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ISOLATION AND CHARACTERIZATION OF RHIZOBIA AND EVALUATION OF THEIR EFFECT ON RADISH (*RAPHANUS SATIVUS*) GROWTH

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Abstract

This study focused on isolating and characterizing rhizobia from alfalfa root nodules and assessing their impact on Radish (*Raphanus sativus* L) growth. Fifteen isolates underwent morphological and biochemical analyses, revealing consistent traits with variations in colony elevation. Confirmatory tests identified all isolates as gram-negative, rod-shaped, catalase, and oxidase positive. Notably, 13 isolates exhibited citrate positivity, 14 urease positivity, and 2 gelatinase positivity. Ten isolates produced indole-3-acetic acid, nine solubilized phosphate, and all produced ammonia. R12 displayed the highest seed germination (100%). Significant effects ($P < 0.05$) on radish growth were observed, with R37 yielding the highest values for root length (19 cm), R5 for shoot length (9 cm), R5 for leaf number (12.6), R36 for fresh weight (8 g), and R37 for dry weight (5 g). These superior isolates hold potential as biofertilizers for enhancing radish growth.

Introduction

The global surge in population, particularly in the post-industrial era, has heightened the demand for life essentials, leading to resource depletion and agricultural challenges Di Benedetto *et al*. (2017). Severe erosion processes, coupled with outdated farming practices, have adversely impacted soil fertility, exacerbating agricultural productivity decline and contributing to food insecurity and poverty I. Singh. (2018). Amidst these challenges, technology emerges as a potential solution for sustainable development and increased agricultural yield. Ethiopia, recognizing the urgency, has outlined comprehensive policies to combat land degradation and enhance agricultural productivity for food security and economic development Tsegaye *et al*. (2015). Addressing ecological imbalance and economic insecurity necessitates an environmentally friendly approach, emphasizing soil fertility improvement and sustainable land productivity. Although synthetic fertilizers offer short-term gains, their long-term negative impact on farmlands is a concern, especially in regions like Tigray with a history of overused agricultural area. The study proposes biological soil fertility improvement through the application of plant growth promoting rhizobacteria, specifically rhizobia, as a promising and environmentally acceptable method Beshah and Assefa (2019).

Rhizobia, known for their positive impact on plant growth, are a well-studied group with various beneficial traits as plant growth promoters. The focus of this investigation is on the isolation and characterization of rhizobia, aiming to evaluate their capacity in promoting the growth and development of radish seedlings. Radish, belonging to the family Brassicaceae, genus Raphanus sativus, is a versatile vegetable cultivated worldwide for its tuberous roots and leaves, known for their antimicrobial properties and health benefits Kaneko and Matszawa (1993). Given the arid and nutrient-depleted soils of Tigray, the study aims to contribute to soil improvement

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through the application of rhizobial inoculants, offering a potential solution to enhance radish seedling growth and development.

Materials and Methods

Isolation of rhizobia and purification of the isolates: Alfalfa plants (*Medicago Sativa*) provided by the College of Veterinary Sciences at Mekelle University were carefully uprooted, and nodules were transported to the microbiology laboratory at Mekelle University's College of Veterinary Science (CVS). After removing attached soil through washing and surface sterilization, nodules were incubated on agar plates at 28 ± 2°C for 24 hrs, yielding pure isolates stored at 4°C for further investigation Baye *et al*. (2015).

Confirmatory tests of rhizobium: Three confirmatory tests (Growth on YEMA with Congo red, Keto-lactose Test, and Growth on Glucose-peptone agar) were conducted to confirm the isolates as Rhizobia and distinguish them from other contaminants.

Biochemical characterization of rhizobium: Pure isolates, grown in YEM broth, were subjected to various biochemical tests, including catalase, oxidase, citrate utilization, urease, and gelatin liquefaction tests Baye *et al.* (2015).

In vitro testing plant growth promoting traits: Isolates' IAA production, ammonia generation, and phosphate solubilizing ability were determined using specific assays Kumar *et al*. (2015).

Germination assay of radish: The impact of IAA on radish germination was studied using seeds from Holleta Agricultural Research Institute, treated with IAA-producing isolates, and germination was assessed Guragain *et al.* (2023). Fifteen seeds of each treatment placed in sterilized Petri plates with moist filter paper, incubated at 28°C. Calculated seed germination, percent seedling emergence using formula Armi *et al*. (2017).

$$\%\ \text{seed emergence} = \frac{\text{number of emergied seedling}}{\text{number of seed sown}} \times 100$$

Pot trial experiment for radish growth: A randomized complete-block design experiment with radish seeds treated with bacterial cultures was conducted using Mekelle University soil. Plant metrics, including root length, shoot length, number of leaves, fresh weight, and dry weight, were recorded after 28 days Jaiswal *et al. (*2021).

