THE EFFECT OF MYCORRHIZA GROWTH AND EXPRESSION OF SOME GENES IN BARLEY

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Barley is one of the most important crops for food consumption. Soil fertilization using minerals and chemical compounds are not safe for human health beside beingas expensive. Therefore, It is important to find suitable alternatives. The arbuscular mycorrhiza (AM) is characterized by the formation of arbuscules (sometimes vesicles). The fungus occupies various proportions of the volume of host cells but is separated from the cell cytoplasm by the elaboration of the host plasma membrane forming a fungal membrane and an apoplectic compartment between the fungus and host cytoplasm. Mycorrhiza changes whatever source of phosphorus into phosphoric acid which acidifies the rhizosphere and thus increases the availability of elements for plants uptake. The increase of the availability of elements specially, calcium which plays the main role in cell signaling which could lead to switch on or accelerate the function of several genes such as protein kinases and other genes related with photosynthesis and eventially, affect the plant growth. Inoculation with mycorrhiza increased all plant growth variables such as number of leaves, stem fresh and dry weights and root dry weight Shockley *et al*. (2004). Gahoonia and Nielsen (2004) reported that the inoculation with mycorrhiza enhanced the release of organic acids and higher acid phosphatase activity in the rhizosphere which may be useful for increasing phosphorus acquisition from inorganic and organic phosphorus pools to the plant. Al-Karaki *et al*. (2004) found that biomass and grain yields were higher in mycorrhizal than non-mycorrhizal plants. Wu *et al*. (2005) reported biofertilizer as an alternative to chemical one to increase soil fertility and crop production.

Glassop *et al*. (2005) reported that a very large number of plant species are capable of forming symbiotic associations with arbuscular mycorrhizal (AM) fungi. Moreover, they reported that a large number of phosphate (P) transporters have been identified in plants; tissue expression patterns and kinetic information supports the roles of some of these in the direct root uptake pathways. Chen *et al*. (2007) indicated that the beneficial impacts of mycorrhizal colonization on plant growth could be largely explained by improved P nutrition. On the other hand, Baltruschat *et al*. (2008) found that the root

endophytic basidiomycete *Piriformospora indica* increase the tolerance against abiotic stress as in many plants.

Gryndler *et al*. (2009) concluded that mycelia of AM fungi are influenced by organic matter decomposition via release of the compounds during the decomposition process and also by secondary metabolites produced by microorganisms involved in organic matter decomposition. Araim *et al*. (2009) reported that AM colonization significantly increased the mass of shoots and roots and the concentrations of proteins and most of the phenolics in the roots. Hence, the selected trait of mycorrhiza could play an important role in optimizing the growth of *E. purpurea* by inducing the production of secondary phytomedicinal metabolites.

The impact of mycorrhizaon gene expression has been addressed by some recent studies, Hause *et al*. (2002) reported that colonization of barley (*Hordeum vulgare* Cv. Salome) roots by an arbuscular mycorrhizal fungus, led to elevated levels of endogenous jasmonic acid (JA) and its amino acid conjugate JA-isoleucine, whereas the level of the JA precursor remained constant. The rise in jasmonate was accompanied by the expression of genes coding for an enzyme of JA biosynthesis. Delp *et al.* (2003) performed relative quantitative RT-PCR and western blotting to investigate the expression of three genes with potentially regulatory functions from the arbuscular mycorrhizal fungus Glomus intraradices in symbiosis with barley.

Weidmann *et al*. (2004) identified 29 plant genes which were upregulated in response to *Medicago truncatula* root inoculation with mycorrhiza. Twenty-two protein spots, 14 upregulated and 8 downregulated were found changed in mycorrhiza inoculated plants as compared with the non inoculated ones by Liang *et al*. (2007). Abo Doma *et al*. (2008) indicated that the treatment with mycorrhiza significantly increased plant growth in two cultivars of wheat using different yield related traits. Moreover, transcriptome analysis using real time- reverse transcriptase indicated that mycorrhiza treatment resulted in an increase in the gene expression of proline 5 carboxilate synthetase (P5CS) on the transcription level. Roldán *et al*. (2008) indicated that differential gene expression for genes conferring superoxide dismutase and peroxidases were recorded between mycorrhizal and non-mycorrhizal shoots of *juniperus oxycedrus* seedling.

