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ORIGINAL ARTICLE

Arbuscular mycorrhizal symbiosis enhances virus accumulation and attenuates resistance-related gene expression in tomato plants infected with *Beet curly top Iran virus*

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Abstract
Beet curly top Iran virus (BCTIV), a member of the genus *Becurtovirus*, is one of the causal agents for curly top disease in tomato plants. Arbuscular mycorrhizal (AM) fungi provide nutrients for host plants and are associated with their improved growth. However, the impact of AM on infection by plant viruses is not well understood. In this study, the interaction between *Funneliformis mosseae* and BCTIV in a susceptible tomato cultivar (Early Eurbana) was investigated. In a completely randomized design experiment, tomato seedlings were inoculated with *F. mosseae*, and after 4 weeks, they were inoculated with an infectious clone of BCTIV. Four treatments were included: untreated control plants (C), BCTIV-infected plants (V), mycorrhizal plants (M) and BCTIV-infected mycorrhizal plants (MV). Results of symptom evaluation based on a disease severity index showed a higher disease severity in MV plants compared to V plants. Supporting this result, a higher level of virus accumulation was observed in MV plants and this became more significant after long-term infection. The expression of three defense-related genes including HSP90, RLK and PRP1 was attenuated in MV plants compared to V plants, which may explain the enhanced symptom production and viral accumulation in these plants. A similar percentage of root colonization by *F. mosseae* in M and MV plants indicated that root colonization was not affected by BCTIV infection. These results show that mycorrhizal symbiosis increases the susceptibility of tomato plants to virus infection and favors BCTIV accumulation and symptom production.

Keywords *Becurtovirus* · *Funneliformis mosseae* · *Geminiviridae* · Gene expression · Mycorrhization

Introduction

Symbiosis with arbuscular mycorrhiza (AM) often enables plants to improve their growth and show a higher tolerance to both biotic and abiotic stresses (Hildebrandt et al. 2007; Pozo et al. 2010).

AM symbiosis plays an important role in plant–pathogen interactions. Reducing the damage caused by some soil-borne pathogenic fungi and nematodes in mycorrhizal plants has been increasingly reported (Whipps 2004; De La Peña et al. 2006; Li et al. 2006). Several mechanisms such as competition with other microorganisms for colonization sites, improvement of plant nutrition, changes in the population and types of root microbial communities and activation of plant defense systems may explain the protective role of AM fungi (Pozo and Azcón-Aguilar 2007; Wehner et al. 2010; Whipps 2004). Activation of plant defense may result in accumulation of reactive oxygen species in plant tissues (Blee and Anderson 2002), regulation of pathogen-related proteins such as glucanase and chitinase (Dumas-Gaudot et al. 2000) and alteration of hormone levels in mycorrhizal plants (Hause et al. 2007; López-Ráez et al. 2010). For example, induction of defense response genes, *PR1*, *PR2* and *PR3*, as well as defense-related genes, *LOX*, *AOC* and *PAL*, in mycorrhizal plants was more rapid and much higher than that in non-mycorrhizal plants in the presence of pathogen infection (Song et al. 2015).

The possible effect of AM symbiosis on pathogens attacking shoots is still uncertain. There are examples of AM

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symbiosis which show reduction of shoot symptoms in mycorrhizal plants infected by phytoplasma (García-Chapa et al. 2004; Lingua et al. 2002), the necrotrophic fungi including *Alternaria solani* (Fritz et al. 2006), *Botrytis cinerea* (Fiorilli et al. 2011; Pozo et al. 2010) and the bacterial pathogen *Xanthomonas campestris* (Liu et al. 2007). The effect of AM symbiosis on plant virus infection is controversial and less studied. A higher titer of RNA viruses has been reported in mycorrhizal plants (Daft and Okusanya 1973; Dehne 1982; Miozzi et al. 2011). For example, Shaul et al. (1999) showed that in mycorrhizal tobacco plants infected by *Tobacco mosaic virus*, more severe symptoms appeared as compared to non-mycorrhizal plants. Similarly, Miozzi et al. (2011) tested the interactions between *Tomato spotted wilt virus* (TSWV) and *Funneliformis mosseae* (syn. *Glomus mosseae*) in tomato plants and found that the level of defense-related genes was attenuated by mycorrhization, causing a higher virus titer in mycorrhizal plants. However, the AM symbiosis has been found to attenuate virus symptoms and reduce virus accumulation in tomato infected with the DNA virus, *Tomato yellow leaf curl Sardinia virus* (TYLCSV) (Maffei et al. 2014).

