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Genetics of Resistance to Root-Knot Nematode and Fusarium Wilt in Cowpea Germplasm From Mozambique

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ABSTRACT OF THE DISSERTATION

Genetics of Resistance to Root-Knot Nematode and Fusarium Wilt in Cowpea Germplasm From Mozambique

by

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Cowpea is a multi-purpose leguminous crop, and its importance as a resource to address food security issues plus production constraints imposed by biotic and abiotic stresses has attracted significant research. Aligned with these efforts, this dissertation describes the resistance found among 53 cowpea genotypes from Mozambique, to root-knot nematodes (RKN) (*Meloidogyne incognita* and *M. javanica*) and Fusarium wilt (FW) [*Fusarium oxysporum* f. sp. *tracheiphilum* (Fot races 3 and 4)]. In the first chapter, an overview is provided about the significance of cowpea as a food security resource, constraints limiting production and research progress and status of cowpea production in Mozambique. Also, available genetic and genomic resources and their utility for cowpea breeding are described. In addition, the concept of plant resistance, types of resistance, mechanisms of plant resistance, disease quantification to RKN and FW, the genetic control of some cowpea diseases and practical example of successful cowpea breeding for diseases is discussed. The second chapter, describes a series of experiments that led to the discovery of seven

cowpea genotypes with broad-based resistance to RKN using nematode reproduction and root-galling phenotypes. The effectiveness of resistance in FN-2-9-04 relative to virulence levels in RKN isolates and the relationship between resistances to different RKN isolates is described. In the third chapter, the genomic architecture of resistance to RKN in FN-2-9-04 is determined through a series of genetic analyses and quantitative trait locus (QTL) mapping. Two QTLs on chromosomes (Vu) 1 and 4 were associated with the strong RKN resistance in FN-2-9-04. The fourth chapter describes the resistance found among the test cowpeas to Fot3 and Fot4 based on wilting and vascular discoloration phenotypes. The virulence profiles of Fot3 and Fot4 are compared, and the effectiveness of FW resistance in FN-2-9-04 and the relationship between wilting and vascular discoloration responses are discussed. In the fifth chapter, the genomic architecture of resistance to Fot4 in FN-2-9-04, determined through a series genetic analyses and QTL mapping, is described. Two QTL on Vu03 and Vu08 were associated with Fot4 resistance in FN-2-9-04. These novel sources of nematode and Fusarium resistance are important for cowpea genetic improvement.

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CHAPTER I - General Introduction

Cowpea – A Versatile Legume Crop

Cowpea (*Vigna unguiculata* (L). Walp.) is a worldwide cultivated legume crop (Quin, 1997; Ehlers & Hall, 1997, Coulibaly and Lowenberg-DeBoer, 2002; FAOSTAT, 2013), and its wide agroecological adaptability, resistance to abiotic and biotic stresses (Ehlers & Hall, 1997; Lombat, 2002), and its multipurpose use as food and fodder make it the most popular legume crop in Africa (Quin, 1997; Rowland, 1993; Ehlers & Hall, 1997; Singh *et al.*, 2002; Lombat, 2002; Hall, 2012; Singh, 2014). The considerable high drought tolerance of cowpea allows it to thrive in marginal agro-ecological conditions prone to drought, especially in the semi-arid and arid tropics and subtropics (Thiaw et al., 1993; Quin, 1997; Ehlers and Hall, 1997; Mortimore et al. 1997; National Research Council, 2006; Singh, 2014) where other legume crops such as common bean and groundnut are less resilient (Thiaw et al., 1993; Ehlers and Hall, 1997; Singh et al., 1997; National Research Council, 2006; Lambot, 2002). Cowpea provides good fodder for livestock (Mortimore et al. 1997; Ehlers and Hall, 1997; Coulibaly and Lowenberg-DeBoer, 2002; National Research Council, 2006) and can be used to replenish soil fertility via nitrogen fixation through its symbiotic association with the *Bradyrhizhobium* spp. bacteria (Quin, 1997; Mortimore et al. 1997; Akyeampong, 1986, Singh *et al.*, 2002; Singh, 2014). This association contributes up to 40 – 80 kg nitrogen/ha to the soil (Awonaike et al., 1990; Quin, 1997; Fening and Danso, 2002; National Research Council, 2006) and in many cropping systems particularly in Africa, cowpea is grown in rotation with or intercropped with cereals which takes advantage of the residual

nitrogen in the soil fixed by cowpea (Quin, 1997; Hall, 2012). Cowpea as a cover crop, especially semi-determinate and indeterminate cowpea types, has a suppressive effect (Quin, 1997; Wang & McSorley, 2004; Roberts *et al*., 2005; Harrison *et al.*, 2006; Hall, 2012) against some plant parasitic nematodes (Ehlers and Hall, 1997; Wang & McSorley, 2004; Roberts *et al*., 2005; Harrison *et al.*, 2006; Hall, 2012), plant pathogenic fungi (Hall, 2012) and weeds (Quin, 1997; Harrison *et al.*, 2006; Hall, 2012), thereby reducing their impact on the current or subsequent crop in the cropping system.

Cowpea – A Strategic Crop for Food Security and Income

In Africa, cowpea is mainly grown as a subsistence crop; however, its potential provides a good opportunity for rural households to improve their incomes because fresh leaves, fresh pods, dry grain, derived processed foods and haulms can be traded for cash (Quin, 1997; Singh *et al*., 2002). Cowpea grains are rich in minerals and have high-quality digestible protein content (Coulibaly and Lowenberg-DeBoer, 2002; Quin, 1997; Lombat, 2002; National Research Council, 2006) of about 20-30% (Quin, 1997; Quin, 1997; Lombat, 2002; National Research Council, 2006; Singh, 2014), which makes this crop an inexpensive source of protein, in particular to resource-poor households (Quin, 1997; Speedy, 2003; National Research Council, 2006; Hall, 2012), who cannot afford beef, fish, poultry or other sources of animal protein (Speedy, 2003; Hall, 2012). In addition, cowpea grains are often less expensive than other leguminous crops, such as common bean and groundnut, making it a strategic resource to address food insecurity issues in many African countries by

complementing starchy foods such as cassava, yam, maize, rice, plantain, millet and sorghum (Singh *et al*., 2002; National Research Council, 2006). Unlike other common legumes, cowpea leaves, either fresh or dried, are used for consumption which adds value to the crop (National Research Council, 2006). The leaves are mainly harvested from indeterminate cowpea types and provide food earlier in the season before grain is mature; thereby, extending the period of food availability. Although leaf consumption is not a generalized habit in all cowpea growing areas (Ehlers and Hall, 1997; National Research Council, 2006) due to regional cultural preferences and production factors, in many parts of eastern Africa fresh leaves are sold to generate income. Generally, in subsistence cowpea production systems dry grain is seldom traded due to lack of production surplus, with harvested grain dedicated entirely for household consumption. Enhanced cowpea productivity and production could allow growers to sell surplus dry grain and leaves to contribute to food security and income generation.

Cowpea Yield Advance and Constraints

Worldwide cowpea is cultivated on about 12.5 million hectares with an estimated global average yield of about 1.4 ton/ha (FAOSTAT, 2013) which is about 25-50% of the known yield potential (Quin, 1997). In Africa cowpea is grown under conditions of significant abiotic and biotic stresses, including drought, insect pests and diseases, plant parasitic nematodes and plant parasitic weeds, which constrain cowpea production (Singh et al., 1997). In the semi-arid and arid tropic and subtropic regions of Africa, cowpea is grown as a

rainfed crop (Quin, 1997; Ehlers and Hall, 1997; Mortimore et al. 1997; Onwuene & Sinha, 1991, Rowland, 1993; Thiaw et al., 1993; National Research Council, 2006) under low input farming systems (Mortimore et al. 1997), generally intercropped with corn, millet, sorghum or cassava (Thiaw et al., 1993; Ehlers and Hall, 1997; Mortimore et al. 1997; National Research Council, 2006). In these systems, average cowpea dry grain yield is extremely low ranging from 100-500 kg/ha (Westphal, 1974; Rowland, 1993; Ehlers and Hall, 1997; National Research Council, 2006; Singh, 2014). However, cowpea yields of 1000-4000 kg/ha have been reported when improved cowpea cultivars and adequate pest and disease management strategies and other crop management inputs are satisfied (Westphal, 1974; Rowland, 1993; Ehlers and Hall, 1997; Singh et al., 2002; Hall et al., 2003; Hall, 2004; National Research Council, 2006).

Intercropping cowpea with companion crop species is the predominant cropping strategy used by resource-poor farmers to reduce the likelihood of crop failure and to guarantee food availability. Although intercropping cowpea has added value for farmers, the portion of land allocated to cowpea is often relatively small compared to the companion crop (Mortimore et al. 1997; National Research Council, 2006), which diminishes cowpea yield (Mortimore et al. 1997; National Research Council, 2006). Frequently, cowpea growing areas are highly prone to drought occurrence due to erratic rainfall patterns during the growing season (Manrique, 1993; Thiaw et al., 1993) which are inadequate to satisfy crop water requirement for optimal growth and yield (National Research Council, 2006). Poor weed management, intercropping,

pests and diseases contribute to low yields under these production conditions. For instance, Striga and Alectra are serious parasitic weeds in some cowpea growing areas in Africa (Quin, 1997; Singh & Emechebe, 2002; Singh, 2014) where they directly compete with cowpea for residual water and nutrients in the soil and for light.

Among other biotic stresses insect pests, bacterial and fungal diseases, and plant-parasitic nematodes limit cowpea yield and can result in complete crop failure. For example, weevils and bruchids are the most problematic postharvest insects on cowpea, causing substantial damage on stored cowpea grain (Murdock et al., 1997; Coulibaly and Lowenberg-DeBoer, 2002; National Research Council, 2006; Singh, 2014). Postharvest insects not only affect the selling price of cowpea by reducing grain quality, but also force farmers to sell grain earlier right after harvest at considerably lower market price, to avoid loss due to bruchid damage (Murdock et al., 1997).

Substantial research advances particularly in West Africa have contributed to the development of improved cowpea cultivars and new technologies for field and postharvest pest management (Coulibaly and Lowenberg-DeBoer, 2002). For example, joint research efforts between cowpea breeders and entomologists have led to development of novel technologies, to protect grain, including solar disinfection, improved breeding lines with seed and pod-wall resistance to insect damage, air-tight containers (metallic drums and triple bagging), use of wood ashes from cooking fires in cowpea storage and treatment of cowpea grain with plant derived oil extracts (Murdock et al., 1997).

Current research efforts on cowpea improvement take into account farmer and market preferences. This goal is still aligned with cowpea research goals which aim to develop cowpea germplasm and cultivars carrying pyramided traits of agronomic interest, such as maturity class and resistance to the major biotic stresses including foliar and flower thrips, nematodes, viral diseases, aphids, bruchids and Striga (Singh et al., 1997; Singh et al., 2002; Hall, 2004). Since subsistence farmers cannot afford sophisticated inputs such as irrigation, fertilizer or synthetic pesticides, cultivar development aims to combine multiple traits in a single cowpea background to optimize and maximize cowpea productivity in target agroecological zones (Quin, 1997; Singh et al., 1997; Ehlers and Hall, 1997; Singh et al., 2002; Hall et al., 2003; Hall, 2004). For example, because cowpea is mostly grown under rain-fed conditions and as a companion crop to cereals, early-maturing cowpea cultivars are preferred by subsistence farmers since they fit well under these low input agricultural settings (Singh et al., 1997; Ehlers and Hall, 1997), whereas medium-and late-maturing cultivars are relevant in geographic regions where cowpea is used as a cover crop, vegetable or as animal fodder (Singh et al., 1997).

Some technical aspects of cowpea production constraints are somewhat understood; for example, the determinants underlying earliness, flowering, yield ability, drought tolerance, resistance to aphids, root-knot nematodes and fungal diseases. However, the integration and consolidation of desired and complex key biological phenomena underlying the ideal cultivar suitable for specific cropping practices and production areas remain significant challenges. Furthermore, local agricultural policies and socio-economic factors (Coulibaly

and Lowenberg-DeBoer, 2002) are often not well aligned with the technical knowledge achieved so far from cowpea research, and renewed efforts are needed on the entire cowpea value-chain.

Cowpea Production in Mozambique

Mozambique is located in southern East Africa with an estimated 36 million hectares of arable land, of which less than 50% is exploited for crop production (FAO, 2013) The agriculture sector employs about 80% of the country's labor force, about 65% of whom are women, and about 70% of the 26-million population lives in rural areas.

Cowpea plays a substantial role in food security for many rural, suburban and low income urban households, and is ranked fourth in crop production after maize, cassava and groundnut (Chiulele et al., 2011). Average cowpea yield is estimated at less than 500 kg/ha (INIA, 2002). Despite its market potential, cowpea is grown mainly as a rain-fed crop for subsistence by resource-poor farmers under intercropping with maize, cassava and other crops in less than one hectare of land per farmer. Lack of access to improved cowpea cultivars, erratic rainfall which leads to drought, intercropping pattern and pest and diseases account for the typically low harvested yield. In general, most of the crop surplus sold in the market comes from farmers with holdings of about 3 hectares (Tostao and Mlay, 2003).

In an on-farm study conducted in the southern region of Mozambique in 2009, farmers reported drought, crop attack by aphids, post-harvest insects, viral diseases, weeds and low soil fertility as the major local cowpea production

constraints (Chiulele, 2010). For those farmers, an ideal cowpea cultivar must produce high grain yield with large seed size and earliness, plus high leaf yield; however, drought tolerance, resistance to postharvest insects, aphids and viral diseases were not ranked as decisive traits for selecting preferred cowpea cultivars. In addition, seed traits such as size and color are also important traits for farmers with white seeds being preferred over other seed colors.

The survey conducted in 2009 in the southern region of Mozambique (Chiulele, 2010) indicated that the relative preference for leaves, fresh pods or dry grain is highly dependent on the production area, which is linked to ease of access to market and on the storability of the harvested product. Farmers located near urban areas focus on leaf production, whereas those in rural areas produce mainly grain which can be stored. Only 16 % of cowpea growers sell their harvested grain in the market (Tostao and Mlay, 2003). A poor infrastructure system, weak local research investment, lack of local agricultural policies advocating for cowpea promotion and prioritization as a strategic legume crop for food security are constraints to market expansion. Adequate agricultural policies aimed at promoting cowpea production as a nutritionally valuable crop and to structure the local cowpea market would enhance small scale farmer household incomes and guarantee food availability.

Cowpea Breeding - Genetic and Genomic Resources

Cowpea (*Vigna unguiculata*) originated in southern Africa (Singh, 2014), and three distinct centers of diversification are indicated: the primary center is located in southeastern Africa, and the secondary and the tertiary centers are

located in West-Central Africa and the Indian subcontinent, respectively (Baudoin and Marechal, 1985; Singh and Rachie, 1985; Padulosi and Ng, 1997; Singh, 2014). Cultivated cowpea (*Vigna unguiculata* subsp. *unguiculata*) is a diploid species with 2n = 22 chromosomes (Faris, 1964; Singh, 2014), and comprises four distinct morphological cultigroups - unguiculata, biflora, sequipedalis and textilis (Baudoin and Marechal, 1985; Singh, 2014).

Cowpea has large and diverse collections of genetic resources with four notable germplasm banks: (i) the largest cowpea collection is at the International Institute of Tropical Agriculture (IITA), Nigeria, with over 15000 cultivated cowpea entries (Quin, 1997; Ehlers and Hall, 1997) and about 1646 wild cowpea accessions (Ehlers and Hall, 1997); (ii) the United States Department of Agriculture (USDA), Griffin – GA, USA, cowpea collection has about 7400 accessions; (iii) the University of California Riverside cowpea germplasm collection holds about 5 600 accessions of cultivated cowpea and 50 wild cowpea genotypes (Hall et al., 2003; Roberts, personal communication); and (iv) the National Bureau of Plant Genetic Resources (NBPGR) in India also maintains cowpea germplasm (Munoz-Amatriaın et al., 2017).

Cowpea genetic improvement has been advanced significantly in the last few years with the development of genome-based technologies and resources. Early cowpea genetic linkage maps were based on RAPD, AFLP, RFLP, biochemical and morphological markers and comprised 10-12 linkage groups spanning 717-2670 cM (Menendez et al, 1997; Fatokun, et al. 1997; Ouédraogo, et al., 2002). Although these early genetic maps were of limited

resolution due to relatively low marker density and marker types, they facilitated identification and mapping of genome regions housing traits of interest to cowpea breeders.

Major advances were made through the development of a 1536-expressed sequence tags (EST)-derived SNP genotyping platform for cowpea (Muchero *et al.,* 2009) which was applied to construct cowpea consensus genetic maps and for analysis of genome synteny between cowpea and reference legumes, common bean and soybean (Muchero et al*.,* 2009; Lucas *et al.,* 2011). These additional genomic resources and associated tools have provided opportunity for further identification and mapping of cowpea genomic regions harboring candidate genes or QTL governing resistance to several biotic stresses including bacterial blight, ashy-stem blight, fusarium wilt, thrips, aphid, Striga, root-knot nematode, (Agbicodo et al., 2010; Muchero et al, 2011; Pottorff et al., 2012; Pottorff et al., 2014; Lucas et al., 2012; Huynh et al., 2015; Huynh et al., 2016;) and yield related traits (Lucas et al., 2013), Description of the diversity of cowpea genetic resources, using SNP genotyping has enabled analysis of cowpea genepools (Huynh et al., 2013). Recently, additional genomic resources were developed, including a cowpea whole-genome shotgun assembly, a BAC physical map, and assembled BACs sequences, which led to the development of a high-density Illumina iSelect SNP genotyping assay of more than 51000 SNP markers. The new SNP assay was utilized to construct a substantially improved version of the cowpea consensus genetic map with more than 37000 mapped SNPs (Munoz-Amatriaın et al., 2017). Also, currently a complete cowpea genome sequence is available through Phytozome.

Host-Plant Resistance to Diseases in Cowpea

In this section, the concepts of host-plant disease resistance, tolerance, field resistance and non-host resistance are defined. In addition, the protocols used to quantify the resistance and susceptibility to these diseases in cowpea are briefly introduced. Also, the status of cowpea breeding for resistance to these diseases is described.

The term host-plant resistance is used to describe the ability of cowpea genotypes to limit infection by a pathogen, proliferation in plant tissues and disease development in plants. Plant resistance to disease is a condition that limits the suitability of the host to the pathogen and limits its ability to reproduce and cause disease in the host (Strauss and Aggarwal, 1999; Agrios, 2005; Mehrotra and Aggarwal, 2013). Plant tolerance to disease is the ability of the host-plant to grow and yield under pathogen infection (Trudgill, 1991; Roberts, 1992; Strauss and Agrawal, 1999; Agrios, 2005). Plant resistance to diseases can be subdivided into three categories. Category one is the *genetic resistance* – when host-plant response to a particular pathogen is governed by at least one resistance gene expressed against avirulence genes present in the pathogen (Agrios, 2005; Mehrotra and Aggarwal, 2013). This resistance is of two types: vertical and horizontal. Resistance is considered vertical when it shows specificity to certain races of a pathogen, while horizontal resistance exhibits a broad spectrum against all races of a pathogen (Agrios, 2005; Mehrotra and Aggarwal, 2013); category two is *non-host resistance* – the interaction between the host-plant and the pathogen is incompatible, and it results in unsuccessful host-plant infection by the pathogen and no disease

development due to the fact that the host-plant belongs to a taxonomic group of plants outside of the pathogen host range; and category three is *field resistance* – pathogen infection of the host plant is restricted due to environmental factors, especially timing required for infection to occur (Agrios, 2005).

The ultimate outcome of the response mounted by host-plant resistance mechanisms against a pathogen is the suppression of disease progress, and such response is associated with suppression of pathogen growth and development (Jenkins et al., 1995; Agrios, 2005; Das et al., 2008). The strength of resistance against pathogens (Roberts et al., 1997) can be quantified or defined on the basis of the symptomatic interaction between host-plant and pathogen. For example, resistance to nematodes can be gauged by the ability of the host-plant to halt nematode reproduction (production of egg-masses or eggs per root system) (Fery et al., 1980; Swanson and Van Gundy, 1984; Trudgill, 1986; Roberts et al., 1997; Cabasan et al., 2012) and root-galling (Fery et al., 1980; Fery et al., 1994; Zhou et al., 2000; Cabasan et al., 2012); a resistant cowpea genotype supports less galling and less nematode reproduction, whereas a susceptible genotype exhibits a severely galled root system and supports high nematode reproduction.

The interaction between susceptible cowpea genotypes and *Fusarium oxysporum* f. sp. *tracheiphilum* results in extensive vascular tissue colonization by the fungus which leads to vascular necrosis, wilting and yellowing, stunting and eventual plant death (Rigert and Foster, 1987; Roberts et al., 1995; Hall and Frate, 1996). These symptoms are used as metrics to determine the

severity of Fusarium wilt disease and resistance to Fusarium wilt (Rigert and Foster, 1987; Roberts et al., 1995).

The practical utility of host-plant resistance to diseases in cowpea and other crop production systems has been documented (Shepherd, 1974; Roberts, 1992; Jenkins et al., 1995; Vos et al., 1998; Plowright et al., 1999; McPherson et al., 2004; National Research Council, 2006; Ulloa et al., 2006; Ehlers et al., 2009). Thus, one of the main goals of cowpea breeding is to develop cultivars with multiple disease resistance combined with favorable agronomic traits (Ehlers and Hall, 1997; Fery and Singh, 1997; Hampton et al., 1997; Singh et al., 2002; Hall et al., 2003; Roberts et al., 2013; Singh, 2014). The genetic control of resistance to some diseases such as bacterial blight, (Agbicodo et al., 2010; Fery and Singh, 1997), scab (Fery and Singh, 1997), Fusarium wilt (Rigert and Foster, 1987; Pottorff et al., 2012; Pottorff et al., 2014), ashy-stem blight (Muchero, et al., 2011), root-knot (Amosu and Franckowiak, 1974; Singh and Redy, 1986; Fery et a., 1994; Roberts et al., 1996; Roberts et al., 1997; Ehlers, 2000;) and viral diseases (Orawu et al., 2013) in some cowpea backgrounds has been elucidated previously, and the knowledge generated from these studies has been applied to assist the introgression of genetic factors controlling these diseases into new cowpea cultivars (Ehlers and Hall, 1997; Singh et al., 1997; Hall et al., 2003; Roberts et al., 2013; Singh, 2014). Several breeding lines and cultivars carrying multiple disease resistance have been developed with the aim of reducing the impact of diseases on cowpea production (Singh et al., 2002). For example, cultivars developed with resistance include Melakh, Lori Niebe, Mouride (Hall et al., 2003), CB27, CB46

(Helms et al., 1991; Hall et al., 2003), Vuli-2 (Mligo and Singh, 2007) and CB50 (Ehlers et al., 2009).

Cowpea cultivars CB27, CB46 and CB50 are some of the successful examples of host-plant resistance breeding; however, most cultivars developed in cowpea breeding programs carry only a narrow spectrum of resistance traits. Also, the dynamics of pathogen populations and shifts in virulence require changes in cowpea breeding objectives to incorporate novel resistance factors into elite cultivars. Shifts in pathogen virulence require identification of novel sources of genetic resistance and introgression of genetic factors of interest into elite cultivar backgrounds (Roberts et al., 2013). For example, CB46 was developed with resistance to RKN and Fusarium wilt race 3, and it has been used by the California cowpea industry for many years (Helms et al., 1991; Hall and Frate, 1996; Hall et al., 2003), but the emergence of new RKN pathotypes and Fusarium wilt race 4 with enhanced virulence to CB46 has prompted the need to broaden the resistance base to RKN and Fusarium wilt in this popular cultivar (Hall and Frate, 1996; Roberts et al., 1997; Ehlers et al., 2000; Petrillo et al., 2006; Roberts et al., 2013).

CB27 carries a broad-based resistance to RKN that controls RKN pathotypes with enhanced virulence (Ehlers et al., 2009; Roberts et al., 2013). In addition to exhibiting significant levels of resistance to virulent RKN species isolates, CB27 also carries strong resistance to the most virulent race of Fusarium wilt (race 4), and the resistance has been transferred into CB46 background to broaden the resistance to this disease in this elite cultivar (Ehlers et al., 2003; Roberts et al., 2013). In 2012, an outbreak of a new Fusarium wilt race was

reported in Tulare County, California, where CB46 carrying Fot3 resistance was heavily infested by a new Fusarium wilt identified as Fusarium wilt race 4 (Fot4) (Frate, 2012). Under these infestation conditions, CB50, a new blackeye cowpea cultivars carrying both Fusarium wilt race 3 and 4 resistance (Ehlers et al., 2009) was considered as an excellent cowpea cultivar option to be grown in fields where Fot4 is prevalent due to its excellent performance under Fot4 infestation (Frate, 2012).

Mechanisms of Resistance to Root-Knot Nematodes in Cowpea

Natural resistance to nematodes in crop plants is conferred by a wide range of resistance genes (Roberts, 1992), with different mechanisms. Some *R* genes inhibit nematode penetration into host roots and block infection (Pegard et al., 2005); host root exudates can be incompatible to promote hatching of infective juveniles (J_2) the eggs (Tomczak et al., 2008; Yang et al., 2016) or hatched J_2 can be repelled from finding host roots (Yang et al., 2016) and consequently die before they enter host roots (Yang et al., 2016). The surviving J_2 can be attracted to the roots by chemotaxis, where they meet mechanical and chemical barriers imposed by cell-wall structure (Davis et al., 2004). In some resistant plants, hard plant cell-wall structure can limit the J_2 from entering roots or dissolving cell-wall components with gland secretions through the stylet (Simonds et al., 1994; Davis et al., 2004). In addition, chemical compounds such as phenols released from roots of resistant plants during root penetration can have repellent activity or be fatal to infective J_2 (Pegard et al., 2005; Yang et al., 2016). Reports from several host plant-nematode interaction studies

indicated that mechanical and chemical resistances to nematode penetration at the cell-walls are not effective resistance mechanisms, but Pegard et al. (2005) reported that in a pepper line resistant to root-knot nematode (RKN) penetration inhibition occurs although it can vary with the RKN species. In cowpea, Das et al. (2008) reported that *Meloidogyne incognita* penetrated equally into resistant and susceptible plant roots, and similar results were reported in cotton, cucumber and *Medicago truncatula* (Creech et al.,1995; Walters et al.,2006; Dhandaydham et al., 2008). Although J_2 can penetrate both resistant and susceptible plant roots, the rate of penetration and total number of J_2 that establish feeding sites was different between resistant and susceptible plants (Ferris et al., 1982; Jenkins et al., 1995; Proite et al., 2008; Faske, 2013). Another common host-plant resistance mechanism is the inability of J_2 to establish effective feeding sites which are required to provide nutrients to sustain their growth and reproduction. According to Das et al. (2008), after the J_2 penetrated successfully both resistant and susceptible cowpea roots, and they were able to establish feeding sites which developed into giant-cells; however, the giant-cells in roots of resistant plants collapsed 14 – 21 days post-inoculation, whereas in susceptible plants the feeding sites developed into huge giant-cells. A similar response was also observed in resistant wild species of peanut inoculated with *M. arenaria* (Proite et al., 2008). In other pathosystems such as grape, cotton, *Medicago truncatula* and cucumber, most J_2 were unable to establish feeding sites in roots of resistant plants (Ferris et al., 1982; Jenkins et al., 1995; Dhandaydham et al., 2008; Faske, 2013).

A third common type of resistance mechanism involves J_2 establishing feeding sites in resistant plants, but the feeding sites collapse with time, as reported in cowpea and peanut (Das et al., 2008; Proite et al., 2008). In cowpea, cucumber and *M. truncatula* this phenomenon was not associated with a hypersensitive response (HR) (Das et al., 2008; Walters et al., 2006; Dhandaydham et al., 2008); contrary to what was observed in tomato, pepper and peanut, (Huang et al.2004; Pegard et al., 2005; Proite et al., 2008). In tomato and peanut the resistance response was delayed, whereas in pepper resistance to nematode infection was triggered earlier in the interaction. This type of resistance mechanism can also be associated with reduced size of established feeding sites in resistant compared to susceptible plants (Walters et al., 2006). The few nematodes that successfully establish feeding sites in resistant plants can either experience slow or arrested development and either fail to grow or take longer to grow into the adult female stage (Creech et al.,1995; Pegard et al., 2005; Walters et al., 2006; Dhandaydham et al., 2008; Proite et al., 2008; Faske, 2013). Furthermore, those that advance to the adult stage typically reproduce poorly (Creech et al.,1995; Jenkins et al 1995; Faske, 2013; Huang et al., 2004).

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CHAPTER II - Broad-Based Root-Knot Nematode Resistance in the Cowpea Germplasm from Mozambique

Abstract

Cowpea (*Vigna unguiculata* L. Walp) is an affordable source of protein and strategic legume crop for food security in Africa; however, infection and root damage by root-knot nematodes (RKN) suppress cowpea yield. Host-plant resistance is an effective strategy to limit the damage by RKN on cowpea, but possible selection for virulence within RKN, and occurrence of more aggressive isolates threatens the effectiveness of the current RKN resistance gene, *Rk*, in commercial cowpea cvs. A cowpea germplasm collection from Mozambique of 48 genotypes was screened to determine the variability of response to RKN infection, the effectiveness and specificity of genetic resistance and the relationship between root-galling (RG) and nematode egg-mass production (EM) through a series of replicated experiments under field, greenhouse and controlled-growth conditions. ANOVA of data for RG and EM per root system identified seven genotypes with broad-based resistance to *Meloidogyne javanica* and avirulent and virulent *M. incognita* isolates, and one of the 48 genotypes exhibited specific genetic resistance to avirulent and virulent *M. incognita* isolates. Resistant genotypes outperformed CB46, a cultivar which carries the gene *Rk,* based on RG and EM by *M. javanica* and virulent *M. incognita* infection ($P < 0.05$). F_1 derived from crosses of some susceptible x new resistant genotypes were also more resistant to *M. javanica* than CB46 ($P < 0.05$). RG responses were correlated across isolates indicating that they are likely under control by the same genetic factors. In addition, the RG and EM responses were correlated ($r = 0.60$, $P < 0.05$), although in some genotypes

these traits appear to be controlled by independent genetic factors. In summary, sources of broad-based genetic resistance to RKN were identified in the Mozambican cowpea collection, and the genetic control of resistance and its relationship to known resistance sources are under investigation to determine its usefulness in resistance breeding.

Introduction

Cowpea (*Vigna unguiculata* (L). Walp.) is one of the most widely grown crops in the world (Ehlers & Hall, 1997, FAOSTAT, 2013) and the most popular legume crop in sub-Saharan Africa (Lombat, 2002) due to its agronomic versatility (Ehlers & Hall, 1997; Lombat, 2002), biological, nutritional (Akyeampong, 1985; Rowland, 1993; Ehlers & Hall, 1997; Lombat, 2002; Quin, 1997; 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 25-50% of the known yield potential, and particularly in Africa the yield ranges between 300 – 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 and diseases, parasitic weeds and plant parasitic nematodes) stresses with very little use of improved crop management strategies (Onwuene & Sinha, 1991, Rowland, 1993).

Root-knot nematode (RKN) species, in particular, *M. incognita* and *M. javanica* (Treub) Chitwood, 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 usually grown (Fery et al., 1994). These plant parasites can 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 translocation and partitioning of photosynthates (Bird and Loveys, 1975; McClure,1977; Taylor and Sasser, 1978). Root-knot nematode management presents challenges not only in cowpea production but also in many cropping systems for several reasons: (i) RKN species are cosmopolitan (Sasser, 1980); (ii) they share common plant host species (Roberts, 1995a; Sasser, 1980); (iii) their populations are highly dynamic and can shift in virulence (Petrillo and Roberts, 2005; Petrillo et al, 2006); and (iv) in some cases the genetic resistance in host plants can be specific to a particular RKN isolate (Swanson & Van Gundy, 1984; Ehlers et al., 2002). Therefore, these and other RKN biological attributes undoubtedly 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 cultivar in the USA, California Black-eye 46 (CB46) is conferred by a single dominant resistance gene, *Rk* (Roberts et al., 1995b; Roberts et al., 1996; Roberts et al., 1997; Ehlers et al., 2000; Ehlers et al., 2002; Ehlers et al., 2009). Recent evidence has 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 & Van Gundy, 1984; Roberts et

al., 1997). Breakdown of genetic resistance in crops is a well-known phenomenon. For example, the *Mi*-resistance gene in tomato known to confer a broad-based resistance to RKN species (Williamson and Hussey, 1996) was ineffective against a virulent population of *M. javanica* in Spain (Ornat, et al., 2001). Resistance breakdown in Florida of a resistant tomato cultivar to *M. incognita* was reported by Noling (2000). Numerous *Mi*-gene breaking isolates have been reported in California and elsewhere (Roberts et al., 1990; Kaloshian et al., 1996; Eddaoudi et al., 1997; Huang et al., 2004). A study on *Mi*-tomato cultivars reported that the resistance was effective against several *M. incognita* isolates, but not resistant to a substantial number of tested *M. javanica* isolates (Roberts and Thomason, 1986). Contrarily, in Florida *Mi*-resistance breaking *M. javanica* isolates were not identified in tested *Mi*-tomato cultivars (Rich and Olson, 1999).

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). An extensive search has found very few additional sources of broad-based resistance (Ehlers et al., 2002). A few sources of effective resistance to RKN carrying genes Rk^2, *rk3* and root *galling gene* (*gg*) have been identified and their biological activity and function for RKN management, singularly and/or as gene pyramids are being investigated for their future application in the development of commercial cowpea cultivars with broad-based RKN resistance (Roberts et al., 1996; Roberts et al., 1997; Ehlers et al., 2000; Ehlers et al., 2002). Results from ongoing research and breeding efforts have shown that broad-based genetic resistance based on complex sets of genes; for instance,

RkRk/Rk²Rk², *RkRk/Rk²Rk²/gg* and *RkRk/rk3rk3* provides robust and effective resistance against diverse RKN populations under field and greenhouse experiments (Roberts et al., 1997; Ehlers et al., 2002; Ehlers et al., 2009; Roberts et al., 2013 - unpublished data). 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. For instance, the *Rk* gene is not effective against virulent isolates of *M. incognita*, but it confers only moderate resistance to aggressive isolates of *M. javanica* (Roberts et al., 1997). A root-galling resistance gene (*gg*), derived from cowpea cultivar CB3, provides resistance to root-galling by avirulent and virulent *M. incognita* populations, but has no resistance to *M. javanica* (unpublished data). The effectiveness of RKN resistance can be classified as broad-based if it provides resistance to a wide range of nematode species and populations (Roberts et al., 1996; Williamson and Hussey, 1996; Ehlers et al., 2000). Host-plant resistance to RKN is defined by the ability of the host to suppress root-galling (Thomason and McKinney, 1960) and nematode reproduction (Thomason and McKinney, 1960; Roberts et al., 1997; Taylor and Sasser, 1978).

