Research Article**Water fern induced mitogen-activated protein kinase signalling in *Sesamum orientale* L. var. *thilarani* involved in systemic resistance against *Alternaria* leaf spot disease****A. S. Lubaina^{1*}, K. Murugan²**¹Department of Botany, Christian College, Kattakada, Thiruvananthapuram, Kerala, India²Plant Biochemistry and Molecular Biology Laboratory, Department of Botany, University College, Thiruvananthapuram, Kerala 695 034, India

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Abstract

Objective: The perception of inductive signals from phytohormones coupled with developmental processes has received considerable attention in defence mechanism. Cytokinins are phytohormones that are involved in multiple physiological processes including plant immunity. *Alternaria* leaf spot is a major biotic pressure in *Sesamum orientale* leads to loss of yield. Plant extract based induction of defence is one of the challenged areas in plant disease management. An attempt was made to evaluate the efficacy of aqueous extract of *Salvinia molesta* against *Alternaria sesami* in sesame under *in vitro* condition. **Materials and methods:** Cytokinin isolated from the fronds of aquatic fern *S. molesta* and subjected to bioassay. The fractionation and quantification of isolated cytokinin by HPLC followed by light microscopic analysis of sesame pre-treated with 10% aqueous extract of *S. molesta*. The molecular mechanism of signalling pathway analysed by semi-quantitative RT-PCR and the pathogen related protein profile by SDS-PAGE. **Results:** The isolated cytokinin showed positive results in the soybean callus bioassay and its fractionation resulted sixteen isoprenoid cytokinins including *trans*CK, *cis*CK, dihydroCK and iP (isopentenyladenine) forms. Further, light microscopic analysis revealed delayed fungal penetration and spreading of hyphae in the leaf tissue pre-treated with water fern extract. This data further substantiated the results of reduction in disease incidence and severity in the sesame. Semi-quantitative RT-PCR analysis revealed the significant expression of MAPK-4 during the early periods of infection. Subsequently, the SDS-PAGE analysis showed the formation of new pathogen related protein bands and also over expression of other bands. **Conclusion:** The results suggests that cytokinin present in *S. molesta* extract up regulates plant immunity via MAPK-4 signalling which in turn induce the formation of pathogen resistant proteins related to resistance in sesame against *Alternaria* leaf spot disease.

Keywords: *Alternaria*, sesame, disease score, mitogen-activated protein kinase signalling, cytokinin

Introduction

Plants have developed defensive and protective responses to various stresses encounter in their environment. The cytokinin signaling cascade is widely considered to be key regulator of plant resistance against herbivores as well as diverse pathogens. Salicylic acid mediates plant resistance against biotrophic and hemibiotrophic pathogens (Meldau et al., 2012). The role of the salicylic – jasmonic/ethylene core defense signalling in plant-pathogen interactions has long been proved. There is still a

lacuna in the influence of growth-promoting hormones like auxin/cytokinin on modulating the major defence pathway. Search on this aspect of plant immunity will reveal important biological inferences concerning the trade-off between defence and development in plants. The effect of auxin has already been established with respect to immune dynamics of plant-pathogen interactions. Phytohormones play diverse role in terms of growth and developmental processes as well as against diverse biotic and abiotic stress responses in plants. Infection of plants with plant pathogens alters the level of these hormones. The identification and characterization of many mutants related with the biosynthesis, perception and signal transduction of these hormones has been instrumental in understanding the role of individual components of each hormone signaling pathway

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in plant defence (Bari and Jones, 2009). Cui et al. (2005) isolated coronatine induced by *Pseudomonas syringae* pv. *tomato* (Pst), triggers the activation of JA-dependent defence signals leading to the suppression of SA-dependent defence responses and promotion of disease symptoms. However, still only limited knowledge is available on complex regulatory networks where multiple hormonal pathways interact and influence plant defence responses. In this juncture, the present study is an attempt to evaluate the effect of exogenous application of *Salvinia molesta* water extract containing cytokinin on *Sesamum orientale* infected with *A. sesami* in terms of MAPK-4 signaling pathway induced plant resistance.

