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# Differential Expression of Defense-Related Genes in *Sinapis alba* and *Brassica juncea* upon the infection of *Alternaria brassicae*

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***ABSTRACT:*** *Alternaria black spot of rapeseed mustard caused by the necrotrophic fungal pathogen Alternaria brassicae is one of the most important plant diseases in India. A comparative study on defense gene induction was undertaken to explore the signaling mechanisms that govern the defense responses of susceptible Brassica juncea and moderately resistant Sinapis alba, belonging to the family Brassicaceae, to alternaria leaf spot disease. The expression of five selected defense-related genes viz., pathogenesis related (PR)-1, PR-2, PR-3, non-expresser of PR-1(NPR-1) and plant defensin (PDF1.2) was examined after inoculation of the seedlings of B. juncea and S. alba. Transcripts of all five defense-related genes accumulated at a greater level and earlier in S. alba than in B. juncea upon challenge inoculation with A. brassicae locally as well as systemically. Although PDF1.2 is known to be induced only by jasmonic acid (JA) and PR-1 by salicylic acid (SA) in the model plant Arabidopsis thaliana belonging to the family Brassicaceae, PDF1.2 was induced also by SA and PR-1 by JA in both B. juncea and S. alba in the present study. These results suggest that defense signaling pathways in B. juncea and S. alba are different from A. thaliana. Although JA responsive genes are predominantly induced in most crop plants and model plants against necrotrophic fungal pathogens, both JA and SA responsive genes seem to play an important role in S. alba conferring some resistance against A. brassicae.*

***Keywords:*** *Brassica juncea, defense gene, jasmonic acid, salicylic acid, Sinapis alba*

## INTRODUCTION

Indian mustard [*Brassica juncea,* (L.) Czern and Coss.] is one of the premier oil seed crops in India and fulfils nearly 27% of vegetable oil requirements of the country. Alternaria blight disease caused by the necrotrophic fungal pathogen *Alternaria brassicae* (Berk.) Sacc.is one of the most common and destructive diseases of the oilseed mustard. The yield losses due to alternaria blight have been estimated to range from 35 to 46% (Mishra *et al.,* 2010).

Conventional breeding to develop resistant cultivars in *B. juncea* against *A. brassicae* is confounded due to non-availability of suitable resistance sources within the available germplasm of cultivated and wild species of *Brassica.* However, resistance to *A. brassicae* has been identified in other wild species in the family Brassicaceae such as *Sinapis alba* (white mustard) (Kolte, 1985; Ripley *et al.,* 1992; Hansen and Earle, 1997; Sharma *et al.,* 2002). The resistance of *S. alba* to *A. brassicae* has been linked partially to the production of

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phytoalexins (Pedras and Smith 1997; Pedras and Zaharia, 2000). Pedras and Zaharia (2000) reported the elicitation, detection, and synthesis of two phytoalexins, sinalbins A and B produced by leaf and stem tissues of *S.alba* upon infection with *A. brassicae*.

Apart from phytoalexin mediated local defense, a large number of defense-related genes are up or down-regulated during plant-pathogen interactions locally as well as systemically (Glazebrook, 2005). The expression levels of pathogenesis related (PR) proteins are low or absent in mature healthy plants but become elevated after pathogen attack (Thomma *et al.,* 2001). Moreover, PR genes might be expressed also in susceptible species but their expression may be seen at the late stage of disease progression or in low concentration that is not sufficient to arrest the lesion development (Thomma *et al.,* 2001). Some key modulators of the plant defense response are salicylic acid (SA) and jasmonic acid (JA) (Glazebrook, 2005). Works by various groups have strongly suggested that resistance to *Alternaria brassicicola* in *Arabidopsis thaliana*, belongs to the family Brassicaceae, requires an intact JA signaling pathway and the production of a phytoalexin, camalexin (Thomma *et al.*, 1998; Van Wees *et al.,* 2003*).* Expression of *PR* genes provides useful markers for systemic acquired resistance (SAR) (Glazebrook, 2005). In particular, *pathogenesis related-1*(*PR-1*), *β 1,3- glucanase (PR-2),* and thaumatin like (*PR-5*) have been extensively used as markers for the onset of SA mediated SAR in Arabidopsis and tobacco (Durrant and Dong, 2004) while plant defensin (*PDF1.2*) and *chitinase* (*PR-3*) have been used as markers for the JA mediated SAR (Thomma *et al.*, 1998). However, the SA and JA mediated pathways interact in a complex fashion including mutual inhibition between JA and SA signaling (Glazebrook, 2005).