Beet curly top Iran virus (BCTIV) is a member of the genus *Becurtovirus* (Family *Geminiviridae*) with a circular single-stranded DNA genome (2.8 kb in length) encapsidated within twinned, icosahedral particles (Heydarnejad et al. 2013). BCTIV is transmitted by a leafhopper, *Circulifer haematoceps*, and produces leaf curling, vein swelling, yellowing and stunting in host plants (Kardani et al. 2013; Soleimani et al. 2013). This virus causes a significant yield loss in economically important crops including sugar beet and tomato (Khoshnazar and Eini 2016; Yazdi et al. 2008). The common tomato cultivars are either susceptible or show a range of resistance to BCTIV infection (Khoshnazar and Eini 2016).

One of the host defense responses to geminivirus infection is phosphorylation of viral pathogenesis proteins by host kinases (Shen et al. 2012). Plant kinases affect geminiviral infection by reducing viral DNA accumulation. Phosphorylation of geminiviral pathogenesis proteins such as βC1 protein was found to negatively impact their function (Shen et al. 2012). Similarly, the nuclear shuttle protein from cabbage leaf curl virus interacts with receptor-like kinases to inhibit their kinase activity (Fontes et al. 2004). Therefore, suppression of this antiviral response positively correlates with viral infection rate (Fontes et al. 2004). Other host factors that regulate plant response to biotic stresses are heat shock proteins (HSPs). HSP90 is a conserved and abundant molecular chaperone that was shown to play a role in geminivirus–host interaction (Moshe et al. 2016; Gorovits et al. 2017).

To investigate the impact of the AM symbiosis on infection by DNA viruses, we examined the effect of AM symbiosis on the infection of tomato with BCTIV by testing the effect of root colonization by *F. mosseae*, an AM fungus largely scattered in agricultural and natural ecosystems, on BCTIV accumulation, symptom production and expression of some defense-related genes in tomato plants.

Materials and methods

Biological materials and experimental design

Tomato seeds (cultivar Early Eurbana) were received from Behta Company (Tehran, Iran). This cultivar is susceptible to BCTIV infection (Khoshnazar and Eini 2016) and is a suitable candidate to test the possible effect of AM on BCTIV infection. An infectious clone of BCTIV has been described before (Eini et al. 2016), and fungal spores of *F. mosseae* (strain BEG 119) were provided by Shahrood-Biotech Company (Semnan, Iran).

Tomato seeds were grown in germination trays containing sand, peat moss and perlite. After 3 weeks, seedlings were transferred to 1 L pots containing sterile loamy soil and sand (1:1). Based on a completely randomized design experiment, four treatments were established: control plants that were mock inoculated with *Agrobacterium* containing the empty pBin20 vector (C), BCTIV-infected plants (V), mycorrhizal plants (M) and BCTIV-infected mycorrhizal plants (MV). The M and MV treatments were inoculated with *F. mosseae* by mixing the inoculum (20 spores per gram) with soil. For each treatment, 12 plants in three replicates were tested. Plants were maintained in a green house under 14 h light/10 h dark at 23 ± 3 °C and watered twice a week, once with modified Long Ashton nutrient solution. Four weeks after inoculation with AM fungi, two groups of plants (V and MV) were agroinoculated with *Agrobacterium* cells (OD_{600} = 0.2) containing an infectious clone of BCTIV as described (Khoshnazar and Eini 2016). Plants were monitored for symptom development and sampled at two stages, 21 and 35 days post-inoculation (dpi).

