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RESEARCH ARTICLE

Resistance genes expression pattern analysis of *Serendipita indica*-root colonized sour orange plants in challenging with citrus bacterial blast (*Pseudomonas viridiflava*)

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ABSTRACT: Citrus bacterial blast disease is one of the prevalent diseases in most citrus-growing regions in the world. Plants use a wide range of mechanisms to defend against pathogens, and the plant-pathogen interaction induces the expression of genes involved in the plant resistance. Furthermore, symbiotic association between plant and mycorrhizal fungi could effectively promote growth and protect the plant against adverse environmental conditions. In the present study, *Serendipita indica*-root colonized sour orange seedlings were infected by *Pseudomonas viridiflava* and the expression patterns of *PR1*, *PR2*, *PR3*, *PR4*, *PR5*, *PAL*, *POX*, and *LOX* genes in the early stages of citrus blast disease were investigated using the qRT-PCR at different sampling times. According to the results, the response of defense genes to bacterial infection was time dependent. In the *S. indica*-colonized sour orange plants, the highest level of *PR1*, *PR4*, *PAL*, *POX*, *PR3*, and *PR5* genes expression was observed at 48 h after infection, but the expression of *PR2* and *LOX* genes was increased at 72 h after infection compared to the control plants. It seems that *S. indica* can induce systemic effects and prepare the host plant to increase the expression of defense genes more rapidly once it receives a signal for the presence of the pathogen.

KEYWORDS: Citrus blast disease, *Piriformospora indica*, PR genes, *Pseudomonas viridiflava*, *Citrus aurantium*

INTRODUCTION

Citrus blast bacterial disease is one of the most common diseases in the citrus-growing regions, except in the tropics. The disease was first reported in northern California in 1916. The *Pseudomonas syringae* pv. *syringae* and *Pseudomonas viridiflava* have been reported as citrus blast causes in northern Iran [42]. Blast pathogens invade plants through wounds on young tissues. The symptoms on the leaf blade and petiole usually appear as burnt spots or black areas and spread on all sides. The infection of phloem leads to the wilt and fall of leaves. The disease symptoms will be more severe in young shoots during the growing season, particularly in rainy and windy areas with humid and cold weather conditions. Due to high humidity and suitable temperature in the climatic conditions of northern Iran, blast disease imposes significant damage as wilting and drying of branch tips [2].

Plants use a wide range of mechanisms to defend against pathogens. The plant-pathogen interaction results in the induction of genes involved in the host plant resistance, followed by the reduction of wounds and pathogen damage [11]. Pathogenesis-related proteins (PRs) are a group of proteins produced in plants by pathogens or pathogen-related substances (e.g. elicitors) and plant

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hormones such as salicylic acid (SA), jasmonic acid (JA), and ethylene [49].

Pathogens cause metabolic changes such as the production of reactive oxygen species (ROS), induction of systemic acquired resistance (SAR), and induced systemic resistance (ISR). The activity and presence of SAR-related proteins are directly related to the level of resistance in the plant. Pathogenicity-related proteins, such as chitinase and glucanase with antibacterial and antifungal roles, are associated with SAR [37].

*Serendipita indica (*synonym *Piriformospora indica)* is an endophytic fungus that was first isolated from the rhizosphere of xerophyte plants, *Prosopis juliflora* and *Zizyphus nummularia*, from the Thar Desert of Rajasthan, India [41]. The importance of *S. indica* symbiosis with different plants in stimulating their growth and yield, as well as increasing plant tolerance to biotic and abiotic stresses has been reported [4,26,33,34]. Furthermore, this fungus can stimulate systemic resistance against plant pathogens. The higher levels of defense genes expression in *S. indica*-colonized rice plants after infection by *Magnaporthe oryzae* in comparison with control plants has been observed [29].

