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Abstract

The current restrictions on the use of chemical nematicides have contributed to increase root-knot nematode problems in horticultural crops. In this context, plant resistance appears as the most effective method of control, but the possible occurrence of virulent nematodes able to reproduce on *R*-plants may constitute a severe threat to this control strategy. In *Capsicum annuum*, resistance to RKN is controlled by several dominant genes — the *N* and *Me* genes. To implement a rational management of the *R*-lines increasing the durability of the *R*-genes, we tested several *R*-gene deployment strategies. Experiments were conducted in climate-controlled rooms, in greenhouses, and under 3-years-field agronomic conditions to compare i) the succession of the same *R*-genes every year, when introgressed in a partially resistant vs. a susceptible genetic background, ii) the alternance, ii) the mixed cultivation and iv) the pyramiding of two *R*-genes with different modes of action in a single genotype. At the plant level, we previously showed that the choice of the *R*-genes and the genetic backgrounds in which they are introgressed can lower the frequency of resistance breakdown, and that the pyramiding of two different *R*-genes in one genotype totally suppressed the emergence of virulent isolates. Here, at the field and rotation level, we confirmed these results and showed that i) alternating different *R*-genes in rotation is efficient to reduce the selection pressure of *R*-genes on the pathogens and allows to recycle broken *R*-genes, and ii) optimal cultivation practices of *R*-plants increase their "trap" effect and may decrease the amount of pathogens in the soil, below their damage threshold. These results are in good agreement with concepts recently developed from the analysis of other plant-pathogen interactions. The root-knot nematode model could thus contribute to generalize strategies for the breeding and management of *R*-cultivars strengthening and increasing the durability of qualitative resistances.

Keywords: Sustainable crop protection, breeding strategy, resistance gene deployment, virulence emergence, root knot nematodes, *Meloidogyne* spp., *Capsicum* spp.

Introduction

Plant-parasitic nematodes are among the most damaging and uncontrollable pests of cultivated crops causing severe economic losses in world agriculture, estimated to $US 121 billion per year and affecting 12.3 per cent of the word crop production (Chitwood, 2003). The specialized and intensive vegetable crops agriculture is becoming particularly vulnerable to a few species belonging to the group of root-knot nematodes (RKNs, *Meloidogyne* spp.), obligate plant endoparasites, found throughout the world, mainly in tropical, subtropical and warm-temperate areas in which several nematode generations can be completed per year. These polyphagous nematodes are one of the

main pathogens on many Solanaceous crops throughout the world (Khan and Haider 1991). The parasite pressure due to these soilborne pests in vegetable crops has increased steadily following the changes in pesticide legislation and the new regulations that have withdrawn the use of most chemical nematicides. These microscopic parasites are difficult to control, particularly because of their highly polyphagous nature and their ability to remain hidden in the soil or in plants. Host resistance is considered as an important component of integrated management of RKNs. Because few *R*-genes acting against these pests are currently available, it is urgently needed to protect them and promote their durability. In pepper, the *Me* genes, identified in local populations, control the main species of *Meloidogyne* (*M. arenaria, M. incognita, M. javanica, M. hapla*) (Hendy et al. 1985; Djian-Caporalino et al. 1999, 2001, 2007). Although they have only recently been used in plant breeding, a risk of some of these genes being overcome has already been demonstrated under laboratory experiments with high inoculum pressure of nematodes (Castagnone-Sereno et al. 2001; Djian-Caporalino et al. 2011). On this pathosystem, it was also shown that virulence is highly specific to a determined *R*-gene on which selection has occurred and that a reproductive fitness cost is associated to nematode virulence (Castagnone-Sereno et al. 2007; Djian-Caporalino et al. 2011). The adaptative significance of trade-offs between virulence traits and fitness-related traits suggests that, although the resistance can be broken, it may be preserved in some conditions. Beside the improvement of knowledge that is essential for a better sustainable management of plant resistance, the interest of the RKN model system is based on its originality compared to other plant pathogens. First, the parasitic pressure that is applied by RKNs to their host plants is theoretically low: small population size (a few hundreds of juveniles in one egg-mass as the total progeny of a female), long biological cycle (about eight weeks at 20°C). Second, the biological features of RKNs that govern their evolutionary potential should not favour the emergence of virulent populations: lack of sexual reproduction (obligatory mitotic parthenogenesis), active dispersal capacities reduced in soil. However, our previous studies in artificial conditions (Djian-Caporalino et al. 2011; Barbary et al. submitted) have shown that breaking resistance is dependent on the major resistance gene used, its allelic status (homozygous *versus* heterozygous) and the genetic background it has been introgressed in. These results are in good agreement with concepts recently developed from the analysis of very different plant-pathogen interactions: pepper-virus (Palloix et al. 2009) or rapeseed-*Leptosphaeria* (Brun et al. 2010). The RKN model studied here could thus contribute to the generalization of strategies for the breeding and management of resistant cultivars.

