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Bacillus cereus a potential strain infested cereal cyst nematode (*Heterodera avenae*)

S. Ahmed†, Q. Liu and H. Jian

Department of Plant Pathology, China Agricultural University, No 2. Yuanmingyuan West Road, Beijing 100193, People's Republic of China

†Corresponding author: nematologist@gmail.com

Abstract

Bacterial strains were isolated from infested *Heterodera avenae* cysts and screened against the second stage juvenile (J_2) mortality assay *in vitro*. About 40.16 % isolates showed 40.0% J_2 mortality during 12 hours. Significantly increased upto 86.0% mortality was observed in 24 hour exposures. Among them twenty six *Bacillus* strains were identified by morphological, physiological and biochemical carbon source utilization using API 50CHB strips that belonged to *Bacillus cereus* group. *Bacillus cereus* strain B48 secondary metabolite with protease and chitinase activity was used as seed bacterization in green house experiments. In green house significant reduction in white female development was observed for avermectin (84.53%) followed by *B. cereus* strain B48 (78.10%) as compared to results of the untreated control treatment ($P \leq 0.05$).

Keywords: *Heterodera avenae*, *Bacillus*, biological control, secondary metabolites, green house

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 first described as *H. latipons* Franklin, 1969 from the Mediterranean region; *H. hordecalis* Andersson, 1975 from Northern Europe and *H. filipjevi* (Madzhidov) Stelter, 1984 from Eastern Europe, including more than 62 species that infect cereals and grasses. Crop losses from soil-borne cereal cyst nematode (*Heterodera avenae*) varied between cultivars, locations and seasonal conditions, causing 10.0% yield losses worldwide (Whitehead, 1998), whereas $100 billion of global crop losses was caused by plant parasitic nematodes (Gabler *et al*., 2000; Andres *et al*., 2001; Nicol, 2002; Bird & Opperman, 2009). In China, *H. avenae* was found in 16 provinces and caused 25% wheat annual yield losses (Peng *et al*., 2015; Qiao *et al*., 2016). *Bacillus* strains have been studied as biological management of soil-borne plant parasitic nematode. Among them *B. subtilis* and *B. thuringiensis* are more thoroughly being used in biopesticides industry, biotechnology and in integrated pest management (IPM) programmes (Crickmore *et al*., 1998; Kerbs *et al*., 1998; Siddiqui & Mahmood, 1999). *Bacillus thuringiensis* (Bt) produces one or more parasporal crystal inclusions (Cry or δ-endotoxins). The endotoxins are known to be toxic to a wide range of insect species and have direct antagonistic effects to pathogenic nematodes belonging to genera *Heterodera* and *Meloidogyne* (Feitelson *et al*., 1992). Other *Bacillus* strains *viz*., *B. amyloliquefaciens, B. cereus, B.licheniformis, B. megaterium, B. mycoides* and *B. pumilus* and their catabolic enzymes like protease, chitinase and gluconase and small molecules or peptides secreted by *Bacillus* spp. might contribute to their activity against soil-borne pathogenic nematodes (Priest, 1993; Siddiqui & Mahmood, 1999; Gardner,

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2004). *B. megaterium* isolated from sugar beet roots, significantly inhibited the egg hatching and second stage juveniles (J_2) root infection by beet cyst nematode *Heterodera schachtii* Schmidt (Neipp & Barker, 1999).

Metabolites based biopesticides like *Streptomyces avermitilis* (Abamectin), *Burkholderia cepacia* (Deny), *B. amyloliquefaciens* and *Paenobacillus macerans* (BioYield^{TM}) are commercially being used as bionematicides (Tian *et al*., 2007). In Israel, bionematicides commercially formulated from the indigenous strain *Bacillus firmus* as active agents and has been sold under the trade name BioNem-WP for integrated management of root-knot nematode in vegetable crops with drip irrigation system (Keren-Zur *et al*., 2000). In China, integrated pest management (IPM) practices for management of CCNs mainly focused on seed coating with different biocontrol agents like Gannong I, II, III, abamectin AVI, AV2 and emamectin benzoate, all are environmentally safe, have lower toxicity and are suitable for wide applications (Peng *et al*., 2015).

