Isolation and identification of Triazole group fungicide degrading isolates from grape rhizosphere of major grape growing districts of Maharashtra, India

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Abstract

Grape (Vitis vinifera L.) is the most important temperate fruit crop that has acclimatized to the subtropical and tropical agro-climatic conditions. Thirty soil samples were collected from grape vineyards rhizosphere of different locations of Nashik, Ahmednagar, Pune, Solapur and Sangli districts of Maharashtra during 2017-18. The 17 isolates (FDB1 to FDB17) were obtained from soil samples which had ability to degrade difenoconazole, myclobutanil and fluopyram+ tebuconazole fungicides. On the basis of colony characteristics as well as biochemical test, eight Gram +ve strains and nine Gram -ve strains were identified up to the generic level. 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., eight strains of Pseudomonas spp. and a strain of Stenotrophomonas spp. were identified which has ability to use sole carbon source from fungicide for its growth.

Keywords: Difenoconazole, myclobutanil and fluopyram+ tebuconazole, Micrococcus spp., Bacillus spp., Staphylococcus spp., Inquilinus spp., Lysinibacillus spp., Pseudomonas spp. and stenotrophomonas spp

Introduction

Grape is commercially cultivated in the tropical wet and dry or the arid and semiarid climatic regions of peninsular India where the weather is conducive to support insect pest attacks on grapevines during both the vegetative and fruiting seasons. The important grapevine diseases are powdery mildew, downy mildew, anthracnose, dead arm, gray mold or bunch rot, black rot, crown gall and bacterial leaf spot. Various fungicides are commonly used to manage diseases of grapevine.

Powdery mildew, caused by the biotrophic fungus Erysiphe necator (Schw.) Burr (earlier Uncinula necator), is one of the most important fungal diseases of grapevines. In table grape vineyards located in Maharashtra state, India, powdery mildew infections can be seen almost throughout the year, except during the hot and dry months of summer season and for a brief period during the cold winter months. Thompson Seedless and other commercial table grape varieties grown in this region are highly susceptible to powdery mildew (Sawant and Sawant, 2012). Frequent pesticide applications are necessary to control the different pests and prevent qualitative and quantitative losses. Powdery mildew management, thus, is very challenging and constraints the grape growers to apply the recommended fungicides at frequent intervals to achieve the desired level of disease control. Difenoconazole, myclobutanil and fluopyram + tebuconazole are relatively broad spectrum systemic fungicides, belonging to the triazole family of chemicals, and are commonly used for the control of powdery mildew in vineyards. Triazole group fungicides are known to be fairly soluble in water, although they are not readily degradable and have a limited sorption tendency. Triazole fungicides are toxic and persist in the soil for long periods of time, thus affecting soil fertility and microflora (Elmholt, 1992 and Munier and Borde, 2000). Microorganisms are most desirable biological tools, because of their ability to resist various pesticides and their metabolic capacity to degrade toxic compounds into nontoxic forms.
Materials and Methods

Identification of isolates

Isolation, incubation and purification

Isolation, incubation and purification
Thirty soil samples were air dried at room temperature and were allowed to retain 20% (w/w) moisture content. These soil samples were passed through sieve with 2 mm mesh. Mineral salt medium (Seubert, 1960) [22] was prepared by a composition are K_{2}HPO_{4} (6.30 g), KH_{2}PO_{4} (1.82 g), NH_{4}NO_{3} (1.00 g), MgSO_{4}.7H_{2}O (0.20 g), CaCl_{2}.2H_{2}O (0.10 g), FeSO_{4}.7H_{2}O (0.10 g), Na_{2}MoO_{4}.2H_{2}O (0.06 g), MnSO_{4}.7H_{2}O (0.06 g) and Distilled water (1000 ml). The MSM was boiled, filtered and the pH was adjusted to 7.0. The medium was then dispensed in 100 ml quantities in 250 ml Erlenmeyer flasks. It was sterilized by autoclaving at 121 °C temperature, 15 psi pressure for 20 min. Solid media contained 1.5 - 2.0 per cent agar in mineral salts medium. One gram of each soil sample was added to each 250 ml Erlenmeyer flask containing 100 ml of sterilized mineral salt medium (MSM) to which 5 mg/L or ml/L concentration of difenoconazole, myclobutanil and fluopyram + tebuconazole were added separately. These flasks were incubated using rotary shaker cum incubator at 30 °C and 150 rpm for a period of 7 days. One ml of this 7 days old culture was transferred to sterilized flask with fresh mineral salt medium (MSM) containing 10 mg/L concentration of above mentioned fungicides and incubated for another 7 days. Further one ml of this culture was transferred to fresh MSM with 10 mg/L fungicide concentration and incubated at 30 °C and 150 rpm for another week. These procedure was followed up to 28 days.

After 28 days of incubation, one ml enriched sample were separately pipette out and diluted using serial dilution technique up to ten fold dilutions aseptically and spread on sterilized mineral salt agar medium plates containing 5 mg/L fungicides individually. The plates were incubated at 37 °C for 96 hrs. Individual colonies were streaked and re-streaked repeatedly and the pure cultures were stored at 4 °C till further experimentation.