Biotechnological methods can successfully be utilized to develop resistant variety of *B. juncea* provided that the molecular mechanism of defense in resistant species such as *S. alba* is delineated. Although significant transcriptome data from Arabidopsis is available in response to challenge inoculation with *A. brassicicola*, information on defense gene induction in *B. juncea* and *S. alba* after challenge inoculation with *A. brassicae* is scarce. In this context, *S. alba* and *B. juncea* plants infected with *A. brassicae* in one set of experiments and treated with SA or JA in another set of experiments were analyzed for the induction of some known defense-related genes *viz.,PR-1, PR-2, PR-3, non-expresser of PR-1(NPR-1)* and *PDF1.2* by semi- quantitative reverse transcriptase polymerase chain reaction (RT-PCR). The present investigation explores, for the first time, the induction of above mentioned defense-related genes in *S. alba* upon challenge inoculation with *A. brassicae* or defense inducers (JA or SA) and thereby deciphering some defense-related signaling pathways occurring in *S. alba*.

## MATERIALS AND METHODS

### Plant material and *A. brassicae* inoculation

*B. juncea and S. alba* plants were raised from seeds in pots containing a mixture of soil and organic manure (2: 1) in a net house at the National Research Centre on Plant Biotechnology, Indian Agricultural Research Institute (IARI), during the winter season. The seedlings were grown for 45 days to the six-leaf stage. The plants were then transferred to a growth chamber with a temperature of 22$^{o}$C and illuminated with compact fluorescent lamps (light intensity of 12.5 µmol/m$^{2}$/s$^{1}$) for a 16h/8h light/dark cycle. These plants were allowed to acclimatize for 3-4 days before they were taken for inoculation or defense inducer treatments.

*A. brassicae* was isolated from an infected leaf of *B. juncea* variety `*Varuna'* collected from the fields of IARI, New Delhi and was identified (I.D. No. 81651) at the Indian Type Culture Collection, IARI. Cultures were maintained for inoculum production on Radish Dextrose Agar (RDA) medium (Thakur and Kolte, 1985) in a growth chamber at 22$^{o}$C under dark condition. Conidial suspensions were prepared by scraping mycelium from 21-day old cultures and suspending in sterilized distilled water (SDW). The conidial suspensions were centrifuged at 3000 rpm for 5 min, the residue re-suspended in SDW and the concentration was adjusted to 5 x $10^3$ conidia/ml using a haemocytometer before application.

Prior to inoculation, 20 plants, each from *B. juncea* and *S. alba* were sprayed with SDW and kept covered with transparent polythene bags for 24h in order to maintain the turgidity. Plants were arranged in a complete randomized design (CRD) in the growth chamber. Randomly selected 10 plants from each species were inoculated by placing 4-6 drops (10µl each) of suspension of conidia onto the upper surface of the first and second true leaves. Rest of the plants was used as the control by placing 4-6 drops (10µl each) of SDW onto the upper surface of the first and second true leaves. Inoculated and mock treated plants were covered with polythene bags with 100% interior relative humidity provided by placing water-soaked cotton balls inside each bag. Both inoculated and control plants were kept in dark for 24h after which they were provided with 16 h light/ 8 h-dark cycle.

**Treatments with SA or JA**

In another set of experiments, a batch of twenty plants (45 days old), each from *B. juncea* and *S. alba* were sprayed with SA (2 mM) (Sigma-Aldrich), pH 6.8, until run-off and another batch of 20 plants was similarly sprayed with a solution of methyl jasmonate [(jasmonic acid (JA), 100 µM) (Sigma-Aldrich)in 0.1% (v/v) ethanol)], until run off. Control plants were treated with 0.1% ethanol or SDW. After treatment, plants were kept at 100% RH by covering them with transparent polythene bags.

**Pretreatment of plants with SA or JA prior to inoculation**

In a separate experiment, after 24 h, the plants treated with SA (2mM), JA (100 µM), a combination of SA and JA (SA + JA) (0.1mM + 50 µM) and the controls were inoculated with the conidia suspension of *A. brassicae,* covered with polythene bags and were placed in a growth chamber as described earlier.

**Sample collection and disease scoring**

Leaf samples from defense-inducer treated plants were collected at 2, 4, 8, 12, 24, 48 and 72 h post treatments (hpt). Leaf samples from plants inoculated with *A. brassicae* were collected at 2, 4, 8, 12, 24, 48, 72 and 96 h post inoculation (hpi). Only the green tissue, about 5 mm distance surrounding the spot/inoculation droplet was collected. The un-inoculated 3$^{rd}$/4$^{th}$ leaf of the infected plants (as for the systemic gene induction) were also collected at different time intervals as mentioned above and all the samples were immediately frozen in liquid nitrogen and stored in -80$^{0}$C.

Diameter of the lesions of *B. juncea* and *S. alba* developed by the pathogen was measured five days post inoculation (dpi) from at least 15 plants. Change in lesion size due to various pretreatments of SA, JA and SA + JA, when compared with that obtained by inoculation alone, was also measured 5 dpi as an estimate of the efficacy of the pretreatment in controlling infection. The experiments were repeated three times and the data were

statistically analyzed using Student's t-test and LSM test using Statistical Analysis System (SAS Version 9.1).

