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Research Article

Genetic Study on JS399-19 Resistance in Hyphal Fusion of *Fusarium graminearum* by Using Nitrate Nonutilizing Mutants as Genetic Markers

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Abstract: Twenty-two nitrate nonutilizing (*nit*) mutants were recovered from five wild-type isolates of *Fusarium graminearum* and fifty *nit* mutants were recovered from three JS399-19-resistant mutants of *F. graminearum* cultured on MMC medium. Some biological properties were compared between *nit* mutants and their parental isolates. The results showed that there were no significant differences in growth rate, cultural characters or pathogenicity between JS399-19-resistant *nit* mutants and their parental isolates. But the conidial production and the sexual reproduction ability changed to some extent. There was no cross resistance toward chlorate and JS399-19 in *F. graminearum* and the resistance could be stable through 20-time subcultures. Therefore, the *nit* could be used as a genetic marker for studying the genetics of JS399-19 resistance in *F. graminearum*, which was used to study JS399-19 resistance transferability in hyphal fusion. Resistance in JS399-19 could not be transferred by hyphal fusion or could be transferred with low chance between two compatible isolates, which would delay the development of JS399-19 resistance in the field.

Keywords: *Fusarium graminearum*; JS399-19 resistance; biological properties; genetic marker; hyphal fusion

Fusarium graminearum (teleomorph *Gibberella zeae*) is the main pathogen of Fusarium head blight (FHB) in wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.). Fusarium head blight is one of the most economically important diseases worldwide especially in the United States[1] and China[2]. This fungal disease caused an estimated three-billion dollar loss to wheat and barley farmers in the United States alone in the 1990s[3] and reduced wheat baking quality[4]. In China, FHB generally occurs in the middle and lower reaches of the Yangtze River, Huaihe River valley and the eastern coastal region. However, an increase in the disease in the North and West wheat growing areas of China has occurred in the last decade. The disease not only resulted in the loss of grain yield by 5%–15% in moderate epidemic years and up to 40% in severe epidemic years, but also caused reduction in grain quality because of the mycotoxin content[5], such as deoxyinvalenol(DON, vomitoxin)[6, 7], which could inhibit amino acid incorporation and protein production in plant tissues[8], and also, caused emesis and feed refusal in animals[9, 10]. Carbendazim is the major fungicide used in controlling FHB in China. However, *F. graminearum*-resistant isolates

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exist in the field.

JS399-19 (Fig. 1), 2-cyano-3-amino-3-phenylancryic acetate, which was introduced by Jiangsu Branch of National Pesticide Research & Development South Center in 1998 is a unique and new-configurate chemical, which exhibits specific activity against the fungal plant pathogens of *Fusarium* spp. and can greatly restrain the mycelial growth specially with the EC_{50} value about 0.15 μg/mL (data under publication) and has a potential market favorably controlling FHB. This antifungal compound has been tested in fields in 2001 and a pesticide temporary registration as an independent and new-style fungicide had been completed in 2005 in China. The Chinese patent right of this product has been obtained. Therefore, it is crucial to research the mode of action, genetics of resistance and the resistance risk assessment of the new fungicide. Nitrate nonutilizing mutants (*nit* mutants), which can not convert chlorate to chlorite (a fungi-toxic substance), become resistant to chlorate. Bowden and Leslie[11, 12] obtained the *nit* mutants of *F. graminearum* for the research of VCG identification and genetic analysis of sexual recombination successfully. Also, the same method had been widely used in determining *Fusarium* diversity, vegetative compatibility and population structure in recent researches[13–16]. The objectives of this article were to 1) investigate whether the *nit* mutation could change the biological properties of *F. graminearum*; 2) determine the stability of the resistance to JS399-19 and chlorate in *F. graminearum*; 3) explore whether the JS399 resistance could be transferred through hyphal fusion in *F. graminearum* by using *nit* as a genetic marker.

