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Evaluation of Biocontrol Efficacy of Herbal and Bioformulations against Root Rot Pathogen *Fusarium Solani* in Tomato

David Paul Raj R.S, Beena Kanimozhi R, Levin Anbu Gomez, Rohini S

ABSTRACT: Background/Objectives: Fusarium solani cause various plant diseases that usually weaken or destroy plant tissues and reduce crop yields varying from 30-40% and root diseases are estimated to cause 10-15% yield losses annually in the world Soil borne pathogen Fusarium solani which causes root rot at the seedling stage itself leading to wide loss of the crop. Solanum lycopersicum (tomato) is one of the most versatile vegetable with wide usage in Indian culinary tradition. Since the use of chemical fungicides cause environmental pollution and adverse effect on human health, there is need to shift to biological control.

Methods: Bacterial isolates were obtained from forest nursery soil samples and screened against F. solani. Of which one isolate showed promising efficacy against the pathogen. Preparation of oil based liquid bioformulation using glycerol, coconut oil and culture filtrate of biocontrol agent and herbal formulation using neem, garlic, ginger and clove blended together with water in the ratio 1:2 showed inhibitory effect on F.solani.

Findings: Biocontrol agent was identified by 16S rDNA sequencing as Bacillus velezensis FZB42. Tomato seeds were coated with Gum Arabic as adhesive along with liquid bioformulation and herbal formulation showed great inhibitory effect against the F. solani in invitro conditions, increased the germination percentage of seeds and also the germination was faster when compared with the control.

Applications: Technology transfer of application of this formulation to the farmers will be initiated which will help them to raise the seedlings in an ecofriendly method.

Keywords: Fusarium solani, biocontrol, Solanum lycopersicum, bioformulation

I. INTRODUCTION

In recent years, the World's land base has been subject to urban expansion, poor management practices and increasing pressure to provide resources for a growing population. Expanding temperatures have expanded infection frequency in tomato [15].

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* Correspondence Author
David Paul Raj R.S, Assistant Professor, Department of Biotechnology, Karunya Institute of Technology and Sciences, Karunya nagar, Coimbatore- 641 114, Tamil Nadu, India
Beena Kanimozhi R, Research Scholar and Student, Department of Biotechnology, Karunya Institute of Technology and Sciences, Karunya nagar, Coimbatore - 641 114, Tamil Nadu, India
Levin Anbu Gomez, Assistant Professor, Department of Biotechnology, Karunya Institute of Technology and Sciences, Karunya nagar, Coimbatore- 641 114, Tamil Nadu, India
Rohini S, Research Scholar and Student, Department of Biotechnology, Karunya Institute of Technology and Sciences, Karunya nagar, Coimbatore - 641 114, Tamil Nadu, India

Tomato plants are affected by a soil borne fungal pathogen *Fusarium solani* that causes major diseases like root rot and crown rot [1]. *Fusarium* sp. is a diverse genus of ascomycete fungi. The major diseases caused by *Fusarium* sp. are rots, blights and wilts and cause threats in both agricultural field and ecosystems.
It also produces a varied mycotoxins that can pollute agricultural products, and make the food or feed unsuitable [19]. *Fusarium* sp. is a most prevalent pathogen which holds accountable for the major losses in tomato production around the world.

Most of the chemically treated pesticides and fungicides have been used by the farmers in agriculture to prevent the mortality rate of the plant and reduce the huge loss caused by the soil-borne pathogens. These chemical exposure could poisoned the young seedlings and also pollute the soil environment. Since because of some limitations in both physical and chemical treatments, biological treatment played a major role in eradicating the diseases caused by the pathogens. [7]. Hence there is a need for environment friendly and cost effective method to control these disease incidences.

