Available online at www.ijpab.com

DOI: http://dx.doi.org/10.18782/2320-7051.2684

ISSN: 2320 – 7051

Int. J. Pure App. Biosci. **5 (2):** 652-660 (2017)

Research Article

International Journal of Pure & Applied Bioscience

OPEN ACCESS

Characterization of Plant Growth Promoting Rhizobacteria from Rhizosphere of Shisham *(Dalbergia sissoo)* and their Effect against Fungal Pathogens

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Received: 14.03.2017 | Revised: 18.03.2017 | Accepted: 19.03.2017

ABSTRACT

The present study was conducted at Chaudhary Charan Singh Haryana Agricultural University (CCS HAU) Hisar, Haryana in which rhizospheric soil samples of Dalbergia sissoo were collected ten each from field & campus of CCS Haryana Agricultural University, to isolate rhizospheric bacteria. A total of 100 rhizobacterial isolates were isolated from all the collected soil samples. It was observed that 66.66 per cent of the Gram positive isolates were spore formers with terminal or central spore, 80 per cent isolates were IAA producers, 25 per cent were phosphate solubilizers. A total of 14 per cent isolated strains showed antifungal activity exclusively against Fusarium oxysporum, 15 per cent isolates showed against Ganoderma lucidium and 16 per cent of the isolates were found to have both the activities (antagonistic to both Fusarium oxysporum & Ganoderma lucidium). The effect of 18 selected isolates were observed on the plant growth under pot house conditions. Out of the 18 isolates the highest nodule number was reported in the plants inoculated with SD97, shoot length enhancement was maximum observed in the plants inoculated with isolate SD99 & root length enhancement was maximum reported with isolate SD6.

Key words: *Dalbergia sissoo, PGPR, Ganoderma lucidium, Fusarium oxysporum*

INTRODUCTION

Shisham (*Dalbergia sissoo* Roxb.) is an important multipurpose tree species under the family Leguminosae (Papilionaceae). It is a medium to large sized, fast growing and gregarious deciduous tree with a spreading crown. The tree is considered to be native of Bengal and found in India, Nepal, Bhutan, Bangladesh, Myanmar, Malaysia, Pakistan and Afghanistan[10]. It is grown as monoculture crop or in agroforestry systems, because of the quality of its wood and its effects on soil fertility through N_2 fixation. Low soil fertility in tropical regions results in poor plant growth. This is significant in the case of forest trees, since they are generally transplanted without considering the fertility status of soil[2]. Plant growth promoting rhizobacteria (PGPR) offer the suitable and sustainable solution for promoting growth of plants and antifungal activity.

Cite this article: Padder, S.A., Pathak, D.V., Bhat, Z.A. and Kuldeep, Characterization of Plant Growth Promoting Rhizobacteria from Rhizosphere of Shisham *(Dalbergia sissoo)* and their Effect against Fungal Pathogens, *Int. J. Pure App. Biosci.* **5(2):** 652-660 (2017). doi: http://dx.doi.org/10.18782/2320-7051.2684

During the late 19th and early 20th centuries inorganic compounds containing nitrogen, potassium and phosphorus (NPK) were synthesized and used as fertilizers. Due to the growth in human populations, fertilizers were used to increase crop production and meet the rising demands for food. Increases in the production cost, and the hazardous nature of chemical fertilizers for the environment has led to a resurgence of interest in the use of biofertilizers for enhanced environmental sustainability, lower cost production and good crop yields. Numerous species of soil bacteria which flourish in the rhizosphere of plants, may grow in, on, or around plant tissues, stimulate plant growth by a plethora of mechanisms. These bacteria are collectively known as PGPR (plant growth promoting rhizobacteria)[1]. In other words plant growth promoting rhizobacteria are a heterogeneous group of bacteria that can be found in the rhizosphere, at root surfaces and in association with roots, which can improve the extent or quality of plant growth directly or indirectly. A large array of bacteria including species of *Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Ralstonia, Bacillus* and *Serratia* have been reported as PGPR to enhance plant growth[12]. The direct growth promotion of plants by PGPR entails either providing the plant growth promoting substances that are synthesized by the bacterium or facilitating the uptake of certain plant nutrients from the soil. The indirect plant growth promotion occurs by PGPR due to their ability to prevent the imposition of deleterious effects of phytopathogenic microorganisms. The exact mechanisms by which PGPR promote plant growth are not fully understood, but are thought to include (i) the ability to produce or change the concentration of plant growth regulators like indole acetic acid, gibberellic acid, cytokinins and ethylene[5], (ii) asymbiotic N_2 fixation[3] (Boddey and Dobereiner, 1995), (iii) antagonism against phytopathogenic microorganisms by production of siderophores[19], antibiotics[20] and cyanide. The variability in the performance of PGPR may be due to various environmental factors that may affect their growth and exert their effects on plant. The environmental factors include climate, weather conditions, soil characteristics or the composition or activity of the indigenous microbial flora of the soil. To achieve the maximum growth promoting interaction between PGPR and nursery seedlings it is important to discover how the rhizobacteria exert their effects on plant and whether the effects are altered by various environmental factors, including the presence of other microorganisms. Therefore, it is necessary to develop efficient strains from field conditions. One possible approach is to explore soil microbial diversity for PGPR having combination of PGP (plant growth promoting) activities and well adapted to particular soil environment. However under Indian conditions, very few studies on these plant microbe interactions have been carried out.