The main objective of this research was to evaluate the biological control potential of the bacterial strains against cereal cyst nematodes on wheat. In this study the inhibitory activity of bacterial strains was investigated isolated from infested CCN cysts and screened against second stage juveniles of *H. avenae* under *in vitro* conditions. The identification of inhibition activity of the bacterial strains may provide important information beneficial to use as environmental friendly management strategy against CCN in natural conditions. Besides the potential bacterial isolate screening on wheat root the cyst development in green house conditions was also studied.

Materials and Methods

Nematode and bacterial isolates: *H. aveane* cysts were collected from susceptible wheat cultivar "Aikang-58" monoculture (≥7y) soil from Agriculture Research Area Shuang Zhong, China Agriculture University Beijing. Cysts were extracted by Fenwick method and kept for four months in the refrigerator at 4^{o}C before hatching (Fenwick, 1940; Riggs *et al*., 2000). Bacterial isolates were found associated with infested CCN cysts, isolated by conventional dilution plate technique and the culture-dependent method. These were differentiated by the Gram staining, cell shape and specific colony appearances according to Schaad (1988). All bacterial isolates were stored in microbial culture banks under low temperature -80°C at Nematology Laboratory Department of Plant Pathology, College of Plant Protection (CPP), China Agriculture University, Beijing, China.

***In vitro* inhibition assay:** Eggs were separated from the cysts by crushing with the help of a rubber cork on a series of sieves, (0.15mm top sieve, 0.05mm middle sieve and 0.038mm bottom sieve). The bottom sieve was backwashed to collect eggs. Second stage juveniles (J_2s) were obtained from eggs kept in sterile distilled water at 15-18^{o}C from 2-10 days, used for the *in vitro* mortality test. The second stage juveniles (J_2s) were disinfected by a series of antibiotic dilutions with centrifugation at 8000 rpm for 2 min. (i) Streptomycin sulphate (1.0%) for 1-2 min. (ii) Ampicillin sodium salt (1.0%) for 1-2 min (iii) Amphotericin-B (1.0%) for 1-2 min. and (iv) Cetyl-trimethyle ammonium bromide (1.0%) for 1-2 min. followed by three time washing and centrifugation at 8000rpm for 2 min. with sterile distilled water (SDW).

The bacterial strains were revived on Lysogenic Broth with addition of agar media at 25^{o}C for 24 h and were further subjected to fermentation in 20ml Lysogenic Broth (L.B) medium at 28^{o}C and under 200rpm shaking condition for 24 h (Sambrook & Russell, 2001; Bertani, 2004). Each bacterial culture broth was centrifuged at 12000rpm for 5min and filtered through 0.22µm membrane filters as described by Nitao *et al*., (1999). Bacterial culture filtrates (CF) were tested in 24-well corning plates. *H. avenae* juveniles @ 50 juveniles/50µl with the ratio of 850µl water (SDW) and 100µl of bacterial culture filtrates (CF) was pipetted into each well of cell culture plates. Sterile culture medium (L.B) containing 100µl sample served as control

for the *H. avenae* mortality assay (Walker *et al*., 1996). Cell culture plates were kept for incubation at 25°C and observed after 12 h and 24 h interval for the dead juveniles. Maximum motionless *H. avenae* juveniles appeared in the wells, which were treated with different bacterial culture filtrates (CF) compared to untreated sterile culture medium (L.B) control wells. *H. avenae* cadaver mortality mathematically calculated according to the equation:

Mortality of J_2 (%) = (Dead cadaver / Total number of Juvenile) x 100

Each treatment was replicated four times and experiments thrice. Results presented are the means of three experiments.

Physiological and biochemical test of selected *Bacillus* strains: Potential *Bacillus* strains culture filtrates (CF) showed maximum inhibitory activity during 12 h and 24 h exposures were further characterized for their morphological, physiological and biochemical characters. Morphology of colonies were studied on LB plates at 30°C for 24 hours, however other distinguished tests, including Voges-Proskauer reaction, gelatin hydrolysis, growth at pH5 and growth at 5 and 40°C were carried out as earlier described (Staley *et al*., 1989). The API50CHB strips were used to determine the carbon source utilization from substrates and other physiological and biochemical characteristics, according to the bioMerieux instructions manual (Logan & Berkeley, 1984).

