IJFANS INTERNATIONAL JOURNAL OF FOOD AND NUTRITIONAL SCIENCES
ISSN PRINT 2319 1775 Online 2320 7876
 UGC CARE Listed (Group -I) Journal Volume 11, Iss 10, 2022

In-vitro Evaluation of Antibacterial Potential of Biocontrol Agents Against Pathogens of *Punica granatum* L.

[1]Sontakke K. S[*]. [2]Bulbule V. M., Kulkarni R. L.
[1*]Assistant Professor, Department of Botany, G.S.G. College Umarkhed, Yavatmal-445206, Maharashtra, India
[2]Research Scholar, Department of Botany, Lal Bahadur Shastri Mahavidyalaya, Dharmabad, Nanded - 431809, Maharashtra, India.
[3]Assistant Professor Department of Botany, Smt. Radhabai Sarda College, Anjangaon Surji, Amravati -444705, Maharashtra, India.

ABSTRACT

Pomegranate (*Punica granatum* L.) is one of the highly valued table fruits, grown in various parts of the world as it has great commercial, medicinal and nutritive value. India is one of the major producers of pomegranates that are exported to various parts of the world and Maharashtra leads in pomegranate production in the country. Thus, in India it is a serious economic threat to pomegranate growers due to lack of effective control measures. Biological control is the most viable approach for the treatment of plant diseases. Thus, there is a need for a safe biocontrol agent. In view of this background, several samples of leaves, fruits, buds and fruits were collected from Pomegranate orchards of Maharashtra for the evaluation of bacterial population and isolation of *Trichoderma species*. Thus, to evaluate the possibility of their extendable use as biocontrol agents in pomegranate. Pomegranate crop is attacked by several bacterial and fungal pathogens. Thus, these diseases of pomegranate decrease the market value of fruits. Use of bactericides and fungicides is a conventional method to control infections which has hazardous impact on public health and these agrochemicals are not cost effective. Biological control is the most viable approach for the treatment of plant diseases. Thus, there is a need for a safe biocontrol agent and the Trichoderma fungi belong to this category. The biocontrol potential of different *Trichoderma species Viz. Trichoderma viride VMB21, Trichoderma reesei VMB23 and Trichoderma stercorarium VMB25.* has been demonstrated on Pomegranate bacterial pathogens *Viz. Xanthomonas axonopodis VMB13, Xanthomonas campestris VMB15* and *Xanthomonas vesicatoria VMB17*.

Keywords: Pomegranate, *Xanthomonas species, Trichoderma species,* Plants extract, Antibacterial activity.

INTRODUCTION

Plant diseases need to be controlled to maintain the quality and abundance of food, feed, and fiber produced by growers around the world. Different approaches may be used to prevent, mitigate or control plant diseases. Beyond good agronomic and horticultural practices, growers often rely heavily on chemical fertilizers and pesticides. Such inputs to agriculture have contributed significantly to the spectacular

ISSN PRINT 2319 1775 Online 2320 7876
 UGC CARE Listed (Group -I) Journal Volume 11, Iss 10, 2022

improvements in crop productivity and quality over the past 100 years. However, the environmental pollution caused by excessive use and misuse of agrochemicals, as well as fear-mongering by some opponents of pesticides, has led to considerable changes in people's attitudes towards the use of pesticides in agriculture. Today, there are strict regulations on chemical pesticide use, and there is political pressure to remove the most hazardous chemicals from the market. Additionally, the spread of plant diseases in natural ecosystems may prevent successful application of chemicals, because of the scale to which such applications might have to be applied. Consequently, some pest management researchers have focused their efforts on developing alternative inputs to synthetic chemicals for controlling pests and diseases. Among these alternatives are those referred to as biological control.

Biological control or biocontrol is defined as the use of organisms in the management of plant diseases. These organisms are referred to as biocontrol agents. In plant pathology, the term applies to the use of antagonistic microbes to suppress the diseases of plants. The use of host specific pathogens for the control of weeds also comes under this field. The biocontrol agents should be safe from humans and environment point of view.

The biocontrol potential of different *Trichoderma species Viz. Trichoderma viride VMB21, Trichoderma reesei VMB23 and Trichoderma stercorarium VMB25.* has been demonstrated on Pomegranate bacterial pathogens *Viz. Xanthomonas axonopodis VMB13, Xanthomonas campestris VMB15* and *Xanthomonas vesicatoria VMB17* by agar well diffusion assay.