In this study, we evaluated several resistance-gene deployment strategies to implement a rational use of pepper *R*-cultivars, with the objective to improve the sustainable management of RKNs. Experiments were conducted in climate-controlled rooms, in greenhouses, and under 3-years-field conditions comparing i) the succession of the same *R*-gene every year, when introgressed in a resistant vs. a susceptible genetic background, ii) the alternance of single *R*-genes in rotation, iii) the mixture of genotypes bearing single *R*-genes sown in the same plot, and iv) the pyramiding of two *R*-genes in one genotype.

Materials and Methods

Plant material

The five pepper (*Capsicum annuum*) genotypes used in this work are inbred lines with differential resistances to RKN. Doux Long des Landes (DLL) is a susceptible cultivar. The two resistant haplo-diploid lines, DH149 and DH330 produced through *in vitro* androgenesis were previously described (Djian-Caporalino et al. 1999); they are homozygous for the *Me3* and *Me1* genes, respectively.The *Me3* gene induces early cellular necrosis in the root epidermis adjacent to the juveniles (Bleve-Zacheo et al. 1998). The selection of virulent variants against the *Me3* gene was achieved through strong selection pressure on avirulent *M. incognita* isolates. *Me1* induces a late hypersensitive reaction in the vascular cylinder of infected roots, thus inhibiting the

development of egg-laying females (Bleve-Zacheo et al. 1998). Under laboratory conditions, *Me1* prevents the emergence of *Me1*-virulent nematode genotypes, despite the implementation of drastic levels of inoculum (Djian-Caporalino et al. 2011). *Me3* and *Me1* are currently being introgressed by breeders into cultivars but they are not yet commercialized. Two F1 hybrid lines were also used, one carrying *Me1* in its heterozygous state in the DLL susceptible genetic background (F1 [DH330 x DLL]), and one combining the two mechanisms of resistance from *Me3* and *Me1* (F1 [DH149 x DH330]). All the lines were produced independently in insect-proof cages to eliminate outcrossing. Pepper seedlings were grown individually in 100 ml pots containing steam-sterilized sandy soil covered by a 1 cm layer of loam in climatic chambers maintained at 24°C (±2°C) with a 12-h light cycle and a relative humidity of 60–70%. Seven to eight-week-old plants (8-10 true leaves) were transplanted in the plots.

Design of the 3-yr field experiment

The experiment was carried out in a plastic tunnel belonging to the Chamber of Agriculture of Alpes-Maritimes (technical institute) in La Gaude (SE France). The tunnel was 224 m2 (28m x 8m). The soil had a pH of 8.2 with 46.68% of sand, 27.99% of loam, and 25,33% of clay. Total limestome was 171 g/kg. The soil temperature in the tunnel varied from 15°C in winter (December to April) to 25°C in summer (June to September) at 15 cm depth (Mediterranean climate). During the whole experiment, the tunnel received no phytosanitary treatment. It was subdivized in 52 microplots of one scare meter each, separated by one meter of bare soil between each plot. Before starting the experiment, nematode-susceptible tomatoes were cultivated for three consecutive years in non disinfected soil which was naturally infested with a mixture of *Meloidogyne incognita* and *M. arenaria*. The experiment was performed on 4 rows (1 meter apart) with two lines of fertirrigation drips (16 mm diameter tubes with 10 holes/ m^2 providing 2 L/h) by rank and the establishment of a non-degradable plastic mulch to prevent contamination between plots. The first year, the experiment received an organic amendment before the establishment of the plastic mulch. The third year, the experiment was only irrigated but not fertilized by the grower.

