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Mycorrhizal symbiosis produces changes in specific flavonoids in leaves of pepper plant (*Capsicum annum* L.)

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ABSTRACT

In this research, experiments were performed to investigate if mycorrhizal plants grown under optimal growth conditions would improve pepper plant quality compared to the plants that had no mycorrhizal application (untreated control). The results clearly showed that while plants inoculated with mycorrhizae and grown under an optimal nutrient supply did not increase the growth of the pepper plant, they did improve crop quality as several health benefitting polyphenols were found in greater amounts in mycorrhizal plants compared to the untreated controls. Using ultrahigh high performance liquid chromatography (UHPLC) coupled with mass spectrometry, three derivatives of luteolin and two derivatives of apigenin were identified. These two types of compounds significantly ($P<0.05$) increased in plants grown in symbiotic association with mycorrhizae compared to non-mycorrhizal plants, thus confirming the beneficial effects of mycorrhizal symbiosis even in plants grown under non-stressed conditions.

Additional index words: Leeks, mycorrhizae, polyphenols, peppers, *Capsicum annum*, flavonoids.

Various species of arbuscular mycorrhizae [AM] fungus are uniquely competent to form symbiotic relationships with many diverse species of terrestrial plants (Douds and Millner, 1999; Helgason and Fitter, 2005; Remy et al., 1994; Smith and Read, 1997). The protruding hyphae of arbuscular mycorrhizae fungi from the roots of symbiotic plants almost universally help plants to scavenge water and nutrients under drought and/or low nutrient availability (Auge, 2004). Phosphorous (P) is one of the important soil nutrients that mycorrhizae are well-known to provide to plants when phosphorous levels are low in the soil or growth medium (George, 2000). In addition to phosphorus supply, increased uptake of several other plant nutrients have been reported in mycorrhizal plants (Hirata et al., 1988; Smith and Read, 1997). Published data have shown that arbuscular mycorrhizae (AM) fungi could also have positive effects on leguminous plants; including improved nodulation, especially in phosphate deficient soils, by attracting rhizobia to plant roots (Chalk et al 2006). This increase in the availability of water and nutrients under stressful conditions results in increased productivity compared to plants with no mycorrhizal symbiosis, which has been observed in several different plant species (Douds et al., 2007, 2008; Farzaneh et al., 2009; Hirata et al., 1988; Wu and Xia, 2006).

In many cases, plant interactions with symbionts or pathogens (microbes or insects) have resulted in changes in polyphenol levels (Ceccarelli, et al., 2010; Kosuge, 1969; Lattanzio et al. 2006; Rhoades, 1985). Polyphenols and their various derivatives have also been implicated in plant defense mechanisms. Such possibilities for the enhancement of plant defense have been mentioned for mycorrhizal symbiosis (Abdel-Fattah, 2011; Feeny, 1976; Felton et al., 1992; Jones & Klocke, 1987; Kashiwagi et al., 2005). In addition to being involved in plant defenses, a multitude of polyphenol compounds in plants are well known for many health benefits to humans (Cardona, 2013; Duthie et

al, 2003; Yao et al., 2004). In many instances, it is the presence of polyphenols that gives special nutritional value to the fruit or vegetable (Covas et al 2006; Manna et al 2002; Tripoli et al 2005).

Bell peppers are a cherished vegetable throughout the world (The world healthiest foods http://www.whfoods.com/genpage.php?dbid=50&tname=foodspice). In addition to their nutritive value in the form of proteins, carbohydrates, and vitamins, they are well-known to contain compounds such as polyphenols and flavonoids (e.g., quercetin, luteolin, apigenin, caffeic acid, kaempferol etc) which are renowned for health benefitting properties (Bae et al 2014; Ghasemnezhad et al 2011; Park et al 2012). In addition to well-known edible pepper fruits, leaves of pepper plants are also consumed as food in different cultures (Guo et al., 2014).