**RNA Isolation and semi quantitative RT-PCR**

Total RNA was extracted from leaves using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. RNA integrity was checked on a formaldehyde-agarose gel, quantified by NanoDrop 1000 (NanoDrop Technologies, Inc., DE, USA) and was treated with DNAse 1 enzyme (Thermo Scientific, USA). First strand cDNA was synthesized from 1µg of total RNA and primed with Oligo (dT) (Thermo Scientific, USA) according to the manufacturer's instructions. Five known defense-related genes *viz.,PR-1, PR-2, PR-3, NPR-1* and *PDF1.2* were selected out of nine defense-related genes*viz., PR-1, PR-2, PR-3, PR-5, NPR-1, PDF1.2, glutathione S transferase (GST), Thionin* and *Thioredoxin* used for initial screening prior to use them in the present study. PCR primers were designed based on the cDNA sequences of *B. juncea* (for *PR-1, PR-2, PR-3,* and *NPR-1*) available at NCBI (http://www.ncbi.nlm.nih.gov) database using Primer 3 software. Primers for *PDF1.2* were from *B. rapa*cDNA sequences and *α-tubulin* were designed from cDNA sequences of Arabidopsis. The corresponding primers and EST accession numbers are listed in Table 1. All primers used were submitted to NCBI database for BLASTn search and confirmed to specifically anneal only with their corresponding genes.

PCR was performed using a thermocycler (Biometra) in a 50-µL final volume including 1µL of ten times diluted cDNA template, 5 µL of 10X amplification buffer (Thermo Scientific, USA), 1 µL of 200 µMdeoxynucleotide triphosphates (Thermo Scientific, USA), 1 µL of 10 pico-molar of each primer, and 0.2 µL (1 U) of Taq DNA polymerase (Bangalore-Genei, Bangalore, India) and 40.8 µL of PCR grade water. PCR conditions included an initial denaturing step at 94 °C for 4 min, followed by 30 cycles of 94 °C for 30 s, 55-57 °C for 30 s, 72°C for 1 min with a final extension at 72 °C for 10 min. PCR products were separated using 1% agarose gels, stained with ethidium bromide and observed on a UV trans illuminator. PCR was performed for three times for each primer using the same cDNA sample in order to confirm the reproducibility of the results.

**Table 1. List of primer pairs used for semi quantitative RT-PCR amplification**

| Gene | Accession No. | Sequence | Annealing Temperature (ºC) | Expected band size |
|---|---|---|---|---|
| ***PR-1*** | DQ359128.1 | Sense 5'- GTCACTAACTGTTCTCGACTTCT -3'<br>Antisense 5'- CCATTGTTACACCTCGCTTTG -3' | 55 | 416 |
| ***PR-2*** | DQ359126.1 | Sense 5'- GCTTCGGACAGGTTGGAAATA -3'<br>Antisense 5'- CCGTTCTTGTAGCCGAAGTAAG -3' | 55 | 532 |
| ***PR-3*** | EF586206.1 | Sense 5'- AAGTTCGGTGCTTCCATCTC -3'<br>Antisense 5'- TCCGGTACACTCCCTACTATTC -3' | 56 | 387 |
| ***NPR-1*** | DQ359129.3 | Sense 5'- GGATGGTAGAACTGCTCTCTTG-3'<br>Antisense 5'- GACTTTCCACGCTCCTCATT-3' | 55 | 621 |
| ***PDF1.2*** | AF528180.1 | Sense 5'- AAGCACCAACAATGGTGAAAG -3'<br>Antisense 5'- TGACACAGACTTATTGAACGTAAA -3' | 57 | 254 |
| ***α-Tubulin*** | NM_100360.3 | Sense 5'- CGTGCTGTCTTTGTTGATCTTG -3'<br>Antisense 5'- TGAAGTGGATTCTTGGGTATGG -3' | 57 | 613 |

## RESULTS

### Disease scoring

Development of disease symptoms was observed in the inoculated leaves of both *B. juncea* and *S. alba*. Un-inoculated controls remained free of symptoms. Yellowing of the tissues surrounding the inoculation droplet appeared as early as 24 h post inoculation in *B. juncea* while it was appeared in *S. alba* for 48 h of post inoculation. *B. juncea* was the earliest to produce necrotic lesions (48 h) followed by *S. alba* (96 h). The lesions appeared as grey circular areas at the site of inoculation in both the species (Fig. 1). However, the lesion diameter of *S. alba* was significantly lower (5.86 ± 0.43) compared to that in *B. juncea* (8.03 ± 0.65) after 5 days of inoculation and the lesions did not progress much beyond the boundaries of the inoculum drop even at the 10$^{th}$ day post inoculation. However, the lesions in *B. juncea* leaves continued to expand forming concentric rings and ultimately died by the 10$^{th}$ day after inoculation.

