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Evaluation of Maize Hybrids for Resistance to Ear Rot Caused by Dominant *Fusarium* Species in Northeast China

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Abstract: Ear rot caused by the *Fusarium* species has led to a decline in maize yield and kernel quality worldwide. The changes in the population structure of pathogens and the widespread planting of susceptible maize varieties have exacerbated the occurrence and harm of ear rot in China. Therefore, it is very important to establish the species composition of *Fusarium* and evaluate the resistance of the main cultivated hybrids. In this study, 366 single conidial isolates of *Fusarium* spp. were obtained from three provinces of Northeast China. *F. verticillioides*, *F. subglutinans*, *F. proliferatum*, *F. oxysporum*, and *F. graminearum* species complex (FGSC) were identified, with *F. verticillioides* being the most prevalent with a frequency of 44.0%. Based on the *TEF-1α* gene sequences analysis, the FGSC populations consisted of two independent species: *F. boothii* and *F. graminearum*, which account for 23.8% and 5.7% of the total isolates, respectively. Additionally, the resistance to ear rot by 97 maize hybrids commonly planted in Northeast China was evaluated by inoculation with *F. verticillioides* during 2021 and 2022. The results showed that the disease parameters of different hybrids varied significantly ($p < 0.05$). Approximately half of the hybrids had damage rates ranging from 0 to 15%, and 79.4% of the hybrids had a severity rating of less than 5.5. In total, 49 (50.5%) hybrids were rated as moderately resistant, which was the dominant resistance category, and 71 hybrids (73.2%) were identified as moderately to highly resistant to ear rot. Current research confirms that Fusarium ear rot in maize is mainly caused by *F. verticillioides* in Northeast China, and many hybrids are resistant to the disease. This study will guide growers to scientifically deploy resistant commercial hybrids to control ear rot.

Keywords: maize; ear rot; population structure; resistance; *Fusarium verticillioides*

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1. Introduction

Maize (*Zea mays* L.) is an important cereal crop extensively used as a raw material in forage and edible oil production worldwide. Multiple fungal pathogens can infect maize ears and kernels, causing whole ears or part of the kernels to rot during the late growth stage, harvest, or storage. Not only does infection seriously affect maize yield and kernel quality, it also reduces germination and seedling survival rates [1–3]. Furthermore, fungi in maize kernels synthesize toxic secondary metabolites, which are a major food and feed safety hazard, directly threatening the health of humans and livestock [4–7].

In recent years, *Fusarium* species have threatened maize cultivation, with ear rot frequently occurring in the maize-growing areas of China [8]. *Fusarium* species represent the main pathogenic fungi causing maize ear rot globally, including *F. verticillioides*, *F. graminearum*, *F. subglutinans*, *F. proliferatum*, and *F. temperatum*. *Fusarium verticillioides* is one of the most commonly isolated pathogens on maize ears throughout the world [9–12]. The population structure of *Fusarium* species changes with environmental conditions [13]. In Europe, *F. verticillioides* and *F. proliferatum* are mainly distributed in drier, warmer regions such as

Italy, France, Belgium, Switzerland, and Spain, while *F. subglutinans* and *F. temperatum* are more dominant in colder, wetter climates [14–20]. Moreover, countries including Canada, Brazil, South Africa, Ethiopia, and Nepal have also reported that maize ear rot, mainly caused by *F. verticillioides*, has caused serious crop losses [21–25]. Previous investigations have shown that *F. verticillioides* is the most prevalent of the *Fusarium* species, followed by *F. graminearum* and *F. proliferatum*, while *F. subglutinans*, *F. temperatum*, *F. equiseti*, and *F. oxysporum* have only been isolated in a limited number of regions in China [26–29]. Significantly, some newly described toxigenic species (including *F. concentricum*, *F. sacchari*, *F. miscanthi*, and *F. sporotrichioides*) that cause ear rot have been observed in maize kernels from China in 2021 and 2022 [30–33].

Currently, the most effective and environmentally safe method of controlling maize ear rot is cultivating genetically resistant hybrids [34,35]. The search for resistant inbred lines and hybrids has been the focus of many studies [36–40]. Due to the effects of global warming, conservation tillage technology, and changes to crop rotation systems, the occurrence of Fusarium ear rot damage has shown a tendency to increase worldwide [41,42]. Although China is the world's largest maize producer and consumer after the United States, few maize hybrids with high resistance to ear rot have been identified in the region [43–46]. Moreover, almost all commercial hybrids in China are developed and sold by private companies that do not disclose genetic information, which makes it impossible for growers to predict the resistance of hybrid plants to various diseases. Therefore, this study was undertaken to clarify the population structure of *Fusarium* spp., and screen maize hybrids with superior resistance to ear rot caused by the dominant *Fusarium* species in Northeast China under artificial inoculated conditions.

2. Materials and Methods

2.1. Fungal Isolation and Species Identification

In the autumn of 2020, 298 samples of maize ear rot were collected from 15 regions in Northeast China, including: 101 samples from Yichun (23), jiamusi (22), Qiqihar (16), Harbin (19), Mudanjiang (21) in Heilongjiang Province; 93 samples from Baicheng (20), Songyuan (21), Changchun (19), Siping (18), Tonghua (15) in Jilin Province; and 104 samples from Tieling (22), Huludao (23), Yingkou (21), Dandong (19), and Dalian (19) in Liaoning Province where severe ear rot has occurred (Figure 1). The samples collected from different plots (at least three plots per site) were individually packed in kraft paper bags and stored in a ventilated area at 25 °C. To isolate pathogens, one infected maize kernel on each ear was surface disinfected by soaking in 5% sodium hypochlorite solution for 5 min, rinsed with sterile water 3 times, dried, and cultivated on potato dextrose agar (PDA: 200 g potato, 20 g glucose, and 20 g agar in 1 L distilled water) with streptomycin (100 μg/mL). The hyphae growing inside the kernels were transferred to a spezieller närstoffarmer agar (SNA: 1 g KNO_3, 1 g KH_2PO_4, 0.5 g KCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.2 g sucrose, 0.2 g glucose, and 20 g agar in 1 L distilled water) to promote sporulation. Based on the monospore separation method proposed by Leslie et al. [11], a single spore was transferred onto a Petri dish containing PDA under a 40× upright microscope (Eclipes E100, Nikon, Tokyo, Japan) using a simple homemade needle. All isolates were cultured at 25 °C under 12:12 h light:dark cycles for 5 days, and morphological and cultural characterizations were used to identify *Fusarium* isolates to the species level [11]. Finally, the mycelial discs were suspended in 20% glycerol solution and stored at −80 °C until plant resistance was evaluated. In total, 366 isolates (132 from Heilongjiang, 105 from Jilin, and 129 from Liaoning) were isolated from samples and preserved at the Institute of Plant Immunology, Shenyang Agricultural University.

