotypes and in some cases higher membrane stability could be associated with abiotic stress tolerance in vegetable crops (Rouphael et al., 2008a; Colla et al., 2010a). The present study showed that the grafting combinations Ikram/Unifort and Ikram/Maxifort reduced the amount of ion leakage (Table 3), indicating that grafting Ikram onto the selected tomato rootstocks has facilitated the maintenance of membrane functions (i.e., semipermeability). Calcium increases structural stability of cell membrane because of electrostatic interactions with membrane phospholipids and proteins and of its role as fundamental component of the cell wall (Borer et al., 2005). Ni applications in the nutrient solution reduced leaf content of Ca leading to a reduction of cell membrane stability of leaf tissues. However, Unifort and Maxifort rootstocks were able to mitigate the detrimental effect of Ni on membrane stability by improving the Ca uptake and translocation in leaf tissues of tomato plants.

The production and accumulation of ROS in plant cells is another common damage under heavy metal stress conditions (Seregin and Ivanov, 2002). In the present study, the activities of antioxidant enzymes like APX and GPX were enhanced especially under severe Ni stress conditions, and more considerably with plants grafted onto Maxifort than in Black Beauty (Table

4). The above findings indicate that certain rootstocks mitigated the Ni toxicity through a better detoxification process in plant cells. The increase in the leaf APX and GPX activities were also observed in cucumber, rice, and *Luffa cylindrica* under Ni stress conditions (GajewskaandSklodowska, 2007; Maheshwari and Dubey, 2009; Wang et al., 2010). Contrary to APX and GPX, CAT activity in the tomato leaves decreased in response to Ni concentration, with no significant differences among grafting combinations. Since CAT are metalloenzymes containing Fe, Cu or Zn (Chen et al., 2009), the decreasing in CAT activity could be expected as excess Ni decreased micronutrients (Fe, Cu and Zn) in the leaf tissues. The decreasing in CAT activity recorded in the current study, is in line with the findings of Pandey and Sharma (2002) and Gajewska and Sklodowska (2008) on cabbage and wheat, respectively.

Another system of protection against Ni toxicity includes the synthesis of osmolytes, such as proline, which contribute to stabilization of protein molecules and membranes (Seregin and Kozhevnikova, 2006). The proline content decreased with increasing the Ni concentration from 0 to 50 µM in disagreement with previous studies, where proline enhancement was observed in response to heavy metal toxicity (Gajewska et al., 2006; Panday and Sharma, 2002). The decrease in proline contents may be related with the oxidative stress resulting in hydrolysis of protein (Azmat and Khan, 2011). Finally, leaves of Ikram/Maxifort combination in Ni-stressed plants accumulate less proline than those of Ikram/Ikram, Ikram/Unifort and Ikram/Black Beauty.

The changes in the membrane lipid composition, have been also observed in the current study, since malondialdehyde (MDA, a lipid peroxidation product) content, which is an important indicator of oxidative stress, increased linearly, when tomato plants were treated with 50 µM Ni as observed earlier on rice, pigeonpea and cucumber (Ros et al., 1992; Madhava Rao and Sresty, 2010; Khoshgoftarmanesh et al., 2014). The lowest MDA accumulation recorded in the Ikram/Maxifort combination confirmed that Maxifort rootstock play an important role in mitigating the oxidative damage caused by nickel by enhancing the antioxidant capacity and reducing the proline and MDA content in leaf tissues.

4.3. Mineral nutrition, translocation and assimilation

The uptake and distribution of Ni in plants is still a debatable issue for plant scientists. Seregin and Khozevnikova (2006) observed that more than 50% of the Ni absorbed by plants is accumulated in the roots, and they attributed the accumulation of this heavy metal in the underground organ to sequestration in the cation exchange sites of the walls of xylem parenchyma cells. Roots of grafted and self-grafted tomato plants accumulated higher amounts of Ni than in the above-ground parts (leaves, and fruits). Our results are in agreement with those reported by Brune and Deitz (1995) and Baccouch et al. (2001) who demonstrated more Ni accumulation in roots than in shoots of barely and maize, respectively. The Ni concentration in fruit was not affected by grafting combination, and that concentration (avg. 5.2 mg kg^{-1}dw) was within the recommended international standards (Poulik, 1999). Moreover, the Ni content in leaf tissue of tomato plants grafted onto Unifort and especially Maxifort had less Ni when compared to self-grafted and plants grafted onto eggplant rootstock. This suggests that some rootstocks of tomato more efficiently reduce the Ni uptake and the transport to leaves. The reduction in accumulation of Ni in leaves of Ikram/Maxifort combination was the principal mechanism that decreases the detrimental effect of Ni toxicity on crop yield and growth. The current results were supported by the findings of Savvas et al. (2013) who demonstrated the ability of four commercial *Cucurbita maxima* × *Cucurbita moschata* rootstocks to reduce the Ni concentration in leaves of cucumber when compared to self-grafted and ungrafted plants.

Ni above a threshold level (5-10 µg g^{-1}dw) may inhibit the absorption, uptake and accumulation of essential macro and micro elements such as K, Ca, Mg, Fe, Cu, Zn, and Mn, leading to their deficiency in plants (Chen et al., 2009; Yusuf et al., 2011). The decrease in the uptake may also be attributed to Ni-induced metabolic disorders that affect root function (Seregin and Kozhevnikova, 2006). This was the case in the current experiment since a significant reduction of macro- (N, K, Ca, and Mg) and microelements (Fe, Mn, and Cu) in leaf tissue of tomato was observed under severe Ni stress (Tables 5 and 6). These results are consistent with the findings of Palacios et al. (1998) who reported that high concentration of Ni decreased significantly the uptake of several divalent cations (Mg^{2+}, Fe^{2+}, Mn^{2+}, Cu^{2+}, and Zn^{2+}). Many rootstocks used in vegetable grafting were able of enhancing the uptake rates of some nutrients even under abiotic stress conditions (e.g. heavy metals) because they are characterized by more vigorous root systems (Savvas et al., 2010).

5. Conclusions

As a summary, in both self-grafted and grafted plants, yield and biomass production decreased, in response to an increase of Ni concentration in the nutrient solution. This significant reduction of crop performance under Ni stress was attributed to a decrease in leaf pigments, SPAD index, F_v/F_m ratio, macro and microelements, and increase in EL and lipid peroxidation. Our results also demonstrate that grafting commercial cv. Ikram onto appropriate rootstocks could minimize the Ni toxicity. Plants grafted onto tomato rootstocks Unifort and especially Maxifort were capable of maintaining higher chlorophyll content (SPAD index), higher F_v/F_m ratio, increased the APX and GPX activities, a better nutritional status (i.e. higher Ca, Fe, and Cu, and lower Ni) in the leaf tissue and higher a membrane selectivity (lower accumulation of MDA and EL) in comparison with self-grafted and Ikram/Black Beauty combination.

Chapter 4

Insight into the role of grafting and arbuscular mycorrhiza on cadmium stress tolerance in tomato

Pradeep Kumar[a,b], Luigi Lucini[c], Youssef Rouphael[d], Mariateresa Cardarelli[e], Raviraj M. Kalunke[b], Giuseppe Colla[b]

[a]*ICAR-Central Arid Zone Research Institute, Jodhpur, 342003 Rajasthan, India*

[b]*Department of Agriculture, Forestry, Nature and Energy, University of Tuscia, Viterbo, Italy*

[c]*Institute of Environmental and Agricultural Chemistry, UniversitàCattolica del Sacro Cuore, 29122 Piacenza, Italy*

[d]*Department of Agricultural Sciences, University of Naples Federico II, 80055 Portici, Italy*

[e]*Consiglio per la Ricerca in Agricoltura e l'analisi dell'economia agraria, Centro di ricerca per lo studio delle Relazioni tra Pianta e Suolo, 00184 Roma, Italy*

*Corresponding author. Tel.: +39 (0)761 357536; fax: +39 (0)761 357453

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E-mailaddress:giucolla@unitus.it (Giuseppe Colla)

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Chapter 4

Insight into the role of grafting and arbuscular mycorrhiza on cadmium stress tolerance in tomato

Abstract

Physiological, biochemical, metabolite changes and gene expression analysis of greenhouse tomato (*Solanum lycopersicum* L.) were investigated in two grafting combinations (self-grafted 'Ikram' and 'Ikram' grafted onto interspecific hybrid rootstock 'Maxifort'), with and without arbuscular mycorrhizal (AM), exposed to 0 and 25 µM Cd. Tomato plants responded to moderate Cd concentration by decreasing yield and crop growth parameters due to the accumulation of Cd in leaf tissue, inhibition of the PS II activity, reduced nutrients translocation, and also to the oxidative stress as evidenced by enhanced hydrogen peroxide (H_2O_2) generation, ion leakage and lipid peroxidation. AM inoculation significantly enhanced the metal concentration in shoots and reduced growth and yield. The Ikram/Maxifort combination induced higher antioxidant enzymes, higher accumulation of proline and reduction of lipid peroxidation products. This suggests that the use of Maxifort rootstock in tomato has a high reactive oxygen species scavenging activity since lower H_2O_2 concentrations were observed in the presence of Cd. The higher crop performance of Ikram/Maxifort in comparison to Ikram/Ikram combination was also due to the improved nutritional status (higher P, K, Ca, Fe, Mn, and Zn) and increased availability of metabolites involved in cadmium tolerance (phytochelatin PC2 and fructansinulins). The up-regulation of *LeNRAMP3* gene in leaf of Ikram/Maxifort could explain the better nutritional status of interspecific grafting combination (higher Fe, Mn, and Zn).

1. Introduction

Cadmium (Cd) is the most hazardous heavy metal element for plant growth with high toxicity even at low level (5-10 µg g^{-1}dry weight; Dong et al., 2006; White and Brown, 2010). Cd contamination in agricultural soils may come from various sources, including the use of phosphate fertilizers, pesticides, organic manures, and sewage sludge (Dong, 2007; Uraguchi et al., 2009). Owing to its high solubility and mobility, Cd is readily absorbed by plant roots and translocated to the shoots (Dong et al., 2007; Gallego et al., 2012) and even to the fruits through

phloem mediated transport (Hediji et al., 2010; Bertoli et al., 2012) thereby, endangering not only plant productivity, but also human health via the food chain (Dong et al., 2007). Cd is a non-essential element for plants and is taken up by plants through transporters of certain essential elements due to their low metal specificity and/or through Ca transport mechanism (Gallego et al., 2012).

In vegetable crops, Cd stressful conditions have been reported to disturb physiological, biochemical and metabolic processes leading to growth inhibition (López-Millánet al., 2009; Hediji et al., 2010; Sharma et al., 2010). Particularly in tomato plants, excess Cd causes inhibition of chlorophyll and carotenoids biosynthesis and substantial inhibition of the photosystem (PS) II activity. Further, it causes significant alteration in nutrient uptake and translocation of K, Ca, Mg, Mn, Fe, and Zn (Djebali et al., 2005; Dong et al., 2006; Hédiji et al., 2010; Bertoli et al., 2012). Therefore, Cd-exposed plants show visible symptoms such as reduction of shoot and root biomass and leaf chlorosis and necrosis (Smeets et al., 2005; Shahabivand et al., 2012). At low Cd concentrations in soil or water, these visible symptoms are less marked, but various cellular processes may still be affected (Smeets et al., 2005; Gratao et al., 2008; Hediji et al., 2010). At cell level, Cd has been demonstrated to induce oxidative stress in plants by producing reactive oxygen species (ROS) that disrupt cellular homeostasis and damage proteins, pigments, lipid peroxidation and ion leakage, which in turn leads to membrane damage and enzymes inactivation (Pitzschke et al., 2006; Sharma and Dubey, 2005; Gratao et al., 2008). In order to cope with such toxic effects derived from ROS, plants may employ integrated antioxidant defense mechanisms (Gallego et al., 2012). The antioxidant defense system comprises antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and guaiacol peroxidase (GPX), as well as non-enzymatic antioxidants such as reduced glutathione (GSH), ascorbic acid (AsA), α-tocopherol and carotenoids (Gill and Tuteja, 2010; Gratao et al., 2008; 2012; Gallego et al., 2012). Moreover, plants exposed to toxic heavy metals have also been shown to regulate some metabolites to cope with the toxic effects of Cd. These include, among others, proline, ascorbate, carotenoids, glucosinolates and phytochelatins as well as phytohormones.