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*.,* 2000; Ehlers et al*.,* 2002). In this study a cowpea collection of 48 genotypes

(Huynh et al, 2013) from Mozambique comprising landraces and accessions was investigated for resistance to *Rk*-avirulent and *Rk*-virulent *M. incognita* isolates (“Beltran or Project 77”, and ”Muller”, respectively) and by aggressive *M. javanica* isolate “Project 811” with the aim to: (i) determine the variability of response to root-galling and reproduction compared to known resistant and susceptible controls; (ii) determine the virulence of the nematode isolates and the spectrum of resistance in putative resistant genotypes, and (iii) determine the effectiveness of resistance in putative resistant genotypes and the relationship between the genetic factors controlling root-galling and nematode reproduction. The relative response of the genotypes for root-galling and egg-mass production was used to distinguish highly resistant genotypes from known resistant and susceptible controls (Table 2.1). The known controls consisted of cowpea landrace, California blackeye cvs. and near-isogenic breeding lines (Table 2.1). Virulence indexes of RKN isolates were computed using galling and reproduction data to measure the ability of resistant genotypes to suppress root-galling and nematode reproduction. Correlation analysis of root-galling and nematode reproduction was performed to determine the specificity of the genetic resistance to manage RKN isolates.

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 accessions and landraces from the Mozambique Institute of Agricultural Research (IIAM) collected across Mozambique. 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. Genotypes used as control in this study are indicated on Table 2.1. CB46-Null, NIL-2 and NIL-3 are near-isogenic breeding lines derived from the CB46 background (Huynh et al., 2016) and CB3-gg was derived from California blackeye cv. CB3.

Table 2.1. *Rk* gene sets in control genotypes and their response to avirulent and virulent *M. incognita* and *M. javanica*. The response was measured using root-galling.

		Root-knot nematode Response		
Genotype	***Rk* gene set**	Avr. *M. incognita*	Vir. *M. incognita*	*M. javanica*
UCR779	No resistance	Susceptible	Susceptible	Susceptible
CB46-Null	No resistance	Susceptible	Susceptible	Susceptible
CB46	*RkRk*	Resistant	Susceptible	Susceptible
CB27	*RkRk/rk3rk3*	Resistant	Resistant	Resistant
IT84S-2049	*RkRk/Rk^{2}Rk^2*	Resistant	Resistant	Resistant
NIL-2 genes	*RkRk/Rk^{2}Rk^2*	Resistant	Susceptible	Resistant
NIL-3 genes	*RkRk/Rk^{2}Rk^2/gg*	Resistant	Resistant	Resistant
CB3-gg	*gg*	Susceptible	Resistant	Susceptible

Root-Knot Nematode Resistance Screening

Nematode Isolates

Screening for RKN resistance was conducted in three environments: (i) growth chamber – using a seedling growth-pouch test; (ii) greenhouse – pot test, and (iii) infested field plots (Table 2). In the seedling growth-pouch test, an avirulent *M. incognita* isolate “Project 77” and an aggressive *M. javanica* isolate “Project 811” were used for the resistance assays. The *M. javanica* isolate was also used for the pot tests. All nematode isolates were maintained on susceptible tomato plants in a greenhouse at UC-Riverside. *M*. *incognita* isolate “Project 77” is avirulent to cowpeas carrying the *Rk* resistance gene (Roberts et al*.,* 1995b), and an incompatible interaction between this nematode isolate and a genotype carrying the *Rk* gene, or any genetic resistance factor equivalent to this gene would be expected*.* Genotypes lacking *Rk* genes are susceptible and indicate a compatible interaction. The *M. javanica* isolate “Project 811” is aggressive and able to induce galling and reproduce successfully on plants carrying resistance gene *Rk* at a level of 50% or more of that of susceptible plants (Roberts et al*.,* 1995b; Ehlers et al*.,* 2002). Cowpea genotypes carrying effective genetic resistance against *M. javanica* would be expected to perform at a level significantly below that of genotypes carrying only the *Rk* gene, and would indicate this phenotype would be conferred by novel (non-*Rk*) RKN resistance genes or by additive effects of *Rk* complementary genes which enhance the performance of gene *Rk*. The virulent *M. incognita* isolate “Muller” is highly virulent to cowpea genotypes carrying gene *Rk*, inducing excessive galling and reproducing successfully on root systems of such backgrounds, but

resistant genotypes carrying *Rk* plus other genes in combination provide effective resistance against this nematode isolate (Table 2.1 and unpublished data).

Table 2.2. RKN resistance screening experiments: test environments and locations, nematode isolate used and screening year.

Exp.	Environment	Location	Nematode isolate	Year
1	Seedling growth pouches	UC-Riverside	Avr. *M. incognita, M. javanica*	2012
2	Field	SCREC	Avr. *M. incognita, M. javanica*	2012
3	Greenhouse	UC-Riverside	*M. javanica*	2013
4	Seedling growth pouches	UC-Riverside	Avr. *M. incognita, M. javanica*	2013
5	Field	SCREC	Avr. and Vir. *M. incognita, M. javanica*	2014
6	Seedling growth pouches	UC-Riverside	Avr. *M. incognita*, *M. javanica*	2014
7	Greenhouse	UC-Riverside	*M. javanica*	2014
8	Greenhouse	UC-Riverside	*M. javanica*	2015
9	Field	KARE	Avr. *M. incognita, M. javanica*	2015
10	Seedling growth pouches and Greenhouse	UC-Riverside	*M. javanica*	2016
11	Field	SCREC	Avr. and Vir. *M. incognita* and *M. javanica*	2016

Exp. – experiment; SCREC - South Coast Research and Extension Center, Irvine, CA; CVARS -Coachella Valley Agricultural Research Station, Thermal, CA; KARE - Kearney Agriculture Research and Extension Center, Parlier, CA; Avr – avirulent.

Nematode Reproduction: Seedling Growth Pouch Test

Screening for RKN resistance using plastic seedling growth-pouches (Ehlers et al., 2000; Atamian et al., 2012) was conducted in a growth chamber with day and night temperatures set at 28 ^{o}C and 22 ^{o}C, respectively, under 16 hours day-length. Five pouch tests were conducted. Each of the 60 tested genotypes (including controls) was considered as a single treatment and replicated four

times. Using the protocol of Ehlers et al (2000) and Atamian et al. (2012), 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. Twelve – fourteen days after emergence, the plants were inoculated with freshly hatched second-stage juveniles (J_2), at a density of 1500 J_2/plant, and laid on a table horizontally for 24 hours in the dark. J_2 were hatched from nematode eggs placed in an incubator at 26-27 ^oC for 7 days. Every 2-3 days emerged J_2 were collected, counted and concentrated to the desired inoculum density for the test. After inoculation, the plants were fertilized for 3-5 days with half-strength Hoagland's solution (Hoagland and Arnon, 1950) and additional fertilizer was applied as needed for the remainder of experiment**.** At 30-35 days after inoculation the roots were infused with erioglaucine solution (1g/l) (Sigma Chemical Co., St. Louis, MO, USA), and 24 hours later the solution was poured off and the stained egg-masses counted under 10X magnification light.

Root Galling Response - Pot Test

Assays in pots were conducted in a greenhouse (Table 2.2) under 28 ^oC day and 22 ^oC night temperatures to assess the response of cowpea genotypes to root-galling induced by *M. javanica* isolate "Project 811". In the first two experiments, the entire test collection of 48 genotypes was screened for resistance to *M. javanica* isolate "Project 811". As a confirmation, two additional tests were conducted on the highly resistant genotypes identified in the two initial screenings.

Pot tests were conducted with four replicates arranged in a randomized complete block design. Two seeds of each genotype were sown in fiber pots (15 cm diameter), containing a soil mixture of 80% sand and 20% peat, and thinned to one plant per pot seven-days after emergence. Water was provided as needed using a drip-irrigation system. Fifteen days after emergence, 10 ml of egg suspension in water containing about 1000 egg/ml were pipetted per pot, and 60 days-after inoculation, plant tops were cut 2 – 3 cm above the soil line and the roots washed and rated for root-galling response under 10X magnification.

Root-galling followed a 0 to 9 rating scale 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 on the main and secondary roots, respectively; 8 = generally huge galls and huge coalesced galls on secondary and main roots, respectively, very few feeder roots can be seen; 9 = huge galls and coalesced galls, generally no feeder roots visible. In this modified scale, ratings of 0 to 3 were assigned to root systems with no or visible small galls on secondary roots only, and no damage seen on tap-root. Ratings of 4 to 6 were assigned to roots showing light to moderately damaged tap-root, while ratings of 7 and above

were assigned to root systems with damaged to severely damaged tap-root. A cut-off between resistant-susceptible genotypes for root-galling response was established at gall index (GI) = 3; thus, genotypes with GI ≤ 3 were considered resistant, and those with GI > 3 were considered susceptible.

Root Galling Response - Field Test

Field trials were conducted in California during June – October of 2012, 2014, 2015 and 2016 (Table 2.2). The field screenings conducted in 2012, experiment 2, were non-replicated, and only twenty-four of the 48 genotypes were tested due to limited space. In 2014 (exp. 5, Table 2.2), all test genotypes were screened in 4 blocks in a randomized complete block design. In the 2015 and 2016 experiments (Exps. 9 and 11), only the highly resistant genotypes identified in 2014 were tested using the same experimental design as in 2014. In all field trials, 20-25 seeds per treatment were planted in a 1.5 m-long single-row plot, and 60 days after emergence plant tops were cut at about 2 – 3 cm above the soil line and root systems dug and evaluated for root-galling response in the lab following the same procedure as in the pot assay. Water and fertilizer were supplied as needed through drip-tape.

Data for root-galling (GI) and nematode reproduction (EM) from all experiments were analyzed by ANOVA. Data analysis for RKN resistance screening experiments, 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 experiments where a single

nematode isolate was used, data were analyzed following the ANOVA for a single experiment. In all cases, the data analysis was performed using SAS University Edition 3.2.2 following the mixed procedure (Proc Mixed) where the blocks or replications were considered as the random factor while the nematode isolates and cowpea genotypes were both considered as fixed factors.

Nematode Virulence and Resistance Specificity

RKN virulence on cowpea can be variable between isolates of the same nematode species and isolates of distinct species (Petrillo et al., 2006). Nematode virulence can be defined as the ability of a nematode to successfully establish a feeding site, induce root-galling and reproduce on root system of resistant plants (Roberts et al., 1997; Petrillo et al., 2006). Virulence index for each nematode isolate was estimated as the proportion between galling or reproduction on the root systems of resistant genotypes and susceptible genotypes (Petrillo et al., 2006).

Relationship between Galling and Nematode Reproduction

To further validate the efficacy and to infer about the heritability of resistance in highly resistant genotypes, additional experiments were conducted in 2015 and 2016 (Exps. 8, 9, 10 and 11; Table 2.2). The genotypes were tested for root-galling (field and greenhouse) and nematode reproduction phenotypes (seedling growth pouches). In addition, F1 plants derived by crossing a selected highly resistant cowpea genotype (FN-2-9-04) to susceptible genotypes (Ecute, INIA-41, CB46 and CB46-Null) were tested for root-galling and nematode reproduction by *M. javanica* isolate “Project 811” (Exps. 8 and 10; Table 2.2). The relationship between root-galling and nematode reproduction was determined by correlation analysis to infer whether the two traits are under control by the same or distinct genetic factors.

Results

Genotype Response to Root-Galling – Field experiments

In a preliminary study conducted at the University of California-SCREC in 2012 (Exp. 2, Table 2.2), a subset of 24 genotypes plus controls were screened in non-replicated trials for resistance to root-galling in separate blocks infested with avirulent and virulent *M. incognita* ("Beltran" and "Muller", respectively) and aggressive *M. javanica* isolate "Project 811" (Figs. 2.1A, 2.1B and 2.1C).

Figure 2.1A shows that 16 of the 24 tested genotypes and controls CB46, CB27, NIL-2 and NIL-3 genes and CB3-gg were resistant (GI ≤ 3) to avirulent *M. incognita* isolate "Beltran" compared to susceptible control CB46-Null. Three of the test genotypes (VAR-3A, FN-2-9-04 and Namuesse-D) were resistant (G ≤ 3) to the virulent *M. incognita* isolate "*Muller*", and similar response was observed for controls NIL-3 genes, CB3-gg and CB27 (Fig. 2.1B), while CB46 and CB46-Null were susceptible. The control NIL-2 genes showed moderate resistance response to the virulent *M. incognita* "Muller" (Fig. 2.1B). Of the 24 test genotypes, four (VAR-3A, FN-2-9-04, Namuesse-D and FAEF-14-INE) were resistant (G ≤ 3) to the aggressive *M. javanica* isolate "Project 811", while controls CB27, NIL-2 and NIL-3 genes were moderately resistant, and CB46 and CB46-Null were susceptible (Fig. 2.1C). The average root-galling scores of the genotypes ranged from 1.3 – 6.1, 1.9 – 5.5, and 1.3 – 6.9 under infection by avirulent *M. incogita*, virulent *M. incognita* and *M. javanica*, respectively.

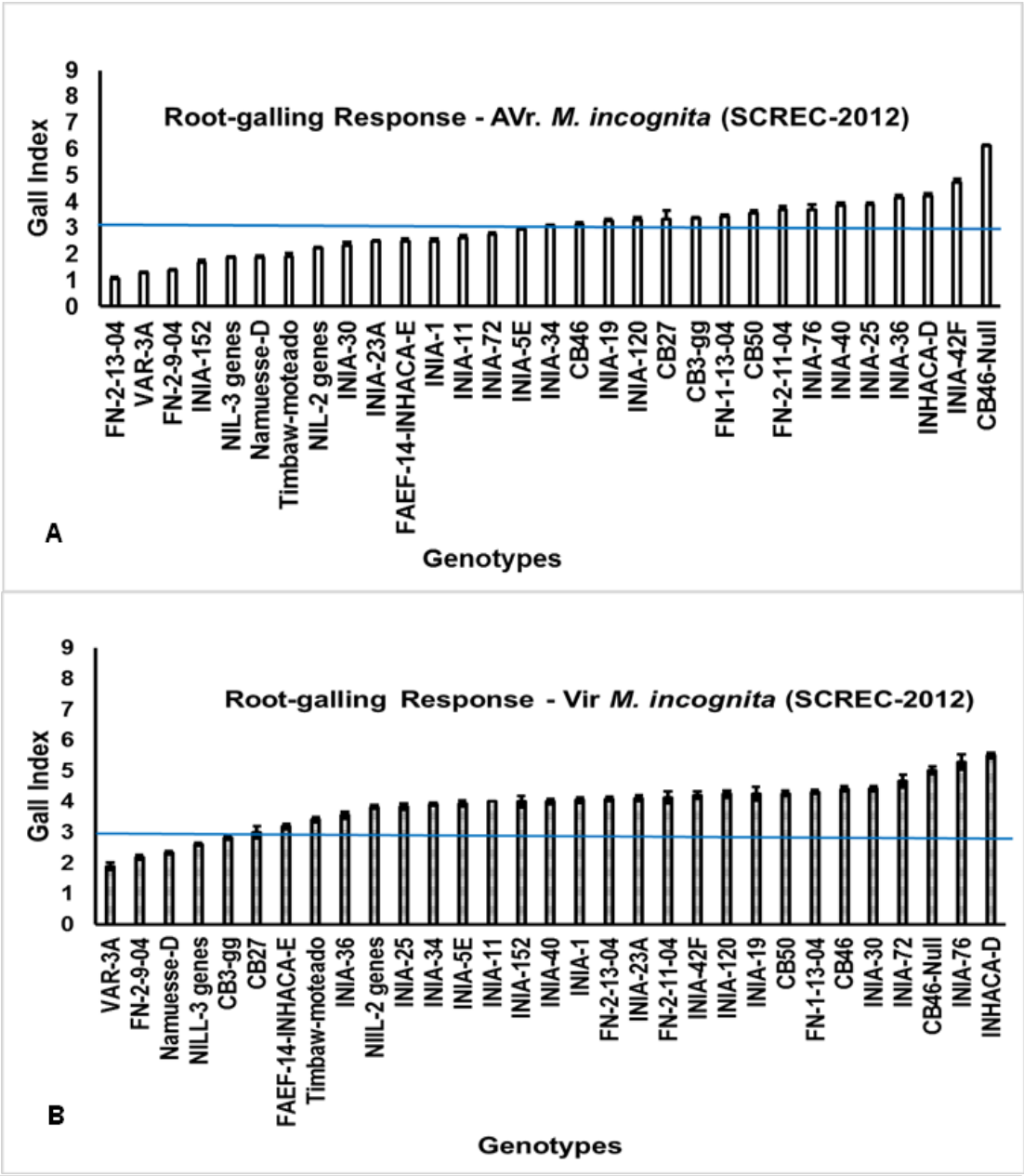

Fig. 2.1. Response to root-galling of 24 genotypes under infestation by (A): avirulent *M. incognita* isolate "Beltran" and (B): virulent *M. incognita* isolate "Muller". Horizontal line is GI = 3 representing cut-off between resistant and susceptible genotypes.

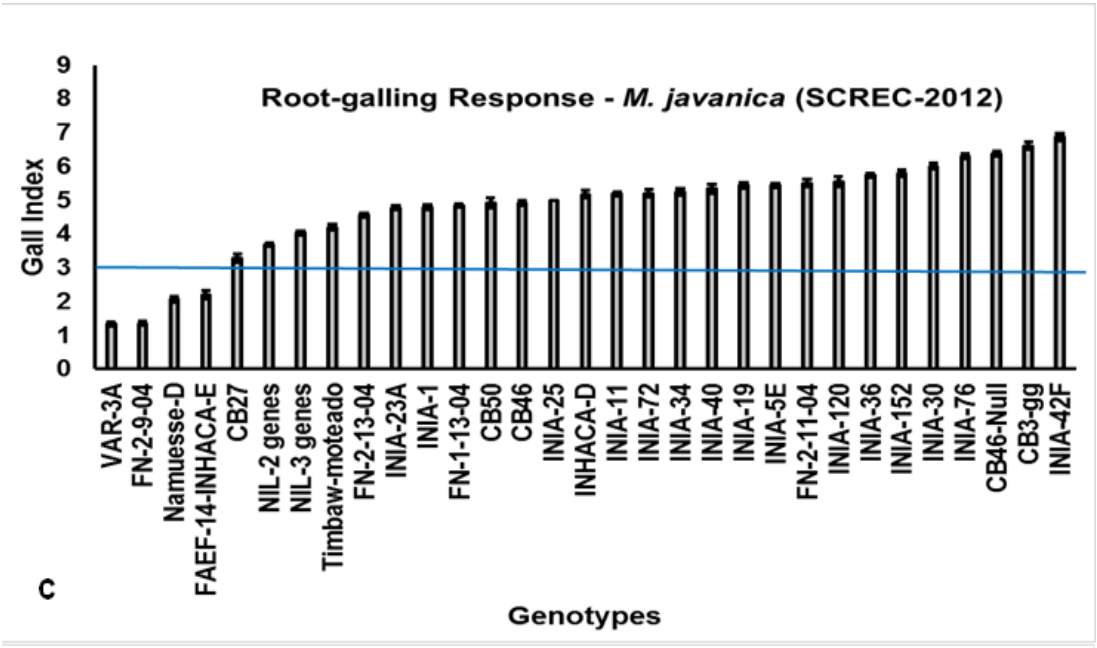

Fig. 2.1 C. Response to root-galling of 24 genotypes under infestation by aggressive *M. javanica* isolate "Project 811". Horizontal line is GI = 3 representing cut-off between resistant and susceptible genotypes.

In 2014, a set of 48 genotypes from Mozambique was screened at SCREC (Exp. 5, Table 2.2), and the results are presented in Table 2.3. The ANOVA showed significant effects of the genotypes, nematode isolates and the interaction genotype x nematode isolate on the observed variability in root-galling response ($P < 0.0001$). Significant differences among genotypes mean root-galling induced by each nematode isolate were detected at GI = 1.4 using Tukey multiple comparison test ($P < 0.05$, Table 2.3). All test genotypes, except one (FN-2-11-04, GI = 3.9), were resistant to root-galling induced by avirulent *M. incogita* isolate "Beltran" (G ≤ 3), and all controls, except those lacking resistance genes (CB-46-Null and UCR779, GI = 5.2 and 6.0, respectively), were also resistant to this nematode isolate (Table 2.3). Most of the differences

in root-galling response under this nematode isolate infestation were not significant (*P > 0.05*). Root-galling phenotypes with avirulent *M. incogita* isolate "*Beltran*" infestation ranged from 0 – 6.

In the test with virulent *M. incogita* "Muller" (Exp. 5, 2014, Table 2.2), several of the test genotypes that were resistant to *M. javanica "*811", were also resistant to this nematode isolate, including FN-2-9-04 and VAR-3A. These two genotypes were also resistant to virulent *M. incogita* in the 2012 test. Of the controls, CB27, NIL-3 genes and CB46-gg were resistant, as they were in the 2012 test, contrary to NIL-2 genes which tested moderately resistant and resistant in 2012 and 2014, respectively under virulent *M. incogita* field infestation. The differences in response to root-galling among controls CB27, NIL-3 genes and CB3-gg, and their differences to that of the resistant test genotypes were not statistically significant (*P > 0.05*). However, the root-galling phenotype of the resistant control NIL-2 (GI = 2.6) was different from that observed in 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) (*P < 0.05*). Genotypes INIA-41, Maputo, and Muinana-Lawe were also resistant to virulent *M. incognita* "Muller", but their response to root-galling was not different to 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 (*P > 0.05*). Root-galling phenotypes with virulent *M. incogita* "Muller" ranged from 0.5 – 5.2 (Table 2.3).

In the test with *M. javanica* “811”, eight of the test 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 were also resistant in the 2012 field test (VAR-3A, FN-2-9-04, Namuesse-D and FAEF-14-INE), showing reproducibility of the results. Of the controls, CB27, NIL-2genes and NIL-3 genes were also resistant to *M. javanica* “811”, but controls carrying only the *Rk* gene (CB46 and CB50) or no gene were susceptible. The responses of the resistant test genotypes, FAEF-14-INE, FN-1-14-04, FN-2-9-04, Namuesse-D and VAR-3A, were different from that of the *Rk* controls ($P < 0.05$), but the differences in response observed among them were not significant. Root-galling phenotypes (0.3 – 5.5) induced by aggressive *M. javanica* “811” were in the same range as those induced by virulent *M. incogita* “Muller”.

Table 2.2. Response to root-galling of 48 genotypes following infection by avirulent and virulent *M. incognita* isolates “Beltran” and “Muller”, respectively and by aggressive *M. javanica* isolate “Project 811” under field infestation - Exp. 5, 2014.

	Nematode isolate		
	Avr *M. incognita* "Beltran"	**Vir *M. incognita* "Muller"**	***M. javanica* "811"**
Genotype	**Gall index (0-9)**	**Gall index (0-9)**	**Gall index (0-9)**
CB27	0.2 ± 0.0	0.8 ± 0.2	2.1 ± 0.2
CB46	1.5 ± 0.7	3.7 ± 0.4	4.8 ± 0.3
CB3-gg	2.5 ± 0.1	1.8 ± 0.1	5.3 ± 0.1
CB46-Null	5.2 ± 0.1	4.3 ± 0.6	5.5 ± 0.2
CB50	0.6 ± 0.2	2.5 ± 0.9	4.6 ± 0.5
Ecute	2.9 ± 0.2	-	5.3 ± 0.3
FEAF14INE	0.0 ± 0.0	1.4 ± 0.4	0.6 ± 0.2
FN-1-13-04	0.5 ± 0.2	2.7 ± 0.7	2.5 ± 1.0
FN-1-14-04	0.1 ± 0.0	1.7 ± 0.6	0.6 ± 0.3
FN-2-11-04	3.9 ± 0.2	4.1 ± 0.4	4.9 ± 0.7
FN-2-13-04	0.0 ± 0.0	3.4 ± 0.8	3.2 ± 1.0
FN-2-9-04	0.0 ± 0.0	0.5 ± 0.1	0.4 ± 0.0
Gile-K-Local	0.1 ± 0.1	0.9 ± 0.2	1.3 ± 0.3
Inhaca-D	0.9 ± 0.7	3.3 ± 0.3	2.9 ± 0.6
Inhaca-I	0.6 ± 0.2	2.3 ± 0.7	3.9 ± 1.3
INIA-1	0.7 ± 0.4	3.3 ± 0.9	3.4 ± 0.4
INIA-11	1.5 ± 0.7	2.6 ± 0.6	5.1 ± 0.6
INIA-120	2.2 ± 1.1	-	4.7 ± 0.5
INIA-152	0.1 ± 0.1	3.4 ± 0.3	3.4 ± 0.7
INIA-19	0.1 ± 0.0	3.1 ± 0.4	3.2 0.6
INIA-19F	0.5 ± 0.3	3.6 ± 0.9	3.4 ± 0.3
INIA-23A	0.4 ± 0.3	3.0 ± 0.3	3.2 ± 0.8
INIA-24	0.5 ± 0.3	2.6 ± 0.5	3.8 ± 1.2
INIA-25	0.7 ± 0.3	3.0 ± 0.8	3.6 ± 0.5
INIA-3	0.1 ± 0.1	2.4 ± 0.7	3.2 ± 0.8
INIA-30	0.5 ± 0.2	3.0 ± 0.5	4.5 ± 0.7
INIA-31	0.4 ± 0.3	3.7 ± 0.7	2.9 ± 0.8
INIA-34	0.4 ± 0.4	2.2 ± 0.7	4.3 ± 0.2
Mean ± SE	0.83 ± 0.09	2.73 ± 0.10	3.31 ± 0.11
LSD*	1.39 (*P = 0.042*)	1.40 (*P = 0.041*)	1.43 (*P = 0.037*)

*LSD = Least significant mean differences, multiple comparison test of means (*P < 0.05*); SE = Standard error.

Table 2.3. (Continued).

	Nematode isolate		
	Avr *M. incognita* "Beltran"	Vir *M. incognita* "Muller"	*M. javanica* "811"
Genotype	**Gall index (0-9)**	**Gall index (0-9)**	**Gall index (0-9)**
INIA-36	0.0 ± 0.0	3.2 ± 0.5	4.0 ± 1.1
INIA-40	0.9 ± 0.8	3.6 ± 0.7	2.1 ± 0.5
INIA-41	2.1 ± 1.0	1.3 ± 0.5	4.9 ± 0.4
INIA-42F	1.4 ± 0.8	3.4 ± 0.9	4.1 ± 0.4
INIA-51	0.9 ± 0.9	2.2 ± 0.5	2.5 ± 0.5
INIA-51A	0.3 ± 0.3	3.0 ± 0.8	4.4 ± 0.6
INIA-5A	0.0 ± 0.0	1.8 ± 0.4	1.7 ± 0.1
INIA-5E	0.1 ± 0.0	3.6 ± 0.5	3.1 ± 0.4
INIA-72	1.2 ± 0.6	2.9 ± 0.3	2.9 ± 0.4
INIA-73	0.5 ± 0.2	2.8 ± 0.7	4.3 ± 0.8
INIA-76	2.4 ± 0.8	3.1 ± 0.9	4.6 ± 0.6
IT-18	0.0 ± 0.0	2.9 ± 0.4	3.2 ± 0.4
Maputo	0.3 ± 0.1	1.6 ± 0.5	3.2 ± 0.8
Massava-11	0.2 ± 0.1	2.7 ± 0.7	1.9 ± 0.7
Muinana-Lawe	-	1.2 ± 0.2	3.4 ± 1.1
Namuesse	0.2 ± 0.1	3.3 ± 0.2	3.9 ± 0.6
Namuesse-D	0.0 ± 0.0	-	0.6 ± 0.1
Namurua	0.1 ± 0.1	3.4 ± 0.3	4.4 ± 0.4
NIL-2 genes	1.1 ± 0.2	2.6 ± 0.3	2.8 ± 0.5
NIL-3 genes	0.7 ± 0.2	1.1 ± 0.3	2.4 ± 0.3
SP-860	0.0 ± 0.0	5.2 ± 0.7	2.7 ± 0.6
SP-866	0.3 ± 0.3	4.0 ± 0.7	2.2 ± 0.5
Timbaw-Monteado	0.2 ± 0.1	2.2 ± 0.5	2.8 ± 0.6
UCR 779	6.0 ± 0.5	4.9 ± 0.5	5.4 ± 0.3
VAR-10B	0.8 ± 0.4	2.5 ± 0.7	3.8 ± 0.4
VAR-11D	0.0 ± 0.0	1.9 ± 0.7	1.0 ± 0.4
VAR-3A	0.0 ± 0.0	0.6 ± 0.1	0.3 ± 0.1
Vita-7	1.3 ± 0.7	-	3.9 ± 0.8
Mean ± SE	0.83 ± 0.09	2.73 ± 0.10	3.31 ± 0.11
LSD*	1.39 (*P = 0.042*)	1.40 (*P = 0.041*)	1.43 (*P = 0.037*)

*LSD = Least significant mean differences, multiple comparison test of means (*P < 0.05*); SE = Standard error.

Genotype Response to Root Galling – Greenhouse experiments

In 2013 and 2014 (Exps 3 and 7, Table 2.2), the Mozambican genotypes were also screened for resistance to root-galling induced by *M. javanica* "811" in pots under greenhouse conditions. In these experiments, the genotypes were fixed as treatment and distributed in 4 replicated blocks in a randomized complete block design. Based on the ANOVA, there were significant effects of the genotypes on root-galling induced by *M. javanica* ($P < 0.0001$). The average root-galling index ranged from 1 to 8 (Fig. 2.2). Consistent with the results observed in the 2012 and 2014 (Exps 2 and 5) in the field (Fig 2.1C and Table 2.3), 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 root-galling phenotypes when challenged with aggressive *M. javanica* "811". 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* "811" infestation. Significant differences in root-galling phenotypes were detected at GI = 1.75 ($P < 0.05$).

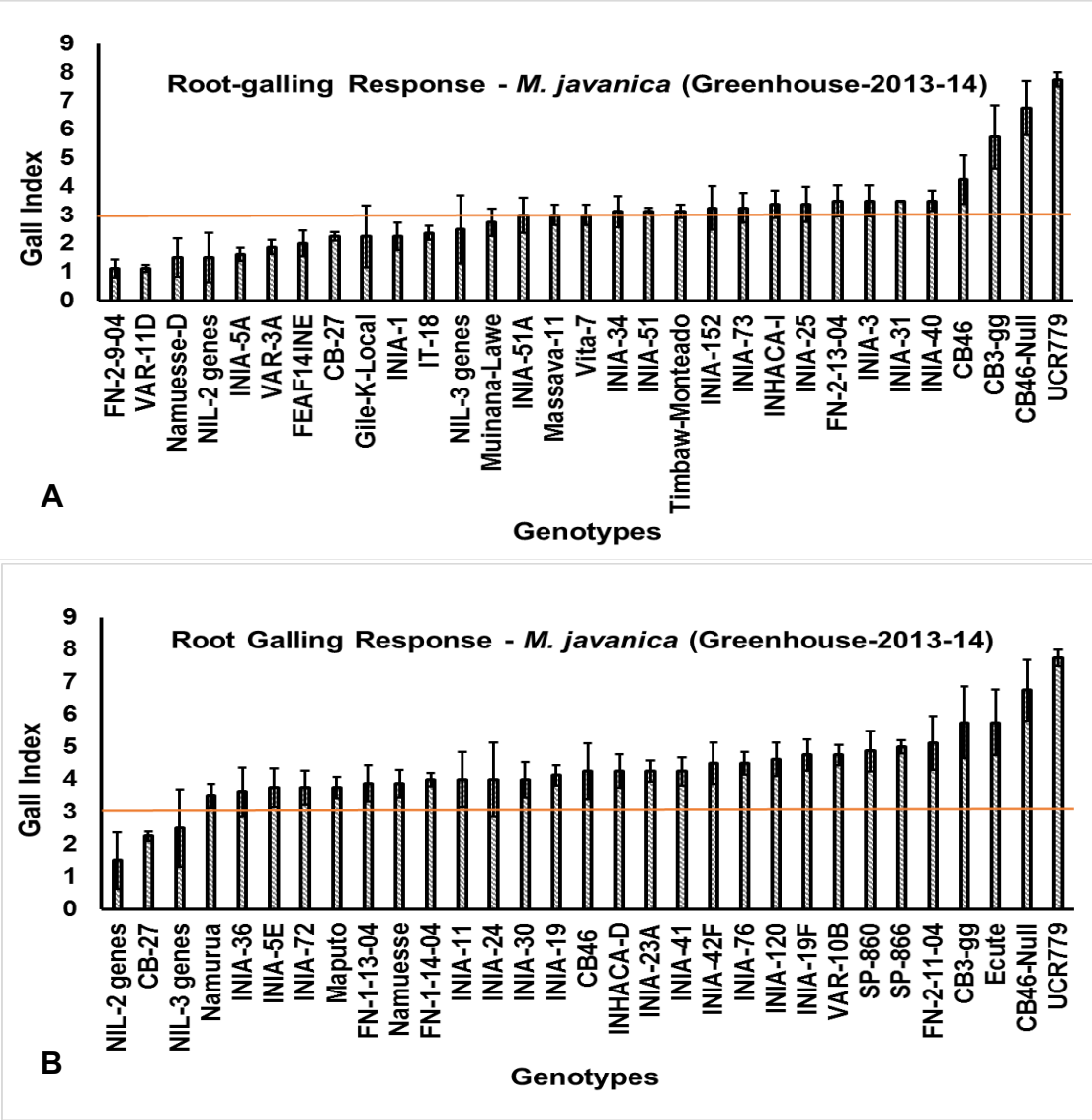

Fig. 2.2. Response to root-galling induced by aggressive *M. javanica* isolate "Project 811" under greenhouse conditions – Exps. 3 and 7 (Table 2.2). Horizontal line is GI = 3 representing the cut-off between resistant and susceptible genotypes.

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 different ($P < 0.05$) from those of CB46, CB46-Null, CB3-gg and UCR779. The root-galling

responses among resistance test genotypes were not different, nor were root-galling responses between CB27, NIL-2 genes and NIL-3 genes.

Responses to Nematode Reproduction

The test cowpea genotypes were also evaluated for the ability to suppress reproduction of avirulent *M. incognita* "Project 77" and *M. javanica* "Project 811" assessed by egg-masses (EM) per root system. Three seedling growth-pouch experiments with 4 replications each were conducted under growth-chamber conditions (Exps. 1, 4 and 6, Table 2.2). To distinguish resistant from susceptible response a cut-off of 30 EM per root system was established, so genotypes with ≤ 30 EM per root system were classified as resistant, and genotypes with EM count > 30 were classified as susceptible. This threshold, was established based on the mean EM production of 3 experiments and reproduction levels on susceptible controls.

The genotypes had significant effects on EM produced by both nematode isolates ($P < 0.0001$). Also, both nematode isolates and the interaction genotype x nematode isolate had significant effect on the EM data ($P < 0.0001$). The variability in EM production per root system is shown in Table 2.4, and the average EM per root system for the avirulent *M. incognita* and *M. javanica* isolates ranged from 0 – 64 and 1.31 – 105, respectively. Significant differences in EM production per root system between genotypes were detected at LSD = 20.7 and 18.4 ($P < 0.05$) for avirulent *M. incognita* and *M. javanica*, respectively.

Table 2.4. Egg-mass production by avirulent *M. incognita* isolate “Project 77” and by aggressive *M. javanica* “Project 811” on root systems of 48 cowpea genotypes - Exps. 1, 4 and 6, 2012-2014 (see Table 2.2).

