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SEED BACTERIZATION WITH *PSEUDOMONAS* ISOLATES AGAINST WHEAT CYST NEMATODE (*HETERODERA AVENAE*)

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Abstract

The indigenous *Pseudomonas* strains were screened in greenhouse and in field experiments against wheat cyst nematode *Heterodera avenae. Pseudomonas fluorescens* and *P. putida* isolates were identified by the 16S rDNA based molecular technique, root colonization and secondary metabolite siderophores, with protease, hydrogen cyanide HCN and chitinase activity also being detected. In green house noteworthy reduction in white female development was observed for *P. putida* Ps190 (75.2%) followed by *P. fluorescens* Ps104 (67.6%), *P. putida* Ps197 (66.7%), *P. putida* Ps196 (58.2%), *P. fluorescens* Ps109 (57.7%) and avermectin 50.0% as compared to results of the untreated control treatment ($p \leq 0.05$). However, in the field experiment results showed avermectin 70.0%, followed by *P. putida* Ps190 (66.8%), *P. putida* Ps196 (66.3%), *P. putida* Ps197 (66.0%), *P. fluorescens* Ps104 (55.9%) and *P. fluorescens* Ps109 (55.4%) compared to the untreated control treatment ($p \leq 0.05$). The seed bacterization also increased wheat yields by *Pseudomonas* isolates as compared to the untreated control ($p \leq 0.05$).

Introduction

The soil-borne cereal cyst nematode (CCN) pathogen of the family *Poaceae* or *Gramineae* belongs to the genus *Heterodera*, and forms a complex of closely related species. *Heterodera avenae* Wollenweber 1924 was described in the 20th century followed by *H. latipons* Franklin1969 from the Mediterranean region, *H. hordecalis* Andersson 1975 from north Europe and H. *filipjevi* (Madzhidov) Stelter 1984 from eastern Europe. The genus includes 62 species that infect cereals and grasses; and are responsible up to more than 10.0% yield losses worldwide (Evans and Rowe 1998, Wouts and Baldwin 1998, Gabler *et al*. 2000, Nicol *et al*. 2002). In China, the occurrence of *H. avenae* is distributed in 16 provinces and approximately on 20 million ha with the annual wheat yield losses being estimated at more than 25.0% (Peng *et al*. 2015).

The integrated pest management (IPM) approach exploits bacteria for the management of CCNs, including obligate parasites such as *Pasturia* spp. *P. nishizawae*, plant growth promoting bacteria and endophytic bacteria like *Bacillus*, *Pseudomonas* spp., *Actinomycetes, Azotobacter chroococcum*, *Azospirillum lipoferum* for the augmentation control of the second stage juvenile (J2) *in vitro* screening as well as *in vivo* screening experiments (Sayer *et al*. 1991, Kloepper *et al*. 1992, Hallmann *et al*. 1997, Davies 1998, Bansal *et al*. 1999, Li *et al*. 2011, Yavuzaslanoglu *et al*. 2011). In China, the management of CCNs mainly focused on seed coating with different biocontrol agents like Gannong I, II, III, abamectin AVI, AV2 and emamectin benzoate. These are all environmentally safe, have lower toxicity and are suitable for wide applications (Peng *et al*. 2015).

The present authors studied the effects of *Pseudomonas* isolates biocontrol activity on wheat root infection as well as cyst development in green house conditions and in field experiments against *H. avenae.* The ability of root colonization, secondary metabolite and enzyme production was also observed.

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Materials and Methods

The five indigenous *Pseudomonas* isolates (*P. fluorescens* Ps104; *P. fluorescens* Ps109; *P. putida* Ps190, *P. putida* Ps196, and *P. putida* Ps197) were selected among the 400 bacterial isolates, those were previously screened against second stage juvenile (J2) mortality *in vitro* in Plant Nematology Laboratory, Department of Plant Pathology at China Agriculture University, Beijing. These were identified by 16S rDNA molecular method (Marchesi *et al*. 1998). The secondary metabolite and enzyme like HCN production was determined by Miller and Higgins (1970), siderophores production was detected (Shin *et al*. 2001), protease and chitinase were detected (Wiedmann *et al*. 2000) and (Hernandez-Torres *et al*. 2004), respectively. The ability of root colonization was determined as described by Sharma (2001).

