

Assessment of regeneration and application of PGPRs on the growth of *Pinus roxburghii* in Ghoragali Forest Sub-Division, Pakistan



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Abstract

This study was conducted to assess the regeneration and application of PGPR's on the Growth of *Pinus roxburghii* in Ghoragali Forest Sub-division, Pakistan. Assessment of regeneration was done in five respective compartments of Ghoragali (52, 53, 57, 58 and 59) using fixed area plot method with 1 percent sampling intensity to assess the regeneration and each sample plot was 1/10th of hec which is equal to 17.84m radius. All those seedlings which were 1-5ft in height were taken as un-established regeneration and from 6 to 10ft were taken as established regeneration. Assessment results were statistically analyzed by regression and correlation. Maximum un-established regeneration per hectare was in compartment 58 which was 23250 (11.4%) and minimum in compartment 59 which was 12500 (6.2%). The seedlings of *Pinus roxburghii* were brought from compartment 59 for the application of PGPR (Plant Growth Promoting Rhizobacteria) and growth was observed with different treatments. Rhizospheric soil bacteria of *Albezzia lebbek* (Black Siris) as it is fast growing species were inoculated on *Pinus roxburghii* by using serial dilution technique. Luria Bertani (L.B) media was used for as growth media. Five treatments T1 (controlled), T2 (water stressed), T3 (1μL PGPR), T4 (2μL PGPR), T5 (3μL PGPR) was observed in two groups. One group was watered twice a week and other was remaining water stressed throughout the period of observation. Growth was observed by Post Hoc (Tukey's test) statistically. The result was significant and positive growth was observed between the seedlings of Chir pine. So, PGPR can be used to enhance the plant growth.

1. Introduction

Chir pine naming as *Pinus roxburghii* or long leaf Indian pine belongs to family Pinaceae. A substantial Chir pine tree is 21 to 33 m tall and the normal measurement is 0.6 m. The crown is adjusted or round about fit as a fiddle. The needles are in threes which are 20 to 30 cm long and the stem is straight or erects (Tiwari, 1994). *Pinus roxburghii* is monoecious. The male blooms are many in number and packed in head like bunches, called cones which are 1.3 to 1.8 cm long. The female blooms are erect or opposite and unaccompanied towards the finish of branches with 2 to 5 bunches. The sprouting period of cones is between Januarys to April. The fruit is the female cone and as it matures it changes into glossy ruddy dark colored shading. The seed after preparation takes a whole year to create and requires 2 years to complete the regenerative cycle. The seeds are two in number and are winged underneath each cone scale. Seeds are shed during September and October (Sheikh, 1993). *Pinus roxburghii* is local to Pakistan, Bhutan, Nepal, India, and Afghanistan. In Pakistan, it is particularly found in the Himalayas, Azad Kashmir, Murree, Swat, Dir, Khyber and Malakand (Sheikh, 1993).

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Regeneration assumes critical part being developed process which shows composition, structure, stand distribution and future product of the forests. It is the procedure of Forest working through which woods and trees get by after some time (Halle *et al.*, 1978). The potential recovery status of tree species inside a forest remain in space and time is most imperative for the future association of forests (Ayyaapan and Parthasarathy, 1999 and Henle *et al.*, 2004). For the support of forests, regeneration of forests species is the most obvious phenomena. Regeneration can be shown by assuming the age, size and structure of individual species of that forest stand. Vegetation in the mountainous ranges is influenced by different factors in which elevation, aspect, slope and soil acuity are dominant that they adjust the establishing of humidity and introduction to sun. Similarly, the contrasts between the microclimate, angle and height extremely influences the vegetation inside the forest area (Pande *et al.*, 2000). It has been seen that the pine forests have serious anthropogenic unsettling influences. The types of disturbances influences were fundamentally included deforestation, animal grazing, cutting, surface consuming and litter expulsion. (Oliver and Larson 1990). Different species that are happening in Chir pine forests normally neglects to recover (Singh and Singh 1987). If the regeneration status of chir pine reduced due to any anthropogenic activities then it can be increase with the help of proper management, silvicultural operations and with the use of growth promoting rhizobacteria. Plant growth promoting rhizobacteria (PGPR), is the gathering of useful plant microscopic organisms that are exceptionally valuable for the expansion in plant development and hence in product yield. In a soil containing aggressive microflora when about 2 to 5% of rhizobacteria are reintroduced by plant inoculation, relate a useful impact on plant development and are named plant growth promoting rhizobacteria (PGPR) (Kloepper and Schroth, 1978). PGPR are free-living microbes and some of them attack the tissues of living plants and cause unobvious and asymptomatic diseases (Kloepper *et al.*, 1989 and Sturz and Nowak, 2000).

