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CHANGES IN PIGMENT COMPOSITION, ACID METABOLISM, ETC. IN *PEDILANTHUS TITHYMALOIDES* LEAF FOLLOWING POWDERY MILDEW INFECTION

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Abstract: The development of powdery mildew in the leaf of *Pedilanthus tithymaloides* var. *caculatus* by *Oidium mangiferae* and associated changes in the host were studied. The disease development occurred through a series of distinct changes in symptoms. Initial whitish, powdery patches of mildew infections became gradually colonized by a mycoparasite, *Ampelomyces quisqualis* (*Syn. Cicinnobolus*) giving the affected areas a greyish appearance. Further advancement of the disease rendered the tissue chlorotic which subsequently transformed into a bright pinkish-red colour. Towards the end of the dry season, certain infected areas became corky and suberized. Higher rainfall reduced both the intensity of powdery mildew and the growth of the mycoparasite. Typical powdery mildew symptoms were induced by artificial inoculation of young leaves with conidia of *O. mangiferae*. A significant alteration of leaf pigment composition occurred when the infected areas turned to a pinkish-red colour. These areas lacked chlorophyll-a but contained other photosynthetic pigments plus several additional anthocyanins that were absent in healthy tissues. The healthy leaves of *P. tithymaloides* showed a clear pattern of diurnal fluctuation of acidity and stomatal aperture on a scale similar to a known CAM plant, *Kalanchoe sp*. The major acid was found to be malic. The infection by *O. mangiferae*, however, reduced the morning acidity in the leaf by about 35% and altered the normal acid fluctuation pattern. The overall results indicated that powdery mildew infection induces a range of disturbances to leaf metabolism, the most obvious being the activation of synthesis of new anthocyanins, degradation of chlorophyll-a and disturbance to malic acid metabolism.

Key words: *Ampelomyces quisqualis, Oidium mangiferae, Pedilanthus tithymaloides* powdery mildew.

INTRODUCTION

Powdery mildews are probably the most common, conspicuous, widespread and easily recognizable plant diseases. Species of *Oidium, Erysiphe,*[14] *Sphaerotheca,*[4] *Uncinula,*[1] *Microsphaera*[8] and *Laveillula* are among the major causes of powdery mildews. Numerous reports indicate that the infection by powdery mildew fungi alters host physiology.[6] For example, alteration of fructose-2,6-biphosphate content and sucrose to starch ratio in leaf of wheat plants actively forming grains,[13] CO_2 metabolism in young rye plants, polyamine levels and their biosynthetic enzyme activities[17] have been reported. The powdery mildew infected areas are generally characterized by

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[1] We sadly record the untimely demise of Ganga Mailewa prior to preparation of this manuscript.

reduced photosynthesis and increased respiration. When the fungus sporulates it makes the highest demand for food. A reduction in net photosynthesis occurred in green islands and surrounding senescing tissue of detached barley leaves infected with *Erysiphe graminis* f.sp *hordei*.[3] A significant increase in peroxidase activity was observed in seedlings of susceptible wheat cultivar artificially inoculated with *E. graminis* f. sp. *tritici*.[18]

Powdery mildew is most common in the ornamental hedge plant, *Pedilanthus tithymaloides*, in Sri Lanka. However, no previous records could be traced of this disease in Sri Lanka. Powdery mildew infection induces striking changes in the colour of *P. tithymaloides* leaves and this paper reports changes of pigment composition and acid levels etc. following powdery mildew infection.

METHODS AND MATERIALS

Examination of diseased leaves: Diseased leaves of *P. tithymaloides* were collected from plants growing in hedges within the Peradeniya University Campus and brought to the Botany Department Laboratory for examination.

Symptom development study: Young and mature leaves showing different stages (stages 1, 2 & 3) of disease development were examined and visual symptoms were recorded. The sequence of symptom progression was followed from the beginning to the advanced stage of infection.

