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Original article

ANALYSIS OF GENE EXPRESSION CHANGES IN CANOLA IN RESPONSE TO INOCULATION WITH BIOTIC AND ABIOTIC ELICITORS TO *ALTERNARIA BRASSICAE*

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Abstract

Background. *Alternaria brassicae* is one of the most destructive pathogens of oilseed rape growth and production. Oilseed rape roots treated with biotic and abiotic elicitors have shown resistance to *A. brassicae* as a result of induced systemic resistance (ISR). In this experiment, we aimed to identify resistance genes in two rapeseed genotypes (*Raphanus brassica* and *Brassica napus*).

Methods. *Pseudomonas fluorescens* PF83 as a biotic elicitor and with Salicylic acid (SA) as an abiotic elicitors were used induced systemic resistance in rapeseed. The expression levels of six defense-related genes (*VSP2*, *MYC2*, *PR-2*, *PR-3*, *PR-4* and *PR-5*) by Quantitative real-time PCR (qRT-PCR) at three times 24, 48 and 120 hpi.

Results. *VSP2*, *MYC2*, *PR-3* and *PR-4* gene expression levels increased in infected leaves by *A. brassicae* and in oilseed genotypes treated by PF83, while the expression levels of PR-2 and PR-5 were increased in leaves treated with SA. The results indicate that the induction of resistance genes depends on the type of elicitor, the biotic and abiotic elicitors interferes with phytohormones SA- and JA-pathways to *A. brassicae*, through the increase in the transcription of resistance-related genes. The abiotic elicitor is key to inducing resistance genes associated with SA-pathways, while the biotic elicitor is key to inducing resistance genes associated with JA-pathways. The results of our study provide a new understanding of the control mechanisms of *A. brassica*.

Conclusion. This study provides a new understanding of the control mechanisms of *A. brassica*. The abiotic elicitor is key to inducing resistance genes associated with SA-pathways, while the biotic elicitor is key to inducing resistance genes associated with JA-pathways. This study explained, that the RR genome is responsible for disease resistance in oilseed *R. brassica* (AARR) to infection processes of *A. brassicae*, so we recommend that using R. brassica as resistance genotype to produce oilseed.

Keywords: *Alternaria brassica; Brassica napus* L; *Raphanus brassica; Pseudomonas fluorescens* PF83; Salicylic acid

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Introduction

Oilseed crop is one of the important sources of oil upon which the industry has grown in many countries such as China, Canada, Australia and others, the researchers continually trying to find varieties capable of producing the largest amount of oil compared to previous varieties, which are considered as source for animal feed, nutrition of humans, soil conditioners, biofuels and other significant socioeconomic importance [1].

*Brassica napus (*AACC, 2n= 4*x* =38) is formed through hybridisation between *B. oleracea* (CC, 2n = 2 *x* = 18) and *B. rapa* (AA, 2n = 2 *x* = 20), and is one of the most important oilseed crops in the world as it provides oils for human consumption along with soybean oil and palm oil [2]. *B. rapa/ Raphanus sativus* (AARR, 2*n* = 38) is called Rapa radish, amphidiploid between *B. rapa* and *R. sativus* is a good seed produce and used as new fodder crop additionally is resist to the beet cyst nematode [3].

Alternaria brassicae is a necrotrophic fungal pathogen that causes major damage to aerial plant parts at all the stages of growth in oilseed rape, to leading to various losses in production and yield worldwide [4]. Although synthetic chemical pesticides are widely used by farmers and have been successful in controlling various plant diseases, their excessive use has caused numerous environmental and health problems for humans. Therefore, specialists in plant disease management and those concerned have sought to find alternative methods.

