School of Agriculture, Policy and Development

Aspects of the epidemiology of *Ditylenchus gigas* on faba bean and its interactions with *Botrytis fabae*

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Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

Anthony Isadeha

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Abstract

The supply of faba bean in UK is unreliable. This may be linked to many factors, including biotic pressure especially from pathogens. The study investigated the interaction between a biotrophic nematode (*Ditylenchus gigas*) and a necrotrophic fungus (*Botrytis fabae*). In faba bean, *B. fabae* causes chocolate spot disease while *D. gigas* causes stem lesions.

D. gigas is the dominant species of *Ditylenchus* on faba bean in the UK. Its spread is controlled in part by certification schemes. To understand how fast isolated infections of *D. gigas* evading certification will spread spatially, field experiments building on the work in other species of *Ditylenchus* were conducted over two seasons. These suggest that spatial spread of the nematode on beans through the growing season is dependent on distance of inoculum from the plant, the orientation of the inoculum from the plant and environmental factors in the field. Nematodes spread from a centrally pre-inoculated plant to a distance of 1.6 m within a season.

B. fabae often co-exists in the presence of *D. gigas* and therefore glasshouse investigations were designed to test the hypothesis that *B. fabae* (as a necrotroph) will decrease susceptibility to *D. gigas* (a biotroph) and together they will reciprocally affect each other's population density and reduce the productivity of faba bean. An initial experiment involved nematodes alone, while a second experiment involved *D. gigas* and *B. fabae* co-inoculations. Similar data from the two experiments were pooled together for analysis using the mixed model for multiple experiments in GenStat. Antagonistic interaction on the population of *D. gigas* was observed, as the number of nematodes extracted from lone inoculation of *D. gigas* was more than those from co-inoculation of the two organisms, suggesting an induced response. However, a synergistic interaction was observed on grain yield, as the reduction in grain yield was more with co-inoculation than lone inoculation of either organism.

In the light of work on induced defence mechanisms to plant pathogens and pests, the hypothesis that the order of infection (i.e., whether *D. gigas* infects before *B. fabae* or vice versa) will have an effect on induced resistance and affect crop production was then tested. Potted faba bean (cv. Fuego) were first inoculated with either *B. fabae* or *D. gigas* at 2 weeks after planting. A second inoculation was done 2 weeks after the first one. The second *B. fabae* inoculation was applied on the upper leaves with protection to prevent spill over to the previously inoculated leaves. Inoculation of *B. fabae* did not induced defence response

against *D. gigas*. However, pre-inoculation of *B. fabae* reduced subsequent *B. fabae* infection, suggesting an induced defence response specific to this species.

Two successive field trials were conducted to determine whether the glasshouse results obtained previously were applicable to the field situation and tested the hypothesis that plant nutrition (application of fertilizers) and/or plant variety affect crop production. None of the results were significant at the 5 % level. The results from the first season were consistent with those from the controlled environment experiment but those from second season differed. The trend indicated that cv. Babylon was more resistant to chocolate spot than cv. Fuego. Also, potassium sulphate fertilizer very slightly but consistently reduced the severity of chocolate spot and rust compared to potassium chloride.

It became increasingly apparent that the methods of monitoring and quantifying *D. gigas* in the studies were time consuming. Therefore, a qPCR assay was developed for the simultaneous quantification and identification of *D. gigas* and *B. fabae* in faba bean, although specificity of the assay at species level was not tested.

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Chapter 1: Introduction

Plants are constantly involved in interactions with organisms with which they occupy the same ecological niche. While some of these interactions are beneficial to the plants, many of them are detrimental to the growth, development and eventual productivity of the plant. The dependence of man and animal on plants for survival cannot be over emphasised. It has been estimated that direct yield losses in crops due to pathogens, animals, and weeds, is between 20 and 40 % of word food production (Oerke, 2006).

Faba bean (*Vicia faba* L.) is an important grain legume widely cultivated in the temperate region and high elevation areas in the subtropical regions. The global production of faba bean in 2019 was 5.43 million tons http://www.fao.org/faostat/en/#data/QC [Accessed 15 March 2021]. Their seeds are mostly consumed by humans in the developing countries (Bond *et al.*, 1985), while they are widely used as animal feed, fodder and to improve soil fertility in Europe. Faba bean can fix nitrogen through symbiosis relationship with *Rhizobium leguminosarum* and has an advantage of higher yield over other grain legumes (O'Sullivan and Angra, 2016).

The bulk of the faba bean produced in the United Kingdom is destined for export or used as animal feed (https://www.pgro.org/downloads/PGROBLUEPRINTFORPULSES.pdf accessed 11 February 2021). Most of the exported beans go to North Africa (mostly Egypt) and the Arabian countries (The Andersons centre, 2015). A very small amount is consumed locally, as the immature “broad beans”, presumably because its taste or texture does not appeal to UK consumers (The Andersons centre, 2015). Rather small amounts of faba bean are used locally as animal feed, mainly because the supplies are not reliable (The Andersons centre, 2015). The unreliability of its supply may be due to several factors including drought and biotic pressure from pests and diseases.

Faba bean is parasitised by an array of pests and diseases. Notable among these are *Ditylenchus* and *B. fabae*. Both organisms occupy overlapping niches on faba bean. *Ditylenchus* is a migratory endo-parasitic nematode which causes stem lesions. Two species of *Ditylenchus* affect faba bean: *D. gigas* and *D. dipsaci*. The former is referred to as the giant race and is more destructive than the latter (Sikora and Greco, 1990, Sturhan and Brzeski, 1991). Its large body size may be the reason why it is more destructive on faba bean than *D. dipsaci*.

D. gigas is the most common species in the UK (Stawniak, 2011). It was recovered from 93 % of commercial seed-lots of faba bean sampled whereas *D. dipsaci* was recovered from 7 % (Stawniak, 2011).
D. gigas is classified as an A2 quarantine pest by the European and Mediterranean Plant Protection Organization, while it is classified as A1 quarantine pest in Azerbaijan, Egypt, Brazil, Uruguay, Argentina and Chile https://gd.eppo.int/taxon/DITYDI/categorization [Accessed 19 November 2020). It is regulated by quarantine measures.

B. fabae on the other hand is a necrotrophic fungus responsible for causing chocolate spot disease in faba bean. Both organisms are reported wherever faba bean is cultivated.

1.1. Faba bean

Faba bean *(Vicia faba* L.) belongs to the family Fabaceae (including other legumes such as peas, peanut, cowpea, phaseolus, alfalfa) in the order Fabales. Faba bean cultivars in the UK are generally classified as winter beans and spring beans. As the name implies winter beans are sown in the late autumn (between mid-October and early November) and grow during the winter months. Spring beans, on the other hand, are usually sown from February, if the condition of the soil is conducive. Crop sown in February usually avoid summer drought when the crop is flowering but unsuitable sowing conditions can greatly reduce potential yield (PGRO, 2017).
Spring beans generally have characteristics such as a white hilum which make them more acceptable in the export market (The Andersons Centre, 2015). Up until the 1970s, row spacing for faba bean was around 50 cm to keep an open structure and reduce disease spread and allow weeding. When fungicides were introduced the spacing was reduced to about 25 cm. Currently, spring beans are sown at a population of 50 -55 plants/ m^2 for varieties with typical growth and 35-45 plants /m^2 for varieties with vigorous growth (PGRO, 2017).

1.1.2. Faba bean varieties

Different varieties of faba bean have been developed by breeders over the years to improve the yield and quality of the plant and in response to challenges posed by pests, disease, drought, frost and other constraints. Available faba bean cultivars are fewer compared to cereals due to less investment (Fouad *et al.,* 2013, Duc *et al.,* 2015b). The greater cereal investment may be because many cereal crops like wheat, rice and maize are staple food for

many people worldwide. Some legumes are also staple foods in some parts of the world. For instance, faba bean is a staple food among the Middle east and some North African countries.

Popular spring varieties of faba bean among UK growers include Fuego, Fury, Maris Bead, Lynx, Vertigo, Fanfare, Boxer and LG cartouche. Winter bean varieties include: Tundra, Bumble, Wizard, Honey and Arthur.

Two spring varieties of faba beans were used in this study (Fuego and Babylon).
Fuego is widely cultivated in UK and valued for its food qualities https://www.gardenorganic.org.uk/growing-field-beans-human-consumption. Accessed 2 September 2019). It has been reported to be susceptible to *Ditylenchus* (Stawniak, 2011). It is moderately resistant to chocolate spot, with a score of 6.5 of 9 (PGRO, 2017). It is on the list of recommended cultivars of spring beans published by PGRO in 2019. It was first featured in 2005. It possesses good agronomic qualities, a high thousand grain weight and a big grain desired by exporters https://www.lgseeds.co.uk/products/pulses/fuego/# Accessed 2 September 2019). The final character is particularly important since most of the grain produced in UK is for export.
The variety Babylon is resistant to chocolate spot; on a scale of 1-9, it was rated 8 (PGRO, 2017). It was last featured on the recommended list of spring beans by PGRO in 2015 and was moved to the outclassed category in 2016 (PGRO, 2016).

1.1.3. Constraints to faba bean production

The major biotic constraints to faba bean production are pressures from pests, diseases and weeds. The three main fungal pathogens that affect faba bean are *B. fabae* which causes chocolate spot, *Ascochyta fabae* which causes *Ascochyta* blight and *Uromyces viciae-fabae* which causes rust (Torres *et al.*, 2006, Stoddard *et al.*, 2010). Also, *Sclerotinia trifoliorum* (mostly on winter bean), *Peronospora viciae*, *Cercospora zonata and Alternaria alternata* are important pathogenic organisms of faba bean. *D. gigas* and *D. dipsaci* are the major nematode pests of faba bean. They cause considerable yield losses to the crop. In severe cases yield losses may be up to 70 % https://www.pgro.org/downloads/2StemNematodesandFieldBeanRotations.pdf. Accessed 20 November 2020). Major insects that affect faba bean are black bean aphid (*Aphis fabae* Scop.), pea leaf weevil (*Sitona lineatus* L.) and Bruchid beetle (*Bruchus rufimanus* Boheman).

1.2. *Ditylenchus*

Ditylenchus (stem and bulb nematode) is an important genus of plant parasitic nematodes. They are migratory endoparasites that infest a wide variety of plants including beans, oats, alfalfa, clovers, tulip, narcissus, onion, garlic, strawberry, and many weed species. The genus comprises between 60 (Siddiqi, 2000) to 80 species of nematodes (Brzeski, 1991). It has a close resemblance to *Tylenchus* and *Anguina* (Southey 1978, Plowright *et al.*, 2002). All species in the genus possess a stylet which enables them to break barriers formed by the host plant cuticle and/or cell walls which serve as a barrier to some fungal pathogens. They are filiform (threadlike), transparent, and straight or slightly curved when heat killed.

1.2.1. Distinguishing features between *D. gigas* and the closely related species.

Morphologically, *D. gigas* is close to *D. dipsaci* and *D. weischeri* (Volvas *et.al*., 2011). *D. weischeri* has been reported on creeping thistle (*Cirsium arvense*) and is not a nematode of economic importance (Tenuta *et.al*., 2014). The most distinguishing feature of *D. gigas* is the longer body length (1.5- 2.22 mm) compared to *D. dipsaci* (1.0- 1.7 mm) and *D. weischeri* (1.3- 1.6 mm). It is for this reason it is referred to as the giant race. 'Gigas' is a Greek word meaning giant. It has a longer vulva-anus distance of 202- 266 µm compared to *D. dipsaci* (132–188 µm) and *D. weischeri* (172–240 µm) [Volvas *et.al*., 2011].

1.2.2. Symptoms caused by *D. gigas* on faba bean

Symptoms caused by *D. gigas* are obvious, unlike those caused by root inhabiting nematodes (e.g., cyst and root knot nematode), which are sometimes attributed to other organisms. One of the early symptoms of *D. gigas* on faba bean is swelling on the leaves (Figure 1), which can be noticeable about 48 hours after inoculation. Swellings also occur on the stem. Swellings contain clumps of nematodes (Campbell and Griffin, 1973). They result from the enlargement of epidermal and cortical cells. Eventually galls are formed, the cavities of which are filled with nematode eggs (Krusberg, 1961).

Swelling on leaf	Swelling on stem	Bulged leaf

Cracked stem	Twisted stem	Shortened internode

Shortened petiole	Blackened stem	Infested seed (Up) Clean seed (Down)

Figure 1.1. Symptoms of *D. gigas* on faba bean

1.2.3. Life cycle of *Ditylenchus*

Ditylenchus species are amphimictic (reproduce sexually). The population growth can be very rapid. Stawniak (2011) reported over fourteen-fold increase in the population of *D. gigas* on the Fuego variety of faba bean in a season. The nematode can lay about 500 eggs in its lifetime (Yuksel, 1960). The life cycle occurs in five stages and takes about 19 – 23 days in onion (egg to egg) under favourable conditions. When the conditions are not favourable, the nematode is able to slow down its life cycle (Bridge and Starr, 2007). The lifecycle begins with the laying of eggs (Figure 1.1). The first moult occurs in the egg. The second-stage juvenile (J2) emerge from the egg after 7 days (Yuksel 1960). The third moult occurs in the soil. Although all life stages of the nematode can infest plants, the fourth stage juvenile (J4) is considered as the main infectious stage (Sturhan & Brzeski, 1991) due to its ability to survive desiccation. The female nematodes start deposition of eggs from 3 to 7 days after the final moult, to begin the cycle again.

D. gigas can also affect other plants including lentil (*Lens culinaris*), Pea (*Pisum sativum*), Corn buttercup (*Ranunculus arvensis*), field bindweed (*Convolvulus arvensis*), White dead nettle (*Lamium album*), Red dead nettle (*Lamium purpureum*), Dead nettle (*Lamium amplexicaule*), Sterile oat (*Avena sterilis*), vetch (*Vicia* and *Lathyrus* spp) [Stawniak, 2011].

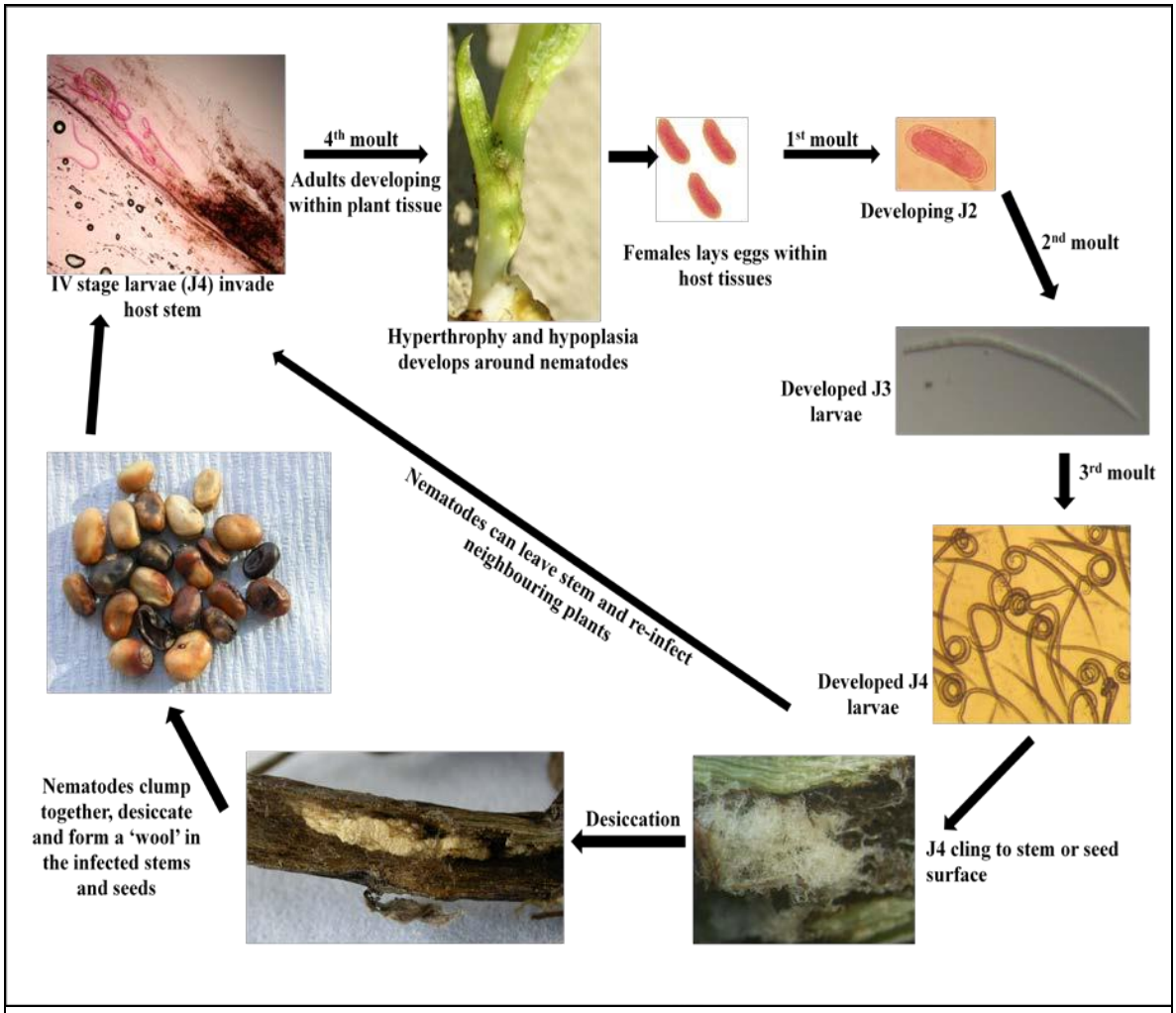

Figure 1.2. Life cycle of stem nematode on faba bean (Stawniak, 2011)

1.2.4. Losses induced by *Ditylenchus* species

Reductions in yield as a result of *Ditylenchus* attack depend on the susceptibility of the host crop, initial population density, pathogenicity, and environmental factors such as soil they are moisture, soil type and the climate (McDonald and Nicol, 2005). *Ditylenchus* spp. can multiply fast in successive crops when introduced in small amounts into an uninfected area (Hooper, 1984). Species in the genus have a complex biology, hence clear economic damage thresholds are difficult to establish (Janssen, 1994). Available figures mostly refer to crops other than faba bean and pre-date the distinction between *D. dipsaci* and *D. gigas*. Bridge and Starr (2007) established a damage threshold level of two *D. dipsaci* per gram of soil in onion. In Germany, a tolerance threshold of 2-3 *D. dipsaci* / 250 cm^3 of soil is used to indicate risk of crop infestation (Knuth, 1993). Varied yield losses caused by *Ditylenchus* on various crops have been reported. In England. Whitehead *et al.* (1983) reported a yield loss of 37 % on oat due to *D. dipsaci* infestation. In field trials in Syria, Hanounik (1983) noted that 650 *D. dipsaci* in 100 ml of soil resulted in 68 % faba bean yield losses and caused 20 % seed infestation. Greco and Di Vito (1994) and Bridge and Starr (2007) noted that *D. dipsaci* could cause yield reduction to legumes such as broad bean, pea and lentil in humid seasons. Biddle and Cattlin (2007) found yield loss of up to 70 % in faba bean in UK.

The interactions of *Ditylenchus* species with other pathogenic organisms such as fungi and bacteria could also result in further yield reduction. This will be an important topic later in this thesis. Some fungi and bacteria usually rely on wounds created by nematodes to access the plant. Whitehead (1998) noted that wounds created by plant parasitic nematodes could expedite secondary infection of fungi and bacteria, causing further reduction in yield, particularly in susceptible cultivars (Griffin, 1990). For instance, inoculation of *D. dipsaci sensu stricto* and *Fusarium oxysporum* f. sp. *medicaginis* caused synergistically shoot and root weight reduction in susceptible *Medicago sativa* plants (Griffin, 1990). Hillnhutter *et al.* (2011) also reported a synergistic interaction between *D. dipsaci* and the fungus *Rhizoctonia solani* in potato plants, which resulted in great damage. *Ditylenchus* has also been implicated in the transmission of plant disease. Hawn (1963) reported the spread of *Corynebacterium insidiosum* by *D. dipsaci* in alfalfa. He reported that the nematode conveyed the bacterium on its cuticle and resulted in a surge in wilting in susceptible alfalfa varieties.

1.2.5. Geographical distribution of *Ditylenchus* species

D. dipsaci occurs naturally in temperate and subtropical countries of the world and in high altitude areas in Africa. *D. dipsaci* has been reported in every continent (Figure 1.2) of the world (Europe, Asia, Africa, North America, South America and Australia).

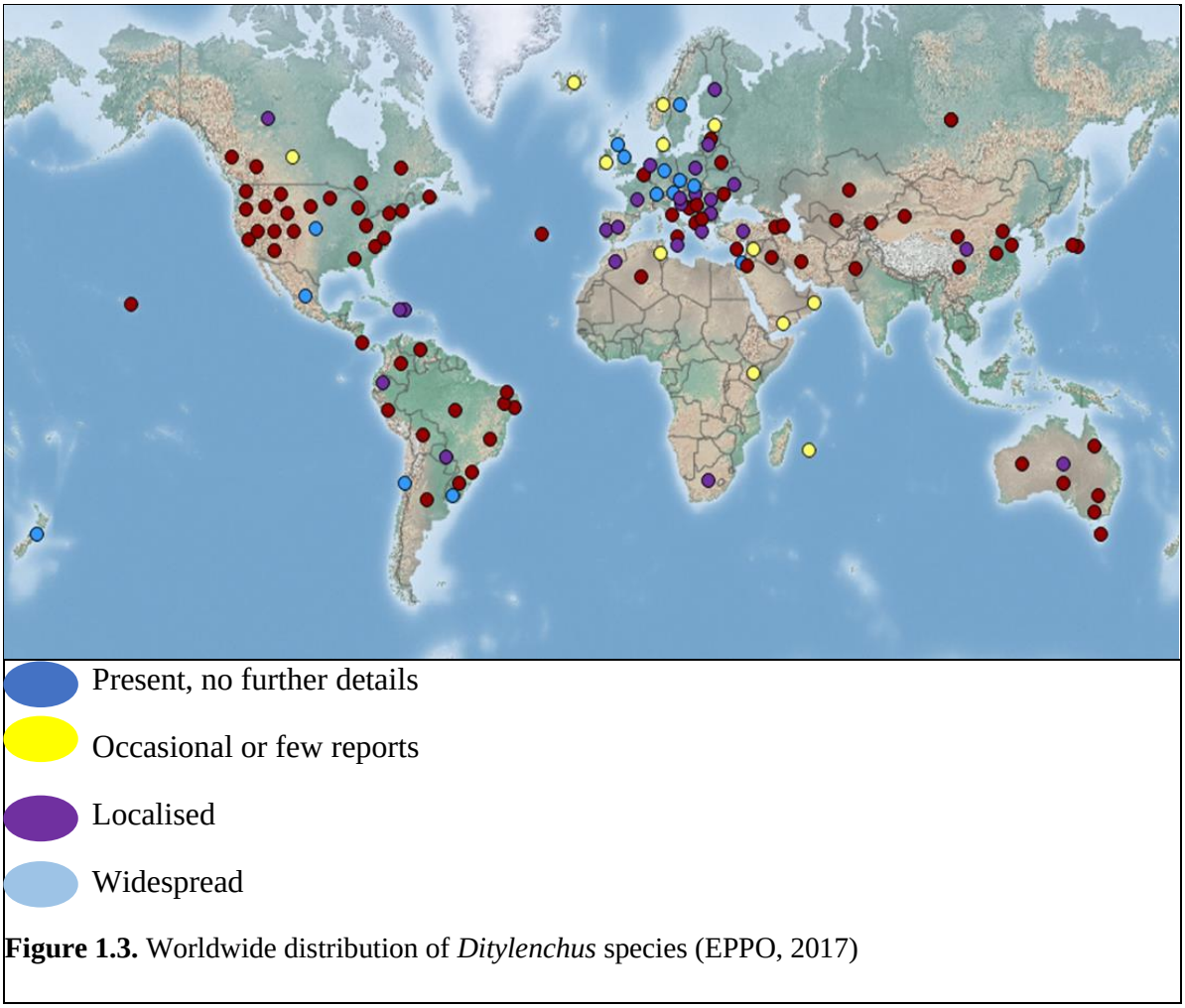

Figure 1.3. Worldwide distribution of *Ditylenchus* species (EPPO, 2017)

Ditylenchus can survive in unfavourable conditions in a desiccated form (eel worm wool) in plant tissue. This may have enabled its widespread distribution (Evans and Perry, 1976). *D. dipsaci* is fast assuming a worldwide distribution.

Information on how *Ditylenchus* is spreads in the field are scarce. It is not unlikely that some nematodes may evade detection at the ports of export. This is likely, as the nematode has been reported for the first time in some new locations.Some examples of reported cases are: Indarti *et al.* (2018) reporting the first record of *D. dipsaci* on garlic in Indonesia; Alabi *et al.*

(2017) established the presence of the *Ditylenchus* (Unspecified species) on yam in Nigeria; and Talwana, *et al.* (2008) also reported the occurrence of *Ditylenchus* (unspecified species) on yam, cassava, sweet potato and tannia in Uganda. Maafi *et.al.* (2013) reported the first case of *D. gigas* infestation on broad bean in Iran.

1.2.6. Spread of *Ditylenchus*

In water films, *D. dipsaci* can traverse 10 cm of soil in 2 hours, enabling them to infect plants immediately adjacent to an infected host (Kort, 1972) but not longer distances. The nematodes, therefore, rely on external factors to aid their movement. In the soil, they could be spread by bulk water movement such as runoff, irrigation water, or flood water. Infested soil, infested plant materials, animal vectors and wind may also enable long distance movement. The widespread occurrence of *D. dipsaci* on the Pacific coast of the U.S was attributed to infected seed of *Hypochaeris radicata* (false dandelion) blown by wind Godfrey (1924). *D. dipsaci* can be moved in irrigation water and spread to remote fields in the same irrigation basin. For example, 11,910 nematodes were recovered in 500 litres of water from an infected field in Utah (Thorne, 1962). Animals can also spread *Ditylenchus*. Livestock feed infected hay can pass out faeces infected with *Ditylenchus*. Migrating birds can also be implicated in the spread of *Ditylenchus*, particularly in spreading the nematodes to new areas https://www.apsnet.org/edcenter/disandpath/nematode/intro/Pages/IntroNematodes.aspx (Accessed online on 16 May 2019). *D. dipsaci* can also be spread by infested host propagules such as seeds and bulbs, and by contaminated farm equipment (Perry and Moens, 2013).

1.3. Management of *Ditylenchus* in faba bean

It is necessary to avoid the introduction of *Ditylenchus* on the field, as their elimination is hard. There are no chemicals available for the control of *Ditylenchus* in faba bean https://www.fwi.co.uk/arable/pulses/how-a-new-test-helps-bean-growers-tackle-stem-nematode (Accessed 27 January 2021). Methyl bromide which has been a nematicide used in the management of plant parasitic nematodes was banned in 2005 due to its contribution to the depletion of the ozone layer. Because of the lack of chemical control of *Ditylenchus*, efforts are geared towards cultural means of managing the nematode.

Soil solarization has been reported to be effective in the control of *D. dipsaci* and other soil borne pathogens in garlic fields (Pinkerton *et al.*, 2000). Temperatures above 40° C sustained

for about two or more hours can cause the death of nematodes (Greco & Brandonisio, 1990). Mean maximum temperature in solarised pots at 10 cm depth reached 45- 47 °C and killed *D. dipsaci* in garlic fields (Siti *et al.*, 1982). Soil solarisation may not be cost effective on a large scale, as the method involve spreading polyethylene mulch on soil surface over a period, until the temperature becomes high to kill the nematode.

Crop rotation with a non-host crop can also be effective in management of *Ditylenchus.* Lorbeer *et al.* (1997) reported that 4-year rotation with lettuce eliminated *D. dipsaci*. PGRO recommend that if nematodes are detected on the field, faba bean should not be cultivated for ten years (https://www.pgro.org/downloads/TU09StemandBulbNematodeinFieldBeans1.pdf [Accessed 22 January 2021].

Use of clean planting material (seed) is necessary as *Ditylenchus* is spread through seed. Using infested planting material will not only produce infested plant but will contaminate the soil making eradication hard.

1.3.1. Chocolate spot

Chocolate spot has been reported wherever faba bean is cultivated. It is one of the most devastating diseases of faba bean. Yield losses of up to 68 % were reported in an unsprayed plot in Ethiopia in a two-season trial in 2004 and 2005 (Sahile *et al.*, 2010). Wilson (1937) attributed chocolate spot in the Eastern counties of the United Kingdom to *B. cinerea* and *B. fabae*. Many researchers (Leach 1955, Mansfield and Widdowson, 1973, Mansfield & Deverall 1974a) have established that *B. fabae* causes more severe lesions on faba bean than *B. cinerea.* Mansfield & Deverall (1974a) discovered that *B. cinerea* did not produce a spreading lesion on faba bean, but *B. fabae* did. Zhang *et al.* (2010) reported *B. fabiopsis* as a new species responsible for chocolate spot on faba bean in China.

The symptoms of chocolate spot infection on faba bean start as small spots on leaves which subsequently coalesce to form blight and can result in outright blackening of the entire plant. The infection can also spread to flowers, pods and seeds under suitable conditions (Richardson and Horsham, 2008). The pathogen can infect the leaves, stems, flowers and pods of faba bean (Gaunt, 1983). However, the flowers and pods are the most susceptible parts of the plant (Griffiths and Amin, 1977).

Reddish brown spots

Spots coalesce to form a dark blight

Figure 1.4. Symptoms of chocolate spot on faba bean

Chocolate spot usually occurs in two stages i.e., the non-aggressive and the aggressive phase (Harrison 1980, Richardson and Horsham 2008). The non-aggressive lesions occur as small regular reddish-brown or chocolate brown spots (1-3 mm in diameter) on the leaves "peppered" over the leaves (Ellis and Waller, 1974b). The lesions occur on most faba bean plants each season and become abundant as the season advances (Harrison, 1984; Figure 1.3). Little yield loss is caused by the non-aggressive phase. However, under favourable conditions (continuous high relative humidity) the lesions rapidly increase in size (5-10 mm diameter) and then coalesce to form a dark blight that covers the stem and leaves (Ellis and Waller, 1974a). This can result in defoliation. *B. fabae* is also able to grow saprophytically on dead host debris, producing conidia and thereby re-infecting new leaves and extending stems (Jellis *et al.,* 1998). A wide variation in pathogenicity of different isolates of *B. fabae* has been reported. Sahile *et.al*. (2012) reported variability in morphological characters on potato dextrose agar, virulence on detached leaves and genetic diversity (using AFLP analysis) of isolates of *B. fabae* collected from different agro-ecological zones of Ethiopia.

1.3.2. Epidemiology of *B. fabae*

Mycelium of *B. fabae* can survive for over a year on soil surface or crop debris and can survive for 4 months when buried at 20 cm (Gorfu, 1999). Other means of survival for *B. fabae* include alternative hosts, volunteer faba bean plants and infected seeds (http://agriculture.vic.gov.au/agriculture/pests-diseases-and-weeds/plant-diseases/grains-pulses-and-cereals/chocolate-spot-of-faba-bean (Accessed 22 October, 2019). Although seeds are recognised as a possible source of infection, the frequency is low and the infection is only viable on seed for 9 months (Harrison, 1978). Seed stock containing 92 % infected seeds

gave rise to 5.4 % plants from which *B. fabae* was isolated at 12 – 39 days after planting Harrison (1978).

The life cycle of *B. fabae* starts with the production of asexual spores (conidia) from the tips of conidiophores (Figure 1.4). The spores could emanate from overwintering sclerotia in crop residue or from infected seeds (the latter usually in places where faba beans have not been cultivated for a long time). When spores land on a suitable host, they are transported by wind, rain splash (spores are not easily miscible with water) or insects - germination occurs when the conditions are favourable. Favourable conditions include temperatures between 15 and 22 °C and about 90 % relative humidity or above (Richardson and Horsham, 2008). Films of water or bright light are not necessary for sporulation, although some exposure to near ultraviolet light may be necessary to induce spore production in some isolates. For example, it is necessary to alternate 12 hours of near ultra-violet light with 12 hours of darkness to induce sporulation of *B. fabae in vitro* (Hanounik and Maliha, 1986, Dhingra and Sinclair, 1995).

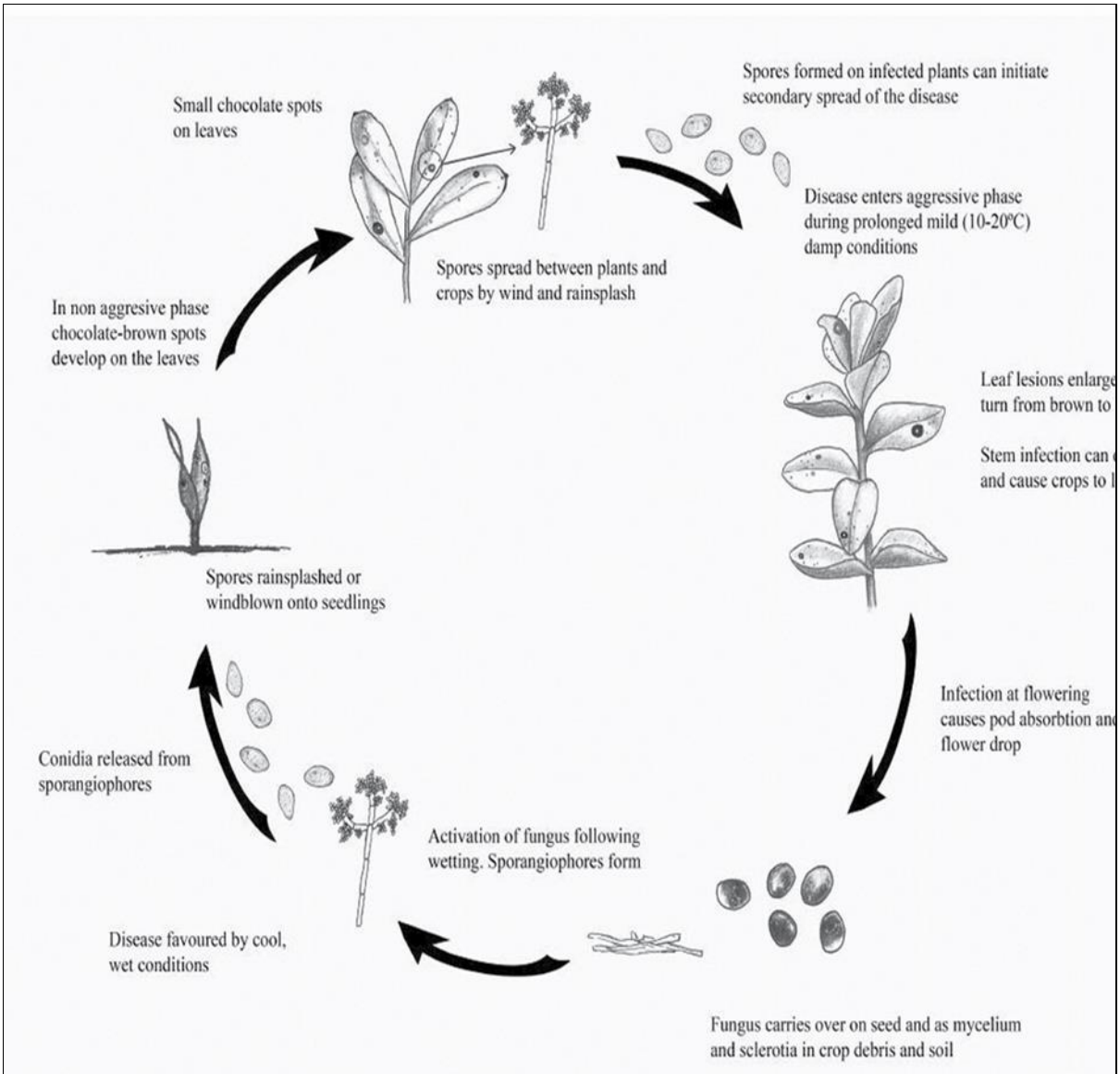

Figure 1.5. Life cycle of *B. fabae* on faba bean https://www6.inrae.fr/quantipest/layout/set/print/Pest-and-pest-injury-identification/Arable-crops/Faba-bean/Chocolate-spot-disease (Accessed 8 November 2018).

