Environmental and Experimental Botany 130 (2016) 181–188

Contents lists available at ScienceDirect

Environmental and Experimental Botany

journal homepage: www.elsevier.com/locate/envexpbot

Tuber borchii Vitt. mycorrhiza protects *Cistus creticus* L. from heavy metal toxicity

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ARTICLE INFO

Article history:
Received 21 December 2015
Received in revised form 14 May 2016
Accepted 10 June 2016
Available online 14 June 2016

Keywords:
Heavy metal damage
Mycorrhiza protection effect
DNA damage reduction
Zinc
Lead
Chromium

ABSTRACT

Heavy metals (HMs), such as copper, zinc, lead, mercury and cadmium, are the most abundant and dangerous inorganic environmental pollutants. Growing pieces of evidence suggest that mycorrhizal fungi can alleviate metal toxicity in plants. In this study, we focused attention on the ectomycorrhizal (ECM) fungus *Tuber borchii* Vitt., which is widespread in Italy and is of great ecological interest because of the mutualistic associations and the advantages it provides to host plants. Seedlings of the Mediterranean shrub *Cistus creticus* L., mycorrhized and non mycorrhized with the ECM fungus *T. borchii*, were treated with HMs (zinc, lead and chromium). HMs induced leaves' chlorosis in non mycorrhized seedlings; while no significant difference was observed in pigmentation of mycorrhized seedlings' leaves. This observation was confirmed by Euclidean Distance of color measurements in L*a*b* units from RGB digital images of leaves. The decrease in leaves pigmentation observed in HM treated non mycorrhized seedlings strongly correlated with a reduced expression of key genes associated with chlorophyll biosynthesis; instead, no significant variation of gene expression was detected in mycorrhized seedlings treated with HMs.

Finally, a comet test on nuclei isolated from leaves of mycorrhized and non mycorrhized *C. creticus* seedlings, treated or not with HMs, confirmed that the DNA damage induced by HMs stress was lower in mycorrhized than in non mycorrhized seedlings. Taken together, the data suggest the involvement of the mycorrhizal fungus *T. borchii* in the improvement of HM stress tolerance in *C. creticus* host.

1. Introduction

Soil contamination by heavy metals is one of the main permanent environmental problems for human health and ecosystems (Kabata-Pendias and Pendias, 2001), due to their carcinogenic and mutagenic effects (Tchounwou et al., 2012). Several heavy metals, e.g. Zn, Cu and Mn, are essential at low concentrations but become toxic at increasing concentrations, others, e.g. Hg, Cd, Pb, have never been proven to be essential for living organisms and are toxic even at very low concentrations (Hall, 2002).

A high concentration of HMs adversely affects plant growth and development. Several physiological, biochemical and molecular processes are negatively modified as a result of HM stress in soil: plant growth rate, seed germination, cell turgor loss, cell-to-cell adhesion, chlorosis, necrosis, senescence, etc., leading ultimately to plant death (Sanità di Toppi et al., 2012; Bano and Ashfaq, 2013).

It is well known that HMs reduce the rate of photosynthesis acting at different levels, including reduction in plastid content of chlorophylls and carotenoids (Mishra and Dubey, 2005). Carotenoids are often used as biomarkers of metal contamination of agricultural soils (Andrianos et al., 2016). This often correlated to a reduced expression of genes essential for isoprenoid and carotenoid accumulation (Mishra and Dubey, 2005). These chemicals are synthesized in plant cells via two distinctly localized cross-talking routes: the cytosolic mevalonate (MVA) and plastidial 2-C-methyl-D-erythritol 4-phosphate (MEP) pathways, (Fig. 1; see Grassi et al., 2013 for a more detailed picture). The key enzimes of these two pathways are: 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR) that is the rate limiting enzyme along the MVA pathway (Chappell et al., 1995) and 1-deoxy-D-xylulose-5-phosphate synthase (DXS) and 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) that are, respectively, the first and the second enzymes in the MEP pathway. It has been demonstrated that DXS, the first enzyme of this route, plays a major role in the overall regulation of the pathway. In addition to DXS, experimental evidence in plants suggest that the second enzyme of this pathway

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http://dx.doi.org/10.1016/j.envexpbot.2016.06.007
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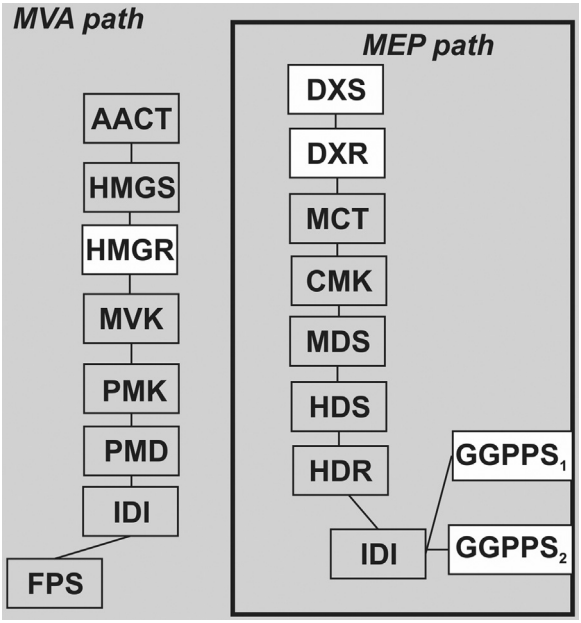

