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GENETICALLY MODIFIED COMMON BEANS WITH RECOMBINANT DEFENSIN *pdf1.2* TOLERANT TO ROOT ROT

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ABSTRACT

Pathogenic fungi are responsible for root rot in common beans. However, an efficient control of this situation has not been possible, either because the genetic diversity of common beans lacks resistance genes or because control methods have a limited effectiveness. Lines of common bean (*Phaseolus vulgaris* L.) cv. Flor de Mayo Anita (FMA) —genetically modified with the defensin gene (*pdf1.2*) from *Arabidopsis thaliana*— were evaluated under confinement conditions to determine their protective capacity against the *Fusarium oxysporum* and *F. lateritium* fungi. Twenty-five plants from five homozygous lines (T4 FMA-*pdf1.2* L2, L3, L4, L7, and L9) were placed in 1-L pots containing Sunshine Mix® No. 6. Then they incubated with 2.0×10^5 conidia mL^{-1} of *Fusarium oxysporum* and, 21 days later, with 2.5×10^5 conidia mL^{-1} of *F. lateritium*. The non-genetically modified controls were FMA common bean plants and cv. Montcalm, incubated and non-incubated with pathogens. The plants were evaluated 21 d after each inoculation, based on a semiquantitative evaluation scale. The evaluation of the plants inoculated with *F. oxysporum* indicated that the five FMA-*pdf1.2* lines showed a significantly lower severity than the control plants; likewise, lines L3 and L9 had a better performance. Meanwhile, *F. lateritium* damaged up to 25 % of the hypocotyl and root tissues of all the FMA-*pdf1.2* common bean lines; this percentage is significantly lower ($p \leq 0.05$) than the damage percentage in the control plants, which reached 50 % of the tissues. There was an inverse correlation between the transcriptional expression levels of the *pdf1.2* gene and the protection degree conferred ($R^2 = -0.93$): the five FMA-*pdf1.2* lines were less severely damaged by *F. oxysporum* and *F. lateritium* (within the tolerance margin).

Keywords: tolerance, protection, broad-spectrum, pathogenic fungi, *Phaseolus vulgaris* L.

INTRODUCTION

The root rot disease of common beans (*Phaseolus vulgaris* L.) is caused by one or more pathogenic fungi; including *Fusarium solani* f. sp. phaseoli (Burk.) Snyd. & Hans., *F. oxysporum*, *F. lateritium*, and *Rhizoctonia solani*, among the most important. They invade the plant through the epidermis, stomata, and wounds (Eke *et al*., 2020). Because of the infection, red lines usually are observed along the base of the hypocotyl; discoloration

and deterioration of both the main and secondary roots can also be observed. This process culminates in the rotting of the infected tissue. In severe cases of the disease, the plant shows visible signs in the aerial part (*e.g.*, chlorosis, defoliation, and low size) (DGSV-CNRF, 2020) and up to 80 % losses in seed yield are recorded (SIAP, 2020).

Derived from the collection of common bean samples carried out in five common bean-producing states in Mexico (Zacatecas, Aguascalientes, San Luis Potosí, Guanajuato, and Querétaro), Montiel-González *et al*. (2005) reported that the three most frequent *Fusarium* species are *F. oxysporum* (39 %), *F. solani* (27 %), and *F. lateritium* (13 %); the latter was reported for the first time as an important pathogen. It was initially mistaken for a low-impact pathogen, because it is a slow-growing species. Additionally, its development and symptoms were hidden by other species with fast-growing characteristics (Sánchez-García *et al*., 2006).

Common bean is a crop that has overcome the recalcitrance condition to obtain complete plants from a single cell under *in vitro* conditions. The most efficient regeneration method is direct organogenesis. Based on these efforts, several researchers have published reports about the development of genetically modified common beans with characteristics such as: tolerance to the glufosinate ammonium herbicide (Aragão *et al*., 2002); resistance to *BGMV* (Bean Golden Mosaic Virus) through iRNA (Bonfim *et al*., 2007); tolerance to *Colletotrichum lindemuthianum* (Espinosa-Huerta *et al*., 2013); tolerance to water stress (Cadena-Hernández *et al*., 2019); and cisgenic lectin rhizosecretion in *P. acutifolius* (Martínez-Alarcón *et al*., 2019), among the most relevant.