Once the spores germinate, germ tubes are produced within a few hours, followed by the development of an appressorium. Enzymes such as cutinases and lipases are produced to break the cuticle and ensure penetration. Host tissues are damaged as a result of the production of chemicals and metabolites. Spores are produced on the damaged tissues as the crop grows. As the disease is established, it spreads within a crop and spores are formed on infected plants within 4 – 5 days and this can start another cycle of infection (Richardson and Horsham, 2008). The appressoria rupture the plant surface largely by producing enzymes, such as lipase and cutinase (van Kan *et al.*, 1997, Reis *et al.*, 2005). At the tip of the penetration pegs, hydrogen peroxide is produced. This results in an oxidative burst in the

cells beneath the cuticle subsequently destroying it (Tenberge *et al.*, 2002). Once penetration is completed, a penetration peg usually grows into the anticlinal wall of underlying epidermal cells and generates pectinases in the right amount to break down pectin in the cell wall (van Kan, 2006). The continuous oxidative burst and production of phytotoxic metabolites such as botrydial and botcinolides ensures successful infection process (van Kan, 2005).

1.3.3. Management of chocolate spot in faba bean

Different strategies are employed in the management of chocolate spot in faba bean. The use of synthetic fungicides is one of the most dependable and effective management measures. PGRO (2017) recommended the use of Tebuconazole, azoxystrobin, metconazole, chlorothalonil + cyproconazole or boscalid + pyraclostrobin. However due to their detrimental effects on the environment, their use is being discouraged. Chlorothalonil has been banned from usage https://www.fwi.co.uk/arable/eu-chlorothalonil-ban-another-hammer-blow-for-growers (Accessed online on 29 September 2020). Other control measures include the use of fertilizers, intercropping, use of resistance varieties and biological control. Zengpeng *et al.* (2020) reported that nitrogen application at the rate of 90 kg per hectare and intercropping faba bean with wheat effectively reduced the severity of chocolate spot in faba bean.

1.4. Nematode fungus interactions

Disease symptoms observed in plants result from the interplay between the host plant, pathogens, and the prevailing environmental conditions. Plants are usually affected by arrays of pathogens which may interact with one another in the process of disease initiation. Atkinson's (1892) report of the interactions between root knot nematode (*Meloidogyne* spp) and *Fusarium oxysporum* was probably the first experiment on the interactions between a nematode and fungus. He noted that *F. oxysporum*, responsible for wilt of cotton, was more severe in the presence of root knot nematode. Since then, several interaction experiments involving nematodes and other plant pathogens have been conducted. The findings from Atkinson gave an insight to the role nematodes could play in an interaction with fungi. They can make plants more susceptible to fungi and other pathogens. This may be due to morphological and physiological changes in plants infected by nematodes (Sikora, 1992; Stirling, 1991).

Nematodes are well endowed to parasitize plants. They possess a stylet which is similar to proboscis in insects, an elongated tubular and flexible mouthpart used for sucking and

feeding. With the aid of the stylet, insects and nematodes can penetrate plant tissue. The stylet is struck back and forth to exert pressure on the cell wall. This is followed by the injection of effector molecules into the plant cell to access nutrients. Cell destruction by *Ditylenchus* can be accomplished by feeding on the cell for about 5 minutes (Doncaster, 1971). The process of feeding of the fourth stage juvenile and adults of *D. gigas* and *D. dipsaci* are the same on a faba bean leaf (Doncaster,1976). The entrance of the nematode also creates openings which can aid the entrance of plant pathogens that are unable to enter the plant on their own.

There have been very few reported cases of interactions between *Ditylenchus* and other pathogens. Perry and Moens (2013) opined that true interactions do not occur between *Ditylenchus* and other pathogens. Vrain (1987) reported that *D. dipsaci* and *Verticilium albo-atrum* wilt did not significantly reduce forage yields below that of *D. dipsaci* alone. However, Griffin (1990) and Hillnhuetter (2011) reported synergistic damage between *D. dipsaci* and *Fusarium oxysporum* f. sp. *medicaginis* on alfalfa, and *D. dipsaci* and *Rhizoctonia solani* on sugar beet respectively.

1.5. Other important pests/pathogens of faba bean

1.5.1. Rust (*Uromyces viciae-fabae*)

Uromyces viciae-fabae is a biotrophic pathogen producing the symptom commonly known as rust of broad bean. It is typified by the production of reddish-brown pustules on the leaves (Figure 1.5). Other species of rust also affect many other crops (Sillero *et al.*, 2006). It is more devastating on spring bean, especially at flowering and pod set, and all varieties are susceptible (PGRO, 2017). Bean rust does not complete its life cycle on nonhosts, but other rust species may need two phylogenetically distant hosts. For example, *Puccinia striiformis* f. sp. *tritici* needs *Berberis* spp. to complete its sexual cycle (Zhang *et al.*, 2011, Cheng *et al.*, 2012).

Figure 1.6. *Uromyces vicia fabae* on faba bean.

Rust can be controlled with the application of fungicides. Three applications of oxycarboxin or copper-mancozeb provided a significant yield increase in faba bean (Emeran, *et.al*., 2011)

1.5.2. *Ascochyta fabae*

Ascochyta fabae is a seed borne pathogen that affects faba bean. It is characterised by brown lesions containing pycnidia (Figure 1.6). It is more devastating on winter bean (PGRO, 2017). The bean seeds can serve as a source of inoculum for subsequent infection (Torres *et al.*, 2006). Its wide distribution is attributed to its ability to survive on the seed. *A. fabae* has a sexual and asexual stage. The sexual stage (*Didymella fabae*) was first discovered in the UK

(Jellis and Punithalingam, 1991). The teleomorph (sexual stage) can occasionally over-winter on crop debris in the spring and then produce ascospores that can start infection https://research.libraries.wsu.edu/xmlui/bitstream/handle/2376/13078/FS302E.pdf?sequence=1&isAllowed=y Accessed, 3 February 2021).

Figure 1.7. *A. fabae* (PGRO, 2017).

Disease free seed is an efficient way to manage the disease.

1.5.3. Black bean aphid (*Aphis fabae*)

Black bean aphid (Figure 1.7) is one of the most important insect pests that parasitise faba bean. It is the most common aphid in Europe due to its preference for *Vicia* and *Phaseolus* (PGRO, 2017). Damage is a result of feeding on the phloem, causing reduced growth and yield (Parker and Biddle 1998; Shannag and Ababneh 2007). This aphid species is also noted for the transmission of virus diseases in plants (El-Amri 1999, Neeraj *et al.*, 1999). Aphids can reproduce asexually (parthenogenesis and vivipary) and for this reason, their population can explode within a short period, resulting in serious damage (El-Amri 1999). Aphid infestation can be more devastating if the colonies are formed pre-flowering (PGRO, 2017). For this reason, adequate observations and monitoring measures should be put in place. This

will ensure that aphid's population are constantly monitored, and efficient control measures are put in place to prevent population build up.

Figure 1.8. *Aphis fabae* in faba bean, PGRO (2017).

To control aphids in UK bean crops, application of pirimicarb is recommended as soon as 5 % of the plants in the crop have been colonised (PGRO, 2017).

1.6. Induced resistance

Plants have evolved mechanisms to defend themselves against pest and pathogen invasion. Parts of the mechanisms deployed by plants include preformed defences (physical and biochemical) and induced responses that are triggered at the initiation of the infection process (Van Loon, 2000). Physical preformed defences include thick cuticle which may prevent pathogen penetration, especially for pathogens that access the plant directly; waxes present on leaves that prevent the build-up of moisture (as a result of their water repellent properties) required for pathogen propagules germination. Preformed biochemical defences including phenolic compounds, tannins and fatty acid-like compounds (dienes) have been suggested to be responsible for the resistance observed in developing tissues to pathogens such as *Botrytis* (Agrios, 2005).

Other defence responses are induced and are activated when plants are invaded by pathogenic organisms. Induced resistance is heightened resistance in a plant against pathogens due to pre-treatment with a pathogen, an attenuated pathogen or chemical inducers (Deverall and Dann, 1995). Many authors have reported that induced resistance, either natural or artificial, is effective against diverse pests such as fungi, viruses, bacteria and insects (Heil and Bobstock, 2002, Graham and Meyers, 2011, Elsharkawy *et.al*. (2013).

Induced resistance is a promising alternative to synthetic pesticides, with their attendant detrimental effects on the environment and the users, leading to some pesticides being banned from use. For instance, one of the most widely used pesticides in UK (chlorothalonil) was recently banned from use by the European Union. Therefore, other promising means of pest management must be explored. The quest for alternative methods of pest management other than pesticides prompted interest in induced resistance.

Rahe *et. al.* (1969) demonstrated the first evidence of induced resistance in bean. There has been increased interest in induced resistance since the 1980s. because induced resistance is both an environmentally friendly means of pest management and a model for the study of host defence genes in plants. Induced resistance includes systemic acquired resistance and induced systemic resistance. They are mainly distinct by their signalling pathways. Systemic acquired resistance can be activated by treating plants with necrotic pathogens and chemicals (Spoel and Dong, 2012). Induced systemic resistance is also the outcome of colonisation of plant roots by some growth promoting hormone rhizobacteria (Spoel and Dong, 2012).

Many substances, especially chemical inducers, have been used to induced resistance in plants against different categories of pests. However, studies on the use of pathogens for the inducement of resistance in plants are very scanty.

1.7. Influence of plant nutrition on plant diseases

Plants require nutrients for adequate growth and development and essential in disease control (Agrios 2005). Plants require 14 essential nutrients alongside oxygen, carbon and hydrogen to be able to complete their life cycles. The primary macro-nutrients are: nitrogen, phosphorus, and potassium; the secondary macronutrients are calcium, sulphur, and magnesium; while the micronutrients are: boron, chlorine, manganese, iron, zinc, copper, molybdenum, and nickel. All the essential nutrients are capable of altering disease severity (Huber and Graham, 1999). However, some incidence of some diseases may be decreased by a particular nutrient while the same nutrient may increase the severity of others (Marschner, 1995). Plant cell walls possess antimicrobial compounds which are released when cell walls are degraded (Vorwerk *et al*., 2004). Two macronutrients are relevant to the studies in this thesis, potassium and sulphur.

Potassium (K) is needed in high quantities by plants. It is the most abundant cation in plant cell, accounting for about 10 % of the plant dry weight (White and Karley, 2010). It is also involved in the metabolism and utilization of water by controlling the osmotic and salt balance (Gooding and Davies, 1997). Many research findings have reported the efficacy of potassium in the control of plant diseases. Mitchell and Walters (2004) reported that sprayed application of potassium phosphate was 70 % effective against powdery mildew of barley. Walters and Bingham (2007) noted that potassium fertilization can reduce plant diseases caused by both necrotrophic and biotrophic organisms.

Sulphur is an important macro-nutrient in the growth and development of plants. It is essential in the production of protein because it is part of the amino acids methionine and cysteine. Among many other effects, insufficiency leads to poor utilization of other nutrients such as nitrogen, reduced production of chlorophyll and poor protection of plants against pathogenic organisms. Hell (1997) noted that a lot of compounds containing sulphur are involved directly or indirectly with the protection of plants from pathogenic organisms. Such compounds include phytoalexins, thionins, defensins, glucosinolates, alliin, and glutathione. Schnug *et al.*, (1995) coined the term Sulphur induced resistance. Sulphur induced resistance

is aimed at application of sulphur to the soil to stimulate metabolic process involving sulphur in order to boost natural resistance of plants against pathogenic organisms. Many authors have affirmed the potential of sulphur in reducing the severity of plant diseases. Chandramohan and Shaw (2013) reported reduction in the percentage germination of conidia of *Parastagonospora nodorum* in wheat by sulphurous acid at pH4. Agrios (2005) noted that elemental sulphur in the form of wettable powder, paste, or liquid can be used to control powdery mildews, certain rusts, leaf blights, and fruit rots. Foliar and soil application of Sulphur, magnesium and micronutrients resulted in reduction in tuber infection caused by *Streptomyces scabies* and *Rhizoctonia solani* (Klikocka, 2009).

1.8. Project outline

The overall aim of this study is to determine if the two organisms interact and to determine their significance for faba bean productivity. It also seeks to understand how *D. gigas* that evades detection in a port is spread in the field. Chapter 3 builds on the work based on other species of *Ditylenchus* and suggests that spatial spread of the nematode on beans through the growing season is dependent on distance of inoculum from the plant, the orientation of the inoculum from the plant and environmental factors in the field. We, therefore, hypothesise that these factors will affect the spread of *D. gigas* from the infected plant to the adjacent ones.

B. fabae often co-exists in the presence of *D. gigas* and therefore, glasshouse investigations (Chapter 4) were designed to test hypothesis that *B. fabae* (as a necrotroph) will decrease susceptibility to *D. gigas* (a biotroph) and together they will reciprocally affect each other's population density and reduce the productivity of faba bean.

The next chapter (5) builds on the glasshouse experiments. In the light of work on induced defence mechanisms to plant pathogens and pests, the hypothesis that the order of infection (i.e., whether *D. gigas* infects before *B. fabae* or vice versa) will have an effect on induced resistance and affect crop production was tested.

Chapter 6 assesses whether the glasshouse results obtained previously are applicable to the field situation and tests the hypothesis that plant nutrition (application of fertilizers) and/or plant variety affect crop production.

Throughout these studies it became increasingly apparent that the methods of monitoring and quantifying *D. gigas* were time consuming. It was therefore decided to attempt to develop a molecular approach that would help in such studies (Chapter 7).

1.9. Objectives of the study

1. To quantify the dispersal of *D gigas* from a single source to plants at different distances away.

2. To determine the interactions between *D. gigas* and *B. fabae* and their reciprocal effects on population development of each organism and their synergistic and or otherwise effects on yield.

3. To determine induced response in faba bean after inoculation with either *B. fabae* or *D. gigas* in a controlled environment.

4. Assessing if early inoculation of *B. fabae*, fertilizers and/or plant variety affect crop production in field conditions.

5. To develop a species-specific qPCR assay to identify and quantify *D. gigas* and *B. fabae* in faba bean.

Chapter 2: General methodology

This chapter describes the general methodology used in this study. Procedures specific to a particular experiment are reported in the appropriate chapter.

2.1. Inoculum/seed sources

The *B. fabae* isolate was obtained from the School of Biological Science, University of Reading UK. *D. gigas* culture was provided by Becky Howard of the Processors and Growers Research Organisation (PGRO) Peterborough, UK. Faba bean seeds (Fuego and Babylon) were procured from Limagrain UK Ltd, Rothwell, Lincolnshire.

2.2. B. *fabae culture*

B. fabae was cultured on Malt Extract Agar (MEA, Oxoid, UK). Routine sub-culturing and production of spores of *B. fabae* was also done with MEA. One litre of MEA contained malt extract 30 g, mycological peptone 5 g, agar 15 g MEA (50 g) suspended in 1 litre of distilled water and autoclaved (SANYO MLS-3751L) at 121 °C for 21 minutes. Cultures were incubated in the dark at 20 °C for 3-5 days to enhance mycelial growth and transferred to a near ultraviolet light incubator (combination of white light and UV light: SYLVANIA, Black light-Blue, F8W/BLB-T5) with a day length of 16 hours to facilitate production of fungal spores.

2.2.1. Production of *B. fabae* spore suspension

B. fabae inoculum was prepared by flooding the surface of 10 - 14 days old culture of *B. fabae* with deionised water in a 9 cm diameter petri dishes. A bent sterile glass rod was used to dislodge the mycelium. The suspension was transferred into a bottle and vortexed for 1 minute, in order to break the mycelium fragments. The suspension was sieved with a sterile cheese cloth. The concentration of the spores was determined using a haemocytometer (Weber Scientific International Ltd, England).

2.3. Extraction of *D. gigas* from infested plant tissue

Fresh stems of faba bean with symptoms of *D. gigas* were chopped into small pieces (approximately 0.5 to 1 cm) and submerged in tap water. The suspension was left for about 2 hours on the laboratory bench to enable the nematodes migrate into the water. It was sieved to separate the nematodes from the plant tissues. The nematode suspension was left

undisturbed to allow the nematodes to settle and later decanted. The nematode suspension was stored at 4° C until use.

2.3.1. Extraction of stem nematodes from soil

Nematodes were extracted from soil samples using the modified Baermann method (Figure 2.1). Each soil sample was thoroughly mixed. Extraction tissue was placed on a plastic sieve. The base of the sieve was fully covered by the tissue. 200 ml soil was placed in the tissue. About 200 ml water was added to the extraction plate. The set up was left undisturbed for 24 hours. Excess water was drained from the sieve and the soil into the extraction. The sieve was removed, and soil disposed. The suspension was poured into a beaker and allowed to settle for about 2 hours. The majority of the liquid was decanted to leave a small volume of concentrated nematode.

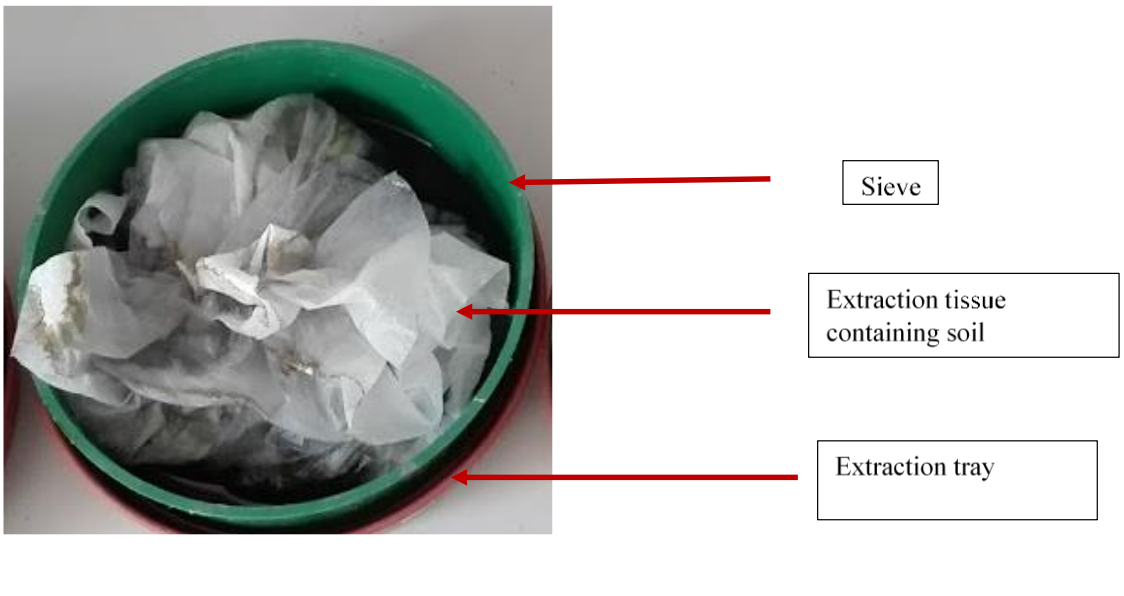

Figure 2.1. Modified Baermann extraction tray. Plastic sieve was placed on extraction tray and covered with extraction tissue filled with 200 ml soil. Water (200 ml) was added to the extraction tray.

2.3.2. Identification and enumeration of *D. gigas* from infested faba bean tissue

Nematodes extracted from infested plant tissue (2.3 above) were examined and identified under the microscope using the morphological and morphometric characteristics of the adult nematode. Identification was later confirmed using qPCR assay (Chapter 7). Nematodes were counted by taking a representative aliquot (1 ml) from a known volume of suspension and

counting in a graticule under the microscope 25 x magnification). The mean number of nematodes in the sample was computed from three aliquots of nematode suspension.

2.3.3. Multiplication of *D. gigas*

D. gigas was multiplied on faba bean plants (cv. Fuego) throughout the duration of the experiment. The cultivar has been reported to be susceptible to *D. gigas* (Stawniak, 2011). Faba bean seed were sown in a pot size of 23 cm diameter filled with John Innes No. 2 compost (JFC Monro, UK). A suspension containing three hundred mixed life stages of *D. gigas* was pipetted on the leaf surface of individual plant and covered with polythene for 3 days. The inoculated plants were kept in the glasshouse, at a temperature of 15- 20° C. The plants were harvested at maturity, before senescence (at about 3-4 months). Nematodes were extracted as stated in section 2.3 above.

2.4. Data analysis

Statistical analyses of the collected data were carried out using the GenStat statistical package GenStat for Windows (18th Edition; VSN International, 2017). The choice of a particular analysis is indicated in the materials and methods of appropriate chapters.

Chapter 3: Spatial spread of *D. gigas* on faba bean in the field

3.1. Abstract

D. gigas is the dominant species of *Ditylenchus* on faba bean in the UK. Its spread is controlled in part by certification schemes. To understand how fast isolated infections of *D. gigas* evading certification will spread spatially, field experiments building on the work in other species of *Ditylenchus* were conducted over two seasons. These suggest that spatial spread of the nematode on beans through the growing season is dependent on distance of inoculum from the plant, the orientation of the inoculum from the plant and environmental factors in the field. The experiments were laid out in a randomised complete block design and replicated 3 times in a plot size of 4 m^2 (2017) and 16 m^2 (2018) with a spacing of 20 cm between plants. The central plant was pre -inoculated with mixed life stages of *D. gigas*. At physiological maturity, the plants were uprooted along transects oriented north, east, south and west. Nematodes were extracted using a modified Baermann technique and counted. Extractions were made from the shoot, root, pod and seed in 2017, but only from the shoot and root in 2018, because the plants did not mature. *D. gigas* spread from the inoculum source up to a distance of 100 cm (the scale of the plots in 2017). In 2018, it spread to a distance of 160 cm (scale of 200 cm in 2018) on the shoot, but only detectably to a distance of 100 cm on the root. There were no directional effects on spread in 2017, but in 2018, the spread towards the south was more than in other directions (P=0.01). Average incidence on shoots (2017) decreased very slowly with distance beyond 40 cm, suggesting a different dispersal mechanism, presumably wind-blown droplets rather than ballistic splash. These results indicate that a single *D. gigas* infected plant could lead to a patch with sporadic seed infection on a scale of over 1.6 m within one season.

Keywords: Faba bean, *Ditylenchus gigas*, spatial spread, rain, splash, dispersal, nematode

3.2. Introduction

Many exotic species have been accidentally or deliberately introduced to different places with the attendant detrimental effects on the native species (McGeoch *et al.*, 2010). The presence of such pests and pathogens can have a negative effect on food production. Such newly introduced species may become established and reduce crop yield as a consequence of their activities.

Quarantine measures have been introduced in different nations to halt the spread of pests and pathogens. Despite the introduction of these measures, international trade and natural means of dissemination have encouraged spread of plant pathogens (Anderson *et al.,* 2004). Travellers are also known to carry plant materials and propagules in their luggage. McCullough *et.al*., (2006) reported that over 725,000 pests were intercepted and recorded in the Port information network in US from 1984 to 2000. These pests were made up of insects, mites, molluscs, nematodes, plant pathogens and weeds. Some pathogens in these taxa will have also made their way to new places undetected.

Ditylenchus spp. are known to naturally occur in temperate and subtropical countries where they affect crops such as faba bean, onions, garlic, alfalfa etc. They are rarely reported in tropical countries. However, some recent reports have recorded the presence of *D. gigas* and other *Ditylenchus* spp. in areas where they do not naturally occur (Section 1.2.5).

The spread of *Ditylenchus* to new regions may be due to their ability to survive extreme conditions (Jones, *et al*., 2013). A single infected faba bean seed can harbour up to 19,000 nematodes and there can be as much as 18,000-fold increase in *Narcissus* in a season (Hesling, 1970). Stawniak (2011) reported 14 -fold increase in the population of *D. gigas* on Fuego variety of faba bean. Seeds are easily transported and moved around, thus seed is an important means of dissemination of the nematode to new places.

Information on how *D. dipsaci* spread in the field is available in literature (1.2.6) but little is known on the spread of *D. gigas*. *D. gigas* is capable of spreading through soil from a point source. The experimental design will quantify how far it does so**.** As experiments were to be conducted over different seasons, data on edaphic factors that might influence nematode spread (e.g., temperature and rainfall) will be considered.

3.3. Materials and methods

The experiments were conducted between April and July in 2017 and from July to November in 2018. The difference in the sowing dates was because in 2018 inoculated plants did not establish on the field due to prolonged period of dry spells. *D. gigas* cultures were raised on faba bean (cv. Fuego) as described in Chapter 2 (2.3.3). Prior to seed sowing, soil samples were collected separately from each plot. Half a meter was avoided from the edge of plot on all sides and samples were taken at 1 m apart (Figure 3.1). At a depth of 20- 30 cm, about 1 kg soil was collected per sample using a hand trowel. Six core samples were collected in an area of 4 m^2 and twelve samples in an area of 16 m^2. The soil samples were bulked and used to fill one litre size pots (top diameter: 28 cm, bottom diameter: 23 cm, depth: 25 cm). One faba bean (cv. Fuego) seed was planted in each of the pots. The plants were kept in a glasshouse and observed for nematode symptoms. The plants were harvested at maturity and modified Baermann extraction trays (Chapter 2, section 2.3) set up for nematode extraction.

At the experimental site *Vicia faba* (cv. Fuego) was sown on three replicates of a square grid pattern at a spacing of 20 cm. Each replicate had sides of 2 m (2017) or 4 m (2018). Two weeks after emergence, two-week-old seedlings (grown in glasshouse) previously inoculated at the growing tip with 300 nematodes/plant was transplanted into the centre of each plot. At physiological maturity (19-20 weeks in 2017, 17-18 weeks in 2018 following inoculation) plants along transects oriented north, east, south and west from the centre were uprooted. Nematodes were extracted from the plants using the modified Baermann extraction method (Chapter 2, section 2.3). Meteorological data was obtained from the University of Reading Atmospheric Observatory.

3.3.1. Statistical analysis

Analysis used GenStat 18th edition. A linear model including direction and distance and their interactions was fitted to the log10 transformed nematode counts.

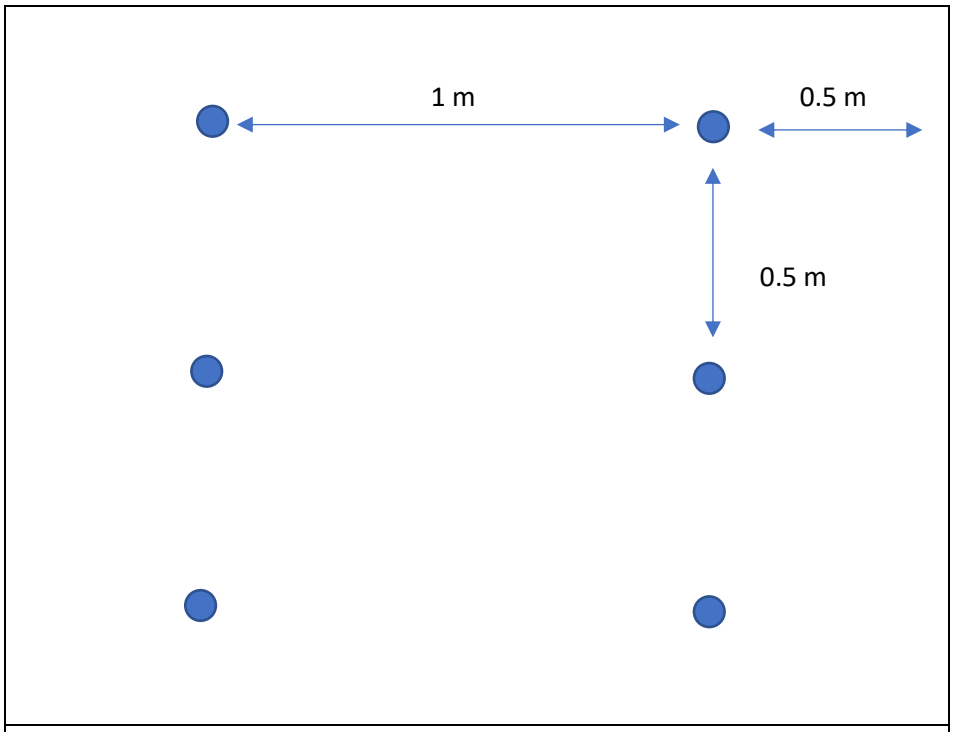

Figure 3.1. Method of sampling soil for nematode in the field. Samples were taken 1 m apart. Half a meter was avoided from the edges of the plot on all sides. Circles represents sampling points.

3.4. Results

3.4.1. Spread of *D. gigas* from single source plants (2017)

The spread of *D. gigas* from the infected source plant to the adjacent plants was detected at a maximum distance of 20 cm on the seed, 40 cm on the pods, 60 cm on the roots and 100 cm on the shoots (Figure 3.2).

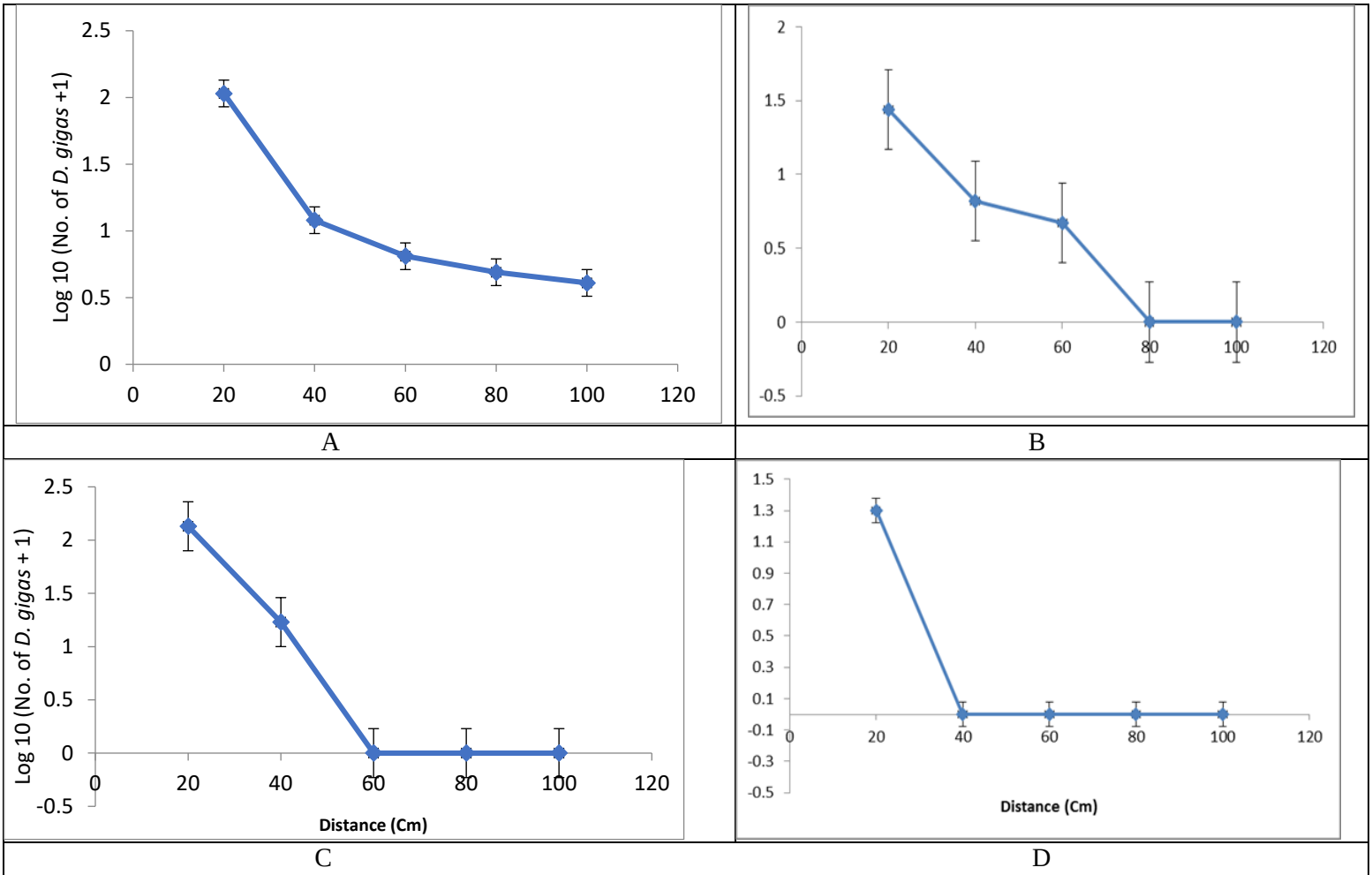

Figure 3.2. *D. gigas* recovery from shoot (A), root (B), pod (C) and seed (D) samples at different distances from a single initially infected source plant (2017). Data were log10 transformed and evaluated 19- 20 weeks following inoculation. Error bars denotes standard error of difference.

