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Screening groundnut rhizobia for multiple plant growth promoting activities in Ethiopia

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Rhizobia could exhibit plant growth-promoting mechanisms besides nitrogen fixation. In search of efficient rhizobia with multiple PGP traits, 72 groundnut nodulating rhizobia isolates were screened in vitro for their plant growth promoting traits like phosphate solubilization, production of indole acetic acid, ammonia, hydrogen cyanide, production of different hydrolytic enzymes and antifungal activity. About 72% of the isolates produced IAA, varying from 7.4 to 78.8 µg.ml^{-1} and 23.6% of the isolates were able to solubilize tri-calcium phosphate. Even if all isolates could produce ammonia, two isolates were strongly produced. Only two isolates were able to produce hydrogen cyanide. The enzymatic production study revealed that 50 and 48% of the tested isolates showed protease, and cellulase activities respectively. Only 13.8% of the isolates were found to be inhibitory against the test pathogen, *Fusarium oxysporum*, but the maximum inhibition potential was exhibited by GNR-07. The present study also demonstrated that 100% of the isolates exhibited multiple PGP (plant growth promotion) properties, with isolates GNR-37 and GNR-28 being superior, acquiring the highest number of PGP properties (87.5% each). These isolates can be potential candidates as a PGPR inoculant after evaluation of their performance under greenhouse and field conditions.

Key words: Rhizobia isolates, plant growth-promoting traits, enzymes and some chemical compound production, antifungal activity.

INTRODUCTION

Plant growth promoting rhizobacteria (PGPR) are a heterogeneous group of bacteria that can be found in the rhizosphere, at root surfaces and in association with roots, which can improve the extent or quality of plant growth directly and or indirectly (Meena et al., 2012; Moustaine et al., 2017). PGPR include different genera of bacteria such as *Acetobacter, Acinetobacter, Alcaligenes, Arthrobacter, Azoarcus, Azospirillum, Azotobacter, Bacillus, Beijerinckia, Burkholderia, Derxia, Enterobacter, Gluconacetobacter, Herbaspirillum, Klebsiella, Ochrobactrum, Pantoae, Pseudomonas, Rhodococcus, Serratia, Stenotrophomonas* and *Zoogloea* (Jha and Saraf, 2015).

PGPR can promote plant growth by both direct and indirect mechanisms. Direct mechanisms are defined as employing those bacterial traits that result in the direct promotion of plant growth. It includes the production of ACC deaminase, auxin, gibberellin, cytokinin, phosphorous solubilization, nitrogen fixation and impounding of iron by siderophore producing bacteria.

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The bacteria have characteristics that involve inhibiting the functioning of one or more plant pathogenic microorganisms, including fungi and bacteria. Indirect mechanisms include the production of cell wall degrading enzymes, antibiotics, competition, induced systemic resistance, hydrogen cyanide, quorum quenching, and siderophores (Olanrewaju et al., 2017). PGPR antagonize phyto-pathogens through various mechanisms; like competition for nutrients and space, production of antibiotics, production of lytic enzymes and hydrogen cyanide (Hamid et al., 2021; Jayaprakashvel et al., 2019; Kaur et al., 2021). Some bacteria produce enzymes such as chitinases, cellulases, proteases, and lipases that lyse and destroy cell walls of bacteria and fungi that are pathogenic for the plant. PGPR are capable to synthesize one or more of these enzymes have been found to have biocontrol activity against a range of pathogenic fungi (Sathya et al., 2017).

Rhizobia that form root nodules and fix atmospheric nitrogen with leguminous plants are one of the PGPR that possess many distinct plant growth-promoting traits that enhance plant growth of both leguminous and non-leguminous crops (Jaiswal et al., 2021; Knežević et al., 2022). Rhizobia can enhance plant growth directly through production of different metabolites having a property of plant growth promotor and biocontrol by production of different lytic enzymes (Gopalakrishnan et al., 2015).

