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Comparison between the *N* and *Me3* genes conferring resistance to the root-knot nematode (*Meloidogyne incognita*) in genetically different pepper lines (*Capsicum annuum*)

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Abstract Genetic resistance to *Meloidogyne incognita* in pepper (*Capsicum annuum*) has been well characterised for the *N* and *Me3* resistance genes. However, there are no studies comparing the effects of these two genes directly or investigating the combined effects when both genes are present together. Several studies were undertaken to investigate the relationship of the *N* and *Me3* gene systems to one another and to assess whether these two genes are allelic or truly separate genes. Two genotypes homozygous for the *N* gene ('Carolina Wonder' and 'Charleston Belle') and two genotypes homozygous for the *Me3* gene (HDA 149 and PM 687) were compared in a replicated greenhouse test for reaction to *M. incognita* race 3. There were no significant differences between the resistant reactions of genotypes possessing the *N* or *Me3* gene. Allelism tests were performed using the F2 populations of the parental genotypes HDA 149 × 'Charleston Belle' and HDA 149 × 'Carolina Wonder'. The results of these studies clearly show the *N* and *Me3* genes to be distinct, separate dominant resistance genes conferring resistance to *M. incognita* race 3 and not alleles of the same gene.

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Abbreviations

DH	double haploid
HR	hypersensitive response
wk	week

Introduction

The southern root-knot nematode (*Meloidogyne incognita*) is a major pest of peppers (*Capsicum annuum*) in the southern regions of the USA and worldwide. The primary control measure consists of pre-plant fumigation with methyl bromide; however, due to increasingly stricter policies on methyl bromide use and its potential loss of registration for agricultural use, genetically-based resistance becomes of even greater importance in the management of root-knot nematodes for successful pepper production (Thies and Fery 2002). The use of resistant pepper cultivars in rotational and double-cropping systems has been shown to reduce root-knot nematode severity in subsequent plantings of susceptible hosts, thus providing growers with the added benefit of protecting highly susceptible crops in their rotation (Thies et al. 1998; 2004).

Many reports of dominant resistance genes exist for the pepper—*Meloidogyne* spp. system. The mode of resistance is often reported as a hypersensitive response (HR) of the host plant in response to recognition events incited by *Meloidogyne* spp. (Kaplan and Keen 1980; Hendy et al. 1985a; Williamson 1999; Bleve-Zacheo et al. 1998; Pegard et al. 2005). The *N* gene and *Me* genes have been reported to control resistance to root-knot nematodes in *C. annuum* (Hare 1956; Hendy et al. 1985a; Fery and Dukes 1996; Castagnone-Sereno 2002; Djian-Caporalino et al. 2001; 2007). Hendy et al. (1985b) observed five genes, designated *Me1* to *Me5* that control resistance to various *Meloidogyne* spp. One of these genes, *Me3*, confers broad spectrum resistance to *M. incognita*, *M. arenaria*, and *M. javanica* (Hendy et al. 1985b; Djian-Caporalino et al. 1999) and is located on the pepper P9 chromosome (Djian-Caporalino et al. 2001). Similarly, the *N* gene confers high resistance to *M incognita*, *M. arenaria* races 1 and 2, and *M. javanica* (Thies and Fery 2000). Allelism tests and fine mapping demonstrated that six *Me* resistance genes (*Me1*, *Me3*, *Me4*, *Me7*, *Mech1*, and *Mech2*) which condition resistance to several *Meloidogyne* spp. in *C. annuum* are different but linked (Djian-Caporalino et al. 2007). These six *Me* genes were shown to be clustered on the P9 chromosome (Djian-Caporalino et al. 2007).

Although the *N* and *Me* gene systems have been individually well characterised, resistance controlled by the two genetic systems has not been compared in a single study; e.g. there is no information about the relationship of the *N* and *Me3* gene systems to each other or whether the *N* and *Me3* genes are allelic to each other. The objectives of these studies were: (i) to characterise resistance to *M. incognita* race 3 in pepper genotypes carrying the *N* or *Me3* genes and (ii) to determine if the *N* and *Me3* genes are independent genes or alleles present at a single locus.