Numerous attempts have been made to enhance heavy metal tolerance of vegetable crops by traditional breeding and genetic engineering with limited success (Savvas et al., 2010).

Consequently, to alleviate the negative effects of Cd-toxicity, various strategies have been suggested. Of these is a grafting strategy involving rootstocks that are known to enhance plants' ability in mitigating the adverse environmental conditions and it can be a faster and sustainable tool (Colla et al., 2010; Savvas et al., 2010; Schwarz et al., 2010). Evidence of exploiting grafting against heavy metal in vegetable came to light only a few years ago from the study done by Takeda et al. (2007). The authors demonstrated that grafting eggplant onto *Solanum torvum* was effective in reducing Cd concentrations in the eggplant fruit by up to 25% when compared to those of ungrafted plants. This phenomenon was, further studied and reported to be attributed by either reduced Cd flux or lower xylem loading in *S. torvum* (Arao et al., 2008; Mori et al., 2009; Yamaguchi et al., 2011). In cucumber, Savvas et al. (2013) demonstrated that the pumpkin rootstock 'Power' could efficiently restrict Cd concentration in plant tissues by 12-50% in comparison with other grafting combinations. The proposed mechanism for reduced aerial Cd contents was found to be related to the higher exclusion efficiency of 'Power' rootstock for Cd at the plasma membrane of root cells.

Another, promising and environment friendly tool to overcome Cd toxicity in contaminated soils would be through inoculation with beneficial microorganisms such as arbuscular mycorrhizal (AM) fungi. AM are beneficial associations between soil fungi and plant roots which is found in 83% of higher plants (Ma et al., 2001). The association of AM fungi with plant roots can enhance plant growth and resistance to stressful edaphic conditions including toxicity produced by heavy metals (Ouziad et al., 2005; Shahabivand et al., 2012). Improved nutritional status, reduced metal uptake, immobilization of metals in fungal biomass, dilution by increased shoot and root growth and metal compartmentalization into plastids or other membrane rich organelles are among the most common Cd-tolerance mechanisms induced by AM fungi (Kaldorf et al., 1999; Gaur and Adholia, 2004; Andrade et al., 2008).

There is scarce or null information about the influence of grafting and AM fungi on physiological and metabolic changes under heavy metal stress in vegetable crops such as tomato. Our hypothesis was that grafting tomato onto vigorous rootstock with or without AM inoculation may raise Cd tolerance through limiting heavy metal uptake by roots and /or its translocation to the shoots, or by facilitating metal detoxification process by regulating the level of antioxidants and of certain metabolite concentrations in plants.

The aim of this study was to investigate the effects of grafting and AM fungi inoculation, alone or in combination, on physiological, biochemical, metabolite, and gene expression changes of greenhouse tomato plant under moderate external Cd concentration, which is common in current agriculture practice as stated by Uraguchi et al.(2009)and Lopez-Millan et al.(2009).

2. Materials and methods

2.1. Growth conditions and treatments

Tomato (*Solanum lycopersicum* L. cv. Ikram, Syngenta, Milan, Italy) plants self-grafted, or grafted onto a vigorous interspecific hybrid rootstock Maxifort (*S. lycopersicum* × *S. habrochaites*, De Ruiter/Monsanto, Bergschenhoek, The Netherlands) were produced using the splice graft technique. During the culture, plants were grown in a greenhouse from April 24 to August 25, 2014.At four true leaf stage tomato seedlings were transferred into pots (6 L) containing quartziferous sand. Pots were arranged in double rows in separate plastic covered channel providing sufficient slope to enable drainage collection into tanks fixed at the lateral end of each channel. The plant density was 3.7 plants m^{-2}. Pollination was facilitated by bumble bees.

The experiment was designed as a factorial combination of two Cd concentrations [0 (control), or 25 μM], two grafting combinations (self-grafted Ikram/Ikram, or Ikram/Maxifort), and two mycorrhizal treatments (with AM, +AM or without AM, –AM). The treatments were arranged in a randomized complete-block design with four replicates per treatment. Each experimental unit consisted of three plants. Prior to transplanting, half of the transplants received a mycorrhizal inoculum carrying 50 $sporesg^{-1}$ of *Rhizophagus irregularis* (formerly *Glomus intraradices*). Inoculum was applied in the transplanting hole at a rate of 5 g $plant^{-1}$.

Plants were fed with a basic nutrient solution containing: 14.0 mM $N–NO_3$, 1.6 mM S, 0.3mM P, 6.0 mM K, 4.5 mM Ca, 1.5 mM Mg, 20 μM Fe, 9 μM Mn, 0.3 μM Cu, 1.6 μM Zn, 20 μM B, and 0.3 μM Mo. After an initial growth period of 28 (May 21), Cd was added as chloride to the basic nutrient solution at a concentration of 25μM. The pH of the nutrient solution for all treatments was 5.6 ± 0.2. Deionized water was used for the preparation of all nutrient solutions. Nutrient solution was pumped from independent supply tanks through a drip irrigation system, with one emitter per plant of 2 L h^{-1} flow rate. The duration of each irrigation event was tuned to

provide at least 35% of the nutrient solution draining from the pots (Rouphael et al., 2004; Rouphael and Colla, 2005).

2.2. Plant biomass

Fruits were harvested from July 2 until August 22 and marketable fruits were weighed. At final plant harvest (August 25, 124 days after transplanting DAT), morphological parameters were recorded. Each plant was divided in three fractions i.e., leaves, stems and roots. Fresh and dry weights of each fraction and the root to shoot ratios were determined. Shoot biomass was equal to the sum of aerial vegetative plant parts (leaves + stems). Leaf area was measured with Li-3000 area meter (Li-CorDelta-T Devices Ltd., Cambridge, UK).

For the biochemical and enzyme assays, fresh leaf samples from the 3rd leaves from top were harvested from each plant and immediately frozen in liquid nitrogen and stored at -80°C for later antioxidant enzyme activity analysis.

2.3. Root colonization

A small fraction of the root system was carefully washed in tap water, cut into 1 cm root pieces. Root samples were cleared with 10% KOH, stained with 0.05% trypan blue in lactophenol as described by Phillips and Hayman (1970), and microscopically examined for AM fungi colonization by determining the percentage of root segments containing arbuscules + vesicles using a gridline intercept method (Giovannetti and Mosse, 1980).

2.4. Chlorophyll fluorescence measurements

The chlorophyll fluorescence was recorded on 20minutes dark-adapted leaves (in 3rd - 4thfrom top; three measurements per plant) by means of a chlorophyll fluorometer Handy PEA (Hansatech Instruments Ltd, King's Lynn, UK) with an excitation source intensity higher than 3000 μmol$m^{-2}s^{-1}$ at the sample surface. The minimal fluorescence intensity (F_0) in a dark-adapted state was measured in the presence of a background weak light signal (about 2-3 μmol photons $m^{-2}s^{-1}$). The maximal fluorescence level in the dark-adapted state (F_m) was induced by 0.8 s saturating light pulse (3000 μmol photons $m^{-2}s^{-1}$). The maximum quantum yield of open photosystem II (PSII) (F_v/F_m) was calculated as ($F_m - F_0$)/F_m, as described by Arena et al. (2005).

2.5. Chlorophyll and carotenoids determination

Total chlorophyll and carotenoid (mg g^{-1} FW) were extracted by homogenization of fresh leaf tissues (0.5 g) in acetone (80%). The resulting extracts were centrifuged at 4,800 *g* for 20 min. Chlorophyll a, chlorophyll b, and carotenoid contents were determined by taking absorbance of the supernatant at 470, 647, and 664 nm by a UV–Vis spectrophotometer (Perkin Elmer, Norwalk, CT, USA) following the method of Lichtenhaler and Wellburn (1983).

2.6. Lipid peroxidation and electrolyte leakage determination

The Cd-induced oxidative damage (membrane lipid peroxidation) in fresh leaf tissue was estimated by measuring the malondialdehyde (MDA) concentrations. Leaf tissues were homogenized in 0.1% (w/v) trichloroacetic acid (TCA) solution in 1:3 ratio. After centrifugation (15 min, 12,000 *g* at 4°C), an aliquot of the supernatant was added to 0.5% thiobarbituric acid (TBA) made in 20% TCA and heated at 95°C for 30 min. After rapid cooling on ice, the mixture was centrifuged at 10,000 g for 10 min. The absorbance was recorded at 532 and 600 nm using UV-Vis spectrophotometer. The MDA concentration was calculated from the difference between the absorbance values at 532 and 600 nm (Ben Amor et al., 2005).

The electrolyte ion leakage (EL) was determined as described by Lutts et al. (1995). Ten randomly chosen mature leaves per experimental unit were taken and cut into 1-cm segments. Leaf samples were placed in individual stoppered vials containing 10 mL of distilled water. The samples were incubated at room temperature (25 ºC) on a shaker (100 rpm) for 24 h. Electrical conductivity of the bathing solution (EC_1) was read after incubation. The same samples were then placed in an autoclave at 120 ºC for 20 min and a second reading of the EC (EC_2) was made after cooling the solution to room temperature. The EL was calculated as EC_1/EC_2 and expressed as percentage.

2.7. Hydrogen peroxide content

Hydrogen peroxide content was determined according to Sergiev et al. (1997).Tissue powder was homogenized in ice bath by adding 0.1% (W/V) TCA in 1:3 ratio (W/V). After centrifugation (12,000 *g* for 30 min) of the homogenate, supernatant (0.5 mL) was added to 0.5 mL 10 mM K-phosphate buffer (pH 7.0) and 1 mL 1 M potassium iodide. The absorbance was

measured at 390 nm. H_2O_2 was used as a standard and the content was expressed as µmol H_2O_2 g^{-1} fresh wt. tissue.

2.8. Antioxidant enzyme analyses

Frozen leaf tissues were homogenized with three volumes of an ice-cold extraction buffer (0.05 M potassium phosphate buffer, pH 7.0) containing 0.1% (w/v) ascorbic acid, 1% (w/v) polyvinilpolypirrolidone, 1 mM Na_2-EDTA and 0.1% (v/v) Triton X-100. After centrifugation (15,000 × *g*, 30 min, 4°C) the supernatant was set aside for the determination of the enzymes activity by a spectrophotometer (Perkin Elmer, Norwalk, CT, USA). Catalase (CAT, EC 1.11.1.6) activity was measured according to Havir and McHale (1989). Enzyme activity was evaluated by following the decomposition of H_2O_2 at 240 nm for 1 min and calculated using the extinction coefficient (0.036 mM^{-1} cm^{-1}). Activity of ascorbate peroxidase (APX, EC 1.11.1.11) was measured following the decrease of absorbance at 290 nm for 1 min (Nakano and Asada, 1981) corresponding to the oxidation of ascorbic acid. APX activity was calculated using its extinction coefficient (2.8 mM^{-1} cm^{-1}). Activity of guaiacol peroxidase (GPX, EC 1.11.1.7) was measured according to with Chance and Maehly (1955). Enzyme activity was evaluated following the increase of absorbance at 470 nm for 40 s due to guaiacol oxidation and calculated using the extinction coefficient (26.6 mM^{-1} cm^{-1}). The specific enzyme activity for all enzymes was expressed as mmol mg^{-1} protein min^{-1}.

