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Research Article

Exploiting Plant Growth Promoting Rhizobacteria for the Conservation and Regeneration of the Near Threatened Chilgoza Pine (*Pinus gerardiana*) In Dir Upper, Khyber Pakhtunkhwa, Pakistan

[a]Shazia, [b]Lubna Ansari, [a]Muhammad Nawaz Rajpar, [a]Abdul Rab, [b]Irfan Ali, [b]Syed Ali Abbas, [c]Noshin Ilyas, [d]Mushtaq Ahmed, [b]Samiullah, [b]Saeed Gulzar

[a] *Forestry Department, Shaheed Benazir Bhatto University, Sheringal, Dir (Upper), Khyber Pakhtoonkhwa, Pakistan.*
[b] *Department of Forestry and Range Management, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi, Pakistan.*
[c] *Department of Botany, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi, Pakistan.*
[d] *Department of Plant Sciences, Quaid-e-Azam University, Islamabad, Pakistan.*

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ABSTRACT

Chilgoza pine (*Pinus gerardiana*) forests in Upper Dir are facing serious threats due to poor natural regeneration and slow growth. This study was conducted to isolate and identify plant growth-promoting rhizobacteria (PGPR) from the rhizospheric soils and root saplings of Chilgoza pine, evaluate their effects on seed germination and seedling growth, and compare their performance with phytohormones. The study area was Narkoon, located in the Pathrak forest range of Upper Dir district. The isolated bacterial strains were *Enterobacter* (from soil) and *Klebsiella* spp. (from roots). Chilgoza pine seeds were treated with gibberellic acid (150 ppm), PGPR, and their combinations. The experiment was carried out under controlled conditions in a growth chamber (CRD) and in a forest nursery (RCBD). Germination was recorded when the radicle reached 2 mm in length, and growth parameters were measured after one growing season. One-way ANOVA was applied to assess significant differences among treatments. The results showed that seeds treated with *Klebsiella pneumoniae* + 150 ppm gibberellic acid exhibited the highest germination energy (83%). Both *Enterobacter cancerogenus* and *Klebsiella pneumoniae*, alone or in combination with gibberellic acid, significantly enhanced germination percentage, mean daily germination, germination index, and seedling growth traits. Notably, (*E. cancerogenus* + 150 ppm gibberellic acid) resulted in the greatest root length (7.8 cm) and shoot length (14.6 cm). Statistical analysis confirmed significant treatment effects on germination and growth parameters ($p < 0.05$). Overall, the study demonstrates that PGPR inoculation combined with gibberellic acid can substantially improve seed germination and seedling growth in *P. gerardiana*.

Corresponding Author: Lubna Ansari
Email: Lubna.ansari@uaar.edu.pk
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Introduction

Pinus gerardiana, commonly known as Chilgoza pine, is a tree species endemic to the northwestern Himalayas, distributed across eastern Afghanistan, Pakistan, India, and restricted patches in the Hindu-Kush Himalayan ranges (Akbar et al., 2014). It grows

at elevations ranging from 1,800 to 3,350 m in dry temperate forests (Malik et al., 2012; Akbar et al., 2014; Khan et al., 2015; TRI, 2020).

In Pakistan, Chilgoza pine is predominantly found in Khyber Pakhtunkhwa, Balochistan, Gilgit-Baltistan, and Kashmir valleys, covering an area of approximately 260 km^2 (Malik et al., 2012). These forests occur both in pure stands and in association with conifers such as Deodar and Blue Pine, as well as broad-leaved species like Oak and Walnut. Although Deodar and Blue Pine are valued for timber, Chilgoza pine is prized for its edible nuts, which are highly demanded locally and internationally (TRI, 2020).

P. gerardiana is a hardy species capable of tolerating harsh conditions, including drought, freezing winters, dry summers, and strong winds. It thrives in areas with limited precipitation, mainly derived from monsoon rains or heavy winter snowfall (Akbar et al., 2014). In addition to providing ecological services, Chilgoza trees stabilize soil and prevent erosion (Malik et al., 2012).

Approximately 20% of Pakistan's forests are composed of Chilgoza pine, contributing significantly to local livelihoods. Its nuts are rich in essential nutrients and healthy fats and command a high price in international markets. Pakistan ranks as the second-largest global producer of Chilgoza nuts after China, with individual trees yielding 20-40 kg of nuts annually (Urooj and Jabeen, 2015; Sharma et al., 2020). In productive seed years, Chilgoza nut trade has the potential to generate billions of rupees (millions of dollars).

