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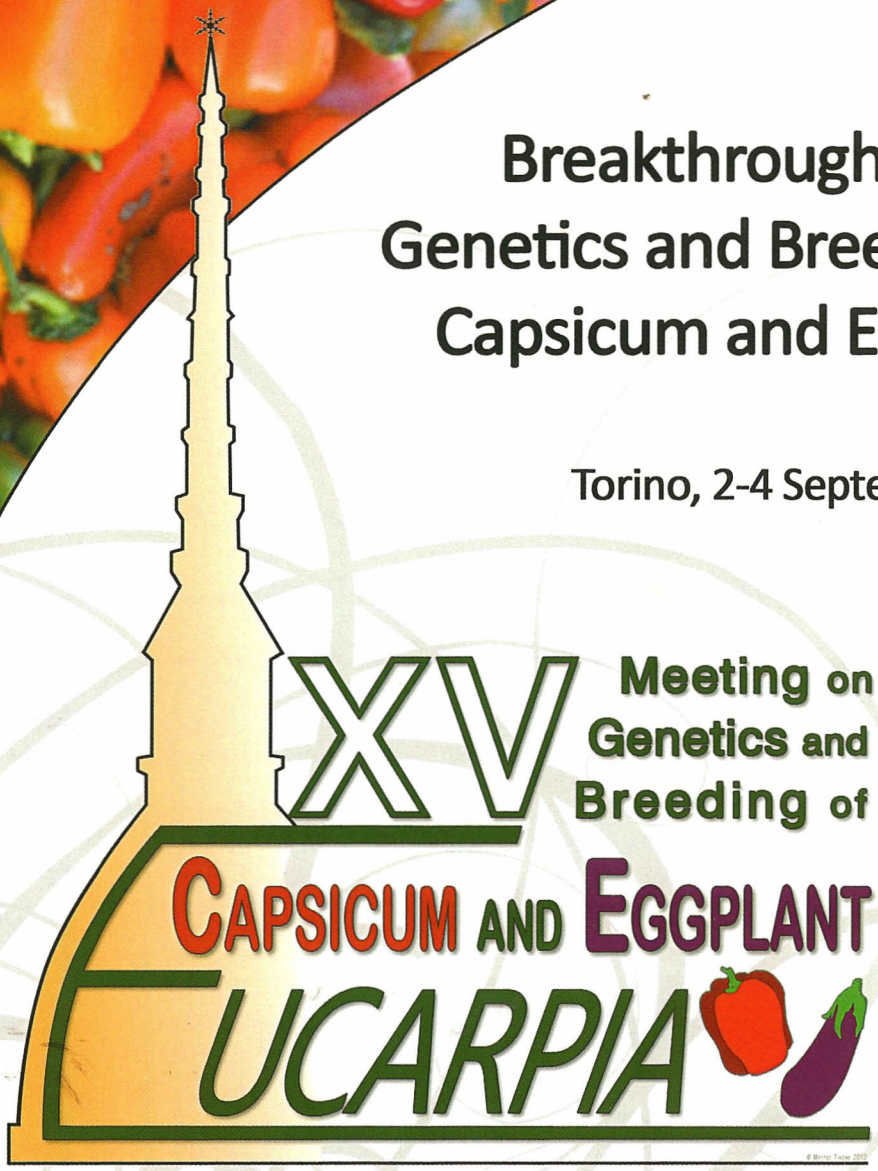

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Towards the deciphering of the genetic factors involved in durability of plant major resistance genes to root knot nematodes in pepper

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Abstract

Root-knot nematodes (RKNs), *Meloidogyne* spp., are extremely polyphagous plant parasites worldwide. Since the use of most chemical nematicides is being prohibited, genetic resistance is an efficient alternative way to protect crops against these pests. However, few resistance genes (R-genes) are available and some nematode populations may become able to overcome them with time. Sustainable management of these valuable resources is thus a key point of R-gene durability. In pepper (*Capsicum annuum*), *Me3* is a dominant major R-gene, currently used in breeding programs, that controls *M. arenaria*, *M. incognita* and *M. javanica*, the three main RKNs species. It was introgressed in either a susceptible or a partially resistant (i.e., that shows reduced symptoms) genetic background in either homozygous or heterozygous allelic status. Doux Long des Landes (DLL) was used as susceptible recipient pepper line and Yolo Wonder (YW) as a partially resistant one. Challenging all these genotypes with a high inoculation pressure of an avirulent *M. incognita* isolate demonstrated that i) the efficiency of the R-gene in reducing the reproductive potential of RKNs is strongly affected by the plant genetic background, ii) the allelic status of the R-genes has no effect on nematode reproduction. These results highlight the primary importance of the choice of both the R-gene and the genetic background into which it is introgressed during the selection of new elite cultivars by plant breeders.