2.9. Proline analysis

Proline content (mg g^{-1} FW) was determined as described by Bates et al. (1973). Leaf tissue (0.5 g) was homogenized in 10 mL of 30 g L^{-1} sulfosalicylic acid and the homogenate was filtered through Whatman No. 2 filter paper. Then 2 mL of filtrate was reacted with 2 mL of acid-ninhydrin (1.25 g of ninhydrin in 30 mL of glacial acetic acid and 20 mL of 6 mol L^{-1} phosphoric acid) and 2 mL of glacial acetic acid in a test tube at 100 °C for 1 h. The reaction was terminated in an ice bath and then 4 mL of toluene was added and the product of the reaction was extracted by vortex mixing. The absorption of the upper phase was read at 520 nm using toluene as a blank.

2.10. Cadmium and mineral analysis

The dried leaf, fruit and root tissues were ground in a Wiley mill to pass through a 20-mesh screen, then 0.5 g samples were analyzed for the following macro- and micro-nutrients and toxic element: P, K, Ca, Mg, Fe, Mn, Zn, and Cd. The Cd concentration in the fruit tissue was also determined. The elements were determined by dry ashing at 400 ºC for 24 h, dissolving the ash in 1:20 HNO_3, and assaying the solution obtained using an inductively coupled plasma emission spectrophotometer (ICP Iris; Thermo Optek, Milano, Italy; Karla, 1998).

2.11. Metabolomic analysis

The screening of plant metabolites was carried out on a hybrid quadrupole-time-of-flight mass spectrometer coupled to an UPLC chromatographic system (UPLC/Q-TOF). The instrument was run in the positive scan mode and operated to acquire spectra in the range of 100–1600 m/z in extended dynamic range mode. A 1290 liquid chromatograph system, equipped with a binary pump and a Dual Electrospray Jet Stream ionization system, then coupled to a G6550 mass spectrometer detector (all from Agilent technologies Santa Clara, CA, USA) was used. Reverse phase chromatographic separation was performed using an Agilent Zorbax Eclipse-plus column (75 x 2.1 mm i.d., 1.8 μm). The LC mobile phase A consisted of water while mobile phase B was methanol. Formic acid 0.1% (v/v) and ammonium formate (5 mM) were added to both phases. The gradient started with 5% B and increased to 90% B within 30 min, then held for 5 minutes. The mobile phase temperature was set to 35 °C, the injection volume was 3μl and the flow rate was 220 μl/min. Samples were grinded in liquid nitrogen, then an aliquot (0.5 g) was extracted in 80% methanol with 0.05% HCOOH using an Ultra Turrax (Ika T-25, Staufen, Germany), diluted in 40% methanol and transferred to a vial for analysis by UPLC/Q-TOF.

Raw data gained from the time-of-flight analyzer were processed by the Mass Hunter Qualitative Analysis B.05 software (from Agilent Technologies) using the naïve "find-by-molecular-feature" algorithm. Unidentified molecular features were subjected to a recursive analysis workflow using Mass Profiler Professional B.12.05 (from Agilent Technologies) for features alignment and filtering after the initial deconvolution. Features that were not present in 80 % of replications within a treatment were discarded. Compound identification was based on

accurate mass and isotope pattern (accurate spacing and isotopes ratio) and expressed as overall identification score, using the database exported from PlantCyc9.5 (Plant Metabolic Network, http://www.plantcyc.org; released November 2014) as reference.

The peak volume of those compounds identified with a mass accuracy below 5 ppm and above 85/100 as overall identification score, were extracted from the total ions current and exported for statistics and data interpretation.

2.12. RNA extraction and RT-PCR analysis

Total RNA was extracted using RNAeasy® Plant Mini Kit (Qiagen, Italy) according to manufacturer's instructions. The RNA concentration was determined both spectrophotometrically and by densitometric analysis of rRNA fragment following agarose gel electrophoresis. QuantiTect® Reverse Transcription Kit (Qiagen) was used to remove genomic DNA contamination and to synthesize cDNA. Elimination of genomic DNA from cDNA preparation was verified by PCR with primers aligned in different exons for gene EF α 1 (elongation factor α1) as described by Expósito-Rodríguez et al. (2008). The quantitative real-time PCR experiments were performed using the iCycler (Bio-Rad, Italy) and using master mix iQTMSYBER Green Supermix (Bio-Rad, Italy), containing the SYBR Green I DNA binding dye. Each reaction was made in triplicate. Primers were used in this study as described by Hartke et al. (2013) and following sequences (sense andantisense, respectively): *LeNRAMP3* F 5′-TGGTTAACTGGATTGTTGGCTGCTGGAC -3′ and *LeNRAMP3* R 5′-ATGAATTCAAAGAAGGGGGATCAGGGCA-3′ for *LeNRAMP3* (Natural Resistance-Associated Macrophage Protein 3); *LeFER* (Ferritin) F 5′-TGGTTAACTCCAACAAGCAAAGGCACGA-3′and *LeFER* R 5′-ATGAATTCAGAAGCTGCAATGTGTCGCC-3′for *LeFER*; UBI3 F 5′-TGCAGATCTTCGTGAAAACC -3′ and UBI3 R 5′-AGCGAGCTTAACCTTCTTCT -3′for UBI3 (Ubiquitin). Total reaction volume was 20 µl that included 10 µl (2X) mastermix, 100 ng of cDNA, 0.5 µl (10 µM) of each forward and reverse primers and volume was adjusted with water. The PCR reaction conditions were: one cycle at 50°C for 2 min,94°C for 15 min, then 40 cycles at 95°C for 15 sec, 60°Cfor 50 sec and 72°C for 50 sec. Primer specificity was confirmed by nucleotide sequencing (MWG, Germany)of amplicon. The Ct values of target genes (*LeNRAMP3* and *LeFER*) and reference gene (UBI3)were used for further relative expression

analysis by using the $2^{-\Delta\Delta CT}$method (Livak and Schmittgen, 2001). Calculation and statistical analyses were performed by Gene Expression Macro™ Version 1.1 (Bio-Rad, Italy).The qRT-PCR experiments performed for three biological replicas containing pooled samples of leaves and roots of three plants for each treatment.

2.13. Statistical analysis

All data were statistically analyzed by ANOVA using the SPSS software package (SPSS 10 for Windows, 2001). To separate treatment means within each measured parameter, Duncan's multiple range test was performed at P = 0.05.

Interpretation of metabolomic analysis was carried out using Mass Profiler Professional B.12.05 (from Agilent technologies). The identified compounds were log2 normalized, filtered (only those having a peak volume above 5000 counts and appearing in 80% of samples in at least one condition, were considered), normalized at 75th percentile and base lined versus the median of each compound in all samples. Statistics and interpretations were performed on this latter dataset: ANOVA analysis (P = 0.001, Bonferroni multiple testing correction) and fold-change analysis (cut-off = 5) were combined into volcano plots.

The "find-by-minimal-entities" naïve Bayesian approach in Mass Profiler Professional, aimed to identify those compounds (target size 30 compounds, forward selection algorithm) that better explain differences among treatments, was also carried out.

The third statistical approach was the multivariate Partial Least Square Discriminant Analysis, a covariance-based prediction model. The score plot for the first latent vector was generated from the model, and those entities having a score higher than 0.1 or lower than -0.1 were considered.

3. Results

3.1. Root mycorrhizal colonization

Neither hyphae nor vesicle were observed in roots of non-inoculated tomato plants. The rates of mycorrhizal colonization in all inoculated treatments were generally high ranging from 64% to 82% (data not shown). Irrespective of grafting combination (Cd × mycorrhizal

interaction, Table 1), the percentage of root colonization was higher at 0 µM (76%) than at 25 µM Cd (68%). Moreover, when averaged over Cd concentration (mycorrhizal × grafting interaction, (Table 1), tomato plants grafted onto Maxifort rootstock had more root colonization (77%) than self-grafted inoculated ones (67%).

Table 1. Mean effects of solution Cd concentration, arbuscular mycorrhizal (AM) inoculation, and grafting combination on root mycorrhizal colonization, fruit yield, leaf area, dry weight biomass and root to shoot ratio of tomato plants.

Treatment	Root AM colonization (%)	Fruit yield (kg plant^{-1})	Dry biomass (g plant^{-1})		Root to Shoot ratio	Leaf area (m^2 plant^{-1})
			Shoot	Root		
Cd level (µM)						
0	38.2	2.82	214.9	25.4	0.122	2.16
25	34.3	2.48	163.6	20.5	0.122	1.83
AM fungi						
-AM	0.0	2.70	192.8	22.0	0.120	2.01
+ AM	72.5	2.59	185.8	24.1	0.123	1.98
Grafting combination[a]						
I/I	33.7	2.49	181.8	21.4	0.116	1.90
I/M	38.8	2.81	196.7	24.7	0.128	2.09
Significance[b]						
Cadmium (Cd)	*	***	***	***	NS	***
AM fungi (M)	***	NS	NS	*	NS	NS
Grafting (G)	**	***	**	**	*	***
Cd × M	*	*	**	NS	NS	NS
Cd × G	NS	NS	NS	NS	NS	NS
Cd × M	**	NS	NS	NS	NS	NS
Cd × M × G	NS	NS	NS	NS	NS	NS

[a]I/I = self-grafted Ikram cultivar; I/M = Ikram cultivar grafted onto Maxifort rootstock.

[b]NS, *, **, ***Nonsignificant or significant at $P< 0.05$, 0.01 or 0.001, respectively.

3.2. Plant biomass accumulation, leaf area, leaf pigments and chlorophyll fluorescence

Irrespective of grafting combination (Cd × mycorrhizal interaction, Table 1), the marketable yield and shoot biomass remained unchanged in both +AM and –AM plants at 0 µM Cd (avg. 2.8 kg plant^{-1} and 214.9 g plant^{-1}, respectively). At 25 µM Cd the yield and shoot dry weight were significantly higher by 11% and 15%, respectively in –AM (avg. 2.6 kg plant^{-1} and

174.8 g plant^{-1}, respectively) in comparison to those recorded with +AM plants (avg. 2.3 kg plant^{-1} and 152.6 g plant^{-1}, respectively). Long-term Cd treatment caused significant decrease in the root dry weight, and leaf area (Table 1). The yield, shoot and root biomass, root-to-shoot ratio, and the leaf area were 13%, 8%, 16%, 10%,and 10%higher with tomato grafted onto Maxifort than self-grafted plants (Table 1).

Table 2. Mean effects of solution Cd concentration, arbuscular mycorrhizal (AM) inoculation, and grafting combination on pigment content and maximum quantum use efficiency of PSII (F_v/F_m) in tomato leaves.

Treatment	Pigments (µg g^{-1}fw)			Fv/Fm
	Chlorophyll a	Chlorophyll b	Carotenoids	
Cd level (µM)				
0	490.3	208.2	183.7	0.833
25	383.9	166.5	149.4	0.810
AM fungi				
- AM	422.7	180.5	164.3	0.821
+ AM	451.5	194.1	168.8	0.819
Grafting combination[a]				
I/I	420.6	184.0	156.4	0.815
I/M	453.6	190.7	176.7	0.824
Significance[b]				
Cadmium (Cd)	***	***	***	***
AM fungi (M)	NS	NS	NS	NS
Grafting (G)	*	NS	**	*
Cd × M	NS	NS	NS	NS
Cd × G	NS	NS	NS	NS
Cd × M	NS	NS	NS	NS
Cd × M × G	NS	NS	NS	NS

[a]I/I = self-grafted Ikram cultivar; I/M = Ikram cultivar grafted onto Maxifort rootstock.

[b]NS, *, **, ***Nonsignificant or significant at $P< 0.05$, 0.01 or 0.001, respectively.

Chlorophyll *a*, chlorophyll *b*, carotenoid contents, and maximum quantum use efficiency of PSII measured as the F_v/F_m ratio showed significant reduction (by 22%, 20%, 19%, and 3% respectively) in cadmium-exposed plants. Moreover, the chlorophyll a, carotenoid contents, and

F_v/F_m ratio increased by 8%, 13% and 2% respectively, when tomato plants were grafted onto Maxifort (Table 2).

