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Developed *Rhizobium* Strains Enhance Soil Fertility and Yield of Legume Crops in Haryana, India

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ABSTRACT

Three strains of Gram-negative bacterium, *Rhizobium*, were developed by gamma (γ)-irradiation random mutagenesis. The developed strains were evaluated for their augmented features for symbiotic association, nitrogen fixation, and crop yield of three leguminous plants—chickpea, field-pea, and lentil—in agricultural fields of the northern Indian state of Haryana. Crops treated with developed mutants exhibited significant improvement in plant features and the yield of crops when compared to the control-uninoculated crops and crops grown with indigenous or commercial crop-specific strains of *Rhizobium*. This improvement was attributed to generated mutants, MbPrRz1 (on chickpea), MbPrRz2 (on lentil), and MbPrRz3 (on field-pea). Additionally, the cocultured symbiotic response of MbPrRz1 and MbPrRz2 mutants was found to be more pronounced on all three crops. The statistical analysis using Pearson's correlation coefficients revealed that nodulation and plant biomass were the most related parameters of crop yield. Among the effectiveness of developed mutants, MbPrRz1 yielded the best results for all three tested crops. Moreover, the developed mutants enhanced macro- and micronutrients of the experimental fields when compared with fields harboring the indigenous rhizobial community. These developed mutants were further genetically characterized, predominantly expressing nitrogen fixation marker, *nifH*, and appeared to belong to *Mesorhizobium ciceri* (MbPrRz1) and *Rhizobium leguminosarum* (both MbPrRz2 and MbPrRz3). In summary, this study highlights the potential of developed *Rhizobium* mutants as effective biofertilizers for sustainable agriculture, showcasing their ability to enhance symbiotic relationships, crop yield, and soil fertility.

1 | Introduction

Gram-negative bacteria Rhizobia are devoted to biological N_2 fixation (BNF) through symbiosis with leguminous plants. These bacteria fix atmospheric nitrogen (N_2) in the form of ammonia (NH_3) that plants can utilize and in turn supply a carbon-rich environment as an energy source [1]. This symbiotic interaction is of agronomic and ecological importance as the substantial amount of N_2 to the total N_2 budget in the terrestrial ecosystem [2]. It gains extreme practical importance

Abbreviations: BNF, biological N_2 fixation; CFU, colony-forming unit; CMC, carboxymethyl cellulose; CTAB, cetyltrimethylammonium bromide; EPS, exopolysaccharides; Kr, kiloroentgen; OD, optical density; YEMA, yeast extract mannitol agar.

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as chemical fertilizers are both costly and hazardous to the environment. The BNF has become one of the most attractive strategies for the development of sustainable agriculture [3]. Use of the biofertilizers can also preclude the depletion of soil organic matter [4]. The BNF ability of Rhizobia varies greatly among the host plants and the strains used [5].

Molecular codes are exchanged among the symbionts in the rhizospheric soils to select the crops specific to Rhizobia [6, 7]. Presently, there are about 17 genera of Rhizobia, belonging to either α- or β-proteobacteria classes; these can actively induce nodulation in the leguminous plants [6, 8]. The establishment of this symbiosis requires recognition of bacterial nodulation factors for successful infection of the respective legume roots [9]. This is a multidimensional developmental process that is driven by the bacterial exopolysaccharides (EPS) but is ultimately under the control of the host (plant) membrane-bound nodulation factor receptors [10]. The detection system for the compatibility between symbiotic partners includes an exchange of signaling molecules [11, 12]. Rhizobia penetrate plant roots through epidermal cells and are deposited inside the cell of a newly formed symbiotic organoid, the nodule [13]. Thus, Rhizobia–plant compatibility must be considered while selecting the best strain as a bioinoculant [14].

There are several changes in the extracellular oligosaccharide signals that are produced by Rhizobia, close to legume roots that can cause changes in legume host range [15, 16]. The carbohydrate component of the plant cell wall is usually predominant and it has been suggested that hydrolytic enzymes, such as cellulase and pectinase, are involved in the nodulation process [17, 18]. A central event in the development of Rhizobia–legume (root-nodule) symbiosis is the localized erosion of the cellulosic plant cell wall by which the bacterial symbiont passes to establish nitrogen fixation; the intracellular endosymbiotic state within the legume [19]. However, this activity (localized intracellular erosion) tends to be low in most of the *Rhizobium* species, and consequently, its impact on the nodulation event is hampered. One way to address this limitation is to have faith in the engineered Rhizobia, equipped with a high level of hydrolytic enzyme/s, whose activity could help to achieve maximum localized intracellular erosion and ultimately symbiotic nitrogen fixation.

Some studies show that genetic mutations induce infection and extend the host range to nonleguminous plants [20, 21]. Genetic exchange and mutations are the proven alternatives for the development of ideal inoculants with enhanced symbiotic properties [22, 23]. These types of promiscuous *Rhizobium* strains could be used as multi-legume biofertilizers [24]. The additional qualities required for a strain to be an optimum inoculant is that it must improve nodulation and consequently BNF rate. To outcompete the native or indigenous rhizobial community, it is also necessary to increase their competence in the rhizospheric soil. Therefore, in the present study, we aimed to develop superior strains of the bacterium *Rhizobium* by gamma (γ)-irradiation random mutagenesis and examined the effects of developed mutants on the crop yield of three leguminous plants (chickpea, field-pea, and lentil) and soil fertility in the experimental fields.

2 | Materials and Methods

2.1 | Soil Sampling, Isolation, and Screening of Indigenous *Rhizobium* Strains

The indigenous Rhizobia ($n = 295$) were isolated from the root nodules of three crops, chickpea (*Cicer arietinum* L.; $n = 160$), field-pea (*Pisum sativum*; $n = 70$), and lentil (*Lens culinaris*; $n = 65$) (Table 1). The root-nodule samples of these crops were collected from four different sites (Figure 1) in Haryana, India: (1) Bhiwani, 28°47′56.5656″ N, 76°8′0.6504″ E; (2) Hisar, 29°9′6.6996″ N, 75°43′ 16.0428″ E; (3) Pataudi, 28°19′31.69″ N, 76°46′42.89″ E; and (4) Siwani, 28° 9084° N, 75°6121″ E. Both soil and nodule samples were collected before the flowering season, from December 2019 to March 2020. From each sampling field, four soil samples were collected at a depth of 20 cm across two diagonals of the field and mixed per diagonal. Soil samples were initially characterized for the pH, total N, C (%), and P contents (Table 6).

To isolate indigenous Rhizobia, root samples of each crop were washed with tap water, and individual nodules were surface sterilized with 70% ethanol and 0.2% $HgCl_2$. After surface disinfection, root nodules were crushed with a sterile glass rod on a sterile glass plate, containing 1 mL sterile milli-Q water. *Rhizobium* colonies from the crushed nodules were isolated by streak-plate method on the modified yeast extract mannitol agar (YEMA) medium [25], containing per liter of distilled water: 0.5 g yeast extract, 10 g mannitol; 0.5 g KH_2PO_4; 0.2 g $MgSO_4 \cdot 7H_2O$; 0.1 g NaCl; 0.5 g K_2HPO_4; 0.06 g $CaCO_3 \cdot 2H_2O$; 15 g agar; and pH adjusted to 7.0. The plates were incubated at 28°C and single individual colonies were isolated by repeated streaking on the YEMA medium. All single bacterial colonies ($n = 295$) were analyzed for their morphology by Gram staining as per Alexandre, Laranjo, and Oliveira [26].