For greenhouse screening CCN susceptible wheat cultivar cv. "Aikang 58" seeds were surface sterilized with 2.5% (NaOCl) while *Pseudomonas* strains were grown on King's medium B and suspended in a solution of 1.0% gelatin as a sticking agent coated on wheat seeds (Weller and Cook 1983). Coated seeds were generally contained about 10^8 CFU per seed. The nematicide, avermectin 1.8% (Syngenta Investment Co., Ltd, Beijing, China) was dressed on wheat seeds as the nematicide control, while only surface sterilized seeds were used as the untreated control. Autoclave sterilized sandy soil was used for greenhouse experiments. *H. avenae* cysts were kept for four months in the refrigerator at 4^{o}C before hatch and second stage juveniles (J2) were obtained from cysts kept in sterile distilled water at 15 - 18^{o}C from 2 - 10 days, collected intermittently for the greenhouse experiments. Individual wheat seed was sown in 30 PVC tubes (2 inch dia. with 7 inch length/PVC tube) of each treatment and 250 second stage juvenile (J2) of *H. avenae* were inoculated soon after seedling germination. After 10 days post inoculation (DPI), wheat plants from 15 tubes per treatment were observed. Roots were stained with acid fuchsin and observed for nematode infection (Byrd *et al*. 1983). Wheat plants and soil of remaining 15 PVC tubes of each treatment were washed in a 300 µm sieve and observed after 60 days post inoculation (DPI) for *H. avenae* white female development on roots. The greenhouse environment was maintained at 16 – 20^{o}C over a 15 : 9 hrs light/dark photoperiod and 80% relative humidity (RH). Quantitative data of white female attached on roots and detached from roots in soil were determined under the stereoscope. The reduction rates of cysts of white female were calculated as; RRWF (%) = (CWF – TWF)/CWF × 100. Where RRWF implies for the reduction rate of white females, CWF denotes the number of white female per plant in the control and TWF signifies the number of white females per plant/treatment. Each experiment was repeated thrice in a completely randomized experimental design (CRD).

Field experiment was performed in Agriculture Research Area, Shuang Zhong China Agriculture University, Beijing in the growing season of 2016. Wheat seeds coated as described above were sown on 15October. The trial was fertilized with 220 kg/ha carbamide (Urea) and 120 kg/ha diammonium phosphate (DAP) 3 days before sowing. Plots were 1.5 × 7 m and arranged in randomized complete block design with three replicates of each bacterial isolate, avermectin and the untreated control treatment. Wheat seeds were sown using no-tillage at a rate of 185 kg/ha. Plots were irrigated and hand weeded as needed throughout the season. The white female cyst development observations were recorded from whole plant roots and soil around the roots at the grain filling stage. About 20 wheat plants were collected from three points in each treatment in each plot. The effects of bacterial strains on wheat plant growth were observed according to plant fresh weight at the time of white female counting and final yield were recorded at harvest time. One-way analysis of variance (ANOVA) was performed on green house and field experiments. Comparisons among means were made via Least Significance Difference (LSD) test using SPSS package (SPSS V16.0. SPSS Ltd., Chicago, IL) at the 0.05% probability level.

Results and Discussion

The selected bacterial isolates belonged to *Pseudomonas* spp. Based on the 16rs DNA sequencing, the isolates Ps104 and Ps109 had 99.0% similarity with *P. fluorescens* (GeneBank accession KJ027533.1, AB862147.1). Isolates Ps190, Ps196 and Ps197 had 99.0% similarity with *P. putida* (GeneBank accession CP015202.1, DQ182328.1 & HQ324912.1); a plant growth promoting rhizobacteria (PGPR) (Table 1). All selected isolates significantly produced siderophores, hydrogen cyanide (HCN), had protease activity but chitinase activity was not detected (Table 1). The biocontrol mechanism in *Pseudomonas* was associated with the production of secondary metabolites such as antibiotic, siderophores, hydrogen cyanide HCN, protease etc. (Weller and Thomashow 1993). The siderophores and protease were higher in all isolates while the HCN was similar, with chitinase activity absent. This factor may affect in the greenhouse bioassay and in field results obtained against *H. avenae*.

Table 1. Extracelluar metabolites and enzymes by selected isolates.

