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يعتبر إنتاج عزلات البكتيريا المثبطة للمرض *P. fluorescens* مواد تحمل عنصر الحديد siderophores (مكونة من شيلات الحديد و عناصر معدنية) بمواصفات مبيد فطري خطوة هامة أسهمت في زيادة الفعالية التضادية وبالتالي خفض نمو وتطور مرض الذبول الفيوزاريومي على الحمص. تعتبر إمكانية تكامل التأثير/الفعالية بين بكتيريا منطقة الجذور وعجينة ثمار النيم طريقة هامة في التطبيق مستقبلاً كإحدى مكونات برنامج المكافحة الحيوية لمرض ذبول الحمص.

كلمات مفتاحية: مكافحة حيوية، *Pseudomonas fluorescens*، *Azadirachta indica*، ذبول الحمص، *Cicer arietinum*.

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Neem cake has advantages over other organic amendments as it possesses several biologically active compounds, mainly alkaloids such as isoprenoids that control various pests, including fungi (5). Combining the rhizobacterial isolates with neem gave an additional beneficial effect on disease control both in glasshouse and field. Isolates I-65 and I-5 when both were applied together they were surprisingly ineffective unless in combination with neem cake.

In some studies on biological control, multi strain treatments were significantly better than when each was used alone (6, 21). Efficacy improvement of the mixture may be due to the different modes of action of the biocontrol agents (7, 12). In our study, one mode of action may have been an iron (Fe^{+3}) chelating siderophore produced by the bacterial biocontrol population resulting in the sequestering of the available iron thereby limiting the pathogen growth and proliferation. Similar increases in the biocontrol potential of the fluorescent siderophore production against *Rhizoctonia solani* in chickpea has been reported (11). Thus, this study has proved siderophore production as a mechanism of biocontrol of the Fusarium wilt of chickpea when using the selected *P. fluorescens* isolates.

Amending soil with neem cake improved the biocontrol activity of the *P. fluorescens* isolates, suggesting that the antifungal activity of the bacteria may be modified by the application of organic amendments or that there is an additive effect of the neem antifungal substances and that of the biocontrol strains. Little attention has been given to the formulation of organic amendments as possible biocontrol agents and determining the modes of action (23). One successful example is the control of *Botrytis* infection of begonias by combining composted amendments with *Trichoderma hamatum* (17). Application of a suitable organic amendment with biocontrol agents may contribute to the induction of systemic resistance in plants (22).

Further research is needed to determine the reliability of the biocontrol inoculum and neem cake in multilocational field trials. The minimum amount of neem cake required to obtain a response is also needed as the amount used in the present study is much greater that would be possible to use economically in field production systems. Biological control of *Fusarium* wilt of chickpea in the economically poor, rainfed areas of Pakistan and India would be of much benefit to farmers as it would be a small cost input to their production system.

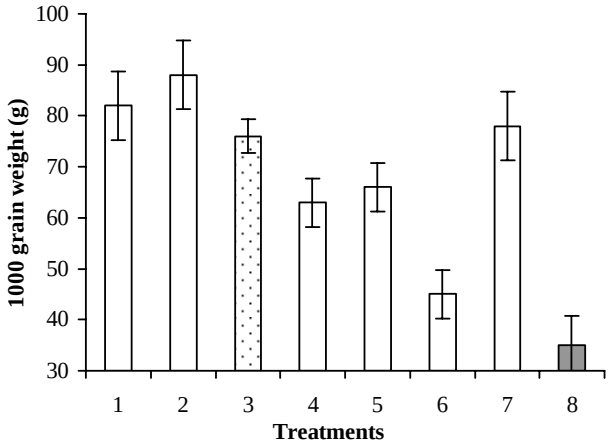

Figure 4. Effect of individual and combined application of two isolates of *Pseudomonas flourescens* (I-5 & I-65) and neem cake (NC) on thousand seed weight of chickpea under field conditions. Data of each treatment are the means of 3 replications. Vertical error bars represent standard errors of differences of means. Means with the same letter are not significantly different from each other according to LSD comparison test (P= 0.05). Treatments are: 1= I-5+NC, 2= I-65+NC, 3= NC, 4= I-5, 5= I-65, 6= I-5+I-65, 7= I-5+I-65+NC, 8= Control.