The common bean cultivar Flor de Mayo Anita (FMA) was genetically modified with the defensin *pdf1.2* from *Arabidopsis thaliana*. Five lines of FMA-*pdf1.2* were previously evaluated and were resistant to *C. lindemuthianum*, unlike the non-transformed isoline (Espinosa-Huerta *et al.*, 2013). The *pdf1.2* gene encodes a defensin from *Arabidopsis thaliana*. It has a high homology (> 85 %) with genes encoding antifungal proteins and acts at the site of infection or in remote, non-inoculated regions of the tissue. Defensins have a four-stage action mechanism: the electrostatic attraction between the cationic peptide and the anionic membrane of the microorganism; the insertion of the peptide in the membrane of the pathogen; the permeabilization of the membrane through pore formation and cell lysis; and finally, the induction of Ca^{+2} influx and K^{+} efflux when they adhere to fungal hyphae (Iqbal *et al*., 2019; Espinosa-Huerta *et al*., 2019).

Based on the fact that the *pdf1.2* defensin gene can provide protection against pathogenic fungi, the objective of this work was to evaluate five independent lines of FMA-*pdf1.2* common beans, which had initially been characterized by their resistance to anthracnose; in order to establish the resistance (minor damage to the plant and arrival at the flowering stage) or tolerance (damage by the disease and a decrease in the flowering potential) to the development of the infection by the *F. oxysporum* and *F. lateritium* pathogenic fungi, which cause root rot. Then, the broad-spectrum protection of this biotechnological model can be defined, for the control of various species of pathogenic fungi that affect common beans wirh economically important effects.

MATERIALS AND METHODS

Plant material

Five homozygous lines (FMA-*pdf1.2* L2, L3, L4, L7, and L9) of three generations of the cultivar Flor de Mayo Anita (FMA) (Castellanos *et al*., 2003), genetically transformed with the defensin gene *35Sprom:pdf1.2*, were genotypically and phenotypically characterized (tolerance to *Colletotrichum lindemuthianum*) (Espinosa-Huerta *et al*., 2013), as part of the identification of the main characteristics of this biotechnological model in development. These same lines were used in this study (T4) to develop the bioassay with pathogenic fungi that cause root rot.

Characterization and collection of fungal isolates of *Fusarium oxysporum and F. lateritium*

Samples of *Fusarium oxysporum* were collected from the soil of a plot cultivated with maize and common beans, during the 2013 spring-summer cycle, in the Campo Experimental Bajío (CEBAJ) of the Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP), Celaya, Guanajuato (20° 34′ 33.44″ N, 100° 4′ 19.24″ W, altitude 1775 m). Sánchez-Garcia *et al.* (2017) described the sampling method, sample processing, purification and identification, characterizing the type, and level of pathogenicity, as well as the conservation of the pathogenic strains of *F. oxysporum*. The *F. lateritium* pathogen was obtained through sampling carried out in 17 fields cultivated with common beans, in the state of Guanajuato (Montiel-González *et al*., 2005).

Inoculation of common bean plants with *Fusarium oxysporum* and *F. lateritium*

From each FMA-*pdf1.2* common bean line —as well as from the non-genetically modified FMA and Montcalm cultivars—, 25 seeds were used for the bioassay. The seeds were first superficially disinfested with 5 % sodium hypochlorite, followed by three rinses with sterile distilled water, and then they were sown in 50-cavities germinating trays. Finally, the cavities were filled with sterilized vermiculite and moistened with tap water; one seed was placed per cavity. Each seed was covered with 1 cm of vermiculite and incubated in a greenhouse at a temperature between 15 and 32 °C. The seeds received an incidence of solar luminosity through the greenhouse glass roof.