Further, these bacterial colonies were screened for the presence of hydrolytic enzymes (cellulase and pectinase) by analyzing

TABLE 1 | Collected crop samples (295) to isolate diverse indigenous *Rhizobium*.

Site of *Rhizobium*	Crop field	Harvested plants
Bhiwani	Chickpea	70
	Field-pea	20
	Lentil	25
Hisar	Chickpea	15
	Field-pea	20
	Lentil	10
Pataudi	Chickpea	40
	Field-pea	15
	Lentil	20
Siwani	Chickpea	35
	Field-pea	15
	Lentil	10

Note: Total samples of chickpea (160), field-pea (70), and lentil (65).

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FIGURE 1 | Isolation and screening of native *Rhizobium* strains from the root nodules of three leguminous crops from four different sites in Haryana, India. The root-nodule samples were collected from (1) Bhiwani, (2) Hisar, (3) Pataudi, and (4) Siwani. Native isolates mentioned on each site displayed superior hydrolytic enzymes (*cellulase* and *pectinase*) activity. The PRCP24 and PRCP37 were identified as *Mesorhizobium ciceri*, which were isolated following screening of 70 nodules of chickpea at Site 1. PRLN17 was identified as *Rhizobium leguminosarum*, which was isolated following the screening of 20 nodules of lentil at Site 1. PRCP6 and PRCP7 were identified as *M. ciceri*, which were isolated following screening of 40 nodules of chickpea at Site 2. PRP12 was identified as *R. leguminosarum*, which was isolated following screening of 25 nodules of field-pea at Site 3. PRP14 was identified as *R. leguminosarum*, which was isolated following screening of 35 nodules of field-pea at Site 4. PRCP11 was identified as *M. ciceri*, which was isolated following screening of 70 nodules of chickpea at Site 4.

TABLE 2 | Cellulose and pectin utilization ability of six *Rhizobium* isolates, selected for Gamma-irradiation random mutagenesis.

	Colony diameter (mm) on different bacteriological medium				
***Rhizobium* isolate**	**YEMA**	**GSY**	**MM**	**CMC**	**Pectin**
CP6A	6	6	5	5	5
CP7C	6	6	5	5	5
CP11A	7	6	5	5	5
FP12B	7	7	6	5	5
FP14A	8	7	7	5	5
LN7D	8	8	7	5	5

Abbreviations: CMC—carboxymethyl cellulose medium; CP—chickpea; FP—field-pea; GSY—glucose salts yeast extract; LN—lentil; MM—minimal medium; YEMA—yeast extract mannitol agar.

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their respective ability to utilize cellulose and pectin as sole carbon sources. For cellulase activity, colonies were initially scrutinized by their growth behavior on the cellulose agar media, composed of (per liter) carboxymethyl cellulose (CMC), 3 g; glucose, 0.2 g; yeast extract, 1 g; K_2HPO_4, 0.5 g; KH_2PO_4, 0.3 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $CaCl_2 \cdot 2H_2O$, 0.06 g; agar, 15 g; and pH adjusted to 6.8–7.2. The cellulose-degradation ability of each bacterial isolate ($n = 295$) was carried out by spotting on cellulose agar medium supplemented with Congo red dye with a composition (per liter), KH_2PO_4, 0.5 g; $MgSO_4$, 0.25 g; cellulose, 2 g; agar, 15 g; Congo red, 0.2 g; gelatine, 2 g; and pH adjusted to 6.8–7.2. Congo red dye was used as an indicator for cellulose degradation in the agar medium. Similarly, the pectin-degradation ability of each bacterial isolate was also checked out by spotting on the pectin agar medium with a composition (per liter), KH_2PO_4, 0.3 g; K_2HPO_4, 0.5 g; $MgSO_4$, 0.1 g; pectin, 3 g; agar, 15 g; glucose, 0.2 g; yeast extract, 0.1 g; $CaCl_2 \cdot 2H_2O$, 0.06 g; and pH adjusted to 6.8–7.2. Growth pattern and colony diameter were used as an indicator for pectin degradation in the agar medium. Colonies displaying a relatively bigger size of the clear zone on the Congo red plate ($n = 6$) were considered cellulase and pectinase producers (Table 2) as suggested by Lu et al. [27].

These six native isolates were further screened on different bacteriological media (Table 2) and tested for purity by analyzing intrinsic antibiotic sensitivity on the YEMA medium supplemented with different antibiotics as per Eaglesham's technique [28]. The selected antibiotics were chloramphenicol (20 and 50 μg/mL), ampicillin (50, 100, and 200 μg/mL), streptomycin (50, 100, 200, and 300 μg/mL), gentamicin (20 and 50 μg/mL), kanamycin (20 and 50 μg/mL), tetracycline (2 and 5 μg/mL), cephalotaxine (20 and 50 μg/mL), rifampicin (20 and 50 μg/mL), erythromycin (20 and 50 μg/mL), and sulfanilamide (20 and 50 μg/mL). Filter-sterilized aliquots of each antibiotic were added aseptically to the sterile YEMA medium at 50°C to give the final concentrations. The pure cultures showing sensitivity to these antibiotics were maintained on YEMA slants and preserved at 4°C for temporary storage.

2.2 | γ-Irradiation Random Mutagenesis on Selected Indigenous Rhizobia and Their Screening

Following the rigorous bacteriological screening of 295 isolates, the aforenoted six scrutinized isolates (Table 2) were chosen for γ-irradiation random mutagenesis. For mutagenesis, the aliquots containing these bacterial isolates were exposed to γ-irradiations for 2 h, 3 h 30 min, and 6 h 30 min at an emission rate (in kiloroentgen; Kr) of 1 Kr per 20 min. These irradiations correspond to 6, 10, and 20 Kr [29]. Following these irradiations, viable bacterial counts of all these treatments were performed and represented as the percentage of survival or colony-forming unit (CFU). Longer exposure to γ-irradiations yielded fewer viable cells (Table 3).

TABLE 3 | Features of Gamma-irradiated developed mutants of *Rhizobium*.

Gamma-radiation dose (kiloroentgen; Kr)	Gamma-irradiated mutant	CFU/mL	Survival (%)	Colony diameter (mm) on different media		
				CMC	Pectin	YEMA
20	H-PrRz6A	12×10^6	1.57	7	7	7
	H-PrRz7C	20.6×10^6	1.19	7	7	7
	H-PrRz11A	25.4×10^6	1.36	7	6	6
	H-PrRz12B	21×10^6	1.98	7	6	6
	MbPrRz2	17.8×10^6	1.56	8	7	6
	MbPrRz3	5.6×10^6	0.29	8	7	6
10	M-PrRz6A	29.3×10^6	3.85	7	6	6
	M-PrRz7C	31×10^6	1.79	7	6	6
	MbPrRz1	36×10^6	1.93	8	7	6
	M-PrRz12B	20.9×10^6	1.97	7	6	6
	M-PrRz14A	23×10^6	2.01	6	6	6
	M-PrRz7D	14×10^6	0.72	6	6	6
6	L-PrRz6A	117×10^6	1.53	6	6	6
	L-PrRz7C	130×10^6	0.75	7	6	6
	L-PrRz11A	97×10^6	5.21	6	6	6
	L-PrRz12B	48×10^6	4.52	6	6	6
	L-PrRz14A	82×10^6	7.19	7	6	6
	L-PrRz7D	27×10^6	1.39	6	6	6

Note: Highlighted mutants were selected for field experimentation and subsequent characterization.

