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STRENGTHENING *PISUM SATIVUM* L. GROWTH THROUGH TRIPARTITE SYMBIOSIS WITH RHIZOBIA AND ARBUSCULAR MYCORRHIZAL FUNGI: A SUSTAINABLE BIO-FERTILIZER APPROACH

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Abstract

Growing reliance on chemical fertilizers has reduced soil fertility and damaged the environment, necessitating the development of sustainable options to accelerate agricultural expansion. Arbuscular Mycorrhizal Fungi and Plant Development-Promoting Rhizobacteria (PGPR) provide environmentally friendly solutions by enhancing nutrient uptake and encouraging organic plant development. The purpose of this study is to isolate and describe Rhizobium strains and Arbuscular Mycorrhizal Fungi (AMF) from pea plants in the Charsadda District in order to develop biofertilizers that work effectively for crops and soil development. Using the Yeast-Mannitol Agar Medium (YMA), eight rhizobium strains were isolated and identified from root nodules of *Pisum sativum* L in ten different areas of Charsadda District. Rhizobium strains actively produced an indole-3-acetic acid (IAA) ranging from 09.48 μgml^{-1} to 18.84 μgml^{-1}. The qualitative evaluation solubilization of phosphate was determined by the diameter of zones of halo around the colonies on agar medium of Pikovskaya, measuring between 03.30 mm and 08.50 mm. Zinc solubilization was quantified by the diameter zones of halo surrounding the colonies on agar media, measuring between 00.46mm and 04.36mm. All isolates exhibited negative findings in the urease test conducted on Christensen's Urea Agar medium. AMF spore density and colonization of the root also gave positive results on the ten samples of the survey from the study area. The *Sclerocystis* species have a maximum concentration of 12.66 gm^{-100} of other AMF spores, which exhibited 90% of root colonization. Furthermore, all inoculants (PGPR and AMF) had a favorable influence on pea development and growth, as well as a substantial rise in root length of the shoot and dry and fresh pea weight. This was shown by the tripartite symbiosis of the plant *Pisum sativum* L. with Rhizobia and AMF as a sustainable biofertilizer strategy. A future study should concentrate on optimizing the use of Rhizobium and AMF as biological fertilizers among diverse crops and environmental circumstances to augment their efficacy. Additionally, it is essential to investigate the long-term impacts of these natural fertilizers on soil health and ecosystem sustainability.

Key words: *Pisum sativum* L., Root nodules, Rhizobium, AMF, Phosphate solubilization, and sustainable agriculture.

Introduction

The *Pisum sativum* L. (Pea) originated in Ethiopia and Afghanistan before spreading to the Mediterranean area. Subsequently, the pea was disseminated to other regions, including Europe and Asia (Cousin, 1997). The pea is a perennial herbaceous plant belonging to the legume family, classified as three varieties: dry beans, the green variety, and snow peas (Thakur *et al.*, 2018). West Asians have long cultivated it in cold, rainy climatic zones, including the Mediterranean region, Ethiopia, portions of southwestern Asia, and the South Caucasus, because of its resistance to winter, drought, and aridity (Sahi *et al.*, 2018). Multiple psychological, morphological, biochemical processes, and molecular changes are produced by salinity and drought (Shahid *et al*., 2024; Ahmad *et al*., 2024), which alters the metabolism of plants' cells (Gupta & Huang, 2014). Pulses are a notable source of nutrients (Noreen *et al*., 2023). Punjab produced 112267 tons of peas, covering an area of 17.644 square kilometers. Plant growth-promoting rhizobacteria (PGPR) have become significant in agriculture, as they can enhance plant growth directly or indirectly, thereby improving overall growth efficiency (Jha *et al.*, 2012). Approximately 90% of plant species, particularly plants with flowers, bryophytes, and ferns, are capable of forming interdependent relationships with arbuscular mycorrhizal fungi (AMF) (Zhu *et al.*, 2010). Root vesicles, arbuscular, and hyphae, as well as rhizosphere spores and hyphae, contain AMF (Wang *et al.*, 2022). The formation of the AMF hyphal network in conjunction with the root system significantly increases root access to an extensive soil area, thereby promoting plant growth. AMF enhances soil nutrient content by increasing the availability and immobilization of various nutrients (Kamran *et al*., 2025). AMF can enhance the availability of inorganic nutrients, particularly phosphate, in nearly all plant species (Nell *et al.*, 2010). Many people use AMF and PGPR to boost seed yield and boost crop nutrient efficiency, while biofertilizers can enhance other methods of fertilization or plant nutrition, reduce the usage of chemical fertilizers, enhance nutrient absorption, and preserve organic matter in the soil (Yaseen *et al*., 2013; Yaseen *et al*., 2016a; Musa *et al*., 2023; Shahid *et al*., 2023). The Leguminosae family categorizes *Pisum sativum L*., an annual herb, into dry peas, green peas, and snow peas. Western Asia, the Mediterranean region, and southwest Asia have developed it, with cold and wet climate zones historically producing it.

This research aims to isolate plant growth-promoting bacteria from the pea root nodules and arbuscular mycorrhizal fungi from the rhizospheric soil. We will screen the isolate for its capacity to produce indole acetic acid, hydrogen cyanide, catalase test, urease test, zine test, nitrogen-fixing ability, and phosphate solubilization, before storing the subculture at 4°C. We reserved purified stocks of the isolates on 50% glycerol and kept them at -80°C. Ultimately, we will examine their effectiveness in stimulating plant growth and AMF spores.