Once the seedlings had fully-opened cotyledonary leaves (approximately 10 d), they were carefully removed from the tray and the root system of the plants was cut by one third. The roots of the seedlings were immersed in a concentration of 2×10^5 conidia mL^{-1} of *F. oxysporum* for 5 minutes; subsequently, they were transferred to 1-L Styrofoam pots with sterilized Sunshine Mix® No. 6 substrate. The plants were fertilized every week with 3 g of urea per pot. Control treatments consisted of FMA and Montcalm plants, either inoculated or submerged in sterile distilled water for 5 min and grown in the same substrate Sunshine Mix® No. 6.

In order to subject common bean lines to high pathogen pressure, while simultaneously distinguishing the damage caused by each *Fusarium* species in successive infections, two inoculations were performed on the same plant. Twenty-one days after inoculation with *F. oxysporum*, the time stipulated to define the potential of a plant against a root pathogen attack, the plants were evaluated according to the severity of the damage; then inoculated with 50-mL of *F. lateritium* (strain Fla-21) inoculum, with a 2.5×10^5 conidia mL^{-1} concentration at the base of the hypocotyl. Some non-modified plants were also inoculated, while others received 50 mL of distilled water (absolute control). The plants were maintained under the same germination conditions and were evaluated 21 d after inoculation. Finally, the surviving plants were maintained until they reached physiological maturity and T5 seed collection.

Evaluation of the damage caused by root pathogens to common bean plants

A semiquantitative evaluation was performed according to the Van Schoonhoven and Pastor-Corrales scale (1987). The severity of the damage to the hypocotyl and root tissue with lesions was evaluated as follows: 1 = No visible symptoms of the disease (0 %); 3 = Slight discoloration, without necrotic lesions or with 10 % of the hypocotyl and root covered with lesions; 5 = Approximately 25 % of the hypocotyl and root tissues are covered with lesions, with severe discoloration, although the tissues remain firm; 7 = Approximately 50 % of the hypocotyl and root tissues are covered with lesions that are combined with softening, rotting, and considerable reduction of the root system; 9 = Approximately 75 % or more of the hypocotyl and root tissues are affected by advanced rotting stages, in combination with a severe reduction of the root system. The severity values of hypocotyl and root tissue with lesions are interpreted as follows: 1 to 3 as resistant (the plant completely overcame and reached the flowering stage); 4 to 6 as tolerant (the plant resisted the effects of the disease without dying, but its flowering potential was reduced by up to 50 %); and 7 to 9 as susceptible (the plant perished in the inoculation bioassay without reaching the flowering stage).

Expression analysis of the *pdf1.2* gene

Total cellular RNA was extracted from the leaf tissue of at least five plants per line in three replicates after inoculation with *F. oxysporum*, using the method with Trizol® (Reagent, Carisbald, CA, USA) according to the manufacturer's instructions. First cDNA strand was synthesized by RT-PCR with One-Step RT-PCR Master Mix from Applied Biosystems (Catalog No. 4309169). The TaqMan® system consisted of the primers and the probe which were designed using the Primer Express v2.0 program (Applied Biosystems, Foster City, CA, USA), based on the *pdf1.2* gene sequence (NCBI, NM_123809).

Specific primers and probes used to detect the *pdf1.2* gene were: sense 5'-AGT TGT GCG AGA AGC CAA GT-3', antisense 3'-GCA TGC ATT ACT GTT TCC GCA AA-5', and the TaqMan® probe 5'-CCC TGA CCA TGT CCC -3', with a FAM™ dye label and minor groove binder (MGB) at the 5' end, and nonfluorescent quencher (NFQ) at the 3' end. The 18S ribosomal internal control (4319413E, Applied Biosystem) was labelled

with the VIC-fluorophore. Amplification conditions consisted of 48 °C for 30 min (reverse transcription), 95 °C for 10 min (denaturation), 45 cycles (PCR amplification), 95 °C for 15 s, and 60 °C for 1 min (extension) (ABI PRISM®, Sequence Detection Systems 7000, Applied Biosystems). The relative expression ratio was calculated normalizing the cycle threshold (Ct) of the target gene (*pdf1.2*), with reference to the Ct of the control gene (*18S*), in order to determine only the efficiency of the defensin gene (ΔCt= Ct *pdf1.2*-Ct18Sr) (Basu *et al*., 2019).