3.4.2. Spread of *D. gigas* from single source plants (2018)

D. gigas spread from the single initial source plant to the adjacent plants. It was detected up to a distance of 160 cm on the shoots, and 80 cm on the root (Figure 3.3). The 2018 experiment was conducted between the months of July to November, so the plants did not pod, hence nematodes were only extracted from the shoot and the root. The maximum number of *D. gigas* was recorded at a distance of 20 cm from the source. Beyond 160 cm no *D. gigas* was extracted from either the shoot or the root.

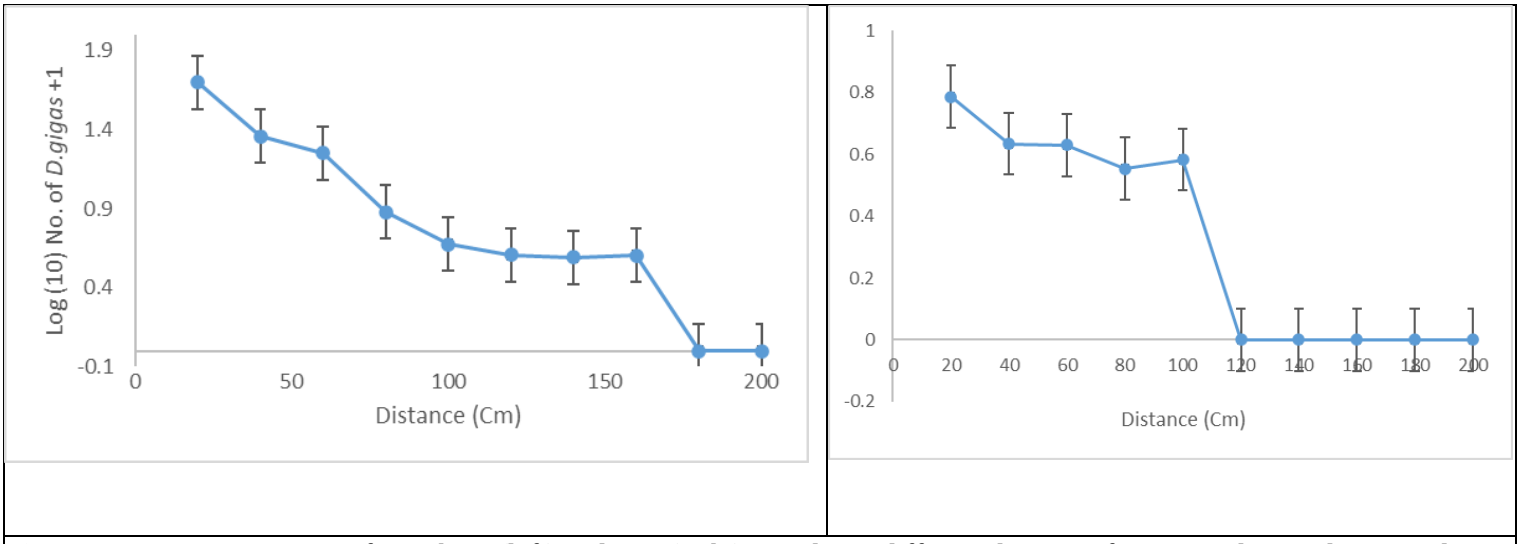

Figure 3.3. *D. gigas* recovery from shoot (left) and root (right) samples at different distances from a single initial source plant (2018). Data were log10 transformed and evaluated 17- 18 weeks following inoculation. Error bars denote standard error of difference.

3.4.3. Direction of spread of *D. gigas* from a single initially infected source plant (2017 and 2018)

The total spread of *D. gigas* along each transect in 2017 and 2018 is presented in Figure 3.4. Differences between directions were not significant (P= 0.9) in 2017 (A), although most nematodes were recovered along the east transect and least along the south. However, in 2018, there was a significant (P= 0.001) effect of direction. Most nematodes were recovered from the north and south transects, while the east and west had the fewest.

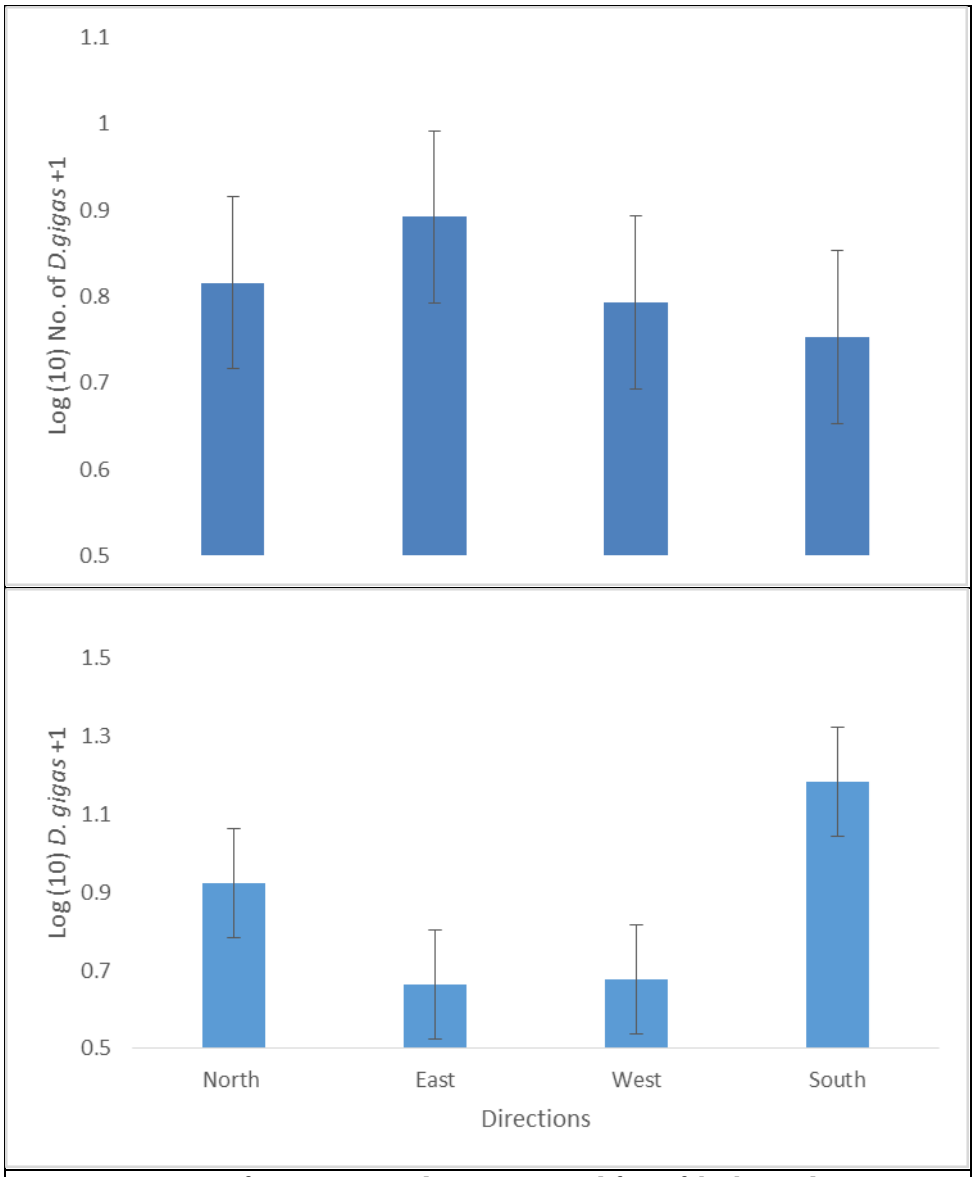

Figure 3.4. Means of *D. gigas* population extracted from faba bean along transects in four orthogonal directions, 2017 (upper panel), 2018 (lower panel). Data were log10 transformed and evaluated 19- 20 (2017), 17-18 (2018) weeks following inoculation. Error bars denote standard error of difference.

3.4.4. Mean number of plants infested by *D. gigas*

In 2017, 11.3 plants were infested with *D. gigas* in a plot size of 4 m^2, compared to 14 plants in 2018 (Table 3.1). The mean incidence in 2017 was 71 %, compared to 44 % in 2018.

Table 3.1: Number of plants infested by *D. gigas*. Data were evaluated 16-17 weeks following inoculation

Year	Plot size	No. of plant sampled	No. of plant infested	Mean incidence
2017	4 m^2	16	11.3	71 %
2018	16 m^2	32	14	44 %

3.4.5. Mean air temperature during 2017 and 2018

The experiments were conducted at different times of the year. The mean air temperature increased from March to July 2017 (Figure 3.5). The lowest mean air temperature (9.45 °C) was recorded in April, while the highest (17.3 °C) was recorded in July. Conversely, the temperature in 2018 decreased progressively from July to November 2018. The highest mean temperature (20.7 °C) was observed in July, while the least (8.4 °C) was recorded in November. In 2017, the minimum temperature of 5.0 °C was recorded in April, while the highest (24.3 °C) was recorded in July. In 2018, the maximum temperature (17.5 °C) was recorded in July, while the lowest (1.3 °C) was observed in November.

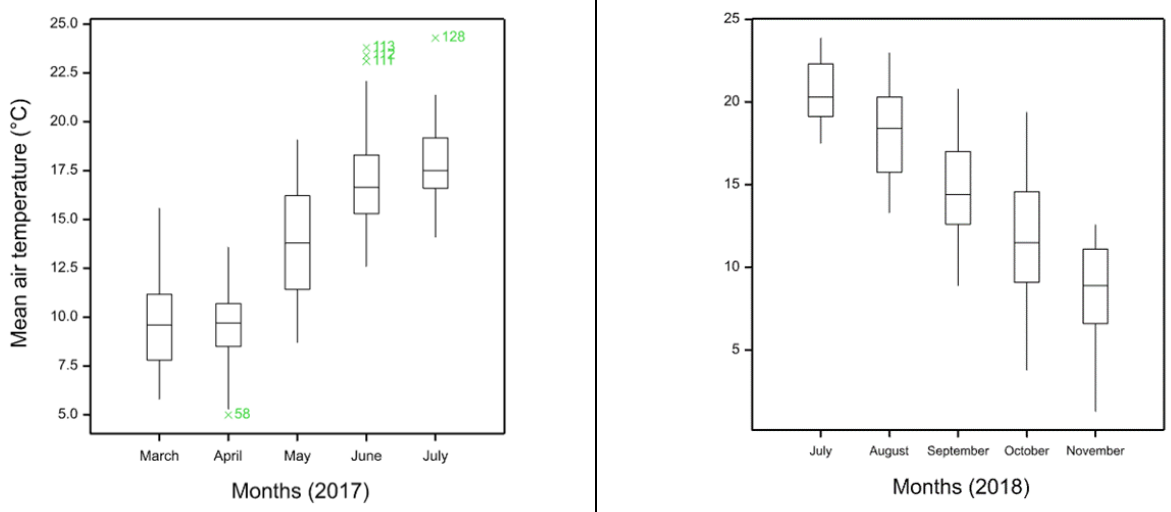

Figure 3.5. Mean air temperatures recorded by the University of Reading Atmospheric Observatory for March- July 2017 and July – November 2018. (Source: https://research.reading.ac.uk/meteorology/atmospheric-observatory/atmospheric-observatory-data/). X represents the outliers

3.4.6. Mean soil temperature during 2017 and 2018

The mean soil temperature at 10 cm increased progressively in 2017 while the reverse was the case in 2018 (Figure 3.6). The highest mean 10 cm soil temperature in 2017 (19.2 °C) was observed in July while the least (7.6 °C) was observed in March. In 2018, the highest soil temperature (22.8 °C) was observed in July, while the least (7.5 °C) was recorded in November. The minimum soil temperature in 2017 was 4.0 °C (March) while the maximum was 24.2 °C (July). The maximum soil temperature in 2018 was 25.1 °C, in July, while the minimum (2.0 °C) was observed in November.

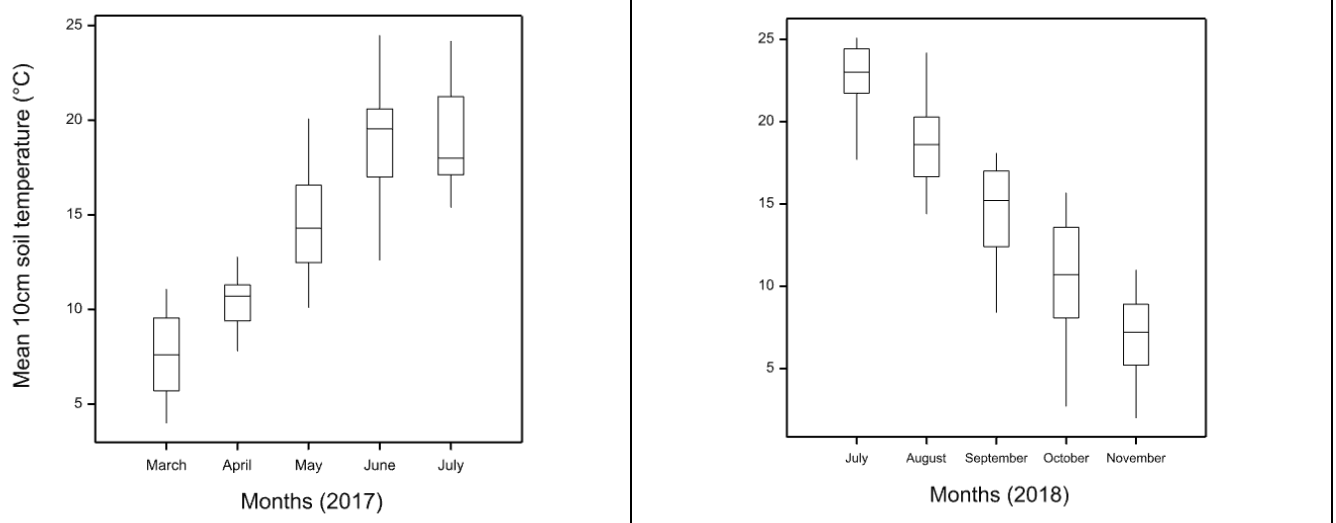

Figure. 3.6. Mean soil temperature at 10 cm depth recorded by the University of Reading Atmospheric Observatory for March- July 2017 and July – November 2018 (Source: https://research.reading.ac.uk/meteorology/atmospheric-observatory/atmospheric-observatory-data/)

3.4.7. Mean relative humidity during 2017 and 2018

The mean relative humidity fluctuated in 2017 while it progressively increased in 2018 (Figure 3.7). The highest mean relative humidity in 2017 was 82.3 % recorded in March, while the least was 64.5 % (April). In 2018, the highest relative mean humidity was 88.2 % (November), while the least was 57.3 % (July). The minimum mean relative humidity in 2017 was 50 % (June), while the maximum was 97 % (March and May). In 2018, the minimum mean relative humidity was 32 % (July), while the least was 98 % (October and November).

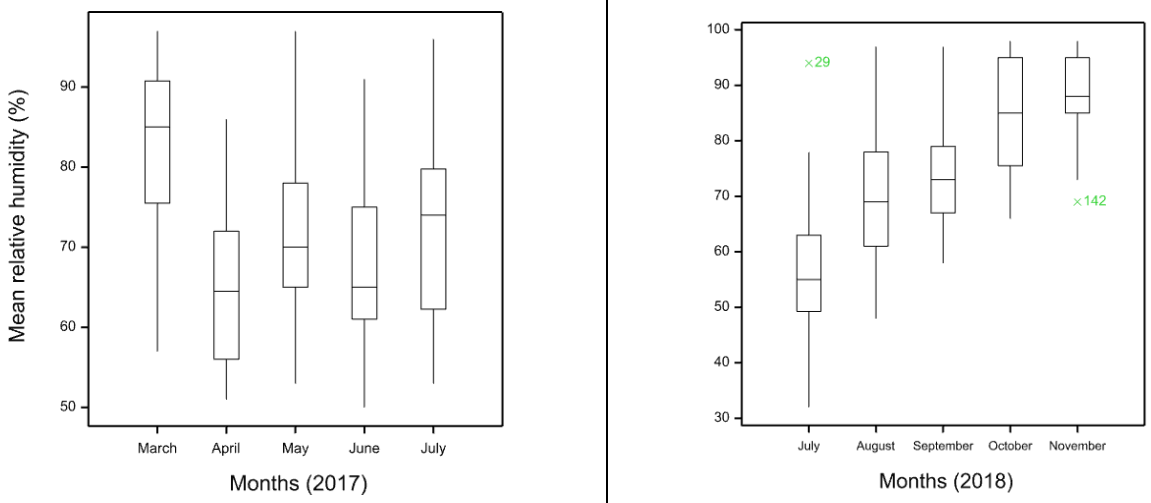

Figure 3.7. Mean relative humidity for March- July 2017 and July – November 2018. (Source: https://research.reading.ac.uk/meteorology/atmospheric-observatory/atmospheric-observatory-data/)

3.4.8. Rainfall during 2017- 2018

The highest rainfall in 2017 was 45 mm (July), while the least (2.5 mm) was recorded in April (Figure 3.8). In 2018, the highest rainfall (25.6 mm) was recorded in October, while the least (5.5 mm) was recorded in July.

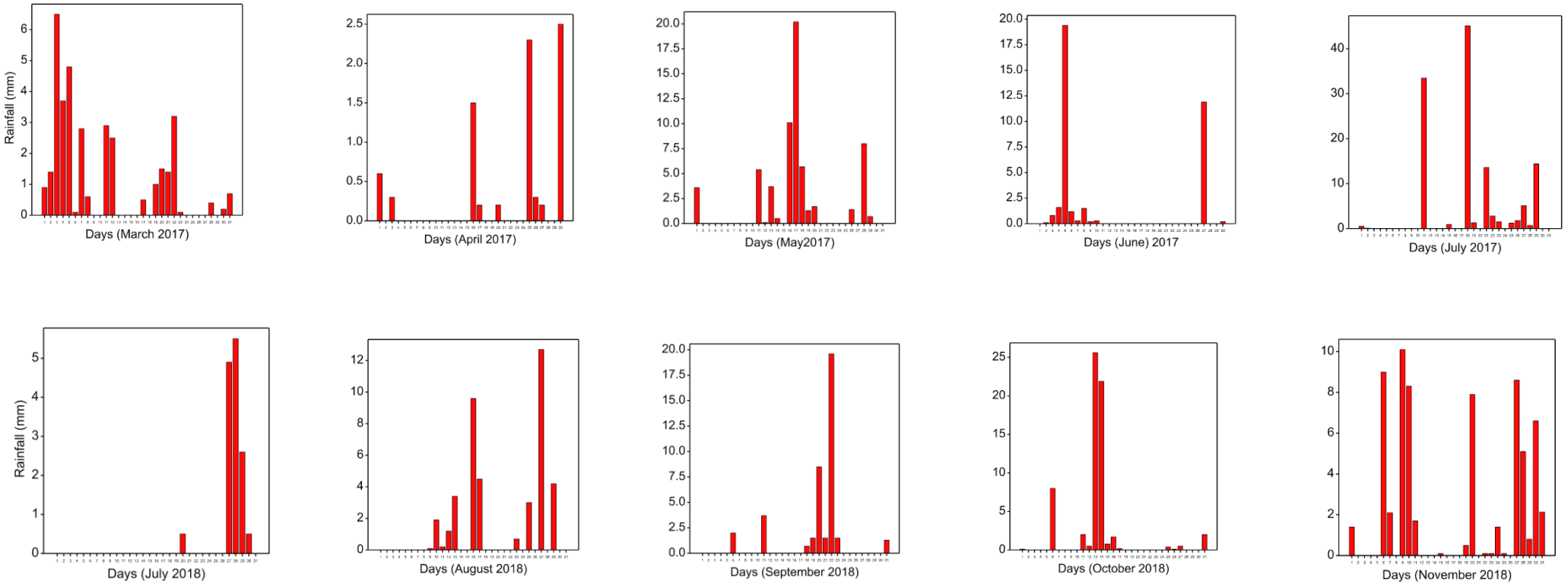

Figure 3.8. Rainfall for March- July 2017 and July – November 2018. (Source: https://research.reading.ac.uk/meteorology/atmospheric-observatory/atmospheric-observatory-data/).

3.5. Discussion

The study established that *D. gigas* infection can spread to a distance of 1.6 m within a season (2018 season). This is consistent with the 2017 data in which the nematode spread to the edge of the plots (1.0 m). The increase in plot size in 2018 to a scale of 16 m^2 was because the nematode spread to the maximum measurable distance of the experiment in 2017. The maximum distance of spread was observed on the shoot in both seasons. On the root, the nematode spread to a distance of 60 cm in 2017 and 40 cm in 2018.

D. gigas may have spread from the infected source to other plants in several ways in this study. Firstly, spread may have occurred when plant leaves touch one another. *D. gigas* are migratory endo-parasites and can move in films of water within and outside the plant tissue. Movement is encouraged especially when there are depleted resources on the infested plant. Although the spacing in this experiment was 20 cm, the plant leaves were able to touch one another later in the growing season.

Secondly, it is also possible for the nematode to have been spread through soil movement. Kort (1972) reported that *D. dipsaci* traverse 10 cm of soil in 2 hours. This means that it would take the nematode 4 hours of continuous movement from an infected plant to the adjacent ones.

Thirdly, the nematodes may have also spread through runoff as they were recovered from the root. Nematodes, with the help of their stylets can pierce plant cell walls to gain entry. Thorne (1962) noted that *D. dipsaci* can be moved in irrigation water, for example 11,910 nematodes were recovered in 500 litres (as stated in section 1.2.6) of wastewater from an infected field in Utah. The recovery of the nematode from the root may be attributed to the spread in run-off water, as *D. gigas* unlike root inhabiting nematodes (e.g., cyst and root knot nematode) does not permanently inhabit the root, although, they may use the root as a means of entering the plant.

Finally, McKenry & Roberts (1985) noted that *D. dipsaci* can be blown in soil particles or infested propagules. Godfrey (1924) hypothesised that the prevalent occurrence of *D. dipsaci* on the Pacific Coast of the U.S. was a result of wind -blown infected seed of *Hypochaeris radicata* (false dandelion).

Since *D. gigas* does not reproduce parthenogenetically, it requires individual nematodes of both sexes to start a new reproducing colony. This will result in uneven and aggregated distribution of nematodes in the field, as witnessed in this study. Uneven nematode distribution also results due to limited locomotion of nematodes.

Faba bean seeds can harbour large number of nematodes. The nematodes are capable of surviving and can remain viable in extreme conditions, hence seeds remain an important inoculum source. Green and Sime (1979) established that a single infected faba bean seed can harbour up to 19, 000 nematodes. Even at low nematode populations, *Ditylenchus* can cause mild, but detectable symptoms. A dose response experiment (Chapter 4) established that as few as 30 *D. gigas* per plant caused a mild but detectable symptom on faba bean. A spacing of 20 cm per plant was used which translates to 25 plants/m^2 or 250 000 plants per hectare. If one infested seed is planted per m^2, then you would initially have about 10 000 infested plants per ha and at the end of the season essentially all plants in the field would be likely to have infection.

Most nematodes were recovered from the shoot, while the fewest came from the seeds. This may be because the nematode usually enters the plant through the stomata and moves, feeds (on parenchymatous tissue, i.e., in the shoot) and reproduces within plant cells, moving to other parts of the plant when the population builds up. The life cycle of the nematode takes about 19-23 days under favourable conditions (Yuksel, 1960), so the observed spread represents several population cycles. The nematodes only migrate to fresh tissue and then act as new sources under heavy infestation or when resources are otherwise limited. This is unlikely in this experiment as low levels of infestation were recorded on the plants other than the source.

There was no directional effect of the spread of the nematode in 2017, while the nematode spread more towards the south and north in 2018. The nematode may have been spread by different means in the field, including run-off and rain splash.

In 2018, the plants did not pod, because the experiment was conducted between the months of July to November and the cultivar tested (cv. Fuego) is a spring variety. This cultivar is normally cultivated between late February and July. The experiment in 2017 was conducted in the spring (March to July) and flowered at about 7 to 8 weeks after sowing. Although the 2018 experiment flowered almost at the same duration as that of 2017, the flowers in 2018 were scanty and there was much abscission. As a result, no pod was produced.

The sparse flower production and abscission observed in 2018 may be due to reduction in day length as well as temperature. Faba bean is regarded as either day neutral or long day plant. The photoperiod requirement for flowering varies. Ellis *et al*. (1990) reported that 9.5 hours was the minimum photoperiod for a Mediterranean landrace. The mean daily temperature was 14.5 and 11.4 °C in September and October 2018 respectively. The optimum temperature for flowering in faba bean is 19.9-26.5 °C (Ellis *et al*. 1988b), 17-23 °C (Ellis *et al*., 1988b).

3.6. Conclusion

The result from this study showed that *D. gigas* could spread from an infected source to adjacent plants. It can spread up to 1.6 m in a season. The population of the nematode can build up over the years and persist in the soil, thus affecting future crops. Knowledge of how the nematode spreads in the field is important for adequate management practices and detection thresholds for regulatory control.

Chapter 4: Interactions between *D. gigas* and *B. fabae* and their effects on population development of each organism

4.1. Abstract

B. fabae often co-exists in the presence of *D. gigas* and therefore glasshouse investigations were designed to test the hypothesis that *B. fabae* (as a necrotroph) will decrease susceptibility to *D. gigas* (a biotroph) and together they will reciprocally affect each other's population density and reduce the productivity of faba bean. An initial experiment involved nematodes alone, while a second experiment involved *D. gigas* and *B. fabae* co-inoculation. Similar data from the two experiments were pooled together for analysis using the mixed model for multiple experiments in GenStat. Antagonistic interactions on the population of *D. gigas* were observed, as the number of nematodes extracted from lone inoculation of *D. gigas* was more than those from co-inoculation of the two organisms, suggesting an induced defence response. However, a synergistic interaction was observed on grain yield, as the reduction in grain yield was more with co-inoculation than lone inoculation of either organism.

Keywords. *D. gigas*, *B. fabae*, faba bean, synergistic, reciprocal effects, detectable.

4.2. Introduction

Quantitative knowledge of plant parasitic nematodes and fungi is vital in their management. The level of damage induced by *Ditylenchus* on their host plants is dependent on the population density at planting, host and susceptibility and the prevailing environmental factors e.g., soil moisture and weather conditions (McDonald and Nicol, 2005). The complex biology of *Ditylenchus* species makes the establishment of a threshold of economic damage extremely complicated (Janssen, 1994). Similarly, the severity of chocolate spot infestation is dependent on the amount of inoculum and the prevailing environmental conditions.

Since the work of Atkinson (1892) which reported that Fusarium wilt of cotton was more devastating on cotton in the presence of root knot nematode (*Meloidogyne* spp), several reports have been published on nematode/fungus interactions (Sikora and Carter 1987, Griffin 1990, Back *et al*., 2002, Hillnhuttter *et.al*., 2011). Information on the interactions between *Ditylenchus* and fungus is very limited. Griffin (1990) reported that simultaneous and sequential inoculation of *D. dipsaci* affected alfalfa growth but not its reproduction; Hillnhütter *et al*. (2011) reported a synergistic increase in damage between *Ditylenchus dipsaci* and *Rhizoctonia solani*. Information on the interaction between *D. gigas* and *B. fabae* is not available in the literature.

Interaction between *D. gigas* and *B. fabae* is possible due to their overlapping niches on the host plant. Both are frequently encountered on faba bean on the field. *D. gigas* can enter the plant through the stomata moving and feeding within the plant cells. All life stages of the nematode have been recovered from the parenchymatous tissues (Perry and Moens, 2013). *B. fabae*, on the other hand, kills the host cells and then colonises them. This may disrupt the movement and proliferation of *D. gigas*. *D. dipsaci* cannot proliferate in a rotting tissue (Perry and Moens, 2013). *D. gigas* requires living cells to carry out its life function but can cause mechanical wounding on plants. These openings created by nematodes predispose the plants to other pathogens, including fungi which are otherwise unable to breach the cell walls of plants.

The different possible outcomes of the in-planta interactions between nematode and fungus are additive, antagonistic and synergistic (Figure 4.1). Additive interactions occurs when the sum of the damage caused by the two organisms equals the sum of the individual damage.

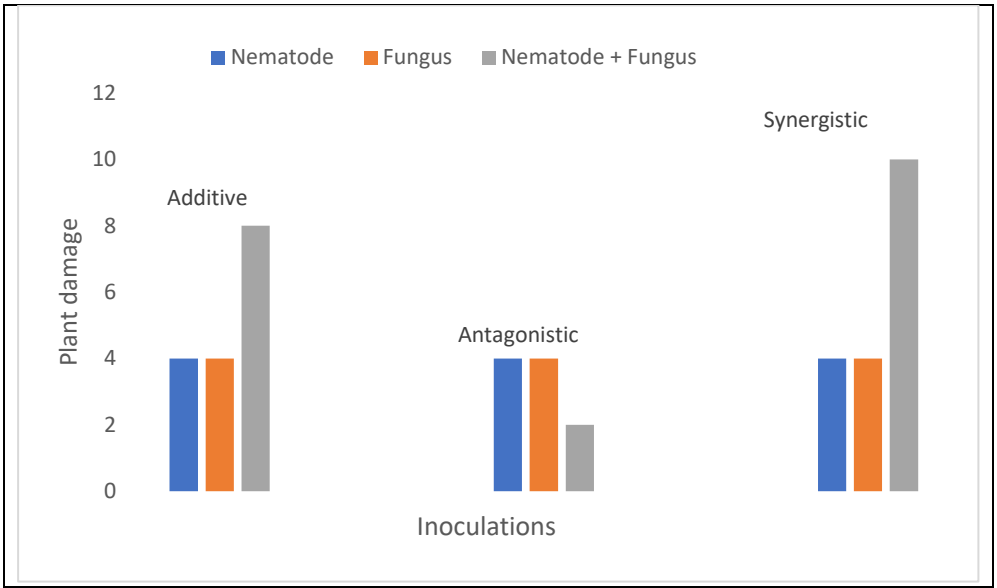

Figure 4.1. Types of interactions between nematode and fungus

Antagonistic interactions occur when the sum of the plant damage by the two organisms is less than that of individual damage by either of the organisms. Synergistic interactions occur when the sum of the plant damage by the nematode and fungus exceeds the sum of individual damage by either of the two organisms.

B. fabae often co-exists in the presence of *D. gigas* and therefore glasshouse investigations (Chapter 4) were designed to test the hypothesis that *B. fabae* (as a necrotroph) will decrease susceptibility to *D. gigas* (a biotroph) and together they will reciprocally affect each other's population density and reduce the productivity of faba bean.

4.3. Materials and methods

Faba bean seeds were sown in plastic 12 L pots (top diameter: 28 cm, bottom diameter: 23 cm, depth: 25 cm) filled with John Innes No 2 compost. The plants were kept in a glasshouse, where temperature ranged between 14 – 21°C. The experiment in 2017 was sown on 14 March and terminated on 6 July, while that of 2018 was sown on 7 March and terminated 2 July. In 2017, two weeks after planting (two leaf stage) the growing tips of the plants were inoculated with 1 ml mixed life stages of five *D. gigas* suspensions (to give 0, 30, 100, 300, 1000 or 3000 nematodes per plant). The concentrations of inoculum were adjusted to their respective doses by counting under the microscope (Section 2.3.2)

In 2018, *B. fabae* conidia (0, $2x10^3$ /ml and $2x10^6$/ml) were co-inoculated with the five populations of *D. gigas* (0, 30, 100, 300, 1000 or 3000 nematodes). One ml of inoculum of *B. fabae* was applied with a paint brush on the leaf surface. The inoculated plants were covered with polythene bags for 24 hours to maintain a humid condition. The experiments were laid out in a randomised complete block design with five replications, blocked parallel to the glasshouse axis. Sachets of *Amblyseius cucumeris,* Oudemans (BioLine, Syngenta) were used to prevent western flower thrips (*Frankliniella occidentalis*, Pergande) and bamboo sticks were used to support individual plants (Figure 4.2)

Plants were left to reach full maturity before the experiment were terminated. This was achieved when stems turned black, pods dried and black and seed hard. Plants were removed for nematode extraction at the junction of the stem and root. The soil in the pot was thoroughly mixed and 200 ml samples were taken for assessment of the nematode population in the soil. The soil samples were extracted and nematode numbers assessed as described in Section 2.3.1. Data collected were number of tillers, percentage chocolate spot severity (inoculated leaves) stem lesion length, lesion girth, nematode population in plant tissue and grain yield. *D. gigas* reproduction factor was calculated by dividing final nematode population by initial nematode population.

A scale of 1-9 (Hanounik and Sikora, 1980) was used in assessing severity symptoms of *D. gigas* (SSI) with index points:

1 = no visible attack of the plant

3 = traces of necrosis at base of the stem

5 = leaves and stem deformed on 10 to 25 % plant

7 = stem with necrosis, swelling and distortion reaching 75 % plant

9 = plant dead or heavily infested

4.4. Statistical analysis

The data obtained from the first experiment was pooled and analysed together with the data including nematode inoculation alone (0, 30, 100, 300, 1000 and 3000 per plant) in the second experiment. The analysis was done using the mixed model for multiple experiments in GenStat 18th edition (VSN, UK) with appropriate blocking. Exponential regression models were fitted for the *D. gigas* only experiment. Exponential models were fitted to have an asymptote at maximal values of the measurement. Data were log10 transformed to stabilize the residual variance and aid interpretation. The second experiment involving *D. gigas* and *B. fabae* was analysed using analysis of variance using GenStat 18th ed, (VSN, UK) with appropriate blocking.

Figure 4.2. Plant supported with bamboo stick and protected from flower thrips with Sachet of *Amblyseius cucumeris,* Pergande.

4.5. Results

4.5.1. Final *D. gigas* population (*D. gigas* inoculated alone)

Final *D. gigas* populations increased with increasing dose of *D. gigas* dose inoculated (Figure 4.3: exponential regression, P<0.001, d.f. = 59, log10 *D. gigas* population extracted = 7.26 - 7.30 x ($0.73^{\log10\ (1+\ \text{number of nematode inoculated})}$). Treatments with the highest initial dose inoculated (3000 nematodes per plant) had the highest concentration of nematodes extracted, although this was similar to plants inoculated with 1000 nematodes per plant. Percentage variance accounted for by the regression was 79.9.

4.5.2. Reproduction rate of *D. gigas* in *V. faba* plants (*D. gigas* inoculation alone)

Plants inoculated with an initial nematode dose of 100 nematodes per plant had significantly higher (P = 0.01) reproduction rates (Table 4.1). The highest initial nematode dose (3000 nematodes per plant) had the lowest reproduction rate.