Biological control agents from diverse bacterial population produces number of secondary metabolites and antibiotics which are potential enough to combat against the pathogen [6] [7]. *Bacillus* species proved to be an important biological control agent in eradicating diseases caused by fungal pathogens [12] and production of active antifungal metabolites inhibit the germination of fungal spores [8] [9] [3]. Reports states that root rot of cotton, caused by *F.solani* was significantly reduced (17.16 per cent) by T.harzianum. Bohra and Mathur (2000) [2] reported effective control of root rot of soyabean (*F. solani*) by *T.harzianum* (JH-2) [5] Ghasolia and Jain (2003) [4] reported that seed treatment with biological agents i.e Trichoderma hazarianum and Trichoderma viride was found effective against Fusarium disease of cumin under in-vitro condition.

The development of liquid formulation has several advantages including high cell count, zero contamination, longer shelf life, greater protection against environmental stresses and increased field efficacy. In liquid formulation, the microbial organisms are present in a dormant form and after application in the field, the dormant form give rise to active cells. This helps to increase the shelf life of liquid bioformulation for more than one year [16].

II. MATERIALS AND METHODS

A. *The Fungal Pathogen*

Fusarium solani is the main causative agent of damping-off, root rot and seed rot disease was obtained from Tamil Nadu Agricultural University,

Coimbatore, Tamil Nadu. *F. solani* was grown on Potato dextrose agar (PDA) plates and incubated at 27± 2° C for 3 days and the slant cultures were stored at -20°C for future use.

B. *Isolation of bacteria from soil samples*

Six soil samples were collected from Boluvampati forest nursery (Longitude of 10.9458°N, latitude of 76.7697°E) of Coimbatore district, Tamil Nadu. Bacteria were isolated from the soil samples by serial dilution technique. 10g of soil was suspended in 90ml sterile distilled water and shaken at 120rpm on a rotary shaker for 15mins. Soil mixture was diluted with sterile distilled water up to 10^{-7}. An aliquot of 100 µl from 10^{-4} to 10^{-7} was gently spread with a sterile glass rod spreader on nutrient agar amended plates. One colony of each morpho-species was selected and purified bacterial culture were maintained at 20% glycerol stock at -80° C [10].

C. *In vitro screening of bacterial isolates for antagonistic activity*

In vitro screening for antagonistic activity was performed by dual culture technique on a Potato dextrose agar (PDA) plates. A 5 mm agar disc of actively growing culture of *R.solani* was placed in the centre of each plate. After 12 hours, each bacterial isolate was streaked 3 cm away from the fungal disc. In the control plate, no bacterial isolates were inoculated. Plates were then sealed with parafilm and incubated at 30 ± 2°C for 3-5 days until the fungal mycelia reached the edge in the control plates. The mycelial growth inhibition towards the direction of the bacterial isolate was the indication of antagonistic activity [14].

D *Molecular identification of isolate F KI 2.10*

i Extraction of genomic DNA, PCR and sequencing

Genomic DNA was extracted using phenol: chloroform: iso-amyl alcohol method. PCR was performed from the genomic DNA using 16S rDNA universal bacterial primer. The amplified PCR product was sequenced in 3130×l Genetic analyzer (Applied Biosystems, CA, USA). The sequences were manually edited using Sequence Scanner Software v. 1.0 (Applied Biosystems, CA, USA) and full length sequences were assembled.

ii *Sequence analysis and phylogeny interpretation*

The sequence was related for comprision with sequences existed in the gene bank database using BLASTn in the NCBI (National Center for Biotechnology Information; http://blast.ncbi.nlm.nih.gov/Blast.cgi). Greater similarity sequences of 16S rRNA gene of the strain were recovered and bring into line with the 16S rRNA gene sequence of isolate F KI 2.10 in ClustalW2 software and subjected to phylogenetic tree construction in MEGA7 using neighbor-joining method.