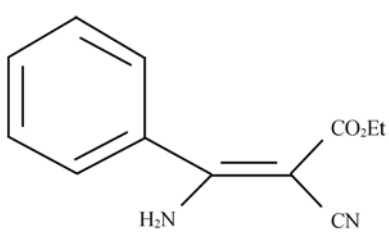

Fig.1 Chemical structure of JS399-19

1 Materials and Methods

1.1 Fungal isolates

Five single-spore strains (S1, S2, S3, S7, and 2021) of *F. graminearum* were isolated from FHB infected wheat ears and determined in the same VCG (vegetative compatibility group) as in the previous experiment. Isolates Y2021, Y2021A, and Y2021B, resistant to JS399-19 were generated from the wild-type strain 2021 by fungicide treatment.

1.2 Media

Potato sucrose agar (PSA) was used in isolating and identifying the tested isolates; Mung bean broth (MBB), 1 L H_2O amended with 50 g mung bean, was used to produce conidia. The media MMC (potassium chlorate medium), MM (minimal medium), MO_2 (nitrite medium), MH (hypoxanthine medium), and MA (ammonium medium) were used for isolating *nit* mutants and determining the types of mutation, respectively. MM consisted of 30.0 g of sucrose, 1.0 g of KH_2PO_4, 0.01 g of $FeSO_4 \cdot 7H_2O$, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of KCl, 2.0 g of $NaNO_3$, 0.2 mL of trace element solution, 20.0 g of agar, and 1 liter of deionized water. The defined MM medium amended with 30 g/L of potassium chlorate and 1.6 g/L L-asparagine was known as MMC medium. Medium MM amended with 0.2% of $NaNO_2$ was MO_2 medium. Medium MM amended with 0.2% hypoxanthine was the MH medium. Medium MM amended with 1.0% ammonium tartrate was the MA medium[11].

1.3 Seeds/cultivars

Yangmai 56 is a local planted wheat cultivar susceptible to FHB.

1.4 Recovery of *nit* mutants

After being cultured on PSA for 3 days, four mycelial plugs of parental isolates (5 mm in diameter) were taken from colony margins and placed equidistantly apart on a plate of MMC. Any spontaneous and

fast-growing sectors from the restricted colonies on the MMC plates were picked up for identification[11].

1.5 *nit* mutant phenotype determination

The phenotypes of the spontaneous and fast-growing sectors, which could be resistant to chlorate, were identified as reference [17-19].

1.6 Research on the characterization of *nit* mutant

1.6.1 Culture characterization of *nit* mutant

After being cultured on PSA for 3 days, mycelial plugs of *nit* mutants and their parental strains (5 mm in diameter) were transferred to PSA plates amended with 2.5% chlorate at 25℃ for 3 days. Colony diameter (in cm) was measured to compare mycelial linear growth. The color of the isolates and the growth of the aerial mycelium were also observed synchronously.

1.6.2 Asexual production ability of *nit* mutant

After being cultured on PSA for 3 days, five mycelial plugs of *nit* mutants and their parental isolates (5 mm in diameter) were transferred to each conical flask containing 100 mL MBB for shake culture. Sporulation capacity was compared by using haemacytomete after 7 days.

1.6.3 Sexual reproduction ability of *nit* mutant

Mycelial plugs of resistant *nit* mutants and their parental isolates (5 mm in diameter) were inoculated onto the autoclaved seeds of Yangmai 56 and cultured at 25℃ for 7 days. Then, the seeds were covered with sterile wet sand and cultured in a humid room at 25℃ in a photoperiod of 12 h light and 12 h darkness. The perithecia production was measured after 3 days.

1.6.4 Pathogenicity of *nit* mutant

The experiment was conducted in both 2005 and 2006 as previously described[20]. Pathogenicity was compared between JS399-19-resistant *nit* mutants and their parental isolates.

