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Effect of zeatin on the infection process and expression of MAPK-4 during pathogenesis of *Alternaria brassicae* in non-host and host *Brassica* plants

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Recent studies have revealed an important role of hormones in plant immunity. Cytokinins are phytohormones that are involved in various regulatory processes including plant defense. Zeatin a cytokinin antagonizes the effect of *Alternaria brassicae* pathotoxin in cell culture of *Brassica juncea*. Phytohormones are also the inducers of MAP Kinase signaling pathways which are the important signaling modules in eukaryotic cells. In this paper, an attempt was made to study the exogenous application of zeatin on the disease score, infection behavior of *A. brassicae*, expression pattern of MAPK-4 in the non host *Sinapsis alba*, host *B. juncea* susceptible c.v Varuna, *B. juncea* moderately tolerant c.v. Divya and transgenic *Brassica* to confer its role in plant immunity. We observed that high concentration of zeatin led to increased defense responses by delaying the infection process as well as significantly reducing the disease score. Semi-quantitative RT-PCR reveals that zeatin also increases the expression of MAPK-4 at early hours of infection. Our result supports that zeatin up regulates plant immunity via an elevation of MAPK-4 and clearly reflects that it antagonizes the effect of *A. brassicae*. The crosstalk between zeatin and MAPK signaling pathway may help plants fine-tune defense responses against *A. brassicae* in the disease Alternaria blight.

Key words: Alternaria blight, *Brassica*, disease score, MAPK, pathogenesis, infection process.

INTRODUCTION

Phytohormones have long been implicated in both biotic and abiotic interactions. Among the plant hormones, ethylene (ET), salicylic acid (SA) and jasmonate (JA) are known for differentially regulating defense responses against biotrophic and necrotrophic pathogens and are considered as the immunity hormones (Grobkinsky et al., 2011). The balance of hormonal crosstalk strongly influences the outcome of plant-pathogen interactions. In addition to these hormones, many researchers uncovered the role of several other hormones in plant defense like auxin, gibberellins, abscisic acid, brassinosteroid and cytokinins (CKs) (Spoel and Dong, 2008; Robert- Seilaniantz et al., 2007). CKs play an essential role in sustaining juvenility of plant tissues and have been investigated to understand the relationship between senescence and susceptibility towards several plant pathogens (Pogany et al., 2004). CKs also promote resistance against biotrophs by enhancing salicylic acid response (Choi et al., 2010). These are perceived by membrane-bound histidine kinase proteins similar to the two-component system of bacteria (Muller and Sheen, 2007; To and Kieber, 2008).

CKs are also produced by a range of various microbial pathogens, including *Pseudomonas syringae* (Akiyoshi et al., 1987; Morris et al., 1991), *Alternaria brassicae* (Tiwari 1993; Pandey et al., 2001) and leaf-mining insects

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(Engelbrecht, 1968) causing the formation of green islands, galls, growth abnormalities and manipulation of primary carbon metabolism (Balibrea et al., 2004). For several pathogens, it was shown that this CK production is essential for infection (Crespi et al., 1994; Hwang et al., 2010). Based on the induction of sink metabolism by CKs (Ehness and Roitsch, 1997; Walters et al., 2008), it has been suggested that the host physiology is altered in response to CKs to allow the pathogen maximum access to nutrients in hemi biotrophic interactions (Walters et al., 2008). In contrast, the role of plant-derived CKs in defense responses against pathogens that are unable to produce CKs is largely unknown. Compared to auxin and abscisic acid signaling, our understanding of the roles of cytokinin in disease, and its interactions with other hormones, is relatively limited. Exogenous application of CKs has been reported to enhance plant resistance during some viral and fungal infections (Dekker, 1963; Clarke et al., 1998). However, the effect of zeatin on the tolerance of plants to disease caused by the necrotrophic fungus *A. brassicae* (Berk.) Sacc. has not been previously investigated. *A. brassicae* cause Alternaria blight (AB) disease in the economically important *Brassica* species, *Brassica juncea*. AB disease causes chlorotic and necrotic lesions mostly on aerial parts of the plant (Verma and Saharan, 1994) and is responsible for substantial reduction in seed yield and oil quality (Meena et al., 2010). Over the years, much effort has been made to understand the plant-pathogen/host specific toxin interaction during pathogenesis of AB of *Brassica*. Interestingly, *A. brassicae* toxin mediated signal transduction pathway was antagonized by a cytokinin (zeatin) in cell culture studies (Pandey et al., 2001). Phytohormones are also known to induce mitogen activated protein kinase (MAPK) cascade, a more conserved signalling pathway involved in plant response against fungal attack (Meskiene and Hirt, 2000). MAPK pathways are minimally comprised of three tiers MAP Kinase Kinase Kinase (MAPKKK), MAP Kinase Kinase (MAPKK) and MAP Kinase (MAPK). The *Arabidopsis thaliana* genome contains 20 MAPKs, 10 MAPKKs and 80 MAPKKKs which can engage in various MAPK modules to regulate responses of plant to different environmental and developmental signals. Recently, some members of the MAPK cascade have been reported to be involved in mediating defense response against various plant pathogens (Pitzschke et al., 2009). The best characterized MAPKs 3, 4 and 6 were activated by a diversity of stimuli including abiotic stresses and pathogens. Flagellin derived peptide flg22 triggers a rapid and strong activation of MAPK3, MAPK4 and MAPK6 (Droillard et al., 2004). MAPK4 and MAPK6 are also activated by harpin protein followed by the induction of pathogenesis- related (PR) genes (Desikan et al., 2001). A number of studies have been identified where MAPK4 is component of pathogen signalling cascade (Gao et al., 2008; Qiu et al., 2008; Pitzschke et al., 2009).