PR1 is the most important PR protein in resistance to various pathogens [39,49] with antimicrobial properties [35,40]. PR2 and PR3 proteins display β-1,3-glucanase and chitinase activities, respectively [18,51]. Although chitinases often possess the antifungal activity and play a major role in the interactions between pathogenic fungi and plants, a lysozyme role has also been reported for chitinases, thereby breaking down the bacterial cell wall [13]. An increase in the expression of the chitinase gene and some PR genes in the pepper plants led to increased resistance to *Xanthamonas campestris* pv. *vesicatoria* [23]. Simultaneous expression of two or more PR genes with a synergistic effect improved resistance and thus better disease control in tobacco [56]. Proteins belonging to the PR5 family (known as Thaumatin-like (TL) proteins) are isolated from *Arabidopsis*, rice, wheat, and many other plants [28,53]. Accumulation of these proteins in plants has been observed in response to stressful conditions such as salinity, wounds, or pathogen invasion. These proteins alter the permeability of the pathogen's cell membrane [25].

Phenylalanine ammonia lyase (PAL) has been identified as one of the first plant defense genes stimulated by environmental stresses and pathogens [5,23,36]. Numerous studies have shown that an increase in the PAL gene expression is involved in pathogen resistance by an effect on increasing SA, activation of the *NPR1* gene, and subsequent expression of *PR* genes, as well as rapid induction of apoptosis [8,12].

Lipoxygenases (LOXs) are a family of enzymes involved in the synthesis of oxygenated fatty acids that play a critical role in plant defense against microbial and insect infections [21].

On the other hand, peroxidase (POX) catalyzes the lignin production process and thereby strengthens the plant cell wall and resistance against many pathogens [9,18,48]. POX has a direct effect on plant resistance and plays an important role in the host defense response through the production of hydrogen peroxide free radicals.

In the present study, to get more understanding of the effect of *S. indica* colonization on the defense mechanisms of sour orange plants against citrus bacterial blast causing by *P. viridiflava*, the expression pattern of pathogensis-related (*PR1, PR2, PR3, PR4*, and *PR5*), *POX*, *PAL*, and *LOX* genes has been investigated.

MATERIALS AND METHODS

Plant material and the pathogen

Sour orange (*Citrus aurantium*) was used as the host of blast-causing bacteria and the fungal plant symbiont. The outer shell of sour orange seeds was separated and they were disinfected in 70% ethanol and 1% NaClO for 1 and 10 min, respectively. Then, seeds were thoroughly washed with sterile distilled water and cultured in a slightly modified MS medium [30]. The seed containers were stored in a dark place for 4 days and then incubated at 25 °C, 16 h light and 8 h dark for 1 month.

The pathogenic bacterium, *Pseudomonas viridiflava*, was isolated from infected citrus fruits with blast symptoms in Mazandaran province and identified with conventional diagnostic bacteriological tests [42].

Preparation and propagation of mycorrhizal fungus

The isolate of *S. indica* (donated by Professor Kogel, President of the Institute of Pathology and Applied Zoology, University of Giessen, Germany) was cultured in CM medium [50] and incubated at 27 °C for 27 days for sporulation.

Plant inoculation and growth condition

One-month-old sour orange seedlings were immersed in a 5×10^5 spore ml^{-1} *S. indica* spore suspension for 4 h

and then kept in a vessel containing wet filter paper for 12 h. Sterile distilled water was used for the control plants. The seedlings were then planted in pots containing sterile light-textured soil (soil: perlite: leaf soil, 1:1:2) and incubated at 26-28 °C.

Four weeks after the inoculation of plants with *S. indica*, the roots of some plants were removed and washed with distilled water, cut into pieces of a few mm, and stained [52]. The root pieces were placed on a slide and the presence of the fungus and its chlamydospores in the root cortex tissue was examined using a microscope. For molecular analysis, the surface of treated and control roots was disinfected with 70% ethanol, washed with sterile distilled water, and DNA was extracted [31]. The presence of *S. indica* EF-1α (*tef* gene) was evaluated by PCR using specific primers (Table 1) [10].

P. viridiflava infection and sampling

Four weeks after planting a 10^7 cfu ml^{-1} suspension of *P. viridiflava* was injected into the leaf of control and root-colonized plants. Control plants were injected by sterile distilled water. At three sampling times, 24, 48, and 72 h after infection, 3-4 upper leaves of plants were flash frozen in liquid N_2 and then stored at −80°C for further experiments.