MATERIALS AND METHODS

Isolation

Soil samples were collected from the rhizosphere of *Dalbergia sissoo*. The rhizospheric soil samples were collected randomly ten from campus and ten from field of CCS Haryana Agricultural University, Hisar. Five samples were collected from each site and pooled together to make the composite sample. The serial dilutions [multiple tube dilution plate technique[8] of soil samples (up to 10^{-7}) were made in 9.0 ml sterilized water blank and 0.1 ml of diluted soil suspension was plated on medium plates. The plates were incubated at 28±2°C in BOD incubator for 3-4 days.

Study of morphological characters of rhizospheric bacterial isolates

All the bacterial isolates were observed for colony morphology during purification process. The colony morphology was studied on plates after streaking a loopful of isolated bacterial colony and colony color, colony size, colony texture and gum production were observed. All the bacterial isolates were used for gram and spore staining. A smear was

prepared from isolated colonies and stained with Gram's stain and spore stain. Slides were observed under Geytnor microscope at 100X. Cell shape, size, Gram's reaction and presence of spore were observed and these were photographed.

Estimation of Indole-3-acetic acid (IAA)

IAA was estimated by Salkowski's method[22]

Reagents

1) Salkowski's reagent- 1 mL of 0.05 M $FeCl_3$ in 50 mL of 35 per cent of perchloric acid ($HClO_4$).
2) IAA stock solution 100 mg mL^{-1} in 50 per cent ethanol.

Selected rhizobacterial isolates were inoculated in 25 mL of their respective broth supplemented with 0.1 g L^{-1} DL-tryptophan. These flasks were incubated at 28$\pm$2°C in a shaking BOD incubator. After 4 days of incubation, 2 mL of culture broth was centrifuged at 7,000 rpm for two minutes and then IAA was determined in culture supernatant by following method: To 2 ml of supernatant, an equal volume of Salkowski's reagent was added. The contents were mixed by shaking and allowed to stand at room temperature for 30 minutes for development of pink colour which was estimated colorimetrically at 500 nm using spectrophotometer. Indole- 3- acetic acid was used as a standard.

P-solubilization

All the selected rhizobacteria were inoculated in 25 mL respective broth. These flasks were incubated at 28$\pm$2°C in a BOD incubator for 3 days. Five µL of each culture was taken and spotted on Pikovskaya's plates. These plates were incubated at 28$\pm$2°C for 3-4 days. Formation of solubilization zone indicates P-solubilizing bacteria. Zone of solubilization was measured and coloney size was also measured & these values were used to calculate solubilization index by the following formula[4].

SI = colony diameter + halozone diameter/colony diameter

Antagonistic activity

The interaction of rhizobacterial isolates with *Ganoderma lucidium* and *Fusarium oxysporum* was studied by the spot test method of Sindhu *et al*[21]., on PDA medium plates. The cultures of fungi *G.lucidium* and *Fusarium oxysporum* were grown on PDA slants. Spore suspension of fungi was prepared in 3 ml sterilized water. About 0.2 ml of fungal spore suspension was spread over PDA medium plates. A loopful of 48-hour old growth of the rhizobacterial isolates was spotted on pre-seeded plates. Five cultures were spotted on each plate. Plates were incubated for 48 hours at 28$\pm$2°C. Detection of antagonistic activity of rhizobacterial isolates was based upon the ability of rhizobacterial strains to inhibit fungal growth on PDA medium plates.

