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Genomics of bio-control agent tested against *Fusarium oxysporum* and *Ganoderma lucidium* in shisham (*Dalbergia sissoo*)

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Abstract
Eighteen bacterial isolates were screened for antagonistic activity against *F. oxysporum and G. lucidium in vitro* conditions. Five cultures showed prominent activity against the test pathogens. These cultures were also characterized for HCN production, siderophore formation and chitinase activity. On the basis of antagonistic activity against two test pathogens and biochemical characterization; five best cultures were identified as SD-25, SD-30, SD-87, SD-97 and SD-99. Maximum seed germination (100%) *in vitro* was observed in the treatment having (SD-99 only); followed by SD-99+ *G. lucidium*. These cultures were also tested for plant growth parameters in *Dalbergia sissoo* under pot house conditions. Isolate SD-99 showed maximum shoot dry weight (0.463g) and root dry weight (0.228g). This culture showed ninety nine percent similarity to *Bacillus sp. strain A2733* on the basis of 16s rRNA sequencing.

Keywords: HCN, PGPR, *Fusarium oxysporum*, *Gandoerma Lucidium, Dalbergia sissoo, Bacillus sp*

Introduction

Dalbergia sissoo Roxb. next to teak (*Tectona grandis* L.), is the most planted tree in South Asia. Its Timber finds multipurpose uses in different fields and has a high economical impact on many South Asian countries. It is one of the most valuable tree species of the sub-Himalayan area from Assam to Afghanistan. *Dalbergia sissoo* (Indian rosewood) is a deciduous tree, commonly known as sissoo, sheesham, tahli and Taland. Its mortality is spread throughout the shisham zone, more severe in the Eastern Uttar Pradesh, Bihar, Panjab and Haryana in India. It is highly susceptible to a disease called wilt caused by fungi which target particular species. Two pathogens have been responsible for shisham mortality - *Fusarium solani* f. sp. *dalbergiae* causing vascular wilt and *Ganoderma lucidium* causing root rot. The former was found wide spread throughout shisham growing areas, whereas, *G. lucidium* is associated in drier localities such as Haryana and Punjab. Khan & Khan (2000) [7] mentioned that *Polyporus* and *Fusarium oxysporum* cause root rot and wilting, respectively; while wilt of shisham was only observed in trees of about 15 – 25 years old. Various terms have been used for the disease such as ‘Mortality of sissoo’ (Baksha & Basak, 2000) [5], ‘Top dying disease’, ‘Shisham decline’ or ‘Dieback of sissoo. The characteristic symptoms are yellowing and necrosis of the leaves, mostly starting from the top of the tree. Successive loss of leaves leads to the ‘stag headed’ symptom, followed by occasional bark lesions with flow of dark red gum from the trunk.

There are several PGPR inoculants currently commercialized that seem to promote growth through at least one mechanism; suppression of plant disease (termed “Bioprotectants”), phytohormone production (termed “Biostimulants”), or improved nutrient acquisition (termed “Biofertilizers”). These bacteria are also capable to suppress the growth of deleterious microorganisms by production of siderophores, β 1,3 - glucanases, chitinases and antibiotics (Cattelan *et al*. 1999) [6]. PGPR beneficial effects have been exploited in many areas including biofertilizers, microbial bioremediation and biopesticides (Adesemoye *et al*. 2008) [1]. Generally, the rhizospheric bacteria can be more effective for control of plant diseases and promotion of plant growth. Keeping in view, the economic importance of shisham and to understand the role of PGPRs for control of its mortality disease and promotion of plant growth, the present investigations were undertaken.

Materials and Methods

Bacterial isolates were collected from the Department of Microbiology. These isolates had been isolated from rhizosphere of *Dalbergia sissoo* plantations. A total of eighteen bacterial isolates were tested for various biochemical tests. Bacterial cultures used in the study were: -

SD-2, SD-3, SD-6, SD-8, SD-10, SD-13, SD-14, SD-22, SD-25, SD-30, SD-31, SD-49, SD-86, SD-87, SD-89, SD-93, SD-97, and SD-99. The fungal cultures namely *Fusarium oxysporum* and *Ganoderma lucidium* were obtained from the Deprtment of Plant Pathology, CCS HAU, Hisar.