1.7 Stability of *nit* mutant

All the *nit* mutants and their parental isolates were transferred 20 times onto new PSA plates and MMC plates, and also conserved on the slants of MMC, MM, and PSA for 60 days in a refrigerator at 4℃. To check whether they were still *nit* mutants and JS399-19-resistant mutants, they were transferred onto MM plates and PSA plates amended with 10 μg/mL JS399-19, respectively (JS399-19-resistant *nit* mutants could not grow on MM plates, and if they could grow on PSA plates amended with 10 μg/mL JS399-19, they remained JS399-19-resistant.).

1.8 Research on influence of JS399-19 resistance on vegetative compatibility

Vegetative compatibility of *nit* mutants recovered from strain 2021, Y2021A and Y2021B was tested as reference[11] so that it could be determined whether the JS399-19 resistance mutation changed the vegetative compatibility of *F. graminearum*.

1.9 Genetic study on JS399-19 resistance in hyphal fusion

A JS399-19-sensitive *nit* mutant and a JS399-19-resistant *nit* mutant were taken as one pair for the complementary test as previously described [11] and pairings between vegetative compatible isolates with different *nit* mutant phenotypes were repeated. A myclial plug was picked up from the hyphal fusion zone where wild-type mycelia growth had appeared after 7–15 days and transferred to MBB for shake culture for 7 days. The progenies of the myclial plug (conidia) were collected and the conidia suspension was adjusted to 10^3 conidia per mL. Two hundred microliters of conidia suspension was spread onto a water agar plate and incubated at 25℃ for 10 hours and then over 300 germinated spores were picked up for further determination of *nit* mutant phenotypes and sensitivity to JS399-19 as described above. Moreover, a *nit* mutant phenotype and sensitivity to JS399-19 combination different from its parents, was

observed.

1.10 Data analysis

Analysis of variance by using the SAS GLM (SAS Institute Inc., Cary, NC) procedure was performed on all the data to test significant differences. The least significant difference (LSD) test was used to determine significant difference in some of the biological properties of *F. graminearum*.

2 Results

2.1 Isolation of JS399-19-resistant *nit* mutants and determination of phenotypes

The eight parental strains could hardly grow on the MMC plates during the first 3 or 4 days. Only a few spontaneous sectors of parental isolates could grow fast in over 4 days. Most of the spontaneous and fast-growing sectors, which were the real *nit* mutants, could hardly grow with little or no aerial mycelium on the MM plates but could grow as unrestricted colonies on MMC plates. A total of 77 *nit* mutants were derived from eight parental strains with the frequency of 8.77%. The phenotype of the *nit* mutants was identified and four phenotypes were obtained including *nitM*, *nit1*, *nit3*, and *nitA* (Table 1).

2.2 Mycelial linear growth and sensitivity to JS399-19

All the *nit* mutants could grow on the PSA plates amended with 2.5% potassium chlorate with thick aerial mycelium but the growth of their parental strains was strongly restrained. The growth of all the *nit* mutants was consistent with their parental strains on the PSA plate with or without JS399-19. This indicated that the *nit* gene and the JS399-19-resistant gene were mutually independent and thus there was no cross resistance toward chlorate and JS399-19 in *F. graminearum* (Tables 1 and 2).

2.3 Conidiophore production

Sporulation capacity of eight parental strains and some of their *nit* mutants was compared after 7-day shake culture within MBB. All the strains produced conidiophore. However, sporulation production changed to some extent, although it had no relationship with *nit* mutations (Table 2).

2.4 Perithecigerous capacity

All *nit* mutants and their parental strains produced perithecia after culturing for 3 days and mature ascospores were observed after a period of time except the isolate Y2021A-5, but their perithecigerous capacity varied, which had no relationship with *nit* mutations (Table 2).

2.5 Comparison of pathogenicity

Mean values of FHB severity were compared after three weeks of inoculation, which indicated that there were no significant differences between the *nit* mutants and their parental strains (Table 2). Therefore, the *nit* phenotypes had no direct relationship with the pathogenicity.

