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Azadirachta indica leaf extract induces resistance in sesame against *Alternaria* leaf spot disease

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Abstract

Induced resistance is an important component of disease-resistance response of plants, and is accompanied by increased capability for activating defense responses upon pathogen ingress or elicitor treatment. Aqueous leaf extract of neem *(Azadirachta indica Juss.)* provided the control of *Alternaria* leaf spot pathogen *(Alternaria sesami)* of sesame (*Sesamum indicum* L: *Syn. S. orientale* L). Treatment with this extract led to the changes in plant metabolism as leaves of the treated plants exhibited significantly high level of enzymes phenylalanine ammonia-lyase (PAL), peroxidase (PO) and content of phenolic compounds. Further more, germination of A. *sesami* spores was not significantly inhibited by neem extract. It is therefore, suggested that, protection of sesame plants against A. *sesami* by neem extract might be due to stimulation of plants natural defence response.

Key Words: *Azadirachta indica*, *Alternaria sesami*, Peroxidase, Phenylalanine ammonia-lyase, phenolic compounds

Azadirachta indica yaprak ekstrelerinin susamda *Alternaria* yaprak lekesi hastalığına karşı direnci uyarması

Özet

Uyarılmış direnç bitkilerin hastalığa-dirence cevabında önemli bir komponenttir ve patojen ya da elisitor uygulamasına bağlı savunma cevaplarının aktivasyon kabiliyetinin artışı ile uyumluluk gösterir. *Azadirachta indica* Juss yapraklarının suda hazırlanan ekstreleri susamda (*Sesamum indicum* L: *Syn. S. orientale* L.) *Alternaria* yaprak lekesi patojeninin (*Alternaria sesami*) kontrolü için uygulandı. Bu ekstrenin uygulanması bitki metabolizmasında değişikliklere yol açtı, uygulanan bitkilerin yapraklarında kayda değer bir şekilde fenil amonya-liyaz (PAL), peroksidaz (PO) enzimlerinin düzeyleri ve fenolik bileşiklerin içeriklerinde artış meydana geldi. Bundan başka, *A sesami* sporlarının çimlenmesi *Azadirachta indica* ekstresi tarafından kayda değer bir şekilde inhibe edilmedi. Bu nedenle, susam bitkilerinin *A. sesami*'ye karşı *Azadirachta indica* ekstreleri ile korunmasının bitkilerdeki doğal savunma cevaplarının teşvik edilmesi ile meydana geldiği ileri sürüldü.

Anahtar Sözcükler: *Azadirachta indica*, *Alternaria sesami*, peroksidaz, fenil amonya-liyaz, fenolik bileşikler

Introduction

Alternaria leaf spot disease of sesame caused by *Alternaria sesami* appear mainly on leaf blades as small, brown, round to irregular spots and is responsible for losses in grain yield of the crop. The disease is usually controlled by conventional fungicides applied as foliar spray. However, high costs

of fungicides and the problems of environmental pollution have stimulated investigations of alternative strategies for the control of pests and pathogens (Kuc, 1987; Lyon et al., 1995). Apart from conventional fungicides and microbial biocontrol agents, plant products/extracts have been found effective against a wide range of pathogens (Amadioha, 2003; Bowers and Locke, 2004). Furthermore, plant product based biofungicides are systemic, specific in action, non-phytotoxic and have poor environmental retention (Singh, 1994). Studies on the mechanisms of disease control by plant extracts /products have revealed that the biologically active constituents present in them may have either direct antimicrobial activity (Amadioha, 2000; Ansari, 1995) or induce host plants defence response resulting in reduction of disease development (Schneider and Ullrich, 1994).