It is reported that rhizobia nodulating groundnut host possess plant growth promoting (PGP) properties such as solubilization of inorganic phosphate (Afzal and Asad, 2019) phytohormone production such as Indole Acetic Acid (IAA) (Kumar et al., 2014; Panigrahi et al., 2020) and siderophore production (Vargas et al., 2017).

In general, root nodule rhizobia possessing multiple PGP traits confer additional advantage to serve as inoculants for both legume and non-legume crops grown rotationally or simultaneously. However, the number of studies on groundnut rhizobia is limited compared to other pulses, despite the growing commercial interest in crop production in Ethiopia. Hence, to improve the yield of groundnut, there is a need to collect, isolates, and screen more groundnut rhizobia for their plant growth promotion characteristics from the major growing areas of the country. In this study, groundnut rhizobia isolates were collected from major prospective groundnut growing areas of Ethiopia using groundnut variety Babile 1. The isolates were then evaluated for multiple plant growth promotion (PGP) traits under laboratory and greenhouse conditions to determine their potential and effectiveness for future inoculant production.

MATERIALS AND METHODS

Source of rhizobial isolates

A total of 72 groundnut rhizobia isolates were used for PGP tests. All of them were trapped by induction method under greenhouse condition from soil samples collected from major groundnut growing areas Oromia Babile, Benishangul and Berhet wereda by using improved groundnut variety called Babile 1 as a trap host. This variety was introduced from India by International Crop Research Semi-Arid and Tropics (IRISAT). It is well adapted in some areas of the country like that of Werer, Miesso, Assosa, Pawe and Babile with an altitude range between 569 to 1100 mm and having an average potential productivity of 24 qt/ha. The majority of isolates were slow growing rhizobia identified as *Bradyrhizobium* species.

Screening for plant growth promoting (PGP) characteristics of groundnut rhizobia

Solubilization of inorganic phosphates

A loopful of fresh rhizobial culture (10µL; 10^8 cells mL^{-1}) was spot inoculated on Pikovskay's medium containing Yeast Extract 0.5 Dextrose 10.0 Calcium Phosphate 5.0 Ammonium Sulphate 0.5 Potassium Chloride 0.2 Magnesium Sulphate 0.1 Manganese Sulphate 0.0001 Ferrous Sulphate 0.0001 Agar 15.0 (in g l^{-1} of distilled water): and incubated at 28-30°C for one week. Clear zone formation around the colonies was recorded as positive for inorganic phosphate solubilization. Phosphate solubilization index (the extent of phosphate solubilizing ability of bacterial isolates) was also determined (Paul and Sinha, 2017).

Phosphate Solubilization Index (SI) = B/A

Where; A = Colony diameter B = Total diameter (colony + halo zone)

Quantitative estimation of indole acetic acid (IAA) production

The ability of rhizobial isolates to produce indole acetic acid (IAA) was determined colorimetrically accordingly. The rhizobial cultures were grown at 28°C in YEM broth (containing K_2HPO_4 - 0.5 g, $MgSO_4$. $7H_2O$ - 0.2 g, NaCl - 0.1g Mannitol - 10.0 g Yeast extract 1 lit distilled water) supplemented with filter sterilized L-tryptophan (2 g l^{-1}) for 72 h on a shaker at 150 rpm, and centrifuged at 6,000 rpm for 15 min at 4ºC. 2 ml of the supernatant was mixed with 4 ml of Salkowski reagent (1 ml of 0.5M $FeCl_3$ solution in 50 ml of 35% perchloric acid) and the mixture was reserved at room temperature for 25 min in darkness. IAA production was confirmed due to the development of pink color. The intensity of pink color was read at 530 nm spectrophotometrically and the amount of IAA produced was extrapolated from the standard curve constructed from pure IAA (Loba Chemie) in the range of 5 to 100 µg/ml. Non-inoculated L-tryptophan supplemented YEM broth medium was used as control (Md Hoirul Azri, 2018).