Materials and Methods

Soil sample collection: In the Charsadda District, samples of soils were collected from bean farms in a number of locations, including sample-01 (Rajjar), sample-02 (Utmanzai), sample-03 (Turang Zai), sample-04 (Umer Zai), sample-05 (Tangi), sample-06 (Sherpho), sample-07 (Ziam), sample-08 (Dakki), sample-09 (Behlola), and sample-10 (Mandani). After digging the root structure to a depth of fifteen centimeters, samples of rhizospheric soil were gathered in bags of plastic, labeled, and kept for four days at 4°C in preparation for further investigation.

Isolation of Rhizobacteria: The root of pea plants was cleaned by using sterilized water, and the pea root nodules were collected and utilized to develop the Rhizobium strain in growth medium. The sterilized nodules were removed for five-minutes using 00.10% (w/v) sodium hypochlorite and soaked them for ten-seconds in a 95% (v/v) solution of Ethanol. Now, solution the removed nodules six times with autoclaved distal water before curding them, allowing a milky fluid to emerge and streak on YMA medium comprising 00.0025% red Congo (w/v). After the initial culture was streaked on YMA ager, it was poured onto Petri plates and solidified, resulting in single Rhizobium colonies. Three days of incubation at 30°C produced a single colony, which we polished with YMA (Schütz *et al.*, 2018).

Morphological, phenotypic characterization and Gram staining analysis: An inoculum of 10^8 mL^-1 cells was generated after third day in YMA by an early pH of 06.80. Following the streaking of a loop of the initial inoculum on YMA, the bacteria were incubated in the dark at 30°C for 3, 5, and 7 days. The colony morphology was then analyzed, focusing on color, mucoid characteristics, clarity, and boundaries (Sinclair & Eaglesham, 1984). Gram staining on pure cultures of bacterial strains was conducted in a laminar airflow hood for more accurate colony identification. Colonies were marked on slides using an inoculating needle, followed by heat-fixing through the Gram method after washing with ethanol (Arora & Bala, 2020; Bajpai *et al*., 2017).

Indole-3-acetic acid (IAA): The cultured rhizobium strains in yeast mannitol broth YMB (Asghar *et al.*, 2002), and then shortly evaluate their IAA output. After 72 hours, we associated the microplates with the cultures of bacteria to respond with 40.00µL of Salkowski's reagent (02.00mL of 00.50mol-L^{-1} $FeCl_3$+45.00mL of 35% $HClO_4$). The mixture was unloaded at a normal temperature for thirty minutes overnight, resulting in red coloration indicative of health, and the standard curve was applied (Brick *et al.*, 1995).

Production of hydrogen cyanide (HCN): We investigated each of the seven strains for HCN (hydrogen cyanide) production utilizing the method described by Lorck (1948), cultivating each strain of bacteria on the modified agar pad and adjusting the nutritional content of the medium to 4.4 g of lysine L-1. The filter papers (Whatman) were immersed in a 00.50% picric acid mixture and a 00.20% solution of sodium carbonate prior to placement in Petri plates and then coated with para-film and allowed for development at 36.2°C for a period of four days (Bashan & Holguin, 1997).

Phosphate solubilization and solubilization index: Rhizospheric soil may contain significant total phosphorus, yet only a minor portion is typically readily available to plants (Stevenson & Cole, 1999), utilizing Pikovskaya's media. We inoculated isolates on sides by 200.00µl of fresh t inoculation and incubating them at 28.00°C with shaking at 120.00rpm for three to seven days. Phosphorus solubilization is indicated by the formation of a clear zone related to the colonies of bacteria (Kokalis-Burelle *et al*., 2006). Pikovskaya agar plates were incubated with newly grown cultures at pH 7.2. The plates were incubated for seven days to promote interaction, followed by the application of an equation for calculating S.I. (Edi-Premono *et al*., 1996):

S.I = Colony diameter + Halo zone diameter

Nitrogen and zinc test: Maintaining the dehydrated media in a tightly sealed container below 30°C and the prepared medium between 2°C and 8°C is recommended. Prevent overheating and freezing conditions. Utilize prior to the expiration date indicated on the label. To prevent hydration, ensure the powder medium remains closed after opening (Okon *et al*., 1977). To assess the efficiency of bacteria in dissolving zinc, they were evaluated on a Tris-minimal agar culture medium enriched by glucose and various in-soluble Zn complexes. The newly cultivated cultures of bacteria were injected in triplicate into the medium using sterile toothpicks, according to sterilization and plating protocols. For seven days at 28°C, the marked plate was placed in the dark to look for any observable halo development around the colonies. The diameters of the colonies and the halos surrounding them were measured after a period of 7 days. Calculated the zinc solubility efficiency (SE) using the formula proposed by Nguyen *et al*., (1992):

S.I = Colony diameter + Halo zone diameter

Catalase test: We examined the bacteria isolated for the catalase existence, an enzyme that lets strains of Rhizobium to convert peroxide of hydrogen into oxygen and water. We separately determined each loop of the isolates being studied on a clean, sterilized glass slide, following the application of several droplets of H_2O_2. The formation of bubbles of gas and effervescence signified a positive test (Javed & Bano, 2008).

