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ARTICLE

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EXPLORING THE ROLE OF RICE-DERIVED ENDOPHYTES IN CHICKPEA GROWTH PROMOTION

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Endophytic microorganisms, residing in plant tissues, play a vital role in enhancing nutrient absorption, stimulating growth-promoting substances, and fortifying disease resistance. This symbiotic relationship contributes to overall plant health, making these microorganisms valuable allies in sustainable agriculture practices which have garnered significant attention in recent years. This study focuses on isolating and evaluating the potential of endophytes to enhance plant growth in chickpea plants (Cicer arietinum L.). Different endophytes were isolated from stem and root samples of rice plant (Oryza sativa) to investigate their potential in enhancing plant growth. Endophytes obtained from rice plants were introduced to seven to eight-day-old chickpea seedlings cultivated in autoclaved soil for monitoring their growth and condition. The inclusion of isolated microorganisms from rice plants shows a positive impact on the growth and health of the seedlings. Morphological and biochemical characterization of the isolated microbes was done to understand microbial characteristics. In this context the strains R2 and 6F found to be more efficient among the 5 isolated strains. This comprehensive approach provides valuable insights into the potential effects of harnessing endophytes as potential biofertilizers for sustainable agricultural practices and maintaining eco-sustainability.

Introduction

Endophytes are microorganisms that reside within plant tissues without causing harm. They are broadly classified into fungal and bacterial types and further distinguished by their obligate or facultative relationships with host plants[1]. While traditionally categorized based on function[2], propose a broader definition that includes commensals, latent pathogens, saprotrophs, and mutualists. A well-known example is the rhizobia-legume symbiosis, which evolved approximately 60 million years ago and is critical for nitrogen fixation. Endophytes contribute significantly to plant health by enhancing stress tolerance, promoting growth, and improving yields[3]. Though endophytes are thought to have originated from rhizosphere or seed-borne microbes, genome studies indicate that they possess versatile traits beneficial to their host plants[4]. These microbes are capable of producing metabolites that promote plant growth and resilience, confirming their significant role in maintaining plant health[5]. Endophytes play a vital role in the biological control of pathogens, plant growth promotion, and the production of valuable compounds. They play a crucial role in plant health, influencing growth, ecosystem function, and resilience to stress. Primarily they are found in roots while their presence decreases from stem to leaves, with diverse species coexisting in a single plant[6]. To sustain symbiotic relationships endophytes produce compounds that help promote plant growth and environmental adaptation.

India's agriculture is more than just a crop-growing industry; it is integral to its economy, culture, and social structure. Agriculture accounts for 17% of India's GDP, ranking among the most important sectors. Millions of people are employed in rural areas by agriculture, which provides a living for more than 50% of the population[7]. But in recent times the major problems for farmers are the

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excessive use of fertilizer for enhancing crops productivity and quality. These synthetic fertilizers have negative effects on both the ecosystem and human health. Where agriculture must enhance yields despite abiotic and biotic stresses, as we need to address the challenges of feeding a global population projected to reach 9.7 billion by 2050[8]. Endophytes offer an eco-friendly alternative for promoting plant growth and producing novel bioactive compounds. These microbes, which evolved over 60 million years ago, form a significant part of the plant microbiome and contribute to plant health by mitigating stress and enhancing growth[9]. Endophytes originating from seed or rhizosphere microbial communities are highly adaptable, containing genetic traits beneficial to their host plants. They play a very crucial role in plant health by eliciting immune responses, producing antioxidants, and suppressing pathogens. The vast and diverse capability of endophytes includes the production of phytohormones like IAA and gibberellic acid, which protect plants against pests and pathogens, and also the synthesis of novel metabolites, which makes them useful for lowering pesticide usage in agriculture and medicine[10]. Modern omics technologies, such as genome sequencing and metagenomics, provide deeper insights into the endophytic lifestyle and its impact on plant health. Hence, endophytes, lead to the discovery of novel, beneficial metabolic compounds in plants making them potent biofertilizers offering promising and eco-friendly alternatives to synthetic fertilizers[11]. Thus, it is imperative to understand the biology of beneficial endophytes and their interactions with host plants. This work aims to explore the different types of endophytes present in a rice plant (*Oryza sativa*) variety obtained from the field of West Bengal and their potential to enhance Chick pea plant (*Cicer arietinum*) growth. The study observed that the rice plant endophytes are capable of enhancing plant root and shoot development compared to the control chickpea plants.