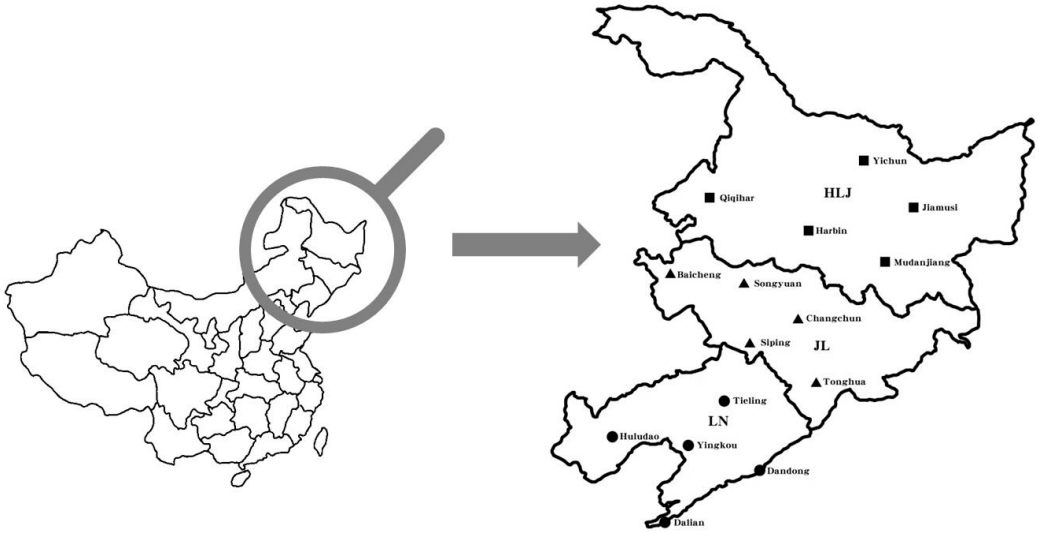

Figure 1. Regions for collection of maize ear rot samples in Northeast China. Squares, triangles, and dots indicate the sites in three different provinces, respectively. HLJ = Heilongjiang; JL = Jilin; LN = Liaoning.

Fungal morphological identification was based on the morphology of conidia. Subsequently, the isolates were validated by species-specific polymerase chain reaction (PCR) to support morphological identification. To extract DNA, the purified isolate was transferred to a sterile conical flask containing 100 mL potato dextrose broth (PDB: 200 g potato and 20 g glucose in 1 L distilled water) and incubated at 25 °C with 150 rpm shaking for 5 days. The collected mycelia were filtered with sterile gauze, washed three times with sterile distilled water, transferred to a centrifuge tube, dried with a vacuum freeze dryer (Modulyo-D, Labconco, Kansas City, MO, USA) for at least 24 h, poured into a cold mortar, and ground with a pestle in liquid nitrogen. The mycelial genomic DNA was extracted using the Plant Genomic DNA Kit (Tiangen, Beijing, China), and the relative purity and concentration of DNA were determined by Ultramicro spectrophotometer (NanoDrop 2000c, Thermo, Waltham, MA, USA). The specific primer sequences were described in Table 1 and each PCR reaction system (25 µL) contained 1 µL template DNA, 12.5 µL 2× Power Taq PCR MasterMix (Takara, Dalian, China), 1 µL each primer, and 9.5 µL double distilled H_2O. PCR amplification conditions were as follows: initiation at 94 °C for 4 min; 35 cycles with denaturation at 94 °C for 40 s, annealing (the temperature depended on the primer pairs) for 40 s, and extension at 72 °C for 1 min, and final extension at 72 °C for 10 min. The amplified products and DL2000 DNA marker (Takara, Dalian, China) were electrophoresed on a 1% agarose gel containing 1× TAE and 4S Green Plus Nucleic Acid Stain (Sangon, Shanghai, China), and visualized by the gel imaging analysis system (FireReader V10, UVItec, Cambridge, UK).

Table 1. Specific primer pairs for *Fusarium* species.

Species	Primer	Sequences (5′-3′)	Annealing Temperature (°C)	Product Size (bp)	Reference
Fusarium spp.	ITSF ITSR	AACTCCCAAACCCCTGTGAACATA TTTAACGGCGTGGCCGC	58	431	[47]
F. oxysporum	FoF1 FoR1	ACATACCACTTGTTGCCTCG CGCCAATCAATTTGAGGAACG	58	340	[48]
F. verticillioides	VER1 VER2	CTTCCTGCGATGTTTCTCC AATTGGCCATTGGTATTATATATCTA	56	578	[49]

Table 1. *Cont.*

Species	Primer	Sequences (5′-3′)	Annealing Temperature (°C)	Product Size (bp)	Reference
F. proliferatum	PRO1 PRO2	CTTTCCGCCAAGTTTCTTC TGTCAGTAACTCGACGTTGTTG	56	585	[49]
F. subglutinans	SUB1 SUB2	CTGTCGCTAACCTCTTTATCCA CAGTATGGACGTTGGTATTATATCTAA	56	631	[49]
F. culmorum	Fc01F Fc01R	ATGGTGAACTCGTCCTGGC CCCTTCTTACGCCAATCTCG	59	570	[50]
F. graminearum species complex	Fg16NF Fg16NR	ACAGATGACAAGATTCAGGCACA TTCTTTGACATCTGTTCAACCCA	57	280	[50]

A partial gene fragment of translation elongation factor 1-alpha (*TEF-1α*) was amplified and sequenced to determine *Fusarium* species in isolates of *F. graminearum* species complex (FGSC). The *TEF-1α* region was amplified using EF1 (5′-ATGGGTAAGGAGGACAAGAC-3′) and EF2 (5′-GGAAGTACCAGTGATCATGTT-3′) as primers [51]. The PCR reaction system was the same as in the previous section. PCR amplifications were performed for 94 °C for 2 min; 35 cycles of 94 °C for 30 s; 53 °C for 30 s; and 72 °C for 1 min; and final extension at 72 °C for 10 min. Amplified products were sequenced by Sangon Biotech (Shanghai, China), and sequence data was assembled using DNAMAN v. 7.0. The final sequences were BLAST compared to sequences of *Fusarium* species from GenBank (http://blast.ncbi.nlm.nih.gov (accessed on 25 October 2021)) and a phylogenetic tree was constructed using MEGA v. 4.0.