Detection of virus by PCR and testing the viral DNA accumulation by real-time PCR

Total DNA was extracted by the Gem-CTAB method (Rouhibakhsh et al. 2008) from V and MV plants at 21 dpi and then tested for the BCTIV infection using the polymerase chain reaction (PCR) with a specific primer pair, BCP-F/BCP-R (Table 1), to amplify the full-length coat protein gene.

To compare the viral DNA accumulation in V and MV plants by real-time quantitative PCR (qPCR), total DNA was extracted at 21 and 35 dpi from infected plants. For each sample, 100 ng of DNA was used in a reaction containing

Table 1 Oligonucleotide primers used in this study

Primers	Sequences (5′ to 3′)	Accession number
BCP-F	CCAAGCTTAAGGTTAGTTTTAAGCG	KP410285
BCP-R	AAAAGCTTCAGCAATTTCTTCACTTC	
BC4-F	CAACACCAAGGAGGAGTTC	KP410285
BC4-R	TTACGAAATATATATTTTG	
SlHSP90-F	GCACAGGCACTTAGGGACTC	AY368907
SlHSP90-R	CTGAGGTGAGAAGGGCAGTC	
SlKinas-F	TAAAGATGGTGATGGCATGG	XM_010324869
SlKinas-R	CAGGCCTCTTTGGAGTATTC	
PRP-F	CTCATGGTCAATACGGCGAAAAC	XM_019399677
PRP-R	CCTAGCACATCCAACACGAAC	
GAPDH-F	GGCTGCAATCAAGGAGGAA	NM_001279325.2
GAPDH-R	AAATCAATCACACGGGAACTG	

26.6 pmol of BC4-F and BC4-R primers and Absolute QPCR SYBR Green buffer (ABgene). The reactions were carried out in a Rotor Gene 2000 qPCR instrument (Corbett Research). A melting curve was recorded at the end of each run to assess reaction specificity. PCR efficiency was determined using standard curves prepared by serial dilution of specific PCR product from the BCTIV genome. The level of viral accumulation was normalized to that of a reference gene, UBC, using Sl UBC primers (Table 1). The relative accumulation of virus for each sample was calculated using the $2^{-\Delta\Delta Ct}$ method as described by Livak and Schmittgen (2001). Three biological repeats were tested for each treatment. For statistical analysis, the mean of biological replicates was tested by a *t* test ($P < 0.5\%$) using SAS software.

Disease evaluation and data analysis

Symptom production in the inoculated plants was monitored from the second week and evaluated at 21 dpi in both V and MV plants. In the infected plants, disease symptoms were scored using the following scale as suggested by Friedmann et al. (1998): zero for symptomless; one for mild leaf thickening; two for leaf thickening and mild leaf curling; three for yellowing, leaf thickening and severe leaf curling; four for yellowing, leaf thickening, severe leaf curling and stunted plants.

The coefficient of infection (CI), plant disease incidence (PDI) and plant disease severity (PDS) index were calculated as previously described (Arunachalam et al. 2002; Khoshnazar and Eini 2016). In this system of disease evaluation, PDS = sum of numerical rating/(total number of observed × maximum disease grade) × 100; PDI = number of infected plants/total number of plants observed and CI = PDI × PDS. Based on the calculated CI, infected plants (V and MV) were grouped into a specific group of resistant/susceptible plants as suggested by Kanakala et al. (2013). In addition, an analysis of variance for the calculated and normalized (Arc $\sin x^{0.5}$) PDI was used to statistically differentiate (Duncan's multiple range test, $P \leq 0.05$) the reaction of each treatment to BCTIV infection using SAS software (version 9.1) by applying a general linear model.

