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An initial assessment of a novel nematicide.

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Abstract

Potato (*Solanum tuberosum*) is an essential crop in the UK and around the world. The most important soil pests of potato production are potato cyst nematodes (PCN), *Globodera pallida* and *G. rostochiensis*, which are subject to regulation and quarantine by the European Union and are present in 48% of ware potato growing sites in the UK. A novel control method has been proposed for PCN; the formulation BGT. This study investigated the effect of BGT and its components on the hatching, mortality and root invasion of infective *G. pallida* juveniles, and found that BGT had a significant detrimental effect on the hatching and mortality of juveniles. The active ingredient in BGT was identified and a second formulation was developed; UBA. The investigations described in this study suggest that the novel formulation UBA could potentially prevent yield loss caused by potato cyst nematodes, and could be an ideal candidate for inclusion into integrated pest management systems.

Declaration and Acknowledgements

I declare that the work submitted in this thesis is my own.

I would like to thank my supervisors Dr Matthew Back and Dr Ivan G Grove for their guidance and encouragement. I would also like to thank my colleagues at Harper Adams for the advice and coffee shared. My sincere thanks to Arcis Biotechnology for their support and sponsorship in undertaking this research, particularly Dr Jan Rogers, Jill Wilkinson and Lucy Martin. Heartfelt thanks go to my friends for their endless patience and cheerleading. I am profoundly grateful to my family, to whom this thesis is dedicated to. Diolch yn fawr.

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1.0 General Introduction

Potatoes *(Solanum tuberosum)* are an important food crop and are susceptible to a number of pests and pathogens, including potato cyst nematodes. The potato cyst nematodes (PCN) *Globodera rostochiensis* and *G. pallida* are currently the most important nematode pest species in the UK and a major factor limiting the efficient production of potatoes (Twining et al., 2009). The management of PCN focuses on suppressing multiplication of PCN populations and preventing crop yield loss, and usually consists of a number of control methods, including crop rotation, the use of resistant and tolerant cultivars and chemical nematicides. The nematicides currently in use are under threat due to concerns about their toxicity to non-target organisms, and there is demand for novel nematicides to be developed for use in integrated pest management systems. A formulation of surfactants with broad spectrum antimicrobial activity has been proposed as a novel nematicide, and will be investigated throughout this project.

1.1 Potato

Potato (*Solanum tuberosum*) is a member of the Solanaceae, an economically important family that includes tomato, pepper, aubergine, petunia and tobacco (Xu *et al*., 2011). It is the world's most important non-grain food crop and was the 5th most produced commodity in the world in 2016, behind sugar cane, maize, wheat and rice (FAO, 2018; Xu *et al*., 2011).

1.1.1 The potato industry in the UK

Potatoes make up the largest share of the UK carbohydrates market, which in 2016/2017 was 46% (Agriculture & Horticulture Development Board (AHDB), 2017a). In 2016, 5.4 million tonnes of potatoes were produced in the UK, and 377 million tonnes were produced globally (FAO, 2018).

In 2017, 1,925 growers (of more than 3 ha) managed 121,000 ha of potatoes with an average of 63 ha per grower, 10 ha per grower more than 5 years ago (AHDB 2017). Over half of the total area planted was managed by just 15% of growers, growing 100 ha or more each (AHDB, 2017). Consolidation of the industry has meant that the number of smaller-scale growers are in decline. Although the amount of registered growers has reduced by >97% and the area planted has declined by 56% from 1960 to 2016, the average yields per hectare have increased over the last 5 decades, from just under 23 tonnes ha^{-1} in 1960 to 39 tonnes ha^{-1} in 2016 (AHDB, 2017; FAO, 2018). This increase in yield has been driven by research into agronomy and improvement of crop protection, mechanization, irrigation and fertiliser regimes varieties used.

1.1.2 Major diseases and pests of potatoes

Potatoes are susceptible to over 30 bacterial, fungal, oomycete and viral diseases, as well as 12 common disorders, all of which are of economic significance in the UK (Bradshaw *et al*., 2001). These diseases account for approximately 15% crop losses annually according to the latest survey performed, in 2001 (Bradshaw *et al*., 2001). The crop is vulnerable to disease at every stage of growth, with pathogens targeting the haulm, roots, stolons and tubers (Twining *et al*., 2009). The main routes of infection are through infected seed, air-borne pathogens infecting foliage and soil-borne pathogens infecting roots.

Late blight (*Phytophthera infestans*) is the most economically important disease of potatoes, with annual crop losses of over 7%. Preventative fungicide treatments are made up to 18 times in a season to reduce the severity and incidence of late blight, even when there is a low risk of disease pressure (Bradshaw *et al*., 2001; Twining *et al*., 2009). The most common surface blemish diseases found in the 2001 survey were common scab, silver scurf, black dot and black scurf; 43% of crops surveyed were found to contain these diseases, which most affect seed and fresh ware markets, although they account for only 1% yield loss (Bradshaw *et al*., 2001). The restrictions on seed health are much greater than those of ware crops, and of the ware markets, the fresh packed sector is the most restrictive, demanding a marketable (larger) size and skin finish.

Yield losses in potatoes due to weeds average around 1%, due to interference with harvesting or encouraging pest attacks (Twining *et al*., 2009). Information on the effect of weeds on potato yields and quality is limited, and more research is required to improve the reliability of available information and improve the use of herbicides (Twining *et al*., 2009).

Several pests also affect both seed and ware production of potatoes. Aphids are one of the most important pests of seed production, with the peach-potato aphid responsible for 6% losses annually (Twining *et al*., 2009). Slugs affecting both seed and ware production, with up to 40% of crops being affected and a 2% reduction in yield potential in both sectors (Twining *et al*., 2009). The most important soil pests of both seed and ware potato production are potato cyst nematodes, *Globodera pallida* and *G. rostochiensis*.

1.2 Potato Cyst Nematodes

Nematodes are vermiform microorganisms that mostly live in fresh or salt waters or in the soil, and are viewed as beneficial contributors to many ecosystems. There are a number of species, however, that parasitize humans, animals and plants. It is estimated that plant parasitic nematodes are responsible for 9 - 15% yield losses in several economically

important crops, including bananas, sugar beet, soybeans and potatoes (Agrios, 2005; Nicol *et al*., 2011).

Potato cyst nematodes (PCN) mainly parasitize potatoes – *Solanum tuberosum* – but are also pests of tomatoes, aubergine and other solanaceous crops (Agrios, 2005). Potato cyst nematodes coevolved alongside their preferred host, *Solanum* spp. from South America (Brodie *et al*., 1993). In 1881, Kühn first recorded cyst-forming nematodes on potatoes and they have since been reported on all continents where potatoes are grown (Moens *et al*., 2018; Turner and Evans, 1998). They are responsible for an estimated 4% in lost yield potential, costing the industry £25 million annually (Twining *et al*., 2009). Potato cyst nematodes are one of many potato pests and diseases subject to regulation and quarantine by the European Union, as listed in Annex I and Annex II of the European Union's Council Directive 2000/29/EC (The Council of the European Union, 2000). The potato cyst nematodes *G. rostochiensis* and *G. pallida* are currently the most important nematode pest species in the UK and a major factor limiting the efficient production of potatoes (Twining et al., 2009). All land used for seed potato production must be proven free of potato cyst nematodes. Potato cyst nematodes are present in 48% of ware potato growing land and of the populations found, 89% were *G. pallida*, 5% were *G. rostochiensis* and 6% contained both species (Dybal-Lima *et al*., 2016).

Each PCN species is further categorized into pathotypes; a group of individual nematodes with common genes for virulence. Virulent populations are able to reproduce on potato cultivars without a corresponding resistance gene (Chapter 1.3.2) (Moens *et al*., 2018). The identification of pathotypes is based on the interactions between differential clone host plants and PCN, where a nematode population multiplication rate of >1.0 indicates susceptibility and a multiplication rate of <1.0 indicates resistance (Turner and Subbotin, 2013). Though some populations are homozygous for virulence, e.g. Ro1 and Pa1, several *G. pallida* populations are heterozygous. These populations are referred to as virulence groupings within pathotypes (Turner and Subbotin, 2013). At the time of writing, a collaborative project between Harper Adams University, The James Hutton Institute and Science and Advice for Scottish Agriculture (SASA), funded by Agriculture and Horticulture Development Board (AHDB) is investigating the virulence of *G. pallida* populations of Great Britain (Dybal-Lima *et al*., 2013).

An additional (or alternative) system to pathotyping is under development at the time of writing, which focuses on the mitochondrial gene cytochrome B. This gene has been used to differentiate three genetically distinct groups of *G. pallida*, consistent with three main introductions of the pathogen into Europe (Plantard *et al*., 2008). These three 'mitotypes' represent a promising opportunity to study the diversity of *G. pallida* in the UK and to examine their distributions (Eves-Van Den Akker *et al*., 2015).

1.2.1 The life cycle of the Potato Cyst Nematode

The active, infective stage of the life cycle of *Globodera* spp. begins with the hatching of second-stage juveniles (J2) from the dormant eggs within cysts (Figure 1.1). Hatching is stimulated by hatching factors present in the chemicals exuded from the host plant roots, known as potato root diffusate (PRD). Potato root diffusate is produced along the length of plant roots, most potently at the root tip, and can stimulate up to 60-80% of eggs to hatch (Rawsthorne and Brodie, 1986). Hatching factors within PRD induce a change in permeability in the egg-shell via interaction with Calcium cations in the lipoprotein membrane (Clarke and Hennessy, 1983; Clarke and Perry, 1985). Not all eggs hatch simultaneously; hatching is affected by a number of factors, including the host species, host photoperiod, soil temperature and the species of PCN (Evans, 1982a; Rawsthorne and Brodie, 1986; Robinson *et al*., 1984). *Globodera rostochiensis* juveniles have a higher optimum hatch temperature (15-25°C) than *G. pallida* juveniles (10-18°C), and *G.pallida* hatch at a slower rate than *G. rostochiensis* (Brodie *et al*., 1993). The longer hatching and emergence period of *G. pallida* allows emerging juveniles to avoid nematicides applied during planting, until their levels have decreased to sub-lethal levels (Whitehead, 1992).

Prior to hatching, juveniles in eggs are surrounded with perivitelline fluid, which contains a high concentration of the disaccharide trehalose. Trehalose increases osmotic pressure within the egg, preventing movement of the juvenile inside (Perry and Curtis, 2013). The change in membrane permeability, initiated by hatching factors in PRD, allows trehalose to leach out of the egg, and water to enter (Clarke *et al*., 1978; Ellenby and Perry, 1976). The rehydration of the juvenile allows movement and exploration of the egg. The juvenile cuts a slit in the eggshell using its stylet, and exits the cyst through any available opening, such as the fenestral region or where the head of the female has broken away (Turner and Rowe, 2006). *Globodera pallida* J2s are vermiform and measure up to 0.5 mm in length (Jones & Northcote 1972). They are able to detect the concentration gradient of the PRD using chemoreceptors (amphids), and are attracted towards the roots (Farnier *et al*., 2012). Juveniles are able to move up to one metre through soil to locate a suitable host (Turner and Evans, 1998).

Globodera pallida J2s invade both main and lateral roots during the primary growth of potatoes (Turner and Evans, 1998). Various proteins are released from the oesophageal glands which alter the cell wall and soften it, allowing penetration by the stylet (Jones and Northcote, 1972). After penetrating the rhizodermis, the juvenile migrates intracellularly to the cortex or endodermis, creating a pathway of destroyed cells (Jones and Northcote, 1972). In the cortex or endodermis, the juvenile uses its stylet to probe and select a feeding site (syncytium) (Sobczak and Golinowski, 2009). After the stylet pierces the cell wall, a feeding plug and a feeding tube are formed and saliva is pumped into the cell and

mixes with the cytoplasm (Jones and Northcote, 1972). The feeding plug anchors the nematode in place, whilst the tube acts as a molecular sieve (Sobczak and Golinowski, 2009), and the cell contents are then ingested by the nematode. The cytoplasm proliferates and the multinucleate syncytium enlarges by dissolution of cell walls (Sobczak and Golinowski, 2009).

The infective J2 of PCN are not sexually differentiated. Epigenetic factors control the proportions of females and males, such as the amount and quality of food provided by the syncytia (Trudgill, 1967). If there is adequate nutrition and space, then the juvenile will mature into a female. If food is scarce, then the juvenile will mature into a male, as they require less nutrition (Trudgill, 1967). Physiological differences between the sexes begin to develop at the latter period of the juvenile life-cycle. (Sobczak and Golinowski, 2009). As well as the availability of nutrition, other environmental factors also affect the maturation of larvae, for example if more lateral roots are present in the root system there will be in a greater proportion of male nematodes developing (Trudgill, 1967). Males are also more likely to develop when there are high initial population densities of PCN (Trudgill, 1967). Overall, the determination of sex due to environmental conditions maintains the population, by increasing the proportion of females (and therefore eggs) when the population density diminishes, and by decreasing the proportion of females when the population density increases (Trudgill, 1967).

Male juveniles feed from the syncytium during the J2 and J3 developmental stages, whereas female juveniles feed throughout (J2, J3, J4 and adult). Sobczak and Golinowski (2009) calculated that a female consumes 29 times more nutrients than a male throughout all feeding stages. After the J2 and J3 stages, males undergo two more moults to adult, before exiting the root and searching for a female to fertilise (Sobczak and Golinowski, 2009). Adult males measuring 1 – 1.5 mm in length emerge from the roots between 20-28 days after juveniles first invade the roots, and are active for up to approximately 11 days (Evans, 1970; Jones and Northcote, 1972; Robinson et al., 1987).

Adult females begin to develop into a saccate shape at J3 and become sedentary (Jones and Northcote, 1972). Their gonads continue to grow until they rupture the root tissue, with their head and neck region cemented into the syncytium (Turner and Evans, 1998). Eruption through the root occurs approximately 28 days after the J2s first invade the roots (Evans, 1970). Unfertilised females produce a chemical attractant, which attracts males up to a distance of 15 cm (Evans, 1970). Females are able to be fertilised for at least 40 days, and fertilisation is usually completed approximately 6 weeks after invasion of the roots (Evans, 1970). This extended fertilisation window allows for the delayed emergence of males which may occur where the soil moisture is low (Evans, 1970, 1969). Once fertilised, 200-500 embryos develop inside the female's body, which measures 0.5 – 0.8 mm (Mulder, 1994), and then the female dies. The female's body wall dries and tans

(cutinizes), forming a protective layer around the egg mass, and the cysts become detached from the root as the plant cells die. During development in the eggs, juveniles undergo the first moult, after which the stylet forms at the anterior end of the second-stageJ2 (Turner and Evans, 1998; Turner and Rowe, 2006). It is at this stage that the juveniles can become dormant.

Potato cyst nematodes are able to undergo 2 forms of dormancy – diapause and quiescence. Diapause is a state of arrested development, and delays hatching until environmental conditions are optimal. During the year after cysts were formed, PCN undergo obligate diapause until soil temperatures rise, host plants are abundant and soil moisture is favourable during late spring (Turner and Rowe, 2006). Quiescence is the spontaneous reversible response to unfavourable environmental conditions such as drought, which results in a delay in hatch until the return of more favourable conditions (Wright and Perry, 2006).

Potato cyst nematodes are active in the soil at temperatures of approximately 7°C and above (Grainger, 1964); soil temperature influences both the rate of PCN development and the rate of PCN population decline. In temperate regions, there is usually one complete generation per growing season, however a second generation may can also develop (Greco *et al*., 1988; Mimee *et al*., 2015). The life cycle of PCN can differ from 29 days (Greco *et al*., 1988) in Southern Italy to 66 days in Croatia (Bacic *et al*., 2011). The length of the life cycle is dependent on a number of factors, including soil type, moisture, temperature and host cultivar (Ebrahimi *et al*., 2014). It is often discussed in degree days, the mean daily temperature minus the base temperature needed for nematode development, which is 6°C for *G. rostochiensis* and 4°C for *G. pallida* (Mugniery, 1978). It is estimated that for these base temperatures, *G. rostochiensis* requires 398 degree-days to complete its life cycle and *G. pallida* requires 450 degree-days, though the basal temperature of *G. pallida* has been estimated up to 7°C (Back *et al*., 2018).

Potato cyst nematodes are unable to move long distances through the soil, however local dissemination can occur due to farming activities, such as movement of farm machinery, livestock and farm workers (Turner and Evans, 1998). Other means of dissemination both locally and further afield include the movement of contaminated seed potatoes, soil and water. High winds may also blow cysts in sandy soils to other areas of fields and onto neighbouring fields (Brodie *et al*., 1993; Turner and Evans, 1998).

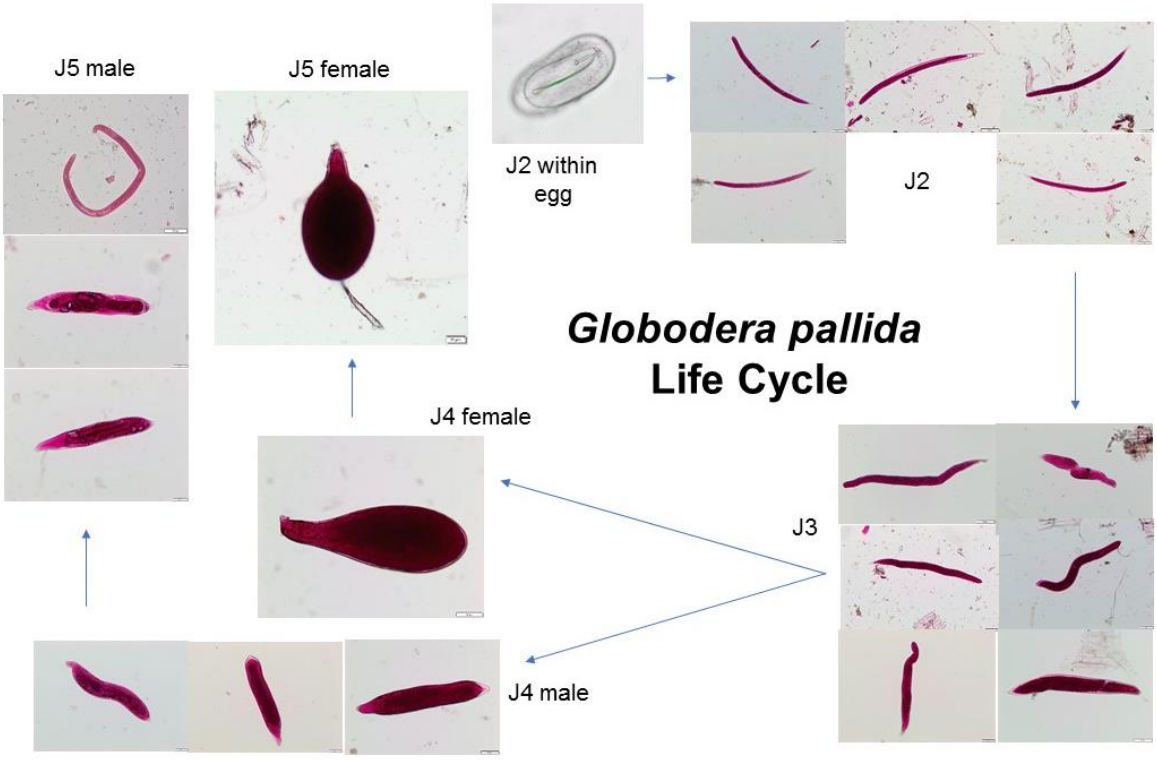


FIGURE 1.1. LIFE CYCLE OF THE POTATO CYST NEMATODE *GLOBODERA PALLIDA*. CYSTS CONTAIN APPROXIMATELY 200 - 500 EGGS, EACH ONE CONTAINING A SECOND-STAGE JUVENILE (J2). AFTER POTATO ROOT DIFFUSATE-STIMULATED HATCHING, THE J2 MOVES THROUGH THE SOIL AND INVADES A HOST ROOT, WHEREIN IT MOVES THROUGH THE ROOT AND ESTABLISHES A FEEDING SITE. THIS FEEDING SITE IS WHERE THE JUVENILE DEVELOPS INTO EITHER FEMALES OR MALES. FEMALES BECOME SACCATE AND RUPTURE THE ROOT, WHILST MALES REMAIN VERMIFORM AND LEAVE THE ROOT TO LOCATE FEMALES TO FERTILISE. ONCE FERTILISED, THE FEMALE DIES AND ITS BODY WALL CUTINIZES TO FORM THE CYST. IMAGES AUTHOR'S OWN OF *G. PALLIDA* RECOVERED FROM ACID FUCHSIN STAINED POTATO ROOTS. IMAGE OF 'J2 WITHIN EGG' FROM UNSTAINED EGG SUSPENSION (AUTHOR'S OWN).

1.2.2 Damage caused by potato cyst nematodes

The damage caused by invading PCN juveniles is a major factor affecting potatoes grown in soil infested with PCN (Trudgill *et al*., 1975a). The effects of infection of potato plants with PCN are varied and stem from the invasion and damage of roots, and interference with ion transport mechanisms at the root surface (Trudgill *et al*., 1975b). The visible symptoms of infection include stunted shoot growth, smaller leaves and wilting (Turner and Evans, 1998). Infested plants are found to have a reduced percentage ground cover and total leaf areas (as shown in Figure 1.2), and also senesce earlier than plants that are not infested (Trudgill *et al*., 1975a). Decreased yield from both reduced tuber number and tuber size is also a common symptom of infestation with PCN. Decreased growth and leaf surface area may be attributed to a reduction in the uptake of potassium, magnesium and phosphorus; chronic deficiency of potassium can also lead to premature senescence, especially in a plant that is also water stressed (Trudgill *et al*., 1975a, 1975b). The systemic changes that occur within host plants during invasion and feeding of PCN can also make host plants more susceptible to attack by fungal pathogens (Back *et al*., 2002). The syncytia formed by female PCN during feeding provides a nutrient rich base for not only the nematode, but also for fungal colonization. Plants with prior resistance to a fungal disease may also become susceptible due to modifications to the normal function of surrounding cells, as demonstrated by France and Abawi (1994). Storey and Evans (1987) found that the wilt fungus *Verticillium dahliae* used the invasion channels of *G. pallida* juveniles to enter and colonize cultivars Pentland Javelin, Maris Peer and Maris Anchor when both organisms are introduced to the plants simultaneously. Back *et al*. (2006) observed that the emergence of cultivar Désirée was significantly reduced when both *G. rostochiensis* and *Rhizoctonia solani* were present, with a positive relationship observed between the percentage of plant emergence and initial population density of the nematode, although plant emergence was not affected by nematodes alone. Back et al. (2006) also found that the incidence of stolons infected with *R. solani* increased with increasing initial population density of *G. rostochiensis* and that interactions between *G. rostochiensis* root invasion and stolon infection by *R. solani* had a significant effect on final tuber yield.

FIGURE 1.2. A POTATO FIELD INFECTED WITH AN AVERAGE OF 20 POTATO CYST NEMATODE EGGS PER GRAM OF SOIL. ON THE RIGHT SIDE OF THE FIELD, POOR PLANT GROWTH IS VISIBLE WHERE APPLICATION OF THE GRANULAR NEMATICIDE WAS AFFECTED BY A MALFUNCTION IN EQUIPMENT. HOTSPOTS IN PCN POPULATION CAN ALSO BE IDENTIFIED BY AREAS OF POOR POTATO PLANT GROWTH ACROSS THE FIELD. IMAGE COURTESY OF IVAN G. GROVE (HARPER ADAMS UNIVERSITY, TELFORD).

1.3 Management of Potato Cyst Nematodes

Management of potato cyst nematodes is complex and multifaceted. The aims of control measures are to reduce PCN population beneath the damage threshold, avoid high multiplication rates of the PCN population to protect future crops and to prevent yield loss. Management of potato cyst nematodes may include cultural control methods such as crop rotation and the use of resistant and tolerant cultivars as well as application of biological or chemical control products such as nematophagous fungi or granular nematicides respectively. Non-host crops may be used as trap crops to promote hatching of nematodes, or be incorporated into the soil in order to kill nematodes. The potential for managing cyst nematodes by combining two or more control strategies in an integrated programme has been widely demonstrated (Roberts, 1993).

1.3.1 Crop Rotation

Crop rotation is the most widely practised method of pest and disease control in agriculture (Back *et al*., 2018). By growing non-host, resistant or no crops in between

seasons of susceptible crops, the population of the pest falls below the damage threshold and low populations are prevented from increasing to damaging levels. The length of rotation and the crops used in controlling potato cyst nematodes are dependent on many factors; the nematode species and initial population density, the host range of the nematode, the soil type, the economic threshold of the susceptible crop and any other pathogens and pests present in the soil (Back *et al*., 2018). Several economic factors also affect the rotation schedule, such as the equipment available, the market value of specific rops and stipulations in contracts when renting land (Back et al., 2018), as 39% of potato growing land in Great Britain is rented (Independent Business Resource, 2017).

Rotation is an important tool in the management of potato cyst nematodes that takes advantage of their narrow host range, which is limited to plant species belonging to the family Solanaceae. The prolonged persistence of PCN cysts in soil for up to 28 years (Turner, 1996) is partially due to a relatively low rate of natural decline. The mean natural decline of *G. pallida* and *G. rostochiensis* was recorded to be 20% and 22% respectively over four years in spring barley (Whitehead, 1995). Decline was mainly caused by spontaneous hatch in the spring, regardless of the initial population density (Whitehead, 1995). The average rotation length of 5 years (or less) in the UK (Minnis *et al*., 2002) may not be long enough to adequately control populations of *G. pallida*. Using the decline rate of 26% found by Trudgill *et al*. (2014) for *G. pallida*, a rotation of at least 8 years is recommended (Trudgill *et al*., 2014). A PCN calculator was developed by Elliott *et al*. (2004) and is currently available through AHDB Potatoes. It is an interactive education tool for potato growers to compare the impact of management decisions such as length of rotation, cultivar choice and nematicide usage on PCN population densities and the yield of potatoes.

1.3.2 Resistance and Tolerance

The use of resistant and tolerant cultivars is important in reducing the impact of PCN on yield and they are important components in any rotation schedule. Resistant plants do not prevent infection by juveniles, but prevent multiplication of nematodes by interfering with the life cycle following infection, by restricting development of the syncytium (Moens *et al*., 2018; Sobczak *et al*., 2005). Tolerant plants do not affect the life cycle of PCN, but their yields are less affected by the presence of PCN than non-tolerant varieties at the same infestation level (Keer, 2007). Varieties may be resistant, tolerant, or both to varying degrees (Back *et al*., 2018; Evans and Haydock, 1990; Turner and Subbotin, 2013).

Resistance to plant parasitic nematodes can be conferred by a number of mechanisms. Many potato cultivars resistant to *G. rostochiensis* have been successfully commercialised, and several of these contain a single dominant resistance gene (R gene), *H1*. The R gene resistance is achieved by interaction with a corresponding avirulence (Avr) gene in the nematode, triggering a hypersensitive response (Fuller *et al*., 2008).

The hypersensitive response is a host defence mechanisms where the syncytium is induced as normal but is then isolated by a layer of necrotic cells, and subsequently degrades (Sobczak *et al*., 2005). Due to the reduced availability of nutrition and the epigenetic determination of sex in PCN mainly males develop, suppressing the proliferation of the population (Trudgill, 1967). The *H1* gene was first discovered in 1952 (Ellenby, 1952) and is still very effective against *G. rostochiensis* after decades of use, however the extensive use of cultivars resistant to *G. rostochiensis* in the UK has led to an increase in the incidence of *G. pallida* (Turner and Subbotin, 2013). Of the top ten varieties planted in the UK in 2016, six were resistant to *G. rostochiensis*, but none were resistant to *G. pallida* (AHDB, 2016). The cultivar Maris Piper accounted for 14% of the total planted area of potatoes in 2016 (AHDB, 2017), and remains the most popular variety due to its' flexibility in use and brand recognition. Maris Piper is resistant to *G. rostochiensis*, but is susceptible to *G. pallida*.

Monogenic resistance to *G. pallida* has been identified, such as the *H2* locus and *Gpa2* (Adillah Tan *et al*., 2009). The *H2* gene confers resistance to *G. pallida* Pa1, and is derived from *Solanum multidissectum*, whilst *Gpa2* confers resistance to a distinct population of *G. pallida* Pa2 and is derived from *S. tuberosum* spp. andigena CPC 1673, the source of the *H1* gene (Adillah Tan *et al*., 2009; Ellenby, 1952). The compound locus *Grp1* confers resistance against both *G. rostochiensis* (Ro5-22 line) and *G. pallida* (Pa2-D383 and Pa3 Rookmaker) (Rouppe Van Der Voort *et al*., 1998). Resistance to *G. pallida* is more commonly found to be polygenic than monogenic, and the combination of partial resistance loci can often provide additive resistance. This is true of *Gpa5* and *Gpa6*, as well as $GpaV^{s}_{spls}$ and $GpaXI^{s}_{spl}$, which when combined not only increases the effects of individual genes on the sex ratio of *G. pallida*, but also give a necrotic reaction in infected roots (Caromel *et al*., 2005). The use of resistant cultivars is particularly successful if the resistant cultivar has the same attributes as commercially successful susceptible cultivars already in use, though the specificity of resistance mechanisms limits the efficacy of resistant cultivars when virulent PCN populations are present.

In a study investigating both resistance and tolerance, Keer (2007) found that the varieties Cara, Maris Piper, Markies, Vales Everest and Melody exhibited the highest levels of tolerance to *G. pallida*, whilst Maris Peer, Marfona, Sante, Lady Rosetta, Saturna, Lady Claire, Vivaldi and Emerald were the least tolerant. Of the tolerant varieties tested, the first early Vales Everest also limited the multiplication of *G. pallida* in test plots, and decreased the population when used in conjunction with the nematicide Vydate (Keer, 2007). Maris Piper, Markies and Melody were all within the top 5 planted varieties in the UK in 2017, and account for 22% of the total area planted in the UK that year (Agriculture & Horticulture Development Board (AHDB), 2017b).

There are a number of mechanisms that may confer tolerance of PCN attack to potato varieties, and tolerance may vary depending on not only the cultivar but the species of PCN, other pathogens present and soil type (Evans and Haydock, 1990). Tolerance is likely to be caused not by a single mechanism, rather by a combination of mechanisms that may vary between cultivars. Any mechanism that reduces the stress caused by developing potato cyst nematodes on plant growth will increase the tolerance of that plant to infection (Evans and Haydock, 1990). These mechanisms can include reducing the number of juveniles hatching and infecting the root system, increased root vigour and tolerance to other plant pathogens such as *Verticillium dahliae* (Evans and Haydock, 1990). Another mechanism of tolerance may be water use efficacy; PCN stunt development of the root system, which can cause water stress, and water shortage during tuber bulking has a particularly adverse effect on yield (Evans and Haydock, 1990). Evans and Franco (1979) suggested that differences in water use efficacy by potato plants may be responsible for varied tolerance between cultivars. As potato cyst nematodes attack roots, the ability of plants to increase root growth under stress is an important mechanism of tolerance. Evans (1982) found that the tolerant cultivar Cara produced twice the root density of Maris Peer and Pentland Dell when the initial population density of *G. rostochiensis* was 10 eggs per gram of soil, and when the population was increased to 100 eggs per gram of soil the rooting density of Cara was doubled, whilst that of Maris Peer and Pentland Dell halved. The ability to produce more roots when attacked by nematodes can significantly limit yield loss, as a robust root system is able to sustain good top development, and avoids premature senescence of the tops, which is a symptom of nematode attack (Trudgill and Cotes, 1983). Not only does the total root mass improve tolerance to PCN, but the reaction of root cells at the point of penetration can increase or decrease tolerance. Some resistance genes cause the plant to isolate areas of attack by surrounding with necrotic tissue, however Huijsman *et al*. (1969) found that tolerant cultivars showed little necrosis, but formed calluses around feeding cells.

1.3.3 Agrochemical control

Nematicides have a significant role in controlling nematode population densities and protecting crop yield and quality. They may be used alone or, increasingly, as part of an integrated pest management system. The cost of granular nematicides to UK potato growers in 2004 was in excess of £9 million (DEFRA, 2004). Approximately 350,000 tonnes of active nematicidal substances are applied globally per annum, at a cost of €755 million ($1 billion); and potatoes make up 25% of the market share (Haydock *et al*., 2013). In 2016, 20% of the total planted ware crop in the UK was treated with either ethoprophos or fosthiazate, the two most common nematicides used on potatoes (Garthwaite *et al*., 2017).

Nematicides are classed as plant protection products (PPP) by The Food and Environment Protection Act (*The Food and Environment Protection Act*, 1985). The manufacture, sale and use of PPP in the UK are regulated by both EU and local (UK) legislation, including Regulation (EC) No 1107/2009 (European Commission, 2009). Regulation (EC) 1107/2009 changed the evaluation of plant protection products from a risk-based evaluation (used in previous legislation 91/414/EEC) to a hazard-based evaluation based on substances' instrinsic properties, and excludes active substances that are deemed as; category 1 or 2 mutagens, category 1 or 2 carcinogens (unless exposure is negligible), category 1 or 2 reproductive toxins (unless exposure is negligible), endocrine disrupters which may cause adverse effects in humans or non-target organisms (unless exposure in negligible), persistent organic pollutant, persistent, bioaccumulative and toxic substances (European Commission, 2009). Some active substances that fall within these categories may be approved under derogation for up to five years. Three nematicides currently approved for use in the UK – oxamyl, ethoprophos and fosthiazate – are possible 'candidates for substitution' and may be eliminated where safer alternatives are available (European Commission, 2015). At the time of writing, oxamyl is registered until January 2019, ethoprophos until July 2019 and fosthiazate until October 2018 ("EU Pesticide Database," 2018).

The organophosphates ethoprophos and fosthiazate, and the oxime carbamate oxamyl are all acetylcholinesterase inhibitors, and are applied mostly in granular formulations. Acetylcholinesterase (AchE) is an enzyme found at cholinergic synapses in the nervous system and is responsible for the hydrolysis of the major excitatory neuromuscular transmitter acetylcholine (Haydock *et al*., 2013; Segerberg and Stretton, 1993). Acetylcholinesterase inhibitors bind with the enzyme, found in the region of the nerve ring (Haydock *et al*., 2013; Wright, 1981). The inhibition of AchE disrupts chemoreception and motor functions in free living stages of potato cyst nematodes (Segerberg and Stretton, 1993). This impairs the ability of the juvenile nematode to detect and locate the host plant roots and of the adult male to detect and locate females (Evans and Wright, 1982). The effects of some AchE inhibitors such as oxamyl are reversible, however sub-lethal doses may disorientate juvenile nematodes sufficiently that they are unable to locate or penetrate host plant roots (Nelmes *et al*, 1973; Bunt, 1975; McLeod and Khair, 1975; Wright and Womack, 1981). Whilst oxamyl inhibits AchE by reversible carbamoylation of active sites (Yu *et al*., 1972), the action of fosthiazate is irreversible due to the stronger bonding of phosphorylation with AchE active sites (Woods *et al*., 1999). Acetylcholinesterase inhibitors have also been found to delay or inhibit hatching of potato cyst nematode juveniles (Brodie, 1983; Woods *et al*., 1999).

Degradation of nematicides in the soil occurs as soon as they are applied, and may be caused by microbial breakdown, adsorption to organic matter in the soil or physically lost

due to run-off or leaching. The persistence and efficacy of nematicides in the field can be affected by a number of variables in the soil system, including temperature, pH, organic matter content and treatment history. Enhanced degradation of nematicides by microbes in the soil can also occur when repeated applications are made of the same active ingredient (Karpouzas *et al*., 1999). Oxamyl is weakly adsorbed to sandy loam soil, thus mobile in the soil profile (Bromilow and Lord, 1979). In soils with high organic matter content, non-polar lipophilic compounds such as oxamyl are less effective due to sorption onto soil organic matter (Bromilow *et al*., 1980; Whitehead *et al*., 1985). The half-life of oxamyl is estimated to be 2-3 weeks, but may be as little as 1 week (Whitehead, 1992). Bromilow *et al*. (1980) found that in sandy loam soil, an increase in temperature from 5°C to 15°C reduced the half-life of oxamyl from 58 days to 21 days, though the rate of degradation was not obviously correlated with any single soil property. If the half-life is 2 weeks, by 10 weeks after application only 3% of the initial dose remains in the soil, although *G. pallida* juveniles may still be emerging (Whitehead, 1992). The degradation of agrochemicals in various soil types must be considered when recommending an application rate, so that the chemical is active in the soil for long enough to protect the plant but is not present in the crop yield.