3.3. Oxidative damage

As shown in Figure 1, the leaves of tomato cv. Ikram grafted onto Maxifort displayed lower Cd-induced hydrogen peroxide (H_2O_2) accumulation (-17%) than those of self-grafted plants, whereas no significant difference of H_2O_2 production was recorded between the two grafting combinations under control conditions (0 µM Cd). The Cd treatment produced a consistent increase in the level of lipid peroxidation in leaves determined by measuring the amount of MDA and also an increase in electrolyte leakage (EL). The MDA content and EL recorded on plants receiving 25 µM Cd was significantly higher by 28% and 9% respectively in comparison to tomato plants treated with 0 µM Cd (Table 3). The MDA content and EL were also affected by grafting combinations with the maximum values recorded in self-grafted than in Ikram/Maxifort combination (Table 3).

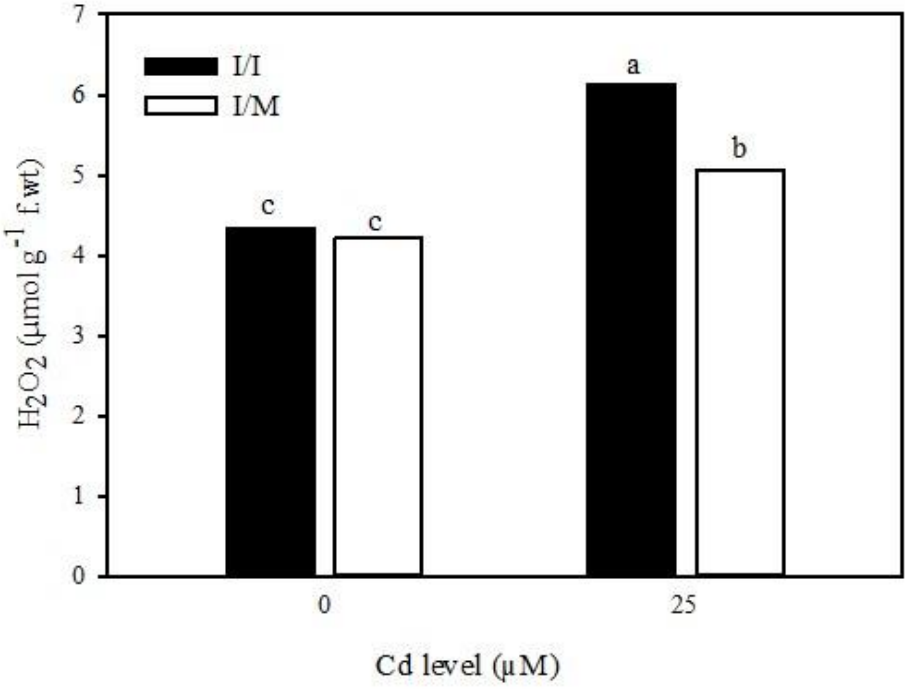

Fig. 1. Effects of grafting combination (I/I = self-grafted Ikram cultivar; I/M = Ikram cultivar grafted onto Maxifort rootstock) and solution Cd concentration on H_2O_2 content in leaves of tomatoes.

Table 3. Mean effects of solution Cd concentration, arbuscular mycorrhizal (AM) inoculation, and grafting combination on hydrogen peroxide (H_2O_2), malondialdehyde, and electrolyte leakage in tomato leaves.

Treatment	H_2O_2 (µmol g^{-1}f.w.)	Malondialdehyde (nmolg^{-1}f.w.)	Electrolyte leakage (%)
Cd level (µM)			
0	4.28	4.65	68.18
25	5.59	5.95	74.10
AM fungi			
- AM	4.84	5.22	70.71
+ AM	5.03	5.39	71.57
Grafting combination[a]			
I/I	5.23	5.40	73.23
I/M	4.64	5.21	69.05
Significance[b]			
Cadmium (Cd)	***	***	**
AM fungi (M)	NS	NS	NS
Grafting (G)	*	*	*
Cd × M	NS	NS	NS
Cd × G	*	NS	NS
Cd × M	NS	NS	NS
Cd × M × G	NS	NS	NS

[a]I/I = self-grafted Ikram cultivar; I/M = Ikram cultivar grafted onto Maxifort rootstock.

[b]NS, *, **, ***Nonsignificant or significant at $P< 0.05$, 0.01 or 0.001, respectively.

3.4. Effects on antioxidative enzymes

Stress response of grafted and self-grafted plants with and without AM exposed to moderate Cd-stress was assessed by analyzing changes in capacity of several antioxidative enzymes on primary leaves (Table 4). Irrespective of grafting combination (Cd × mycorrhizal interaction), CAT activity in leaves remained unchanged in both +AM and –AM plants at 25 µM Cd (622.1mmol mg^{-1}protein min^{-1}), whereas the CAT activity was higher by 44% in –AM (570.1mmol mg^{-1}protein min^{-1}) in comparison to the one recorded with +AM plants (396.2 mmol mg^{-1}protein min^{-1}). APX and GPX activities in the leaves of moderate Cd-exposed tomato plants

were higher by 9% and lower by 15%, respectively than in the control (Table 4). Among the grafting combinations, the lowest APX and GPX activities were recorded in the Ikram/Ikram combination. The proline content in leaves of tomato cv. Ikram grafted onto Maxifort was higher by 7% than the one recorded on self-grafted plants under Cd-stress conditions, whereas no difference in proline was observed between the two grafting combinations under control treatment (Fig. 2).

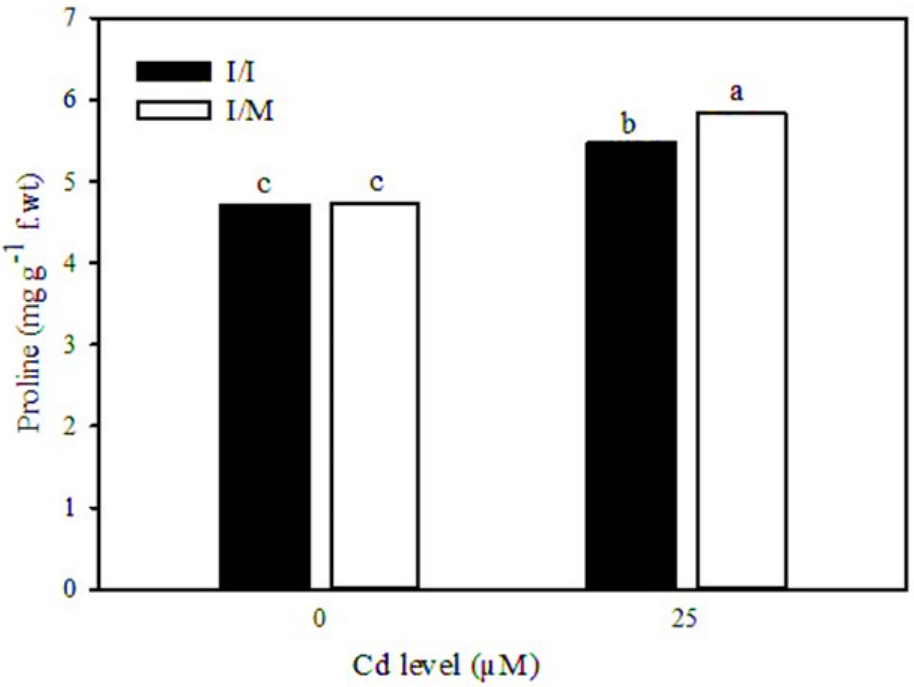

Fig. 2. Effects of grafting combinations (I/I = self-grafted Ikram cultivar; I/M = Ikram cultivar grafted onto Maxifort rootstock) and solution Cd concentration on proline content in leaves of tomatoes.

3.5. Metabolic profiling of leaves

The combination of a comprehensive database like PlantCyc together with proper data handling (mass and retention time alignment, filtering by frequency and following recursive analysis) was effective in removing false positives while ensuring a thorough dataset. Indeed, starting from a total of 2242 compounds detected, recursive analysis and the following filters in Mass Profiler Professional dramatically reduced the number of compounds in the dataset and provided better interpretations.

Table 4. Mean effects of solution Cd concentration, arbuscular mycorrhizal (AM) inoculation and grafting combination on antioxidant enzymes catalase (CAT), ascorbate peroxidase (APX), and guaiacol peroxidase (GPX) and proline contents in tomato leaves.

Treatment	Antioxidant enzymes (mmol min^{-1} mg^{-1} protein)			Proline ($mg\ g^{-1}fw$)
	CAT	APX	GPX	
Cd level (µM)				
0	483.0	11.6	2.61	4.72
25	621.9	12.7	2.17	5.65
AM fungi				
- AM	601.6	12.6	2.47	5.20
+ AM	503.4	11.7	2.31	5.17
Grafting combination[a]				
I/I	51.69	11.6	2.24	5.09
I/M	58.80	12.8	2.55	5.28
Significance[b]				
Cadmium (Cd)	***	*	***	***
AM fungi (M)	***	*	NS	NS
Grafting (G)	**	**	*	NS
Cd × M	**	NS	NS	NS
Cd × G	NS	NS	NS	*
Cd × M	NS	NS	*	NS
Cd × M × G	NS	NS	NS	NS

[a]I/I = self-grafted Ikram cultivar; I/M = Ikram cultivar grafted onto Maxifort rootstock.

[b]NS, *, **, ***Nonsignificant or significant at $P< 0.05$, 0.01 or 0.001, respectively.

The unsupervised cluster analysis (Fig.3) performed on the dataset gave good clustering, considering that the treatments were properly grouped according to the stress applied (Cd-stressed versus control) and then to grafting combination and mycorrhizal inoculation. The effect of the latter was secondary to grafting. Furthermore, the output of PLS-DA multivariate analysis carried out on grafting combinations (samples position in the model hyperspace, together with score plot for first and second later vectors) is given in Figure 4. The class prediction models gave good accuracies for both grafting and mycorrhization (overall accuracy was 96.9% and 100% respectively). The compounds selected from Volcano analysis (using a fold-change cut off

= 5 and a p-value of 0.001), PLS-DA scores and naïve Bayesian analysis were summarized in Tables 5 and 6, grouped in classes according to their biological role.

The main classes of compounds identified were ascribed to hormones (indole acetic acid derivatives, the phytoalexinsmedicarpin and sativan, some inactive forms of gibberellins and a brassinosteroid, glucosinolates, indole and benzylisoquinoline alkaloids, hydroxycinnamic acids amides, and several products of lipoperoxidation (oxo- and hydroxyl-derivatives of lipids). Phenolic compounds (flavonoids and lignans), together with terpenes, were also altered in response to the treatments. Some additional compounds, not directly ascribable to the previous classes, were also pointed out. These latter included other metabolites, such as glutamate derivatives (*N*-acetyl-glutamate, *N*,*N*-dihydroxyglutamate, α-ethyl-glutamate), phytochelatin PC2, fructaninulins, a melatonin intermediate (N^1-acetyl-N^2-formyl-5-methoxykynuramine) and quinones/quinols (dopaxanthinquinone, ubiquinone-8 and menaquinol-7).

Interestingly, the outcome of the multivariate Partial Least Square Discriminant Analysis provided a ranked list of metabolite features in good agreement with the previous Volcano analyses. Hence, a few additional compounds still belonging to the functional classes reported above were added by this approach. Finally, a few compounds were pointed out through the naïve Bayesian analysis, mainly regarding the contribution of grafting, namely the methyl-THF (hence a methyl-donor group) and the two forms of ascorbate.

