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Studies on Symbiotic Association of Mycorrhiza with *Burkholderia multivorans* for Sustainable Agriculture

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This study deals the symbiotic association of *Scutellospora nigra* an arbuscular mycorrhizal fungus with and without the presence of *Burkholderia multivorans* with *Allium cepa* as host plant. The inoculum consisted of AM fungus *Scutellospora nigra* and the *Burkholderia multivorans* bacterial isolates were prepared and used for this study. Identification of *Burkholderia multivorans* based on physical and biochemical characters were not sufficient for species identification. Amplification of 16S rRNA gene was a suitable technique for molecular level identification. The sequence of 16S rRNA gene of *Burkholderia multivorans* was submitted to NCBI database. *Burkholderia multivorans* was gave antibiotic activity against the antibiotic such as chloramphenicol and tetracycline. Dual inoculation of *Scutellospora nigra* and *Burkholderia multivorans* significantly increased the association of AMF *Scutellospora nigra* with *Allium cepa*.

Key words: Arbuscular mycorrhizal fungi, *Scutellospora nigra*, *Burkholderia multivorans,* 16SrRNA, NCBI.

Arbuscular Mycorrhical (AM) fungi are soil microorganisms and obligate symbionts of plants which forms link between the soil & plant roots to enhance nutrients, uptake water by their hosts, toxic metal resistance and also enhances production of plant growth regulations. AM fungi can reduce the plant root disease caused by pathogen in mycorrhizal plants[1, 2]. According to Leigh *et al*. (2011)[3], AMF are the most common type of mycorrhiza, formed by a fungal group that occurs in most soils. The most characteristic feature of AM Fungi is the Arbuscules. Arbuscules are formed within the plant cell.

Interactions between bacteria with plant roots are able to stimulate plant growth such kind of bacteria have been classified as plant growth promoting rhizobacteria (PGPR). Mycorrhizal fungi are colonized plant roots with their presence of bacteria. These dual effects have been showed combined beneficial impacts on plants[4].

Burkholderia multivorans is a gram negative, motile and rod shaped bacteria found abundantly in the environment, especially within the region of rhizosphere[5]. Many species of Burkholderia have been considered as symbiotic rhizospheric or endophytic plant groth promoters[6]. They can act as a symbiotic partners between the roots of plants including Onions, Sugarcane, Maize, Wheat and Legumes by fixing nitrogen and solubilize metals[5, 7- 10].

For species identification, sequencing of 16S rRNA is a molecular technique for characterization of bacteria and various molecular tools has been raised to analyse the phylogentic relationship of an organism[11]. In this article, we will focus on mutualistic interaction between *Burkholderia multivorans* and *Scutellospora nigra* leads to stimulation of plant growth. This has been performed to test the effect of these inoculants upon mycorrhizal colonization. The *Burkholderia multivorans* a soil bacteria enhance the growth of the symbiotic fungus in the *Allium cepa*. The

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antibacterial activity of *Burkholderia multivorans* prevents the growth of the other soil bacteria in the trap culture.

MATERIALS AND METHODS

The soil samples in the coastal region such as Mahabalipuram and ECR near Chennai, TamilNadu, India were collected.

Isolation and Identification of *Scutellospora nigra*

By Wet sieving and decanting method[12], the spores were extracted. The extracted AMF spores were identified by based on size, shape, color and surface structure, hyphal attachment and wall details of spores[13].

Trap Culture development

Allium cepa was used as host plant in this study. With the host *Allium cepa*, the spores were multiplied

Isolation of *Burkholderia multivorans*

Burkholderia multivorans were isolated from soil sample by serial dilution method. Identification of *Burkholderia multivorans* were based on physical characterization such as gram staining and the biochemical characterization such as Catalase test, Oxidase test, Methyl red test, Indole test, Nitrate reduction test, Citrate utilization test outlined in Bergey's Manual of determinative bacteriology[14].

Identification of *Burkholderia multivorans* at Molecular Level

Sequencing of 16S rRNA gene region was performed to identify the organism at species level.

DNA Extraction

DNA from *Burkholderia multivorans* was extracted based on the method described by Pitcher *et al*., (2008)[15]. At constant voltage of 100V the extracted DNA sample was run on 1% agarose gel and it was examined on UV tranilluminator.