Materials and methods

Salvinia molesta extract as fungicide

The aquatic fern, *Salvinia molesta* was collected fresh from the Botanical garden pond, University College, Thiruvananthapuram, Kerala, India and was kept in plastic bucket containing tap water at the green house for 7 days. Chemicult, commercially available hydroponic supplement containing full range of macro and micro nutrients was added to the tap water. This was done to ensure vigorous growth of the water fern for the experiment. 30 g fresh weight (FW) plant samples were surface dried using paper towel and homogenized in 100 ml 80% ethanol for 24 h at 10°C. The extracts were further sonicated and then filtered using whatman No. 1 filter paper, rinsed further with 100 ml 80% ethanol and dried under vacuum at 35°C. The crude extract was partially purified following the protocol of Dobrev and Kaminek (2002), where each sample was re-dissolved in 3 ml 80% ethanol and passed through an activated Sep-Pak® Plus C18 (SPE) cartridge. The eluate was collected and dried under vacuum. The samples were re-dissolved in 5 ml 1 M formic acid and loaded onto another activated Oasis® MCX (150 mg/6 cc) cartridge. Cytokinin eluted from the cartridge using 5 ml 0.35 M NH₄OH in water followed by 5 ml 0.35 M NH₄OH in 60% methanol. These two fractions were mixed and dried. The extracts were resuspended in 2.5 ml 80% ethanol and loaded onto Whatman No. 1 chromatography paper and developed in a descending manner in isopropanol:ammonia:water (10:1:1, v/v/v) until the solvent front had moved approximately 30 cm. After drying at 60°C for 24 h, the chromatograms were fractionated into 10 equal R_f zones and placed in individual flasks. These were further evaluated for cytokinin activity using the soybean callus bioassay (Miller, 1965).

Miller's solidified culture media (excluding the kinetin) added to the flasks containing the cut-up chromatograms. Three pieces of approximately 1 mg 3-4 week old soybean callus were placed in each flask under aseptic conditions. The callus grew for 28 days in continuous 1 µmol m⁻²s⁻¹ light at 24°C after which

the callus was weighed. Triplicate set of kinetin standards was included where kinetin was added to the medium at 5, 10 and 50 µg l⁻¹ concentrations, as well as a control in which no kinetin was added to the medium.

High-performance liquid chromatography (HPLC)

After 3 h of extraction, the homogenate was centrifuged (15000 x g, 4°C) and the pellets were re-extracted. The combined supernatants were concentrated to approximately 1 ml under vacuum at 35°C, diluted to 20 ml with ammonium acetate buffer (40 mM, pH 6.5) and purified using a combined DEAE-Sephadex (1.0 cm × 5.0 cm)-octadecyl silica (0.5 cm × 1.5 cm) column and immunoaffinity chromatography based on generic cytokinin (Faiss et al., 1997). The methanolic eluate from immunoaffinity chromatography columns were evaporated to dryness and dissolved in 75 µl of the mobile phase for HPLC analysis. Cytokinin O-glucosides and nucleotides were hydrolyzed to free bases and ribosides by action of α-glucosidase or alkaline phosphatase respectively before subsequent HPLC-MS analysis (Novak et al., 2003).

The samples were analysed by HPLC (Waters Alliance 2690) linked to a Micromass ZMD 2000 single quadrupole mass spectrometer equipped with an electrospray interface [LC(+)ES-MS] and photodiode array detector. Isopentenyladenine (iP) and ribotide used as internal standards. Using a post column split of 1:1, the effluent was introduced into an electrospray source (source block temperature 100°C, desolvation temperature 250°C, capillary voltage +3.0 V, cone voltage 20 V) and PDA (scanning range 210-300 nm; with 1.2 nm resolution) and quantitative analysis of the different cytokinins was performed in selective ion recording mode. Quantification performed by Masslynx software using a standard isotope dilution method. The ratio of endogenous cytokinin to appropriate labelled standard was determined and further used to quantify the level of endogenous compounds in the original extract, according to the known quantity of added internal standard (Novak et al., 2003).