In our previous study, we discovered that certain flavonoids increase in leeks during plant growth, even under optimal P levels, where the effect of mycorrhiza on growth and yield was minimal (Malik et al., 2015). This study is an extension of our previous work and therefore, the objective of this research is to investigate if mycorrhizal symbiosis would increase or change the levels of different flavonoids in other plants. Therefore, for this purpose we chose a common vegetable plant; i.e., pepper plants which can be easily grown in a limited period of time and space.

MATERIALS AND METHODS

Mycorrhizae inoculum and colonization. Inoculum of *Rhizophagus intraradices* (arbuscular mycorrhizal fungus) was prepared as described previously (Malik et al., 2015). The inoculum was grown for four months on Ri T-DNA transformed carrot roots, using a split-plate culture system (Douds, 2002). The spores were harvested by blending the agar in 10 mM sodium citrate (pH 6.0) and then collected on a 38 µm sieve (Doner and Becard, 1991). The spores from the sieves were removed and quantified by counting under a dissecting microscope at x20 magnification (Using Nikon SMZ 1500 microscope). The pepper roots were stained with trypan blue (at 0.5% w/v) and assessed for fungal colonization under the dissecting microscope, as described by Phillips and Hayman (1970).

Plant Growth. Seeds of peppers, *Capsicum annum* (cv Colossal), were purchased from Siegers Seed Company (Holland, MI). Approximately fifty seeds were planted in a 6 inch diameter pot filled with sterilized vermiculite and moistened with RO (purified by reverse osmosis) water. The seeds were covered with a half inch 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 seedlings were supplied with Hoagland solution (Hoagland and Arnon, 1939) containing low P levels ($1/10^{th}$ the strength of Hoagland solution), once a week.

Twenty-one days after sowing, the seedlings were transferred from the germination trays to 66 ml plastic cones (2.5 cm top diameter and 16 cm deep; purchased from Stuewe & Sons Tangent, Oregon). The cones were 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 mixture was autoclaved (twice sterilized, for 60 min) before use. 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 cones; the control cones were given water without spores. After a week, 12 cones of plants with similar growth pattern were selected, for each of the treated and control sets. Plant nutrient solution (Hoagland and Arnon, 1939) was added every five days until harvest at 8 weeks.

The experiment was a completely randomized design with two treatments as mentioned above. Six replicate plants were used for each treatment. At the time of harvest, the leaves were cut from the stem and weighed for individual plants. Leaves of two replicate plants were combined to obtain 3 composite replicate samples of leaves, which were stored at -80°C for sample preservation until extraction and analysis were conducted. The remaining roots, from both treatments, were stained to determine colonization of roots by the mycorrhizae.

Samples Preparation and Extraction of flavonols. The frozen leaf samples were pulverized in liquid nitrogen, as described earlier (Malik and Bradford, 2005). Polyphenol/flavonoids from the pulverized plant material were extracted in 80% methanol, as described before (Malik, and Bradford, 2005). Finally, the extracts in methanol were spun at 26000 rpm in a refrigerated Eppendorf microfuge (model 5417R, Eppendorf, North America) for a half hour and then the supernatant liquid was stored at -80°C until extraction.

Chromatographic analysis. The chromatographic separation of the 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 40°C and running at 60µl/minute. The UHPLC-UV chromatogram was obtained at 280 nm with an Acquity TUV (Waters) as the detector. The method used for analysis and sample preparation was as previously reported (Malik et al., 2015). Briefly, the UHPLC 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 column wash of water-acetonitrile 20:80 (0.1%) formic acid and returning to the initial condition at 20 minutes, while allowing 10 minutes for stabilization. Samples for the treated and control experiment were individually combined with internal standard by mixing 10µl of each sample 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 in the UHPLC system connected with UV detector.

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, Milford, Massachusetts). This was operated in the W mode (resolving power of 18,000) and with an electrospray ionization (ESI) probe, operated in the positive mode and controlled by MassLynx v.4.1 software (Waters). The instrument parameters and calibration procedures were as reported before (Malik et al., 2015).