Experimental design

The experimental design was completely randomized. Two pathogenic microorganisms were analyzed in each of the *pdf1.2*-genetically-modified common bean lines, as well as its non-modified FMA comparator and the Montcalm cultivar, as a pathogenicity control. Each homozygous line consisted of 25 plants. The experimental unit was a pot with a plant. The statistical analysis consisted of an analysis of variance of the severity values and comparison of means through the Tukey test ($p \leq 0.05$), using the Minitab 17 statistical package (Minitab Statistical Software).

RESULTS AND DISCUSSION

Damage levels within the population of unmodified FMA and Montcalm plants inoculated with the evaluated concentrations of *F. oxysporum* or *F. lateritium* were identified, in order to standardize the evaluation of the populations of inoculated lines. Thus, the complete range of semiquantitative damage was determined throughout the 21 d of incubation and the scale was validated for the evaluation model used in this study (Figure 1). Sánchez-Garcia *et al*. (2017) evaluated *F. oxysporum* using this system and determined that common bean plants cv. Montcalm inoculated with this strain were susceptible, which confirmed our observations.

Figure 1. Severity levels caused by *Fusarium oxysporum* and *F. lateritium* in inoculated common bean plants. A: no damage (1); B: 10 % of the hypocotyl and root with lesions and coloration (3); C: 25 % of the hypocotyl and root with lesions and loss of root mass (5); D: 50 % of the hypocotyl and root with rotting and reduced root mass (7); E: more than 75 % of the tissue with rot and loss of root tissue (9).

Inoculation of common bean plants with *Fusarium oxysporum* and *F. lateritium*

The damage caused by *F. oxysporum* to the plants of the FMA-*pdf1.2* lines, 21 d after their inoculation, indicated that the L3, L4, and L9 lines stood out for having an average damage of 3.4 (equivalent to 10 % of the surface with damage or injury); according to the evaluation scale they are located among the most resistant plants. The L2 and L7 lines showed a severity value of 4 (10 - 25 % of the hypocotyl and root surface covered by lesions), while the FMA and Montcalm control plants showed an average severity of 5 and were considered moderately susceptible (Figure 2). This first inoculation block indicates a quantitative differential in the damage caused by *F. oxysporum* to the FMA-*pdf1.2* lines; however, these differences were not statistically significant between lines, which establishes homogeneity in the response derived from the expression of the *pdf1.2* gene with high levels of root inoculum. On the contrary, a significant difference was observed between the lines population and the control plants of FMA and Montcalm (Figure 2).

Analysis of plant populations 21 d after inoculation with *F. lateritium* (42 d after inoculation with *F. oxysporum*) indicated that all FMA-*pdf1.2* lines reached 4.8-5.2 damage values, equivalent to 25 % of the hypocotyl and root surface covered by lesions and with noticeable discoloration. This indicates that, although the L2 and L7 lines showed a lower quantitative level of *pdf1.2* expression and had moderate susceptibility values to *F. oxysporum.* The increase in damage observed, after *F. lateritium* inoculation, was not significant and the plants kept their degree of tolerance.