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The live bacterial mutants ($n = 800$) were further screened for their growth on the YEMA medium, and enzyme activities of cellulase and pectinase on the corresponding CMC and pectin media. The cellulase activity of these mutants was examined quantitatively by calculating the ratio of the diameter of the clearing zone to the size of the colony as per Hendricks, Doyle, and Hugley [30].

The majority of the mutants showed less growth on both CMC and pectin media. Out of 800 mutants, 18 superior growing isolates were tested and compared with crop-specific commercial strains, *Mesorhizobium* CH-21 (for chickpea), *Rhizobium* LN-65 (for lentil), and *Rhizobium* P-34 (for field-pea) again on CMC and pectin media for the size of colony and diameter of the clearing zone with respect to growth on the YEMA medium (Table 3). As per the ratio of the diameter of the clearing zone to the colony size, three mutants, MbPrRz1, MbPrRz2, and MbPrRz3, were selected for the field experimentation. These mutants were developed at corresponding doses of γ-irradiation, at 10 Kr (MbPrRz1) and 20 Kr (MbPrRz2 and MbPrRz3), and originated from native rhizobial communities of chickpea, field-pea, and lentil, respectively.

2.3 | Inoculum of Commercial and Developed Strains for Field Experimentation

Culture inoculum for the developed mutants (MbPrRz1-3) and for the commercial crop-specific strains (*Mesorhizobium* CH-21 for chickpea, *Rhizobium* LN-65 for lentil, and *Rhizobium* P-34 for field-pea) was prepared by growing them individually in the YEM medium on a rotary shaker with agitation (150 rpm) for 2–3 days at 28°C. For field experimentation, the optical density (OD 600 nm) of each culture was adjusted to 0.3 by using a spectrophotometer (ThermoFisher Scientific, A5119500C).

2.4 | Field Experimental Design

To evaluate the performance of three selected mutants, a field experiment was conducted from November to April (known as Rabi season) of the year 2019–2020. The agricultural field (40 m^2) was marked at Chaudhary Charan Singh Haryana Agricultural University (CCS HAU), Hisar, India. The location of this agricultural field was 29°8′36.9″ N, 75°42′26.6″ E at an altitude of 215.2 m above mean sea level in the northwest plain region of Haryana, India. The field experiment was laid out in a random block design with three replicates. A total of 63 plots (1.8 m^2 each) were made with 0.5 m spacing. A standard package of agricultural practices as per the Government of Haryana, India was followed throughout. The experimental field was plowed twice after harvesting the previous crop. No basal dose of any fertilizer was applied at the time of field preparation. Three crops—chickpea, field pea, and lentil—were raised in respective rows with 30 seeds per line for each replicate.

2.5 | Commercial Crop Seeds and Crop-Specific Rhizobia

Seeds of chickpea (HC1), field-pea (HFP4), and lentil (Garima) were procured from the pulse section at CCS HAU, Hisar, India. These seeds were surface sterilized with 70% ethanol for 10 s, followed by 5 min treatment with 0.1% $HgCl_2$ solution. All seeds were then washed with sterile distilled water. The in vitro grown culture of the developed mutants (10^8 cells/mL) and the crop-specific strains were separately mixed with a 10% sterile solution of jaggery. The slurry was then poured over the pre-sterilized seeds and mixed in a way to make a thin layer around the seeds. Uninoculated seeds of each crop served as negative controls. The treated seeds were sown in the experimental field area.

2.6 | Measurement of Plant Growth Features

Five plants (random selection) per plot, resulting in 15 plants (of each crop) per treatment, were examined for the plant growth parameters, and the yield of each crop was evaluated from the entire corresponding plot. Sampling was performed at two stages of the growth cycle: (1) in the vegetative phase, corresponding to approximately 65 days after planting, and (2) in the reproductive phase, corresponding to approximately 90 days after planting. Plant growth features, that is, height, fresh shoot and root weight, number of nodules, number of branches, and number of pods were counted during each crop cycle. The number of nodules was determined immediately after the sampling. Shoots and roots were subsequently dried at 65°C for 3 days to determine their dry weight. Crops were harvested when plants and seeds were dried, approximately 110 days after planting. Crops were removed completely and the number of plants per plot was counted. Seeds were sun-dried for 2 days and seed dry weight per plot was determined.

2.7 | Molecular Characterization of Developed Rhizobia

Genomic DNA (gDNA) from the selected isolates of three developed mutants was isolated and purified using the CTAB lysis method [31]. The concentration of isolated gDNA was measured by a Nanodrop spectrophotometer (NanoDrop 2000/2000c ThermoScientific) and stored at −80°C till further use. The gDNA was used for PCR amplification of the *nifH* gene with primer pair, *nifH*19F and *nifH*407R [32]. Subsequently, a second PCR reaction was set up to amplify the 16S rRNA gene from the same gDNA using primer pair, 27F and 1492R [33].

For each PCR, a 25 μL reaction was set up on ice that contained 10× *Taq* DNA polymerase reaction buffer, 100 mM of dNTP mix (dATP, dCTP, dTTP, and dGTP), 100 ng of respective forward and reverse primer, and 1.0 U of *Taq* DNA polymerase (Promega). The PCR was carried out in a Thermal cycler (MyCyler, Bio-Rad) with the following parameters, initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 35 s, annealing at 52°C for 40 s, extension at 72°C for 50 s, and a final extension step (one cycle) at 72°C for 7 min. The amplified PCR product was electrophoresed on agarose gel (1.5%) and a 16S rRNA band was excised from the gel at ~1.5 kb and purified using a gel extraction kit (ThermoFisher Scientific).

Purified DNA was sequenced using 27F and 1492R sequencing primers (Eurofins Genomics, India). The obtained nucleotide

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sequences were analyzed by the NCBI BLASTn server. Similar sequences ($n = 60$) up to the species level were retrieved and further aligned using CLUSTALW of the MEGA 11.0 software [34]. A phylogenetic tree was constructed using the maximum likelihood method with 1000 bootstrap values. All analyzed sequences were submitted to NCBI GenBank with accession numbers OQ121148, OQ121149, and OQ121150.

2.8 | Statistical Analysis

Analyzed data represents the average of three biological replicates. Values from each experiment were initially subjected to the normalization test and the homogeneity of variance for each variable. Subsequent analysis of the variance (ANOVA) was calculated using SPSS statistical software (IBM, USA). A p-value ≤ 0.05 was considered statistically significant. One-way ANOVA was carried out for each parameter. Treatment means were compared using Tukey's HSD as a post hoc significance test (SPSS24.0, Lead Technology, Inc). The correlation coefficient among the growth parameters was determined using Pearson's method.

