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CELL WALL CHANGES AND H⁺ATPASE ACTIVITY ON THE INFECTION PROCESS OF *ALTERNARIA SESAMI IN SESAMUM INDICUM L.*

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Alternaria leaf spot disease of sesame caused by *Alternaria sesami*, which appear mainly on leaf blades as small, brown, round to irregular spots leads to loss of yield of *Sesamum indicum* L. in the tropical and subtropical regions of the world. The aim of this study was to investigate the cell wall changes and H⁺ATPase activity of sesame against the invading pathogen *A. sesami*. The inter and intra cellularly spreading hyphae and its colonization results in marked alterations in the host tissues, including disorganization of cytoplasm, cell organelles and disintegration of cell walls and collapse of host cells. The degradation of cell wall components suggests that the pathogen may secrete cell wall degrading enzymes (CWDEs) such as cellulases, xylanases and pectinases during infection and subsequently its spreading in the sesame leaf tissues. Fungal infection affects decreased growth rate, membrane stability and also the photosynthetic pigments in the host. The K^+ efflux was stimulated with infection. Correspondingly, the infection produced a reduction in the content of pectin and cellulose fraction of leaves. On the other hand, the hemicellulose and lignin contents were stimulated. H^+ ATPase activity showed a decline in the infected cells suggesting the impairment of transportation of nutrients in the affected cells. Further studies are warranted to trace the oxidative burst and hypersensitive reaction in the host cells due to the sesame fungal interaction.

Keywords : *Alternaria sesami;* Cellulase; H⁺ATPase; Sesame; Xylanase.

Introduction

Sesame *(Sesamum indicum* L; Pedaliaceae), an ancient oil seed crop, nicked as queen of oils, has probably originated in Africa. India ranks first in both area and production and also enjoys the paramount position for export of white seeded type; which are in great demand. It is one of the most important oil seed crop and has been cultivated in tropics from ancient times and considered to be the oldest oil seed crop known to man[1]. *Alternaria* Nees. is a cosmopolitan fungal genus that includes saprophytic, endophytic and pathogenic species. Plant pathogenic species of *Alternaria* infect economically important plants such as *Malus domestica*[2] and *Lycopersicon esculentum*[3]. Alternaria blight of sesame has been identified as a predominant biotic pressure of single origin, that limits seed yield both qualitatively and quantitatively. Prevention of penetration by pathogens during plant infection is generally dependent on an accurate time course of the pathogen perception by host cells and activation of a signaling cascade triggering a network of coordinated responses.

Multiple hydrolytic enzymes act in concert to depolymerize and hydrolyze cell wall components during host cell wall degradation by pathogens. The enzyme polygalacturonase hydrolyzes the α-1,4-d galacturonan backbone of pectic polysaccharides, and this increased activity coincides with pectin depolymerization[4]. De-esterification significantly affects the physical properties of pectin, and the creation of free carboxyl groups increases the charge density in the wall, influencing the activities of wall enzymes such as polygalacturonase and pectic lyase[5] . Endo-β-1,4-glucanase can hydrolyze the 1,4-β-d linkages between unsubstituted linear glucans, and thus cellulose and xyloglucan within plant cell walls are potential substrates[6]. Expansin enzymes weaken glucan-glucan interactions by disrupting hydrogen bonds at the cellulose-cross-linking glucan interface and appear to act in wall disassembly as well as in cell growth[7]. Similarly, cleavage and re-ligation of xyloglucans through the activity of xyloglucan endotransglycosylase may also function during cell wall degradation. In addition to these and other enzymes, nonenzymatic factors such as reactive oxygen species (ROS) : hydroxyl radicals and hydrogen peroxide (H_2O_2) may disrupt pectin and hemicellulose backbones.

ROS degrade cell wall polysaccharides *in vitro*. Thus the aim of this paper is to provide ultrastructural cell wall changes, its composition and H^+ATPase activity during the infection process of sesame by *A. sesami* using scanning electron microscopic and biochemical methods.