3.6. Cd accumulation in tomato tissues

In the absence of added Cd in the nutrient solution, very low concentrations of Cd accumulated in leaves (avg. 0.35 mg kg^{-1}, Table 7), roots (avg. 0.38 mg kg^{-1}, data not shown), and fruits (0.08 mg kg^{-1}, data not shown). Addition of Cd in the nutrient solution greatly increased the Cd concentration in roots (475.1 mg kg^{-1}, data not shown), leaves (119.1 mg kg^{-1}, Table 7), and fruits (5.1 mg kg^{-1}, data not shown), confirming that roots are the primary site of Cd accumulation. In the aerial tissues, the Cd concentration changed with AM fungi inoculation. At 25 µM Cd, the Cd concentration was higher by 14% in leaves of +AM than in leaves of -AM plants (Fig. 5).

Table 5. Variation of Differential metabolites in tomato leaves under Cd stress (25 µM) and absence of mycorrhization, in response to grafting combination (Ikram/Maxifort *vs* Ikram/Ikram). Data are gained from Volcano analysis (alpha 0.01, fold-change = 5) and PLS-DA multivariate analysis.

Metabolites	Variation
Phenolics	
(+)-sesaminol 2-*O*-β-D-gentiobioside	+
quercetin-3,3'-bissulfate	+
Ponciretin	-
Alkaloids	
Harmalol	+
(*S*)-norcoclaurine	-
Glucosinolates	
indole-3-acetyl-aspartate-N-β-D-glucose	+
8-methylthiooctyldesulfoglucosinolate	-
2-(6'-methylthio) hexylmalate	-
Lipoperoxidation productsand membrane lipids	
6-cis-3-oxo-tridecenoyl-CoA	-
22-hydroxydocosanoate	-
1-18:3-2-16:2-monogalactosyldiacylglycerol	+
Heptanal	-
Hormones	
indole-3-acetyl-phenylalanine	-
Hydroxycinnamic acids amides	
dihydroxyferuloyl-sinapoylspermidine	-
Feruloylserotonin	-
Fructaninulins	
1,1-kestotetraose	+
Kestotriose	+
Others	
Anthranilate	+
Dopaxanthinquinone	-
ubiquinone-8	-
D-galactosylononitol	+
N^1,N^2-formyl-5-methoxykynuramine	+
(1*E*,6*E*)-1-(4-hydroxyphenyl)-7-phenylhepta-1,6-diene-3,5-dione	+
L-asparagine	+
precorrin-2	-
PC2 (γ-Glu-Cys-γ-Glu-Cys-β-Ala)	+

Table 6. Differential metabolites in tomato leaves of self-grafted plants (Ikram/Ikram) under Cd stress (25 uM) in response to arbuscular mycorrhizal (AM) root inoculation (+AM *vs* -AM). Data are gained from Volcano analysis (alpha 0.01, fold-change = 5) and PLS-DA multivariate analysis.

Metabolites	Variation
Phenolics	
apigenin 7-*O*-β-*D*-glucoside	-
7-*O*-β-*D*-glucosyl-7-hydroxyflavone	-
quercetin-3,3'-bissulfate	+
Alkaloids	
Harmaline	-
Harmalol	+
Glucosinolates	
4-methoxy-3-indolylmethylisothiocyanate	+
indole-3-acetyl-aspartate-*N*-β-*D*-glucose	+
Tetrahomomethionine	+
6-hydroxy-indole-3-acetyl-phenylalanine	-
Lipoperoxidation products and membrane lipids	
1-18:3-2-16:2-monogalactosyldiacylglycerol	+
1-dodecanol	-
1-18:2-2-16:0-monogalactosyldiacylglycerol	-
Hormones	
(-)-medicarpin	-
(-)-sativan	-
indole-3-acetyl-phenylalanine	-
Hydroxycinnamic acids amides	
dihydroxyferuloyl-sinapoylspermidine	-
Feruloylserotonin	+
Phytochelatins related compounds	
acryloyl-CoA	-
γ-glutamyl-ethylamide	-
Others	
Anthranilate	+
N-acetyl-L-glutamate	+
α-ethyl-*L*-glutamate	-
Dopaxanthinquinone	-
ubiquinone-8	-
N-δ-(phosphonoacetyl)-L-ornithine	-
D-galactosylononitol	+
precorrin-2	-
1,7,8-trihydroxy-6-methoxy-2-methylanthraquin	+

one	
1,7-dihydroxy-5,6,8-trimethoxyanthraquinone	-
N,*N*-dihydroxy-L-tryptophan	+
4-methylumbelliferone 6'-*O*-malonylglucoside	-
4-coumaroylhexanoylmethane	-

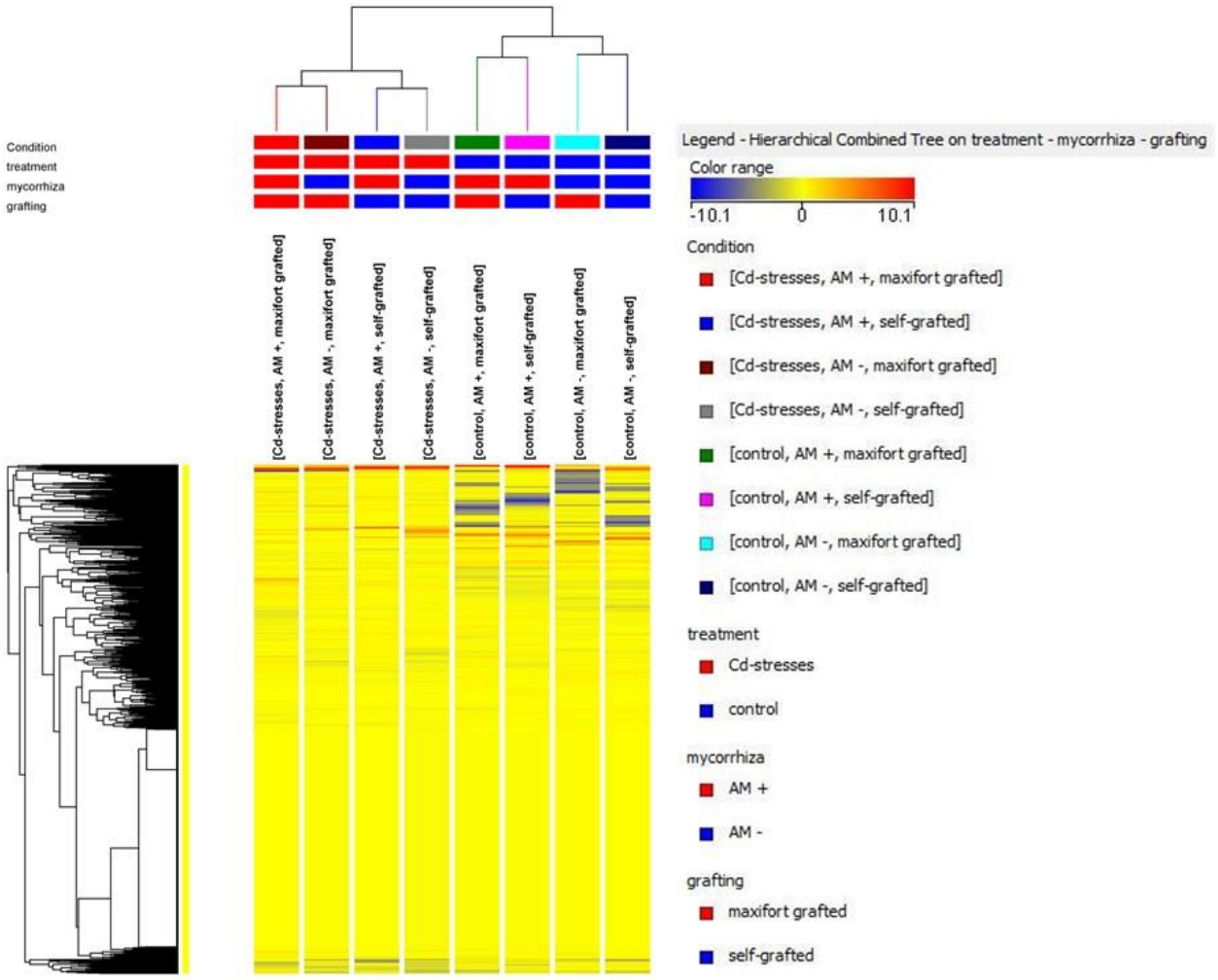

Fig. 3. Unsupervised hierarchical cluster analysis of metabolite contents in all treatments.

3.7. Mineral concentrations in leaf tissue

Increasing the Cd concentration in the nutrient solution from 0 to 25 µM, decreased the leaf mineral composition in particular K, Ca, Mg, Fe, Mn, and Zn by 7%, 13%, 13%, 49%, 12%, and 30% respectively (Table 7). The effect of AM inoculation on mineral concentration in leaf tissues was less pronounced. Except for Fe concentration, the better nutritional status (higher P,

Mg and Zn) was recorded with +AM than in –AM plants (Table 5). Macro and micronutrient concentrations were also affected by grafting combination, with the highest values of P, K, Ca, Fe, Mn, and Zn observed in shoot tissues of Ikram grafted onto Maxifort rootstock (Table 7).

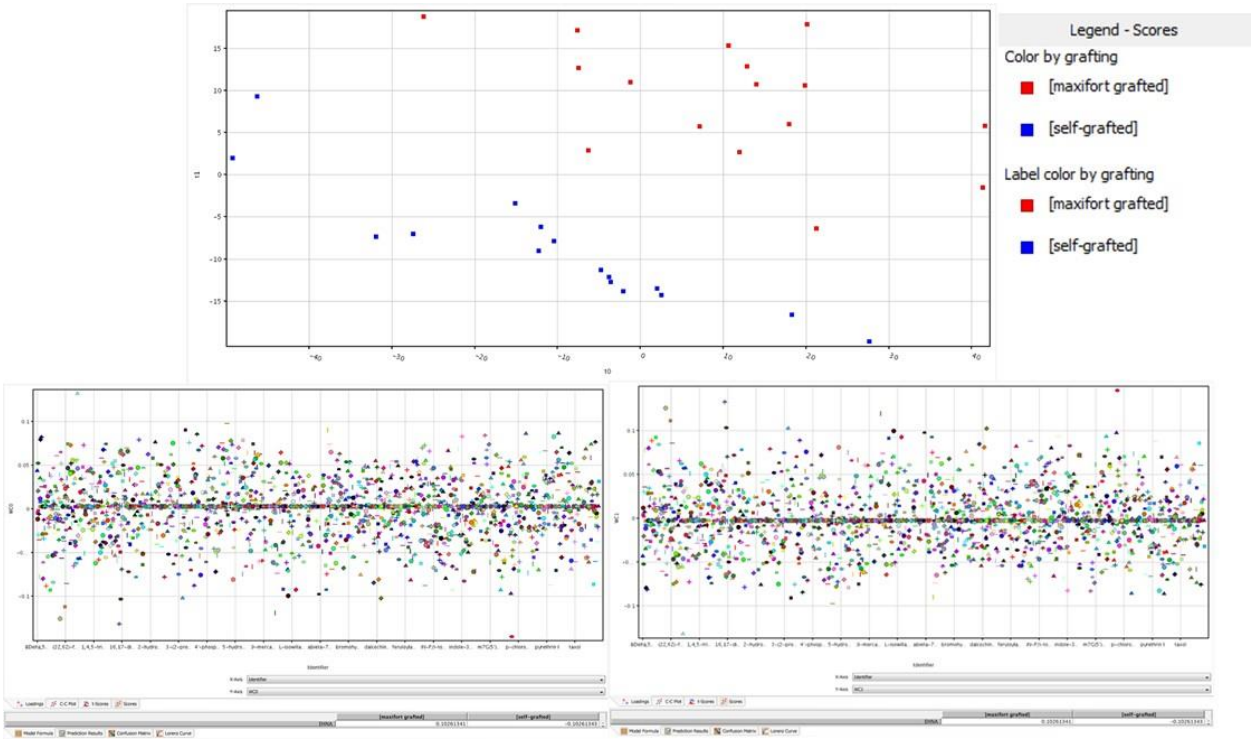

Fig. 4. Partial Least Squares Discriminant Analysis (PLS-DA) identification of metabolites being significantly altered by grafting. Upper pane is the hyperspace plot from the PLS-DA model, while lower panes are the class prediction loadings on first (left) and second (right) hyperspace components.