Materials and Methods

Collection and Sterilization of Plant Sample: The rice plant from the field of Nadia, West Bengal was collected at its vegetative stage and cleaned under running tap water properly. The stem and root portion of the plant were taken after removing the leaves. A solution with 0.5 percent of Tween 20 and 2% sodium hypochlorite combined with autoclaved distilled water (dH, O) was used to sterilize the plant's surface under a laminar airflow. Samples were washed with Bavistin powder to remove unwanted fungal growth. It was kept for 10 mins, followed by washing with 70% ethanol wash and water wash[12].

Isolation and Purification of Endophytes: The sterilized stems were peeled and the roots were cut into small pieces (1-2mm) and placed on different agar media like nutrient agar (NA), Luria agar (LA), potato dextrose agar (PDA) for isolation. The plates were incubated at 37ºC and 30ºC for 24 -72 h until microbial growth was observed. After the incubation period colonies with distinguished visual characteristics were isolated and streaked further in NA media to obtain a pure culture of the microbial growth[13]. From the pure culture, samples were taken to grow in Nutrient broth for further tests.

Characterization of Isolated Endophytes: The isolated microbes were tested for preliminary characterization using the following procedure

Microscopic Analysis: The normal staining procedure was followed for the characterization. Gram staining techniques were used to classify bacteria as Gram-positive or Gram-negative. Drops of microbial pure cultures were used for making smears over an open flame. After the smear dried, a drop of ammonium oxalate crystal violet was added, and the mixtures were left to settle for 1 minute. With the help of distilled water, the extra stains were carefully removed. Next, one drop of Gram's iodine was added. After 1 minute, gently wash away the mordant, and a drop of 90% Ethanol was added and allowed to sit for 15 seconds. A single drop of safranin was added and allowed to sit for 30 seconds, and then the mixture was cleaned up with distilled water and observed under a microscope[14].

Biochemical Analysis: *i) Catalase Test:* A few drops of hydrogen peroxide (H_2O_2) were added to the culture in a glass slide to detect the presence of catalase enzymes in the isolated microbes. The presence of bubbles indicated positive results; while their absence indicated negative outcomes[16].

ii) Oxidase test: This test shows the presence of the cytochrome oxidase enzyme. During aerobic respiration, cytochromes facilitate the transfer of electrons to oxygen, producing water. A reagent p-phenylenediamine dihydrochloride (PPDD) dye is used; which substitutes oxygen for an artificial electron acceptor. In the presence of cytochrome oxidase, a dark purple coloured end product was formed as the dye is oxidized to indophenol blue[17].

iii) Citrate test: Simmons citrate agar was made by dissolving a suitable mass of distilled water into compounded media (24.28 g of media to 1 L of distilled water). The mixture was then distributed into tubes and sterilized at 121 °C for 15 minutes at 15 psi.

The tubes were arranged and solidified in a slanting pose. Then samples were inoculated in the slant and incubated for 24 h at room temperature. A positive test result shows a shift in the medium‘s color from green to blue (Labrador et al.[14]).

Antibiotic Resistance Test: The antibiotic resistance test was performed following Kirby-Bauer disc diffusion technique. 0.1 ml of each pure culture was taken from different media (broth) and spread in NA plates. After the addition of culture, different antibiotic discs of 10 mg/ml concentration were prepared for Ampicillin, Erythromycin, Streptomycin & Tetracycline. Discs were then added into newly spread culture plates and incubated at 37ºC for 24h. The zone of inhibition was measured to ascertain the antibiotic resistance pattern[15].

