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A Method for Selection and Characterization of Rhizosphere-Competent Bacteria of Chickpea

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Abstract. A greenhouse assay was developed to evaluate the root-colonizing capability of the native chickpea rhizospheric bacterial population. In this assay system, screening time was reduced on two counts. First, spontaneous chromosomal rifampicin-resistant (Rif^r) strains were directly inoculated to seeds without any check for the stability of the mutation, and second, no attempts were made to taxonomically identify all the strains being screened for chickpea rhizosphere competence. Only two chickpea rhizosphere-competent Rif^r strains from the group of six good chickpea rhizosphere colonizers forming 10^7 to 10^8 colony-forming units (cfu)/g root were taxonomically identified as *Pseudomonas fluorescens* NB13R and *Pseudomonas* spp. NB49R, after screening 49 bacteria. Both the strains showed no difference from their corresponding wild-type strains *P. fluorescens* NB13 and *Pseudomonas* spp. NB49 in terms of chickpea rhizosphere competence. Isogenic or equally rhizospheric competitive second non-isogenic bacterial isolate, when present in tenfold higher amount, pre-empted the colonization of the soil by the bacterium, which was present in smaller ratio. These findings indicate that the isogenic or equally rhizospheric competitive second non-isogenic Rif^r strains should be compared for their survival and competition with that of the isogenic parent and with each other for specific ecological niche, before using a mixture of isolates, for stable and consistent biological seed treatment to control soilborn pathogens or pests or to promote plant growth.

A major factor in the unsuccessful commercialization of rhizosphere bacteria has been the inconsistency of field test results. Reasons for the reported variability include nonpersistence on seed before it is planted and poor bacterial establishment on seed and roots [2, 4–6, 10, 13, 17, 18, 21, 25]. The introduced microorganism must colonize plant roots and demonstrate rhizosphere competence before its further utilization as biological control and/or plant growth-promoting agent. When the proper bacterial strain is used, plant roots are extensively colonized by the introduced strain, which suggests a close bacteria–plant association that allows for beneficial plant growth or disease protection [11, 20].

The current techniques that are required to ascertain bacterial root colonization capacity are laborious and often produce highly variable results. Bennett and Lynch [1] developed a closed test tube assay for measuring root colonization capacity of bacteria under gnotobiotic conditions, which proved useful for studying specific microbial interactions in the rhizosphere. It is not possible to extrapolate results obtained in sterile soils to those expected under field conditions [8]. Scher et al. [19] measured the root colonization capacity of bacteria on maize in raw soil-sand closed test tube assay and demonstrated that root population densities determined in the soil–sand assay were comparable to those determined with plants grown in soils under greenhouse conditions. However, Scher et al. [19] did not compare the competitive fitness of Rif^r mutants with the wild-type strain. Compeau et al. [3] have demonstrated that colonization of soil by a species that is isogenic to a challenging organism may preempt the colonization of the soil by the second organism. This is true even when organisms display identical fitness. This interaction may be important in the failure of introduced strains to increase in number when introduced into their own environment.

The objectives of these studies were to develop a raw soil assay for quick, large-scale screening of native rhizosphere microflora of chickpea to identify and characterize naturally occurring rhizosphere bacteria that could

