EARLY DETECTION OF LEAF SPOT DISEASE IN *TINOSPORA CORDIFOLIA* THROUGH CHLOROPHYLL FLUORESCENCE OJIP ANALYSIS

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Abstract. The aim of the present work was to study the impact of fungal pathogen *Phoma putaminum* Speg. on various photosynthetic parameters of tissues adjacent to the infection sites on leaves of *Tinospora cordifolia* which visually appear healthy. Changes in chlorophyll fluorescence data, specific and phenomenological fluxes, density of active reaction centers and maximal photochemical activity of PSII (*F*v/*F*m) indicate that toxins produced from *P. putaminum* impair photosynthesis before the appearance of visual symptoms of infection. The chlorophyll fluorescence OJIP analysis (The OJIP or JIP-test name comes from the specific points on the induction curve that is formed by the recorded chlorophyll fluorescence signals) revealed that changes of the photosynthetic apparatus at an early stage can be used as measurable markers for detection of leaf spot disease in *T. cordifolia*.

Key words: *Tinospora cordifolia*, leaf spot disease, chlorophyll florescence, JIP-test.

INTRODUCTION

Plants are exposed to numerous biotic stresses, caused by other living organisms, which finally inhibit photosynthesis by altering primary photochemistry, electron transport, Calvin-Benson-Bassham cycle, gas exchange and photosynthetic leaf area. It has been estimated that pathogens (virus, bacteria or fungi) cause on average 15% reduction in crop plants [10]. Fungal pathogens of plants are often well established in the host long before disease symptoms appeared. There is evidence that the fungal disease symptoms in plants result mainly from the effects of toxins produced by the fungus [19, 21]. *Tinospora cordifolia* (*Thunb*.) *Miers* (*Menispermaceae*), widespread in the tropical Asian countries like India, Sri Lanka and Bangladesh, is an important medicinal climber, which is widely used in folk and Ayurvedic systems of medicine to treat various ailments [15] due to its antidiabetic, anticancer, antiinflammatory, antimalarial,

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antimicrobial, antispasmodic, antiulcer, free-radical scavenging, hepato-protective, immune-stimulating and radio-protective activities [6, 9, 16]. Recent report [8] shows that *T. cordifolia* ameliorates anxiety-like behavior and improves cognitive functions in acute sleep deprived rats. Unfortunately, the growth and productivity of the plant is severely infected by leaf spot disease caused by a fungal pathogen *P. putaminum* [14]. The disease is characterized by initial appearance of small, yellow spots that turned into dark brown-to-black lesions surrounded by a yellow halo (Fig. 1).

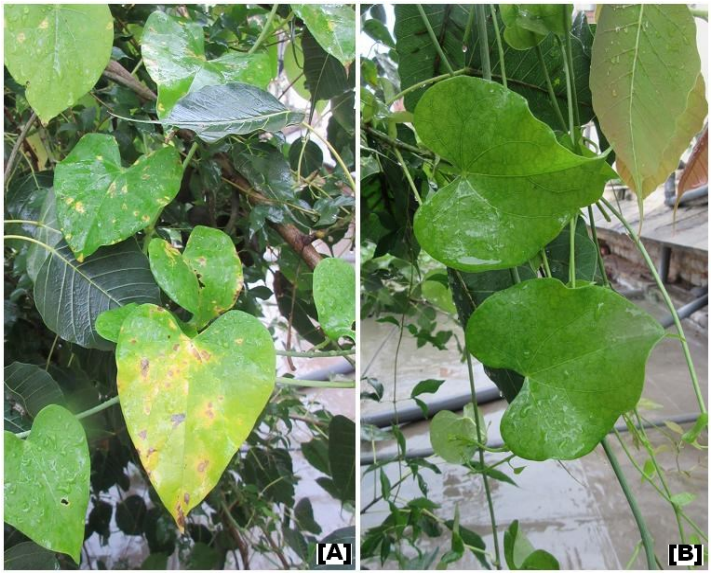

Fig. 1. (A) Infected and (B) healthy leaves of *T. cordifolia*.

