Mycopath (2018) 16(1): 1-5 **Research Article**

Effect of nitrozist bio-fertilizer on melon resistant to wilt disease caused by *Fusarium oxysporum* f.sp. *melonis* and expression of melon defense-related genes

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Abstract

Fusarium oxysporum f. sp. *melonis* (*Fom*) is causal agent of the *Fusarium* wilt of melon, with a reported loss of 100% on melon (*Cucumis melo* L.). This research was conducted to study the effect of nitrozist bio-fertilizer (containing *Enterobacter* sp.) on melon resistant to wilt disease caused by *Fom*. This experiment was carried out as seed treatment with nitrozist bio-fertilizer under greenhouse conditions. Furthermore, the expression of *peroxidase* (*POX*), *polyphenol oxidase* (*PPO*), *phenylalanine ammonia-lyase* (*PAL*) and *lipoxygenase* 2 (*LOX2*) genes was evaluated using Real-Time PCR (qRT-PCR) technique. Treated plants with nitrozist bio-fertilizer had a significant difference with regard to the severity of the disease. The percentage of disease reduction of nitrozist bio-fertilizer was 41%. The level of expression of *POX, PPO, PAL* and *LOX2* genes was significantly increased by nitrozist bio-fertilizer. Therefore, the use of a nitrozist bio-fertilizer containing *Enterobacter* sp. is efficient in induction of resistance in melon plants against to *Fom*.

Keywords: Bio-fertilizer, *Fusarium oxysporum*, Induced resistant, *Peroxidase*.

Introduction

Fusarium oxysporum f. sp. *melonos* (*Fom*) is causal agent of Fusarium wilt of melon melon (*Cucumis melo* L.), which has been reported in America (Chupp, 1930), Europe and Asia (Sherf and MacNab, 1986). This pathogen attacks the melon plant at all stages, and the loss to melon has been reported to be 100% (Sherf and MacNab, 1986). The disease was first reported in Iran in 1347 from Mashhad (Banihashemi, 1968). The intensive use of chemical fungicides creates problems such as fungicide resistance, soil contamination and high toxicity for microbial communities (Yacine *et al*., 2014).

Enterobacter cloacae is a nitrogen-fixing bacterium (Ladha *et al*., 1983). *E. cloacae* has reduced seed rot and pre-emergence damping-off caused by *Pythium* spp. (Nelson, 1988), rot of peach caused by *Rhizopus stolonifer* (Wilson *et al.,* 1987) and has inhibited chlamydospore germination of *F. oxysporum* f.sp. *cucumerinum* (Sneh *et al*., 1984).

Studies have shown that *peroxidase* (*POX*), *phenylalanine ammonia-lyase* (*PAL*) and *lipoxygenase* 2 (*LOX2*) genes play an important role in induced resistance in plants against pathogens and increase their expression during treatment with antagonistic agents (García-Gutiérrez *et al*., 2013; Chandrasekaran *et al*., 2017). Therefore, the aim of this research was to investigate the effect of nitrogen bio-fertilizer on melon resistance to Fusarium wilt caused by *Fom* and understand the resistance pathways induced by this bio-fertilizer.

Materials and Methods

Pathogen and bio-fertilizers

F. oxysporum f.sp. *melonis* was isolated from melon plants and its pathogenicity was confirmed. In this research, we used nitrozist bio-fertilizer containing *Enterobacter* sp. with a concentration of 1.5×10^8 (Keshtkar Gostar Nojan Company).

Effect of bio-fertilizers on melon resistant to Fusarium wilt disease in greenhouse conditions

Melon seeds were soaked in nitrozist solution for 2 h (Burgess and Hepworth, 1996). Seeds of control plants were soaked in distilled water. Seedlings were grown in sterilized soil in greenhouse conditions for 7 days. The roots of 7-day-old seedlings were removed from soil and were dipped in the conidial suspension (2×10^6 mL^{-1}) and transplanted into pots, in a greenhouse at 20–25 °C (Banihashemi, 2010).

Disease severity was scored on a 1–5 scale as described by Perchepied and Pitrat (2004). Plants were examined at 3- to 4-day intervals for 3 weeks after appearance of symptoms (Perchepied and Pitrat 2004). The disease severity was calculated using the formula described by Lak *et al.* (2018). Disease severity = [Σ(numerical value of each category × number of plants in each category) / (the highest number in the scale × total number of plants)] × 100.