Phytohormone Production: *Indole acetic acid production:* Five µL of LB (Luria Bertani) broth were combined with 100 ìg/ml of tryptophan added to the LB broth medium. The test tubes were placed on a rotating shaker and incubated for 24 hours at 28-degree celsius with paper covering them. For fifteen minutes, the broth was centrifuged at 10,000 rpm. After gathering two milliliters of supernatant, 2-3 drops of o-phosphoric acid were added. The aliquots were vigorously vortexed after being agitated and 4 ml of reagent (1 ml of 0.5M $FeCl_3$ in 49 ml of 35% perchloric acid ($HClO_4$)) were added. After 25 minutes of room temperature incubation, the samples' absorbance at 530 nm was measured. The quantification value of auxin was obtained by extending the calibration curve created with the IAA standard (10-100 µg/ml) (Phetcharat et al.[16]).

Gibberellin Production: Gibberellin production was measured by incubating isolated microbes in nutrient broth for 7 days at room temperature. The samples were centrifuged at 8000 rpm for 10 min. After that 2 ml of zinc acetate and 2 ml of potassium ferrocyanide was added and centrifuged at 8000 rpm for 10 min. After adding 5 ml of 30% hydrochloric acid to the obtained supernatant, the mixture was incubated for 75 minutes at 27°C. Then the gibberellin production was measured by a spectrophotometric analysis at 254 nm[17].

Morphological and Physiological Analysis: **Plant Growth Analysis:** Chickpea seeds were washed by soaking them in sterile water. 0.1% mercury chloride ($HgCl_2$) and 70% ethanol were used to sterilize the seeds and allowed to germinate in a plant growth chamber. Two-leaf stage chickpeas were harvested and put in a autoclaved soil container. 100 ml of culture broth containing endophytes was added to individual tubs. A tub containing only water was maintained as control. After 30 days, the effectiveness of plant growth promotion was assessed by measuring the length of shoot and root and comparing the data with control plants[18].

Relative Water Content (RWC): To assess the water status of the plants, RWC was evaluated by the gravimetric method (Oukarroum et al[23]). Fresh mass (FM) of the compound leaf of both control and endophyte treated was measured. After rehydration in distilled water for 24 h, the samples were dried thoroughly with paper towels and weighed to obtain the turgid mass (TM). They were then dried at 80°C for another 24 h to obtain the dry mass (DM). RWC [%] was calculated as follows:

$$RWC\ [100\%] = 100 \times (FM - DM)/(TM - DM) \quad (1)$$

Chlorophyll Content: Chlorophyll content was measured using UV-spectrophotometer**.** One gram of leaf tissue was weighed and incubated at 50°C for 3 days. The incubated tissues were crushed in 80% acetone with a pinch of calcium carbonate and centrifuged at 10,000×g for 10 min. The absorbance of the supernatant was measured at wavelengths of 663 nm (A_{663}) and 645 nm (A_{645}) and the concentration of total chlorophyll content of the leaf samples was calculated using the following equation (2)[19].

$$\text{Mg Total Chl per gm tissue} = \{20.2(A_{645}) + 8.02(A_{663})\} \times V\ (W/1000) \quad (2)$$

where V and W were the total volume and fresh leaf weight respectively.

Statistical Analysis: SPSS v17 was used to analyze the data statistically. ANOVA was used to identify the effect of endophyte growth-promoting capability in the selected plants (Ali et al.[18]).

Results and Discussion

Stem and root samples of rice plants were used to isolate the endophytes associated with the plant parts. Among different endophytes isolated from Nutrient agar

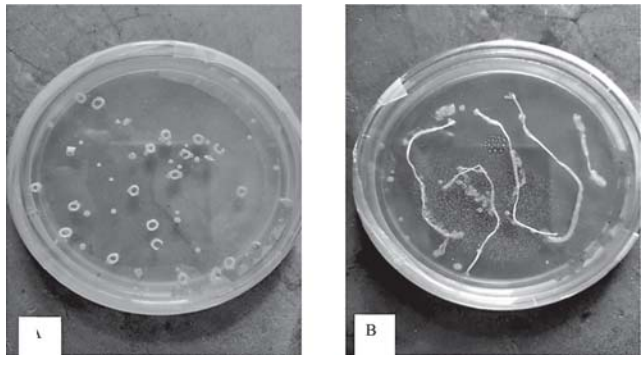


Fig. 1. Nutrient Agar plates showing endophytes growing in close vicinity of stem (A) and root (B) explants of rice plants

(NA), Luria agar (LA), and Potato dextrose agar (PDA), five endophytes named R1, R2, 6F, 5F, and 1F were selected based on their different colony pattern from NA plates. The isolated endophytes are shown in Fig.1. Further characterization studies were carried out using these five isolated endophytes.