3 | Results

3.1 | Isolation and Characterization of Native Rhizobia

Native Rhizobia ($n = 295$) from four different sites in the state of Haryana, India (Figure 1) were isolated from the nodules of three leguminous crops—chickpea, field-pea, and lentil (Table 1). These isolates, 160 (from chickpea), 70 (from field-pea), and 65 (from lentil) were characterized for their fundamental physiological and biochemical properties as per Bergey's Manual of Systematic Bacteriology [35]. Eighteen of 295 isolates showed cellulase and pectinase activity as assessed by a clear zone around each bacterial colony. Based on their superior growth features, that is, large colony size and a clear zone around respective colony—an indicator of presence of hydrolytic enzymes, especially cellulase and pectinase on complex carbohydrate medium (CMC and pectin), six isolates—three from chickpea (CP6A, CP7C, and CP11A), two from field-pea (P12B and P14A), and one from lentil (LN7D) were selected (Table 2) for the subsequent purity analysis by checking antimicrobial sensitivity profile of each. These isolates were found sensitive to ampicillin (50, 100, and 200 μg/mL), tetracycline (2 and 5 μg/mL), cephalotaxine (20 and 50 μg/mL), and rifampicin (20 and 50 μg/mL), but resistant to chloramphenicol (20 and 50 μg/mL), streptomycin (50, 100, 200, and 300 μg/mL), gentamicin (20 and 50 μg/mL), kanamycin (20 and 50 μg/mL), erythromycin (20 and 50 μg/mL), and sulfanilamide (20 and 50 μg/mL). All these six isolates were subjected to γ-irradiation random mutagenesis.

3.2 | Development and Characterization of *Rhizobium* Mutants

To develop better *Rhizobium* mutants for each crop, the aforementioned six indigenous isolates were exposed to γ-irradiation with three doses: 6, 10, and 20 Kr. Following these irradiations, viable bacterial counts of all treatments were performed. The survival rate of these isolates went down as the radiation dose and/or exposure time increased; roughly > 95% killing was observed in the highest radiation dose and the longer exposure (Table 3). The live bacterial mutants ($n = 800$) were further screened for their growth on the YEMA medium enzymatic activity (for cellulase and pectinase) on respective CMC and pectin medium.

The cellulase activity of these mutants was examined quantitatively by calculating the ratio of the diameter of the clearing zone to the size of the colony as per Hendricks, Doyle, and Hugley [30]. The majority of these mutants showed inferior growth on both pectin and cellulose medium. Out of 800 mutants, 18 relatively superior growing mutants were retested on the CMC and pectin medium for the colony size and diameter of the clearing zone with respect to the growth on the YEMA medium (Figure 2 and Table 3). Based on the ratio of the diameter of the clearing zone to the size of the colony, three mutants, MbPrRz1−3 from the aforementioned 18 mutants, were selected for subsequent studies. These mutants were developed at corresponding doses of γ-irradiation, at 10 Kr (MbPrRz1) and 20 Kr (MbPrRz2 and MbPrRz3), and originated from native Rhizobia of chickpea, field-pea, and lentil, respectively. Before evaluating their efficacy either in the greenhouse or in the experimental fields, these mutants were scrutinized again for the retention of enzymatic activity (for cellulase and pectinase) in comparison to the corresponding native isolate. The developed mutants showed better growth and enhanced enzymatic activity (for both pectinase and cellulase) in comparison to respective native isolates (Figure 2B–D). Following confirmation of enhanced enzymatic activity, the symbiotic infection behavior and cross-infectivity of each mutant were evaluated on three selected legume plants under controlled greenhouse conditions (data not shown) and then in the experimental fields.

3.3 | Developed *Rhizobium* Mutants Improved Nodulation and Plant Features of the Legume Crops

Symbiotic infection behavior and cross-infectivity of the developed mutants were evaluated on chickpea, field-pea, and lentil plants under field conditions. The chosen legume hosts were inoculated with developed mutants, MbPrRz1, MbPrRz2, and MbPrRz3, and crop-specific commercial strains of Rhizobia (*M. ciceri* CH-21 for chickpea, *Rhizobium leguminosarum* P-34 for field-pea, and *R. leguminosarum* LN-65 for lentil) as respective positive control. Following 65 days of sowing, the plant's height was recorded. The average height of each plant treated with mutant strains was found higher than that of the negative control (uninoculated plants). The evaluated plant-growth parameters showed the positive effect of the inoculum that contained developed mutants. In the case of primary branch numbers, all crops showed more or comparable results in all treatments. Additionally, the plant's height was most significantly increased in the lentil crop (Table 4). Nodule numbers from the control plants (uninoculated) indicate the efficacy of the indigenous community of Rhizobia to nodulate roots of the leguminous crops. However, nodulation

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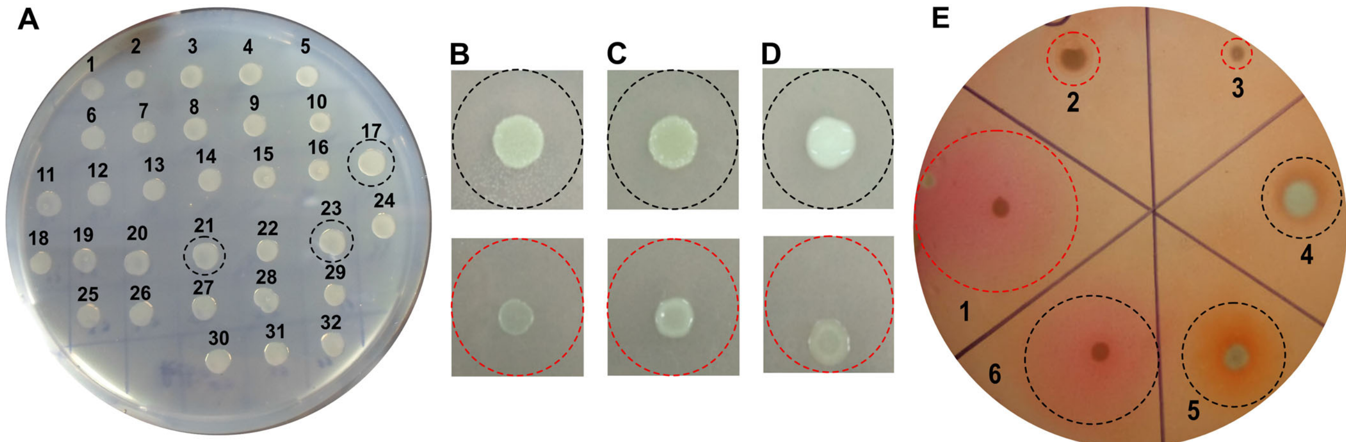