	Nematode Isolate	
	Avr *M. incognita* "Project 77"	***M. javanica* "Project 811"**
Genotype	**Egg Masses**	**Egg Masses**
CB27	0.3 ± 0.2	21.0 ± 4.5
CB3-gg	47.1 ± 9.9	44.9 ± 17.1
CB46	1.5 ± 1.1	71.3 ± 11.2
CB46-Null	56.5 ± 17.3	78.5 ± 6.9
CB50	1.5 ± 0.8	44.3 ± 10.7
Ecute	22.7 ± 3.2	81.6 ± 10.3
FEAF-14-INE	0.9 ± 0.3	1.3 ± 0.3
FN-1-13-04	0.4 ± 0.2	18.4 ± 3.6
FN-1-14-04	0.1 ± 0.1	25.3 ± 9.7
FN-2-11-04	47.9 ± 17.5	35.3 ± 10.4
FN-2-13-04	0.0 ± 0.0	28.5 ± 6.4
FN-2-9-04	0.4 ± 0.2	1.9 ± 0.5
Gile-K-Local	1.2 ± 0.8	16.6 ± 3.4
Inhaca-D	1.8 ± 1.1	42.8 ± 4.5
Inhaca-I	0.8 ± 0.4	25.7 ± 5.4
INIA-1	2.5 ± 0.5	64.9 ± 8.1
INIA-11	2.7 ± 1.5	37.9 ± 6.9
INIA-120	3.1 ± 0.8	54.9 ± 7.2
INIA-152	14.3 ± 11.2	9.6 ± 2.3
INIA-19	1.9 ± 1.5	22.4 ± 3.3
INIA-19F	0.5 ± 0.3	17.8 ± 4.5
INIA-23A	0.4 ± 0.3	21.1 ± 3.7
INIA-24	0.3 ± 0.2	13.9 ± 3.7
INIA-25	0.8 ± 0.4	17.4 ± 6.8
INIA-3	1.4 ± 1.2	16.1 ± 2.5
INIA-30	1.3 ± 0.4	35.0 ± 6.2
INIA-31	0.4 ± 0.1	15.1 ± 3.9
INIA-34	1.6 ± 0.3	39.5 ± 5.0
Mean ± SE	5.45 ± 1.0	27.45 ± 1.5
LSD*	20.7	18.4

*LSD = Least significant mean differences, multiple comparison test of egg mass means ($P < 0.05$); SE = Standard error.

Table 2.4 (continued).

	Nematode Isolate	
	Avr *M. incognita* "Project 77"	***M. javanica* "Project 811"**
Genotype	**Egg Masses**	**Egg Masses**
INIA-36	0.3 ± 0.1	17.0 ± 3.7
INIA-40	2.0 ± 1.0	36.6 ± 3.6
INIA-41	16.6 ± 4.0	69.5 ± 6.6
INIA-42F	0.5 ± 0.3	28.0 ± 4.7
INIA-51	1.3 ± 0.8	26.3 ± 4.6
INIA-51A	2.4 ± 0.8	26.4 ± 6.7
INIA-5A	0.1 ± 0.1	2.5 ± 0.6
INIA-5E	0.6 ± 0.4	18.1 ± 3.6
INIA-72	0.3 ± 0.2	12.1 ± 1.2
INIA-73	2.6 ± 1.2	11.4 ± 3.2
INIA-76	47.5 ± 17.6	53.6 ± 13.3
IT-18	0.9 ± 0.4	15.9 ± 4.6
Maputo	2.4 ± 1.0	21.0 ± 4.3
Massava-11	0.7 ± 0.3	12.4 ± 3.2
Muinana-Lawe	0.3 ± 0.1	37.2 ± 11.6
Namuesse	0.5 ± 0.4	14.3 ± 2.9
Namuesse-D	0.5 ± 0.3	1.4 ± 0.7
Namurua	4.2 ± 0.9	33.0 ± 6.3
NIL-2 genes	0.0 ± 0.0	23.3 ± 3.2
NIL-3 genes	1.3 ± 0.8	24.1 ± 7.1
SP-860	1.0 ± 0.9	11.3 ± 4.1
SP-866	0.3 ± 0.2	1.7 ± 0.6
Timbaw-Monteado	0.2 ± 0.2	33.6 ± 11.7
UCR779	64.0 ± 10.8	105.2 ± 22.3
VAR-10B	0.9 ± 0.4	27.8 ± 1.9
VAR-11D	0.3 ± 0.2	3.3 ± 2.5
VAR-3A	0.1 ± 0.1	1.3 ± 0.5
Vita-7	2.0 ± 0.3	36.4 ± 3.2
Mean ± SE	5.5 ± 1.0	27.5 ± 1.5
LSD	20.7	18.4

*LSD = Least significant mean differences, multiple comparison test of egg mass means ($P < 0.05$); SE = Standard error.

The avirulent *M. incognita* isolate reproduced poorly on the roots of most test genotypes with the exception of FN-2-11-04 and INIA-76 (EM = 47.9 and 47.5, respectively) (Table 2.4). Among the control genotypes, CB3-gg, CB46-Null and UCR779 were susceptible to this nematode isolate (EM = 47.1, 56.5 and 64.0, respectively). Most of the test genotypes (for example, FN-2-9-04, VAR-3A, Namuesse-D, INIA-5A, VAR-3A, FAEF-14-INE and Gile-K-Local) had very low EM, indicating they were resistant to reproduction by this nematode isolate, and the differences among them in EM phenotypes were not significant ($P > 0.05$). Also, their differences in response to that of control genotypes carrying the *Rk* genes (CB46, CB27, CB50, NIL-2 genes and NIL-3 genes) were not significant ($P > 0.05$). These control genotypes supported fewer EM than controls CB3-gg, CB46-Null and UCR779 ($P < 0.05$) (Table 2.4).

Nematode Virulence and Resistance Spectrum

The virulence of the nematode isolates used in this study was estimated using RG and EM data. 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. In addition, variability in RG and EM production in the test genotypes was examined to identify genotypes with broad-based or narrow-based resistance to the isolates used in this study.

Estimates of virulence presented in Figs. 2.3A and 2.3B are expressed based on average RG and EM production per root system of all test genotypes, respectively. The avirulent *M. incognita* isolate was less virulent to the test cowpea genotypes compared to the virulent *M. incognita* and *M. javanica*

isolates, and the virulence indexes of the avirulent *M. incognita* isolate based on RG and EM data were 12% and 5%, respectively. Based on RG data, the *M. javanica* (VI = 50%) and the virulent *M. incognita* (VI = 42%) isolates were about twice as virulent as the avirulent *M. incognita* isolate (Fig. 2.3A). The differences in RG levels between *M. javanica* and virulent *M. incognita* isolates (Table 2.3) were not statistically significant ($P > 0.05$), but their RG levels were significantly different than that of avirulent *M. incognita* ($P < 0.05$).

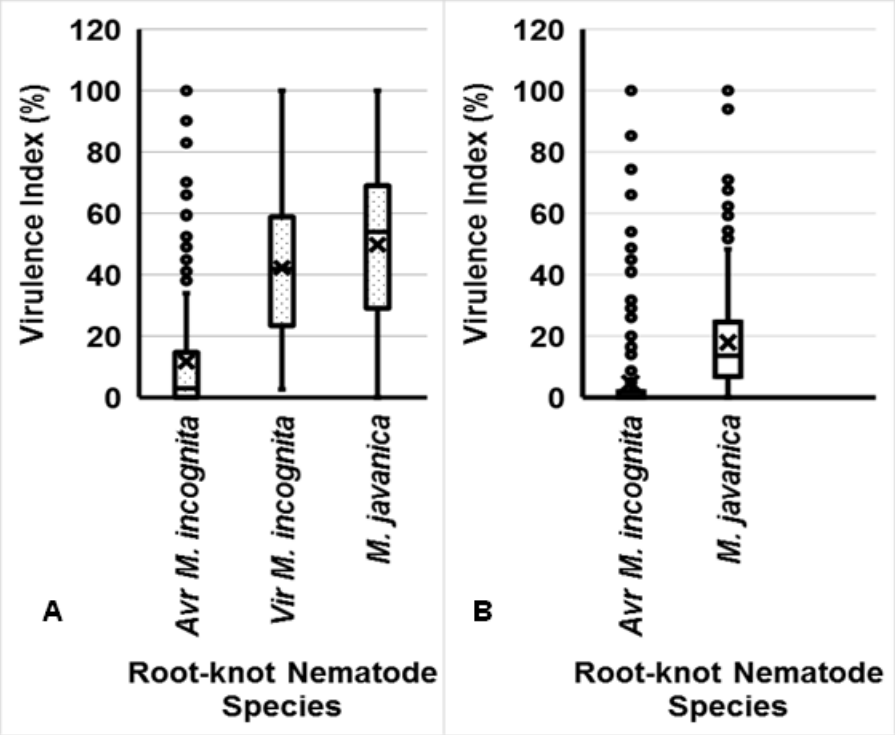

Fig. 2.3. (A): Average virulence index estimates of *M. javanica* "Project 811" and Avr and Vir *M. incognita* "Beltran" and "Muller", respectively. Estimates based on the average root-galling data – Exp. 5, 2014 (Table 2.2); (B): Average virulence index estimates of *M. javanica* "Project 811" and Avr *M. incognita* "Project 77". Values are the average of egg-mass data from Exps. 1, 4 and 6, 2012-2014 (see Table 2.2).

Using EM data, estimates of virulence revealed that *M. javanica* “Project 811” was more virulent than the avirulent *M. incognita* “Project 77” (Fig. 2.3B). The average virulence index of *M. javanica* “Project 811” was 18% compared to 5% for avirulent *M. incognita* “Project 77”.

A summary of the spectrum of resistance is given in Table 2.5 (R = resistant and S = susceptible), based on the response of test genotypes to root-galling incited by Avr *M. incognita* "Beltran", virulent *M. incognita* isolate "Muller and aggressive *M. javanica* "811". Seven test genotypes (FEAF-14-INE, FN-2-9-04, Gile-k-local, INIA-5A, Namuesse-D, VAR-11-D and VAR-3A) exhibited broad-based resistance effect against all three RKN isolates; in contrast, INIA-41 and Maputo were only resistant to the avirulent and virulent *M. incognita* isolates "Beltran" and "Muller”, respectively.

Table 2.5. Root-knot nematode resistance spectrum of selected test cowpea genotypes, based on root-galling phenotypes – field Exp. 5, 2014, (see Table 2.2). R = resistant and S = susceptible.

	Nematode isolate		
	Avr *M. incognita* "Beltran"	**Vir *M. incognita* "Muller"**	***M. javanica* "811"**
Genotype	**Response**	**Response**	**Response**
CB27	R	R	R
CB46	R	S	S
CB46-gg	R	R	S
CB46-Null	S	S	S
FEAF14INE	R	R	R
FN-2-9-04	R	R	R
Gile-K-Local	R	R	R
INIA-41	R	R	S
INIA-5A	R	R	R
INIA-5E	R	S	S
Maputo	R	R	S
Namuesse-D	R		R
NIL-2 genes	R	R	R
NIL-3 genes	R	R	R
UCR 779	S	S	S
VAR-11D	R	R	R
VAR-3A	R	R	R

Relationship between Galling and Nematode Reproduction

The relationship between root-galling and nematode reproduction responses was examined to determine whether the response to both traits in the test cowpea collection is under control by the same or different genetic factors. This analysis was performed using root-galling and egg-mass production data collected in greenhouse and seedling growth-pouch tests (Exps. 1, 3, 4, 6, and 7; see Table 2.2). Also, root-galling phenotypes observed under field infestation by the Avr *M. incognita* isolate "Beltran", virulent *M. incognita* "Muller and

aggressive *M. javanica* "811" were analyzed to determine the relationship between genetic determinants for resistance against the three RKN isolates.

The root-galling and egg-mass production responses under infestation by *M. javanica* “Project 811” were moderately correlated ($r = 0.60$, $P < 0.0001$). This relationship is illustrated in Fig. 2.4, and it was weakly ($R^2 = 0.34$) explained by variability of response of the test genotypes to root-galling and egg-mass production. Based on this relationship, three classes of genotype responses were identified in the cowpea test genotypes: (i) genotypes with low root-galling and low egg-masses per root system (GI from 0 to 3; for example, FN-2-9-04); (ii) genotypes showing moderate root-galling and moderate egg-mass production (GI from 3 to 4; e.g., Timbawene-Monteado), and (iii) heavily galled genotypes with high egg-mass numbers (GI > 5; e.g., INIA-76).

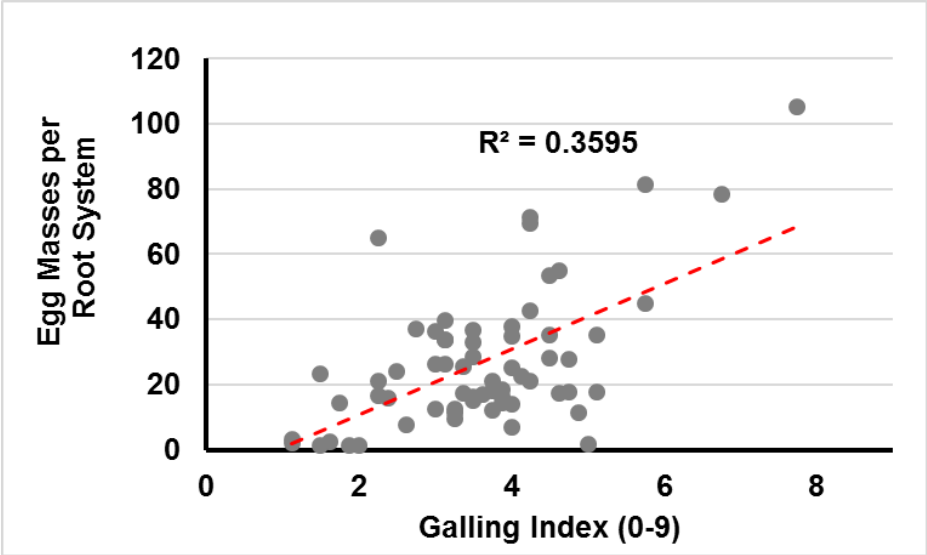

Fig. 1.4. Relationship between root-galling and egg-mass production under *M. javanica* infestation.

Root-galling phenotypes induced by virulent *M. incognita* "Muller" and by *M. javanica* "Project 811" were highly and significantly correlated ($r = 0.98$, $P < 0.0001$) as illustrated in Fig. 2.5A, and the relationship was explained ($R^2 = 0.97$) by the variability in response of test genotypes to root-galling under infestation by both nematode isolates.

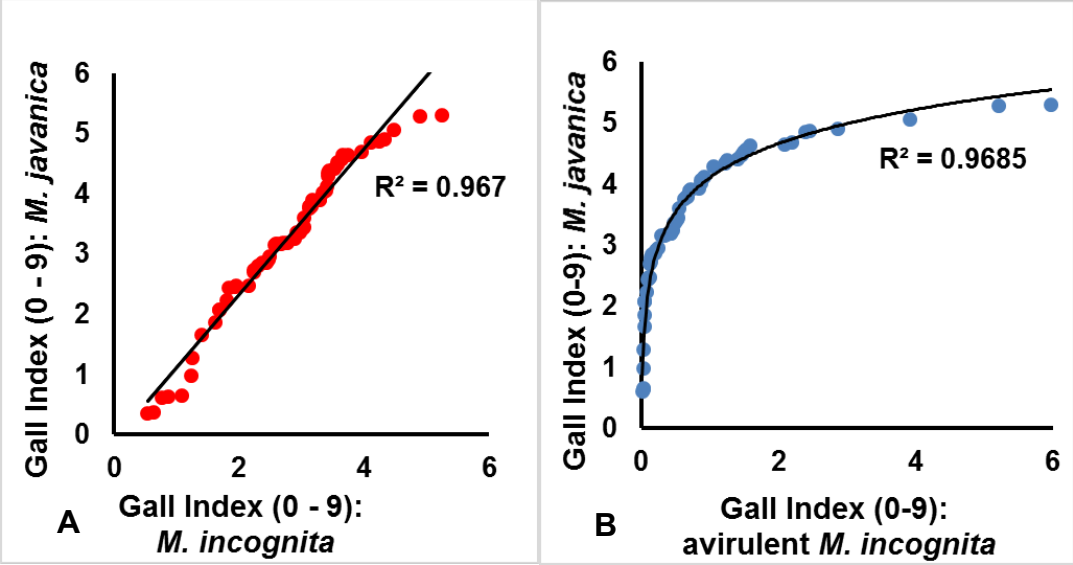

Fig. 2.5. Relationship between root-galling induced by (A): virulent *M. incognita* "Muller" and *M. javanica* "Project 811" and by (B): avirulent *M. incognita* "Beltran" and *M. javanica* "Project 811".

Root-galling responses to avirulent *M. incognita* "Beltran" and to *M. javanica* "Project 811" were highly correlated ($r = 0.72$, $P < 0.0001$) (Fig. 2.5B), and the relationship was largely explained ($R^2 = 0.97$) by the observed variability in root-galling among the test genotypes under infestation by both nematode isolates. In this relationship, the test genotype could be classified into three groups: (i) *M. javanica* resistant genotypes (GI = 0 to 3) that show low or no root-galling under avirulent *M. incognita* infestation, (ii) moderately resistant genotypes that

are resistant to root-galling under avirulent *M. incognita* (GI = 3 – 4), and (iii) genotypes that are susceptible to both RKN isolates.

Resistance Effectiveness

The highly resistant test genotypes were further screened for response to root-galling induced by avirulent *M. incognita* ("Beltran" and "Project 77"), virulent *M. incognita* "Muller" and *M. javanica* "Project 811" under greenhouse (Exps. 8 and 10, see Table 2.2), growth chamber and field (Exps. 9 and 11) conditions. Analysis of resistance and the relationship between root-galling and nematode reproduction was conducted on data from greenhouse and seedling growth-pouch tests (Table 2.6).

The test genotype FN-2-9-04 was selected to develop F_1 populations by hybridizing it with test genotypes INIA-41, CB46-Null, Ecute and CB46, which were used as female parents. Ten F_1 plants per cross were phenotyped together with their parents and control genotypes (Table 2.6) for root-galling response and egg-mass production by *M. javanica* "Project 811" under greenhouse and growth-chamber conditions, respectively. The F_1 populations were phenotyped for resistance to egg-mass production in seedling growth-pouches, then transplanted into 5-liter pots containing sandy soil (UC-mix 80:20) and re-inoculated with 10^4 eggs of *M. javanica* isolate "Project 811".

Table 2.6. Effectiveness of the genetic resistance to root-galling and nematode reproduction by *M. javanica* isolate "Project 811 in resistant cowpea genotypes and in F_1 populations.

	Root Galling	Nematode Reproduction
Genotype	**Gall Index (0-9)**	**Egg Masses**
CB46	6.5[a]	41.3[c]
CB46 x FN-2-9-04 F1	1.6[b]	9.3[d]
Ecute x FN-2-9-04 F1	2.0[b]	14.2[dc]
FAEF-14-INE	0.0[b]	---
FN-2-9-04	0.3[b]	1.7[d]
INIA-41	7.1[a]	44.0[cf]
INIA-41 x FN-2-9-04 F1	1.5[b]	4.0[d]
INIA-5A	0.0[b]	---
Namuesse-D	0.1[b]	---
CB46-Null	7.7[a]	46.7[c]
CB46-Null x FN-2-9-04 F1	3.0[b]	1.8[d]
UCR779	7.6[a]	77.4[e]
VAR-11D	0.0[b]	---
VAR-3A	0.0[b]	---
CB3-gg	7.0[a]	64.2[cef]
Ecute	5.8[a]	81.6[e]
Mean	4.1	31.7
LSD (*P < 0.05*)*	1.34	31.4

*LSMD = Least square mean differences, means with same letter are not significantly different.

The genotypes had significant effect on both root-galling and egg-mass production (*P < 0.0001*). The test genotypes FAEF-14-INE, FN-2-9-04, INIA-5A, Namuesse-D, VAR-11D and VAR-3A were consistently resistant to root-galling induced by *M. javanica* "Project 811", but the observed differences in response to root-galling among these test genotypes were not different. However, they were more resistant than the control genotypes CB46, INIA-41, CB46-Null, UCR779, CB3-gg and Ecute (*P < 0.05*).

The F_1 plants (average root-galling range 1.63 - 3) suppressed root-galling induced by *M. javanica* "Project 811", compared to female parents (CB46, CB46-Null, INIA-41 and Ecute) and the genotype CB3-gg (*P < 0.05*), but did

not differ from test genotypes FAEF-14-INE, FN-2-9-04, INIA-5A, Namuesse-D, VAR-11D and VAR-3A ($P > 0.05$). The F_1 populations also showed an overall similar root-galling response under this nematode isolate ($P > 0.05$) (Table 2.6). The test genotype FN-2-9-04 was more resistant to egg-mass production by *M. javanica* "Project 811", than the controls CB46, CB46-Null, Ecute and INIA-41 ($P < 0.05$). The responses of test genotypes to egg-mass production were similar to that of F_1 populations ($P > 0.05$). The F_1 populations supported lower egg-mass production than female parents (CB46, CB46-Null, INIA-41 and Ecute) ($P < 0.05$).

Discussion

This research was conducted to: (i) determine the variability of response to root-galling and nematode reproduction by avirulent and virulent *M. incognita* isolates and by aggressive *M. javanica* in a cowpea collection from Mozambique, compared to susceptible and resistant controls; (ii) determine the virulence of the nematode isolates and the spectrum of resistance in putative resistant test genotypes; and (iii) determine the effectiveness of resistance in putative resistant test genotypes and the relationship between the genetic factors controlling root-galling and nematode reproduction in the test cowpea collection.

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. Additional field, greenhouse and seedling growth-pouch screens consistently indicated that these seven genotypes carry strong genetic resistance to avirulent *M. incognita* and *M. javanica*. In the greenhouse experiment with *Rk*-virulent *M. incognita*, of the seven resistant test genotypes only FN-2-9-04 and VAR-11D were resistant to root-galling. However, in the greenhouse test with the virulent *M. incognita*, 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 of that observed in CB46 and the susceptible near isogenic line (CB46-Null) lacking any RKN resistance gene,

respectively, which indicated effective resistance to reproduction by this nematode isolate.

Some inconsistency was observed among the virulent *M. incognita* experiments (field vs greenhouse). This might be explained by differences in virulence between the field and greenhouse isolates. Estimated virulence indices of this nematode in the field and in the greenhouse on the basis of root-galling data were 42 and 75%, respectively. Differential virulence between field and greenhouse maintained populations of *M. incognita* (the same isolate as used in this study) was reported by Petrillo et al (2006) and Petrillo and Roberts (2005). It is likely that the virulence in the greenhouse isolate is fixed genetically compared to the field isolate.

Analysis of nematode virulence confirmed that both virulent *M. incognita* and *M. javanica* have greater ability to cause damage on cowpea root systems than avirulent *M. incognita* as expected, based on root-galling. Although the average virulence index of virulent *M. incognita* was lower than that of *M. javanica*; based on field data both nematode isolates had similar root-galling impact on the test genotypes. 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 resistance of these seven genotypes than the commercial cultivar CB46 (which

carries the *Rk* gene) 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 test with virulent *M. incognita*, and this genotype consistently tested resistant to this nematode isolate on the basis of root-galling and egg production per gram of root.

The effectiveness of genetic factors in conferring resistance to the RKN isolates in this study could be influenced by at least three factors: (i) resistance specificity; (ii) the level of resistance (low vs high); and (iii) the composition of resistance (single vs additive gene effect). Resistance specificity has been reported in other studies; for example, in tobacco (NG' ambi et al.,1999). Resistance specificity is found in cowpea, the *Rk* gene being highly effective against avirulent *M. incognita* populations and susceptible to 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 against several *M. incognita* isolates, but did exhibit differential response under *M. javanica* infestation. The strength of the *Rk* gene can partially suppress 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 modifies *Rk* resistance to a level that makes the gene combination effective in

controlling both *M. javanica* and virulent *M. incognita* (Ehlers et al., 2000; Ehlers et al., 2002).

The effectiveness of the genetic resistant in test genotypes was further validated by additional screening of their derived F_1 populations. The response of F_1 populations was used test the heritability of resistance found in test genotype FN-2-9-04. The results confirmed that the resistance in this genotype is effective, dominant and heritable. These results indicated that the novel sources of genetic resistance in the Mozambican genotypes can be successfully bred into elite cowpea cultivars. The relatively large seed of FN-2-9-04 would allow transfer of the RKN resistance into elite cultivars without negative effects on seed size, contrary to the reduction in seed size observed when RKN resistance from a West African breeding line, IT84S-2049, was bred into California blackeye cultivar CB46 (carrying only the *Rk* gene) to broaden its genetic resistance.

The relationship between root-galling and nematode reproduction in the test genotypes was indicated by positive correlation, which suggested that both responses are under control by the same resistance determinants. The *R* genes in RKN pathosystems are resistance genes that suppress nematode development and nematode reproduction in root systems, and in the process 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 strength of the observed relationship between root-galling and egg-mass production in the present study led to the hypothesis that in some backgrounds root-galling and nematode reproduction responses might

be under control by at least some independent genetic factors. For example, the control genotype CB3-gg was resistant to root-galling incited by avirulent *M. incognita*, but was susceptible to reproduction by the same nematode isolate, which suggests that the RKN resistance gene in this breeding line is limited to root-galling response. This may be similar to the situation in lima bean, where root-galling and nematode reproduction responses were reported to be under independent genetic controls (Roberts et al., 2008).

Root-galling incited by *M. javanica* in the test cowpea genotypes was strongly and positively correlated to root-galling incited by both avirulent and virulent *M. incognita*, suggesting that broad-spectrum resistance occurs in some of the test genotypes, probably promoted by additive effects of gene sets or by genetic factors dedicated to response to specific nematode isolates in test cowpea backgrounds. A practical example of broad-based genetic resistance to RKN is found in the cultivar CB27 with a gene set comprised of the *Rk* gene and a minor effect recessive gene, *rk3*, which enhances the response of *Rk* under infestation by virulent *M. incognita* and *M. javanica* (Ehlers et al., 2000). The *Rk* gene alone does not provide effective resistance to these RKN isolates. The association between root-galling response under infestation by virulent *M. incognita* and *M. javanica* is not absolute because the resistance response in the test genotype INIA-41 was highly effective against both avirulent and virulent *M. incognita*, but not effective against *M. javanica*. Also, the resistance in breeding line CB3-gg was exclusively effective in suppressing root-galling by avirulent and virulent *M. incognita* isolates, but not to *M. javanica*. Association

between root-galling induced by *M. incognita* and *M. javanica* was also reported in tomato accessions and cultivars by Ammati et al. (1985).

In summary, this study identified novel sources of broad-based genetic resistance to 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 particularly to avirulent *M. incognita* and *M. javanica*, and their responses to both root-galling and nematode reproduction were effective.

Field populations of virulent *M. incognita* "Muller" and *M. javanica* "Project 811" were more virulent than the avirulent *M. incognita* isolate "Beltran", and the greenhouse-maintained isolate of *M. javanica* ("Project 811") allowed validation of the effectiveness of the test resistance genotypes. However, differential virulence level between field and greenhouse-maintained isolates of virulent *M. incognita* ("Muller") led to inconsistent results between the two test conditions in the effectiveness of resistance of test genotypes to this nematode isolate. The seven identified resistant test genotypes exhibited a broad-based genetic resistance, although some appear to carry a low level of resistance to virulent *M. incognita* with fixed virulence (e.g. FAEF-14-INE and VAR-3A). The genetic resistance in INIA-41 was specific to both avirulent and virulent *M. incognita*. Most test cowpea genotypes were resistant to avirulent *M. incognita* isolates "Beltran" and "Project 77" indicating that the *Rk* gene or a gene equivalent to it is predominant among in them. Also, the observed variability in response among the tested cowpea genotypes suggested that the test collection is likely

to contain minor and major effect resistance genes, which are of value for breeding cowpea cultivars with broad-based resistance.

The RKN resistance in FN-2-9-04 was found to be heritable and dominant. In some backgrounds among the test genotypes the resistance to root-galling and nematode reproduction might be under control by the same resistance factors. Also, in some test genotypes resistance to root-galling induced by avirulent *M. incognita* and *M. javanica* might be under control by the same genetic factors or by sets of genetic factors acting in additive fashion.

This study identified novel sources of resistance to RKN; however, the genetics underlying resistance to RKN isolates in this study and the genetic architecture of resistance in the resistant test genotypes is under investigation, and its uniqueness and its potential value for improvement of RKN resistance in cowpea commercial cultivars currently available in the market is still to be determined.

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CHAPTER III - A Novel Root-Knot Nematode Resistance QTL in Cowpea Accession FN-2-9-04 from Mozambique

Abstract

The root-knot nematode (RKN) species, *Meloidogyne incognita* and *M. javanica* cause substantial root system damage and suppress yield of susceptible cowpea cultivars. The narrow-based genetic resistance conferred by the *Rk* gene, present in some commercial cultivars, is not effective against *Rk*-virulent populations found in several cowpea production areas. The dynamics of virulence within RKN populations demand a broadening of the genetic base of resistance in elite cowpea cultivars. As part of this goal, F_1 and F_2 populations from the cross CB46-Null (susceptible) x FN-2-9-04 (resistant) were phenotyped for *M. javanica* induced root-galling (RG) and egg-mass production (EM) in controlled growth chamber and greenhouse infection assays. In addition, $F_{2:3}$ families of the same cross were phenotyped for RG on field sites infested with *Rk*-avirulent *M. incognita* and *M. javanica*. The response of F_1 to RG and EM indicated that resistance to RKN in FN-2-9-04 is partially dominant, as supported by the degree of dominance in the F_2 and $F_{2:3}$ populations (D/A = 0.4 – 0.5). Two resistance QTLs associated with RG and EM were detected on chromosomes Vu01 and Vu04 ($P < 0.05$) of the cowpea consensus genetic map, and the QTL on Vu01 (PVE = 34% - 94%) was more effective against the aggressive *M. javanica* isolate, whereas both QTLs (Vu01 - PVE = 27.9% and Vu04 - PVE = 73.4%) were effective against avirulent *M. incognita* isolate. Allelism tests indicated that CB46 and FN-2-9-04 share the same RKN resistance locus on Vu04, but that strong, broad-based resistance in FN-2-9-

04 is conferred by the additive effect of a novel resistance QTL on Vu01. This novel resistance in FN-2-9-04 is important for broadening RKN resistance in elite cowpea cultivars.

Introduction

Root-knot nematode (RKN) species, particularly *Meloidogyne incognita* and *M. javanica*, cause substantial damage to root systems of susceptible cowpea cultivars, which impairs water and nutrient uptake, remobilization, partitioning and translocation of photo-assimilates (Bird and Loveys, 1975; McClure,1977; Taylor and Sasser, 1978; Williamson and Hussey, 1996; Sikora et al., 2005). This damage suppresses yield of susceptible cowpea cultivars. Host-plant resistance is an important strategy to mitigate the impact of nematode infestation (Hall and Frate, 1996; Roberts, 1992; Ehlers et al., 2000; Castagnone-Sereno, 2002; National Research Council, 2006), particularly in Africa where the accessibility to sophisticated agronomic inputs including nematicides is limited (Sasser, 1980; Luc et a., 2005). Even in developed agriculture, such as in the U.S.A, reliance on nematicides for RKN management in cowpea is not an option due to high cost (Ehlers et al., 2000) and environmental concerns (Hall and Frate, 1996; Roberts, 1992; Castagnone-Sereno, 2002; Luc et al., 2005).

Narrow-based genetic resistance to RKN, conferred by the gene *Rk*, has provided protection against RKN in cowpea agricultural systems worldwide (Amosu and Franckowiak, 1974; Singh and Reddy, 1986; Helms et al., 1991; Fery et al., 1994; Roberts et al., 1995; Roberts et al., 1996; Roberts et al., 1997;

Ehlers and Hall, 1997; Ehlers et al., 2009). The resistance conferred by gene *Rk* is highly effective against avirulent forms of RKN populations (Roberts et al., 1995; Hall and Frate, 1996; Roberts et al, 1997; Ehlers et al., 2000; Roberts et al., 2013), but its weak effectiveness against virulent isolates of common RKN species raises concern about its future sustainability for RKN management in cowpea production. *Rk*-virulent nematode populations of *M. javanica* and *M. incognita* have been reported in California (Swanson & Van Gundy, 1984; Roberts et al., 1995; Hall and Frate, 1996; Roberts et al.; 1997; Petrillo et al., 2006). Selection for virulence in RKN populations (Roberts et al, 1997; Petrillo and Roberts, 2005; Petrillo et al., 2006) has prompted a broadening of the genetic base of resistance in elite cowpea cultivars which is based on the *Rk* gene (Hall and Frate, 1996; Roberts et al., 1996; Roberts et al., 1997; Ehlers et al., 2000; Roberts et al., 2013). The threat imposed by virulence plasticity of RKN species led to the discovery of new resistance genes, Rk^2, rk^3 and *gg*, to broaden the genetic base of resistance, and advanced breeding materials with one or more of these genes have shown promising performance under RKN infestation (Roberts et al., 1996; Roberts et al., 1997; Ehlers et al., 2000; Ehlers et al., 2002; Roberts et al., 2013). Broad-based genetic resistance can be developed through effective gene pyramiding of independent sets of strong major and minor genes from distinct genetic sources (Ehlers et al., 2002; Roberts, 2013).

The RKN resistance currently deployed in cowpea cultivars is mainly governed by a single dominant gene, *Rk* (Fery et al., 1994; Singh and Reddy, 1986), but additional resistance genes Rk^2, with a dominant effect, (Roberts et al., 1996;

Roberts et al., 1997; Ehlers et al., 2000), *rk*^{3}, with a recessive effect, (Roberts et al., 1996; Ehlers et al., 2000) and *gg* with presumably recessive effect (Ehlers et al., 2002), have been identified in cowpea backgrounds (Roberts et al., 1997; Ehlers et al., 2000). The action of gene *Rk*^{2} alone is not clearly understood, but in breeding line IT84S-2049 (which also carries gene *Rk*) its additive effect contributes substantially to an enhanced response to virulent populations of *M. incognita* and to a partial extent to *M. javanica* compared to gene *Rk* alone (Roberts et al., 1996; Roberts et al., 1997; Roberts et al., 2005). The gene *rk*^{3} was characterized as a modifier gene which improves resistance of cowpea cultivars carrying *Rk* when challenged with virulent RKN isolates (Ehlers et al., 2000). The gene *gg*, a galling resistance gene, was identified in cv. California Blackeye 3 (CB3); alone it reduces root-galling incited by virulent *M. incognita*, but it provides no resistance to nematode reproduction, and its activity under *M. javanica* infestation is minimal (Ehlers et al., 2002).

The *Rk* locus has been mapped on chromosome Vu04 (previous cowpea linkage group 11) of the cowpea consensus genetic map (Huynh et al., 2016). This genomic region and flanking markers associated with RKN resistance within this region are important resources for introgressing this resistance into elite cowpea cultivars. Also, markers flanking the resistance in this genomic can be utilized as a reference to decipher the genetic relationship between the resistance conferred by gene *Rk* and potential novel sources of resistance to RKN.

