



## Evaluation of synthetic antimicrobial peptides for the control of mulberry brown leaf spot disease incited by *Myrothecium roridum*

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### ABSTRACT

Brown leaf spot of mulberry is caused by necrotrophic pathogen *Myrothecium roridum* (ascomycete) and results in the foliage loss (12-15%) during June-September in West Bengal. The management practice for brown leaf spot (BLS) includes application of dithio-carbamate fungicides; which are combative for user-health and the environment. The current study evaluates application of synthetic antimicrobial peptides (AMPs) as an alternative for BLS suppression. Eight AMPs (cecropin-mellitin derivatives) were screened initially for anti-*M. roridum* activity in vitro (0-72hrs at 24h interval). Four AMPs (<50µM) inhibited significant conidial growth suppression and the minimum inhibitory concentration (MIC<sub>50</sub>) recorded for most effective AMP (PRE-2) was ~23µM after 24h. LDH (lactate dehydrogenase) activity of *Bombyx mori* haemocytes treated with PRE-2 (50 to 175µM) showed negligible cytotoxicity post-4h treatment: 0.68% at 50µM and 9.4% at 175µM. The effectiveness of PRE-2 (100µM) was assessed by spraying on BLS inflicted mulberry leaves ex vivo. Around 42% BLS suppression was recorded 20 days post-treatment. DNA isolated from PRE-2 treated and untreated samples infected with BLS were profiled with 7 SSRS and 5 ITS primers. ITS 1&4 and ITS 3&4 primer pairs revealed significant variation in band intensities between control and AMP treated samples.

**Keywords:** Antimicrobial peptides, brown leaf spot, cytotoxicity, mulberry

Mulberry (*Morus* spp.; Family: Moraceae; Order: Rosales; Chase *et al.*, 2009) leaves are primary food source of the domesticated silkworm *Bombyx mori* L. As a highly nutritious food source, mulberry is prone to several foliar diseases. One such foliar disease is brown leaf spot (BLS) caused by an ascomycete necrotrophic pathogen *Myrothecium roridum*. This fungal pathogen is a facultative parasite which causes disease of aerial plant parts of many species of vegetables, fruits and ornamental plants including mulberry (Murakami and Shirata 2005; Okunowo *et al.*, 2010). Primary symptoms of the disease are appearance of small round or oval shaped tiny brown spots surrounded by dark brown margin on leaves of infected plant. BLS is predominant during summer in Eastern and North-Eastern India causes foliage loss of ~12% to 15%. The disease affects three major commercial crops of mulberry in West Bengal. At present, carbamate fungicides are most frequently used chemical to control BLS of mulberry which arise both health and the environment issues. Hence an alternate method of BLS suppression by antimicrobial peptides (AMPs) is being explored in the present study.

AMPs are produced as part of innate immunity to fight against invading microbes / pathogens in almost all species (Nawrot *et al.*, 2014). These are usually short peptides (< 50 amino acids), cationic, dominated by cysteine or glycine with molecular mass of 3 to 10 kDa. AMPs show a wide range of antimicrobial activity with immunomodulatory functions (Stotz *et al.*, 2013). They

possess the ability to acquire an amphipathic assembly in membrane surroundings as a prerequisite to initiate lytic function (Shai and Oren 2001). Majority of the AMPs rapidly infuse and disintegrate the membrane. On contrary, most of the conventional pesticides, act on specific targets like enzymes and/or DNA (Makoviitzki *et al.*, 2006). Recently designed or synthetic AMPs have been investigated for control of pathogens in plant, animals and humans. MSI-99 (substituted analogue of Magainin) suppressed the mycelial growth and spore germination of *F. oxysporum* f.sp. *ubense*, *A. alternata*, *B. cinerea* and *S. sclerotiorum* (Alan and Earle, 2002). D4E1 and MB39 (two analogues of cecropin-B) inhibits *V. dahlia* and *F. moniliforme*, (DeLucca and Walsh, 1999). However, AMP based control approach of BLS pathogen control is scant. Therefore, objectives of this investigation were to evaluate the ability of selected eight synthetic / modified AMPs to control *M. roridum*.

### MATERIALS AND METHODS

#### Culture of *myrothecium roridum*

The pathogen (*Myrothecium roridum*) was isolated from the experimental garden of the Institute. Pure pathogen mycelium was cultured on potato dextrose (PD) agar (Himedia) medium in dark at 4°C and sub-cultured at an interval of 20 days. Analyses were carried out either with PD broth or solidified with 15g L<sup>-1</sup> agar and 20mg L<sup>-1</sup> streptomycin sulphate, amended by specific dose of synthetic AMPs.