Arabidopsis MAPK4 has been involved in osmotic stress response pathway (Droillard et al., 2004) as well as implicated in plant defense regulation as *mpk4* knockout plants exhibited constitutive activation of salicylic acid (SA) dependent defenses, but failed to induce jasmonic acid (JA) defense marker genes in response to JA (Brodersen et al., 2006). Considering these observations, it was hypothesized that *A. brassicae* and its toxin may affect these MAPKs. Phytohormones are also the inducers of pathogenesis related proteins which also play important role in defense. Earlier, our laboratory has reported that overexpression of osmotin PR-5 in *Brassica* develop tolerance against *A. brassicae* (Taj et al., 2004). Since it is well known that CKs antagonize the effects of abscisic acid (ABA) and delay senescence and induce the expression of defense-related genes, we investigated whether CK (zeatin) can be used to develop the tolerance against *A. brassicae* in *B. juncea* c.v. Varuna, Divya, non host *Sinapsis alba* and transgenic *B. juncea* c.v. Divya harbouring osmotin gene. We also investigated the effect of exogenous zeatin on the infection process of *A. brassicae* and the expression pattern of MAPK4.

MATERIALS AND METHODS

Selection of non host/host genotypes

Seeds of a non-host (*S. alba*), susceptible host *B. juncea* cultivar (Varuna), moderately tolerant host *B. juncea* cultivar (Divya) and tolerant transgenic *B. juncea* c.v. Divya harbouring osmotin gene (Taj et al., 2004) were sown in plastic inserts (7.5 × 5 cm; 2 seeds per insert) containing mixture of soil, sand and vermicomposte in the ratio of 2:1:1. Plants were grown in the greenhouse (22°C day/18°C night; 16 h photoperiod), and watered at appropriate amount and time.

Alternaria brassicae

A. brassicae (Berk.) Sacc. was isolated from a diseased leaf of *B. juncea* cultivar 'Varuna' at Crop Research Centre (CRC), Pantnagar Uttarakhand, India. Pure single spore culture of *A. brassicae* was developed with the help of stereo microscope (Nikon make) and maintained on potato dextrose agar (PDA) slants at 4°C.

Inoculum preparation

A. brassicae was subcultured from the 7-day old culture on V-8 agar medium (10% V-8 juice, 0.02% $CaCO_3$ and 2% agar) and incubated at 22°C. A conidial suspension was prepared by scraping the mycelia and spores from surface of the actively growing fungal culture into autoclaved distilled water and filtered using four layered cheese cloth to remove most of the mycelia. The filtered spore suspension was centrifuged at 2000 x *g* for 5 min and resuspended in deionized water. This centrifugation was repeated one more time in order to ensure a clear spore suspension free of metabolites. After the final wash, supernatant was discarded and spores were resuspended in water containing 0.05% Tween-20 as an adhesive. The concentration of spore suspension was adjusted to 5×10^4

Table 1. The list of primers used for semiquantitative RT-PCR analysis.

Name of gene	Sense primer	Antisense primer	Expected product size (bp)
MAPK-4	GCTCTAACCAACCCTTAACTG	GTACCAGCGTGTAACAACGTA	228
Actin	ATTCTTACCCTCAAGTATCC	CATGATCTGAGTCATCTTCT	200

spores ml^{-1} using a haemocytometer (Sharma et al., 2007).

Sample preparation for light microscopy

10 µl suspension of *A. brassicae* spores were deposited separately on the leaf surface of all the plants (45 days old) with the drop plus agarose method (Giri, 2013, personal communication). For the control, 10 µl of sterile deionized water was applied. The inoculated plants were placed in humidity chamber (90 to 100% relative humidity). Inoculated plants were sampled at 3, 6, 9 and 12 h post-inoculation (hpi), and then daily for 4 day post-inoculation (dpi). Sampled leaves (area cut where drop was given) were decolourized in an acetic acid : ethanol : water (2:2:1) solution at 25°C for overnight. It was then washed with two changes of deionized water and stained with 1% cotton blue in lactophenol (Garg et al., 2010). Whole wet mounts of leaf piece on microscope glass slides were examined and photographed using a fluorescent microscope Nikon Eclipse 80i. The experiment was also done with exactly same procedure with *A. brassicae* spore suspension along with 1 ppm zeatin, to show the effect of zeatin on the infection process.

Sample preparation for molecular studies

The 45 days old plants were subjected to three kinds of treatments. In the first treatment, 10 µl volume of *A. brassicae* spore along with 10 µl of 1 ppm zeatin was applied onto the plants. In the second and third treatment, *A. brassicae* spore suspension (10 µl) and zeatin (10 µl) were applied individually, respectively. Inoculation was performed by drop plus agarose method (Giri, 2013, personal communication) of true leaves (2 leaves/plant) with a pipette tip. For the uninoculated controls, 10 µl of sterile deionized water was applied. The inoculated plants were incubated at 25°C with 90 to 100% relative humidity for 3 days in humid chamber and then transferred into growth room. Inoculated leaves were collected from each plant for each treatment at 3, 6 and 9 h post-inoculation for RNA isolation and stored at -80°C until use. Disease severity was assessed using a scale described by Conn et al. (1990) where a score of 0 indicates no symptoms; 1, small irregular spots covering <5% leaf area; 2, small irregular brown spots covering 5 to 10% leaf area; 3, symptom covering 10 to 20% leaf area; 4, symptom covering 20 to 30% leaf covering; and 5, symptom covering 30 to 50% leaf area covering. For RT-PCR analysis, leaves samples were subjected to RNA isolation using RNEasy plant minikit (Himedia Laboratories Private Limited India) as per the manufacturer instructions. RT-PCR analysis was performed with the help of One-Step RT-PCR (Qiagen, USA) using the gene specific primer of MAPK4 and internal control actin under the following PCR conditions: reverse transcription at 50°C for 30 min, initial PCR activation step at 95°C for 15 min followed by 35 cycles of amplification (94°C for 1 min, 59°C for 1 min and 72°C for 1 min) with final extension at 72°C for 10 min. After the completion of RT-PCR, the amplicons were analyzed by electrophoresing them in 1.8% agarose gel electrophoresis followed by quantification by using the spot densitometry tool of AlphaImager software. The sequences of all primers are described in Table 1.