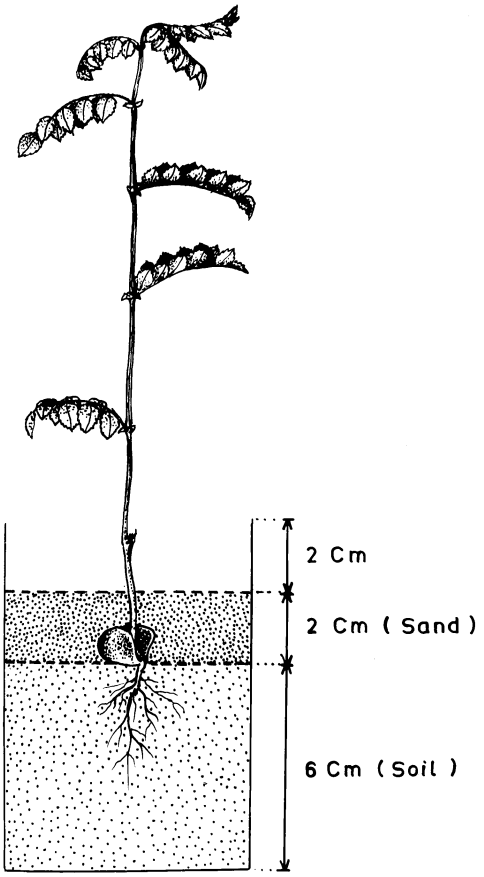

Fig. 1. Sand-nonsterilized soil assay system for screening chickpea rhizosphere-competent bacteria.

effectively colonize chickpea roots. These bacteria would then be available for genetic manipulation and eventual use as biological control agent or plant growth-promoting bacteria applied as seed inoculants. Fitness of the genetically marked strains was confirmed by their ability to successfully compete with wild-type parents. The fate of Rif^r rhizosphere-competent bacteria of chickpea, either as a single isolate or as a mixture of two isogenic or equally competitive non-isogenic isolates, was monitored after their introduction into nonsterilized soil.

Materials and Methods

Bacterial strains were isolated from the roots of field-grown chickpea (*Cicer arietinum* L.) grown in chickpea fungal (*Fusarium oxysporum* f. sp. ciceri, *Rhizoctonia bataticola*, and *Pythium* sp.) disease-conducive plot maintained for the past 60 years at C.S. Azad University of Agriculture and Technology, Kanpur, India. Roots were sampled after 6 weeks of plant growth in the fungal disease-conducive soil, because the bacteria that are present at this stage would be organisms that had colonized roots during initial root development. Roots were thoroughly washed with tap water to remove all loosely adhering soil particles, followed by washing with sterile 0.85% (wt/vol) saline Milli Q water (MQW). The roots were then macerated in 0.85% saline MQW with a mortar and pestle. Serial dilutions of the homogenate were then plated on *Pseudomonas* isolation agar, Nutrient agar, and Tryptone-Glucose-Yeast extract (TGY) agar (from HI-MEDIA Laboratories Pvt. Ltd., Bombay, India). Colonies representative of different morphological types present on the plates were selected and purified on minimal media based on AT salts [16]. Microflora associated with the rhizosphere of chickpea were identified as described earlier [15].

Spontaneous bacterial Rif^r strains were isolated on TGY agar plates containing 100 µg rifampicin (from Sigma Chemical Co., St. Louis, Missouri, USA)/ml by plating 100 µl overnight grown culture per plate. Resistance to rifampicin was used because it is mediated by a mutation in β subunit of RNA polymerase [22], unusual among soil bacteria. The chromosomal nature of the mutation affords greater stability than occurs with plasmid-borne markers and is also advantageous since the mutation is not transferable [3]. This popular technique is simple, rapid, sensitive, inexpensive, and has been used successfully with samples from various environments [10].

Spontaneous bacterial Rif^r strains showing growth comparable to wild type, on TGY agar plates containing 100 µg rifampicin, were selected for further studies. Serial dilution plating of the Rif^r strains was done on TGY agar plates containing 0, 5, 25, 50, and 100 µg rifampicin/ml. No significant differences in the viable counts were observed in different plates. Thus, TGY agar plates containing 50 µg rifampicin/ml, an amount sufficient to inhibit the growth of other organisms in non-sterilized soil, were used to recover Rif^r strains from the rhizosphere.

Bacterial inoculum for chickpea (*Cicer arietinum* L.) seeds was prepared by scraping 48-h grown culture from AT plates with 10 ml of 0.85% saline MQW. Chickpea seeds were surface sterilized by gently shaking (80 rpm, on a reciprocal shaker at 28°C) with 70% ethanol (5 min), 20% bleach Chlorox (10 min), followed by three rinses in sterile MQW. After surface sterilization seeds were soaked in the bacterial suspension for 4 h at 28°C on a reciprocal shaker at 100 rpm. Control seeds (uninoculated) were soaked in 0.85% saline MQW washed from uninoculated AT plates. Inoculum levels of seeds were determined by agitating four seeds from each treatment and plated after serial dilution on AT agar plate containing 50 µg rifampicin/ml. Mean colony-forming units (CFU)/seed were determined by averaging the CFU/g values of three populations in three replicates per treatment after 48-h incubation of the plates at 28°C.