Treatment of sesame with *S. molesta* extract and *Alternaria sesami*

In the treatment experiment initially, the sesame seeds were pre-treated with *Salvinia* extract (10%) containing cytokinin like compounds by dip method for a period of 2 h. Subsequently, 30 days old seedlings were treated again with *S. molesta* extract (10%) containing cytokinin thrice with an interval of 7 days. 7 days after the final application of the extract, the treated plants were inoculated with 20 µl conidial suspension of *A. sesami* (1×10³ conidia/ml) prepared from 10 days old pure culture. Control was

sprayed with distilled water followed by 20 µl conidial suspension of *A. sesami* only (no plant extract treatment). The plant extract treated and untreated sesame leaves after inoculation with the pathogen were harvested at different time intervals for light microscopic as well as molecular analysis in terms of MAPK-4 signalling pathway by RT-PCR using gene specific primers of MAPK-4 with the internal control actin.

Sample preparation for light microscopic analysis

Seven days after the final application, the *S. molesta* extract treated and untreated control plants were inoculated with 20 µl conidial suspension of *A. sesami* by drop method. The inoculated plants were covered with plastic bags to maintain high humidity for rapid penetration of the pathogen. Inoculated plants were sampled at 6, 12, and 24 h post inoculation (hpi). Thin sections of the leaf samples were taken, stained with 1% cotton blue in lactophenol and were examined using fluorescent microscope (Garg et al., 2010).

Sample preparation for molecular studies

The 30 days old sesame plants in pots were subjected to three kinds of treatments. (i) plants were treated with *S. molesta* extract containing cytokinin only (ii) plants were inoculated with 20 µl conidial suspension of *A. sesami* alone (iii) plants were treated initially with *S. molesta* extract containing cytokinin followed by 20 µl conidial suspension of *A. sesami*. Leaves were collected from plants of each treatment for RNA isolation at 6, 12 and 24 h post inoculation (hpi) for RT-PCR analysis. RT-PCR analysis was performed with the help of One-Step RT-PCR (Qiagen, USA) using the gene specific primer of MAPK -4 and actin as internal control 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 using the spot densitometry tool of Alpha Imager software. The sense and antisense primer sequence of MAPK -4 and actin were G C T C T A A C C A A C C C T T A A C T G , G T A A C C A G C G T G T A A C A A C G T A (228 bp) A T T C T T A C C C T C A A G T A T C C and CATGATCTGAGTCATCTTCT(200bp) respectively.

Pathogenesis related proteins (PR)

Total protein from two samples viz., *S. molesta* pre-treated sesame plants challenged with *A. sesami* and sesame + *A. sesami* inoculated were isolated and subjected to SDS-PAGE to know the polypeptide band pattern of PR proteins as per the methodology described by Laemmli, (1970).

Disease assessment by pot and field study

The disease assessment parameters namely the disease incidence (number of spots per leaf) and disease severity (% of surface infected area) were recorded after *S. molesta* and a synthetic fungicide Mancozeb (0.1%) pre-treatments separately on sesame seeds for a period of 2 h followed by mature plants and subsequently inoculated with *A. sesami* in pot and field conditions during the growing seasons of 2012 and 2013 and each trial replicated thrice.

Pot study

One month old sesame plants in pots were treated with 10% aqueous plant extract of *Salvinia molesta* and 0.1% Mancozeb separately using a hand sprayer and the treatment was repeated up to three times at one week interval. Seven days after the final treatment, the plants were inoculated with 20 µl conidial suspension of *A. sesami* by drop method. Control plants sprayed with 20 µl conidial suspension of *A. sesami* and distilled water. The experimental plants, along with their respective controls were covered with polyethylene bags for maintaining high humidity to ensure successful penetration of the pathogen into the host tissue. The disease severity was recorded one week after inoculation with the pathogen following 0-5 scale of Shrestha et al. (2005) with some modifications where 0= no infection, 1= up to 5% area covered by the disease, 2= 6-10% area covered, 3= 11-20% area covered, 4= 21-30% area covered, 5= 31-100% area covered.

