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Some Phytochemical Prosperities Affected by the Infection of Leaf Spot Disease of *Cucumis sativus* (Linnaeus) Caused by *Penicillium notatum*

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Abstract: The present investigation deal with the leaf spot diseases of *Cucumis sativus*: Infected leafs was collected around Thanjavur, Tamil Nadu, India. The isolated fungus from the infected leaves was identified as *Penicillium notatum*. In both healthy and infected leaves, Terpenoids, Steroids, Saponins, phenols were found to be present. But in infected leaves the quantities of Phenols was more. The amount of chlorophyll-a and chlorophyll-b were found to be lesser in infected leaves than those of healthy ones. Physiological constituents such as chlorophyll changed significantly ($P<0.001$) at different days after infection as compared to healthy tissue. Phenol content was found to be increased simultaneously in diseased leaf tissues in comparison to the healthy once with the increase in the period of infection. The result of peroxidase, polyphenol oxidase and total phenol were found to be higher in infected leaves compared with healthy ones.

Key words: Cucumber leaf spot · Peroxidase · *Cucumis sativus* · Polyphenol oxidase

INTRODUCTION

Cucumber *Cucumis sativus* is one of the major vegetable crops in most of the countries. In October, 1966 a new leaf spot of Ashley cucumber *C. sativus*, was observed in two locations south of Immokalee. The leaf spot disease in *C. sativus* causes browning/blackening of the leaves resulting in enormous loss to the total photosynthates of the plant followed by reduced yield of the plant. Increased activity of polyphenol oxidase and peroxidase in response to infection by the pathogen has been reported by many workers [1-4] and is considered to play an active role in contributing to disease resistance in certain plant host-pathogen interaction following infections. Higher levels of total phenols following infection with the pathogens have been reported by previous workers [5-8] where phenols may play an important role as post-infectional factors in the disease resistance.

The interaction was also expressed in terms of biochemical and morphological changes induced in the cucumber host by the Trichoderma, among which the increased growth response was the most salient, as described in earlier studies with soil [9,10]. Several hypotheses, including the control of minor pathogens [11], have been put forward to explain the effects of Trichoderma on plants. Their results of our study in an aseptic environment with Trichoderma monocultures suggest that a direct plant-fungus interaction is responsible for the increased growth response as well as other responses in the plant [12].

Extraction of cucumber leaf tissue expressing induced resistance against powdery mildew fungi revealed the presence of two new major *C*-glycosyl flavonoid products: vitexin-6-(4-hydroxy-1-ethylbenzene) (cucumerin A,) and isovitexin-8-(4-hydroxy-1-ethylbenzene) (cucumerin B,). In addition, the known *C*-glycosyl flavonoids apigenin-8-*C*-β-d-glucopyranoside (vitexin,), apigenin-6-*C*-β-d-glucopyranoside (isovitexin), luteolin-8-*C*-β-d-glucopyranoside (orientin,) and luteolin-6-*C*-β-d-glucopyranoside (isoorientin,), as well as 4-hydroxycinnamic acid (*p*-coumaric acid,) and its methyl ester (*p*-came,), were found in higher quantities within resistant plants [13]. The structures were elucidated using spectroscopic methods and unambiguously confirmed for using co-chromatography experiments with authentic standards [13]. On the basis of the results of this study and the reported biological activities of *C*-glycosyl flavonoids, these compounds would play a vital role in the defense strategy of this species by acting as phytoalexins [13].

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Effects of two biotrophic cucumber powdery mildews, *Erysiphe cichoracearum* and *Sphaerotheca fuliginea*, on photosynthetic pigment content and chlorophyll (Chl) fluorescence parameters were studied in cucumber cotyledons (*Cucumis sativus* cv. Marketer) within 35 days after inoculation. No marked changes in the followed parameters were found in case of *E. cichoracearum* so that this infection had no pronounced effect on the thylakoid function.

A decrease in F_V/F_M, slowing down of the induction kinetics of fluorescence quenching coefficients q_P and q_N and a slower recovery of F_V/F_P after photoinhibitory treatment were observed in case of *S. fuliginea*. The Chlorophyll and carotenoid content per leaf area, Chlorophyll *a/b* and Chlorophyll (a+b)/Car (x+c) ratios were slightly lower in these cotyledons [14]. The results indicated that the infection by *S. fuliginea* caused an acceleration of cotyledon senescence including a decrease in function of photosystem II, an inhibition of electron and proton transfer processes and a slower regeneration from photo inhibition [14].

The objectives of the present research were: i) to isolate and identify the causal organism of the leaf spot disease of cucumber, ii) to test the susceptibility of common cucumber cultivars grown under local conditions to infection with the disease, iii) to asses the efficacy of the qualitative phytochemical analysis and biochemical changes and iv) to estimate enzymes and total phenol content in the infected leaf *C. sativus*.