Gene expression analysis

Total RNA was isolated using RNX-plus kit (Cat. No. RN7713C, Sinaclon, Iran). After treatment with DNaseI (Cat. No: EN0525, Thermo Fisher), synthesis of 1st strand cDNA was carried out using RevertAid First Strand cDNA Synthesis Kit according to the manufacturer's instructions (cat. No. RT5201, Thermo Fisher). All the reactions were done on a StepOnePlus Real-Time PCR System (Applied Biosystems). Diluted cDNA samples were used as template and mixed with 200 nM of each primer pairs and Maxima SYBR Green/ROX qPCR Master Mix (2x) (Cat. No. K0221, Thermo Fisher). The qRT-PCR reactions condition was as 95 °C for 10 min; 95 °C for 15 s and 60 °C for 1 min repeated for 40 cycles. The ubiquitin gene was used as endogenous control to normalize the samples and the relative gene expression was computed by $2^{-(\Delta\Delta Ct)}$ method [27]. The list of genes and primer sequences are shown in Table 1.

Table 1. Names of genes and their primer sequences.

Gene	Primer sequence (5'→ 3')	Ref.
PR1	ccctaagcttacaaacacacatctccgaaa gcatgaattcttgaaatgagcagcagcaaa	[47]
PR2	gacgtcgtcgtatctcatgg gagttgggcgtcaaaaagg	AJ000081
PR3	acagaattgtggaagcgg agcaagtcttcaaacatctcc	AF090336
PR4	aatgatgaacgatgccctgcca ccacttgatgctgtctccaa	[54]
PR5	taggaccaattctgtctctcac atatctcatttggcttccct	[54]
POX	gatcttcgtgctcgtgttca tgcgaatgttttgctgtctc	[54]
PAL	cacaaattgaagcaccatcc ttctcagggcataacgatcc	AY681119
LOX	gtcgttctggaacttgtcggcact ctgtgattgcaccaggcgtccc	[54]
UBI	gatcccaccagaccagcaa accaaatgaagggttgattcctt	[20]
Tef	acc gtc ttg ggg ttg tat cc tcg tcg ctg tca aca aga tg	[27]

RESULTS

Establishment of fungal symbiosis with sour orange roots

Round to pear-shaped and chained fungal chlamydospores were observed in the stained root cortex tissues of inoculated plants. Fungal hyphae were also visible on the root surface, while chlamydospores and hyphae were not seen in the control plant roots (Fig. 1.) In the molecular examination with specific *tef* gene primers, a *S. indica*-related band with 160 bp was seen in inoculated plants, but no band was observed in the controls.

Gene expression analysis

The highest *PR1* gene expression in the *S. indica*-colonized plants was observed 48 h after infection with *P. viridiflava* which was 33.12 times higher than control plants. In the non-colonized plants infected with

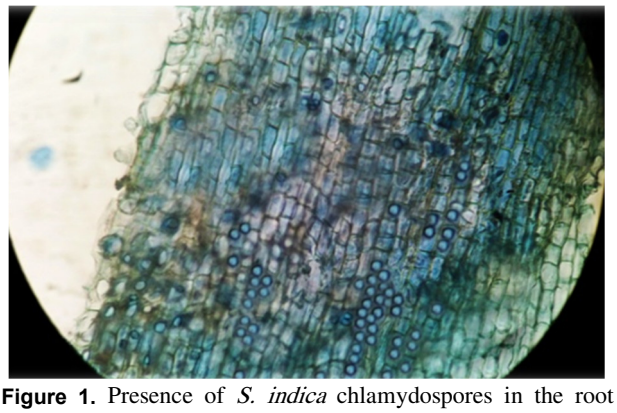

Figure 1. Presence of *S. indica* chlamydospores in the root cortex tissue of sour orange.

P. viridiflava, the expression was as 7.49, 4.43 and 1.03 at 24, 48 and 72 h after infection in comparison with non-infected control plants, respectively (Fig. 2a).