Nematicides have been in use since the late 19th century when the fumigant carbon disulphide was introduced (Haydock *et al*., 2006). Modern fumigant nematicides consist of either halogenated hydrocarbons (such as methyl bromide) or active ingredients that release methyl isothiocyanate, such as metam sodium and dazomet (Haydock *et al*., 2006). Methyl bromide has non-selective toxicity, and has been classified as an ozone-depleting compound, thus is no longer registered in the EU for use against PCN (Haydock *et al*., 2006). Metam sodium decomposes rapidly in water and forms the secondary product methyl isothiocyanate, which, once inside the nematode body interferes with enzymatic, nervous and respiratory systems (Back *et al*., 2018). The action of isothiocyanates on nematodes is described in Chapter 1.3.4. Metam sodium 510 (Certis Europe) and Basamid (Certis Europe) are two fumigant nematicides registered for use in the EU (Back *et al*., 2018). Metam sodium is a liquid formulation and is injected into the soil profile, where it quickly volatilizes and spreads both laterally and vertically. Following treatment, the soil is sealed using a contra-rotating roller or a tarpaulin to prevent early loss of the fumigant. Basamid is a granular formulation that contains the active ingredient Dazomet, and is spread and rotovated into soils prior to sealing. Both products are applied up to 8 weeks prior to planting due to the phytotoxicity of the volatile products released (Back *et al*., 2018). Metam sodium is a candidate for substitution under EC 1107/2009, due to its endocrine disruption properties (binding with receptors for hormones such as oestrogen and androgen and change how the body functions), though Dazomet is not yet listed (European Commission, 2015; The Andersons Centre, 2014).

1.3.4 Biofumigation

Biofumigation refers to the use of biologically active compounds released from macerated tissue of *Brassica* plants in the management of weeds, nematodes, insects, bacteria and soil borne fungi (Back and Askew, 2012). Biofumigant crops such as *Brassica juncea* and *Sinapis alba* are grown to flowering before being macerated and incorporated after ca 8-14 weeks (Back *et al*., 2018) (Figure 1.3). Each Brassica species has a unique glucosinolate profile, which can vary in different plant tissues (Back *et al*., 2018). Glucosinolates are stable, water soluble substances that are not directly biocidal (Fahey *et al*., 2001). Endogenous thioglucosidases (myrosinases) released by cell damage (caused by bruising, freeze-thawing, maceration or pest attack) hydrolyse glucosinolates to yield a number of biologically active, volatile substances including isothiocyanate, thiocyanates, nitriles, epithionitriles and oxazolidines (Back *et al*., 2018; Lord *et al*., 2011). Isothiocyanates are the most toxic of the hydrolysis products, and their activity results from interaction with proteins and by increasing oxidative stress by depleting glutathione (Brown and Morra, 1997; Kawakishi and Kaneko, 1985). Buskov *et al*. (2002) demonstrated that intact glucosinolates did not cause mortality of *G. rostochiensis* J2s, however in the presence of myrosinase, 100% mortality of *G. rostochiensis* J2s was observed within 16 h for phenethylglucosinolate (gluconasturtiin), 24 h for benzylglucosinolate (glucotropaeolin) and 40 h for prop-2-enylglucosinolate (sinigrin). Lord *et al*. (2011) investigated the effect of incorporation of chopped *B. juncea* tissue in pots and found that the viability of encysted *G. pallida* juveniles was reduced by >95%. The primary glucosinolate present in *B. juncea* cultivars is 2-propenyl glucosinolate (sinigrin), whose hydrolysis products include allyl-isothiocyanate. The efficacy of biofumigation is dependent on glucosinolate production by Brassicas, and can be affected by a number of factors, including the timing of cultivation. Biomass and glucosinolate production was higher in summer cultivated Brassicas than in overwintered crops, with 78% less glucosinolates found in overwintered *B. juncea* (Ngala *et al*., 2014). The summer cultivated Brassicas demonstrated a significant reduction in the viability of encysted *G. pallida* juveniles, whereas the overwintered crops did not (Ngala *et al*., 2014). The equipment used for maceration and incorporation of biofumigant crops can also affect the efficacy of biofumigation, and is currently under investigation at Harper Adams (Watts *et al*., 2014). Glucosinolates are not only released from plants due to cell destruction, but have also been found to leach from plant roots. These leached glucosinolates are hydrolysed by myrosinase-producing organisms in the soil, releasing isothiocyanates during plant growth (Sakorn *et al*., 1999). Ngala *et al*. (2015) demonstrated that partial biofumigation (the term used to describe biofumigant effects during plant growth) with *Raphanus sativus* significantly reduced the viability of encysted *G. pallida* juveniles. Alternatives to traditional and partial biofumigation are currently being developed, including dried pellets and liquid formulations (De Nicola *et al*., 2013; Lazzeri *et al*., 2004).

FIGURE 1.3. DESTRUCTION OF BIOFUMIGANT CROP AT FLOWERING. IMAGE AUTHOR'S OWN, TAKEN AT HARPER ADAMS UNIVERSITY AT THE 5TH INTERNATIONAL BIOFUMIGATION SYMPOSIUM.

1.3.5 Solarization

Solarization is a hydrothermal process that increases soil temperature by placing plastic sheeting over moist soil, and causes a number of physical and chemical changes within the soil system, including mortality of plant-parasitic nematodes. It is not a control strategy currently used in the UK due to the requirement for high air and soil temperatures, though it is a suitable strategy in hotter countries and may become feasible within the UK due to climate change (The Environment Agency, 2018). The broad-spectrum lethal effects of solarization affect pests, pathogens and beneficial microorganisms. Soil-borne organisms are more likely to rapidly recolonize a sterilized soil system than plant parasitic organisms, and this shift in the biological equilibrium provides a healthier environment for plant growth (Stapleton, 2000).

Solarization has been found to be effective in PCN control in Italy, where maximum temperatures of 37 – 44°C at 10 cm were reached over an 8 week period. Plots were irrigated and covered with transparent polyethylene sheets, and after 4 weeks a reduction of 93% viability of *G. rostochiensis* eggs was achieved, comparable to the use of the nematicide 1,3-Dichloropropene in the study. The yield was also found to have increased in solarized plots by 32%, with 1,3-Dichloropropene increasing yield by 38% (Greco, 2000). Currently, no studies have looked at the effects of solarization on *G. pallida*,

although the viability of encysted *G. pallida* eggs was found to be reduced by 60% after 30 minutes in water at 37°C, and at 42 – 52°C viability was zero (Spaull and McCormack, 1988). In the UK, the temperature increases achieved in fields under plastic sheeting is insufficient for practical applications at the present time, however there is potential for achieving sufficient temperatures in glasshouses for control of other plant pathogens (Garibaldi and Gullino, 1991).

1.3.6 Anaerobic soil disinfestation and inundation

Anaerobic soil disinfestation (ASD) is the term used to describe the induction of anaerobic conditions in the soil by incorporation of fresh organic matter to wet soil and covering with airtight plastic for several weeks (Blok *et al*., 2000). Runia *et al*. (2014) found that the addition of organic matter increased the mortality of *G. pallida* 8-29 times that of anaerobic conditions only in a variety of soils by increasing CO_2, decreasing O_2, and increasing organic acids including acetic, propionic and butyric acid (Runia et al., 2014).

Inundation involves the induction of anaerobic conditions in the soil by flooding for a prolonged period of time. This leads to various physical and chemical changes in the soil including the depletion of O_2, increase in CO_2, reduced soil pH and production of organic acids, methane, ammonia and hydrogen sulphide (Runia *et al*., 2012). The efficacy of inundation as a control method depends on a number of factors, including duration of flooding and temperature. Spaull *et al*. (1992) found that 14 weeks inundation at 20°C produced a 98% reduction in viability of *G. pallida*, but at 10°C 50 weeks were required for comparable efficacy. Ebrahimi *et al*. (2016) found that the addition of agro-industrial waste products (including steamed potato peels, fresh potato peels, calcium sulphate, a mixture of steamed potato peels and calcium sulphate and green leaves of leek) reduced the required inundation time from 8 weeks to 4 weeks, and increased the control of *G. pallida* from 72% to 99.9%. Inundation has also been utilised in the control of other pests and pathogens present in the soil, such as *Pratylenchus penetrans*, *Meloidogyne hapla*, *Verticillium dahlia* (Runia *et al*., 2012) and *Ralstonia solanacearum* (van Overbeek *et al*., 2014). Unlike *P. penetrans*, which can be controlled by anaerobic conditions (Runia *et al*., 2012), Spaull *et al*. (1992) found that reduction of PCN viability was more effective when soil was flooded. The increased efficacy of inundation over ASD has been attributed to formation of fatty acids such as acetic and propionic acids (Hollis and Rodriguez-Kabana, 1966; López-Robles *et al*., 2013), and hydrogen sulphide (Spaull *et al*., 1992). The volatile fatty acids (including acetic acid and propionic acid) produced by liquid swine manure in an anaerobic system caused 99% mortality in *G. rostochiensis* cysts after 30 days treated soil (López-Robles *et al*., 2013). Hydrogen sulphide is highly toxic to *G. pallida*, 90 min of exposure resulted in complete loss of viability (from 99.9% to 0%) (Spaull *et al*., 1992).

1.3.7 Trap Crops

Trap crops, such as sticky nightshade, *Solanum sisymbriifolium*, stimulate hatch in soil, but do not allow PCN to complete their life cycle (FERA, 2010). Trap crops are usually a sacrificial crop or a plant species that has no intrinsic economic value other than this sole purpose (Kerry *et al*., 2003). Kerry *et al*. (2009) found that extracts from *S. sisymbriifolium* stimulated hatch of PCN, and may be of use in combination with other control methods. The use of trap crops - or break crops - is, however, an unpopular method of control due to cost, increase in labour and inconsistency of effects on different populations of PCN (Evans and Haydock, 2000; Kerry *et al*., 2003), though the technique does not require nematicide if tolerant cultivars are used, making it appealing for organic growers (Back *et al*., 2018). *Solanum sisymbriifolium* expresses hatching factors that stimulates hatch of PCN, though the invading juveniles are unable to develop a satisfactory feeding site and are unable to multiply (Back *et al*., 2018; Kerry *et al*., 2003). The efficacy of trap crops can be affected by a number of agronomic variables and are grown following specific agronomic guidelines according to the temperature and soil type (Back *et al*., 2018). Sasaki-Crawley (2012) found that extracts from foliage of *S. sisymbriifolium* also induce hatching of *G. pallida in vitro*, which could be exploited by incorporation into the soil to promote further hatching.

1.3.8 Plant extracts

A garlic based product (Nemguard DE) is currently registered as a nematicide in the UK for use on carrots, parsnips and potatoes (Certis, 2018). The essential oils extracted from garlic (*Allium sativum*) contain sulphuric compounds such as allicin, which have been shown to have nematicidal activity against a number of nematode species including PCN (Chitwood, 2002; Danquah *et al*., 2011; Park *et al*., 2005). Allicin is produced by the enzyme allinase's action on allin when garlic cells are lysed or damaged (Danquah *et al*., 2011). Danquah *et al*. (2011) found that *in vitro*, garlic extract caused mortality of *G. pallida* J2s after 24 h and, at higher concentrations and after eight weeks treatment, prevented hatching completely and reduced the percentage viability of unhatched eggs to 24%. At low concentrations of the garlic extract, the author observed a stimulant effect on hatching. Danquah *et al*. (2011) suggested that the stimulation of hatch may be utilized to provoke hatching of PCN between potato crops to lower population. Induction of hatching by synthetic (and natural) hatching stimulants has been discussed by several other authors, including Byrne *et al*. (2001), Devine & Jones (2000) and Whitehead (1977).

Danquah *et al*. (2011) assessed the efficacy of salicylaldehyde, a secondary plant metabolite and precursor of salicylic acid, on mortality, hatching and viability of *G. pallida*, and found this chemical to be effective at reducing hatching and viability of *G. pallida* and toxic to hatched *G. pallida* juveniles. Similar results were found by Zinov'eva *et al*.(2011), who found that treatment of tomato plants with salicylic acid resulted in increased

nematode resistance. Increased salicylic acid content in plant tissues has a downstream role in effecting Mi-1-mediated resistance to both aphids and nematodes, and significantly influenced nematode development (Fuller *et al*., 2008).

Saponins are typical secondary plant metabolites, found in plants such as soapwort and alfalfa (*Medicago sativa*). Most of these surface-active glycosides are haemolytic and are utilised for their molluscicidal, anti-inflammatory, antimicrobial and cytotoxic activity (Argentieri *et al*., 2007). Vito *et al*. (2007) found that hatch of *G. rostochiensis* was stimulated at low concentrations and suppressed at higher concentrations by extract of pokeweed fruit, rich in potassium and saponins. (D'Addabbo *et al*., 2010) also found that saponins from *M. sativa* were toxic to *G. rostochiensis* J2s. Although the specific mode of this interaction is unknown, many saponins protect plants from insect attack and their biological effects have been ascribed to their efficacy in increasing membrane permeability by creating pores (Francis *et al*., 2002).

1.3.9 Biological control

Nematode populations may be suppressed by microorganisms found in the rhizosphere. The modes of action of these microorganisms may be direct or indirect, from physical interaction with mobile stages of PCN to interaction with the host plant, causing increased resistance to PCN. Over 150 pathogens of nematode cysts, females and eggs have been isolated from sites, although <10% have been tested for parasitism (Kerry *et al*., 2003).

Fungi such as *Arthrobotrys oligopora* have been found to 'trap' juvenile and adult *G. pallida* within complex mycelial structures. The mode of action, however, is poorly understood, and manipulation of these interactions and commercialization is unlikely (Kerry *et al*., 2003). Other fungi produce small spores that attach to the nematode cuticle. *Drechmeria coniospora* infects the anterior chemoreceptors, rendering the nematode unable to sense food sources. This organism, however, is outcompeted by the residual microflora, and is therefore difficult to exploit (Jansson and Nordbring-Hertz, 1984; Kerry *et al*., 2003). Nematophagous fungi that paratize eggs and females, such as *Pochonia chlamydospora* and *P. lilacinum*, use appressoria, a specialized penetration peg or lateral mycelia branches to invade colonize the nematode and have been found to have antagonistic activity against *Globodera* spp. (Davies *et al*., 2018). The inoculation of potato plants with arbuscular mycorrhizal fungi was found to stimulate hatching of *G. pallida* by 25% over non-inoculated plants, and initiated hatching sooner than in the control (Ryan *et al*., 2000). This effect was not seen in *G. rostochiensis*, however, and may be specific to *G. pallida*.

The bacteria *Pasteuria* spp. parasitise a range of nematodes, and produce spores that attach to the cuticle of nematodes. *Meloidogyne* spp females infected with *Pasteuria penetrans* produced few, if any, eggs. The bacterial spores are multiplied inside the

female and released into the soil as the female's cadaver degrades (Karssen & Moens, 2006). The presence of *Bacillus sphaericus* and *Agrobacterium radiobacter* in the rhizosphere reduces root penetration by *G. pallida* by inducing systemic resistance in host plants (Hasky-Günther *et al*., 1998). Several bacteria and fungi produce metabolites that may also adversely affect PCN. One such fungus, *Myrothecium* spp., has been utilized in the nematicide DiTera® (Valent Biosciences Corp., Chicago) (Kerry *et al*., 2003). Acetic acid extracted from fungal culture filtrates was also identified as having nematicidal properties (Chitwood, 2002).

Due to the length of rotations between each potato crop, and the mixing of rhizosphere soil during harvest, the population of antagonistic microorganisms may not build up to, or retain, an effective dose level to suppress PCN replication and damage (Kerry *et al*., 2003). Several factors continue to limit commercial development of biocontrol products for PCN; mass production of certain microorganisms remains difficult, effective application rates may be impractical on a field scale and individual isolates of the bacterium have restricted host ranges that may limit commercial development (Kerry *et al*., 2009, 2003). It is likely that the initial PCN population density in soil is critical in the ability of biological control to succeed, and biological control agents have produced much less reliable effects than granular nematicides, with differences in efficacy observed between sites (Kerry *et al*., 2009).

1.3.10 Integrated pest management and future management strategies

Integrated pest management relies on minimising pesticide use through the complementary adoption of alternative methods to control pests, diseases and weeds (Hillocks, 2012). Combining several management methods for control of PCN, is likely to improve yields, reduce the population and prevent multiplication. For example, the three strategies of resistance, rotation, and non-fumigant nematicide treatment have been effectively combined in managing PCN, though the use of *G. rostochiensis*-resistant cultivars such as Maris Piper has lead to selection of *G. pallida*, which is also less responsive to treatment with non-fumigant nematicides (Roberts, 1993). Due to the European regulation EC 1107/2009 and additional legislation to protect water quality, a large number of pesticides will be banned or heavily restricted (Twining *et al*., 2009). This reduction in availability and diversity of chemical crop protection products will require farming systems to change in order to comply with regulations and maintain commercial viability (Hillocks, 2012). The nematicides currently in use were developed over 20 years ago, and control of PCN has depended on the use of a small number of active ingredients. As these active ingredients are withdrawn, growers will be left with less reliable methods of suppressing the exponential population increases of PCN each crop rotation, and rotations will become longer. Growers are increasingly looking to alternative control

methods to nematicides, and combining alternative control methods to maintain PCN populations under the damage threshold levels. For these methods (and integration of various methods) to be effective, research must focus on how PCN interact with both host plants, non-host plants and other microorganisms found in the rhizosphere, and the mode of action of novel active ingredients within this system, to protect future crops and prevent further multiplication of this pest.

1.4 The Novel Formulation, BGT

The novel mixture being studied consists of a blend of surfactants and a biguanide. Surfactants, or surface-active agents, are amphiphilic chemicals (Merianos, 2001) utilised for their solubilisation and cleaning purposes (Ying, 2006). Surfactants influence the properties of surfaces and interfaces (Schramm *et al*., 2003), and are widely used as cleaning, wetting, dispersing, emulsifying and foaming agents in a wide range of markets, including personal care and agriculture (Ying, 2006). Surfactants comprise two regions, one hydrophobic, the other hydrophilic (McDonnell and Russell, 1999), and combine both hydrophobic and hydrophilic properties in one molecule (Ying, 2006). The hydrophobic chain is usually a long-chain hydrocarbon, and the hydrophilic group an ionic or highly polar group (Castro *et al*., 2013). Surfactants may be classified as cationic, non-ionic or anionic depending on the presence or absence of ionization of the hydrophilic group, and the charge of this ionization (Castro *et al*., 2013; Merianos, 2001).

The dual nature of amphiphiles causes self-association when in polar solvents, for example water. The surfactant monomers arrange themselves into organized molecular assemblies known as micelles (Domínguez *et al*., 1997). In aqueous-surfactant solutions, the hydrophobic portion forms the core of the micelle, while the hydrophilic head groups face outwards, towards the water molecules. These colloidal solutions may be homogenous on a macro scale, but are heterogeneous on a microscopic scale (Domínguez *et al*., 1997). The concentration of surfactant above which monomers form micelles is called the critical micelle concentration. Above this concentration, surfactant monomers and micelles exist in dynamic equilibrium. The formation of micelles induces changes in the physiochemical properties of the solution, such as an increase in enolic absorption and, in ionic surfactant solutions, a change in the electrical conductance (Domínguez *et al*., 1997).

1.4.1 Cationic Surfactants

Cationic surfactants are surface-active agents with a long-chain hydrocarbon hydrophobic group and a positively-charged hydrophilic group. Cationic surfactants have the greatest antimicrobial activity of all surfactants (McDonnell, 2009). They are compatible with non-ionic surfactants and are commonly co-formulated to increase efficacy. The activity of cationic surfactants can be affected by water-hardness of the diluent, the presence of

organic matter and lipid-containing substances and the presence of anionic surfactants (McDonnell, 2009).

Quaternary Ammonium Compounds

Quaternary Ammonium Compounds (QACs) are derivatives of ammonium salts, where alkyl groups replace hydrogen atoms (Russell, 1990). Quaternary ammonium compounds are cationic surfactants with at least one hydrophobic hydrocarbon chain linked to a positively charged nitrogen atom, the other alkyl groups being mostly short-chain substituents such as methyl or benzyl groups (García *et al*., 2001; Ying, 2006). The hydrocarbon chain length affects disinfectant ability via protein binding and displacement (Fisher, 2003; Lien and Perrin, 1976). As the chain length of QACs increases, so does the critical micelle concentration (Lien and Perrin, 1976). Lien and Perrin (1976) found that the optimal chain length for binding of alkyl dimethyl benzalkonium chloride compounds (ADBAC) to bovine serum albumin (BSA) is C16.

The common properties of QACs include attraction to surfaces of negative charge (including bacteria), reduction of surface tension, increasing solubility, inhibition or stimulation of enzymatic activity and denaturation, precipitation and complex formation of proteins (Merianos, 2001). Quaternary ammonium compounds are poor detergents, but good wetting agents. Their surface active properties stem from the cationic portion of the molecule, the substituted nitrogen, which can bind to oxidised surfaces (Fisher, 2003).

Quaternary ammonium compounds were initially used as an adjunct to surgery, in preoperative disinfection of both patient and surgical team, as well as the surgical instruments. Use of the compounds as disinfectants on floors, walls and surfaces of hospitals, nursing homes and other public places lead to the development of formulations including both cationic and non-ionic surfactants, to optimise cleaning and antimicrobial properties (Merianos, 2001). They are regularly used to treat large bodies of water in various industrial processes, including in paper mills and swimming pools, to prevent the proliferation of microorganisms that interfere with processes, are aesthetically undesirable or cause disease. They may also be applied to fabric, and are used in nappies to prevent growth of ammonia-producing bacteria associated with ammonia dermatitis and nappy rash. Quaternary ammonium compounds are also used as ophthalmic and cosmetic preservatives (Ittoop *et al*., 2014; Merianos, 2001), and as topical antiseptics for the skin, conjunctivae and mucous membranes (Springthorpe 1990). The most common use for QACs is in hard surface disinfection, such as in food processing facilities (Ittoop *et al*., 2014). The QACs are most active against gram positive bacteria, and less active against spores and non-enveloped viruses, due to their activity being focused on lipid membranes (Fisher, 2003). The activity of QACs on spore-forming bacteria is restricted to the vegetative stages of development and the inhibition of germination from spores (McDonnell and Russell, 1999). Cationic surfactants also have non-biocidal uses, such as

in ophthalmic preparations where they are included due to their ability to solubilize the intercellular junctions within the corneal epithelium to enhance drug delivery (Ittoop *et al*., 2014).

Quaternary ammonium compounds cause cell death by interacting with the cytoplasmic membrane of bacteria, the plasma membrane of yeasts and the envelope of viruses (McDonnell, 2009; McDonnell and Russell, 1999). The membrane consists of both lipids and proteins, arranged in a phospholipid bilayer. The interior of the phospholipid bilayer contains hydrophobic fatty acid chains, rendering the membrane impermeable to water-soluble molecules (Cooper, 2000). The fatty acid chains move freely within the membrane, allowing the membrane to be flexible and for phospholipids and proteins to diffuse laterally along the membrane (Cooper, 2000). Proteins are found inserted into the lipid bilayer and bound indirectly via protein-protein interactions (Cooper, 2000). Integral membrane proteins require disruption of the phospholipid bilayer to be released, such as treatment with surfactants (Cooper, 2000). Protons (H^+) are separated from hydroxyl ions (OH^-) across the surface of the cytoplasmic membrane. This charge separation is a major site of energy conservation called the proton motive force, and is responsible for driving functions such as biosynthesis of adenosine triphosphate (ATP), as well as motility and solute transport (Madigan *et al*., 2009; McDonnell and Russell, 1999).

The positively charged QAC and the negatively charged microbial cell are attracted to one another, and the compound is adsorbed onto the cell surface with the cationic head group facing outwards and the hydrophobic tails inserted into the lipid bilayer (Maillard, 2002). The hydrophobic portion of a cationic surfactant displaces the membrane lipids and binds to the hydrophobic portions of integral membrane proteins. The hydrophilic tail of the surfactant renders the surfactant-protein complex soluble in aqueous solutions (Cooper, 2000; Maillard, 2002). Both peripheral and integral membrane proteins are dissociated from the membrane and released (Cooper, 2000). The insertion of the hydrophobic tail into the phospholipid bilayer causes the rearrangement and disruption of the cytoplasmic membrane, and a loss of membrane integrity (Hancock, 1984; Maillard, 2002). The generalised membrane damage and loss of structural organisation promotes uptake of the surfactant (Maillard, 2002) allows the release of cytoplasmic constituents and ions from the cell (Ioannou *et al*., 2007) and leakage of intracellular low molecular weight material (Salton, 1968). Cell death may be mediated by a build-up of the damage caused by the cationic components, which also includes coagulation of intracellular material and disruption of replication (Hugo, 1965; Maillard, 2002), as well as promotion of the cell's autolytic enzymes (Salton, 1968). Cationic compounds may also cause dissipation of the transmembrane proton motive force; disrupting oxidative phosphorylation, inhibition of active transport across the membrane and ATP synthesis (Hugo, 1965; Maillard, 2002; Vasilev *et al*., 2009).

Silane quaternary ammonium compounds

The addition of a silane-containing group to a quaternary ammonium compound produces a silane quaternary ammonium compound (Si-QAC), or organosilane. Organosilanes are able to covalently attach to a variety of substrates, including hydroxylated and hydrophilic surfaces (Vasilev *et al*., 2009). The silane groups crosslink (=Si-O-Si=) and bind covalently to the surface, whilst the quaternary ammonium group confers antimicrobial activity (Daniels and Hourani, 2009). Organosilanes are used in many different industries, from treatment of textiles to antimicrobial coating of rubber, due to their durability. They remain on a treated surface for a prolonged period, and can withstand wear, rinsing with water and laundering (Gao and Cranston, 2008).

1.4.2 Non-ionic Surfactants

Non-ionic surfactants are composed of hydrocarbon chains attached to a non-polar hydrophilic group. They are used in various industries, including in domestic detergents and cleaners, personal care products such as shampoo and cosmetics, pharmaceutical products, oil recovery and as adjuvants in a range of agricultural products (Iglauer *et al*., 2010; Maillard, 2002). Non-ionic surfactants are used in many fields of technology and research, due to a number of their properties, including the ability to facilitate solubilisation and to enhance the dissolution rate of active ingredients (Cserháti, 1995). Non-ionic surfactants increase the stability of pharmaceutical drug emulsions and alter the stability of various emulsions in the food industry (Maillard, 2002). They also decrease foaming of formulations, including those containing ionic surfactants (Cserháti, 1995). Non-ionic surfactants readily bind to various proteins, stimulating or inhibiting enzyme activity and modify the adsorption capacity of proteins and peptides (Cserháti, 1995; Maillard, 2002). They are known to increase the permeability of phospholipid membranes and vesicles by insertion of the hydrophobic portion of the surfactant into the apolar fatty acid domain of phospholipids (Cserháti, 1995). Non-ionic surfactants are commonly used as co-formulants or adjuvants to ionic surfactants or other active ingredients. For example, the hard surface cleaning and disinfection by QACs is potentiated by the addition of non-ionic surfactants (Merianos, 2001). Non-ionic additives possess good penetrating, good solubility and spreading behaviour that are utilised to increase efficacy of a number of active ingredients (Bergstrom and Johansson, 2005). Non-ionic surfactants such as Alkyl polyglucoside (APG) are also ideal co-formulants due to their water solubility and stability in the presence of electrolytes (Bergstrom and Johansson, 2005). Alkyl polyglucosides have a hydrophilic saccharide group and a hydrophobic hydrocarbon tail (Castro *et al*., 2013; Iglauer *et al*., 2010). Other non-ionic surfactants differ in their hydrophilic group, such as alcohol ethoxylates, that consist of an ethylene oxide unit combined with a hydrophobic alkyl chain (Castro *et al*., 2013; Cserháti, 1995; Maillard, 2002). Both alkyl polyglucosides and alcohol ethoxylates are used extensively in agrochemicals (Chapter 1.4.4).

1.4.3 Polyhexamethylene biguanide

Polyhexamethylene biguanide (PHMB) is a cationic polymer with a biguanide functional group. It is a broad spectrum bioicide of low mammalian toxicity, commonly used in wound dressings, to sanitize swimming pools, in cosmetics and personal care products, fabric softeners, handwash and contact lens solutions (Broxton *et al*., 1983; SCCS (Scientific Committee on Consumer Safety), 2017), and is used in cleaning medical and dental utensils, animal drinking water and farm equipment (Moore and Payne, 2004). It is also a component of hard surface disinfectants used in food handling facilities and in breweries, where non surface-active agents are preferable as they do not affect head retention on ales and beers (Moore and Payne, 2004). Polyhexamethylene biguanide displays excellent activity against a wide range of gram positive and gram negative bacteria, fungi, yeasts and is particularly effective against microorganisms such as *Pseudomonas* species, which are difficult to control and commonly colonise wounds and water lines (SCCS, 2017). Polyhexamethylene biguanide is soluble in polar solutions such as water, but insoluble in non-polar solvents such as toluene (Moore and Payne, 2004). The positively charged polymer attracts and adsorbs to negatively charged bacterial cell walls (Russell, 2001). It causes immediate and irreversible non-specific damage to the cytoplasmic membrane, resulting in leakage and precipitation of cytoplasmic constituents, including loss of potassium ions (Broxton *et al*., 1983; Ikeda *et al*., 1984). The interaction between PHMB and cytoplasmic membrane brings about a loss of selective permeability, enabling uptake of the polymer and further interaction of PHMB with the hydrocarbon interior of the membrane (Ikeda *et al*., 1984). The antimicrobial activity of PHMB is directly linked to the polymer chain length. As the chain length increases, so does the antimicrobial efficacy (Moore and Payne, 2004).

1.4.4 Surfactants in Agriculture

Surfactants are the 2nd largest group of adjuvants in agriculture, behind solvents (Cserháti, 1995; Krogh *et al*., 2003). They have many functions, from improving solubility and delivery of formulation to uptake and efficacy of active ingredients (Castro *et al*., 2013). They can be found built in to formulations, or as tank additives (Seaman, 1990). By increasing the efficacy of potentially toxic and/or expensive active ingredients, lower concentrations can be used while maintaining biological effectiveness (Knoche, 1994). The ecological and economic advantages of this are becoming more important as the regulations concerning active ingredients tighten and the number of registered active ingredients decreases.

Surfactants are able to lower surface tension of formulations, and this physiochemical property is utilised in agrochemicals to increase the efficacy of foliar sprays. The equilibrium surface tension of a solution affects its' leaf wetting ability (spreading active ingredients evenly across the surface), and the dynamic surface tension affects the

retention of drops on the leaf surface (Knoche, 1994). The addition of cationic and non-ionic surfactants can increase the retention of droplets on leaves, particularly when sprayed on plants with crystalline epicuticular waxes rather than smooth cuticular surfaces (Rutter *et al*., 1990). Organosilanes have also been found to enhance the uptake of active ingredients by two mechanisms; by increasing cuticle penetration of the active ingredients, and by inducing mass flow through the stomatal pore. This is possible due to the equilibrium surface tension being equal to or lower than the critical surface tension of the leaf surface (Knoche, 1994). Organosilanes have been found to improve the rain fastness of herbicides and increase the efficacy of herbicides (including glyphosate), insecticides, fungicides and fertilizers (Knoche, 1994).

Quaternary ammonium compounds are currently registered as adjuvants, microbiocides and algaecides under the American Enviroment Protection Agency (EPA). The QAC Benzalkonium chloride (BAC) is currently registered with the EPA as a microbiocide and algaecide, as well as a food and feed area disinfectant. Benzalkonium chloride is not currently registered in Europe as an active plant protection product ingredient under regulation 1107/2009 (European Commission, 2014) but may be used as an adjuvant. The QAC didecyldimethyl ammonium chloride (DDAC) was approved as an active ingredient in Europe as a plant protection product for use on ornamental crops, however this approval was revoked in 2013 due to insufficient further confirmatory information on the specification of the active substance (European Commission, 2014, 2013a).

Daniels and Hourani (2009) evaluated the influence of the Si-QAC 3-(trimethoxysilyl)propyl dimethyl octadecyl ammonium chloride (a component of BGT) on the compaction, strength, swell, erosive and hydraulic properties of several soils. The authors found that addition of the Si-QAC to soil resulted in “modest changes to strength and swell potential and a dramatic reduction in infiltration capacity, as well as reducing soil erosion”. These findings were attributed to hydrophobicity conferred to silicone surfaces by the Si-QAC (Daniels and Hourani, 2009). Soils contain between 5 and 40% silicone, found as liquid, adsorbed and solid phase fractions (Matichenkov and Bocharnikova, 2001; Tubana *et al*., 2016). Silicone is found in crystalline forms such as primary and secondary silicates and silica materials, as well as in complexes with heavy metals and organic matter (Matichenkov and Bocharnikova, 2001; Tubana *et al*., 2016). Formulations containing silicone are used in agriculture as fertilisers and as adjuvants, due to their good wetting properties (Tubana *et al*., 2016). Silicone fertilisers are used in several agricultural systems, including in sugarcane and rice in Florida, US (Tubana *et al*., 2016). Silicone fertilisers have been found to increase the efficacy of other fertilisers such as phosphate and potassium, decrease aluminium toxicity, change heavy metal mobility in soil, initiate soil mineral formation processes and improve adsorption properties and water-air regime of soils (Matichenkov and Bocharnikova, 2001). Matichenkov and

Bocharnikova (2001) found that that the addition of silicone rich materials to sand, clay-sand, sand-clay and clay soil types increased the water-holding capacity of the soil.

Non-ionic surfactants are an integral part of pesticide formulation (Cserháti, 1995). One of the most commonly used surfactants in agricultural applications are alcohol ethoxylates (Knoche, 1994). Alcohol ethoxylates (AEO) are wetting agents and spreading agents, and increase the foliar uptake of herbicides (Hazen, 2000). Arriaga *et al*. (2009) and Cooley *et al*. (2009) found that the use of AEO on potato growing land reduced nitrogen leaching and improved water distribution, reducing the impact of dry zones that often appear on sandy soils. Alkyl polyglucosides (APG) are another group of widely used non-ionic surfactants, commonly co-formulated with AEO due to their synergistic activity (Bergstrom and Johansson, 2005). Alkyl polyglucoside is highly water-soluble, and is used to solubilize non-ionic wetting agents into solutions containing high concentrations of electrolytes, such as fertilisers (Bergstrom and Johansson, 2005). The combination of AEO and APG reduces surface tension, decreases the critical micelle concentration and increases wetting properties of formulations towards hydrophobic surfaces (Bergstrom and Johansson, 2005). It is a useful combination in preventing phase separation, and has been found to increase the efficacy in field of a number of fertilizers and active ingredients, including the herbicide glyphosate (Bergstrom and Johansson, 2005; Castro *et al*., 2013). Non-ionic surfactants have also been found to increase the activity of a number of plant enzymes (Xiaoli *et al*., 2000). When sprayed with a non-ionic surfactant, soybean plant enzymes invertase and superoxide dismutase activity was increased, increase cleavage of sucrose and removal of toxic superoxide radical anions respectively (Xiaoli *et al*., 2000).

1.4.5 Surfactants and Nematodes

Surfactants may be used as adjuvants in the application of entomopathogenic nematodes, such as the addition of potassium fatty acid salts to suspensions of *Steinernema carpocapsae* and *Heterorhabditis bacteriophoria* for control of the larvae of the spotted cucumber beetle *Diabrotica undecimpunctata* in soil and all feeding stages of the cabbage aphid *Brevicoryne brassicae* on foliage (Kaya *et al*., 1995). It is recommended that the nematode/surfactant mixture is applied immediately, however, as storage for over 24 h reduces the viability of nematodes (Kaya *et al*., 1995). The non-ionic polyol polymer Pluronic-F127 has been used extensively in studies of nematodes (Reynolds *et al*., 2011; Sasaki-Crawley *et al*., 2012; Wang *et al*., 2009; Williamson *et al*., 2009). Mutwakil *et al*. (1997) found that supplementation of agar with several non-ionic surfactants stimulated growth of *Caenorhabditis elegans*, but most also caused a generalized, low-level stress response, possibly due to the surface-active nature of the additives.

Some surfactants have been found to be toxic or have deleterious effects on nematodes. The hydroxynaphthoate salt of a QAC (bephenium) and related compounds were found to

be effective against a number of parasitic nematodes in the gastrointestinal tract of humans and animals (Burrows *et al*., 1960; Copp, 1959; Copp *et al*., 1958). The anthelmintics bephenium and thenium act as agonists at nicotinic acetylcholine receptors, and are cholinergic ganglionic blockers (Martin *et al*., 2002). Riedel *et al*. (1949) found no anthelmintic activity in chickens when fed a combination of alkyl dimethyl benzalkonium chloride (ADBAC) and alkyl dimethyl dichlorbenzalkonium chloride. Sodium lauryl sulfate-citric acid derived from coconut oil was found to be nematostatic against *Pratylenchus penetrans* both *in vitro* and when applied to strawberry plants, although phytotoxicity was also observed (Pinkerton and Kitner, 2006). SoyFast™ Manufacturer's Base is a blend of bio-derived surfactants including ethoxylates castor oil and soybean oil methyl ester. When applied as a soil drench to loamy sand, it was found to reduce the number of viable citrus nematodes (*Tylenchulus semipenetrans*) from 4672 to 1 per 50ml soil over 5 days (Arbogast *et al*., 2009). Lole & Binks (2001) found that the QAC-containing product "Antec Ambersil" reduced viability of *Ditylenchus dipsaci* up to 70% in solution. A composition comprising two BACs, dimethyl sulfoxide, ammonium lauryl sulfate, papain, grapefruit peel and seed extracts in an aqueous solution has been found to reduce the number of nematodes (no species specified) in soil from 29,000 per 10 grams soil to zero (Rajamannan, 2011). The products described above, however, are not commercially available at the time of writing. Currently, no surfactants are registered for use against plant parasitic nematodes in the UK or the EU.