The induction of resistance in plants against plant pathogens by chemicals is the result of number of mechanisms. Salicylic acid (Chen et al., 1993; Guleria et al., 2005), 2, 6-dichloroisonicotinic acid (Dann et al., 1996) and benzothiadiazole (Gorlach et al., 1996) are among such chemicals which induce systemic acquired resistance in plants. Execution of SAR is brought about by the expression of genes coding for pathogenesis related (PR) proteins (Granell et al.,1987), increase in activity of enzymes such as PAL and peroxidase and level of fungitoxic phenols (Kagale et al., 2004).

Several studies report the direct effect of neem leaf and fruit extracts on target pests and pathogens (Eppler, 1995; Amadioha, 2000). However, limited information is available on the effect of leaf extracts of neem on chemical constituents of host plant leaves involved in inducing resistance against pests and pathogens. Neemazal a product of neem (*Azadirachta indica* Juss) has been found to induce resistance in pea (*Pisum sativum* Linn) against *Erysiphe pisi* (Singh and Prithiviraj, 1997). Aqueous leaf extract of *Azadirachta indica* induced resistance in barley against *Drechslera graminea* through biochemical changes in the host plant (Paul and Sharma, 2002).

The aim of this study was to study the potential for induction of systemic resistance in sesame by aqueous leaf extract of *Azadirachta indica* against *Alternaria sesami*. The results are presented here.

Materials and methods

Sesame cv. LTK-4 (susceptible to *A. sesami*) seeds were surface sterilized with two changes of sterile distilled water before sowing in 800 ml (16 cm height) pots containing a medium of soil:sand:FYM (Farm Yard Manure [decomposed cow dung] (2:1:1 v/v/v)). Plants were maintained under controlled conditions. Irrigation was applied in the pots every third day with a beaker without touching the plant foliage.

Preparation of neem leaf extract

Neem leaf extract was prepared according to Paul and Sharma (2002). 400 g (fresh wt) of mature leaves were homogenized in a pre-chilled pestle and mortar using chilled, sterilized distilled water. The extract was filtered through four layers of moistened muslin cloth. The final volume was adjusted to 1000 ml with distilled water. The filterate was centrifuged at 2000 g, 4 °C for 15 min. The supernatant thus obtained was designated as concentrated leaf extract. Dilution of 1:2 was made from this concentrated extract.

Neem leaf extract treatment and determination of biochemical constituents

The neem leaf extract was sprayed on 4-week-old sesame seedlings using hand driven sprayer in the form of fine droplets. The control plants were sprayed with sterile distilled water only. Leaves from control and treated plants were sampled after 24, 48, 72 and 96 h of treatment for estimation of Phenylalanine ammonia lyase (PAL), Peroxidase (PO) and free and wall bound phenolic content. Phenolics, PAL and PO experiments were performed twice; each experimental treatment was replicated three times.

Incidence of Alternaria leaf spot disease and spore germination of A. sesami

Neem leaf extract (1:2 dilution) treated and control (water sprayed) plants were inoculated with spores of *A. sesami* 24 h after treatment. For plant inoculation, aqueous spore suspensions (4-5x10^5spores/ ml) were prepared from 10 days old pure culture of the pathogen. The development of disease was assessed one week after inoculation following 0-5 scale (Karunanithi, 1996), where 0=No visible symptoms, 1=1-10% leaf area infected (LAI), 2=11-25% LAI,

3=26-50% LAI, 4=51-75 % LAI, 5=76-100 % LAI. The experiment was repeated once and similar results were obtained.

The effect of neem leaf extract on germination of spores of *A. sesami* was studied *in vitro* by following the method of Kumar et al. (1998). For the spore germination inhibition studies, spore suspension was prepared in the sterilized distilled water from 10 days old pure cultures of the pathogens. 25 µl of spore suspension ($2x10^5$) was poured in cavity slides along with 25 µl of undiluted extract and incubated at 24 ± 2 °C. After 12 h of incubation, spore germination was examined under microscope. Appropriate controls were maintained with sterilized distilled water and 0.2% Indofil M-45(Mancozeb). Experiment was replicated thrice. Spore germination was considered as the extension of germ tube to a length equal to one half of the diameter of the spore from any cell (Medwid and Grant 1984). The percentage inhibition of spore germination was calculated as per the formula (Vincent 1947). Three independent experiments were performed with similar results.