MATERIALS AND METHODS

Preliminary identification and isolation of the pathogen from affected parts of Pomegranate plant

Ooze test was performed as per description of Sharma *et al*., (2010) which is based on the microscopic observations for confirmation of presence of the pathogen in infected leaves. Pomegranate leaves which show typical symptoms of oily spot (bacterial blight) were collected from the field. In this test, the infected leaves were washed with the sterile distilled water. Further, all the selected infected leaves were sterilized with 0.1% mercuric chloride ($HgCl_2$) for 10 minutes and followed by repeatedly washing with sterile distilled water and next blot dried. Then a fresh young lesion containing a leaf was selected. The small bits of infected tissue were cut down from affected portions of plants such as leaves and pericarp of the fruits with the help of a sharp sterile scalpel. Then it was placed in a drop of sterile saline taken on a glass slide and observed from cut ends under the high power objective of the compound microscope. Jet of bacterial cells started oozing out from the cut pieces of the tissue in water were observed under a microscope called ‘ooze’ (Schaad, 1992).

Further, suspension of bacteria prepared from infected tissues taken from affected leaves, twigs and fruits were used for isolation of well separated bacterial colonies on suitable medium. Singh *et al*., (2015) stated the disease diagnosis depends on visual symptoms on plant parts and ooze test.

ISSN PRINT 2319 1775 Online 2320 7876
 UGC CARE Listed (Group -I) Journal Volume 11, Iss 10, 2022

Isolation of bacteria exudes out from ooze

The leaf having the infected part was washed and further crushed in sterile distilled water. The extract obtained was then streaked inoculated on the surface of a sterile Glucose Yeast Extract Calcium Carbonate Agar (GYECC Agar) (Yenjerappa, 2009). The plate was further incubated at 30^0C for 3 days. As per the literature, a well isolated, gummy, mucous, yellow colored colony was selected for further study (Yenjerappa, 2009).

Isolation of *Trichoderma species*

Soil samples were collected from different ecological habitats of Latur district, (India) for the isolation of *Trichoderma spp*. These soil samples were brought to the laboratory and stored in a refrigerator at 4^0C until used. Four-fold serial dilutions of each soil sample were prepared in sterilized distilled water. 0.5 ml of this diluted sample was poured on the surface of Potato Dextrose Agar (Elad *et al*., 1982). Plates were incubated at 28 ± 2^0C for 96 hours. Morphologically distinct colonies appearing on the agar plates were purified on Potato Dextrose Agar (PDA). The purified promising isolates were further preserved at 4^0C and used during the course of study.

Preparation of Solvent Extracts of Selected Medicinal Plants

Twenty grams of dried powder of each plant was filled in the thimble and extracted consecutively with the solvents *Viz*. methanol, ethanol and chloroform by using a Soxhlet extractor (Soxhlet Complete Borosil, Code 3840) for 48 hours. All these extracts were separately prepared in Soxhlet extractor. Further, all these extracts were concentrated using a rotary flash evaporator (Superfit Rotary Vacuum Flash Unit PBU-6D) and preserved at 4^0C in airtight bottles for further use. Afterwards, all these extracts were subjected to antibacterial activity assay (Raghavendra *et al*., 2006).

Determination of Antibacterial Activity

The antibacterial activity of aqueous extracts, solvent extracts and the mixture of all plant extracts under study were determined by agar well diffusion method (Cruickshank *et al*., 1975) on the sterile nutrient glucose agar medium. Cell density was adjusted to 106 -107 CFU/ml on the basis when culture reaches 0.1 optical units at 600nm with spectrophotometer (Schaad, 1992).The inoculum containing 106-107 CFU / ml of 72 hours old culture of *Xanthomonas axonopodis* pv. *Punicae*VMB13, *Xanthomonas campestris VMB15* and *Xanthomonas vesicatoria VMB17*was spread inoculated on medium with the help of sterile glass spreader. Wells were prepared in sterile nutrient glucose agar plate by using sterile cork borer (5mm). 100µl of aqueous extract and solvent extract was put in the wells prepared in the inoculated plates

RESULTS AND DISCUSSIONS

Trichoderma is a soil-borne filamentous fungus that is most widely used as a biocontrol agent; since it is capable of parasitising several plant pathogenic fungi. *T. harzianum, T. viride* and *T. asperellum* have been mostly reported as biocontrol agents against plant pathogens (Harman *et al*., 2004, 2011; Kumar *et al*., 2012; Cuervo-Parra *et al*., 2011).