The only data available on the activity of BGT components against nematodes has been found on the QAC benzalkonium chloride (BAC). Höss *et al*. (2010) determined that the EC_{50} of BAC to *Caenorhabditis elegans* was 15.1 mg/L, whilst Hasan *et al*. (2001) found that 24 h treatment with 10% BAC had no effect on motility or infectivity of *Toxocara canis*. Treatment of banana plants infested with *Meloidogyne* spp. and *Radophilus similis* with BAC 0.1% reduced the percentage of infested plants after 92 days from 60% to 30% (*Meloidogyne* spp.) and from 25% to 10% (*R. similis*) (Peachey and Hooper, 1963).

1.5 Research Questions

The overall aim of this research is to evaluate the nematicidal potential of the novel surfactant formulation BGT. The cationic surfactants contained in the formulation are known for their broad spectrum antimicrobial activity and their actions on cell membranes. One of the cationic components of BGT, Benzalkonium chloride, has previously been found to cause mortality of the model organism *Caenorhabditis elegans,* which suggests that the formulation may also cause mortality in juveniles of other nematodes. Benzalkonium chloride has also been found to reduce infestation of banana plants by the economically important plant-parasitic nematodes *Meloidogyne* spp. and *Radophilus similis*. Due to the increasing pressure of growers to maintain the size and quality of

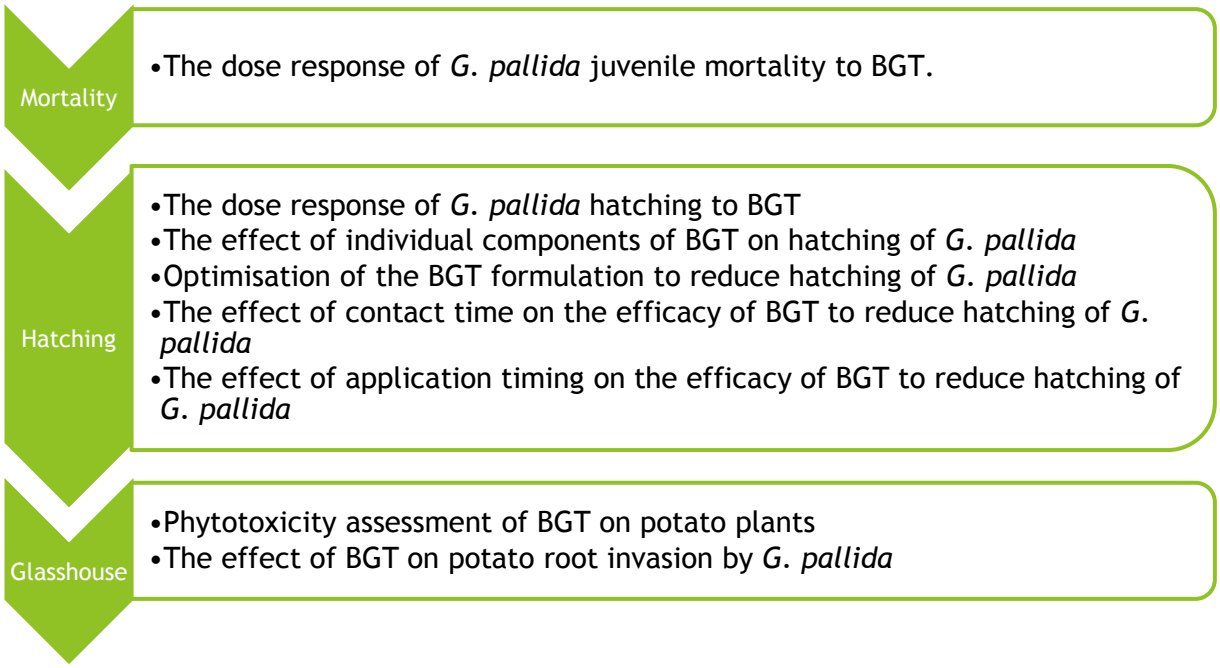
yields, the increased incidence of *Globodera pallida* due to selection pressure caused by use of cultivars with the *H1 gene* and the potential loss of current agrochemical control agents it is vital that investigations into alternative control measures are conducted.

Objectives

The objectives of this research project are to:

1. Determine the effect of BGT and its components on the mortality, hatching and infectivity of the potato cyst nematode *Globodera pallida*.
2. Determine the optimum formulation and concentration of BGT for maximum nematicidal activity.
3. Determine the mode of action of BGT on mortality, hatching and infectivity of *Globodera pallida*.
4. Determine the effects of BGT on *Solanum tuberosum*.
5. Determine a suitable protocol for BGT *in situ*.

Experiment plan



Chapter 2 : Determining the effect of BGT on the mortality of *Globodera pallida*

2.1 Introduction

Potato *(Solanum tuberosum)* is the world's most important non-grain food crop, and is susceptible to over 30 bacterial, fungal, oomycete and viral diseases, as well as 12 common disorders, all of which are of economic significance in the UK (Bradshaw *et al*., 2001; Xu *et al*., 2011). The potato cyst nematodes *Globodera pallida* and *G. rostochiensis* are the most important nematode pest species in the UK and are a major factor limiting the efficient production of potatoes, costing the industry £25 million annually (Twining *et al*., 2009). A survey in 2016 found that potato cyst nematodes (PCN) were present in 48% of ware potato growing land and of those positive samples, 89% contained only *G. pallida* (Dybal-Lima *et al*., 2016).

Second-stage juveniles of potato cyst nematodes *G. pallida* and *G. rostochiensis* can cause significant damage to the roots of *Solanum tuberosum* plants. Once hatched from encysted eggs found in the soil, the juveniles migrate towards potato roots following the concentration gradient of PRD released from the roots and penetrate root tissue (Farnier *et al*., 2012). Second-stage juveniles are able to survive for approximately 6-11 days in soil (Robinson *et al*., 1987) and are found in the soil up to 8 weeks after plant emergence (Ryan and Devine, 2005). The J2 damages potato plant roots by penetrating root tissue and migrating through the root system to locate and establish a suitable feeding site (Jones and Northcote, 1972). This damage reduces the roots' ability to take up water and nutrients (Trudgill *et al.*, 1975a) and allows entry of opportunistic pathogens into the root system (Back *et al*., 2006; Storey and Evans, 1987).

A novel formulation with broad spectrum antimicrobial activity has been proposed as a nematicide. The formulation, BGT, contains of a blend of cationic and non-ionic surfactants. Surfactants are amphiliphilic chemicals that influence the properties or surfaces and interfaces (Merianos, 2001; Schramm *et al*., 2003). They combine hydrophobic and hydrophilic properties in one molecule, and are classed as cationic, non-ionic or anionic depending on the presence or absence of ionization of the hydrophilic group (Castro *et al*., 2013; Merianos, 2001). Non-ionic surfactants are widely used in agriculture (Cserháti, 1995), though cationic surfactants are not. Cationic surfactants are the most active surfactants against microorganisms, and cause cell death by interacting with the cytoplasmic membrane of bacteria, the plasma membrane of yeasts and the envelope of viruses (McDonnell, 2009). The insertion of the hydrophobic tail into the phospholipid bilayer causes the rearrangement and disruption of the cytoplasmic membrane, and a loss of membrane integrity (Hancock, 1984; Maillard, 2002).

Second-stage juveniles of *G. pallida* are dependent on lipid reserves for survival until they are able to establish a feeding site on host roots (Robinson *et al*., 1987). They are also dependent on the internal pressure created by the pseudocoelom for mobility (Decraemer and Hunt, 2013). If the cationic and non-ionic surfactants in BGT are able to disrupt the internal membranes of the J2, they may render it immobile or cause mortality of the nematode.

If it is possible to reduce the number of J2s in the soil, the severity of infection of and damage to the potato plants could be limited. To determine the effect of the novel formulation BGT on J2s of *G. pallida*, mortality assays were conducted *in vitro*.

Aim

To determine the effect of BGT on the mortality of second-stage juveniles of *G. pallida*.

Objectives

1. Determine the mortality of *G. pallida* second-stage juveniles after 24h contact with BGT.
2. Determine the LC_{50} for 24 h contact with BGT in relation to the mortality of *G. pallida* second-stage juveniles.

Null Hypothesis

BGT has no effect on the *in vitro* mortality of *G. pallida* second-stage juveniles.

2.2 Materials and methods

Preparation of potato root diffusate

Potato root diffusate (PRD) was obtained from 4 week old potato plants (cv. Estima) using the methods described by Fenwick (1949). Plants were grown in horticultural sharp sand in 20 cm pots for 4 weeks, and left un-watered for 48 h prior to collection of PRD. Potato root diffusate was obtained by adding 250 ml water to the potting medium and collecting the washings in a beaker beneath the pot. The washings were passed through the same pot three times, before being filtered through Whatman No. 1 Paper and then refrigerated until required. Prior to use in assays, PRD was diluted to 25% v/v with tap water.

Preparation of nematodes.

Globodera pallida cysts were extracted from soil from a field in Shropshire in which potato crops had been grown at least once in the past 5 seasons, using a Fenwick can (Shepherd, 1986). The population tested was 'VTBN' and was extracted from a field in Shropshire. Cysts were dried and refrigerated until required.

The species of PCN was confirmed as *G. pallida* by performing quantitative polymerase chain reaction (qPCR) using a Bio-Rad CFX-96 (Bio-Rad Laboratories, California, United

States). DNA was extracted from crushed cysts by boiling in TE buffer with activated carbon for 5 minutes, following the qPCR protocol adapted from Nakhla *et al*. (2010). 5 µl of extracted DNA was added to 0.05µl each primer (100 µmol μl^{-1}), 0.025 µl probe (100 µmol μl^{-1}), 12.5 µl Takyon No Rox Probe Mastermix (Eurogentec, Liége, Belgium) and 7.375µl nuclease free water. The qPCR programme used consisted of takyon activation at 95°C for 180 seconds followed by 40 cycles of 95°C for 10 seconds and 58°C for 60 seconds. Table 2.1 contains the primers and probes used. Positive controls were obtained from Fera Science Ltd (York, UK). The viability of *G. pallida* eggs in samples was determined by a hatching assay (method in Chapter 3) based on that described by Kroese *et al*. (2011).

TABLE 2.1 PRIMERS AND PROBES USED IN THE QPCR ASSAY TO IDENTIFY THE SPECIES OF POTATO CYST NEMATODE POPULATIONS. FROM NAKHLA *ET AL*. (2010)

	Primer/probe name	Sequence (5' –> 3')
Primers	PITSpf	ACGGACACATGCCCGCTA
	PITSp4	ACAACAGCAATCGTCGAG
	PGrtf	TCTGTGCGTCGTTGAGC
	Prostor	CGCAGACATGCCGCAA
Probes	G FAM p	ACATGAGTGTTGGGGTGTAAC
	G YY p	CGCAGATATGCTAACATGGAGTGTAG

Preparation of test solutions

Test solutions of BGT were obtained from Arcis Biotechnology Ltd (Sci-Tech Daresbury, WA4 4FS). All dilutions were performed by volume in tap water. For mortality assays, the test solutions were diluted to 90% of the final volume used in the assay to allow for the addition of the nematode suspension. Initial mortality assays using concentrations of 12.5% - 0.2% BGT resulted in 100% mortality of *G. pallida* J2s, thus concentrations of 0.2% - 0.003% BGT were investigated (Table 2.2).

Mortality assay protocol

Globodera pallida cysts were placed in hatching baskets in a 24-well plate (Corning™ Costar™ Flat Bottom Cell Culture Plates) (Chapter 3 Figure 3.1). Potato root diffusate at 25% was added to each well and the plates were incubated in an LMS cooled incubator (LMS Technology, Warrington, United Kingdom) at 15°C for up to 3 weeks in darkness. To recover hatched juveniles, the baskets were removed from each well and the juvenile suspensions pooled into a glass vial. The suspension was adjusted to approximately 100 juveniles per 100 µl suspension with tap water.

TABLE 2.2 CONCENTRATIONS OF BGT USED IN MORTALITY ASSAYS TO DETERMINE THE EFFECT OF BGT ON MORTALITY OF *GLOBODERA PALLIDA* SECOND-STAGE JUVENILES.

Treatment Number	BGT Concentration (%)	BGT Concentration (ppm)
1	0.000	0
2	0.003	17
3	0.006	33
4	0.012	66
5	0.025	133
6	0.05	266
7	0.10	531
8	0.20	1063

100 µl of hatched juvenile suspension was pipetted into a well of a 24 well plate. 900 µl of test solution was added to the nematode suspension using an Eppendorf Research variable adjustable volume pipette (Eppendorf, Hamburg, Germany) and incubated in darkness at 15°C for 24 h. 900 µl tap water was added to untreated control juveniles. After 24 h, the number of live (mobile) and dead (immobile) nematodes in each well were counted using a Leica Wild M3Z microscope (Leica Camera, Wetzlar, Germany).

All mortality assays were performed in a randomized block design, and the experiment was performed three times.

Statistical analysis

Statistical analyses were performed using Genstat 18th Edition (VSN International, Hemel Hempstead, United Kingdom). Tests of data distribution were used to support the appropriate choice of statistical analysis, including histogram of residuals, fitted-value plot, normal plot and half-normal plot. Analysis of Variance and Tukey's multiple range test were performed to determine any significant differences between treatments. Probit analysis was performed and the lethal concentration for 50% mortality of J2s after 24 h of exposure (LC_{50}) was estimated from the best fitted regression curve between BGT concentrations and percentage of dead nematodes.

Additional Microscopy

Second-stage juveniles incubated in 0.2% BGT at 15°C for three days were pipetted onto a glass slide and examined for any physical symptoms of mortality using light and phase contrast microscopy on an Olympus IX73. Images were taken using the Olympus SC100 camera and cellSens standard 1.9 software (Olympus Corporation, Tokyo, Japan).

2.3 Results

Mortality assay

Mortality assessments were performed on *G. pallida* J2s after 24 h treatment with BGT 0.2% - 0.003% (Figure 2.1). At the lowest concentration tested (0.003%), BGT caused 44% mortality in *G. pallida* J2, over five times the mortality of 8% observed in untreated control juveniles. The percentage of immobile juveniles increased with increasing concentrations of BGT until 100% mortality was achieved at 0.2% BGT. Analysis of variance was performed and determined that juveniles treated with BGT had significantly ($F_{(7,28)}$=86.84, P<0.001) higher mortality than the control juveniles that were incubated in water. Tukey's multiple range test (P=0.05) demonstrated significant differences between all treatments and the control, as well as significant differences between treatments. Probit regression analysis was performed to determine the LC_{50} of BGT, which was found to be 0.004%.

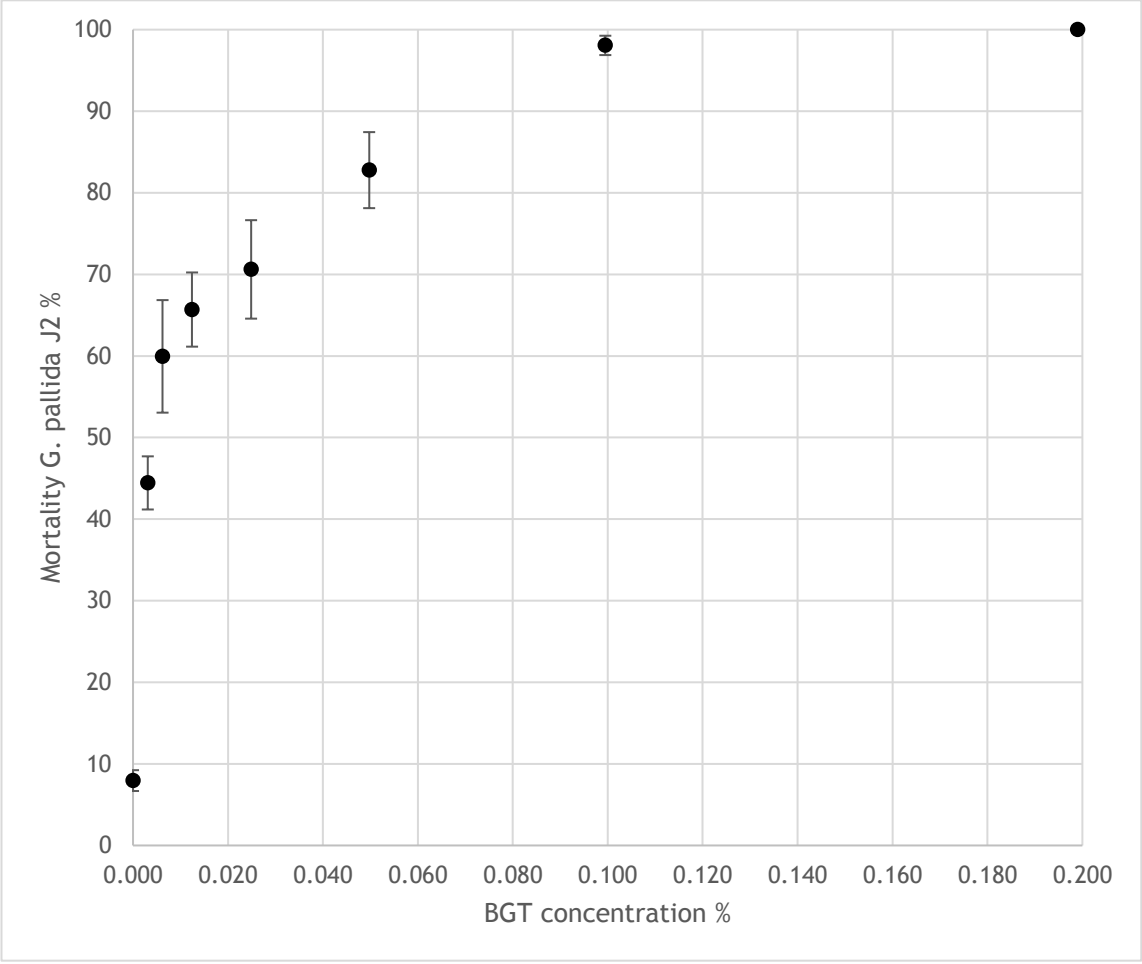


FIGURE 2.1 PERCENTAGE MORTALITY OF SECOND-STAGE JUVENILES OF *GLOBODERA PALLIDA* AFTER 24 H CONTACT WITH BGT AT CONCENTRATIONS OF 0.003% TO 0.2%. LC_{50} AS CALCULATED BY PROBIT REGRESSION ANALYSIS WAS DETERMINED AS 0.004% BGT. ERROR BARS SHOW THE STANDARD ERROR OF THE MEAN. MORTALITY WAS SIGNIFICANTLY (P<0.01) HIGHER IN TREATED JUVENILES THAN IN CONTROL JUVENILES, WHERE MORTALITY WAS 8%. TREATMENT WITH 0.2% BGT CAUSED 100% MORTALITY IN *G. PALLIDA* JUVENILES. THE COEFFICIENT OF VARIANCE WAS 10.9%

Microscopy

Assessments of mortality were made after 24 h contact with BGT, although qualitative observations were made at various timepoints during pilot assays where *G. pallida* J2 were treated with BGT at concentrations of 0.2% to 12.5%. After 1 h contact with 6% BGT, movement of juveniles had changed from smooth and undulating to very little movement with erratic spasms. Figure 2.2B shows the awkward angles made by juveniles after treatment with 6% BGT. After 24 h, motility had ceased completely. Juveniles immersed in 0.2% BGT for 3 days were observed to have complete disorganization of internal organs, and visible separation of the cuticle from the internal organs (Figure 2.3). These juveniles were found to be fairly straight, with a slight bend to one side. The morphology of an untreated *G. pallida* juvenile can be seen in Figure 2.3A, with a visible stylet and clear distinction between the pharynx and digestive system. The lipid reserves are visible as granular mass and the cuticle is intact. Figure 2.3B shows the loss of differentiation between *G. pallida* organs following immersion in BGT for 3 days. The stylet and metacorpus are barely visible, the lipid reserves are no longer restricted to the posterior region, the inner membrane has disassociated from the cuticle and there is a thin channel seen within the entire length of the juvenile. There is also a change in the basal zone at the tail region, where the inner membrane can be seen shrinking away from the cuticle.

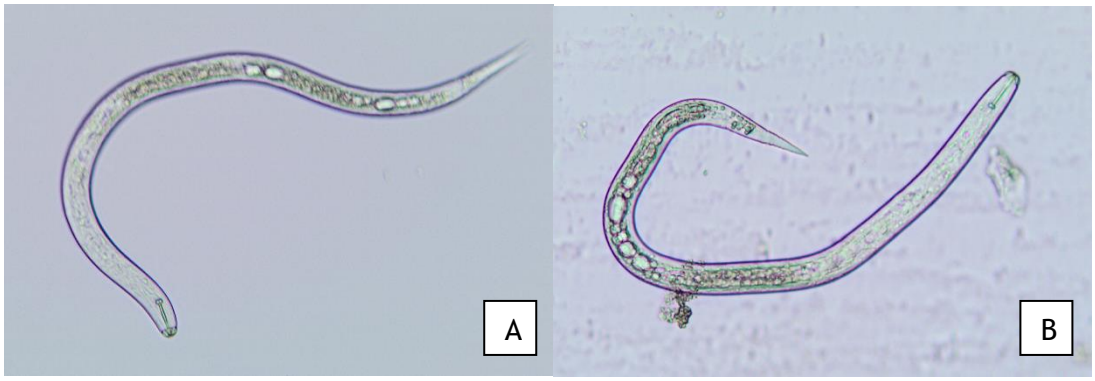


FIGURE 2.2 *GLOBODERA PALLIDA* JUVENILES IN WATER (A) AND AFTER 1 H CONTACT WITH 6% BGT (B). THE MOVEMENT OF UNTREATED JUVENILES IN WATER (A) WAS SMOOTH AND UNDULATING, WHILST TREATED JUVENILES (B) MOVED ERRATICALLY, WITH SPASMS OF MOVEMENT IN BETWEEN LONGER PERIODS OF INACTIVITY.

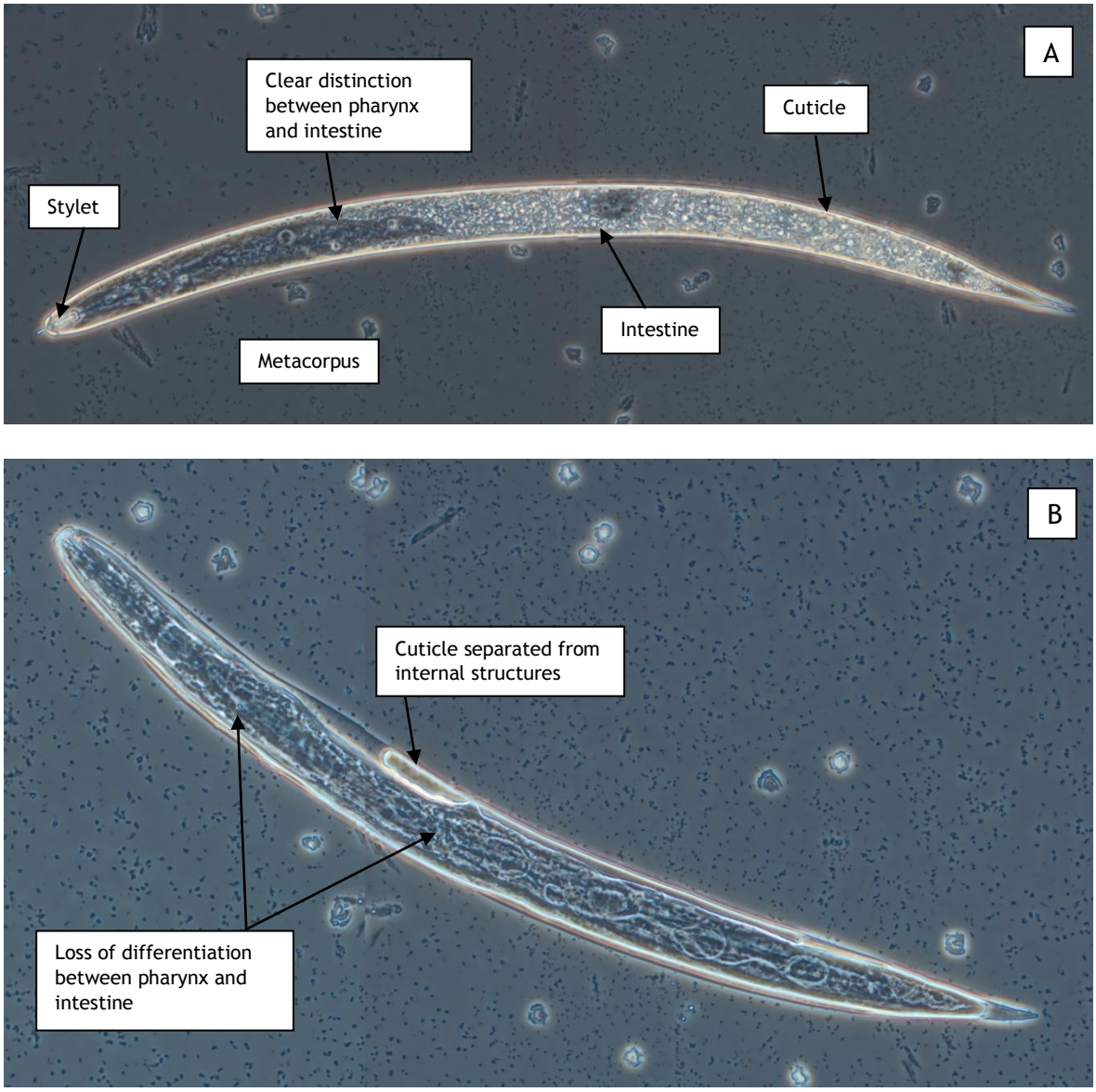


FIGURE 2.3 PHASE CONTRAST MICROSCOPY OF *GLOBODERA PALLIDA* SECOND-STAGE JUVENILES. (A) *G. PALLIDA* SECOND-STAGE JUVENILE STORED FOR 3 DAYS IN WATER AT 15°C. (B) *G. PALLIDA* SECOND-STAGE JUVENILES STORED FOR 3 DAYS IN BGT 0.2% AT 15°C. UNTREATED JUVENILES (A) DISPLAY CLEAR DIFFERENTIATION BETWEEN PHARYNX AND DIGESTIVE SYSTEM, WITH A VISIBLE STYLET AND METACORPUS, WHILST TREATED JUVENILES (B) SUFFERED DISSOCIATION OF THE INTERNAL MEMBRANES AND LIPID RESERVES.

2.4 Discussion

The second-stage juvenile of *G. pallida* damages potato plant roots by penetrating the root tissue, migrating through the root system and establishing a feeding site, where it develops into an adult male or female (Jones and Northcote, 1972). Prior to infecting root tissue, J2s of *G. pallida* must migrate through the soil from their hatching site to a prospective host. A novel formulation with broad spectrum antimicrobial activity has been proposed as a nematicide. If this formulation, BGT, is able to cause mortality in *G. pallida* juveniles, then it may reduce the number of juveniles infecting roots, reducing the damage to root tissue and limit the loss of yield.

Second-stage juveniles of *G. pallida* were immersed in BGT for 24 h prior to mortality assessments by microscopy. The mortality of *G. pallida* J2 was significantly increased after 24 h contact with BGT *in vitro*. The non-linear response to BGT at concentrations between 0.003% and 0.2% is dose dependent; juvenile mortality increases as the concentration of BGT increases.

BGT caused mortality of juveniles at all concentrations tested. The lowest concentration, 0.003%, contains 17 ppm of the components and caused 44% mortality. 0.1% BGT caused 98% mortality and the highest concentration tested, 0.2%, caused 100% mortality and contains 1063 ppm of the components. Danquah *et al*. (2011) found that the LC_{50} of the garlic extract tested was 983.0 µl L^{-1} (0.9 ppm), far lower than that of BGT (21 ppm). The flavanoid (E)-Chalone was also found to have a, LC_{50} of 6 ppm against both *G. pallida* and *G. rostochiensis* (Gonzalez and Estevez-Braun, 1998). The LC_{50} of active ingredients/products is useful in comparing the biological activity of compounds. BGT is less biologically active against *G. pallida* than both G8014S and (E)-Chalcone, though BGT is a formulation of five components, which may not all be active against *G. pallida*. Mortality testing of single components would elucidate which of the components is active against *G. pallida*, and may have an LC_{50} comparable to the single actives (E)-Chalcone and garlic extract.

Although BGT may not be as biologically active *in vitro* against *G. pallida* as compounds such as garlic extract or (E)-Chalcone, this may not be the case *in situ*, as the variables introduced in the soil may affect active substances differently. Plant protection products including nematicides are primarily used to protect the quality and quantity of yield, and must be of economic benefit to the grower to be successful. Acetylcholine esterase inhibitors such as oxamyl are currently registered as active substances in plant protection products in the EU. Though the EC_{50} of oxamyl for juveniles of *G. rostochiensis* was 0.5 µg ml^{-1}, treatments up to 1.0 µg ml^{-1} were not able to completely arrest movement in juveniles (Evans and Wright, 1982), whereas 100% mortality was observed when *G. pallida* juveniles were exposed to 0.2% BGT. According to Ibrahim & Haydock (1999),

there is no evidence to suggest any difference in the inherent susceptibility of J2s of *G. pallida* and *G. rostochiensis* to nematicides, therefore the results of Evans & Wright (1982) should be applicable to *G. pallida*.

The differences in biological activity may be explained by the different modes of action of these very different products. Although the mode of action of (E)-Chalcone is not known, it is thought that it may act as an uncoupler of the oxidative phosphorylation processes in the mitochondria (Gonzalez and Estevez-Braun, 1998). Acetylcholinesterase inhibitors cause temporary paralysis of juveniles by impairing neuromuscular and sensory activity (Evans and Wright, 1982). This mode of action is not species specific and is the cause for acetylcholinesterase inhibitors being listed as candidates for submission by EC 1107/2009 (European Commission, 2009). The garlic extract contains the thiosulfinate allicin (Danquah *et al*., 2011), which decomposes to other organosulfur molecules including diallyl polysulfides (DAPS) (Anwar *et al*., 2017) which have a broad suite of biological effects, such as interaction with lipophilic structures, perturbing homeostasis of metal cations and disruption of enzyme function (Anwar *et al*., 2017). BGT is unlikely to affect the nervous system or metabolic function of *G. pallida* juveniles, but may cause physical disruption of internal membranes, possibly in a similar manner to the mode of action of DAPS on lipids (Anwar *et al*., 2017). The observations made using light and phase contrast microscopy suggest that the effect of BGT on *G. pallida* is disruption of internal membranes. As shown in Figure 2.3, the dissociation of internal membranes and lipid stores would not be reversible, thus the mortality caused by BGT is permanent.

The observations made via light and phase contrast microscopy (Figure 2.3) give insight into the mode of action of BGT on *G. pallida*. After treatment with BGT, there appeared to be no degradation of the cuticle, with damage apparently restricted to the inner tissues of the nematode. Juveniles treated with BGT showed a separation of the internal body cavity and the cuticle and a breakdown of internal membrane and lipid reserves. Infective *Globodera* spp. juveniles depend on neutral lipid reserves to survive, and depletion of lipid reserves during storage leads to reduced motility and infectivity of hatched *G. rostochiensis* juveniles (Robinson *et al*., 1987). Surfactants solubilize lipids and proteins, and can insert themselves into lipid membranes (Maillard, 2002). The insertion of the hydrophobic tail of cationic surfactants into a lipid membrane causes rearrangement and disruption of the membrane, and a loss of membrane integrity (Maillard, 2002). The disruption of lipid reserves by BGT would reduce the motility and infectivity of *G. pallida*, reducing the damage caused to potato roots. The internal structure of the treated juveniles appeared to be completely disorganised, with loss of differentiation between the pharynx and digestive system (Figure 2.3B). The loss of internal membrane integrity and structural organisation caused by the surfactants in BGT would cause a loss of internal turgor pressure in the pseudocoelom, rendering the juvenile unable to move. In bacteria,

cell death may be mediated by a build-up of the damage caused by cationic components, as coagulation of intracellular material occurs and autolytic enzymes are promoted (Hugo, 1965; Salton, 1968). It is unknown whether these mechanisms (or similar) may also be triggered in *G. pallida* juveniles.

Although mortality assays can provide information on the mode of action of a product and are an obvious first step in evaluating nematicidal products, there are several limitations, including the sterility of the method. It is well known that the efficacy of surfactants is reduced in the presence of organic matter (McDonnell, 2009), which is present in 45% agricultural soils in Europe at 2 – 6% (Rusco *et al*., 2001), but can be 13% or more (Johnston, 1986). Standard methods for testing antimicrobial activity include the use of interfering substances such as bovine serum albumin (BSA) to determine the efficacy of disinfectants in the presence of organic matter (European Committee for Standardization, 2009). The addition of an interfering substance such as BSA may increase the robustness of the assay.

The longevity of BGT in soil is unknown, therefore one cannot assume juveniles will be in contact with the test solution for a minimum of 24 h. Though the results of microscopy on juveniles after 72 h contact suggest the effect of BGT are permanent, these morphological changes are not seen at 24 h. It is possible that juveniles may recover after less than 72 h contact, this could be investigated further by rinsing juveniles in water or PRD after various contact times to determine the time point at which the irreversible damage occurs.

By reducing the number of infective PCN juveniles in the soil, the potential damage to plant roots is decreased. To ensure that any product is able to protect plants, it is essential that the active ingredients are present at a lethal concentration when juveniles are active in the soil, between hatch and infection. Second-stage juveniles are only active for up to eleven days (Robinson *et al*., 1987) but are found in the soil up to eight weeks post plant emergence as they are continuously hatching (Ryan and Devine, 2005).

The longevity of BGT in soil is likely to be adversely affected by the organic matter present in the rhizosphere and as it is water-soluble, it may leach out of the soil profile. Nematicides should persist for long enough to offer good control of the nematode, but their degradation must be rapid enough to not leave residues in the crop or environment (Back *et al*., 2018). Many commercial nematicides are applied in granular format and gradually release active ingredients from the inert material (Vidhyasekaran, 2004), and though this is not a feasible option for a surfactant-based formulation such as BGT, application via drip tape may be utilised to increase the longevity of the product in the soil and offer a target application method (Kiezebrink, 2009).

Whilst BGT was shown to cause mortality in *G. pallida* J2s *in vitro*, it may not be effective as a plant protection product *in situ*. Prior to hatching, *G. pallida* juveniles develop within

encysted eggs. The infective J2s can survive in the soil for up to 11 days (Robinson *et al*., 1987), but unhatched juveniles in eggs (within cysts) are able to survive for up to 30 years in the soil (Turner, 1996). As they are present in the soil prior to (and remain in the soil following) host plants being grown, they are an obvious target for plant protection products. If BGT could prevent the infective juveniles hatching, it would provide more protection to plants than by killing a proportion of juveniles that have successfully hatched and may damage plant roots.

2.5 Conclusion

Globodera pallida is an important pest of potatoes, and it is the J2 that causes damage to potato root tissue, causing a reduction in yield. The surfactant formulation BGT caused mortality of J2s of *G. pallida* after 24 h contact, and after 72 hr contact caused irreversible disruption of internal organs and structures. It is likely that the cationic surfactants in the BGT caused membrane disruption and breakdown of the neutral lipid reserves that enable the survival of the juveniles in the soil. It is also likely that this mechanism also contributed to the loss of internal pressure required for motility of J2s. To further evaluate the suitability of BGT as a nematicidal treatment, the effect of BGT on hatching of *G. pallida* juveniles should be investigated.

Chapter 3 : The effect of BGT on hatching of second-stage juveniles of *Globodera pallida*.

3.1 Introduction

The potato cyst nematode *Globodera pallida* causes significant yield loss to potato crops through its destructive parasitism of root tissue. Chapter 2 investigated the effect of the novel formulation, BGT, on the mortality of the infective J2s of *G. pallida* and found that BGT caused up to 100% mortality within 24 h of contact.