The expression level of *PR2* gene in *S. indica*-colonized sour orange plants peaked at 72 h after infection, which was 6.72 times than control, its changes at 24 and 48 h were 3.71 and 1.46, respectively. In the non-colonized plants infected with *P. viridiflava*, the maximum expression of this gene was observed 24 h after infection, which was 2.72 more than control and then gradually decreased (Fig. 2b).

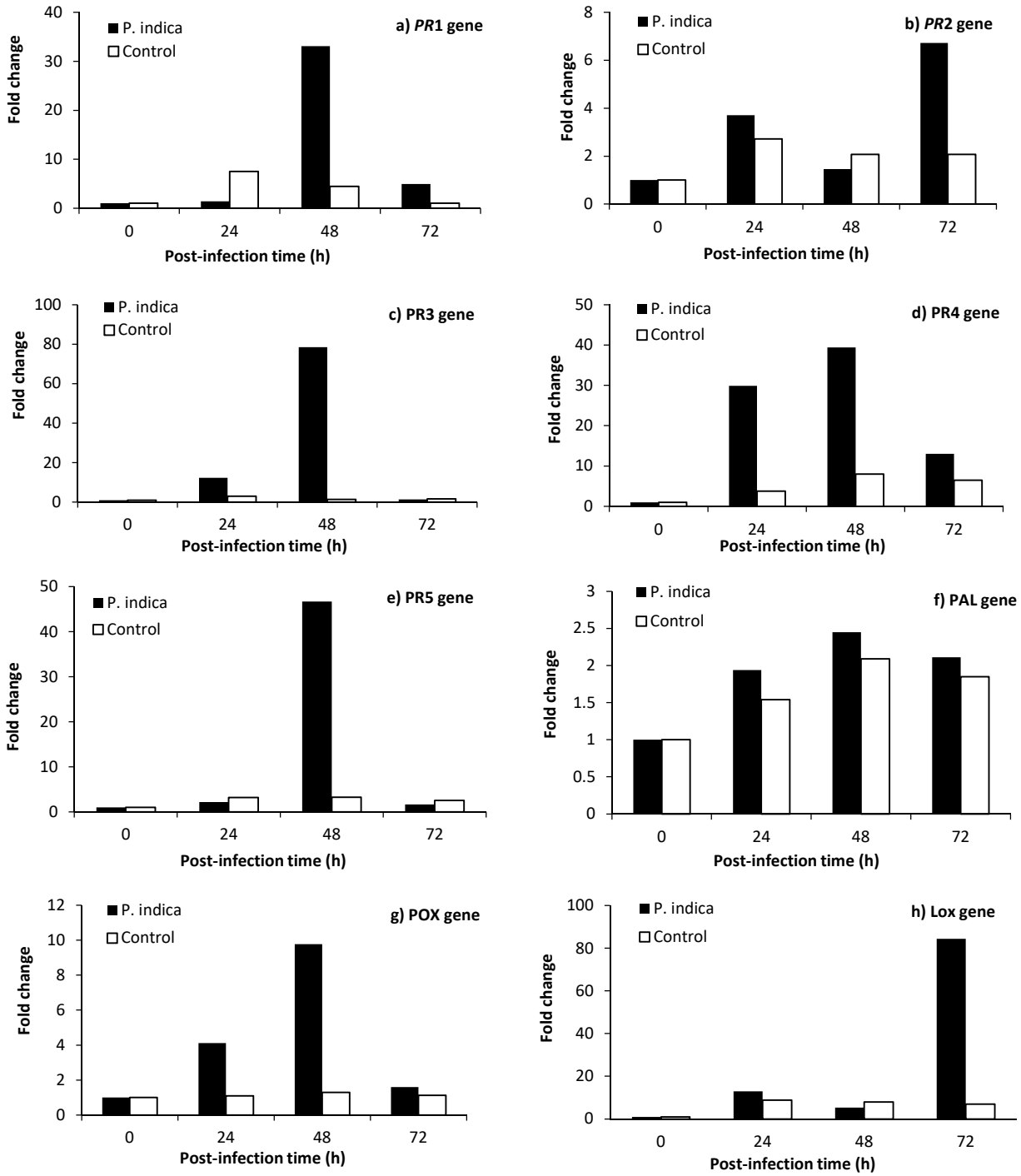

Figure 2. The relative gene expression of a) *PR1*, b) *PR2*, c) *PR3*, d) *PR4*, e) *PR5*, f) *PAL*, g) *POX* and h) *Lox* genes at 24, 48 and 72 h after *P. viridiflava* infection in *S. indica*-colonized (*P. indica*) and non-colonized (Control) sour orange plants.