RESULTS AND DISCUSSION

Conidial germination and fungal development on four different plants and effect of zeatin on the infection process of *A. brassicae*

The initial steps in the infection process of *A. brassicae* on four different plants: *S. alba*, *B. juncea* c.v. Varuna & Divya and transgenic *Brassica* were compared using light microscopy (Table 2). The effect of zeatin was also observed on the infection process of *A. brassicae.* It was observed that conidial germination was not evident during the interplay between *A. brassicae* and the leaf tissue of the non-host *S. alba* even upto the second day of infection (Figure 2: 1A to 1F). It suggests that non host might produce certain antifungal/antifungistatic compounds which are not suitable for the conidial germination. This is also supported by Kowalska and Niks (1999) for a resistant flax (*Linum usitatissimum*) genotype against *Melampsora lini* and by Blakeman and Sztejnberg (1973) in beetroot (*Beta vulgaris*) against *Botrytis cinerea*. At 3 days post inoculation (dpi), epidermal penetration was observed in *S. alba* (Figure 2: 1G), but it was not evident in zeatin plus pathogen treated leaves (Figure 2: 1N); the mycelium only grew at the surface of the plant. The penetration of fungal mycelium was much delayed; it might be due to the incompatible interaction, which showed resistance to *A. brassicae* attack (Sharma et al., 2002). Moreover, it also correlated with the disease scoring data of *S. alba* where no spot was observed at 1 day of post inoculation, but as the disease progresses, the penetration might have occurred as a result symptoms that appeared from 5 days onwards (Figure 1). In the case of Varuna cultivar, conidial germination began within 3 hpi (Figure 2: 2A) but conidial germination was not evident up to 6 hpi (Figure 2: 2H and 2I) in zeatin plus pathogen treated leaves. From this time point onwards, the conidial germination began and the infection proceeded. At initial hour of infection, multiple germ tubes appeared (Figure 2: 2B and 2C) but a bit late due to the exogenous application of zeatin in Varuna c.v. (Figure 2: 2J and 2K). Appresorium and infection thread are the two hyphal modifications observed in *A. brassicae* infection. Penetration of infection thread into the intercellular spaces was observed at 12 hpi (Figure 2: 2D) whereas, no such penetration was evident in the case of zeatin treated leaves in Varuna c.v. The process of infection behavior followed the same steps but at different time intervals. Exogenous application of zeatin delayed the infection process, as appresorium penetration through stomata

Table 2. Description of growth of *A. brassicae* and effect of zeatin on the infection process in the leaf surface of different plants.

Genotype		3 hpi	6 hpi	9 hpi	12 hpi	1 dpi	2 dpi	3 dpi
S. alba	*A. brassicae*	No spore germination	No SPore germination	No spore germination	No spore germination	No spore germination	No spore germination	Penetration of infection thread into intercellular spaces
	A. brassicae + *zeatin*	No Spore germination	No spore germination	No spore germination	No spore germination	No spore germination	No spore germination	No penetration
B. juncea c.v. Varuna	*A. brassicae*	Spore germinated	Germ tube elongation	Formation of several germ tubes	Penetration of infection thread into intercellular spaces	Penetration through stomata	Mycelial network	Proliferation of spores
	A. brassicae + *zeatin*	No spore germination	No Spore germination	Spore germinated	Formation of several germ tubes	No penetration	Penetration through stomata	No proliferation
B. juncea c.v. Divya	*A. brassicae*	Spore germinated	Germ tube elongation	Germ tube elongation	Penetration of infection thread into intercellular spaces	Sporulation on the surface	Penetration through stomata	Mycelial network
	A. brassicae + *zeatin*	No spore germination	Spore germinated	Germ tube elongation	Penetration of infection thread into intercellular spaces	Differentiation of hypha into infection thread	Appresoria approaches towards stomata	Penetration through stomata
Transgenic *Brassica*	*A. brassicae*	No spore germination	Spore germinated	Formation of several germ tubes	Formation of several germ tubes	Penetration of infection thread into intercellular spaces	Penetration through stomata	Mycelial network
	A. brassicae + *zeatin*	No spore germination	No Spore germination	Spore germinated	Germ tube elongation	Penetration of infection thread into intercellular spaces	Penetration through stomata	Penetration through stomata

was observed at 2 dpi (Figure 2: 2M) as compared to without zeatin where it was evident at 1 dpi (Figure 2: 2E) only. In both cases, penetration of appresorium was evident only through stomata. At late hours of infection process, proliferation of spores was observed (Figure 2: 2G) in contrast to the zeatin treated leaves where only mycelial network were observed (Figure 2: 2L and 2N). In the case of Divya cultivar, conidial germination also began within 3 hpi (Figure 2: 3A), but the length of the germ tube was significantly less as compared to the germ tube that emerged on Varuna cultivar (data not shown). Here also, zeatin delayed the conidial germination which was eventually initiated from 6 hpi onwards (Figure 2: 3I). Penetration of epidermal cells by fungal hyphae was observed at 12 hpi in both cases (Figure 2: 3D and 3K). At 1 dpi, the spores multiplied by budding on the host surface (Figure 2: 3E). This is a typical feature of fungi belonging to phylum ascomycota. This is an adaptation to augment the inoculums density, thereby enhancing the inoculums potential. But this type of augmentation of inoculums density was not evident on zeatin treated leaves suggested that zeatin somehow interferes in the infection process of *Alternaria brassicae.* At 2 dpi, appresorium penetration was observed (Figure 2: 3F) which is clearly evident at 3 dpi in zeatin treated leaves in Divya c.v. (Figure 2: 3N). In the case of transgenic *Brassica* harbouring PR-5 protein osmotin (Taj et al., 2004), there was a consistent delay in conidial germination which eventually started from 6 hpi onwards (Figure 2: 4B) and even later, that is, at 9 hpi in zeatin treated transgenic *Brassica* leaves (Figure 2: 4J). The infection process was slower as compared to Varuna cultivar. Here, appre-sorium penetration through stomata was evident at 2 dpi (Figure 2: 4F) and no proliferation of spores were observed. The findings of the present study suggested that zeatin obstruct the interplay of host and pathogen