For biomass evaluation, the aboveground parts of plants from all treatments were harvested at 55 dpi to measure and analyze the fresh and dry weight statistically (Duncan's multiple range test, $P \leq 0.05$).

Mycorrhiza evaluation

Root tissues from mycorrhizal plants (M and MV) were first washed with water and cleared with 10% KOH and then stained with 0.1% (w/v) lactophenol cotton blue overnight and then washed with lactic acid as described by Phillips and Hayman (1970). Randomly selected root segments were cut into one cm pieces and observed under a light microscope. The percentage of root colonization with *F. mosseae* was determined by observing the mycelium, arbuscules or vesicles in these root segments and then dividing the number of colonized root segments to the total number of root segments in each subsample. From each plant, three subsamples were measured. The average of percentage for each treatment was then analyzed statistically.

RNA extraction and RT-qPCR assays

About 150 mg of shoot tissue was ground in liquid nitrogen and used for total RNA extraction. Total RNA was extracted from leaf tissues using RNX-Plus kit (Sinaclon, IRI). DNA contamination was removed using the *DNase* I kit (Ambion, USA) following the manufacturer's instructions. The purified RNA was used for oligo-dT-primed first-strand cDNA synthesis with SuperScript III reverse transcriptase (Vivantis Technologies, Malaysia). Reverse-transcription qPCR (RT-qPCR) assay for the defense-related genes including HSP90, pathogenesis-related protein 1(PRP-1) and a receptor-like protein kinase (RLK) was performed using the prepared cDNA and their specific primers (Table 1). Each reaction

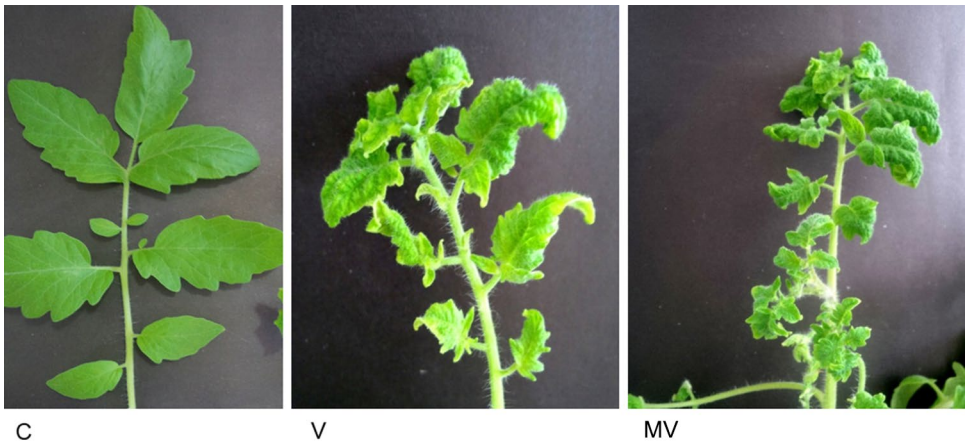

Fig. 1 Tomato plants infected with BCTIV show severe leaf curling symptoms 28 days after infection. A newly emerged leaf from a healthy (c), infected plant (V) and mycorrhizal + virus-infected plant (MV) is shown

contained 7.5 µl SYBR Green Supermix (Bio-Rad), 3 µM of each primer and 1.5 µl of cDNA template. The PCR cycling program consisted of 95 °C for 10 min, followed by 40 cycles at 95 °C for 20 s, 56 °C for 30 s and 72 °C for 30 s ending with a melting curve from 60 to 95 °C with a heating rate of 0.5 °C for 5 s. PCR efficiency was tested by preparing serial dilutions of pooled cDNAs and drawing a standard curve for each gene. The expression level of these genes for each sample was normalized to that of reference gene, GAPDH (glyceraldehyde 3-phosphate dehydrogenase). The relative amount of gene expression for each sample was calculated and analyzed as described above.