Despite its ecological and economic value, the Chilgoza ecosystem faces severe threats from overexploitation, mismanagement, and climate change. *P. gerardiana* is listed as Near Threatened on the IUCN Red List (Sharma et al., 2020). Over 70% of coniferous forests in its natural range have been depleted due to fuelwood collection and timber extraction, resulting in poor regeneration (Kumar et al., 2013). Additional factors, including uncontrolled grazing, insect pests, drought, irregular seed years, intense heat, low soil fertility, and soil erosion, further hinder natural regeneration. Consequently, mature and over-mature trees dominate these forests, while saplings and young poles are scarce, reflecting regeneration failure (Kumar et al., 2013, 2016).

Improving seed germination and seedling growth is critical to addressing these regeneration challenges (Kumar et al., 2014). Understanding germination patterns and developing novel regeneration strategies are essential to restore the species' ecological function and secure sustainable economic benefits.

A novel approach in this regard involves the use of Plant Growth-Promoting Rhizobacteria (PGPR), which have shown promising results in agriculture but remain underexplored in forestry. For example, studies on Brazilian *Araucaria* trees demonstrated PGPR's effectiveness in disease control (Riberio and Cardoso, 2012). However, no prior research has evaluated PGPR inoculation for enhancing *P. gerardiana* germination or regeneration. Utilizing PGPR as seed inoculants may provide a transformative strategy for conserving Chilgoza pine forests, rehabilitating degraded areas, and promoting sustainable forestry practices.

This pioneering study explores the potential of PGPR in enhancing seed germination and seedling growth of Chilgoza pine. It establishes the foundation for a strategic conservation framework by isolating and characterizing PGPR from Chilgoza rhizospheric soils and root saplings, evaluating their impact on seed germination and seedling growth, and comparing their effects with those of phytohormones. Advancing knowledge of PGPR's role in forestry will not only support the regeneration of *P. gerardiana* but also contribute to sustainable utilization of this ecologically and economically valuable species.

The objectives of this study were to isolate and characterize bacteria from the rhizospheric soil and roots of *P. gerardiana* and to evaluate the effects of phytohormones and PGPR inoculation on its seeds to enhance germination and seedling growth.

Materials and Methods

Study area

The study was conducted in the Narkoon, Pathrak Forest Range, Dir Upper, Khyber Pakhtunkhwa, Pakistan. The site covers an area of 2,970 hectares, located at 35.3583°N and 71.9583°E, with an elevation of 2,057 m as shown in Figure 1 and 2. The region is characterized by a dry temperate climate, with temperatures ranging from -2°C in winter to 33°C in summer. The dominant tree species include *Pinus gerardiana*, *Cedrus deodara*, and associated flora. The terrain has a slope gradient of 40-66° (Saddozai, 1995).

Isolation and purification of bacteria from soil samples

Soil samples (15) were collected from Narkoon, Pathrak forest range, an area characterized by dense mixed

vegetation dominated by Chilgoza pine. Rhizospheric soil and root samples were stored at -4°C and transported to the Forest Ecology Laboratory, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi. Bacteria were isolated using the serial dilution plate technique on Luria Bertani (LB) agar medium and incubated at 28°C for 48 h (Vincent, 1970).

Bacteria associated with Chilgoza pine roots were isolated using Yeast Extract Mannitol (YEM) agar medium. Roots were surface-sterilized with 5% sodium hypochlorite and 70% ethanol, followed by maceration and plating on YEM medium. After incubation at 28°C for 48 h, repeated streaking under sterile laminar flow conditions yielded pure colonies. These isolates were subjected to biochemical and molecular characterization to identify potential Plant Growth-Promoting Rhizobacteria (PGPR).