FIGURE 2 | Screening of Gamma-irradiated developed *Rhizobium* strains on complex carbohydrate media. (A) Screening of 32 mutants on CMC medium for colony size and carbohydrate utilization ability. Dotted encircled represents *Rhizobium* strains, MbPrRz1 (17), MbPrRz2 (21), and MbPrRz3 (23). (B–D) Representative growth profile (in black dotted circle) of developed mutant strains, MbPrRz1 (B), MbPrRz2 (C), and MbPrRz3 (D), on pectin media, along with respective commercial crop-specific control strain (in red dotted circle) of Rhizobia, *Mesorhizobium ciceri* CH-21 for chickpea, *Rhizobium leguminosarum* P-34 for field pea, and *R. leguminosarum* LN-65 for lentil. (E) Representative growth profile of developed MbPrRz1 (4), MbPrRz2 (5), and MbPrRz3 (6) mutants, along with respective commercial crop-specific strains (control; 1–3) of *Rhizobium* on Congo red plate indicating clear zones (encircled in dotted lines) upon utilization of complex carbohydrates.

was improved most strongly by the developed mutants. The developed strains showed a significant increase ($p < 0.05$) in the nodule numbers and plant biomass when compared to those inoculated only with the commercial strains and the uninoculated plants. There was a clear difference between the developed mutants and the crop-specific commercial strains in their response to the plant growth. Furthermore, the positive effect of the developed mutants on chickpea was much higher than that of field-pea and lentil (Table 4).

3.4 | Developed *Rhizobium* Mutants Augmented the Yield of Legume Crops

As observed for other evaluated parameters, it was consistently found that mutant-inoculated treatments performed better for the nodule numbers and yield per nodule as compared to uninoculated crops (having only native rhizobial community) or crops grown with commercial crop-specific Rhizobia (*M. ciceri* CH-21 for chickpea, *R. leguminosarum* P-34 for field-pea, and *R. leguminosarum* LN-65 for lentil). In general, crops inoculated with developed mutants were more productive. An interesting profile of the crop yield per nodule was observed (Figure 3). The developed mutants, MbPrRz1 and MbPrRz2, significantly elevated the crop yield for field-pea in comparison to negative control (uninoculated crop), and the chickpea crop yield was boosted by both MbPrRz2 and MbPrRz3 mutants; this is also again in comparison with uninoculated chickpea crop (Figure 3). The lentil crop yield was significantly enhanced by all three developed mutants when compared with the yield from the commercial strains tested on each crop (Figure 3). Additionally, the chickpea yield was more pronounced by MbPrRz3 mutant when compared with commercial strains for the field-pea crop. The yield of field-pea was highest by MbPrRz2 mutant when compared with negative and/or commercial strains (Figure 3).

Statistical analysis using Pearson's correlation coefficients, between crop yield and plant parameters (evaluated during the growth cycle), showed the impact of nodule numbers on yield variation of each tested crop (Table 5). As per the obtained data, the shoot weight and number of pods and nodules were found the most related parameters of the crop yield. This could indicate that nodulation contributes to the growth at an early developmental stage of the plant. All the parameters were significantly correlated in chickpea and field-pea but in the case of lentils, only the root weight and number of pods were significantly correlated. It was observed that at the end of the growth cycle, nodules had disappeared. However, they showed more positive correlation values at 90 days of the growth cycle instead of 65 days. In general, better results were obtained by MbPrRz1 and MbPrRz2. In addition, it was obvious that for all three crops, mutant MbPrRz1 generated the highest response. The respective highest plant height and shoot weight for each crop were as follows: 73.66 cm and 55.40 g (for chickpea), 52.26 cm and 18.07 g (for lentil), and 82.83 cm and 40.22 g (for field-pea). Moreover, this treatment helped to obtain the highest crop yield for two of the three tested crops. For chickpea, the yield was about 2.78 kg/plot while for field-pea the yield was about 2.08 kg/plot. Hence, it can be concluded that MbPrRz1 was foremost among all the treatments for nearly all evaluated parameters of three crops (Figure 3 and Tables 4 and 5).

3.5 | Developed *Rhizobium* Mutants Augmented Fertility of the Experimental Soils

A total of four soil samples were collected and analyzed for their physiochemical properties, including available organic carbon (C), potassium (K), phosphorus (P), salinity, and pH (Table 6). The initial organic matter and nitrogen (N) were found lower than standards, representing poor fertility of the experimental soils (Table 6). The post-experimental values (after plantation) of the field showed an increased total nitrogen and other

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TABLE 4 | Effect of developed *Rhizobium* strains on the growth features of chickpea, lentil, and field-pea.

	Height (cm)	Number of branches	Shoot weight (g)	Root weight (g)	Number of pods	Number of nodules	Yield (kg/plot)
Chickpea							
Negative control	67.13 ± 1.76	6.93 ± 0.43	44.30 ± 1.97	1.91 ± 0.20	35.26 ± 2.53	27.06 ± 4.83	2.06 ± 0.21
Chickpea commercial	70.73 ± 2.32	7.46 ± 0.58	50.94 ± 1.60	2.42 ± 0.33	43.40 ± 3.11	32.86 ± 2.83	2.36 ± 0.17
MbPrRz1	73.66 ± 1.71	7.63 ± 0.72	55.40 ± 1.98	2.79 ± 0.36	45.00 ± 2.49	41.53 ± 2.16	2.78 ± 0.15
Lentil commercial	68.26 ± 0.83	6.96 ± 0.44	41.96 ± 1.61	1.91 ± 0.22	36.40 ± 1.00	26.13 ± 2.90	2.17 ± 0.10
MbPrRz2	71.46 ± 1.63	7.10 ± 0.40	46.87 ± 1.82	2.16 ± 0.20	40.60 ± 2.21	33.33 ± 1.10	2.80 ± 0.12
Field-pea commercial	66.20 ± 1.10	6.46 ± 0.52	44.44 ± 1.47	2.00 ± 0.11	35.93 ± 1.23	30.80 ± 1.20	2.15 ± 0.35
MbPrRz3	67.92 ± 0.96	6.85 ± 0.27	45.36 ± 2.24	2.57 ± 0.15	39.70 ± 0.36	29.53 ± 3.01	2.42 ± 0.29
F-test	*	NS	*	NS	NS	NS	*
LSD 0.05	0.062		0.08				0.47
Lentil							
Negative control	44.40 ± 2.54	6.20 ± 0.80	14.97 ± 0.93	0.28 ± 0.01	69.60 ± 2.98	12.13 ± 2.63	1.06 ± 0.13
Chickpea commercial	43.53 ± 1.16	6.26 ± 0.66	14.41 ± 0.94	0.25 ± 0.01	66.46 ± 1.24	15.46 ± 3.34	1.01 ± 0.11
MbPrRz1	52.26 ± 1.74	7.06 ± 0.99	18.07 ± 1.02	0.32 ± 0.01	79.00 ± 1.44	18.06 ± 0.63	1.36 ± 0.14
Lentil commercial	48.40 ± 1.17	7.93 ± 0.54	13.92 ± 1.08	0.30 ± 0.02	75.20 ± 3.17	17.00 ± 0.50	1.06 ± 0.12
MbPrRz2	51.43 ± 1.18	6.86 ± 0.74	15.62 ± 1.95	0.34 ± 0.02	79.46 ± 1.23	23.93 ± 4.30	1.12 ± 0.07
Field-pea commercial	44.00 ± 0.70	7.53 ± 0.40	13.26 ± 1.06	0.26 ± 0.02	68.60 ± 2.00	16.13 ± 2.17	1.04 ± 0.02
MbPrRz3	45.80 ± 0.71	7.19 ± 0.50	13.20 ± 0.61	0.26 ± 0.11	66.48 ± 1.57	12.66 ± 2.25	1.09 ± 0.82
F-test	*	NS	NS	*	*	NS	NS
LSD 0.05	0.32			0.015	0.11		
Field-pea							
Negative control	70.93 ± 3.18	5.40 ± 0.11	34.10 ± 0.76	0.60 ± 0.07	24.00 ± 2.22	28.46 ± 3.12	1.36 ± 0.08
Chickpea commercial	79.06 ± 2.78	5.73 ± 0.13	36.78 ± 1.83	0.72 ± 0.05	25.53 ± 2.07	25.40 ± 4.54	1.55 ± 0.16
MbPrRz1	82.53 ± 2.14	5.93 ± 0.24	40.22 ± 1.28	0.74 ± 0.05	31.06 ± 2.52	33.13 ± 3.58	2.08 ± 0.07
Lentil commercial	75.66 ± 0.92	5.40 ± 0.11	34.33 ± 2.35	0.69 ± 0.06	26.80 ± 3.60	30.73 ± 3.39	1.72 ± 0.19
MbPrRz2	79.40 ± 0.87	6.10 ± 0.26	36.03 ± 2.95	0.74 ± 0.03	32.46 ± 3.29	22.26 ± 1.68	1.76 ± 0.09
Field-pea commercial	76.53 ± 3.12	5.36 ± 0.12	33.61 ± 2.36	0.63 ± 0.09	28.06 ± 2.65	29.80 ± 3.81	1.62 ± 0.18
MbPrRz3	82.54 ± 1.77	7.36 ± 0.29	38.89 ± 1.45	0.74 ± 0.51	32.54 ± 1.58	41.06 ± 3.06	1.92 ± 0.16
F-test	*	NS	NS	NS	NS	*	NS
LSD 0.05	0.043					0.025	