In recent years, several successful methods for control of plant diseases have emerged, including the application of systemic resistance, there are

varieties of induced resistances which vary according to the different signaling. Induced Systemic Resistance (ISR) and Systemic Acquired Resistance (SAR) are two forms of systemic resistance; in both SAR and ISR, plant defenses are released by previous infection or treatment that lead to systemic resistance against subsequent challenge by a pathogens or the application of biotic and abiotic elicitors, with most agents urged to reduce disease in plants infected by between 20 and 85%, this resistance can be using as an alternative to chemical pesticides [2; 5; 6].

Many defensive compounds used to induce systemic resistance in plants against both pathogens and herbivores and the SA pathway dependent can be activated when the plants are treated with chemical/abiotic inducers such as salicylic acid (SA), quinolizidine alkaloids, gossypol, nonproteinogenic amino acids, glucosinolates, furanocoumarins andβ_ aminobutyric acid [7].

Plant growth-promoting rhizobacteria (PGPR) directly or indirectly stimulate local or systemic resistance in plants against a wide range of different pathogens. This depends on their establishment, survival and continuity, in addition to the network of interactions in plant roots [8]. There are two types of beneficial interactions between rhizo-microbiomes and plant roots, i.e. symbiotic alliances where microbes and plants share costs and benefits [9]. There are many bacterial genera that form the PGPR, such as: *Acinetobacter*, *Arthobacter*, *Azotobacter*, *Azospirillum*, *Agrobacterium*, *Bacillus*, *Burkholderia*, *Bradyrhizobium*, *Frankia*, *Pseudomonads*, *Rhizobium*, *Serratia* and *Thiobacillus* [10]. PGPR strains such as *Pseudomonas fluorescens* have been reported to activate systemic protection by induced systemic resistance in plant such as in radish against *Fusarium oxysporum* [11], rice against *Rhizoctonia solani* [12], Tomato against *Alternaria solani* [13], grapevine against *Botrytis cinerea* [14], *Arabidopsis thaliana* against *Pseudomonas syringae* pv. *Tomato* [15], maize against *Rhizoctonia solani* [16]. Hence the present experiment was conducted to evaluate the induced systemic resistance ability of *P. fluorescens* and Salicylic acid in oilseed rape to *Alternaria brassicae* by studying the gene expression of resistance genes associated with hormonal pathways in two genotypes of oilseed rape.

Materials and methods

1. The Pathogen and the elicitors

The *Alternaria brassicae* is used as a pathogen and *P. fluorescens* PF 83 and salicylic acid were used as biotic and abiotic elicitors in this experiment were obtained from the Plant Protection department, College of Agriculture,

Wasit University, Wasit, Iraq. *A. brassicae* was maintained and cultured on PDA medium (200 g peeled potato, 20 g dextrose, 15 g agar, and 1 L distilled water) in the dark at 20 ± 2°C, and 5-mm-diameter mycelia agar plugs were punched from the growing margin after 4 days. For leaves inoculation, ascospores were harvested in sterile distilled water using a sterile brush and filtered through four layers of cheesecloth to remove the mycelia of the culture. The resulting spore suspension was adjusted to 1×10^4 spores/ml.

Fluorescent *Pseudomonas* was grown in King's broth medium and incubated at 30± 1°C [17]. Salicylic acid was dissolved in ethanol for use in subsequent experiments.

2. Plant Materials and Growth

Two spring-type genotypes *Brassica napus* (AACC) and *B. raphanus* (AARR) were used in this research and were obtained from the Field crop department, College of Agriculture, Wasit University, Wasit, Iraq. Oilseed rape seeds were surface-sterilized in 70% ethanol for 1, 10 min in 10% v/v sodium hypochlorite, and rinsed 5 times with sterile distilled water. The seeds were sown and grown in a growth chamber under 12 h of light at 25°Cand 12 h of dark conditions at 22°C. Each genotype independent line of oilseed rape was grown in one of conjoined pots (9 × 12 cm, height × diameter) containing 1 kg a mixture of peat moss and sand at a ratio of 1:1, that had been autoclaved twice for 1 h with a 24 h interval. Each pot was planted with 8 seedlings, each pot was covered with transparent film for 4 days to avoid rapid water loss. After germination, only five plants were allowed to grow in each pot. Irrigation was applied by drenching twice a week.