PCR amplification and Sequencing of 16S rRNA

Amplification of 16S rRNA was performed with the universal primers. The following reaction mixture was added into the PCR tube, for setting up PCR. The reaction mixture were 5µl of template, Primers: 1µl of forward primer- 518F, 1µl of reverse primer- 800R, 6µl of assay buffer, 2µl of Taq DNA polymerase and 5µl of dNTP mix (Applied Biosystems Acme Progen Biotech (India) Pvt. Ltd, Salem, TamilNadu, India). Thermal cycler for 40 cycles was adjusted for amplification using the following reaction conditions, DNA was denatured at 94°C for 1 minute, annealing of primer at 56°C for 30 seconds and extension of primer at 72°C for 1 minute. Agarose gel was casted at 1.5% and 5µl of gel loading buffer was mixed with the amplified PCR product. 5 µl of 1 Kb DNA ladder (HIMEDIA, Mumbai, Maharastra, India) as a molecular marker were loaded along with the sample. The gel was examined on UV transilluminator to visualize the bands. PCR product was sequenced with an ABI Prism 3700 DNA analyzer (Acme Progen Biotech (India) Pvt, Ltd., Salem, TamilNadu, India).

Nucleotide Sequence and BLAST analysis

16S rRNA gene region data was submitted to NCBI nucleotide sequenence database. Using BLAST tool, Phylogenetic tree was designed from NCBI database search tool.

Antibiotic Susceptibilty Test of *Burkholderia multivorans*

Burkholderia multivorans bacterial antibiotic resistance was determined on Muller Hington agar using agar disc diffusion method[16] against 5 different type of antibiotic disc such as Amikacin, Chloramphenicol, Erythromycin, Tetracycline and Vancomycin. The resistance of the *Burkholderia multivoran* was defined by the zone formation.

Assessment of symbiotic association of *Scutellospora nigra* with *Burkholderia multivorans* in *Allium cepa* as host plant

At 12 days after inoculation, the roots of *Allium cepa* were stained in tryphan blue. The symbiotic association with and without the presence of *Burkholderia multivorans* was assessed by calculating the percentage of mycorrhizal colonization in the roots by the gridline intersect method[17].

RESULTS AND DISCUSSION

AMF spores were collected from Mahabalipuram and ECR near Chennai, TamilNadu, India (per 1kg of soil) and *Scutellospora nigra* spores were identified (www.invam.com). Based on the information provided in the Bergeys Manual of determinative bacteriology the bacteria were identified with reference to colony morphology, physical characterization and biochemical test,

Table 1. Physical and Biochemical characterization of *Burkholderia multivorans*

Cultural characterization	*Burkholderia multivorans*
Colony Morphology	Small, Pigmented circular, Flat, Entire, Dry colonies
Gram's Staining	Gram Negative
Motility	Active Mobile
Catalase	-
Oxidase	-
Methyl Red	-
Indole	-
Citrate Test	+
Nitrate Test	+

+ Positive, - Negative

Table 2. Antibiogram activity of *Burkholderia multivorans* isolated from trap culture

S. No	Antibiotics				
	Amikacin	Cholramphenicol	Tetracycline	Erythromycin	Vancomycin
1.	NA	10mm	6mm	NA	NA

NA- No activity

the organism was conformed as *Burkholderia multivorans* and the results were presented in Table1 and Figure 1. To confirm the *Burkholderia multivorans* at molecular level, sequencing of 16S rRNA gene region was performed using universal primer, the resulted PCR product was about 1491bp (Figure 2). The sequence was submitted to NCBI database and accession number was **KF534712**. Similarity between and within the organisms was analyzed using BLAST tool, based on the NCBI data the *Burkholderia multivorans* gave 99% similarity was obtained with 100 number of organisms (Figure 3). Antibacterial activity of *Burkholderia multivorans* was performed using Antibiotics such as Amikacin, Chloramphenicol, Erythromycin, Tetracycline and Vancomycin. The *Burkholderia multivorans* showed antibiotic resistance against the antibiotics such as Chloramphenicol and Tetracycline (Table 2, Figure 4). Interaction between rhizospheric bacterial and AMF species were studied. According to Avram *et al*. (2003)[18], Burkholderia spp. has the ability to enter the AM Fungus spores *Gigospora decipiens*. This bacterium extends the interaction with *Scutellospora nigra*. In this study, trap culture of *Allium cepa* was developed in Glass house condition. In the soil microbiota, AMF associated with bacterial combination are the essential living components[19]. In trap culture, the symbiotic association of *Scutellospora nigra* within the root cell of *Allium cepa* was observed by staining the roots of *Allium cepa* using Tryphan blue stains. Arbuscules, Vesicles and hyphae were observed in Figure 5 and the percentage of association of