Ammonia (NH3) production

The isolates were tested for ammonia production by inoculating a loopful of freshly grown cells in to 10 ml of pre-sterilized peptone water tubes and incubated at 28^0C for 3 days. The tubes were treated with 0.5 ml of Nessler's reagent (potassium iodide -50 g, saturated mercuric chloride -35 mL, distilled water -25 mL, potassium hydroxide (40%) -400 mL) to detect development of brown to yellow color as a positive test for ammonia production (Manasa et al., 2017).

Production of hydrogen cyanide (HCN)

The rhizobial isolates (100 µL; 10^8 cells mL^{-1}) were inoculated into

YEMA plates supplemented with 4.4 g L^{-1} of glycine to detect HCN production. Stripes of filter paper (Whatman filter paper No.1) were soaked in the yellow picric acid solution (2.5 g of picric acid and 12.5 g of Na_2CO_3 dissolved in 1L of distilled water) and fixed to the underside of the upper lids and sealed with parafilm (to avoid the escaping of volatiles like HCN) and incubated at 28°C for 5 days. A color change of the yellow filter paper to brown was recorded as positive for HCN production (Mir et al., 2021a).

In vitro antagonistic activity against test pathogenic fungus (Fusarium oxysporum)

The inhibitory effect of the rhizobia isolates against the pathogen, *F. oxysporum*, was evaluated *in vitro* on YEMA plates using the dual culture technique as described by Kumar et al. (2016). A loopful (10 µL; 10^8cells mL^{-1}) of each rhizobial culture was spot-inoculated on YEMA plates amended with 0.5% sucrose at a distance of 2.0 cm from the center at four equidistant points and incubated at 28°C for 5 days. Then, five days old mycelial discs (4 mm diameter) of *F. oxyporum* was positioned at the center of the Petri dishes and incubated at the same temperature until the test pathogen in the control plates (plates without rhizobia isolate) reached the edges of the plates. The radial growth of fungal mycelium and the inhibition percentage was compared with control using the formula, I = [C − T/C] × 100, where I is the percent inhibition and C and T are the pathogen radial growth in control and treatment, respectively.

Test for the production of different lytic enzymes

Cellulase production activity

Isolates were tested for their ability to produce cellulase according to the method. A loopful of broth culture (10 µL; 10^8 cells mL^{-1}) of each isolate was streaked on a cellulose agar media containing composition of KH_2PO_4 0.5 g, $MgSO_4$ 0.25 g, cellulose 2 g, agar 15 g, and gelatin 2 g; distilled water one liter and at pH 6.8–7.2. The plates were incubated at 28°C for 72 h and flooded with Gram's iodine for 5 min to detect clear zone formation around colonies (Dar et al., 2015).

Protease production activity

Protease activity was assayed with YEM agar containing 5% skimmed milk (Mohamad et al., 2018). After incubation at 28°C for 5–7 days, formation of clear halo zone around the bacterial colonies was positive reaction for milk casein hydrolysis.

Production of chitinase

Chitinase activity of the isolates was tested on chitin agar plates constituting (g l^{-1}) colloidal chitin (4), $MgSO_4.7H_2O$ (0.5), K_2HPO_4 (0.7), KH_2PO4 (0.3), $FeSO_4.7H_2O$ (0.01), $MnCl_2$ (0.001), NaCl (0.3), yeast extract (0.2) and agar (20). After adding iodine the development of clear zone around the colony was reflected as positive for the enzyme chitinase production (Madison et al., 2017).

RESULTS

Phosphate solubilizing ability of isolates

Phosphate-solubilizing bacteria were identified characteristically by the formation of a clear zone around the colonies after 48 h of incubation following spot inoculation in Pikovskaya's agar plate were taken as positive tests (Figure 1). Accordingly, out of the 72 rhizobial isolates, seventeen (17) isolates (23.6%) were capable of solubilizing tri-calcium phosphate. There was variation in the diameter of the halo zones surrounding each colony, indicating that these seventeen phosphate solubilizing bacteria exhibited differing capacities for phosphate solubilization (Table 1). The clear zone diameter ranged from 1 to 3 mm, with isolates GNR-67 and GNR-28, respectively. The phosphate solubilization indices formed by these isolates also varied between 1.7 and 2.45. The solublization index was calculated as: Phosphate Solubilization Index (SI) = B/A