PGPR action has been accounted for strains having a place with a few other genera, for example, *Azospirillum*, *Serratia*, *Clostridium*, *Bacillus*, *Hydrogenophaga*, *Azotobacter*, *Enterobacter* and *Arthrobacter* (Rovira 1969; Kloepper *et al.*, 1986, 1989 and Chanway and Holl, 1992). These days, scientists utilize them effectively in field analyzes over and over. It has been accounted for that PGPRS expanded the development and harvest yields of sugar beet, potato, sweet potato and radish (Farzana *et al.*, 2009). Soil conditions influence the productivity of bacterial inoculants. These inoculants have superior impact on plant development where the soil is nutrient insufficient. Rhizosphere is the association between soil, microorganisms and plants. Lynch, 1990 observed that rhizosphere can be isolated into three sections, the ecto-rhizosphere, which is characterized as the soil neighboring living roots; the root surface or rhizoplane and the internal region of the root (rhizodermal and cortical cells). Free-living beneficial microscopic organisms can be found among these microorganisms which are harmful to the host plant (Chanway *et al.*, 1991). Application of PGPRs are: Biological nitrogen fixation, Symbiotic nitrogen fixers (*Rhizobium*, *Bradyrhizobium*), Non-symbiotic nitrogen fixers (*Azotobacter*, *Azospirillum*, *Acetobacter*, *Azoarcus*), *Bacillus* and *Pseudomonas* (Egamberdiyeva, 2007). The specific objectives of the study were to assess the regeneration of *Pinus roxburghii* and the application of PGPRs on the growth of its seedlings in Ghoragali forest subdivision.

2 Materials and Methods

2.1 Study Area

The study area was Ghoragali forest subdivision Tehsil Murree district Rawalpindi and is located at 33.83°N 73.35°E in the northeastern tip of province of Punjab, Pakistan. The tract dealt with is bounded in the east by river Jhelum in the North West by the KPK in the south by the sub mountainous areas of Rawalpindi and Gujjar khan tehsil. Two important towns namely, Murree and Kahuta which are also tehsil headquarter, fall within the jurisdiction of this tract (Working plan for coniferous forests of Murree and Kahuta 1994-95 to 2023-24). Climate of the study area falls in sub-tropical continental highlands. Variations in climate are caused by the differences in altitude, amount of winter snowfall and the duration and depth of snow accumulation. Generally, the climate is cooler at high altitude and warmer at lower ones. Spring and autumn are short. Ghoragali receives 1500-1700mm of annual precipitation in the form of rain and snowfall in approximately 89 mean rainy days, however, different parts of the area receive varying amount of rainfall. Chir pine trees grow well in rainfall of 450 to 1625mm/year (champion, 1965). The average maximum and minimum temperature is 18°C and 6°C respectively. Normally temperature is high in the month of May and June every year. The temperature goes up slowly from January to June till the start of monsoon in the Goragali forest sub division Murree. Then, its starts falling steadily till a very rapid fall of temperature in December and January. Chir pine trees grow well in temperature range of -5 to 40°C at elevation between 500-2500m (Sheikh, 1993).

2.2 Regeneration Assessment Methodology

2.2.1 Sampling Intensity

The forest sub division had twelve compartments and assessment was done in five of them (52, 53, 57, 58 and 59). Their area according to working plan of Murree forest division and number of plots to be sampled are also mentioned in table 1. Random sampling was done and 1% sampling intensity was decided as per inventory time, spatial coverage and available resources. Each sample plot was 2×2m (Walayat, 1998). Total number of plots in each compartment to be sampled was obtained by area in hectare multiply with sampling intensity divided by plot size.