Seeds for treatments in which mixtures of two isolates were used were inoculated by using the same total number of bacteria for the inoculum as was used for the single-isolate treatments. Thus, one-half the normal amount of each isolate in the mixture was used.

Trays (35 × 35 cm) with 16 (4 × 4) places per tray (each space was of 7 cm width, 10 cm depth, and 1 cm apart from each other) were used to grow plants. Each place was filled up to 6 cm. with either nonsterilized or sterilized (1 h at 121°C for 3 consecutive days) soil, when stated. Tap water (25 ml) was added to each hole before planting seeds to adjust the soil to 20% moisture. One bacteria-treated seed was added per hole and covered with 2 cm sterile coarse sand (Fig. 1).

Plants were grown in a greenhouse and were carefully removed at the specified times from the pots, and all root segments 5 mm below seed remnants were excised. This was done to ensure that only the bacteria that colonized the roots and not the bacteria that remained on the seed coat were assayed. Roots were washed thoroughly to remove all the sand particles and then macerated in 0.85% saline MQW with a mortar and pestle. Rhizosphere bacterial population was quantified by serial dilution plating of the homogenate on TGY media, in the presence or absence of 50 µg rifampicin/ml, as and when required. Recovery of wild-type *Pseudomonas fluorescens* and *Pseudomonas* spp. from the rhizosphere of the sterile soil was done by initial plating onto *Pseudomonas* isolation agar followed by replica plating on *Pseudomonas* isolation agar containing 50 µg rifampicin/ml to enumerate the Rif^r subpopulation.

Table 1. Rifampicin-resistant bacteria as seed inoculants on chickpea root population assayed after 30 days post-planting[a] in sand-nonsterilized soil assay

Isolate	CFU/seed[b]	CFU/g root[c]	Isolate	CFU/seed	CFU/g root
NB01R	1.2×10^4	2.0×10^6	NB30R	5.7×10^5	1.3×10^3
NB02R	1.3×10^5	3.2×10^4	NB31R	1.5×10^5	2.0×10^5
NB03R	4.2×10^4	3.3×10^2	NB32R	3.4×10^3	3.2×10^6
NB05R	1.8×10^3	1.5×10^7	NB33R	7.2×10^5	4.9×10^4
NB06R	1.0×10^6	3.5×10^5	NB34R	8.5×10^6	ND
NB08R	1.2×10^4	ND[d]	NB35R	6.3×10^4	2.6×10^7
NB09R	1.2×10^5	7.3×10^7	NB36R	1.5×10^5	4.8×10^6
NB10R	1.2×10^4	5.7×10^2	NB37R	3.5×10^4	2.3×10^3
NB11R	1.5×10^5	2.9×10^3	NB38R	5.7×10^4	7.9×10^5
NB13R	1.6×10^4	2.7×10^8	NB40R	6.4×10^5	4.2×10^5
NB14R	1.2×10^3	3.8×10^6	NB41R	6.1×10^3	5.0×10^6
NB15R	1.7×10^5	3.3×10^2	NB42R	8.3×10^5	ND
NB16R	1.0×10^5	7.6×10^7	NB44R	2.5×10^5	3.2×10^4
NB17R	2.4×10^4	ND	NB45R	3.0×10^4	4.3×10^6
NB18R	3.8×10^5	8.2×10^5	NB46R	1.2×10^4	6.4×10^2
NB20R	5.0×10^5	3.6×10^4	NB47R	4.3×10^5	5.6×10^5
NB21R	9.1×10^3	4.7×10^6	NB48R	2.6×10^3	6.5×10^6
NB22R	1.2×10^5	2.0×10^4	NB49R	1.1×10^5	2.3×10^8
NB23R	1.8×10^4	6.5×10^5	NB50R	6.8×10^4	7.3×10^6
NB24R	1.2×10^6	3.2×10^4	NB51R	3.3×10^5	8.2×10^4
NB25R	9.4×10^5	2.6×10^2	NB52R	6.2×10^6	ND
NB26R	1.2×10^5	6.7×10^6	NB53R	7.3×10^5	8.2×10^4
NB27R	3.0×10^5	5.6×10^5	NB54R	5.6×10^3	2.7×10^6
NB28R	8.7×10^4	2.3×10^6	NB56R	2.5×10^5	3.6×10^4
NB29R	3.5×10^5	8.9×10^5	Control[e]	0.0	ND