Note: Negative ctrl—crop with indigenous *Rhizobium*; commercial—crop grown with plant-specific commercial strain of *Rhizobium*.
*The mean difference is significant at the 0.05 level.

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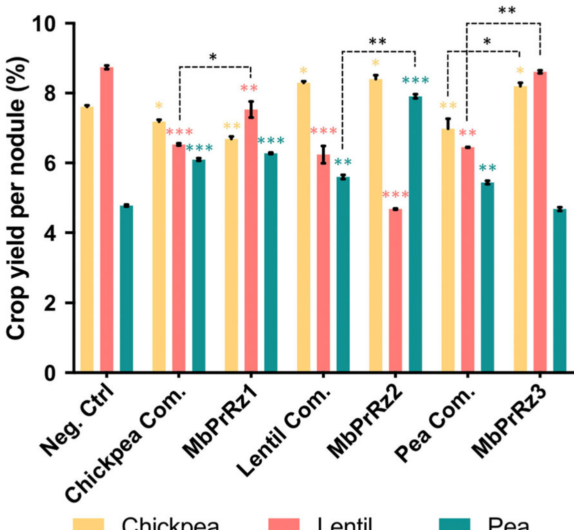

FIGURE 3 | Effect of commercial crop-specific and developed Rhizobia mutants on the crop yield of three leguminous plants. The crop yield of the indicated plants (in percentage) was calculated per nodule following growing each crop with respective commercial or developed rhizobial strains. Neg. ctrl: crops grown with indigenous/native community of *Rhizobium*; MbPrRz1, MbPrRz2, and MbPrRz3: gamma-irradiated developed rhizobial mutants*;* comr.*:* commercial rhizobial strain for the corresponding crop. Two-tier analysis was performed to calculate the crop yield; Tier-1: effect of developed and commercial rhizobial mutants and strains in comparison with crops grown within the indigenous/native community of Rhizobia (neg. ctrl), and Tier-2: enhanced crop yield by the developed strains in comparison with respective commercial (comr.) strains. For clarity, only the significantly enhanced yield of the respective crop by the developed strains is indicated. Bars represent the mean ± standard deviations. Two-tailed correlation significance at $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

factors in the soils treated with either commercial or three developed rhizobial mutants (Table 6). Results also revealed that crop rotation enhanced soil's carbon content and other features in comparison to continuous-crop monoculture.

3.6 | Genetic and Molecular Characterization of Developed *Rhizobium* Mutants

Genetic and molecular characterization of the developed rhizobial mutants was carried out by PCR amplification of *nifH* (molecular marker for the BNF) and 16S *rRNA* (phylogenetic marker) genes (Figure 4). The developed mutants showed consistent expression profiles for both genetic markers, indicating an efficient BNF ability (*nifH*-mediated) and phylogenetic closeness (16S-mediated) to other strains of *Rhizobium* (Figure 4). Based on the nucleotide sequence similarity of the 16S rRNA gene, available on the NCBI nucleotide GenBank, the phylogeny of developed MbPrRz1-3 strains was analyzed. A phylogenetic tree was constructed with evolutionary closed bacterial strains (Figure 5). The MbPrRz1 strain was found to be *M. ciceri* (98.8% nucleotide identity) and the MbPrRz2 and MbPrRz3 strains appeared to belong to *R. leguminosarum* with corresponding 98.4% and 97.8% nucleotide identity. All these sequences were submitted to NCBI GenBank with accession numbers, OQ121148 (*R. leguminosarum* PWT; MbPrRz2), OQ121149 (*R. leguminosarum* LWT; MbPrRz3), and OQ121150 (*M. ciceri* CWT; MbPrRz1).

4 | Discussion

Three *Rhizobium* mutants (MbPrRz1, MbPrRz2, and MbPrRz3) were developed and characterized to enhance the growth and crop yields of chickpea, field-pea, and lentil. These mutants originated from the corresponding crop's native *Rhizobium* community and were developed using γ-irradiation at 10 Kr (MbPrRz1) and 20 Kr (MbPrRz2 and MbPrRz3). γ-Rays are high-energy electromagnetic ionizing radiations that can induce gene mutation/s and chromosomal breaks. The frequency of these aberrations is directly proportional to the applied dose. In the present study, lower doses were found favorable while higher doses were detrimental as the survival percentage of the bacterial colonies went significantly down as the radiation dose increased. Thus, it can be concluded that the effect of radiation was accumulative disruptive. The developed mutants exhibited improved growth and increased hydrolytic enzymatic activity (for pectinase and cellulase) compared to their native isolates (Figure 2B–D), indicating enhanced biological/symbiotic nitrogen fixation (BNF) ability of these mutants. While hydrolytic enzymes (pectinase and cellulase) play a crucial role in the central event of infection during symbiosis [36], host choice and BNF behave as independent traits [37].