Materials and methods

Characterisation of host resistance (Test I)

Pepper genotypes Eight pepper (*C. annuum* and *C. chinense*) genotypes differing in presence or absence of the *N* and *Me3* genes that confer resistance to *M. incognita* were evaluated in the greenhouse. *N* gene: 'Charleston Belle' and 'Carolina Wonder' are homozygous for the *N* gene (*NN*) (Fery et al. 1998). *Me*3 gene: HDA 149 and PM 687 possess the *Me*3 gene. Seeds of these two *C. annuum* breeding lines were obtained from Dr. Alain Palloix (Centre de Recherche Agronomique d'Avignon, INRA, France). HDA 149, a double haploid (DH) developed through *in vitro* androgenesis from the F1 hybrid (PM 687 × 'Yolo Wonder') contains only the *Me3* gene. PM 687 is an inbred line developed through selfing U.S. Plant Introduction (PI) 322719, which contains at least two genes, the *Me3* and *Me4* genes (Dr. Alain Palloix, personal communication). Susceptible genotypes: 'California Wonder', 'Yolo Wonder B', and 'Keystone Resistant Giant' are *C. annuum* genotypes susceptible to *M. incognita*. PA-350 is a *C. chinense* susceptible to *M. incognita.* These genotypes were included in the test as susceptible reference checks.

Inoculum production and infestation Meloidogyne incognita race 3 was cultured on 'Polinas' tomato (*Solanum lycopersicum*) in isolated soil benches in the greenhouse. Egg inocula were extracted from infected tomato roots using 0.5% sodium hypochlorite (NaOCl) (Hussey and Barker 1973). Five 2 week-old seedlings of each of the eight pepper genotypes were transplanted in a 10-cm square planting pattern in greenhouse benches containing steam-pasteurised 2 washed river sand : 1 sandy loam soil (vol:vol). Five replicates of each genotype were planted. The pepper seedlings were inoculated 9 days after transplanting with 3,000 eggs of *M. incognita* race 3 in 3 ml tap water. Eight weeks post-infestation, roots were lifted and washed, and scored for root galling and egg masses using a 1 to 5 scale where 1 = 0 to 3% of root system galled or covered with egg masses, 2 = 4 to 25%, 3 = 26 to 50%, 4 = 51 to 80%, and 5 = 81 to 100% of root system galled or covered with egg masses (Thies and Fery 2000). Root systems were also rated for fibrous root mass using a 1 to 5 scale where 1 = large amount of fibrous roots (best); 3 = moderate amount of fibrous roots, and 5 = no fibrous roots present (worst). Nematode eggs were extracted from the entire fibrous root sample from each five-plant plot using the NaOCl method (Hussey and Barker 1973). Three aliquots of each egg sample were counted using a stereomicroscope and the mean number of eggs g^{-1} fresh root was reported.

Experimental design and data analysis The experimental design was a randomised complete block with 5 replicates. Each replicate consisted of 5 plants per genotype. Nematode egg data were log $_{10}$ (x+1)-transformed to normalise the data before analysis and back-transformed data were reported. Data were analysed using the GLM procedure of SAS for Windows System Version 6.12 (SAS Institute, Cary, NC) and means were separated using Duncan's multiple range test. Differences reported in the text were significant at the $P<0.05$ level.

Allelism tests (Tests IIa and IIb)

Plant populations The DH population HDA 149 was crossed with the cvs 'Carolina Wonder' and 'Charleston Belle' to create F1 generations. Reciprocal crosses (F1R) were also created ('Carolina Wonder' × HDA 149 and 'Charleston Belle' × HDA 149). F2 generations were the result of selfing a single F1 plant of each test cross and bulking the seed. Seed for the parental populations used in the allelism tests was generated from selfing the original parental plant.

Inoculum production and infestation Egg inocula were produced as described for Test I. Four to 5 week-old seedlings from the representative generations of both crosses were inoculated with 5,000 eggs per plant. Eight weeks after nematode infestation, individual plants were rated susceptible or resistant based on severity ratings of galling and egg mass coverage per root system using the scale described in Test I. The galling index required for a plant to be scored as resistant was <3.

Experimental design and data analysis A completely randomised design was used for Tests IIa and IIb. Each test was repeated once; results of the repetitions were similar and therefore data from the repetitions were combined for analysis. Segregation data on resistance to *M. incognita* race 3 infection obtained from the F2 progeny were tested for departures from expected Mendelian segregation ratios using chi-square analyses. Initial observations suggested a two dominant gene model. Hence, the data obtained from the F2 progenies for each parental cross were tested against the following gene models: single dominant gene (allelism between *Me3* and *N* genes), two dominant genes (*Me3* and *N* genes), three dominant genes and four dominant genes. All chi-square tests for specific proportions for goodness of fit were performed using the PROC FREQ function of SAS (SAS Institute Inc., Cary, NC) and invoking the testp option. Departures from expected segregation ratios for the various gene models were considered significant at the $P=0.05$ level.

