Exploitation and characterisation of resistance to the root-knot nematode *Meloidogyne incognita* in soybean

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Abstract

Meloidogyne incognita (Kofoid and White) is a major pest of soybean in South Africa and due to its high level of pathogenicity to the crop it is quintessential that research in this regard should receive priority. Root-knot nematode control has in the past mostly included the use of nematicides, while crop rotation and inclusion of cultivars with genetic host plant resistance (henceforth referred to as resistance only) to these pests were also used. Since no synthetically-derived and/or biological agents are registered locally as nematicides on soybean, the use of resistant cultivars represents one of the most viable and environmentally-friendly strategies to protect local soybean crops against damage resulting from parasitism by *M. incognita.*

Although numerous exotic soybean cultivars have been identified with resistance to *M. incognita*, only a few locally adapted ones have proved to exhibit resistance to the latter species. Moreover, at present Egret is the only cultivar still available for commercial use in South Africa. Little and fragmented information is, however, available on the use of plant enzymes, that are interrelated in biochemical pathways that are expressed in root-knot nematode resistant cultivars, for its use as an additional parameter to exploit such a trait. Therefore, the present study was undertaken to identify *M. incognita* resistance in selected, locally adapted soybean cultivars by quantifying and exploiting the latter trait by using enzyme activities as an additional parameter. In addition, resistance to *M. incognita* in selected resistant soybean cultivars was also verified by means of histopathological studies to identify cellular changes associated with the trait.

In the first part of the present study, 31 locally adapted soybean cultivars of which 23 were commercially available in the 2012 growing season were evaluated for resistance to *M. incognita*. The latter was done by means of traditional screening protocols for which *M. incognita*-gall rating, egg and second-stage juvenile as well as the reproductive factor data per root system for each cultivar screened were recorded. Two greenhouse experiments were subsequently conducted concurrently, one of which the abovementioned nematode parameters were recorded 30 and the other 56 days after inoculation. Reproduction factor values were used as the main criterium to identify *M. incognita* resistance in local soybean cultivars since it is considered as a more reliable parameter for this specific type of evaluations. Reproduction factor values equal to and lower than one, indicating resistance to the *M. incognita* population used in this study, were recorded only for

cultivar LS5995, as well as seven pre-released GCI cultivars. These eight cultivars also had very low egg, as well as egg and second-stage juvenile counts per root system, all of which differed significantly from the susceptible control, as well as a number of other cultivars. Root gall indices, on the other hand, did not show consistent results in terms of the identification of the host status of the 31 cultivar screened during this study. Using reproduction factor values, local farmers can thus be supplied with information on the resistance of commercially-available soybean cultivars. Eventually, such *M. incognita*-resistant cultivars can be used to reduce population levels of this nematode pest in fields of producers and also as valuable germplasm sources in breeding programs to introgress/stack this trait in newly-developed soybean cultivars.

The second part of the study aimed to verify and exploit *M. incognita*-resistance in soybean either identified as resistant or susceptible during the screenings experiments, using enzymatic activity as biochemical markers. Cultivar LS5995 was included as the resistant and Dundee as the susceptible standard. The activity of three enzymes, namely guaiacol peroxidase, lipoxygenase and catalase were recorded at different time intervals in roots and leaf samples of the latter cultivars, of both nematode-inoculated and nematode-free plants of each cultivar. Significant ($P \leq 0.05$) increases in guaiacol peroxidase activity in leaf and root samples of the *M. incognita*-resistant cultivars GCI7 and LS5995 (inoculated with J2) were recorded 24 hours (h) after onset of the experiment. Use of this enzyme thus emanated as a useful parameter to identify soybean cultivars that exhibit resistance against *M. incognita,* especially in leaves, which could substantially reduce the time needed to screen cultivars. In terms of lipoxygenase activity recorded, substantial variation existed between the cultivars tested. The *M. incognita*-susceptible cultivar Egret was the only cultivar for which a significant ($P \leq 0.05$) increase in lipoxygenase activity in the roots was evident 24 h after inoculation. However, during the 48 h sampling time, significant ($P \leq 0.05$) differences in lipoxygenase activity were also recorded for the two *M. incognita* resistant cultivars GCI7 and LS5995. Although the increase in lipoxygenase activity for the susceptible cultivar Egret was unexpected, it may indicate that some level of resistance is present in the latter cultivar, which has in previous studies been identified as resistant to *M. incognita*. Other factors such as a different *M. incognita* populations used and temperature differences in greenhouse conditions that applied in this study compared to that for an earlier study may, however, serve as explanations for the latter differences in host status identification of cultivar Egret. In terms of catalase activity recorded in leaf samples of the *M. incognita*-resistant cultivar LS5995, substantial reductions of as much as

35.6 % were recorded for J2-inoculated plants compared to those of the J2-free control plants. In leaf samples of the susceptible cultivars, Egret and Dundee, catalase was also reduced, but to a lesser extent and ranged from 6 to 26 %. Conversely, catalase activity in the leaves of J2-inoculated plants of the highly susceptible cultivar LS6248R was substantially increased by as much as 29.3 %. Enzyme data obtained as a result of the current study thus generally complemented those of traditional screening assays in which resistance in locally adapted cultivars were identified to a certain degree. It is, however, recommended that enzyme activity, to be used as bio-markers, still needs further refinement and more investigation to optimise their use in identification, verification and exploitation of *M. incognita* resistance in soybean cultivars.

The third and final part of the study encompassed a comparison of cellular changes induced by *M. incognita* in resistant and susceptible soybean cultivars to verify the resistant reactions expressed in the enzyme data. According to light- and transmission electron microscope observations, distinct differences in the appearance and development of giant cells in roots of the *M. incognita*-resistant cultivars LS5995 and GCI7 existed when compared to those in roots of the susceptible cultivars Dundee and LS6248R. In the latter cultivars, giant cells that formed were characteristically large and contained a dense cytoplasm, with thick irregularly surfaced cell walls. Cell walls also displayed thick aggregations that appeared to be cell-wall ingrowths. These giant cells are optimal to facilitate *M. incognita* development and reproduction. In contrast, giant cells that were associated with the resistant cultivars LS5995 and GCI7 were small, irregularly shaped and contained increased amounts of deposited cell-wall material in the cytoplasm known as cell wall inclusions. Necrosis was also present in *M. incognita*-infected root cells of both cultivars. Such giant cells have been associated with retarded feeding, development and reproduction of the latter root-knot nematode species. However, it was evident that neither GCI7 nor LS5995 are immune to *M. incognita* since J2 survived and developed to third- and fourth and ultimately mature females that reproduced in their roots. Optimal giant cells that were formed in the roots of the *M. incognita*-susceptible cultivars Dundee and LS6248R thus supported the nutritional needs of the developing *M. incognita* individuals and led to significant increases in *M. incognita* populations 56 days after inoculation as was evident from the high reproduction factor values that were obtained for such cultivars during host status assessments that represented the first part of this study. The opposite was recorded the *M. incognita*-resistant cultivars LS5995 and GCI7 since sub-optimal giant cells in their roots could not sustain high offspring from such mature females. The presence of necrotic root

tissue adjacent to giant cells, furthermore, indicated that hypersensitive reactions occurred in the latter resistant cultivars. Enzyme data obtained in the second part of this study supported the presence of hypersensitive reactions in root cells of the latter resistant cultivars. Guaiacol peroxidase and lipoxygenase inductions in particular in plant tissues have been reported to play integral roles in hypersensitive reactions that are exhibited by cultivars that are resistant to pests and diseases.

Finally, results obtained from the different parts of this study complemented each other. It resulted in the resistance that was identified in the GCI7 pre-released cultivar being verified and exploited against that of the resistant standard LS5995. Research that was done during this study also represented the first investigations into the use of enzymes as biochemical markers of resistance against *M. incognita* in soybean in South Africa.

Keywords: *Meloidogyne incognita*, Lipoxygenase, Peroxidase, Catalase, Nematodes, Soybean.

Uittreksel

Meloidogyne incognita (Kofoid & White) is ’n ekonomies belangrike plaag organisme van sojabone in Suid-Afrika. Dit is daarom belangrik dat navorsing op hierdie gebied prioriteit geniet. Die beheer van knopwortelaalwurms op sojabone in ander wêrelddele was en is steeds tot ’n groot mate afhanklik van die gebruik van Klas 1, chemiese nematisiede. Die verbouing van eksotiese sojaboonkultivars wat genetiese weerstand (voortaan slegs verwys na as weerstand) teen hierdie plaagorganisme besit, asook in ’n mindere mate wisselbou-praktyk word ook aangewend om *M. incognita* bevolking in sojaboonrotasiestelsels drasties te verlaag. Sodanige beheerstrategieë is egter nie van toepassing in Suid-Afrika nie aangesien geen sintetiese of biologiese middels as nematisiede op sojaboon geregistreer is nie. Wisselbou is ook meestal ’n onsuksesvolle en beperkende aalwurmbeheerstrategie weens die wye gasheerreeks van *M. incognita*. Die gebruik van weerstandbiedende kultivars is dus ’n omgewingsvriendelike strategie om plaaslike sojaboonoeste te beskerm teen skade as gevolg van parasitering deur *M. incognita*. Die probleem is egter dat slegs enkele plaaslik-aangepaste sojaboonkultivars reeds geïdentifiseer is met gasweerstand teen hierdie knopwortelaalwurmspesie. Voorts is daar tans slegs een kultivar wat vroeër geïdentifiseer is as *M. incognita*-weerstandbiedend, nl. Egret, en is kommersieël beskikbaar vir gebruik in Suid-Afrika.

Beperkte inligting bestaan in terme van die gebruik van interafhanklike ensieme wat integrale rolle in die biochemiese sisteme van gasheerplante vervul. Dus is laasgenoemde benadering in hierdie studie as 'n potensiële, addisionele parameter ondersoek om weerstandsmeganismes en –vlakke in geselekteerde *M. incognita* weerstandbiedende kultvars te probeer kwantifiseer en verifieër. Plaaslike kultivars is dus aanvanklik geëvalueer vir hul *M. incognita*-gasheerstatus deur gebruik te maak van konversionele, glashuis-siftingseksperimente. Vervolgens is die aktiwiteit van drie ensieme, wat bekend is vir hul betrokkenheid in gasheer/plaag/siekte-interaksies, vasgestel in blaar- en wortelmonsters van geselekteerde *M. incognita*-weerstandbiedende sojaboonkultivars. Laasgenoemde kultivars se gasheerstatus is vasgestel tydens voorafgaande, konvensionele siftingseksperimente. Die laaste faset van hierdie studie het behels dat die sellulêre veranderinge in die geselekteerde weerstandbiedende sojaboonkultivars deur middel van histopatologiese studies ondersoek en geverifieer is.

In die eerste deel van hierdie studie is 31 plaaslik aangepaste sojaboonkultivars, waarvan 23 kommersieel beskikbaar en sewe voor-vrygestelde GCI-kultivars vir weerstand teen *M. incognita* geëvalueer. Laasgenoemde is gedoen deur middel van konvensionele siftingseksperimente waartydens *M. incognita*-wortelgalindekse, eier en tweede jeugstadiumgetalle (J2) asook voortplantingsdata per wortelstelsel vir elke kultivar, aangeteken is. Twee glashuiseksperimente is vervolgens gelyktydig uitgevoer waartydens die wortelgalindekse 30 en 56 dae na nematoodinokulasie aangeteken is. Bykomende voortplantingsdata is ook tydens die laasgenoemde monsternemingsinterval aangeteken. Laasgenoemde parameter is gebruik as die belangrikste kriterium om *M. incognita* weerstand te identifiseer in die plaaslike sojaboonkultivars. Die rede hiervoor is dat voortplantingsdata algemeen beskou word as ŉ meer betroubare parameter om die gasheerstatus van knopwortelnematode te bepaal. ŉ Waarde laer as een vir laasgenoemde parameter toon weerstand aan teen die spesifieke *M. incognita*-bevolking wat gebruik was in hierdie studie. Weerstand teen *M. incognita* is slegs aangeteken vir kultivar LS5995, wat die weerstandbiedende standaard verteenwoordig het, asook die sewe voor-vrygestelde GCI-kultivars. Egret het ŉ relatiewe hoë reproduksie faktor gekry en is dus in hierdie studie as vatbaar geïdentifiseer. Dundee en LS6248R was hoogs vatbaar as gevolg van baie hoë reproduksie-faktore. Wortelgal-indeksdata vir die verskillende kultivars het min ooreenstemming getoon ten opsigte van hul gasheerstatus met betrekking tot *M. incognita* tydens die twee monsternemings-intevalle.

Die agt voor-vrygestelde kultivars het ook laer eier sowel as eier en tweede jeugstadiums van *M. incognita* getalle per wortelstelsel gehad en het beduidend verskil van die vatbare standaard asook ŉ aantal ander kultivars. In teenstelling het wortelgalindekse nie konsekwente resultate getoon in terme van gasheerstatus-identifisering van die 31 kultivars tydens hierdie studie nie. Dus word voorgestel dat voortplantingsdata gebruik word om plaaslike boere te voorsien van inligting rakende *M. incognita*-weerstand wat teenwoordig is in kommersieel-beskikbare sojaboonkultivars. Gevolglik kan sodanige *M. incognita*-weerstandbiedende kultivars gebruik word om bevolkingsvlakke van hierdie nematoodplaag in die lande van produsente te beheer. Voorts kan sulke kultivars ook gebruik word as waardevolle kiemplasmabronne in teelprogramme om sodoende *M. incognita*-weerstand te integreer in nuut-ontwikkelde sojaboonkultivars.

Die tweede deel van die studie was daarop gerig om *M. incognita* weerstand te kwantifiseer en/of te verifieer in sojaboonkultivars wat geïdentifiseer as weerstandbiedend of vatbaar gedurende die

siftingseksperimente. Vir hierdie doel is ensiemaktiwiteit as biochemiese merkers ingespan. Kultivar-LS5995 is ingesluit as die *M. incognita*-weerstandbiedende en Dundee as die vatbare standaard. Die aktiwiteit van drie ensieme, naamlik guaiakolperoksidase, lipoksigenase en katalase is aangeteken op verskillende tydsintervalle in wortel- en blaarmonsters van die verskeie kultivars. Beide *M. incognita*-geïnokuleerde en -vrye plante van elke kultivar is vir laasgenoemde doel gebruik. Beduidende ($P \leq 0.05$) verhogings in guaiakolperoksidase-aktiwiteit in blaar- en wortelmonsters van die *M. incognita*-weerstandbiedende kultivars GCI7 en LS5995 wat geïnokuleer is met tweede jeugstadiums van *M. incognita* is 24 uur na aanvang van die eksperiment aangeteken. Hierdie ensiem kan dus moontlik as ŉ nuttige parameter ingespan word om sojaboonkultivars te identifiseer wat weerstand besit teen *M. incognita*. Dit geld veral vir data wat verkry is ten opsigte van die blare van die weerstandbiedende kultivars, wat dus die tyd wat nodig is kultivars te sif aansienlik kan verminder.

In terme van die lipoksigenase-aktiwiteit wat aangeteken is in blaar- en wortelmonsters van *M. incognita*-weerstandbiedende kultivars het dit geblyk dat aansienlike variasie bestaan het vir hierdie parameters ten opsigte van nematood-geïnokuleerde versus –vrye plante van alle kultivars wat getoets is. Die *M. incognita*-vatbare kultivar Egret was die enigste kultivar wat ŉ beduidende ($P \leq 0.05$) toename in lipoksigenase-aktiwiteit in die wortels getoon het 24 uur na nematoodinokulasie. Betekenisvolle ($P \leq 0.05$) verskille is egter ook aangeteken vir lipoksigenase aktiwiteit vir die twee *M. incognita*-weerstandbiedende kultivars GCI7 en LS5995 48 uur na aanvang van die eksperiment. Hoewel die toename in lipoksigenase aktiwiteit vir die vatbare kultivar Egret verassend was, kan dit daarop dui dat ŉ sekere vlak van weerstand teenwoordig is in hierdie kultivar. Dit bevestig data van ŉ vorige studie waarin Egret met weerstand teen *M. incognita* geïdentifiseer is. Ander faktore, soos die gebruik van ŉ ander *M. incognita* bevolkings en temperatuurverskille in die glashuis, in vergelyking met dié van ŉ vorige studie kan egter dien as verklarings vir die laasgenoemde verskille in gasheerstatus van kultivar Egret.

In terme van katalase-aktiwiteit wat aangeteken is in blaarmonsters van die *M. incognita*-weerstandbiedende kultivar LS5995, is ŉ aansienlike verlaging in aktiwiteit aangemeld van tot soveel as 35.6 % vir plante wat met J2 van *M. incognita* geïnokuleer was in vergelyking met dié wat nematoodvry was. In blaarmonsters van die vatbare kultivars Egret en Dundee was katalase-aktiwiteit ook verlaag maar tot ŉ mindere mate en het gewissel vanaf 6 tot 26 %. In teenstelling was

katalase-aktiwiteit in die blare van die *M. incognita*-geïnokuleerde plante van die hoogs vatbare kultivar LS6248R met soveel as 29.3 % verhoog. Samevattend het dit geblyk dat ensiemdata wat verkry van *M. incognita*-weerstandbiedende en –vatbare kultivars in die huidige studie, resultate van die konvensionele siftingseksperimente aangevul en ondersteun het. Dit word egter aanbeveel dat indien ensiemaktiwiteit gebruik sou word as bio-merkers vir weerstand, protokolle vir ensiembepalings verder verfyn moet word om die gebruik daarvan te optimaliseer en sodoende *M. incognita* weerstand identifisering en verifikasie in sojaboon kultivars te fasiliteer.

Laastens is 'n vergelykende studie gedoen ten opsigte van sellulêre veranderinge veroorsaak deur *M. incognita*-parasitisme van die wortels in geselekteerde weerstandbiedende en vatbare sojaboonkultivars. Volgens lig- en transmissie-elektronmikroskopiese waarnemings was merkbare verskille in die voorkoms en ontwikkeling van reuseselle in die wortels van *M. incognita*-weerstandbiedende kultivars LS5995 en GCI7 sigbaar in vergelyking met die wat in wortels van vatbare kultivars Dundee en LS6248R teenwoordig was. In laasgenoemde kultivars is reuseselle van kenmerkende grote, digte sitoplasma en dik selwande met ingroeiings aangetref. Hierdie tipe reuseselle is optimaal om ontwikkeling en voortplanting van *M. incognita*-individue te fasiliteer. In teenstelling met laasgenoemde optimale reuseselle is selle wat met die *M. incognita*-weerstandbiedende kultivars LS5995 en GCI7 geassosieer was, beduidend kleiner as die wat teenwoordig was in wortels van die vatbare kultivars. Hierdie sub-optimale reuseselle het ‘n onreëlmatige voorkoms getoon en het groot hoeveelhede gedeponeerde selwandmateriaal in die sitoplasma bevat. Nekrose was ook teenwoordig in beide weerstandbiedende kultivars in die area rondom die reuseselle waar *M. incognita*-individue besig was om te voed. Sulke sub-optimale reuseselle was verantwoordelik vir vertraagde ontwikkeling van en 'n beduidende afname in voortplanting van knopwortelaalwurmwyfies van *M. incognita.* Dit het egter geblyk uit hierdie studie dat GCI7 of LS5995 nie immuun is teen *M. incognita* nie aangesien tweede jeugstadiums in staat was om te oorleef en te ontwikkel tot die derde en vierde jeustadiums en uiteindelik volwasse, eierproduserende wyfies.

Optimale reuseselle wat gevorm is in die wortels van die *M. incognita*-vatbare kultivars Dundee en LS6248R het dus voldoen aan die voedingsbehoeftes van die ontwikkelende nematoodlewenstadiums. Uiteindelik het voeding van *M. incognita* op laasgenoemde reuseselle gelei tot 'n beduidende toename in hul bevolking 56 dae na nematoodinokulasie, soos aangedui is

deur die hoë voortplantingsdata vir laasgenoemde kultivars gedurende die gasheerstatus-siftingseksperimente in die eerste deel van die studie. Die teenoorgestelde is aangeteken vir *M. incognita*-weerstandbiedende kultivars LS5995 en GCI7 aangesien sub-optimale reuseselle in hul wortels aangetref is en lae vlakke van nematoodvoortplanting verkry is. Die teenwoordigheid van nekrotiese weefsel langs reuseselle in wortels van laasgenoemde kultivars het verder dui op die teenwoordigheid van hipersensitiewe reaksies. Ensiemdata wat in die tweede deel van hierdie studie vervat is, ondersteun ook die teenwoordigheid van die hipersensitiewe reaksies in wortelselle van hierdie kultivars. Die induksie van guaiakolperoksidase en lipoksigenase in plantweefsel van kultivars wat weerstand het teen peste en siektes is in die verlede aangemeld en speel dus 'n integrale rol in die hipersensitiewe reaksie soos hierbo aangedui.

Ten slotte blyk dit dat die resultate verkry vir die verskillende studies wat in die huidige navorsing omvat is, mekaar aanvul. Weerstand teen *M. incognita* wat dus geïdentifiseer is in die voor-vrygestelde kultivar GCI7, is geverifieer teen dié van die weerstandbiedende standaard LS5995. Navorsing wat tydens hierdie studie gedoen is, dien ook as die eerste ondersoek na die gebruik van ensieme as biochemiese merkers van weerstand teen *M. incognita* in sojabone in Suid-Afrika.

Sleutelwoorde: *Meloidogyne incognita*, Lipoksigenase, Peroksidase, Katalase, Nematode, Sojaboon.

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CHAPTER 1

Introduction and literature review

1.1. Introduction

The dissertation represents data that emanated from studies that were conducted regarding the relationships that exist between locally adapted, soybean (*Glycine max* (L.) Merr) cultivars (acting as the hosts) and the root-knot nematode (RKN) species *Meloidogyne incognita* (Kofoid & White) Chitwood (representing the pathogen). A general, condensed overview about the soybean crop is given that ranges from its origin to the plant-parasitic nematodes (PPN) that parasitise such crops. In addition, focus is particularly placed on RKN that are regarded as economic constraints of local soybean production. The latter specifically accentuated the role of *M. incognita* in this regard. Research studies that were conducted furthermore included i) the host status of locally adapted soybean cultivars to *M. incognita*, ii) exploitation and quantification of enzyme activities in selected *M. incognita*-resistant and -susceptible cultivars to investigate the mode of resistance at biochemical level and iii) histopathological studies to confirm the changes that occur at cellular level for the selected cultivars used.

1.2. Literature review

1.2.1. Soybean

Soybean is an important oilseed crop in South Africa (Liebenberg, 2012). An estimated 787 100 tonnes (t) of soybean had been produced in South Africa during the 2012/2013 growing season (Anon., 2013a), while the estimated world production was 268 million t during the same period (Ash, 2013). The progressive increase in soybean production is due to an increasing demand for protein-rich food world-wide (Liebenberg, 2012), especially in developing countries such as South Africa.

As a result of the global human population being estimated to reach 9.1 billion in 2050 (FAO, 2009), world soybean production is required to increase by 140 % to approximately 515 million t during the same year (Bruinsma, 2009) to meet the growing demand for protein-rich food sources.

By 2050, the contribution of developing countries such as South Africa to world soybean production is estimated to be in excess of 70 % (Bruinsma, 2009).

1.2.2. Origin

Soybean represent subtropical plants and are considered to be one of the oldest cultivated crops that are native to north and central China (Hymowitz, 1970). It is a member of the pea family (*Fabaceae*) and has been a dietary staple food in Asian countries for at least 5 000 years. During the 1700s and 1800s soybean was introduced to Europe and the United States, respectively (Shurtleff and Aoyagi, 2010). With the discovery of this source of oil and protein by the western world, production escalated and by 2010 more than 250 million t was being produced worldwide. The United States of America (USA) produced approximately 36 %, Brazil 26 %, Argentina 20 % and China 6 % (Liebenberg, 2012).

The first recorded cultivation of soybean in South Africa was in 1903 when it was reported that the crop was grown at Cedara in the KwaZulu-Natal Province and also in the Gauteng Province (Shurtleff and Aoyagi, 2010, Liebenberg, 2012). The late 1930s showed commercialisation of soybean products in Africa, with South Africa introducing soybean flour in 1937. Such flour was used by several gold mines in the Gauteng Province to fortify the diets of mine workers (Shurtleff and Aoyagi, 2010).

1.2.3. Soybean production in South Africa

Soybean production in South Africa has increase substantially from 1991 to 2013 (Fig 1.1) (Grain SA, 2013). The main soybean-producing areas (Figure 1.2) are located in the Mpumalanga and Free State Provinces, while soybean is also grown to a lesser extent in the Gauteng, KwaZulu-Natal, Limpopo, North West, Northern Cape and Eastern Cape provinces (DAFF, 2011). The Mpumalanga Province had the highest soybean production figures in South Africa, with 396 000 t harvested in the 2012/2013 growing season (Figure 1.3). An estimated 205 000 hectares (ha) were planted to soybean in the latter province during the 2012/2013 growing season (Figure 1.3), with a relative low yield obtained per ha (1.8 t) (Figure 1.4). The Free State Province represents the second largest producer of soybean (Figure 1.3). Although more agricultural land (215 000 ha) is used for cultivating soybean in this province, production of the crop was substantially lower (226 000 t)

during the 2012/2013 season than that for the Mpumalanga Province (Fig 1.3). Soybean yields obtained in this area during the previous season were estimated at 1 t per ha (Figure 1.4).

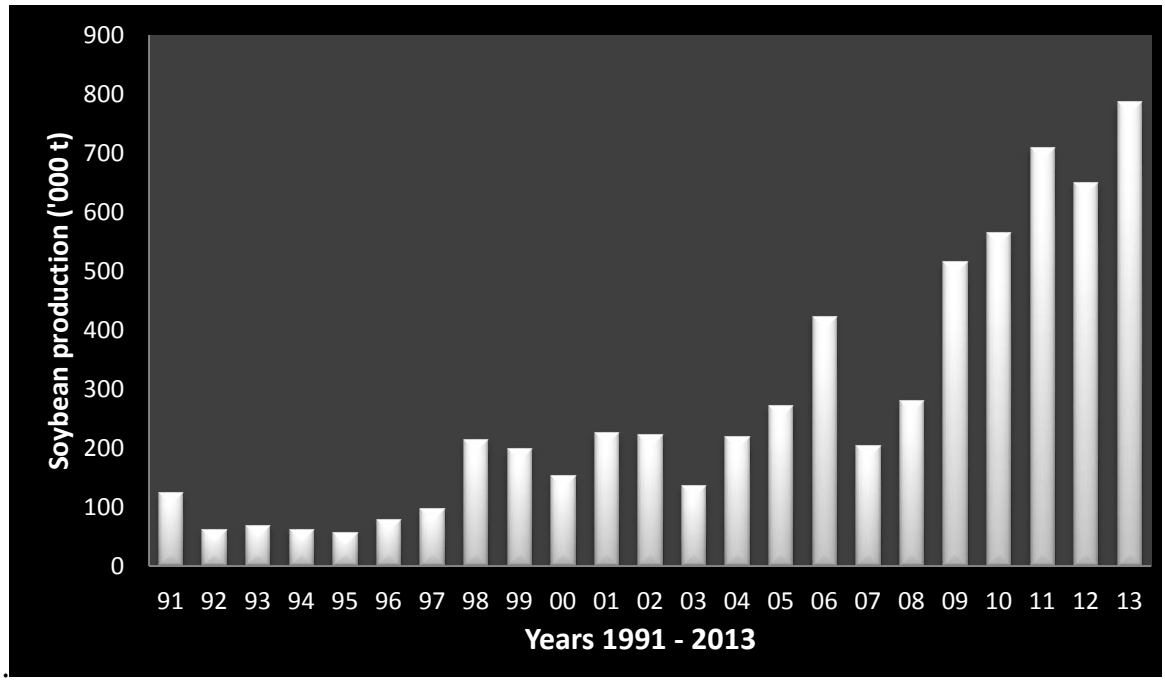


Figure 1.1. Annual soybean production (‘000 t) over a period of time in South Africa (Adapted from Grain SA, 2013).

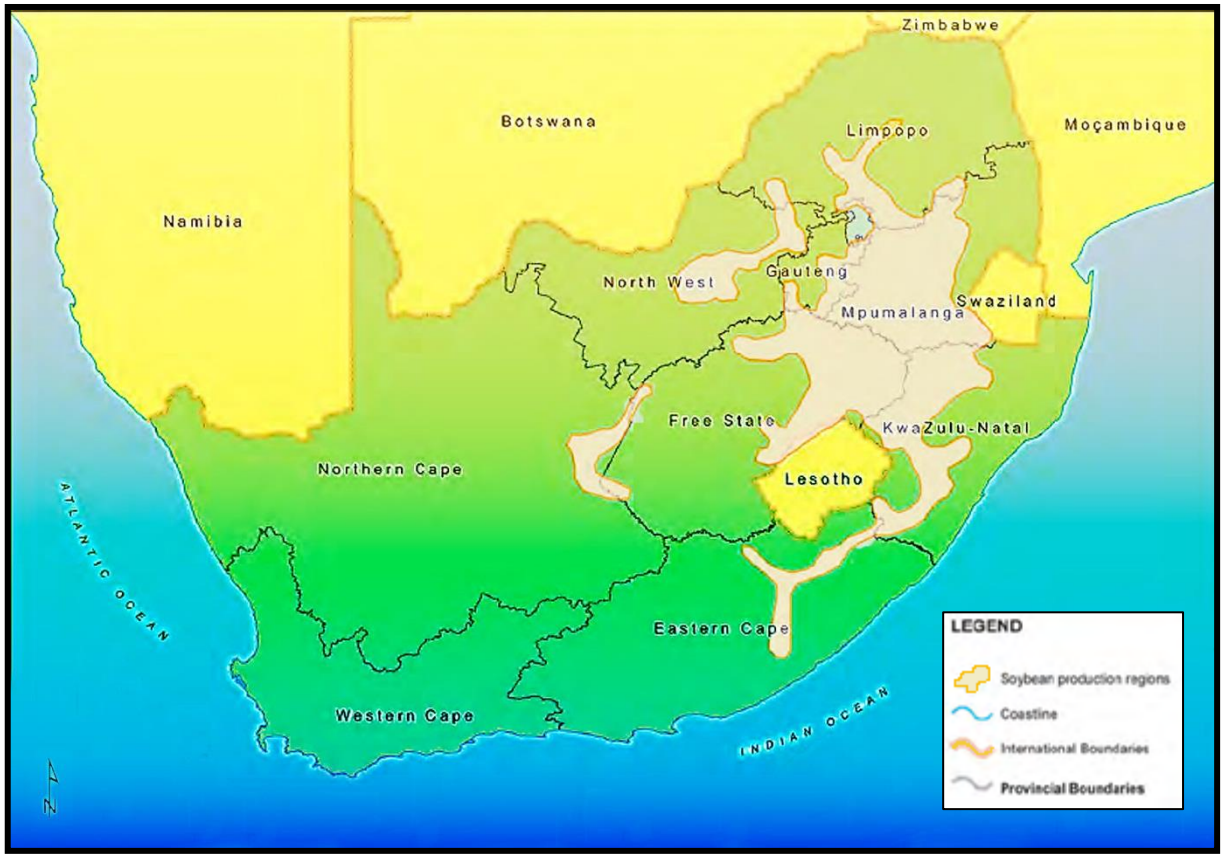


Figure 1.2. A map that indicates the main regions where soybean are cultivated only in South Africa (Adapted from Blignaut and Taute, 2010).