Determination of urease: Combine the first six parts in 100.00mL of distilled water in order to produce the urea base. Stir well and sterilize by means of a 0.45-mm pore size filter. Dissolve the agar well in 900 milliliters of

purified water and sterilize for fifteen minutes at 121 degrees Celsius and 15 pressure. Permit the agar mixture to cool to a temperature range of 50-55°C. In the chilled agar solution, add one hundred milliliters of filter-sterilized urea base and stir gently. Dispense 4-5 mL into each sterilized tube (13 x 100 mm) until solidified. Consider using a long slant and a short butt as alternatives (AL-Joda & Jasim, 2021).

Roots are collected and stored: The plant root was cut, gathered, and stored at laboratory of Bacha Khan University Charsadda (BKUC), to stimulate and maintained the KOH solution at room temperature by combining 10g of potassium hydroxide in 90mL of distilled water with 0.025g of acid fuchsine dissolved in 220mL of lactic acid. The mixture was combined with 10 mL of water that was filtered and sixteen mL of glycerin to produce an acidic fuchsine stain. The staining solution had been effectively designed and executed through the application of two staining procedures. We slightly modified Phillips & Hayman (1970) staining approach for unpigmented roots to stain the fungal structure. The process involves the isolation and extraction of AMF spores, followed by root colonization by using a rapid clearing and staining procedure for root colonization. We calculated the infection rate of root colonization using the following formula:

$$\text{Colonization of root (\%)} = 100$$

If the root segment had any of the three elements—hyphae, arbuscules, or vesicles—it was considered mycorrhizal (Giovannetti & Mosse, 1980). The spore density was calculated by using Stahl and Christensen's 1982 technique. This technique filters and retains the soil's coarse particles, whereas 10g of soil combine with AMF spores and organic particles using sieves of varying diameters. Following a vigorous shake to discharge the soil mixture containing 100 milliliters of water, the 500 milliliter conical flask is used to decant the supernatant using standard sieves after allowing the soils-derived AMF spores to settle for fifteen to forty-five minutes. Spores were collected using a needle system and a dissecting microscope; spore density can be defined as an average amount of spores per one milligram of soil. Throughout numerous enlargements, the spores persisted in the image (4x10as). We characterized them using the latter method (Hall & Fish, 1979).

Soil analysis: A 2 mm sieve separates stones, plant remnants, and small beings (earthworms, etc.). We then carefully clean, grind, and mix the samples. The test was conducted at a temperature of 4°C. McLean *et al.,* (1982) determine the pH of the soil, and electrical conductivity using the methods of Rhoades & Booth (1982). Organic matter content and AB-DTPA extraction process for the calculation of macronutrients and micronutrients, including Nitrate-N, Available Phosphorus, Extractable Potassium, and Sodium, was determined following the procedure of Walkley (1947).

Pot experiments: The pot investigations were carried out at BKUC during the 2019–2021 session, while all investigations examining the effects of PGPR and the Rhizobium strains on pea seed germination utilized a control condition. This trial was conducted in the Randomized Complete Block Design method (RCBD), to grow bacterial isolates, sterilize pea seeds for 1 minute with H_2SO_4, wash them 2-3 times with double-distilled water, and then gently cover them with water. We utilize sterilization-treated water to safeguard the seeds from contamination (Amiri *et al*., 2017) and plant the seeds in pots that include 00.70kg (700 g) of sterilized soil within the development chamber for a duration of one month. After 6 days, the seeds germinated, and each pot received autoclaved distilled water every day. We collected the plants after a month and noted the following growth statistics (Amiri *et al*., 2017). The plant growth and root duration (cm) was measured using a scale after one month of planting. One month after planting, we measured the dry and fresh biomass of root and shoots in milligrams.

Statistical analysis

Computed variance (ANOVA) and the least significant distinction (LSD) using Statistics 8.1 (Statistical Analysis).

Results

Isolation of rhizobacteria: The strains of rhizobium were obtained by the use of yeast mannitol agar (YMA) media from the root nodule of pea plants. Following the incubation of samples from ten regions, eight strains are selected for every replicated sample based on their size, shape, and color. A total of eighty strains were then sent to perform additional refinement, as illustrated in Fig. 1.

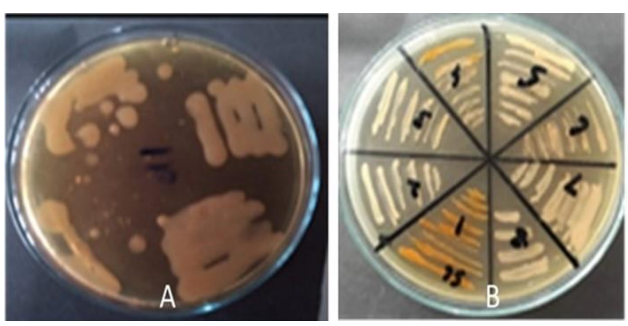

Fig. 1. Rhizobacteria's isolation from pea plants' root nodules.

Morphological, phenotypic, and gram-stained microscopic observations: Morphological and phenotypic Characterization of bacteria is shown by zooming loops full of the original inoculum on YMA media and the colony morphology (color, lucidity, transparency, boundaries, and shape). The Gram-staining findings from the bacterial isolates collected in various places indicate a continuous predominance of gram-positive bacteria, with minor changes in specific samples (Table 1). All site samples exhibited consistent Gram-positive responses across all tested colonies, suggesting a significant presence of Gram-positive bacteria in these areas. This consistency indicates that environmental or ecological variables in these regions may promote the proliferation of gram-positive bacteria. However, Sample 2 (Utmanzai), and Sample 6 (Sherpho) exhibited one gram-negative colony, indicating a mixed bacterial community, perhaps impacted by localized environmental differences and the development of gram-negative species.