E. *Preparation of Plant extracts*

The leaves of neem tree (*Azadirachta indica*), bulbs of garlic (*Allium sativum*), rhizome of ginger (*Zingiberofficinale*) and cloves (*Syzygium aromaticum)* were washed with distilled water. 30 grams of each plant materials were blended together adding double amount of distilled water (1:2 w/v) and heated at 80°c for 10 minutes in hot water bath. The extracts were filtered by syringe filtration [17]

F. *Screening of antifungal activity of plant extracts against Fusarium solani*

As per poisoned food technique, 10ml of the plant extracts were mixed with the sterilized PDA medium, to obtain a homogenous mixture of the extracts and medium, under aseptic condition. 20 ml medium was poured into each Petri dishes, and two replicates were maintained. The discs of *F. solani* were cut with sterilized cork borer and placed in the centre of Petri dish which contains plant extracts amended PDA medium. The fungus grown on PDA without plant extracts served as control. The plates were maintained at room temperature. In well plate technique, 1 ml of the plant extract was added in the well on one side of the Petri dishes and on the other side, the discs of *R. solani* was placed. Petri plates containing sterile water in the well serves as a control [17].

G. *Preparation of liquid bioformulation*

Bacillus velezensis was grown on nutrient broth for 18-20 hrs, on an orbital shaker at 150 rpm at 37 °C. The culture broth was adjusted to ≥10^8 CFU/ml (OD value of 1.0). The oil based bioformulation was prepared using Gum Arabic, coconut oil, glycerol and culture broth of *Bacillus velezensis* (BCA) in a ratio of 2:20:76:8 respectively per 100ml of bioformulation. Gum Arabic is used as an adhesive material. Coconut oil was used as a carrier material and glycerol to maintain the viability of cells for long period. Treatment design and treatment details were tabulated in Table 4 and Table 5

Table 1: Experimental setup of bacterial liquid bioformulation

Experimental setup	Treatment design	Code name
1	15% Gum Arabic + coconut oil+ glycerol+ culture broth	1A
2	30% Gum Arabic + coconut oil+ glycerol+ culture broth	1B
3	45% Gum Arabic + coconut oil+ glycerol+ culture broth	1C
4	Pre treated overnight with 15% Gum Arabic + coconut oil+ glycerol+ culture broth	2A
5	Pre treated overnight with 30% Gum Arabic + coconut oil + glycerol + culture broth	2B
6	Pre treated overnight with 45% Gum Arabic + coconut oil +glycerol +culture broth	2C

Table 2: Treatment details of bacterial liquid bio formulation

Treatments	Gum arabic	Coconut oil	Glycerol	Culture broth
1A	3.15 g	4.2 ml	15.12ml	2.5 ml
1B	6.3 g	4.2 ml	15.12ml	2.5 ml
1C	9.5g	4.2 ml	15.12 ml	2.5 ml
2A	3.15 g	4.2 ml	15.12 ml	2.5 ml
2B	6.3 g	4.2 ml	15.12 ml	2.5 ml
2C	9.5 g	4.2 ml	15.12 ml	2.5 ml

H. *Preparation of Herbal formulation*

Herbal formulation of plant extracts was prepared by mixing plant extracts with Gum Arabic, to improve the adhesiveness of bio formulation on seeds. The seed coating efficiency was optimized from various treatments by varying the quantity of Gum Arabic (15%, 30% and 45%) [18]. Treatment design and treatment details were tabulated in Table 6 and Table 7.

Table 3: Experimental setup of bio formulation of plant extracts

EXPERIMENTAL SETUP	TREATMENT DESIGN	CODE NAME
1	15% Gum Arabic + herbal extract	1A
2	30% Gum Arabic + herbal extract	1B
3	45% Gum Arabic + herbal extract	1C
4	Pretreated overnight with 15%Arabic gum+ herbal extract	2A
5	Pretreated overnight with 30%Arabic gum+ herbal extract	2B
6	Pretreated overnight with 45%Arabic gum+ herbal extract	2C

Table 4: Treatment details of herbal formulation

TREATMENT	GUM ARABIC	PLANT EXTRACTS
1A	3.15 g	25 ml
1B	6.3 g	25 ml
1C	9.5 g	25 ml
2A	3.15 g	25 ml
2B	6.3 g	25 ml
2C	9.5 g	25 ml