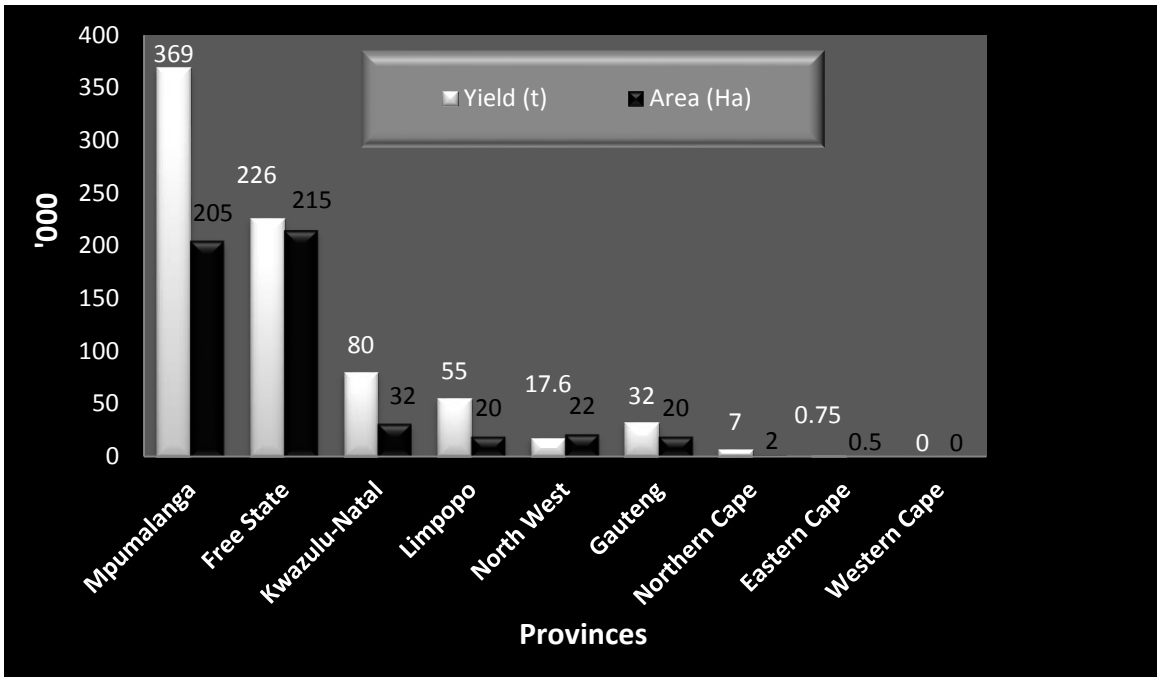


Figure 1.3. Soybean production figures for yield (t) and area (Ha) planted in each of the provinces of South Africa during 2012 (Adapted from Grain SA, 2013).

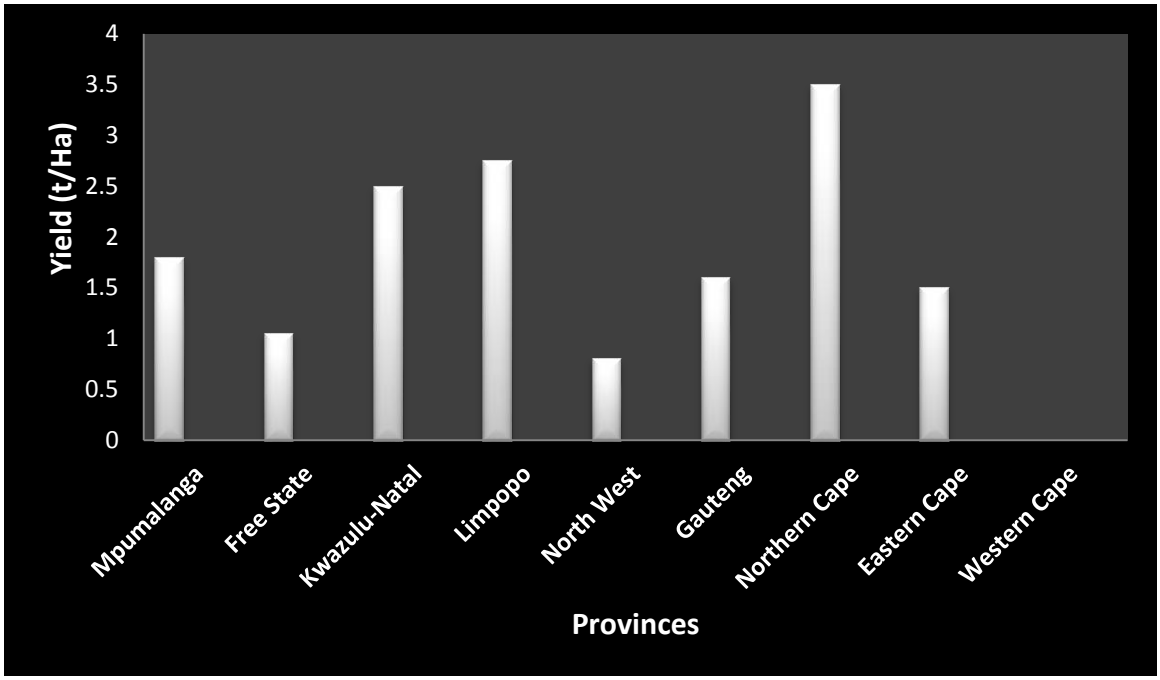


Figure 1.4. Soybean yield figures per hectare for 2013 for each of the provinces in South Africa where the crop is grown (Adapted from Grain SA, 2013).

1.3. Classification

The taxonomic classification of soybean is as follows (NRCS, 2013):

Kingdom	Plantae – Plants
Subkingdom	Tracheobionta – Vascular plants
Super division	Spermatophyta – Seed plants
Division	Magnoliophyta – Flowering plants
Class	Magnoliopsida – Dicotyledons
Subclass	Rosidae - Eudicots
Order	Fabales – Legume family
Family	Fabaceae – Bean family
Genus	*Glycine* Willd. – soybean
Species	*Glycine max* (L.) Merr. – soybean

1.4. Anatomy

1.4.1. The soybean plant

Cultivated soybean is an erect, bushy herbaceous annual that grows from 0.3 to 1.5 m high (DAFF, 2010). The soybean inflorescence on each node of the plant may contain numerous pods, from one to as many as 20. A mature plant may in total have as much as 400 pods. Each pod generally contains between two to four seeds (DAFF, 2010). Soybean may be grown for use as high-protein forage for grasing, haying or ensiling. However, when the monetary value of oil seed increased substantially during the 1960s and 1970s, soybean breeding shifted almost exclusively to supply seed rather than forage genotypes (Blount *et al*., 2009).

Soybean is considered a self-pollinated species that is propagated commercially by seed and artificial hybridisation (Liebenberg, 2012). Soybean growth and duration of pod maturation respond positively to day length, thus cultivars are generally classified in 12 maturity groups (MG) according to how responsive they are to the latter stimuli (Kinloch, 1998). Thus, based on their photoperiodic requirements, cultivars can be differentiated accordingly (Smit, 2000). South Africa has a shorter day length and thus MG IV – VII cultivars are most commonly produced locally (Appendix 1). Also, the vegetation period for soybean in South Africa is approximately 70-170 days, but cultivars that are grown at higher altitudes can have longer vegetation periods (Liebenberg, 2012). The development of the crop is divided into six main stages, namely sprouting, ramification, blossoming, bean formation, full ripening of the seeds and full maturity (DAFF, 2010). When the latter stage commences, leaves start to yellow and drop, while seeds begin to lose moisture. Ideally, soybean should be harvested when the water content of the seed is less than 14%, which represents the optimum moisture level for long-range storage of seeds (Liebenberg, 2012).

In terms of cultivars, soybean cultivars are genetically differentiated into either determinate or indeterminate growth habits (Liebenberg, 2012). An indeterminate growth habit results in continued growth during flowering and pod formation, while determinate cultivars stop growing once flowering starts and the growth tip ends in a pod bearing raceme (Liebenberg, 2012). Several registered determinate and indeterminate cultivars with varying levels of adaptability according to regions or provinces in terms of day-length requirements are available commercially. The latter cultivars also vary in terms of their resistance levels to diseases, nematodes, other pests,

environmental conditions, weeds and also other factors that affect production (DAFF, 2010; Liebenberg, 2012).

1.4.2. Vegetative and reproductive stages

The vegetative (V) stages of soybean plants commence with seedling emergence (VE) (Table 1.1) and are followed by the VC stage that is characterised by the cotyledons and unifoliate leaves on the first node (McWilliams *et al*., 1999). The preceding stages, V1-4 are characterised according to the uppermost fully developed trifoliate leaves. A fully developed leaf node is characterised as when the leaf above it has leaflets of which the edges are separated. Reproductive (R) stages in soybean plants commence during flowering. These stages describe the development of flowers (R1 & R2), pods (R3 & R4), seed formation (R5 & R6), as well as maturity (R7 & R8). Vegetative growth is evident in some of the reproductive stages (McWilliams *et al*., 1999).

Table 1.1. A description of the vegetative (V) and reproductive (R) growth stages that are typical of soybean plants during their development (McWilliams *et al*., 1999).

Vegetative Stages		**Reproductive Stages**	
Stage	**Description**	**Stage**	**Description**
VE	Seedling emergence	R1	Open flower present at any node on main stem
VC	Cotyledons present	R2	Open flower at one of the two uppermost nodes on the main stem with a fully developed leaf
V1	Unifoliolate and first fully developed trifoliolate leaves present	R3	Pod is 5 mm long at one of the four uppermost nodes on the main stem with a fully developed leaf
V2	Unifoliolate and first two fully developed trifoliolate leaves	R4	Pod is 2 cm long at one of the four uppermost nodes on the main stem with a fully developed leaf
		R5	Seed is 3 mm long in the pod at one of the four uppermost nodes on the main stem with a fully developed leaf
V3	Unifoliolate and first three fully developed trifoliolate leaves	R6	Pod containing a green seed that fills the pod cavity at one of the four uppermost nodes on the main stem with a fully developed leaf
V(n)	Unifoliolate and number of fully developed trifoliolate leaves	R7	One normal pod on the main stem that has reached its mature pod colour
		R8	95% of the pods have reached their mature pod colour

1.4.3. Root system

The root system of a soybean plant consists of a taproot that may exceed 1, 5 m in length. Lateral roots extend from the taproot into the soil at a depth of up to 300 mm (Liebenberg, 2012). In new areas of soybean production or when grown in greenhouse trials, inoculation of seed during planting with the rhisosphere bacterium *Bradyrhizobium japonicum* is necessary for optimum efficiency of the nodulated root system to fixate available soil nitrogen independently (Liebenberg, 2012).

1.4.4. Leaves

The primary leaves are unifoliate, opposite and ovate, while the secondary leaves are trifoliolate and alternate. Compound leaves may also be present with four or more leaflets. Typically, leaf colour can vary from dark green to tinted, with brown, red or blue lesions (DAFF, 2010).

1.4.5. Flowers

Originating in leaf axils, flowers develop on short racemes, with each inflorescence bearing up to twenty small purple or white flowers that usually undergo self-pollination (cross pollination is usually less than one percent) (DAFF, 2010). Pods are usually formed by less than 70 % of the flowers (Liebenberg, 2012).

1.4.6. Fruit

Pods are short and hairy, varying in size, usually with some brown or black shade but can be tinted shades of green, red or purple. The pods usually contain three hard, round or ovoid seeds, with smooth and shiny testa as well as a small distinct hilum (DAFF, 2010).

1.4.7. Seeds

Seeds are typically round to ovoid in shape, with their mass varying from 12 to 25 g per 100 seeds. Seed colour vary, with the most common colours being yellow, green, red, brown, black, slightly mottled or occasionally bi-coloured depending on the variety. The hilum colour also varies and usually ranges from colourless to black. For commercial processing of soybean seeds as products to be used by humans, the most acceptable hilum colour is pale yellow (Liebenberg, 2012). A soybean seed generally contains 17 to 22 % oil and 36 to 42 % protein, which serve as nutrition for the

developing seedling during the first two weeks of growth (Liebenberg, 2012). Seeds will typically germinate when they have absorbed approximately 50 % of their own mass in moisture and the soil temperature reaches 10°C. Emergence time for seedlings, however, varies between four to 14 days under favourable conditions (Liebenberg, 2012).

1.5. Economic and social importance of soybean

Soybean is undeniably of great economic and social importance worldwide (Liebenberg, 2012). The crop represents a major source of primary raw materials used by global feed and food industries. Soybean crops provide approximately 58 % of the world's oilseed meal supply. Moreover, soybean is the world's second most important source of edible plant oil after palm oil, accounting for about 29 % of total production worldwide during 2010 (Soystats, 2011; Liebenberg, 2012). Soybean is of key importance in the global animal feed industry, since soybean meal supplies high performance diets. In contrast with grain feeds, soybean meal is a rich source of protein and amino acids (FAO, 2002; Liebenberg, 2012).

For a number of developing countries, especially in impoverished areas, soybean represents the best protein source available for improving the nutritional value of traditional foods (Bressani, 1974; Vaidehi *et al*, 1985; Verma *et al*., 1987; Seralathan *et al*., 1987; Akpapunam *et al*., 1996; Seralathan and Thirumaran, 1998). Also, the crop has revolutionised many rural economies, such as in parts of India. This has been achieved by raising the living standards of soybean farmers. (Paroda, 1999). Ultimately, introduction of soybean to several countries has led to a shift from mono-cropping systems to soybean-based intercropping systems. The latter has resulted in improved cropping intensity, as well as an increase in the profitability per unit area (Paroda, 1999).

1.5.1. The soybean market and production in South Africa

The South African soybean market is dominated by commercial farmers, with small-scale farmers representing only about 2 % of the market. South Africa is a net importer of soybean, with production of 787 100 t in the 2012/2013 season and consumption of 1.8 million t of soybean for cake during 2013 (Anon., 2013b). The soybean-cake market has increased due to growth in the poultry industry, which drives the demand for poultry feed.

Although soybean is an attractive crop to be grown by commercial farmers in South Africa due to the strong market, shortfalls exists in terms of its production. Almost all farmers in South Africa use genetically modified (GM) soybean seeds and *Rhizobium*/*Bradyrhizobium* inoculants have to be applied annually (NAMC, 2011). Also, approximately 40 % of local farmers use fertiliser and lime during soybean production due to prevailing soil conditions. Another constraint is high production costs in rain-fed production areas, which makes soybean production only marginally attractive to commercial farmers. However, commercial farmers that plant the crop under irrigation can obtain larger profits due to higher yields (NAMC, 2011).

South African farmers are also able to legally plant genetically modified (GM) soybean cultivars that are herbicide resistant (Roundup Ready - RR) in accordance to the GMO Act of 1997 (Sadie, 2012). This has led to the widespread use of GM RR soybean cultivars, which constitutes 47 % of commercial cultivars that are currently available (Sadie, 2012). This makes South Africa the only nation on the African content that is allowing the production and use of RR soybeans. However, neighbouring African countries do not allow the import of GM RR soybean, which adversely impacts on South Africa's potential to export raw soybean seeds. Processed soybean products made from GM RR soybean are, on the other hand, currently acceptable and legal (NAMC, 2011).

Another restraint for local soybean production is the area of land available to grow the crop (NAMC, 2011). In order to meet projected demands for 2015, the area on which soybean has to be cultivated is estimated to increase by approximately 69 %. This situation would require the reallocation of land that is currently used for maize (*Zea mays* L.) production to that for growing soybean. Also, land reforms could displace current commercial soybean farmers, which could result in a decrease in future soybean production (NAMC, 2011).Ultimately, soybean is prone to infection by various diseases and pests, (Liebenberg, 2012; Sikora, *et al*., 2005) such as plant-parasitic nematodes (PPN),in particular RKN (Liebenberg, 2012). The latter has recently been reported as the number one nematode pest of a wide range of agri- and horticultural crops (Jones *et al*., 2013). It has also been reported that *M. incognita*, followed by *M. javanica* (Treub) Chitwood are the predominant species that infect soybean crops locally (Fourie *et al*., 2001). The latter scenario often causes extensive yield losses, with such RKN species constituting an important economical production constraint of soybean in South Africa (Keetch, 1989; Fourie, 2010). As a result, RKN are of major importance to local commercial and subsistence soybean farmers due to a wide range

of RKN-susceptible crops such as maize, potato, sunflower and vegetable crops being included in local cropping systems.

1.5.2. Plant-parasitic nematode pests of soybean

Numerous reports exist about PPN that have become well adapted over time to parasitise a wide range of host plants (Moens *et al.,* 2009), including soybean (Sikora *et al*., 2005). Globally more than 100 PPN species have been reported to parasitise soybean roots, however only a few are economically important (Sikora, *et al*., 2005; Holshouser, 2011). To date 18 plant-parasitic nematode genera and 48 species have been associated with soybean in South Africa (Van der Linde, 1959; Coetzee, 1968; Keetch and Buckley, 1984; Kleynhans *et al*., 1996; Fourie *et al*., 2001; Marais, 2012). Predominant endoparasites found include *M. incognita, M. javanica, M. hapla, M. ethiopica, Pratylenchus zeae* and *P. brachyurus*. *Meloidogyne* species occurred in 91 % of all root samples.

From an agronomic perspective the interactions of PPN with leguminous crops can be devastating. Soybean production, kernel quality (Kinloch, 1982; Shane and Barker, 1986; Liebenberg, 2012) and yield (Lordello, 1955; Ibrahim *et al*., 1972; Kinloch, 1980; Lewis *et al*., 1993; Liebenberg, 2012) have all been reported to be negatively affected by parasitising PPN species. Being obligatory parasites, they have developed various modes of actions that vary from relatively simplistic feeding strategies to highly complex relationships with their host plant. The majority of PPN are soil-dwelling and feed in and/or on various below-ground plant organs such as roots (in the case of soybean), tubers [e.g. *Solanum tuberosum* L. (potato), *Daucus carota* (carrot) and *Beta vulgaris* (beetroot)], rhizomes [e.g. *Manihot esculenta* (cassava)], as well as pods and seeds [e.g. *Arachis hypogaea* (groundnut)] (Koltai *et al*., 2002). Parasitism is established when the infective stage of a PPN pierces the cell wall of a plant part of a host plant using its stylet. Eventually, the cell contents of plant cells such PPN feed on are liquidised by means of enzymes they secrete into the cell. After feeding on the cell contents, the plant cell usually dies or is transformed in such a way, depending on the parasitising PPN group, that it cannot perform its basic tasks optimally (Davis *et al*., 2004).

Genera/species of PPN differ substantially in terms of their parasitic behaviour (Lambert and Bekal, 2002). Migratory PPN damage plant tissue through which they migrate, frequently causing necrosis and cell death. Evolutionarily more advanced PPN species become sedentary (e.g. RKN, cyst nematodes and citrus nematodes) and feed from a single cell or a group of cells for a prolonged period of time (Hussey *et al*., 2002; Lambert and Bekal, 2002). To sustain this feeding strategy, such sedentary PPN substantially modify root cells of susceptible hosts into elaborate, optimal feeding cells that include modulating complex changes in plant cell gene expression, physiology, morphology, and function (Bird, 1996; Gheysen and Fenoll, 2002; Lambert and Bekal, 2002).

1.5.3. Root-knot nematodes

Of the wide range of PPN genera, RKN are one of the most important and most devastating PPN genera that infect and parasitise soybean worldwide (Jones *et al*., 2013). Although the soybean cyst nematode *Heterodera glycines* also represents an economical constraint to soybean in some world countries (Davis *et al*., 1996; Riggs *et al*., 1998; Wrather and Koenning, 2006), it is not yet been found in local production areas (Fourie *et al*., 2001; Keetch and Buckley, 1984; Marais, 2013).

According to literature, *M. incognita* is recognised as the most damaging RKN species that parasitise soybean worldwide (Shane and Barker, 1986; Moens *et al*., 2009). In South Africa, *M. incognita* and *M. javanica* are regarded as the economically most important and predominant PPN in soybean production areas (Fourie *et al*., 2001; Liebenberg, 2012), as well as areas where maize was traditionally grown. These RKN species thus pose a threat to production of soybean and maize staple food crops (Riekert, 1996; Riekert and Henshaw, 1998). Also, increased parasitism of other rotation crops being included in soybean-maize rotations by these RKN species is imminent. In terms of soybean, total destruction of an experimental soybean trial of the National Soybean Cultivar Trials, being conducted annually by the Agricultural Research Councils' Grain Crops Institute (ARC-GCI), was experienced in 1998 due to infection and parasitism by RKN pests (Smit and De Beer, 1998). Also, Riekert and Henshaw (1998) reported substantial soybean yield losses in fields where *M. incognita* and *M. javanica* occurred concomitantly. During the past few years, diagnostic nematode analyses often suggested that RKN most probably are the reason why producers recorded substantial yield losses in soybean crops (Fourie *et al*., 2011). Therefore, for the

purpose of this chapter emphasis will be placed on the biology, pathogenecity, control measures and related issues concerning *M. incognita*.

The taxonomic position of the RKN *M. incognita* is as follows (Subbotin and Moens, 2006):

Kingdom	Animalia
Subkingdom	Metazoa
Phylum	Nematoda
Class	Secernentea
Order	Tylenchida
Family	Meloidogynidae
Genus	*Meloidogyne*
Species	*incognita*

1.5.3.1. Biology

The RKN *M. incognita* is an obligatory endoparasite that has developed a specialised and complex feeding relationship with its host plant (Jones *et al*., 2013), in this case soybean. Within the egg, the first-stage juvenile (J1) moults to become a second-stage juvenile (J2) that represents the infective stage of the genus (Jones *et al*., 2013). It migrates through the soil and explores the root surface of soybean plants by pressing and rubbing with its mouth area between the tip and root-hair zone. Ultimately, J2 penetration takes place behind the root cap at the elongation or meristematic area (Hussey, 1985; Jones *et al*., 2013).

Several studies have reported that RKN J2 do not locate roots by random movement but rather infect roots/other plant parts due to factors such as temperature signals together with optimal soil moisture levels (Robinson and Perry, 2006). Recently, it has also been reported that RKN J2 infect

plant roots in response to stimuli initiated by root exudates of host plants (Teillet *et al*., 2013). Having well-developed chemoreceptors (six labial papillae and two lateral amphids), J2 are thus also well adapted to locating roots of their host plants through root exudates (Robinson and Perry, 2006) as was illustrated for *M. incognita* J2 penetration of *Pisum sativum* (pea) root tips (Zhao *et al*., 2000).

The J2 generally invade the roots of soybean host plants within 24 – 72 h after inoculation (Gourd *et al*., 1993; Fourie *et al.,* 2013b). After penetration, J2 migrate through the root cortex to the apex of the host plant root at which point the direction of migration is reversed (Caillaud *et al*., 2008). The J2 then move upward within the cortex towards to the vascular cylinder of the roots of the host plant (Christie, 1936; Bird, 1961; Krusberg, 1963; Bird, 1996; Potenza *et al*., 1996; Gravato-Nobre, 1996; Kyndt *et al*., 2013). Migration of the J2 is largely intercellular and although it causes separation of the cortical cells along the middle lamella, it rarely results in rupturing of such cells (Davis *et al*., 2000). Compared to damage caused by migration of other PPN, mechanical disruption of cells caused by J2 of *Meloidogyne* spp. is almost insignificant (Endo and Wergin, 1973; Endo, 1987; Bird and Wilson, 1994; Hussey, 1985; Hussey *et al*., 1994; Gheysen and Fenoll, 2002). The invasion strategy of RKN has the advantage of attenuating the production of signals that are likely to be detected by the host-plant defense mechanism that alert the plant of an invading pathogen (Robertson, 1996).

Once a J2 reaches the vascular cylinder of its host plant it becomes sedentary and starts to modify several root cells on which it feeds simultaneously to form an elaborate feeding site that consists of several multinucleate giant cells (GC) (Hussey, 1985; Jones, 1981; Kyndt *et al*., 2013). The latter strategy ensures that food sources that are needed for the development and reproduction of the different parasitising RKN life stages are obtained. The GC are larger than normal root cells and contain multiple nuclei, thickened walls with extensive ingrowths and a dense cytoplasm in which an increased numbers of organelles are suspended (Bird, 1974; Caillaud *et al*., 2008; Jones, 1981). The metabolical activity of the GC is increased and such cells serve as metabolic sinks to feed the developing RKN (Bird and Loveys, 1980; Caillaud *et al*., 2008; McGlure, 1977). The latter pathogen is completely reliant on GC for its development and reproduction (Davis and Mitchum, 2005; Huang, 1985).

Successful completion of the life cycle of the RKN is ultimately dependent on the effective induction and maintenance of specialised GC (Potenza, *et al*., 1996; Gheysen and Fenoll, 2002). Enzyme secretions from the esophageal glands of feeding RKN are reported to play a very important role in the induction and formation of GC (Potenza, *et al*., 1996; Gheysen and Fenoll, 2002). According to literature, *M. incognita* injects secretions into GC through a unique tube-like structure called a feeding tube (Robertson, 1996; Jones and Goto, 2011; Fourie *et al*., 2013b). The latter acts as a filter that enables the feeding RKN to remove cytosol without damaging the cytoplasm. Ultimately, *Meloidogyne* spp. feed on GC in cycles, creating a new feeding tube each time the stylet is removed and reinserted into such cells. As a result, several feeding tubes may be present in each GC in which RKN feed (Hussey *et al*., 1994). Distinctive galls that are visible on the roots of RKN-infected host plants also exists. The number and size of galls present on root systems of host plants, however, varies according to the plant host and RKN species involved (Bird, 1974) but should never be used for identification of RKN species.

After a feeding period of about 10-12 days in roots of susceptible host plants, J2 cease to feed and during the next 48 h moult three times into third- (J3) and fourth stage juveniles (J4) and ultimately mature females (Eisenback *et al*., 1980). The J2, female and male RKN all have stylets but only J2 and female RKN use them to initiate feeding sites and feed on root cells of their hosts (Eisenback *et al*., 1980). Female RKN continue to feed and develop into considerably larger, obese-like individuals, while the males remain vermiform. The latter phenomenon is referred to as sexual dimorphism. Swollen RKN females (Eisenback *et al*., 1980) remain in the roots of their host, while mature males of amphimictic RKN species leave the roots of their host to fertilise females. However, parthenogenesis is encountered in several RKN species, with fertilisation not being obligatory for egg development even when sperm is present in the spermotheca (Caillaud *et al*., 2008; Taylor and Sasser, 1978). In terms of *M. incognita*, males generally migrate out of the roots of host plants and play no role in reproduction (Caillaud *et al*., 2008). On the other hand, mature RKN females deposit their eggs in a gelatinous matrix that normally protrudes from the root surface of its host (Caillaud *et al*., 2008; Singh and Sitaramaiah, 1994), which in this case is soybean. The latter RKN life stages generally live for approximately three months after which they die and the GC they fed on degenerate (Bird, 1961; Dropkin, 1969; Potenza *et al*., 1996).

1.6. Control measures

The high reproductive capacity of *M. incognita*, together with its optimal population development potential in roots of susceptible soybean cultivars pose an increasing threat to production of soybean in South Africa (Fourie *et al*., 1999; Fourie *et al*., 2006; 2010). The latter is of particular significance due to the progressive trend in local soybean production by which the crop is expanded to lighter textured soils where maize was traditionally grown as the dominant crop (Anon., 2011). Moreover, the presence of *M. incognita* (Riekert and Henshaw, 1998) as a major constraint in the latter production areas emphasise the magnitude of the problem.

To minimise soybean yield losses as a result of RKN parasitism, it is thus crucial that proper management strategies are applied. Although the use of Class I, chemically-derived nematicides are predominantly used by producers in other countries to protect their crops against RKN such as *M. incognita*, no such chemically or biologically-derived nematicides are registered locally for use on soybean in South Africa (CropLife, 2013). Another common management strategy that is used to combat RKN pests of soybean is crop rotation (Viaene *et al*., 2006). The latter is, however, complex and limited in most cases since the RKN have a wide host range (Radewald, 1978; Boerma and Hussey, 1992; Potenza *et al*., 1996).

Other RKN management strategies such as the use of biological control agents and/or soil amendments have been reported for soybean in other world countries (Karssen and Moens, 2006), but are not commonly or at all used by local producers (CropLife, 2013; Fourie *et al*., 2013b). The use of *M. incognita*-resistant soybean cultivars, however, offers a sustainable strategy to combat such pests in soybean-based cropping systems and will be debated in the discussions that follow.

1.6.1. Genetic host plant resistance (GHPR)

The use of GHPR to *M. incognita* is currently one of the few cost-effective and environmentally-friendly management strategies to minimise yield losses to local soybean crops (Fourie *et al*., 2006; 2008; 2010). Significant yield increases have been obtained in *M. incognita* resistant soybean in comparison to susceptible cultivars (Niblack *et al*., 1986). In addition, Ichinohe (1952) reported that during a 5 year period the resistant soybean cultivar Forrest saved growers more than $400 million

in southern USA. Fourie *et al*. (2010) also reported the economic viability of using a locally adapted, *M. incognita*-resistant cultivar LS5995.

Since no nematicides (synthetic and/or biological) are currently registered and/or are not foreseen to be registered in the near future for use on soybean locally (CropLife, 2012), the need for genetic resistance in locally-adapted cultivars is further emphasised. The progressive withdrawal of Class 1, synthetically-derived nematicides from world markets (Atkin and Leisinger, 2000) furthermore limits the potential registration of such products on soybean.