Determination of IAA: The figure 2 shows the concentration of IAA across ten samples, comparing eight bacterial strains regarding IAA production, quantified in micrograms per milliliter (µg/ml). The data presents evidence regarding the variation of IAA production among bacterial strains and samples, with percentage values consistently ranging from 6% to 18%. Strains 1, 3, 5, and 6 exhibit higher IAA production in samples 4, 6, and 8 (Fig. 2). Strains 2 and 8 have reduced IAA concentration in most samples, indicating variability in their IAA synthesis capacities. Strain 5 in sample 6 exhibits a peak IAA concentration exceeding 18%, signifying its considerable auxin-producing capacity, which may have important implications for the enhancement of plant growth and development. These results reveal the competitive performance of different strains in IAA production, highlighting specific strains as intriguing possibilities for further investigation in bio-fertilization and crop growth enhancement strategies.

Hydrogen cyanide (HCN) production: The HCN production test results indicate that all strains (Strain-1 to Strain-8) from the sampled sites exhibited positive HCN production. All strains of 1-10 samples exhibited a positive response for HCN production across the various locations, indicating a consistent capacity among the strains to produce HCN in these environments (Table 2).

Phosphate solubilization of the isolate from the root nodule: The phosphate solubilization test, as depicted in the graph, assesses the efficacy of eight distinct strains (strain 1-8) across ten samples. The solubilization percentages, typically range from 0% to 9%. In Sample 1, the majority of strains demonstrate moderate solubilization, with percentages ranging from 4% to 7%. Sample 2 exhibits greater variation, with strains 3 and 5 achieving performance levels near 7%, whereas other strains show solubilization rates between 4% and 6%. In Sample 3, strains 4 and 6 demonstrate greater efficiency, achieving approximately 8%, while the remaining strains solubilize between 3% and 7%. In Sample 4, strains 4 and 5 exhibit marginally superior performance compared to the other strains, which all fall within the 3% to 7% range (Fig. 3).

The trend persists in Samples 5 to 10, with solubilization percentages ranging from 3% to 8%. Strain 6 in Sample 6 and strain 8 in Sample 9 exhibit elevated solubilization levels, consistently surpassing several other strains. The graph illustrates variability in phosphate solubilization efficiency among samples and strains, with strains 4 and 6 often exhibiting superior solubilization potential relative to others.

Nitrogen fixation test: Nitrogen fixation occurred in Jensen's medium, with the 80 strains selected from 10 samples exhibiting optimal growth (Table 3). Numerous studies have demonstrated the ability of various microorganisms to fix nitrogen.

Evaluation of *In vitro* zinc solubilization potential: The figure 4 presents the results of a zinc solubilization test quantified in centimeters across ten samples (sample1 to sample10), with eight strains (strain1 to strain8) evaluated for their zinc solubilization efficacy. Each bar indicates the solubilization percentage of a specific strain for a given sample. Sample8 exhibits the highest levels of zinc solubilization, with strain6 achieving approximately 4.5%, representing the maximum in the dataset. Other samples exhibit moderate solubilization rates, typically ranging from 1.5% to 3%, with strains displaying differing levels of activity among the samples. Strain performance varies significantly, with certain strains (e.g., strain2, strain6) demonstrating consistent efficacy across samples, whereas others (e.g., strain3 and strain5) exhibit lower or more variable performance.

Table 1. Gram-stained microscopic analysis of different sites of Charsadda District.

Sample No.	Site	S-1	S-2	S-3	S-4	S-5	S-6	S-7	S-8
S-1	Rajjar	+	+	+	+	+	+	+	+
S-2	Utmanzai	+	+	+	−	+	+	+	+
S-3	Turang Zai	+	+	+	+	+	+	+	+
S-4	Umer Zai	+	+	+	+	+	+	+	+
S-5	Tangai	+	+	+	+	+	+	+	+
S-6	Sherpho	+	−	+	+	+	+	+	+
S-7	Zaim	+	+	+	+	+	+	+	+
S-8	Dakki	+	+	+	+	+	+	+	+
S-9	Behlola	+	+	+	+	+	+	+	+
S-10	Mandani	+	+	+	+	+	+	+	+

Table 2. Hydrogen cyanide (HCN) production.

Sample No.	Site	S-1	S-2	S-3	S-4	S-5	S-6	S-7	S-8
S-1	Rajjar	+	+	+	+	+	+	+	+
S-2	Utmanzai	+	+	+	+	+	+	+	+
S-3	Turang Zai	+	+	+	+	+	+	+	+
S-4	Umer Zai	+	+	+	+	+	+	+	+
S-5	Tangai	+	+	+	+	+	+	+	+
S-6	Sherpho	+	+	+	+	+	+	+	+
S-7	Zaim	+	+	+	+	+	+	+	+
S-8	Dakki	+	+	+	+	+	+	+	+
S-9	Behlola	+	+	+	+	+	+	+	+
S-10	Mandani	+	+	+	+	+	+	+	+

Table 3. Results of nitrogen fixation test various strains of microorganisms.