Microscopic observation study: Scrapings and transverse sections taken from diseased regions at different stages of disease development were mounted in lactophenol/ cotton blue and observed under a light microscope. The length and width of 100 randomly selected conidia was measured using a stage micrometer attached to the eyepiece of a light microscope. Towards the end of the dry season, the lower surface of the diseased areas of certain leaves were transformed into light brown, corky thickenings. To test for suberih, transverse sections taken from these areas were immersed in drops of concentrated potassium hydroxide on glass slides for 4 hrs. The slides were then warmed before observation under the light microscope.[14]

Disease development in relation to rainfall: The disease in naturally infected plants was also monitored weekly during 1993 - 96. The rainfall data for the above months were obtained from the Horticultural Research and Development Institute, Sri Lanka Department of Agriculture at Gannoruwa, Peradeniya.

Artificial inoculation study: For artificial inoculation studies, healthy leaves were selected from *P. tithymaloides* plants grown in the greenhouse at 28-30°C. Conidia scraped from freshly infected leaves were applied onto the upper leaf surface using a fine paint brush. The inoculated leaves were covered with polyethylene bags for

the first 24 hours and which were then removed. Inoculated leaves were examined regularly and the period of time taken for different stages of symptom development was noted.

Paper chromatographic separation of chlorophyll and anthocyanin pigments: Powdery mildew-infected leaves showing red pigmentation underneath and healthy leaves of corresponding age were collected separately. Ten grams of tissue from each were immersed separately in 10ml volumes of acetone in reagent bottles overnight. The acetone extracts were condensed by evaporation in a water bath at 50°C. The concentrated extracts were taken up in 1 ml acetone. Aliquots (0.1ml) were spotted on chromatography paper (Whatman No. 2) and the chromatographs were developed in petroleum ether: benzene (8:2). The presence or absence of chlorophyll-a, chlorophyll-b, carotene and xanthophyll in the two extracts was recorded.

For extraction and separation of other pigments, diseased and healthy leaves (1.22g), collected as described previously, were kept immersed separately in 20ml portions of 1% methanolic hydrochloric acid for 3 days. The extracts were partitioned by adding 20 ml ether in a separating funnel followed by distilled water. The solvents were shaken thoroughly and allowed to settle. The aqueous layer was separated out and washed with petroleum ether and benzene. A small portion of each aqueous extract was tested separately by adding a few drops of Na_2CO_3 and then dilute HCl for colour changes. The extracts were evaporated to dryness in a rotary evaporator at 37°C. The residue was dissolved in 1.2 ml of methanol and 0.1 ml was chromatographed on paper (Whatman No. 2) using butanol: acetic acid: water (4:1:2) and also acetic acid: HCl: water (5:1:5) as solvent systems.

Diurnal changes in titratable acidity: Diseased and healthy leaves of *P. tithymaloides* were collected separately at 2 hourly intervals commencing at 8.00 a.m. and ending with a sampling at 6.00 a.m. the following day, into small pieces and immediately stored in a freezer (-20°C). Frozen leaves (15 g) were separately extracted in 200 ml of boiling water for 10 minutes, until the extracts had turned into a brown to brownish-green colour. The extracts were filtered and diluted to 300 ml by adding distilled water. Aliquots (10 ml) were pipetted into 100 ml conical flasks and titrated against 0.04N NaOH using phenolphthalein as indicator. Four replicates from each extract were titrated and the titratable acidity was calculated by using the formula,

$$\text{Titratable acidity} = \frac{\text{Molarity of NaOH X Average volume of NaOH expended}}{\text{Volume of extract (10ml)}}$$

In order to identify the acids present, the aqueous extracts (20 ml) of leaf collected at 6 a.m. were concentrated by freeze drying (Edwards Modulyo, U.K.) at -60°C for 72 hours. The residue was divided into two and one portion was dissolved in 40 µl distilled water. Aliquots (10 µl) were chromatographed on four Thin Layer Chromatography (TLC) plates (20x20 cm^2, 0.5mm thick, Kieselgel 254 PF60 MERCK)

using solvent systems, methanol:water (1:1), methanol:water (1:2) methanol:water (2:1) and methanol:water (2:3). The plates were air-dried and held under a UV lamp (wave length 254) to observe fluorescent regions.