4.5.3. Reproduction rate of *D. gigas* (co- inoculation)

B. fabae inoculation significantly (P=0.01) reduced the reproduction rate of *D. gigas* (Table 4.2). The reduction in the reproduction rate was greater at the highest concentration of *B. fabae* inoculum (2×10^6 conidia/ml). There was no difference between 2×10^3 and 2×10^6 conidia/ml except at 100 nematodes per plant alone.

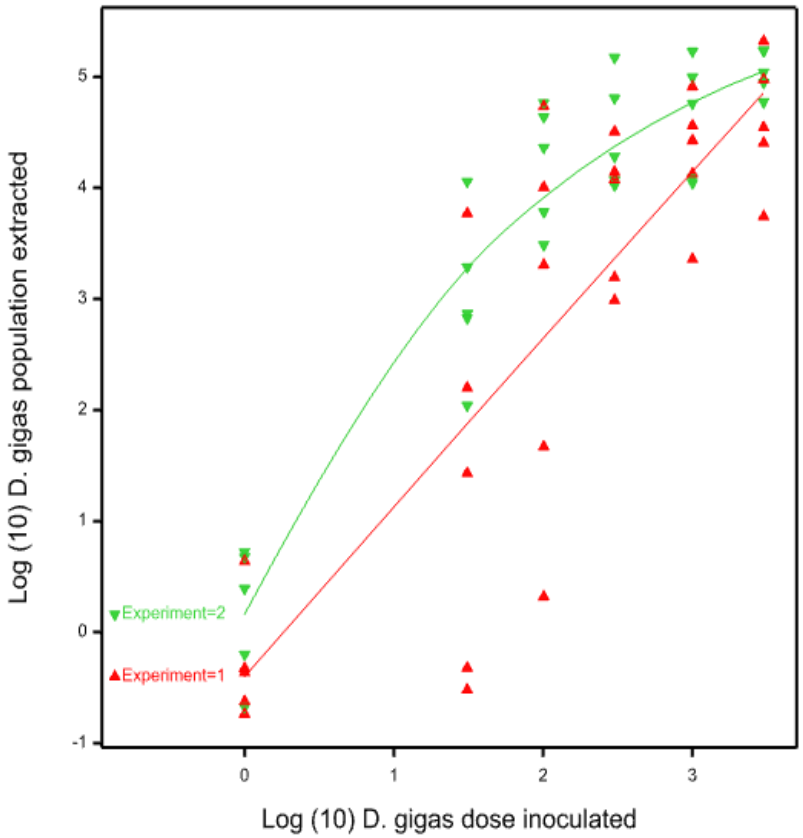

Figure 4.3. The relationship between log10 (number of *D. gigas* inoculated +1) and log10 (number of *D. gigas* extracted +1) in experiments in 2017 and 2018. Best fitting exponential shown in red 2017 (Experiment 1) and green 2018 (Experiment 2). Data were evaluated from five replicates at 14- 15 weeks following inoculation.

Table 4.1. Reproduction rate of *D. gigas* in *V. faba* plants (*D. gigas* alone) 14-15 weeks following inoculation. Data were evaluated from five replicates and back transformed from log10 (x+1)						
D. gigas dose inoculated	0	30	100	300	1000	3000
Reproduction rate	0	38.7	83.1	64.1	34.3	12.4
LSD: 33.1 P value: 0.01 C.I: 50.4 (Upper), 27.1 (Lower)						

Table 4.2. Reproduction rate of *D. gigas* in *V. faba* plants (co-inoculation) 14-15 weeks following inoculations. Data were evaluated from five replicates and back transformed from log10 (x+1)						
	D. gigas dose inoculated					
B. fabae concentration	0	30	100	300	1000	3000
	Reproduction factor					
0	0	54.6	110.9	75.1	32.4	11.3
2×10^3	0	32.5	94.6	69.7	23.4	3
2×10^6	0	30.3	98.2	52.1	19.6	1.4
LSD: 30.4 P value: 0.01 C.I: 48.2 (Upper), 30.60 (Lower)						

4.5.4. Stem lesion length of *D. gigas* (*D. gigas* inoculated alone)

The stem lesion length increased with increasing dose of *D. gigas* inoculum (Figure 4.4, exponential regression P<0.001, d.f. = 59, stem lesion length (cm) = -9.11+ 8.76 x ($1.57^{\log10\ (1+\ \text{number of nematode inoculated})}$). Stem lesion length was highest in plants with 3000 nematodes per plants. Percentage variance accounted for by the regression was 69.9.

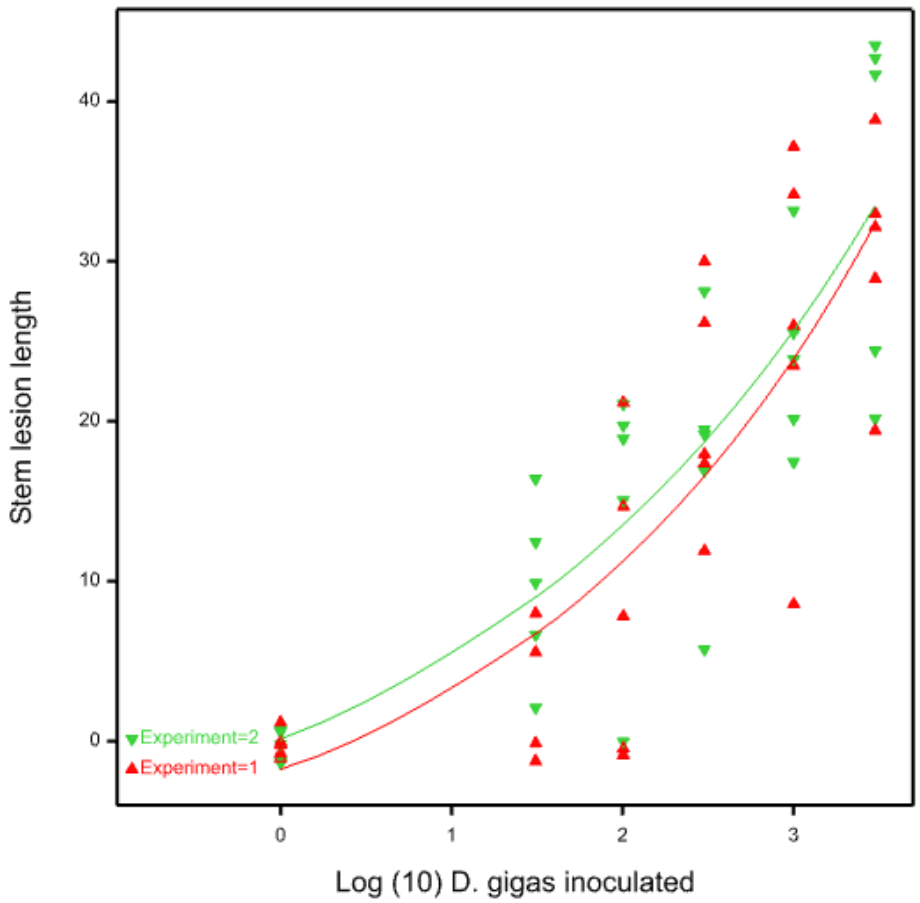

Figure 4.4. Relationship between log10 (number of *D. gigas* inoculated +1) and stem lesion length. Best fitting exponential shown in red 2017 (Experiment 1) and green 2018 (Experiment 2). Data were evaluated from five replicates 8-9 weeks following inoculation.

4.5.5. *D. gigas* severity symptom index (*D. gigas* inoculated alone)

D. gigas severity symptom index score increased with increasing dose of *D. gigas* inoculum (Figure 4.5, exponential regression, P<0.001, d.f. = 59, *D. gigas* severity symptom index = -4.13- 5.13 × (1.26 $^{\log 10}$ ($^{1+ \text{ number of nematode inoculated}}$). Treatments with the highest number of nematodes had the highest severity symptoms index. Percentage variance accounted for by the regression was 82.6.

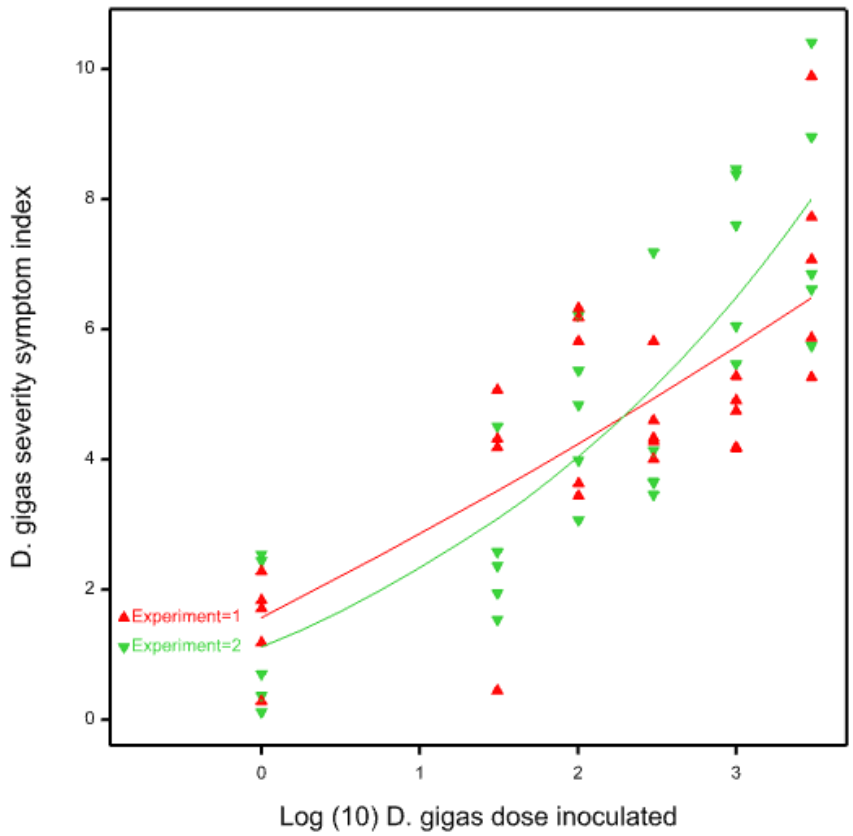

Figure 4.5. The relationship between log 10 (number of *D. gigas* inoculated +1) and *D. gigas* severity symptoms index. Best fitting exponential shown in red 2017 (Experiment 1) and green 2018 (Experiment 2). Data were evaluated from five replicates 8-9 weeks following inoculation.

4.5.6. Number of tillers (*D. gigas* inoculated alone)

The number of tillers on a plant decreased with increasing dose of *D. gigas* inoculum (Figure 4.6, exponential regression P<0.001, d.f. = 59, number of tillers = 3.02 -0.68 × (1.4 × $^{\log10\ (1+\text{number of nematode inoculated})}$). Maximum mean number of tillers was obtained from treatments un-inoculated with nematodes, while treatments with 3000 nemaodes per plant had the fewest tillers. Percentage variance accounted for by the regression was 28.8.

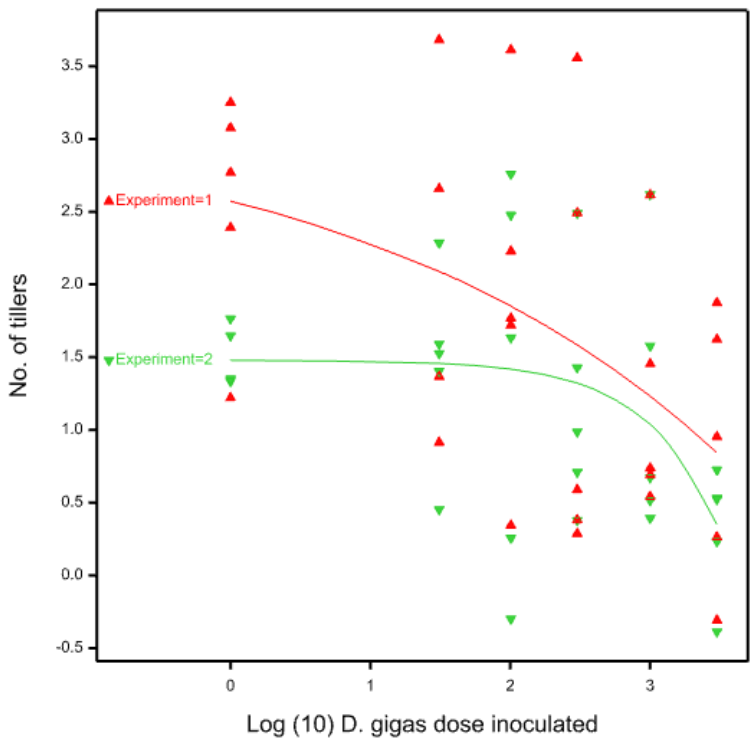

Figure 4.6. Relationship between log10 (number of *D. gigas* inoculated +1) and number of tillers. Best fitting exponential shown in red 2017 (Experiment 1) and green 2018 (Experiment 2). Data were evaluated from five replicates 8-9 weeks following inoculation.

4.5.7. Stem lesion girth (*D. gigas* inoculated alone)

Stem lesion girth increased with increasing dose of *D. gigas* inoculation (Figure 4.7, exponential regression, P<0.001, d.f. = 59, stem lesion girth = -2.64 – 2.73 × (1.35 × $^{\log10\ (1+\text{number of nematode inoculated})}$). Plants inoculated with 3000 nematodes per plants had the highest stem lesion girth. Percentage variance accounted for by the regression was 60.3.

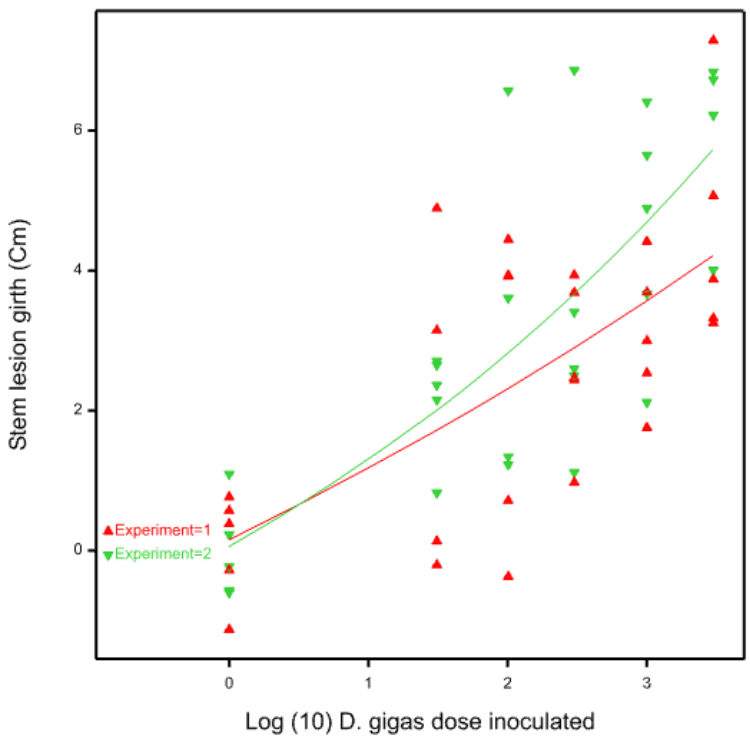

Figure 4.7. Relationship between log10 (number of *D. gigas* inoculated +1) and lesion girth. Best fitting exponential shown in red 2017 (Experiment 1) and green 2018 (Experiment 2). Data were evaluated from five replicates 8-9 weeks following inoculation

4.5.8. Grain yield (*D. gigas* inoculated alone)

Grain yield was reduced with increasing dose of *D. gigas* inoculum (Figure 4.8; exponential regression P<0.001, d.f. = 59, grain yield = 16.99 – 1.55 × (1.85 × $^{\log10\ (1+\ \text{number of nematode inoculated})}$). Uninoculated plants had the highest grain yield, while treatments with the highest number of nematodes inoculated had the least grain yield. Percentage variance accounted for by the regression was 28.8.

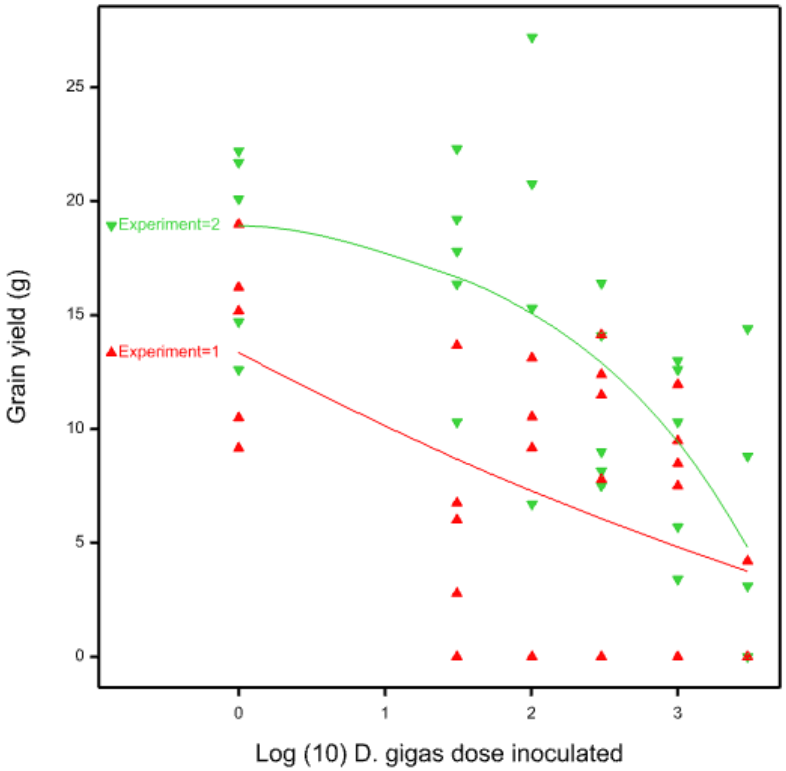

Figure 4.8. Relationship between log10 (number of *D. gigas* inoculated +1) and grain yield. Best fitting exponential shown in red 2017 (Experiment 1) and green 2018 (Experiment 2). Data were evaluated from five replicates 8-9 weeks following inoculation.

4.5.9. Relationship between grain yield and *D. gigas* severity symptom index (*D. gigas* inoculated alone)

There was a negative relationship between grain yield and *D. gigas* severity symptom index. (Figure 4.9, exponential regression, P<0.001, d.f. = 59, grain yield (g) = 21.5 – 5.8 x (1.15 x $^{(\text{severity symptom index})}$). Percentage variance accounted for by the regression was 44.8.

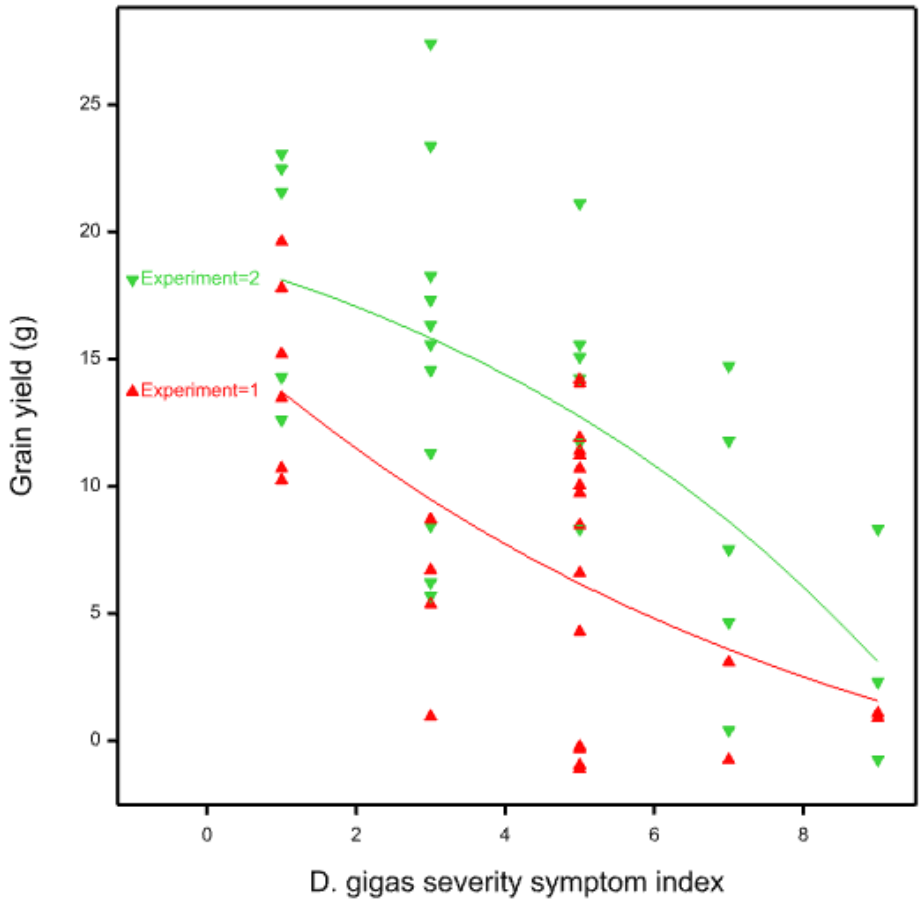

Figure 4.9. Relationship between *D. gigas* severity symptom index and grain yield. Best fitting exponential shown in red (Experiment 1) and green (Experiment 2). Data were evaluated from five replicates 8-9 weeks following inoculation.

4.5.10. *D. gigas* population in soil (*D. gigas* inoculated alone)

Low nematode population was extracted from the soil (Figure 4.10, exponential regression, P<0.001, d.f. = 58, log 10 *D. gigas* population in 200 ml soil = -0.562- 0.458 × (1.495 × $^{\log 10 (1+ \text{number of nematode inoculated})}$). No nematode was extracted from soil samples at very low initial dose (30 nematodes per plant). Percentage variance accounted for by the regression was 34.8.

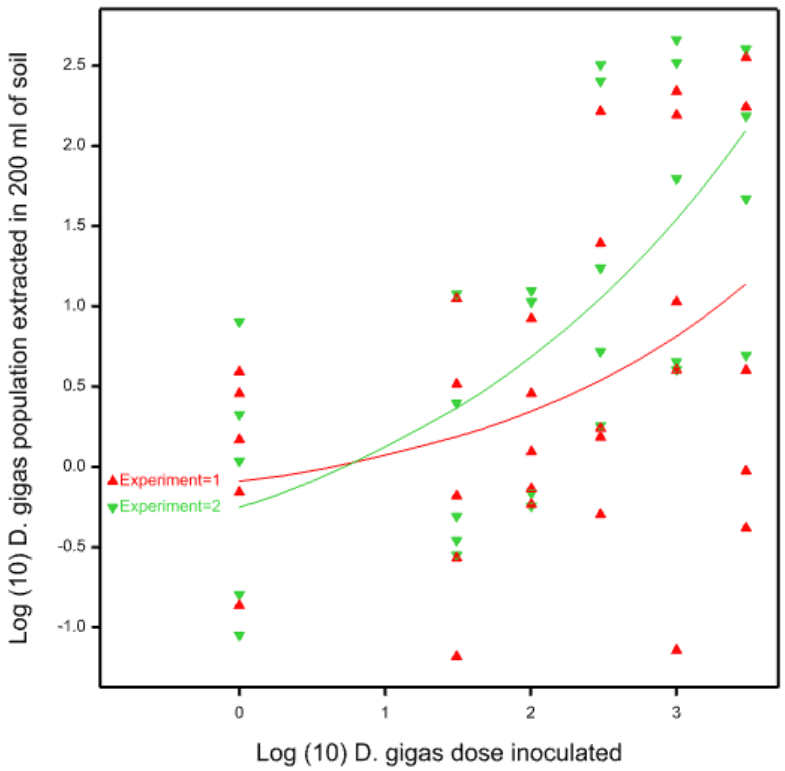

Figure 4.10. Relationship between log10 (number of *D. gigas* inoculated +1) and log10 (number of *D. gigas* extracted from 200 ml soil +1). Best fitting exponential shown in red (Experiment 1) and green (Experiment 2). Data were evaluated from five replicates 14-15 weeks following inoculation.

4.5.11. *D. gigas* population (co-inoculation of *B. fabae* and *D. gigas*)

Co-inoculation of *B. fabae* and *D. gigas* resulted in an antagonistic relationship as the number of *D. gigas* extracted from plants inoculated with nematode alone was significantly (P= 0.001, Appendix table 1) higher than those with co-inoculation of both organisms (Figure 4.11). Treatments at the highest dosage of the two organisms had fewer nematodes than other treatments except treatments with doses of 30 nematodes and 2×10^6 /ml *B. fabae* inoculum.

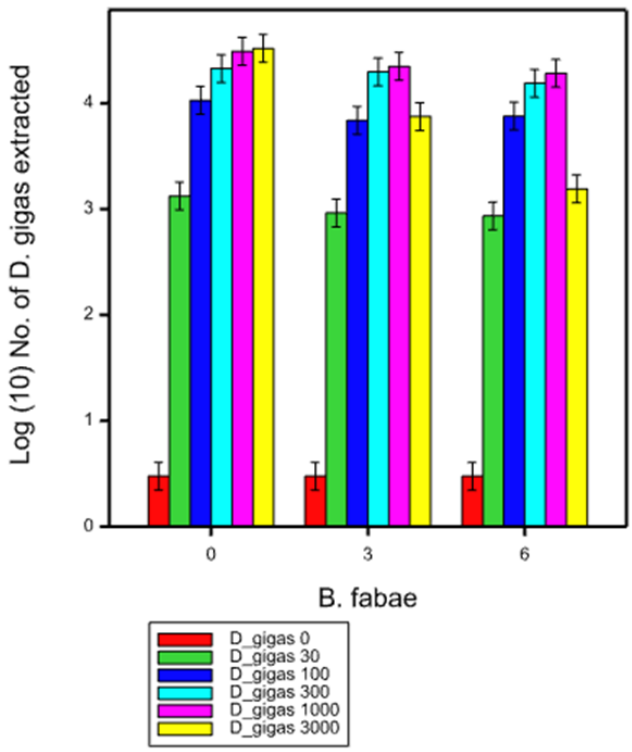

Figure 4.11. The effects of co-inoculation of *B. fabae* and *D. gigas* on population of *D. gigas* plotted on a log scale (log10 (x+1). Data were evaluated from five replicates 8-9 weeks following inoculation. 0 = Uninoculated, 3 = 2×10^3 *B. fabae* spores inoculated, 6= 2×10^6 *B. fabae* spores inoculated. Error bars denotes standard error of difference.

4.5.12. Lesion length (co- inoculation of *D. gigas* and *B. fabae*)

There was a reduction in lesion length (Figure 4.12, P<0.001, Appendix table 2) caused by *D. gigas* when co-inoculated with *B. fabae*, compared, with lone inoculation of *D. gigas*. This indicates an antagonistic relationship between the nematode and the fungus. The interaction with *B. fabae* was substantial at 2×10^3 conidia/ml lesion length was decreased at 30, 1000 and 3000 *D. gigas*/plant but increased at 100.

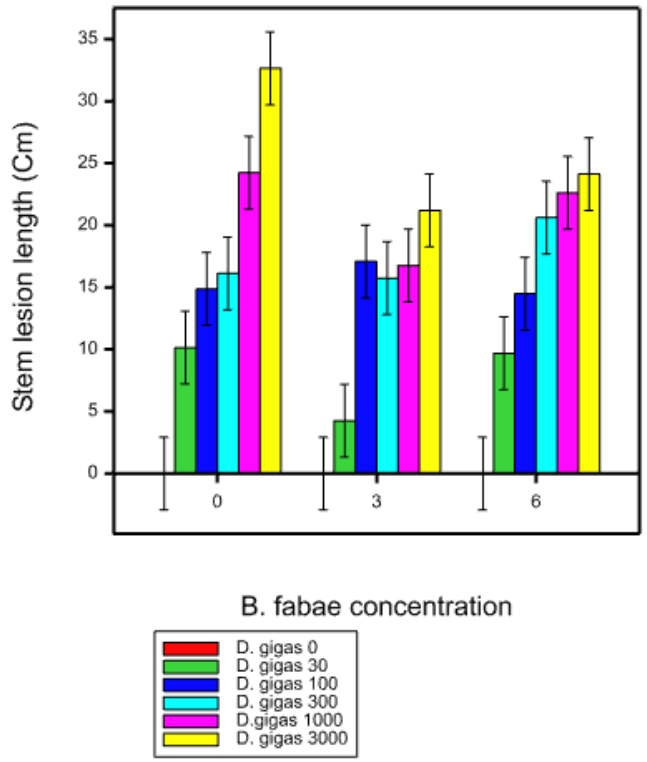

Figure 4.12. The effects of co-inoculation of *B. fabae* and *D. gigas* on stem lesion length. Data were evaluated from five replicates 8-9 weeks following inoculation, 0 = Uninoculated, 3 = 2×10^3 *B. fabae* spores inoculated, 6= 2×10^6 *B. fabae* spores inoculated. Error bars denotes standard error of difference.

4.5.13. Number of tillers (co-inoculation of *D. gigas* and *B. fabae*)

Co-inoculation of both organisms resulted in an antagonistic relationship as the number of tillers from plants inoculated with nematode alone was significantly (P= 0.001, Appendix table 1) higher than those with co-inoculation of both organisms (Figure 4.13). The antagonistic interaction was more evident at higher dosage of *B. fabae* spore concentration (2×10^6) than 2×10^3 spores/ml. At 1000 nematodes per plant, number of tillers decreased with increasing *B. fabae* inoculation.

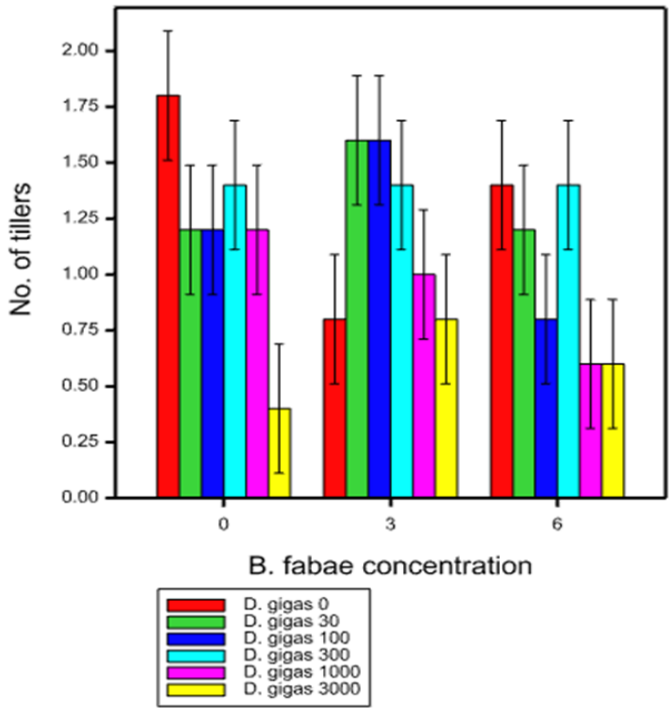

Figure 4.13. The effects of co- inoculation of *B. fabae* and *D. gigas* on number of tillers. Data were evaluated from five replicates 8-9 weeks following inoculation. 0 = Uninoculated, 3 = 2×10^3 *B. fabae* spores inoculated, 6= 2×10^6 *B. fabae* spores inoculated. Error bars denotes standard error of difference.

4.5.14. Stem lesion girth (co-inoculation of *D. gigas* and *B. fabae*)

Stem lesion girth was significantly higher (Figure 4.14, P<0.001, Appendix table 4) when *D. gigas* was inoculated alone than when co-inoculated with *B. fabae*, except for treatments with 30 nematodes per plant. At 3000 nematodes per plant, stem lesion girth decreased with increasing *B. fabae* inoculation.

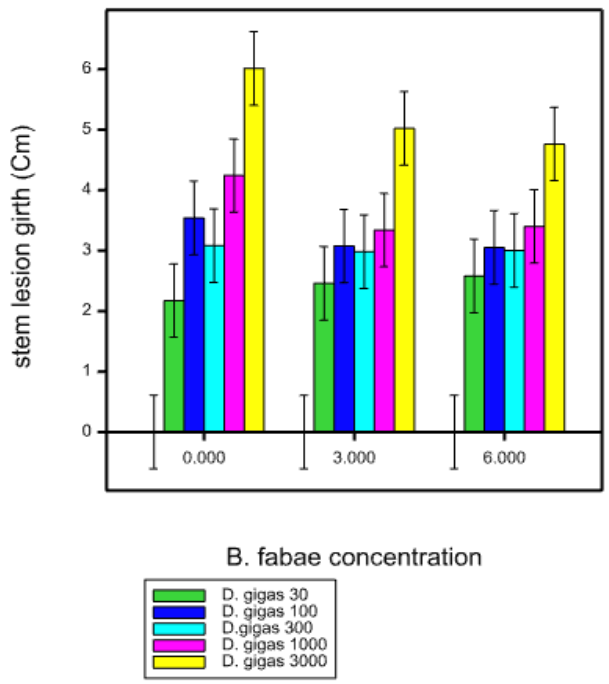

Figure 4.14. The effects of co-inoculation of *B. fabae* and *D. gigas* on stem lesion girth. Data were evaluated from five replicates 8-9 weeks following inoculation, 0 = Uninoculated, 3 = 2×10^3 *B. fabae* spores inoculated, 6= 2×10^6 *B. fabae* spores inoculated. Error bars denotes standard error of difference.

4.5.15. Grain yield (co- inoculation of *D. gigas* and *B. fabae*)

There are indications of synergistic interaction between *D. gigas* and *B. fabae* (Figure 4.15). There was greater reduction ($P<0.001$, Appendix table 5) in grain yield in the treatment with co-inoculation of *D. gigas* and *B. fabae* compared with treatments with either organism alone or left uninoculated. Grain yield decreased with increasing rates of both *D. gigas* and *B. fabae* inoculum. Treatments with the highest dose of *D. gigas* and *B. fabae* had the least grain yield, while the uninoculated plants had the highest grain yield.