Resistance that is exhibited to a RKN species, for example *M. incognita* by a host plant such as soybean can be described as the ability of the host plant to suppress development and reproduction of the latter nematode species when compared to that of a susceptible host plant that allow optimal nematode development and reproduction (Boerma and Hussey, 1992; Davis and Mai, 2003; Potenza *et al*., 1996). Such resistance depends on the host-plant's ability to resist penetration by the J2 and/or post-penetration development of such a RKN pest (Caillaud *et al*., 2008; Williamson and Roberts, 2009). Defense responses that are represented by typical reactions of GHPR are either quick and strong and characteristic of resistant or incompatible hosts due to the presence of resistant genes within the host. Weak and/or slow responses by the host plant often result in successful infection by the RKN J2 and are characteristic of susceptible hosts (Williamson and Roberts, 2009). Changes in gene expression in resistant hosts due to wounding or defense responses have been recorded as soon as 12 h after J2 inoculation of RKN (Gheysen and Fenoll, 2002).

Biochemical mechanisms of GHPR to PPN, including *M. incognita*, were first discussed by Giebel (1973). The latter author and Rohde (1965) distinguished between four characteristic expressions of resistance to PPN by host plants, namely:

1) Production of lethal toxins that are fatal to PPN i.e. those present in *Asparagus officinalis* have been reported as toxic to the stubby-root nematode *Trichodorus christei*.
2) The host plant is incompatible for development and reproduction of PPN due to insufficient nutrients and/or substances being available to the nematode pests for feeding, development and reproduction.
3) Certain mechanisms needed for the attraction of PPN juveniles may be absent in resistant plants.

4) The host-nematode interaction triggers histopathological and metabolic changes in the host that results in hypersensitive reactions (HR), killing cells needed for PPN feeding, development and reproduction.

In general, GHPR can be divided into two functional types, *viz.*:

i) Pre-infectional, antixenosis resistance (passive) (Giebel, 1982; Horber, 1980; Painter, 1951; Walters, 2010), which is highly dependent on physical and also chemical barriers (Horber, 1980; Kogan and Ortman, 1978) that are exhibited by the host plant and aim to deter and/or prevent RKN J2 penetration and/or negatively affect the development and reproduction of these pests (Giebel, 1982; Walters, 2010).

ii) Post-infectional, antibiosis resistance (active) (Horber, 1980; Painter, 1941) which, on the other hand, is characterised by successful infection of the host plant by RKN J2, followed by non-optimal development and reproduction of the subsequent development stages of such pests (Trudgill, 1991). This type of resistance is generally dependent on morphological or biochemical factors but also the response of the host to RKN J2 infection (Giebel, 1982; Veech, 1982; Walters, 2010). Post-infectional resistance to *M. incognita* often involves a HR, however timing and localisation thereof varies with regard to the level of GHPR that is exhibited by the particular cultivar (Williamson and Kumar, 2006). Dropkin (1969), however, suggested that the HR was not entirely necessary for expression of plant resistance genes, even though it is the most common type of resistance against *Meloidogyne* and *Heterodera* spp. Ultimately the subsequent, non-optimal development of the GCs may lead to an increase of male RKN and reduction in reproducing females (Dropkin and Nelson, 1960*;* Walters, 2010). The latter has been confirmed in a study done by Fourie and co-workers (2013b) who reported an increase of *M. incognita* J3, J4 males, as well as mature males as a result of post-infectional, antibiosis resistance exhibited by the local soybean cultivar LS5995. Conversely, the latter authors reported an increase in adult female numbers in roots of the susceptible standard cultivar Prima2000. Other examples of post-infectional resistance that have been reported for *M. incognita* are for pea (*Pisum sativum*), bean (*Phaseolus vulgaris*), tomato (*Lycopersicum esculentum)* (Hadisoeganda and Sasser, 1982) and tobacco (*Nicotiana tabacum*) (Sosa-Moss *et al*., 1983).

Another aspect of host-plant interactions with PPN is the occurrence of tolerance or intolerance of the host to the pest. The latter refers to the sensitivity of the host to specific populations of RKN and is determined mostly by assessing plant growth and/or yield of RKN-infected cultivars. Tolerance can effectively be used in RKN management by reducing RKN populations and this way maintaining their populations below the economic injury level. Tolerance is greatly affected by initial population levels as host efficiency is directly correlated to nematode density per unit available host tissue (Cook and Starr, 2006). The ideal situation for RKN management would, therefore, be a tolerant, resistant cultivar, as this would allow for adequate population control, as well as acceptable crop yield.

Furthermore, quantitative and qualitative resistance are regarded as measures of the durability of the resistant trait. Quantitative resistance is based on complex, polygenic inheritance patterns with many genes, all having an effect on the resistant phenotype. This type of resistance is often more durable than qualitative resistance, which is expressed by only a few resistance genes (Van der Plank, 1982). The latter resistance is therefore, also known as monogenic resistance and is based on a gene-for-gene interaction. Breeders often rely on major genes for resistance, which is pathotype specific. Thus, for each major resistance (R) gene in the host plant there is an avirulence gene in the pathogen, such as RKN (Flor, 1971).

1.6.2. Traditional and/or classical RKN screening to identify GHPR

Several *M. incognita*-resistant soybean cultivars have been identified in various countries to minimise yield losses and optimise sustainable production of crops in areas where this nematode pest poses problems (Fourie *et al*., 2008; Fazal *et al*., 2002; Allen *et al*., 2005). This has predominantly been done by using traditional, greenhouse screening protocols. These classical evaluations are used to assess resistance levels present in soybean cultivars to RKN such as *M. incognita* and are generally based on root galling, egg-laying female indices (ELF) (Hussey and Boerma, 1981) and the calculation of nematode-reproduction (Rf) values (Windham and Williams, 1987). In spite of traditional assays being elaborate, laborious and time-consuming, they proved to be successful for this specific purpose (Hussey and Boerma, 1981; Windham and Williams, 1987; Fourie *et al*., 2006). The restrictions experienced when using traditional screening protocols, as well as a strong influence of environmental conditions and nematode genetic variability, complicate the

comparison of results between experiments and seasons. In addition, the use of traditional screening protocols also make it difficult to accurately measure the level of RKN resistance exhibited by the germplasm of crops being evaluated (Abd-Elgawad and Molinari, 2008). Therefore, other approaches to exploit/identify/verify/quantify RKN resistance in crop cultivars have been investigated (Abd-Elgawad and Molinari, 2008).

This includes biochemical markers that form part of recent deoxyribonucleic acid (DNA) technology and represent phenotypic markers that are based on proteins and/or enzymes associated with the resistance level or response to RKN infection (Molinari and Abd-Elgawad, 2007). Enzymes have been used and exploited to assist in the identification of RKN resistance in various crops (Molinari and Abd-Elgawad, 2007). These enzymes include guaiacol peroxidase (GPX) in *M. incognita*-inoculated resistant and susceptible cotton (*Gossypium*) cultivars (Aryal *et al*., 2011), lipoxygenase (LOX) in maize genotypes that are identified with resistant and -susceptibility to *M. incognita* (Gao *et al*., 2008) and catalase (CAT) in *M. incognita*-inoculated tomato plants of resistant and susceptible cultivars (Molinari and Abd-Elgawad, 2007). Such enzyme-based approaches have been argued as being more accurate, suitable, simple and rapid than traditional greenhouse bioassays in terms of identifying RKN resistance in crop cultivars (Molinari and Abd-Elgawad, 2007). Furthermore, enzyme assays may be performed on root and/or leaf tissue of crop seedlings as soon as a few days after planting and RKN inoculation and do not require particular expertise and equipment according to Molinari and Abd-Elgawad (2007). Advanced approaches such as the use of enzymes to identify/verify RKN resistance in crop cultivars will thus be emphasised from here onwards.

1.6.3. Advanced enzyme bio-markers as a tool to identify resistance

A variety of host defence responses at biochemical level are induced in soybean cultivars that exhibit GHPR to RKN (Jones and Goto, 2011**)**. Such responses include but are not restricted to generation of reactive oxygen species (ROS) (Mehdy, 1994; Melillo *et al*., 2006; Almagro *et al*., 2009). A variety of ROS exists, such as superoxide (O_2^-), singlet oxygen ($^1O_2$), hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2). Under stress conditions a host plant is able to produce a burst of ROS that is primarily made up of H_2O_2 and is collectively referred to as the oxidative burst

(Bolwell *et al.*, 2002; Scandalios, 2005). When ROS levels exceed the defensive ability of the host plant, affected cells are said to be in a state of oxidative stress (Sharma *et al*., 2012).

Being described as chemically reactive, ROS can react with proteins, deoxyribonucleic acid (DNA) and membrane lipids (Scandalios, 2005). The latter process result in reduced photosynthesis, increased electrolyte leakage, accelerated senescence and ultimately cell death (Scandalios, 2005). In order to survive in a constantly fluctuating environment, plants regulate the homeostasis between ROS and antioxidants (Van Breusegem *et al*., 2001; Vanová *et al*., 2002). Antioxidants that naturally occur in plants are enzymes such as GPX and CAT. ROS such as H_2O_2 is regulated enzymatically in almost all cell compartments by CAT and GPX, with the former catalysing dismutation and the latter catalysing dismutation and formation of H_2O_2 (Bhattachrjee, 2005). For example, CAT may prevent plant cell damage by removing, neutralising, or scavenging free radicals and reactive oxygen intermediates such as H_2O_2 (Vishnevetskaia and Roy, 1999; Panda, 2012). Another function of ROS is the activation of oxidation signalling molecules (Dat *et al*., 2000; Foyer and Noctor, 2009). Serving as an important ROS signal of oxidative stress, H_2O_2 for example, activates a signalling chain of enzyme activities as a result of the early response of the host plant to biotic stress (Mejía-Teniente *et al*., 2013). Signals that originate from different organelles due to production of ROS such as H_2O_2 have been studied by Foyer and Noctor (2009) and were reported to result in large transcriptional alterations, as well as cellular reprogramming. These changes serve either to protect plant cells against invading pathogens such as RKN, or lead to programmed cell death (Foyer and Noctor, 2009). The LOX reaction, which is involved in the hydroperoxidation of polyunsaturated fatty acids (PUFA) is another possible source of ROS, specifically H_2O_2 (Spiteller, 2003). A concise explanation of each of the three enzymes that have been and were used in this study to characterise/exploit/verify RKN resistance in crop cultivars, namely GPX, LOX and CAT is given below.

1.6.3.1. Guaiacol peroxidase

Peroxidases are heme-containing, monomeric glycoproteins that usually catalyse the reduction of H_2O_2 through the single one-electron oxidation of several substrates: $2RH + H_2O_2$ yields $2ROX + 2H_2O$ (RH = organic reductant; ROX = residual oxygen consumption) (Scandalios, 2005). However, peroxidases can be divided in two functional groups based on their function and localisation, namely APX (EC 1.11.1.11) and GPX (EC 1.11.1.7). The first group, ascorbate

peroxidases (APX) are located in cell organelles such as chloroplasts, microbodies and the cytosol and its main function is to scavenge H_2O_2. On the other hand GPX, which is the main focus area of this study, is involved in various physiological and biochemical processes in plants. These include responses to abiotic and biotic stresses, including auxin oxidation, lignin and suberin biosynthesis, cross-linking of cell wall structural proteins (Durner and Klessig, 1995), production of phytoalexins (antimicrobial metabolites) and the metabolism of reactive nitrogen species (RNS) and ROS (Fig. 1.5) (Almagro *et al*., 2009). The latter both activating the HR that occur in host plants as a result to pathogen parasitism, limiting pathogen development at the infection site (Almagro *et al*., 2009; Gazaryan *et al*., 1996; Mehdy, 1994). GPX are found throughout the cell, but not in organelles and are proposed to play an important role in regulating extracellular H_2O_2 (Bindschedler *et al.,* 2006; Bolwell *et al.,* 2002). There are currently several models proposed in the understanding of incompatible responses induced by host plants in reaction to pathogen invasion, feeding and development. These include but are not restricted to peroxidase-generated radicals and ROS as catalysts of covalent cell wall modifications (Ostergaard *et al*., 2000; Ros Barcelo, 1995), as well as signals for HR (Passardi *et al*., 2005).

Activation of GPX in response to biotic and abiotic stress agents has been established and often plants that induce higher levels of this enzyme are able to withstand stress conditions (Hammerschmidt *et al*., 1982; Stein and Loebenstein, 1976; Melgar *et al*., 2006; Wang *et al*., 2008). Several plant peroxidase isoenzymes are induced following pathogen infection and wounding, illustrating their importance in plant defence responses (Van Loon *et al.*, 2006).

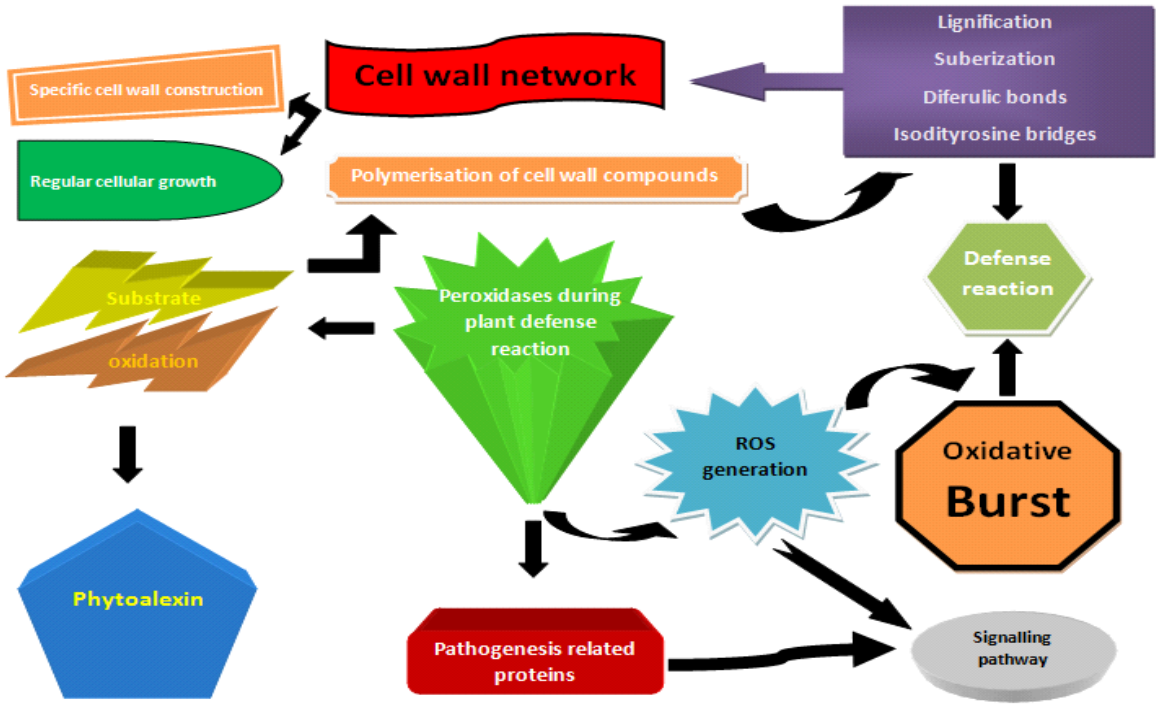


Figure 1.5. A schematic overview of the role that guaiacol peroxidase (GPX) play in the defense reaction of host plants following infection by pathogens (Adapted from Almagro *et al*., 2009)

1.6.3.2. Lipoxygenase

LOX (EC 1.13.11.12) constitutes a family of structurally related non-heme, iron containing enzymes that catalyse the hydroperoxidation of PUFA (Ali and Alqurainy, 2006; Karuppanapandian *et al*., 2011). The pathways in which LOX are actively involved in have been implicated in several physiological processes such as plant development, senescence and resistance to environmental factors (Feussner and Wasternack, 2002; Howe and Schilmiller, 2002; Porta and Rocha-Sosa, 2002). The oxygenation of PUFA is also another possible source of ROS species (Karuppanapandian *et al*., 2011).

In plants, linolenic and linoleic acids are the most common substrates for LOX (Porta and Rocha-Sosa 2002). By a reaction called lipid peroxidation, LOX transform PUFA to lipid hydroperoxides. These lipid-peroxidation products represent biological signals and a non-specific response (Spiteller, 2003). Lipid peroxidation is a typical metabolic process under ordinary aerobic conditions and is an important consequence of ROS action on membrane composition and function (Ali and Alqurainy, 2006). Lipid peroxy radicals cause peroxidative damage to cell membranes,

which contain large amounts of PUFA, resulting in a decrease of lipid unsaturation and subsequently, membrane fluidity (Mao *et al*., 2007). Increased LOX activity may contribute to resistance in a number of ways. For example, oxidative damage to membranes may lead to leakage of cellular contents, and ultimately plant cell death (Hildebrand, 1989). The involvement of LOX in defence reactions became evident as a response to environmental stress and pathogen exposure (Spiteller, 2003; Blée, 2002). The resistance of tobacco to fungal infections was, for example, reported to be dependent on a specific inducible LOX (Blée, 2002).

Important products of LOX action include plant defense activators such as jasmonates (Blée, 2002), lipid peroxides, as well as anti-microbial and -fungal compounds such as leaf aldehydes or divinyl ether fatty acids (Babitha *et al*., 2004), which all are of significant importance in the plants' defence against insect and pathogen infections (Blée, 2002). Plant hormones such as salicylic acid (SA), ethylene (ET) and jasmonic acid (JA) are signalling molecules known to play key roles in initiating plant response to both biotic and abiotic stresses. These three compounds function together through a complex set of regulatory interactions (Shah, 2005; Smith and Boyko, 2006; Kunkel and Brooks, 2002). In pea roots, LOX activity was induced (Leone *et al*., 2001), as well as soybean roots (Alkharouf *et al*., 2006) after infection with cyst nematodes. These studies demonstrate the role of lipid metabolic pathways in the defense responses of plants to pathogen stress.

1.6.3.3. Catalase

Catalases (EC 1.11.1.6) are tetrahedral proteins constituted by four heme groups and are responsible for rapid degradation of H_2O_2 (Asada, 1992; Zacheo and Bleve-Zacheo, 1988). These enzymes are present in peroxisomes of virtually all aerobic cells that protect them from the damaging effects of ROS by catalysing the dismutation of O_2^- and H_2O_2 to water and molecular oxygen (O_2). By reducing these damaging ROS, cellular damage to plant cells upon which RKN feed is limited (Asada, 1992; Zacheo and Bleve-Zacheo, 1988). However, it is proposed that the affinity of CAT for H_2O_2 is very low and that the two part reaction of CAT is highly dependent on H_2O_2 concentrations. Ultimately, CAT activity increases linearly with an increase in H_2O_2 concentrations being produced in plant cells infected by pathogens, such as RKN. Saturation of CAT by H_2O_2 may be reached at high H_2O_2 concentrations, inhibiting its activity (Lardinois *et al*., 1996; Switala and Loewen, 2002).

These various enzymes, *viz.* CAT, GPX and LOX, thus play an important role in regulating ROS concentrations in plants by either synthesising or metabolising ROS and have in several studies been found to play an integral part in host plant resistance mechanisms to PPN, whether they are expressed or inhibited varies (Molinari and Abd-Elgawad, 2007; Rani *et al*., 2008; Zacheo *et al.*, 1982; Aryal *et al*., 2011; Sundararaju and Suba, 2006). In soybean, however, few studies thus far have been conducted to verify GHPR to RKN using enzyme activity. Alkharouf *et al*. (2006) recorded increased levels of LOX in roots of *Heterodera glycines*-infected soybean cultivars that exhibit GHPR to this nematode pest when compared with its uninfected, susceptible counterpart.

Changes in plant enzyme activities, such as those above have been described for RKN-inoculated, susceptible and resistant varieties of various crops such as tomato (Molinari and Abd-Elgawad, 2007; Rani *et al*., 2008; Shukla and Chakraborty, 1988; Zacheo *et al.*, 1982, 1983 & 1993), cotton (Aryal *et al*., 2011; Noel and McClure, 1978), coffee (Mazzafera, 1989), banana (Sundararaju and Suba, 2006) and rice (Anita and Samiyappan, 2012). From these reports it is evident that the time after RKN penetration by J2 seems to be an important factor in detecting the enzymes that react as a result. For example, studies done by Zacheo *et al.*, (1983) reported that GPX activity increased five-fold in roots of resistant tomato cultivars 10 days after inoculation (DAI) with *M. incognita* compared to the nematode-free controls and reduced to normal levels 24 DAI.

Except for traditional screening protocols and the application of advanced techniques such as enzymes to identify GHPR in soybean and/or other crop cultivars, histopathological techniques can be implemented. This way that RKN impact at cellular level of their host plants can be determined and is discussed next.

1.7. Cellular changes in RKN-resistant soybean cultivars

The use of histopathological studies to qualify *M. incognita*-resistance and susceptibility in soybean roots have extensively been used in the past (Barker and Hussey, 1976; Dropkin, 1959; Dropkin and Nelson, 1960; Veech and Endo, 1970). Pedrosa *et al.* (1996) also reiterated on the impact of *M. incognita* at cellular level in roots of exotic resistant and susceptible cultivars. Also, Fourie *et al*. (2013b) confirmed distinct changes that this RKN species inflicted in roots of the local resistant cultivar LS5995 compared to those in the susceptible cultivar Prima2000. Confirmed by these authors, *M. incognita* is a sedentary endoparasite (Abad *et al*., 2009), spending the majority of its

active life cycle in roots of soybean plants (Dropkin and Nelson, 1960; Fourie *et al*., 2013b). Feeding by J2 and females requires the establishment of a complex feeding site, causing substantial damage to the host plants' vascular system. These specialised feeding sites, characterised by large, multinucleate cells are accordingly referred to as GC and are formed to sustain development and reproduction of parasitising RKN (Bird, 1974; Caillaud *et al*., 2008; Jones and Goto, 2011).

Soybean cultivars that are susceptible to RKN often have characteristically larger GC (Dropkin and Nelson, 1960; Fourie *et al*., 2013b) compared to the non-infected, neighbouring vascular cells. Except for their multinucleate nature, GC have thick cell walls with multiple cell wall ingrowths that are proposed to enhance uptake of nutrients (Jones, 1981; Jones and Goto, 2011). The latter are also characteristic of transfer cells (Jones, 1981; Jones and Goto, 2011). Optimal GC are characterised by having high metabolic activities and dense cytoplasm that replace the central vacuole (Caillaud *et al*., 2008). Increased amounts of organelles and smaller vacuoles are also suspended in the cytoplasm (Caillaud *et al*., 2008; Jones and Goto, 2011). The classification system of Dropkin and Nelson (1960) distinguishes between GC that characteristically form in roots of soybean cultivars that have been identified as resistant and susceptible to *M. incognita*. This is based on four morphologically distinguishable types of GC, with Type 1 being those that are generally associated with the hypersensitive response. Type 2 and 3 GC represent those that are associated with retarded, non-optimal development of *M. incognita* individuals, while Type 4 GC represent optimally developed ones that are generally present in highly susceptible soybean cultivars.

1.8. Aims of the study

Since Class 1, red-band nematicides are detrimental to the environment (Hussey and Boerma, 1992) are progressively being withdrawn from world markets, GHPR is gaining more interest as a useful and applicable PPN management strategy. Controlling RKN with GHPR has also been found to be cost effective and environmentally friendly (Starr *et al*., 2002) and as such the current study aimed to gain further insights into the mechanisms and levels of resistance that are present in locally adapted soybean cultivars to *M. incognita* in particular.

Research objectives of this study thus included:

i) the screening of current locally adapted soybean cultivars for resistance to *M. incognita,*

ii) the application of enzyme activity to determine the role of GPX, LOX and CAT in exploiting, characterising and/or verifying such resistance and

iii) the use of a comparative histopathological approach that included both *M. incognita*-resistant and -susceptible cultivars to verify resistance identified in the first and second part of the study and possibly determine additional information on such resistance mechanisms.

A schematic illustration of the latter objectives are represented in Fig. 1.6.

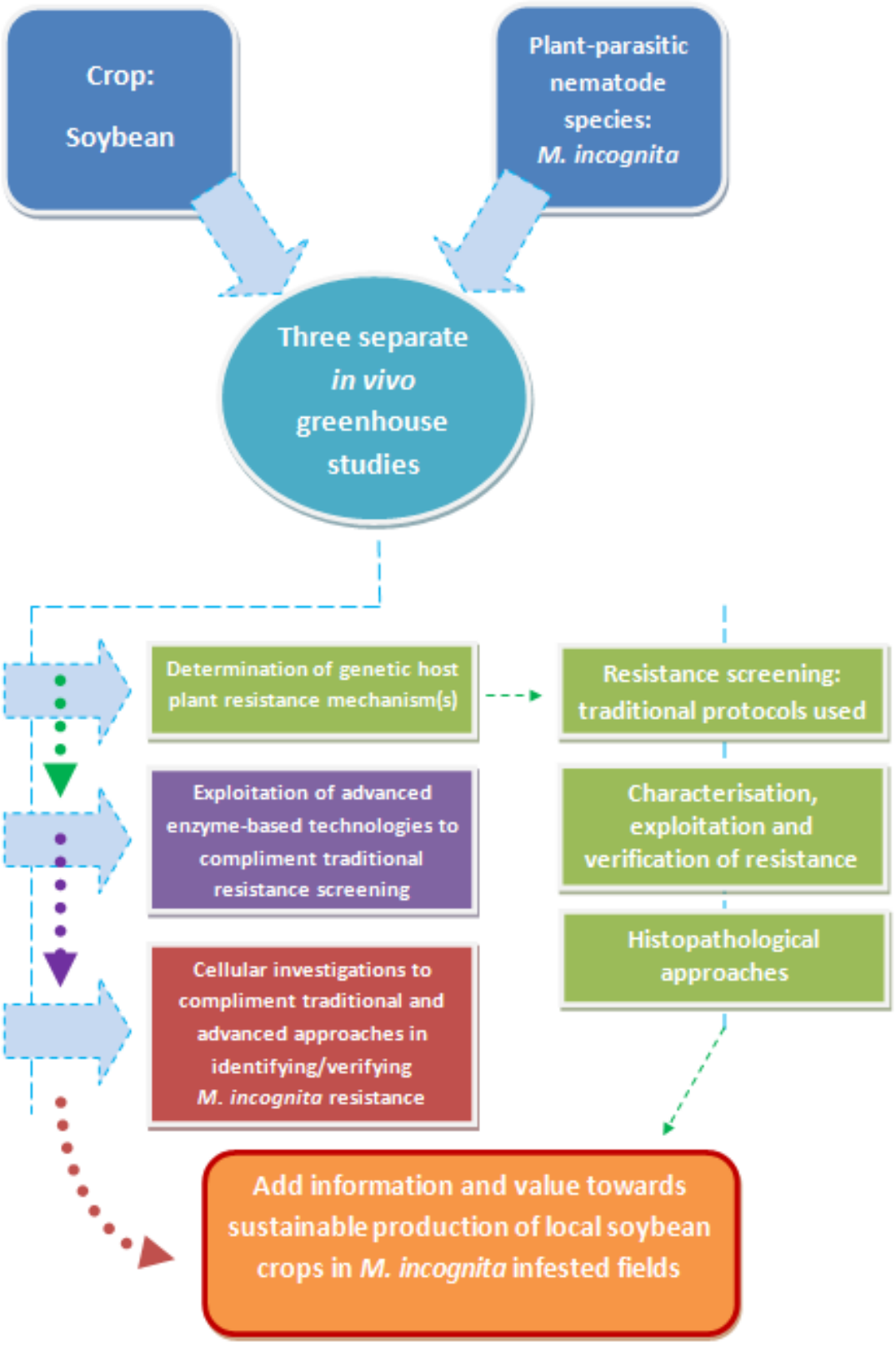


Figure 1.6. A schematic representation of the outline of the present study.

CHAPTER 2

Host suitability of local soybean cultivars to *Meloidogyne incognita*

2.1. Introduction

Soybean (*Glycine max* (L.) Merr.) production in South Africa increased by approximately 25 % from the 2011/12 to the 2012/13 growing season. According to statistics, 650 000 t soybean has been produced on 516 500 hectares (ha) during the latter season (Anon., 2013b). Plant-parasitic nematodes (PPN), of which root-knot nematodes (RKN; *Meloidogyne* spp.) are the predominant nematode pest, adversely affect soybean production in various world countries (Sikora *et al*., 2005), including South Africa (Keetch, 1989; Fourie *et al*., 2001, Fourie *et al*., 2006). Yield losses that resulted from RKN parasitism of local soybean crops have been reported, ranging from nine (Keetch, 1989) to 100 % (De Beer *et al*., 2002; Fourie *et al*., 2001; Riekert and Henshaw, 1998).

M. incognita is the predominant RKN species, followed by *M. javanica* (Fourie *et al*., 2001; Liebenberg, 2012) that reduce yield in local soybean producing areas. In the past local soybean production has primarily been practised in areas with heavier soils. Therefore, little or insignificant RKN damage has been experienced by producers (Fourie *et al*., 2006). This situation is, however, no longer applicable since soybean production has been, and still is being extended and/or included in crop rotation systems where light sandy soils occur and where maize was a major crop in the past (Fourie *et al*., 2006). Since the dominant RKN species in local maize production areas are *M. incognita* and/or *M. javanica* (Riekert, 1996; Riekert and Henshaw, 1998) expansion of soybean production poses an increased risk of this crop being infected and damaged by these nematode pests (Fourie *et al*., 2006).

Since no synthetically-derived and/or biological agents are registered locally as nematicides on soybean, genetic host plant resistance (GHPR) represent one of the most viable and environmentally-friendly strategies to protect soybean crops against RKN infections such as *M. incognita* (Fourie *et al*., 2013a). Although numerous soybean cultivars have been identified by authors across the world as being resistant to *M. incognita* (Table 2.1), they are often not adapted to the unique South African climate and, therefore, cannot perform optimally when grown locally

(Liebenberg, 2012). Exotic soybean cultivars thus cannot necessarily compete with locally adapted cultivars that are selected for the unique growing conditions that prevail in South Africa. Furthermore, the intensity of RKN damage to susceptible and resistant soybean cultivars is often aggravated by other biotic and abiotic factors (Shane and Barker, 1986; Fourie *et al*., 2013a). Ultimately, the level of GHPR exhibited by a given soybean cultivar is a determining factor in the damage potential of RKN to the soybean crop (Williamson and Roberts, *2009;* Fourie *et al*., 2013a). Although various local soybean cultivars exhibit varying levels of GHPR to *M. incognita* (Fourie *et al*., 1999; 2006), none of them except Egret are still commercially available.