2.2. Field Experiment Design and Inoculum Preparation

The test materials included 97 commercial hybrids mainly planted in Northeast China, and 2 inbred lines, X178 (resistant) and B73 (highly susceptible), as controls. Evaluation of the resistance to maize ear rot was carried out at the research base of Shenyang Agricultural University, which is located at 41.84° N and 123.58° E, at an elevation of 89.36 m AMSL. The soil of the test field was loam with a pH of 6.8, and the organic matter content was 2.5%. The study was repeated twice, with maize seeds sown in experimental fields on 27 April 2021, and 30 April 2022. Hybrid seeds were randomly distributed, and each row was 4 m long and repeated three times, with 60 cm between rows and 10 equally spaced hills per row. Three seeds of the same hybrid were sown in the hills of each plot, and one plant per hill was maintained for two weeks after emergence. Field management measures, including timely manual weeding and pesticide spraying, were implemented throughout the growing season. The preparation of inoculum followed the method proposed by Xu et al. [44]: wooden toothpicks were washed in boiling water until the water did not contain color solubles, and then autoclaved and spread over the coagulated PDA medium. The inoculated pathogen was an isolate of *F. verticillioides* named TL1803, a proven aggressive toxin-producing isolate (with a disease index of 89.30 after inoculating the variety Xianyu335) adapted to the environment of Northeast China. Five mycelium discs with a diameter of 8 mm from the initially isolated and purified TL1803 isolate were evenly inverted onto toothpicks, and then the toothpicks were incubated until they were completely covered by mycelia and conidia.

2.3. Inoculation and Evaluation

One ear from each plant was randomly marked for inoculation approximately 7 days after silking. Two toothpicks with mycelia were inserted into the cob from the outside of the bract at the middle part of the ear. Bracts of the 10 inoculated ears of each hybrid were removed, and the damage rates (DR: ratio of infected kernels areas to total kernels areas) were recorded at growth stage R6 of the maize plants. The severity rating (SR) of maize ear rot was measured on a five-point scale (1 = 0–1%; 3 = 2–10%; 5 = 11–25%; 7 = 26–50%;

and 9 = 51–100% of total kernels showing visual symptoms of infection) proposed by Yang et al. [45]. Five resistance categories (RC) were evaluated based on the average SR of the 10 ears from each hybrid, where values between 0.1 and 1.5, 1.6 and 3.5, 3.6 and 5.5, 5.6 and 7.5, and 7.6 and 9.0 were defined as highly resistant (HR), resistant (R), moderately resistant (MR), susceptible (S), and highly susceptible (HS) responses, respectively. When control B73 showed HS to ear rot in the field test, inoculation was considered effective. Where the average SR was inconsistent between the two planting years, the higher value was used as the criterion for evaluating the final resistance category. The frequency and distribution of different *Fusarium* species, as well as the average values of DRs and SRs of each hybrid, were calculated using Microsoft Excel 2007. SAS version 9.4 was used for statistical analysis, and the mean comparisons were performed using $LSD_{(p = 0.05)}$ test. Separate analysis was conducted on the annual disease parameters data due to the interaction between genotype and year.

3. Results

3.1. Weather Conditions

During the maize growth season (May to September), the average temperature in 2021 and 2022 were 22.08 °C and 21.75 °C, respectively, and the temperature fluctuated slightly in different months of the same year (Figure 2). The total precipitation in the summer (June to August) of 2022 was 347.32 mm, which was significantly lower than the 527.90 mm in 2021. Furthermore, the precipitation in August had increased significantly compared with other months in the past two years, and it was in the R2 and R3 stages of maize plants, which was conducive to the occurrence and prevalence of ear rot.

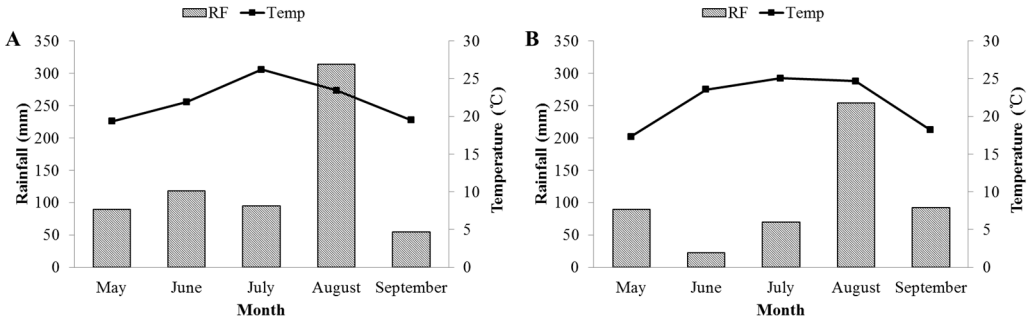

Figure 2. Mean monthly rainfall and temperature during the maize growth season in 2021 (**A**) and 2022 (**B**). RF = Rainfall; temp = Temperature.

3.2. Occurrence Frequency and Distribution of Different Species of Fusarium

In this study, *F. verticillioides*, *F. subglutinans*, *F. proliferatum*, *F. oxysporum*, and FGSC were identified through morphological structural analysis and species-specific PCR, respectively, and no other *Fusarium* species were detected in Northeast China (Figure 3). The *TEF-1α* gene sequences of 108 FGSC isolates were BLAST compared with the standard reference isolates of *Fusarium* in GenBank and a phylogenetic tree was constructed, indicating FGSC isolates that were divided into two distinct clades, with 87 isolates (e.g., LN011, JL032, and HLJ053) expressing 99% to 100% homology with *F. boothii* (KX269073.1 and KX269077.1), while the other 21 isolates (e.g., LN004, LN078, and HLJ019) exhibited 99% to 100% homology with *F. graminearum* (KX269094.1 and MW620073.1) (Figure 4). However, the isolation frequency of each species varied greatly (Table 2). *Fusarium verticillioides* was the dominant species with a frequency of 44.0%, followed by *F. boothii* with a frequency of 23.8%. In addition, *F. subglutinans*, *F. proliferatum*, *F. graminearum* and *F. oxysporum* were only found at a few study sites, accounting for 12.0%, 9.8%, 5.7% and 4.6% of total isolates, respectively.