Results

Phenotypic responses of mycorrhizal plants to BCTIV infection

Various symptoms were observed in both mycorrhizal and non-mycorrhizal plants infected with BCTIV. Infected plants showed leaf thickening, yellowing, leaf curling and stunting. Only slightly more severe symptoms were observed in the MV plants as compared with V plants (Fig. 1).

In the inoculated plants, V and MV plants, virus accumulation was tested by PCR in the newly emerged leaf tissues at 21 dpi (Fig. S1). Figure S1 shows a representative PCR result for detection of BCTIV in the inoculated V and MV plants. PCR results showed that BCTIV was detected in 83.3% and 75% of MV and V plants, respectively. This means that BCTIV replicates and spreads efficiently to the new leaf tissues in both V and MV treatments. Based on the PCR results, PDI was calculated and then three infected plants were selected for analyzing viral DNA accumulation by qPCR.

A higher CI was recorded for MV plants (Table 2); however, both V and MV plants were grouped as susceptible to the virus infection, based on the grouping system suggested by Kanakala et al. (2013). In this system, the calculated CI for susceptible group was in the range of 30.1–60. In addition, a slightly higher PDS was observed for MV plants as compared to the V plants. The ANOVA for the calculated and normalized PDS index showed no significant ($P < 5\%$) difference between V and MV plants.

Table 2 Coefficient of infection rate (CI) based of the PDI and PDS for virus-infected (V) and BCTIV-infected mycorrhizal (MV) plants obtained at 21 dpi

Treatment	CI	PDI	PDS
V	46.5	75	62
MV	54.9	83.3	66

The CI value between 30 and 60% indicates moderately susceptible plants to the virus infection (Kanakala et al. 2013)

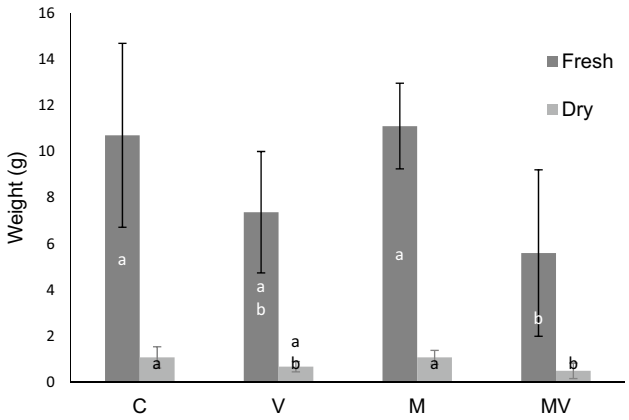

Fig. 2 Biomass of aboveground portions of plants in control plants (C), BCTIV-infected plants (V), mycorrhizal plants (M) and BCTIV-infected mycorrhizal plants (MV), measured 55 days after virus inoculation. The same letters on columns indicate no statically difference ($P < 0.5\%$) for the obtained weight using Duncan's multiple range test. Error bars represent standard deviation

Figure 2 shows that mycorrhization improved the biomass only slightly (not significantly) in tomato plants as compared to control plants. This may indicate that the

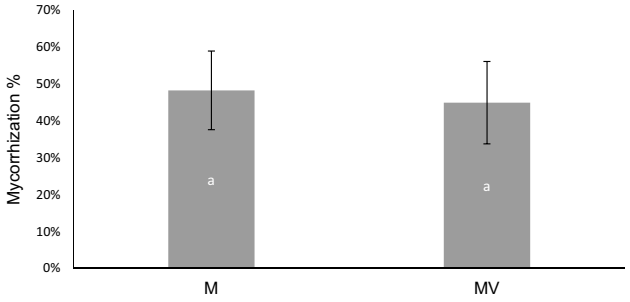

Fig. 3 The percentage of root colonization with *F. mosseae* in M and MV plants. Vertical lines on each bar represent standard deviation. The same letter on the bars shows no statically difference between these groups