Extraction and determination of phenolics

Free and cell wall-bound phenolics were determined according to Kofalvi and Nassuth (1995). Fresh leaves (0.5 g) were extracted in 50% methanol (12 v/v) for 90 min at 80 °C. The extract was centrifuged at 14000 g for 15 min and the supernatant was taken for free phenolic determination using the Folin-Ciocalteu's phenol reagent. The pellet was sponified with 2 ml of 0.5 N NaOH for 24 h at room temperature to release the bound phenolics, neutralized with 0.5 ml 2 N HCl and centrifuged at 14000 g for 15 min. The supernatant was taken for bound phenolic determination using the Folin-Ciocalteu's assay. One hundred microliters of the methanol and NaOH extracts were diluted to 1 cm^3 with water and mixed with 0.5 ml 2.0 N Folin-Ciocalteu's reagent and 2.5 ml of 20% Na_2CO_3. After 20 min at room temperature, absorbance of samples was measured at 725 nm with a T117 spectrophotometer (Systronics, India). Phenolic concentration in the extracts was determined from standard curve prepared with gallic acid.

Extraction and determination of peroxidase

Peroxidase was determined according to the method based on Shannon et al. (1966). Sesame leaves (0.2 g fresh weight) were ground with a pestle in a mortar in 5 ml ice-cold 0.1 M Tris-HCl buffer (pH 7.5) containing $5x10^{-3}$ M 2-mercaptoethanol. The extract was centrifuged at 14,000 g for 25 min at 4 °C. Samples (100 µl) of the supernatant were added to 3 ml of a solution of 0.1 M sodium phosphate buffer (pH 6.5), 0.8 mM H_2O_2 and 0.05 M O-methoxyphenol (guaicol). Peroxidase activity was determined by measuring the absorbance at 470 nm with a T117 spectrophotometer that averaged readings at 15, 30, 45 and 60 sec after solution addition. Enzyme activity was expressed as change in absorbance expressed as units g^{-1} fresh wt.

Extraction and determination of phenylalanine ammonia-lyase

PAL was determined according to the procedure of Burrell and Rees (1974). Leaf samples (0.2 g) were ground with a pestle in a mortar in 5 ml 0.05 M borate extraction buffer (pH 8.8) at 4 °C. The homogenate was centrifuged at 14,000 g for 25 min and the supernatant dialyzed overnight at 4 °C against 5 L of 0.05 M borate extraction buffer (pH 8.8). A 0.2 ml sample of the remaining mixture in the dialysis tube was transferred to a test tube containing 0.03 M L-phenylalanine in 2.5 ml 0.05 M sodium borate buffer (pH 8.8). The reaction mixture was placed in a 37 °C water-bath for 1 h. Tubes were removed from the water-bath and placed in ice. The production of cinnamic acid in extracts was determined by measuring the absorbance of a sample containing L-phenylalanine against that of an identical sample containing D-phenylalanine. Absorbance was measured at 290 nm using the T117 spectrophotometer. The amount of cinnamic acid produced was determined from a *trans*-cinnamic acid standard curve and PAL activity was expressed as n mole cinnamic acid produced h^{-1} g^{-1} fresh wt.

Data analysis

The data were analyzed using students t-test (Gupta and Kapoor 1993). Values in figures are the means and the bars indicate the standard deviations.

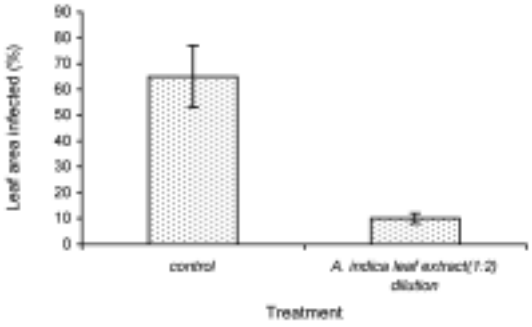

Figure 1. Percentage of area infected in leaves treated with *A. indica* leaf extract and water.