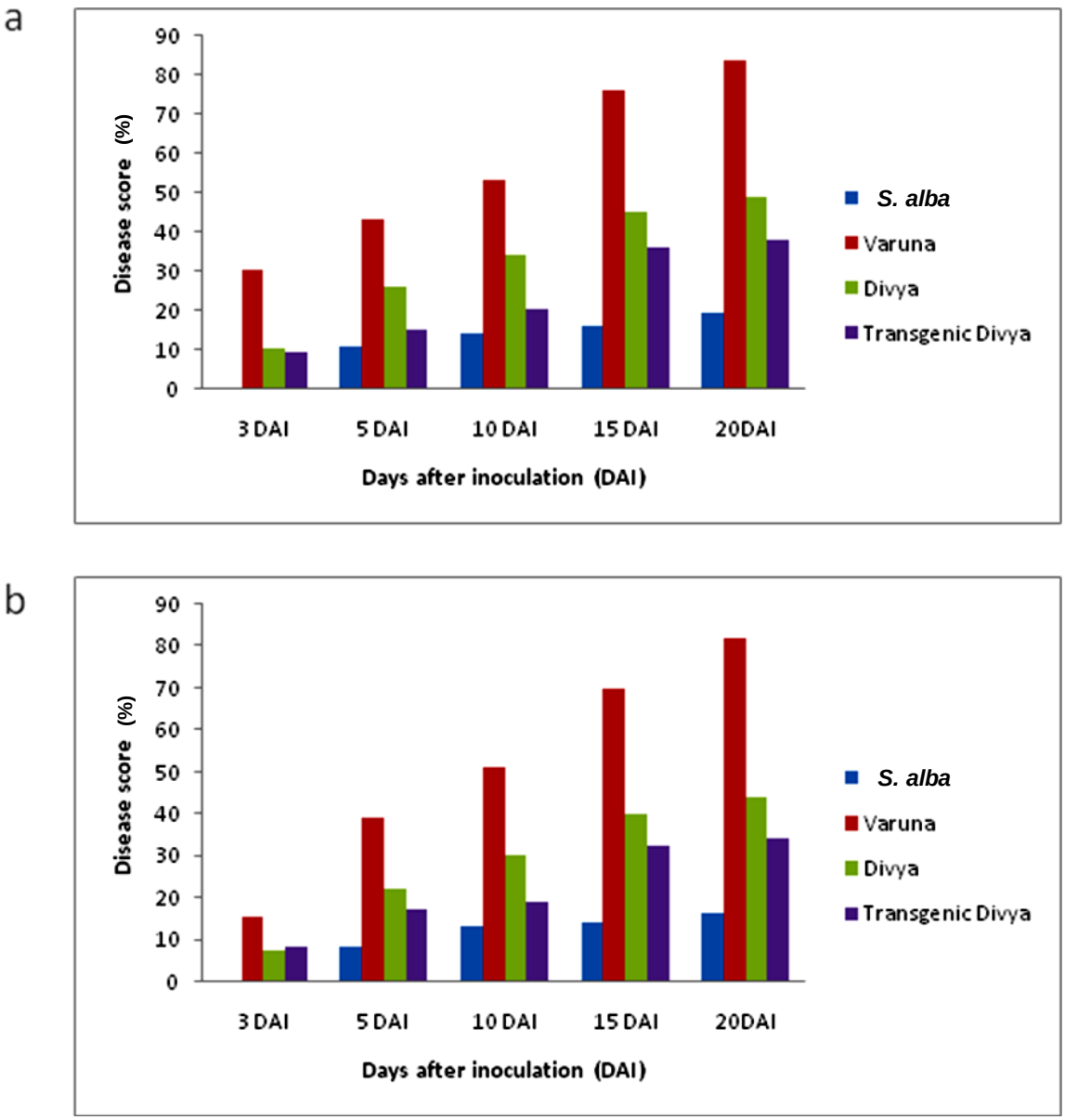
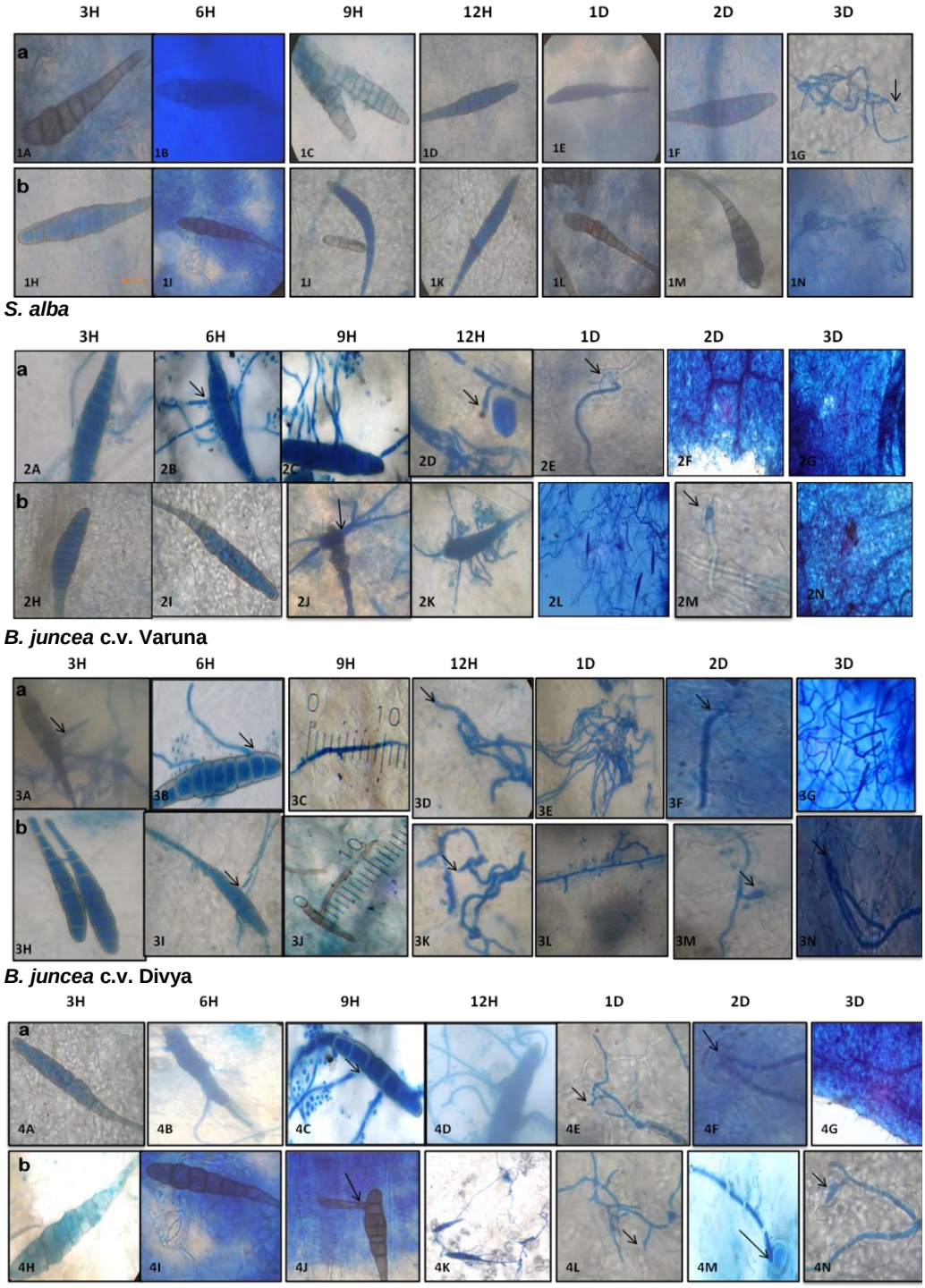


