Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
www.phytojournal.com
JPP 2020; 9(6): 201-206
Received: 15-09-2020
Accepted: 19-10-2020

VR Bangar
Ph.D. Scholar, Department of Plant Pathology & Agril. Microbiology, Post Graduate Institute, Mahatma Phule Krishi Vidyapeeth, Rahuri. Dist. Ahmednagar, Maharashtra, India

SV Kolase
Associate Professor, Department of Plant Pathology & Agril. Microbiology, Post Graduate Institute, Mahatma Phule Krishi Vidyapeeth, Rahuri. Dist. Ahmednagar, Maharashtra, India

SB Sable
Ph.D. Scholar, Department of Plant Pathology & Agril. Microbiology, Post Graduate Institute, Mahatma Phule Krishi Vidyapeeth, Rahuri. Dist. Ahmednagar, Maharashtra, India

SB Latake
Scientist (Plant Pathology), Pulses Improvement Project, Mahatma Phule Krishi Vidyapeeth, Rahuri. Dist. Ahmednagar, Maharashtra, India

Corresponding Author:
VR Bangar
Ph.D. Scholar, Department of Plant Pathology & Agril. Microbiology, Post Graduate Institute, Mahatma Phule Krishi Vidyapeeth, Rahuri. Dist. Ahmednagar, Maharashtra, India

Screening for fungicide degrading potential of isolated bacterial strains and identification of potent degrading strains

VR Bangar, SV Kolase, SB Sable and SB Latake

Abstract
The total 17 fungicide degrading isolated strains (FDB1 to FDB17) were tested for fungicide degrading potential. All the strains were able to utilize the sole carbon source from fungicides *viz*., difenoconazole, myclobutanil and fluopyram+ tebuconazole at different concentrations for its growth and development but all strains showed variation in their tolerance level of fungicide. In mineral salt agar medium spiked with difenoconazole at 0.5 ml/L, myclobutanil (0.4 g/L) and fluopyram+ tebuconazole (0.5 ml/L), the highest degree of tolerance was visually observed in FDB5, FDB8, FDB13 and FDB16 strains which exhibited moderate growth. In another experiment, all the bacterial strains exhibited the growth in MSM amended with either fungicide or glucose as a sole carbon source throughout the period of study. All the strains were able to utilize a sole carbon source from fungicides as compared with glucose. The turbidity of inoculated broth was increased day by day and recorded as in absorbance. Among all the strains, the bacterial strains *viz*., FDB5, FDB8, FDB13 and FDB16 were showed maximum absorbance on 2 days of incubation and it was approximately near about the absorbance of glucose amended medium. The results indicated that, the absorbance was recorded on 0 days of incubation was approximately ~1.5 × 10^8 CFU/ml counts as compared with McFarland standard. After 1 day of incubation, the absorbance was increase and their count was approximately ~3×10^8 CFU/ml. More than 3×10^8 CFU/ml population was recorded on 2nd day of incubation. It was found that all the fungicide degrading strains had the capability to break down each of the three triazole fungicides and was utilized the carbon for their growth. These fungicide degrading strains were sent to the National Centre for Microbial Resources, Pune for identification. These strains were identified on the basis of 16S rRNA gene sequencing 1200 bp and the results obtained are as; FDB5 (*Lysinibacillus macrolides*), FDB8 (*Inquilinus limosus*), FDB13 (*Stenotrophomonas acidaminiphila*) and FDB16 (*Bacillus subtilis*).

Keywords: Difenoconazole, myclobutanil, fluopyram+ tebuconazole, *Lysinibacillus macrolides*, *Inquilinus limosus*, *Stenotrophomonas acidaminiphila* and *Bacillus subtilis*

Introduction
Biodegradation is associated with environmental bioremediation. Therefore, biodegradation is nature's way of recycling wastes, or breaking down organic matter into nutrients that can be used and reused by other organisms. In the microbiological sense, "biodegradation" means that the decaying of all organic materials is carried out by microorganisms comprising mainly bacteria, yeast, fungi and possibly other organisms. Many soil microorganisms have the ability to act upon pesticides and convert them into simpler non-toxic compounds. This process of degradation of pesticides and conversion into non-toxic compounds by microorganisms is known as “biodegradation”. Biodegradation and bioremediation are matching processes to an extent that both of these are based on the conversion or metabolism of pesticides by microorganisms. The difference between these two is that, the biodegradation is a natural process whereas bioremediation is a technology. In bioremediation, we use microbes to degrade the pesticides *in situ*.

