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Broad-based root-knot nematode resistance identified in cowpea gene-pool two

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Abstract

Cowpea (*Vigna unguiculata* L. Walp) is an affordable source of protein and strategic legume crop for food security in Africa and other developing regions; however, damage from infection by root-knot nematodes (RKN) suppresses cowpea yield. The deployment through breeding of resistance gene *Rk* in cowpea cultivars has provided protection to cowpea growers worldwide for many years. However, occurrence of more aggressive nematode isolates threatens the effectiveness of this monogenic resistance. A cowpea germplasm collection of 48 genotypes representing the cowpea gene-pool from Eastern and Southern Africa (cowpea has two major pools of genetic resources – Western Africa and Eastern/Southern Africa) was screened in replicated experiments under field, greenhouse and controlled-growth conditions to identify resistance to RKN, to determine the spectrum of resistance to RKN, the relative virulence (Vl) among RKN species and isolates, and the relationship between root-galling (RG) and egg-mass production (EM). Analysis of variance of data for RG and EM per root system identified seven genotypes with broad-based resistance to *Meloidogyne javanica* (Mj), avirulent (Avr-Mi), and virulent (Mi) *M. incognita* isolates. Two of the 48 genotypes exhibited specific resistance to both Mi isolates. Most of the genotypes were resistant to Avr-Mi indicating predominance of *Rk* gene in the collection. Based on RG data, both Mj (Vl = 50%) and Mi (Vl = 42%) were fourfold more virulent than Avr-Mi (Vl = 12%). Resistant genotypes had more effective resistance than the *Rk*-based resistance in cowpea genotype CB46 against Mj and Mi. Root-galling was correlated across isolates (Avr-Mi/Mj: $r = 0.72$; Mi/Mj: $r = 0.98$), and RG was correlated with EM ($r = 0.60$), indicating resistance to RG and EM is under control by the same genetic factors. These new sources of resistance identified in cowpea gene-pool two provide valuable target traits for breeders to improve cowpea production on RKN-infested fields.

Key words

Broad-based resistance, Cowpea, Cowpea gene-pool *two, Meloidogyne* spp., Root-knot nematode, *Vigna unguiculata.*

Cowpea (*Vigna unguiculata* (L). Walp.) is one of the most widely grown crops in the world (Ehlers and Hall, 1997, FAOSTAT, 2013) and the most popular legume crop in sub-Saharan Africa (Lambot, 2002) due to its agronomic versatility (Ehlers and Hall, 1997; Lambot, 2002) and its nutritional (Akyeampong, 1986; Rowland, 1993; Ehlers and Hall, 1997; Quin, 1997; Lambot, 2002; Singh et al., 2002; Hall, 2012) and economic values (Quin, 1997; Singh et al., 2002; Speedy, 2003; Hall, 2012). Worldwide, the average cowpea yield is low, at about 25 to 50% of the known yield potential, and particularly in Africa the yield ranges between 300 and 500 kg/ha (Quin, 1997; FAOSTAT, 2013) because the crop is mainly grown under harsh environmental conditions of severe abiotic (drought, high temperature, and low soil fertility) and biotic (pest, diseases,

and parasitic weeds) stresses with very little use of improved crop management strategies (Onwuene and Sinha, 1991; Rowland, 1993).

Root-knot nematode (RKN) species, in particular, *Meloidogyne incognita* and *M. javanica*, are cosmopolitan plant parasites (Taylor and Sasser, 1978; Sasser, 1980), and one of the major cowpea yield suppressors in the semi-arid tropics and subtropics where the crop is grown (Fery et al., 1994). These plant parasites cause serious damage to cowpea root systems and impair crucial physiological (Taylor and Sasser, 1978; Williamson and Hussey, 1996) and biochemical plant functions (Williamson and Hussey, 1996) for growth and yield, including water and nutrient uptake, and partitioning and translocation of photosynthates (Bird and Loveys, 1975; McClure,1977; Taylor and Sasser, 1978). Root-knot nematode management presents challenges in cowpea and other cropping systems for several reasons: RKN species are cosmopolitan (Sasser, 1980); they share common plant host species (Roberts, 1995; Sasser, 1980); their populations are highly dynamic and can shift in virulence (Petrillo and Roberts, 2005; Petrillo et al., 2006); and in some cases the genetic resistance in host plants can be specific to a particular RKN isolate (Swanson and Van Gundy, 1984; Ehlers et al., 2002). These factors threaten the effectiveness and durability of resistance deployed in commercial cowpea production (Roberts et al., 1997).

Resistance to RKN in the most commonly grown commercial cowpea cultivars in the USA, including California Blackeye 46 (CB46) is conferred by a major dominant gene, *Rk* (Roberts et al., 1995, 1996, 1997; Ehlers et al., 2000a, 2002, 2009). Studies have shown that frequent use of gene *Rk* to manage RKN can lead to selection for virulence to *Rk* (Petrillo and Roberts, 2005; Petrillo et al., 2006). In California, for example, *Rk*-virulent and aggressive populations of *M. incognita* and *M. javanica* have been reported (Swanson and Van Gundy, 1984; Roberts et al., 1997). Breakdown of genetic resistance in crops is a common phenomenon. For example, the *Mi*-resistance gene in tomato which confers broad-based resistance to RKN species (Williamson and Hussey, 1996) has been reported to be ineffective against virulent populations where *Mi*-resistant tomatoes are grown frequently (Roberts and Thomason, 1986; Roberts et al., 1990; Kaloshian et al., 1996; Eddaoudi et al., 1997; Noling, 2000; Ornat, et al., 2001; Huang et al., 2004).

In general, RKN management in cowpea cropping systems relies on a narrow genetic base of resistance (Roberts et al., 1997; Fery et al., 1994; Ehlers et al., 2002) derived from cowpea gene-pool one which comprises mostly cowpea genotypes from West Africa (Huynh et al., 2013). An extensive search has found very few additional sources of resistance (Ehlers et al., 2002) in this cowpea gene pool. A few sources of effective resistance to RKN carrying genes *Rk*^{2}, *rk3*, *QRk-vu9.1*, and a root-galling resistance gene have been identified. Their biological activity and function for RKN management, singularly or as gene pyramids are being investigated for their future application in the development of cowpea cultivars with broad-based resistance (Roberts et al., 1996, 1997, 2014; Ehlers et al., 2000a, 2002; Santos et al., 2018). Results from ongoing research and breeding efforts have shown that broad-based resistance based on complex sets of genes, for example, *RkRk/QRk-vu9.1*, *RkRk/QRk-vu9.1/*root-galling gene, and *RkRk/rk3rk3* provide robust and effective resistance against diverse RKN populations (Roberts et al., 1997, 2014; Ehlers et al., 2002, 2009; Santos et al., 2018). However, the biological function of these genes is not yet fully understood. In addition, some of these genes exhibit resistance specificity which limits their effectiveness to particular RKN species; the *Rk* gene is not effective against virulent isolates of *M. incognita* and it confers only moderate resistance to aggressive isolates of *M. javanica* (Roberts et al., 1997).