Figure 1. Disease severity score (%) induced by a) *A. brassicae*; b) Effect of zeatin on the disease severity score on leaves of non host *S. alba*, *B. juncea* c.v. Varuna & Divya and transgenic *Brassica* at 3, 5, 10, 15 and 20 days after inoculation (DAI).

and delays the infection process by inhibiting the conidial germination and fungal development. This is in accordance with the findings of Pandey et al. (2001) who reported that increased zeatin concentration antagonizes the effect of *A. brassicae* pathotoxin in cell culture of *B. juncea* c.v. Divya.

Response of host and non host plants against *A. brassicae* and *A. brassicae* along with zeatin treatment

The effect of zeatin was investigated on the disease score of all four different plants. A significant reduction in the disease score and on the appearance of the symptoms was observed when compared with the plants only sprayed with *A. brassicae* suspension and plants sprayed with *A. brassicae* suspension along with zeatin (Figures 1 and 3). *B. juncea* c.v. Varuna exhibited severe chlorosis and necrosis that spread from the spot that had been inoculated with *A. brassicae* spores, had irregular margins extending towards the periphery of the leaf suggesting that it was quite susceptible to *A. brassicae*. In contrast, the leaves of *S. alba*, transgenic *Brassica* and *B. juncea* c.v. Divya exhibited localized cholorosis and necrosis only around the inoculated region which clearly indicated that these hosts were more tolerant to the



S. alba

***B. juncea* c.v. Varuna**

***B. juncea* c.v. Divya**

Transgenic *Brassica*

Figure 2. Light microscopic study of the leaves of non-host *S. alba.* 1A-1G, *A. Brassicae;* 1H-1N, *A. brassicae* together with zeatin; 2A-2G, *B. juncea* susceptible cultivar Varuna with *A. Brassicae;* 2H-2N, *A. brassicae* together with zeatin; 3A-3G, *B. juncea* moderately tolerant cultivar Divya with *A. Brassica;* 3H-3N, *A. brassicae* together with zeatin; 4A-4G, transgenic *Brassica* with *A. Brassicae;* 4H-4N, *A. brassicae* together with zeatin. (1A-1F), (1H-1M), (2H and 2I), 3H, (4A, 4H, 4I, conidium on the leaf surface; 2A, 2J, 3A, 3I, 4B, 4J, germ tube emerged from conidium; 2B, 3B, 3C, 3J, 4C, 4K- elongation of germ tube; 2C, 2K, 4D, formation of several germ tubes and their elongation;1N, fungal hyphae continued growth on the leaf surface; 1G, 2D, 3D, 3K, 4E, 4L, differentiation of hyphae into infection thread and its penetration through intercellular spaces; 2E, 2M, 3F, 3N, terminal hyphae leading to formation of appresoria and its penetration through stomata; 2F, 2L, 2N, 3G, 4G, mycelial network; 2G, excessive proliferation of the spores. Scale bar: 50 µm.

