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ORIGINAL ARTICLE

Differential of *PAL* and *PR2* Expressions in Barley Plants Challenged with Seed (*Pyrenophora graminea*) and Soil-Borne (*Cochliobolus sativus*) Fungal Pathogens

Eyad Al-Shehadah, Huda Alek, Mohammed Jawhar

[1] *Department of Molecular Biology and Biotechnology, AECS, P. O. Box 6091 Damascus, Syria.*

*E-Mail: ***ascientific@aec.org.sy***

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The seedborne *Pyrenophora graminea* and the soilborne *Cochliobolus sativus* are economical fungal pathogens of barley worldwide. To better understand barley mechanisms to resist these two pathogens, expression of two well known defense-related genes *PAL* and *PR2* were monitored in resistant and susceptible barley cultivars at early points of infection using quantitative real-time PCR (qPCR). Data showed significant variance in the expression patterns of both genes between barley *P. graminea* or *C. sativus* interactions as compared to the non-inoculated controls. It is also notable that *PAL* and *PR2* genes have a higher expression and faster induction in the resistant cultivar as compared with the susceptible one after infection with each pathogen. However, qPCR analysis revealed higher gene expression in resistant barley plants inoculated with seedborne *P. graminea* as compared with soilborne *C. sativus*, with a maximum expression for *PAL* (15 and 6.8-fold) at 72 hours post inoculation and *PR2* (13.2 and 5.3-fold) at 96hpi, respectively. Our data suggest that *PAL* and *PR2* genes, positively regulate *P. graminea* and *C. sativus*—resistance in barley plants during disease progress, which can provide useful information for a deeper molecular research on barley defense responses against pathogens with different infection styles.

Key words: Barley, defense response, soilborne - seedborne pathogens, RT-PCR

Plants have developed immune systems that recognize the presence of fungal pathogens involving seed and soil-borne species and initiate their necessary defense responses. A soil borne pathogen is a disease causing agent which lives both in soil and in a plant host, and has an ability to infect healthy plants growing in that soil. Whereas, a seed borne pathogen is transmitted in embryo which is an essential place for the fungus life cycle. These soil-seedborne pathogens are globally distributed in crops growing area causing significant yield losses (Delgado-Baquerizo *et al.,* 2020; Goko *et al.,* 2021). Therefore, increasing our understanding of the crop resistance mechanisms is required.

Leaf stripe caused by the seedborne pathogen, *Pyrenophora graminea* S. Ito & Kurib. (anamorph *Drechslera graminea* (Rabenh. ex. Schltdl.) S. Ito), and common root rot (CRR) caused by the soilborne [*Cochliobolus sativus* Drechs. ex Dastur] of barley are among the most devastating fungal diseases causing significant yield losses across the world (Fernandez *et al.*, 2009; Ghazvini, 2012). Barley plants infected with *P. graminea and C. sativus* pathogens excite defence response which is regulated by various signalling pathways (Ghannam *et al.,* 2016; Jawhar *et al.,* 2017). Hence, it is highly challenging to control *these both pathogens* due to a poor understanding of the barley defense mechanisms towards them.

A large number of pathogenesis-related (*PR*) genes are up or down-regulated during plant-pathogen interactions. However, many of their precise roles are still unknown. *PR2* is predicted to encode a β-1, 3-glucanase, an enzyme hydrolyzing β-1, 3-glycosidic bonds in β-1, 3-glucans, and has been reported to degrade the fungal pathogen cell wall (Akiyama *et al.,* 2009). In addition, phenylalanine ammonia-lyase (*PAL*) has an essential function in secondary phenylpropanoid metabolism and considered to play a role towards fungal pathogens attack (Hyun *et al.,* 2011). The *PAL* and *PR2* have been found to be involved in the resistance mechanisms towards different plant diseases, such as in rice (Heydari nezhad *et al.,* 2016), soybean (Zhang *et al.,* 2010) and grapevine (Lakkis *et al.,* 2019). Quantitative PCR (qPCR) would be an efficient technique for measuring the plant relative expression genes after being exposed to biotic stresses, such as infection by fungal pathogens (Derveaux *et al.,* 2010).

We hypothesized that the *PAL* and *PR2* genes could drive contrasted levels of interactions in barley resistant and susceptible genotypes challenged with seed - and soil-borne fungal pathogens. Thus, the present study aimed at evaluating the changes in induction of *PAL* and *PR2* expressions in two barley cultivars infected with different infection styles *P. graminea* and *C. sativus* pathogens using qPCR approach.