Where; A = Colony diameter B = Total diameter (colony + halo zone)

Indole acetic acid (IAA) production

The isolates showed marked differences in their ability to produce IAA in tryptophan supplemented YEM broth media (Figure 2). Of the 72 isolates, 72% (52 isolates) produced a detectable amount of IAA, which ranged from 7.4 to 78.8 µg.ml^{-1}, while the auxin levels produced by the remaining 20 isolates were below the detection limit. A large amount of IAA was produced by isolate GNR-43 (78.8 µg.ml^{-1}) followed by isolates GNR-37 (77.2 µg.ml^{-1}), GNR-28 (67.8 µg.ml^{-1}), GNR-34 (66.8 µg.ml^{-1}), GNR-03 (65.8 µg.ml^{-1}), GNR-07 (58.8 µg.ml^{-1}) and GNR-54 (56.8 µg.ml^{-1}) (Table 1).

Ammonia (NH_3) production

Screening the rhizobial isolates for ammonia production was an important trait of plant growth-promoting rhizobacteria (PGPR) that indirectly influences plant growth and performance. Of all the tested Rhizobium isolates, two isolates were (GNR-28, GNR37) exhibited as strong (+++) ammonia producer, six isolates (GNR-3.GNR-7, GNR-19, GNR-34, GNR-43 and GNR-54) were produce moderately (++) .Whereas the rest isolates were scored as weak (+) for Ammonia production (Figure 3).

Antagonistic activity of *Rhizobium* against fungal pathogen

In vitro antagonistic potential of the *Rhizobium* isolates were tested against *F. oxysporum* in dual culture under *in vitro* conditions and the percentage of inhibition were recorded. After five days of incubation, inhibition zone was clearly visible. Hence, ten isolates were found to be inhibitory against fungal strain; yet, maximum inhibition potential was exhibited by the rhizobial isolates GNR-07

Figure 1. Clear zones of phosphate solublization for the isolates AAUR19, AAUR34 and AAUR37 on Pikovskaya's agar plate.

followed by GNR-03 and GNR-28 with growth inhibition of 42.3, 28 and 25.8% respectively against *F. oxysporum* (Table 1).

Production of different lytic enzymes

Screening of bacterial isolates for lytic enzymes production was done. Lytic enzymes include Protease, cellulase and chitinase positive isolates showed distinct, clear, and prominent zones of clearance around the colonies showing lytic enzymes production (Figure 4). In this study, of the tested isolates, 35 and 36 isolates showed positive for cellulase and protease activity, respectively. The majority (90%) of the isolates that produce cellulase enzyme also produce protease enzyme. However, all the tested isolates were negative for chitinase activity (data not shown).

Production of Hydrogen Cyanide (HCN)

HCN production is ascribed as one of the mechanisms of biocontrol activity of rhizobia, the ability of the 72 isolates to produce HCN was determined by the picric acid assay (Figure 5). Only two *Rhizobium* cultures, were produced HCN scored as moderate (++) for GNR-37 and weak for GNR-28 whereas the redcxw<nbvcxwdsfw<nbcw< sqmaining 70 isolates not changed the yellow color of the picric acid solution treated filter paper, implying majority of the groundnut isolate were failed to produce HCN (Table 1).

DISCUSSION

Among the tested rhizobial isolates, 17 (23.6%) isolates produce a halo zone around the colonies after 24 h of incubation on Pikovskaya's agar medium, which gradually increased up to 72h. The solubilisation efficiency (SE) of *Rhizobium* strains on solid media ranged between 71 to 150%. The *Rhizobium* strain GNR-28 showed maximum solubilisation efficiency followed by GNR-19 and GNR-34. In the study by Adnan et al. (2017), it was observed that 21% of the tested rhizobia were phosphate-solubilising bacteria, with solubilisation efficiencies ranging between 38 and 270%. Similarly, phosphate-solubilizing microorganisms Bacillus subtilis and Bacillus cereus, isolated by Satyanandam et al. (2021) from groundnut rhizosphere soil, exhibited maximum phosphate solubilizing zones. The isolates in the current study varied in their intrinsic ability to produce IAA as the production varied under the same condition. Hence, 72% of the tested isolates produced a detectable

Table 1. Summary of multiple plant growth promoting traits of groundnut rhizobial isolates.