Keywords: *Meloidogyne* spp., *Capsicum annuum* (pepper), *Me* resistance genes, dosage allele effect, resistance durability, quantitative resistance

Introduction

Root-knot nematodes (RKNs), *Meloidogyne* spp., are considered as one of the most damaging pathogen in the world (Trudgill and Blok 2001). A reliable way of controlling these polyphagous endoparasitic worms is the use of chemical nematicides. However, the use of such compounds was drastically restricted in the past years because of environmental and public health issues. Now, one of the best alternatives to cope with nematode infestations relies on the deployment of resistance genes (R-genes), which represent an efficient, environmentally safe and economically sustainable method of control (Djian-Caporalino et al. 2009). As a consequence, many breeding programs are being developed in order to introgress the desired R-genes into elite cultivars and/or rootstocks. However, not only the R-gene itself may completely account for the observed resistance phenotype. Indeed, effects linked to the plant genetic background have been recognized to modify levels of nematode resistance in several crops (Jacquet et al. 2005; López-Pérez et al. 2006; Wang et al. 2008). Additionally, in several pathosystems, including plant-nematode interactions, it was shown a dosage effect of the R-gene alleles on the pathogen multiplication (Collmer et al. 2000; Jacquet et al. 2005; Chintamanani et al. 2008).

Since resistance sources against RKNs are limited, management of the available R-genes is of crucial importance. As RKNs exhibit noteworthy capacities of adaptation to their environment,

including R-genes, the emergence and spread of virulent nematode populations constitutes a severe threat to R-gene durability (Castagnone-Sereno 2002, 2006; McDonald and Linde 2002).

In pepper (*Capsicum annuum* L.), several dominant R-genes have been identified and well characterized for their spectrum of resistance against RKNs, i.e., the *Me* genes and the *N* gene (Hare 1956; Hendy et al. 1985a, 1985b; Djian-Caporalino et al. 1999; Thies et Fery 2000). Some of them have been mapped and co-localised in a cluster on the pepper P9 chromosome (Djian-Caporalino et al. 2001, 2007; Fazari et al. 2012). One of these dominant R-genes, *Me3*, displays a broad spectrum of resistance to the three main RKN species, i.e., *M. incognita*, *M. arenaria* and *M. javanica*. Currently, it is being actively exploited in breeding programs.

The present work aims at evaluating the influence of the genetic background of pepper genotypes on the expression of the resistance to RKNs conferred by the *Me3* R-gene, using either a susceptible or a partially resistant genetic background. Allele dosage effect of this gene was also tested to evaluate the relevance of hybrid varieties *versus* inbreed lines on R-genes efficiency.

Materials and Methods

Plant material

Pepper (*Capsicum annuum* L.) genotypes used in this work were Yolo Wonder (YW), Doux Long des Landes (DLL) and DH149. These inbred lines were selected for their differential resistance to *Meloidogyne incognita*. DLL is a highly susceptible pepper cultivar; YW is a partially resistant (i.e., shows reduced symptoms) cultivar. DH149 is a resistant doubled haploid (DH) lines produced through *in vitro* androgenesis (Dumas de Vaulx et al. 1981) from the intraspecific F1 hybrids (PM687 x YW). DH149 carries the single dominant resistance allele *Me3* (Hendy et al. 1984).

In order to introgress the *Me3* allele into the DLL (very susceptible) or YW (partially resistant) genetic backgrounds, the resistant parental line DH149 was crossed with the susceptible parental lines DLL and YW (recurrent parents) and the two F1 hybrids were backcrossed (BC) with their respective recurrent parental lines. At each backcross cycle, the heterozygous resistant plants were sorted using molecular markers linked to the *Me3* allele and self-pollinated to generate a segregating population for the R-gene (BC-S1). The progenies issued from the first backcross (BC1 and BC1-S1) were used.