Significant yield increases have been obtained in *M. incognita* resistant soybean in comparison with susceptible cultivars (Niblack *et al*., 1986). Therefore, advantages of using resistant cultivars include that they substantially reduce nematode reproduction and subsequently the population size, no need generally exists for nematicide application (Trudgill, 1991), little or no technology or specialised equipment are required for planting soybean (Trudgill, 1991), this is cost effective since seed of resistant cultivars are generally the same price as that of their susceptible counterparts (Boerma and Hussey, 1992; Westphal, 2011), less disease complexes are generally associated with the RKN-infected soybean roots (Mai and Abawi, 1987) and ultimately sustainable production of subsequent crops (Roberts, 2002). Although RKN-resistance breeding is a timely and laborious process, it is an attractive alternative for managing these pests (Roberts, 2002).

The present study thus focused on the screening of soybean cultivars that have been released for commercial use during the 2012-growing season to determine their host suitability to a local population of *M. incognita*. In addition, seven pre-released cultivars that were derived from a cross between the *M. incognita*-resistant LS5995 and the –susceptible Prima2000 (Fourie *et al*., 2008) were also included in these experiments.

Table 2.1. A list of various soybean genotypes that exhibit resistance to *Meloidogyne incognita* as identified by various researchers worldwide.

Genotype	**Reference**
Anderson, Monroe, Blackhawk and Laredo	Crittenden (1952)
Laredo, Delmar	Ibrahim *et al*. (1972)
D69-6344, Hill, Laredo	Williams *et al*. (1973)
F_1& F_2 breeding material	Boquet *et al*. (1976)
D69-6344	Saichuk *et al*. (1976)
Cobb	Hussey and Boerma (1981)
Hutton	Ibrahim and El-Saedy (1982)
LA75-1794	Birchfield and Harville (1984)
Numerous cultivars	Kinloch *et al*. (1985)
Coker 156	*Windham and Barker* (1986)
Amredo,PI96354,PI408088,PI417444	Luzzi *et al*. (1987)
TN 4-86	Allen *et al*. (1988)
Sharkey	Hartwig *et al*. (1988)
50 cultivars	Riggs, *et al*. (1988)
G80-1515,G83-559,G93-9106,G93-9223	Davis *et al*. (1989)
Asgrow 3307, FFR 398, Pioneer 9442	Kirkpatrick and May (1989)
Stonewall	Weaver *et al*. (1989)
Colquitt	Boerma *et al*. (1990)
Pearl	Carter *et al*. (1995)
MACS-212,MACS-124,JS-79-277	Chawla *et al*. (1990)
Davis, Williams, Bragg, Forrest	Mohammed and Elgindi (1990)
Lamar	Hartwig *et al*. (1990)
PK-946, PK-948, JS340, JS-2, KB-32, PBN-110	Reddy *et al*. (1990)
D82-3298	Hartwig *et al*. (1991)
Numerous cultivars	Hussey *et al*. (1991)
D83-3349	Hartwig *et al*. (1996)
G93-9009	Luzzi *et al*. (1996a)
G93-9106	Luzzi *et al*. (1996b)
Benning	Boerma *et al*. (1997)
JS 7981, MSCS-1	Jayant *et al*. (1998)
Monetta © AVT-II(E), NRC-2©AVT-II(N)	Pramila *et al*. (1999)
Prichard	Boerma *et al*. (2001)
S96-2692	Anand *et al*. (2002)
PK-628,DS393	Fazal *et al*. (2002)
PI 594753A and PI 594775A	Harris *et al*. (2003)
N96-180, LS98-1782, ExF11-1, TN96-58 and ExF20-32	Allen *et al*. (2005)
LS5995	Fourie *et al*. (2006)
TGM344, TGM1784 and TGX1448-2E	Adegbite *et al*.(2007)
S99-2281	Shannon *et al*. (2008)
GCI 1,2,3,4,5,6,7	Fourie *et al*. (2008); Venter *et al*. (2013)

2.2. Materials and methods

2.2.1. Soybean germplasm

Twenty-three commercially available soybean cultivars were obtained from the germplasm bank of the Agronomy Unit of the Agricultural Research Council – Grain Crops Institute (ARC–GCI) in Potchefstroom, South Africa. The selected cultivars represented the most popular locally released cultivars for commercial production during the 2012 growing season (De Beer, 2013). These cultivars were included in the National Soybean Cultivar Trials during the 2012 growing season for comparison and evaluation of their yield potential and various other general agronomical characteristics such as standing ability, shattering, oil percentage and others (De Beer, 2013). Their maturity groups ranged from 4 to 7 (Appendix 1). In addition, seven pre-released cultivars were included in the screenings experiments to assess their host status to the latter RKN species in a temperature-regulated greenhouse. These cultivars were developed from a cross between the *M. incognita*-resistant cultivar LS5995 and Prima2000 –susceptible. These ranged from maturity groups 5 to 6.5 (Liebenberg, 2012). Cultivars LS5995 (MG 6), added as the 31st cultivar, was the resistant and Dundee the susceptible standard during the present study (Venter *et al*., 2013; Fourie *et al*., 2006). In total, 31 entries were thus included in each of the soybean screening trials that have been done during this study.

2.2.2. *In vivo* mass rearing of a *M. incognita* population used as the inoculum source

The *M. incognita* population used was originally obtained during 2011 from a groundnut farm near Vryburg in the Northern Cape Province and was used as the inoculation source throughout the present study. A monoculture *M. incognita* population was established by removing single egg masses from maize roots sampled at the abovementioned locality and inoculating such individual egg masses on the roots of individual tomato seedlings (cv. Rodade) that has been identified as being susceptible to this RKN species (Fourie *et al*., 2012).

The *M. incognita* population established was maintained *in vivo* in roots of the abovementioned tomato plants in a greenhouse of which the temperature and light requirements were regulated as indicated in paragraph 2.2.4 Plastic pots (25-ℓ capacity) were filled with Telone II-fumigated (150 ℓ/ha) and solarised soil (5.3 % clay, 93.6 % sand, 1.1 % silt and 0.47 % organic matter content).

The pH (H_2O) of the soil was 7.47. Nutrients were added by application of Multifeed®Classic [19:8:16 (43)] at a dosage of 5 g/ℓ tap water (pH 6.98) once every month.

Nematode eggs and second stage juveniles (J2) were extracted 56 days after inoculation (DAI) from root systems of tomato plants that were initially inoculated with single egg masses as described above. This was done by using a modified sodium hypochlorite (NaOCl)-method (Riekert, 1995). Eggs and J2 obtained were subsequently pipetted on exposed roots of seedlings of another RKN-susceptible tomato cultivar Moneymaker. Roots of these seedlings were covered with the Telone-II fumigated soil and plants were watered as needed. Plants were grown in the same greenhouse at a temperature range of 18 to 26°C and a 14L:10D hour photoperiod being maintained during the *in vivo* mass-rearing of the *M. incognita* population.

2.2.3. Preparation of soybean seedlings and nematode inoculum

Five seeds of each of the 24 soybean cultivars as well as the seven pre-released cultivars were planted in plastic pots (4 000 cm^3 capacity) that contained a Telone II-fumigated (equivalent of 150 ℓ/ha; 1,3 dichloropropene/soil) sandy loam soil (5.3 % clay, 93.6 % sand, 1.1 % silt and 0.47 % organic matter content). Soil pH (H_2O) was 7.47. Nitrogen fixing bacteria were obtained from Soygro Pty Ltd. (Potchefstroom) and inoculated at planting to optimise nitrogen fixation. The *Bradyrhizobium japonicum* (10 g) was suspended in 150 mℓ distilled water and a subsequent 300 µℓ was titrated per seed. Nutrients were applied weekly by administering 250 mℓ of Hoagland's solution (Arnon and Hoagland, 1950) to each potted soybean plant. Plants were further watered two times a week using tap water. Fourteen days after seedling emergence they were thinned to one per pot and *M. incognita* eggs and J2 obtained as discussed below for inoculation of two-leaf-stage seedlings.

For inoculum preparation *M. incognita* eggs and J2 were extracted from at least four infected tomato root systems (described in paragraph 2.2.1) using the modified NaOCl-method of Riekert (1995). Nematode eggs and J2 extracted from each of the four tomato root systems were divided and rinsed into a six separate glass beakers (250 mℓ) using clean tap water. The inoculum was kept in suspension throughout the inoculation process using a magnetic stirrer with a speed not exceeding 750 rpm. The eggs and J2 to be used for inoculating seedlings that represented each of the six replicates were prepared from those present in each of the corresponding six glass beakers.

This was done to optimise the accuracy of obtaining the required number of eggs and J2 to be inoculated per seedling, which was approximately 5 000.

The total amount of nematode eggs and J2 that were extracted and contained in each of the six glass beakers were determined by drawing six aliquots of 20 µℓ each of the inoculum solution, using a micropipette. The eggs and J2 contained in each of these aliquots were subsequently pipetted into a De Grisse counting dish (De Grisse, 1963) and counted using a dissection microscope (40 x magnification). After calculation of the required number of eggs and J2 per aliquot, the eggs and J2 counted from each of the aliquots were collected again from the counting dish and pipetted into the appropriate glass beaker from which it was initially drawn.

The 5 000 eggs and J2 to be inoculated per plant were always suspended in 5 mℓ aliquots of tap water in 10 mℓ plastic tubes. Roots of soybean seedlings were exposed by removing the surrounding soil, using the handle of a spatula. The egg and J2-inoculum was subsequently carefully pipetted directly onto the exposed roots and the tubes that contained the inoculum were rinsed with tap water and the content poured on the roots to ensure that all eggs and J2 were used. After completing inoculation, exposed roots of each soybean seedling were covered with the soil that was originally removed.

2.2.4. Experimental layout, nematode reproduction assessments and data analysis

Two screening experiments were conducted concurrently and represented assessment of nematode parameters 30 and 56 DAI, respectively. Each of these experiments was repeated twice at different times. For each experiment the cultivars were arranged in a complete randomised block design (CRBD), including six replicates of each cultivar (Appendix 2). A 14L:10D photoperiod and an ambient temperature range of 23 - 27ºC were maintained throughout the duration of the experiments. Pots representing each repetition were rotated biweekly to ensure that each of the plants were exposed to similar conditions in the glasshouse since temperature, light and air-movement gradients may have occurred throughout the duration of the experiments.

Thirty and 56 DAI, respectively, plants were removed from the pots and their root systems were cut from the aerial plant parts. The roots were subsequently rinsed free of adhering soil and debris with running tap water, blotted on towel paper and weighed. For roots of plants that were removed 30

DAI, the severity of root galling was determined by using an adapted gall rating method (Fig. 2.1) that was originally described by Hussey and Jansen (2002). Ratings in the present study represented the degree of visibly galled root system. Gall rating values for each soybean plant root system were determined by calculating the average for the root systems of the six replicates.

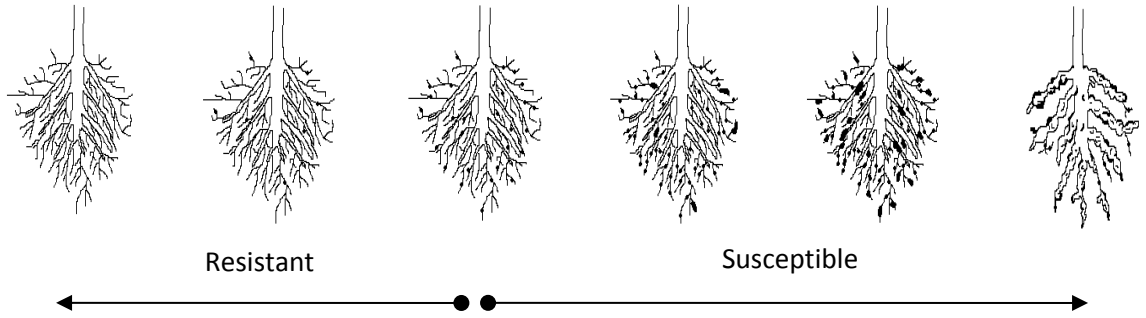


Figure 2.1. Root-knot nematode gall rating indices used as a parameter to quantify genetic host plant resistance in soybean cultivars, where 0 = no galls, 1 = few galls, difficult to locate, 2 = small galls more visible and abundant but main roots clean of galls, 3 = many small galls visible with some larger galls appearing but main roots clean of galls, 4 = many small and large galls of which some were larger and were present on main roots, 5 = root system reduced with well-developed galls appearing on main roots [adapted from the method of Hussey and Jansen (2002)].

The roots of plants that were removed 56 DAI were rated for *M. incognita*-galling using the same method. In addition, eggs and J2 were extracted from each root system using Riekert's (1995) adapted NaOCl method, after root-galling indices were recorded and counted using a stereomicroscope. Nematode reproductive potential was assessed using Oostenbrink's Rf value [RF = final egg and J2 numbers (Pf) / initial egg and J2 numbers (Pi)] (Windham and Williams, 1988). Finally, the number of eggs and J2 per gram root was determined. Egg-laying females (ELF) indices could not be determined as a parameter during these experiments since the Phloxin B staining chemical was not available from suppliers at the time. As an added parameter of resistance the percentage susceptibility (total amount of J2 and eggs per cultivar root system/highest number of eggs and J2 obtained in the group x 100) (Hussey and Jansen, 2002) was also calculated for each cultivar.

Nematode data for each parameter obtained from each of the two experiments were subjected to an analysis of variance (Statistica, Version 11) and means were separated by the Tukey test ($P \leq 0.05$). Degrees of freedom (error) > 18 (Van Ark, 1981) were always pursued and in this case represented

155 being calculated as follows: (number of cultivars -1) x (number of replicates – 1). Nematode data given in this chapter represented those of initial trials since similar trends were observed in repeat trials.

2.3. Results

Although substantial variation existed among the 31 soybean cultivars used in both screening experiments in terms of all host response parameters measured, none of the cultivars evaluated were immune to *M. incognita* (Tables 2.2 and 2.3). This applies as the *M. incognita* population used produced galls or eggs and J2 on roots of all soybean cultivars screened during both experiments.

2.3.1. Experiment 1: 30 DAI

Significant ($P \leq 0.05$) differences were evident among the cultivars in terms of *M. incognita* root gall rating indices recorded 30 DAI (Table 2.2). This indicated that substantial variation existed for root gall rating indices between different cultivars. Gall rating indices ranged from 0.5 for cultivar LS5995 to 4.2 for cultivar LS6248R (Table 2.2). The latter cultivar represented the highest gall rating index, followed by that for Marula and LS6444 R (4.0) (Table 2.2). The susceptible standard cultivar Dundee had a rating of 3.7. In the present study, all cultivars that had indices of 2 and higher were considered susceptible to the *M. incognita* population used in this experiment. Cultivars LS5995 (resistant standard), GCI7, GCI1, GCI3, PHB95Y20 and GCI6 were resistant according to the adapted gall rating index used (Hussey and Jansen, 2002) since they had values < 2.

Table 2.2. Gall rating indices recorded for *Meloidogyne incognita* on roots of local soybean cultivars 30 days after inoculation with 5 000 eggs and J2 in a greenhouse experiment.

Cultivars	**Gall rating indices**	
LS5995[1]	**0.5**	**a**
GCI7	1.3	ab
GCI1	1.5	ab
GCI3	1.7	abc
PHB95Y20	1.8	bcd
GCI6	1.8	bcd
GCI2	2.0	bcde
PHB95Y40	2.2	bcdef
GCI4	2.2	bcdef
Ibis2000	2.3	bcdef
Egret	2.3	bcdef
GCI5	2.8	cdefg
Highveld Top	3.0	defgh
LS6146R	3.0	defgh
Heron	3.2	efgh
LS6150R	3.2	efgh
PAN1664R	3.3	fgh
PAN737R	3.3	fgh
Knap	3.3	fgh
PAN1454R	3.3	fgh
PHB95B53	3.7	gh
PAN1666R	3.7	gh
A5409RG	3.7	gh
LS6164R	3.7	gh
Dundee[2]	**3.7**	**gh**
PAN1583R	3.8	gh
Sonop	3.8	gh
LS6161R	3.8	gh
LS6444R	4.0	gh
Marula	4.0	gh
LS6248R	4.2	h
P value	0.0000	
F ratio (30, 147)	17.835	

Means in the same column followed by the same letter do not differ significantly ($P \leq 0.05$) according to the Tukey test.

[1] = resistant standard

[2] = susceptible standard

2.3.2. Experiment 2: 56 DAI

Significant ($P \leq 0.05$) variation existed for all nematode parameters determined 56 DAI to establish the host status of the cultivars screened against *M. incognita* (Table 2.3).

For root gall rating indices, the highest rating recorded was for cultivars LS6248R (5.0), followed by LS6164R (4.5) and the susceptible standard Dundee (4.3) (Table 2.3). Twenty-one of the cultivars screened had gall rating indices higher than two, indicating susceptibility (Hussey and Jansen, 2002) to *M. incognita*. The lowest gall rating index was recorded for the resistant standard cultivar LS5995 which was zero, followed by that for GCI1 (0.3), GCI7 and GCI3 (both 0.5). Ten cultivars had gall rating values lower than 2, indicating resistance to the *M. incognita* population that were used during this study.

A significantly lower amount of eggs and J2 per root system and eggs and J2 per gram roots were also maintained by these cultivars compared to the susceptible standard Dundee (Table 2). The number of eggs and J2 that were extracted from the root systems of the respective cultivars, ranged from 62 for GCI7 to 386 750 for LS6248R (Table 2.3). The same trend was evident for the number of eggs and J2 per gram root, which ranged from the lowest of 2 for GCI7 to the highest of 9 232 for LS6248R.

In terms of Rf values, the data ranged from the lowest of 0.01 for cultivar GCI7 to 68.35 for cultivar LS6248R (Table 2.3). Eight cultivars, including the resistant standard LS5995 (0.02) had Rf values lower than 1, indicating resistance to the *M. incognita* population used in this study. The other 23 cultivars had Rf values above 1, ranging from 1.62 for PHB95Y40 to 68.35 for LS6248 which were even more susceptible than the susceptible standard Dundee with an Rf value of 34.90.

The percentage susceptibility data indicated that 17 cultivars, including the resistant standard LS5995, maintained less than 10 % of the *M. incognita* population in their roots compared to that recorded for the most susceptible cultivar LS6248R (Table 2.3). The remaining cultivars maintained between 10 and 51 % of the RKN population in their roots compared to that recorded for LS6248R.

Table 2.3. Reproduction parameters for *Meloidogyne incognita*-infected root systems of on local soybean genotypes determined 56 days after inoculation with 5 000 eggs and J2 in a greenhouse experiment.

Cultivars	Gall rating indices		No. of eggs and J2/root system		No. of eggs and J2/ g root		Rf values		Susceptibility (%)
GCI7	0.5	ab	62	a	2	a	0.01	a	0.02
LS5995[1]	**0.0**	**a**	**99**	**a**	**3**	**a**	**0.02**	**a**	**0.03**
GCI1	0.3	ab	151	a	4	a	0.03	a	0.04
GCI4	1.3	abcd	297	a	7	a	0.05	a	0.08
GCI3	0.5	ab	554	a	12	a	0.10	a	0.14
GCI2	1.0	abc	687	a	16	a	0.12	a	0.18
GCI6	1.0	abc	845	a	37	a	0.15	a	0.22
GCI5	2.5	cdefg	2 968	a	84	a	0.52	a	0.77
PHB95Y40	1.3	abcd	9 188	a	350	a	1.62	a	2
LS6146R	2.8	defgh	11 638	a	393	a	2.06	a	3
PHB95Y20	1.0	abc	11 900	a	375	a	2.10	a	3
PAN1583R	1.8	bcde	17 150	ab	646	a	3.03	ab	4
PAN1454R	3.3	efghi	22 050	abc	838	ab	3.90	abc	6
LS6150R	2.8	defgh	23 713	abc	486	ab	4.19	abc	6
Egret	2.3	cdef	25 025	abc	731	ab	4.42	abc	6
LS6161R	3.0	efghi	26 338	abc	827	ab	4.65	abc	7
Highveld Top	2.3	cdef	32 375	abc	1167	ab	5.72	abc	8
PAN1664R	3.3	efghi	37 975	abc	952	ab	6.71	abc	10
Heron	3.0	efghi	38 500	abc	889	ab	6.80	abc	10
PHB95B53	2.8	defgh	39 900	abc	1168	ab	7.05	abc	10
Ibis2000	3.3	efghi	40 338	abc	1174	ab	7.13	abc	10
LS6444R	3.8	fghij	44 888	abc	2451	ab	7.93	abc	12
PAN1666R	2.8	defgh	46 375	abc	1318	ab	8.20	abc	12
A5409RG	3.3	efghi	47 075	abc	2055	ab	8.32	abc	12
PAN737R	3.0	efghi	51 450	abc	1378	ab	9.09	abc	13
Knap	3.5	fghij	51 888	abc	1442	ab	9.17	abc	13
Sonop	4.0	ghij	57 838	abc	1521	ab	10.22	abc	15
Marula	3.5	fghij	94 063	bc	2914	abc	16.62	bc	24
LS6164R	4.5	ij	99 050	c	3638	bc	17.51	c	26
Dundee[2]	**4.3**	**hij**	**197 488**	**d**	**5695**	**c**	**34.90**	**d**	**51**
LS6248R	5.0	j	386 750	e	9232	d	68.35	e	100
P value	0.000		0.000		0.000		0.000		
F ratio (30, 90)	20.12		25.63		12.48		25.63		

Means in the same column followed by the same letter do not differ significantly ($P \leq 0.05$) according to the Tukey test. Cultivars arranged according to Rf values.

[1] = resistant standard

[2] = susceptible standard

2.4. Discussion

Although gall ratings have been and are still used in field evaluations as a criterion for determining RKN resistance in soybean (Acosta and Negrón, 1982; Birchfield and Harville, 1984; Dropkin, 1959; Kinloch *et al*., 1985), it should not be the preferred parameter to apply. Gall rating indices have been and often still are combined with other criteria such as egg or egg mass production by RKN females to identify GHPR in crops such as soybean (Allen *et al.*, 2005; Davis, 1996). Ultimately, Rf values have been found to be a more reliable measure to identify and/or verify RKN resistance for various crops (Windham and Williams, 1988; Fourie *et al*., 2006; Fourie *et al*., 2012). Results from this study complimented and substantiated data by the latter authors since Rf values provide a basic measurement of the reproduction potential of RKN. This parameter is thus a valuable tool since it gives a good indication of the usefulness of GHPR that is exhibited by a crop cultivar. Additionally, the use of susceptibility percentages can complement Rf data and be used in combination with it.

Although considerable variation existed for gall rating indices that were recorded 30 and 56 DAI, respectively, for each of the cultivars screened, as well as between these two sampling dates, this parameter might in some cases serve as a more time and resource-effective method to differentiate between susceptible and RKN resistant soybean cultivars. For example before cultivars are evaluated in field trials. Results from this study, however, indicated that cultivars that met the requirements for resistance 30 DAI by means of gall ratings were not necessarily identified as being resistant to *M. incognita* 56 DAI according to Rf values. This could be illustrated using cultivar PHB95Y20 as an example, which had a gall rating index of 1.8 and 1, 30 and 56 DAI, respectively, indicating resistance. In contrast, the latter cultivar had an Rf value of 2.1 recorded 56 DAI, indicating it as being susceptible to the *M. incognita* population used during this study. This indicates that although cultivar PHB95Y20 had a low root gall index, *M. incognita* females reproduced well in the roots as has been reflected in the Rf value that was greater than 1. The latter scenario has been documented in exotic and local soybean cultivars (Hussey and Boerma, 1981; Fourie *et al*., 2001, 2006). This supports the suggestion that Rf values are a better and more accurate indication of GHPR to RKN, in this case *M. incognita*.

Based on results obtained for Rf during this study as well as number of eggs and J2/root system and eggs and J2/g roots, RKN resistance exhibited by the seven GCI lines, previously identified with the use of molecular markers (Fourie *et al*., 2008), was verified. These lines were derived from an initial cross between the *M. incognita*-resistant standard LS5995 and the high-yielding, RKN susceptible cultivar Prima2000 and were advanced through eight progenies. The background of LS5995 that contains USA germplasm with GHPR to exotic populations of *M. incognita, M. javanica* and *M. arenaria* (personal communication, P. Herbst, Link Seed, 24 February 2005) were thus seemingly introgressed, to a certain degree, into the GCI pre-released cultivars. Ultimately, the level of GHPR to *M. incognita* in the pre-released cultivar GCI7 in particular, proved to be more superior than that present in LS5995 from which it has been derived. Also, yield of the latter pre-released cultivar compared well to those of commercially available cultivars that have been included in the National Cultivar Trials that are conducted annually by the ARC-GCI (Smit and de Beer, 1998). However, all seven pre-released GCI cultivars have the potential to be included as a major source of resistance to *M. incognita* in conventional breeding programmes for further selection, introgression and stacking of the resistance trait into local, high-yielding soybean cultivars. The latter will particularly be valuable for round-up ready (RR) cultivars since no RKN resistance has been identified in those that are commercially available (Venter *et al*., 2013) and that are dominating the local market (De Beer, 2013). Should this intervention be realised, it will add considerable value to producers and the soybean industry in South Africa.

Except for the role that the seven pre-released, *M. incognita*-resistant GCI cultivars can play in resistance breeding programmes, they have the potential to decrease *M. incognita* population levels in soybean-based cropping systems once they become commercially available. This way he latter germplasm can contribute towards sustainable crop production in areas where *M. incognita* in particular poses problems.

Emanating from this study also is the report that cultivar Egret, previously identified with *M. incognita*- resistance by Fourie *et al*. (2006), had a Rf value of 4.4 and a gall rating index of 2.3 that indicated it as being susceptible. This phenomenon could be explained by fluctuations in greenhouse temperatures which may have occurred during the duration of the host status experiments in relation to those reported by Fourie *et al*. (2006). It has been demonstrated that RKN-resistance is temperature-dependent (Mitkowski and Abawi, 2003), which could have resulted

in higher final population levels of *M. incognita* being reached 56 DAI. In addition, the *M. incognita* population used in this study was not the same one used by Fourie *et al*. (2006). It has also been documented that different populations of RKN can result in varying outcomes in terms of resistance screening (Anwar and McKenry, 2007) since some populations are more virulent than others. Ultimately, the possibility also exists that the genetic integrity of cultivar Egret could have been compromised during seed production and therefore may explain the contradictory results obtained in terms of its host status to *M. incognita*. It is, however, important to bear in mind that the *M. incognita*-resistance that has been identified in cultivar LS5995 by Fourie *et al*. (2006) proved to be stable albeit the variables listed above that may have impacted on Egret's resistance being compromised.

Except for the seven pre-released GCI cultivars and the resistant standard LS5995, no other cultivars that are currently commercially available have been identified with *M. incognita* resistance in this study. Commercially available cultivars such as PHB95Y40, LS6164R, PHB95Y20, PAN1583R, PAN1454R, LS6150R, Egret and LS6161R, which were identified as good hosts to the *M. incognita* population used, since they exhibited Rf values than ranged between 1 and 5 (Windham and Williams, 1988), should rather be used by producers than their excellent-host counterparts (Rf values > 5). Should none of the cultivars that exhibit Rf values below 5 be adapted to a certain production area, producers can also choose from those that had susceptible percentages lower than 10 compared to the most susceptible cultivar LS6248R. According to Hussey and Jansen (2002) such cultivars could also be considered for cultivation, since they will reduce the target RKN populations substantially. Growing of highly susceptible cultivars will result in substantial increases of *M. incognita* in soybean fields and resultantly adversely affect the production of follow-up crops to be planted there. The susceptible standard cultivar Dundee, as well as LS6248R, for example had extremely high Rf values for *M. incognita* and should not be grown on fields where these nematode pests are present.

The screenings of soybean cultivars and pre-released ones that were conducted during the present study has ultimately led to generation of knowledge and information about the host status of such germplasm to *M. incognita*. Similar studies should be conducted annually to update producers on the host status of commercially available soybean cultivars in terms of *M. incognita* and *M. javanica* since these two RKNs pose as a real problem in local production areas. It is, however,

crucial that GHPR that has been identified in soybean cultivars be verified and exploited to optimise its use.

CHAPTER 3

A comparative study to investigate the use of enzyme activity in roots and leaves of *M. incognita*- resistant and –susceptible soybean cultivars as a tool to identify and exploit genetic host plant resistance

3.1. Introduction

Plants are subjected to various abiotic and/or biotic stress conditions (Hammond-Kosack and Jones, 2000). The latter are induced by pests and diseases such as bacteria, viruses, herbivorous insects and plant-parasitic nematodes (PPN) (Hammond-Kosack and Jones, 2000). Root-knot nematodes (RKN) inflict substantial damage to crops such as soybean (Sikora *et al*., 2005) and have recently been rated as the predominant nematode pests of a wide range of agri- and horticultural crops worldwide (Jones *et al*., 2013). The RKN *Meloidogyne incognita* is recorded as one of the predominant PPN that parasitise soybean worldwide (Sikora *et al.,* 2005), including South Africa (Fourie *et al*., 2001). RKN individuals spend most of their active life cycle sedentary in the vascular cylinder of roots of soybean plants and concurrently induce permanent and specialised feeding sites called giant cells (GC). RKN-infestations severely affect physiological processes such as the translocation of water and nutrients to the aerial parts of cultivars that are susceptible to these pests (Jones and Goto, 2011). The induction of sub-optimally developed and functioning GC in RKN-resistant cultivars, however, does not result in restricted translocation of water and nutrients to aerial plant parts (Fourie *et al*., 2013b).

Genetic host plant resistance (GHPR) to RKN is directly correlated to the effect of a soybean cultivar on the development and reproduction of the parasitising pathogen (Williamson and Roberts, 2009). Ultimately, soybean cultivars that exhibit GHPR generally induce reduced development and reproduction rates of RKN compared to those of susceptible cultivars where optimal development and reproduction of such pathogens occur (Williamson and Roberts, 2009). A variety of host defence responses, that include but are not restricted to the generation of reactive oxygen species (ROS), are induced in soybean cultivars that exhibit GHPR to RKN (Mehdy, 1994; Melillo *et al*., 2006; Almagro *et al*., 2009; Jones and Goto, 2011**)**. A variety of ROS exist, such as superoxide (O_2^-), singlet oxygen ($^1O_2$), hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2). Under stress conditions a host plant produces a burst of ROS that primarily consists of H_2O_2 and that is

collectively referred to as the oxidative burst (Bolwell *et al.*, 2002; Scandalios, 2005). When ROS levels exceed the defensive ability of the host plant, affected cells suffer oxidative stress (Sharma *et al*., 2012).