Pot house studies

The plant growth promoting efficiency of selected 18 rhizobacterial isolates were assessed under pot culture conditions using *Dalbergia sissoo* as test host. Seeds of *Dalbergia sissoo* were surface sterilized by using 0.2 per cent mercuric chloride and alcohol. They were sown in the poly bags of one kg capacity and were inoculated with the respective isolates (control was inoculated with only broth) and 15 days after germination they were transplanted in pots (one plant per pot). Three kg of soil was taken in each earthen pot. Five replicates of each treatment were kept. All the transplanted plants were inoculated with 3 ml of inoculum of rhizobacterial isolates. Control was also mentioned by inoculating with broth only devoid of culture. Pots were irrigated every day. After 60 days of growth, plants were uprooted and observations on nodule number, nodule, root and shoot fresh weight and then dry weight were recorded after drying the samples in oven at 80°C till constant weight was observed.

RESULTS AND DISCUSSION

Morphological characters of the rhizobacterial isolates

The present study was planned with the objective to isolate and characterize the plant growth promoting rhizobacteria from the rhizosphere of *Dalbergia sissoo*. A total of twenty rhizospheric soil samples of *Dalbergia*

sissoo were collected from campus & field (ten from each location) of CCS HAU Hissar. One hundred bacterial isolates were isolated from the collected soil samples based on morphological characteristics by dilution plate method using King's B, YEMA, LB & nutrient agar media. Similarly, a number of researchers have successfully isolated different numbers of bacterial isolates from the rhizosphere of various plants. Khot *et al*[11]., isolated 36 rhizobacteria from rhizosphere of chickpea using fungal cell wall material as carbon source for growth. Gupta *et al*[6]., isolated rhizobacteria from the rhizotic zones of green gram using 7 selective and 4 non-selective media. Kumar *et al*[13]., isolated thirty bacterial isolates from the six rhizospheric soil samples of French bean. All the rhizobacterial isolates were examined to study the different morphological characters. Colony morphology was studied during purification of isolates and simultaneously, Gram's staining was done by making smears from isolated colonies, shape and size of the cells was also monitored. Spore staining was also done to know the presence and position of spores. Morphology of all the isolates varied from gummy to non-gummy, flat to umbonate, rough to smooth, entire to lobate and colony size varied from minute to large. Coloration of the colonies varied from white, creamy white, pinkish white, pink, yellow brown, yellowish orange etc. Cell size varied from minute to small, small to medium and medium to long, cell shapes were variable from cocci, oval to rod. There were 27 per cent Gram positive & 73 per cent Gram negative isolates & out of the 27 per cent Gram positive isolates 66.66 per cent (18 per cent of the grand total) isolates were spore formers & it was found that the total isolates with terminal position of the endospore were 8 per cent of the total isolates and 44.44 per cent of the spore formers, total isolates with central position of the endospore were 7 per cent of the total isolates & 38.88 per cent of the spore formers finally the isolates with central and terminal endospore position were 2 per cent of the total isolates & 11.11 per cent of the spore formers. In the same way sixty five (65) isolates were identified and characterized for their morphological, cultural, staining and biochemical characteristics by Rani *et al*[16]. Rawat *et al*[17]., also in the same way selected rhizobacterial isolates from wheat rhizosphere on the basis of the colour of their colonies and morphological characters. Joshi *et al*[9]., also in the same way selected 93 isolates from the rhizosphere of rice on the basis of their colony morphology. A greater percentage of Gram negative bacteria was observed in the present study i.e. 73 per cent isolates were Gram negative and 27 per cent were gram positive. Almost similar results were observed by Megersa and Aseffa[15] who found 69 per cent Gram negative and 31 per cent Gram positive rhizobacteria from the rhizosphere of *Erythrina brucei*. Upon gram staining, the cell morphology of majority of the isolates was rod shaped and few were round shaped. Megersa and Aseffa[15] also confirmed the dominance of rod shaped bacteria in the rhizosphere.