Table 1 Recovery and identification of different *nit* mutant phenotypes and their sensitivity to JS399-19

Strains	Identification of *nit* mutants					Total *nit* mutants	Sensitivity to JS399-19	
	nit1	*nit3*	*nit8*	*nitA*	*nitM*		Resistant	Sensitive
S1	3	1	0	0	0	4	0	4
S2	1	0	0	0	2	3	0	3
S3	1	1	0	0	0	2	0	3
S7	2	0	0	1	0	3	0	2
2021	5	3	0	1	1	10	0	10
Y2021	11	9	0	2	3	25	25	0
Y2021A	5	4	0	3	1	13	13	0
Y2021B	6	3	0	1	2	12	12	0

Table 2 Comparison of biological properties between resistant *nit* mutants and their parental isolates

Isolate (*nit* phenotype)	Mycelial linear growth (mm)	Sporulation capacity (10^6 spores/mL)	FHB severity (%)		Perithecigerous capacity
			2005	2006	
S1	51.04±0.84Aa[1)]	0.69±0.23Aa	38.71±4.33Aa	36.56±3.69Aa	++[2)]
S1-1[3)] (*nit1*)	50.83±0.89Aa	0.71±0.19Aa	35.46±4.59Aa	36.54±4.27Aa	++
S1-3 (*nit3*)	49.27±1.07Aa	0.70±0.16Aa	34.76±4.73Aa	38.26±5.73Aa	+
S2	52.34±0.84Aa	1.59±0.34Aa	38.26±6.16Aa	36.12±5.47Aa	++
S2-1 (*nitM*)	53.28±0.81Aa	0.68±0.12ABab	37.12±5.54Aa	34.83±5.68Aa	+
S2-2 (*nitM*)	53.87±0.92Aa	1.73±0.41Aa	34.68±6.77Aa	35.48±4.82Aa	+
S3	60.22±1.10 Aa	0.73±0.08Aa	33.47±4.83Aa	36.45±3.71Aa	+++
S3-1 (*nit1*)	59.48±1.38 Aa	0.35±0.02ABab	34.27±5.87Aa	31.68±5.51Aa	+++
S3-2 (*nit3*)	59.12±1.13 Aa	0.69±0.11Aa	32.43±6.36Aa	33.74±3.53Aa	++
S7	64.76±2.13Aa	0.63±0.09ABab	45.65±7.46Aa	42.46±5.32Aa	++
S7-1 (*nit1*)	62.35±2.56Aa	0.27±0.03BCbc	41.23±6.54Aa	45.72±4.63Aa	+
S7-2 (*nitA*)	65.62±1.87Aa	0.89±0.12Aa	40.92±7.92Aa	43.68±6.47Aa	++
Y2021	55.60±1.01Aa	0.35±0.04Bb	33.12±3.88Aa	34.58±4.07Aa	+
Y2021-2(*nit1*)	55.67±0.76Aa	0.46±0.19Bb	39.76±5.89Aa	35.40±3.45Aa	+++
Y2021-4(*nitA*)	56.27±0.76Aa	0.35±0.07Bb	33.27±6.95Aa	31.48±4.51Aa	+
Y2021-7(*nit3*)	55.80±0.82Aa	0.34±0.17Bb	32.55±6.47Aa	33.43±1.53Aa	++
Y2021-11(*nitM*)	56.30±0.80Aa	0.67±0.31ABab	36.74±6.40Aa	34.67±3.68Aa	+++
Y2021-15(*nit3*)	55.87±0.85Aa	0.36±0.01Bb	34.99±6.74Aa	33.54±3.70Aa	++
Y2021-20(*nit3*)	56.57±1.20Aa	0.64±0.29ABb	35.18±4.11Aa	38.45±4.17Aa	++
Y2021-22(*nit1*)	55.77±0.83Aa	1.20±0.24Aa	37.01±4.83Aa	37.84±3.22Aa	+++
Y2021-25(*nit3*)	56.13±0.65Aa	0.54±0.08ABb	33.65±4.53Aa	36.05±3.11Aa	++
Y2021A	53.67±0.68Aa	0.67±0.14Aa	34.84±5.43Aa	36.41±4.23Aa	+
Y2021A-1(*nit3*)	53.13±0.73Aa	0.71±0.08Aa	35.61±5.41Aa	34.12±3.23Aa	++
Y2021A-5(*nitM*)	53.30±0.63Aa	0.35±0.12ABab	37.12±4.46Aa	33.72±4.29Aa	–
Y2021A-9(*nit1*)	53.87±0.65Aa	0.65±0.19Aa	33.68±5.17Aa	35.48±3.22Aa	+
Y2021B	50.10±0.71Aa	0.65±0.21Aa	33.68±4.18Aa	36.36±3.19Aa	+
Y2021B-2(*nitA*)	49.83±0.79Aa	0.71±0.17Aa	35.46±4.21Aa	37.54±3.13Aa	++
Y2021B-5(*nitA*)	49.27±0.67Aa	0.70±0.19Aa	36.42±5.17Aa	35.70±4.28Aa	+