A broad-based resistance to RKN has been identified through a series of field, greenhouse and growth pouch tests in a cowpea accession, FN-2-9-04, from Mozambique (see Chapter II). This accession carries stronger resistance to avirulent *M. incognita* and *M. javanica* than that conferred by the *Rk* gene alone. The performance of FN-2-9-04 under *M. javanica* infestation was contrasted to cowpea breeding lines and cowpea cultivars carrying sets of RKN resistance genes, including *RkRk/Rk^{2}Rk^2*, *RkRk/rk^{3}rk^3*, *RkRk/Rk^{2}Rk^2/gg* and IT84S-2049 which indicated that the RKN resistance in accession FN-2-9-04 is unique. Therefore, to characterize the resistance in FN-2-9-04, genetic analyses were conducted to: (i) examine the uniqueness of the genetic resistance; and (ii) to determine its genomic architecture and localization through genetic linkage analysis and QTL mapping.

Materials and Methods

Plant material

Four F_1, three F_2 and one $F_{2:3}$ populations (Table 3.1) were developed under greenhouse conditions (University of California Riverside – UCR). The accession FN-2-9-04 was crossed with CB46-Null, CB46, Ecute and INIA-41. A single F_1 seed from crosses CB46-Null x FN-2-9-04, CB46 x FN-2-9-04 and INIA-41 x FN-2-9-04 was grown to derive three independent F_2 populations, and 150 F_2 lines of populations CB46-Null x FN-2-9-04 were advanced to generate 150 $F_{2:3}$ families (Table 3.1). Subsets of each F_2 population were phenotyped for root-galling and egg-mass production following infection with nematode isolates listed in Table 3.1. Four F_1 populations (CB46-Null x FN-2-9-04, CB46 x FN-2-9-04, INIA-41 x FN-2-9-04, Ecute x FN-2-9-04) and the F_2 populations were phenotyped for root-galling and egg-mass production in greenhouse and seedling-growth pouch screens, respectively. Five to ten seeds per F_1 population were used in each test.

CB46 is a California blackeye cultivar, and the CB46-Null genotype is a near-isogenic breeding line (NIL) derived from cowpea cultivar CB46. This breeding line has the CB46 background, but differs at the genomic region harboring the *Rk* resistance locus (Huynh et al., 2016). Ecute and INIA-41 are a cowpea landrace and accession, respectively, from Mozambique. The accession FN-2-9-04 is resistant to both avirulent *M. incognita* isolates and *M. javanica* isolate "Project 811" used in this study, whereas CB46-Null, CB46, Ecute and INIA-41 are all susceptible to *M. javanica*. In addition, CB46-Null and Ecute are susceptible to the avirulent *M. incognita* isolates (Beltran and Project 77).

Table 3.1. Populations used for inheritance studies and QTL mapping, their sizes, phenotyping conditions, target trait, nematode isolate used and testing period.

Exp.	Population	Size	Environment	Trait	Nematode isolate	Year
1	CB46-Null/FN-2-9-04 (F_2)	163	SGP-UCR	EM	*M.j*	2015
2	CB46/FN-2-9-04 (F_2)	172	SGP-UCR	EM	*M.j*	2015
3	INIA-41/FN-2-9-04 (F_2)	126	GH-UCR	RG	*M.j*	2015
4	CB46-Null/FN-2-9-04 (F_2)	177	GH-UCR	RG	*M.j*	2016
5	CB46/FN-2-9-04 (F_2)	197	GH-UCR	RG	*M.j*	2015
6	CB46/ FN-2-9-04 (F_2)	400	Field-CVARS	RG	Avr. *M.i*	2015
7	CB46/FN-2-9-04 (F_2)	162	Field-KARE	RG	Avr. *M.i*	2015
8	CB46-Null/FN-2-9-04 ($F_{2:3}$)	150	Field-SCREC	RG	*M.j*	2016
9	CB46-Null/FN-2-9-04 ($F_{2:3}$)	150	Field-SCREC	RG	Avr. *M.i*	2016

Exp. – experiment; SGP – seedling growth pouches; GH – greenhouse; RG – root galling; EM – egg masses; Avr *M.i* – avirulent *M. incognita* and *M.j* – *M. javanica* Project 811; UCR = University of California Riverside; CVARS = University of California Coachella Valley Agricultural Research Station; KARE = University of California Kearney Agricultural Research and Extension Center

Root-knot nematode isolates

Three RKN isolates were used to phenotype plant materials for response to infection. Two *M. incognita* isolates, Beltran and Project 77 are avirulent to the *Rk* gene, with little or no galling and EM production on root system of plants carrying gene *Rk* (Roberts et al*.,* 1995; Roberts et al*.,* 1996; Roberts et al*.,* 1997), whereas *M. javanica* isolate Project 811, is an aggressive isolate due to its enhanced virulence (Ehlers et al., 2000; Ehlers et al., 2009), inducing galling and reproducing successfully on roots of plants carrying gene *Rk* (Thomason and Mckinney, 1960; Roberts *et al.,* 1997; Ehlers et al., 2009).

Resistance phenotyping: egg-mass production

The F_1 and F_2 populations (Table 3.1) plus parental genotypes were phenotyped for EM production of *M. javanica*, in seedling growth-pouches according to Ehlers et al. 2000 and Atamian et al., 2012. Briefly, a single seed of each F_1 and F_2 was planted per plastic pouch, and plants grown in a controlled environment chamber with day/night temperatures set at 28/22 ^{o}C under 16 h day-length. Plants were inoculated two weeks after germination with 1500 freshly hatched second-stage juveniles (J_2) of *M. javanica*. Two days after inoculation, plants were supplied daily with fertilizer for 3-5 days using half-strength Hoagland's solution (Hoagland and Arnon, 1950). Thirty-five days after inoculation the pouches were irrigated with erioglaucine dye (Sigma Chemical Co., St. Louis, MO, USA) to stain egg-masses, which were counted under 10X magnification.

Resistance phenotyping: Root galling

Phenotyping for resistance to root-galling was conducted under greenhouse and field conditions in 2015 and 2016 (Table 3.1). In the greenhouse, the F_1 and F_2 populations and parental genotypes phenotyped for response to *M. javanica* isolate Project 811 egg-mass production in seedling growth-pouches (in growth chamber conditions) were transplanted into 4L pots containing soil UC-mix 3 and maintained at 28/22 ^{o}C day/night temperatures. After 21 days, each plant was inoculated with 10 ml of *M. javanica* egg suspension in water adjusted to 1000 egg/ml. The F_2 population INIA-41 x FN-2-9-04 (Table 3.1) also was phenotyped for root-galling response under greenhouse conditions.

All greenhouse-grown plants were irrigated twice a day by drip-irrigation for about 90 days to allow seed production, and $F_{2:3}$ seeds were collected from each F_2 plant. After seed collection, the plant tops were cut at 2 – 3 cm above the soil line and the roots were washed and scored for root-galling response, under 10X magnification, using a 0 - 9 gall index (GI) (Bridge and Page, 1980): 0 = no galls on root system; 1 = very few, small galls and hard to see; 5 = generally large galls can be seen on the root system and the taproot slightly bumped, with bumps of different sizes; 9 = large galls on the root system, and most lateral roots lost.

Field experiments were conducted in 2015 and 2016 at three sites (Table 3.1). At CVARS and KARE, 400 and 162 F_2 lines respectively of CB46 x FN-2-9-04 were phenotyped for root-galling response to avirulent *M. incognita* isolate Project 77. In 2016 at SCREC parental genotypes, F_2 and $F_{2:3}$ populations were phenotyped for root-galling response in separate fields infested with avirulent *M. incognita* isolate Beltran or *M. javanica* isolate Project 811 (Table 3.1). In both experiments (Exps. 8 and 9), $F_{2:3}$ families with 25 – 30 plants/family were planted as single replicate.

The *M. javanica* isolate Project 811 used in the pot and seedling growth pouch tests was the same isolate used to infest field sites. In the field evaluations, 25-30 plants were evaluated per $F_{2:3}$ family and F_2 population. For both F_2 and $F_{2:3}$ generations, 30 seeds were planted on a 1.5 m-long bed, and 60 days after plant emergence plant tops were cut at about 2 – 3 cm above the soil line, and the root systems dug and evaluated for root-galling using the same root-galling index described for the pot tests (Bridge and Page, 1980).

Inheritance of resistance and allelism test

Segregation for resistance to root-galling and reproduction of *M. javanica* and avirulent *M. incognita* isolates in FN-2-9-04 was determined using phenotypic (root-galling and egg-masses) and genotypic data. In addition, phenotypic data of F_1, F_2 and $F_{2:3}$ populations, and SNP marker genotypes of F_2 populations at mapped QTL regions were processed for goodness-of-fit analysis to determine the genetic model underlying resistance to RKN in FN-2-9-04. In addtition, the numbers of genes determining resistance were estimated using the Castle-Wright (1921) estimator of gene number, $n = \frac{(P1-P2)^2}{8Vg}$, where *n* is the estimated number of genes influencing the trait, *P1 and P2* are the mean phenotypic values of the parents of the population and *Vg* is the genetic variance of the trait. To estimate the number of genes governing response to root-galling and egg-mass production, the *Vg* influencing these traits was derived as the genetic variance in the mapped QTL regions, flanked by known SNP markers.

Broad-sense heritability ($H^2 = V_g/V_p$) of resistance was estimated using two methods, midparent-offspring regression analysis (Fernandez and Miller, 1985; Falconer and Mackay, 1996) and the phenotypic variation among F_2 lines and among $F_{2:3}$ families accounted for by V_g at the QTL regions. The phenotypic variance, V_p, in root-galling or egg-masses attributed to genetic factors, V_g, was estimated using SNP marker genotype scores at the mapped QTL regions. To estimate the narrow-sense heritability ($h^2 = V_a/V_p$), the genetic variance ($Vg = V_a + V_d$) was partitioned into additive and dominance variances, and the V_a component was used to compute the h^2 of the trait. Root-galling data of 7 F_2 populations and parental genotypes were used to perform midparent-offspring

regression analysis, and four mapping populations (2 F_2 and 2 $F_{2:3}$) were used to derive genetic variances within the QTL regions, influencing the response to galling and egg-mass production. Allelic relationship between the *Rk* locus present in CB46 (Roberts et al., 1995; Hall and Frate,1996; Roberts et al., 1996; Roberts et al., 1997; Ehlers et al., 2009; Huynh et al., 2016) and the genetic determinants of resistance in FN-2-9-04 was determined using the 4 F_2 population sets of CB46 x FN-2-9-04 phenotyped with *M. incognita* isolate Project 77 and *M. javanica* infestation (Table 3.1).

Linkage and QTL mapping

Leaf samples were collected from parents and each of 119 F_2 lines of population CB46-Null x FN-2-9-04 30 days after transplanting and dried in plastic ziploc bags containing silica gel packs. Genomic DNA was extracted from dried leaves using Plant DNeasy (Qiagen protocol) and quantified using Quant-iTTM dsDNA Assay Kit and fluorescence measured using a microplate reader. In addition, each F_2 plant was selfed to generate $F_{2:3}$ seeds for field phenotyping (Table 3.1). This F_2 population is the same that was evaluated for resistance to *M. javanica* (isolate “Project 811”) reproduction and root-galling in seedling-growth pouch and greenhouse tests.

Each DNA sample was assayed for single nucleotide polymorphism (SNP) using the 51128 Illumina iSelect SNP genotyping platform (Munoz-Amatriain et al., 2017). The SNP data were filtered for quality as follows: (i) elimination of SNPs with missing data > 20%; (ii) elimination of monomorphic SNPs; (iii)

elimination of SNPs with minor allele frequency (MAF) < 40%; iv) and elimination of duplicate lines. No loci were detected with non-parental alleles.

A linkage-map of F_2 population CB46-Null x FN-2-9-04 was constructed with MSTmap program (Wu et al., 2015), and linkage groups were determined at LOD threshold = 10 and marker placement followed the Kosambi mapping function. The options “no mapping size threshold” and “no mapping distance threshold” were fixed at 2 units and 10 cM, respectively. In addition, the no mapping distance threshold option was set at 15 cM and the detection of genotyping errors was not solicited. The linkage groups of the final genetic map were numbered and ordered following the cowpea consensus genetic map order (Munoz-Amatriain et al., 2017) and following the new linkage group naming (Lonardi et al., 2017).

QTL mapping was performed using four phenotypic data sets comprising 2 F_2 populations and 2 $F_{2:3}$ populations of cross CB46-Null x FN-2-9-04 (Table 3.1). QTL analysis was performed following the mixed-model for QTL mapping described by Xu (2013) using the SAS University Edition 3.2.2, and significant QTL presence was declared using Bonferroni adjusted threshold at $P < 0.05$.

Results

Inheritance of resistance to RKN in FN-2-9-04

The Figs. 3.1A and 3.1B show the response of four F_1 populations and their parental genotypes to root-galling (RG) and egg-mass (EM) production, respectively by the aggressive *M. javanica* isolate Project 811. All recurrent parents (Ecute, CB46, INIA-41 and CB46-Null) exhibited susceptible phenotypes for RG and EM, and their mean RG scores and EM scores ranged from 5.8 to 7.7 and 41 to 82, respectively, whereas the resistant parent, FN-2-9-04 had mean RG and EM scores of 0.4 and 4, respectively.

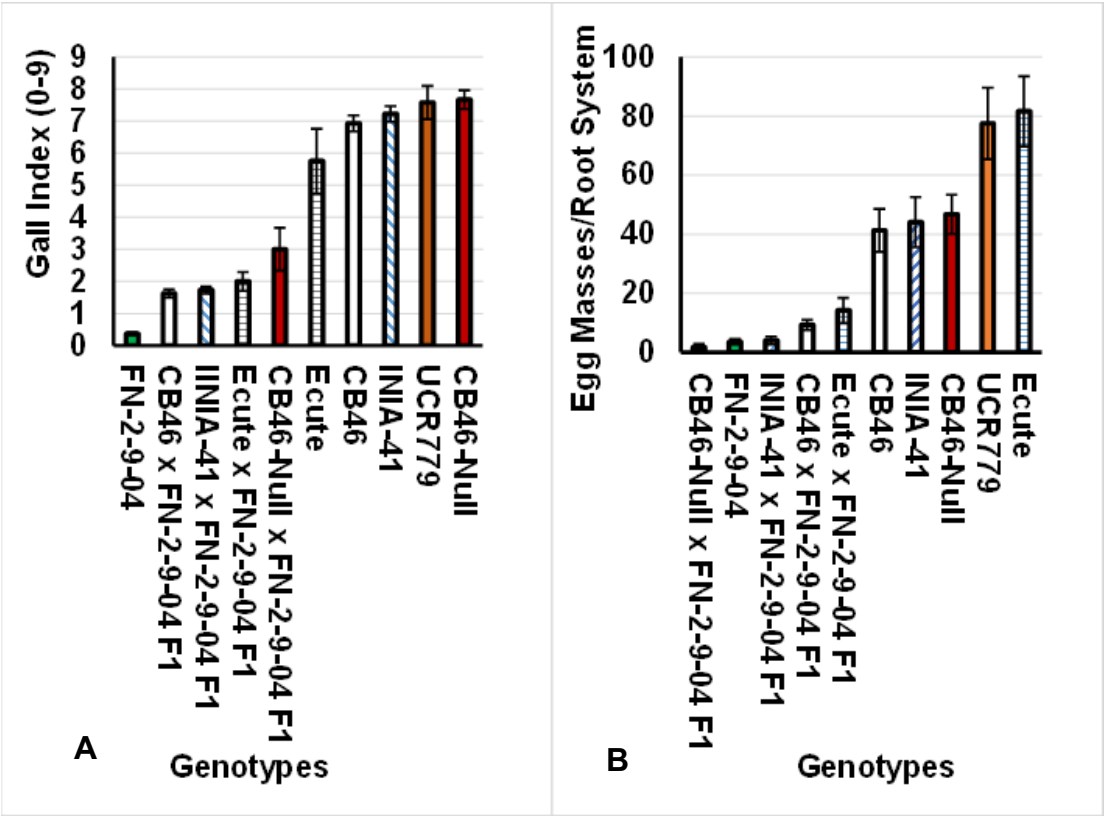

Fig. 3.1. Response of F_1 populations to: (A) root-galling and (B) egg-mass production by *M. javanica* isolate Project 811 in pot and seedling-growth pouch inoculations, respectively.

All F_1 populations were resistant to *M. javanica* RG and EM production (Figs. 3.1A and 3.1B), with mean RG and EM scores below the mid-parent RG and EM score (GI = 6.9 and EM = 53). The F_1 population of CB46-Null x FN-2-9-04 had the highest mean RG (GI = 3) of the four F_1 populations. The observed differences in RG and EM between the resistant and susceptible parents were significant ($P < 0.05$), but the resistant parent and F_1 populations phenotypes were not different.

The segregation of F_2 (Fig. 3.2A) and $F_{2:3}$ (Fig. 3.2B) populations for *M. javanica* RG appeared to follow a bimodal distribution, and in all populations the response to *M. javanica* was skewed toward lower RG, and the segregation of RG in F_2 and $F_{2:3}$ populations of CB46-Null x FN-2-9-04 induced by avirulent *M. incognita* Beltran followed a similar pattern (Fig. 3.3). A bimodal segregation pattern also was observed for EM in F_2 populations of CB46-Null x FN-2-9-04 and CB46 x FN-2-9-04 (Fig. 3.2C). This segregation pattern was consistent across all phenotyping environments (greenhouse, field and seedling-growth pouches) and traits (RG and EM). EM ranged from 0 – 180 (Fig. 3.2C), and RG across environments and generations ranged from 0 – 9 (Figs. 3.2 and 3.3). The resistant parent had consistently lower ($P < 0.05$) RG compared to all susceptible parents.

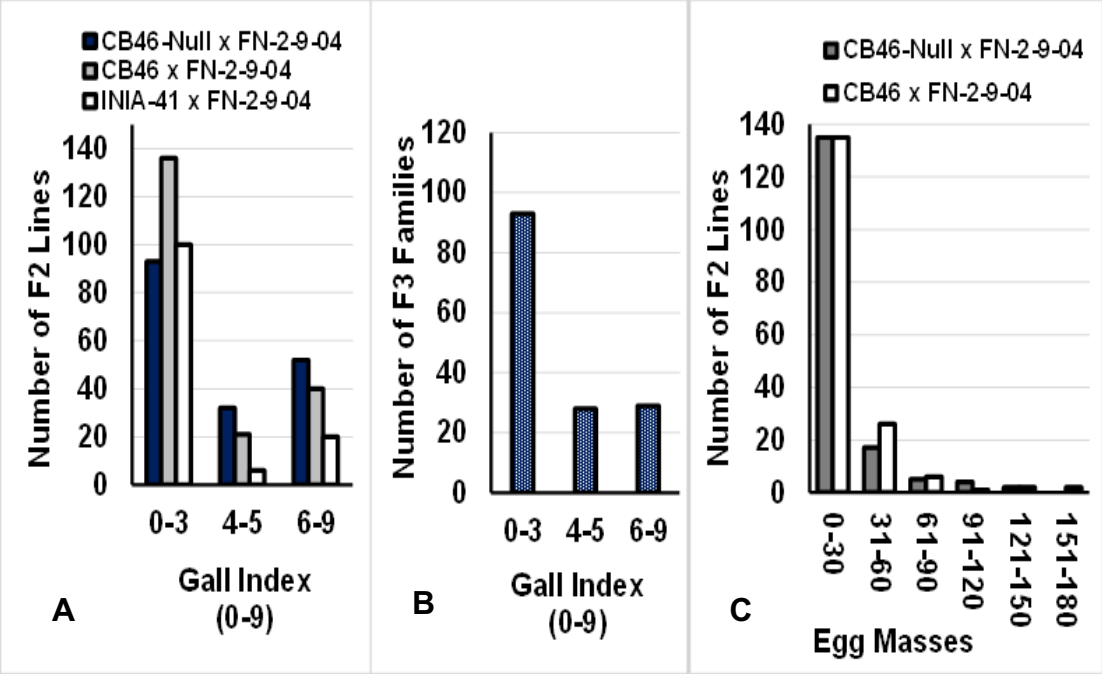

Fig 3.2. Distribution of root galling responses (A) in F2 populations (greenhouse, 2015/16), (B) $F_{2:3}$ population CB46-Null x FN-2-9-04 (field, 2016), and (C) egg mass production in F2 populations CB46-Null x FN-2-9-04 and CB46 x FN-2-9-04 (seedling-growth pouch test, 2015) under *M. javanica* isolate Project 811 infestation.

RG and EM responses in F_2 populations CB46 x FN-2-9-04 and CB46-Null x FN-2-9-04 were highly correlated ($r = 0.78$, $P = 0.008$ and $r = 0.62$, $P = 0.06$, respectively), although the correlation between these traits in the F_2 population CB46-Null x FN-2-9-04 was not significant ($P > 0.05$).

The segregation ratio between resistant-susceptible lines in the F_2 was determined through marker-trait association analysis performed using marker genotype within mapped QTL regions (Table 3.2) and phenotypic response of F_2 and $F_{2:3}$ populations.

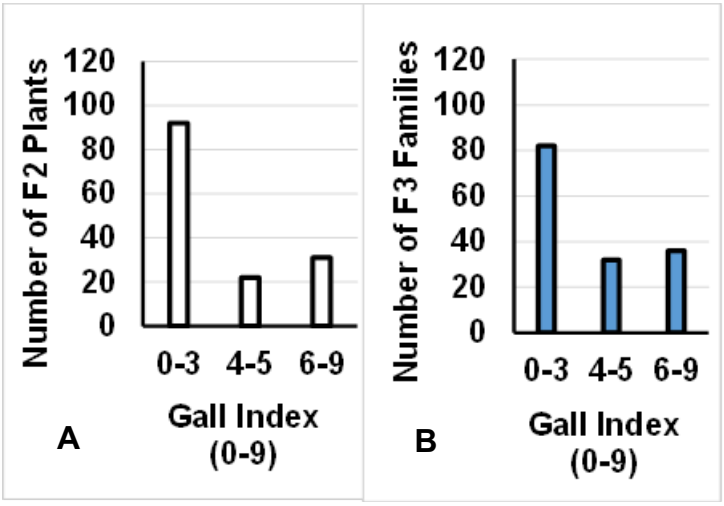

Fig. 3.3 Distribution of root galling response in the F2 (A) and F2:3 (B) populations of CB46-Null x FN-2-9-04 under field infestation with avirulent M. incognita isolate Beltran.

Each F_2 plant was scored for presence of parental genotypes at each locus, and scores 2,1 and 0 were assigned to homozygous resistant allele (BB = resistant parent), heterozygous (AB) and homozygous susceptible allele (AA = susceptible parent), respectively. The genotype of each F_2 plant, within the QTL region, was determined as the mean score across all marker loci, and it was associated with its RG or EM phenotypic response determined at the F_2 and $F_{2:3}$ generations. The data for frequency distribution of genotypes (BB, AB and AA) (Table 3.2) were processed for goodness-of-fit analysis, and the chi-square values were determined following Yates correction for continuity (Little and Hills; 1978). The 119 F_2 plants assayed for 51128 SNP markers segregated for resistance-susceptibility, and closely fit a ratio of 13:3 within each mapped QTL regions (Table 3.2), and also a 3:1 ratio was significant, suggesting that the resistance to RKN at both QTL regions is mainly governed by one dominant gene or a combination of genes acting under dominant-recessive interaction.

The fit to a 13:3 ratio could also indicate genetic distortion for a single dominant gene.

Table 3.2. Best fit segregation ratios (resistant : susceptible) in 119 F_2 plants derived from cross CB46-Null x FN-2-9-04 determined using SNP marker loci at the RKN QTL regions.

F_2 Population	Genotypes (Observed) BB + AB	AA	Exp	X^2	P value	Trait	Vu	Nema
CB46-Null x FN-2-9-04	97	22	13:3[a]	0.002	0.95-0.99	RG	1	Avr
	93	26	13:3[a]	0.56	0.25-0.50	RG	4	*M.i*
CB46-Null x FN-2-9-04	98	21	13:3[a]	0.04	0.50-0.75	RG	1	*M.j*
CB46-Null x FN-2-9-04	98	21	13:3[a]	0.04	0.50-0.75	EM	1	*M.j*

BB = alleles from resistant parent, AB = heterozygous, AA = alleles from susceptible parent; Exp. = expected ratio; RG = root galling, EM = egg masses per root system; Vu = cowpea chromosome (Chr) naming (Lonardi et al., 2017); Nema = Nematode isolate; Avr = avirulent *M. incognita* isolate Beltran, *M.j* = *M. javanica* isolate Project 811. [a] Also fit a 3:1 ratio.

The broad-sense heritability (H^2) of resistance to *M. javanica* root-galling (RG) estimated through regression of 7 field phenotyped F_2 populations to the mean performance of their parents (CB46-Null and FN-2-9-04) was high ($b = 0.76 \pm 0.07$, $P = 0.00004$) (Fig. 3.4); however, estimates of H^2 for the same trait computed using the genetic variance directly derived from the QTL region located on chromosome 1 (Vu01) yielded moderate (0.47) and high (0.94) H^2 estimates for greenhouse and field phenotyped F_2 and $F_{2:3}$ populations, respectively. For these populations, the estimates of h^2 for RG were 0.34 and 0.67, respectively. Egg mass production (EM) response in the F_2 had low H^2 (0.34) (Table 3.3) and h^2 (0.24). The estimated H^2 and h^2 of resistance to RG

induced by avirulent *M. incognita* were 0.28 and 0.19 on Vu01 and 0.73 and 0.49 on Vu04, respectively.

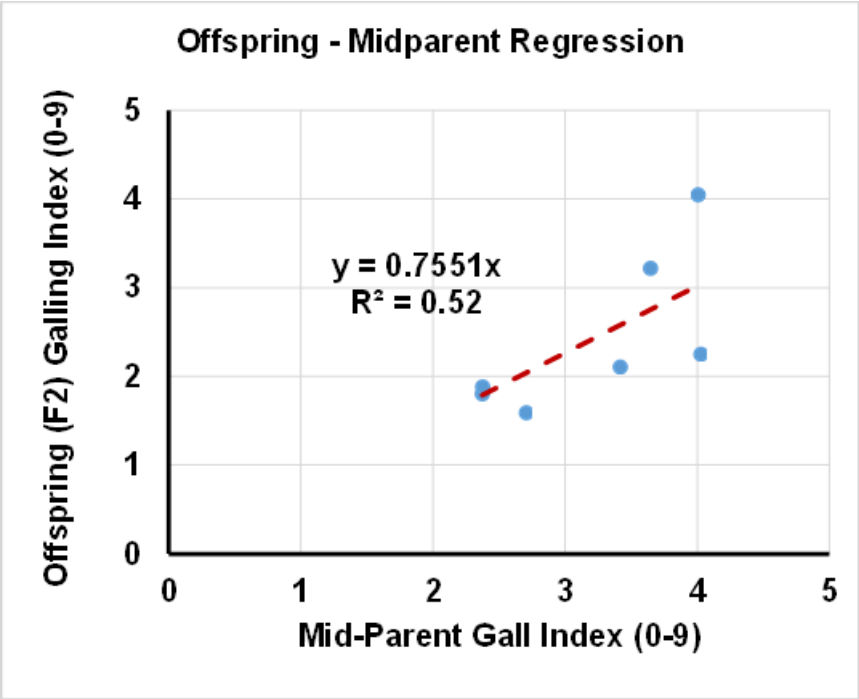

Fig 3.4. Midparent – offspring regression for F_2 population means regressed on the midparent root-galling values.

RKN resistance relationship: CB46 and FN-2-9-04

The relatedness between the resistance controlling root-galling (RG) and nematode reproduction (EM) in accession FN-2-9-04 and the *Rk* gene in CB46 was determined through allelism tests using F_2 populations of CB46 x FN-2-9-04. In addition, analysis of similarity was performed between FN-2-09-04, CB46 and CB46-Null within the mapped QTL regions harboring resistance to RKN to identify putative haplotypes associated with resistance in FN-2-9-04. In 2015 (Table 3.1), 400 and 162 F_2 plants plus parents were phenotyped for resistance to RG induced by avirulent *M. incognita* Project 77 under field infestation at

CVARS and KARE, respectively. At both sites (Fig. 3.5), all F_2 plants were resistant with no obvious segregation for resistance to RG between plants, indicating that FN-2-9-04 carries a resistance locus allelic to or equivalent to the *Rk* gene found in CB46.

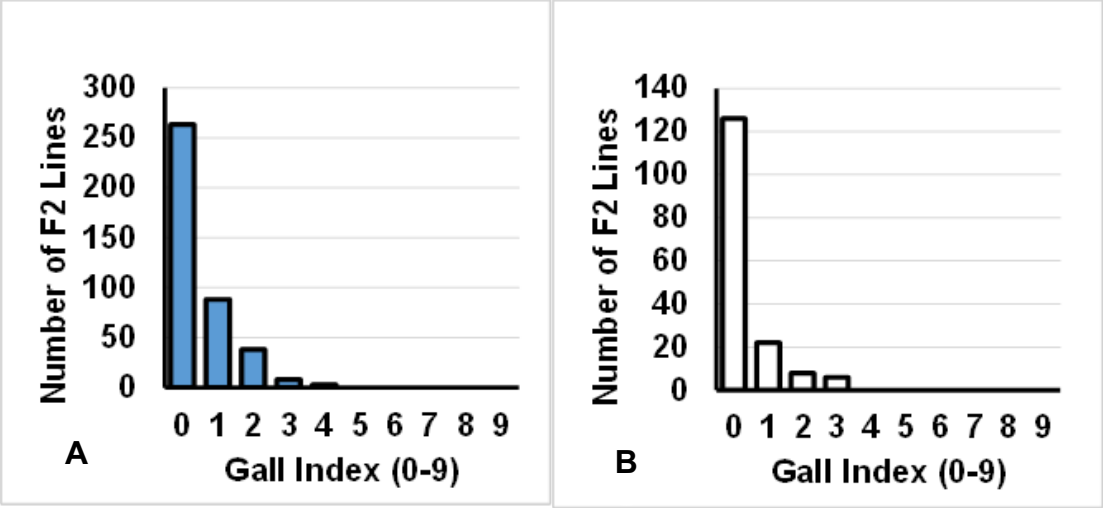

Fig. 3.5. Distribution of root-galling response in the F_2 populations CB46 x FN-2-9-04 under field infestation by avirulent M. incognita Project 77 (A): CVARS and (B): KARE, 2015.

F_2 populations of CB46 x FN-2-9-04 were also phenotyped for resistance to *M. javanica* RG and EM to validate the allelic relationship between CB46 and FN-2-9-04, since these parents exhibited significant differences in RG and EM production responses (Figs. 3.1A and 3.1B). Using data from phenotyping 197 and 177 F_2 lines for RG and EM, respectively, segregation occurred for *M. javanica* RG and EM in these F_2 populations (Figs. 3.2A and 3.2C).

Analysis of similarity between FN-2-09-04 and CB46 within the genomic region associated with resistance to RG mapped on Vu04 (Table 3.3) revealed a putative haplotype associated with the resistance (Fig. 3.6). The location of the *Rk* locus on Vu04 of the cowpea consensus genetic map identified in CB46

(Huynh et al., 2016) overlapped with the resistance region on the same chromosome in FN-2-9-04 by 1.59 cM, equivalent to 116579 bp on the cowpea physical map. Within this region these genotypes are nearly 39% identical based on the SNP haplotypes. However, within the same genomic region, FN-2-09-04 is completely different from CB46-Null (identity = 0%) which is 60% identical to CB46. Conversely, in region of Vu01 where an additional resistance QTL was detected in FN-2-09-04 (Table 3.3), FN-2-09-04 shares no similarity with either CB46 or CB46-Null (identity = 0%), whereas CB46 and CB46-Null are 100% identical.

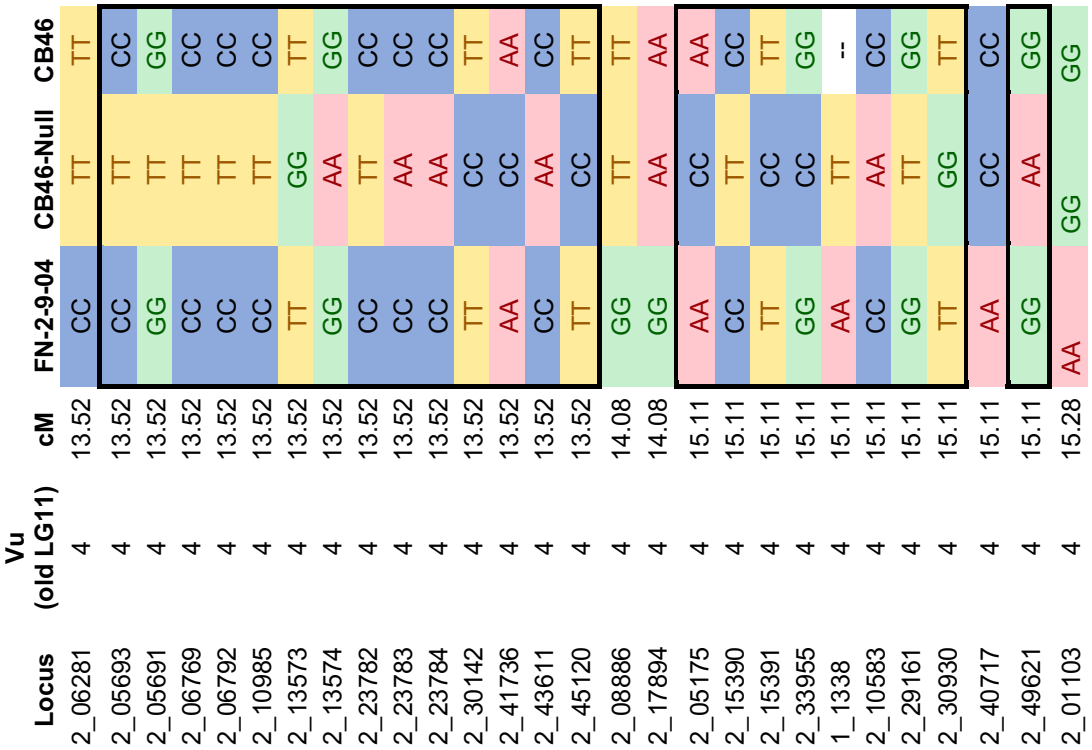

Fig. 3.6. Haplotype associated with RKN resistance on Vu04 and similarity within chromosomal regions harboring RKN resistance in FN-2-9-04 and CB46 on Vu04. Identical loci are highlighted in rectangules.

Linkage and QTL mapping

The linkage map of the F_2 population CB46-Null x FN-2-9-04, derived from 119 individuals and 51128 SNP markers, harbors 17209 polymorphic SNP markers distributed on 11 chromosomes which span 985.89 cM, and of these 17209 SNPs, 90.79% (15624) are mapped on the cowpea consensus genetic map (Munoz-Amatriain et al., 2017), and 9.21% (1585) are unique to this specific population. In addition, this population's specific linkage map comprises 1392 bins distributed at an average density of 1 bin per 0.71 cM and with an average marker spacing of approximately 1 cM. For QTL mapping, marker order and map distances on the population specific genetic map of CB46-Null x FN-2-9-04 were oriented based on the cowpea consensus genetic map, and linkage groups or chromosome numbering followed the new nomenclature adopted from the common bean (*Phaseolus vulgaris*) chromosome numbering scheme (Lonardi et al., 2017).