Characterization of Isolated Endophytes: **Microscopic Analysis:** Endophytes isolated from rice plants showed different characteristics during the gram staining procedure. Among the five isolated endophytes, four isolates (R1, 6F, 5F, 1F) were gram-positive bacteria and only one (R2) was gram-negative (Table 1). Similar results were obtained by studies on rice endophytes.

Biochemical Analysis: After microscopic analysis of the endophytes different biochemical analyses were conducted to understand the basic characteristics of the isolates. Table 1 summarizes the results of the characterization of the five endophytes in the light of different biochemical tests like catalase, oxidase, and citrate tests.

Catalase Test: Three out of five bacterial strains (R2, 5F, 1F) showed positive catalase test while strain R1 and 6F showed negative results. Similar results were observed in wheat endophytes. In another report, endophytes isolated from rice exhibited enhanced catalase activity in all the bacterial strains isolated[20].

Oxidase Test: In the case of oxidase test, 6F and 5F strains showed positive oxidase activity while the other three isolates showed negative oxidase activity. Similar results were also reported in rice endophytes[21].

Citrate Test: In the citrate test, a positive result was obtained in the R1, R2, and 6F strains while the other two strains 5F and 1F exhibited negative activity.

Table 1. Summary of the biochemical and microscopic analyses of isolated endophytes

Isolated Endophytes					
Biochemical Analysis	**R1**	**R2**	**6F**	**5F**	**1F**
Catalase Test	-	+	-	+	+
Oxidase Test	-	-	+	+	-
Citrate Test	+	+	+	-	-
Microscopic Analysis	+	-	+	+	+

(+/- indicate positive/negative activity for biochemical activity)

Antibiotic Assay: To test the antibiotic sensitivity or resistance of the isolated endophytic strains, Kirby-Bauer disc diffusion technique was performed. The sensitivity or resistance of endophytic strains against ampicillin, tetracycline, streptomycin, and erythromycin was tested. The R1 strain showed no reaction (neither sensitivity nor resistance) in the antibiotic test. The R2 strain exhibited resistance against ampicillin, and sensitivity against tetracycline, streptomycin, and erythromycin. The 6F strain showed resistance against ampicillin & erythromycin and sensitivity against tetracycline and streptomycin. 5F strain had shown resistance against ampicillin and sensitivity against tetracycline, streptomycin, and erythromycin. Lastly, 1F strains had shown resistance against ampicillin & streptomycin and sensitivity against streptomycin & erythromycin. Disc diffusion by the Kirby-Bauer method is a standardized technique to determine the sensitivity or resistance of a bacterial strain against an antimicrobial agent[15].

Table 2: Summary for antibiotic resistant test

Bacterial Strain	Ampicillin	Tetracycline	Erythromycin	Streptomycin
R1	-	+	-	+
R2	-	+	+	+
6F	+	+	+	+
5F	-	-	+	+
1F	-	+	+	+

+= antibiotic sensitive and -= antibiotic resistant

Phytohormone Production: Endophytes support plant growth by several methods, which are well understood. Plant development may be influenced by Plant growth promoting Bacterias (PGPBs), either directly or indirectly. PGPB promotes plant growth directly by either acquiring resources from the environment, such as nitrogen, phosphate, and iron, or by controlling plant hormones like auxin, cytokinin, or ethylene. Whereas PGPBs indirectly promote plant growth through mechanisms such as antibiotic synthesis and cell wall degradation or by inhibiting the growth of harmful bacteria, fungus, and nematodes, which indirectly promoted plant growth[22].