Reduction in disease severity was calculated as (Chandrasekaran *et al*., 2017):

Disease reduction (%) = [(disease severity control – disease severity treatment)/disease severity control] × 100.

Effect of nitrozist bio-fertilizer on expression of melon defense- related genes

The leaves were sampled for 3 consecutive days with one day interval. Total RNA was extracted from leaves using the Top Plant and Fungi RNA purification kit (Topaz Gene Research, Iran) based on the method described in the kit's manufacturer's instructions. The first strand of cDNA was synthesized using the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, Waltham, Massachusetts, USA). Expression of genes was evaluated by using real-time PCR (qRT-PCR) technique with Eva Green fluorescent dye and their specific primers. The qRT-PCR was performed with a Rotor-Gene 3000 (Corbett Robotics, Australia). The process was performed with 20 µL of reaction mixture consisting of 1 × HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis BioDyne, Riia, Estonia), 1 µL cDNA, and 10 µM of each forward and reverse primer (Mehraban *et al.,* 2018). The following protocol was applied: initial polymerase activation: 15 min at 95 °C; 40 cycles of 20 s at 95 °C, 20 s at 58 °C and 30 s at 72 °C. The characteristics of the primers used for qRT-PCR are listed in Table 1. The relative changes in the expression of target genes were normalized to the *ADP ribosylation factor* (*ADP*) housekeeping gene (Kong *et al*., 2014). Analysis of relative gene expression data were carried out using the Relative Expression Software Tool (REST) 2009 software version 2.0.13 (Qiagen, Hilden, Germany) (Pfaffl *et al*. 2002).

Statistical analysis

In this experiment, three replicates were considered for each treatment and the means of disase severity were compared with *t-test* and other means were compared with least significant difference (LSD) using MSTAT-C (v.1.42). The differences with $P < 0.05$ were considered significant.

Results

Effect of nitrozist bio-fertilizer on Fusarium wilt of melon

Effect of nitrozist bio-fertilizer on disease severity Fusarium wilt of melon is shown in Fig. 1. Treated plants had significantly lower disease severity than the untreated control. The percentages of disease reduction in nitrozist bio-fertilizer was 41%.

Effect of nitrozist bio-fertilizer on expression of melon defense- related genes

The optimal conditions were provided so that no non-specific product was produced during the reaction, which was confirmed using a melting curve (observing a single melt peak) (Fig. 2).

Results of determination of expression of *POX* gene in nitrozist-treated and non-treated (control) melon plants after inoculation with *Fom* is shown in Fig. 3. The results showed significant differences between the expression of *POX* gene in infected nitrozist-treated and control melon plants at 24, 48 and 72 h after inoculation. Treatment with nitrozist bio-fertilizer increased the level of *POX* gene expression at 24, 48 and 72 h after inoculation, and maximum expression of *POX* gene was 24 h after inoculation. Results of determination of expression of *PPO* gene in nitrozist-treated and non-treated (control) melon plants after inoculation with *Fom* is shown in Fig. 3. The results showed significant differences between the expression of *PPO* gene in infected nitrozist-treated and non-treated (control) melon plants at 24, 48 and 72 h after inoculation. Treatment with nitrozist bio-fertilizer increased the level of *PPO* gene expression at 24 and 48 h after inoculation, and maximum expression of *PPO* gene was 24 h after inoculation. Results of determination of expression of *PAL* gene in nitrozist-treated and non-treated (control) melon plants after inoculation with *Fom* is shown in Fig. 3. The results showed significant differences between the expression of *PAL* gene in infected nitrozist-treated and non-treated (control) melon plants at 24, 48 and 72 h after inoculation. Treatment with nitrozist bio-fertilizer increased the level of *PAL* gene expression at 24, 48 and 72 h after inoculation, and maximum expression of *PAL* gene was 24 h after inoculation. Results of determination of expression of *LOX2* gene in nitrozist-treated and non-treated (control) melon plants after inoculation with *Fom* are shown in Fig. 3. The results showed significant differences between the expression of *LOX2* gene in infected nitrozist-treated and non-treated (control) melon plants at 24, 48 and 72 h after inoculation. Treatment with nitrozist bio-fertilizer increased the level of *LOX2* gene expression at 24 and 48 h after inoculation, and maximum expression of *LOX2* gene was 24 h after inoculation.