QTL analysis revealed two major QTLs associated with resistance to RG and EM in the FN-2-9-04 (Table 3.3, Figs. 3.7 and 3.8), and these QTLs were mapped and positioned on chromosomes Vu01 and Vu04 of the cowpea consensus genetic map (Munoz-Amatriain et al., 2017; Lonardi et al., 2017). The QTL region on Vu01 was consistently mapped to almost the same position using F_2 and $F_{2:3}$ populations phenotyped under greenhouse, seedling-growth pouch and field conditions using two RKN isolates (Table 3.3).

Table 3.3. Chromosome locations of root-knot nematode (RKN) resistance determinants in cowpea accession FN-2-9-04, mapped using F_2 and $F_{2:3}$ populations.

Mapping population (♀ x ♂)	Trait	RKN	Vu	Position	Flanking markers	-log(p)	PVE (%)	A	D/A
CB46-Null x FN-2-9-04 $F_{2:3}$	RG	Avr *M.i* - field	1	22.59-22.76	2_04038-2_23260	5.40	27.9	-1.2	0.5
CB46-Null x FN-2-9-04 $F_{2:3}$	RG	Avr *M.i* - field	4	13.52-16.06	2_06281-2_18980	20	73.4	-2.0	0.5
CB46-Null x FN-2-9-04 $F_{2:3}$	RG	*M.j* - field	1	19.10-29.06	2_26171-2_42871	20	94.1	-2.4	0.4
CB46-Null x FN-2-9-04 F_2	RG	*M.j* - GH	1	19.10-25.89	2_26171-2_16798	20	47.3	-2.7	0.4
CB46-Null x FN-2-9-04 F_2	EM	*M.j* - SGP	1	19.10-25.63	2_26171-2_51288	10.58	34.1	-17.1	0.4

RG = root-galling; EM = egg-masses per root system; Avr. *M.i* avirulent *M. incognita* isolate Beltran; *M.j* = *M. javanica* isolate Project 811; GH = greenhouse; SGP = seedling-growth pouches; Vu = cowpea chromosome (Chr) naming (Lonardi et al., 2017); PVE = percent of total phenotypic variation explained; A = additive effect of favorable alleles from the resistant parent (negative values indicate the extent of average reduction in RG or EM production due to the presence of favorable alleles; D = dominance effect due to substitution of favorable allele; and D/A = degree of dominance. Thresholds of QTL significance values indicated by horizontal dashed lines (Figs 3.7 and 3.8.

Two QTLs controlling resistance to RG by avirulent *M. incognita* Beltran were detected and mapped on Vu01 and Vu04 ($P < 0.05$) (Fig. 3.7A) of the cowpea consensus genetic map (Munoz-Amatriain et al., 2017; Lonardi et al., 2017) in the $F_{2:3}$ of CB46-Null x FN-2-9-04. The resistance QTL on Vu01 spanned 0.17 cM (22.59 - 22.76) between flanking markers 2_04038 and 2_23260, and it accounted for 27.91% of the total phenotypic variation (V_p) of the RG resistance response (Table 3.3).

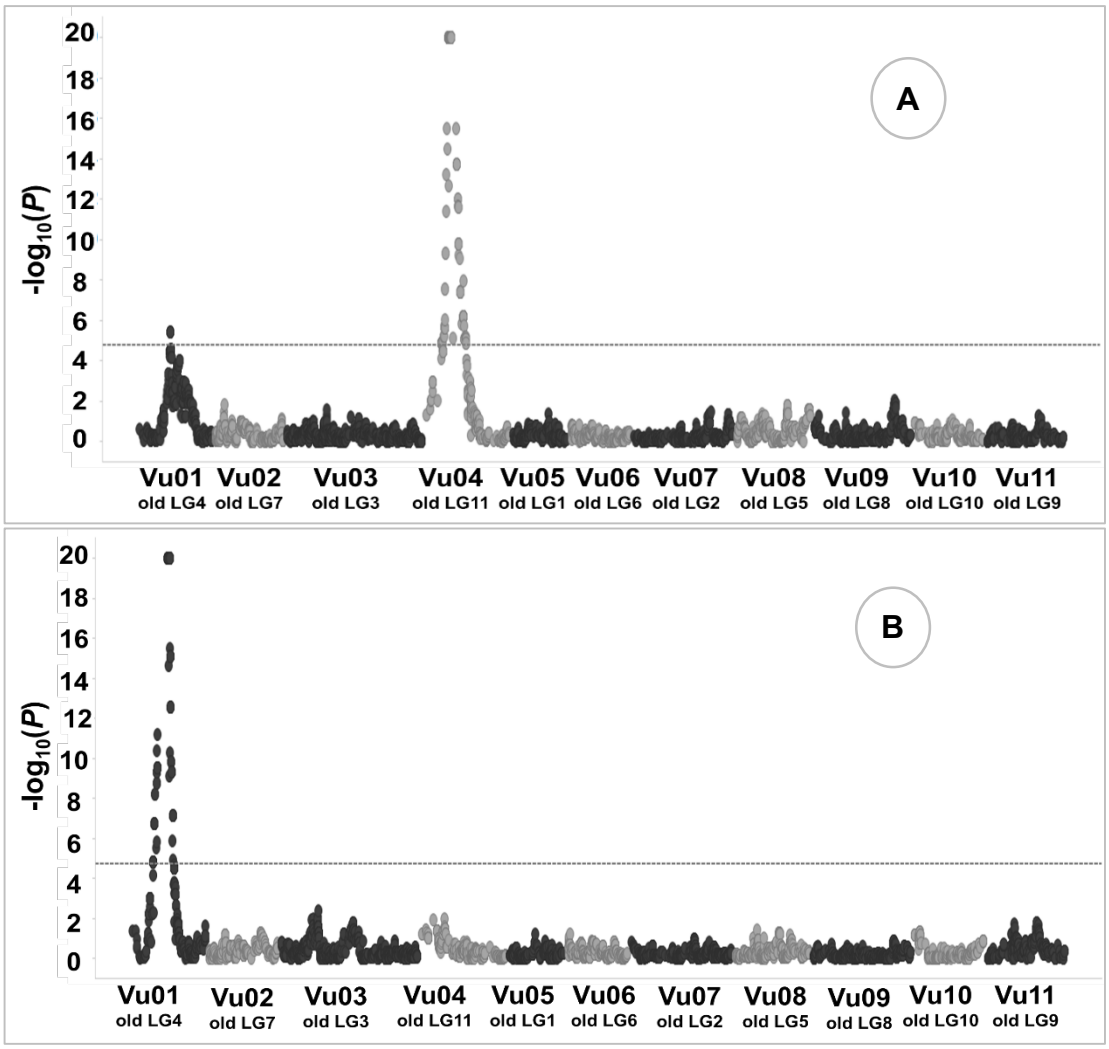

Fig. 3.7. Genomic localization on the cowpea consensus genetic of QTLs associated with resistance to root-galling (RG) by: (A) avirulent *M. incognita* and (B) the aggressive *M. javanica* isolate Project 811. The QTLs were detected in the $F_{2:3}$ population CB46-Null x FN-2-9-04 phenotyped for RG under field infestation. Horizontal dashed line represents the Bonferroni threshold of significance at $P < 0.05$. Old LG stands for former cowpea linkage group naming and Vu indicates the new cowpea linkage group naming based on cowpea chromosome pseudomolecules (Lonardi et al., 2017).

This resistance QTL on Vu01 exhibited additive and dominance effects of 1.2 and 0.6, respectively, and the degree of dominance, measured as a ratio between dominance and additive effects (D/A), indicated that the resistance in this QTL has partial dominant effect (D/A =0.5). The resistance QTL detected

on Vu04 is located at Chr position 13.52 - 16.07 cM of the cowpea consensus genetic map and spanned 2.54 cM with flanking SNP markers 2_06281 and 2_18980 (Table 3.3).

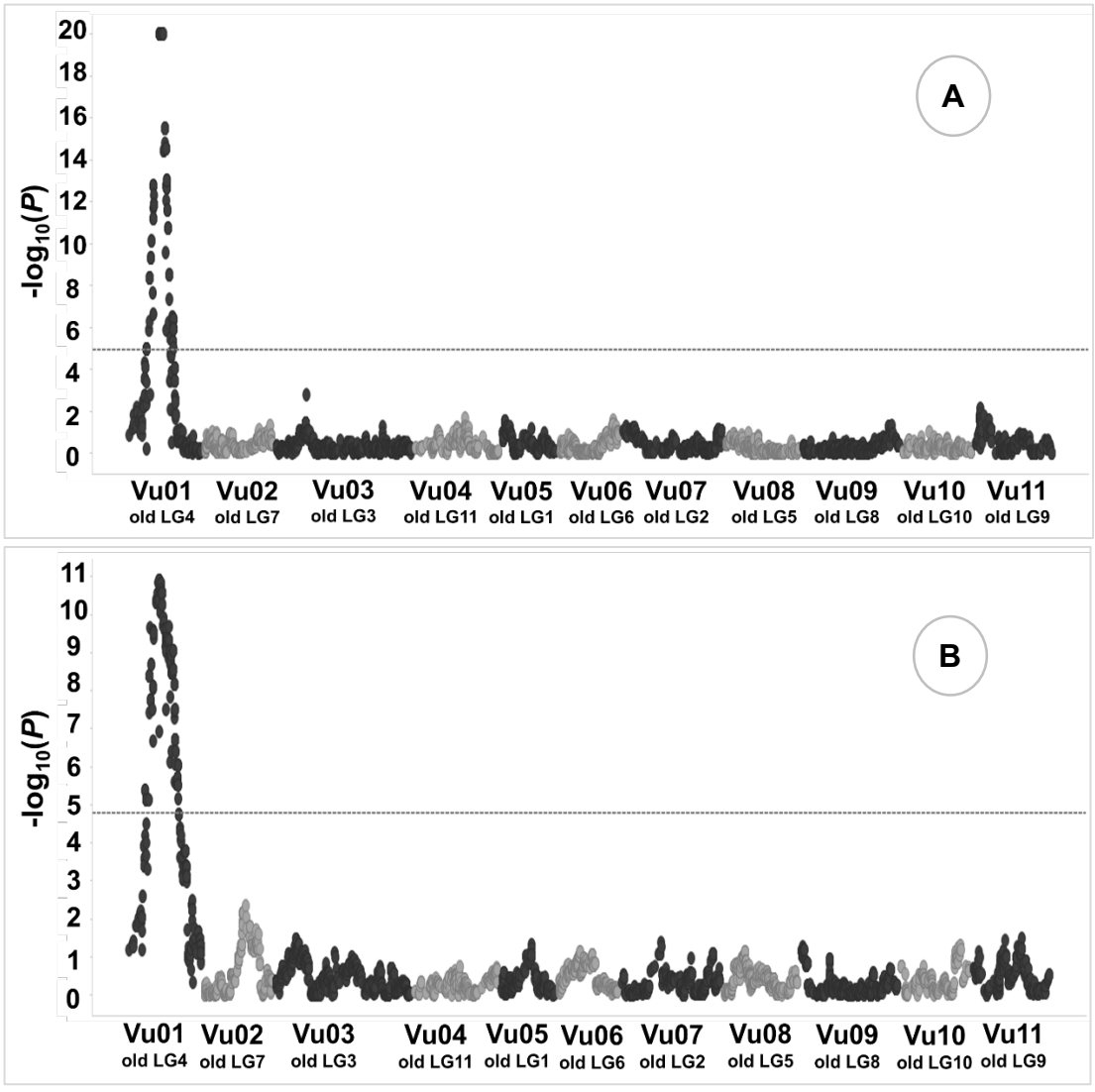

Fig. 3.8. Genomic localization on the cowpea consensus genetic of QTL associated with resistance to (A) root-galling (RG) and (B) egg-mass production (EM) by the aggressive *M. javanica* isolate Project 811. The QTLs were detected in the F_2 population CB46-Null x FN-2-9-04 phenotyped for RG in greenhouse and for EM in seedling-growth pouches inoculations, respectively. Horizontal dashed-line represents the Bonferroni threshold of significance at $P < 0.05$. Old LG stands for former cowpea linkage group naming and Vu indicates the new cowpea linkage group naming based on cowpea chromosome pseudomolecules (Lonardi et al., 2017).

This QTL explained 73.35% of the total V_p of the resistance response, and it had an infinite likelihood of occurrence which was represented by $-\log_{10}(p) = 20$ (Table 3.3). In addition, the additive (A = -2) and dominance (D = -1) effects of the QTL on Vu04 were higher than those of the QTL on Vu01, but both QTLs showed the same degree of dominance (D/A = 0.5).

On Vu01, an additional genomic region controlling resistance to RG (Fig. 3.7B and 3.8A) and EM production (Fig. 3.8B) by the aggressive *M. javanica* isolate "Project 811" was consistently mapped in the F_2 and $F_{2:3}$ populations of CB46-Null x FN-2-9-04 using RG and EM phenotypic data from field, greenhouse and seedling-growth pouch experiments (Table 3.3). The resistance QTL associated with *M. javanica* RG mapped at positions 19.1 - 25.89 cM and 19.1 - 29.06 cM on Vu01 of the cowpea consensus genetic map in F_2 (greenhouse experiment) and $F_{2:3}$ (Field experiment) populations, respectively. These genomic regions spanned 6.79 and 9.96 cM, with flanking markers 2_26171 - 2_16798 and 2_26171 - 2_42871, respectively (Table 3.3). In both F_2 and $F_{2:3}$ populations, the resistance QTL was detected with infinite likelihood represented by $-\log_{10}(p) = 20$ (Fig. 3.7B and 3.8A); however, the percent of total phenotypic variation explained by the QTL effect in the $F_{2:3}$ (PVE = 94.1%) was higher than in the F_2 (PVE = 47.3), but the contribution of the additive and dominance effects in the total phenotypic variation in the F_2 and $F_{2:3}$ were similar. Also, the degree of dominance in both generations was equal, D/A = 0.4 (Table 3.3), both indicating resistance with partial dominance.

The QTL on Vu01 associated with resistance to *M. javanica* reproduction was mapped to position 19.1-25.63 cM of the cowpea consensus genetic map using

F_2 population CB46-Null x FN-2-9-04 (Table 3.3, Fig. 3.8B). This resistance QTL spanned 6.53 cM between flanking SNP markers 2_26171 and 2_51288. This genomic region accounted for 34.1% of the total phenotypic variation with additive and dominance effects of 17.1 and 7.5, respectively, and the gene action measured within the same QTL region indicated partial dominance (D/A = 0.4). Although this QTL was detected with high likelihood, $-\log_{10}(p) = 10.5$ (critical threshold = 4.78) (Fig. 3.8B), it was lower than that observed for the RG QTL (Table 3.3).

Gene enumeration estimated at the mapped genomic regions associated with resistance following the Castle-Wright (1921) algorithm indicated that the resistance to avirulent *M. incognita* RG is under control primarily by 2 and 5 genes residing in QTL regions mapped on Vu04 and Vu01, respectively; whereas, the responses to *M. javanica* RG and EM production mapped on Vu01

are governed mainly by 2 genes each. However, the extent of genetic distortion in these regions or multiallelic effects requires further study.

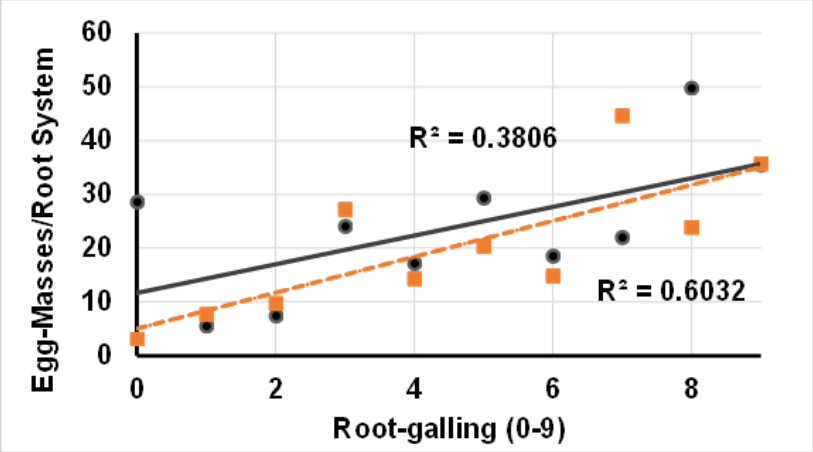

Fig. 3.9. Correlation between *M. javanica* root-galling, RG, (greenhouse test) and egg-mass production, EM, (seedling-growth pouch test) in F_2 populations ●CB46-Null x FN-2-9-04 and ■ CB46 x FN-2-9-04.

The relationship between *M. javanica* RG and EM production responses in the F_2 population CB46-Null x FN-2-9-04 and CB46 x FN-2-9-04 is shown in Fig 3.9. In both populations, RG and EM were significantly correlated: $r = 0.62$ ($P = 0.058$; $R^2 = 0.381$) and $r = 0.78$ ($P = 0.008$, $R^2 = 0.603$) for CB46-Null x FN-2-9-04 and CB46 x FN-2-9-04, respectively, although, only 38.1% of the relationship RG-EM in CB46-Null x FN-2-9-04 was explained by genetic factors compared to 60.3% in CB46-Null x FN-2-9-04.

Discussion

Genetic studies on the inheritance of strong and broad-based resistance to avirulent *M. incognita* and aggressive *M. javanica*, present in cowpea accession FN-2-9-04, revealed that the resistance is determined by two major QTLs which were mapped on crhomosomes Vu01 (old LG4) and Vu04 (old LG11) of the cowpea consensus genetic map. The response of F_1 populations to RG and EM production relative to the resistant parent and the skewed segregation of F_2 populations for RG and EM production and $F_{2:3}$ populations for RG indicated that these responses are under control by major genes with partial dominance effect. Resistance to RKN under control by major genes with partial dominance effect has been reported in several studies (Ali et al., 2014; Huynh et al., 2016). Analysis of segregation for resistance through marker-trait association also suggested that the major genes controlling resistance are putatively aided by minor/recessive genes, and collectively in a dominant-recessive interaction to confer substantially stronger, broad-based resistance than that conferred by the *Rk* gene alone. A similar genetic phenomenon of major gene and minor/recessive gene interaction was described in cowpea cultivar CB27, where gene *Rk* acts together with a recessive gene to enhance and broaden the resistance against RKN (Ehlers et al., 2000).

The allelism test between CB46 and FN-2-9-04, revealed a lack of resistance segregation in the CB46 x FN-2-9-04 F_2 population for resistance under avirulent *M. incognita* infection, indicating that both parents putatively carry the same major RKN resistance locus previously mapped by Huynh et al (2016) on Vu04 of the cowpea consensus genetic map (Munoz-Amatriain et al., 2017).

This was the first known RKN resistance locus in to cowpea, and it has been bred into many commercial cowpea cultivars (Fery and Dukes, 1980; Helms et al., 1991; Ehlers et al., 2009). In contrast, the segregation found in F_2 population CB46 x FN-2-9-04 for *M. javanica* RG and EM production, and the mapping of resistance QTLs for RG and EM production confirmed that the heightened and broad-based resistance response in FN-2-9-04 relative to CB46 is conferred by additional resistance factors located on Vu01.

Estimates of heritability of resistance in FN-2-9-04 to avirulent *M.* and aggressive *M. javanica* in the F_2 generation and from greenhouse experiments were lower than those estimated in the $F_{2:3}$ generation and under field conditions. This can be accounted for by the segregation in both populations and because greenhouse phenotyping is less variable compared to field testing. The estimates of narrow-sense heritability of resistance to RG induced by both RKN species were in the range 0.34 – 0.68, indicating that the resistance in FN-2-9-04 can be transferred successfully into elite cowpea cultivars to broaden the genetic base of resistance to RKN which currently relies on the *Rk* gene. The resistance response to *M. javanica* EM production had lower heritability estimates ($H^2 = 0.34$ and $h^2 = 0.24$) compared to those for *M. javanica* RG, which could be due to EM production data being generally more variable compared to RG data. The result that RG was highly correlated to EM production response and that the resistance response to RG and EM mapped within the same genomic region would suggest that both traits may be governed by the same genes determining resistance. Similar to these results, significant correlation between RG and EM production in cowpea recombinant inbred

populations was reported by Huynh et al (2016). In contrast, in lima bean (*Phaseolus lunatus* L.) the responses to RG and nematode reproduction were reported to be under control by independent genetic factors (Roberts et al., 2008). Since genetic factors explained 38.1 and 60.3 % of the correlation between RG and EM in this study, these data suggest that although the genomic regions governing both traits are collocated, the two traits may be under distinct regulatory mechanisms or that the resistance to both traits may reside within a multi-allelic locus or tandemly arranged loci.

The heritability of resistance to *M. incognita* RG comprised two components, one on Vu01 ($h^2 = 0.19$, $H^2 = 0.28$) and the other on Vu04 ($H^2 = 0.73$, $h^2 = 0.49$) indicating that the major locus for this resistance in FN-2-9-04 is housed on Vu04, and it is aided by the additional locus on Vu01 with low resistance heritability. Also, the differential activity between the resistance loci on Vu01 and Vu04 points to specificity of resistance. Huynh et al (2016) reported that, although the QTL harboring the *Rk* gene had a significant effect on controlling both avirulent *M. incognita* and *M. javanica*, its resistance activity was lower against *M. javanica*.

The resistance to *M. javanica* in FN-2-9-04 consistently mapped to Vu01 using RG and EM phenotypic data from F_2 and $F_{2:3}$ populations. This major *M. javanica* resistance QTL mapped to position 19.1 – 29.06 cM in Vu01 spanning 9.96 cM, based on several mapping data sets for RG and EM phenotypes. Therefore, this distinct genomic region on Vu01 compared to the *Rk* locus (*QRk-vu4.1* - old *QRk-vu11.1*, Huynh et al, 2016) which was mapped on Vu04

of the cowpea consensus genetic map (Munoz-Amatriain et al., 2017) represents a novel RKN resistance QTL here designated *QRk-vu1.1*.

Two significant avirulent *M. incognita* resistance QTLs ($P < 0.05$) for RG were mapped on Vu01 and Vu04 at positions 22.59 – 22.76 and 13.52 – 16.07 cM, respectively, and are flanked by SNP markers 2_04038 – 2_23260 and 2_06281 – 2_18980 on the cowpea consensus genetic map, respectively. The QTL mapped on Vu04 overlaps by 1.59 cM (13.52 – 15.11 cM) with the previously mapped genomic region on the same Chr which harbors the *Rk* resistance locus (Huynh et al., 2016), suggesting that gene *Rk* is located within this genomic region. In previous RKN resistance QTL mapping (*QRk-vu4.1* - old *QRk-vu11.1*, Huynh et al., 2016), the *Rk* locus spanned 8.35 cM of the cowpea consensus genetic map compared to 2.54 cM in this study. This difference in mapping resolution is attributed to the current availability of the high-density SNP genotyping platform and high-density cowpea consensus genetic map (Munoz-Amatriain et al., 2017). If the genomic region where the *Rk* locus resides is a multi-allelic or multi-gene locus, the overlap between *QRk-vu4.1* and the QTL mapped in this study on Vu04 indicates the resistance alleles are within a 1.59 cM interval which provides effective resistance against avirulent *M. incognita* populations.

The segregation for resistance supported that 2 genes on Vu01 and 2 genes on Vu04, are responsible for the resistance against *M. javanica* and avirulent *M. incognita*, respectively, as estimated by the Castle-Wright (1921) formula. However, for avirulent *M. incognita* the estimates of genes involved in resistance on Vu01 disagreed with the observed segregation for resistance.

The data also fit a 3:1 ratio expected for a single major gene and the better fit to the 13:3 of the SNP haplotypes could represent genetic distortion within each locus. These estimates regarding the number of genes directly involved in resistance can be verified by determining putative candidate genes within the mapped QTLs and tests for their function.

Flanking markers associated with these genomic regions on Vu01 and Vu04 housing resistance to the target RKN isolates can be used to assist the introgression of this resistance in to elite cowpea cultivars. In particular, the resistance detected on Vu01 which was highly effective against *M. javanica*, and also effective against avirulent *M. incognita*, was for the first time identified and mapped in this study. The resistance on Vu01 seems to be more specifically effective against aggressive *M. javanica*, as confirmed by the absence of a QTL peak on Vu04 when F_2 and $F_{2:3}$ populations were challenged with *M. javanica* isolate. Conversely, both these QTLs had obvious activity against avirulent *M. incognita*, but the higher QTL peak observed on Vu04 under this nematode infestation suggested that the QTL on Vu04 plays the major role in resistance. Both RKN resistance QTLs on Vu01 and Vu04 are responsible for the strong and broad-based resistance observed in FN-2-9-04 which differs from the narrow-based resistance provided by the *Rk* gene alone. The allelism test indicated that FN-2-9-04 also carries the *Rk* gene which would be very beneficial in resistance breeding to pyramid the *R* genes. The mechanism of resistance displayed by this novel broad-based resistance, and the interaction between the resistance loci on Vu01 and Vu04 are yet to be determined. The linkage map of the F_2 population CB46-Null x FN-2-9-04 is an

additional valuable genetic resource especially because it is the first linkage map constructed using a cowpea genotype from the cowpea gene-pool II from southeastern Africa (Huynh et al., 2013), and because 9.2% of 17209 SNP markers were unique to this population and are not mapped on the current version of the cowpea consensus genetic map (Munoz-Amatriain et al., 2017). In addition, accession FN-2-9-04 is a multi-trait genotype which enables it to be used in other relevant studies for cowpea research.

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CHAPTER IV - Cowpea Genetic Resources for Resistance to Fusarium Wilt races 3 and 4

Abstract

Cowpea (*Vigna unguiculata* L. Walp) is susceptible to several biotic stresses including Fusarium wilt caused by *Fusarium oxysporum f. sp. tracheiphilum* (Fot). Host-plant resistance provides an efficient Fot management, but few sources of resistance are available. Through a series of greenhouse replicated experiments, eleven novel sources of broad-based resistance to Fot3 and Fot4 were identified among 53 genotypes from Mozambique. Resistance was phenotyped based on response to wilting (disease index – DI), and vascular discoloration length (%VDL). The eleven genotypes exhibited similar response to that of resistant controls CB27 and IT93K-503-1 under both Fot3 and Fot4 infection ($P > 0.05$). Most genotypes were resistant to Fot3 indicating the predominance of Fot3 resistance in this cowpea germplasm. The response of 4 F_1 populations derived from resistant genotype FN-2-9-04 confirmed that this genotype carries dominant resistance with high heritability. Moderate correlation ($r = 0.52, P < 0.0001$) between Fot3 and Fot4 DI data suggested that resistance to these races is controlled by distinct resistance mechanisms. Strong correlation between DI and %VDL ($r = 0.95, P < 0.05$) indicated that interrelated mechanisms control both responses; however, weak regression ($b = 0.056$) suggested that the association depends on the cowpea background. Fot4 was four-fold more virulent than Fot3, which explains the high susceptibility of cv. CB46 to Fot4. These novel sources of resistance can be utilized for disease diagnosis and in breeding Fot resistant cultivars.

Introduction

Globally, cowpea (*Vigna unguiculata* L. Walp) yield is below the known potential (Adegbite and Amusa, 2008), and this undermines its potential as food and a source of income for many households in developing countries, particularly in Africa. Cowpea yield is mainly lost to abiotic and biotic stresses. Fusarium wilt of cowpea (FW), caused by *Fusarium oxysporum* f. sp. *tracheiphilum* (Schl.) (Fot), a soil-borne vascular wilt fungus, is a common disease in several cowpea growing areas (Hall and Frate, 1996). The fungus can infect plants early or in mid-season and cause seedling death, plant wilting and stunting, which result in severe plant stand reduction and yield loss (Smith et al., 1999; Hall and Frate, 1996). The impact of Fusarium wilt disease on susceptible cowpea cultivars can be exacerbated by root-knot nematode (RKN) infection in fields where both pathogens are predominant (Harris and Ferris, 1991; Roberts et al.,1995); the two pathogens form a disease complex in which nematode root infection predisposes and weakens the plants to infection by the fungus (Sidhu and Webster, 1974; Harris and Ferris, 1991; Roberts et al.,1995).

Breeding Fot resistant cowpea cultivars has been a major goal for cowpea improvement. Fot populations are dynamic which leads to the emergence of novel Fot pathotypes or races with enhanced virulence (Hall and Frate, 1996; Smith et al., 1999; Ehlers et al., 2009), while chemical options are costly and in general not suitable for cowpea production systems. Therefore, breeding for host-plant resistance is one of the most efficient strategies to counteract the impact of the disease in cowpea. Four Fot races of cowpea have been reported

- races 1 and 2 (Armstrong & Armstrong, 1950; Armstrong & Armstrong, 1980; Smith et al*.,* 1999), race 3 (Armstrong & Armstrong, 1980; Smith et al*.,* 1999) and race 4 (Smith et al., 1999). Currently, the most damaging races in cowpea growing areas are races 3 and 4, and Fot3 is the most wide-spread race (Hall and Frates, 1996; Smith et al., 1999; Ehlers et al., 2009). The virulence differences between Fot3 and Fot4 can be substantial depending on the cowpea cultivar and other environmental conditions. For example, in California Fot4 is currently the most virulent and problematic race for the cowpea industry (Ehlers et al., 2009; Frate, 2012). Fusarium wilt resistance in commercial cowpea cv. CB46, developed in California (Helms et al., 1991) is only effective against Fot1 and Fot2 (Smith et al*.,* 1999) and Fot3 (Helms et al., 1991; Hall and Frate, 1996; Smith et al*.,* 1999), and this cultivar has been grown widely in many cowpea production areas. However, with the emergence of Fot4, severe plant wilt of this cultivar has been reported under field conditions (Ehlers et al., 2009; Frate, 2012).

Relatively few genetic resources of effective resistance to Fot3 and Fot4 have been identified in cowpea (Ehlers et al., 2000), although resistance to the most virulent race, Fot4, has been bred into new commercial blackeye cultivars CB27 and CB50 (Ehlers et al., 2000; Ehlers et al., 2009) and the resistance is effective under field conditions (Frate, 2012). Recently, the resistance in currently available cowpea lines and cultivars was genetically mapped to three distinct genomic locations on the cowpea consensus genetic map, including two loci

for Fot4 resistance (Pottorff, et al., 2014) and one for Fot3 resistance (Pottorff, et al., 2012).

The resistance to Fusarium wilt in cowpea, common bean, tomato and cucumber has been reported as being under control by one or a few genes either with dominant or partially dominant effects (Netzer et al., 1977; Ribeiro and Hagedorn, 1979; Rigert and Foster, 1987; Scott and Jones, 1989; Salgado et al., 1995; Cross et al., 2000). Modifier genes that enhance the resistance conferred by dominant genes also have been documented (Scott and Jones, 1989). However, polygenic control of resistance to Fusarium wilt also was reported in common bean, and it was found to vary with the genotypic races of common bean (Cross et al., 2000; Salgado et al., 1995).

In cowpea, the genetic base of resistance to FW is extremely narrow due to the simple nature of inheritance and limited known sources of resistance to this disease. Eventual emergence of novel Fusarium pathotypes with enhanced virulence than the currently prevalent FW races (races 3 and 4), might render the currently available sources of resistance ineffective. Therefore, searching for additional unique sources of genetic resistance to this pathogen in cowpea, and understanding the relative virulence between Fusarium wilt races are important goals. Novel sources of resistance to FW would allow broadening the genetic base of resistance through breeding FW resistant commercial cowpea cultivars with pyramided genes for effective disease management and less crop

loss. The present study was conducted to: (i) determine the variability of response to Fot3 and Fot4 in a unique cowpea collection from Mozambique comprising 53 genotypes; (ii) estimate the relative levels of virulence between Fot3 and Fot4 and determine the specificity of resistance to both Fusarium wilt races; and (iii) determine the effectiveness of resistance in genotypes and the relationship between plant wilting and vascular discoloration incited by the fungus.

Materials and Methods

Plant Materials: Resistance screening for Fot3 and Fot4 resistance was conducted on a cowpea collection from Mozambique which comprises 53 genotypes. These genotypes are part of a diverse cowpea germplasm of about 350 accessions and landraces, which lack information on their responses to biotic stresses including Fusarium wilt.

Fusarium Wilt Isolates and Inoculum Preparation

Dried cultures of Fot3 and Fot4 isolates T89-15 and T97-30, respectively stored at -80 ^{o}C on potato dextrose agar (PDA) plates, were re-cultured to generate inoculum. A single dried plug, 1cm^2, was cut from each petri dish containing each Fot race and transferred into new petri dishes containing fresh PDA. Both petri dishes were incubated under room temperature for 3-4 days, and from these PDA plates a 1cm^2 fresh plug was cut aseptically and transferred into a 500 ml Erlenmeyer flask containing freshly prepared potato-dextrose broth which was then incubated in a shaker for 4 days at 30 rpm, at 27 ^{o}C under light. Post-incubation, the spore solution was filtered through 8 layers of cheesecloth, and the flow-through solution containing spores was collected in a beaker. The spores were counted using a hemocytometer under a light microscope and the concentration adjusted to 10^6 microconidia/ml.

To test inoculum viability, cowpea genotypes known for their Fusarium wilt resistance (CB27 and IT93K-503-1, both resistant to Fot3 and Fot4, and CB46 resistant to Fot3) and susceptibility (24-125B-1, susceptible to both races) were challenged with both Fusarium wilt races separately, following a modified

protocol of Rigert and Foster (1987). Briefly, lateral and tap roots of 7-day-old seedlings were clipped to 3 cm length and dipped for 3 minutes into a spore solution containing 10^6 microconidia/ml, transplanted into 0.95 L foam cups containing UC-Mix 3 soil, grown for 28 days and then evaluated for wilting and vascular discoloration.

Infection Assays

All assays were conducted under greenhouse conditions at UC-Riverside. Following confirmation of inoculum viability, 62 cowpea genotypes including controls were planted under controlled greenhouse conditions (min = 29 ^oC and max = 32 ^oC) in trays containing growing mix. Seven days later, the seedlings were uprooted, and the root system washed to remove excess soil, clipped to 3 cm length, and the roots dipped into the fresh spores suspension. After inoculation five plants per genotype (20 plants/genotype in total) were transplanted into four foam cups (Fig. 4.1A), and grown for 28 days. The seedlings were watered once a day, and about two weeks after inoculation they were supplied with fertilizer.