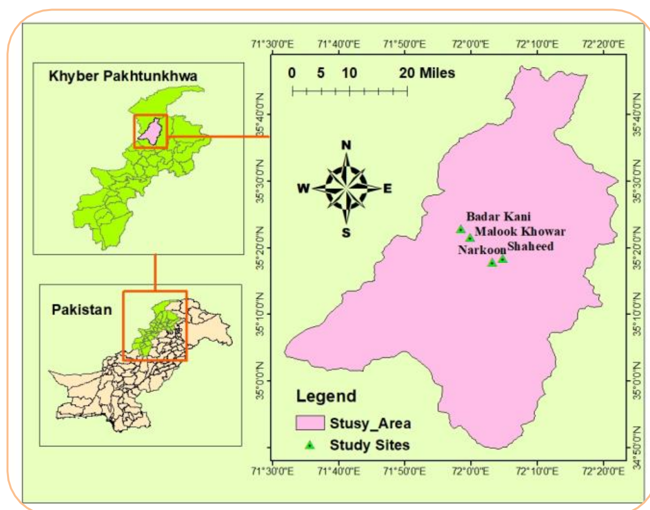

Figure 1. GIS map of the study area showing the Pathrak and Sheringal forest ranges.

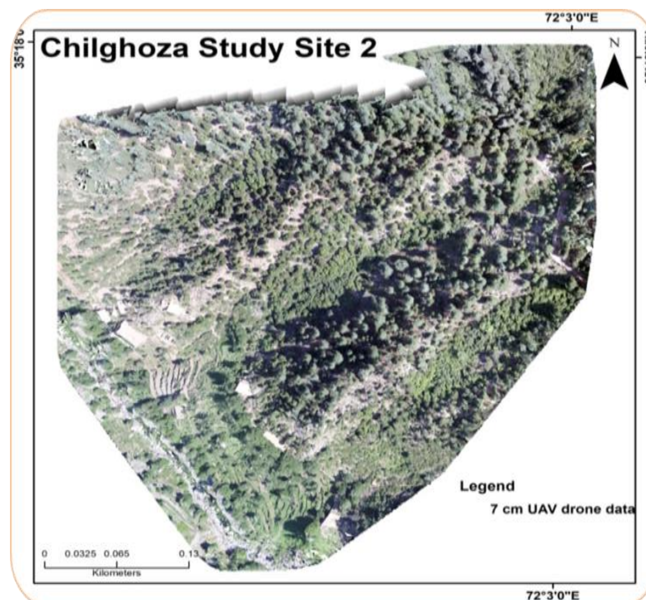

Figure 2. Drone image of the Pathrak Forest Range (Shaheed and Narkoon areas).

Biochemical characterization

Phosphorus solubilization

Bacterial isolates were evaluated for their phosphate-solubilizing ability by inoculating 24-h-old cultures into Pikovskaya's broth medium supplemented with tri-calcium phosphate. After 7, 13, and 18 days of incubation at 28°C, the supernatant was treated with ortho-phosphoric acid and Salkowski reagent to estimate phosphate solubilization. The solubilization index (SI) was calculated using the following formula (Pikovskaya, 1948; Premono et al., 1996):

$$SI = \frac{\text{Colony diameter} + \text{Halozone diameter}}{\text{Colony diameter}}$$

Indole-3-acetic acid (IAA) production

Isolates were tested for IAA production by culturing them on LB agar supplemented with tryptophan at 28°C for 48-72 h. After centrifugation, the supernatant was mixed with Salkowski reagent, and the development of a pink color indicated IAA production. Quantification was carried out spectrophotometrically at 530 nm (Brick et al., 1991).

Siderophore production

Siderophore production was assessed using Chrome Azurol S (CAS) agar plates, where the formation of orange halos around colonies after 24 h indicated siderophore secretion. The type of siderophores was further characterized: catecholates were identified using Arnow's assay, and hydroxamates were identified using Atkin's assay (Schwyn and Neilands, 1987).

Molecular characterization

The following standard procedures were performed for molecular characterization:

DNA extraction

Genomic DNA was extracted from bacterial colonies using a CTAB lysis buffer, followed by incubation and treatment with chloroform:isoamyl alcohol. DNA was precipitated with isopropanol, washed with 70% ethanol, air-dried, and re-suspended in Low TE buffer. The purified DNA was incubated at 60°C and stored at -20°C until further use.

Polymerase chain reaction (PCR)

PCR amplification was carried out using universal primers (forward: CCTAYGGGRBGCASCAG, reverse:

GGACTACNNGGGTATCTAAT) with an annealing temperature of 57°C, yielding a 465 bp product. The reaction mixture contained master mix, PCR-grade water, and template DNA. The cycling conditions included an initial denaturation at 95°C for 10 min, followed by repeated cycles of denaturation, annealing, and extension, and a final hold at 4°C (Poly et al., 2001).