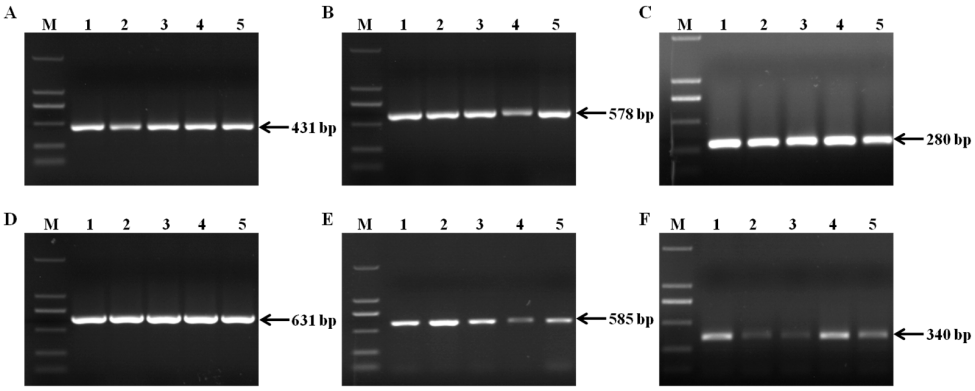

Figure 3. Molecular identification of *Fusarium* species by species-specific polymerase chain reaction (PCR). M: DL2000 DNA marker. (**A**) PCR amplification of *Fusarium* spp., 1–5: LN001, LN017, JL012, JL031, HLJ003; (**B**) PCR amplification of *F. verticillioides*, 1–5: LN001, JL012, JL035, HLJ007, HLJ014; (**C**) PCR amplification of *F. graminearum* species complex, 1–5: LN004, LN011, JL022, JL031, HLJ002; (**D**) PCR amplification of *F. subglutinans*, 1–5: LN007, LN043, JL004, JL018, HLJ032; (**E**) PCR amplification of *F. proliferatum*, 1–5: LN017, LN027, JL006, JL019, HLJ003; and (**F**) PCR amplification of *F. oxysporum*, 1–5: LN044, JL036, JL059, HLJ028; HLJ041.

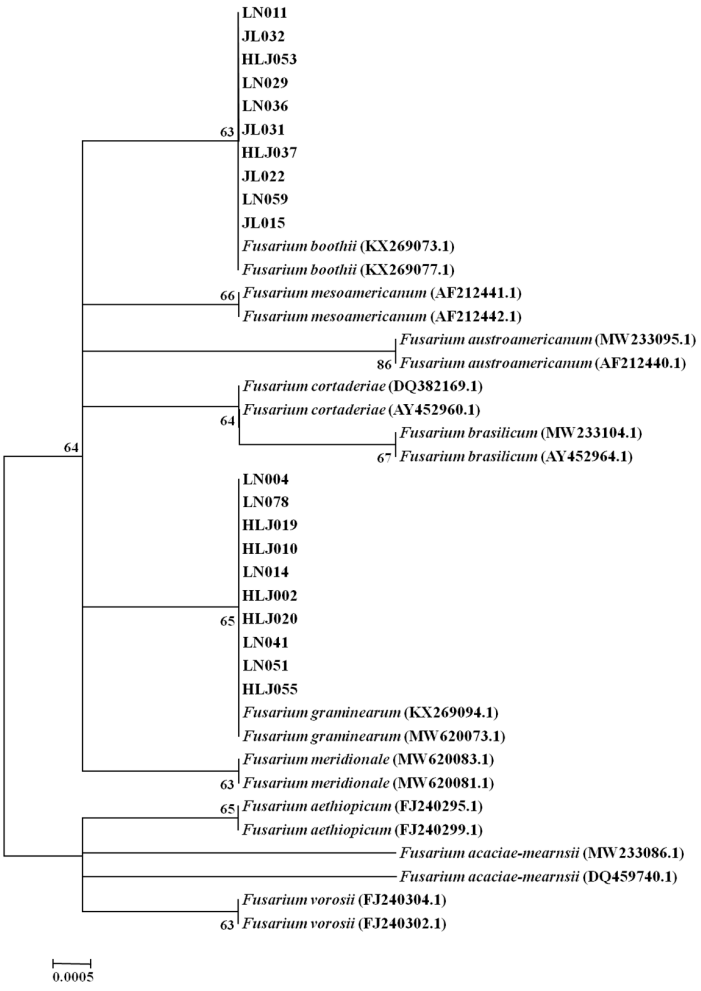

Figure 4. Phylogenetic analysis of some *Fusarium graminearum* species complex isolates based on *TEF-1α* gene sequences. The numbers in parentheses indicate the accession numbers of standard reference isolates *TEF-1α* gene sequences in GenBank and the bootstrap values present at the branch node.

Table 2. Frequency and distribution of different species of *Fusarium* in various regions of Northeast China.

Province Name	Site Name	No. of Species	No. of Isolates [a]						All Species [b]
			Fv	*Fs*	*Fp*	*Fo*	*Fb*	*Fg*	
Heilongjiang	Yichun	4	9	7	8	…	8	…	32
	Jiamusi	5	13	6	5	1	10	…	35
	Qiqihar	4	6	…	…	2	4	4	16
	Harbin	3	9	…	…	…	7	6	22
	Mudanjiang	4	11	3	…	3	10	…	27
	All sites [c]	6	48	16	13	6	39	10	132
	Per site (%)	-	36.4	12.1	9.8	4.5	29.5	7.6	-
Jilin	Baicheng	4	11	9	…	3	2	…	25
	Songyuan	4	7	5	9	…	3	…	24
	Changchun	3	9	6	7	…	…	…	22
	Siping	2	14	…	…	…	5	…	19
	Tonghua	3	9	…	…	2	4	…	15
	All sites [c]	5	50	20	16	5	14	…	105
	Per site (%)	-	47.6	19.0	15.2	4.8	13.3	…	-
Liaoning	Tieling	3	13	…	…	3	9	…	25
	Huludao	4	11	6	…	…	8	6	31
	Yingkou	5	15	2	7	1	…	3	28
	Dandong	4	10	…	…	2	6	2	20
	Dalian	2	14	…	…	…	11	…	25
	All sites [c]	6	63	8	7	6	34	11	129
	Per site (%)	-	48.8	6.2	5.4	4.7	26.4	8.5	-
All provinces [d]		6	161	44	36	17	87	21	366
Per province (%)		-	44.0	12.0	9.8	4.6	23.8	5.7	-

[a] *Fusarium* species. *Fv* = *F. verticillioides*; *Fs* = *F. subglutinans*; *Fp* = *F. proliferatum*; *Fo* = *F. oxysporum*; *Fb* = *F. boothii*; and *Fg* = *F. graminearum*. [b] The total number of isolates from different regions. [c] The total number of isolates of each species in various regions. [d] The total number of isolates of each species in all provinces.

