Valerio-Landa S *et al.* (2020)
Notulae Botanicae Horti Agrobotanici Cluj-Napoca 48(3):1357-1368
DOI:10.15835/nbha48312033
Research Article

Morpho-physiology and *Pht1* gene expressions in native maize plants with AM fungi and phosphorus

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Abstract

Maize is a crop important worldwide, but its production is limited to phosphorus availability in soil. Plants form a symbiotic association to improve their nutrition with arbuscular mycorrhizal fungi (AMF), which increase to absorption phosphorus (P) and the expression of transporters of the family *Pht1*. Few studies have focused on native maize plants and AMF. Thus, the objective of this study was to determine the morpho-physiological response and expression of phosphate *Pht1* transporters in two native maize plants inoculated with *Claroideoglomus etunicatum* and P concentrations. The height, leaf area, dry biomass, CO_2 assimilation rate, stomatal conductance, transpiration rate, intercellular CO_2, water potential, greenness index, total chlorophyll, and *ZEAma; Pht1;3* and *ZEAma; Pht1;6* transporter expressions in maize plants under P (0.01 and 1 mM) concentrations were evaluated. The results showed that each native maize plant had a differential response in morpho-physiology and transporter expressions when they were inoculated with AMF and P. The response of maize plant was related with its genotype and phenotype plus environmental factor that influenced the AMF-host interaction, mycorrhizal colonization and soil nutrient absorption.

Keywords: *Claroideoglomus etunicatum*; endophyte; qPCR; *ZEAma-Pht1; 3* transporter; *ZEAma-Pht1; 6* transporter

Introduction

Phosphorus (P) represents from 0.1-0.5% of plant dry weight, and it is an element that participates in the synthesis of nucleic acids, phospholipids, membranes, adenosine triphosphate (ATP), and forms part of

Received: 10 Aug 2020. Received in revised form: 08 Sep 2020. Accepted: 10 Sep 2020. Published online: 14 Sep 2020.

enzyme activation/inactivation (Malhotra *et al.*, 2018). Inorganic phosphate (Pi) is the chemical way that plants assimilate this macronutrient. Nonetheless, it shows low mobility and availability in soil due to its ion retention in soil particles charged positively (mineral oxides and clays) and/or to its precipitation in insoluble mineral complexes by its combination with calcium, iron and aluminum (Wu *et al.*, 2020). Low Pi availability has turned out to be a limiting factor for agriculture production globally.

Mexico is the center of maize (*Zea mays* L.) origin, because it has the greatest diversity of native species in worldwide. Nevertheless, one of the main limitations in maize production is low availability of Pi in soil in the tropical/subtropical regions (Lie *et al.*, 2019), which has allowed plants to develop different strategies to improve their nutrition. The strategy that stands out is its symbiotic association with arbuscular mycorrhizal fungi (AMF) (Saia *et al.*, 2020).

As a fundamental soil microbial component, AMF belonging to the phylum Glomeromycotina, are the most common type of mycorrhizal fungi and form mycorrhizal symbioses with 80% of all terrestrial plant species (Spatafora *et al.*, 2016; Cheng *et al.,* 2020; Rimington *et al.*, 2020). Within the main AMF species, those that stand out are *Funilliformis mossea*, *Rhizophagus intraradices*, *Claroideoglomus etunicatum*, among others (Higo *et al.*, 2019a, 2019b, 2020a, 2020b). In this symbiotic association between AMF-plant, the benefits that endophytes obtain are carbon sources that used for synthesizing their lipids and cellular membranes (Thirkell *et al.*, 2020), and the plants increase their defenses toward soil or foliage phytopathogens, mitigate environmental stress, increase water absorption and acquire mineral nutrients, mainly Pi though *Pht1* (Attarzadeh *et al.*, 2019; Jain and Pundir, 2019) gene activation. In plants, the *Pht1* gene family codifies phosphate transporters of high affinity, which participate actively in Pi absorption and transport, expressed preferably in root cells (Victor Roch *et al.*, 2019). Several *Pht1* transporters have demonstrated to be regulated in plants by AMF (Zhang *et al.*, 2019).