RESULTS AND DISCUSSION

Effect of Mycorrhizal Symbiosis in full P on Plant growth. It is a general concept among researchers that mycorrhizae benefits plant growth under nutrient and water stress conditions (Auge, 2004; George 2000). On the other hand, under optimum nutrient levels, with full phosphorus supply (100-200 mg/kg of soil), there is generally little improvement in plant growth of inoculated plants when compared to un-inoculated controls (Douds et al., 2008: Lambert, 1982; Levy et al., 1983). Similarly, in our experiments described here there were no significant (*P>0.05*) difference in plant growth between plants inoculated with mychorrhizae and the untreated control pepper plants (Table 1).

Therefore, the results of our experiments are in agreement with previous findings that mycorrhizal symbiosis does not increase plant growth when optimal levels of P are available. It is, therefore, very important to note that several types or species of polyphenols in pepper leaves were significantly (P<0.05) greater in plants grown in associations with mycorrhizal symbiosis compared to controls under optimal availability of P (Table 2). The specific plant polyphenol species that increased with mycorrhizal symbiosis were separated by UHPLC and the specific peaks that increased are identified by numbers on the profile (Figure 1).

Table 1. Measurements of growth parameters of pepper plants, supplied with full Hoagland solution, inoculated or un-inoculated with mycorrhizae after 8 weeks growth.

Treatment	Plant Height (cm)	Leaf Weight (g)	Root Weight (g)
Inoculated	12.6± 0.52a	1.81±0.31b	3.81 ± 0.59c
Control	12.3 ± 0.24a	1.85 ± 0.21b	3.15 ± 0.22c

No significant differences in plant growth (P>0.05) were detected between mycorrizal inoculated and the control (untreated) plants.

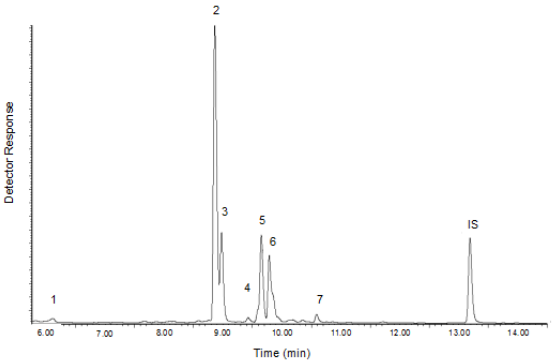


Figure 1. UHPLC elution of polyphenols with UV detection at 280 nm. Kaempferol, labeled as IS, was used as an internal standard for quantification. For peak identification, see Table 3.

Identification of Polyphenols that increased in mycorrhizal plants. Polyphenols eluting under the peaks are numbered in the chromatogram, in Figure 1. These were analyzed using a Q-TOF mass spectrometer. The

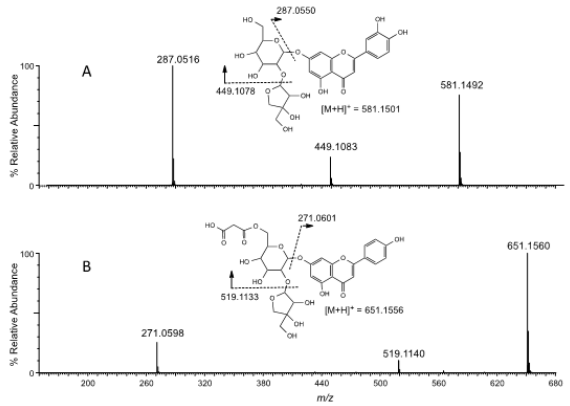


Figure 2. Q-TOF mass spectra and associated structure with the calculated expected fragments mass (dash lines) for A) peak # 2, and B) peak #7.

MS/MS of polyphenols provided direct evidence for the aglycone and the glycosylated sugars. Typically

Table 2. Percentage change in levels of various flavonoids in leaf of mycorrhizal and control pepper plants, which were given Hoagland's solution with Phosphorous at full concentration .

