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Control of Phytopathogens with Application of Vermiwash

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Vermiwash is an economic and ecofriendly formulation having biocidal activities which can replace chemical pesticide employed in agricultural sector. In the present study an attempt has been made to find out in vitro and in vivo effect of vermiwash on common plant pathogens. A total of eight bacteria were isolated from the freshly prepared vermiwash and were identified as *Flavobacterium* sp., *Burkholderia* sp., and six different species of *Bacillus* by using standard biochemical tests. Then the fresh and preserved vermiwash were treated against five fungal and bacterial phytopathogens respectively. Moreover, out of the eight bacterial isolates, *Bacillus* sp.V08 showed a significant antibiosis activity against bacterial phytopathogen *Xanthomonas campestris*. *Bacillus* sp.V08 and *Burkholderia* sp. were most effective against fungal phytopathogens such as *Fusarium oxysporium, Fusarium solani, Rhizoctonia solani, and Alternaria solani.* The bacterial isolates *Bacillus* sp.V08 showed antibiosis against bacterial and fungal phytopathogens was identified by 16S-rRNA gene sequencing and the sequence was submitted to Genbank with accession no. KF543076. Thus, this in-vitro and in-vivo studies indicates that application of vermiwash can control these bacterial and fungal phytopathogens of various plants like tomato, brinjal, and mustard respectively.

Keywords: Vermiwash, Biocidal, Antibiosis, *In-vitro, In-vivo.*

Application of chemical fertilizer and pesticides to increase productivity of crop plants not only deteriorates the environment causing environmental hazards with detrimental effects on human health & environment[1] but also has greatly affects soil health. On account of that significant attempts are being taken in recent past to reduce use of synthetic pesticides, fertilizers, weedicides etc. with emphasis on natural and biological resources. Role played by Earthworms, designated as "intestine of earth" in vermicomposting and soil formation is well documented. Vermicompost and vermiwash are considered as inseparable components of organic farming besides in situ culturing of earthworms in crops[2] and antibiosis. Vermiwash, a collection of excretory products and mucus secretion of earthworms along with micronutrients from the soil organic molecules, stimulate the growth and yield of crops[3] and can be used to cure diseases in plant[4]. Although work pertaining to biocidal activity of vermiwash is very few, there is no report on practical application of vermiwash to control plant diseases. On account of that, an attempt has been made in the present investigation to study application of vermiwash in vitro & in vivo condition for control of plant diseases.

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MATERIALS AND METHODS

Collection and storage of Vermiwash

Vermiwash was prepared by putting adult *Eisenia foetidia* earthworms (weighing 7-9gms each, collected from vermicompost pit of OUAT, BBSR) in 100ml of lukewarm distilled water for 5 minutes. Then the earthworms were removed and the filtrate was taken as 100% vermiwash. Ten percent concentration of five different types of vermiwash viz. fresh, 1 month old, 2 months old, 1

month old freezed and 2 months old freezed were used for the experiment. The storage of vermiwash was done by keeping it in closed sterile bottles sealed with paraffin, and the bottles were stored in cool & dry place for 1 month, 2 months old vermiwash and inside the freeze for freezed vermiwash.

Bacteriological analysis of vermiwash

Bacterial load of fresh and stored vermiwash was studied through serial dilution and isolation was done by spread plate method in Nutrient Agar (NA) plates (HiVeg media). Bacterial isolates with different colony morphology were studied and pure culture of the bacterial isolates (V01, V02, V03, V04, V05, V06, V07, and V08) were made and maintained at 4°C. Identification of bacterial isolates was done on the basis of the colony characteristics on the basal media, Gram's staining, biochemical tests, sugar utilization, and enzymatic activities of the isolates. Molecular characterization of potential isolate was done.

Mycological analysis of vermiwash

The fungal load of fresh and stored vermiwash was studied by using PDA plates.

***In vitro* studies**

Collection and isolation of phytopathogens

Different diseased leaves of tomato, brinjal, and infected seeds of mustard were collected from cultivated fields at Nayagarh, Odisha. Bacterial & fungal phytopathogens were isolated from the diseased leaves & seeds by using NA, Potato Dextrose Agar (PDA), CKTM & Yeast Extract-Dextrose-$CaCO_3$ (YDC) medium. From the samples, fungal phytopathogens like *Fusarium oxysporium, Fusarium solani, Rhizoctonia solani, and Alternaria solani* were isolated and identified by following scotch tape method & their pathogenicity assays.

The bacterial phytopathogen, *Xanthomonas campestris* were isolated and identified by following its morphological, biochemical & pathogenicity assays.

Antibiosis of bacterial isolates against phytopathogens

Lawn culture of *Xanthomonas campestris* was made in NA plates and 50µl of the bacterial cultures of vermiwash (V01-V08) were inoculated in well & incubated at 37°C for 24hrs. Similarly lawn culture of *Fusarium oxysporium, Rhizoctonia solani, Alternaria solani and Fusarium solani* were made on PDA plates and 50µl pure culture of all the bacterial isolates (V01-V08) of vermiwash were inoculated in the well & incubated at 28°C for 48hrs.