Although host plant resistance is considered the most effective strategy for RKN management on cowpea (Ehlers et al., 2002), the dynamic nature of RKN populations and the emergence of virulent pathotypes (Roberts et al., 1997; Petrillo et al., 2006) suggest that additional novel sources of resistance to these pathogens are needed (Fery et al., 1994; Roberts et al., 1996; Ehlers et al., 2000a, 2002). This study was conducted to identify and characterize resistance to root-knot nematodes (*Rk*-avirulent and *Rk*-virulent *M. incognita* isolates and aggressive *M. javanica*) in a cowpea collection of 48 genotypes comprising landraces and accessions representing the cowpea gene-pool two from Southeastern Africa (Huynh et al., 2013) and to compare the putative novel resistance against known resistance phenotypes in controls derived from cowpea gene-pool one. The specific objectives of this study were to: (i) identify resistance to RKN; (ii) determine the spectrum of resistance to RKN and relative virulence among RKN; and (iii) determine the relationship between root-galling and nematode reproduction.

Materials and methods

Plant materials

The test materials were a subset of 48 cowpea genotypes previously selected from a drought tolerance study from a diverse pool of 350 genotypes, which include accessions and landraces from the Mozambique Institute of Agricultural Research (IIAM) and others col-

Table 1. Resistance gene sets in control genotypes and their response status to avirulent (Avr) and virulent *Meloidogyne incognita* and *M. javanica*.

		Root-knot nematode response		
Genotype	***R* gene set**	**Avr. *M. incognita***	***M. incognita***	***M. javanica***
UCR779	None	Susceptible	Susceptible	Susceptible
CB46-Null	None	Susceptible	Susceptible	Susceptible
CB46	*RkRk*	Resistant	Susceptible	Susceptible
CB27	*RkRk/rk3rk3*	Resistant	Resistant	Resistant
CB50	*RkRk*	Resistant	M. Resistant	M. Resistant
NIL-2 genes	*RkRk/QRk-vu9.1*	Resistant	M. Resistant	Resistant
NIL-3 genes	*RkRk/QRk-vu9.1/*[a]	Resistant	Resistant	Resistant
CB3-gg	*Root-galling gene*[a]	Resistant	Resistant	Susceptible

[a]Root-galling resistance gene. M = moderately. The responses were based on root-galling indices.

lected across Mozambique representing the cowpea gene-pool two (Huynh et al., 2013). These cowpea genotypes display very distinct agronomic and morphological traits including seed size, shape and color, stem pigmentation, stem diameter, leaf shape and size, plant architecture, growth habit, biological cycle, yield ability, and drought tolerance. The cowpea genotypes used in all experiments as controls and their responses to *M. incognita* and *M. javanica* are given in Table 1.

The control genotypes CB46-Null, NIL-2 genes and NIL-3 genes are near-isogenic lines (NIL) developed in a California blackeye cultivar CB46 background through multiple backcrosses (Roberts et al., 2014; Huynh et al., 2016). The breeding line CB46-Null is susceptible whereas NIL-2 genes and NIL-3 genes are resistant lines. The genotype CB3-gg is a NIL developed in a CB46 background through successive backcrosses, and it carries a root-galling resistance gene derived from California blackeye cv. CB3 (Roberts et al., 2014). The cultivar CB50 also has CB46 background in its pedigree (Ehlers et al., 2009). California blackeye cultivar CB27 is resistant; in addition to the gene *Rk*, it carries a recessive gene with additive effect (Ehlers et al., 2000a, 2000b). The genotype UCR779 is a cowpea accession originally from Botswana; it lacks resistance to all tested RKN.

Nematode isolates

Three *Meloidogyne incognita* and one *M. javanica* (same isolate used in all experimental conditions) isolates were used in this study. Two of the *M. incognita* isolates ("Project 77" and "Beltran") were avirulent to cowpea genotypes carrying resistance gene *Rk* (Roberts et al., 1995), and an incompatible interaction between these isolates and a genotype carrying *Rk*, or any genetic resistance factor equivalent to this gene would be expected. The term avirulent is used to indicate nematode populations that reproduce poorly on plants on which virulent nematode populations of the same species reproduce significantly and induce substantial root-galling (Roberts et al., 1995b). The third *M. incognita* isolate, "Muller," is highly virulent to cowpea genotypes carrying gene *Rk*, inducing excessive root-galling and reproducing successfully (Roberts et al., 1995), but resistance based on *Rk* plus other genes in combination (Table 1) provides effective resistance against this isolate (Roberts et al., 2014). The "Muller" isolate was obtained from a cowpea field in which selection for virulence to gene *Rk* had occurred following repeated planting of cowpeas with *Rk*-based resistance (Petrillo and Roberts, 2006). The *M. javanica* isolate "Project 811" is aggressive and able to induce root-galling and to reproduce significantly on plants carrying gene *Rk* at a level of 50% or more of that observed on susceptible plants, although this ability is not based on selection for *Rk*-virulence (Roberts et al., 1995; Ehlers et al., 2002). The term "aggressive" refers to the enhanced ability of this *M. javanica* isolate to cause damage to cowpea plants carrying the *Rk* gene (Roberts et al., 1995).

Root-galling assays

Experiments to determine the root-galling response of the test genotypes were conducted in infested field sites and field data were validated in greenhouse pot

experiments. Four field experiments were conducted during June – October of 2012, 2014, 2015, and 2016 to determine the response of the test genotypes to root-galling by *M. javanica*, avirulent, and virulent *M. incognita* isolates in separate field sites infested with each nematode isolate. These sites were established several years ago by injecting nematode eggs extracted from greenhouse-grown tomato plants into the root-zone of susceptible tomato plants, and are maintained by planting susceptible tomato plants to provide high and uniform nematode infestation levels (Huynh et al., 2016). In the 2012 field experiment (conducted at South Coast Research and Extension Center – SCREC), 24 of the 48 genotypes were tested for root-galling by *M. javanica*, avirulent, and virulent *M. incognita* separately in a completely randomized design on each nematode site. In 2014 (at SCREC), all test genotypes were screened in four replicate blocks in a randomized complete block design on each nematode site. In the 2015 and 2016 experiments (at Kearney Agriculture Research and Extension Center – KARE, and SCREC, respectively), only the highly resistant genotypes identified in the 2012 and 2014 experiments plus all controls were tested in each nematode site using the same experimental design as in 2014, to validate the response of these potential RKN resistance donors. The genotype Gile-K-Local was not included in this experiment due to seed shortage. In all field experiments, each replicate plot consisted of 20 to 25 seeds per genotype planted in a 1.5 m-long single-row, and water and fertilizer were supplied as needed through drip-irrigation tape. Sixty-days after emergence, plant tops were cut 2 to 3 cm above the soil line and all root systems (except for the 2012 experiment where plant stand per genotype ranged from 10 to 24) dug and evaluated for root-galling response. The genotype response was assessed as the average of all plants in each plot. The levels of nematode infestation in the field were indicated by root-galling index of susceptible controls CB46-Null and UCR779; these controls in all field experiments were planted at every fifth plot planted to test cowpea genotypes.