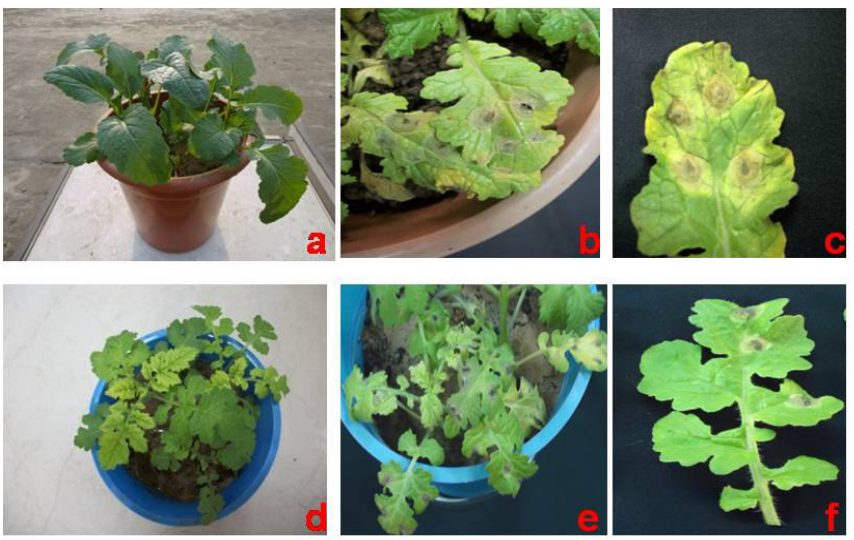


**Fig. 1.** ***In planta* challenge of *B. juncea* and *S. alba* with *A. brassicae*; (a) *B. juncea* mock control, (b and c) *B. juncea*- 5 days after infection with *A. brassicae*, (d) *S. alba* mock control, (e and f) *S. alba*- 5 days after infection with *A. brassicae***

### *A. brassicae* induced genes

To identify a set of defense-related genes induced during the early stage of *A. brassicae* infection, in both *S. alba* and *B. juncea*, a panel of nine defense-related genes *viz., PR-1, PR-2, PR-3, PR-5, NPR-1, PDF1.2, GST, Thionin* and *Thioredoxin* was initially selected for screening. Of them, only five genes encoding *PR-1*, *PR-2 (β-1,3 glucanase)*, *PR-3* (Class IV *chitinase*), *NPR1* and *PDF1.2* gave consistent and reproducible results and hence they were used for further analysis. Using RT-PCR changes in the transcript levels of these genes relative to the reference gene *α-tubulin* were determined at different time intervals as described earlier. Basal expression of all five defense-related genes was observed in *S. alba* leaves to which no inoculums was applied (mock treated plants) (Fig. 2 & 3).

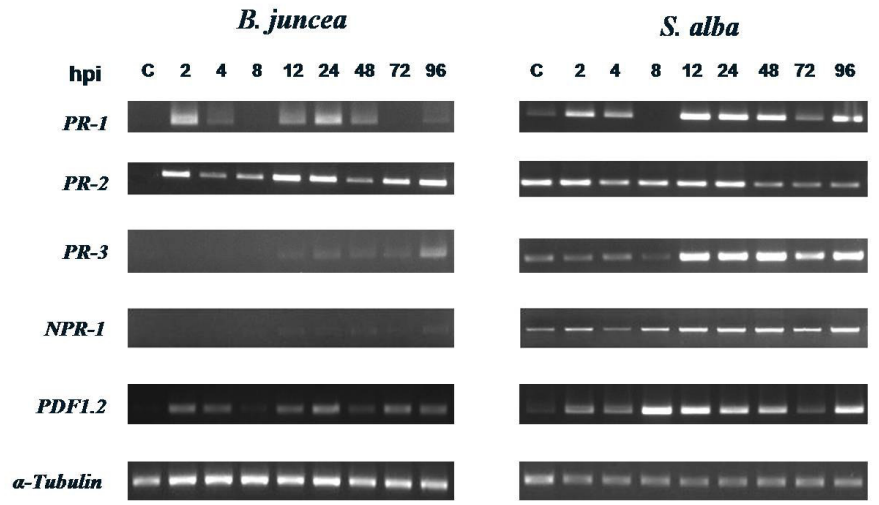


**Fig. 2.** **Time course of *PR-1*, *PR-2*, *PR-3*, *NPR-1* and *PDF1.2* transcript accumulation in *A. brassicae* inoculated local leaves from *B. juncea* (susceptible) and *S. alba* (moderately resistant). Total RNA was isolated from leaves at 0 to 96 h post infection (hpi). Semi quantitative RT-PCR was performed from cDNA made from each RNA sample. *α-Tubulin* transcripts were used to normalize the sample. 30 PCR cycles were performed for all the genes**