In soybean GHPR to *M. incognita* identified in locally adapted cultivars occurs at the cellular level (Fourie *et al*., 2013b). In this study one of the mechanisms that induce such resistance was identified as post-infectional resistance and specifically antibiosis since the development of *M. incognita* life stages are retarded and/or adversely affected. Additionally, a significant reduction in the reproduction rate of the mature females was recorded in roots of *M. incognita*-resistant cultivars compared to their susceptible counterparts. Although the biochemical pathways involved in GHPR in such locally adapted soybean cultivars are not known, several studies have investigated the effect of various enzymes on crop hosts that are resistant to PPN. These include studies that reported activity increases of peroxidase in roots of cabbage (*Brassica oleraceae*) that were inoculated with *Pratylenchus penetrans (*Acedo and Rohde, 1971), while a similar scenario was reported for *Ditylenchus dipsaci*-infected pea (*Pisum sativum*) (Hussey and Krusberg, 1970). Induction of higher peroxidase activity in RKN-resistant crop host plants to protect plant cells from oxidative damage has been also been reported by other authors for banana (*Musa* spp.) (Sundararaju and Suba, 2006), coffee (*Coffea* spp.) (Mazzafera, 1989), cotton (*Gossypium* spp.) (Aryal *et al*., 2011; Noel and McClure, 1978), rice (*Oryza* spp.) (Anita and Samiyappan, 2012) and tomato (*Solanum lycopersicum*) (Zacheo *et al.*, 1982, 1983, 1993; Shukla and Chakraborty, 1988; Molinari and Abd-Elgawad, 2007; Rani *et al*., 2008).

Peroxidases are substrate specific and located in almost every compartment of the plant. Accordingly, peroxidases can be subdivided into ascorbate peroxidases (APX, EC 1.11.1.11) that uses ascorbate as substrate and is a major H_2O_2 scavenging enzyme. The second group uses guaiacol (2-methoxyphenol) as substrate and are accordingly known as guaiacol peroxidases (GPX, EC 1.11.1.7). The latter peroxidases do not only differ in reactivity, but also in respect to physiological roles (Srivastava and Tarafdar, 2008). Contrary to APX, GPX is involved in auxin oxidation, lignin and suberin biosynthesis, cross-linking of cell wall structural proteins (Durner and Klessig, 1995), production of phytoalexins (antimicrobial metabolites) and the metabolism of reactive nitrogen species (RNS) and ROS (Fig. 1.9) (Almagro *et al*., 2009). In terms of RKN,

qualitative differences have been recorded for GPX activity in both *M. incognita*-inoculated and non-inoculated tomato roots (Zacheo *et al.*, 1982 & 1983).

In terms of catalase enzyme activity (CAT), Molinari (1998), Molinari and Loffredo (2006) and Molinari and Abd-Elgawad (2007) reported that reduced levels of the enzyme were recorded in the roots of *M. incognita*-resistant tomato cultivars after nematode inoculation compared to that of the non-infected plants. However, for soybean limited research thus far have been conducted to characterise GHPR to RKN using enzyme activity. Veech and Endo (1969) listed the localisation of enzymes in soybean roots infected with *M. incognita* during the late 1970's. In this study it was indicated that alkaline phosphatase, esterase, peroxidase, adenosine, triphosphatase and cytochrome oxidase were localised most actively in the direct vicinity of the GC. On the other hand, Alkharouf *et al*. (2006) recorded increased levels of lipoxygenase (LOX) in roots of *Heterodera glycines*-infected soybean cultivars that exhibit GHPR to this nematode pest when compared with its uninfected, susceptible counterpart.

According to Molinari and Abd-Elgawad (2007) the use of enzymes to quantify GHPR in crop cultivars holds several advantages, viz. (1) it is more accurate and is not as laborious as greenhouse bio-assays that are traditionally used, (2) it is not elaborate and time-consuming as traditional screening protocols and (3) is ultimately not affected by environmental conditions and the genetic variability of the target PPN as is the case with traditional protocols. Therefore, to add value to the existing knowledge base in terms of the potential use of enzymes to identify, verify and/or characterise GHPR in *M. incognita*-resistant soybean cultivars the aims of this study were to:

i) quantify the activity of GPX, LOX and CAT in roots and leaves of susceptible and resistant cultivars that were inoculated with second stage juvenile (J2) as well as their non-inoculated counterparts and

ii) assess whether the use of such enzymes can be used to replace or complement traditional screening assays to identify GHPR in locally adapted cultivars

3.2. Materials and methods

3.2.1. Chemicals

All solvents used during this study were of analytical or high-performance liquid chromatography (HPLC) grade where applicable.

3.2.2. Soybean germplasm

During this study GPX, LOX and CAT activity levels were determined in roots and leaves of several *M. incognita*-resistant and –susceptible cultivars.

To determine GPX and LOX activity, five locally adapted soybean cultivars were selected according to their reproduction factor (Rf) values for *M. incognita*. This data was recorded during host suitability experiments of this study (Chapter 2, paragraph 2.2.4.). Three of these cultivars were commercially available during the 2012/2013 growing season and were identified with varying levels of susceptibility to *M. incognita*, namely Egret (Rf = 4.42), Dundee (Rf = 34.90) and LS6248 R (Rf = 68.35). Conversely, cultivar LS5995 which is no longer commercially available in South Africa served as the resistant standard (Rf = 0.02) together with the pre-released cultivar GCI7 (Rf = 0.01). The latter cultivar has been derived from crosses between LS5995 and Prima2000 as a result of traditional breeding to introgress *M. incognita*-resistance in high-yielding cultivars (Fourie *et al*., 2010).

CAT activity was determined in root and leaf samples of the commercially available, *M. incognita*-susceptible soybean cultivars LS6146R (Rf = 2.06), PHB95Y20 (Rf = 2.10), Egret (Rf = 4.42.) and LS6248 R (Rf = 68.35). In addition, leaf and root samples from the resistant cultivar LS5995 were also obtained for CAT analyses.

3.2.3. Trial layout, nematode inoculation and sampling of plant material for enzyme analyses

All cultivars used during this study were grown in a temperature regulated greenhouse (18-26°C) with a 14L:10D hour photoperiod being maintained. For each cultivar, three replicates were included in a split-plot design that represented the *M. incognita* J2-inoculated and J2-free treatments (Appendix 3). The latter treatment served as the untreated control, while five-thousand *M. incognita*

J2 and eggs were inoculated on the roots of each seedling for the J2-inoculated treatment. Nematode inoculation was done as described in Chapter 2, paragraph 2.2.3. J2 were obtained by extracting *M. incognita* eggs from at least four infected tomato root systems (described in Chapter 2, paragraph 2.2.2), using the modified sodium hypochlorite (NaOCl)-method of Riekert (1995).

Soybean plants of the various cultivars used were grown in a greenhouse under the same conditions as described for the host suitability experiments in Chapter 2, paragraph 2.2.3. Styrofoam cups (250 cm^3 capacity) were filled with a sandy loam soil (5.3 % clay, 93.6 % sand, 1.1 % silt and 0.47 % organic matter) that was fumigated with Telone II (150 ℓ/ha; a.s. 1,3-Dichloropropene) four weeks before the soybean seeds were planted and the experiments commenced. The pH (H_2O_2) of the soil used in all experiments was 7.47. At planting, soybean seeds were inoculated manually with *Bradyrhizobium japonicum* bacteria (see Chapter 2, paragraph 2.2.3) to optimise nitrogen fixation.

For GPX and LOX analysis (Berner and Van der Westhuizen, 2010), *M. incognita*-J2 inoculation was done 14 days after seedling emergence, while soybean seedlings designated for CAT analyses were inoculated with J2 30 days after seedling emergence (Molinari and Loffredo, 2006). Plants were watered three times per week or as needed. Leaves and roots of each seedling for each cultivar (J2-inoculated and J2-free/control) were obtained 24, 48 and 120 h after inoculation for LOX and GPX assays, while the same plant material was obtained 48 h after inoculation for CAT analyses. Root and leaf samples for enzyme analyses were immediately preserved in liquid nitrogen after they were harvested from the soybean plants. The samples were then transferred to a -80°C freezer where they were kept until the enzyme analyses were conducted.

3.2.4. Enzyme activity assessments

3.2.4.1. GPX and LOX analyses

Frozen soybean leaf and root tissue (0.3 g), were homogenised separately with approximately 50 mg acid-washed sand and 10 % (w/v) polyvinylphenol (PVP) in a pre-cooled pestle and mortar using 5 mℓ of a 0.1 M potassium extraction buffer (pH 7.5) containing 1 mM ethylenediaminetetraacetic acid (EDTA). The samples were centrifuged at 14 000 rpm for 10 min at 4°C. The supernatant was immediately used to determine the enzyme activity of both GPX and LOX. GPX activity was determined by measuring the change in absorbance in the enzyme reaction mixture with guaiacol as the hydrogen donor substrate. The enzyme extract (10 µℓ) was added to a

500 µℓ assay mixture (50 mM Potassium buffer containing 0.2 mM EDTA, pH 5.5), 340 µℓ distilled H_2O, 100 µℓ of 50 mM guaiacol and 50 µℓ of 8.2 mM H_2O_2. The tetraguaiacol that was formed as a result of the latter reaction has a maximum absorbance at 470 nm and the change in absorbance was measured spectrophotometrically using a double-beam spectrophotometer with an electronic temperature control unit for 180 seconds at 30°C against a blank sample. The latter consisted of distilled water only. Specific GPX activity was measured and documented as nmol tetraguaiacol/mg protein/min (Zieslin and Ben-Zaken; 1991).

LOX activity was measured by recording the continuous change in absorbance, using the same spectrophotometer as mentioned above. Diluted enzyme extract (50 µℓ) was added to 1 mℓ of a 0.1 M sodium citrate phosphate buffer (pH 6.2) together with 10 µℓ linoleic acid substrate. Linoleic acid substrate (2.5mM linoleic acid in 0.15 % Tween20) was prepared according to Ocampo *et al*., (1986). Linoleic acid (200 µℓ), 384 µℓ Tween20 and 20 mℓ methanol was mixed in a round bottomed flask and dried by rotary evaporation at 60˚C. Adding 250 mℓ of 0.05 M sodium phosphate buffer (pH 9) to the latter mixture, the residue was redissolved and the further divided into 5 mℓ aliquots, which were stored in air tight containers at -80˚C. During transfer, nitrogen gas was used to saturate the content of round-bottomed flask as well as the air tight containers before addition of the aliquots to minimise contact with air. Excess substrate was stored at -80˚C. After thawing, the substrate was kept on ice during use and not reused. The change in absorbance was monitored at 234 nm for 10 min at 30°C. Specific LOX activity was documented as $HPOD.mg^{-1}$ $protein.min^{-1}$ (Grossmann and Zakut, 1979; Ocampo *et al*., 1986).

3.2.4.2. CAT analyses

CAT activity was measured by homogenising 0.5 g of frozen leaf and root material per plant with acid washed sand and 10 % (w/v) PVP in a pre-cooled pestle and mortar using 5 mℓ 0.1 M Tris-HCl buffer (pH 8.5) that contained 2 mM EDTA and 10 % glycerol. The samples were centrifuged at 15 000 rpm for 15 min at 4°C. The supernatant was stored at –80°C and later used for the enzyme activity measurement, while the pellet was discarded. CAT activity in the leaves and roots, respectively, were determined by recording the disappearance of H_2O_2 in the enzyme reaction mixture (Brennan and Frenkel, 1977). The reaction mixture consisted of 10 µℓ enzyme extract, 765 µℓ assay buffer (50 mM Tris-HCl, pH 6.8) and 100 µℓ 25 mM H_2O_2. The reaction was terminated

after 10 min by adding 125 µℓ of a 20 % (v/v) titanic tetrachloride solution (in concentrated HCl) to the reaction mixture at 20°C. Subsequently, the same method was used to terminate the enzyme activity at time zero in order to determine the change of enzyme activity over time. The absorbance of the reaction mixtures were read at 415 nm using a double-beam spectrophotometer with an electronic temperature control unit against distilled water. CAT activity was determined by comparing absorbance against a standard curve of H_2O_2 from 10 to 60 mM and expressed as H_2O_2 mM/min/mg protein (Gong *et al.*, 2001).

3.2.4.3. Determination of the protein concentrations in enzyme extracts

Soluble protein quantities of the respective supernatants that were obtained after enzyme extraction from each of the leaf and root soybean samples were determined according to the dye-binding assay technique of Bradford (1976). The standard used represented 0.5 $\mu g.\mu\ell^{-1}$ γ-globulin. Protein concentrations in each of the samples were determined using a Biorad Microplate Reader as described by Rybutt and Parish (1982) and the absorbance was measured at 595 nm. The assay mixtures contained 160 µℓ dH_2O, 40 µℓ BioRAD and 10 µℓ of the appropriate enzyme extracts from each leaf samples. However, for the root samples, 20 µℓ enzyme extract was used as protein concentrations were lower in the roots and subsequent alterations were made in the calculations.

3.2.5. Data analyses

Enzyme data for GPX and LOX were subjected to factorial analyses of variance (Statistica Version 11), using treatments (J2-inoculated and J2-free) as factor 1 and time intervals (24, 48 and 120 h) as factor 2. Means for the respective data sets were separated using Tukey's honest significant difference (HSD) test ($P \leq 0.05$). For CAT, data were also subjected to a factorial analyses of variance with treatments (J2-inoculated and J2-free) representing the only factor. Means for the respective data sets were separated using Tukey's honest significant difference (HSD) test ($P \leq 0.05$). All experiments were repeated to verify the results obtained.

3.3. Results

Enzyme activity data for GPX, LOX and CAT subjected to factorial analyses of variance for cultivars x treatments (J2-inoculated vs. J2-free) x sampling times, indicated significant ($P \leq 0.05$)

interactions between the parameters that were evaluated as listed in Tables 1a, b; 2a, b; 3a, b and 4a, b.

3.3.1. Specific GPX activity

GPX activity was substantially higher in root samples of all cultivars, for both the J2-inoculated as well as J2-free treatments, evaluated compared to those in leaf samples (Tables 1a and 1b).

3.3.1.1. Specific GPX activity in roots

A significant interaction was recorded for cultivars x treatments ($P \leq 0.05$; F = 10.84) for the 24 h sampling time (Table 1a). This indicated that the cultivars reacted differently in terms of the GPX activity recorded in their roots when inoculated with J2 compared to the J2-free treatments. This was illustrated particularly for the *M. incognita*-resistant cultivar GCI7 that had significantly ($P \leq 0.05$) higher GPX activity in root samples of J2-inoculated plants 24 h after inoculation compared to those of the J2-free plants.

A significant interaction also existed for cultivars x times ($P \leq 0.05$; F = 3.91), indicating that GPX activity in some cultivars differed significantly ($P \leq 0.05$) between the three time intervals (Table 1a; Fig. 1a). For example, for cultivar GCI 7 GPX activity was significantly ($P \leq 0.05$) higher in root samples during the 24 h sampling times compared to activity levels recorded at 48 and 120 h after the onset of the experiment. Cultivars also differed significantly ($P \leq 0.05$) from each other for all three sampling times with regard to the two treatments (J2-inoculated vs. J2-free) (Table 1a). For example, the GPX activity 24 h after inoculation in roots of resistant cultivar GCI 7 was significantly ($P \leq 0.05$) higher than that of the susceptible LS6248R for the J2-inoculated treatments but similar to those recorded for the other resistant (LS5995) as well as the other two susceptible cultivars Egret and Dundee 24 h after inoculation. For the J2-free treatments, however, at all three time intervals after onset of the experiment, the susceptible cultivar LS6248R had significantly ($P \leq 0.05$) higher activity than the resistant cultivar GCI7.

No significant interaction was, however, recorded for treatments x times ($P \geq 0.05$; F ratio = 0.06). The latter indicating that very little variation existed in terms of GPX activity in roots of the respective cultivars in terms of both the J2-inoculated and J2-free treatments during all three respective sampling times (Table 1a; Fig. 1a).

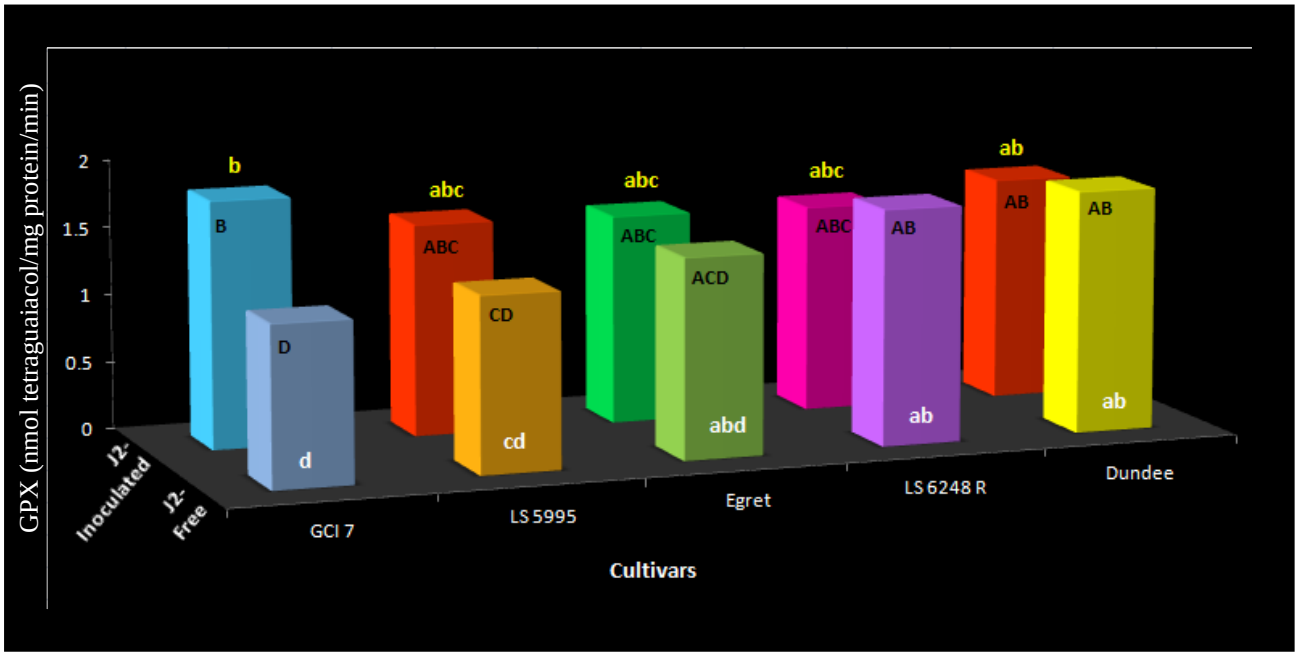


Figure 1a. A graphical illustration of interaction data for cultivars x treatments for three sampling times (24, 48 and 120h after onset of the experiments) in terms of the peroxidase activity (GPX) in roots of *Meloidogyne incognita*-resistant (R) and -susceptible (S) soybean cultivars (n = 3). Means for cultivars for the same treatment (J2-free and J2-inoculated) are represented by lower case letters in yellow (J2-inoculated) and white (J2-free) followed by the same letter do not differ significantly for the Tukey Test ($P > 0.05$); Means to separate the treatments (J2-inoculated and J2-free) for each cultivar are represented by upper case letters and do not differ significantly for the Tukey Test ($P > 0.05$) when followed by the same letter.

Table 1a. Interaction data for cultivars x treatments x sampling times in terms of the guaiacol peroxidase activity (GPX) expressed as nmol tetraguaiacol/mg protein/min in roots of *Meloidogyne incognita*-resistant (R) and -susceptible (S) soybean cultivars (n = 3).

Cultivar	**24 h**		**48 h**		**120 h**	
	J2-Inoculated	**J2-free**	**J2-Inoculated**	**J2-free**	**J2-Inoculated**	**J2-free**
Resistant						
GCI7	1.9687 (± 0.4656) b C	1.0579 (± 0.0343) b A	1.8941 (± 0.0859) a A	1.2728 (± 0.0022) a A	1.6089 (± 0.1395) a ABC	1.2321 (± 0.0851) a AB
LS5995	1.3509 (± 0.0949) ab AB	1.2924 (± 0.1144) ab AB	1.7457 (± 0.3963) a AB	1.4658 (± 0.3145) ab A	1.6005 (± 0.3168) a ABC	1.1343 (± 0.0663) a A
Susceptible						
Egret	1.4968 (± 0.0730) ab ABC	1.3511 (± 0.0204) ab AB	1.7515 (± 0.1566) a AB	1.7493 (± 0.3890) ab AB	1.3835 (± 0.1016) a ABC	1.3367 (± 0.0277) a AB
Dundee	1.6161 (± 0.3752) ab ABC	1.7027 (± 0.0759) ab BC	1.6983 (± 0.2937) a AB	1.8213 (± 0.1238) ab AB	1.6509 (± 0.1448) a BC	1.8495 (± 0.2855) ab C
LS6248R	1.0992 (± 0.0509) a A	1.4226 (± 0.1034) a ABC	2.0217 (± 0.1596) a AB	2.2918 (± 0.4031) b B	1.4682 (± 0.1587) a ABC	1.5114 (± 0.1785) b ABC
Interaction data:						
P values:	0.001		0.069		0.016	
F – ratio's:	8.013		2.575		3.958	

Means for cultivars for each of the two treatments (J2-free and J2-inoculated) are represented by lower case letters and if followed by the same letter do not differ significantly for each of the specific sampling times in each column according to the Tukey Test ($P > 0.05$); Means separating the treatments (J2-inoculated and J2-free) for each cultivar during each sampling time are represented by upper case letters and do not differ significantly for the Tukey Test ($P > 0.05$) when followed by the same letter in that specific row.

3.3.1.2. Specific GPX activity in leaves

A significant interaction existed for cultivars x treatments ($P \leq 0.05$; F = 11.73), indicating that the resistant cultivars GCI7 and LS5995 had significantly ($P \leq 0.05$) higher GPX activity in their leaves of those plants that were inoculated with J2 compared to those of J2-free plants only for the 24 h sampling time (Table 1b; Fig. 1b). However, no significant differences in GPX levels of the latter resistant cultivars were recorded for the two treatments for the 48 and 120 h sampling times. The latter trend was also evident for the susceptible cultivars for all three sampling times in terms of the two treatments.

A significant interaction also existed for cultivars x times ($P \leq 0.05$; F = 6.81), indicating that GPX activity in leaves of the resistant cultivar GCI7 were substantially higher for the J2-inoculated treatment during the 24h time interval compared to those for the 48 and 120 h (Table 1b; Fig. 1b). Furthermore, GPX activity in leaves of the susceptible cultivar Dundee were significantly ($P \leq 0.05$) higher in terms of the J2-free treatment 24 h after onset of the experiment compared to the other cultivars, except LS5995. Also, for the latter treatment 120 h after the experiment commenced, GPX activity was lower in leaves of the resistant cultivar GCI7 and the susceptible cultivar LS6248R compared to those recorded for the other resistant cultivar LS5995 and the susceptible cultivar Dundee but not from the susceptible cultivar Egret.

Ultimately a significant interaction was also recorded for treatments x times ($P \leq 0.05$; F ratio = 11.60), which indicated that the GPX activity in leaves of J2-inoculated and J2-free treatments for all cultivars differed substantially for the respective time intervals (Table 1b).

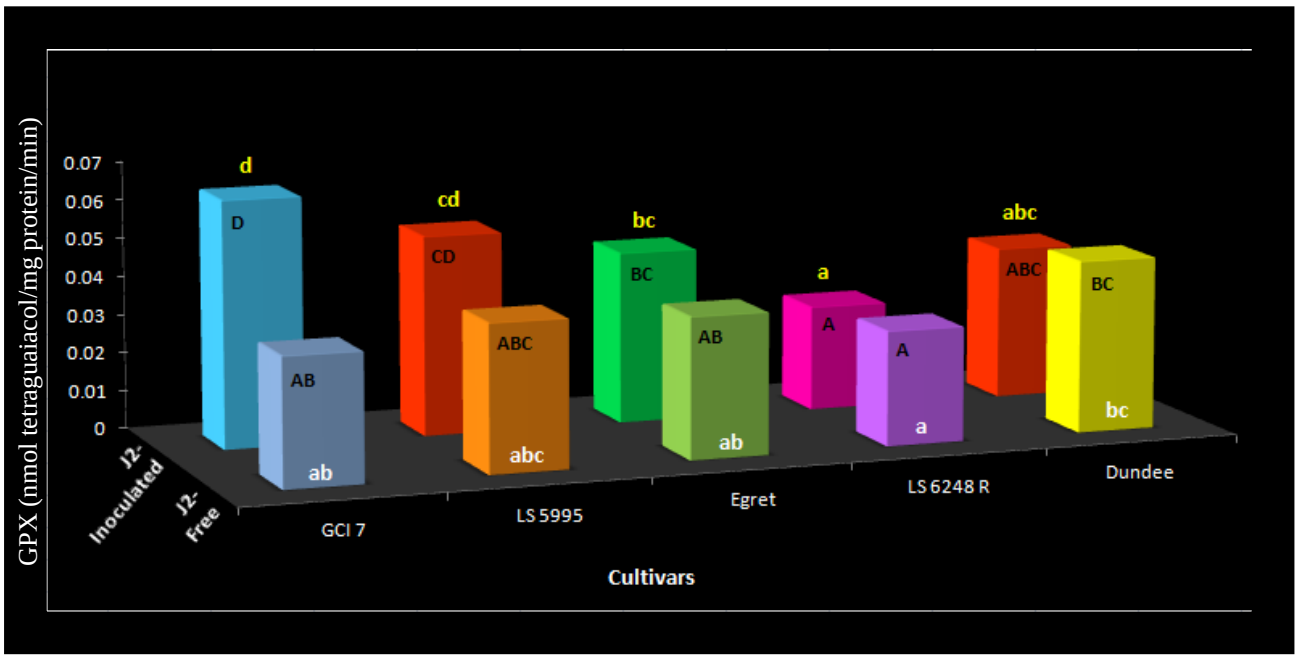


Figure 1b. A graphical illustration of interaction data for cultivars x treatments for three sampling times (24, 48 and 120h after onset of the experiments) in terms of the peroxidase activity (GPX) in leaves of *Meloidogyne incognita*-resistant (R) and -susceptible (S) soybean cultivars (n = 3). Means for cultivars for the same treatment (J2-free and J2-inoculated) are represented by lower case letters in yellow (J2-inoculated) and white (J2-free) followed by the same letter do not differ significantly for the Tukey Test ($P > 0.05$); Means to separate the treatments (J2-inoculated and J2-free) for each cultivar are represented by upper case letters and do not differ significantly for the Tukey Test ($P > 0.05$) when followed by the same letter.

Table 1b. Interaction data for cultivars x treatments x sampling times in terms of the guaiacol peroxidase activity (GPX) expressed as nmol tetraguaiacol/mg protein/min in leaves of *Meloidogyne incognita*-resistant (R) and -susceptible (S) soybean cultivars (n = 3).

Cultivar	**24 h**		**48 h**		**120 h**	
	J2-Inoculated	**J2-free**	**J2-Inoculated**	**J2-free**	**J2-Inoculated**	**J2-free**
Resistant						
GCI7	0.1021 (± 0.0281) c C	0.0354 (± 0.0053) ab A	0.0487 (± 0.0100)a AB	0.0358 (± 0.0023) a B	0.0422 (± 0.0037) a AB	0.0295 (± 0.0005) a BCD
LS5995	0.0757 (± 0.0109) bc BC	0.0437 (± 0.0057) bc A	0.0345 (± 0.0025) a A	0.0282 (± 0.0024) a AB	0.0461 (± 0.0008) a A	0.0438 (± 0.0049) b A
Susceptible						
Egret	0.0465 (± 0.0028) ab AB	0.0268 (± 0.0017) a A	0.0472 (± 0.0066) a AB	0.0452 (± 0.0059) a AB	0.0406 (± 0.0039) a AB	0.0387 (± 0.0011) ab AB
Dundee	0.0430 (± 0.0068) ab A	0.0484 (± 0.0037) c AB	0.0332 (± 0.0069) a AB	0.0386 (± 0.0085) a A	0.0434 (± 0.0067) a A	0.0477 (± 0.0082) b A
LS6248R	0.0300 (± 0.0037) a A	0.0346 (± 0.0020) ab A	0.0260 (± 0.0030) a AB	0.0279 (± 0.0061) a AB	0.0260 (± 0.0045) a C	0.0272 (± 0.0017) a CD
Interaction data:						
P values:	0.000		0.23		0.035	
F – ratio's:	12.63		3.584		3.200	

Means for cultivars for each of the two treatments (J2-free and J2-inoculated) are represented by lower case letters and if followed by the same letter do not differ significantly for each of the specific sampling times in each column according to the Tukey Test ($P > 0.05$); Means separating the treatments (J2-inoculated and J2-free) for each cultivar during each sampling time are represented by upper case letters and do not differ significantly for the Tukey Test ($P > 0.05$) when followed by the same letter in that specific row.

3.3.2. Specific LOX activity

LOX activity was substantially higher in root samples of all cultivars evaluated compared to those in leaf samples (Tables 2a & b).

3.3.2.1. Specific LOX activity in roots

A significant interaction existed between cultivars x treatments ($P \leq 0.05$; F = 4.37). The cultivars thus reacted differently in terms of the LOX activity recorded in their roots when inoculated with J2 compared to the J2-free treatments (Table 2a; Fig. 2a). This was illustrated for the susceptible cultivar Egret that had significantly ($P \leq 0.05$) higher LOX activity in root samples inoculated with J2 as well as the J2-free samples collected at 24 h after onset of the experiment only. In terms of LOX activity in root samples of the other cultivars, the resistant cultivars (GCI7 and LS5995) and susceptible (Dundee and LS6248R) did, however, not differ significantly from each other for the 24 h sampling time. However, during the 48 h interval, significant ($P \leq 0.05$) difference existed for the two resistant cultivars GCI7 and LS5995 after inoculation, compared to their J2-free controls. Ultimately, no further significant differences in LOX activity were recorded during the 48 and 120 h sampling times.

A significant interaction also existed for cultivars x times ($P \leq 0.05$; F = 9.35), indicating that that LOX activity in roots of the cultivars differed substantially for the three respective sampling times (Table 2a). The latter was illustrated for the susceptible cultivar Egret that had significantly higher LOX activity in root samples of J2-inoculated plants during the 24 h sampling time compared to those recorded during the 48 and 120 h sampling times.

No significant interaction, however, was recorded for treatments x times ($P \geq 0.05$; F = 11.60), indicating that very little variation existed for LOX activity in roots of J2-inoculated and J2-free treatments for all cultivars for the three respective time intervals (Table 2a).