Fig. 1. Isoprenoid biosynthetic pathways. A scheme displaying the enzymes participating in the cytoplasmic (MVA path) and plastidial (MEP path) isoprenoid biosynthetic pathways. The enzymes coded from the studied genes are highlighted. MVA path: *AACT* acetoacetyl CoA thiolase, *HMGS* Hydroxymethylglutaryl- CoA synthase, *HMGR* hydroxymethylglutaryl-CoA reductase, *MVK* mevalonate kinase, *PMK* 5-phosphomevalonate kinase, *PMD* 5 diphosphomevalonate decarboxylase, *IDI* isopentenyl diphosphate isomerise, *FPS* farnesyl diphosphate synthase; MEP path: *DXS* 1-deoxy-D-xylulose 5-phosphate synthase, *DXR* 1-deoxy-D-xylulose 5-phosphate reductoisomerase, *MCT* 2-C-methyl-D-erythritol 4 phosphate cytidylyl-transferase, *CMK* 4-(cytidine 50-diphospho)-2-C-methyl-D-erythritol kinase, *MDS* 2-C-methyl-D-erythritol 2,4 cyclodiphosphate synthase, *HDS* (E)-4-hydroxy-3-methylbut-2 enyl diphosphate synthase and *HDR* (E)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase, *GGPS* geranylgeranyl diphosphate synthase.

DXR also has a rate-limiting role in carotenoids biosynthesis (Cordoba et al., 2009). Analysis of Arabidopsis transgenic lines that express higher (over-expressors) or lower (antisense) DXS levels showed that the level of various isoprenoid final products including chlorophyll, carotenoids, tocopherols, and ABA are increased or decreased in the corresponding transgenic plants (Estèvez et al., 2001). Similar results have been obtained in other plants: tomato (Enfissi et al., 2005), potato (Morris et al., 2006) and *Ginkgo biloba* (Gong et al., 2006). The impact on the level of some final products of the pathway (between 2 and 7-fold) reported in these works supports that, in plants, these enzymes catalyse rate-limiting steps for the synthesis and the accumulation of various isoprenoid final products. The rate-limiting function of this enzyme has also been found in eubacteria (Matthews and Wurtzel, 2000). However, the rate limiting role for each of these two enzymes appears to vary among plants and in different conditions. Additional studies are obviously required to determine the precise conditions in which these enzymes are rate-limiting steps for the pathway flow (Cordoba et al., 2009). Finally, of particular interest are GGDPS1 and GGDPS2 (geranylgeranyl diphosphate synthases), two enzymes responsible for geranylgeranyl diphosphate (GGDP) synthesis, the building block of all diterpenoids, carotenoids, gibberellins and ABA (Pateraki and Kanellis, 2008). Hédiji et al. (2010) correlated reduction in the content of leaf pigments with changes in the expression of gene coding for enzymes which are involved in isoprenoid and carotenoid biosynthetic pathway (which produces plant pigments). They reported reduction of DXS and GGDPS transcript level progressively with Cd supply. HMs can also induce oxidative stress which leads to DNA damage in plants; the Comet test is a valuable approach for studying the genotoxic effects of different stress conditions on plants and it is a sensitive method to evaluate DNA damages (Santos et al., 2015).

A major approach to overcome or minimize the adverse effects of HMs is the phytoremediation, which is defined as the use of plant-based processes to remove, decrease or detoxify the environmental pollutants (McGrath et al., 2002). Increasing interest has been focused on native species with the capability of growing in polluted sites and under local climatic conditions, so avoiding invasive species that could threat the local plant diversity. In our study, *Cistus creticus* was chosen as model host plant because: (a) the genus *Cistus* is one of the main constituents of the Mediterranean-type maquis and these shrub species are peculiar in that they have developed a range of specific adaptations to non-friendly environments; (b) more than 200 fungal species belonging to 40 genera have been reported so far to form mycorrhizas with *Cistus*. One of the ectomycorrhizal fungi reported to be associated with *Cistus* spp. is the *Tuber borchii* (Comandini et al., 2006). *Tuber borchii* is one of the most spread species of the genus *Tuber* in Italy (Iotti et al., 2010; Russo et al., 2010). Recent reviews have focused on the impact of mycorrhizal colonization upon the fitness of host plants to natural and anthropogenic disturbances, such as salt stress (Luo et al., 2011), tolerance to heavy metals (Colpaert et al., 2011; Ma et al., 2014; Luo et al., 2014).

Because the development of stress-tolerant plant-mycorrhizal associations may be a promising new strategy for soil reclamation and amelioration, the sensitivity to HMs of *C. creticus* seedlings mycorrhized and non mycorrhized with the ECM symbiont *T. borchii* was compared by evaluating: (1) differences in leaf pigmentation, (2) variation in expression levels of genes coding for enzymes involved in isoprenoid and carotenoid biosynthesis and (3) changes in DNA damage level by the Comet test, in order to verify the hypothesis that the ECM fungus is able to alleviate zinc, lead and chromium stress in this autochthonous host plants. These heavy metals (HMs) are, in fact, those that more usually exceed the allowable limits in Apulia region soil (Brunetti et al., 2009).