Six cultivation modalities were compared during 3 successive years: 1) the succession of the same *R*-gene (*Me1*), when introgressed in a resistant genetic background (DH330), 2) the succession of the same *R*-gene (*Me1*) when introgressed in a susceptible genetic background (F1 [DH330 x DLL]), 3) the alternance of single *R*-genes in rotation (*Me3* (DH149) the first year, *Me1* (DH330) the second year, then *Me3* (DH149) the third year), 4) the mixture of lines bearing single *R*-genes (*Me1* (DH330) or *Me3* (DH149), respectively) transplanted in the same plot, 5) the pyramiding of two *R*-genes (*Me3* and *Me1*) in one line (F1 [DH149 x DH330]) and 6) the susceptible cultivar (DLL) as control. Each scare-meter plot harboured five plants of a given modality from April-May to October, followed by five growing cycles of susceptible salads (*Lactuca sativa* cultivar Dedale-batavia), from November to February. Globally, repeats of 8 to 9 plots and 40 to 45 plants per genotype were tested, respectively.

Infestation parameters

Several infestation parameters were analysed along the 3 years. The gall index (GI) was determined for the roots of each pepper or salad plant using a 0 to 10 scale (Zeck, 1971). The number of infected plants per genotype tested was also recorded. To determine the RKN soil infection potential (SIP), 5 replicates of 1 kg-rhizospheric soil were sampled from each plot at 15 cm depth before and after pepper or salad cultivation. Two-month-old susceptible tomato plants (cv. Saint Pierre) were transplanted in pots filled with these soil samples and maintained in a climatic chamber (24°C±2°C, 14-h photoperiod). After 6 weeks, the number of egg masses (EMs) on the tomato plants was evaluated as previously described. To determine the reproduction rate (RR) of potentially virulent nematodes, EMs, if they were detected on a resistant pepper, were picked and inoculated on a 2-month-old resistant pepper carrying the same *R*-genes(s) and maintained in the climatic chamber. After 6 weeks, the roots were carefully washed with tap-water and examined

under a magnifying glass to detect EMs. If EMs were detected, they were reared by successive re-inoculations on 2-month-old resistant peppers carrying the same *R*-genes(s) according to the procedure of Jarquin-Barberena et al. (1991). After 2 generations, 10 EMs were picked up and the mean number of eggs per EM (i.e., the number of eggs produced by one female) was evaluated.

Statistical analysis

In order to compare the evolution of each data mean (SIP, RI, RR), a Kruskal-Wallis test was firstly carried out. Wilcoxon-Mann-Whithney unilateral tests were then used for comparisons in order to check if differences were significant. Bonferroni correction was consequently applied (significance level at α=0.05). Analyses were performed using the free software R (http://www.r-project.org/).

Results

Results on strength and durability of resistances

The evolution of the root infestation of peppers during the three successive years for the 6 modalities, respectively, is presented in Figure 1. As expected, the susceptible cultivar DLL, cultivated in naturally-infested plots, exhibited high infestation levels over the whole experiment (GI ranging from 9.2 to 9.4). Conversely, the 5 modalities that include R genotypes showed a significant reduction of the number of galls on their root systems, whatever the R gene(s) and the mode of use considered (i.e., alternance, mixture or pyramiding). However, differences were noticed among the 5 modalities. After one year of cultivation, the homozygous line DH330 did not show any gall (in monoculture or in alternation with DH149), while the level of infestation progressively increased during years 2 and 3 (GI raised up to 1.6 in year 3). The same trend was generally observed for the other modalities, the highest infestation level being observed in the case of the heterozygous F1 line [DH330xDLL] after the third year of cultivation (GI=3.7). The only notable exception is reported for the F1 [DH149xDH330] pyramiding *Me3* and *Me1*, which remained almost uninfested over the 3 years (GI ranging from 0 to 0.2). In order to evaluate the possible selection of *M. incognita* isolates virulent against *Me1* or *Me3* during the experiment, eggs recovered from R peppers were hatched and the resulting J2 used to reinoculate the same R genotype. Egg-masses sampled on HD149 contained more than 900 eggs on average, and a virulent line was successfully reared by successive re-inoculation on DH149 peppers. After 3 successive re-inoculations, the mean number of eggs per EM was 866.7±43.1 (18 replicates; data not shown). Numerous egg-masses were recovered from *Me1* peppers, either homozygous (DH330) or heterozygous ([DH330xDLL]), but they contained few eggs (<65 eggs per EM), and the nematodes obtained from these eggs did not survive to a successive inoculation, which impaired the selection of a *Me1*-virulent isolate. Very few EM were recovered from the F1 [DH149 x DH330] peppers combining *Me1* and *Me3*, and again no virulent population could be obtained after re-inoculation on R plants (data not shown).

