RESEARCH ARTICLE

CONTROL OF FUSARIUM WILT DISEASE IN COWPEA PLANT (*VIGNA UNGUICULATA* L.). USING SECONDARY METABOLITES PRODUCED IN *BRADYRHIZOBIUM JAPONICUM*

Kannan, K.[1]*, V. Rajesh Kannan[2], N. Shibinaya[2], and M. Umamaheswari.[2]

[1]Department of Microbiology, The Madura college, Madurai -625 011, Tamil Nadu, India
[2] Rhizosphere Biology Laboratory, Department of Microbiology, School of Life Sciences, Bharathidasan University, Tiruchirappalli - 620 024, Tamil Nadu, India.

ABSTRACT

Rhizobium known for its nitrogen fixation and plant growth promoting capabilities which is symbiotically associative with legume plants. So forth *Rhizobium* used as biofertilizers in the agriculture. The ability of controlling plant diseases by using *Rhizobium* produced secondary metabolites as biocontrol agent is the current open area in the agriculture research. The nodules inhabited *Rhizobium* strains were selected for the production of secondary metabolites and the ability of controlling *Fusarium* was evaluated preliminarily by agar well diffusion assay. Four different *Rhizobium* were isolated, among that S1 cannot showed any inhibition, whereas S2, S3 and S4 were showed 11, 15 and 19mm of inhibition respectively. Among that S4 selected further and DNA isolated and identified using 16S rDNA gene sequencing. The sequences were submitted in genbank and got accession number MH165175. This organism was found to be *Bradyrhizobium japonicum* and mass cultured for compound extraction using organic solvents. The extracted secondary metabolite were purified using different chromatography techniques. The purified fractions were analyzed for the biocontrol of *Fusarium sp.,* isolated from infected cowpea and results showed fraction 4 showed 21mm zone of inhibition. Further the selected fractions were analytically characterized to know the compounds present. Finally the purified compounds were evaluated for its biocontrol behavior against *Fusaium* sp., and plant growth promotion in *in vitro* conditions.

Keywords: Cowpea, Rhizobium, Biocontrol, PGPR and Secondary Metabolites.

1. INTRODUCTION

Cowpea (*Vigna unguiculata* L.), is one of the most important legume crop grown in the tropical belt. This crop provides food, animal feed and cash for the rural population in addition to benefits to farmlands through *in situ* decay of root residues and ground cover due to the spreading habit of the plant. In addition, cowpea grain provides a cheap and nutritious food for relatively poor urban communities. Cowpea's high protein content, its adaptability to different types of soil and intercropping systems, its resistance to drought, and its ability to improve soil fertility and prevent erosion makes it an important economic crop in many developing countries (1). Cowpea due to its wide adaptation across the regions and soils harbours a number of diseases which considerably influence growth and productivity potential. One of the prime cowpea disease is *Fusarium* wilt caused by *Fusarium oxysporum* Schlechtend f.sp. *ciceris* is a major constraint to cowpea production throughout the world and particularly in the Indian subcontinent and the Mediterranean basin (2). *Fusarium oxysporum* can survive in soil several years by means

of chlamydospores, which markedly reduce the potential of crop rotation as a disease management strategy. Efforts must be addressed toward developing new alternatives for more effective disease management. In current scenario use of chemical fertilizer for biocontrol of soilborne plant pathogens including *F. oxysporum* has been shifted to the option of green technology that have an agriculture importance.

Biological control agents for plant diseases are currently being examined as alternatives to synthetic pesticides due to their perceived level of safety and minimal environmental impacts. Strains of several bacterial species such as *Bacillus*, *Pseudomonas* and recently the *Rhizobium* group used as the biocontrol agents (1,3). As compared to the other biocontrol agents, Rhizobia offer the great advantage of symbiotic nitrogen fixation by association with legumes. Among the *Rhizobium* group, *Rhizobium leguminosarum*, *Sinorhizobium meliloti* and *Bradyrhizobium japonicum* have been used successfully against fungal pathogens belonging to the genera *Macrophomina, Rhizoctonia* and *Fusarium*. (4-6).

*Correspondence: Kannan, K., Department of Microbiology, The Madura college, Madurai -625 011, Tamil Nadu, India.
E.mail: kannan.bdu.micro@gmail.com

Induction of host defenses can be local and or systemic in nature, depending on the type, source, and amount of stimuli. Induced systemic resistance is one of the mechanisms of disease suppression by fungal pathogens by many microorganisms (7,8). Rhizobia have several mechanisms of action that allow them to control pathogens. In general, competition for nutrients, niche exclusion, induced systemic resistance and antifungal metabolites production are the chief modes of biocontrol activity in PGPR. The aim of the present work is to characterize and select *Rhizobium* isolates with antagonistic activity against wilt disease in cowpea as well as extraction of their secondary metabolites from selected rhizobia and their effects on *Fusarium* and disease development in Cowpea were assessed *in vitro* nursery conditions.