ISSN PRINT 2319 1775 Online 2320 7876
 UGC CARE Listed (Group -I) Journal Volume 11, Iss 10, 2022

Table 1: *In Vitro* Evaluation of Antibacterial Potential of Biocontrol Agents against Bacterial Pathogens of Pomegranate Diseases

Bacterial Pathogens	*Trichoderma viride VMB21*	*Trichoderma reesei VMB23*	*Trichoderma stercorarium VMB25*
	Zone of Growth Inhibition (mm)		
Xanthomonas axonopodis VMB13	45	40	39
Xanthomonas campestris VMB15	42	35	40
Xanthomonas vesicatoria VMB17	41	38	31

***In Vitro* Evaluation of Biocontrol Agents against Bacterial Pathogens**

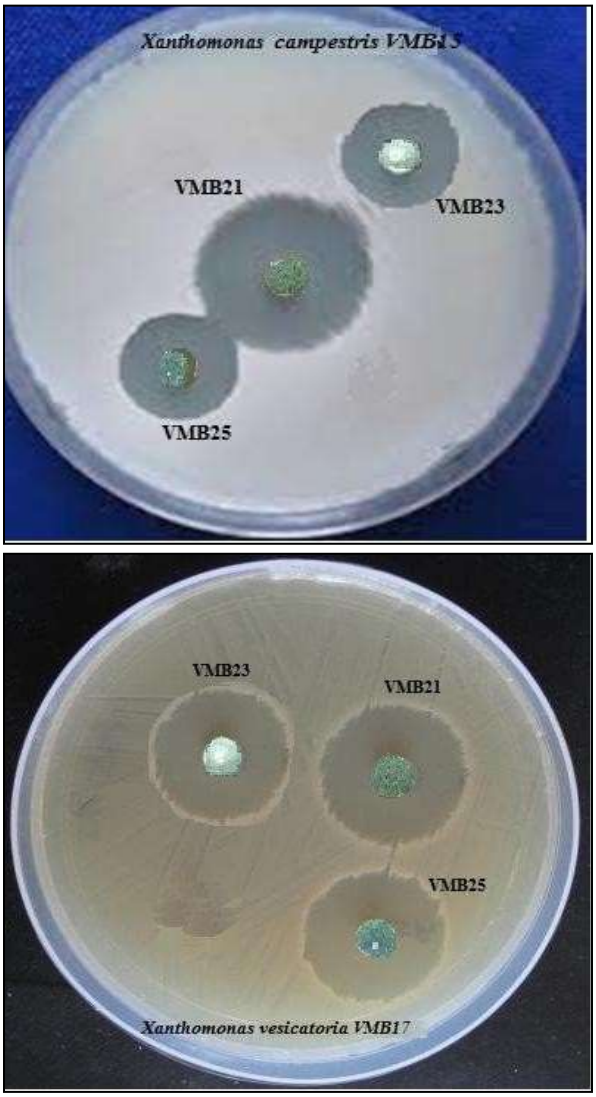
Trichoderma species are frequent inhabitants of the rhizosphere and contribute to control of many soil borne plant diseases which were caused by fungi. The potential of different *Trichoderma species* used as biological agents of plant diseases have been known since the 1930s.Therefore, three species of Trichoderma were selected for their antifungal activity against the targeted pathogens as follows.

1) *Trichoderma viride VMB21*
2) *Trichoderma reesei VMB23*
3) *Trichoderma stercorarium VMB25*.

Three Biocontrol agents *Viz. Trichoderma viride VMB21, Trichoderma reesei VMB23* and *Trichoderma stercorarium VMB25*were tested against three bacterial pathogens *Viz. Xanthomonas axonopodis VMB13, Xanthomonas campestris VMB15, Xanthomonas vesicatoria VMB17* which were isolated from diseased Pomegranate leaves, stem and fruits. Zones of growth inhibition were measured and calculated. The results were tabulated in **Table 1.**

Among the three bioagents tested (**Table 1),** *Trichoderma viride VMB21* showed the maximum inhibition of the test pathogens.

ISSN PRINT 2319 1775 Online 2320 7876
 UGC CARE Listed (Group -I) Journal Volume 11, Iss 10, 2022