The quantification of phytohormones, namely indole acetic acid (IAA) and gibberellic acid (GA), was performed for each isolate in the study. Among the tested strains, isolates R2 and 6F exhibited the highest proficiency in IAA production, yielding substantial amounts with the highest recorded ranges of 0.322 µg/ml and 0.221 µg/ml, respectively. Conversely, strains 5F and 1F demonstrated superior capability in GA synthesis, generating the maximum quantities within the studied strains. The peak ranges for GA production were determined to be 0.102 µg/ml, 0.103 µg/ml and 0.102 µg/ml for isolates R2, 5F and 1F, respectively. This highlights the distinct hormonal

synthesis capacities of the various strains examined in the study. (Fig. 2).

Endophytes like R2 and 6F produce high amounts of phytohormone IAA, crucial for plant root and stem growth regulation[23]. Indole-3-acetic acid (IAA), produced by plant growth-promoting bacteria, is a crucial hormone for plant growth, regulating physiological processes and stimulating cell lengthening, division, and differentiation[24]. The production of IAA varies significantly among species and strains within a single generation and is influenced by growth rates, growth environments, and the availability of substrates such as amino acids[25]. It is the most potent native auxin; which plants produce from the amino acid tryptophan. Both soil and plant-associated microbes have the ability to produce IAA. This suggests multiple pathways or effects that are specific to plants. Whereas it was observed that strain 5F and 1F produced higher amounts of phytohormone, GA, which significantly influenced the root formation and elongation of chickpea plants, promoting plant growth and increased yield[26]. Gibberellic acids are plant growth-promoting hormones involved in seed germination, abiotic stress response, stem elongation, and flowering, derived from plants, fungi, and bacteria. Gibberellic acid is mostly recovered from the rhizosphere or root of plants and is produced by endophytic bacteria, which are known to enhance plant development[27]. As reported by[28] formation of GA12-aldehyde in fungi is facilitated by subsequent intermediates such as geranylgeranyl diphosphate (GGDP), ent-copalyl diphosphate (CDP), ent-kaurene, and ent-kaurenoic acid. A similar biosynthetic pathway is observed in higher plants. However, differences emerge between fungal and plant pathways after the conversion to GA_{12}-aldehyde. GA12-aldehyde in fungus is 3 β-hydroxylated to GA14-aldehyde, which is oxidized to generate GA14. GA14 is then converted to GA4, which is similar to how plants produce GA9 and GA20. In a related study[29] has shown that penicillium is capable of producing GAs. As a result, our findings are consistent with earlier research

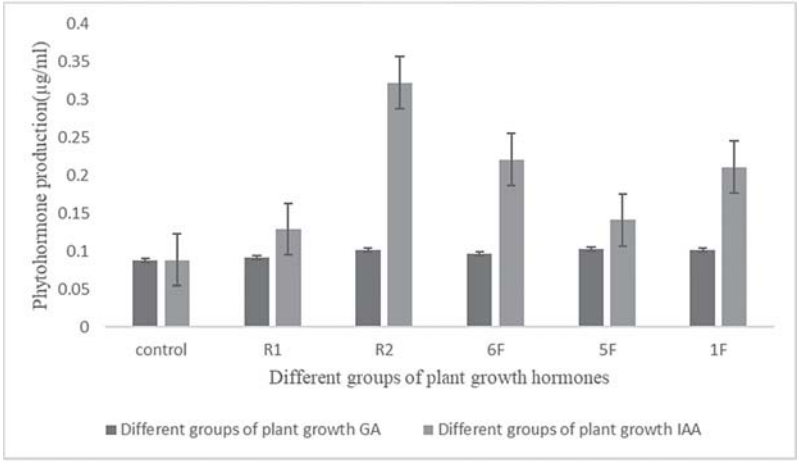


Fig. 2. Data on production of phytohormones: Indole acetic acid (IAA) and Gibberellic acid (GA) by different endophytic strain Each value is the mean ± SD of three independently conducted experiments with three replicates per treatment.