Identification of isolates

Colony characteristics

The efficient fungicide degrading strains were characterized (identified up to generic level) based on colony characteristics and biochemical tests as detailed below. Colony characteristics of the isolates were studied according to Holt et al., (1994) [9]. All the tests were performed using actively growing bacterial cultures. Bacterial colonies were observed for colony morphology and Gram’s staining. Bacterial cultures were grown on Nutrient agar plates to examine the colour (white, cream, yellow, creamish yellow, brown, light brown, pink); size (pinpointed, small, large, very large); shape (circular, irregular, filamentous, rhizoid); margin (entire, undulate, filiform, lobate); elevation (flat, raised, convex, pulvinate, umbonate); surface (smooth, rough, glistening, dull) and pigmentation [present (+), absent (-)] properties determined by visual observation as well as by using light microscope.

Biochemical test

Biochemical tests of the isolates were studied according to Holt et al., (1994) [9]. Bacterial isolates were also subjected to biochemical tests viz., Gram staining Amylease activity, Gelatinase activity Gelatinase activity Catalase test, Hydrolysis of starch, Casein hydrolysis, Acid and gas
production, Citrate utilization, Motility Test, Oxidase Test, Urease Test, Methyl Red Test, Voges-Proskauer (V.P.) Test, Indole Test, Urease test, Nitrate Reduction and Fermentation of carbohydrate were carried out according to Bergey’s Manual of Systematic Bacteriology (Holt et al., 1994) [9].

Results and Discussion

Isolation of fungicide degrading microorganisms

The soil samples were collected from grape orchards of different tehsils as well as locations of Nashik, Ahmednagar, Pune, Solapur and Sangli districts of Maharasthra during 2017 – 2018. The 17 isolates (FDB1 to FDB17) were obtained from soil samples which had ability to degrade difenoconazole, myclobutanil and fluopyram+ tebuconazole fungicides on mineral salt medium (PLATE 1). The obtained fungicide degrading isolates are presented in Table 1.

Table 1: List of obtained fungicide degrading isolates from major grape growing districts in Maharashtra

| Sr. No. | District | Tehsil | Location | GPS Location | Obtained isolates | Assign isolate code |
|---------|----------|--------|----------|--------------|-------------------|---------------------|
| 1       | Nashik   | Niphad | Nandur Madhashwar | 20.018576 | 74.149475 | 3 | FDB1 |
|         |          | Dindori | Mavadi | 20.317182 | 73.933401 | 2 | FDB4 |
|         |          | Nashik | Matori | 20.054277 | 73.737447 | 1 | FDB6 |
| 2       | Ahmednagar | Rahuri | MPKV | 19.350359 | 74.651093 | 1 | FDB7 |
|         |          | Rahata | Loni | 19.583452 | 74.484661 | 1 | FDB8 |
| 3       | Pune     | Junnar | Narayangaon | 19.126099 | 73.968801 | 1 | FDB9 |
|         |          | Ambeagaon | Kalamb | 19.046274 | 73.954312 | 1 | FDB10 |
|         |          | Haveli | Manjri | 18.334663 | 73.995161 | 1 | FDB11 |
| 4       | Solapur  | Malshiras | Paniv | 17.648353 | 74.954313 | 1 | FDB12 |
|         |          | Pandharpur | Kasegaon | 17.614136 | 75.334145 | 1 | FDB13 |
| 5       | Sangli   | Walwa | Walwa | 17.031158 | 74.380154 | 1 | FDB14 |
|         |          | Taugaon | Kunhhe | 16.962895 | 74.657097 | 1 | FDB15 |
|         |          | Jath | Daphalapur | 16.973460 | 75.097541 | 2 | FDB16 |
|         |          | Total       |            |              |                  | 17                        |

It was observed from Table 1, the maximum number of fungicide degrading isolates were obtained from Nashik district (6 isolate) and it was followed by Sangli (4 isolate), Pune (3 isolate), Ahmednagar (2 isolate) and Solapur district (2 isolates).

Identification of isolates

All the 17 fungicide degrading isolates were subcultured on nutrient agar medium and incubated at 30 °C for 2 days. The colony characteristics of fungicide degrading isolates are given in Table 2.

Colony characteristics

Results revealed that, the slight variation among the all isolates were observed in colony characteristics viz., shape, margin, elevation, surface structure, consistency, opacity and pigmentation. Results indicated that, cell shape were irregular, round and uneven; margin were entire, lobed and undulate; elevation of colony were low convex, convex and flat; surface texture of colonies were smooth; consistency were watery, gummy and coarse; opacity of colonies were translucent or opaque and pigmentation such as, light red, light yellow, creamy, light green and grey white were observed in colony characteristics of these isolates.

