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# ASSESSMENT OF INDUCED SYSTEMIC RESISTANCE THROUGH ANTAGONISTIC RHIZOBACTERIAL POTENTIAL WITH SALICYLIC ACID AGAINST KARNAL BUNT OF WHEAT

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**A B S T R A C T**

Wheat is the staple food crop all over the world. *Tilletia indica* causing Karnal bunt of wheat now considered alarming factor due to considerable losses in terms of crop yield. Chemical control of Karnal bunt is yet very difficult so there was need of alternate control especially biological control. Detailed survey was done for the collection of infected grains and the soil for pathogen as well as antagonistic microbe isolation. In-vitro tests were performed to check the antagonistic properties against *Tilletia indica*. Field evaluation of these rhizobacterial isolates with and without salicylic acid was done in BARI Bahawalpur. Already screened two susceptible advance lines and one moderately susceptible variety was selected for further studies. Seeds treated with three antagonistic rhizobacterial isolates were grown in plots already treated with salicylic acid and untreated as control. Treatments were assessed for incidence of karnal bunt in susceptible and moderately susceptible varieties of wheat. Incidence of Karnal bunt was significantly lower in antagonistic rhizobacterial treated seeds sown in salicylic acid treated plots as compared to untreated plots.

**Keywords**: Induced Systemic Resistance, karnal bunt, biological control, artificial inoculation, salicylic acid.

## INTRODUCTION

Wheat (*Triticum aestivum* L.) is the main food crop and major source of nutrition for the people of Pakistan. It is used as a staple food in Pakistan and other countries. It plays a remarkable role in meeting the food requirements and economic stability of the country. In the GDP its share is about 3.1 %. According to Economic Survey of Pakistan, 2011-12 wheat was cultivated on an area of 9.042 million hectares. Production target of wheat was set 25 million tons in 2011-12, but the size of wheat crop was 23.864 million tons (FAOSTAT, 2013).

The pathogen infects the ovaries in the emerging wheat heads and converts the grains partially or completely into dark colored powdery masses of teliospores. The diseased fields emit a foul smell like that of rotten fish due to production of Trimethylamine. Karnal bunt can reduce wheat yields. There is no estimate of losses, due to this disease, occurring in Pakistan; however, survey in India conducted that years of heavy disease revealed a total loss of 0.5 percent, but in some fields where 89 percent of the kernels were infected, the yield losses ranged from 20-40 percent in highly susceptible varieties (Hussain *et al.,* 1988). During studies in 2011-12, maximum disease prevalence was observed in Chakwal (50%) as compared to Rawalpindi (35%). Village Chakri in Rawalpindi and Village Mangwal in Chakwal exhibited highest disease incidence of 9.98% and 10.34% respectively and from advance lines, 'MN-8' and 'MN-26' were found to be susceptible with 10.2 and 19.2 coefficient of infections, and from commercial varieties, 'Kohinoor-83' with 7.73 coefficient of infection was moderately susceptible. (Ahmed *et al.,* 2013).

As the pathogen is soil, seed and air-borne, it can penetrate locally into host plant, so application of spray fungicides is very critical. Epidemiological factors have

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great influence on the epidemic development of karnal bunt disease. Wheat is vulnerable to Karnal bunt fungus only during a 2-3 week windows of its physiological development stages if the environmental conditions happen to be conducive during this short period for successful infection and the weather favorable for the disease development does not exist every year (Workneh *et al.,* 2008). Singh *et al.* (1985) were able to control the infection of common bunt by 100% by soaking wheat seed in plant juices of *Canabis sativa*, *Eucalyptus globulus*, *Thuja sinensis* and *Datura stramonium*.

Conventional approaches for controlling KB include cultural practices such as crop rotation, sowing of disease- free seeds, and adjustment of the time of irrigation to minimize disease infection (Munjal 1974; Singh 1985). Chemical control of the disease is very difficult and is not cost effective. Most of the fungicides tested against teliospores are fungistatic and not fungicidal.