Enzyme activity for *Bacillus cereus* strain B48: *Bacillus cereus* strain B48 was assessed for production protease and chitinase. Protease and chitinase were detected as described by Wiedmann *et al*., (2000) and Hernandez-Torres *et al*., 2004, respectively.

Effect of *Bacillus cereus* strain B48 against CCN (J_2) on root infection and development of *H.avenae*: Wheat cultivar "Aikang 58" seeds were surface sterilized with 2.5% sodium hypochlorite (NaOCl), rinsed in sterile distilled water and dried overnight. *Bacillus cereus* strain B48 were grown on LB, suspended in a solution of 1.0% methyl cellulose and mixed with wheat seeds (Weller & Cook, 1983). Coated seeds were sampled for the number of colony forming units (CFU) by macerating 10 seeds in a mortar and pestle with 100ml of phosphate buffer (pH 7.2) and plating 100µl on plates of LB agar. Coated seeds contained about 10^8CFU per seed. Avermectin 1.8% (Syngenta Investment Co., Ltd, Beijing, China) was dressed on wheat seeds as nematicide control while seeds without any dressing (only surface sterilized) were used as untreated control (CK). Autoclave sterilized sandy soil was used for green house experiments. The second stage juveniles were collected as described earlier. Individual wheat seed was sown in 50 PVC tubes. After germination each plant was inoculated with 300 second stage motile juvenile (J_2).

After 15 days post inoculation (DPI) 25 PVC tubes of each treatment were observed. Roots were stained with acid fuchsin and observed for nematode infection (Byrd *et al*., 1983). Wheat plants and soil of remaining 25tubes of each treatment were washed in a 300µm sieve and observed after 65 days post inoculation (DPI) for *H. avenae* white female development. The greenhouse environment was maintained at 16-20°C with photoperiod of 15:9 h Light: Dark 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 x 100

Where RRWF implies for the reduction rate of white females, CWF denotes to 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 twice in a completely randomized design (CRD).

Statistical analysis: One-way analysis of variance (ANOVA) was performed on the data of percentages of non-motile *H. avenae* juveniles on the 12 and 24 h exposure duration of *in vitro* experiments and green house 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

***In vitro* inhibition assay:** A total of 365 bacterial strains were isolated from infested CCN cyst *H. avenae* and screened for nematicidal activity against second stage juvenile (J_2) of *H. avenae*.

Significant inhibition of juveniles motility by the screened culture filtrates increased with a maximum exposure time duration to the culture filtrates. Mean numbers of non-motile juveniles (18.57±4.30) and (26.29±2.93) were observed simultaneously in sterile LB broth culture medium controls at the first 12 h and 24 h experiment exposure times (Fig. 1). The significant inhibition of second stage juveniles (J_2) were observed in strains 26 during 12 h followed by 24 h exposure time consecutively.

These isolates were identified as gram positive and belonged to *Bacillus* (Fig.1). About 40.16% of the total isolates showed more than 40.0% second stage juvenile (J_2) mortality during the first 12 h exposure time to culture filtrates. The mortality percentage significantly increased up to 60-87% in 47.5% of the total screened strains as compared to the sterile L.B broth culture medium control treatment during 24 h exposure time. There were significant differences in inhibition activity between isolates and the control at both 12h and 24 h exposure time (Fig. 1).

Morphological, physiological and biochemical test of selected *Bacillus* strains: Morphological, physiological and biochemical characterization of twenty six potential *Bacillus* strains indicated a great similarity to *B. cereus* group (Fig. 2). The API 50CHB strips also exhibited 99.9% similarity with *B. cereus* group. Based on the physiological and biochemical characteristics, the carbohydrates utilization biochemical profile from API 50CHB all twenty six *Bacillus* strains were grouped in *B. cereus* (Table 1).

Enzyme activity for *Bacillus cereus* strain B48: *Bacillus cereus* strain B48 significantly showed protease and chitinase activity (Fig. 3). The activity of these enzymes cleared from surrounding zones around the colonies appeared on agar medium contained skim milk (Fig. 3-A) or colloidal chitin (Fig. 3-B). Since the preliminary screening tests showed that this *strain* significantly reduced the *in vitro* inhibition activity of second stage juveniles (J_2) of *H. avenae* hence it was further tested in a green house bioassay.