Plant allelic status determination

Total genomic DNA was isolated from 100 mg of fresh leaf material as described by Fulton et al. (1995). After RNAse treatment, DNA concentration and purity were measured with a NanoDrop 2000 spectrophotometer (Thermoscientific) and adjusted to a final concentration of 20 ng/µL for PCR.

BC1-S1 plants carrying *Me3* were genotyped with SCAR_N, a codominant marker linked to this gene, in order to discriminate *Me3* homozygous susceptible ($Me3^+/Me3^+$), homozygous resistant (*Me3*/*Me3*) and heterozygous ($Me3/Me3^+$) plants in both DLL and YW genetic backgrounds, according to a standard procedure (Fazari et al. 2012).

Nematode material

The RKN isolate used in this study was *M. incognita* Morelos from the collection maintained at INRA research centre in Sophia Antipolis. It is avirulent towards *Me3* gene. Because of the mitotic parthenogenetic mode of reproduction of *M. incognita* (Triantaphyllou 1985), all the second-stage juveniles (J2s) that hatched from a single egg mass were considered as a clonal line. Prior to multiplication, this isolate was specifically identified according to its isoesterase electrophoretic pattern (Dalmasso and Bergé 1978) and/or by SCAR PCR (Zijlstra et al. 2000).

Experimental procedures and evaluation

Pepper seedlings were sown individually in 9-cm plastic pots containing steam-sterilized sandy soil covered by a 1-cm layer of loam. At least twenty replicates (individual plants) were performed for each control genotype (i.e., DLL, YW, DH149 and each F1) and one hundred and twenty BC1-S1 plants were grown. This was done to ensure to obtain at least twenty replicates of each genotype (homozygous susceptible, homozygous resistant and heterozygous). The whole experiment was conducted in a climatic chamber maintained at 24°C (±2°C) with a 12-h light cycle and a relative humidity of 60–70%. Six to seven-week old plants (4-6 true leaves) were inoculated with a water suspension of 5,000 hatched second-stage juveniles (J2s) obtained in a mist chamber, from previously inoculated susceptible tomato roots (cultivar Saint Pierre). Six to seven weeks after inoculation (i.e., a duration that allowed completion of the nematode life cycle), plants were harvested, carefully washed individually with tap-water, and stained for 10 min in a cold aqueous solution of eosin yellow (0.1 g/l water), to specifically stain egg masses (EMs) (Roberts et al. 1990). The roots were rinsed and examined under a magnifying glass. The number of EMs was counted for each plant and the average number of EMs was calculated for the different genotypes, providing their disease severity (DS). In addition, for each genotype, the frequency of plants exhibiting more than five EMs in relation to the number of inoculated plants was computed, giving their disease incidence (DI) ranging from 0 to 1.

Statistical analysis

All the statistical analyses were performed using the free software R (http://www.r-project.org/). First, to check the good fit of the expected segregation of the BC-S1 populations, a χ^2 test was performed. In order to investigate a possible effect of the genetic background and/or a dosage allele effect, non-parametric tests were further applied to compare the DS of the different genotypes. When the Kruskal-Wallis test was significant, Wilcoxon-Mann-Whitney bilateral tests with a significance level at α=0.05 were carried out using Bonferroni correction.

Results

Homozygous susceptible (*Me3*$^+$/*Me3*$^+$), homozygous resistant (*Me3*/*Me3*) and heterozygous (*Me3*/*Me3*$^+$) BC1-S1 plants were sorted using SCAR_N, a codominant marker linked to *Me3*. With both recurrent parents DLL and YW, the observed segregation of *Me3* fitted the expected segregating ratio as revealed by a χ^2 test at α=0.05 (Table 1).