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الملخص

إنعام الحق، محمد، سعيد الحسن، سايمون غاون و نذير جافاد. 2009. تأثير إستخدام عزلتين بكتيريتين وعجينة ثمار النيم في مكافحة ذبول الحمص المتسبب بواسطة *Fusarium oxysporium* f. sp. *ciceris*. مجلة وقاية النبات العربية، 27: 103–110.

تم تقويم المقدرة التضادية لعزلتين بكتيريتين من منطقة الجذور تنتمي لـــ *Pseudomonas fluorescens* وعجينة ثمار النيم (الأزدرخت الهندي *Azadirachta indica*)، فرادى أو في توافيق ثنائية أو ثلاثية، لإعاقة نمو *Fusarium oxysporium* f. sp. *ciceris* الكائن المسبب لمرض ذبول الحمص في غرفة النمو المتحكم بها، الدفيئة الزجاجية و تحت الظروف الحقلية. أظهرت العزلتان I-5 و I-65 من بين 90 عزلة بكتيرية مختبرة خفضاً لأكثر من 32% في النسبة المئوية للذبول الوعائي. تمت التجارب الإختبارية بإضافة هذه العزلات الى نوعين من التربة، إحداهما معاملة بعجينة ثمار النيم كمادة نباتية عضوية والأخرى غير معاملة. في الدفيئة الزجاجية، عندما إضيفت العزلات البكتيرية المختبرة مع عجينة ثمار النيم في أنواع ثلاثة من التربة الحقلية؛ معقمة، غير عقمة وتربة طبيعية، سببت خفضاً في نسبة الذبول تراوحت من 27–31%، 31–38% و 31–36%، على التوالي، بالمقارنة مع معاملة الشاهد المعدى بالفطر الممرض. تبين أن نسبة حدوث المرض كانت أكثر عندما استخدمت تربة لا تحتوي على عجينة ثمار النيم. عندما تمت معاملة التربة بعجينة ثمار النيم، أدى إضافة العزلات البكتيرية الى زيادة في الغلة الحبية تراوحت ما بين 146–155%. لم توجد فروق معنوية في الغلة الحبية ووزن الـــ 1000 حبة بين العزلتين البكتيريتين سواء منفردة أو في توافيق ثنائية، ولكن عندما استخدمت في التجارب التي لا تحتوي على عجينة ثمار النيم الفروق المعنوية كانت قليلة جداً بينهما.

increase (Figure 4). Treatments I-65 and I-5 resulted in 88 and 80% increase over control respectively but NC applied alone was equally good as the two antagonists. The least effective treatment was I-65+I-5 when applied without NC caused only 28%, but when this treatment applied with NC caused 123% increase in 1000-grain weight. And this treatment was statistically on a par with NC, which yielded a 117% increase over the control (Figure 4).

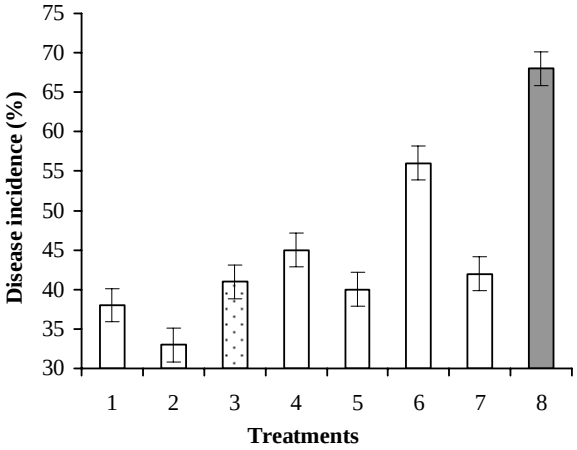

Figure 2. Effect of individual and combined application of two isolates of *Pseudomonas flourescens* (I-5 & I-65) and neem cake (NC) on the wilt disease of chickpea in the field. Data of each treatment are the means of 3 replications. Vertical error bars represent standard errors of differences of means. Means with the same letter are not significantly different from each other according to LSD comparison test (P =0.05). Treatments are: 1= I-5+NC, 2= I-65+NC, 3= NC, 4= I-5, 5= I-65, 6= I-5+I-65, 7= I-5+I-65+NC, 8= Control.