Isolate	IAA produced	Phosphate solublization index	Ammonia Production	Protease	Cellulase	Chitinase	Antifungal activity	HCN	%PGP
GNR03	65.8	2.1	++	+	+	−	28	−	75
GNR05	8	NS	+	+	+	−	0	−	50
GNR08	32.5	2.21	+	−	+	−	12.3	−	40
GNR02	36	NS	+	+	−	−	0	−	37.5
GNR12	0	NS	+	+	+	−	0	−	37.5
GNR07	58.8	2.34	++	+	+	−	42.3	−	75
GNR15	23.4	NS	+	+	−	−	0	−	37.5
GNR17	0	NS	+	+	+	−	0	−	37.5
GNR19	76	2.42	++	+	+	−	22.5	−	75
GNR20	11.2	2.1	+	+	+	−	0	−	62.5
GNR24	32.2	2.24	+	+	+	−	0	−	62.5
GNR25	24.4	1.8	+	−	−	−	0	−	37.5
GNR26	0	NS	+	+	+	−	0	−	37.5
GNR27	0	NS	+	−	+	−	0	−	37.5
GNR28	67.8	2.45	+++	+	+	−	25.8	+	87.5
GNR29	32.4	2.33	+	+	−	−	0	−	50
GNR31	16.5	NS	+	−	+	−	0	−	37.5
GNR35	0	NS	+	+	+	−	12.3	−	50
GNR34	66.8	2.41	++	+	+	−	0	−	62.5
GNR36	28	NS	+	+	−	−	0	−	37.5
GNR37	77.2	2.4	+++	+	+	−	25	++	87.5
GNR38	11.6	NS	+	−	+	−	0	−	37.5
GNR42	42.4	1.8	+	+	−	−	0	−	50
GNR43	78.8	2	++	+	+	−	12.5	−	75
GNR44	33.4	NS	+	+	+	−	0	−	50
GNR46	7	NS	+	−	−	−	15.5	−	37.5
GNR47	33.7	2.22	+	−	+	−	0	−	50
GNR49	18.5	NS	+	+	+	−	0	−	50
GNR50	0	NS	+	+	+	−	0	−	37.5
GNR51	29.8	NS	+	−	+	−	0	−	37.5
GNR53	22	NS	+	+	−	−	0	−	37.5
GNR54	56.8	1.8	++	+	+	−	26.7	−	75
GNR55	0	NS	+	+	+	−	0	−	37.5
GNR58	34	2.31	+	−	+	−	0	−	50
GNR60	0	NS	+	+	+	−	0	−	37.5
GNR61	39.2	NS	+	+	−	−	0	−	37.5
GNR64	20.8	NS	+	+	+	−	0	−	50
GNR65	9.6	NS	+	−	+	−	0	−	37.5
GNR67	41.2	1.7	+	+	−	−	0	−	50
GNR68	7.4	NS	+	+	+	−	0	−	50
GNR71	34.8	1.74	+	+	+	−	0	−	62.5

amount of IAA, which ranged from 7.4 to 78.8 µg.ml^{-1}. The biosynthesis of IAA is widespread among plant-

Figure 2. IAA production of tested isolates AAUR37 (Pinkish color showed IAA production, Creamy color showed no IAA produced and Whitish color only YEM broth as control).