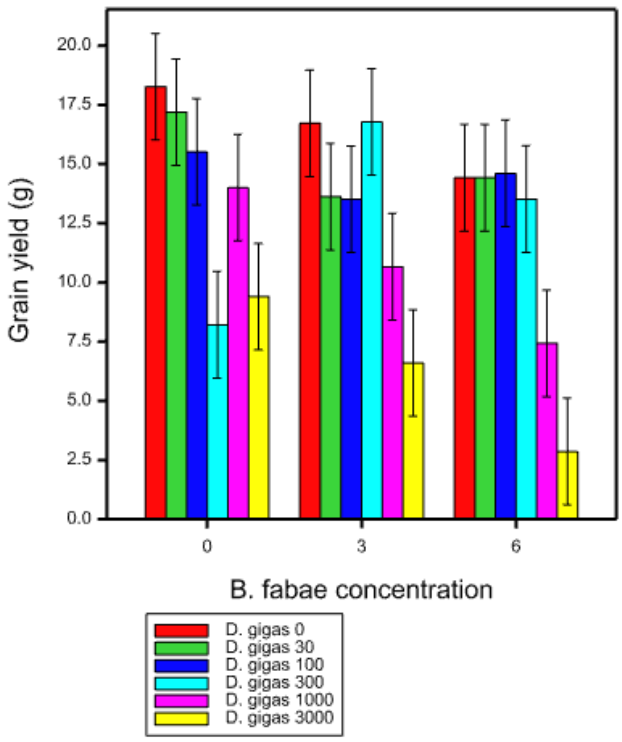

Figure 4.15. The effects of co- inoculation of *B. fabae* and *D. gigas* on grain yield at harvest. Data were evaluated from five replicates 8-9 weeks following inoculation. 0 = Uninoculated, 3 = 2×10^3 *B. fabae* spores inoculated, 6= 2×10^6 *B. fabae* spores inoculated. Error bars denotes standard error of difference

4.5.16. *D. gigas* severity symptom index (co- inoculation of *D. gigas* and *B. fabae*)

D. gigas severity symptom index was higher with *D. gigas* inoculation alone than with *B. fabae* inoculation (Figure 4.16). Treatments with 3000 nematodes per plant had significantly greater severity symptom index than the other treatments ($P < 0.01$, Appendix table 6). At 100 nematodes per plant, severity decreased with increasing *B. fabae* inoculum.

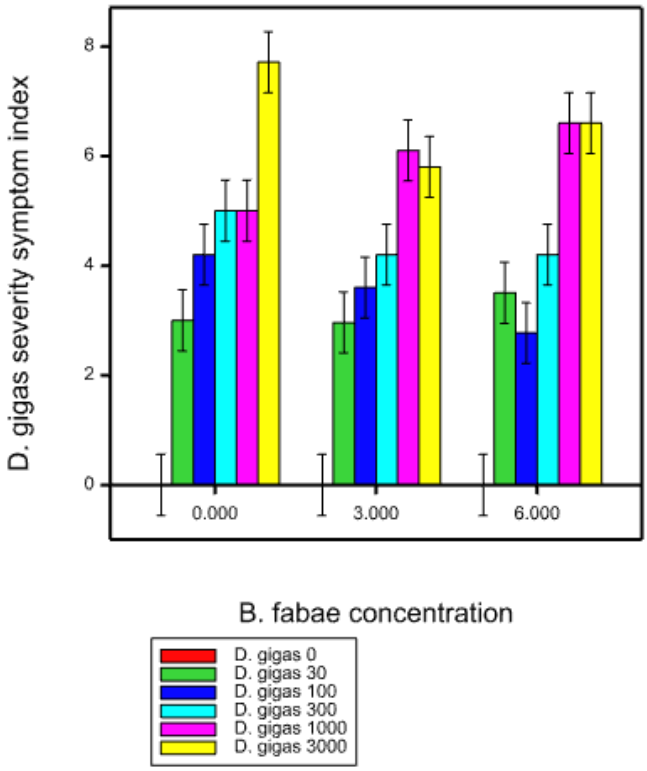

Figure 4.16. The effects of co- inoculation of *B. fabae* and *D. gigas* on *D. gigas* severity symptom index. Data were evaluated from five replicates 8-9 weeks following inoculation. 0 = Uninoculated, 3 = 2×10^3 *B. fabae* spores inoculated, 6= 2×10^6 *B. fabae* spores inoculated. Error bars denotes standard error of difference.

4.5.17. *D. gigas* population in soil (co- inoculation of *D. gigas* and *B. fabae*)

Co-inoculation of *B. fabae* and *D. gigas* resulted in an antagonistic interaction between the two organisms (Figure 4.17). The number of *D. gigas* extracted from 200 ml soil was reduced ($P < 0.01$, Appendix table 7) due to the co-inoculation of both organisms. There was an increase in the number of nematodes extracted in accordance with the incremental dosage of *D. gigas*, except at highest dosage of both organisms.

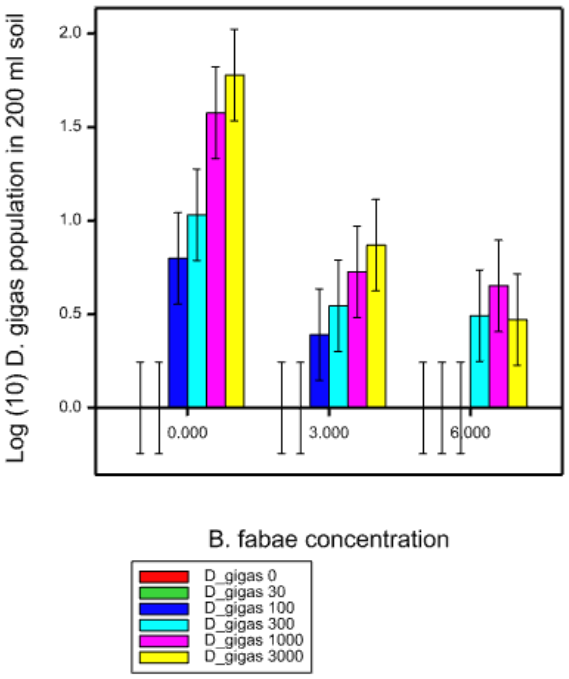

Figure 4.17. The effects of co- inoculation of *B. fabae* and *D. gigas* on *D. gigas* population in 200 ml soil. Data were evaluated from five replicates 8-9 weeks following inoculation. 0 = Uninoculated, 3 = 2×10^3 *B. fabae* spores inoculated, 6= 2×10^6 *B. fabae* spores inoculated. Error bars denotes standard error of difference.

4.5.18. Chocolate spot severity (co- inoculation of *D. gigas* and *B. fabae*)

Inoculation of *D. gigas* at any dose did not significantly (P= 0.97, Appendix, table 8) increase the severity of chocolate spot (Figure 4.18). Also, no significant reduction was observed, suggesting an additive interaction between the two organisms. The trends clearly show an increase in chocolate spot severity due to incremental *B. fabae* spore dosage as expected.

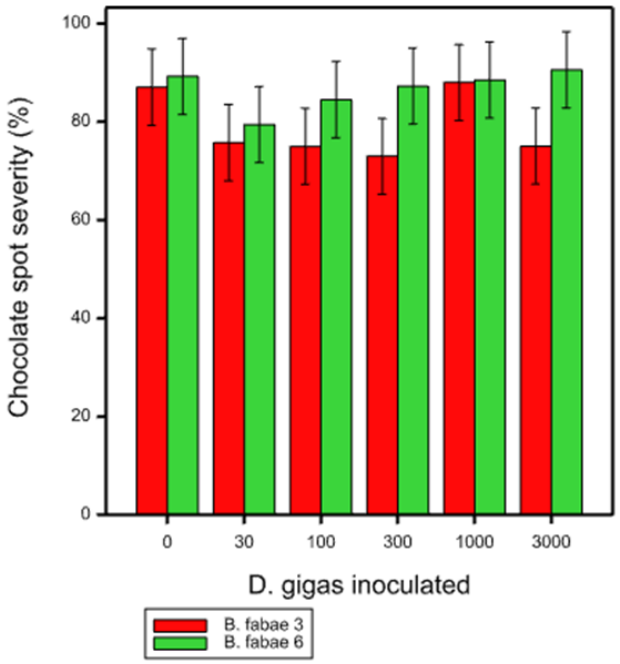

Figure 4.18. The effects of co- inoculation of *B. fabae* and *D. gigas* on chocolate spot severity. Data were evaluated from five replicates 8-9 weeks following inoculation. *B. fabae* 3 = 2×10^3 *B. fabae* spores inoculated, *B. fabae* 6= 2×10^6 *B. fabae* spores inoculated. Error bars denotes standard error of difference.

4.6. Discussion

The results demonstrate that *B. fabae* did not affect the multiplication of *D. gigas* on faba bean. The severity of symptom index of *D. gigas* was higher when *D. gigas* was inoculated alone compared to when co-inoculated with *B. fabae*. Also *D. gigas* did not increase the severity of chocolate spot induced by *B. fabae*. This gives an indication of antagonistic interactions between the two organisms.

However, a greater reduction in grain yield was observed when both organisms were co-inoculated compared with either organism alone or plants left uninoculated. This signifies a synergistic damaging interaction of the two organisms on grain yield. Nematode and fungi interactions have been reported to cause worse damage on crops than either of the organisms. For example, Hillnhuttter *et.al.* (2011) reported synergistic damage between *D. dipsaci* and *Rhizoctonia solani* (AG 2–2IIIB) on sugar beet.

Antagonistic interactions on the population of *D. gigas* observed in plant tissue and in soil. Nematodes extracted from plant tissues and soil in co-inoculation of both organisms were fewer than those from lone inoculation of *D. gigas.* The reduction in the population of *D. gigas* extracted was dependent on the concentration of *B. fabae* conidia inoculated. More reduction in the population of *D. gigas* extracted was observed at 2×10^6 conidia/ml compared to 2×10^3. Very low numbers of nematodes were extracted from the soil compared to the plant tissue population. The low population recovered from the soil may be because the nematode is able to survive in dry conditions such drying or dried leaf tissue.

The reproduction rate of *D. gigas* was reduced by *B. fabae*. The damage done on the leaf by *B. fabae* may have affected the nematode feeding. Also, *B. fabae* produces toxins which may create unfavourable conditions for the nematode.

The reproduction rate of *D. gigas* alone and with co- inoculation with *B. fabae* fell with increasing dose of *D. gigas* only above 100 nematodes per plant. The reproduction rate at 30 nematodes per plant was lower than 100 and 300 nematodes per plant respectively. The low reproduction rate at low inoculation doses may suggest plant defences being effective initially but exhausted as inoculum dose rises. Low reproduction rates of *D. gigas* observed at high initial nematode doses may be due to competition for limited resources (including space and nutrients) among the nematodes. This can result in reduced fecundity.

The low reproduction rate at high inoculum doses reported in this study is similar to the findings of Seinhorst (1970) and Greco and di Vito (2009).

The reduction in reproduction rate of *D. gigas* by *B. fabae* differs from with the finding of Griffin (1990) who reported that the fungus *Fusarium oxysporum* f. sp. *medicaginis* did not reduce reproduction of *D. dipsaci* when sequentially or simultaneously inoculated on alfalfa. The disparity in the results may be because *F. oxysporum* and *D. dipsaci* colonise different tissues of alfalfa, while *B. fabae* and *D. gigas* affect the same tissue of faba bean.

Susceptible genotypes to *D. gigas* in faba bean are characterised by increased stem swelling and brownish stem lesions (Caubel and Leclercq, 1989b). Severity symptom index and rate of reproduction are used as a measure of resistance in faba bean (Hooper, 1984). In the present study stem lesions and stem swelling (stem lesion girth) were observed both when *D. gigas* was singly inoculated and when it was inoculated in combination with *B. fabae*. The brown colour is a result of phenolic compounds produced around the damaged cells (Perry and Moens, 2013). Phenolic compounds are produced in plants when they are challenged by pathogens or stressed. The length and girth of the lesion produced was dependent on the number of *D. gigas* inoculated. A reduction in lesion length and girth were observed when *D. gigas* was co-inoculated with *B. fabae*. This may be because the fungus reduced the reproduction rate of the nematode, and hence reduced the extent of stem lesions and girth. Both stem lesion length and stem lesion girth contributed to the reduction in grain yield.

D. gigas did not increase the severity of chocolate spot induced by *B. fabae.* This result is similar to the work reported in some other systems. Vrain (1987) reported that the severity of wilt induced by *Verticillium albo-atrum* on alfalfa did not increase with inoculation of *Pratylenchus penetrans.* However, Griffin (1990) reported that *D. dipsaci* increased the severity of wilt induced by *Fusarium oxysporum* f. sp. *Medicaginis* on alfalfa. One likely reason for non-increase in the severity of chocolate spot by *D. gigas* is that *B. fabae* causes cell death and colonises dead substrate. *Ditylenchus* can only propagate in a living host and not in dead tissue (Perry and Moens, 2013).

4.7. Conclusion

The present study has established that *B. fabae* partly suppresses the reproduction of *D. gigas* on faba bean. *D. gigas* did not increase the severity of chocolate spot induced by *B. fabae*. The grain yield of faba bean was worsened due to co-inoculation of both organisms.

Chapter 5: Induction of defence response in faba bean after inoculation with *D. gigas* and *B. fabae*

5.1. Abstract

Plants have the potential to repel pathogens when infected. This may be due to their pre-existing defences or to increased production of defence responses after infection. In the light of this, the hypothesis that the order of infection (i.e., whether *D. gigas* infects before *B. fabae* or vice versa) will have an effect on induced resistance and affect crop production was tested. Potted faba bean (cv. Fuego) plants were inoculated with either *B. fabae* or *D. gigas* at 2 weeks after planting. A second inoculation was done 2 weeks after the first one. The second *B. fabae* inoculation was applied on the upper leaves with protection to prevent spill over to the previously inoculated leaves. Inoculation of *B. fabae* did not induced a defence response against *D. gigas*. However, pre-inoculation of *B. fabae* did reduce subsequent *B. fabae* infection, suggesting an induced defence response specific to this species

Keywords: *D. gigas, B. fabae,* faba bean, induced defence, controlled environment.

5.2. Introduction

This chapter builds on the findings from the glasshouse experiment (Chapter 5) where lone *D. gigas* inoculation had more nematodes extracted than co-inoculation with *B. fabae*. Also *D. gigas* inoculation did not increase the severity of chocolate spot when co-inoculated with *B. fabae*. This indicates an antagonistic interaction between *D. gigas* and *B. fabae.* This suggests that an induced response may have been triggered by either the fungus or the nematode.

It is well-established that plants resist most pathogens that exist in nature (non-host resistance). This process is linked with defence response (Heath, 2000b). However, some plants are susceptible to some pathogens or isolates or races of those pathogens. Such plants lack the means to detect the pathogens and therefore, unable to defend themselves against the pathogens. When plants are induced or infected, their ability to defend themselves increases (Conrath *et al*., 2002).

Plants respond to attack from pathogens in many ways. When challenged by nematodes, plants produce anti-nematode enzymes and compounds, reinforce cell walls and trigger hypersensitive responses (Sato *et.al*., 2019). Some nematodes can infest plants without the induction of a strong immune response from the host plant. This is due to the possession of a number of virulence traits (Warmerdam *et al*., 2018). For example, the possession of stylets by nematodes makes it easier for them to penetrate and infest plants.

Some types of infection or other treatments can induce disease resistance in plants (Vallad and Goodman, 2004). For example, infection of a lower leaf of some plants with certain pathogens resulted in systemic resistance to the same or different pathogens (Kuc 1987). Increased production of reactive oxygen species such as hydrogen peroxide, hydroxyl radicals and superoxide anions have been frequently observed upon infection of plants by fungi (Patykowski and Urbanek, 2003). Chemical inducers can also be used to trigger induced defence response in plants. Many studies have reported the increased production of pathogen inhibitors upon inoculation of faba bean with *B. fabae* or application of chemical inducers. Hassan *et. al.* (2006) reported that application of citric, benzoic and salicylic acids resulted in reduction of chocolate spot disease caused by *B. fabae* in faba bean. El-Komy (2014) reported increased production of reactive oxygen species (ROS), lipid peroxidation and antioxidant enzymes (superoxide dismutase, catalase and ascorbate peroxidase) upon

infection of faba bean by *B. fabae*. Balthazar *et al*. (2020) noted that five putative defence genes were induced at the infection site of *B. cinerea* on cannabis.

Due to the negative effects of chemical pesticides (detrimental effects on the health of the users, their persistency in the environment and development of resistance to pesticides by pathogens), there is a need for alternative measures of disease management. Biopesticides are less toxic and have reduced risks of expression of resistance against pathogens compared to chemical pesticides. However, they have lower efficacy, slow rate of control and are highly susceptible to adverse environmental conditions. Induced resistance presents an alternative measure that is promising and has broad spectrum effects.

There is a scarcity of information in the literature on how induced responses produced in plants as a result of necrotrophic fungi inoculation affect biotrophic organisms, or vice-versa. The objective of this study, therefore, is to determine if an induced defence response is produced in faba bean after inoculation with either *B. fabae* or *D. gigas* and how it affects productivity. This chapter test the hypothesis as to whether or not the order of infection (i.e., whether *D. gigas* infects before *B. fabae* or vice versa) has an effect on triggering induced resistance and so an effect on crop production.

5.3. Materials and methods

Potted faba bean (cv. Fuego) were first inoculated with 300 individuals of mixed life stages of *D. gigas* (applied on the growing tips) or a spore suspension of *B. fabae* (2×10^5 spores / ml applied with a paint brush). A second inoculation was done 2 weeks later (4 weeks after sowing). The second inoculation of *B. fabae* was applied on the upper leaves carefully to prevent spill over to the previously inoculated leaves. The experiment was a 3 x 3 factorial laid out in a randomised complete block design with five replicates and 9 treatments (Table 5.1). The experiment was maintained at a temperature of 15/12 ^{o}C (day /night), relative humidity 70 / 90 % (day / night) and photoperiod of 16 hours / day. Data were collected on the percentage severity of chocolate spot induced by *B. fabae* and number of nematodes extracted using the modified Baermann extraction method.

The experiment was repeated.

Table 5.1. Treatment combinations

First Inoculation	**Second Inoculation**
B. fabae	*D. gigas*
B. fabae	*B. fabae*
B. fabae	Water
Water	*B. fabae*
Water	Water
Water	*D. gigas*
D. gigas	*B. fabae*
D. gigas	Water
D. gigas	*D. gigas*

5.4. Statistical analysis

Data were analysed using the mixed model for multiple experiments in GenStat 18th edition (VSN, UK) with appropriate blocking. Data were log10 transformed to stabilize the residual variance and aid interpretation. Although the experiment was set up as a 3 x 3 factorial, the treatments without either pathogen (procedural controls to show that there was no spill-over of inoculum or cross-infection during the experiment) were exempted from the analysis, by using anova contrasts appropriate to the hypotheses under test (Tables 5.2, 5.3,5.4).

Table 5.2. Contrast matrix for the number of *D. gigas* and associated hypotheses					
Treatment 1	Treatment 2	Does pre-treatment with *B. fabae* affect *D. gigas*?	Does post-treatment with *B. fabae* affect *D. gigas*?	Does late treatment with *D. gigas* increase nematode count?	Which treatments have more *D. gigas*: early or late treatment with *D. gigas?*
B. fabae	*B. fabae*	0	0	0	0
B. fabae	Water	0	0	0	0
B. fabae	*D. gigas*	1	0	0	1.5
Water	*B. fabae*	0	0	0	0
Water	Water	0	0	0	0
Water	*D. gigas*	-1	0	0	1.5
D. gigas	*B. fabae*	0	1	-1	-1
D. gigas	water	0	-1	-1	-1
D. gigas	*D. gigas*	0	0	2	-1

Table 5.3. Contrast matrix for *B. fabae* severity on lower leaves			
Treatment 1	Treatment 2	Does pre-treatment with *D. gigas* affect score?	Does pre-treatment with *B. fabae* affect *B. fabae*?
B. fabae	*B. fabae*	0	1
D. gigas	*B. fabae*	1	0
Water	*B. fabae*	-1	-1

Table 5.4. Contrast matrix for *B. fabae* severity on upper leaves			
Treatment 1	Treatment 2	Does pre-treatment with *D. gigas* affect score?	Does pre-treatment with *B. fabae* affect *B. fabae*?
B. fabae	*B. fabae*	0	1
D. gigas	*B. fabae*	1	0
Water	*B. fabae*	-1	-1

5.5. Results

5.5.1. Population of *D. gigas* on leaves and stem

The population of *D. gigas* extracted from the stem was higher than that extracted from the leaves (Figure 5.1). Plants with double inoculation of *D. gigas* had significantly (P=0.01) higher number of nematodes in the stem. These plants also had significantly (P=0.01) more nematodes in the leaves but is similar to treatments that had both pre- inoculation of *D. gigas* and post inoculation of *B. fabae*. Plants pre-inoculated with *B. fabae* and post- inoculated with *D. gigas* had the fewest nematodes in the leaves, but the concentration was similar to plants pre-inoculated with water and post- inoculated with *D. gigas*. No nematodes were extracted from the stem of plants pre-inoculated with *B. fabae* and post- inoculated with *D. gigas*.

The contrast (Table 5.5, row 8) revealed that only the difference between early and late treatment with *D. gigas* was significant (P= 0.001). Early treatment with *D. gigas* resulted in increased number of *D. gigas* on the stem.

The contrast (Table 5.6, row 8) showed that only the early vs late treatment with *B. fabae* had significant (P= 0.001) effects on number of nematodes on stem. Early treatment with *D. gigas* resulted in a non-significantly increased number of *D. gigas* on the leaves

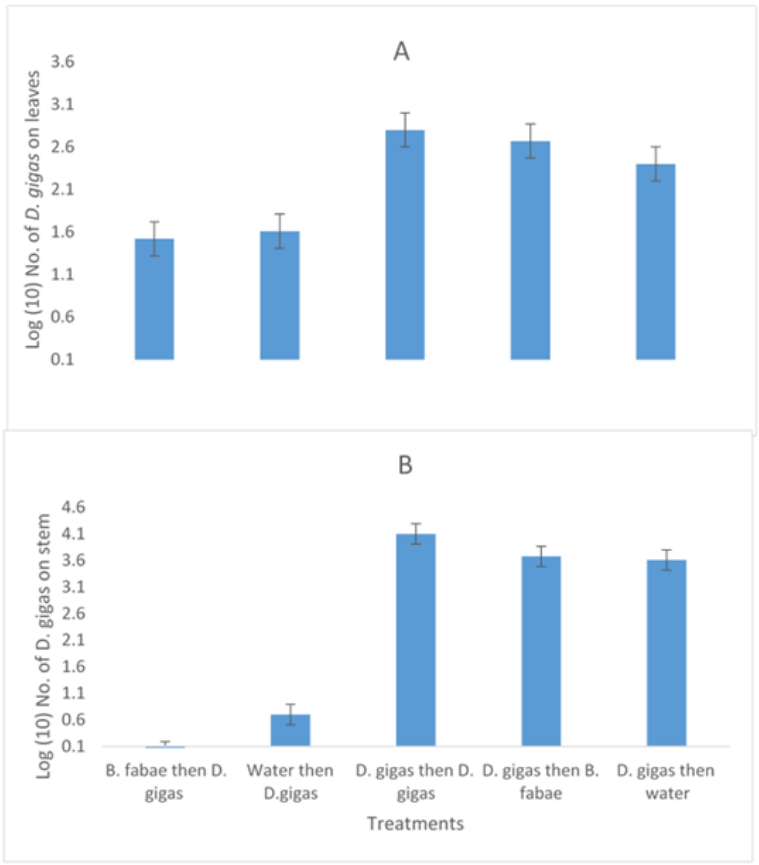

Figure 5.1. Number of *D. gigas* extracted on leaf (A) and stem (B) of faba bean leaves six weeks after the first inoculation. Plants were kept in the controlled environment and maintained at a temperature of 15/12 ºC (day /night), relative humidity 70/90 % (day/night) and photoperiod of 16 hours per day.

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Table 5.5. Analysis of variance table showing contrasts for the number of *D. gigas* extracted from leaves, six weeks after the first inoculation Tables 5.2-5.4)**.** Plants were kept in the controlled environment and maintained at a temperature of 15/12 ºC (day /night), relative humidity 70/90 % (day/night) and photoperiod of 16 hours per day for 4 weeks. Data were log10 transformed. Dg = *D. gigas*, Bf = *B. fabae*.

Source of variation	Df	s.s	m.s	v.r	F pr.
Experiment- stratum	1	6.8	6.8	9.6	-
Experiment. Treatment-stratum					
Treatment	4	16	4	5.7	0.06
If pre-treatment with Dg affects Bf?	1	0.1	0.1	0.2	0.7
If post-treatment with Bf affects Dg?	1	0.3	0.3	0.6	0.5
Does late treatment with Dg increase Dg.	1	0.6	0.6	0.9	0.4
Early vs late treatment with Dg.	1	14.9	14.9	21	0.01
Residual	4	2.8	0.7	0.7	-
Experiment. treatment. units-stratum	40	42.6	1.06	-	-
Total	49	68.2	-	-	-

Table 5.6. Analysis of variance table showing contrast for the number of *D. gigas* extracted from stems six weeks after the first inoculation**.** Plants were kept in the controlled environment and maintained at a temperature of 15/12 ºC (day /night), relative humidity 70/90 % (day/night) and photoperiod of 16 hours per day for 4 weeks. Data were log10 transformed**.** Dg = *D. gigas*, Bf = *B. fabae*.

Source of variation	Df	s.s	m.s	v.r	F pr.
Experiment stratum	1	13.7	13.7	6.2	-
Experiment. treatment-stratum					
Treatment	4	73.7	18.4	8.3	0.03
If pre-treatment with Dg affect Bf?	1	0.3	0.3	0.13	0.7
If post-treatment with Bf affect Dg?	1	0.02	0.02	0.01	0.9
Does late treatment with Dg increase Dg.	1	2.3	2.3	1.03	0.3
Early vs late treatment with Dg.	1	71.1	71.1	31.9	0.005
Residual	4	8.9	2.2	3.2	-
Experiment. treatment. units-stratum	40	28.18	0.7	-	-
Total	49	124.5	-	-	-

5.5.2. Relationship between *D. gigas* on leaves vs stems

There was a moderate positive linear relationship (r= 0.53, P= 0.001) between the number of *D. gigas* extracted from the stems and those extracted from the leaves (Figure 5.2). The number of *D. gigas* extracted from the stem of faba bean outnumbered those from the leaves.

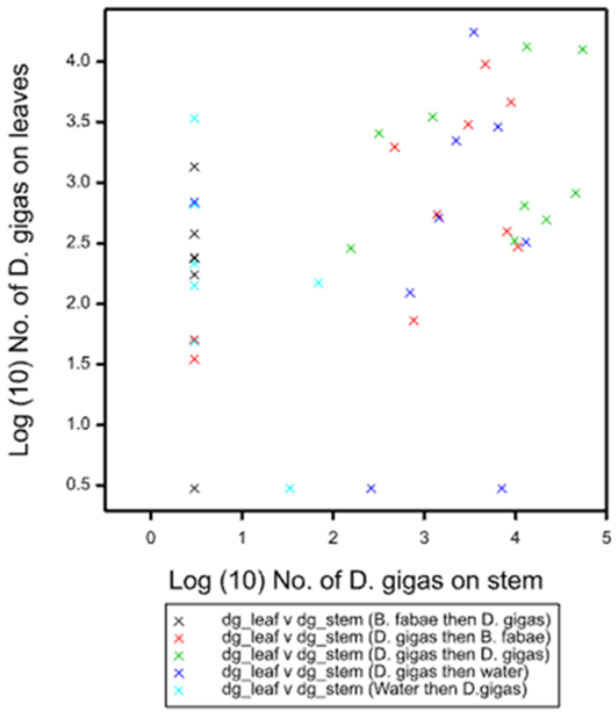

Figure 5.2. Correlation between number of nematodes extracted from the stem and number extracted from the leaves, six weeks after the first inoculation. Plants were kept in the controlled environment and maintained at a temperature of 15/12 ºC (day /night), relative humidity 70/90 % (day/night) and photoperiod of 16 hours per day.

5.5.3. Severity of chocolate spot in faba bean lower leaves

Post treatment with *D. gigas* or *B. fabae* did not increase the severity of chocolate spot in faba bean lower leaves inoculated on the first occasion (Figure 5.3). The trend of the severity of chocolate spot was similar in the 3 treatments (P= 0.17). The contrast (Table 5.7, 5.8, 5.9 and 5.10) showed that early vs late treatment with *D. gigas* was significant for week 2 alone. Early treatment with *B. fabae* resulted in an increased chocolate spot score.

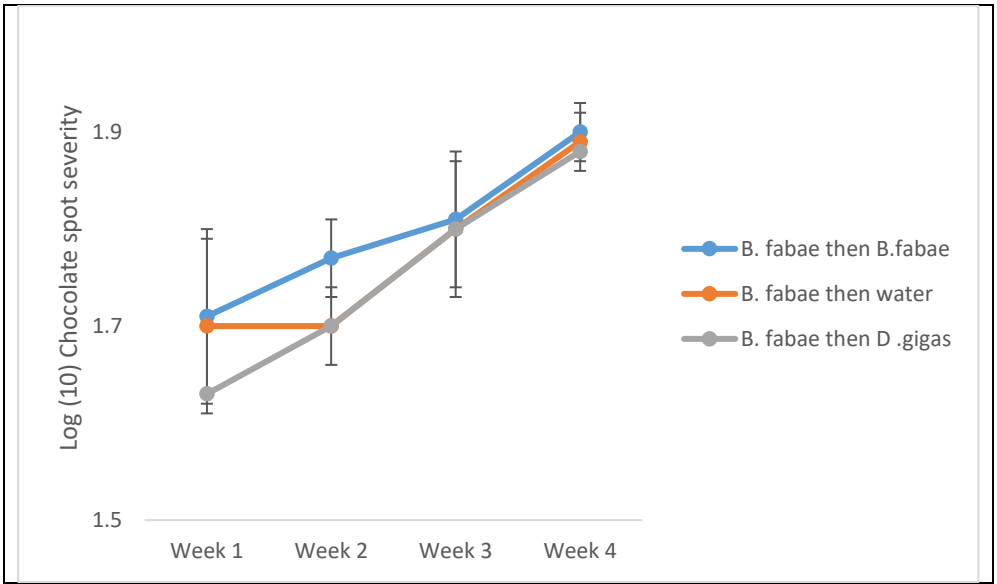

Figure 5.3. Chocolate spot severity on faba bean lower leaves. Plants were kept in the controlled environment and maintained at a temperature of 15/12 ºC (day /night), relative humidity 70/90 % (day/night) and photoperiod of 16 hours per day. Data were log10 transformed.

Table 5.7. Analysis of variance table showing contrast for the severity of *B. fabae* on lower leaves (week 1). Plants were kept in the controlled environment and maintained at a temperature of 15/12 ^{o}C (day /night), relative humidity 70/90 % (day/night) and photoperiod of 16 hours per day for 4 weeks. Data were log10 transformed. Dg = *D. gigas*, Bf = *B. fabae*.

Source of variation	df	s.s	m.s	v.r	F pr.
Experiment-stratum	1	1.16986	1.16	65.34	-
Experiment. treatment -stratum					
Treatment	2	0.0158	0.0079	0.44	0.7
If pre-treatment with Dg affect Bf?	1	0.0006	0.0006	0.03	0.9
If pre-treatment with Bf affect Bf?	1	0.0142	0.0142	0.79	0.5
Residual	2	0.0358	0.0179	1.58	-
Experiment. Treatment-units-stratum	24	0.2726	0.0113	-	-
Total	29	1.4941	-	-	-

-

Table 5.8. Analysis of variance table showing contrasts for the severity of *B. fabae* on the lower leaves (week 2). Plants were kept in the controlled environment and maintained at a temperature of 15/12 ºC (day /night), relative humidity 70/90 % (day/night) and photoperiod of 16 hours per day for 4 weeks. Data were log10 transformed. Dg = *D. gigas*, Bf = *B. fabae*.

Source of variation	df	s.s	m.s	v.r	F pr.
Experiment. stratum	1	1.54337	1.54337	2156.9	-
Experiment. treatment-stratum					
Treatment	2	0.03062	0.01531	21.4	0.04
If pre-treatment with Dg affect Bf?	1	0.00159	0.00159	2.2	0.2
If pre-treatment with Bf affect Bf?	1	0.02806	0.02806	39.2	0.02
Residual	2	0.00143	0.00072	0.07	-
Experiment. treatment. units-stratum	24	0.25933	0.01081	-	-
Total	29	1.83476	-	-	-

Table 5.9. Analysis of variance table showing contrast for the severity of *B. fabae* on the lower leaves (week 3). Plants were kept in the controlled environment and maintained at a temperature of 15/12 ºC (day /night), relative humidity 70/90 % (day/night) and photoperiod of 16 hours per day for 4 weeks. Data were log10 transformed. Dg = *D. gigas*, Bf = *B. fabae*.

Source of variation	df	s.s	m.s	v.r	F pr.
Experiment stratum	1	1.6	1.6	527.69	-
Experiment. treatment-stratum					
Treatment	2	0.04	0.02	6.71	0.13
If pre-treatment with Dg affect Bf?	1	0.0004	0.0004	0.14	0.74
If pre-treatment with Bf affect Bf?	1	0.03	0.02	8.82	0.09
Residual	2	0.006	0.003	0.37	-
Experiment. treatment. units-stratum	24	0.19	0.008	-	-
Total	29	1.8	-	-	-

Table 5.10. Analysis of variance table showing contrast for the severity of *B. fabae* on the lower leaves (week 4). Plants were kept in the controlled environment and maintained at a temperature of 15/12 ºC (day /night), relative humidity 70/90 % (day/night) and photoperiod of 16 hours per day for 4 weeks. Data were log10 transformed. Dg = *D. gigas*, Bf = *B. fabae*.

Source of variation	df	s.s	m.s	v.r	F pr.
Experiment-stratum	1	1.65388	1.65388	311.83	-
Experiment. treatment-stratum					
Treatment	2	0.05517	0.02758	5.2	0.161
If pre-treatment with Dg affect Bf?	1	0.00022	0.00022	0.04	0.856
If pre-treatment with Bf affect Bf?	1	0.03823	0.03823	7.21	0.115
Residual	2	0.01061	0.0053	0.5	-
Experiment. treatment. units-stratum	24	0.257	0.01071	-	-
Total	29	1.97665	-	-	-

5.5.4. Severity of chocolate spot in faba bean upper leaves

The inoculation of *B. fabae* did not detectably alter the severity of chocolate spot (P= 0.61, Figure 5.4). Treatments with pre-inoculation of *B. fabae* had clearly reduced chocolate spot severity at week 3 (P = 0.01) on the upper leaves compared to treatments with *D. gigas* and water as their first inoculation. The contrast (Tables 5.11, 5.12 and 5.13) revealed that pre-treatment with *B. fabae* resulted in an increased chocolate spot score at week 2 (P= 0.03) and week 3 (P=0.02) after inoculation.