**MATERIALS AND METHODS**

**Isolation of Pathogen and Antagonistic Microbes:** A survey of Wheat growing areas and grain markets was done and karnal bunt infected grain samples were collected from Punjab and KPK during 2012-2013. Teliospores were collected by culturing infected wheat samples first on water agar and then primary cultures were obtained on PDA. The growing liquid cultures of *T. indica* were harvested.

Isolation of rhizobacterial cultures were also done from the soil samples collected from wheat growing areas of Punjab and KPK using Nutrient agar media with incubation of 2 -3 days at 25±2$^0$C and then cultures were purified by picking single colony and mass cultured.

**Siderophore Production of Bacterial Isolates:** Production of siderophore was measured using method as described by Alexander and Zuberer (1991). Universal chrome azurol S (CAS)-agar plate assay was used to detect siderophore production by microorganisms using CAS medium (Merck, Germany). 10 µL of the bacterial suspension was inoculated on each plate in twice. After incubation at 28 $^0$C for 24 h orange color indicated siderophore production and the diameter was measured. Ratio of orange halo diameter on media to colony diameter shown siderophore production as described by Sung *et al*., (2001).

**Auxin Production of Bacterial Isolates:** Auxin production was analyzed by the method described by Patten and Glick (1996). Briefly, the test bacterial cultures were inoculated into broth with tryptophan (0,1 g / L) or without tryptophan was incubated at 30 °C. Cultures were centrifuged at 3000 rpm for 30 minutes. Two milliliter of supernatant was mixed with 2 drops of phosphoric acid and 4 ml of reagent Salkowski (50 mL, 35% perchloric acid, 1 ml of 0.5% $FeCl_3$). After 20 min, the samples were considered positive blushed, aapsorbancija mixture was measured at 535 nm with a spectrophotometer.

**Hydrogen Cyanide (HCN) Production of Bacterial Isolates:** Qualitative cyanide determination was carried out by Lorck method modified by Alstrom. Isolates sub cultured on NA medium were supplemented with glycine (4.4 $gL^{-1}$). The production of cyanide was detected 48 h after inoculation, using picrate/$Na_2CO_3$ paper fixed to the underside of the petri dish lids which were sealed with parafilm before incubation at 28˚C. A change from yellow to orange, red, brown, or reddish brown was recorded as an indication of weak, moderate, or strongly cyanogenic potential, respectively. Then, cyanogenic isolates were determined and identified.

**In-vitro Evaluation of Rhizobacterial Isolates:** The efficacy of five selected isolates of rhizobacteria collected from wheat growing areas of Pakistan in inhibiting the colony growth of *Tilletia indica* was tested through inhibition zone techniques at 40 ppm, 60 ppm and 80 ppm (Khan and Ilyas, 2007). Sporidial suspension was spread on the surface of the Petri plates containing PDA. Each isolates of bacteria was streaked in each replicated Petri plates. After incubation at 15˚C for 6 days, zone of the inhibition of mycelial growth made around the vicinity of the bacterial colony was recorded.

**Field Evaluation of Rhizobacterial Isolates and Salicylic Acid against artificial inoculation of *Tilletia indica:*** From 119 wheat advance lines and 11 commercial wheat verities already screened, Two susceptible advance lines (MN-8 and MN-26) and one moderately susceptible variety (Kohinoor-83) (Ahmed *et al.,* 2013) were selected for field evaluation. Experiment was carried out in 2 m × 1 m microplots at BARI Bahawalpur in 2012 and then repeated in 2013. The beds were treated with salicylic acid and untreated beds served as control. After treatment with rhizobacterial strains (2.0–2.5 × $10^6$ CFU/seed), wheat seeds were sown in these plots. Then about 4 ml bacterial suspensions were inoculated during last week of March, when wheat plants were at booting stage. Following boot inoculation technique about five spikes

per line were inoculated with the help of hypodermal syringe and water was used as control (Aujla *et al.,* 1983). Harvesting and then hand threshing was done to check the incidence of karnal bunt using formula:

$$\text{Disease incidence (\%)} = \frac{\text{Total No. of infected grains}}{\text{Total No. of grains}} \times 100$$

Disease severity was checked using 0-9 disease rating scale. Modified disease rating scale (0-9) was used to determine level of resistance based on Aujla *et al.* (1989) and Bonde *et al.* (1996).