Sample No.	Site	S-1	S-2	S-3	S-4	S-5	S-6	S-7	S-8
S-1	Rajjar	+	+	+	+	+	+	+	+
S-2	Utmanzai	+	+	+	+	+	+	+	+
S-3	Turang Zai	+	+	+	+	+	+	+	+
S-4	Umer Zai	+	+	+	+	+	+	+	+
S-5	Tangai	+	+	+	+	+	+	+	+
S-6	Sherpho	+	+	+	+	+	+	+	+
S-7	Zaim	+	+	+	+	+	+	+	+
S-8	Dakki	+	+	+	+	+	+	+	+
S-9	Behlola	+	+	+	+	+	+	+	+
S-10	Mandani	+	+	+	+	+	+	+	+

Table 4. AMF root colonization of various district Charsadda.

S. No.	Site	EH%	IH%	A%	V%	AMF root colonization%
1.	Sample 1	10	15	20	30	75
2.	Sample 2	0	10	30	20	60
3.	Sample 3	15	20	10	30	75
4.	Sample 4	20	0	15	20	55
5.	Sample 5	10	15	30	40	75
6.	Sample 6	20	10	0	10	40
7.	Sample 7	30	20	0	40	90
8.	Sample 8	20	5	10	20	55
9.	Sample9	5	20	10	30	65
10.	Sample10	10	10	20	40	80

Key: EH (External hyphae), V (Vesicles), A (Arbuscules), IH (Internal hyphae)

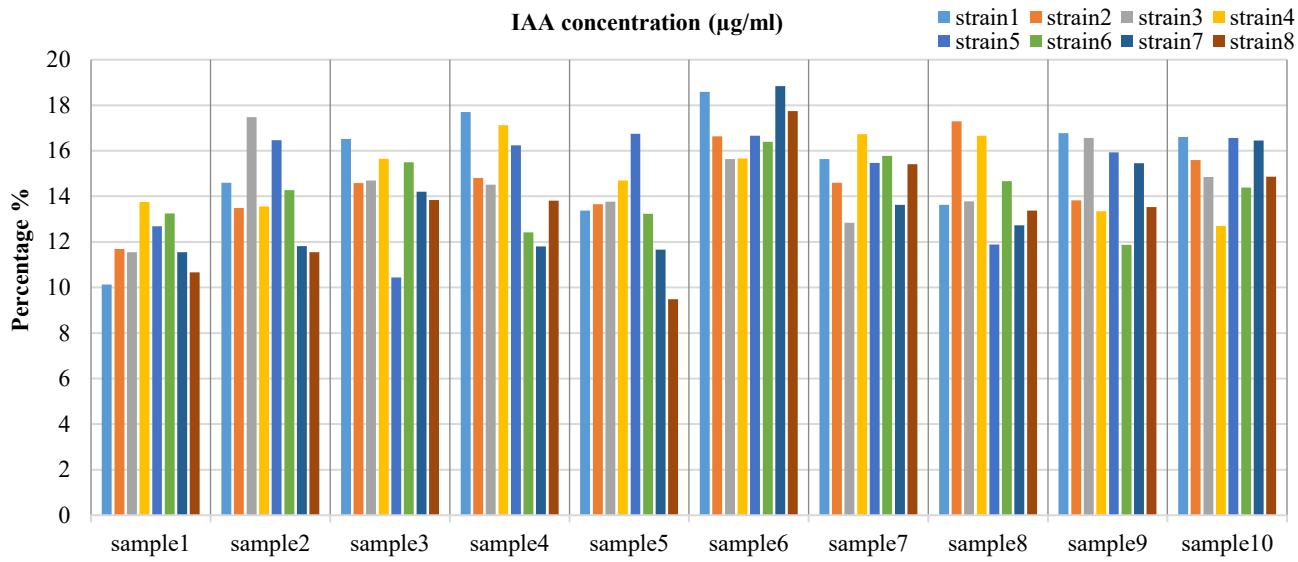

Fig. 2. The percentage of IAA concentration varies across different samples.