The trend of *PR3* gene expression in plants treated with the *S. indica* was initially 12.25, and then reached the highest level (78.52) at 48 h, after which it showed a downward trend (1.37). The expression level was also variable in the sour orange plants infected with blast-causing bacteria and the highest expression level was 3 times at 24 h, which then reached 1.38 and 1.55 in the 48 and 72 hours, respectively (Fig 2c).

The peak of *PR4* gene expression in plants treated with the endomycorrhizal fungus was measured at 48 h being 39.39 times higher than control. The gene expression level increased to 29.85 at 24 h, but it reached 12.99 at 72 h. Bacterial-treated control plants also reached 3.73 at 24 h, showed the highest expression (8) at 48 h after infection, and reached 6.49 at 72 h (Fig 2d).

In the sour orange plant, the expression level of *PR5* gene peaked in the *S. indica*-treated plants at 48 h after infection, which was 46.68 times than control. This expression level was 2.17 times higher at 24 h, and it dropped to 1.63 at 72 h. In the control plant, PR5 gene expression reached 3.23 at 48 h after infection, while it showed an upward trend (3.22) at 24 h, and then reached 2.54 at 72 h (Fig 2e).

The *PAL* gene expression in sour orange plants in symbiosis with *S. indica* reached1.94 at 24 h and peaked after 48 h after infection, being 2.45 times than control, which decreased to 2.11 at 72 h. In *P. viridiflava*-treated control plants, the expression of this gene peaked at 48 h, which was 2.09 times than control, and values of 1.54 and 1.85 times were recorded at 24 and 72 h, respectively (Fig 2f).

The *POX* gene expression level in the *S. indica*-treated plant reached a peak at 48 h after infection, with a 9.78-fold increase compared to the control plants. This increase occurred after a 4.12-fold increase at 24 h and decreased to 1.6 at 72 h after infection. This gene was also induced in plants infected with *P. viridiflava*, and the highest expression was observed at 48 h after infection with values of 1.1 and 1.12 at 24 and 48 h, respectively (Fig 2g).

According to the results, the highest expression of the *LOX* gene reached 84.44 at 72 h, which belonged to the root endomycorrhizal fungus treatment. In this treatment, the expression level of *LOX* gene was initially 12.99 and then declined to 5.27 at 48 h. Expression levels of 8.87, 8, and 6.96 were measured in sour orange plants treated with the citrus blast causing bacteria at 24, 48, and 72, respectively (Fig 2h).

DISCUSSION

PR1s are a large family of cysteine-rich proteins found in various plant tissues such as cell walls, vascular bundles, and vacuoles [19]. PR1 proteins are used as an indicator for SAR [39] and their direct effect prevents the growth and spread of pathogens in the host plant. In this study, the symbiosis of sour orange plants with *S. indica* was first confirmed and then they were inoculated with *P. viridiflava*. Our results revealed that the expression of this gene peaked in the resistant plant by fungal treatment at 48 h after infection, and this increased expression level prevents further spread of the pathogen and thus leads to resistance in the cultivar. A study on the expression pattern of the *PR1* gene in wheat resistance in response to surface blight infection showed that this gene played the greatest role in the pathogen-plant interaction [3].