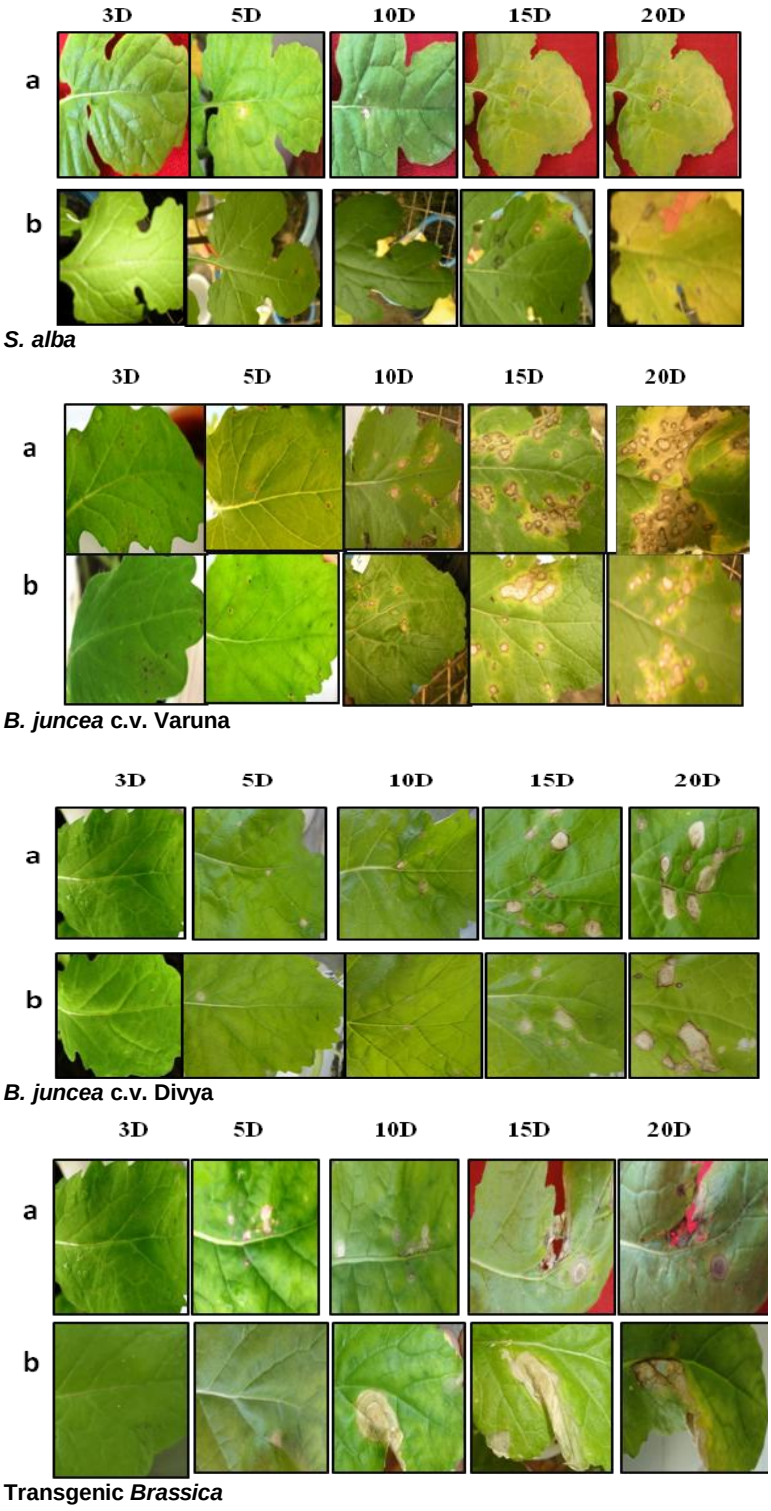


Figure 3. Response of non host *S. alba*, *B. juncea* susceptible c.v. Varuna, *B. juncea* moderately tolerant c.v. Divya and transgenic *Brassica* to (a) *A. brassicae* and (b) *A. brassicae* plus zeatin at 3, 5, 10, 15, 20 days after inoculation.

pathogen as compared to *B. juncea* c.v. Varuna. We can conclude that exogenous application of zeatin antagonizes the effect of *A. brassicae* and hence decreases the disease score imparting role in defence.

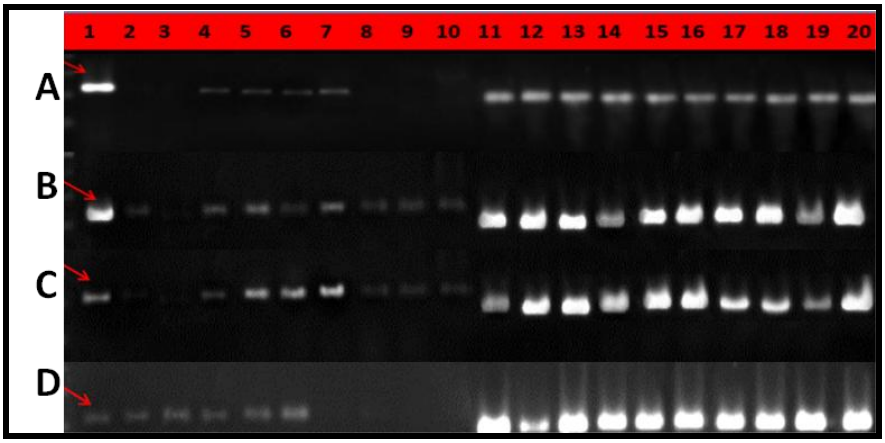


Figure 4. Determination of change in transcript profiling of MAPK4 gene by semi quantitative RT-PCR from *A. brassicae* infected leaves, zeatin sprayed leaves and *A. brassicae* along with zeatin sprayed leaves of A) *S. alba*, B) transgenic *Brassica*, C) *B. juncea* c.v. Divya, D) *B. juncea* c.v. Varuna. Lanes 1 to 4, Healthy, *A. brassicae* infected leaves, zeatin sprayed leaves and *A. brassicae* along with zeatin sprayed leaves at 3 h; lanes 5 to 7, *A. brassicae* infected leaves, zeatin sprayed leaves and *A. brassicae* along with zeatin sprayed leaves at 6 h; lane 8-10, *A. brassicae* infected leaves, zeatin sprayed leaves and *A. brassicae* along with zeatin sprayed leaves at 9 h; lanes 11 to 20, actin gene expression from *A. brassicae* infected leaves, zeatin sprayed leaves and *A. brassicae* togethjer with zeatin sprayed leaves at 3, 6, 9 h, respectively.