Biodegradation is a process that involves the complete rupture of an organic compound in its inorganic constituents (Paul *et al*., 2005) [6]. It is well known that microorganisms play a major role in degrading pesticides. The higher than MRL levels of pesticide residues on fruits indicate the need for a safe, convenient, and economically feasible method of *in situ* detoxification. Microbial degradation of pesticides has been recognized as a potential solution for elimination of residues. Hence, they can be used to facilitate faster degradation of pesticide residues, by applying them on the fruits subsequent to the pesticide spray.

As earlier, Fang *et al*., (2010) [3] tested the carbendazim degrading ability of *Pseudomonas* strains. Biodegradation of miticide propargite was reported by Sarkar *et al*., (2010) [11] *in vitro* with selected *Pseudomonas* strains isolated from tea rhizosphere on the basis of their tolerance level. Moawad *et al*., (2014) [5] studied the thiram fungicide degradation was assessed by

colorimetric method by peanut rhizobia isolate 8 in broth media amended with 2000 ppm of thiram fungicide (1000 ppm a.i.). Salunkhe *et al*., (2014) [9] conducted an enrichment culture experiment of culture tubes and observed that for all the four *B. subtilis* strains, the cell density in MSM containing carbendazim as the sole carbon source, indicating that each of them was utilized carbendazim as a carbon source for their growth. Salunkhe *et al*., (2015) [8] carried out an enrichment culture experiment to observed utilization of triazole fungicides (myclobutanil, tetraconazole and flusilazole) as sole carbon source by *Bacillus* spp. strains. The *Pseudomonas aeruginosa* PS-4 was investigated by Satapute and Kaliwal, (2016) [12] for its propiconazole degradation ability.

Keeping in view the economic importance of the environmental fates due to extensive use of fungicides the experiments were planned and conducted on screening for fungicide degrading potential of isolated bacterial strains and identification of potent degrading strain.

Materials and Methods

Fungicides

The tested fungicides were obtained from the local market. Particulars of evaluated fungicides are as Difenoconazole 25% EC, Score, Syngenta India Ltd.; Myclobutanil 10% WP, Systhane, Dow Agro Sciences Ltd. and Fluopyram 17.7% + Tebuconazole 17.7 w/w SC, Luna Experience, Bayer Crop Science Ltd.

Fungicide degrading strains

The fungicide degrading potential of previously isolated 17 bacterial strains by Bangar *et al*., 2020. The Gram +ve strains were 3 strains of *Micrococcus* spp., 2 strains of *Bacillus* spp., a strain of *Staphylococcus* spp., *Inquilinus* spp. and *Lysinibacillus* spp. In Gram -ve strains *viz*., 8 strains of *Pseudomonas* spp. and a *Stenotrophomonas* spp. Two experiment were conducted at Department of Plant Pathology and Agricultural Microbiology, Post Graduate Institute, MPKV, Rahuri to identify the potent fungicide bacterial strain.

Screening of fungicide degrading potential of strains for fungicide tolerance

Mineral salt medium was prepared by a composition are K_2HPO_4 (6.30 g), KH_2P0_4 (1.82 g), NH_4N0_3 (1.00 g), $MgS0_4.7H_20$ (0.20 g), $CaCl_2.2H_20$ (0.10 g), $FeS0_4.7H_20$ (0.10 g), $Na_2Mo0_4.2H_20$ (0.06 g), $MnS0_4.7H_20$ (0.06 g) and Distilled water (1000 ml). Mineral salt agar medium was prepared, boiled, filtered and the pH was adjusted to 7.0. The medium was then dispensed in 250 ml quantities in 500 ml Erlenmeyer flasks and sterilized by autoclaving at 121 0C temperature, 15 psi pressure for 20 min. After sterilization, three concentrations of fungicides i.e. 0.1, 0.3 and 0.5 ml/L for difenoconazole and fluopyram + tebuconazole; while 0.1, 0.2 and 0.4 g/L concentrations of fungicide myclobutanil mixed in each flask prior to pouring, separately. Then 20 ml mineral salt agar medium spiked with fungicides was pour aseptically into each sterilized petri plates separately. Seventeen bacterial strains were screened for fungicide tolerance in sterilized MSM petri plates containing three concentrations 0.1, 0.3 and 0.5 ml/L of fungicides *viz*., difenoconazole and fluopyram + tebuconazole; while 0.1, 0.2 and 0.4 g/L of fungicide myclobutanil as a sole carbon source separately. Mineral salt agar plates without fungicides as well as any carbon source were maintained as a control. Each treatment was repeated three times. The individual bacterial strains were streak aseptically with the help of bacterial inoculating loop and incubated at 30 ^{0}C in incubator.