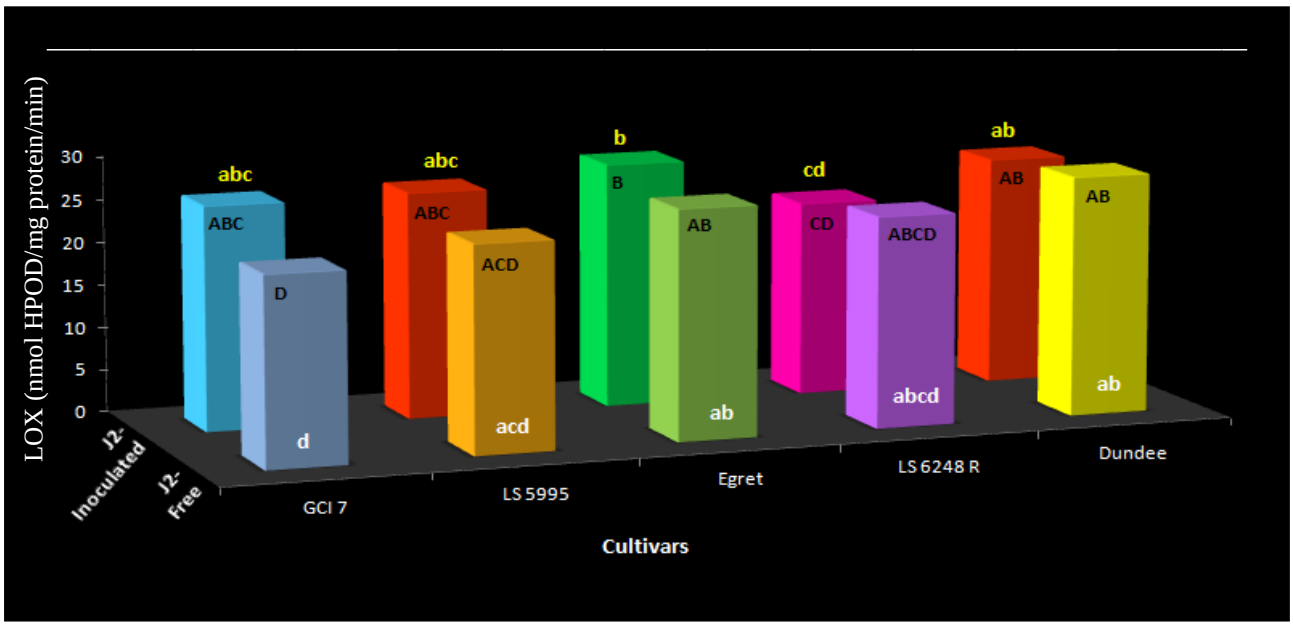


Figure 2a. A graphical illustration of interaction data for cultivars x treatments for three sampling times (24, 48 and 120h after onset of the experiments) in terms of the lipoxygenase activity (LOX) in roots of *Meloidogyne incognita*-resistant (R) and -susceptible (S) soybean cultivars (n = 3). Means for cultivars for the same treatment (J2-free and J2-inoculated) are represented by lower case letters in yellow (J2-inoculated) and white (J2-free) followed by the same letter do not differ significantly for the Tukey Test ($P > 0.05$); Means to separate the treatments (J2-inoculated and J2-free) for each cultivar are represented by upper case letters and do not differ significantly for the Tukey Test ($P > 0.05$) when followed by the same letter.

Table 2a. Interaction data for cultivars x treatments x sampling times in terms of the lipoxygenase activity (LOX) expressed as nmol HPOD/mg protein/min in roots of *Meloidogyne incognita*-resistant (R) and -susceptible (S) soybean cultivars (n = 3).

Cultivar	**24 h**		**48 h**		**120 h**	
	J2-Inoculated	**J2-free**	**J2-Inoculated**	**J2-free**	**J2-Inoculated**	**J2-free**
Resistant						
GCI7	26.0 (± 2.7) bc BC	21.4 (± 2.1) ab AB	28.4 (± 2.2) a A	21.8 (± 0.6) a B	24.0 (± 1.2) aA	22.8 (± 0.3) aA
LS5995	23.8 (± 1.1) ab AB	23.6 (± 0.4) ab AB	31.1 (± 3.3) a A	25.3 (± 3.8) a B	24.3 (± 0.6) aA	23.3 (± 2.4) aA
Susceptible						
Egret	36.4 (± 2.4) d D	30.0 (± 2.8) c C	26.8 (± 2.5) a AB	27.3 (± 2.5) a AB	22.3 (± 1.6) aA	22.9 (± 3.1) aA
Dundee	25.0 (± 3.0) abc ABC	25.2 (± 1.7) b ABC	29.0 (± 2.8) a AB	30.4 (± 4.2) a A	26.2 (± 2.9) aA	28.2 (± 5.8) aA
LS6248R	19.4 (± 0.9) a A	20.1 (± 1.3) a AB	26.6 (± 1.9) a AB	28.9 (± 1.7) a AB	22.0 (± 0.9) aA	24.7 (± 2.3) aA
Interaction data:						
P values:	0.018		0.023		0.635	
F – ratio's:	3.860		3.584		0.648	

Means for cultivars for each of the two treatments (J2-free and J2-inoculated) are represented by lower case letters and if followed by the same letter do not differ significantly for each of the specific sampling times in each column according to the Tukey Test ($P > 0.05$); Means separating the treatments (J2-inoculated and J2-free) for each cultivar during each sampling time are represented by upper case letters and do not differ significantly for the Tukey Test ($P > 0.05$) when followed by the same letter in that specific row.

3.3.2.2. Specific LOX activity in leaves

Although a significant interaction ($P \leq 0.05$) existed when cultivar x treatment (J2-inoculated vs. J2-free) x sampling times were analysed, no significant interactions existed between cultivars x treatments (J2-inoculated vs. J2-free), cultivars x times and/or treatments x times for LOX activity in leaves of the various cultivars for each of the respective time intervals (Table 2b). This ultimately indicated that over the three sampling intervals, LOX activity in leaf samples of the resistant cultivar GCI7 only were significantly ($P \leq 0.05$) higher in J2-inoculated than J2-free plants (Fig. 2b).

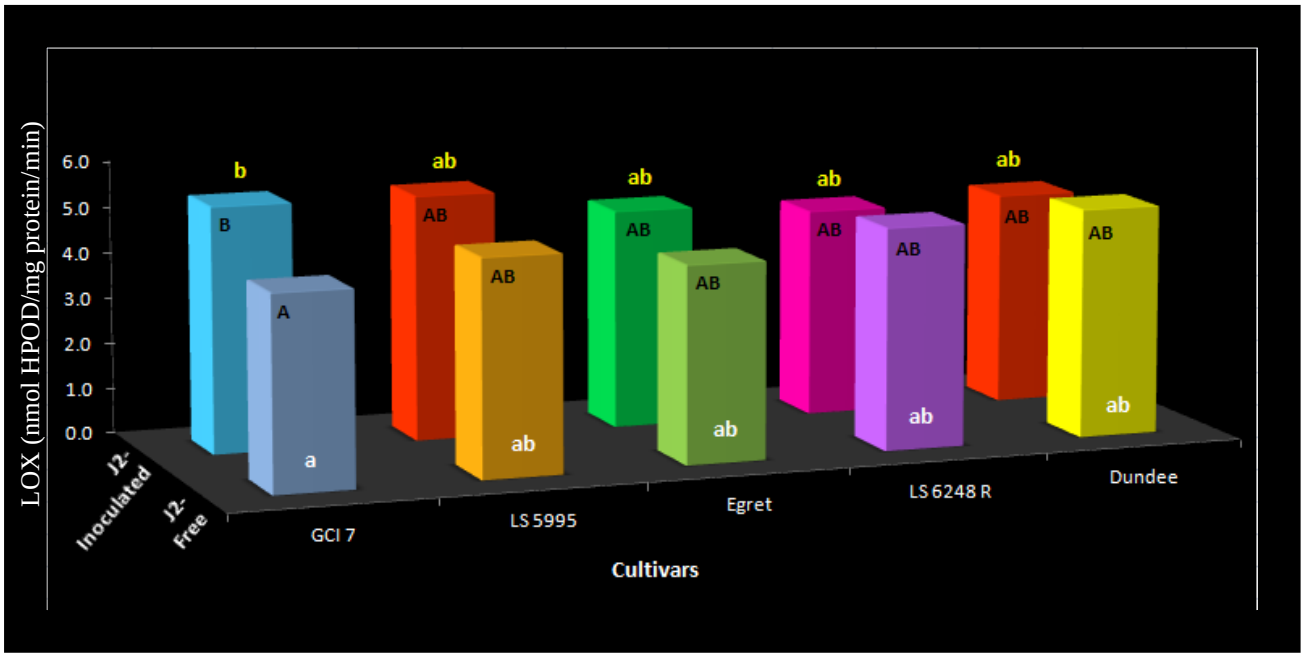


Figure 2b. A graphical illustration of interaction data for cultivars x treatments for three sampling times (24, 48 and 120h after onset of the experiments) in terms of the lipoxygenase activity (LOX) in leaves of *Meloidogyne incognita*-resistant (R) and -susceptible (S) soybean cultivars (n = 3). Means for cultivars for the same treatment (J2-free and J2-inoculated) are represented by lower case letters in yellow (J2-inoculated) and white (J2-free) followed by the same letter do not differ significantly for the Tukey Test ($P > 0.05$); Means to separate the treatments (J2-inoculated and J2-free) for each cultivar are represented by upper case letters and do not differ significantly for the Tukey Test ($P > 0.05$) when followed by the same letter.

Table 2b. Interaction data for cultivars x treatments x sampling times in terms of the lipoxygenase activity (LOX) expressed as nmol HPOD/mg protein/min in leaves of *Meloidogyne incognita*-resistant (R) and -susceptible (S) soybean cultivars (n = 3).

Cultivar	**24 h**		**48 h**		**120 h**	
	J2-Inoculated	**J2-free**	**J2-Inoculated**	**J2-free**	**J2-Inoculated**	**J2-free**
Resistant						
GCI7	3.7 (± 0.6) a A	3.2 (± 0.7) a A	4.4 (± 0.4) b B	3.7 (± 0.3) a AB	8.0 (± 1.5) a A	5.8 (± 0.5) a A
LS5995	4.5 (± 0.1) a A	3.8 (± 0.6) a A	3.4 (± 0.4) ab AB	2.9 (± 0.2) a A	8.2 (± 0.8) a A	7.5 (± 1.2) a A
Susceptible						
Egret	3.7 (± 0.2) a A	3.6 (± 0.3) a A	3.4 (± 0.4) ab AB	3.3 (± 0.3) ab AB	7.2 (± 0.9) a A	6.1 (± 0.7) a A
Dundee	3.2 (± 0.5) a A	3.7 (± 0.8) a A	3.8 (± 0.6) ab AB	3.9 (± 0.7) ab AB	7.0 (± 0.8) a A	7.5 (± 0.4) a A
LS6248R	3.8 (± 0.2) a A	3.9 (± 0.5) a A	4.0 (± 0.5) ab AB	4.1 (± 0.7) ab AB	5.9 (± 0.7) a A	6.7 (± 1.9) a A
Interaction data:						
P values:	0.259		0.498		0.135	
F – ratio's:	1.435		0.871		1.988	

Means for cultivars for each of the two treatments (J2-free and J2-inoculated) are represented by lower case letters and if followed by the same letter do not differ significantly for each of the specific sampling times in each column according to the Tukey Test ($P > 0.05$); Means separating the treatments (J2-inoculated and J2-free) for each cultivar during each sampling time are represented by upper case letters and do not differ significantly for the Tukey Test ($P > 0.05$) when followed by the same letter in that specific row.

3.3.3. Specific CAT activity in roots and leaves

Specific CAT activity recorded in soybean root samples 48 h after J2 inoculation was inconclusive since negative values were recorded for the J2-inoculated samples of Egret during the initial and repeat experiments (Table 3). Furthermore, the standard deviations of some samples were larger than the recorded CAT values. The data thus warrant no further discussion.

For leaf samples, however, a significant ($P \leq 0.05$) interaction existed for cultivars x treatments (J2-inoculated vs. J2-free) in terms of CAT activity in leaf samples (Table 3; Fig. 3). This indicated that cultivars reacted differently in terms of CAT activity for the two treatments with the resistant cultivar LS5995 in particular having significantly lower CAT activity (34.9 %) in leaves of plants that were inoculated with J2 compared to those that were J2-free. The latter trend was, however, not recorded for the other cultivars but CAT activity inhibition in J2-inoculated compared to the J2-free treatments were as follows for three of the susceptible cultivars: LS6248R (29.3 %), PHB95Y20 (25.6 %), LS6146 (16.6 %) and Egret (5.6 %).

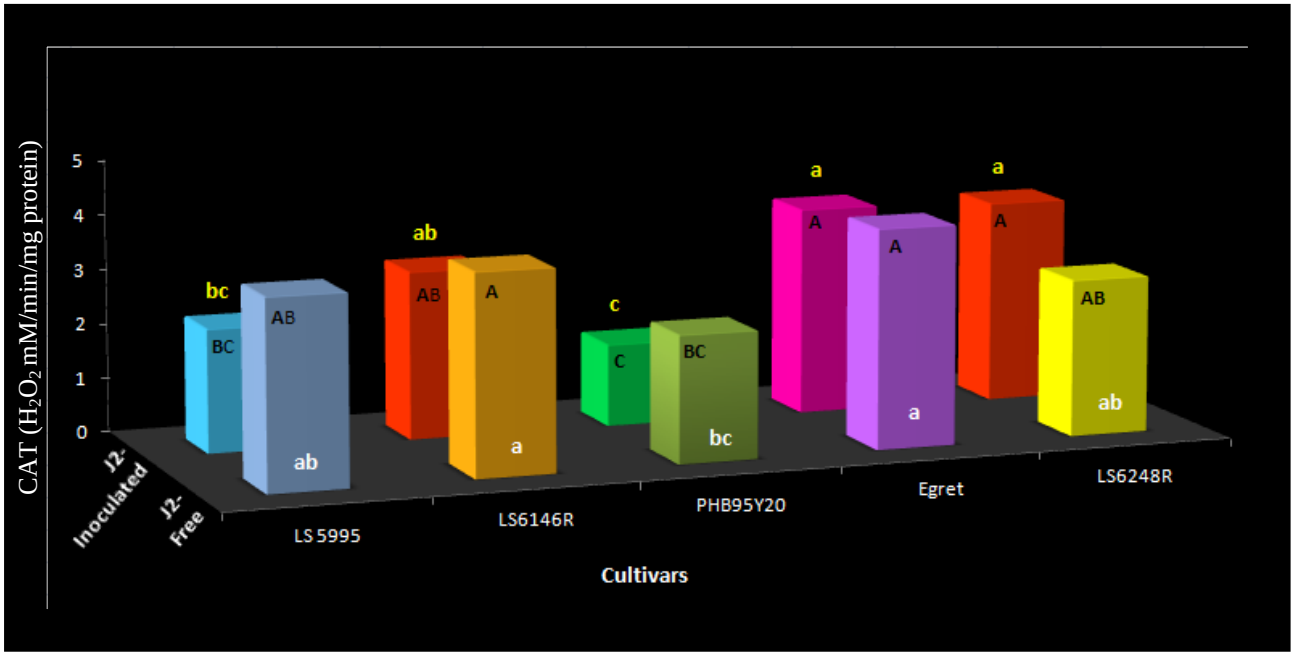


Figure 3. A graphical illustration of interaction data for cultivars x treatments for one sampling times (24, after onset of the experiment) in terms of the catalase activity (CAT) in leaves of *Meloidogyne incognita*-resistant (R) and -susceptible (S) soybean cultivars (n = 3). Means for cultivars for the same treatment (J2-free and J2-inoculated) are represented by lower case letters in yellow (J2- inoculated) and white (J2-free)

followed by the same letter do not differ significantly for the Tukey Test ($P > 0.05$); Means to separate the treatments (J2-inoculated and J2-free) for each cultivar are represented by upper case letters and do not differ significantly for the Tukey Test ($P > 0.05$) when followed by the same letter.

Table 3. Catalase (CAT) activity (H_2O_2 mM /min /mg protein) in leaves and roots of resistant (R) and susceptible (S) soybean cultivars at 48 h.

Cultivar	**Roots**		**Leaves**	
	J2-inoculated	**J2-free**	**J2-inoculated**	**J2-free**
Resistant				
LS5995	0.42 (± 0.16) aA	0.38 (± 0.07) aA	2.26 (± 0.11) ab AB	3.47 (± 0.20) bc C
Susceptible				
LS6146R	0.33 (± 0.00) aA	0.33 (± 0.07) aA	3.07 (± 0.09) a A	3.68 (± 0.29) ab AB
PHB95Y20	0.07 (± 0.04) aA	0.06 (± 0.06) aA	1.50 (± 0.07) bc BC	2.33 (± 0.10) c C
Egret	-0.08 (± 0.18) aA	0.12 (± 0.55) aA	3.80 (± 0.06) a A	4.02 (± 0.16) a A
LS6248R	0.28 (± 0.10) aA	0.35 (± 0.00) aA	3.71 (± 0.13) ab AB	2.87 (± 0.11) a A
Interaction data:				
P values:	0.842		0.008	
F – ratio's:	0.349		4.63	

Means for cultivars for each of the two treatments (J2-free and J2-inoculated) are represented by lower case letters and if followed by the same letter do not differ significantly for each of the specific sampling times in each column according to the Tukey Test ($P > 0.05$); Means separating the treatments (J2-inoculated and J2-free) for each cultivar during each sampling time are represented by upper case letters and do not differ significantly for the Tukey Test ($P > 0.05$) when followed by the same letter in that specific row.

3.4. Discussion

Data obtained for enzyme activity in leaves and roots of *M. incognita*-resistant and –susceptible cultivars were not always significantly accountable for all enzymes that were investigated during this study. However, potential exists in using such biochemical parameters as a tool to identify and/or verify GHPR that are employed in soybean cultivars to RKN such as *M. incognita*.

The latter is substantiated by significant increases in GPX activity in leaf and root samples of two locally adapted *M. incognita*-resistant soybean cultivars GCI7 and LS5995 that were inoculated with J2. The latter data complements reports by other authors that accentuated the integral role that enzymes play in host plant resistance mechanisms that are deployed as a result of RKN parasitism (Aryal *et al*., 2011; Molinari and Abd-Elgawad, 2007; Rani *et al*., 2008; Zacheo *et al*., 1982 & 1993). Thus, during this study, GPX activity recorded 24 h after J2 inoculation emanated as a useful parameter to identify soybean cultivars that exhibit GHPR against *M. incognita*.

Induction of higher GPX activity in RKN-resistant crop host plants to protect plant cells from oxidative damage (Blokhina *et al*., 2003) has been reported in previous studies, *viz*. banana (Sundararaju and Suba, 2006), coffee (Mazzafera, 1989), cotton (Aryal *et al*., 2011; Noel and McClure, 1978), rice (Anita and Samiyappan, 2012) and tomato (Zacheo *et al.*, 1982, 1983 & 1993; Shukla and Chakraborty, 1988; Molinari and Abd-Elgawad, 2007; Rani *et al*., 2008). However, the time after root penetration of RKN J2 has been accentuated as an important factor in detecting the enzymes that are induced as a protective measure by the host plant. For example, studies done by Zacheo *et al.*, (1983) reported that GPX activity increased five-fold in roots of *M. incognita*-resistant tomato cultivars 10 days after inoculation (DAI) with J2 compared to the J2-free controls. During the present study, however, the most pronounced induction of GPX in *M. incognita*-resistant soybean cultivars was 24h after J2 inoculation. Ultimately, increased GPX activity in *M. incognita*-resistant tomato cultivars used in the study by Zacheo *et al*. (1983) was reduced to normal levels 24 DAI, while those in leaf samples of the locally adapted cultivars used during this study were substantially lower 48 and 120 h after J2 inoculation.

GPX has many proposed methods of protecting plant cells from oxidative damage of which scavenging ROS are predominant. However, the signalling agent, salicylic acid (SA), inhibits only APX, which has been found to rapidly metabolise H_2O_2 (Durner and Klessig, 1995), and not GPX (which was tested for in this study). It can this be presumed that these enzymes have different roles as sited in literature (Srivastava and Tarafdar, 2008). Notably, cell wall GPX has been found to produce apoplastic H_2O_2 in the presence of reductants (Blokhina *et al*., 2003; Bolwell *et al.*, 1998; Jiang and Miles, 1993). A study done by Kawano and Muto (2000) also reported that SA-induced O_2^- and H_2O_2 production by GPX in tobacco cell cultures. The latter ROS production was further enhanced by the addition of H_2O_2 to the GPX prior to SA addition, indicating its role rather in production than scavenging. These authors thus proposed that the presence of H_2O_2 may be a prerequisite to catalyse the GPX oxidation of SA, which in turn produces O_2^- and H_2O_2 in the extracellular space. These findings are supported by previous studies that proved that H_2O_2 and extracellular GPX are prerequisites for SA-dependent production of O_2^- (Kawano *et al*., 1998). Thus it is proposed in this study that GPX plays a defensive role extracellularly by production of ROS in the area where J2 penetrated root cells of the resistant cultivars. This is supported by results obtained in the current study, representing an increase of 86 % in GPX activity in root and as much as 188 % in leaf samples of *M. incognita* J2-infected plants of the resistant cultivar GCI 7. Increased GPX activity in leaf samples due to J2 inoculation could indicate that systemic acquired resistance (SAR) coupled with a sudden increase in ROS such as O_2^- and H_2O_2 (Lamb and Dixon, 1997) is the biochemical mechanism of *M. incognita*-resistance present in GCI7. Therefore, it is proposed that RKN-resistant soybean host plants generate ROS as signalling molecules that are used to manipulate the environment and subsequently rendering it to be unfavourable for the development and reproduction of such pathogens by inducing cell death. Such RKN-resistant host plants may, as indicated by previous studies done by Ogallo and McClure (1996) and Aryal *et al*. (2011), use ROS as signals to induce SAR after J2 infection.

In addition to GPX, increased LOX activity has also been recorded in *M. incognita*-inoculated compared to nematode-free treatments of *M. incognita*-resistant cultivars used in this study. Although significant increases in LOX activity were evident in J2-inoculated root samples of the local commercially available, *M. incognita*-susceptible cultivar Egret 24 h after J2 inoculation, this was no longer evident 48 h after inoculation and a significant difference was rather recorded for the *M. incognita* resistant cultivars GCI7 and LS5995. Also, substantial increases in LOX activity were recorded in roots and leaves of the resistant

GCI7 and in leaves of the resistant cultivar LS5995 after J2 inoculation. For example, substantial increases in LOX activity of 21 and 38 % for resistant cultivar GCI7 in root and leaf samples 24 and 120 h after J2 inoculation, respectively, compared to their J2-free counterparts. These findings furthermore emphasise the potential use of LOX in this regard. It should, therefore, be borne in mind that LOX may also be used as an alternative parameter to identify GHPR to RKN in soybean after further experiments have to be done to exploit and refine the use of this biochemical parameter.

According to literature, LOX initiates one of the earliest events that precede the oxidative burst, namely a change in membrane permeability of the affected cells (Maccarrone *et al*., 2001). The resultant changes in cell membrane permeability subsequently lead to a pH change in the cell apoplast, which is a prerequisite for the complete oxidative burst to take place (Bindschelder *et al*., 2006; Bolwell *et al*., 2002). LOX has also been found to contribute to oxidative stress by the oxidation of pyridine nucleotides, producing O_2^- (Roy *et al*., 1994; van Gestelen *et al*., 1997). Furthermore, H_2O_2 can be converted by LOX to stable aldehydes, hydroxy- and epoxy-fatty acids, that exhibit antimicrobial activity and by means of the octadecanoid pathway produces a diverse group of jasmonates such as jasmonic acid (JA) and methyl jasmonates (MJ) (Feussner and Wasternack, 2002). Serving as signalling agents, JA and MJ have been proven to be genetically involved in plant defense reactions as a result of pathogen infection (Bate and Rothstein 1998; Birkett *et al.* 2000; Engelberth *et al*. 2007; Kishimoto *et al*. 2006). Studies that were conducted by Ali *et al*. (2006) reported that MJ induce oxidative stress by an increase in lipid peroxidation and subsequent increase of O_2^-. Another interesting discovery was the decrease in CAT activity following the application of MJ to the roots of *Panax ginseng* (Ali *et al*., 2006). LOX also produces various other signalling agents such as ethylene (ET) and SA in response to biotic stresses such as the tobacco mosaic virus (Enyedi *et al*., 1992; Klessig and Malamy, 1994). SA is also widely known for its nematicidal properties (Abd-alla *et al*., 2013; Maheshwari and Anwar, 1990). Furthermore, a study conducted by Smith-Becker *et al*. (1998) on *Pseudomonas syringae* pv. *syringae* inoculated leaf material of cucumber plants confirmed that it signalled the production of phenylalanine ammonia lyase (PAL). During the latter study, an increase in SA activity commenced in phloem fluids of the host plant at approximately the same time PAL activity increased. Ultimately SA activity continued to increase even when *Pseudomonas syringae* pv. *syringae* inoculated leaves were removed from the plant (Smith-Becker *et al*., 1998). Another study by Vasyukova *et al*. (2007) found *M. incognita* inoculated resistant

tomato induce PAL in leaves, stems and roots while SA level in the leaves were mostly unchanged, root levels doubled.

The knowledge that CAT is a H_2O_2 detoxifying enzyme of which the activity is inhibited during unfavourable conditions experienced during pest/disease-resistant crop host plant interactions, is an additional useful tool to identify GHPR (Klessig *et al.* 2000). Molinari (2001) first reported that CAT was induced in RKN-resistant tomato leaf samples, but that the opposite was true for roots as a result of recent studies (Molinari and Abd-Elgawad, 2007). In this study, however, CAT activity in leaf samples of the *M. incognita*-resistant cultivar LS5995 were substantially reduced by 35.6 % in J2-inoculated plants compared to those of the J2-free plants. In leaf samples of the susceptible cultivars, Egret and Dundee, CAT was, however, reduced to a lesser extent that ranged from 6 to 26 %, while the CAT activity in the leaves of J2-inoculated plants of the highly susceptible cultivar LS6248R was substantially increased, by 29.3 %. Therefore, it could be proposed that the latter highly susceptible cultivar could potentially allow for the more effective metabolism of H_2O_2 and substantial deactivation of peroxide radicals, resulting in a more favourable environment within the host plant for development and reproduction of *M. incognita* life stages compared to those in the resistant cultivars. These suggestions are supported by reports on the inhibition of CAT in roots of RKN-resistant tomato cultivars (Mohammed *et al*., 1999; Molinari, 1999; Molinari, 2001) as soon as 24 h after J2 inoculation. In contrast to CAT data obtained in leaves of *M. incognita*-resistant cultivars after J2 inoculation, data recorded for roots of the same resistant cultivars were, however, inconclusive. This was due to negative CAT values being recorded for cultivar Egret as well as large standard deviations recorded for cultivars PHB95Y20 and Egret in the nematode-inoculated samples. Personal communication with Dr. Sergio Molinari (CNR, Istituto per la Protezione delle Piante, Bari, Italy; 26 February 2013) revealed the following actions should be implemented to improve CAT analyses in future research:

- the use of young soybean seedlings since enzyme activity, including CAT, decrease with increasing plant age and this could vary between crops,
- CAT enzyme extracts should be ultra-filtered at a 10,000 molecular weight cut-off and

- phenols should be removed from CAT enzyme extracts since such chemical substances markedly inhibit activity of this specific enzyme and can adversely affect the data.

The refinements indicated above should be incorporated into the protocol that has been used during this study to facilitate these recommendations and optimise the use of CAT in future experiments to serve as a tool to identify GHPR to RKN in soybean.

Ultimately, enzyme data gathered as a result of this study shed more light on the GHPR mechanisms that protect *M. incognita*-resistant soybean cultivars against RKN, as well as the biological pathways that contribute to such resistance. It is important to note that the *M. incognita*-resistant and susceptible cultivars that were used during this study exhibited different levels of GHPR or susceptibility to this pathogen. This can serve as an explanation for activity of the three respective enzymes being increased in J2-inoculated root and/or leaf samples of some of the resistant cultivars but not in those of other. Although thus not conclusive at this stage, the data that were generated during this study can in future be used to develop enzyme markers to identify RKN resistance in soybean and other crop cultivars. However, it should be emphasised that enzyme studies can only partially explain the GHPR mechanisms that are employed by different host plants against RKN. Therefore, more experiments should be conducted to gain a more comprehensive understanding of the specific role of enzymes in this regard. Future studies on soybean should for example include how, when and where other important enzymatic pathways are involved in GHPR against RKN. In addition, the involvement of other important enzymatic pathways such as PAL and APX, as well as signalling agents such as ROS, SA, JA and H_2O_2 should be determined to confirm the findings of the current study. It would also be valuable to confirm whether JA/MJ serves as a CAT inhibitor in soybean and what the exact functions of GPX are. Also, split-root experiments can, for example, be performed to determine whether SAR is responsible for low levels of RKN infection in soybean cultivars that have been identified with resistance to such pathogens. Ultimately, enzyme studies should be done using multiple pathogens such as PPN and for example mites or fungi, which feed on different plant organs, to determine the extent of SAR after nematode inoculation.

The ultimate outcome of this particular study, however, illustrated that the use of the three enzymes used in this study generally confirmed host suitability data (see Chapter 2) that categorised the host status of the cultivars used. However, LOX data obtained for Egret that

has been identified with resistance to *M. incognita* earlier (Fourie *et al*., 2006), but conversely as a susceptible host during host suitability studies by Venter *et al.* (2013), indicate that this specific enzyme pathway might be involved predominantly in the resistance mechanism of this cultivar. Furthermore, the use of enzymes to identify/verify GHPR in soybean cultivars is not necessarily a quicker, more accurate or cost-effective way. In addition, a particular high level of expertise in terms of plant physiology methodology is a prerequisite to ensure that such experiments are conducted accurately. Enzyme activity can, however, be used as a complementary tool to assist researchers to identify and verify RKN resistant crop varieties such as soybean if the infrastructure needed for such an intervention is available.

CHAPTER 4

Cellular changes in *Meloidogyne incognita*-infected resistant and susceptible soybean cultivars: A comparative study

4.1 Introduction

Meloidogyne incognita (Kofoid and White) is a major pest of soybean in South Africa (Fourie *et al*., 2001; Liebenberg, 2012). Due to the wide geographic distribution, high reproduction capability and damage potential of this root-knot nematode (RKN) pest it is quintessential that research in this regard should receive priority (Fourie *et al*., 1999; Fourie *et al*., 2001). RKN are sedentary endoparasites (Abad *et al*., 2009) and thus spend most of their active life cycle in roots of a host plant such as soybean (*Glycine max* (L.) Merr) (Liebenberg, 2012). These nematode pests establish complex and long-lasting interactions in roots of susceptible soybean plants, causing major damage to the vascular system of its host by altering it into a permanent feeding site, referred to as a giant cell (GC) complex (Jones and Goto, 2011). Moreover, the presence of visible characteristic galls on soybean roots, ultimately indicates RKN are parasitising such crops (Liebenberg, 2012).