The frequencies and distributions of *Fusarium* species varied within and among provinces. All six identified species were found in Heilongjiang and Liaoning, while all except *F. graminearum* were found in Jilin. *Fusarium verticillioides* was the most prevalent species in Heilongjiang, Jilin, and Liaoning provinces, and its frequency of occurrence was 36.4%, 47.6%, and 48.8%, respectively. The number of *F. boothii* isolates detected in Jilin was only higher than that of *F. oxysporum* and *F. graminearum*, while in the other two provinces, this species was the second most prevalent species after *F. verticillioides*. The population structure was also different within and between each site. *Fusarium verticillioides* was present in all 15 sites, whereas *F. boothii* was described for 13 sites, *F. subglutinans* and *F. oxysporum* for 8 sites, and *F. proliferatum* and *F. graminearum* for 5 sites. Significantly, five species of *Fusarium* were distributed in Jiamusi of Heilongjiang and Yingkou of Liaoning, while only *F. verticillioides* and *F. boothii* were detected in Siping of Jilin and Dalian of Liaoning.

3.3. Evaluation of Maize Hybrids

All hybrids showed visible symptoms of ear rot after being inoculated with *F. verticillioides*, and the disease parameters DR and SR of different hybrids planted in the same year varied significantly ($p < 0.05$; Table 3). In 2021 and 2022, the lowest disease parameters occurred in the Huanong887 hybrid, which had DR of 1.07% and 0.54%, and SR of 1.20 and 1.00, respectively. The Jiudan318 hybrid had the highest disease parameters in 2021 (DR and SR were 64.07% and 8.20, respectively), while the Jinyuan15 hybrid demonstrated the highest disease parameters in 2022 (DR and SR were 59.97% and 8.07, respectively). The proportion distribution of maize hybrids in the two disease parameters in different years is shown in Figure 5. The DR values of the tested hybrids were distributed between 1.07 and 64.07% in 2021, and 0.54 and 59.97% in 2022, of which approximately half varied

within a range of 0 to 15%. The resistance response of each hybrid was sorted into different categories based on the SR value investigated during the R6 growth stage of the maize plants. The RC distribution results indicated that 79.4% of the hybrids had values below 5.5 in 2021 and 2022, which were determined to be resistant to ear rot (evaluated as HR, R and MR). The resistance categories of all hybrids fluctuated slightly between 2021 and 2022, and 52.6% of hybrids were observed to have the same resistance response in both sowing years. Significant correlation existed between disease parameters ($r > 0.95$), and the control inbred lines B73 and X178 achieved theoretical HS and R responses, respectively. This indicated that the field environment was suitable for the occurrence of ear rot, and the evaluation results were true and effective.

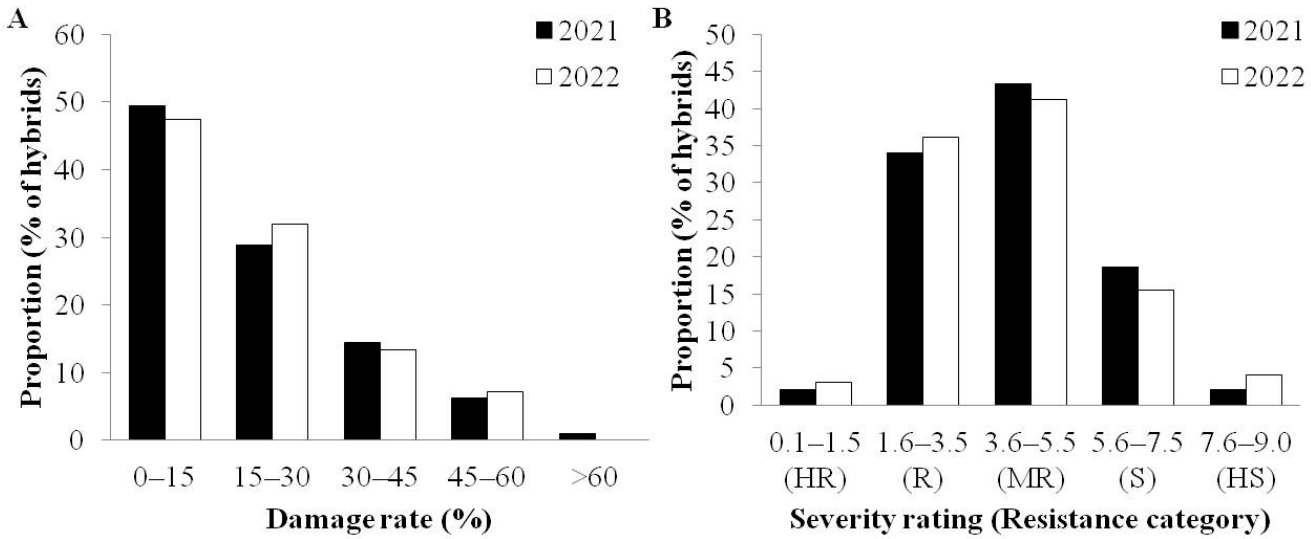

Figure 5. Proportion distribution of maize hybrids in disease parameters. (**A**) Damage rate; (**B**) severity rating (resistance category). HR = highly resistant; R = resistant; MR = moderately resistant; S = susceptible; and HS = highly susceptible.

Statistical analysis of the resistance category for all tested hybrids in this study confirmed that MR hybrids were most common (n = 49; 50.5%), followed by R (n = 20; 20.6%), and S (n = 20; 20.6%) hybrids. In contrast, only six HS and two HR hybrids were observed, with identification frequencies of 6.2% and 2.1%, respectively. Overall, 71 hybrids (73.2%) were identified as resistant (HR, R, MR) to ear rot, and 26 hybrids (26.8%) were rated as HS or S. The distribution of resistance categories for the planted maize hybrids varied between each province (Figure 6). Compared with other provinces, more hybrids in Heilongjiang were classified as MR and HS, accounting for 53.1% and 9.4% of the total, respectively. Among the planted hybrids in Jilin, 27 were identified as resistant to ear rot (including 10 R and 17 MR hybrids), with a resistance level of 77.1%, which was significantly higher than that of the other two provinces. Conversely, up to 30% of the hybrids in Liaoning were designated as S or HS.

Table 3. Disease parameters and resistance evaluation of maize hybrids in Northeast China infected by *Fusarium verticillioides*.