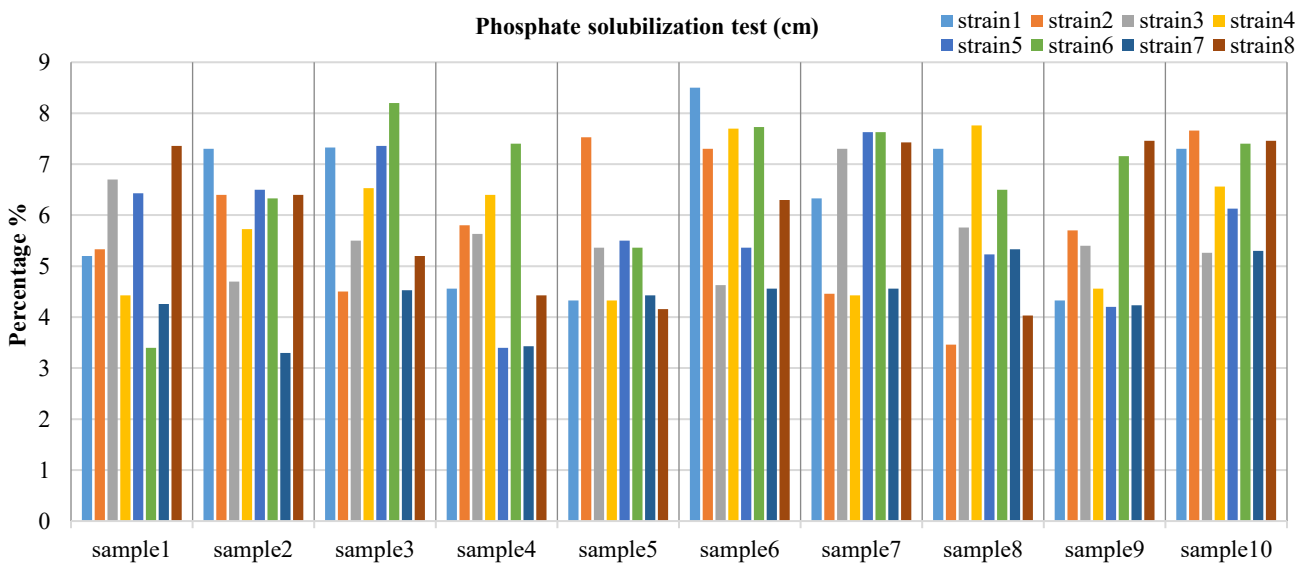

Fig. 3. Solubilization of strain 1-8 on different mean values.

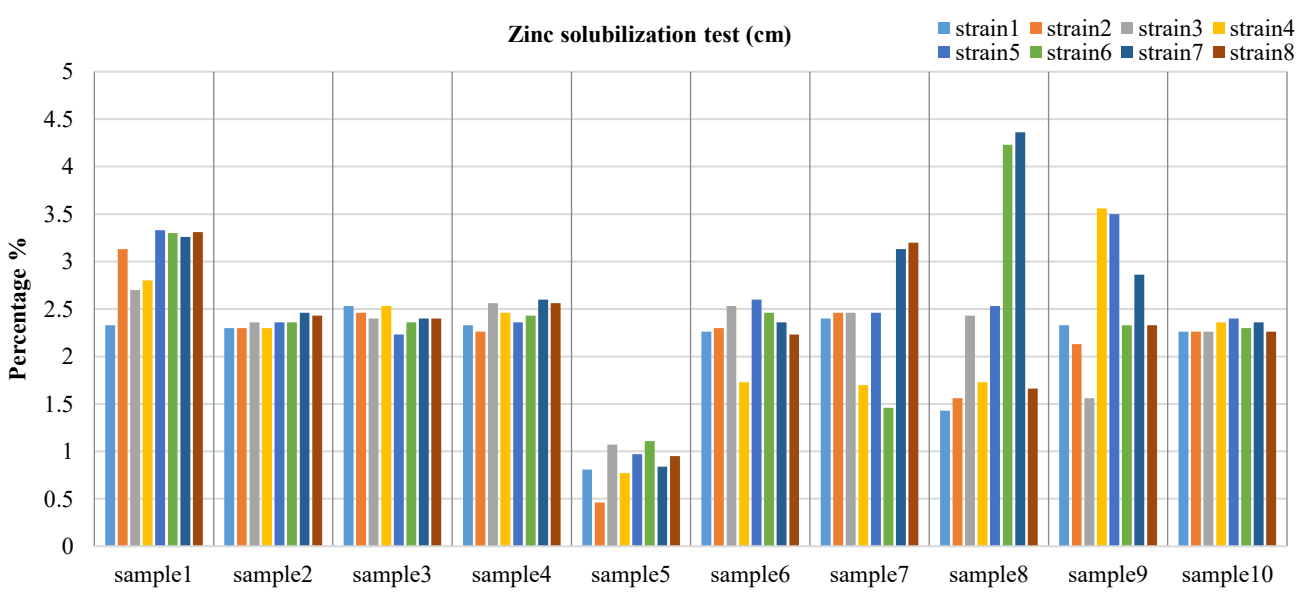

Fig. 4. Result of Zinc solubilization of ten samples of Charsadda district.