Six Pht1 transporters have been characterized in maize plants, of which those that stand out are *ZEAma;Pht1;3* related with direct Pi soil absorption (DPU, Direct Pathway Uptake) and the transporter *ZEAma;Pht1;6* associated with Pi soil absorption through mycorrhizal symbiosis (MPU, Mycorrhizal Pathway Uptake) (Smith *et al.*, 2011). In maize varieties or hybrids, different studies have assessed responses in growth, physiology and expressions of the family of *Pht1* genes associated to AMF colonization (Liu *et al.*, 2016; Liu *et al.*, 2018). Nevertheless, no information has been found on the morpho-physiological expressions of the *Pht1* genes in native maize plants. Therefore, the objective of this study was to determine the differential response in morpho-physiology and expressions of *Pht1* transporters of two native maize plants inoculated with *Claroideoglomus etunicatum* and Pi concentrations in soil.

Materials and Methods

Maize seed and AMF inoculum

Zea mays L. seeds of two native maize plants -catalogued as PEE004-AC and LPO-km100 that belong to the germplasm collection of the School of Agriculture at Universidad Veracruzana, Xalapa, Veracruz, México- were used. The AMF (*Claroideoglomus etunicatum*) inoculum -provided by Universidad Veracruzana- contained as substrate sand and soil (5:1, v/v) and 20 spores g^{-1} of dry soil.

Zea mays seedlings

The seeds were treated with NaClO at 10% for 20 min, then washed thrice with sterile distilled water and deposited in a bedding with sterile sand for 10 days. Subsequently, seedlings with two true leaves were transplanted to 10 L pots previously disinfected with NaClO at 10%, which contained 7 L of a substrate sand and peat moss (5:2, v/v) previously sterilized by autoclaving for 15 min at 120 °C. The amount of phosphorus in the substrate was 59 mg kg^{-1} and determined with the method proposed by Jackson (1976). Plants were kept in a greenhouse for 75 days.

Inoculation of maize with AMF

During transplant, each plant was inoculated with 10 g of substrate (sand and soil, 5:1, v/v) containing *C. etunicatum* and irrigated with K_2HPO_4 using phosphorus as source. The concentrations of 0.01 and 1 mM, equivalent to 0.309 mg L^{-1} and 30.9 mg L^{-1} of P were used. As control treatment, a group of plants was not inoculated with AMF and only fertilized. Ten replicates were used per treatment and the experiment was repeated twice.

Irrigation

Irrigation was performed every other day with Hogland nutritional solution with the following modifications; KNO_3 (6.0 mM), $Ca(NO_3)_2 \bullet 4H_2O$ (4.0 mM), NH_4NO_3 (1.0 mM), $MgSO_4 \bullet 7H_2O$ (2.0 mM), $FeSO_4 \bullet 7H_2O$ (0.15 mM), $Na_2EDTA_2 \bullet H_2O$ (0.09 mM), $MnCl_2 \bullet 4H_2O$ (0.009 mM), H_3BO_3 (0.046 mM), $ZnSO_4 \bullet 7H_2O$ (0.0008 mM), $CuSO_4 \bullet 5H_2O$ (0.0005 mM) and $Na_2MoO_4 \bullet 2H_2O$ (0.0005).

Morpho-physiological variables

At 75 days, the height (cm), leaf area (cm^2), root length (cm) and dry biomass (g) were determined. Furthermore, CO_2 assimilation rate (Λ= μmol $CO_2 m^{-2} s^{-1}$), stomatal conductance (gs= mol $H_2O m^{-2} s^{-1}$), transpiration rate (E= mmol $H_2O m^{-2} s^{-1}$) and intercellular CO_2 (Ci = μmol $CO_2 mol^{-1}$) were measured with a gas exchange analyzer (LCi Photosynthesis System, ADC BioScientific Ltd®). The water potential (MPa) was quantified with a psychrometer (WP4-T, DecagonDevices, Inc.). The greenness index (SPAD units) and total chlorophyll (μg cm^{-2}) were determined with a portable double wavelength-measuring instrument (Minolta® SPAD-502).