Materials and Methods

Sesamum indicum wild and cultivar named Thilarani were raised from seeds in healthy conditions in a glass house. For *in vitro* fungal inoculation studies, mature plants were inoculated with 20 µl of *Alternaria sesami* conidial suspension (1×10^3 conidia ml^{-1}) or 20 µl of water (mock inoculation). The inoculated plants, along with their respective healthy controls, were then maintained at 30°C in a temperature controlled glass house under a photoperiod of 12/12 h (light/dark) and 60% RH. After the development of symptoms in infected plants the experiment was terminated and the plants are harvested for analysis.

Sample of sesame leaf pieces after infection collected were prefixed in 3 % glutaraldehyde in 0.05 M. Sodium cacodylate buffer at pH 7.2 for 2 h, at room temperature. The samples were then washed three times with a 0.05 M sodium cacodylate buffer solution for 10 min. Samples were dehydrated in graded alcohol series, followed by critical point drying. The dried materials were adhered on to aluminium specimen mounts with colloidal silver paste and then sputter coated with gold palladium. The specimens were examined and photographed on a scanning electron microscope at 15-30 kv.

Estimation of Pectic substance: Pectic substance was quantified following the method of Southgate[8]. 5g of sesame leaf powder is extracted with 0.5% ammonium oxalate for 2 h at 85 °C. The extraction is repeated for four times. The filtrates are combined and make it slightly acidic by 1N HCl. Four volumes ethanol is added to the filtrate by stirring. After the precipitate had settled, decant the supernatant through a weighed glass filter crucible. The precipitate is transferred to the crucible and washed with 71% methanol. The difference in weight showed the quantity of pectic substance.

Estimation of Hemicellulose: The residue after the extraction of pectic substance is treated with KOH at different concentrations (5% and 24%) for 24 h. The flask is flushed with nitrogen during alkaline treatment. The KOH extraction is repeated thrice. Filter the residue on a glass crucible. Acidify the combined filtrate with acetic acid and add four volumes of methanol. The precipitate is filtered in a weighed glass crucible. Wash the precipitate with acetone. The crucible is dried and re-weighed. The difference in weight is taken as hemicellulose.

Estimation of Cellulose: The residue dissolved in 24% KOH is washed thoroughly with water followed by acetone, dried and weighed. The weighed residue is hydrolysed in 1N H_2SO_4 for 2 h and filter the residue in a weighed ashless filter paper. The paper is washed with water, acetone and then dried and re-weighed. The paper is ignited and the ash was weighed. The weight of ash is deducted from the weight of residue after dilute acid hydrolysis and record the value as cellulose.

Quantification of lignin: Lignin content of the samples was estimated by acetyl bromide method of Kenji Iiyama and Adrian Wallis[9]. 0.5g tissue was homogenized in 10 ml methanol. The homogenate was centrifuged at 5000 rpm for 5min at room temperature. After decanting the supernatant, the pellet was subjected to sequential washing with methanol, ethyl alcohol and water with three washes in each followed by centrifugation. The pellet was taken and dried at 70°C in a hot air oven over night. 10 mg of dried tissue was solubilized with 3 ml 25% acetyl bromide in acetic acid. The mixture was kept at 70°C in a water bath for 30 min, followed by cooling to room temperature. 0.9 ml 2N NaOH was added to it, followed by 0.1 ml hydroxylamine hydrochloride. The resulting reddish brown solution was diluted with 1% acetic acid and centrifuged at 5000 rpm for 5 min and the absorbance at 280 nm was measured immediately. A standard graph of lignin was prepared with dehydroconiferyl alcohol polymerizate (DHAP).

Isolation and assay of H^+ATPase: Plasma membrane H^+ATPase was isolated from leaf samples of control and infected *Sesamum indicum* using the protocol of Gallagher and Leonard[10]. The isolation buffer used was 0.1M phosphate buffer (pH 7). The reaction mixture consists of 3 mM ATP, 3 mM $MgSO_4$, 50 mM KCl and 30 mM Tris HCl. The pH of the buffer is 7. The reaction was initiated by 100 µl extract to the reaction mixture at 25°C and was terminated after 30 min. by the addition of 0.9 ml of 5% Sodium dodecyle sulphate (SDS) to the reaction mixture. In control the extract was added after termination of the reaction. The release of inorganic phosphate (Pi) was determined according to the method of Fiske and Subbarow[11]. After termination the reactive solution was mixed with 0.1 ml of 1-amino-1-naphthol-sulphonic acid(0.125% in 15% $NaHSO_3$ 1% Na_2SO_3). This mixture was shaken at 25°C for 30 min. The absorbance was recorded at 750 nm. The protein content of the enzyme was estimated by Bradford method[12].