Results and discussion

Characterisation of *Me3* and *N* resistance genes

All genotypes ('Charleston Belle' and 'Carolina Wonder') carrying the *N* gene (or alleles of the *N* gene) exhibited high resistance to *M. incognita* (Table 1). The gall and egg mass indices were 1.0 (0% to 3% of root system galled or covered with egg masses) for both 'Charleston Belle' and 'Carolina Wonder'. Numbers of *M. incognita* eggs were very low (≤139 eggs g^{-1} fresh root) (Table 1). The fibrous root index was 1.9 for 'Carolina Wonder' and 2.3 for 'Charleston Belle'.

The genotypes HDA 149 (*Me3*) and PM 687 (*Me3* and *Me4*) that carry the *Me3* and/or *Me4* genes exhibited high resistance. The gall and egg mass indices were 1.0 (0% to 3% of root system galled or covered with egg masses) for both HDA 149 and PM 687. The fibrous root index was 2.1 for PM 687 and 2.4 for HDA 149.

All four of the susceptible check cultivars ('Yolo Wonder B', PA-350, 'California Wonder', and 'Keystone Resistant Giant') exhibited susceptible reactions to *M. incognita*, as expected. Root galling was severe (gall indices ranged from 3.9 to 4.5) and nematode reproduction was high (numbers of *M. incognita* eggs g^{-1} fresh root ranged from 13,390 to 61,944). The fibrous root index varied from 2.6 for 'Keystone Resistant Giant' to 4.1 for PA-350. PA-350 had the least amount of fibrous roots of all entries evaluated in this test. Overall, effects of the *N* and *Me3* genes were comparable with regard to resistance to *M. incognita* race 3. Although there were no significant differences among populations possessing the *N* and *Me3* genes, the populations possessing the *N* gene had slightly higher levels of reproduction as evidenced by higher eggs g^{-1} fresh

Table 1 Gall and egg mass indices, *Meloidogyne incognita* eggs g^{-1} fresh root, and fibrous root index for pepper entries with resistance to root-knot nematodes conditioned by the *N*, *Me3*, and *Me4* genes inoculated with *M. incognita* race 3 in a greenhouse test

Pepper cultivar	Gall index	Egg mass index	Eggs g^{-1} fresh root	Fibrous root index
N gene[a]				
Charleston Belle	1.0 a[b]	1.0 a	91 a–c	2.3 a–c
Carolina Wonder	1.0 a	1.0 a	139 a–c	1.9 a
Me genes[c]				
HDA 149	1.0 a	1.0 a	55 a	2.4 a–c
PM 687	1.0 a	1.0 a	96 a–c	2.1 ab
Susceptible checks				
Yolo Wonder B	3.9 d	3.8 b	13,390 e	2.7 c
PA-350	4.3 e	4.3 c	61,944 f	4.1 d
California Wonder	4.4 e	4.1 bc	18,230 e	2.8 c
Keystone Resistant Giant	4.5 e	4.1 bc	16,459 e	2.6 c

[a] Resistance to root-knot nematodes conferred by *N* gene

[b] Mean separation within a column by Duncan's multiple range test, $P<0.05$

[c] Resistance to root-knot nematodes conferred by *Me3* gene (HDA 149) or *Me3* and *Me4* genes (PM687)

root, perhaps suggesting the mode of resistance among the two genes are not entirely comparable.

Allelism tests

The high proportion of phenotypically-resistant individuals in the F2 generations of HDA 149 × 'Charleston Belle' and HDA 149 × 'Carolina Wonder' clearly indicates that additive or complementary gene action did not significantly contribute to the *M. incognita* resistance observed in these populations (Table 2). The patterns of inheritance of resistance to *M. incognita* race 3 in these F2 populations also suggest that the *Me3* and *N* genes are not allelic. In both allelism tests, the susceptible check populations, 'Keystone Resistant Giant' and 'Yolo Wonder', displayed a very high proportion of susceptible individuals (data not shown).