Field trials

Similar to pot culture, field trials in plots of 2.0 m x 2.0 m (4 m²) and separated by a clean space of 0.4 m² in a Randomized Complete Block Design (RCBD) were also conducted on growing seasons of 2012 and 2013 and replicated three times in a field at Vembayam, Trivandrum, Kerala. Plant extract pre-treated sesame seeds (20 g) mixed with 100 g sandy soil were sown uniformly in the plots. The 4 weeks old sesame plants were treated with 10% aqueous plant extract of *Salvinia molesta* and 0.1% Mancozeb separately and the treatment was repeated at one week interval until 6 WAS (weeks after sowing) using a hand held garden sprayer. Subsequently, after the final application of plant extracts the plants sprayed with spore suspension of *A. sesami*. Plants sprayed with sterilized water followed by conidial suspension of *A. sesami* served as control. Disease assessment done one week after pathogen spray from two permanent randomly placed quadrants (50 cm x 100 cm) per plot. The total number of plants and number of infected in a quadrant were counted and the disease incidence was calculated. Disease severity recorded according to percentage of area covered following

0-5 scale of Shrestha et al. (2005) by assessing 10 randomly tagged plants per plot after stipulated treatments. The following formula used in determining the severity of infections.

$$\text{Disease severity} = \frac{\text{Sum of all scores} \times 100}{\text{Number of plants scored} \times \text{N} \times \text{Highest score (5)}}$$

Where N is the total number of assessments and 5 the maximum score on the scale.

Statistical analysis

All statistical analysis were performed using ANOVA in CRD to compare the effectiveness of the treatments (Snedecor and Cochran, 1968). In the treatment trials, after confirming the significance of F values, the significance of the differences between the mean values of 6 replications were tested using ANOVA. Significant differences were considered at $P < 0.01$ probability levels.

Results and discussion

Cytokinin like compounds in *S. molesta* aqueous extract

Remarkable multiplication and increase in biomass of *S. molesta* was noticed under the experimental conditions. The aqueous extract isolated from the fronds of *S. molesta*, grown under experimental conditions revealed cytokinin activity in the soybean callus bioassay. The partial purification of crude aqueous extract of *S. molesta* followed by its fractionation and quantification exhibited sixteen isoprenoid cytokinins including *trans*CK, *cis*CK, dihydroCK and iP (isopentenyl-adenine) forms i.e., significant amount of cytokinin content in the extract (Table 1). 10% aqueous extract of *S. molesta* was used for the pre-treatment of sesame seeds by dip method and subsequently at the three pair leaf stage thrice with an interval of 7 days. One week after the final application of the extract, the treated plants were

inoculated with 20 μ l conidial suspension of *A. sesami* (1×10^3 conidia ml^{-1}) prepared from 10 days old pure culture. The microscopic and molecular analysis carried out at 6, 12 and 24 hour post inoculation (hpi) periods.

Table 1. Quantification and fractionation of cytokinin isolated from *Salvinia molesta*

Hormones	Quantity (pmol/g tissue)
tCK	6.32 $\pm$ 0.18
tCKR	4.6 $\pm$ 0.11
tCKOG	5.6 $\pm$ 0.23
tCKROG	3.91 $\pm$ 0.09
tCKR5MP	4.1 $\pm$ 0.32
Total tCK derivatives	24.52
cCK	1.7 $\pm$ 0.01
cCKR	3.4 $\pm$ 0.21
cCKOG	1.8 $\pm$ 0.02
cCKROG	6.1 $\pm$ 0.42
cCKR5MP	10.6 $\pm$ 0.39
Total cCK derivatives	23.6
dihydroCK	1.21 $\pm$ 0.04
dihydroCKR	1.78 $\pm$ 0.06
dihydroCKOG	2.1 $\pm$ 0.03
Total dihydro CK derivatives	5.09
iP	10.3 $\pm$ 0.45
iPR	9.4 $\pm$ 0.24
iPR5MP	13.2 $\pm$ 0.63
Total iP derivatives	32.9
Total cytokinins	86.11