3. Screening of elicitors based on ISR-eliciting potential and effects on plant growth

For screening the biotic and abiotic elicitors capable of eliciting ISR on thirty days old oilseed plants root of genotypes were treated with suspensions separately by 100 ml / pot (*P. fluorescens* suspension, salicylic acid (200 ppm) by mixing the upper soil surface of each pot in the greenhouse experiments. After 24 hours of treatment, the leaves and stems of plants were inoculated by suspension of *A. brassicae* were separated in 100 ml / pot (reported in 2.1). Each treatment consisted of three replicates.

4. Gene Expression Assay of *A. brassicae* growth

The total RNA from the leaves of genotypes treated or non-treated with elicitors at different incubation times (24, 72 and 120 hpi) with pathogen and first-strand cDNA synthesis and quantitative real-time polymerase chain reaction (qRT-PCR) were used as described in detail previously [5]. Six primers

(Table 1) were used for AACC and AARR, including the *PR-2* and *PR-5* genes were used as markers for SA pathways, whereas *VSP2* and *MYC2* were used as marker JA signaling pathways, whereas for the ETH signaling pathway used the *PR-3* and *PR-4*, in addition to the GAPDH gene.

Table 1.

PCR primers used in the present study

Gene	Gene description	Pathway	Plant	Primer sequence (5′-3′)
PR-2	Beta 1,3 glucanase	SA	AACC AARR	GCTCTGCGAGGCTCTAAC TTGACCCATCATTGACTACA
PR-5	Pathogenesis-related protein 5	SA	AACC	ACCGCCACCATCTTCGTT GCCAGGGCAAATCTCGTT
			AARR	CGGCAACAGCACCACCACA GCAATCTCCAGTTCCTCCC
VSP2	Vegetative storage protein 2	JA	AACC AARR	TACCTCACTTCCGACCAG TTCTCAGTCCCGTATCCA
MYC2	Hypothetical protein	JA	AARR AACC	GTAATCACTTTCTCACCCTCG CGTTTGTCCCTTCAATCAG
PR-3	Basic chitinase	ET	AACC AARR	CCTCAGCCGAGCAATGTG CCGAAGCCAGGGAAAGAC
PR-4	Hevein-like protein	ET	AACC AARR	GCCACCTACCATTACTACAAC TCCAAATCCAATCCTCCA
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	-	AACC AARR	CGCTTCCTTCAACATCATTCCCA TCAGATTCCTCCTTGATAGCCTT

5. Statistical analysis

The experimental design of the greenhouse was completely randomized, with three replicates for all treatments. Data of the results were subjected to analysis of variance using GenStat software, and the means ($P < 0.05$) were compared between treatments of oilseed genotypes, biotic, abiotic treatments and alternria leaf spot disease using least significant difference tests [18].

Results

Plants respond actively to different pathogen attacks by deploying a range of defense-response mechanisms that eventually result in plant resistance. To determine the ability of *B. napus* and *R. brassica* genotypes treated or non-treated with biotic (PF83) and abiotic (SA) elicitors to *A. brassica* infection

during different time periods (24, 48 and 120 hpi), by qRT-PCR we investigated the expression profile of resistance defense-related genes in all genotypes. Six primers for each genotype were used (Table 1), including *PR-2*, *PR-3*, *PR-4, PR-5 VSP2* and *MYC2* genes.

1. Expression levels of gene-related pathogen of healthy and infected *B. napus* and *R. brassica* leaves by *A. brassicae* fungi

The qRT-PCR results showed that the *PR-2* and *PR-5* expression levels increased in *A. brassicae* infected leaves, but the expression levels of *PR-3, PR-4*, *VSP2* and *MYC2* decrease.