During a compatible interaction between RKN and susceptible soybean host plants the infective second stage juvenile (J2) finds and penetrates the roots of its host just behind the root tip (Caillaud *et al*., 2008). The J2 then migrate intercellularly from the infection site toward the root apex and then turn around to establish an optimal feeding site in the form of a multinucleate GC complex in the vascular cylinder (Caillaud *et al*., 2008). Ultimately a mature, egg-producing female will develop and feed on the GC to sustain a high fecundity rate (Pedrosa *et al.*, 1996).

GC are initiated by secretory proteins that are injected through the stylet of the RKN while they are feeding on the roots of their host (Caillaud *et al*., 2008). Such RKN-infected cells subsequently become multinucleate after nuclear division occurred, increasing in size and content (Caillaud *et al*., 2008). During the latter process no new cells are formed and no cell wall degradation occurs, instead they increase in size and nuclear material. Metabolically active GC produce large amounts of proteins that are consumed by feeding RKN J2 and/or females (Mitkowski and Abawi, 2003). These GC also serve as highly metabolic nutrient sinks to facilitate development and reproduction of the parasitising RKN (Hussey, 1985; Davis and Mitchum, 2005; Caillaud *et al*., 2008; Jones and Goto, 2011).

In susceptible soybean cultivars GC are characteristically larger (in some cases up to 400 times) (Caillaud *et al*., 2008) than the non-infected surrounding vascular cells. Except for being multinucleate, they also consist of thick cell walls, with many cell wall ingrowths that enhance uptake of nutrients, typical of transfer cells (Jones, 1981; Jones and Goto, 2011). Another characteristic feature of the GC is a dense cytoplasm in which an increased amount of organelles such as well-developed Golgi apparatus, smooth endoplastic reticulum, mitochondria, plastids and ribosomes are suspended (Caillaud *et al*., 2008; Jones and Goto, 2011). Furthermore, the central vacuole, that is present in normal plant cells, usually disappears/degenerates in the GC and gives rise to many small vacuoles (Bird, 1974; Caillaud *et al*., 2008; Jones and Goto, 2011).

In contrast, the reaction of a poor-host and/or soybean cultivar that exhibit genetic host plant resistance (GHPR) to RKN infection and parasitism, is directly correlated to the host plant's effect on nematode development and subsequent reproduction of the RKN (Taylor and Sasser, 1978). GHPR, therefore, does not allow any RKN reproduction or low levels thereof, whereas susceptible plants allow optimal development and reproduction of these pests (Williamson and Roberts, 2009).

Ultimately, GHPR can be divided into two functional types, pre-infectional, antixenosis resistance (passive) and post-infectional, antibiosis resistance (active) (Giebel, 1982; Horber, 1980; Painter, 1951; Walters, 2010). Passive, antixenosis resistance (Kogan and Ortman, 1978) is highly dependent on physical and also chemical barriers that are exhibited by the host plant and aim to deter and/or prevent RKN J2 penetration and/or negatively affect the development and reproduction of these pests (Giebel, 1982; Walters, 2010).

Active, antibiosis resistance (Painter, 1941) on the other hand, is characterised by successful infection of the host plant by RKN J2, followed by non-optimal development and reproduction of the subsequent development stages of these pests (Trudgill, 1991). This type of resistance is also most commonly known as post-infectional resistance and is generally dependent on morphological or biochemical factors, but also the response of the host to RKN J2 infection (Giebel, 1982; Veech, 1982; Walters, 2010). Post-infectional resistance to *M. incognita* often involves a hypersensitive reaction (HR), however, timing and localisation thereof varies with regard to the level of GHPR that is exhibited by the particular cultivar (Williamson and Kumar, 2006). This subsequent non-optimal development of the GC may lead to an increase of male RKN and reduction in reproducing females (Dropkin and Nelson,

1960*;* Walters, 2010). This has also been confirmed in a study done by Fourie and co-workers (2013b), who reported an increase of *M. incognita* J3 and males as a result of the resistance exhibited by soybean cultivar LS5995, while an increase in adult females numbers were recorded in roots of the susceptible cultivar Prima2000. Generally, *M. incognita* males migrate out of the roots of host plants and play no role in reproduction (Caillaud *et al*., 2008).

Dropkin and Nelson (1960) classified four morphologically different types of GC that have been reported as a result of *M. incognita*- infection in soybean roots, namely:

Type 1: Characterised by HR of cells surrounding the anterior region of the infecting J2 with no further development of the nematode. Necrosis is evident with minor enlargement or incorporation of cells into the GC.

Type 2: Moderate fusion of cells to form GC, however they remain relatively small. Such GC also contain an abundance of cell wall inclusions deposited in the cytoplasm, varying in shape and staining positive for cellulose and pectin. Some HR may, however, also be present in Type 2 GC.

Type 3: These GC are large and almost equal to the size of those that are characterised as Type 4. However, cell walls differ from those found in Type 4, since they lack the dense aggregations of granular cell wall material characteristic of Type 4. Type 3 GC also has a highly vacuolated cytoplasm, which appears to be less dense than in the optimal Type 4 GC. Nuclear clusters such as those that are present in Type 4 GC may also be present in GC characterised as Type 3.

Type 4: The GC that are grouped as Type 4 are considered to be optimal for RKN development and reproduction. In soybean, the mature GC complex usually consists of between five to nine multinucleate GC, with a dense cytoplasm that contain few cell wall material inclusions. The cell wall comprises of two clearly distinguishable parts, namely an outer layer which may contain areas resembling pit fields and is also first to thicken in early GC development. The inner layer of the GC varies in thickness and has a beaded appearance which is aggregated symmetrically on both sides of adjacent GC.

Types 1, 2 and 3 GC have previously always been associated with poor RKN development, reproduction as well as reduced fecundity. Conversely, type 4 is associated with optimal RKN development, reproduction and fecundity (Dropkin and Nelson, 1960).

Recently, Fourie *et al*. (2013b) published findings on the penetration and subsequent development of the different life stages of *M. incognita* in the South African soybean cultivars Prima2000 (susceptible) and LS5995 (resistant). Histopathological observations in the latter study confirmed optimal development of GC that facilitated J2, J3 and J4 development up to mature, reproducing females in roots of Prima2000. However, in roots of LS5995 juvenile development as well as reproduction and fecundity of mature females were restricted as a result of sub-optimally developed GC in association with HR (Fourie *et al*., 2013b). Ultimately, Fourie *et al*. (2013b) suggested that one new characteristic was associated with GC in the resistant cultivar LS5995. This finding indicated that the thickness of cell walls of sub-optimally developed GC in roots of LS5995 varied substantially and ranged from thin as reported for soybean by other authors (Dropkin and Nelson, 1960; Pedrosa *et al.,* 1996) to thick (Fourie *et al*., 2013b).

The aims of this study were to investigate whether differences in GC formation that are induced by *M. incognita* J2 in resistant and susceptible soybean cultivars complement results obtained in screenings- and enzyme experiments in which resistant and susceptible cultivars were identified.

4.2. Materials and methods

4.2.1. Soybean cultivars

The *M. incognita*-resistant cultivars LS5995 (representing the resistant standard) (Rf = 0.02) and GCI7 (Rf = 0.01) together with the susceptible cultivar Egret (Rf = 4.42) and the highly susceptible cultivars Dundee (represented the susceptible standard) (Rf = 34.90) and LS6248R (Rf = 68.35) were selected for this study as a result of the outcome of the screenings- and enzyme experiments (see Chapters 2 and 3). Seed of the latter five cultivars used in this study were supplied by the germplasm bank of the Agronomy Unit of the Agricultural Research Council – Grain Crops Institute (ARC–GCI) in Potchefstroom, South Africa.

4.2.2. Greenhouse experiment

Soybean were grown in a glasshouse with a 14L:10D photoperiod and ambient temperature range of 23 ± 1ºC (minimum) and 26 ± 1ºC (maximum) that were maintained throughout the 30-day duration of the experiment. Five seeds of each cultivar were planted in plastic pots (4,000 cm^3 capacity) containing Telone II-fumigated (equivalent of 150 ℓ/ha; 1,3 dichloropropene a.s.) sandy loam soil (5.3 % clay, 93.6 % sand, 1.1 % silt & 0.47 % organic matter content). Seedlings of each of the four cultivars were thinned to one per pot five days after emergence (DAE). The soil pH (H_2O) was 7.47. Addition of nutrients were done once weekly by administering 250 mℓ of a freshly prepared Hoagland's solution (Arnon and Hoagland, 1950) to each potted soybean plant, while the plants were additionally watered two times per week using tap water.

Bradyrhizobium japonicum bacteria inoculation, at the same dosage as used for the screenings and enzyme experiments (see Chapters 2 and 3), was done at planting to optimise nitrogen fixation. *M. incognita* eggs and J2 were obtained from the same population that was used for the screenings and enzyme experiments (see Chapters 2 and 3). Approximately 5 000 eggs and J2 were inoculated on the roots of each soybean seedling 12 DAE as described in Chapter 2 (paragraph 2.2.3). Roots of each soybean plant for each cultivar were collected 30 days after inoculation (DAI) by removing it from the aerial plant parts, washed free of adhering soil particles and prepared for microscopic observations as described in paragraph 2.2.4.

4.2.3. Preparation of *M. incognita*-infected root material for light- and transmission electron microscopy (TEM) studies

Root systems of the different cultivars were inspected under a stereomicroscope to accurately identify galled areas that represented the areas of parasitism by *M. incognita* females. Small sections (< 5 mm) of the RKN-galled root areas were excised using a scalpel and immediately fixed in Todd's fixative (pH 7.2) (Todd, 1986) for 12 hours (h) at 4 ºC. After 12 h the fixative was replaced with 0.05 M sodium cacodylate buffer in which the root samples were washed three times for 15 min each using a slow rotator at 25 rpm. The galled root pieces were then post-fixed in 1 % osmium tetroxide for 1 h and again washed three times with the cacodylate buffer for 15 min each. Dehydration was done by exposing the galled root material to an ethanol series of 50, 70, 90 and 100 % for 15 min each. The galled root

pieces were then saturated with LR® white resin and each individual piece then placed in a resin filled gelatine capsule and polymerised in an oven at 70 °C for at least 12 h (O'Brien and McCulley, 1981).

Under a stereomicroscope capsules containing the galled root pieces were cut into trapezoid shaped pyramids to ensure optimal positioning of the mature, egg-laying *M. incognita* females for sectioning using razor blades. Subsequently, an Ultracut Microtome was used to prepare ultra- (90-95 nm) and semi-thin (0.5 - 1 µm) root sections from each of the galled pieces. Both transverse and longitudinal sections were made of galled root pieces. The semi-thin sections were heat-fixed on individual microscope slides and stained with 0.5 % aqueous toluidine blue, followed by 0.05 % neofuchsin. Each section that was prepared for each of the six replicates of the four cultivars was examined and photographed using a Leica DM light microscope at 100, 200, 400 and 1000x magnification. If the section contained GC and the head of *M. incognita* females, the same capsule from which it was prepared were again fitted to the Ultracut Microtome and ultra-thin sections then cut and prepared for transmission electron microscopy (TEM) also. The ultra-thin sections were stained with a 2 % aqueous uranyl acetate solution and lead citrate (Reynolds 1963). The ultra-thin sections were examined with a FEI Tecnai G2 high resolution TEM at 100 kV.

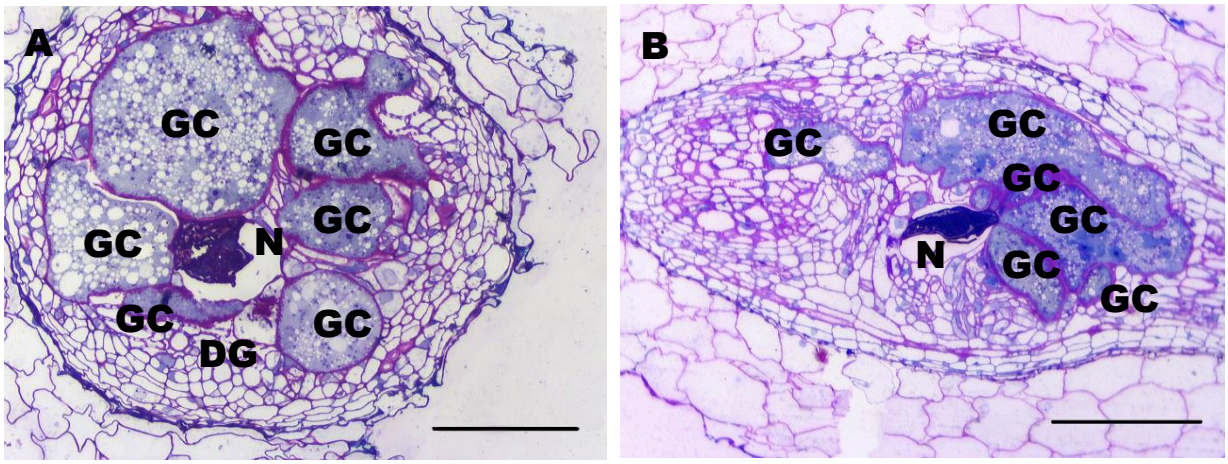
4.2.4. Experimental design

The five cultivars used during this study were arranged according to a complete randomised block design (CRBD), including six replicates of each cultivar (Appendix 4). To ensure that plants from all replicates were equally exposed to all possible variables (mainly temperature and light) that could influence their growth, as well as the development and reproduction of the RKN the pots in each replicate were rotated every second week. As the aim of the study was to qualify morphological differences in resistant and susceptible GC, no statistical analysis could be done.

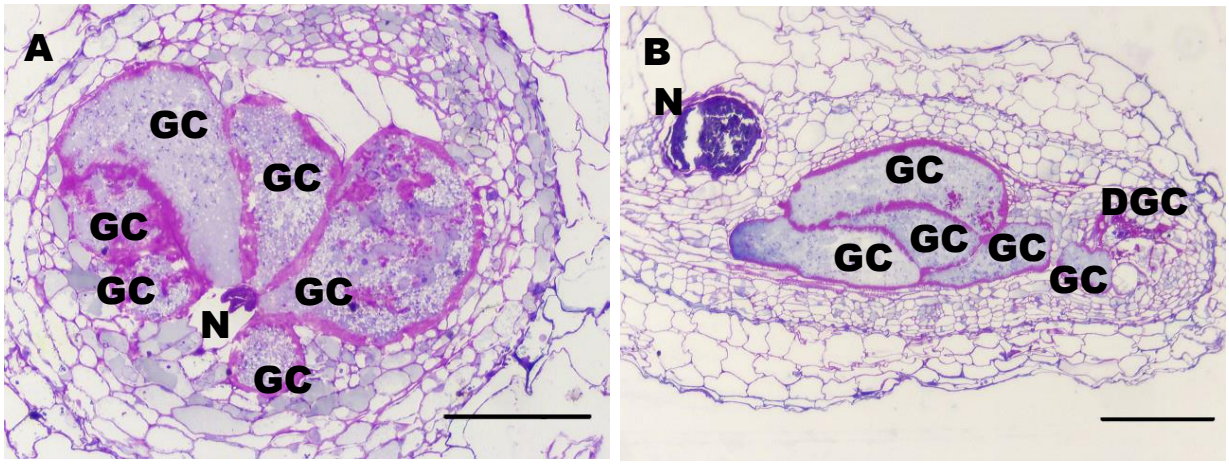
4.3. Results

Results obtained were consistently observed in all six replicates of each cultivar. In roots of the susceptible (Egret, Dundee and LS6248R), as well as the resistant (LS5995 and GCI7) cultivars GC were formed in the vascular cylinder.

In terms of GC development and appearance, distinct differences between those present in roots of susceptible (Figs. 1 - 6) and resistant (Figs. 7 - 10) cultivars were visible 30 DAI. Although groups of six to eight optimally-developed GC were present (Figs. 1 A, B & 2 A, B) in roots of the highly susceptible cultivars Dundee and LS6248R, some GC were already visibly degenerating while others were empty (Figs. 1 A & 2 B). Ultimately, GC in roots of the highly susceptible cultivars were generally larger (Figs. 1 A, B & 2 A, B) than those in the resistant ones (Figs. 7 A, B & 8 A, B). This scenario also applied to uninfected cells that were adjacent to GC in roots of the highly susceptible cultivars Dundee and LS6248R (Figs. 1 A, B & 2 A, B) compared to those in roots of the resistant cultivars LS5995 and GCI7 (Figs. 7 A, B & 8 A, B). Furthermore, GC in roots of the susceptible cultivars Dundee (Figs. 1 A & B) and LS6248R (Fig 2 A & B) largely replaced the vascular tissue and were associated with hyperplasia and hypertrophy of the cells surrounding them.

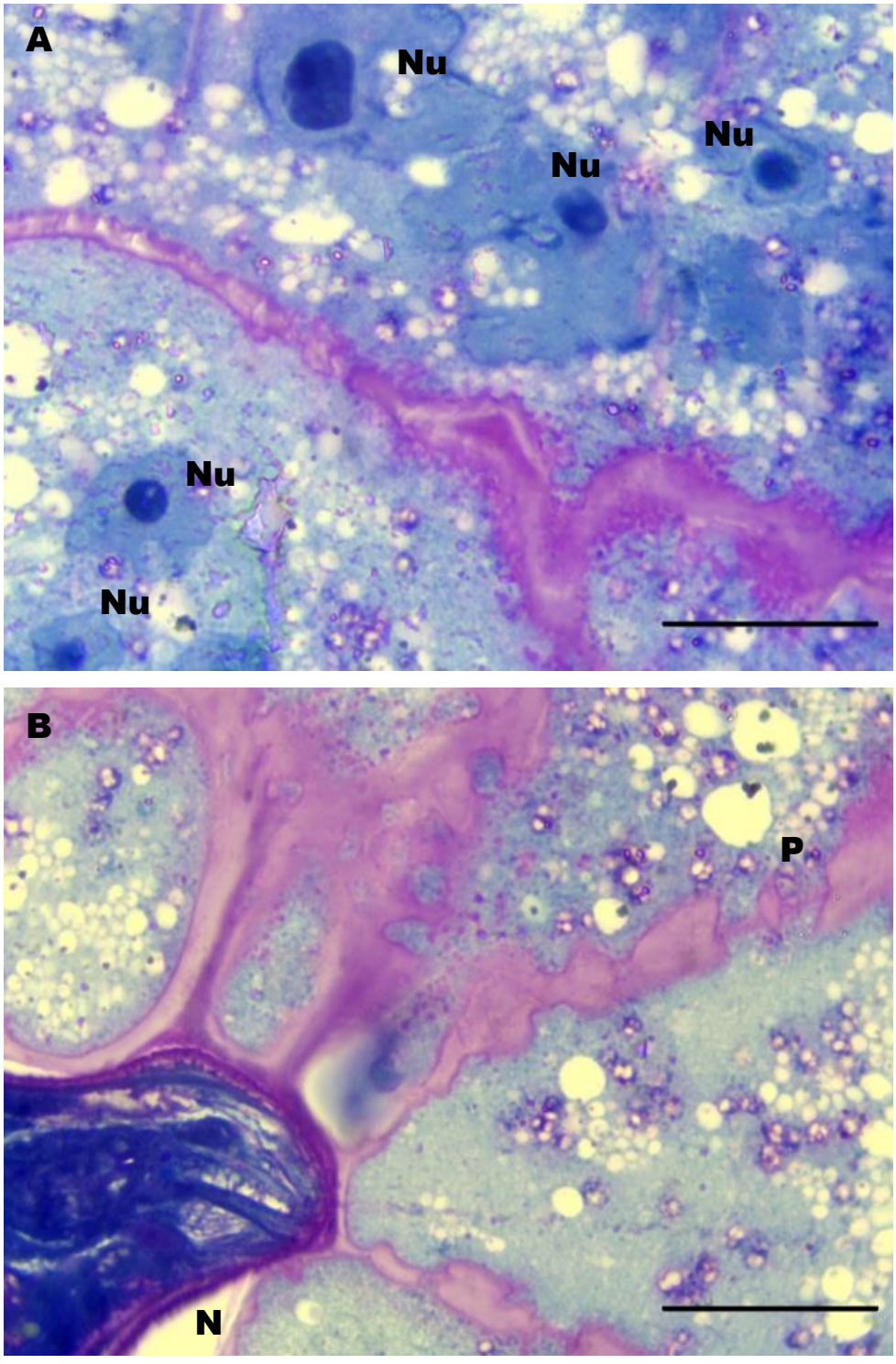


Figures 1 – Giant cells in the vascular cylinder in the roots of the highly susceptible cultivar Dundee, 30 days after inoculation with second-stage juveniles of *Meloidogyne incognita*, indicating (A) a transverse light micrograph and (B) a longitudinal light micrograph. N = nematode, DGC = degrading giant cell, GC = giant cell. Bars: A – 100 µm, B – 200 µm.



Figures 2A & B - Giant cells in the vascular cylinder in the roots of the highly susceptible cultivar LS6248R, 30 days after inoculation with second-stage juveniles of *Meloidogyne incognita*, indicating (A) a transverse **light** micrograph and (B) a longitudinal **light** micrograph. N = nematode, DGC = degrading giant cell, GC = giant cell. Bars: A – 100 μm, B – 200 μm.

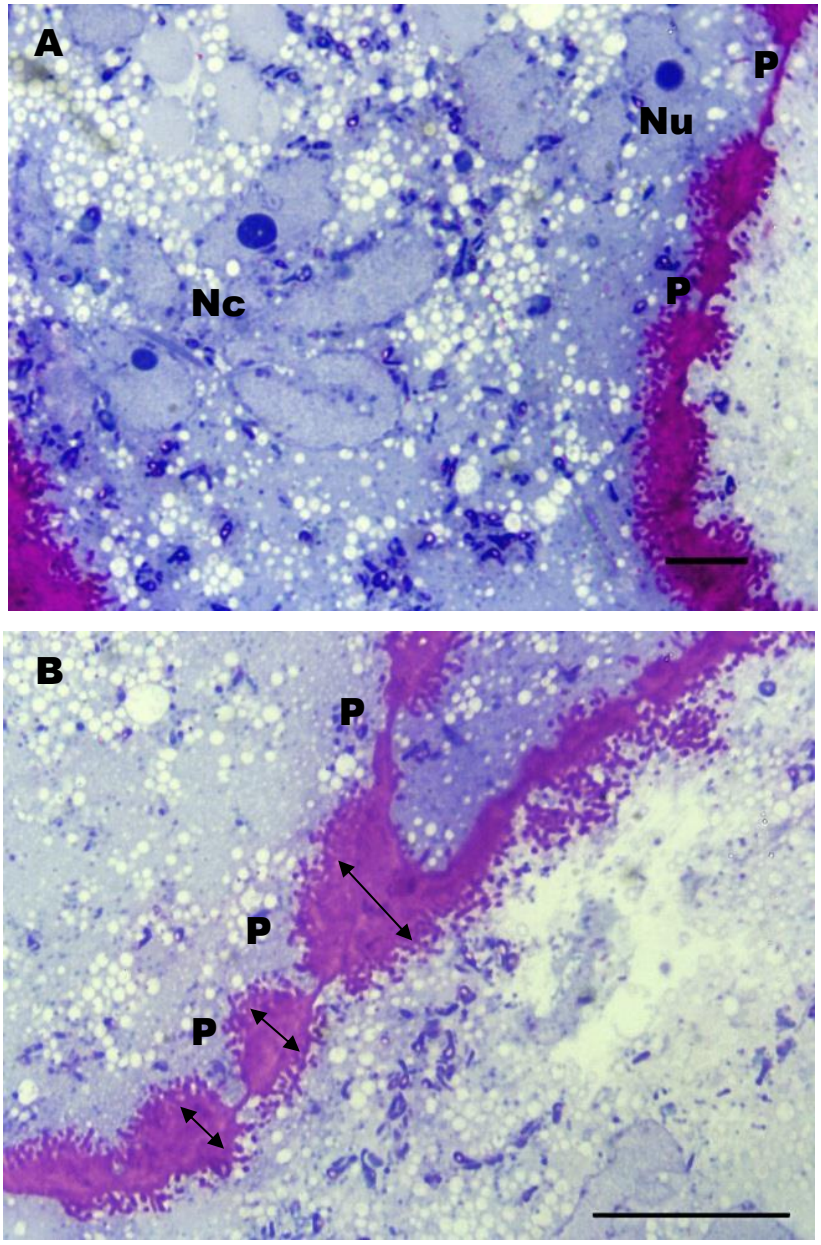
In roots of Dundee GC were represented by large, multinucleate cells with cell walls that varied in thickness. In these GC, minor aggregations of granular material were deposited on the cell walls. The latter GC were also highly vacuolated and contained a dense cytoplasm in which hypertrophied nuclei, containing large nucleoli (Fig. 3 A) were suspended.



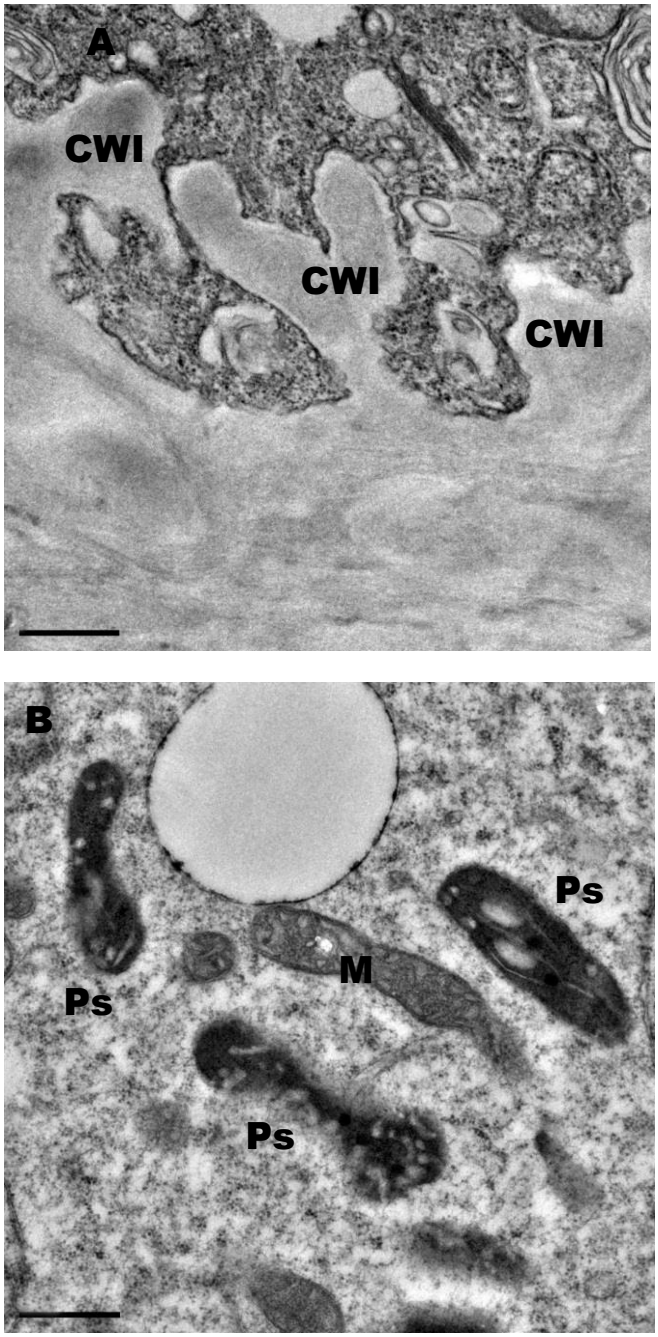
Figures 3A & B - Longitudinal light micrographs demonstrating giant cells in mature vascular cylinders in the roots of the highly susceptible cultivar Dundee, 30 days after inoculation with second-stage juveniles of *Meloidogyne incognita*, indicating (A) hypertrophied nuclei, containing large nucleoli and (B) abnormal pits or pit fields. N = nematode, Nu = nuclei, P = pits. Bars: 20 μm.

GC in roots of LS6248R were also observed to be large (Figs. 2 A & B) and multinucleate (Fig. 4 A) but contained a more dense cytoplasm in which the proliferation of the smooth

endoplasmic reticulum (ER), ribosomes, mitochondria (Fig. 5 B), Golgi apparatus and plastids were clearly visible. Cell walls of these GC were thick (Fig. 5 A) and also characterised by dense aggregations of granular cell wall material that were deposited on adjacent sides of the cell wall (Figs. 4 B) as was also evident for GC in roots of cultivar Dundee (Fig. 3 B) but to a lesser extent. Such cell wall aggregations gave a beaded appearance to cell walls of such GC. Furthermore, what appeared to be abnormal pits were also present in both cultivars (Figs. 3 B, 4 A & B).

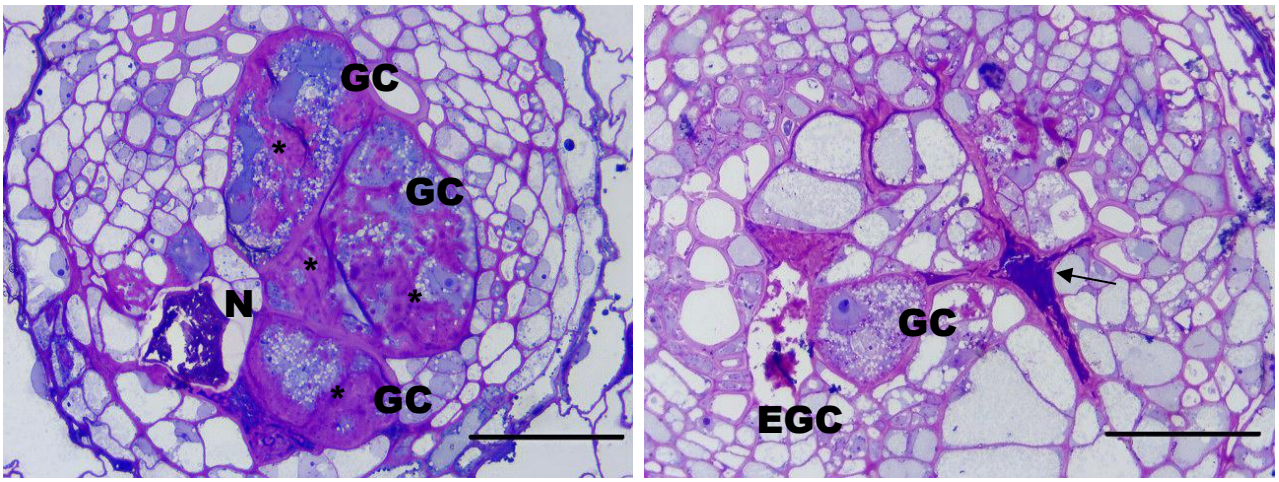


Figures 4A & B - Longitudinal sections demonstrating giant cells in mature vascular cylinders in the roots of highly susceptible cultivar LS6248R, 30 days after inoculation with second-stage juveniles of *Meloidogyne incognita*, indicating (A) hypertrophied nuclei, containing large nucleoli in a clustered appearance and (B) abnormal pits or pit fields with dense aggregations of granular cell wall material. Nc = nuclei cluster, Nu = nuclei, P = pits, Arrows = aggregations. Bars: A – 10 µm, B – 20 µm.