Discussion

In this research, the effect of nitrozist bio-fertilizer was investigated on melon resistance against Fusarium wilt caused by *Fom* and expression of defense genes of *POX, PPO, PAL* and *LOX2*. According to several reports, *E. cloacae* has induced resistance in plants against pathogens. Nelson (1988) evaluated *E. cloacae* as biological seed treatment on cotton. All strains reduced the incidence of *Pythium* seed rot and preemergence damping-off in naturally infested soil. Wilson *et al*. (1987) reported that *E. cloacae* reduced the development of rot in artificially wounded peaches inoculated with *R. stolonifer*.

In this study, the nitrozist bio-fertilizer increased expression of *POX, PPO, PAL* and *LOX2* genes. Expression of *PAL* gene in tomato plants were upregulated after treatment with *Bacillus subtilis* against *Xanthomonas campestris* pv. *vesicatoria* (Chandrasekaran *et al*., 2017) and *Erwinia carotovora* subsp. *carotovora* (Chandrasekaran and Chun, 2016). García-Gutiérrez *et al*. (2013) reported that expression of the *LOX2* and especially *PR-1* and *PR-9 (POX)* genes increased in melon plants treated with *B. subtilis* UMAF6639 and inoculated with *Podosphaera fusca*.

Conclusion

The results of this study conclude that the nitrozist bio-fertilizer reduces the severity of Fusarium wilt caused by *Fom* and increased the expression of *POX, PPO, PAL* and *LOX2* genes in melon plants and this reduction of the disease severity by nitrozist bio-fertilizer treatment was probably due to induction of melon defense- related genes, therefore, it is recommended that bio-fertilizer be used to minimize the use of chemical fertilizers, along with increasing yield, and the resistance of melons to the Fusarium wilt caused by *Fom* increase.

Table 1: Nucleotide sequences of primers used in this study.

Gene	Forward primer 5-3	Reverse primer 5-3	Accession number
POX	ATTCAAAAATGGCCTCCGCC	CGAGGGCAGGTTTGATCGTA	XM_008451553.2
PPO	TGCGGGCAGTTTTGTGAATG	CGTCAAGTTCGATCTTTAAGCC	XM_008444052.2
PAL	CCCATCTTTTCGTTCAACTCTG	CCCAGTTGAGTGGATCCTG	XM_008455916.2
LOX2	GAATAGGCATGGAGCTGGTG	AAATGGGAGATGAAAGACACACA	XM_008451532.2
ADP	ATATTGCCAACAAGGCGTAGA	TGCCCGTAAACAAGGGATAAA	

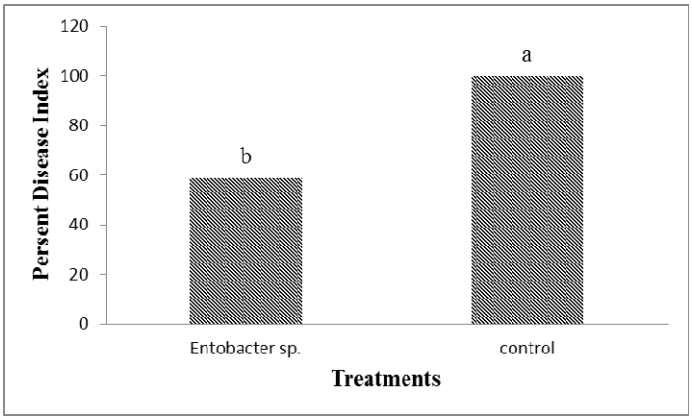

Fig. 1: The effect of nitrozist (*Entrobacter* sp.) bio-fertilizer on disease severity of infected melon with *Fusarium oxysporum* f.sp. *melonis*.