All the 100 rhizobacterial isolates were screened for the production of IAA by Salkowski's method. It wsa found that 80 per cent of the isolates were IAA producers however their production amount varied considerably (Fig.1). Maximum IAA was produced by rhizobacterial isolate SD99 (70.135 µg mL^{-1}) while minimum by SD56 (2.292 µg mL^{-1}). Similar results were observed by Salamone *et al*[18]., who found that 80 per cent of the rhizobacteria produce IAA. But Kumar *et al*[13]., observed that 100 per cent of the isolated rhizobacteria produced IAA. Phosphate solubilization is an important character of the rhizobacteria to make the availability of phosphorus to plants. Therefore, all the rhizobacterial isolates were screened for the presence of phosphate solubilization activity on Pikovskaya's medium. A considerable variation was observed among the isolates in their ability to solubilize phosphate (Table 1). Depending upon the radius of solubilization zone, the isolates were categorized as very good (10.1-12 mm), good (8.1-10 mm), moderate (6.1-8 mm), low (4.1-6 mm) and very low (4 & < 4 mm) solubilizers

of phosphate so as to compare the different isolates . Screening for phosphate solubilization showed that overall 25 per cent bacterial isolates were phosphate solubilizers out of 100 isolates. Very good phosphate solubilizers account for 8 per cent of the total phosphate solubilizers, good phosphate solubilizers account for 8 per cent of the total phosphate solubilizers, moderate phosphate solubilizers account for 12 per cent of the total phosphate solubilizers, low phosphate solubilizers account for 24 per cent of the total phosphate solubilizers & very low phosphate solubilizers account for 48 per cent of the total phosphate soubilizers.The highest phosphate solubilizer identified was SD25 and SD93 (11 mm solubilization zone) & lowest phosphate solubilizer identified was SD3, SD11, SD19, SD88 (2 mm solubilization zone each). The solubilization index varied from 1.25 in SD11 to 2.89 in SD25. However, Kumar *et al*[13]., found 40 per cent of the isolated rhizobacterial isolates to be phosphate solubilizers. Nearly Similar to our findings Joshi & Bhatt[9] observed that 22.56 per cent rhizobacterial isolates solubilize phosphate.

Screening of bacterial cultures for growth inhibition of fungi

All the 100 rhizobacterial isolates/strains were screened for their antagonistic interaction against two fungi i.e., *Fusarium oxysporum* and *Ganoderma lucidium* on PDA medium plates. It was observed that 45 per cent of the total isolates were having antifungal activity. Further it was found that 31 per cent of the antifungal activity possessing isolates were antagonistic to *Fusarium oxysporum* exclusively, 33 per cent of the antifungal activity possessing isolates were antagonistic to *Ganoderma lucidium* exclusively & 15 per cent of the antifungal activity possessing isolates were antagonistic to both *Ganoderma lucidium* & *Fusarium oxysporum* (Table 2). *Fusarium oxysporum* was maximum inhibited by the isolate SD30 likewise, maximum inhibition of *Ganoderma lucidium* was observed to be caused by SD93. Idris *et al*[7]., demonstrated effective biological control by the rhizobacterial isolates against *Fusarium oxysporum*. Out of 78 isolates tested, 23 isolates inhibited the fungal mycelium of *F. oxysporum* accounting about 29.4 per cent of the selected isolates. Which is almost same as confirmed by us.

Promotion of plant growth by rhizobacterial isolates under pot house conditions

To assess this, an experiment under pot culture conditions was conducted using *Dalbergia sissoo* as test crop and rhizobacteria were used as inoculants. An absolute control was also kept containing field soil without inoculation. The worth of all outstanding 18 rhizobacterial isolates (making 19 treatments) was observed while considering their impact on root length, shoot length, dry weight of shoots and dry weight of roots (Table 3). In the same way their impact on nodule number, nodule fresh weight & nodule dry weight was observed (Table 3). It was observed that the shoot length promotion was highest caused by SD99 (T_{19}) (fig. 4.1g) followed by SD6 (T_4). In the same way root length promotion was highest caused by SD6 (T_4) followed by SD99 (T_{19}). Shoot fresh and dry weight was highest observed in T_{19} while as highest root fresh weight was observed in T_4 but highest root dry weight was observed in $T_{19.}$ In case of nodule number, SD97 (T_{18}) caused the highest increment with the nodule no of 35 (only 21 nodules in control) & same trend was observed in nodule fresh and dry weight by SD97. Tizzard *et al*[23]., reported almost the same findings under pot culture conditions while observing the effect of different plant growth promoting rhizobacteria on growth parameters of various legume forest trees. In treatment T_4 (inoculated with isolate SD6) the root length enhancement was found to be highest contradicting the expected results because under *in vitro* conditions SD99 caused the highest root growth enhancement, therefore, the treatment T_{19} inoculated with SD99 should have caused the maximum root length enhancement. It becomes clear from the above findings that SD99 could not perform well under pot house conditions as compared to SD6. So we conclude that many bacterial strains exert their

beneficial effects in laboratory culture and a much lower number are functioning under practical conditions, i.e., in a commercial greenhouse or in the field. Same findings were reported by Lugtenberg and Kamilova[14] while assessing the performance of various rhizobacterial isolates under pot culture and field conditions. Yadav *et.al*[24]. 2010 also isolated and screened various rhizobacteria from the rhizosphere of chickpea and found most of isolates resulted in a significant increase in shoot length, root length and dry matter production of shoot and root of chickpea seedlings under pot house conditions, which is parallel to our findings.