Microbiological variables

The number of *C. etunicatum* spores were determined following the technique described by INVAM (2016), and for the mycorrhizal colonization percentage, McGonigle *et al.* (1990) method was used. From each plant per treatment, twenty root fragments of 1 cm were taken, previously stained with trypan blue (Phillips and Hayman, 1970) and observed in the compound microscope (Nikon Eclipse, Model E200). The estimation of the mycorrhizal colonization percentage (%CM) was determined with the following formula: %CM = (# of observations with AMF/# of total observations) x 100.

Pht1 gene expressions in maize

Five grams of root tissue were collected from each treatment; they were washed with sterile distilled water, wrapped in aluminium foil and stored in liquid nitrogen at -80 °C. RNA extraction was performed by the TRIzol® Reagent (Thermo Fisher Scientific) method. To eliminate the genomic DNA residual, RNA was purified by DNase I (Thermo Fisher Scientific) enzyme. RNA visual integrity was assessed with the methodology described by Aranda (2012). Total RNA concentration ng μL^{-1} was performed by spectrophotometry (NanoDrop, ThermoScientific); 100 ng of total RNA were used for cDNA synthesis starting from the reverse transcriptase enzyme SuperScript® II (Thermo Fisher Scientific); cDNA was stored at -20 °C.

Pht1 gene expression

For the real time quantitative polymerase chain reaction (RT-qPCR) gene expression assays, the reactive SsoFastTMEvaGreen®SuperMix (Bio-Rad) was used. Each reaction contained 5 μL of SsoFastTMEvaGreen®SuperMix (2X); 0.3 μL of each primer (10 μm of *ZEAma:Pht1;3* and *ZEAma:Pht1;6*), 1.5 μL cDNA and the final volume was adjusted to 10 μL with sterile ultrapure H2O. The amplification was one cycle at 95 °C for 30 s, 40 cycles at 95 °C for 5 s and 60 °C for 30 s. To confirm that the primers did not generate unspecific products, a dissociation curve was performed with a cycle of 65 at 95 °C with increments

of 0.5 °C each 5 s, using a real time thermocycler "C1000TouchTM" (Bio-Rad CFX96TM Real-Time System). To normalize expression levels, the eukaryotic initiation factor-4A (EIF4A) (Lin *et al.*, 2014) (Table 1) was used. Amplification efficiency was confirmed with the following formula:

PCR efficiency percentage = $10^{-1/slope}$ x 100

For the relative expression analysis, the $2^{-\Delta\Delta Cq}$ (Livak and Schmittgen 2001) method was performed. The results were expressed as the change in units of a target gene expression level in a group of treatments related to a calibrator group.

Change in expression level = $2^{-\Delta\Delta Cq}$

Where $\Delta\Delta Cq = (Cq_{objetive} - Cq_{endogenous})$ calibrator - $(Cq_{objetive} - Cq_{endogenous})$ treatment

Table 1. Primer list of *Pht1* genes and constitutive gen used in RT-qPCR test

Gene	5'-3' forward primer	5'-3' reverse primer	Size (bp)	Reference
ZEAma;Pht1;3	tgtttccgttctgtctggtgcttgtg	tcccgacggtgacctccgattattta	180	Deng *et al.,* 2014
ZEAma;Pht1;6	cggacgtgagcaaggatgacaa	ggattccacacccccctgtgtagt	180	
EIF4A	cgtccagaggttctacaa	catccttcgccacaatac	180	Lin *et al.,* 2014

Statistical analysis

Data were processed by a one-way analysis of variance (ANOVA). The statistical data analysis was performed using the software STATISTICA v. 10.0 (StatSoft software package, Tulsa, OK, U.S.A.) and Tukey's test ($P<0.05$) was used for comparison of means. Prior to ANOVA, mycorrhization percentages were arcsine-square root transformed.

