Research Article

Performance of Cereal Varieties against Cereal Cyst Nematode (*Heterodera avenae*)

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Abstract | The soilborne pathogen cereal cyst nematode (CCN) *Heterodera avenae* is one of the biotic stress factors that directly affects the crop physiology from seed germination to crop maturity and limits the cereal production in different agro-ecologies of the world. In China, this pathogen is widespread in more than 20 provinces and infects the major cereal varieties and germplasm being adopted for cereal crop production annually. We tested number of cereal accessions to see the host pathogen interaction; among 15 wheat lines from Zhonnguyuan Sun (CAAS) CIMMYT Beijing and 10 lines of oats from Jilin Province were screened against *H. avenae*. The results showed that 5 wheat and 7 oat lines ranked as resistant genotypes while 9 wheat and 3 oat lines were ranked as moderately resistant and 1 wheat line and the local check were highly susceptible to *H. avenae*. These genotypes need to be exploited in breeding program to introduce the resistance gene pool in local cereal cultivars for resistance to *H. avenae* in China.

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Introduction

Wheat (*Triticum aestivum* L.) and their ancestor crops are the initial domesticated and essential food crop of the major civilizations of Asia, North Africa and Europe for last more than 8000 years. These crops are widely cultivated on about 240 million ha, more than any other crop and estimated as one sixth of the total arable land in the world under wheat cultivation (Anon., 2014). The soil born pathogenic cereal root nematodes including cereal cyst nematodes (CCN) *Heterodera* spp., and root-lesion nematodes (RLN) *Pratylenchus* spp., are considered as unseen emerging threat for the cereal crop production around the world. Along with other foliar biotic factors like different leaf rusts, various strains of yellow dwarf viruses (YDV); these soil borne root parasitic eelworms are also responsible for severe damage to cereal roots infection, low grain setting and reduced seed size in cereals during inflorescence as well as they reduce the crop yield in many agro-ecologies (Cook and Noel, 2002; Dababat *et al*., 2015; Smiley and Nicol, 2009). The economic yield losses are varied and were reported ranges from 30.0% to 100% in cereal crops by cyst nematode (*Heterodera avenae*) (Rivoal and Cook, 1993). Lately the attention of the world scientists have been diverted and focused by the International Cereal Cyst Nematode Initiative (ICCNI) of CIMMYT with special reference to soil-borne cereal root nematodes.

It was observed that this nematode has been widely distributed in European countries; where about more than 50.0% of the fields have been infested by different pathogenic species of *Heterodera* spp. *and Pratylenchus* spp. causing approximately US$ 3.0 million yield losses annually (Nicol and Rivoal, 2008). In United States, the cereal yield losses were estimated to be about 24.0% on wheat being cultivated in spring season (Smiley *et al*., 2005). In the year 1989, the prevalence and distribution of *H. avenae* was initially reported from Hubei region of China. However, the extensive research and development was observed in other wheat growing agro ecologies with subsequent observation of this nematode in other 16 provinces viz., Henan, Beijing, Anhui, Inner Mongolia, Gansu, Jiangsu, Hebei, Qinghai, Shandong, Xinjiang, Tianjin, Shaanxi and Tibet (Chen *et al*., 1992; Liu *et al*., 2009; Li *et al*., 2010, 2012; Peng, 1995; Peng *et al*., 2008, 2012; Zheng *et al*., 1996). Today more than 4,000,000ha wheat producing lands were infested by these nematodes and about 40.0% yield losses of wheat annual production was observed from China (Peng *et al*., 2007, 2009). In China, various sources of resistance from Gramineae family including wheat, oat, barley and other relatives, have been identified against cereal cyst nematode, *H. avenae*.

While it was envisaged that the cereal cyst nematode (CCN) pathotypes originated from South Asia and Europe, *H. avenae* pathotype of South-eastern Australia is commonly known as Ha13 (Yuan *et al*., 2010; Brown, 1969). In China the pathotype of *H. avenae* population is being considered to be possibly different from those of other regions of the world because of no arrangement and allocation of pathotypes specification according to the scheme of Anderson and Anderson (1982) and Peng and Cook (1996). Hence, uses of tolerant and resistant cereal varieties with their efficient application are environmentally and economically stable and safe methods for sustainable control of this soil borne cereal cyst nematode (CCN). For durable resistance in wheat crop against CCN, the characterization of CCN and pathotypes is imperative to produce nematode resistance in cereal crop breeding programme and CCN nematode threshold level (NTL) controlling strategies. In present study investigation was undertaken on some winter wheat lines and oats for resistance identification to *H. avenae* population of the Beijing P.R. China.