2. MATERIALS AND METHODS

2.1. Sample Collection

Root from young and healthy seedling of cowpea (*Vigna unguiculata* L.) plants was collected from Viralimalai village, Pudukkottai district of Tamil Nadu, India. The collected samples were kept in plastic bags without affecting the root with nodules, brought to the laboratory under sterile conditions and stored in refrigerator for further processing.

2.2. Isolation and Identification of Rhizobium

Healthy cowpea nodules were detached from the root and further isolation of root nodulating rhizobia was carried out. The detached root nodules were washed in tap water to remove the adhering soil particles from nodule surface. Nodules were dipped in 0.1% mercuric chloride (HgCl$_2$) solution for 30 sec and later washed successively ten times with sterilized distilled water to remove the traces of toxic HgCl$_2$. Surface sterilized nodules were transferred in test tube containing 5 ml sterilized distilled water. These nodules were crushed with the help of sterilized glass rod to obtain a milky suspension of bacteroides. These were streaked on YEMA medium containing congo red. The plates were sealed by parafilm to avoid contamination and incubated at 28ºC for 7 days and make daily observations for the appearance of typical colonies of rhizobia (9). After the incubation period from the pool of well grown colonies were picked up and selected four different colonies named as S1, S2, S3 and S4. Further the cultures subjected to gram staining for the shape, size, and arrangement of bacterial isolates (10).

2.3. Biochemical Characterization and Molecular Identification

Various biochemical tests were carried out were indole test, methyl red test, Voges Proskauer test and citrate utilization tests based on standard microbiological manual in order to confirm primarily by the *Rhizobium* genus for all the four isolates. In order to confirm the *Rhizobium* genus secondarily by subjected to Bromothymol blue test and Ketolactose test, in which the Yeast Extract Mannitol was enriched with 1% (w/v) Bromothymol blue to selectively identify fast *Rhizobium.* All the four samples S1, S2, S3 and S4 were subjected to grow on BTB added YEM media for 48 hrs at 28ºC. Positive sample showed yellow color to acid production after incubation. Whereas in Ketolactose test, a loopful of the inoculums from a fully grown culture slant (7 days old culture) was transferred to a petri plate containing the ketolactose agar medium. After incubation for 5 days at 27ºC the plates were flooded with a shallow layer of Benedict's solution. After pouring the plates were incubated for one hour at 30ºC without any disturbance. The absence of yellow coloration around bacterial colonies confirms the presence of *Rhizobium* (11).

2.4. Molecular Identification

Overnight fresh bacterial broth cultures (2 ml) were taken and centrifuged at 10000 rpm for 5 minutes and the supernatant was discarded. To the pellet, 570 μl of TE buffer, 30 μl of 10% SDS were added, mixed thoroughly and incubated at 37°C for 1 hour. After incubation, 5M NaCl was added and mixed thoroughly by vortex. 80 μl of CTAB/NaCl solution was added, mixed thoroughly and incubated at 65°C for 10 min. Equal volume (0.7 – 0.8ml) of chloroform or isoamyl alcohol was added, mixed and centrifuged at 10000 rpm for 5 min. The viscous supernatant was transferred to a fresh centrifuge tube (without disturbing the interface); equal volume of phenol/chloroform or isoamyl alcohol was added and spun at 10000 rpm for 5 min. The aqueous supernatant was transferred to a fresh centrifuge tube and 0.6 volume of ice cold isopropanol was added and incubated for 20 min at -20°C. Later, the tubes were centrifuged at 10000 rpm for 5 minutes. To the pellet, 70% of ethanol was added and centrifuged. The pellet was stored at -20°C after adding TE buffer or sterile mQ water. For running the sample, 0.8% of agarose was prepared in TE buffer. To 50 ml of agarose 3 μl of ethidium bromide was added and casted in the boat which contains comb. After cooling, the comb was removed and the sample was loaded. To 5 μl of the sample 2 μl of the gel loading dye was added and loaded in the

respective well, and electrophoresis was done it 50 V (12).

