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Tomato plants (*Solanum lycopersicum* L.) with mycorrhizal application show specific changes in leaf polyphenol levels

N. S. A. Malik*, A. Nuñez and L. C. McKeever

Eastern Regional Research Center, Agricultural Research Services, US Department of Agriculture, 600 E Mermaid Lane, Wyndmoor, PA, 19038-8598 USA

*Corresponding author e-mail: nasir.malik@ars.usda.gov

ABSTRACT

The objective of this study was to determine if different polyphenols increase in tomato plants treated with mycorrhizal fungi and grown under optimal growth conditions. Three weeks old tomato seedlings were inoculated with AM fungus *Rhizophagus intraradices*. After 8 weeks, the plant height and weights of roots and shoots were measured, and leaf samples were taken for extraction, analysis, identification and quantitation of polyphenol. Our studies showed that under optimal water and nutrient availability, there was no difference in plant growth between mycorrhizal and non-mycorrhizal plants, but several polyphenols were significantly ($P<0.05$) increased in mycorrhizal plants compared to uninoculated control plants. The greatest increase was observed in rutin-O-hexoside (47%) while chlorogenic acid levels increased minimally (9%) but significantly. Other polyphenols that included derivatives of kaempferol, rutin, quercetin, and naringen increased in the range 15-30% but each was significant at $p<0.05$. The results prove our hypothesis that inoculation with mycorrhizal fungi increases polyphenol levels, and possibly the quality of produce even when the crop is grown under normal agricultural practices.

Additional index words: Tomato, *Solanum lycopersicum*, polyphenols, polyphenol derivatives, flavonoids, mycorrhizal fungi.

Arbuscular mycorrhizae [AM] species are symbiotic fungi that are ubiquitous to terrestrial plants (Douds et al., 1999; Smith et al., 1997). As plant symbionts, they are known to provide extra water and nutrients to plants through their protruding hyphae that could extend several inches into the soil surrounding plant roots (Auge, 2004). Thus, these plant symbionts are especially considered useful under drought or nutrient deficiencies, especially phosphorus (Baslam et al., 2011; Farzaneh et al., 2009, Hirata, et al., 1988). The increased availability of water and nutrients in soils under various levels of drought or nutrient deficiencies results in increased crop yield compared to uninoculated plants (Allen, et al., 1983; Nelsen et al., 1982; Ruiz-Lozano et al. 1995). In addition to providing extra water and nutrients, AM symbiosis with plants could also enhance the levels of phenolic compounds in host plants (Ceccarelli, et al., 2010; Simmonds, 2003; Shreenivasa, et al., 2011). Increased levels of phenolic compounds in turn could increase resistance to pest and pathogens in mycorrhizal plants (Liu et al. 2007; Pozo and Azcon-Aguilar 2007; Watanarojanaporn, 2011). Thus, there are several reports indicating increased defense response in tomato from the symbiotic association of mycorrhizae (Cordier, et al., 1998; Hussey and Roncadori, 1982; Ren et al. 2010). Also, tomato plants inoculated with mycorrhizal fungi showed increased resistance to pathogens in addition to increased levels of polyphenols (Isman and Duffe, 1982, Pearce et al., 1998).

Besides producing defense responses in plants, polyphenols from various edible portions of plants are known to act as antioxidants and help to reduce or counter cancer risks, cardiovascular ailments, bacterial and viral infections, and boost immune response (Caderon-Montano et al., 2011; Fraga, et al. 2010; Mladenovic et al., 2011; Rio et al., 2010; Soler-Rivas et al. 2000; Yao et al., 2004). Tomato plants also con-

tain a large variety of polyphenols and their derivatives such as, caffeic acid, ferulic acid, naringenin, rutin, kaempferol, and quercetin (Vallverdu-Queralt, et al., 2010). Although increases in total polyphenols in tomato from symbiotic association with mycorrhizae have been reported, changes in the levels of specific polyphenols were not reported (Ulrichs et al., 2008). Therefore, this study was conducted to determine changes in individual or specific polyphenols that occur in tomato plant as result of its symbiosis with arbuscular mycorrhizae (*Rhizophagus intraradices*).

MATERIALS AND METHODS

Mycorrhizae inoculum and colonization. Inoculum of *Rhizophagus intraradices*, was prepared as described previously (Malik et al., 2015a). To determine levels of AM fungi colonization, the roots were stained with trypan blue (0.5%) and checked under the dissecting microscope by the established method in our laboratory (Malik et al., 2014; Phillips, and Hayman, 1970).

Plant Growth. Hybrid tomato seeds (BHN589) of determinate type were purchased from Siegers Seed Company, Holland, Michigan 49424. Approximately, fifty seeds were planted in a 6 inch diameter pot filled with vermiculite moistened with RO (reverse osmosis) water. The seeds were covered with a 1 cm layer of additional moist vermiculite and placed in a growth chamber for germination. The growth chamber was maintained at 14 hr photoperiod; daytime temperatures set at 25°C and nighttime temperature set at 18°C. After germination, the plants were supplied with Hoagland solution (Hoagland et al., 1939) once a week.