Under greenhouse conditions (28°C day and 22°C night temperatures), the entire cowpea collection including all controls was tested for root-galling induced by *M. javanica* in two separate experiments each with four replicates arranged in a randomized complete block design. Two seeds of each genotype were planted in fiber pots (15-cm-diameter), containing a soil mixture of 80% sand and 20% peat, and thinned to one plant per pot 7 d after emergence. Water and fertilizer were provided as needed using a drip-irrigation system. Fifteen-days after emergence, a 10 ml egg suspension in water containing about 1,000 egg/ml were pipetted per pot in four 3 cm-deep holes around the plant stem. The eggs for these experiments were extracted from roots of susceptible tomato plants maintained in a greenhouse at UC-Riverside. Sixty-days after inoculation, plant tops were cut 2 to 3 cm above the soil line and the roots washed and indexed for root-galling response under 10X magnification following the protocol described next. The greenhouse experiments focused mainly on evaluating the response of the cowpea collection to *M. javanica* because there was a need to identify a highly effective source of resistance to this nematode isolate for breeding purposes and to validate the results from field experiments with *M. javanica*. However, a subset of six *M. javanica* resistant genotypes (including all controls) identified in the 2012 and 2014 field experiments plus a genotype identified with resistance to virulent *M. incognita* in the 2014 experiment were also evaluated for egg production per root system and root-galling by virulent *M. incognita* in a separate greenhouse experiment. This was a single experiment with four replications; the genotypes were inoculated with virulent *M. incognita* isolate "Muller" (following the protocol described above) obtained from greenhouse cultures mantained on tomato plants.

Root-galling assessment followed a 0 to 9 index (GI) modified from Bridge and Page (1980), where 0 = no galls on root system; 1 = very few, small galls and difficult to see; 2 = very few and small galls can be seen; 3 = galls can be easily seen on most roots except the main root, the size varies from very small to small; 4 = root system is obviously galled, some large galls can be seen on secondary roots and very few bumps can be seen on the main root; 5 = generally large galls can be seen on the root system and the main root is slightly galled with galls of different sizes; 6 = large galls, main root heavily galled; 7 = large galls and large coalesced galls on the main and secondary roots, respectively; 8 = generally huge galls and huge coalesced galls on secondary and main roots, respectively, very few secondary roots can be seen; 9 = huge galls and coalesced galls, generally no secondary roots visible. A cut-off between resistant-susceptible genotypes for root-galling response was set at GI = 3; this threshold was established based on the average root-galling on resistant and susceptible controls, and the average root-galling of all tested genotypes; thus, genotypes with GI $\leq$ 3 were considered resistant, and those with GI > 3 were considered susceptible (modified from Fery et al., 1994; Roberts et al., 2008). In cowpea, the size, location, and the relative number of galls allows identification of resistant from susceptible reactions to RKN. Also, cowpea resistant genotypes do not show galls on main roots, so this phenotypic response has been used to easily distinguish resistant from susceptible plants.

Nematode reproduction assays

Egg-mass production (EM) by *M. javanica* "Project 811" and avirulent *M. incognita* "Project 77" was assessed in the test cowpea collection using seedling growth-pouch tests (Ehlers et al., 2000a; Atamian et al., 2012) in a growth chamber with day and night temperatures set at 28 and 22°C, respectively, under 16 hr day-length. A single seed of each genotype was planted in a plastic pouch, and pouches were minimally watered using a wash bottle to allow seed germination and seedling emergence. After seedling emergence pouches were watered as needed. Second-stage juveniles (J_2) were hatched from nematode eggs placed in an incubator at 26 to 27°C for 7 d. Every 2 to 3 d emerged (J_2) were collected, counted and concentrated to the desired inoculum density. Approximately, 12 to 14 d days after emergence, the plants were inoculated with freshly hatched (J_2) at a density of 1,500 J_2/plant and laid on a table horizontally for 24 hr in the dark. After inoculation, the plants were fertilized for 3 to 5 d with half-strength Hoagland's solution (Hoagland and Arnon, 1950) and additional fertilizer was applied as needed for the remainder of the experiment. At 30 to 35 d after inoculation the roots were infused with erioglaucine solution (1 g/l) (Sigma Chemical Co., St. Louis, MO, USA), and 24 hr later the solution was poured off and the stained egg-masses counted under 10X magnification. The distinction between resistant and susceptible reactions to nematode reproduction measured by egg-masses per root system (EM), was determined using EM = 30, which was based on the average EM production on resistant and susceptible controls, and the average EM production in the entire collection (modified from Roberts et al., 1996, 2008). Genotypes showing ≤30 EM per root system were classified as resistant, and genotypes with EM count >30 were classified as susceptible. This assay was conducted to determine the response of the test cowpea genotypes to nematode reproduction, and to determine the relationship between root-galling and nematode reproduction responses (to infer on the relationship between the genetic determinants controlling both phenotypes). Five separate experiments each with four replicates were conducted. In the first three experiments, the entire cowpea collection including controls was screened for nematode reproduction by each nematode isolate, whereas in the additional two experiments only resistant genotypes plus controls were tested to validate their response. These experiments focused mainly on *M. javanica* and avirulent *M. incognita* due to the reason stated previously.

Relationship between root-galling and nematode reproduction

Data for root-galling and egg-mass production by *M. javanica* from greenhouse and seedling growth-pouch experiments were correlated to infer whether both traits are under control by the same or distinct genetic factors.

Nematode virulence and resistance spectrum

Nematode virulence herein is defined as the ability of a nematode to successfully establish a feeding site, induce root-galling and reproduce on roots of resistant plants (Roberts et al., 1997; Petrillo et al., 2006). Nematode virulence was determined using virulence index (VI) estimates for each nematode isolate, calculated as the proportion between galling or reproduction on the root systems of resistant genotypes and susceptible genotypes (Petrillo et al., 2006). The spectrum of resistance of tested cowpea genotypes was defined as the relative response of tested genotypes to each RKN isolate. In addition, the relationships between root-galling data for *M. javanica*, avirulent *M. incognita*, and virulent *M. incognita* were analyzed for correlation to infer on the relationship among the resistances underlying response to root-galling by these nematodes using root-galling data from the 2014 field experiment. Virulence indices were computed using root-galling data from the 2014 field experiment and egg-mass data from growth chamber experiments.

Data analysis

Raw data for root-galling and nematode egg-mass reproduction from all experiments were analyzed by ANOVA. Data analysis comprising separate testing within a season to more than one nematode isolate was performed following the procedure for analysis of series of experiments described by Gomez and Gomez (1984), where the nematode isolates were considered as environments. In the first step the data were analyzed separately for each nematode isolate, and in the second step combined ANOVA was performed to test the genotype × environment effect. For separate greenhouse experiments with *M. javanica* and virulent *M. incognita*, one-way ANOVA was performed using the average genotypic responses of two separate experiments within each replication and genotypic responses from a single experiment in four replications, respectively. For all experiments (field, greenhouse and growth chamber), the data analysis was performed using SAS University Edition 3.2.2 following the mixed procedure (Proc

Mixed) where the blocks were considered as the random factor while nematode isolates and cowpea genotypes were considered as fixed factors. The 2012 field experiment followed the same procedure as the other experiments; 10 to 24 plants of each genotype were evaluated for root-galling under each nematode isolate infestation. This experiment was arranged in a complete randomized design (CRD). The 2014, 2015, 2016, and greenhouse experiments were arranged in a randomized complete block design (RCBD) with four replicate blocks. The growth chamber experiments were conducted five times (three with all test genotypes and two with resistant genotypes), and each was arranged in a RCBD with four replicate blocks. Mean separation in all experiments was performed using Tukey's multiple comparison test at $p < 0.05$. The relationship between *M. javanica* root-galling (greenhouse data) and egg-mass production responses was determined through Pearson correlation following procedure Corr, and procedure Reg was used to fit a linear model using SAS University Edition 3.2.2. Also, the relationships between root-galling by *M. javanica*, avirulent *M. incognita*, and virulent *M. incognita* were analyzed for correlation following the same procedures. However, the relationship between root-galling by *M. javanica* and avirulent *M. incognita* was best fit using a logarithmic model.