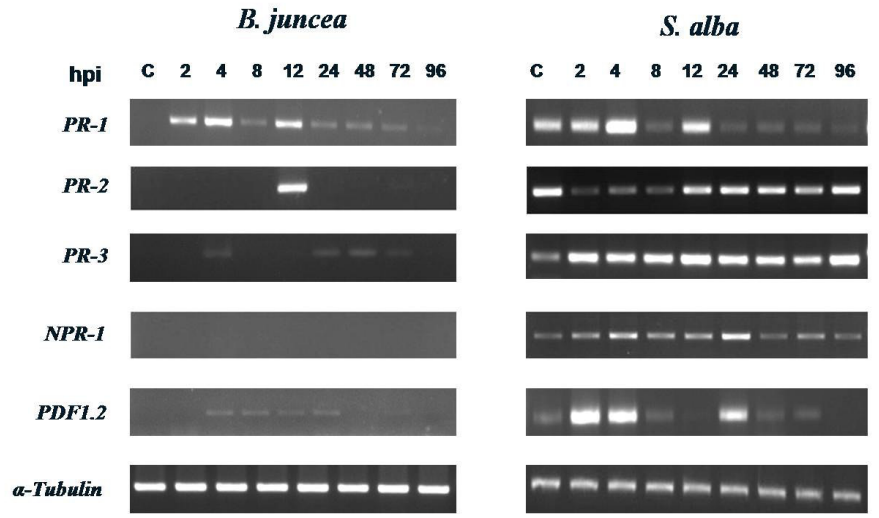


**Fig. 3.** **Time course of *PR-1*, *PR-2*, *PR-3*, *NPR1* and *PDF1.2* transcript accumulation in un-inoculated distal (systemic) leaves from *B. juncea* (susceptible) and *S. alba* (moderately resistant) plants infected with *A. brassicae*. Total RNA was isolated from leaves at 0 to 96 h post inoculation (hpi). Semi quantitative RT-PCR was performed from cDNA made from each RNA sample. *α-Tubulin* transcripts were used to normalize the sample. 30 PCR cycles were performed for all the genes**

Interestingly, basal expression of *PR-2* was predominant than the other defense-related genes in *S. alba.* However, in *B. juncea* no basal level induction of defense-related genes was observed in mock treated plants (Fig. 2 & 3). In locally challenged leaves of *B. juncea* with *A. brassicae*, *PR-1* and *PR-2* transcripts accumulated to a significant level while a slight induction of *PDF1.2* was observed as early as 2 h post inoculation (Fig. 2). However, in locally challenged leaves of *S. alba* with *A. brassicae,* all five defense-related genes except *PR-2* showed an increased expression at different time intervals than at the basal level expression (Fig. 3). Nevertheless, in both plant species, none of the defense-related genes showed an increase or decrease expression pattern but rather fluctuations with increasing time intervals. Northern blot analysis further confirmed this same expression pattern for both plant species (results not shown).

In systemic leaves (distal un-inoculated leaves) of *B. juncea, PR-1* induction was observed similar to that in locally challenged leaves while *PR-2* gene induction occurred only at 24 h post inoculation. However, in systemic leaves of *S. alba*, all five defense-related genes except *PR-2* expressed to great extent similar to that observed in locally challenged leaves (Fig. 3).

**JA or SA induced genes**

In order to investigate further the defense signaling pathways, *B. juncea* and *S. alba* plants were also spray treated with JA or SA. In response to JA treatment, transcripts of *PR-1* and *PR-3* (at 12 h) and *PDF1.2* (at 24 h) genes accumulated to great extent as compared to basal expression in *S. alba*. However, in *B. juncea*, *PR-1* and *PR-3* started to express prominently after 24 h post treatment (Fig. 4 & 5) while PR-3 expressed only at 24h after treatment. Interestingly, PDF 1.2 expressed as early at 2h post treatment while NPR-1 expressed slightly after 24h post treatment. Interestingly, the genes generally induced by SA such as *PR-1* and *PR-2* were also induced by JA treatment in both plant species.

Higher concentrations of SA, 4mM and above, were phytotoxic and led to yellowing of leaves of both *B. juncea* and *S. alba* (results not shown). However, SA treatment at 2 mM did not show any phytotoxicity or yellowing. At this concentration, transcripts accumulated in *B. juncea* as early as 2 h after treatment for *PR-1*, *PR-2* and *NPR-1* genes while in *S. alba,* increased expression of *PR-1* and *PR-2* was observed 8 h after SA treatment. Interestingly the genes generally induced by JA such as *PR-3* and *PDF1.2* were also induced by SA to great extent in *S. alba* (Fig. 4 & 5).

**Effect of JA, SA and SA+JA on lesion developed by *A. brassicae***

Because JA and SA activated a set of defense genes that were also induced by *A. brassicae*, we investigated whether the application of these chemicals 24h prior to inoculation could alter the development of leaf spot symptoms in both *B. juncae* and *S. alba*. The results presented in Fig. 6 show the effect of pretreatment with defense regulators on mean diameter of the lesions developed 5 days after inoculation.