Materials and Methods

Cereal cyst nematode (CCN) Heterodera avenae inoculum

He experiments were performed in a controlled environmental condition in green house at 18°C temperature at Plant Nematology Lab, China Agricultural University, Beijing, China. The cereal cyst nematode (CCN) Beijing isolate culture was previously established by mono culturing on local susceptible wheat cultivar "Wenmai 19" at Shang Zhuang Agriculture Research Experimental Area of China Agriculture University, Beijing. The CCN soil samples were collected; washed thoroughly and mature cysts were obtained by "Fenwick-Can" laboratory method (Fenwick, 1940). The CCN inoculums were kept for 3-4 months in refrigerator at4°C temperature. Eggs of CCN were discharged from the cyst by crushing with the help of rubber cork on the series of sieves: 0.15mm followed by 0.05mm and 0.0308mm with continuous washing with tap water. The bottom sieve (0.0308mm) was backwashed to collect the eggs. These eggs were kept in sterilized water at the temperature of 15-18°C for the period of 3-10 days for hatching of juveniles. The second stage infective juveniles were collected intermittently and frequently used in vitro screening of resistance/ susceptible reactions against wheat accessions.

Cereal germplasm/ varieties

The wheat entries were acquired from the CIMMYT office located at Chinese Academy of Agricultural Sciences (CAAS) Zhonguyuansun, Beijing, however the oats varieties were requested and obtained from Jilin Province. A total of 15 wheat entries and ten oats varieties were screened for resistance/susceptibility reactions against the *H. avenae*.

Nematode extraction from soil

Composite soil samples were gathered from monoculture wheat fields that were infested with *H. avenae,* at Shang Zhuang Agriculture Research Area of China Agriculture University, Beijing after wheat harvest in year 2016-17. Cyst nematode infested soil was thoroughly mixed with sufficient water gently stirred and passed on series of sieves (710-, 425- and 250µm- pore sieves from top to bottom). Sieving and decanting method described by Ingham (1994) was used to collect dark brown cysts from the soil. The cysts accumulated on the 250µm sieve were dissected with a sharp needle for estimation of eggs count/ cyst. The eggs were carefully observed under

stereomicroscope. The collected cysts were stored in sterilized water in 50ml centrifuge tube and stored in 4˚C for identification of nematodes and incubation.

DNA extraction from nematodes

Cysts were gently crushed by sharp needle and nematode DNA was extracted from the juveniles and eggs. Some 24 individual cysts were selected randomly and were transferred to Nanopure water using a pipette and crushed with sterilized pipette tip. The genomic DNA from these cysts were extracted as described (Yan and Smiley, 2010; Waeyenberge *et al*., 2000).

The suspension containing juveniles and eggs (10µl) was shifted to a 0.5-ml sterile eppendrof tube with 8µl of worm lysis buffer (WLB) (500 mM KCL, 100 mMTris-HCL [pH 8.3], 15 mM $MgCl_2$, 10mM dithiothreitol, 4.5% Tween 20 and 0.1% gelatin). The extracted genomic DNA were frozen in liquid nitrogen and kept at -20˚C for at least 20 minutes. The nematode extracted genomic DNA was gently thawed and in each eppendrof tube containing nematode sample, 2µl of Proteinase K at 600µg/ml was added. This Proteinase K was removed by keeping genomic DNA at 65˚C for 1h followed by 95˚C for 10 min. The extracted genomic DNA mix was centrifuged at 16000 x g for 4 min, shifted in to clean 0.5-ml eppendrof tube and frozen at -20˚C till PCR amplification was carried out. The extracted genomic DNA was quantified in NanoDrop ND-1000 Spectrophotometer.

The PCR amplification was performed by adding the genomic DNA suspension (1µl) to a PCR mixture containing 25µl of 2x Taq PCR master mix 1µl each of forward and reverse primer(HaITS-F1/HaITS-F2) 1µM, and double-distilled water (ddH_2O)to make a final volume of 25µl. The PCR amplification was carried out in a MyCycler Thermal Cycler (Bio-Rad) using the following program as (one initial cycle of denaturation at 95˚C for 3 min; 35 cycles of 95˚C for 30 seconds, 58˚C for 30 seconds, and 72˚C for 7 min). The PCR product was screened on 2.0% agarose gel and 0.5x Tris-borate-EDTA buffer (45 mMTris-borate and 2mM EDTA, pH 8.0). It was stained with ethidium bromide and visualized under UV light for different band pattern.

Cereal germplasm resistance testing against cereal cyst nematode (CCN) H. avenae

Wheat and oats accessions were thoroughly surface sterilized and disinfected by immersing in 95.0% Ethanol for four to five minutes. These were soaked in a solution of 5.0% NaOCl for five minutes and then progressively rinsed in sterile distilled water (ddH_2O). These were kept on moist sterilized filter paper on sterilized glass Petri plates and incubated at 20˚C for 2-5 days for germination. Soon after germination seedlings were transplanted into PVC tubes (4.5cm diameter and 16cm length) filled with hot air oven sterilized soil. Each accession was replicated ten times.