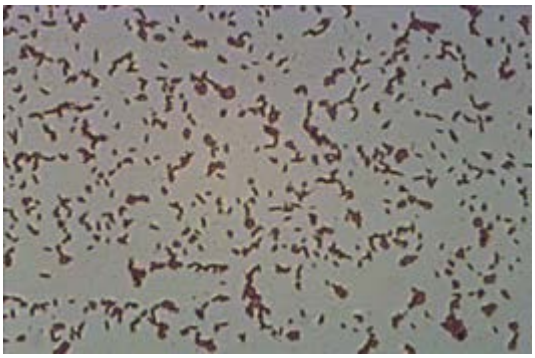


Fig. 1. Gram Staining of *Burkholderia multivorans*

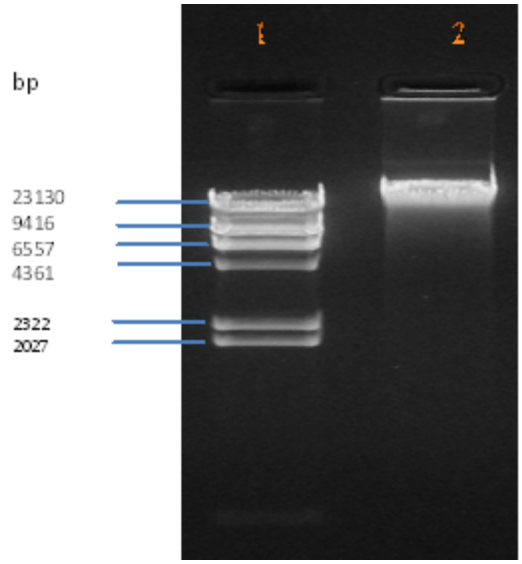


Fig. 2(a). Genomic DNA of *Burkholderia multivorans* isolated from trap culture, Lane 1-Lamba DNA/ Hind III; Lane 2- Genomic DNA

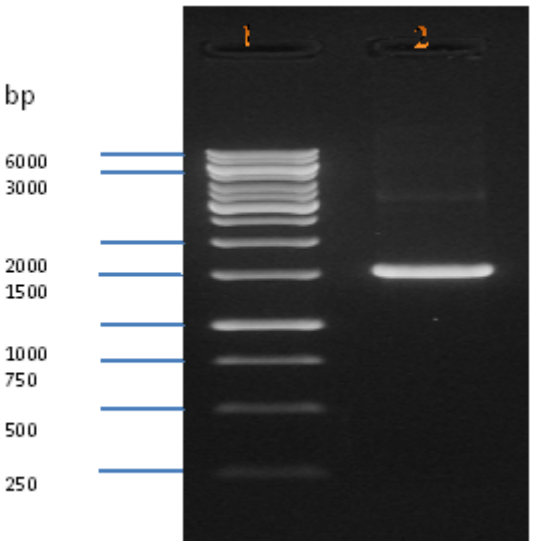


Fig. 2(b). PCR of *Burkholderia multivorans* isolated from trap culture, Lane 1- 1kb DNA Ladder; Lane 2- PCR Product