Gel electrophoresis

A 1.5% agarose gel was prepared by dissolving 0.45 g of agarose in 30 ml of 1× TBE buffer. After boiling and cooling, 5 µl of ethidium bromide was added. PCR products (5 µl) were mixed with 2 µl of loading dye and electrophoresed on the gel.

16S rRNA gene sequencing

The amplified 16S rRNA gene products were sequenced by Macrogen, Korea.

Collection of seeds

High-quality Chilgoza pine seeds were collected from the Dir Kohistan Forest Division, specifically from Badar Kani, Shaheed, and Narkoon, during September-October 2020. After careful screening for size, health, and vigor, only the best seeds were selected. The seeds were dried and stored in zipper bags. Since P. gerardiana seeds possess hard and impermeable seed coats, dormancy-breaking treatments were required to enhance germination.

Conventional practices for breaking seed dormancy of *P. gerardiana*

Seed dormancy was broken through stratification, where seeds were soaked in cold water (-4°C to 10°C) for 2-3 months, or through hot-water treatment. Furthermore, mechanical scarification was applied to some seeds to promote germination. All treatments were carried out in the forest nursery of the Forestry Department, Dir Kohistan.

Laboratory analysis of *P. gerardiana* seed germination

Seeds were surface-sterilized using 5% sodium hypochlorite (Chlorox) and 95% ethanol, then soaked in water for 4 h. Subsequently, seeds were inoculated with the prepared inoculum for 8 h. They were sown in polythene bags filled with a composite soil mixture consisting of soil from the study sites, sand, and farmyard manure in a 2:1:1 ratio (soil:sand:FYM). The bags were placed in a growth chamber at 25 ± 1°C for 48 days. The first seedlings emerged on January 24, 2021, and the last on February 18, 2021. No further germination was observed until the experiment concluded on February 21, 2021.

A completely randomized design (CRD) was used, involving 350 seeds of *P. gerardiana* with six treatments and one control, each replicated five times (10 seeds per replication). The treatments were as follows:

T0 = Control
T1 = Gibberellic acid (GA_3) 150 ppm only
T2 = *Klebsiella pneumoniae* only
T3 = *Enterobacter cancerogenus* only
T4 = *Klebsiella pneumoniae* + Gibberellic acid (150 ppm)
T5 = *Enterobacter cancerogenus* + Gibberellic acid (150 ppm)
T6 = *Enterobacter cancerogenus* + *Klebsiella pneumonia*

Determination of germination parameters under laboratory conditions

After 48 days, the following germination and growth parameters were recorded:

Germination percentage (GP)

$$GP = \frac{GN}{\text{Total Number of seeds in an experiment}} \times 100$$

Where, GP = germination percentage
GN = number of germinated seeds.

Germination energy percentages

$$GE\% = \frac{\text{No. of seeds germinated on day (peak germination)}}{\text{Total number of seeds tested}} \times 100$$

Germination index

$G_I = ((G_1/1) + (G_2/2) + (G_3/3) + \ldots\ldots\ldots + (G_i/i))$

Where G1, G2, G3 … Gi = number of seeds germinated on the 1st, 2nd, 3rd … ith day, and i = number of days (Maguire, 1962).

Mean daily germination

$$MG = \frac{\text{Cumulative \% of full seeds germination}}{\text{Time period from sowing to end of test}}$$

Germination value

GV = PV × MG
Where, GV = germination value
PV = peak value of germination
MG = mean daily germination (Czabator, 1962).

Hypocotyl length

The length of the hypocotyls (cm) of uprooted seedlings was measured.

Root length

The root length (cm) of uprooted seedlings was recorded.

Seedling Length

Total seedling length (cm) was measured using a ruler.

Mortality percentage

Of the 10 seeds sown per replication, germinated seeds were counted, and the remainder was used to calculate mortality percentage.

Analysis of seed germination in nursery at Sheringal, Dir Upper

P. gerardiana seeds were pre-treated and sown in a nursery experiment arranged in a Randomized Complete Block Design (RCBD) with factorial arrangement. The experiment comprised seven treatments, including a control, applied to four blocks with 10 seeds per block. A composite mixture of soil, sand, and farmyard manure (FYM) in a 2:1:1 ratio was used as the growth medium. Seeds were sown in polythene bags, irrigated, and weeded regularly. The experiment was conducted from April 1, 2021, to June 15, 2022. The first sprouting was observed on May 10, 2021, and germination data were recorded until May 30, 2021. Seedlings were monitored during the active growing period (March-October), while no growth was observed during dormancy (November-March 15). After one growth season, seedling growth parameters were recorded by uprooting seedlings between June 7-10, 2022.