The expression of the *PR-2* gene was found to be more and peaked at 120 hpi in infected *B. napus* (AACC) and 48 hpi in infected *R. brassica* (AARR) genotypes, which were up-regulated by 2.34-fold (from 1.00- to 2.34-fold), 6.32-fold (from 1.24- to 4.69-fold), respectively (Fig. 1-A).

The expression levels of *PR-5* gene increased clearly with time and reached its peak at 120 hpi in *B. napus* and at 48 hpi in *R. brassica* infected compared with the non-infected (healthy) ones; the expression levels were up-regulated by 2.41 -fold (from 1.37- to 3.31-fold) and 5.58-fold (from 1.63- to 9.11-fold), respectively (Fig. 1-B).

Thus, the response of defense was late in AACC, where the pathogenic fungus can grow quickly before activation of resistance unlike in AARR, where the response of defense is very quick.

VSP2 and *MYC2* are anti-pathogenic genes. Thus, the expression levels of the *VSP2* genes increased in *B. napus* infected than the non-infected (healthy) ones, which was up-regulated by 7.16-fold (from 0.85-fold to 6.09 -fold), but in *R. brassica* was up-regulated at 48 hpi by 22.46-fold (from 1.00-fold to 22.46 -fold) (Fig. 1-C). The *MYC2* gene expression levels increased in infected both genotypes than in the non-infected ones, which was up-regulated by 6.13-fold (from 1.12- to 6.87-fold) and peaked at 120 hpi in *B. napus*, whereas up-regulated by 47.62-fold (from 1.20- to 57.17-fold) and peaked at 24 hpi in *R. brassica* (Fig. 1-D).

The expression of the *PR-3* gene increased and peaked at 120 hpi in infected *B. napus* and 24 hpi in infected *R. brassica* genotype, which were up-regulated by 5.51-fold (from 1.15- to 6.34-fold) and 13.05-fold (from 1.10- to 14.35-fold), respectively (Fig. 1-E).

The expression levels of the *PR-4* gene showed significant differences between infected and non-infected *B. napus* and were up-regulated by 2.60-fold (from 1.20- to 3.13-fold) at 120 dpi, and reached in infected *R. brassica* genotype 2.94-fold (from 1.45- to 4.27-fold) at 120 dpi (Fig. 1-F).