2.4.1. 16S rRNA PCR Amplification

Amplification of the 16S rRNA gene was conducted using universal primers: 27F(5'-GAGAGTTTGATCCTGGCTCAG-3'), 1495R (5'-CTACGGCTACCTTGTTACGA-3'). The final products were analyzed through electrophoresis on 1.2% agarose gel and stained with $0.5\mu g$ ml^{-1} ethidium bromide. The PCR products obtained were purified and sequenced using Sanger's dideoxynucleotide chain termination method and were sequences at Amnion sequencing Pvt. Ltd. India (Bangalore). The sequences obtained were aligned with previously published sequences available in NCBI using BLAST (13). The phylogenetic and evolutionary analyses were conducted using MEGA 5 software (14).

2.5. Isolation and Identification of Pathogenic Fungi

Infected stem (Brick red tissue in stem indicates wilt disease) region of cowpea plants were collected and submerged in 5% sodium hypochlorite for five minutes. After this treatment, they were extensively washed with sterile distilled water and placed on Petri dishes containing potato dextrose agar (PDA, Difco) and incubated at 22°C for 48 hrs. Morphological identification are done according to the standard taxonomic key included colony diameter, texture, colour identified in their sporulation state by staining with lactophenol cotton blue.

2.6. Identification of Potential Rhizobium
2.6.1. Antifungal Activity of Rhizobium Extracts against Fungal Pathogen

The *Rhizobium* strain S1, S2, S3, S4 were subjected to antifungal activity using agar well diffusion method against the *Fusarium* sp. on Muller Hinton Agar plates. The test fungi was spread over the agar plate using the sterile cotton swab and well were created on the agar. 100µl bacterial culture was added to into the well and incubated for 4 days at room temperature. Finally plates are observed for zones of inhibition and their diameter was measured with the help of antibiotic zone scale. Among the four isolate S3 and S4 showed better inhibition than the other isolates.

2.7. Extraction and Purification of Secondary Metabolites from Rhizobium Extracts

The *Rhizobium* isolates S4 was mass cultivated in Yeast Extract Mannitol broth and incubated at 37°C for 10 days with periodical shaking at 150 rpm. After the incubation period, the cultures are taken out and then centrifuged at 5000 rpm for 10 mins, the cell free supernatant was transferred to another flask. To this equal volume of different solvents such as ethyl acetate, diethyl ether, chloroform, and hexane was added and the compound was extracted using solvent extractor. The crude extracts were tested for their antimicrobial activity against *Fusarium* sp. The extracted compound was separated by column chromatography using silica gel as the packing material. The fraction was eluted by using chloroform: methanol: water (5:6:4) as the solvent system. Different fractions were eluted from column chromatography and checked for antifungal activity (15).

2.8. Characterization of Pure Compound

The collected fraction from *Rhizobium* isolate (S4) with higher antifungal activity were subjected to FT-IR (Perkin Elmer, Spectrum MRX-1 model) and also analyzed using GC-MS (model Q-Mass 910, Perkin Elmer) (16).

2.9. In vitro Seed Germination

The viability of the seeds were tested by using Tetrazollium chloride test (TZ test). The seeds were soaked in 0.1 % of 2, 3, 5 triphenyl tetrazollium chloride for few minutes and the seeds were evaluated for the viability. Sound tissues produce a normal red colour and resist the penetration of tetrazollium, and those seeds were said to be viable. After the viability test cowpea seeds were sterilized by using 70% ethanol followed by 0.1% mercuric chloride before rinsing with sterilized water. The experiments were carried out in a positive, negative control and preventive method. Positive control (cowpea seed+ pathogen + carboxy methyl cellulose), negative control (cowpea seed+ distilled water + carboxy methyl cellulose) and preventive test using 5 dose of fractions from nodule compound as well as from the *Rhizobium* culture compound (10, 20, 30, 40, and 50 µl) on seed (cowpea seed + pathogen + compound doses + carboxy methyl cellulose). The seeds were coated with respective materials and air dried overnight. The seed were placed on a sterile filter paper in petriplates. Water is sprinkled at regular intervals and kept at a place receiving sunlight for 7 days. The germination rate was assessed in triplicates after 7 days of incubation using the formula below (17).

$$\text{Germination rate } (\%) = \frac{\text{Number of germinated seeds}}{\text{Number of total seed tested}} \times 100$$

3. RESULTS AND DISCUSSION

3.1. Collection of Nodule and Isolation of Rhizobium

Healthy cowpea (*Vigna unguiculata*) plants were carefully uprooted from the cowpea field, Viralimalai village, Pudhukottai district of Tamil Nadu, India. The roots along with nodules were stored in sterile polythene bags at 4° C for further analysis. The surface sterilized root nodules were crushed and the suspension was streaked over the YEMA medium with the addition of congo red. Growth of large opaque mucoid elevated glistening colonies in the medium shows the presence of *Rhizobium.* Four different colonies S1, S2, S3 and S4 were selected for further study (Fig. 1).