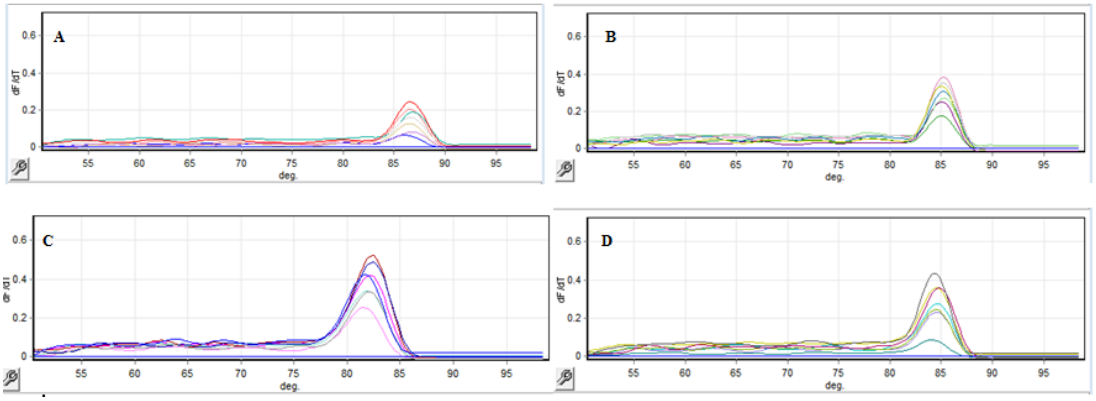

Fig. 2: Analysis of the melting curve for *peroxidase* (A), *polyphenol oxidase* (B), *phenylalanine ammonia-lyase* (C) and *lipoxygenase 2* (D) genes. Each peak represents the melting temperature of a PCR product.

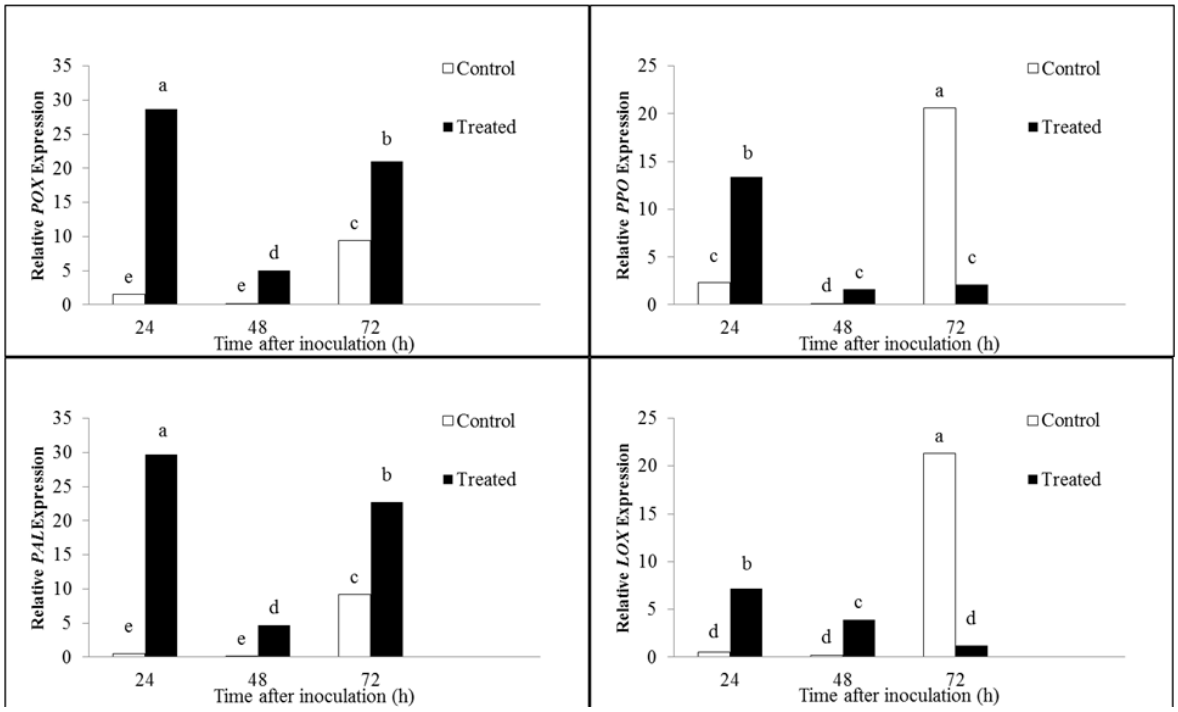

Fig. 3: Level of mRNA expression of *peroxidase (POX), polyphenol oxidase (PPO), phenylalanine ammonia-lyase (PAL)* and *lipoxygenase 2 (LOX 2)* in nitrozist-treated and non-treated (control) melon plants after inoculation with *Fusarium oxysporum* f.sp. *melonis*. Each sample is normalized for the amount of the template to the levels of *ADP ribosylation factor 1*. Means with different letters are significantly different according to the least significant difference (LSD) test ($P < 0.05$).

References

Banihashemi Z, 1968. The existence of Fusarium wilt of melon in Iran. *Proc. First Nat. Cong. Plant Med. Iran,* pp. 47-48.

Banihashemi Z, 2010. Reaction of *cucumis melo* cultivars to races of *Fusarium oxysporum* f. sp. *melonis* the cause of melon vascular wilt. *Int. J. Plant Pathol.,* **46**:11-22.