Figures 5A & B - Transmission electron micrographs of transverse sections of the highly susceptible cultivar LS6248R, 30 days after inoculation with second-stage juveniles of *Meloidogyne incognita*, indicating (A) abnormally thick cell wall, cell wall ingrowths and a dense cytoplasm. (B) Mitochondria of highly metabolic giant cells (GC). CWI = cell wall ingrowths, M = mitochondria, Ps = peroxisomes. Bars: 5 μm.

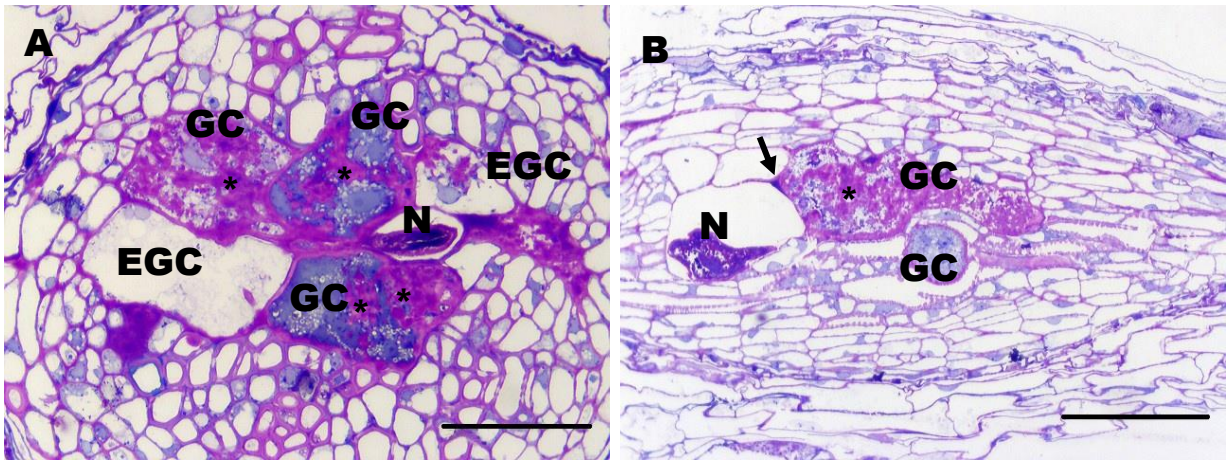
The susceptible cultivar Egret contained groups of three to five GC (Fig. 6 A) of which some were visibly degrading and/or empty (Fig. 6 B). These GC were generally larger than those found in roots of the resistant cultivars LS5995 and GCI7, but smaller than GC that were present in roots of the highly susceptible cultivars Dundee and LS6248R. Lacking the dense aggregations of granular cell wall material deposited on the cell walls, characteristic of the highly susceptible cultivars (Fig. 4 B), some GC did, however, contain some cell-wall inclusions deposited within the cytoplasm (Fig. 6 A). Ultimately, necrosis of some root cells adjacent to GC in roots of the susceptible cultivar Egret indicated a HR that is generally associated with resistant cultivars (see Figs. 7 and 9).



Figures 6A & B - Transverse light micrographs demonstrating giant cells in mature vascular cylinders in the roots of the susceptible cultivar Egret, 30 days after inoculation with second-stage juveniles of *Meloidogyne incognita*. N = nematode, EGC = empty giant cell, Arrow = necrosis, Asterisk = cell wall inclusions. Bars: 50 µm.

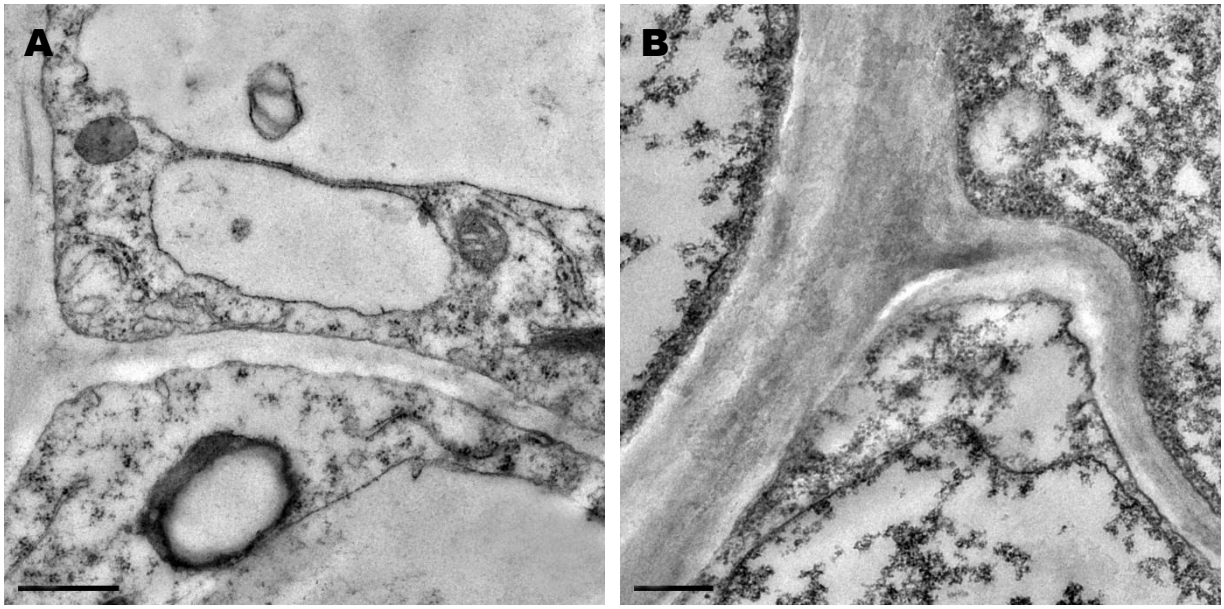
In the two *M. incognita*-resistant cultivars GC formation differed markedly from those in the susceptible hosts. The GC were generally characterised by HR with necrosis being visible (Figs. 7A, B & 9 A, B). In addition, the GC in the roots of both resistant cultivars were smaller and fewer in numbers, ranging between one to four in a group (Figs. 7 A, B & 9 A, B), than those present in roots of the susceptible cultivars (Figs. 1 A, B & 2 A, B). Furthermore, more GC in the two *M. incognita*-resistant cultivars were degenerated and/or empty 30 DAI (Figs. 7 A & 9 A, B) compared to the susceptible cultivars. In both resistant cultivars, all GC were underdeveloped, had irregular outlines and only occupied a limited area of the central vascular cylinder (Figs. 7 A, B & 9 A, B). The uninfected cells surrounding the GC in both resistant cultivars seemed normal in terms of their appearance, with limited hypertrophy and hyperplasia (Figs. 7 A, B & 9 A, B). Cytoplasm of GC in the resistant cultivars was generally filled with supposed cell-wall inclusions described by Dropkin and Nelson (1960), which complicated identification of cell organelles that were

suspended in the cytoplasm (Figs. 7 A, B & 9 A) and such cell wall inclusions generally filled the whole area of the cell (Figs. 7 A & B). Non-optimal GC within the roots of the two resistant cultivars also contained numerous small vacuoles between the cell wall inclusions.



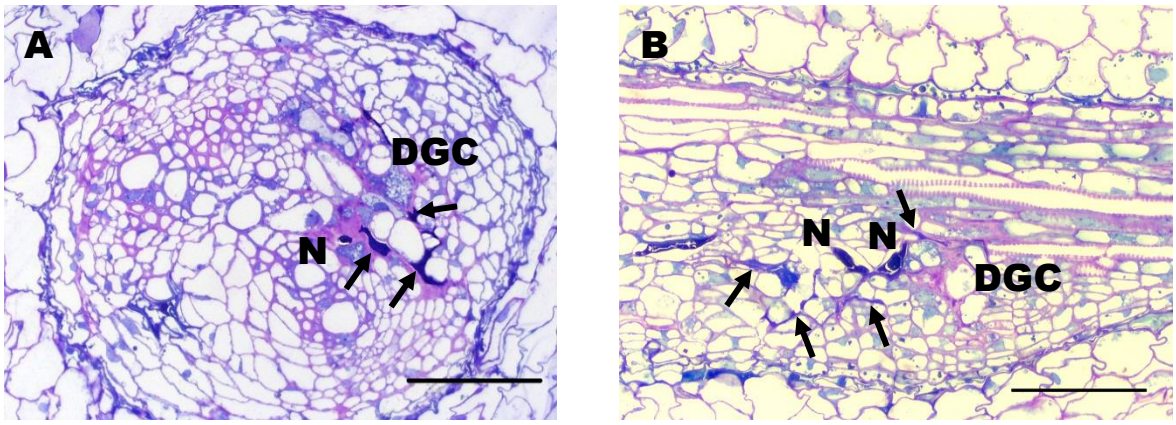
Figures 7A & B - Giant cells in mature vascular cylinders in the roots of resistant cultivar LS5995, 30 days after inoculation with second-stage juveniles of *Meloidogyne incognita*, indicating (A) a transverse section and (B) a longitudinal section. Arrow = necrosis, Asterisk = cell wall inclusions, EGC = empty giant cell, GC = giant cell, N = nematode. Bars: A – 50 µm, B – 100 µm.

In LS5995 all GC were generally smaller in comparison to those present in the two susceptible cultivars as mentioned above. However, some GC in roots of LS5995 were relatively large and contained multiple nuclei with enlarged nucleoli (Fig. 7 A). Cell walls of GC in LS5995 were generally thin with some areas of thickening and limited cell wall aggregations (Figs. 8 A & B), giving the cell walls a smooth appearance in comparison to the susceptible cultivars (Figs. 5 A), which had a beaded appearance. These GC were highly vacuolated with the cytoplasmic density, varying between cells (Figs. 8 A & B). Necrosis was present in the area of GC, generally around the anterior region of the head of the RKN female (Figs. 7 B & 9 A, B).

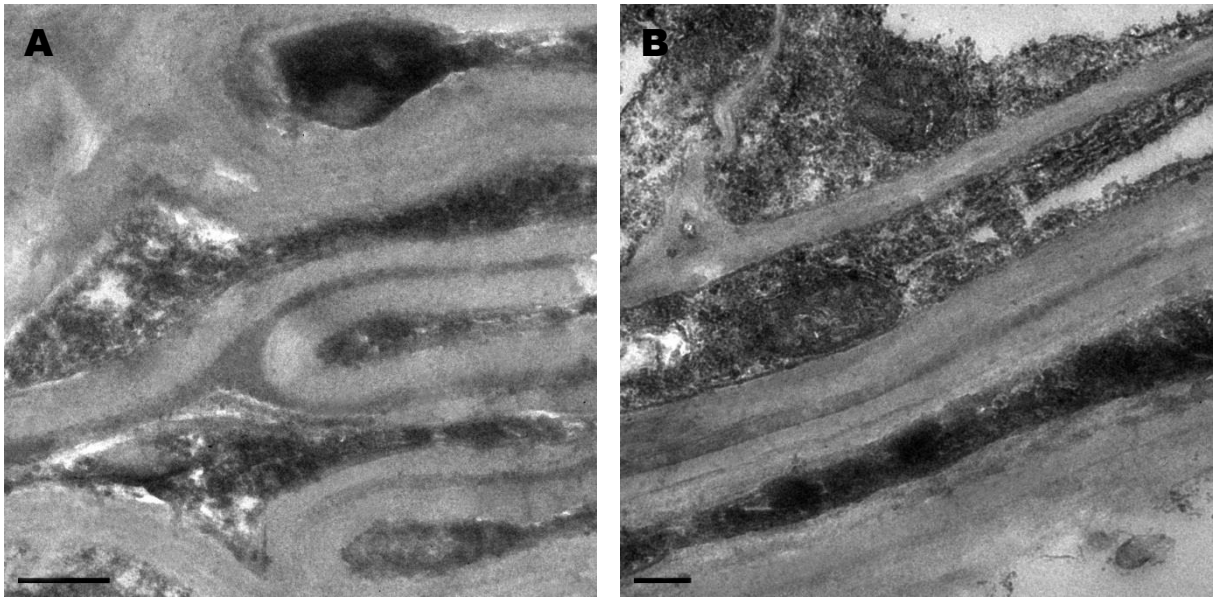


Figures 8. A & B - Transmission electron micrographs of transverse sections of the resistant cultivar LS5995, 30 days after inoculation with second-stage juveniles of *Meloidogyne incognita*, (A) indicating a much less dense cytoplasm and (B) a smooth cell wall with varying cell wall thickness. Bars: A – 10 µm, B – 5 µm.

In roots of the resistant cultivar GCI7, markedly smaller, irregularly shaped GC that contained many small vacuoles (Fig 9 A & B) were observed. Moreover, HR that was associated with large necrotic areas was present in the vicinity of sub-optimally developed GC (Fig 9 A & B). Ultimately, collapsed root tissues and thin cell walls (Fig 10A & B) of GC in roots of the latter resistant cultivar were observed.



Figures 9A & B - Giant cells in mature vascular cylinders in the roots of resistant cultivar GCI7, 30 DAI with second-stage juveniles (J2) of *Meloidogyne incognita*, indicating (A) a transverse section and (B) a longitudinal section. Arrow = necrosis, N = nematode, DGC = degenerating giant cells, GC = giant cell. Bars: 100 µm



Figures 10A & B - Transmission electron micrographs of transverse sections indicating collapsed giant cells of the resistant cultivar GCI7, 30 days after inoculation with second-stage juveniles of *Meloidogyne incognita*. Bars: A – 5 μm, B – 0.2 μm

4.4. Discussion

Light- and TEM observations showed that distinct differences in the appearance and development of GC in roots of the resistant cultivars LS5995 and GCI7 existed when compared to those that were present in roots of the susceptible cultivar Egret and the highly susceptible cultivars Dundee and LS6248R.

In the highly susceptible cultivars Dundee and LS6248R, GC that formed were typically associated with optimal development and reproduction of *M. incognita*. Dropkin and Nelson (1960) described such GC as Type 4 (large, dense cytoplasm, thick irregularly surfaced cell walls with characteristic aggregations), however Type 3 GC (large, highly vacuolated, but lacking the characteristic walls of Type 4 GC) were also present to a lesser extent. This is in agreement with reports by Dropkin and Nelson (1960) and Fourie *et al*. (2013b) who recorded optimally developed GC in roots of exotic and/or South African cultivars that were identified as being susceptible to populations of *M. incognita*.

Also present in GC of these two susceptible cultivars were what appear to be abnormal pits or pit fields that were connected with adjacent cells, facilitating intracellular communication and increased solute import (Hofmann *et al*., 2010), also described by Dropkin and Nelson (1960). These characteristics are common of metabolically active cells such as GC. These

cells have increased metabolism that is stimulated by the feeding RKN which are in need of nutrients and proteins to support their development and promote reproduction by the mature females (Caillaud *et al*., 2008; Jones, 1981).

The susceptible cultivar Egret mostly contained GC typical of Type 3, which are large but smaller than those typical of Type 4 (Dropkin and Nelson, 1960). These cells also did not contain the dense aggregations of cell-wall material on their cell walls, but rather cell-wall inclusions in the cytoplasm. Some of the GC recorded in roots of Egret represented those classified as Type 2, with visible necrosis of cells surrounding such GC (Fig. 6 B). These characteristics are typical of a non-optimal *M. incognita*-soybean host plant response and support findings by Fourie *et al*. (2006), who reported Egret to be resistant to this RKN. It is thus proposed that differences in greenhouse conditions and possibly the *M. incognita* population used could have contributed to the varying levels of resistance/susceptibility reported for Egret during this and the study of Fourie *et al*. (2006).

In contrast, the resistant cultivars LS5995 and GCI7 contained mostly Type 1 and 2 GC while a few Type 3 GC were also recorded. However, Type 2 GC were most abundant in LS5995 and is in agreement with previous studies that reported small, irregular GC that contained increased amounts of deposited cell wall material in the cytoplasm that retarded RKN feeding, development and reproduction (Dropkin and Nelson, 1960; Fourie *et al*., 2013b). These findings were also reported for *M. incognita* parasitising on cowpea (Singh *et al*., 1984) and soybean (Crittenden, 1958). The presence of thicker areas in cell walls of such sub-optimally developed GC also supported findings by Fourie *et al*. (2013b).

In roots of GCI7 more pronounced HR in the vicinity of GC was mostly associated with those classified as Type 1. The visible degeneration in the cytoplasm of affected cells and the restriction of this reaction to the area surrounding the head of the parasitising RKN is consistent with a HR to infection by these pathogens as were reported for tomato (Paulson and Webster, 1972; Bleve-Zacheo *et al.,* 1982) and soybean (Dropkin, 1969). However, even with these resistance responses it is evident that neither cultivar is immune to *M. incognita* as some J2 survive and become either males as recorded in a previous study on LS5995 (Fourie *et al*., 2013b), or develop to mature females as some eggs are produced on both LS5995 and GCI7 (Chapter 2, paragraph 3.1.2). These findings were supported by reports on HR in potato infected with *M. incognita* (Berthou *et al*., 2003; Kouassi *et al*., 2005).

In conclusion, optimal GC were formed in the roots of Dundee and LS6248R to support the nutritional needs of the developing *M. incognita* J2. This lead to significant increases in *M. incognita* populations 56 DAI (Chapter 2, paragraph 2.3.2). In contrast, although the susceptible cultivar Egret developed non-optimal GC in reaction to *M. incognita* parasitism it sustained increased development and subsequent reproduction rates for this RKN species. The resistant cultivars LS5995 and GCI7, however, did not facilitate optimal induction, development and maintenance of GC to successfully support developing nematodes.

These observations are in agreement with the hypothesis that a compatible interaction between the nematode and host plant supports optimal GC formation, while host-plant mechanisms are responsible for reduced RKN fecundity in incompatible interactions.

CHAPTER 5

General conclusions and future prospects

Results that emanated from this study can be concluded as follows:

i. Only the seven pre-released cultivars and LS5995 were identified with *M. incognita* resistance following traditional, greenhouse screening assays.
ii. The use of enzymes such as guaiacol peroxidase (GPX), lipoxygenase (LOX) and catalase (CAT) has potential to be used as a tool to identify, verify and quantify *M. incognita* resistance in soybean cultivars. Refinement of such protocols is, however, a prerequisite to optimise enzyme-based approaches in this regard.
iii. Distinct cellular changes in roots of *M. incognita*-resistant and –susceptible cultivars confirmed the host status of the selected cultivars used in histopathological investigations.
iv. Data obtained from enzyme assays, as well as histopathological investigations generally confirmed *M. incognita* resistance identified in the traditional, greenhouse screenings. Contradiction, however, existed for cultivar Egret that was identified as susceptible to *M. incognita* in screening assays. Enzyme and histopathological studies, however, suggested that the latter cultivar exhibited some resistance to this root-knot nematode (RKN) species. Explanations for the latter phenomena are that differences in greenhouse-temperature settings and the use of another *M. incognita* population could have contributed to such abnormalities. Furthermore, the integrity of the genetic background of cultivar Egret could have been compromised, since it was initially identified with resistance to *M. incognita* in an earlier study by Fourie *et al*. (2006).

Ultimately, the data that were obtained from this study are valuable and could be used to assist producers to grow soybean sustainably in soybean-based cropping systems where *M. incognita* poses a threat.

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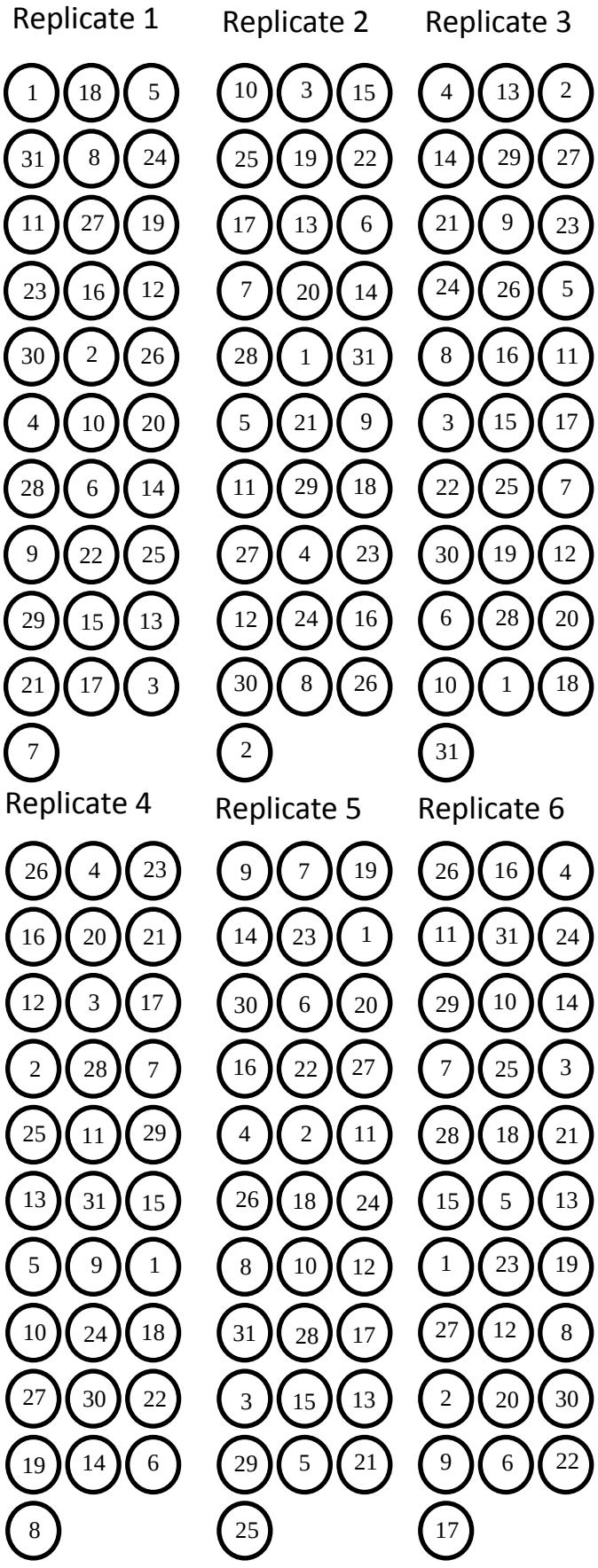
Appendix 1. Maturity group indices for the respective soybean cultivars.

Cultivar number	Name	Maturity group
1	**Sonop**	4.0
2	**LS6444R**	4.0
3	**PAN1454R**	4.4
4	**LS6146R**	4.4
5	**LS6248R**	4.8
6	**PAN1583R**	5.3
7	**Highveld Top**	5.0
8	**Knap**	5.0
9	**PHB95Y20**	5.2
10	**PHB95Y40**	5.4
11	**A5409RG**	5.4
12	**PHB95B53**	5.5
13	**PAN1666R**	6.0
14	**PAN1664R**	5.9
15	**LS6164R**	6.0
16	**Dundee**	6.0
17	**Marula**	6.0
18	**LS6161R**	6.3
19	**LS6150R**	6.5
20	**PAN737R**	7.0
21	**Egret**	7.0
22	**Heron**	7.0
23	**Ibis2000**	7.0
24	**LS5995**	6.0
25	**GCI1**	5.0
26	**GCI2**	6.0
27	**GCI3**	6.0
28	**GCI4**	6.5
29	**GCI5**	5.5
30	**GCI6**	5.5
31	**GCI7**	6.5

Appendix 2: Trial layout for Chapter 2
Complete randomised block design (CRBD)

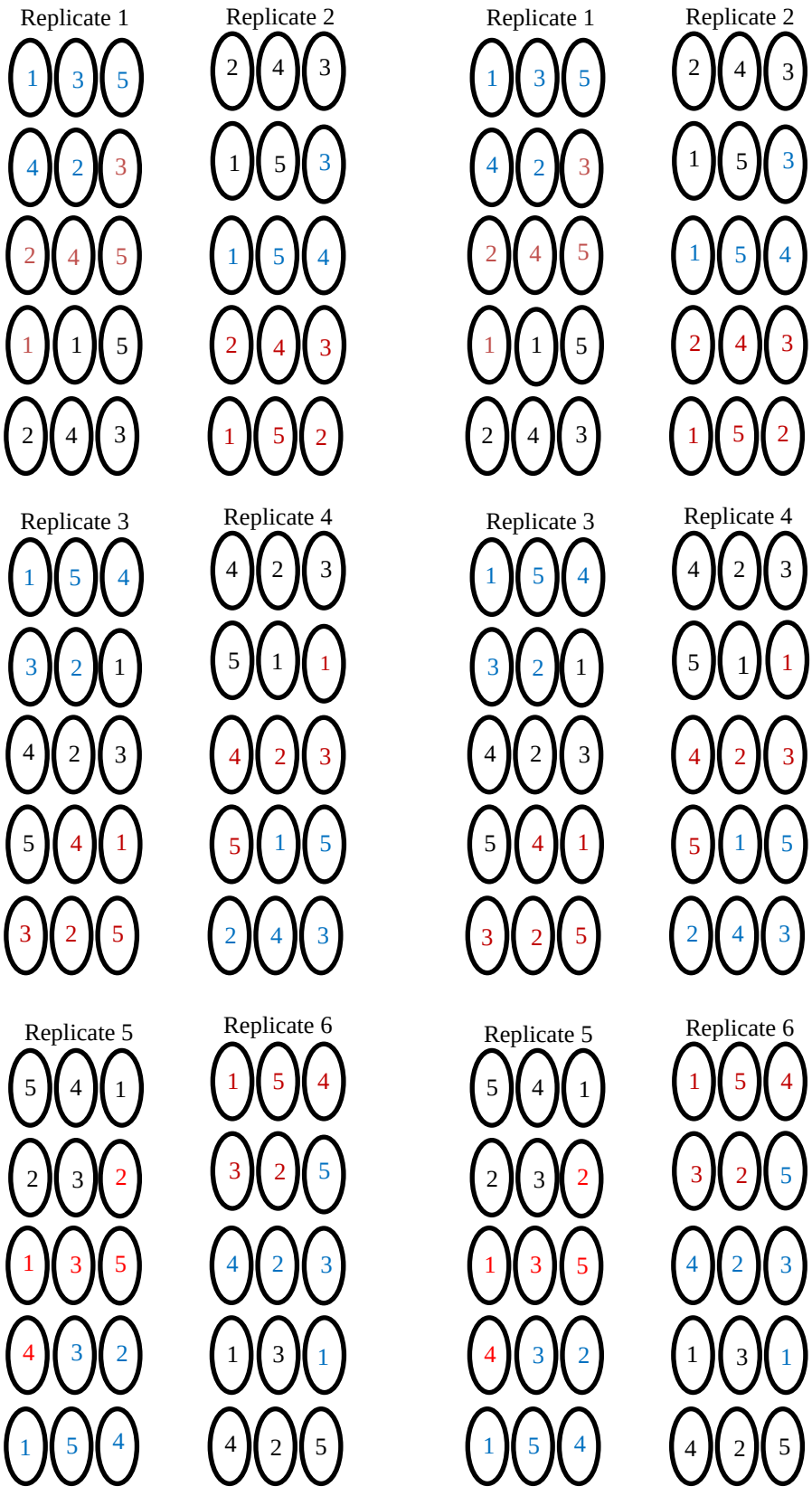


Cultivar number and name:

Cultivar number	Cultivar name
1	Sonop
2	LS 6444 R
3	PAN 1454 R
4	LS 6146 R
5	LS 6248 R
6	PAN 1583 R
7	Highveld Top
8	Knap
9	PHB 95 Y 20
10	PHB 95 Y 40
11	A 5409 RG
12	PHB 95 B 53
13	PAN 1666 R
14	PAN 1664 R
15	LS 6164 R
16	Dundee
17	Marula
18	LS 6161 R
19	LS 6150 R
20	PAN 737 R
21	Egret
22	Heron
23	Ibis 2000
24	LS 5995
25	GCI 1
26	GCI 2
27	GCI 3
28	GCI 4
29	GCI 5
30	GCI 6
31	GCI 7

Appendix 3: Trial layout for Chapter 3
Split-block design, with cultivars in each treatment randomised completely

Treatment 1: J2-inoculated Treatment 2: J2-free (non-inoculated)

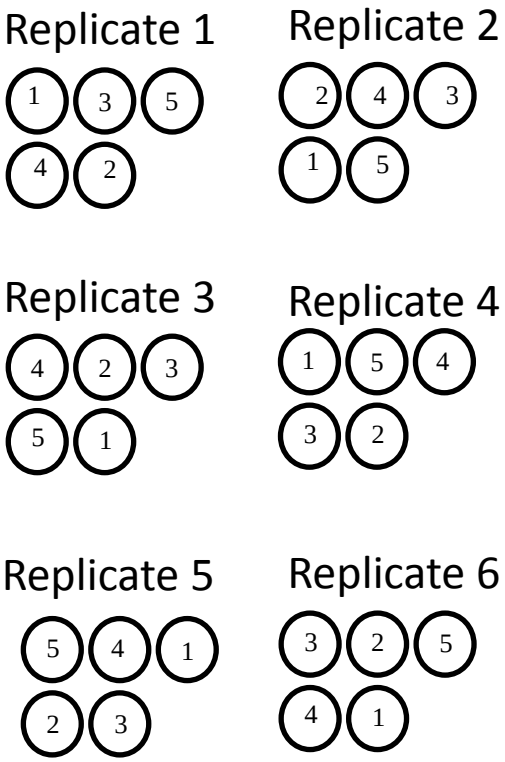


Cultivar number and name:

Cultivar number	Cultivar name
1	GCI7
2	LS5995
3	Egret
4	Dundee
5	LS6248R

Sampling times:
Red = 24 h after J2 inoculation
Blue = 48 h after J2 inoculation
Black = 120 h after J2 inoculation

Appendix 4: Trial layout for Chapter 4
Complete randomised block design (CRBD)



Cultivar number and name:

Cultivar number	Cultivar name
1	CGI7
2	LS5995
3	Egret
4	Dundee
5	LS6248R