Observations

After 7 days of incubation, the strains exhibiting the highest degree of fungicide tolerance were selected on the basis of visual growth observed as below (Sarkar *et al*., 2010) [11].

Good growth	:	+ + +	Poor growth	:	+
Moderate growth	:	+ +	No growth	:	-

Growth profile of bacterial strains in different carbon source amended mineral salt medium (enrichment culture) by spectrophotometer:

The growth profile of the 17 fungicide degrading bacterial strains was tested in mineral salt medium spiked with each fungicide *viz*., difenoconazole, myclobutanil and fluopyram + tebuconazole at 10 ppm concentration, separately. MSM amended with glucose was served as a control to know the utilization of carbon source by bacterial strains. Erlenmeyer flask containing sterilized 100 mL mineral salt medium with 10 ppm as a carbon source either from glucose or above fungicides were inoculated separately with 1ml (1×10^8 CFU mL^{-1}) inoculum of each bacterial strain individually and incubated in rotary shaker cum incubator at 28 °C and 150 rpm for 2 days under natural daylight. The absorbance was recorded at 630 nm on a spectrophotometer at 0, 1 and 2 days after inoculation (Salunkhe *et al*., 2014b) [9]. Absorbance data was compared with the McFarland standard to know approximate bacterial population (cell density). Based on above studies, those strains which was able to utilize, tolerate the maximum concentration of fungicide and exhibited maximum absorbance as a sole carbon source from fungicide for its growth and development that were selected. FDB5, FDB8, FDB13 and FDB16 strains were selected for further studies.

Identification of potent fungicide degrading strains

The four pure bacterial cultures *viz*., FDB5, FDB8, FDB13 and FDB16 were streaked for single colonies on petri plates individually. It was sent to the National Centre for Microbial Resources (NCMR) under National Centre for Cell Science (NCSS), Pune for an identification based on 16S rRNA gene sequencing (~1200bp).

Statistical analysis

The data obtained were statistically analyzed by the methods suggested by Gomez and Gomez (1984) [4]. The standard error and critical difference were worked out and the results obtained were compared statistically.