Genetic background	Allelic status at the *Me3* locus	Number of plants	χ^2 (1:2:1)
BC1-S1 [(DH149 x DLL) x DLL]	Homozygous susceptible *Me3*$^+$/*Me3*$^+$	19	X-quared = 4.1538
	Homozygous resistant *Me3*/*Me3*	23	df = 2
	Heterozygous *Me3*/*Me3*$^+$	62	p-value = 0.1253
BC1-S1 [(DH149 x YW) x YW]	Homozygous susceptible *Me3*$^+$/*Me3*$^+$	35	X-quared = 3.2182
	Homozygous resistant *Me3*/*Me3*	22	df = 2
	Heterozygous *Me3*/*Me3*$^+$	53	p-value = 0.2001

Table 1 Observed segregation ratio at the *Me3* allele in progenies with DLL (upper part) or YW (lower part) genetic backgrounds from a self-pollinated heterozygous resistant backcross 1 plant
BC1-S1=Backcross 1 Self-pollinated; DH149=Double Haploid 149 line (resistant genotype); DLL=Doux Long des Landes (susceptible genotype); YW=Yolo Wonder (partially resistant genotype)

All the genotypes were infested with a high pressure inoculum (5,000 J2s) of *M. incognita* Morelos avirulent isolate. The Kruskal-Wallis test revealed that there was a significant effect of the plant genotype on nematode reproduction (χ^2=363.71, df=10, p-value<10^{-3}). Consequently, the

mean values of the different genotypes were compared each other to determine which one(s) provided the best efficiency against RKNs.

As expected, DLL exhibited a high number of EMs (DS=1579.7) whereas YW showed a moderate one (DS=462.4). For both genotypes, EMs were detected on the root system of all inoculated plants (DI=1.00). The plants of the susceptible BC1-S1 (*Me3*$^+$/*Me3*$^+$) genotypes were all infested (DI=1.00) and they exhibited numerous EMs, but less than their respective susceptible parents DLL and YW. The EM number of susceptible BC1-S1 (*Me3*$^+$/*Me3*$^+$) plants in the DLL genetic background was much higher than in the YW one (DS=490.9 and DS=116.1, respectively) (Fig. 1).

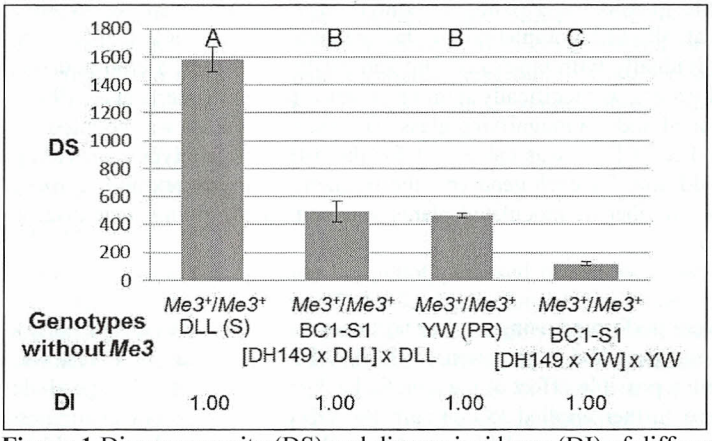

Figure 1 Disease severity (DS) and disease incidence (DI) of different pepper genotypes inoculated with a high pressure inoculum (5,000 J2s) of an avirulent isolate of *M. incognita*. DS=average number of egg masses/plant; DI=number of plants exhibiting more than five egg masses in relation to the number of inoculated plants; *Me3*$^+$/*Me3*$^+$=homozygous susceptible at the *Me3* locus; BC1-S1= Backcross 1 self-pollinated; DLL=Doux Long des Landes (susceptible genotype or genetic background); YW=Yolo Wonder (partially resistant genotype or genetic background); DH149=Double Haploid 149 line (resistant genotype). Bar=standard error. Different letters mean significant differences (Wilcoxon-Mann-Whithney bilateral tests at α=0.05 after Bonferroni correction).