Three weeks after planting, the seedlings were transferred from the pot to 66 ml plastic cones (2.5 cm top diameter and 16 cm deep; purchased from Stuewe & Sons Tangent, Oregon) filled with our standard potting mix (1.5 parts vermiculite: 1.5 parts acid washed sand: 1 part washed Turface (Profile Products, Buffalo grove IL): 1 part sieved farm soil. The mix was autoclaved (for 60 min. sterilization time, twice) before pouring into the cones. A single seedling was transferred into each cone. Approximately, 400 spores of *Rhizophagus intraradices*, were added, in a 1 ml suspension in water to each of the 10 replicate cones. The same numbers of replicate cones were kept as controls in which only water was applied without spores. Nutrient solution was added every five days until harvest at 8 weeks.

At harvest, the roots were separated from the stem. The length of the roots and the plant height of each replicate plant were recorded individually (the plant heights being recorded as the highest stretching leaf tip). The leaves were cut from the stem and weighed. Leaves of two replicate plants were combined separately to obtain 3 composite replicate samples of leaves for each treatment and stored at -80°C. The remaining roots from both treatments were stained to determine colonization by mycorrhizae. At the end of experiment the height of the plants were measured from the junction of root to the maximum spread of top leaf. Shoots and roots were also weighed and recorded before separating leaves for polyphenol analyses.

Samples Preparation and Extraction of Phenolic Compounds. Frozen plant samples were individually pulverized in liquid nitrogen as described earlier (Malik et al, 2005a). Polyphenol/flavonoids from the pulverized plant material were extracted in 80% methanol as described before (Malik et al., 2012). The methanol extracts were finally spun at full speed in refrigerated Eppendorf microfuge for half hour and then stored at -80°C until needed for HPLC-MS analysis.

Chromatographic analysis. The chromatographic separation of the methanol extract was performed with a Nano-Acquity (Waters, Milford, MA) ultrahigh performance liquid chromatographer (UHPLC) equipped with an Acquity UPLC BEH C18, 1.7 µm (1x100 mm) column (Waters) maintained at 40oC and running at 60µl/minute. The UHPLC-UV chromatogram was obtained by attaching to the UHPLC instrument an Acquity TUV detector (Waters) set to scan at 280 nm. The solvent gradient started with waters-acetonitrile 95:5 (0.1% formic acid) for 2 minutes and ramped linearly to water-acetonitrile 60:40 (0.1% formic acid) at a final time of 14 minutes, maintained at that solvent composition for 2 minutes and followed with a columns wash of water-acetonitrile 20:80 (0.1%) formic acid) and returning to the initial condition at 20 minutes. A 10 minutes stabilization time was allowed between injections. Samples for the treated and control experiment were combined by mixing 10µl of each with 10µl of a kaempferol solution (internal standard, 5µg/ml). The solvent was removed under nitrogen, followed by resuspension in 50µl of water-methanol 90:10. Three injections of 4 µl were made for each sample for determination of the concentration change according to the peak-height determined by MassLynx v.4.1 software (Waters). The same chromatographic conditions were used for the mass spectrometry analysis.

Mass Spectrometry Analysis. The mass spectrometry analysis was accomplished by connecting the effluent of the UHPLC instrument to a Synapt G1 quadrupole-time of flight mass spectrometer (Waters) operating in the V mode (resolving power of 8,000) and with an electrospray ionization (ESI) probe operated in the negative mode, [M-H]-, and controlled by MassLynx

v.4.1 software (Waters). The instrument parameters were 3.1 kV capillary voltage, 4 V extractor voltage, 300 L/h desolvation gas (N2) flow, and 200°C and 150°C source and desolvation temperatures, respectively. The MS/MS of the deprotonated precursor ions [M-H]- were obtained by collision induced dissociation with argon gas at 0.9 ml/min with the collision energy ramped between 10 to 25 eV.

RESULTS AND DISCUSSION

Table 1 shows data on plant height and fresh weights of shoot and root system in mycorrhizal and control plants. No significant differences were observed in plant heights or weights. These results are not surprising because plants were grown under optimum conditions for water and nutrients, and we have previously reported that under optimum nutrient and water supply there is no increase in plant growth of mycorrhizal plants as compared with uninoculated controls (Malik et al., 2015b). Increases in plant growth has only been observed under water or nutrient stress conditions where presence of protruding hyphae from mycorrhizal plant roots provide more water and nutrients than what is available for control plants which has been reported in a number of cases (Douds et al., 2008; Hirata et al., 1988; Wu and Xia 2006). However, we intentionally used these conditions to

Table 1. The effect of mycorrhizal inoculation on tomato plant height and weight.