Antibiosis of vermiwash against phytopathogens

For antibiosis study, 50µl of 10% vermiwash of all the five different (fresh, 1 month old, 2 months old, 1 month old freezed and 2 months old freezed) samples were applied in well to the individual fungal and bacterial pathogens followed by incubation at 28°C for 48hrs for fungal and at 37°C for 24hrs for bacterial phytopathogens.

In vivo studies

Plantation

Seeds of Mustard and seedlings of tomato & brinjal were planted in pot (final volume 4.0 Kg) in triplicate under field condition. Pots were watered (soil: water=1:2) and the level of water was maintained. The pots were supplemented with organic manure at the rate of 100 gm per pot. Healthy plants were taken for the experiment.

Injection of phytopathogens

The different phytopathogens were injected in to the leaves of healthy plants for infection of the diseases (Fig. 1, Table 1). *Rhizoctonia solani* was injected in to the germinating seeds of mustard to cause damping off disease.

Application of Vermiwash on diseased seeds and plants

The fresh vermiwash, 1 month old, 2 months old, 1 month old freezed and 2 months old

Table 1. Diseases of Plants

S.No.	Plants	Phytopathogens	Disease
1	Tomato	*Xanthomonas campestris*	Bacterial leaf spot
		Alternaria solani	Early leaf blight
2	Brinjal	*Fusarium oxysporium*	Fusarium wilt
3	Mustard	*Rhizoctonia solani*	Damping off root

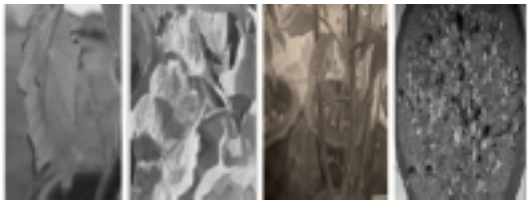
freezed vermiwash were sprayed over the diseased seeds of mustard and diseased plants of tomato & brinjal on an interval of one day for 9 days.

Molecular identification of bacterial isolates

Molecular identification of the potent bacterial isolate was carried out by sequencing of 16S rRNA gene followed by submission of sequence to NCBI GenBank. Genomic DNA was extracted from the isolate[5] and sequencing was done at Xcelris Genomics, India. Raw sequence was analyzed by Bio-Edit software (7.0.5.3) and identification of the isolate was done by BLASTN in NCBI database (www.ncbi.nlm.nih.gov/nucleotide). Multiple alignments of sequence was performed with the ClustalX[6] (1.83). A phylogenetic tree was constructed using the neighbour-joining DNA distance algorithm[7] using distance tree analysis tool.

RESULTS AND DISCUSSION

A total of eight bacterial isolates obtained from the vermiwash sample were identified as; *Flavobacterium* sp., *Burkholderia* sp., *and* six different *Bacillus* sp. No fungal strain was isolated. The antagonistic activities of the bacterial isolates were tested against the bacterial *(Xanthomonas campestris)* & fungal (*Fusarium oxysporium, Fusarium solani, Rhizoctonia solani, and Alternaria solani*) phytopathogens. It was observed that *Burkholderia* sp. *and Bacillus* sp. V08 showed significant zone of inhibition against *Fusarium oxysporium, Fusarium solani, Rhizoctonia solani,* and *Alternaria solani* (Table 2). The proteolytic and lipolytic activities of the



1 2 3 4

1. Early blight in tomato
2. Fusarium wilt in the brinjal
3. Bacterial leaf spot in tomato
4. Damping off in mustard

Fig. 1: Diseases after Injection of phytopathogens**.**

Fig. 2. Healthy plants after 9 days of vermiwash application.

above bacteria may be responsible for the suppression of the fungal diseases has been reported[5]. It was observed that *Bacillus* sp. V08 showed best zone of inhibition against *Xanthomonas campestris* (Table 3). In-vivo studies indicate that two months old vermiwash showed best zone of inhibition against the phytopathogens (Table 4 and Table 5). The antagonism of *Bacillus*

Table 2. Antibiosis of bacterial isolates against fungal phytopathogens

Fungal pathogens	Well Content (in µl)	*V01 Flavobac-terium*	*V02 Bacillus* sp.	*V03 Burkholderia* sp.	*V04 Bacillus* sp.	*V05 Bacillus* sp.	*V06 Bacillus* sp.	*V07 Bacillus* sp.	V08 (Accession-KF543076)
Fusarium oxysporium	50	++	+	+++	++	+	+	+	+++
Fusarium solani	50	++	++	+++	++	+	+	++	+++
Rhizoctonia solani	50	R	+	++	+	R	R	+	+++
Alternaria solani	50	+	+	++	R	R	R	+	++

+++: Sensitive, ++: Moderately Sensitive, +: Mild Sensitive, R: Resistant

spp. against *Xanthomonas campestris* has already been reported[8]. It is opined that, in addition to the lipolytic and proteolytic enzyme there must be the secretion of some bioactive substances by *Bacillus* sp. V08 which inhibited the growth of *Xanthomonas campestris* and bacterial leaf spot in Tomato**.** The study on fungal pathogens indicates that the extracts can be effectively used to control the sporulation of fungal pathogens[9].