Effect of *Bacillus cereus* strain B48 against CCN (J_2) on root infection and development of *H.avenae*: In the green house bioassay, *Bacillus cereus* strain B48 and avermectin coated wheat seeds significantly reduced the J_2 infection in wheat roots, when wheat seedlings were inoculated with 300 J_2s compared to control treatment after 15 days post inoculation DPI ($P \leq 0.05$) (Table 2).

There was no significant difference in root length observed after 15 days post inoculation DPI among the bacterial seed coating treatment, avermectin and untreated control ($P \geq 0.05$), (Fig. 2). Significant reductions in white female cyst development were observed in roots treated with avermectin 84.53% and *B. cereus* strain B48 78.10% as compared to the untreated control treatment ($P \leq 0.05$), (Table 3). The maximum numbers of white female cyst development were observed in the control treatment after 65 days post inoculation DPI (Table 3). The root length was significantly increased in treatment *B. cereus* strain B48 followed by avermectin as compared to the untreated control ($P \leq 0.05$) (Table 3).

Discussion

The *H. avenae* cyst associated soil microbial intensive availability leads to potent and suppressive effect on the soil, which is responsible for the nematostatic condition in the fields (Stirling, 1991). The bacterial strains screened in this study were isolated from inside the CCN cysts of *H. avenae* collected from wheat monoculture soil type (≥7y) in Agriculture Research Area Shuang Zhong,

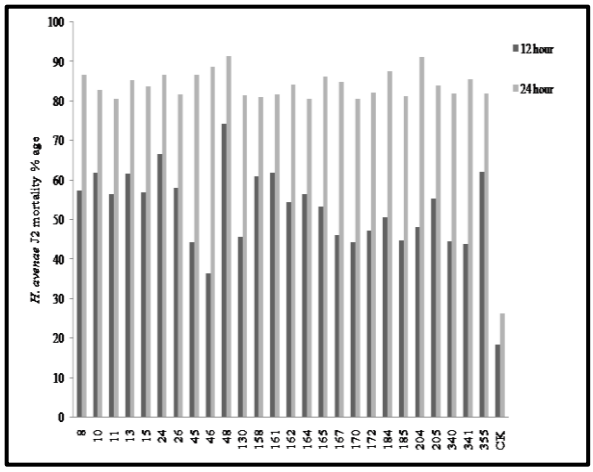

Fig.1. *In vitro* inhibition rates of mortality by *Bacillus* strains culture filtrates isolated from cyst of *H. avenae* relative to the negative control against second stage juveniles (J_2s) of *H. avenae* on 12 hour and 24 hour duration exposure time.

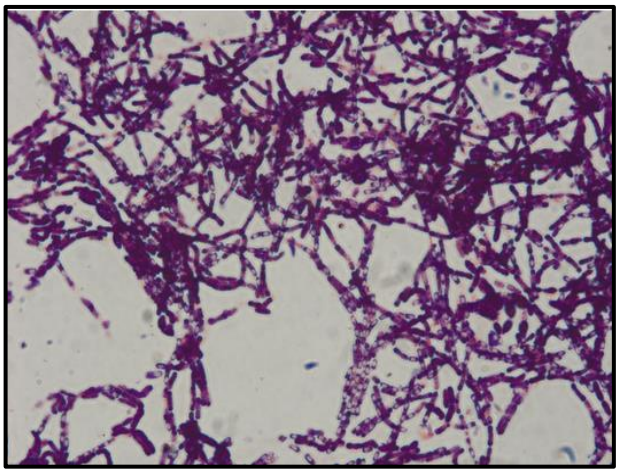

Fig.2. Morphological characteristics of *Bacillus cereus* strain B48. Cell and endospore of *B. cereus* strain B48 with Gram staining and oil microscopy (1600x).