Results

Root-galling responses in the field

In a preliminary study conducted at the UC-SCREC in 2012, a subset of 24 of the 48 cowpea genotypes plus controls were screened for root-galling response on sites infested with avirulent *M. incognita*, virulent *M. incognita* or aggressive *M. javanica* (Fig. 1). A total of 16 of the 24 test genotypes and controls CB46, CB27, NIL-2 and NIL-3 genes, and CB3-gg were resistant (GI ≤ 3) to avirulent *M. incognita* compared to susceptible control CB46-Null (Fig. 1). Three test genotypes (VAR-3A, FN-2-9-04, and Namuesse-D) were resistant (G ≤ 3) to virulent *M. incognita*, as were controls NIL-3 genes, CB3-gg, and CB27 (Fig. 1), while control genotypes CB46, CB50, and CB46-Null were susceptible.

The control NIL-2 genes showed moderate resistance (GI = 3.8) response to virulent *M. incognita* (Fig. 1). Of the 24 test genotypes, four (VAR-3A, FN-2-9-04, Namuesse-D, and FAEF-14-INE) were resistant (G ≤ 3) to aggressive *M. javanica*, while controls CB27, NIL-2, and NIL-3 genes were moderately resistant (GI = 3.3, 3.7, and 4.0, respectively), and CB46, CB50, CB3-gg, and CB46-Null were susceptible (GI = 5.0, 5.0, 6.6, and 6.4, respectively). The average root-galling scores of the genotypes ranged from 1.3 to 6.1, 1.9 to 5.5, and 1.3 to 6.9 under infection by avirulent *M. incognita*, virulent *M. incognita* and *M. javanica*, respectively.

In 2014, the full set of 48 test genotypes was screened for root-galling response on the same infested field sites at SCREC (Fig. 2). There was significant effect by genotypes, nematode isolates and their interaction for root-gall indices ($p < 0.0001$). Significant differences among genotypes for mean root-galling induced by each nematode isolate were detected at GI = 1.4. All test genotypes except one (FN-2-11-04, GI = 3.9) were resistant to root-galling induced by avirulent *M. incognita* (G ≤ 3), and all controls, except those lacking resistance genes (CB-46-Null and UCR779, GI = 5.2 and 6.0, respectively), were resistant to avirulent *M. incognita* (Fig. 2). The average root-galling phenotypes induced by avirulent *M. incognita* infestation ranged from 0 to 6, and most of the differences in root-galling response among test genotypes were not significant ($p > 0.05$).

Several of the test genotypes that were resistant to *M. javanica*, were also resistant to virulent *M. incognita* (Fig. 2), including FN-2-9-04 and VAR-3A. These two genotypes were also resistant to virulent *M. incognita* in the 2012 test (Fig. 1). Of the controls, CB27, NIL-3 genes and CB3-gg were resistant to virulent *M. incognita*, confirming their response in the 2012 test. The control NIL-2 genes were resistant to virulent *M. incognita* in 2014 (Fig. 2), but only moderately resistant in the 2012 test (Fig. 1). Root-galling responses of resistant controls CB27, NIL-3 genes, and CB3-gg were not different ($p > 0.05$) from resistant test genotypes. However, the root-galling phenotype of the resistant control NIL-2 (GI = 2.6) was significantly higher ($p < 0.05$) than that of FN-2-9-04, Gile-K-Local, VAR-3A, CB27, and NIL-3 genes (GI = 0.5, 0.9, 0.6, 0.8, and 1.1, respectively). Genotypes INIA-41, Maputo, and Muinana-Lawe were also resistant to virulent *M. incognita*, but their response to root-galling did not differ ($p > 0.05$) from that of CB3-gg, CB27, NIL-2 genes, NIL-3 genes, and resistant test genotypes FN-2-9-04, Gile-K-Local, VAR-3A, INIA-5A, FAEF-14-INE, Namuesse-D, and VAR-11D. Root-galling phenotypes with virulent *M. incogita* ranged from 0.5 to 5.2 (Fig. 2). Susceptible test genotypes included SP-860, SP-866 and FN-2-11-04. As expected, CB46, UCR779, and CB46-Null were also susceptible.

In the test with *M. javanica*, eight genotypes (VAR-3A, FN-2-9-04, Namuesse-D, INIA-5A, Gile-K-Local, FN-1-14-04, VAR-11D and FAEF-14-INE) were resistant (GI ≤ 3) to root-galling, of which four (VAR-3A, FN-2-9-04, Namuesse-D and FAEF-14-INE) were also resistant in the 2012 field test (Fig. 1). Of the controls, CB27, NIL-2 genes, and NIL-3 genes were also resistant to *M. javanica*, but controls carrying only the *Rk* gene (CB46 and CB50), the root-galling gene (CB3-gg)

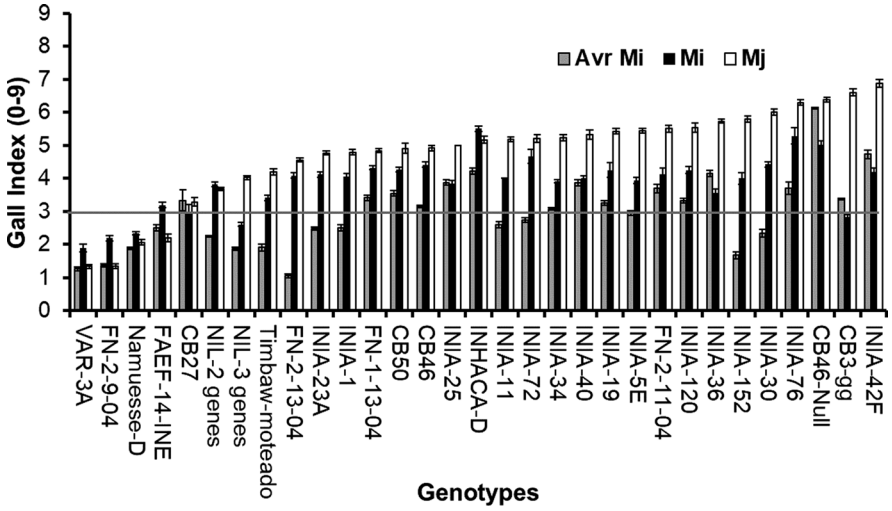

Figure 1: Root-galling response of 24 test plus control genotypes under field infestation (2012) by avirulent (Avr Mi) and virulent *Meloidogyne incognita* (Mi) isolates "Beltran" and "Muller," respectively, and aggressive *M. javanica* (Mj) isolate "Project 811". Control genotypes CB27, NIL-2 and NIL-3 genes, CB50 and CB46 carry gene *Rk*, CB3-gg carries a root-galling resistance gene effective against Avr Mi and Mi, and CB46-Null is susceptible. Horizontal line is GI = 3 representing the cut-off between resistant and susceptible response. Bars indicate the standard error. The data are ranked by response to Mj, Mi, and Avr Mi.

or no gene were susceptible, as expected. The responses of the resistant genotypes FAEF-14-INE, FN-1-14-04, FN-2-9-04, Namuesse-D, and VAR-3A, were similar, and lower ($p < 0.05$) than those of the *Rk*-gene controls. Root-galling phenotypes (ranging from 0.3 to 5.5) induced by aggressive *M. javanica* were in the same range as those induced by virulent *M. incogita*. At KARE, these genotypes were planted in separate fields infested with avirulent *M. incognita* isolate "Project *77*" and *M. javanica*, and at SCREC screening was done on sites infested with avirulent *M. incognita* "Beltran," virulent *M. incognita* and *M. javanica* each. The results from these field experiments were consistent with the 2012 and 2014 experiments (data not shown).