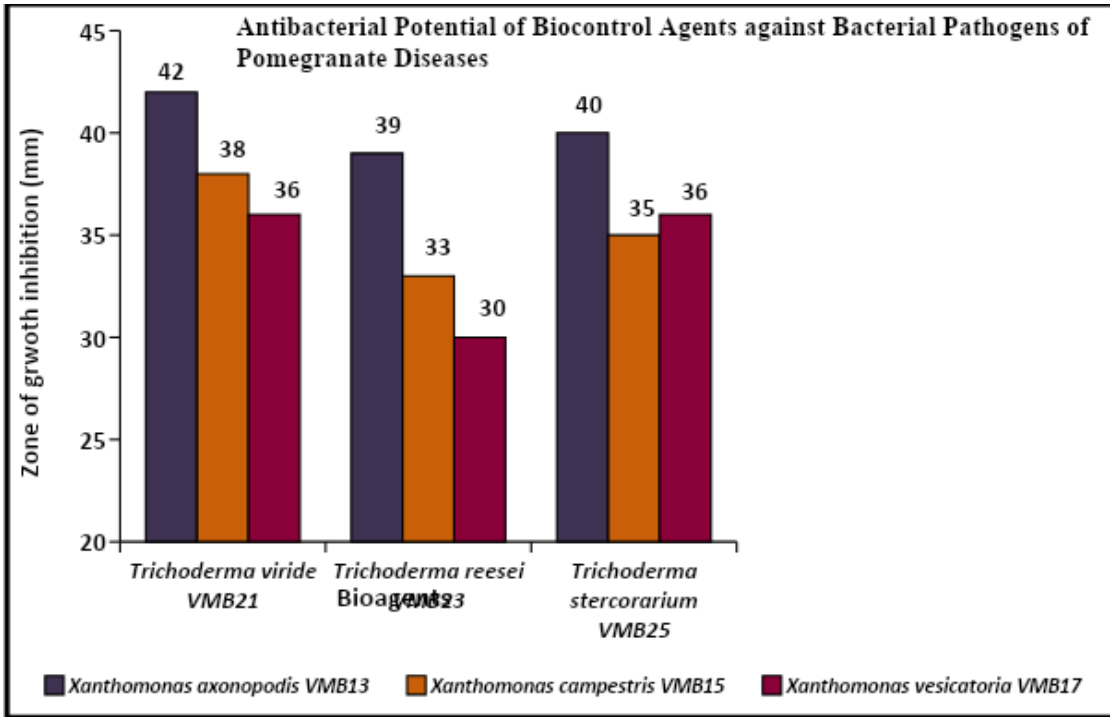
Photoplate:1 Antibacterial Potential of Biocontrol Agents against Bacterial Pathogens of Pomegranate Disease

Evaluation of Efficacy of Biocontrol agents against Bacterial Pathogen

In the light of present day constraints with the utilization of chemical pesticides in the plant disease management, the biological control as an alternate option is gaining very much importance and awareness since the approach is eco-friendly and cost effective. Under biological control of plant diseases, different antagonistic organisms have been identified, which fight against the pathogens by a range of mechanisms *Viz.*, competition, antibiosis, siderophore production, lysis, etc. In the present investigation *Trichoderma viride* showed maximum inhibition of the three bacterial pathogens *Viz. Xanthomonas axonopodis VMB13, Xanthomonas campestris VMB15* and *Xanthomonas vesicatoria VMB17*.

ISSN PRINT 2319 1775 Online 2320 7876
 UGC CARE Listed (Group -I) Journal Volume 11, Iss 10, 2022

Figure 1: Antibacterial Potential of Biocontrol Agents against Bacterial Pathogens of Pomegranate Disease



Antibacterial potential of three biocontrol agents *Viz. Trichoderma viride VMB21, Trichoderma reesei VMB23* and *Trichoderma stercorarium VB25 against* bacterial pathogens *Viz. Xanthomonas axonopodis VMB13, Xanthomonas campestris VMB15* and *Xanthomonas vesicatoria VMB17* of pomegranate disease were evaluated. The results are depicted in **Figure 1.**

It is found from **Figure 1** that the three biocontrol agents *Viz. Trichoderma viride VMB21, Trichoderma reesei VMB23* and *Trichoderma stercorarium VB25* shows the maximum zone of inhibition i.e. 42mm, 39mm and 40mm respectively to *Xanthomonas axonopodis VMB13*.The maximum zone of inhibition shown by the three bioagents *Trichoderma viride VMB21, Trichoderma reesei VMB23* and *Trichoderma stercorarium VMB25* to the *Xanthomonas campestris VMB15* is 38mm, 33mm and 35mm respectively. Similarly, the zone of growth inhibition shown by the same bioagents to the *Xanthomonas vesicatoria VMB17* is 36mm, 30mm and 36mm respectively.

Our results are in strong agreement with the results reported by Nurbailis *et al*., (2019) in which they reported that *Trichoderma viride, Trichoderma harzianum and Trichoderma PP3*reducing the growth of *Xanthomonas axonopodis pv.* caused leaf blight disease on red onion.

Abhishek Gupta *et al*., (2018) reported that the secondary metabolites of *Trichoderma viride* such as cellulase enzyme (this enzyme try to use as a bio-control agent) give strong antibacterial activity against plants pathogenic bacteria *Xanthomonas citri* which are cause disease in lemon plants.

Our results are in contrast with the results reported by Bhure *et al.*, (2019) in which they tested the antagonistic activity of three bioagents and confined that

ISSN PRINT 2319 1775 Online 2320 7876
 UGC CARE Listed (Group -I) Journal Volume 11, Iss 10, 2022

Trichoderma harzianum gives maximum inhibition zone (22.86mm) followed by *Pseudomonas fluorescens* (17.20mm) and *Bacillus subtilis* (15.00mm).