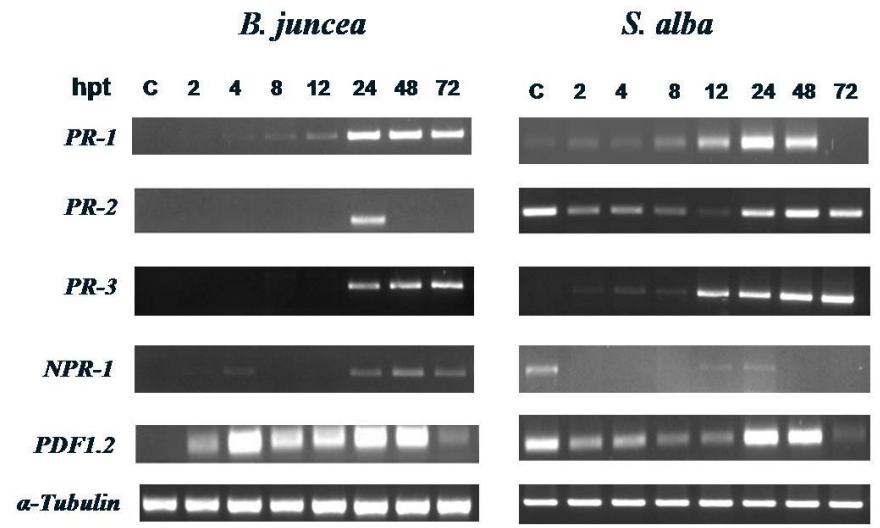


**Fig. 4.** **Time course of *PR-1*, *PR-2*, *PR-3*, *NPR1* and *PDF1.2* transcript accumulation in JA treated leaves from *B. juncea* (susceptible) and *S. alba* (moderately resistant). Total RNA was isolated from leaves at 0 to 72 h post treatment (hpt). Semi quantitative RT-PCR was performed from cDNA made from each RNA sample. *α-Tubulin* transcripts were used to normalize the sample. 30 PCR cycles were performed for all the genes.**

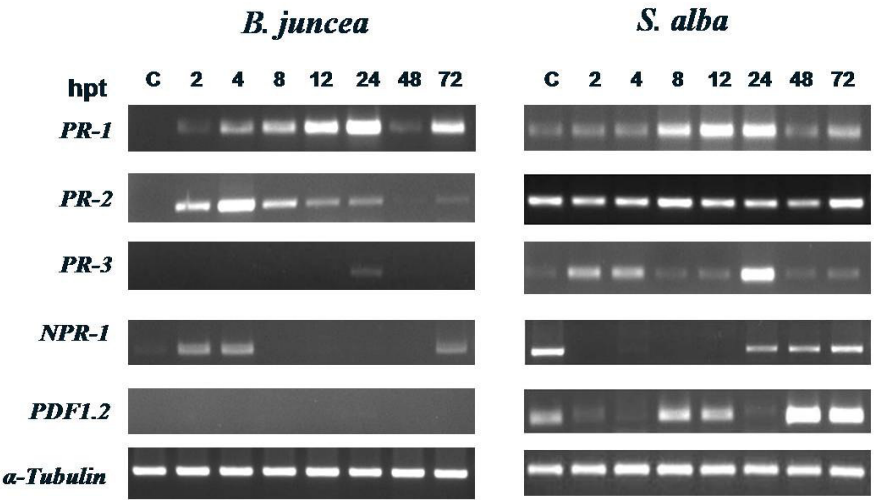


**Fig. 5.** **Time course of *PR-1*, *PR-2*, *PR-3*, *NPR1* and *PDF1.2* transcript accumulation in SA treated leaves from *B. juncea* (susceptible) and *S. alba* (moderately resistant). Total RNA was isolated from leaves at 0 to 72 h post treatment (hpt). Semi quantitative RT-PCR was performed from cDNA made from each RNA sample. *α-Tubulin* transcripts were used to normalize the sample. 30 PCR cycles were performed for all the genes.**

Pre-treatment with SA (2 mM) significantly increased the diameter of the spots caused by *A. brassicae* in both *S. alba* and *B. juncea* 5 days after inoculation but characteristic concentric rings could be seen only in *B. juncea.* Interestingly pre-treatment of both *B. juncea* and *S. alba* plants with JA significantly reduced the diameter of lesions caused by *A. brassicae* compared to controls on 5$^{th}$ day post inoculation. However, SA+JA pretreatment resulted in a significant increase in lesion diameter in *S. alba* compared to the controls but SA+JA pretreatment significantly reduced the lesion diameter in *B. juncea* similar to that of JA alone (Fig. 6). Nevertheless, neither of the chemical inducer pretreatments alone or in combination reduced the lesion development in *B. juncea* 10 days after inoculation.