Root-galling responses in greenhouse experiments

The test genotypes were screened for resistance to root-galling by *M. javanica* in pots under greenhouse conditions. Based on the ANOVA, genotypes differed ($p < 0.0001$) in *M. javanica* root-galling response. Mean root-galling index per genotype ranged from 1 to 8 (Fig. 3). Consistent with the results observed in the field (Figs 1, 2), except for FN-1-14-04, genotypes FN-2-9-04, FAEF-14-INE, VAR-3A, Namuesse-D, Gile-K-Local, INIA-5A, and VAR-11D exhibited resistant *M. javanica* root-galling phenotypes. The controls CB27, NIL-2 genes, and NIL-3 genes also showed consistent resistant root-galling phenotypes as in the 2014 field test. The susceptible phenotypes observed for controls CB46, CB3-gg, UCR779, and CB46-Null were also consistent with the results observed in the field experiments under *M. javanica* infestation. Significant differences in root-galling phenotypes were detected at GI = 1.75 ($p < 0.05$).

Root-galling phenotypes observed among the resistant test genotypes (FN-2-9-04, VAR-3A, Namuesse-D, INIA-5A, VAR-3A, FAEF-14-INE, and Gile-K-Local) and controls CB27, NIL-2 genes, and NIL-3 genes were not different, but root-galling phenotypes of the resistant test genotypes were lower ($p < 0.05$) than those of CB46, CB46-Null, CB3-gg, and UCR779, as expected (Fig. 3). The root-galling responses among the resistant test genotypes were not different, nor were root-galling responses between controls CB27, NIL-2 genes, and NIL-3 genes.

The cowpea subset evaluated in greenhouse inoculations with virulent *M. incognita* included genotypes FN-2-9-04, VAR-3A, Namuesse-D, INIA-5A, VAR-3A, FAEF-14-INE, and INIA-41, plus all controls. The genotype Gile-K-Local was not tested due to seed shortage. The results from this experiment (root-galling responses) were not consistent with those observed in the 2012 and 2014 field experiments. Of these genotypes, FN-2-9-04, VAR-11D, and INIA-41 showed moderate resistance to root-galling similar to CB27, CB46, and NIL-2 genes

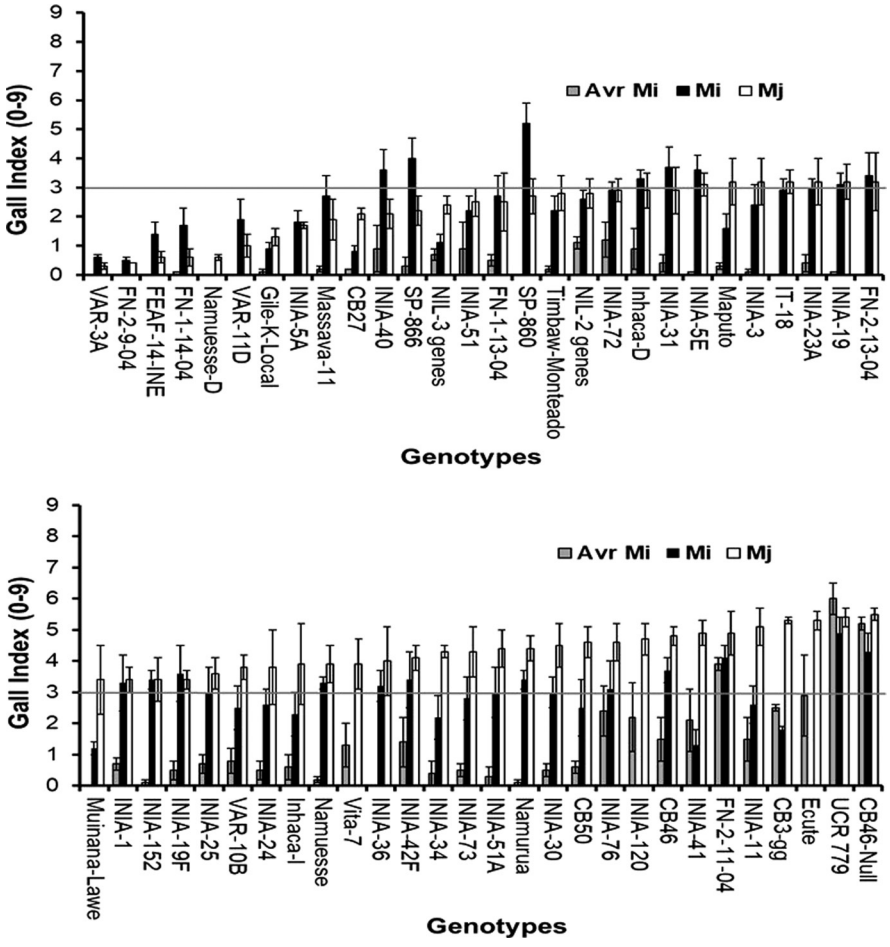

Figure 2: Root-galling response of 48 genotypes following infection by avirulent (Avr Mi) and virulent *Meloidogyne incognita* (Mi) isolates "Beltran" and "Muller," respectively, and by aggressive *M. javanica* (Mj) isolate "Project 811" under field infestation (2014). Control genotypes CB27, NIL-2 and NIL-3 genes, CB50 and CB46 carry gene *Rk*, CB3-gg carries a root-galling resistance gene effective against Avr Mi and Mi, and CB46-Null is susceptible. Horizontal line is GI = 3 representing the cut-off between resistant and susceptible response. Bars indicate the standard error. The data are ranked by response to Mj, Mi, and Avr Mi.

($p > 0.05$) under virulent *M. incognita* greenhouse inoculation (data not shown).

Nematode reproduction responses in growth chamber experiments

The test cowpea genotypes were also evaluated for the ability to suppress reproduction of avirulent *M. incognita* and *M. javanica* assessed by production of egg-masses (EM) per root system in controlled inoculations (Fig. 4). There was significant effect by the nematode isolates, genotypes and their interaction for EM ($p < 0.0001$). The mean EM per root system for avirulent *M. incognita* and aggressive *M. javanica* ranged from 0 to 64 and 1.31 to 105, respectively (Fig. 4). Significant differences in EM production per root system between genotypes were detected at EM = 20.7 and 18.4 ($p < 0.05$) for avirulent *M. incognita* and *M. javanica*, respectively.