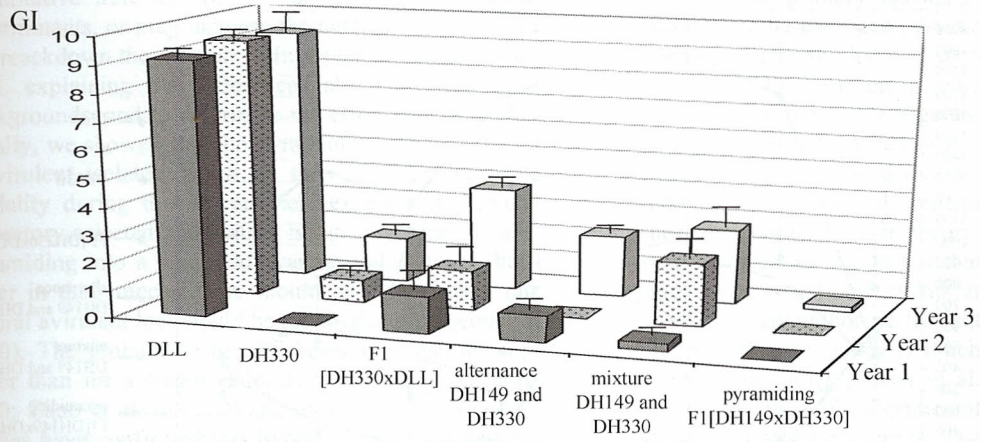

Figure 1: Gall index (GI) on peppers (mean of 40 to 45 replicates ± standard error).

Results on reduction of the soil infection potential ("trap" effect)

In the first year of the experiment, before planting the pepper genotypes, the SIP of the whole plot was moderate to heavy (the mean SIP for 52 microplots reached 546±71 EMs per plant) (Figure 2). A succession of susceptible plants each year (DLL in summer and salads in winter) greatly increased the SIP in corresponding microplots (from 456±140 to 1019±100 EMs in year 3). After 2 months of bare soil, no significant changes in SIP could be observed. Resistant peppers DH330, F1 [DH330xDLL], and mixture DH330 and DH149 did not significantly reduce SIP over three years of experimentation. In contrast, the results highlight the beneficial effects of two management strategies of resistance: the cultivation of hybrids combining two resistance factors and alternating rotation of varieties, each carrying a different resistance. In fact, DH149 doubled SIP (from 412±157 to 823±204) the first year with the developpement of a virulent population. Nevertheless, the rotation with DH330 significantly reduced SIP from 874±218 to 78±76 (91%). F1 [DH149 x DH330] combining *Me1* and *Me3* most strongly reduced SIP the first year (from 596±179 to 2.8±2.8, ie 99.5%), this reduction being almost complete in some microplots, when hairy root peppers were particularly developed through addition of an organic amendment and proper fertirrigation. This "trap plant" effect was maintained over the 3 years. The final level of reduction using this modality was 97.4% of the mean initial rate recorded in the 45 plots. These results are in agreement with those comparing the GI on susceptible salads each year after each pepper modalities (data not shown). After the first cultivation cycle of peppers, salads cultivated after the 5 modalities that include R genotypes showed a significant reduction of GI compared to salads cultivated after the susceptible cultivar DLL (mean GI=0 after pyramiding to 0.9 after DH149 compared to 1.5 after DLL). Alternance and especially pyramiding allowed protecting the salads during the 3 years : GI raised up to 4.3±0.3 in year 3 after DLL, 2±0.3 after alternance DH149, DH330, DH149, and only 0.6±0.1 after F1 [DH149 x DH330] peppers combining *Me1* and *Me3*.