2.2.2 Secondary data collection

Secondary data was collected from the maps and compartment history files taken from Sub Divisional forest office Ghoragali. Fixed area plot method was used for the assessment of regeneration. Plot directions were located with the help of magnetic compass and

from that point movement was started. Diameter of the trees was calculated with Calliper and diameter tape. Plot to plot and within the plot distance was taken with the help of measuring tape and fixed it at one point in the center.

Regeneration was divided into two groups: Un-established regeneration and established regeneration. All those seedlings which were encounter in the plot area, the heights of seedlings which were 1-5ft measured as un-established regeneration and from 6-10ft as established regeneration. Same procedure was applied in all plots of every compartment to assess regeneration status.

2.3 Established and Un-Established Regeneration

Following formulas were used during calculation. (Attri *et al.*, 2017).

2.3.1 Un-Established regeneration was found by

$$= 2500 \times \text{Total no. of un - established plants in sampled plot} \div \text{Total no. of sampled plots}$$

- Established regeneration was found by

$$= 2500 \times \text{Total no. of established plants in sampled plots} \div \text{Total no. of sampled plots}$$

- Establishment index

$$= \text{Average height} \div \text{Established height}$$

- Established Stocking percent

$$= 100 \times (\text{Establishment index} \times \text{Stocking Index})$$

- Stocking Index

$$= \text{Un - Established regeneration (hec)} + \text{Established regeneration (hec)} \div 4$$

2.3.2 Collection of *Pinus roxburghii* seedlings from Ghoragali forest and Application of Plant Growth Promoting Rhizobacteria (PGPR)

Plant growth promoting rhizobacteria (PGPR) were applied to the seedlings of *Pinus roxburghii* which were brought from forest sub division and placed in Forest nursery at PMAS Arid Agriculture University, Rawalpindi. Growth of *Pinus roxburghii* seedlings were checked with five treatments: T1 (Control), T2 (Water stress), T3 (1µl PGPR), T4 (2µl PGPR), T5 (3µl PGPR).

2.3.3. Collection of Rhizospheric Soil

Rhizospheric soil of *Albezzia lebbek* (Black Siris) was collected from forest nursery and was brought to the laboratory for further processing.

2.3.4 Isolation of Bacteria from Rhizospheric soil of *Albezzia lebbek*

Bacteria were isolated from rhizospheric soil by using serial dilution plate technique. Luria Bertani (L.B) media was used as growth media and isolated bacteria were preserved in glycerol stock for future.

2.3.5 Preparation of Inocula

Inocula were prepared with Luria Bertani (L.B) as growth media. Took 1g of soil and centrifuged at 10,000rpm for 20 minutes. Then bacteria was isolated with serial dilution plate technique and streaked and re-streaked until pure colonies of bacteria were obtained. After that, L.B Broth was prepared and inoculated a loop full of colonies and kept at shaker for 48 hours.

After 48 hours, when samples got turbid, with the help of micro pipette bacteria was inoculated in the rhizosphere of seedling of *Pinus roxburghii* with different concentrations according to treatments.

2.3.6 Growth parameters studied

Seedlings were inoculated with PGPR on 9th April 2018 and data was recorded after one month of inoculation of PGPR. Data collection was started on 9th May 2018 with the interval of two weeks. Drought stress was applied when data collection was started. Growth parameter recorded was only height of seedlings.

3.4 Statistical analysis

All the collected data for the analysis of regeneration was transferred to the Microsoft excel sheets for the purpose of compilation and tabulation. Data was interpreted with descriptive statistics. PGPR data was analyzed statistically by using one way ANOVA, Multi comparison Post Hoc (Tukey's test). (Henrique, A. et al., 2006)

$$\text{Formula: } (M1 - M2 \div \sqrt{MSw} \frac{1}{n})$$

(M = treatment/group mean, n = number per treatment/group).