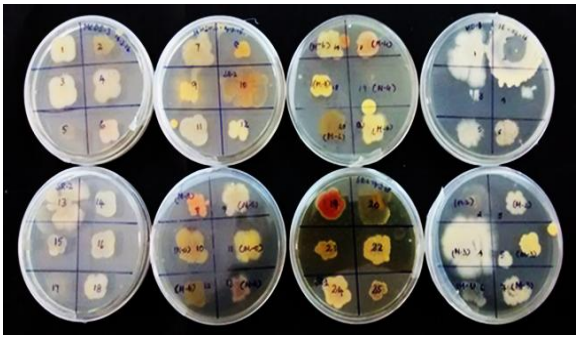
I. *Invitro Studies*

One set seeds of *Solanum lycopersicum* were completely soaked in 25 ml of bio formulations . After 1 hour of treatment, the seeds were shade dried and sowed in the nursery bags containing soil infected with *Fusarium solani*. This process was repeated after 2 and 3 hours of treatment of bioformulation .Seeds not treated with bio formulation served as control treatment. Another set of seeds were first soaked in arabic gum overnight and then immersed in bioformulaion. Then sown at one hour intervals upto three hours. The effectiveness of the treatment was studied by observing the growth of plants in comparison with control plant.

III. RESULTS

A. Isolation of bacteria from soil samples

Around 60 bacterial isolates were isolated (Fig 1) from different soil samples of forest nurseries by serial dilution technique. These isolates were used for the screening for antagonistic activity



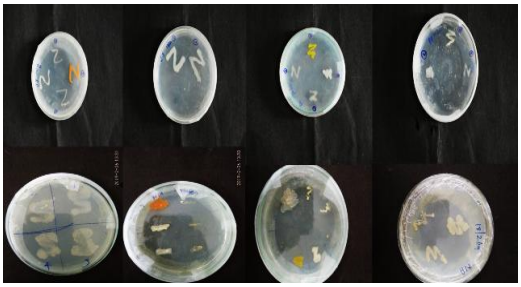


Figure 1: Bacterial isolates isolated from soil samples

B. Screening of bacterial isolates against *F. solani*

Among 245 bacterial isolates, one isolate F KI 2.10 showed promising antagonistic activity against *F. solani* by showing diameter of 1.0 cm of zone of inhibition (Fig). In vitro dual culture test is the initial testing of biological control agents. Antagonistic activity is typically proved by the development of zone of inhibition between bacterial isolate and the fungal isolate [14].

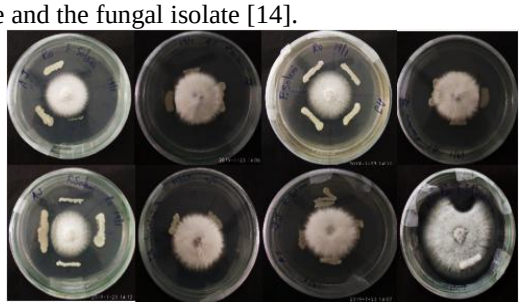


Figure 2: Screening of bacterial isolates for antagonism against *Fusarium solani*

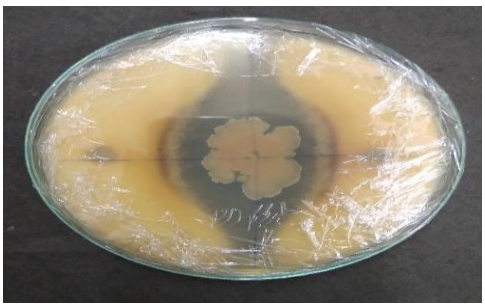


Figure 3: Details of Zone of inhibition of isolate FKI 2.10 against *F. solani*

C. Molecular identification of the isolate

The sequencing was done and sequences were subjected to BLAST and found bacterial isolate FKI 2.10 showed 99% similarity with *Bacillus velezensis* FZB42.