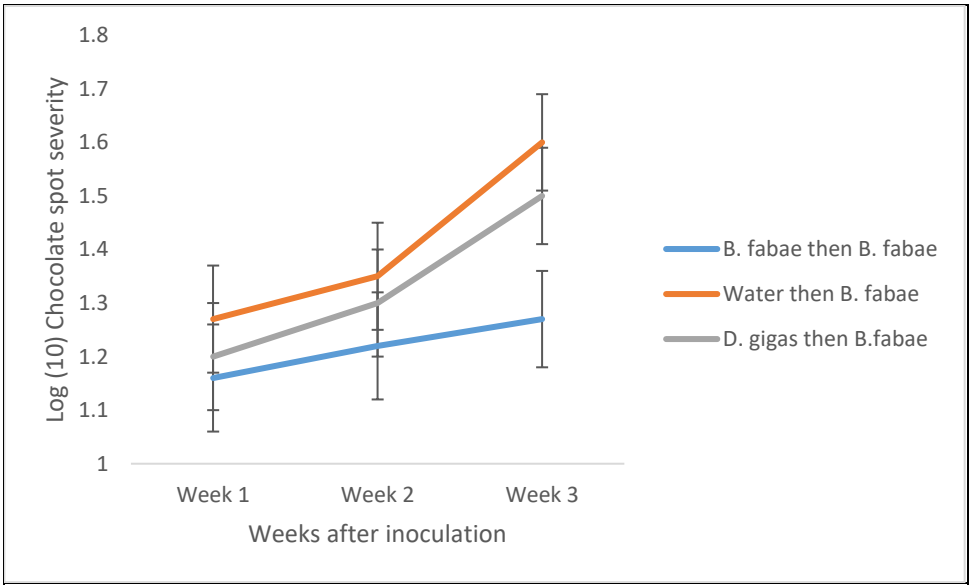

Figure 5.4. Chocolate spot severity on faba bean upper leaves; Plants were kept in the controlled environment and maintained at a temperature of 15/12 ºC (day /night), relative humidity 70/90 % (day/night) and photoperiod of 16 hours per day. Data were log10 transformed

Table 5.11. Analysis of variance table showing contrast for the severity of *B. fabae* on the upper leaves (week 1). Plants were kept in the controlled environment and maintained at a temperature of 15/12 ºC (day /night), relative humidity 70/90 % (day/night) and photoperiod of 16 hours per day for 4 weeks. Data were log10 transformed. Dg = *D. gigas*, Bf = *B. fabae*.

Source of variation	df	s.s	m.s	v.r	F pr.
Experiment- stratum	1	1.2	1.2	132	-
Experiment. treatment-stratum					
Treatment	2	0.06	0.03	3.8	0.2
If pre-treatment with Dg affect Bf?	1	0.0001	0.0001	0.01	0.9
If pre-treatment with Bf affect Bf?	1	0.04	0.04	5.5	0.1
Residual	2	0.01	0.008	0.4	-
Experiment. treatment. units-stratum	24	0.5	0.02	-	-
Total	29	1.7	-	-	-

Table 5.12. Analysis of variance table showing contrast for the severity of *B. fabae* on the upper leaves (week 2). Plants were kept in the controlled environment and maintained at a temperature of 15/12 ºC (day /night), relative humidity 70/90 % (day/night) and photoperiod of 16 hours per day for 4 weeks. Data were log10 transformed. Dg = *D. gigas*, Bf = *B. fabae*

Source of variation	df	s.s	m.s	v.r	F pr.
Experiment stratum	1	1.29	1.29	569.9	-
Experiment. treatment-stratum					
Treatment	2	0.1	0.05	22.7	0.04
If pre-treatment with Dg affect Bf?	1	0.0009	0.0009	0.4	0.6
If pre-treatment with Bf affect Bf?	1	0.07	0.07	30.1	0.03
Residual	2	0.005	0.002	0.19	-
Experiment. treatment. units-stratum	24	0.28	0.01	-	-
Total	29	1.68	-	-	-

Table 5.13. Analysis of variance table showing contrast for the severity of *B. fabae* on the upper leaves (week 3). Plants were kept in the controlled environment and maintained at a temperature of 15/12 ºC (day /night), relative humidity 70/90 % (day/night) and photoperiod of 16 hours per day for 4 weeks. Data were log10 transformed. Dg = *D. gigas*, Bf = *B. fabae*.

Source of variation	df	s.s	m.s	v.r	F pr.
Experiment-stratum	1	0.74	0.74	100.2	-
Experiment. treatment-stratum					
Treatment	2	0.5	0.25	33.9	0.03
If pre-treatment with Dg affect Bf?	1	0.0002	0.0002	0.03	0.9
If pre-treatment with Bf affect Bf?	1	0.36	0.36	49.5	0.02
Residual	2	0.01	0.007	0.41	-
Experiment. treatment. units-stratum	24	0.43	0.017	-	-
Total	29	1.7	-	-	-

5.6. Discussion

The study revealed that pre or post inoculation of *B. fabae* did not induce a defence response against *D. gigas*. Treatments with double inoculation of *D. gigas* had more nematodes extracted. The more nematodes were inoculated in total, the more nematodes were extracted. Treatments with a double inoculation of *D. gigas* had a similar number of nematodes extracted from the leaves to treatments that had a water or *B. fabae* second inoculation.

D. gigas is the most important nematode parasite of faba bean. The nematode readily infests and multiply on the plant. The plant upon infection is able to mobilise its defence arsenals against the nematode. Production of anti-nematode enzymes and compounds, reinforcement of cell walls and triggering of hypersensitive responses are ways in which plants respond to nematode attack (Sato *et.al*., 2019). Nematodes on the other hand can infest compatible host plants without the induction of a strong immune response from the host plant (Warmerdam *et al*., 2018). Possession of the stylet makes it easier for nematodes to breach the formidable plant cell wall.

Pre- inoculation of *B. fabae* seems to induce a defence response against subsequent *B. fabae*. This was evident in this study as post -inoculation of *B. fabae* was less severe in the treatments with pre-inoculation of *B. fabae*, compared with treatment with pre-inoculation of *D. gigas* or water.

D. gigas is a biotrophic organism and has a long-lasting relationship with the host plant, as it requires the host plant to complete its life cycle. However, *B. fabae* is a necrotrophic pathogen that does not have an enduring relationship with the host plant. It produces toxins that kill the host cells and then colonises them. On general grounds, the effect of *B. fabae* on host cells could impede the movement and probably the reproduction of the nematode.

More nematodes were extracted on the stem than on the leaves when *D. gigas* was the first inoculum, while more nematodes were extracted on the leaves than on the stem when *B. fabae* was the second inoculum. This gives an indication that the nematode will migrate from the leaves to the stem after some time, especially when food is depleted on the leaves. The nematode was inoculated on the growing tips in this study.

5.7. Conclusion

The results from this study show that *D. gigas* inoculation did not result in the induction of defence response against *B. fabae*. Also, *B. fabae* did not induce defences against *D. gigas*. However, pre- *B. fabae* inoculation resulted in induced resistance against subsequent *B. fabae* inoculation. This was tested on the field (Chapter 6).

Chapter 6: Effects of early inoculation of *B. fabae* and fertilizers on two varieties of faba bean

6.1. Abstract

Two successive field experiments (2017 and 2018) were conducted to determine whether the glasshouse results obtained previously were applicable to the field situation and tested the hypothesis that plant nutrition (application of sulphate fertilizer) and/or plant variety affect crop production. Faba bean seeds were sown in a plot size of 1.9 x 5 m at a population of 36 plants/ m^2. The 2017 experiment had a factorial treatment combination of inoculated plants (inoculated and un-inoculated) and fertilizer applications (potassium sulphate or chloride) and was laid out in a split plot design, while the 2018 experiment was a 2 x 2 x 2 factorial laid out in a randomized complete block design, comprising: sprays (*B. fabae* and water), varieties (Fuego and Babylon) and fertilizers (potassium sulphate or chloride). Six plants were sampled per plot. None of the results were significant at the 5 % level. The results from the first season were consistent with those from the controlled environment experiment but those from second season differed. The trend indicated that cv. Babylon was more resistant to chocolate spot than cv. Fuego. Also, potassium sulphate fertilizer, compared to potassium chloride, very slightly but consistently reduced the severity of chocolate spot and rust.

Key words: Field experiment, *B. fabae*, faba bean, severity, sulphate, chloride

6.2. Introduction

This study was a follow up to an observation from a controlled environment experiment (Chapter 5) in which pre-treatment of faba bean with *B. fabae* resulted in reduced severity of subsequent infections of *B. fabae*. The inclusion of sulphate fertilizer was because of a dramatic effect of sulphate fertilizer on faba bean observed the previous year at the University of Reading research farm at Sonning, Berkshire, UK.

Plant nutrients are vital for the growth and development of plants. The uptake and utilization of nutrients may be affected as a result of pathogen attack. Some pathogens exploit appreciable amounts of plant nutrient for their growth, thereby depriving the plants of the nutrients and so making the plants vulnerable to attack (Timonin, 1965).

Sulphur fertilizer has been reported by many authors to reduce severity of diseases. Bloem *et al*. (2004) reported reduction in severity of *Pyrenopeziza brassicae* on *Brassica napus.* Khaldoon (2016, unpublished thesis, pp. 58-94) reported that sulphur fertilization reduced disease severity of *Zymoseptoria tritici* and *Parastagonospora nodorum* in wheat.

Two varieties of faba bean were evaluated (cv. Fuego and Babylon). These two varieties are widely grown in Europe, under varying climatic conditions (Karkanis *et.al*., 2018). Babylon is resistant to chocolate spot. It was for this reason it was included in the experiment. In a 3-year trial involving 8 faba bean cultivars, cv. Babylon, alongside cv. Fury variety had the highest rating (8 of 9), while Fuego was rated 6.5 (PGRO, 2017).

Controlled environment experimental design is artificial. It removes some variables that interfere with crops in the field, thus allowing a close examination of the imposed factors. Wind and rain are two important factors that are involved in the spread of pathogens, but these are eliminated in a controlled environment. This will reduce the spread of diseases in the enclosure. Results obtained in a controlled environment may not reflect the situation of things in the field and need validation with complementary field experiments due to the artificial nature of the controlled environment.

Environmental factors play a major role in plant disease development. Disease development will be hampered in an unfavourable condition. Virulent pathogen will not be able to infect an otherwise susceptible host if the environmental conditions are not favourable for the

pathogen. Both plant defence mechanisms and the pathogen virulence mechanisms are affected by environmental factors.

Breeding for disease resistance is one of the most effective means of managing plant diseases. Resistance in plants against pathogens can either be horizontal or vertical, so-called. Horizontal resistance is controlled by many genes while vertical resistance is mediated by one major gene. Horizontal resistance is often moderately stable. It can be built up by breeding either for constitutive or induced resistance, but both are similar. Up till now, no variety of faba bean fully resistant to *B. fabae* is available for growers (https://www.fwi.co.uk/arable/pulses/how-a-new-test-helps-bean-growers-tackle-stem-nematode (Accessed 27 April 2021). Induced resistance might be able to fill the gap. The objective of the experiment reported here was to determine the extent of induction of defence responses by early inoculation of *B. fabae* in field conditions. The hypothesis tested was that early inoculation of *B. fabae* would result in less severe disease infection later in the season.

6.3. Materials and methods

Faba bean seeds were sown in a plot size of 1.9 x 5 m at a population of 36 plants/ m^2 at the University of Reading's Sonning farm, Sonning, UK (0°54' W, 51°29' N). The soil is a free-draining deep sandy loam. The 2017 experiment was conducted between March and July, while the 2018 experiment was conducted between April and August. The 2017 experiment had a factorial treatment combination of inoculated plants (*B. fabae* or water sprayed) and fertilizer application (potassium sulphate or potassium chloride) and was laid out in a split plot design with fertilizer as the main plot and spray as the sub plot. The 2018 experiment used a factorial combination of spray (*B. fabae* or water), fertilizer application (potassium sulphate or potassium chloride), and variety (Fuego or Babylon). Potassium sulphate was applied at the rate of 40 kg/ ha, while potassium chloride was applied at the rate of 44 kg/ ha, representing equal potassium doses. *B. fabae* spore inoculation and fertilizer applications were done at three weeks after planting.

6.3.1. Spore suspension and spraying

B. fabae spore suspension was prepared as described in section 2.2.1. A haemocytometer was used to count the number of spores present in the suspension and was adjusted to 2 x 10^5 spores per ml. It was sprayed using air pressure sprayer (12 L, Hozelock Knaspack sprayer/home base/UK). The spore suspension of *B. fabae* (10 ml) was added 0.5 litres of tap water.

6.3.2. Disease measurement: Percentage severity of chocolate spot and rust was recorded by visual observation. Half a meter was avoided from the edge of plot on all sides. Samples were then collected from 6 plants per plot taken along a zigzag path through the plot.

6.3.3. Aphid assessment: Aphid infestation was assessed by visually counting the aphids on 6 pre- tagged plants. Half a meter was avoided from the edge of the plot.

Grain yield per plant was recorded from six plants per plot.

6.3.4. Statistical analysis

The two experiments were analyzed separately because of the differences in the design. Analysis was done using analysis of variance with GenStat 17th edition (VSN, UK). Where necessary, data were log10 transformed to stabilize the residual variance.

6.4. Results

6.4.1. Severity of chocolate spot on faba bean (2017)

The effects of variety and spray in 2017 are presented in Figure 6.1. The main effect and interactions were not significant (P= 0.8, 0.1, 0.9 for fertilizer, spray and fertilizer x spray, Appendix table 9) although chocolate spot was reduced in samples with pre-treatment of *B. fabae*. The trends indicated that plots with potassium sulphate fertilizer also had less severe chocolate spot.

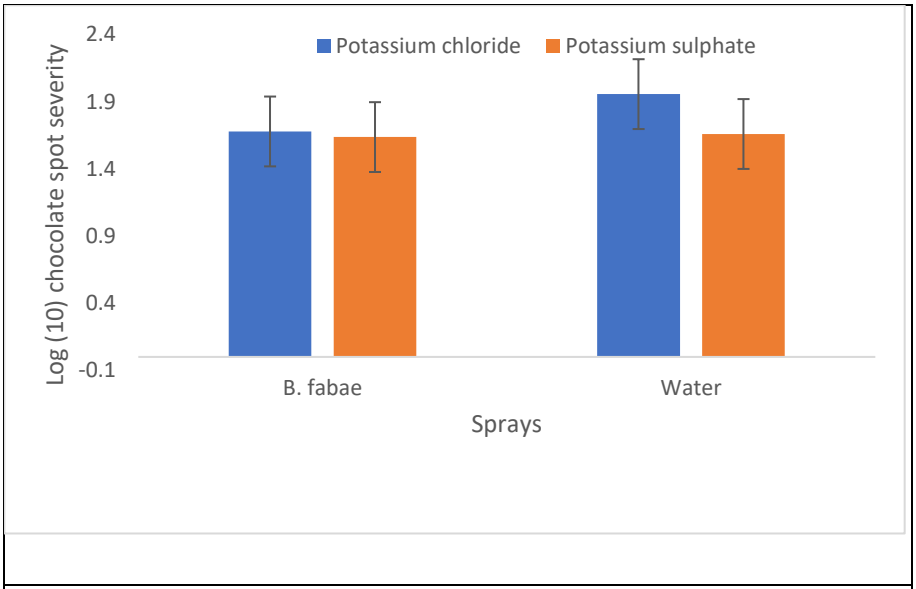

Figure 6.1. Interactions of *B. fabae* and fertilizers on percentage severity of chocolate spot on faba bean in 2017. Six plants were sampled in a plot size of 1.9 x 5 m. Plants were arranged in a split plot design with variety as main plot and inoculation as sub-plot. Error bars denotes standard error of difference.

6.4.2. Severity of chocolate spot on faba bean (2018)

The main effects of variety, fertilizer and spray and interactions were not significant (P= 0.5, 0.2, 0.5, 0.3, 0.9, 0.4, 0.3 for fertilizer, spray, variety, fertilizer x spray, fertilizer x variety, spray x variety, fertilizer x spray x variety respectively; Appendix table 10), Figure 6.2) and were quite small compared to the overall mean. Chocolate spot was more severe in samples that received initial inoculation of *B. fabae*. Plots with potassium sulphate fertilizer were on average less severely infected with chocolate spot than plots with potassium chloride fertilizer.

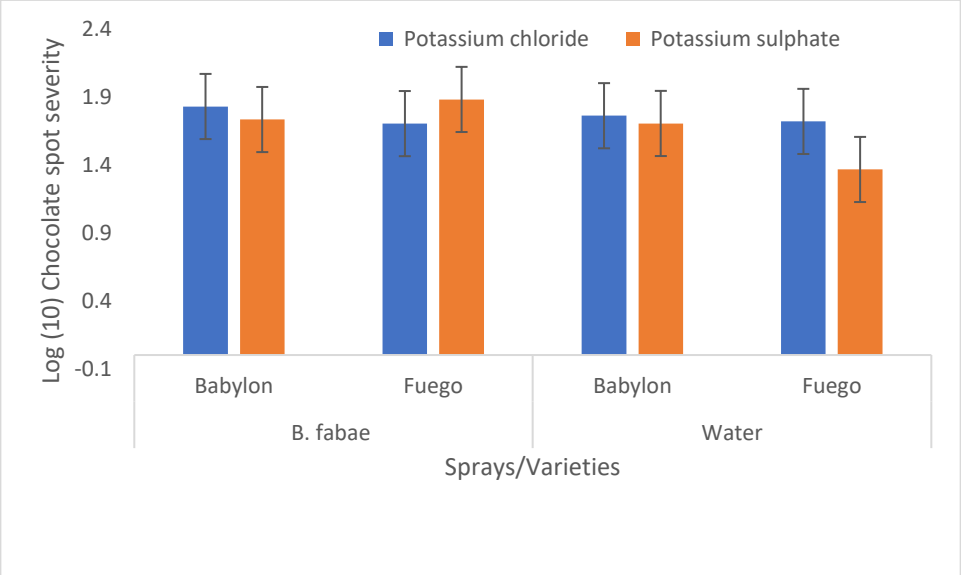

Figure 6.2. Interactions of varieties, fertilizers and *B. fabae* inoculation on percentage severity of chocolate spot (2018). Six plants were sampled in a plot size of 1.9 x 5 m. Plants were arranged in a randomised complete block design. Error bars denotes standard error of difference.

6.4.3. Severity of rust on faba bean (2017)

The main effects and interaction of *B. fabae* inoculation and fertilizer were not significant (P= 0.4, 0.4 and 0.1, for spray, fertilizer and spray x fertilizer, Appendix table 11) Figure 6.3. Rust was slightly less severe in plots with initial pre-treatment of *B. fabae*. Potassium sulphate fertilizer very slightly but consistently reduced the severity of rust.

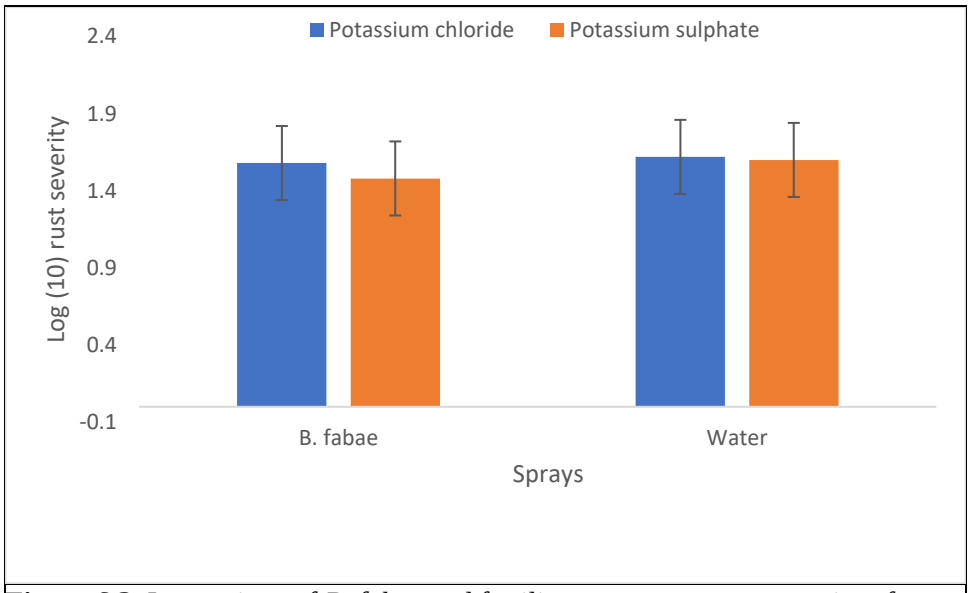

Figure 6.3. Interactions of *B. fabae* and fertilizers on percentage severity of rust on faba bean (2017). Six plants were sampled in a plot size of 1.9 x 5 m. Plants were arranged in a split plot design, with variety as main plot and inoculation as sub-plot. Error bars denotes standard error of difference.

6.4.4. Severity of rust on faba bean (2018)

The main effect and interactions between the effects of variety, fertilizer and *B. fabae* inoculation on rust severity are presented in Figure 6.4. There were no significant 2- or 3-way interactions or main effects. (P= 0.6, 0.5, 0.1, 0.9, 0.1, 0.2, 0.1 for fertilizer, spray, variety, fertilizer x spray, fertilizer x variety, spray x variety, fertilizer x spray x variety respectively; Appendix table 12). The trends indicated that rust severity was more in uninoculated plots except for Babylon inoculated with *B. fabae*. Overall, potassium sulphate application reduced percentage rust severity compared to the potassium chloride plots.

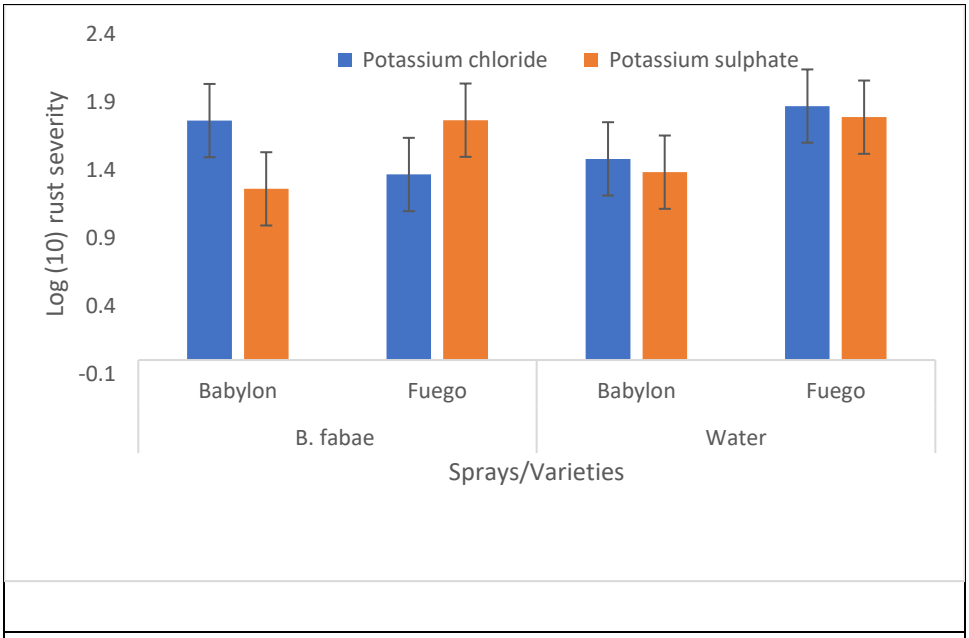

Figure 6.4. Interactions of varieties, fertilizers and *B. fabae* inoculation on percentage severity of rust (2018). Six plants were sampled in a plot size of 1.9 x 5 m. Plants were arranged in a randomised complete block design. Error bars denotes standard error of difference.

6.4.5. Aphid counts (2017)

Plots with initial inoculation of *B. fabae* had fewer aphids on them (Figure 6.5) but the main effect and interaction with fertilizers were not significant (P= 0.5, 0.3 and 0.3 for sprays, fertilizers and sprays x fertilizers, Appendix table 13). The trends were for plots receiving potassium sulphate to have fewer aphids when sprayed with *B. fabae* than when sprayed with water.

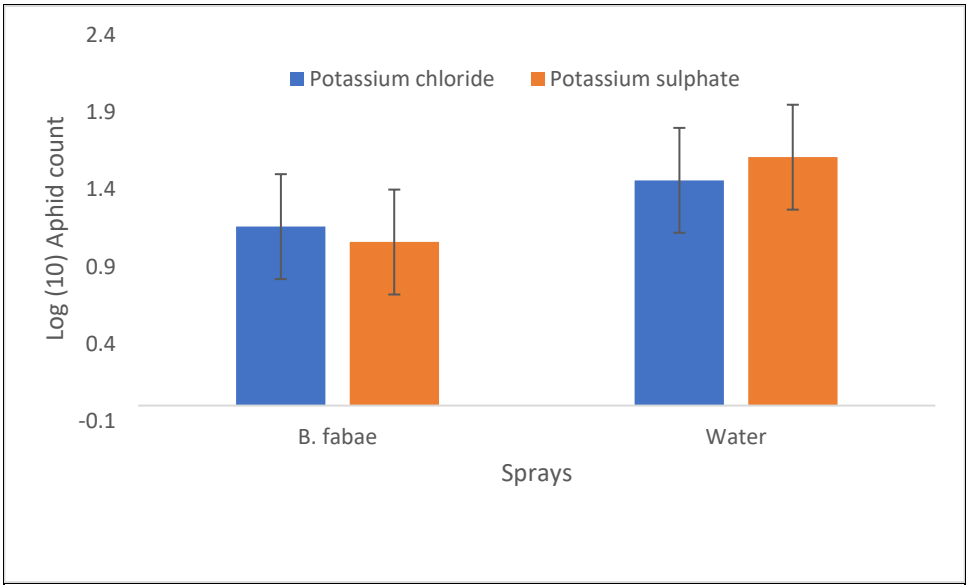

Figure 6.5. Interactions of *B. fabae* and fertilizers on number of aphids on faba bean (2017). Six plants were sampled in a plot size of 1.9 x 5 m. Plants were arranged in a split plot design, with variety as main plot and inoculation as sub-plot. Error bars denotes standard error of difference.

6.4.6. Aphid count (2018)

The interactions between the effects of variety, fertilizer and *B. fabae* inoculation on aphid number were very variable (Figure 6.6). There were no significant 2- or 3-way interactions or main effects (P=0.7, 0.6, 0.9, 0.8, 0.9, 0.2, 0.4 for fertilizer, spray, variety, fertilizer x spray, fertilizer x variety, spray x variety, fertilizer x spray x variety respectively, Appendix table 14). Overall, aphids were fewer on plots with early *B. fabae* inoculation.

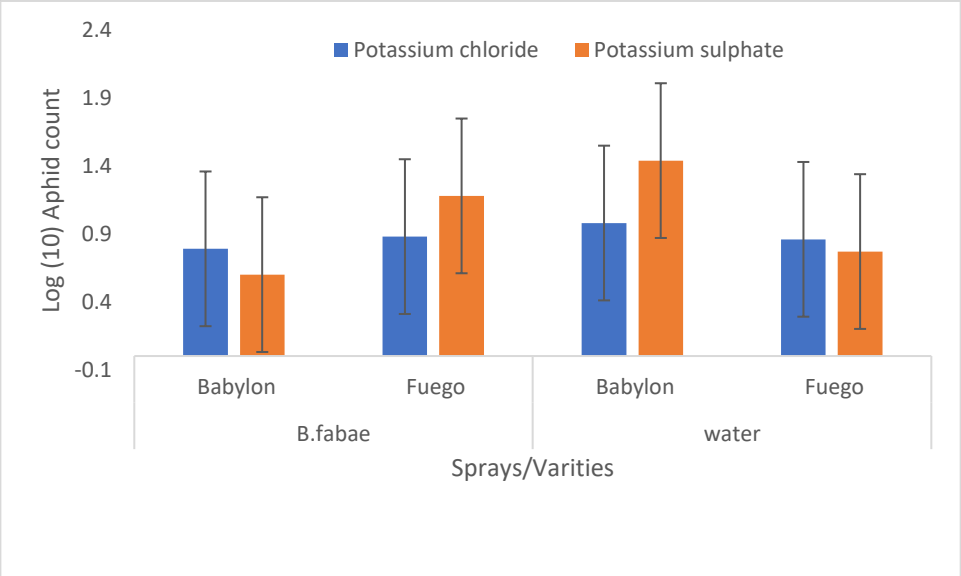

Figure 6.6. Interactions of variety, fertilizers and *B. fabae* inoculation on number of aphids (2018). Six plants were sampled in a plot size of 1.9 x 5 m. Plants were arranged in a randomised complete block design. Error bars denotes standard error of difference.

6.4.7. Grain yield (2017)

The grain yield from plots with pre-treatment of *B. fabae* was higher than those without prior inoculation of *B. fabae* (Figure 6.7). However, there was no significant main effect or interaction (P= 0.4, 0.7 and 0.3 for fertilizer, spray and fertilizer x spray, Appendix table 15). Potassium sulphate treated plots had a higher grain yield with prior inoculation of *B. fabae*, while potassium chloride treated plots had a higher grain yield without the inoculation.

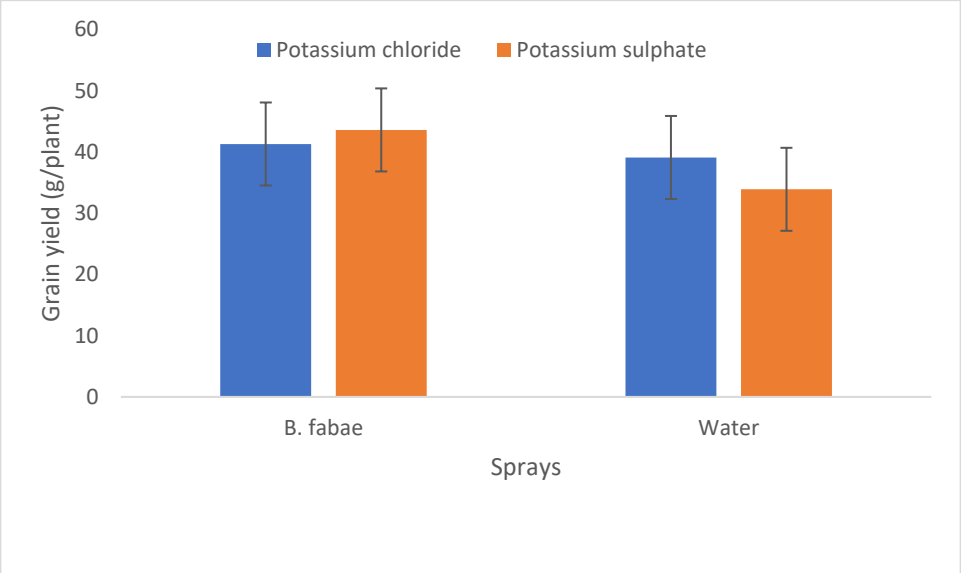

Figure 6.7. Interactions of *B. fabae* and fertilizers on grain yield of faba bean (2017). Six plants were sampled in a plot size of 1.9 x 5 m. Plants were arranged in a split plot design with variety as main plot and inoculation as sub-plot. Error bars denotes standard error of difference.

6.4.8. Grain yield (2018)

The interactions between the effects of variety, fertilizer and *B. fabae* inoculation on grain yield are presented in Figure 6.8. The main effects and interactions were not significant (P = 0.6, 0.3, 0.5, 0.5, 0.3, 0.3, 0.9 for fertilizer, spray, variety, fertilizer x spray, fertilizer x variety, spray x variety, fertilizer x spray x variety respectively, Appendix table 16). On the average, grain yield was higher in plots initially inoculated with *B. fabae.*

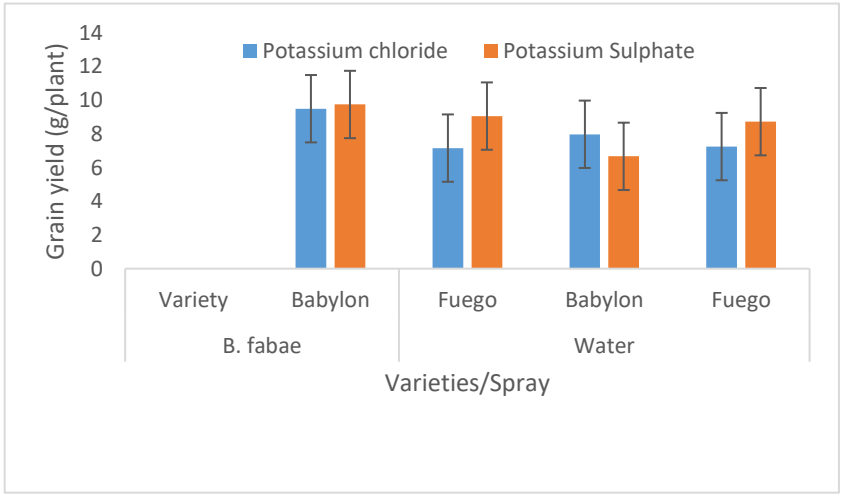

Figure 6.8. Interactions of variety, fertilizers and *B. fabae* inoculation on grain yield of faba bean (2018). Six plants were sampled in a plot size of 1.9 x 5 m. Plants were arranged in a randomised complete block design. Error bars denotes standard error of difference.

6.4.9. Mean relative humidity during 2017-2018

The experiments were conducted at deferent time of the year, hence the data on weather conditions in the field**.** The mean relative humidity during 2017 and 2018 is presented in Figure 6.9. The highest relative humidity recorded was 97 % in 2017 (March and May), 98 % in 2018 (May). The minimum relative humidity was 50 % in 2017 (June) and 32 % in 2018 (June and July).