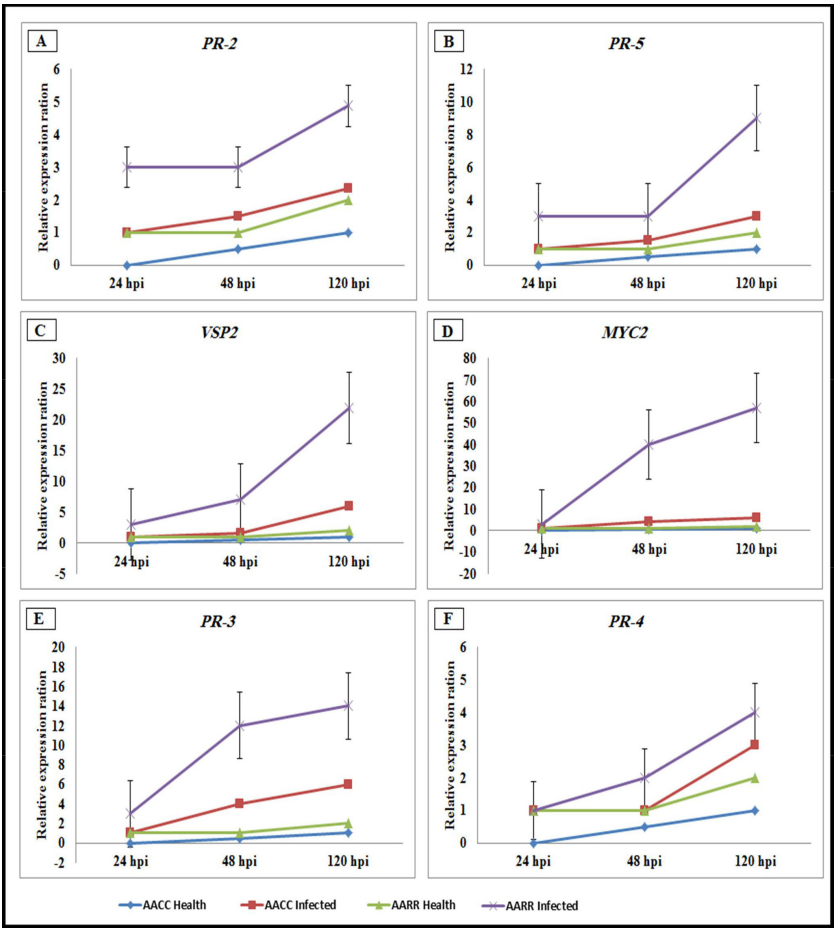

Fig. 1. The expression of defense-related genes in three potted for each time of *B. napus* and *R. brassica* genotypes of fifteen days old inoculated by suspension of *A. brassicae* onto leaves. Leaves were collected 24, 48 and 120 hour post infection. Total RNA was extracted, and cDNA was synthesized. Expression levels of the *PR-2*, *PR-3*, *PR-4, PR-5 VSP2* and *MYC2* genes were monitored by RT q- PCR.

2. P. fluorescens PF83 mounts ISR in genotypes to A. brassicae

The expression levels of *VSP2, MYC2, PR-3* and *PR-4* increased in leaves that were treated with PF83 compared with non-treated without infection of the pathogen.

The expression level of the *PR-2* genes in *B. napus* peaked at 120 hpi and up-regulated by 23.83 -fold (from 0.85- to 20.26-fold) and in *R. brassica* which peaked at 48 hpi and up-regulated by 28.91-fold (from 1.21- to 34.99-fold) (Fig. 2-A).

The expression level of the *PR-5* genes reached in *B. napus* which peaked at 120 hpi and up-regulated by 19.22 -fold (from 1.22- to 23.45-fold), whereas in *R. brassica* which peaked at 48 hpi and was up-regulated by 36.07-fold (from 1.14- to 41.13-fold) (Fig. 2-B).

