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The effect of arbuscular mycorrhizal fungi on the phenolic compounds profile, antioxidant activity and grain yields in wheat cultivars growing under hydric stress

Running title: AM fungal effects on phenolics and antioxidants in wheat grains under hydric stress

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Abstract:

BACKGROUND: Hydric stress affects the production of wheat (*Triticum aestivum L.*) worldwide, making some tools necessary to cope with the decrease in rainfall. A sustainable alternative is the use of arbuscular mycorrhizal fungi (AMF) as biofertilisers. Here, we analysed the effects of AMF strains adapted or non-adapted to hyper-arid conditions on the phenolic profiles and antioxidant activities of wheat grains from two cultivars with contrasting tolerance to osmotic stress (Ilustre, moderately tolerant, and Maxi, tolerant) grown with and without hydric stress.

RESULTS: Eight phenolic compounds were detected, being apigenin-C-pentoside-C-hexoside I the most abundant and showing an increase of 80.5% when inoculated with the fungus *Funneliformis mosseae* (FM) obtained from Atacama Desert under normal irrigation with respect to non-mycorrhizal (NM) plants. NM treatments were associated with the higher grain yields. FM showed a noticeable effect on most phenolic compounds, with an increase up to 30.2% in apigenin-C-pentoside-C-hexoside III concentrations under hydric stress with respect to the normal irrigation, being also responsible of high antioxidant activities as the ABTS and DPPH activities.

CONCLUSION: We conclude that inoculation with FM adapted to hydric stress produced improvements in phenolics composition and antioxidant activities in grains of wheat plants growing under hydric stress conditions, improving their food quality and supporting the development of further studies to determine whether the use of adapted AMF could be a realistic tool to improve grain quality in a scenario of increasing hydric stress conditions.

Keywords: Antioxidant activities, apigenin, Arbuscular mycorrhizae, Water stress, *Triticum aestivum*

INTRODUCTION

Wheat (*Triticum aestivum* L.) is one of the most cropped cereals worldwide. In Chile, wheat production is centred in the central and southern areas, where approximately 1.34 million tons year^{-1} are produced in an area of 266,000 ha.[1] Wheat crops are currently strongly affected by climate change, which has led to significantly increasing reductions in rainfall over recent years,[2] resulting in large losses in agricultural production.[3] In this scenario, it is necessary to look for new tools to fulfil the functions that are naturally affected.

Arbuscular mycorrhizal fungi (AMF) are organisms that generate symbioses with the roots of most plant species, with the plants taking advantage of the absorption of nutrients and water and the fungi obtaining carbohydrates from photosynthesis.[4] Moreover, AMF exert several beneficial effects that increase interest in their use as a tool to improve crop productivity given the current climate change.[5] One of the most noticeable benefits of the use of AMF is the increased tolerance to drought based on the improved water absorption, since hyphae can act as a complimentary root system.[6] For instance, in mung bean plants, inoculation with AMF increased the water use efficiency in plants subjected to severe hydric stress,[7] whereas AMF-colonised palm plants were less sensitive to changes in soil moisture.[8] It has also been reported that colonisation by AMF can provide a greater tolerance to drought stress in maize[9] as well as in numerous other species. Whereas, in wheat, the simultaneous application of AMF and water deficit decreased the number of seeds per spike without affecting the biomass of grains, but grains accumulated higher contents of copper, iron, manganese, zinc and gliadins.[10,11] Other benefits of AMF include the improvement of P limitations by reducing the N:P molar uptake ratio,[12] the increase in selenium content in wheat grains,[13] a greater Al tolerance in wheat crops[14] and protection of corn flats against stress due to high temperatures.[15]

In this context, it is necessary to study the response of wheat plants to stress conditions, such as hydric stress. Under unstressed conditions, five classes of phenolic compounds can be found in wheat grains: flavonoids, phenolic acids, lignans, stilbenes and other polyphenols.[16] Specifically, hydroxybenzoic acids (syringic, vanillic, and p-hydroxybenzoic acids), hydroxycinnamic acids (*trans*-caffeic, *trans*-p-coumaric, *trans*-ferulic and sinapic acids), and flavone-C-glucoside derived from apigenin and luteolin have been identified in bran and wheat flour.[17] Under drought conditions, metabolic and biochemical responses lead to an increase in antioxidant enzymes and their substrates,[18] highlighting phenolic compounds that present antioxidant activity.[19] These compounds are normally synthesized during plant development but can also be produced during periods of environmental stress, such as during responses to protect tissues against oxidative damage.[20] Inoculation with AMF can increase the concentration of phenolic compounds, as has been reported in strawberries,[21] onion[22] and lettuce.[23] An increase in antioxidant capacity attributed to phenolic compounds under saline stress and water deficit has been described in lettuce plants.[23,24,] Likewise, under conditions of hydric stress, an increase in phenolic compounds has been reported in corn

and wheat,[25] but these findings have not been focused on the effects that may occur in wheat grains, which is of importance to the food supply. In this regard, we aimed to study the effect of inoculation with AMF strains presumably adapted to aridity obtained from the Atacama Desert on the profiles of phenolic compounds and antioxidants in two wheat cultivars growing under hydric stress conditions. We hypothesised that AMF root colonisation would increase both the antioxidant response and the presence of phenolic compounds even in wheat grains, thus also contributing to the food quality of this essential commodity.

MATERIAL AND METHODS

Reagents

The reagents ABTS (2,2´-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), DPPH (2,2-diphenyl-1-picrylhydrazyl), TROLOX (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxilic acid), neocuproine (≥98%), gallic acid, sodium carbonate and apigenin (≥95%, HPLC grade) were obtained from Sigma-Aldrich (Steinheim, Germany). The reagents water, methanol, ammonia, acetonitrile (HPLC grade), formic acid (p.a. grade), Folin-Ciocalteu, and copper (II) chloride (p.a. grade) were obtained from Merck (Darmstadt, Germany). Ammonium acetate was acquired from J.T. Baker (USA).