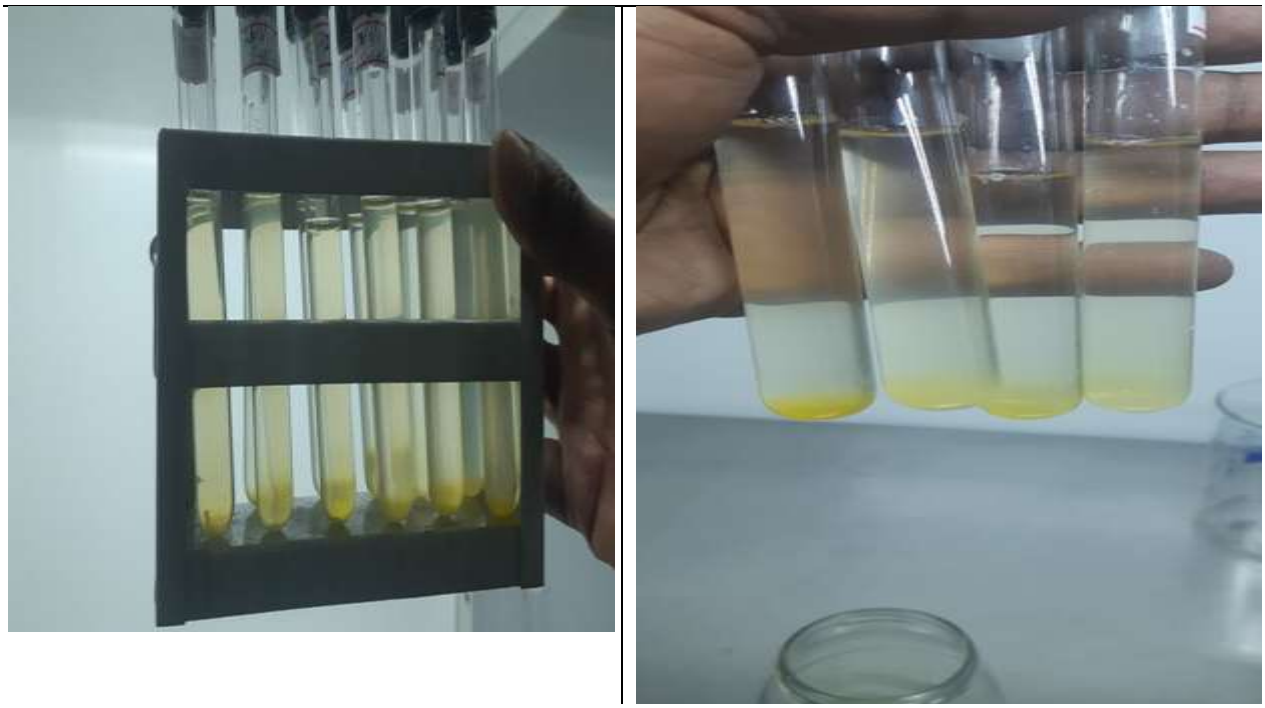

Figure 3..Test for ammonia production of rhizobial isolates AAUR19, AAUR28,AAUR37 and AAU43 from left to right(yellowish color indicates ammonium production).

associated bacteria (Ulrich et al., 2021). Several studies also showed that many soybean rhizobia produced IAA irrespective of the type of rhizobia nodulating groundnut host varieties (Dlamini et al., 2021; Ibny et al., 2019). Ibny et al. (2019) working with 89 rhizobial strains and found that 39% of the strains produce IAA. The IAA produced by some of the isolates in the present study was higher than previously reported for both fast and slow growing