Determination of cell membrane: Cell membrane stability was determined according to the method of Premachandra *et al.*[13]. Leaf discs (number of 10, each 1 cm diameter)

was rinsed three times with deionized water and placed in 30 cm^3 deionized distilled water for 24 h at 10°C. The electrical conductivity (conductimeter) of the bathing solution was measured at 25°C. Following the measurements, leaf discs were autoclaved for 15 min, cooled to 25°C, and the electrical conductivity of the bathing solution was measured for the second time. The degree of injury was calculated according to the equation:

Percentage injury (%) = $1- (1-T_1/T_2) / (1-C_1/C_2) \times 100$

Where T_1 and T_2 are the first (before autoclaving) and second (after autoclaving) conductivity measurements of the treatment, respectively, C_1 and C_2 are the first and second conductivity measurements of the control. The flame photometric method of Williams and Twine[14], was used for the determination of potassium leak.

Results and Discussion

Scanning electron microscopic analysis - The hyphae of *A. sesami* grew extensively over abaxial surface and fill the whole leaf epidermal cell. Conidia of *A. sesami* were small (6-28 µm) and septate with filiform beaks. Multiple branched germ tubes (length 9 - 240 µm) are produced by the conidium and grew profusely and randomly across the surface (Fig. 1). Appressoria are not formed directly on the cuticle or on stomata. Most germ-tubes grew directly into stoma without forming an appressorium over the stomata while others showed direct penetration into the host tissue (Fig. 2, 3). Occasionally, profuse growth of germ tubes formed a hyphal mesh on the host tissue (Fig. 4) leading to a centeral darkned area. Conidia are produced primarily on the surface of lesion on mature or senescent leaves and also on wilted twigs (Fig. 5). Some of hyphal branches were ramifying intercellular along mesophyll and paranchyma tissues (Fig. 6, 7). The toxin produced by the hyphae may cause cell damage and cell wall disruption (Fig. 8).

Conidium production was highest under moisture or humidity condition but lowest in wet leaves. Conidia germinate quickly if moisture is present and begin to produce toxin even before they penetrate the tissue. Penetration has been consistently associated with formation of appressoria in most species, however in the present study, penetration occurs through stomata on the abaxial surface of the leaf without the formation appressoria. Sporulation does not occur on lesion until the plant tissue mature. The presence of these discoloured cells indicates that the cells have been disrupted. Most of the sporulation occurs on leaves than fruits or twigs. The optimum temperature for infection is 27°C.

The mode of infection process of *A. sesami* observed is the present study was generally similar to that of *A. alternata* on Minneola tangelo[15] and also on a range of hosts[16-18]. The present results are in consistent with that of Mimes *et al.*[16]. Production of multiple germ-tubes that grew randomly across the leaf surface in *A.sesami* correlate with that of *Alternaria citri.*

Electron microscopic studies in *Cassia* infected by *Alternaria cassiae*[16] and *A. porri* in *Citrus*[17] have shown appressoria associated with germ tube may have an adhesive function. In the present study, germ tubes and their growth were extremely variable, but this is not unusual because similar response have been reported in cowpea[19]. Similarly, SEM observations in sesame showed both direct and indirect penetration without the formation of appressoria suggesting that appressoria are not always necessary for infection. The hyphal penetrations were seldom observed prior to necrosis and that the death of mesophyll cells in advance of fungal penetration suggests the action of diffusible fungal toxins by *A.sesami.* Secondary hyphae produced from primary hyphae penetrate and grew intercellulary.