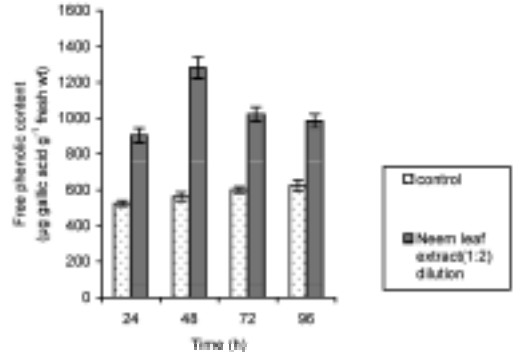

Figure 2. Effect of *A. indica* leaf extract treatment on free phenolic content of sesame leaves.

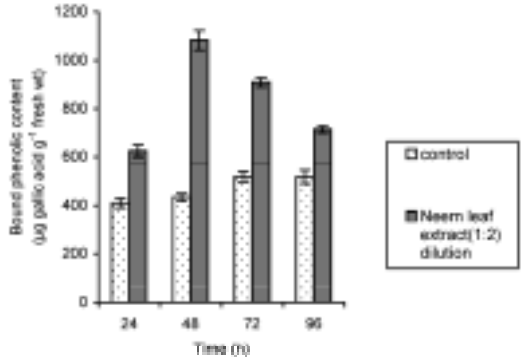

Figure 3. Effect of *A. indica* leaf extract treatment on bound phenolic content of sesame leaves.

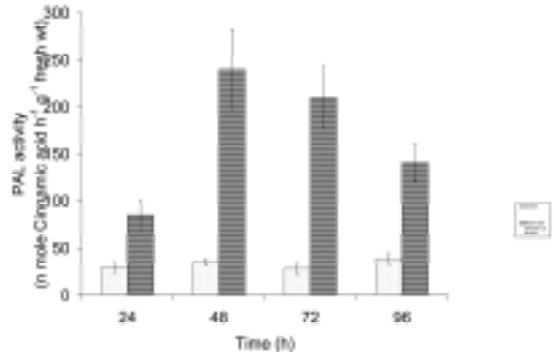

Figure 4. Effect of *A. indica* leaf extract treatment on PAL activity in sesame leaves.

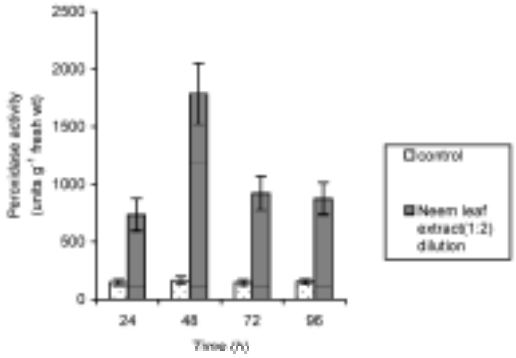

Figure 5. Effect of *A. indica* leaf extract treatment on peroxidase activity in sesame leaves.

Results

Incidence of leaf spot disease and germination of A. sesami spores

A significant control of *Alternaria* leaf spot disease was observed in the plants treated with neem leaf extract (Fig. 1). Maximum severity of disease was observed in control (65%), whereas, in plants treated with neem leaf extract (1:2 dilution) it was only 10%.

Germination of *A. sesami* spores was 98% in aqueous neem leaf extract (1:2 dilution) as compared to 92% in case of water control. Indofil M-45(0.2%) completely inhibited the germination of *A. sesami* spores.

Free and wall bound phenolic content

It is evident from the Figs. 2 and 3 that free and wall bound phenolic content is significantly affected by the neem leaf extract (1:2 dilution) treatment (P>0.01). Maximum free phenolic (1283 µg gallic acid g^{-1} fresh wt) and bound phenolic (1082 µg gallic acid g^{-1} fresh wt) content in sesame leaves treated with 1:2 dilution of neem extract was observed after 48 h of treatment.