2. Material and methods

2.1. Fungus and plant growth conditions

The *T. borchii* culture (identified as strain ATCC 96540), obtained from ascocarps collected in natural "truffiére" of the Salento area, Apulia (Southern Italy), was grown on Malt Extract Agar (Duchefa Biochemie, Haarlem, Netherlands). Seeds of *C. creticus* (provided by the Botanical Garden of the University of Salento, Lecce, Italy) were sterilized for 30 min in aqueous solution containing 30% NaClO and 10% Tween20, heated at 100 °C for 3 min to increase germination performance (Tilki, 2008) and subsequently placed on MS-agar (Difco, Sparks, USA) part of the dual culture Petri dishes (fifteen seeds per dish); the malt extract side of the Petri dishes was inoculated with a 1.0 cm mycelial plug of the *T. borchii* strain ATCC 96540 (taken from the colony margin of an approximately 1-month-old colony grown on malt extract) or with a malt extract agar plug (mock control) (according to Sabella et al. (2015)). The experiments were carried out in a growth chamber at 23 ± 2 °C with a 16 h photoperiod and a 95 $\mu mol\, m^{-2}\, s^{-1}$ light intensity. After 7 weeks of growth, hyphal compartment of the Petri dish was removed and substituted with liquid Malt Extract Broth (Duchefa Biochemie, Haarlem, Netherlands). Half of the mycorrhized and not mycorrhized seedlings (for a total of 10 Petri dishes each) were subjected to HMs stress by enriching the malt extract with a mixture of 50 μM $ZnSO_4$, plus 50 μM $Pb(NO_3)_2$ and 50 μM Cr $(NO_3)_3$; the remaining seedlings (mycorrhized and not mycorrhized) were used as untreated controls. Mycorrhizal development was confirmed by trypan blue staining of the roots (Kagan-Zur et al., 1994) followed by laser scanning microscope (Zeiss LSM 700)

observations according to the method of Ventura et al. (2006). Trypan blue fluorescence was detected with a 559 nm longpass filter, employing an excitation wavelength of 555 nm. Evaluation of colonisation level was carried out by spreading out the complete root system between two transparencies in a scanner. Total root length was calculated using ImageJ software. Root colonisation was detected under a light microscope and marked on the transparency with subsequent measurement by ImageJ. Percentage of root length colonised by *T. borchii* was calculated from total root and colonised root lengths (Ventura et al., 2006). Analyses on leaves and roots were performed after 6 days of HMs treatment.

2.2. Leaf color measurements

Digital RGB color images of the Petri dishes containing *C. creticus* seedlings (mycorrhized and non mycorrhized; treated and untreated with HMs) were vertically acquired, at the highest image quality settings, using a Canon EOS 1100D digital camera (2012 Canon Inc., Japan) placed at 25 cm distance from the object and analyzed by the Adobe Photoshop CS5 Extended version 12.0 × 32 (2010 Adobe Systems Inc. USA). The Lab color space dimensions L* (luminance), a* (a channel) and b* (b channel) were obtained through the software, where L*, also known as lightness component, ranges from 0 to 100, and the two chromatic components a* (from green to red) and b* (from blue to yellow) range from −120 to 120 (Yam and Papadakis, 2004). The L*a*b space is perceptually uniform, i.e., the Euclidean distance between two different colors corresponds approximately to the color difference perceived by the human eye (Hunt, 1991). The Euclidean distance was calculated from mean L*, a* and b* values by using the *dist* function in R-2.12.1 Software.

2.3. RNA isolation

Total RNA was extracted according to the protocol of Pateraki and Kanellis (2004) optimized for *C. creticus* tissues.

The described methods were appropriate for nucleic acid isolation independently of the sampling period, plant age, or plant cultivation method and worked efficiently for other medicinal plant species containing high amounts of metabolites that interfere with nucleic acid isolation (Pateraki and Kanellis, 2004).

2.4. Expression analysis of HM-induced genes

Aliquots of two micrograms of total RNA of each sample were reverse transcribed using oligo (dT) primer with TaqMan® Reverse Transcription Reagents (Applied Biosystems) according to the manufacturer's standard protocol. The reaction mixture was incubated at 25 °C for 5 min, then at 48 °C for 30 min. Subsequently, the cDNAs were quantified using a Qubit Fluorometer (Invitrogen, Oregon, USA) and used for real-time PCR amplifications with specific primers. Primers used for 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (HMGR, Accession num. EF062866.1), 1-deoxy-D-xylulose-5-*syn*thase (DXS, Accession num. EF062865.1), 1-deoxy-D-xylulose-5-reductois*omer*ase (DXR, Accession num. AY297794.2), Geranylgeranyl diphosphate synthase 1 (GGDPS1, Accession num. AF492022.1), Geranylgeranyl diphosphate synthase 2 (GGDPS2, Accession num. AF492023.1) and for the constitutively expressed *C. creticus* eukaryotic translation elongation factor-1a (EF1a, Accession num. EF062868) were designed on coding sequence by using Primer Express Software 3.0 and are shown in Table 1.

Real-time PCR was performed with SYBR Green fluorescence detection in a real-time PCR thermal cycler (ABI PRISM 7900 HT Fast Real-Time PCR System, Applied Biosystems). The PCR mix was prepared using 5 μl from cDNA derived from the reverse transcriptase reaction, 12.5 μl of Power SYBR Green RT-PCR Master mix (Applied Biosystems), 5.0 μM forward and reverse primers, in a total volume of 25 μl. The cycling conditions were: 2 min at 50 °C and 10 min at 95 °C, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Melting curve analysis was performed after PCR to evaluate the presence of non-specific PCR products and primer dimers.