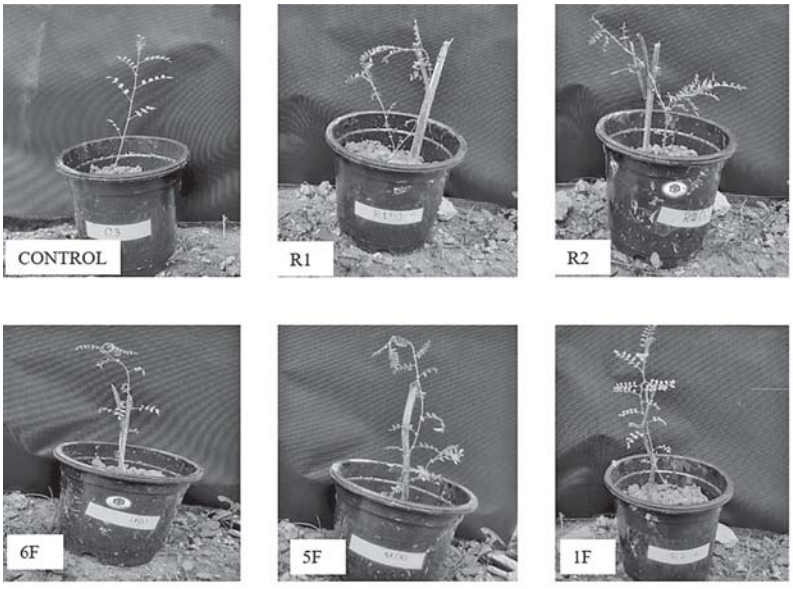


Fig. 3. Experimental set up of Chickpea seedlings treated with isolated endophytes during 7-8 days oled showed improvement in plant growth and branch formation in comparison to control seedlings grown in only autoclaved soil.

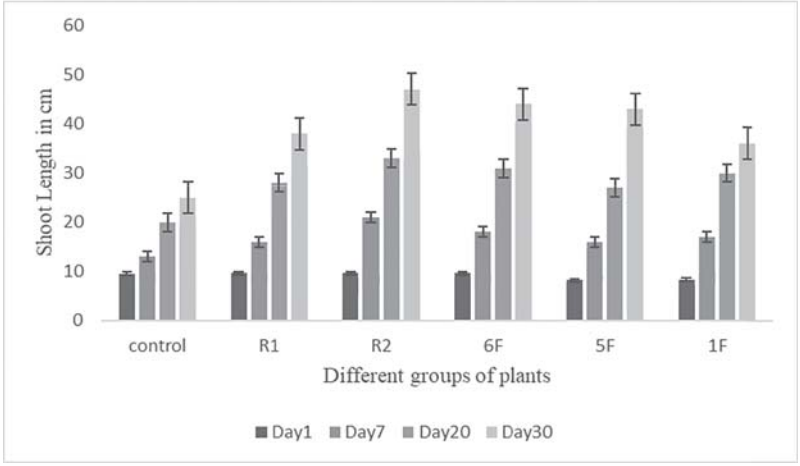


Fig. 4. Data showing shoot length measured on different days after endophytic treatment. Control growth was measured in the same manner with autoclaved soil. Each value is the mean ± SD of three independently conducted experiments with three replicates per treatment.

suggesting that endophytes are capable to produce phytohormones.

Plant Growth Analysis: The isolated endophytes were cultured on nutrient broth. Liquid cultures of endophytes were used to study the growth of chick pea seedlings. was introduced to 7-8-days old chickpea seedlings cultivated in the soil to monitor their growth length, branching pattern, root development (Fig.3), chlorophyll content and relative water content. Endophytic species often belong to the α-, β-, and γ-proteobacteria subgroups. These endophytic communities provide an important role in agroecosystems by modulating plant metabolism, encouraging growth, and signaling phytohormones to regulate environmental stressors[30]. The phytohormone IAA is mainly involved in the regulation of root and stem growth in plants. Microorganisms linked with soil and plants are widely capable of producing IAA. In every isolated endophytic strain that we choose from Rice was found to have this capability.