MATERIALS AND METHODS

Plant Materials

The resistant cv. Banteng and susceptible cv. Furat1 barley cultivars to *P. graminea* and *C. sativus* were used in this work (Arabi *et al.,* 2004; Jawhar *et al.,* 2017). 'Banteng' is a German cultivar, and 'Furat-1' is a local Syrian cultivar.

Inoculation with *P. graminea*

The most Syrian virulent strain Sy3 (Arabi *et al.,* 2004) was used. The fungus was grown on PDA media and incubated for 7 days at 20 ± 1 °C in the darkness. Inoculation was performed according to Hammouda *et al.* (1986). Inoculated and control seeds were planted in plastic 20-cm pots filled with sterilized peat moss with five replicates. Each replicate consists of five pots each of ten seeds. Plants were grown under temperature at 12 ^{o}C with a daylength of 12 h (Delogu *et al.,* 1989).

Inoculation with *C. sativus*

The Syrian virulent isolate of *C. sativus* (*Cs*16) (Arabi and Jawhar, 2002) was used in the experiments. The fungus was grown on Petri dishes containing potato dextrose agar (PDA, DIFCO, Detroit, MI, USA) for 10 days under 20 °C in the dark. Conidial suspension was adjusted to 2 x 10^4 conidia/mL. Seeds were inoculated by mixing them thoroughly with a natural Arabic gum (8 drops/L)-peat-conidia inoculums, and 40 ml of spore suspension was mixed in a plastic Petri dish with 50 gr sterile neutralized peat, and planted in pots at a depth of 6 cm to promote long subcrown internodes (Fetch and Steffenson, 1999) in three replicates with 10 seeds per

replicate. Based on *in vitro* preliminary tests on PDA media, *CRR-free seeds were used* as controls.

RNA isolation and cDNA synthesis

Barley leaves were collected after 24, 48, 72 and 96 hours post inoculation (hpi) with each pathogen. mRNA was extracted using the QuantiTect Reverse Transcription Kit (Qiagen) following the manufacturer's instructions. RNA was used for cDNA which was stored at −20 °C. Control samples were collected at each time point from plants spread with pathogen-free water.

Quantitative real time PCR (qPCR)

PAL and *PR2* were assayed using SYBR Green Master kit (Roche, USA) according to the method described by Derveaux *et al.* (2010). The RT-PCR primers sequences are given in Table 1. The fluorescence readings of three replicated samples were averaged, and the blank value was subtracted. The expression levels were measured using the average cycle threshold (Ct). Average Ct values were calculated from the triplicate experiment conducted for each gene, with the ΔCT value determined by subtracting the average Ct value of genes from the Ct value of the *EF1α* gene as a reference gene. Finally, the equation $2^{-\Delta\Delta CT}$ was determined as described by Livak and Schmittgen (Livak and Schmittgen, 2001), with *EF1α* as a reference (housekeeping control) gene. Statistical analysis was performed via the Tukey's test at the 0.05 level.

RESULTS AND DISCUSSION

In this work, two barley cultivars with different resistance levels to *P. graminea* and *C. sativus* infections were used. As shown in Fig. 1, both pathogens caused more severe infection on the susceptible cv. 'Furat1' as compared with the resistant cv. 'Banteng' one. These results are in agreement with our previous observations under natural field conditions (Arabi and Jawhar, 2002; Arabi *et al.,* 2004).

Further studies of barley interactions with *P. graminea* and *C. sativus* by measuring *PAL* and *PR2* expression at four early time points after pathogen challenge, demonstrated that these genes displayed different expressions at $P = 0.001$ in both barley cultivars, and these expressions were higher and faster induction for both pathogens in the resistant cultivar as compared with the susceptible one (Figures 2 and 3).

Data showed that PCR (qPCR) analysis revealed higher gene expression in resistant barley plants inoculated with seedborne *P. graminea* as compared with soilborne *C. sativus*, with a maximum expression for *PAL* (15 and 6.8-fold) at 72 hpi and *PR2* (13.2 and 5.3-fold) at 96hpi, respectively (Figures 2 and 3).

The both genes *PAL* and *PR2* were higher up-regulated in *P. graminea* inoculated barley plants as compared to *C. sativus* inoculated plants, which might indicate that these genes might play significant roles in barley resistance against *P. graminea* which is in line with Ghannam *et al.* (2016).