PR2 proteins (β-glucanases) show β-1,3-glucanase (glucan endo-1, 3- β-glucosidase) activity, which breaks down the 1, 3- β-D-glucosidase units in β-1, 3-glucans. This compound is present in plant tissues and is involved in callus formation, the hairy growths of stems and leaves, root hairs, pollen grains, ovule, and wound parenchymal cells [51]. Their molecular weight is about 33 to 36 kDa [49]. The major role that β-glucanases play in the bacterial-plant interaction is the disintegration of the pathogen cell wall, which in turn leads to the death of the pathogen. The dissipation of cell walls fragments resulting from the bacterium disintegration acts as an elicitor and activates the plant defense system [51]. Research has proven the synergistic activity of proteins in this group with the PR3 group [45]. Additional research has shown that this protein is in some cases involved in the release of plant elicitor molecules, such as phenolic compounds, phytoalexins, and other PRs, and the increase in resistance does not result from the direct function of this protein [51]. β1,3-glucan is the substrate of the enzyme glucanase, which is found in plant tissues and is involved in the callus formation, hairy growths of stems and leaves, root hairs, and wound parenchymal cells. This enzyme directly causes the death of the pathogen [6], and the fragments of cell wall destruction as an eliminator will indirectly induce resistance [51]. Glucanase proteins have antifungal and antibacterial activities and are associated with SAR [44]. The increased expression of glucanase genes produced highly resistant plants to pathogens [7,38].

In the present study, the *PR2* gene expression level decreased initially and then increased in sour orange plants. The peaked expression level of this gene at 72 h seems to indicate the importance of the *PR2* gene expression in the early hours after inoculation and its efficacy in resistance. High expression levels of this gene against bacterial and fungal pathogens were also reported in previous studies [43]. Heidarinejad et al. (2015) investigated the role of *PR2* and *PAL* genes in rice plant resistance to the bacterium *Acidovorax avenae*. subsp. *avenae* and found that these two genes significantly affected the resistance of rice plants to the bacterium causing brown strip disease [17].

PR3 protein is of special importance among PR proteins. This protein is a linear homopolymer of N-acetyl-glucosamine units linked by β-1,4 bonds [22]. In the present study, the *PR3* gene expression level initially increased with a peak at 48 h and then showed a decrease. Khaksari et al. (2017) examined the *PR3* gene expression pattern in citrus resistance against blast-causing bacteria on Okitsu, sour orange, and a limequat hybrid and found an increase in citrus resistance after the gene application [24].

The first PR4 protein was reported from the potato plant, which was named win1 and win2 (wound-inducible proteins), and then reports emerged from several hosts such as tomatoes, tobacco, wheat, barley, corn, peppers, cabbage, and *Arabidopsis* [14]. These proteins were then classified as endokitinase [32]. Our results indicated that the symbiotic fungus affected the *PR4* gene expression level, which increased in the plant within 24 h after infection and then peaked at 48 h, leading to the plant resistance to the pathogen. The expression of this gene increased in the rice plant in symbiosis with fungi against the causative agent of Bakanae disease of rice *Fusarium proliferatum* [15].

The PR5 protein alters the permeability of the structural components of the bacterial cell wall and ultimately causes the death of the bacterial cell [51]. The present results showed an increase in the *PR5* gene expression at 48 h after infection in the plant resistant by the fungal treatment. In a study on barley, the expression of this gene was measured 24 h after powdery mildew infection [55]. An increase in the expression of this gene was reported in rice in symbiosis with fungi against F. *proliferatum* [15].

Phenylalanine ammonia lase (PAL) catalyzes the deamination process and conversion of L-phenylalanine into trans-cinnamic acid. This conversion is the first step in the phenylpropanoid pathway, which provides precursors to phenolics, lignin, and phytoalexins [51]. An increase in the amount of the *PAL* mRNA has been shown to underlie an increase in its activity. Inactivity of the *PAL* gene, which is required for the synthesis of salicylic acid (SA), leads to a decrease in SAR. *PAL* is involved in the SA biosynthesis pathway and other defense-related compounds and is a key signaling compound for activating defense-dependent genes, catalysts, receptor-like proteins, and transcription factors. Therefore, *PAL* plays an important role in plant resistance to disease [46]. In the present study, the *PAL* gene expression level peaked in fungus-treated sour orange plants at 48 h after infection. Similarly, the expression of this gene increased in wheat in symbiosis with fungus against the powdery mildew pathogen *Blumeria graminis* f. sp. *tritici* [3].