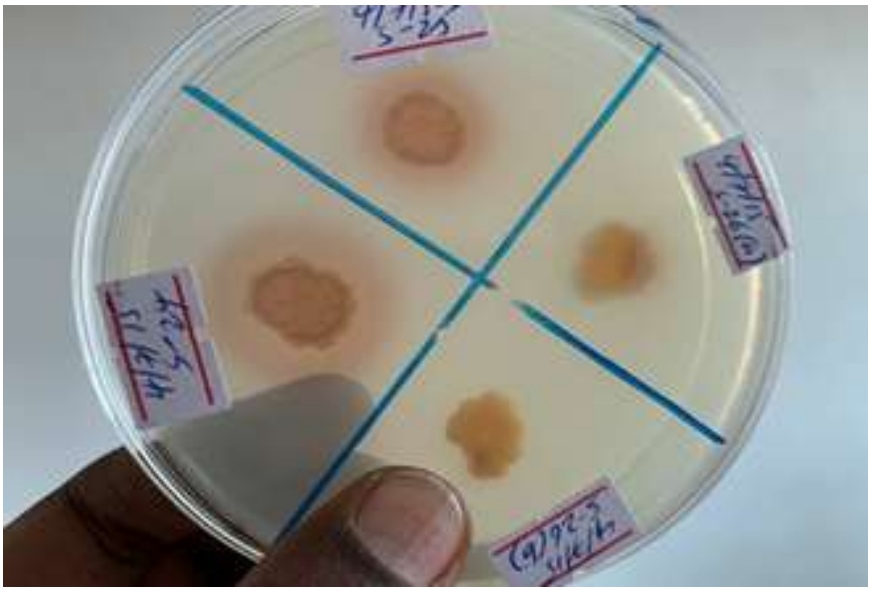

Figure 4. Protease enzyme production by rhizobial isolates AAUR19, AAUR28, and no protease production by isolates AAUR37and AAUR43 on SMA media.

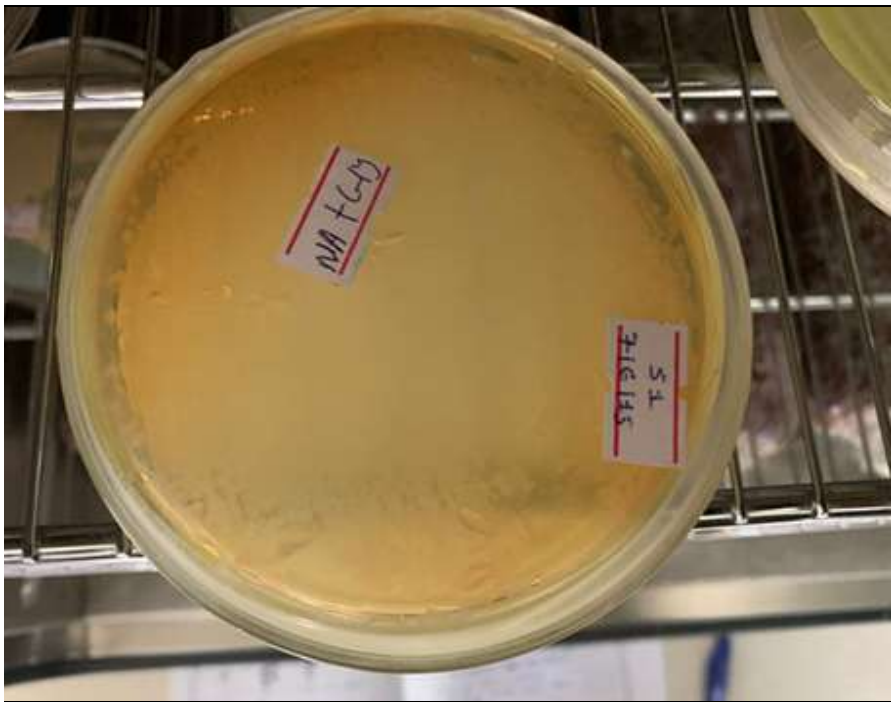

Figure 5. Plate based qualitative assays showing HCN production.

groundnut rhizobia in Pakistan (0.02 to 69.71 µg.ml−1). Ibny et al. (2019) and lower than which has been reported from Ghana (56 to 290 µg ml^{-1}) (Khalid et al., 2020). The production of IAA by PGPR can be differ with in species and strains, and influenced by culture conditions, substrate availability and growth stage (Ashrafuzzaman et al., 2009; Kumar et al., 2012; Ngoma et al., 2013).