The remaining residue was dissolved in 30 µl of methanol and aliquots (10µl) were spotted on three TLC plates. The plates were developed using solvent systems, dichloromethane:methanol (4:1), dichloromethane:methanol (9:1). The plates were air-dried and compounds were visualized by spraying one plate each with the following reagents: 5ml of anisaldehyde in ethanol, 1% 2,6 dichlorophenol indophenol sodium salt and I_2 vapour (sublimation of I_2 crystals).

Diurnal changes in the size of stomatal aperture in healthy leaves: Epidermal peel segments were removed from healthy leaves collected at 2 hourly intervals commencing at 8.00 a.m. and ending with a sampling at 6.00 a.m. the following day and fixed in absolute alcohol in sample tubes. The peel segments were stained using Heath's reagent (I_2/KI in phenol). The width of stomatal aperture was measured in 25 randomly selected stomata using a micrometer eyepiece and average width calculated.

RESULTS

Disease symptoms

Three distinct stages could be recognized in the development of powdery mildew in *P. tithymaloides*. The disease first appeared on newly expanding, young leaves, stems and young twigs. Initial symptoms were the appearance of whitish, circular (0.2 - 0.6 mm diameter), raised powdery patches on both the upper and lower leaf surfaces and on one side along the stem and the young twigs. These spots gradually enlarged and became irregular as the disease progressed (stage 1).

Most of the whitish, irregular areas turned grey about a week later and the powdery appearance of the infected areas in most leaves disappeared gradually (stage 2) although in some leaves, the whitish powdery appearance remained longer. Where the mildew patches are found on the lower leaf surface, the corresponding upper surface turned a diffused yellow colour. With the advancement of the disease (stage 3), most of the infected areas turned yellow. The yellow areas on the upper surface of young and mature leaves then turned into a bright pinkish red colour. The corresponding lower surfaces either remained as blackish/greyish mildew areas or developed into raised, light brown, irregular, corky thickenings. Occasionally, the infected areas showed necrosis. Most of the leaves remained attached to the plant for a long period in spite of infection but severely infected leaves dropped. The substance associated with thickened areas first turned yellow on reaction with concentrated KOH and then dark yellow following heating.

Microscopic observation of diseased leaves

Microscopic examinations of scrapings taken from whitish powdery patches revealed the presence of *Oidium sp.* From the whitish surface mycelium, erect, un-branched and septate conidiophores arose. Average size of conidia, 38.43 ± 3.34 µm (length) 21.71 ± 3.24 µm (width), was compared with other recorded species of *Oidium*[9] and found to fit exactly with *O. mangiferae.*

Transverse sections taken from greyish spots were examined under the microscope. In addition to the mycelium of *Oidium*, large, spherical and brown coloured pycnidia (length 55.49 µm, width 35.80 µm) of the mycoparasite, *Ampelomyces quisqualis* (*Syn. Cicinnobolus sp.*), were found within the enlarged base of conidiophores of *Oidium sp.* Pycnidia of *A. quisqualis* were partially immersed, spherical, brown coloured, occurred at different development stages and contained numerous small (length 8.09 µ, width 4.65 µ), hyaline, thin-walled conidia. The enlarged base of conidiophore of *Oidium sp.* represented the initial stage of colonization by *A. quisqualis.* The mycoparasite was located at the base of the interior conidiophore of *Oidium sp.* which was still attached to the mycelium. In the greyish black regions of infected leaf, only the pycnidia of *A. quisqualis* and the enlarged base of conidiophore of *Oidium sp.* were seen.

Disease development in relation to rainfall

Fresh infections mostly appeared during July and August as whitish, powdery and isolated patches (Fig.1). The disease was less intense with increasing rainfall, particularly during October and November which received the highest rainfall. Greater incidence of disease was found during December and January when cold and dry weather conditions prevailed (Fig. 1).