Molecular Identification of Biocontrol Agents

Molecular identification of the biocontrol agents was carried out by 18s rRNA sequencing. Full length 18s rRNA region was amplified using the universal primers. The amplified PCR generated sequences were then analyzed at National Centre for Biotechnology information (NCBI), Bethesda, MD. www.nbi.nlm.nih.Gov/BLASTfor closed homology using BLASTn algorithm.

The related sequences for fungal isolates were downloaded from the NCBI database and then were aligned by using CLUSTAL X2 and Dambe multiple sequence alignment tool. The Phylogenetic evolutionary relationship was then inferred using the Kimura-2- parameter and Neighbor Joining Method analysis.

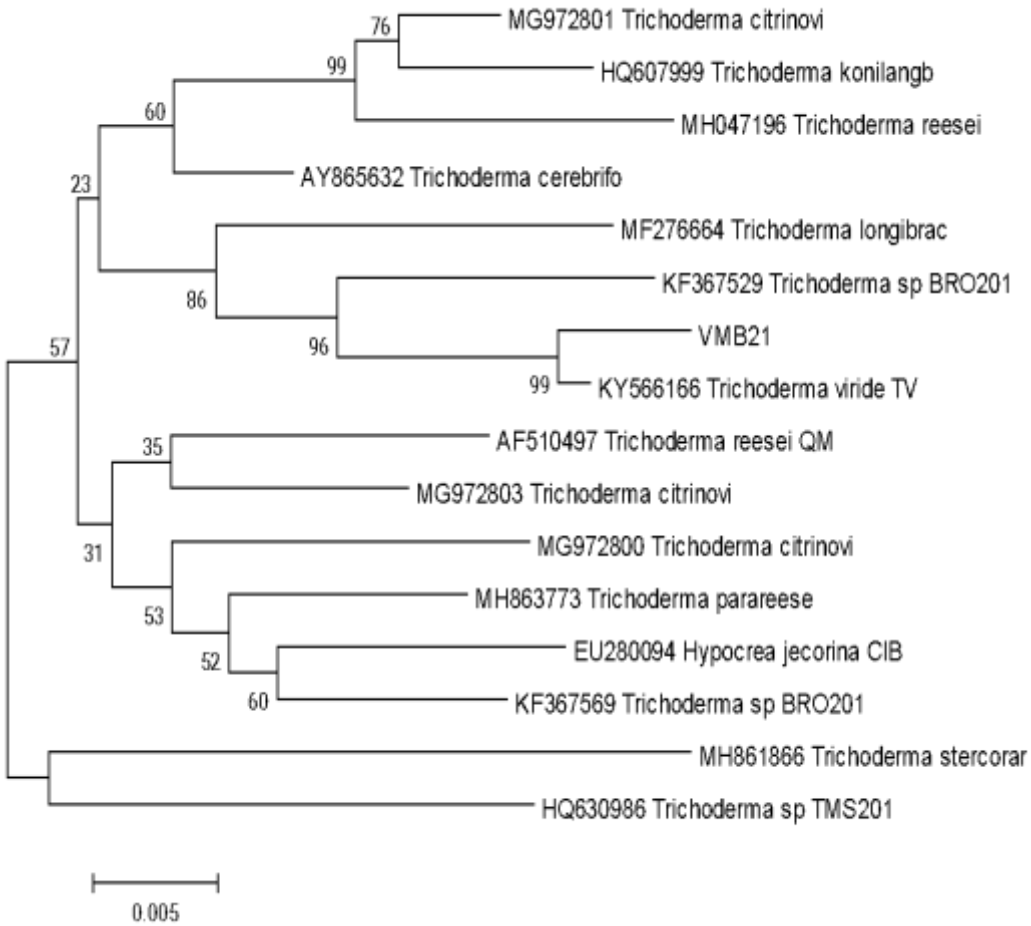


Figure 2: Phylogenetic placement of VMB21 (DDBJ Accession NumberLC535970) based on 18S rRNA analysis

Phylogenetic tree of *Trichoderma viride VMB21*.Phylogenetic analysis of 18s rRNA gene sequence of *Trichoderma viride VMB21*. The percent numbers at the nodes indicate the levels of bootstrap support based on neighbor-joining analyses of 1,000 replicates. The scale bar (0.005) indicates the genetic distance.