Globodera pallida juveniles develop within encysted eggs (see Chapter 1.3 for the life cycle of *G. pallida*) and these eggs protect juveniles from environmental stresses and enable survival for up to 30 years in the soil (Turner, 1996). The eggshell itself consists of three layers; the outer lipoprotein layer derived from the vitelline layer of the fertilized oocyte, the middle chitinous layer providing structural integrity, and the innermost lipid layer, which represent the main permeability barrier (Jones *et al*., 1998). Hatching of J2s of *G. pallida* from encysted eggs in the soil is stimulated by hatching factors present in potato root diffusate (PRD). Potato root diffusate is produced during the vegetative phase of plant growth along the length of the roots, most potently at the root tip, and can stimulate 60-80% of eggs to hatch (Rawsthorne and Brodie, 1986). Though J2s of *G. pallida* are only able to survive for up to 11 days in the soil, they do not all hatch simultaneously, and are found in the soil prior to and up to eight weeks after plant emergence (Robinson *et al*., 1987). The efficacy of any treatment targeting J2s of *G. pallida* is dependent on achieving a nematicidal concentration in the soil when the juveniles are active. *Globodera pallida* juveniles within eggs are present in the soil far longer than active J2s, therefore the unhatched juveniles within eggs are an obvious target for plant protection products.

BGT is a blend of cationic and non-ionic surfactants and a biguanide and has been proposed as a novel PCN control agent. Surfactants, or surface-active agents, are amphiphilic chemicals (Merianos, 2001) utilised for their solubilisation and cleaning purposes (Ying, 2006). Cationic surfactants have the greatest antimicrobial activity of all surfactants (McDonnell, 2009); they are compatible with non-ionic surfactants and are commonly co-formulated to increase efficacy. Two of the components in BGT are quaternary ammonium compounds (QACs), which act upon lipid membranes (Fisher, 2003) and denature, precipitate and complex proteins (Merianos, 2001). The positively charged surfactant adsorbs onto the negatively charged cell membrane, with the cationic head group facing outwards and the hydrophobic tails inserted into the lipid bilayer (Maillard, 2002). The hydrophobic portion of a cationic surfactant displaces the membrane lipids and binds to the hydrophobic portions of integral membrane proteins.

The hydrophilic tail of the surfactant renders the surfactant-protein complex soluble in aqueous solutions (Cooper, 2000; Maillard, 2002). Both peripheral and integral membrane proteins are dissociated from the membrane and released (Cooper, 2000). The insertion of the hydrophobic tail into the phospholipid bilayer causes the rearrangement and disruption of the cytoplasmic membrane, and a loss of membrane integrity (Hancock, 1984; Maillard, 2002). Non-ionic surfactants readily bind to and modify the adsorption capacity of proteins and peptides (Cserháti, 1995; Maillard, 2002), and are known to increase the permeability of phospholipid membranes and vesicles by insertion of the hydrophobic portion of the surfactant into the apolar fatty acid domain of phospholipids (Cserháti, 1995).

Results from Chapter 2 showed that BGT caused up to 100% mortality in J2s of *G. pallida*, and mass disruption of the internal organs and structure of the nematode. BGT also disrupts lipid energy reserves within the juveniles. It is postulated that BGT may be able to interact with and disrupt the lipid membranes of the eggshell of *G. pallida*, increasing permeability of the eggs, promoting uptake of itself into the egg, thus causing mortality of the *G. pallida* juveniles within.

To determine the effect of BGT on unhatched eggs of *G. pallida*, hatching assays were performed. Hatching assays immerse *G. pallida* cysts in PRD, which is changed weekly for eight weeks. The number of J2s emerging each week is counted, and the number of eggs remaining in the cysts counted at the end of the experiment to determine the percentage hatched. Hatching assays are commonly used as a marker of viability as they mimic the exposure of encysted nematodes to the hatching factors released by the roots of growing host plants. Although hatching assays underestimate the viability of nematodes as they do not include juveniles in dormant stages, they are indicative of the number of juveniles that could hatch when in contact with a growing host plant. Therefore, they are a good predictor for the severity of infection that would occur in the field and are an effective method to compare treatments that are intended to affect hatching of nematodes, such as BGT.

Aim

To evaluate the effects of BGT on the hatching of *Globodera pallida* second-stage juveniles from encysted eggs.

Objective

Determine the percentage of *Globodera pallida* second-stage juveniles hatching from encysted eggs in potato root diffusate after 24 h contact with BGT.

Null Hypothesis

BGT has no effect on *in vitro* hatching of *G. pallida* second-stage juveniles.

3.2 Materials and methods

For methodology on preparation of potato root diffusate (PRD) and extraction and identification of PCN, see Chapter 2. The *G. pallida* populations tested are listed in Table 3.1. All *G. pallida* populations were extracted from potato growing sites in Shropshire.

Preparation of test solutions

Test solutions of BGT were obtained from Arcis Biotechnology Ltd (Sci-Tech Daresbury, United Kingdom). BGT was diluted to concentrations between 1.0E+02% - 1.0E-06% by volume with tap water (Table 3.1). Water was used as a control.

Hatching assay protocol

The viability of cysts and activity of PRD was confirmed by means of a hatching assay based on that described by Kroese *et al*. (2011) prior to the experimental assays. In triplicate, batches of ten cysts were placed in hatching baskets made from 1ml pipette tips and silk (Figure 3.1A). Baskets were placed in 24-well cell culture plates (Corning™ Costar™ Flat Bottom Cell Culture Plates) (Figure 3.1B) and soaked in 1.5 ml water for seven days at 15°C before being transferred to new plates containing 1.5 ml PRD (diluted 1:3 with water) and incubated in darkness in an LMS Cooled incubator (LMS Technology, Warrington, United Kingdom) at 15°C. Every seven days, the baskets were transferred to fresh PRD and the number of juveniles hatching from each batch of cysts were counted on a Leica Wild M3Z microscope (Leica Camera, Wetzlar, Germany) at x40 magnification. Once baskets were removed to another plate the well was filled to the brim with water (total volume 3 ml), and a 1 ml subsample was counted on a nematode counting slide (Microscope Service & Sales Ltd, Bourne End, United Kingdom).

After eight weeks, the cysts were removed from baskets to 1.5 ml Eppendorf tubes and crushed in 200 µl water with a micropestle. The nematode egg suspension was added to 3.8 ml water and 1 ml of the diluted suspension was counted on a nematode counting slide. All counts were performed on a Leica Wild M3Z microscope at 40x magnification. Juvenile counts were multiplied by three to give final counts and remaining egg counts were multiplied by four. The percentage hatch of juveniles in each sample was calculated using the following equation:

$$Percentage\ hatch = \frac{hatched\ juveniles}{hatched\ \ juveniles + remaining\ eggs} x\ 100$$

The percentage hatch of juveniles in each population was determined to predict the likely percentage hatch in juveniles in the control group of experimental assays. Experimental

assays were performed immediately after the end of the initial hatching assay to limit any possible effects of storage on the cysts and PRD.

Experimental hatching assay protocol

Six experiments were performed throughout the research period on different concentration ranges of BGT between 10% - 1E-06%. The BGT concentrations tested, *G. pallida* populations and PRD batches used are detailed in Table 3.1.

Experimental hatching assays were performed as above, with the addition of a transfer to 1.5 ml test solution for 24 h (Table 3.1) following soaking cysts in water and prior to transfer to PRD. Assays were performed in replicated randomized block design. To calculate the percentage reduction of test results from the control, the following equation was used:

$$Percentage\ reduction\ from\ control = \frac{control\ \%\ hatch - treated\ \%\ hatch}{control\ \%\ hatch}\ x\ 100$$

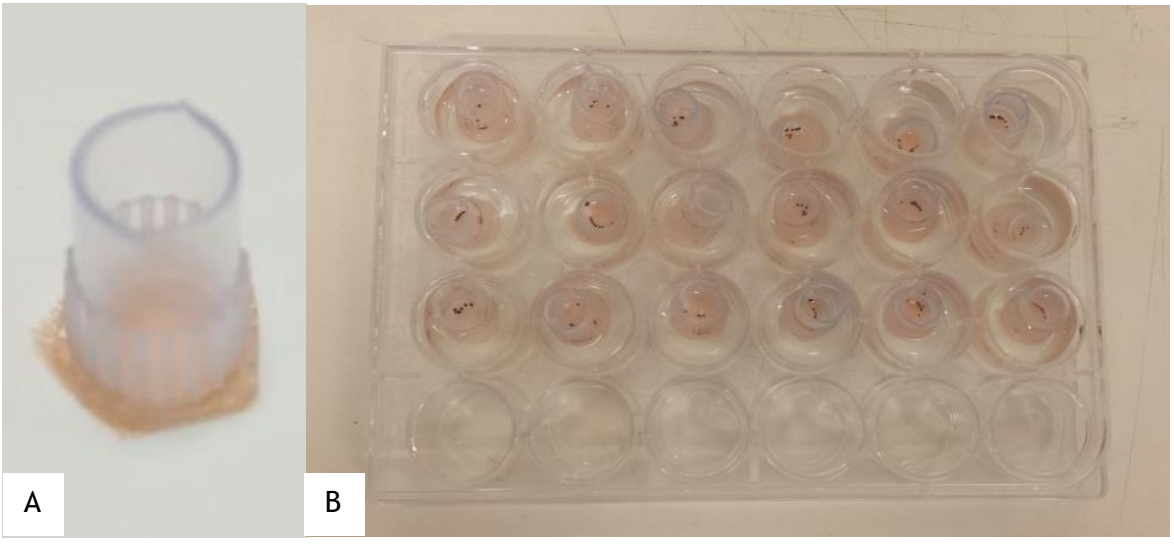


FIGURE 3.1 SETUP FOR *GLOBODERA PALLIDA* HATCHING EXPERIMENTS. (A) HATCHING BASKET THAT CYSTS ARE PLACED IN FOR IMMERSION IN TEST SOLUTIONS OR HATCHING SOLUTIONS. (B) HATCHING BASKETS IN 24-WELL PLATE. CYSTS ARE COUNTED INTO HATCHING BASKETS, WHICH ARE PLACED IN WELLS OF A 24-WELL CELL CULTURE PLATE. WATER, TEST PRODUCT OR HATCHING SOLUTION (POTATO ROOT DIFFUSATE) IS THEN ADDED AND THE LID SEALED ON WITH PARAFILM.

TABLE 3.1 DETAILS OF *GLOBODERA PALLIDA* POPULATIONS, POTATO ROOT DIFFUSATE BATCHES AND BGT BATCHES TESTED IN EACH OF THE SIX BGT DOSE RESPONSE EXPERIMENTS AND THE PERCENTAGE OF SECOND-STAGE JUVENILES HATCHING FROM UNTREATED CYSTS IN PRD IN EACH EXPERIMENT.

Experiment Reference	Year experiment performed	PCN population tested	PRD batch used	BGT batch tested	BGT concentrations tested	Percentage hatch of control
1	2014	MiBu	Estima Feb 2014	130326A	6%, 3%, 1.5%, 0.8%, 0.4%	34%
2	2015	MiBu	Estima March 2015	150512A	0.4%, 0.2%, 0.1%, 0.05%, 0.02%, 0.01%, 0.005%	73%
3	2016	MiBu	Jersey Dec 2015	150512A	0.8%, 0.4%, 0.2%, 0.1%, 0.05%	37%
4	2016	VTBN	Jersey Dec 2015	150512A	0.8%, 0.4%, 0.2%, 0.1%, 0.05%	26%
5	2017	Shrop	Estima March 2017	161031A	1E-02%, 1E-03%, 1E-04%, 1E-05%, 1E-06%	65%
6	2017	Shrop	Estima March 2017	161031A	10%, 1%, 0.1%, 0.01%, 0.001%	37%

Statistical analysis

Analysis of variance (ANOVA) and Tukey's ad hoc test were performed using Genstat v18 (VSNI International Ltd., Hemel Hempstead, United Kingdom) on data from each experiment to determine significant differences at $P < 0.05$ between population and treatments. Tests of data distribution were used to support the appropriate choice of statistical analysis, including histogram of residuals, fitted-value plot, normal plot and half-normal plot. To combine experimental data for final analysis, control values were normalised to 100% and treated values expressed as percentage of control hatch as shown in Table 3.2. Normalised percentage hatch values for the six experiments were combined and an analysis of an unbalanced design using Genstat regression was performed with Fisher least significant difference ad hoc test, using experiment as a blocking factor.

3.3 Results

The normalised mean values (Table 3.2) from six hatching experiments (Table 3.1) were combined to observe the effect of BGT at 10% - 1E-06% (Figure 3.2). Analysis of variance performed on the combined normalised data found a highly significant ($F_{(17,151)}=4.2$, $P < 0.001$) difference between experiments. The ANOVA also found a highly significant ($P < 0.001$) difference between treatments. Cysts treated with BGT at 0.02% and above hatched significantly less than the control, with the exception of 1% and 1.5%, which were not significantly different from the control. Three of the concentrations of BGT tested stimulated hatch of *G. pallida* juveniles; 0.01%, 0.001% and 1E-06% increased hatch by 5%, 17% and 4% from the control respectively. Treatments of 0.02% - 3% reduced hatch by 9 – 49% from control. BGT 10% had the greatest effect on hatching of *G. pallida* juveniles, reducing percentage hatch by 76% from control.

The number of juveniles hatching weekly are shown for Experiment 1 in Figure 3.3, which shows the majority of hatch occurring in week two. After the peak in hatch, hatching declined throughout the remainder of the experiment. Cysts treated with BGT followed the same hatching pattern as the control, with suppression of hatch in week two. The results shown in Figure 3.2 are representative of all six experiments performed, which produced a similar pattern of peak hatch and a period of declining hatch for the remainder of the experiment.

TABLE 3.2 MEAN VALUES FOR PERCENTAGE OF SECOND-STAGE JUVENILES OF *GLOBODERA PALLIDA* HATCHING FROM CYSTS IMMERSED IN WATER OR BGT FOR 24 H PRIOR TO 8 WEEKS HATCHING IN POTATO ROOT DIFFUSATE (EXPERIMENT 2). NORMALISED VALUES SHOW DATA EXPRESSED AS PERCENTAGE OF CONTROL HATCH.

BGT	**Percentage hatch of juveniles**	
Concentration (%)	Mean from experiment 2	Normalised values
0	73	100
0.005	61	84
0.01	58	79
0.02	48	65
0.05	49	68
0.1	56	78
0.2	37	51
0.4	47	65

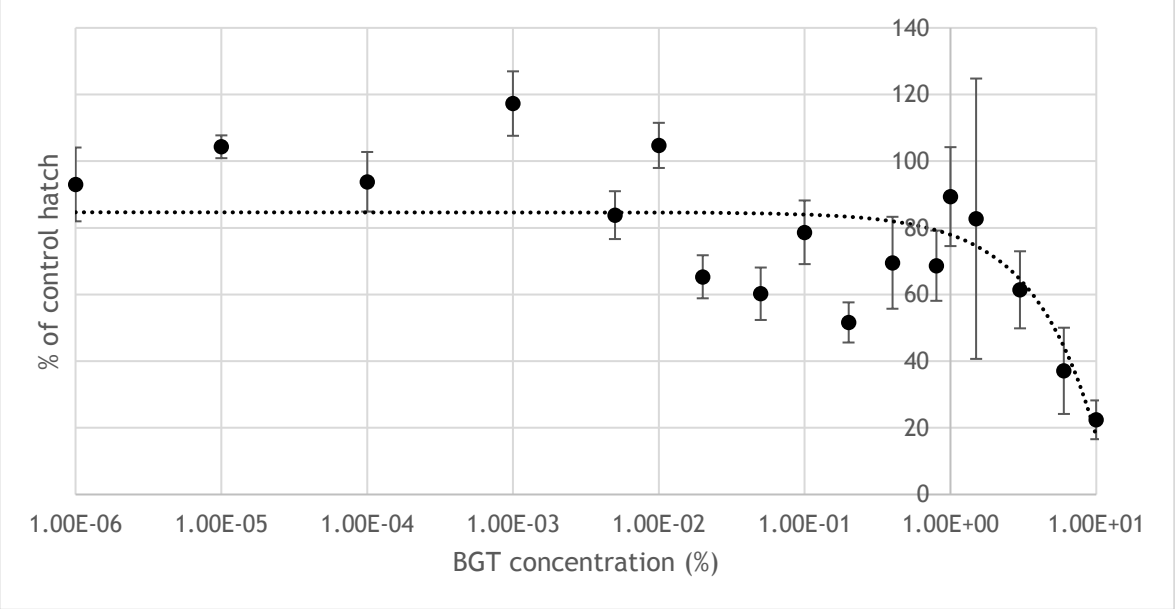


FIGURE 3.2 PERCENTAGE OF TOTAL *GLOBODERA PALLIDA* SECOND-STAGE JUVENILES HATCHED OVER EIGHT WEEKS IN POTATO ROOT DIFFUSATE AFTER 24 H EXPOSURE TO BGT. VALUES EXPRESSED AS PERCENTAGE OF CONTROL HATCH. RESULTS OF PREDICTION FROM REGRESSION MODEL OF ANALYSIS OF VARIANCE OF DATA FROM SIX EXPERIMENTS COMBINED. ERROR BARS SHOW THE STANDARD ERROR OF THE MEAN. R SQUARED VALUE OF THE TRENDLINE = 0.54.

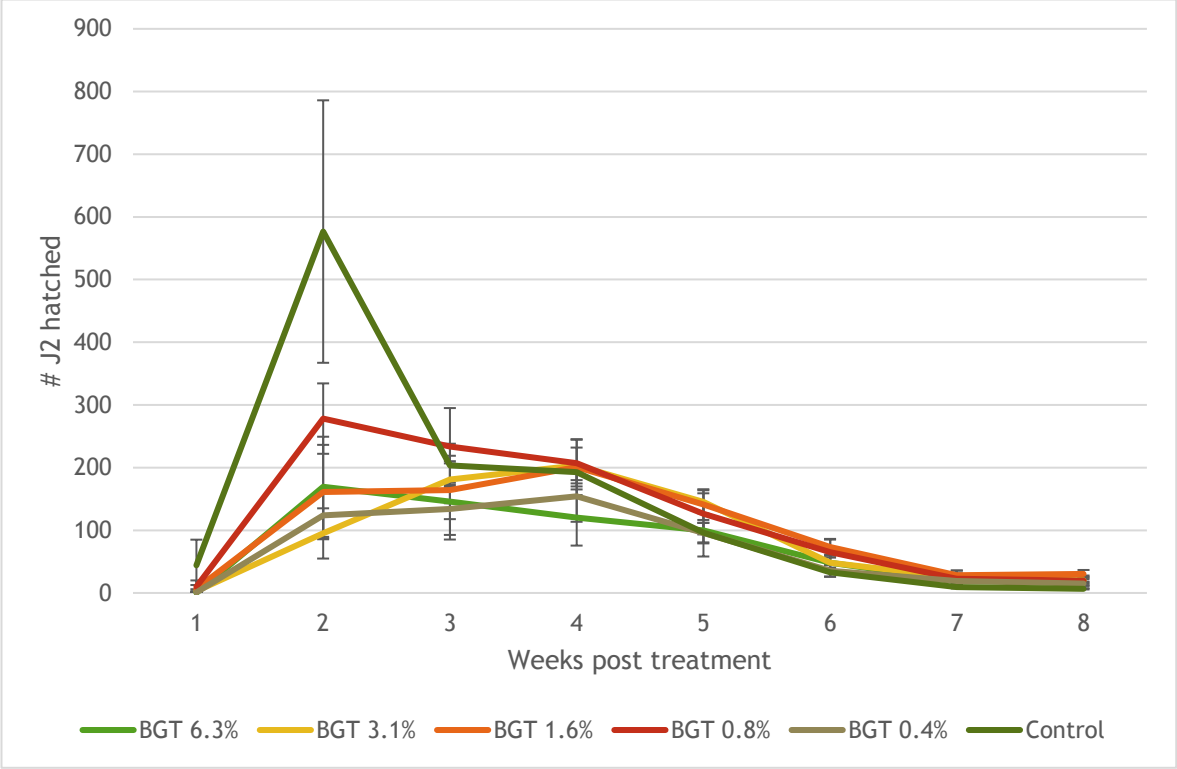


FIGURE 3.3 NUMBER OF SECOND-STAGE JUVENILES HATCHING FROM 25 *GLOBODERA PALLIDA* CYSTS OVER EIGHT WEEKS IN POTATO ROOT DIFFUSATE AFTER 24 H EXPOSURE TO BGT. RESULTS FROM EXPERIMENT 1. ERROR BARS SHOW THE STANDARD ERROR OF THE MEAN.

3.4 Discussion

The effective control of *G. pallida* is important in commercial potato production. Current management methods such as fumigant and non-fumigant chemical control, particularly the acetylcholinesterase inhibitors, are under threat by EU legislation (Twining *et al*., 2009). The development of nematicidal management strategies that are less toxic to non-target organisms and the environment is vital to protect future growers from significant yield loss by PCN, which could cost the industry up to £55 million if untreated (Twining *et al*., 2009). BGT is a blend of cationic and non-ionic surfactants, and is proposed in this thesis as a potential nematicide. In Chapter 1, BGT was found to cause mortality in J2s of *G. pallida*, and in this study, BGT was found to reduce hatching of J2s of *G. pallida* from encysted eggs.

Globodera pallida juveniles generally hatch within the first three weeks after initial exposure to potato root diffusate *in vitro* (Woods *et al*., 1999). Hatching assays were conducted over eight weeks to observe any change to typical hatching behaviour, such as a delay in peak hatch or a significant reduction/increase in hatching. Second-stage juveniles of *G. pallida* within cysts treated with 1E-06% - 10% BGT hatched less than those in untreated cysts within the initial three weeks of the assay, with no increase in hatching throughout the duration of the experiment (Figure 3.3). The results show that the

suppression of *G. pallida* hatching by 24 h contact of cysts with BGT is not reversible within eight weeks of stimulation by PRD and suggest that BGT could afford some protection to plant roots *in situ,* at least during the initial eight weeks of growth when plant growth is most vulnerable.

The results between experiments varied significantly ($P < 0.001$), with control hatch varying between 26 - 72%. Some variation between experiments is to be expected, as even under favourable conditions, a proportion of a single population of viable potato cyst nematode eggs will not hatch (Hominick *et al*., 1985). A proportion of eggs require a second period of stimulation by potato root diffusate to induce hatch in a later season to ensure persistence of the population over a number of growing season (Forrest and Farrer, 1983). Variation in hatching provides the potato cyst nematode with greater adaptive flexibility, and decreases the competition for feeding sites between hatched juveniles (Jones *et al*., 1998). Turner *et al*. (2009) investigated the variation in hatching of potato cyst nematodes and found that the hatching of different populations in the same PRD varied between 53% - 83%. They also found that the hatching of a single population of *G. pallida* Pa3 varied between 51% - 97% in PRD from different *Solanum* genotypes. Hominick *et al*. (1985) found that a single population of *Globodera rostochiensis* showed different hatching patterns when tested using standard protocols in two different laboratories.

To compare results from several experiments (Table 3.1), results were normalised as shown in Table 3.2. The data were combined to observe effect on hatching over a wide range of concentrations. Figure 3.2 shows the linear relationship between BGT concentration and percentage hatch between 1-10%, which plateaus at 1% and less. Cysts treated with BGT at 0.02% to 10% hatched significantly less than the control, with the exception of cysts treated with 1% and 1.5% BGT. The correlation between the concentration of BGT and the percentage hatch is strong (R=0.73), though there was a high level of variance both within and between treatment, especially at concentrations of 1.5% BGT and less. 1.5% BGT had a particularly high level of variance; the standard error of the mean was 42.06, compared to 14.84 for BGT 1% and 12.94 or less for all other treatments. BGT was tested at 1.5% in one experiment (Experiment 1) which had the highest variability of all the experiments. The coefficient of variance of Experiment 1 was 85.5%, all other experiments had a coefficient of variance between 19% and 32%. Experiment 1 tested the MiBu population, which was also used in Experiments 2 and 3 (Table 3.1), though different PRD was used in each experiment as they were performed over a 3-year period. The coefficient of variance of Experiments 2 and 3 were 23% and 32% respectively, far lower than that of Experiment 1. It is likely that the increased variance observed in Experiment 1 could be due to the batch of PRD used, as the

standard error of the mean was >8.0 for both control cysts and for cysts treated with BGT at concentrations of 1.5% or less.

Much higher concentrations of BGT were required to significantly reduce hatching of *G. pallida* juveniles than were found to cause mortality of J2s in Chapter 2. The lowest concentration of BGT that reduced hatching of *G. pallida* eggs by 50% was 0.2%, fifty times higher than the LC_{50} for J2s of *G. pallida*. Other studies have shown that nematicidal components required higher concentrations to reduce hatching than cause mortality of J2s. For example, the concentration of a garlic based product, G8014S, required to reduce viability of *G. pallida* eggs by 44% in 2 weeks was over a hundred times that of the LC_{50} for *G. pallida* J2s in 24 h (Danquah *et al*., 2011). This might be expected since the structural integrity of the middle chitin layer and the lipoprotein permeability barrier inner layer of the eggshell serve to protect juveniles from environmental stresses and chemical treatments (Jones et al., 1998; Turner, 1996). Second-stage juveniles migrating from the cyst to the roots are the most susceptible life stage of *G. pallida*, as they are not protected by eggs or root tissue and may be found free in the soil for up to 11 days (Robinson *et al*., 1987).

BGT had the greatest effect on hatch of second-stage *G. pallida* juveniles at 10%, reducing hatch by 76% from the control. The carbamate oxamyl, the active ingredient of the commercial nematicide Vydate (Du Pont), significantly suppressed hatch of *G. rostochiensis* juveniles when included at concentrations of 1 µg ml^{-1} and above in potato root diffusate (Evans and Wright, 1982). The number of juveniles hatching from cysts in PRD only was 5738, while only 5 juveniles hatched from cysts immersed in 16.0 µg ml^{-1} oxamyl (Evans and Wright, 1982). This effect was temporary, however, as hatch in oxamyl treated cysts increased the following week when transferred to fresh PRD, and no significant differences were found between the total number of juveniles hatching overall (Evans and Wright, 1982). The organophosphate fosthiazate, the active ingredient in the commercial nematicide Nemathorin (Syngenta Ltd), performed far better at reducing hatch than oxamyl *in vitro*. Two weeks contact (one at week 1, the second at week 6) with the active ingredient in potato root diffusate reduced hatch of *G. pallida* juveniles by 90% at 2 µg ml^{-1} (Woods *et al*., 1999). The effect of fosthiazate on *G. pallida* eggs was also temporary; an increase in hatch in week 5 prompted the second week of treatment. The concentration of fosthiazate tested had a significant effect on hatching; 6 weeks immersion in 0.05 µg ml^{-1} fosthiazate had no impact on hatch of *G. pallida* (Woods *et al*., 1999). The garlic extract and salicylaldehyde mixture G8014S completely reduced hatch of viable *G. pallida* juveniles after two weeks contact with concentrations of 1376 µl L^{-1} and above (Danquah *et al*., 2011). The viability of treated eggs was determined by hatching and staining remaining eggs with Meldola's blue, and found that treatment with 1376 µl L^{-1} G8014S for 2 weeks reduced viability of *G. pallida* by 40% (Danquah *et al*.,

2011). The results presented in this chapter suggest that BGT has a greater effect on hatching of *Globodera pallida* juveniles than oxamyl does on hatching of *G. rostochiensis* juveniles. According to Ibrahim & Haydock (1999), there is no evidence to suggest any difference in the inherent susceptibility of the J2s of *G. pallida* and *G. rostochiensis* to nematicides, therefore the results of Evans & Wright (1982) should be applicable to *G. pallida*. In comparison with fosthiazate and G8014S, the effect of BGT on hatching of *G. pallida* juveniles is lower, 76% reduction compared with over 90%, although the contact time in this study was much shorter, only 24 h not 2 weeks. The effects of prolonged treatment of *G. pallida* cysts with BGT on hatching of juveniles is investigated in Chapter 5.

Three of the concentrations of BGT tested stimulated hatch of *G. pallida* juveniles; 0.01%, 0.001% and 1E-06% BGT increased hatch by 5%, 17% and 4% respectively. The stimulation of hatching of *G. pallida* juveniles by BGT could be due to an interaction with the lipoprotein membranes found in the eggshell. Lower concentrations might contain enough active ingredient to increase eggshell permeability, but not enough to cause mortality to the juveniles within the egg. Stimulation was observed at concentrations of 0.001% and 1E-06% BGT, which are lower than the LC_{50} of BGT (0.004%) found in Chapter 2. Chemicals capable of disrupting lipoprotein membranes and affecting membrane permeability may be expected to stimulate hatch (Jones *et al*., 1998). This can occur by triggering different mechanisms to that of hatching in response to stimulation by hatching factors in potato root diffusate, such as increasing membrane permeability and releasing trehalose (Jones *et al*., 1998). Atkinson & Ballantyne (1979) reported that ionophores increased hatching of *G. rostochiensis*, but Clarke & Hennessy (1983) found that the ionophores tested in their study inhibited hatching of *G. rostochiensis*. Ionophores are lipophilic compounds that are able to sequester calcium and pass freely through lipid membranes, controlling permeability of the membrane to Ca^{2+} (Atkinson and Ballantyne, 1979). Another class of chemicals that can induce hatching of *G. rostochiensis* are the glycoalkaloids α-solanine and α-chaconine (Devine *et al*., 1996). Glycoalkaloids interact with lipid membranes, causing disruption to the membrane and a change in permeability, enabling the leakage of trehalose from the egg (Jones *et al*., 1998). A number of studies have observed stimulation of PCN hatch by low concentration of nematicidal compounds. The garlic extract tested by Danquah *et al*. (2011) reduced hatch by 45% at 0.3 ppm, but increased hatch by 8% at 0.1 ppm. Fosthiazate was also found to increase hatch of *G. pallida* at 0.05 – 0.08 ppm, but decreased hatch at concentrations of 0.09 ppm and above (Woods *et al*., 1999). The authors do not discuss the possible mechanisms of hatch stimulation, though Danquah *et al*. (2011) suggests the stimulatory effect of the garlic extract may be caused by unknown components released during extraction of garlic cells. The stimulation of *G. pallida* hatching by BGT could be explored and exploited for use prior to the growing season, by promoting hatch when no

host plants are in the ground. Artificial hatching factors have been previously examined against potato cyst nematodes (Clarke and Hennessy, 1987; Clarke and Shepherd, 1968). Although picrolonic acid hatched over 95% of the total viable eggs of *G. rostochiensis in vitro* (Hominick *et al*., 1985), and increased hatch when used in combination with a trap crop, it did not increase hatch when used alone, possibly due to strong adsorption by the soil (Whitehead, 1977). To the knowledge of the author, there are no commercial products available in the EU that stimulate hatching of PCN in the absence of a host.

3.5 Conclusion

The economically important potato pest *Globodera pallida* is able to survive many years in soil due to its' protective cyst and eggshell, its ability to undergo diapause and the requirements for numerous environmental hatching stimuli. This chapter found that 24 h contact with BGT can significantly affect hatching of *G. pallida* juveniles for up to eight weeks *in vitro*. If BGT can prevent hatching of *G. pallida* juveniles *in situ*, the potential yield loss of potato crops due to PCN damage could be prevented or reduced. The effect of BGT on hatching of *G. pallida* varied significantly over the concentrations tested. To reduce variability in efficacy and to deduce the mode of action of BGT on the *G. pallida* eggshell, it would be beneficial to investigate the effects on *G. pallida* hatching of individual components of BGT and identify any synergistic or antagonistic interaction between components.

Chapter 4 : The effect of components of BGT on hatching of second-stage juveniles of *Globodera pallida*.

4.1 Introduction

The typical process of nematicide discovery and development begins with screening of thousands of active ingredients from various sources, such as biological extracts or chemicals (Haydock et al., 2013). Once active ingredients have been identified, adjuvants may be included in the formulation to aid performance. Active ingredients must be approved by the European Commission, all member states of the European Union (EU) and the European Food Safety Authority (EFSA) by submission of a technical dossier (European Commission, 2018a). This dossier includes physical and chemical properties of the active ingredient as well as results of toxicological and environmental fate studies. The process of approval takes up to three and a half years and must be completed for each active ingredient in a plant protection product (PPP) prior to authorisation (European Commission, 2018a, 2013b). Authorisation of a PPP is made by the same authorities, and takes up to 18 months (European Commission, 2018b, 2013c). The development of a nematicide from initial screen to launch takes eight to ten years and costs approximately €47 million (Haydock *et al*., 2013). The novel formulation BGT has been found to reduce hatching of *G. pallida* juveniles by up to 76%, though it is unknown which of the five components are responsible. To determine the mode of action, and fulfil regulatory requirements (should the product be registered in the European Union as a plant protection product), the active ingredient(s) must be identified.

BGT is a blend of cationic and non-ionic surfactants that are used extensively in a number of industries, including agriculture. Cationic surfactants have the greatest antimicrobial activity of all surfactants (McDonnell, 2009) and are compatible with non-ionic surfactants; they are commonly co-formulated to increase efficacy. BGT contains the quaternary ammonium compounds (QACs) benzalkonium chloride (BAC) and 3-(trimethoxysilyl)propyl dimethyl octadecyl ammonium chloride (Si-QAC). The activity of QACs is focused on lipid membranes (Fisher, 2003); the insertion of the hydrophobic tail into the phospholipid bilayer causes the rearrangement and disruption of the cytoplasmic membrane, and a loss of membrane integrity (Hancock, 1984; Maillard, 2002). BGT also contains polyhexamethylene biguanide (PHMB), a cationic polymer with a biguanide functional group. It is a broad spectrum bioicide of low mammalian toxicity, and displays excellent activity against a wide range of gram positive and gram negative bacteria, fungi and yeasts (SCCS, 2017). Non-ionic surfactants are known to increase the permeability of phospholipid membranes and vesicles by insertion of the hydrophobic portion of the surfactant into the apolar fatty acid domain of phospholipids (Cserháti, 1995). The non-

ionic surfactants found in BGT – alcohol ethoxylate (AEO) and alkyl polyglucoside (APG) – are used as adjuvants in agriculture and are commonly co-formulated due to their synergistic activity (Bergstrom and Johansson, 2005). Alcohol ethoxylates (AEO) are wetting agents and spreading agents, and increase the foliar uptake of herbicides (Hazen, 2000). Alkyl polyglucosides (APG) are highly water-soluble, and are used to solubilize non-ionic wetting agents into solutions containing high concentrations of electrolytes, such as fertilisers (Bergstrom and Johansson, 2005). The combination of AEO and APG reduces surface tension, decreases the critical micelle concentration and increases wetting properties of formulations towards hydrophobic surfaces (Bergstrom and Johansson, 2005).

It was reported in Chapter 3 that 24 h contact with BGT inhibits hatch of *Globodera pallida* juveniles in PRD by 76% for up to eight weeks, though a high level of variation was observed. To determine which of the components of BGT inhibit *G. pallida* hatch and identify any synergistic or antagonistic relationships between components, individual components and mixtures/combinations of individual components were investigated in hatching assays identical to those reported in Chapter 3.

Aim

To evaluate the effects of individual and combined components of BGT on the hatching of *G. pallida* second-stage juveniles from encysted eggs.

Objectives

1. Determine the effects of individual components of BGT on the hatching of *G. pallida* second-stage juveniles from encysted eggs.
2. Determine the effects of combinations of individual components of BGT on the hatching of *G. pallida* second-stage juveniles from encysted eggs.
3. Identify the active ingredient(s) in BGT that affect hatching of *G. pallida*.

Null Hypothesis

Individual or combined components of BGT have no effect on *in vitro* hatching of *G. pallida* second-stage juveniles from encysted eggs.

There is no synergistic action between components of BGT in reducing *in vitro* hatching of *G. pallida* second-stage juveniles from encysted eggs.

4.2 Materials and methods

Potato root diffusate

For methodology on preparation of potato root diffusate (PRD) see Chapter 2.2. The PRD batch used in this study was ‘Estima Feb 2016’.

Preparation of potato cyst nematodes

For methodology on extraction and identification of PCN, see Chapter 2.2. Hatching assays were performed on batches of 25 cysts from populations 'MiBu' and 'VTBN' (Chapter 2.2) simultaneously.

Preparation of test solutions

Individual components of BGT (BAC, Si-QAC, AEO, APG and PHMB) were obtained from Arcis Biotechnology Ltd (Sci-Tech Daresbury, United Kingdom). Individual components were diluted by volume with tap water to the concentrations found in BGT 100% before further dilution to 0.2% (Table 4.1). Combinations of individual components were also made in tap water by volume and diluted to 0.2% (Table 4.2). Tap water was used as a control.