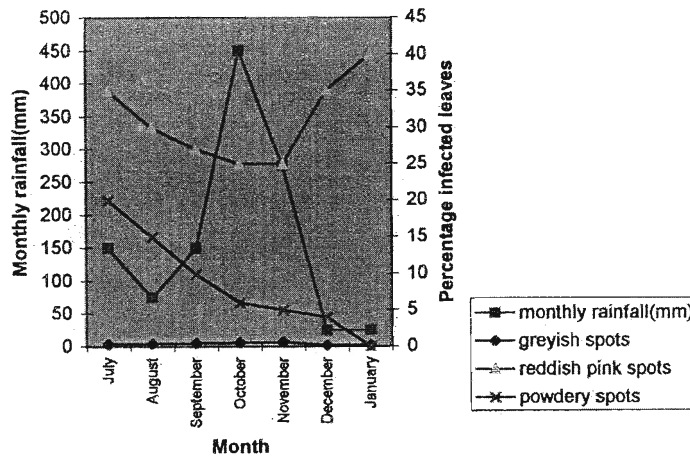

Figure 1: Disease symptoms in relation to rainfall

Artificial inoculation study

First symptoms appeared on artificially inoculated leaves 2-3 days after inoculation which in another 2-3 days progressed into whitish, powdery areas. The yellowing could be seen on the corresponding upper surface 10-11 days after inoculation. Some inoculated leaves turned completely yellow within 16-18 days. Other yellowish areas developed greyish black mycelium with further development of the disease. Inoculated areas did not produce brown corky thickenings as on naturally infected leaf. Most of the leaves which turned yellow, dropped about 3 weeks after inoculation.

Pigment composition of diseased tissue

Paper chromatography of leaf extracts showed the absence of a green band corresponding to chlorophyll-a in the diseased tissues whereas the healthy extract had bands corresponding to chlorophyll-a, chlorophyll-b, carotene and xanthophyll. The number of yellow/orange bands was similar in both chromatograms.

Table 1: The pigments visible in the chromatograms of extracts from infected and healthy plants and colour reaction after exposure of chromatograms to NH_3.

Solvent system	Rf & the colour of pigment		Colour after exposure to NH_3	
	Infected	Healthy	Infected	Healthy
Butanol: acetic	0.90 (brown)	0.90 (brown)	Dark brown	Dark brown
acid: water	0.80 (yellow)	-	Dark yellow	-
	0.65 (pink)	-	Blue	-
	0.42 (brown)	-	Dark yellow	-
2N HCl	0. 90 (Yellow)	0.90 (Yellow)	Yellow	Yellow
	0.25 (pink)	-	Blue	-
Acetic acid:	0.90 (brown)	0.90(Brown)	Dark brown	Dark yellow
HCl: Water	0.80 (yellow)	0.80 (Yellow)	Dark yellow	Yellow
	0.64 (pink)	-	Blue	-
	0.58 (yellow)	-	Dark yellow	-
	0.50 (pink)	-	Blue	-

Paper chromatographic separation of anthocyanin pigments using different solvent systems indicated the presence of at least 3 additional pigment bands in the infected tissue extracts compared to healthy leaves (Table 1). When the

chromatogram was exposed to ammonia fumes, the pink bands turned blue and the other bands became darker. Further, the aqueous extracts of infected tissue when treated with dilute Na_2CO_3 gave a green colour which turned into red when drops of dilute HCl were added.

However, such a change was not observed with extracts from healthy leaf tissue. When the aqueous extracts of infected and non-infected areas were treated separately with Na_2CO_3, the extracts taken from infected leaves turned green. The addition of HCl to aqueous extracts of infected areas resulted in a red colour. Such a change was not observed with extracts from healthy tissues.

Titratable acid content and stomatal width

The healthy leaf of *P. tithymaloides* showed a clear fluctuation in acid levels during the course of the day. The acid level was highest in the morning and dropped to its lowest level during the evening (Tables 2 & 3). The pattern of diurnal fluctuation of acidity in the healthy leaf of *P. tithymaloides* was similar to that of the leaf of *Kalanchoe*, a plant well known for Crassulacean Acid Metabolism (CAM) (Table 3). The titratable acid content was about 35% less in the extract of diseased leaves collected in the morning than that of healthy leaves, which increased slightly during the mid-day and declined slowly towards the evening (6 p.m). A fluctuation of acid levels was not observed in the diseased leaves (Fig. 2). The stomatal opening in *P. tithymaloides* followed a pattern similar to that of acidity where the stomata opened early morning and closed in the evening (Fig.3).

