JOURNAL OF PURE AND APPLIED MICROBIOLOGY, June 2013. Vol. 7(2), p. 1141-1144

Red-ochre Pigment Production by *Fusarium* spp. on Co-cultivation with *Bacillus subtilis*

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(Received: 18 September 2012; accepted: 09 November 2012)

Bacillus **strains offer several advantages over other bacteria for protection against plant pathogens because of their ability to form endospore and to produce broad spectrum antibiotics. The aim of this work was to study the interaction of *Bacillus subtilis* with rice pathogen, *Fusarium*. Eleven aerobic endospore forming bacilli were isolated from Bhitarkanika mangrove sediment, Odisha, India. Among them one strain was found to be antagonistic to the tested rice pathogens. The isolated bacterium was Gram-positive rod having sub-terminal endospore. The analysis of partial sequences containing 700 to 900 bases of the 16S ribosomal DNA gene showed 99% identity with *B. subtilis.* Strong inhibitory activity against *Fusarium* spp. was found on co-cultures with *B. subtilis* strain, particularly on Sabouraud dextrose agar (SDA) and Potato dextrose agar (PDA). Their interaction resulted in the secretion of red and red-ochre color pigments, probably due to melanization after seven days of co-culturing.**

Key words: Antibiosis, *Bacillus subtilis, Fusarium* spp., mangrove, Red-ochre pigments.

B. subtilis have capacity to produce more than two dozen antibiotics and have been used widely in biotechnological applications as biocontrol agent. Antibiosis against the phytopathogenic microorganisms has been associated with production of secondary metabolites[1]. Co-cultivation of fungi and *Bacillus subtilis*. can lead to increased bacteriocin production that is inhibitory to the growth of fungi [2,3]. It has also been reported that *Bacillus* spp. can induce morphological changes in some fungi [4].

Fusarium spp. occurs widely in nature as saprophytes and plant parasites. The presence of Fusarium toxins found on naturally occurring rice emphasizes increasing need of research on the biocontrol of *Fusarium* spp. [5, 6]. It is also considered as a major pathogen of the Gramineae, particularly in tropical and subtropical regions, where it causes economic losses as high as 50%, depending on the crop[7].

A natural characteristic of many *Fusarium* spp. is the production of dark cellular pigments, which is the result of a melanization process. Melanin has been linked to high virulence, resistance in adverse environmental conditions and related to low susceptibility to antifungal drugs[8, 9].

The objective of this experiment was to study the interaction of rice pathogen, *Fusarium* spp. with a Gram-positive *B. subtilis* strain as a biocontrol agent, *in vitro*. The isolated bacterium was identified up to species level through phenotypic and genotypic profile study.

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MATERIALS AND METHODS

Study site

Geographically Bhitarkanika is located between 20° 4' N and 86° 45' E and is the second largest mangrove ecosystem of India having an area of 650 km^2. Samples were collected from three different sites, covering a distance of five kilometers (Fig-1).

Collection and maintenance of rice pathogens

Eleven *Fusarium* spp., all identified as rice pathogen were collected from CRRI, Cuttack and maintained on SDA (HiMEDIA Ltd., Mumbai, India) and PDA (HiMEDIA Ltd., Mumbai, India) slants at Department of Microbiology, OUAT.

Isolation and total DNA extraction

Bacteria were isolated by 10-fold serial dilution technique followed by spread plating. Diluents were prepared with sterilized phosphate buffer having pH 7.2. From each dilution tube 100µl of samples were used for spread plating on pre-sterilized Nutrient Agar (NA- (HiMEDIA Ltd., Mumbai, India) plates in triplicate. All the plates were incubated at 37°C for 24-48 hr. Selection was done on the basis of their antifungal activity against the tested pathogen. Total DNA was isolated by Kit method using HiPurA™ Bacterial Genomic DNA Purification Spin Kit – MB 505.

Biochemical characterization of bacterial strain

Identification was assisted by biochemical analysis. The Gram stain, Schaeffer-Fulton spore stain, oxidase, catalase, nitrate and nitrite reduction, starch hydrolysis, casein hydrolysis, and gelatin hydrolysis tests were carried out as described by Collins *et al* 1995 [10].

PCR amplification of ribosomal 16S gene

The amplified 16S ribosomal DNA (rDNA) gene was obtained from *Bacillus* strain, using thermal amplifier, with the universal primers, F (5'-AAG AGT TTG ATC CTG GCT CAG -3') and R (3'-GGT TAC CTT GTT ACG ACT T 5'). These primers are targeted to universally conserved regions and permit the amplification of an approximately 1500 bp fragment. PCR amplification was carried with a thermal program, which comprised of 35 cycles at 94°C for 1min, 55°C for 45 sec and 72°C for 1min 30 sec in a thermal cycler (Minicycler, MJ Research).

Analysis of 16S rDNA gene sequence

The sequences obtained were aligned with those in GenBank by using BLAST [11] and showed 99% identity with *B. subtilis*.

Detection of antagonistic activity

The well diffusion assay was used for the in-vitro test of antagonistic activities of bacterial isolates [12]. The *Fusarium* culture (7 d old) was spread over SDA and PDA plates and was inoculated with 0.1ml broth culture of *B. subtilis* strain (24hr old) by well diffusion method, without antibiotic. The plates were incubated at 28°C and examined daily for one week. The assay was carried out in duplicate for all the test organisms.