Antagonistic activity of Isolates against fungal pathogens

The interaction of rhizobacterial isolates with *Ganoderma lucidium* and *Fusarium oxysporum* was studied by spot test method of Sindhu *et al*. (1997) [14] on PDA medium plates.

Biochemical characterisation of bacterial isolates from Dalbergia sissoo

The bacterial cultures were biochemically characterized for HCN production, chitinase activity and siderophore formation.

(a) HCN production

King's B medium amended with 0.44% L-Glycine was used for detection and quantification of hydrogen cyanide production following the method of Schippers *et al*. (1987) [12].

(b) Chitinase activity

For screening of chitinase activity the nutrient agar medium amended with colloidal chitin was used. The medium consisted of (g/l) Na_2HPO_4 6.0; K_3NH_4Cl 1.0; NaCl 0.5; yeast extracts 0.05; agar 15.0; and colloidal chitin 1% (wt /volume). Unbleached chitin was used for the preparation of colloidal chitin using the method of Morrisey *et al*., 1976 [9]. The colonies showing clearance zone on creamish back ground were considered as chitinase producing bacteria.

(c) Siderophore production

Bacterial isolates were screened for siderophore production using universal chemical assay of Schwyn and Neilands (1987) [13] on Chrome azurol S (CAS) agar plates.

Plant growth promotion *in vitro* by selected isolates

Ten seeds for each treatment in triplicates were placed on soaked filter paper under sterilized conditions and the plates were incubated at 25^0C in seed germinator. The first count of germinated seeds was taken on fifth day of incubation and finally on eleventh day. Observations were made in terms of % seed germination, root length and % plant mortality.

Evaluation of five best isolates under pot house conditions

To evaluate the effect of selected bacterial isolates on plant growth of *Dalbergia sissoo*; an experiment was conducted in pot house conditions. Different treatments used for pot house experiment (Table 1) included control and combination of five selected cultures with *F. oxysperum or G. lucidium*. Each treatment had three replications. The experiment was conducted using CRD design.

For pot experiment, seeds of *Dalbergia sissoo* were sown in polythene bags containing 2.0 kg unsterilized soil. Polythene bags were inoculated with1.0 ml of log phase of best five cultures along with 1.0 ml of fungal culture. The Five seeds were sown initially and one plant per polythene bags was maintained after 20 days of sowing. Different plant growth parameters i.e. nodule number, root weight, shoot weight and plant height were recorded at 45 DAS.

Statistical analysis

The experimental data was analyzed by the application of OPSTAT software.

Sequencing of 16S rDNA of bacterial isolates

The best strain SD-99 was identified by partial 16S rDNA sequencing. For PCR product, 2% and for genomic DNA, 0.8% agarose in 1X TBE buffer was prepared and used for gel preparations. One kb ladder was used as molecular weight marker for comparing PCR amplification product. For each loading, 1.0 µl of the ladder was mixed with 4.0 µl of loading dye.

Taq DNA polymerase at a concentration of 3U μl^{-1} was added to storage buffer containing 20 mM Tris-HCl (pH 8.0), 100 mM KCl, 0.1 mM EDTA, 1mM DDT, 0.5% Tween 20 (v/v), 0.5% Igepal and 50% Glycerol (v/v). To prepare dNTP's mix, 1 µl of each dNTP was added into 36 µl of distilled water. The forward and reverse primers used for 16S rDNA amplification were:

16s rDNA specific primer (The PCR product size 1.5 kb)

16s Forward Primer: 5'-AGHGTBTGHTCMTGNCTCAS -3'

16s Reverse Primer:5'-TRCGGYTMCCTTGTWHC GACTH -3'

(The PCR product size ~1.3 kb)