Mechanism of action of antagonistic rhizobacterial isolates

Phenazine antibiotic production - The two rhizobacterial isolates I-65 and I-5 did not produce detectable quantities of phenazine-1-carboxylate. In the plate bioassay, there was no colony pigmentation or presence of a dark zone in the bacterial colonies. Even after 2 weeks no significant discoloration in the bacterial colonies with crystalline deposits in the centers of the bacterial cultures was noticed. The filter paper assay for cyanide production showed that these bacteria did not produce cyanide on NA supplemented with glycine. In the assay for siderophores production, discoloration of the bacterial colonies to orange occurred after 48 h of incubation with coloured zones of varying widths around the colonies indicating positive siderophore production.

Discussion

The majority of the rhizobacteria isolated from the three crops varied in their biological activity against chickpea Fusarium wilt in growth room and glasshouse conditions. *Pseudomonas fluorescens* isolates, I-5 and I-65, from chickpea and soybean rhizosphere which were used individually or in combination with neem cake affected the development of disease symptoms caused by *F. oxysporum* f. sp. *ciceris* in a glasshouse bioassay in unsterilized soil at levels that suggest they might be useful as a biological control agent in the field. Addition of recommended quantity of neem cake (2%, w/w) enhanced this biocontrol effect. Rhizobacteria may suppress the pathogens by various mechanisms such as competition, antibiosis and induced resistance and then positively affect plant growth with their plant growth promoting (PGP) properties (7, 34).

Disease incidence was greater in sterilized soil possibly because sterilization created conditions more favourable to Fusarium development by affecting other microflora. Neem cake had little effect on the disease in the sterile soil, possibly because sterilization killed the microorganisms responsible for breakdown and/or release of the active compounds. In contrast, disease reduction in the unsterilized or natural field soil was different. Neem cake was effective either applied alone or combined with rhizobacterial isolates, and presumably the conditions enabled breakdown and release of fungitoxic metabolites which did not affect the biocontrol activities of I-65 and I-5.

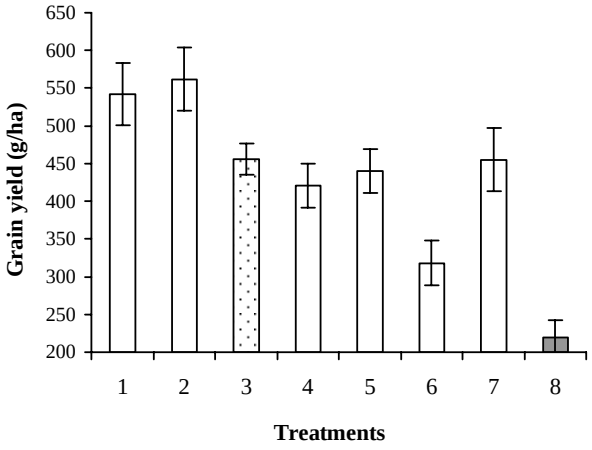

Figure 3. Effect of individual and combined application of two isolates of *Pseudomonas flourescens* (I-5 and I-65) and neem cake (NC) on grain yield under field conditions. Data of each treatment are the means of 3 replications. Vertical error bars represent standard errors of differences of means. Means with the same letter are not significantly different from each other according to LSD comparison test (P= 0.05). Treatments are: 1= I-5+NC, 2= I-65+NC, 3= NC, 4= I-5, 5= I-65, 6= I-5+I-65, 7= I-5+I-65+NC, 8= Control.

Microbial activity is partly responsible for disease control (16) and suppressive organic matter possesses higher microbial activity than organic matter that is conducive (1, 16). Large microbial activity is likely to cause depletion in nutrients essential for the survival and multiplication of the pathogen. Neem cake could provide such a source of nutrients for beneficial microflora and antagonists and might enhance the production of antibiotics by the antagonists (15, 20).

(NC) or neem cake alone, decreased the disease incidence compared to control. However, the most effective and statistically similar treatments were I-65 and I-65+NC causing 33 and 32% reduction in disease incidence, respectively. Strain I-5 with and without NC reduced disease incidence by 26 and 19%, respectively (Figure 1). The combined treatment I-5+NC was significantly different from the I-65 and I-65+NC. The least effective treatments were those with the combined strains I-5+I-65+NC and I-5+I-65 which resulted in 15 and 13% reduction in wilt incidence.