Table1. Rating scale used to determine level of resistance/ susceptibility.

| Rating | % Grain infection | Response |
|---|---|---|
| 0 | Healthy | 0 |
| 1 | 25 % seed bunted. | 0.25 |
| 2 | 25 % seed bunted. | 0.25 |
| 3 | 50 % seed bunted. | 0.50 |
| 4 | 75 % seed bunted. | 0.75 |
| 5 | 100 % seed bunted. | 1.0 |

Table 2. An example of the calculation of coefficient of infection (modified from Aujla *et al*., 1989).

| Scale | 0 | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|---|
| Num. values | 0 | 0.25 | 0.25 | 0.50 | 0.75 | 1.0 |
| Grains | 200 | 75 | 75 | 50 | 25 | 10 |
| Multiplication with Num. | 0 x 200 | 0.25 x 75 | 0.25 x 75 | 0.50 x 50 | 0.75 x 25 | 1.0 x 10 |
| Values after multiplication | 0 | 18.75 | 18.75 | 25 | 18.75 | 10 |

Gross total = 0.00 + 18.75 + 18.75 + 25 + 18.75 + 10 = 91
Total grains = 200 + 75 + 75 + 50 + 25 + 10 = 435
Coefficient of infection = 91 x 100 ÷ 435 = 20.91

## RESULTS AND DISCUSSIONS

**Isolation and purification:** Total forty five rhizobacterial isolates were isolated from soil samples and were purified on nutrient agar by picking single colony and then mass cultured for further studies. Out of total forty five isolates five isolates were selected on the basis of its antagonistic effect for further studies. Khot *et al.* (1996) isolated 36 rhizobacteria from rhizosphere of chickpea and five bacteria were found to inhibit the growth of *Fusarium oxysporum* and *Rhizoctonia bataticola*. Siddiqui *et al.* (2001) showed that *Pseudomonas aeruginosa* and *Bacillus subtilis* strains produced inhibition zones by inhibiting the radial growth of *Macrophomina phaseolina*, *Fusarium oxysporium* and *Rhizoctonia solani*.

**Production of Siderophore by Bacteria:** All the five isolates were capable of producing siderophore (Table 3). Larger diameter orange halo around the colonies formed by isolate Rh-14 (2.07 cm) and then in case of Rh-3 the diameter was 2.04 cm.

**Production of Auxin by Bacteria:** Auxin (IAA) production was observed in all the isolates. As shown in Table 3, Rh-7 produced more auxin (24µg $mL^{-1}$) as compared to other isolates.

**Production of Hydrogen Cyanide (HCN) by Bacteria:** HCN generating bacteria were classified in 4 groups with very high, high, medium and low ability. All the isolates showed medium ability in producing HCN except Rh-7 (3.01) shown high ability in producing HCN (Table 3).

Table 3. Production of siderophore in CAS medium, HCN production and auxin concentration in the isolates.

| Isolates | Auxin concentration (µg $ml^{-1}$) | Siderophore production (Halo diameter cm) | HCN* |
|---|---|---|---|
| Rh-3 | 21 b | 2.04 a | 2.00 a |
| Rh-4 | 19 b | 2.00 a | 2.03 a |
| Rh-7 | 24 b | 2.01 a | 3.01 b |
| Rh-9 | 20 b | 2.00 a | 2.45 a |
| Rh-14 | 21 b | 2.07 a | 2.09 a |

Similar letters in each column represent insignificant difference in LSD test at ($P<0.05$). Data are means of 3 replications. * 0: No ability, 1: low ability, 2: medium ability, 3: high ability, 4: very high ability of HCN production.