No.	Hybrid Name	Province [a]	2021			2022			FRC [e]	No.	Hybrid Name	Province [a]	2021			2022			FRC [e]
			DR [b] (%)	SR [c]	RC [d]	DR [b] (%)	SR [c]	RC [d]					DR [b] (%)	SR [c]	RC [d]	DR [b] (%)	SR [c]	RC [d]	
1	Damin3307	HLJ	41.73	6.40	S	40.04	6.20	S	S	52	Jinongda598	JL	46.17	7.07	S	49.17	7.47	S	S
2	Demeiya1	HLJ	11.54	3.67	MR	6.26	3.07	R	MR	53	Jinongyu1881	JL	5.30	2.80	R	4.34	2.47	R	R
3	Demeiya3	HLJ	6.03	3.07	R	4.61	2.47	R	R	54	Jinongyu719	JL	14.73	4.33	MR	13.08	4.00	MR	MR
4	Dika517	HLJ	53.54	7.60	HS	38.75	6.53	S	HS	55	Laike818	JL	7.72	3.40	R	7.10	3.07	R	R
5	Dongnong254	HLJ	21.67	4.87	MR	23.68	5.20	MR	MR	56	Liaoke38	JL	26.31	5.00	MR	22.32	5.07	MR	MR
6	Dongnong259	HLJ	5.11	3.13	R	18.13	4.53	MR	MR	57	Limin33	JL	14.63	3.73	MR	18.64	4.33	MR	MR
7	Dunyu213	HLJ	15.23	4.00	MR	8.39	3.13	R	MR	58	Nonghua101	JL	8.47	3.33	R	14.46	4.00	MR	MR
8	Fuer116	HLJ	16.24	4.00	MR	33.21	6.07	S	S	59	Pingan169	JL	11.75	3.60	MR	11.11	3.47	R	MR
9	Hetian4	HLJ	18.86	4.47	MR	13.80	4.00	MR	MR	60	Tianyu108	JL	7.24	3.07	R	16.24	3.73	MR	MR
10	Heyu27	HLJ	19.08	4.27	MR	38.06	5.47	MR	MR	61	Xiangyu998	JL	32.34	5.67	S	53.36	7.87	HS	HS
11	Huamei2	HLJ	4.94	2.73	R	3.62	2.27	R	R	62	Xiongyu581	JL	6.32	3.07	R	12.37	3.53	MR	MR
12	Huanong887	HLJ	1.07	1.20	HR	0.54	1.00	HR	HR	63	Yinghe165	JL	30.25	5.40	MR	22.93	4.53	MR	MR
13	Jingnongke728	HLJ	55.91	7.07	S	56.42	7.53	HS	HS	64	Youdi519	JL	6.87	3.20	R	6.43	3.00	R	R
14	Jinongda935	HLJ	17.04	4.20	MR	10.38	3.20	R	MR	65	Youdi599	JL	39.12	5.73	S	44.15	6.47	S	S
15	Jiudan318	HLJ	64.07	8.20	HS	55.55	7.60	HS	HS	66	Youdi919	JL	55.93	7.00	S	48.97	6.60	S	S
16	Keyu16	HLJ	14.65	3.93	MR	8.26	3.13	R	MR	67	Zeyu517	JL	25.95	5.07	MR	17.90	4.47	MR	MR
17	Longdan86	HLJ	16.03	4.47	MR	22.03	5.20	MR	MR	68	Danyu402	LN	22.43	5.07	MR	15.61	4.13	MR	MR
18	Longfuyu9	HLJ	8.42	2.80	R	3.47	2.27	R	R	69	Danyu405	LN	31.75	6.07	S	24.08	5.20	MR	S
19	Longken10	HLJ	11.74	3.73	MR	29.79	5.53	S	S	70	Dongdan118	LN	30.16	5.33	MR	22.15	4.73	MR	MR
20	Longyu10	HLJ	12.40	3.87	MR	11.08	3.13	R	MR	71	Dongdan1501	LN	6.19	3.00	R	9.13	3.47	R	R
21	Longyu828	HLJ	3.85	2.47	R	3.45	2.33	R	R	72	Dongdan60	LN	10.16	3.27	R	7.54	2.53	R	R
22	Lvdan2	HLJ	23.54	4.73	MR	34.54	6.00	S	S	73	Dongdan6531	LN	27.73	5.67	S	21.88	4.73	MR	S
23	Nendan18	HLJ	35.67	6.07	S	29.97	5.27	MR	S	74	Dongdan70	LN	36.71	6.13	S	40.79	6.67	S	S
24	Ruifuer1	HLJ	17.69	4.13	MR	11.33	3.67	MR	MR	75	Dongtiannuo100	LN	10.72	3.07	R	15.70	3.67	MR	MR
25	Suiyu23	HLJ	5.72	3.00	R	18.75	4.13	MR	MR	76	Hongkai49	LN	1.63	1.47	HR	0.93	1.13	HR	HR
26	Xianyu335	HLJ	54.02	7.13	S	43.32	6.20	S	S	77	Hongshuo1798	LN	13.84	4.07	MR	7.64	3.20	R	MR
27	Xianyu696	HLJ	11.81	3.20	R	8.24	2.73	R	R	78	Hongshuo899	LN	3.62	1.80	R	1.36	1.40	HR	R
28	Xianzhengda408	HLJ	4.84	2.47	R	18.09	4.07	MR	MR	79	Jiaduoxing939	LN	11.80	3.13	R	10.28	2.80	R	R
29	Xinkeyu1	HLJ	6.21	2.73	R	22.21	4.60	MR	MR	80	Jinshi566	LN	20.55	4.53	MR	14.57	4.27	MR	MR
30	Yinongyu10	HLJ	17.60	4.27	MR	13.17	3.20	R	MR	81	Jinyuan15	LN	50.43	7.33	S	59.97	8.07	HS	HS
31	Zhitai3	HLJ	15.64	4.27	MR	11.07	3.47	R	MR	82	Lianda288	LN	13.57	4.20	MR	9.34	3.13	R	MR
32	Zhongdan909	HLJ	8.85	3.33	R	16.82	4.27	MR	MR	83	Liangyu88	LN	32.83	6.20	S	26.05	5.40	MR	S
33	Changdan551	JL	24.67	5.07	MR	17.68	4.40	MR	MR	84	Liangyu911	LN	10.38	3.87	MR	7.13	2.87	R	MR
34	Deyu919	JL	5.85	3.00	R	5.34	2.53	R	R	85	Liangyu99	LN	20.23	4.67	MR	25.25	5.20	MR	MR
35	Dika159	JL	8.69	3.33	R	14.63	4.00	MR	MR	86	Liaodan565	LN	7.34	3.47	R	13.37	3.93	MR	MR
36	Dika516	JL	28.33	5.07	MR	21.37	4.47	MR	MR	87	Liaohe308	LN	43.79	6.73	S	43.72	6.80	S	S
37	Fulai77	JL	20.48	4.47	MR	11.56	3.33	R	MR	88	Shenhai49	LN	31.81	5.80	S	48.84	7.73	HS	HS
38	Fulai818	JL	22.72	4.53	MR	33.73	5.73	S	S	89	ShennongT100	LN	2.73	1.67	R	7.76	2.47	R	R
39	Fumin105	JL	43.07	6.60	S	38.04	6.20	S	S	90	Shennuo18	LN	27.84	5.33	MR	14.21	3.40	R	MR
40	Fumin108	JL	23.61	5.27	MR	27.82	5.53	S	S	91	Shenyu35	LN	26.27	5.13	MR	33.20	5.73	S	S