DH149 confirmed its resistant status with a mean number of EMs close to zero (DS=4.4) and a few number of plants affected (DI=0.17). There was no significant differences between the average EM number of the two F1 hybrids (DH149 x YW) and (DH149 x DLL) which appeared resistant (DS=0.2 and DS=0.9, respectively). Different results were obtained with the BC1-S1 plants heterozygous or homozygous at the *Me3* allele. In the YW genetic background, the number of EMs of the BC1-S1 plants, heterozygous or homozygous at the *Me3* allele, did not significantly differ from DH149 and both genotypes had a similar rate of infested plants. In the DLL genetic background, the number of EMs was significantly much higher than in the DH149 one (DS=30.8 and DS=49.1, respectively) and the rate of infested plants was important (DI=0.68 and DI=0.61, respectively). Comparing *Me3* heterozygous to homozygous resistant BC1-S1 plants within the same genetic background (DLL or YW) did not reveal significant differences (Fig. 2).

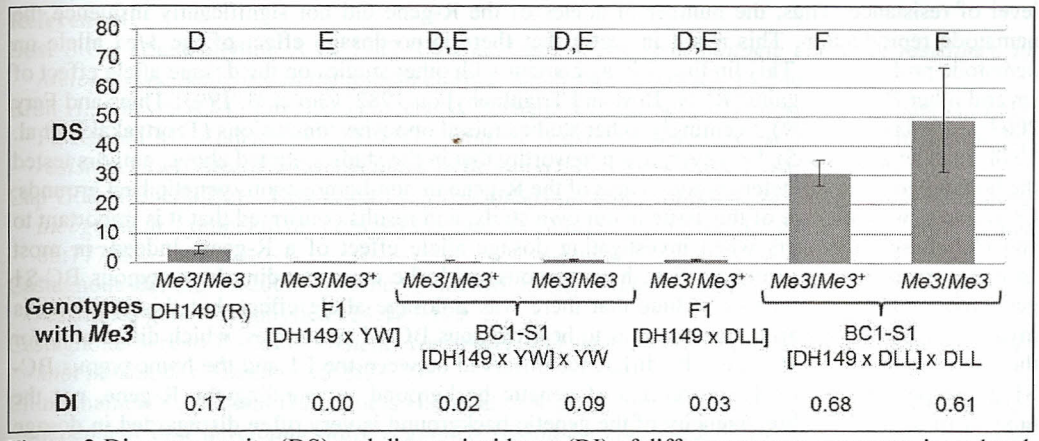

Figure 2 Disease severity (DS) and disease incidence (DI) of different pepper genotypes inoculated with a high pressure inoculum (5,000 J2s) of an avirulent isolate of *M. incognita*. DS=average number of egg masses/plant; DI=number of plants exhibiting more than five egg masses in relation to the number of inoculated plants; *Me3/Me3*=homozygous resistant at the *Me3* locus; *Me3/Me3*$^{+}$=heterozygous at the *Me3* locus; BC1-S1= Backcross 1 self-pollinated; F1=Hybrid F1; DLL=Doux Long des Landes (susceptible genetic background); YW=Yolo Wonder (partially resistant genetic background); DH149=Double Haploid 149 line (resistant genotype). Bar=standard error. Different letters mean significant differences (Wilcoxon-Mann-Whithney bilateral tests at α=0.05 after Bonferroni correction).

Discussion

In order to explore the effect of the plant genetic background on R-gene efficiency, *Me3* was introgressed into a susceptible (i.e., DLL) or a partially resistant (i.e., YW) pepper genetic background. Compared with the donor resistant parental lines, the DLL or YW genetic background surrounding the R-gene was increased of 50% in F1 hybrids and of 75% in BC1-S1 plants. The different genotypes were challenged with a high inoculation pressure of *M. incognita* and their ability to resist to this pathogen was evaluated. The main observation was that plants with *Me3* in a susceptible genetic background were more easily attacked than in a partially resistant one. Nematode reproduction was higher when the part of susceptible genetic background surrounding the R-gene was increased. This result is in agreement with studies on other pathosystems. Influence of the plant genetic background on R-gene efficiency to nematodes was shown in tomato (López-Pérez et al. 2006) and cotton (Wang et al. 2008). It was also demonstrated that the genetic background was able to modulate the expression of a R-gene in rice, conferring more or less resistance efficiency against a bacteria (Zhou et al. 2009). Further research needs to be conducted to determine the genetic factors, within the plant genetic background, that may explain the discrepancies from a pepper genotype to another. The homozygous susceptible BC-S1 plants at the *Me3* locus but carrying a residual genome part of the resistant donor parental line always showed a lower number of EMs than their respective parental recurrent lines DLL and YW. This difference may indicate the presence of resistance quantitative trait loci (QTLs) controlling the quantitative differences in the level of resistance, as observed between YW and DLL genetic backgrounds. To date, no QTLs were found against RKN in pepper. In that respect, a QTL analysis is currently on-going on this biological material, as we strongly suppose that the protective effect of the plant genetic background on R-genes is provided by such quantitative resistance factors.