[a] Number of days after planting when roots were sampled.
[b] Values represent the average of three replications.
[c] Values represent the average of four replications. Dry weights (g) of root systems were sampled.
[d] ND, not detectable.
[e] Uninoculated seeds were used as control.

No naturally occurring Rif^r bacteria were seen when root homogenates of uninoculated controls were plated from sterile or nonsterile soils. Macerated root segments were dried for 4 days in an oven at 80°C before measuring the weight. Average CFU/g (dry weight) of roots were made as previously described for seeds, except with four replications per treatment.

Results

Fifty-six bacterial strains, representing different morphological types, were isolated from the rhizosphere of chickpea. Forty-nine spontaneous Rif^r strains showing growth comparable to its wild type, on TGY agar plates containing 100 µg rifampicin, were selected. No Rif^r strains could be obtained on the TGY agar plates containing 100 µg rifampicin for three cultures, while the size of Rif^r strains in four bacterial cultures was too small by 48 h as compared with its wild type. Initial bacterial population densities on seeds ranged from 10^3 to 10^6 cfu/seed (Table 1). The average root colonization values CFU/g (dry weight) of roots of the Rif^r strain, after 30 days of post planting, ranged from nondetectable to 10^8 CFU/g root. Five Rif^r strains failed to colonize roots (Table 1). From the CFU/g root values, 49 Rif^r strains could broadly be divided into three different groups. First group consisted of six good chickpea rhizosphere colonizers with 10^7 to 10^8 CFU/g root; the second group consisted of 30 medium chickpea rhizosphere colonizers with 10^4 to 10^6 CFU/g root; and the third group consisted of 13 poor chickpea rhizosphere colonizers with nondetectable to 10^3 CFU/g root (Table 1). Two Rif^r strains NB13R and NB49R from the first group of good chickpea rhizosphere colonizers were selected for further detailed studies. These two strains were identified as *P. fluorescens* NB13 and *Pseudomonas* spp. NB49 respectively.

To evaluate the ecological fitness of the wild-type *P. fluorescens* NB13 and *Pseudomonas* spp. NB49, their Rif^r derivatives *P. fluorescens* NB13R and *Pseudomonas* spp. NB49R were inoculated 1:1 with the respective wild-type parent. *P. fluorescens* NB13R and *Pseudomonas* spp. NB49R increased in titer at the same rate and achieved the same final density of about 1×10^8 CFU/g root by 3 weeks and decreased by about 1.0 $\log_{10}$ units when sampled after 9 weeks (Fig. 2A and B). When the *P. fluorescens* NB13 was inoculated to the seed at a tenfold excess over *P. fluorescens* NB13R, the strains maintained the same ratio up to 9 weeks and vice versa (Fig. 2C and