Statistical analysis: Statistical analyses, including ANOVA and LSD test, were performed using Minitab 19 software to determine significant treatment effects at a 5% significance level

Results and Discussion

Fifteen bacteria were isolated from alfalfa plants collected at Mekelle University, College of Veterinary Sciences in Tigray, Ethiopia. These rhizobial isolates exhibited optimal growth on YEMA medium at pH 7.0 after 24 hrs of incubation at 28 ± 2°C. Colonies displayed uniform color, form, and margin (Fig. 1A), consistent with previous studies, though a slight difference in colony elevation was noted Simbine *et al.* (2021).The rhizobium cultured on YEMA medium produced small to medium-sized colonies with diameters ranging from 0.7 to 3 mm. Under a light microscope at 1000X magnification, motile and rod-shaped cells were observed (Fig. 1B), confirming Gram-negative characteristics, consistent with findings by other researchers Ali *et al*. 2019). In conclusion, this experiment determined that all bacterial isolates from Alfalfa plants were identified as Rhizobium spp. (Table 1).

Table 1. Morphological classification of rhizobial isolates.

Bacterial Isolates	Colony Form	Colony Margin	Colony Elevation	Colony Color	Cell Shape	Gram Staining	Cell arrangement
R5	Circular	Entire	13 Raised 2 Convex	White	Rod	-Ve	Single

Biochemical characters of selected rhizobial isolates: The biochemical characterization revealed distinctive traits confirming the identity of the bacterial isolates as *Rhizobium* spp. In the Congo red test, isolates did not absorb the dye (Fig. 1C), consistent with rhizobial characteristics and distinct from agrobacterium Qudratullah Oryakhil (2020). Similar results were observed in various studies, reinforcing the rhizobial classification.

Keto-lactose tests showed no yellow zones around colonies, a rhizobium trait supported by other findings (Fig. 1D). Growth on glucose peptone agar further affirmed rhizobial features, consistent with previous reports. From the above observations we could conclude that all the bacterial isolates were *Rhizobium* spp.

Additional biochemical tests, including catalase and oxidase (Fig. 1G), confirmed the rhizobial identity. Positive catalase and oxidase results, indicated by bubble formation and dark purple stain, aligned with findings by Singha *et al.* (2018).

Citrate utilization test demonstrated carbon source utilization by the majority of isolates (Fig. 1F), while urea hydrolysis test showed positive results in 14 out of 15 isolates, indicated by color change to yellow/orange. Notably, no gelatinase enzymes were produced, consistent with the observed negative gelatinase activity of rhizobium Wang *et al.* (2022). In conclusion, the comprehensive biochemical tests support the classification of all bacterial isolates as *Rhizobium* spp. (Table 2).

Table 2. Biochemical characteristics of the root nodule bacteria.

Isolates	Cat	Oxid	Urease	Cit	Gel	CR	KL	GPA
R5	+	+	+	-	+	-	-	+
R7	+	+	+	+	-	-	-	+
R8	+	+	-	+	-	-	-	+
R9	+	+	+	+	-	-	-	+
R10	+	+	+	+	-	-	-	+
R11	+	+	+	-	+	-	-	+
R12	+	+	+	+	-	-	-	+
R25	+	+	+	+	-	-	-	+
R35	+	+	+	+	-	-	-	+
R36	+	+	+	+	-	-	-	+
R37	+	+	+	+	-	-	-	+
R41	+	+	+	+	-	-	-	+
R42	+	+	+	+	-	-	-	+
R45	+	+	+	+	-	-	-	+
R47	+	+	+	+	-	-	-	+

Key: (+) Positive; (-) Negative; Cat: Catalase; Cit: Citrate; Gel: Gelatinase; CR: Congo red; KL: Ketolactose; GPA: Glucose Peptone Agar.