Most of the damages by the *Alternaria* may be due to the production of cell wall lytic enzymes such as polyglacturonase, pectin lyase, pectin methylestrase, cellulase and toxins - host specific toxins (HST) and non host specific toxins (NHST).

Membrane stability clearly showed a highly significant decrease with fungal infection in the cultivar Thilarani than its control (Fig. 9). Interestingly, in the wild infected species the magnitude of K^+ efflux was less. A similar reduction in membrane stability induced by fungal infection stress has also been observed by Hashem and Hamada[20]. The K^+ leakage in the infected cells was stimulated with fungal infection. Such leakage of ionic solutes and potassium efflux as well as leakage of cellular metabolites are frequently used to asses membrane integrity. In this respect, Nemec[21] reported that fungal phytotoxins may enhance membrane permeability. These results are also in accordance with wheat root rot disease[22].

Cell wall structure and its properties determine to a large extent the magnitude of cell division and elongation, and hence plant growth. Therefore, cell wall components of the studied plants were estimated to evaluate how far they could have been affected by fungal infection stress. The pectin and cellulose fractions in sesame cultivar leaves were significantly lowered by fungal infection stress compared to wild species, whereas hemicellulose and lignin were stimulated (Table 1). Furthermore, the cell wall composition data in wild infected sesame species clearly demonstrate the capability of it in retarding the fungal infection in the plants.

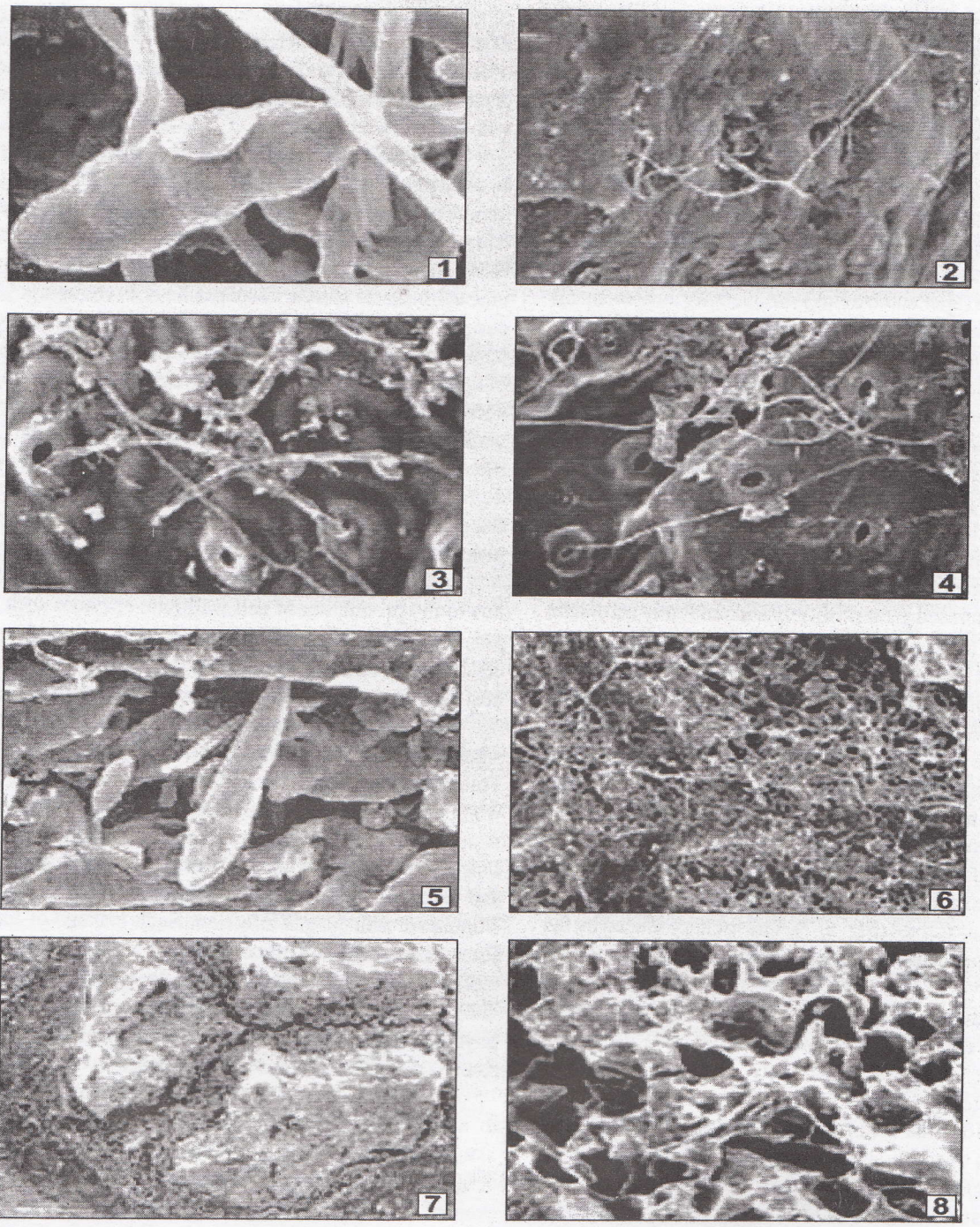
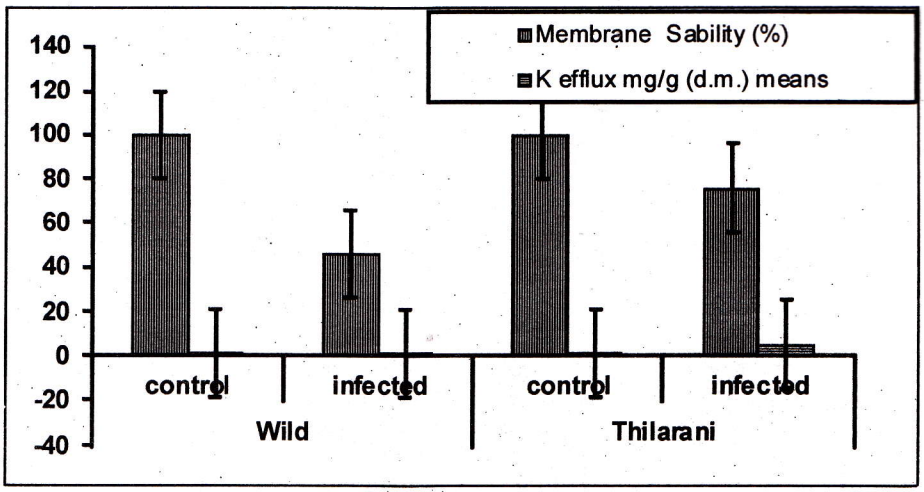