4 Results and Discussions

4.1 Regeneration status

Table 1 shows total established and un-established regeneration per hectare. Maximum un-established regeneration per hectare is in compartment 58 which is 23250 and minimum is in compartment 59 which is 12500 whereas maximum established regeneration per hectare is in compartment 58 which is 5250 and minimum is in compartment 59 which is 3000 while in comparison study maximum un-established recruits was 6333 and maximum established recruits was 1083 were found in PB I (periodic block I) (Attri *et al.*, 2017).

Table 1: Established and un-established regeneration per hectare

Compartment No.	Area (hec)	No. of plots to be sampled	Un-Established Regeneration		Established Regeneration	
			Total Reg.	Total established reg.	Total Reg.	Total established reg.
52	165.6	16	112	17500	21	3281
53	189.2	18	135	18750	28	3888
57	146.8	14	103	18393	21	3750
58	108.4	10	93	23250	21	5250
59	50.4	5	25	12500	6	3000

Table 2 shows that in compartment 52, the mean values of diameter and height are 4.99 ± 2.27 and 2.58 ± 1.46 , in compartment 53, the mean values of diameter and height are 5.36 ± 2.41 and 2.82 ± 1.54 , in compartment 57, the mean values of diameter and height are 4.28 ± 2.02 and 2.12 ± 1.30 , in compartment 58, the mean values of diameter and height are 5.98 ± 2.34 and 3.22 ± 1.49 and in compartment 59, the mean values of diameter and height are 5.29 ± 2.17 and 2.78 ± 1.40 respectively.

Table 2: Summary statistics of compartment growth data

Parameters	Comp 52		Comp 53		Comp 57		Comp 58		Comp 59	
	Dia	Ht.	Dia	Ht.	Dia	Ht.	Dia	Ht.	Dia	Ht.
Mean	4.99	2.58	5.36	2.82	4.28	2.12	5.98	3.22	5.29	2.78
Standard Error	0.19	0.12	0.18	0.12	0.18	0.11	0.21	0.14	0.39	0.25
Standard Deviation	2.27	1.46	2.41	1.54	2.02	1.30	2.34	1.49	2.17	1.40
Sample Variance	5.17	2.14	5.81	2.38	4.11	1.71	5.48	2.23	4.74	1.97

Table 3 demonstrates that correlation and regression summary among the diameter and height of the inventoried seedlings. The correlation was observed in compartments was 0.99 coefficient of determination (R-squared). Linear models were adopted for all relationships.

Table 3: Summary of linear regression model of the compartments

Compartments	Attributes	Equation	Model	R2	S.E	F
Comp 52	Dia& ht.	$Y=0.5204x + 0.2171$	Linear	0.99	0.02	783274
Comp 53		$Y=0.543x + 0.0651$	Linear	0.99	0.03	733028
Comp 57		$Y=0.5115x + 0.2024$	Linear	0.99	0.03	478747
Comp 58		$Y=0.4908x + 0.7921$	Linear	0.99	0.03	480350
Comp 59		$Y=0.478x + 0.7708$	Linear	0.99	0.02	209775

Table 4 demonstrates that total established index is highest in compartment 58 (0.8) and lowest in compartment 59 (0.65) whereas stocking index and established stocking percent is also highest in compartment 58 which is 0.114 and 9.12 respectively and lowest in compartment 59 which is 0.062 and 4.03 respectively while in contrast study maximum stocking index (0.58) Percent Regeneration (57.78) and Established stocking Percent (32.29) were found in PB I as compared to Others PB's (Attri et al., 2017).