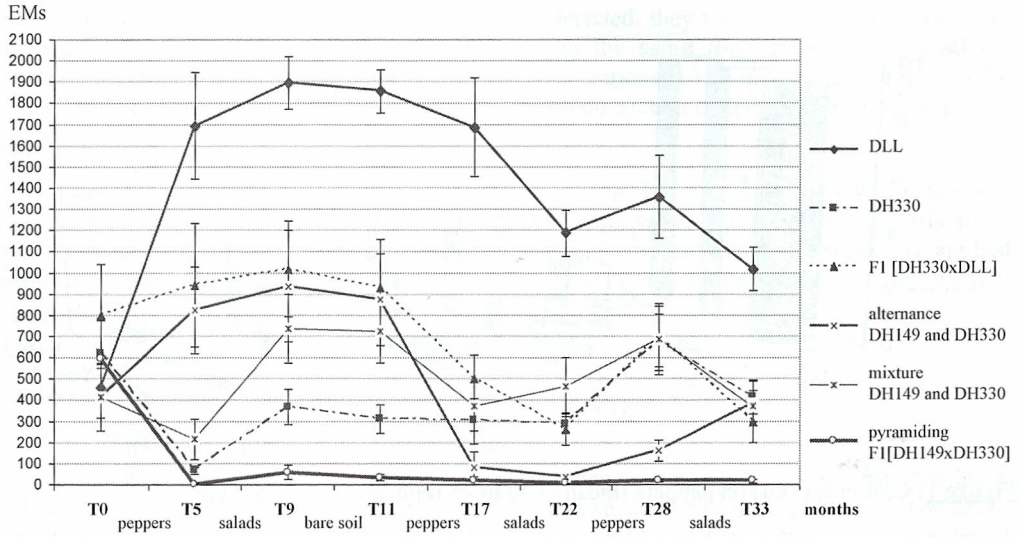

Figure 2: RKN soil infection potential (SIP) corresponding to the number of egg-masses (EMs) on susceptible tomato plants maintained 6 weeks in pot filled with 1 kg-rhizospheric soil sampled from each plot at 15 cm depth (mean of 8 to 9 replicates ± standard error).

Discussion

Our experimental data allow the identification of conditions strengthening the durability of qualitative resistances by lowering the emergence of virulent soil-borne pests and assess the time required for the sustainable improvement of soil health (reduction of parasite populations under their damage threshold) using the *R*-plants as "traps". Since resistance sustainability is influenced by the variation in (a)virulence and host range, we showed that two primary attributes of host resistance for resistance breeding and management are relevant: i) the value of resistance in crop self-protection, based on the level of resistance of the plant to injury caused by infection, and ii) the rotational value of different resistances in cropping systems for protecting subsequent crops, by reducing the selection pressure of each *R*-gene on the pathogens or by decreasing the amount of pathogens in the soil.

At the plant level, we showed, first, that the choice of the *R*-gene is of crucial importance. In fact, even in natural field conditions, one of the RKN *R*-gene (*Me1)* conferred a high level of resistance without being overcome (no virulent population obtained), while another one (*Me3*) was easily overcome and *Me3*-virulent natural isolates were generated by successive re-inoculation on *Me3*-peppers. Nevertheless, we showed that the genetic background in which the major *R*-gene is introgressed is important. In fact, the *Me1* *R*-peppers with fifty percent of susceptible DLL background (F1 [DH330xDLL]) had more EMs compared to DH330. These results are confirmed by another study in which *Me3* and *Me1* were introgressed in either a susceptible or a partially resistant genetic background in either homozygous or heterozygous allelic status (Barbary et al. submitted). Confronting these genotypes to the high inoculation pressure of an avirulent *M. incognita* isolate demonstrated that the genetic background plays indeed an important role, whatever the allelic status (homo- or heterozygous) of the *R*-genes. These results are in agreement with laboratory experiments on other pathosystems such as tomato-*M. incognita* (Williamson and Roberts 2009), cotton-*M. incognita* (Wang et al. 2008), potato-*Globodera pallida* (Fournet et al. 2013), pepper-virus (Palloix et al. 2009), and in field experiments on rapeseed-*Leptosphaeria maculans* interaction (Brun et al. 2010). The authors suggested the presence of additional genes or