Table 1. Screening of rhizobacterial isolates for the biological control of *F. oxysporum* f. sp. *ciceris* in natural field soil under glasshouse conditions.

Disease incidence (%)	Rhizobacterial isolate used
58 - 68	I-5, I-65
69 - 77	I-1, I-2, I-3, I-4, I-6, I-7, I-8, I-9, I-10, I-11, I-16, I-17, I-18, I-19, I-21, I-22, I-23, I-24, I-25, I-26, I-27, I-28, I-29, I-30, I-31, I-32, I-33, I-34, I-41, I-42, I-44, I-47, I-51, I-53, I-55, I-56, I-57
78-95	I-12, I-13, I-14, I-15, I-20, I-35, I-36, I-37, I-38, I-39, I-40, I-43, I-45, I-46, I-48, I-49, I-50, I-52, I-54, I-58, I-59, I-60, I-61, I-62, I-63, I-64, I-66, I-67, I-68, I-69, I-70, I-71, I-72, I-73, I-74, I-75, I-76, I-77, I-78, I-79, I-80, I-81, I-82, I-83, I-84, I-85, I-86, I-87, I-88, I-89, I-90
100	Control

Data were calculated on the basis of percent disease incidence. Isolates I-1 to I-30 were from chickpea, I-31 to I-60 from soybean and I-61 to I-90 from maize.

Un-sterilized soil experiment - The most effective treatments for reducing disease incidence were I-65+NC and I-5+NC causing 38% and 32% reduction, respectively. Combination of I-65 and I-5 with NC were the most effective treatments and significantly (P = 0.05) better than NC alone. The I-5+I-65 treatment was the least effective (Figure 1).

Preliminary evaluation of rhizobacteria and neem cake on Fusarium wilt in the field

In the field conditions, the most effective treatments were I-65+NC, I-5+NC and I-65 which caused 36, 31 and 29% reduction in wilt incidence, respectively (Figure 2). Both isolates I-65 and I-5 were effective, but when combined with NC their performance was slightly increased. In contrast, I-65+I-5 was the least effective treatment and caused only 13% reduction, but when both I-65+I-5 were mixed with NC caused 27% reduction (Figure 2).

As far as yield is concerned, the highest increase was obtained from I-65+NC, which yielded 562% over control, followed by I-5+NC with a 146% increase, but I-65+NC and I-5+NC were significantly different (P=0.05). Individual application of I-65, I-5 and NC caused 100, 91 and 107% increase in yield over the control respectively and statistically I-65 and I-5 were the same, but significantly differed from NC (P = 0.05). Combinations of I-65+NC and I-5 +NC gave better results and were the most effective treatments (Figure 3). The least effective treatment was I-65+I-5, which caused only 44.5% increase, but when applied with NC it was more effective and caused 107% increase in yield.