Hatching assay

Batches of 25 *G. pallida* cysts in hatching baskets (see Chapter 3, Figure 3.1) were immersed in test solutions for 24 h prior to hatching. Baskets were transferred to potato root diffusate (PRD) for seven days. Each week, baskets were transferred to fresh PRD and the number of hatched juveniles counted. After eight weeks of hatching, the number of remaining eggs in each batch of cysts was counted and the percentage of juveniles hatched calculated. The hatching assay method used is described in more detail in Chapter 3.2. In this study, two populations, 'MiBu' and 'VTBN' were tested simultaneously. To calculate percentage reduction, the following equation was used:

$$Percentage\ reduction = \frac{Control\ \%\ hatch - treated\ \%\ hatch}{Control\ \%\ hatch}\ x\ 100$$

Statistical analysis

Analysis of variance (ANOVA) and Tukey's ad hoc test were performed using Genstat v18 (VSNI International Ltd., Hemel Hempstead, United Kingdom) to determine significant differences at $P < 0.05$ between populations and treatments. Tests of data distribution were used to support the appropriate choice of statistical analysis, including histogram of residuals, fitted-value plot, normal plot and half-normal plot.

TABLE 4.1 DETAILS OF BGT COMPONENTS AND THEIR CONCENTRATIONS TESTED IN HATCHING ASSAYS DESCRIBED IN CHAPTER 4. *GLOBODERA PALLIDA* CYSTS WERE IMMERSED IN TEST SOLUTIONS FOR 24 H PRIOR TO HATCHING IN POTATO ROOT DIFFUSATE FOR EIGHT WEEKS.

Chemical ID	Chemical name	Category	Concentration (ppm)
BAC	Benzalkonium chloride	Quaternary ammonium compound (Cationic surfactant)	312.5
Si-QAC	3-(trimethoxysilyl)propyl dimethyl octadecyl ammonium chloride	Quaternary ammonium compound (Cationic surfactant)	250
AEO	Alcohol ethoxylate	Non-ionic surfactant	125
APG	Alkyl polyglucoside	Non-ionic surfactant	250
PHMB	Polyhexamethyl biguanide	Polymeric biguanide (Cationic polymer)	125

TABLE 4.2 A LIST OF COMBINATIONS OF COMPONENTS TESTED AGAINST *G. PALLIDA* IN THE HATCHING ASSAYS DESCRIBED IN CHAPTER 4. *GLOBODERA PALLIDA* CYSTS WERE IMMERSED IN TEST SOLUTIONS FOR 24 H PRIOR TO HATCHING IN POTATO ROOT DIFFUSATE FOR EIGHT WEEKS.

#	Cationic surfactant	Non-ionic surfactant
1	None (Control)	None (Control)
2	Si-QAC	None
3	Si-QAC	APG
4	Si-QAC	AEO
5	Si-QAC	APG and AEO
6	PHMB	None
7	PHMB	APG
8	PHMB	AEO
9	PHMB	APG and AEO
10	BAC	None
11	BAC	APG
12	BAC	AEO
13	BAC	APG and AEO
14	Si-QAC, PHMB and BAC	APG and AEO

4.3 Results

Cysts of two populations of *G. pallida* were treated with individual components of BGT (Table 4.1) for 24 h prior to eight weeks hatching in potato root diffusate. Each week, the number of hatched juveniles were counted and the remaining eggs at week eight were counted to determine the percentage hatch (Figure 4.1). Two-way ANOVA found a significant difference in the percentage of juveniles hatching from encysted eggs between the two populations, 'MiBu' and 'VTBN' ($F_{(1,39)}$=43.2, P<0.001). A significantly higher proportion of juveniles from the population 'MiBu' hatched when compared to those from the population 'VTBN', with the untreated cysts (control) hatching at 52% and 35% respectively.

Individual components

Figure 4.1 shows the percentage hatch of two populations of *G. pallida* after 24 h treatment with individual components of BGT. To find the significant differences between treatments, two-way ANOVA was performed with Tukey's ad hoc test on each population. In both populations, encysted eggs treated with BAC and BGT had a significantly lower hatch than the control (Figure 4.1). Benzalkonium chloride reduced the hatch of the population 'MiBu' ($F_{(6,18)}$=3.58, P=0.016) by 39% and the population 'VTBN' ($F_{(6,18)}$=10.7, P<0.01) by 69%. BGT reduced the hatch of the population 'MiBu' by 42% and the population 'VTBN' by 70%. No other treatments were significantly different from the control.

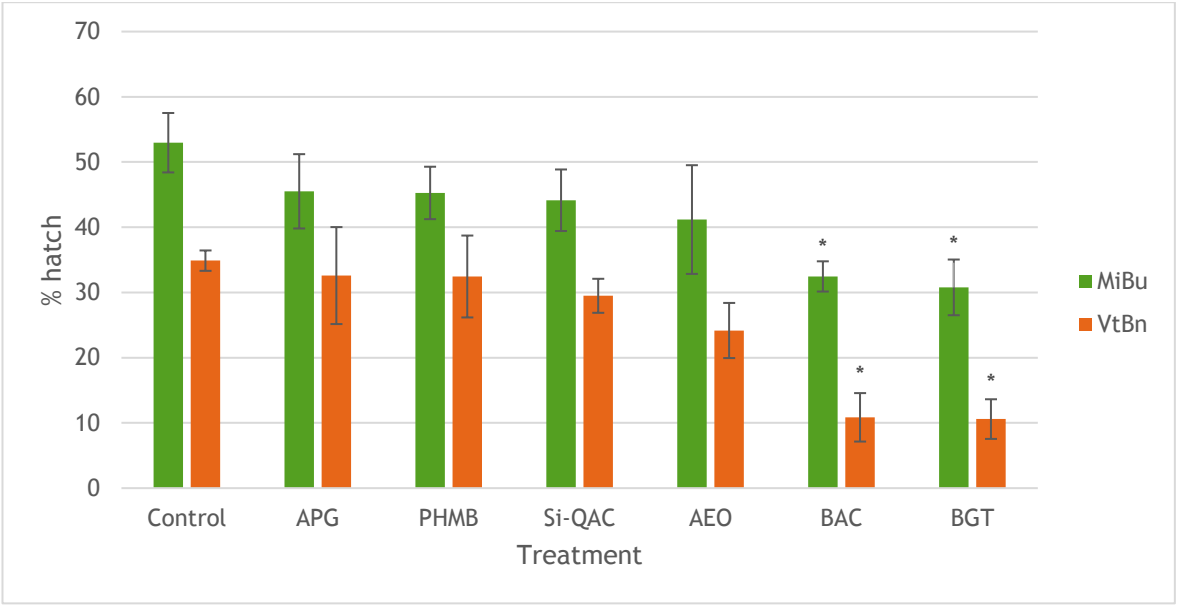


FIGURE 4.1 RESULTS OF HATCHING ASSAYS PERFORMED ON TWO DIFFERENT POPULATIONS OF *GLOBODERA PALLIDA*. PERCENTAGE HATCH OF JUVENILES FROM ENCYSTED EGGS TREATED WITH INDIVIDUAL COMPONENTS OF BGT FOR 24 H PRIOR TO EIGHT WEEKS HATCHING IN POTATO ROOT DIFFUSATE. '*' DENOTES TREATMENTS WITH A SIGNIFICANTLY LOWER HATCH THAN THAT OF THE CONTROL (TAP WATER). ERROR BARS SHOW THE STANDARD ERROR OF THE MEAN. THE COEFFICIENT OF VARIANCE WAS 19.7% FOR THE POPULATION MIBU AND 25.2% FOR THE POPULATION VTBN.

Combinations of components

Figure 4.2 shows the mean percentage hatch for two population of *G. pallida* after 24 h treatment with individual and combined components of BGT. To determine any significant differences between treatments, two-way ANOVA was performed with Tukey's ad hoc test on each population. Only two treatments significantly ($F_{(15,45)}$=2.51, P=0.009) reduced the percentage hatch of the population 'MiBu' from the control; BAC + APG (46% reduction) and BGT (42% reduction). Hatch of the population 'VTBN' was significantly ($F_{(15,45)}$=5.36, P=0.001) reduced by BAC (69% reduction), BAC + APG (69% reduction), BAC + AEO (62% reduction) and BGT (70% reduction).

Analysis of variance and Tukey's ad hoc test was also performed on the combined data (with the population as a blocking factor) to produce Figure 4.3. Treatment with Si-QAC did not significantly lower the hatching of *G. pallida* juveniles. The addition of one or more non-ionic surfactants to Si-QAC did not increase the efficacy of Si-QAC as a hatch inhibitor. Treatment with PHMB did not significantly lower hatching of *G. pallida* juveniles. The addition of APG reduced hatch to 32% but was not significantly lower than PHMB alone (29% hatch) or the untreated control (44% hatch). Addition of AEO to PHMB reduced hatch to 30%, which was significantly lower than the untreated control and PHMB alone. The combination of PHMB, APG and AEO reduced hatch by 37% from the untreated hatch, though this was not statistically significant from the control.

Treatment with BAC reduced hatching of *G. pallida* juveniles by 51% compared to the untreated control. The addition of APG to BAC increased efficacy to a 55% reduction. Addition of AEO to BAC lowered efficacy to a 47% reduction. Combining both non-ionic surfactants with BAC further lowered efficacy to 37% reduction, though there was no significant difference between treatments, and all five treatments (including BGT) containing BAC hatched significantly lower than the untreated control.

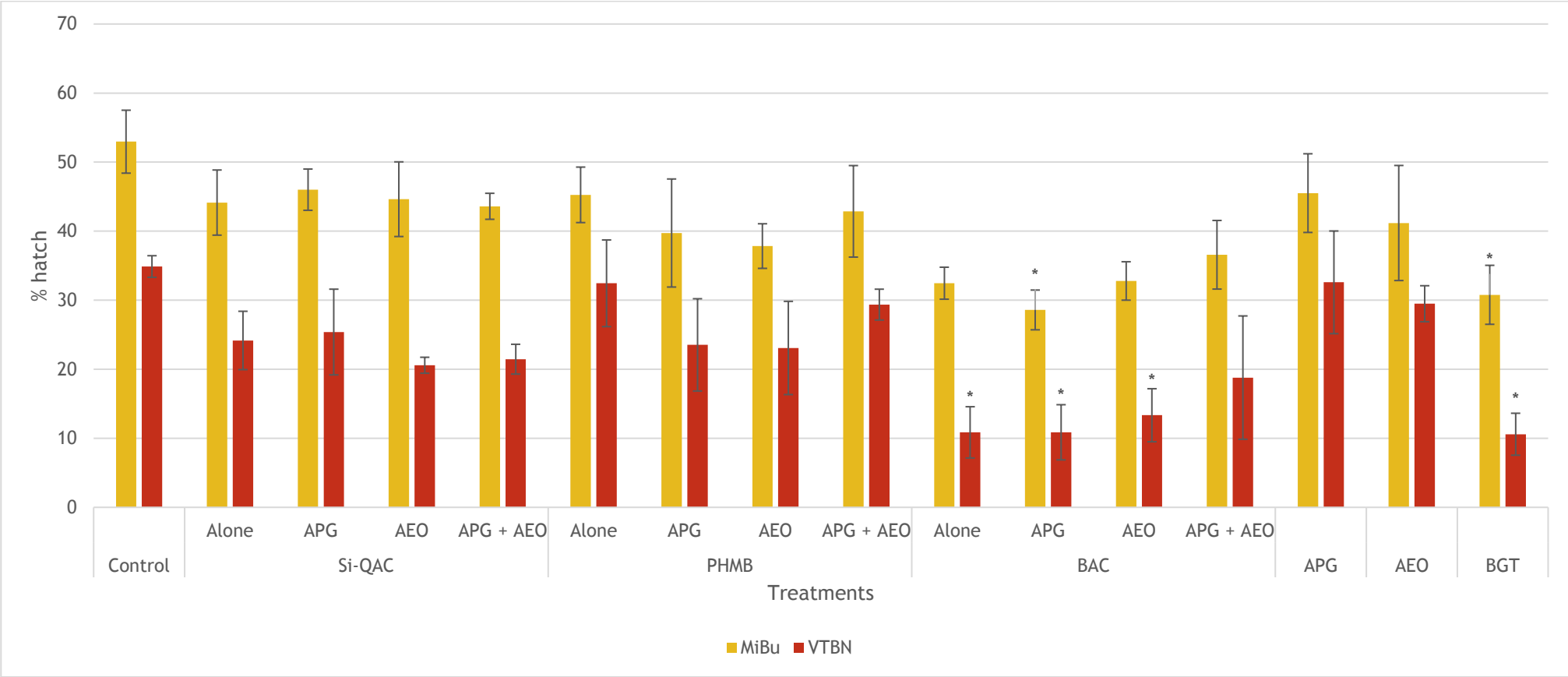


FIGURE 4.2 RESULTS OF HATCHING ASSAYS PERFORMED ON TWO POPULATIONS OF *GLOBODERA PALLIDA*. PERCENTAGE HATCH OF JUVENILES FROM ENCYSTED EGGS TREATED WITH COMBINATIONS OF THE COMPONENTS OF BGT FOR 24 H PRIOR TO EIGHT WEEKS HATCHING IN POTATO ROOT DIFFUSATE. '*' DENOTES TREATMENTS THAT ARE SIGNIFICANTLY LOWER THAN THE UNTREATED CONTROL (TAP WATER). ERROR BARS SHOW THE STANDARD ERROR OF MEAN. THE COEFFICIENT OF VARIANCE WAS 20.8% FOR THE POPULATION MIBU AND 30.9% FOR THE POPULATION VTBN.

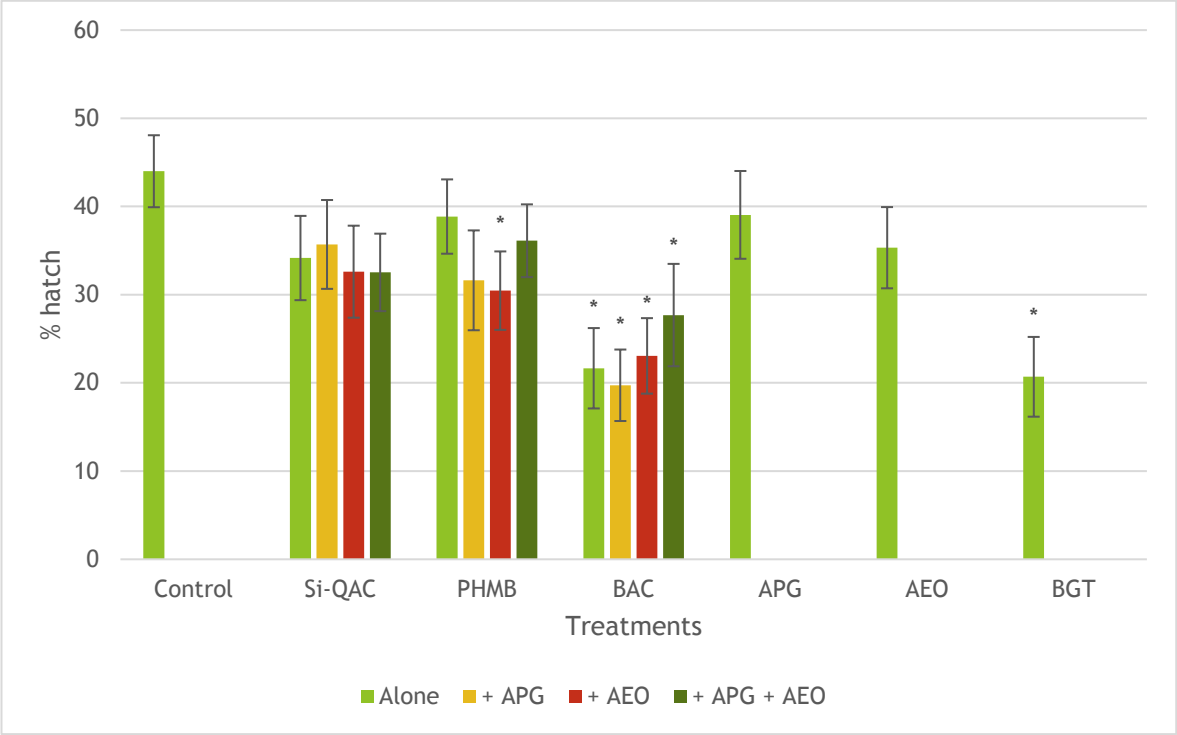


FIGURE 4.3 COMBINED RESULTS OF HATCHING ASSAYS PERFORMED ON TWO POPULATIONS OF *GLOBODERA PALLIDA*. PERCENTAGE HATCH OF JUVENILES FROM ENCYSTED EGGS TREATED WITH COMBINATIONS OF THE COMPONENTS OF BGT FOR 24 H PRIOR TO EIGHT WEEKS HATCHING IN POTATO ROOT DIFFUSATE. '*' DENOTES TREATMENTS THAT ARE SIGNIFICANTLY LOWER THAN THE UNTREATED CONTROL (TAP WATER). ERROR BARS SHOW THE STANDARD ERROR OF MEAN. THE COEFFICIENT OF VARIANCE WAS 23.6%.

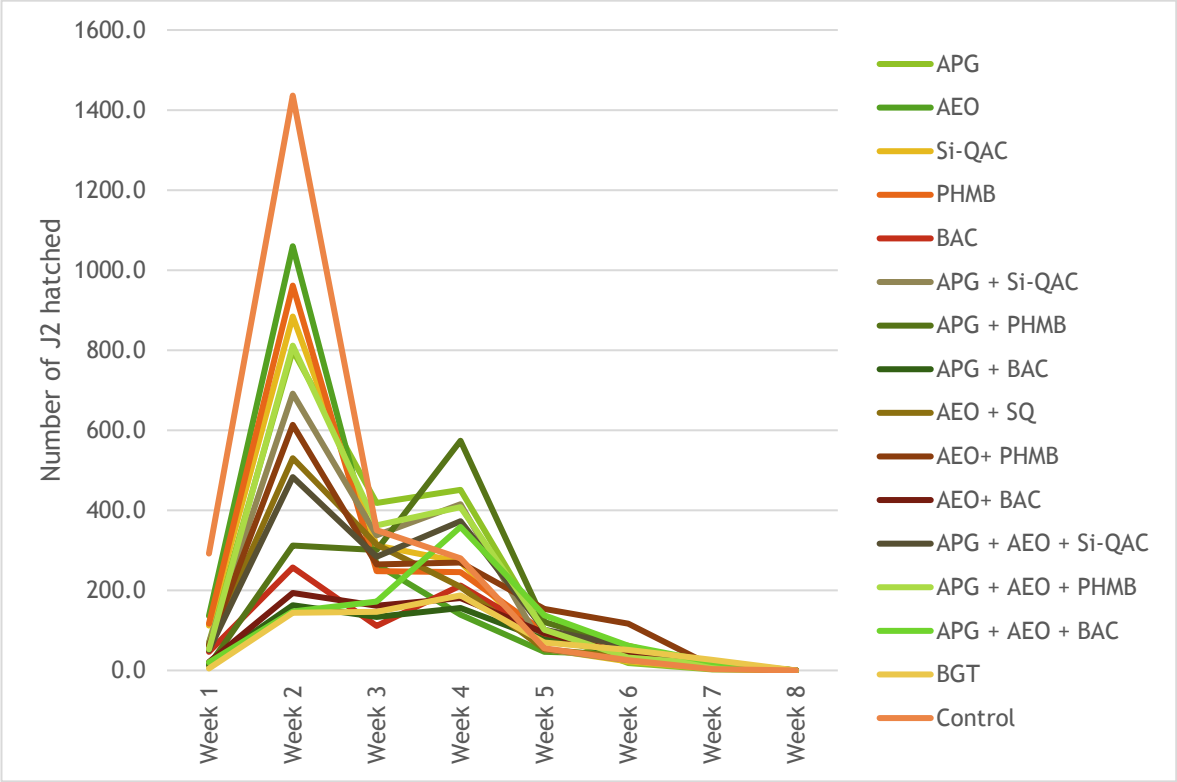


FIGURE 4.4 THE NUMBER OF SECOND-STAGE JUVENILES HATCHING FROM *GLOBODERA PALLIDA* CYSTS OVER EIGHT WEEKS IN POTATO ROOT DIFFUSATE AFTER 24 H EXPOSURE TO INDIVIDUAL COMPONENTS AND COMBINATIONS OF COMPONENTS OF BGT. RESULTS SHOWN ARE OF POPULATION VTBN.

4.4 Discussion

Plant protection products in Europe are authorised by the European Commisision, member states of the EU and EFSA, and all active ingredients within the formulation must also be approved by the same authorities (European Commission, 2018a, 2013b). BGT is a blend of five components; this chapter investigated its components individually and in various combinations to identify the active ingredients in the formulation and to determine any synergistic or antagonistic action between components. Identification of the active ingredients in the formulation is vital in order to understand the mode of action and successfully register a plant protection product. In this study, BAC was found to be the only active ingredient against *G. pallida* in the formulation BGT.

Hatching from cysts of the population 'VTBN' was significantly lower than from cysts of the population 'MiBu'. This was observed previously in Chapter 3, where the control hatch of the population 'MiBu' was 37% and the population 'VTBN' was 26% in PRD 'Jersey Dec 2015' (see Chapter 3 Table 1, experiments 3 and 4). In this study, the hatch of untreated cysts was 52% for the population 'MiBu' and 35% for the population 'VTBN'; higher than the untreated cysts in experiments 3 and 4 in Chapter 3. The PRD used in this study was 'Estima Feb 2016'. Is it likely that the cultivar used for obtaining PRD is responsible for the difference in hatching of untreated cysts between the two studies. Experiments 1 and 2 of Chapter 3 also used PRD derived from Estima and the population 'MiBu', as in this study. The hatch of untreated 'MiBu' cysts in experiment 1 of Chapter 3 was 34%, whilst the hatch of untreated 'MiBu' cysts in experiment of Chapter 3 was 73%. These experiments were performed with different batches of PRD (see Chapter 3 Table 1) and demonstrate the variation that can occur when hatching the same population in different batches of PRD. Variation in hatching between populations of PCN and with different sources of PRD has been examined by several authors (Byrne *et al*., 2001; Rawsthorne and Brodie, 1986; Turner *et al*., 2009). Turner *et al*. (2009) found that different pathotypes of *G. pallida* hatching in the same batch of PRD (cv. Desiree at 50%) can vary from 30% to 86%. Turner *et al*. (2009) also found that hatching varies within a population when cysts are exposed to PRD from different cultivars and clones (between 26% and 92%) and according to dilution of the PRD (between 53% and 2%). Rawsthorne & Brodie (1986) examined PRD collected over several weeks of growth and found that *Globodera rostochiensis* hatching peaked in PRD collected three weeks after emergence. The authors also found that hatching activity of PRD is correlated with root weight and length. *Globodera pallida* and *G. rostochiensis* respond to different hatching factors released at various points through the host plant growing season (Byrne *et al*., 2001). Byrne *et al*. (2001) found that *G. pallida* (Pa2/3) hatched preferentially in leachate obtained from four to seven day old plants, whereas *G. rostochiensis* (Ro1) hatched preferentially in leachate

obtained in plants over thirty eight days old, five and a half weeks after planting, similar to the results of Rawsthorne & Brodie (1986). Byrne *et al*. (2001) also found that hatching of *G. pallida* pathotypes Pa1 and Pa2/3 were different, with Pa1 falling in between the hatch responses of *G. pallida* Pa2/3 and *G. rostochiensis* Ro1. The variation in response to hatching stimuli between the two PCN species and between pathotypes of *G. pallida* may infer a variation in response to other chemicals, such as nematicides.

Though there are many studies that compare the hatching of different populations of PCN to PRD, there are none at the time of writing that look at the difference in response to nematicidal treatments *in vitro*. Differences seen in results between field trials at similar sites may be due to different populations of PCN, though there are several other factors that may affect the multiplication rate of *G. pallida in situ*, such as soil moisture and temperature (Robinson *et al*., 1987, 1984). Norshie *et al*. (2016) found that in two sites with sandy clay loam soils, similar soil pH and initial *G. pallida* population density, Fluensulfone G (5.05 kg ha^{-1}) significantly decreased the multiplication of *G. pallida* in Woodcote in 2010 but not Howle in 2011. Conversely, Fluensulfone EC (4.05kg ha^{-1}) was more effective at reducing multiplication of *G. pallida* in the Howle 2001 trial than at Woodcote in 2010. This variation in efficacy could have been caused by different populations of *G. pallida* at each site, or due to other unknown, uncontrolled variables.

By calculating the percentage reduction for each treatment (Chapter 4.2) the results from both populations can be compared. Of the five individual components tested, only BAC significantly reduced hatching, in either population. Benzalkonium chloride (BAC) reduced hatching in the population ‘MiBu’ from 52% (control) to 32%, a 39% reduction. In the population ‘VTBN’, hatching was reduced from 35% (control) to 11%, a 69% reduction. None of the treatments affected the hatching profile of the *G. pallida* juveniles, all treatments had peak hatch within the first three to four weeks of the hatching assay (Figure 4.4), as seen previously in Chapter 3 Figure 3.2. This suggests that the action of BGT components on *G. pallida* eggs is irreversible by the presence of PRD for at least eight weeks. Three of the treatments tested were more effective at reducing hatch of the population ‘VTBN’ than the population ‘MiBu’; BAC, AEO and BGT. Alcohol ethoxylate (AEO) reduced the population ‘MiBu’ by 22% and the population ‘VTBN’ by 31%, and BGT reduced the population ‘MiBu’ by 42% and the population ‘VTBN’ by 70%. The remaining treatments, PHMB and APG were more effective for the population ‘MiBu’ than the population ‘VTBN’, though the standard error of the mean for the population ‘VTBN’ treated with PHMB and APG is higher than other treatments for the same population (Figure 4.1), at 6.277 for PHMB and 7.434 for APG, compared to 1.563 for the control. When taking this variability into account and observing Figure 4.1, the trend in both populations appears similar, with the population ‘VTBN’ more susceptible to all

treatments, and overall less viable than the population 'MiBu'. The viability of eggs remaining in cysts after the hatching assay was not examined. Hatching assays were performed in preference to viability assays such as staining with Meldola's blue (Ogiga and Estey, 1974) or trehalose measurement (van den Elsen *et al*., 2012) as hatching is more representative of the level of infection a host plant would be subjected to. Though hatching assays are subject to variation due to the variable nature of PRD, Kroese *et al*. (2011) found that estimates of viability made by Meldola's stain and hatching assays were similar on cysts extracted from the field. By standardising results as 'percentage reduction from control', separate experiments using different PCN populations and PRD can be compared.

Both populations tested were significantly affected by BAC. Cationic surfactants such as BAC are more active against microorganisms than non-ionic surfactants (McDonnell, 2009). The increased activity against lipid membranes would suggest that the cationic surfactants in BGT are more effective at reducing hatching of *G. pallida*, however the results in Figures 4.1 and 4.2 demonstrate that this is not necessarily the case. The cationic compounds reduced hatch by 12 – 51%, and the non-ionic compounds by 12 – 20%. When tested against bacteria, PHMB is the most effective of the cationic compounds in BGT, with a minimum biocidal concentration (MBC) of <0.05 mg L^{-1} for *Staphyloccocus aureus* and *Escherichia coli* (Rembe *et al*., 2016). The MBC of BAC for the same two organisms was almost one hundred times higher, at 45 mg L^{-1} (Fazlara and Ekhtelat, 2012), and although the MBC of Si-QAC for *S. aureus* was 3.9 mg L^{-1}, its MBC for *E. coli* was 62.5 mg L^{-1} due to the additional polysaccharide layer on the cell wall of gram negative organisms such as *E. coli* (Li *et al*., 2015). The high toxicity of PHMB observed against microorganisms was not seen in this experiment. Treatment with PHMB reduced the percentage hatch of *G. pallida* by 12% and was less effective than all other components except APG (12% reduction). Though the biocidal mode of action of PHMB is widely reported to be interaction between PHMB and cytoplasmic membrane (Ikeda *et al*., 1984), Chindera *et al.* (2016) found that PHMB did not cause mortality in *E. coli* by increasing membrane permeability, rather it selectively bound to and condensed bacterial chromosomes and was excluded by mammalian nuclei. This recent insight into the mode of action of PHMB would account for the lower efficacy of PHMB against *G. pallida*. Polyhexamethylene biguanide (PHMB) is included in the formulation at the lowest concentration of the cationic compounds (Table 4.1) and its efficacy against *G. pallida* may be increased at a higher concentration of PHMB, though it has recently been listed as a potential candidate for substitution by the ECHA after Creppy *et al*. (2014) found it to be a potential carcinogen. As the compound had little effect on hatch of *G. pallida*, its removal from the formulation should be considered.

Treatment with Si-QAC did not significantly reduce hatch of *G. pallida* juveniles. It reduced hatch by 22% and was more effective than PHMB and the non-ionic compounds but less effective than BAC or BGT. The addition of one or more non-ionic surfactants did not improve the efficacy of Si-QAC in reducing hatch. The silane groups of Si-QAC bind covalently to hydroxylated and hydrophilic surfaces such as silt and sand (Vasilev *et al*., 2009), which may confer some residual activity to the formulation. Daniels & Hourani (2009) found that addition of a 1-1.5% solution of Si-QAC to silty clay loam soil resulted in a reduction in soil erosion and an increase in strength and swell potential. Though Si-QAC did not have a significant effect on hatching of *G. pallida*, it may have beneficial effect on the soil, particularly in soil with a high silt or clay content. The potential benefits of Si-QAC to soil and its effect on longevity of the formulation in soil will be investigated in soil media pot trials.

Benzalkonium chloride (BAC) was the most effective component at reducing hatch and the only single component to reduce hatch significantly below that of the control. Benzalkonium chloride is an effective broad-spectrum antimicrobial and has previously been found to have toxicity against the nematode *Caenorhabditis elegans* (Hoss *et al*., 2012), though the mechanism of toxicity was not discussed. There was no significant difference between the efficacy of BAC and BGT, where hatch was reduced by 69% and 70% respectively. This suggests that BAC is the sole active ingredient responsible for the reduction of hatch of *G. pallida* within the formulation BGT, and that the other components do not have any synergistic or additive action.

The non-ionic surfactants AEO and APG had little effect on hatching of *G. pallida*, reducing hatch by 20% and 12% respectively. The addition of either or both non-ionic surfactants to the cationic compounds did not increase the efficacy of the cationic compounds in reducing hatch of *G. pallida*. Although the percentage hatch from cysts treated with PHMB with AEO was statistically lower than the control, there was no significant difference between PHMB alone and PHMB with any combination of non-ionic surfactants. Alcohol ethoxylate (AEO) and APG are commonly used as adjuvants to help spray homogeneity, stability of active ingredients and as wetting agents (Bergstrom and Johansson, 2005; Hazen, 2000). Their inclusion in the formulation is as adjuvants to increase stability and distribution of the formulation in the soil. Their benefit to the formulation should therefore be investigated within soil media pot experiments.

Chapter 2 reported that BGT disrupts the lipid membranes inside the J2s of *G. pallida*. The egg shells of *G. pallida* consist of a lipoprotein permeability barrier inner layer, a middle chitin layer and an outer vitelline layer (Jones *et al*., 1998; Turner *et al*., 2009). It is proposed that BGT and its membrane-active components are able to increase egg shell permeability and cause mortality of the juveniles within. Chemicals capable of disrupting

lipoprotein membranes and affecting membrane permeability may be expected to stimulate hatching (Jones *et al*., 1998). BGT was found to stimulate hatch of *G. pallida* at concentrations of 0.001% and 1E-06% in Chapter 3, possibly due to increasing lipoprotein membrane permeability at a sublethal concentration. This study tested components at the equivalent concentration found in 0.2% BGT. None of the components caused stimulation of hatch, thus any disruption of the egg shell allowed mortality of the juvenile within, or prevented hatching by another mechanism. To confirm that in-egg juvenile mortality is the mode of action of BGT, microscopy on treated eggs should be performed, to compare with microscopy of treated hatched *G. pallida* J2s (Chapter 2.3).

The hatching assays were based on 24 h exposure to BGT and its components followed by eight weeks in PRD, as the longevity of BGT in soil is currently unknown. No other studies on hatch inhibitors of *G. pallida* have been found with a contact time as short as 24 h. Twomey *et al*. (2000) treated *G. pallida* cysts for five weeks in DiTera prior to two weeks in PRD, and found that the inhibition of hatch by >80% caused by the chemical was irreversible by exposure to PRD. Woods *et al*. (1999) treated cysts with Fosthiazate for one week, and found that although hatching was completely suppressed for four weeks, it occurred in week five, prompting a second week treatment. The experiment was ended at week seven therefore it is unknown how long the hatch suppression would last after two treatments. Danquah *et al*. (2011) found that the efficacy of salicylaldehyde was significantly improved by increasing contact time from two to eight weeks, reducing hatch in PRD after treatment from 37% after two weeks treatment to 29% after eight weeks treatment. This increase in efficacy was not seen in the garlic extract tested nor in the mixture G8014S. Hatching studies with extended exposure to BGT should be performed to determine the effect of contact time on the efficacy of BGT. Potato root diffusate (PRD) should also be included in the treatments as it will be found *in situ* following treatment of potatoes with BGT, and may interact with the formulation.

4.5 Conclusion

All active ingredients within a plant protection product must be approved by the European Commission, member states of the EU and EFSA prior to registration of the product itself. Identification of active ingredients is vital to understand the mode of action of a plant protection product. This chapter found that the cationic surfactant Benzalkonium chloride (BAC) was the only individual component in BGT that significantly reduced hatch of *G. pallida* and was comparable in efficacy to BGT. The addition of non-ionic surfactants to BAC did not improve efficacy against *G. pallida*, however these components are included for their stability and wetting properties, and should be assessed as such. Similarly, Si-QAC did not significantly reduce hatching of *G. pallida*, but may improve soil structure or provide longevity to the formulation. Conversely, PHMB has not been used previously in

agricultural chemicals, and its behaviour in and action on the soil is unknown. To further evaluate the efficacy of BGT, investigations should focus on glasshouse studies in soil media. The effect of BGT and its constituents on hatching of *G. pallida* has so far only been tested by a discrete contact time of 24 h followed by exposure to PRD for eight weeks. To design an efficient treatment schedule for use *in situ*, the effects of timing and duration of contact between BGT and *G. pallida* cysts should be explored.

Chapter 5 : The effect of treatment timing and duration on hatching of *Globodera pallida*

5.1 Introduction

Infective second-stage juveniles of *Globodera pallida* develop within eggs found in the cutinized body wall (known as cyst) of fertilised females (Turner and Evans, 1998; Turner and Rowe, 2006). The cysts protect the eggs from physical, biological and chemical stresses, but are not watertight, and allow moisture through gaps in the cyst wall, predominantly at the neck and vulva openings (Masler and Perry, 2018). These opening not only allow moisture in, but serve as exit routes for hatched juveniles (Moens *et al*., 2018). Although approximately 20% of *G. pallida* juveniles will hatch spontaneously in water, hatching generally requires stimulation by chemicals released from host plant roots, known as potato root diffusate (PRD) (Hominick *et al*., 1985; Rawsthorne and Brodie, 1986). The hatching mechanism of *G. pallida* is stimulated by as little as 5 min contact with PRD, which is found in the soil within a week of planting potatoes (Forrest and Perry, 1980). In *in vitro* hatching assays, the majority of juveniles hatch within the first three weeks of the experiment (as seen in Chapter 3), though these are commonly performed using a single batch of PRD collected up to five weeks after plant emergence (Danquah *et al*., 2011; Ibrahim and Haydock, 1999; Kiezebrink, 2009; Twomey *et al*., 2000; Woods *et al*., 1999). Rawsthorne and Brodie (1986) showed that the composition of PRD changes throughout the growing season, with a peak in its ability to stimulate hatch at 3 weeks after emergence. The relationship between root weight and the ability of PRD to stimulate PCN hatch was linear until 3 weeks, but not after 3 weeks (Rawsthorne and Brodie, 1986). Hatching factors found in PRD are not only species selective but may also be pathotype- and population-selective (Byrne *et al*., 2001). Byrne *et al*. (2001) found that *G. pallida* hatching was affected by the number of days after plant emergence PRD was extracted. They collected PRD from host plants 0 – 56 days after emergence and found initial hatching peaks at 8 – 10 days for a population of *G. pallida* Pa2/3 and 11 – 14 days for a population *G. pallida* Pa1, though only one population of each pathotype was tested, and little research into the differences in hatching is available to date. Turner *et al*. (2009) found no significant differences in the total percentage hatch of six populations of Pa2, Pa2/3 or Pa3 hatched in the same batch of PRD, though one of two Pa1 populations tested had a significantly lower hatch than the Pa2/3 populations tested.