Photosynthesis is a major physiological process of plants affected by various kinds of biotic stresses caused by a multitude of pathogens like fungi, viruses and bacteria [5]. Chlorophyll *a* fluorescence imaging is a non-invasive, non-destructive technique which has also been used to examine the impact of various biotic stresses on the photosynthetic activity of host plants [3, 18]. There is evidence that the disease symptoms result mainly from the effects of toxins produced by the fungus [19, 21]. The evaluation of the photosynthetic performance of plants infected by pathogens provides an opportunity to understand the mechanisms of pathogenesis, and gives novel strategies to alleviate diseases [7, 11, 12, 13]. Therefore, in the present investigations; efforts were done to study the impact of fungal pathogen *P. putaminum* on various photosynthetic parameters of adjacent tissues which visually appear healthy and to understand physiological basis of resistance against fungal infection in *T. cordifolia.*

MATERIALS AND METHODS

PLANT MATERIALS AND MEASUREMENT OF POLYPHASIC CHLOROPHYLL FLUORESCENCE KINETICS

Infected and healthy leaves of *T. cordifolia* were collected during rainy season from University College of Science Campus, Udaipur (India) and were kept in the dark for one hour (Fig. 2 A). Chlorophyll *a* fluorescence OJIP transients were recorded in green tissues of dark-adapted leaves with a Plant Efficiency Analyzer, PEA (Hansatech Instruments, Kings Lynn, Norfolk, U.K.). Fluorescence transients were induced over a leaf area of 4 mm diameter by a red light (peak at 650 nm) of 3000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (sufficient excitation intensity to ensure closure of all PSII RCs to obtain a true fluorescence intensity of F_m) provided by a high intensity LED array of three light emitting diodes. A total measuring time of one second was used throughout the experiments.

JIP-TEST

The Chlorophyll *a* fluorescence transient OJIP was analyzed according to the JIP-test [17]. The fluorescence intensities determined at 50, 100 and 300 μs ($F_{50\mu s}$, $F_{100\mu s}$ and $F_{300\mu s}$, respectively), 2 and 30 ms ($F_{2ms} = F_J$ and $F_{30ms} = F_I$) and at F_m (maximum fluorescence) were used to calculate the JIP-test parameters [17]. The intensity measured at 50 μs was considered to be the initial fluorescence (F_0). The Chl *a* fluorescence transient was analyzed by the JIP-test using Biolyzer software (Laboratory of Bioenergetics, University of Geneva, Switzerland). The extracted and technical parameters, specific energy fluxes (per reaction center), phenomenological energy fluxes (per cross section), quantum efficiencies or flux ratios, density of reaction centers and performance indexes were calculated by using the equations of JIP-test (Table 1).

Table 1

Description of the chlorophyll fluorescence parameters used in the text
Data extracted from the recorded fluorescence transient OJIP

$F_0 \cong F_{50\mu s}$	minimal fluorescence, when all PS II reaction centers are open (at $t = 0$)
$F_m = F_P$	maximal fluorescence, when all PS II reaction centers are closed
$F_{100\mu s}$	fluorescence at 100 μs
$F_{300\mu s}$	fluorescence at 300 μs
$F_J \equiv F_{2ms}$	fluorescence at the J-step (2 ms) of OJIP

Table 1

(*continued*)

Quantum efficiencies or flux ratios

$\Phi_{Po} = TR_0/ABS = F_V/F_m$	Maximum quantum yield for primary photochemistry (at $t = 0$)
$\psi_0 \equiv ET_0/TR_0 = (1 - V_J)$	probability (at time 0) that a trapped exciton moves an electron into the electron transport chain beyond Q_A^-

Specific energy fluxes (per Q_A-reducing PSII reaction center – *RC*)

$ABS/RC = M_0 \cdot (1/V_J) \cdot (1/\Phi_{Po})$	absorption flux per reaction center
$TR/RC = M \cdot (1/V_J)$	trapped energy flux per reaction center
$ET/RC = M \cdot (1/V_J) \cdot \psi_0$	electron transport flux per reaction center
$DI/RC = (ABS/RC) - (TR/RC)$	dissipated energy flux per reaction center

Phenomenological energy fluxes (per excited cross section – *CS*)

ABS/*CS*	absorption flux per cross section
$TR/CS = \Phi_{Po} \cdot (ABS/CS)$	trapped energy flux per cross section
$ET/CS = \Phi_{Po} \cdot \psi_0 \cdot (ABS/CS)$	electron transport flux per cross section
$DI/CS = (ABS/CS) - (TR/CS)$	dissipated energy flux per cross section

Density of reaction centers

$RC/CS = \Phi_{Po} \ (V_J/M_0) \cdot ABS/CS$	density of reaction centers (Q_A–reducing PSII reaction centers)

EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS

For each experimental category, five leaves of each plant were measured. Three independent experiments have been performed. The average fluorescence values were evaluated using Handy PEA software version 1.20 (Hansatech, Kings Lynn, Norfolk, U.K.).