[1)] Difference analysis between every parent and its *nit* mutants respectively (a: p =0.05; A: p =0.01);

[2)] "+++", "++", "+" and "–" indicate the perithecia covering wheat grain surface more than 2/3, between 1/3 and 2/3, less than 1/3 and none perithecia, respectively;

[3)] S1-1 means the 1st *nit* mutant was recovered from the parental strain S1.

2.6 Stability of resistant phenotype

Of all *nit* mutants, only three mutants (Y2021-6, Y2021-13, and Y2021-18) were restored to their parental phenotype after being transferred onto new PSA plates 20 times. All of the others maintained their sensitivity to potassium chlorate and JS399-19 after being transferred onto new MMC plates 20 times and conserved on the slants of MMC, MM, and PSA for 60 days in a refrigerator at 4℃. This indicated that the sensitivity to JS599-19 of most *nit* phenotypes were stable through subcultures.

2.7 Influence of JS399-19 resistant mutation on vegetative compatibility

The three selective strains could complement each other and therefore JS399-19 resistant mutation did not change the vegetative compatibility of *F. graminearum*. Furthermore, the results also suggested that the JS399-19-resistant gene had no direct relationship with the gene controlling the ability of vegetative compatibility.

Table 3 *Nit* phenotype and sensitivity of the progeny of the mycelial plug from the hyphal fusion zone to JS399-19

Paired strains (*nit* phenotype and sensitivity to JS399-19)		Total number of single spore tested	*Nit* phenotype and sensitivity to JS399-19 (number of the progeny)
Paired mutant 1	Paired mutant 2		
Y2021-11 (*nitM*-R)*	2021-3 (*nit1*-S)*	371	*nitM*-R (257); *nit1*-S (114)
Y2021-11 (*nitM*-R)	S7-3 (*nit1*-S)	355	*nitM*-R (81); *nit1*-S (274)
Y2021-2 (*nit1*-R)	2021-2 (*nitM*-S)	373	*nit1*-R (261); *nitM*-S (112)
Y2021-2 (*nit1*-R)	S3-2 (*nit3*-S)	414	*nit1*-R (158); *nit3*-S (256)
Y2021A-1 (*nit3*-R)	S2-1 (*nitM*-S)	382	*nit3*-R (236); *nitM*-S (146)
Y2021B-5 (*nitA*-R)	S2-1 (*nitM*-S)	342	*nitA*-R (108); *nitM*-S (234)

*Y2021-11: the 11th *nit* mutant recovered from Y2021; 2021-3: the 3rd *nit* mutant recovered from 2021; *nitM*: the nit phenotype of the mutant; R and S: resistant and sensitive to JS399-19.