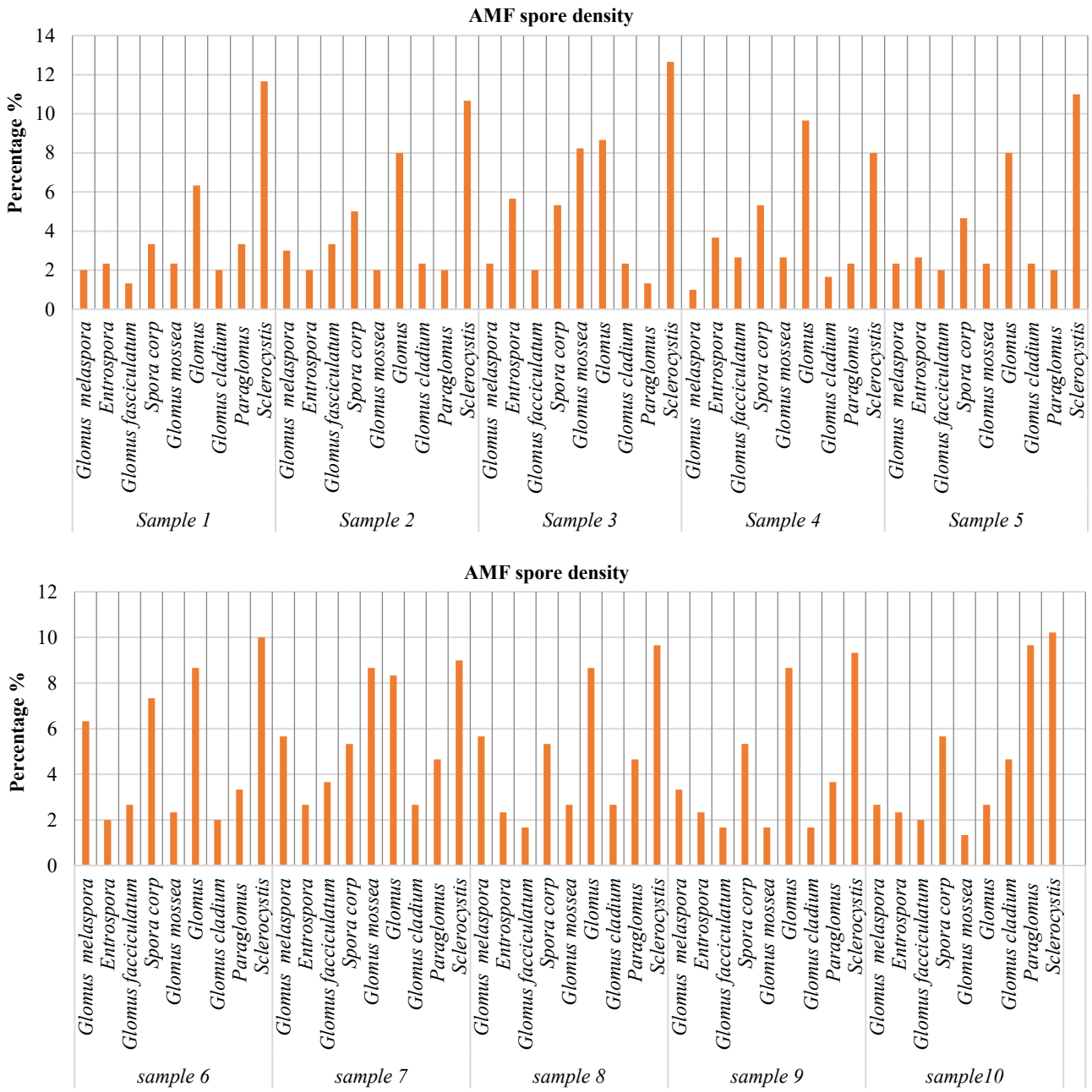

Fig. 5. Isolation and identification of AMF spore from pea plant of Charsadda District.

Catalase and urease test: There were bubbles indicating positive results in each of the 10 isolate and strain samples totaling eighty. The eighty strains yielded favorable outcomes.

All 10 samples yielded a negative result in the biochemical urease test (Christensen's Urea Agar). Despite optimal inoculum growth across all tubes, the enzyme responsible for hydrolyzing urea into the gases carbon dioxide and ammonia did not exist in eighty strains.

Density of mycorrhizal spores: The investigation focused on the quantity of mycorrhizal spores present in ten samples of pea plants under the influence of arbuscular mycorrhizal fungi (AMF). Species such as *G. melanosporum*, *G. fasciculatum*, *G. mosseae*, *Sclerocystis* spp., and *Entrospora* spp., are represented, with each sample showing different levels of spore density (Fig. 5). In Sample 1, the spore density is evenly distributed across species, while Sample 2 highlights peaks in *G. melanosporum* and *Entrospora* spp., indicating their higher prevalence. Sample 3 shows a more balanced distribution, though *G. fasciculatum* dominates slightly. Sample 4 has pronounced peaks in *Entrospora* spp. and *Sporocarp*, and Sample 5 shows an increase in *Sclerocystis* spp. The overall trends suggest that *Entrospora* spp. and *Sclerocystis* spp. have the highest spore densities, particularly in Samples 2 and 5. Other species like *G. fasciculatum* and *G. mosseae* maintain moderate densities across the samples.

Collection and conservation of roots: Sample seven exhibited the highest severity of infection, approximately 90%. External infection was identified in 30% of the root segments examined. Straight, thin-walled hyphae and thickly walled, slightly strained hyphae were observed (Table 4). 20% of cases involving internal infection

(hyphae) were documented. Internal hyphae exhibited thin walls, branching structures, and slight elongation. The majority were heterosexual. Sample seven exhibited no sign of arbuscular infection. Vesicular infection was identified in seven root samples (40%).

Pot experiment

Root/shoot length (cm): The plants' root and shoot lengths were assessed one month after planting. All bacterial strains and AMF considerably enhanced root and shoot length compared with control (un-inoculated) plants (Fig. 6). The mean root length was recorded at 29 cm for plants infested with the Rhizobium and bacterial strains, while the mean shoot length was noted at 35.66 cm. The average shoot length in the AMF the inoculum range is 28.66 cm, while the mean root length is 22.66 cm. The shortest length of the shoot in the untreated (un-inoculated) the group was 12 cm, and the mean root length has 0.6 cm (Fig. 6). The control group was greatly outperformed by all duplicates infected with bacterial strains and AMF inoculum.