Fig. 1. Cowpea plant root with fresh nodules and rhizobium colonies on the YEMA plate

3.2. Identification of Rhizobium
3.2.1. Morphology of Isolates

All the four isolates S1, S2, S3 and S4 were found to be circular, mucoid and raised colonies. The shapes of the isolates were identified by the staining techniques. Colony size, shape, margin, elevation and opacity of all the four bacterial isolates were tabulated (Table 1).

3.3. Biochemical characterization

The biochemical characterization is carried out to identify the genus of the isolated bacteria and along with two confirmatory tests (Table 2). All isolates were streaked on Bromothymol blue added YEM selective media for further confirmation. Strains S1, S3, S4 showed growth in 2 days and turned YEM media from blue to yellow confirming their nature of being fast growers and acid producers whereas strain S2 was a slow grower and acid producer (Fig. 2). Whereas in the ketolactose test the plates were flooded with Benedict's reagent and incubated for one hour. The excess reagent is drained off and the plates were checked for colour change. All the four isolates used lactose from the medium and showed growth. No yellow colourations were found around the colonies after adding Benedict's reagent. Absence of yellow colouration around the colonies denotes the presence of *Rhizobium.*

3.4. Molecular Identification

The Rhizobium isolates having higher antagonistic S3 and S4 were found to be the potential isolates for acting against the fungal pathogen. Hence the genomic DNA was isolated for those particular two isolates (S3 and S4) by using C-TAB method. The isolated DNA was identified using 0.8% agarose gel followed by observation on ultraviolet trans illuminator which revealed sharp high molecular weight bands of DNA. This indicated that the DNA was of good quality and suitable for PCR analysis.

Table 1: Morphological characteristics of the four isolates

S. No.	Strain characters	S1	S2	S3	S4
1.	Colony Shape	Circular	Circular	Circular	Circular
2.	Size	4mm	6mm	6mm	5mm
3.	Colour	Pinkish white	White	White	Milky white
4.	Opacity	Translucent	Transparant	Transparant	Translucent
5.	Motility	Motile	Motile	Motile	Motile
6.	Bacterium shape	Rod	Rod	Rod	Rod
7.	Gram staining	Gram negative	Gram negative	Gram negative	Gram negative

Table 2: Biochemical test of the four isolates

Isolates	Indole	MR	VP	Citrate	Catalase	Oxidase	Urease	Nitrate Reduction	Lipase
S1	-	-	+	-	+	-	+	+	+
S2	-	-	+	+	+	-	+	+	+
S3	-	-	+	+	+	-	+	+	+
S4	-	-	+	-	+	-	-	+	+

**Fig. 2. Bromothymol blue test and Ketolactose test**

Notes: In the first plate colour change of the medium from blue to yellow shows the presence of fast growing acid producers and in the second plate addition of Benedict's reagent does not cause any change in the medium.

The DNA of the bacterial isolates S3 and S4 were amplified by performing 16S rRNA gene of the isolated DNA using forward primer 27F (5'-GAGAGTTTGATCCTGGCTCAG-3') and the reverse primer 1495R (5'-CTACGGCTACCTTGTTACGA-3'). The conformation of amplification was done by agarose gel electrophoresis by running 5µl of PCR reaction mixture on 1% agarose gel. The amplified product was sequenced in AMNION Pvt. Ltd., Bangalore. The obtained sequences were compared with NCBI databases through BLAST analysis. 16S rRNA gene of the bacterial isolate S4 showed 99.9% similarity with the *Rhizobium* genus. Then the sequence was submitted in Gene Bank for the accession number MH165175 and the organism found to be as *Bradyrhizobium japonicum.*