Peroxidase (POX) directly affects plant resistance and plays an important role in the host defense response through the production of hydrogen peroxide free radicals. On the other hand, it lignifies the cell wall, creates cross-links with cell wall proteins, and makes the plant resistant to the pathogen [9]. Our data indicated that the *POX* gene expression level increased in symbiotic sour orange plants at 48 h after blast infection. An increase in the expression of this gene was reported in tomatoes in symbiosis with fungi against salinity stress [1].

These enzymes are involved in the synthesis of oxygenated fatty acids and include jasmonic acid and aldehydes, which play a critical role in plant defense against microbial and insect infections [21]. According to our results, the expression of this gene increased in the symbiotic sour orange plant at 72 h after infection. Mousavi et al. (2014) obtained a similar result on the increased expression of this gene in symbiotic rice against the blast agent *Magnaporthe oryzae* [29]. The present results revealed the peak expression levels of *PR1, PR4, PAL, POX, PR3*, and *PR5* genes in sour orange plants in symbiosis with *S. indica* at 48 h after infection. This early and rapid increase prevents further spread of the pathogen and thus leads to plant resistance. However, the expression levels of *PR2* and *LOX* genes peaked in the plant at 72 h after infection.

The results of this study demonstrate that the symbiotic fungus can induce systemic effects to prepare the host plant to rapidly increase defense genes once it receives a signal for the presence of the pathogen. In another study,

barley plants in symbiosis with this fungus showed systemic resistance against barley root rot disease compared to control plants, while resistance was associated with an increase in the expression of genes involved in resistance [10]. The symbiosis of this fungus with barley was also reported to induce resistance to root rot disease caused by *Fusarium culmorum*, which was associated with increased levels of antioxidant enzymes in the plant [16].

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بررسی تغییر بیان ژن‌های مقاومت گیاه نارنج همزیست شده با قارچ *Serendipita indica* در برابر باکتری عامل بلاست مرکبات (*Pseudomonas viridiflava*)

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چکیده

بیماری باکتریایی بلاست مرکبات (*Pseudomonas viridiflava*) ازجمله بیماری‌های شایع در بیشتر نقاط مرکبات خیز جهان است. گیاهان برای مقابله با عوامل بیماری‌زا از طیف وسیعی از مکانیسم‌ها استفاده می‌کنند که نتیجه برهمکنش گیاه با عوامل بیماری‌زا، القای بیان ژن‌های درگیر در مقاومت و کاهش زخم و آسیب است. علاوه بر این، ارتباط همزیستی بین گیاه و قارچ میکوریزا می‌تواند به‌طور مؤثر رشد را تقویت کرده و گیاه را در برابر شرایط نامطلوب محیطی محافظت کند. در مطالعه حاضر گیاهچه های نارنج همزیست شده با قارچ میکوریز *Serendipita indica* به باکتری *Pseudomonas viridiflava* آلوده شدند. نمونه‌برداری از گیاهان تیمار شده در بازه‌های زمانی ۲۴، ۴۸ و ۷۲ ساعت پس از تلقیح انجام شد و الگوی بیان ژن‌های *PR1*، *PR2*، *PR3*، *PR4*، *PR5*، *PAL*، *POX* و *LOX* در مراحل اولیه بیماری بلاست مرکبات با روش qRT-PCR بررسی شد؛ بنابراین بر اساس نتایج به‌دست‌آمده میزان بیان ژن‌های *PR1*، *PR4*، *PAL*، *POX*، *PR3* و *PR5* در ۴۸ ساعت پس از اعمال آلودگی در گیاه نارنج همزیست شده با قارچ *S. indica* افزایش داشت اما سطح بیان ژن‌های *PR2* و *LOX* در ۷۲ ساعت پس از اعمال آلودگی افزایش نشان داد. به نظر می‌رسد قارچ همزیست قادر است با القای اثرات سیستمیک سبب آمادگی گیاه میزبان در جهت افزایش سریع بیان ژن‌های دفاعی به‌محض دریافت سیگنال حضور عامل بیمارگر بلاست شود.

کلمات کلیدی: بیماری بلاست مرکبات، نارنج، *Piriformospora indica*، ژن‌های PR، *Pseudomonas viridiflava*