This investigation aimed to: 1-study the effect of barley root inoculation with mycorrhiza on some plant traits and 2-determine the changes in gene expression of the genes conferring calcium dependent protein kinases (CDPKs), phosphoenol pyrovate carboxylases (PEPCs) and proline-5 carboxylate synthetase (P-5CS) in barley plants in response to their inoculation with

mycorrhiza at the molecular level as quantified using RT-PCR.

MATERIALS AND METHODS

A greenhouse experiment was conducted in the successive season of 2008/2009 using two barley cultivars (*Hordeum Vulgare*s L.) namely Viola and Wady Otbah$_1$ which were obtained from the Agriculture Research Center, Tsawah campus, Libya. The plants were grown up to 90 days after germination as mycorrhiza treated and none treated ones (control). Mycorrhiza inoculants were obtained from Biofertilizers Unit, Faculty of Agriculture, Ain Shams University. The experiment was terminated after ninety days of germination.

The effect of barley roots inoculation with mycorrhiza on the plant growth was estimated at the end of the experiment using six different yield related traits. The traits were plant height (P.H.), number of tillers (N.T.), shoot fresh weight (S.F.W.), shoot dry weight (S.D.W.), root dry weight (R.D.W.) and weight of the youngest elongate blade leaf (Y.E.B.). Data were and statistically analysed according to Steel and Torrie (1980).

Molecular analysis

RNA was extracted from the plants of the four aforementioned samples (the two cultivars, mycorrhiza treated and control) according to Ashoub *et al*. (2006) the resolved RNAs are shown in Fig. (1)**.** Reverse transcription (RT) was performed to produce cDNA from equal amounts of RNA of the four samples using the one-step RT-PCR requires gene-specific primers. For this application, the QIAGEN one step RT-PCR Kit, which contains all the required components for RT-PCR were used.

The range of QuantiTect Kits and Assays guarantee highly specific and sensitive results in real-time RT-PCR on any real-time cycler and require no optimization of reaction and cycling conditions. QuantiTect Kits are available for two-step and one-step RT-PCR and are compatible with detection by SYBR® Green I dye or by sequence-specific probes (e.g., Taq Man and FRET probes). Multiplex RT-PCR of up to 4 targets is also contained.

RT-PCR conditions for (CDPKs), (PEPCs) and (P5CS)

RNA of each of the four samples was reverse transcribed (RT), to produce the first strand of cDNA in the presence of 5 mM MgCl2, 1X PCR Buffer, 1 mM dNTPs, 25 u MuLV reverse transcriptase, 4 u RNA-guard ribonuclease inhibitor, the mixture was prepared as described three times in three different PCR tubes and 2.5 µl of 20 P mol of CDPKs reveres primer with the following sequence (AAT TGA TGG CCA TGG CCT GAC TTT C) was added to the mixture in one of the three PCR tubes and to the second tube 2.5 µl of 20 P mol of PEPCs reveres primer with the following sequence (GCC GGC TTG CTC GTG TCC AT), Finally, 0.5 µl of 20 P mol of P5CS reveres primer with the following sequence (GTA AAG CGT ATC CGC ACT AAC GC) was added in

a final reaction volume of 30 µl in each tube. Reactions were carried out at 42°C for 30 min, followed by a 10 min step at 94°C to denature the enzyme, then was cooling at 4°C.