Studies evaluating the efficacy of active ingredients to reduce hatching of PCN have combined the test chemical with PRD for up to eight weeks to determine the effects on hatching of *G. pallida*. Twomey *et al*. (2000) treated *G. pallida* cysts with DiTera for five weeks prior to hatching in PRD for two weeks (reducing hatch by over 70%), and Woods

et al. (1999) found that one week treatment with Fosthiazate completely suppressed hatch of *G. pallida* in PRD for four weeks. Danquah *et al*. (2011) found that increasing the contact time between *G. pallida* and salicylaldehyde at 24 µl L^{-1} significantly improved the efficacy of the chemical, reducing hatch from the control of 50% to 37% after two weeks treatment and 29% after eight weeks treatments. This effect, however, was not replicated when testing the formulation G8014S, which contains salicylaldehyde (Danquah *et al*., 2011). Ibrahim and Haydock (1999) found that hatching was completely inhibited when *G. pallida* cysts were incubated in PRD with cadusafos for one week, but hatching resumed soon after removing the chemical. Re-incubation with cadusafos for a week at weeks 6 and 8 completely inhibited hatch in those weeks, though hatching again resumed when the chemical was removed in weeks 7 and 9. The formulation BGT has been found to irreversibly reduce the percentage of juveniles hatching from cysts (Chapters 3 and 4). The hatching studies reported in Chapters 3 and 4 were conducted by immersing cysts in BGT for 24 h, then moving to fresh PRD weekly to stimulate hatching over eight weeks.

The longevity of BGT in soil is currently unknown, though it is likely to remain in the soil longer than 24 h without significant rainfall or irrigation, due to adhesion to silt particles. Longevity of the formulation may be increased by frequent application, for example via drip tape. Treatment with BGT at 0.2% and above for 24 h significantly reduced hatching of *G. pallida* juveniles by up to 76%. Chapter 4 investigated the individual components of BGT, and using the same hatching assays as Chapter 3, found that the cationic surfactant benzalkonium chloride (BAC) was the only individual component that significantly reduced hatching of *G. pallida*, and was comparable in efficacy to BGT. This chapter examines not only the formulation BGT, but also a second formulation, UBA. The formulation UBA consists of BAC and the adjuvants alcohol ethoxylate (AEO) and alkyl polyglucoside (APG). UBA will also be tested with the addition of polyhexamethyl biguanide (PHMB) and 3-(trimethoxysilyl)propyl dimethyl octadecyl ammonium chloride (Si-QAC) to determine whether these two components provide any value to the formulation.

Whilst the results of these hatching studies have suggested that the formulations may reduce hatching of G. pallida in the field, glasshouse soil media pot studies must be performed to confirm this and optimise the treatment schedule. To design glasshouse experiments, additional information is required on the contact time and timing of treatment, as well as the impact of PRD on the efficacy of the formulation. This chapter seeks to investigate the effect of increasing contact time between the formulations and G. pallida, as well as the timing of exposure to chemicals. This chapter will also include PRD in treatments, to better represent the conditions in situ and discover any interaction between PRD and the test formulations.

In order to improve the design of treatment schedules for glasshouse experiments and optimise the efficacy of the formulations for reducing hatching of G. pallida, it is important to gain a greater understanding of the mode of action. Microscopy performed on second-stage G. pallida juveniles in Chapter 2 demonstrated that BGT disrupts internal lipid membranes and destroys the lipid energy stores within, rendering the juveniles unable to infect host plants. BGT may also disrupt and increase permeability of the inner lipid membrane of the G. pallida eggshell and cause in-egg mortality of the juvenile within using the same mechanism as on hatched juveniles. To confirm this proposed mode of action, microscopy will be performed on eggs exposed to BAC, UBA and BGT.

Aim

To evaluate the effect of increasing contact time and timing of exposure of *G. pallida* to BGT and/or UBA to design an application schedule for soil growth media pot studies.

Objective

1. Determine optimal contact time for the formulations under investigation to reduce hatching of *G. pallida.*
2. Investigate the effect of the formulations under investigation on hatching of *G. pallida* in the presence of PRD.
3. Investigate the effect of application timing on efficacy of the formulations under investigation in reducing hatching of *G. pallida.*
4. Investigate the physical effects of the formulations under investigation on unhatched *G. pallida* eggs.

Null Hypothesis

1. Increasing contact time between the formulations under investigation and *G. pallida* will not affect the hatching of *G. pallida*.
2. The timing of exposure between the formulations under investigation and *G. pallida* will not affect the hatching of *G. pallida*.
3. The formulations under investigation will not have any physical effect on unhatched *G. pallida* juveniles.

5.2 Materials and methods

For methodology on preparation of potato root diffusate (PRD) and extraction and identification of PCN, see Chapter 2.2. The standard hatching assay method used is described in Chapter 3.2.

Potato cyst nematodes

Potato cyst nematodes (PCN) were extracted using the method in Chapter 2.2. The population 'MiBu' was used for all hatching experiments in this chapter. The population 'Shrop' was used for microscopy.

Potato root diffusate

Potato root diffusate (PRD) was prepared using the method in Chapter 2.2. Experiment 1 used the PRD batch 'VTKDL'. Experiments 2 and 3 used the PRD batch 'VT170321'. PRD was diluted to 25% for hatching assays.

Preparation of test solutions

Test solutions of UBA, UBA + PHMB, UBA + Si-QAC and BGT were obtained from Arcis Biotechnology Ltd (Sci-Tech Daresbury, United Kingdom). Tap water was used as a control. The solutions tested in each experiment are listed in Table 5.1 (Experiment 1), Table 5.2 (Experiment 2) and Table 5.3 (Experiment 3).

Experiment 1

BGT batch 161031A, UBA batch 161031B, UBA + PHMB batch 161031C and UBA + Si-QAC batch 161031D were diluted with tap water to 0.2% for 24 h treatments. For continuous treatment (hatching in PRD + treatment), PRD 'VTKDL' was mixed with each treatment to give a final concentration of 25% PRD and 0.1% treatment.

Experiments 2 + 3

UBA batch 161031B was diluted with tap water to 0.2%. For PRD + UBA, PRD 'VT170321' and UBA 161031B were combined to give a final concentration of 25% PRD and 0.2% UBA.

Hatching assays

Experiment 1 (Table 5.1)

Batches of 25 *G. pallida* cysts were immersed in 1.5 ml UBA, UBA + PHMB, UBA + Si-QAC, BGT or water (control) for 24 h at 15°C in darkness in LMS cooled incubator (LMS Technology Ltd, Warrington, United Kingdom). After 24 h treatment, cysts from each treatment were rinsed in water six times by immersing in 1.5 ml fresh tap water for 1 minute under agitation. The remaining cysts were transferred immediately into hatching solution. After rinsing, cysts were transferred into 1.5 ml hatching solution. Cysts in hatching solution were incubated at 15°C in darkness in an LMS cooled incubator, and each week transferred to fresh hatching solution. The number of hatched juveniles was counted each week and the number of remaining eggs were counted at the end of the eight-week hatching period, as described in Chapter 2.2. The percentage hatch and percentage reduction in hatch were calculated using the following equations;

$$Percentage\ hatch = \frac{hatched\ juveniles}{hatched\ \ juveniles + remaining\ eggs} x\ 100$$

$$Percentage\ reduction\ from\ control = \frac{control\ \%\ hatch - treated\ \%\ hatch}{control\ \%\ hatch}\ x\ 100$$

TABLE 5.1 A LIST OF TREATMENTS TESTED IN EXPERIMENT 1. *GLOBODERA PALLIDA* CYSTS WERE IMMERSED IN ONE OF FOUR TEST PRODUCTS OR WATER (CONTROL) FOR 24 H, THEN RINSED OR TRANSFERRED DIRECTLY TO EITHER PRD OR PRD + UBA FOR HATCHING.

Treatment No.	24 h treatment	Rinse	Hatching solution
1	Water	-	PRD
2	UBA	Rinse	PRD
3	UBA	No rinse	PRD
4	UBA	No rinse	PRD + UBA
5	UBA + PHMB	Rinse	PRD
6	UBA + PHMB	No rinse	PRD
7	UBA + PHMB	No rinse	PRD + UBA + PHMB
8	UBA + Si-QAC	Rinse	PRD
9	UBA + Si-QAC	No rinse	PRD
10	UBA + Si-QAC	No rinse	PRD + UBA + SiQAC
11	BGT	Rinse	PRD
12	BGT	No rinse	PRD
13	BGT	No rinse	PRD + BGT

Experiment 2 (Table 5.2)

Batches of 25 *G. pallida* cysts were immersed in PRD, UBA or PRD + UBA for 24 h at 15°C in darkness in LMS cooled incubator. Cysts were then transferred to either PRD or PRD + UBA for hatching. Cysts in hatching solution were incubated at 15°C in darkness in LEC incubator, and each week transferred to fresh hatching solution. The number of hatched juveniles was counted each week and the number of remaining eggs were counted at the end of the eight-week hatching period, as described in Chapter 2.2. The percentage hatch and percentage reduction in hatch were calculated using same equations as described in Experiment 1 (above).

TABLE 5.2 A LIST OF TREATMENTS TESTED IN EXPERIMENT 2. *GLOBODERA PALLIDA* CYSTS WERE IMMERSED IN PRD, UBA OR PRD + UBA FOR 24 H PRIOR TO HATCHING IN EITHER PRD OR PRD + UBA.

Treatment No.	24 h treatment	Hatching solution
1	PRD	PRD
2	PRD	PRD + UBA
3	UBA	PRD
4	UBA	PRD + UBA
5	PRD + UBA	PRD
6	PRD + UBA	PRD + UBA

Experiment 3 (Table 5.3)

Batches of 25 cysts were immersed in PRD for up to seven days before transferring to PRD + UBA for hatching for eight weeks. The control cysts were hatched in PRD only. Cysts in hatching solution were incubated at 15°C in darkness in LMS cooled incubator, and each week transferred to fresh hatching solution. The number of hatched juveniles was counted each week and the number of remaining eggs were counted at the end of the eight-week hatching period, as described in Chapter 2.2. The percentage hatch and percentage reduction in hatch were calculated using same equations as found in Experiment 1 (above).

TABLE 5.3 A LIST OF TREATMENTS TESTED IN EXPERIMENT 3. *GLOBODERA PALLIDA* CYSTS WERE IMMERSED IN PRD FOR UP TO 7 DAYS PRIOR TO HATCHING IN PRD + UBA. CONTROL CYSTS WERE HATCHED IN PRD.

Treatment No.	Days in PRD	Hatching solution
1	0	PRD + UBA
2	1	PRD + UBA
3	2	PRD + UBA
4	3	PRD + UBA
5	4	PRD + UBA
6	7	PRD + UBA
7	-	PRD

Statistical analysis

Analysis of variance (ANOVA) and Tukey's ad hoc test were performed using Genstat v18 (VSNI International Ltd., Hemel Hempstead, United Kingdom) to determine significant differences at $P < 0.05$ between populations and treatments. Tests of data distribution were used to support the appropriate choice of statistical analysis, including histogram of residuals, fitted-value plot, normal plot and half-normal plot.

Microscopy of treated eggs

Batches of 10 *Globodera pallida* cysts in hatching baskets were immersed in water or BAC, UBA or BGT at 1% for seven days at 15°C in the dark in a LEC incubator. Cysts were transferred to microscope slides and gently pressed open in a drop of water to release eggs. Eggs were examined for any physical symptoms of damage, and the juveniles within were examined for symptoms of mortality at X10 - 20 magnification on an Olympus BX61 and photographs taken with an Olympus SC100 camera and cellSens standard 1.9 software (Olympus Corporation, Tokyo, Japan).

5.3 Results

Three experiments were performed to investigate the effect of formulation, timing, contact time and presence of PRD in treatments. The first experiment focused on the contact time between treatments and cysts, the second on the formulation UBA and the initial 24 h treatment and the third on pre-treatment with PRD.

Experiment 1

Globodera pallida cysts were immersed in UBA, UBA + PHMB, UBA + Si-QAC, BGT or water (control) for 24 h (Table 5.1). The cysts were then either transferred directly to PRD

(Figure 5.1 - no rinse), PRD and treatment (Figure 5.1 - continuous) or rinsed six times in water and then transferred to PRD (Figure 5.1 - 6 rinses). The control cysts were treated with water for 24 h and hatched in PRD, and 30% of eggs hatched over the eight-week hatching period. All of the treated cysts hatched less than the control cysts, though only the cysts in continuous treatment were significantly lower ($F_{(12,48)}$=12.18, P<0.001)than the control. The continuous treatment samples hatched between 2 – 5%, up to 93% reduction from the control. For each formulation there was no significant difference between no rinsing and rinsing; though all continuous treatment samples hatched significantly lower than those treated for 24 h (for each treatment). 24 h treatment with both UBA and BGT reduced hatch to 19%, though after rinsing hatch increased to 23% (UBA) or 28% (BGT). Hatching in cysts treated with UBA + Si-QAC also increased after rinsing, from 22% to 25% hatch. Rinsing after 24 h treatment with UBA + PHMB did not increase the hatching of *G. pallida*, but reduced hatch from 20% to 18%.

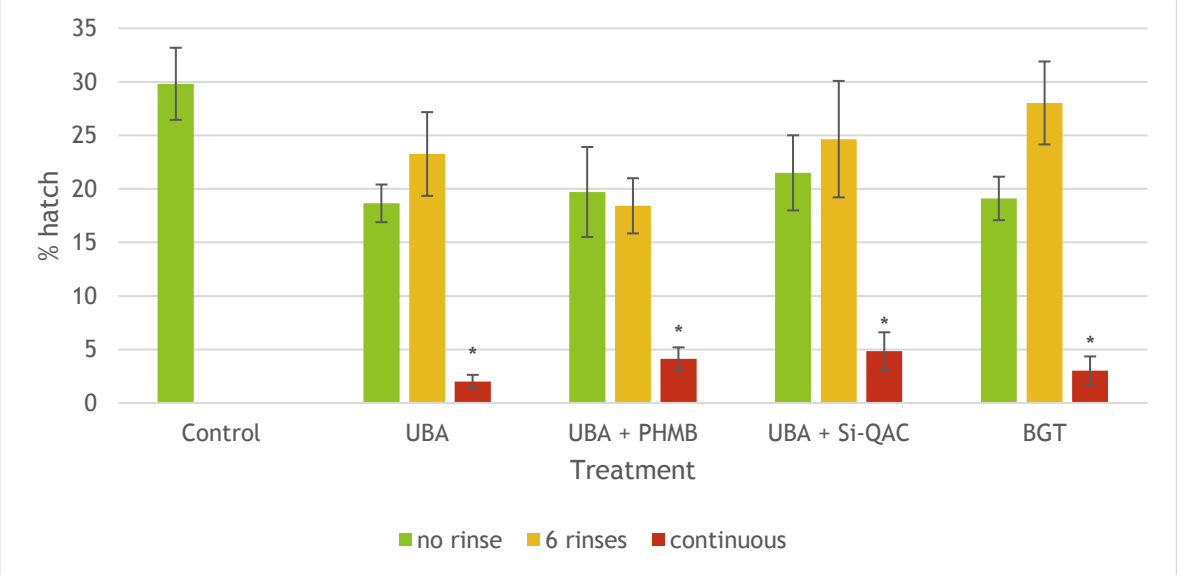


FIGURE 5.1 PERCENTAGE OF *GLOBODERA PALLIDA* SECOND-STAGE JUVENILES HATCHING OVER EIGHT WEEKS IN POTATO ROOT DIFFUSATE OR POTATO ROOT DIFFUSATE WITH UBA, UBA + PHMB, UBA + SI-QAC OR BGT AFTER 24 H EXPOSURE TO THE SAME TEST TREATMENTS. ERROR BARS SHOW THE STANDARD ERROR OF THE MEAN. '*' SIGNIFIES RESULTS THAT ARE SIGNIFICANTLY (P<0.01) LOWER THAN THE CONTROL HATCH. THE COEFFICIENT OF VARIANCE WAS 37.6%.

Experiment 2

Cysts were immersed in PRD, UBA or PRD + UBA for 24 h prior to hatching in either PRD or PRD + UBA for eight weeks. The control hatch (cysts exposed to PRD only) was 57%. 24 h treatment with UBA reduced hatch by 28% of the control to 41% hatch, but was not significantly lower ($F_{(5,25)}$=37.17, P<0.001) than the control. Treatment with PRD + UBA reduced hatch by 46% to 31% hatch, and was significantly lower than the control. All cysts hatching in PRD + UBA had a significantly lower hatch than the PRD only control, and were also significantly lower than other treatments hatched in PRD. Hatching in PRD

+ UBA was <3%, >95% reduction from the control. Exposure for 24 h in UBA followed by hatching in PRD + UBA produced the lowest percentage hatch (1.5%), a 97% reduction from the control.

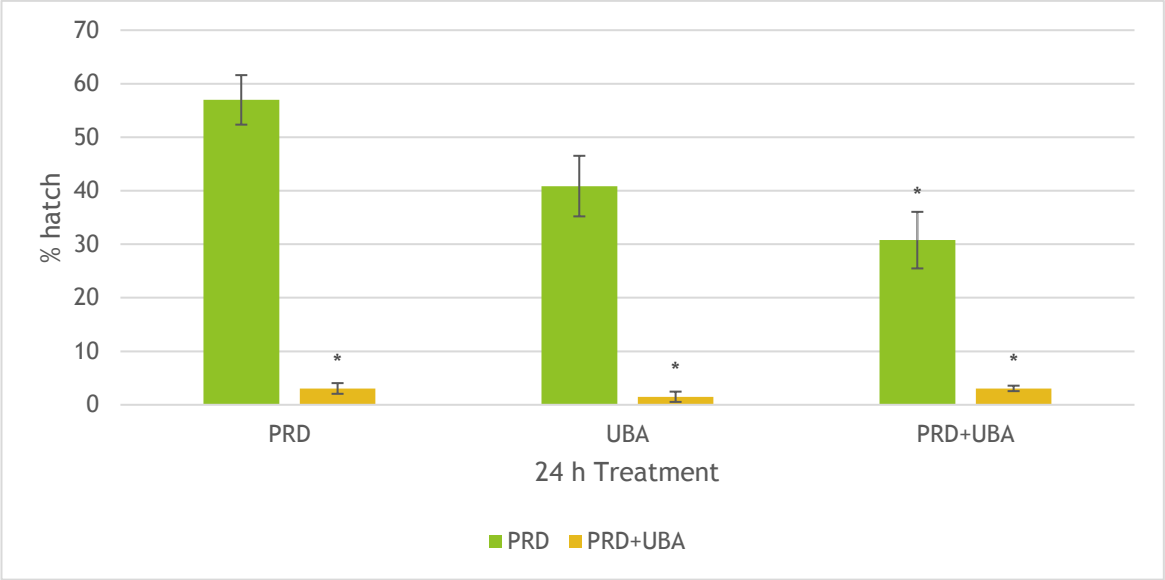


FIGURE 5.2 PERCENTAGE OF *GLOBODERA PALLIDA* SECOND-STAGE JUVENILES HATCHING OVER EIGHT WEEKS IN POTATO ROOT DIFFUSATE (PRD) OR PRD + UBA, AFTER 24 H EXPOSURE TO PRD, UBA OR PRD + UBA. ERROR BARS SHOW THE STANDARD ERROR OF THE MEAN. '*' SIGNIFIES RESULTS THAT ARE SIGNIFICANTLY ($P<0.01$) LOWER THAN THE CONTROL HATCH. THE COEFFICIENT OF VARIANCE WAS 40.7%.

Experiment 3

Cysts were treated with PRD for up to 7 days prior to hatching for 8 weeks in PRD + UBA (Figure 5.3). Control cysts were hatched in PRD only. The control cysts hatched 50%, as did cysts exposed to PRD for seven days prior to hatching in PRD + UBA. All cysts exposed to PRD for up to 4 days prior to hatching in PRD + UBA hatched significantly ($F_{(6,30)}$=19.1, P<0.001) less than the control and those exposed to PRD for 7 days. Hatching in PRD+UBA (0 days in PRD) hatched 9%, an 83% reduction from the control, and 24 h contact with PRD increased hatch to 16%.

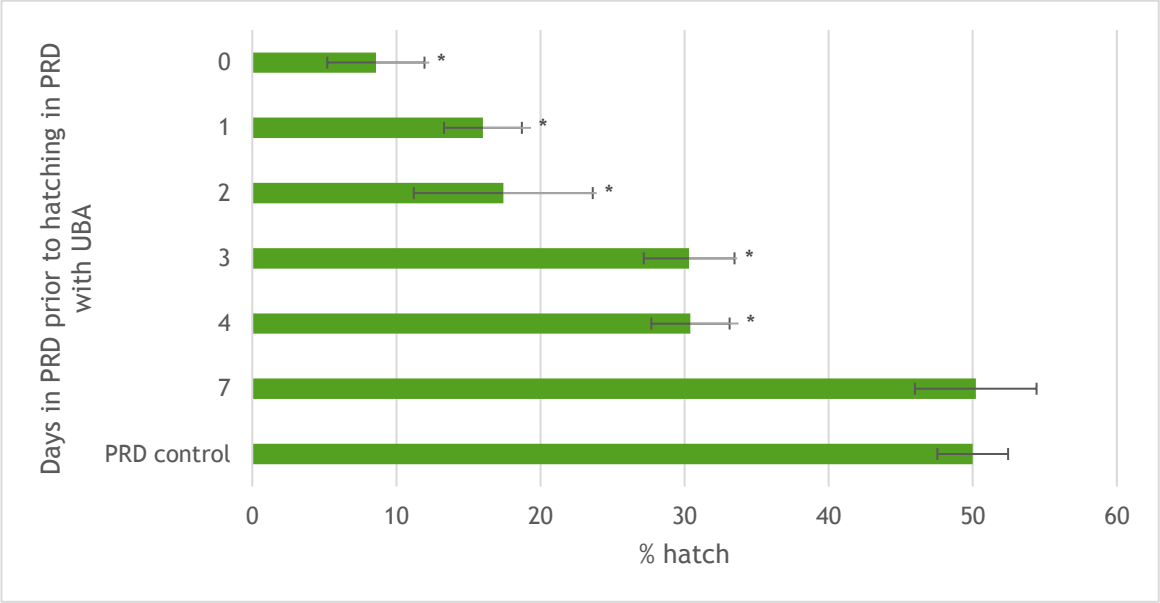


FIGURE 5.3 PERCENTAGE OF *GLOBODERA PALLIDA* SECOND-STAGE JUVENILES HATCHING OVER EIGHT WEEKS IN POTATO ROOT DIFFUSATE (PRD) OR PRD + UBA, AFTER UP TO 7 DAYS EXPOSURE TO PRD. ERROR BARS SHOW THE STANDARD ERROR OF THE MEAN. '*' SIGNIFIES RESULTS THAT ARE SIGNIFICANTLY ($P<0.01$) LOWER THAN THE CONTROL HATCH. THE COEFFICIENT OF VARIANCE WAS 31.7%.

Microscopy of treated eggs.

Globodera pallida cysts were immersed in test solutions for 7 days prior to being gently broken open and the eggs examined (Figure 5.4). Figures 5.4A and 5.4B show a J2 within an egg immersed in water at two depths of focus. The stylet is clearly visible in Figure 5.4A and the digestive system in Figure 5.4B. The lipid reserves are restricted to the posterior region of the juvenile and the eggshell appears intact with the juvenile folded inside. Figures 5.4C, 5.4D and 5.4E show eggs treated with BAC, UBA and BGT respectively. No differences were observed between control and treated eggs. Treated juveniles also demonstrate a clear distinction between the pharynx and digestive system with lipid reserves restricted to the posterior region. Treated eggshells also appear intact with juveniles folded within. Juvenile cuticle striation was also observed on treated and control (water) samples.

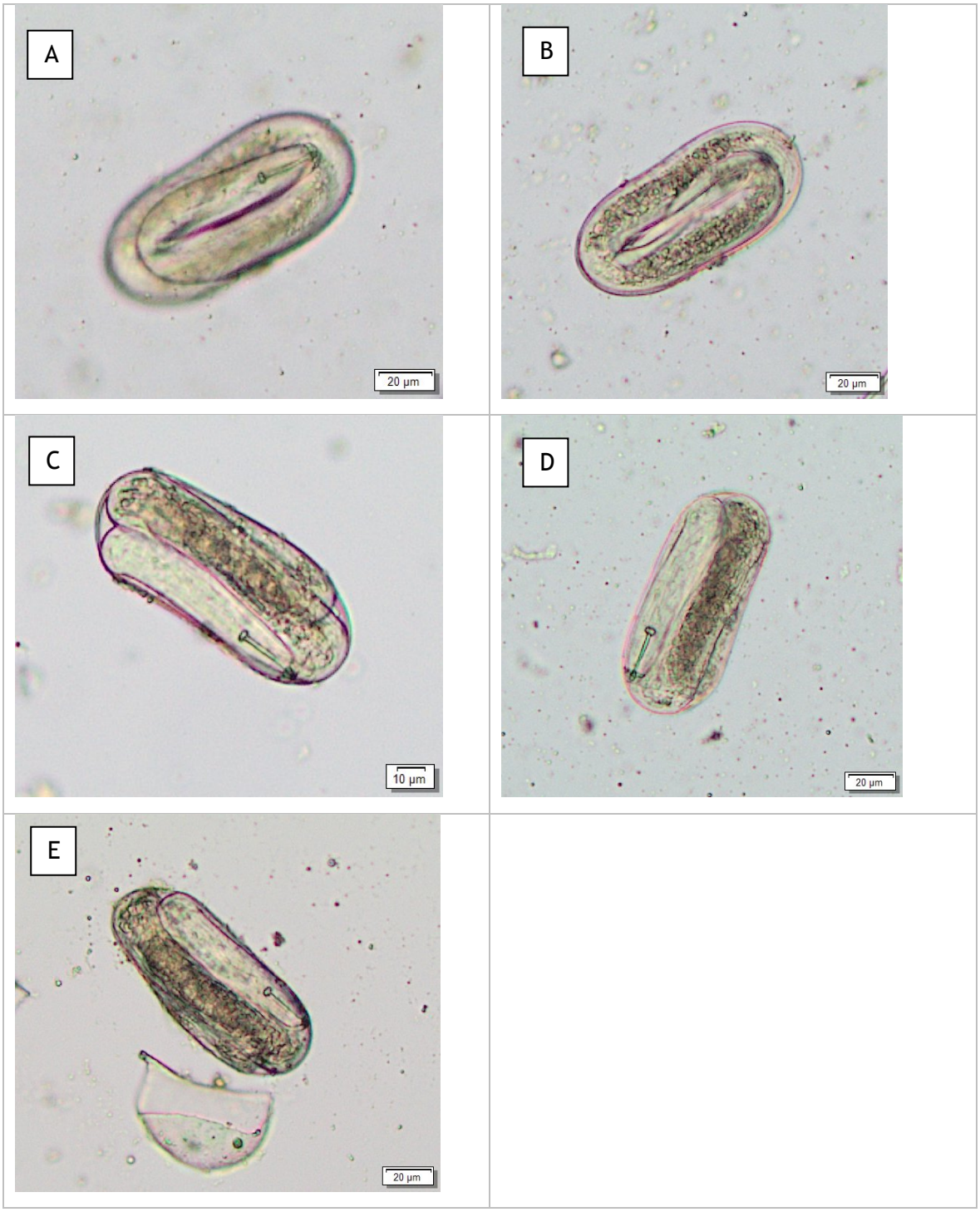


FIGURE 5.4 *GLOBODERA PALLIDA* EGGS EXTRACTED FROM CYSTS IMMERSED IN TEST SOLUTIONS FOR 7 DAYS AT 15°C IN DARKNESS. (A) + (B) *G. PALLIDA* EGGS FROM CYST IMMERSED IN WATER (C) *G. PALLIDA* EGG FROM CYST IMMERSED IN BENZALKONIUM CHLORIDE (BAC) (D) *G. PALLIDA* EGG FROM CYST IMMERSED THE FORMULATION UBA (E) *G. PALLIDA* EGG FROM CYST IMMERSED IN THE FORMULATION BGT. ALL IMAGES TAKEN AT X10 MAGNIFICATION ON OLYMPUS BX61.

5.4 Discussion

In general, the efficacy of a chemical to inhibit hatch can be increased by increasing its concentration or by increasing the exposure time. In Chapters 3 and 4, the formulation BGT and its components were examined *in vitro* to determine their efficacy in reducing hatch of *G. pallida* juveniles. The hatching assays performed in previous chapters have exposed *G. pallida* cysts to test chemicals for 24 h prior to hatching in PRD. This chapter examined the effect of both timing of exposure and length of exposure on the efficacy of BAC, UBA and BGT. The efficacy of the test products in reducing hatch was significantly increased when the exposure time was increased from 24 h to 8 weeks, and the formulation UBA was found to significantly reduce hatch of *G. pallida* J2s after exposure to PRD for up to 4 days.

The experiments performed in Chapter 3 found that 24 h exposure to BGT significantly reduced hatching of *G. pallida*. To determine whether hatch inhibition was caused within the 24 h period or if the test product was being carried over into the hatching assay, cysts were rinsed with water after treatment with the test solutions and prior to hatching for eight weeks in potato root diffusate (PRD). Chapter 4 determined that benzalkonium chloride (BAC) was the most effective component at reducing hatching of *G. pallida*. The formulation UBA combines BAC with the adjuvants alcohol ethoxylate (AEO) and alkyl polyglucoside (APG), commonly used agrochemicals included as wetting and spreading agents. The addition of poly hexamethylbiguanide (PHMB) and 3-(trimethoxysilyl)propyl dimethyl octadecyl ammonium chloride (Si-QAC) to UBA was included to determine if either of these components would improve the efficacy or longevity of the active ingredient BAC after rinsing.

In Experiment 1 (Figure 5.1), all cysts treated with the test products listed in Table 5.1 had a lower percentage hatch than the 30% hatch of the untreated cysts. In all but one treatment (UBA + PHMB), the proportion of juveniles hatching increased after rinsing. The increase in percentage hatch was not statistically significant in any treatment but suggests that in the assays performed in previous chapters, the test solution was carried over in the cysts or baskets into the hatching solution, PRD. The decrease in efficacy after rinsing was not significant, and may be due to the standard error of the mean being higher in rinsed cysts. Hatching in samples treated with UBA + PHMB decreased slightly from 20% to 18% after rinsing, though UBA + PHMB was less effective at reducing hatch than either UBA or BGT. The addition of Si-QAC did not significantly improve the longevity of UBA, as hatch increased from 22% to 25% after rinsing. The inclusion of PHMB and Si-QAC may not offer any residual activity within the cyst, but may still offer benefits to the formulation in soil, and should be evaluated using soil media pot experiments. UBA (BAC, AEO + APG) and BGT both reduced hatch by 37% from the

control hatch of 30% after 24 h contact. Hatch in both treatments increased slightly after rinsing, though no significant difference was found. BGT was affected more than UBA by rinsing, again demonstrating that the addition of Si-QAC does not confer residual activity as previously thought. The hatch of samples treated with BGT increased from 19% to 28% after rinsing, whilst hatching in samples treated with UBA increased from 19% to 23%. The percentage hatch of cysts in continuous contact with all treatments was significantly lower than the control. All continuous treatments had <5% hatch and were significantly lower than 24 h contact with the same treatment. The formulation UBA was the most effective treatment in each series (24 h exposure, 24 h exposure then rinsed and continuous exposure) and reduced the percentage hatch by 93% when in continuous contact with *G. pallida* cysts in PRD. This experiment demonstrated that rinsing cysts prior to hatching does not significantly impact results, thus previous results obtained in Chapters 3 and 4 were achieved in 24 h. This experiment also demonstrated that increasing contact time from 24 h to 8 weeks significantly increased the efficacy of the products tested, and that UBA is comparable to BGT in efficacy.

Experiment 2 (Figure 5.2) also demonstrated a significant increase in efficacy when contact with UBA was increased from 24 h to eight weeks. 24 h treatment with PRD, UBA or PRD + UBA was followed by hatching in either PRD or PRD+UBA to further investigate the effect of UBA on hatching of *G. pallida*. The formulation UBA was less effective at reducing hatch of *G. pallida* after 24 h contact in Experiment 2 (28% reduction) than Experiment 1 (37% reduction) or Chapter 4 (37% reduction). Although all experiments used the same population of *G. pallida* (and the same experimental conditions such as incubation temperature and duration), different batches of PRD were used in each experiment. The control hatch for Experiment 1 was 30%, while Experiment 2 had a higher control hatch of 57% and in Chapter 4 44% of the control cysts hatched. The efficacy of PRD to stimulate hatch could affect the efficacy of test solutions to reduce hatching by lowering the 'maximum' percentage hatch a population can achieve (thus skewing the percentage reduction). The use of synthetic hatching factors could improve reliability of hatching assays, though they would be less representative of the conditions *in situ* and may interact differently with both the nematodes hatching and the chemicals under investigation.

Cysts exposed to PRD for the initial 24 h of the experiment hatched 57% in PRD, but only 3% In PRD + UBA. *Globodera pallida* hatch can be stimulated after as little as 5 mins contact between eggs and PRD (Forrest and Perry, 1980), though subsequent exposure to PRD +UBA was able to significantly suppress hatching after initial stimulation. Initial 24 h treatment with PRD+UBA significantly reduced hatching of *G. pallida* in PRD by 46% after 24 h contact, though UBA alone did not (28% reduction). This may be due to

interaction between PRD and UBA. Potato root diffusate contains hatching factors that stimulate hatch by triggering a cascade of events resulting in changes in the eggshell, activation of the unhatched juveniles and eclosion (Masler and Perry, 2018). Hatching factors displace internal Ca^{2+} from binding sites in the lipoprotein layer of the eggshell, causing increased permeability (Clarke and Perry, 1985). The glycoalkaloids α-solanine and α-chaconine are able to destabilise lipid membranes and can induce hatch of *G. rostochiensis* (Devine *et al*., 1996). Benzalkonium chloride (BAC) is also able to interact with and destabilise lipid membranes (Maillard, 2002). When tested at the equivalent concentration found on 0.2% BGT, BAC did not induce hatch of *G. pallida* juveniles, but it was not tested alone at the equivalent concentrations of BGT found to stimulate hatch (Chapter 4), and may increase hatching at very low concentrations.

In Chapter 4 it was suggested that BAC could increase membrane permeability of the eggshell and cause mortality of the juvenile within prior to hatching, as vigorous movement of the J2 does not begin until at least 3 days after initial exposure to root diffusate (Masler and Perry, 2018), however microscopy performed on eggs exposed to the test solutions demonstrates that this is not the case (Figure 5.4). If BAC increased membrane permeability of the eggshell and caused mortality of the juvenile in the eggs exposed to BAC, UBA and BGT, it would be expected that those juveniles would sustain the same damage as seen in Chapter 2 when second-stage *G. pallida* juveniles were exposed to BGT for 3 days. Exposed juveniles suffered loss of differentiation within the body cavity, no clear distinction between the pharynx and digestive systems and disruption of the lipid reserves (Figure 2.3). When cysts were exposed to BGT, UBA or BAC no damage to the juveniles within eggs was observed. Exposed encysted juveniles do not display any of the symptoms observed in Chapter 2. These results demonstrate that BAC does not directly affect the juveniles within eggs, and does not cause in-egg mortality of *G. pallida* juveniles. It is possible that the cationic compound BAC interferes with the cationic calcium ion mediated change in eggshell lipid layer permeability, thus preventing the juvenile from hatching but not causing mortality. It is unknown whether the active ingredient interacts with the eggshell directly and / or the PRD; to determine this would require further investigation using techniques such as atomic force microscopy or X-ray microanalysis which are used to view interactions of chemicals on cell membranes (Alakomi et al., 2006; Atkinson and Taylor, 1983). The increase in efficacy of UBA from 29% to 46% reduction (Experiment 2) when PRD is present during 24 h treatment and a loss of efficacy after rinsing (Experiment 1) suggest that there is some interaction between the PRD and UBA. Twomey *et al*. (2000) found that the product DiTera prevented changes in eggshell permeability in both *G. pallida* and *G. rostochiensis*, possibly by binding to or blocking hatching factor binding sites on the eggshell. DiTera reduced hatching of both potato cyst nematodes by >70% after 5 weeks contact.

To further investigate the effect of timing of application and to examine the efficacy of UBA in disrupting the hatching cascade, cysts were exposed to PRD for up to seven days prior to hatching in PRD + UBA. The presence of UBA in PRD significantly reduced hatching of *G. pallida* after up to four days stimulation with PRD. After seven days in PRD, UBA was unable to reduce hatching, and the total percentage hatch was the same as in the PRD only control. Hatch was lowest when cysts were exposed to PRD + UBA for the entire experiment, with 9% of eggs hatching. This was higher than in Experiment 2, where 3% of *G. pallida* eggs hatched after the same treatment, though both experiments were performed under the same experimental conditions with the same population of *G. pallida* and the same batches of PRD and UBA. The control was also different between the two experiments, with 57% of control eggs hatching in Experiment 2 and 50% in Experiment 3. This may indicate a loss in efficacy of PRD, though experiment 3 was started one week after experiment 2. Hatching increased when cysts were exposed to PRD prior to PRD + UBA. These results show that to effectively reduce hatching of *G. pallida*, the product must be applied within a week of PRD production, preferably prior to the appearance of hatching factors in the soil. The results also demonstrate that prolonged contact with UBA most effectively reduces hatching of *G. pallida*.

