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Research Article

CAN BACTERIA - BE A SUPERVISED CONTROL SYSTEM FOR CERCOSPORIOSIS?

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ABSTRACT: Experiments on cercosporin toxin produced by Cercospora species have revealed an important role for this toxin in pathogenesis of host plants. When the attempts to control the disease were made by regular cultural practices and fungicide application, the results were not satisfactory and attempts to develop cercosporin resistant plants were also failed. In present study in an attempt to develop a supervised control system for the disease, bacteria were collected from different sources like soil, leaf tissue and leaf surfaces of infected groundnut plants and screened them for their ability to degrade cercosporin. Their degrading capability was tested by inoculating them onto definite amount of cercosporin containing medium (NACE). Further these bacterial strains were applied to healthy groundnut plant leaves (TMV_2 and K_6 host plants) in an attempt to study the possible role of these bacterial isolates in reducing primary inoculum in the soil, the major source of disease.
Key words: Leaf spot of Groundnut, Cercosporin degradation, Photosensitizers, Phytotoxicity, Perylenequinones.

INTRODUCTION

Cercosporin has been the subject of investigation since its discovery over 50 years ago. Cercosporin has been extensively studied due to its interesting mode of action. It was unique among the plant pathogen toxins due to its requirement for light for activity [15, 9, 2]. Cercosporin, "sensitize" cells to normally harmless levels at visible wavelengths of light in the presence of oxygen. [12]. Cercosporin produced by Cercospora personata, a major causative pathogen of leaf spot of groundnut, is functionally similar to biologically important photosensitizers, like riboflavin and porphyrin compounds. Beyond their role in nature, photosensitizers are being investigated for use as insecticides and pharmaceuticals where they show potential for treatment of tumors and as antiviral agents [4, 5]. Present investigations were aimed to develop a supervised control system for Cercospora diseases by targeting the toxin directly. We have initially focussed to the detection and isolation of bacteria capable of degrading cercosporin, and the possibility of their implication in minimizing the effects of cercosporin on crop plants. Bacterial populations were harvested from the soil, groundnut leaf surfaces, and cercospora infected leaf tissues and inoculated on cercosporin containing medium. Bacteria that are able to degrade cercoporin were identified by a clear halo surrounding the colony on the red plates, which were further isolated & purified. These bacterial cultures able to degrade cercosporin were applied in combination with cercosporin and alone on fresh healthy groundnut leaves. Promising results were observed giving a hope of, these bacterial culture filtrates as a supervised control system for leaf spot diseases of groundnut.

MATERIALS AND METHODS

Cercosporin Stocks

The cultures were grown on PDA petriplates overlaid with cellophane discs. Crude extracts from the PDA grown isolates were prepared by air drying. Air dried cultures were ground and their components extracted with ethyl acetate. About 75g of mycelial tissue extracted twice with 50ml ethyl acetate, dried, residue dissolved in 20ml chloroform. Cercosporin was crystallized directly from the chloroform layer. Dried crystals were stored in the dark at -20^0C. To get working stocks crystals were dissolved in 0.1N NaOH which was then added to medium with a corresponding equal volume of 0.1N HCl.

Collection of Mixed Bacterial populations from different sources:

Mixed bacterial populations from Cercospora infected plants were obtained. Soil surrounding the root system of infected groundnut plant was collected. Necrotic spots on the leaves were rubbed with swabs moistened in 0.9% saline. The swab was swirled into a small volume of saline to dislodge the bacteria from swab. An aliquot from the resulting solution was added to 5gms of sterile soil in a vial. Pieces of leaf tissue bearing necrotic leaf spots were suspended in sterile saline (0.9%) using a tissue homogenizer. From the resulting suspension, an aliquot was added to 5 gms of sterile soil in a vial.

Cercosporin in 0.1N NaOH at a concentration of 1mg/5gms soil was added to each soil sample. Amphotericin B (3.5*μg*/ml) was also added to suppress the growth of eukaryotic organisms. Samples were incubated in dark at 25^0C. Cercosporin in 0.1N NaOH (1mg/5gm soil) was added again at two week interval. Moisture content was readjusted to 20% water with each addition of cercosporin. pH was maintained at 7.