Real time PCR conditions

For real time PCR quantification of cDNA encoding for CDPKs, PEPCs and P5CS, all PCR processes were performed using commercially available reagents that included a thermostable DNA polymerase, dNTPs, $MgCl_2$, and other salts and buffering agents necessary for optimum performance. One µl of cDNA of the four samples was used as template in the reaction mix two in a final volume of 25 µl in all assays. Conventional PCR, using CDPKs, PEPCs and P5CS forward and reverse primers with the following sequences [CDPKs forward (TGA GTA AGG CCG ACA AGG AGG ATA), reverse (AAT TGA TGG CCA TGG CCT GAC TTT C)] and [PEPCs forward (TGG CCC CAC TCA TCT TGC TAT CTT), reverse (GCC GGC TTG CTC GTG TCC AT)] and [P5CS forward (ATT CCG ACC TTG TGT AAC CGG C), reverse (GTA AAG CGT ATC CGC ACT AAC GC)], respectively, were employed to define the detection limit of the assay. Cycling was carried out in a Stratagene Mx-3000 Real-time PCR system which allows the detection of most commercially available dyes including FAM, SYBR® Green I, TET, HEX™, JOE™, VIC™, TAMRA™, TexasRed®, ROX™, Cy5™, Cy3™ and ALEXA Fluor® 350. The system supports 96-well plate format and can perform multiple sub-experiments up to four dyes in the same well. Bioron product, SYBR® Green I Real Time QPCR (cat No. 119205) master mix for (100 rcs) detection protocol was used in this investigation as described in Bioron manual. Data from fluorescence thresholds were statistically analyzed using Microsoft Excel (Office 2000). PCR products were dissolved in TBE agarose gel.

RESULTS AND DISCUSSIONS

Assessment of mycorrhizal effect on plant growth

In general, plant growth of the two cultivars was elevated in response to root inoculation with mycorrhiza, as detected from the some of the yield related traits with varied responses between traits or cultivars. Details of these results are shown in (Table 1 and Fig. 2).

Regarding plant height, the average was increased from 20.88 cm in the non inoculated plants to 27.67 cm in the mycorrhizal inoculated plants of Cv. Viola with increasing of 32.83% relative to the control. On the other hand, PH was increased from 21.33 cm in the non inoculated plants to 26.50 cm in the mycorrhiza inoculated plants of Cv. Wady $Otbah_1$ with an increase of 24.22%. These results indicated that Viola plants exhibited a greater response for mycorrhizal inoculation than Cv. Wady $Otbah_1$.

For number of tillers/plant, the average was increased from 3.00 in the non inoculated plants to 4.67 in the mycorrhizal inoculated plants of Cv. Viola

with an increase of 55.56% relative to the control. On the other hand, the trait was increased from 2.50 in the non inoculated plants to 3.17 in the mycorrhiza inoculated plants of Cv. Wady Otbah$_1$ with an increasing of 26.67%. These results indicated that Viola plants had a greater response for mycorrhizal inoculation than Cv. Wady Otbah$_1$.

As for shoot fresh weight, the average was increased from 1.37 g in the non inoculated plants to 2.37 g in the mycorrhizal inoculated plants of Cv. Viola with an increase of 73.17% relative to the control. On the other hand, the trait was increased from 1.40 in the non inoculated plants to 2.45 gm in the mycorrhiza inoculated plants of Cv. Wady Otbah$_1$ with an increasing of 75.00%. These results indicated that Viola plants had a greater response for mycorrhizal inoculation than Cv. Wady Otbah$_1$.

In shoot dry weight, the average was increased from 0.22 g in the non inoculated plants to 0.37 g in the mycorrhizal inoculated plants of Cv. Viola with an increase of 69.10% relative to the control. On the other hand, the trait was increased from 0.15 g in the non inoculated plants to 0.25 g in the mycorrhiza inoculated plants of Cv. Wady Otbah$_1$ with an increasing of 66.67%. These results indicated that Viola plants had a greater response for mycorrhizal inoculation than Cv. Wady Otbah$_1$.

In root dry weight, the average was increased from 0.065 g in the non inoculated plants to 0.108 g in the mycorrhizal inoculated plants of Cv. Viola with an increase of 60.00% relative to the control. On the other hand, the trait was increased from 0.057 g in the non inoculated plants to 0.133 g in the mycorrhiza inoculated plants of Cv. Wady Otbah$_1$ with an increase of 42.50%. These results indicated that Viola plants had a greater response for mycorrhizal inoculation than Cv. Wady Otbah$_1$.