Meanwhile, the severity levels of the L3, L4, and L9 lines with a *F. oxysporum* severity value of 3 increased from 4.8 to 5.3 when they were inoculated with *F. lateritium*, *a* value considered within the tolerance spectrum (Figure 3). Therefore, the FMA-*pdf1.2* population plants reached the maximum tolerance threshold, as a consequence of the expression levels of the defensin gene. Finally, the damage to the FMA and Montcalm control plants populations exceeded the FMA-*pdf1.2* lines, with 6-7 damage values: *i.e.*, 21 d after inoculation with *F. lateritium*, approximately 50 % of the hypocotyl and

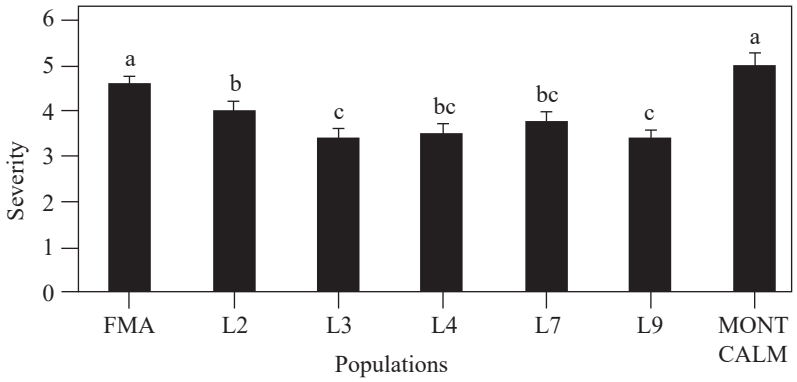

Figure 2. Degree of severity of root rot induced by *Fusarium oxysporum* in common bean plants, 21 d after inoculation (Means with different letters in a row are statistically different, Tukey, $p \leq 0.05$). The bars represent the standard error.

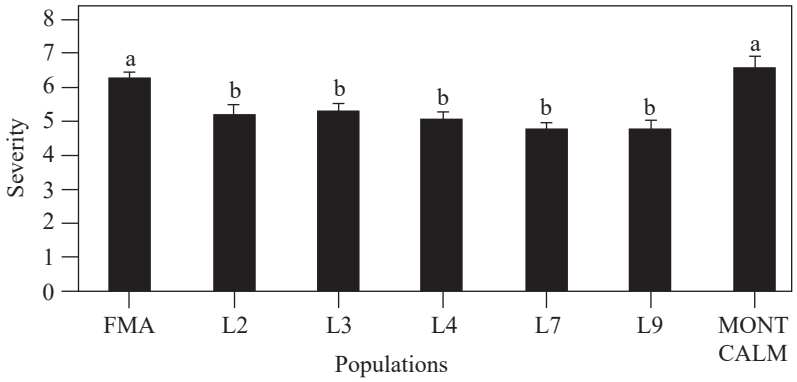

Figure 3. Degree of root rot severity induced by *Fusarium lateritium* in common bean plants, 21 d after inoculation (Means with different letters in a row are statistically different, Tukey, $p \leq 0.05$).

root tissue was covered with lesions that were combined with softening, rotting, and considerable reduction of the root system (Figures 3 and 4).

Although the response of the plants of the FMA-*pdf1.2* lines can be considered moderate in terms of tolerance, it opens the discussion about the causes of this difference in

Figure 4. Analysis of damage to common bean plants, 21 d after inoculation with *Fusarium lateritium.* A: non-inoculated FMA (vermiculite); B: inoculated FMA; C: Inoculated Montcalm (susceptible control); D: to the left: inoculated FMA-*pdf1.2*-L9 (severity 3); to the right: inoculated FMA (severity 7).

contrast to the control plants and the possible signaling of other induced resistance proteins. On the one hand, the reduction in the severity of the damage in the plants of the FMA-*pdf1.2* lines is probably related to the activity of the PDF1.2 antimicrobial peptide which directly affects *Fusarium* spp., since the unmodified plants (FMA and Montcalm) showed signs of more severe damage than the FMA-*pdf1.2* plants.

Other recombinant defensin models have had similar responses. From moderate protection, such as the MsDef1defensin of *Medicago truncatula*, which provided tolerance to tobacco leaves inoculated with 0.3×10^6 spores mL^{-1} of saline medium against the invasion by the *Aspergillus niger* and *Rhizoctonia solani* pathogenic fungi (Deb *et al*., 2020). Up to more robust level of protection, provided by the bean (*Phaseolus lunatus*) defensin to barley (*Hordeum vulgare* L.), inhibiting chlorophyll loss in foliage, 15 d after being sprayed with *Fusarium oxysporum* (OD = 0.7 in LB liquid medium) (Rehorova *et al*., 2018).