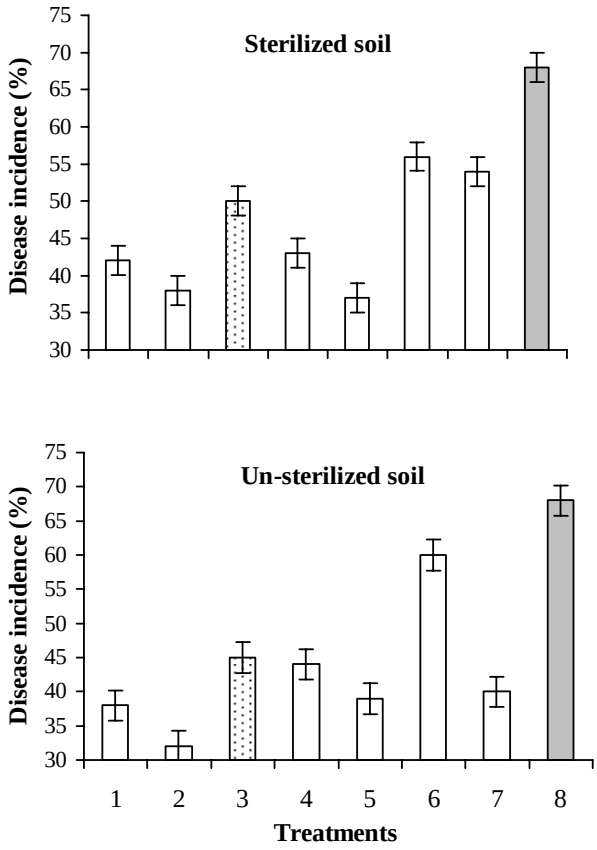

Figure 1. Effect of individual and combined application of two isolates of *Pseudomonas flourescens* (I-5 & I-65) and neem cake (NC) on chickpea wilt disease in sterilized and unsterilized (natural field) soil in the glasshouse. Data of each treatment are the means of 3 replications. Vertical error bars represent standard errors of mean differences. Means with the same letter are not significantly different from each other according to LSD comparison test (P= 0.05). Treatments are: 1= I-5+NC, 2= I-65+NC, 3= NC, 4= I-5, 5= I-65, 6= I-5+I-65, 7= I-5+I-65+NC, 8= Control.

As far as 1000-grain weight is concerned, the two rhizobacterial isolates when amended individually with neem cake significantly increased 1000-grain weight (P = 0.05). Among these two, the combination of I-65 with NC resulted in a 151% increase in grain weight which is highest and significantly better than all treatments (P=0.05) followed by the treatment I-5+NC which caused 134%

20±5°C. Neem cake (NC) was mixed at the rate of 2% (w/w) by shaking the soil thoroughly in a plastic drum, moistened and allowed to decompose for one week before filling the trays in the glasshouse (23). Ten seeds were planted in each tray; each rhizobacterial treatment (3 replications of each 3 trays) contained 90 plants. Seedling trays were set up in a randomized complete block design (RCBD) of eight treatments including control in each experiment. Fungal inoculum was added 2 weeks after sowing the seeds to avoid early seedling stage mortality (3) and allow the rhizobacteria to establish in the rhizosphere. Pathogen inoculum rate was applied in 6x1 cm deep holes besides the seedlings. Then the holes were covered to avoid desiccation and care was taken to avoid over watering and flushing of the pathogen inoculum for several days following application. Plants were watered every 3-5 days depending on the needs. At flowering stage, stress was given to the plants to develop wilt symptoms by watering every 7-9 days and increasing the temperature 2 degrees over the average. Percent of disease incidence was calculated after 60 days as described above.

Preliminary evaluation of rhizobacteria and neem cake on Fusarium wilt in the field

Field trials were conducted using a standard Fusarium-diseased plot, used regularly for screening chickpea cultivars over 15 years. The inoculum level of *F. oxysporum* f. sp. *ciceris* was 3.6 x 10^5 CFU/g of clay-loam soil which is sufficient for field screening. The neem cake amendment was mixed at the above rate (2%, w/w) in 6 x 1.2 m plots by spreading neem cake powder on the soil and then mixing manually at depth of 10 cm approximately and allowing it to decompose for 1 week before sowing. The 160 bacteria-treated seeds were sown by drill at 30 cm row-to-row and 15 cm plant-to-plant distances. Seedlings were inoculated, 14 days after planting, with *F. oxysporum* at the above concentration by spreading the inoculum in furrows beside the seedlings. The trial was laid out in RCBD with three replications and the experiment repeated twice. One hundred plants were selected randomly from each plot. Wilt incidence data per plot were recorded after 60 days as described above. Chickpea yield (kg/ha), 1000-grain weight (g) were also recorded at crop maturity.

Determination of mechanism of action of antagonistic rhizobacteria

Pseudomonas spp. control plant pathogens by various mechanisms such as production of antibiotic, siderophores, niche competition and by induced systemic resistance. Assessment of phenazine antibiotic, cyanide and siderophore production were undertaken to assess which of these mechanisms could be contributing to the biocontrol properties of the selected isolates. The test for phenazine antibiotic was done *in vitro* in Petri dishes. The two selected biocontrol bacteria (I-5 and I-65) were grown in shake cultures of nutrient broth supplemented with 2% glucose for 24 h at 20°C. After separating the cells, the supernatant was measured spectrophotometrically at 349.5 nm (31). Bacterial cell suspensions (50 µL) were plated on NA plates and incubated at 28°C for 48 h. After 2 days, the cultures were screened for production of phenazine-1-carboxylate under a UV light. Colonies positive for phenazine production were dark yellow and deposited yellow crystals. Each isolate was assessed for cyanide production by streaking cultures onto 9 cm Petri plates of NA supplemented with 4.4 g glycine/l. A sterilized Whatman No.1 filter paper was soaked in 5 mg/ml copper ethylo acetate and 5 mg/ml 4, 4-methylene-bis-N, N-dimethylalanine in chloroform, air-dried and placed on agar surface (36). The culture dishes were sealed with Parafilm, incubated at 28°C for 2 days and examined for the colour changes on the filter paper. Siderophore production was assessed by suspending bacterial cells in a 10 mM $MgSO_4$ which was spot-inoculated (5 µL) at two points on NA plates supplemented with 100 µM $FeCl_3$and incubated at 27°C for 48 h.