Results

Effect of P, AMF and native maize plants on morphologic variables

P, AMF, native maize plants and the interaction between AMF-plant increased morphologic variables (Table 2). The highest Pi (+) concentration increased leaf area, root length, dry stem weight, and aerial biomass in the two native maize plants compared to those fertilized with the lowest Pi (-) dose. Moreover, the inoculation of both maize with AMF only increased the leaf area when compared to the plants without AMF. Among the native maize plants, LPO-km100 increased leaf area, dry stem, leaf and root weight, and total aerial biomass compared with maize PEE004-AC. In the AMF × Native Maize Plant interaction, the maize LPO-km100 only increased leaf dry weight (Figure 1). The treatments Pi × AMF, Pi × Native Maize Plant and Pi × AMF × Native Maize Plant did not show differences in morphologic variables.

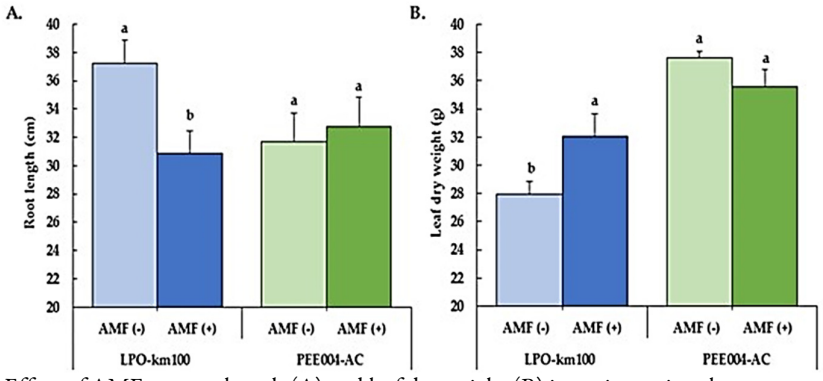

Figure 1. Effect of AMF on root length (A) and leaf dry weight (B) in native maize plants

Data are shown as the mean ± standard deviation (SD) (n = 10). The experiment was repeated twice under greenhouse conditions. Columns with different letters were significantly different according to Tukey's test ($P<0.05$)

Table 2. Effect of P and AMF on morphologic variables of native maize plants

Factor	Height (cm)	Leaf area (cm^2)	Root length (cm)	Dry biomass (g)				
				Stem	Leaf	Root	Air biomass	Total biomass
Pi (mM)								
0.01	156.6a*	5735.6b	28.9b	53.0b	32.4a	35.1a	90.0b	120.5a
1.0	144.2a	6259.0a	37.8a	58.6a	34.1a	32.3a	97.2a	125.0a
AMF								
With	142.1a	6300.4a	33.3a	55.9a	33.8a	34.1a	93.9a	123.8a
Without	158.6a	5694.1b	33.3a	55.7a	32.8a	33.3a	93.4a	121.7a
Native Maize Plant								
LPO-km100	153.6a	5420.3b	34.2a	51.4b	30.0b	26.4b	90.5b	107.8b
PEE004-AC	147.2a	6574.3a	32.4a	60.2a	36.6a	41.0a	96.8a	137.8a
Interaction: Pi×AMF								
Reason-*F*	0.16	0.25	0.68	2.64	0.19	1.98	0.8	3.67
*P*value	0.6915	0.6203	0.4158	0.1137	0.6684	0.1687	0.3789	0.0643
Interaction: Pi×Native Maize Plant								
Reason-*F*	0.34	3.7	0.68	1.58	2.83	2.16	0.25	0.86
*P*value	0.5644	0.0637	0.4158	0.2182	0.1023	0.1516	0.6194	0.3612
Interaction: AMF×Native Maize Plant								
Reason-*F*	0.36	2.36	5.21	0.17	8.38	1.49	1.29	3.4
*P*value	0.554	0.1343	0.0292	0.6867	0.0068	0.2313	0.2652	0.0745
Interaction: Pi×AMF×Native Maize Plant								
Reason-*F*	0.23	0.1	0.04	0.02	3.09	0.01	0.15	0.26
*P*value	0.6313	0.7514	0.8503	0.897	0.0884	0.9421	0.6974	0.6162