Organophosphates such as fosthiazate and cadusafos have been found to completely inhibit hatching of *G. pallida in vitro*. This inhibition is not permanent, as removal of these compounds results in *G. pallida* hatch resuming (Ibrahim and Haydock, 1999; Woods *et al*., 1999). Due to implementation of regulations such as EU 1107/2009 the use of acetylcholinesterase inhibitors, including organophosphates, is under constant threat of licence revocation (Back *et al*., 2018). Several non-acetylcholinesterase inhibiting chemicals have been evaluated for their activity against *G. pallida* in order to provide control methods if the use of organophosphates and carbamates is prohibited. Danquah *et al*. (2011) found that the allicin-based formulation G8014S reduced hatching of *G. pallida* by 46% after 8 weeks contact. Brolsma *et al*. (2014) found that the biofumigant product 2-propenyl isothiocyanate reduced *G. pallida* hatch by 50% after 2 h contact with the volatile compound. The product DiTera reduced hatching of *G. pallida* by 70% after 5 weeks contact and is obtained by submerged fermentation of *Myrothecium verrucaria* (Twomey *et al*., 2000). The formulation under investigation, UBA, provided greater efficacy than G8014S, 2-propenyl isothiocyanate and DiTera *in vitro*, reducing hatching of *G. pallida* by 97%. UBA also provided greater efficacy than the 90% reduction caused by fosthiazate (Woods *et al*., 1999) and was comparable in efficacy (98%) to Rugby 100ME at 0.25 µg L^{-1}, which contains cadusafos (Ibrahim and Haydock, 1999).

The formulations UBA and BGT are water soluble and will not persist in the soil profile for eight weeks due to leaching, therefore multiple applications will be required to maintain an

effective concentration in the soil and reduce *G. pallida* hatching *in situ*. Cationic surfactants are negatively affected by the presence of organic matter found in abundance in the soil, which could affect the efficacy of the formulations in reducing *G. pallida* hatching *in situ*. Glasshouse studies will be conducted to introduce the variables found within soil growing media and determine which of the formulations is most effective.

5.5 Conclusion

Repeat application or prolonged presence of active ingredients may increase their efficacy *in situ*, as hatching of *G. pallida* occurs until at least eight weeks after host plant emergence. This chapter investigated the effect of contact time and the presence of PRD on the efficacy of the formulations and found that by increasing the contact time of BGT and UBA, hatch of *G. pallida* can be reduced by >90%. The efficacy of UBA increased when treatments included PRD, indicating an interaction between the two solutions. Contact with UBA significantly reduced hatching of *G. pallida* up to 4 days after cysts were in contact with PRD, reversing or halting the hatching cascade. The formulation UBA is as (or more) effective at reducing hatching of *G. pallida in vitro* as the organophosphate nematicides cadusafos and fosthiazate. The results from this experiment will be used to design soil media pot experiments, and indicate that frequent treatments from planting onwards will give the best efficacy for both BGT and UBA. Root invasion analysis will be performed to investigate the efficacy of BAC, BGT and UBA on hatching and invasion of *G. pallida in situ*.

Chapter 6 : The effect of BAC, UBA and BGT on potato plant growth and the infectivity of *Globodera pallida*

6.1 Introduction

Hatching assays are commonly utilised in screening agrochemicals to identify active ingredients and examine their mode of action, but do not guarantee the efficacy of an active ingredient in the field (Brolsma *et al*., 2014). There are several variables that can affect active ingredients in the soil, including soil type, pH, moisture and temperature (Nieder *et al*., 2018). The amount of organic matter present in the soil can also affect the efficacy of an active ingredient. The more organic matter present, the stronger the adsorption of active ingredients in the soil (Morris *et al*., 2018). When active ingredients do not adsorb strongly to soil, due to low organic matter or pH, the risk of leaching is increased (Karpouzas *et al*., 2007). Degradation of agrochemicals may also be accelerated by the presence of certain microorganisms. Several microorganisms have been isolated and identified that exhibit enhanced biodegradation of nematicides including fenamiphos (Ou and Thomas, 1994), ethoprophos (Karpouzas *et al*., 2000) and oxamyl (Rousidou *et al*., 2016).

Root invasion assays are a useful method of screening the efficacy of products against *G. pallida* (Whitehead, 1973). Though hatching assays assess a product's efficacy in preventing hatch of juveniles, root invasion assays also include assessment of the infectivity of juveniles hatching and their development within the roots. Ibrahim and Haydock (1999) examined the effect of cadusafos at reducing invasion of *G. pallida* by exposing J2s to the test solution prior to applying to young established plants. Evans and Wright (1982) used similar methodology to assess oxamyl against *Globodera rostochiensis* in tomato plants. Whilst these studies demonstrated that treatment with organophosphates significantly impairs the infectivity of juveniles, they are not representative of the situation in the field, where juveniles are protected from chemical control agents by the eggshell prior to hatching. In eight field trials investigating the efficacy of oxamyl in granular and liquid formulations, performed at four sites over three years, only one trial showed any significant differences between treatments (Kiezebrink, 2009). It was concluded that the variation between replicates and drought stress experiences obscured any significant differences between treatments. The experiment described in this chapter combines the methodologies used by the aforementioned researchers, by inoculating pots with *G. pallida* cysts at planting and reducing variation by growing host plants under controlled glasshouse conditions.

The timing of application of agrochemicals is crucial to ensure optimal efficacy, along with concentration and application method. Granular agrochemicals impregnate molten active ingredients into the pores of a preformed granule formed from compounds such as clay (Surgant and Deming, 1990). Dry granular formulations have a number of benefits over liquid formulations; shipping costs are lower, stability is increased allowing storage for longer, and containers are less expensive to purchase and dispose of. Dry formulations also limit the use of solvents, used to stabilise and solubilise active ingredients (Surgant and Deming, 1990). Granular nematicides need incorporation into the soil and are designed to be active for at least 28 days (Back *et al*., 2018). The nematicide Vydate (active ingredient oxamyl) is degraded in soil by hydrolysis of the carbamate group (Bromilow *et al*., 1980). Oxamyl has a half-life in soil of ten to twenty four days, depending on the soil temperature, moisture and pH (Haydock *et al*., 2012). Increasing the temperature, moisture or pH in soil can expedite hydrolysis of active ingredients, as can prior use of nematicides (Osborn *et al*., 2010). Karpouzas *et al.* (1999) found that when applied to a field with thirty years of nematicide use, the nematicidal activity of ethoprophos persisted for 14 days. When ethoprophos was applied to an adjacent field with no known history of nematicide use, the nematicidal activity persisted for over 35 days. The longevity of an active ingredient in the soil can be increased by changing the application method. Kiezebrink (2009) found that application of oxamyl using drip irrigation was as effective as using the granular formulation, and that the concentrations used in the drip irrigation could be reduced to 75% of the standard concentration of the granular formulation without compromising nematicidal efficacy. Application by drip irrigation reduces the exposure of non-target organisms and growers to the active ingredient and allows targeted application of agrochemicals throughout the season, increasing the longevity of their activity.

The longevity of the formulations BGT and UBA in soil is unknown to date. Quaternary ammonium compounds (QACs) such as benzalkonium chloride (BAC), the active ingredient in reducing hatch of *G. pallida* in BGT, are biodegradable in biological systems such as soil. The half-lives vary from hours to months depending on the QAC structure and concentration, the soil type, temperature and moisture and the presence of QAC degrading microorganisms (Tezel, 2009). Although BAC is an antibacterial compound, microorganisms such as *Aeromonas hydrophilia* are capable of utilizing BAC as a sole source of carbon (Patrauchan and Oriel, 2003). Due to its positive charge, BAC strongly adsorbs on negatively charged soil particles and the long hydrophobic carbon chain facilitates its adsorption on soil organic matter (Khan *et al*., 2017). Humic acids in soil severely reduce the bactericidal activity of benzalkonium chloride (BAC) due to the strong adsorption of cationic surfactants to humic substances (Araújo *et al*., 2013). Benzalkonium chloride and the two formulations that contain the active ingredient, UBA

and BGT, are all aqueous solutions, and will be applied directly to the soil in the glasshouse experiment described in this chapter. The experiment will be performed on a sand soil mix with low organic matter content to reduce the impact of adsorption and degradation on the results.

Chapter 5 examined the effect of contact time on the efficacy of two formulations, BGT and UBA on reducing hatching of *G. pallida* juveniles, and found that the efficacy of both formulations was significantly improved by increasing contact time from 24 h to 8 weeks. 24 h contact with BGT reduced hatching of *G. pallida* by 36%, but when BGT was present in PRD for the entirety of the hatching assay, hatch was reduced by 90%. In Chapter 5, UBA demonstrated a marginally superior efficacy against *G. pallida* hatching (93% reduction) than BGT (90% reduction) and was assessed further. UBA significantly reduced the percentage of *G. pallida* hatching when applied up to 4 days after cysts were exposed to PRD, but not after 7 days, thus application of product should occur prior to or just after PRD is being released from the roots. The results from Chapter 5 have been used to design a glasshouse experiment to assess the efficacy of the active ingredient BAC and the formulations UBA and BGT in reducing invasion of potato plants by *G. pallida* juveniles. This experiment will assess the benefits of inclusion of the non-ionic surfactants alcohol ethoxylate (AEO) and alkyl polyglucoside (APG), the biguanide polyhexamethyl biguanide (PHMB) and the quaternary ammonium compound 3-(trimethoxysilyl)propyl dimethyl octadecyl ammonium chloride (Si-QAC) to the active ingredient BAC. In order to maintain an effective concentration in the soil throughout the experiment, the test chemicals will be applied weekly.

Aim

To assess the efficacy of BAC, UBA and BGT in reducing root invasion by *G. pallida* of potato plants in soil.

Objectives

1. Determine the effect of BAC, UBA and BGT on potato plant growth.
2. Determine the effect of BAC, UBA and BGT on potato plant growth in the presence of *G. pallida*.
3. Determine the effect of BAC, UBA and BGT on root invasion of potato plants by *G. pallida*.
4. Determine which formulation (BAC, UBA or BGT) is the most effective at reducing root invasion of potato plants by *G. pallida*.

Null Hypotheses

1. The test chemicals will have no effect on the growth of potato plants.
2. The test chemicals will have no effect on the growth of potato plants in the presence of *G. pallida*.
3. The test chemicals will have no effect on root invasion of potato plants by *G. pallida*.
4. The addition of non-ionic surfactants will have no effect on the efficacy of the active ingredient BAC.
5. The addition of a biguanide and cationic surfactant will have no effect on the efficacy of the formulation UBA.

6.2 Materials and methods

For methodology on preparation of potato root diffusate (PRD) and extraction and identification of PCN, see Chapter 2.2.

Potato cyst nematodes

Globodera pallida cysts 'VT May' were used in this experiment. The population originated from Shropshire.

Preparation of test solutions

Test solutions were obtained from Arcis Biotechnology Ltd (Sci-Tech Daresbury, United Kingdom). Benzalkonium chloride (BAC), UBA, BGT were diluted to 1% using tap water. Tap water was used as a control (Table 6.1).

TABLE 6.1 A LIST OF TREATMENTS TESTED IN THE GLASSHOUSE EXPERIMENT. POTS OF SOIL WERE INOCULATED EITHER WITH OR WITHOUT *G. PALLIDA* CYSTS, AND TREATED WITH WATER, BAC, UBA OR BGT FOUR TIMES OVER A FOUR-WEEK PERIOD.

Treatment Number	Presence of PCN	Treatment
1	PCN	Control (water)
2	PCN	BAC
3	PCN	UBA
4	PCN	BGT
5	No PCN	Control (water)
6	No PCN	BAC
7	No PCN	UBA
8	No PCN	BGT

Glasshouse experiment

The glasshouse experiment (Table 6.1, Figure 6.1) was conducted in the main glasshouse at Harper Adams University, Telford. Horticultural sharp sand and the potting compost John Innes No 2 were mixed in a 70:30 ratio and used to fill VCH 9F pots (0.37L capacity). Fifty *G. pallida* cysts were added to pots requiring infection and mixed into soil thoroughly, an inoculation of approximately 20 eggs g^{-1} soil. Maris Peer microtubers (18-20mm) were obtained from TLC potatoes (Wardend Farm, Banchory) and planted approximately 1" deep.

Pots were watered and allowed to stand for 4 hours prior to treatment with 1 ml 1% BAC, UBA, BGT or water (Table 6.1) before being watered briefly. Pots were placed on capillary matting and watered twice a week (Figure 6.1). Each week, plants were treated with 1 ml 1% BAC, UBA, BGT or water prior to watering for three weeks, receiving four treatments in total. The experiment was designed in randomised blocks, with 10 replicates for each treatment.

Four weeks after planting plants were removed from pots and any remaining soil washed off roots. Plants were cut at the stem and haulm and roots weighed separately. Roots were labelled and placed in freezer bags and stored at -20°C until required for root invasion analysis.

Root invasion analysis

Acid fuchsin stain was prepared and roots were stained following the Harper Adams University standard operating protocol Potato Cyst Nematode Root invasion: sampling, preserving, staining and counts (Woods *et al*., 1997). Stained roots were cut into small sections and blended in 50 ml glass distilled water using a Waring blender (Waring Commercial, United Kingdom) for 60 seconds on low and 30 seconds on high. The roots were transferred to measuring cylinder and made up to 100 ml with glass distilled water. After thorough mixing, 2 ml of each sample was transferred to a De Grisse counting tray and the counted using a Leica Wild M3Z microscope (Leica Camera, Wetzlar, Germany) at x10-40 magnification. Juveniles were categorised into life stages using the reference images in Figure 1.3 (Chapter 1). To determine the number of juveniles per gram of root tissue, the following calculation was performed:

$$Number\ of\ juveniles\ per\ gram\ of\ root\ tissue = \frac{Number\ of\ juveniles\ in\ 2\ ml \times 50}{Root\ weight\ (g)}$$

FIGURE 6.1 PHOTOGRAPH OF GLASSHOUSE EXPERIMENT. MARIS PEER POTATO PLANTS FOUR WEEKS AFTER PLANTING, PRIOR TO FINAL MEASUREMENTS AND ROOT INVASION ANALYSIS. HALF OF THE POTS CONTAINED *G. PALLIDA* CYSTS AND THE OTHER HALF DID NOT. POTS WERE TREATED WITH WATER, BAC, UBA OR BGT AT PLANTING AND WEEKLY THEREAFTER. IMAGE AUTHOR'S OWN.

Statistical analysis

Analysis of variance (ANOVA) and Tukey's ad hoc test were performed using Genstat v18 (VSNI International Ltd., Hemel Hempstead, United Kingdom) to determine significant differences at $P < 0.05$ between treatments. Tests of data distribution were used to support the appropriate choice of statistical analysis, including histogram of residuals, fitted-value plot, normal plot and half-normal plot.

6.3 Results

The effects of the test chemicals on potato plants

Maris Peer microtubers were planted in 0.37 L pots containing horticultural sharp sand and John Innes No. 2 (70:30 ratio), half of which were infected with *Globodera pallida* cysts and the other half contained no PCN. Four weeks after planting, plants were removed and fresh haulm weight (Figure 6.2) and root weight (Figure 6.3) were recorded.

There was no significant difference ($F_{(1,72)}=0.01$, $P=0.982$) in haulm weight (Figure 6.2) between pots containing *G. pallida* and those not containing *G. pallida*, though the mean

haulm weight of infected plants was 4% lower than uninfected plants. There was also no significant difference ($F_{(3,72)}$=0.86, P=0.467) in haulm weight between treatments. Benzalkonium chloride (BAC) decreased the haulm weight by 3% without the presence of *G. pallida*. Treatment with UBA increased the haulm weight by 7% in pots without *G. pallida* and by 10% when *G. pallida* was present. BGT reduced haulm weight by 11% when *G. pallida* was not present, but increased haulm weight by 2% when *G. pallida* was present.

There was no significant difference ($F_{(1,72)}$=0.05, P=0.821) in root weight (Figure 6.3) between pots containing *G. pallida* and those not containing *G. pallida*, though the presence of *G. pallida* decreased root weight by 8%. There was also no significant difference ($F_{(3,72)}$=1.18, P=0.322) in root weight between treatments. Benzalkonium chloride (BAC) increased root weight by 8% in pots without *G. pallida*, and by 7% when *G. pallida* was present. Treatment with UBA increased the root weight by 23% when *G. pallida* was present. BGT reduced root weight by 7% when no *G. pallida* was present, but increased root weight by 2% when *G. pallida* was present.

The effects of the test chemicals on infection of potato plants by *Globodera pallida*

Root invasion assessments were made after staining roots with acid fuchsin and the number of juveniles per gram of root calculated (Figure 6.4). *Globodera pallida* juveniles were categorised into life stages using the reference images in Figure 1.1 (Chapter 1). In control pots, 476 juveniles per gram of root were counted, 88% of which were second-stage juveniles (J2). Treatment with BAC and UBA significantly reduced ($F_{(3.36)}$=3.91, P=0.016) the number of juveniles per gram of root recovered, by 29% and 27% respectively. The proportion of J2 in BAC samples was 87% and samples treated with UBA contained 75% J2. Both BAC and UBA had significantly lower ($F_{(3,36)}$=4.53, P<0.01) numbers of J2 than the control. BGT reduced the number of juveniles by 21% to 374 per gram of root, with 83% J2. The number of J3 g^{-1} root was significantly lower ($F_{(3,36)}$=3.48, P=0.029) in plants treated with BAC, but not in those treated with UBA or BGT.

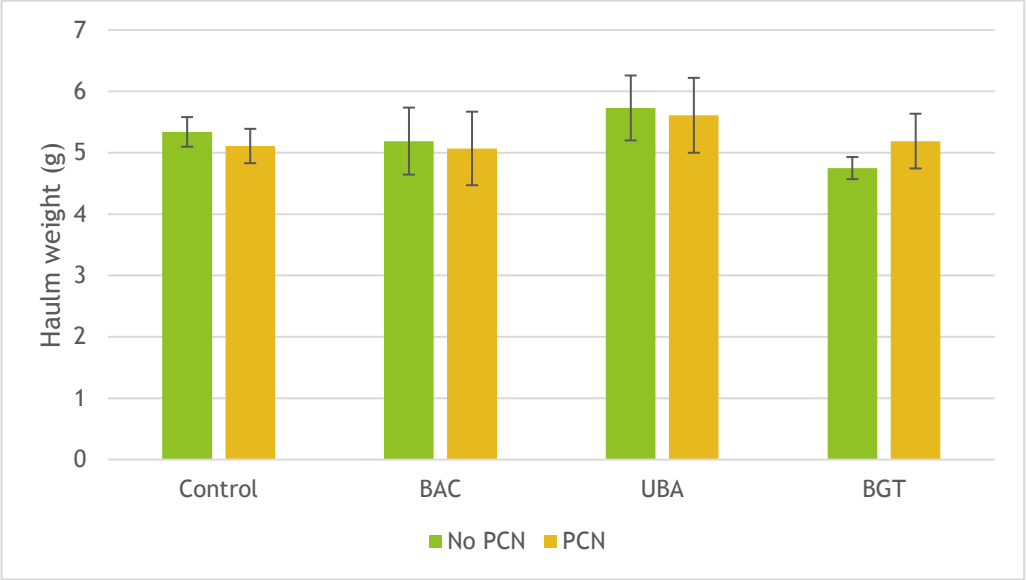


FIGURE 6.2 FRESH HAULM WEIGHT OF MARIS PEER PLANTS FOUR WEEKS AFTER PLANTING WITH AND WITHOUT *G. PALLIDA* CYSTS. POTS WERE TREATED FOUR TIMES WITH BENZALKONIUM CHLORIDE (BAC), UBA AND BGT OR WATER (CONTROL). NO SIGNIFICANT DIFFERENCES ($P>0.05$) WERE FOUND BETWEEN TREATMENTS. THE ERROR BARS SHOW THE STANDARD ERROR OF THE MEAN. THE COEFFICIENT OF VARIANCE WAS 27.6%.

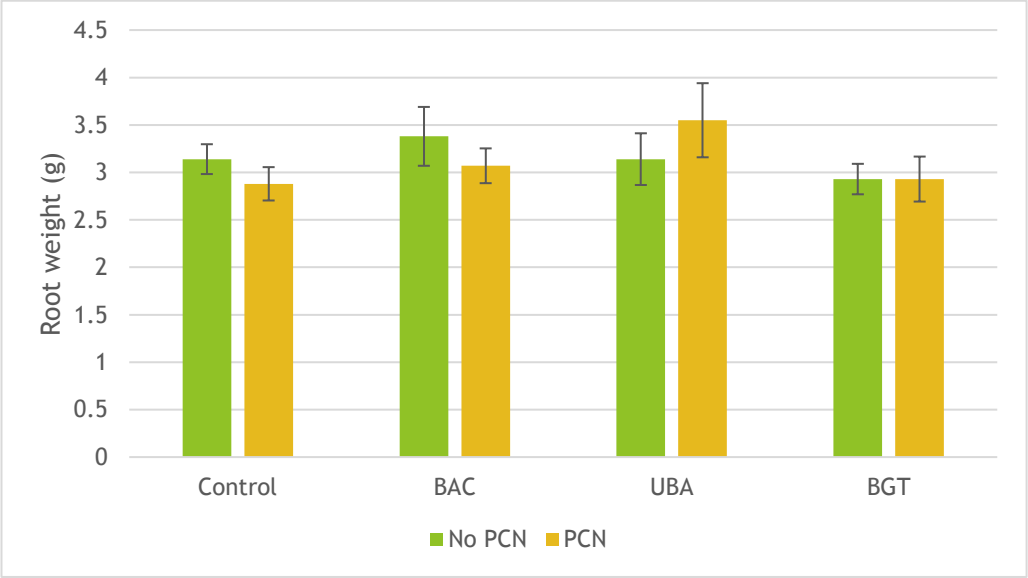


FIGURE 6.3 FRESH ROOT WEIGHT OF MARIS PEER PLANTS FOUR WEEKS AFTER PLANTING WITH AND WITHOUT *G. PALLIDA* CYSTS. POTS WERE TREATED FOUR TIMES WITH BENZALKONIUM CHLORIDE (BAC), UBA, BGT OR WATER (CONTROL). NO SIGNIFICANT DIFFERENCES ($P>0.05$) WERE FOUND BETWEEN TREATMENTS. THE ERROR BARS SHOW THE STANDARD ERROR OF THE MEAN. THE COEFFICIENT OF VARIANCE WAS 25.1%

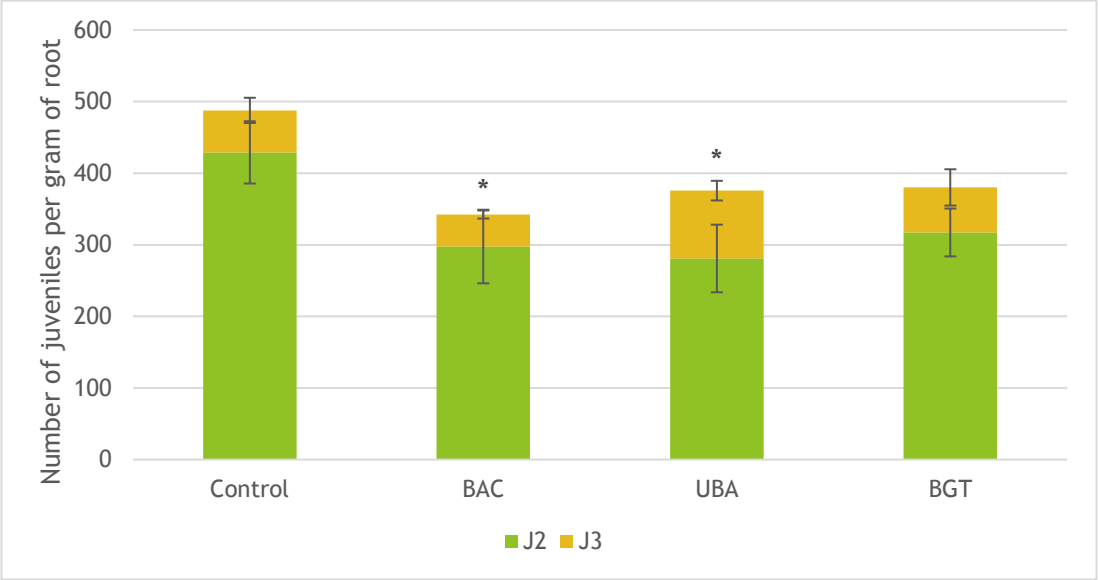


FIGURE 6.4 THE NUMBER OF JUVENILES PER GRAM OF ROOT RECOVERED FROM MARIS PEER PLANTS FOUR WEEKS AFTER PLANTING WITH *G. PALLIDA* CYSTS. POTS WERE TREATED FOUR TIMES WITH BENZALKONIUM CHLORIDE (BAC), UBA, BGT OR WATER (CONTROL). JUVENILES WERE CATEGORISED INTO SECOND-STAGE JUVENILES (J2) OR THIRD STAGE JUVENILES (J3). '*' DENOTES TOTAL JUVENILE COUNTS THAT ARE SIGNIFICANTLY (*P*=0.016) LOWER THAN THOSE RECOVERED FROM UNTREATED PLANTS. THE ERROR BARS SHOW THE STANDARD ERROR OF THE MEAN FOR THE COUNTS OF INDIVIDUAL LIFE STAGES OF *G. PALLIDA*.

6.4 Discussion

In vitro assays such as mortality and hatching assays are useful in screening the efficacy of chemicals against plant parasitic nematodes such as *G. pallida*, and in determining the mode of action of active ingredients. The *in vitro* assays performed in Chapters 2 to 5 found that BAC, UBA and BGT cause mortality in J2s and reduce hatching of juveniles from encysted eggs. Efficacy of an active ingredient *in vitro* does not guarantee efficacy *in situ*, due to the variables introduced in the soil such as soil type, temperature, pH and the presence of other microorganisms that may cause accelerated degradation (Brolsma *et al*., 2014; Karpouzas *et al*., 2007, 2000; Morris *et al*., 2018; Nieder *et al*., 2018; Ou and Thomas, 1994; Rousidou *et al*., 2016). To determine whether active ingredients and formulations will be efficacious against nematodes in the field, glasshouse studies are commonly used prior to larger, more expensive field experiments. Glasshouse studies enable the researcher to control a number of variables such as soil type, temperature, pH and moisture and reduce variability between replicates, as well as being able to screen a number of treatments in a smaller area than if field experiments were used.

A glasshouse experiment was conducted to determine the effects of benzalkonium chloride (BAC) and the formulations UBA and BGT on potato plants in the presence and absence of the potato cyst nematode *G. pallida*. Maris peer microtubers were planted in a mix of sharp sand and potting compost that contained *G. pallida* cysts. Pots were treated

weekly with BAC, UBA, BGT or Water for 3 weeks prior to removal 4 weeks after planting. Pots without *G. pallida* were also planted and treated identically, to detect any phytotoxicity caused by the test chemicals and determine whether results in pots with *G. pallida* were caused by interaction with the host plant or the pest. The cationic surfactant BAC and the formulation UBA significantly reduced potato root invasion by *G. pallida* juveniles, and the root and haulm growth of potato plants were increased by treatment with UBA.

The presence of *G. pallida* in pots decreased the haulm weight by 4% and root weight by 8%, though these results were not significantly different. Root mass and haulm growth are generally correlated to yield (Allen and Scott, 1980; Beukema *et al*., 1990; Iwama, 2008; Lahlou and Ledent, 2005; Whitehead, 1977). Potato plants infested with potato cyst nematodes (PCN) grow slower, are dwarfed with smaller leaves and senesce earlier than plants that are not infected with nematodes (Trudgill et al., 1975a). By reducing the effectiveness of the root system, the haulm expansion rate is slowed down, reducing the efficiency of light intercepted by the crop canopy, which ultimately reduces yield (Trudgill, 1986). Maris Peer was the third most popular variety in terms of planted area in the UK in 2017, accounting for 4% of the total area planted (AHDB, 2017), and has low tolerance for *Globodera* spp. (Evans, 1982b; Evans and Franco, 1979; Keer, 2007; Trudgill et al., 2003). Evans (1982) found that yield of Maris Peer plants reduced from 24.8 t ha^{-1} to 11 t ha^{-1} when the initial number of *G. rostochiensis* eggs per gram of soil increased from 8 to 105. Evans and Franco (1979) found that at 100 *G. rostochiensis* eggs g^{-1} soil, yield was reduced by 95% compared to an uninfected field. The initial population in this study was 20 eggs g^{-1} soil, far lower than the studies of Evans (1982) and Evans and Franco (1979). The cultivar Maris Peer is more affected by *G. pallida* than *G. rostochiensis* (Fatemy and Evans, 1986) and initial population densities of potato cyst nematodes considered to result in economic damage to potato are usually fewer than 20 eggs g-1 soil (Bird *et al*., 2018), thus it was expected that 20 eggs g^{-1} soil would have a detrimental effect on the plants in this experiment. Keer (2007) found that when Maris Peer plants were infected with 11 – 14 *G. pallida* eggs g^{-1} soil, treatment with the nematicide Vydate increased yield by 18%. Trudgill and Cotes (1983) found that Maris Peer had a threshold for yield reduction of 36 – 55 eggs g^{-1} soil, approximately double the infection used in this experiment. Further investigations could increase the egg count in pots, although high populations such as those tested by Trudgill and Cotes (1983) and Woods *et al*. (1999) may not be representative of the populations found in the field. More recent data is not available, but Minnis *et al*. (2002) found that about 65% of infested fields had <10 eggs g^{-1} soil and only 10% had >60 eggs g^{-1} soil, thus most growers do not have infestations with high population densities.

Treatment of Maris Peer plants with BAC, UBA and BGT affected the fresh haulm and root weights, though these differences were not statistically significant. Benzalkonium chloride (BAC) increased the root weight of Maris Peer plants in both the presence and absence of *G. pallida* by up to 8%, though the haulm weight was reduced by up to 3%.

The reduction in haulm weight four weeks after planting may be due to the increase in root weight. As more resources are used in establishing a larger root system, the expansion of the canopy is delayed (Beukema et al., 1990). A longer glasshouse experiment would demonstrate whether the haulm growth would recover and increase, though this is likely when the root system is established, as root growth and haulm growth are correlated (Beukema *et al*., 1990). The increase in root growth may be due to the broad spectrum antimicrobial properties of BAC causing mortality in microorganisms in the soil and releasing nutrients, though as the pots were 70% sand, it is more likely that the effect was caused by the amphiphilic nature of the surfactant. Sand-based soils are more likely to be hydrophobic due to the small specific surface area of sand particles (Song *et al*., 2014). Surfactants improve moisture retention in hydrophobic soils by adhering to the soil particles with their non-polar hydrophobic region, and interacting with water in its polar hydrophilic region (Song *et al*., 2014). By improving uniformity in soil moisture throughout the pot, the surfactant may increase root growth by allowing roots to utilise the entire pot.

Treatment with UBA had the most positive affect on Maris Peer plants. Pots treated with UBA had an increase in haulm weight both in the presence and absence of PCN (10% and 7% respectively), and in the presence of PCN increased root weight by 23%. The addition of the non-ionic surfactants alkyl polyglucoside (APG) and alcohol ethoxylate (AEO) increased root growth from 3.07g in *G. pallida* infested plants treated with BAC to 3.55g in *G. pallida* infested plants treated with UBA. The increase in root growth between BAC treated plants and UBA treated plants may be due to an overall increase in percentage active ingredients, as the total w/v% increased from 0.15% to 0.33%. It is more likely, however, that the wetting properties of non-ionic surfactants have increased the effect seen in pots treated with BAC. Cationic surfactants adsorb more strongly to soil organic matter than non-ionic surfactants due to the positive charge (Khan *et al*., 2017), which reduces their efficacy as wetting agents. Non-ionic surfactants have previously been shown to increase plant root growth in cucumber seedlings (Parr and Norman, 1964). Further evaluation of the formulations could include the non-ionic surfactants without the cationic surfactant BAC, though they did not demonstrate any efficacy *in vitro* against *G. pallida* in Chapter 4.

Of the three products tested in this experiment, BGT was the least effective. It reduced both haulm and root weight by up to 11% in the absence of *G. pallida*, and it increased both by only 2% in the presence of *G. pallida*. The cause of the phytotoxicity may be

PHMB, though it has been used to stabilize silver nanoparticles for treatment of oilseed rape (Gusev *et al*., 2014) and fodder beet (Gusev *et al*., 2016). The organosilane Si-QAC could also cause phytotoxicity as is not commonly found in agrochemical formulations, though it has been found to decrease erosion in soil (Daniels and Hourani, 2009), possibly due to its high soil adsorption coefficient, which indicates very strong adsorption to soil particles (ECHA, 2018; Khan *et al*., 2017). Either of these compounds may be antagonistic to the functions of BAC, AEO and APG in the soil. The high affinity for adsorption of Si-QAC may lock in other components, so that they are unable to function in the soil.

Root invasion assessments were made on roots removed from infected pots. Roots were stained with acid fuchsin and the number of juveniles of each life stage counted. The control plants were infected with 476 juveniles per gram of root, similar to those of Norshie *et al.* (2016) (259 and 358 juveniles g^{-1} root from an initial population density of 18 and 11 eggs g^{-1} soil respectively) but much lower than the infection in experiments by Woods *et al.* (1999) (3767 juveniles g^{-1} root from an initial population density of 140 eggs g^{-1} soil) and Woods and Haydock (2000) (1700-1850 juveniles g^{-1} root from an initial population density of 37 eggs g^{-1} soil). The invasion of roots by juveniles is density dependent, if a small population is present then a large proportion of that population will infect the roots as space and food is not limited (Whitehead, 1973). The haulm and root growth of the *G. pallida* susceptible Maris Peer was not significantly reduced by infection with *G. pallida*, though this may be caused by the low level of infection. Trudgill and Cotes (1983) found that Maris Peer had a threshold for yield reduction of 36 – 55 eggs per gram of soil with haulm and root growth and yield being impacted at over 300 juveniles per gram of root tissue. At 600 eggs per gram of soil the root invasion assays performed by Trudgill and Cotes (1983) found approximately 3,000 juveniles per gram of root in three week old plants. Woods *et al.* (1999) found that application of Fosthiazate at rate of 2 mg kg^{-1} yielded a 99.5% reduction in root invasion of *G. pallida*, though when tested at an equivalent rate by Norshie *et al.* (2016), the efficacy was reduced to 63% at the Woodcote site and 87% at the Howle site (Shropshire, UK). This may be due to different levels of infestation in the soil; the control counts for root invasion in Woods *et al.* (1999) trial was 3767 juveniles per gram of root, whereas Norshie *et al.* (2016) had controls of 258.5 and 358 juveniles per gram of root. Norshie *et al.* (2016) also tested fluensulfone which reduced root invasion by 76% at Woodcote and 61% at Howle; and oxamyl which reduced root invasion by 81% at Woodcote and 77% at Howle. In both experiments, no significant difference in root or haulm weight was found, nor between either ware or total yields between treatments. Kiezebrink (2009) also found that treatment with oxamyl did not significantly change root invasion, multiplication of *G. pallida* or potato yield in several experiments performed over three years at three different sites. The controls for root

invasion assessments made 27 – 34 days after planting ranged between 200 – 400 juveniles per gram of root. The reduced efficacy observed by Kiezebrink (2009) may be due to the low number of juveniles invading roots in the control plots, which are comparable to those obtained in this experiment. Further glasshouse experiments should contain a range of infection levels, to determine whether the test products are able to reduce invasion at high and low infection pressures and may improve statistical significance of results. In this experiment only second-stage juveniles (J2) and third stage juveniles (J3) were recovered; 88% of the juveniles recovered were J2 and the remainder were J3. These results differ to those of Kiezebrink (2009), who found all life stages in roots recovered 27 – 34 days after planting Maris Piper and Saturna. Maris Piper, Saturna and Maris Peer all score 2 for resistance to *G. pallida* according to the AHDB varieties database (http://varieties.ahdb.org.uk/), though the *G. pallida* population, seed tuber size and growing conditions (glasshouse versus field experiment) could account for the differences found. To determine the effect of the test products on the development of *G. pallida* in the roots, and proportion of females to males, the length of further glasshouse experiments should be increased beyond four weeks.

Soil moisture measurements were not taken during the experiment as the pots used (0.37 L) were too small to fit a soil moisture probe into without damaging plant roots. At four weeks after planting when plants were removed from their pots, plant roots had filled the containers and were growing out of drainage holes at the base of the pots. Root and shoot growth, plant water relations and nutrient uptake are affected by root restriction and container size (NeSmith and Duval, 1998). Further glasshouse experiments should be performed in larger containers, which would enable the measurement of soil moisture as well as monitoring plant growth over a longer time period. By using very large containers such as tonne bags, the yield would also be able to be measured.