16s Forward Primer: (Pair 2) 5'– CMGSCVTDACACAWG CHAGYC – 3'

16s Reverse Primer: (Pair 2) 5' – GGCGSMTGWGTNCAA GSV – 3'

Isolation of Genomic DNA (Modified method as described by Ausubel et al. 2001)

Genomic DNA was extracted from bacterial isolate SD 99 by using CTAB method. The genomic DNA was diluted 100 times and quantified by measuring the absorbance at 260 nm and 280 nm using uv-vis spectrophotometer. The amount of DNA was estimated using the relationship that O.D. of 1.0 corresponds to 50 µg ml^{-1}. The purity of DNA was assessed by measuring A260/A280 ratio; A260: A280 = 1.5 - 1.8 for pure DNA. Purity of DNA was also checked on 0.8% agarose gel for bands. In case of faint or no bands, DNA extraction was repeated.

Amplification of 16S rDNA sequences

Amplification of 16S rDNA sequence was carried out by polymerase chain reaction using a thermal cycler. The primers 27F and 1378R enabled the amplification of 16S rDNA sequences present in bacterial DNA.

Results and Discussion

Dalbergia sissoo is an important commercial plant with many uses, mainly the wood is important. However, wilting of this tree is a serious problem. To overcome this problem investigations were undertaken to find out the probable biocontrol agent which also promote plant growth along with its antagonistic activity.

Screening of different isolates for inhibition zone formation against test fungi *(Fusarium oxysporum* and *Ganoderma lucidium)*

Out of eighteen cultures, ten bacterial isolates exhibited inhibition zone formation against *F. oxysporum in vitro* conditions. The inhibition zone formation ranged from 0.1cm to 1.7cm. The maximum inhibition zone formation (1.7cm) was shown by SD-99. It was followed by SD-30 (1.6cm) and SD-97 (1.5cm). Table 2 also depicts that ten bacterial isolates exhibited inhibition zone formation against *G. lucidium*. The zone formed varied from 0.1cm to 2.2cm. The maximum inhibition zone was shown by bacterial isolate SD-99 (2.2cm); followed by SD-97 (2.0cm) and SD-87(1.6cm).

Biochemical characterization of bacterial isolates from rhizosphere of *D. sissoo*

a) HCN production

Bacterial isolates were characterized for hydrogen cynide production by growing in modified King's B agar medium amended with 0.44% of L-glycine. Eight bacterial isolates namely SD-2, SD-6, SD-10, SD-13, SD-14, SD-49, SD-89 and SD-93 did not show any HCN production (Table 3). Four cultures namely SD-3, SD-22, SD-31 and SD-86 showed very low HCN production. Three cultures namely SD-8, SD-25 and SD-87 showed low HCN production while SD-30 and SD-99 were moderate HCN producers. SD-97 showed highest HCN production.

(b) Chitinase activity

Eight bacterial isolates did not show any chitinase activity (Table 3). Five cultures namely SD-2, SD-6, SD-14, SD-31 and SD-93 were low chitinase producers. Three cultures namely SD-25, SD-30, SD-97 were moderate chitinase producers. The maximum chitinase activity in terms of clear zone around bacterial colony was detected in SD-99.

(c) Siderophore production

The presence of iron chelator (siderophore) was indicated by the decolorization of blue-coloured ferric dye complex, resulting in yellow halo zones around the colonies. Eight bacterial isolates did not show any siderophore formation. Five cultures namely SD -2, SD-10, SD-30, SD-49 and SD-89 were very low siderophore producers (Table 3). Only one culture SD-25 was low siderophore producer, while SD-30 and SD-97 were detected as moderate producers. Maximum siderophore production was observed in SD-99; followed by SD-87. The best five cultures on the basis of biochemical characters were concised in Table 4. These cultures were further used to study their effect on plant growth parameters in vitro and in vivo conditions.