The results are presented as mean $\pm$ S.D of duplicate measurements

Figure 1. Light microscopic analysis of sesame leaves inoculated with *A. sesami* (1a-1c) and sesame leaves pre-treated with *S. molesta* extract followed by *A. sesami* inoculation (2a-2c) at 6, 12 & 24 hour post inoculation (hpi)

Effect of *S. molesta* aqueous extract on penetration and growth of *A. sesami* in sesame

Invasion of *A. sesami* on *S. orientale* (Thilarani) leaves pre-treated with *S. molesta* extract was analysed using light microscopy. Conidial germination, germ tube formation and penetration into the host tissue were observed in the control sesame (not treated with plant extract) whereas the exogenous application of *S. molesta* aqueous extract delayed the penetration of appressorium, the production and spreading of mycelia network (Figure. 1). The results suggest that *S. molesta* extract containing cytokinin like compounds that inhibits the cross talk between host and pathogen and delays the infection process. Further, the extract suppresses the conidial germination and fungal hyphae proliferation. Marmath et al. (2013) reported similar impact of zeatin dose against *A. brassicae* pathotoxin in cell culture of *B. juncea*. Truman et al. (2007) revealed jasmonic acid (JA) signaling has been implicated in the long-distance information transmission leading to systemic immunity in *Arabidopsis*. Further, rapid accumulation of JA in phloem exudates of leaves challenged with an avirulent strain of *Pseudomonas* species with over expression of JA biosynthetic gene transcripts as well as JA levels in systemic leaves suggests that JA could act as a mobile signal in *Arabidopsis*.

MAPK-4 expression profile in the experimental and control sesame by semi quantitative RT-PCR

Expression profile of mitogen activated protein kinase -4 (MAPK -4) was evaluated at 6, 12 and 24 hpi on three

experimental samples of sesame (i) treatment with *S. molesta* extract containing cytokinin only, (ii) sesame with *A. sesami* inoculation alone (iii) sesame pre-treated with *S. molesta* extract containing cytokinin followed by *A. sesami* inoculation (Figure 2). MAPK -4 (228 bp) showed an up regulation from 6 to 12 hpi and then decreased marginally at 24 hpi. The transcript expression level was found to be highest at 12 hpi. The constitutive expression of actin (control) was normal. These observations imply that MAPK-4 initiated its expression as signalling molecule in the plant to induce or strengthen plant defense and there by restrict conidial germination and its growth.

Expression of MAPK- 4 and the conidial germination are initiated at 6 hpi. This strongly suggests the resistance induced by *S. molesta* treatment in sesame i.e., cytokinin like compounds in the *S. molesta* further enhanced the expression of MAPK- 4 at 6 and 12 hpi. The RT-PCR data related with the expression of MAPK- 4 further corroborates the infection behaviour visualized under light microscopy. Indeed, it is well known that CKs activates numerous plant defense response genes (Schafer et al., 2000). Cytokinin present in the *S. molesta* enhanced the expression of MAPK-4 from 6 to 12 hpi in treated sesame. Meanwhile, at 24 hpi, the expression of MAPK- 4 was reduced comparatively, which further suggest that apart from MAPK- 4, there are other signal cascades which provides additional defense against pathogen invasion. This tolerance may be due to induction of osmotin or other related defence related genes. Further, during disease

Figure 2. Change in transcript profiling of MAPK-4 & Actin genes by RT-PCR analysis (i) Sesame with *S. molesta* extract containing cytokinin (ii) Sesame with *A. sesami* inoculation (iii) Sesame pre-treated with *S. molesta* extract containing cytokinin followed by *A. sesami* inoculation at 6, 12 & 24 hour post inoculation (hpi)

progression slightly larger chlorotic and necrotic lesions exhibited around the inoculated region with no new additional spots which clearly indicates that the osmotin or other defence gene imparts some level of defence against *A. sesami*. In the treated sample inoculated with pathogen, maximum expression of MAPK -4 was observed at 12 hpi which implies that cytokinin present in the extract inhibits the conidial germination by enhancing the MAPK -4 expression, which further supports that both cytokinin and MAPK -4 imparts defense.