The concentration of certain nutrients (Fe, Zn, and Mg) in the root tissue was significantly increased with 25 µM Cd (avg. 375.6 mg kg^{-1}, 96.4 mg kg^{-1}, and 2.13 g kg^{-1}, respectively) compared to control treatment (avg. 255.4 mg kg^{-1}, 77.9 mg kg^{-1}, and 1.76 g kg^{-1}, respectively), whereas Mn decreased with Cd treatment (from 110.8 to 98.0 mg kg^{-1}). Finally, Fe, Mn and Cu concentrations were significantly higher in Ikram/Maxifort (avg. 330.7, 108.7, 80.1 mg kg^{-1}, respectively) compared to Ikram/Ikram combination (avg. 300.2, 100.1, 60.3 mg kg^{-1}, respectively).

Table 7. Mean effects of solution Cd concentration, arbuscular mycorrhizal (AM) inoculation, and grafting combination on major and trace elements in tomato leaves.

Treatment	Major elements (g kg^{-1} of dw)					Trace elements (mg kg^{-1}dw)			
	N	P	K	Ca	Mg	Fe	Mn	Zn	Cd
Cd level (µM)									
0	26.4	3.38	24.1	37.2	7.54	74.5	300.3	33.9	0.35
25	25.3	3.28	23.9	32.2	6.55	38.0	265.1	23.7	119.14
AM fungi									
- AM	25.7	3.20	23.2	33.7	6.82	60.3	280.6	27.1	55.73
+ AM	26.1	3.46	24.9	35.7	7.27	52.1	284.8	30.5	63.75
Grafting combination[a]									
I/I	26.1	3.20	22.0	31.9	7.01	53.6	251.6	26.7	58.06
I/M	25.6	3.46	26.1	37.4	7.08	58.8	313.8	30.9	61.42
Significance[b]									
Cadmium (Cd)	NS	NS	**	**	***	***	***	***	***
AM fungi (M)	NS	*	NS	NS	*	**	NS	**	***
Grafting (G)	NS	*	***	***	NS	*	***	**	NS
Cd × M	NS	NS	NS	*	*	**	NS	NS	***
Cd × G	NS	NS	NS	NS	NS	NS	NS	*	NS
Cd × M	**	NS	NS	NS	NS	NS	NS	NS	NS
Cd × M × G	***	*	NS	NS	NS	*	NS	*	NS

[a]I/I = self-grafted Ikram cultivar; I/M = Ikram cultivar grafted onto Maxifort rootstock.

[b]NS, *, **, ***Nonsignificant or significant at $P< 0.05$, 0.01 or 0.001, respectively.

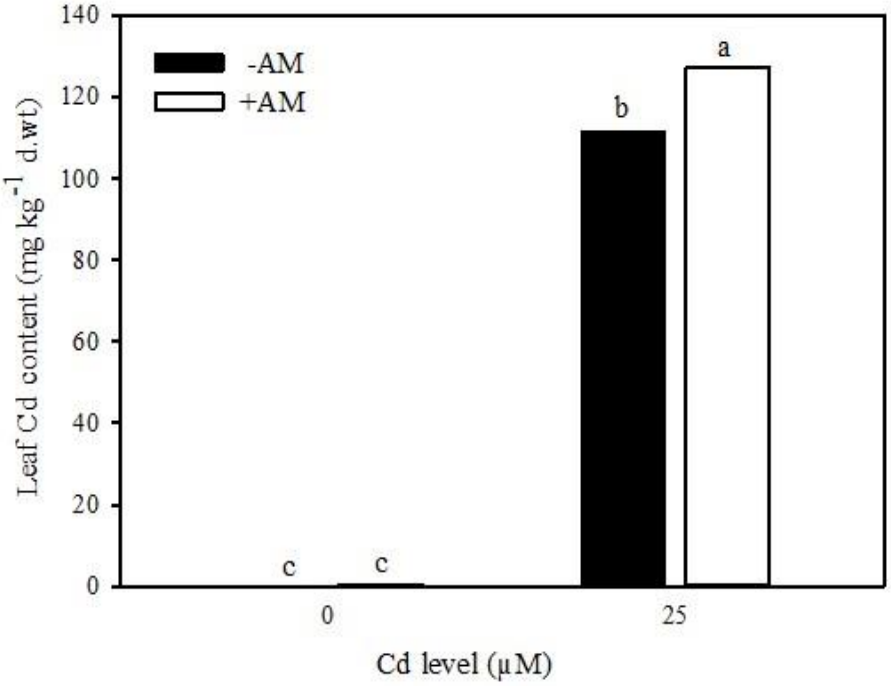

Fig. 5. Effects of arbuscular mycorrhizal (AM) inoculation and solution Cd concentration on Cd content in leaves of tomatoes.

3.8. Gene expression

The relative gene expression analysis of two important metal transporters *LeFER* (root specific transporter) and *LeNRAMP3* genes (a wide range metal transporter)was performed. Under Cd-stress, the *LeFER* and *LeNRAMP3* genes were up-regulated in non-inoculated roots (about two-fold) of both Ikram/Ikram, and Ikram/Maxifort combinations. This seems to be related to root Cd concentrations, which was similarly increased in both grafting combinations (Table 8). When *LeNRAMP3*was analyzed in leaves, it showed up-regulation only in Ikram/Maxifort combination, with and without AM inoculation (two and three-fold, respectively),whereas a down-regulation trend was observed in self-grafted plants (Ikram/Ikram)with and without AM inoculation (Table 8). Moreover, at 25 µM Cd, the *LeFER* and *LeNRAMP3* genes showed down-regulation in mycorrized roots of both grafting combinations (Table 8).

Table 8. Relative gene expression of *LeFER* and *LeNRAMP3* gene in self grafted (I/I) and Maxifort rootstock grafted (I/M) plants with (+AM) or without (-AM) mycorrhizal inoculation. qRT-PCR analysis was performed in root and leaf tissues following the Cd treatment and mycorrhization in self grafted and Maxifort rootstock grafted plants. Quantification of gene expression was performed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen). Values are means of three technical replicates and two biological replicates.

Grafting combination	Relative gene expression					
	LeFER		*LeNRAMP3*			
	Root		Root		Leaf	
	-AM	+AM	-AM	+AM	-AM	+AM
I/M	1.77 ± 0.2	0.61 ± 0.35	2.05 ±0.69	0.72 ± 0.19	2.70[a] ± 0.2	2.01[a] ± 0.32
I/I	1.78 ± 0.2	0.34 ± 0.17	1.84 ± 0.43	0.63 ± 0.31	0.46 ± 0.2	0.59 ± 0.30

[a]Means that significantly differed from the calibrator by *t* student test ($p< 0.05$).

4. Discussion

4.1. Plant biomass accumulation and root colonization

Plants respond to elevated Cd concentrations in water or soil by decreased yield and crop growth parameters due to Cd-induced inhibition of physiological and metabolomic processes and/or impairment of root activity, nutrient uptake, and accumulation (Lopez-Millan et al., 2009; Djebali et al., 2010; Sharma et al., 2010). However, the response of crops to Cd-stress conditions during the growing cycle may vary in relation to several interacting variables such as species, the phenological stage, the cultural environment and the magnitude of stress (i.e. time of exposure and Cd concentration; (Lux et al., 2010 and references cited therein). In the current experiment, significant decrease in root colonization and agronomical responses (Table 1) in Cd-treated tomato plants was observed; and that effect varied in relation to AM fungi inoculation and grafting combination. The expected positive effect of mycorrhizal fungus colonization on plant growth and productivity was not evident under moderate Cd-stress conditions. At 25 µM Cd, the reduction of yield, shoot and leaf area production was more pronounced with AM inoculation. This significant reduction in plant performance was attributed to the higher accumulation of Cd in shoot tissues (63.7 vs. 55.7 mg kg^{-1}, for +AM and –AM, respectively), which is likely the result of active substrate-Cd transport to the roots via extraradical hyphal network (Burleigh et al., 2003; González-Guerrero et al., 2005). These results are consistent with the findings of Prasad et al. (2011), who reported that AM fungal inoculation decreased the shoot and root yield of sweet basil at low dose of Cd (25 mg kg^{-1}), whereas at elevated levels (50 mg kg^{-1}) of Cd in soil, AM fungal inoculation showed an opposite behavior. In a recent meta-analysis study on the dynamic roles of AM symbiosis in heavy metal phytoremediation, Audet and Charest (2007) demonstrated a transition role of the AM shifting from ‘enhanced uptake’ at low soil heavy metal levels, to ‘metal binding’ at high soil heavy metal levels. Contrarily to AM symbiosis, the negative effect of moderate Cd on yield, shoot and root development was clearly attenuated, when tomato cv. ‘Ikram’ was grafted onto the vigorous rootstock Maxifort. Cd tolerance of tomato plants grafted onto Maxifort may be due to the better uptake and translocation of macro and microelements in particular P, K, Ca, Fe, Mn, and Zn to the leaves. The improved nutritional status of vegetables grafted onto vigorous rootstocks under adverse chemical soil conditions may

be associated to the higher root-to-shoot ratio and also to the greater surface area necessary for absorption of water and nutrients (Colla et al., 2010; Savvas et al., 2010).

4.2. Leaf pigments and chlorophyll fluorescence

Cd stress can cause damage at physiological level by interfering with several aspects of plant biochemistry including pigment synthesis and photosynthesis (Djebali et al., 2005; Hédiji et al., 2010). In the current experiment, Cd-induced degradation of leaf pigments in particular chlorophyll and carotenoids, as well as inhibition of their biosynthesis (Sandalio et al., 2001; Boswell et al., 2002), was probably a consequence of Cd induced nutrient deficiency (i.e. Fe, Mg; Vazquez et al., 1987; John et al., 2009). Excess Cd concentrations also create disturbance in electron transport rates of the photosystem II, therefore generating reactive oxygen species (Djebali et al., 2005; Herbette et al., 2006). Furthermore, the reduction in chlorophyll is a direct consequence of reduced carotenoids content, the latter is known to protect chlorophyll and other macromolecules by quenching excited triplet state of chlorophyll to avoid generation of free radicals (Bartley and Scolnik, 1995; Hédiji et al., 2010). The negative impact of Cd on leaf pigments and F_v/F_m ratio was clearly mitigated when Ikram/Maxifort combination was used (Table 2). In self-grafted plants, the higher plant biomass accumulation and yield in Ikram/Maxifort seem to be related to the capacity of maintaining higher photochemical activity in response to Cd-stress. Similar results were observed on grafted tomato and cucumber plants, which were able to delay photo-inhibition under salt stress in comparison to ungrafted plants (He et al., 2009; Colla et al., 2013).

4.3. Oxidative damage and defense mechanisms

Elevated Cd concentration in nutrient solution induced oxidative stress at cellular level as evidenced by enhanced hydrogen peroxide (H_2O_2) generation, ion leakage and lipid peroxidation (Table 3; Djebali et al., 2005; Sharma and Dubey, 2005; Smeets et al., 2005; Rodriguez-Serrano et al., 2006).The Ikram/Maxifort combination was more tolerant to Cd stress than the self-grafted plants; this was determined based on lowest values of ROS accumulation and malondialdehyde (MDA, a lipid peroxidation product). Our results also demonstrated that plants grafted onto Maxifort reduced the amount of ion leakage in Cd stressed tomato thus facilitating the maintenance of membrane functions (i.e., semipermeability). Calcium increases structural

stability of cell membrane because of electrostatic interactions with membrane phospholipids and proteins and of its role as fundamental component of the cell wall (Borer et al., 2005). Cd applications in the nutrient solution reduced root calcium uptake leading to a reduction of cell membrane stability of leaf tissues; however, Ikram/Maxifort combination was able to mitigate the detrimental effect of Cd on membrane stability by improving the Ca uptake in leaf tissues of tomato plants.