In Test IIa (HDA 149 × 'Carolina Wonder' crosses), the F2 progenies of HDA 149 and 'Carolina Wonder' fit segregation ratios of 15 (resistant):1 (susceptible) in their reaction to *M. incognita* (Table 2), regardless of which parental plant was used as the maternal or paternal parent. The chi-square analysis combined with the data from the parental generation indicates that two distinct genes conditioning resistance exist, one potentially contributed from each parent. This result is in accordance with the proposed *Me3* gene originating from HDA 149 and the proposed *N* gene originating from 'Carolina Wonder'. Based on the segregation ratios obtained from Test IIa, we concluded that the *N* gene and the *Me3* gene are not alleles located at the same locus.

Results of Test IIb, which evaluated HDA 149 × 'Charleston Belle' progenies, showed that there were no plants in the parental generations expressing the susceptible phenotype, as expected. However, a single plant in the F1 generation was categorised as exhibiting the susceptible phenotype and a very few F2 individuals were categorised as the susceptible phenotype. In the case of the susceptible F1, the single plant exhibiting susceptibility may be the result of incomplete penetrance in the heterozygous of either of the genes in question. While the chi-square analysis suggests these data conform to a four gene model (256:1) (Table 2), it is also possible that the *N* gene and the *Me3* gene may be linked. As in Test IIa, the segregation patterns of the F2 generations of HDA 149 × 'Charleston Belle' and its reciprocal cross were very similar, indicating that there were no significant maternal or paternal effects.

Based on the F2 segregation ratios (Table 2) of the two experimental crosses HDA 149 × 'Carolina Wonder' and HDA 149 × 'Charleston Belle', the *Me3* gene and the *N* gene are not allelic. In both tests, the F2 segregation ratios of R:S far exceeded the expected proportion of 3:1 that would indicate the *N*

Table 2 Reaction of parents, F1 and F2 progenies of experimental crosses of pepper lines HDA 149, 'Carolina Wonder' and 'Charleston Belle' to infection by *Meloidogyne incognita* race 3

Parent or cross	Number of plants			Expected ratio (R:S)	χ^2 (df=1, P=0.05 $\chi^2 \geq 3.84$)	P value
	Total	R	S			
Test IIa: HDA 149 X 'Carolina Wonder'						
HDA 149	34	32	2	1:0	–	–
'Carolina Wonder'	40	39	1	1:0	–	–
F1 (HDA 149 X 'Carolina Wonder')	63	61	2	1:0	–	–
F1R ('Carolina Wonder' X HDA 149)	54	51	3	1:0	–	–
F2	493	466	27	3:1	172.17	<0.0001
				15:1	0.50	0.4781
				64:1	49.25	<0.0001
				256:1	328.20	<0.0001
F2R	498	477	21	3:1	190.00	<0.0001
				15:1	3.51	0.0609
				64:1	22.89	<0.0001
				256:1	90.40	<0.0001
Test IIb: HDA 149 X 'Charleston Belle'						
HDA 149	49	49	0	1:0	–	–
'Charleston Belle'	49	49	0	1:0	–	–
F1 (HDA 149 X 'Charleston Belle')	29	29	0	1:0	–	–
F1R ('Charleston Belle' X HDA 149)	26	25	1	1:0	–	–
F2	498	496	2	3:1	243.06	<0.0001
				15:1	29.07	<0.0001
				64:1	4.35	0.0370
				256:1	0.615	0.4368
F2R	538	536	2	3:1	263.06	<0.0001
				15:1	31.73	<0.0001
				64:1	4.95	0.0261
				256:1	0.80	0.3724

gene and the *Me3* gene were actually alleles of the same gene.

As evidenced by the very high levels of resistance in the HDA 149 × 'Charleston Belle' progeny, multiple resistance genes deployed in a cultivar could provide superior protection against root-knot nematode infestation in field situations and ameliorate the effects of any heat-instable resistance genes that may fail to hold up under high temperatures. Additionally, multiple sources of resistance in a host minimises the likelihood of further race development in *M. incognita* populations. As resistance genes to root-knot nematodes operate in a gene-for-gene fashion (Castagnone-Sereno 2002), the continued deployment of a few specific genes conferring resistance selects for pathogens able to overcome the source of genetic resistance. *Meloidogyne incognita* has been documented to overcome the *Me3* gene in laboratory studies (Castagnone-Sereno et al. 1992, 1994). Through the use of multiple dominant resistance genes, selection for virulent *M. incognita* pathotypes able to overcome specific genes is theoretically minimised.

To our knowledge, this is the first report confirming the *Me3* and *N* genes as separate, distinct genes. We are currently initiating test crosses to develop populations to further elucidate the additional sources and patterns of inheritance of *M. incognita* resistance found in this study.

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