Results and Discussion

Screening of fungicide degrading potential of strains for fungicide tolerance

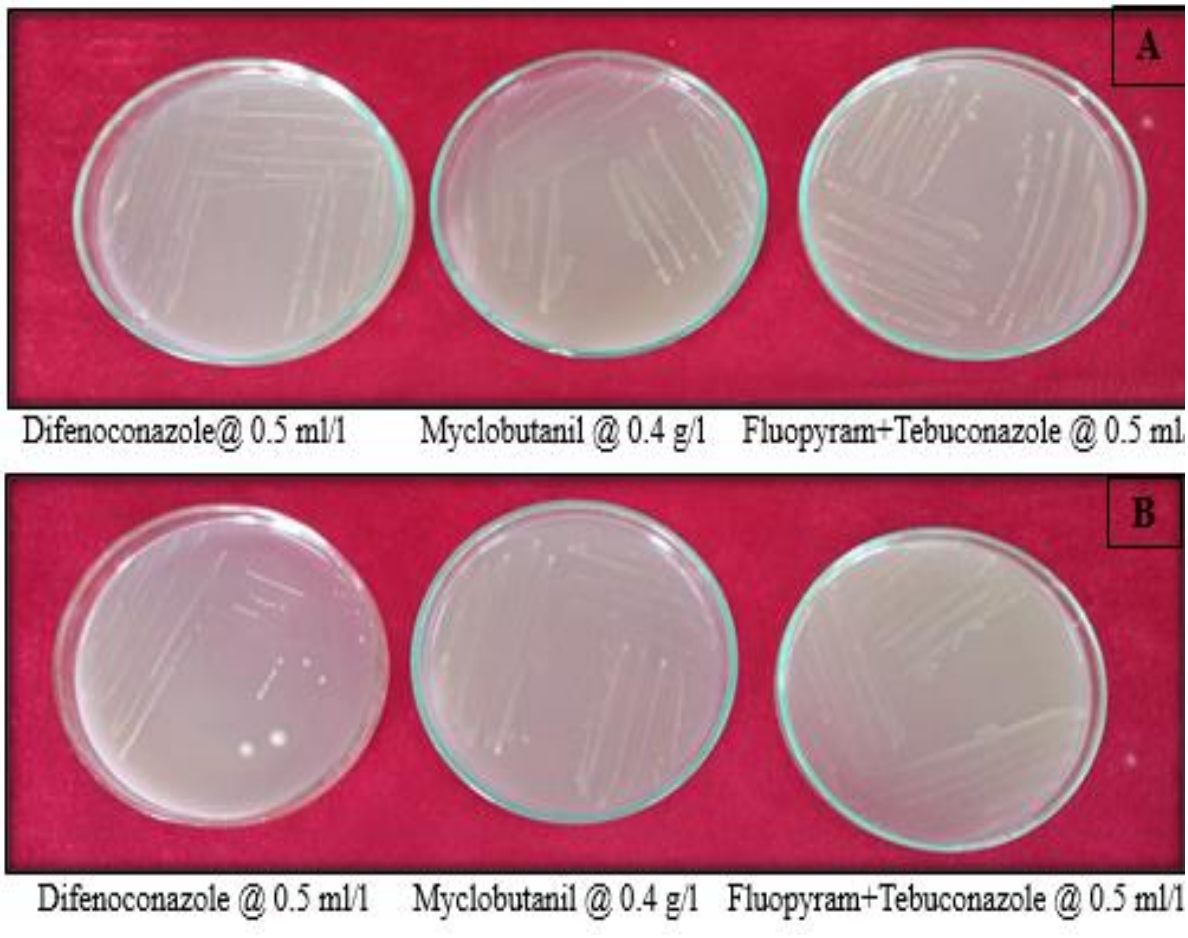
Seventeen strains were screened to identify their fungicide degrading potential based on their highest degree of fungicide tolerance. The visual colony growth of bacterial strains was observed in fungicide amended MSM agar plates as a sole carbon source for its growth and development. The result regarding fungicide tolerance level exhibited by strains was obtained and is presented in Table 1. The results revealed that, all the strains were able to utilize the sole carbon source from fungicides *viz*., difenoconazole, myclobutanil and fluopyram+ tebuconazole for its growth and development but all the strains showed variation in their tolerance level of fungicide.

There was no growth observed in control. In mineral salt agar medium spiked with difenoconazole at 0.5 ml/L concentration, the highest degree of tolerance was visually observed in FDB5, FDB8, FDB13 and FDB16 strains which showed moderate growth (PLATE I).

Table 1: Screening of bacterial strains for fungicide tolerance on mineral salt agar medium spiked with different concentrations of fungicides

Strains	Bacterial growth on MSAM spiked with fungicides at different concentration (ml/L or g/L)								
	Difenoconazole			Myclobutanil			Fluopyram+Tebuconazole		
	0.1	0.3	0.5	0.1	0.2	0.4	0.1	0.3	0.5
FDB-1	+ +	+	-	+ +	++	+	+ +	+ +	+
FDB-2	+ +	+	+	++	+	+	+	+	-
FDB-3	+ +	++	+	++	++	+	++	+	+
FDB-4	+ +	+	-	++	+	+	+ +	+	+
FDB-5	+ + +	+ +	+ +	+ + +	++	+ +	+ + +	+ +	+ +
FDB-6	+	+	+	+ +	+	+	+ +	+	+
FDB-7	+ +	+	+	+ +	+	+	+	+	+
FDB-8	+ + +	+ +	+ +	+ + +	+ +	+	+ + +	+ +	+ +
FDB-9	+	+	+	+	+	-	+	-	-
FDB-10	+	+	+	+	-	-	+	-	-
FDB-11	+	+	-	+	-	-	+	-	-
FDB-12	+	-	-	+	-	-	+	+	-
FDB-13	+ + +	+ +	+ +	+ + +	+ +	+ +	+ + +	+ +	+ +
FDB-14	+	+	-	+	-	-	+	+	-
FDB-15	+	-	-	+	-	-	+	-	-
FDB-16	+ + +	+ +	+ +	+ + +	+ +	+ +	+ + +	+ +	+ +
FDB-17	+ +	+	+	+ +	+	+	+ +	+	+