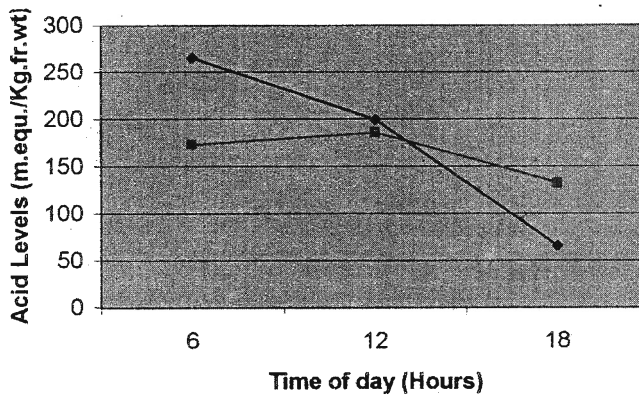

Figure 2: Titratable acidity of healthy (■) and diseased (◆) tissue of *Pedilanthus*.

Thin Layer Chromatography of extracts taken from *P. tithymaloides* leaf when sprayed with 1% 2,6 dichlorophenol indophenol sodium salt gave a pink band on a blue background at Rf 0.46 for the leaf extract and at Rf 0.51 for a sample of standard malate.

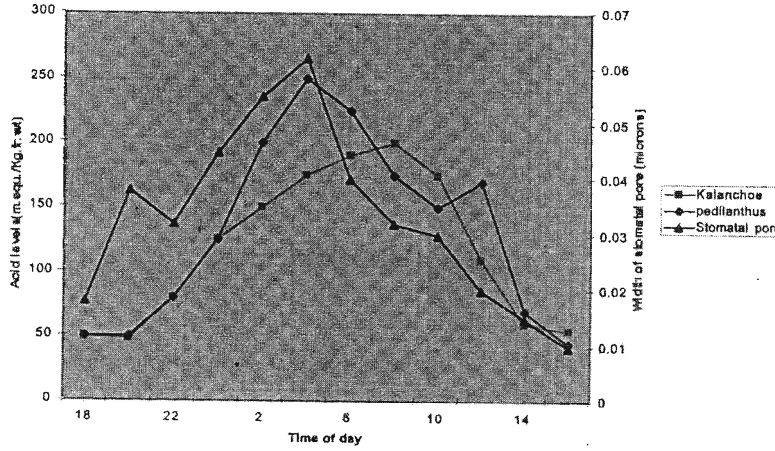

Figure 3: Diurnal change in stomatal aperture (▲) and acid levels of leaf of *Pedilanthus* (◆) and *Kalanchoe* (■).

DISCUSSION

During the period 1993 -1996, the development of powdery mildew and associated metabolic changes in the host were investigated in *P. tithymaloides* var. *caculatus*. The causal organism was similar to *Oidium mangiferae* identified based on its morphology and size of conidia. After sometime, the initial whitish, powdery spots turned into a greyish black due to the colonization by a mycoparasite, *Ampelomyces quisqualis* (*Syn. Cicinnobolus*). *A. quisqualis* is a naturally occurring pycnidial hyperparasite of powdery mildew.[5,15] It produces brown coloured spherical pycnidia within the enlarged base of the conidiophore. *A. quisqualis* is commonly found within the mycelium, conidiophore, conidia and ascospores of several species of Erysiphaceae, including *Uncinula necator* (causing the powdery mildew of grapevine), *Erysiphe cichoracearum*, *Podosphaera leucotricha* (in apple), *Sphaerotheca pannosa* (in rose).[5] After infection occurs, the mycoparasite ramifies within the host hyphae reducing growth, and eventually causing death of the mildew colony.[5] The pycnidia of the fungus produce large numbers of small pycnidiophores which are released by the breaking of the pycnidial wall after water absorption. An isolate of *A. quisqualis* has been developed into a biocontrol agent and found to be effective against powdery mildew in various crops.[15]