To calculate the Fold Changes (FC) the following formula was used: FC = 2^-ΔΔCT where

$$\Delta\Delta CT = (CT_{targetgene} - CT_{referencegene})\ \ treatedsample - (CT_{targetgene} - CT_{referencegene})\ controlsample$$

CT means Threshold Cycle. *Control samples* were HMs untreated non mycorrhized and mycorrhized seedlings respectively for HMs treated non mycorrhized and mycorrhized seedlings.

The CT data are expressed as average of three biological replicates. According to Chen et al. (2007), a gene is said to be differentially expressed, for biological significance, if the Fold Change exceed a value of 2 (up-regulation) or if it is minor of 0,5 (downregulation).

2.5. Comet assay

The method of Comet assay (according to Zhang et al. (2006)) was used to detect DNA damage in the leaves of *C. creticus* L. After harvest, the fresh leaves were washed three times with double-distilled water, blotted dry with a filter paper and used in the Comet assay. All operations were conducted under dim or red light to avoid photoinduced DNA damage. Plant material was placed in a 60-mm Petri dish on ice and covered with 250 μl of cold 1X PBS (NaCl 130 mmol/L, Na_2HPO_4 7 mmol/L, $NaHPO_4$ 3 mmol/L, EDTA 50 mmol/L, pH 7.5). Using a new razor blade, each leaf was gently sliced into pieces. The pieces were washed with the PBS by repeated pipetting using micropipette. The nuclei were released

Table 1
Primers used for *DXR, DXS, GGDPS1, GGDPS2* and *HMGR* expression studies.

Gene	Primers' Name	Primers' sequence	Accession number
DXR	DXR.For1	5′ AGACCTCAACTGGTTGCCATAAG 3′	AY297794.2
	DXR.Rev1	5′ GCCAAAGCCTCTCTCAATTCA 3′	
DXS	DXS.For1	5′ GGCGATTAGCAGCTGAGCAT 3′	EF062865.1
	DXS.Rev1	5′ AGAGCCGAAACCTCCAATAGAA 3′	
GGDPS1	GGPPS1.For1	5′AAATCGATTGGTTCGGAAGGA 3′	AF492022.1
	GGPPS1.Rev1	5′GGCCCTCACTGCAGATATCC 3′	
GGDPS2	GGPPS2.For1	5′ TGAGGCCGCTTTTGTGTATTG 3′	AF492023.1
	GGPPS2.Rev1	5′ GCATCGCCACGGAAACAT 3′	
HMGR	HMGR.For1	5′ CTGGAAATTTCTGCTCGGATAAG 3′	EF062866.1
	HMGR.Rev1	5′ TGCCCCGTCCCTCAATC 3′	
EF1α	EF1a.For1	5′ ATGAACCACGCAGGACAGATT 3′	EF062868.1
	EF1a.Rev1	5′ TGAGAGGTGTGACAGTCGAGAAC 3′	

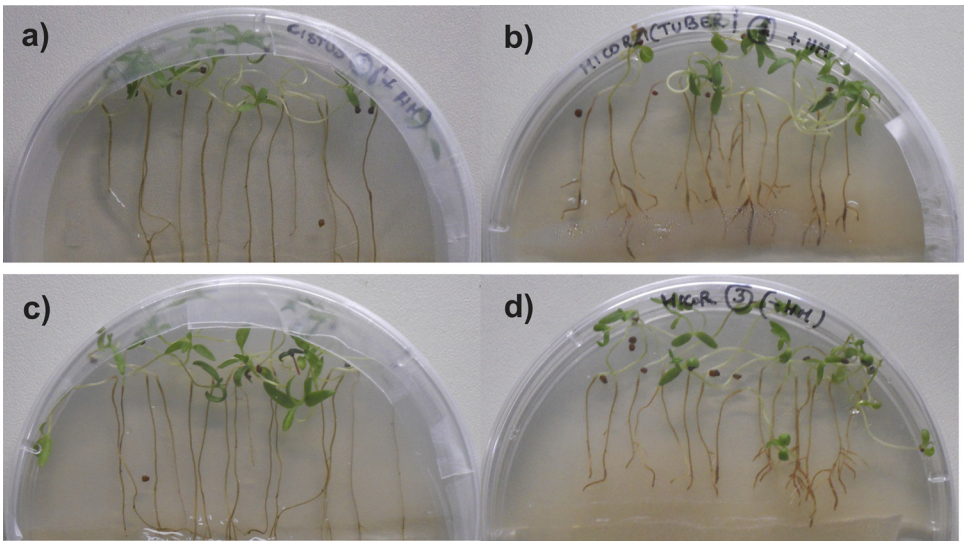

Fig. 2. Dual culture Petri dishes. Seedlings of *C. creticus*, 8-weeks-old, grown in dual culture Petri dishes: (a) non mycorrhized seedlings treated with HMs; (b) seedlings mycorrhized with *T. borchii* mycelium treated with HMs; (c) non mycorrhized seedlings HMs untreated; (d) seedlings mycorrhized with *T. borchii* mycelium HMs untreated.

and collected in the buffer. The nuclei suspension was used for the alkaline Comet assay, as described by Gichner (2003) with some modifications (Lin et al., 2005). After preparation, microscope slides were put in freshly prepared cold alkaline buffer (300 mmol/L NaOH, 1 mmol/L Na_2EDTA, pH > 13) at 4 °C to allow DNA denaturation. Electrophoresis was conducted for 15 min at 300 mA. After electrophoresis the slides were immersed in 0.4 mol/L Tris–HCl (pH 7.5) solution at room temperature for further 15 min.