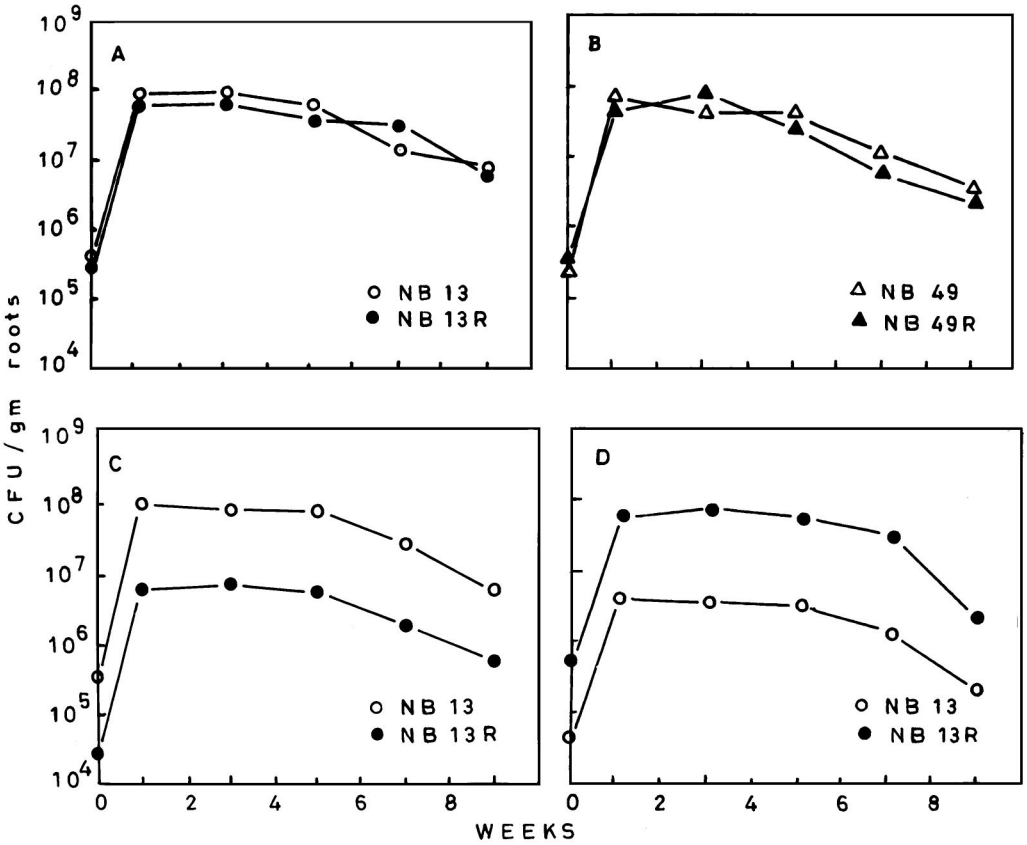

Fig. 2. Survival and competition of *P. fluorescens* NB13, *P. fluorescens* NB13R, *Pseudomonas* spp. NB49, and *Pseudomonas* spp. NB49R in sterile soil. Bacteria were distinguished by Rif^r (A and B) 1:1 initial ratio; (C and D) 1:10 and 10:1 initial ratio, respectively. Four observations per mean.

D). Similar results were obtained for *Pseudomonas* spp. NB49 and *Pseudomonas* spp. NB49R (data not shown).

P. fluorescens NB13R and *Pseudomonas* spp. NB49R were further studied in nonsterilized soil based on rhizosphere competence (Table 1), to evaluate the effect of indigenous microbial population on the introduced bacteria and on each other. Titer of both *P. fluorescens* NB13R and *Pseudomonas* spp. NB49R increased to about 1×10^8 CFU/g root by 30 days and decreased by about 2.0 $\log_{10}$ units when sampled after 90 days (Table 2).

Mixing of two non-isogenic isolates, *P. fluorescens* NB13R and *Pseudomonas* spp. NB49R, together in the ratio of 1:1 resulted in the successful colonization of chickpea rhizosphere, indicating that the two isolates examined were compatible with each other (Table 2). When the *P. fluorescens* NB49R was inoculated to the seed at a tenfold excess over *P. fluorescens* NB13R, the strains maintained the same ratio up to 30 days, but by 90 days the counts were comparable and vice versa (Table 2).