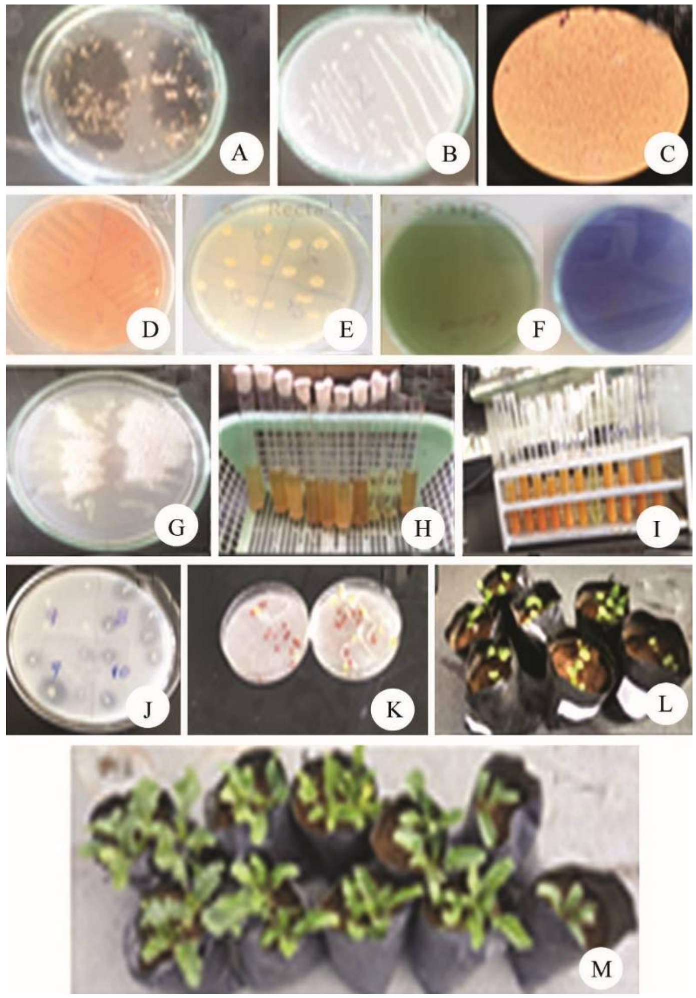

Fig. 1(A) Root nodules, (B) Purified cultures of rhizobium on YEMA medium, (C) Gram's staining, (D) Congo red test, (E) glucose peptone agar test, (F) Citrate utilization test, (G) Catalase test, (H) IAA production, (I) Ammonia production, (J) Phosphate solubilization, (K) Germination, (L) Radish plant 10 days old and (M) Radish plant 21 days old.

Plant growth promoting features of the test isolates: In this experiment, all isolates underwent characterization for various plant growth-promoting activities (Table 3). Color variation, ranging from deep pink to pale pink, served as an indicator of the isolates' potential to produce indole acetic acid (IAA). Out of the 15 rhizobial isolates, ten demonstrated the ability to produce IAA (Fig. 1H).

This aligns with previous findings of auxin-producing Mesorhizobium species isolated from chickpeas Shoukry *et al.* (2018). The development of a yellow to brown color after adding Nessler's reagent to fully grown bacterial suspensions in peptone water indicated ammonia production (Fig. 1I), a trait observed in all rhizobial isolates.

This finding is consistent with the ammonia production observed in other studies Hussein and Joo (2018). Regarding phosphate solubilization, nine out of 15 isolates exhibited clear halo zones around their colonies on Basal Sperber Agar plates impregnated with tri-calcium phosphate (Fig. 1J). These results align closely with findings by other researchers Alemneh *et al.* (2020).

Table 3. Production of plant growth promoting substances.

Isolates	IAA	AMM	PS
R5	+	+	+
R7	-	+	-
R8	+	+	+
R9	+	+	+
R10	-	+	+
R11	+	+	-
R12	+	+	-
R25	+	+	-
R35	+	+	+
R36	+	+	+
R37	+	+	+
R41	-	+	+
R42	-	+	-
R45	-	+	-
R47	+	+	+
Control	-	-	-

Effects of rhizobium on seed germination: In this experiment, IAA-producing rhizobial isolates significantly enhanced radish seed germination, with treated seeds (coated with Rhizobium culture) showing higher rates and longer sprouts compared to the non-inoculated control group (Table 4). This supports findings from previous studies, such as Francis *et al.* (2022). The treated group also exhibited increased shoot and root lengths in radish seedlings, consistent with observations by Khan *et al*. (2020) highlighting the positive impact of rhizobacteria on seedling vigor and crop growth.