Following the confirmation of enhanced enzymatic activity, the ability for symbiotic infection behavior and cross-infectivity of these mutants were examined on three selected legume plants in the greenhouse and subsequently in field conditions. The developed mutants showed significant potential to increase the yield of legume crops under field conditions, potentially reducing the need for chemical fertilizers for sustainable agriculture. These mutants were genetically characterized based on the gene-expression profile of invariant *nifH* and 16S rRNA genes. The highly conserved nature of *nifH* makes it an ideal molecular marker for the BNF ability of Rhizobia isolated from diverse plants in different environments [38, 39]. Notably, in the public domain, particularly NCBI GenBank, *nifH* constitutes the largest number of *Rhizobium* sequences. Robust expression of both molecular markers, *nifH* and 16S *rRNA* genes, among these mutants indicate that these mutants could be equipped with a durable BNF ability. Based on the sequence similarity of the 16S rRNA gene, MbPrRz1 was identified as *M. ciceri* (98.8% nucleotide identity), while MbPrRz2 and MbPrRz3 both were classified as *R. leguminosarum* with corresponding 98.4% and 97.8% nucleotide identity. The *M. ciceri* strain Ca181 has been previously isolated from soils in Hisar, Haryana, India, and has been completely sequenced [39].

For field experimentation, fields with no legume crops in the past 5 years were selected, as continuous cultivation of legumes builds up the indigenous *Rhizobium* community in the soil [40–43]. The soil's physiochemical properties were determined

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TABLE 5 | Pearson's correlation coefficient analysis of different growth parameters of tested crops.

Crop	Parameters	Height	Branches	Shoot weight	Pods	Root weight	Nodules	Yield
Chickpea	Height	1						
	Branches	0.440*	1					
	Shoot weight	0.623**	0.314	1				
	Root weight	0.477*	0.290	0.662**	1			
	Pods	0.628**	0.513*	0.624**	0.653**	1		
	Nodules	0.332	0.317	0.532*	0.565**	0.492*	1	
	Yield	0.483*	0.138	0.264	0.299	0.441*	0.563**	1
Lentil	Height	1						
	Branches	0.123	1					
	Shoot weight	0.274	0.057	1				
	Root weight	0.612**	−0.151	0.493*	1			
	Pods	0.684**	0.151	0.392	0.783**	1		
	Nodules	0.549**	−0.109	−0.087	0.414	0.508*	1	
	Yield	0.187	0.205	0.501*	0.359	0.526*	0.199	1
Field-pea	Height	1						
	Branches	0.431	1					
	Shoot weight	0.341	0.484*	1				
	Root weight	0.417	0.272	0.413	1			
	Pods	0.660**	0.379	−0.027	0.283	1		
	Nodules	0.019	0.441*	0.127	0.222	−0.004	1	
	Yield	0.479*	0.493*	0.333	0.254	0.631**	0.124	1

Note: Two-tailed correlation significance at 0.05 (* $p < 0.05$) and 0.01 (** $p < 0.01$).

TABLE 6 | Physiochemical properties of the experimental field soil samples.

Soil's features	Soil sample	pH	EC (mS/cm)	C (%)	N (kg/ha)	P (kg/ha)	K (kg/ha)
Before plantation	Sample-1	8.0	0.16	0.52	119	25	200
	Sample-2	8.0	0.16	0.67	112	22	181
	Sample-3	8.3	0.14	0.60	112	27	152
	Sample-4	7.8	0.19	0.45	105	25	133
After plantation with	Commercial *Rhizobium*	8.0	0.87	0.67	133	22	300
	MbPrRz1	7.8	1.67	0.60	140	25	200
	MbPrRz2	7.7	0.22	0.75	154	27	162
	MbPrRz3	8.0	3.18	0.67	147	25	388

Abbreviations: C—carbon; EC—electrical conductivity; K—potassium; N—nitrogen; P—phosphorus.

before and after field experimentation to assess the impact on soil fertility. The mean nitrogen varied from 133 to 154 kg/ha of soil at the end of experimentation, explaining the improvement in soil nitrogen (N) levels after treatment with developed mutants (Table 6). Earlier studies support the necessity of legume inoculation with effective *Rhizobium* inoculants, especially in nitrogen-deficient soils [41–46]. Our field data demonstrate the promising effects of these isolates on leguminous crop yields, indicating the favorable symbiotic efficiency of these mutants (Figure 3 and Table 4).

Improved nodule numbers serve as a reliable indicator of symbiotic efficiency or BNF [47]. Variations in nodule numbers per plant and yield per plot in our study underscore the varying response of each legume variety to *Rhizobium* strains. Farid and Navabi also found a positive correlation between increased nodule numbers and symbiotic efficiency [48]. Our developed mutants exerted a constructive influence on the yield of the three tested crops (Figure 3). Despite not irrigating the plants throughout the growth cycle, considering the relatively low average precipitation, enhanced nodulation may be linked to increased nitrogen content and grain yield. Specifically, in chickpea, the number of nodules significantly correlated ($p \leq 0.01$) with yield, confirming its vital role in plant growth. Similarly, Yang et al. observed inconsistent nitrogen fixation in field-pea plants inoculated with Rhizobia, suggesting the need

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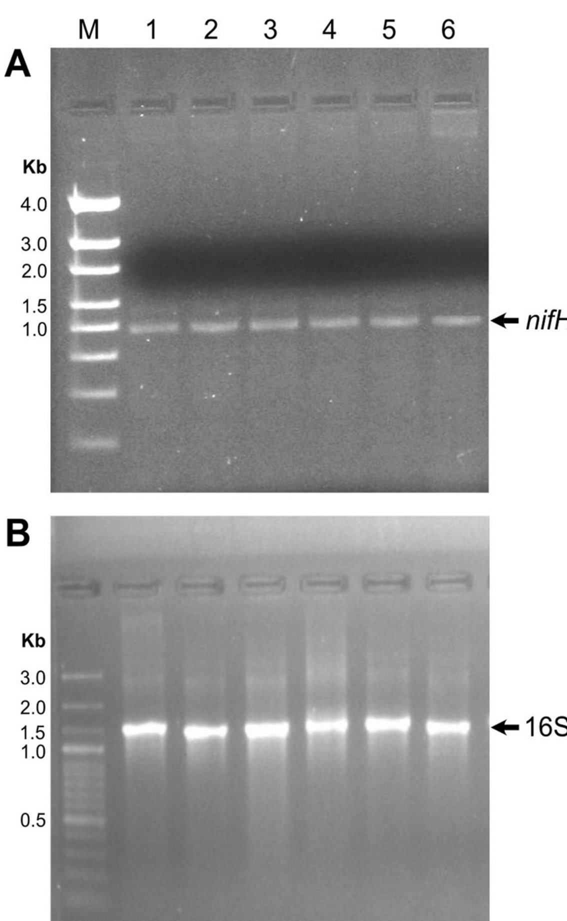

FIGURE 4 | PCR amplification of *nifH* (A) and 16S *rRNA* (B) genes. M: DNA marker (New England Biolabs; 1 kb DNA ladder). Lanes 1–6: Amplified PCR product from the developed strains, 1. MbPrRz1; 2. MbPrRz2; 3. MbPrRz3; and commercial strains, 4. *Mesorhizobium ciceri* CH-21 for chickpea; 5. *R. leguminosarum* LN-65 for lentil; and 6. *R. leguminosarum* P-34 for field-pea. The molecular size (in kb) of *nifH* and 16S *rRNA* is indicated by respective arrows.

for crop breeding programs alongside biofertilizer application to improve legume crop yields [49]. Selection and inoculation of the predominant chickpea Rhizobia have also been shown to enhance nodule occupancy, nitrogen fixation, and grain yield of chickpea under field conditions [50].