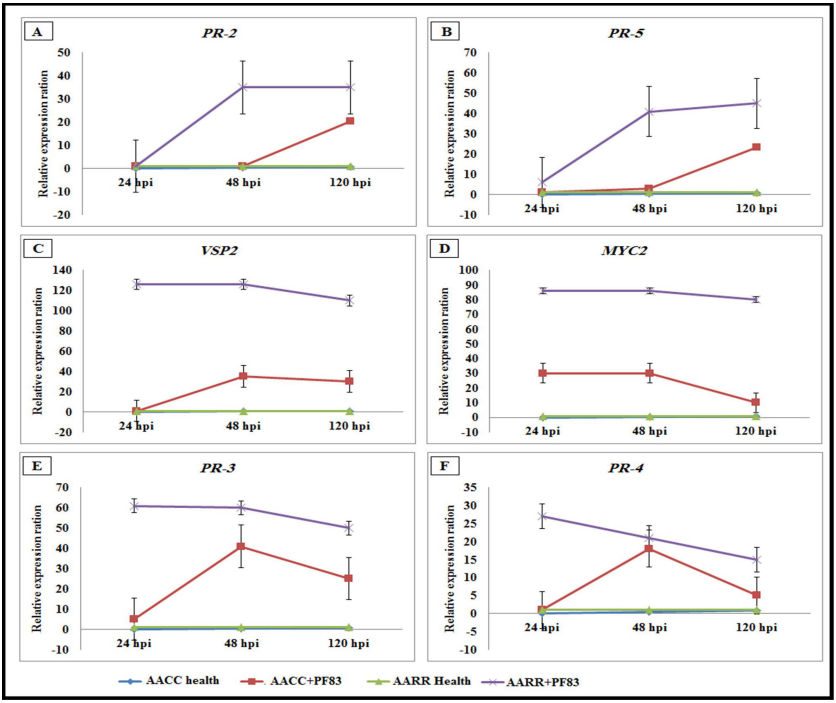

Fig. 2. Expression of defense-related genes in three potted for each time of *B. napus* and *R. brassica* genotypes of 30 days old were drenched about 100 ml / pot of *P. fluorescens* PF83 suspensions and after 1 day inoculated by 5 mm mycelium disk of *A. brassicae* onto leaves. Leaves were collected 24, 48 and 120 hpi. Total RNA was extracted, and cDNA was synthesized. Expression levels of the *PR-2*, *PR-5, Vsp2,* MYS3, *PR-3* and *PR-4* genes were monitored by RT q- PCR.

The expression level of *VSP2* gene peaked at 48 hpi in *B. napus*, and 24 hpi in *R. brassica* respectively, which were up-regulated by 34.52 -fold (from 1.02- to 35.22-fold) and 112.34-fold (from 1.13- to 126.94-fold), respectively (Fig. 2-C).

The expression level of the *MYC2* genes also increased in *B. napus* and *R. brassica* which peaked at 24 hpi and up-regulated by 27.26 -fold (from 1.13- to 30.80-fold) and 52.76-fold (from 1.63- to 86.00-fold), respectively (Fig. 2-D).

The expression level of the *PR-3* genes in *B. napus* which peaked at 48 hpi was up-regulated by 40.44 -fold (from 1.02- to 41.25-fold) and in *R. brassica* which peaked at 24 hpi was up-regulated by 55.45-fold (from 1.10- to 61.10-fold) (Fig. 2-E).

The expression level of *PR-4* gene peaked at 48 hpi in *B. napus*, and 24 hpi in *R. brassica*, which were up-regulated by 12.74 -fold (from 1.02- to 18.10-fold), and 21.34-fold (from 1.27- to 27.10-fold), respectively (Fig. 2-F).