Recognizing the significant influence of rhizobacteria on germination and seedling vigor in agricultural crops, this experiment aligns with the broader understanding of the positive effects of rhizobacteria, including their contribution to early seedling establishment and overall crop growth and development Hussein and Joo (2018). The isolated rhizobacteria demonstrated notable activities in promoting seedling growth and nitrogen fixation, suggesting their suitability for use in biofertilizers (Fig. 1K).

Pot trial experiment for radish growth: In a pot culture experiment assessing the impact of Plant Growth Promoting Rhizobacteria (PGPR) on radish (*Raphanus sativus*), the growth and yield were examined (Table 5). Similar results were reported by Flores Clavo *et al.* (2023). The pot experiment aimed to evaluate the effect of PGPR isolates on various parameters such as root length, shoot length, number of leaves, shoot fresh weight, and shoot dry weight of radish plants Bhardwaj *et al.* (2023). All tested strains demonstrated a significant increase in these parameters compared to the control group (Fig. 1L, M). This experiment reaffirms that the isolated rhizobacteria have notable beneficial effects on radish plant growth, making them suitable candidates for biofertilizers with activities related to rhizobium production and nitrogen fixation Umar Ibrahim Iro and Obidola (2019).

Table 4. Germination radish in laboratory.

RV	RT	GS	NGS	GP(%)	RS	SL
R5	R/T	13	2	86.6	5.8^{AB}	2.5^{DE}
R11	R/T	13	2	86.6	3.5^{CDE}	4.7^{A}
R12	R/T	14	1	93	5^{AB}	4.7^{A}
R25	R/T	13	2	86.6	4^{BCD}	3^{CD}
R35	R/T	14	1	93	5.9^{A}	4^{AB}
R36	R/T	14	1	93	4.8^{ABCD}	4.6^{A}
R37	R/T	15	0	100	3.5^{CDE}	3^{BC}
R41	R/T	13	2	86.6	5^{ABC}	3^{CD}
R42	R/T	13.	2	86.6	3^{DE}	2.9^{CDE}
R47	R/T	13	2	86.6	2.7^{EF}	2.8^{DE}
Control	N/R	9	3	60	1.5^{F}	2^{E}

RV: Radish vegetables; RT: Rhizobium Treated; GS: Germinating Seeds; NGS: Non germinating Seed: GP: Germination Percentage (%); RT: Root seedling; SL: Shoot seedling

Table 5. The effect of the treatments on radish root length system.

T	RT	RL (cm)	SL (cm)	NOL	FW (g)	DW (g)
R5	R/T	18^{A}	9^{A}	12.6^{A}	$6.6A^{B}$	4^{BC}
R11	R/T	17.6^{A}	5^{B}	12^{A}	3.6^{DE}	2^{DE}
R12	R/T	14.5^{CD}	4.6^{B}	11.6^{AB}	5.6^{BC}	2^{DE}
R25	R/T	15.6^{BC}	4^{BC}	11^{ABC}	4^{CD}	2^{DE}
R35	R/T	15^{C}	5^{B}	$9B^{CD}$	5.6^{BC}	2.7^{CD}
R36	R/T	18^{A}	6.6^{B}	$9B^{CD}$	8^{A}	4^{AB}
R37	R/T	19^{A}	5.6^{B}	9^{BCD}	4^{CD}	5^{A}
R41	R/T	18^{A}	5^{B}	9^{BCD}	3.7^{DE}	2^{DE}
R42	R/T	16^{BC}	4^{BC}	8.6^{CD}	5^{BCD}	3^{CD}
R47	R/T	17^{AB}	4^{BC}	8^{CD}	$.5^{CD}$	3^{CD}
Control	R/T	13^{D}	4^{C}	7^{D}	2^{E}	1^{E}

T: Treatments; RT: Rhizobia Treated; RT: Root Length; SL: Shoot Length;
No: Number of Leaves; FW: Fresh Weight; DW: Dry Weight

The Finding Regarding, The rhizobacteria isolated from root nodule of Alfalfa cultivated in Mekelle University College of Veterinary Science. Different preliminary tests and biochemical tests confirmed that the isolates were rhizobia.

Superior isolates were selected and tested for plant growth promoting traits and 10 isolates showed indole-3-acetic acid production, 9 isolates showed phosphate solubilization and all the isolates were positive for ammonia production. The radish seeds were treated with the plant growth promoting isolates.

As a result, most of the isolates promote seed germination, seedling growth and development of radish. The superior rhizobial isolates can be used as a bio inoculants/ bio fertilizer not only for leguminous plants but also for non-leguminous plants like radish. Further studies may be important to investigate the effect of PGPR on seedling radish.

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