Treatment	Plant Height	Plant Weight	Root Weight
Non- Mycorrhizal	20.65 ± 0.62	5.82 ± 0.32	3.15 ± 0.24
Mycorrhiza	19.92 ± 0.88	6.15 ± 0.38	4.17 ± 0.40

No Significant Differences (P<0.05) between Mycorrhizal and Non-mycorrhizal plants.
All values are Average ± SEM

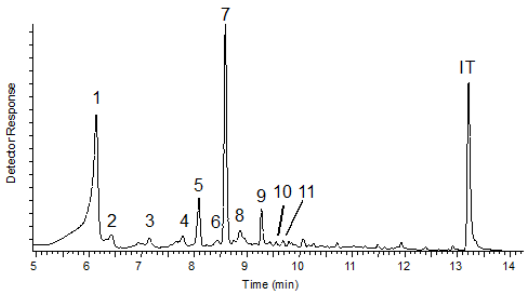

Figure 1. UHPLC elution of polyphenols with UV detection at 280 nm. Kaempferol, labeled as IT, was used as an internal standard for quantification. For peak identification, see Table 2.

Table 2. Identification of polyphenols compounds by mass spectrometry and the percentage increase in their levels in mycorrhizal tomato plants compared to uninoculated controls.

Retention time	Peak #	Compound identified	Percent* increase in mycorrhizal plants	[M-H]-	ΔMass	MS/MS
6.14	1	Neochlorogenic acid	28.83	353.09	-0.01	191
6.44	2	Rutin-O-Hexoside	47.21	771.18	-0.02	609
7.15	3	Chlorogenic acid	9.39	353.09	-0.01	191
7.78	4	Feruloylquinic acid	22.72	529.14	-0.01	367; 191
8.07	5	Rutin-O-pentoside-1	20.85	741.14	-0.03	609; 300
8.44	6	Rutin-O-pentoside-2	16.38	741.19	-0.03	609; 300
8.57	7	Rutin-(quercitin 3-O-rhamnosyl-glucoside)	30.18	609.13	-0.02	300
8.84	8	Quercitin-O-hexoside	20.35	463.08	-0.01	300
9.25	9	Kaempferol-3-O-rutinoside	43.35	593.14	-0.01	285
9.53	10	Kaempferol-3-O-glucoside	17.65	447.1	0.01	281
9.77	11	Naringenin-O-dihexoside	20.79	595.19	0.02	271

Identification was based on the previously published data by Vallverdu-Queralt et al., 2010 & 2011.
*All percent increases are significantly different from controls at P<0.05

demonstrate that mycorrhizal plants may contain more polyphenols even when there is little apparent increase in their plant growth compared to uninoculated control plants.

The UV-chromatogram shown in Figure 1 corresponds to the eluting profile of polyphenols in the sample. In order to identify the compound under the corresponding peak a Q-TOF mass spectrometer was connected to the UHPLC instrument. The analysis was conducted in negative mode and the MS and MS/MS of the specific compound eluting under the peak were compared with the previously identified polyphenols in tomato (Vallverdu-Queralt et al., 2010, and 2011). Accordingly, Table 2 shows the identified compounds with the [M-H]- ion mass and its error (ΔMass), and the main characteristic fragments produced by the MS/MS analysis. We found that peaks # 5 and # 6 have the same spectra and are reported as rutin-O-pentoside 1 and 2 respectively.

The data obtained on changes in polyphenol levels in mycorrhizal plants compared to uninoculated control tomato plants shows that the majority of polyphenols, except for two species, significantly($P<0.05$) increase in mycorrhizal plants (Table 2). The polyphenol rutin-O-hexoside showed the greatest increase (47%) while the rise in chlorogenic acid was minimal (9%) but significant at $p<0.05$. This data supports our hypothesis that mycorrhizal symbiosis could increase specific phenolic compounds even under optimal growth conditions when plant growth in not increased in mycorrhizal plants and is in line with our previous studies on peppers and leeks (Malik et al., 2015a &b).

In general, our findings confirm our hypothesis that several polyphenol species in crop plants increase in mycorrhizal plants even when plants are grown under optimal conditions. These findings are important because polyphenols are known to play important role in plant defenses against pests (Liu et al. 2007; Pozo and Azcon-Aguilar 2007; Watanarojanaporn, 2011). In addition, increased polyphenol levels improve crop quality because polyphenols are known for several health benefits to humans (Caderon-Montano et al., 2011; Fraga, et al. 2010; Mladenovic et al., 2011; Rio et al., 2010; Soler-Rivas et al. 2000; Yao et al., 2004). Thus, while mycorrhizal plants could improve plant productivity under poor water and nutrient availabilities, mycorrhizal plants could also improve crop quality through increased levels of polyphenols under normal growth conditions. The increased levels of polyphenols could increase the nutritive quality of tomatoes.

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