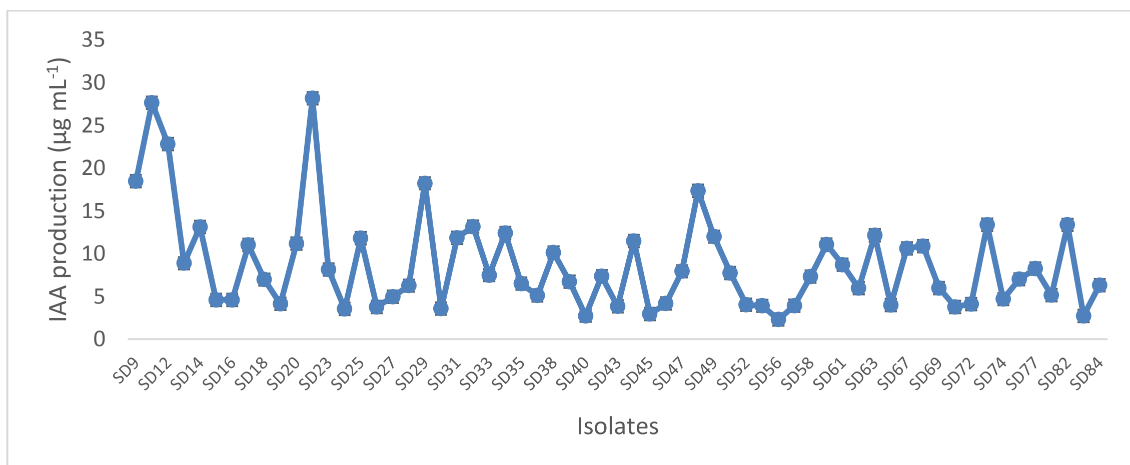

Fig. 1: IAA production (µg mL^{-1}) various rhizobacterial isolates

Table 1: Phosphate solubilization activity of isolated rhizobacterial strains

Bacterial isolate	Solubilization zone (Radius in mm)	Solubilization index
SD2	8.0	2.71
SD3	2.0	1.30
SD4	2.5	1.66
SD5	4.5	2.25
SD6	4.0	2.00
SD11	2.0	1.25
SD13	6.5	2.60
SD14	4.5	2.25
SD19	2.0	1.33
SD21	3.5	1.75
SD25	11	2.89
SD31	6.5	2.60
SD47	4.5	2.25
SD49	5.0	2.50
SD59	5.0	2.00
SD64	2.5	1.5
SD65	3.0	2.00
SD66	4.5	2.25
SD69	3.0	1.50
SD70	4.0	2.00
SD86	9.0	3.00
SD87	8.5	2.83
SD87	8.5	2.83
SD88	2.0	1.33
SD92	4.0	2.00
SD93	11	2.88
Mean	5.05	2.13
C.D. ($p \leq 0.05$)	0.73	0.34
SE(m)	0.07	0.11
C.V per cent	3.12	1.73