Determination of germination and growth parameters under nursery conditions

Germination Percentage

$$G\% = \frac{\text{Number of germinated seeds}}{\text{otal Number of seeds in an experiment}} \times 100$$

Seedling growth measurements

Seedling height (cm)

Seedling height was measured from the ground level to the shoot apex.

Collar diameter (mm)

Collar diameter was measured at the collar region using a Vernier caliper.

Root length (cm)

Root length was measured from the collar region to the tip of the taproot using a ruler.

Root-to-shoot ratio (RSR)

RSR was determined for each plant separately by using following formula

$$RSR = \frac{\text{dry shoot weight}}{\text{dry root weight}}$$

Total biomass of seedling

Total biomass of seedling was calculated by adding the dry weights of root and shoots of Chilgoza pine seedlings.

Total biomass (g) = Root weight + Shoot weight

Vigor index

VI = Total biomass × GP

Statistical analysis

Statix 8.1 was used to study the effects of various treatments on germination and growth parameters.

Results

Isolation and purification of bacteria from soil and root samples

The strains SN and RN isolated from the rhizospheric soil and roots of *P. gerardiana* were identified as *Enterobacte*r and *Klebsiella* species, respectively from Narkoon.

Biochemical characterization

The results obtained from Phosphorus solubilization, Indole acetic acid and Siderophore production were positive.

Molecular characterization

DNA extraction and gene sequencing were carried out for molecular characterization. The isolates SN and RN, obtained from soil and roots respectively, were used for DNA extraction. These isolates were identified as *Enterobacter cancerogenus* and *Klebsiella pneumoniae*. Gel electrophoresis was performed using a 1 Kb ladder as shown in Figure 3.

The isolates SN (*E. cancerogenus*) and RN (*K. pneumoniae*) were analyzed using BLAST at the NCBI website, and their sequences were submitted to GenBank. The following accession numbers were assigned:

1. SUB13924077 SeqSN - OR717528
2. SUB13924077 SeqRN - OR717529

The bacterial isolate showed 99.78% homology with *E. cancerogenus* as reported by Dickey and Zumoff (1988).

Application of Isolated PGPR and Gibberellic Acid on Chilgoza Seeds in the Laboratory

The results presented in Table 1 clearly revealed that seed germination was enhanced when treated with gibberellic acid (150 ppm) and both isolated PGPR strains, *K. pneumoniae* and *E. cancerogenus*. Germination parameter values were significantly higher when Chilgoza seeds were treated with *K. pneumoniae* and *E. cancerogenus* individually. The results were also improved when both treatments were combined with 150 ppm gibberellic acid. However, under control conditions, the values of germination parameters were significantly lower.

The germination percentage (GP) was found to be maximum (96%) when seeds were treated with *K. pneumoniae* (isolated from sapling roots), followed by *E. cancerogenus* (isolated from rhizospheric soil) (92%).

GP was also higher (90%) when seeds were treated with *K. pneumoniae* + 150 ppm gibberellic acid. In comparison, GP was lower (88% and 82%) when seeds were treated with *E. cancerogenus* + 150 ppm gibberellic acid and *K. pneumoniae* + *E. cancerogenus*, respectively. The minimum GP (50%) was observed under control conditions; however, treatment with gibberellic acid (150 ppm) increased GP to 74%.

Similarly, germination energy (GE%) was highest (88% and 84%) in seeds treated with *K. pneumoniae* and *E. cancerogenus*, respectively. GE% was also higher (83%) when seeds were treated with *K. pneumoniae* + 150 ppm gibberellic acid. By contrast, GE% was lower (74% and 76%) in seeds treated with *E. cancerogenus* + 150 ppm gibberellic acid and *K. pneumoniae* + *E. cancerogenus*, respectively. The minimum GE% (45%) was recorded under control conditions; however, treatment with gibberellic acid (150 ppm) increased GE% to 66%.

Figure 3. Phylogenetic tree of *E. cancerogenus* and *K. pneumonia*.