Table 2. Effect of rifampicin-resistant bacterial isolates in various combinations as seed inoculants on chickpea root population assayed after 30 and 90 days[a] post-planting in nonsterilized soil

		CFU/g root[c]	
Isolate(s)	CFU/seed[b]	30 days	90 days
P. fluorescens NB13R	6.3×10^5	1.1×10^8	1.7×10^6
Pseudomonas spp. NB49R	8.5×10^5	9.2×10^7	8.9×10^5
P. fluorescens NB13R+	3.8×10^5	8.5×10^7	7.3×10^5
Pseudomonas spp. Nb49R (1:1)	4.4×10^5	7.8×10^7	5.3×10^5
P. fluorescens NB13R+	6.3×10^4	2.2×10^6	1.1×10^5
Pseudomonas spp. NB49R (1:10)	7.0×10^5	3.4×10^7	2.0×10^5
P. fluorescens NB13R+	5.6×10^5	8.4×10^7	1.9×10^5
Pseudomonas spp. NB49R (10:1)	4.8×10^4	1.7×10^6	8.6×10^4
Control[e]	0.0	ND[d]	ND

[a] Number of days after planting when roots were sampled.
[b] Values represent the average of three replications.
[c] Values represent the average of four replications. Dry weights (g) of root systems were sampled.
[d] ND, not detectable.
[e] Uninoculated seeds were used as control.

Discussion

An efficient sand-nonsterilized soil assay is described here for evaluating rhizospheric colonization potential of diverse group of bacteria isolated from the rhizosphere of chickpea. In this assay system, 44 of 49 Rif^r strains tested were able to compete with native soil microorganisms and colonize chickpea roots. The advantage of this system over a sterile or closed-test-tube raw soil assay is

that the capacity to compete in native soil as well as the ability to grow on roots with regular irrigation is required for rhizospheric colonization. The rhizospheric bacteria used in this study were sampled 4 weeks after harvesting of the chickpea shoots. During this period the plant roots were subjected to temperature and water stress, because maximum daytime temperature during this period in the unirrigated field ranged from 46°C to 50°C. Past results with sugar beet [23], potato [9], and wheat [26] demonstrated that most bacterial strains that colonized roots immediately after plant emergence continued to colonize the developing root system throughout the season. Therefore, it can be presumed that the bacteria that are present at this stage would be the most competitive rhizospheric bacteria that had colonized chickpea roots during initial root development and survived throughout the 5 months of growing season of chickpea and 1 month post-harvest high temperature and low water availability stress. Any useful competitive rhizospheric bacteria, to be available for the next chickpea growing season, will have to undergo these stressful conditions (high temperature and drought).

Current techniques require much time to screen the native microorganisms for rhizospheric competence. In the present study the screening time, compared with previous reports [3, 7, 12–14, 19, 24] has been reduced on two counts. First, Rif^r strains in the present assay system were directly inoculated to seeds without any check for the stability of the mutation, which usually requires at least 25 passages through nonselective media. It is anticipated that any Rif^r strain that shows high CFU/g root after 30 days of inoculation should be able to do so only if it is capable of sustained growth and competing against the native microorganisms. Secondly, no attempts have been made to taxonomically identify all the 49 strains (Table 1). Only the two chickpea rhizosphere-competent strains, selected for further detailed studies after screening 49 bacteria strains, were taxonomically identified as *P. fluorescens* NB13 and *Pseudomonas* spp. NB49.

It has been demonstrated in a simple sand-nonsterilized soil assay system that two strains, *P. fluorescens* NB13R and *Pseudomonas* spp. NB49R, were equally competitive compared with the wild type (Fig. 2A and B). It was also demonstrated that colonization of soil by the isogenic (Fig. 2C and D) or equally rhizospheric competitive second non-isogenic bacterial isolate (Table 2) may preempt the colonization of the soil by the bacterium, which is present in the higher ratio. These observations have important implication for the commercial biofertilizer preparations consisting of single or more than one bacterial isolates for ''super'' seed treatment. Thus, to ensure the success of the introduced strains, it will be desirable to compare their survival and competition with those of the isogenic parent and with each other in a specific ecological niche, for stable and consistent biological seed treatment.

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