A substantial increase in the dry weight of roots and shoots was observed in the presence of developed mutants (Tables 4 and 5). Notably, MbPrRz1 exhibited the highest average dry weight for both shoots and roots compared to commercial *Rhizobium* inoculation. Significant variations in plant growth in response to *Rhizobium* inoculation have been observed in legumes under different growth conditions [51–55]. Previous reports also highlight the positive effects of rhizobial inoculations on plant growth and legume yield [41–43].

Comparison of our developed strains with the negative control and commercial crop-specific rhizobial inoculant strains (*M. ciceri* CH-21 for chickpea, *R. leguminosarum* P-34 for field-pea, and *R. leguminosarum* LN-65 for lentil) revealed a significant difference in yield at a 95% confidence level. This suggests

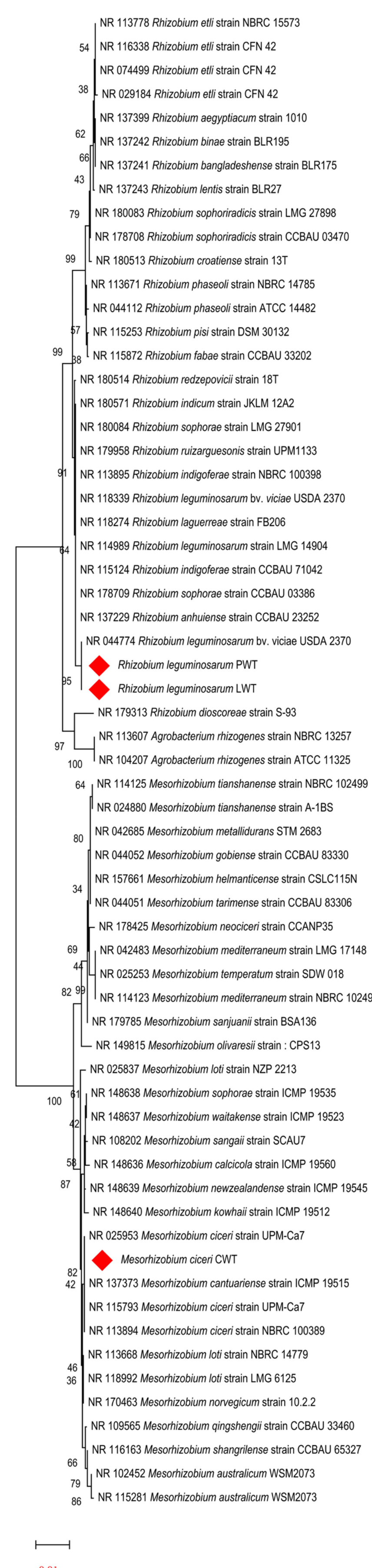

FIGURE 5 | Legend on next page.

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variations in host responsiveness to developed strains among legumes and soil types, with potential implications for agronomic production. Our results clearly show that the developed strains/mutants achieved higher mean scores in terms of crop yield. In earlier tests on CMC and pectin media, we observed better growth in terms of colony size and clearing diameter for the developed mutants compared to commercial strains. The increased plant biomass and yield further confirm the competitiveness of promiscuous strains against commercial and indigenous strains. However, Tena, Wolde-Meskel, and Walley demonstrated that native *Rhizobium* communities typically compete with inoculant *Rhizobium* [56]. Some growth parameters did not show a constructive influence between inoculated and control, non-inoculated plants. Aziz et al. also reported the failure of inoculation to improve all growth parameters [57].

The effectiveness of *Rhizobium* strains is attributed to the variation in grain yield and other growth parameters. Previous studies have shown that a greater number of nodules enhance nitrogen uptake, leading to improved nutrient uptake [54, 58]. The positive impact of *Rhizobium* spp. inoculation on nodulation and growth parameters is often attributed to the increased accumulation of nitrogen through BNF [59, 60]. The variation among *Rhizobium* strains in BNF emphasizes the need for the selection of efficient strains for biofertilizer development [45]. In this study, MbPrRz1 was found to be the most effective strain and yielded the best results for all three crops. This suggests that there may not be a need to select a specific strain for a particular crop in a region, highlighting its compatibility with different varieties of legumes. However, previous studies have concluded that specific *Rhizobium* spp. is required for specific legume species [61–64].

The incorporation of more efficient strains of *Rhizobium* for sustainable agricultural practices in India has the potential to cut down the reliance on chemical fertilizers while simultaneously mitigating the adverse effects on the environment. A rational dose of biofertilizer use enhances crop yield and improves soil biochemical properties, promoting colonization by crop-specific Rhizobia [65]. This approach offers maximum economic and ecological benefits. In the context of sustainable agriculture, gaining a broader perspective on other agriculturally important microbial communities requires conducting multiomics on postharvest soil samples from these leguminous crops [66].

In conclusion, three *Rhizobium* mutants were developed in our study, which demonstrated enhanced nodulation ability as indicated by an increased number of nodules per plant compared to the native strains under field conditions. These mutants significantly improved plant yield and productivity. Additionally, rhizospheric colonization and survival of these mutants were higher than that of native controls, indicating improved nodule occupancy and positive effects on plant growth. Our findings could contribute to establishing an inoculum for enhancing legume yield in a regional environment. While clear benefits were observed in Haryana (India) soils, further extensive field trials are necessary to validate these promising results on a larger scale.

FIGURE 5 | Phylogenetic similarity of the developed *Rhizobium* mutants as per 16S rRNA sequence. Maximum likelihood trees with 1000 bootstraps were generated. The analysis involved 63 nucleotide sequences and all ambiguous positions were removed for each sequence pair (pairwise deletion option). Evolutionary analyses were conducted in MEGA11. Developed queried mutant strains, MbPrRz1 (*Mesorhizobium ciceri* CWT), MbPrRz2 (*Rhizobium leguminosarum* PWT), and MbPrRz3 (*R. leguminosarum* LWT), are indicated by the red squared box.

Author Contributions

Ikbal Shah: conceptualization, data curation, formal analysis, investigation, methodology, software, validation, visualization, writing–original draft, writing–review and editing. **Khan M. Sarim:** data curation, investigation, methodology, software, visualization, writing–review and editing. **Virendra K. Sikka:** conceptualization, formal analysis, funding acquisition, project administration, resources, supervision, validation, writing–review and editing. **Surjit S. Dudeja:** formal analysis, supervision, validation, writing–review and editing. **Dharmender K. Gahlot:** conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, software, supervision, validation, visualization, writing–original draft, writing–review and editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Nucleotide sequence data of the developed *Rhizobium* strains is submitted to NCBI GenBank with accession numbers, OQ121148 (*R. leguminosarum* PWT; MbPrRz1), OQ121149 (*R. leguminosarum* LWT; MbPrRz2), and OQ121150 (*M. ciceri* CWT; MbPrRz3).

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