3.5. Isolation and Identification of Pathogenic Fungi

The wilted stem of cowpea plant showed the presence fungal growth in the PDA plate. The fungal colony is macroscopically and microscopically identified (Fig. 3). Macroscopical colony morphology shows cottony mycelium with pale brown to yellowish brown zonation. The isolated fungi subjected to lactophenol cotton blue staining were observed under microscope. The spore formation and hyphal structures were identified and confirmed to be *Fusarium* strain. The staining shows the presence of sickle shaped conidiospores and septate spores which is the characteristic feature of *Fusarium* sp. The isolated *Fusarium* strains were stored at 5°C in tubes containing PDA.
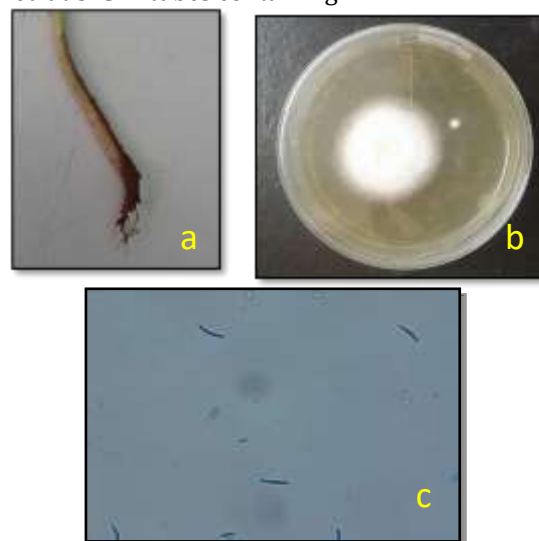
Fig. 3. Isolation and identification of fungi from infected cowpea plant

Notes: a. Brick red tissue in stem indicates wilt disease, b. Pure culture of plant pathogen on PDA plate showed white cottony colony, c. Lactophenol cotton blue staining showed sickle shaped spore and septate.

3.6. Identification of Potential Rhizobium Antifungal Activity by Agar Well Diffusion Method

All the isolates were cultured in YEM broth and the supernatant was collected by centrifugation. The supernatant was then tested for antifungal activity against *Fusarium* sp., (Fig. 4.). The zone of inhibition was found around the wells containing the supernatants. There was no zone clearance found around the S1 supernatant, whereas the other three cultures showed zone of inhibition against the pathogen. Among them S2 showed 11mm, S3

showed 15mm and S4 showed 19mm of inhibition against *Fusarium* sp.,

Fig. 4. Antifungal test of the culture supernatants, extraction from *Bradyrhizobium japanicum* against *Fusarium* sp.

Notes: a. S1, S2, S3 and S4-Culture supernatants, b. F1-F5-Rhizobium Fractions, C- Ethyl acetate

3.7. Extraction of Secondary Metabolites from Bradyrhizobium japanicum

The collected supernatant from the isolates S3 and S4 were extracted with equal volume of different solvents such as water, ethyl acetate, diethyl ether, chloroform, and hexane. The crude extract is tested for antifungal activity by agar well diffusion method. Among the two isolates the crude extract of S4 isolate obtained from ethyl acetate extraction showed maximum inhibition of 19mm and S3 by 12 mm of inhibition than in the other solvents used (Fig.4). So the ethyl acetate extract of S4 only taken for further purification of the secondary metabolite using column chromatography.

3.8. Purification of the Secondary Metabolite from Bradyrhizobium japanicum by Column Chromatography

In column chromatography 10 different fractions were collected at regular time intervals to get pure compounds. All the obtained fractions were tested for antifungal activity by agar well diffusion method in MHA plates and among them, fractions F4 and F5 showed inhibition against *Fusarium.* The zones of inhibition were measured to be 21 and 18mm for F4 and F5. The pure extract F4 were dried and further subjected for structure elucidation.

### 3.9. Characterization of extracted compounds

Purified compounds further characterized using FT-IR, IR spectrum and The FT-IR spectrum showed various peaks at different wavelengths which indicate the presence of various chemical groups in the extracted sample (Fig. 5). Several chemical groups also existed in the extracts from *Bradyrhizobium japanicum* which are represented in the FT-IR result showed absorption at 3441.85 (O—H), 2922.0 (COOH), 2851.93 (COOH) broad, 1541.58 (N=O), 1095.00 (C—O), 1462.67 cm^{-1} confirmed the presence of C—C=C (Fig.3). GC-MS results of bacterial extract compound showed that major peak formed at the retention time 4.235, 17.2 and 22.5 were Propenemide , Hexadecanoic acid methyl ester and 1,2 benzenedicarboxylic respectively which has responsible for antimicrobial and antifungal character (Fig.6).