Seed germination under *in vitro* condition

The maximum percent seed germination i.e 100% was observed with SD-99 alone. It was followed by SD-99+ *G. lucidium* (80%). The average percent germination was 50.5%; while in the control, it was 40% (Table 5). With *F. oxysporum* and *G. lucidium* alone, the percent germination was less than control, indicating that both pathogens inhibited percent germination *in vitro* conditions. Ten treatments affected germination positively over the control. SD-99 was found best culture under *in vitro* conditions; followed by SD-97. Response of SD-25 with both the test pathogens was not satisfactory.

In the control, root length was 0.67cm; in case of SD-99 alone it was 3.94cm. SD-99+ *F. oxysporum* showed root length as 2.24cm and SD-99+ *G. lucidium* showed 2.76cm (Table 4). The mean root length was 1.85cm. All the treatments, except *F. oxysporum* alone and *G. lucidium* alone, positively affected mean root length. SD-87+ *F. oxysporum* was adjudged as second best; followed by SD-97+ *G. lucidium* (2.00cm). The percent mortality varied from zero to eighty percent. Maximum mortality was observed with *F. oxysporum* alone (80%) followed by *G. lucidium* alone (70%). The least mortality *i.e.* zero percent was observed in SD-99 alone, while in the treatment having SD-99+ *G. lucidium* this value was 20%. The average percent mortality was 49.4 percent.

Plant growth parameters under pot house conditions

The effect of best five isolates namely SD-25, SD-30, SD-87, SD-97, SD-99 was observed on plant growth parameters in *Dalbergia sissoo* under pot house conditions when co-inoculated with *F. oxysporum* or *G. lucidium.* Data in terms of fresh shoot weight indicated that shoot weight varied from 0.338gm to 1.335gm (Table 6 & Fig 1). The minimum fresh shoot weight was recorded in control (0.338gm); while fresh shoot weight in the treatment having SD-99+ *F. oxysporum* was maximum (1.335gm). It was followed by SD-97+ *F. oxysporum* (1.070gm). The similar trends were observed in terms of shoot dry weights. It varied from 0.165gm in the control to 0.463gm in SD-99+ *F. oxysporum*. The data was statistically significant indicating that all the cultures positively affected plant shoot weight when co-inoculated with *F. oxysporum*.

The fresh root weight ranged from 0.128gm to 0.403gm (Table 6). In terms of root weight, SD-97+ *F. oxysporum* was adjudged as the second best treatment while minimum root weight (0.128gm) was recorded in control. Number of nodules varied from 0.3 in the un-inoculated control to 11.6 in SD-99+ *F. oxysporum*. SD-87+ *F. oxysporum* was adjudged as second best. It was followed by SD-97+ *F. oxysporum* (7.0). These values indicate that PGPRs positively affected the nodule number. The plant height at 45 days varied from 15.66cm to 23.33cm. Maximum plant height (23.33cm) was observed in treatment having SD-99+ *F. oxysporum*. It was followed by SD-30+ *F. oxysporum* (23.16cm). All inoculated treatments were significantly higher over the control. Similar trends were followed when co-inoculated with *G. lucidium* (Table 7 and Fig 2). In this case also, isolate SD-99 was adjusted as the best.

Identification of promising bacterial isolate

In the present study, DNA of the most promising isolate (SD-99) was extracted and approx 1500 bp DNA fragment was amplified from 16S rRNA gene. After purification the amplified fragment then got sequenced from CHROMOUS Biotech PVT LTD., Bangalore. The promising strain was identified as *Bacillus sp.* Strain (Fig 3).