Fig.1. A conidium producing several germ-tube at random points on the conidium body and at the tip of the filiform beak. Bar=100 µm; **Fig.2.** Brannched germ-tubes passing open stomata without forming appressoria. Bar=10 µm; Fig.3. Germ tube entering the stomata; **Fig.4.** Inner surface of the leaf, showing direct penetration of hyphae though the epidermis. Bar=10 µm, **Fig.5.** A young conidiophore developing through a stoma of leaf. Bar=10µm, **Fig.6.** Hyphae are visible within the mesophyll cells. Bar=10 µm, **Fig.7.** Hyphae growing intercellularly, passing through adjacent tissues. Bar=10 µm, **Fig.8.** The cell wall disrupted by the fungal toxin.



*values are mean of six replication (n=6) ±S.D

Fig. 9. Effect of fungal-infection on membrane stability and K^+efflux.

Table 1. Cellulose, hemicellulose, pectin and lignin content(mg/g) in the wild and cultivar control and infected *Sesamum indicum* L.

	Wild control	Wild infected	Cultivar control	Cultivar infected
Cellulose	235±1.2	177±1.6	132±1.2	80±1.0
Hemicellulose	255±1.4	360±0.7	225±0.9	315±0.65
Pectin	114±0.4	96±0.2	64±0.74	46±0.24
Lignin	180±0.7	517±0.67	118±0.2	387±0.3

*values are mean of six replication (n=6) ±S.D

Table 2. H^+ATPase activity (μ/mg protein) in sesame cultivar-Thilarani and wild species.