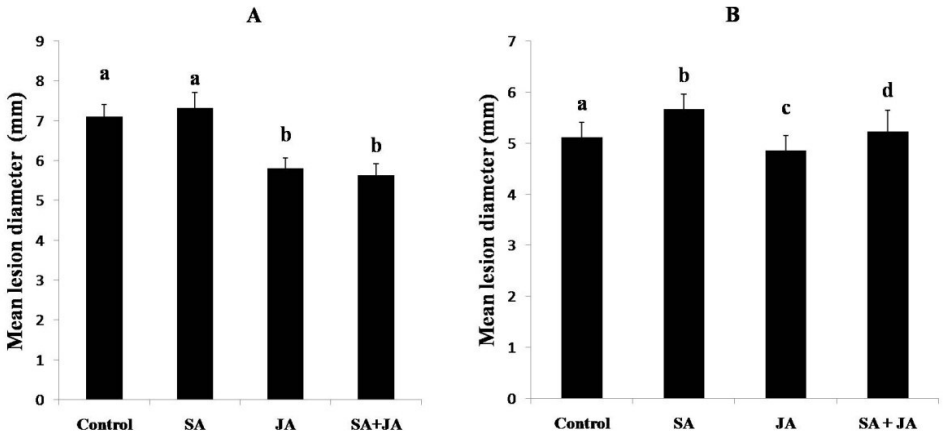


**Fig. 6. Protective effect of exogenously applied SA or JA on infection by *A. brassicae* in *B. juncea* (A) *and S. alba* (B). Prior to inoculation, separate sets of plants were sprayed for 24 h with a JA solution at a concentration of 100 µM or a SA solution at a concentration of 2mM or a combination of SA and JA (SA+JA) at a concentration of 2mM and100µM respectively. A: Average diameter of lesions formed after 5 d on 45-day-old *B. juncea* inoculated with a spore suspension of *A. brassicae* B: Average diameter of lesions formed after 5 d on 45-day-old *S. alba* inoculated with a spore suspension of *A. brassicae.* Data points represent averages with SEM of measurements from forty lesions on ten different plants. Bars with different letters represent data that are significantly different at 95 % confidence, as determined by LSM test using SAS**

## DISCUSSION

Alternaria leaf spot caused by *A. brassicae* is a serious disease of *B. juncea*. Despite this, very little is known about the molecular defense responses activated following challenge by this pathogen and its potential for manipulating *B. juncea* to improve resistance. No *Brassica* species are known to be resistant to Alternaria black spot. However, black spot resistance is found within the family Cruciferae (syn. Brassicaceae), for instance in white mustard, *S. alba*. Apart from phytoalexin mediated local defense exist, the nature of local and systemic gene induction in *S. alba* in response to *A. brassicae* infection remains unknown. Nevertheless, there were occasional reports of defense gene induction in *B. juncea* in response to *A. brassicae* challenge (Kamble and Bhargava, 2007).

*A. brassicae* successfully colonized both *S. alba* and *B. juncea* plants and developed symptoms 48-96 h after inoculation. However, *S. alba* was reported to be resistant to *A. brassicae* challenge in previous studies (Kolte, 1985; Ripley *et al.,* 1992; Sharma and Singh, 1992; Hansen and Earle, 1997). Nevertheless, Sharma *et al.* (2002) described *S. alba* as moderately resistant to *A. brassicae*. In the present study, older leaves of both *B. juncea* and *S. alba* were found susceptible to infection, however, the young leaves of *S. alba* were resistant. Hansen and Earle (1995) reported a high degree of variability in reaction to *A. brassicae* among 16 genotypes of *S. alba*. Therefore, together with the results of Sharma *et al.* (2002) and Hansen and Earle (1995), moderately resistant nature of *S. alba* to *A. brassicae* might be due to the difference in genotype used in the present study. Further, Ghose *et al.* (2008) described *S. alba- A. brassisicola* as an incompatible interaction although visible lesion formation was observed in infected leaves after 72 hours of infection.

In the present study, the transcripts of five defense response genes encoding *PR-1, PR-2, PR-3*, *NPR-1*, and *PDF1.2* accumulated at a greater level and earlier in the moderately resistant *S. alba* than in the susceptible *B. juncea* leaf tissues upon challenge inoculation with *A. brassicae*. Interestingly, basal expression of all five defense genes was observed in *S. alba* but not in *B. juncea*. These defense genes induced by *A. brassicae* in *S. alba* have not been reported previously and hence they might represent parts of the preformed resistance mechanism which can be directly attributed to the degree of resistance of *S. alba* to *A. brassicae* infection. Although *PR-1* is a marker gene for SA-dependant signaling pathways and SAR against biotrophic pathogens (Pieterse *et al.,* 2009) expression of *PR-1* in plants challenged by necrotrophic pathogens has also been observed (Thomma *et al.,* 2001; Govrin and Levin, 2002). Nevertheless, *PR-1* has been shown to be induced in Arabidopsis-*A. brassicicola* compatible (Mukherjee *et al.,* 2009) as well as incompatible interactions (Schenk *et al.,* 2003). Correlation between *β-1,3 glucanase (PR2)* and *Chitinase* type IV *(PR3)* activity and pathogen resistance has been reported in a number of plant species including *B. juncea* (Mondal *et al.,* 2003). An Arabidopsis defensin, activated after necrotrophic fungi invasion, is encoded by *PDF1.2* gene and is a predominant marker gene for JA pathway (Thomma *et al.,* 2000).