Benzalkonium chloride and UBA significantly reduced the number of juveniles infecting roots by 29% and 27% respectively. BGT again demonstrated the least efficacy, reducing invasion by 21%. The proportions of J2 and J3 were similar in the control, BAC and BGT, though UBA lowered the proportion of J2 to 75% (from 88%). This may be indicative of the product building up in the soil or an increase in longevity, as fewer juveniles are invading later in the experiment. These results suggest that one or both of the components Si-QAC and PHMB have a detrimental effect on the formulation, as haulm growth, root growth and infection by *G. pallida* juveniles were least improved by the BGT formulation and most improved by the UBA formulation.

Although BAC and UBA significantly reduced the number of juveniles infecting roots, the number of juveniles only reduced from 476 juveniles g^{-1} root to 348 juveniles g^{-1} root tissue. Though this may reduce disease pressure on the young root system, it may not be

beneficial to the grower in the long run. The multiplication rate of *G. pallida* is inversely proportional to the initial population density, and the determination of sex is affected by the amount and quality of food provided by the syncytia (Trudgill, 1967). If there is adequate nutrition and space, then the juvenile will mature into a female. If food is scarce, then the juvenile will mature into a male, as they require less nutrition (Trudgill, 1967). Males are also more likely to develop when there are high initial population densities of PCN (Trudgill, 1967). A longer field or glasshouse experiment would be required to determine the effect on the multiplication rate of *G. pallida* after treatment with BAC, UBA or BGT.

Of the three products tested in this experiment, UBA demonstrated the greatest efficacy by increasing haulm and root weights and significantly reducing the number of juveniles invading roots. Inoculation of pots with *G. pallida* caused a 4% loss of haulm weight and 8% loss of root weight. Treatment with UBA increased haulm weight in infested plants to 5% more than that of the uninfested untreated plants. The root weight in infested plants treated with UBA was also higher than the uninfested untreated control, by 13%. As root mass and haulm growth are generally correlated with yield (Allen and Scott, 1980; Beukema *et al*., 1990; Iwama, 2008; Lahlou and Ledent, 2005; Whitehead, 1977), these results may suggest that treatment of infested plants with UBA could negate yield loss caused by *G. pallida*.

Although the test products reduce hatching of *G. pallida* and invasion of potato roots, they do not appear to cause mortality in unhatched juveniles (Chapter 5), thus hatch is effectively being delayed. It is unknown whether hatching would recommence if cysts were reintroduced to potato root diffusate the following season (without further treatment). Whitehead (1973) stated that if hatching is delayed, performing root invasion analysis four weeks after planting is not a useful tool in determining *in situ* efficacy and pre- and post-season viability of recovered eggs gives the best estimation of nematicidal activity. Peachey and Hooper (1963) found that the same treatments were most effective at reducing hatching, invasion, the number of cysts on roots and multiplication, so root invasion should give a good estimation of efficacy *in situ*. As the test products BAC, UBA and BGT do not appear to act as nematicides, viability assessments may not demonstrate efficacy, as hatching may be delayed until the potato root system is established, thus allowing invasion of the roots without causing a loss in yield. Further testing should include large pot or field plot experiments that are able to measure marketable yield, as that is the benefit to the grower of using nematicidal products.

6.5 Conclusion

A glasshouse experiment was conducted to measure the effects of the cationic surfactant BAC and the formulations UBA and BGT on the haulm weight and root growth of potato plants and invasion of potato plant roots by *G. pallida* juveniles. Benzalkonium chloride reduced the number of *G. pallida* juveniles invading Maris Peer roots by 29%. The efficacy of the cationic surfactant was increased when non-ionic surfactants alkyl polyglucoside (APG) and alcohol ethoxylate (AEO) were added to BAC to form UBA. The formulation UBA also reduced the number of *G. pallida* juveniles invading Maris Peer roots, by 27%, and also increased the haulm and root growth by up to 23%. The addition of a biguanide (PHMB) and a silyl quaternary ammonium compound (Si-QAC) to UBA to form BGT reduced the efficacy of the formulation. Further investigations are required to determine the effect of the formulations on potato yield and on the multiplication rate of *G. pallida*, and the results of this chapter indicate that UBA is the best candidate for further investigations into the effect of this formulation on *G. pallida* and potato plant growth *in situ*.

Chapter 7 : General Discussion

Introduction

Potato (*Solanum tuberosum*) is an essential crop in the UK and around the world. The starch-rich tuber was the 5th most produced commodity in the world in 2016, behind sugar cane, maize, wheat and rice (FAO, 2018). The most important soil pests of potato production are potato cyst nematodes, *Globodera pallida* and *G. rostochiensis*, which are subject to regulation and quarantine by the European Union (The Council of the European Union, 2000). A survey in 2016 found that potato cyst nematodes were present in 48% of sites sampled, and of the populations found, 89% were *G. pallida*, 5% were *G. rostochiensis* and 6% contained both species (Dybal-Lima *et al*., 2016). Potato cyst nematodes are responsible for an estimated 4% in lost yield potential, costing the industry £25 million annually (Twining *et al*., 2009). The infectious life stage of *Globodera* spp. is protected from physical and chemical stresses in eggs within cysts in the soil until prompted to hatch by favourable environmental conditions and hatching factors contained in diffusate from potato plant roots. After hatching, the infective J2s invade plant roots to feed and develop, causing a number of symptoms of damage in potato plants, including decreased growth, increased susceptibility to pathogens and reduced yield (Back et al., 2002; Trudgill et al., 1975b).

Management of potato cyst nematodes (PCN) is complex and multifaceted. Several management methods may be implemented together in an integrated pest management (IPM) program to reduce their population and prevent yield loss. These include the use of resistant and tolerant cultivars, crop rotation and the use of chemical nematicides (Back *et al*., 2018). Plant protection products such as nematicides are regulated by both EU and local legislation, including Regulation (EC) No 1107/2009, which has listed several of the nematicides currently used in the UK as 'candidates for substitution' and may revoke registration of those products where safer alternatives are available (European Commission, 2009). Without these products, Twining *et al*. (2009) estimated that yield losses from PCN could increase from £25 million to £55 million. Development and commercialisation of novel nematicides can take up to 10 years and costs up to US$60 million (Haydock *et al*., 2013). This timescale and cost are prohibitive to the development of novel chemical control agents for PCN, particularly for small to medium enterprises (SME). Current research into alternative control measures for PCN include biofumigation, trap crops, biocontrol and plant extracts (Back *et al*., 2018). There is demand for plant protection products that are low in toxicity to non-target organisms such as operators, bees and other wildlife, and the environment that provide an economic benefit to growers

and are complementary to other control measures in an integrated pest management programme.

The novel formulation BGT consists of a blend of cationic and non-ionic surfactants. Surfactants are amphiphilic chemicals utilised for their solubilisation and cleaning purposes (Merianos, 2001; Ying, 2006) and are commonly utilised in agrochemical formulations due to their wetting properties (Iglauer *et al*., 2010). The cationic surfactants quaternary ammonium compounds (QACs) are broad spectrum antimicrobial agents and cause disruption to lipid membranes by insertion of their hydrophobic tail into the membrane (Maillard, 2002). Several surfactants have been found to have deleterious effects on nematodes including benzalkonium chloride (BAC), a component of BGT (Höss *et al*., 2010; Peachey and Hooper, 1963). The formulation BGT may have an effect on the viability, hatching and infection of the potato cyst nematode *G. pallida* by disrupting lipid membranes found within the juveniles and egg shell. The components of BGT interact with and disrupt the phospholipid bilayer of cell membranes, and bind to proteins (Cooper, 2000; Maillard, 2002; McDonnell and Russell, 1999). The proposed targets of BGT in *G. pallida* juveniles are the internal membranes that separate the internal organs and maintain pressure for movement, and the lipid reserves. The innermost lipoprotein layer of *G. pallida* eggshells is the main permeability barrier of the eggshell (Jones *et al*., 1998), and may be affected by BGT.

This study aims to evaluate the potential of the formulation BGT as a control method for *G. pallida*. Several experimental methods were used to determine the effect of BGT on the mortality, hatching and infectivity of *G. pallida in vitro* and in soil. Mortality assays demonstrated that BGT caused up to 100% mortality in J2s of *G. pallida* after 24 h contact, and microscopy of treated juveniles revealed that BGT caused disruption of internal membranes and lipid reserves within 72 h contact (Chapter 2). Hatching assays demonstrated that BGT reduced hatching of *G. pallida* juveniles in PRD after 24 h contact by up to 76% (Chapter 3), and that the active ingredient responsible for the suppression of hatch was the cationic surfactant BAC (Chapter 4). Further hatching studies found that the suppression of hatch by BAC, UBA (a formulation comprising BAC and two non-ionic surfactants) and BGT could be improved by increasing the contact time from 24 h to 8 weeks (the length of the hatching assay) (Chapter 5). Microscopy performed on eggs treated with BAC, UBA and BGT revealed that no physical damage was caused to unhatched *G. pallida* juveniles, which suggests that the mode of action of the test products on suppressing hatch is by interfering with the hatching cascade, and not by increasing eggshell permeability and causing mortality in unhatched juveniles (Chapter 4). To investigate the effect of BAC, UBA and BGT on the infectivity of *G. pallida* juveniles *in*

situ, a glasshouse experiment was performed, and the results indicate that the formulation UBA may have potential as a plant protection product.

The effect of BGT on mortality of second-stage juveniles of *G. pallida*

The initial mortality assays in Chapter 2 focused on the effect of BGT on the infective stage of *G. pallida*, the second-stage juvenile. After 24 h contact with BGT, up to 100% of *G. pallida* juveniles were immobile (Figure 2.1). BGT was observed to affect the movement of juveniles in solution within an hour of contact (Figure 2.2). Juveniles exposed to BGT for 72 hours experienced disruption of internal membranes and breakdown of the lipid reserves (Figure 2.3). This damage would render the juvenile immobile and unable to utilise the lipid energy stores.

The physical symptoms observed during microscopy of treated juveniles - disruption of internal membranes vital for movement and loss of lipid energy stores - suggest that the results obtained from the mortality assays are irreversible and that BGT is able to prevent hatched juveniles from infecting plant roots. The J2s of *G. pallida* damage potato plant roots by penetrating root tissue and migrating through the root system to locate and establish a suitable feeding site (Jones and Northcote, 1972), which reduces the roots' ability to take up water and nutrients (Trudgill et al., 1975a) and allows entry of opportunistic pathogens into the root system (Back *et al*., 2006; Storey and Evans, 1987). If it is possible to reduce the number of J2s in the soil, the severity of infection of and damage to the potato plants could be limited. The results of this study suggest that the mode of action of BGT on J2s of *G. pallida* may be transferrable to other nematodes species including other economically important plant parasitic nematodes such as *Pratylenchus* spp.. Nematodes are relatively conserved in morphology, despite having diverse lifestyles. Their filiform body consists of an external wall, a pseudocoelomic cavity maintaining pressure for movement and containing organs such as the reproductive system, and an internal digestive system, and contains a number of membranes that could be disrupted by BGT (Decraemer and Hunt, 2013).

The effect of BGT on hatching of *G. pallida*

Although BGT caused mortality in J2s of *G. pallida*, they are only present in the soil for a limited time period. Unhatched *G. pallida* juveniles remain viable within encysted eggs for up to 30 years (Turner, 1996), whilst hatched juveniles are only found in the soil up to eight weeks after plant emergence, and are only able to survive for up to 11 days in the soil after hatching (Robinson *et al*., 1987). Second-stage juveniles of *G. pallida* hatch in response to stimulation by hatching factors found in potato root diffusate, or in fewer numbers as a spontaneous response in water (Hominick *et al*., 1985; Rawsthorne and

Brodie, 1986). When designing a product to reduce nematode population, the encysted eggs are the most obvious target as they are in the soil much longer than the infectious J2s. If BGT can target unhatched juveniles or prevent juveniles hatching, it would provide more protection to plants than by killing a proportion of juveniles that have hatched and could be used at any time in the crop rotation.

The effect of BGT on hatching of *G. pallida* juveniles was investigated in Chapter 3 by conducting *in vitro* hatching assays. Cysts were immersed in BGT for 24 h prior to hatching over 8 weeks in potato root diffusate (PRD). The number of juveniles hatching each week and the number remaining within cysts at the end of the hatching assay were counted and the percentage hatch calculated. Percentage hatch is a good measure of viability as it gives the number of juveniles that are likely to hatch when in contact with the host plant diffusate and is commonly used when evaluating potential products for management of potato cyst nematodes (Danquah *et al*., 2011; Ibrahim and Haydock, 1999; Twomey *et al*., 2000; Woods *et al*., 1999). Hatching assays were performed in preference to viability assays such as staining with Meldola's blue (Ogiga and Estey, 1974) or trehalose measurement (van den Elsen *et al*., 2012) as hatching is more representative of the level of infection a host plant would be subjected to. Although hatching assays are subject to variation due to the variable nature of PRD, Kroese *et al*. (2011) found that the estimated viability made by Meldola's stain and hatching assays were similar on cysts extracted from the field. By standardising results as 'percentage reduction from control', different experiments can be compared, reducing the variation caused by different populations and PRD.

In the hatching experiments described in Chapter 3, exposure to BGT for 24 h reduced hatching of *G. pallida* by up to 76% at concentrations of 0.02% to 10%, in contrast to low concentrations of BGT (1E-06%, 0.01% and 0.01%), which caused stimulation of hatch by up to 17% more than the control (Figure 3.2). Chemicals capable of disrupting lipoprotein membranes and affecting membrane permeability may be expected to stimulate hatch (Jones *et al*., 1998). This can occur by triggering similar mechanisms to that of hatching in response to stimulation by hatching factors in potato root diffusate, such as increasing membrane permeability and releasing trehalose (Jones *et al*., 1998). Stimulation of hatch at low concentrations has been observed in a number of studies into nematicidal and nematostatic compounds, including the garlic extract tested by Danquah *et al*. (2011) and fosthiazate (Woods *et al*., 1999). The stimulation of *G. pallida* hatching by BGT could be explored and exploited for use prior to the growing season, by promoting hatch when no host plants are in the ground. Both artificial hatching factors and those derived from plant leachates have been investigated in stimulating hatch of *G. pallida* in the absence of host plants (Byrne *et al*., 2001; Devine and Jones, 2001; Whitehead, 1977). Single hatching

factors such as picrolonic acid may not be an effective control method, as they can be species-selective, but the use of mixed preparations such as potato or tomato root diffusate reduces the risk of selection of PCN populations (Byrne *et al*., 2001); application of tomato root diffusate to *G. pallida* infested soil resulted in up to 79% reduction in the number of viable eggs per cyst recovered 12 weeks after treatment (Devine and Jones, 2001).

Investigating the formulation of BGT

Development and registration of nematicides and other plant protection products generally focuses on a single active ingredient (Haydock *et al*., 2013). BGT, however, is a blend of five components, and therefore to investigate the mode of action and improve the efficacy of the formulation, the individual components were examined alone and in combinations using hatching assays (Chapter 4).

The microscopy performed on J2s of *G. pallida* in Chapter 2 indicate that mortality is caused by disruption of the lipid membranes (Figure 2.3). This suggests that cationic surfactants such as BAC, which are more active against lipid membranes than non-ionic surfactants (McDonnell, 2009), would be more effective in reducing hatching of *G. pallida*. The results of Chapter 4 suggest that the mode of action may be more complex than simply rupturing lipid membranes as treatment with the cationic compound Si-QAC, which produced a 22% reduction in hatch, was similar in efficacy to that of the non-ionic alcohol ethoxylate (AEO), which produced a 20% reduction in hatch. The most effective component however was BAC which reduced hatch by 69% (Figure 4.1). The efficacy of BAC in reducing hatching of *G. pallida* was comparable to that of the formulation BGT, which gave a 70% reduction, suggesting that BAC is the sole active ingredient responsible for the reduction of hatch of *G. pallida* within the BGT, and that the other components have little beneficial, synergistic or additive action.

The polymeric biguanide PHMB only reduced hatching of *G. pallida* by 12%, comparable to the non-ionic alkyl polyglucoside (APG), though it is the most effective component of BGT against bacteria. The minimum biocidal concentration (MBC) of PHMB is <0.05 mg L^{-1} for *Staphyloccocus aureus* and *Escherichia coli* (Rembe *et al*., 2016) much lower than the MBC of BAC for the same organisms, which is 45 mg L^{-1} (Fazlara and Ekhtelat, 2012). The biocidal mode of action of PHMB is widely reported to be interaction with the cytoplasmic membrane (Ikeda *et al*., 1984), but Chindera *et al.* (2016) found that PHMB did not cause mortality in *E. coli* by increasing membrane permeability, rather it selectively bound to and condensed bacterial chromosomes and was excluded by mammalian nuclei. The results of Chindera *et al*. (2016) on the mode of action of PHMB on *E. coli* explain

why it did not significantly reduce hatching in *G. pallida*, as it does not cause disruption of and increased permeability of lipid membranes.

The results of Chapter 4 (Figure 4.1) demonstrated that BAC is the active ingredients responsible for reducing hatch of *G. pallida*. Further hatching experiments included a second formulation, UBA, which contained the cationic surfactant BAC and the non-ionic surfactants AEO and APG. The components Si-QAC and PHMB were removed from the evaluation for a number of reasons: their low efficacy in reducing *G. pallida* hatch, the cost of production of Si-QAC and the potential carcinogenic properties of PHMB (Creppy *et al*., 2014). The non-ionic surfactants AEO and APG were retained in the UBA formulation with BAC to evaluate their efficacy as adjuvants. The non-ionic surfactants AEO and APG are two of the most commonly used surfactants in agriculture, and are commonly co-formulated due to their synergistic activity (Bergstrom and Johansson, 2005; Knoche, 1994). The non-ionic surfactants decrease foaming of formulations, help spray homogeneity, increase the stability of active ingredients and act as wetting agents (Bergstrom and Johansson, 2005; Cserháti, 1995; Hazen, 2000). Non-ionic additives possess good penetrating, good solubility and spreading behaviour that are utilised to increase efficacy of a number of active ingredients (Bergstrom and Johansson, 2005; Castro *et al*., 2013). Arriaga *et al*. (2009) and Cooley *et al*. (2009) found that the use of alcohol ethoxylates on potato growing land reduced nitrogen leaching and improved water distribution, reducing the impact of dry zones that often appear on sandy soils.

The effect of increasing contact time with treatment on hatching of *G. pallida*

The hatching studies performed in Chapters 3 and 4 exposed *G. pallida* cysts to test products for 24 h prior to hatching in potato root diffusate for eight weeks. Other studies on hatch inhibitors of *G. pallida* have used contact times far longer than 24 h, and included the test chemicals with PRD (Danquah *et al*., 2011; Twomey *et al*., 2000; Woods *et al*., 1999). To determine whether the effect of the formulations BGT and UBA on *G. pallida* hatch could be improved by increasing the contact time beyond 24 h a series of hatching assays was performed. When the contact time between *G. pallida* cysts and the test chemicals was extended by including BGT or UBA with PRD throughout the hatching assays, suppression of hatch reached 97%. In comparison with other products, prolonged contact with the formulation UBA provided greater efficacy at reducing *G. pallida* hatching than the garlic-based formulation G8014S, 2-propenyl isothiocyanate and DiTera, which reduced hatching of *G. pallida* by 46%, 50% and 80% respectively (Brolsma *et al*., 2014; Danquah *et al*., 2011; Twomey *et al*., 2000). UBA also provided greater efficacy than the 90% reduction caused by fosthiazate as reported by Woods *et al*. 1999 and was

comparable in efficacy (98%) to Rugby 100ME at 0.25 µg L^{-1}, which contains cadusafos (Ibrahim and Haydock, 1999).

In Chapter 4 it was suggested that BAC could increase membrane permeability of the eggshell and cause mortality of the juvenile within the egg prior to hatching of the juvenile, as vigorous movement of the J2 does not begin until at least 3 days after initial exposure to root diffusate (Masler and Perry, 2018), though microscopy performed on eggs exposed to the test solutions demonstrated that this is not the case (Figure 5.4). If BAC increased membrane permeability of the eggshell and caused mortality of the juvenile, it would be expected that the juvenile would sustain the same damage as seen in Chapter 2 (Figure 2.3) when second-stage *G. pallida* juveniles were exposed to BGT, which contains BAC. Second-stage juveniles of *G. pallida* exposed to BGT 6% for 72 h suffered loss of differentiation within the body cavity, no clear distinction between the pharynx and digestive systems and disruption of the lipid reserves (Figure 2.3). When cysts were exposed to BGT, UBA or BAC no damage to the juveniles within eggs was observed (Figure 5.4). The juveniles within eggs exposed to BGT do not show any of the symptoms of membrane disruption described in Chapter 2 such as disruption of the lipid reserves, and do not appear different to the control (exposed to water). These results demonstrate that BAC does not directly affect the juveniles within eggs and does not cause in-egg mortality of *G. pallida* juveniles. Hatch of *Globodera* spp. is stimulated by hatching factors found in potato root diffusate that trigger a cascade of events resulting in changes in the eggshell, activation of the unhatched juveniles and eclosion (Masler and Perry, 2018). Hatching factors displace internal Ca^{2+} from binding sites in the lipoprotein layer of the eggshell, causing increased permeability (Clarke and Perry, 1985). It is possible that BAC interacts with the eggshell membrane and prevents the interaction between the hatching factors and the eggshell membrane, as cationic surfactants such as BAC interact with integral membrane proteins and affect the charge separation across the surface of membranes (Cooper, 2000). Alternatively, BAC may interact with the potato root diffusate and render the hatching factors (HF) ineffective, though it is unlikely that the surfactant acts in the same way as natural hatch inhibitors (HI) found in potato root diffusate as the HI inhibition of HF-induced hatch was reversible (Byrne *et al*., 1998), whilst the inhibition of hatch by BAC, UBA and BGT were irreversible. Twomey *et al*. (2000) found that the product DiTera prevented changes in eggshell permeability in both *G. pallida* and *G. rostochiensis*, possibly by binding to or blocking hatching factor binding sites on the eggshell. The mode of action of BAC may be similar. It is unknown whether BAC interacts with the eggshell directly and / or the PRD; to determine this would require further investigation using techniques such as atomic force microscopy to view the membrane in greater detail. Investigation of other cationic surfactants such as didecyldimethylalkonium chloride (DDAC) might also assist to further elucidate the mode

of action. The increase in efficacy of UBA from 29% to 46% reduction when PRD is present during 24 h treatment and a loss of efficacy after rinsing suggest that there is some interaction between the PRD and UBA.

The fungal fermentation product DiTera prevents hatching of *G. pallida* and *G. rostochiensis* without causing mortality of the juvenile within (Twomey *et al*., 2000), as does UBA. DiTera not only affects hatching of *G. pallida* and *G. rostochiensis* but has also been found to prevent sensory perception of *G. rostochiensis* and reduce stylet thrusting of *G. rostochiensis* and *Ditylenchus dipsaci*. Sensory studies such as those performed by Twomey *et al*. (2002) would help to elucidate whether the sensory functions of *G. pallida* are similarly affected by UBA at sub-lethal concentrations, and inhibit hatching of the nematode. The mode of action of DiTera in reducing hatch of *Globodera* spp. is thought to be by interference with the hatching cascade, as it was unable to reduce hatching of *Meloidogyne incognita*, which hatches readily in water (Twomey *et al*., 2000). As both DiTera and UBA are thought to interfere with the Ca^{2+}-mediated hatching cascade, the mode of action of UBA on *G. pallida* eggs may not be transferrable to all plant parasitic nematodes; not all plant parasitic nematodes require stimulation from hatching factors (Jones *et al*., 1998). There are, however, a number of other economically important species of plant parasitic cyst nematodes that hatch preferentially in host plant root diffusates in addition to *Globodera* spp., including Soybean cyst nematodes (*Heterodera glycines*), Sugar beet cyst nematodes (*H. schachtii*) and Carrot cyst nematode (*H. carotae*). Generally, species with a narrow host range tend to have a strong dependency on the presence of diffusates from host plant roots in order to hatch (Moens *et al*., 2018). Twomey *et al*. (2000) used a fluorescent dye to confirm that the DiTera prevented Ca^{2+}-dependent alteration of eggshell permeability and therefore further investigation of UBA and *G. pallida* could include a similar assay to confirm that UBA also prevents increased eggshell permeability and would support the observations made following microscopy of treated eggs found in Chapter 5.

The effect of BAC, UBA and BGT on potato plant growth and root invasion by *G. pallida*

Hatching assays are commonly utilised in screening agrochemicals to identify active ingredients and examine mode of action, but do not guarantee the efficacy of an active ingredient in the field (Brolsma *et al*., 2014). There are several variables that can affect active ingredients in the soil, including soil type, pH, organic matter content, moisture and temperature (Nieder *et al*., 2018). Root invasion assays are a useful method of screening the efficacy of products against *G. pallida* in soil whilst being able to control a number of the aforementioned variables (Whitehead, 1973). Although hatching assays assess the efficacy of products in preventing hatch of juveniles, root invasion assays also include

assessment of the infectivity of juveniles hatching and their development within the roots and give insight into the efficacy of the test chemical *in situ*. In addition, root invasion assays may also indicate effects on host finding or hatch suppression if done using the 'cysts in sachet' technique, where cyst can be recovered and the remaining eggs counted and examined. The timing of application of agrochemicals is crucial to ensure optimal efficacy, along with concentration and application method. Application by drip irrigation reduces the exposure of growers to the active ingredient and allows targeted application of agrochemicals into the soil throughout the season, increasing the longevity of their activity. The liquid formulations BGT and UBA are water soluble and thus good candidates for application by drip irrigation. The longevity of BGT and UBA in soil is currently unknown, therefore multiple applications were applied to pots in order to increase the contact time between the test products and the *G. pallida* cysts. Maris Peer potato tubers were planted in sandy soil, either with or without *G. pallida* cysts. Fresh haulm weight, fresh root weight and root invasion assessments were made four weeks after planting, after four treatments with either water, BAC, UBA or BGT. The presence of *G. pallida* at 20 eggs g^{-1} soil in untreated pots decreased the haulm weight by 4% and root weight by 8%. This damage may have been increased if the initial population density had been higher. Pots were infected with 20 eggs g^{-1} soil, which could be considered to result in economic damage to potatoes (Bird *et al*., 2018) and is representative of the populations found in the field (Minnis *et al*., 2002), though is lower than the damage threshold determined by Trudgill & Cotes (1983) of 36 – 55 eggs g^{-1} soil.

Treatment with UBA had the most positive affect on Maris Peer plants. Pots treated with UBA had an increase in haulm weight of 10% in the presence of PCN and 7% in uninfected plants, and increased root weight by 23% in the presence of PCN, though there were no significant differences between any haulm or root weight measurements. The increase in root weight when non-ionic surfactants AEO and APG were added to the cationic surfactant BAC (to form UBA) is likely to be caused by the wetting properties of the non-ionic surfactants. Sand-based soils are more likely to be hydrophobic due to the small specific surface area of sand particles (Song *et al*., 2014). Surfactants improve moisture retention in hydrophobic soils by adhering to the soil particles with their non-polar hydrophobic region, and interacting with water in its polar hydrophilic region (Song *et al*., 2014). By improving uniformity in soil moisture throughout the pot, the surfactant may increase root growth by allowing roots to utilise the entire pot. Non-ionic surfactants have previously been shown to increase plant root growth in cucumber seedlings (Parr and Norman, 1964). Soil moisture readings were not made during the experiment due to the small pot size used, but should be included in any further studies to identify any differences in soil moisture between untreated and treated pots. Further evaluation of the formulations could include the non-ionic surfactants without the cationic surfactant BAC,

though they did not demonstrate any efficacy *in vitro* against *G. pallida* in chapter four. Of the three products tested in this experiment, BGT was the least effective and did not increase haulm nor root weight of plants, whether infected with *G. pallida* or not. This may be due to phytotoxicity of PHMB and / or Si-QAC, or due to the adsorption of Si-QAC. Cationic surfactants adsorb more strongly to soil organic matter than non-ionic surfactants due to the positive charge (Khan *et al*., 2017), which reduces their efficacy as wetting agents. In this glasshouse experiment, 30% of John Innes No. 2 compost was added to 70% sand, creating a soil mix that was relatively low in organic matter. Further studies should include soils with a range of organic matter content to observe any reduction in efficacy caused by increasing the organic matter content. The silane groups of Si-QAC bind covalently to hydroxylated and hydrophilic surfaces such as silt and sand (Vasilev *et al*., 2009), which may further reduce the formulation's efficacy in moving through the soil and may also lock in the other components.

Root invasion assessments were made on roots four weeks after planting, and untreated control plants contained 476 juveniles g^{-1} root. Norshie *et al.* (2016) had similar root invasion counts in their control samples (259 and 358 juveniles g^{-1} root from an initial population density of 18 and 11 eggs g^{-1} soil respectively), though these are much lower than the infection in experiments by Woods *et al.* (1999) (3767 juveniles g^{-1} root from an initial population density of 140 eggs g^{-1} soil) and Woods and Haydock (2000) (1700-1850 juveniles g^{-1} root from an initial population density of 37 eggs g^{-1} soil). Trudgill and Cotes (1983) found that haulm and root growth and yield of Maris Peer was impacted at infection over 300 *G. rostochiensis* juveniles per gram of root tissue, and as Maris Peer is more affected by *G. pallida* than *G. rostochiensis* (Fatemy and Evans, 1986) it would be expected that loss of yield would occur with >400 *G. pallida* juveniles per gram of root tissue. Although Benzalkonium chloride and UBA significantly reduced the number of juveniles infecting roots by up to 29%, the number of juveniles within the roots was reduced by 128 juveniles g^{-1} root, from 476 juveniles g^{-1} root to 348 juveniles g^{-1} root tissue. Though this may reduce disease pressure on the young root system, it may not be beneficial to the grower in the long run; the multiplication rate of *G. pallida* is inversely proportional to the initial population density, and the determination of sex is affected by the amount and quality of food provided by the syncytia (Trudgill, 1967). If there is adequate nutrition and space, then the juvenile will mature into a female. If food is scarce, then the juvenile will mature into a male, as they require less nutrition (Trudgill, 1967). Males are also more likely to develop when there are high initial population densities of PCN (Trudgill, 1967). A longer glasshouse or field experiment would be required to determine the effect on the multiplication rate of *G. pallida* after treatment with BAC, UBA or BGT.

PCN caused 4% loss of haulm weight and 8% loss of root weight in untreated plants. Treatment with UBA increased haulm weight in PCN plants by 5% over the non-PCN control and root weight by 13%, thus PCN infected plants treated with UBA were larger than plants not infected with PCN (and not treated). Root mass and haulm growth are generally correlated to yield (Allen and Scott, 1980; Beukema *et al*., 1990; Iwama, 2008; Lahlou and Ledent, 2005; Whitehead, 1977), therefore if the results of this experiment are representative of yield, treatment of infected pots with UBA could negate any yield loss caused by PCN. The benefit to growers of using agrochemicals is to increase yield and quality. To determine whether UBA increases the yield and quality of a potato crop, further glasshouse and field experiments are required. These experiments would also give insight into the long-term effects of UBA on *G. pallida*, including long term viability and the hatching activity when exposed to potato root diffusate the following growing season.

The effect of BGT on non-target organisms

BGT is broad spectrum antimicrobial formulation, and may have deleterious effects on non-target organisms in the soil, such as beneficial bacteria, fungi and nematodes. Bryan (2017) conducted a number of studies into the ecological impacts of BGT. The authors found that no significant chances in total abundance of bacterial communities were detected over a growing season when BGT (100%) was applied four times at a rate of 4L/ha or less. The author also found that in controlled toxicity assays, BGT was found to be “of relatively low toxicity” to earthworms. Germination assays were also performed on wheat and tomato seeds, and though a reduction in seedling growth was found, there were no phytotoxic effects on wheat plants in glasshouse trials after multiple applications.

Broad spectrum pesticides have the potential to kill beneficial organisms (Timper, 2014), and the results of the mortality assay in Chapter 2 suggest that BGT and UBA may cause mortality of beneficial entomopathogenic nematodes and nematodes that feed on bacteria and fungi. Nematicides are toxic to all nematodes, though some genera may be more sensitive than others (Bongers, 1990). Larger parasitic nematodes such as *Globodera* spp. have longer life cycles and are less fecund than smaller bacterial feeders (Bongers, 1990), for example the model organism *Caenorhabditis elegans* lays eggs approximately 55hr after hatching (Byerly 1976), whilst the life cycle of *G. pallida* females is approximately 30 days from hatching to egg laying (Moens *et al*., 2018). The smaller beneficial species therefore recover faster from mass mortality than larger, plant parasitic species (Bongers, 1990). To determine the effect of UBA on non-plant parasitic nematodes a glasshouse or field experiment could be performed to categorise any decline or change in the population over a growing season or longer.

Further investigations of the test formulations

Further investigation into BAC, UBA and BGT should include plot or field experiments to determine the effects of the test products on development of infected juveniles, and on yield. As surfactants are affected by organic matter, trials should be conducted on several soil types, as the application rate on soils rich in organic matter may need to be increased. Optimisation of the application rate and treatment schedule would increase efficacy, as only one concentration was tested in pots in this study. As the test products are water soluble, tank mixing with other agrochemicals such as fertilisers is a possible application method, as the wetting properties of the formulations may improve fertiliser application.

It is unknown what the long-term effects of BAC, UBA and BGT are on *G. pallida*, the decline rate could be affected as could the hatchability of *G. pallida* cysts in the following season. The effect of the test products on other nematodes is also unknown. Several species of entomopathogenic, bacterial and fungal-feeding nematodes are beneficial to soil health. Though the number of economically important nematodes that require stimulation by root diffusates is limited, the effect of the cationic surfactant BAC on *G. pallida* juveniles is likely to be transferable to other life stages of plant parasitic nematodes that are found in the soil.

The results of this study suggest that BAC (and the two formulations containing the active ingredient) prevents hatching of *G. pallida* in response to stimulation by potato root diffusate by interfering with the Ca^{2+} mediated hatching cascade initiated by hatching factors in PRD. Confirmation of the mode of action could be established by a number of methods, including the use of fluorescent dyes such as acridine orange (as used by Twomey *et al*. (2000a)) to demonstrate that BAC prevents permeability of eggshell membranes. Microscopy techniques such as atomic force microscopy are used to identify changes in membranes of bacteria after treatment with cell permeabilizers, and could be utilized in determining the effect of BAC on nematode cell membranes (Alakomi *et al*., 2006). X-ray microanalysis was used to demonstrate that a calcium binding sialoglycoprotein on the innermost region of the eggshell is an important component of the calcium mediated hatching mechanism and that the hatching inhibitors lanthanum and ruthenium red compete with calcium for binding sites on the eggshell (Atkinson and Taylor, 1983). These techniques could also elucidate whether the BAC interacts with the eggshell directly or with hatching factors in potato root diffusate.

This study is the first to investigate the use of cationic surfactants in the management of potato cyst nematodes. Benzalkonium chloride is one of many cationic surfactants currently utilized for their antimicrobial properties, which could be investigated for activity against potato cyst nematodes and other plant parasitic nematodes.

Conclusion

Potato cyst nematodes *G. pallida* and *G. rostochiensis* are an economically important pest of potatoes, with *G. pallida* occurring in 48% of fields surveyed in England (Dybal-Lima *et al*., 2016). Due to the toxicity and ecotoxicity concerns surrounding nematicidal chemicals currently registered for use in the UK, research into alternative methods is imperative.

The surfactant-based formulation BGT contains cationic compounds which alter cell membrane permeability, and has been found to cause mortality in the J2s of *G. pallida*. The formulation BGT was also found to reduce hatching of *G. pallida* from encysted eggs after 24 h contact. The cationic surfactant benzalkonium chloride was found to be the active ingredient in the formulation BGT, and was combined with two non-ionic surfactants to create the second formulation tested in this study, UBA. In the glasshouse experiment, *G.pallida*-infected plants that were treated with UBA had haulm and root weights greater than the uninfected control. UBA also significantly reduced the number of *G. pallida* juveniles invading Maris Peer plant roots. The formulation UBA should be investigated further using plot and field experiments to determine whether the results of the pot experiment can be achieved in a field setting, and to determine whether UBA can protect potato yield from infection with *G. pallida*.

This study is an initial investigation into a novel method for control of *G. pallida*, and the information gained throughout may be transferrable to other cyst nematodes and free-living nematodes, and opens up a new class of chemicals for investigation, with less toxicity and ecotoxicity concerns than current chemical nematicides. The investigations described in this study suggest that the formulation UBA could potentially prevent yield loss caused by potato cyst nematodes, and could be an ideal candidate for inclusion into integrated pest management systems.

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