2.8 Genetic of JS399-19 resistance in the hyphal fusion

Direct hyphal fusion was observed in six pairs in which wild-type mycelia growth appeared over the hyphal fusion zone as evidence of heterokaryon formation. All the progeny of the mycelial plug from the hyphal fusion zone exhibited their parental phenotype and no asexual recombinants were observed, which indicated that JS399-19 resistance could not be transferred by hyphal fusion or with low chance between two compatible isolates (Table 3). Thus, hyphal fusion might take little part in the development of JS399-19 resistance in the field.

3 Discussion

The benzimidazole fungicides, particularly carbendazim, have been used during each period of wheat heading and flowering in areas with warm and moist weather to control FHB in China for over 30 years. But in the last 10 years, the frequency of carbendazim-resistant populations increased dramatically and FHB control decreased markedly in many areas all over China. Yuan and Zhou[16] concluded that a single gene controlled resistance to carbendazim in *F. graminearum* isolates from China, and was generally expressed as a high degree of resistance to this fungicide. China is now facing a challenge to find alternative fungicides for controlling FHB on wheat. JS399-19 is a new cyanoacrylate fungicide, which exhibits specific activity against the fungal plant pathogens of *Fusarium* spp. and can strongly restrain the mycelial growth specially, showing efficacy in controlling FHB [22–24]. Although, resistance risks with this fungicide may not be as great as with carbendazim, strategies to manage the resistance risk should be developed and implemented to avoid unexpected control failures and to sustain the usefulness of the new product. In this article, a total of 77 *nit* mutants were recovered from five JS399-19-sensitive strains and three JS399- resistant strains in *F. graminearum*. The *nit* mutation did not appear to change some of the biological properties of *F. graminearum* such as mycelial growth rate, cultural characters and pathogenicity. However, the conidial production and the sexual reproduction ability changed to some extent, which seemed not to have the direct relationship with the *nit* mutation. Furthermore, the sensitivity to JS399-19 was stable through 20 subcultures with the resistance to chlorate and thus a genetic study on the JS399-19 resistance in the hyphal fusion in *F. graminearum* was done by using the *nit* genes as genetic makers, and concluded that *nit* mutation hardly changed the vegetative compatibility of *F. graminearum* and the JS399-19 resistance could hardly be transferred by hyphal fusion or could be transferred with low chance. Therefore, the JS399-19 resistance might not take place and spread in the field by hyphal fusion. These results might be important for assessment of the resistance risk of this new fungicide. Furthermore, a

study on the unknown mode of action of this new fungicide was required. Hence, this series of fungicides could be ameliorated and more effective strategies of resistance management could be made.

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以 *nit* 为遗传标记研究禾谷镰刀菌对氰烯菌酯的抗药性在菌丝融合过程中的遗传

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摘 要：分别从对氰烯菌酯敏感和抗性禾谷镰刀菌菌株中诱导了 22 个和 50 个 *nit* 突变体。通过比较它们的生物学特性表明，*nit* 突变体在菌丝生长速率、培养形状以及致病性方面与其亲本没有显著差异，但是无性繁殖和有性繁殖能力有所改变。同时实验还表明，禾谷镰刀菌对氰烯菌酯和氯酸盐不存在交互抗性，且对氰烯菌酯和氯酸盐的双重抗性能够稳定地遗传。因此，可以将 *nit* 作为一个优良的遗传标记研究禾谷镰刀菌对氰烯菌酯的抗药性遗传。另外成功运用 *nit* 作为分子标记研究了禾谷镰刀菌对氰烯菌酯的抗药性在菌丝融合过程中的遗传和变异。研究结果表明，抗药性基因不能通过菌丝融合传递给另一个菌株或发生的概率极低，这将不利于对氰烯菌酯的抗性群体的发展。因此，菌丝融合在禾谷镰孢菌对氰烯菌酯的抗药性群体发展中的作用较小。

关键词：禾谷镰刀菌；氰烯菌酯抗药性；生物学特性；遗传标记；菌丝融合

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