In the youngest elongate leaf (YEB), the average was increased from 0.20 g in the non inoculated plants to 0.35 gm in the mycorrhizal inoculated plants of Cv. Viola with an increase of 75.00% relative to the control. On the other hand, the trait was increased from 0.27 g in the non inoculated plants to 0. 37 g in the mycorrhiza inoculated plants of Cv. Wady Otbah$_1$ with an increasing of 37.5%. These results indicated that Viola plants had a greater response for mycorrhizal inoculation than Cv. Wady Otbah$_1$.

The results were in agreement with those of Al-Karaki *et al*. (2004), who reported that biomass and grain yields were higher in mycorrhizal than non-mycorrhizal plots irrespective of soil moisture, and *G. etunicatum* inoculated plants generally had higher biomass and grain yields than those colonized by *G. mosseae* under either soil moisture condition. The mycorrhizal inoculated plants had higher shoot fresh weight than the non-mycorrhizal plants. The improved growth, yield and nutrient uptake in wheat plants were noticed and demonstrate of the potential of mycorrhizal inoculation to

reduce the effects of drought stress on wheat grown under field conditions in semiarid areas of the worldAbo-Doma *et al.* (2008), reported comparable results in a studying of the effect of mycorrhiza inoculation on wheat plant growth using two wheat cultivars, one hexaploid and the second was Tetraploid. . Langenfeld-Heyser *et al*. (2007) reported that mycorrhiza stimulated the growth of the poplar hybrid Populus x canescens and the expression of genes conferring peroxidase but not superoxide dismutase activities. Moreover, element analyses suggested that improved performance of mycorrhizal poplar under salt stress and increased supply of K+.

Molecular analysis

The recorded changes in plant growth and productivity in response to root inoculation with mycorrhiza led to study the internal changes in the plant on the molecular level. The fungus, as detected from the previous studies, occupied various proportions of the inter cellular spaces in the cortical region of plant roots, while its effects extended to the whole plant. This fact led to study the way by which the effects of mycorrhiza inoculation is transferred from roots to different parts of the plant. Cell signaling is probably the way by which the effects of mycorrhiza inoculation are transferred from roots to different parts of the plant. Calcium dependent protein kinase (CDPKs) is a membranous protein which acts as secondary messenger. It is activated by receipting calcium and then it is activates different protein molecules by phosphorelation. The activated proteins could affect finally the expression of different genes. The affected genes could lead to increased photosynthesis rate in the inoculated plants and thus increase plant growth. Other genes could increase the plant tolerance for abiotic stress by increasing the solid substances in plant cells of the inoculated plants.

Calcium dependent protein kinase (CDPKs) .

Calcium dependent protein kinase (CDPKs) is a membrane protein which acts as secondary messenger. It is activated by binding with calcium and then it activates different protein molecules by phosphorelation. The activated protein could affect finally the expression of different genes. The affected genes could leads to increase photosynthesis rate in the inoculated plants and thus enhances plant growth. Other genes could agument the plant tolerance for abiotic stress by increasing the solid substances in plant cells of the inoculated plants. The products of RT-PCR were resolved in 1.4% agarose/ TBE gel. Bands with a length of 675 bp occurred in the four samples with different intensities. As shown in Table (2) and Fig. (3), the results indicated that the expression of the gene conferring calcium dependent protein kinase increased in the inoculated plants as compared to non inoculated ones in both cultivars Viola and Wady Otbah$_1$. The results also showed that the increase was greater in cultivar Viola than Wady Otbah$_1$, reflecting higher

response for mycorrhiza inoculation in viola cultivar than Wady Otbah$_1$. Quantification of the gene expression of this gene using semi quantitative RT-PCR protocol revealed that, in Viola cultivar the gene expression increased by 2.68 folds compared to the control. On the other hand, in the cultivar Wady Otbah$_1$ the gene expression increased by 2.47 folds over the control. These results indicated that mycorrhiza inoculation stimulates cell signaling of the secondary messenger calcium dependent protein kinase (CDPKs).