*The values are the means ± standard deviation (SD) of ten replicates. Different letters indicate a significant difference (P<0.05) according to Tukey's test

Effect of P, AMF and native maize plants on physiologic variables

The response of the native maize plants and interactions between P-plant and AMF-plant increased physiological variables (Table 3). The maize EPP004-AC with P (+) dose and AMF only increased total chlorophyll (Figure 2a, 2d) with respect to maize LPO-km100. The CO2 assimilation rate and stomatal conductance were increased in the maize LPO-km100 with the lowest Pi (-) dose (Figure 2b, 2c).

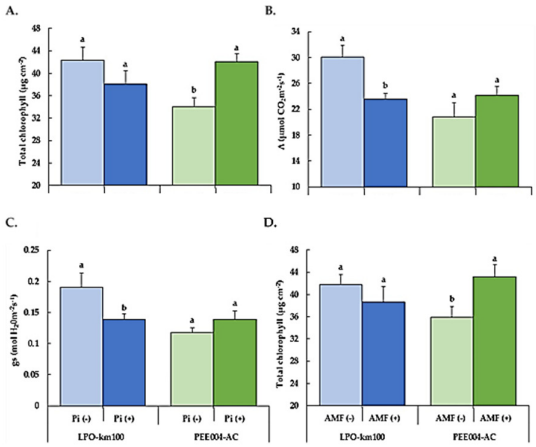

Figure 2. Effect of P and AMF on physiologic variables of native maize plants. Content of total chlorophyll in plants with P (A) and AMF (D). CO_2 assimilation rate (B). Stomatal conductance (C)

Data are shown as the mean ± standard deviation (SD) (n = 10). The experiment was repeated twice under greenhouse conditions. Columns with different letters were significantly different according to Tukey's test (P<0.05)

Table 3. Effect of P and AMF on physiologic variables of native maize plants

Factor	Total chlorophyll ($\mu g\ cm^{-2}$)	Greenness index (SPAD units)	Water potential (MPa)	CO_2 assimilation rate (Λ)	Stomatal conductance (gs)	Transpiration rate (E)	Intercellular CO_2 (Ci)
Pi (mM)							
0.01	40.09a*	42.6a	-2.24a	24.5a	0.150a	3.14a	20.9a
1.0	40.10a	45.3a	-2.23a	24.4a	0.139a	3.01a	14.5a
AMF							
With	40.45a	43.0a	-2.30a	24.4a	0.149a	3.15a	20.5a
Without	39.74a	45.0a	-2.17a	24.5a	0.141a	3.00a	14.9a
Native Maize Plant							
LPO-km100	41.11a	42.7a	-2.21a	26.4a	0.160a	3.37a	17.2a
PEE004-AC	39.08a	45.3a	-2.26a	22.5b	0.129b	2.78b	18.2a
Interaction: Pi×AMF							
Reason-F	0.72	0.22	2.01	0.66	2.05	1.05	2.3a
P value	0.4031	0.6457	0.1662	0.424	0.1623	0.3142	0.1395
Interaction: Pi×Native Maize Plant							
Reason-F	7.69	0.49	1.51	4.38	4.21	3.69	0.04
P value	0.0092	0.4881	0.2277	0.0444	0.0485	0.0637	0.8386
Interaction: AMF×Native Maize Plant							
Reason-F	6.57	0	3.41	1.68	1.53	0.97	0
P value	0.0153	0.9641	0.0739	0.2037	0.2258	0.3312	0.9527
Interaction: Pi×AMF×Native Maize Plant							
Reason-F	0.68	0.01	0	1.59	1.23	1.26	0.12
P value	0.4151	0.909	0.9589	0.217	0.2765	0.2697	0.7342