The second objective of this study was to evaluate an eventual dosage effect of the *Me3* allele on the reproductive potential of RKN. Heterozygous and homozygous genotypes at the R-gene locus, in the same genetic background and at the same level of introgression, exhibited the same

level of resistance. Thus, the number of alleles of the R-gene did not significantly influence the nematode reproduction. This result indicates that there is no dosage effect of the *Me3* allele on nematode proliferation. This finding is in agreement with other studies on the dosage allele effect of several other R-genes against RKN (Bost and Triantaphyllou 1982; Cap et al. 1993; Thies and Fery 2002; Cortada et al. 2009). Seemingly, other studies raised opposite conclusions (Tzortzakakis et al. 1998; Jacquet et al. 2005). However, it is noteworthy that in the studies quoted above, authors tested the homozygous *versus* heterozygous status of the R-gene in non-homogenous genetic backgrounds. Conversely, we took care of this issue in our own study, and results confirmed that it is important to consider these parameters when investigating dosage allele effect of a R-gene. Indeed, in most cases, comparing a F1 genotype (i.e., heterozygous) with the corresponding homozygous BC-S1 genotype would have led to conclude that there was a dosage allele effect, but this assertion is invalidated when comparing homozygous to heterozygous BC-S1 genotypes, which differ only for the allelic status of the R-gene. The difference observed between the F1 and the homozygous BC-S1 genotype was due to the proportion of genetic background surrounding the R-gene, not the number of alleles. The homogeneity of the genetic background is very often disregarded in dosage allele studies, whereas it is of major importance.

The same kind of experiment was performed on *Me1*, another pepper dominant major R-gene used in breeding programs since it controls *M. arenaria*, *M. incognita* and *M. javanica*. As for *Me3*, *Me1* efficiency was influenced by the plant genetic background, with higher level of infestation on genotypes with a DLL genetic background than with a YW one. Similarly to *Me3*, no dosage effect of the *Me1* allele was shown on the reproductive potential of RKNs (data not shown).

In the present study, the crucial role of the plant genetic background in resistance to RKNs was clearly demonstrated using the *M. incognita*/pepper pathosystem as a model. This point can have direct practical implications on breeding strategies. It is of major importance for breeders to take into account the genetic background into which they introgress major R-genes, in order to increase their efficiency and likely improve the lifetime of new elite varieties released on the market. Thus, one of the best alternatives to avoid nematode damages without impairing *Me3* (and *Me1*) efficiency would be to combine them with partial resistance factors (i.e. QTLs). This strategy would take simultaneous advantage of these R-genes, which provide total resistance to the three main RKN species, and of QTLs, which theoretically reduce the level of infestation. In addition to increased resistance efficiency, we suspect that a partially resistant genetic background may have a protective role on *Me3* and may prevent it from being quickly overcome. One might expect that the reduction of the nematode reproduction due to the partially resistant genetic background surrounding *Me3* (or *Me1*) may decrease the risk of resistance breakdown by RKNs and may increase its durability. This hypothesis is supported by several studies on different pathosystems which proved that the durability of R-genes was dependent on the plant genetic background into which they were introgressed (Palloix et al. 2009; Brun et al. 2010; Fournet et al. 2012).

Another point that can have direct practical implications on breeding strategies relies on the absence of dosage effect of *Me3* (and *Me1*) allele on the reproductive potential of RKNs. Consequently, since the proportion of hybrids in commercial cultivars has been increasing, taking advantage of the possibility to cumulate R-genes against different pathogens in an heterozygous status, using *Me3* (or *Me1*) in hybrid varieties is not an issue, as long as it is into a suitable genetic background.

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