Breeding crops resistant to various diseases is difficult due to the absence of sources of resistance in the genomes, as is the case of common beans. Previously, our group demonstrated the resistance of the FMA-*pdf1.2* lines to anthracnose (*Colletotrichum lindemuthianum*) (Espinosa-Huerta *et al.,* 2013) which, together with the results of this report, provides evidence of broad-spectrum protection against fungal pathogens in a constitutive expression system. Su *et al.* (2020) reported similar protection results in wheat (*Triticum aestivum* L.) using the DmAMP1W defensin from dahlia (*Dahlia merckii*). The authors were able to demonstrate a significant increase in broad-spectrum resistance to common root rot *(Bipolaris sorokiniana*) and eyespot (*Rhizoctonia cerealis*) in the T1 and T2 generations. Their broad-spectrum and stable resistance condition makes antimicrobial peptides, such as defensin *pdf1.2*, good candidates for inclusion in plant breeding programs. This is, to our knowledge, the first report consolidating the expression and protection of common beans against several species of pathogenic fungi through an antimicrobial peptide.

Another possible cause that explains why the FMA-*pdf1.2* lines showed significant differences in the severity of root rot compared to control plants may include the induction of other antimicrobial peptides. It has been reported that the expression of one antimicrobial peptide can induce the expression of others, either by infection or by chemical compounds such as methyl jasmonate, silver nitrate, etc. The chili defensin gene (*J1–1*) expressed in tobacco not only supressed root rot caused by *Phytophthora parasitica* var. *nicotianae* and *Pythium aphanidermatum*. But also induced the expression of *PR* genes, which had synergistic effects with defensin (Lee *et al*., 2018). In the case of *pdf1.2*, the co-expression of some defense proteins exerts an additive effect on the immune response, constitutively stimulating the defense pathways even before the invasion of the pathogen takes place (Swaminathan *et al*., 2021). It is highly possible that several genes associated with resistance proteins in the FMA-*pdf1.2* lines are constitutively active and influence the timely defense response to pathogen attacks. Kashyap *et al*. (2019) corroborated this fact; they reported that the difference between susceptible and resistant wheat genotypes is subject to the duration and magnitude of expression of *pdf1.2*, *PR1*, and *PR5*.

In this study, two types of simultaneous response were observed in the control of *Fusarium* spp. in FMA-*pdf1.2* plants. On the one hand, regarding the reduction of the signs of infection, the FMA-*pdf1.2* lines showed less severity in the incidence of *F. oxysporum* and *F. laterirtium*; and on the other hand, there was a delay in their appearance compared to the control plants (FMA and Montcalm). Most of the tolerant plants reached physiological maturity under greenhouse conditions; however, yield data under confinement conditions were not considered because they were not considered representative.

The delay in the appearance of signs and reduction of damage was also reported in the tomato transcriptome analysis with the locus of resistance to the TSWV virus (*Sw-7*). The participation of PR-5 was found through its overexpression, which conferred increased resistance and delayed virus accumulation and the expression of symptoms (Padmanabhan *et al*., 2019). Similarly, olive (*Olea europaea* L.) plants transformed with the *NPR1* gene and inoculated with *Rosellinia necatrix* showed a slight delay in disease development, with a means under the disease development curve, 7 – 15 % lower than the control (Narváez *et al*., 2020).

The foregoing is especially relevant because, unlike fungicides, this biotechnological model does not entail the application of chemical controls; thereby eliminating environmental pollution and preventing reduction in micro-biological diversity. Neither does it has an impact on the antagonistic microbiota, as reported by Granados-Vallejo *et al.* (2019), who concluded that the presence of defensin did not pose a risk factor against microorganisms that did not invade the inter and intracellular space of modified plants, nor against microorganisms not included in the action spectrum of *pdf1.2*. These microorganisms include *Trichoderma harzianum* (pathogen antagonist fungus), *Rhizobium tropici* (nitrogen-fixing bacteria), and *Rhizophagus intraradices* (mycorrhizal fungus).