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 which are necessary for the biosynthesis of SA and JA (Sano et al., 1996). Cytokinins are also known to delay senescence and can affect sensitivity of plants to pathogens. Moreover, *A. sesami* and several other necrotrophic fungi are known to infect senescing plants due to its increased susceptibility towards the senescing tissue. Exogenous application of CKs may facilitate delay and reduction of disease. Transgenic tobacco lines with higher CK levels were more tolerant to tobacco necrosis virus (Pogany et al., 2004). Sharma et al. (2010) reported that cytokinin antagonizes the effects of ABA produced by *Alternaria* species and delayed the senescence and induces the expression of defense-related genes. The present study also demonstrates that cytokinin inhibits the *in vivo* growth of *A. sesami* on the leaf surface and also delays the infection process. Taj et al. (2011) reported that during the initial stage of pathogen infection, all MAPKs viz., MAPK-3, MAPK-6 and MAPK-4 have been expressed and these kinases play an important role in defense pathway. The expression of MAPK-3 and 6 is governed by the salicylic acid pathway and MAPK-4 is governed by the jasmonic acid pathway. *A. sesami* infection requires green tissue for sporulation, which supports the view that this fungus is hemi biotrophic and behaves as both bio-trophic as well as necrotrophic pathogen. At the initial stage of infection, *A. sesami* behaves like a biotroph but at later stages, it changes into necrotrophic pathogen. From this study, it is not possible to suggest firmly that, at early stage which kinase is playing a

pertaining role in providing sustainable disease resistance. But, 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 (MAPK-4) pathway. It could be hypothesized that resistance against *A. sesami* may be carried out by the jasmonic acid pathway, as it is a hemi biotrophic fungus. The preliminary findings related with MAPK-4 expression suggest that LOX, AOC and OPR3, which are the upstream enzymes of the jasmonate biosynthesis pathway, were expressed after pathogen infection and also observed the co-expression of MAPK 3with LOX, which again support that SA and JA pathway have crosstalk at early stage of infection with *A. sesami*. This study reveals the possibility that cytokinin and MAPK-4 play role in early defense. Further studies are warranted to unravel the role of cytokinin related with induction of defence gene in the pathosystem.

Effect of *S. molesta* extract containing cytokinin on disease incidence and severity

The impact of *S. molesta* extract containing cytokinin was further analysed on the disease score under pot and field conditions in *S. orientale* infected with *A. sesami*. Remarkable decline in the disease score and also on the appearance of the symptoms were found in sesame pre-treated with plant extract. Meanwhile, the control plants (inoculated with *A. sesami* spores alone) showed severe discolouration, irregular margins and dead spots suggesting its susceptibility towards the pathogen. The treated plants in pots recorded a significant reduction of disease incidence (number of lesions per leaf). Similarly, under field condition also DI recorded was more or less similar with pot studies. However, the control plants displayed a high DI of 57.72 and 58.17 under pot and field conditions respectively (Table 2). The disease severity (percentage of surface infected area) of pre-treated and control sesame challenged with *A. sesami* under pot conditions was also significant. Disease severity in field condition was more or less at par

Table 2. Disease assessment of Sesame variety Thilarani in terms of disease incidence and disease severity from pot and field study after *Salvinia molesta*, Mancozeb treatments followed by *A. sesami* infection and control (Treated with *A. sesami* alone)