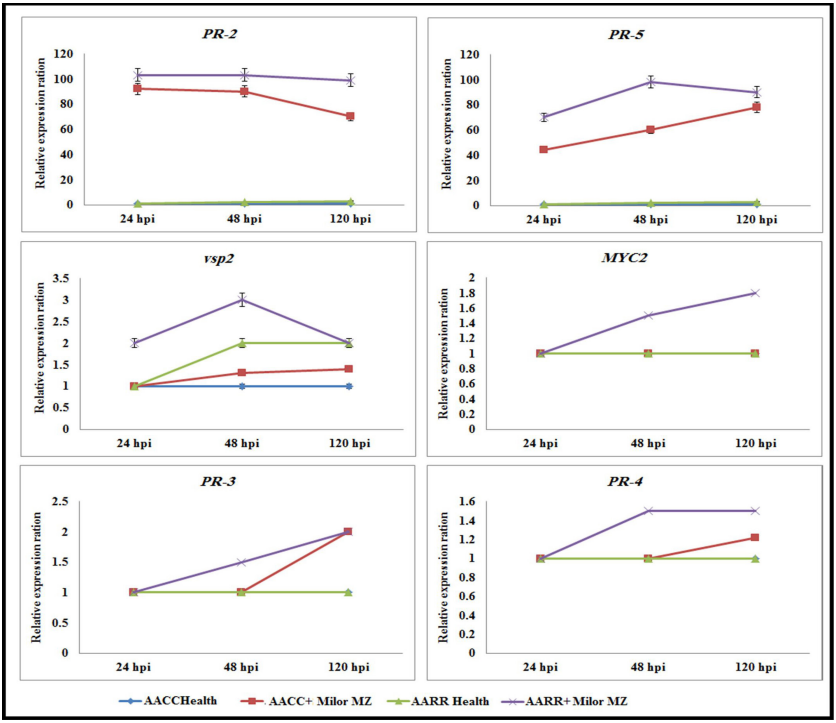

Fig. 3. Expression of defense-related genes in three potted for each time of *B. napus* and *R. brassica* genotypes of 30 days old were drenched about 100 ml / pot of Salicylic acid SA after 1 day from inoculated by 5 mm mycelium disk of *A. brassicae* onto leaves. Leaves were collected 24, 48 and 120 hpi. Total RNA was extracted, and cDNA was synthesized. Expression levels of the *PR-2*, *PR-5, Vsp2,* MYS3, *PR-3* and *PR-4* genes were monitored by RTq- PCR.

The expression level of the *PR-2* gene in plants treated with SA at 24 hpi were up-regulated by 64.83-fold (from 1.42- to 92.05-fold) in *B. napus*, 90.57-fold (from 1.14- to 103.24-fold) in *R. brassica*, compared with the non-treated plants (Fig. 3-A).

The expression level of *PR-5* gene in *R. brassica* was up-regulated at 24 hpi by 51.20-fold (from 1.93- to 98.83-fold), in *B. napus* was up-regulated at 48 hpi by 65.91-fold (from 1.10- to 72.50-fold) compared with the non-treated plants (Fig. 3-B).

The expression levels of *VSP2* and *MYC2* (related with JA) and *PR-3* and *PR-4* (related with ET pathway) were non-increased or slight increase when the plants treated with salicylic acid (SA) in both genotypes.

Dissection

The quantitative real time-polymerase chain reaction (qRT-PCR) results showed that the *PR-2* and *PR-5* expression levels increased in *A. brassicae* infected leaves of AARR, but the expression levels of *PR-3, PR-4*, *VSP2* and *MYC2* decreased. Low and delayed levels of expression of*, PR-3*, *PR-4*, *VSP2* and *MYC2* genes as well as being affected by JA/ET pathway and the fact that *A. brassicae* is saprophytic does not stimulate this pathway, and the high level of gene expression of *PR.1* and *PR.2* may be due to being linked to SA pathway which is motivated by pathogenic micro-organisms and in turn stimulates the so-called systemic acquired resistance (SAR) in host, whereas SAR is accompanied by the induction of pathogenesis-related proteins [19].

Several studies have shown the ability of some of the varieties canola to resist different types of pathogens by the induction of pathogenesis related proteins, and its resistance to diseases may be attributed to the presence of various plant antifungal proteins that are believed to be involved in plant protection through growth inhibition of pathogens, such as *Rs-AFP1* and *Rs-AFP2* in *R. sativus* (RR) [20; 21] and through the production of high peroxidases, which have important roles in plant protection because they reduce pathogenic attack [22].

The expression levels of some genes resistance were increased in *B. napus* and *R. alboglabra* infected compared with non-infected plants, the qRT-PCR results were showed that the *PDF 1.2, AOC3* and *ERF2* genes (marker for the JA/ET signal pathway) expression levels increased in early in plants infected, additionally to *PR-1* gene, but the expression levels were decreased of *TGA5* and *TGA6* (marker for the SA signal pathway) [23]. The resistance gene expression increased over time in *B. napus* and *R. alboglabra* genotypes inoculated with the fungus pathogen *E. curciferarium* caused by powdery mildew disease compared

with non-inoculated plants. It showed that the *PR1* and *PR2* gene expression levels increased early, but the *PR-3* and *PDF1.2* resistance genes which were used as markers for the JA/ET pathway increased later, the reason was due to the lifestyle of a pathogenic fungus in obligate parasite [5].

The results indicated that most of the amplified genes were affected by a combined inoculation with PF83, SA. This result indicates the ability of the biotic and abiotic elicitor to induce systemic resistance in all genotypes against a pathogen.