Biochemical test

Different biochemical test were performed to characterized and identify strains up to genera on the basis of Bergy’s Manual of Determinative Biology (Holt et al., 1994) [9]. The results of biochemical test of 17 fungicide degrading isolates are presented in Table 3.

On the basis of colony characteristics as well as biochemical test, eight Gram +ve strains and nine Gram -ve strains were identified up to generic level. 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 streptotrophomonas spp. were identified.

Results revealed that, the maximum number of isolates were obtained as Pseudomonas spp. All the strains were showed MR and VP test negative. In case of indole production test, only strain FDB17 (Bacillus spp.) was showed positive while rest of the all strains were negative to indole production test. FDB1 to FDB17 strains were for positive to catalase test.

Table 2: Colony characteristics of different fungicide degrading isolates

| Sr. No. | Isolates | Shape | Margin | Elevation | Consistency | Opacity | Pigmentation |
|---------|----------|-------|--------|-----------|-------------|---------|-------------|
| 1       | FDB1     | Irregular | Lobed  | Low convex | Watery      | Translucent | Light Yellow |
| 2       | FDB2     | Uneven  | Entire | Flat      | Gummy      | Opaque    | Light Yellow |
| 3       | FDB3     | Round   | Entire | Convex    | Gummy      | Opaque    | White       |
| 4       | FDB4     | Uneven  | Entire | Flat      | Gummy      | Opaque    | Light Yellow |
| 5       | FDB5     | Round   | Entire | Flat      | Gummy      | Opaque    | Creamy      |
| 6       | FDB6     | Irregular | Lobed  | Low convex | Watery      | Translucent | Light yellow |
| 7       | FDB7     | Uneven  | Entire | Flat      | Gummy      | Opaque    | White Yellow |
| 8       | FDB8     | Uneven  | Entire | Flat      | Gummy      | Opaque    | White Yellow |
| 9       | FDB9     | Uneven  | Entire | Flat      | Gummy      | Opaque    | Light Yellow |
| Identified genera | FDB1 | FDB2 | FDB3 | FDB4 |
|------------------|------|------|------|------|
| Micrococci spp.  | +    | +    | +    | +    |
| Pseudomonas spp. | +    | +    | +    | +    |
| Staphylococcus spp. | + | + | + | + |
| Pseudomonas spp. | + | + | + | + |
| Bacillus spp. | + | + | + | + |
| Bacillus subtilis | + | + | + | + |
| Bacillus licheniformis | + | + | + | + |

The findings of present investigation are also in accordance with Pawar and Mali, (2014a) [13] and Pawar and Mali, (2014b) [14] who collected the soil samples from grape rhizosphere and isolated the dichlorovos degrading strain of *Bacillus* spp. and Quinolphos degrading *Pseudomonas* spp., respectively. Results of present study are in the conformity with earlier findings of Satapute and Kaliwal, (2016) [10] that isolated the propiconazole fungicide degrading *Pseudomonas aeruginosa* strains from paddy field. Salunkhe et al. (2015) [15] reported that phenopentos, carbendazim, myclobutanil, flusilazole and tetraconazole in MSM as well as on grape berries degrade effectively by *Bacillus subtilis* strains. Yadav et al., (2016) reported that *Staphylococcus* spp., *Micrococcus* spp. and *Pseudomonas* spp. strains had degrading Malathion and Dichlorvos insecticides. Results of present study are in the conformity with earlier findings of Mercadier et al., (1997) [11] who isolated the iprodione degrading bacteria viz., *Pseudomonas fluorescens, Pseudomonas* spp. and *Pseudomonas paucimobilis* from soil. Eizuka et al., (2003) [6] isolated ipconazole triazole fungicide degrading microorganisms consisting a bacterial, 12 actinomycetous and 7 fungal strains from paddy soil. Sarkar et al., (2009) [17] isolated propargite degrading *Pseudomonas* strains from tea rhizosphere soil on MSM. Sehrem, et al., (2009) [21] reported that tebuconazole biodegradation by bacteria isolated from contaminated soils. Dwivedi, et al., (2010) [13] isolated and characterized butachlor-catabolizing bacterial strain *Stenotrophomonas acidaminiphila* JS-1 from soil. Fang et al., (2010) [13] isolated the *Pseudomonas* spp. on MSM from carbendazim contaminated soil in vegetable cultivation under greenhouse. Cycon et al., (2011) [13] isolated *Bacillus* spp. TDS-2 strain from sandy soil previously treated with thiofanate methyl (TM) in mineral salt medium (MSM) and soil. Earlier, Youness et al., (2018) [24] isolated the tebuconazole degrading *Bacillus* strains and *Pseudomonas* spp. Samadi, et al., (2019) [16] isolated *Lysinibacillus macrolides* and *Bacillus firmus* strains from contaminated soil which had biodegrading ability of polychlorinated biphenyls.
Conclusion
Fungicide degrading isolates (FDB1 to FDB17) were obtained from all major grape growing districts of Maharashtra. It was naturally occurring in rhizosphere soil due to better adaptability of microbes to pesticide contaminated sites over many years.

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