Treatments	Pot study		Field Study	
	Disease incidence	Disease severity	Disease incidence	Disease severity
<i>Salvinia molesta</i>	13.05 ^a	6.34 ^a	13.47 ^a	6.62 ^a
Mancozeb	5.24 ^b	3.61 ^b	6.15 ^b	4.23 ^b
Control	57.72 ^c	35.29 ^c	58.17 ^c	35.82 ^c
F ratio	5180.731 **	2364.821 **	6837.175 **	3148.917 **

** $p < 0.01$

with that of pot study (Table 2). The obtained results are comparable with the synthetic fungicide Mancozeb. Therefore, it can be suggested that exogenous application of plant extract can decrease the disease score by providing induced defense. This strongly corroborates with the infection behavior, where the plant extract interfere with the conidial germination and hyphal growth. The disease assessment data showed significant difference among the treated and untreated sesame after pathogen inoculation. Successful reduction in disease incidence and severity of *Alternaria* leaf spot disease after plant extract application under pot and field conditions indicated the efficacy of them to suppress the pathogen in an eco-friendly method.

Pathogen related protein analysis by SDS-PAGE

Protein banding pattern by SDS-PAGE from leaves of experimental (sesame + *S. molesta* treated + *A. sesami* inoculated) showed variation, compared with the control

(sesame + *A. sesami* inoculated) (Figure 3). Control and the experimental plants showed 9 and 13 polypeptide bands respectively, of which 9 bands were common. Unique pathogen related protein bands (PR) of 7, 10, 16.5 and 54 kDa were formed newly in sesame, treated with *S. molesta* extract containing cytokinin. In addition, certain bands like 6.5, 14.3, 19, 20.1 and 45 kDa were over expressed, compared to the control. Temporal and spatial coordination of induced defense related responses are required to effectively kill or reduce the invading pathogen or minimize damage of tissues in the host. The up regulated protein may be β -1, 3 glucanase, phenylalanine ammonia lyase (PAL), chitinase, peroxidase or other antioxidant enzymes.

Pathogen resistance associated proteins are synthesized in many fungi-host interactions (Rep et al., 2002). Phytopathogens like viruses, bacteria, fungi and nematodes induce the formation of defense related proteins, which help in inhibiting the multiplication and invasion of pathogens in the normal cells (Zamani et al., 2012). Infection of *Piper nigrum* with *Phytophthora capsici* led to the accumulation of β -1, 3-glucanases, chitinase, POX and PPO (Nazeem et al., 2008). Similarly, Zamani et al. (2012) reported the expression of the *Secale cereal* thaumatin like protein in transgenic *Brassica*, against stem rot caused by *Sclerotinia sclerotiorum*. Protein content of *Lycopersicon esculentum* was dramatically altered upon infection with the vascular wilt fungus *Fusarium oxysporum*. PR-5 protein family was accumulated during this interaction (Rep et al., 2002). So the differential expression of protein bands and the formation of new bands noticed in the experimental sesame, compared with the control strongly suggest the induced resistance offered by the cytokinin of *S. molesta* in the extract against the fungus - *A. sesami*.

Conclusion

In the present study the consequences of maintaining signaling elements of MAPK-4 that mediate early induced defense responses by RT-PCR analysis in sesame was substantiated by light microscopic studies. The reduction in the disease incidence and severity further supports defense responses with increased growth and fitness levels of the plant. The new PR proteins and over expression of other proteins suggest the induced resistance offered by the cytokinin in the *S. molesta* extracts against *Alternaria* leaf spot disease in *Sesamum orientale*. Further studies involving field analysis with more samples and detailed time course experiment will be necessary to extend the understanding of the complex regulatory mechanisms operating between plant hormone signaling and plant defense responses. A better understanding of phytohormone-mediated plant defense responses is important in designing

Figure 3. Pathogenesis related (PR) protein profile of sesame with *A. sesami* alone treated (C) and sesame pre-treated with *S. molesta* followed by *A. sesami* inoculation (Ex). M- marker C-control Ex-experimental

effective strategies for engineering crops for disease and pest resistance.

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Conflict of interest

The authors are declaring no conflict of interest.

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