RESULTS AND DISCUSSION

The use of non-destructive imaging methods holds great promise for early, efficient and objective detection of plant responses to various stresses [1, 2]. Based on the physiology of photosynthesis, chlorophyll fluorescence imaging allowed detection of the initial phase of tissue damage. Infection may lead to a complete inhibition of the metabolic activity, including a pronounced disturbance of photosynthetic performance. This can be easily identified by a rapid decline in the photochemical efficiency in the infected leaves, even before visible chlorophyll degradation occurs. In the present study, the values of F_0 were found constant in all healthy as well as infected leaves (Fig. 2 B). Constant value of F_0 shows that pathogenic fungi *P. putaminum* could not alter the composition of LHCII in

healthy tissues of *T. cordifolia*. On the other hand, increased F_0 value, due to the dissociation of LHCII from reaction centers was observed in response to heat pulses [20]. This result indicates that *T. cordifolia* has a more effective protective system against damages to pigments caused by the fungal infection. Low *F*m values indicate the accumulation of inactive PSII reaction centers in infected leaves of *T. cordifolia*. All phenomenological fluxes *i.e. ABS*/*CS*, *TR*/*CS* and *ET*/*CS* declined with increasing severity of infection (Fig. 3 A, B). Decrease in phenomenological fluxes, described as PIcs, may be due to the inactivation of reaction centers.

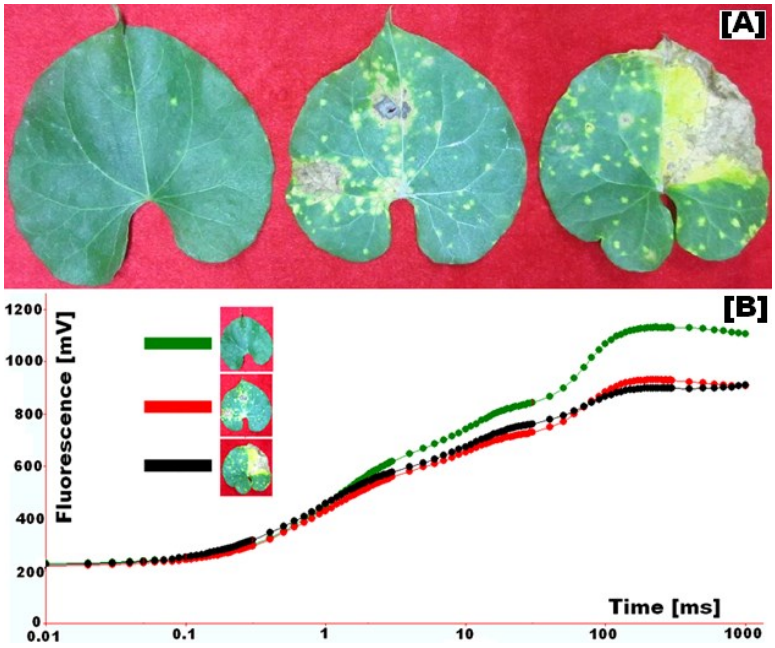

Fig. 2. (A) Healthy, mild and severely infected leaves, (B) polyphasic chlorophyll fluorescence OJIP induction in healthy, mild and severely infected leaves of *T. cordifolia*.