Mean daily germination (MG) was higher (4.63 and 4.32) for seeds treated with *K. pneumoniae* and *E. cancerogenus* separately, while the lowest MG (0.21) was recorded under control conditions. Germination index (GI) was also higher (4.86 and 4.62) in seeds treated with *K. pneumoniae* and *E. cancerogenus*, respectively, and reached 4.32 when treated with *K. pneumoniae* + 150 ppm gibberellic acid. The minimum GI was observed in seeds treated with gibberellic acid (150 ppm) only.

Germination value (GV) was highest (4.22 and 4.08) in seeds treated with *K. pneumoniae* and *E. cancerogenus*, respectively. The lowest GV among treatments (1.82) was recorded for gibberellic acid (150 ppm) alone, while the control exhibited the minimum GV overall (0.22).

Mean hypocotyl length was maximum (6.8, 6.7, and 6.5 cm) for seeds treated with *K. pneumoniae*, *E. cancerogenus*, and *K. pneumoniae* + gibberellic acid (150 ppm), respectively. Under control conditions, the mean hypocotyl length was lowest (3.5 cm). Mean root length was highest for *K. pneumoniae* (7.8 cm), followed by *E. cancerogenus* (7.6 cm), *K. pneumoniae* + gibberellic acid (150 ppm) (7.5 cm), and *E. cancerogenus* + gibberellic acid (150 ppm) (7.4 cm).

Similarly, mean shoot length was maximum (14.6 cm) in seeds treated with *K. pneumoniae*, followed by 14.3 cm with *E. cancerogenus* and 14.0 cm with *K. pneumoniae* + gibberellic acid (150 ppm). Seed mortality percentage was also reduced under these treatments compared to the control. It was 4% for seeds treated with *K. pneumoniae* and 8% for those treated with *E. cancerogenus*, whereas under control conditions, the mortality percentage was 50%.

Application of isolated PGPR and gibberellic acid on Chilgoza seeds in forest nursery

The results in Table 2 revealed that Chilgoza pine seeds exhibited improved germination when treated with isolated PGPR (*K. pneumoniae* and *E. cancerogenus*) as well as with gibberellic acid (150 ppm).

The treatments also resulted in significant increases in shoot length, root length, collar diameter, total biomass, and vigor index of seedlings after completion of one growing season compared to the control. The maximum germination percentage (87.5%) was recorded for seeds treated with *K. pneumoniae*, followed by 82.5% for those treated with *E. cancerogenus*, whereas the minimum germination percentage (35%) was observed in the control.

After one growing season, seedling height increased to 44.49 cm for seeds treated with *K. pneumoniae* and 38.56 cm for those treated with *E. cancerogenus*. The minimum increase in seedling height (23.28 cm) was observed for seeds treated with gibberellic acid (150 ppm) alone. Collar diameter increased to 4.88 mm and 4.55 mm with *K. pneumoniae* and *E. cancerogenus*, respectively. Similarly, root length increased to 32.58 cm and 31.00 cm with the respective treatments.

The maximum total seedling biomass (5.17 g and 4.44 g) was also recorded for *K. pneumoniae* and *E. cancerogenus*, respectively. Vigor index (VI) was highest for seeds treated with *K. pneumoniae* (452.73) followed by *E. cancerogenus* (366.47) after completion of one growing season.

Table 1. Germination parameters of Chilgoza pine seeds under laboratory conditions.

Treatment	GP	GE	MG	GI	GV	mHL (cm)	mRL (cm)	SL (cm)	M %
Control	50±1.01	45±2.28	0.21±2.11	1.19±4.01	0.22±3.04	3.88±1.12	4.5±4.04	8.38±2.26	50±1.1
T1 (GA=150ppm)	74±3.12	66±4.06	1.86±1.17	2.43±4.03	1.82±2.11	4.77±1.19	5.7±4.00	10.47±3.0	26±7.1
T2 (*K. pneumoniae*)	96±1.16	88±2.03	4.63±0.04	4.86±5.50	4.22±2.12	6.8±2.16	7.8±4.03	14.6±2.11	4±3.03
T3 (*E. cancerogenus*)	92±1.01	84±2.15	4.32±3.77	4.62±6.16	4.08±2.31	6.7±4.01	7.6±5.51	14.3±6.1	8±2.12
T4 (*K. pneumoniae* + GA150 ppm)	90±2.02	83±1.12	3.88±0.04	4.32±4.24	3.96±2.11	6.5±1.16	7.5±3.13	14±4.18	10±1.1
T5 *(E. cancerogenus* +GA150 ppm)	82±1.14	74±3.11	2.92±2.26	3.81±1.19	3.38±5.11	5±1.17	7.4±1.18	12.4±6.68	18±2.2
T6 (*E. cancerogenus* + *K. pneumonia*	88±1.19	76±3.07	2.86±4.44	3.89±6.61	3.87±3.38	5.5±4.04	6.8±1.18	12.3±9.10	12±5.1