MATERIALS AND METHODS

Collection of Plant Materials and Disease Symptoms: During January 2009, a new leaf spot disease of *cucumis sativus,* (Linnaus) was observed in two locations around Thanjavur, Tamil Nadu, India (Fig. 1 and 2): The leaf flecks were of indefinite shape, yellow at first, later becoming angular with a definite outline. As the disease progressed the spots became somewhat circular with light brown centers surrounded by dark brown margins. Diameters of the individual lesions varied from 4 to 10 mm, but in many cases the lesions coalesced and produced larger irregular necrotic areas with subsequent drying and shedding of leaves. Spotting was only observed on the leaves. No petiole or fruit spotting was observed. A fungus was repeatedly isolated from the leaf spots. It fitted the description of *Corynespora* (Melonis) *Cassiicola* (Berk and Curt.) blight of sesame, *Sesamum indicum* L, Cotton, *Gossypium hirsutum*.

Fig. 1: Infected leaf

Fig. 2: Healthy leaf

Culture of Fungus from the Infected Leaf: Isolations were made by surface sterilization of leaf spots with a 3% of sodium hypochlorite prepared from 0.5% Clorox solution and plating in Czapek Dox Agar (CDA). Cultures were maintained on CDA medium at 37°C for 48-72 hours. The fungus was identified by standard methodology [12].

Qualitative Phytochemical Analysis: The healthy and infected leaves were air-dried and powdered using a Thomas-willey milling machine. The aqueous extract of each sample was prepared by soaking 100g of dried powered materials in 200ml of distilled water for 12h. The extracts were filtered using whatman filter paper No.IV. The residue thus obtained was used for phytochemical analysis.

Phytochemical Screening: Various active phytochemical constituents were investigated both in the aqueous extract and powdered samples of *cucumis sativus* using standard procedure as essentially described by Harborne and Trease [15,16].

Test for Saponin: About 2g of the powdered samples of healthy and infected leaves were boiled individually in 20ml of distilled water in a water bath and filtered. 10ml of the filtrate was mixed with 5ml of distilled water and shaken vigorously for a stable persistent froth. The froth was mixed with 3 drops of olive oil and again shaken vigorously and observed for the formation of emulsion [15, 16].

Test for Terpenoids: Five ml of each healthy and infected leaves extract were mixed in chloroform and concentrated sulphuric acid (2:3 v/v) and noted a layer formation. A reddish brown colour formation show positive results for the presence of terpenoids [15,16].

Test for Steroids: Two ml of each acetic anhydride and sulphuric acid were added to 0.5g ethanol extract of healthy and infected leaves samples. The colour changed from violet to blue or green indicating the presence of steroids [15, 16].

Biochemical Changes

Estimation of Chlorophyll: The aim of the present investigation was to study the biochemical changes in *Cucumis* leaves associated with leaf spot at different stages of diseases development. Plants were artificially inoculated by spraying with spore suspension of Penicillium *notatum*. Plants sprayed with sterilized distilled water served as control.

Sample Stage: 1-Immediately after infection
Sample Stage: 2-After two days when brown leaf spot had developed and were scattered irregularly on leaves
Sample Stage: 3-After four days when this spot had enlarged with yellowish halo
Sample Stage: 4-After six days when there was extensive leaf spot development
Sample Stage: 5-After eight days when spots had coalesced together becoming necrotic patches followed by defoliation.

Chlorophyll pigments were extracted from healthy and infected leaves in 80% acetone According to Mahadevan [17] and chlorophyll a and b were estimated using the equation of Arnon [18].

Estimation of Enzymes and Total Phenol

Extraction and Assay of Peroxidase: To extract the enzyme, 100 mg of each of infected and healthy leaf tissues were ground separately with a pinch of neutral sand in 20 ml of cold distilled water in a mortar at 0°C. The extract was obtained by filtering off the debris with a clean cloth and centrifuging at 3000 rpm for 15 minutes in a refrigerated centrifuge. The supernatants were recovered and kept in a tube in an ice bath until assayed. Peroxidase activity was estimated following the method of [19]. Briefly 5 ml of freshly prepared pyrogallol reagent (prepared by mixing 10 ml of 0.5 M pyrogallol solution and 12.5 ml of 0.66 M phosphate buffer and the volume made to 100 ml. with distilled water) and 1.5 ml of the enzyme extract were mixed in a spectrophotometer tube and the mixture was immediately adjusted to zero absorbance of a spectrophotometer. A volume of 0.5 ml of 1% H_2O_2 solution was added to it and the content was mixed by inverting the tube. The reaction was initiated by the addition of H_2O_2. Enzyme activity was recorded as the change in absorbance per minute (ΔA / mint/Δ) at 430 nm immediately after the addition of substrate. Similarly, control of non-enzymatic oxidation was maintained by heating the extract at 100°C where the activity was always measured zero indicating its complete inactivation by the heat treatment.