Table 3. *Cont.*

No.	Hybrid Name	Province [a]	2021			2022			FRC [e]	No.	Hybrid Name	Province [a]	2021			2022			FRC [e]
			DR [b] (%)	SR [c]	RC [d]	DR [b] (%)	SR [c]	RC [d]					DR [b] (%)	SR [c]	RC [d]	DR [b] (%)	SR [c]	RC [d]	
41	Heyu301	JL	25.60	4.93	MR	26.67	5.20	MR	MR	92	Tieyan120	LN	12.10	3.53	MR	6.38	2.80	R	MR
42	Heyu9	JL	9.07	3.07	R	15.03	3.53	MR	MR	93	Tieyan358	LN	13.32	3.73	MR	19.33	4.47	MR	MR
43	Huadan398	JL	10.32	3.20	R	9.75	3.00	R	R	94	Tieyan38	LN	9.75	3.67	MR	14.74	4.27	MR	MR
44	Jidan1402	JL	7.13	2.53	R	1.76	1.40	HR	R	95	Tieyan58	LN	35.68	5.80	S	33.46	5.40	MR	S
45	Jidan551	JL	8.75	3.33	R	8.29	3.13	R	R	96	Xindan336	LN	17.91	4.07	MR	23.99	4.67	MR	MR
46	Jidan558	JL	9.11	3.47	R	8.64	3.27	R	R	97	Zhengdan958	LN	9.82	3.20	R	15.82	3.93	MR	MR
47	Jidan56	JL	5.32	2.80	R	6.73	3.20	R	R		B73	Control	66.17	8.27	HS	69.13	8.40	HS	HS
48	Jidan96	JL	24.85	5.47	MR	11.34	2.80	R	MR		X178	Control	4.82	2.73	R	4.48	2.53	R	R
49	Jingke968	JL	6.54	2.93	R	5.78	2.87	R	R		Mean	…	19.86	4.32	…	20.22	4.29	…	…
50	Jinkai7	JL	30.72	5.53	S	28.58	5.13	MR	S		$LSD_{(p=0.05)}$ [f]	…	2.98	0.30	…	3.01	0.32	…	…
51	Jinongda585	JL	27.76	5.40	MR	21.76	4.73	MR	MR		CV (%)	…	75.27	34.87	…	74.57	37.74	…	…

[a] HLJ = Heilongjiang; JL = Jilin; LN = Liaoning. [b] DR = Damage rate: infected kernels area as a percentage of the total kernels area. [c] SR = Severity rating: the damage rates between 0 and 1%, 2 and 10%, 11 and 25%, 26 and 50%, and 51 and 100% were recorded as severity levels of 1, 3, 5, 7, and 9, respectively. [d] RC = Resistance category: the severity rating mean values between 0.1 and 1.5, 1.6 and 3.5, 3.6 and 5.5, 5.6 and 7.5, and 7.6 and 9.0 were defined as highly resistant (HR), resistant (R), moderately resistant (MR), susceptible (S), and highly susceptible (HS) responses, respectively. [e] FRC = Final resistance categories: the higher severity rating mean value in 2021 and 2022 was used as the standard for the final evaluation of the resistance category. [f] LSD = least significant difference.

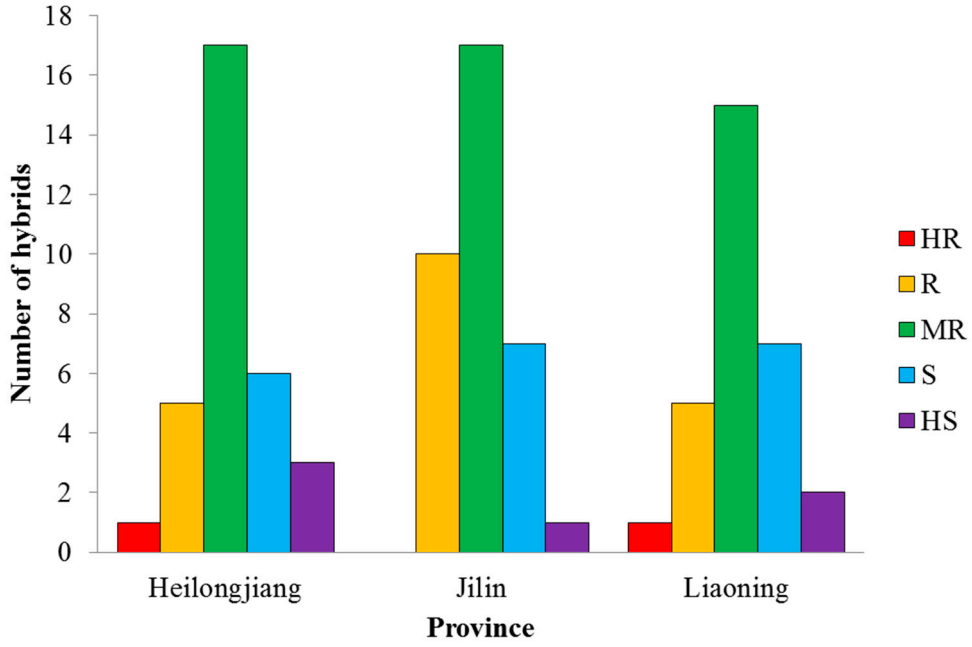

Figure 6. Quantitative distribution of resistance categories of maize hybrids in different provinces. HR = highly resistant; R = resistant; MR = moderately resistant; S = susceptible; and HS = highly susceptible.

4. Discussion

Ear rot caused by *Fusarium* spp. poses a serious threat to maize yield and kernel quality. *Fusarium verticillioides* was the most frequently observed and widely distributed *Fusarium* species in this study, which concurs with a previous report on the isolation and identification of maize ear rot pathogens in Northeast China [52]. However, the population structure of *Fusarium* that causes ear rot in maize varies widely across countries and regions. The dominant species was *F. verticillioides* in Switzerland, Poland, Ethiopia, Iran, and Eastern China; *F. graminearum* in France and Belgium; and *F. subglutinans* in the Harbin area of China [16,18,20,25,28,41,53–55]. The frequency and distribution of *Fusarium* species varied among provinces and sites, which was closely related to environmental conditions [41]. In general, dry and hot conditions are conducive to infection by *F. verticillioides* and *F. proliferatum*, while *F. subglutinans* and *F. graminearum* prefer frequent rainfall and cold temperatures [1,3]. In summer, high temperature lasted for an extended period in the Liaoning Province (http://ln.cma.gov.cn/zfxxgk/zwgk/zcwj/gfxwj/202106/t20210607_3387749.html (accessed on 16 March 2022)), which may be a reason for the highest isolation frequency of *F. verticillioides* being observed in this area. The population structure of *Fusarium* has changed significantly over time, demonstrated by a change in the dominant fungus in Northeast China from *F. semitectum* in 2011 [56], to *F. verticillioides* in this study. The isolation frequency of various species in the same region also fluctuated in different years. In 2015, ear rot caused by *F. subglutinans* did not occur in maize-producing areas of Liaoning [57]; however, by 2020, *F. subglutinans* was the fourth most dominant species in the area with a frequency of 6.2%, which may have been influenced by cultivated hybrids, location and time of sample collection. The population structure of *Fusarium* was influenced by the sources of collected maize ear rot samples. Furthermore, the samples were only collected from 15 regions in 2020, which could have led to a bias in the results, and more samples across regions and years should be examined in future studies.