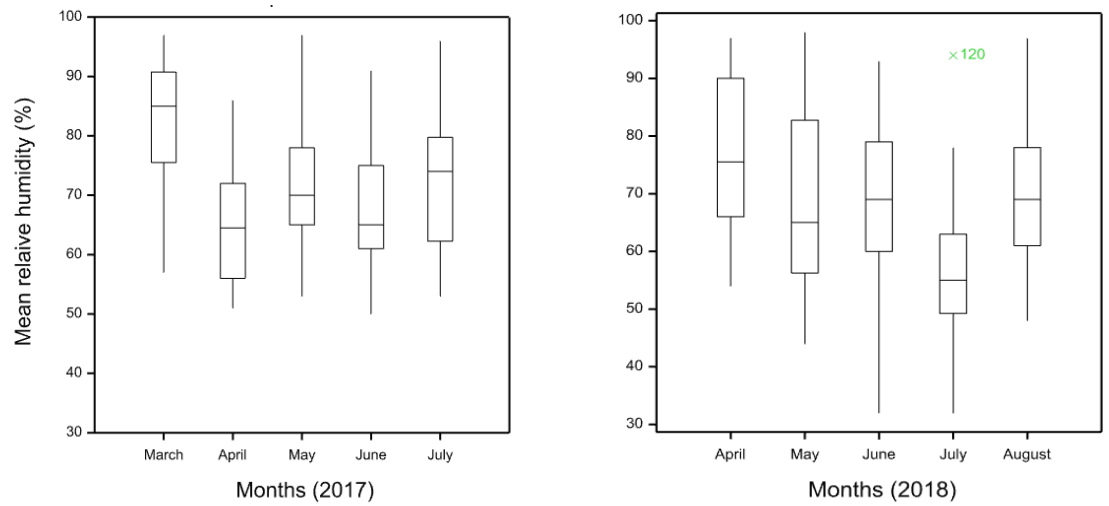

Figure 6.9. Reading mean relative humidity for March- July 2017 and April – August 2018. (Source: https://research.reading.ac.uk/meteorology/atmospheric-observatory/atmospheric-observatory-data/)

6.4.10. Mean air temperature during 2017 and 2018

The mean air temperature increased progressively in both seasons, except for a slight drop in April 2017 and at the end of the season (August) in 2018 (Figure 6.10). The highest mean air temperature was recorded in July for both seasons. Also, the lowest temperature for each season was recorded in April.

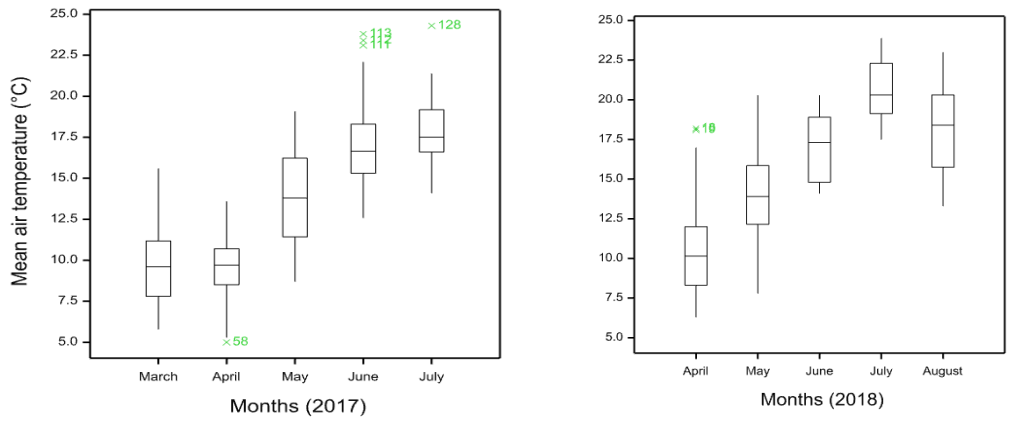

Figure 6.10. Reading mean air temperature for March- July 2017 and April – August 2018. (Source: https://research.reading.ac.uk/meteorology/atmospheric-observatory/atmospheric-observatory-data/). X represents an outlier

6.4.11. Rainfall during 2017- 2018

It was wetter in 2017 compared to 2018 (Figure 6.11). However, the first 2 months in 2018 (April and May) were wetter compared to the first two months in 2017 (March and April). The highest rainfall in 2017 was recorded in July, while the highest rainfall in 2018 was recorded in April.

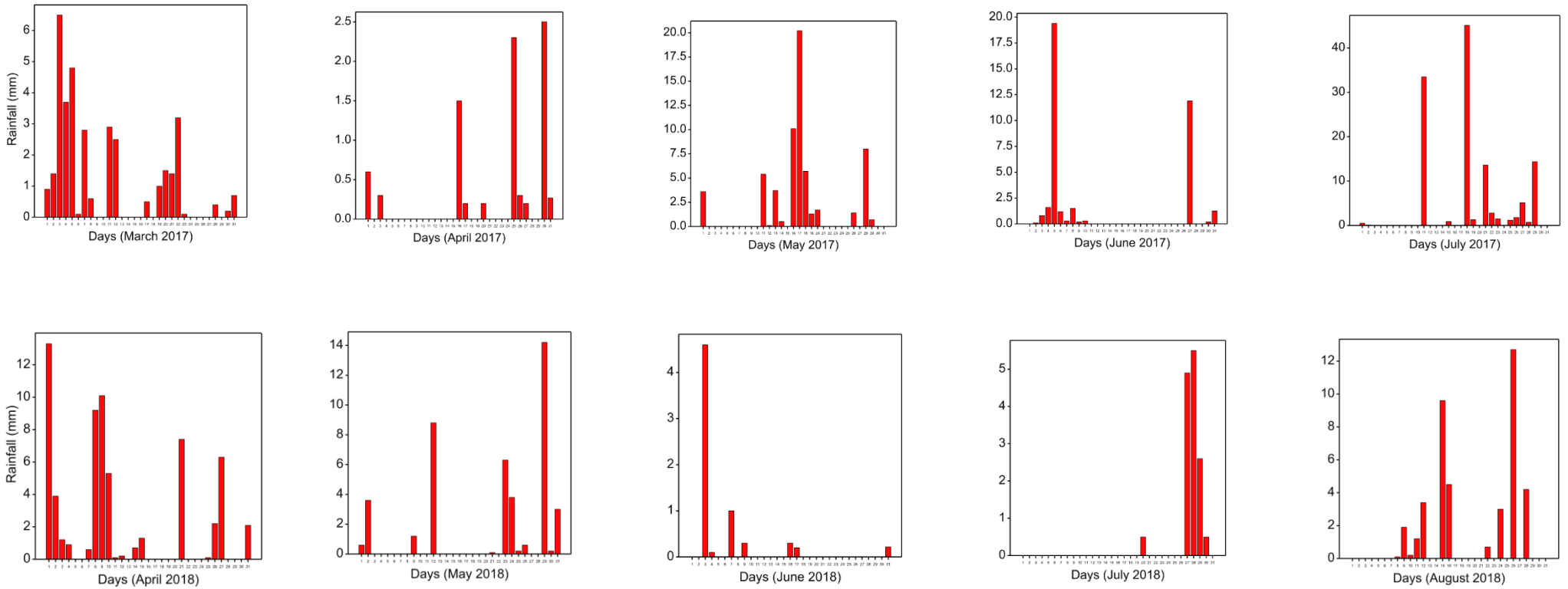

Figure 6.11. Rainfall for March- July 2017 and April – August 2018. (Source: https://research.reading.ac.uk/meteorology/atmospheric-observatory/atmospheric-observatory-data/).

6.5. Discussion

This study was done as a follow up to an earlier controlled environment experiment (chapter 5) which indicated that earlier inoculation of *B. fabae* resulted in less severe infection of subsequent inoculation. The experimental design in 2017 was a split plot, while in 2018 a randomised complete block design was employed. The difference in design was largely due to constraints in space. The space available in 2017 could not accommodate a randomised complete block design.

The study revealed that pre-treatment of faba bean with *B. fabae* and fertilizer application reduced the severity of chocolate spot in 2017, although not statistically significant. The 2017 results agreed with the controlled environment experiments. The results in 2018 deviated from the 2017 results. The inconsistencies in the field experiment results may be an indication that the induced resistance observed in the laboratory experiments does not hold strongly enough to be applied in the field.

Rust (*Uromyces viciae-fabae*) was slightly less severe in plots with initial pre-treatment of *B. fabae* in 2017. This result was consistent with the observation in 2018, as rust severity was less on pre-inoculated plots than the uninoculated plots (although not significantly). One explanation for the reduced severity of rust in the inoculated plot is that rust is a biotrophic pathogen that does not quickly cause the death of the host plant cells attacked. They require the host plant to complete their life cycle (Staples, 2000). *B. fabae* on the other hand produces toxin which cause the death of the host cells attacked. Plants with early inoculation of *B. fabae* would have suffered local necrosis, which will impede rust growth, hence reduced rust infection.

On the average, aphid incidence was lower on pre-inoculated plants in both seasons. Aphids are usually non- uniformly distributed on the field. Aphids feed by sucking the phloem sap with the aid of their stylet. Necrosis, which infection is a product of *B. fabae* infection will reduce their feeding and their population on pre-inoculated plants.

The Babylon variety was more resistant to chocolate spot than Fuego in both seasons. Although this was not statistically significant it is consistent with their resistance ratings from PGRO trials (8 of 9 for cv. Babylon and 6.5 of 9 for cv. Fuego, PGRO, 2017). The trend of

the results also indicated that Babylon had a better yield compared to Fuego. This was expected, as the variety had reduced chocolate spot severity.

Potassium sulphate fertilizer very slightly but consistently reduced the severity of chocolate spot and rust compared to potassium chloride (but again non-significantly). This is in line with previous published works on the reduction of disease severity due to sulphate application, as detailed in the chapter introduction.

The difference between years observed in the results may be due to the prevailing weather conditions during the period of inoculation. On the average, the weather condition in 2017 was more favourable for the pathogen development, but the conditions at the time of inoculation in 2018 were better. The average relative humidity in the growing months was 71.80 % in 2017 and 71.69 % in 2018. More rainfall was observed in 2017 than 2018. The mean temperature was 13.62 ℃ in 2017 and 13.69 ℃ in 2018. Optimum conditions for the *B. fabae* infection are a temperature of between 15 ℃ and 22 ℃, relative humidity of about 90 % and films of water for sporulation (Richardson and Horsham, 2008). Overall, the weather conditions can be said to be better for the pathogen in 2017 than 2018, mainly because it was wetter in 2017. This may have encouraged more chocolate spot infection following inoculation in 2018 and hence the difference from the 2017 result. Also, since controlled environment is artificial, variables that interfere with crops (wind, rain and insects are responsible for the spread of pathogens) in field situations are eliminated.

6.6. Conclusion

The results obtained are inconclusive. Therefore, further studies are required to identify which of the proposed mechanisms is operating behind the observed results, and whether the striking controlled environment results can have any practical application.

Chapter 7: Development of qPCR assay to identify and quantify *D. gigas* and *B. fabae* simultaneously in faba bean

7.1 Abstract

It became increasingly apparent that the methods of monitoring and quantifying *D. gigas* in the studies were time consuming. Therefore, there was a need to develop a method that is fast. Internal transcribed spacer (ITS) gene of *D. gigas*, heat shock protein 60-like (HSP60) gene of *B. fabae* and elongation factor 1-alpha (EF1-a) of *V. faba* were used to design primers for conventional and real-time PCR. The assays had linear relationships to *D. gigas* numbers and to *B. fabae* concentration in plants. The assay will be useful in detection and quantification of *D. gigas* on faba bean. As an example of its utility, it was tested in the experiments on the interaction of *D. gigas* and *B. fabae* and had good correlation with the visual scores and nematode counts.

Key words: *D. gigas*, *B. fabae*, *V. faba*, ITS, HSP60, EF1-a, PCR, Primers, assay.

7.2. Introduction

Throughout these studies it became increasingly apparent that the methods of monitoring and quantifying *D. gigas* were time consuming. The method used (modified Baermann) also relies on morphological characters of the nematode using the microscope. Nematodes can sometimes be similar morphologically, but genetically different. Also, adult nematodes are sometimes required for precise identification. This was certainly the case in the experiments described in Chapters 3, 4 and 5. Vast knowledge from a trained expert is also required (Capote *et al*., 2012). Fungi on the other hand rely on visual estimation of the symptoms or in vitro investigation of the spores for the identification and quantification of diseases. Some biotrophic fungi like rust and powdery mildew are difficult to culture *in vitro.* Molecular based identification will overcome these drawbacks. Molecular identification is fast, accurate and reliable. It was therefore decided if a molecular approach could be developed that would help in such studies

Precise identification and quantification of nematodes and fungi in plant propagules or soil prior to planting is important to reduce yield loss and the spread of pathogens to other regions. It is an important strategy in the management of plant diseases. Precise identification is especially important for organisms like *Ditylenchus* which is a pest of quarantine importance. Its spread to other areas devoid of natural enemies can be devastating.

D. gigas and *B. fabae* occupy overlapping niches on faba bean. Both are found together on the plant. Identification can be difficult where both organisms occur together. PCR methods have been developed for the identification of *B. fabae* (Zhang, *et al*., 2010) and *D. gigas* (Volvas *et al*., 2011, Saadi, *et al*., 2019) in faba bean. These methods considered each organism in isolation. There are no published reports on the simultaneous identification of *D. gigas* and *B. fabae* on faba bean. The method developed in this research identified each organism in association with the other. This ensures simultaneous identification of individual organisms.

The use of DNA-based techniques is becoming essential for plant disease identification and quantification, especially with the drawbacks associated with the use of the traditional methods. The real- time PCR or quantitative polymerase chain reaction (qPCR) is a technique used for many diverse purposes in laboratories (Pabinger *et.al*., 2014). It was used in this

experiment for the detection and quantification of *D. gigas* and *B. fabae* in faba bean. The method is also very useful when a pathogen is present in an asymptomatic condition.

Two techniques are commonly used with real-time PCR; these are SYBR- green and Taq-man. SYBR- green is cost- effective and simple to use (Donia, *et al*., 2010), so it was employed in this study

The objective of this study was to develop a species-specific qPCR assay for the simultaneous identification and quantification of *D. gigas* and *B. fabae* in faba bean. The hypothesis tested in this chapter was that nematodes estimated using the modified Baermann technique and visual chocolate spot severity would be consistent with those estimated from the qPCR method

7.3. Materials and methods

7.3.1. Primer design for species identification

The nucleotide sequences for internal transcribed spacer (ITS) gene (GenBank: KJ653267.1) of *D. gigas*; heat shock protein 60-like (HSP60) gene, (GenBank: EU365876.1) of *B. fabae* and elongation factor 1-alpha (EF1-a) of *V. faba* (GenBank: AJ222579.1) were retrieved from NCBI website (https://www.ncbi.nlm.nih.gov/nucleotide/). The sequences were used to design species specific primers using the PRIMER BLAST tool from NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast). The primers were procured from Invitrogen (Thermo Scientific, Life Technologies Ltd, UK).

7.3.2. DNA extraction

DNA was extracted from *D. gigas*, *B. fabae* and *Vicia faba* using DNeasy Plant Mini kits (QIAGEN, UK) following the manufacturer's instructions**,** with modification. To isolate total genomic DNA from *D. gigas*, *B. fabae* and *Vicia faba*, the suspension was centrifuged in an Eppendorf tube and ground in a TissueLyser (QIAGEN, UK) for 5 minutes. After grinding, 400 µl of buffer AP1 supplemented with 4 µl RNase was added to the tube, vortexed and incubated for 10 min at 65 ^{o}C in a thermo mixer (Eppendorf, UK). 5 µl proteinase K was added to the digestion, vortexed, and incubated for 10 min at 55 ^{o}C. (This was done for *D. gigas* only) The tube was inverted three times during incubation. After incubation, 300 µl buffer P3 was added and centrifuged for 5min at 14,000 revolution per minute (RPM). The lysate was pipetted into QIAshredder spin column placed in a 2 ml collection tube and centrifuged for 2 minutes at 14,000 RPM. The flow-through was transferred into a new tube without disturbing the pellet, supplemented with 1.5 volumes buffer AW1 and mixed by pipetting. The mixture (600 µl) was transferred into DNeasy mini spin column placed in a 2 ml collection tube and centrifuged for 1 minute at 6000 RPM. The flow-through was discarded. The process was repeated for the remaining sample. Then, for washing, the spin column was placed in a new 2 ml collection tube. 500 µl buffer AW2 was added and centrifuged for 1 minute at 8000 RPM. The washing step was repeated once. The spin column was carefully removed from the collection tube and transferred to a new 1.5 µl micro centrifuge tube. Finally, 75 µl buffer AE was added to the column, incubated for 5 minutes at room temperature and centrifuged at 14000 rpm for 5 minutes to elute the DNA. Quantity and quality of the eluted DNA was determined by measuring the 260/280 absorbance ratio using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, UK).

7.3.3. Polymerase Chain Reaction (PCR)

A 20 µl PCR reaction was assembled containing 1.5 µl each of 5 µM forward and reverse primers, 10 µl of Biomix (Bioline, UK), 6 µl of PCR water and 1 µl of 10 ng/µl DNA template. PCR reactions were performed in a thermal cycler (Veriti, Applied Biosystems, UK) programmed to one cycle of 94 °C for 5 minutes, followed by 35 cycles of 94 °C for 30 seconds, 60 °C for 15 seconds and 72 °C for 30 seconds. Final extension was performed at 72 °C for 5 minutes. PCR products were mixed with 1/5 volume of 6 x Ficoll dye and resolved in 5 % agarose gels containing ethidium bromide. The gel was run at 90 volts for 40 minutes and visualized under ultraviolet light (Gene Flash, Syngene Bio Imaging). Hyper Ladder I (BioLine) was used as a size standard. The PCR products were sent to Source Bioscience (http://www.sourcebioscience.com/) for sequencing to verify their specificities.

7.3.4. Synthetic standard for Real time PCR

The oligonucleotides were ordered from Integrated DNA Technologies BVBA (https://eu.idtdna.com/pages). The synthetic standard with inverted primer size on both ends were cloned in vector *(*PCR 4 BLUNT TOPO) and restricted with Not I. The synthetic standards usually contain incomplete fragments and tend to disintegrate from both ends. Hence, cloning provides a constant supply of single intact template for qPCR studies. Purified PCR product (6.5 ng) was mixed with PCR4 BLUNT TOPO vector (Thermo Scientific, Life Technologies Ltd, UK) in 1:4 vector template ratio. The tube was supplemented with 1 µl of salt solution and enough deionised water to bring the volume to 6 µl. The contents were mixed gently and spun briefly for 5 seconds to collect the contents. The mixture was incubated at 22 °C for 40 min and chilled on ice for 3-5 minutes. For transformation, 2 µl of the ligation mixture was added to Top10 chemo-competent cells (Invitrogen, UK), mixed gently and left on ice for 40 minutes. The transformation was conducted by the heat shock method. For this purpose, the cells containing ligation mixtures were incubated at 42 °C for 60 seconds and immediately chilled on ice for 2 minutes. After chilling, 250 µl of broth medium was added to the tube. The cells were incubated at 37 °C for one hour in an orbit shaker at 250 RPM. The culture was spread on Luria-Bertani (LB) agar medium supplemented with 50 mg/ml of kanamycin at 20 or 200 µl per 9 cm petri plate. Plates were incubated at 37 °C overnight. Eight colonies were picked from the plate and grown in 5ml of LB broth containing the selection antibiotic (kanamycin). The cells were incubated over night at 37 °C with 250 RPM shaking. The plasmid DNA was isolated from the cells using GeneJet mini prep kit (Thermo Scientific, Life Technologies Ltd, UK)

following manufacturer's instructions. The isolated DNA was subjected to restriction as well as sequencing confirmation. The positive plasmid was restricted with NOT I enzyme to linearize it and quantified on NanoDrop. The copy number was calculated using online calculator (https://www.thermofisher.com/uk/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/dna-copy-number-calculator.html) and 10 X serial dilutions were prepared with starting copy number of the plasmid containing synthetic standards 1×10^9 µl per reaction.

7.3.5. Determination of optimum annealing temperature

The optimum annealing temperature of each of the three primers in PCR was assessed. The reaction contained 10 µl master mix (Taq PCR Master Mix Kit, QIAGEN), 1.5 µl each of forward and reverse primers, 1 µl DNA sample and 6 µl molecular grade water. The conditions for the PCR cycle were 95 °C for 5 minutes initial denaturation, followed by 30 cycles of denaturation at 95 °C for 15 seconds annealing temperatures (56 °C, 58 °C, 60 °C and 62 °C for 15 seconds, an extension of 72 °C for 15 seconds and final elongation of 72 °C for 5 minutes). The primer sets were accessed separately with their respective pure DNA samples (*D. gigas*, *B. fabae* and tissue of *V. faba*).

7.3.6. Determination of specificity of primers

In other to check that there was no cross reactivity, the specificity of each primer set was separately tested. Genomic DNA was isolated from pure cultures of *D. gigas*, *B. fabae* and tissue of *V. faba*. Each primer set was used with each source DNA. Amplification or lack of it was confirmed by electrophoresis of an aliquot of the PCR products in 2 % agarose gel in 1x TAE buffer.

7.3.7. Real time PCR assay

The quantity of *D. gigas* and *B. fabae* in each sample was quantified by real-time quantitative PCR (qPCR). DNA from each sample was adjusted to 5 ng/ µl. The qPCR was carried out in a 20 µl final reaction volume. It was done by mixing 5 µl DNA solution with 1.5 µl each of forward and reverse primers, 10 µl of power up SYBR Green (Thermo Scientific, UK https://www.thermofisher.com/uk/en/home.html) and 2 µl molecular grade water. Thermal cycling was done with one cycle of 95 °C for 2 minutes, 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute, followed by melt curve analysis. The assay included a no template

control and genomic DNA standards in duplicate. Pure genomic DNA of *D. gigas*, *B. fabae* and *V. faba* were previously serially diluted in order to determine the calibration curve and PCR efficiency for each 96-well microplate. Quantification used a standard curve technique.

7.3.8. Statistical analysis

ANOVA was used to analyse all data using GenStat 18th edition (VSN, UK). Where applicable, data were $\log_{10}$ transformed to stabilize the residual variance and aid interpretation.

7.4. Results

7.4.1. Primer design for qPCR

The primers designed from internal transcribed spacer (ITS) gene (GenBank: KJ653267.1) of *D. gigas*; heat shock protein 60-like (HSP60) gene (GenBank: EU365876.1) of *B. fabae* and elongation factor 1-alpha (EF1-a) of *V. faba* (GenBank: AJ222579.1) were employed to conduct a PCR using DNA from their respective species. The primers sequence is depicted in Table 7.1. The PCR yielded an intact band of the expected size from *D. gigas*, *B. fabae* and *V. faba* respectively. Each band was eluted from the gel and sent for sequencing from both ends.

Table 7.1. Primers and sequences		
Species	Size (bp)	Sequence
D. gigas	422	Aacggctctgttggcttctatgattctctgagcagttgtatgcctacgtccgtggctgcgttgaagaga atcgacaggtggtcttcgtgatcgctagaatcaatgagtaccagataggtgccgccaacaaaaaccc cattttgaacttttttacaagaaaacatttctagtcttatcggtggatcactcggttcatagatcgatgaa gaacgcagccaactgcgatatatggtgtgaactgcagatattttgaacaccaagaattcgaatgcaca ttgcgccactggatatctatcctttggcacatctggctcagggtcgtaaataccaaacgaaagcaaatt cgttgattatgacagattcatggcaaaactagcgggtgcttttccgctggtgtcatgtttttgtgaaggg acttgcc
B. fabae	647	Ccacaaccgctactgtccttgctaaatctattttctccgagaccgtaaagaacgtcgccgcaggatgc aacccaatggacttgcgcagaggtacccaagccgccgtggaggccgttgttgantttgcaaagaa caagcgtgatatcacaacangcgagaaatcgcacaagttgcgactatcantgcaagcggtgatacc cacatcggaaaattgattgccaacgctatggaaaaggttggaaaggaaggtgttatcacagttaagg aaggaaagaccatggaggagcaactcgatattaccgagggaatgagatttgaccgcggttatgtttc ccatacttcatcaccgataccaagtcgc Aaaaggtggaattcgagaagccattgattctcctttctgagaagaagatttcaaacgtccaagatatta tcccagcacttgaggcgtctactcaacttcgtcgtcctttggtcatcattgctgaagatatcgatggaga agctctcgctgtatgcattcttaacaagctccgtggtcagctccaagttgccgctgtcaaggcccccg gtttcggtgataaccgaaagtctatcctcggcgatctcggtatcttgaccaatgctaccgtcttcactga cgagctt
V. faba	721	Tggttttgagggtgacaacatgattgagaggtccaccaaccttgactggtacaagggaccaactctc cttgatgctcttgacaacatcaatgagcccaagagaccctcagacaagccactcaggcttccattgca agatgtttacaagattggtggtattggaactgtgccagtgggacgagttgaaaccggtgttgtgaagc ctggtatgcttgtgacttttgctcctactggtttgacaactgaggttaagtccgttgagatgcaccacga ggctctcactgaggctcttccaggagacaatgtcggattcaatgttaagaatgttgcagtcaaggatct caagcgtggttttgttgcatccaactccaaggatgaccctgccaaggaagctgccaacttcacatccc aagtcatcatcatgaaccatcctggacagatcggtaacggttatgcaccagtgctagattgccacacc tctcacattgctgtgaagtttgctgaacttatcaccaagattgacagacgatctggtaaggagattgag aaggagcccaagtttttgaagaatggtgatgccggtatggttaagatgattcccactaagcccatggtt gtggaaacttttgctgagtatcctcctcttggtcgttttgctgttagggacatgagacaaactgttgctgt cggagtcatcaagagtgtggagaagaaggaccca

Two primer sets were designed each for *D. gigas*, *B. fabae* and *V. faba* for quantitative PCR (Table 7.2). The following primer sets were used in the remainder of the work because they gave bright bands in gel electrophoresis and produced the expected amplicon size of PCR products which is favourable for qPCR:

Table 7. 2. Standard PCR and qPCR sequence			
Gene		Name of the primer	Sequence
ITS	Standard PCR	Nema F1	AACGGCTCTGTTGGCTTCTA
		Nema R1	GGCAAGTCCCTTCACAAAAA
	qPCR	Nema F1 qPCR	AACGGCTCTGTTGGCTTCTA
		Nema R1 qPCR	GCGGCACCTATCTGGTACTC
Heat shock protein 60-like	Standard PCR	BFi	CCACAACCGCTACTGTCCTT
		BFii	AAGCTCGTCAGTGAAGACGG
	qPCR	Bot qPCR F2	GAAAGACCATGGAGGAGCAA
		Bot qPCR R2	ATTCCACCTTTTGCGACTTG
Elongation factor 1-alpha	Standard PCR	Primer F	TGGGTCCTTCTTCTCCACAC
		Primer R	TGGTTTTGAGGGTGACAACA
	qPCR	FVQ_F2	TCCGACAGCAACAGTTTGTC
		FVQ_R2	AATGGTGATGCGGTATGGTT

7.4.2. Determination of optimum annealing temperature and primer specificity

The PCR products were examined at 56, 58, 60 and 62 °C annealing temperatures. The brightest bands were produced at 60 °C. Therefore, 60 °C was used as the annealing temperature in qPCR. The electrophoresis of the PCR products of the 3 primer sets for qPCR showed that primers did not amplify the spp. to which they were not designed (Figure 7.1)

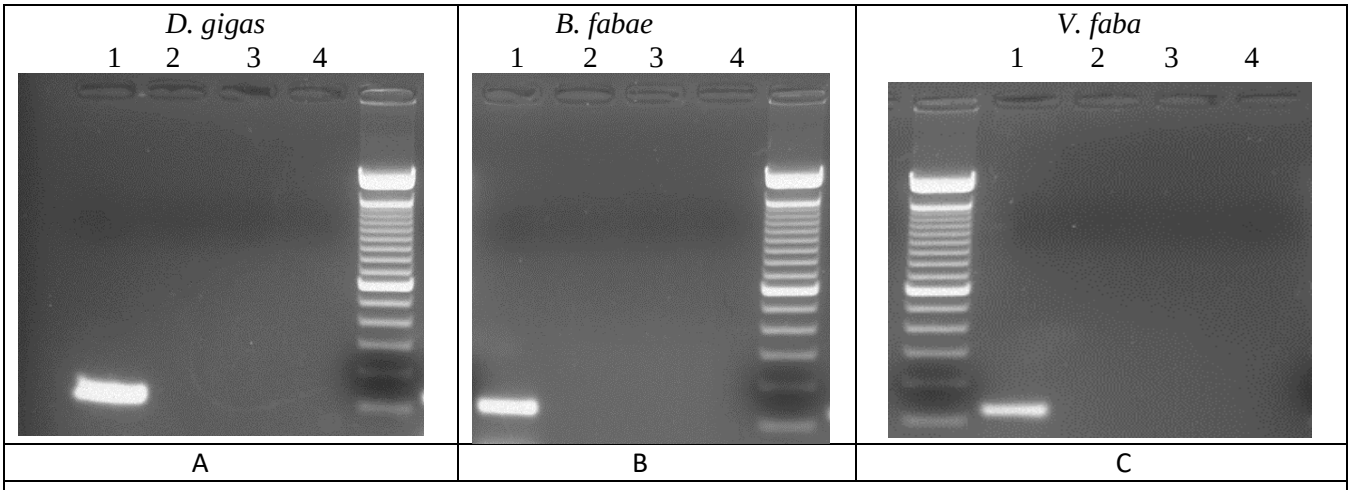

Figure 7.1. Gel electrophoresis of PCR amplicons of the test DNA samples for the specificity of the three primers. (A) Specificity of *D. gigas* primer against the DNA of 1- *D. gigas*, 2- *B. fabae*, 3- *V. faba*, 4 control without DNA. (B) Specificity of *B. fabae* primer against the DNA of 1- *B. fabae*, 2- *D. gigas*, 3- *V. faba*, 4 control without DNA. (C) Specificity of *V. faba* primer against the DNA of 1- *V. faba*, *2- D. gigas*, 3- *B. fabae*, 4 control without DNA.

7.4.3. Relationship between number of *D. gigas* extracted by the modified Baermann method and real- time PCR

There was a linear relationship between the number of *D. gigas* extracted by the modified Baermann method and real- time PCR (Figure 7.2, r = 0.63, P= 0.001). The number of *D. gigas* DNA strands recovered from the real-time PCR outnumbered the count of *D gigas* individuals from modified Baermann method. The zero values are from the Modified Baermann method.

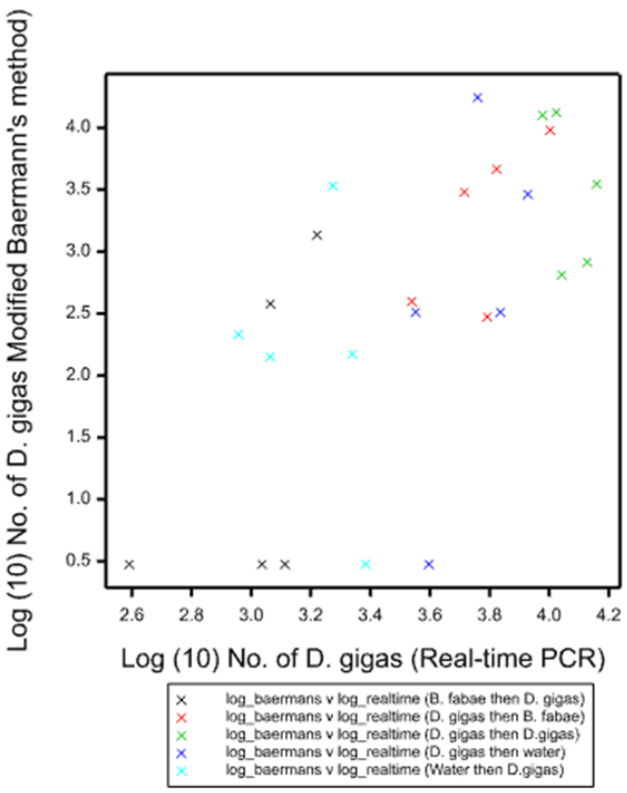

Figure 7.2. Correlation between Number of *D. gigas* extracted by the modified Baermann method and real- time PCR. The values are averages of two biological replicates, and three technical replicates.

7.4.4. Relationship between number of *D. gigas* DNA copy numbers and faba bean DNA copy numbers

There was a linear relationship between the number of *D. gigas* DNA copy numbers and ratio of *D. gigas* DNA/ faba bean DNA (Figure 7.3, r = 0.76, P= 0.001).

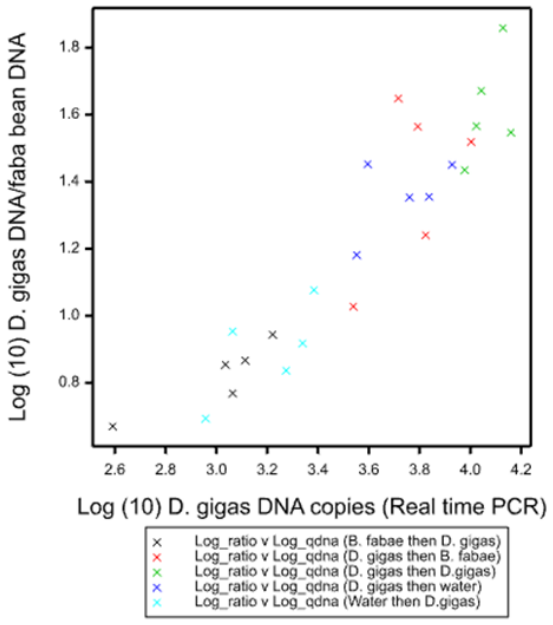

Figure 7.3 Correlation between *D gigas* copy numbers and its ratio to *V. faba* copy numbers. The values are averages of two biological replicates and three technical replicates.

7.4.5. Relationship between number of *D. gigas* extracted from modified Baermann method and faba bean DNA.

A weak and non-significant correlation existed between number of *D. gigas* extracted from the modified Baermann method and faba bean DNA (Figure 7.4, r = 0.32, P= 0.1). This was done as a check of the method with real samples.