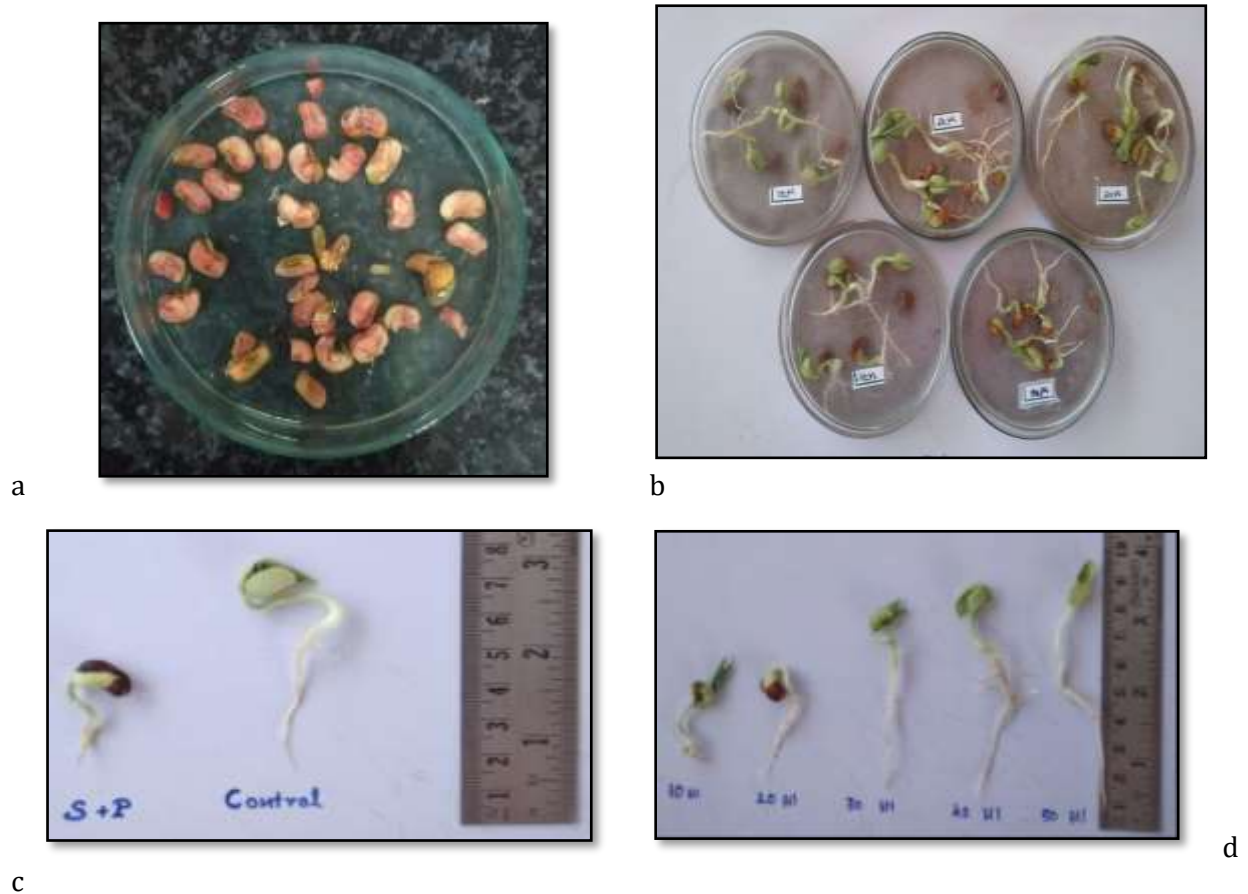### 3.10. In vitro Seed Germination in cowpea seeds