*The values are the means ± standard deviation (SD) of ten replicates. Different letters indicate a significant difference (P<0.05) according to Tukey's test

Table 4. Microbiological variables of AMF on native maize plants in two concentration of P

Factor	AMF colonization (%)	Number of spores (10 g/soil)
Pi (mM)		
0.01	33.9a	8.1a
1.0	24.7b	5.9a
AMF		
With	58.4a	13.8a
Without	0.2b	0.3b
Native Maize Plant		
LPO-km100	32.0a	8.3a
PEE004-AC	26.6a	5.7a
Interaction: Pi×AMF		
Reason-*F*	8.78	1.57
P value	0.0092	0.2283
Interaction: Pi×Native Maize Plant		
Reason-*F*	0.35	0.88
P value	0.5617	0.3614
Interaction: AMF×Native Maize Plant		
Reason-*F*	2.47	2.45
P value	0.1355	0.1369
Interaction: Pi×AMF×Native Maize Plant		
Reason-*F*	0.24	0.39
P value	0.6311	0.5399

*The values are the means ± standard deviation (SD) of ten replicates. Different letters indicate a significant difference (P<0.05) according to Tukey's tes

Pht1 gene expression in native maize plants inoculated with AMF

ZEAma;Pht1;3 and *ZEAma;Pht1;6* gene expressions in EPP004-AC were significantly greater in fertilized plants with the lowest Pi (-) dose when compared with the fertilized plants with the highest Pi (+) dose (Figure 4). The expression of both genes was not conditioned to the presence or absence of AMF in plants. The lowest expression levels of the *ZEAma;Pht1;3* and *ZEAma;Pht1;6* genes was in the plants fertilized with the highest Pi (+) dose. With respect to LPO-km100, the *ZEAma;Pht1;3* and *ZEAma;Pht1;6* gene expressions were significantly greater with the presence of AMF in plants and the lowest Pi (-) (Figure 5). The lowest expression levels for the *ZEAma;Pht1;3* gene was in the plants fertilized with the highest Pi (+) dose.

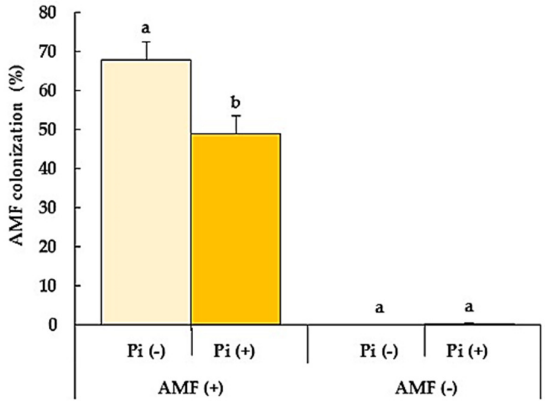

Figure 3. Interaction of P-AMF on mycorrhizal colonization in root of native maize plants

Data are shown as the mean ± standard deviation (SD) (n = 10). The experiment was repeated twice under greenhouse conditions. Columns with different letters were significantly different according to Tukey's test (P<0.05)