Table 2. Germination percentage and seedling growth of Chilgoza pine in the nursery after one growing season.

Treatments	GP	Seedling Height	Collar Diameter	Root Length	Total Biomass	VI
Control	35 ± 0.04	17.25±1.12	3.38±2.04	15.65±2.12	0.109±4.04	3.815±0.06
T1 (GA = 150 ppm)	65 ± 3.13	23.28±1.12	3.65±0.01	26.36±3.03	2.705±1.14	175.825±13.04
T2 (*K. pneumoniae*)	87.5 ± 5.14	44.49±2.46	4.84±1.13	32.58±4.46	5.174±3.32	452.725±8.24
T3 (*E. cancerogenus*)	82.5 ± 4.46	38.56±5.25	4.55±1.12	31±2.24	4.442±2.01	366.465±6.48
T4 (*K. pneumoniae* + GA150 ppm)	77.5 ± 2.26	28.15±4.44	3.89±3.13	29.37±8.15	3.853±2.21	298.608±10.11
T5 (*E. cancerogenus* + GA150ppm)	80 ± 4.00	29.55±3.66	4.52±1.12	30.17±7.11	4.263±2.46	341.04±12.24
T6 (*E. cancerogenus* + *K. pneumonia*	75 ± 6.24	27.64±1.14	3.57±2.01	28.65±7.01	3.374±1.16	253.05±13.33

Discussion

Our study confirmed that pre-sowing treatments, such as stratification (cold and hot water treatments) at temperatures ranging from -4°C to 10°C for 2-3 months, were effective in breaking dormancy, consistent with Malik's findings who reported that Chilgoza pine seeds exhibit both physiological and morphological dormancy Malik (2007). Similar results were reported by Kumar (2015) and Malik et al. (2013), who found that stratification of Chilgoza seeds for 50 days enhanced germination. Singh et al. (1973 also observed improved germination in spruce seeds after 60 days of stratification compared to non-stratified seeds. In our study, mechanical scarification was also effective in breaking seed dormancy and improving germinability. These findings are supported by Tadros et al. (2011), who reported that scarification and hot water soaking effectively broke seed dormancy and improved germination in *Leucaena leucocephala* and *Acacia farnesiana*. Similarly, Gilani et al. (2019) found that hot water treatment enhanced germination in *A. nilotica* and *Faidherbia albida*. This study also revealed that treating seeds with 150 ppm gibberellic acid (GA_3) significantly increased germination percentage, germination energy, germination index, mean daily germination, germination value, root length, and hypocotyl length compared to untreated control seeds. These findings are consistent with Mugloo et al. (2017), who reported that soaking spruce seeds in 200 ppm GA^3 for 48 h improved germination and seedling growth. Mukhametshin et al. (2020) similarly found that pre-sowing treatments with gibberellin and biohumus improved seed germination and seedling growth in Scots pine and common spruce. In the present study, 150 ppm GA_3 resulted in the best germination of Chilgoza seeds.

Our results are further supported by Kumar et al. (2014) and Sharma et al. (2020), who also reported that GA_3 treatment enhances seed germination and seedling growth in Chilgoza pine. While our study used 150 ppm GA_3, previous studies demonstrated positive effects even at 75 ppm, suggesting that the optimal concentration may vary depending on conditions.PGPR enhance plant growth through mechanisms such as phosphate solubilization, siderophore production, phytohormone production, nitrogen fixation, and biocontrol activity (Maougal et al., 2021).