Statistical analysis

The bioassay experiments were repeated three times apart from field trial; there was no significant difference of error of the variances of the three data sets on disease incidence and other studied parameters. This allowed the data to be pooled and analyzed according to standard analysis of variance procedures by GenStat Sixth Edition package (Lawes Agricultural Trust, Rothamsted Experimental Station, Herts, UK). The disease incidence data were arc-sine transformed prior to analysis and contrasts were calculated to compare the significance between the treatments. Mean separations were performed on disease incidence, yield and 1000 grain weight with Fischer's least significance difference (LSD) at $P \leq 0.05$.

Results

Screening of rhizobacterial isolates on Fusarium wilt in the growth room

The efficacy of the 90 rhizobacterial isolates (recorded as I-1, I-2, I-3, I-4, I-5, … I-90) on the suppression of Fusarium wilt disease of chickpea under growth room conditions were evaluated (Table 1). The bacterial isolates were selected as follows: I-1 to I-30 from chickpea, I-31 to I-60 from soybean and I-61 to I-90 from maize. The selected isolates were identified according to the methods described by somewhere (37) as different morphological strains of *Pseudomonas fluorescens.* All the 39 rhizobacterial isolates significantly decreased wilt incidence of chickpea in the growth room bioassay (first two groups, Table 1). Two of them, I-5 and I-65; from chickpea and soybean rhizosphere, were shown to be antagonistic against *F. oxysporum* f. sp. *ciceris* causing 32-42% reduction in disease incidence in the glasshouse (Table 1). These isolates were selected for glasshouse and field experiments and their potential use was tested further by applying them individually or combined with neem cake.

Evaluation of rhizobacteria and neem cake on Fusarium wilt in the glasshouse

Sterilized soil experiment - Seven treatments comprising the 2 bacterial strains alone or combined, with neem cake

Material and methods

Preparation of rhizobacteria and fungal pathogen inoculum

The dilution plate technique was used to isolate 90 bacterial strains from samples of rhizosphere soil. Samples (1 kg) were collected separately from different fields growing chickpea, soybean and maize (30 samples per crop) at the Department of Plant Pathology Research Station, University of Agriculture, Faisalabad, Pakistan. A 1 g subsample of each air-dried soil was placed in 99 ml sterilized distilled water (SDW), stirred at 100 rpm for 10 min and 0.2 ml soil suspension was then evenly distributed by a glass rod on the surface of a glucose peptone agar medium (GPAM; 10 g glucose, 20 g peptone, 15 g agar, 1000 ml SDW). Thirty bacterial isolates from each crop soil showing different morphological characteristics on GPAM were selected for studying their bio-control efficacy against *F. oxysporum*. Single colony isolates were obtained by repeated streaking on fresh medium and pure cultures were maintained and stored on nutrient agar (NA; 25 g/l, Oxoid, Basingstoke, UK) tubes in the refrigerator at 4°C for routine use.

The culture of the fungal pathogen *F. oxysporum* f. sp. *ciceris* was originally isolated from the stem of a chickpea infected plant collected from the field of the Department of Plant Pathology. A single spore isolate of *F. oxysporum* culture was obtained, using sterilised glass needle to pick up a small piece of Komada's Fusarium-selective medium containing germinated spore, and subcultured on potato dextrose agar medium (PDA; 39 g/l, Oxoid, Basingstoke, UK) in a growth room at 25±2°C for 7 to 10 days with a 12-h photoperiod regime. Pathogen inoculum for soil application was prepared by adding three 5-mm-diameter agar discs of 10 days fungal growth of *F. oxysporum* to 250 ml of sterilized potato dextrose broth (PDB; 25 g/l, Oxoid, Basingstoke, UK) and incubating the flasks at 25°C on a rotary shaker (100 rpm). After 7 days of incubation, 250 ml of SDW was added to each flask, homogenized for 1 min and then filtered through a six layers of sterilized cotton cloth. The conidial suspension population density was determined using a Fuchs Counting Chamber (Scientific Laboratory Supplies Ltd., Hawksley, UK) and adjusted with SDW to give a final concentration of 3.5×10^5 conidia/ml and used as inoculum added to a hole in the soil beside the seedling 14 days after sowing. A stock culture of *F. oxysporum* was maintained on PDA and stored in the refrigerator at 4°C for routine use.