### Peptides

Eight designed peptides of >60% purity (all are modified cecropin or melittin in nature) were synthesized from Biolinkk (New Delhi). Peptides were solubilized in nuclease free water with 200 $\mu$ L DMSO (SRL, India), filter sterilised and stored as 500 $\mu$ M stock at -40°C for further use.

### Assessment of pathogen growth suppression ability of selected AMPs

Fungal mycelia and conidial active growths were ascertained preliminarily using a Trinocular microscope (Radical model RXLr-4). Spore concentration was determined by haemocytometer following the method of Aneja (2014).

*In vitro* growth suppression assays were conducted in sterile 96-well flat bottom plates (Himedia, India) according to Zeitler *et al.* (2013) with minor modifications. Spores of pathogenic fungus were collected fresh and actively dividing mycelial colonies and adjusted to 1.5- 2.0 x 10<sup>4</sup> conidia mL<sup>-1</sup>. Each reaction was conducted with total 200 $\mu$ L of incubation mix per well. About 10  $\mu$ L conidial suspensions were transferred into appropriate PD broth contacting specific doses of AMP (10 $\mu$ M - 175 $\mu$ M) in plates on a rotary shaker (300rpm) and incubated at 25°C  $\pm$  1°C in dark up to 72h. The mycelial growth was estimated at 24h intervals at A<sub>450nm</sub> in a microplate reader (model-iMark Bio-Rad, USA). The minimal peptide dose, where fungal growth was lacking was considered as minimum inhibitory concentration (MIC).

### Cytotoxicity assay

Cytotoxicity of AMPs on silkworm hemocytes was assessed by measuring lactate dehydrogenase (LDH) activity *in vitro* as per the protocol described by manufacturer (Thermo Scientific™ Peirce™). For assay, hemocytes were collected from fifth instar *Bombyx mori* larvae and washed with anticoagulant solution at 4 °C under centrifugation (5000 rpm) for 10 min. The pellet containing hemocyte was suspended in Graces' insect medium (Himedia) and incubated at 27 °C for 24h. Cell count was adjusted to 20000 cells/100  $\mu$ L media in 96-well microplate in triplicates. Hemocytes were exposed to AMPs (50 to 175  $\mu$ M) for 45 min at 27 °C. Activity of LDH was assessed by using the  $\Delta$ A (A<sub>490</sub> minus A<sub>680</sub>). Cytotoxicity (%) was calculated following the formula of Decker and Lohmann-Matthes (1988).

### Isolation of DNA from pathogen

Fungal DNA was isolated from small active lump (~100-200mg) of fresh culture incubated with 10  $\mu$ M,

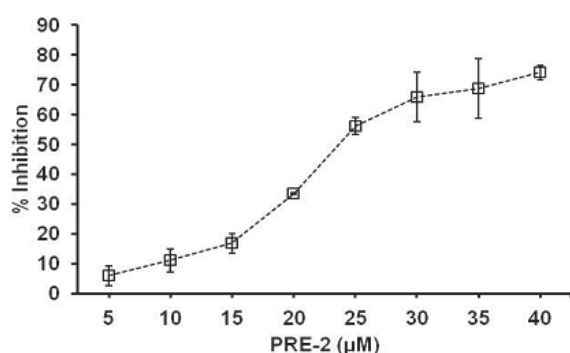
50  $\mu$ M and 100 $\mu$ M AMP against untreated control following the method described by Cenis (1992). About 50mg of fresh culture was homogenized by using a micro-pestle in 500 $\mu$ l of freshly prepared extraction buffer in a 1.5ml microfuge tube. To this 3 $\mu$ l of RNAase (150  $\mu$ g. $\mu$ l<sup>-1</sup>; Merck, India) was added with gentle shaking and incubated for 30 minutes at 37°C. Subsequently, prechilled Phenol: Chloroform: Isoamyl alcohol (25:24:1) mix was added in equal volume to the homogenized mixture, vortexed in cyclomixer. Then mixture was centrifuged (10,000 rpm) for 15 minutes at 4°C. The supernatant was collected in a fresh tube. An equal volume of Chloroform: Isoamyl alcohol (24:1) and supernatant was mixed with vigorous mixing. After settlement at room temperature, the solution was centrifuged at 10,000 rpm for 10 minutes at 4°C. The DNA was precipitated by adding 2 volumes of Isopropanol to supernatant and centrifuged further at 10,000 rpm for 10 minutes at 4°C. DNA pellet was washed with 100 $\mu$ l of 70% ethanol and centrifuged at 8000 rpm for 5 minutes. Finally DNA pellet (at room temperature) was dissolved in deionised H<sub>2</sub>O and the concentration was adjusted to 50ng/ $\mu$ l.