As *F. oxysporum* and *G. lucidium* are two important pathogenic fungi which are causing heavy economic losses due to mortality of shisham trees; hence these two fungi have been studied *in vitro* as well as *in vivo* conditions. PGPRs (Plant growth promoting rhizobacteria) can be an alternative for biocontrol of these two plant pathogens as chemical control was not so effective, it also causes soil pollution and economically, it is not feasible due to high cost of chemical fungicide. Many bacterial species, mostly associated with plant rhizosphere have been tested and found beneficial for plant growth (Sureshbabu *et al*. 2016) [15]. These bacteria are capable of suppressing the growth of deleterious microorganisms by production of siderophore, glucanase, chitinase and antibiotics (Cattelen *et al*. 1999). The PGPRs are the natural habitat of rhizosperic soil of healthy shisham trees. Hence, the bacterial isolates isolated from the rhizosphere of 3-4 years old tree species from two locations Hisar and Bawal, were screened for biochemical tests. Eighteen cultures selected were further screened for antagonistic activities against two test pathogens.

The antifungal activity of the isolates might be due to synergistic interaction of these three biochemical characters.

Similar observations have been made by Ahmad *et al*. (2008) [3]. Siderophore production enables bacteria to compete with pathogens by removing iron from the environment (Persello-Cartieaux *et al.* 2003) [10]. As SD-99 showed maximum siderophore formation qualitatively, it also served as best PGPR and also inhibited both the pathogens under *in vitro* conditions. The HCN production is found to be a common trait of *Pseudomonas* (88.89%) and *Bacillus* (50%) in the rhizospheric soil and plant root nodules (Ahmad *et al*. 2008) [3]. These strains suppress disease by synthesis of HCN. The best five cultures (SD-25, SD-30, SD-87, SD-97, SD-99) selected on the basis of biochemical characterization and antagonistic activity showed moderate to high production of HCN production, siderophore production and chitinase activity.

Seed germination under *in vitro* conditions when co-inoculated with selected five best isolates and two fungal pathogens showed germination percentage varied from 20% to 100%. SD-99 was found to be the best isolate as it shown 100% seed germination and 80% and 70% when co-inoculated with *F. oxysporum* and *Ganoderma lucidium*, respectively. Similar observations were made by Rajput *et al.* (2010) [11], Kumar (2014) [8]. They also observed that seed germination percentage also reduced to 50% when soil was infested with *F. solani*. In our results *F. oxysporum* reduced seed germination from 40 percent in control to 20 percent; while *G. lucidium* reduced it to 30 percent.

All the five best isolates in combination with *F. oxysporum* or *G. lucidium* positively affected the plant growth parameters. As indicated by the results maximum nodule number was observed with SD-99+ *G. lucidium* (17.3) followed by SD-99+ *F. oxysporum* (11.8). As SD-99 was adjudged as the best isolate among the five isolates tested under pot house conditions. Hence, this isolate was identified using 16s rRNA gene sequencing as *Bacillus sp.* which showed 99.99% similarity with *Bacillus sp.* strain *A2733*. Most of the PGPRs belong to either *Psuedomanas* or *Bacillus sp.* (Ahemad and Kibret, 2014) [2]. SD-99 identified as closely related to *Bacillus sp*. can serve as a good biocontrol agent against *F. oxysporum* and *G. lucidium.* If this isolate is tested further, it can serve as a good PGPR promoting plant growth as well as biocontrol agent against *F. oxysporum* and *G. lucidium*. Promotion of plant growth and increase in nodule biomass in by PGPRs was also noticed by some other workers (Sureshbabu *et al*., 2016) [15].

Table 1: Details of treatments used for pot experiment

Treatment No.	Details
T1	Control
T2	SD-25+ *F. oxysporum*
T3	SD-30+ *F. oxysporum*
T4	SD-87+ *F. oxysporum*
T5	SD-97+ *F. oxysporum*
T6	SD-99+ *F. oxysporum*
T7	SD-25+ *G. lucidium*
T8	SD-30+ *G. lucidium*
T9	SD-87+ *G. lucidium*
T10	SD-97+ *G. lucidium*
T11	SD-99+ *G. lucidium*

Table 2: Inhibition zone formation by bacterial isolates against *F. oxysporum a*nd *G. lucidium.*