Pseudomonas isolates	Siderophores [w]	Protease[x]	HCN [y]	Chitinase[z]
P. fluorescens Ps.104	++	++	++	-
P. fluorescens Ps.109	++	++	++	-
P. putida Ps.190	++	++	++	-
P. putida Ps.196	++	++	++	-
P. putida Ps.197	++	++	++	-

Isolates were tested for ability to produce siderophores[w] as described by Shin *et al.* (2001), Protease[x] by Wiedmann *et al.,* (2000), HCN[y] by Miller and Higgins (2001) and Chitinase[z] by Hernandez-Torres *et al*., 2004; - represents no inhibition zone and +, ++ represent zones of 0 to 5 and 5 to 10 mm in width, respectively.

In the greenhouse bioassay, *P. fluorescens* Ps104, *P. fluorescens* Ps109, *P. putida* Ps190, *P. putida* Ps196, *P. putida* Ps197 and avermectin coated wheat seeds significantly reduced the second stage juvenile (J2) infection in wheat roots, when wheat seedlings were inoculated with 250 second stage juvenile (J2) compared to control treatment after 10 days post inoculation DPI ($p \leq 0.05$) (Table 2). There was no significant reduction in mean numbers of juvenile infection on roots observed among different bacterial treatments and avermectin ($p \geq 0.05$) (Table 2). There was also no significant difference in root length observed after 10 days post inoculation DPI among the bacterial seed coating treatments, avermectin and untreated control ($p \geq 0.05$) (Table 2). Noteworthy reductions in white female cyst development were observed in roots treated with *P. putida* Ps190 75.2% followed by *P. fluorescens* Ps104 67.6%, *P. putida* Ps197 66.7%, *P. putida* Ps196 58.2% and *P. fluorescens* Ps109 57.7% and avermectin 50.0% as compared to the untreated control treatment ($p \leq 0.05$) (Table 2). The maximum numbers of white female cyst development were observed in the control treatment after 60 days post inoculation DPI (Table 2). The root length was significantly increased in treatment *P. putida* Ps190, followed by *P. fluorescens* Ps109, *P. fluorescens* Ps104 and *P. putida* Ps196 simultaneously, as compared to the avermectin treatment and the untreated control ($p \leq 0.05$), (Table 2). In addition, *P. putida* Ps196 and Ps196 were best at root colonization followed by *P. fluorescens* Ps109, *P. putida* Ps197 and *P. fluorescens* Ps104, respectively (Table 2). Different extracellular secondary metabolites and enzymes secreted by *Pseudomonas* spp. (including siderophores, hydrogen cyanide HCN, protease) have inhibitory results against soil-borne phytopathogens (Rodriguez and Fraga 1999, Zukerman and Esnard 1994, Siddiqui 2006) with the induction of plant defense mechanisms. In

the field experimentation, there were significant reductions in white female cyst development observed on wheat roots before harvesting in different treatments and showed to be as avermectin 70.0%, followed by *P. putida* Ps190 66.8%, *P. putida* Ps196 66.3%, *P. putida* Ps197 63.0%, *P. fluorescens* Ps104 55.9% and *P. fluorescens* Ps109 55.4% as compared to the untreated control treatment ($p \leq 0.05$) (Table 3). Wheat plant fresh weight was observed to be non-significant in bacterial seed coating treatments, avermectin and the untreated control ($p \geq 0.05$) (Table 3). However, a noteworthy difference was observed in yield/hectare calculated in bacterial isolates, avermectin seed coating as compared to the untreated control ($p \leq 0.05$) (Table 3). The nematostatic or nematode control ability of all five *Pseudomonas* isolates was shown clearly by

Table 2. Effects of bacterial isolates on the second stage juveniles (J2) of *H. avenae* penetration in wheat roots after 10 days post inoculation, fresh root length and white female cyst development after 60 days post inoculation, fresh root length and root colonization in green house.

Treatment	After 10 DPI		After 60 DPI			Root colonization
	H. avenae (J2)	Root length (cm)	White female	R.R (%)	Root length (cm)	
P. fluorescens Ps104	12.47 ± 5.63a	20.27 ± 5.72a	10.67 ± 4.51bc	67.6	28.47 ± 3.32ab	1.2 ×103
P. fluorescens Ps109	11.33 ± 2.27a	20.60 ± 5.21a	13.60 ± 2.87b	57.7	27.60 ± 5.58abc	1.3 × 104
P. putida Ps190	11.47 ± 2.12a	22.27 ± 6.13a	8.40 ± 4.67c	75.2	30.27 ± 4.37a	1.4 × 103
P. putida Ps196	12.67 ± 5.49a	19.20 ± 3.23a	13.47 ± 3.98b	58.2	27.67 ± 5.15abc	1.4 × 104
P. putida Ps197	13.73 ± 4.97a	18.53 ± 5.07a	10.93 ± 5.43bc	66.7	26.47 ± 6.12bcd	1.3 × 104
Avermectin	13.80 ± 3.94a	19.13 ± 4.81a	7.80 ± 3.76c	50.0	24.60 ± 2.18cd	-
Control (CK)	31.87 ± 4.69b	18.60 ± 4.67a	30.80 ± 6.97a	0	24.20 ± 2.29d	-