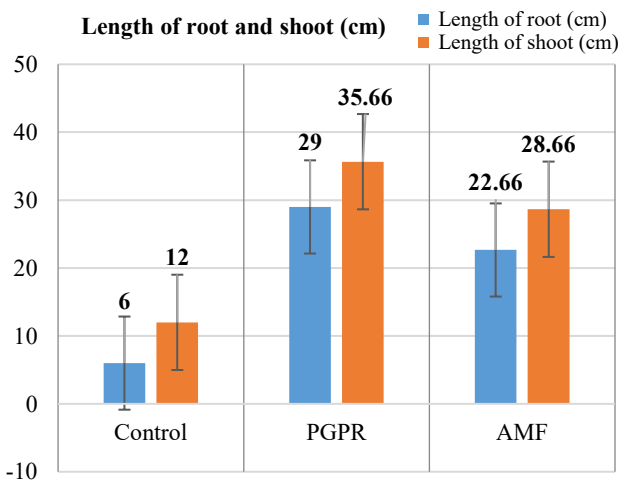

Fig. 6. The effect of PGPR and AMF on the length of the root and shoot (cm).

Discussion

This research examined Rhizobium strains obtained from the root nodules of pea plants in the Charsadda district. The nodules underwent sterilization through a standardized procedure, which involved immersion in 0.1% sodium hypochlorite for five minutes, a subsequent brief treatment with 95% ethanol, and multiple rinses with distilled water. Subsequently, the nodules were crushed, and the extracts were inoculated onto yeast mannitol agar (YMA) medium enriched with 0.0025% Congo red for the purpose of bacterial isolation. Colonial development was assessed following a 24-hour incubation period. The procedures aligned with those documented by Afzal & Bano (2008). The initial inoculum, comprising approximately 10^8 cells/mL, was cultured in YMA at pH 6.8 subsequent to isolation. The study of colony morphology, including color, transparency, boundary definition, and shape, involved streaking the novel inoculum on YMA medium and incubating the bacteria in the dark for three, five and seven days, respectively. The observations are consistent with the findings of Arora & Bala (2020). Gram staining confirmed the presence of Gram-negative bacteria in the majority of isolates, with the exclusion of samples second, which exhibited pinkish, bacteria of rod-shaped, and sample 6th, which also tested as Gram-negative, displaying a pinkish tint and rod-shaped morphology, which are align with the findings of Gebremedhin (2019). Indole-3-acetic acid (IAA), an essential hormone in plant development, was identified in all eighty Rhizobium strains from ten samples. The highest concentration of IAA was observed in sample six (strain six), measuring 18.84 g/mL, while the lowest concentration was recorded in sample five (strain eight), at 9.48 g/mL. Spectrophotometric absorbance at 530 nm was used to quantify the generation of IAA. The IAA secretion was quantified using a reference curve, confirming consistency with prior research that shows plant growth-promoting bacteria (PGPR) produce IAA (Asghar *et al.*, 2002; Asad *et al.*, 2021; Dilawar *et al.*, 2021). All eighty Rhizobium strains produced hydrogen cyanide (HCN), a secondary metabolite with potential for biocontrol. This is consistent with earlier research indicating that HCN production occurs frequently among various bacterial taxa, such as Rhizobium (Ahmad *et al.*, 2008; Paray *et al.*, 2018). Phosphate solubilization was observed in all isolates, exhibiting varying degrees of clearing zones surrounding the colonies. The solubilization index (SI) was determined by the ratio of the clearing zone to the diameter of the colony. Nitrogen fixation, essential for plant growth, was evaluated using Jensen's medium. All isolates exhibited nitrogen-fixing capabilities, producing a white to cream-colored gel after four days of incubation at 30–35°C, in accordance with prior studies (Upadhyay *et al.*, 2009. Zinc solubilization was evaluated, and all isolates demonstrated significant zone formation around colonies after 9 to 11 days. The solubilization index was determined in a manner analogous to phosphate solubilization, yielding results that align with those reported by Rana *et al.*, (2012). Catalase activity, as evidenced by the decomposition of H_2O_2 and subsequent oxygen generation, was observed in all isolates. The results are consistent with the studies conducted by Datta *et al.*, (2015). Urease activity was measured, with pH adjustments implemented to optimize enzyme activity and distinguish between positive and negative outcomes. All ten pea plant root samples exhibited infection rates of arbuscular mycorrhizal fungi (AMF). *Sclerocystis*, a significant genus, was observed in high quantities, supporting earlier research by Yaseen *et al.*, (2016), Naz *et al.*, (2019), indicating that seasonal variations affect spore densities and the diversity of mycorrhizal species.

Conclusion

The isolation and characterisation of rhizobium and AMF strains from pea plant root nodules in different locations within Charsadda District demonstrated notable differences in their morphological, phenotypic, and biochemical characteristics. Predominantly gram-positive bacteria were identified, with a minor occurrence of gram-negative strains in select samples, suggesting a diverse bacterial population influenced by specific environmental factors. The research revealed significant production of IAA, HCN, phosphate solubilization, nitrogen fixation, and

zinc solubilization among the bacterial isolates, underscoring their potential as plant growth-promoting rhizobacteria (PGPR). Furthermore, AMF spore density differed across species, with *Entrospora* spp. and *Sclerocystis* spp. demonstrating the greatest concentrations. Pot studies validated the advantageous effects of both Rhizobium and AMF on pea growth, markedly increasing root and shoot lengths relative to control plants. The results indicate that the isolated strains are promising for bio-fertilization and sustainable agriculture, enhancing plant growth and soil fertility.

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