In case of myclobutanil (0.4 g/L) and fluopyram+ tebuconazole (0.5 ml/L) as a carbon source, the bacterial strains *viz*., FDB5 (*Lysinibacillus macrolides*), FDB8 (*Inquilinus limosus*), FDB13 (*Stenotrophomonas acidaminiphila*) and FDB16 (*Bacillus subtilis*) were showed moderate growth. These strains were tolerate the higher concentration of all fungicides used in this study and utilize the carbon source from these fungicides for its growth. Hence, these four strains showed highest potential to grow by utilizing a sole carbon source from fungicide amended medium. These results are in accordance with the findings of Sarkar *et al*., (2009) [10] and Diwedi *et al*., (2010) [1]. Earlier, Eizuka *et al*., (2003) [2] reported the ipconazole fungicide tolerance at 1, 10 and 100 mg/L concentration with degrading microorganisms. These results are similar with the findings of Yadav *et al*., (2015) [13].



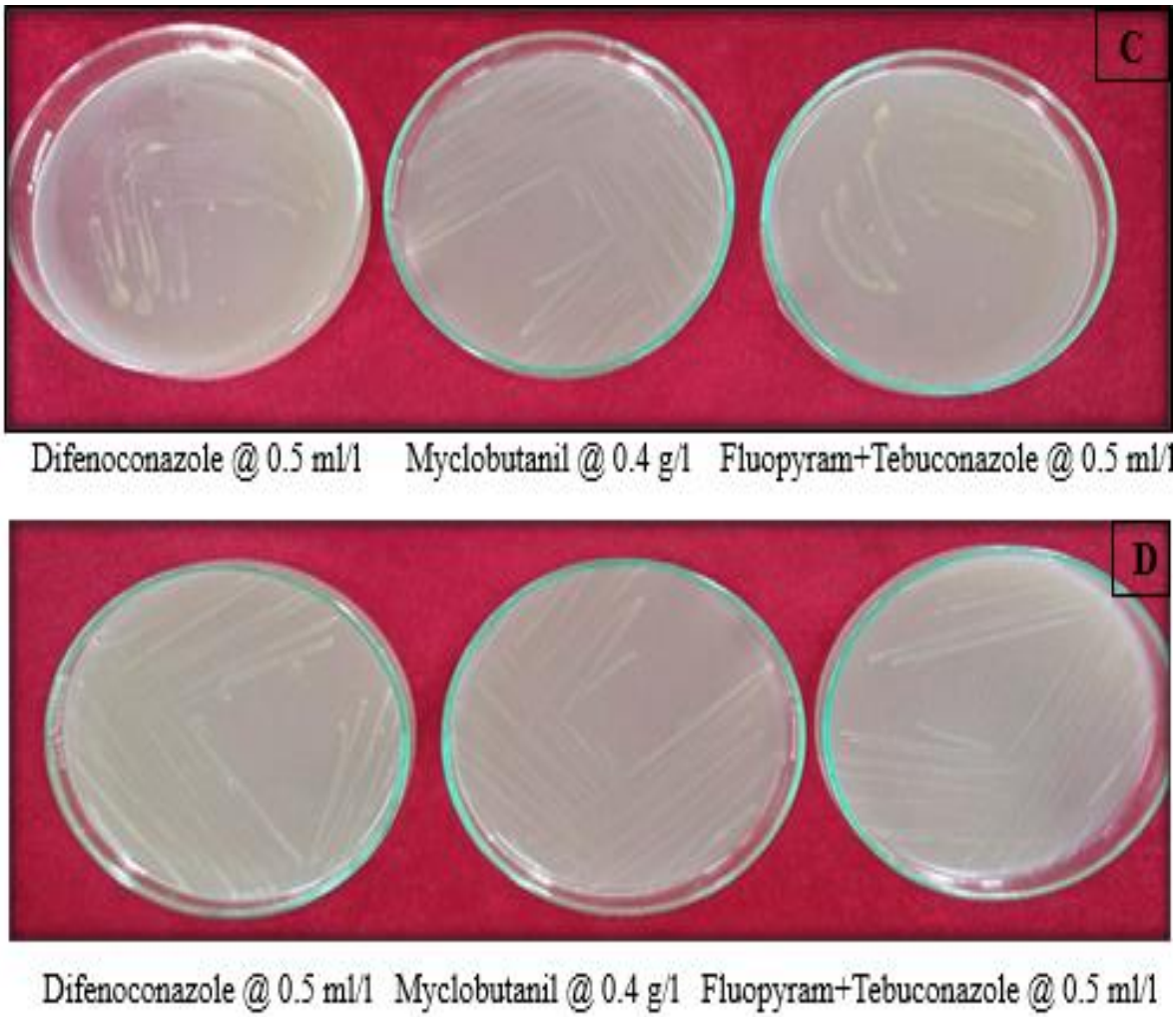


Plate I: Growth of fungicide degrading strains; A) FDB 5 (*Lysinibacillus macrolides*), B) FDB 8 (*Inquilinus limosus*), C) FDB 13 (*Stenotrophomonas acidaminiphila*) and D) FDB 16 (*Bacillus subtilis*) on MSM amended with fungicide at 7 DAI

Growth profile of bacterial strains in MSM either amended with fungicide or glucose (enrichment culture) by spectrophotometer

Another laboratory experiment was conducted for screening of bacterial strains to check the fungicide degrading potential on the basis of utilization of carbon source from fungicide. In time frame observations on 0, 1 and 2 days after incubation were recorded on spectrophotometer at 630 nm.