Table 4 Regeneration percentage

Comp	Established index	Stocking index	Established percent	stocking	Regeneration percentage
52	0.73	0.083	6.06		8.31
53	0.76	0.090	6.88		9.05
57	0.71	0.088	6.28		8.85
58	0.8	0.114	9.12		11.4
59	0.65	0.062	4.03		6.2

Present study observed the maximum regeneration percent recorded was in compartment 58 (11.4%) and minimum was in compartment 59 (6.2%). The total regeneration success followed the sequence 58 (11.4), 53 (9.05), 57 (8.85), 52 (8.31) and 59 (6.2) which was also described in other study in such a way that maximum regeneration percent (41.71) was recorded in the plots under E4 and minimum regeneration percent (13.82) was recorded in the plots under E1. The total regeneration success followed the sequence E4 (41.71), E3 (38.86), E2 (30.78) and E1 (13.82). However, from different periodic blocks, PB-I recorded a higher regeneration percent (39.02); whereas, minimum regeneration percent (23.14) was recorded in PB-IV (Mahajan, A. (2016).

Table 5 Watered plants

Control	Initial Heights (cm)	1 st week	2 nd week	3 rd week	4 th week	Height Increment (cm)
C1	8	11	13.5	15	16	8
C2	8.5	12	13	13	14	5.5
C3	8	10	13	13	13.5	5.5
1 μ l PGPR	9	17	18	19	20	11
	8.5	13	14	15	16	7.5
	9	15	16	18	19	10
2 μ l PGPR	8	14	16	16	16.5	8.5
	8.5	16	17	17	18	9.5
	9.5	17	18	19	19.5	10
3 μ l PGPR	8.5	14	14	16	17	8.5
	9	16	17	18	19.5	10.5
	9.5	15	18	19	20	10.5

The seedlings of *Pinus roxburghii* was brought from compartment 59 for the application of PGPR (Plant Growth Promoting Rhizobacteria) and then growth was observed with different treatments.

4.2 Application of PGPR's on the growth of *Pinus roxburghii*

The initial height of plants was recorded at the time of application of Plant Growth Promoting Rhizobacteria (PGPR) was 8 to 10 cm. Data was collected in form of tables.

Table 5 and 6 demonstrates the initial readings and total height increment (cm) of each plant along with the readings of all four weeks of both groups which were watered or kept as water stressed.

In this study, Plant Growth Promoting Rhizobacteria (PGPR) was applied in different proportion i-e 1 μ l, 2 μ l and 3 μ l and evaluations were performed after 30 days of application and data was recorded with the interval of two weeks with 6 treatments. Experiment was carried out as one way ANOVA with 3 replications with 12 factors. Growth was maximum in 3 μ l PGPR seedlings and means were compared by (Post-Hoc) Tukey's test while another research shows that *P. caribaeavar. hondurensis* cuttings treated with IBA produced a higher percentage of rooted cuttings than those treated with NAA, and the experiment was carried out as a completely randomized design with 13 treatments containing 4 replicates with 8 cuttings each. The means were compared by Tukey's test (Henrique et al., 2006).

Table 6 Water stressed plants

Control	Initial Heights (cm)	1 st week	2 nd week	3 rd week	4 th week	Height Increment (cm)
C1	8	13	13.5	14	14	6
C2	8.5	12	13	13.5	15	6.5
C3	8	11	12	13.5	15	7
1μl PGPR	9	14	15.5	16.5	17.5	8.5
	8.5	15	16	17	18	9.5
	9	14	15.5	16	17	8
2μl PGPR	8	10	10.5	11	12	4
	8.5	9	10	10.5	11	2.5
	9.5	10	11	12	12.5	3
3μl PGPR	8.5	8	9	9.5	10.5	2
	9	16	17	18	18.5	9.5
	9.5	15	15.5	16	17	7.5

Table 7 demonstrate that the comparison of control with 1ul and 3ul PGPR, Comparison of 1ul with 2ul PGPR and comparison of 2ul with 3ul PGPR is significant ($p < 0.05$) which mean that PGPR has a positive effect on height of Chir pine while the difference between control and 2ul PGPR and comparison of 1ul with 3ul PGPR is non-significant ($p > 0.05$) which means that growth increment is due to any other factor whether due to climatic or genetic and not due to application of PGPR. Table 8 shows that the comparison of week 1 with week 3 and week 4 is significant ($p < 0.05$) while with week 2 is non-significant ($p > 0.05$). The comparison of week 2 with week 3 is non-significant ($p > 0.05$) while with week 4 is significant ($p < 0.05$). The comparison of week 3 with week 4 is non-significant ($p > 0.05$).