Screening and Selection of cercosporin degrading microorganisms with NACE medium (Robeson et al**, 1990) [11]**

0.1 gm of the soil from each enriched soil sample was added to 10ml of sterile saline. Serial dilutions of the soil suspension were prepared to 10^{-3} using 0.9% sterile saline. 50 µl of each dilution was plated on Nutrient agar (containing amphotericin 3.5µg/ml) and the plates were incubated at 25^0C until bacterial growth was evident as colonies. Plate with suitable number of colonies was replica plated on NACE medium.(Nutrient agar containing 50µM cercosporin) Crystalline cercosporin was dissolved in 0.1N NaOH to get a concentration of 10mg of cercosporin/ ml of solution.5ml of 0.1 N HCl/lit of medium was added to molten, sterile NA medium. 5ml of the cercosporin solution was then added per liter of the NA/HCl medium, to get a final concentration of 50 µM/ml of medium. The molten medium was mixed by gentle swirling and asceptically dispensed into pertriplates, at a volume of 25 ml/ plate. The plates had a distinctive, uniform red coloration due to the presence of cercosporin.

Clear zone surrounding bacterial colonies were indicative of degradation of cercosporin by isolate. Candidate colonies showing discoloration were streaked on fresh NACE medium to get pure cultures. Then their characteristics were studied for their identification by gram's staining.

Measurement of % loss of Cercosporin

Cercosporin can be easily detected in the medium as a red pigment. It can be extracted, by removing plugs of agar media with a no.3 cork borer (6mm diameter) and soaking in 5N KOH. The absorbance of the soaking solution was measured at 480 nm. [6] spectrophotometrically. Plain agar plugs were included as controls.

Effect of bacterial culture filtrates and cercosporin on healthy groundnut leaves

Healthy fresh groundnut leaves were collected from the field to test the effects of cercosporin and/or bacterial culture filtrates on plant tissue. Cercopsorin and bacterial culture filtrates were inoculated into the healthy leaves by lightly touching the surface of the tissue using a needle. A tray with wet Whatman no. 1 filter paper was used to keep the leaves (to prevent drying off) and incubated in dark and under constant fluorescent light. After incubation the treated areas were assessed. 5-6 leaves were used for each treatment and the experiment repeated twice.

Host Plants

Commercial varieties of groundnut, TMV_2 (Thindivanam 2, Tamil Nadu) and K_6 (Kadiri 6, Andhra Pradesh) were employed in the study. The seeds were obtained from crop specialist, ICRISAT, Hyderabad, India.

Plants were grown in a green house in earthenware pots (15 cm diam) filled with clay, red soil mixture (2:1) and were watered on alternate days. The temperature in the green house ranged between 28-33ºC and relative humidity was 50-80%. Light intensity at plant height was maintained at 5-6000 lux. Supplemented light was provided during winter months using banks of incandescent and mercury lamps (Philips, India) from 8.00 a.m. to 6.00 p.m.

Effect of bacterial culture filtrates on cercosporin:

Bacterial culture filtrates were added to cercosporin (100 µg/ml) and mixture was incubated for 0, 5, 10, 20, and 30 min at 37^0 C. UV-Visible absorption spectra was determined using spectrophotometer.

RESULTS

Screening and Selection of Cercosporin degrading microorganisms

Identification of cercosporin degrading bacteria was carried out initially by looking for the production of a cleared zone surrounding colonies in cercosporin containing solid medium. A few colonies have shown a permanent and expanded clear zone in the medium, suggesting the degradation of the cercosporin. A limited clear area restricted to the medium immediately under the colony and concurrently turned purple, indicating that cercosporin adsorption occurred. Cercosporin degrading isolates were then re screened.

Measurement of % loss of cercosporin

Isolate 1

Decoloration of the medium was observed as a distinct pale yellow halo. The amount of cercosporin present in agar plugs taken from the discolored section of the plate of isolate 1 was about 78% lower than that in the control plugs taken from colored NACE medium. G+ve bacilli were observed and further analysis demonstrated that isolate was *Bacillus subtilis.*

Isolate 2

Decoloration of the medium was observed as a clear zone. Initially the discoloration was reversible; however after an extended period of time, cercosporin-degradation became irreversible. The amount of cercosporin present in agar plugs taken from the discolored section of the plate of isolate 2 was about 83% lower than that in the control plugs taken from colored NACE medium. Gram positive bacilli were observed and further analysis demonstrated that isolate was Bacillus cereus.