The density of active reaction centers (*RC*/*CS*) declined drastically in infected leaves (represented as black dots in leaf models). Decline in *RC*/*CS* may be due to the lethal interaction between PSII and toxins produced by *P. putaminum.* Although the density of active reaction centers (*RC*/*CS*) was low in infected leaves, all specific fluxes (*ABS*/*RC*, *TR*/*RC*, *ET*/*RC*) were very high in infected leaves which maintained the photosynthetic performance during infection. The high activity of reaction centers, despite their low density in infected leaves, shows that the reaction centers have increased their activity to cope up with their meagre number (Fig. 3 A, B). We suggest that the accumulation of inactive reaction centers is associated with the increased efficiency of dissipation of absorbed light as heat,

as shown by the significantly higher values of those parameters indicating the efficiency of non-photochemical de-excitation processes (*DI*/*RC*, *DI*/*ABS*, Φ*Do*).

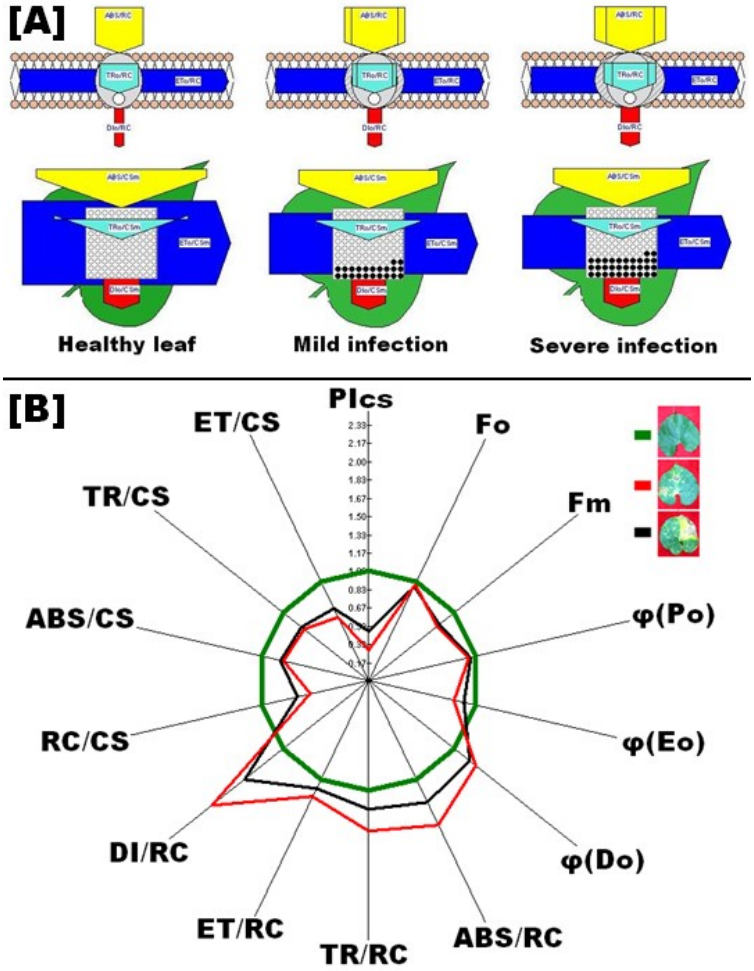

Fig. 3. (A) Thylakoid membrane and leaf phenomenological models showing specific (*ABS*/*RC*, *TR*/*RC*, *ET*/*RC*, *DI*/*RC*) and phenomenological fluxes (*ABS*/*CS*, *TR*/*CS*, *ET*/*CS*, *DI*/*CS*), and (B) radar plot showing relative values of various photosynthetic parameters in healthy, mild and severely infected leaves of *T. cordifolia*.

In majority of higher plants, maximal photochemical activity of PSII (calculated as *F*v/*F*m) is close to 0.83 [4] and under controlled conditions this parameter is often proportional to photosynthetic rate. Changes in *F*v/*F*m also could be caused by non-photochemical quenching [20]. In the present study, infected leaves showed a slight reduction in *F*v/*F*m, which may be due to the

increased activity of remaining active centers to compensate impaired reaction centers. The study suggests that fungal toxins diffuse from infected to healthy tissues of leaves and change various photosynthetic parameters in *T. cordifolia*.

CONCLUSIONS

An important aim of the work was to clarify whether or not photosynthetic performance of adjacent green tissues decline before visual symptoms appear. This study clearly demonstrates that the fungal toxins transport from infected areas to healthy tissues to impair photosynthesis before the appearance of visual symptoms.

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