Bacterial isolate	Halo zone formed (cm) against *F. Oxysporum*	Halo zone formed (cm) against *G. lucidium*
SD-2	0.1	-
SD-3	0.2	-
SD-6	-	0.3
SD-8	0.3	-
SD-10	-	-
SD-13	-	0.1
SD-14	-	-
SD-22	0.2	-
SD-25	0.5	1.2
SD-30	1.6	0.9
SD-31	-	-
SD-49	-	0.4
SD-86	-	-
SD-87	0.2	1.6
SD-89	-	0.5
SD-93	0.4	0.8
SD-97	1.5	2.0
SD-99	1.7	2.2

Table 3: Biochemical characterization of different bacterial isolates

Bacterial samples	Level of HCN Production	Level of Chitinase activity	Level of Siderophore Production
SD-2	-	±	±
SD-3	±	-	-
SD-6	-	±	-
SD-8	+	-	-
SD-10	-	-	±
SD-13	-	-	±
SD-14	-	±	-
SD-22	±	-	-
SD-25	+	++	+
SD-30	++	++	++
SD-31	±	±	-
SD-49	-	-	±
SD-86	±	-	-
SD-87	+	+	+++
SD-89	-	-	±
SD-93	-	±	-
SD-97	+++	++	++
SD-99	++	+++	++++

*Qualitative group = (-) = No, (±) =Very low, (+) = Low, (++) = moderate, (+++) = significant, (++++) = highly significant.

Table 4: Best five isolates selected on the basis of inhibition zone formation and biochemical characteristics

SN	Bacterial sample	Inhibition zone formation (Halo zone size in cm)		HCN production	Chitinase activity	Siderophore production
		F. oxysporum	*G. lucidium*			
1	SD-25	0.5	1.2	+	++	+
2	SD-30	1.6	0.9	++	++	++
3	SD-87	0.2	1.6	+	+	+++
4	SD-97	1.7	2.2	+++	++	++
5	SD-99	1.5	2.0	++	+++	++++

*Qualitative group = (-) = No, (±) =Very low, (+) = Low, (++) = moderate, (+++) = significant, (++++) = highly significant.

Table 5: Effect of different treatments on seed germination *in vitro* conditions

Seed Treatment	Germination (%)	Mean root length (11 DAS) (cm)	Mortality (%)
Control	40	0.67	60
F. oxysporum	20	0.60	80
G. lucidium	30	0.62	70
SD-25	60	1.28	40
SD-30	50	1.54	50
SD-87	60	2.46	40
SD-97	60	1.91	40
SD-99	100	3.94	0
SD-25+ *F. oxysporum*	30	1.36	70
SD-30+ *F. oxysporum*	40	1.95	60
SD-87+ *F. oxysporum*	40	2.05	60
SD-97+ *F. oxysporum*	50	1.92	50
SD-99+ *F. oxysporum*	70	2.24	30
SD-25+ *G. lucidium*	30	1.66	70
SD-30+ *G. lucidium*	50	1.90	50
SD-87+ *G. lucidium*	40	1.90	60
SD-97+ *G. lucidium*	60	2.00	40
SD-99+ *G. lucidium*	80	2.76	20
Mean	50.55	1.85	49.44

Table 6: Effect of bacterial isolates on plant growth parameters when co-inoculated with *F. oxysporum*

Treatment	Fresh Weight (g/plant)		Dry Weight (g/plant)		Nodule number	Plant Height (cm)
	Shoot	Root	Shoot	Root		
SD25+ *F. oxysporum*	0.453	0.190	0.258	0.095	3.0	17.00
SD-30+ *F. oxysporum*	0.511	0.280	0.343	0.137	4.3	23.16
SD-87+ *F. oxysporum*	0.646	0.267	0.215	0.110	8.3	23.03
SD-97+ *F. oxysporum*	1.070	0.345	0.332	0.176	7.0	22.36
SD-99+ *F. oxysporum*	1.335	0.403	0.463	0.201	11.6	23.33
Control	0.338	0.128	0.165	0.059	0.3	15.66
CD @ 5 %	0.130	0.086	0.066	0.039	1.9	5.14