***In-vitro* Evaluation of Rhizobacterial Isolates:** Rh-4 and Rh-9 were less effective than Rh-14 as antagonistic rhizobacterial isolates under *in-vitro* conditions that produced 2.31, 2.10, 2.01 mm diameter inhibition zones at 2.0 × $10^6$ CFU, 2.3 × $10^6$ CFU and 2.5 × $10^6$ CFU respectively. Ahmad *et al.* (2008) reported that siderophore production and antifungal activity was exhibited by 10 to 12.77% of *Azotobacter* and *Pseudomonas* isolates. Pseudomonas Ps5 and Bacillus B1 isolates showed broad-spectrum antifungal activity on Muller-Hinton medium against *Aspergillus*, *Fusarium* and *Rhizoctonia bataticola*. Similarly, Karuppiah and Rajaram (2011) showed that eight Bacillus sp. out of 63 different Bacillus isolates exhibited plant growth promoting activities and six of these Bacillus isolates also inhibited the growth of *Penicillium sp., Cerco-spora sp.* and *Fusarium oxysporum*. However, there was statistically difference between the effectiveness of Rh-4 and Rh-9. Rh-7 at 2.0 × $10^6$ CFU dosage rates and Rh-4 at 2.5 × $10^6$ CFU produced statistically same results like 1.93 mm and 1.94 mm in diameter inhibition zone of fungus. Rh-14 produced statistically good results at 2.0 × $10^6$ CFU and 2.3 × $10^6$ CFU dosage but there was less and not good result of Rh-4. Control of all the replications was zero because there was not applied any antagonistic bacterial isolate in Petri plates and Fungus growth was maximum and no movement of zone towards the fungus was shown.

Table 4. Mean inhibition zones of colony of *Tilletia indica* by antagonistic microbes at different rates in PDA medium.

| Isolates | Mean Inhibition zone (mm) at 3 dosage rates | | |
|---|---|---|---|
| | 2.0 × 106 CFU | 2.3 × 106 CFU | 2.5 × 106 CFU |
| Rh-3 | 2.07 h* | 2.12 e | 1.98 e |
| Rh-4 | 1.71 f | 1.30 c | 1.94 ef |
| Rh-7 | 1.93 ef | 1.80 d | 1.74 a |
| Rh-9 | 1.88 l | 1.81 l | 1.50 l |
| Rh-14 | 2.31 h | 2.10 e | 2.01 e |
| Control (Water) | 0.00 | 0.00 | 0.00 |

Table 5. ANOVA of Mean inhibition Zone *Tilletia indica* by Antagonistic Microbes.

| SOV | D.F | S.S | M.S | F value | Prob>F |
|---|---|---|---|---|---|
| Treatment | 3 | 1.487 | 0.496 | 9.2969$^{S*}$ | 0.003 |
| Replication | 2 | 0.485 | 0.242 | 4.5469 | 0.0212 |
| Treatment x Replication | 6 | 0.335 | 0.056 | 1.0469 | 0.4226 |
| Error | 24 | 1.280 | 0.05 | | 0.053 |
| Total | 35 | 3.587 | | | |

Coefficient of Variation: 12.89 % P=0.05

**Field Evaluation:** Karnal bunt disease of wheat has assumed on alarming situation in the Punjab during the previous two to three decades and has been reported to cause, depending upon the cultivar affected, up to 30 percent grain losses (Ilyas *et al*., 1989). This calls for control of disease either by the use of host resistance or through the use of chemotherapy and by other means especially biological control. In field condition (microplots) the efficacy of antagonistic bacteria alone and in combination with salicylic acid had great impact against karnal bunt of wheat as the results (Table 6) show that the highest decrease in mean disease incidence was 0.303 with coefficient of infection was 0.116 (Table 6) when antagonistic bacteria (*Rh-3*) was applied in combination with salicylic acid. But in the case of alone salicylic acid applied without antagonistic bacteria the effect was not as better as applied in combination. Bacteria alone and in combination with amendments not only reduced the disease incidence of karnal bunt, it also affected the yield of the wheat by increasing its seed weight, however, bacteria with organic amendments were more effective than alone. As seed treatment for organic agriculture it is recommended to use mustard or milk powder (Borgen and Kristensen, 2001), milk-powder in combination with bio-agents (Borgen and Davanlou, 2000), acetic acid (Borgen, 2001) or hot water treatment (Nielsen *et al.,* 2000), which are more efficient than the ones tested in this experiment.