ISSN PRINT 2319 1775 Online 2320 7876

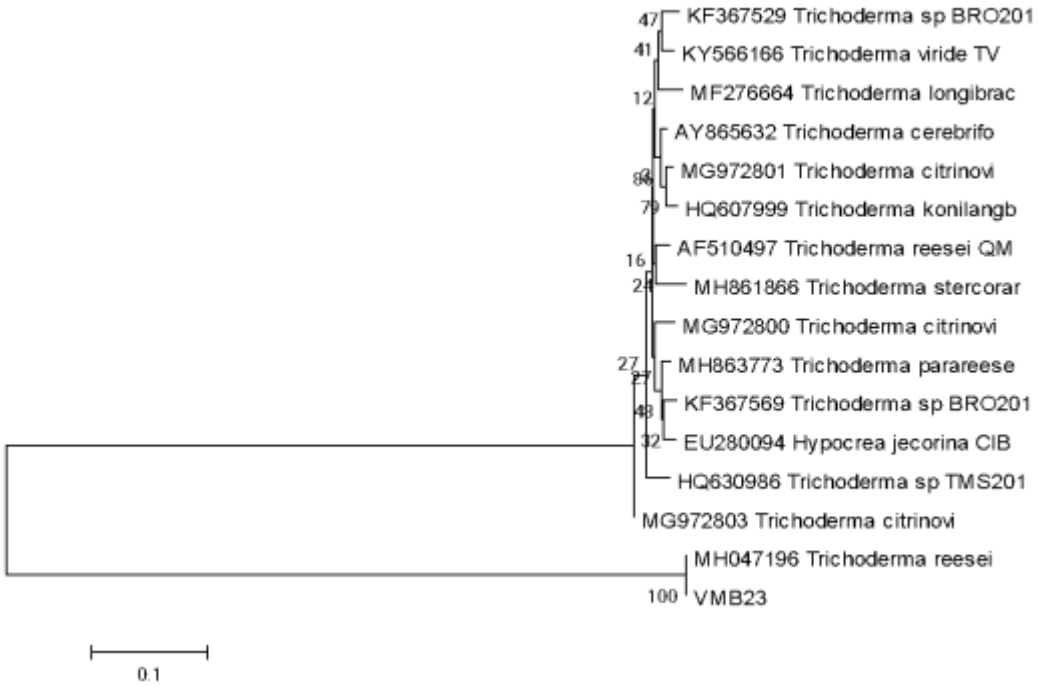


Figure 3: Phylogenetic placement of VMB23 (DDBJ Accession NumberLC535971) based on 18S rRNA analysis

Phylogenic tree of *Trichoderma reesei VMB23*.Phylogenetic analysis of 18s rRNA gene sequence of *Trichoderma reesei VMB23*. The percent numbers at the nodes indicate the levels of bootstrap support based on neighbor-joining analyses of 1,000 replicates. The scale bar (0.1) indicates the genetic distance.

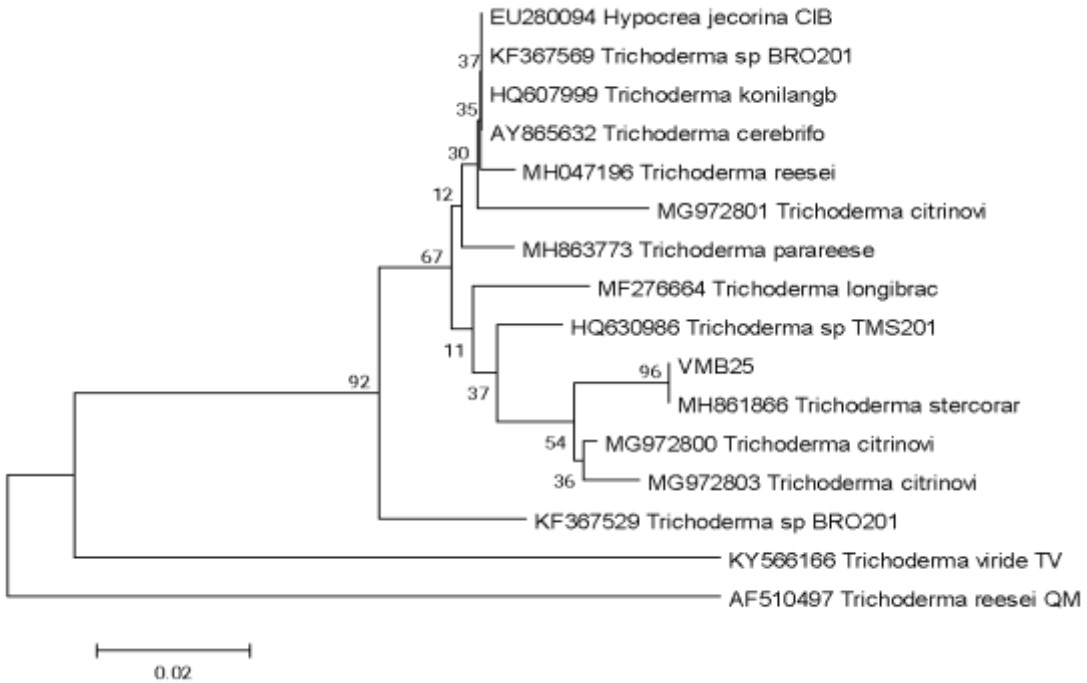


Figure 4: Phylogenetic placement of VMB25 (DDBJ Accession NumberLC535972) based on 18S rRNA analysis