### Fungal DNA profiling using ITS primers

DNA isolated from fungal mycelia from AMP treated and untreated samples were subjected to PCR amplification (Mastercycler, Eppendorf, USA) with ITS primers (ITS1, ITS2, ITS3, ITS4 and ITS5). PCR amplification was conducted in a reaction mix of 20 $\mu$ l reaction mix containing 10mM tris-HCl buffer, 0.12 units of Taq DNA polymerase and 2.0mM each dNTPs with 50 ng of template DNA and ITS primer pair. PCR conditions were: 94°C for 2 mins, 35 cycles of 94°C (30 secs), 50°C (30 secs) and 72°C (2 mins) with a single step of 10 mins for final extension at 72°C. Amplified DNA products with the standard ladder (50 bp; Himedia), were loaded on 2% agarose gel in 1x TBE (Tris-base, Boric acid, EDTA) buffer. Electrophoresis (Bio Rad, USA) was run at 70V for 2h to get proper separation of bands. Gel bands were visualised with ethidium bromide (10 mg/ ml) and photographed under a Gel Documentation System (Vilber Lourmat, France, model Infinity 3000). The difference in band intensities was measured using Bio1D software.

### Statistical analysis

Data generated by percentage growth inhibition experiments and ITS primers was used for analysis. The mean of two independent experiments with three replications per sample for each occasion was calculated by using the 'Statistica' version AXA-9 software. Fisher's least significant differences were also calculated.



**Fig. 1:** Dose response (5μM to 40 μM) of curve of PRE-2 on the *M roridum* mycelial growth after 24h of incubation.

## RESULTS AND DISCUSSION

Mycelial growth suppression ability of eight synthetic peptides to the BLS pathogen fungus *M. roridum* was determined in a plate reader. Out of eight AMPs, four (PRE1, PRE2, PRE3 and PRE5) showed conidial germination inhibiting activity at 50 μM and 100 μM concentration after 24h incubation while other four AMPs were unable to suppress the growth of *M roridum* (Table 1). The pathogen growth suppression was 48.96% more after 24h of incubation with 50 μM of PRE-2 over PRE-5. The most effective AMP (PRE-2) based growth suppression pattern of *M. roridum* was evaluated with a plate reader at 24h interval with seven datum points for 72 h in different doses (5 μM to 175 μM) of antimicrobial peptides (range: 11% to 100%). The minimum inhibitory concentration (MIC<sub>50</sub>) for 50% inhibition was found at ~23 μM (Fig. 1).

The MIC obtained for PRE-2 for mulberry BLS suppression corroborate the view of cecropin originated AMP based mycelial growth suppression of various ascomycetes pathogens of Strawberry and Blueberry (Hammerschlag, 2004).

Cytotoxicity of AMP was assessed by measuring LDH (lactate dehydrogenase) activity in haemocytes of *Bombyx mori* treated with PRE-2 (10 to 175μM). PRE-2 showed negligible cytotoxicity post-4h treatment with 0.68 to 9.4% at concentrations 50 μM to 175μM. Cytotoxicity of PRE2 increased linearly with increase in concentration. Several AMPs have been reported to show minimal or no hemolytic activity upto 200 μg/mL (Zeitler et al., 2013; Kishi et al., 2018).

The effectiveness of PRE-2 was assessed by spraying two different concentrations (50 μM & 100 μM) on BLS inflicted mulberry leaves *ex vivo*. PRE2 suppressed 43 and 52% of the BLS disease severities over the peptide

untreated control after 20 days of treatment. Similarly, spraying of potential AMPs on tomato leaves suppressed bacterial pathogens (*Pseudomonas syringae*, *Pectobacterium carotovorum*, and *Xanthomonas vesicatoria*) and fungal spore germination (Zeitler et al., 2013).

Disease suppression ability of PRE2 at molecular level was assessed by semi-quantitative PCR. Pathogenic DNA isolated from control and PRE-2 treated sets were amplified using ITS primer pairs (ITS1/4 and ITS3/4). PCR profiling of fungal DNA showed variation in amplification of control sets and PRE-2 treated samples at concentration 10 μM, 50 μM and 100 μM. In control sets amplified DNA band intensity was significantly higher than in PRE2 treated samples.

Several AMPs have been evaluated by researchers to control plant pathogens. Synthetic peptides tested in this study revealed PRE-2, a lysine rich (50%) linear peptide (12-mer), showed maximum mycelial growth suppression of *M roridum in vitro* and *ex vivo*, possessed no cytotoxicity to hemocytes of silkworm has potential to be utilized for BLS management in mulberry.

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