It is well established that under stress conditions, multiple antioxidant enzymes are expected to play a crucial role in the scavenging of ROS and thus protect cells from oxidative damage (Xu et al., 2012). Antioxidant enzymes, such as.CAT, APX and GPX, responded differently under various Cd conditions. The activities of CAT and APX, the major enzymes responsible for H_2O_2 degradation (Vanacker et al., 1998) increased at 25 µM Cd whereas an opposite trend was observed for GPX (Table 4). The activities of CAT and APX were significantly higher with –AM in comparison to +AM plants. The Ikram/Maxifort combination induced higher antioxidant enzymes, which suggested that the use of Maxifort rootstock in tomato has a high ROS scavenging activity. This result may explain the lower H_2O_2 concentrations that were observed in the presence of Cd (Fig. 1). Similar results were observed in grafted plants under thermal and salt stress (He et al., 2009; Colla et al., 2013) where the antioxidant enzymes activity was higher in rootstock-grafted plants than in self-grafted plants.

Another system of protection against Cd toxicity includes the synthesis of osmolytes, such as proline, which contribute to stabilization of protein molecules and membranes (Matysik Alia et al., 2002; Yilmaz and Parlak, 2011). In our study, the higher accumulation of proline in Ikram/Maxifort combination supports the observed higher Cd tolerance in grafted than self-grafted plants (Table 4).

4.4. Metabolic changes in leaves

The results gained from the statistics, carried out on the metabolomic dataset, were consistent with previous assessments and pointed out secondary metabolites that are known to be related to abiotic stress. Glucosinolate biosynthesis was stimulated by mycorrhiza, being up-accumulated in this treatment, while phenolics were up-accumulated in Maxifort-grafted plants. As expected, the whole phytohormone network was imbalanced by both root colonization and

grafting in a complex way probably related to hormones cross-talking. Although a clear role of hormone changes could not be identified, the involvement of our differential hormones is consistent with previous literature (Choudhary et al, 2012; Khan et al, 2012).

As an overall consideration, several metabolites can be linked to oxidative stress. In particular, products from lipid peroxidation are reported to be down-accumulated in Maxifort-grafted plants confirming the role of grafting in supporting the quenching of the oxidative stress generated by cadmium exposure. Consistently, diacylglycerols and diacylglycerolglucosydes were up-accumulated in this treatment indicating a possible preservation of membrane lipids. The reduction in flavonoids in Maxifort grafted plants, indicate a less stringent need to cope with ROS and oxidative stress, therefore enforcing the previous assumption. Some other compounds can be related to the improved ability of Maxifort-grafted plants for coping with Cd stress, like the case of phytochelatins PC2 (γ-Glu-Cys-γ-Glu-Cys-β-Ala) or the fructaninulinskestotriose and1,1-kestotetraose.

Other abiotic stress related compounds included some alkaloids, and mainly indole and benzylisoquinoline alkaloidsand the melatonin derivative N^1-acetyl-N^2-formyl-5-methoxykynuramine. All these latter compounds were up-accumulated in Maxifort-grafted plants,

The increased accumulation of melatoninisis also supported by literature. The most frequently mentioned functions of melatonin are related to abiotic stresses including chemical stresses. Environmental stress can increase the level of endogenous melatonin in plants, and this compound was demonstrated to alleviate the stress (Zhang et al, 2015). Experiments in tomato, specifically indicated the role of melatonin in coping plant stress and oxidative stress in particular (Arnao and Ruiz, 2013). Monoterpeneindole alkaloids are also reported to be active against oxidative stress (Matsuura et al, 2014), and therefore, they probably acted in this direction in our experiments. The role of *N*-acetyl-L-glutamate in contrasting abiotic stress, though less obvious, has been previously reported in literature (Kalamaki et al, 2009), whereas the role of phytochelatins and inulin in cadmium stress tolerance is more obvious. Hydroxycinnamic acid amides were found to be related to both mycorrhiza and abiotic stress (Jin et al, 2003; Choudhary et al, 2012) thus supporting their presence among differential metabolites.

The naïve Bayesian analysis, mainly regarding the contribution of grafting, pointed out the involvement of methyl-THF (hence a methyl-donor group) and the two forms of ascorbate. Although these compounds were not selected by fold-change analysis, they resulted as differential through this complementary approach.

4.5. Cd and nutrient accumulation and interaction in plants

Cd may interfere with nutrient uptake by affecting the permeability of plasma membranes and thereby nutrient composition in plant tissues (Dong et al., 2006; Bertoli et al., 2012). A significant reduction of essential elements - in particular those with the same valence as Cd, such as, Ca, Mg, Fe, Mn, and Zn - was found at 25 µM Cd (Table 7). A decrease in the contents of such nutrients could be attributed to the competitive inhibition between these cations and Cd(Bertoli et al., 2012). At 25 µM, the Cd concentration in roots was five-fold higher than in the aerial part suggesting that Cd transport to the xylem was restricted in most plants (Lux et al., 2011 and references cited therein). The amount of Fe in roots increased with increasing contents of Cd, suggesting synergetic effect of Cd by stimulating Fe absorption (López-Millán et al., 2009; Bertoli et al., 2012), whereas an antagonistic effect was observed for Mn in line with the findings of Dong et al.(2006) and Wu et al.(2007). It was also noteworthy that fruit developing on 25 µM-treated plants accumulated low amounts of Cd (avg. 5.1 mg kg^{-1}). This is consistent with previous results showing limited accumulation in tomato fruits grown on contaminated soils (Gupta et al., 2008) and in hydroponic conditions at 20 µM Cd (Hédiji et al., 2010).

Data of this study also showed that Cd concentration in tomato leaves was higher in inoculated than non-inoculated plants grown under moderate Cd-stress (Table 7). This indicated that Cd was not retained in intra-radical AM fungi or compartmentalized in the root cell vacuoles leading to translocation of Cd in the aerial parts, with detrimental effect on plant performance. Our results are in agreement with the meta-analysis of Audet and Charest (2007)who demonstrated that heavy metal uptake is enhanced at low to intermediate soil heavy metal concentrations.

Grafting experiments have suggested that root characteristics (i.e. length, density, surface area) of several rootstocks act as a barrier in restricting Cd (exclusion mechanism) to shoots in eggplant and cucumber (Arao et al., 2008; Mori et al., 2009; Yamaguchi et al., 2011; Savvas et

al., 2013). This was not the case in the current study since similar Cd concentration was recorded in both grafting combinations (Table 7). Nevertheless, plant tolerance to heavy metals can vary with specific metal and plant species in question and more than one mechanism can be involved in mitigating the toxic effect (Hall, 2002). For instance, it is reported that plant tolerance strategy can not completely rely on exclusion mechanism as in the case of Cd due to possible exclusion of certain essential cations sharing similar transmembrane for uptake (Hall, 2002). The higher crop performance of Ikram/Maxifort in comparison to Ikram/Ikram combination was due to the improved nutritional status (higher P, K, Ca, Fe, Mn, and Zn, Table 7). The higher root to shoot ratio and the higher selective root uptake of elements in Ikram/Maxifort combination than in self-grafted plantscan explain the better leaf nutritional status.

4.6. Gene expression

Finally, the plant response to Cd stress was also evaluated at molecular level by gene expression analysis (Table 8). Under Cd-stress, *LeFER*and *LeNRAMP3* genes were up-regulated in roots in both grafting combinations - as also observed by Hartke et al. (2013). However, the up-regulation of *LeNRAMP3* gene only occurred in leaf of Ikram/Maxifort combination that was related to the better nutritional status (higher Fe, Mn, and Zn;Table 7). Previous studies have revealed similar functions of some NRAMP genes e.g., *OsNRAMP3* for Mn(Yang et al. 2013) and *OsNRAMP5* for Fe, Mn and Zn (Ishimaru et al., 2012a,b and Sasaki et al., 2012) in rice, and *LeNRAMP3* for Fe in tomato (Bereczky et al. 2003). The down-regulation of both the genes observed in +AM roots could be attributed to dilutive effects or transcriptional and proteome changes (Ouziad et al., 2005; Aloui et al. 2011).

5. Conclusions

Growth and yield of tomato was restricted by the application of moderate levels of Cd which could occur in vegetable production systems. The deleterious effects of Cd on plant performance reflect changes in physiological, biochemical and metabolic processes. AM inoculation was not able to alleviate the detrimental effect of Cd on growth and productivity because Cd was not retained in intra-radical AM fungi or compartmentalized in the root cell vacuoles, leading to translocation of Cd in the aerial parts. Our study showed that grafting tomato, involving vigorous rootstock such as Maxifort, could effectively mitigate adverse effects

of Cd stress by improving plant nutritional status, photosynthetic pigments, photochemical activity of PSII, increase the capacity of antioxidant enzymes (CAT, APX), proline and metabolites still linked to oxidative stressor clearly related to Cd tolerance (i.e. phytochelatin, and fructansinulins). Hence, the contribution of Maxifort rootstock minimized the level of Cd-induced oxidative injury by decreasing the level of hydrogen peroxide, lipid peroxidation, and electrolyte leakage in tomato leaves, thus promoting the performance of tomato plants.

Chapter 5

Summary

In the advent of faster industrialization and intensive agriculture practices, soil and water pollution by heavy metals have become a greater concern among plant scientists as their consequences endanger plants productivity and human health via food chain. Among the heavy metals, cadmium (Cd) and nickel (Ni), are the two most hazardous metals. Their excess concentrations in root ambient adversely affect plant performance of vegetable crops, including tomato, are frequently reported to be affected by heavy metal pollution in soil or water, especially in urban and sub-urban areas of different parts of the world. Plants tolerance to heavy metals are ascribed to the ability of plants to restrict the entry of toxic metals ions into roots and/or their transfer to shoots, and to stimulate the antioxidative systems to detoxify oxidative injury, while maintaining the optimum nutrient availability in plants. However, most of the commercial cultivars of vegetables are lacking such behavior's. One way to improve the ability of commercial cultivars of fruit vegetables to alleviated heavy metals stress would be grafting them onto robust rootstocks. The experiments conducted herein aimed i) to evaluate the performance of different grafting combinations under Cd and Ni stress conditions, ii) to understand underlying mechanisms in plant tolerance of grafted plants, iii) to evaluate the performance of selected rootstock-grafted plants with integration of arbuscular mycorrhizal fungi (AMF), as another sustainable tool in further enhancement of plant tolerance, and to study the plant tolerance mechanisms at physiological, and metabolomic level. In the first step, the performance of grafting combinations was tested under long term exposure to three levels (0, 25 and 50 µM) each of Cd and Ni in the nutrient solution, in separate greenhouse experiment under hydroponic system. Fruit yield and plant biomass of all five tested grafting combinations under Cd stress and four grafting combinations under Ni stress invariably decreased in response to their levels in nutrient solution. The rootstocks were common in both Cd and Ni experiments such as Maxifort and Unifort (tomato) and Black Beauty (eggplant) and the tomato cv. Ikram was used as scion.