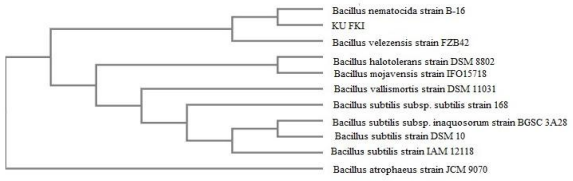


Figure 4. Phylogenetic tree of bacterial isolate FKI 2.10

D. Screening of Antifungal activity of herbal extracts against *F. solani*

By comparing the radial growth of *F. solani*, the inhibition effect of plant extracts were observed in poisoned food technique whereas well plate technique did not show inhibition effect because only 700 µl was added in well plate technique in the other hand 5 ml was added in poisoned food technique. The study stated that the plate with the PDA medium amended with the homogenous mixture of plant extracts, controlled the growth of soil borne pathogen *F. solani* when compared to the control plate.

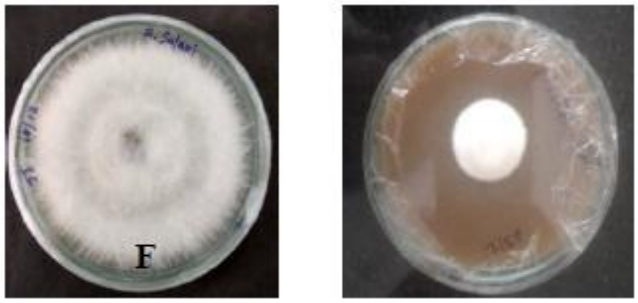


Figure 5: Screening of antifungal activity of herbal formulation against *F. solani*

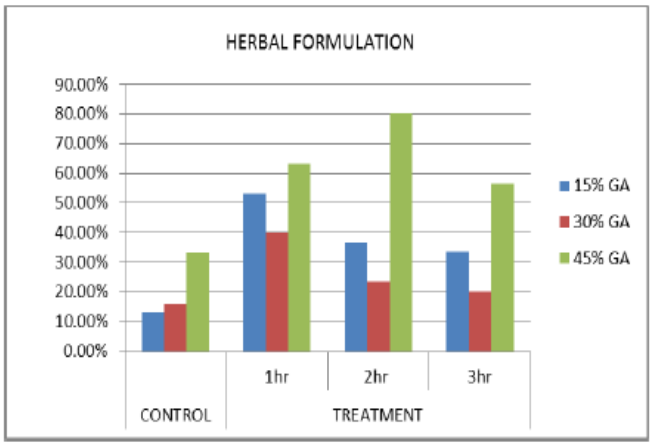
E. Seed germination of *Solanum lycopersicum*

Initial stages of germination started third day after the seeds were sown. Almost 720 seeds were sown in seed trays 628 seeds have germinated. The germination percentage {(No. of seeds germinated /Total no. of seeds sown) x100} in each treatment was tabulated [11]

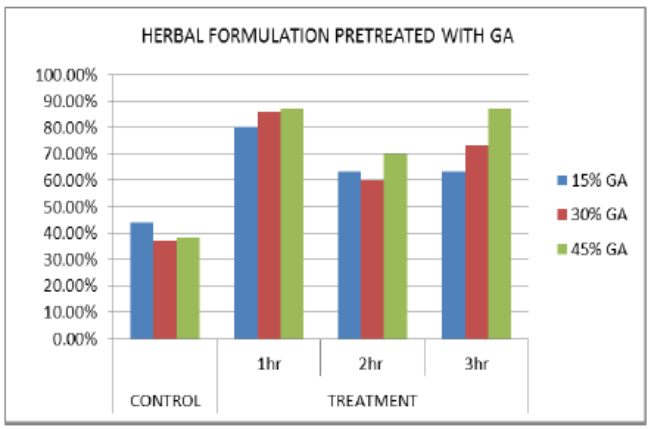
Figure 6: Seed germination in seed trays