Data in column are standard error of 15 replicates. Means followed by the same letter are not significant different by LSD at p = 0.05; R.R = Rate of reduction of white female %.

Table 3. Effects of bacterial isolates on *H. avenae*, wheat plant growth and on yield in field experiment.

Treatment	White female	R.R (%)	Fresh weight / plant (g)	Yield/hectare (kg)
P. fluorescens Ps104	13.95 ± 3.84b	55.9	4.97 ± 1.29a	2413.66 ± 27.36b
P. fluorescens Ps109	14.10 ± 3.31b	55.4	4.86 ± 1.33a	2431.66 ± 57.53b
P. putida Ps190	10.75 ± 3.88cd	66.8	4.89 ± 1.15a	2543.33 ± 45.63a
P. putida Ps196	12.65 ± 3.79bc	66.3	4.84 ± 1.27a	2480.00 ± 61.73ab
P. putida Ps197	11.85 ± 4.70bcd	63.0	4.94 ± 1.24a	2553.33 ± 22.57a
Avermectin	9.80 ± 3.38d	70.0	4.99 ± 1.11a	2546.66 ± 28.54a
Control (CK)	30.40 ± 6.30a	-	4.82 ± 0.89a	2324.00 ± 36.60c

Data in column are standard error of three replicates. Means followed by the same letter are not significant different by LSD at p = 0.05; R.R = Rate of reduction of white female %.

the reduction of white female of *H. avenae* development on wheat plant roots in greenhouse experiments and in field observation. On either side by inducing systemic resistance, the plant system can be protected from various diseases and pests (Zehnder *et al*. 2001). Furthermore, *Pseudomonas* isolates may also improve the plant growth by suppressing other related soil-borne

parasitic and non-parasitic root pathogens (Oostendrop and Sikora 1989). The study demonstrates the potential of *Pseudomonas* isolates to be as sustainable biological management system of *H. avenae* by exploitation of indigenous biocontrol agents. Although health of wheat plants grown from bacteria treated wheat seeds were presumably similar to that of avermectin and untreated control, the white female cyst development was consistently suppressed by seed bacterization. The results of our study reveals a suppression fraction of possible more potent isolates, while continuous further screening may lead to isolate more effective strains that provide significant control measures for cereal crops.

Cereal cyst nematodes have become more severe in more than 16 provinces in China. Biological control through seed bacterization offers a new and sustainable approach to management. Because of vulnerable endoparasitic nature, the root parasite nematode *H. avenae* could be augmented by seed treatment with antagonistic, root colonizing bacteria. The wheat seed bacterization is being targeted for the management of soil-borne nematodes of different cereal crop genera for different agro-ecologies in China. In this study the *Pseudomonas* isolates secondary metabolites produced a significant control against *H. avenae* in green house experimentation and in the field study when applied as a seed coating. They improved plant and in particular root growth. Application of indigenous bacterial isolates could be an alternate solution for the management of soil borne cereal cyst nematode *H. avenae*.

Wheat is the major cereal whose yields have a significant impact on global productivity and thus an influence on food security targets that have been projected for 2050 by when at least 300 million more tons of grain will be required to sustain a populace of about 9.4 billion on this planet. China is a crucial producer and thus addressing all wheat production stresses is paramount to combat this food demand. Wheat varietal production relies heavily on use of genetic diversity to meet yield increase goals but alternate ways are equally important. In this study with CCN and wheat as the model crop, we have shown what integrated management systems have to offer and the outputs are encouraging. For major global impact such strategies need to be integrated with the main breeding systems so an over all methodology can be infused into breeding across a wide range of wheat germplasm efforts. The current positive findings warrant widespread application in Chinese provinces affected and we suggest the protocol applications by researchers in those countries where CCN is problematic.

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