It is revealed from the data presented in Table 2 that, all the bacterial strains showed significant growth in MSM amended with either fungicide or glucose as a sole carbon source throughout the period of study. All the strains were able to utilize a sole source of carbon from fungicides as compared with glucose. The turbidity of inoculated broth was increased day by day and observed in terms of absorbance. Results indicated that, the absorbance were recorded on 0 days of incubation was approximately ~ 1.5×10^8 CFU/ml counts as compared with McFarland standard. After 1 days of incubation, the absorbance was increased and their count was approximately ~ 3×10^8 CFU/ml. More than 3×10^8 CFU/ml bacterial population were recorded at 2 days of incubation. This shows that all the fungicide degrading strains had the capability to break down each of the three triazole fungicides and was able to utilize the carbon for their growth. Among all the strains, the bacterial strains *viz*., FDB5, FDB8, FDB13 and FDB16 were showed maximum absorbance on 2 days of incubation and it was approximately near about the absorbance of glucose amended medium. Hence, the FDB5, FDB8, FDB13 and FDB16 strains were selected for further study (PLATE II).

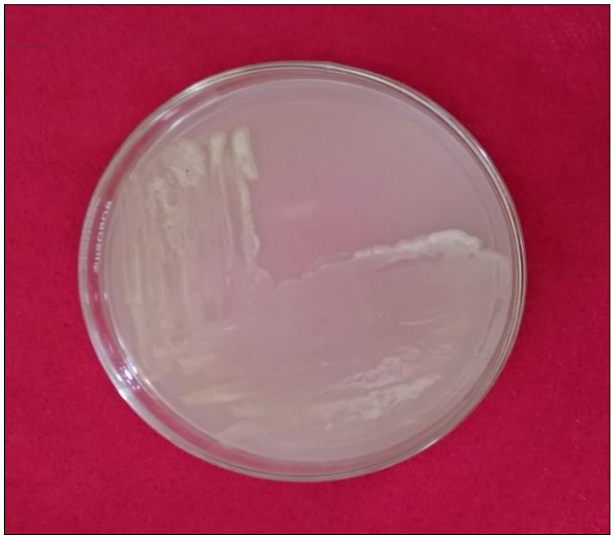
The results are similar to earlier findings of Yadav *et al*., (2015) [13]. The above findings are in conformity with the result of Salunkhe *et al*., (2015c) [8] and found that absorbance data for bacterial growth of *Bacillus* strains in the range of 0.254 – 0.225, 0.213 – 0.261, 0.221 – 0.261 and 0.233 – 0.291 for tetraconazole, myclobutanil, flusilazole and glucose, respectively as a sole carbon source in broth after 1 DAI. The results of present study are also in agreement with the findings of Salunkhe *et al*., (2013a) [7] and Salunkhe *et al*., (2014b) [9] in profenophos and carbendazim amended MSM inoculated by different *Bacillus subtilis* strains, respectively.