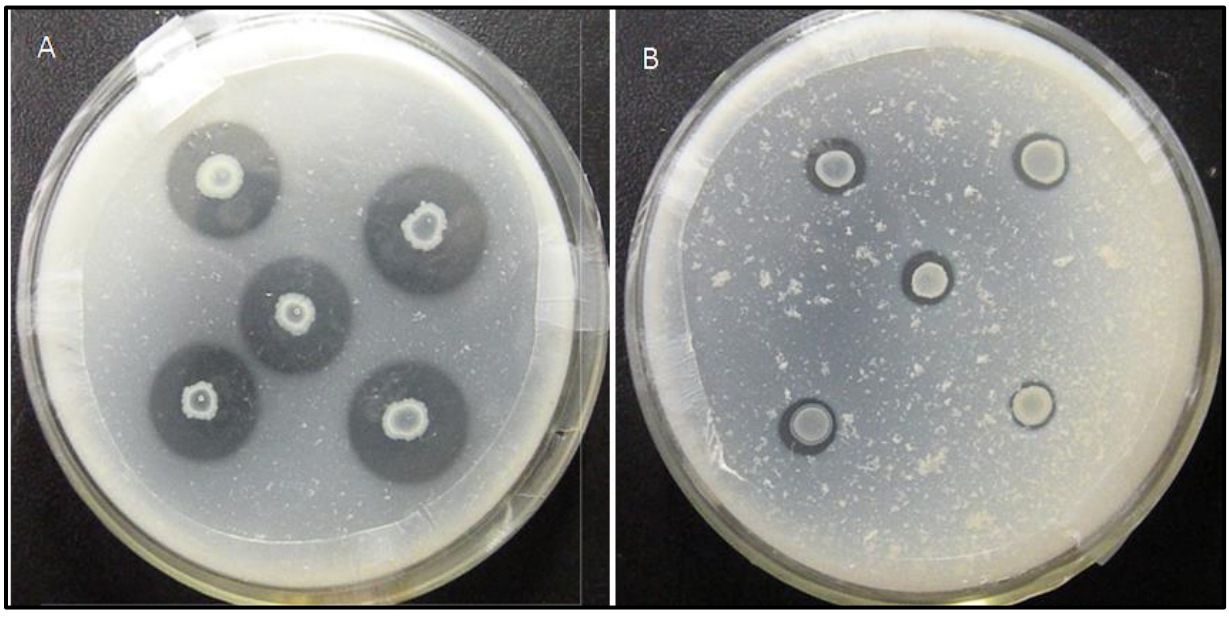

Fig.3. Enzyme activity of *Bacillus cereus* strain B48. Colonies produced clear zone on agar medium plates contained skim milk for protease (A) or colloidal chitin for chitinase (B) after 5 days of incubation at 28^{o}C.

Table 1. Morphological, physiological and biochemical characteristics of *Bacillus* strains[y] (No. of strains 26).

S.No.	Biochemical test	*B. cereus*	S.No.	Biochemical test	*B. cereus*
1.	Cell width (µm)	1.4	21.	API 50CHB tests[z]	
2.	Chains of cells	26	22.	Glycerol	+
3.	Motility	+	23.	Ribose	+
4.	Parasporal crystal	-	24.	Galactose	+
5.	Catalase	+	25.	D-Glucose	+
6.	Anaerobic growth	+	26.	D-Fructose	+
7.	Voges-Proskauer	+	27.	D-Mannose	-
8.	Hydrolysis of		28.	Inositol	+
9.	Starch	+	29.	α-Methyle-D-Glucoside	+
10.	Casein	+	21(	*N*-Acetylglucoseamine	+
11.	Gelatin	+	21	Amygdalin	+
12.	Utilization of citrate	+	21	Arbutin	+
13.	Nitrate reduction	d	21	Aesculin	+
14.	Growth at		21	Salicin	+
15.	5ºC	-	21	Cellobiose	+
16.	10ºC	d	21(	Maltose	+
17.	40ºC	+	21	Lactose	+
18.	Acid from:		21	Sucrose	+
19.	Glycerol	+	21	Trehalose	+
20.	Glycogen	+	22(	Starch	+

[y] *Bacillus* spp. according to Shivaji *et al*. 2006; + and – represents positive and negative reactions.
[z] Data were obtained by use of API50CHB test strips.