This strongly correlates with the infection behavior study where zeatin interferes with the conidial germination. Earlier, one group (Sharma et al., 2010) also reported that cytokinin provides resistance to canola plants against *Alternaria* pathogen.

Expression profiling of MAPK4 at different time intervals in different treatments by semi quantitative RT-PCR

Expression of mitogen activated protein kinase 4 (MAPK 4) was analyzed in three time points after infection with *A. brassicae* alone, *A. brassicae* along with zeatin and zeatin alone in four different plants namely *S. alba*, *B. juncea* cultivar Varuna & Divya and transgenic *Brassica* (Figure 4). In all the host plants, the expression level of MAPK 4 (228 bp) increased from 3 to 6 hpi and then decreased at 9 hpi. The transcript level of MAPK 4 was found to be highest at 6 hpi. In all the samples, actin was found to be constitutively expressed (Figure 5). This observation implies that at the early phase of infection, MAPK-4 gradually increases to strengthen plant defence, so as to restrict conidial germination. Upon looking up the expression pattern of MAPK 4 in all the plants, it was observed that the non-host *S. alba* and moderately tolerant Divya has the highest expression of MAPK 4 in the healthy samples in comparison with the treated samples indicating the involvement of MAPK 4 in other functions apart from the defense. Whereas, in transgenic *Brassica* and susceptible Varuna, the expression of MAPK 4 was found to be highest at 6 hpi in comparison with healthy samples. If we correlate the expression pattern of MAPK 4 with the conidial germination, we can say that in transgenic *Brassica* as the conidia started germinating at 6 hpi, the MAPK 4 expression was highest, although in susceptible Varuna, the conidial germination started at 3 hpi. In both host plants, Varuna had more expression of MAPK 4 at 3 hpi. This gives a clue that MAPK 4 expression triggers the conidial germination. This further supports that MAPK 4 playing role in defense as its expression started with the conidial germination. The exogenous application of zeatin in Varuna further enhanced the expression of MAPK 4 in comparison with healthy samples as well as pathogen treated samples at 3 and 6 hpi. This shows that zeatin enhanced the expression of MAPK 4 which in turn was not allowed to germinate the conidium as shown in the infection behavior of Varuna (Figure 2: 2H and I). Indeed, it is well known that CKs activates numerous plant defense response genes (Memelink et al., 1987; Smigocki et al., 1993; Schäfer et al., 2000). Surprisingly, no expression of MAPK 4 was found at 9 hpi in Varuna; it might be due to the fact that from this point of time, susceptible genes are expressed. In transgenic *Brassica* also, zeatin enhanced the expression of MAPK 4 at 6 hpi but at 9 hpi, the expression of MAPK 4 was low as compared to healthy sample; this further suggest that apart from MAPK 4, there is something which provides