The results were further co related with the previous finding of biological control of karnal bunt of wheat as Kollmorgen (1976) observed that Bacillus species

reduced disease incidence of common bunt under field conditions. McManus *et al.* (1993) reported that some strains of *Pseudomonas fluorescens* inhibited the germination of *T. laevis* teliospores and reduced bunt incidence by 65% when wheat seeds were inoculated with these strains. Hokeberg *et al.* (1997) and Johnsson *et al.* (1998) found that one *P. chlororaphis* isolate, MA 342, is a potent inhibitor of *T. caries* in the greenhouse and in the field. This strain has been developed into the commercial biopesticides, Cedomon and Cerall. So in the results shown that highest decrease in the incidence of karnal bunt in susceptible advance line MN-08 was 0.303 % with coefficient of infection 0.116 due to use of Rh-3 (Antagonistic Bacteria) in combination with salicylic acid, and the result lies in resistant line according to the scale (Table 1) and similarly in case of MN-26 Rh-14 in combination of salicylic acid gave best results and again in case of Kohinoor-83, Rh-3 with salicylic acid shown best results.

Table 6. Evaluation of rhizobacterial isolates alone and with Salicylic acid against Karnal bunt of wheat on three varieties.

| Treatment | MN-8 | | MN-26 | | Kohinoor-83 | |
|---|---|---|---|---|---|---|
| | Mean D I | C I | Mean D I | C I | Mean D I | C I |
| Control | 11.1491 | 6.375 | 8.642 | 5.027 | 6.566 | 5.664 |
| Salicylic acid | 8.3196 | 4.654 | 8.4551 | 4.88 | 5.3951 | 2.915 |
| Rh-3 | 1.105 | 0.3623 | 1.2009 | 0.3787 | 4.2884 | 4.076 |
| Rh-4 | 4.361 | 1.403 | 2.7954 | 0.9259 | 3.9662 | 2.4611 |
| Rh-7 | 1.19 | 0.8458 | 1.2728 | 0.988 | 0.4081 | 0.4132 |
| Rh-9 | 5.3864 | 2.327 | 1.7157 | 0.956 | 4.361 | 1.403 |
| Rh-14 | 1.489 | 0.845 | 1.0141 | 0.391 | 1.032 | 0.344 |
| Rh-3 + SA | 0.303 | 0.116 | 0.8163 | 0.609 | 0.4651 | 0.125 |
| Rh-4 + SA | 2.0587 | 1.351 | 1.8007 | 1.1467 | 1.9264 | 0.5067 |
| Rh-7 + SA | 0.588 | 0.649 | 1.5909 | 0.741 | 0.6557 | 0.586 |
| Rh-9 + SA | 1.7157 | 0.956 | 1.0167 | 0.4484 | 1.554 | 0.5 |
| Rh-14+ SA | 0.606 | 0.119 | 0.4347 | 0.2403 | 0.7547 | 0.246 |

DI means Mean Disease incidence (%), CI means Coefficient of infection.

As results were closely related with the previous findings like treatment of Jasmonic acid (JA) that the effect was more pronounced in susceptible lines than the resistant lines. The infected and JA-treated plants had short spikes and reduced number of spikelets. JA-treated but pathogen inoculated spikes showed reduction in KB infection especially at their basal portion. The spikes were compact but not shriveled like in the pathogen inoculated varieties. The spikes of JA treated control plants were more compact and healthy than those in other treatments (Mandal *et al.,* 2006). According to Borgen (2001) besides JA which acts as defense activator molecule, some other molecules such as salicylic acid (SA) and ethylene also regulate the expression of defense related genes in a spatio-temporal manner and thus act as signals for induced systemic resistance (ISR).

**CONCLUSION**

To control common bunt in organic agriculture it is recommended to use a combination of different measures. This includes discarding the most infested seed lots, use of resistant varieties, organic amendments (use of salicylic acid, Jasmonic acid etc.) and use of antagonistic microbial potential. With a combination of these control measures, Karnal bunt can be controlled in the future.

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