Table 2: Screening of bacterial strains for its growth (turbidity) in MSM broth spiked with either fungicides or glucose

Strains	Absorbance @ 630 nm (MSM spiked with different carbon sources @ 10 ppm)											
	0 DAI				1 DAI				2 DAI			
	D	M	F+T	G	D	M	F+T	G	D	M	F+T	G
FDB1	0.105	0.096	0.111	0.086	0.222	0.206	0.212	0.235	0.412	0.354	0.408	0.553
FDB2	0.103	0.097	0.109	0.086	0.218	0.205	0.217	0.239	0.424	0.370	0.423	0.574
FDB3	0.103	0.099	0.122	0.091	0.232	0.219	0.220	0.244	0.392	0.343	0.417	0.542
FDB4	0.102	0.094	0.112	0.084	0.238	0.229	0.224	0.256	0.383	0.361	0.414	0.565
FDB5	0.110	0.102	0.117	0.100	0.285	0.295	0.273	0.309	0.681	0.613	0.649	0.784
FDB6	0.110	0.094	0.117	0.091	0.232	0.226	0.218	0.255	0.408	0.422	0.381	0.557
FDB7	0.109	0.093	0.117	0.086	0.219	0.213	0.223	0.243	0.393	0.421	0.408	0.584
FDB8	0.105	0.096	0.119	0.099	0.244	0.244	0.270	0.292	0.522	0.541	0.644	0.774
FDB9	0.098	0.093	0.110	0.088	0.213	0.209	0.204	0.234	0.388	0.354	0.394	0.513
FDB10	0.108	0.091	0.113	0.097	0.236	0.219	0.208	0.249	0.352	0.374	0.387	0.504
FDB11	0.109	0.097	0.117	0.094	0.234	0.236	0.217	0.255	0.381	0.414	0.370	0.575
FDB12	0.106	0.092	0.112	0.088	0.229	0.219	0.217	0.245	0.421	0.435	0.383	0.619
FDB13	0.109	0.101	0.120	0.102	0.253	0.263	0.235	0.274	0.623	0.632	0.582	0.711
FDB14	0.105	0.093	0.114	0.094	0.217	0.205	0.206	0.238	0.382	0.414	0.452	0.660
FDB15	0.108	0.095	0.119	0.099	0.229	0.222	0.217	0.258	0.378	0.393	0.434	0.573
FDB16	0.108	0.103	0.121	0.103	0.261	0.277	0.249	0.294	0.635	0.681	0.619	0.744
FDB17	0.108	0.102	0.115	0.099	0.211	0.203	0.220	0.232	0.511	0.513	0.472	0.643
SEm(±)	0.02	0.01	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
CD at 1%	0.06	0.05	0.06	0.07	0.06	0.06	0.06	0.07	0.06	0.07	0.06	0.07

DAI, Days after inoculation; D, Difenoconazole; M, Myclobutanil; F+T, Fluopyram+Tebuconazole; G, Glucos

FDB 5 (*Lysinibacillus macrolides*)



FDB 8 (*Inquilinus limosus*)

FDB 13 (*Stenotrophomonas acidaminiphila*)

FDB 16 (*Bacillus subtilis*)

Plate II: Potent fungicide degrading bacterial strains

Identification

These fungicide degrading strains were sent to the National Centre for Microbial Resources, Pune for identification. These strains were isolated on the basis of 16S rRNA gene sequencing 1200 bp and the results obtained are as, FDB5 (*Lysinibacillus macrolides*), FDB8 (*Inquilinus limosus*), FDB13 (*Stenotrophomonas acidaminiphila*) and FDB16 (*Bacillus subtilis*).