ISSN PRINT 2319 1775 Online 2320 7876
 UGC CARE Listed (Group -I) Journal Volume 11, Iss 10, 2022

Phylogenic tree of *Trichoderma stercorarium* VMB25. Phylogenetic analysis of 18s rRNA gene sequence of *Trichoderma stercorarium* VMB25. The percent numbers at the nodes indicate the levels of bootstrap support based on neighbor-joining analyses of 1,000 replicates. The scale bar (0.02) indicates the genetic distance.

Shreeshail, S. *et al*., (2015) used seven botanicals *Viz.* Neem leaf extract, Garlic bulb extract, Onion bulb extract, Datura leaf extract, Ocimum leaf extract, Eucalyptus leaf extract and Rhizome extract and four bioagents *Viz. Bacillus subtilis, Pseudomonas fluorescens*, *Trichoderma viride* and *Trichoderma harzianum* were evaluated *In Vitro* against the causal agent of wilt of pomegranate *i.e. Ceratocystis fimbriata*. The results of their study showed that *a*mong four bioagents *Trichoderma harzianum* and *Trichoderma viride* showed 100%inhibition of the test fungus *i.e. Ceratocystis fimbriata* followed by *Pseudomonas fluorescens* (42.33%) within 4 days and completely inhibited the perithecium production with growth over the pathogen.

Our results are in contrast with the results reported by Shreeshail, S. *et al*., (2015) in that among the different bio agents *Trichoderma harzianum* showed the maximum inhibition of the test fungus i. e. the causal agent of wilt of pomegranate *Ceratocystis fimbriata* (100%) but remains on par with *Trichoderma viride* (100%).

Antibiotic-mediated suppression is the major mechanism that is noticed during biocontrol. Biocontrol agents produce one or several antimicrobial compounds that inhibit the growth of several plant pathogens. For example, *Bacillus cereus* strain is known to produce zwittermycin (Silo-Suh *et al*., 1994) and kanosamine (Silo-Suh *et al*., 1998). The ability to produce multiple antibiotics enables the biocontrol agents to inhibit a wide range of plant pathogens, which enhances the efficacy of biological control. *Pseudomonas putida* WCS358r, a genetically engineered strain produces phenazine and 2, 4-Diacetylphloroglucinol (DAPG), which exhibits improved suppression of plant diseases in fields (Glandorf *et al*., 2001).

CONCLUSION

In all these interactions, the pathogens are antagonized by other organisms which indicate that there are several mechanisms involved in biological control. These mechanisms are hyperparasitism or predation, production of antibiotics, lytic enzymes, unregulated waste products and induction of host resistance. In hyperparasitism or predation, a specific biocontrol agent kills the pathogens by directly attacking it. Biocontrol agents produce one or several antimicrobial compounds that inhibit the growth of several plant pathogens. The ability to produce multiple antibiotics enables the biocontrol agents to inhibit a wide range of plant pathogens, which enhances the efficacy of biological control. The biocontrol potential of different *Trichoderma species Viz. Trichoderma viride VMB21, Trichoderma reesei VMB23* and *Trichoderma stercorarium VMB25* has been effectively demonstrated on Pomegranate bacterial pathogens *Viz. Xanthomonas axonopodis VMB13, Xanthomonas campestris VMB15* and *Xanthomonas vesicatoria VMB17*.