Table 7 Data was analyzed by the Post Hoc test. Multiple comparisons for application of PGPR's by Tukey's HSD are following:

Multiple Comparisons							
Increase Tukey's HSD (I) Application of PGPR	in	height	of	plant	in	cm	
(J) Application of PGPR	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	Lower Bound	Upper Bound	
Control	1ul PGPR	-3.14	0.556	0.00	-4.61	-1.68	
	2ul PGPR	-0.70	0.555	0.58	-2.17	0.75	
	3ul PGPR	-2.37	0.556	0.00	-3.84	-0.90	
1ul PGPR	Control	3.14	0.556	0.00	1.68	4.61	
	2ul PGPR	2.43	0.556	0.00	0.97	3.90	
	3ul PGPR	0.77	0.556	0.51	-0.69	2.23	
2ul PGPR	Control	0.70	0.556	0.58	-0.75	2.17	
	1ul PGPR	-2.43	0.556	0.00	-3.90	-0.97	
	3ul PGPR	-1.66	0.556	0.02	-3.13	-0.20	
3ul PGPR	Control	2.37	0.556	0.00	0.90	3.84	
	1ul PGPR	-0.77	0.556	0.51	-2.23	0.69	
	2ul PGPR	1.66	0.556	0.02	0.20	3.13	

Research concluded that PGPR have incredible potential for use in plant nurseries where they can help in creating sound seedlings for commercial utilization and in the rebuilding of corrupted soils and also help in direct mechanism of PGPR like N₂ fixation, better impact on root development and morphology and helpful plant organisms symbioses while other studies revealed that methods of application of PGPR incorporate settling of N₂, expanding the accessibility of supplements in the rhizosphere, emphatically impacting root development and morphology and advance other helpful plant organism symbioses. They also concluded that PGPR application expands nutrient accessibility in the rhizosphere or indirectly by repressing the plant pathogens and stimulates the development of different microorganisms gainful to plant improvement (Vessy 2003 and Bran et al., 2011).

Table 8 multiple comparisons for interval of reading during study were also analyzed by this test