quantitative trait loci (QTL) that may have epistatic interactions with the primary resistance determinants, or may increase the number of virulence mutations required in the pathogen genome to breackdown the resistance. In pepper, experiments are now underway to detect and localize such QTL explaining the differences observed between susceptible and partially resistant genetic backgrounds, and to determine the effectiveness of their « protective » role on the major *R*-genes. Finally, we showed that the pyramiding of two different *R*-genes totally suppressed the emergence of virulent isolates, based on their complementary mode of action and was the more durable modality during the 3-years-field experiment. Moreover, this modality also controlled virulent laboratory selected and natural isolates overcoming one of both genes (data not shown). In theory, pyramiding into a single cultivar several *R*-genes that have the same spectrum of action but that differ in their mechanisms should provide a more durable resistance since mutational events at several avirulent loci would be required simultaneously to produce a new virulent pathotype (Mundt 1990). The probability of simultaneous mutations for virulence to two effective genes is much lower than for a single gene, as suggested by several simulation modeling studies (Porter et al. 2000; Zhao et al. 2003; Wang et al. 2007). However, a rather limited number of experimental studies have confirmed this hypothesis in plant pathosystems, especially for vegetable crops. The reason probably being that genotyping the pyramiding population needs reliable molecular markers that are not always at hand. The availability of molecular markers closely linked to each of the *Me* *R*-genes (Djian-Caporalino et al. 2007; Fazari et al. 2012) makes the identification of digenic genotypes possible and will help breeders to construct novel resistant pyramid genotypes.

At the field and rotation level, we further demonstrated that alternating different *R*-genes in rotation is efficient to reduce the selection pressure of the *R*-genes on the pathogens and to decrease virulent populations in fields (Figure 2). Previous studies (Castagnone-Sereno et al. 2001; Djian-Caporalino et al. 2011) showed indeed that neither natural nor selected *Me3*-virulent RKN isolates were able to reproduce on *Me1*-peppers. Such strict specificity of virulence could explain that, once virulent isolates are selected on a determined *R*-gene, alternance in the rotation with a different gene reduces the number of nematodes in the soil under their damage threshold, improving soil health. This finding offers the possibility of 'recycling' broken resistance genes in successive cycles of cultivation. We did not observed a significant protection of *Me3* *R*-lines by *Me1* *R*-lines when sown together in the same plot, except the first year, when the roots were highly developed due to organic matter (Fig. 2). Soil borne pathogens, including RKNs, have limited dispersion ability. So, mixture of *R*-lines could only be effective if the roots are intertwined. In this case, it can minimize the probability of resistance breakdown by decreasing the amount of pathogens in the soil. Implementing a root growth stimulation when using *R*-plants could increase the "trap plant" effect and thus decrease the amount of pathogens in the soil. Finally, we showed that the pyramiding of two different *R*-genes appears very promising as RKN "traps" plants, reducing up to 90% the infestation rate of the soil. When pyramiding remains difficult, the other *R*-genes deployment strategy - alternating – may benefit yields in the long-term by increasing the durability of the qualitative resistances.

To decrease the amount of pathogens and increase the durability of *R*-genes, the combination of *R*-plants and cropping techniques should also be tested. It is currently underway at INRA on RKN in protected vegetable cropping systems with financial support from the European Commission and from the French Ministry of Agriculture, Food and Fisheries (Gedunem project, launched in the framework of the INRA metaprogramme SMaCH – Sustainable Management of Crop Health). Results are expected to suggest rules for breeders and farmers for the sustainable management of disease resistance, re-engineering the agroecosystem to increase overall host diversity, at the species level as well as at the gene level, to reduce directional selection and present an evolutionary dilemma to the pathogen.

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