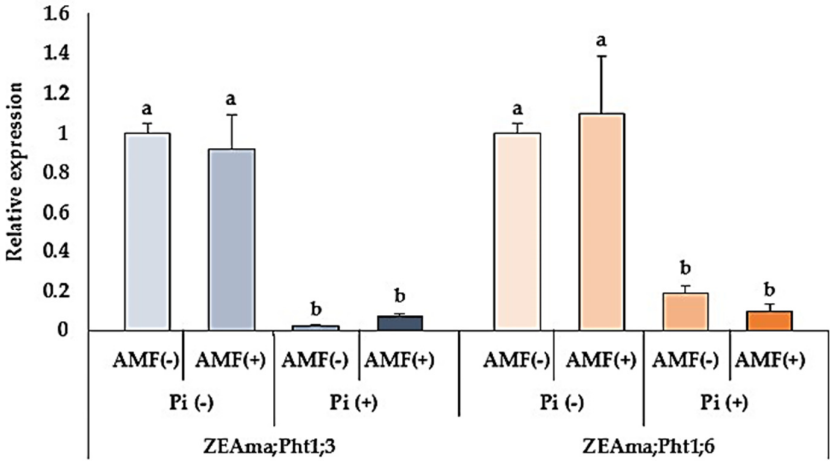

Figure 4. *ZEAma;Pht1;3* and *ZEAma;Pht1;6* genes expression in native maize EPP004-AC plants inoculated with AMF.

Data are shown as the mean ± standard deviation (SD). Columns with different letters were significantly different according to Tukey's test (P<0.05)

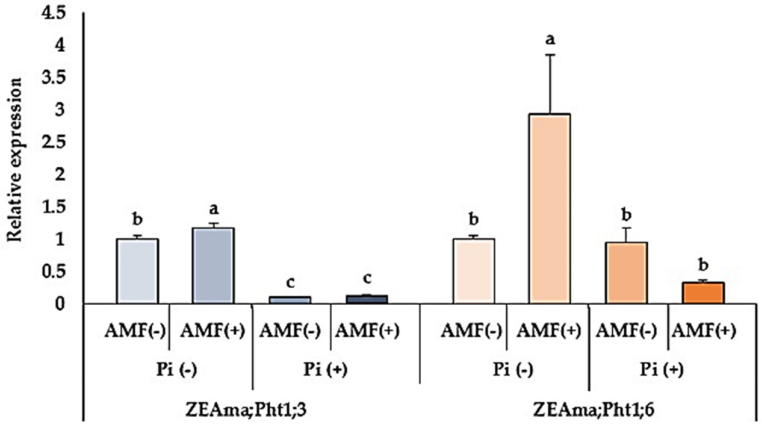

Figure 5. *ZEAma;Pht1;3* and *ZEAma;Pht1;6* genes expression in native maize LPO-km100 plants inoculated with AMF

Data are shown as the mean ± standard deviation (SD). Columns with different letters were significantly different according to Tukey's test (P<0.05)

Discussion

Studies have shown the benefits that AMF contribute to plants, mainly in promoting growth, productivity (Rocha *et al.*, 2019; Scrase *et al.*, 2019), and gene expressions related with P and N absorption, among others (Wu *et al.*, 2020). When plants are colonized by AMF, they increase their capacity of exploring the soil surface and achieving crossing boundaries beyond the nutrient depletion zone, allowing them to absorb more water, macro and micronutrients (especially P, Zn, and Cu) that improve plant vigour (Liu *et al.*, 2018). Nevertheless, the benefits of AMF colonized plants depend mainly on the interaction plant genotype, soil, environment and AMF species, which could affect the symbiotic root-endophyte relationship directly, observing differences in growth response among plants of the same species (Kim *et al.*, 2017). Moreover, in agricultural management practices, tillage and cover cropping such as crop histories can alter the indigenous AMF community structure and diversity in soil and roots (Higo *et al.*, 2018a, 2018b; Morimoto *et al.*, 2018).