Relative expression of the *pdf1.2* gene from inoculated common bean plants

The common bean plants of the FMA-*pdf1.2* lines showed differential values of relative transcriptional expression of the *pdf1.2* gene. The L2 (0.49) and L7 (0.63) lines had lower values than the L3 (0.73), L4 (0.68), and L9 (0.90) lines; however, no statistically significant differences were obtained in the expression values of the five lines (Figure 5). The expression values were contrasted with the severity values and an inverse correlation was found (R^2 = -0.93). Regarding inoculation with *F. oxysporum*, the L2 and L7 lines had lower *pdf1.2* gene expression values and higher severity values than L3, L4, and L9.

In the case of the damage values caused by *F. lateritium* to the FMA-*pdf1.2* lines, it was observed that, although these increased compared to the first inoculation (*F. oxysporum*), the lesions were restricted and kept at lower values than the control plants. Similar results were observed in the characterization of sweet pepper defensin J1-1, whose gene was overexpressed in ripe pepper fruit. The overexpression of that gene not only provided resistance to *Colletotrichum gloeosporioides*, but it also induced restriction of symptoms and inhibition of fungal colonization (Seo *et al*., 2014).

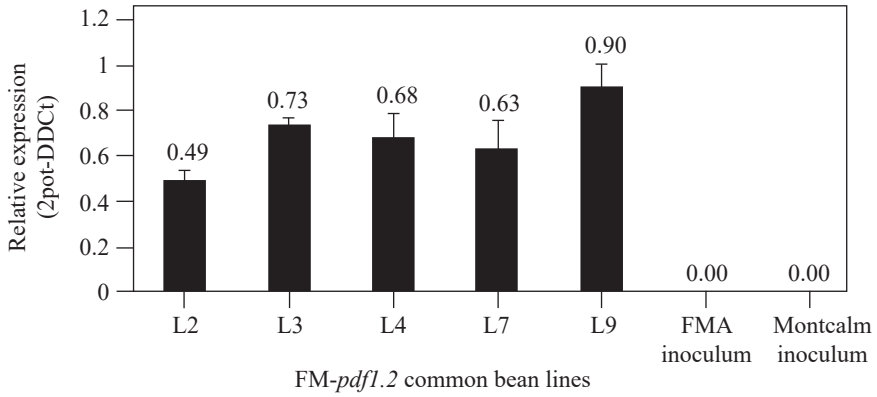

Figure 5. Analysis of the *pdf1.2* gene expression by Q-PCR of common bean plants from FMA-*pdf1.2* lines and control plants inoculated with *Fusarium oxysporum* and *F. lateritium*.

Therefore, the plants with the lowest level of expression reached the same level of tolerance to pathogens as the plants with the highest level of expression. Consequently, a window of opportunity is seen, where the combination of other root rot control systems, such as the use of antagonistic microorganisms, can provide a synergistic effect and thus a reduction in the effects of the disease (Granados-Vallejo *et al*., 2019).

CONCLUSIONS

Tolerance to *Fusarium* spp. shown by plants of the FMA-*pdf1.2* lines established that the expression of the defensin gene protected plants through the coded protein against root pathogens. The advantage of the *pdf1.2* gene consisted in the reduction of the severity of the damage, as well as in an initial restriction of the symptoms of the disease. The damage caused by *F. lateritium* contributed to the progress of the severity in the affected tissues, demonstrating that it is a major pathogen in root rot.

The expression levels of the *pdf1.2* gene in the different FMA-*pdf1.2* lines were related to the degree of protection provided to the plants of the evaluated lines and were sufficient to reduce the incidence and severity of the disease caused by *Fusarium* spp. As a result of the broad-spectrum protection provided by the defensin gene, the reduction in the severity of root rot contributes to an ecologically efficient agronomic system.

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