Before the seed germination test the seeds subjected to Tetrazolium chloride test (TZ test) to check the viability of the seeds. Color change of seeds from normal to red colour indicates that the seed is viable various color change into brown indicates dead tissue. After 7 days of incubation, the plant indexes such as root length, shoot height, and germination percentage have been observed and compared with positive and negative control (Fig. 7) (Table 3).
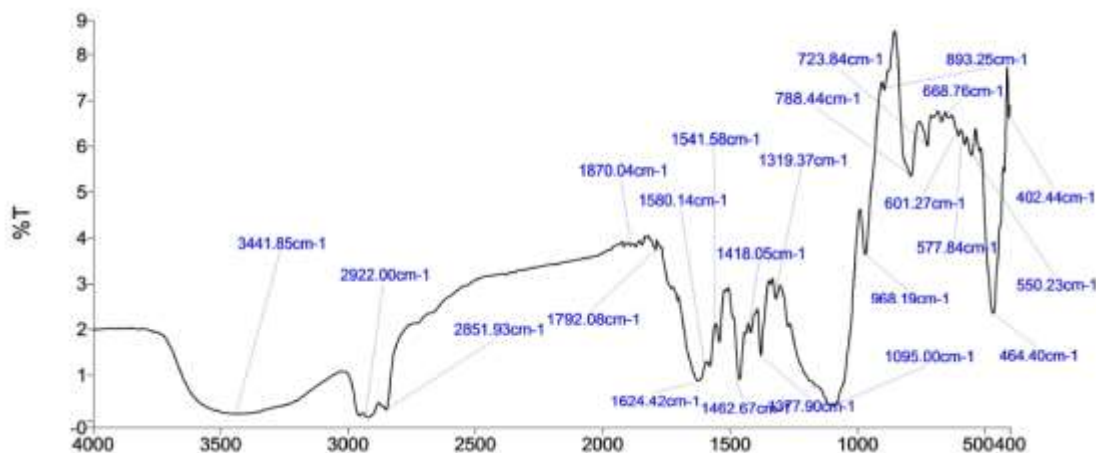
Fig. 5. Detection of compounds from bacterial extract using FT-IR
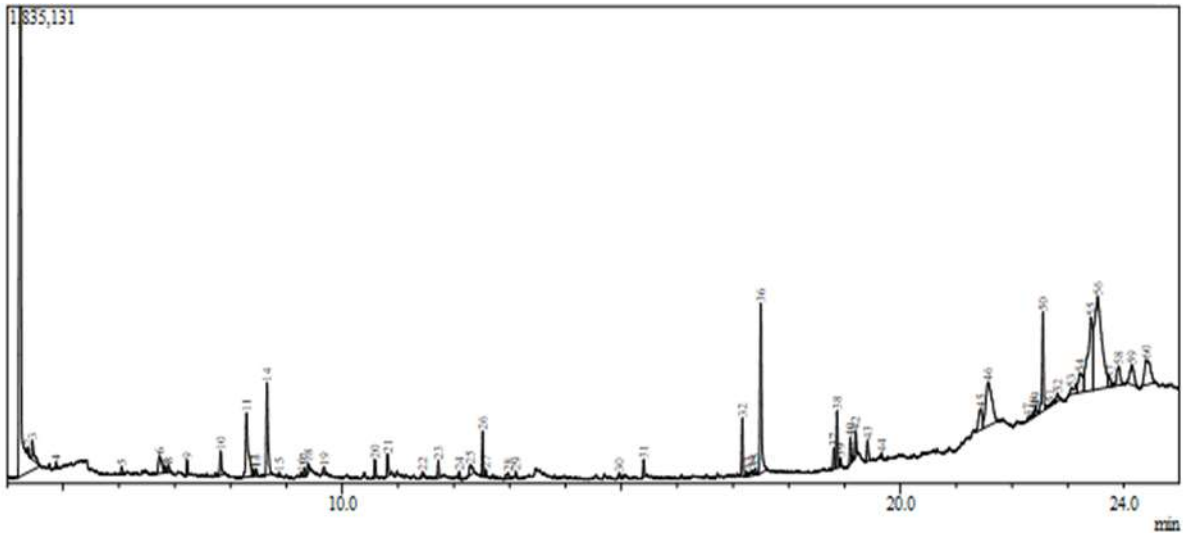
Fig. 6. GC-MS analysis of bacterial extract

a

b

c

d

Fig. 7. *In vitro* seed germination in cowpea seeds

Notes : a – Tetrazollium chloride seed viability test, b - Seeds treated with different concentration (10, 20, 30, 40, and 50 µl) of the compound obtained from Rhizobium. c – Positive control (seed + pathogen) and negative control (seed alone), d – Germinated seeds

In comparison it is clearly seen that the compound untreated seeds show less or no growth as they cannot combat with the pathogen. But the compound treated seeds had grown well combating

34

the pathogen. Moreover as the concentration of the compound increases, the germination also increases to a greater extent. This shows that the compound has a good antifungal activity in *in-vitro* conditions.

4. CONCLUSION

Rhizobium was isolated from the root nodules of cowpea plant and identified using various biochemical and morphological characterization. The isolates were checked for its antagonistic nature by agar well diffusion methods. From those experiments, potential Rhizobium (S4) isolates were identified among the four *Rhizobium* isolates (S1, S2, S3 and S4) and secondary metabolites were extracted from the *Bradyrhizobium japonicum* (S4) using solvent extraction and their antifungal activity checked against *Fusarium* sp., Which causes wilt disease in the cowpea. The pure extracts were then analyzed using FT-IR and GC-MS for the identification of compounds. The identified compounds were treated with seeds and the biocontrol ability was checked by the *in vitro* seed germination methods.

From this study, it is proved that the production of the secondary metabolite from the antagonistic *Rhizobium* strain *Bradyrhizobium japanicum* isolated from cowpea root nodule control the growth of the plant pathogenic fungi *Fusarium* sp., and improves the growth.