Extraction and Assay of Polyphenol Oxidase: Hundred mg of healthy and infected leaf tissues were homogenized separately with a pinch of sand in 6 ml. Phosphate buffer of 0.1 M at pH 7.0 at 0°C. The extract was filtered with a clean cloth, centrifuged at 3000 rpm for 15 minutes and stored in an ice-bath until used. Polyphenol oxidase activity was measured by the method of [20]. Briefly 2 ml of enzyme extract and 3 ml. of distilled water were mixed together in a spectrophotometer tube and adjusted to zero absorbance of a spectrophotometer. One ml of catechol solution (0.4 mg / ml) was added to the above mixture and the reactants were quickly mixed. The enzyme activity was measured as the change in absorbance per minute (ΔA / mint.) at 490 nm immediately after the addition of catechol solution which initiated the reaction. Control in similar manner was maintained by heating at 100°C which always showed zero absorbance.

Extraction and Estimation of Total Phenol: One gm of healthy and infected leaf tissues were cut into pieces of 1-2 cm. They were kept in 5-10 ml. Ethyl alcohol (80%) immediately and allowed to boil for 5-10 min in a hot water bath. The extract was cooled in a pan of cold water. The tissues were crushed thoroughly in a mortar and pestle for 5-10 minutes, then passed through a double-layered cloth. The ground tissue was extracted in boiling of ethyl alcohol (80%), then cooled and passed through

Whatman's No. 1 filter paper. Total phenol was estimated by the method of [19]. Briefly 1ml. of alcoholic extract was pipetted in graduated tubes. Then 1 ml. of folin-ciacalteau reagent was added followed by 2 ml. of 10% Na2CO3 solution. The tube was shaken and heated in boiling water bath for 1-2 minutes. The tube was cooled under running tap water. The blue solution was diluted to 25ml with distilled water and absorbance of it was measured at 650 nm in spectrophotometer. A control containing all the reagent except plant extract was used to adjust the absorbance at zero.

RESULTS AND DISCUSSIONS

Identification of the Fungus: The isolated fungus was identified as *Penicillium notatum* according to morphological characters described by [12].

Qualitative (Phytochemical) Analysis

Saponins: Many saponins exhibit potent antifungal activity and are often present in relatively high levels in healthy plants; these molecules have been implicated as determinants of a plants resistance to fungal attack [21]. A number of other properties are also associated with these compounds, including pesticidal, insecticidal and molluscicidal activity: allelopathic action; and antinutritional effects [22, 23].

Saponins are glycosylated compounds that are widely distributed in the plant kingdom and can be divided into three major groups, depending on the structure of the aglycone, which may be a triterpenoid, a steroid, or a steroidal glycoalkaloid. Triterpenoid saponnins are found primarily in dicotyledonous plants but also in some monocots, whereas steroid saponins occur mainly in monocots, such as the liliaceae, dioscoraceae and agavaceae and in certain dicots, such as fosglove, which contains the saponin digitonin [23]. Oats (Genus Avena) are unusual because they contain both triterpenoid and steroid saponins [24]. Steroidal glycoalkaloids are found primarily in members of the family solanaceae, which includes potato and tomato, but also in the liliaceae [23]. The saponins produced by oats and tomato have been studied in the greatest detail in relation to their potential role in the defense of plants against phytopathogenic fungi [21].

Table 1: Results for qualitative analysis of healthy and disease leaf

S.No	Phytochemical test	Normal leaf	Diseased leaf
1	Saponin	+	+
2	Steroids	+	+
3	Terpinoids	+	+
4	Phenolic compounds	+	++

Table 2: Changes in chlorophyll content (μg g^{-1} fresh weight of C. sativus infected leaf P. notatum at different days of infection)

	Chlorophyll-a		Chlorophyll-b	
Days after infection	Healthy	Infected	Healthy	Infected
0	1104 ±1.7X*	1100 ± 1.7X	405±1.3 X	405±1.3Y
2	1103±1.7X	904 ± 2.9 Y	409± 1.1X	343 ± 1.7Y
4	1102±1.7X	708 ± 3.1 Y	405±1.2 X	281 ± 1.2Y
6	1100±1.7X	363±3.1 Y	408±1.1 X	211±1.7Y
8	1103±1.7X	223±2.1 Y	409± 2.3X	131 ±1.4Y

* Data are averages and standard errors of five replicates different letter denote a significant difference (P<0.001) between healthy and infected leaves