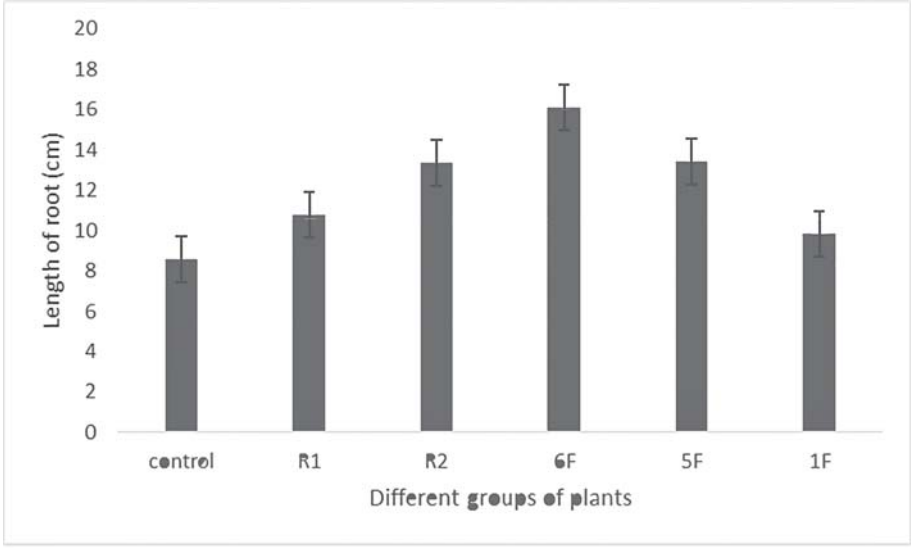


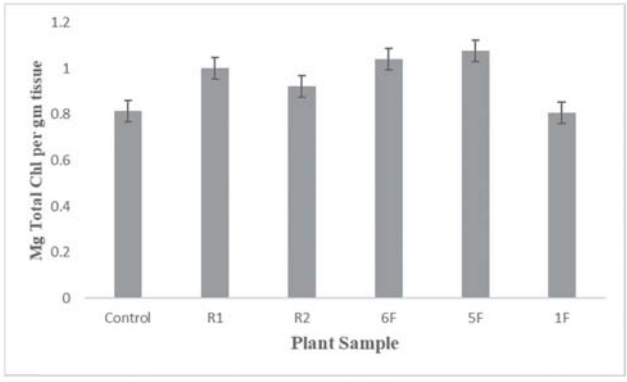
Fig.5. Data of root length by taking the plants out of soil after grown for 30 days with endophytic treatment. Control comprised only sterilized soil. Each value is the mean ± SD of three independently conducted experiments with three replicates per treatment.

Shoot Length: In Fig. 4, a higher shoot length was observed in strains R2 (27.67 cm) and 6F (25.67 cm), while lower values were recorded in the control group (16.9 cm) without endophytes. However, the remaining strains exhibited shoot lengths in the range of 5 cm and 24.9 cm on average. The values were found to be statistically significant as compared to the control ones. We also evaluated the strains' influence on the growth of several chickpea varieties because they were bioactive enough to create phytohormones. It was proposed that the presence of bioactive metabolites will enhance the growth of the chickpea variety in addition to increasing shoot growth. Growth, dry weight, height, and yield increases in plants corroborated the notion that endophytic bacterial consortia contribute to improved crop health and productivity[31]. The seed endophytic microbial consortium sometimes may imitate the natural soil environment, but the essential soil microbial community may diverge. As a result, the current study enhanced the soil's physicochemical composition as well as the growth and yield of the endophytic bacteria on chickpea plant.