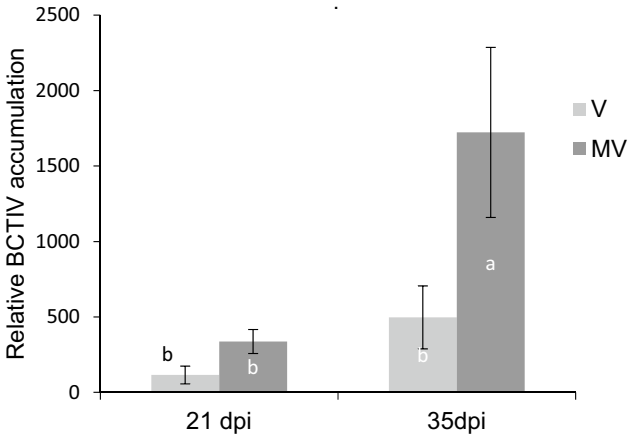

Fig. 4 Analysis by qPCR showed accumulation level of BCTIV in the mycorrhizal plants (MV) and non-mycorrhizal plants (V) at two stages of infection (21 dpi and 35 dpi). For each treatment, three infected plants were tested by qPCR. The error bar shows standard deviation for the three biological replicates for each treatment. The same letters on columns indicate no statically difference ($P < 0.5\%$) for virus accumulation

phosphate content in the provided soil mixture was sufficient to avoid the effect of phosphate shortage in control plants. The biomass of aboveground parts was reduced significantly ($P < 0.05$) in both V and MV plants (Fig. 3). A lower biomass (although not significant) was recorded for MV plants as compared to the V plants (Fig. 3), which is supported by a higher PDS in these plants.

AM fungal colonization was assessed in M and MV plants at the end of the experiment. A similar percentage, 48% and 45%, of root colonization by *F. mosseae* was observed in M and MV plants, respectively (Fig. 3).

Effect of mycorrhiza on the viral DNA accumulation

Comparison of BCTIV accumulation in the V and MV plants by qPCR showed a higher, but not significant ($P < 0.05$) level of viral DNA accumulation in MV plants as compared to V plants at 21 dpi (Fig. 4). However, a clearly higher virus accumulation was observed in MV plants at 35 dpi. The ratio of viral DNA accumulation was 2.9 and 3.5-fold at 21 and 35 dpi, respectively. This result indicates more virus accumulation in long-term infection in MV plants.

Gene regulation in mycorrhizal plants infected with BCTIV

Regulation of selected genes involved in defense mechanisms was tested in shoot of C, V and MV plants by RT-qPCR at 21 dpi when the first BCTIV symptoms became evident. RT-qPCR results show a clear induction of expression of HSP90, RLK and PRP1 in V plants compared to the control plants. The level of change in expression for these genes was reduced in MV plants as compared with that of V plants (Fig. 5).

Discussion

Symbiosis with AM often enables plants to improve their tolerance to both biotic and abiotic stresses (Gernns et al. 2001; Hildebrandt et al. 2007; Pozo and Azcón-Aguilar 2007; Pozo et al. 2010). In a natural ecosystem, tomato plants interact with AM fungi (Beckers and Conrath 2007; Maffei et al. 2014) and it has been reported that among the tested AM fungi, *F. mosseae* has a strong protective role in various pathosystems (Ozgonen and Erkilic 2007; Pozo et al. 2002; Veresoglou and Rillig 2011). Therefore, in this study *F. mosseae* was used to investigate the effects of mycorrhization on the BCTIV infection in tomato. Establishment of mycorrhiza on the plant roots prior to the challenge with pathogens was found essential for bioprotection (Khaosaad et al. 2007; Rosendahl 1985; Slezack et al. 2000). Therefore, in this study tomato seedlings were first inoculated with *F. mosseae* to establish the symbiosis and then inoculated with an infectious clone of BCTIV.