Avirulent *M. incognita* reproduced poorly on most test genotypes compared to *M. javanica*, with the exception of FN-2-11-04 and INIA-76 (EM = 47.9 and 47.5, respectively) (Fig. 4). Among the control genotypes, CB3-gg, CB46-Null, and UCR779 were susceptible to avirulent *M. incognita* (EM = 47.1, 56.5, and 64.0, respectively) (Fig. 4). Most of the test genotypes (FN-2-9-04, VAR-3A, Namuesse-D, INIA-5A, VAR-3A, FAEF-14-INE, and Gile-K-Local) had very low EM by avirulent *M. incognita* and *M. javanica*, indicating they were highly resistant, and the EM phenotypes among them were not different ($p > 0.05$). Also, their phenotypes were not different from control geno-

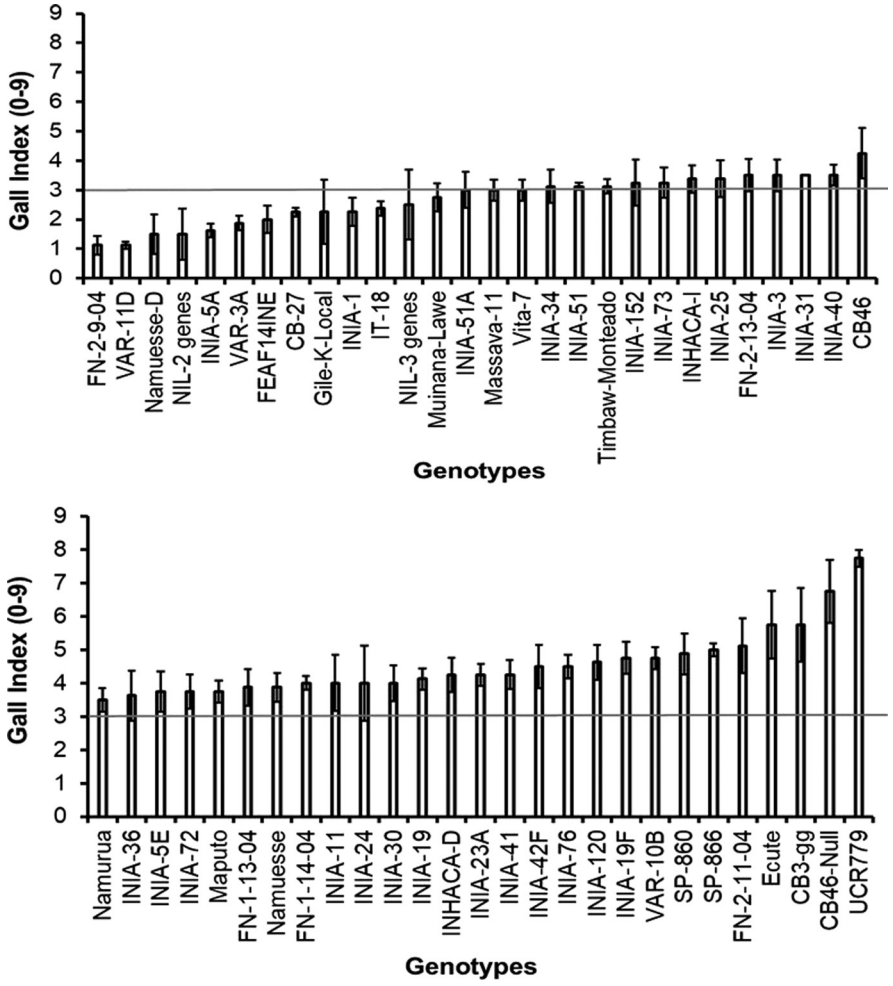

Figure 3: Root-galling response of 48 genotypes to aggressive Meloidogyne javanica isolate "Project 811" in greenhouse inoculations. Control genotypes CB27, NIL-2 and NIL-3 genes, CB50 and CB46 carry gene *Rk*, CB3-gg carries a root-galling resistance gene effective against Avr Mi and Mi, and CB46-Null is susceptible. Horizontal line is GI = 3 representing the cut-off between resistant and susceptible response. Bars indicate the standard error.

types carrying the *Rk* genes (CB46, CB27, CB50, NIL-2 genes, and NIL-3 genes). These control genotypes supported fewer EM than controls lacking *Rk* (CB3-gg, CB46-Null, and UCR779) ($p < 0.05$) (Fig. 4).

Relationship between root-galling and nematode reproduction

The root-galling and egg-mass production responses under infestation by *M. javanica* were moderately correlated ($r = 0.60$, $p < 0.0001$) (Fig. 5), and a small proportion ($R^2 = 0.36$) of the variability in this relationship was explained by the response of the test genotypes to root-galling and egg-mass production. Based on this relationship, three classes of genotype responses were identified (Fig. 5): (i) genotypes with low root-galling and low EM production (GI from 0 to 3; e.g., FN-2-9-04); (ii) genotypes with moderate root-galling and moderate EM production (GI from 3 to 5; e.g., Timbawene-Monteado); and (iii) heavily galled genotypes with high EM production (GI > 5; e.g., INIA-76).

Root-galling phenotypes induced by virulent *M. incognita* and *M. javanica* were highly correlated ($r = 0.98$, $p < 0.0001$) (Fig. 6A), and a large proportion ($R^2 = 0.97$) of the variability in root-galling was explained by the response of the genotypes under infestation by both nematodes.

Root-galling responses to avirulent *M. incognita* and to *M. javanica* also were highly correlated ($r = 0.72$, $p < 0.0001$) (Fig. 6B), and the relationship was largely explained ($R^2 = 0.97$) by the observed variability in root-galling among the test genotypes. In this re-