Similarly, Cardoso et al. (2011) reported that PGPR promote coniferous tree growth, improve seedling health, and aid soil restoration. In the present study, two PGPR strains isolated from rhizospheric soil and seedling roots significantly improved seed germination and seedling growth under both laboratory and nursery conditions. These findings are supported by Ribeiro (2012) and Barriuso et al. (2008), who reported that *E. aerogenes* R43, a PGPR isolated from rhizospheric soil, significantly promoted growth and biocontrol in *Araucaria angustifolia* and improved mycorrhization in *Pinus pinea*. Similarly, García et al. (2004) found that four PGPR strains enhanced the growth of *Quercus ilex* and *P. pinea* seedlings by increasing stem length, neck diameter, and shoot dry weight.

Our study also showed that PGPR treatment improved seed emergence, biomass, and root-shoot length in Chilgoza pine seedlings in the nursery. These findings are supported by studies on loblolly pine and slash pine, where PGPR inoculation enhanced seed emergence. However, Enebak et al. (1998) reported contrasting results, observing reduced biomass and root-shoot growth in loblolly and slash pine seedlings treated with different bacterial strains. Similarly, Reis et al. (2021) reported that bacterial isolates induced root modifications in *Arabidopsis thaliana*, such as suppression of primary root growth, stimulation of lateral roots, and formation of root hairs. Isolates such as *Bacillus megaterium* supported plant development, while *Serratia quinivorans* and *B. cereus* showed biocontrol potential.

Zul et al. (2022) found that a PGPR consortium increased *Eucalyptus pellita* seedling growth by 46.92% in height and 22.88% in stem diameter when combined with fungicide. The consortium also produced IAA, siderophores, and solubilized phosphate, while inhibiting pathogens. These results further support our findings on the positive role of PGPR inoculation in enhancing Chilgoza pine growth. Kotiyal and Sharma (2024) also reported that PGPR improve plant growth, stress tolerance, and sustainability in tree species such as *Quercus suber*, *Haloxylon ammodendron*, and *A. gerrardii*. For example, *B. subtilis* inoculation enhanced salinity and drought tolerance in *A. gerrardii*. Although studies on PGPR effects in pine species, particularly Chilgoza, are limited, research by Heredia-Acuña et al. (2019) showed that ten bacterial strains isolated from *P. pseudostrobus* promoted plant growth through auxin production and phosphate solubilization, leading to significant growth enhancement. Similarly, Flores-Núñez et al. (2018) found differences in PGPR diversity and soil properties between pine forest and agricultural soils, highlighting correlations between bacterial distribution and soil physicochemical characteristics.

Regarding biosafety, most studies have shown that the PGPR strains isolated in this study are generally safe for agricultural use. However, Demir et al. (2014) reported a rare case of community-acquired pneumonia caused by *E. cancerogenus* in a patient with bronchial asthma, indicating its potential opportunistic pathogenicity. Hart et al. (2006) reported that *K. pneumoniae* infections are mostly opportunistic and nosocomial, with specific subspecies such as *K. pneumoniae* subsp. *rhinoscleromatis* causing chronic rhinoscleroma. Nonetheless, recent research by Yankey et al. (2022) demonstrated that strains of *E. cloacae*, *K. pneumoniae*, and *Klebsiella* sp. exhibited plant growth-promoting traits and cadmium tolerance while remaining safe in mouse biosafety studies, indicating their potential as biofertilizers for sustainable agriculture.

Conclusion

The results of this study demonstrated that seed germination in *Pinus gerardiana* depends on pre-sowing treatments. Germination was significantly enhanced when seeds were treated with PGPRs (*Enterobacter cancerogenus* and *Klebsiella pneumoniae*), with positive effects observed on germination percentage, germination energy, mean daily germination, germination index, germination value, root length, and hypocotyl length under both laboratory and nursery conditions. Seedling growth, root and shoot length, and biomass were also enhanced when seeds were treated with PGPRs, either alone or in combination with gibberellic acid.

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Authors' Contributions

Shazia conceived and designed the research idea, conducted the experiments, and prepared the original draft of the manuscript; LA provided overall supervision, guided the research process, and critically reviewed the draft; RMN supervised the statistical analyses and assisted in the interpretation of results; NI contributed to proofreading and improving the clarity of the manuscript; MA and SG served as members of the supervisory committee, providing academic guidance and constructive feedback, in addition to assisting with proofreading.

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Conflict of Interest

The authors declare no conflict of interest.

Sustainable Development Goals Targeted

SDG 12: Responsible Consumption and Production

SDG 13: Climate Action

SDG 15: Life on Land

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