Table 5: Seed germination percentage of Tomato plant

Herbal formulation				
	1hr	2hr	3hr	Control
15% GA	53.30%	36.60%	33.30%	16.60%
30% GA	40%	23.33%	20%	13.30%
45% GA	63.30%	80%	56.60%	23.30%
Herbal formulation pretreated with GA				
	1hr	2hr	3hr	Control
15% GA	80%	63.30%	63.30%	33.30%
30% GA	86.60%	60%	73.30%	30%
45% GA	86.60%	70%	86.60%	26.60%
Liquid bio formulation				
	1hr	2hr	3hr	Control
15% GA	56.60%	60%	60%	43%
30% GA	27%	46.60%	43.30%	30%
45% GA	80%	43.30%	86.60%	33.30%
Liquid bio formulation pretreated with GA				
	1hr	2hr	3hr	Control
15% GA	60%	46.60%	44%	23.30%
30% GA	46.60%	60%	56.60%	30%
45% GA	80%	50%	63%	36.60%



(a)



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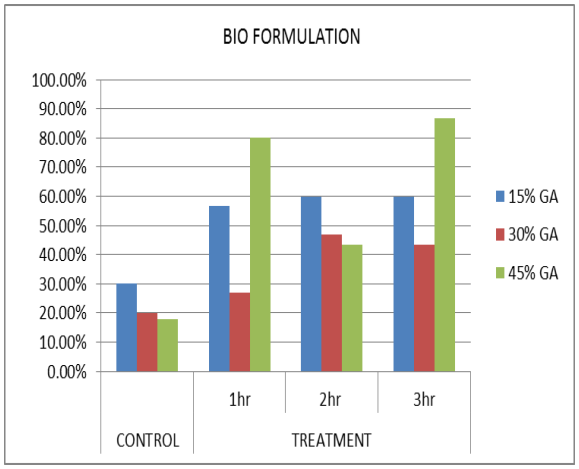

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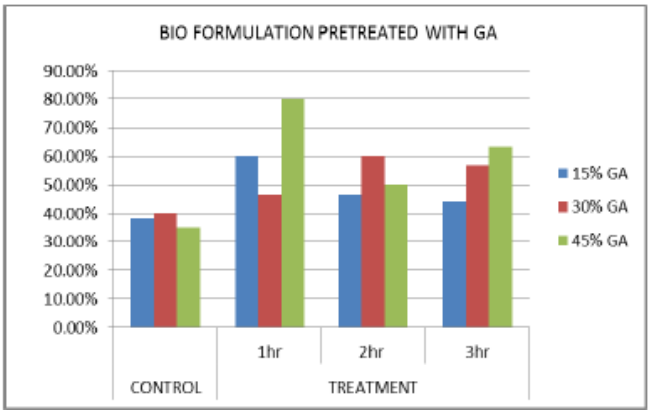

(d)

Figure 7. Germination percentage of (a) seeds treated with herbal formulation, (b) seeds in herbal formulation pretreated with gum arabic, (c) seeds treated with bio formulation and (d) seeds in bio formulation pretreated with Gum Arabic

F. Disease Incidence

Damping of was observed in control and some treated seeds. Damping off disease incidence was found in certain treatments. The disease incidence percentage was calculated using the formula-Disease incidence percentage= (No. of infected plants /Total no. of plants) x100 [11].

Figure 8. Damping off diseased seedlings and healthy seedlings of tomato

Table 6: Disease incidence percentage of Tomato plant

Herbal formulation				
	Treatment		Control	
	Seed germination %	Disease incidence %	Seed germination %	Disease incidence %
15% GA	57%	23%	16.60%	6.66%
30% GA	53.30%	18.75%	13.30%	3.33%
45% GA	54%	12.50%	23.30%	26.60%
Bio formulation				
15% GA	60%	33.30%	60%	23.30%
30% GA	36%	23.50%	43.30%	33.30%
45% GA	56%	37.50%	46.60%	36.60%