RESULTS

Biochemical and molecular characterization

The bacteria out of all the isolate showing antagonistic effect against rice pathogen was a gram positive, motile rod having terminal endospores. The biochemical tests were positive for Voges-Proskauer, methyl red, mannitol, catalase,

Fig. 1. Bhitarkanika mangrove forest

oxidase, urease, amylase, caseinase, gelatinase, nitrate utilization and citrate utilization,

The 16S rDNA sequence (1.4kb) exhibited maximum identity to the *Bacillus* genus in all the bank databases. However, a crossed identity of ribosomal genes over 99% was detected among the species of *B. subtilis*.

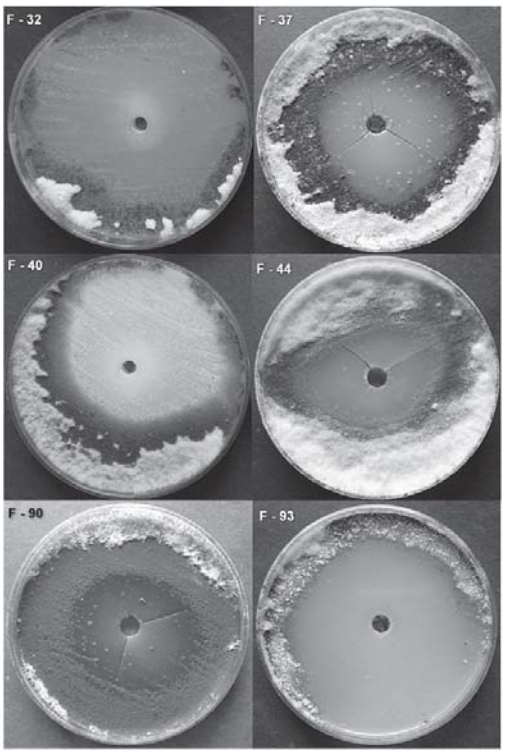

Fig. 2: *In vitro* co-cultures between rice pathogen *Fusarium* species *and B. subtilis* on SDA medium

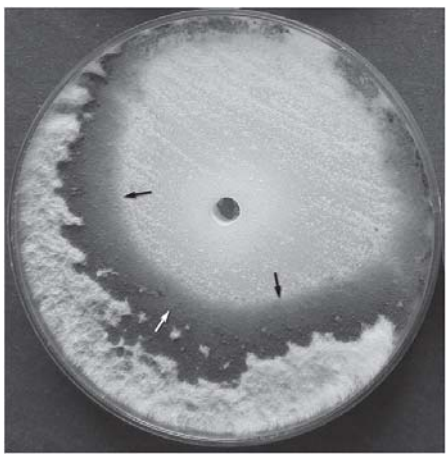

Fig. 3: Red-ochre Pigment Production by *Fusarium* spp. after seven days on Co-cultivation with *B. subtilis*

***In vitro* interactions**

Antibiosis of *Bacillus* sp was observed in co cultures against *Fusarium* species by co-cultivation method. Antifungal activity was more pronounced on SDA medium in comparison to PDA. In many instances, a line of precipitation was observed in co-cultures plates near the bacterial colony (Fig. 2, Fig. 3).

The diffusion of red and red-ochre pigments, on agar plates by all fungal colonies were observed after one week of co-culture with *B. subtilis*.

DISCUSSION

Gram-positive bacilli and fungi are important bio-regulators and widespread in soil[13]. Microbial antagonisms have been studied due to their numerous biotechnological applications, particularly those related in search of new biocontrol variety[14]. Many *Bacillus* species possess natural biocidal activities against fungi and other bacteria, and are described as biocontrollers of phytopathogens [8].

In the present study, an environmental isolate of *B. subtilis* having excellent antagonistic property against rice pathogen was observed. Antifungal activity was more pronounced on SDA, suggesting increased production of inhibitory substances in acidic pH. In control neither the fungus nor the bacteria are producing any pigment. The red and red-ochre color pigments secretion by the *Fusarium* spp. resulted after seven days of co-cultivation with *Bacillus subtilis* which may be due to the process of melanization. The ability of microbes to synthesize melanins is related to virulence and pathogenicity[15]. Reports are available regarding transformation of pathogenic forms of fungi to melanized froms in order to increase antifungal resistance or host defense mechanism 9-15. Melanin production and secretion also has been linked to offer resistance in adverse environmental condition and related to low susceptibility to antifungal drugs. The melanin production by *Fusarium* spp. is an extracellular secretion and is associated with bacterial antibiosis or that metabolic substances are capable of inducing the melanogenesis pathway; however, more studies are necessary to confirm these hypotheses.

CONCLUSION

The above study needs further experiment for isolation of the active molecule working against the rice pathogens.

ACKNOWLEDGMENTS

The authors are grateful to the management and staff of Department of Microbiology, O.U.A.T., Bhubaneswar, Odisha and Fish Health Management Division, CIFA, Odisha, for technical and laboratory assistance.

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