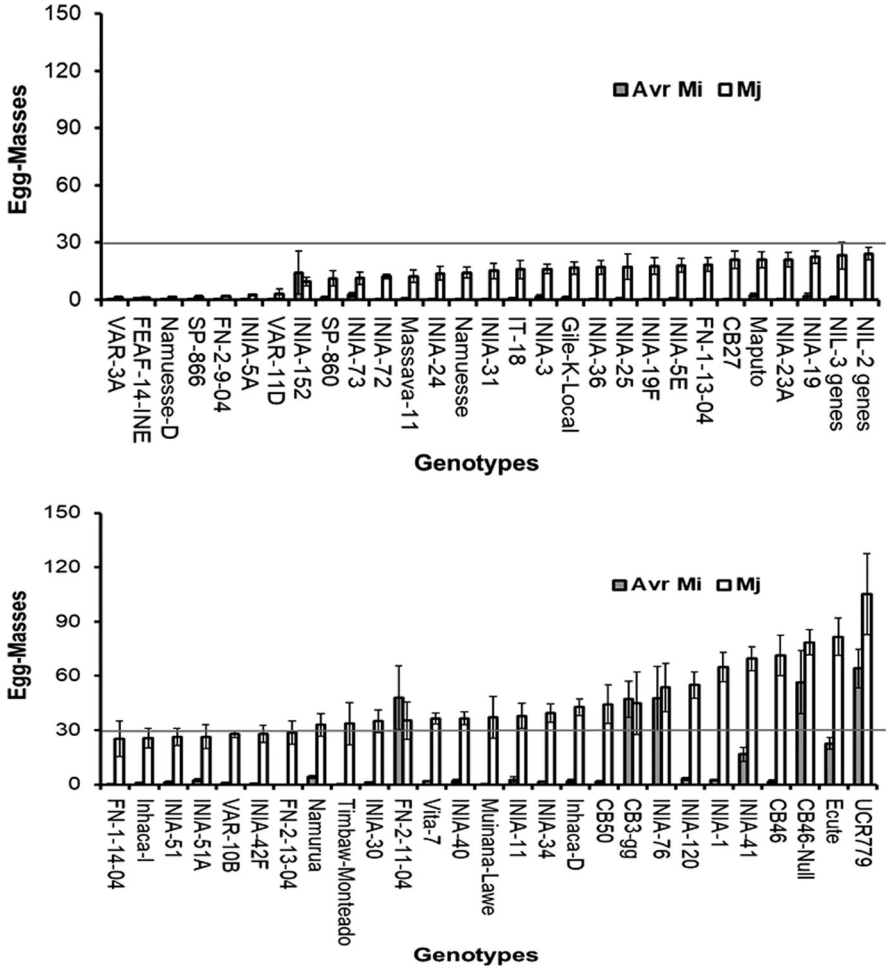

Figure 4: Egg-mass production by avirulent Meloidogyne incognita (Avr Mi) isolate "Project 77" and by aggressive M. javanica "Project 811" on root systems of 48 cowpea genotypes screened using seedling growth-pouches in a growth chamber. Control genotypes CB27, NIL-2 and NIL-3 genes, CB50 and CB46 carry gene *Rk*, CB3-gg carries a root-galling resistance gene effective against Avr Mi and Mi, and CB46-Null is susceptible. Horizontal line is EM = 30 representing the cut-off between resistant and susceptible response. Bars indicate the standard error. The data are ranked by response to Mj and Avr Mi.

lationship, the test genotypes could be classified into three groups: (i) *M. javanica* resistant genotypes (GI = 0 to 3) with low or no noticeable root-galling symptoms under avirulent *M. incognita* infestation; (ii) moderately resistant genotypes that were resistant to root-galling by avirulent *M. incognita* (GI = 3–4); and (iii) genotypes that were susceptible to both RKN isolates.

Nematode virulence and resistance spectrum

Virulence index (VI) for each nematode isolate was estimated as the ratio between RG or EM production of a test genotype and susceptible control lacking RKN resistance. The genotype UCR779 was used as the reference susceptible control to estimate VI values because it had the highest average RG and EM production. Estimates of virulence presented in Figure 7 are expressed based on average RG and EM production per root system of all test genotypes. As expected, the avirulent *M. incognita* isolate was less virulent to the test cowpea genotypes compared to the virulent *M. incognita* and aggressive *M. javanica* isolates (Fig. 7), with VI values based on RG and EM data of 12 and 5%, respectively. Based on RG data, *M. javanica* (VI = 50%) and virulent *M. incognita* (VI = 42%) were about fourfold more virulent than the avirulent *M. incognita* isolate (Fig. 7A). Estimated virulence index for virulent *M. incognita* using field RG data was lower (VI = 42%) than that estimated based on greenhouse data (VI = 75%) (data not

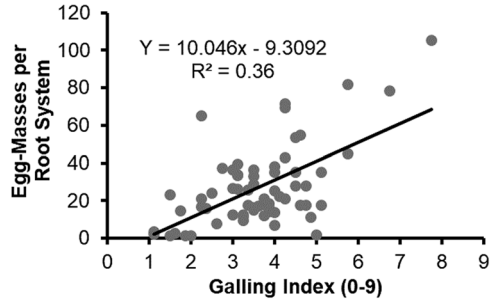

Figure 5: Relationship between root-galling and egg-mass production under Meloidogyne javanica "Project 811" infestation. Correlation established using average root-galling and egg-mass data from two greenhouse and three growth chamber experiments, respectively.

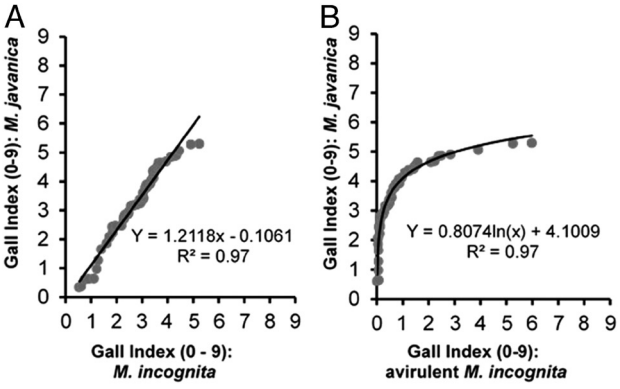

Figure 6: Relationship between root-galling induced by (A): virulent *Meloidogyne incognita* "Muller" and *M. javanica* "Project 811" and (B): avirulent *M. incognita* "Beltran" and *M. javanica* "Project 811", based on average root-galling data from 2014 field experiment.

shown). The RG levels between *M. javanica* and virulent *M. incognita* were not different, but their RG levels were lower ($p < 0.05$) than that of avirulent *M. incognita*.

Using EM data, the average virulence index of aggressive *M. javanica* was 18% compared to 5% for avirulent *M. incognita* (Fig. 7B).

Analysis of the spectrum of resistance to root-galling induced by avirulent and virulent *M. incognita* and aggressive *M. javanica* in field experiments showed that seven test genotypes (FEAF-14-INE, FN-2-9-04, Gile-k-local, INIA-5A, Namuesse-D, VAR-11-D, and VAR-3A) exhibited resistance to all three isolates. In contrast, INIA-41 and Maputo were only resistant to avirulent and virulent *M. incognita*, respectively.

Discussion

The analysis of variability in response to root-galling and egg-mass production induced by *M. incognita* and *M. javanica* in the test cowpea genotypes identified valuable sources of resistance. In particular, genotypes FAEF-14-INE, FN-2-9-04, INIA-5A, Namuesse-D, VAR-11D, Gile-K-local, and VAR-3A exhibited broad-based resistance. Field, greenhouse and seedling growth-pouch screens consistently indicated that these seven genotypes carry genetic resistance to *Rk*-avirulent *M. incognita* and to *M. javanica*. In the greenhouse experiment with *Rk*-virulent *M. incognita*, although only test genotypes FN-2-9-04 and VAR-11D were moderately resistant to root-galling, the test genotypes FAEF-14-INE, FN-2-9-04, INIA-5A, Namuesse-D, VAR-11D, and VAR-3A supported only 23 and 17% of nematode reproduction observed on CB46 (carrying gene *Rk*) and the susceptible near-isogenic line CB46-Null. These results demonstrated that effective resistance to reproduction by this nematode isolate is present in cowpea gene-pool two.