Phenylalanine ammonia-lyase activity

The activity of PAL was significantly higher (P>0.01) as compared to control in neem leaf extract treated leaves of sesame (Fig. 4). Peak activity of PAL in 1:2 dilution neem leaf extract treated leaves was observed after 48 h of treatment (240 n mole cinnamic acid h-1 g-1 fresh wt), whereas, activity after 72 and 96 h of treatment was 210 n mole cinnamic acid h^{-1} g^{-1} fresh wt and 141n mole cinnamic acid h^{-1} g^{-1} fresh wt respectively.

Peroxidase activity

Neem leaf extract treatment (1:2 dilution) significantly affect (P>0.01) the level of PO as compared to control in sesame leaves at all harvest times. Ten fold increase in PO activity as compared to control was observed after 48 h of treatment (1785 units g^{-1} fresh wt), whereas, increase in PO activity was five, six and six fold as compared to control after 24 (734 units g^{-1} fresh wt), 72 (922 units g^{-1} fresh wt) and 96 h (875 units g^{-1} fresh wt) after neem leaf extract treatment.

Discussion

It is evident from the above results that neem leaf extract (1:2 dilution) protected the sesame plants from *Alternaria* leaf spot pathogen (*Alternaria sesami*) and induced changes in the host plant metabolism. Moreover the spores of *A. sesami* were not significantly inhibited by neem extract indicating towards the involvement of plants natural defence response in protection conferred by neem extract. We have demonstrated higher level of PAL, PO and phenols in the leaves of sesame cv. LTK-4 as a result of neem leaf extract treatment. The optimal time of 48 h at which appreciable increase in activity of PAL, PO and level of phenolics was observed represents a programmed response period. The decrease in level of PAL, PO and phenolics after 48 h might be due to some mechanism that down regulates sensitivity of cells to bioactive constituents present in aqueous neem extract after certain time. Paul and Sharma (2000) reported a time dependent induction of activities of PAL, PO and phenolics in barley upon treatment with aqueous neem leaf extract. They observed a decrease in level of PAL, PO and total phenols in barley leaves with increasing length of elicitor treatment, and their maximum level was observed after 48 h of elicitor treatment. Similarly, time dependent changes in PAL activity in pea leaves following treatment with aqueous neem extract has been reported (Singh and Prithviraj, 1996).

Mauch-Mani and Slusarenko (1996) reported that resistance induction in an incompatible interaction between *Arabisopsis* and *Peronospora parasitica* was largely due to the production of the signal molecule salicylic acid via increased PAL activity. Treatment of sesame leaves with neem extract significantly increased PAL activity, which might result in higher salicylic acid synthesis resulting in induction of resistance.

Peroxidase contributes to resistance by catalyzing oxidative polymerization of simple phenols to lignin and synthesis of antimicrobial oxidized phenols (Misaghi 1992). Protection of tobacco and cucumber plants by leaf extracts of R. *sachalinesis* against powdery mildew have been reported to be accompanied by increased activities of polyphenol oxidase, β-1,3-glucanase, peroxidase and PAL (Schneider and Ullrich 1994). Similarly, Kagale et al., (2004) reported higher activity of PAL, peroxidase, chitinase, β-1,3-glucanase and increase in level of phenols in rice leaves treated with *Datura metel* leaf extract and inoculated with *Rhizoctonia solani* or *Xanthomonas oryzae* pv. *oryzae*.

Further investigations are needed to establish which bioactive constituents of neem are involved in the induction of resistance in sesame against *A. sesami*.

It is suggested that neem leaf extract induced activity of PAL and PO enzymes in sesame leaves, which resulted in increased biosynthesis and metabolism of phenols might have protected the sesame plants from *A. sesami*.

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