ISSN PRINT 2319 1775 Online 2320 7876
 UGC CARE Listed (Group -I) Journal Volume 11, Iss 10, 2022

REFERENCES

1. Abhishek Gupta, Prabhat Jatav, Sonu Singh Ahirwar, Kirti Kushwaha and Sadhna Jatav (2018). Antagonistic activity of cellulase enzyme produced by *Trichoderma Viride* against *Xanthomonas Citri*. *Indian J. Agric. Res*., 52(5): 497-504
2. Bhure S.S., S.B. Bramhankar, K.D. Thakur, A.A. Labhasetwar, S.S. Isokar, G.T. Dinkwar, C.A. Sarode and D.G. Tathod (2019). *In Vitro* bio efficacy of different antibiotics, bioagent and botanical against (*Xanthomonas axonopodis pv. citri*.) causing bacterial canker of acid lime. *International Journal of Chemical Studies*. 7(1): 1789-1782
3. Cruickshank R, Duguid JP, Marmion BP, Swain RH (1975). Medical Microbiology (12 th ed) Longman group Ltd. Edinburgh, London pp. 180-188.
4. Cuervo-Parra J.A., Ramirez-Suero M., Sanchez-Lopez V., Ramirez-Lepe M. (2011). Antagonistic effect of *Trichoderma harzianum VSL291* on phytopathogenic fungi isolated from cocoa (*Theobroma cacao L*.) fruits. *Afr J Biotechnol* 10: 10657-10663.
5. Elad Y, Chet I. and Henis Y. (1982). Degradation of plant pathogenic fungi by *Trichoderma harzianum*. *Can J Microbiol* 28:719–725
6. Glandorf DCM, Verheggen P, Jansen T, Jorritsma JW, Smit E, Leeflang P, Wernars K, Thomashow LS, Laureijs E, Thomas-Oates JE, Bakker PAHM & LC van Loon (2001) Effect of genetically modified *Pseudomonas putida* WCS358r on the fungal rhizosphere microflora of field grown wheat. *Appl. Environ. Microbiol*. 67: 3371–3378.
7. Harman G.E., Howell C.R., Viterbo A., Chet I. and Lorito M. (2004). Trichoderma plant symbionts species—Opportunistic, avirulent plant symbionts. *Nature Reviews. Microbiology*. 2:43-56
8. Kumar K., Amaresan N., Bhagat S., Madhuri K. and Srivastava R.C. (2012). Isolation and characterization of *Trichoderma spp*. for antagonistic activity against root rot and foliar pathogens. *Indian J Microbiol* 52: 137-144.
9. Nurbailis, Akmal Djamaan, Haliatur Rahma and Yenny Liswarni (2019). Secondary Metabolite Production by *Trichoderma spp*. and its Potential as Antibacteria. *Int. J. Curr. Microbiol. App. Sci*. 8(4): 196-201
10. Raghavendra M. P., Satish S. and Raveesha K. A. (2006). *In Vitro* evaluation of anti-bacterial spectrum and photochemical analysis of *Acacia nilotica*. *Journal of Agricultural Technology*. 2(1):77-88.
11. Schaad N. W. (1992). Laboratory guide for the identification of plant pathogenic bacteria. 2nd ed. *American Phytopathol. Soc*., 138 pp.
12. Sharma K. K., Sharma J., Jadhav V. T. (2010). Status of bacterial blight of pomegranate in India. In: Fruit, vegetable, cereal science and biotechnology. 4(2). *Global Science Books publication*, Japan: 102–105.
13. Shreeshail Sonyal, Manjunath, S. Hurakadli, Mahesha, H. S, K. B. Palanna. Madhu S. Giri and Anil Pappachan (2015). Effect of Botanicals and Bio

ISSN PRINT 2319 1775 Online 2320 7876
 UGC CARE Listed (Group -I) Journal Volume 11, Iss 10, 2022

agents on Growth of *Ceratocystis fimbriata ELL*. and Halst. Causing Wilt in Pomegranate. *Int. J. Pure App. Biosci*. 3 (4):42-48

14. Silo-Suh, L. A., Lethbridge, B. J., Raffel, S. J., He, H., Clardy, J., & Handelsman, J. (1994). Biological activities of two fungistatic antibiotics produced by Bacillus cereus UW85. *Applied and environmental microbiology*, *60*(6), 2023-2030.
15. Silo-Suh, L. A., Stabb, E. V., Raffel, S. J., & Handelsman, J. (1998). Target range of zwittermicin A, an amino polyol antibiotic from *Bacillus cereus*. *Current microbiology*, *37*, 6-11.
16. Singh N. V., Abburi V. L., Ramajayam D. Ravinder Kumar, Ram Chandra, Sharma K. K.,Sharma J. K., Dinesh Babu, Pal R. K., Mundewadikar D.M., Thangasamy Saminathan T., Cantrell R., Nimmakayala P., Reddy U.K. (2015). Genetic diversity and association mapping of bacterial blight and other horticulturally important traits with microsatellite markers in pomegranate from India. *Mol Genet Genomics*: 1-10.
17. Yenjerappa S. T. (2009). Epidemiology and management of bacterial blight of pomegranate caused by *Xanthomonas axonopodis pv. punicae* (Hingorani and Singh) vauterin. Th 9936 (accession no) submitted to University of Agricultural Sciences, Dharwad, Karnataka State, India.