Each slide was stained with 50 μl of 2 mg/ml of 4′,6-diamidin-2-fenilindolo (DAPI) and observed using a Carl Zeiss LSM 700 laser scanning microscope with an excitation filter of 338–385 nm and a barrier of 590 nm. Images of 50 randomly chosen cells were acquired for each slide and analyzed by the Comet Assay Software Project (CASP). Various comet parameters were measured such as tail length (a rough estimate of DNA migration expressed in arbitrary units), tail DNA (the percentage of DNA in the comet tail), Tail Moment (TM – the integrated value of DNA density in the tail multiplied by the migration distance) and Olive Tail Moment (OTM – product of the distance in × direction between the center of gravity of the head and the center of gravity of the tail and the percentage of the tail DNA) (Konca et al., 2003).

2.6. Statistical analysis

Kruskal-Wallis test was used to:

– reveal significant colour difference, measured as the Euclidean Distance in CIE 1976, L*a*b* (L = Luminance, a = a channel, b = b channel; Hunt, 1991), between leaves of *C. creticus* seedlings: non mycorrhized (−HMs) vs mycorrhized (+HMs); non mycorrhized (−HMs) vs mycorrhized (−HMs); mycorrhized (−HMs) vs mycorrhized (+HMs) and non mycorrhized (−HMs) vs non mycorrhized (+HMs).;

– reveal significant increase in DNA da9mage induced by heavy metals in cells from leaves of non mycorrhized and mycorrhized *C. creticus* seedlings.

The Student *t*-test was used to test the statistical significance of the differences found in the expression profile of genes coding for enzymes involved in the biosynthesis of isoprenoids in roots and leaves of *C. creticus* seedlings treated with heavy metals.

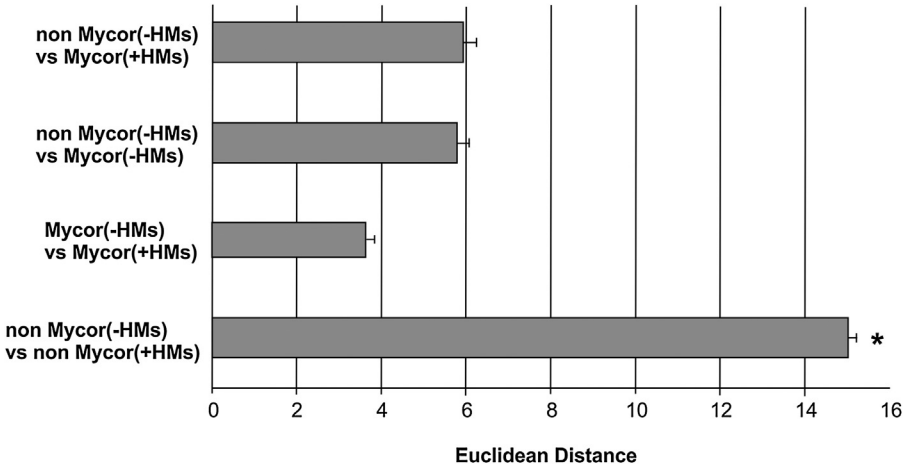

Fig. 3. Colour difference between leaves of *Cistus creticus* seedling mycorrhized/non mycorrhized with *Tuber borchii* mycelium and treated/untreated with heavy metals. Colour difference (measured as the Euclidean Distance in CIE 1976, L*a*b*, L = Luminance, a = a channel, b = b channel; Hunt, 1991) between leaves of *C. creticus* seedlings: non mycorrhized (−HMs) vs mycorrhized (+HMs); non mycorrhized (−HMs) vs mycorrhized (−HMs); mycorrhized (−HMs) vs mycorrhized (+HMs) and non mycorrhized (−HMs) vs non mycorrhized (+HMs). The only significant difference was revealed for the comparison between non mycorrhized (−HMs) vs non mycorrhized (+HMs) according to Kruskal–Wallis test (* p-value <0,05).