Twenty eight days after inoculation, the plants were evaluated for wilting (disease index - DI), growth [plant height (PH) and shoot-weight (SW)] and vascular discoloration, expressed as vascular discoloration length (%VDL). Each plant was cut at the soil line and rated for disease index using a 0 to 5 rating scale (Fig. 4.1A and 4.1B) to record the level of plant wilting/yellowing/stunting symptoms, where 0 indicated a healthy plant with no yellowing/wilting symptoms; 1 = about 10% of the plant canopy shows

wilting/yellowing symptoms; 2 = 25% of the plant canopy shows wilting/yellowing/stunting; 3 = 50% wilting/yellowing and canopy loss is obvious and dark-brown spots can be seen on the insertion of fallen leaf; 4 = 75% wilting/yellowing, severe canopy loss, and stem dark colored; 5 = the plant is dead and no green tissue present (Fig. 4.1B). The level of vascular discoloration (%VDL) incited by fungal vascular colonization was computed as the ratio between the length of vascular discoloration and the total plant height, ($VDL\ (\%) = \frac{VDL}{PH} x\ 100$). Wilting and vascular discoloration were considered as independent response traits and evaluated separately. The threshold for resistant responses was established at DI ≤ 3 and %VDL ≤ 30% based on the response of the susceptible control, so plants showing wilting/yellowing and vascular discoloration phenotypes, respectively above these values were considered as susceptible. Four experiments were conducted for data validation (1 for Fot3 and 3 for Fot4). Genotypes CB27 and IT93K-503-1 were used as resistant controls for both Fot3 and Fot4 while CB46 and 24-125B-1 were used as susceptible controls for Fot4. CB46 and 24-125B-1 were also used as resistant and susceptible controls, respectively in Fot3 test (Table 4.1).

Table 4.1. Control genotypes used in infection assays and their known Fusarium wilt resistance genes.

Genotype	*Fot* gene set	Fusarium Wilt Response	
		Fot-Race 3	Fot-Race 4
CB46	*Fot3Fot3*	Resistant	Susceptible
CB27	*Fot4-2Fot4-2/Fot3Fot3*	Resistant	Resistant
IT93K-503-1	*Fot4-1Fot4-1/Fot3Fot3*	Resistant	Resistant
24-125B-1	No resistance	Susceptible	Susceptible

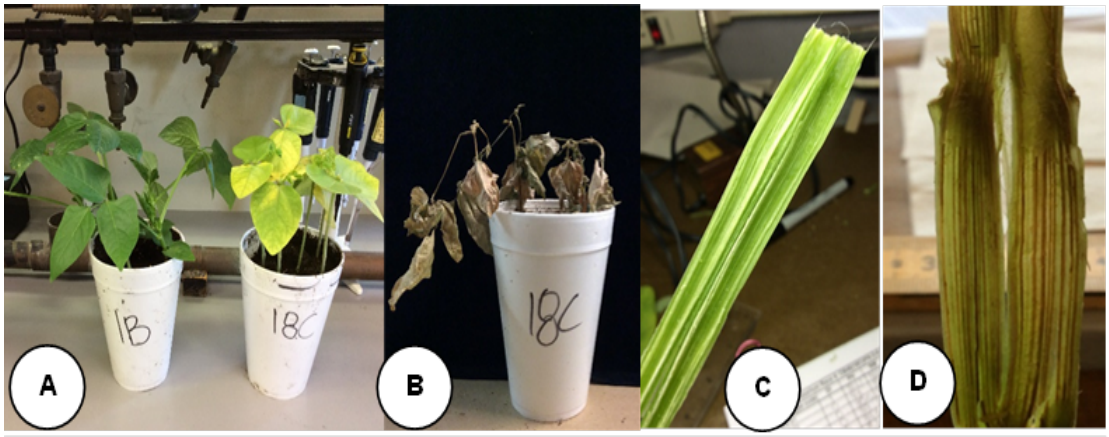

Fig. 4.2. Fusarium wilt disease symptoms on cowpea, (A) and (B) - Fot race 4 induced leaf yellowing (A - pot 18C, DI = 5) and wilting symptoms (B - pot 18C, DI = 5) on susceptible plants and Fot4 resistant plants (A - pot 1B, DI = 0); Fig. 4.1C and 4.1D - vascular discoloration symptoms on resistant (%VDL = 0) and susceptible (%VDL = 100%) plants, respectively.

Statistical Analysis

Data for DI and VDL (%) were processed for ANOVA following the Proc Mixed procedure using SAS University Edition 3.2. The cowpea genotypes were considered as fixed factor each with four replications, and each experimental unit comprised five seedlings per genotype which were transplanted into the same cup right after clipping and dipping the roots into the inoculum suspension. The replications were considered as random factors, and each Fusarium race was considered as a separate environmental condition on which the genotypes were evaluated. Both separate and combined ANOVA were performed using the same procedure (Proc Mixed). Differences between means were detected at $P < 0.05$ through multiple comparisons between means following the option DIFF.

To determine the effectiveness of resistance, four F_1 populations were developed by crossing a resistant genotype with each of 4 different susceptible genotypes. These hybrids were evaluated for wilting/yellowing and vascular discoloration together with their parents. The data for DI and VDL (%) were processed for simple ANOVA following the Proc Mixed procedure where the test F_1 progeny and control genotypes were fixed as treatments, and the replications were considered as random factor.

Pathogen virulence is defined as the degree (severity) with which a certain pathogen can infect and cause disease (Read, 1994; D'Arcy, 2001). In this study, Fusarium wilt virulence was estimated as a ratio between plant wilting (DI) or vascular discoloration [VDL (%)] observed on test cowpea genotypes and that observed on known Fusarium wilt susceptible genotypes. Thus, the differential level in virulence between Fot3 and Fot4 was based on the rate of plant wilting and vascular discoloration observed under each race. Resistance specificity was determined based on the differential response of the test cowpea genotypes to infection by each Fot race. To determine the association between plant wilting, plant growth and vascular discoloration induced by the each Fot race, data for DI, plant growth (SW) and VDL (%) were subjected to regression analysis.

Results

Response to Fot3 and Fot4

Data for disease index (DI) and vascular discoloration length (%VDL) were subjected to ANOVA. The results indicated that the response of genotypes to wilting, growth and vascular discoloration were all affected by the genetic background of each genotype, Fusarium wilt race, and by the interaction between genotype and Fusarium wilt race ($P < 0.0001$). The DI under Fot3 and Fot4 infection (Table 4.2) varied from 0 – 5, with average plant witling index of 0.9 and 3.0, respectively, and %VDL varied from 0 – 100% with average %VDL of 19.8 and 53.4, respectively. Significant differences among genotypes in response to infection by both Fot3 and Fot4 were detected at DI = 1.0 and %VDL = 18.7.

Analysis of wilting induced by Fot4 (Table 4.2) showed that twenty genotypes were resistant to wilting/yellowing (DI ≤ 3), with average DI ranging from 0 – 2.9, and the differences in response among them were not significant ($P > 0.05$). Genotypes Maputo, FN-2-9-04, FN-2-11-04, Massava-11, VAR-3A, INIA-72 and Nhacoongo-2 had the lowest DI, similar to controls IT93K-503-1 and CB27 ($P > 0.05$). Genotypes INIA-42F, INIA-51, INIA-25, SP-373, INIA-36 and SP-860 were highly susceptible to Fot4 wilting similar to the Fot4 susceptible controls CB46 and 24-125B-1 (DI = 5).

Table 4.2. Responses of 53 cowpea genotypes from Mozambique to wilting and vascular discoloration induced by *Fusarium oxysporum* f. sp. *tracheiphilum* races 3 and 4.

	Fusarium Wilt			
	Race 3		Race 4	
Genotype	**DI (0-5)**	**VDL (%)**	**DI (0-5)**	**VDL (%)**
24-125B-1	4.9 ± 0.1	97.5 ± 2.5	4.8 ± 0.2	88.7 ± 11.3
CB-27	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	10.8 ± 2.9
CB46	0.3 ± 0.3	2.6 ± 2.4	4.8 ± 0.1	90.2 ± 5.9
Ecute	0.0 ± 0.0	9.2 ± 2.2	3.4 ± 0.4	55.3 ± 4.8
FEAF-14-INE	0.1 ± 0.1	18.5 ± 1.8	3.5 ± 0.7	53.4 ± 16.4
FN-1-13-04	4.0 ± 0.4	56.5 ± 15.1	3.9 ± 0.3	57.4 ± 9.4
FN-1-14-04	0.3 ± 0.3	10.6 ± 1.8	2.2 ± 0.2	34.9 ± 4.7
FN-2-11-04	0.0 ± 0.0	4.8 ± 2.9	0.5 ± 0.3	16.8 ± 1.1
FN-2-13-04	0.7 ± 0.5	16.3 ± 4.2	0.9 ± 0.3	19.9 ± 3.1
FN-2-9-04	0.0 ± 0.0	5.5 ± 0.5	0.0 ± 0.0	13.4 ± 2.4
Gile-K-Local	0.3 ± 0.2	8.4 ± 3.0	1.8 ± 0.3	29.5 ± 4.5
INHACA-D	0.7 ± 0.2	7.1 ± 0.9	4.1 ± 0.2	64.6 ± 4.8
INHACA-I	0.0 ± 0.0	0.0 ± 0.0	1.5 ± 0.4	30.5 ± 5.8
INIA-1	0.0 ± 0.0	6.6 ± 2.4	2.1 ± 0.6	40.4 ± 7.3
INIA-11	2.9 ± 0.3	49.9 ± 7.5	4.5 ± 0.3	83.2 ± 9.8
INIA-120	2.0 ± 0.5	46.1 ± 6.6	4.4 ± 0.2	76.5 ± 9.1
INIA-152	0.0 ± 0.0	6.4 ± 0.8	3.7 ± 0.2	54.1 ± 1.9
INIA-19	0.1 ± 0.1	7.1 ± 2.0	3.5 ± 0.2	48.6 ± 5.2
INIA-19F	1.2 ± 0.8	25.6 ± 4.1	3.9 ± 0.3	57.5 ± 9.3
INIA-23A	0.0 ± 0.0	5.3 ± 1.8	1.1 ± 0.3	26.5 ± 2.7
INIA-24	3.8 ± 0.7	71.4 ± 13.7	4.3 ± 0.2	75.8 ± 4.6
INIA-25	2.8 ± 1.0	51.1 ± 19.1	4.9 ± 0.1	91.1 ± 8.9
INIA-3	0.3 ± 0.3	3.2 ± 2.0	2.9 ± 0.2	51.1 ± 4.0
INIA-30	5.0 ± 0.0	100.0 ± 0.0	4.3 ± 0.2	71.7 ± 10.8
INIA-31	0.5 ± 0.5	23.3 ± 6.3	3.2 ± 0.4	50.9 ± 9.0
INIA-34	0.5 ± 0.3	14.2 ± 6.4	3.9 ± 0.3	61.4 ± 10.4
INIA-36	2.4 ± 0.5	24.0 ± 4.6	5.0 ± 0.0	100.0 ± 0.0
INIA-40	0.0 ± 0.0	0.0 ± 0.0	2.0 ± 0.2	35.8 ± 3.4
INIA-41	1.1 ± 0.1	26.2 ± 2.3	2.2 ± 0.8	51.4 ± 8.0
Mean ± SE	0.9 ± 0.2	19.8 ± 4.1	3.0 ± 0.3	53.4 ± 6.2
LSD ($P < 0.05$)	1.0 125	18.73	1.0187	18.72

DI = disease index, VDL = vascular discoloration length (%), SE = standard error.

Table 4.2 (continued).

	Fusarium Wilt			
	Race 3		Race 4	
Genotype	**DI (0-5)**	**VDL (%)**	**DI (0-5)**	**VDL (%)**
INIA-42F	0.0 ± 0.0	17.6 ± 2.2	4.9 ± 0.1	96.7 ± 3.3
INIA-51	2.0 ± 0.5	27.8 ± 6.8	5.0 ± 0.1	100.0 ± 0.0
INIA-51A	4.8 ± 0.3	89.5 ± 10.5	4.5 ± 0.2	80.5 ± 7.2
INIA-5A	1.0 ± 0.4	32.1 ± 1.5	2.7 ± 0.5	50.8 ± 8.6
INIA-5E	0.3 ± 0.3	7.6 ± 2.3	2.1 ± 0.2	33.6 ± 2.7
INIA-72	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.1	28.2 ± 3.0
INIA-73	3.0 ± 0.6	56.6 ± 10.0	4.4 ± 0.2	75.3 ± 9.4
INIA-76	0.7 ± 0.4	14.1 ± 5.3	4.3 ± 0.2	67.3 ± 13.8
IT-18	2.5 ± 1.1	48.8 ± 18.0	3.9 ± 0.3	63.2 ± 6.4
IT93K-503-1	0.0 ± 0.0	13.9 ± 3.2	1.0 ± 0.1	31.2 ± 2.2
Maputo	0.2 ± 0.2	13.8 ± 5.4	0.5 ± 0.1	18.9 ± 2.4
Massava-11	0.0 ± 0.0	5.6 ± 5.6	0.7 ± 0.2	17.2 ± 1.8
Muinana-Lawe	0.2 ± 0.1	4.6 ± 2.8	4.0 ± 0.2	59.9 ± 5.6
Namuesse	0.3 ± 0.2	20.0 ± 5.0	1.9 ± 0.1	32.9 ± 3.1
Namuesse-D	0.3 ± 0.3	4.7 ± 3.4	2.5 ± 0.4	32.3 ± 5.6
Namurua	0.0 ± 0.0	1.7 ± 0.6	4.4 ± 0.3	80.7 ± 7.9
Nhacoongo-1	0.0 ± 0.0	6.4 ± 2.2	3.1 ± 0.2	38.5 ± 2.8
Nhacoongo-2	0.0 ± 0.0	0.2 ± 1.5	0.5 ± 0.2	18.1 ± 3.2
Nhacoongo-3	0.0 ± 0.0	3.0 ± 0.2	4.2 ± 0.5	70.0 ± 14.0
SP-373	1.9 ± 0.7	17.3 ± 11.9	4.9 ± 0.1	88.5 ± 11.5
SP-860	1.3 ± 0.6	7.1 ± 3.4	4.8 ± 0.2	86.8 ± 13.2
SP-866	0.4 ± 0.4	2.1 ± 1.9	3.2 ± 0.4	30.2 ± 7.3
Tete-2	0.0 ± 0.0	5.7 ± 2.3	3.9 ± 0.3	62.2 ± 4.6
Timbawene-Monteado	0.0 ± 0.0	1.4 ± 1.0	3.3 ± 0.4	53.6 ± 8.7
VAR-10B	0.4 ± 0.4	7.6 ± 7.1	3.4 ± 0.4	53.6 ± 2.4
VAR-11D	0.0 ± 0.0	0.0 ± 0.0	4.0 ± 0.5	64.1 ± 13.7
VAR-3A	0.0 ± 0.0	0.2 ± 0.2	0.3 ± 0.2	24.6 ± 2.3
Xingove	-	-	3.4 ± 0.3	45.6 ± 2.0
Mean ± SE	0.9 ± 0.2	19.8 ± 4.1	3.0 ± 0.3	53.4 ± 6.2
LSD ($P < 0.05$)	1.0 125	18.73	1.0187	18.72

DI = disease index, VDL = vascular discoloration length (%), SE = standard error.

Intermediate responses to wilting under Fot4 infection were observed for genotypes INIA-3, INIA-5A, Nhacoongo-1, Tete-2, Ecute, INIA-19, VAR-10B and SP-866.

The test genotypes were also evaluated for vascular discoloration (%VDL) caused by colonization of vascular tissues by Fot4. In this assay, plant stems were cut open to measure the height of discoloration of vascular tissues using a ruler. The threshold for resistance to vascular discoloration was set at %VDL ≤ 30% and the susceptible reaction set at %VDL ≥ 30%. Based on this threshold, resistant genotypes included Maputo, FN-2-9-04, FN-2-13-04, FN-2-11-04, INIA-23A, Gile-K-Local, Massava-11, VAR-3A, INIA-72, Inhaca-I and Nhacoongo-2, and they did not differ from each other ($P > 0.05$) (Table 4.2). These resistant genotypes had similar responses to that of resistant controls CB27 and IT93K-503-1. The differences between these controls were significant, with CB27 more resistant than IT93K-503-1 ($P < 0.05$). Genotypes INIA-42F, INIA-51, INIA-25, SP-373, INIA-36 and SP-860 supported the most vascular discoloration, similar to susceptible controls CB46 and 24-125B-1, whereas genotypes INIA-3, FN-1-13-04, INIA-5A, FN-1-14-04, INIA-152, INIA-1, INIA-36 and INIA-19, VAR-10B and SP-866 exhibited an intermediate response to vascular discoloration induced by Fot4. Genotypes Ecute, FN-1-14-04 and INIA-41, although resistant to wilting, exhibited susceptible reactions to vascular discoloration under Fot4 infection (Table 4.2).

Most of the test genotypes exhibited resistant responses to wilting induced by Fot3 (Table 4.2), with 19 genotypes showing similar responses to resistant

controls CB46, CB27 and IT93K-503-1. Most of the differences in response to wilting by Fot3 among genotypes were not significant (*LSD = 1.0*, *P > 0.05*). Fot3 wilt susceptible genotypes included INIA-30, FN-1-13-04, INIA-51A and INIA-24 (DI > 3.0).

The test genotypes exhibited variable responses to Fot3 induced vascular discoloration, as measured by %VDL (Table 4.2), although most genotypes were resistant. However, slight vascular discoloration symptoms were detected on most resistant genotypes, and they did not differ in symptom level (*P > 0.05*). No discoloration symptoms were detected on vascular tissues of genotypes VAR-3A, INIA-40, Inhaca-I, INIA-72, VAR-11D and Nhacoongo-2, similar to that observed in control CB27. CB46 and IT93k-503-1 sustained vascular discoloration of 3 and 14%, respectively, but this was not different from CB27 (*P > 0.05*). Fot3 induced susceptible vascular discoloration phenotypes on eight genotypes. Extensive vascular discoloration induced by Fot3 was recorded in genotypes INIA-30, INIA-51A and INIA-24 (%VDL = 82.3, 79 and 63.5%, respectively).

Relationship Between Plant Wilting and Vascular Discoloration

The relationship between plant wilting and vascular discoloration was examined to determine whether plant wilting is independent from vascular discoloration induced individually by each Fusarium wilt race. An overall positive and strong relationship between both responses was found for both Fot3 and Fot4 infection symptoms (*r = 0.95, P < 0.0001* and *r = 0.94, P < 0.0001*, respectively (Figs.

4.2A and 4.2B). Although this relationship was largely explained by the response of the genotypes (R^2 = *0.91* - Fot3 and R^2 = *0.88* - Fot4), the regression analysis (Figs 4.2A and 4.2B) indicated an extremely week association between plant wilting and vascular discoloration under each Fusarium wilt infestation (*b* = *0.05* for Fot3 and *b* = *0.06* for Fot4).

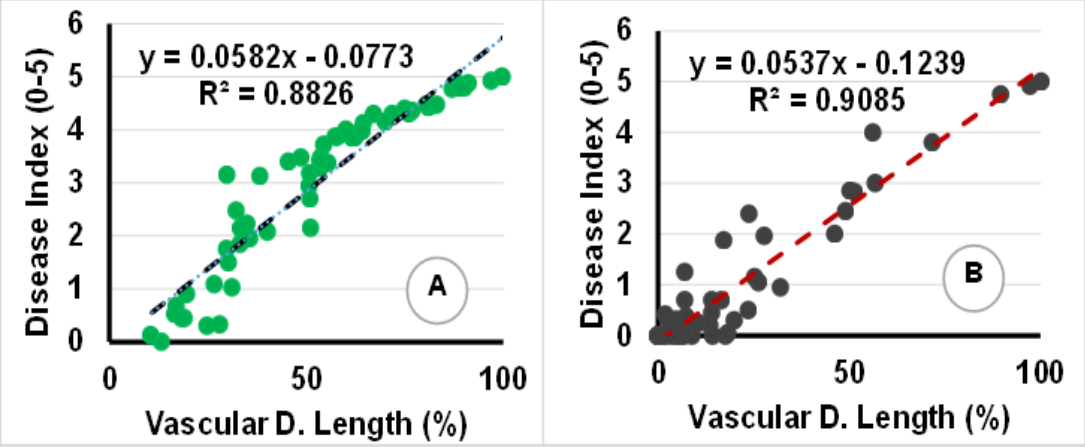

Fig. 4.2. Relationship between response to vascular discoloration and plant wilting induced by (A) Fot4 and (B) Fot3 infection.

Plant growth (expressed by shoot-weight) was suppressed under both Fot3 and Fot4 infection and was strongly correlated with both wilting (*r = 0.87, P < 0.0001* and *r = 0.97, P < 0.0001*, respectively) and vascular discoloration (Figs 4.3A and 4.3B). Since plant wilting and vascular necrosis were strongly correlated (Figs 4.2A and 4.2B), only the relationship between shoot-weight and wilting/yellowing is presented. Both wilting/yellowing and vascular discoloration were strongly associated with plant growth decline in Fot3 and Fot4 susceptible plants.

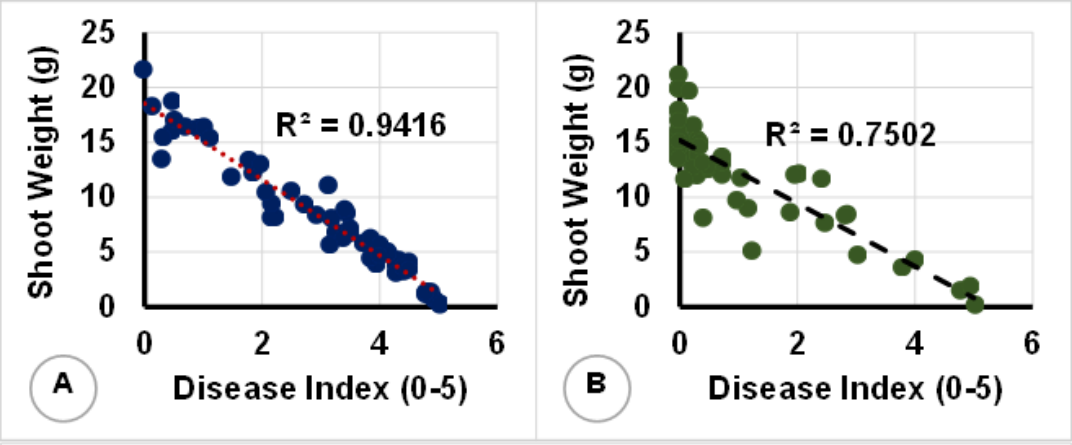

Fig. 4.3. Relationship between plant wilting (DI) and growth (shoot weight) under (A) Fot4 and (B) Fot3 infection.

Fusarium Wilt Virulence and Resistance Specificity

Differences in virulence between Fot3 and Fot4 in the test genotypes were analyzed to determine the potential damage of the two races. Virulence indexes (VI) were estimated utilizing phenotypic data for plant wilting. In addition, data for plant wilting/yellowing of each Fusarium wilt race were examined to determine the relationship between genetic determinants underlying resistance to Fot3 and Fot4 among the test cowpea genotypes.

The ANOVA of data for wilting/yellowing and vascular discoloration indicated that both races Fot3 and Fot4 induced significant responses on the test cowpea genotypes ($P < 0.0001$), and the differences between both in the ability to induce wilt/yellowing and vascular discoloration were significant ($P < 0.0001$).

The differences in virulence between Fot3 and Fot4, as measured by wilting (disease index) are illustrated in Figs. 4.4A and 4.4B. On average, Fot4 (VI = 67.7%) was four-fold more virulent than Fot3 (VI = 17.3%) (Fig. 4.4A).

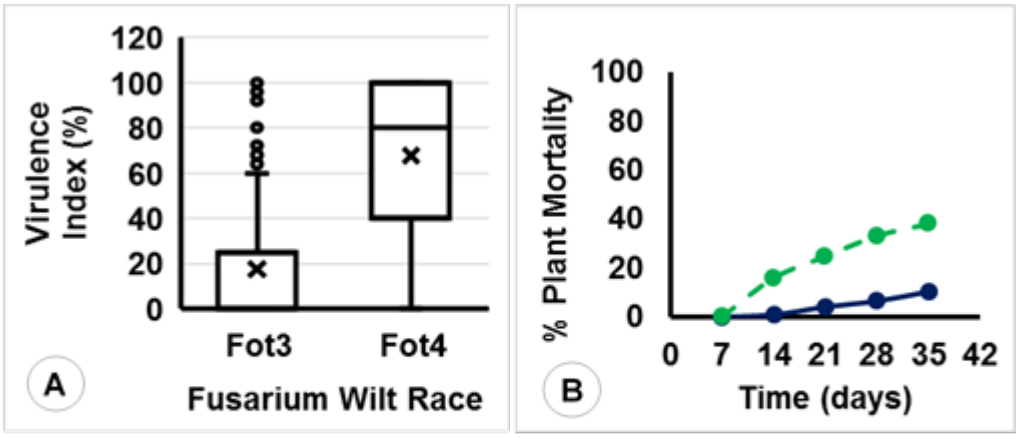

Fig. 4.4. (A) Differential virulence between Fot3 and Fot-r4 based on plant wilting symptoms; (B) disease progress and plant death caused by (—) Fot3 and (- - -) Fot4 days after plant inoculation.

Disease progress (as measured by percent plant mortality) under Fot3 and Fot4 infection is presented in Fig. 4.4B. The difference in slope between Fot4 and Fot3 suggests an enhanced virulence of Fot4 compared to Fot3. Data for plant wilting were recorded every 7 days after plant inoculation by counting the number of plants showing wilting/yellowing symptoms. Plant wilting/yellowing symptoms first appeared on susceptible genotypes about 9 – 12 days after inoculation regardless of Fusarium wilt race, but by 14 days after inoculation plant death incited by Fot4 was sixteen-fold greater than that caused by Fot3, and 35 days after inoculation, the difference in plant death between both races was four-fold.

There was a moderate but significant correlation (*r= 0.52, P < 0.0001*) between plant wilting responses induced by Fot4 and Fot3 infections (Fig. 4.5). Most Fot4 resistant genotypes showed cross resistance to Fot3 (Table 4.2), and resistance specificity was observed for Fot3. For example, genotypes INIA-19F, Muinana-Lawe, SP-373, INIA-42F, INIA-31 and VAR-10B were resistant to Fot3 but susceptible to Fot4, similar to the response observed in control CB46, which only has Fot3 resistance. Genotypes INIA-73, FN-1-13-04, INIA-24, INIA-30 and INIA-51A were susceptible to both Fot3 and Fot4, and their responses were similar to susceptible control 24-125B-1 (Table 4.2).

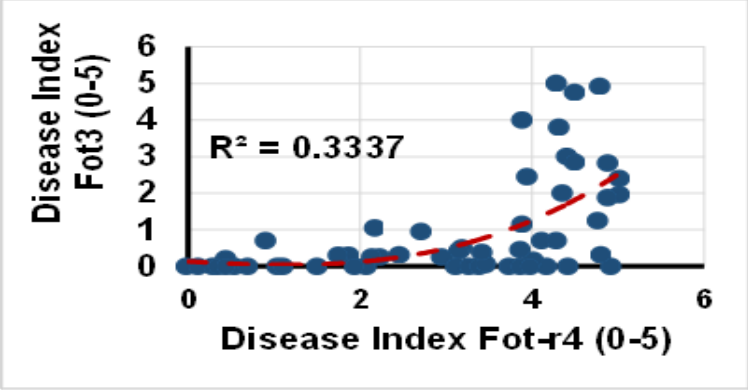

Fig. 4.5. Correlation between plant wilting caused by Fot3 and Fot-4 infections.

Effectiveness of Resistance in Fot4 Resistant Genotypes

The effectiveness of resistance to Fot4 in resistant genotypes was investigated to determine the heritability of resistance. For this analysis, four F_1 populations were developed by crossing the resistant test genotype FN-2-9-04 to four Fot4 susceptible genotypes (CB46, INIA-73, Ecute and 24-125B-1) (Table 4.2). The F_1 populations together with parental genotypes were assayed for resistance to

wilting/yellowing (DI) and vascular discoloration (%VDL) incited by Fot4 infection. The data for DI and %VDL were collected from 3 independent experiments, and in each test 10 plants of each F_1 population were phenotyped for DI and %VDL.

The response of F_1 populations, parental genotypes and additional controls to wilting/yellowing (Fig. 4.6A) and vascular discoloration (Fig. 4.6B) induced by Fot4 is shown in Fig. 4.6. The ANOVA revealed significant effects of the tested genotypes in response to wilting and vascular discoloration ($P < 0.05$). Significant differences ($P < 0.05$) in response to wilting and vascular discoloration among the tested genotypes were detected at DI = 0.97 and 16.63, respectively, indicated by the horizontal dashed lines (Fig. 4.6). The four F_1 populations did not differ ($P > 0.05$) in their responses to both phenotypes, and their responses were similar to that of the resistant parent (FN-2-9-04). However, all F_1 populations were more resistant ($P < 0.05$) than their susceptible parents. No obvious wilting symptoms were detected in the F_1 plants and in the resistant parent, but vascular discoloration symptoms restricted to a few centimeters of the total plant height were detected on both FN-2-9-04 and F_1 plants. The average length of vascular discoloration in the F_1 populations ranged from 0.3 – 22% of the plant height, and their level of vascular discoloration was the same as the resistant parent, but less than that of their susceptible parents ($P < 0.05$) (Fig. 4.6).

Overall, the average performance of all 4 F_1 populations in response to wilting/yellowing and vascular discoloration incited by Fot4 was DI = 0 and VDL = 8.6%, respectively, compared to DI = 2.8 and VDL = 56.9 of their parents

(average of all parents), respectively. The phenotypic responses in the F_1 populations to wilting and vascular necrosis induced by Fot4 were not different from those observed in resistant controls CB27 and IT93k-503-1 ($P > 0.05$). The genotype Ecute exhibited a similar reaction to wilting and vascular necrosis as in previous experiments (Table 4.2). This genotype was resistant to wilting but showed some susceptibility measured by vascular discoloration (Table 4.2 and Fig. 4.6).

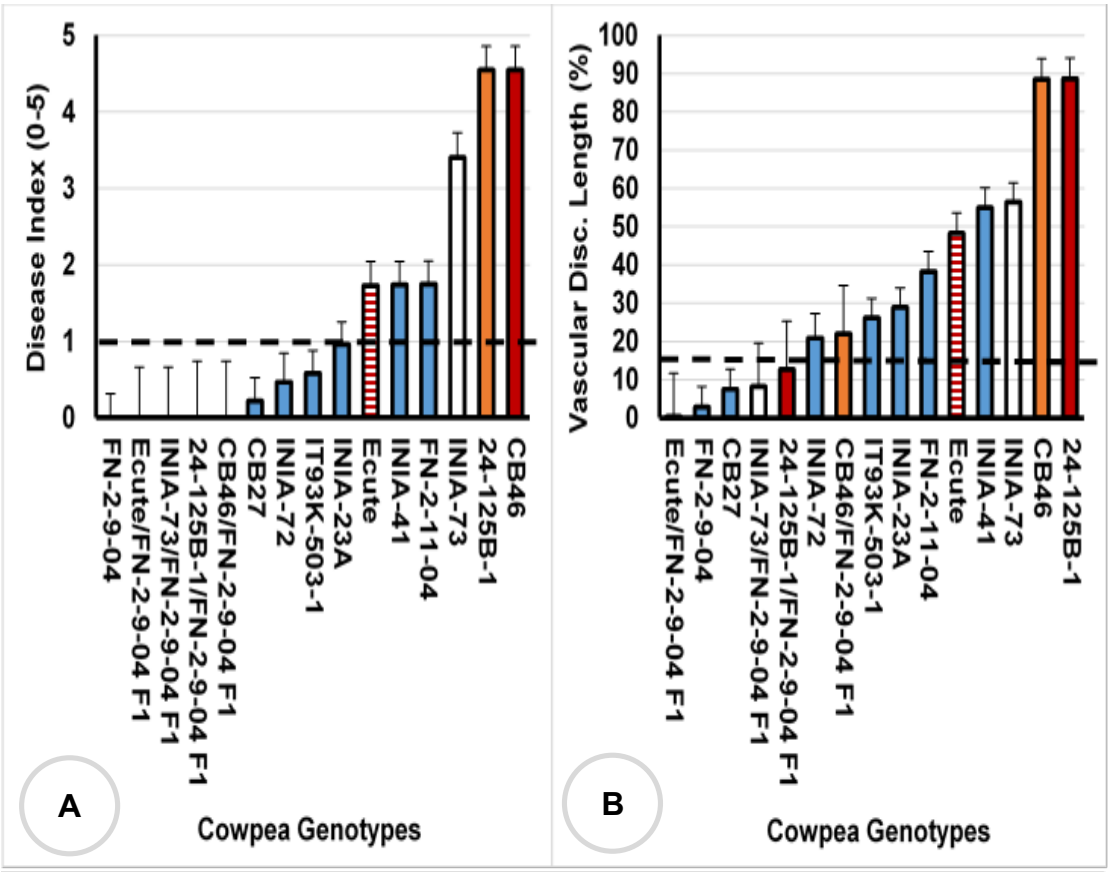

Fig. 4.6. (A) Wilting and (B) vascular discoloration after Fot4 infection in FN-2-9-04 (and additional controls) and four F_1 populations derived from FN-2-9-04 and 4 susceptible genotypes. Horizontal lines represent LSD ($P = 0.05$) = 0.97 (disease index – 4.6A) and 16.63 (vascular discoloration length – 4.6B), genotypes with mean differences higher than these thresholds were different in response.

Discussion

Significant variation in response to Fot3 and Fot4 infection based on wilting and vascular discoloration were found among the set of 53 cowpea accessions and landraces from Mozambique. Genotypes Maputo, FN-2-9-04, FN-2-13-04, FN-2-11-04, Massava-11, VAR-3A, INIA-72, INIA-23A, Gile-K-Local, Inhaca-I and Nhacoongo-2 were identified as potential novel resistance donors for breeding Fot3 and Fot4 resistant cowpea cultivars due to their consistently high levels of resistance. The performance of these novel sources of Fot3 and Fot4 resistance was statistically equivalent to that of the resistant controls, IT93K-503-1 and CB27, whose joint resistance to Fot3 and Fot4 is being bred into new cultivars. These novel sources of resistance may carry the same or distinct genetic resistance factors as those identified previously in CB27 and IT93K-503-1 (Pottorff et al., 2012; Pottorff et al., 2014). The differential phenotypic responses between these two controls to vascular discoloration induced by Fot4 suggested that CB27 carries stronger resistance than that in IT93K-503-1 ($P < 0.05$) (Table 4.2). The resistance to Fot4 present in CB27 (*Fot4-2*) and IT93K-503-1 (*Fot4-1*) mapped to distinct chromosomes of the cowpea consensus genetic map (Pottorff et al., 2014); however, the difference in resistance strength conferred by these genes was not recorded in the earlier studies. The response of the genotypes from Mozambique to Fot3 indicated that most of them carry resistance to this Fusarium wilt race, suggesting that Fot3 resistance might be widespread in cowpea. The known differential responses to Fot3 and Fot4 observed here among the test cowpea genotypes

provide a useful set of cowpea genotypes that can be used as a tool for routine field diagnosis of Fusarium wilt disease in cowpea.