Experimental design, growth conditions and sampling

The seed germination of two wheat cultivars (Ilustre Baer and Maxi Baer) was carried out with seedlings in 200 wells, each of which was 10 millilitres (n=36). In a previous screening, we categorised Ilustre as moderately tolerant to osmotic stress, while Maxi was categorised as tolerant to osmotic stress (in preparation). The seed were surface-sterilized using a chloramin-T solution (2%, w:v) during 5 min and washed using sterile distilled water for three times. For the AMF differentiation treatment, three grams of *Funneliformis mosseae* (FM) or *Claroideoglomus claroideum* (CC) inoculum were incorporated into the substrate with the seedlings. Each inoculum was obtained from monosporic cultures using trap pots with a mix of *Tagetes* sp., *Trifolium repens*, and *Avena sativa* as the host plants. After six months, the pots were air-dried, and the substrate was sieved though 500 and 36 µm sieves. With this pre-concentration procedure, we obtained an inoculum with approximately 600 spores per gram. The growth substrate for

germination and for the experiment consisted of a mix of sand, coarse vermiculite and sterile soil at a 25:25:50 (v:v:v) ratio. The soil used was an Andisol from the Mahuidache localty, Freire commune (30° 50' 32'' S, 72° 30' 43'' W; 88 m a.s.l.; Araucanía region, southern Chile). The growth substrate was sterilized by using an autoclave. Pots with a capacity of 1.5 L were filled with the abovementioned mix, and healthy homogeneous plantlets with 21 days of growth after germination (to ensure AMF colonisation) were transferred into the pots. At the transplanting time, a filtrate containing the non-AMF propagules was used to restore the microbial community. For this, a mix of ten gram of each inoculum and non-sterilized soil was mixed with 300 mL sterile distilled water, shaken in an orbital shaker at 120 *xg* for 30 min, and filtered using Whatman filter paper, grade 1. Finally, 10 mL of suspension was applied to each pot.

The treatments established were determined from the full factorial combination of the two cultivars (Ilustre and Maxi), three AMF categories (no AMF, CC and FM), and two types of irrigation (without or with hydric stress), for a total of 2 x 3 x 2 = 12 treatments, with each treatment being performed in triplicate (n=3). Throughout the cropping stage, a normal daily irrigation routine was maintained using a pressurized water sprayer with distilled water by weighing the pots to maintain field capacity (without stress) and the equivalent to 70% of the humidity at field capacity (equivalent to 40% of the water holding capacity). The experiment was maintained under greenhouse conditions, with a 16/8 h light/dark photoperiod and day/night temperatures of 25°C/18°C, in the Departamento de Ciencias Químicas y Recursos Naturales, Universidad de La Frontera, Temuco, Chile. Harvest was carried out once the ears were dry, and the ears were stored at -80°C until analysis. To determine the yield, the number of spikes per plant was counted, and the weights of the spikes and the grains were obtained after separation from the spikes.

Measurement of spores, extraradical hyphae and AMF colonisation

The determination of AMF spores was carried out by wet sieving and decanting using 20 g of rhizosphere substrate through 250 and 53 µm sieves with tap water, according to the methodology described by Sieverding.[26] The number of spores per sample was determined using a stereoscopic magnifying glass at 40-90X magnification. Total extraradical hyphae were assayed using 3 g of substrate after filtering through 250 and 38 µm sieves and following the methodology described by Borie and Rubio.[27] Intersections between

extraradical mycelium and the grid were quantified under a microscope at 100-400X. To quantify the total density of hyphae, Newman's formula was used for the grid line intersection method.[28]

Root colonisation determination was performed after cleaning and staining the roots with trypan blue, as described by Phillips and Hayman.[29] For colonisation, the stained roots were spread on methacrylate plates with a 1 x 1 cm grid, and the intersections between the roots and the grid were counted with a 40-90X stereoscopic magnifying glass. The percentage of root colonisation was obtained from the ratio between the number of roots that had intraradical mycelia and the total number of roots that crossed the grid, according to Giovanetti and Mosse.[30]

Extraction of phenolic compounds

An aliquot of 0.3 g of grains was mashed and mixed with 3 mL of the extraction solvent (methanol:formic acid (95:5, v:v)) in the dark. Then, the samples were sonicated with a 130 Watt Sonics & Materials (Connecticut, USA) ultrasonic processor for 60 seconds at 40% amplitude, shaken for 30 minutes at 200 x*g*, and centrifuged for 10 minutes at 4000 x*g,* and the supernatant were stored at -20°C until use. The prepared extracts were treated in a rotary evaporator (BUCHI Flawil, Switzerland) to remove solvent, resuspended in a mix of water:acetonitrile:formic acid (92:3:5, v:v:v) and filtered through 0.45 μm filters.

Identification and quantification of phenolic compounds

The identification of phenolic compounds was performed by HPLC-DAD-ESI-MS/MS using an HPLC-DAD system coupled to an Applied Biosystem MDS Sciex system QTrap3200 LC/MS/MS mass spectrometer (Foster City, CA, USA), and the quantification was carried out by HPLC-DAD using a Shimadzu HPLC system (Tokyo, Japan) equipped with an LC-20AT quaternary pump, a DGU20A5R degassing unit, a CTO-20A oven, a SIL-20A autosampler and a UV-vis diode array detector (SPD-M20A). The equipment and data were managed by Lab Solutions software (Shimadzu Duisburg, Germany). The analysis was carried out as reported by Santander *et al.*[24] using a Kromasil ClassicShell C_{18} column (100 x 4.6 mm, 2.5 μm) and a Waters Novapak C_{18} pre-column (22x3.9x4 μm). The mobile phases were water:acetonitrile:formic acid (92:3:5, v:v:v) and water:acetonitrile:formic acid (45:50:5, v:v:v) as A and B, respectively, with an elution

gradient between 6% and 50% B over 30 min at 0.55 mL/min and 40°C. Phenolic compounds were quantified at 360 nm using apigenin as standard.