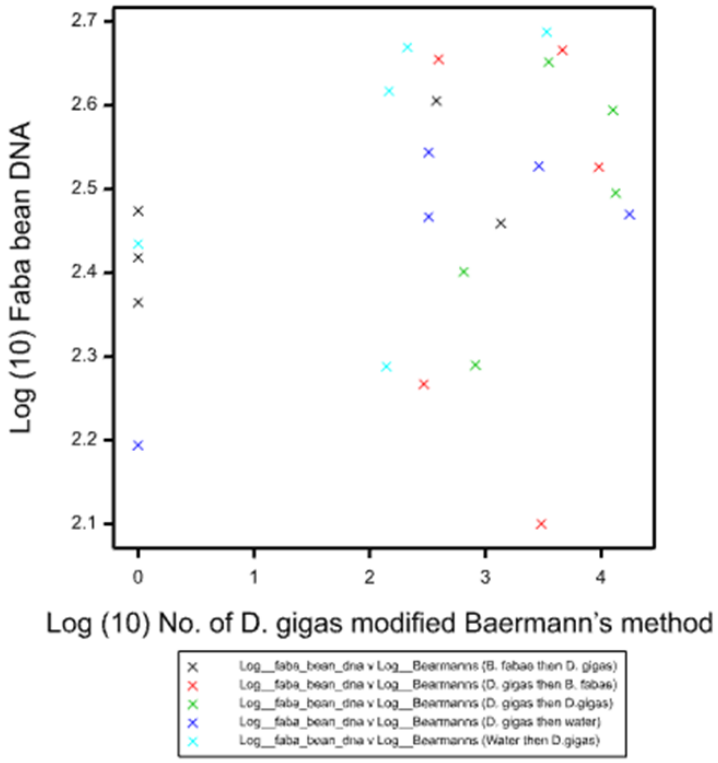

Figure 7.4. Correlation between number of *D. gigas* extracted from modified Baermann method and faba bean DNA. The values are averages of two biological replicates and three technical replicates

7.4.6. Ratio of nematode copy numbers to plant copy numbers

Plant samples with double inoculation of nematodes had significantly ($p < 0.001$) higher ratio of *D. gigas*: *V. faba* DNA, while plants with first inoculation of *B. fabae* and second inoculation of *D. gigas* had the least (Figure 7.5.) as expected from the Baermann count

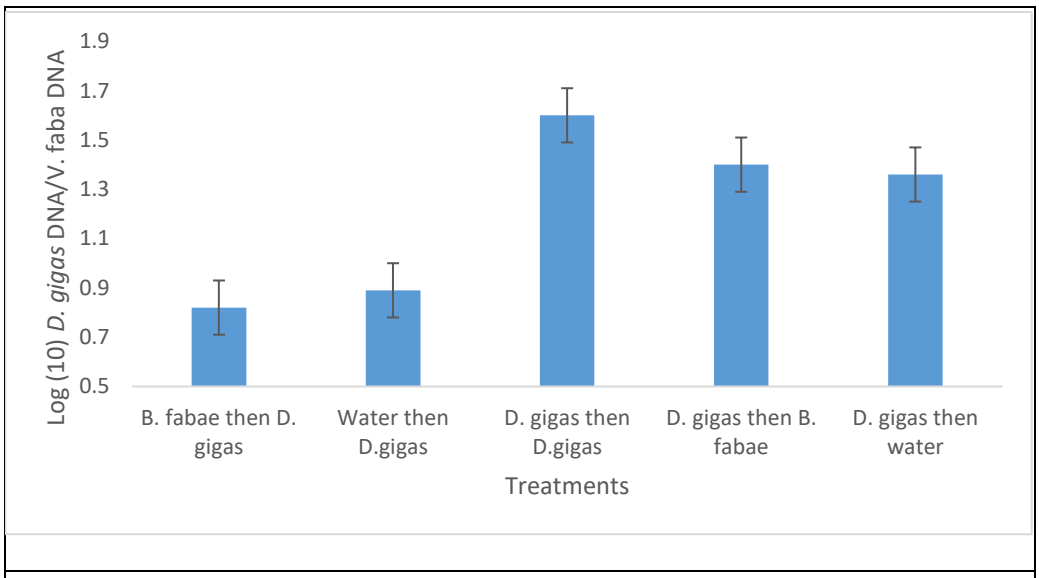

Figure 7.5. Ratio of *D. gigas* DNA to *V. faba* DNA estimated by qPCR. The values are average of two biological replicates and three technical replicates. Error bars denotes standard error of difference.

7.4.7. DNA copy numbers from *B. fabae*

Plant samples with first and second inoculation of *B. fabae* had significantly higher (P<0.001) DNA copy numbers than the treatment with first inoculation of *D. gigas* and second inoculation of *B. fabae* (Figure 7.6.). This is in line with visual observation. Plants with post-treatment of *B. fabae* had the least DNA copy numbers.

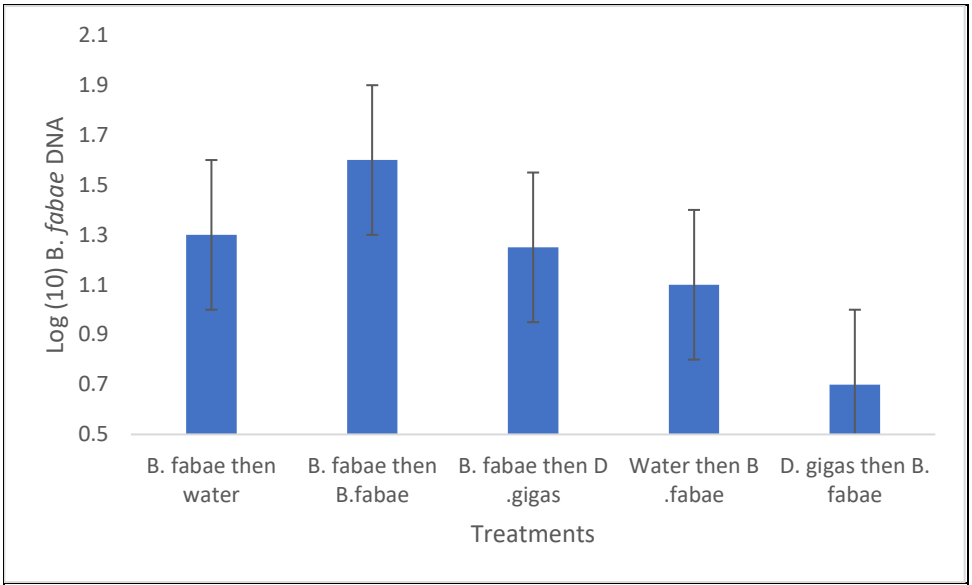

Figure 7.6. Copy number of *B. fabae* estimated by qPCR. The values are averages of two biological replicates, and three technical replicates. Error bars denotes standard error of difference.

7.5. Discussion

A qPCR assay for the identification and quantification of *D. gigas* and *B. fabae* in faba bean was successfully developed in this study. Identification of the genus *Ditylenchus* to spp level is quite difficult, as they are conserved in gross morphology (Brzeski, 1991). Both primers produced a single amplicon in melt curve analysis and there was no cross contamination. Both primers were specie specific when blasted on NCBI site (https://www.ncbi.nlm.nih.gov/nucleotide/). Although the primers were not tested against *D. dipsaci* (a close relative of *D. gigas*) and *B. cinerea* (a close relative of *B. fabae*). *D. gigas* can be distinguished morphologically from *D. dipsaci* using a number of features which include larger body size (1.6- 2.29 mm) compared to *D. dipsaci* (1-7- 2.0 mm), longer vulva-anus distance of 202- 266 mm compared to *D. dipsaci* (202- 266 mm) (Volas *et.al*., 2011). *B. cinerea* grows faster on nutrient media (17-19 d^{-1}mm) compare to *B. fabae* (13 mm d^{-1}mm).

The ability to detect *D. gigas* and *B. fabae* DNA within a mixture with faba bean tissue of host plant and nematode DNA eliminates the need for *D. gigas* extraction and *B. fabae* isolation prior to DNA extraction. The sensitivity of the assay was 78.2 DNA copy numbers for *D. gigas* and 84 DNA copy numbers for *B. fabae*. The sensitivity of the modified Baermann extraction method was not determined. This could have been determined by inoculating plants with a known number of nematodes and extracting the nematodes 1 to 2 days after inoculation.

The DNA yield in the extractions made from preserved tissue from the experiment in chapter 5 for both *D. gigas* and *B. fabae* was very low. There are many reasons that may be responsible for this. Firstly, the samples were oven dried at 70° C and kept in a desiccator, while the assays were being developed. This may have altered the DNA contents of the tissues. Secondly, washing the samples with AW1 and AW2 buffers (Section 8.2.1) enables the removal of salts that will be precipitated during elution with buffer AE. This process needs to be done thoroughly so that the salts are properly get rid of.

The extraction method was modified to accommodate the complexity of the template (*D. gigas*, *B. fabae* and faba bean). Two extraction kits were tried (DNeasy plant mini kit and DNeasy blood and tissue kit). The DNeasy plant mini kit would give a good DNA yield of *B. fabae* and faba bean DNA, but not *D. gigas*, while the DNeasy blood and tissue kit would

give a good DNA yield for *D. gigas* and not *B. fabae* and *V. faba*. DNeasy plant mini kit gave a better DNA yield for the three templates.

The number of nematodes estimated from real time PCR was higher than the modified Baermann method. This agrees with the works of (Toyota *et al*., 2008, Yan *et al*., 2012, Huang, and Yan, 2017). The reason for the higher number of nematodes estimated by the real time PCR was because the real time PCR estimates included the adult, juvenile and the eggs, while the modified Baermann method assessment did not include the eggs. Each cell has two nuclear DNA molecules with lots of ITS copies and hundreds of mitochondria. In addition, in the modified Baermann method, the nematode must be active for it to be extracted, whereas dead nematodes may be detected by real-time PCR (Yan, *et.al*., 2013). The nematode must be mobile as it must move from the plant tissue through the extraction tissue to the water.

Real time PCR estimates of *D. gigas* population were in line with the observation from the modified Baermann method. Thus, the real-time PCR is a better alternative to the traditional modified Baermann method. It is faster and less prone to error. The assay will be useful for accurate detection, identification and quantification of *D. gigas* and *B. fabae* in diagnostic laboratories.

7.6. Conclusion

qPCR assay for the identification and quantification of *D. gigas* and *B. fabae* in faba bean was successfully developed although the assays were not tested against *D. dipsaci* and *B. cinerea*. Each are morphologically different from *D. gigas* and *B. fabae* respectively. The results from this chapter are a good start for the molecular diagnostic and quantification of *B. fabae* and *B. fabae* in faba bean.

Chapter 8: General discussion

Plant diseases are hardly ever caused solely by one organism. Diseases are usually the result of interactions between different microorganisms. In nature, each plant species is affected by about a hundred species of microorganisms (Agrios, 2005). *D. gigas* and *B. fabae* are among the important organisms that cause disease in faba bean. This thesis has explored the interactions between these two organisms on faba bean. The overall aim was to determine if *D. gigas* and *B. fabae* interact and what implications this may have for faba bean productivity. The research also sought to understand the spread of *D. gigas* from a single source of infection on faba bean in field conditions. Finally, the research investigated whether the development of a qPCR assay could aid the identification, detection and quantification of *D. gigas* and *B. fabae* in faba bean?
The main hypotheses examined in this study were:

1. Spatial spread of the nematode on beans through the growing season is dependent on distance of inoculum from the plant, the orientation of the inoculum from the plant and environmental factors in the field. We, therefore, hypothesise that these factors will affect the spread of *D. gigas* from the infected plant to the adjacent ones.

2. *B. fabae* often co-exists in the presence of *D. gigas* and therefore, glasshouse investigations were designed to test the hypotheses that *B. fabae* (as a necrotroph) would decrease susceptibility to *D. gigas* (a biotroph), and that they will reciprocally affect each other's population density and reduce the productivity of faba bean.

3. In the light of work on induced defence mechanisms to plant pathogens and pests, the hypothesis that the order of infection (i.e., whether *D. gigas* infects before *B. fabae* or vice versa) will have an effect on induced resistance and affect crop production was tested.

4. The hypothesis that the glasshouse results obtained previously are applicable to the field situation was tested in two varieties. In the same experiment the hypothesis that increased plant sulphate nutrition would improve yield was tested.

5. Finally, the hypothesis that nematodes loads estimated using the modified Baermann technique would be consistent with those estimated from a newly developed qPCR method was tested.

D. gigas was found to spread from a source plant to adjacent ones and beyond (Chapter 3) in the field. A single *D. gigas* spread to a distance of 1.6 m within one season.

One of the major means of spread of *D. gigas* is through the seed. International trade plays a major role in disseminating *D. gigas*. A single infected faba bean seed can harbour up to 19,000 nematodes (Stawniak, 2011). Quarantine measures are usually put in place in ports to halt the spread of pests of quarantine importance. Despite this measure, some pests may evade detection and find their way into new areas. McCullough *et al*. (2006) analysed the data from the port information network between 1984 and 2000. They revealed that 725, 000 pests were intercepted, with baggage (62 %) and cargoes (30 %) responsible for most of the interception, while 7 % were recovered from plant propagules. This data revealed the extent to which pests are moved about in international trade. It is possible that some of these pests will evade detection, especially in developing countries where state of the art facilities are not available to ensure detection of pests of quarantine importance.

Ditylenchus naturally are adapted to the temperate and subtropical climate. Recent surveys have reported *Ditylenchus* in some more humid conditions. Indarti *et al.* (2018) reported the first record of *D. dipsaci* on garlic in Indonesia. Alabi *et al.* (2017) established the presence of the *Ditylenchus* (Unspecified species) on yam in Nigeria, Maafi *et.al.* (2013) reported the first case of *D. gigas* infestation on broad bean in Iran, while Talwana, *et al.* (2008) also reported the occurrence of *Ditylenchus* (Unspecified species) on yam, cassava, sweet potato and tannia in Uganda. The specific species were not identified in some cases, probably due to absence of a molecular means of identification that could identify the nematode to species level. In most developing countries, nematodes are identified based on morphological characteristics using a microscope. This type of identification is tedious and takes a long time. The real time PCR assays which were developed in this study (Chapter 7) may be employed in the identification of the nematode. Although, the assays were not tested against *D. dipsaci*, *D. gigas* is distinguishable morphologically from *D. dipsaci*, as the former is larger, hence referred to as gigas (giant in Greek). The method developed should ensure efficient and timely identification and quantification of *D. gigas*. The method eliminates the stress of extracting and counting nematodes using the microscope. It also prevents the

occupational hazards associated with the regular use of microscopes. The assay could identify *D. gigas* in the presence of *B. fabae*.

The plot size for the experiment was 2m × 2m (2017) and 4m × 4m (2018) with a spacing of 20 cm between plants. An average of 14 plants were infected per m^2 (20 plants/m^2). If one faba bean seed from each infected plant (on average) were used to plant the next season's crops, this would result in a 14-fold increase per season. The population would continue to increase over the year unless drastic measures were put in place to halt the build-up.

There are no chemicals available for the control of *Ditylenchus* in faba bean (https://www.fwi.co.uk/arable/pulses/how-a-new-test-helps-bean-growers-tackle-stem-nematode , accessed 27 April 2021). This may be due to the cost of applying nematicide. Use of nematicide is also difficult because *D. gigas* inhabits both the soil and plant tissue and are migratory (Lilley *et al.*, 2007a). Nematicides are also persistent in the environment and pests may develop resistance with consistent use. Therefore, growers rely on cultural means of managing the nematode.

Some of the measures that can be employed to manage the nematode include:

1.Use of clean seeds: Only seeds free of nematodes should be sown. Sowing infected seeds or farmers saved seeds may introduce nematode to the site. Farmers' saved seeds can be sent to laboratories such as PGRO for testing before planting.

2. Crop rotation: Unlike *D. dipsaci*, *D. gigas* has a relatively narrow host range. Crop rotation may be ideal to rid land of these nematodes. PGRO (2017) recommend 10 years of rotation with a non-host crop where *D. gigas* is detected. This must be done alongside proper weed control, as the nematode does have some weed hosts. Common weed hosts according to (Stawniak, 2011) are Field bindweed (*Convolvulus arvensis*), White deadnettle (*Lamium album*), Red deadnettle (*Lamium purpureum*), Deadnettle (*Lamium amplexicaule*) and Sterile oat (*Avena sterilis*).

3. Destruction of infected plant debris: Infested plant material should be removed and destroyed. This is necessary as the nematode can persist and remain viable in infected plant material for a long time.

4. Use of resistant varieties: Where available, resistant varieties are one of the best options for the management of *Ditylenchus*. Resistant varieties prevent the multiplication of nematode and thereby supress their population. However, there are no varieties of faba bean resistant to

D. gigas and there are no fully resistant varieties of faba bean against chocolate spot (https://cropscience.bayer.co.uk/threats/diseases/pea-and-bean-diseases/chocolate-spot-beans/ [Accessed 22 May 20.

The glasshouse dose-response experiments established a synergistic reduction in grain yield between *D. gigas* and *B. fabae*. However, an antagonistic interaction was observed between *B. fabae* and populations of *D. gigas* in plant tissue and soil. The synergistic interaction resulted in greater reduction in the yield of faba bean. This implies that the co-infection of both pathogens on the field will cause yield reduction greater than that caused by either of the pathogens alone. 21]. These experiments also revealed that the nematode did not alter the severity of chocolate spot induced by *B. fabae* even though nematode interactions with fungi can cause diseases in a several ways. In particular, nematodes' stylets are used to puncture the plant during ingress. The hole created can be exploited by other pathogens which are unable to breach the plant structural defences on their own. *B. fabae* can penetrate the plant on its own without nematode assistance but the hole created could make the entry of the fungal easier.

In a field situation, the severity of chocolate spot may be more severe than observed in the glasshouse. One explanation is that in field conditions *Botrytis* propagules can be dispersed by several means, including rain, wind and insects. Raindrops can dislodge conidia from infected leaves and splash them on other parts of the plant or nearby plants. Dried conidia can be dispersed by wind. This is the most important means of *Botrytis* propagule spread (Elad *et al*., 2007) but the conidia of *B. cinerea* can also be dispersed by insects. Insects that have been implicated in the spread of chocolate spot include Mediterranean fruit fly (*Ceratitis capitata*, Engelbrecht, 2002) and the vinegar fly (*Drosophila melanogaster*, Louis *et al*., 1996).

Reproduction of *D. gigas* was suppressed by *B. fabae* when co-inoculated. Virulence of *D. gigas* is measured by their rate of reproduction (Stawniak, 2011). *B. fabae* appears to suppress the multiplication of *D. gigas*. *B. fabae* is a necrotrophic pathogen, while *D. gigas* is a biotrophic organism. While the cell death caused by *B. fabae* is noticeable about 24 hours after inoculation, lesions resulting from *D. gigas* infestation may take a longer time to manifest. The reduction in the reproduction rate of the nematode after co-inoculation with *B.*

fabae may be due to reduction in food supplies, as the fungus injects toxins to kill the plant cells. At this point, the options available to the nematode is to migrate to another part of the plant not colonised by the fungus, or to explore new foliage as it emerges. Despite the reduction in the reproductive potential of the nematode, the faba bean yield was lowest at the highest doses of both nematode and fungus.

In the controlled environment chamber experiment (Chapter 5), chocolate spot severity was not detectably altered by prior *B. fabae* inoculation. This was similar to the result obtained in the dose response experiment (Chapter 4) although the time of inoculation was different (in the dose response experiment, the two organisms were co-inoculated, while there are two weeks intervals between the two inoculations in the controlled environment).

Also, in the controlled environment experiment, the severity of chocolate spot on the upper leaves was reduced as a result of the initial *B. fabae* inoculation on the lower leaves, suggesting induced resistance. Induced resistance is a mechanism by which plants protect themselves against intruding pests and pathogens. Constitutive defences are present before plants are attacked, while induced defenses are ignited upon attack by plants. Individual plant defence mechanisms may function both as constitutive and inducible resistance (Ahman, 2009).

Plant defence inducing agents can either be biotic or abiotic. *B. fabae* acts as a biotic inducing agent in this study. Chemical inducers are now commercially produced and can be used to induce defence responses in plants; as such they may be used in place of synthetic pesticides in the control of plant diseases. Examples of such chemical inducers are salicylic, citric, ascorbic and oxalic acids. The efficacy of each of these against chocolate spot in faba bean was at par with carbendazim (Mbazia, Omri Ben Youssef *et al*., 2016).

Recently chlorothalonil, which is one of the most widely used pesticides worldwide, was banned from usage in 2020 by the European Union states after a review by European food safety authority, due to health and environmental concerns. The pesticide has been commercialised since 1964, used especially on cereal crops (barley and wheat), legumes (peas and beans) as well as potatoes. It is a broad-spectrum pesticide which can be used as a fungicide, bactericide and nematicide. Chlorothalonil in mixture with cyproconazole was one

of the fungicides used by farmers and recommended by the PGRO in UK to combat chocolate spot in faba bean. The recent ban has left farmers looking for alternative means of controlling pests on their crops.

Two season field experiments were conducted to see if the induced resistance observed in the controlled environment (Chapter 6) could be substantiated in field conditions. In the first season, the result obtained (early inoculation of *B. fabae* resulted in reduced severity in subsequent infection) was in line with the controlled environment observations, though the significance was low. The results in the second season differed from those in the controlled environment, though again the significance of treatment differences was very low. One reason for this may have been the differences between the conditions in the controlled environment and the field. Successful pathogen infection occurs in a favourable environment (Temperature, relative humidity, light, water, etc). In particular, the conditions in the controlled environment were fixed (Chapter 4) but fluctuated in the field.

Sulphur also improved the yield of faba bean in both seasons (though not significantly in either). This was expected, as obvious effects of sulphur were first observed at the student demonstration plots at the University of Reading experimental site at Sonning prior to this experiment. Sulphur is a constituent of plant proteins and is required by plants to efficiently utilize nitrogen and other nutrients. Its deficiency symptoms are similar to those of nitrogen, reducing crop growth and yield. Because sulphur is a constituent of plant protein, the quality of the product is also severely affected. Plant nutrient-induced resistance is receiving interest owing to its efficacy in controlling plant diseases. Schnug *et al*. (1995) introduced the term sulphur induced resistance. It signifies boosting of the innate resistance of plants to fungal pathogens by activating metabolic processes involving sulphur through soil applied fertilizers. Concentrations of reactive oxygen species (a major signalling molecule) are connected to sulphur metabolism (Foyer and Halliwell, 1976). Sulphur containing fertilizer can be used alongside chemical inducers in the control of chocolate spot and nematode induced stem lesion in faba bean.

There are no faba bean cultivars completely resistant to *D. gigas.* Stawniak (2011) reported that some genotypes thought to be resistant were susceptible to UK isolates of *D. gigas*. In my field experiments the variety Babylon had less severe chocolate spot severity. This was

expected, as the cultivar had a better disease rating than Fuego in a trial conducted by PGRO (2017). Hence, the cultivar can be used to reduce the severity of chocolate spot.

8.1. Conclusion

The results from this study have shown that *D. gigas* did not detectably alter the severity of chocolate spot caused by *B. fabae*, while co-inoculation of both organisms further reduced the yield of faba bean. Induced resistance could be a viable alternative in the control of chocolate spot in faba bean. Inducers are environmentally friendly and a potentially cheaper alternative to fungicides.

D. gigas have the potential to spread as far as 1.6 m within a season. They do not necessarily rely on passive movement such as farm equipment, machinery etc. It is necessary to plant only clean seeds to prevent their spread, but threshold needs to be effectively zero, which is clearly unachievable. *D. gigas* has the potential to spread in the field and can persist for a long time, even in the absence of the host plant. They are also resistant to desiccation; this can make their eradication harder. Therefore, it is better to prevent the nematode from entering the field. This requires detection of very low concentrations of nematode. PCR assays for the identification and quantification of *D. gigas* and *B. fabae* were developed. These assays, after further validation, could be employed by diagnostic laboratories in the identification and quantification of the two organisms. Where the organisms are endemic, the population density can be managed by different means including the use of cultural and biological methods (Chapter 1).

8.2. Future work

The specificity of the primers designed should be checked against close relatives, including at least *D. dispaci* in case of *D. gigas* and *B. cinerea* in case of *B. fabae*.

The detection threshold of the modified Baermann method could be determined by inoculating plants and nematode extracted after 1- 2 days post inoculation.

Co-inoculation of *D. gigas* and *B. fabae* in the glasshouse greatly reduced the yield of faba bean. However, the conditions (temperature, relative humidity, photoperiod) in the glasshouse were fixed. In field situations, these conditions vary and the results are likely to

differ. Larger scale field experiments to determine the interactions of the two organisms would be worthwhile.

Infection of faba bean resulted in an induced defence response, and synthetic chemical inducers have been reported to be effective against chocolate spot in faba bean. Studies to assess the efficacies of synthetic inducers against co-infection of *D. gigas* and *B. fabae* should be conducted.

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APPENDICES

Table 1: Summary of ANOVA table of the effects of co-inoculation of *B. fabae* and *D. gigas* on population of *D. gigas* plotted on a log scale (log10 (x+1). d.f = degree of freedom, s.s = sum of square, v.r = variance ratio, F.pr = F-test probability associated with variance ratio.

Source of variation	d.f.	m.s.	v.r.	F pr.
Rep stratum	4	0.10133	1.18	
Rep.*Units* stratum				
B. fabae	2	0.84631	9.86	<.001
D. gigas	5	32.50722	378.68	<.001
B. fabae x *D. gigas*	10	0.30710	3.58	<.001
Residual	68	0.08584		
Total	89			

Table 2: Summary of ANOVA table of the effects of co-inoculation of *B. fabae* and *D. gigas* on stem lesion length. d.f = degree of freedom, s.s = sum of square, v.r = variance ratio, F.pr = F-test probability associated with variance ratio.

Source of variation	d.f.	(m.v.)	m.s.	v.r.	F pr.
Rep stratum	4		0.05792	1.38	
Rep.*Units* stratum					
B. fabae	2		0.05458	1.30	0.280
D. gigas	5		1.87469	44.60	<.001
B. fabae. D. gigas	10		0.04087	0.97	0.476
Residual	63	(5)	0.04204		
Total	84	(5)			

Table 3: Summary of ANOVA table of the effects of co-inoculation of *B. fabae* and *D. gigas* on number of tillers. d.f = degree of freedom, s.s = sum of square, v.r = variance ratio, F.pr = F-test probability associated with variance ratio.

Source of variation	d.f.	m.s.	v.r.	F pr.
Rep stratum	4	0.008273	1.64	
Rep.*Units* stratum				
B. fabae	2	0.005138	1.02	0.367
D. gigas	5	0.016453	3.26	0.011
B. fabae x D. gigas	10	0.006348	1.26	0.272
Residual	68	0.005050		
Total	89			

Table 4: Summary of ANOVA table of the effects of co-inoculation of *B. fabae* and *D. gigas* on stem lesion girth. d.f = degree of freedom, s.s = sum of square, v.r = variance ratio, F.pr = F-test probability associated with variance ratio.

Source of variation	d.f.	m.s.	v.r.	F pr.
Rep stratum	4	15.780	8.55	
Rep.*Units* stratum				
B. fabae	2	1.358	0.74	0.483
D. gigas	5	44.809	24.29	<.001
B. fabae x *D. gigas*	10	0.539	0.29	0.981
Residual	64	1.845		
Total	85			

Table 5: Summary of ANOVA table of the effects of co-inoculation of *B. fabae* and *D. gigas* on grain yield. d.f = degree of freedom, s.s = sum of square, v.r = variance ratio, F.pr = F-test probability associated with variance ratio.

Source of variation	d.f.	m.s.	v.r.	F pr.
Rep stratum	4	0.10685	3.33	
Rep.*Units* stratum				
B. fabae	2	0.04199	1.31	0.277
D. gigas	5	0.31157	9.72	<.001
B. fabae x *D. gigas*	10	0.06359	1.98	0.049
Residual	68	0.03207		
Total	89			

Table 6: Summary of ANOVA table of the effects of co-inoculation of *B. fabae* and *D. gigas* on *D. gigas* severity symptom index. d.f = degree of freedom, s.s = sum of square, v.r = variance ratio, F.pr = F-test probability associated with variance ratio.

Source of variation	d.f.	m.s.	v.r.	F pr.
Rep stratum	4	0.006571	0.83	
Rep.*Units* stratum				
B. fabae	2	0.006011	0.76	0.474
D. gigas	5	0.446454	56.18	<.001
B. fabae x *D. gigas*	10	0.009781	1.23	0.291
Residual	60	0.007947		
Total	8			

Table 7: Summary of ANOVA table of the effects of co-inoculation of *B. fabae* and *D. gigas* on population of *D. gigas* in 200 ml soil plotted on a log scale (log10 (x+1). d.f = degree of freedom, s.s = sum of square, v.r = variance ratio, F.pr = F-test probability associated with variance ratio.

Source of variation	d.f.	m.s.	v.r.	F pr.
Rep stratum	4	0.2596	0.87	
Rep.*Units* stratum				
B. fabae	2	2.8622	9.58	<.001
D. gigas	5	3.2098	10.75	<.001
B. fabae x *D. gigas*	10	0.3878	1.30	0.249
Residual	68	0.2986		
Total	89			

Table 8: Summary of ANOVA table of the effects of co-inoculation of *B. fabae* and *D. gigas* on chocolate spot severity. d.f = degree of freedom, s.s = sum of square, v.r = variance ratio, F.pr = F-test probability associated with variance ratio.

Source of variation	d.f.	m.s.	v.r.	F pr.
Rep stratum	4	496.1	1.65	
Rep.*Units* stratum				
B. fabae	1	867.8	2.89	0.096
D. gigas	5	203.7	0.68	0.641
B. fabae x *D. gigas*	5	102.8	0.34	0.884
Residual	42	299.8		
Total	57			

Table 9: Summary of ANOVA table of the Interactions of *B. fabae* and fertilizers on percentage severity of chocolate spot on faba bean (2017). Six plants were sampled in a plot size of 1.9 x 5 m. Plants were arraigned in a split plot design, with variety as main plot and spray as sub-plot

Source of variation	d.f.	m.s.	v.r.	F pr.
Block stratum	5	6.50	0.85	
Block*fertilizer*stratum				
Fertilizer	1	0.35	0.05	0.839
Residual	5	7.66	0.92	
Block*fertilizer*units*stratum				
Spray	1	24.44	2.92	0.118
Fertilizer*spray	1	0.00	0.00	0.995
Residual	10	8.37		
Total	23			

Table 10: Interactions of varieties, fertilizers and *B. fabae* inoculation on percentage severity of chocolate spot (2018). Six plants were sampled in a plot size of 1.9 x 5 m. Plants were arraigned in a randomised complete block design.

Source of variation	d.f.	m.s.	v.r.	F pr.
Rep stratum	3	0.0341	0.28	
Rep*units*stratum				
Fertilizer	1	0.0539	0.45	0.511
Spray	1	0.1784	1.48	0.238
Variety	1	0.0643	0.53	0.474
Fertilizer*spray	1	0.1210	1.00	0.328
Fertilizer*variety	1	0.0003	0.00	0.963
Spray*variety	1	0.0815	0.67	0.421
Fertilizer*spray*variety	1	0.1638	1.36	0.257
Residual	21	0.1208		
Total	31			

Table 11: Summary of ANOVA table of the Interactions of *B. fabae* and fertilizers on percentage rust severity on faba bean (2017). Six plants were sampled in a plot size of 1.9 x 5 m. Plants were arraigned in a split plot design, with variety as main plot and spray as sub-plot

Source of variation	d.f.	m.s.	v.r.	F pr.
Block*stratum	5	336.67	2.36	
Block*spray stratum				
Spray	1	150.00	1.05	0.352
Residual	5	142.50	1.83	
Block*spray*fertilizer stratum				
Fertilizer	1	66.67	0.86	0.377
Spray*fertilizer	1	204.17	2.62	0.137
Residual	10	77.92		
Total	23			

Table 12: Interactions of varieties, fertilizers and *B. fabae* inoculation on percentage rust severity (2018). Six plants were sampled in a plot size of 1.9 x 5 m. Plants were arraigned in a randomised complete block design.

Source of variation	d.f.	m.s.	v.r.	F pr.
Rep stratum	3	0.4146	2.87	
Rep*units*stratum				
Fertilizer	1	0.0395	0.27	0.607
Spray	1	0.0671	0.46	0.503
Variety	1	0.4073	2.82	0.108
Fertilizer*spray	1	0.0030	0.02	0.888
Fertilizer*variety	1	0.4229	2.92	0.102
Spray*variety	1	0.2354	1.63	0.216
Fertilizer*spray*variety	1	0.3928	2.72	0.114
Residual	21	0.1446		
Total	31			

Table 13: Summary of ANOVA table of the Interactions of *B. fabae* and fertilizers on aphid count (2017). Six plants were sampled in a plot size of 1.9 x 5 m. Plants were arraigned in a split plot design, with variety as main plot and spray as sub-plot

Source of variation	d.f.	m.s.	v.r.	F pr.
Block stratum	5	3210.3	1.81	
Block*spray stratum				
Spray	1	970.3	0.55	0.493
Residual	5	1773.4	1.98	
Block*spray*fertilizer stratum				
Fertilizer	1	950.0	1.06	0.327
Spray*fertilizer	1	980.5	1.10	0.320
Residual	10	893.7		
Total	23			

Table 14: Interactions of varieties, fertilizers and *B. fabae* inoculation on aphid count (2018). Six plants were sampled in a plot size of 1.9 x 5 m. Plants were arraigned in a randomised complete block design.

Source of variation	d.f.	m.s.	v.r.	F pr.
Rep stratum	3	0.5089	0.78	
Rep*units*stratum				
Fertilizer	1	0.1059	0.16	0.692
Spray	1	0.1857	0.28	0.600
Variety	1	0.0070	0.01	0.919
Fertilizer*spray	1	0.0339	0.05	0.822
Fertilizer*variety	1	0.0019	0.00	0.957
Spray*variety	1	1.0618	1.62	0.217
Fertilizer*spray*variety	1	0.5411	0.82	0.374
Residual	21	0.6560		
Total	31			

Table 15: Summary of ANOVA table of the Interactions of *B. fabae* and fertilizers on grain yield of faba bean (2017). Six plants were sampled in a plot size of 1.9 x 5 m. Plants were arraigned in a split plot design, with variety as main plot and spray as sub-plot

Source of variation	d.f.	m.s.	v.r.	F pr.
Block stratum	5	126.1	0.56	
Block*spray*stratum				
Spray	1	211.2	0.94	0.378
Residual	5	225.5	2.25	
Block*spray*fertilizer stratum				
Fertilizer	1	11.8	0.12	0.739
Spray*fertilizer	1	84.4	0.84	0.381
Residual	10	100.3		
Total	23			

Table 16: Interactions of varieties, fertilizers and *B. fabae* inoculation on grain yield (2018). Six plants were sampled in a plot size of 1.9 x 5 m. Plants were arraigned in a randomised complete block design.

Source of variation	d.f.	m.s.	v.r.	F pr.
Rep stratum	3	0.01303	0.98	
Rep.*units*stratum				
Fertilizer	1	0.00291	0.22	0.645
Spray	1	0.01711	1.29	0.270
Variety	1	0.00483	0.36	0.553
Fertilizer*spray	1	0.00641	0.48	0.495
Fertilizer*variety	1	0.01348	1.02	0.326
Spray*variety	1	0.01110	0.84	0.371
Fertilizer*spray*variety	1	0.00039	0.03	0.866
Residual	20	0.01327		
Total	30			