Isolate 3

Decoloration of the medium was observed as a pale yellow halo around the colony. The amount of cercosporin present in agar plugs taken from the discoloured section of the plate of isolate 3 was about 50% lower than that in the control plugs taken from colored NACE medium. Gram positive bacilli were observed and further analysis demonstrated that isolate was Pseudomonas species (species not identified)

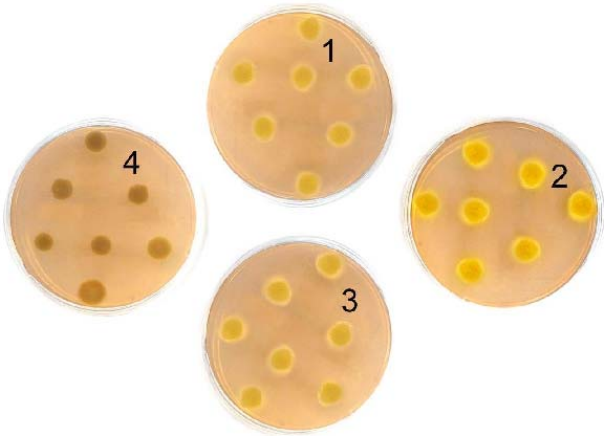
Isolate 4

Discoloration was not observed surrounding the bacterial colony suggesting that it is a non cercosporin degrading species. Gram negative bacilli were observed and further analysis demonstrated that isolate was Escherichia coli. (Table 1)

The Cercosporin in the agar plugs extracted was measured by reading the absorbance spectrophotometrically at 480nm. Maximum degrading ability was observed with isolate 2 i.e., B. cereus (83%) followed by isolate 1 i.e., B. subtilis (78%) followed by isolate 3 i.e., Pseudomonas (50%) where as isolate 4 i.e., E.coli was unable to degrade cercosporin. B. cereus with maximum degrading ability and E.coli with no degrading ability were chosen as +ve and -ve controls for further investigation. (Fig 1).

Table-1: Cercosporin degrading bacterial characteristics

Source	Isolate	Cleared halo(+/-)	Gram reaction	Loss of cercosporin (%)	Organism
Soil leaf tissue	Isolate 1	+	+	78%	*B. subtilis*
Soil leaf tissue	Isolate 2	+	+	83%	*B. cereus*
Leaf Surfaces leaf tissue	Isolate 3	+	+	50%	*Pseudomonas species*
Soil	Isolate 4	-	-	-	*E. coli*



1. B.subtilis
2. B. cereus
3. Pseudomonas species
4. E. coli

Figure-1: Bacterial isolates showing Cercosporin degradation after 48 h of incubation on NACE medium

Effect of bacterial culture filtrates and cercosporin on Healthy groundnut leaves:

Various combinations were demonstrated by using cercosporin and cercosporin degrading and non degrading bacterial culture filtrates under dark and light. Leaves treated with cercosporin and incubated under constant light showed dark spots surrounded by circular to irregular yellow-brown margins Leaves treated with cercosporin + bacterial culture filtrates have shown, reduced necrotic zone. Leaves exposed only to bacterial culture filtrates and controls (maintained without cercosporin and bacterial culture filtrates but with needle injury) have shown no lesions (Fig 2). This suggested that the toxicity of cercosporin can be reduced by the addition of bacterial culture filtrates (capable of degrading cercosporin). When cercosporin was inoculated along with bacterial culture filtrates (cercosporin non degrading) there was no change in the size of necrotic lesions compared to that of lesions caused by cercosporin alone. Similar results were also explored by Khan et al, 2008. [8] (Table 2).

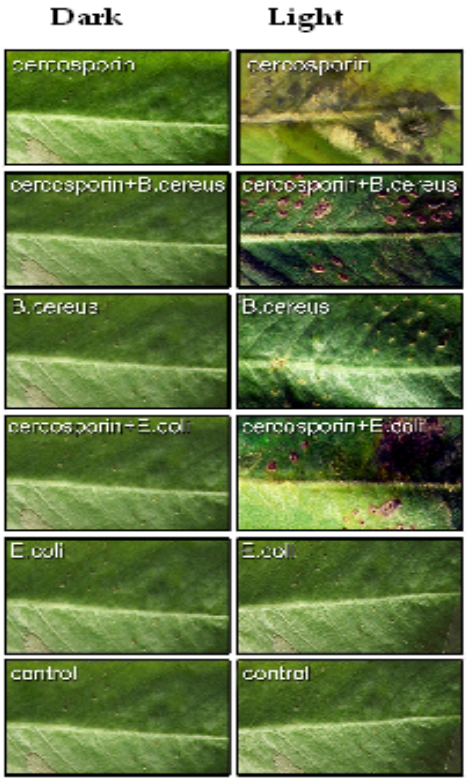


Figure-2: necrotic symptoms of fresh groundnut leaf surfaces induced by cercosporin (50 µg/ml) from CP_4 and or/bacterial culture filtrates of *B.cereus* and *E.Coli,* incubation was for 48 h at 26^0C in the dark or under constant light