Table 2: Antifungal activity of isolated rhizobacterial strains

Rhizobacterial isolates	Inhibition of *Fusarium oxysporum* (Halo zone size (mm)	Inhibition of *Ganoderma lucidium* (Halo zone size)(mm)	Rhizobacterial isolates	Inhibition of *Fusarium oxysporum* (Halo zone size (mm)	Inhibition of *Ganoderma lucidium* (Halo zone size)(mm)	Rhizobacterial isolates	Inhibition of *Fusarium oxysporum* (Halo zone size (mm)	Inhibition of *Ganoderma lucidium* (Halo zone size)(mm)
SD 1	5.0	-	**SD39**	7.0	9.0	**SD82**	5.0	6.0
SD 8	6.0	-	**SD40**	6.0	5.0	**SD83**	6.0	-
SD12	5.0	-	**SD46**	-	**10**	**SD84**	5.0	5.0
SD16	-	5.0	**SD47**	6 .0	6.0	**SD 85**	-	6.0
SD19	7.0	-	**SD48**	-	6.0	**SD86**	6.0	9.0
SD20	-	7.0	**SD56**	8.0	**12**	**SD87**	-	19
SD21	6.0	-	**SD58**	5.0	7.0	**SD89**	-	9.0
SD23	5.0	-	**SD61**	-	8.0	**SD90**	5.0	-
SD24	6.0	-	**SD62**	-	6.0	**SD92**	-	7.0
SD25	-	**10**	**SD63**	-	5.0	**SD93**	8.0	**16**
SD 27	5..0	-	**SD64**	6.0	9.0	**SD97**	17	9.0
SD28	6 .0	5.0	**SD67**	6.0	6.0	**SD99**	6.0	8.0
SD29	5.0	6.0	**SD69**	-	7.0	**SD100**	-	8.0
SD30	**18**	**10**	**SD70**	-	8.0			
SD31	5.0	-	**SD71**	5.0	-			
SD33	6 .0	-	**SD73**	6.0	-			
			Mean	**6.73**	**8.03**			
			C.D. (p≤0.05)	**0.45**	**0.32**			
			SE(m)	**0.02**	**0.17**			
			C.V per cent	**1.11**	**0.89**			

Table 3: Impact of isolated rhizobacterial strains on various growth parameters of *Dalbergia sissoo*

Treatments	Shoot length (cm)	Shoot Fresh wt. (g)	Shoot dry wt. (g)	Root length (cm)	Root Fresh wt. (g)	Root dry wt. (g)	Nodule number	Nodule fresh weight (g)	Nodule dry weight (g)
(T_1) = Control	12.1	1.612	0.795	06.31	0.295	0.155	21	0.191	0.063
(T_2) = Control + SD2	14.62	1.662	0.837	10.42	0.531	0.274	29	0.294	0.102
(T_3) = Control +SD3	15.0	1.807	0.895	09.59	0.570	0.286	25	0.248	0.085
(T_4) = Control+ SD6	15.68	1.917	0.983	12.65	0.769	0.388	27	0.281	0.095
(T_5) = Control+ SD8	14.54	1.709	0.863	08.34	0.487	0.268	28	0.288	0.098
(T_6) = Control +SD10	13.26	1.551	0.765	07.96	0.446	0.243	22	0.235	0.079
(T_7) = Control+ SD13	14.78	1.825	0.918	08.24	0.476	0.246	26	0.263	0.089
(T_8) = Control + SD14	14.3	1.633	0.818	09.75	0.497	0.259	23	0.218	0.076
(T_9) = Control + SD22	15.66	1.909	0.944	11.46	0.697	0.331	23	0.223	0.071
(T_{10}) = Control + SD25	15.6	1.855	0.920	09.42	0.551	0.289	29	0.343	0.113
(T_{11}) = Control + SD30	13.2	1.607	0.812	08.43	0.472	0.271	24	0.236	0.075
(T_{12}) = Control+ SD31	12.64	1.183	0.747	06.42	0.381	0.196	30	0.316	0.107
(T_{13}) = Control + SD49	13.36	1.372	0.773	09.45	0.552	0.283	29	0.338	0.115
(T_{14}) = Control + SD86	14.38	1.539	0.853	07.54	0.448	0.230	30	0.303	0.105
(T_{15}) = Control +SD87	13.25	1.497	0.830	09.03	0.549	0.292	31	0.318	0.109
(T_{16}) = Control + SD89	13.42	1.509	0.841	06.72	0.389	0.176	29	0.275	0.098
(T_{17}) = Control + SD93	13.16	1.467	0.816	06.88	0.385	0.211	29	0.294	0.103
(T_{18}) = Control + SD97	13.26	1.589	0.833	08.24	0.501	0.253	35	0.366	0.126
(T_{19}) = Control + SD99	18.02	2.093	1.149	12.58	0.765	0.405	29	0.294	0.107
Mean	14.22	1.64	0.86	8.91	0.51	0.26	27.31	0.28	0.09
C.D. (p≤0.05)	1.67	0.48	0.07	0.91	0.005	0.05	2.33	0.01	0.04
SE(m)	0.21	0.07	0.12	0.23	0.06	0.04	1.05	0.01	0.04
C.V per cent	0.67	0.23	0.09	0.34	0.08	0.02	0.23	0.02	0.22

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