The determination of total phenols was performed using a microplate reader (Epoch™, BioTek Instruments, Winooski, USA) based on the method reported by Parada *et al.*,[21] using gallic acid as a standard. The following reagents were added in the following order to an Eppendorf tube: 15 μL of standard or extract, 750 μL of deionized water, 75 μL of Folin–Ciocalteu reagent, 300 μL of sodium carbonate 20% w:v and 360 μL deionized water. The solutions were incubated at 20°C for 30 min in the dark; then, 250 μL of the solution was added to the microplate wells, and the absorbance was read at 750 nm.

Determination of antioxidant activity

The determination of the antioxidant activity was carried out in a microplate reader (Epoch™, Biotek Instruments, Winooski, USA) using the methods optimised for a 96-well plate described by Parada *et al.* (2019).[21] For the ABTS antioxidant activity method, 245 μL of previously diluted $ABTS^{+}$ 7.5 mM (A1) was added, then 5 μL of the standard or the sample was added and the solution was incubated for 30 minutes at 30°C (A2), and measurements were taken at 734 nm. For the copper-reducing antioxidant capacity (CUPRAC) method, we used a mix containing 50 μL of 10 mM $CuCl_2$, 50 μL of 7.5 mM neocuproine, and 50 μL of 1 M ammonium acetate buffer pH 7. This mixture was left to incubate for 15 minutes at 27°C, then the standard or sample was added and allowed to incubate for another 30 minutes at 27°C. The absorbance was measured at 450 nm. For the 2,2-diphenyl-1-picrylhydrazyl radical scavenging method (DPPH), 240 μL of 0.1 mM DPPH radical dissolved in methanol was added to a 96-well plate (A1), 10 μL of sample or standard was incubated for 30 minutes (A2), and the measurements were taken at 517 nm. For all three methods, Trolox was used as the standard, and the results were expressed as Trolox equivalents.

Statistical analysis

The main effects of cultivar, AMF inoculation, hydric stress, and their multiple interactions were statistically analysed by factorial analysis of variance (ANOVA). Data not meeting statistical assumptions for normality and homoscedasticity were transformed using the ln function, but the results are presented in their original scale of measurement. Treatments with significant differences were analysed using Tukey's HSD as a post

hoc test to compare the means between treatments. The data were also subjected to principal component analysis (PCA) to evaluate the multivariate effect of the established treatments and the relationship between variables. For all the procedures, we considered $P < 0.05$ to be statistically significant. SPSS 22.0 (IBM Corp.) software was used for the analyses.

RESULTS AND DISCUSSION

Spikes and grain biomass production

For the number of spikes per plant (Figure 1A), a decreasing trend was observed under water stress, but significant differences were observed only in the Maxi-FM treatment, with a 50% decrease. Considering the spike biomass (Figure 1B) and the grain biomass (Figure 1C), a similar decreasing trend was observed in the treatment under water stress, which was significant for the Ilustre cultivar, with decreases of 44.2% and 63.4% in spike biomass for the Ilustre-NM and Ilustre-CC treatments, respectively. Moreover, decreases in grain biomass of 43.9%, 50.0% and 70.6% were observed in the Ilustre-NM, Ilustre-FM and Ilustre-CC treatments, respectively.

The results of previous research by Haider *et al.*[31] showed that drought stress in cereals decreased yield parameters, such as the number of fertile tillers, spikelets per spike, number of grains per spike, and grain yield. A decrease in grain yield due to drought has also been observed in wheat, rice, and sesame plants.[32-34] Moreover, in a study in which wheat plants were subjected to heat, drought and combined stress treatments, the most affected yield component was the grain yield, with reductions of 45% when plants were subjected to drought stress.[35] This reduction may be caused by the effects that drought has on grain development, inducing sterility of the spikelets as well as the development of sterile and dysfunctional pollen grains, which ultimately result in a decrease in grain size or a reduction in grain quantity.[36,37] It is known that abscisic acid (ABA) increases under drought conditions, and decreases in grain yield in association with the accumulation of ABA in spikes have been reported.[38,39]

AMF spores, extraradical hyphae and root colonisation

Regarding the number of AMF spores present in the soil (Figure 2A), the Ilustre-FM treatment had the highest quantity, with 571 and 702 spores in 20 mL of substrate for the treatments with normal irrigation and

water stress, respectively, while in the other treatments, there were between 13 and 224 spores in 20 mL of substrate. No viable spores were found in the NM treatments. Moreover, a higher density of AMF extraradical hyphae (Figure 2B) was observed in the Maxi cultivar than in the Ilustre cultivar, showing significant increases of 107.1% and 28.6% in the Maxi-FM and Maxi-CC treatments, respectively, under hydric stress. AMF root colonisation (Figure 2C) presented a marked tendency to decrease under hydric stress, reaching decreases of 35% and 44% in the Maxi-CC and Ilustre-FM treatments, respectively, compared with the treatments under normal irrigation. Previous studies in *Medicago truncatula* plants evidenced that a lower humidity in the substrate did not affect colonisation, which remained similar in all treatments,[6] whereas in palm plants, it has been reported that the intensity of root colonisation decreases when water deficiency increases.[8] Moreover, for maize plants growing under water shortage conditions, contradictory results have been reported, with both increases[9] and decreases[40] under increasing stress conditions. Therefore, it can be assumed that the response of AMF root colonisation within an individual plant species is dependent on several factors.