Table 7: Effect of bacterial isolates on plant growth parameters when co-inoculated with *Ganoderma lucidium*

Treatment	Fresh Weight (g/plant)		Dry Weight (g/plant)		Nodule number	Plant height (cm)
	Shoot	Root	Shoot	Root		
SD-25+ *G. lucidium*	0.728	0.313	0.250	0.150	5.0	17.90
SD-30+ *G. lucidium*	0.612	0.633	0.205	0.206	9.3	17.00
SD-87+ *G. lucidium*	0.633	0.268	0.240	0.118	7.3	17.00
SD-97+ *G. lucidium*	0.858	0.301	0.232	0.132	4.3	18.30
SD-99+ *G. lucidium*	1.031	0.779	0.360	0.228	17.3	22.46
Control	0.436	0.225	0.187	0.050	0.3	11.63
CD @ 5 %	0.123	0.135	0.037	0.038	2.39	3.91

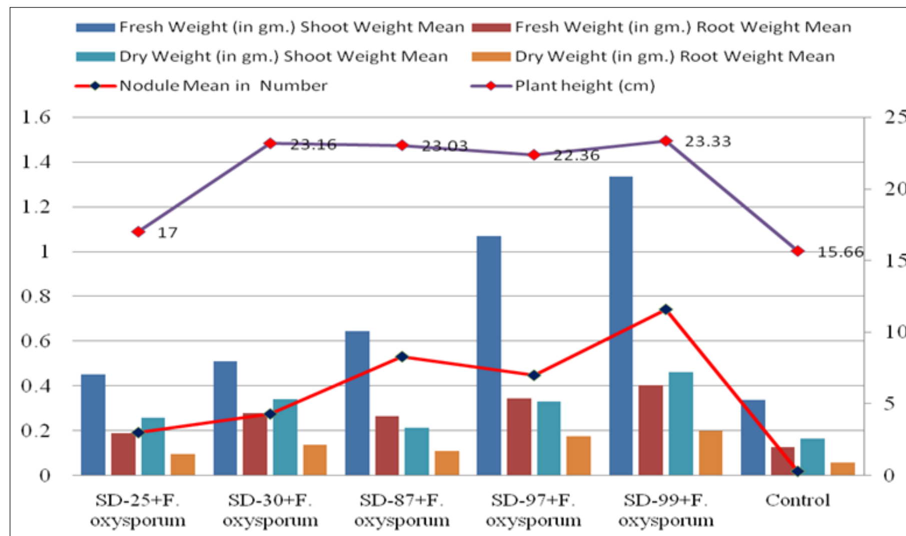

Fig 1: Effect of bacterial isolates on plant growth parameters when co-inoculated with *F. oxysporum*

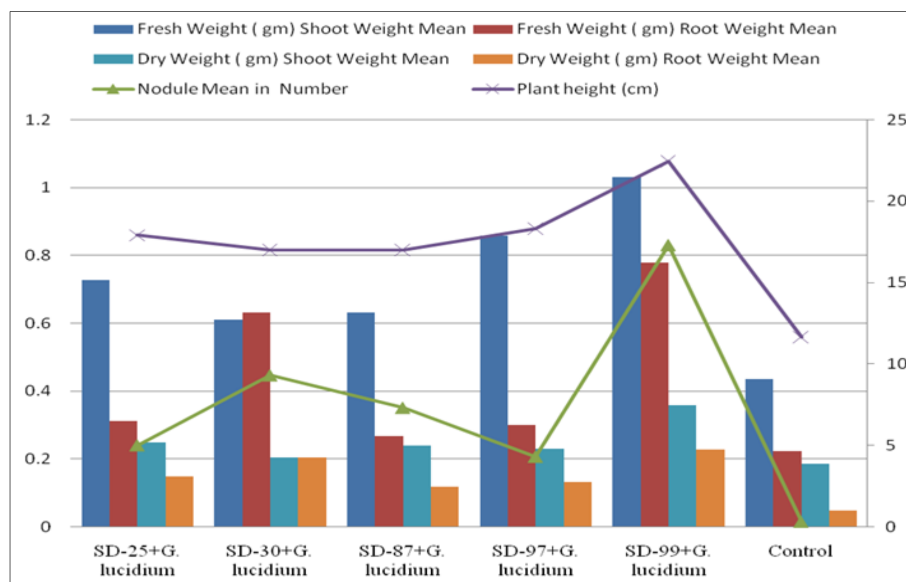

Fig 2: Effect of bacterial isolates on plant growth parameters when co-inoculated with *Ganoderma lucidium*