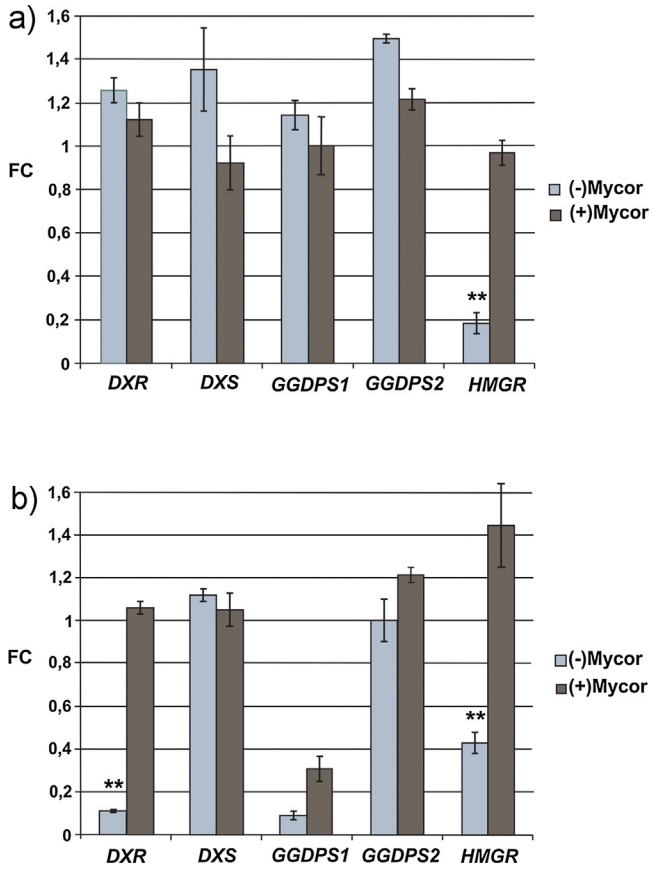

Fig. 4. Expression profile of genes coding for enzymes involved in biosynthesis of isoprenoids in roots and leaves of *Cistus creticus* seedlings treated with heavy metals. Variation of *DXR*, *DXS*, *GGDPS1*, *GGDPS2* and *HMGR* transcript relative abundance in (a) roots and (b) leaves of *C. creticus* seedlings mycorrhized (+Mycor) and non mycorrhized (−Mycor) with *Tuber borchii* mycelium and (both) treated with heavy metals. FC = Fold Change. The only significant differences were revealed for the genes coding for the enzyme: HMGR (in roots and in leaves) and DXR (leaves) according to Student *t*-test (** p-value <0,01).

Statistical tests were performed with the R-2.12.1.

3. Results and discussion

3.1. Influence of HMs on leaf photosynthetic pigments

After 6-days-exposure to the medium enriched with a mixture of zinc (50 μM), lead (50 μM)and chromium (50 μM), a reduction in the content of leaf pigments in non mycorrhized seedlings of *C. creticus* compared to the HMs untreated seedlings was observed; differently, *T. borchii* mycorrhized seedlings (with a percentage of colonised root of 83,24 ± 5,95%) did not show any variation of the leaf color with respect to its control (mycorrhized seedlings HMs untreated) (Fig. 2). Consistently with the visual observations, leaf color difference between treatments was calculated using the Euclidean Distance by digital color analysis (Fig. 3); according to the Kruskal-Wallis test, significant difference was observed only between non mycorrhized *C. creticus* seedlings treated and untreated with HMs.

It is well documented that heavy-metal toxicity has an effect on normal physiological processes. This could be due to their interference with activities of a number of enzymes essential for normal metabolic and developmental processes as well as to their direct interactions with proteins, pigments, etc. (Emamverdian et al., 2015). Exposure of the seedlings to metals resulted in changes in the content of leaf pigments (Bertrand and Guary, 2002). In this work, reduction in leaf pigmentation was compared between leaves of mycorrhized/non mycorrhized seedlings treated/untreated with HMs (zinc, lead and chromium) by using color measurement in L^*a^*b units from RGB digital images; this is the most used color space in the measuring of colors due to the uniform distribution of colors and because it is very close to human perception of color. In this perceptually uniform color space, Euclidean distances highly agree with perceptual color differences (Leon et al., 2006) and in relation to our data, it revealed a statistically significant different pigmentation only in leaves of non mycorrhized seedlings treated with HMs when compared with non