Another important trait of rhizobia is the production of ammonia that indirectly influences the plant growth. The plants can take up ammonia produced by the rhizobia as a source of nitrogen for their growth. The ammonia

production results of the test isolates showed varied outcome (Table 1). All isolates (100%) were able to produce ammonia. Among these, two isolates were exhibited as strong ammonia producer, six isolates were produce moderately the remaining isolates were scored as weak ammonia producer. Similarly Ajilogba et al. (2022) works show that, even if all the tested isolates were positive for NH_3 production, two of them were recorded as moderate and the rest as weak ammonia producers. In general, having such rhizobia characteristics suggests that it is vital to select a nitrogen fixer with ammonia production in a bio-fertilizer consortium for agriculture practices.

To check the efficacy of antagonism of selected rhizobial isolates against soil borne fungal isolates (*F. oxysporum*) infecting groundnut plant, dual culture method was adapted and the percentage of inhibition of growth was recorded (Table 1). As a result of the plate assay, some rhizobial isolates curtailed the growth of pathogenic fungi tested and were found to be highly inhibitory to *F. oxysporum,* whereas other strains showed only nominal antifungal activity. Rhizobial Strains GNR-07, GNR-03 and GNR-28 suppressed the growth of tested fungi at higher percentage when compared to other strains tested. Similar results were observed by Antoun et al. (1998), who found that 49 of his rhizobial strains inhibited the growth of *F. oxysporum*. A significant reduction in damping -off or wilt disease of groundnut plant could be achieved by inoculating the strains that have antifungal ability and capable to reduce the percentage of crop loss. Such biocontrol agents have also been reported to produce toxic metabolites, enzymes or volatile compounds that have inhibitory effects on soil-borne pathogens.

Hydrolytic enzymes act as agents for prevention of plant diseases by causing lysis of pathogenic microbes in the close vicinity of the plant as they secrete increased level of cell wall lytic enzymes (Protease, cellulase and chitinase) (Neeraja et al., 2010). In this study, 50% isolates were positive for protease production and 48% isolates were positive for cellulase enzyme production. Even if there is no work on groundnut rhizobia in production of different lytic enzymes in Ethiopia, similar result reported by Abera et al. (2018) that indicate 33 and 38% of soybean rhizobia isolated from Ethiopian soils showed protease and cellulase activity, respectively whereas, none of them utilized chitin. PGPR that able to produce these lytic enzymes are expected to have biocontrol property against a wide range of fungi and bacteria that are potentially pathogenic for the plant and led the crop to enhance crop yield.

Bacterial isolates for their production ability of hydrogen cyanide (HCN) was also screened. Of all the tested *Rhizobium* isolates (72) for Hydrogen Cyanide, only two isolates showed moderate and weak production (Table 1).

Irrespective of the types of host plants, out of 22-tested soybean isolates which isolated from Ethiopia soil only one isolate produced HCN (Getahun et al., 2022). Earlier, Antoun et al. (1998) have also reported that only 3% of rhizobial strains from different genera and species were found to produce HCN, implying the rare occurrence of HCN production among rhizobia species, indicating that PGP rhizobia are relatively inefficient in the production HCN (Mir et al., 2021b).

In general, presence or absence and intensity of Hydrogen Cyanide production can play a significant role in antagonistic potential of bacteria against phytopathogens.

Conclusion

The current study covers the way for the ideal selection of *Rhizobium* possessing multiple PGPR properties and confirmed antagonistic activity for their consistent performance on the growth and yield of groundnut. Selection of the appropriate rhizobial inoculant improves nitrogen fixation and food production. The present study was mainly taken up to screen and identify multiple PGPR trait producers of rhizobial isolates isolated from the major groundnut growing area of the country and to use it as a biofertilizer for stimulating the growth of groundnut. The ability of tested *Rhizobium* isolates exhibiting some PGP-properties, namely, IAA production, PSB, ammonia, HCN, lytic enzyme (protease, cellulase and chitinase) production and antagonistic activity evaluated under in vitro conditions. Accordingly, isolates such as GNR-37 and GNR-28 proved 85% efficiency followed by GNR-43, GNR-19 and GNR-which showed 75% efficiency performance from the all tested traits. Owing to this, the isolates that showed much multiple traits have a selective advantage for practical application to formulate an inoculant for groundnut.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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