Some inconsistency was observed among the virulent *M. incognita* experiments (field vs greenhouse) possibly due to differences in virulence between the field and greenhouse isolates. Differential virulence between field and greenhouse-maintained populations of

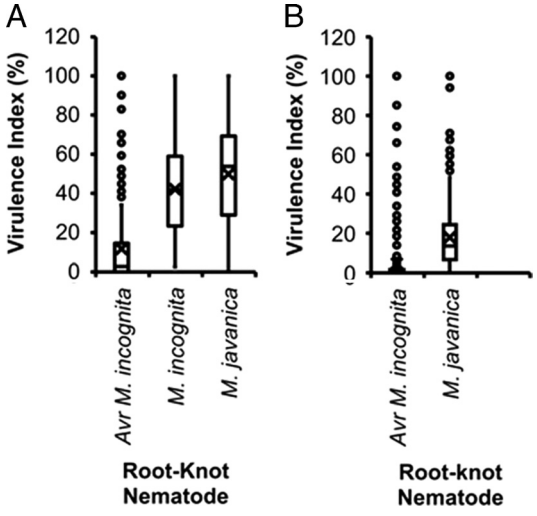

Figure 7: (A): Average virulence index of *Meloidogyne javanica* "Project 811", avirulent (Avr) and virulent *M. incognita* isolates "Beltran" and "Muller," respectively, based on average root-galling data from 2014 field experiment; (B): Average virulence index of *M. javanica* "Project 811" and Avr *M. incognita* "Project 77," based on average egg-mass production in growth chamber experiment.

this isolate was reported by Petrillo et al. (2006) and Petrillo and Roberts (2005). It is likely that the virulence in the greenhouse isolate is genetically fixed compared to the field isolate. A comparative genetic study to elucidate the observed differential virulence between the greenhouse-maintained population of virulent *M. incognita* isolate "Muller" and the equivalent field population used in field experiments would be informative.

Analysis of nematode virulence confirmed that both *Rk*-virulent *M. incognita* and *M. javanica* have greater ability to cause galling damage on cowpea root systems than *Rk*-avirulent *M. incognita,* as expected. Although the average virulence index of virulent *M. incognita* was relatively lower than that of *M. javanica*, both nematode isolates had similar root-galling impact on the test genotypes under field conditions ($p > 0.05$). The strong resistance response of most of the test genotypes to avirulent *M. incognita* indicated that a majority of them carry at least the *Rk* gene or its equivalent. Allelism tests with genotypes carrying the *Rk* gene, for example CB46, will be needed to confirm this hypothesis. Most of the test genotypes were resistant to avirulent *M. incognita* while genotypes FAEF-14-INE, FN-2-9-04, INIA-5A, Namuesse-D, VAR-11D, Gile-K-Local, and VAR-3A showed broad-based resistance to all isolates. The more effective broad-based resistance of these seven genotypes compared to commercial cultivar CB46 (which carries gene *Rk*) indicated that these genotypes probably carry additional resistance factors. The resistance in test genotypes INIA-41 and Maputo appeared to be highly specific to *M. incognita* isolates. The specificity of resistance in INIA-41 to *M. incognita* isolates was further confirmed in the greenhouse assays with virulent *M. incognita*, and this genotype consistently tested resistant on the basis of root-galling and egg-mass production phenotypes.

Resistance specificity and the genetic composition of resistance (single vs additive gene effect) could influence the effectiveness of the novel resistance reported here. In cowpea, the *Rk* gene is highly effective against avirulent *M. incognita* populations but fails to resist virulent *M. incognita* isolates (Roberts et al., 1997). In tomato, resistance specificity to RKN was reported by Roberts and Thomason (1986), where tomato cultivars carrying the *Mi* gene exhibited effective resistance to several *M. incognita* isolates but exhibited differential responses to *M. javanica* populations. The *Rk* gene partially suppresses root-galling by *M. javanica*, which distinguishes phenotypically plants carrying the *Rk* gene from those with no resistance. The broad-based resistance in CB27 is conferred by the combination of gene *Rk* and a recessive resistance gene designated rk^3 which enhances *Rk* resistance to a level that makes the gene combination effective in controlling both *M. javanica* and virulent *M. incognita* (Ehlers et al., 2000a, 2000b; Ehlers et al., 2002). Gene Rk^2 in cowpea provides yet another resistance determinant with broader and stronger resistance expression (Roberts et al., 1996). It is likely that these or additional novel resistance genes regulate or modify resistance to elevated levels in the new sources of resistance identified in this study.

The positive correlation between root-galling and nematode reproduction in the test genotypes indicates that both responses are probably under control by the same resistance determinants. The *R* genes in RKN pathosystems suppress nematode development and nematode reproduction in root systems, and in most cases also limit root-galling. In tomato for example, root-galling response was also found to be associated with nematode reproduction response (Ammati et al., 1985). However, the moderate correlation (r = 0.60, R^2 = 0.36) between root-galling and egg-mass production by *M. javanica* in this study may indicates that root-galling and nematode reproduction responses might be in part under control by at least some independent genetic factors. For example, the control genotype CB3-gg was resistant to root-galling induced by avirulent *M. incognita*, but it was susceptible to reproduction by the same nematode isolate, which indicates that this RKN resistance gene is limited to root-galling response. This may be similar to the observation in lima bean where root-galling and nematode reproduction responses were found to be under independent genetic control (Roberts et al., 2008).

Root-galling induced by *M. javanica* in the test cowpea genotypes was strongly and positively correlated to root-galling induced by both avirulent and virulent *M. incognita*, indicating that broad-spectrum resistance occurs in many of the test genotypes, possibly explained by additive effect of gene sets or by genetic factors that respond to specific nematode isolates. A reported example of broad-based genetic resistance to RKN is found in the cultivar CB27 which carries the *Rk* and rk^3 genes (Ehlers et al., 2000a, 2000b). The *Rk* gene alone does not provide effective resistance to these RKN isolates. Among the test cowpea genotypes, the association between root-galling response under infestation by virulent *M. incognita* and *M. javanica* was not absolute. For example, the resistance in INIA-41 was highly effective to both avirulent and virulent *M. incognita*, but not to *M. javanica*. A similar response was observed in breeding line CB3-gg.

In summary, this study identified novel sources of broad-based genetic resistance in the cowpea genepool two, effective against a range of RKN species and isolates which vary in virulence or aggressiveness on

known sources of resistance in cowpea. Genotypes FAEF-14-INE, FN-2-9-04, INIA-5A, Namuesse-D, VAR-11D, Gile-K-Local, and VAR-3A exhibited consistently high resistance responses to avirulent *M. incognita* and especially *M. javanica*, and their responses to both root-galling and nematode reproduction were effective. The *M. javanica* resistance found in the present study is the most effective compared to that identified previously in other sources. The genetics underlying resistance to RKN isolates in the identified novel sources of resistance and the genetic architecture of resistance in these test genotypes is under investigation, and its uniqueness and its potential value for improvement of RKN resistance in cowpea commercial cultivars is still to be determined. The previously identified RKN resistance in cowpea has come from cowpea gene-pool one. One of the few known effective resistances to RKN is derived from the West African breeding line IT84S-2049, which is a small seed-size cowpea (from cowpea gene-pool one) (Roberts et al., 1996); however, introgression of this resistance into California blackeye cultivar CB46 was affected by a linkage drag effect on seed size which required a series of backcross cycles to rescue the rescue seed size of the recurrent cultivar CB46. In contrast, relatively large seed size of resistant cowpea FN-2-9-04 would allow transfer of this RKN resistance into elite cultivars without negative effects on seed size. The presence of RKN resistance in cowpea gene-pool two offers cowpea breeders opportunities to develop RKN resistant cultivars which also have other desirable traits emanating from gene-pool two.

Acknowledgments

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