Weidmann *et al*. (2004) identified 29 plant genes which were upregulated in response to *Medicago truncatula* root inoculation with mycorrhiza. Eleven genes coding plant proteins with predicted functions in transcription were investigated in their relation to early events of symbiotic interactions. Abo-Doma *et al*. (2008) studied the effect of mycorrhiza root inoculation in Tetraploid and hexaploid wheat cultivars and non inoculated ones on the modification of genes expression of calcium dependent protein kinase using northern blot technique. Their results showed an increasing in gene expression of the gene under investigation in the mycorrhiza inoculated plants compared to the non inoculated ones.

Phosphoenol Pyruvate Carboxylase (PEPCs)

Semi quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) of Phosphoenol Pyruvate Carboxylase (PEPCs) was performed as described previously. The products of RT-PCR were resolved in 1.4% agarose/TBE gel. A band with a length of 225 bp was exhibited in the four samples with different intensities. The results are recorded in Table (3) and Fig. (4). The results showed that the gene expression of the gene conferring (PEPCs) increased in the inoculated plants as compared to non inoculated ones in both cultivars Viola and Wady Otbah$_1$. The results also showed that the increase in gene expression was greater in cultivar Viola than Wady Otbah$_1$, reflecting a higher response for mycorrhiza inoculation in viola cultivar than Wady Otbah$_1$. Quantification of the gene expression of this gene using semi quantitative RT-PCR protocol revealed that, in Viola cultivar increased by 3.05 folds over the control. On the other hand, in cultivar Wady Otbah$_1$ increased by 2.94 folds over the control. These results indicated that mycorrhiza inoculation stimulates the photosynthesis of the electron transmitter compounds whose synthesis is conferred by phosphoenol pyruvate carboxilase (PEPCs), which wasprobably reflected on the rate of photosynthesis and thus plant growth.

The results were in agreement with the findings of (Vance and Gantt, 1992), who reported that phosphoenol pyruvate carboxilase (PEPCs) was induced in nodules, and a corresponding gene was found to be transcriptionally activated. A carbonic anhydrase gene (MtC00156), was highly up-regulated at early and late symbiotic stages, as already documented (Coba de la Pena *et al*., 1997; Galvez *et al*., 2000), which could be related to the control of osmolarity.

Abo-Doma *et al*. (2008) studied the effect of mycorrhiza root inoculation in Tetraploid and hexaploid wheat cultivars and non inoculated ones on the modification of genes expression of phosphoenol pyruvate carboxilase (PEPCs) using northern blotting technique. The results showed an increase in gene expression of the gene under investigation in the mycorrhiza inoculated plants as compared to the non inoculated ones.

Proline-5 Carboxylate Synthetase (P-5CS)

Semi quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) of Proline-5 Carboxylate Synthetase (P-5CS) was performed as described previously. The products of RT-PCR were resolved in 1.4% agarose / TBE gel. Three different bands with lengths of 700, 460 and 245 bp were obtained. Two of them with lengths of 700 and 245 bp bands were present in the four samples with different intensities. On the other hand, a band with length of 460 bp was produced as a newly synthesized band in response to mycorrhiza inoculation was produced in the inoculated plants of the two cultivars but not in the non inoculated ones. The results were recorded in Table (4) and Fig. (5). The results showed that the gene expression of the gene conferring (P-5CS) increased in the inoculated plants as compared to non inoculated ones in both cultivars Viola and Wady $Otbah_1$. The results also showed that the increase in gene expression of the gene conferring (P-5CS) was greater in cultivar Viola than Wady $Otbah_1$, reflecting higher response for mycorrhiza inoculation in Viola cultivar than Wady $Otbah_1$. Quantification of the gene expression of this gene using semi quantitative RT-PCR protocol revealed that, in Viola cultivar the gene expression increased from 13.65 in non inoculated plants to 30.75 in the inoculated ones, (2.25 folds of the control). On the other hand, in cultivar Wady $Otbah_1$ the gene expression increased from 14.35 in non inoculated plants to 31.67 in inoculated ones, (2.21 folds of the control). These results indicated that mycorrhiza inoculation stimulates the bio-synthetic passway of proline by induction of P5CS which conferring the production of proline. Which resulte in better growth.

Porras-Soriano *et al*. (2009) reported that inoculating olive plantlets with the arbuscular mycorrhizal fungi (AMF) increased plant growth and the ability to acquire nitrogen, phosphorus, and potassium. AMF-colonized plants also increased in survival rate after transplant.