Identification and quantification of phenolic compounds

Phenolic compounds were identified based mainly on their spectroscopic characteristics, e.g., in UV spectra and MS and MS/MS spectra, and their fragmentation pattern (Table 1). In total, eight phenolic compounds were detected in wheat grains: five flavones, two phenolic acids and one unidentified compound. Into the flavone group, all C-glycosidic conjugates, four pentoside-hexoside derivatives of apigenin were detected (peaks 3-6), all of which showed a pseudomolecular ion of *m/z* 563 and product ions of 353, 383, 473 and 443 amu[41] and one pentoside-hexoside derivative of luteolin (peak 2) with a pseudomolecular ion of *m/z* 579.[41] The phenolic acids were also identified based on the literature; the pseudomolecular ion and fragmentation patterns of caffeoylhexoside (peak 1) were concordant with those reported by Ruiz *et al.*,[42] whereas the derivative of quinic acid (peak 8) was not identified, presenting a neutral loss of 236 amu. Another signal (peak 7) was tentatively assigned as a phenolic compound (probably phenolic acid) according to its UV spectra (maximum at 328 nm, concordant with a phenolic acid), but its fragmentation pattern was not registered.

The phenolic compounds were quantified using apigenin as standard (Tables 2 and 3). Among the eight detected compounds, apigenin-C-pentoside-C-hexoside I presented the highest concentration, reaching 14.98 μg g^{-1} in the Ilustre-FM treatment under normal irrigation, representing a significant increase (80.5%) in this concentration compared to the NM treatment. A similar trend was observed for apigenin-C-pentoside-C-hexoside II, apigenin-C-pentoside-C-hexoside III and the quinic acid derivative, with increases of 11.7%, 71.4% and 61.5%, respectively, also in Ilustre cultivar under FM inoculation, compared to the NM treatment. In addition, the concentrations of apigenin-C-pentoside-C-hexoside I and the quinic acid derivative showed a high correlation (r=0.91). It has been reported that colonisation with *F. mosseae* can modify the metabolism of different biomolecules, such as sugars, lipids, phytohormones or flavonoids, in wheat plants.[43] Positive effects were detected in corn plants under water stress conditions when inoculated with *F. mosseae*, such as an improvement of growth and absorption of nutrients and a decrease in lipid peroxidation.[40] Nevertheless, inoculation with *F. mosseae* did not increase the concentration of phenolic compounds under water shortage conditions.

Hydric stress produced an increase in caffeoylhexoside and apigenin-C-pentoside-C-hexoside III concentrations in the Ilustre-NM treatment, with increases of 4.7% and 30.2%, respectively, with respect to treatment without stress. Moreover, in the Ilustre-FM treatment, an increase of 18.6% was observed for apigenin-C-pentoside-C-hexoside IV relative to the same combination under normal irrigation. Nevertheless, for grains of the Maxi cultivar, in general, no significant differences were observed due to water stress or under inoculation with AMF, which is contrary to observations of wheat leaves, for which a close relationship between the accumulation of flavonoids and tolerance to water stress has been previously reported.[44] In contrast, it has been reported that the concentrations of phenolic compounds, such as phenolic acids and flavonoids, increased under water stress conditions due to their protective effect on plants, which is mainly due to their structure, as they are composed of hydroxyl groups and double bonds that counteract oxidative stress.[45-47]

Total phenol concentrations

Total phenols reached concentrations between 172.7 and 339.9 μg g^{-1} in the Ilustre-NM treatment under normal irrigation and hydric stress, respectively; however, only an increasing trend, without significant

differences, was observed under water stress between treatments, mainly in Maxi cultivar (Figure 3A). The concentrations of the individual phenols quantified by HPLC-DAD were in the range of 6 to 15 μg g^{-1}; therefore, it is likely that other phenolic compounds not detected by reversed-phase chromatography were also contained in the extracts. Other phenolic compounds that have previously been reported in wheat are caffeic, cinnamic, sinapic, p-coumaric and ferulic acids, in both their free and bound forms,[16] with the bound derivatives being the most abundant phenolic acids in grains.[48,49] In addition, gallic and gentisic acids have been found in the bound derivatives, and 4-methoxycinnamic and 3,4,5-trimethoxycinnamic acid derivatives of cinnamic acid, flavonoids such as chrysoeriol, apigenin, luteolin, quercetin and their glucosides and some anthocyanins have also been reported.[50] On the other hand, it is known that Folin-Ciocalteu reagent is not specific for phenolic compounds and can also react with organic substances (sugars, proteins, etc.) and inorganic substances such as Fe II.[51,52]

Regarding the effect of hydric stress on total phenols in wheat grains, it has been reported that the increases are dependent on the genotype and other factors,[53] such as grain size or the proportions of endosperm and bran, since it is known that phenolic compounds are mainly concentrated in bran.[54] Regarding AMF inoculation, under normal irrigation in the Maxi cultivar, there was a tendency for the phenol concentrations to decrease, in contrast to the observations for the Ilustre cultivar. However, other plants inoculated with AMF have shown increases in total phenols, such as strawberries, *Solanum nigrum* and *Digitaria sanguinalis*.[21,55] In this sense, it remains unclear the reasons why in some cases the AMF colonisation preferentially protects plants against oxidative stress by increasing phenolic compound, but contrarily in other cases the AMF-colonised plants show a diminished phenol concentrations, suggesting that the response against oxidative stress in AMF colonized plants is dependent on the functional compatibility at species-specific level.