Table 2: Effects of bacterial culture filtrates and cercosporin (50 µg/ml 0.1 NaOH) on healthy groundnut leaves after 48 hrs of incubation in dark and under constant light.

Treatment	Dark	Light
Cercosporin	No lesions	crusty necrotic lesions
Cercosporin + bacterial CF able to degrade cercosporin *(B. cereus)*	No lesions	Reduced dark brown lesions
Bacterial CF(B.cereus)	No lesions	No lesions
Cercosporin + non degrading bacteria *(E.coli)*	No lesions	Circular light yellow necrotic lesions
Non degrading bacterial CF *(E.coli)*	No lesions	No lesions
Control	No lesions	No lesions

CF: culture filtrate

Direct effect of bacterial culture filtrates on cercosporin

Decolorization of pigments or dyes has been used as standard assay to rapidly assess the oxidative activity of the culture filtrate. Bacterial culture filtrates used in the present study were able to decolorize and decrease the absorbance of cercosporin. These results indicating that bacterial culture filtrates have the ability to decolorize and show oxidative activity on cercosporin. After 5 min of reaction a more drastic decrease in the absorbance was found. No significant changes were found after first 5 min. The results are in accordance with Wong and Yu (1999). [14] (Fig-3).

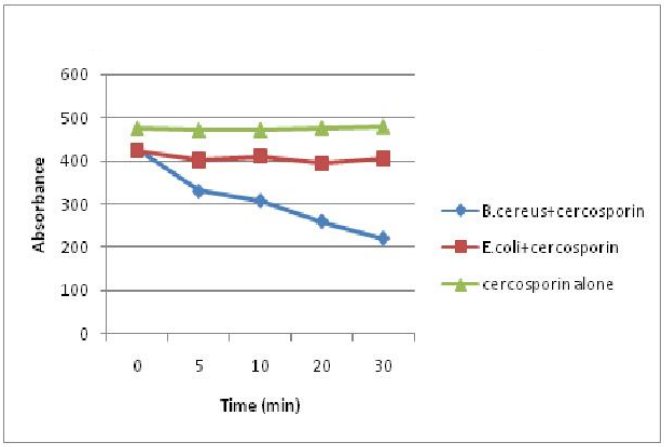


Figure-3: Effect of bacterial Culture filtrates on Cercosporin

DISCUSSION

Many species in the genus Cercospora produce cercosporin that is highly toxic to most organisms including plants animals and micro organisms [3]. Research efforts directed towards minimizing the impact of toxin hold promise for the control of the disease. In spite of extensive efforts to control them. Cercospora species remain a highly successful plant pathogen community and continue to cause severe economic losses. Presently, the control of diseases caused by Cercospora species relies on the use of fungicides and appropriate cultural practices such as tillage and crop rotaion. [7, 1, 13]. Initial attempts were focused mainly on the mechanisms of resistance in the fungal strains and on understanding cercosporin biosynthesis. Neither of these approaches has provided practical disease control yet. Later development of resistant cultivars has been attempted, but high levels of resistance to Cercospora diseases are uncommon. Alternate methods of control would be desirable. One such approach would be application of cercosporin degrading bacteria in minimizing the effects of cercoporin on crop plants. Of the four bacterial isolates screened three were able to degrade cercosporin in the growth medium. The best degrading isolates were Bacillus cereus, B.subtilis and Pseudomonas species. There is no constructive information or reason why these bacteria have this capability. C. personata not known to be significant member of microflora of the leaf surface. However Bacillus species have been identified as capable of degrading the cercosporin the most.

Present results suggested that the toxicity of cercosporin towards groundnut leaf tissue can be reduced by bacterial culture filtrates (capable of cercosporin degradation). A similar study was also involved tobacco leaves. [10]. Soil is a source of primary inocula of leaf spot disease of groundnut. This primary inoculum can be targeted by application of bacterial culture filtrates as an effective approach which might offer the development of supervised control system.

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