Estimated virulence of Fot3 and Fot4 based on data for DI and %VDL indicated that Fot4 was at least four-fold more virulent than Fot3. This difference explains the reported severe damage caused by Fot4 on cv. CB46 in cowpea growing areas in California (Smith et al., 1999; Frate, 2012). Plant wilting induced by Fot4 was moderately correlated with that induced by Fot3, suggesting that the resistance response against both races might be under control by distinct resistance factors or mechanisms, or that Fot4 resistant genotypes carry resistance to both Fot3 and Fot4. This finding is in agreement with the mapping of Fot3 and Fot4 resistance QTLs present in cv. CB27 to distinct genomic regions of the cowpea consensus genetic map (Pottorff et al., 2012; Pottorff et al., 2014). All Fot4 resistant genotypes showed a broad-based resistance to both races. CB46 carries Fot3 resistance (similar to that in CB27), and this resistance is not effective against Fot4 (Table 4.2). Whether the responses of CB27 and IT93k-503-1 to Fot4 are conferred exclusively by *Fot4* alleles remains unknown since both controls carry Fot3 resistance in addition to Fot4 resistance. Likewise, in controls CB27 and IT93k-503-1, the broad-based resistance in the test resistant genotypes might be conferred by the presence of both Fot3 and Fot4 resistance alleles.

The strong correlation between wilting/yellowing and vascular discoloration (Figs. 4.2A and 4.2B) suggested that in most Fusarium wilt resistant test genotypes, both responses might be under control by interrelated or the same

response mechanisms. However, the weak association detected through regression analysis (*b = 0.05* - Fot3 and *b = 0.06* - Fot4) suggested this correlation might be limited to particular cowpea backgrounds, and distinct resistance mechanisms might be present. For example, genotypes FN-1-14-04, INIA-41 and Ecute were resistant to wilting/yellowing induced by Fot4, but susceptible to vascular discoloration caused by the same Fusarium wilt race (Table 4.2 and Fig. 4.6). Probably, the number of vascular vessels, their diameter, distribution and structural composition of the vessels may play a substantial role in resistance expression.

The effectiveness of Fot4 resistance present in test cowpea genotype FN-2-9-04 was confirmed by the response of four distinct F_1 populations. The performance of F_1 progenies relative to their parents indicated that the resistance to Fusarium wilt in FN-2-9-04 is heritable, and the resistance response of F_1 populations relative to their resistant parent indicated that the resistance in this genotype is controlled by genetic factors with dominant effect. Dominant-type resistance to Fusarium wilt has been reported in other crops as well as cowpea (Netzer et al., 1977; Ribeiro and Hagedorn, 1979; Rigert and Foster, 1987; Scott and Jones, 1989; Salgado et al., 1995; Cross et al., 2000).

In summary, this study identified sources of broad-based and strong resistance to Fot3 and Fot4 in cowpea accessions and landraces from Mozambique with potential value for breeding Fusarium wilt resistant cowpea cultivars. Also, these sources of resistance can be useful for field diagnosis of Fot3 and Fot4 in newly infested fields. Although these sources showed a broad-based resistance to this pathogen and potential value for breeding resistant cultivars, the relationship between the resistance factors in these novel sources and those in control genotypes CB27 and IT93K-503-1 remains to be determined through genetic studies. Also, the resistance relationship among the novel sources of resistance still requires investigation. In addition, the relationship between Fot3 resistance in CB27 and IT93K-503-1 is unknown. These controls, although both resistant to Fot4, showed a significant differential response to vascular discoloration which suggests that the allele variants present in these controls might confer resistance of different strengths. Also, it remains unknown whether Fot4 resistance alleles present in these genotypes are specific to Fot4 alone or have a broad resistance spectrum to both Fot3 and Fot4. The resistance in test genotype FN-2-9-04 is heritable and dominant. The data suggested that Fot3 and Fot4 resistances are controlled by distinct resistance mechanisms, although the phenotypic responses of wilting and vascular discoloration incited by Fusarium wilt are likely to be under control by related mechanisms.

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CHAPTER V - Genetics and QTL Mapping of Fusarium Wilt Race 4 Resistance in Cowpea Accession FN-2-9-04

Abstract

Fusarium wilt disease of cowpea caused by *Fusarium oxysporum* f. sp. *tracheiphilum* (Fot) can cause severe crop loss. Fusarium wilt race 4 (Fot4) is the most aggressive form of this pathogen on cowpea, and its effective management relies on host-plant resistance. The genetics and genomic location of the strong Fot4 resistance in cowpea accession FN-2-9-04 was investigated using 3 F_1, 7 F_2 and 1 $F_{2:3}$ populations derived from this donor. Segregation analysis of phenotypic responses to plant wilt (W) and vascular necrosis (VN) induced by Fot4 indicated genetic control of resistance in FN-2-9-04 is conferred by two partially dominant genes and one recessive gene. Two QTLs were detected, one on chromosome Vu03 (PVE = 49.3 and 54.5%, W and VN, respectively) between positions 20.25 – 53.11 cM (W) and 29.55 – 35.46 cM (VN) and one on Vu08 (PVE = 13.4%, W) between positions 26.72 – 33.04 cM of the cowpea consensus genetic map. The QTL on Vu03 and Vu08 overlapped partially with the previously mapped *Fot4-1* and *Fot4-2* loci present in CB27 and IT93K-503-1, respectively. The Vu03 QTL was associated with both W and VN phenotypes, whereas the Vu08 QTL was associated with only W. Recombination fractions of 22.29 and 24.74% in the F_2 CB27 x FN-2-9-04 and IT93K-503-1 x FN-2-9-04 populations, respectively indicated loosely linked resistance loci between FN-2-9-04 and the other parental sources. However, segregation for resistance in these F_2 populations and the resistance location of the Vu03 QTL peak in FN-2-9-04 relative to that in CB27, plus the presence

of two Fot4 QTLs in FN-2-9-04, suggested that this accession carries a multi-allele resistance locus for response to Fot4. The Vu03 and Vu08 QTLs have additive effect, and both are required for effective Fot4 resistance.

Introduction

Globally, cowpea (*Vigna unguiculata* L. Walp) yield is below the known potential (Adegbite and Amusa, 2008) although it has substantial importance as proteinaceous food and a source of income in the developing world. Fusarium wilt disease of cowpea, caused by *Fusarium oxysporum* f. sp. *tracheiphilum* (Schl.) (Fot), a soil-borne fungus, is a common disease in many cowpea growing areas. It can infect young seedlings early in the growing season, and adult plants at mid-season stage, resulting in seedling death, plant wilting and stunting leading to reductions in plant stand and yield loss (Smith et al., 1999). The impact of Fusarium wilt disease on susceptible cowpea cultivars can be exacerbated by root-knot nematode (RKN) infection in cowpea fields where both pathogens are present (Harris and Ferris, 1991; Roberts et al.,1995). Under such conditions, both pathogens can interact with the host plant to form a disease complex, in which root infection by RKN weakens and predisposes the host plant to infection by the fungus and disease development (Sidhu and Webster, 1974; Harris and Ferris, 1991; Roberts et al.,1995).

Breeding for Fot resistant cowpea cultivars has been a major goal in cowpea breeding programs as a primary disease management strategy. Emergence of virulent forms of Fot can compromise the effectiveness of currently available sources of resistance (Smith et al*.,* 1999; Ehlers et al*.,* 2009), while chemical

options are costly and not economically viable for cowpea production systems. Therefore, breeding for host-plant resistance has been pursued as the most efficient strategy to counteract the impact of several cowpea diseases including Fusarium wilt.

Four Fot races of cowpea have been reported (Hall and Frate, 1996), races 1 and 2 (Armstrong & Armstrong, 1950; Armstrong & Armstrong, 1980; Smith et al., 1999), race 3 (Armstrong & Armstrong, 1980; Smith et al., 1999) and race 4 (Smith et al., 1999). Currently, Fot3 and Fot4 are the most problematic races in cowpea production areas (Frate, 2012; Hall and Frate, 1996), and Fot3 is the most wide-spread race in cowpea growing areas (Smith et al., 1999; Ehlers et al., 2009; Hall and Frate, 1996; Frate, 2012). In recent years, Fot4 populations have emerged which are virulent on cowpea cultivars carrying Fot3 resistance. In California, Fot4 is currently the most aggressive and problematic race for the cowpea industry (Ehlers et al., 2009; Frate, 2012). The resistance to Fusarium wilt present in the common commercial cowpea cultivar CB46 (Helms et al., 1991) is effective against Fot1 and Fot2 (Smith et al., 1999) plus Fot3 (Helms et al., 1991; Smith et al., 1999; Hall and Frates, 1996; Frate, 2012), and this cultivar has been grown widely in many cowpea production areas. However, the emergence of Fot4 severe plant wilt of CB46 in fields where this cultivar is frequently grown has stimulated a search of additional sources of resistance (Ehlers et al., 2009; Frate, 2012).

Relatively few sources of effective resistance to Fot3 and Fot4 have been identified in cowpea germplasm (Ehlers et al., 2000). Resistance to the virulent

race Fot4 has been bred recently into new commercial blackeye cowpea cultivars CB50 (Ehlers et al., 2009; Frate, 2012) and CB27 (Ehlers et al., 2000), and it has been effective under field conditions (Frate, 2012). Recently, through quantitative trait locus (QTL) mapping, two Fot4 resistance loci (Pottorff et al., 2014), which include the ones in cowpea cultivars CB50 and CB27, have been mapped and positioned on the cowpea consensus genetic map. These Fot resistance loci, derived from two geographically distinct cowpea genetic resources in cowpea gene-pool I (Huynh et al., 2013), provide effective resistance against the current Fot races.

The genetic basis of resistance to Fusarium wilt in cowpea, common bean, tomato, cucumber and lentil, cotton and cabbage has been reported as being of few resistance factors either with dominant or partially dominant effect (Netzer et al., 1977; Ribeiro and Hagedorn, 1979; Rigert and Foster, 1987; Scott and Jones, 1989; Salgado et al., 1995; Cross et al., 2000; Kamboj et al., 1990; Ulloa et al., 2006; Lv et al., 2014). Some evidence for involvement of modifier genes that enhance the genetic resistance conferred by major genes has also been reported (Scott and Jones, 1989; Ulloa et al., 2006). Polygenic control of resistance to Fusarium wilt has been reported in common bean, and it varies with the genotypic race of common bean (Cross et al., 2000; Salgado et al., 1995), while resistance to Fusarium wilt in chickpea has been reported to be associated with single recessive genes (Tullu et al., 1998; Sharma et al., 2005).

The management of Fusarium wilt disease in cowpea production systems currently relies on a narrow genetic base of resistance. This is due to the limited

availability of sources of resistance and by the narrow-based genetic resistance present in the few known sources. Shifts in virulence within the prevailing Fot races and the emergence of novel and aggressive Fot pathotypes with enhanced virulence would diminish the effectiveness, durability and value of currently available resistance to Fusarium wilt disease. The dynamics in virulence of Fot populations supports a search for novel Fot resistance sources to broaden the genetic base of resistance in commercial cowpea cultivars. A cowpea accession FN-2-9-04, from Mozambique, was identified as a source of strong and broad-based resistance to both Fot3 and Fot4. To examine the genetic determinants of this resistance in FN-2-9-04, genetic analysis was conducted to determine: (i) the inheritance of the resistance to Fot4; (ii) the uniqueness of genetic resistance in FN-2-9-04 relative to known resistance sources; and (iii) the genomic architecture and localization of Fot4 resistance determinants in FN-2-9-04 through genetic linkage analysis and QTL mapping.

Materials and Methods

Plant Materials: The cowpea accession FN-2-9-04 was identified, from a germplasm collection of 53 cowpea accessions and landraces from Mozambique, to possess strong and broad-based resistance to Fusarium wilt disease in a series of greenhouse screening trials for resistance to Fot3 and Fot4. This cowpea germplasm represents a very diverse and distinct collection of accessions and landraces genetically distinct from other known cowpea sources of Fusarium wilt resistance, and these genotypes are part of cowpea gene-pool II from southern and eastern Africa (Huynh et al., 2013).

Three F_1, seven F_2 and one $F_{2:3}$ segregating populations, with FN-2-9-04 as a parent, were developed under greenhouse conditions (University of California Riverside – UCR). For inheritance tests, FN-2-9-04 was crossed to Fot4 susceptible genotypes INIA-73, 24-125B-1, Ecute, Bambey-21 and CB46, to generate F_1 and F_2 and $F_{2:3}$ populations. The $F_{2:3}$ population was derived from F_2 population CB46 x FN-2-9-04. In addition, for allelism tests, two F_2 populations (resistant x resistant) were developed from the crosses IT93K-5031 x FN-2-9-04 and CB27 x FN-2-9-04.

Fusarium Wilt Resistance Screening

Inoculum Preparation and Plant Inoculation: Fusarium wilt race 4 (Fot4) inoculum was re-cultured from dried and stored culture at -80 ^{o}C on potato dextrose agar (PDA). Aseptically, a single dried plug, 1 cm^2, was cut and transferred into a petri dish containing freshly prepared PDA medium, and the

petri dish was incubated under room temperature to grow the fungus vegetatively. Five days after incubation, 3 fresh inoculum plugs, 1 cm^2, were cut aseptically and transferred into an Erlenmeyer flask containing 500 ml of freshly prepared potato-dextrose broth, and the flask was incubated for 4 days in a shaker at 30 rpm and 27 ^oC under light to promote fungus sporulation. Four days after incubation, the spore solution was filtered through 8 layers of cheesecloth, and the flow-through solution containing Fusarium spores was collected in a beaker. The spore density in the solution was determined by counting the number of spores using a hemocytometer under a light microscope, and the spore concentration was adjusted to the desired density of 10^6 microconidia/ml.

The viability of the inoculum was tested on two known Fot4 susceptible controls, CB46 and 24-125B-1 under greenhouse conditions at temperature settings of 29 ^oC min and 32 ^oC max. Ten 7-day-old seedlings of each control were uprooted, and the root system washed to remove soil and inoculated with Fot4 following a modified protocol of Rigert and Foster (1987). Briefly, the root system was clipped to about 3 cm length and dipped for 4 minutes into an Fot4 spore suspension containing 10^6 microconidia/ml. Five inoculated plants of each control were transplanted into 0.9 L foam cups (Fig. 5.1A) containing soil UC-mix 3 and grown for 28 days. The plants were watered every two days. Fifteen days-post inoculation, the plants were fertilized with osmocote (14-14-14) and assessed for wilting/yellowing and vascular necrosis symptoms 28 days-post inoculation.

Fusarium Wilt Disease Assessment: The FW disease symptoms were assessed using two phenotypic responses: (i) plant wilting (W) – scored on a diseases index (DI) and ii) vascular necrosis (VN). The plant wilting was based on a 0 to 5 disease index (Fig. 5.1A) rating chart to quantify the amount of wilting/yellowing/stunting symptoms as follows: DI of 0 = healthy plants with no obvious yellowing/wilting/stunting symptoms; DI of 1 = plants displaying yellowing/wilting symptoms on about 10% of their canopy; DI of 2 = 25% of the plant canopy showed yellowing/wilting; DI of 3 = 50% of the canopy with wilting/yellowing, plant stunting and canopy loss obvious and dark-red spots on the node of fallen leaf; DI of 4 = 75% yellowing/wilting/stunting, severe canopy loss, and DI of 5 = plant dead. Resistance to vascular necrosis (VN) (Fig. 5.1B and Fig. 5.1C) incited by Fot4 was determined by counting the number of necrotic vessels (NNV) which are discolored vascular vessels that eventually die (Fig 5.1C).

Fig. 5.3. Fusarium wilt symptoms on resistant and susceptible cowpea plants – (A-left): no wilting/yellowing/stunting, disease index (DI) = 0; (A-right): yellowing/stunted plants that eventually wilt and die, DI = 5. (B): longitudinally cut stem of a resistant plant showing no vascular necrosis and (C): susceptible plant stem showing extensive vascular necrosis.

Inheritance of Resistance and Allelism Test

The genetics underlying resistance in cowpea accession FN-2-9-04 to Fusarium wilt disease induced by Fot4 was investigated in different populations and generations using phenotypic data for plant wilting/yellowing and vascular necrosis. Parental genotypes were used as controls in each experiment, and the phenotyping and disease assessment followed the procedures described above. The details of the populations used are shown in Table 5.1. Populations 1 through 10 were used for classical genetic studies to investigate the segregation pattern, the genetic control of the trait and to estimate broad-sense heritability, while population 11 and its corresponding F_2 (used for genotyping) were used for linkage mapping and quantitative trait locus (QTL) mapping and analysis.

Table 5.1. Populations used for inheritance studies and QTL mapping of Fot4 resistance in FN-2-9-04.

Order	Population	Cross	Size
1	CB46/FN-2-9-04 F_1	Susceptible x Resistant	15
2	INIA-73/FN-2-9-04 F_1	Susceptible x Resistant	18
3	24-125B-1/FN-2-9-04 F_1	Susceptible x Resistant	18
4	CB46/FN-2-9-04 F_2	Susceptible x Resistant	364
5	INIA-73/FN-2-9-04 F_2	Susceptible x Resistant	353
6	24-125B-1/FN-2-9-04 F_2	Susceptible x Resistant	209
7	Bambey-21/FN-2-9-04 F_2	Susceptible x Resistant	120
8	Ecute/FN-2-9-04 F_2	Resistant x Resistant	145
9	CB27/FN-2-9-04 F_2	Resistant x Resistant	323
10	IT93K-503-1/FN-2-9-04 F_2	Resistant x Resistant	363
11	CB46/FN-2-9-04 $F_{2:3}$	Susceptible x Resistant	175

Segregation Analysis: Data for phenotypic responses to wilting and vascular necrosis of parental genotypes, F_2 and $F_{2:3}$ population were analyzed for goodness-of-fit to various genetic models. In addition, SNP marker genotypes of F_2 population CB46/FN-2-9-04, within the mapped QTL regions were also processed for goodness-of-fit for validation of segregation patterns and the genetic models.

Resistance Heritability: Phenotypic data for plant wilting/yellowing and vascular necrosis of seven F_2 populations (Table 5.1, Populations 4-10) and parental genotypes were used to estimate broad-sense ($H^2 = V_g/V_p$) heritability of resistance to Fot4 in FN-2-9-04 following the midparent-offspring regression analysis (Fernandez and Miller, 1985; Falconer and Mackay, 1996). The estimated V_g at the QTL region associated with wilting phenotypes was used to estimate the H^2 for validation. In addition, the Vg ($Vg = V_a + V_d$) accounting for variability of phenotypic variability of response to Fot4 in the $F_{2:3}$ generation was partitioned into its components to estimate the narrow-sense heritability ($h^2 = V_a/V_p$) of response to both wilting/yellowing and vascular necrosis.

Allelism tests: To investigate the uniqueness of resistance to Fot4 in cowpea accession FN-2-9-04, phenotypic data for wilting/yellowing and vascular necrosis of two F_2 populations derived from crosses IT93K-503-1 x FN-2-9-04 and CB27 x FN-2-9-04 (Table 5.1 - populations 9 and 10) were analyzed for goodness-of-fit to genetic models. Cowpea cv. CB27 and breeding line IT93K-503-1 carry independent Fot4 resistance loci (*Fot4-2* and *Fot4-1*, respectively)

previously mapped in chromosomes Vu03 (old linkage group 3) and Vu08 (old linkage group 5), respectively (Pottorff, et al., 2014); thus, segregation for resistance to Fot4 with presence of susceptible progeny in F_2 populations IT93K-503-1 x FN-2-9-04 and CB27 x FN-2-9-04 would indicate recombination and independence between Fot4 resistance loci present in FN-2-9-04 and those present in CB27 and IT93K-503-1.

Linkage and QTL Mapping

Leaf DNA: Leaf samples of parental genotypes (CB46 and FN-2-9-04) and 175 F_2 lines of population CB46 x FN-2-9-04 were collected into plastic bags containing silica gel 30 days after planting and left to dry at room temperature. Genomic DNA of each dried leaf sample was extracted using Plant DNeasy (Qiagen protocol) and quantified using Nano drop. Each F_2 line was selfed to produce $F_{2:3}$ seeds, and 25-30 seeds per $F_{2:3}$ family were assayed for response to Fot4 infection as described previously.

Plant Genotyping and SNP Data Inspection: The DNA samples (> 50 ng/µl) were genotyped for single nucleotide polymorphism (SNP) using the 51128 iSelect SNP genotyping platform (Munoz-Amatriain et al., 2017). The SNP data were screened for quality by eliminating: (i) SNPs with more than 20% missing; (ii) monomorphic SNPs; (iii) SNPs with minor allele frequency (MAF) below 30%; and (iv) duplicate lines. Since the QTL mapping population was in the F_2 generation, heterozygous lines were not excluded from the data set during the inspection of data quality. No SNP marker loci were detected with non-parental

alleles which indicated that the genotyped parents corresponded to the parental plants used to develop the population.

Linkage mapping: SNP marker data of 137 F_2 lines of population CB46 x FN-2-9-04 were used for linkage mapping utilizing MSTmap program (Wu et al., 2015), and linkage groups were defined at a threshold of LOD = 10. The Kosambi mapping function was selected for marker placement, and mapping options *no mapping size threshold* and *no mapping distance threshold* were selected and fixed at 2 units and 10 cM, respectively. In addition, the option *no mapping distance threshold* was set at 15 cM and the *detection of genotyping errors* was not employed. The linkage map output was optimized by numbering and ordering it based on the cowpea consensus genetic map (Munoz-Amatriain et al., 2017), also the linkage group numbering followed the new linkage group naming based on cowpea chromosome pseudomolecules (Lonardi et al., 2017).

QTL Mapping: The detection and mapping of QTL regions associated with resistance to Fot4 was performed using 137 $F_{2:3}$ families of population CB46 x FN-2-9-04 (Table 5.1 – Population 11). Phenotypic data for wilting/yellowing and vascular necrosis were inputted separately into a mixed-model for QTL mapping described by Xu (2013) using the SAS University Edition 3.2.2, and the presence of significant QTLs was declared using Bonferroni adjusted threshold at $P < 0.05$.

Results

Inheritance of resistance to RKN in FN-2-9-04: Phenotypic responses of three F_1 populations (CB46 x FN-2-9-04, INIA-73 x FN-2-9-04 and 24-125B-1 x FN-2-9-04) and parental genotypes to wilting/yellowing (Fig. 5.2A) and vascular necrosis (Fig. 5.2B) induced by Fot4 are illustrated in Fig. 5.2. The levels of wilting (W) and vascular necrosis (VN) were expressed by disease index (DI) and number of necrotic vessels (NNV), respectively. The three F_1 populations had resistant phenotypic responses to both W and VN, and the average phenotypic response of each F_1 population to both phenotypic responses was similar to but slightly less resistant than the resistant parent, FN-2-9-04 (DI = 0 and NNV = 0.4). The ranges of phenotypic responses of individual F_1 populations were DI = 0.1 – 0.6 and NNV = 1.3 – 2.3, and the average response of all F_1 populations to W and VN were DI = 0.3 and NNV 1.7, respectively, compared to the mid-parent responses of DI = 3.4 and NNV = 9.5, respectively.

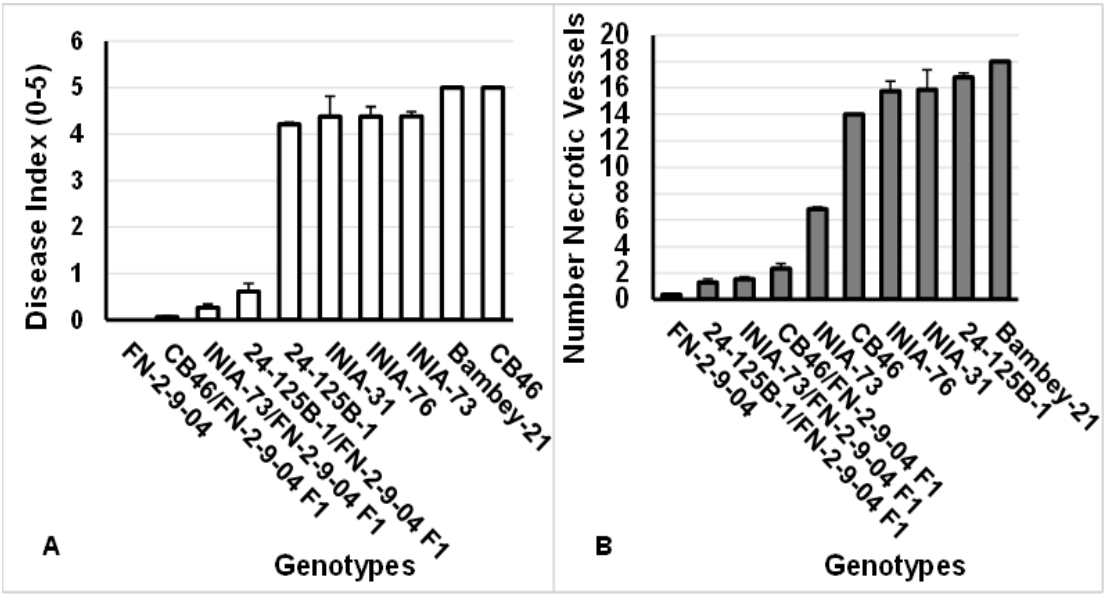

Fig. 5.2. Phenotypic responses of F_1 populations and parents to (A) wilting and (B) vascular necrosis induced by *Fusarium oxysporum* f. sp*. tracheiphilum*

In the F_2 generation of population CB46 x FN-2-9-04, the wilting (Fig. 5.3A) and vascular necrosis (Fig. 5.3B) responses followed bi-modal distribution; this distribution pattern was found in all 7 F_2 populations phenotyped for reaction to Fot4 (Table 5.1, populations 4 – 10). The average wilting and vascular necrosis responses in the F_2 population CB46 x FN-2-9-04 were DI = 2.0 and NNV = 5.4, slightly lower than that of the mid-parent response of the same population, (DI = 2.4 and NNV = 6.7). The average wilting and vascular necrosis responses in the F_2 population were between the resistant parent FN-2-9-04 and the mid-parent response similar, to the F_1 generations (Fig. 5.2). The wilting and vascular necrosis responses in the F_2 populations 5 – 8 (susceptible x resistant) (Table 5.1) were generally the same. However, in F_2 populations 9 and 10 (resistant x resistant) (Table 5.1), the average phenotypic response to infection of the offspring was transgressive to that of the parents.

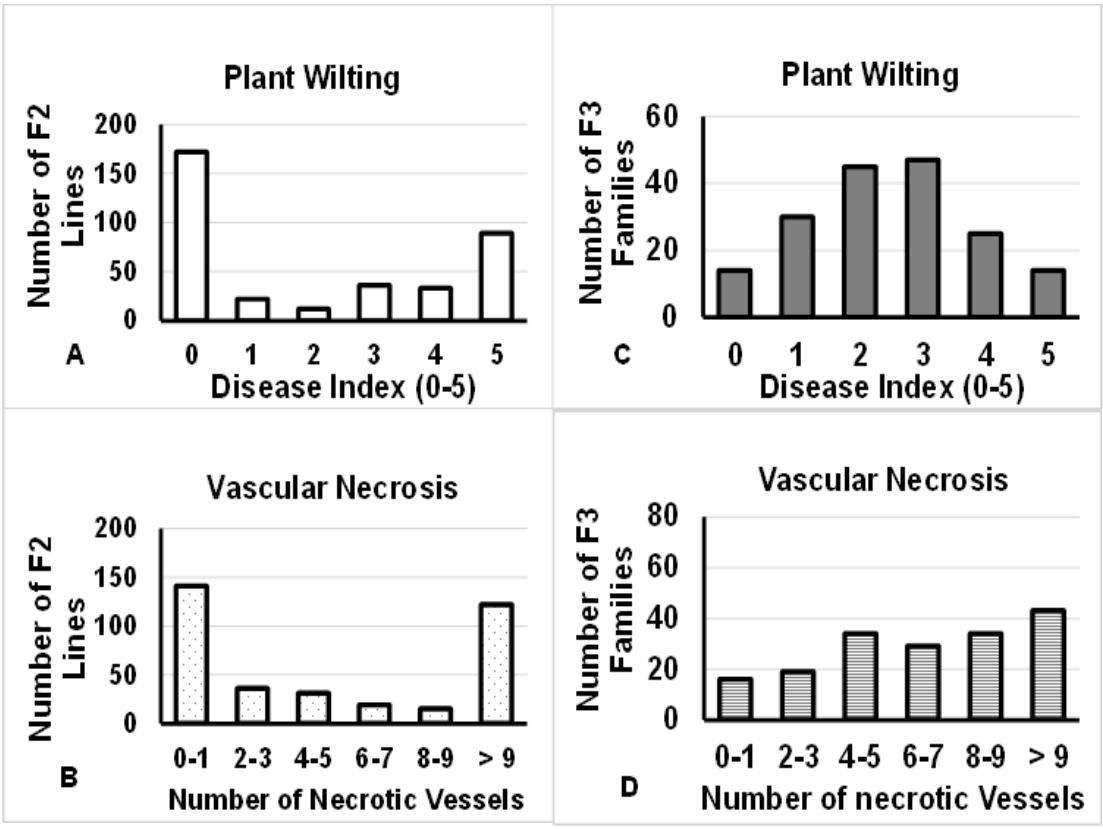

Fig. 5.3. Frequency distribution of response to plant wilting and vascular necrosis in the F_2 (5.3A and 5.3B, respectively) and in the $F_{2:3}$ (5.3C and 5.3D, respectively) generations of population CB46 x FN-2-9-04.

In the $F_{2:3}$ population CB46 x FN-2-9-04, the distribution of wilting and vascular necrosis responses also followed a bi-modal distribution (Figs 5.3C and 5.3D), and in this generation segregating lines could be distinguished. Average wilt and vascular necrosis responses in the $F_{2:3}$ were DI = 2.4 and NNV = 7, respectively, with the average in this generation between the resistant parent (DI = 0 and NNV = 2.0) and the mid-parent phenotypic response (DI = 2.5 and NNV = 6.8).

Resistance Heritability: Strong and positive correlation was found between the 7 F_2 populations and the mean (mid-parent) of their parents for wilting/yellowing and vascular necrosis responses to Fot4 infection (Fig. 5.4). These results indicated high broad-sense heritability (H^2) of Fot4 resistance to wilting and vascular necrosis incited by Fot4 in accession FN-2-9-04. Broad-sense heritability for wilting and vascular necrosis responses were 63% ($P = 0.012$, $R^2 = 0.75$) and 67% ($P = 0.0047$, $R^2 = 0.82$), respectively, and the regression explained a major proportion of the degree of association between the reactions of the parents and the F_2 progenies. The average level of wilting and vascular necrosis of F_2 progenies derived from resistant x resistant crosses was lower than that of F_2 progenies from susceptible x resistant crosses, as expected.

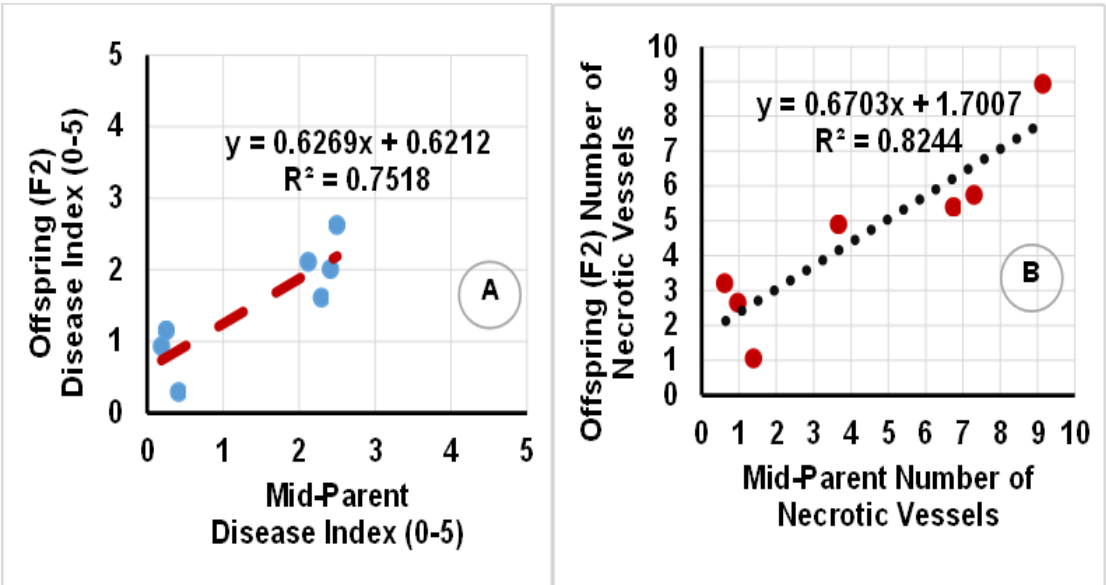

Fig. 5.4. Midparent – offspring regression for 7 F_2 populations means regressed on the mid-parent (A) wilt/yellowing and (B) vascular necrosis phenotypes.

To validate the H^2 of resistance to wilting and vascular necrosis, the genetic variances influencing both phenotypic responses were estimated within the QTL regions (Vu03 and Vu08) (Table 5.2) associated with these phenotypic responses. Based on these estimates, the H^2 of resistance to wilting contributed by Vu03 and Vu08 were 49.3% and 13.4%, respectively, while the H^2 of resistance to vascular necrosis (Vu03) was 54.5%. In addition to these heritability estimates, narrow-sense heritability (h^2) of resistance to wilting was 33.1 and 12.2% for Vu03 and Vu08, respectively, and 49.1% for vascular necrosis on Vu03.

Allelism tests: Allelic relationships of genetic factors associated with resistance to wilting and vascular necrosis between FN-2-9-04 and two Fot4 resistant genotypes CB27 and IT93K-503-1 were investigated in F_2 populations CB27 x FN-2-9-04 and IT93K-503-1 x FN-2-9-04 (Table 5.1, populations 9 and 10).