Antioxidant activity

A decrease in the antioxidant activity under hydric stress conditions, as determined by the ABTS method (Figure 3B), was observed in both cultivars; however, significant differences were observed only in Maxi cultivar under NM and FM treatments. The antioxidant activity determined by the DPPH method (Figure 3C) did not show significant differences between the cultivars or between the treatments, despite also a trend

to decrease in the antioxidant activity was observed under hydric stress; however, for the Ilustre cultivar, a similar trend in total phenols was observed (Figure 3A). This may be due to the higher linear correlation between the Folin-Ciocalteu method and other electron transfer-based assays, such as the DPPH method.[56] Moreover, the CUPRAC method (Figure 3D) presented the lowest antioxidant activity among the three methods used here, being in the range of 0.189-0.334 μmol g^{-1}, without significant differences between the treatments with normal irrigation and hydric stress, while in ABTS and DPPH, the antioxidant activity was found to be between 1-2 μmol g^{-1}. This result suggests that the CUPRAC assay responded better to phenolic compounds, such as flavonols and hydroxycinnamic acid derivatives,[42] while the phenolic compounds detected in wheat grains corresponded mainly to flavones. Similarly, in other studies with durum wheat and wheat bran, strong positive correlations between the total phenol concentrations and antioxidant activity detected with the ABTS and DPPH have been reported.[54,57]

It has been reported that symbiosis with AMF under water stress improves enzymatic and non-enzymatic antioxidant activities to counteract the effects of water deficit in other cereals, such as corn.[40] Enzymatic antioxidant activities also increase in response to stress conditions in wheat plants.[58] On the other hand, under drought stress conditions, antioxidant activity has been shown to increase in mint and cassava plants.[59,60] However, to the best of our knowledge, studies have not focused on the effect of AMF inoculation under hydric stress on non-enzymatic antioxidant activities in wheat grains.

Multivariate analysis

A multivariate analysis was carried out considering both wheat cultivars, AMF inocula and water conditions (Figure 4). Although an association can be observed between five of the identified phenolic compounds, mainly apigenin derivatives, and the antioxidant activities identified by the ABTS and DPPH methodologies (Figure 4A), this trend is not remarkable. This may be because the glycosylated forms of flavonoids have relatively low ABTS and DPPH values.[61] Of the AMF strains inoculated (Figure 4B), the NM treatment was associated with a higher yield, while inoculation with *F. mosseae* was associated with higher concentrations of the individual compounds, mainly the apigenin derivatives and the antioxidant activities identified by the ABTS and DPPH methods. The *C. claroideum* inoculated treatments were mainly associated with CUPRAC activity and phenolic compounds such as luteolin-C-hexoside-C-pentoside and apigenin-C-pentoside-C-

hexoside IV, but this trend was not as clear as that for FM. It has been reported that inoculation with AMF increases the concentration of compounds such as apigenin in chamomile, and this increase is related to the influence of these fungi on plant physiology, because these compounds participate mainly in protection mechanisms, signalling, and defence, among others.[62] Regarding irrigation, the non-stressed condition was associated not only with the concentrations of the individual phenolic compounds and antioxidant activities identified by the ABTS and DPPH methods but also with the yield parameters, while the hydric stress condition was associated with the antioxidant activity identified by the CUPRAC method and a lower number of phenolic compounds (Figure 4C). However, as the antioxidant activity identified by the CUPRAC method was relatively low, it is not clear whether this trend was a response to water stress. Finally, both the wheat cultivars showed a high association with the identified phenolic compounds and antioxidant activities, but there were no clear tendencies regarding the yield parameters (Figure 4D). In general, inoculation with FM under hydric stress had a greater influence on the performance parameters of wheat grains, principally on the concentration of phenolic compounds and on the antioxidant activities determined by the ABTS and DPPH methods, while the cultivar seemed not to significantly influence any of the evaluated parameters. Moreover, there was an evident association between inoculation with FM and root colonisation and spores. Considering that the AMF strain FM used here was isolated from soils in an area with extreme aridity and salinity, our results suggest that its use can provide beneficial effects not only on the vegetative growth of plants but also on the quality of grains. This is an important finding since hydric stress is one of the main effects of climate change on extensive areas of land used to cultivate cereal crops, where the use of efficient biotechnological tools can contribute to partially improving the deleterious effects of hydric stress.

CONCLUSIONS

Among the eight detected phenolic compounds, three apigenin derivatives, including the compound with the highest concentration and a quinic acid derivative, increased in the treatments inoculated with *Funneliformis mosseae*. The colonization with this fungus was also associated with higher antioxidant activities, as determined by the ABTS and DPPH methods. Moreover, the wheat genotype had no influence on yield traits, phenolic compounds or antioxidant activity, despite a slight tendency towards an increase in the Ilustre cultivar (moderately adapted to osmotic stress) compared with that in the Maxi cultivar (adapted

to osmotic stress), which is of importance since the use of efficient fungal strains can modify the quality of wheat grains. Our results suggest that hydric stress was the experimental factor that had the greatest impact on yield, producing a decrease in most of the compounds detected and in the antioxidant activities detected by the ABTS and DPPH methods. Therefore, we suggest that inoculation with arbuscular mycorrhizal fungi (AMF) strains adapted to water shortage (as FM) can benefit wheat grains, an important food source, by increasing their phenolic compound concentrations, also improving beneficial effects in the health of the final consumer. Finally, our results support the need for further studies to validate the finding that certain water stress-adapted AMF have beneficial effects on the profiles of phenolic compounds and antioxidant activities of wheat grains.

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CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interest.