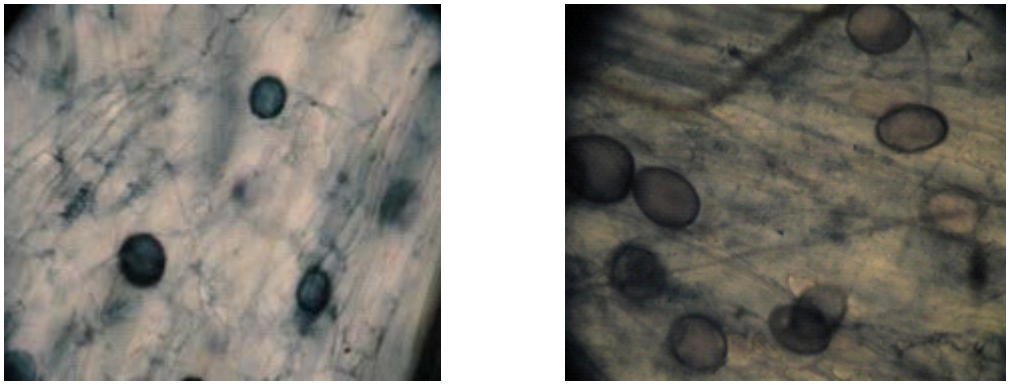
Gene region consist of 1491 bp

CTCAGATTGAACGCTGGCGGCATGCCTTACACATGCAAGTCGAACGGCAGCACGGGTGCT
TGCACCTGGTGGCGAGTGGCGAACGGGTGAGTAATACATCGGAACATGTCCTGTAGTGGG
GGATAGCCCGGCGAAAGCCGGATTAATACCGCATACGATCCACGGATGAAAGCGGGGGAC
CTTCGGGCCTCGCGCTATAGGGTTGGCCGATGGCTGATTAGCTAGTTGGTGGGGTAAAGG
CCTACCAAGGCGACGATCAGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGGACTGAG
ACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGAAAGCC
TGATCCAGCAATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTTGTCCGG
AAAGAAATCCTTGGCTCTAATACAGTCGGGGGATGACGGTACCGGAAGAATAAGCACCGG
CTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTAATCGGAATTACTG
GGCGTAAAGCGTGCGCAGGCGGTCTGTTAAGACAGATGTGAAATCCCCGGGCTCAACCTG
GGAACTGCATTTGTGACTGGCAGGCTAGAGTATGGCAGAGGGGGGTAGAATTCCACGTGT
AGCAGTGAAATGCGTAGAGATGTGGAGGAATACCGATGGCGAAGGCAGCCCCCTGGGCCA
ATACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCC
ACGCCCTAAACGATGTCAACTAGTTGTTGGGGATTCATTTCCTTAGTAACGTAGCTAACG
CGTGAAGTTGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGG
GACCCGCACAAGCGGTGGATGATGTGGATTAATTCGATGCAACGCGAAAAACCTTACCTA
CCCTTGACATGGTCGGAATCCTGAAGAGATTCGGGAGTGCTCGAAAGAGAACCGGCGCAC
AGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGA
GCGCAACCCTTGTCCTTAGTTGCTACGCAAGAGCACTCTAAGGAGACTGCCGGTGACAAA
CCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATGGGTAGGGCTTCACACG
TCATACAATGGTCGGAACAGAGGGTTGCCAACCCGCGAGGGGGAGCTAATCCCAGAAAAC
CGATCGTAGTCCGGATTGCACTCTGCAACTCGAGTGCATGAAGCTGGAATCGCTAGTAAT
CGCGGATCAGCATGCCGCGGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACA
CCATGGGAGTGGGTTTTACCAGAAGTGGCTAGTCTAACCGCAAGGAGGACGGTCACCACG
GTAGGATTCATGACTGGGGGTGAAGTCGTAACAAGGTAGCCGTATCGGAAG

Fig. 3. Amplified 16S rRNA gene region of *Burkholderia multivorans*

Fig. 4. Antibiogram activity of *Burkholderia multivorans* isolated from trap culture, A-Amikacin; B-Chloramphenicol; C-Tetracycline; D-Erythromycin; E-Vancomycin

Scutellospora nigra treated with and without the presence of *Burkholderia multivorans* was recorded in Figure 6. In the control root, 0% of association was found. The *Scutellospora nigra* associated roots treated with showed 56% and without the presence of *Burkholderia multivorans* showed 78%. In this study, *Scutellospora nigra* has shown very good association with the presence of *Burkholderia multivorans*. This shows a mutualistic relationship between *Scutellospora nigra* and *Burkholderia multivorans*.



a) b)

A) *Scutellospora nigra* associated with *Allium cepa* root (100X) B) *Scutellospora nigra* treated with *Burkholderia multivorans* associated with *Allium cepa* root (100X)

Fig. 5. Assessment of *Scutellospora nigra* association with *Allium cepa* root stained in tryphan blue

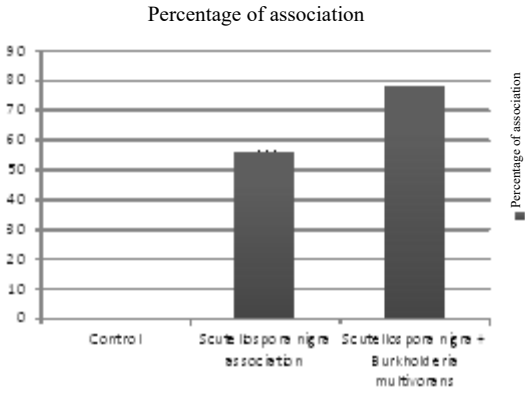


Figure 6: Percentage of association of *Scutellospora nigra* with *Allium cepa*

CONCLUSION

Burkholderia multivorans strains are morphologically similar but different at genetic level. Amplification of 16S rRNA gene region is the suitable technique for identification at molecular level. The antibacterial activity of *Burkholderia multivorans* inhibits the growth of other bacteria in trap culture. In the presence of *Burkholderia multivorans*, *Scutellospora nigra* has shown very good association with *Allium cepa*. Our results emphasize the need for an ecological appraisal of *Scutellospora nigra* by their interaction with *Burkholderia multivorans*.

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