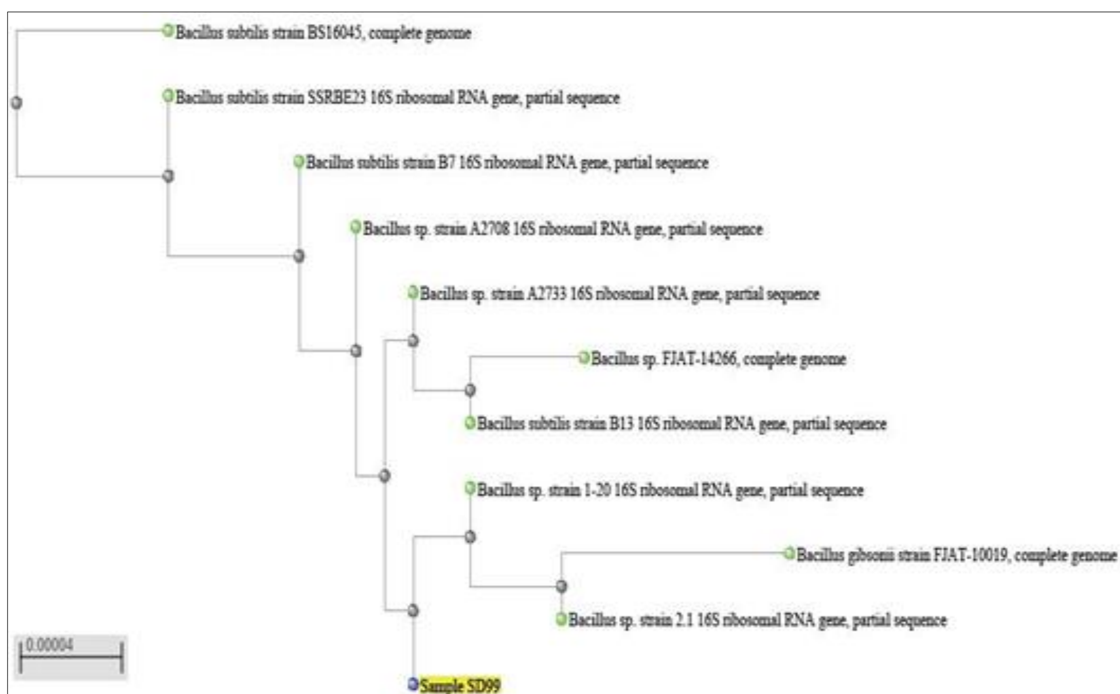

Fig 3: Phylogenetic trees of the isolate prepared by using neighbour joining of BLAST programme

Conclusion

Dalbergia sissoo is an important commercial tree mainly used for timber purpose. However, from last 4-5 decades wilting has become a serious problem. To overcome this problem some biocontrol agents have been tested, which also promote plant growth along with its antagonistic activity; five best cultures were identified as SD-25, SD-30, SD-87, SD-97 and SD-99. Among these cultures SD-99 was found superior in seed germination (100%) *in vitro*, showed maximum antagonistic activity against *F. oxysporum* and *G. lucidium;* also performed better in different plant growth parameters under pot house conditions. This isolate showed maximum shoot dry weight (0.463g) and root dry weight (0.228g). This culture showed ninety nine percent similarity to *Bacillus sp. strain A2733* on the basis of 16s rRNA sequencing.

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