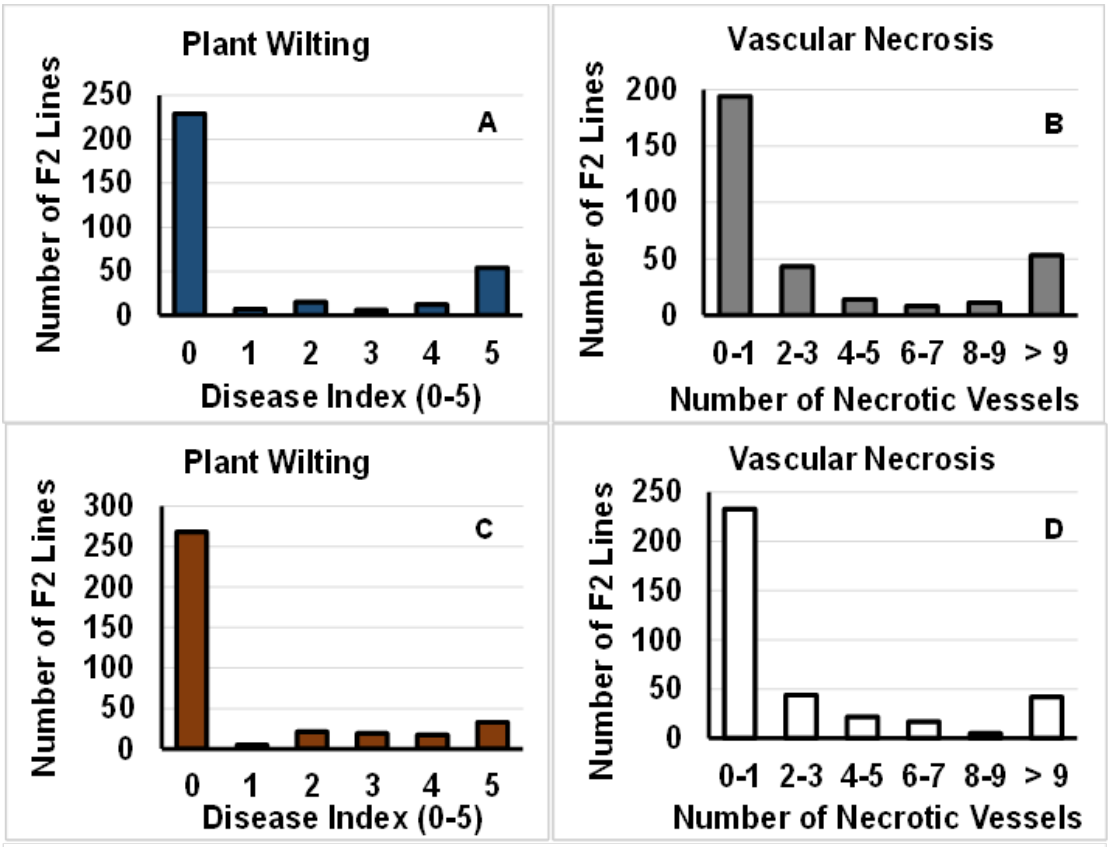

Fig. 5.5. Frequency distribution of plant wilting (A, C) and vascular necrosis (expressed by the number of necrotic vessels) (B, D) induced by Fot4 in the F_2 populations of CB27 x FN-2-9-04 and IT93K-503-1 x FN-2-9-04, respectively.

Both resistant x resistant F_2 populations segregated for resistance to wilting and to vascular necrosis following Fot4 inoculation (Figure 5.5). Based on wilting phenotypes, 22.29% of 323 and 24.74 % of 363 F_2 lines of populations CB27 x FN-2-9-04 and IT93K-503-1 x FN-2-9-04, respectively, were susceptible recombinants, and these lines supported high numbers of necrotic vessels, wilted and died. The best-fit segregation ratios for wilting response between resistant and susceptible plants in these populations were 13:3 (X^2 = 2.43, P = *0.10 – 0.25*) and 3:1 (X^2 = *0.001*, P = *0.95 – 0.99*), respectively. In these F_2

populations; the average wilting responses were DI = 1.2 and 0.9, respectively, compared to the mid-parent wilting responses of DI = 0.2 and 0.1, respectively.

Linkage and QTL mapping: For QTL mapping in the CB46 x FN-2-9-04 population, 137 $F_{2:3}$ families were phenotyped for wilting and vascular necrosis responses to Fot4 infection, and the individual F_2 plants corresponding to these families were genotyped with 51128 SNP markers. The genotypic data were used to construct a linkage map with a total of 17903 SNP markers which were polymorphic between the parents. The linkage map was constructed using MSTmap (http://www.mstmap.org). The map comprised 11 chromosomes which spanned 1158.681 cM, and 9.4% of the 17903 SNP were unique to this population, not mapped on the cowpea consensus genetic map (Munoz-Amatriain et al., 2017). Marker order and map distances in this population were oriented based on the cowpea consensus genetic map, and linkage groups or chromosome numbering followed the new nomenclature adopted from the common bean (*Phaseolus vulgaris*) chromosome numbering scheme for easy reporting (Lonardi et al., 2017).

Table 5.2. Chromosomal location of QTLs for resistance to wilting and vascular necrosis induced by Fot4, mapped in $F_{2:3}$ population CB46 x FN-2-9-04.

Mapping population (♀ x ♂)	Phenotype	Vu	Position (cM)	Flanking markers	-log(p)	PVE (%)	A	D/A
CB46 x FN-2-9-04 $F_{2:3}$	Wilting	3	20.25 - 53.11	2_47771 - 2_52190	20	49.3	-0.9	0.6
	Wilting	8	26.72 - 33.04	2_00858 - 2_53531	5.9	13.4	-0.5	0.2
	Vascular necrosis	3	20.25 - 53.11	2_47771 - 2_52190	16	54.5	-3.6	0.1

Vu = cowpea chromosome (Chr) naming (Lonardi et al., 2017); PVE = percent of total phenotypic variation explained; A = additive effect of favorable alleles from the resistant parent (negative values indicate the extent of average reduction in wilting or vascular necrosis due to the presence of favorable alleles); D = dominance effect due to substitution of favorable allele. D/A = degree of dominance. Threshold of QTL significance: wilting = 5.2 and vascular necrosis = 4.9.

Two QTLs associated with resistance to wilting and vascular necrosis in cowpea accession FN-2-9-04 were detected and mapped on chromosomes Vu03 (one QTL for wilting and vascular necrosis) and on Vu08 (one QTL for wilting) (Table 5.2, Fig. 5.6A and 5.6B). A major QTL effect on Vu03 and a minor QTL effect on Vu08 were found to be significantly ($P < 0.05$) associated with wilting response and were mapped to positions 3.24 – 74.33 cM (flanking markers = 2_29126 – 2_40995) and 26.72 – 33.04 cM (flanking markers = 2_00858 – 2_40518), respectively. The peak of the major QTL on Vu03 associated with wilting response was between positions 20.25 – 53.11 cM with flanking markers 2_47771 – 2_52190 (Table 5.2).

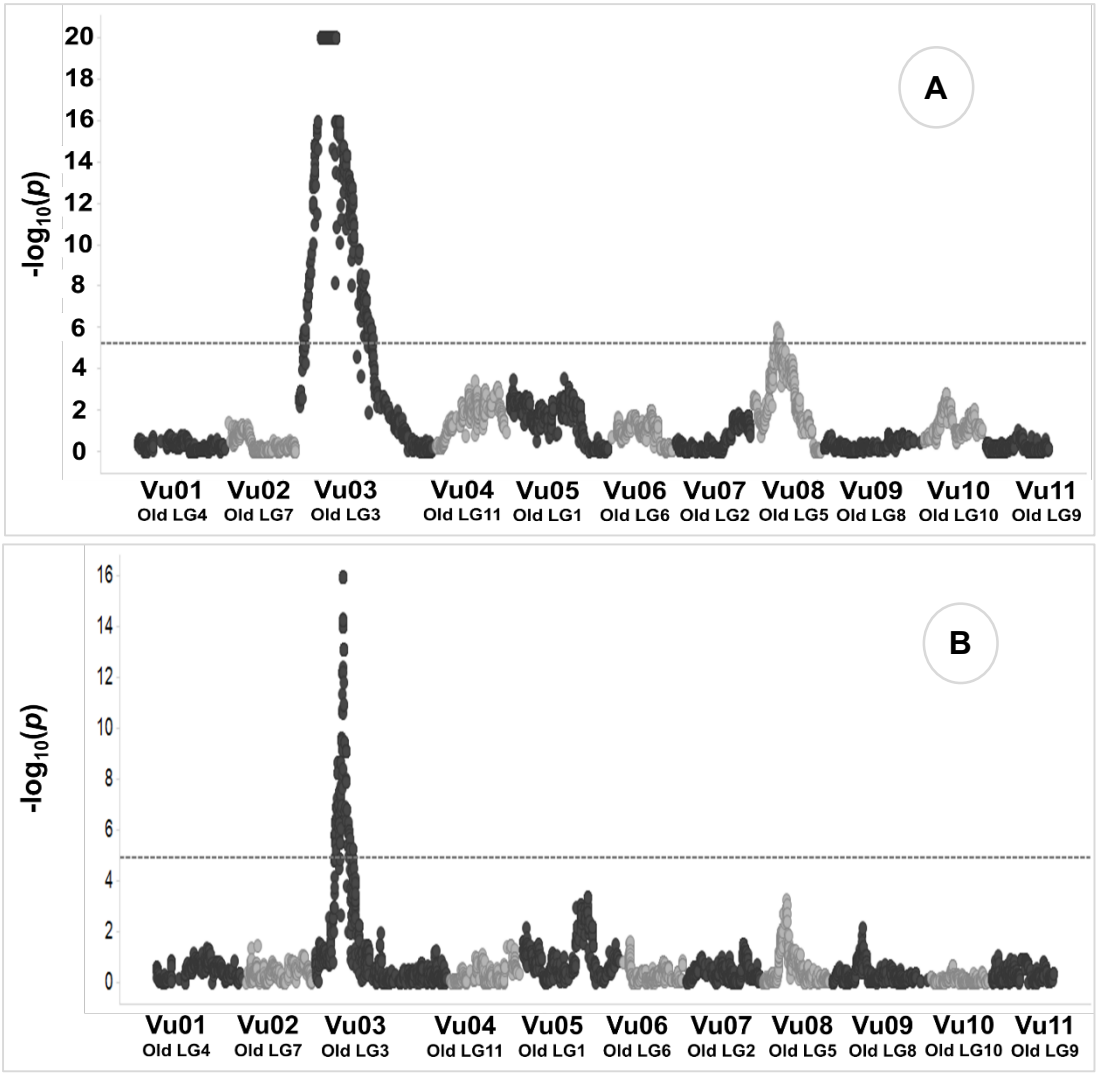

Fig. 5.6. Genomic locations [Chromosomes 3 (Vu03) and 8 (Vu08)] on the cowpea consensus genetic map of QTLs associated with (A) wilting and (B) vascular necrosis responses induced by Fot4 infection. The QTLs were detected using wilt and vascular necrosis phenotypes of 137 $F_{2:3}$ families from the cross CB46 (susceptible) x FN-2-9-04 (resistant). Bonferroni threshold for QTL significance at P < 0.05 is represented by the dashed-line (-logP = 5.2 and 4.9 for wilting and vascular necrosis, respectively). Old LG indicates the former cowpea linkage group nomenclature and Vu indicates the new linkage group naming (Lonardi et al., 2017).

The major QTL peak was defined by highly significant markers [*-log(p) = 20*] associated with wilt phenotype which had significance values above the significance threshold (Fig. 5.6). This genomic region alone explained on

average 49.3% of the total variation in wilt phenotype, while that on Vu08 accounted on average for 13.4% of the total phenotypic variation. Both resistances to wilting on Vu03 and Vu08 exhibited partial dominance effect (D/A = 0.6 and 0.2, respectively) (Table 5.2). An additional QTL region mapped on Vu03, within the region associated with resistance to wilting, (Table 5.2, Fig. 5.6B) was associated with resistance to vascular necrosis and spanned 21.05 to 44.74 cM, with flanking markers 2_04847 – 2_39558; this region overlapped with the region associated with resistance to wilting (Figs. 5.6A and 5.6B). The resistance in this Vu03 region, alone explained on average 54.5% of the total phenotypic variation for vascular necrosis phenotypes (Table 5.2), and the degree of dominance (D/A = 0.1) in this region indicated resistance control with partial dominance.

Segregation ratios: To determine genotypic ratios in the $F_{2:3}$ population CB46 x FN2-2-9-04 segregating for resistance to wilting and vascular necrosis (Table 5.3), each $F_{2:3}$ family was scored for presence of parental genotypes at each SNP marker locus within the mapped QTL (Table 5.2) regions using F_2 genotypic data. Marker loci were scored from 0 to 2 to indicate the complement and zygosity of parental alleles, and scores of 0, 2 and 1 were assigned to homozygous non-favorable allele (AA = susceptible parent), homozygous favorable allele (BB = resistant parent) and heterozygous (AB), respectively. The genotype of each F_2 line was determined as the average of scores across all marker loci within the QTL region, and it was associated with the average phenotypic response (wilting or vascular necrosis) of its corresponding $F_{2:3}$

family. The data for frequency distribution of genotypes (BB, AB and AA) within the QTL region (Table 5.3) was analyzed for goodness-of-fit and the chi-square values were adjusted using Yates correction for continuity (Little and Hills; 1978). The 137 F_2 lines were assayed for 51128 SNPs, and marker segregations for favorable (resistant phenotype) and non-favorable (susceptible phenotype) alleles within the mapped QTL regions were significant (Table 5.3). In the chromosome Vu03 QTL, genotypic segregation for wilt and vascular necrosis phenotypes conformed to 13:3 and 3:1 ratios, respectively, indicating genetic control for wilt phenotypes governed by two genes acting under dominant-recessive interaction mode, and for vascular necrosis phenotypes governed by a single gene with partial dominant effect (Table 5.3).

Table 5.3. Genotypic ratios based on SNP allele calls within the QTL regions associated with Fot4 resistance determined for wilting and vascular necrosis traits using 137 F_2 lines of population CB46 x FN-2-9-04.

	Observed genotypes						
F_2 Population	BB + AB	AA	Expected ratio	X^2	*P value*	Phenotype	Vu
	108	29	13:3[a]	0.38	0.50-0.75	Wilting	3
	101	36	3:1	0.06	0.75-0.90	Wilting	8
CB46 x FN-2-9-04	98	39	3:1	0.70	0.25-0.50	Vascular necrosis	3

BB = favorable alleles from resistant parent, AB = heterozygous between both parents, AA = unfavorable alleles from susceptible parent and Vu = cowpea chromosome naming (Lonardi et al., 2017). Expected genotypes: wilting (Vu03): (AB + BB) = 111.3 and AA = 25.7; wilting (Vu08): (AB + BB) = 102.8 and AA = 34.2; vascular necrosis (Vu03): (AB + BB) = 111.3 and AA = 25.7. [a] Also fit a 3:1 ratio ($X^2 = 0.88, P = 0.25 – 0.50$).

For the QTL region on Vu08, where additional influence on wilting response was found, the segregation between resistant and susceptible genotypes fit a 3:1 ratio, indicating that a dominant gene in this region is also associated with

resistance to wilting. The high fit to a 13:3 ratio for wilting on Vu03 could reflect genetic distortion for a single gene resistance model.

QTL pyramiding: To examine the interaction between the two mapped resistance QTLs and their value in breeding for Fot4 resistance in cowpea, pair-wise comparisons were made between all possible allele combinations of the two QTLs on Vu03 and Vu08 for their associated wilt phenotype (Fig. 5.7). ANOVA of wilt phenotypes associated with each QTL combination was performed in SAS university studio following the Proc Mixed procedure, and differences between means were determined through multiple test comparison using Tukey test ($P < 0.05$). The sample sizes among these combinations varied from 5 to 39, and the average response to wilting associated with these QTL combinations ranged from 0 to 4.9. Significant differences between means were detected at DI = 0.7. The extreme wilt phenotypes, 0 and 4.9, were observed in parents FN-2-9-04 (resistant) and CB46 (susceptible), respectively. Families with genotype Vu03/Vu08 (++/++) carrying favorable haplotypes in both QTLs, showed significant ($P < 0.05$) high suppression of wilting compared to the susceptible parent CB46 (--/--) carrying no resistance to Fot4; although it supported slight wilting compared to the resistance parent FN-2-9-04 (++/++)

which showed no wilt symptoms (Fig. 5.7). Families with genotypes Vu03/Vu08 (--/--), expected to be susceptible to wilting, had less wilting than CB46.

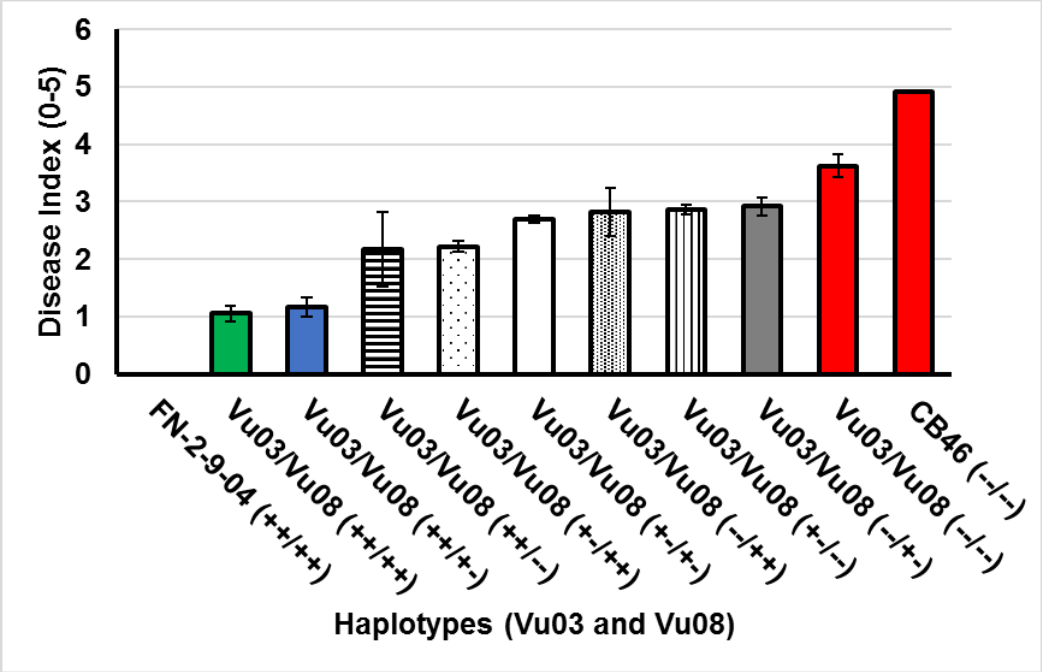

Fig. 5.7. Effect of QTL combinations on plant wilt response to infection by Fusarium wilt race 4 in $F_{2:3}$ families of CB46 x FN-2-9-04. Signs ++ and - - stand for presence and absence of favorable and non-favorable haplotypes, respectively, on chromosomes Vu03 and Vu08 where Fot4 resistance resides.

Two and three-fold more wilting symptoms were observed in families with genotypes Vu03/Vu08 (++/--) and Vu03/Vu08 (--/++), respectively, than families with favorable haplotypes present at both QTLs Vu03/Vu08 (++/++). The heterozygous allele condition at both QTLs Vu03/Vu08 (+-/+-) had similar wilting phenotypes ($P > 0.05$) to families in which favorable alleles are absent in either one of the QTLs, Vu03/Vu08 (++/--) or Vu03/Vu08 (--/++) (Fig. 5.7).

Discussion

Genetic analysis and QTL mapping of resistance to Fusarium wilt race 4 present in cowpea accession FN-2-9-04 revealed that the resistance in this accession is partially dominant. QTL mapping identified two QTLs associated with wilt and vascular necrosis phenotypes which were mapped on Vu03 and Vu08 of the cowpea consensus genetic map. Analysis of segregation for resistance (Table 5.3) suggested that the resistance to wilting in FN-2-904 is under control by up to three main genes, with two located on Vu03 (one partial dominant and another recessive acting under dominant and recessive interaction) and a third main gene with partial dominant effect located on Vu08. The resistance to vascular necrosis incited by Fot4 was detected and mapped in Vu03, and the segregation ratio indicated that this resistance is governed mainly by a single gene with partial dominant effect. The co-location of resistance to wilting and vascular necrosis in Vu03 suggests that these phenotypic responses are under the same gene control located on Vu03. However, the QTL mapping results suggested that the heightened resistance to wilting in FN-2-9-04 is conferred by the additive effect of both resistances on Vu03 and Vu08.

Overall, the resistance to Fot4 present in FN-2-9-04 is highly heritable, with H^2 = 63 and 67% for wilting and vascular necrosis responses, respectively. However, individually the resistance to wilting on Vu08 had lower broad-sense heritability compared to the locus on Vu03 (H^2 = 13.4% v.s 49.3%). These estimates determined using different methods were consistent. The heritability of resistance to vascular necrosis was also high with H^2 = 54.5%. In addition,

both resistances to wilting and vascular necrosis conferred by the locus on Vu03 showed moderate narrow-sense heritability (h^2 = 33.1 and 49.1% respectively), while the additional resistance to wilting on Vu08 had low heritability, h^2 = 12.2%. These values may have been underestimated due to the degree of heterozygosity observed in the F_2 population, and the degree of dominance (D/A) observed in the population, which may have affected the real estimates of additive variances influencing the responses to Fot4.

The QTL regions associated with resistance to wilting and vascular necrosis mapped on Vu03 spanned 71.09 and 23.71 cM, respectively, (Table 5.2, Figs 5.6A and 5.6B) on the consensus genetic map (Munoz-Amatriain et al., 2017; Lonardi et al., 2017), and all SNP markers mapped within this chromosomal region were significant ($P < 0.05$); however, highly significant markers [*-log(p) = 20* and *-log(p) = 16*] for wilting and vascular necrosis responses, respectively, were located between positions 20.25 - 53.11 and 29.55 – 36.46 cM spanning 32.86 and 5.91 cM. Although the resistances to wilting and vascular necrosis were co-located on Vu03, the phenotypic variation explained (PVE) by the QTL on Vu03 for each phenotypic response (PVE = 49.3% and 54.5%, respectively) suggests that these responses may be in part under control by distinct genetic mechanisms. Also, the fact that the resistance to wilting mapped to two distinct genomic regions, on Vu03 and Vu08 (Fig. 5.6A), also support this hypothesis. The QTL region controlling resistance to wilting on Vu08 spanned 6.32 cM from 26.72 to 33.04 cM on the cowpea consensus genetic map, being a much smaller interval than that of the Vu03 QTL.

In previous QTL mapping studies of Fot4 resistance (Pottorff et al., 2014), two resistance loci, *Fot4-2* and *Fot4-1* were identified in cultivar CB27 and breeding line IT93K-503-1, and these loci mapped to Vu03 and Vu08, respectively, on the cowpea consensus genetic map. The resistance on Vu03 conferred by *Fot4-2* mapped to location 64.44 – 80.23 cM, and it was associated with both wilting and vascular necrosis responses. This QTL region on Vu03 overlaps with the Vu03 QTL detected in this study (3.24 – 74.33 cM) by 9.89 cM. Interestingly, the peak region of the QTL identified in this study, containing the most significant SNP markers, is located 11.33 cM away from the *Fot4-2* locus (64.44 – 80.23 cM), suggesting that the Fot4 resistance QTL on Vu03 identified in this study might be a multi-allele locus for Fot4 resistance. The observed overlap in Fot4 resistances between CB27 and FN-2-9-04 indicates that these donors share a common chromosomal region associated with Fot4 resistance. Whether the shared chromosomal region harbors the same gene, the same allele of different alleles or tandemly arranged genes with a role in resistance to Fot4 in both backgrounds will require further fine-mapping and gene function studies. However, evidence supporting a hypothesis of loosely linked loci determining resistance in CB27 and FN-2-9-04 is provided by the segregation of 323 F_2 lines of population CB27 x FN-2-9-04 for wilting and vascular necrosis in ratios of 13:3 and 3:1, respectively, and the recombination fraction estimated based on the frequency of wilt-susceptible recombinant phenotypes was 22.29%, indicating independence of resistance conferred by linked resistance loci on Vu03. Furthermore, in this study the resistance to wilting in FN-2-9-04 was found to be under control by two QTLs on separate chromosomes, Vu03

and Vu08, operating in an additive manner. In CB27, the resistance locus mapped on Vu03 (Pottorff et al., 2014) was characterized as being associated with both wilting and vascular necrosis responses.

Fot4-1 is a second known Fusarium wilt race 4 resistance locus in cowpea found in breeding line IT93K-503-1, which was mapped on Vu08 located at 21.57 – 29.40 cM (7.83 cM distance) on the cowpea consensus genetic map (Pottorff et al., 2014). This resistance was characterized as being associated with both wilting and vascular necrosis responses. This region of *Fot4-1* on Vu08 overlaps with the one mapped in this study (26.72 – 33.04 cM) by 2.68 cM, indicating that a common region for resistance is shared between the donors IT93K-503 and FN-2-9-04. However, segregation among 363 F_2 lines from the cross IT93K-503-1 x FN-2-9-04 for resistance to wilting and vascular necrosis induced by Fot4 suggested that FN-2-9-04 carries an additional resistance gene or allele independent from that in IT93K-503-1. The estimated recombination fraction in the IT93K-503-1 x FN-2-9-04 population based on the frequency of susceptible recombinants indicated that the resistance locus present on Vu08 in IT93K-503-1 is 24.74 cM apart from a resistance locus present in FN-2-9-04 on the same chromosome. The hypothesis of independence between the resistance in IT93K-503-1 and FN-2-9-04 is supported further by the fact that the Fot4 resistance in FN-2-9-04 resides on two chromosomes, Vu03 and Vu08, with each determining resistance to wilting, whereas the resistance determinant for vascular necrosis in FN-2-9-04 is located on Vu03. However, in IT93K-503-1, the resistance determinants for both phenotypic responses were mapped on Vu08.

The analysis of the effect of alleles status on resistance to wilting mapped on Vu03 and Vu08 indicated these loci operate in additive fashion. The strong resistance of lines carrying favorable haplotypes only on Vu03, Vu03/Vu08 (++/--) suggested that the resistance in this QTL plays a main role in resistance to wilting compared to the resistance on Vu08, Vu03/Vu08 (--/++). Surprisingly, lines carrying favorable haplotypes in both QTLs, Vu03/Vu08 (++/++) supported slight wilt symptoms compared to the resistance donor, FN-2-9-04 (++/++) (Fig. 5.7). This phenomenon suggests that the lines Vu03/Vu08 (++/++) might be lacking an additional component of resistance present in the donor parent genome background. Based on the more susceptible responses of heterozygous lines to wilting, complete allele dosage is crucial for effective resistance to Fot4.

In summary, genetic analysis and QTL mapping of Fusarium wilt race 4 induced wilt and vascular necrosis phenotypes revealed that the resistance in cowpea accession FN-2-9-04 is under control by two QTLs residing on Vu03 and Vu08, and that they function in additive manner. The resistance to wilting is determined by both chromosomes, whereas the resistance to vascular necrosis is determined by the QTL on Vu03. Overall, the resistance showed high heritability, and it is controlled by genetic determinants with partial dominance effect, which appear to interact with a genetic determinant with recessive effect. Analysis of the QTL allele status showed that both resistance QTLs are required for effective resistance to wilting. The mapping of two Fot4 resistance QTLs in early generations of the CB46 x FN-2-9-04 population using phenotypic data of 137 $F_{2:3}$ and high-density SNP marker data of the corresponding F_2 population

was a useful approach to confirm the genomic location of Fot4 resistance in cowpea accession FN-2-9-04. In addition, the overlap in chromosomal locations of Fot4 resistances present in FN-2-9-04 and those characterized previously by Pottorff et al. (2014) in CB27 and IT93K-503-1 validated the findings in this study. Analysis of allelism revealed that both resistance loci present in CB27 (Vu03 – *Fot4-2*) and IT93K-503-1 (Vu08 – *Fot4-1*) are linked to resistance loci present in FN-2-9-04 on both chromosomes, but segregation for resistance in resistant x resistant crosses indicated that FN-2-9-04 carries additional resistance loci independent from but linked to *Fot4-1* and *Fot4-2*. This is the first report of QTL mapping of Fot4 resistance in cowpea where more than two resistance loci were found in a single background. Interestingly the Fot4 resistance mapped in this study exhibit a distinct genomic architecture in that the resistance response to wilting mapped to two genomic regions on Vu03 and Vu08, while the response to vascular necrosis only maps to Vu03. Based on the architecture of Fot4 resistance found in FN-2-9-04, it is likely that this accession carries multi-allelic Fot4 resistance loci.

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General Conclusions

Worldwide, cowpea production is limited by abiotic, biotic and socioeconomic factors. Cowpea research plays a crucial role for the development of necessary technologies required to address key constraints hindering cowpea production and its contribution to food security. The availability of extensive collections of cowpea genetic resources and recently developed genomic resources, including cowpea consensus genetic maps, whole genome sequence assembly and a high throughput marker platform, have enabled the understanding of genomic architecture of cowpea traits of agronomic interest, leading to genetic analysis and their expedited introgression into cowpea elite cultivars through breeding. In contribution to the genetic improvement of cowpea, this dissertation reports on the identification of novel resistance to root-knot nematode (RKN) and Fusarium wilt, the genetics underlying resistance, and the genomic localization of determinants governing resistance in cowpea accessions from Mozambique.

A unique cowpea germplasm set from Mozambique comprising 53 genotypes of accessions and landraces was profiled for RKN response in field, greenhouse and growth chamber experiments. Seven cowpea genotypes were highly resistant to root-galling and egg-mass production by avirulent *Meloidogyne incognita* and aggressive *M. javanica* isolates. Most genotypes were resistant to avirulent *M. incognita*, indicating that the gene *Rk*, present in many cowpea cultivars such as CB46, is predominant in the Mozambique cowpea germplasm. Gene *Rk* is highly effective against avirulent *M. incognita*

isolates, and it confers some protection against aggressive *M. javanica* isolates, although more effective resistance is needed.

Analysis of virulence of the aggressive *M. javanica* and virulent *M. incognita* to the Mozambique cowpea germplasm showed that these nematodes were four-fold more virulent than avirulent *M. incognita*, and isolates exhibited equivalent virulence based on root-galling responses in field experiments. In addition, root-galling induced by *M. javanica* and virulent *M. incognita* were highly correlated, indicating that the genetic determinants underlying resistance in the Mozambique cowpea germplasm can provide broad-based resistance against these RKN isolates. Interestingly, two genotypes (INIA-41 and Maputo) showed resistance specificity to root-galling by both avirulent and virulent *M. incognita* isolates. *M. javanica* and avirulent *M. incognita* root-galling were correlated, indicating that resistance to *M. javanica* in the cowpea germplasm is also highly effective against avirulent *M. incognita*. Correlation between resistance to *M. javanica* root-galling and egg-mass production in the cowpea germplasm indicated that both responses are not independent. The response of F_1 populations developed by crossing highly RKN resistant genotype FN-2-9-04 with susceptible genotypes indicated that the resistance in this accession has high heritability. This finding was also confirmed in the study in Chapter II, which aimed to further characterize the resistance.

Further research was conducted on RKN resistance to determine the genomic architecture of and genetic relationship between the broad-based resistance in the Mozambique cowpea germplasm and the *Rk* gene-based resistance in commercial cowpea cultivars (Chapter III). Several breeding populations (F_1, F_2

and $F_{2:3}$ generations) were developed by crossing RKN resistant accession FN-2-9-04 with susceptible genotypes, and phenotyped for root-galling and egg-mass responses. In addition, leaf samples were collected from a selected F_2 population to provide DNA for genotyping on the 51128 iSelect SNP marker array, with > 17000 SNP found to be polymorphic. The SNP genotyping data were combined with derived F_2 and $F_{2:3}$ phenotypic data for QTL mapping.

One major and one minor QTL, on Vu04 and Vu01 respectively, were found associated with resistance to root-galling by avirulent *M. incognita* in FN-2-9-04. The resistance QTL on Vu04 explained 73.4% of the total phenotypic variation (PVE) in root-galling, and the QTL on Vu01 had PVE = 27.9%. Both QTLs were found to be required for effective resistance to avirulent *M. incognita* infection. QTL mapping revealed that *M. javanica* root-galling and egg-mass production are controlled by a QTL located on Vu01. This QTL on Vu01 had PVE = 47.3 to 94.1% for root-galling under different screening assays, and it co-located with resistance to egg-mass production (PVE = 34.1%). Root-galling and egg-mass production were correlated, indicating that both phenotypic responses are under control by the same genetic determinants on Vu01. Analysis of genetic factors controlling resistance to root-galling and egg-mass production within mapped QTL indicated that both phenotypes may be controlled by up to four genes; two on Vu01 and two gene on Vu04.

The resistance conferred by the QTL on Vu04 in FN-2-9-04 was found to be linked to the *Rk* locus present in some commercial cultivars. However, the resistance QTL mapped on Vu01 (designated here *QRk-vu1.1*) is a novel RKN resistance locus not previously identified in cowpea. High heritability estimates

indicated that the resistance in FN-2-9-04 is valuable for broadening RKN resistance in commercial cultivars.

The spectrum of resistance in Mozambique cowpea genotypes to Fusarium wilt races 3 and 4 (Fot3 and Fot4) was investigated, including the relationship between resistance to plant wilting and vascular discoloration responses to infection (Chapter IV). Eleven cowpea genotypes were identified with broad-based resistance to both wilting and vascular discoloration induced by both Fusarium wilt races. Most genotypes were resistant to wilting and vascular discoloration by Fot3, indicating that most genotypes carry at least Fot3 resistance. Fot4 was four-fold more virulent than Fot3. Fot4-resistant genotypes were also resistant to Fot3, but some Fot3-resistant genotypes were susceptible to Fot4. Moderate correlation between Fot4 and Fot3 wilting suggested that resistances to these races are independent, and it is likely that Fot4-resistant genotypes also carry Fot3 resistance. Wilting and vascular discoloration responses were correlated, suggesting that both phenotypic responses to infection are under control by the same genetic determinants. The genotype FN-2-9-04, with broad-based resistance, was considered a valuable resistance donor due to the high levels of heritability of resistance.

In Chapter V, a novel metric termed “number of necrotic vessels” (NNV) was developed to index vascular necrosis, to better determine the amount of vascular discoloration described in chapter IV. The number of necrotic vessels was determined by enumeration of discolored vessels in the plant stem 28 days-post inoculation. F_1, F_2 and $F_{2:3}$ populations were phenotyped for both wilting and vascular necrosis measured as NNV induced by Fot4. F_2 genotypic

data were combined with $F_{2:3}$ phenotypic data for QTL mapping, which detected two genomic regions associated with resistance to wilting and vascular necrosis. A major QTL on Vu03 (PVE = 49.3%) and a minor QTL on Vu08 (PVE = 13.4%) were associated with wilt phenotype induced by Fot4. Resistance to vascular necrosis also mapped to Vu03 (PVE = 54.5%) and overlapped with resistance to wilting. The localization of wilting response to two distinct chromosomes suggests that although the resistance to wilting and vascular necrosis were co-located on Vu03, both phenotypic responses are controlled by distinct genetic mechanisms. Analysis of genetic factors controlling resistance to wilting and vascular necrosis within mapped QTL indicated that resistance to wilting may be controlled by up to three genes; two on Vu03 and one partially dominant gene on Vu08. Pyramiding of QTL positive alleles indicated that the resistance determinants on Vu03 and Vu08 act in an additive manner to confer effective resistance against Fot4. Both resistance to wilting and vascular necrosis present in FN-2-9-04 had high heritability estimates.

Allelism tests indicated that the Fot4 resistance in FN-2-9-04 on Vu03 and Vu08 is controlled by resistance loci loosely linked and independent from the known Fot resistance present in cultivar CB27 and breeding line IT93K-503-1. The localization of Fot4 resistance present in FN-2-9-04 to two distinct genomic regions, Vu03 and Vu08, represents a unique Fot4 resistance architecture in cowpea.

Valuable genetic resources for broadening RKN and Fusarium wilt resistance in commercial cultivars through breeding are reported in this dissertation. Unique and highly heritable resistance to RKN and Fusarium wilt were identified

in cowpea accession FN-2-9-04, which represents an important multi-trait resistance genotype. Furthermore, its relatively large seed size can offer significant gain in seed size in breeding programs. The overlap between RKN resistance QTL mapped in FN-2-9-04 on Vu04 and the *Rk* locus mapped previously in the same chromosomal region can be explored for fine mapping of the *Rk* locus. Candidate genes in mapped QTL regions for both Fusarium and RKN resistance will be determined to further resolve the number and nature of genes controlling resistance.