Conclusion

Among the 17 fungicide degrading strains; FDB5, FDB8, FDB13 and FDB16 strains were found to be more potent to utilize the sole carbon source from the fungicides for its growth. The highest fungicide tolerance as carbon source was observed in FDB5, FDB8, FDB13 and FDB16 strains at recommended doses of fungicide. The strains *viz*., FDB5 (*Lysinibacillus macrolides*), FDB8 (*Inquilinus limosus*), FDB13 (*Stenotrophomonas acidaminiphila*) and FDB16 (*Bacillus subtilis*) showed growth in enrichment culture to utilize the sole carbon from fungicide likely as compared with glucose.

Reference

1. Dwivedi S, Singh BR, Al-Khedhairy AA, Alarifi S, Musarrat J, Isolation and characterization of butachlor-catabolizing bacterial strain *Stenotrophomonas acidaminiphila* JS-1 from soil and assessment of its biodegradation potential. Letters in Applied Microbiology 2010;51:54-60.
2. Eizuka T, Ito A, Chida T. Degradation of Ipoconazole by Microorganisms Isolated from Paddy Soil. J Pestic Sci 2003;28(2):200-207.
3. Fang H, Wang Y, Gao C, Yan H, Dong B, Yu Y. Isolation and characterization of *Pseudomonas* spp. CBW capable of degrading carbendazim. Biodegradation 2010;21(6):939-946.
4. Gomez KA, Gomez AA. Statistical procedures for agricultural research. 2nd ed. Willey, New York, USA, 1984, 880.
5. Moawad H, Abd El-Rahim WM, Shawky H, Higazy AM, Daw ZY. Evidence of Fungicides Degradation by Rhizobia. Agricultural Sciences 2014;5:618-624.
6. Paul D, Pandey G, Pandey J, Jain RK. Accessing microbial diversity for bioremediation and environmental restoration. Trends in Biotechnology 2005;23:135-142.
7. Salunkhe VP, Sawant IS, Banerjee K, Rajguru YR, Wadkar PN, Oulkar DP *et al*. Biodegradation of profenofos by *Bacillus subtilis* isolated from grapevines (*Vitis vinifera*). Journal of Agricultural and Food Chemistry 2013a;61:7195-7202.
8. Salunkhe VP, Sawant IS, Banerjee K, Wadkar PN, Sawant SD. Enhanced Dissipation of Triazole and Multiclass Pesticide Residues on Grapes after Foliar Application of Grapevine-Associated *Bacillus* Species. J Agric. Food Chem, 2015c;63:10736-10746. DOI: 10.1021/acs.jafc.5b03429
9. Salunkhe VP, Sawant IS, Banerjee K, Wadkar PN, Sawan SD, Hingmire SA. Kinetics of degradation of carbendazim by *B. subtilis* strains: possibility of in situ detoxification. Environ Monit Assess, 2014b;186:8599-8610. DOI 10.1007/s10661-014-4027-8
10. Sarkar S, Seenivasan S, Premkumar R. Biodegradation of propiconazole by *Pseudomonas putida* isolated from tea rhizosphere. Plant Soil Environment 2009;55:196-201.
11. Sarkar S, Seenivasan S, Premkumar R, Asir S. Biodegradation of propargite by *Pseudomonas putida*, isolated from tea rhizosphere. Journal of Hazardous Materials 2010;174:295–298. doi:10.1016/j.jhazmat.2009.09.050
12. Satapute P, Kaliwal B. Biodegradation of the fungicide propiconazole by Pseudomonas aeruginosa PS-4 strain isolated from a paddy soil. Annals of Microbiology, 2016;66(4):1355-1365. DOI 10.1007/s13213-016-1222-6
13. Yadav S, Verma SK, Chaudhary HS. Isolation and characterization of organophosphate pesticides degrading bacteria from contaminated agricultural soil. On Line Journal of Biological Sciences 2015;15(1):113-125.