Similarly class V *chitinase* is also predominately activated via JA pathway and beneficial mainly against necrotrophic pathogens (Thomma *et al.,* 1998). In *B. juncea*, *chitinase* was not induced by *A. brassicae* even up to 72 h post infection whereas in *S. alba*, *chitinase* was induced strongly as early as at 12 hpi. Moreover, *PDF1.2* was induced slightly in local tissues of *B. juncea* while in *S. alba*, *PDF1.2* was induced strongly at the earliest stage of infection, both locally and systemically. Therefore, *PDF 1.2* and *chitinase* genes seem to be a part of an early defense response occur in moderately resistance *S. alba* than in susceptible *B. juncea* against *A. brassicae* infection. All five defense genes, except *NPR-1,* were induced in *S. alba* in response to both JA or SA treatments. Interestingly, *PR-1* was induced by JA in both *B. juncea* and *S. alba* in contrary to *Arabidopsis thaliana* where *PR-1* has been shown to be induced only by SA but not by JA or Ethylene (ET) (Thomma *et al.,* 1998; Durrant and Dong, 2004). However, SA independent but JA or ET dependant activation of *PR-1* has been reported in other crops such as tobacco (Xu *et al.,* 1994) and rice (Agarwal, 2000). Moreover, *chitinase* was also strongly induced by both JA and SA in *S. alba* but not in *B. juncea*. Nevertheless, SA induced plant chitinases have also been reported in cotton (Ji and Jin-Yuan, 2003), tomato (Ding *et al.,* 2002) and Pine (Davis *et al.,* 2002). Although *PDF1.2* is known to be induced only by JA (Thomma *et al.,* 1998), in the present study *PDF1.2* was also strongly induced by both SA and JA in *S. alba*. Therefore, unlike in *A. thaliana*, same set of PR genes can be induced by SA or JA in *B. juncea* and *S. alba*. SA induced plant defensin has already been reported in *B. juncea* (Anuradha *et al.,* 2009).

Interestingly, none of the defense-related genes showed an increase or decrease expression pattern but rather fluctuations with increasing time intervals. This could be due to circadian variations of defense gene induction. There is growing realization that circadian rhythms may play an import role in disease outcomes (Wang *et al.,* 2011). Pathogen-inducible genes have been identified that have diurnal and/or circadian rhythms of expression (Weyman *et al.,* 2006; Michael *et al.,* 2008; Wang *et al.,* 2011). The functional significance of this is not yet clear. It was also demonstrated that under defined environmental conditions, 89% of Arabidopsis transcripts are expressed rhythmically, being regulated by the circadian clock or directly by environmental changes in light or temperature (Michael *et al.,* 2008).

Pre-treatment with JA significantly reduced the lesion size in both *B. juncea* and *S. alba*. This suggests that induction of JA-dependant defense gene expression before pathogen challenge may possibly provide increased resistance in both *B. juncea* and *S. alba*. Previous studies have shown that exogenous application of JA improves resistance in *Arabidopsis* against necrotrophic pathogens such as *A. brassicicola*, *Botrytis cinerea* or *Plectosphaerella cucumerina* (Thomma *et al.,* 2000) and against *Fusarium pseudograminearum* (Desmond *et al.,* 2006) and *F. graminearum* (Pritsch *et al.,* 2000) in wheat. On the other hand, SA was not able to induce resistance response in *Arabidopsis* against *A. brassicicola* and *B. cinerea* (Thomma *et al.,* 2000). Conversely, treatment of *B. juncea* at low SA concentrations (0.5mM and 1mM) significantly reduced the size of lesions caused by *A. brassicae* (Kamble and Bhargava, 2007). However, in contrary to the studies of Kamble and Bhargava (2007) and accordance to Thomma *et al.* (2000), application of SA at slightly higher concentration (2mM) favored disease progression of *A. brassicae* in both *B. juncea* and *S. alba* in the present study. This might be due to the antagonistic effect exerted by SA to suppress JA responsive defense genes (Glazebrook, 2005).

El Oirdi *et al.* (2011) demonstrated that the necrotrophic pathogen *B. cinerea* produces an exopolysaccharide, which acts as an elicitor of the SA pathway. In turn, the SA pathway antagonizes the JA signaling pathway, thereby allowing the fungus to develop its disease in tomato. However, in the present study, concomitant activation of both SA and JA pathways, by SA+JA treatment, was favorable for *B. juncea* but not for *S. alba*. Simultaneous activation of both SA and JA pathways resulted in an additive effect on the level of induced protection against *P. syringae* pv. Tomato in *A. thaliana* (Van Wees *et al.,* 2000). Although JA or SA+JA pre-treatment arrested the lesion expansion in inoculated seedlings of *B. juncea* for up to 6-7 days, the reduction in symptom level was not sustained after 10-15 days of inoculation because all infected leaves died irrespective of pre-treatments. However, in the case of *S. alba*, infected leaves survived even after 15 days of infection upon JA pre-treatment. Therefore, a single application of JA at the concentrations used here does not affect a sustained control of Alternaria leaf spot in *B. juncea.*

## CONCLUSION

The present study revealed that defense-related genes are expressed earlier and in a greater magnitude in moderately resistant *S. alba* than in the susceptible *B. juncea* locally as well as systemically in response to challenge inoculation with *A. brassicae*.

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