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Figure Captions

Figure 1. Yield parameters of *Triticum aestivum* grains under normal irrigation and hydric stress. (A) Number of spikes per plant, (B) total spike biomass, and (C) grain biomass. NM: non-mycorrhizal, CC: *Claroideoglomus claroideum*, FM: *Funneliformis mosseae*. Different letters indicate significant differences according to Tukey's multiple range test ($P < 0.05$).

Figure 2. Quantification of arbuscular mycorrhizal fungal (AMF) characteristics in *Triticum aestivum* plants growing under normal irrigation and hydric stress. (A) AMF spores in the substrate, (B) extraradical AMF hyphae, (C) AMF root colonisation. CC: *Claroideoglomus claroideum*, FM: *Funneliformis mosseae*. Different letters indicate significant differences according to Tukey's multiple range test ($P < 0.05$).

Figure 3. Total phenolic compound concentrations and antioxidant activity (AA) in *Triticum aestivum* grains under normal irrigation and hydric stress. A) Total phenols determined by the Folin-Ciocalteu method, B) AA determined by the ABTS antioxidant method, C) AA determined by the DPPH (2,2-diphenyl-1-picrylhydrazyl) method and D) AA determined by the CUPRAC (copper reducing antioxidant capacity) method. NM: non-mycorrhizal, CC: *Claroideoglomus claroideum*, FM: *Funneliformis mosseae*, GAE: gallic acid equivalents, TE: Trolox equivalents. Different letters indicate significant differences according to Tukey's multiple range test ($P < 0.05$).

Figure 4. (A) Principal component (PC) scores for the experimental variables determined in grains of two cultivars of *Triticum aestivum* plants inoculated or not with different strains of arbuscular fungi (AMF) and grown under hydric stress or non-limiting water conditions. The percentage values in parentheses indicate the variation explained by each PC. The plot shows the distribution of the experimental individuals according to the PCs and grouped according to (B) the type of AMF inoculum, (C) the water condition, and (D) the wheat cultivar. CC = *Claroideoglomus claroideum*, FM = *Funneliformis mosseae*, NM = Non-inoculated. Drought conditions: HS = Hydric stress, NI = Normal irrigation.

Table 1: Identifications of phenolic compounds in *Triticum aestivum* grains by HPLC-DAD-ESI-MS/MS

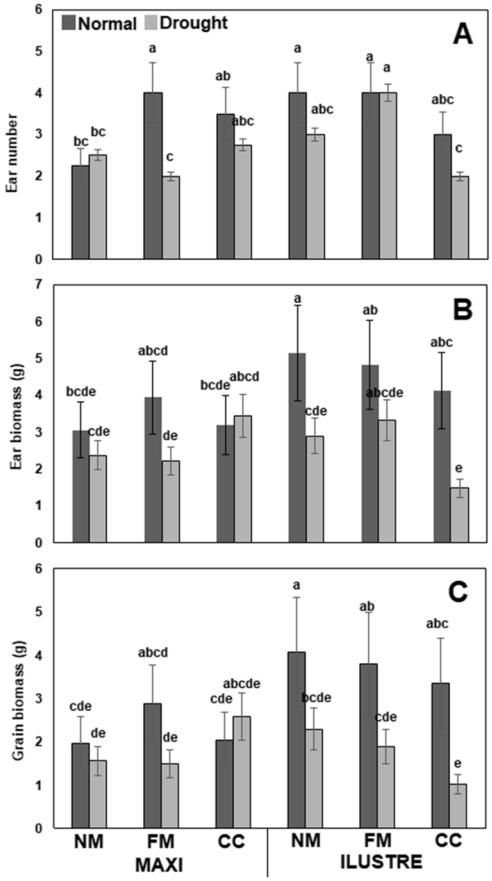
Peak	t_R (min)	Identifications	Λmax (nm)	[M-H]-	Fragment data
1	2.5	Caffeoylhexoside	276	341.0	179.0 (100.0)
2	12.0	Luteolin-C-hexoside-C-pentoside	311	579.1	399.0 (100.0); 369.1 (86.0); 489.0 (50.0); 428.9 (28.0); 458.9 (22.0)
3	12.3	Apigenin-C-pentoside-C-hexoside I	334	563.1	353.0 (66.9); 383.1 (53.7); 473.0 (34.1); 443.1 (12.8); 503.2 (9.6)
4	12.9	Apigenin-C-pentoside-C-hexoside II	334	563.1	353.0 (100.0); 383.2 (48.6); 443.0 (35.6); 473.2 (25.6)
5	13.2	Apigenin-C-pentoside-C-hexoside III	334	563.0	383.1 (100.0); 353.1 (71.0); 443.1 (30.0); 472.9 (23.0); 503.0 (13.0)
6	13.5	Apigenin- C-pentoside-C-hexoside IV	321	563.0	353.0 (82.9); 443.0 (53.6); 382.9 (36.5); 472.9 (21.9)
7	15.3	No identified	328	-	-
8	15.8	Quinic acid derivative	329	427.1	191.0 (100.0)

Table 2. Analytical parameters for HPLC and spectrophotometric methods. Where: DL: detection limit, QL: quantification limit, LR: linear range CV% coefficient of variation.