Table 3: Peroxidase and polyphenol oxidase activity in healthy and *P. notatum* infected leaves of *C. sativus* at different periods of infection

	Peroxidase activity g^{-1} min^{-1}		Polyphenol oxidase g^{-1} min^{-1}	
Days after infection	Healthy	Infected	Healthy	Infected
O days	0.039±0.000	0.041±0.002	0.035±0.001	0.037±0.002
7 days	0.061±0.002	0.220±0.006	0.047±0.001	0.247±0.013
14 days	0.051±0.001	0.281±0.002	0.054±0.001	0.328±0.011
21 days	0.045±0.002	0.229±0.011	0.054±0.001	0.459±0.016

*For Peroxidase: CD at 5% = 0.073, For Polyphenol oxidase: CD at 5% = 0.103, SEM = + 0.025, SEM = + 0.036 * Data are mean values of five replicates

In both healthy and infected leaves, Terpenoids, Steroids, Saponins, phenols were found to be present. But in infected leaves the quantities of Phenols was more (Table 1).

Biochemical Changes

Estimation of Chlorophyll: Chlorophyll content was found to be decreased in infected leaves with the progressive of disease. However, the decrease in chlorophyll-*a* was more pronounced then chlorophyll-b at different stage of infection. Concentration of physiological constituent such as chlorophyll changed significantly ($P<0.001$) at different days after infection as compared to control tissue (Table 2).

When a foliar pathogen establishes infection inside host tissues, the chlorophyll content is usually decreased. This is accompanied by yellowing of the infected leaf [25].

The chlorophyll pigments in leaves decreased significantly due to infection of *Penicillium notatum* and continued with the progress disease. Various plants pathogens are known to produce toxic metabolites, which may destroy the chloroplast resulting into decrease of chlorophyll pigments [26 and 27]. The decrease in chlorophyll pigments due to foliar infection has been reported in many plants-fungus interaction [28].

Estimation of Enzymes and Total Phenol: The results revealed that the activity of both the phenol oxidizing peroxidase and polyphenol oxidase was higher in infected leaf tissues than in uninfected ones and that it increased considerably with the increase in progression of infection (Table 3). Phenol content was found to be increased simultaneously in diseased leaf tissues in comparison to the healthy tissues with increase in the period of infection. Increase in the activity of peroxidase and polyphenol oxidase in host tissues in response to infection by the pathogen has been reported in many cases [12, 29, 30]. Increased peroxidase activity upon infection might be required for an additional deposition of lignin around the lesion court induced by pathogen. Peroxidase is a key enzyme in the biosynthesis of lignin and other oxidized phenols [31]. Peroxidase and polyphenol oxidase mediate the oxidation of phenols and oxidized phenols are highly toxic to the pathogen [32]. PO and PPO catalyse the oxidation of phenolic compounds through a PPO-PO-H2O2 system [33]. A number of studies have found a correlation between PPO and the resistance response [34]. PO itself was also found to inhibit the spore germination and mycelial growth of certain fungi [35]. Peroxidase may be rapidly involved in the peroxidation of substrate molecule, leading to the accumulation of highly toxic compounds (*i.e* phenolic compounds), which may contribute to resistance via their antifungal potential [36]. The role of phenol oxidases in resistance is based on the observations that the activity of these enzymes is increased in infected tissues and that the oxidized phenols *i.e* quinones are more reactive and more toxic to microorganisms compared to their non-oxidized form [37].

Total phenols increased in infected plant than the healthy ones and it is well known that phenolic compounds are fungitoxic. Moreover, they increase the physical and mechanical strength of the host cell wall and thus inhibit fungal invasion. Therefore, from the present observation, the greater activity of PO and PPO, along with higher amount of total phenols enhancing host resistance is in compliance with the previous report.

Confirmation of the results obtained on the efficacy of tested fungicides should be carried out on whole plants under field conditions before being recommended to be applied by the farmers and extension agents. Also, once the results are confirmed at field level, integration of treatments with fungicidal spray and suitable chemicals which control disease were identified. Terramycine. Dithane M-45, Daconil-2787 were found to be suitable and basic Copper sulfate was found to be ineffective. Saponins, Steroids, Terpenoids were found to be present in the cells of both control and infected leaf. The amount of chlorophyll-a and chlorophyll-b were found to be both the cells of the leaves but the amount of the chlorophyll a and b were decreased in infected leaf because of the necrosis of the leaf due to leaf spot disease. The presence of enzymes peroxidase and polyphenoloxidase and total phenol were estimated and found to be high in infected leaves only and increased considerably with increase of progression of infection because they increase physical and mechanical strength of the host cell wall and inhibits fungal invasion.

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