The field application of vermiwash on germinating mustard seeds affected with damping off disease showed recovery from the infection and developed healthy plant without any sign of infection (Fig. 2) caused by the phytopathogens. Similarly the application of vermiwash also prevented the diseases like bacterial leaf spot and early blight in tomato, fusarium wilt in the brinjal. Two months old vermiwash showed a significant result in comparison to others. This indicates that with increase in time period in addition to the enzymes there may be secretion of some secondary metabolites or bioactive compounds by the bacteria present in vermiwash which shows an effective results against the pathogen. Further study is required to find out the bioactive compound.

Sequencing of 16S rRNA gene confirms the molecular identification of the potential bacterial isolate. The bacterial isolates *Bacillus* sp.V08 showed antibiosis against bacterial and fungal phytopathogens was identified by 16S-rRNA gene sequencing and the sequence was submitted to Genbank with accession no.

Table 3. Antibiosis of bacterial isolates against bacterial phytopathogen

Fungal pathogens	Well Content (in µl)	*V01 Flavobac-terium*	*V02 Bacillus* sp.	*V03 Burkholderia* sp.	*V04 Bacillus* sp.	*V05 Bacillus* sp.	*V06 Bacillus* sp.	*V07 Bacillus* sp.	V08 (Accession-KF543076)
Xanthomonas campestris	50	+	+	+	+	R	+	+	++

++: Moderately Sensitive, +: Mild Sensitive, R: Resistant

Table 4. Antibiosis of vermiwash against fungal pathogens

Fungal pathogens	Well Content (in µl)	Fresh vermiwash	1 month old vermiwash	1 month old freezed vermiwash	2 months old vermiwash	2 months old Freezed vermiwash
Fusarium oxysporium	50	R	+	R	+++	R
Fusarium solani	50	R	+	R	+++	R
Rhizoctonia solani	50	R	+	R	+++	R
Alternaria solani	50	R	+	R	+++	R

+++: Sensitive, +: Mild Sensitive, R: Resistant

Table 5. Antibiosis of vermiwash against bacterial pathogens

Bacterial pathogen	Well Content (in µl)	Fresh vermiwash	1 month old vermiwash	1 month old freezed vermiwash	2 months old vermiwash	2 months old freezed vermiwash
Xanthomonas campestris	50	R	R	R	++	R

++: Moderately Sensitive, R: Resistant.

KF543076. The Phylogenetic relationship of *Bacillus* sp. V08 with selected bacteria has been provided. The ancestor of *Bacillus* sp. V08 appears to have obtained the gene from the ancestor of *Bacillus subtilis, Bacillus amyloliquefaciens, Bacillus atrophaeus*. The phylogenetic relationship of Bacillus sp. V08 with selected bacteria has been provided in Fig. 3.

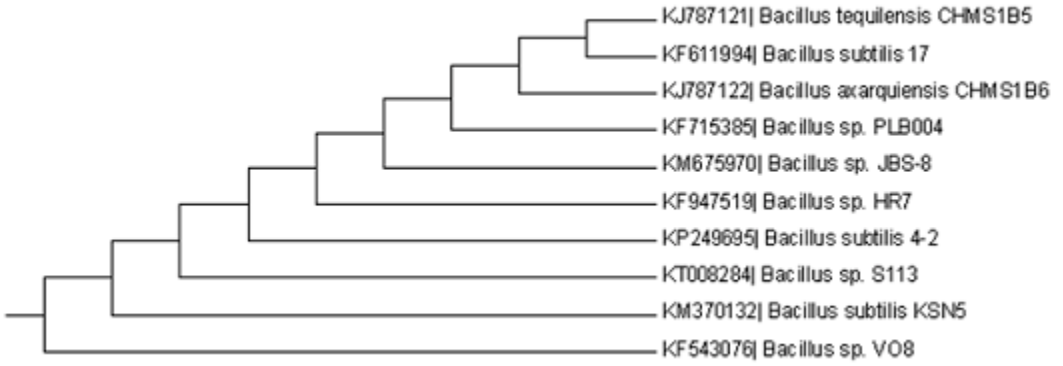


Fig. 3. Phylogenetic relationship of *Bacillus* sp. V08 with selected bacteria

CONCLUSIONS

Bacillus sp. V08 (accession no. KF543076.1) present in vermiwash is found to be effective against the above said bacterial and fungal phytopathogens. Among the five different types of vermiwash is used in the above said experience the two months old vermiwash was found to be most effective in controlling bacterial leaf spot in tomato, early leaf blight in tomato, fusarium wilt in brinjal, damping off root disease in mustard. So it can be used as a potential biocontrol agent for these diseases.

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