Method	Standard	Equation	R^2	DL	QL	LR	CV%
Folin	Gallic acid	y = 0.0007x – 0.0002	0.999	3,5 mg L^{-1}	11.7 mg L^{-1}	11.7 - 100 mg L^{-1}	15.7
TEAC	Trolox	y = 0.3583x + 0.003	0.994	0.02 mM	0.07 mM	0.07 - 0.7 mM	6.4
CUPRAC	Trolox	y = 4.4681x + 0.0105	0.993	0.005 mM	0.016 mM	0.016 - 0,7 mM	16.7
DPPH	Trolox	y = 0.6088x + 0.0162	0.996	0.07mM	0.25 mM	0.25 - 0,7 mM	18.7
HPLC	Apigenin	y = 108574x – 159590	0.999	0.845 mg L^{-1}	2.818 mg L^{-1}	0.845 - 175 mg L^{-1}	2.9

Table 3: Individual phenolic compounds concentration (µg g^{-1}) by HPLC-DAD in wheat grains. Where peak 1: Caffeoylhexoside, peak 2: Luteolin-C-hexoside-C-pentoside, peak 3: Apigenin-C-pentoside-C-hexoside I, peak 4: Apigenin-C-pentoside-C-hexoside II, peak 5: Apigenin-C-pentoside-C-hexoside III, peak 6: Apigenin-C-pentoside-C-hexoside IV, peak 7: n.i and peak 8: quinic acid derivative. NM: no mycorrhizal, FM: *Funneliformis mosseae,* CC: *Claroideoglomus claroideum*. NI: normal irrigation, HS: Hydric stress. Different letters indicate significant differences according to Tukey's multiple range test ($P < 0.05$).

Treatment	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6	Peak 7	Peak 8
Maxi NM NI	6.21 ± 0,14 a	7.24 ± 0.85 ab	12.84 ± 0.92 abc	7.29 ± 0.04 a	10.60 ± 1.15 de	7.03 ± 0.44 c	7.46 ± 0.93 abcd	10.18 ± 2.05 ab
Maxi NM HS	6.47 ± 0.19 a	6.84 ± 0.09 ab	13.37 ± 0.25 abc	7.05 ± 0.20 a	11.20 ± 0.13 cd	6.98 ± 0.15 c	8.16 ± 0.10 a	10.89 ± 0.01 a
Maxi FM NI	6.42 ± 0.10 a	6.40 ± 5x10^{-3} b	13.62 ± 0.50 ab	6.92 ± 0.05 ab	11.84 ± 0.39 bcd	6.62 ± 0.02 c	7.96 ± 0.24 ab	10.62 ± 0.35 ab
Maxi FM HS	6.30 ± 0.05 a	6.46 ± 0.45 ab	12.07 ± 0.98 bc	6.70 ± 0.22 ab	10.33 ± 0.60 de	6.36 ± 0.15 c	7.50 ± 0.44 abc	9.67 ± 0.76 ab
Maxi CC NI	6.21 ± 0.08 a	8.69 ± 0.36 a	12.88 ± 0.15 abc	7.18 ± 0.02 a	10.83 ± 0.04 cde	6.83 ± 0.36 c	7.84 ± 0.07 ab	10.56 ± 0.24 ab
Maxi CC HS	6.34 ± 0.15 a	7.33 ± 1.23 ab	12.70 ± 0.72 abc	6.85 ± 0.03 ab	10.77 ± 0.48 cde	6.98 ± 0.15 c	7.91 ± 0.20 ab	10.60 ± 0.41 ab
Ilustre NM NI	6.03 ± 0.05 b	9.06 ± 1.36 a	8.30 ± 1.84 d	6.39 ± 0.04 b	8.63 1.52 e	7.37 ± 0.44 bc	6.42 ± 0.34 e	6.87 ± 0,91 c
Ilustre NM HS	6.32 ± 0.22 a	10.04 ± 2.46 a	11.45 ± 1.71 bc	6.70 ± 0.27 ab	11.24 ± 1.51 cd	7.34 ± 0.83 bc	6.48 ± 0.34 de	8.69 ± 0.86 bc
Ilustre FM NI	6.94 ± 0.22 a	9.18 ± 1.66 a	14.98 ± 0.17 a	7.14 ± 0.58 a	14.79 ± 0.17 a	7.22 ±0.98 bc	7.39 ±0.33 abcde	11.09 ± 0.20 a
Ilustre FM HS	7.02 ± 0.06 a	7.90 ± 0.74 ab	13.59 ± 1.49 ab	7.26 ± 0.43 a	13.52 ± 0.96 ab	8.57 ± 0.03 a	7.15 ± 0.32 bcde	10.33 ± 0.46 ab
Ilustre CC NI	6.73 ± 0.04 a	6.95 ± 3x10^{-3} b	12.61 ± 0.30 abc	7.27 ± 0.01 a	12.87 ± 0.35 abc	8.28 ± 0.01 ab	7.13 ± 0.11 bcde	9.80 ±0.40 ab
Ilustre CC HS	6.70 ± 0.21 a	7.41 ± 0.73 ab	10.91 ± 1.22 c	6.83 ± 0.36 ab	11.48 ± 1.50 bcd	8.72 ± 0.50 a	6.76 ± 0.55 cde	9.16 ± 1.08 ab