In this study, we observed a different response in the *Pht1* gene expression and morpho-physiological parameters of native maize plants when inoculated with AMF because each genotype showed differences in its phenotype and root architecture, which influenced in mycorrhizal symbiosis and the beneficial effects of AMF on plant growth (Londoño *et al.*, 2019). Additionally, among maize genotypes, different plant-dependence levels exist that influence mainly on the host-endophyte interaction and expression of the genes that codify phosphate transporters (Sawer *et al.*, 2017). The physiological response of the inoculated plants with AMF is generally affected during abiotic stress conditions, such as temperature, soil fertility (Mathur and Jajoo, 2020), salinity (Hashem *et al.*, 2019) or hydric stress (Mickan *et al.*, 2019). These conditions have a bearing on chlorophyll concentration, relative leaf water content, gas exchange capacity carbon demand, enzymatic activity, changes in hormonal level, among others (Chandrasekaran *et al.*, 2019). In particular, maize plants inoculated with AMF have shown to increase photosynthetic efficiency and chlorophyll content associated to an increase in soil Mg absorption by the extraradical mycelium in plants colonized by the endophyte (Londoño *et al.*, 2019). Moreover, morpho-physiological maize cultivars of plants synthetically fertilized with P, particularly those that grow on soils with low P content, have increased their acid phosphatase and phytase, peroxidase and superoxide dismutase activities and AMF colonization, showing an influence on mycorrhized plant morphology and physiology (Lie *et al.*, 2019).

The effect of Pi on AMF colonization in maize plants with the highest Pi (+) dose reduced the colonization. Moreover, the application of low P (-) dose increased the association between AMF and host. The high phosphorus concentration in soil induced direct P absorption in plants through root epidermis cells,

autoregulating mycorrhizal colonization starting from a decrease in endophyte carbon delivery to maintain cellular homeostasis (Chu *et al.*, 2020).

Finally, a differential response was observed in the *Pht1* transporter expressions in native maize plants inoculated with AMF. Nagy *et al.* (2009) and Tian *et al.* (2013) discussed that a high P availability in soil for plants eliminates the transcription of *Pht1* phosphate transporters implied in the MPU route, affecting mycorrhizal colonization indexes. In this study, the differences in *Pht1* transporter expressions assessed in native maize plants inoculated with AMF were related with genotype and environmental factors that have an influence on genomic and phenotypic expression in plants during different growth stages (Loth-Pereda *et al.*, 2011). Additionally, root architecture -one of the structures that characterizes each plant- has a direct influence in AMF-host interaction, mycorrhizal colonization and soil nutrient absorption (Deng *et al.*, 2014).

Conclusions

The results in this study showed a differential response in morpho-physiology and *Pht1* transporter expressions of two native maize (PEE004-AC and LPO-km100) plants inoculated with *Claroideoglomus etunicatum* and under Pi in soil. The response of maize plant was related with its genotype and phenotype plus environmental factor that influenced the AMF-host interaction, mycorrhizal colonization and soil nutrient absorption. Subsequent studies focused on genomic and phenotypic response of each maize plant inoculated with AMF should allow knowing in depth the endophyte-host-environment interactome. Furthermore, future research should investigate how and whether different AMF inoculations show functional diversity in Pi uptake by maize.

Authors' Contributions

SVL, RZR, and LGHM, designed the experiment. SVL, EEQA, LLC, and CA contributed in the assembly of experiment in agricultural area, collection of samples and transfer to the laboratory. SVL, EEQA, and LGHM participated with laboratory analysis, data and preparation of manuscripts. CA and PPR contributed with reagents another laboratory and field materials. RZR, PPR, and LGHM analyzed the data. SVL and LGHM wrote and edited the first draft of the manuscript. LGHM wrote the final version of the manuscript. RZR and LGHM are the main thesis advisors of SVL, a Master of Science student. All authors read and approved the final manuscript.

Acknowledgements

This study was funded by CIBNOR project PAZA. Sergio Valerio Landa (SVL) acknowledges financial support given by the National Council for Science and Technology of México (CONACYT) for graduate study. The authors are grateful to Diana Fischer for editorial service in English.

Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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