The artificial inoculation of *P. tithymaloides* leaves with conidia of the fungus resulted in typical powdery mildew symptoms within 2-3 days and the symptom progression was observed to be similar to those in natural infections. Mildew growth also appeared on the lower surface of leaves, 4-5 days after inoculation. The artificially inoculated leaves turned a yellow colour. However, no reddish pink colouration, normally observed in natural infections, was observed. Higher rainfall reduced the intensity of powdery mildew and colonization by *A. quisqualis*. Histochemical tests

indicated the presence of suberized tissues in the thickened, light brown areas that appear at a subsequent stage of powdery mildew development.

Natural infections of *P. tithymaloides* by *O. mangiferae* led to pinkish red colouration. Three large families of pigments give colour to plants- chlorophylls, carotenoids and flavonoids. Anthocyanins are responsible for most pink, red, mauve and blue colours in higher plants. The number of variations in these colours cannot be explained by the colours of the few known anthocyanins. The extraction followed by paper chromatography revealed that chlorophyll-a was absent in the infected tissue. However, there was no difference observed in the other photosynthetic pigments. Degradation of chlorophyll is a common symptom of leaves following infection by fungal pathogens.

The typical blue-red colour changes exhibited by the aqueous extracts taken from the pigmented areas of infected leaves following addition of acid or base indicated the presence of anthocyanin pigments. Other flavonoid pigments such as flavonols, flavones, chalcones and aurones, etc. do not change their colour in an acidic medium. Similar extracts from healthy tissues did not give such a colour change. Subsequent paper chromatography of extracts confirmed the presence of anthocyanins and certain other unidentified pigments in diseased tissues. Anthocyanins are easily identified by pink to red bands on chromatograms which change into blue following exposure to NH_3 fumes. The colour of anthocyanin pigments may vary due to hydroxylation, pattern of ring structure, co-pigmentation, pH of the cell sap, etc.[10] Anthocyanins form co-pigment complexes with flavonoids and other compounds at pHs ranging from 2-5.[2] The degree of co-pigmentation was a function of the concentration of anthocyanins and molar ratio of co-pigments to anthocyanins.[2] Since *P. tithymaloides* exhibits certain features of a CAM plant , the changes in the cell sap pH could facilitate co-pigmentation. The intensity of chlorophyll bands in the chromatograms was less in the diseased tissue extract. This degradation of chlorophyll with the development of disease may have caused the reddish pink pigments to be more prominent in these areas. Anthocyanin synthesis is believed to share a common pathway from the primary metabolic precursor, phenylalanine. Phenylalanine ammonia-lyase (PAL) (EC 4.3.1.5), the first enzyme in the pathway, has a pivotal role in directing secondary metabolites.[11]

A clear diurnal fluctuation of acid content was found in healthy tissues and this was comparable to the acid fluctuation in the leaf of *Kalanchoe* sp., a well known CAM plant. The acidity in *P. tithymaloides* was shown to be due to high levels of malic acid. The leaf stomata opened during the night and closed during the day. The results indicate that infection by *O. mangiferae* disturbs the CAM pathway. The CAM mechanism enables plants to maximize their water use efficiency by opening their stomata during the cool desert night and by closing them during the hot dry day.[16] The titratable acid level of tissues infected with the pathogen was lower in the morning than that of healthy areas showing that the powdery mildew

infection disturbs acid metabolism. The altered chlorophyll pigment composition in the infected areas would have a negative effect on the photosynthetic rate in the *P. tithymaloides* leaf. This means both the breakdown of malic acid and CO_2 fixation have been affected by the powdery mildew infection. Fungal infections that induce chlorosis in foliage invariably result in drastic reduction of photosynthetic rate. Powdery mildew infection reduces the efficiency of CO_2 fixation and light utilization of sugar beet leaves.[7] However, disturbance to CAM pathway caused by fungal infections has not been reported previously.

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