Under Cd stress, plants grafted onto Maxifort rootstocks performed better based on higher fruit yield and plant growth parameters. The higher fruit yield of Maxifort grafted plant was mainly due to higher mean fruit weight. Next to Maxifort, Unifort grafted plants performed better than non-grafted or self-grafted plants and especially, Black Beauty grafted plants, exhibited the lowest yield, shoot biomass and leaf area. However, Black

Beauty grafted plants were exceptionally better with regard to fruit quality (higher DM, TSS and surface color, less fruit toxicity symptoms, lower Cd content in fruit (i.e., 39 % than of non-grafted). Next to Black Beauty, fruits from Maxifort grafted plants were better with lesser (23%) fruit Cd content and toxicity symptoms. Moreover, the better plant performance of Maxifort followed by Unifort grafted plants was due to their better leaf nutritional status (Ca, Mg, Fe, Mn and Cu) and higher SPAD index, maximum quantum yield of PSII (Fv/Fm) and chlorophyll contents.

Under Ni stress, Maxifort followed by Unifort grafted plants performed better in terms of higher yield and plant biomass production, compared to self-grafted and especially Black Beauty grafted plants. The better performance was related to higher chlorophyll and carotenoids contents, higher SPAD index and Fv/Fv, associated with maintained better leaf nutritional status. Moreover, tomato rootstock grafted plants, especially onto Maxifort experienced lower level of oxidative damage due to better antioxidative defense activities. Again, Black Beauty grafted plants were poorly performed also under Ni stress with lowest yield and biomass production.

In the next step, the performance of outperformed Maxifort rootstock grafted plants with self-grafted plants, with and without AMF inoculation (*Rhizophagus irragularis* formerly, *Glomus intraradices*) were evaluated under moderate Cd stress (25µM Cd) and the mechanisms of plant tolerance to Cd at cellular and molecular levels were assessed. Results demonstrates that highest performance of Maxifort grafted plants were in due part to better ability of Maxifort grafted plants in maintaining physiological and metabolic activities. Besides, maintaining higher leaf chlorophyll and PSII efficiency, keeping lower level of oxidative stress (lower MDA, H_2O_2 and EL) with higher antioxidant enzymes (higher CAT, APX) and carotenoids and proline contents. In addition, stimulation of certain plant metabolites especially, phytochelatin and fructansinulins could contributed grafted plants tolerance ability. Moreover, less interference of Cd, in particular of root-to-shoot translocation of nutrient elements, thus higher maintained mineral status in plants. Concerning the gene expression analysis, we demonstrate that over expression of *LeNRAMP3* gene, which is known to involve nutrient homeostasis, especially of Fe, Mn and Zn could lead to Cd stress tolerance. AM inoculation was not able to alleviate the detrimental effect of Cd on growth of grafted or self-grafted plants at this moderate Cd stress level. In short, grafting represents an effective tool in alleviating plant tolerance to heavy metals stress by involving vigorous rootstock.

Chapter 6

Conclusion

The major conclusions from this research are:

- both Cd and Ni stresses caused depression in plant growth and fruit yield of grafted and non-grafted plants by altering physiological, biochemical and metabolic processes;
- plants grafted onto Maxifort rootstock could effectively mitigate adverse effects of Cd stress by improving plant nutritional status, photosynthetic pigments, photochemical activity of PSII (Fv/Fm ratio), increasing activities of antioxidant enzymes (CAT, APX), proline and certain metabolites linked to oxidative stressor i.e. phytochelatin, and fructansinulins and thus minimizing the level of Cd-induced oxidative stress such as decrease in level of ROS, lipid peroxidation (MDA content) and electrolyte leakage (EL);
- under Ni stress, plants grafted onto tomato rootstocks, Unifort and especially Maxifort were capable of maintaining a better leaf nutritional status, higher chlorophyll content, higher SPAD index and F_v/F_m ratio, increased the APX and GPX activities with decreased MDA content and EL;
- the Cd contents in aerial plant parts (leaves + stems + fruits) of Maxifort rootstock grafted plants was lower than those was in non-grafted or self-grafted plants, indicating better ability of Maxifort rootstock in restricting Cd transfer from roots to the aerial parts. Moreover, the content of Ni was also lower, particularly in leaves of Maxifort grafted plants than self-grafted plants;
- Black Beauty rootstock showed some incompatibility reaction with scion cv. Ikram as reflected by lower nutrient elements transfer to the aerial parts, including toxic heavy metals, and eventually poor plant physiological and biochemical activities and finally, the poor growth and yield of Black Beauty grafted plants;
- AM inoculation was not able to alleviate the detrimental effect of Cd on growth and productivity at the tested moderate Cd level.

In short, grafting tomato onto vigorous rootstock such as Maxifort could minimize the toxic effects of Cd and Ni, and possibly also the other heavy metals.

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8. Appendixes

Photographs (Photo.) taken during experimentation

Foto. 1. Grafted seedlings at transplanting stage (a,b)

Photo. 2. Tomato plants in plastic covered channel under greenhouse

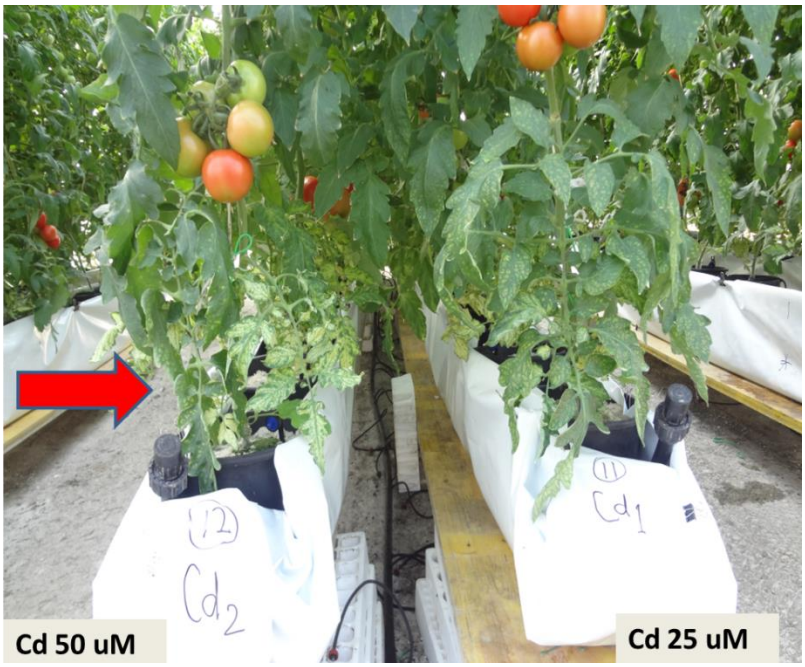
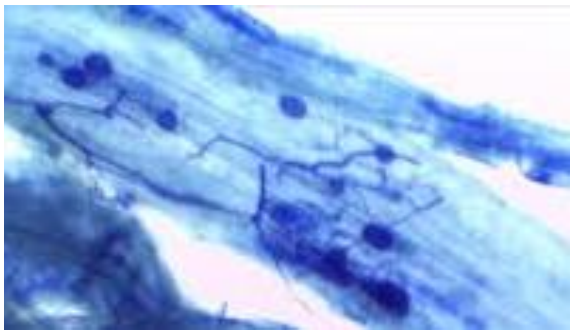
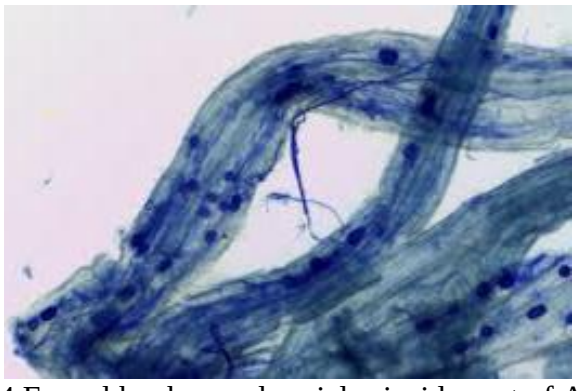


Photo. 3 Foliar symptoms on older leaves under Cd stress



a



b

Photo. 4 Fungal hyphae and vesicles inside root of AM plants (a,b)

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April, 2015 **(PRADEEP KUMAR)**

10. List of publications and presentations

The publication and presentation from thesis work and related activities performed during PhD

Research Paper:

1. **Kumar, P**., Rouphael, Y., Cardarelli, M., Colla., 2015. Effect of nickel and grafting combination on yield, fruit quality, antioxidative enzyme activities, lipid peroxidation and mineral composition of tomato. (Accepted for publication in Jurnal of Plant Nutritiona and Soil Science).

2. Giuseppe Colla, Youssef Rouphael, Rama Jawad, **Pradeep Kumar**, Elvira Rea, Mariateresa Cardarelli (2013). The effectiveness of grafting to improve NaCl and $CaCl_2$ tolerance in cucumber. *Scientia Horticulturae,* 164, 380–391.

Book of Abstracts/Proceedings:

1. Giuseppe Colla, Youssef Rouphael, Mariateresa Cardarelli, Antonio Fiorillo, **Pradeep Kumar**, Alberto Battistelli (2013). Improvement of abiotic stress tolerance through the use of vegetable grafting. In: 1st Meeting of COST Action FA1204 on 'Vegetable Grafting to Improve Yield and Fruit Quality under Biotic and Abiotic Stress Conditions' held during 11-12 March 2013 at Agricultural University of Athens, Greece. p.58.
2. **Pradeep Kumar**, Menahem Edelstein, Mariateresa Cardarelli, Elvira Rea and Giuseppe Colla (2013). Grafting reduces the cadmium translocation from root to shoots and fruits in tomato. In book of Abstract: 1st Annual Conference COST ACTION FA 1204-ROOTOPOWER Workshop held at Murcia (Spain) during 12-14, November, 2013. p 46.
3. Youssef Rouphael, Rama Jawad, **Pradeep Kumar**, Mariateresa Cardarelli, Elvira Rea, Giuseppe Colla (2013). Physiological and growth responses of grafted cucumber grown under different salt sources. In book of Abstract: 1st Annual Conference COST ACTION FA 1204 - ROOTOPOWER Workshop held at Murcia (Spain) during 12-14, November, 2013. p.52.

4. **Pradeep Kumar**, Youssef Rouphael, Mariateresa Cardarelli, Giuseppe Colla. 2014. Grafting response to nickel stress in tomato. In Abstract: 2nd COST Action FA1204 annual conference from 20-22 October 2014 at Carcavelos, Portugal. p. 24.

5. Youssef Rouphael, Mariateresa Cardarelli **Pradeep Kumar**, Giuseppe Colla. 2014. Effectiveness of grafting to improve cucurbitaceae crop performance under salinity. In Abstract: 2nd COST Action FA1204 annual conference from 20-22 October 2014 at Carcavelos, Portugal. p. 46.

Book chapter:

Giuseppe Colla, **Pradeep Kumar**, Mariateresa Cardarelli and Yusuf Rouphael (2013). Grafting an effective tool for abiotic stress alleviation in vegetables. Eds. K.L. Chadha, A.K.Singh, S.K.Singh and W.S. Dhillon. West Ville Publishing House, New Delhi. pp 15-28.

Presentations

Oral:

'Grafting reduces the cadmium translocation from root to shoots and fruits in tomato' during an International Event: 1st Annual Conference of COST Action FA1204 on "Exploiting root-to-shoot communication for improving yield stability and fruit quality in vegetable crops" held in Murcia (Spain) during November, 12-14, 2013.

Poster:

'Grafting response to nickel stress in tomato' during 2nd Annual Conference- COST Action FA1204 organized under a European Union funded project (Cost Action FA1204) from 20-22 October 2014 at Carcavelos, Portugal.

Participation/Attendence in Trainigs/Conferences/Symposia

1. Participated in an International Symposium on 'Advances in Irrigation and Hydroponics (AGRICOM 2013)' organized by AGRICOM consortium of European Union during 19th-20th Sept., 2013 at Tuscia University in Viterbo, Italy.

2. Attended 1st training school on Vegetable grafting organized by Catania University, Catania (Italy) from 23 to 26 Sept., 2014 under a European Union funded project (Cost Action FA1204: 'Vegetable grafting to improve yield and fruit quality under biotic and abiotic stress conditions').