Burgess DR, Hepworth G, 1996. Biocontrol of sclerotinia stem rot (*Sclerotinia minor*) in sunflower by seed treatment with *Gliocladium virens*. *Plant Pathol*., **45**: 583-592.

Chandrasekaran M, Belachew ST, Yoon E, Chun SCh, 2017. Expression of *β-1,3-glucanase* (*GLU*) and *phenylalanine ammonia-lyase* (*PAL*) genes and their enzymes in tomato plants induced after treatment with *Bacillus subtilis* CBR05 against *Xanthomonas campestris* pv. *vesicatoria*. *J. Gen. Plant Pathol*., **83**: 7-13.

Chandrasekaran M, Chun SC, 2016. Expression of PR-protein genes and induction of defense-related enzymes by *Bacillus subtilis* CBR05 in tomato (*Solanum lycopersicum*) plants challenged with *Erwinia carotovora* subsp. *carotovora*. *Biosci. Biotechnol. Biochem.,* **80**: 2277-2283.

Chupp C, 1930. Fusarium wilt of muskmelon. *Plant Dis. Rep*., **14**: 160.

García-Gutiérrez L, Zeriouh H, Romero D, Cubero J, de Vicente A, Pérez-García A, 2013. The antagonistic strain *Bacillus subtilis* UMAF6639 also confers protection to melon plants against cucurbit powdery mildew by activation of jasmonate-and salicylic acid-dependent defence responses. *Microb. Biotechnol*., **6**: 264-74.

Kong Q, Yuan J, Niu P, Xie J, Jiang W, Huang Y, Biem Zh, 2014. Screening suitable reference genes for normalization in reverse transcription quantitative real-time PCR analysis in melon. *PLOS One*, **9**: e87197.

Ladha JK, Barraquio WL, Watanabe I, 1983. Isolation and identification of nitrogen-fixing *Enterobacter cloacae* and *Klebsiella planticola* associated with rice plants. *Can. J. Microbiol.,* **29**: 1301-1308.

Lak F, Sarpeleh A, Shahriyari D, 2018. Genetic Diversity of amony *Fusarium oxysporum* f. sp. *melonis* Isolates, The Causal Agent of Cantaloupe And Muskmelon Wilt in Iran. *J. Appl. Res. Plant Prot.*, **7**: 69-83.

Mehraban A, Abkhoo J, Dahmardeh E, 2018. Effect of extracts of *Capparis spinosa*, *Withania somnifera* and *Peganum harmala* on *Aspergillus flavus* growth and expression of major genes in aflatoxin biosynthetic pathway. *Zahedan J. Res. Med. Sci.,* **20**: e64602.

Nelson EB, 1988. Biological control of *Pythium* seed rot and pre-emergence damping-off of cotton with *Enterobacter cloacae* and *Erwinia*

herbicola applied as seed treatments. *Plant Dis*. **72**:140-142.

Perchepied L, Pitrat M, 2004. Polygenic inheritance of partial resistance to *Fusarium oxysporum* f. sp. *melonis* race 1.2 in melon. *Phytopathology*, **94**: 1331-1336.

Pfaffl MW, Horgan GW, Dempfle L, 2002. Relative expression software tool (REST[a]) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res*., **9**:1–10.

Sherf AF, Macnab AA, 1986. Fusarium wilt of muskmelon,. In: *Vegetable diseases and their control*. Wiley, New York. pp. 334-337.

Sneh B, Dupler M, Elad Y, Baker R, 1984. Chlamydospore germination of *Fusarium oxysporum* f. sp. *cucumerinum* as affected by fluorescent and Lytic bacteria from a *Fusarium*-suppressive soil. *Phytopathology*, **74**:1115-1124.

Tan D, Fu L, Han B, Sun X, Zheng P, Zhang J, 2015. Identification of an endophytic antifungal bacterial strain isolated from the rubber tree and its application in the biological control of banana Fusarium wilt. *PLoS One*, **10**: e0131974.

Wilson CL, Franklin JD, Pusey PL, 1987. Biological control of Rhizopus rot of peach with *Enterobacter cloacae*. *Phytopathology*, **77**: 303-303.

Yacine G, Omrane T, Amine Y, Nasserdine S, Folrence M, Abdelghani A, 2014. Biocontrol of *Rhizoctonia solani* damping-off and promotion of tomato plant growth by endophytic actinomycetes isolated from native plants of *Algerian Sahara*. *Microb. Res*., **169**: 59-65.