The toxicity of *Fusarium* species, the complexity of the population structure, and current production practices strongly influence the occurrence and development of ear rot [58–60]. An aggressive fumonisin-producing *F. verticillioides* adapted to the local environment was selected for artificial inoculation to accurately evaluate the disease resistance of these plants in Northeast China. Experiments conducted in greenhouses do not reflect

the true resistance levels of maize hybrids in the diverse cultivation environments of different regions. Therefore, the experiment conducted by Reid et al. [61] was carried out under field conditions to evaluate the resistance of maize hybrids to ear rot caused by *F. verticillioides*. The selection of an inoculation method suitable for local conditions was particularly important for the identification of resistance to maize ear rot. Since the inoculum was easy to culture, and the amount of inoculum could be accurately controlled, pathogens were in direct contact with the ears and kernels; thus, double toothpick inoculation proved to be a relatively simple and effective method [44,62]. Moreover, climatic conditions in different years have a profound impact on the response of plants to ear rot [13,63]. In our study, the resistance category controls X178 and B73 reached the theoretical level of using double toothpicks to inoculate maize ears in both years.

As a fungal disease commonly occurring in maize-producing areas of the world, ear rot has attracted more and more attention from growers. Planting resistant hybrids is considered to be the most economical and effective way to prevent ear rot, but the resistance of maize hybrids to ear rot differs greatly [64,65]. Many countries and regions have carried out evaluations of the resistance of maize inbred lines and hybrids to ear rot. Inoculation trials undertaken in Urbana, Illinois, evaluated the severity of Fusarium ear rot in 68 food-grade dent maize hybrids, and found 13 had a consistent ear rot rate of less than 5% over two years [38]. A total of 103 maize inbred lines were inoculated with *F. verticillioides* in Ikenne and Ibadan, Nigeria, during 2003 and 2004. Results of the study showed that 02C14683, 02C14624, 02C14606, 02C14603, 02C14593, and 02C14585 were consistently highly resistant to ear rot across years and locations [36]. Maize materials from Canada and the United States have been reported to show significant genotypic differences in resistance to *F. verticillioides* [37]. After analyzing the germplasms from the Misión Biológica de Galicia Bank, Spain, in 2010 and 2011, 61 inbred lines were determined to have the highest resistance to Fusarium ear rot and fumonisin accumulation [40]. Inoculated by the toothpick method, the flint maize proved to be more easily infected by *F. verticillioides* than dent maize [66]. The responses of three maturity groups (early, mid-late, late) of maize inbred lines to ear rot was studied in France, Germany, Hungary, and Italy during the 2003 and 2004 crop seasons. The focus of the analysis was on the correlation between resistance to *F. verticillioides* and *F. graminearum*. The results indicated that the severity of ear rot caused by *F. graminearum* was significantly higher than that caused by *F. verticillioides*, with the moderate correlation occurring in early maturing dent and flint lines [39]. In this study, 71 hybrids were ultimately evaluated as MR, R, and HR in their resistance to ear rot caused by *F. verticillioides*. However, ear rot is often caused by a combination of multiple *Fusarium* species under natural conditions in the field. Thus, it is necessary to increase the inoculation analysis of other *Fusarium* species.

In recent years, the incidence of maize ear rot in China has increased annually, which may be due to changes in climate and cultivation systems, increased insect vector activity, and large-scale planting of susceptible hybrids [34,41,42]. In this study, 97 maize hybrids commonly planted in Northeast China were inoculated with the dominant fungal species *F. verticillioides* under natural field conditions. These trials found that 79.4% of the hybrids were resistant (HR, R, or MR) to ear rot in 2021 and 2022, and response levels were slightly higher than previously reported [43,67]. Only two hybrids were designated as HR, which was consistent with there being almost no reports of maize hybrids with high resistance to ear rot [44,62]. The resistance of maize to ear rot varies under different environmental conditions and management practices [68,69], which may be an important reason for the differences in maize resistance levels among provinces. Multiyear and multisite resistance monitoring should be conducted to evaluate the more accurate resistance level of maize hybrids in future studies. Significantly, up to 30% of the hybrids in Liaoning province were susceptible to ear rot caused by *F. verticillioides*, indicating a higher epidemic risk of ear rot appearing in Liaoning province; therefore, the monitoring of ear rot, and the screening of resistant hybrids should be strengthened.

The data showed that although DR and SR disease parameters differed among hybrids, significant correlations occurred within the same hybrids, one of which can be used to evaluate the resistance of maize hybrids to ear rot. Moreover, despite the monthly average temperature during the maize growing season having remained relatively stable, the rainfall had plummeted from 527.90 mm in 2021 to 347.32 mm in 2022, which may affect the severity of maize hybrid infections. Unexpectedly, individual hybrids evaluated as HS were already widely planted with an expanding range in Northeast China, which may promote outbreaks and increased prevalence of ear rot. In summary, our study suggests that on the basis of strengthening field disease management measures, resistant hybrids should be actively cultivated and promoted to sustainably control ear rot.

5. Conclusions

In conclusion, our study showed that the population structure of *Fusarium* causing maize ear rot in Northeast China had changed significantly over time, with the dominant fungus shifting from the past *F. semitectum* to *F. verticillioides*, the *F. graminearum* species complex populations consisted of *F. boothii* and *F. graminearum*, and the isolation frequency of various species in the same region also fluctuated in different years. Moreover, the field evaluation of common maize hybrids inoculated with the dominant fungus *F. verticillioides* confirmed that disease parameters differed among hybrids, and 73.2% of hybrids were moderately to highly resistant to ear rot. Finally, individual highly susceptible hybrids were widely planted in some areas of Northeast China, which indicated the need to strengthen the screening of resistant varieties and reasonable layout to prevent the further occurrence and spread of Fusarium ear rot.

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