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Table (1): The average of six yield related traits in the two barley cultivars grown as control and under mycorrhiza treatment.

Trait	Viola			Wady Otbah$_1$		
	Control	Mycorrhizal	%increase	Control	Mycorrhizal	%increase
Plant height	20.88	27.67	32.83	21.33	26.50	24.22
No. tillers/ plant	3.00	4.67	55.56	2.50	3.17	26.67
Shoot fresh weight	1.37	2.37	73.17	1.40	2.45	75.00
Shoot dry weight	0.22	0.37	69.10	0.15	0.25	66.67
Root dry weight	0.065	0.108	60.00	0.057	0.133	42.50
YEB	0.20	0.35	75.00	0.27	0.37	37.50

Table (2): Quantification of calcium dependent protein kinase gene expression in the two barley cultivars under mycorrhiza inoculation and non inoculated plants.

Cultivars	Non inoculated	Inoculated	Folds of increasing
Viola	7.64	20.45	2.68
Wady Otbah$_1$	9.56	23.64	2.47

Table (3): Quantification of phosphoenol pyruvate carboxilase (PEPCs) gene expression in the two barley cultivars under mycorrhiza inoculation and non inoculated plants.

Cultivars	Non inoculated	Inoculated	Foldsof increasing
Viola	5.68	17.33	3.05
Wady Otbah$_1$	4.97	14.65	2.94

Table (4): Quantification of proline5 carboxylate synthetase (P5CS) gene expression in two barley cultivars under mycorrhiza inoculation and non inoculated plants.

Cultivars	Non inoculated	Inoculated	Folds of increasing
Viola	13.65	30.75	2.25
Wady Otbah$_1$	14.35	31.67	2.21

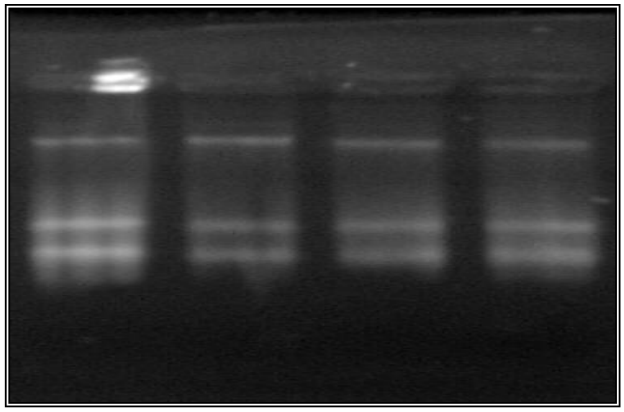

Fig. (1): Electrophoresis on FA denaturing gel for the RNA of the two barley cultivars as mycorrhiza inoculated and non inoculated plants.

Fig. (2): Photos of the two cultivars, (Viola left) and (Wady Otbah$_1$ right) grown as control and under mycorrhiza treatment.

Fig. (3): Quantification of calcium dependent protein kinase gene expression using RT-PCR in the two barley cultivars under mycorrhiza inoculation and non inoculated plants.

Fig. (4): Quantification of phosphoenol pyruvate carboxilase (PEPCs) gene expression using RT-PCR in the two barley cultivars under mycorrhiza inoculation and non inoculated plants.

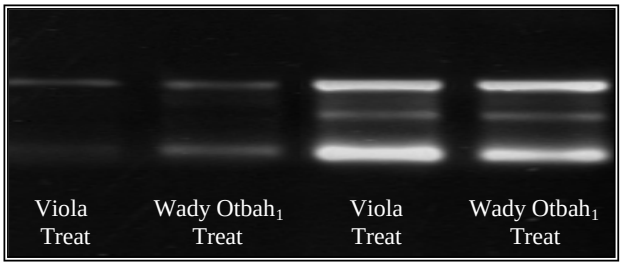

Fig. (5): Quantification of proline 5 carboxylate synthetase (P5CS) in gene expression using RT-PCR in two barley cultivars under mycorrhiza inoculation and in non inoculated plants.