In each tube having the transplanted seedlings, 250 *H. avenae* juveniles (J_{2s}) were immediately added and at three to four days interval another 250 J_{2s} were added to make the final inoculum density of 500 J_2/tube. Wheat and oat plants seedlings were kept in a greenhouse for ten weeks at 16-18˚C; with a day and night period of 16 and 8 hours light and dark respectively. Each plant was carefully irrigated and fertilized. Also these plants were treated with specific chemicals to manage the foliar disease and insect pests to ensure normal plant growth. After ten weeks, the roots of wheat and oats as well as the soil were removed from the PVC tubes. Each plant root was washed under low stream of tap water over 250µm sieves. The female white cysts attached to the roots and those extracted from soil were observed under a stereoscopic microscope as well as compound microscope. The wheat lines and oat cultivars were classified according to scale developed by Nicol *et al*., (2008). The data for the mean number of females cysts population of CCN per plant were calculated and analyzed statistically.

Results and Discussion

The cereal cyst nematode (CCN) *H. avenae* of Beijing population were identified by application of specific sequence characterized amplified region (SCAR) by PCR. Results demonstrated that one single fragment of 1010 bp were amplified from 24 CCN cysts. The SCAR molecular diagnosis species-specific assay method is unique for *H. avenae* (Qi *et al*., 2012).

There were five wheat entries in the present study viz., WM25-INIFAPM 97, WM38-UP2338, WM41-HP1761, WM77-Mino and WM92-Taurum that showed resistance to the *H. avenae* population. Moreover, nine wheat entries including WM-PFAU/VEE#5, WM4-SeriM82, WM6-KAUZ/GEN, WM11-TIA3, WM16-PIRINA, WM18-

CIANOT79, WIM23-BACANORA, WM30-RHEA and WM87-Otus were considered as moderately resistant, however one wheat line WM5-VORONA/GEN showed moderately susceptible reaction as compared to local check variety Wenmai 91 which showed a highly susceptible reaction (Table 1).

Table 1: *The reaction of different wheat accessions from CIMMYT to H. avenae of Beijing population.*

Wheat lines ID	Mean	Reaction
WM-PFAU/VEE#5	7.4 ± 3.4	MR
WM4-Seri M82	6.2 ± 1.1	MR
WM5-VORONA/GEN	10 ± 5.4	MS
WM6-KAUZ/GEN	7 ± 3.1	MR
WM11-TIA 3	9.4 ± 2.8	MR
WM16-PIRINA	5.8 ± 2.7	MR
WM18-CIANOT 79	8 ± 2.0	MR
WIM23-BACANORA	6.4 ± 2.4	MR
WM25-INIFAPM 97	4 ± 0.8	R
WM30-RHEA	7 ± 2.8	MR
WM38-UP2338	5 ± 2.0	R
WM41-HP1761	2.6 ± 0.8	R
WM77-Mino	3 ± 0.6	R
WM87- Otus	7.4 ± 2.4	MR
WM92- Taurum	3.6 ± 1.2	R
Wenmai 91	32.6 ± 8.16	HS

Data for the means of 10 replicates; HS, Highly Susceptible; MS, Moderately Susceptible; MR, Moderately Resistant and R, Resistant. Based on the number of white female cysts per root system: M, Immune (0 females); R, Resistant (0.1-5.0 females); MR, Moderately Resistant (5.1-10.0 females); MS, Moderately susceptible (10.1-15.0 females); S, Susceptible (15.1-25.0 females) and HS, Highly susceptible (>25.0 females).

Among the ten entries of oats; there were seven oat lines viz., Baiyan 3, Baiyan 4, Baiyan 13, Baiyan 16, Baiyan 17, Baiyan 18 and Baiyan 19 demonstrated resistance to *H. avenae* and the rest three lines; Baiyan 8, Baiyan 9 and Baiyan 10 showed moderate resistance to *H. avenae* (Table 2).