The *in vitro* study showed better seed germination in the compound treated seeds to a greater degree of germination when compared to the positive and negative control. The compound extracted from the *Bradyrhizobium japanicum* is not only exhibit biocontrol against *Fusarium* wilt disease but also as improves the growth of the cowpea. These biometabolite compounds from microorganisms act as an alternative for the chemical control agents against wilt diseases in cowpea and which improves the soil fertility for the sustainable agriculture.

REFERENCES

1. Arfaoui, A., B. Sifi, A. Boudabous, I.E. Hadrami and M. Cherif, (2006). Identification of *Rhizobium* isolates possessing antagonistic activity against *Fusarium* oxysporum f. sp. ciceris, the causal agent of *Fusarium* wilt of chickpea. *J. Plant Pathol.* 67-75.
2. Nene, Y.L., M.P. Haware, M.V. Reddy, J.C., Phillips, E.L., Castro, S.R., Kotasthane and R.P. Sah, (1989). Identification of broad based and stable resistance to wilt and root-rots in chickpea. *Indian Phytopathol.* **42**(4), 499-505.
3. Kannan, K., and V. Rajesh Kannan, (2019). Characterization of the bioactive metabolite from a plant growth promoting rhizobacteria *Pseudomonas aeruginosa* VRKK1 and exploitation of antibacterial behaviour against *Xanthomonas campestris* a causative agent of bacterial blight disease in cowpea. *Arch Phytopathol. Plant Protect.*
4. Huang, H.C., and R.S. Erickson, (2007). Effect of Seed Treatment with *Rhizobiumleguminosarum* on *Pythium* Damping-off, Seedling Height, Root Nodulation, Root Biomass, Shoot Biomass and Seed Yield of Pea and Lentil. *J. Phytopathol.* **155**(1): 31-37.
5. Akhtar, M.S., and Z.A. Siddiqui, (2008). Biocontrol of a root-rot disease complex of chickpea by *Glomus intraradices*, *Rhizobium* sp. and *Pseudomonas straita*. *Crop protect.* **27**(3-5): 410-417.
6. Arora, N.K., S.C. Kang and D.K. Maheshwari, (2001). Isolation of siderophore-producing strains of *Rhizobiummeliloti* and their biocontrol potential against *Macrophomina phaseolina* that causes charcoal rot of groundnut. *Curr. Sci.* 673-677.
7. Mavrodi, D.V., R.F. Bonsall, S.M. Delaney, M. Soule, J., G. Phillips, and L.S. Thomashow, (2001). Functional analysis of genes for biosynthesis of pyocyanin and phenazine-1-carboxamide from *Pseudomonas aeruginosa* PAO1. *J. bacterial.* **183**(21): 6454-6465.
8. Yu, X.Q., Y.F. Zhu, C. Ma, J.A. Fabrick and M.R. Kanost, (2002). Pattern recognition proteins in Manduca sexta plasma. *Insect Biochem. Mol. Biol.* **32**(10): 1287-1293.
9. Vincent, J.M. (1970). A manual for the practical study of the root-nodule bacteria. *A manual for the practical study of the root-nodule bacteria.*
10. Graham, P.H. and C.A. Parker, (1964). Diagnostic features in the characterisation of the root-nodule bacteria of legumes. *Plant and soil*, **20**(3): 383-396.
11. Bernaerts, M.J., and I. Deley, (1963). A biochemical test for crown gall bacteria. Nature **197**: 406-7.
12. Saitou, N., and M. Nei, (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**: 406–425.
13. Altschul, S.F., T.L., Madden, A.A. Schäffer, J. Zhang, Z. Zhang, W. Miller and D.J. Lipman, (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**(17): 3389-3402.
14. Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei and S. Kumar, (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **28**(10): 2731-2739.

15. Raviraja, N.S., G.L. Maria and K.R. Sridhar, 2006. Antimicrobial evaluation of endophytic fungi inhabiting medicinal plants of the Western Ghats of India. *Eng. Life Sci.* **6**: 515-520.

16. Rispail, N., P. Morris and K.J. Webb, (2005). Phenolic compounds: extraction and analysis. In *Lotus japonicus handbook* (pp. 349-354). Springer, Dordrecht.

17. Ng, L.C., M. Sariah, O. Sariam, O. Radziah and M.A. Zainal Abidin, (2012). Rice seed Bacterization for promoting germination and seedling growth under aerobic cultivation system. *Aus. J. Crop Sci.* **6**: 170-175.