Neem cake preparation

Neem cake (NC), a by product left after the extraction of oil from neem seed, was imported from India, dried, crushed and converted into fine powder using a Glen Creston grinder (Dalton Garden, Stanmore, UK) fitted with a 2 mm pore size sieve. The moisture content of dry cake powder was 6.34%. The powder was stored in metal containers at 4°C for experimental use. From preliminary experiments, it was decided to apply neem cake powder at 2 % w:w of soil.

Screening of rhizobacterial isolates on Fusarium wilt in the growth room

Plastic trays (35 x 21 x 6 cm) were filled with clay-loam natural field soil. The fresh soil had been air-dried in a shed for 8 h, sieved (2-mm/10-mesh) and characterized as having pH 8; electrical conductivity of saturated soil extract, 1.58 dS/m; cation exchange capacity 7.70 cmol (+)/ kg and organic matter, 1.10%.

All bacterial isolates were grown in GPAM broth (200 ml in 500 ml conical flasks) on a rotary shaker at 28 ± 2°C and 100 rpm for 48 h. Two-day-old bacterial broth (10^8 CFU/ml) was injected in sterile peat bags (50 g/bag) at 400 ml/kg and bags incubated for 24 h at the same temperature. Chickpea seeds of AUG-480, a susceptible variety, were surface sterilized for 1 min with 70% ethanol and 20 min in 5% v/v commercial bleach (NaOCl) and then rinsed 5 times with SDW. The seeds were then dried in an aseptic environment for 2 h prior to dressing with rhizobacterial isolates. Seeds were treated by mixing with 1 kg of this peat based bacterial inoculum amended with 100 ml of 10% sugar solution. The control consisted of seeds treated with peat containing GPAM broth only. The treated seeds were dried in a laminar airflow cabinet for 6-8 h and then ten chickpea seeds were sown in each tray. For each isolate, 90 seeds (10 seeds per tray) in 9 trays and three trays as pathogen only inoculated control were sown. After 14 days, seedlings were infested with *F. oxysporum* f. sp. *ciceris* by inoculating the trays with 60 ml suspension containing 3.5×10^5 conidia/ml around the plants.

The experimental plants were grown in a growth room at 16 h photoperiod (using 400 W mercury halide lamps), 25/20°C day/night and 80% relative humidity (close to the saturation range). Data were recorded on the basis of percent disease incidence which was determined by counting the number of plants showing typical symptoms caused by the Fusarium, which included yellowing and wilting of leaves followed by general chlorosis and complete wilting of the plants and confirmed by observing vascular discoloration as described by Haq and Jamil (13). Out of 90 rhizobacterial isolates, the two most effective ones, I-5 and I-65, were selected for glasshouse and field experiments.

Determination of colony forming units (CFU) on seeds

One gram sample of seeds treated with each isolate was taken and placed in separate 99-mL sterile PBST solution (Phosphate Buffered Saline, pH 7.0 plus 0.05% vol/vol Tween 20) and stirred at 100 rpm for 30 min. Five dilutions were made from seed washings and 0.2 ml were plated on GPAM media (5 plates). Each rhizobacteria colony was counted and these were expressed as CFU/g of seed. The counts indicated that the initial viable cells of bacteria on seed varied between 6.5×10^7 to 5.5×10^8 CFU/g.