Table 7: Detemination of shoot length and root length of germinated seeds

Herbal formulation				
	Treatment		Control	
	Shoot length(cm)	Root length(cm)	Shoot length(cm)	Root length(cm)
15% GA	6.5±1.4	2.9±.35	4.5±1.72	2.1±0.5
30% GA	7.3±1.53	3.1±.801	5.2±1.86	3.1±0.23
45% GA	7.6±1.601	3.3±0.72	5.9±1.93	3.5±.42
Herbal formulation pretreated with GA				
15% GA	6.6±1.909	5.1±1.101	4.9±1.033	1.43±0.47
30% GA	7.2±2.1	5.2±1.21	6.67±0.77	2.7±0.75
45% GA	8.4±2.3	6.1±1.42	5.3±2.03	3.4±1.011
Bio formulation				
15% GA	5.8±1.24	3.1±0.47	4.2±0.53	2.3±0.41
30% GA	7.6±2.11	5.1±0.71	4.76±0.76	3.1±0.81
45% GA	6.5±1.54	4.8±0.54	6.1±1.03	4.1±0.49
Bio formulation pretreated with GA				
15% GA	6.4±1.503	4.3±1.49	5.1±1.52	2.3±0.83
30% GA	5.4±1.744	3.1±0.85	4.3±1.3	2.3±0.72
45% GA	4.3±1.59	2.5±0.473	2.1±0.32	1.1±0.21

IV. DISCUSSION

Although the interactions between many bacteria and fungi have been studied those involving *Fusarium solani* have received less attention. This study presents the data of screening of bacteria isolated from forest nurseries and agricultural fields against *Fusarium solani*, one of the major causal agents of root rot in various vegetable nurseries especially *Solanum lycopersicum*.

Among 60 bacterial isolates one isolate was able to antagonize *F. solani* in dual Petri plate assay. This isolate was chosen for molecular identification and was identified as *Bacillus velezensis* FZB42 by 16SrDNA sequencing. *B.velezensis* was found to be a strong antagonistic isolate which produced a clear zone of inhibition up to 7mm.

Bacillus velezensis isolates (Y6 and F7) retained antagonistic activity against *Fusarium oxysporum* under *in vitro* and field conditions were reported but detailed inhibitory effect of as *Bacillus velezensis* FZB42 strain has not yet been reported elsewhere[20].

The further step of *invivo* studies were attempted with preparation of bio formulation. For the current study in bioformulation, two types of liquid bioformulation were analyzed. One is bacterial bio formulation and the other is herbal bio formulation. In order to increase the seed coating efficiency liquid bioformulation was preferred over solid bioformulation. The regular use of carboxy methyl cellulose CMC was replaced with gum arabic to increase the adhesiveness.

Bioformulation was prepared using gum arabic, coconut oil, glycerol and culture broth of *Bacillus velezensis* FZB42.

Glycerol can be used as an emulsifier as well as it was used to increase the viability of bacterial cells added in the bio formulation. Since coconut oil did not show any antifungal activity against *F.solani* it was chosen as carrier material of the bio formulation. Gum Arabic obtained from natural sources of acacia species increase the adhesiveness and coating efficiency of bio formulation on the surface of the seeds. The herbal formulation was prepared using neem, clove, ginger and garlic as studied by Kumar *et al.,* 2018.

The herbal extracts when tested individually against *F. solani* they showed very less inhibitory effect but when all the herbal extracts were mixed together the inhibition was higher. In well plate technique, where only 700µl of mixed herbal extracts added detailed by, they did not show inhibitory effect but in poisoned food technique where 5 ml of mixed herbal extracts were amended in the media showed great inhibitory effect. This showed that the mixed herbal extracts at high concentration shows inhibitory effect against *F.solani*.

To test this bio formulation in high scale, study was carried out in seed trays. 16 seeds tray setups were made where each treatment set up had thirty seeds. Around seven hundred twenty seeds were sown in the field study. The *Solanum lycopersicum* showed germination in three days. Around 628 seeds germinated in first week. This proves that the formulation not only provides resistance but also increased the germination percentage of *Solanum lycopersicum* seeds. Among the experimental setups, the seeds pretreated overnight with Gum Arabic enhanced the seed coating efficiency of herbal extracts on the surface of the seeds in which 1 hour treatment of herbal formulation with 45% gum Arabic was found to be effective followed by bioformulation in which seeds pretreated with 45% gum Arabic was found to be optimal.