More severe symptom was observed in MV plants compared to V plants. This indicates that tomato plants became more susceptible to BCTIV infection after mycorrhization. This result is in line with a slight reduction in the biomass of MV plant compared to V plants (Fig. 2) and also a higher level of BCTIV accumulation in MV plants compared to V plants (Fig. 4). In addition, in MV plants, a higher level of BCTIV accumulation (118%) was observed at 35 dpi compared to that at 21 dpi by comparing to the level of virus accumulation in V plants in each stage. Therefore, *F. mosseae* symbiosis favors long-term BCTIV accumulation in tomato plants. Similarly, colonization of tomato plants by *F.*

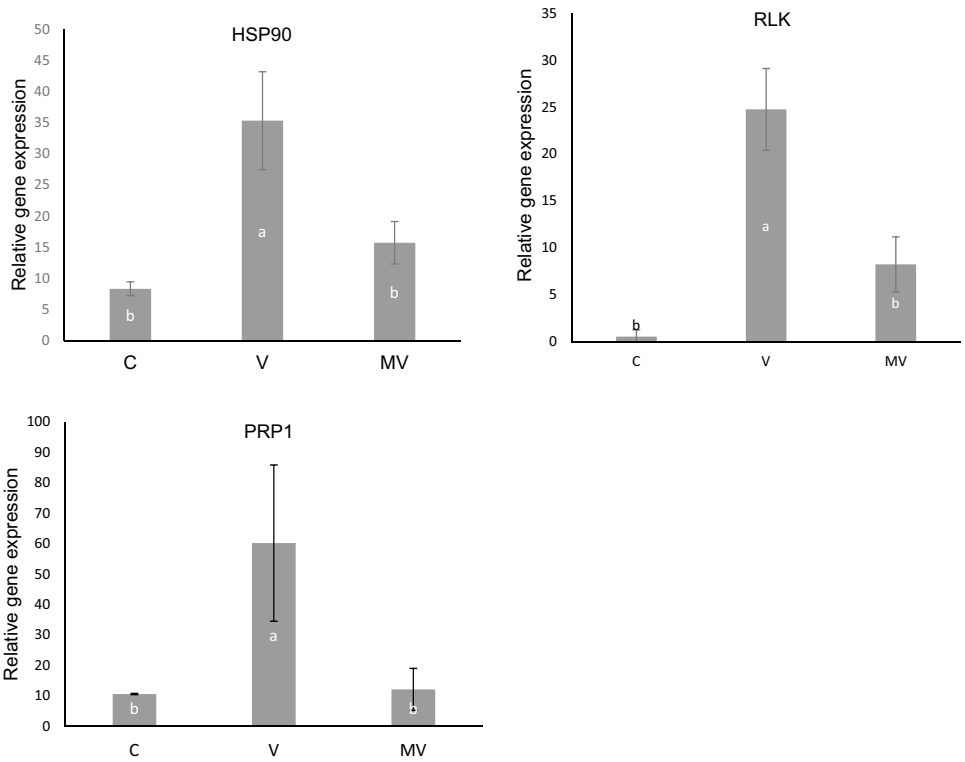

Fig. 5 Expression analysis of resistance-related genes by RT-qPCR in control plants (C), BCTIV-infected plants (V) and BCTIV-infected mycorrhizal plants (MV) at 21 dpi in tomato. Different letters on columns indicate statically different ($P < 0.5\%$) expression levels. Error bars represent standard deviation for the three biological replicates for each sample