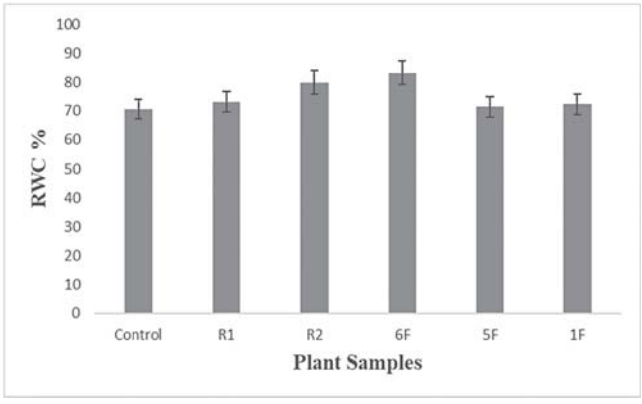
Root Length: To visualize the effects on chickpea seedling roots, we identified control, R1, R2, 6F, 5F, and 1F as numbered treatments. Chickpea plants were treated with endophytes, and their growth was observed at different time scales. Our studies have shown that addition of endophytic strains showed good root or shoot development. Root hair formation enhances plant nutrient and water uptake by increasing root surface area[32]. Root hairs also enhance crops' nutrient acquisition capacity, enhancing the uptake of immobile nutrients like phosphate and potassium. Also, in response to drought stress, endophytic colonization can trigger several physiological functions that support photosynthesis and plant growth. The current study correlates with the data that endophytic bacterial inoculation on chickpea seedlings led to the development of long, abundant root hairs.

Relative Water and Chlorophyll Content: The total chlorophyll content was calculated for both the control and treated plants and it was found to be higher in R1 and 5F among the others (Fig. 6a). When measured separately Chlorophyll a content was found to be significantly higher than chlorophyll b in all the treated plants. The highest chlorophyll content was observed in plants R1, 6F, 5F with the lowest in the control group. The differences in chlorophyll a content between treatments and control are statistically significant. Whereas Chlorophyll b content was lowest in the control group and highest in treated ones. The differences in chlorophyll b content between treated ones were not statistically significant. In chickpea the Total chlorophyll content usually ranged from 0.437 to 1.07 mg/g in matured plants.

Two fundamental factors are typically used to characterize the water status of plants: the amount of water present (or RWC) and the water's energy status, which is



(a)



(b)

Fig. 6(a): Data showing total amount of chlorophyll measured in plants after treatment with different endophytic strains. 6(b) Showing relative water content of plant samples after treatment with the isolated endophytes in comparison to control untreated plants. Each value is the mean ± SD of three independently conducted experiments with three replicates per treatment.

represented by the water's total potential (also known as the leaf water potential). As observed from the Fig. 6(b) the addition of endophytes was found to increase the water content in treated plants as compared to the control one. Out of all the strains R2 and 6F are found to have more RWC percentage among the others. The findings[33], who indicated that endophyte-infected plants have better water content status than the control plants, are similar to our results. According[34], drought stress decrease RWC and constrained the male and female Populus cathayana plants' indices of photosynthesis and chlorophyll fluorescence. On the other hand, endophytic inoculation significantly improved RWC, photosynthesis, and both sexes' intrinsic water use efficiency (WUEi), particularly in drought-stricken conditions. Furthermore[35], found that enhancing osmotic adjustment and controlling antioxidant systems, dual inoculation of fax (Linum usitatissimum) plants with AM fungus and Pseudomonas putida maintained an appropriate plant water status during drought stress.

Conclusion

It is apparent that there is an increasing demand for sustainable agronomy to fulfill the needs of a growing population while enhancing agronomic productivity without disrupting ecological components. The need for biostimulants as growth promoters has been rising for a number of years, and this has resulted in an increase in publications and research interest in this field. Plant growth and development have been discovered to be enhanced by microorganism inoculation. Researchers found that the identification and isolation of advantageous microorganisms for plant cross-protection is quite valuable. In the current investigation, endophytes isolated from rice plant stems and roots demonstrated a substantial ability to enhance chickpea plant growth. When compared to untreated control plants, all isolated endophytes developed more successfully. Since we employed autoclaved soil for plant growth, we may conclude that the isolated endophytes have the ability to improve plant morphological and physiological development. Although the effectiveness of these strains as biofertilizers in agricultural fields still needs validation, further research is essential to identify unique microbial species through gene sequencing and enhance their efficiency by integrating other growth-promoting components to develop them into robust biofertilizers.

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