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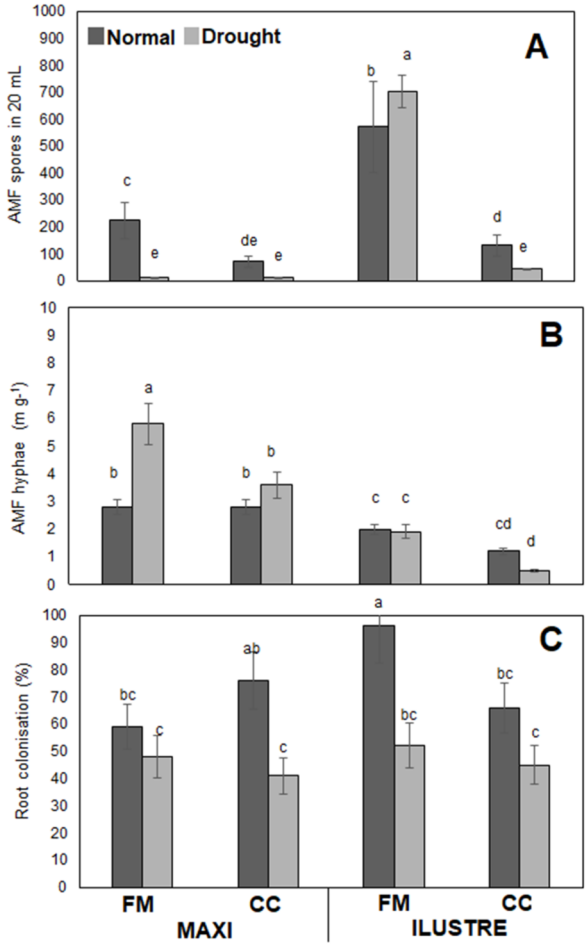

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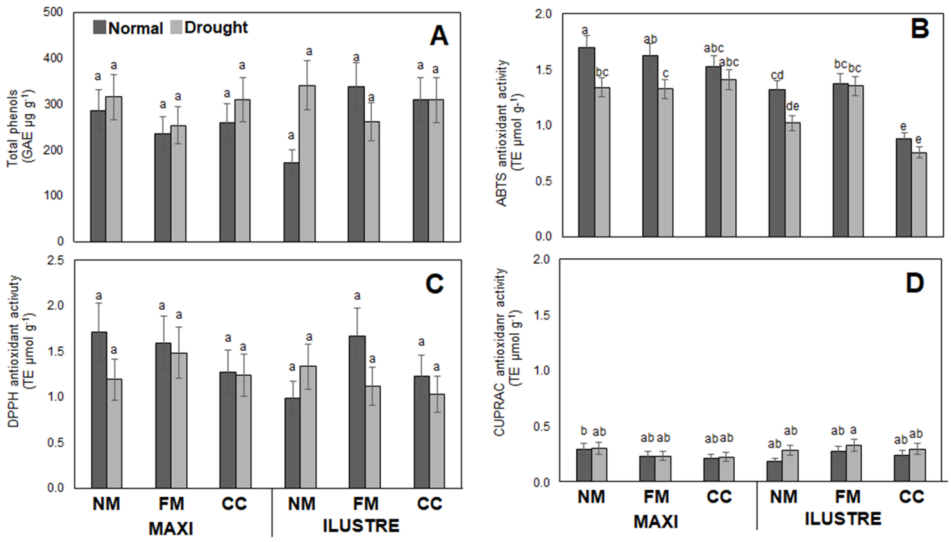

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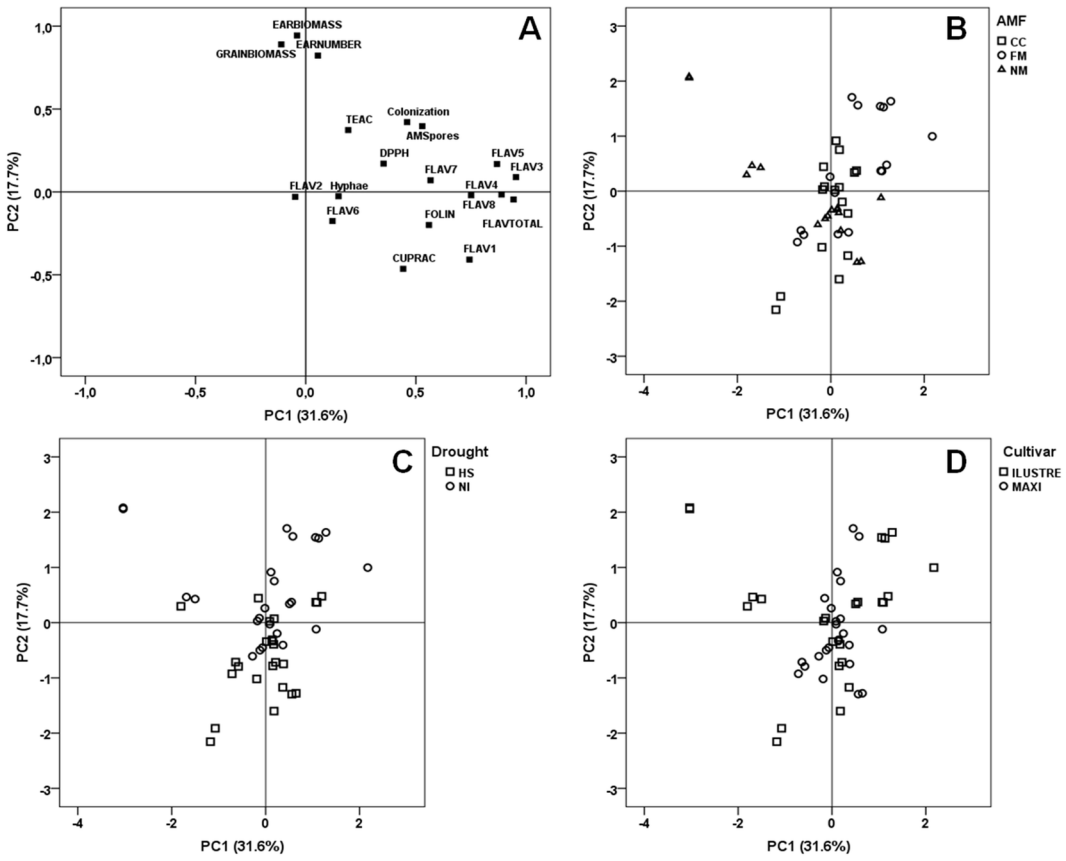

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