mosseae and *Piriformospora indica* was found to enhance long-term accumulation of TSWV (Miozzi et al. 2011) and *Pepino mosaic virus* (Fakhro et al. 2010). Moreover, a higher level of virus accumulation has been observed in mycorrhizal petunia, tomato and tobacco plants infected with *Alfalfa mosaic virus*, *Potato virus x* and TMV, respectively (Daft and Okusanya 1973). Therefore, the higher virus accumulation in MV plants can be a common effect for mycorrhization in plants. Exceptionally, the accumulation of TYLCSV was shown to be attenuated in tomato plants colonized with *F. mosseae* (Maffei et al. 2014), which can be explained by a specific interaction between virus and host cultivar. BCTIV and TYLCSV are from two separated genera in the family *Geminiviridae* and have a clear difference in transmission, host range and pathogenesis (Soleimani et al. 2013; Kardani et al. 2013). Tomato cultivar also can moderate the effect of mycorrhization on virus accumulation. It needs to be noted that in Super Chief, a tomato cultivar resistance to BCTIV infection (Khoshnazar and Eini 2016), root colonization by *F. mosseae* only slightly enhanced the virus accumulation (data not shown). Accordingly, application of AM for improving plant tolerance to abiotic and other biotic stresses can be still applicable by growing more resistant tomato plants to virus infections including BCTIV.

The higher uptake of nutrients, especially phosphorous and nitrogen, into the AM plants is well known (Guether et al. 2009; Javot et al. 2007). It has been demonstrated that increasing phosphorus content in mycorrhizal plants associates with an increase in virus infection in mycorrhizal plants (Borer et al. 2010; Daft and Okusanya 1973). However, providing tobacco plants with phosphorus artificially could not reproduce the increased susceptibility observed in TMV-infected mycorrhizal plants (Shaul et al. 1999). This means that phosphorus uptake is not the sole determinant for susceptibility of mycorrhizal plants to the virus infections.

BCTIV invades all parts of host plants and develops symptoms in the aboveground parts. Mycorrhiza colonization only occurs in roots, but it has been demonstrated to affect non-colonized aboveground parts of plants. This systemic effect was proven by molecular evidences such as gene expression analysis in the aboveground parts of mycorrhizal plants which affects regulation of several genes in leaf (Fiorilli et al. 2009; Liu et al. 2007; Taylor and Harrier 2003) and fruits tissues (Salvioli et al. 2012). For example, a large number of genes which have a role in stress or defense have been shown to be up-regulated in the shoots of mycorrhizal *Medicago truncatula* (Liu et al. 2007). Similarly, RNA-seq analysis in leaf tissues from tomato leave shows that 742 genes including genes in defense priming mechanism displayed differential expression between the mycorrhizal and non-mycorrhizal conditions (Cervantes-Gamez et al. 2015). Searching the differentially expressed genes in this RNA-seq analysis shows that the tested defense-related genes (HSP90, PRP1 and RLK) in our study are not significantly regulated in leaves from mycorrhizal plants compared with non-mycorrhizal plants. However, in tomato plants colonized by *F.*

mosseae, some defense-related genes including genes encoding for PR proteins and WRKY-type binding proteins have been reported to be down-regulated (Fiorilli et al. 2009). Our RT-qPCR results show that the tested defense-related genes (HSP90, PRP1 and RLK) were down-regulated in MV plants compared to V plants. This may at least in part explain a higher viral accumulation (Fig. 4) and infectivity in MV plants. Similarly, in TSWV-infected mycorrhizal plants, several defense-related genes (i.e., genes coding for PR proteins, WRKY transcription factors, HS-related proteins, chitinases and GST) (Catoni et al. 2009) were also attenuated or not activated. This is in line with a lower accumulation and a delay in activation of PR proteins (i.e., PR1 and PR3) in mycorrhizal tobacco (Shaul et al. 1999) which was suggested to be associated with the higher virus infectivity.

Measuring the percentage of root colonization by *F. mosseae* at the end of experiment showed no significant difference between M and MV plants. This indicates that BCTIV infection has no effect on the extension of mycorrhiza colonization. Similarly, it has been found that the percentage of tomato root mycorrhization by *F. mosseae* was not affected by TYLCV (Maffei et al. 2014) and TSWV infection (Miozzi et al. 2011).

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest. The authors of this manuscript had no financial or personal relationships with other people or organizations that could inappropriately influence the contents of this manuscript.

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