Through these results we can deduce the success of PF83 to induce systemic resistance in inoculated and infected with pathogen. The results showed that the PF83 depended on JA and ET pathways and are independent from SA pathway because the resistance genes related with JA/ET pathway *VSP2, MYC2, PR3* and *PR-4* were up-regulated in plants treated with biotic elicitors and genes resistance related with SA-accumulation as *PR-2* and *PR-5* were down-regulated, these results lead to knowledge about the PF83 elicitors to ISR, no SAR, the reason may be due to the fact that PF83 from the plant growth promoting rhizobacteria (PGPR) group that leads to activation of the ISR, which in turn depends on JA pathway and ET pathway. Results also showed that there is a slight increase in genes associated with SA-pathway; this discrepancy suggests that PF87 produced SA not exuded into the rhizosphere of genotypes.

In a previous study has shown that bacterial *P. fluorescens* is able to produce some of the materials capable of stimulating the systemic resistance, such as SA, pseudobactin, pseudomonine and a siderophore containing SA [24].

Psedomonas spp. was capable of inducing resistance to *Botrytis cinerea* in grapevine by inducing low phytoalexin host levels in cells, as SA, pyochelin, DAPG and/or pyoverdin are potentially effective in inducing or priming defence responses in grapevine cells [25].

These results indicate the ability of PF83 to elicited ISR in some cases by dependent of SA pathway and the regulatory gene *PR-1* and *PR-2,* These results were consistent with the Kloepper et al [26]. The mechanisms of the bacteria associated with the roots (PGPR) to stimulate systemic resistance to the output of compounds able to induce resistance in treatment plants, some reports that point to a cooperative than antagonist activity of JA/ET and SA pathways in plant protection treated with bacteria [27; 28].

The expression levels of *PR-2* and *PR-5* were related with SA pathway which increased when the plants treated with salicylic acid (SA) in all genotypes. Salicylic acid (SA) plays an important role to stimulating systemic resistance in plants to different pathogens attack by activating some genes associated with

resistance, there are many studies have pointed to this topic, on peanut against *Alternaria alternata* [29], in *B. napus* cv. GSC 5 and *B. juncea* cv. ELM 079 against alternaria blight disease caused by *Alternaria brassicae* [30].

SA plays an important role to development of systemic acquired resistance (SAR) in hosts to defend themselves to pathogens; the SAR response causes rapid accumulation of several pathogenesis related proteins (*PR-Protein*).

Through these results we can note the success of SA to induce systemic resistance in inoculated and infected with pathogen. The results showed that the SA depended on SA pathway and is independent from JA/ET pathway because the resistance gene related with SA pathway as *PR-2* and *PR-5* was up-regulated in plants treated with SA and gene resistance related with SA-accumulation as *VSPE, MYC2, PR3* and *PR-4* were down-regulated, the reason may be due to the fact that SA as abiotic elicitors that lead to activate the SAR, which in turn depends on SA pathway.

PR-1, PR-2 and *PR-5* genes considered as markers for systemic acquired resistance (SAR) dependent on salicylic acid (SA) were examined in the shoots and roots of tomato plants infected by root-knot nematodes (RKNs) and, the results showed the expression of genes was up-regulated in plants pre-treated with SA and increased the resistance to pathogen [31].

Conclusion

This study explained, that the RR genome is responsible for disease resistance in oilseed *R. brassica* (AARR) to infection processes of *A. brassicae*, so we recommend that using *R. brassica* as resistance genotype to produce oilseed, and we recommend application of useful resistance biotic and abiotic elicitors for protection and increasing oilseed production is now under the practice of agriculture.

Our study showed that the signaling pathways in oilseed were depended on the type of elicitor. The SA signaling hormonal pathway was activated in the early stage of *R. brassica* after being treated with biotic elicitors, but the JA and ET signaling hormonal pathways were activated in the early stage after treatment with abiotic elicitors.

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