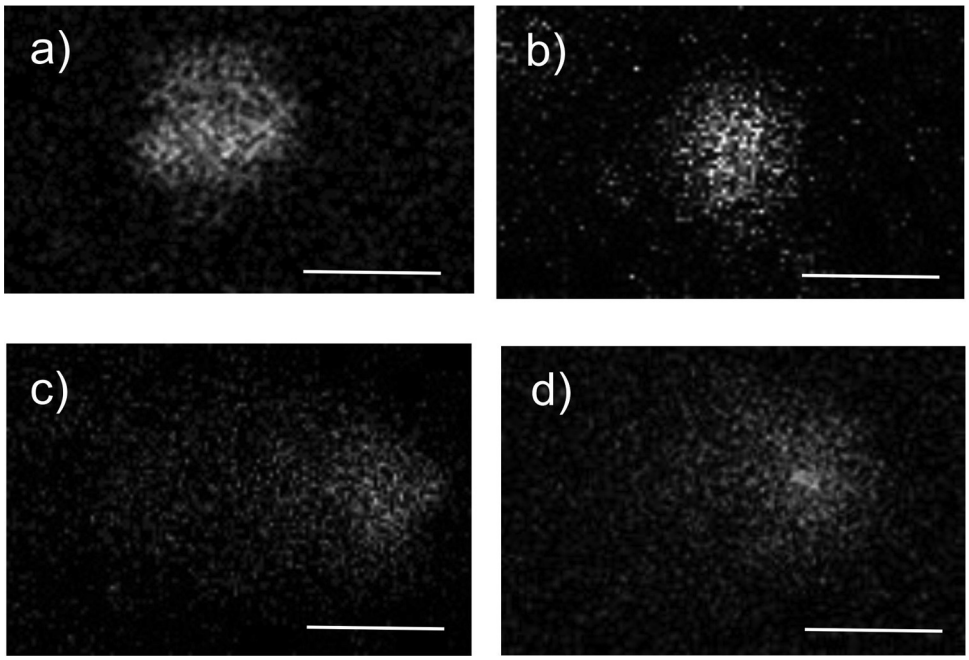

Fig. 5. Comet images of DNA from leaves of *Cistus creticus*. Comet images formed by DNA migration in electrophoresis field in cells of *C. creticus* leaves excised from (a) seedlings non mycorrhized (−HMs); (b) seedlings mycorrhized (−HMs); (c) seedlings non mycorrhized (+HMs); (d) seedlings mycorrhized (+HMs). Bar: 50 μm.

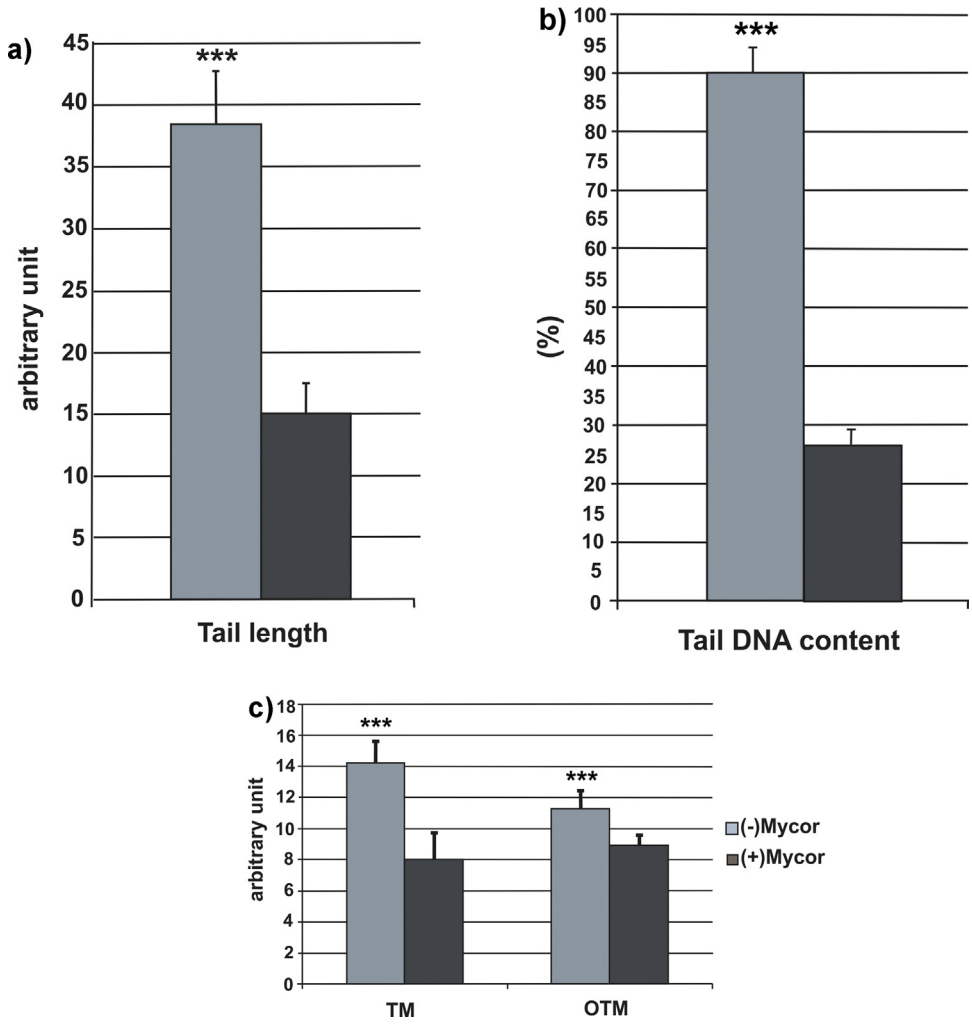

Fig. 6. Tail length, Tail DNA content, TM and OTM from Comet assay. Evaluation of DNA damages in cells from leaves of *Cistus creticus* induced by heavy metals in non mycorrhized (−Mycor) and in mycorrhized (+Mycor) seedlings. (a) Tail length; (b) Tail DNA content; (c) TM and OTM. A significant increase (*** p-value <0.001, according to Kruskal–Wallis test) in DNA damage was observed in non mycorrhized seedlings treated with heavy metals.

mycorrhized seedlings untreated with HMs, indicating that mycorrhization confer to *C. creticus* tolerance to HM induced stress.