It is well known that *PR2* encodes glucanase enzyme which hydrolyses the glycosidic bond of glucan in plant cell walls that led to cell wall loosening and expansion (Leubner-Metzger and Meins, 1999). Pecchioni *et al.* (1999) found that the infection of barley rootlets by *P. graminea* lead to induce the accumulation of seven PR genes of mRNA families. On the other hand, the other gene *PAL* plays an important role in catalyzing the non-oxidative domination of phenylalanine to *trans*-cinnamate as a first step in the phenylpropanoid pathway which is an important regulation point between primary and secondary metabolism (Vogt *et al.,* 2010). These events may be the cause of barley cell wall damage during infection with the seed and soilborne pathogens.

The two seed and soilborne pathogens used in this study have several different morphological and biological aspects, such as symptomatology, systemic growth and ability to produce successful secondary infections on barley plants (Porta-Puglia *et al.,* 1986; Kumar *et al.,* 2002). Therefore, we believe that analysis of *PAL* and *PR2* expression might provide useful information for a deeper molecular research on barley defense responses against pathogens with different soil infection styles. Our results are in agreement with previous works in chickpea (Raju *et al.,* 2008) and in grapevine (Lakkis *et al.,* 2019).

Table 1: Properties and nucleotide sequences of primers used in this study

Gene	Gene description	Sequence	Amplified fragment (bp)
PAL	Phenyl alanine amino lyase	CCATTGATGAAGCCAAAGCAAG	123
		ATGAGTGGGTTATCGTTGACGG	
PR2	Beta1,3-glucanase2	TGGTGTCAGATTCCGGTACA	193
		TCATCCCTGAACCTTCCTTG	
EF1α	Elongation foctor-1 Alapha	TGGATTTGAGGGTGACAACA	167
		CCGTTCCAATACCACCAATC	

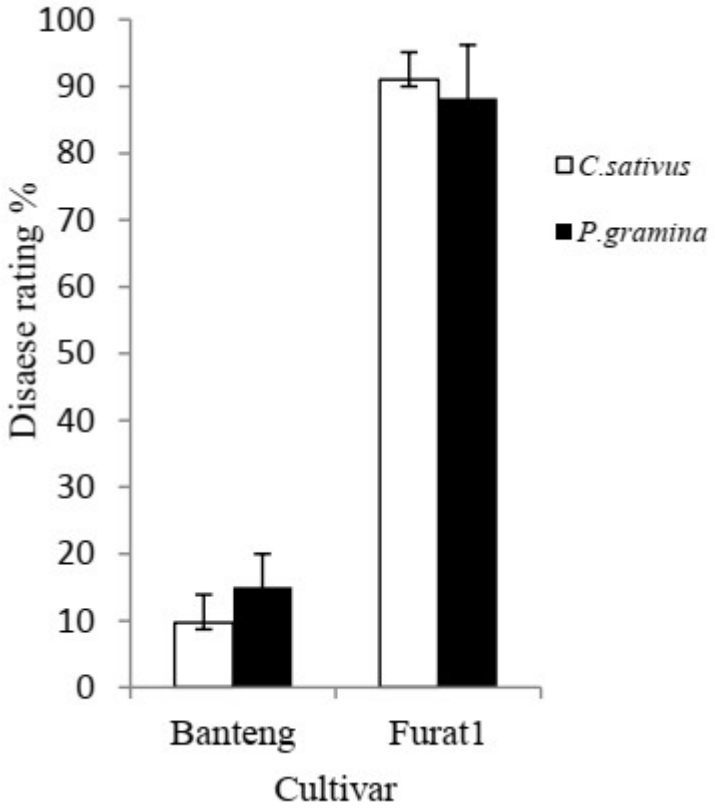

Figure 1: Frequency of disease reactions on the barley resistant cv. Banteng and susceptible cv. Furat 1 by *P. graminea* and *C. sativus*. Significance at ***$P < 0.001$ between the cultivars for each disease