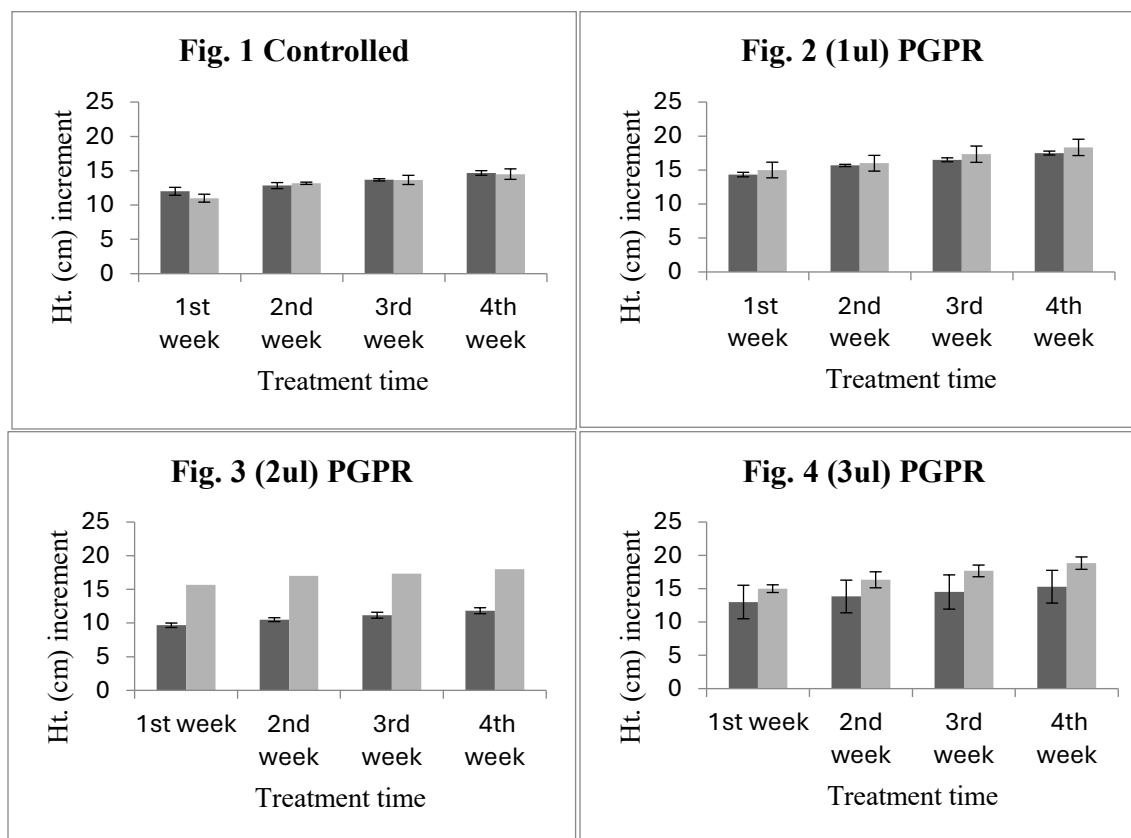
Multiple Comparisons						
increase Tukey HSD (I) interval of reading during study period	in (J) interval of reading during study period	height of Mean Difference (I-J)	plant Std. Error	Sig.	95% Confidence Interval	cm
					Lower Bound	Upper Bound
week 1	week2	-1.208	0.556	0.14	-2.674	0.258
	week3	-2.021	0.557	0.00	-3.487	-0.555
	week4	-2.917	0.556	0.00	-4.383	-1.451
week2	week1	1.208	0.556	0.14	-0.258	2.674
	week3	-0.812	0.556	0.46	-2.278	0.653
	week4	-1.708	0.556	0.01	-3.174	-0.242
week3	week1	2.021	0.556	0.00	0.555	3.487
	week2	0.812	0.556	0.46	-0.653	2.278
	week4	-0.896	0.556	0.37	-2.362	0.570
week4	week1	2.917	0.556	0.00	1.451	4.383
	week2	1.708	0.556	0.01	0.242	3.174
	week3	0.896	0.556	0.37	-0.570	2.362

Application of PGPRs on the seedlings of *Pinus roxburghii* in different proportions have different positive effects on the growth and height of plants and are also beneficial for the rapid increase in growth of different seedlings of plants while other study concluded that naturally soil occurring plant growth promoting rhizobacteria (PGPR) are beneficial to plants as they increase their growth and application of PGPRs at the initial stage of plants will increase total crop yield and its application on different trees can get many benefits as well as increase crop yield rapidly by noticing the quick increase in germination of seed, density of crop, timely growth and plant height (Saharan and Nehra 2011).

Table 9 Means of water stressed and watered plants

Treatments means	1st week	2nd week	3rd week	4th week
0	12	12.83	13.67	14.67
0	11	13.17	13.66	14.5
1	14.33	15.67	16.5	17.5
1	15	16	17.33	18.33
2	9.67	10.5	11.16	11.83
2	15.66	17	17.33	18
3	13	13.83	14.5	15.3
3	15	16.33	17.67	18.83

Table 9 shows the means of all the treatments which were watered and water stressed and their graphs are mentioned in Fig. 1, 2, 3 and 4.



- Black bars represent water stressed plants and grey represents watered plants growth.

Figures 1.2.3.4 indicate means and standard errors of watered and water stressed plants

Conclusion

It is concluded that the regeneration was maximum in compartment 58 and lowest in 59. To increase the growth of *Pinus roxburghii* (Chir pine) PGPR (plant growth promoting rhizobacteria) was applied to the seedlings which indicate that the PGPR has the positive role in growth parameters, have incredible potential for use in plant nurseries, can create sound and vivacious seedlings for commercial utilize and can also rebuilt eroded and corrupted soils. So, it is recommended that the isolates of PGPR can be applied to enhance the plants with stunted growth.

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