Cultivar control	Cultivar infected	Wild control	Wild infected
14.5±0.01	6.6±0.03	26.1±0.02	18.7±0.04

*values are mean of six replication (n=6) ±S.D

Plants have generally evolved complex mechanism including both passive (preformed) and active (inducible) defense responses for protection against pathogenic agents. The active defense responses include rapid modification of existing cell wall material, and deposition of new cell wall material including lignins, callose, phenolics and hydroxyproline-rich glycoproteins. The results also clearly indicate the capability of wild species in mitigating partially the depressive effect of fungal infection on the cell wall component of the test plants which should be considerably helpful in defense and concomitant fortification of the plant via the cell wall.

In addition to the wall polysaccharides described above, H^+ATPase activity was assayed in both the cultivar and wild sesame. 50% reduction in H^+ATPase activity was observed in wild species compared to cultivar suggesting the active closure mechanism and thereby minimizing the fungal infection through stomatal pores (Table 2). The low H^+ATPase activity in cultivar species strongly supports the SEM data of fungal penetration into the leaves *via* the stomata.

All plant pathogens must invade host tissue during infection. Foliar pathogens frequently enter leaves through wounds or stomata. However, stomata are not

merely openings at the leaf surface, passively allowing pathogen access to the interior of leaves during infection. The guard cells that make up stomatal pores react dynamically to prevent pathogen entry[23]. Furthermore, the present data indicate a key role for the plasmamembrane (PM) H^+ATPase working together with plant immune signaling components to regulate stomata movements during fungal invasion[24]. The function of the PM H^+ATPase in stomatal opening is well established and has been reviewed in detail[25]. During stomatal opening, PM H^+ATPases are activated, resulting in hyperpolarization of the guard cell membrane. This event activates inward rectifying voltage-gated K^+ channels. The efflux of K^+ is accompanied by an influx of anions (Cl^- and NO_3^-) and production of osmotically active solutes from starch. Ion and solute accumulation in the cell interior lowers water potential and drives the uptake of water into guard cells, increasing turgor and widening the stomatal aperture. Inhibition of the PM H^+ATPase is also important for ABAmediated stomatal closure. Lines expressing constitutively active alleles of Arabidopsis AHA1 result in an open stomata phenotype and are completely insensitive to ABA induced stomata closure but not other stimuli[26]. This result suggests that down-regulation of PM H^+ATPase activity is an important component of the ABA mediated stomatal closure pathway. Upon perception of pathogens, guard cells of diverse plant species rapidly lose turgor, resulting in stomatal closure that prevents pathogen invasion. Importantly, plant mutants with stomata that do not close in response to fungal inoculation are more susceptible than wild-type plants[24].

Given the numerous roles of H^+ATPase.activity in plant cell physiology and induced responses to environmental stimuli, it is no wonder that pathogens have evolved mechanisms to target this enzyme. Alterations in PM H^+ATPase activity, via stimulation or inhibition, can have drastic effects on plant cell function. PM H^+ATPases generate cellular membrane potential and therefore influence the transport of numerous molecules into and out of the plant cell[27]. Manipulation of membrane potential by pathogens could result in significant nutrient accumulation in host tissue. Microbes may exploit host membrane energization to facilitate pathogen membrane transport processes in order to acquire nutrients from host tissue. Pathogen acidification of the apoplastic space could also promote plant cell wall loosening that may facilitate fungal movement in host tissue[28]. In addition, over-activation of PM H^+ATPases can lead to cell death[29].

In conclusion, the present study showed that A.sesami infection occur either through stomata and also by direct penetration. The content of hemicellulose and lignin were antagonistically increased with concomitant decrease in cellulose and pectin. PM H^+ATPases are dynamically regulated during plant immune responses. Proteomics studies suggest complex spatial and temporal modulation of PM H^+ATPase activity during early pathogen recognition events. Due to its established importance in regulating stomatal apertures, it is not surprising that the PM H^+ATPase also function in stomata-based immune responses. Understanding how these complex interactions lead to resistance or susceptibility will likely keep researchers engaged for years to come.

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