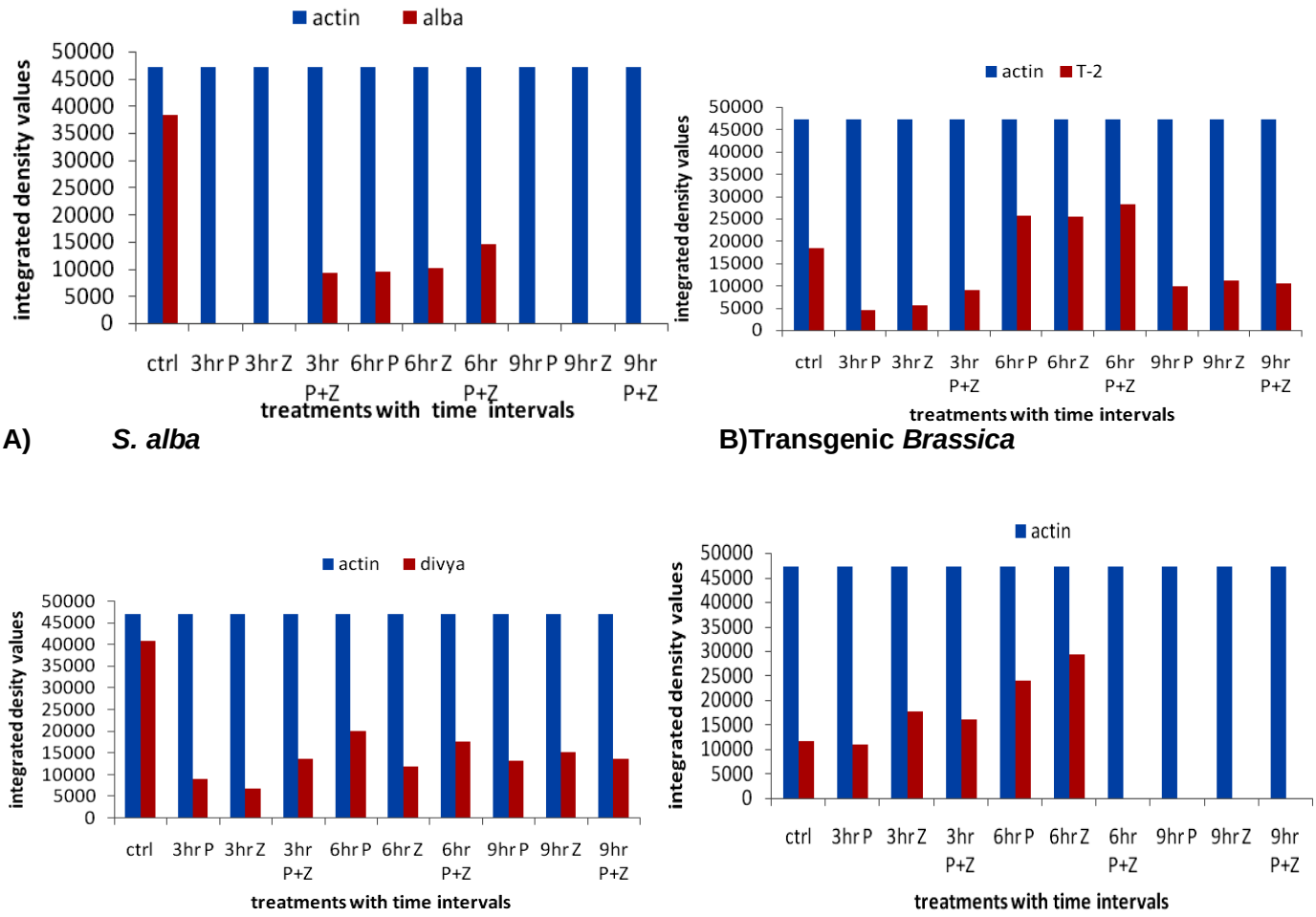


Figure 5. Densitometric analysis of the expression profile of MAPK4 gene from *A. brassicae* infected leaves, zeatin sprayed leaves and *A. brassicae* together with zeatin sprayed leaves of A) *S. alba*, B) transgenic *Brassica*, C) Divya, D) Varuna at different time intervals (3, 6 and 9 h).

tolerance in transgenic *Brassica*. This tolerance could be due to osmotin gene because at later stages of disease progression, transgenic *Brassica* exhibited localized bigger chlorotic and necrotic lesions around the inoculated region with no new spot (Figure 3) and it clearly indicates that the osmotin gene imparts some level of tolerance to *B. juncea* against *A. brassicae.* This was also reported earlier in our laboratory (Taj et al., 2004). In the treated samples, maximum expression of MAPK 4 was observed at 6 hpi in pathogen along with zeatin treatment and this implies that zeatin inhibits the conidial germination by enhancing the MAPK 4 expression, which further supports that both zeatin and MAPK 4 play role in defense. Zeatin also decreases the disease score in all the plants. The role of cytokinins has been investigated earlier and it was observed that cytokinins, serving as endogenous inducers for distinct classes of pathogenesis-related (PR) proteins, are necessary for the biosynthesis of SA and JA (Sano et al., 1994, 1995, 1996). Cytokinins are also known to delay senescence and can affect sensitivity of plants to pathogens. Moreover, *A. brassicae* and several other necrotrophic fungi are known to infect senescing plants due to increased susceptibility of the senescing tissue; exogenous application of CKs may aid in the delay and reduction of disease. Transgenic tobacco lines with higher CK levels were observed to be more tolerant to tobacco necrosis virus (TNV) (Pogany et al., 2004). CK also had a suppressive effect on the wildfire disease of tobacco caused by the bacterium *Pseudomonas tabaci* (Van Hall) (Lovrekovich and Farkas, 1963). In another study, CKs were observed to induce resistance of *Phaseolus vulgaris L.* to the white clover mosaic potexvirus (Clarke et al., 1998) and they also affected the growth of the fungus *Erysiphe cichoracearum* DC on leaf discs of tobacco (Cole and Fernandes, 1970). CKs have also been reported to enhance the resistance of barley to the fungal pathogen *Erysiphe graminis* f. sp. *hordei.* It is

also supported by Sharma et al. (2010) who showed that zeatin antagonizes the effects of ABA produced by *A. brassicae* and delays the senescence and induces the expression of defense-related genes. The present study demonstrates that zeatin inhibits the *in vivo* growth of *A. brassicae* on the leaf surface and delays the infection process. To the best of our knowledge, even though CKs have been implicated in other host-pathogen interactions, this is the first direct demonstration of a protective role of zeatin against *A. brassicae- B. juncea* pathosystem. Apart from playing role in defense, MAPK 4 might also play role in other activities because in *S. alba* and Divya healthy samples, it showed maximum MAPK 4 expression. Our laboratory has identified that at the initial stage of *Alternaria* infection, all three well studied MAPKs viz. MAPK3 (Taj et al., 2011), MAPK6 (Tiwari, 2012, personal communication) and MAPK4 have been expressed, it is also well documented that these three kinases plays an important role in defense pathway. The expression of MAPK 3 and 6 is governed by the salicyclic acid pathway and MAPK4 is governed by the jasmonic acid pathway (Petersen et al., 2000). *A. brassicae* infection requires green tissue for sporulation which supports the view that this fungus is hemi biotrophic and behaves as both biotrophic as well as necrotrophic pathogen. At initial stage of infection, *A. brassicae* behaves like a biotroph but at later stages, it behaves as a necrotrophic pathogen. From this study, it is very difficult to say, at early stage which kinase among them is playing a prominent role in providing sustainable disease resistance; it might be a crosstalk among all these MAPKs and at later stages it could be a switch over from SA induced pathway (MAPK3/MAPK6) to JA induced (MAPK4) pathway. It could be hypothesized that resistance against *A. brassicae* may be carried out by the jasmonic acid pathway as it is a hemibiotrophic fungus. The earlier findings of our laboratory shows that LOX, AOC and OPR3 which are the upstream enzyme of the jasmonate biosynthesis pathway, were expressed after pathogen infection (Tej, 2012, personal communication) and also observed the co-expression of MAPK3 with LOX (Taj et al., 2011) which again support that SA and JA pathway have crosstalk at early infection of *A. brassicae.* This study gives a clue that cytokinin (zeatin) and MAPK4 both plays role in early defense. Our efforts are now directed towards understanding the role of zeatin induced defense genes in this pathosystem.

In conclusion, a more intensive study has to be carried out at the molecular level by taking later stages of disease progression.

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