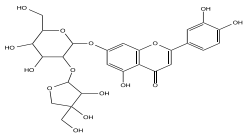
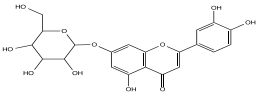
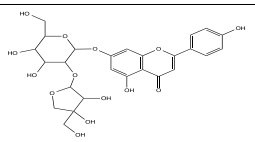
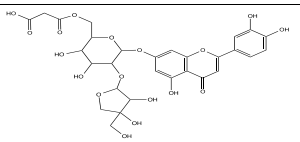
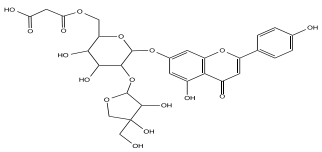
Peak #	Percentage change in mycorrhizal plants compared to un-inoculated controls	
	%increase	%decrease
1	39.35	-
2	9.72	-
3	6.35	-
4	25.84	-
5	7.75	-
6	20.85	-
7	20.62	-

All numbers were significantly different from controls (at P<0.01). The symbol '-' indicates no decrease was recorded.

the spectrum presented the protonated molecule, $[M+H]^+$, with product ions indicating the loss of hexose, 162 Da, or pentose, 132 Da, and a lower product ion that provided the identity of the aglycone moiety after the elimination of the sugars.

For simplicity in this discussion the nominal masses were used, but the spectra and data presented were obtained with a high-resolution Q-TOF instrument with an error <5 ppm, that allowed for the use of the calculated masses of the compounds for identification. Figure 2A is the spectrum of the peak #2 identified in Figure 1, showing a $[M+H]^+$ at *m/z* 581 with the subsequent loses of 132 Da and 162Da, to generate the product ion at *m/z* 449 and 287, respectively, where

Table 3. Retention times and peak # from Figure 1 with the corresponding identification information and the proposed structures from mass spectral analyses.

RT (Peak#)	$[M+H]^+$	Aglycone	Hexose	Pentose	Malonyl	Proposed structure[x]
6:12 (1)	333.158	Unknown				ND
8.86(2)	581.1492	Luteolin	1	1		
8:98 (3)	449.1090	Luteolin	1			
9:42 (4)	479.126	Unknown	1			ND
9:65 (5)	565.1548	Apigenin	1	1		
9:78 (6)	667.1481	Luteolin	1	1	1	
10:58 (7)	651.1560	Apigenin	1	1	1	

[x]Proposed structure based on Mass spectroscopy.

this last product ion is the protonated aglycone. Our literature search indicated that the ion at *m/z* 287, corresponds with luteolin, and the spectrum in Figure 2A is consistent with luteoilin 7-*O*-(2-apiosyl) glucoside compound, whose structure is included in the figure 2 showing the calculated fragments masses. Our determination is consistent and matches the spectrum data previously described (Wahyuni, et al., 2011).

Similarly, the spectrum in Figure 2B, corresponding to the peak # 7 in the chromatogram in Figure 1, shows the $[M+H]^+$ at *m/z* 651 and loss of 132 Da and of 248 Da, indicating a pentose and a 6'-*O*-malonyl-hexose was detected as reported for other polyphenols (Malik et al., 2015; Di Donna et al., 2014). The ion at *m/z* 271 is consistent with apigenin that was reported before in pepper as C-glycosides 40. In this paper, we are proposing that the apigenin is *O*-glycosylated in a similar manner as the luteolin derivative, as shown in the structure in Figure 2B as apigenin 7-*O*-(2-apiosyl)-*O*-6'-malony-glucoside.

Table 3 lists the peaks on the chromatogram in Figure 1, with the proposed structure according to the mass spectrometry data and based on similar structures reported in the literature (Wahyuni et al., 2011). For peaks # 1 and #4, no structures were possible to match the spectra and are therefore reported as unknown compounds. The luteolin derivative was also detected on peak #3, with only one hexose, and on peak # 6, where the hexose has the malonic acid glycosylated at the C6 of the sugar. Apigenin was also identified under peak #5 and is proposed as apigenin 7-*O*-(2-apiosyl)-*O*-glucoside.

In general, our results confirm that even under optimum nutrient supply, mycorrhizal plants may not show significant increase in plant growth and yield compared to un-inoculated control plants. However, the mycorrhizal plants exhibited higher levels of polyphenols. Since polyphenols are well known for their significant health promoting effects, it appears that the mycorrhizal plants grown under non-stressed condition may not increase yield, but would improve crop quality.

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