Evaluation of rhizobacteria and neem cake on Fusarium wilt in the glasshouse

Two experiments were conducted with a neem cake soil amendment and rhizobacteria in both steam sterilized and un-sterilized clay-loam soil under glasshouse conditions at

Effects of Two Rhizobacterial Isolates and Neem Cake Application on Control of Chickpea Wilt Caused by *Fusarium oxysporum* f. sp. *ciceris*

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Abstract

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Two rhizobacterial isolates of *Pseudomonas fluorescens* and neem cake, individually and in combination, were evaluated for their ability to antagonize *Fusarium oxysporum* f. sp. *ciceris* the causal organism of a chickpea wilt disease in a growth room, glasshouse and under field conditions. A reduction of >32% in wilt incidence was achieved by two strains I-5 and I-65 out of 90 rhizobacterial isolates tested. These bacterial isolates were applied to soil amended with or without neem cake, as an organic amendment in all of the experiments. The rhizobacterial isolates when applied with neem cake caused reductions in wilt incidence from 27 to 31%, 31 to 38% and 31 to 36% over the pathogen inoculated control in glasshouse sterilized, unsterilized and field soil, respectively. Disease incidence was higher when isolates were applied without neem cake. When soil was amended with neem cake, each isolate resulted in an increase in grain yield of 146 to 155%. The yield and 1000 grain weight in plots treated with each bacterial isolate either singly or combined, but without neem cake was significantly lower. The production of siderophores with fungicidal properties, by disease-suppressive isolates of *Pseudomonas fluorescens*, is an essential step for the improvement of their effectiveness and reducing the growth and development of Fusarium wilt disease of chickpea. The potential of combining the rhizobacteria and neem cake is worthy of further evaluation as a biocontrol system for chickpea wilt.

Keywords: Biological control, *Pseudomonas fluorescens*, *Azadirachta indica*, chickpea wilt, *Cicer arietinum*.

Introduction

Chickpea (*Cicer arietinum* L.) is one of the most important food legume crops grown worldwide (29). Chickpea Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *ciceris* (Padwick) Matuo & Sato, is a serious soil-borne plant disease in Pakistan and worldwide, resulting in considerable yield losses (2, 14, 18, 33). In Pakistan, chickpea is grown in rainfed areas and the livelihoods of many farmers are reliant on the success of this crop.

The difficulty in controlling Fusarium wilt has stimulated research in biological control independently of the recent concern for environmental protection from using broad spectrum fungicides (10). Microbial antagonism is an important component in biological control of soil-borne plant pathogens (4). Application of *Pseudomonas fluorescens* Migula to chickpea seeds significantly reduced Fusarium wilt incidence and increased grain yields over the control by more than 100% (35). Similar studies showed that isolates of *Bacillus subtilis* and *Trichoderma hamatum* were highly effective against vascular wilt of lentil caused by *F. oxysporum* f. sp. *lentis* (8, 9).

Of the numerous soil microorganisms reported to be antagonistic to plant pathogens few are available as commercial products (27). Several obstacles hinder commercialization; these include lack of correlation between bioassay and field performance, consistency in efficacy, specificity for target organisms, rhizosphere competition and abiotic factors (27, 28, 34). Even so, there could be opportunities for developing rhizobacteria as part of a low cost control strategy appropriate for farmers in arid regions.

In many crop-pathogen systems, the primary mechanism of biocontrol by fluorescent pseudomonads is production of anticbiotics such as 2,4-diacetylphloroglucinol, pyoluteorin, pyrrolnitrin and phenazine-1-carboxylate (32). Under certain conditions, antibiotics improve the ecological fitness of these bacteria in the rhizosphere, which can further influence long-term biocontrol efficacy (26). Siderophores, including salicylic acid, pyochelin and pyoverdine, which chelate iron and other metals, also contribute to disease suppression by conferring a competitive advantage to biocontrol agents for the limited supply of essential trace minerals in natural habitats (25). Antibiotics and siderophores may further function as stress factors or signals inducing local and systemic host resistance (24).

Some organic amendments are effective in controlling a variety of soilborne plant diseases, plant parasitic nematodes and weeds (23). More than 100 compounds from various parts of the neem tree (*Azadirachta indica*) have activity against insects and plant pathogens (5, 19, 30).

The main objective of this study is to evaluate rhizobacterial isolates as disease control agents and to see whether addition of a neem cake formulation, singly or combined, enhanced their antagonistic activity against chickpea wilt pathogen (*F. oxysporum* f. sp. *ciceris*) in a series of growth room and glasshouse bioassays and in a preliminary field trial.