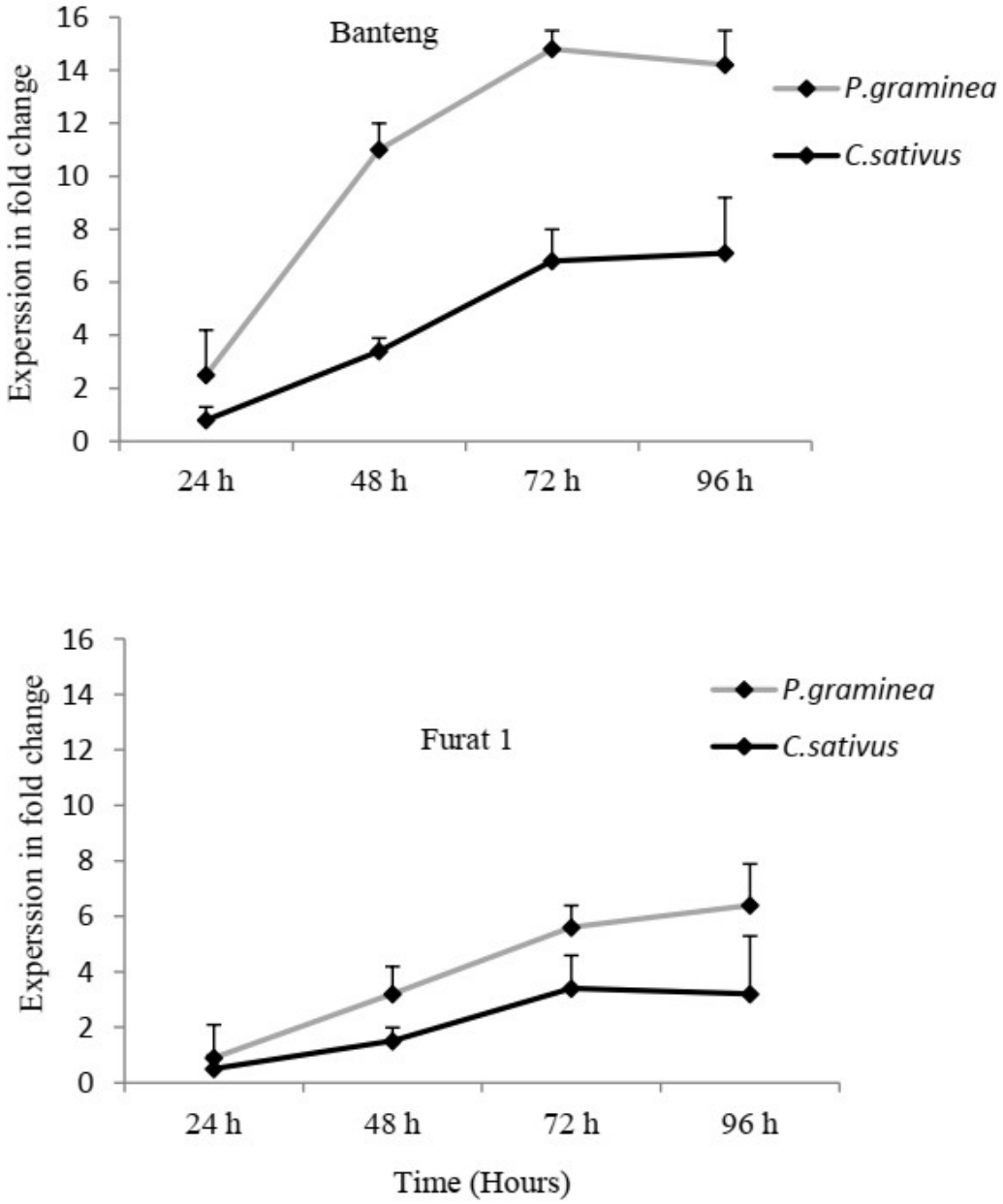

Figure 2: Relative expression profiles of *PAL* gene in the resistant cv. 'Banteng' and in the susceptible cv. 'Furat1' during the time course following *P. graminea* and *C. sativus* infections. Error bars are representative of the standard error (mean ± SD, n = 3). Data are normalized to Elongation factor 1α (EF-1α) gene expression level (to the calibrator, Control 0 h, taken as 0).

CONCLUSION

Seedborne and soilborne diseases pose a significant risk to barley cropping in world. In this work, our data demonstrated that, significant increases in *PAL* and *PR2* expression were found upon barley challenged with the two seed - soilborne fungi *P. graminea* and *C. sativus* respectively, with values being consistently higher in plants inoculated with *P. graminea*. It is also notable that *PAL* and *PR2* have higher expressions and faster induction in the resistant cultivar as compared with the susceptible one. This might be a useful complementary option for the management of these both pathogens in barley.

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CONFLICTS OF INTEREST

The authors declare that they have no potential conflicts of interest.

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