Since the reduction of photosynthetic pigments under HMs toxicity appears related to the sensitivity of several enzymes of isoprenoid pathways, involved in chlorophyll and carotenoid synthesis (Mishra and Dubey, 2005; Hédiji et al., 2010), Quantitative RT-PCR analyses were performed for *DXR*, *DXS*, *GGDPS1*, *GGDPS2* and *HMGR* on leaves and roots excised from HM treated mycorrhized and non mycorrhized seedlings. In roots, only *HMGR* (MVA pathway) resulted significantly downregulated in non mycorrhized seedlings (Fig. 4a) according to the fact that roots seem to produce low amounts of volatile mono- and sesquiterpenes compared to leaves (Schnee et al., 2002). In leaves (Fig. 4b), *HMGR* confirmed a downregulation in non mycorrhized seedlings, further, *GGDPS1* resulted downregulated in mycorrhized and non mycorrhized seedlings; *DXS* and *GGDPS2* showed a similar stable expression profile. *DXR* resulted downregulated only in non mycorrhized seedlings. Obtained data are in keeping with the *C. creticus* tissue distribution of terpenoid biosynthetic genes reported by Pateraki and Kanellis (2010); they found that *HMGR* has a similar expression pattern in the analyzed tissues (leaves, stems, flower buds, fruit, seeds and roots) suggesting that this specific gene is not tissue regulated. *DXS* and *GGDPS2* show a similar expression profile with maximum accumulation in flower buds and lower in leaves and roots. *DXR* and *GGDPS1* exhibit high abundance in leaves. Therefore, in our work, we found a detectable reaction to heavy metals treatment in the expression pattern of the genes that are mostly abundant in leaves or, as for *HMGR*, in leaves and roots. Previous works have used expression data of genes involved in isoprenoids and carotenoids biosynthetic pathway to assess biological plant response to heavy metals stress. Hédiji et al. (2010) evaluated the response of tomato plants to long-term cadmium exposure; in their experiment, carotenoid and chlorophyll content decreased in HM treated leaves correlated with a reduction of the expression of genes essential for isoprenoid and carotenoid accumulation. In our work, the down-regulation of the key enzymes *DXR* and *HMGR* only in non mycorrhized seedlings treated with HMs, suggests that root colonization by the ECM fungus *T. borchii* affects seedlings tolerance to selected heavy metals.

3.2. DNA damage in Cistus creticus L.

Another method used to test the influence of *T. borchii* ectomycorrhizas in HMs stress tolerance in host plants is the Single Cell Gel Electrophoresis (SCGE) which is also called comet assay, it was used to assess DNA damage caused by HMs. Typical comet images of DNA in the leaves of *C. creticus* are summarized in Fig. 5. In leaves of mycorrhized seedlings treated with heavy metals the comet images had smaller comet tails and more feeble

fluorescent density with respect to the control (Fig. 5a, c) corresponding to a low DNA migration from the nuclei. On the contrary, much more damaged DNA was observed in the comet images obtained from non mycorrhized seedlings treated with HMs (Fig. 5c, d). All the parameters, measured by using the image analysis system CASP (the tail length, tail DNA content, TM value and OTM value which were used to quantify DNA damage), were significantly lower in mycorrhized compared to non mycorrhized seedlings (Fig. 6a–c) indicating that plant colonization by the ectomycorrhizal fungus *T. borchii* decreased the DNA damages induced by HMs. These results gave a further evidence that *T. borchii* ectomycorrhizas can affect host plant capacity to tolerate exposition to HMs.

The high concentration of heavy metals in soil adversely affects plant growth and development; several physiological, biochemical and molecular processes are disturbed as a result of heavy metal stress. Among biological methods, mycorrhizal fungi can play a role in bioremediation of heavy metal pollution in soil. Infact, metallothionines – like polypeptides are known to be involved in Cd and Cu detoxification in AM fungal cells (Bano and Ashfaq, 2013). The external mycelium of some AM fungi produces glomalin, an insoluble glycoprotein that efficiently binds HMs at specific sites forming stable complexes thus reducing their toxicity (Bano and Ashfaq, 2013). Most ECM and Ericoid mycorrhizal fungi enhance tolerance of host plants to HMs modulating their cellular, physiological and molecular processes. In the last decade, a number of ectomycorrhizal fungal isolates and host plants have been characterized for their tolerance to heavy metals (Luo et al., 2014). The present study increases knowledge in this branch of research by demonstrating that the hypogeous mycorrhizal fungus *T. borchii* has a role in the mechanisms of HM tolerance for its host plants.

4. Conclusions

In this study, with a combination of different evidences correlated with heavy metals induced stress (reduction of pigments content in leaf, down-regulation of genes coding for key enzymes involved in plant pigments production, measurement of DNA damage induced by heavy metals treatment) we successfully provide a preliminary, but consistent, evidence that the colonization of *C. creticus* roots with the symbiont fungus *T. borchii* can play an important role in improving plant tolerance under HMs stress. *C. creticus* was treated with fungus inocula since it represents an important species in the Mediterranean-type maquis that could be usefully employed in vegetal reconstruction processes. Infact, it is a structuring specie that is commonly employed to restore the original vegetation in the interested area (Russo et al., 2010). In addition, *Cistus* can form ectomycorrhizas and arbuscular mycorrhizas with more than 200 fungal species (Comandini et al., 2006) and, considering the ecological niches occupied by this shrub, it can also be evaluated as a potential model, for future studies, to assess the role mycorrhizal symbiosis in the ecosystem functioning of Mediterranean area.

Acknowledgements

Authors thank the Italian Ministry of Education, University and Research (MIUR) that provides financial support for the Zeiss LSM 700 laser scanning microscope purchase (Enhancement Project 2HE, Cod. PONa3_00334-CUP F81D11000210007, Project of Structural Enhancement financed by PON R&C 2007–2013, Axis I Operative 4.1.1.4, Action I “Structural Improvement”).

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