V. CONCLUSION

In this study, sixty bacterial isolates were obtained from soil samples of forest nurseries. All isolates were screened for their *in vitro* antifungal activity against *Fusarium solani*. One of that had great pathogen inhibitory capacity was subsequently given for 16S rDNA sequencing.The results of molecular characterization shows that the bacteria belongs to the *Bacillus* species i.e. *Bacillus velezensis* FZB42. Oil based bioformulation was prepared to test the *in vitro* biocontrol potential of *Bacillus velezensis* against *Fusarium solani* by coating the bioformulation on the seeds of *Solanum lycopersicum*. Coated seeds with antagonistic bacteria significantly reduced the severity of damping off in *Solanum lycopersicum* seedlings. Also evaluated the aqueous extracts of commonly available plant species such as neem, garlic, ginger and clove, blended together in a ratio of 1:2, for their inhibitory effect on *Fusarium solani*. *In vitro* studies of the extracts showed inhibitory effect on *Fusarium solani*. *In vivo* studies were carried out by developing a bioformulation using the extracts and coating the bioformulation on the seeds of *Solanum lycopersicum* which showed great inhibitory effect against *Fusarium solani*. Around 720 hundred seeds were sown in the field study. Around 628 seeds germinated. This proves that the formulation not only provides resistance but also increased the germination percentage of *Solanum lycopersicum* seeds. the seeds pretreated overnight with Gum Arabic enhanced the seed coating efficiency of herbal extracts on the surface of the seeds in which 1 hour treatment of herbal formulation with 45% gum Arabic was found to be effective followed by bioformulation in which seeds pretreated with 45% gum Arabic was found to be optimal.

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AURHORS PROFILE

Dr. R. S. David Paul Raj completed his PhD from University of Madras, Chennai in 2003 with specialization in Molecular Plant Pathology and Plant Breeding. He has 15 years of teaching and research experience in the field of biotechnology. He has a credit of a patent entitled "Enhanced production of anti-snake venom compound (Taraxerol) from suspension cultures systems of euphorbia hirta l" Patent No. 201941017991 A. He has received the best teacher award and several best research presentations award. He holds National and International publications in peer reviewed journal with a cumulative H index of 5. He has developed two bioformulations for plant growth and biocontrol of root rot pathogens. He has organized several national level conferences, Symposium and workshops in the field of biotechnology. He is currently working as Assistant Professor (SG) in Department of Biotechnology, Karunya Institute of Technology and Sciences, Coimabtore, Tamilnadu, India.

R. Beena Kanimozhi pursuing PhD in Department of Biotechnology, Karunya Institute of Technology and Sciences, Coimabtore, Tamilnadu, India. She completed her B.Tech Bioinformatics in Karunya Institute of Technology and Sciences, Coimabtore, Tamilnadu, India. And completed her M.Tech Genetic Engineering in SRM Institute of Science and Technology, Kattankulathur, Chennai, Tamil Nadu, India. Her research work is on developing a bioformulation for biocontrol of root rot pathogen.

Dr. Levin Anbu Gomez is working as an Assistant Professor with 12 years of experience in teaching and 8 years of research proficiency. Major part of research was on Immunotechnology. Had numerous projects handled for both undergraduate and post graduate students with a publication as an outcome in peer reviewed journals which further led to process for a patent to be filed. Avidly taking part in Conferences, Seminars, Workshops and Hands on training. Had also organized workshops and national level conferences to make science abound to the younger generation. As part of my Ph.D work I focused on raising chicken antibodies against Rabies Viral Antigen which could be used for passive immunization. I have 9 publications till date in both peer reviewed National and International Journals.

S. Rohini has completed her B.Tech Biotechnology, Karunya Institute of Technology and Sciences, Coimabtore, Tamilnadu, India. Her project work is on developing a bioformulation for the biocontrol of *Fusarium solani* in tomato plants.