The soil born cereal cyst nematode (CCN) *Heterodera* spp. is widely associated with cereal roots in the dry-land and rain-fed cereal production system and responsible for significant yield losses on common cultivars annually. The effects of global warming and climatic changes have been enhancing dramatically the noxiousness pathogens in cereal production regions as well as in intensive wheat production agro-ecologies in Western European countries. There are different management strategies being practiced for *H. avenae* such as the crop cultural control methods comprised of crop rotation of non-host crops or varieties cultivation. It also includes sanitary fallows, although in the winter wheat production areas of different provinces of China, this option is limited. It was experiential that the cereal cyst nematode population compactness reduced by 75.0% with progressive crop rotation with non-host field crops (Singh *et al.*, 2009). In Australia the cereal germplasm is extensively deployed with moderate to strong resistance to *H. avenae* (Riley and McKay, 2009). Some resistance genes were identified for managing the *H. avenae* infection in cereals and described as *Cre*2, *Cre*5 and *Cre*6; these are from *Aegilops ventricosa*; *Cre*7 is from *Aegilops triuncialis*;*Cre*3 and *Cre*4 from *Triticum tauschii*; *Cre*1 and *Cre*8 from *Triticum aestivum*; and *Cre*R is from *Secale cereals* (Barloy *et al.*, 2007).

Table 2: *The reaction of different oat varieties from Jilin Province to H. avenae of Beijing population.*

Oats lines ID	Mean	Reaction
Baiyan 3	3.8 ± 0.78	R
Baiyan 4	4.4 ± 1.01	R
Baiyan 8	5.6 ± 0.48	MR
Baiyan 9	5.4 ± 1.35	MR
Baiyan 10	5.8 ± 0.74	MR
Baiyan 13	4.6 ± 1.01	R
Baiyan 16	1 ± 0.0	R
Baiyan 17	1 ± 0.0	R
Baiyan 18	2.4 ± 0.48	R
Baiyan 19	1.8 ± 0.74	R

Data for the means of 10 replicates; HS, Highly Susceptible; MS, Moderately Susceptible; MR, Moderately Resistant and R, Resistant. Based on the number of white female cysts per root system: M, Immune (0 females); R, Resistant (0.1-5.0 females); MR, Moderately Resistant (5.1-10.0 females); MS, Moderately susceptible (10.1-15.0 females); S, Susceptible (15.1-25.0 females) and HS, Highly susceptible (>25.0 females).

It was observed that the integrated crop management (ICM) supports mainly on genetic host resistance, which seems to be significant when two or more soil borne pathogens initiate infection in the roots of host in the soil at the same time (Nicol and Rivoal, 2008). The results of evaluation and screening of thousands of wheat lines / accessions, the resistant germplasm was relatively scarce in the pool of CCN *H. avenae* resistant wheat germplasm. The present study findings support the above assumption and showed similar results. The incredible and highly susceptible

wheat accession comprised 68.0% of the total wheat varieties, however only 20.0% are resistant against *H. avenae*. It is assumed that the mechanization and use of agriculture machinery in different provinces of China as well as irrigation system might be major source of the dispersal and dissemination of CCN inoculums (Wang *et al*., 2012). Alternatives like application of different kinds of fertilizers and soil amendments with suitable additives of micro or macro elements/ nutrients may reverse back the low production of wheat yields but their use is limited by economic and environmental factors (Rivoal and Nicol, 2009). Other management strategies include; cultural practices, application of different types of soil chemicals, exploitation of genetic pool (resistance/tolerance) and use of microorganisms as biological control agents. To minimize and restrict *H. avenae* population densities below the damage thresholds level (DTL), their net effect should be planned to sustain the possible wheat yield. It was estimated that *H. avenae*is responsible for decrease of wheat yield amounting to 1.9 billion RMB in China only and has acknowledged considerable awareness (Peng *et al*., 2009; Yuan *et al*., 2010). The possible measure for sustainable management of *H. avenae* is the farming with resistant cereal cultivars to the *H. avenae* virulent pathotypes; and the population complexity also affects the pathotypes (Cook and Rivoal, 1998). In the results of studies on CCN pathotype characterization of Qinghai Huangyuan as well as Beijing Daxing populations, it was found that the Beijing Daxing population pathotype was Ha91 (Cui *et al*., 2015). The CCN biotypes from wheat have been identified that can infect resistant plants of cereal accessions/germplasm (Valerie, 1999). The research studies that actively or passively deployed the resources upon host-pathogen interaction and host resistance must have a series of resistance genes recognized and being deployed into wheat cultivars available for use of farmers (Nicol, 2002). Hence to develop resistant wheat cultivars we must understand the CCN nematode population pathotypes and work on morphological, molecular identification as well as host-pathogen interaction tests. Our results provide a data reference for scientists interested in resistance crop breeding to improve cereal production in Northern and Northwestern China agro ecologies.

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Novelty Statement

The cereals and related relative of grass family can provide the source of resistance against cereal cyst nematode pathogens.

Authors Contribution

Shahid Ahmed: Conducted the research as part of his PhD.
Liu Qian: Co supervised the research.
Heng Jian: Supervised the research and guided overall.

Conflict of interest
The authors have declared no conflict of interest.

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