Table 2. Effect of *Bacillus cereus* strain B48 on the second stage juveniles (J2) penetration of *H. avenae* after 15 days post inoculation (DPI).

Bacterial isolates	*H. avenae* penetration	Root length (cm)
Bacillus cereus B48	11.52±3.69 b	19.48±5.24 a
Avermectin	9.76±3.23 b	18.12±3.59 a
Control (CK)	37.88±6.01 a	17.06±3.32 a

Data in column are standard error of 25 replicates. Means followed by the same letter are not significant different by LSD at P=0.05

Table 3. Effect of *Bacillus cereus* strain B48 white female cyst development after 65 days post inoculation (DPI), fresh root length in green house.

Bacterial isolates	White female	Reduction rates (%)	Root length (cm)
Bacillus cereus B48	6.72±2.19 b	78.10	28.68±3.66a
Avermectin	5.04±1.60 b	84.53	25.76±3.35b
Control (CK)	27.12±5.30 a	-	22.84±3.31c

Data in column are standard error of 25 replicates. Means followed by the same letter are not significant different by LSD at P=0.05

Beijing, China Agriculture University. Among them, 40.16% strains showed 40.0% mortality of second stage juvenile (J_2) in an initial 12 h exposure duration and subsequently 47.5% strains reduced more than 60 -87 % of second stage juvenile (J_2) mortality *in vitro* bioassay during 24 h exposure time. Second stage juvenile (J_2) nematode cuticle is rigid and composed of proteins and chitins. The possible activity of hydrolytic enzyme of bacterial strains might be involved in the penetration process to help bacteria to kill the juveniles *in vitro* screening. Rhizospheric *Bacillus* strains exhibited hyperplastic activity and by excreting hydrolytic enzyme can easily attacks the soil-borne nematodes (Ahman *et al*., 2002). Among 365 *Bacillus* strains, 26 were specifically identified for morphological, physiological and biochemical characterization. These belonged to *B. cereus* group. This group comprised of *Bacillus* species as *B. cereus*, *B. thuringiensis B. mycoides* and *B. weihenstephansis*. The API 50CHB strips also exhibited 99.9% similarity with *B. cereus*. Based on the physiological and biochemical characteristics, the carbohydrates utilization biochemical profile from API 50CHB all twenty six *Bacillus* strains were grouped in *B. cereus*. The *B. cereus* strains were isolated inside the infested CCN cyst showed significant *in vitro* biocontrol potential against second stage juvenile (J_2) mortality and reduced *H. filipjevi* nematode population up to 30.0% in *in vivo* screening (Ashrafi *et al*., 2015).

In the present study, potential bacterial strain *Bacillus cereus* strain B48 was assessed for their biocontrol significance against soil-borne cyst nematode *H. avenae*. The nematostatic or nematode control ability was showed clearly by the rate of reduction of white female and their development on the wheat roots in greenhouse experiments. Our results demonstrate that the root length of wheat plants in green house conditions was significantly increased by the application of *B. cereus* strain B48. This might be due to the strain's ability of inducing systemic resistance in plant system to be protected from various soil-born nematodes as earlier reported by (Zehnder *et al*., 2001). Among *Bacillus* spp. *B. subtilis*, *B. licheniformis*, *B. amyloliquefacies* and *B. cereus* have been reported effective against soil borne nematodes (Carneiro, 1998; Hassan *et al*., 2010). The *Bacillus* strains associated with cyst and wheat roots showed 70.0-80.0% *in vitro* inhibition of juveniles of *H. avenae* (Unpublished data). The *Bacillus* strains from sugar beet roots reduced hatch of the cyst nematode *H. schachtii* and potato cyst nematode, *Globodera rostochinensis* and *G. pallida* (Ryan & Jones, 2004).

Conclusion

Wheat is the world major and widely cultivated cereal; its yield has a significant impact on global productivity, thus an influence on food security targets projected for 2050 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 any threat to wheat production in China. 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 that an overall methodology can be infused into breeding efforts across a wide range of wheat germplasm. The current positive findings warrant widespread application in Chinese provinces affected with CCN; and we suggest the protocol applications by researchers in those countries where CCN is problematic.

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